

Effects of Vitamin E on airway responses and biochemical parameters in guinea pigs sensitized to ovalbumin

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Abstract

The effect of dietary supplementation with Vitamin E was studied in sensitized guinea pigs. After measurement of baseline airway reactivity and sensitization with ovalbumin, the animals were randomized into two groups: Group A, on a commercial feed and Group B, on dietary supplementation with oral Vitamin E (0.7 IU/kg). These were challenged with inhaled ovalbumin after 4 weeks. The following outcomes were studied: airway responses to ovalbumin inhalation, airway reactivity, sodium and calcium ion influx in isolated tracheal cells, Na⁺ K⁺ ATPase and Ca²⁺ ATPase activity in tracheal homogenate and plasma malonaldehyde. Sensitization increased airway reactivity in Group A but not in Group B. The tracheal cells of animals in Group B showed significantly lower rates of ⁴⁵Ca and ²²Na influx and lower activities of tracheal Na⁺ K⁺ ATPase and Ca²⁺ ATPase as compared to Group A. Plasma malonaldehyde was similar between two groups. We concluded that Vitamin E suppresses the increase in airway reactivity following sensitization and has membrane stabilizing actions.

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1. Introduction

Diets deficient in antioxidants have been blamed for increasing prevalence of asthma (Weiss, 1997). In one study, the lowest intakes of Vitamin C were shown to be associated with a more than five-fold increased

risk of airway hyper-reactivity (Soutar et al., 1997). Lung lining fluid ascorbic acid (Vitamin C) and alpha-tocopherol (Vitamin E) concentrations were reported to be low in patients with mild asthma even though blood levels were normal or increased (Kelly et al., 1999). Schunemann et al. (1997) proposed that dietary antioxidants may play a role in respiratory health. They observed a strong association between Vitamin E intake and FEV₁% predicted (Schunemann et al., 2002). Increased oxidative stress has been proposed to play

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an important role in the genesis and perpetuation of the inflammatory response in the airways (Barnes, 1990). We have recently shown that there is an increased oxidative stress in asthmatics (Nadeem et al., 2003).

Dietary supplementation with antioxidants may have a potentially useful role in the management of asthma. Among the possible candidates for such a supplementation is alpha-tocopherol (Vitamin E). It has been shown to have anti-inflammatory (Jialal et al., 2002; van Dam et al., 2003 and Rocksen et al., 2003) and membrane stabilizing properties (Erin et al., 1984). However, its role as a therapeutic agent in asthma remains to be explored.

The guinea pig model of asthma produced by sensitization with ovalbumin is a convenient tool to study the effects of therapeutic interventions as it resembles clinical asthma (Santing et al., 1995). In studies on the biochemical correlates of airway hyper-reactivity in our laboratory, tracheal $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$, both key enzymes involved in the regulation of intracellular calcium levels, were shown to have a positive relationship with airway reactivity to histamine in guinea pigs (Nath et al., 1983; Jain et al., 2001). Recently, we have reported that sensitization with ovalbumin increases influx rates of ^{45}Ca and ^{22}Na in intact cells (Jain et al., in press). The present study was carried out to evaluate the effect of dietary supplementation with Vitamin E on airway responses and these biochemical parameters in guinea pigs sensitized to ovalbumin.

2. Materials and methods

Specific pathogen-free male guinea pigs weighing from 250 to 400 g were used. These were housed in climate-controlled animal quarters in the animal house of the Institute and were given water and food ad libitum. The study was approved by the Board of Research Studies in Medical Sciences, University of Delhi.

Baseline specific airways conductance (sGaw) and airway responsiveness to histamine were measured using a non-invasive body plethysmographic technique (Agrawal, 1981). The dose of histamine that produced a 35% fall in sGaw was determined and was labeled as ED35 histamine. This represented the airway reactivity. The animals were sensitized with ovalbumin and alternately segregated into two groups: Group A animals ($n = 10$) received a commercial feed, Group

B animals ($n = 10$) received the same commercial diet along with dietary supplementation with oral Vitamin E (0.7 IU/kg).

Sensitization was carried out by injecting intraperitoneally and at sites of peripheral lymph nodes, 100 mg aluminium hydroxide and 100 μg ovalbumin per ml of normal saline, as described (Santing et al., 1995). At the end of 4 weeks, airway reactivity to inhaled histamine was determined again as described above. On the next day, animals were challenged with 0.2% ovalbumin aerosol. Airway responses were continuously monitored to observe the occurrence of the early asthmatic response (EAR) that was defined as a 35% fall in sGaw from the pre-challenge baseline value. Following this sGaw was monitored at 1 h interval for 8 h to detect any late asthmatic response (LAR) after the animal had recovered from the EAR. The LAR usually occurred between 6 and 8 h after the ovalbumin challenge. Airway reactivity to histamine was again measured at the time of the occurrence of LAR and finally at 24 h.

The animals were then anaesthetized with an intraperitoneal injection of pentothal sodium (50 mg/kg) and blood was drawn into heparinized syringes directly from the heart. The trachea was exposed and cannulated, and the lungs were lavaged with normal saline warmed at 37 °C. The returning fluid was processed and total and differential cell counts in the bronchoalveolar lavage (BAL) were obtained using standard techniques.

Sodium and calcium ion uptake was measured in tracheal strips as described (Simcowitz and Spilberg, 1982). After dissecting out the trachea, 2–3 mm wide strips were cut free from the cartilage and cleaned of the surrounding connective tissue under a dissecting microscope. These tracheal strips were equilibrated at 37 °C for 15 min before addition of the radiolabelled tracer. Subsequently, these were incubated at 37 °C in the presence of 0.4 μCi ^{22}Na or ^{45}Ca for 7.5 min, and then washed twice in buffer, and finally separated from the aqueous phase by centrifugation over a layer of di-butyl phthalate at 12,000 rpm. The tissue was dissolved with 0.1% Triton X-100 in 0.1 N sodium hydroxide at 51 °C for 1 h and the counts in the lysate were taken on a Beckman β -scintillation counter. The ion influx was expressed as pmoles of ion/mg tissue min. A preliminary time course determination showed that ion fluxes reached steady state at 15 min. The time point of 7.5 min was selected arbitrarily to have the measurements before the steady state was reached. As the two

groups were being compared, any time point before attainment of steady state would be expected to provide the information on whether the fluxes were different or not.

Na^+ K^+ ATPase and Ca^{2+} ATPase activity was measured in tracheal homogenate by the method of Schmalzing and Kushera, (1982). In a basal medium containing 100 mM Tris-Cl and 5 mM MgCl_2 , we added (a) 100 mM Na^+ , 20 mM K^+ and 2 mM EGTA to estimate Na^+ K^+ /Mg²⁺ ATPase activity or (b) 100 mM Na^+ , 0.15 mM Ca^{2+} to measure Ca^{2+} /Mg²⁺ ATPase activity. In another set of tubes containing the basal medium with (c) 100 mM Na^+ and 2 mM EGTA, we measured Mg²⁺ ATPase activity. All enzymatic reactions were initiated by the addition of tissue homogenate equivalent to 100–150 μg protein. Control reactions without the enzyme (tissue homogenate) were set up in parallel. Reactions were terminated with 10% trichloroacetic acid. Inorganic phosphorous (P_i) in the reaction supernates as well as controls was estimated by a colorimetric assay (Lebel et al., 1978). Na^+ K^+ ATPase and Ca^{2+} ATPase activities were calculated by subtracting the coupled Mg²⁺ ATPase activity (c) from each of the remaining two reaction sets. Both the enzyme activities were expressed as μg P_i liberated from ATP/mg protein/30 min at 37 °C.

Lipid peroxidation was estimated in plasma as thiobarbituric acid reactivity of malonaldehyde (MDA), an end product of fatty acid peroxidation (Jain et al., 1989) and expressed in nmoles/ml plasma.

The following outcomes were studied to evaluate the effect of Vitamin E supplementation: Airway responses to ovalbumin inhalation; changes in airway reactivity following sensitization and ovalbumin challenge; sodium and calcium ion influx in tracheal strips, tracheal Na^+ K^+ ATPase and Ca^{2+} ATPase activity and lipid peroxidation (MDA).

Statistical analysis was carried out using SPSS10.0 for windows and GraphPad Prism 3.0 for windows 95. Unpaired *t*-test was used for comparing the biochemical parameters between groups. Baseline airway reactivity in the two groups was compared using the Mann–Whitney *U*-test. Evaluation of changes in airway reactivity that was measured at four points in time was done using non-parametric analysis of variance, the Kruskal–Wallis test followed by the Dunn's test for between-group comparisons. A '*p*' value of less than

0.05 was considered as significant to indicate differences between the two groups.

3. Results

Eight sensitized animals fed on a normal commercial diet (Group A) showed an early phase bronchoconstriction (early asthmatic response, EAR) within 20 min of ovalbumin inhalation. Six of these recovered from bronchospasm within 1–2 h while in 2, the EAR continued to persist at 8 h. Four of the six animals that recovered showed a second phase of bronchoconstriction (late asthmatic response, LAR) at 6–8 h post-challenge. Thus, these animals had a dual response while the two animals that did not show the LAR had an isolated EAR. Two of the animals in the group did not show any bronchoconstrictive response to ovalbumin. Animals fed on Vitamin E supplemented diet (Group B) demonstrated the following types of responses to ovalbumin inhalation: isolated EAR (*n* = 3), dual response (*n* = 2), isolated LAR (*n* = 1) and no response (*n* = 4).

Comparison of baseline airway reactivity in the animals in the two groups did not reveal any significant differences (*p* > 0.05). Sensitization with ovalbumin produced an increase in airway reactivity to histamine (decreased ED35 histamine) in Group A. The median ED35 histamine values were as follows: baseline (pre-sensitization), 3.23 mg/ml; post-sensitization (but before challenge with ovalbumin), 0.9 mg/ml; 6 h post-challenge, 0.89 mg/ml; 24 h post-challenge, 1.49 mg/ml (Fig. 1). Non-parametric one-way analysis of variance using the Kruskal–Wallis test revealed these to be significantly different (*p* < 0.01). Between-group comparisons with the Dunn's multiple comparison test showed that the baseline ED35 histamine was significantly higher as compared to post-sensitization value (*p* < 0.01) as well as the 6 h post-challenge (*p* < 0.01) and 24 h post-challenge (*p* < 0.05) values. However, the post-sensitization, 6 h post-challenge and 24 h post-challenge airway reactivity were not significantly different among themselves (*p* > 0.05),

Sensitization with ovalbumin did not produce an increase in airway reactivity to histamine in Group B. The median ED35 histamine values were as follows: baseline (pre-sensitization), 1.54 mg/ml;

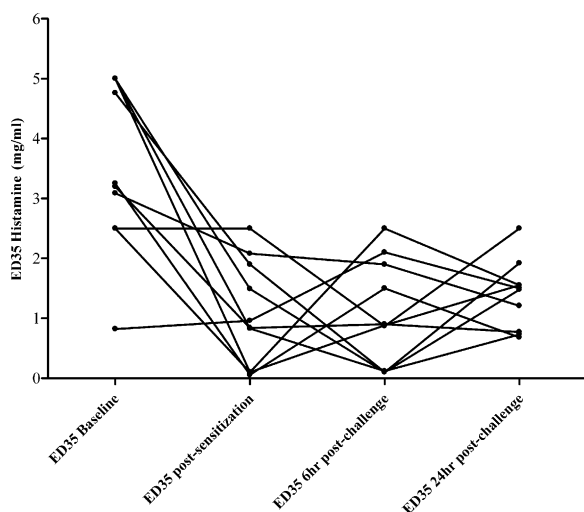


Fig. 1. Airway reactivity (ED35 histamine, mg/ml) at baseline, after sensitization and after challenge with inhaled ovalbumin in Group A (Kruskal–Wallis non-parametric ANOVA $p < 0.01$).

post-sensitization (before challenge with ovalbumin), 0.51 mg/ml; 6 h post-challenge, 1.55 mg/ml; 24 h post-challenge, 1.00 mg/ml (Fig. 2). The Kruskal–Wallis test revealed that the ED35 histamine not significantly different at these time points ($p > 0.05$).

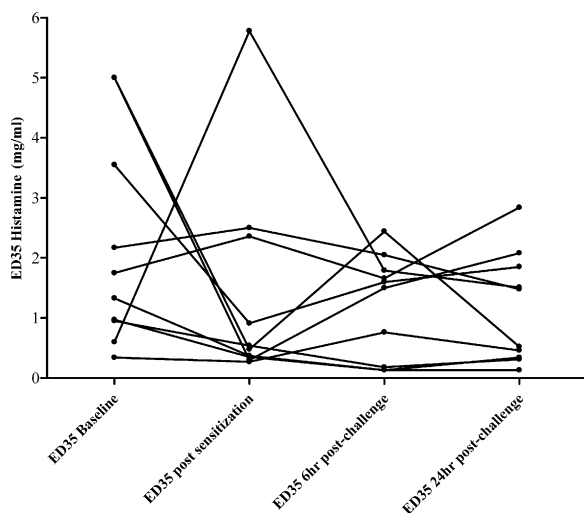


Fig. 2. Airway reactivity (ED35 histamine, mg/ml) at baseline, after sensitization and after challenge with inhaled ovalbumin in Group B (Kruskal–Wallis non-parametric ANOVA $p > 0.01$).

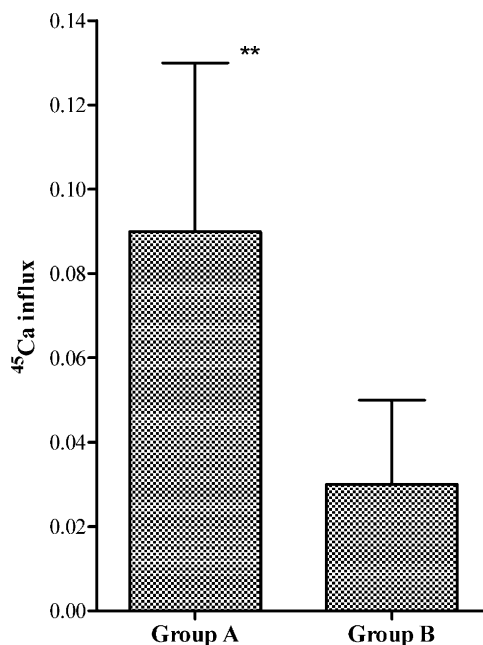


Fig. 3. Rates of ^{45}Ca influx in pmoles of ion/mg tissue/min in the isolated tracheal cells in the two groups (Group A, $n = 7$; Group B, $n = 10$, **: $p < 0.01$).

3.1. Effect on transmembrane ionic fluxes

The ion fluxes were expressed in pmoles of ion/mg tissue/min. Data is presented as mean $\pm$ S.D. Sensitized challenged animals on Vitamin E supplemented diet (Group B) showed a significantly lower rate of ^{45}Ca influx (0.03 ± 0.02) as compared to the animals on normal diet (Group A) (0.09 ± 0.04) ($p < 0.01$) (Fig. 3). The former also had lower ^{22}Na influx (1.63 ± 1.16) as compared to the latter (3.29 ± 1.83) ($p < 0.05$) (Fig. 4).

3.2. Effect on tracheal ATPases

The ATPases were expressed in $\mu\text{g P}_i$ liberated from ATP/mg protein/30 min at 37°C . Data is presented as mean $\pm$ S.D. Tracheal $\text{Na}^+ \text{K}^+$ ATPase activity in Group B was lower at 2.10 ± 1.07 as compared to 5.69 ± 5.46 in Group A. ($0.05 < p < 0.1$). The Ca^{2+} ATPase activity in the former group was also lower than the latter, the values being 0.89 ± 0.79 and 4.91 ± 2.45 , respectively ($p < 0.001$) (Fig. 5).

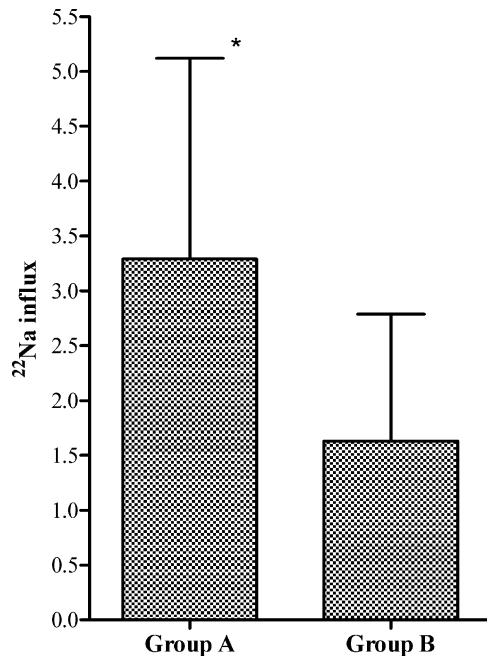


Fig. 4. Rates of ^{22}Na influx in pmoles of ion/mg tissue/min in the isolated tracheal cells in the two groups (Group A, $n=7$; Group B, $n=10$, *: $p<0.05$).

3.3. Effect on pulmonary inflammation

There were no significant differences in the total cell counts ($\times 10^6 \text{ ml}^{-1}$) on BAL between the two groups: Group A, 26.8 ± 5.5 and Group B,

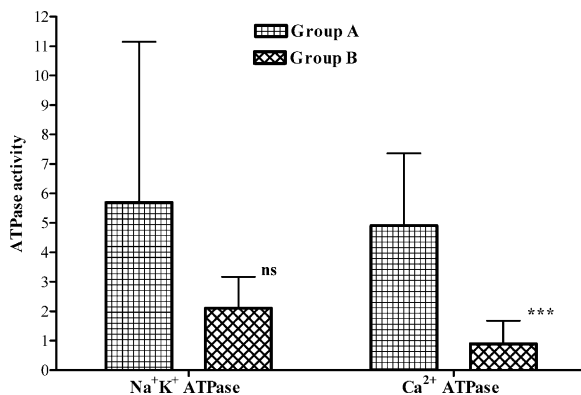


Fig. 5. Activities of $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ ($\mu\text{g P}_i$ liberated from ATP/mg protein/30 min at 37°C) in the tracheal homogenate in the two groups (Group A, $n=10$; Group B, $n=10$, ns: $p>0.05$, ***: $p<0.001$).

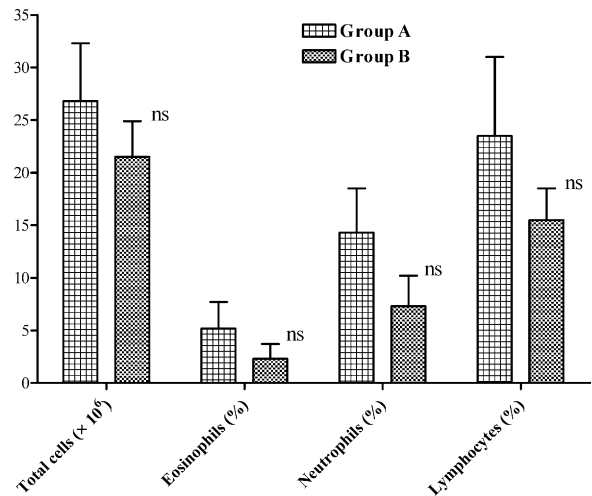


Fig. 6. Bronchoalveolar lavage cytology after ovalbumin challenge in the two groups (Group A, $n=10$; Group B, $n=10$, ns: $p>0.05$).

21.5 ± 3.4 ($p>0.05$). Although the percentage of eosinophils, neutrophils and lymphocytes was approximately 1.5–2-fold lower in the Vitamin E supplemented Group B, the differences were not statistically significant between the two groups ($p>0.05$). The percentage of eosinophils in the BAL fluid was 5.2 ± 2.5 and 2.3 ± 1.4 in Groups A and B, respectively, while neutrophils were 14.3 ± 4.2 and 7.3 ± 2.9 , and lymphocytes 23.5 ± 7.5 and 15.5 ± 3.0 (Fig. 6).

3.4. Effect on lipid peroxidation

The mean $\pm$ S.D. plasma MDA levels in animals in Group A were 0.63 ± 0.31 nmoles/ml as compared to 1.09 ± 0.58 in Group B. The difference was not significant ($p>0.05$).

4. Discussion

The present study shows that dietary supplementation with 0.7 IU/kg Vitamin E for 4 weeks modulates airway responses in sensitized guinea pigs. It prevented the increase in airway reactivity that follows sensitization and ovalbumin challenge. The tracheal cells of animals in the Vitamin E supplemented diet showed significantly lower rates of ^{45}Ca influx and ^{22}Na influx as compared to the animals on normal diet suggesting a membrane stabilizing effect. The activities of tracheal

$\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase were lower in animals on Vitamin E supplemented diet.

While the number of animals in the two groups was small, and therefore a statistical comparison of the pattern of airway responses to ovalbumin challenge could not be carried out, the limited data available suggested that there was an amelioration of the response to ovalbumin in animals on Vitamin E supplemented diet. Both the early and the late airway responses were observed in fewer animals on Vitamin E. While the total cell counts in bronchoalveolar lavage fluid were not affected, the number of lymphocytes, eosinophils and neutrophils was approximately 1.5–2-fold lower in the Vitamin E supplemented group. These observations point towards a possible anti-inflammatory effect of Vitamin E.

We have recently reported that sensitization of guinea pigs is associated with an increase in the activity of $\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase activity, and higher rates of ^{45}Ca and ^{22}Na influx in leukocytes as compared to non-sensitized animals (Jain et al., in press). The increase in the activities of these enzymes was explained as a homeostatic mechanism to lower the intracellular concentrations of sodium and calcium cations that would rise due to increased influx of these cations induced by sensitization. As the membrane fluxes of calcium and sodium were decreased in the Vitamin E supplemented animals, the homeostatic increase in the activities of these enzymes was not observed in these animals. A similar effect of Vitamin E on peroxynitrite-induced increase in calcium influx and Ca^{2+} ATPase activity has recently been described in bovine pulmonary smooth muscle cells (Chakraborti et al., 2004).

There is some evidence in literature to suggest that Vitamin E has anti-inflammatory actions. Especially at high doses, it shows a benefit with regards to LDL oxidation, isoprostanes and a decrease in inflammatory markers such as C-reactive protein, pro-inflammatory cytokines and PAI-1 levels (Jialal et al., 2002). However, antioxidants do not alter inflammatory markers in the absence of inflammation. This was shown in healthy men in whom combined supplementation with alpha-tocopherol and Vitamin C for 3 years had no effect on circulating levels of TNF-alpha, IL-6 or CRP (Bruunsgaard et al., 2003). Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells (van Dam et al., 2003). It reduces transendothelial migration of neutrophils and prevents

lung injury in endotoxin-induced airway inflammation (Rocksen et al., 2003).

Vitamin E has other actions that may be relevant in asthma. It has a protective action against cytotoxic action of different agents (Bolt et al., 2001). Boscoboinik et al. (1991) described non-antioxidant cell signaling functions for alpha-tocopherol, demonstrating that Vitamin E regulates protein kinase C activity in smooth muscle cells. In cultured cells, it has been demonstrated that Vitamin E inhibits inflammation, cell adhesion, platelet aggregation and smooth muscle cell proliferation. Recent advances in molecular biology and genomic techniques have led to the discovery of novel Vitamin E-sensitive genes and signal transduction pathways (Rimbach et al., 2002).

It has been shown that Vitamin E has a protective effect against lipid peroxidation induced by oxidative stress. Supplementation of mice with exogenous Vitamin E before influenza virus inoculation was observed to protect lung and blood against lipid peroxidation (Mileva et al., 2002). In the present study, however, the levels of lipid peroxides were not significantly different in the two groups. One possible explanation is that the dose of Vitamin E used in the present study was not adequate to decrease lipid peroxidation. Another possible explanation is that this model of sensitization does not increase the levels of lipid peroxides significantly. Although we did not include a control group of healthy animals to address this question in the present study, we have shown in our previous work (Jain et al., in press) that sensitized animals had significantly higher levels of lipid peroxides as compared to healthy control animal. Therefore, this explanation for lack of effect of Vitamin E can be ruled out.

Accumulating evidence suggests that dietary antioxidant vitamins are positively associated with lung function. Increased oxidative stress measured by increased lipid peroxidation products in plasma has been shown to be inversely related to lung function, measured as forced expiratory volume in 1 s ($\text{FEV}_1\%$) (Schunemann et al., 1997). Among all antioxidant vitamins, the strongest association was observed for Vitamin E with $\text{FEV}_1\%$ (Schunemann et al., 2002). This finding lent support to the hypothesis that dietary antioxidants may play a role in respiratory health (Schunemann et al., 1997).

Direct estimations of antioxidants including Vitamin E in bronchoalveolar lavage fluid and serum of

clinically stable atopic asthmatic children have not shown any significant differences (Schock et al., 2003). Whether there are any changes in acute exacerbations or otherwise symptomatic asthma is not known. Moreover, estimations of a single antioxidant may be of little value. There are several cellular and extracellular antioxidants that likely respond in concert, either simultaneously or sequentially, to an increased oxidant load. Alterations in levels and activities of several non-enzymatic and enzymatic antioxidants have been described by us recently in symptomatic asthma (Nadeem et al., 2003).

There has been no study on the effect of dietary supplementation with Vitamin E in asthma. In the present study, we used an ovalbumin-sensitized guinea pig model. Kilic and Erol, (2003) have recently shown that 25 and 50 mg/(kg day) intraperitoneal doses of Vitamin E reduced the contractile effects of ovalbumin in isolated tracheal preparations from ovalbumin-sensitized guinea pigs. Our results show that Vitamin E at a comparable dose (0.7 IU/kg equals 40 mg/kg of alpha-tocopherol) suppresses the increase in airway reactivity following sensitization and has membrane stabilizing actions. It has been shown that Vitamin E is incorporated into the lipid bilayer in an amount proportional to the amount of PUFAs in the biological membranes (Sekharam et al., 1990). Taken together this would suggest that oxidant-stress induced hyper-responsiveness is abrogated by the direct stabilization of biological membranes at a dose that is much lower than that required for its effects as a free-radical scavenger. Vitamin E plays an important regulatory role in ROS-induced autonomic imbalance between muscarinic receptor-mediated contraction and β -adrenergic-mediated relaxation of the airway smooth muscle (Doelman and Bast, 1990). This autonomic imbalance is suggested to be important in the development of BHR. Thus, these properties may be exploited therapeutically in asthma. There is sufficient indirect evidence from literature in other contexts that Vitamin E may help in reducing airway inflammation and its consequences. The present study provides the first direct evidence of such a beneficial action in an animal model resembling clinical asthma. Our results should provide impetus for controlled studies of therapeutic intervention with Vitamin E in asthma as an adjunct to the current therapy.

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