



A novel cinnamate derivative attenuates asthma features and reduces bronchial epithelial injury in mouse model

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ABSTRACT

Airway epithelial injury is the hallmark of various respiratory diseases and therapeutic targeting of epithelial injury could be an effective strategy for controlling these diseases. We recently reported that a novel cinnamate, ethyl 3',4',5'-trimethoxythionocinnamate (ETMTC) derived from *Piper longum* derivative, was most potent among various cinnamate derivatives in inhibiting inflammatory cell adhesion molecules (CAMs). In this study, we investigated the effects of ETMTC on the features of allergic asthma and epithelial injury in a murine model. ETMTC treatment to ovalbumin sensitized and challenged mice during ovalbumin challenge reduced airway hyperresponsiveness, and airway inflammation. This attenuation of asthma features was associated with the reduction in the expressions of various CAMs, NF-κB activation, Th2 cytokines, eotaxin and 8-isoprostane that were estimated in lung homogenates. Further, it increased activities of mitochondrial complexes I and IV in lung mitochondria and reduced cytochrome c and caspase 9 activities in lung cytosol. In addition, it reduced the levels of oxidative DNA damage marker in bronchoalveolar lavage fluid and DNA fragmentation of bronchial epithelia in lung sections. Further, ETMTC not only increased the levels of 15-(S)-hydroxyeicosatetraenoic acid, suppressor of airway remodeling, but also inhibited goblet cell metaplasia and sub-epithelial fibrosis. These results demonstrate that ETMTC reduces epithelial injury and mitochondrial dysfunction associated with allergic asthma and thus ETMTC could be useful to develop efficient therapeutic molecule against asthma.

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1. Introduction

Asthma is a chronic airway inflammatory disease which is characterized by airway inflammation including airway eosinophilia, increased IgE synthesis, mucus hypersecretion and airway hyperresponsiveness, and repeated allergen exposures lead to airway remodeling changes [1]. Most of these pathophysiological features are mediated by Th2 dominant response which recruits various inflammatory cells such as eosinophils, lymphocytes, and macrophages into the airway and creates a local 'oxidative milieu' and leads to airway injury [2]. The successful recruitment of inflammatory cells into the airway requires systematic expression of adhesion molecules on both vascular endothelium and inflammatory cells and various chemoattractants such as eotaxin [3]. The initiation and perpetuation of allergic inflammation and consequent oxidative stress require a coordinative expression of pro-inflammatory cytokines, chemokines, adhesion molecules, and other pro-inflammatory lipid

mediators. It is important to note that most of this broad array of genes contains the NF-κB binding site within their promoters, suggesting that NF-κB could be a master switch [4]. To support this view, p50 NF-κB knockout mice are not able to produce Th2 cytokines and develop airway eosinophilia [5]. However, permanent knockdown may not be ideal as NF-κB involved in maintaining normal immune response against foreign invaders.

At earlier stages of asthma, bronchial epithelia secrete various anti-inflammatory molecules to protect themselves from allergic inflammation induced injury. Due to repeated allergen exposures, however, epithelia lose their protective mechanisms including antioxidant defenses which lead to denudation from the basement membrane [6,7]. To confirm this view, epithelial clumps have been found in sputum and bronchoalveolar lavage (BAL) of asthmatic patients [8]. Though the exact mechanism for the denudation is not clear, evidence suggests that the probable reason might be apoptosis and we have shown the involvement of mitochondria in this context [9]. Moreover, human asthmatic bronchial epithelial cells showed increased caspase 9 activity and DNA fragmentation [10]. This epithelial injury activates epithelial mesenchymal trophic unit which leads to airway remodeling changes with transformation of attenuated fibroblast into myofibroblast that secretes collagen. 15-S-hydroxyeicosatetraenoic acid (15-(S)-HETE) is secreted

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by epithelia of normal airways and suppresses the activation of attenuated fibroblast sheath which exists underneath the epithelium [11]. In addition, epithelial injury is followed by repair or goblet cell metaplasia depends on the microenvironment [12]. Thus, epithelial injury seems to act as a bridge between airway inflammation and airway remodeling. Thus it has been suggested that different therapeutic approaches can be devised by focusing on allergen induced epithelial injury rather than suppressing airway inflammation or manipulating the immune response [13]. Though other therapeutic strategies such as anti-cytokine and gene therapy seem to be exciting, majority of existing effective therapies were derived from either natural substances or hormones [14]. In our earlier study, we found that cinnamate derivatives of natural plant source, *Piper longum*, were effective in inhibiting TNF- α induced expression of cell adhesion molecules in human endothelial cells [15]. Further, we synthesized various novel analogs of cinnamates, thiocinnamates and

thionocinnamates; and structure–activity relationship studies of these analogs revealed that ethyl 3',4',5'-trimethoxythionocinnamate (ETMTC) was most efficient in inhibiting TNF- α induced expression of cell adhesion molecules [16]. Indeed, ETMTC was 200 times more potent than its natural derivative [16], and this could be due to the presence of sulfur group in ETMTC (Fig. 1A). With this view, we have chosen ETMTC, to determine its effects on epithelial injury and its mechanism of action in mice model of asthma.

2. Materials and methods

2.1. Animals

8–10 week old male BALB/c mice were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for a week

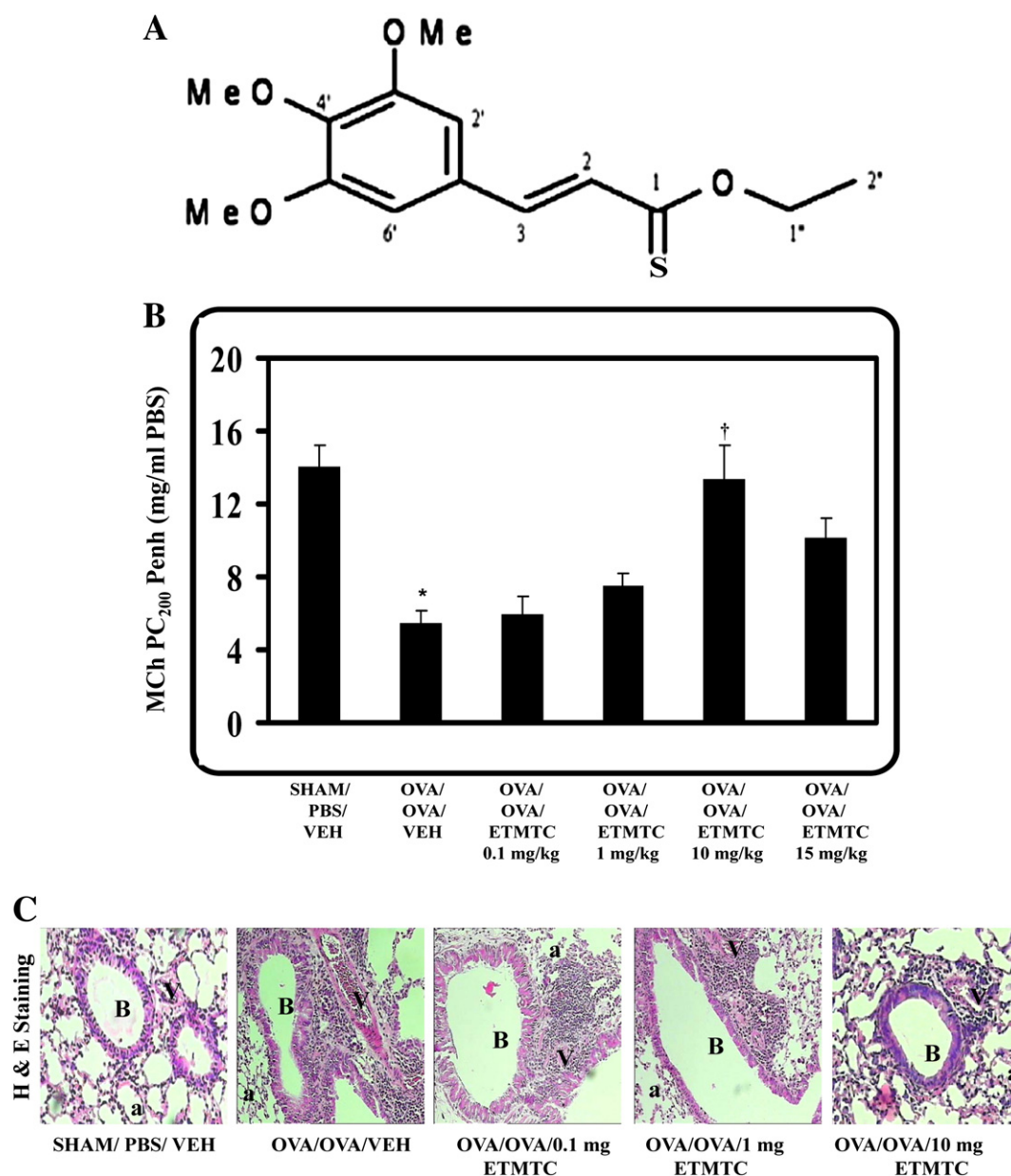


Fig. 1. The structure of ethyl 3',4',5'-trimethoxythionocinnamate (ETMTC) and the effects of different doses of ETMTC on airway hyperresponsiveness (AHR) to methacholine and airway inflammation. A) The structure of ETMTC. B) Mice were randomly divided into six groups of mice, each group had five mice and named as per status of sensitization/challenge/treatment: SHAM/PBS/VEH, OVA/OVA/VEH, OVA/OVA/ETMTC 0.1, 1, 10 and 15 (ETMTC 0.1, 1 10 and 15 mg/kg wt). AHR was determined with increasing concentrations of methacholine on day 33 using single chamber plethysmography and MCh PC₂₀₀ Penh values (Partial concentration of methacholine which produces 200% of baseline Penh) were determined. Results are shown as mean $\pm$ SEM. *P < 0.05 vs. SHAM/PBS/VEH †P < 0.05 vs. OVA/OVA/VEH. n = 6 mice per group. C) Representative photomicrographs of hematoxylin and eosin stained lung sections of different groups of mice.

prior starting the experiments. All animals utilized in this study were under protocols approved by Institutional Animal Ethics Committee.

2.2. Grouping, sensitization, challenge and treatment of mice

For dose titration experiments, mice were divided after acclimatization into 6 groups and each group (5 mice in each group) was named according to sensitization/challenge/treatment: SHAM/PBS/VEH (VEH—vehicle, 50% ethanol or 10 % dimethylsulfoxide), OVA/OVA/VEH (OVA—chicken egg ovalbumin grade V, Sigma, USA), and OVA/OVA/ETMTC 0.1, 1, 10 and 15 (ETMTC 0.1, 1, 10 and 15 mg/kg wt). As described earlier [9], mice were sensitized by three intraperitoneal injections of 50 µg OVA in 4 mg aluminum hydroxide (OVA groups such as OVA/OVA/VEH, OVA/OVA/ETMTC) or 4 mg aluminum hydroxide (SHAM/PBS/VEH) on days 0, 7, and 14. Mice were challenged from day 21 to day 32 (30 min per day) with 3% OVA in PBS (OVA groups) or PBS (SHAM/PBS/VEH) using a nebulizer with a flow rate of 9 L/min (OMRON CX3 Model). ETMTC at different doses (0.1, 1, 10 and 15 mg/kg wt) was administered orally from days 21 to 32 twice a day in 20 µL volume per dose. From the dose titration experiments, 10 mg/kg dose was selected for further experiments.

In the confirmation experiments, mice were divided after acclimatization into 3 groups and each group (6 mice in each group) was named according to sensitization/challenge/treatment: SHAM/PBS/VEH (VEH—vehicle, 50% ethanol or 10 % dimethylsulfoxide), OVA/OVA/VEH (OVA—chicken egg ovalbumin grade V, Sigma, USA), and OVA/OVA/ETMTC (ETMTC 10 mg/kg weight). ETMTC was derived as described earlier [16] and was administered orally from days 21 to 32 twice a day in 20 µL volume per dose. As described earlier [9], mice were sensitized by three intraperitoneal injections of 50 µg OVA in 4 mg aluminum hydroxide or 4 mg aluminum hydroxide on days 0, 7, and 14. Mice were challenged from day 21 to day 32 (30 min per day) with 3% OVA in PBS or PBS alone.

2.3. Airway hyperresponsiveness (AHR) measurement, bronchoalveolar lavage (BAL) and sera separation

In dose titration experiments, AHR measurement was performed on day 33 in unrestrained conscious mice by single chamber body plethysmography respectively as described earlier [9] with little modifications. Final results were expressed as MCh PC₂₀₀ values (Partial concentration of methacholine which produces 200% of baseline Penh while Penh measured with PBS aerosol was considered as 100%).

In the confirmation experiments, AHR in the form of airway resistance was determined on day 33 with invasive method using flexiVent system (Scireq), as described previously [17]. After AHR measurement, each mouse was sacrificed, bronchoalveolar lavage (BAL) was performed, BAL fluid was processed to obtain supernatants and serum was separated as described previously [9].

2.4. Measurements of interleukin-4, IL-5, eotaxin in lung homogenates and OVA specific IgE, and IgG₁ in sera

Lung tissue homogenates was prepared [9] and were used for ELISA (BD Biosciences for IL-4 and IL-5 and R&D systems for eotaxin). Measurements were performed as per manufacturer's protocol. Results were expressed in picograms and normalized by protein concentrations. OVA specific IgE, and IgG₁ in sera were determined and performed as described earlier [9,17] with little modifications.

2.5. Preparation of cytoplasmic and nuclear extracts from lung tissue of mice and nuclear factor κB (NF-κB) activation assay

The cytoplasmic and nuclear extracts were prepared as described earlier [18]. NF-κB activation assay was performed by electrophoretic mobility shift assay (EMSA) as described earlier [19]. Briefly, 10 µg of

nuclear extract was added to ³²P-end labeled double-stranded NF-κB oligonucleotide (5'-AGTTGAGGGGACTTCCCAGG-3', 40–80 fmol/rxn) and the mixture was incubated in binding buffer for 30 min at RT. Specificity of binding was ascertained by cold competition with an excess of unlabeled NF-κB oligonucleotides, and nonspecific competition was assessed by incubation with an excess of a double-stranded unlabeled Oct-1 or Sp-1 probe. The reaction mixture was electrophoresed through 5% polyacrylamide gels containing tris-glycine buffer. The gel was then dried and subjected to autoradiography.

2.6. Western blot analysis

The expression of cell adhesion molecules in the lung homogenates was determined by Western blot with ICAM-1, VCAM-1 and E-selectin antibodies (Santa Cruz Biotechnology, Inc.) and detected with HRP conjugated anti-mouse secondary antibodies and DAB-H₂O₂. β-Tubulin was used for loading control. Signals were detected by spot densitometry (Alpha EaseFC software from Alpha Innotech).

2.7. Histological examination of lung tissue of mice

For histological examination, one portion of the harvested lung was fixed with 10% buffered formalin. Paraffin-embedded lungs were cut into 5 µm sections for hematoxylin and eosin staining, periodic acid staining, Masson Trichrome staining to assess inflammatory changes, goblet cell metaplasia and sub-epithelial fibrosis, respectively and stained sections were photographed with Nikon microscope with camera (Model YS-100). Inflammation scoring and quantitative morphometry were performed by experimentally blind experts with the lung sections as described earlier to determine airway mucin content and collagen deposition [9,17].

2.8. 8-Isoprostane and 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay

Since peroxidation is the common consequence of airway inflammation, oxidative markers such as 8-isoprostane and 8-OHdG were measured by competitive ELISA in lung homogenates and BAL fluid supernatants and the results were expressed in pg/25 µg protein and pg/100 µL BAL fluid supernatants, respectively.

2.9. Isolation of whole lung mitochondria and cytosolic separation

One more portion of the lung was used to prepare mitochondrial and cytosolic fractions as described previously [9] with mitochondria isolation kit (MITO-ISO1; Sigma Chemical Co). Protein estimation was done for both mitochondrial and cytosolic fractions by bicinchoninic acid BCA [Sigma Chemical Co] assay.

2.10. Cytochrome c and 15-(S)-HETE measurements in cytosol

The levels of cytochrome c were measured in lung cytosol by ELISA as per instructions (R&D systems). 50 µg cytosol per well in duplicate was used for ELISA of 15-(S)-HETE (Cayman Biochemical Corp.). Measurements were performed as per manufacturer's instructions without further extraction. Results were expressed in ng/50 µg protein.

2.11. Estimation of activities of complex I and complex IV in lung mitochondria

Activities of complex I (NADH-ubiquinone oxidoreductase) and complex IV (cytochrome c oxidase) were estimated in isolated mitochondria with complex I enzyme activity assay kit (MitoSciences) and cytochrome c oxidase activity assay kit (Sigma, USA) according to the manufacturer's instructions [9].

2.12. Caspase 9 assay

Since cytochrome c in cytosol activates caspase 9, we have assayed it in lung cytosol. It was based on colorimetric detection of the cleavage of *para*-nitroaniline-labeled peptide (Leu-Glu-His-Asp) substrates specific for caspase 9 (R&D systems). Briefly, 150 μ g lung cytosol was incubated with substrate conjugates at 37 °C for 2 h, detected at 405 nm and results were expressed in arbitrary units.

2.13. TUNEL apoptotic assay

Since increased cytochrome c and caspase 9 lead to induce apoptosis, TUNEL assay of apoptosis was performed on lung tissue sections using an in situ apoptosis detection kit (Dead End Colorimetric TUNEL system; Promega) [20].

2.14. Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Since we conducted hypothesis driven comparisons between two

selected groups, we used unpaired Student's 't' test using JMP software and statistical significance was set at $P \leq 0.05$. ANOVA with post-hoc correction was used to compare group-wise data using JMP software.

3. Results

3.1. ETMTC reduced airway hyperresponsiveness to methacholine (MCh)

In dose titration experiments, MCh PC₂₀₀ was significantly lower in OVA/OVA/VEH mice compared to SHAM/PBS/VEH (Fig. 1B) which indicated that OVA induced mice had developed airway hyperresponsiveness. However, ETMTC administration to OVA-sensitized and challenged mice had shown an increase in MCh PC₂₀₀ values and reduction in the airway inflammation (Fig. 1B, C). While low doses of ETMTC were partially effective, 10 mg/kg was found to be an optimal dose (Fig. 1B, C). So, further experiments were performed with 10 mg/kg. In the confirmation experiments, AHR was measured after allergen challenge with increasing concentrations of methacholine with invasive airway mechanics. Percentage baseline airway resistance was significantly higher in OVA/OVA/VEH mice

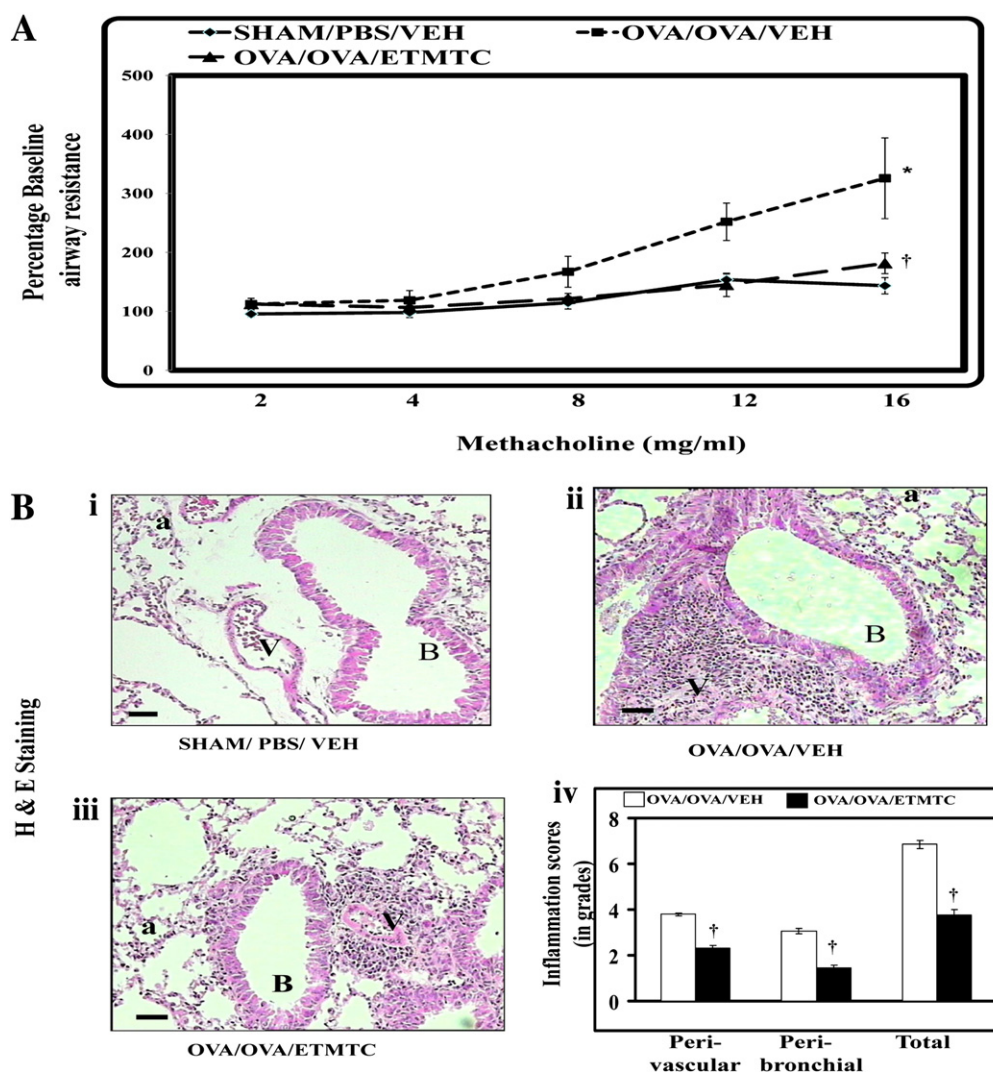


Fig. 2. ETMTC at 10 mg/kg reduced airway resistance and airway inflammation. A) Mice were randomly divided into three groups and named as per status of sensitization/challenge/treatment: SHAM/PBS/VEH, OVA/OVA/VEH, OVA/OVA/ETMTC (ETMTC 10 mg/kg wt). AHR in the form of percentage baseline airway resistance using invasive instrument was determined with increasing concentrations of methacholine on day 33 $^*P < 0.05$ vs. SHAM/PBS/VEH $^{\dagger}P < 0.05$ vs. OVA/OVA/VEH. (n = 6 mice per group) B, i–iii) Representative photomicrographs from three independent experiments have been shown. Bars, 50 μ m. B, bronchus; V, vessel; a, alveolus. iv) Peribronchial, perivascular and total airway inflammation scoring was performed on lung tissue sections. Data were mean $\pm$ SEM of three separate experiments. $^{\dagger}P < 0.05$ vs. OVA/OVA/VEH group, n = 6 mice per group.

compared to SHAM/PBS/VEH (Fig. 2A). However, ETMTC administration to OVA-sensitized and challenged mice had shown increased attenuation in percentage baseline airway resistance compared to OVA/OVA/VEH mice (Fig. 2A).

3.2. ETMTC reduced airway inflammation

As ETMTC showed a significant reduction in AHR (Fig. 2A), the effect of ETMTC on airway inflammation was determined. SHAM/PBS/VEH mice showed normal architecture of lung without any sign of inflammation (Fig. 2B, i) and OVA/OVA/VEH mice showed infiltration of inflammatory

cells including eosinophils, mononuclear cells such as monocytes and lymphocytes, neutrophils in perivascular and peribronchial regions, however alveolar regions were free from any infiltrated cells (Fig. 2B, ii). Interestingly, OVA/OVA/ETMTC showed a reduction in both perivascular and peribronchial inflammation (Fig. 2B, iii, iv).

3.3. ETMTC attenuated the expressions of CAMs (ICAM-1, VCAM-1 and E-selectin) in lungs

Since CAMs play a major role in the migration of inflammatory cells such as leukocytes from blood stream to the site of inflammation, we

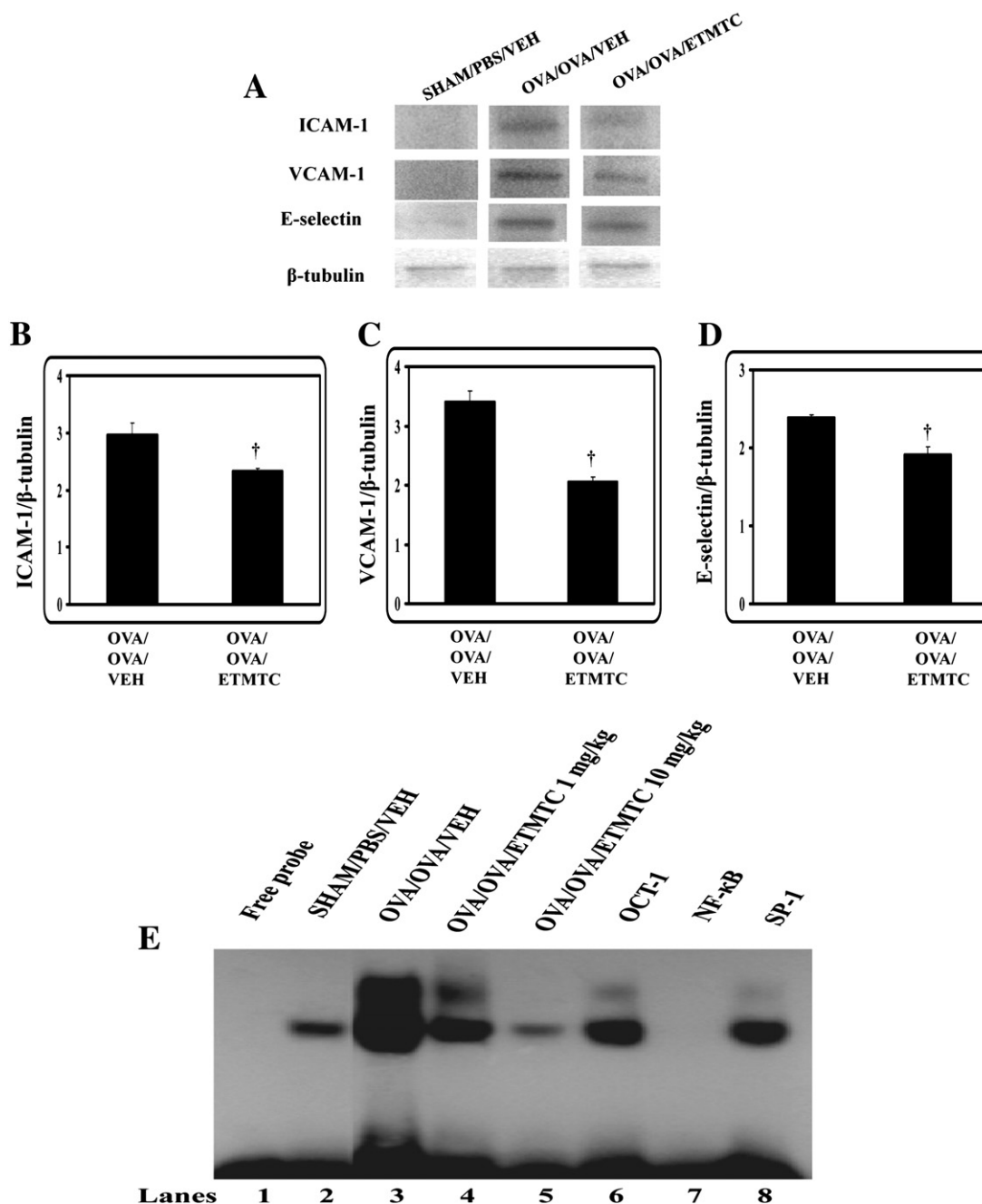


Fig. 3. ETMTC reduced the expressions of cell adhesion molecule and NF-κB activation in lungs. A) The expressions of ICAM-1, VCAM-1 and E-selectin were determined by Western blot in lung homogenates. β-Tubulin was used as loading controls. B, C, D) Signals were estimated with spot densitometry and β-tubulin signals divided by signals of ICAM-1 (B), VCAM-1 (C) or E-selectin (D). Results are shown as mean ± SEM. [†]P < 0.05 vs. OVA/OVA/VEH. The data presented are representative of three separate experiments and n = 6 mice per group. E) Lungs were harvested and lung nuclear extracts from different groups of mice were prepared and EMSA was performed as described in Materials and methods.

have determined the expressions of key CAMs in lung. As shown in Fig. 3A, there was a significant increase in the expressions of ICAM-1, VCAM-1 and E-selectin in OVA/OVA/VEH mice compared to SHAM/PBS/VEH. In contrast, ETMTC treatment had shown a significant reduction in the expressions of these CAMs. Equal loading of protein amount was visualized by β -tubulin (Fig. 3A). Densitometry of Western blot confirmed these findings (Fig. 3B–D).

3.4. ETMTC attenuated NF- κ B activation in lungs

NF- κ B is one of the major transcription factors which control the expression of CAMs. The effect of ETMTC on NF- κ B activation was studied by using EMSA as mentioned in Materials and methods. As shown Fig. 3E, NF- κ B expression was low in the nuclear extracts of lungs of sham control mice whereas it was significantly increased in asthmatic control mice (lane 2 vs. lane 3). Interestingly, OVA/OVA/ETMTC (10 mg dose) mice had shown a significant decrease in the level of NF- κ B binding compared to 1 mg/kg treatment. The specificity of the NF- κ B-DNA complex was confirmed in control experiments. Incubation with excess

unlabelled NF- κ B oligonucleotide inhibited the formation of the complex, whereas competition with an excess of an irrelevant oligonucleotide, Oct-1 or SP-1 did not inhibit the complex (compare lane 7 with lanes 6 and 8). These results demonstrated that ETMTC inhibited NF- κ B activation in asthmatic control mice.

3.5. ETMTC reduced Th2 cytokines in lung and OVA-specific IgE levels in sera

To determine the effects of ETMTC on Th2 cytokines, we measured the levels of IL-4, and IL-5 in lung tissue as described in Materials and methods. As shown in Fig. 4A and B, the levels of these cytokines were increased in OVA/OVA/VEH compared to SHAM/PBS/VEH mice. However, treatment with ETMTC showed the significant reduction of both the cytokines (Fig. 4A, B).

To determine the effects of ETMTC on OVA specific immunoglobulins, we measured IgE, and IgG1 in sera as described in Materials and methods. As shown in Fig. 4C and D, the levels of OVA specific IgE and IgG1 were increased in OVA/OVA/VEH compared to SHAM/PBS/VEH mice. ETMTC

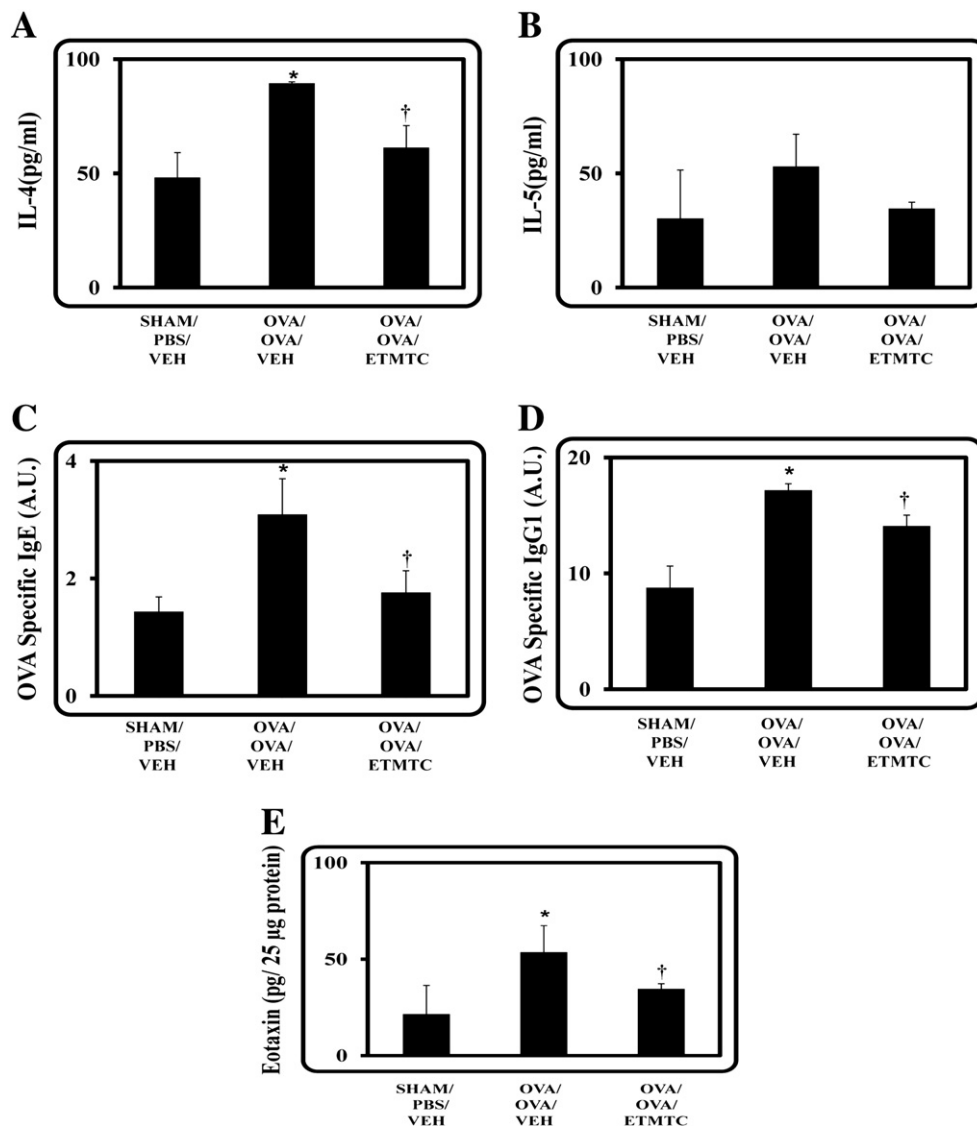


Fig. 4. ETMTC reduced the levels of Th2 cytokines in lungs and OVA specific IgE in sera. The levels of IL-4 (A), and IL-5 (B), in lungs were determined by ELISA. OVA specific IgE (C) and OVA specific IgG1 (D) were determined by ELISA in sera. The levels of eotaxin in lungs were determined by ELISA. Results are shown as mean $\pm$ SEM. * P <0.05 vs. SHAM/PBS/VEH, † P <0.05 vs. OVA/OVA/VEH. The data presented are representative of three separate experiments and n =6 mice per group.

treatment showed a significant reduction in both OVA specific IgE and IgG1 (Fig. 4C, D).

3.6. ETMTC attenuated eotaxin levels in lungs

As Th2 cytokines such as IL-4 induces eotaxin through NF- κ B activation and eotaxin plays a significant role in the migration of eosinophil which is critical in causing epithelial injury in asthma we measured eotaxin levels in the lung homogenates as described in [Materials and methods](#). As shown in Fig. 4E, eotaxin levels were significantly higher in OVA/OVA/VEH mice compared to SHAM/PBS/VEH. Interestingly, ETMTC treatment reduced the eotaxin levels significantly compared to OVA/OVA/VEH mice (Fig. 4E).

3.7. ETMTC reduced lipid peroxidation

Since oxidative stress is a definite consequence of airway inflammation, 8-isoprostane was estimated in lung homogenates. As shown in Fig. 5A, 8-isoprostane levels were found to be significantly increased in OVA/OVA/VEH mice compared to SHAM/PBS/VEH mice. However, ETMTC treatment significantly reduced it (Fig. 5A).

3.8. ETMTC increases activities of mitochondrial complex I and cytochrome c oxidase and reduced the levels of cytochrome c in cytosol

Since increased pro-inflammatory mediators and lipid peroxidation lead to epithelial injury and we showed the mitochondrial dysfunction in asthmatic mice, and the activities of cytochrome c oxidase and complex I were determined in lung mitochondria. As shown in Fig. 5B and C, the activities of complex I and complex IV were found to be reduced in

OVA/OVA/VEH mice compared to SHAM/PBS/VEH. However, these reductions were restored with ETMTC treatment (Fig. 5B and C).

3.9. ETMTC reduced the levels of cytochrome c and caspase 9 in cytosol

As there was a reduction in mitochondrial complex activities, cytochrome c levels and caspase 9 activities were determined. The levels of cytochrome c (Fig. 5D) and caspase 9 (Fig. 6A) activities were significantly increased in OVA/OVA/VEH mice compared to SHAM/PBS/VEH mice. However, treatment of asthmatic mice with ETMTC showed a significant reduction of both cytochrome c and caspase 9 in the cytosol (Figs. 5D and 6A).

3.10. ETMTC reduced DNA fragmentation in bronchial epithelial cells

Since increased cytochrome c and caspase 9 activities in cytosol lead to initiate apoptosis, we performed apoptosis assay in lung sections by TUNEL and 8-OHdG in BAL fluid supernatants. There was an increase in the levels of 8-OHdG in BAL fluid supernatants of asthmatic mice compared to normal control mice. However, it was reduced by ETMTC treatment (Fig. 6B). As shown in Fig. 6C, asthmatic mice showed increased DNA fragmentation especially in bronchial epithelial cells. ETMTC treatment to asthmatic mice showed a reduction in DNA fragmentation as well as TUNEL positive bronchial epithelia (Fig. 6C, i–iv).

3.11. ETMTC reduced goblet cell metaplasia

To determine the effect of ETMTC on goblet cell metaplasia, periodic acid-Schiff staining was performed. As shown in Fig. 7A, asthmatic mice have shown a significant goblet cell metaplasia compared to normal

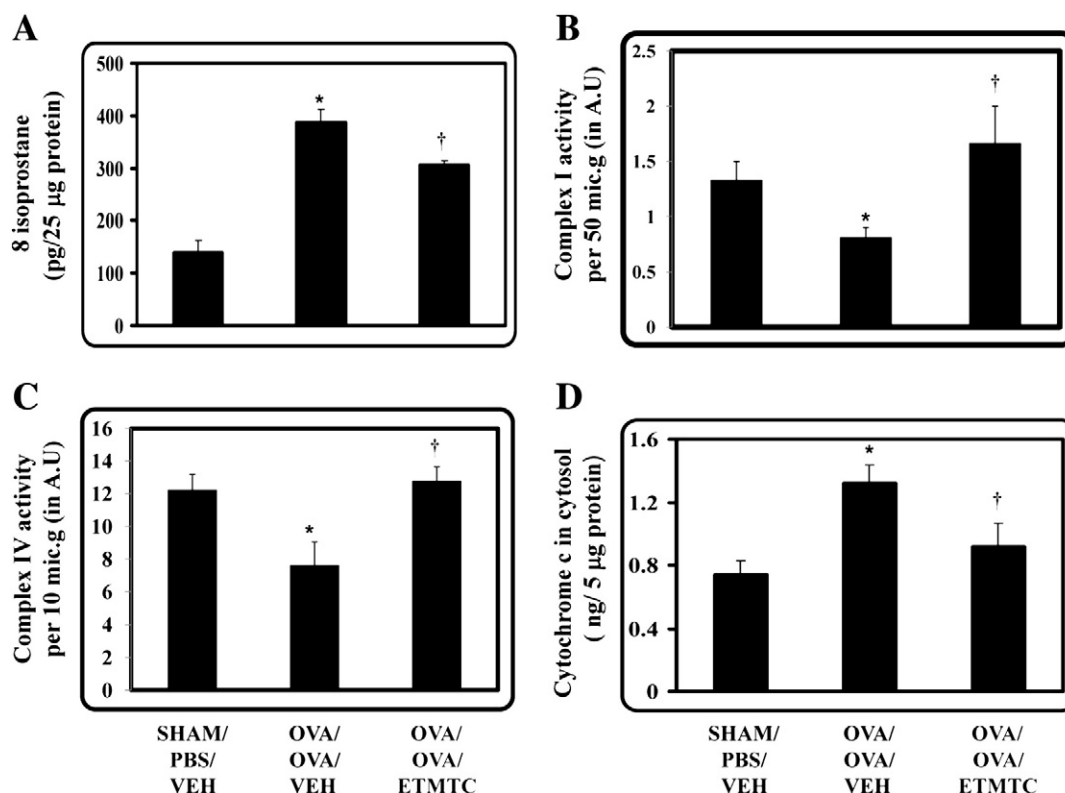


Fig. 5. ETMTC reduced lipid peroxidation and cytochrome c and restored activities of complexes I and IV. A) The levels of 8-isoprostane, a marker of lipid peroxidation, were measured in lung as described in [Materials and methods](#). Data were mean $\pm$ SEM of three separate experiments. B) Complex I and C) complex IV activities were determined in lung mitochondria. D) Cytochrome c levels were measured in cytosolic fractions of lung. * $P < 0.05$ vs. SHAM/PBS/VEH, † $P < 0.05$ vs. OVA/OVA/VEH group, $n = 6$ mice per group.

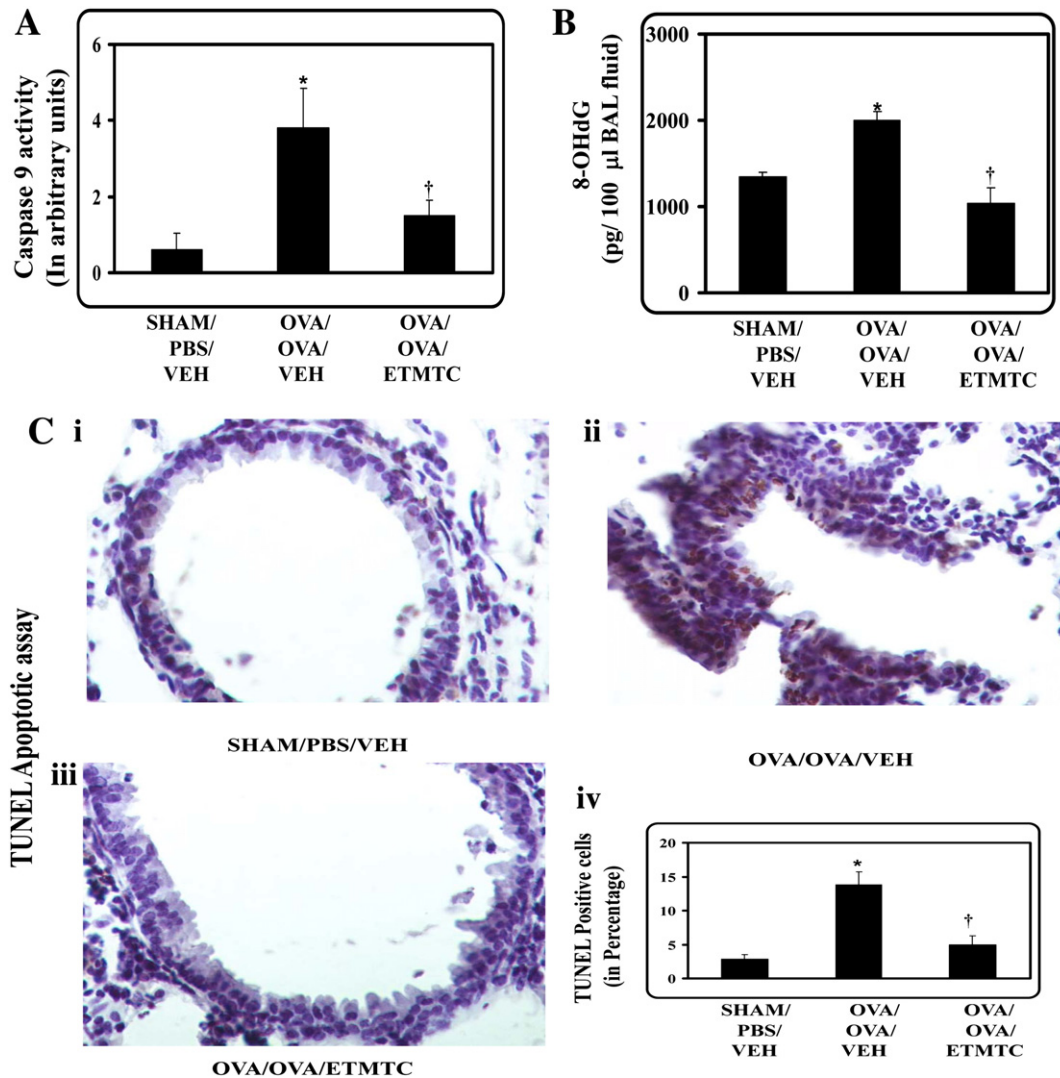


Fig. 6. ETMTC reduced caspase 9 activity in lung cytosol, DNA fragmentation in bronchial epithelial cells and oxidative DNA damage in lung. A) Caspase 9 activity was measured in lung cytosols. To determine the effect of ETMTC on DNA damage, B) 8-hydroxydeoxyguanosine (8-OHdG) in BAL fluid supernatants, C) TUNEL apoptosis assay (i–iii) and apoptotic index (iv) in lung tissue sections were performed. Brown color indicates the DNA fragmentation. Data were mean $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. SHAM/PBS/VEH, † $P < 0.05$ vs. OVA/OVA/VEH group, $n = 6$ mice per group. All images are at $20\times$ magnifications.

control mice. ETMTC treatment to asthmatic mice had significantly reduced goblet cell metaplasia. Quantitative morphometry also confirmed this (Fig. 7A, iv).

3.12. ETMTC reduces sub-epithelial fibrosis

To determine the effect of ETMTC on sub-epithelial fibrosis, Masson's Trichrome staining was performed. As shown in Fig. 7B, dense accumulation of collagen was found in sub-epithelial regions of bronchi and also around vascular regions compared to none in normal control mice. However, ETMTC administration to asthmatic mice had significantly reduced collagen deposition in the bronchovascular regions compared to control asthmatic mice. Quantitative morphometry also confirmed this (Fig. 7B, iv).

3.13. ETMTC restores 15-S-HETE levels

As 15-(S)-HETE is important in the suppression of sub-epithelial fibrosis, we next determined the possible effect of ETMTC on this by ELISA. As shown in Fig. 7C, OVA/OVA/VEH mice showed a

reduction in the levels of 15-(S)-HETE and ETMTC treatment significantly restored it (Fig. 7C).

4. Discussion

Asthma features are mainly due to the increase in Th2 cytokines and decrease in Th1 cytokines [2]. Epithelial injury is one of the end products of allergic airway inflammation which is predominantly mediated by Th2 cytokines such as IL-4, IL-5 and IL-13. IL-4 is known to activate NF- κ B in allergic lungs [21]. On the other hand, NF- κ B knockout mice not only couldn't produce Th2 cytokines but also abolish airway eosinophilia in allergic lung indicating the possible vicious cycle between Th2 cytokines and NF- κ B activation [5]. The observation of reduction in both Th2 cytokines and NF- κ B activation with ETMTC treatment in this study indicated that ETMTC is potent in breaking this vicious cycle. Similar blockade of NF- κ B pathway by non-steroidal small molecules has been considered as a potential strategy for management of various inflammatory diseases including asthma and allergic diseases [22].

NF- κ B, a pivotal transcription factor, stimulates the expression of a broad number of CAMs and cytokines to recruit and activate inflammatory cells in asthmatic airway [6]. Various mice and human studies have

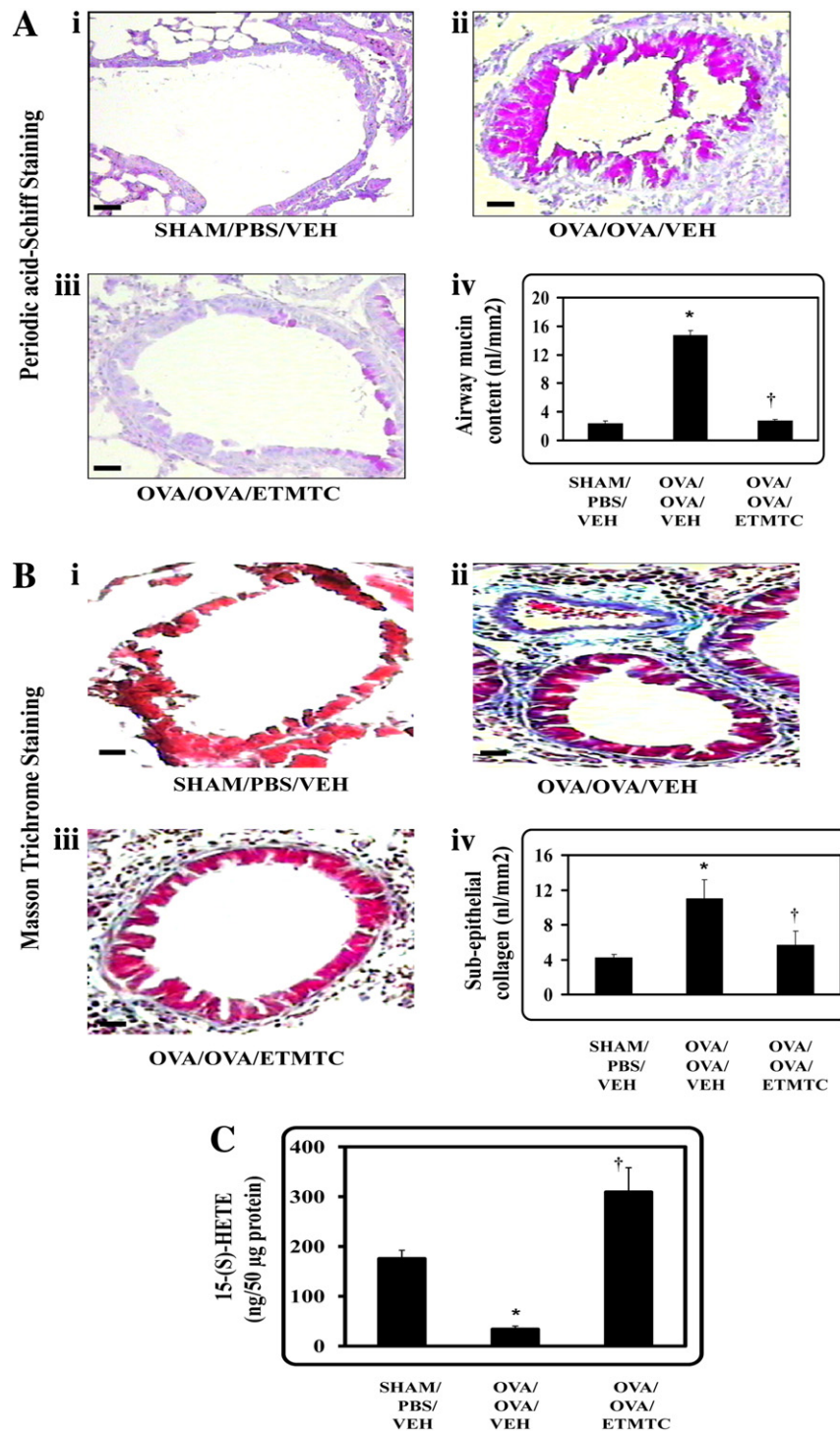


Fig. 7. ETMTC reduced airway remodeling changes. A) Periodic acid and B) Masson Trichrome stainings were performed in lung tissue sections to determine goblet cell metaplasia and sub-epithelial fibrosis, respectively. i) to iii) indicate different groups and morphometry (iv) was performed as described in [Materials and methods](#). * $P < 0.05$ vs. SHAM/PBS/VEH, $^{\dagger}P < 0.05$ vs. OVA/OVA/VEH group. Data were mean $\pm$ SEM of three separate experiments, $n = 6$ mice per group. Bars, 50 μm . C) The level of 15-(S)-HETE was determined by ELISA in lungs. Results are shown as mean $\pm$ SEM. * $P < 0.05$ vs. SHAM/PBS/VEH, $^{\dagger}P < 0.05$ vs. OVA/OVA/VEH. The data presented are representative of three separate experiments and $n = 6$ mice per group.

shown the up regulation in the expressions of E-selectin, ICAM-1 and VCAM-1, which involve in the infiltration of inflammatory cells to the endothelium, along with airway eosinophilia in allergic airway inflammation [23]. The inhibition of NF- κ B activation in ETMTC treated mouse lungs is interesting. However, the mechanism by which ETMTC inhibits NF- κ B in mice is not known. It is likely that ETMTC may act through the inhibition of I κ B α degradation. However, the

possibility of inhibition of IKK α kinase in non-canonical pathway of NF- κ B activation could not be ruled out [24].

Among various recruited inflammatory cells in asthmatic airway, Th2 cells are dominant with a reduction in Th1 cells and regulatory T cells (Treg) [25]. This cellular microenvironment leads to an increase in pro-inflammatory cytokines and decrease in anti-inflammatory cytokines such as IL-10 [26]. Recently Th17 cell have been considered to be a crucial

cell type in causing severe asthma phenotype as it leads to neutrophilia, mucus hypersecretion and steroid resistant phenotype [27]. Eosinophils cause maximum airway injury due to their toxic granular proteins such as eosinophil peroxidase, eosinophil cationic protein etc. In this study, ETMTC treatment reduced the expression of cell adhesion molecules with a concomitant decrease in the infiltration of cells including eosinophils. Importantly, ETMTC treatment reduced Th2 cytokines such as IL-4 and increased Th1 response with a decrease in IgG1 in sera. However, the effects of ETMTC on other immune cells such as Th17, Treg cells and their cytokines such as IL-17, IL-10 are yet to be investigated. In addition, cinnamate derivatives of *P. longum* are a potent inhibitor of TNF- α induced expression of cell adhesion molecules in human endothelial cells [15]. Previously, we have reported the structure–activity relationship studies of these analogs and discussed that position and number of methoxy group in the aromatic ring of the cinnamoyl moiety or replacement of oxygen with sulfur is critical for their biological activities [16]. It is important to note that ETMTC was 200 times better than its natural *P. longum* analog [16].

Recruited inflammatory cells in the airway release various oxidative free radicals to produce oxidative microenvironment in the lung. NF- κ B also induces oxidative stress; on the other hand, ROS directly activates NF- κ B by oxidizing its cysteine–SH groups indicating another vicious loop between NF- κ B and oxidative stress. In this study, we found that ETMTC treatment reduced oxidative stress along with the reduction in NF- κ B activation. Importantly, oxidative stress causes the epithelial injury due to the reduction of antioxidant defense of airway epithelium. This stress leads to mitochondrial structural alteration and its dysfunction [9]. In this study, ETMTC reversed key features of mitochondrial dysfunction such as decreased activities of complexes I and IV in lung mitochondria, increased levels of cytochrome c and caspase 9 activities in lung cytosols. In this context, it is interesting to note that NF- κ B activation also leads to mitochondrial dysfunction through oxidative stress [28,29]. In addition, 8-OHdG was found to be increased in BAL fluid supernatants along with DNA fragmentation in bronchial epithelial cell (Fig. 6B, C). Epithelial injury activates epithelial mesenchymal trophic unit to initiate airway remodeling changes such as goblet cell metaplasia, and subepithelial fibrosis. ETMTC treatment reduced these features of airway remodeling along with an increase in the levels of 15-S-HETE which protects epithelia by suppressing the myofibroblast transformation that is crucial in airway remodeling. Thus, the inhibition of oxidative stress, inhibition of NF- κ B activation, restoration of mitochondrial function, reduction of eotaxin levels, inhibition of Th2 cytokines etc. may be implicated in the mechanism of action of ETMTC in mice lungs. In conclusion, our findings reveal for the first time that ETMTC reduces epithelial injury and alleviates asthma features; and thus it could be useful in the development of better therapeutic strategy in allergic airway diseases.

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