

Esculetin Restores Mitochondrial Dysfunction and Reduces Allergic Asthma Features in Experimental Murine Model¹

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We recently showed that IL-4-dependent oxidative stress and mitochondrial dysfunction are associated with allergic asthma. IL-4 also induces a prooxidant enzyme, 15-lipoxygenase, which predominantly expresses in asthmatic bronchial epithelium and degrades mitochondria. Esculetin (6,7-dihydroxy-2H-1-benzopyran-2-one), a plant-derived coumarin and immunomodulator, was found to have potent bronchodilating property in carbachol-induced bronchoconstriction and also reduces mitochondrial dysfunction in neurological diseases. In this study, we evaluated its potential in restoring mitochondrial dysfunction and structural changes and anti-asthma property in a mouse model of experimental asthma. In this study, we found that esculetin treatment reduced airway hyperresponsiveness, Th2 response, lung eotaxin, bronchoalveolar lavage fluid eosinophilia, airway inflammation, and OVA-specific IgE. It also reduced the expression and metabolites of 15-lipoxygenase and lipid peroxidation which is an essential prerequisite for mitochondrial dysfunction. Interestingly, esculetin treatment restored the activity of cytochrome *c* oxidase of electron transport chain in lung mitochondria and expression of the third subunit of cytochrome *c* oxidase of electron transport chain in bronchial epithelium. It reduced the cytochrome *c* level and caspase 9 activity in lung cytosol and restored mitochondrial structural changes and lung ATP levels. In addition, esculetin reduced subepithelial fibrosis and TGF- β 1 levels in the lung. These results suggest that esculetin not only restores mitochondrial dysfunction and structural changes but also alleviates asthmatic features. *The Journal of Immunology*, 2009, 183: 0000–0000.

Asthma is a complex syndrome characterized by a variable degree of airflow obstruction, bronchial hyperresponsiveness, and airway inflammation (1). These features are commonly associated with increased IgE synthesis, airway eosinophilia, and repeated allergen exposures, leading to airway remodeling changes such as subepithelial airway fibrosis and goblet cell metaplasia (2). These pathophysiological features are due to an imbalance in the Th1/Th2 paradigm and the Th2 dominant response predominantly causes this imbalance. Increased Th2 cytokines lead to the recruitment and activation of inflammatory cells into the airway by systematic expression of various molecules such as eotaxin and adhesion molecules on both vascular endothelium and inflammatory cells (3). Recruited inflammatory cells injure the bronchial epithelium by local oxidative stress and the epithelial injury activates the epithelial mesenchymal trophic unit to initiate the airway remodeling changes (4, 5). Furthermore, oxygen free radicals alone are able to activate the epidermal growth factor receptor to develop goblet cell metaplasia (6). Hence, it seems that oxidative stress-induced epithelial injury is the bridge between airway inflammation and airway remodeling. Therefore, inhibition of oxidative stress is crucial to inhibit the

airway remodeling features and targeting oxidative mechanisms which causes epithelial injury could be a fruitful approach to inhibit the features of asthma as well as to develop efficient therapeutic strategies. Although other therapeutic strategies such as anti-cytokine and gene therapy seem to be exciting, existing effective therapies are derived from either natural substances or hormones (7). The role of mitochondria in this process has not been well studied, although they are very essential in the generation of endogenous reactive oxygen species. However, recent studies have shed some light in this aspect. For example, mitochondrial metabolism has been shown to be essential in the maturation of dendritic cells; mitochondrial dysfunction has been observed in experimental asthma (8).

Esculetin (6,7-dihydroxy-2H-1-benzopyran-2-one; ESC), is one of the potent coumarin-derived antioxidants. ESC reduces oxidative stress in various modes such as reduction of neutrophil-dependent superoxide anion generation (9), free radical scavenging, reduction of the lipid peroxidation (10), and so forth. It has been isolated from various plants such as *Artemisia capillaries*, *Citrus limonia*, and *Euphorbia lathyris*. An in vitro study suggests that ESC shows bronchodilating property on carbachol-induced airway spasm (11). However, its anti-asthma property in vivo has not yet been explored. ESC shows immunomodulatory effects which could be responsible for its various pharmacological properties such as antiviral and antitumor responses (12). Very importantly, ESC reduced the mitochondrial dysfunction to prevent the dopamine-induced neurotoxicity (13). 15-Lipoxygenase (LOX)³ and its

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³ Abbreviations used in this paper: LOX, lipoxygenase; AHR, airway hyperresponsiveness; COX_{ETC}, cytochrome *c* oxidase of electron transport chain; CYTO, cytosolic fraction; ESC, esculetin; HODE, hydroxyoctadecaenoic acid; HETE, hydroxy-eicosatetraenoic acid; L-OOH, lipid hydroperoxide; LOX, lipoxygenase; Penh, enhanced pause; sGAW, specific airway conductance; sRAW, specific airway resistance; VEH, vehicle; MCh, methacholine; SHAM, saline sensitized.

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metabolites, especially linoleic acid-derived metabolites such as 13-(*S*)-hydroxyoctadecaenoic acid (13-(*S*)-HODE), are known to cause mitochondrial degradation and they are increased both in human and murine asthmatic conditions (14–16). ESC has been shown to inhibit 15-LOX and reduce linoleic acid-induced oxidative stress (17) which is crucial in causing mitochondrial dysfunction (18). In humans, 15-LOX has two different forms: 15-LOX-1 and 15-LOX-2. 15-LOX-1, a human counterpart of murine 12/15-lipoxygenase (12/15-LOX), preferentially metabolizes linoleic acid to form HODEs such as 9 and 13-(*S*)-HODE and minimally metabolizes arachidonic acid to form hydroxyeicosatetraenoic acids (HETEs) such as 12 and 15-(*S*)-HETEs. In contrast, 15-LOX-2 preferentially metabolizes arachidonic acid into 15-(*S*)-HETE. In addition, IL-4 stimulates 15-LOX through acetylation of nuclear histones and STAT6 (19). With this view, we have hypothesized that ESC could have a role in restoring mitochondrial dysfunction and thus it may alleviate asthma features. To determine this, ESC was administered to asthmatic mice and its effects on key mitochondrial structural changes and functions were determined.

Materials and Methods

Animals

Male BALB/c mice (8–10 wk old) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for 1 week before starting the experiments. All animals were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines and protocols and were approved by the Institutional Animal Ethics Committee.

Grouping of mice

Mice were divided into three groups and each group ($n = 6$) was named according to sensitization/challenge/treatment: SHAM/PBS/vehicle (SHAM, saline sensitized; VEH; normal controls), OVA/OVA/VEH (asthmatic controls, OVA, chicken egg OVA grade V; Sigma-Aldrich), and OVA/OVA/ESC (Sigma-Aldrich). ESC was soluble in DMSO, a VEH.

Sensitization, challenge, and treatment of mice

Mice were sensitized and challenged as described earlier (8). Briefly, mice were administered three i.p. injections of 50 μ g of OVA in 4 mg of aluminum hydroxide (OVA groups such as OVA/OVA/VEH and OVA/OVA/ESC) or 4 mg of aluminum hydroxide (SHAM/PBS/VEH) on days 0, 7, and 14. Mice were challenged from days 21 to 32 (30 min/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). DMSO or ESC (1 mg/kg) was given orally from days 19 to 32 once a day in a 10- μ l volume per dose. We have selected this dose since it was an optimal effective dose in the reduction of airway hyperresponsiveness (AHR) among the different concentrations of ESC (0.1, 1, and 10 mg/kg) tested in our pilot experiments (data not shown). To determine the basal effects of ESC on lung function and key mitochondrial functions, unchallenged mice were administered either 1 mg/kg ESC (OVA/ESC) orally from days 19 to 32 once a day in a 10- μ l volume per dose or VEH (SHAM/VEH) ($n = 6$ each).

AHR measurement

AHR to methacholine (MCh; Sigma-Aldrich) was determined in unrestrained and restrained conscious mice by single- and double-chamber whole-body plethysmography, respectively (models PLY 3211 and PLY 3351; Buxco Electronics) as described earlier, with little modifications (8). Single-chamber plethysmography was performed for the estimation of enhanced pause (Penh) and double-chamber plethysmography was performed for the estimation of both specific airway resistance (sRAW) and specific airway conductance (sGAW). The mice were acclimatized to the plethysmographic chambers before OVA challenge. Final results for single-chamber plethysmography were expressed in percent baseline Penh with increasing concentrations of MCh while the PBS aerosol value was considered as baseline and results for sRAW and sGAW were expressed in percent baseline sRAW and sGAW respectively.

Bronchoalveolar lavage and sera separation

On day 33, each mouse was sacrificed, bronchoalveolar lavage was performed, and bronchoalveolar lavage fluids (BALF) were processed to sep-

arate cell pellets and supernatants as described earlier (20, 21). Total cell and differential counts were performed with resultant cell pellets to determine the absolute number of cells (8). Blood was withdrawn by cardiac puncture and serum was separated as described previously (20, 21).

Transmission electron microscopy and lung histopathology

Combined in situ whole-body perfusion and immersion fixation was performed as described earlier (8), and the fixed lungs were dissected under an Olympus SZX-12 dissection microscope to locate the first-generation bronchi and those were cut into many slices of the same thickness. Each slice was made into three blocks and they were further processed and the stained sections were viewed under a transmission electron microscope.

Formalin-fixed, paraffin-embedded lung tissue sections were stained with H&E, periodic acid-Schiff and Masson's trichrome to assess the airway inflammation, goblet cell metaplasia, and subepithelial fibrosis, respectively, and observed and microphotographs were taken with a Nikon microscope with camera (model YS-100). Quantitative morphometry to determine the airway mucin content was performed as described earlier (8).

Measurements of IL-4, IL-13, eotaxin, and TGF- β 1 in the lung

Lung tissue homogenates (prepared by homogenization of 50 mg of tissue with 500 μ l of PBS and centrifugation at $10,000 \times g$ for 30 min) in duplicate were used for IL-4 and TGF- β 1 ELISA (BD Pharmingen) and IL-13 and eotaxin ELISA (R&D Systems). Results are expressed in picograms and normalized by protein concentrations.

OVA-specific IgE, IgG1, and IgG2a measurement

This was done as described earlier (8), with modifications. Briefly, each well of the microtiter plate was coated with 2 μ g of OVA (chicken egg OVA grade V; Sigma-Aldrich) in a 100- μ l volume overnight at 4°C. After blocking and sera adding, bound IgE or IgGs were detected with biotinylated anti-mouse IgE, anti-mouse IgG1, or anti-mouse IgG2a and streptavidin-HRP conjugates (BD Pharmingen). The absorbances were read at 450 nm and they were converted to arbitrary units (AU).

Isolation of whole lung mitochondria and cytosolic separation

After sacrifice, the lung portion below the trachea was removed and processed to separate mitochondrial and cytosolic fractions (CYTO) as described previously (8) and protein estimation was done in those fractions by the bicinchoninic acid (Sigma-Aldrich) assay.

15-LO Western blot

CYTO proteins were separated on 8% SDS-polyacrylamide gel, transferred onto polyvinylidene fluoride membranes (Millipore) which were blocked with blocking buffer (3% skim milk), incubated with a 1/100 dilution of the Ab which was cross-reactive also with 12-LO isoforms (Santa Cruz Biotechnology) and detected with HRP-conjugated anti-mouse secondary Ab and diaminobenzidine- H_2O_2 . α -tubulin was used as a loading control.

Measurements of 9-(*S*)-HODE, 13-(*S*)-HODE, and 12-(*S*)-HETE in CYTO

One hundred micrograms of cytosolic protein per well in duplicate was used for ELISA of 9-(*S*)-HODE (Oxford Biomedical Research), 13-(*S*)-HODE, and 12-(*S*)-HETE (Assay Designs). Measurements were done as per the manufacturer's instructions without extraction.

Lipid hydroperoxide (L-OOH) and 8-isoprostane assay

Lipid peroxidation was determined in lung tissues by measuring L-OOH with a L-OOH assay kit (Cayman Chemical) as described earlier (8). 8-Isoprostane was measured in lung homogenates and the results are expressed in pg/25 μ g of protein. Briefly, 50 mg of lung tissue was homogenized as per the manufacturer's instructions (Cayman Chemical) and centrifuged at $600 \times g$ at 4°C and supernatants were further centrifuged at $10,000 \times g$ for 30 min at 4°C. Resultant supernatants were used for 8-isoprostane measurements by competitive ELISA.

Total cytochrome c oxidase activity measurement

Cytochrome c oxidase of electron transport chain (COX_{ETC}) activity and total citrate synthase activity were measured and calculated as described earlier (8). Briefly, COX_{ETC} activity was measured based on the oxidation of ferrocytochrome c to ferricytochrome c by COX_{ETC} present in the mitochondria treated with *n*-dodecyl- β -D-maltoside. Total citrate synthase activity was measured by the hydrolysis of acetyl CoA to thiols which reacts with 5',5'-dithio-bis-(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoic

acid and the ratio between total COX_{ETC} and net citrate synthase activities was calculated.

Immunohistochemistry

Commercial goat polyclonal Ab for COX_{ETC} subunit III (Santa Cruz Biotechnology) and anti-goat HRP-conjugated secondary Ab (Sigma-Aldrich) were used for immunohistochemistry (8). Negative control experiments were performed by using either γ -globulin as isotype control (Jackson ImmunoResearch Laboratories) or omission of primary Ab.

Cytochrome *c* estimation

The levels of cytochrome *c* were measured in CYTO and BALF supernatants by ELISA as per the manufacturer's instructions (R&D Systems). Briefly, 5 μ g of CYTO protein or 100 μ l of BALF supernatants per well in duplicate was used to measure cytochrome *c* with a rat/mouse cytochrome *c* enzyme immunoassay kit.

ATP and caspase 9 activity assays

ATP measurement was done with an ATP bioluminescence assay kit as described earlier (8) and caspase 9 activity was estimated (R&D Systems) in CYTO using caspase 9-specific peptide (Lou-Glu-His-Asp) conjugated to the color reporter molecule *p*-nitroaniline. The cleavage of the peptide by the caspase releases the chromophore *p*-nitroaniline, which was measured at 405 nm and expressed in AU.

Statistical analysis

Data are expressed as means $\pm$ SEM. Since we conducted hypothesis-driven comparisons between selected groups, we used the unpaired Student *t* test and statistical significance was set at $p \leq 0.05$.

Results

ESC reduces AHR

AHR was measured after allergen challenge with increasing concentrations of MCh (Fig. 1). The percentage of baseline Penh and sRAW was increased significantly with MCh in a dose-dependent manner in asthmatic controls (OVA/OVA/VEH) compared with normal control mice (SHAM/PBS/VEH) (Fig. 1, A and B). On the other hand, the percentage of baseline sGAW was decreased significantly in asthmatic controls compared with normal controls (Fig. 1C). However, treatment with ESC (OVA/OVA/ESC) attenuated the increase in the percentage of baseline Penh and sRAW and the decrease in sGAW compared with control asthmatic mice (Fig. 1, A–C).

ESC reduces airway inflammation, goblet cell metaplasia, lung eotaxin, and BALF eosinophilia

Histopathological analysis of formalin-fixed, paraffin-embedded lung sections was performed and the following features were observed (Fig. 2, A and B): 1) SHAM/PBS/VEH mice showed normal architecture of the lung and no goblet cell metaplasia; 2) OVA/OVA/VEH mice showed dense infiltration of inflammatory cells including eosinophils, mononuclear cells such as monocytes and lymphocytes, neutrophils in perivascular and peribronchial regions but alveolar regions were free from any infiltrated cells and also showed a significant goblet cell metaplasia (airway mucin content: mean $\pm$ SEM; OVA/OVA/VEH vs SHAM/PBS/VEH: 12.9 ± 0.5 vs 1.2 ± 0.1 , $p < 0.05$); 3) OVA/OVA/ESC showed the reduction in both perivascular and peribronchial inflammation and also goblet cell metaplasia (airway mucin content: mean $\pm$ SEM; 7.5 ± 0.7 , $p < 0.05$ compared with OVA/OVA/VEH).

Since eosinophil migration is one of the crucial events in asthmatic airway inflammation and eotaxin plays a significant role in this process, we measured eotaxin levels in the lung homogenates. As shown in Table I, eotaxin levels were significantly higher in OVA/OVA/VEH mice compared with SHAM/PBS/VEH. Interestingly, ESC treatment reduced the eotaxin levels significantly compared with OVA/OVA/VEH mice. Also,

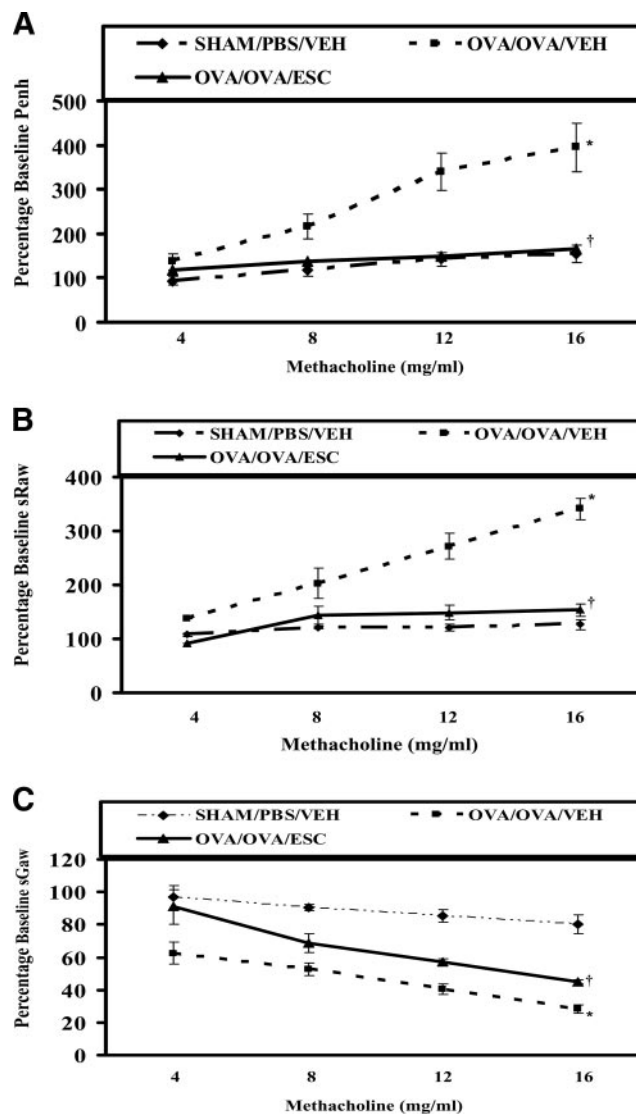


FIGURE 1. ESC treatment reduced AHR in asthmatic mice. Percentage baseline Penh (A), sRAW (B), and sGAW (C) indicators of bronchoconstriction were determined for the three groups of mice: SHAM/PBS/VEH (saline sensitized and saline challenged and orally administered VEH), OVA/OVA/VEH (OVA sensitized and challenged and orally administered VEH), and OVA/OVA/ESC (OVA sensitized and challenged and orally administered ESC). Each mouse was challenged with increasing concentrations of MCh (0–16 mg/ml) and the final results for single-chamber plethysmography are expressed in percent baseline Penh; double-chamber plethysmography results are expressed in percent baseline sGAW and sRAW, respectively, while the PBS aerosol value was considered as baseline. Values plotted are mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH.

the absolute number of cells was determined in the BALF from total cell count and differential cell count (Fig. 2C). The distribution of inflammatory cells like eosinophils, neutrophils, mononuclear cells (monocytes and lymphocytes), and macrophages were significantly increased in OVA/OVA/VEH mice. However, ESC treatment caused a significant reduction in recruitment of these inflammatory cells.

ESC reduces Th2 cytokines and OVA-specific IgE

To determine the effects of ESC on Th2 cytokines, we measured the levels of IL-4 and IL-13 in lung tissue. As shown in Table I, the

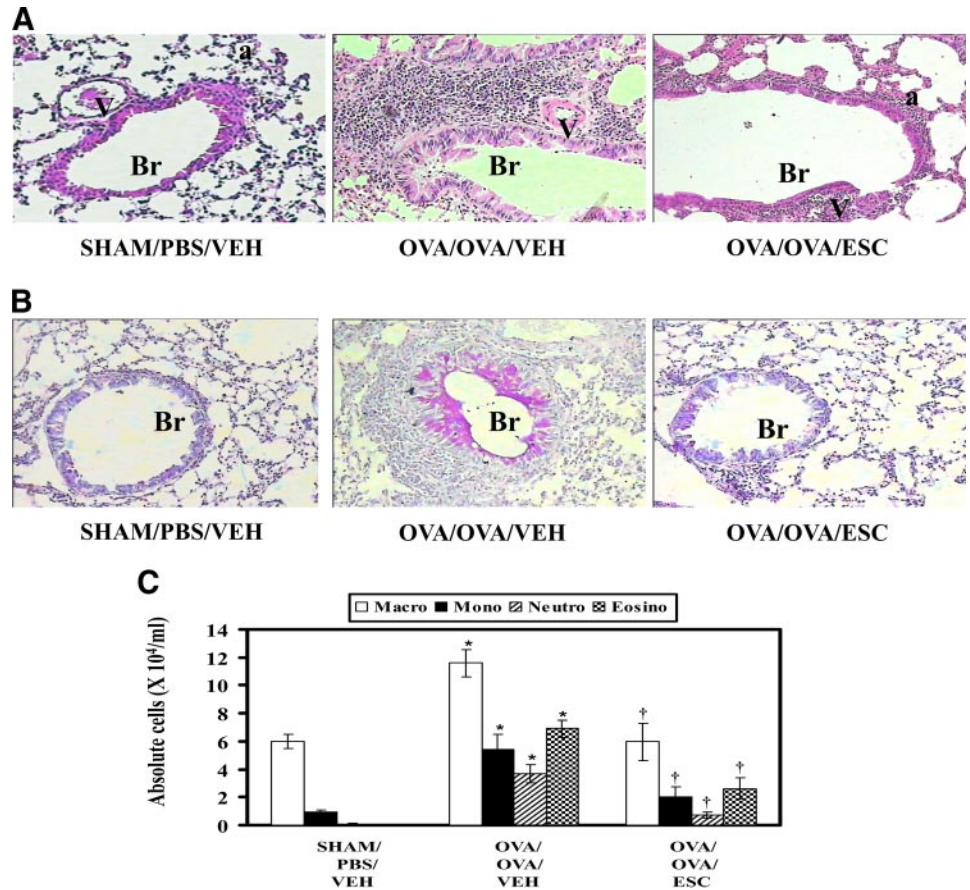


FIGURE 2. ESC treatment reduced airway inflammation and goblet cell metaplasia. *A* and *B*, Representative photographs of H&E and periodic acid-Schiff stainings were shown. Br, Bronchus; V, vessel; a, alveolus. All photographs were at $\times 10$ magnification. *C*, Absolute number of macrophages (Macro), mononuclear agranulocytes (Mono), neutrophils (Neutro), and eosinophils (Eosino) in BALF were determined from total cell count and differential cell count. Values plotted are mean $\pm$ SEM. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH.

levels of both IL-4 and IL-13 were increased in OVA/OVA/VEH compared with SHAM/PBS/VEH mice. However, treatment with ESC showed a significant reduction of both the cytokines.

To determine the effects of ESC on OVA-specific Igs, we measured IgE, IgG1, and IgG2a in sera as described in *Materials and Methods*. As shown in Table I, the levels of OVA-specific IgE and IgG2a were increased in OVA/OVA/VEH compared with SHAM/PBS/VEH mice. ESC treatment produced a significant reduction of OVA-specific IgE and further increased IgG2a (Table I). However, there was no effect on IgG1 levels with ESC treatment, although it was increased in asthmatic controls (data not shown).

ESC reduces 15-LOX expression, its metabolites, and lipid peroxidation

To determine the effect of ESC on the expression of 15-LOX in this study, Western blot analysis was done in CYTO. As shown in Fig. 3A, 15-LOX was almost absent in SHAM/PBS/VEH mice. In contrast, OVA/OVA/VEH showed a significant increase in its expression. Interestingly, ESC treatment of asthmatic mice showed a significant reduction of 15-LOX.

To determine the effect of ESC on the metabolites of 15-LOX, we measured the levels of 13-(*S*)-HODE, 9-(*S*)-HODE, and 12-(*S*)-HETE by ELISA. As shown in Fig. 3, C–E, all of these metabolites were significantly increased in OVA/OVA/VEH compared with SHAM/PBS/VEH mice. ESC treatment reduced the levels of these metabolites significantly.

Because 15-LOX is one of the potent prooxidant enzymes and oxidative stress is a definite consequence of airway inflammation, L-OOH and 8-isoprostane were estimated in the lung. As shown in Fig. 4, L-OOH and 8-isoprostane levels were found to be significantly increased in OVA/OVA/VEH mice compared with SHAM/PBS/VEH mice. However, ESC treatment significantly reduced both of them (Fig. 4).

ESC restored the activity of COX_{ETC} and the expression of COX_{ETC} subunit III

Since lipid peroxidation is a prior event for causing mitochondrial dysfunction and also 15-LOX itself causes lipid peroxidation and mitochondrial degradation, we measured COX_{ETC} activity and it was normalized by respective citrate synthase activity. The results

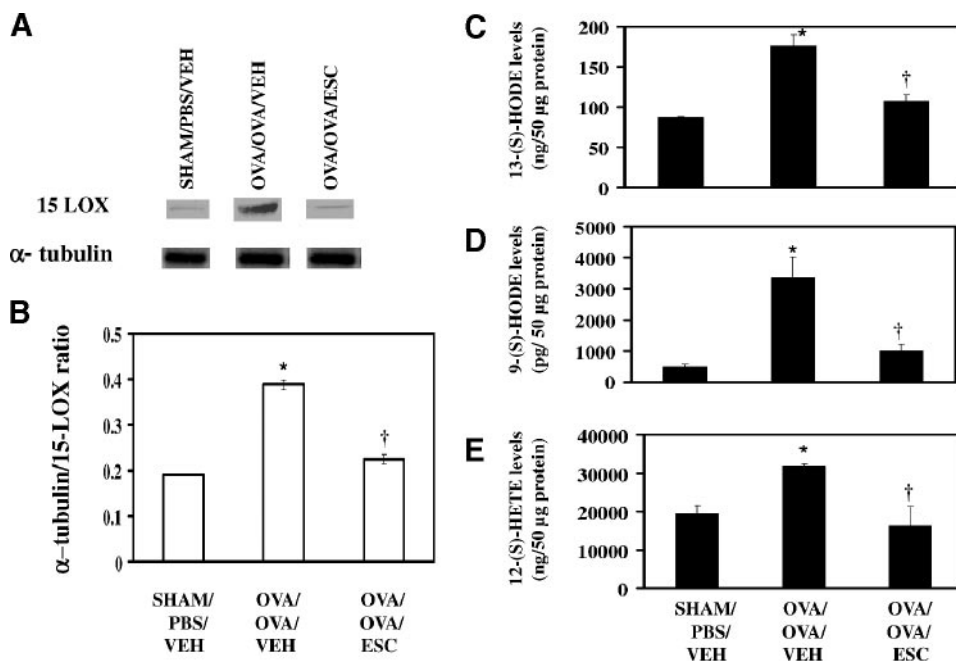
Table I. Effects of ESC on IL-4, IL-13, OVA-specific IgE, IgG2a, and eotaxin levels^a

	IL-4 (pg/100 μ g protein)	IL-13 (pg/100 μ g protein)	OVA-Specific IgE (AU)	OVA-Specific IgG2a (AU)	Eotaxin (pg/25 μ g protein)
SHAM/PBS/VEH	65.6 $\pm$ 5.0	8.0 $\pm$ 1.1	1.1 $\pm$ 0.06	1.7 $\pm$ 0.5	19.4 $\pm$ 7.6
OVA/OVA/VEH	128.7 $\pm$ 15.1*	50.6 $\pm$ 7.6*	2.1 $\pm$ 0.06*	4.9 $\pm$ 0.5*	50.2 $\pm$ 3.9*
OVA/OVA/ESC	83.7 $\pm$ 7.1†	28.1 $\pm$ 0.8†	1.4 $\pm$ 0.23†	6.3 $\pm$ 0.9†	33.2 $\pm$ 6.7†

^a Data are mean $\pm$ SEM of three independent experiments.

*, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH group.

FIGURE 3. ESC treatment reduced the expression and levels of 15-LOX metabolites. After sacrifice, lungs were harvested, CYTO were separated, and Western blot was performed for 15-LOX and α -tubulin (A). Signals were estimated with spot densitometry and divided by respective α -tubulin signals (B). Levels of 15-LOX metabolites such as 13-(S)-HODE (C), 9-(S)-HODE (D), and 12-(S)-HETE (E) were estimated in CYTO. Data are the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH.



indicated that the COX_{ETC}/citrate synthase was decreased in OVA/OVA/VEH mice compared with SHAM/PBS/VEH (Fig. 5A). Interestingly, ESC treatment significantly restored the activity of COX_{ETC}.

To examine the effect of ESC on the expression of subunit III of COX_{ETC}, which is specifically affected by oxidative insults, immunohistochemistry was performed as described in *Materials and Methods*. As shown in Fig. 5B, subunit III expression was found to be predominantly expressed in bronchi of SHAM/PBS/VEH mice and it was found to be significantly decreased in OVA/OVA/VEH mice. On the other hand, ESC treatment significantly restored the expression of COX_{ETC} subunit III.

ESC reduces the appearance of cytochrome *c* in cytosol and BALF supernatants

Since COX_{ETC} activity was found to be reduced in asthmatic mice lung, we further analyzed the cytochrome *c* levels by ELISA both in cytosol and BALF supernatants. As shown in Fig. 5, C and D, the levels of cytochrome *c* were significantly increased in OVA/OVA/VEH mice compared with SHAM/PBS/VEH mice. Interestingly, treatment of asthmatic mice with ESC demonstrated a significant reduction of cytochrome *c* in the cytosol and BALF supernatants (Fig. 5, C and D).

ESC restores mitochondrial ultrastructural changes

To evaluate the morphological changes of the mitochondria in bronchial epithelium, we performed transmission electron microscopy of the lung samples. As shown in Fig. 6A, maximal mitochondrial damage was observed in OVA/OVA/VEH mice in the form of loss or disruption of cristae, swelling, and thinned outer membrane. Interestingly, when mitochondrial morphology was evaluated after ESC treatment, it significantly restored the mitochondrial structural changes.

ESC reduces caspase 9 levels in the lung

As we observed the reduction in COX_{ETC} activity and cytochrome *c* release in cytosol, we assessed the status of caspase 9 activity in lung cytosol. Interestingly caspase 9 activity was found to be increased in OVA/OVA/VEH mice compared with SHAM/PBS/VEH mice (Fig. 6B). However, ESC treatment significantly reversed caspase 9 activity (Fig. 6B).

ESC increases ATP levels in the lung

To evaluate the effect of ESC on one of the vital functions of mitochondria, we measured ATP levels in lung tissue. As shown in Fig. 6C, ATP levels were found to be decreased in the lungs of

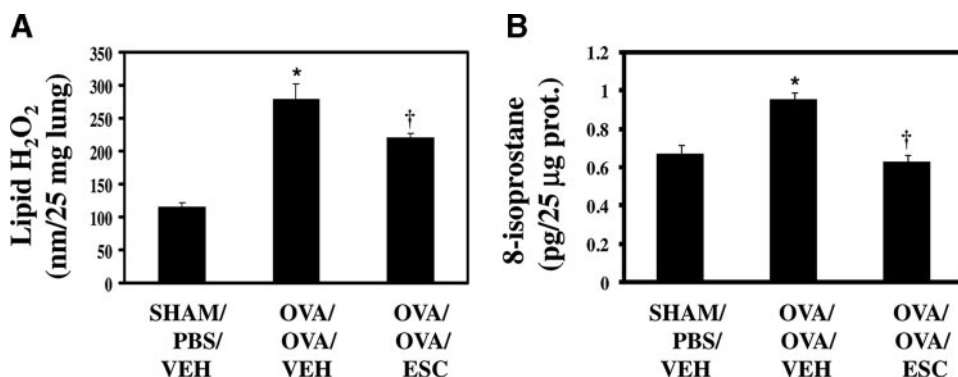
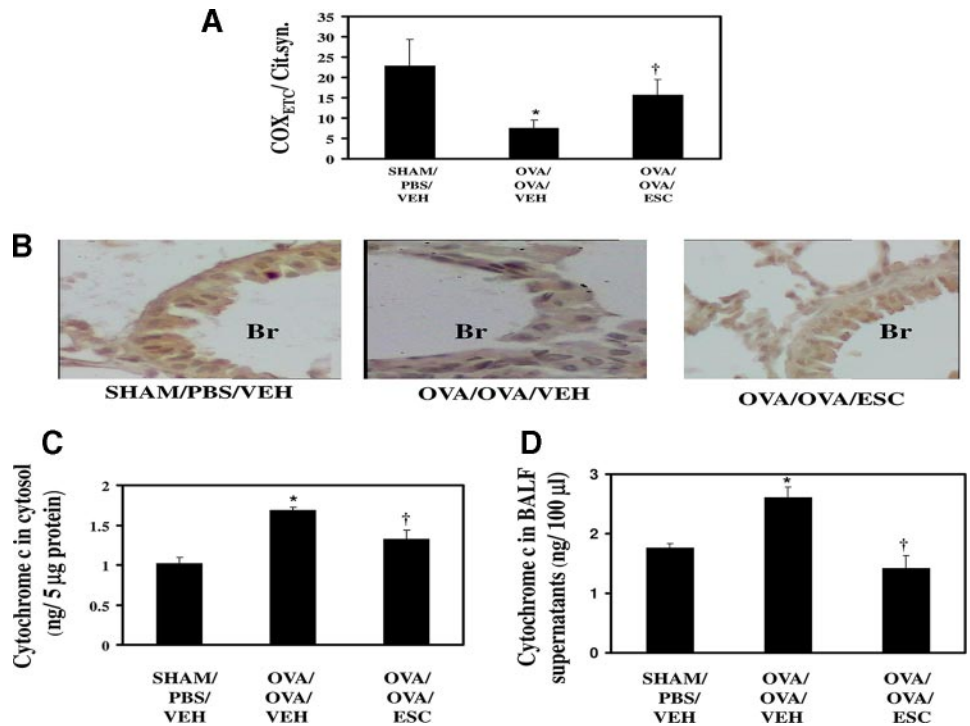


FIGURE 4. ESC treatment reduced L-OOH (A) and 8-isoprostane (B) levels in the lung. After sacrifice of mice, the levels of L-OOH and 8-isoprostane were measured in lung tissue and lung homogenates, respectively. Data are the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH.

FIGURE 5. ESC treatment restored the activity of COX_{ETC} and expression in the third subunit of COX_{ETC}. **A**, After sacrifice, mitochondria were isolated from fresh lung tissue; COX_{ETC} activity was estimated and normalized by dividing with respective citrate synthase (Cit.syn.) activity. Data are the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH. **B**, Immunohistochemistry was performed for subunit III of cytochrome *c* oxidase in lung tissue sections. Brown color indicates the positive expression of the respective subunit. Br, Bronchus. Representative photomicrographs from three independent experiments are shown here in $\times 40$ magnification. Cytochrome *c* levels were measured in CYTO (**C**) and BALF supernatants (**D**) by ELISA. Data are the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH.



OVA/OVA/VEH mice compared with SHAM/PBS/VEH mice. Interestingly, a significant reversal of ATP levels was found with ESC treatment (Fig. 6C).

ESC does not affect mitochondrial functions and lung function in unchallenged mice

To determine the basal effects of ESC on mitochondrial functions and lung function, unchallenged mice were administered either ESC or VEH. There was no significant difference between ESC or VEH administered mice either in key mitochondrial functions (VEH administered vs ESC administered, mean $\pm$ SEM; COX_{ETC}: citrate synthase ratio: 26.8 ± 1.8 vs 24.7 ± 1.9 , $p = 0.5$; ATP levels (in relative light units): 4898.1 ± 119.6 vs 5230.1 ± 230.5 , $p = 0.19$) or in MCh PC₂₀₀ Penh values (provocative concentration of MCh which increases the Penh to 200% of the baseline

value, in mg/ml, VEH administered vs ESC administered, mean $\pm$ SEM; 15.4 ± 0.4 vs 14.5 ± 0.9 , $p = 0.37$).

ESC reduces subepithelial fibrosis and TGF- β 1

Because mitochondrial dysfunction is associated with fibrosis and subepithelial fibrosis is one of the critical components of airway remodeling, Masson's trichrome staining was performed. As shown in Fig. 7A, dense accumulation of collagen was found in control asthmatic mice, especially in the subepithelial regions of bronchi and also around vascular regions compared with tiny deposition in normal controls. ESC administration to asthmatic mice significantly reduced the collagen deposition in the bronchovascular regions compared with control asthmatic mice (Fig. 7A).

Because TGF- β 1 is the crucial growth factor in subepithelial fibrosis and it has a functional cross-talk with 15-LOX, its levels

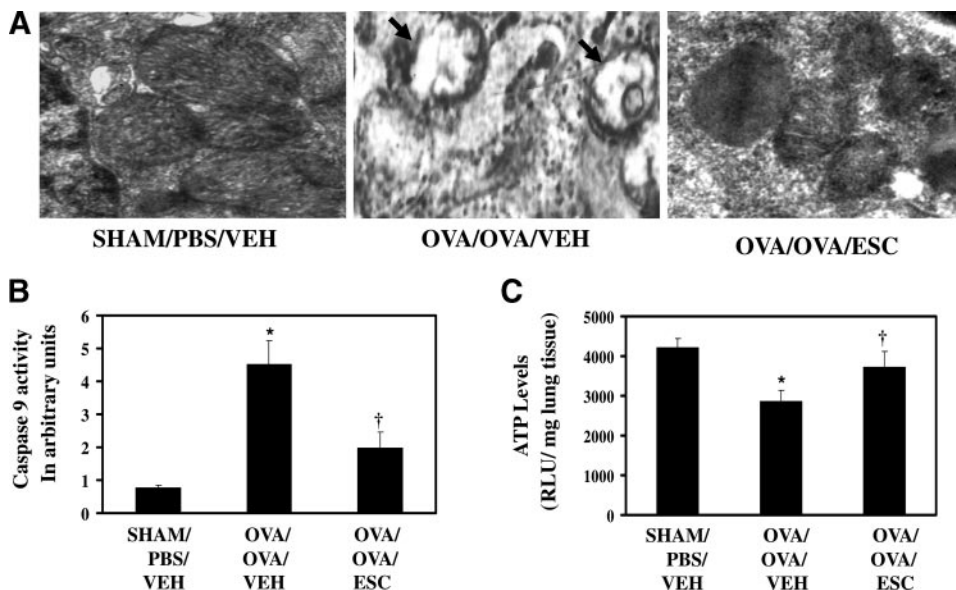


FIGURE 6. ESC treatment restored ultrastructural changes of mitochondria, caspase 9, and lung ATP levels. **A**, To determine the morphological changes of the mitochondria with ESC treatment, transmission electron microscopy was performed with first-generation bronchi. Randomly 50 epithelial cells were visualized and a representative photograph is from each group. Black arrow indicates the swelled mitochondria. **B**, Caspase 9 activity and ATP (**C**) were measured in lung cytosol and lungs, respectively. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH. Data are the mean $\pm$ SEM of three independent experiments.

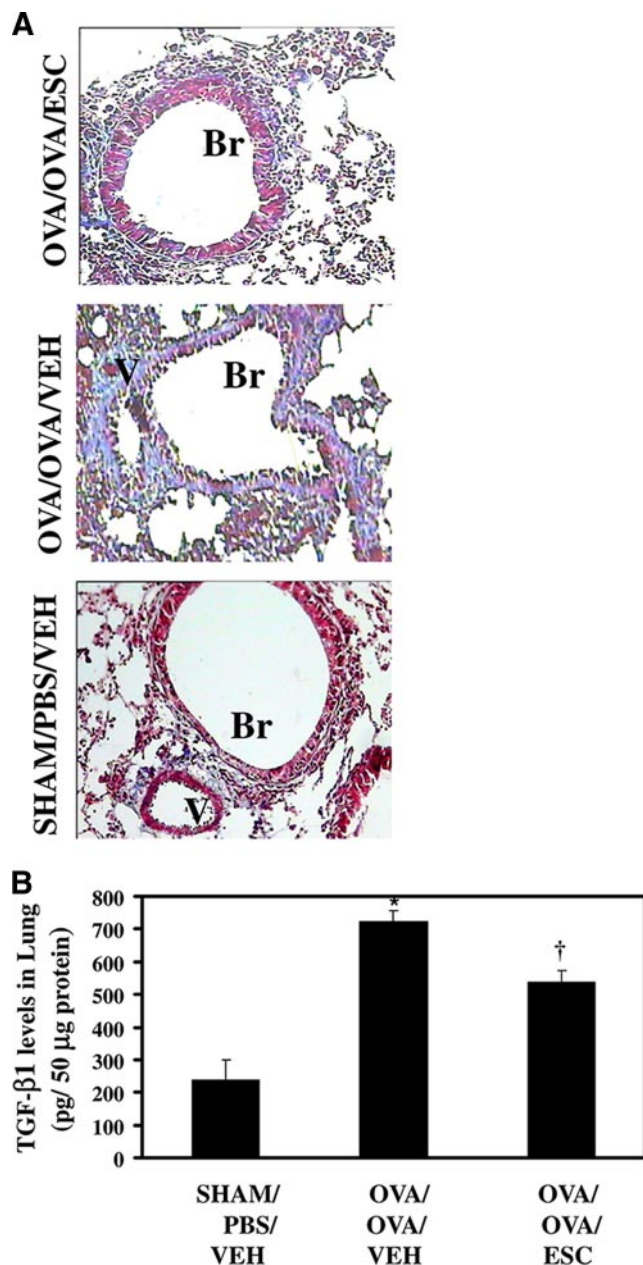


FIGURE 7. ESC treatment reduced subepithelial fibrosis and TGF- β 1 levels. **A**, To determine the effect of ESC on subepithelial fibrosis Masson's trichrome (A) staining was performed in lung tissue sections. Br, Bronchus; V, vessel. All photographs were at $\times 10$ magnification. **B**, TGF- β 1 levels were determined in lung tissue homogenates by ELISA. Data are the mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs SHAM/PBS/VEH and †, $p < 0.05$ vs OVA/OVA/VEH group.

were determined in lung tissue homogenates by ELISA. As shown in Fig. 7B, TGF- β 1 levels were markedly increased in asthmatic controls compared with normal controls. However, ESC treatment reduced its levels significantly (Fig. 7B).

Discussion

Recent evidences indicate that oxidative stress involving mitochondrial degeneration and dysfunction is important in airway inflammation and airway remodeling (8). Thus, it is hypothesized that inhibition of such processes could be useful in developing therapeutic modalities for asthma. In this study, we

have evaluated the potency of ESC, a coumarin antioxidant, in reducing mitochondrial dysfunction and alleviating asthma features.

In this study, we observed that ESC was not only capable of reducing IL-4, IL-13, OVA-specific IgE, and eotaxin levels, but also significantly able to reduce the infiltration of various inflammatory cells such as macrophages, eosinophils, mononuclear granulocytes like lymphocytes and monocytes, and neutrophils into the airway and also goblet cell metaplasia (Table I and Fig. 2). Earlier studies showed that ESC suppressed the production of pro-matrix metalloproteinase 1/interstitial procollagenase and pro-matrix metalloproteinase 3/prostromelysin 1 which are required for the migration and recruitment of inflammatory cells (22). Inhibition of these processes could be attributable to the reduction of airway inflammation and AHR by ESC. It is also noted that these reductions were comparable to asthmatic mice treated with dexamethasone (data not shown). Also, ESC is a nontoxic molecule ($LD_{50} = 2000$ mg/kg based on mouse acute toxicity tests (23)) and it was shown to reduce the liver toxicity induced by paracetamol and carbon tetrachloride (24).

The oxidative stress is an afferent and efferent component of airway inflammation. Oxidative free radicals directly oxidize various macromolecules including lipid (25). Lipid peroxidation is an essential prior component for mitochondrial dysfunction (8). Among various oxidative metabolites of lipids, 8-isoprostanes are found to be reliable oxidative and stable lipid peroxides (25). Oxidative stress also favors various lipoxygenases, particularly 15-LOX, which is a prooxidant enzyme and importantly inducible by IL-4 and IL-13. 15-LOX has been shown to be increased in asthmatic patients and it is predominantly expressed in bronchial epithelial cells (15). Among two forms of 15-LOX, 15-LOX-1 is mainly up-regulated in airway inflammation compared with 15-LOX-2 (15, 26). In addition, the deficiency of murine 12/15-LOX, a human counterpart of 15-LOX-1, alleviates the features of asthma (27, 28). Importantly, 15-LOX-1 is a crucial enzyme in causing mitochondrial degradation in reticulocytes by directly oxygenating esterified polyenoic fatty acids of membranes, including mitochondrial membranes, without prior help of phospholipase A₂ (29). These indicate that linoleic acid metabolism of 12/15-LOX is crucial both in asthma pathogenesis and mitochondrial degradation. In addition, ESC is known for its property of reducing linoleic acid-induced oxidative stress (17). In this study, ESC potentially reduces 12/15-LOX expression and its metabolites such as 9 and 13-(S)-HODE and 12-(S)-HETE in the lung cytosol, L-OOH, and 8-isoprostane (Figs. 3 and 4). These properties of ESC might have decreased the oxygenation of mitochondrial membrane and may account for its activity in restoring mitochondrial dysfunction. It is also important to note that since ESC is a known competitive non-specific inhibitor of 15-LOX (23); its antiasthmatic activities could be due to various other mechanisms not studied here. In addition, 15-LOX also produces lipoxins such as LXA4. 15(S)-HETE and lipoxins are reported to have paradoxical pro- and anti-inflammatory effects (15, 30). Various ex vivo studies have shown their anti-inflammatory properties (15, 30). However, in vivo studies related to bronchodilation of 15-(S)-HETE are controversial (15). Interestingly, available reports suggest that both metabolites are high in mild asthmatic patients (15). Severe asthmatics showed a deficiency in LXA4 synthesis; however very little is known about 15(S)-HETE in this context (15). These data indicate the necessity of further studies to explore the exact role of various 15-LOX metabolites in asthma. Similarly, further investigations are required to find the effects of ESC on 15-(S)-HETE and lipoxins.

COX_{ETC} is the terminal enzyme in the mitochondrial electron transport chain and it is inhibited by NO, peroxynitrite, and carbon monoxide which have been shown to be increased in asthma (8). The decrease of COX_{ETC} activity has also been reported in Alzheimer disease and the initial stages of diabetes mellitus (31–33). Lymphocytes isolated from smokers were also found to have reduced COX_{ETC} activity (34). This enzyme has 13 subunits: 3 are coded by mitochondria and 10 by the nucleus. The activity of COX_{ETC} is mainly due to mitochondrially coded catalytic subunits (I, II, and III). Also, earlier reports, including ours, suggest that subunit III of COX_{ETC} is mainly affected by oxidative insults including asthma (35, 8). In this study, ESC treatment restored both the COX_{ETC} activity and expression of subunit III (Fig. 5, A and B). Reduction in the activity of COX_{ETC} is mostly associated with the release of cytochrome *c* in the cytosol to initiate apoptosis by activating caspase 9 (36). We also found that decreased COX_{ETC} is correlated not only with the appearance of cytochrome *c* in lung cytosol but also in the increased activity of caspase 9 (Figs. 5C and 6B). It is important to note that ESC treatment reduced both the cytochrome *c* and caspase 9 activities in cytosol. Furthermore, ESC treatment restores mitochondrial ultrastructure and ATP levels in the lung (Fig. 6, A and C). However, ESC treatment to unchallenged mice did not affect key mitochondrial functions and lung function.

Various studies have shown that mitochondrial damage is associated with fibrosis although the reasons are not clear (37, 38). Also, 15-LOX has a functional cross-talk with TGF- β 1 (39). Since ESC reduces mitochondrial dysfunction and 15-LOX, we further checked its effect on subepithelial fibrosis, one of the crucial components of airway remodeling. ESC treatment not only reduces subepithelial fibrosis, it also reduces TGF- β 1 levels in the lung (Fig. 7). ESC inhibits neoangiogenesis, a feature of airway remodeling, by various mechanisms such as the inhibition of the vascular smooth muscle cell proliferation and inhibition of the activation of signaling molecules like p42/44 MAPK, NF- κ B, AP-1, PI3K, and also Ras (40). It also reduced leukotriene B₄ secretion (41) which has an important role in airway remodeling by increasing the collagen accumulation (42). 15-LOX-1 is also known to induce MUC5AC, which is the marker of goblet metaplasia (43) that was reduced by ESC (Fig. 2B). The reduction of airway remodeling by ESC in this study might be due to these mechanisms. Taken together, these results suggest that ESC attenuates asthma features and it is associated with restoration of the mitochondrial dysfunctions and structural changes, and thus it could be useful in the development of future safer and efficient asthma therapeutics.

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Disclosures

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