



L-Arginine reduces mitochondrial dysfunction and airway injury in murine allergic airway inflammation

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ABSTRACT

Bronchial epithelial injury is the hall mark of asthma which is a chronic airway inflammatory disease. We have shown the mitochondrial ultrastructural changes and dysfunction in bronchial epithelia of OVA induced mice. Reduced L-arginine bioavailability in asthma leads to increased formation of peroxynitrite which could induce mitochondrial dysfunction. We have also shown that L-arginine administration attenuates experimental asthma and reduces peroxynitrite. In this study, we wanted to determine the effect of L-arginine on mitochondrial dysfunction and airway injury in allergic airway inflammation. To determine this, L-arginine was administered to ovalbumin sensitized and challenged mice during allergen challenges. Mitochondrial and cytosolic fractions were purified from the lung to determine key mitochondrial functions, and mitochondrial ultrastructural changes in bronchial epithelia of first generation bronchi were determined. It was found that L-arginine administration increased mitochondrial cytochrome c oxidase activity, reduced cytosolic cytochrome c, increased lung ATP levels, reduced DNA fragmentation in bronchial epithelia and restored the ultrastructural changes of mitochondria of bronchial epithelia. In addition, L-arginine administration reduced the widening of intercellular spaces between adjacent bronchial epithelia. These findings indicated that L-arginine administration reduced airway injury and restored mitochondrial dysfunction in murine allergic airway inflammation.

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1. Introduction

Allergic airway inflammation, epithelial injury, airway remodeling and hyperresponsiveness (AHR) are important features of human and experimental models of asthma [1]. Nitric oxide (NO) has a controversial role in asthma and it seems that dual properties of NO depend on its enzyme source, availability of L-arginine, and local biological context [2,3]. Recent evidence suggest that alteration in the metabolism of arginine, NO donor, may importantly contribute to the development and progression of asthma. L-Arginine metabolism has been shown to be dysregulated due to increased expressions of inducible NOS (iNOS) and arginase [2] in inflammatory cells such as macrophages in asthmatic mice. Both these enzymes consume endogenous L-arginine and cause reduced L-arginine bioavailability in the airway [3,4]. This leads to reduced availability of L-arginine to eNOS and increases bronchoconstriction

since eNOS derived NO causes bronchodilation through the cGMP pathway [3]. On the other hand, iNOS derived NO produces peroxynitrite which is known for its properties of causing cell injury and mitochondrial dysfunction by degrading heme centers of cytochrome c oxidase [5,6]. Interestingly, mitochondrial dysfunction induced by either ragweed pollen exposure or down-regulation of mitochondrial protein, ubiquinol cytochrome c reductase core II, with antisense oligonucleotide has been shown to increase the severity of allergic airway inflammation [7]. We have previously shown such reduction in the activity of cytochrome c oxidase in OVA sensitized and challenged mice [8–10]. In addition, we have shown that asymmetric dimethyl L-arginine (ADMA), an L-arginine analog, is increased in both mitochondrial and cytosolic fractions of allergic murine lung and causes increased oxo-nitrosative stress [11]. ADMA also leads to cause mitochondrial dysfunction in chronic inflammatory diseases [12,13]. L-Arginine treatment reduces both peroxynitrite and ADMA in the lung [4,11]. In the current study, we determined the effect of L-arginine on mitochondrial dysfunction and airway injury in murine allergic airway inflammation, and found that it restored mitochondrial dysfunction and reduced airway injury.

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2. Materials and methods

2.1. Animals and grouping

Male BALB/c mice (8–10 weeks old, National Institute of Nutrition, Hyderabad, India) were acclimatized for a week prior to starting the experiments. All animals were maintained as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Ministry of Environment and Forests, New Delhi, India) guidelines and protocols were approved by the Institutional Animal Ethics Committee, Institute of Genomics and Integrative Biology, Delhi, with approval number 09/1999/CPCSEA.

Mice were divided into 3 groups and each ($n=6$) was named according to sensitization/challenge/treatment: SHAM/PBS/VEH (normal controls, VEH=vehicle), OVA/OVA/VEH (allergic controls, OVA, chicken egg ovalbumin, Grade V, Sigma), and OVA/OVA/L-ARG 250 (L-ARG, L-arginine 250 mg/kg, Sigma). L-Arginine was soluble in water (vehicle).

2.2. Development of mouse model of asthma

Mice were sensitized and challenged as described earlier [8,9]. Briefly, mice were injected with 50 μ g OVA in 4 mg aluminium hydroxide or only 4 mg aluminium hydroxide by intraperitoneal route on days 0, 7, and 14. Mice were challenged with 3% OVA in PBS or PBS from Day 21 to Day 32 (30 min per day). Vehicle or L-ARG was given orally from Day 19 to 32 once a day in 30 μ l volume per dose.

2.3. Transmission electron microscopy

The whole body perfusion fixation was performed as described earlier [8,9] and the fixed lung was processed and stained sections were viewed under a transmission electron microscope (Morgagni 268D, Fei Electron Optics).

2.4. Isolation of whole lung mitochondria and cytosolic separation

After sacrifice, the lung portion below the trachea was removed, processed to separate mitochondrial and cytosolic fractions (CYTO) as described previously [8,9] and protein estimation was performed in those fractions by Bicinchoninic acid (Sigma) assay.

2.5. Immunohistochemistry (IHC)

Commercial rabbit polyclonal antibody for cytochrome c oxidase subunit III (Santa Cruz) was used as the primary antibody and HRP conjugated secondary antibody (Sigma) was used. Either gamma globulin as isotype control (Jackson ImmunoResearch Laboratories) or omission of primary antibody was used for negative control experiments.

2.6. Measurements of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

The levels of 8-OHdG (a marker of oxidative DNA damage) were measured in lung homogenates by competitive ELISA and results were expressed in ng/50 μ g protein.

2.7. Total cytochrome c oxidase activity measurement and cytochrome c estimation

Cytochrome c oxidase of electron transport chain (COXETC) activity and total citrate synthase activity were measured and calculated as described earlier [8,9]. Since citrate synthase activity is not altered by pathological conditions [14] COXETC activity was normalized by respective citrate synthase activity. The levels of cytochrome c were measured in cytosolic fractions by ELISA as per instructions (R&D systems).

2.8. ATP assay

This was done with ATP Bioluminescence Assay kit (Sigma) as described earlier [8–10] using Bioluminometer (Berthold AG model)

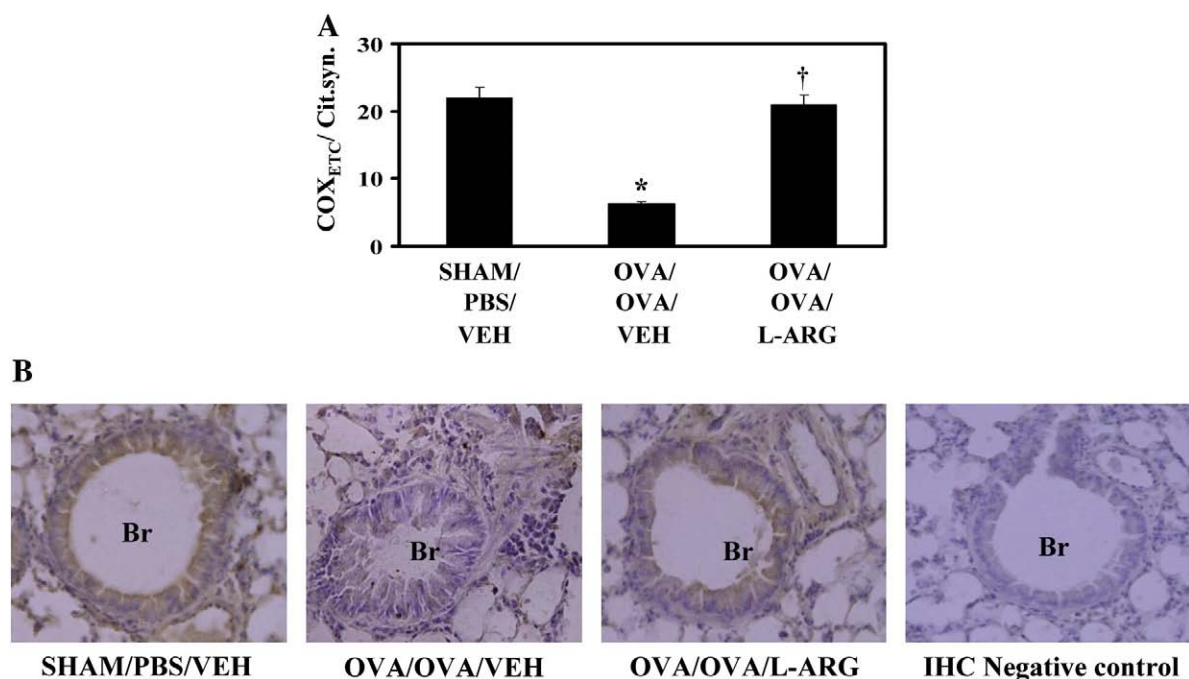


Fig. 1. L-Arginine restores cytochrome c oxidase (COXETC) activity and expression of its third subunit. Mitochondria were isolated from the fresh lung tissue after sacrifice, COXETC activity was estimated and normalized by citrate synthase activity. Data were mean $\pm$ SEM of three independent experiments. * $P<0.05$ vs. SHAM/PBS/VEH, and † $P<0.05$ vs. OVA/OVA/VEH. B) Immunohistochemistry (IHC) was performed for subunit III of COX_{ETC}. Brown color indicates the positive expression of respective subunit. Br indicates the bronchi. Representative photomicrographs from three independent experiments have been shown.

and the amount of bioluminescent signal was recorded and expressed as relative light units (RLU) per mg lung tissue.

2.9. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptotic assay

Since increased cytochrome c leads to apoptosis, TUNEL assay of apoptosis was performed on lung tissue sections using an *in situ* apoptosis detection kit (Dead End Calorimetric TUNEL system; Promega) as described earlier [15]. Bronchial epithelial cells per lung section were examined by investigators blind to the conditions and the numbers of apoptotic cells are expressed as a percentage of the whole epithelial cell population (apoptotic index).

2.10. Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Significant differences between selected two groups were estimated using unpaired Student *t*-test and ANOVA with post-hoc correction. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. L-arginine increased the activity of COXETC

We had shown earlier that high dose L-arginine (250 mg/kg) administration twice a day reduced the features of asthma such as airway hyperresponsiveness, parameters of airway inflammation such as increased Th2 cytokines, OVA specific IgE, lipid peroxidation, iNOS expression, ADMA levels and peroxynitrite formation [3,11]. Since peroxynitrite causes the inhibition of cytochrome c oxidase,

COXETC activity was estimated. As shown in Fig. 1, COXETC/citrate synthase was found to be reduced in OVA/OVA/VEH mice compared to SHAM/PBS/VEH. However, L-ARG treatment increased the activity of cytochrome c oxidase.

3.2. L-arginine restored the reduction in the expression of COX_{ETC} subunit III

It is known that cytochrome c oxidase is coded by 10 nuclear subunits and 3 mitochondrial subunits. Amongst these, mitochondrial coded subunits (subunits I, II, and III) are crucial for its catalytic activity. We had shown earlier that subunit III was specifically reduced in OVA induced mice [8]. In this study also, subunit III expression was found to be reduced in OVA/OVA/VEH mice compared to SHAM/PBS/VEH mice in which predominant expression was found in bronchial epithelia (Fig. 1B). Interestingly, L-ARG significantly restored the expression of COX_{ETC} subunit III (Fig. 1B).

3.3. L-arginine reduced the cytochrome c in the lung cytosol

As COXETC activity was found to be reduced in asthmatic mice lung and peroxynitrite causes the mitochondrial pore formation, we further analyzed the cytochrome c levels in the lung cytosol. As shown in Fig. 2A, the levels of cytochrome c were significantly increased in allergic control mice compared to normal control mice. However, treatment of allergic mice with L-ARG showed a significant reduction of cytochrome c in the lung cytosol (Fig. 2A).

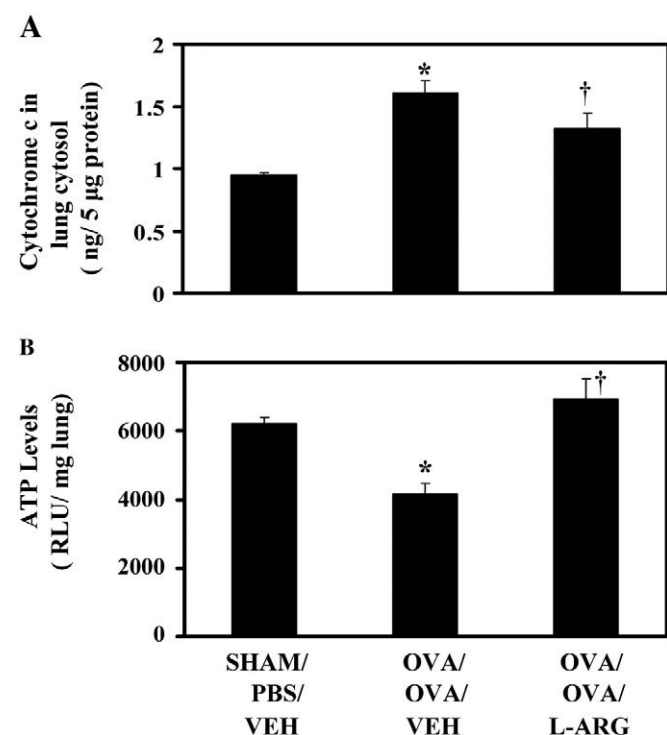


Fig. 2. L-Arginine restores cytochrome c and lung ATP levels. Data were mean $\pm$ SEM of three independent experiments. *P < 0.05 vs. SHAM/PBS/VEH and †P < 0.05 vs. OVA/OVA/VEH. A) Cytochrome c levels were measured in lung cytosolic fractions by ELISA. B) Lung ATP levels were estimated by bioluminescence and expressed as relative light units per mg.

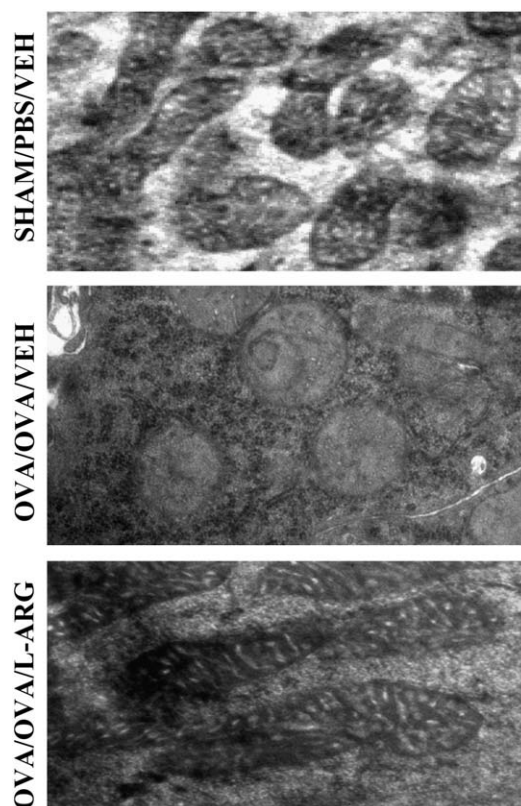


Fig. 3. L-Arginine restores mitochondrial ultrastructural changes in bronchial epithelia. To determine this, whole body perfusion fixation was performed and then first generation bronchi were dissected by dissection microscopy and processed, stained and viewed under a transmission electron microscope. Approximately 50 epithelial cells were randomly visualized and representative photograph of bronchial epithelial mitochondria from each group is shown.

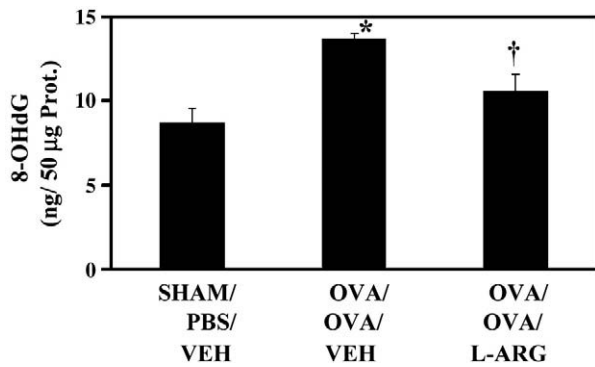


Fig. 4. L-Arginine prevents oxidative DNA damage of the lung. To determine this 8-OHdG (8-hydroxy deoxyguanosine) levels were determined in the lung tissue. Data were mean $\pm$ SEM of three independent experiments. * P <0.05 vs. SHAM/PBS/VEH, † P <0.05 vs. OVA/OVA/VEH.

3.4. L-Arginine increased ATP levels in the lung

To evaluate the effect of L-ARG on one of the vital functions of mitochondria, ATP levels were measured in the lung tissue. As shown in Fig. 2B, ATP levels were found to be decreased in lungs of allergic control mice compared to normal control mice. However, significant reversal of ATP levels was found with L-ARG treatment (Fig. 2B).

3.5. L-Arginine restored mitochondrial ultrastructural changes in bronchial epithelia

It was found that bronchial epithelia had shown more apoptosis, and earlier we had shown increased caspase 9 in asthmatic lungs [9]. In addition, caspase 9 activity was prominent in asthmatic bronchial epithelia in human studies. These indicate the possible involvement of mitochondria in bronchial epithelial injury. So we determined the mitochondrial ultrastructural changes in bronchial epithelia by transmission electron microscopy. In normal control mice, matrix was found to be dense with tightly packed granules and also well developed cristae were seen in mitochondria of bronchial epithelia (Fig. 3). In contrast, in allergic control mice, the matrix was less dense

with loss of cristae due to the swelling of mitochondria. However, when mitochondrial morphology was evaluated after L-ARG treatment, it significantly restored the mitochondrial structural changes (Fig. 3).

3.6. L-Arginine decreased oxidative DNA damage in lung

To determine the effect of L-ARG on oxidative stress in the lung, 8-OHdG levels were measured in the lung tissue. As shown in Fig. 4, 8-OHdG levels were found to be increased in lungs of allergic control mice compared to normal control mice. However, L-ARG treatment significantly decreased its levels (Fig. 4).

3.7. L-Arginine reduced bronchial epithelial cell injury

Since oxidative stress, mitochondrial dysfunction in bronchial epithelia and increased cytosolic cytochrome c lead to initiate apoptosis, we determined apoptosis (Fig. 5A), and apoptotic indices (Fig. 5B) in lung sections. Expectedly, allergic control mice showed increased DNA fragmentation, especially in bronchial epithelial cells. These events were significantly reversed by L-ARG treatment (Fig. 5).

3.8. L-Arginine reduced the widening of intercellular space between adjacent bronchial epithelia

Since it is known that epithelial damage leads to an increase of intercellular space between adjacent bronchial epithelial cells and L-ARG treatment reduced the epithelial injury, we sought to determine the effect of L-ARG on this feature. As shown in Fig. 6, there was a widening of intercellular space in OVA/OVA/VEH mice compared to SHAM/PBS/VEH mice and L-ARG treatment significantly reduced it.

4. Discussion

L-Arginine, a semi essential amino acid, has various pharmacological properties and is being used for various disease conditions. A recent study reported that L-arginine bioavailability is strongly associated with airflow obstructions in humans [16]. We have recently shown that high dose L-arginine administration improves

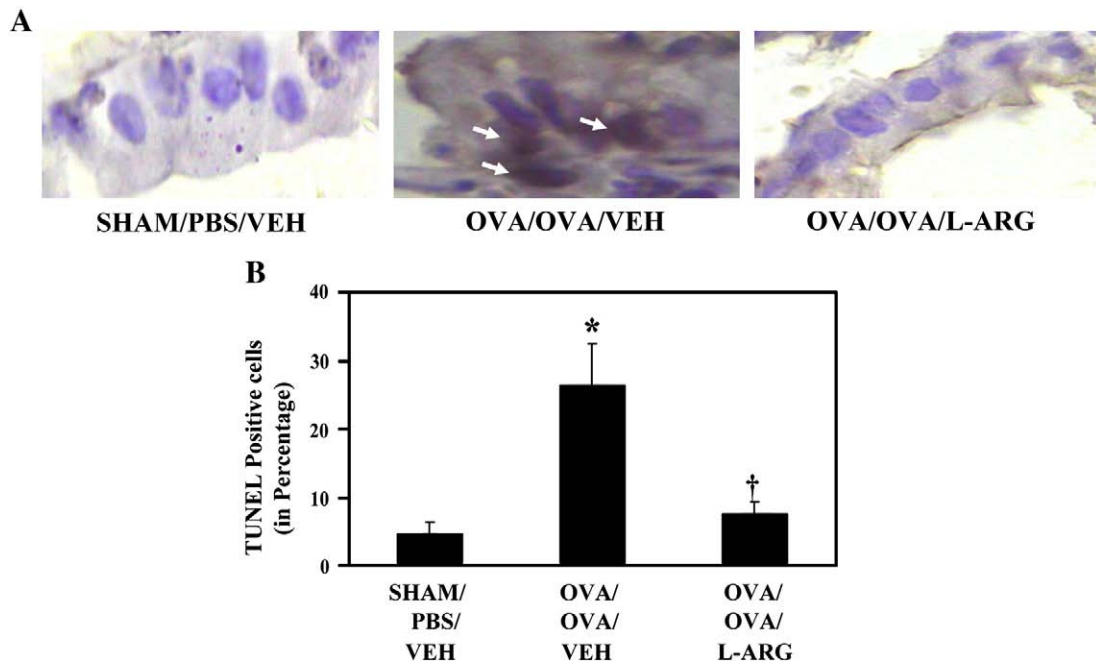


Fig. 5. Effect of L-arginine on apoptosis in the lung. To determine this: A) TUNEL apoptosis assay (white arrows indicate the apoptotic nuclei), B) apoptotic index in lung tissue sections were determined. Data were mean $\pm$ SEM of three independent experiments. * P <0.05 vs. SHAM/PBS/VEH, † P <0.05 vs. OVA/OVA/VEH group.

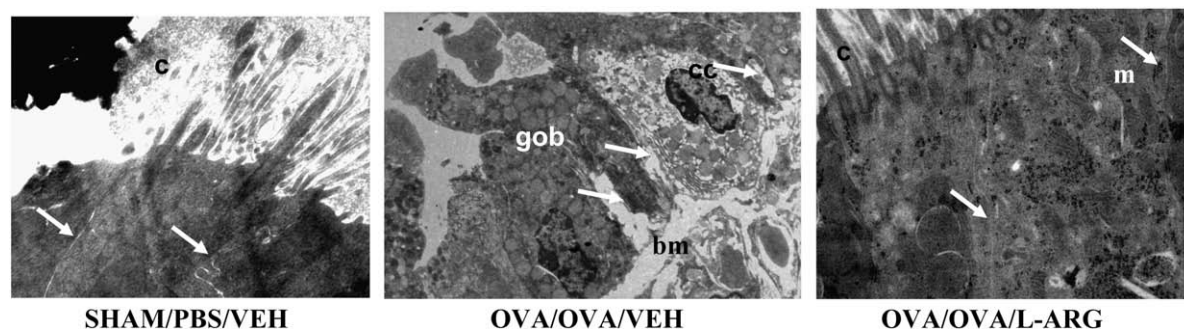


Fig. 6. L-Arginine restores widening of intercellular spaces between adjacent bronchial epithelia. White arrows indicate the intercellular space. c, cilia, cc, clara cell, gob, goblet cell, m, mitochondrion and bm, basement membrane.

airway hyperresponsiveness and reduces the features of allergic airway inflammation such as airway eosinophilia, increased levels of Th₂ cytokines, OVA specific IgE, eotaxin and TGF- β_1 and structural changes like goblet cell metaplasia, and subepithelial fibrosis by regulating arginine NO metabolism in bronchial epithelia [3].

NO originating from iNOS leads to recruitment of inflammatory cells in the airway including eosinophils [17]. L-ARG treatment reduced eosinophils in BAL fluid along with the reduction in the expression of iNOS [3,11]. It is known that arginine depletion leads to the production of peroxynitrite [18]. Peroxynitrite is a cytotoxic molecule which causes heme degradation to inhibit the cytochrome c oxidase. Further L-ARG treatment reduces the expression of iNOS, peroxynitrite and ADMA which are all shown to be responsible for mitochondrial dysfunction and airway injury. With this view, we wanted to determine the effect of L-ARG on mitochondrial dysfunction. L-ARG treatment not only reduced the levels of peroxynitrite [4] but also restored the activity of cytochrome c oxidase (Fig. 1). The reduction of COXETC, a terminal enzyme in mitochondrial electron transport chain, has been observed in many inflammatory oxidative diseases such as Alzheimer's disease and diabetes mellitus [19,20]. It is also interesting to note that L-arginine preconditioning of the liver has been shown to reduce the mitochondrial injury including the restoration of cytochrome c oxidase activity [21]. Further, L-ARG treatment restored the expression of the third subunit of COXETC which was reduced in the bronchial epithelia of asthmatic mice. Reduction in the activity of COXETC is mostly associated with the release of cytochrome c in the cytosol to initiate apoptosis. There is a tight physical interaction between cytochrome c oxidase, cytochrome c and cardiolipin in the inner mitochondrial membrane and reduction in cytochrome c oxidase activity leads to dissociation of this interaction to release cytochrome c to the cytosol. L-ARG treatment reduced the cytochrome c in the lung cytosol along with the restoration ATP levels in the lung (Fig. 2A and B).

L-ARG treatment not only reduces the mitochondrial dysfunction but also restores the mitochondrial ultrastructural changes in bronchial epithelia (Fig. 3). Optimal mitochondrial bioenergetics and function are required for the integrity of cilia of bronchial epithelia [22,23]. Importantly, eNOS has been shown to be important in ciliary functions through the NO-cGMP signaling pathway as well as L-arginine which promotes the functions of cilia [24,25]. Earlier we had shown that L-ARG treatment enhances eNOS, ENO, and cGMP production [3]. In this study, L-ARG treatment reduced not only the loss of cilia on bronchial epithelia (data not shown) but also oxidative DNA damage (Fig. 4) and DNA fragmentation in the bronchial epithelia (Fig. 5). These findings indicate the protective mechanisms of L-ARG against bronchial epithelial injury. It is also important to note that asthmatic bronchial epithelia have been shown to be inherently susceptible for oxidative stress, this could be due to the reduction in antioxidative defenses such as labile Zn and heat shock proteins [26,27]. However, it is not clear at this moment whether epithelial injury is a cause and/or an effect in asthma pathogenesis though it has

been suggested earlier that asthma results from epithelial injury which alters neural reflexes in the airway to cause narrowing of the airways [28]. Epithelial damage in the form of widening of intercellular spaces of bronchial epithelia has been well correlated with increased bronchial hyperreactivity [29]. In this study, we also found that L-ARG treatment decreased the widening of the intercellular space. Indeed, it is believed that therapeutic strategies to reduce the epithelial injury could be promising approaches in asthma [30].

Whether the effect of L-arginine on mitochondrial dysfunction is independent of its effect on airway inflammation, is less clear. It has been shown that the presence of existing mitochondrial dysfunction increases allergic airway inflammation [7]. However, in at least some studies reduction of allergic airway inflammation [31,32], didn't reduce the key mitochondrial dysfunction (Supplementary Fig. 1). These indicate that possibly the observed mitochondrial dysfunction in bronchial epithelia may exist independent of inflammation and may even enhance the inflammation. However, this hypothesis needs further investigations in the future. Since L-arginine has been shown to have pleiotropic effects, it may ameliorate airway injury by various other mechanisms which have not been investigated in this study or in our earlier study [3]. In conclusion, our findings reveal that L-arginine could reduce the bronchial epithelial injury and restore key mitochondrial dysfunctions in allergic airway inflammation. It seems therefore that L-arginine supplementation to susceptible persons might improve the health of bronchial epithelia and thus have beneficial effects against asthma.

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Supplementary Text

L-Arginine Reduces Mitochondrial Dysfunction and Airway Injury in Murine Allergic Airway Inflammation

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Methods

Animals and grouping. 8 weeks old male BALB/c mice (National Institute of Nutrition, Hyderabad, India) were acclimatized for a week prior starting the experiments. All experimental protocols were approved by Institutional Animal Ethics Committee. After acclimatization, mice were randomly divided into 3 groups and each group was named according to sensitization/challenge/treatment: SHAM/PBS/VEH (VEH-Vehicle), OVA/OVA/VEH, and OVA/OVA/MEP (MEP-Mepacrine in the form of dihydrochloride 1 mg/kg, Sigma Chemical Co, USA). Water was used as a vehicle for dissolving MEP.

The development of mouse model of asthma and MEP treatment. Mice were sensitized by three intraperitoneal (i.p.) injections of 50 µg OVA (grade V chicken egg ovalbumin, Sigma Chemical Co, USA) adsorbed in 4 mg alum (OVA/OVA/VEH and OVA/OVA/MEP groups) or 4 mg alum alone (SHAM/PBS/VEH group) on days 0, 7 and 14. From Day 21, the mice were challenged for 30 minutes with an aerosol of 3 % (wt/vol) OVA in PBS (OVA/OVA/VEH and OVA/OVA/MEP groups) or PBS alone (SHAM/PBS/VEH group) for 12 days consecutively using a nebulizer with a flow rate of 9 L/min (OMRON CX3 Model, Japan). MEP was given orally from day 19 to 32 twice a day (12 hrs interval between two doses) in the volume of 10 µl per dose. After the measurement of airway hyperresponsiveness (on day 33), mice were sacrificed and lung tissues were processed as described earlier to prepare mitochondrial and cytosolic fractions [1, 2, 3]. Protein estimation for both mitochondrial and cytosolic fractions was determined by Bicinchoninic acid (BCA) assay (Sigma Chemical Co, USA).

Total cytochrome c oxidase activity measurement and cytochrome c estimation

Cytochrome c oxidase of electron transport chain (COX_{ETC}) activity and total citrate synthase activity were measured and calculated as described earlier [1, 2]. The levels of cytochrome c were measured in cytosolic fractions by ELISA as per instructions (R&D systems).

Results

MEP neither increase COX_{ETC} activity nor reduce cytochrome c in lung cytosol

As shown in Suppl. Fig. 1A, COX_{ETC}/citrate synthase was found to be reduced in OVA/OVA/VEH mice compared to SHAM/PBS/VEH. However, MEP treatment didn't increase the activity of cytochrome c oxidase. As COX_{ETC} activity was found to be reduced in asthmatic mice lung, we further analyzed the cytochrome c levels in lung cytosol. As shown in Fig. 1B, the levels of cytochrome c were significantly increased in allergic control mice compared to normal control mice. However, treatment of allergic mice with MEP didn't reduce cytochrome c in the lung cytosol (Fig. 1B).

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Figure legends

Supp. Figure 1. Effect of Mepacrine on cytochrome c oxidase (COX_{ETC}) activity in lung mitochondria and cytochrome c in the lung cytosol. Data were mean $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. SHAM/PBS/VEH, and [†] Nonsignificant vs. OVA/OVA/VEH. A) Mitochondria were isolated from the fresh lung tissue after sacrifice, COX_{ETC} activity was estimated and normalized by citrate synthase activity. B) Cytochrome c levels were measured in lung cytosolic fractions by ELISA.