



House dust mite allergen causes certain features of steroid resistant asthma in high fat fed obese mice

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ABSTRACT

Obesity is a high risk factor for diseases such as cardiovascular, metabolic syndrome and asthma. Obese-asthma is another emerging phenotype in asthma which is typically refractive to steroid treatment due to its non-classical features such as non-eosinophilic cellular inflammation. The overall increased morbidity, mortality and economical burden in asthma is mainly due to steroid resistant asthma. In the present study, we used high fat diet induced obese mice which when sensitized with house dust mite (HDM) showed steroid resistant features. While the steroid, dexamethasone (DEX), treatment to high fat fed naïve mice could not reduce the airway hyperresponsiveness (AHR) induced by high fat, DEX treatment to high fat fed allergic mice could not reduce the HDM allergen induced airway remodeling features though it reduced airway inflammation. Further, these HDM induced high fat fed mice with or without DEX treatment had shown the increased activity and expression of arginase as well as the inducible nitric oxide synthase (iNOS) expression. However, DEX treatment had reduced the expressions of high iNOS and arginase I in control chow diet fed mice. Thus, we speculate that the steroid resistance seen in human obese asthmatics could be stemming from altered NO metabolism and its induced airway remodeling and with further investigations, it would encourage new treatments specific to obese-asthma phenotype.

1. Introduction

Obesity has emerged as a major risk factor for developing asthma over the decade [1]. Recent epidemiological studies suggest an association between obesity and asthma prevalence, morbidity as well as mortality. Various studies and views remark that the percentage of obese to overweight people among asthmatics could range from 30 to 50% [1]. In the light of the emerging research, obese-asthma has been classified among difficult to treat asthma sub-group due to its presentation of non-classical phenotype [2]. Akin to asthma, obese-asthma also has a heterogeneous phenotype. Obesity, in itself introduces pertinent physical changes making the disease multifactorial. For instance, abdominal and thoracic fat in obese individuals, leads to stiffening of lung movement which results in reduction of expiratory reserve volume (ERV) and functional residual capacity (FRC), creating smaller high resistance airways. However, the exact mechanism of airway hyperresponsiveness in obesity is unknown [3]. Obese-asthma is also consistently associated with lower amounts of fraction of exhaled nitric oxide (eNO), eosinophil independent inflammation, generalized

inflammatory mediators such as TNF- α and IL-6, leptins and unresponsiveness towards steroids [2–7]. It has been shown that elevated body mass index (BMI) has blunted response to dexamethasone [8,9]. The cellular phenotype of obese-asthma is considered to be dominated by neutrophils and Th-17 dependent inflammation, further indicating a plausible mechanism of steroid resistance. Thus, it can be concluded that while obesity has its own repercussion on lung health, with comorbidity to asthma, it can worsen the existing symptoms of asthma. With non-Th2 cell types dominating the asthma phenotypes and its refractiveness towards steroids, there is a need of understanding the pathobiology of obese-asthma steroid resistance.

One of the potential mechanistic links between asthma and obesity is nitric oxide (NO) metabolism [10–13]. Cluster studies which show the link between late onset asthma and obesity also show an inverse association between BMI and exhaled NO (eNO) [4,5]. Reduced bioavailability of L-arginine and increased arginase activity has been associated with reduced forced expiratory volume1 (FEV1) and greater airway obstruction [6]. Hence, it could be possible that in later onset asthma, obesity could induce L-arginase methylation causing changes

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in NO metabolism leading to oxo-nitrative stress. Evidently, we have shown the reduced arginine bioavailability and increased ADMA associated oxo-nitrative stress in high fat fed obese mice [13]. In the present study, we have demonstrated that high fat diet fed obese mice which when sensitized with house dust mite (HDM) developed steroid resistant asthma like features. We further showed that these HDM-treated obese steroid resistant mice have altered NO metabolic status with an increased expression of both arginase and iNOS enzymes. So this study highlights that the steroid resistance seen in obese-asthma could be stemming from the altered NO metabolism.

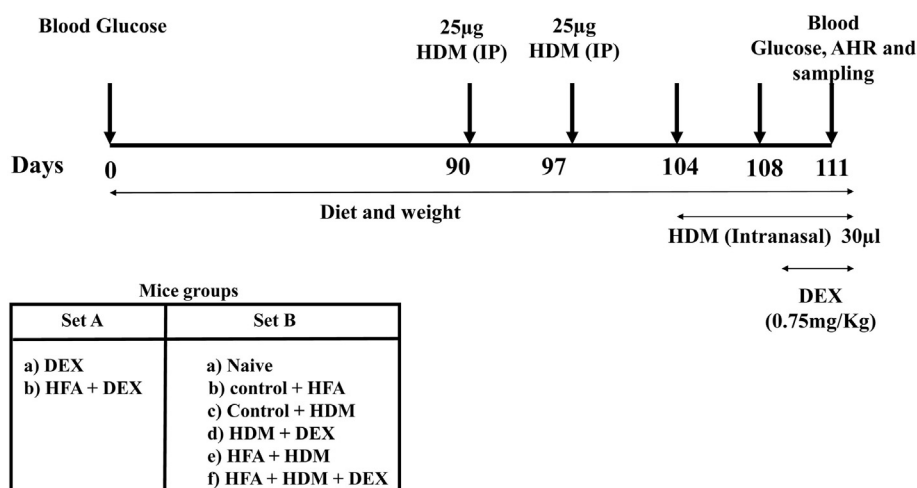
2. Materials and methods

2.1. Animals and grouping

All mice experiments were as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines) and all experimental protocols were approved by the Institutional Animals Ethics Committee. For this study, 5–6 weeks old male C57BL/6 mice were obtained from breeding facility at CSIR-IGIB, Delhi and were acclimatized for a week to holding room environment with regular 12 h light cycle (temperature: $23^{\circ} \pm 2^{\circ}$, humidity 50–60%, Light intensity: 300 lx at floor level). There were two sets of experiments: Set A) naïve mice experiments to determine the effect of steroid, dexamethasone (DEX), on high fat diet induced airway hyperresponsiveness and Set B) house dust mite induced mice experiments to determine the effects of steroid on high fat diet induced allergic airway inflammation, airway remodeling and expressions of arginase and iNOS. In the former set, mice were divided randomly into two groups and were named as: DEX (control chow diet fed mice treated with dexamethasone), and HFA + DEX (high fat diet fed mice treated with dexamethasone). In the latter set, 36 mice were divided randomly into six groups and were named as: Naïve (control chow diet fed mice with PBS sensitization and challenge), control + HFA (High fat diet fed mice with PBS sensitization and challenge), Control + HDM (control chow diet fed mice with house dust mite allergen sensitization and challenge and vehicle treatment), HDM + DEX (control chow diet fed mice with house dust mite allergen sensitization and challenge and dexamethasone treatment), HFA + HDM (high fat diet fed mice with HDM sensitization and challenge and vehicle treatment), and HFA + HDM + DEX (high fat diet fed mice with HDM sensitization and challenge and dexamethasone treatment).

2.2. Allergen sensitization and challenge and treatment (Fig. 1)

After acclimatization to animal house and random classification,



mice (both set A and B) were fed either control chow diet (Altromin 1324) or high fat diet (Research Diet Inc., D12492) according to their groups with periodical measurement of body weight. The blood glucose level was measured on day zero and on day 111 using Accucheck active⁺ glucose strip [13]. On Day 90 and 97, mice (Set B) were sensitized with either phosphate buffered saline (PBS) or 25 µg HDM (house dust mite, *Dermatophagoides pteronyssinus*, obtained from Greer Laboratories, USA or All Cure Pharma Pvt. Ltd., India) in 200 µl PBS by intraperitoneal injections. Then, these (Set B) mice were challenged with either PBS or HDM allergen (25 µg in 30 µl PBS) intranasally from Day 104 to Day 111. DEX groups (from both sets) were administered dexamethasone treatment (0.75 mg/kg weight) orally from Day 108 to Day 111.

2.3. AHR estimation

On Day 111, AHR was determined as described earlier for set A mice [16,17]. Briefly, every individual mouse that was anaesthetized was intubated and ventilated by a mechanical ventilator (Sciencelab, flexivent, Canada) and airway resistance was estimated with increasing concentrations of methacholine (Mch) and expressed as % baseline airway resistance assuming PBS aerosols derived values are 100%.

2.4. Lung histopathology

Set B mice were euthanized with higher dose of sodium pentothal, and lung tissues were excised and fixed in 10% buffered formalin. The fixed paraffin embedded tissue were dissected into 4 µm sections and stained with Haematoxylin and Eosin (HE), Masson Trichrome (MT) and periodic acid-Schiff (PAS) staining to assess lung inflammation, collagen fiber content and goblet cell metaplasia, respectively [16]. The images of stained sections were microphotographed (Nikon microscope, Model YS-100). The inflammation score was estimated by experimentally blind investigators from HE stained slides as described earlier [16]. There were separate scores for both peri-vascular (PV) and peri-bronchial (PB) and Total score was the sum of both PV and PB. Similarly PAS stained lung sections were used for determining PAS staining intensity [16].

2.5. Immunohistochemistry

The immunohistochemistry was performed in lung sections to determine the expressions of arginase and iNOS (Santa Cruz Biotechnology, Texas, USA) with respective secondary antibodies (Sigma, St. Louis, MO, USA or Genei, India) as described by us earlier [16]. The negative control experiments were performed using either

Fig. 1. Experimental design: After acclimatization and random division, mice were fed either control chow diet or high fat (HFA) diet with periodical measurement of body weight. On Days 90 and 97, mice were intraperitoneally injected with PBS or 25 µg HDM and these mice were challenged with PBS or HDM allergen from Day 104 to Day 111. Some mice were treated with vehicle or dexamethasone from Day 108 to Day 111 and all mice were euthanized on day 111 after the airway hyperresponsiveness measurement.

isotype control or omission of only primary antibody. It is to be noted that in all IHC experiments, negative control experiments did not show any brown color (data not shown).

2.6. Arginase activity

The activity of arginase was performed using colorimetric assay (Abcam) in the total lung lysates. First 50 mg lung tissue was homogenized in 500 μ l RIPA (radioimmunoprecipitation assay) buffer and centrifuged at 5000g in refrigerated centrifuge and resultant supernatants, total lung lysate proteins (TLP), were used for protein quantitation by bicinchoninic acid (Sigma) assay. The 25 μ g TLP were taken for the arginase activity assay. The TLP that contain arginase reacts with the substrate, arginine, to form an intermediate which further reacts with the probe and form a colored product which can be detected at optical density of 570 nm and arginase activity was expressed as nmol/min/25 μ g TLP.

2.7. Statistical analysis

All the data were represented in mean $\pm$ SEM with $n = 5$ –6 in each group. The significance between two and more groups was estimated using two-tailed Student's *t*-test (comparing two groups) or analysis of variance (comparing 3 or more groups) with PRISM software. A *p*-value of < 0.05 was considered as non-significant (NS).

3. Results

3.1. Steroid treatment to high fat fed mice did not reduce airway hyperresponsiveness

We and others had demonstrated that high fat fed mice developed spontaneous airway hyperresponsiveness (AHR) even without allergen induction and these mice also showed the stressed mitochondria in airway epithelia [10,11]. In this study, we wanted to determine the effects of steroid (DEX) on HFA induced AHR. To determine this, we used high fat fed obese model as we described earlier (Fig. 1) [13]. In this experiment, there were two groups: DEX and HFA + DEX. DEX group mice were fed normal chow diet and HFA + DEX mice were fed high fat diet. As shown Fig. 2, HFA + DEX mice showed an increase in methacholine induced AHR compared to DEX alone. This indicates that steroid could not reduce high fat diet induced AHR.

3.2. Steroid treatment to high fat fed mice did not reduce airway remodeling

As we observed that steroid treatment could not reduce high fat diet

induced AHR, it is important to determine the effects of steroid treatment in human relevant obese-asthma model. So we determined the effects of steroids in HDM induced obese mice. In this experiment, there were 6 groups of mice: Naïve, Control + HFA, Control + HDM, HDM + DEX, HFA + HDM and HFA + HDM + DEX as described in Materials and Methods and Fig. 1. Among these groups, Naïve, Control + HDM, and HDM + DEX group mice were fed with control chow diet and remaining groups were fed with high fat diet. The high fat diet (HFA) fed mice had shown the increased body weight (Fig. 3A) and random blood glucose levels (data not shown) compared to control chow diet (Naïve) fed mice. Interestingly, HFA + HDM + DEX mice also had increased body weight compared to Naïve mice (Fig. 3A). Although, prolonged glucocorticoid treatment is known to decrease body weight and increase blood glucose levels [14,15], we did not find any change due to dexamethasone (DEX) treatment and this could be due to short duration of DEX treatment.

Among all groups, Naïve and control + HFA group mice were sensitized and challenged with PBS and remaining all groups were sensitized and challenged with HDM as described in Materials and Methods and Fig. 1. Then to determine the effects of HFA on HDM induced allergic airway inflammation, haematoxylin and eosin staining was performed in the lung sections. Expectedly, Control + HDM mice showed the significant infiltration of inflammatory cells both around the bronchi (peri-bronchial, PB) and vessel (peri-vascular PV). However, Control + HFA mice also had shown significant bronchovascular inflammation even without HDM induction. In any event, HDM + DEX group had a significant reduction in airway inflammation in. Similarly, HFA + HDM + DEX mice also showed a significant reduction in inflammation compared to HFA + HDM mice (Fig. 3B, C). Though DEX reduced inflammation in HFA + HDM albeit not to the level of HDM + DEX, the difference between HDM + DEX and HFA + HDM + DEX is not significant. These indicate that steroid resistance in high fat fed mice may be independent of airway inflammation.

Then to determine the effects of DEX on high fat fed HDM induced mice on airway remodeling features, Periodic acid-Schiff and Masson's trichrome stainings were performed as described in Materials and Methods. Expectedly, HDM induced naïve mice had shown the increased goblet cell metaplasia. However, HFA fed mice had also shown the similar increased goblet cell metaplasia even without allergen induction indicate the possible effects of obesity on airway remodeling feature (Fig. 4A, B). While DEX treatment could reduce the goblet cell metaplasia in HDM induced control mice, there was no reduction in HFA fed HDM induced allergic mice (Fig. 4A, B). Similarly, DEX treatment did not reduce the sub-epithelial fibrosis in HFA fed HDM induced allergic mice though it could reduce the same in control diet fed HDM induced allergic mice (Fig. 5).

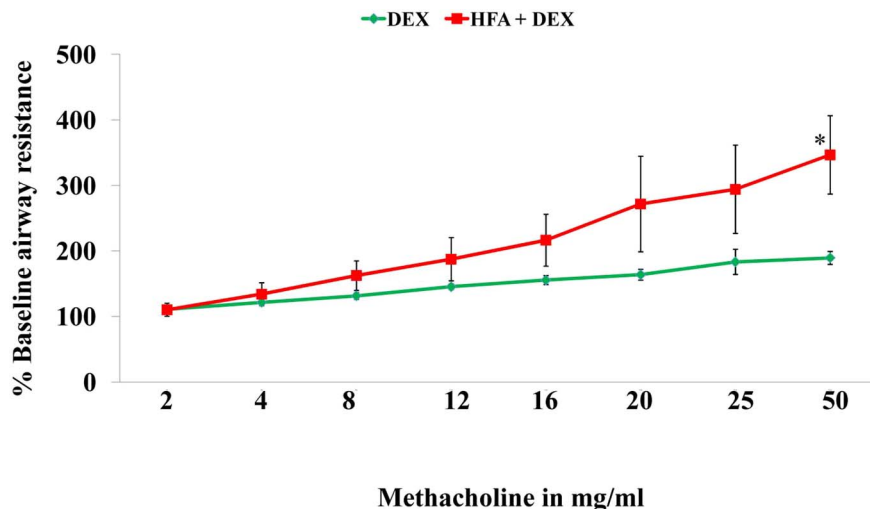


Fig. 2. Steroid treatment could not reduce airway hyperresponsiveness (AHR) in HFA mice. On Day 111, AHR was determined using a mechanical ventilator attached with airway mechanics. Anaesthetized and intubated mice were mechanically ventilated and airway resistance was determined with increasing concentrations of methacholine (Mch) and expressed as % baseline airway resistance assuming PBS aerosols derived values are 100%. Data were mean $\pm$ SEM. * $P < 0.05$ Vs DEX group. $N = 4$ –5 mice per group.

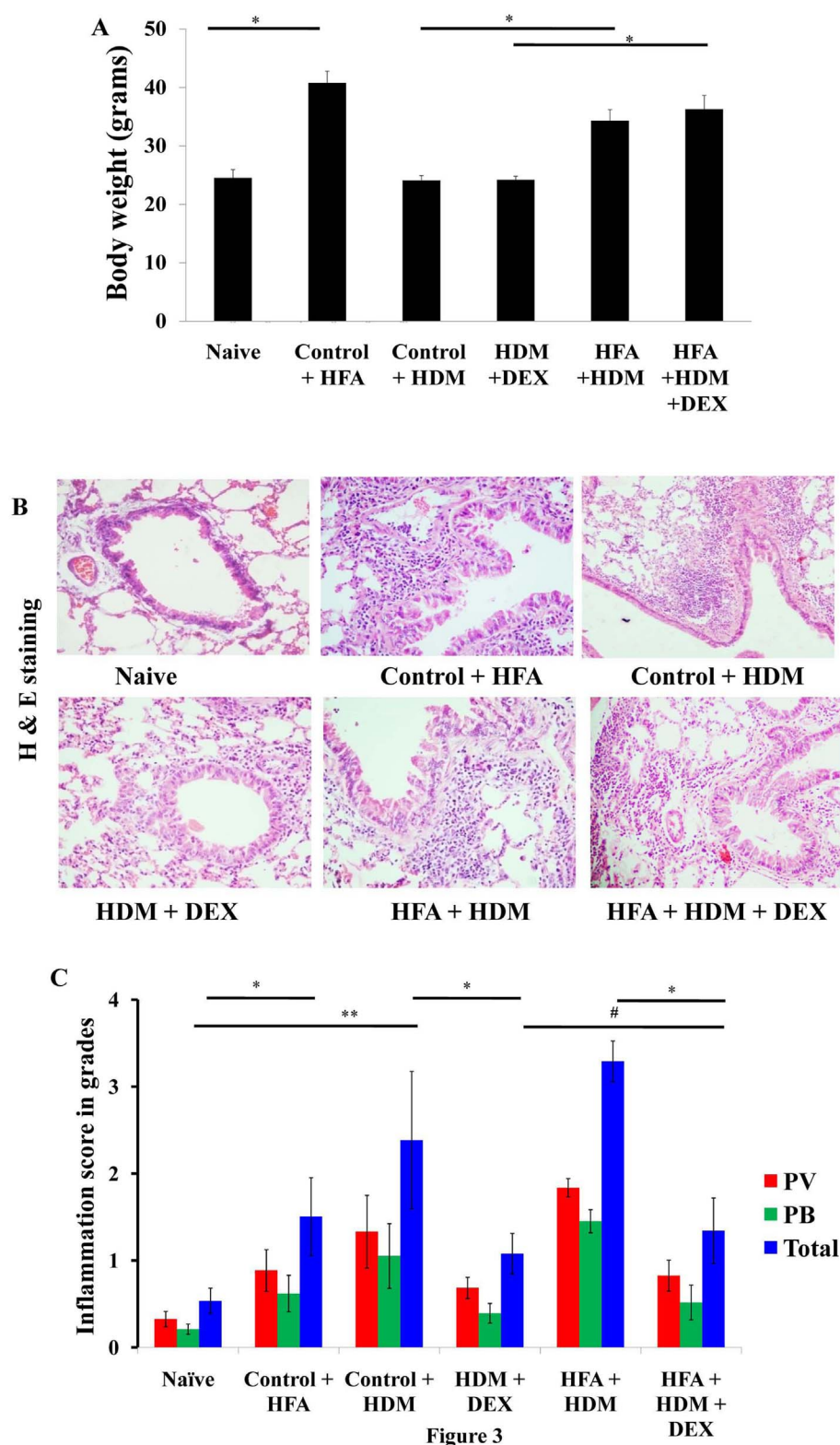


Fig. 3. Effects of steroid treatment on airway inflammation in HFA fed allergic mice. A) The body weight of different groups. B) After the AHR estimation, lungs of mice were removed and fixed with 10% buffered formalin and further paraffin embedded lung sections were stained with haematoxylin and eosin (H & E) staining to assess the airway inflammation. C) These stained sections were examined by experimentally blind expert to score the inflammation and scores were expressed as grades. PV: peri-vascular, PB, peri-bronchial, total; the sum of both peri-vascular and peri-bronchial inflammation. The shown statistical symbols are for total inflammation scores. Data were mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$ and # Non-significant. $N = 6$ mice per group.

3.3. Steroid treatment to high fat fed mice showed increased iNOS and arginase expressions

Our previous report suggests that HFA diet fed obese mice had reduced arginine bioavailability and increased oxo-nitrate stress. It is known that increased expressions of arginase and iNOS lead to reduction in arginine bioavailability [13,16–18]. In addition, increased arginase might lead to initiate airway remodeling due to the abnormal

accumulation of polyamine in the airway [19] and it has been shown that polyamine administration is linked with increased asthma features [19,20]. So we hypothesize that the HFA fed mice might have developed an alteration in NO metabolism upon HDM induction and this could be attributed to failure in the reduction of airway remodeling with DEX treatment. Therefore, we checked the expression of arginase-1 in HDM induced HFA fed steroid resistant mice. Expectedly, HDM treated control mice had shown the increased arginase expression and

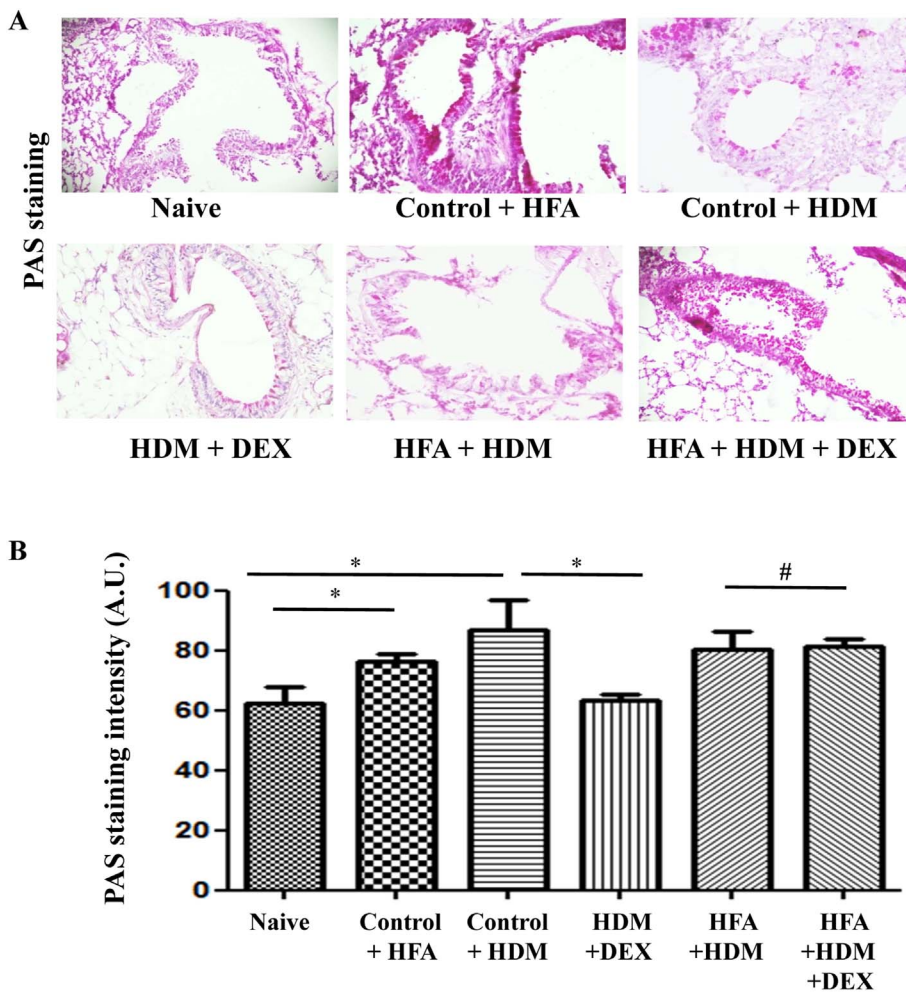


Figure 4

Fig. 4. Effects of steroid treatment on goblet cell metaplasia in HFA fed allergic mice. A) The paraffin embedded lung sections were stained with periodic acid-Schiff staining to assess the goblet cell metaplasia. B) The intensities of PAS positive regions in bronchial epithelia were determined using ImageJ software. Data were mean $\pm$ SEM. * $P < 0.05$, and # non-significant. N = 6 mice per group.

activity (Fig. 6). Higher magnification revealed that the arginase positive cells were mostly inflammatory cells. Surprisingly, control HFA mice also had shown the increased arginase activity and this is correlated with increased goblet cell metaplasia in these mice. In addition,

DEX treatment to control HDM induced mice had shown a reduction in the expression and activity of arginase (Fig. 6). However, dexamethasone was unable to reduce the arginase expression in HDM-treated obese mice. Similarly, arginase activity assay also showed that

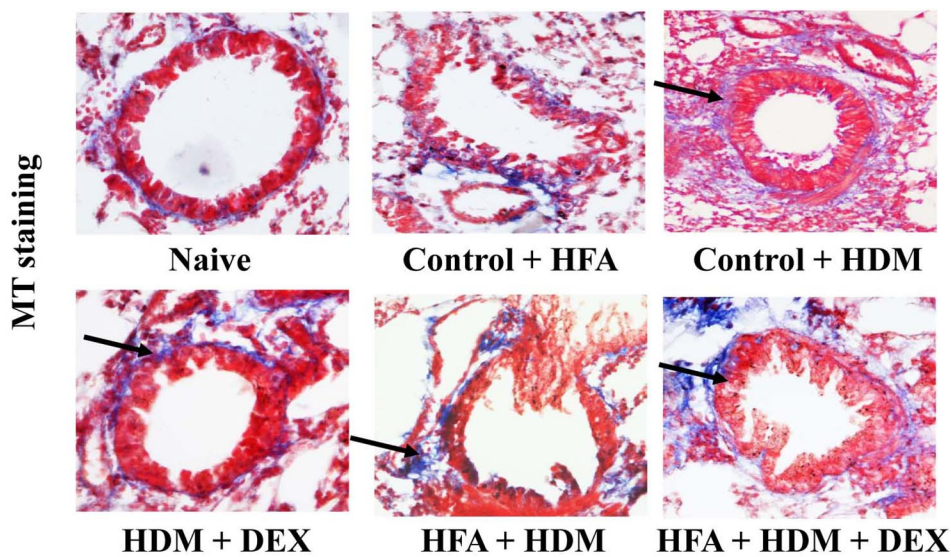


Figure 5

Fig. 5. Effects of steroid treatment on sub-epithelial fibrosis in HFA fed allergic mice. The paraffin embedded lung sections were stained with Masson's trichrome staining to assess the sub-epithelial fibrosis. N = 6 mice per group.

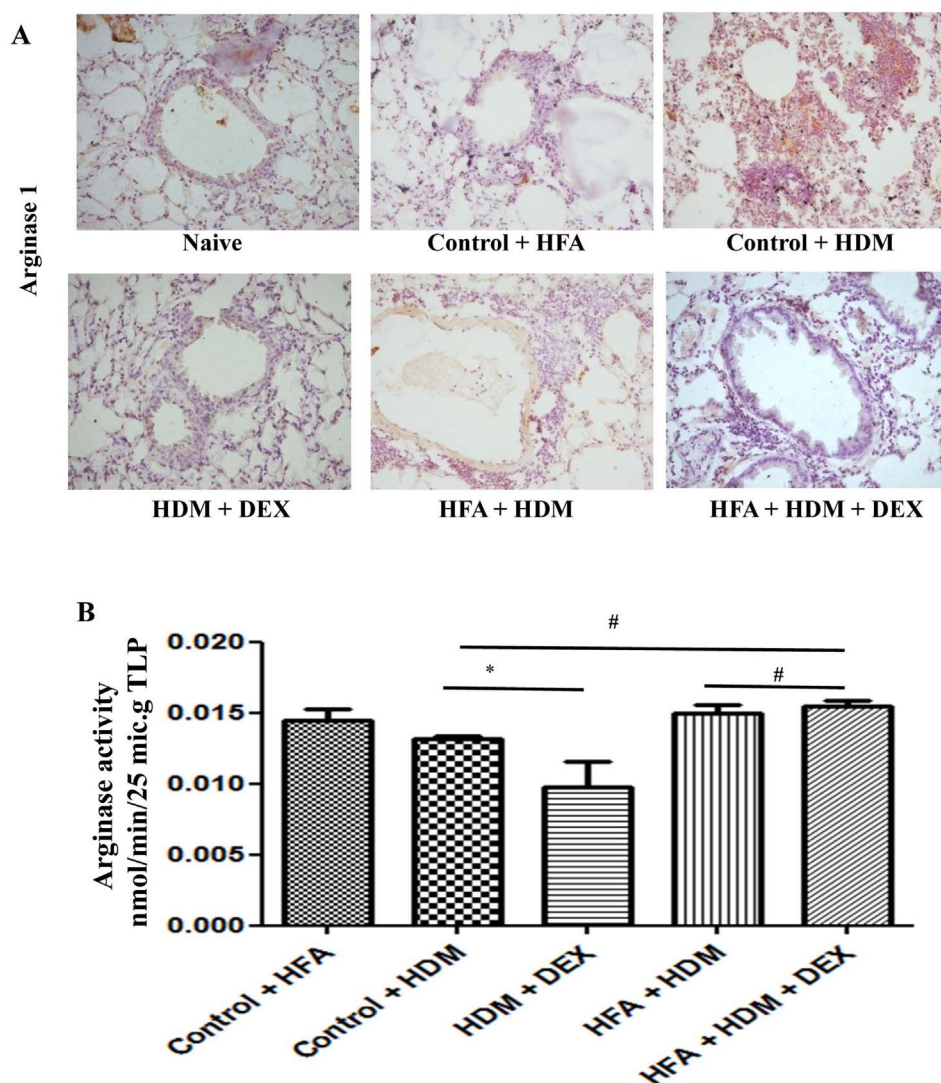


Fig. 6. Effects of steroid treatment on the expression and activity of arginase in HFA fed allergic mice. A) The arginase expression in paraffin embedded lung sections were determined by Immunohistochemistry. Arrows indicate the positive expression. B) The arginase activity was performed in the total lung lysates. Data were mean $\pm$ SEM. * $P < 0.05$ and # non-significant. $N = 6$ mice per group.

Figure 6

dexamethasone was able to reduce arginase activity in HDM-treated naïve mice but not in HDM-treated obese mice.

Further, we investigated the expression of iNOS in the lung sections of HDM-treated obese mice. In our earlier study, we found that high fat fed mice had shown increased iNOS expression [13]. In this study also we validated the same findings and in addition, we found the increased expression of iNOS in bronchial epithelia (Fig. 7). As compared to HDM-treated naïve mice, dexamethasone was unable to reduce the iNOS expression in HDM-treated obese mice (Fig. 7). This indicates that steroid unresponsiveness in obese mice could be attributed to shift in NO metabolism, where low levels of L-arginine tilts the reaction pathways towards production of oxidative and nitrosative species.

4. Discussion

Recent studies have demonstrated the ever increasing complexity of asthma confirming the syndromic nature of asthma [21,22]. These studies have classified asthmatic patients in various clusters based on multiple factors like atopy etc. These clusters included early onset mild allergic asthma, severe non atopic asthma with frequent exacerbations and later-onset asthma associated with obesity [21,22]. This classification not only emphasized the different pathophysiologies of these different clusters but also indicate the necessity of multiple cluster oriented therapeutic strategies. In the present study, we have developed a

steroid resistant model which mimics the obese-asthma phenotype with the potentiation of airway remodeling and alteration in NO metabolism.

Recently, a lot of studies have been carried out to develop a suitable mouse model for steroid resistant asthma, which would not only present the physiology but also the cell types observed in steroid resistance. While some mouse models have been developed using the allergens such as long dose of ovalbumin (OVA), triple allergen combination of OVA, HDM and cockroach extract, some have been developed by exploiting the cell types seen in steroid resistance, such as adoptive transfer of Th17 cells [23–25]. However, there exists no model which would explain the obese-asthma phenotype. In our previous study we had shown that HFA diet fed mice developed spontaneous asthma features. We had also shown that these diet induced asthmatic mice had reduced L-arginine/ADMA ratio, and increased oxo-nitrate stress [13]. Based on our earlier findings, we developed a HFA fed obese mice which were sensitized with HDM to mimic the clinical obese-asthma condition. Further, these HFA fed obese mice were treated with DEX to determine these mice are sensitive to steroids. But these mice had shown the insensitivity to steroids. Although these mice showed a reduction in airway inflammation, there was no reduction in the airway remodeling features such as goblet cell metaplasia and sub-epithelial fibrosis. These indicate that steroid resistance in these mice could be independent of airway inflammation. In addition, there is a poor correlation between airway inflammation and airway remodeling and this

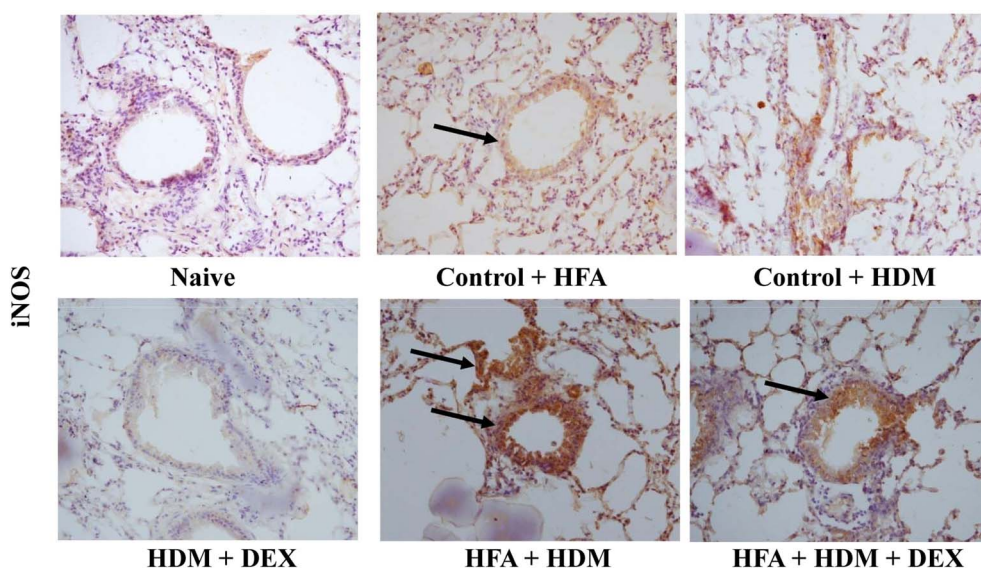


Fig. 7. Effects of steroid treatment on the iNOS expression in HFA fed allergic mice. A) The iNOS expression in paraffin embedded lung sections were determined by immunohistochemistry. Arrows indicate the positive expression. N = 6 mice per group.

Figure 7

could be because of parallel events rather than subsequent events of airway inflammation and airway remodeling [26,27]. This implies that the phenotype seen in this steroid resistant model could be obesity induced rather than allergen mediated. In human obese-asthmatics too, it has been seen that there are reduced Th2 or Th17 cell markers.

Role of L-arginine and NO metabolism is now well established in the maintenance of airways. It is considered to be a potential link between asthma and obesity. Cluster studies which have revealed the association of females with late onset asthma and obesity, also showed the association between lower airway eosinophils and exhaled NO and inverse association of BMI with exhaled NO [4,5]. L-arginine acts as a substrate for nitric oxide synthase (NOS), producing NO, which helps in the cGMP activation, ultimately maintaining the airway tone with bronchodilation. These beneficial effects are mostly mediated by eNOS. In contrast, pathological inflammatory conditions which increase arginase and iNOS expressions, shifts the beneficial bronchodilatory actions towards the bronchoconstrictory action with the consumption of endogenous L-arginine by both arginase and iNOS to generate polyamines and peroxynitrates. These byproducts lead to cause irreversible airway remodeling and airway constriction. The superoxides indeed uncouple NOS to cause the formation of peroxynitrates instead of NO. As we had already shown the shift of this reaction mechanism in HFA induced obese mice, we checked the expression of arginase and iNOS in HDM-treated obese mice. We found that dexamethasone failed to alleviate HFA and HDM mediated increase of both iNOS and arginase. As we already know that HFA diet reduced L-arginine/ADMA ratio in naïve mice [13], it could be further concluded that steroid resistant features seen in HDM-treated obese mice could be due to the imbalance in the NO metabolism reaction. While researchers have used 0.01 mg/kg to 3 mg/kg doses of dexamethasone in various mice models of asthma [28,29], most of the studies have used 0.75 mg/kg to 1 mg/kg in both steroid sensitive and resistant models of asthma [30–34]. It would be interesting to see the effects of higher dose like 2 to 3 mg/kg dexamethasone in high fat fed allergic mice.

The present steroid resistant mouse model reveals the involvement of NO metabolism in the obese-asthma phenotype. While this phenotype has not been well dissected so far, this study would open new avenues for studying new molecular targets and development of personalized and phenotype specific treatments.

Disclosure of potential conflict of interest

The authors have declared that they have no conflict of interest.

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