Oxidative airway inflammation leads to systemic and vascular oxidative stress in a murine model of allergic asthma

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A B S T R A C T
Oxidant–antioxidant imbalance plays an important role in repeated cycles of airway inflammation observed in asthma. It is when reactive oxygen species (ROS) overwhelm antioxidant defenses that a severe inflammatory state becomes apparent and may impact vasculature. Several studies have shown an association between airway inflammation and cardiovascular complications; however so far none has investigated the link between airway oxidative stress and systemic/vascular oxidative stress in a murine model of asthma. Therefore, this study investigated the contribution of oxidative stress encountered in asthmatic airways in modulation of vascular/systemic oxidant–antioxidant balance. Rats were sensitized intraperitoneally with ovalbumin (OVA) in the presence of aluminum hydroxide followed by several intranasal (i.n.) challenges with OVA. Rats were then assessed for airway and vascular inflammation, oxidative stress (ROS, lipid peroxides) and antioxidants measured as total antioxidant capacity (TAC) and thiol content. Challenge with OVA led to increased airway inflammation and oxidative stress with a concomitant increase in vascular inflammation and oxidative stress. Oxidative stress in the vasculature was significantly inhibited by antioxidant treatment, N-acetyl cysteine; whereas hydrogen peroxide (H2O2) inhalation worsened it. Therefore, our study shows that oxidative airway inflammation is associated with vascular/systemic oxidative stress which might predispose these patients to increased cardiovascular risk.

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1. Introduction

It has been well established now that oxidant–antioxidant imbalance plays an important role in repeated cycles of airway inflammation observed in asthma. Endogenous or exogenous environmental oxidants are critical to the inflammatory response through perpetuation and amplification of pro-inflammatory signaling pathways [1–4]. At the same time, endogenous antioxidant mechanisms are present to attenuate this ROS-mediated inflammatory response. It is when oxidative stress overwhelms antioxidant defenses that severe inflammatory state becomes apparent and may manifest in the form of mucus hypersecretion, increased vascular permeability, and airway remodeling [3–5].

Several studies have shown oxidant burden in different components of the lung and blood. Inflammatory cells such as eosinophils, neutrophils, and lymphocytes from the lung/blood, and also pulmonary resident cells such as bronchial epithelial/smooth muscle cells, have been shown to produce oxidants in response to various stimuli [1, 5–7]. For example, asthmatics have shown increased ROS generation such as superoxide and H2O2 from their airways as compared to controls [5,8–10]. Furthermore, ROS generation in asthmatic airways is associated with an increase in airway responsiveness and negatively correlated with FEV1 [7,10]. Oxidative stress in the blood of asthmatics has been shown to have a pattern similar to the lung by us and others [1, 11,12].

ROS-mediated airway responses and tissue injury depend on the nature of antioxidant defenses available in the lung/blood. ROS mediated depletion of antioxidants may result in amplification of inflammatory loop which results in insufficient protection of biomolecules [3,13]. Evidence for antioxidant imbalance in asthmatic airways has been shown in several studies which show alteration not only in the non-enzymatic but also in enzymatic antioxidants in different components such as BAL and sputum [14–16]. Similar derangements in antioxidants have been reported in different components of the blood of asthmatics as well [1, 11,17,18]. Of note, total antioxidant capacity (TAC) has been consistently shown to be positively correlated with lung function [1,18,19].
However, it needs further investigation whether oxidative stress observed in asthmatic airways also contributes to systemic/vascular oxidative stress.

Several studies in humans are beginning to show an association between chronic airway inflammation and cardiovascular complications [20–22]. Studies in animals and humans show that airway inflammation causes enhanced systemic inflammation and cardiovascular complications such as myocardial ischemia–reperfusion injury, endothelial dysfunction and inflammatory cell recruitment [10,23–26]. This might be a result of airway oxidants gaining entry into the systemic circulation due to ROS mediated dysfunction of pulmonary endothelial/epithelial barrier [5,27,28]. ROS produced in asthmatic inflammation such as hydrogen peroxide, hypochlorous acid and superoxide radical are known to increase airway epithelial and microvascular endothelial permeability in vitro and in vivo [29,30]. However, no study so far has attempted to establish a link between oxidative airway inflammation, and systemic/vascular oxidant–antioxidant balance in vivo. Therefore, this study was undertaken to investigate the effects of inhaled allergen/hydrogen peroxide on systemic and vascular oxidative stress in the context of allergic airway inflammation. The data from our study show that airway oxidative stress is concurrently associated with vascular and systemic oxidative stress in a murine model of asthma.

2. Materials and methods

2.1. Animals

Female Wistar rats (150–160 g), free of specific pathogens, were used in the experiments. The animals were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were kept under standard laboratory conditions of 12-h light–dark cycle and 24–26 °C ambient temperature. All experimental animals used in this study were under a protocol approved by the Animal Care and Research Committee of College of Pharmacy, King Saud University.

2.2. Rat sensitization and challenge

Sensitization was performed according to the protocol described earlier by us with some modifications [31,32]. Rats were sensitized on days 1 and 8 with intraperitoneal (i.p.) injections of 200 μg ovalbumin (grade V) adsorbed to 4 mg alum. Non-sensitized control animals received phosphate-buffered saline was introduced into the lungs via the tracheal cannula and the total cells were counted manually in a hemocytometer chamber followed by spinning of cells onto glass slides for differential count. A differential count of at least 300 cells was made according to standard morphologic criteria on cyt centrifuged Diff-Quik stained slides. The number of cells recovered per rats was calculated and expressed as mean ± SE per ml for each group.

2.3. Bronchoalveolar lavage (BAL)

The trachea was cannulated to perform BAL one day after the final allergen challenge; phosphate-buffered saline was introduced into the lungs via the tracheal cannula and the total cells were counted manually in a hemocytometer chamber followed by spinning of cells onto glass slides for differential count. A differential count of at least 300 cells was made according to standard morphologic criteria on cyt centrifuged Diff-Quik stained slides. The number of cells recovered per rats was calculated and expressed as mean ± SE per ml for each group.

2.3.1. Protein carbonyl assay

The content of protein bound carbonyls in the trachea/aorta/plasma, an indicator of protein oxidation, was measured at 380 nm using 2,4-dinitropheny lhydrazine (DNPH) by the method of Levine et al. [34] as described by us previously [1]. Briefly, after precipitation of proteins in sample supernatant/plasma by trichloroacetic acid, pellet was dissolved in DNPH followed by precipitation again after a waiting period of 1 h. The resulting pellet was dissolved in 6 M guanidine solution after several washes with ethanol:ethyl acetate solution. Absorbance of the sample was taken at 380 nm and carbonyl content was calculated using molar absorption coefficient of 22,000/M/cm. The final results were expressed as nmol/mg protein.

2.4. Thiol content assay

For reactive oxygen species generation, the harvested trachea/aorta were incubated with 100 μM 6-carboxy-2,7′-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37 °C. DCFH-DA forms a fluorescent product, DCF (dichlorofluorescein) upon oxidation with ROS. Fluorescence caused by DCF in each well was measured and recorded for 30 min at 485 nm (excitation) and 530 nm (emission) by the method of Wang and Joseph [37] using a multi-mode fluorescent microplate reader (FLUOstar Omega, BMG LabTech, USA) with temperature maintained at 37 °C as described earlier by us [38]. The background fluorescence caused by buffer and DCF was subtracted from the total fluorescence in each well caused by the trachea/aorta in the presence of DCF. Fluorescence intensity was expressed as ROS generation (% control).

To assess the location of ROS generation in the aortas of allergen and oxidant challenged animals, the aorta was cut into equal pieces followed by mechanical removal of endothelium (this was done by placing a piece of thin wire in the lumen and rubbing the aortic ring over a wet blotting paper) in one half and leaving the other half intact as described previously by us [39]. This procedure leads to lack of relaxation in the endothelium denuded aorta in response to acetylcholine in an organ bath [39]. Afterwards, ROS generation in the endothelium intact and denuded aortas was measured as described above.

2.6. Lipid peroxide assay

Plasma/tissue lipid peroxides were measured as malondialdehyde (MDA)–thiobarbituric acid (TBA) adducts by the method of Jentzsch et al. [40] as described earlier by us [36]. Briefly, the sample was incubated with butylated hydroxy tolune, ortho-phosphoric acid and TBA at 90 °C for 45 min, followed by ice-cooling and extraction of MDA–TBA adducts in n-butanol. Absorption was read at 535 and 572 nm for baseline correction in a multimeter plate reader. MDA–TBA adducts
were calculated using the difference in absorption at the two wavelengths compared to the standard curve generated by the use of tetraethoxypropane. Results were expressed in μmol/l or nmol/mg protein.

2.7. Total antioxidant capacity assay

Total antioxidant capacity of low molecular weight non-enzymatic antioxidants in the BALF, plasma and aorta was measured by the method of Benzie and Strain [41] as described earlier by us [1,36]. Briefly, ferric tripyridyltriazine (FeIII-TPTZ) complex reduced by antioxidants present in the sample supernatant is monitored by measuring the change in absorbance at 593 nm. The change in absorbance is directly related to the combined or total reducing power of electron donating non-enzymatic antioxidants present in the reaction mixture. The components of sample that contribute to total antioxidant capacity are ascorbate, alpha-tocopherol, uric acid, bilirubin and the remaining antioxidants. Results were expressed in μmol/l or nmol/mg protein.

2.8. Western immunoblotting

Aliquots of the supernatants isolated from the endothelium intact and denuded aorta (30 μg protein/well) from different groups were separated on 10% SDS-PAGE as described previously by us [38,42]. Proteins

Fig. 1. Effect of allergen challenge and oxidant/antioxidant treatment on airway inflammation. A) Total leukocyte count, B) eosinophil count, and C) H&E staining of lung sections. Airway inflammation in BAL was assessed 24 h after the final allergen challenge through total cell and eosinophil cell counts. Values are expressed as mean ± SE, n = 6–8/group. *P < 0.05, vs. SEN + CHAL group. Each photomicrograph is a representative image from every group (n = 5–6/group; magnification, 100×).
were transferred to nitrocellulose membranes and then probed either with NADPH oxidase-2 (NOX-2) polyclonal goat antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:1000, or GAPDH goat polyclonal antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:5000. This was followed by the incubation with the secondary horseradish peroxidase-conjugated antibody (anti-goat IgG; Santa Cruz Biotechnology, USA) for 1 h at room temperature. For detection of bands, the membranes were treated with enhanced chemiluminescence reagent (Amersham ECL, GE Healthcare) for 0.5–1 min and subsequently exposed to ECL Hyperfilm. The relative expression of the protein bands was quantified by densitometric analysis. Western blot values are expressed in percentage after normalization to GAPDH levels.

2.9. Hepatic, renal and cardiac functions

Serum was separated from the blood for different biochemical analyses. Biochemical estimations to assess hepatic, renal and cardiac functions were carried out in serum by an autoanalyzer (Dimension® RxL Max®, Siemens, USA).

2.10. Lung histology

The lungs were removed from the thorax and fixed with formalin for histological analysis. Formalin fixed lung and aorta were sectioned at a thickness of 5 μm followed by staining with hematoxylin and eosin (H&E) for inflammation related morphology. Sections were examined by brightfield microscopy.

2.11. Chemicals

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (USA).

2.12. Statistical analysis

The data were expressed as mean ± SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey’s multiple comparison tests. A P value of less than 0.05 was considered significant for all statistical tests. All the statistical analyses were performed using GraphPad Prism statistical package.

3. Results

3.1. Effect of oxidant/antioxidant treatment on allergen induced airway and systemic inflammation

As shown in Fig. 1A, allergen challenge led to significant increase in airway inflammation as reflected by an increase in total cell and eosinophil counts (Fig. 1A–B). Treatment with antioxidant, i.e. NAC attenuated, whereas oxidant, i.e. H2O2 enhanced allergen induced airway inflammation (Fig. 1A–B). Histopathological analysis showed trends similar to that observed in BALF, i.e. there was a blockade of inflammatory cell infiltrate into airways and perivascular regions in NAC treated group, whereas H2O2 treated group had greater airway inflammation than observed in allergen sensitized and challenged rats (Fig. 1C).

Fig. 2. Effect of allergen challenge and oxidant/antioxidant treatment on airway oxidative stress. A) ROS production, B) lipid peroxides, and C) protein carbonyls. Values are expressed as mean ± SE, n = 6–8/group. *P < 0.05, vs. SEN + CHAL group.
These data show that antioxidant treatment attenuates whereas oxidant treatment enhances the existing airway inflammation.

3.2. Effect of oxidant/antioxidant treatment on allergen induced airway and systemic/vascular oxidative stress

To know whether oxidant generation in asthmatic airways is associated with systemic/vascular oxidative stress; we investigated airway oxidative stress along with systemic/vascular oxidative stress simultaneously in all the groups. Allergen sensitized and challenged rats had higher airway oxidative stress as assessed by ROS, lipid peroxides, and protein carbonyls (Fig. 2A–C). Increased oxidative stress in airways got reflected in systemic and vascular components as well as of allergen sensitized and challenged rats. The aorta and plasma of allergen sensitized and challenged rats had higher lipid peroxides and protein carbonyls than control group (Fig. 3A–D). Antioxidant treatment decreased whereas oxidant treatment enhanced allergen induced changes in lipid peroxides and protein carbonyls both in the lung and vasculature. Since ROS generation is thought to be a major factor in causing vascular oxidative stress, we measured the source and location of ROS generation in the aortas of different groups. Western blot analysis showed increased expression of NOX-2 in allergen sensitized and challenged aorta as compared to control group (Fig. 4A). Antioxidant treatment decreased whereas oxidant treatment enhanced allergen induced increase in the expression of NOX-2 in the aorta (Fig. 4A). Higher expression of NOX-2 led to higher ROS generation in allergen sensitized and challenged aorta compared to control group (Fig. 4B). The endothelium denuded aorta showed significantly decreased NOX-2 expression (Fig. 4C) as well as ROS generation compared to the endothelium intact aorta in allergen and oxidant challenged animals (Fig. 4D). This suggests that the predominant source of NOX-2 derived ROS is the aortic endothelium in allergen and oxidant challenged animals. Overall, these data show that oxidant generation in asthmatic airways is reflected by systemic/vascular compartment and antioxidant treatment has the potential to dampen oxidant generation in both the lung and vasculature.

3.3. Effect of oxidant/antioxidant on allergen induced airway and systemic antioxidants

Next we wanted to assess the effect of airway oxidative stress on systemic/vascular antioxidants, therefore we measured total antioxidant capacity (TAC) along with thiols to depict overall oxidant–antioxidant load. Allergen sensitized and challenged rats had lower airway antioxidants as assessed by TAC and protein sulfhydryls (Fig. 5A–B). Decrease in antioxidants in airways got reflected in systemic and vascular components as well as of allergen sensitized and challenged rats (Fig. 6A–D). Plasma and aorta of allergen sensitized and challenged rats had lower TAC and thiols than control group. Antioxidant treatment increased whereas oxidant treatment lowered allergen induced changes in TAC and thiols both in the lung and vasculature. These data show that oxidant generation in asthmatic airways decreases antioxidants in both systemic and vascular compartments.

3.4. Effect of oxidant/antioxidant on allergen induced modulation on hepatic, renal and cardiac parameters

Finally to evaluate whether allergen sensitization and challenge also affect other organ systems, we measured biochemical parameters

![Graphs showing the effect of oxidant/antioxidant treatment on various parameters](image-url)
related to renal, cardiac and hepatic functions. Biochemical parameters of renal and cardiac functions were similar among all groups, although hepatic function biochemistry showed elevated levels of albumin and total protein in allergen sensitized and challenged group as compared to control group (Table 1). Oxidant treatment further enhanced allergen induced changes in albumin and total proteins whereas antioxidant...
treatment normalized them. This data shows that airway inflammation and oxidant–antioxidant imbalance not only affect the blood/vasculature but also the other organs as well such as the liver.

4. Discussion

The current study shows that allergen challenge in a murine model of asthma leads to systemic and vascular oxidative stress. Oxidative stress in the vasculature is enhanced by an airway oxidant challenge and attenuated by an antioxidant treatment. To the best of our knowledge, this is the first study to show direct association between airflow and vascular/systemic oxidant–antioxidant imbalance in a murine model of asthma.

ROS are constantly generated in asthmatic airways from various sources such as inflammatory cells or resident cells. These ROS have the capacity to deplete or inactivate antioxidants due to the chronicity of the inflammatory cascade in asthmatic lungs [1,3,15,18,31,42]. ROS have also been shown to be intimately connected to lung inflammation as they have negative correlation with lung function in asthmatics [1,6–8]. Recent studies from our lab have also shown that depletion of antioxidant in the lung may enhance airflow inflammation [31,42]. Therefore, all of these studies suggest a close association between airflow oxidative stress and airflow inflammation. It is also to be noted here that several studies have also shown increased oxidative stress in the systemic circulation of asthmatics [1,11,12,43]. However, whether lung inflammation and oxidative stress have direct relationship with systemic and vascular oxidative stress needs to be investigated.

We further extended these observations in the present study to investigate the oxidant antioxidant profile in the systemic circulation/vasculature in a murine model of asthma. Lipid peroxides/protein carbonyls/ROS generation and TAC/thiol content (combined non-enzymatic antioxidant potential) in combination show overall oxidant load in airways/vasculature [3,44]. Our study showed an increase in ROS generation, lipid peroxides and protein carbonyl levels and a decrease in TAC and thiol content levels in the vasculature after H2O2 inhalation to allergic rats. Vascular and systemic oxidative stress paralleled airway oxidative stress. This was confirmed by both oxidant and antioxidant treatments. Oxidant inhalation to the allergic airways worsened systemic/vascular oxidative stress and depleted antioxidants. On the contrary, NAC treatment to the allergic rats had the opposite effect on antioxidants of the blood and vasculature since TAC and thiol content got increased and oxidative stress decreased. ROS formation can deplete non-enzymatic antioxidants or oxidize proteins leading to the formation of lipid peroxides or protein carbonyls [3,45]. This notion is strengthened by studies in human asthmatic subjects where lipid peroxides/ROS and TAC have been shown to have negative and positive correlations respectively with lung function [1,8,18,19]. Therefore, earlier indirect observations along with our study support the notion that ROS produced in the lung have the capacity to deplete systemic and vascular antioxidants.

Accumulating evidence indicates that asthma-related inflammation impacts vasculature in a negative way and is associated with increased events of cardiovascular complication. Other airway inflammatory disorders such as COPD also have systemic/vascular complications [20–22,46]. For example, arterial thickness and inflammation are increased in asthmatics and there is increased risk of developing atrial fibrillation in asthmatics [20,21,47]. Systemic inflammation in murine model of asthma has been shown previously by us and others. For example, Ponnoth et al. [23] showed increased markers of systemic inflammation along with endothelial dysfunction in a murine model of asthma. Hazarika et al. [25] showed an increase in neutrophils and eosinophils in the bronchoalveolar lavage and blood in a murine model of asthma. Allergen challenge in asthmatics has been shown to increase priming of inflammatory cells in the blood indicating systemic inflammation [24]. These studies along with our study suggest that airway inflammation is associated with systemic inflammation. Our study has attempted to establish a link between oxidative airway inflammation and its effects on vascular/systemic oxidant–antioxidant balance for the first time.

Of all the NADPH oxidase (NOX) isoforms found in the vasculature, NOX-2 is predominantly involved in increased ROS generation under diseased conditions. ROS generated from NOX-2 may be involved in dysregulation of vascular responses through several pathways [48,49]. For instance, ROS produced by immune or endothelial cells can cause endothelial dysfunction by removing nitric oxide from vasculature which is considered to be a vasorelaxant. Our study showed that aortic ROS generation largely originated from endothelial NOX-2 in allergen and oxidant challenged groups as depicted by significantly decreased NOX-2 expression/ROS generation after endothelial denudation in both groups. Ponnoth et al. [23] previously showed decreased endothelial relaxation in allergic mice as compared to normal mice. This could be due to scavenging of NO by NOX-2 derived ROS in the endothelium. Increased vascular ROS may also react with nitric oxide to produce peroxynitrite thereby leading to oxidation of proteins, and increased inflammation, vascular permeability and endothelial dysfunction [26,45,50,51]. Our earlier studies have shown the involvement of NOX-2 derived ROS in dysregulation of airway responses in allergic mouse model [31,38]. This study highlights that allergic airway inflammation also leads to increased ROS generation through NOX-2 upregulation in the vasculature.

ROS generated from NOX-2 could contribute towards the plaque formation and development of atherosclerosis as shown in previous...
studies [48,49,52]. For example, ROS may lead to oxidation of lipids and protein in the vasculature thereby causing injury to the endothelium which is considered to be an initiating event in the development of atherosclerosis [26,48,52]. Our study adds new information in that ROS produced in the vasculature in asthmatics might predispose these patients to increase risk of cardiovascular complications.

We also assessed renal and hepatic functions by measurement of common biochemical tests in serum. There was not any major alteration in any of the biochemical parameters of renal and hepatic functions except albumin and total proteins. Albumin and total proteins were increased in allergen sensitized and challenged rats with a further increase by H2O2 inhalation. On the contrary, NAC treatment normalized these parameters. This seems to be an adaptive change by the liver to counteract increased systemic oxidative stress and to provide the blood and vasculature with continuous supply of antioxidants. It is well known that albumin accounts for >50% of total plasma proteins and provides >2/3 thiol groups to the plasma. Therefore, albumin thiol group is considered to be an important first line of defense against oxidants such as hydrogen peroxide, peroxynitrite, hypochlorous acid and superoxide [53,54]. These data suggest that the liver plays an important role in modulation of vascular/systemic oxidative stress due to asthmatic inflammation as albumin accounts for 10% of the total liver protein synthesis [50]. It also emphasizes the point that airway inflammation induces changes beyond vasculature.

Oxidants produced in the lung may be able to leak into the systemic circulation due to their ability to cause dysfunction of pulmonary endothelial/epithelial barrier. Several studies in vitro and in vivo have shown that oxidants generated during asthmatic inflammation are able to increase airway epithelial and microvascular endothelial permeability [5,27,28,50]. For example, H2O2 increases endothelial protein permeability and also causes human bronchial epithelial barrier dysfunction which is associated with reduced level of tight junction protein in vitro [55,56]. Human and animal studies in vivo also suggest that airway epithelial and endothelial barriers may be compromised by ROS [5,33]. On the other hand, antioxidant treatments reduce epithelial/endothelial dysfunction induced by common airway oxidants [29,33]. Our study corroborates these observations and suggests that oxidative inflammation encountered in asthmatic airways may spill into the systemic circulation which could lead to vascular oxidative stress as well.

In conclusion, the current study shows that ROS produced in airways of asthmatics are involved in vascular/systemic oxidant antioxidant
imbalance. Therefore, there is a need to revisit the notion of antioxidants as an adjunct therapy to asthmatics to cut down the risk of cardio-vascular complications. Newer antioxidants should be designed to address this issue which not only dampen airway but also systemic/vascular oxidative stress and hence inflammation. Whenever that happens, it will be killing two birds with one stone.

Disclosures

The authors declare no conflict of interest.

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