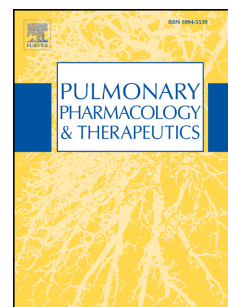


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Andrographolide prevented toluene diisocyanate-induced occupational asthma and aberrant airway E-cadherin distribution via p38 MAPK-dependent Nrf2 induction

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Title Page**Andrographolide Prevented Toluene Diisocyanate-induced Occupational Asthma and Aberrant Airway E-cadherin Distribution via p38 MAPK-dependent Nrf2 Induction**

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Running Title Page

Running Title: Andrographolide prevents occupational asthma

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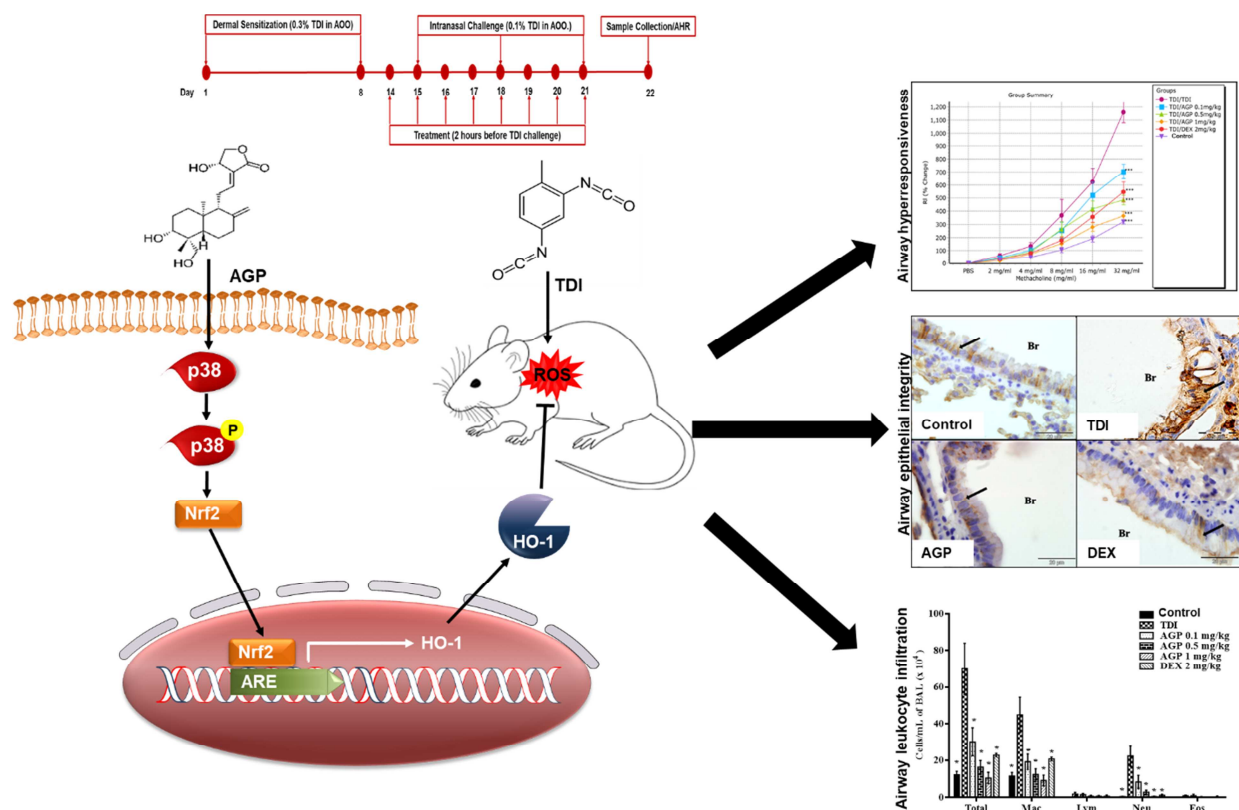
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Abstract

Toluene diisocyanate (TDI) is a major cause of chemical-induced occupational asthma, which contributes about 15% of global asthma burden. Resistance and compounded side effects associated with the use of corticosteroid in asthma necessitate the search for alternative drugs. Andrographolide (AGP), a naturally occurring diterpene lactone is known to exhibit various bioactivities. Its ability to ameliorate cardinal features of allergic asthma was previously suggested in an eosinophilic asthma endotype. However, its potential antiasthma activity and mechanism of action in a neutrophilic occupational asthma model, as well as its effect on epithelial dysfunction remain unknown. BALB/c mice were dermally sensitised with 0.3% TDI or acetone olive oil (AOO) vehicle on day 1 and 8, followed by 0.1% TDI intranasal challenge on days 15, 18 and 21. Endpoints were evaluated via bronchoalveolar lavage fluid (BALF) cell analysis, 2',7'-dichlorofluorescein diacetate (DCFDA) assays, immunoblotting, immunohistochemistry and methacholine challenge test. Decreases in total and differential leukocyte counts of BALF were recorded in AGP-treated animals. The compound dose-dependently reduced intracellular de-esterification of DCFDA, thus suggesting AGP's potential to inhibit intracellular reactive oxygen species (ROS). Mechanistically, the treatment prevented TDI-induced aberrant E-cadherin distribution and restored airway epithelial β -catenin at cell to cell contact site. Furthermore, AGP ameliorated TDI induced pulmonary collagen deposition. In addition, the treatment significantly upregulated pulmonary HO-1, Nrf2 and phospho-p38 levels. Airway hyperresponsiveness was markedly suppressed among AGP-treated animals. Collectively, these findings suggest AGP's protective function against TDI-induced airway epithelial barrier dysfunction and oxidative lung damage possibly through the upregulation of adherence junction proteins and the activation of p38/Nrf2 signalling. This study elucidates the therapeutic potential of AGP in the control and management of chemical-induced allergic asthma. To the best of our knowledge, the potential anti-asthma activity of AGP in TDI-induced occupational asthma has not been reported previously.

Keywords: Asthma; Toluene diisocyanate; Andrographolide; Airway epithelial dysfunction; p-38 MAPK.

Graphical abstract



Introduction

The use of diisocyanate-rich chemicals in several manufacturing processes such as paint and polyurethane foam production has contributed significantly to the rise in work-related respiratory disorders in industrialised nations [1]. Occupational asthma (OA) is the leading work-related respiratory disorder commonly caused by exposure to highly reactive diisocyanate chemicals such as toluene diisocyanate (TDI), hexamethylene diisocyanate (HDI) and diphenylmethane diisocyanate (MDI) [2]. TDI-induced OA (TDI-OA) is characterised by airway inflammation, hyperresponsiveness and remodelling [3]. TDI is considered the most common cause of OA, it induces airway inflammation by interacting with the airway epithelia to trigger cytokine and chemokine productions, thereby enhancing airway inflammatory cell recruitment and survival [4]. Although, the generation of ROS and reactive nitrite species (RNS) is part of normal physiological mechanisms, the levels of these oxidising molecules are usually maintained at low concentrations [5]. The pathogenesis of TDI-OA has been characterised by an imbalance in oxidant and anti-oxidant systems [6–8]. The end target of several signalling pathways affected by TDI exposure commonly involves the suppression of anti-oxidant defence proteins such as heme oxygenase 1 (HO-1), catalases (CAT) and prevention of Nrf2 nuclear translocation [8].

Airway exposure to pro-oxidants has been associated with airway epithelial cell necrosis [9]. Impairment of epithelial barrier function occurs through defective adherens and tight junction formation, thereby enhancing the penetration of potentially toxic elements [10]. Airway epithelial cells are tightly regulated to form an impermeable barrier by junctional proteins such as junctional adhesion molecule, zonula occludens, claudin and occludin, as well as adherens junction (AJ) proteins such as E-cadherin and β -catenin [10]. The stability of epithelial barrier is well maintained by E-cadherin and it has been described as the

‘gatekeeper’ of airway mucosa and allergic sensitisation [11]. Similarly, E-cadherin’s expression is significantly lower in bronchial epithelial cells of asthmatics, thereby compromising the stability of airway epithelial barrier [12,13]. The disruption of the epithelial barrier and loss of airway epithelial integrity contributes to asthma pathogenesis possibly by facilitating easy allergens invasion and presentation to allergen-presenting cells [11,14]. Airway remodelling in TDI exposed animals has been associated with an aberrant distribution of E-cadherin and β -catenin in airway epithelia [3,15,16].

The efficacy of andrographolide (AGP) in the treatment of ovalbumin-induced asthma was previously described [17,18]. Similarly, AGP suppressed epithelial mesenchymal transition of lens epithelial cells by rescuing E-cadherin and inhibiting mitogen-activated protein kinase (MAPK) signalling pathway in growth factor-treated fetal human lens (FHL-124) cell line [19]. Interestingly, AGP induced Nrf2 activation and upregulated HO-1 in primary astrocytes by activating p38 MAPK signalling [20]. These imply that administration of AGP may present a valuable strategy for control and management of oxidative damage and epithelial impairment in TDI-OA. Furthermore, the observed inefficacies of most OA treatments is attributable to oxidative stress. Thus, targeting oxidising molecules alongside inflammatory mediators is considered an indispensable approach to the management of OA [21]. Although the effectiveness of AGP in eosinophilic asthma model has been reported, to the best of our knowledge, there is no report on AGP’s efficacy in chemical-induced asthma model with characteristic airway neutrophilia. This study objectively determined the effectiveness of AGP in the management of chemically-induced OA model and specifically explored its potential mechanism of action in TDI-OA model.

Materials and methods

Materials

Glycine, sodium chloride, N,N'-Methylenebisacrylamide ($\geq 99.9\%$) and tris (≥ 99.8) were obtained from Affymetrix/USB (Cleveland, OH, USA). β -mercaptoethanol, acrylamide and tween 20 were purchased from Merck (Darmstadt, Germany). Ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad (Bio-rad Laboratories, Hercules, CA, USA). Ketamine and xylazine were obtained from Ilium, (Smithfield, NSW, Australia). Quantikine ELISA kits for IL-4, IL-5, IL-13, eotaxin and IFN- γ were acquired from R&D Systems (Minneapolis, MN, USA), whereas IgE and IgG ELISA kits were purchased from Abnova (Taipei City, Taiwan). RNeasy mini kit, RT2 first strand kit, RT2 SYBR® Green ROX qPCR mastermix and custom RT2 profiler array PCR (Custom Catalog Number CLAM23846) were obtained from Qiagen (Hilden, Germany). RNeasy® and T-PER pierce tissue protein extraction reagent were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Methacholine and TDI (98.0%) were obtained from Nacalai Tesque, Inc. (Japan). Rabbit anti-HO-1 (Cat no: 82206), anti-Nrf2 (Cat no: 12721), anti-E-cadherin (Cat no: 3195), anti- β -catenin (Cat no: 8480), anti-p38 MAPK (Cat no: 8690), anti-phospho p38 MAPK (Thr180/Tyr182) (Cat no: 4511), anti-GSK-3 β (Cat no: 9322), anti-phospho GSK-3 β (Ser 9) (Cat no: 9323), anti-Akt (Cat no: 4685) and anti-phospho Akt (Ser 473) (Cat no: 4060) antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). AGP was isolated from *Andrographis paniculata* and its identity was confirmed by direct comparison with standard AGP obtained from Sigma via spectroscopy techniques. Dexamethasone was purchased from Ilium (Smithfield, NSW, Australia).

Animals

Female Balb/c mice, 6 to 8 weeks old (20 – 22 g) were obtained from Institute of Medical Research (IMR), Malaysia. They were maintained in 12-h light/dark cycle (temperature $25 \pm$

2°C) with food and water were given *ad libitum*. All animal experiments were conducted with strict adherence to institutional guidelines specified and approved by Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-R027/2015).

Asthma induction and experimental design

Human exposure to TDI occurs mainly through inhalation and dermal sensitisation [22]. Acetone olive oil (AOO) vehicle used for dissolution of TDI prior to dermal sensitisation and intranasal challenge. An AOO mixture comprising two volumes of acetone and three volumes of olive oil was used for dermal sensitisation, whereas a mixture comprising one volume of acetone and four volumes of olive oil was used for the intranasal challenge. Asthma induction was performed as previously described [23]. Briefly, animals were dermally sensitised (0.3% TDI or AOO vehicle) on day 1 and 8 via the dorsum of both ears (20 µL each). Intranasal challenge (0.1% v/v TDI or AOO vehicle) was conducted on day 15, 18, and 21. AGP (0.1, 0.5 and 1 mg/kg), dexamethasone (2 mg/kg) and vehicle (2% DMSO) were administered (i.p) following prophylactic regimen from day 14 through day 21 (Fig. 1A). Mice were randomised into the following groups: (i) AOO-sensitised, AOO-challenged, and DMSO-treated (AOO/AOO group), (ii) TDI-sensitised, TDI-challenged, and DMSO-treated (TDI/TDI group); (iii) TDI-sensitised, TDI-challenged, and 0.1 mg/kg AGP-treated (TDI/ TDI 0.1AGP); (iv) TDI-sensitised, TDI-challenged, and 0.5 mg/kg AGP-treated (TDI/ TDI 0.5AGP); (v) TDI-sensitised, TDI-challenged, and 1 mg/kg AGP-treated (TDI/ TDI 1AGP); (vi) TDI-sensitised, TDI-challenged, and 2 mg/kg dexamethasone-treated (TDI/ TDI Dex). The doses of AGP used in this study are below its non-observable adverse effect level (NOAEL). The lethal dose (LD₅₀) of AGP was documented to be 11.46 g/kg (i.p) in mice (Bothiraja et al., 2013).

Blood and bronchoalveolar lavage fluid analyses

Mice were anaesthetised (ketamine 100 mg/kg, xylazine 10 mg/kg, i.p) 24 h after the last challenge (day 22), blood samples were collected, allowed to coagulate (3 h, room temperature) and then centrifuged (4,000 rpm, 10 minutes). The serum (supernatant) was harvested and stored at -20°C. The total serum IgE and IgG were measured by ELISA (Abnova, Taipei City, Taiwan) according to the manufacturer's recommendation. Tracheotomy was performed, 500 µL of ice-cold PBS (containing 2.6 mM EDTA) was instilled into the lungs and the bronchoalveolar lavage fluid (BALF) was collected (repeated three times). The total and differential BALF leukocyte count were performed as previously described [26]. The acellular BALF supernatant was stored at -80°C for cytokine analysis. The BALF IL-4, IL-5, IL-13 and IFN- γ were analysed by ELISA as previously described [26].

Intracellular reactive oxygen species assay

To determine the intracellular reactive oxygen species (ROS) generation, BALF cells were harvested 24 hours after last challenge, the cells were rinsed with Hank's balanced salt solution (HBSS) and incubated with 20 µM 2',7'-dichlorofluorescein diacetate (DCFDA) reagent for 60 minutes. DCFDA forms a fluorescent product upon oxidation of dichlorofluorescein by ROS. The fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 535 nm, respectively. The viability of the cells was determined as previously described [27]. The fluorescence intensities were normalised with corresponding cell viabilities, while the ROS level was expressed relative to the AOO controls.

Airway hyperresponsiveness test

To examine the effects of the treatments on airway hyperresponsiveness (AHR) in TDI-OA, the AHR was measured 24 h after the last challenge. Tracheotomy was performed on anaesthetised animals, the trachea was intubated with 19G blunt cannula, the airway resistance (RI) and dynamic lung compliance (C_{dyn}) in response to graded concentrations of nebulised methacholine (0, 2, 4, 8, 16, 32 mg/mL) were recorded using FinePointe™ RC System (Buxco Research Systems, New Brighton, MN, USA). The results were analysed as a percentage relative to baseline PBS response (% change).

Histopathological staining and immunohistochemistry

The left lung was collected, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin for histopathological analysis. Examination of inflammatory cell infiltration, collagen deposition and mucus production were performed by H&E, Masson trichrome and periodic acid Schiff (PAS) stains, respectively. The stained tissue sections (5 µm) were scored as described previously [17,28–30]. Immunohistochemical staining for E-cadherin and β-catenin proteins were conducted as described previously [31]. Briefly, lung sections were deparaffinised, rehydrated, subjected to heating for antigen retrieval and then treated with 3% H₂O₂ for 10 min to block endogenous peroxidase. Upon incubation in recommended dilutions of anti-E-cadherin and anti-β-catenin antibodies (4°C, overnight), the sections were probed with secondary antibody, signals were developed using diaminobenzidine (DAB) chromogen and counterstained with haematoxylin.

Western blotting

Lung tissues from the superior and middle lobes were homogenised in protease and phosphatase inhibitor supplemented T-PER tissue protein extraction reagent. The protein extracts were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane.

The blots were probed for E-cadherin, β -catenin p-38, p-p-38, Akt, p-Akt, GSK-3 β , p-GSK-3 β , Nrf2 and HO-1. Bands were detected using WesternBright™ ECL substrate (Advansta, Inc). Blots were stripped and re-probed for loading control (β -actin).

RT2 qPCR array

Isolation of RNA from the inferior and postcaval lobes was performed using Qiagen RNeasy mini kit (Hilden, Germany) according to manufacturer's instruction. The quantity, purity and integrity of the RNA samples were determined. cDNA was synthesized from 400 ng of RNA using the RT² First Strand cDNA synthesis kit (Qiagen, Hilden, Germany). Custom RT² qPCR array (SABiosciences, Qiagen, Frederick, MD, United States, Cat #: CLAM23846) containing pre-validated primers for 17 genes of interest (GOI) comprising of oxidative stress-related, Th2, Th1, extracellular matrix related and muc5ac genes was used for this analysis. β -actin and GAPDH were used as reference genes. Real-time PCR was performed on an Eppendorf Mastercycler *ep* realplex 4S (Eppendorf, Hamburg, Germany) using RT² SYBR® Green ROX qPCR Mastermix (Qiagen, Hilden, Germany). The PCR amplification assay, melting curve analysis, determination of PCR efficiency, genomic DNA contamination and reverse transcription efficiency were conducted as described previously [32].

Statistics

Data analysis was performed by one-way ANOVA using GraphPad Prism 6 (GraphPad Software, San Diego, CA). AHR data were evaluated by repeated measures ANOVA. Non-parametric data were analysed by Kruskal–Wallis Test. RT2 qPCR data were analysed on SABioscience PCR Array Data Analysis Web Portal (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Bonferroni post-hoc test was performed for multiple comparisons. Mean scores for all measurements were expressed as the mean $\pm$ SD. Significance level (α) was set at $p < 0.05$.

Results

AGP Inhibited airway inflammatory cell recruitment in TDI-OA models

To determine the extent of TDI induced airway leukocyte infiltration, BALF leukocyte analysis was conducted. Airway TDI sensitisation and challenge resulted in about eight-fold increase in total BALF leukocytes relative to AOO control (Fig. 1B). Among the four different categories of leukocyte that were identified and counted, the absolute count of macrophages and neutrophils were prominently increased. A transient increase in neutrophil distribution (31%) was observed in TDI-OA animals. AGP administration significantly decreased both the total and differential BALF leukocyte counts, therefore substantiating the anti-inflammatory activity of the intervention.

Administration of AGP downregulated BALF intracellular ROS level in TDI exposed mice

An investigation into the tendency of AGP to inhibit TDI-induced generation of intracellular ROS revealed that TDI exposure induced 2.3 fold increase in DCFDA fluorescence intensity relative to BALF cells collected from AOO control animals (Fig. 1C). When treated with AGP, ROS level was dose-dependently reduced by a maximum of 50% relative to TDI-induced levels.

Effect of AGP on AHR

Airway hyperresponsiveness, an important cardinal feature of asthma, was assessed in TDI-OA. Relative to PBS challenge, TDI-exposed animals had an unprecedented increase in RI following methacholine challenge (Fig. 1D). This increase was statistically significant when compared to AOO controls. Administration of AGP dose-dependently attenuated TDI-induced RI amplification. Percentage change in Cdyn was marginally decreased by TDI exposure ($p=0.051$ relative to AOO, Fig. 1E). The administration of AGP notably improved

the Cdyn without any statistical relevance. These results indicate the antagonistic role of AGP against TDI-induced AHR.

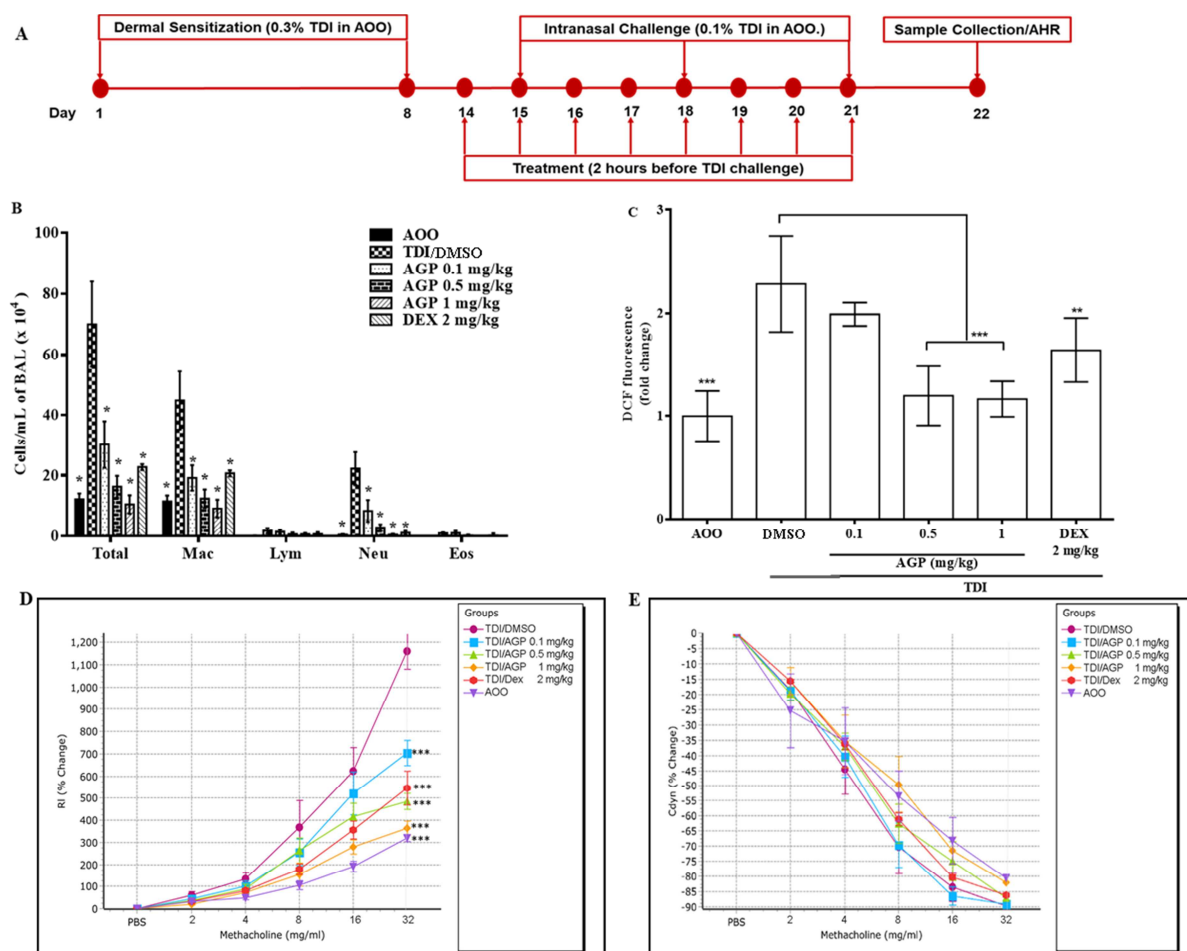
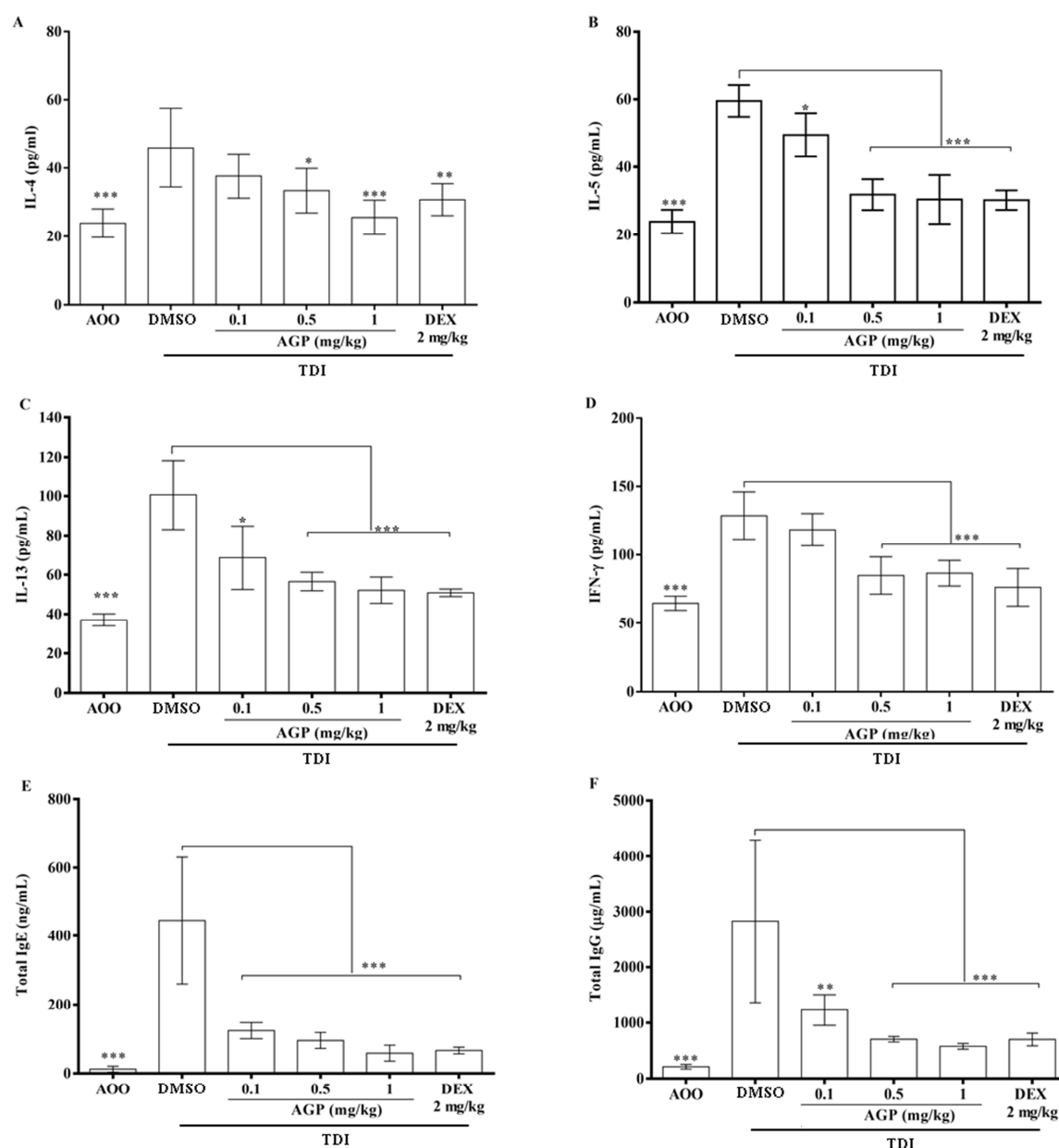


Fig. 1: AGP decreased airway hyperresponsiveness, BALF leukocyte counts and ROS production in TDI-sensitised and challenged mice. (A) Animals were dermally sensitised and intranasally challenged with TDI in a 22-day asthma induction protocol, (B) Dose-dependent reversal of TDI-induced increase in total and differential leukocyte counts was observed, (C) The TDI-induced transient surge in intracellular ROS production was reversed by administration of AGP. (D) Airway resistance (RI) and (E) dynamic lung compliance (Cdyn) were dose-dependently improved in AGP treated animals. Values are expressed as mean $\pm$ SD ($n = 5 - 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ level of significance in comparison to untreated TDI controls.

Effects of AGP on BAL fluid cytokines and serum immunoglobulins

Evaluation of BALF cytokine profile was conducted using ELISA technique. TDI sensitisation and challenge notably increased BALF IL-4, IL-5, IL-13 and IFN- γ (Fig. 2). However, TDI exposure induced greater increase in BALF IL-13 level. A dose-dependent

288 decrease in the concentrations of all the cytokines was observed in AGP treated animals. TDI
289 exposure also triggered a greater increase in serum total IgE and IgG. These serum
290 immunoglobulins were dose-dependently downregulated by AGP.



291

292 **Fig. 2: AGP downregulated BALF th2 cytokines and serum immunoglobulin levels in TDI-**
293 **sensitized and challenged mice.** Administration of AGP dose-dependently decreased (A) BALF IL-
294 4, (B) IL-5, (C) IL-13 and (D) IFN- γ as well as (E) total serum IgE and (G) IgG levels. Data are
295 expressed as mean $\pm$ SD (n= 4 – 6). *p<0.05, **p < 0.01, ***p < 0.001 imply level of significance
296 relative to TDI control.

297

298 **AGP attenuated pulmonary inflammation, airway mucus secretion and collagen**
299 **deposition in the lungs of TDI exposed mice**

300 TDI sensitisation and challenge triggered pulmonary leukocyte infiltration in the
301 peribronchial and perivascular spaces. This was affirmed by semi-quantitatively scoring H&E
302 stained lung sections from both treated and untreated animals. AGP inhibited both the
303 peribronchial and perivascular inflammation (Fig. 3A and 3B respectively). Intranasal
304 exposure to TDI induced moderate goblet hyperplasia with an average of 30% PAS-positive
305 cells in the bronchial lining (Fig 4). AGP dose-dependently and significantly ($p<0.001$)
306 ameliorated airway mucus production and goblet metaplasia. In addition to airway
307 inflammation and mucus secretion, TDI also enhanced collagen deposition in bronchial
308 subepithelia (Fig. 5). Semi-quantitative analysis of collagen deposition showed about 9-fold
309 increase in peribronchial collagen score relative to AAO controls. Treatment with AGP
310 decreased collagen deposition around the bronchioles of TDI exposed animals. Athletes

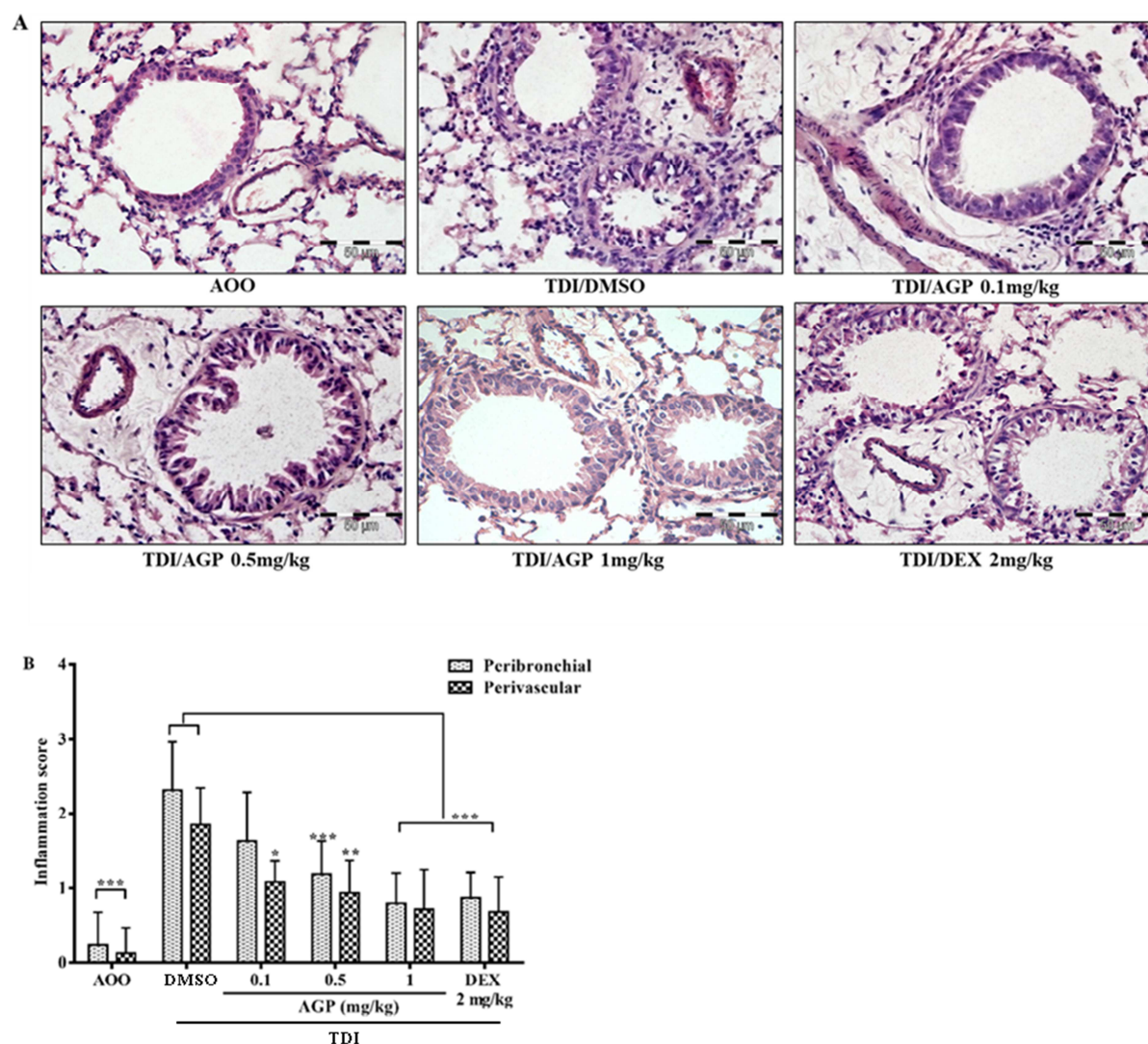


Fig. 3: Photomicrograph of haematoxylin and eosin (H&E) stained lung sections of TDI sensitised and challenged animals. (A) TDI induced accumulation of inflammatory cells in the peribronchial and perivascular spaces. (B) Administration of AGP significantly inhibited peribronchial and perivascular leukocyte infiltration as determined by semi-quantitative scoring. Interpretation of scores: 0= no detectable inflammation, 1= mild inflammation (occasional, <20 inflammatory cells in peribronchial or perivascular space), 2= moderate inflammation (1- 2 layers of 20 – 100 inflammatory cells in peribronchial or perivascular space), 3= severe inflammation (> 2 layers of more than 100 inflammatory cells in peribronchial or perivascular space). Data are presented as mean $\pm$ SD (n=6). * p <0.05, ** p <0.01, *** p <0.001 shows significance level in comparison to TDI controls.

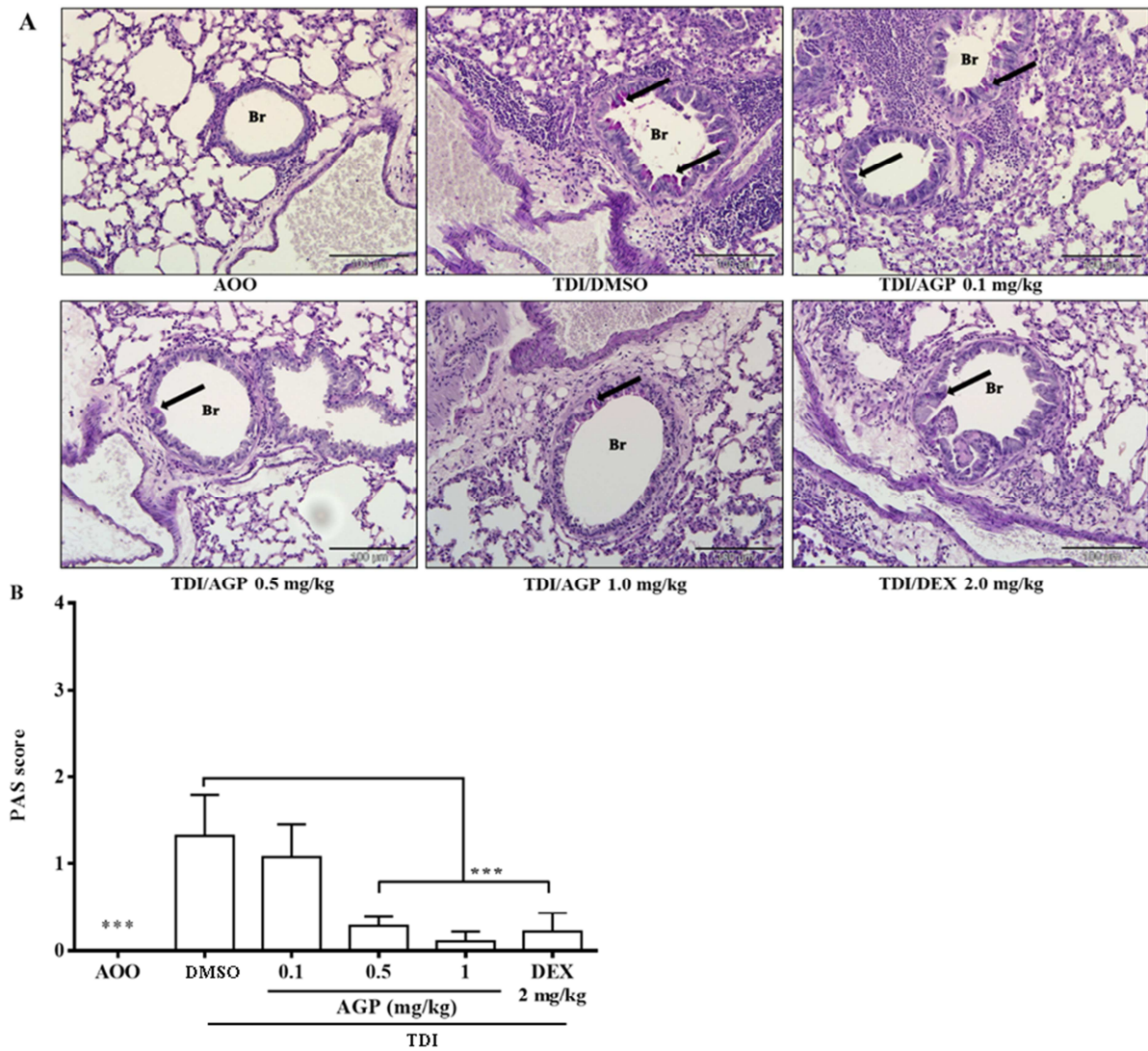


Fig. 4: Photomicrographs showing AGP-mediated suppression of airway mucus secretion in TDI sensitised and challenged animals. TDI exposure resulted in (A) increased bronchial (Br) mucus secretion (black arrow). (B) Semi-quantitative scoring showed that administration of AGP (especially 0.5 and 1 mg/kg) decreased the amount of bronchial PAS-positive cells (magenta coloured cells in the airway). Interpretation of scores: 0= no goblet metaplasia, 1= < 25% PAS positive airway goblet cells, 2= 25%- 49% PAS positive airway goblet cells, 3= 50%-75% PAS positive airway goblet cells, 4= > 75% PAS positive airway goblet cells. (n=6). Data are presented as mean ± SD. ***p<0.001 shows significance level in comparison to TDI controls.

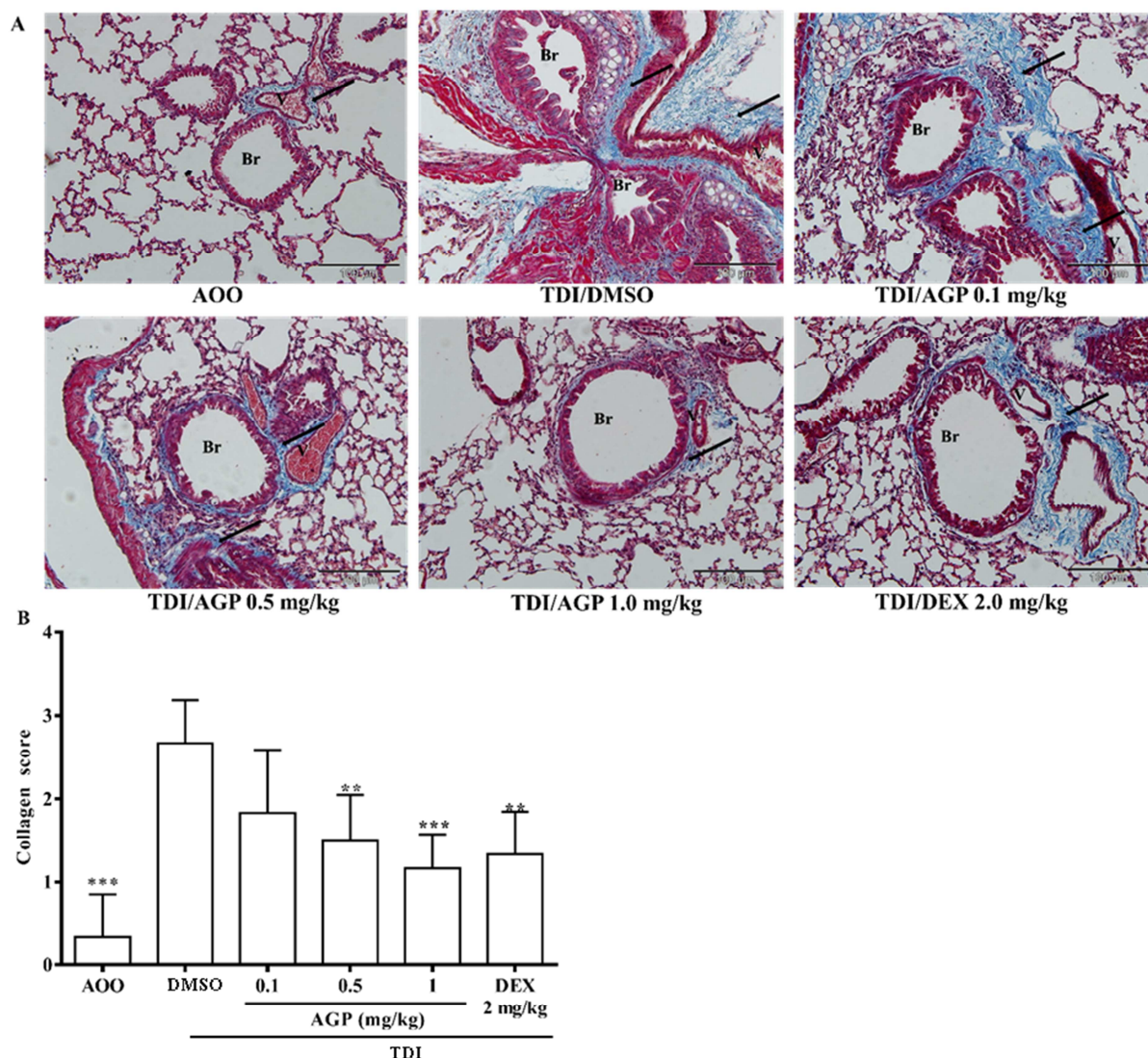


Fig. 5: Photomicrographs showing AGP-mediated reduction in airway collagen deposition in TDI-induced asthma. (A) Lung sections were stained with Masson's trichrome to assess collagen deposition in treated and untreated animals. Black arrows indicate collagen deposits (stained blue), cytoplasm stained pinkish red, smooth muscles, basement membrane and red blood cells stained red. (B) Semi-quantitative scoring showed that AGP administration significantly reduced subepithelial collagen deposition around both the bronchioles (Br) and blood vessels (V). Score interpretation: 0= no collagen in peribronchial space, 1= a thin layer of peribronchial collagen deposits, 2= a cluster of peribronchial collagen deposits, and 3= a thick layer of peribronchial collagen deposits. Data are presented as mean $\pm$ SD (n=6). * p <0.05, ** p <0.01, *** p <0.001 shows significance level in comparison to TDI controls.

AGP restored the apicolateral distribution of E cadherin and β -catenin in bronchial epithelia of TDI-exposed mice

Epithelial barrier function was assessed based on the morphology of adjacent bronchial epithelial cell to cell contact and the apicolateral distribution of E-cadherin and β -catenin. The cell to cell contact of AOO controls appeared intact, whereas that of untreated TDI-exposed animals was obviously disrupted (Figs. 6 and 7). The administration of AGP narrowed the TDI-induced widening of airway epithelial intercellular space. Furthermore, as revealed by immunohistochemical staining, the examined AJ proteins are localised at the lateral and apicolateral borders of airway epithelial cells in unexposed animals (AOO). TDI sensitisation and challenge decreased the immunoreactivity of both airway epithelial β -catenin (Fig 6) and E-cadherin (Fig. 7) at the cell to cell contact site. TDI particularly triggered cytoplasmic diffusion of E-cadherin and downregulation of β -catenin in airway epithelial cells. Furthermore, the alveoli showed abundant expression of β -catenin in both AOO and TDI-exposed animals (Fig. 6). Administration of AGP, especially 0.5 and 1 mg/kg doses, rescued TDI-induced aberration of airway E-cadherin and β -catenin distribution. The treatments substantially restored airway epithelial apicolateral distribution of the proteins by upregulating epithelial β -catenin levels and averted cytoplasmic diffusion of E-cadherin. Western blotting was performed to evaluate the effect of TDI and AGP administration on total pulmonary E-cadherin and β -catenin levels. Relative to the AOO controls, the TDI exerted no marked effect on the total amount of E-cadherin in the lungs. However, AGP seemed to upregulate the basal total E-cadherin levels by up to two folds in 1 mg/kg treated animals (see Fig. 8). Interestingly, TDI exposure slightly lowered total β -catenin in the lungs. This slight reduction was averted by AGP administration (Fig. 8).

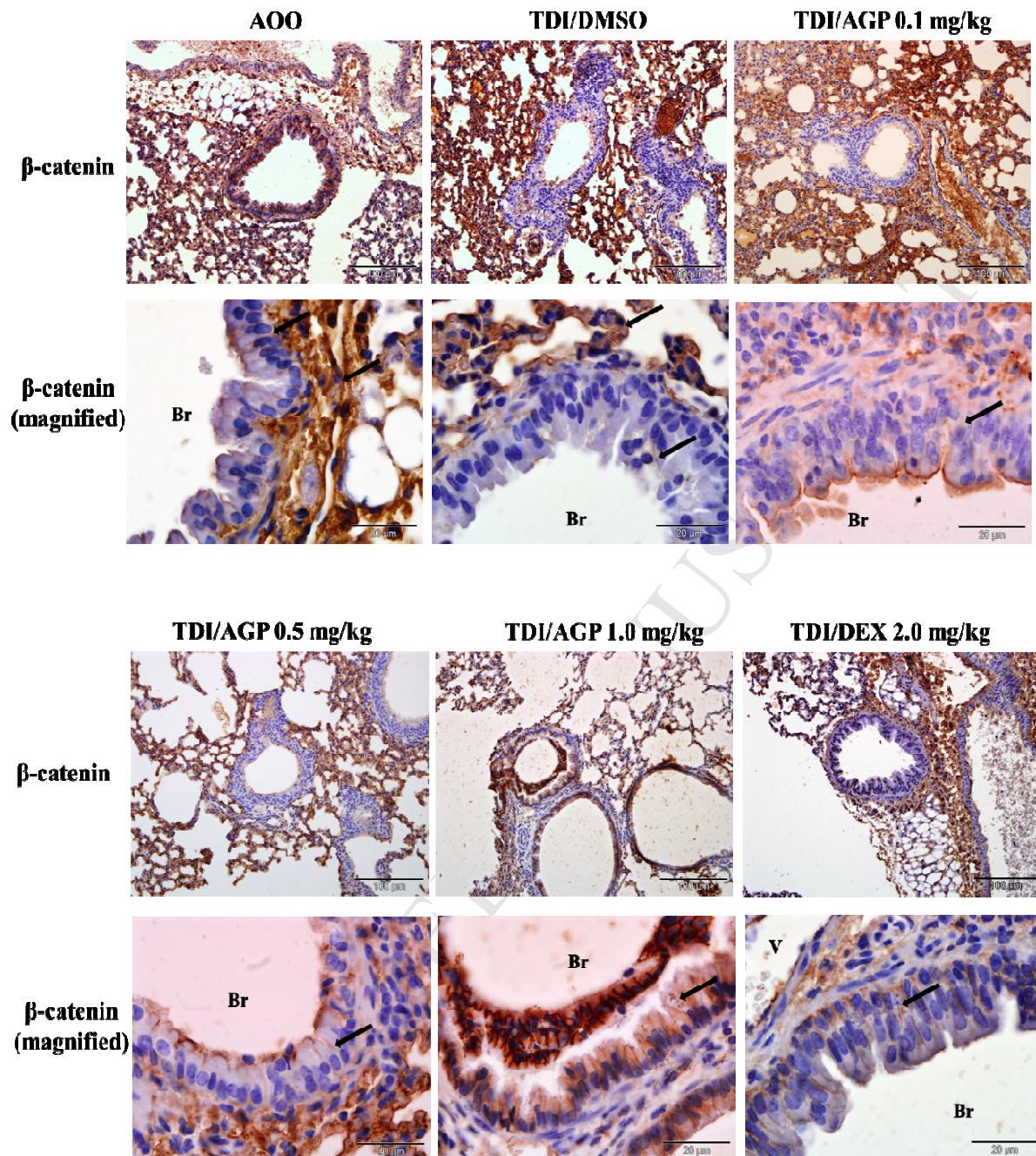


Fig. 6: Immunoreactivity of β -catenin in lungs of AGP treated animals. Low (rows 1 and 3) and high (rows 2 and 4) magnification photomicrographs showing the alveolar and apicolateral distribution of β -catenin. Note the marked downregulation of epithelial β -catenin in TDI/DMSO group and its restoration in the treatment groups. Acetone-olive oil control-(AOO); dexamethasone-(DEX).

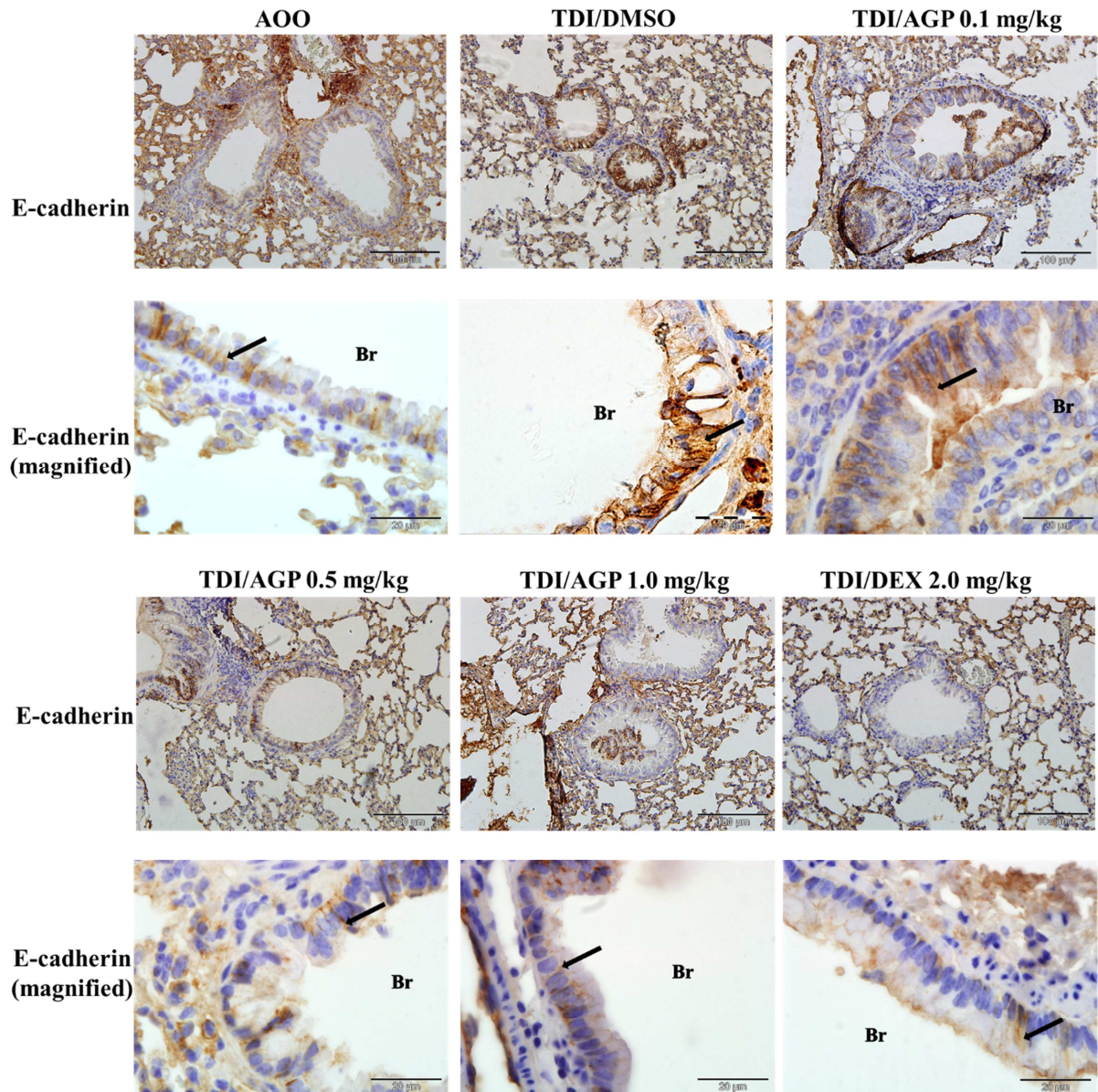


Fig. 7: Immunoreactivity of E-cadherin in lungs of andrographolide (AGP) treated animals. Low (rows 1 and 3) and high (rows 2 and 4) magnification photomicrographs showing the alveolar and apicolateral distribution of E-cadherin. Note the substantial cytoplasmic diffusion of airway E-cadherin in TDI/DMSO group. Administration of AGP restored apicolateral distribution and reversed the cytoplasmic diffusion of airway epithelial E-cadherin.

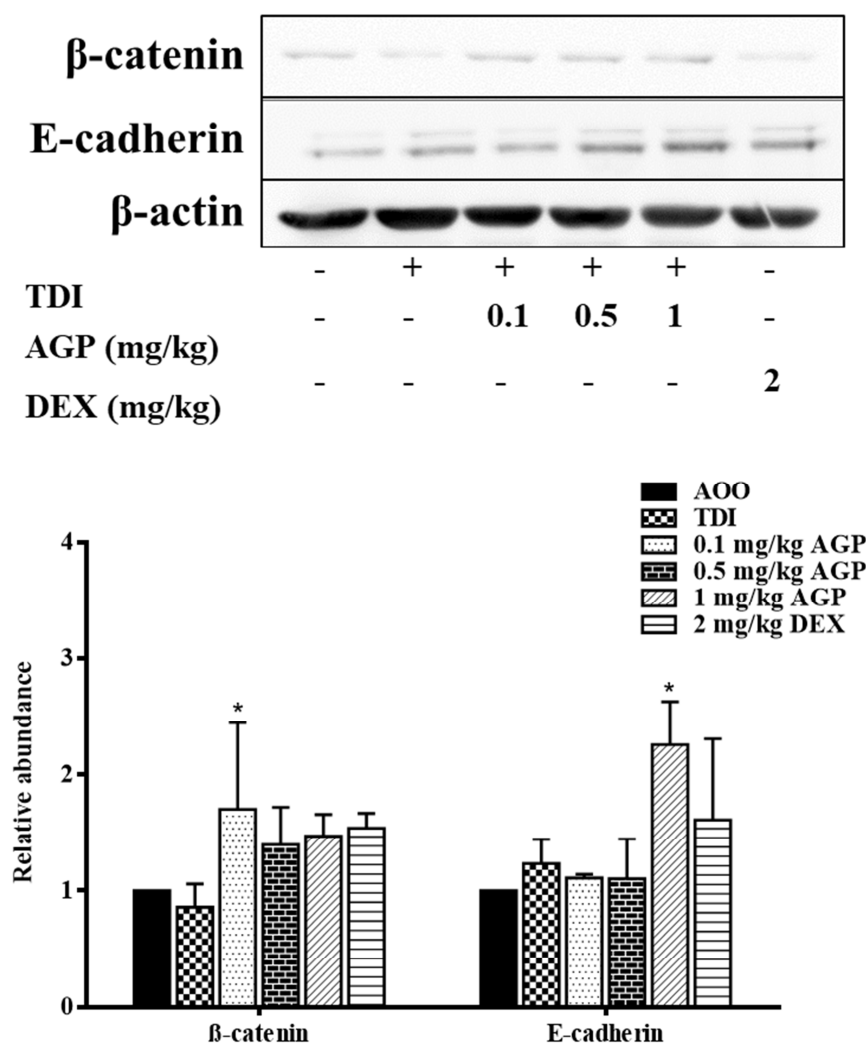


Fig. 8: Upregulation of total pulmonary E-cadherin and β-catenin in AGP treated animals. TDI sensitisation and challenge mildly decreased total β-catenin levels, this was improved by AGP administration. Although TDI sensitisation and challenge did not affect total E-cadherin levels, AGP administration increased total pulmonary E-cadherin level beyond the basal level. The blot is a representative of three different animals. Densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA). Data are presented as mean ± SD (n=3). *p<0.05 relative to TDI.

AGP upregulated Nrf2 and HO-1 through Akt and p38 dependent pathways

To further elucidate the mechanism of AGP's inhibition of TDI-induced airway oxidative damage in OA model, the pulmonary levels of HO-1, a phase II enzyme that protects against oxidative stress and its regulatory transcription factor, Nrf2, were evaluated. Selected upstream Nrf2 regulatory proteins (Akt, p38 MAPK, and GSK-3β) were also evaluated by

western blotting. TDI exposure upregulated pulmonary HO-1 level, whereas it induced only a slight increase in total Nrf2 level (Fig. 9). Treatment with AGP increased p38 MAPK activation, total Nrf2 and HO-1 levels. Relative to AOO, the level of Akt appeared unaffected by TDI exposure. However, a slight increase in pAkt level was observed at low and median doses of AGP, whereas 1 mg/kg AGP marginally inhibited Akt phosphorylation. TDI exposure induced mild reduction in p38 phosphorylation, interestingly, AGP stimulated a dose-dependent increase in p38 phosphorylation at Thr180/Tyr182 residue, Nrf2 and HO-1 levels. The results showed p38 mediated induction of Nrf2 and HO-1 in AGP treated animals (Fig. 9). Slight induction of GSK-3 β phosphorylation at serine residue 9 was witnessed in AGP treated animals.

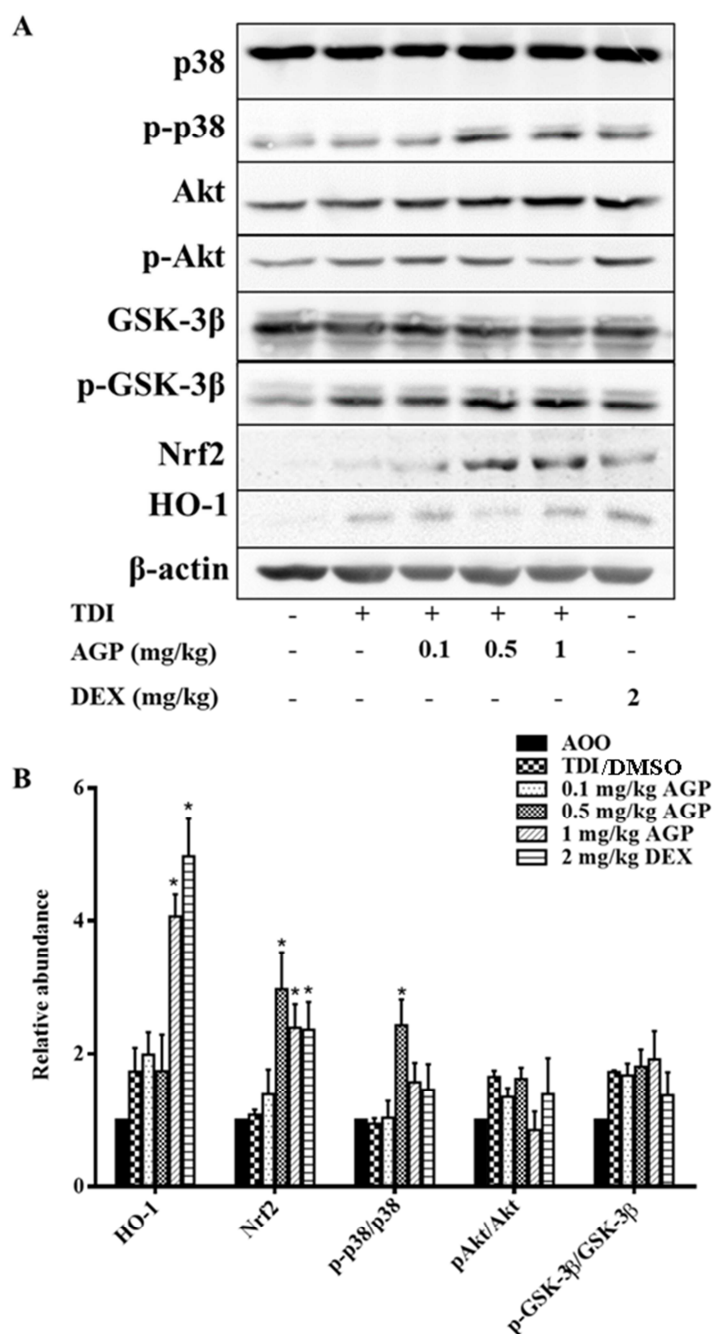


Fig. 9: Andrographolide (AGP) mediated upregulation of HO-1. (A) Levels of HO-1, Nrf2, GSK-3β, p-GSK-3β, Akt, p-Akt, p38 and p-p38 in TDI induced asthma. (B) Immunoblot analysis showed a dose-dependent increase in p-p38 and Nrf2. The blot is a representative of three different animals. Densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA). Data are presented as mean ± SD (n=3). *p<0.05 relative to TDI, #p<0.05 relative to AOO.

Effects of AGP administration on expression of inflammatory and oxidative stress-related genes in TDI induced asthma models

Sequel to the role of Nrf2 in AGP-mediated inhibition of TDI-OA, the pulmonary expression of antioxidant (*Nfe2l2* and *Hmox1*) and pro-oxidant (*Duox1*) genes were evaluated. TDI exposure resulted in slight reduction in *Hmox1* expression (Fig. 10A). However, it did not show statistical significance relative to AOO controls. Interestingly, basal *Nfe2l2* expression was not affected by TDI exposure. Furthermore, the expression of *Duox1* was mildly increased in TDI-exposed animals. Administration of AGP dose-dependently increased both *Nfe2l2* and *Hmox1* expressions, whereas a decrease in *Duox1* expression occurred independent of AGP dose (Fig. 10A). The role of specific Th2 inflammatory genes (*Il4*, *Il5*, *Il13*, *Serpinb2* and *Clca3a1*) in TDI-induced OA were further analysed. There was a notable increase in the expressions of *Il4*, *Il5* and *Il13* in untreated TDI sensitised and challenged mice (Fig. 10B). The expression of these genes was downregulated following eight-day AGP treatment regimen. *Serpinb2* and *Clca3a1* expressions were only slightly increased in TDI exposed animals. Although, AGP treatment downregulated the expression of *Serbinb2*, *Clca3a1* remained unaffected in AGP-treated animals. Highest downregulation of TDI-induced *Il5*, *Il13* and *Serpinb2* gene expressions were observed in 1 mg/kg AGP treated animals.

Considering the mixed Th1/Th2 BALF cytokine profile observed in TDI-exposed animals (Fig 2), the expressions of specific Th1-related genes (*Ifng*, *Tnf*, *Ccl5* and *Cxcl2*) were evaluated by RT2 qPCR. TDI sensitisation and challenge increased the expressions of these Th1 genes by 3 to 6 folds (Fig. 10C). *Cxcl2* expressions was dose-dependently downregulated ($p < 0.05$). Although *Ifng* showed marked downregulation in AGP (1 mg/kg) treated animals relative to untreated TDI controls, the downregulation was not statistically significant. As illustrated in Fig. 10C, the expression of *Tnf* gene was only slightly modulated

by the treatment. The genetic expression of extracellular matrix (*Mmp9*, *Timp*, *Cldn1* and *Chil4*) and Mucus (*Muc5ac*)-related genes were assessed in the lungs of TDI exposed animals. The results showed a remarkable dose-dependent decrease in the expressions of *Muc5ac* and *Chil4* genes in AGP-treated animals relative to TDI groups (Fig. 10D). The expression of *Mmp9* was slightly increased by TDI exposure and it was not reversed by AGP administration. Although the expression of *Timp* seemed unaffected by TDI exposure, administration of AGP mildly upregulated its expression particularly at 0.5 and 1 mg/kg doses. *Cldn1* expression was only slightly downregulated by TDI exposure. Administration of AGP resulted in considerable increase in *Cldn1* expression (Fig. 10D).

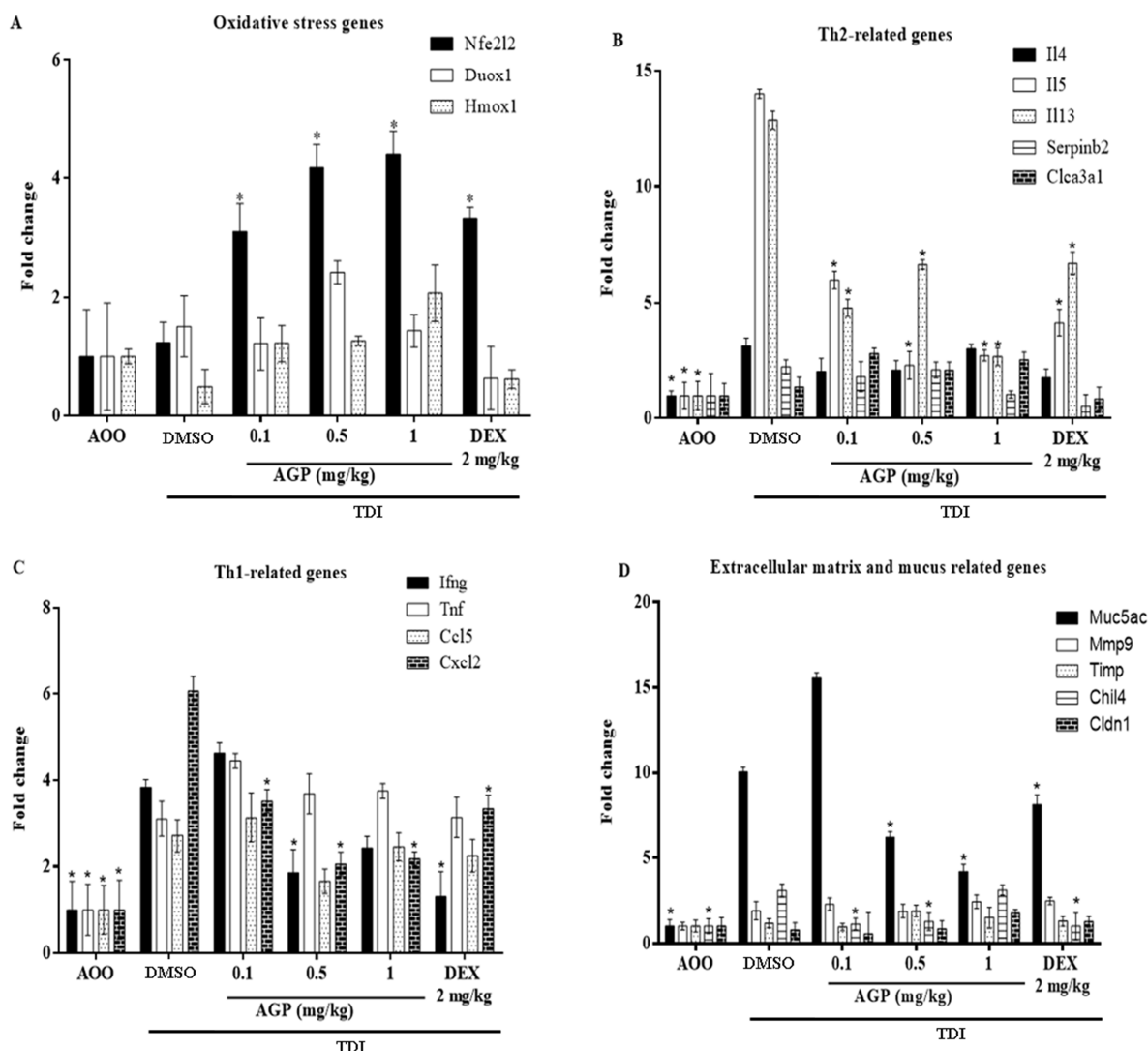


Fig. 10: Expression of oxidative stress, Th2, Th1, extracellular matrix and mucin genes in TDI exposed animals. Administration of AGP improved the expression of *Nfe2l2*, and *Hmox1* genes (A). Furthermore, AGP dose-dependently reversed TDI-induced upregulation of Th2 (B) and Th1 (C) related genes. The expression of *Muc5ac* and *Chil4* genes were significantly upregulated by TDI (D) in asthmatic mice as determined by RT2 qPCR arrays analysis. Results are presented as mean $\pm$ SEM, * $p < 0.05$, (n=3 to 4). DEX: dexamethasone.

Discussion

The role of AGP in the control and management of asthma has been suggested by numerous researchers [17,18,33,34]. However, most of these studies only evaluated the activity of AGP in OVA asthma models and LPS/IFN- γ -induced airway inflammatory models. The potential role of AGP in ameliorating occupational asthma has not been reported to date. TDI, a highly reactive chemical, has been identified to be a low-molecular-weight trigger of allergic

asthma, especially among individuals that are regularly exposed to it in their working environment [35,36]. This study evaluated the therapeutic potential of AGP in a TDI-induced asthma model.

As illustrated in Fig. 1B, TDI exposure triggered airway inflammation characterised by neutrophilia and peribronchial leukocyte infiltration in BALB/c mice. Depending on the adopted induction protocol and the choice of mouse strain, TDI could trigger varying BALF cellular phenotypes [35–37]. According to Ban and colleagues [35], TDI sensitisation by topical application and tracheal instillation, followed by tracheal challenge with 0.1% TDI at days 18, 28 and 31 resulted in BALF eosinophilia with characteristic Th2 dominance. On the contrary, Tarkowski and colleagues reported high (40%) neutrophilia in a 10 day TDI asthma model that was established by repetitive dermal sensitisation (0.3% TDI) on day 0 and 7, coupled with an intranasal challenge with 0.1% TDI on day 10 [37]. In addition, the time-point at which the BALF was collected seems to play an essential role in determining the percentage distribution of BALF eosinophils and neutrophils. The use of dermal sensitisation and intranasal challenge, alongside BALF collection at 24 hours after the last TDI challenge had consistently produced asthma with characteristic airway neutrophilia [23,37–39]. In our model, the BALF was collected 24 hours after the last TDI challenge, and airway neutrophilia was the resultant effect.

Aggravation of Th2 responses due to Th1-Th2 imbalance is typically thought to be the major developmental pattern for allergic diseases [40,41]. It was however observed, that TDI exposure triggered mixed Th2/Th1 immune responses and concomitant upregulation of their corresponding genetic expressions. The slight increase in the expression of Th2 determinants (*Serpinb2* and *Clca3a1*) coupled with that of *Il4*, *Il5* and *Il13* (Fig. 10) in TDI sensitised and challenged animals confirmed that the model is Th2 skewed. Using three-gene signature

pattern, asthma has been categorised into Th2-high and Th2-low asthma [42]. These genes include the *Periostin*, *Serpinb2* and *Clca3a1*, both of which are tightly regulated by *IL-13* activity [42–45]. Apart from these genes, Tregs have proven to be an important determinant of Th1, Th2 and Th17 polarisation in inflammatory diseases [41]. An elucidation of the function and kinetics of Tregs in TDI sensitisation revealed the suppression of both Th1 and Th17 mediated responses by expanded Tregs cells from TDI-sensitised mice, perhaps, influencing Th2 inflammatory responses in TDI-exposed animals [41].

The decrease in both BALF Th1 (IFN- γ) and Th2 (IL-4, IL-5 and IL-13) cytokines among AGP treated animals could partly be due to the downregulation of their corresponding genes. Previous studies have demonstrated AGP's potential to prevent induction of IFN- γ , IL-4, IL-5 and IL-13 genes in various models of inflammatory disorders including asthma and acute lung injuries [17,34,46]. Apart from the heterogeneity of the inflammatory responses, TDI sensitisation and challenge also triggered desquamation of airway epithelium, collagen deposition and intracellular ROS surge. These suggest the presence of airway remodelling and oxidative damage in TDI-exposed animals [3,8,47–49].

Airway epithelium is directly damaged by exposure to TDI, thereby resulting in increased airway epithelial permeability and loss of epithelial integrity. Apart from the mechanistic activation of inflammatory responses in airway epithelium, the permeability of bronchial epithelial cells was reportedly influenced by TDI-induced ROS surge [50]. In our study, TDI exposure did not only increase BALF intracellular ROS, but it also affected the airway epithelial distribution of AJ proteins (E-cadherin and β -catenin). Loss of proteins responsible for the maintenance of airway integrity and survival has been connected to TDI-induced airway inflammation. These include proteins such as folliculin, zonula occludens, claudins, occludin, junctional adhesion molecule, E-cadherin and β -catenin [3,15,51]. The attenuation

of aberrant E-cadherin and β -catenin distribution in the airways of TDI-exposed animals possibly occurred through the antioxidant activity of AGP [52]. However, there is need for future time point evaluation of the direct effect of AGP on the distribution of membrane E-cadherin and β -catenin in isolated cells/normal bronchial epithelial cell line.

Suppression of cigarette smoke-induced airway oxidative damage was observed following the administration of AGP [53]. This was achieved by enhancing Nrf2 nuclear retention and binding to antioxidant response element (ARE) of the DNA double helix. In agreement with our findings, AGP augmented the pulmonary level of HO-1 and Nrf2 transcription factor [53–55]. HO-1, a Nrf2 regulated protein catalyses the degradation of haem, a potent oxidant, to generate biliverdin, iron and carbon monoxide [56]. Biliverdin is converted to bilirubin, an endogenous anti-oxidant, while the iron is sequestered by ferritin, leading to an additional anti-oxidant effect [56]. Carbon monoxide possesses anti-inflammatory properties, suggesting that HO-1 plays a vital role in defending the lung against inflammatory and oxidative damage [56,57].

It was recently suggested that AGP increases HO-1 protein and its mRNA expressions through a p38 MAPK-Nrf2 dependent pathway [58]. Moreover, AGP was shown to modulate the PI3K/Akt pathway, an important upstream regulator of p38 [59,60]. In the present study, the level of Akt activation (p-Akt/Akt ratio) was slightly increased in the TDI-exposed group, which tallies with previous findings [61,62]. Besides, p-Akt induction has been linked to oxidative stress and inflammatory responses in asthma [63–65].

Attenuation of TNF- α induced ROS generation in AGP treated EA.hy926 endothelial cells was postulated to have occurred via upregulation of Nrf2 [59]. Similarly, pre-treatment of human hepatocellular carcinoma cells (HepG2) with 20 μ M AGP prevented H₂O₂ induced

oxidative damage by upregulating Nrf2 through Akt phosphorylation [54]. This indicated the possible role of TDI-induced ROS generation in steering AGP's activity toward the upregulation of Nrf2 and HO-1. Although it is unclear if AGP-induced Akt phosphorylation could directly enhance Nrf2 and HO-1 upregulation, it was established the Akt directly phosphorylates Ser9 residue of glycogen synthase kinase 3 β (GSK-3 β) to render it inactive [54]. GSK-3 β , when active, it inhibits Nrf2 in a Kelch-like ECH-associated protein 1 (Keap1)-independent manner, whereas its phosphorylated form (inactive GSK-3 β) enhances Nrf2 nuclear translocation and retention [54,66]. Thus, inactivation of GSK-3 β will allow binding of Nrf2 to its antioxidant response element and subsequently, transcribe HO-1 activation as witnessed in AGP treated animals. Although we have demonstrated a possible link between Akt phosphorylation and HO-1 upregulation, our findings suggest the involvement of p38 in the activation and upregulation of Nrf2 activity. Kahweol, a diterpene commonly found in coffee beans was observed to induce HO-1 upregulation via p38/Nrf2 signalling in ROS surged SH-SY5Y neuroblastoma cells [67]. The marked decline of p-p38 among TDI controls and its upregulation among AGP treated animals suggests the activation of p38 MAPK by AGP in TDI sensitised and challenged mice. Interestingly, no significant change was observed in total amount of Nrf2 among untreated TDI controls. Time point evaluation of the direct effect of TDI on Nrf2 may show the point at which TDI could have induced Nrf2 downregulation. TDI has been previously shown to time-dependently downregulate HO-1 and Nrf2 abundance in A549 cells [8].

The effect of AGP on Th2 cytokines and their gene expression, especially IL-13, resulted in improvement of TDI-induced dramatic increase in airway resistance. The lowering of airway goblet hyperplasia and collagen deposition occurred as a result of downregulation of *Muc5ac*, *Mmp9*, and *Chil4* expressions. These activities cumulatively averted chances of airway

remodelling and hyperresponsiveness, both of which are important cardinal features of asthma. This study revealed for the first time, that the use of AGP has a puissant anti-inflammatory and anti-oxidant action in a murine model of TDI-induced OA. It exerted its effect by inhibiting intracellular ROS possibly via p38 MAPK-dependent Nrf2 induction. It also restored airway integrity by reversing aberrant airway epithelial E-cadherin and β -catenin distribution.

Conflict of interest

Authors declare no conflict of interest

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