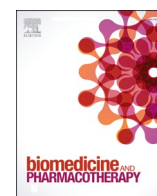




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Mitoquinone ameliorated airway inflammation by stabilizing β -catenin destruction complex in a steroid-insensitive asthma model

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ABSTRACT

Background and purpose: Mitochondrial dysfunction is an essential part of the pathophysiology of asthma, and potential treatments that target the malfunctioning mitochondria have attracted widespread attention. We have previously demonstrated that aberrant epithelial β -catenin signaling played a crucial role in a toluene diisocyanate (TDI)-induced steroid-insensitive asthma model. The objective of this study was to determine if the mitochondrially targeted antioxidant mitoquinone(MitoQ) regulated the activation of β -catenin in TDI-induced asthma.

Method: Mice were sensitized and challenged with TDI to generate a steroid-insensitive asthma model. Human bronchial epithelial cells (16HBE) were exposed to TDI-human serum albumin (HSA) and ethidium bromide(EB) to simulate the TDI-induced asthma model and mitochondrial dysfunction.

Results: MitoQ dramatically attenuated TDI-induced AHR, airway inflammation, airway goblet cell metaplasia, and collagen deposition and markedly protected epithelial mitochondrial functions by preserving mass and diminishing the production of reactive oxygen species (ROS). MitoQ administration stabilized β -catenin destruction complex from disintegration and inhibited the activation of β -catenin. Similarly, YAP1, an important constituent of β -catenin destruction complex, was inhibited by Dasatinib, which alleviated airway inflammation and the activation of β -catenin, and restored mitochondrial mass. In vitro, treating 16HBE cells with EB led to the activation of YAP1 and β -catenin signaling, decreased the expression of glucocorticoid receptors and up-regulated interleukin (IL)-1 β , IL6 and IL-8 expression.

Conclusion: Our results indicated that mitochondria mediates airway inflammation by regulating the stability of the β -catenin destruction complex and MitoQ might be a promising therapeutic approach to improve airway inflammation and severe asthma.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

1. Introduction

Asthma is a chronic airway disease characterized by airway

hyperresponsiveness (AHR), airway inflammation and increased oxidative stress. In asthma, the airway epithelium plays a critical role in orchestrating the allergic inflammation, and immune responses to

Abbreviations: TDI, toluene diisocyanate; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; MitoQ, mitoquinone; EB, ethidium bromide; Gr, Glucocorticoid receptor; LRP6, low-density lipoprotein receptor-related protein 6; OCR, oxygen consumption rate; Cytb, cytochrome b; COX1, cytochrome c oxidase 1; COX2, cytochrome c oxidase 2; TFAM, transcription factor A, mitochondrial; HSA, human serum albumin; BAL, bronchoalveolar lavage; qPCR, quantitative PCR; mtDNA, mitochondrial DNA; AOO, A mixture of 2 vol of acetone and 3 vol of olive oil.

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allergens, viruses and environmental pollutants [1]. Allergens such as toluene diisocyanate (TDI) directly damage the airway epithelium to induce consequent barrier dysfunction and local inflammation, and prominently activate Th2/Th17 responses [2]. In our previous study, we found that abnormal regulation of β -catenin in airway epithelial cells played a critical role in airway inflammation and AHR in TDI-induced asthma, which had been identified as a steroid-insensitive asthma model [2–5]. However, it is unknown how to relieve asthmatic airway epithelium destruction and target anomalous β -catenin signaling in asthma.

β -catenin has dual effects with respect to cell signaling. On the one hand, β -catenin is a vital component of adhesion junctions [6]. On the other hand, cytosolic and nuclear β -catenin is the central effector of the Wnt signaling, which is precisely controlled by the stability of β -catenin destruction complex. Activation of Wnt signaling induces the disintegration of β -catenin destruction complex [6] and then promotes nuclear translocation of β -catenin and YAP/TAZ from the complex. This mechanism is instrumental for Wnt/ β -catenin signaling [7], and is involved in the mediation of different diseases [8–10]. Additionally, knock-out of YAP/TAZ and β -catenin in regulatory T cells inhibited asthmatic Th17 and Th2 inflammation respectively [11], indicating YAP/TAZ and β -catenin signaling played different role in asthmatic airway inflammation. However, the role of YAP/TAZ in airway epithelium and whether YAP/TAZ interacts with β -catenin to promote asthmatic airway inflammation has not been reported.

Mitochondria involves in oxidative stress, which modulates apoptosis, ferroptosis and potentiates the inflammatory activity in the lung [12]. Additionally, oxidative stress plays an important role in steroid insensitivity and the development of asthma [1,13,14]. Hence, drugs targeting mitochondria had attracted much attention with respect to the treatment of asthma and mitochondrially targeted antioxidant showed potential in alleviating airway remodeling and airway inflammation [15,16]. Therefore, we hypothesized that mitochondrial dysfunction was involved in the pathogenesis of TDI-induced asthma model. Thus, the purpose of this study was to determine if mitochondrially targeted antioxidant mitoquinone (MitoQ) could ameliorate airway inflammation by stabilizing β -catenin destruction complex in a steroid-insensitive asthma model.

2. Materials and methods

2.1. Reagents

Acetone, TDI (toluene-2, 4-diisocyanate, $\geq 98.0\%$) and methacholine were bought from Sigma-Aldrich (Shanghai, China). A mixture of 2 vol of acetone and 3 vol of olive oil was used as the vehicle (AOO) to dissolve TDI for cutaneous sensitization, and 1 vol of acetone and 4 vol of olive oil was utilized for airway challenge. Dasatinib, K-975 and MitoQ were obtained from Medchemexpress (New Jersey, USA). ELISA kits for total IgE, IL-4, IL-5, IL-13, IL-17A and IL-17 F were bought from Thermo Scientific (Waltham, Massachusetts, USA). Anti-YAP, anti- β -catenin, anti-GAPDH and anti-TAZ were purchased from Proteintech (Chicago, USA). Anti-Non-phospho (Active) β -catenin (Ser33/37/Thr41) was from Cell Signaling Technology (Boston, USA). Anti-Non-phospho (Active) YAP (Ser127) was from abcam (Cambridge, UK).

2.2. Animals and in vivo treatment with YAP/TAZ inhibitors and MitoQ

Male C57BL/6 J mice (specific pathogen-free), 6–8 weeks old, were purchased from Southern Medical University. The mice were kept in a SPF house with a temperature of $23 \pm 2^\circ\text{C}$ and humidity range of 40–70% and a 12 h dark/light cycle (lighting: 7:00–19:00). Mice were given sterile water and irradiated food at will. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The Animal Subjects Committee of Nanfang Hospital approved the protocols.

50 mice were used in sum ($n = 10$ for each group). Mice were randomized to the following groups: (1) Sensitization and challenge with AOO and treat with DMSO (Control group); (2) Sensitization and challenge with TDI and treat with DMSO (TDI group); (3) Sensitization and challenge with TDI and treat with MitoQ (TDI + MitoQ group); (4) Sensitization and challenge with TDI and treat with Dasatinib (TDI + Dasatinib group); (5) Sensitization and challenge with TDI and treat with K-975 (TDI + K-975 group); Animal models were modified according to our previous reporting [5]. On days 1 and 8, Male C57BL/6 J were dermally sensitized with 0.6% TDI (20 μl each ear) on the dorsum. On days 15, 18, 21 and 24 each mouse was put in a horizontal cylindrical chamber and exposed for three hours to a 3.5% TDI solution diluted in acetone and olive oil (1:4) through the airway using a compressed air nebulizer (NE-C28; Omron, Tokyo, Japan). Mice were sensitized and challenged using the same methods and the same dose of AOO as a control group. K-975 (10 mg/kg, i.p.), Dasatinib (10 mg/kg, i.p.) and MitoQ (2.5 mg/kg, i.p.) dissolved in DMSO were given to the mice immediately before each sensitization and airway challenge. Comparatively, sham mice were given the same amount of vehicle.

2.3. Assessment of airway hyper-responsiveness (AHR)

A barometer plethysmographic chamber (Buxco Electronics, Troy, NY) was used to measure each mouse's airway responsiveness to methacholine on day 22 in accordance with our prior investigation [17]. Lung resistance (RL) were measured in response to increasing dosages of methacholine by means of ultrasonic nebulization (6.25, 12.5, 25 and 50 mg/mL) on anaesthetized and mechanically ventilated mice. Following each nebulization process, measurements of RL were made every 5 min until a plateau phase was reached. Results were shown as a percentage of the RL value at baseline for each methacholine concentration.

2.4. Quantification of total serum IgE

The mice were sacrificed as described previously [18]. Blood samples collected were rested for 1 h at room temperature, then centrifuged (3000g, 20 min) and supernatants were harvested and stored at -80°C . Following the manufacturer's instructions, total serum IgE was measured by ELISA.

2.5. Analysis of bronchoalveolar lavage (BAL) fluids

The total number of cells in the obtained BAL fluids were counted before the BAL fluids were centrifuged (1000 g, 10 min). In accordance with the manufacturer's recommendations, the supernatant was frozen (80°C) for subsequent IL-4, IL-5, IL-13, IL-17A, and IL-17 F ELISA analysis. Hematoxylin and eosin (H&E) staining was performed on a cytospin sample in order to determine the differential cell counts. A total of 200 cells from each sample were counted for the numbers of macrophages, eosinophils, neutrophils, and lymphocytes.

2.6. Pulmonary histopathological examination

Left lungs were dissected from mice, then fixed in 4% neutral formalin, dehydrated, and paraffin-embedded. Sections (4 μm) were cut with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E, periodic acid-Schiff (PAS), Masson's trichrome for blinded histopathological assessment. Inflammation of the lung and goblet cell metaplasia of the airway were semi-quantified as previously described [19,20]. At least 40 image fields of 20 slices from 8 mice per group were scored at a magnification of 200.

2.7. Transmission electron microscopy

Lung tissue was taken and fixed in 1.25% glutaraldehyde/0.1 M phosphate buffer. Ultrathin sections (60 nm) were prepared by a routine procedure and examined under an electron microscope (JEOL JEM-1010).

2.8. Immunohistochemistry

Lung section immunohistochemistry for β -catenin and YAP1 was performed after deparaffinization and antigen retrieval. After being exposed to H_2O_2 for 15 min to inhibit endogenous peroxidase, samples were incubated with anti- β -catenin and anti-YAP1 antibodies in recommended dilutions overnight at 4 °C. Slices were treated with a secondary antibody for 20 min at room temperature after being washed with PBS. DAB was used to visualize the signals.

2.9. Preparation of TDI-HSA(human serum albumin) conjugates

According to our previous study [21], TDI-HSA(human serum albumin) conjugates were prepared.

2.10. Cell culture and treatment

Human bronchial epithelial cell line 16HBE14o-(16HBE) cells (Bio-Rad Laboratories (Shanghai) Co, Ltd, ATCC, Portland, Oregon) were cultured in RPMI-1640 medium (Gibco) containing 10% fetal calf serum (Gibco) at 37 °C and 5% CO_2 . Cells that had passed to five to eight generations, with medium being changed every two days were used for the following experiments. Once reaching 90% convergence, the cells were passaged and seeded to suitable culture plates at a density of 10^4 – 10^5 cells/cm². Various concentrations of TDI-HSA conjugates (0–150 μ g/mL) were then added to the culture medium for the durations indicated, either with or without pretreatment with Dasatinib (10 nM, 1 h), K-975 (0.1 μ M, 1 h) or MitoQ (0.5 μ M, 1 h). The TDI-HSA concentrations used in this experiment were based in part on earlier research [21]. Varying concentrations of ethidium bromide (EB) (0.1–1000 μ M) were then added to the culture medium for durations specified. The genOFFTM st-h-YAP1 CCACCAAGCTAGATAAAGA, and siRNA-mediated knockdown of YAP1 in 16HBE cells was performed using Lipofectamine 3000 transfection reagent (Invitrogen, California, USA). According to the manufacturer's instructions, YAP1 siRNA was transfected into 70% confluent 16HBE cells.

2.11. Western blot

Expression of GAPDH, YAP1, TAZ, β -catenin, α -SMA, Non-phospho (Active)YAP(Ser127) and Non-phospho(Active) β -catenin (Ser33/37/Thr41) in lung or 16HBE cells was detected by Western blot. Whole lung or 16HBE cells extracted protein were blended with 5xSDS loading buffer (Fude Biological Technology, Hangzhou, China), separated by 10% SDS–polyacrylamide gel and then transferred onto PVDF membranes. After incubation with an IRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE) for image capture. Odyssey software (LI-COR Biosciences, Lincoln, NE) was utilized for data analysis. Image J software was used for quantitative image analysis.

2.12. Coimmunoprecipitation

Thermo Scientific Pierce™ Classic Magnetic IP/CO-IP Kit (Waltham, Massachusetts, USA) was used in accordance with the manufacturer's instructions to perform the coimmunoprecipitation among YAP1 and β -catenin.

2.13. Quantitative PCR (qPCR)

Following the manufacturer's instructions, total RNA isolation was extracted with the use of the RNAex Pro Reagent RNA isolation system (Accurate Biology, Hunan, China). Using 1 μ g of RNA in 10 μ l of reaction buffer containing 5 $\times$ Evo M-MLV RT Master Mix (Accurate Biology, Hunan, China) and random primers, the first strand of complementary DNA was generated by sequentially heating the mixture to 37 °C for 15 min and 85 °C for 5 s. Real-time PCR was performed using a Platinum SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Hunan, China). The primer pairs sequences are displayed in TABLE S1. Analysis of mitochondrial DNA (mtDNA) was carried out as reported previously [22]. Briefly, total DNA was extracted with a DNeasy blood and tissue kit (Qiagen) in accordance with the manufacturer's instructions. mtDNA was amplified using primers for the mitochondrial cytochrome c oxidase subunit 2 (COX2) gene and normalized to ribosomal protein s18 (RSP18).

2.14. Immunofluorescence microscopy

After TDI-HSA stimulation, the localization of YAP1 and β -catenin was investigated using immunofluorescence microscopy. 16HBE cells (2×10^5 cells/well) were cultured in RPMI-1640 containing 10% (v/v) FBS on Glass Bottom Cell Culture Dishes (NEST, China), and were treated with TDI-HSA (100 μ g/mL) for 6 h at 37 °C with or without MitoQ (0.5 μ M, 1 h) before being treated. Following this, 16HBE cells were fixed with 4% (v/v) formaldehyde in PBS for 10 min, treated with 0.5% (v/v) Triton X100 for 15 min, blocked with 5% (w/v) BSA for 2 h at room temperature, and then incubated with rabbit anti-YAP antibody and rabbit anti- β -catenin antibody overnight at 4 °C respectively. The cells were incubated in the dark at room temperature with Alexa 488-labeled goat anti-rabbit IgG antibody for 1 h. Then, the cells nuclei were stained with 4', 6-Diamidino-2-phenylindole (DAPI) dihydrochloride (Beyotime Biotechnology, China) for 10 min. The distribution of β -catenin, YAP and TAZ was investigated using a laser-scanning confocal microscope (Olympus, Tokyo, Japan). MitoSOX (Yeasen, Shanghai, China) and Rhodamine 123 staining (Beyotime Biotechnology, China) were used for detection of mitochondrial oxidative stress and mitochondrial membrane potential according to the manufacturer's instructions.

2.15. Measurements of mitochondrial oxygen consumption rate

The rates of mitochondrial oxygen consumption in 16HBE cells were measured with a Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience), using the XF Cell Mito Stress Test Kit (#103015; Seahorse Bioscience). Approximately 3×10^4 cells were seeded in each well of a Seahorse 96-well XF Cell Culture Microplate. Oligomycin (1 μ M), FCCP (0.5 μ M) and antimycin A and rotenone (0.5 μ M each) were added to the XF assays as directed by the manufacturer. Parameters of basal OCR, maximal OCR, ATP-linked OCR, and the reserve capacity were analyzed according to the manufacturer's protocol.

2.16. Statistical analysis

Statistical analysis was performed using SPSS version 16.0 software. Data were presented as mean $\pm$ standard error (SE), and comparisons among groups were analyzed by one-way analysis of variance (ANOVA) accompanied by Bonferroni post hoc testing for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

3. Result

3.1. MitoQ ameliorated TDI-induced AHR and airway inflammation by preserving mitochondrial function

To investigate the relationship between mitochondrion and severe

asthma, the studies were performed in TDI-induced steroid-insensitive asthma model. Firstly, we found TDI-induced asthmatic mice with increased airway reactivity (Fig. 1A), higher serum IgE concentration (Fig. 1B) and elevated levels of IL-4, IL-5, IL-13, IL-17A, IL-17 F in BAL fluid (Fig. 1C). The numbers of total and differential inflammatory cells were counted in BAL fluid. Higher numbers amounts of neutrophils and eosinophils were found after the sensitization and challenge with TDI (Fig. 1D-F). However, treatment with MitoQ significantly reversed these changes.

Next, we then performed a comprehensive analysis of pathological injury of airway epithelium. As shown in the Fig. 2A-B, H&E staining showed that a lot of neutrophils and eosinophils were concentrated in the airway epithelium of asthmatic mice. Additionally, we found notable

goblet cell metaplasia and increased thickening of the epithelial layer and subepithelial collagen fibrils deposition with up-regulation of MUC5AC in asthmatic mice (Fig. 2A and C-E). As expected, the typical pathological features of TDI-induced asthma were ameliorated by the treatment of MitoQ.

Transmission electron microscopy showed TDI-induced mitochondrial swelling and cristae disorganization, metaplasia and exfoliation of epithelial cells, destruction of tight junctions and cell necrosis, which were largely alleviated by MitoQ (Fig. 2F). As shown in Fig. 2G-H, mtDNA content was declined in TDI-induced asthmatic mice and the mRNA expression levels of mtDNA-encoded cytochrome b (Cytb), cytochrome c oxidase 1 (COX1), cytochrome c oxidase 2 (COX2) and transcription factor A, mitochondrial (TFAM) were decreased. Notably,

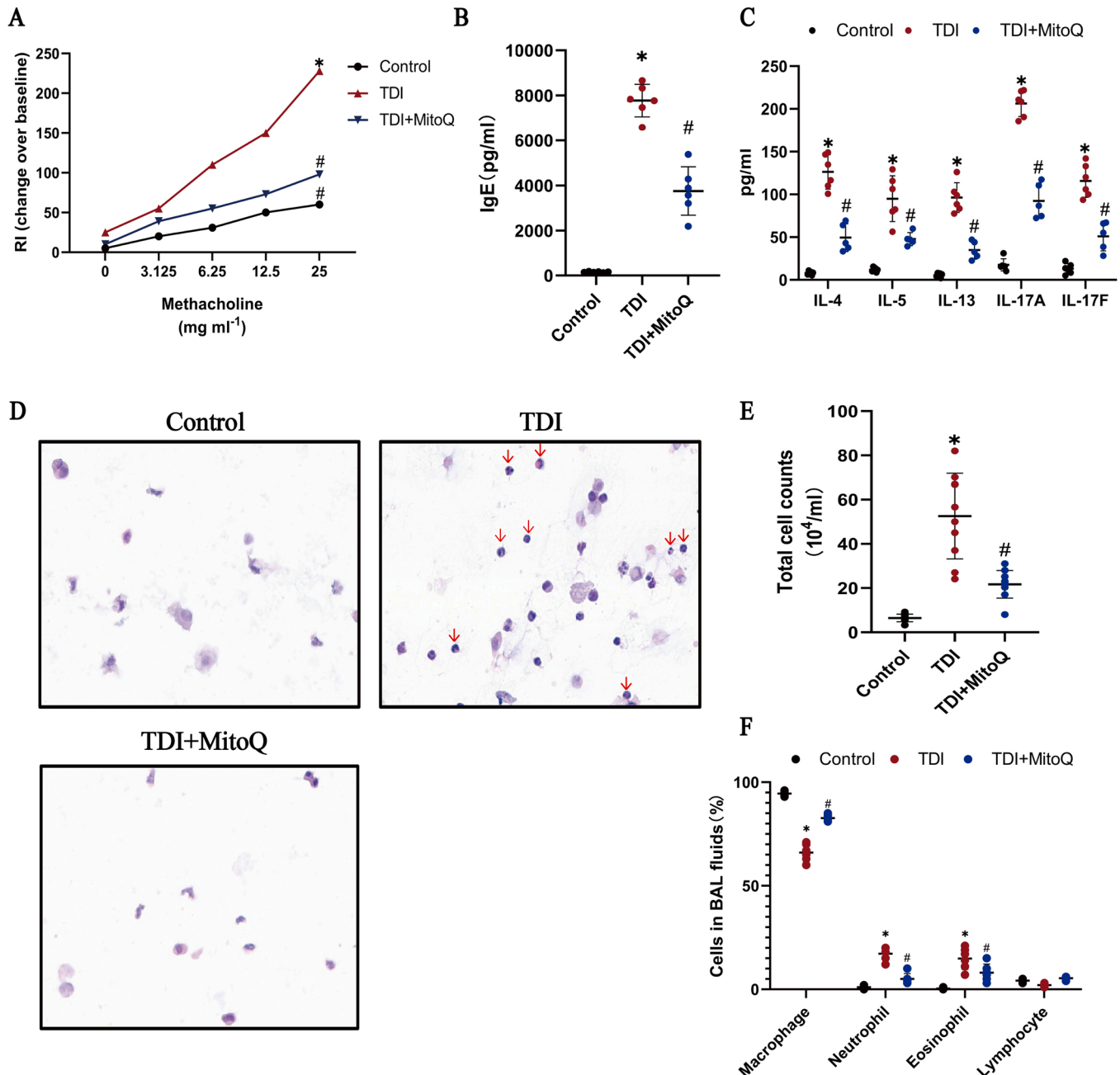


Fig. 1. Mitochondrially targeted antioxidant MitoQ ameliorated airway inflammation and AHR. AHR was measured by lung resistance (RL). Results were shown as percentage of baseline value ($n = 6$). (B) Measurement of IgE in serum by ELISA. (C) Measurement of IL-4, IL-5, IL-13, IL-17A, IL-17 F in BAL fluid by ELISA ($n = 6$). (D,E,F) Total and differential inflammatory cells in BALF ($n = 6-10$). Percentages of different inflammatory cells were calculated by counting a total of 200 cells in H&E stained cytospin samples. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with TDI group.

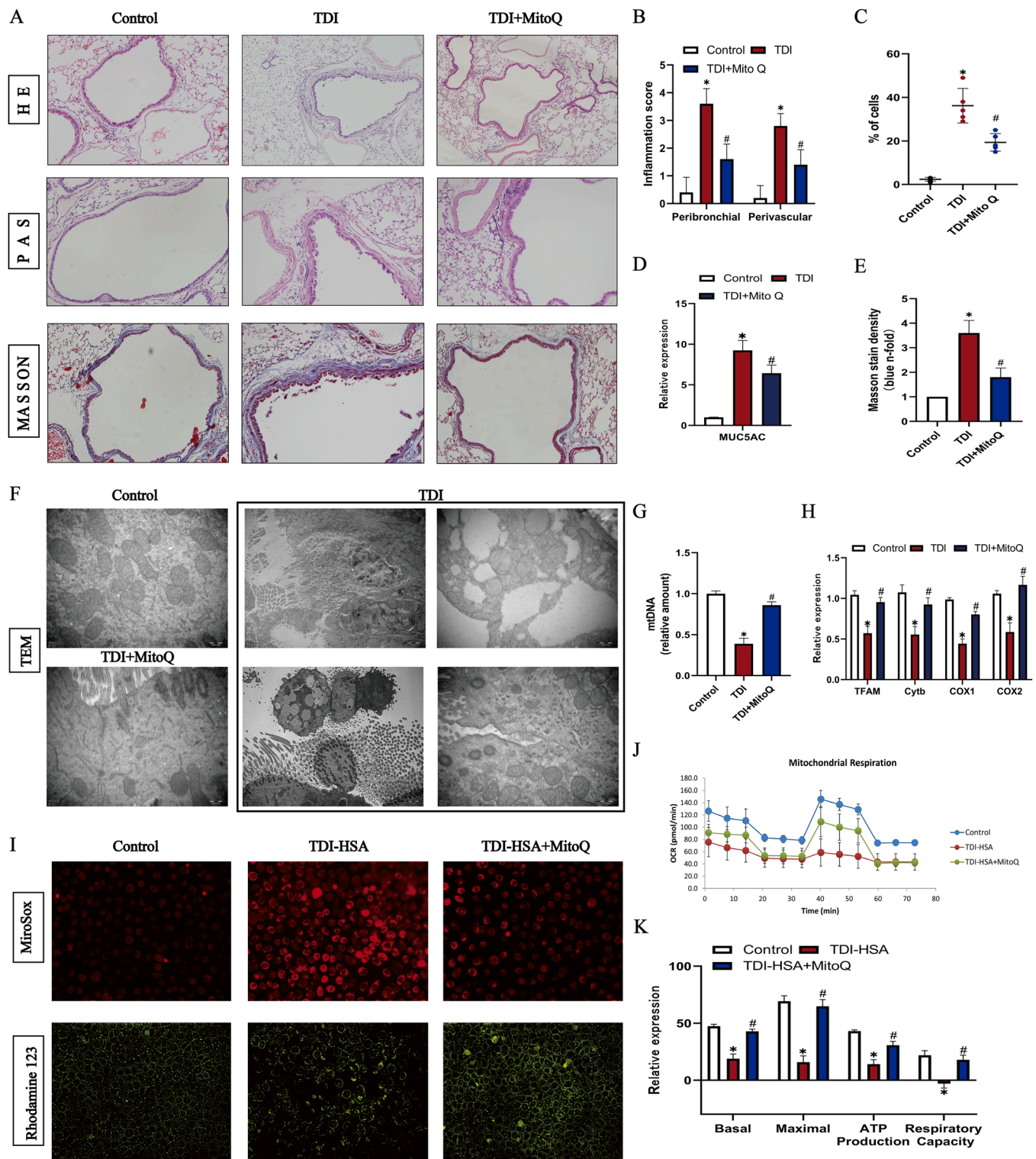


Fig. 2. Effect of MitoQ on airway epithelium of TDI-induced asthma model. Representative H&E stained lung sections of different groups at 200 × original magnification, periodic acid-Schiff base (PAS) stained sections at 400 × original magnification and Masson's trichrome stained sections at 200 × original magnification. (B,C,E) Semi-quantitative analysis of airway inflammation, goblet cell metaplasia and subepithelial collagen fibrils deposition (n = 5). (D) mRNA expression of MUC5AC in the whole lung (n = 5). (F) Transmission electron microscopy detection of different groups at 5000 ×, 10,000 × and 4000 ×. (G) Analysis of mitochondrial DNA (mtDNA) content in lung. Total DNA was extracted and amplified with the primer of mitochondrial cytochrome c oxidase 2 (COX2) and normalized to ribosomal protein s18 (RSP18). (H) mRNA expression of TFAM, Cytb, COX1, COX2 in the whole lung. (I) MitoSOX stained 16HBE cells of different group and Rhodamine 123 stained of different groups. The concentration of MitoQ is 0.5 μM. (J,K) Analysis of oxygen consumption rate (OCR) by Seahorse assay. *P < 0.05 compared with control group; #P < 0.05 compared with TDI group, n = 5–8.

all of the aforementioned changes were largely reversed by MitoQ.

To further study mitochondrial dysfunction induced by TDI, we analyzed mitochondrial function in 16HBE cells stimulated by TDI-HSA. In TDI-HSA treated 16HBE cells, MitoSOX staining showed that mitochondrial ROS production increased, and Rhodamine 123 staining showed mitochondrial membrane potential deficiency (Fig. 2I). We then evaluated mitochondrial oxygen consumption rate (OCR) using the Seahorse assay. As shown in Fig. 2J-K, TDI-HSA decreased the levels of oxygen consumption and ATP production in 16HBE cells. Furthermore, pretreatment with MitoQ almost completely blocked these effects. These results suggested that mitochondrial dysfunction plays an important role in the development of TDI-induced asthma model.

3.2. MitoQ reversed TDI-induced β -catenin destruction complex disintegration

In cytoplasm, YAP/TAZ and β -catenin are bound in β -catenin destruction complex, which prevents them from nuclear translocation. On the one hand, YAP/TAZ is transcriptionally inactivated by sequestration in the destruction complex [7]. On the other hand, cytoplasmic YAP/TAZ inhibits β -catenin at the level of the destruction complex [5,7]. Consistent with our previous study [5], β -catenin was distributed in disorder and diffused in the cytoplasm and nucleus in the airway epithelium of TDI-induced asthmatic mice (Fig. 3A). Treatment with MitoQ not only ameliorated the redistribution of β -catenin, but also

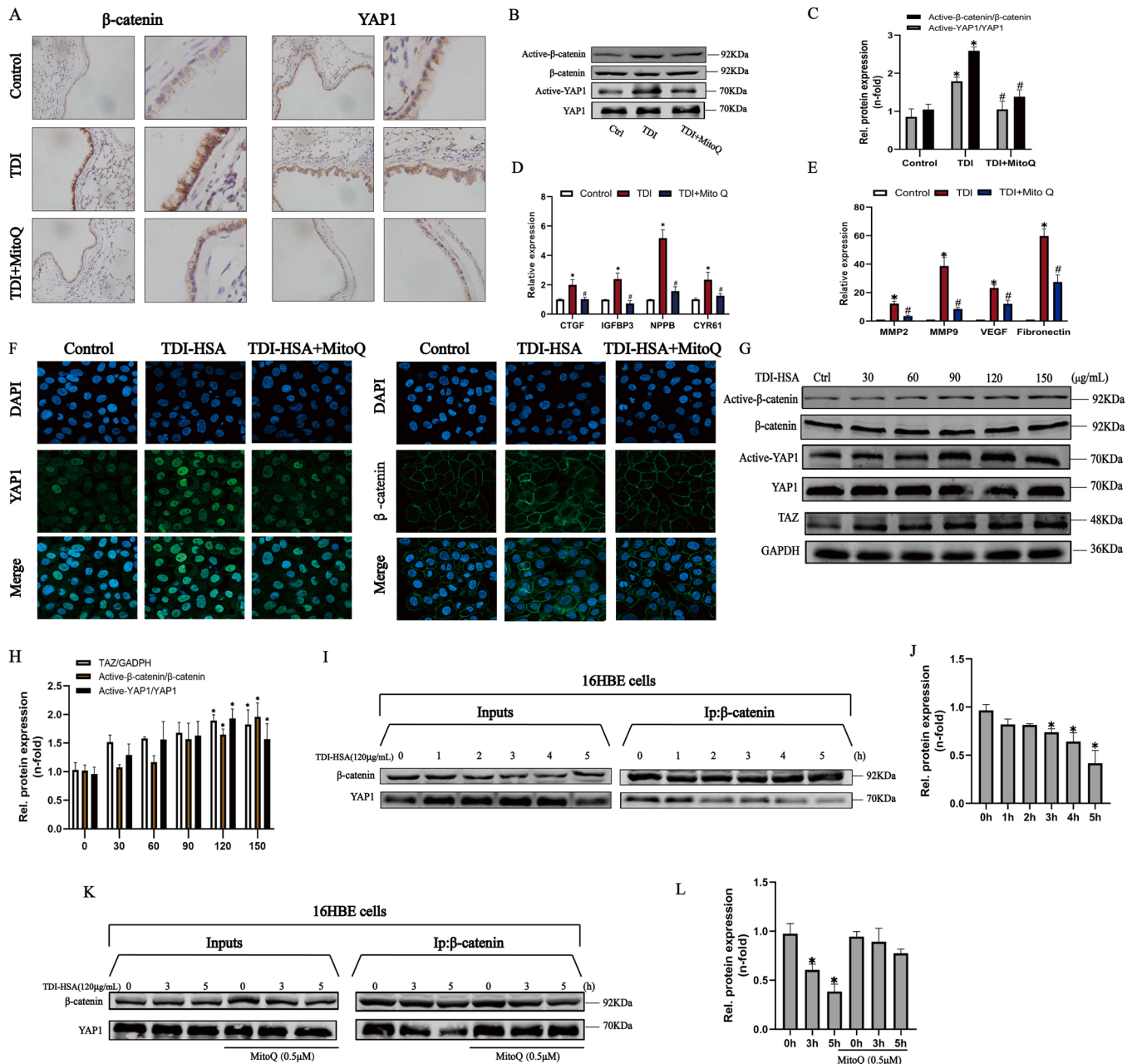


Fig. 3. Effect of MitoQ on β -catenin destruction complex in TDI asthma model. Immunohistochemistry analysis of the expression and distribution of β -catenin and YAP1 in airway epithelium. (B,C) Western blotting of YAP1, Active-YAP1, β -catenin and Active- β -catenin expression in whole lung tissue. (D,E) mRNA expression of CTGF, NPPB, IGFBP3, CYR61, MMP2, MMP9, VEGF and fibronectin. (F) Immunofluorescence staining was used to visualize the distribution of YAP1 and β -catenin. (G, H) Western blotting of TAZ, GAPDH, YAP1, Active-YAP1, β -catenin and Active- β -catenin expression in 16HBE cells. (I,J,K,L) Lysates were subjected to IP using an anti- β -catenin antibody. Coprecipitating proteins were visualized and analyzed by western blot. *P < 0.05 compared with control group; #P < 0.05 compared with TDI group, n = 3–5.

inhibited the diffused expression of YAP1 in TDI-induced asthmatic mice (Fig. 3A). In line with this findings there was an increased level of non-phospho-(Active)YAP1(Ser127) and non-phospho-(Active) β -catenin in(Ser33/37/Thr41) in the lung tissue (Fig. 3B-C). In addition, qPCR demonstrated that a number of β -catenin target genes (MMP-2, MMP-9, fibronectin and VEGF) and YAP/TAZ target genes (NPPB, CTGF, CYR61 and IGFBP3) were up-regulated by TDI exposure (Fig. 3D-E). Notably, excessive activation of YAP/TAZ and β -catenin signaling induced by TDI was largely reversed by MitoQ.

To further explore the relation between β -catenin destruction complex and mitochondria, we evaluated the protein-protein interaction between β -catenin and YAP1 in 16HBE cells. As shown in Fig. 3F, immunofluorescence assay showed that YAP1 and β -catenin redistributed from the cytomembrane and cytoplasm to the nucleus after TDI-HSA stimulation, which were reversed by MitoQ (Fig. 3F). Similar to animal experiments, YAP1, TAZ and β -catenin were activated by TDI-HSA stimulation (Fig. 3G-H). As the coimmunoprecipitation between YAP/TAZ and β -catenin depended on β -catenin destruction complex [7], we evaluated the stability of the β -catenin destruction complex by the coimmunoprecipitation between YAP/TAZ and β -catenin. As shown in Fig. 3I-J, the coimmunoprecipitation between YAP1 and β -catenin decreased in TDI-treated 16HBE cells. However, treatment with MitoQ stabilized the β -catenin destruction complex and prevented it from disintegration (Fig. 3K-L). In summary, these results suggested that mitochondria probably mediated TDI-induced asthma by regulating β -catenin destruction complex.

3.3. Dasatinib, but not K-975 inhibited the disintegration of β -catenin destruction complex

Treatment with Dasatinib largely reversed TDI-induced up-

regulation of non-phospho (Active) YAP1(Ser 127), non-phospho (Active) β -catenin (Ser33/37/Thr41) and their targeted genes transcription, while K-975 inhibited the expression of YAP1 targeted genes and had a minimal effect on the expression of non-phospho (Active) YAP1(Ser 127), non-phospho (Active) β -catenin (Ser33/37/Thr41) and β -catenin targeted genes (Fig. 4B-E). In line with this, immunohistochemistry analysis also showed that Dasatinib reversed the redistribution of YAP1 and β -catenin nuclear translocation after TDI exposure, while K-975 had no effect on this (Fig. 4A). To verify that Dasatinib inhibited the activation of β -catenin by YAP1, we established YAP1 knockout 16HBE cells. As shown in Fig. 4F-G, knock-out of YAP1 significantly weakened the inhibition of Dasatinib on the activation of β -catenin. Additionally, treatment with Dasatinib, but not K-975, inhibited TDI-HSA-induced disintegration of β -catenin destruction complex (Fig. 4H-I). These results indicated that Dasatinib stabilized β -catenin destruction complex by inhibiting YAP1.

3.4. Blockade of YAP/TAZ signaling ameliorated TDI-induced airway inflammation and AHR

To further investigate the role of YAP/TAZ and β -catenin destruction complex in TDI-induced asthma, K-975 and Dasatinib were respectively administered to TDI-exposed mice. K-975 is a novel potent inhibitor of YAP/TAZ-targeted genes transcription [23]. Dasatinib is a tyrosine kinase inhibitor, inhibiting the expression and nuclear translocation of YAP/TAZ [24] and alleviating Th2 asthmatic airway inflammation [25]. As shown in Fig. 5A, treatment with both Dasatinib and K-975 ameliorated TDI-induced AHR. Additionally, ELISA showed that asthmatic mice had higher levels of IgE, IL-4, IL-5, IL-13, IL-17A and IL-17 F (Fig. 5B-C). The numbers of total and differential inflammatory cells were counted in BAL fluid. Higher numbers amounts of neutrophils and

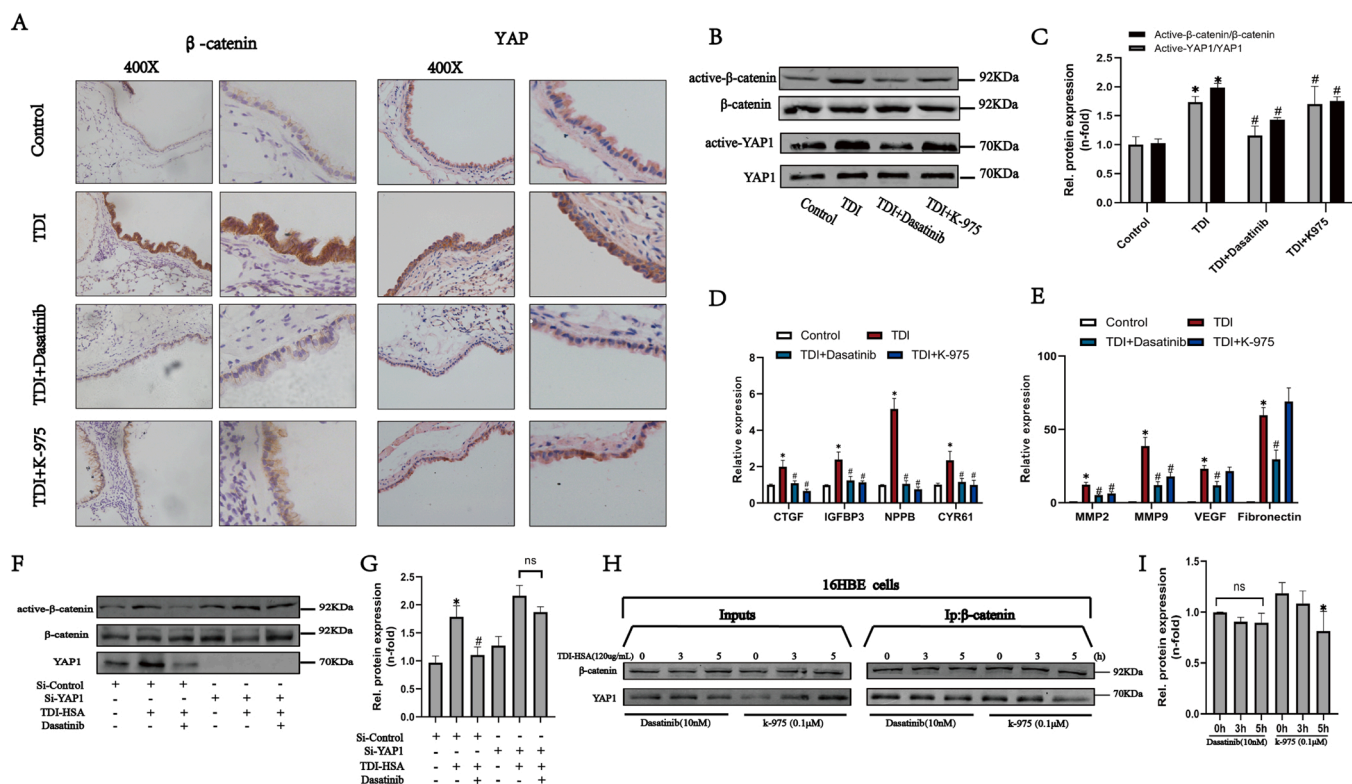


Fig. 4. Effect of K-975 and Dasatinib on β -catenin destruction complex in TDI asthma model. (A) Immunohistochemistry analysis of the expression and distribution of β -catenin and YAP1 in airway epithelium. (B,C) Western blotting of YAP1, Active-YAP1, β -catenin and Active- β -catenin expression in whole lung tissue. (D,E) mRNA expression of CTGF, NPPB, IGFBP3, CYR61, MMP2, MMP9, VEGF and fibronectin. (F,G) Western blotting of YAP1, β -catenin and Active- β -catenin expression in 16HBE cells. (H,I) Lysates were subjected to IP using an anti- β -catenin antibody. Coprecipitating proteins were visualized and analyzed by western blot. *P < 0.05 compared with control group; #P < 0.05 compared with TDI group, n = 3–5.

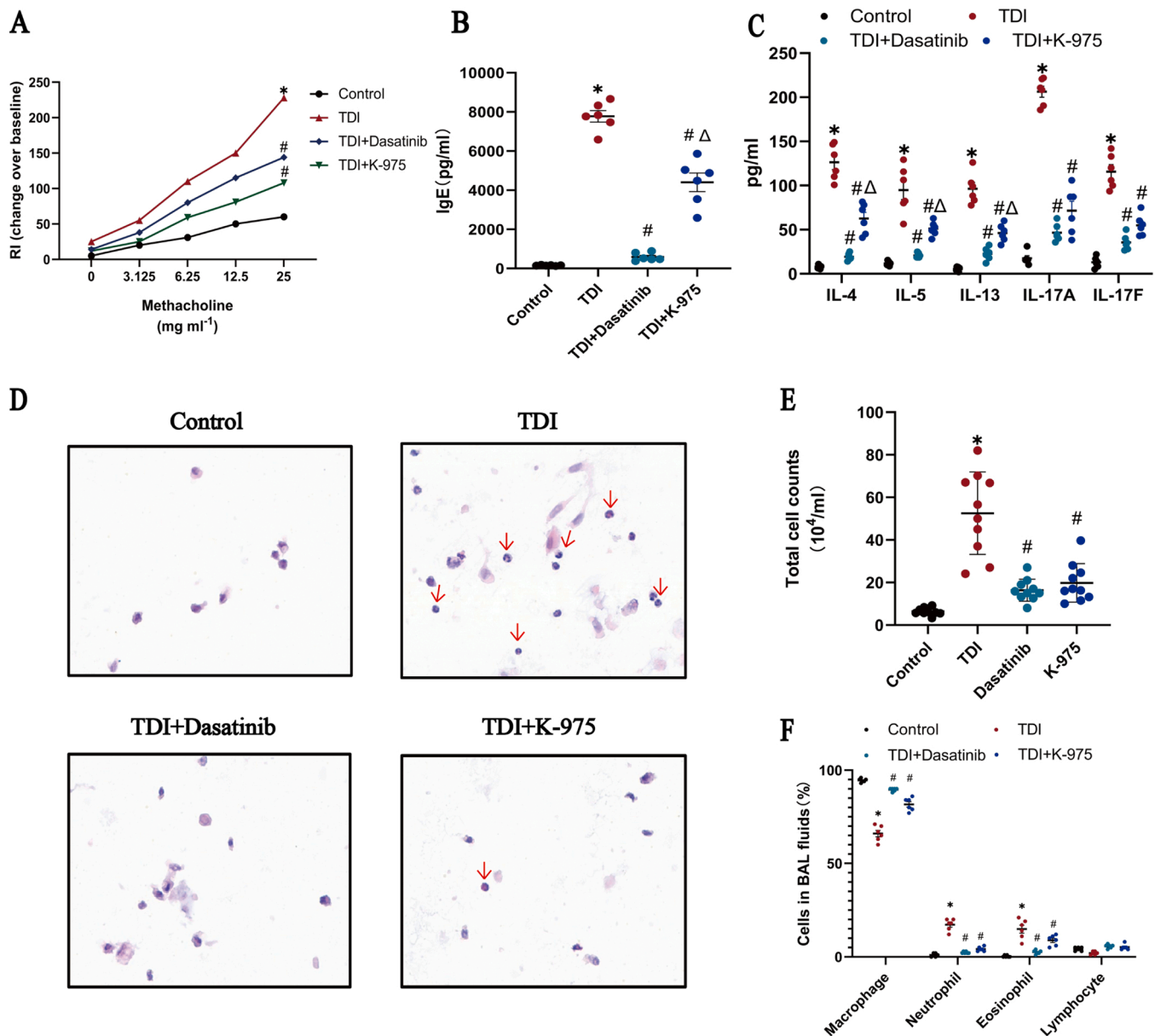


Fig. 5. Dasatinib and K-975 ameliorates airway inflammation and airway AHR. (A) AHR was measured by lung resistance (RL). Results were shown as percentage of baseline value ($n = 6$). (B) Measurement of IgE in serum by ELISA. (C) Measurement of IL-4, IL-5, IL-13, IL-17A, IL-17 F in BAL fluid by ELISA ($n = 6$). (D, E, F) Total and differential inflammatory cells in BALF ($n = 6-10$). Percentages of different inflammatory cells were calculated by counting a total of 200 cells in H&E stained cytopsin samples. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with TDI group; $\Delta P < 0.05$ compared with Dasatinib group.

eosinophils were found after sensitization and challenge with TDI (Fig. 5D-F). It is notable that, these changes were largely reversed by Dasatinib, while K-975 partly ameliorated these changes and showed weaker effect on reducing IgE and Th2 inflammatory cytokines, indicating that blockade of the disintegration of β -catenin destruction complex and YAP/TAZ transcription both improved TDI-induced airway inflammation and AHR. Therefore, blockade of the disintegration of β -catenin destruction complex appeared to be an effective treatment for TDI-induced asthma model, as both of YAP1 and β -catenin signaling were inhibited.

3.5. Blockade of YAP/TAZ signaling reversed TDI-induced airway epithelial injury

As shown in the Fig. 6A-B, H&E stained showed that both Dasatinib and K-975 largely reversed the accumulation of neutrophils and

eosinophils in the airway of TDI-induced asthmatic mice. Additionally, Dasatinib and K-975 also reversed TDI-induced goblet cell metaplasia, increased thickening of the epithelial layer and subepithelial collagen fibrils deposition as well as the up-regulation of MUC5AC (Fig. 6A, C-E). Transmission electron microscopy and qPCR analysis suggested that Dasatinib and K-975 alleviated TDI-induced mitochondrial swelling and cristae disorganization as well as the reduction of mtDNA and its coding genes (Fig. 6F-H). In vitro, the MitoSOX staining and Rhodamine 123 staining showed that Dasatinib and K-975 significantly reduced TDI-HSA-induced mitochondrial ROS production increased and mitochondrial membrane potential deficiency (Fig. 6I). Furthermore, the analysis of OCR showed that pretreatment with K-975 and Dasatinib ameliorated the reduction of oxygen consumption and ATP production in 16HBE cells induced by TDI-HSA (Fig. 6 J-K).

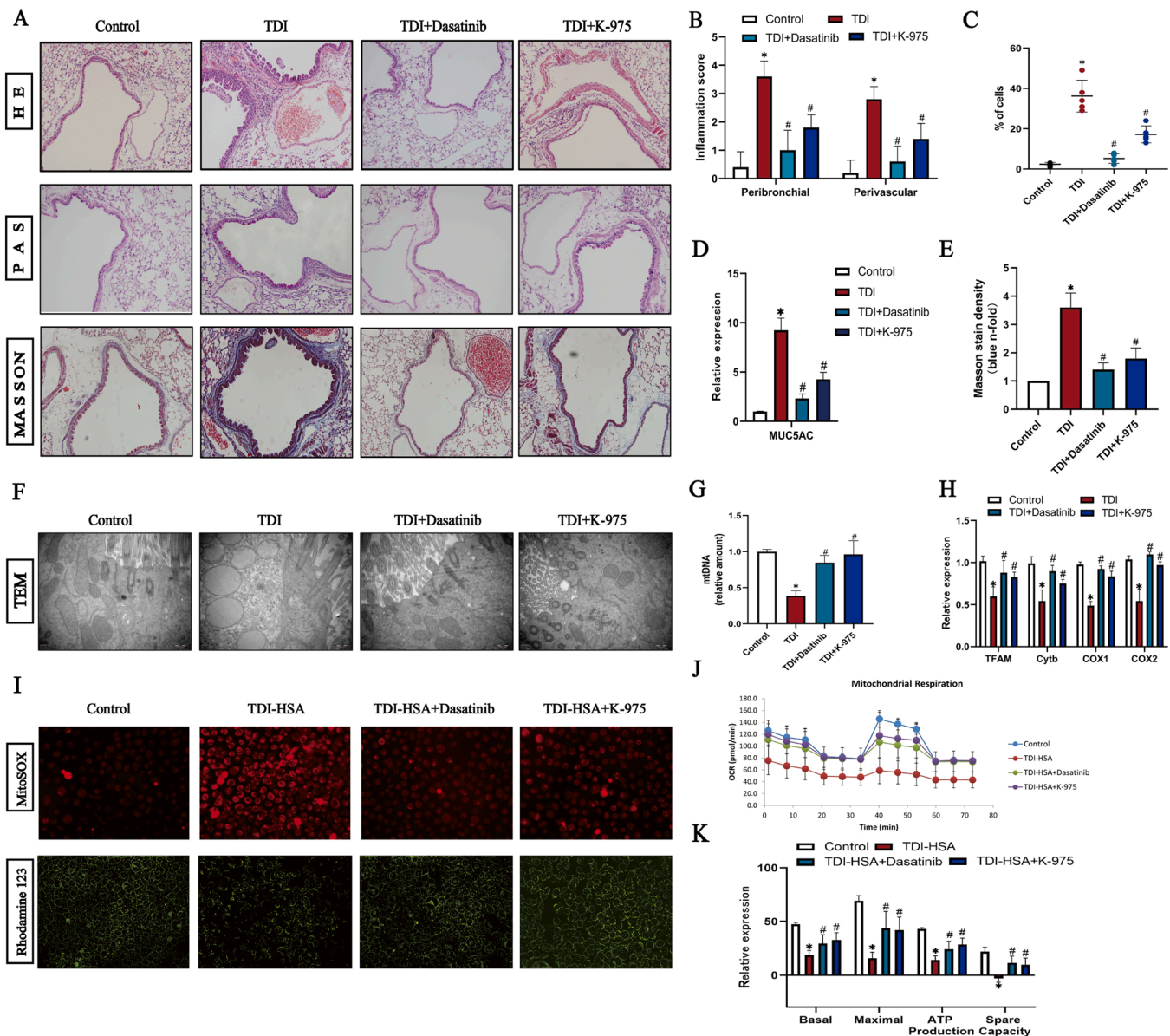


Fig. 6. Effect of Dasatinib and K-975 on airway epithelium of TDI-induced asthma model. Representative H&E stained lung sections of different groups at 200 × original magnification, periodic acid-Schiff base (PAS) stained sections at 400 × original magnification and Masson's trichrome stained sections at 200 × original magnification. (B,C,E) Semi-quantitative analysis of airway inflammation, goblet cell metaplasia and subepithelial collagen fibrils deposition. (D) mRNA expression of MUC5AC in the whole lung. (F) Transmission electron microscopy detection of different groups at 5000 ×, 10,000 × and 4000 ×. (G) Analysis of mtDNA content in lung. Total DNA was extracted and amplified with the primer of mitochondrial cytochrome c oxidase 2 (COX2) and normalized to ribosomal protein s18 (RSP18). (H) mRNA expression of TFAM, Cytb, COX1, COX2 in the whole lung. (I) MitoSOX stained 16HBE cells of different groups and Rhodamine 123 stained of different group. The concentration of MitoQ is 0.5 μM. (J,K) Analysis of oxygen consumption rate (OCR) by Seahorse assay. *P < 0.05 compared with control group; #P < 0.05 compared with TDI group, n = 5–8.

3.6. Deletion of mtDNA activated β -catenin and YAP1 signaling and was related to steroid-insensitivity

To further disclose the role of mitochondria in asthma, different concentrations of mtDNA destroyer EB were co-cultured with 16HBE cells. As shown in Figures S1A–B, YAP1 and β -catenin were significantly activated at a 1 μM concentration of EB. Analysis by qPCR suggested that pretreatment with EB nearly completely inhibited the expression of TFAM, Cytb, COX1 and COX2, indicating that EB caused the destruction of mitochondria (Figure S1C). In addition, treatment with EB resulted in an increase in the levels of IL-1 β , IL-6 and IL-8 (Figure S1D), which was elevated in TDI asthma model [5,26] and related to steroid-insensitive asthma [27,28]. Furthermore, we next evaluated the expression of

glucocorticoid receptor (GR) to determine the relation between mitochondrion and steroid insensitivity. As shown in Figures S1E–G, EB not only decreased the expression of GR α and GR β , but also reduced the ratio of GR α /GR β in 16HBE cells, both of which were vital to the mechanism of asthma steroid insensitivity. Taken together, these results indicate that mitochondrial dysfunction was related to asthma steroid insensitivity and activated β -catenin and YAP1 signaling.

4. Discussion

To our knowledge, this was the first time, we showed that mitochondrially targeted antioxidant alleviated TDI-induced asthmatic airway inflammation by stabilizing β -catenin destruction complex.

Additionally, our data showed that YAP/TAZ signaling was central to the pathogenesis of TDI-induced asthma model and interacted with β -catenin signaling to regulate TDI-induced mixed granulocytic airway inflammation.

Over the past 20 years, the studies of mitochondria in asthma had been mostly limited in airway smooth muscle cells [29–31], while the changes of mitochondria in asthmatic airway epithelium had been overlooked. In this study, we observed morphological disorder of mitochondria, decreased mitochondrial membrane potential, DNA amount, gene transcription, oxygen consumption, and ATP production in the airway epithelium of TDI-induced asthmatic mice, indicating that mitochondrial dysfunction was an important pathological feature of TDI-induced asthma model. Allergens cause airway smooth muscle abnormal hyperactive mitochondrial function [32], while allergens have an adverse effect on the airway epithelium, revealing the differential regulation of allergens on mitochondrion of different lung sections.

Antioxidants that target mitochondria are in clinical development for a number of diseases [33]. In our study, TDI-exposed mice were pre-treated with the mitochondrially targeted antioxidants MitoQ via intraperitoneal injection. At first, we treated the mice with different concentrations of MitoQ. Although MitoQ was used in the treatment to lung injury mice model at a concentration of 10 mg/kg [34], we unexpectedly found that MitoQ at that concentration resulted in the death of 90% of the mice, and it had a poor effect on airway inflammation at a concentration of 5 mg/kg. However, MitoQ significantly attenuated TDI-induced AHR, airway inflammation, airway epithelial metaplasia, airway remodeling and mitochondrial dysfunction at a concentration of 2.5 mg/kg, which was the concentration used in this study. Additionally, MitoQ decreased the level of serum IgE and IL-4, IL-5, IL-13, IL-17A and IL-17 F in BAL fluid, indicating that it had an effect on both Th2 and Th17 asthmatic airway inflammation. A more vivid view from the transmission electron microscope showed that MitoQ ameliorated goblet cell metaplasia, epithelial cell exfoliation, cell necrosis, mitochondrial damage and tight junction destruction induced by TDI, which further confirmed the therapeutic effect of MitoQ. Similar with our research, latest research showed that treatment with MitoQ significantly reversed pathological features of lean and obese allergic asthma in mice [35], which included both of steroid-sensitive asthma model and steroid-insensitive asthma model. However, the changes of mitochondria and the mechanism of MitoQ in asthma had not been verified in their study. Taken together, mitochondrially targeted antioxidants have the potential in treating different types of asthma.

Notably, we found abnormal activation and distribution of β -catenin and YAP1 in the airway epithelium of TDI-exposed mice accompanied by increased expression of β -catenin and YAP1 targeted genes. Further experiments suggested that TDI-HSA induced the disintegration of β -catenin destruction complex, which promoted the phosphorylation and degradation of β -catenin and YAP1. Treatment with MitoQ significantly reversed these changes. Hence, mitochondrially targeted antioxidants may alleviate TDI-induced airway inflammation via stabilizing β -catenin destruction complex.

With Wnt stimulation, Frizzled and Lrp6 bind to Wnt ligands to induce the disintegration of β -catenin destruction complex which leads to nuclear translocation of YAP1 and β -catenin [7]. On the contrary, absence or nuclear translocation of YAP1 signaling also induces the disintegration of β -catenin destruction complex. Our previous study showed that TDI-HSA activated Lrp6 and β -catenin signaling independent of Wnt signaling [5,36], indicating TDI exposure was probably a novel and unique trigger of β -catenin destruction complex disintegration. Hence, we used inhibitors of YAP1 to investigate the relationship between YAP1 and β -catenin destruction complex and the role of β -catenin destruction complex in TDI-induced asthma model. We found that Dasatinib was confirmed to stabilize β -catenin destruction complex, and inhibit β -catenin signaling by the inhibition of YAP1 instead of K-975. However, pretreatment with K-975 or Dasatinib potently

inhibited the YAP1/TAZ transcription and TDI-induced elevation of serum IgE and the levels of IL-4, IL-5, IL-13, IL-17A, IL-17 F in BAL fluid. This was coupled with diminishing airway eosinophilia, neutrophil infiltration, AHR and mitochondrial dysfunction, indicating YAP/TAZ signaling played an important role in TDI-induced steroid-insensitive asthma model.

Inhibition of YAP1 had been shown to caused mitochondrial dysfunction [37] and high concentration of YAP1 inhibitors Dasatinib (200 nM in vitro; 25 mg/kg in vivo) induced cell apoptosis via promoting mitochondrial dysfunction [38,39]. However, our results showed that a low concentration of Dasatinib (10 nM in vitro; 10 mg/kg in vivo) significantly alleviated allergens-induced mitochondrial dysfunction in airway epithelium. As a drug to induce tumor cell death in clinic, Dasatinib showed different effect and mechanism on the treatment to asthma.

Furthermore, our data on inhibition of YAP/TAZ in murine asthma model stands in contrast to previous reports that knockout of YAP/TAZ had a poor effect on decreasing Th2 inflammation and serum IgE [11]. The reason behind this difference may be due to different targeted strategy. Inhibition of abnormal activation of YAP/TAZ provides a better response, while knock-out of YAP/TAZ promotes the activation of β -catenin [7]. However, the mechanism and effect of the cooperation between YAP/TAZ and β -catenin in TDI-induced asthma remain unknown. In this study, we showed that only Dasatinib could significantly improve the stabilization of β -catenin destruction complex by inhibiting YAP1 signaling, which had a better effect on Th2 inflammatory cytokines and serum IgE than K-975. However, we couldn't completely figure out the different effect of Dasatinib and K-975 on asthmatic airway inflammation and AHR in a mixed granulocytic asthma model, more experiments in asthma models with different inflammatory phenotypes were needed. Herein, our study showed that different methods of targeting YAP/TAZ signaling played different roles in regulating β -catenin and provided important experiential evidences for clinical application of targeted drugs for YAP/TAZ.

Changes in mitochondria, led to mitochondria dysfunction or change of the cell culture conditions can activate cellular responses by a mechanism called “mitochondrial retrograde signaling”, where changes in mitochondria cause extensive changes in nuclear gene expression to help the cell adapt to the new conditions [40]. Dysfunctional mitochondria results in decreased expression of nuclear-encoded mitochondrial oxidative phosphorylation genes, and triggers JNK retrograde signaling pathway [41], which can be activated by TDI to regulate β -catenin signaling [42]. Therefore, to investigate if mitochondrial retrograde signaling will aggravate asthma, we adopted targeted EB, which was a destroyer for mitochondrial DNA, to stimulate mitochondrial dysfunction. For the first time, we found that mitochondrial dysfunction activated YAP1 and β -catenin and elevated the expression of IL-1 β , IL-6 and IL-8, revealing mitochondrial retrograde signaling led to activation of YAP1 and β -catenin in airway epithelium and may play an important role in asthmatic airway inflammation. Furthermore, exposed to EB not only decreased the expression of GR α and GR β , but also reduced the ratio of GR α /GR β in 16HBE cells, indicating mitochondrial dysfunction was a potential therapeutic target for steroid insensitivity and severe asthma.

In summary, we have shown that the mitochondrially targeted antioxidant MitoQ ameliorated airway inflammation by stabilizing β -catenin destruction complex in a TDI-induced steroid-insensitive asthma model. As shown in Fig. 7, exposure to TDI led to mitochondrial dysfunction and disintegration of β -catenin destruction complex, while mitochondrial dysfunction and disintegration of β -catenin destruction complex were processes of mutual promotion. Therefore, clarifying the relationship between mitochondrial dysfunction and β -catenin destruction complex may result in novel treatments for TDI asthma model and severe asthma.

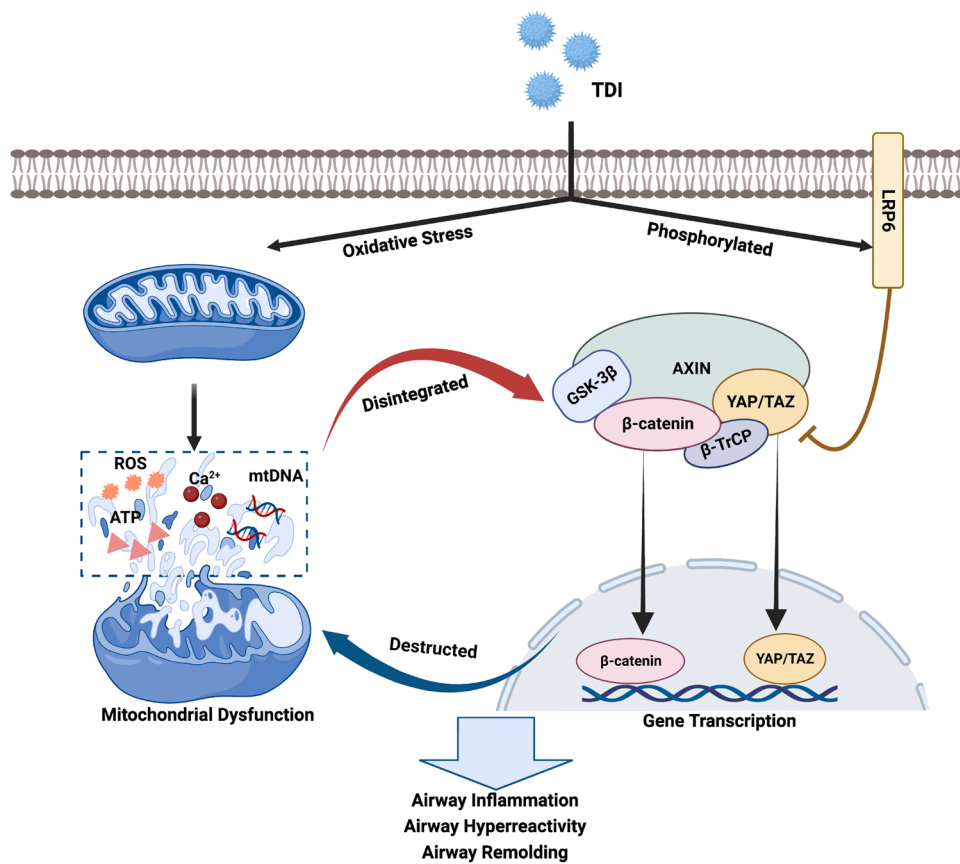


Fig. 7. Model of the mechanism of mitochondrial dysfunction and β -catenin destruction complex in TDI asthma model. On the one hand, exposure to TDI leads to mitochondrial dysfunction to release mitochondrial damage-associated molecular pattern molecules (such as ROS, mtDNA, ATP, calcium) and disintegration of β -catenin destruction complex to induce nuclear translocation of β -catenin and YAP1. On the other hand, TDI promotes the phosphorylation of LRP6, which binds to Wnt ligands to regulate the disintegration of β -catenin destruction complex, to induce the disintegration of β -catenin destruction complex and nuclear translocation of β -catenin and YAP1. It will cause the activation of downstream inflammatory pathway and mitochondrial dysfunction.

Ethics approval

All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital.

CRediT authorship contribution statement

Haijin Zhao and Wenqu Zhao conceived the study, and supervised the scientific work. Junwen Huang and Ying Chen performed the experiments, and wrote the first draft of the manuscript. Xianru Peng, Zhaoqian Gong, Yanhong Wang, Maosheng Xu, Yanyan Ma, Yuemao Li, Changhui Yu, and Shaoxi Cai contributed to the research plan and design. All of the authors contributed to, and approved the final manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The data that has been used is confidential.

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Authors' consent for publication

All authors giving consent for publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.114680](https://doi.org/10.1016/j.biopha.2023.114680).

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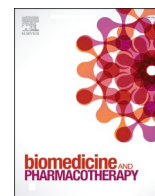
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Update

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