Online Supplementary Material for

Mitochondrial transplantation attenuates airway hyperresponsiveness by inhibition of cholinergic hyperactivity

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The file includes Supplementary Methods, References, Figure S1-S9 and Legends

Supplementary Methods

1. Reactive oxygen species (ROS) measurement and calibration.

ROS monitoring and quantitative estimation using DCFDA in airway epithelial cells (RAECs) performed at room temperature as recently described in detail [1].

The H_2O_2 concentration was calibrated by exploiting the oxidation of DCFDA as conducted in our recent study [1]. The similar method has also been employed successfully in other laboratories [2, 3]. Four independent experiments were performed for each of a series of extracellular H_2O_2 concentrations of 0.1, 1, 10, 20, 30, 50, 100 μM applied to RAECs and the resulting oxidation of DCFDA were plotted again the concentration of H_2O_2 to generate the calibration curve and equation.

Supplementary references

1. Zhang J, Zhou J, Cai L, Lu Y, Wang T, Zhu L, et al. Extracellular calcium-sensing receptor is critical in hypoxic pulmonary vasoconstriction. Antioxid Redox Signal. 2012;17:471-484.

2. Roy S, Parinandi N, Zeigelstein R, Hu Q, Pei Y, Travers JB, et al. Hyperoxia alters phorbol ester-induced phospholipase D activation in bovine lung microvascular endothelial cells. Antioxid. Redox. Signal. 2003;**5:**217-228.

3. Wang QS, Zheng YM, Dong L, Ho YS, Guo Z, Wang YX. Role of mitochondrial reactive oxygen species in hypoxia-dependent increase in intracellular calcium in pulmonary artery myocytes. Free. Radic. Biol. Med. 2007;42:642-653.

Supplementary Figures

Figure S1

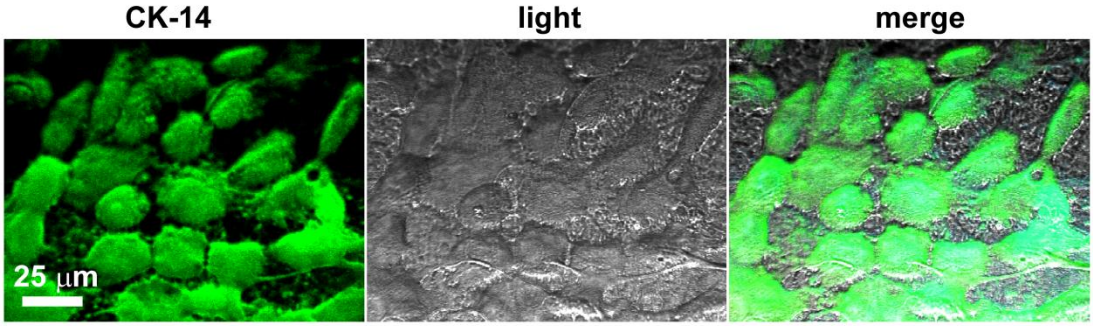

Figure S1. The immunocytochemial identification of the expression of CK-14 in cultured rat airway epithelial cells.

The cultured rat airway epithelial cells (RAECs) were washed by PBS, fixed with cold acetone, then subjected to immunocytochemical staining using monoclonal antibody against cytokeratins-14 (CK-14) and finally examined under confocal microscope with the mode of differential interference contrast (DIC). Shown are the representative stainings of RAECs cultured from at least three individual rats with an averaged positive staining of CK-14 > 95%. Under DIC mode, the light imagings of the cells are identified as the convex, smooth area and the concave and coarse area are the intercellular space.

Figure S2

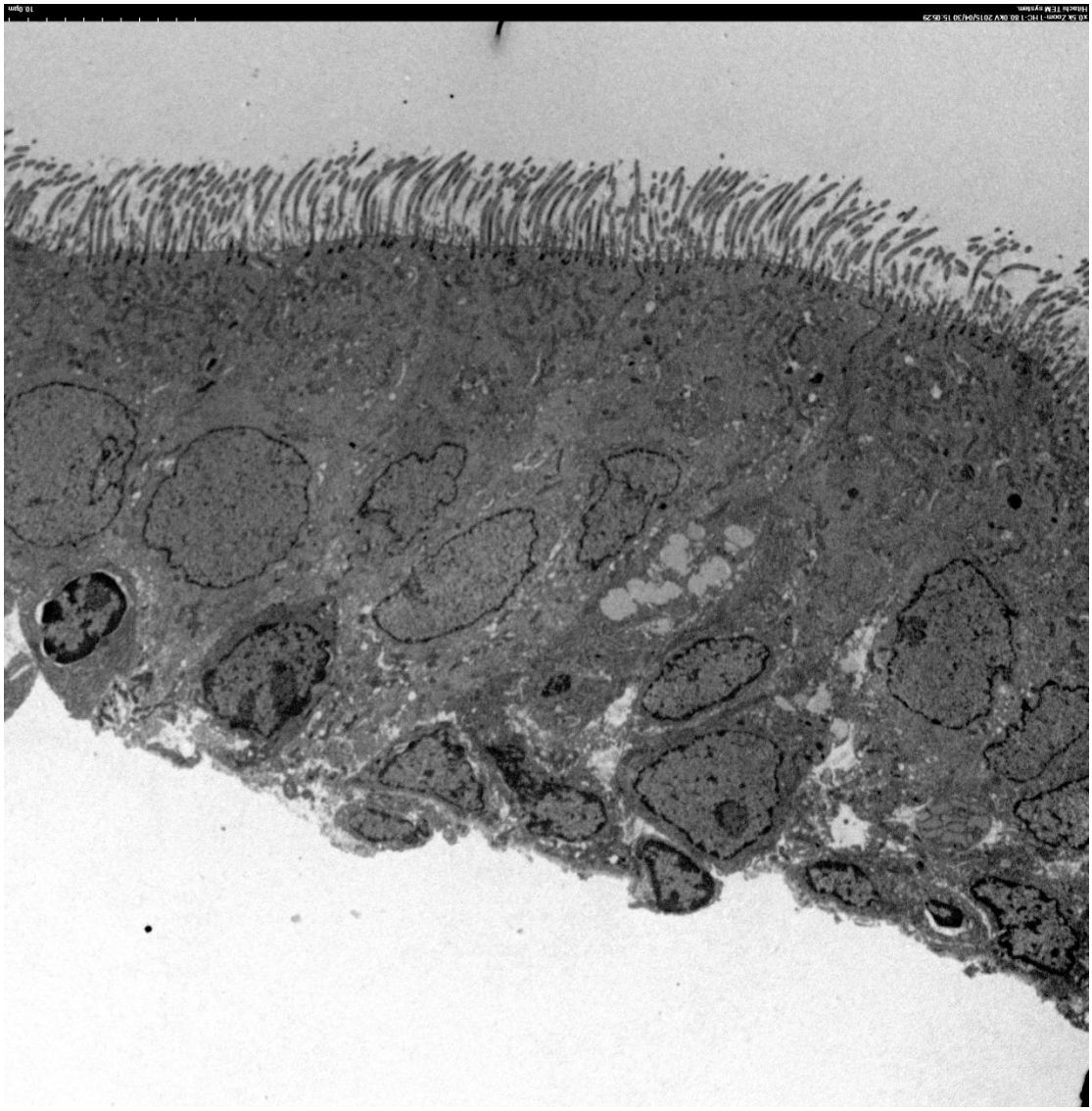

Figure S2. The ultra-structural examination of the tissue of rat tracheal epithelium.

The tissue of tracheal epithelium was freshly stripped from rat airway and subjected to electronic microscopy examination. The representative images of separate tissue preparations from four rats consistently showing the majority of epithelial cells with cilia, the minority of goblet cells and basal cells, however no fibroblasts or smooth muscle cells.

Figure S3

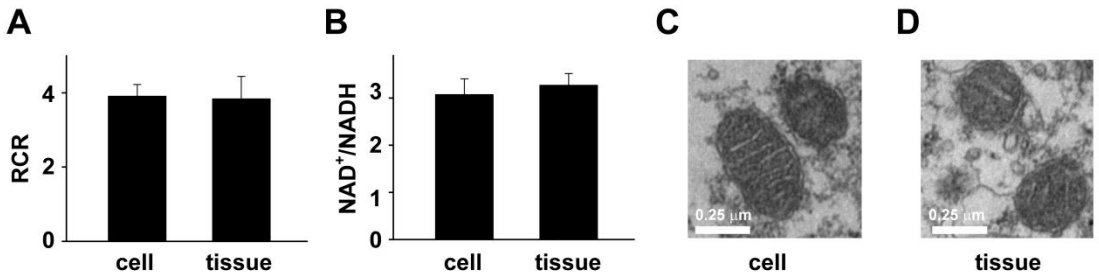

Figure S3. The evaluation of the respiratory function of mitochondria isolated from cultured rat airway epithelial cells and fresh airway epithelium.

The naked mitochondria isolated from cultured rat airway epithelial cells (RAECs, cell) and fresh airway epithelium tissue (tissue) were evaluated for the respiratory control ratio (RCR, **A**) and the NAD+/NADH (**B**) respectively, or prepared for electronic microscopy examination (**C** and **D**) (n=3 for each).

Figure S4

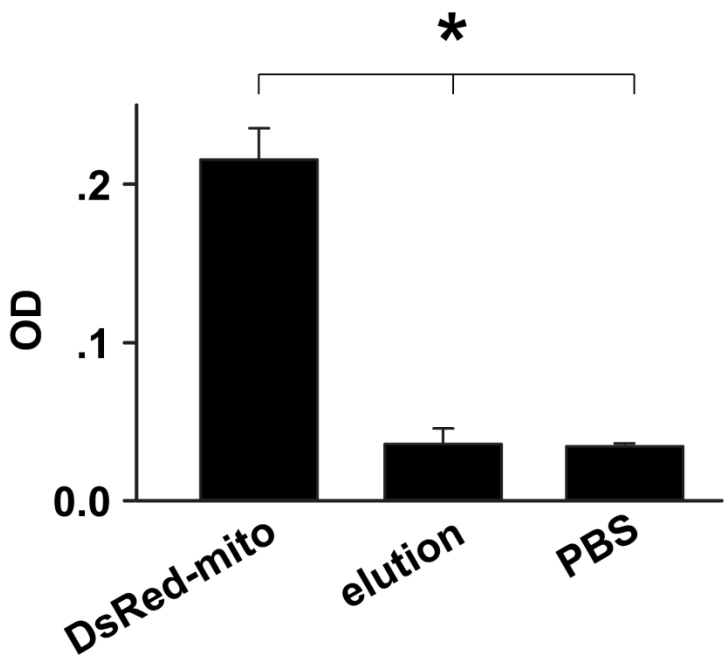

Figure S4. The washout of the incubation medium for exogenous transplantation into RAECs.

The incubation medium for the transplantation of DsRed-labeled exogenous mitochondria into rat airway epithelia cell (RACEs), the final elution after complete washout by PBS and PBS alone were collected for DsRed fluorescence detection (n=3, * $p<0.001$).

Figure S5

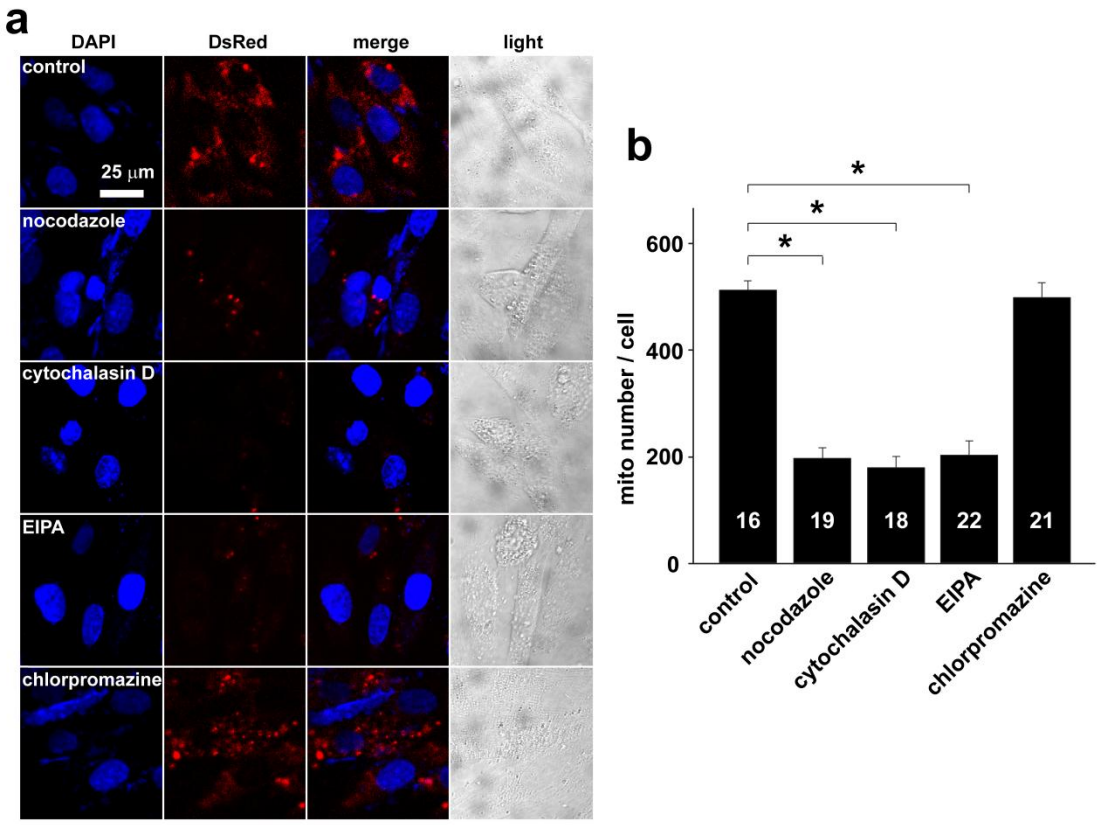

Figure S5. Macropinocytosis-dependent internalization of naked mitochondria.

The confocal images of rat airway epithelial cells (RAECs) after a 24 hours of incubation with DsRed-labeled mitochondria in the absence or the presence of 10 μM nocodazole, 2 μM cytochalasin D, 25 μM 5-(N-ethyl-N-isopropyl)amirolide (EIPA) or 10 μM chlorpromazine, respectively (**a**) and statistical summary (the number in the bar indicates individual RAECs from 3-4 separate experiments, * $p < 0.05$ *vs* control, **b**).

Figure S6

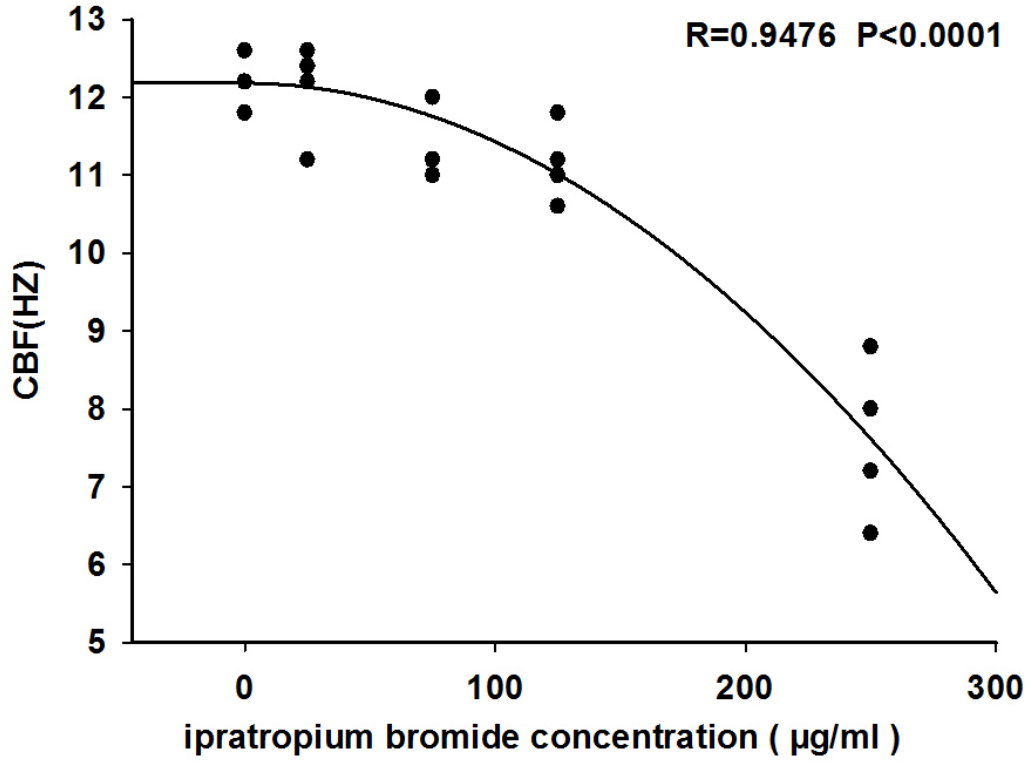

Figure S6. The dose-dependent effects of ipratropium bromide on CBF of airway epithelial cells.

The effects of M receptor inhibitor, ipratropium bromide (IB) in 100, 200 and 300 μg/mL on the ciliary beating frequency (CBF) of cultured rat airway epithelial cells (n=4, R=0.9476, $p < 0.0001$).

Figure S7

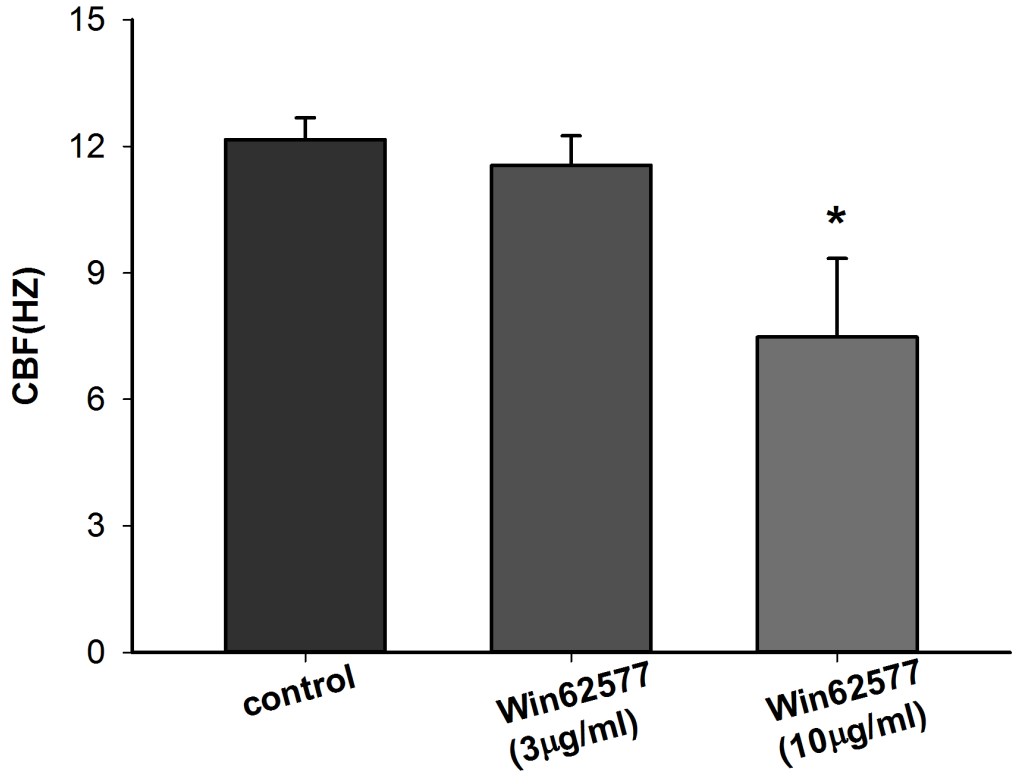

Figure S7. The dose-dependent effects of win62577 on CBF of airway epithelial cells.

The effects of M receptor enhancer, win62577 (WIN) at 3 and 10 μg/mL on the ciliary beating frequency (CBF) of cultured rat airway epithelial cells (n=4-8, * $p < 0.05$).

Figure S8

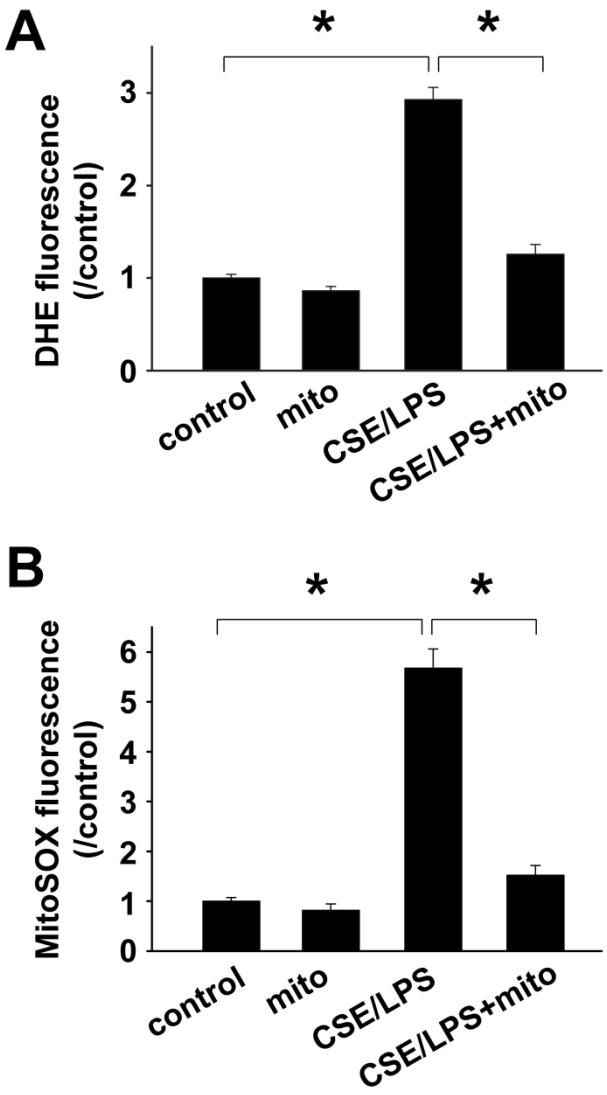

Figure S8. CSE/LPS stimulated the overproduction of superoxide from RACEs.

After incubation with 5 μM Dihydroethidium (DHE, Beyotime Biotechnology) or 5 μM MitoSOX (Molecular Probes) at 37℃for 30min and wash three times, the red fluorescence from RAECs was detected under fluorescent microscope with excitation and emission wavelength of 535 nm and 610 nm, or 510 nm and 580 nm for DHE (**A**) or MitoSOX (**B**), respectively. n=3 for each, $p < 0.05$.

Figure S9

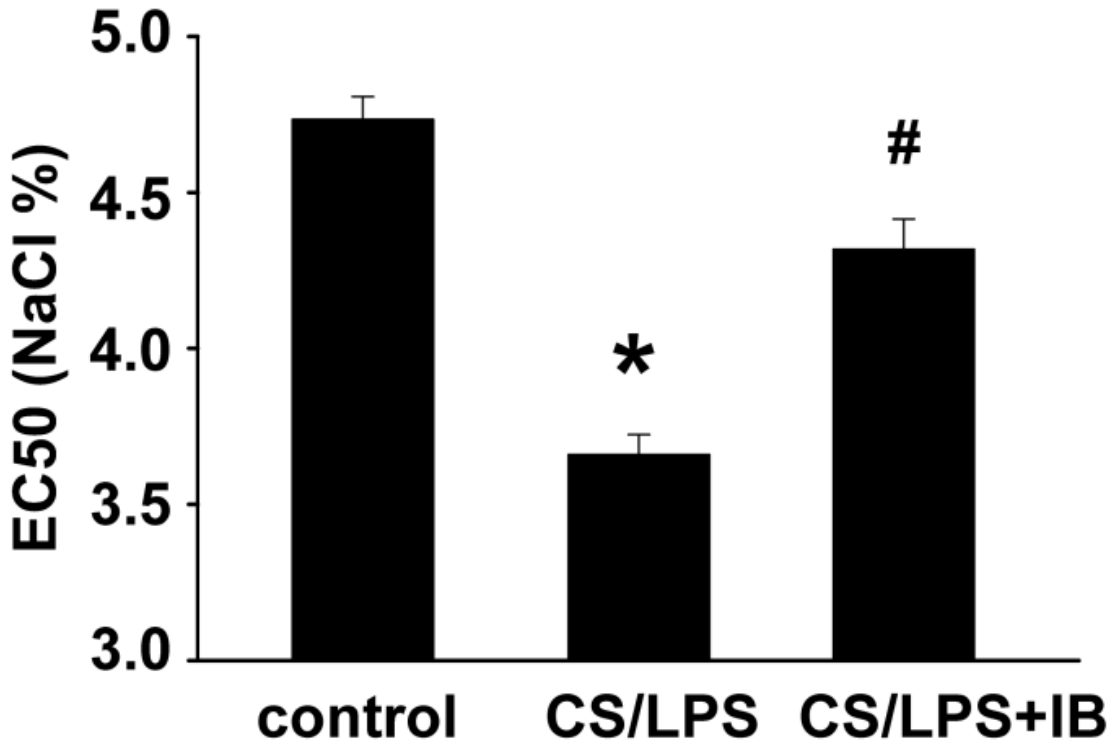

Figure S9. Inhibition of airway hyperresponsiveness to hyertonic NaCl by ipratropium bromide in CSE/LPS-exposed rats.

The airway resistance (R_{RS}) following aerosol challenge successively with the gradient concentrations of NaCl at 1.8%，2.7%，3.6%，4.5%，5%，5.4% and 6.3% each for 5 min was monitored. The EC50 of NaCl concentration were obtained and compared between control (n=6), cigarette smoke plus LPS exposed (CS/LPS, n=4) and ipratropium bromide (IB) treatment in CS/LPS-exposed rats (n=5). * # $p < 0.05$ vs. control and CS/LPS, respectively.