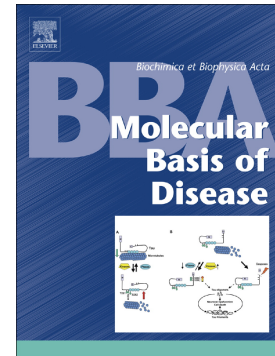


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**IgE Induces Hypotension in Asthma Mice by Down-regulating vascular NCX1
expression Through Activating MiR-212-5p**

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Highlights

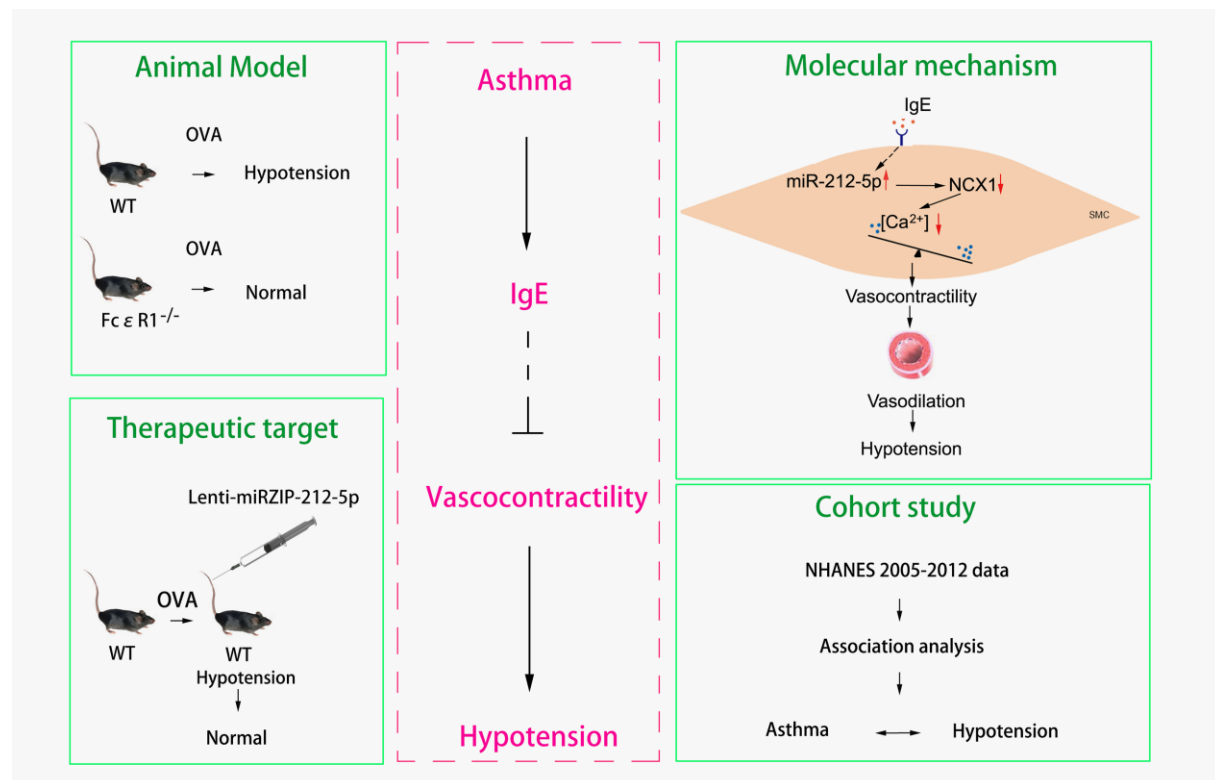
- IgE mediates decrease of vasoconstriction of thoracic aorta, which contributes to the asthma-induced hypotension.
- miR-212-5p mediates IgE-induced downregulation of Na⁺/Ca²⁺ exchanger protein 1 in aortic smooth muscle cells.
- Suppression of miR-212-5p in asthma mice restores blood pressure by increasing Na⁺/Ca²⁺ exchanger protein 1 expression and vasocontractility.
- Asthma is correlated with hypotension in crowd data.

Abstract

Immunoglobulin E (IgE) has been suggested as a risk factor for allergy-induced low blood pressure, which has not been well explained in molecular details. Our current study shows a novel mechanism involving IgE, FcεRI, miRNA-212-5p (miR-212-5p), and sodium/calcium exchanger protein 1(NCX1) for asthma to induce hypotension. In arterial smooth muscle cells, IgE up-regulated miR212-5p via its receptor FcεRI, which resulted in down-regulation of NCX1 that is a regulating factor for blood pressure. In mice, asthma induced hypotension by interfering vasoconstrictive function; knockout of FcεRI kept the asthmatic mice from developing hypotension; knock-down of miR-212-5p in asthmatic mice resulted in a significant restoration of blood pressure. In human, asthma and IgE were positively correlated with hypotension in cohort study on NIH epidemiological data. This study suggests a novel therapeutic target (miR-212-5p) for treatment of asthma-induced hypotension.

Key words: Asthma, IgE, *FcεR1*^{-/-}, Hypotension.

Graphical Abstract



1. Introduction

By far, cardiovascular disease is still the first leading cause of mortality and morbidity. Severe hypotension as a cardiovascular disease can lead to shock by depriving oxygen and nutrients of brain and other vital organs, which often occurs as a complication of some serious allergic reaction like anaphylaxis that induces various cytokines (histamine, mast cell tryptase, and soluble TNF receptor I) [1], and subsequently causing vasodilation and fluid leakage from blood vessels [2, 3]. The mechanism underlying allergic reaction induced-hypotension remains unclear.

Elevated IgE in circulation is the characteristic of allergic responses in circulation. Our and others' previous studies demonstrated that IgE is a new risk factor for cardiovascular diseases [4, 5], and elevated IgE level appears in patients with asthma and cardiovascular diseases [4, 5]. IgE has been shown to promote atherosclerosis and abdominal aortic aneurysm in mice [4, 6]. However, the association of elevated IgE and hypotension has not been well studied.

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1 (NCX1) proteins, encoded by SLC8A1 gene in mammals, is responsible for intracellular Ca^{2+} homeostasis and VSMC contraction [7]. Vascular smooth muscle cell (VSMC) contraction plays an important role in regulating blood pressure. NCX1 belongs to the NCX family which typically containing 930-970 amino acids with two Ca^{2+} -binding domains. The biological function of NCX1 in VSMC is mediating both Ca^{2+} influx and Ca^{2+} efflux by coupling Na^+ in a ratio of $3\text{Na}^+ : \text{Ca}^{2+}$ according to electrochemical gradient across the membrane. This function is closely related to the localization of NCX1 in the arterial smooth muscle. Immunocytochemical studies revealed that NCX1 was confined to regions in

adjacent to sarcoplasmic reticulum on plasma membrane, which makes it an important regulator of calcium concentration and consequently vasoconstriction[8]. It has been demonstrated that NCX1 facilitates a net Ca^{2+} entry in VSMC to maintain normal blood pressure, and disruption of NCX1 expression using smooth-muscle-specific knock out decreases blood pressure [9, 10]. Our current study suggests that IgE reduces NCX1 expression to promote hypotension.

MicroRNAs (MiRNAs), as typical 22-nt small non-coding RNAs, usually bind on 3' untranslated region (UTR) of messenger RNA (mRNA) to regulate its expression. Commonly, this regulation results in suppression or degradation of the targeted mRNA. Thus, miRNA represents another layer of post-transcriptional regulatory networks maintaining homeostasis of the body. Since the discovery of miRNAs in circulation in 2008, numerous studies have been conducted on miRNA expression profile in physiological as well as pathological conditions including asthma, contact dermatitis, and hypertension[11]. In our previous study, we found that miR-212-5p was significantly up-regulated in response to IgE in arterial smooth muscle cells (ASMC) using miRNA array (unpublished data). We hypothesized that IgE is involved in asthma-induced hypotension through regulating miR-212-5p, and verified that it happens in vitro and in vivo.

In this study, we first explored the association between allergy and hypotension using an asthmatic mice model (WT and *FcεRI* knockout (KO) mice). Then, we investigated the mechanism responsible for asthma-induced hypotension using in vitro IgE stimulated ASMC model (WT and *FcεRI* KO ASMC). Further, we established the role of miR-212-5p in asthma-induced hypotension using tail vein injection of miRZIP-212-5p lentivirus. At last, we

epidemiologically verified our hypothesis that asthma is positively associated with hypotension using health survey data from National Institutes of Health. Our study reveals that asthma-induced hypotension depends on IgE-FcεR1 regulated NCX1 expression through regulating miR-212-5p in ASMC. Recover of the blood pressure in our asthma mice with miR-212-5p knock-down suggests that miR-212-5p may be a therapeutic target for asthma-induced hypotension.

2. Materials and methods`

2.1. *Animal model*

Mice at age of six to eight-week with body weight of 20~22g were maintained in a temperature and humidity controlled room with a 12h light-dark cycle. Mice had free access to tap water and standard mouse chow ad libitum. WT and FcεR1^{-/-} littermates (C57BL/6J, Jackson Laboratory, USA) were randomly assigned to the asthma group or control group. Briefly, the experimental mice were sensitized by intraperitoneal injection (i.p.) of 50μg ovalbumin with alum adjuvant [4] on day 0, 7 and 14, while the control mice received i.p. saline with alum. From day 21 onward, the mice were exposed to aerosolized 3.75% OVA (allergen exposure) or saline (sham exposure) 30 min/day for 3 successive days. Blood pressure was recorded by tail-cuff method as described [12] at day 0 and day 23. All procedures were approved by the Animal Care and Use Committee of Peking Union Medical College.

2.2. *Bronchialveolar lavage and lung histopathology*

Mice were anesthetized with ketamine through i.p. (60 mg/kg), and the adequacy of anesthesia was continually monitored by assessing reflexes and respiration. After

exsanguinations via cardiac puncture, bronchoalveolar lavage fluid (BALF) was collected (0.8 ml saline, two times) by tracheal intubation. Allergic inflammatory responses and lung pathology were assessed in situ by lung histopathology staining (H&E and Masson) [13], and ex vivo by Wright-Giemsa staining eosinophils counts of BALF according to previously studies[14].

2.3. Echocardiography

Animals were anesthetized with isoflurane, and underwent M-mode echocardiography at 0 and 23 days after saline or OVA infusion using a 30 MHz probe (Vevo 770 system; Visual Sonics, Toronto, Ontario, Canada)[15]. Body temperature was maintained at 37°C using a thermally controlled surgical table. The diastolic measurements of interventricular septum (IVS), left ventricle[16], internal dimension (LVID), LV posterior wall thickness (LVPW), and the systolic measurement of LV internal dimension (LVID) were obtained from original M-mode tracings. The LV ejection fraction (%LVEF) and LV fractional shortening (%LVFS) were calculated. The LV FS (fractional shortening) was calculated as: $\%FS = [(LVID_d - LVID_s)/LVID_d] \times 100\%$.

2.4. Examination of thoracic aortic vascular tension

Thoracic aortae were dissected from WT and *FcεR1^{-/-}* mice after anesthetized with chloral hydrate (10%) intraperitoneally. Aortas from mice were removed in ice-cold Krebs solution: 118mM NaCl, 4.7mM KCl, 2.5mM CaCl₂, 1.19mM MgSO₄, 25mM NaHCO₃, 1.19mM KH₂PO₄, and 10mM glucose (pH=7.4). The adherent connective tissues of each aorta were

carefully trimmed and the aorta was cut into ring segments of 2mm in length. Aortic rings were suspended between 2 tungsten wires in chambers of a Multi Myograph System (610 M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. Each organ chamber was filled with 5 mL of the modified Krebs-Ringer bicarbonate solution maintained at $37 \pm 0.5^{\circ}\text{C}$ and aerated with 5% CO_2 and 95% O_2 . At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension of 3 mN for 30 minutes by stepwise stretching. After 30 min equilibrium period, each ring was contracted by 60 mM KCl to test its viability, and washed three times in Krebs solution. The functional integrity of the endothelial cells was verified by more than 80% relaxation induced by acetylcholine (ACh, 10 μM) in phenylephrine pre-contracted rings. Then rings were rinsed and contracted again by phenylephrine (10^{-9} – 10^{-5} M) before cumulative addition of ACh ($(3 \times 10^{-8}, 1 \times 10^{-7}, 3 \times 10^{-7}, 1 \times 10^{-6}, 3 \times 10^{-6}, 1 \times 10^{-5} \text{ mol/L})$) to evoke endothelium-dependent relaxations. For data presentation, vasoconstrictive responses induced by phenylephrine were expressed as a percentage of the tone (mN) invoked by 60 mM KCl. Vasorelaxation responses were expressed as percentage of the peak tone invoked by 1 μM phenylephrine.

2.5. Detection of the cytokines of interest in the serum

Blood samples were taken before the experimental animals were sacrificed, then the serum samples were collected. Circulating IgE and $\text{TNF-}\alpha$ were measured using mouse enzyme-linked immunosorbent assay according to the manufacturers' instructions (IgE kit from Cayman Chemical, $\text{TNF-}\alpha$ kit from eBioscience).

2.6. Western blot

Total proteins from cultured cells or vessel tissues were extracted in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.1% EDTA) on ice with cocktail protease inhibitor (Roche, NJ, USA). The concentrations of total proteins were determined using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Equal amounts of proteins (35µg) were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking in 5% milk/Tris buffered saline-Tween (TBST) for 1 h, the membranes were incubated with the specific primary antibodies including NCX1 (1:500, Abcam) and GAPDH (1:5000, Proteintech) followed by horseradish peroxidases (HRP)-labeled secondary antibodies (Invitrogen, 1:2000) for 1.5 h. Immunoreactive bands were visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce).

2.7. RNA isolation and quantitative real-time PCR

Total RNA was isolated from the cells or vessels using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was reversely transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (TransGen Biotech, China), and analyzed by quantitative real-time PCR with a SYBR® Premix Ex Taq™ II RT-PCR Kit (Bio-Rad, USA). All real-time PCR reactions were performed on the iQ5™ Multicolor Real-Time PCR detection system (Bio-Rad, USA). The data were normalized to endogenous GAPDH mRNA and U6 snRNA. The $2^{-\Delta\Delta CT}$ method was used for analysis of the PCR data.

2.8. Cell Culture and Transfection of Artery Smooth Muscle Cell

Primary arterial smooth muscle cell (ASMC) were isolated from WT or FcεR1^{-/-} mice, and cultured in DMEM containing 10% FBS as previously described [17]. Briefly, aortas were isolated from the mice at age of 1 week, and cut into small pieces, then digested using collagenase and elastase at 37°C for 15 min with mild shaking. The enzymatic digestion was stopped by adding DMEM containing 20% FBS, then the tissue explants were subjected to centrifugation. The precipitated cells were resuspended in DMEM containing 10% FBS, and then seeded into fibronectin-coated dishes. ASMC were routinely sub-cultured in growth medium at about 80% confluence. The day before transfection, ASMC were seeded onto 6-well plates (4×10^5 cells per well). The miR-212-5p mimics or inhibitors, and the negative control, which were gained from Ribobio (Guangzhou, China), pMIR-Reporter-NCX1, and its mutants were constructed and transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's procedures. For luciferase assay, cells were seeded onto 24-well plates. For western blot analysis of NCX1, cells were seeded onto 6mm dish, and then transfected as described above [18].

2.9. Dual-Luciferase activity reporter assay

According to the bioinformatics predictions, The WT of miR-212-5p binding sequences on the 3'-UTR of NCX1 gene were amplified by PCR and cloned into pMIR-reporter luciferase reporter plasmid (WT-3'-UTR). Then, mutations of the miR-212-5p binding site on the 3'-UTR of NCX1 (Mutant-3'-UTR) were generated by bridge PCR (The primers were listed in Supplementary Table S1). The resultant mutants were transfected into 293TN cells together with miR-212-5p mimics or scramble control mimic. After 48h of incubation, the cells were

lysed according to the instruction of Dual-Luciferase reporter system Kit (Promega, USA), and renilla fluorescence was used as the internal standard. All transfection assays were performed in triplicates and the ratio of luciferase vs renilla fluorescence of each group was statistically analyzed.

2.10. Study population

The National Health and Nutrition Examination Survey (NHANES) used a stratified multistage probability sampling design. 21390 subjects, who completed interviews, examinations or laboratory tests related to asthma, diabetes mellitus, age, sex, race, smoking status, body mass index, and high density lipoprotein level, were selected from NHANES (2005–2012) in this study. Details of the survey design and implementation of NHANES 2005–2012 are available online (<https://www.cdc.gov/nchs/nhanes/index.htm>). Subjects over age of 18 were included in the analysis. Asthma subjects were selected in those who had asthma history which was recorded by their doctors or other health professionals. Similarly, diabetic subjects were also selected. Hypotension subjects were selected in those with systolic blood pressure <90mmHg. The systolic blood pressure is defined as the mean of the three measurements. Age, sex, race, diabetes, smoking, body mass index (BMI), HDL-cholesterol, were considered as potential confounders in the analysis. Information regarding asthma, sex, race, diabetes, smoking (current smokers currently smoke cigarettes; past smokers smoked at least 100 cigarettes in life but don't smoke at all now.) was obtained by inquiring. BMI and blood pressure were both measured by physical examinations. Serum HDL-cholesterol level was determined by laboratory tests. All analyses were performed with SAS 9.4. Demographic

characteristics of the included participants across populations with or without hypertension were analyzed by Chi-square test or t-test. The Chi-square test was used for analyzing the correlation between hypotension and asthma. The same method was applied to analysis of hypotension with diabetes, smoking, sex, race. The age difference between hypotension and non-hypotension was analyzed by t-test. The same method was used for analyzing body mass index (BMI) and HDL-cholesterol. The sampling weights (WTMEC2YR) and design variables (SDMVSTRA; SDMVPSU) were applied to these survey sampling procedures.

2.11. Statistical analysis

All data were expressed as means $\pm$ SD. Two-way ANOVA was used to determine the statistical significance of the differences among the groups. A level of $p < 0.05$ was considered statistical significant.

3. Results

3.1. Ovalbumin-sensitized C57BL/6J wild-type mice developed hypotension

To test whether asthma induces hypotension or not, the C57BL/6J wild type mice were sensitized with ovalbumin intraperitoneally three times followed by 3 days of inhalation. As shown in supplementary figure 1, ovalbumin-sensitized mice showed significantly higher plasma IgE, inflammation, and airway remodeling compared with control group. The blood pressure of the sensitized mice was measured using tail-cuff method. Compared with saline-sensitized group, the systolic blood pressure of OVA-sensitized mice was much lower (83.4 ± 0.857 mmHg vs. 102.8 ± 11.557 mmHg, $p < 0.05$) despite their similar baseline levels (Fig. 1a). Similarly, as shown in Fig. 1b, the diastolic blood pressure of OVA-sensitized mice

(61.4 ± 1.356 mmHg) was also lower than control group (75.3 ± 3.807 mmHg). In addition, the mean blood pressure of OVA-sensitized mice was also lower than control group (Fig.1c).

3.2. Hypotension development in ovalbumin-sensitized mice is dependent on IgE-FcεR1 signaling.

To investigate the role of IgE in hypotension, we used FcεR1-deficient mice model to test whether disruption of IgE-FcεR1 signaling protects mice from OVA-induced hypotension. As shown in figure 2, the systolic blood pressure (Fig. 2a), diastolic blood pressure (Fig. 2b), and mean blood pressure (Fig. 2c) showed no decrease in OVA-sensitized FcεR1^{-/-} mice compared with WT mice, indicating that IgE-FcεR1 signaling is involved in the blood pressure drop induced by asthma.

3.3. Asthma-induced hypotension is related to the decreased vasocontractility but not cardiac dysfunction

Blood pressure is regulated by complex networks which involved in the heart and the vascular system. In this study, we first examined the effect of OVA sensitization on the cardiac function of WT mice. Compared with saline-treated control group, the echocardiogram data showed that both the left ventricular wall thickness and ventricular internal diameter in a cardiac cycle had no significant alteration in the OVA-treated asthma group (Table S2). Similarly, the calculated ejection fractions of asthma and control group were also similar ($68.826 \pm 3.095\%$ vs $65.135 \pm 0.934\%$). These data suggest that OVA sensitization does not alter cardiac function.

We next investigated the effect of OVA sensitization on vasocontractility by myograph system using thoracic artery ring assay. As shown in Fig. 3a, the thoracic artery from both OVA and saline-treated groups produced a concentration-dependent contraction in response to phenylephrine stimulation. However, the contractile response to phenylephrine was reduced in ova-sensitized group compared with saline group. This reduction in contractile response did not occur when IgE receptor was knocked out (Fig. 3b). In addition, the relaxation of the thoracic artery in response to acetylcholine showed no significant difference between OVA-sensitized group and saline group regardless of the FcεR1 genotype (Fig. 3c, d). Overall, these data suggest that asthma-induced hypotension is mainly due to the defect of vascular contraction, which is IgE-FcεR1 dependent.

3.4. NCX1 is down-regulated in the asthma-induced hypotension mice

Based on previous finding that VSMCs NCX1 mediates net Ca^{2+} influx that is required for the maintenance of agonist-induced vasoconstriction and basal blood pressure [19], we hypothesized that IgE may affect the contractility of blood vessel through regulating NCX1 expression in smooth muscle cells. We first examined the NCX1 expression in OVA/saline-sensitized mice. As shown in Fig. 4a and Fig. 4b, NCX1 mRNA level in wild type asthma mice was only 14.32% of control mice. Accordingly, the protein level of NCX1 in the vessel of asthma mice was also significantly decreased. However, there was no difference in NCX1 expression in FcεR1^{-/-} mice regardless of the treatment. Some previous studies have shown that the NCX1 has important roles in cardiac function[20], so we examined the expression of NCX1 in heart. The data suggested there is no significant difference in NCX1 expression between

saline mice and OVA treated mice (Fig.S2).

We next assessed the effect of IgE on NCX1 using wild type and $Fc\epsilon R1^{-/-}$ aortic smooth muscle cells (ASMC). ASMC were treated with IgE for different period, the transcription level of NCX1 was determined by real-time PCR. As shown in Figure 4c, NCX1 mRNA significantly decreased after IgE treatment in wild type ASMC. Similarly, the protein level of NCX1 was also markedly decreased in western blot (Fig.4d). Consistent with our in vivo observation, there was no significant difference in NCX1 expression of $Fc\epsilon R1^{-/-}$ in ASMC before and after IgE treatment (Fig. 4e, f).

3.5. *miR-212-5p modulates IgE-reduced NCX1 expression in aortic smooth muscle cells*

IgE is a key mediator for asthma-induced hypotension through down-regulation of NCX1 expression in both mRNA and protein level. To explore the molecular mechanism by which IgE reduced NCX1 mRNA level, we examined the post-transcriptional regulation of the gene. MiRNA, as the negative regulator for gene expression, is reported altered in IgE stimulated mast cell[21]. Therefore, we hypothesized that IgE may regulate NCX1 expression through manipulating miRNAs expression in VSMCs. To test our hypothesis, we screened NCX1 3'-UTR binding factors by bioinformatics analysis. The resultant data showed that miR-212-5p seed sequences match 3'-UTR of NCX1 (Fig.5a), and miR-212-5p mRNA is markedly elevated after IgE treatment (Fig.5d). The dual-luciferase assay demonstrated that NCX1 was a direct target of miR-212-5p (Fig. 5b). In addition, over-expression of miR-212-5p decreased the expression of NCX1 at both mRNA and protein levels. In contrast, inhibition of miR-212-5p attenuated IgE-decreased NCX1 expression at both mRNA and protein level (Fig. 5c, 5d, S3),

further suggesting that NCX1 is a target of miR-212-5p. Thus, we hypothesized that IgE upregulates miR-212-5p in OVA-sensitized mice, which decreases NCX1 expression, and subsequently leads to hypotension. Real-time PCR analysis showed that miR-212-5p was significantly increased in OVA-sensitized WT mice. However, there was no difference in miR-212-5p expression between saline and OVA-treated $Fc\epsilon R1^{-/-}$ mice (Fig. 5e). These data are consistent with our in vitro data (Fig. 5f). These results support our hypothesis that miR-212-5p is involved in IgE-mediated NCX1 reduction in asthma-induced ASMC (Fig.S4).

3.6. Inhibition of miR-212-5p rescues hypotension in OVA-sensitized mice.

Based on the above data, we hypothesized that inhibition of miR-212-5p may rescue asthma-induced hypotension. To test this hypothesis, WT mice were divided into three groups for the treatments of saline, OVA with vector-control (OVA-Lenti-GFP), and OVA with miR-212-5p inhibitor (OVA-Lenti-miRZIP-212-5p). As shown in Fig.6a, in agreement with previous data, the systolic blood pressure (SBP) of OVA-sensitized mice was lower than the control of saline group (88.573 ± 0.797 mmHg vs 99.783 ± 1.009 mmHg). However, injection of miRZIP-212-5p inhibitor rescued SBP to 102.642 ± 1.390 mmHg which is similar to that in the saline group. ($p > 0.05$). Similarly, the diastolic blood pressure (DBP) of OVA group was also recovered (65.313 ± 1.798 mmHg vs 74.450 ± 3.494 mmHg, Fig. 6b).

To better understand why miR-212-5p inhibitor rescued the blood pressure, vascular responses of thoracic artery assay was applied. As shown in Fig.6c, the contractile responses to phenylephrine in mice injected with OVA-Lenti-GFP were still impaired in comparison with saline group ($p < 0.05$). However, the contractile responses of OVA-sensitized thoracic artery of

mice injected with Lenti-miRZIP-212-5p were similar to those of the saline-treated group. Consistent with previous data, acetylcholine produced similar relaxation of thoracic arteries in these three groups (Fig. S5).

Our analysis of mRNA and protein level of NCX1 revealed that mice in OVA-Lenti-GFP group exhibited lower NCX1 expression accompanying higher level of miR-212-5p compared with saline group (Fig.6d, 6e). Mice with Lenti-miRZIP-212-5p virus injection showed significantly reduced miR-212-5p expression and increased NCX1 expression (Fig.6d-f). Taken together, these data indicate that inhibition of miR-212-5p recovers asthma-induced hypotension by increasing NCX1 expression and enhancing the artery contractility. These findings provide a potential therapeutic strategy for asthma-induced hypotension.

3.7. Association between asthma and blood pressure in crowd data.

To investigate whether asthma is associated with hypotension in human, the data from 2005-2012 National Health and Nutrition Examination Survey (NHANES) were analyzed. A total of 21390 participants were included, among of them, there were 2922 (14.05%) with asthma, 18468 (85.95%) without asthma. As shown in Table 1, there is significant correlation between blood pressure and asthma ($p=0.0212$). In subjects with asthma, hypotension was more prevalent (17.66%) compared with 15.52% in non-asthma group.

4. Discussion

This report for the first time describes a detailed study on the association of allergic disorder and hypotension, and the mechanism underlying asthma-induced hypotension. In our mouse model, asthma induces hypotension which is mainly due to asthma-induced

vasoconstrictive dysfunction in OVA-treated WT mice. In this event, IgE-FcεR1 signaling down-regulates NCX1 expression in ASMC through activation of miR-212-5p. Knockdown of miR-212-5p in asthma mice prevents hypotension. Our findings strongly support the role of IgE in regulation of blood pressure, and suggest miR-212-5p as a novel target for controlling allergy-related hypotension.

Asthma is usually known as being associated with hypertension, especially in young subjects with obstructive lower airways[22]. Nevertheless, it is also reported as being associated with hypotension in albuterol (β adrenergic receptor agonist) treated asthmatic patients[23-25]. Hypotension may worsen the morbidity or cause sudden death in young patients with asthma[26, 27]. As lack of established explanation for the reason why administration of albuterol may cause hypotension in asthmatic patients, we hypothesized that asthma may also be a trigger for occurrence of hypotension.

Considering that the inherent difficulty of monitoring or examining anaphylaxis for its acuteness and lethality, we designed the mouse model to focus on cardiovascular dysfunction associated with asthma-based chronic allergy. Our in vitro study is designed to elucidate the molecular mechanism by which IgE-mediate hypotension. As both anaphylaxis and asthma are IgE mediated pathological conditions[28], our findings in this study may reveal a common mechanism underlying the effect of allergy including asthma and anaphylaxis on the development of hypotension.

Our discovery of IgE function in regulating blood pressure, which is mainly dependent on its effect on regulating vascular small non-coding RNA, has opened up a new direction for research on blood pressure risk factors and mechanisms underlying allergy-related blood

pressure dysfunction. Some studies from others showed that anaphylactic hypotension in mice is caused by a decrease in cardiac output but not vasodilation[29]. Anaphylactic shock usually accompanies with increased vascular permeability, interstitial capillary leakage and exhibited anaphylactic hypotension[30, 31]. Besides, liver and splanchnic vascular beds are also reported involved in systematic anaphylactic hypotension probably because of decrease in circulating blood volume resulted from portal-hypertension induced splanchnic congestion happened in anaphylactic presinusoidal contraction [32]. This study shows for the first time how IgE directly affects the vasculature system in SMC. Upon challenged with the same allergen, massive production of IgE down-regulates NCX1 which controls the contraction responses.

NCX1 has been intensively investigated for its role in maintaining local Na^+ , Ca^{2+} homeostasis[33]. Wang *et al* demonstrated that arterial NCX1 normally mediates net Ca^{2+} influx, which helps maintain basal vascular tone in small resistance arteries and blood pressure under physiological conditions, and conditional knockout of smooth muscle NCX1 attenuates phenylephrine induced-vasoconstriction in isolated mesenteric small resistance artery and hypotension[19]. These data suggest that NCX1 play an important role in regulating blood pressure. Our results also showed that IgE mediates declined vasoconstriction in the isolated thoracic aorta and decrease of blood pressure in the OVA-sensitized mice model. Meanwhile, both mRNA and protein levels of NCX1 significantly decreased under IgE stimulation in both OVA-sensitized mice and cultured ASMC. These data are consistent with the results published by Wang *et al*. Taken together, our results further support that NCX1 plays a key role in the pathophysiology of asthma related-hypotension.

To explore the molecular mechanism by which IgE reduces NCX1 expression, we focused

on NCX1 mRNA expression because that the 3'-UTR of NCX1 matches the seed sequence of miR-212-5p in bioinformatics analysis. The present study provides favorable evidences showing that IgE down-regulates NCX1 by activating miR-212-5p in asthma-induced hypotension. However, the detail of how IgE regulates miR-212-5p is largely unknown. It is possible that IgE may repress miR-212-5p transcription factor, cAMP response element-binding protein (CREB), which increases miR-212-5p level in a way similar to how TGF- β does in endothelial cells[34]. Our present study only focused on calcium regulation through NCX1. It is very likely that the changes of asthma-induced arterial tone are a net result of the unbalanced intracellular ion regulation, and single microRNA may target more than one genes involved in the vascular system. Future studies may be necessary to assess potential targets of miR-212-5p.

Marseglia *et al* reported that hypotension occurred when IgE level was higher than 7 kU/L in lysozyme-sensitized children [35]. Our present results also showed that low blood pressure occurred in the OVA-sensitized mice with high IgE level. To investigate whether the association of asthma and hypotension exists in human, the NIH 2005-2012 data which include a total of 21390 participants, 2922 (14.05%) with asthma and 18468 (85.95%) without asthma, were analyzed in this study. The analysis results demonstrated that there is correlation between blood pressure and asthma ($p=0.0212$), and hypotension in asthma group is more prevalent compared with non-asthma group (17.66% vs. 15.52%). Therefore, the high level of IgE in patients with asthma may become a biomarker for predicting asthma-induced hypotension.

In conclusion, this study provides molecular detail of a novel mechanism underlying the IgE function in hypotension, in which IgE is a key player in pathogenesis of asthma-related

hypotension. This novel mechanism also suggests that miR-212-5p may be a novel therapeutic target for controlling asthma-related hypotension.

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Conflicts of Interest/ Disclosure

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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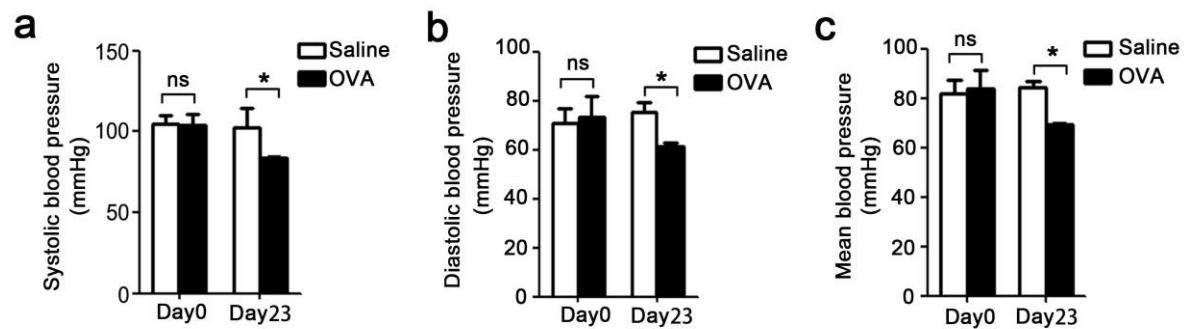


Fig.1 Ovalbumin-sensitized C57BL/6J wild type mice develop hypotension.

a. Systolic blood pressure was determined in asthma group (OVA) and saline group by blood pressure monitor. **b.** Diastolic blood pressure was analyzed in OVA- and saline-treatment wild type mice by blood pressure monitor. **c.** Statistical analysis of mean blood pressure in OVA- and mock-treatment wild type mice. Values are expressed as mean $\pm$ SD. *, $p < 0.05$ vs saline, $n=7$.

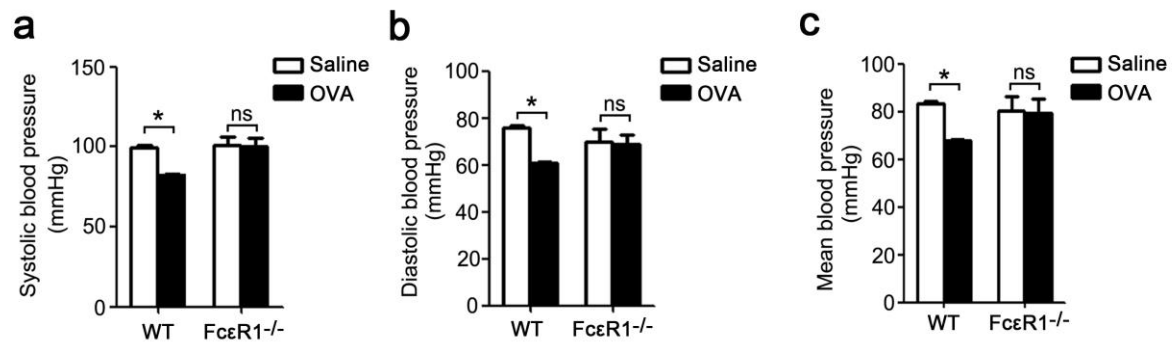


Fig.2 Hypotension development in ovalbumin-sensitized mice is dependent on IgE-FcεR1 signaling.

a. Analysis of systolic blood pressure level in OVA-treated WT and *FcεR1*^{-/-} mice. **b.** Diastolic blood pressure of OVA-induced asthmatic WT and *FcεR1*^{-/-} mice. **c.** Statistical analysis of mean blood pressure in OVA and saline treated mice. Values are expressed as mean ± SD. *, $p < 0.05$ vs saline, $n \geq 4$.

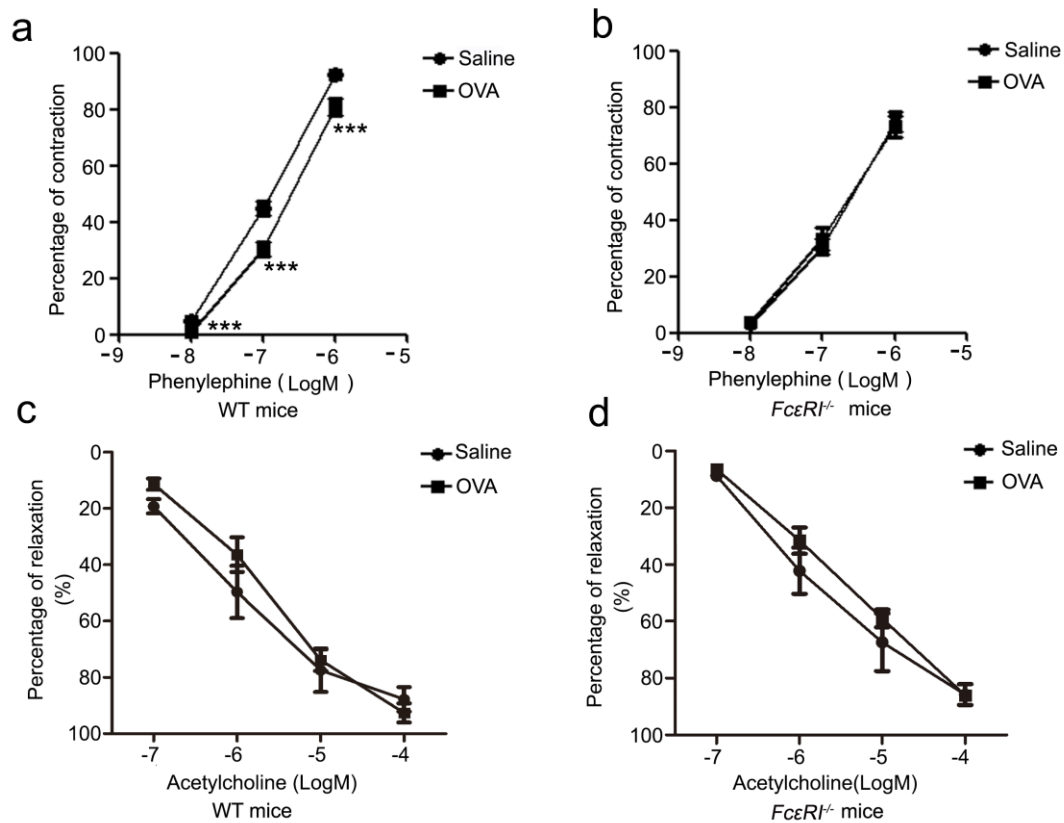


Fig.3 Vascular reactivity of thoracic aorta from WT and *FcεRI*^{-/-} mice. **a.** Percentage of contraction of thoracic arteries from saline (solid circles) and OVA (solid squares) sensitized wild type mice under different concentration of phenylephrine treatment. **b.** Percentage of contraction of thoracic aortic vasculature from saline (solid circles) and OVA (solid squares) treated *FcεRI*^{-/-} mice with different concentration of phenylephrine. **c.** Degree of relaxation of thoracic arteries from saline (solid circles) and OVA (solid squares) treated WT mice in response to the endothelium-dependent agonist, acetylcholine. **d.** Percentage of relaxation of thoracic arteries from saline (solid circles) and OVA (solid squares) immunized *FcεRI*^{-/-} mice in different concentrations of acetylcholine. Values are expressed as mean ± SD. *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs saline, $n \geq 4$ per group.

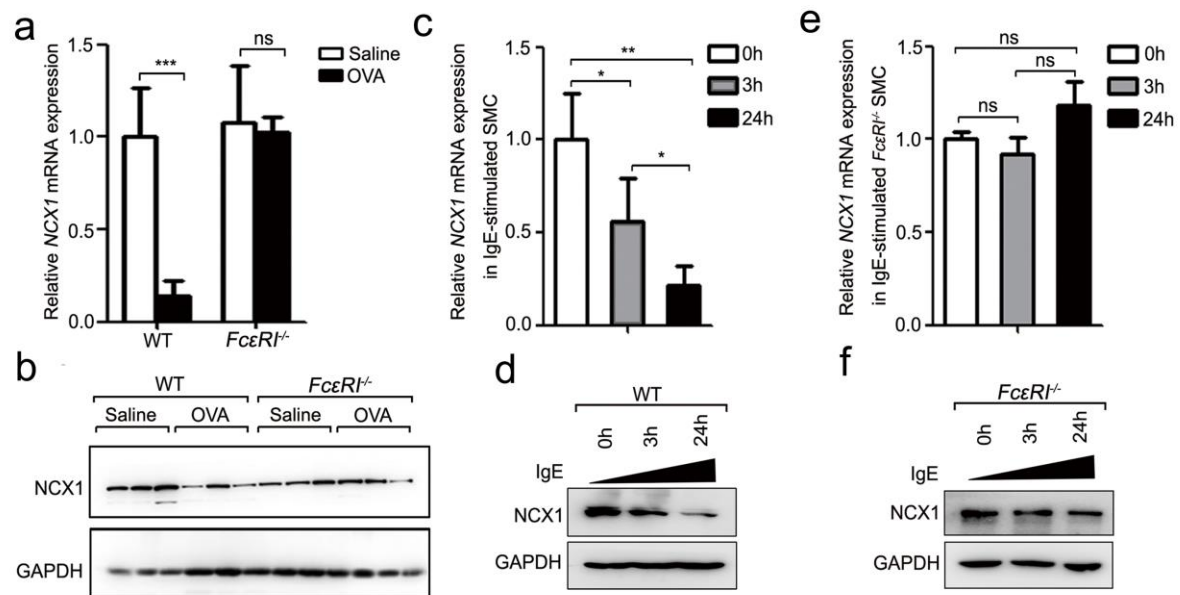


Fig.4 NCX1 is down-regulated in asthma-induced hypotension which is IgE-FcεR1 dependent. **a.** The levels of NCX1 mRNA in thoracic aorta of saline and OVA-treated WT and *FcεRI*^{-/-} mice were detected by qPCR. Values are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs saline, $n \geq 3$ per groups. **b.** Western blot analysis of the NCX1 expression in thoracic aorta from saline and OVA-immunized WT and *FcεRI*^{-/-} mice, $n = 3$. **c.** qPCR analysis of NCX1 mRNA expression in WT ASMC during IgE activation. **d.** The protein level of NCX1 detected by Western blot in WT ASMC during IgE stimulation. **e.** qPCR analysis of NCX1 mRNA level in *FcεRI*^{-/-} ASMC during IgE activation. **f.** Western blot analysis of the protein level of NCX1 in *FcεRI*^{-/-} ASMC during IgE stimulation.

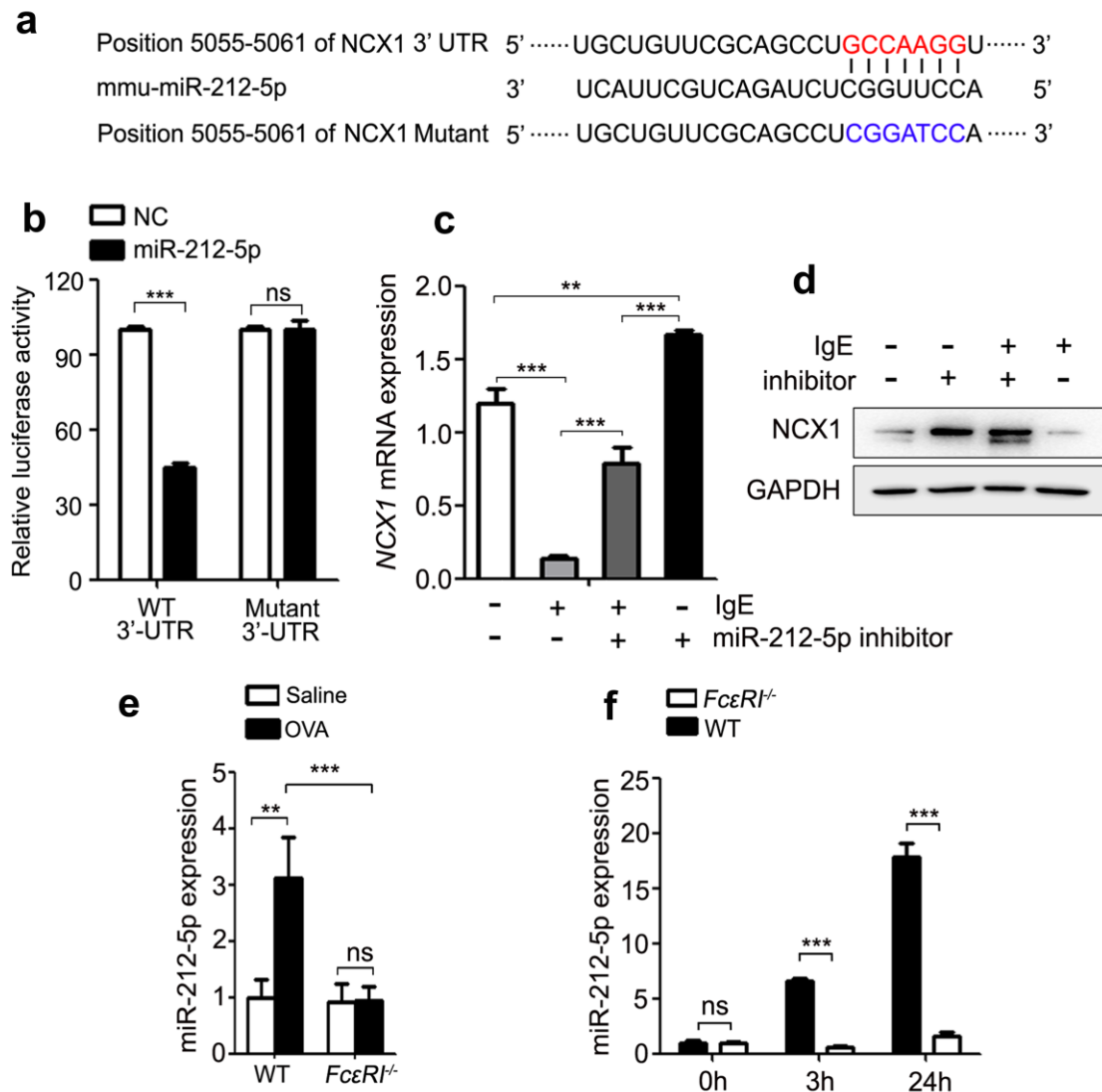


Fig.5 IgE decreases NCX1 expression by up-regulating miR-212-5p. **a.** Sequence in red represents the region in the 3'-UTR of NCX1 targeted by miR-212-5p, the sequence in blue represents mutant of miR-212-5p-matched seed sequence. A 3'-UTR fragment of NCX1 mRNA containing WT or mutant of miR-212-5p binding site was cloned into the downstream of the luciferase gene in pMIR-reporter vector. **b.** 293T cells were transfected with pMIR-reporter vectors containing WT and mutated NCX1 3'-UTR with either miR-212-5p mimic or scramble mimic. Luciferase activity was measured 48h after transfection. Values are expressed as mean $\pm$

SD. *** $p < 0.001$ vs NC. **c.** qPCR analysis of NCX1 expression in ASMC treated with miR-212-5p inhibitor or NC, and subsequently treated with IgE for 3 hours. **d.** Western blot analysis of NCX1 protein expression in ASMC treated with different combinations of miR-212-5p inhibitor and IgE as indicated. **e.** qPCR analysis of miR-212-5p expression in the thoracic aorta of saline and OVA sensitized wild type and FcεR1 knockout mice. Values are expressed as mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ vs saline, $n \geq 4$. **f.** miR-212-5p expression levels in different periods of IgE treatment in wild type or *FcεR1* knockout ASMC. Values are expressed as mean $\pm$ SD. *** $p < 0.001$ vs WT.

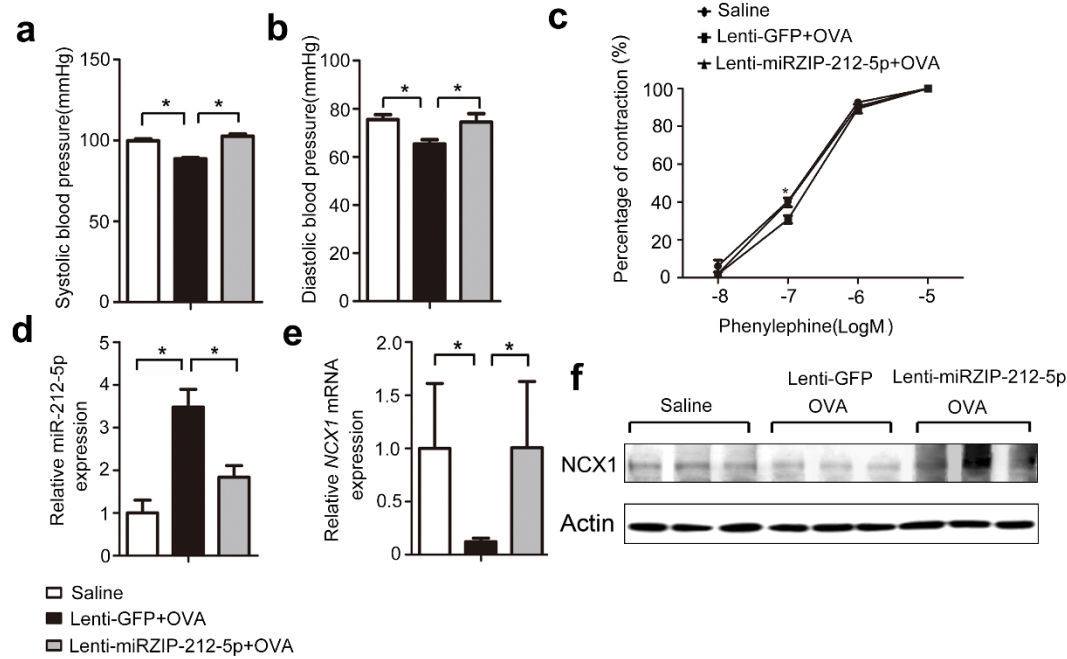


Fig. 6 Inhibition of miR-212-5p prevents hypotension in OVA-sensitized mice.

a. Systolic blood pressures and **b.** diastolic blood pressure (tail cuff) of saline-treated mice or OVA-treated mice with different lenti-virus therapy were measured. **c.** Percentage of contraction of thoracic arteries from each group in different concentration of phenylephrine treatment. **d.** Real-time PCR analysis of the expression of miR-212-5p in each group. **e.** Real-time PCR analysis of the expression of NCX1 mRNA. Values are expressed as mean $\pm$ SD. *, $p < 0.05$, $n \geq 4$ per group. **f.** Western blot analysis of NCX1 protein expression in arteries of mice from the three experimental groups ($n = 3$ per group) as indicated.

Table 1. Association analysis of blood pressure and asthma (n = 21390) .

Characteristics	Hypotension (SBP<90mmHg)	Non-Hypotension (SBP >90mmHg)	p
Asthma, n/%			
Yes	500(17.66)	2422(82.34)	0.0212
No	2992(15.52)	15476(84.48)	
Sex, n/%			
Male	1472(13.66)	8917(86.34)	<.0001
Female	2020(17.81)	8981(82.19)	
Race, n/%			
Mexican American	581(15.54)	2910(84.46)	0.0387
Other Hispanic	259(15.00)	1643(85.00)	
Non-Hispanic White	1508(15.11)	8194(84.89)	
Non-Hispanic Black	870(19.18)	3799(80.82)	
Other - Including Multi-Racial	274(18.34)	1352(81.66)	
Smoking status, n/%			
Never	1979(16.81)	9647(83.19)	0.0071
Former	796(14.43)	4352(85.57)	
Current	717(14.88)	3899(85.12)	
Diabetes mellitus, n/%			
Yes	448(17.03)	2146(82.97)	0.2237
No	3044(15.70)	15752(84.30)	
Age (mean ± SD)	46.78±0.35	46.75±0.15	<.0001
Mean BMI, kg/m ²	29.01±0.16	28.51±0.06	<.0001
Mean HDL, mmHg	1.38±0.01	1.37±0.004	<.0001

Supplemental Table S1. Primers

U6 qPCR up	CTCGCTTCGGCAGCACATATACT
U6 qPCR down	ACGCTTCACGAATTTGCGTGTC
NCX1 qPCR up	AGCCATCACTGTCAGCGCTG
NCX1 qPCR down	AGGCCCAGCCATTCCAGT
MiR-212-5p qPCR Up	ACCTTGGCTCTAGACTGCTTA
MiR-212-5p qPCR down	TCGTATCCAGTGCAGGGTC
GAPDH qPCR up	CTGTGCCGTTGAATTTGCCG
GAPDH qPCR down	CGGGTTCCTATAAATACGGACTG
Oligo dT	TTTTTTTTTTTTTTTTTTT
U6 RT	AAAATATGGAACGCTTCACGAATTTG
MiR-212-5p RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACAGTAAGCA
MiRZip212-5p up	GATCCCCACAGTAAGCAGTCTAGAGCCAA GGTTTCAAGAGA ACCTTGGCTCTAGACTGCTTACTTTTTTTG
MiRZip 212-5p down	AATTCAAAAAAAGTAAGCAGTCTAGAGC CAAGGTTCTCTTGAAACCTTGGCTCTAGACTG CTTACTGTGGG

Supplementary Table S2. Echocardiographic characterization of Saline/OVA-treated mice

	Saline	OVA	P
LV Anterior wall thickness in diastole, mm			
Basal	0.752±0.003	0.760±0.014	NS
Asthma	0.838±0.011	0.844±0.005	
LV Anterior wall thickness in systole, mm			
Basal	1.006±0.038	1.118±0.072	NS
Asthma	1.328±0.044	1.326±0.041	
LV internal diameter in diastole, mm			
Basal	3.572±0.133	3.704±0.143	NS
Asthma	3.853±0.123	4.094±0.157	
LV internal diameter in systole, mm			
Basal	2.488±0.173	2.673±0.112	NS
Asthma	2.382±0.153	2.746±0.146	
LV posterior wall thickness in diastole, mm			
Basal	0.645±0.015	0.684±0.018	NS
Asthma	0.732±0.035	0.894±0.143	
LV posterior wall thickness in systole, mm			
Basal	0.929±0.034	0.890±0.026	NS
Asthma	1.160±0.084	1.094±0.070	
Ejection fraction, %			
Basal	58.522±4.103	54.472±2.433	NS
Asthma	68.826±3.095	65.135±0.934	
Body weight (g)	25.83±0.441	26.1±0.200	S
Heart weight (g)	0.11±0.003	0.11±0.003	S
Heart rate(BPM)	611.04±12.86	606.5±24.44	S

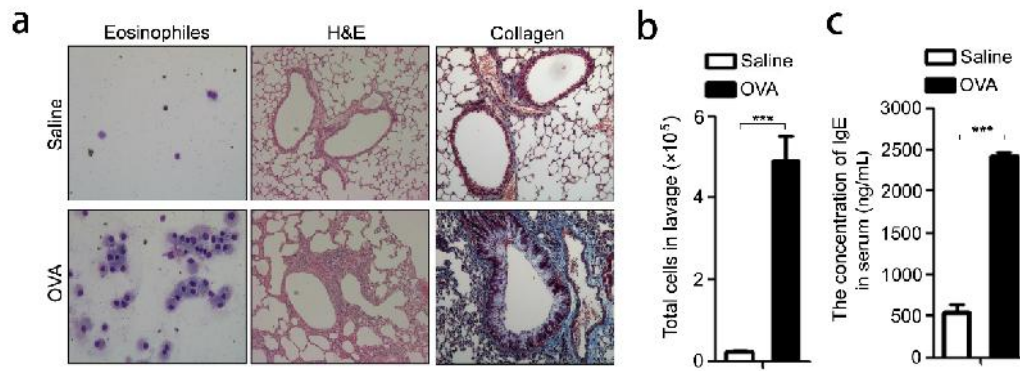


Fig. S1 Assessment of asthma-induced hypotension in mouse model. a. Histological microscopic analysis of asthma (OVA) and control (Saline) mice, included Wright-Giemsa staining for eosinophils in bronchoalveolar lavage fluid, H&E staining for lung structure, and Masson staining for collagen in the lung. Left panel: magnification 400X, middle and left panel: magnification 200X. **b.** Statistic analysis of the total inflammatory cell number in bronchoalveolar lavage fluid from asthma (OVA) and control mice (saline), and **c.** serum IgE levels in asthma-induced hypotension (OVA) and control group (Saline) detected by ELISA. Values are expressed as mean $\pm$ SD. *** $p < 0.001$ vs saline, $n = 7$.

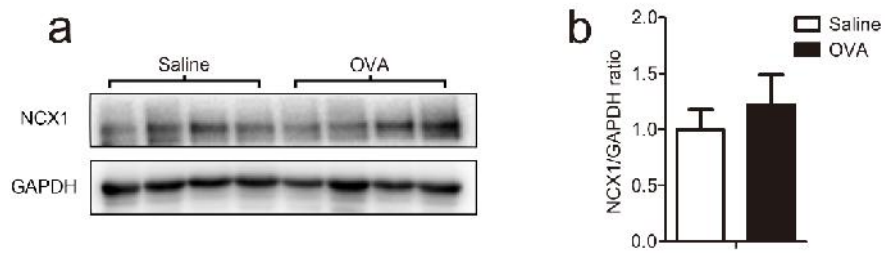


Fig. S2 The expression of NCX1 in heart from saline or OVA treated WT mice. a. Western blot analysis. **b.** quantification of NCX1 to GAPDH of NCX1 expression in heart of saline and OVA-immunized WT mice, n=4 for each group.

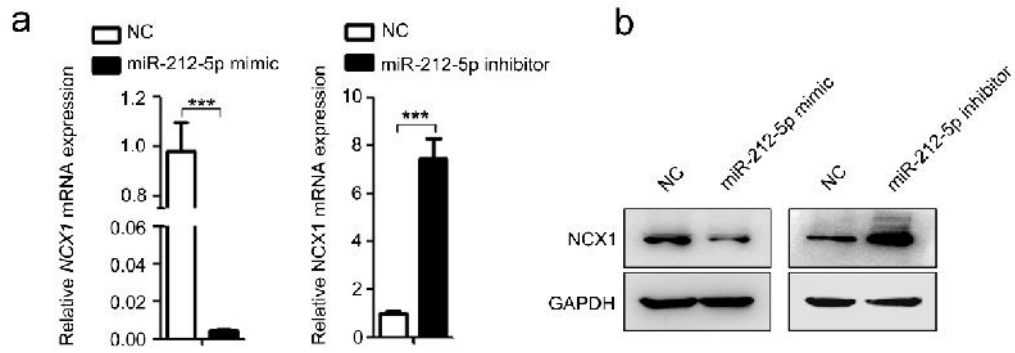


Fig. S3 NCX1 is a direct target of miR-212-5p. a. qPCR of NCX1 mRNA. **b.** Western blot of NCX1 from smooth muscle cells transfected with miR-212-5p mimic or NC or miR-212-5p inhibitor.

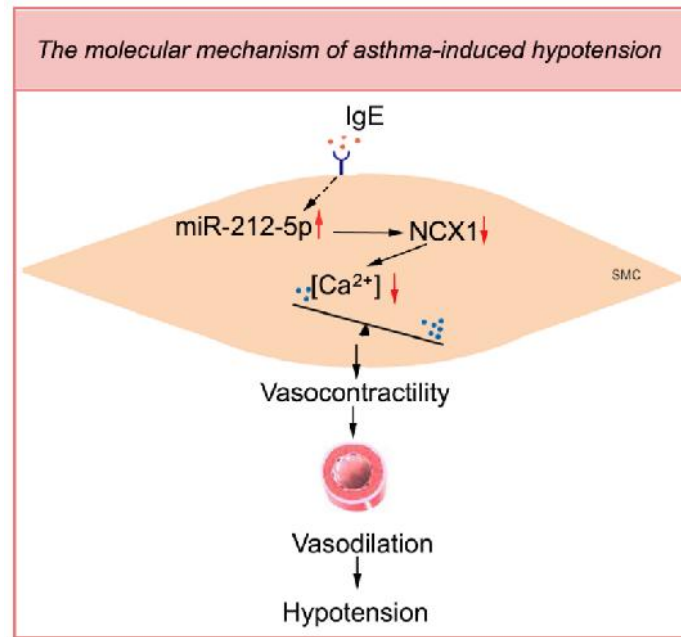


Fig. S4 A schematic representation of the role of IgE in inducing hypotension.

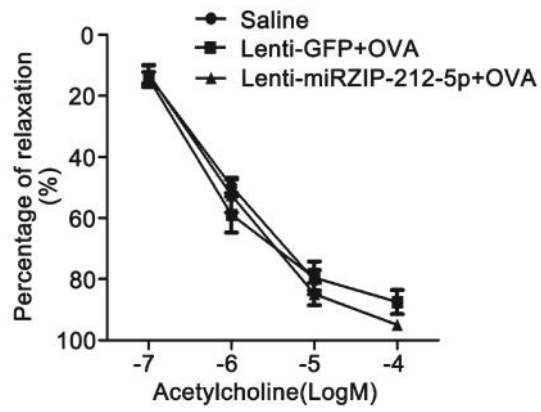


Fig. S5 Degree of relaxation of thoracic arteries in response to acetylcholine in saline (filled circles) or OVA (filled squares) treated WT mice, and inhibition miR-212-5p plus OVA treated WT mice (filled triangle), $n \geq 4$ per group.