

AMPK activation reduces vascular permeability and airway inflammation by regulating HIF/VEGFA pathway in a murine model of toluene diisocyanate-induced asthma

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Abstract

Background Occupational asthma is characterized by airway inflammation and hyperresponsiveness associated with increased vascular permeability. AMP-activated protein kinase (AMPK) has been suggested to be a novel signaling molecule modulating inflammatory responses.

Objective We sought to evaluate the involvement of AMPK in pathogenesis of occupational asthma and more specifically investigate the effect and molecular mechanisms of AMPK activation in regulating vascular permeability.

Methods The mechanisms of action and therapeutic potential of an AMPK activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) were tested in a murine model of toluene diisocyanate (TDI)-induced asthma.

Results AICAR attenuated airway inflammation and hyperresponsiveness increased by TDI inhalation. Moreover, TDI-induced increases in levels of hypoxia-inducible factor (HIF)-1 α , HIF-2 α , vascular endothelial growth factor A (VEGFA), and plasma exudation were substantially decreased by treatment with AICAR. Our results also showed that VEGFA expression was remarkably reduced by inhibition of HIF-1 α and HIF-2 α with 2-methoxyestradiol (2ME2) and that an inhibitor of VEGFA activity, CBO-P11 as well as 2ME2 significantly suppressed vascular permeability, airway infiltration of inflammatory cells, and airway hyperresponsiveness induced by TDI. In addition, AICAR reduced reactive oxygen species (ROS) generation and levels of malondialdehyde and T-helper type 2 cytokines (IL-4, IL-5, and IL-13), while this agent enhanced expression of an anti-inflammatory cytokine, IL-10.

Conclusions These results suggest that AMPK activation ameliorates airway inflammatory responses by reducing vascular permeability via HIF/VEGFA pathway as well as by inhibiting ROS production and thus may be a possible therapeutic strategy for TDI-induced asthma and other airway inflammatory diseases.

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Abbreviations

Ab	Antibody
AHR	Airway hyperresponsiveness
AICAR	5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMPK	AMP-activated protein kinase
BAL	Bronchoalveolar lavage

CMC	Carboxymethylcellulose
DCF	Dichlorofluorescein
DMSO	Dimethylsulfoxide
EBD	Evans blue dye
HIF	Hypoxia-inducible factor
MDA	Malondialdehyde
2ME2	2-Methoxyestradiol
p-AMPK	Phosphorylated AMPK
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
Rrs	Respiratory system resistance
TDI	Toluene diisocyanate
Th2	T-helper type 2
Treg	Regulatory T
VEGFA	Vascular endothelial growth factor A

Introduction

Occupational asthma is the most prevalent work-related lung disease in industrialized countries, accounting for about 10 % of all adult-onset asthma [1]. Toluene diisocyanate (TDI), a widely used low-molecular-weight compound, is one of the leading causes of occupational asthma. Although the clinical, functional, and pathologic features of TDI-induced asthma resemble those of allergic asthma, several important differences such as a low prevalence of specific immunoglobulin E and a low association with atopy have resulted in a research for alternative or complementary mechanisms [2]. Recent advances have been made in characterizing the immune response to TDI, but the pathogenesis of TDI-induced asthma is far from clear.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) and protects cells against ATP depletion [3, 4]. The role of AMPK has been documented in regulating a wide variety of physiological processes such as substrate metabolism, protein synthesis, cell proliferation, and apoptosis [4, 5]. The AMPK signaling is particularly critical for glucose and lipid metabolism and has cardioprotective effects against pathological stress [6, 7]. A potent activator of AMPK, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) has been studied extensively with various cell types and animal models [4, 5, 8]. AICAR enters cells and is quickly converted to the equivalent monophosphorylated nucleotide, ZMP that mimics AMP and activates AMPK without altering the cellular levels of ATP, ADP, or AMP [9]. There is recent evidence that pharmacologic AMPK activation by AICAR modulates inflammatory reactions [4, 5, 10]. AMPK directs signaling pathways in macrophages in a manner that suppresses proinflammatory responses and promotes macrophage

polarization toward an anti-inflammatory phenotype [11]. Previous studies using animal models have demonstrated that AICAR alleviates experimental autoimmune encephalomyelitis, lipopolysaccharide-induced lung injury, and respiratory virus-induced airway inflammation [4, 10, 12]. Although there is little information available on the role of AMPK in the pathogenesis of human asthma, AMPK pathway has been shown to inhibit production of cellular reactive oxygen species (ROS) through modulation of NADPH oxidase in human neutrophils [13]. Oxidative stress plays a crucial role in the development of various airway inflammatory disorders [14]. However, the anti-inflammatory effect of AMPK activation through regulating oxidative stress has not been elucidated.

While major cellular components driving asthmatic reactions include mast cells, eosinophils, and T cells, with a prominent role for T-helper type 2 (Th2) cells, more recently, roles of basophils, invariant natural killer T cells, Th17 cells, and a number of soluble mediators in asthma, even in asthmatic patients have also been proposed [15–17]. The recent discovery of a distinct T helper subset, referred to as Th17 cells based on their IL-17 production, leads to transformation of the Th1/Th2 paradigm of immunity into a novel viewpoint that incorporates Th1, Th2, Th17, and regulatory T (Treg) cells as elements of a complex and mutually interacting network [18]. In fact, several studies have reported the alteration of Th1/Th2/Th17/Treg balance in human asthmatics; the Th1/Th2 cell ratio is decreased [19], peripheral Treg cell prevalence is reduced [20, 21], and Th17 cells and their related cytokines are increased, especially in moderate to severe persistent allergic asthmatics [22]. In addition, there is accumulating evidence that Treg cells are important in maintaining immune homeostasis in the airway and that their function may be altered in asthma, mostly focusing on IL-10, which has broad immunosuppressive and anti-inflammatory actions relevant to the inhibition of asthma pathology [23]. As for human asthma, a number of studies have investigated IL-10 synthesis in allergic and asthmatic patients. A substantial reduction in mRNA and protein levels of IL-10 or IL-10 positive T cells and increased amounts of proinflammatory cytokines have been observed in patients with asthma as compared to control healthy subjects [24–26].

The structural and functional changes in the airway microcirculation including the increased vascular permeability and blood flow, proliferation of new vessels, and increased vascular area of the airway play a prominent role in airway inflammation and remodeling in asthma [27]. Vascular endothelial growth factor A (VEGFA) is a fundamental determinant in these bronchial microvascular changes. Growing evidence has indicated that VEGFA stimulates airway inflammation and hyperresponsiveness, airway remodeling, and Th2 immune responses in asthma

[27, 28]. In fact, VEGFA level and vascular permeability index in asthmatic patients are higher than those in normal control subjects [29]. In human airway smooth muscle cells, VEGFA is produced constitutively and in response to stimulation with a variety of inflammatory mediators, including the Th2 cytokines, IL-4 and IL-13 [30–33]. VEGFA also enhances IL-13 expression in the airways [34], suggesting a positive feedback loop with VEGFA enhancing Th2 sensitization and inflammation and IL-13 subsequently enhancing VEGFA production. Moreover, VEGFA level is strongly correlated with the degree of airway vascular abnormality and asthma severity in asthmatics [29]. Our previous study using a murine model has shown that VEGFA expression is dramatically increased by TDI inhalation and that inhibition of VEGFA reverses all pathophysiological features of TDI-induced asthma, suggesting that VEGFA may be a major determinant of TDI-induced asthma [28].

Expression of VEGFA is modulated by hypoxia-inducible factor (HIF)-1, which is a heterodimer composed of HIF-1 α and HIF-1 β [35]. HIF-1 α expression is precisely regulated by the intracellular condition, while HIF-1 β is constitutively expressed. A structurally related protein, HIF-2 α , has also been described to be induced similarly to HIF-1 α and transcribe *VEGFA* gene [36]. In contrast to widely present HIF-1 α , HIF-2 α is only expressed in certain organs including the lung [37]. These findings have suggested that increased activity of both HIF-1 α and HIF-2 α leads to overexpression of VEGFA in airway inflammation. Levels of VEGFA, HIF-1 α , and HIF-2 α in bronchoalveolar lavage (BAL) fluids are significantly higher in asthmatic patients than those in controls, and VEGFA expression correlates with HIF-1 α and HIF-2 α expression, supporting the important role of HIF/VEGFA pathway in human asthma [38]. However, the role of HIF activation in TDI-induced asthma has not been explored.

On the basis of these observations, we evaluated the involvement of AMPK in pathogenesis of occupational asthma and more specifically investigated the effect and molecular mechanisms of AMPK activation in regulating vascular permeability and ROS generation using a murine model of TDI-induced asthma.

Materials and methods

Animals and experimental protocol

Female BALB/c mice, 10–12 weeks of age and free of murine specific pathogens, were obtained from the Orient Bio Inc. (Seoungnam, Korea) and were housed throughout the experiments in a laminar flow cabinet and maintained on standard laboratory chow ad libitum. Care and use of

experimental animals were conducted in accordance with the ‘Guide for the care and use of laboratory animals’ formulated by the National Research Council. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University. Mice were sensitized by two courses of intranasal administration of 20 μ l of 3 % TDI dissolved in ethyl acetate:olive oil (1:4) under light anesthesia (sodium pentobarbitone, 30 mg/kg, intraperitoneally) once daily for five consecutive days with a 3-week interval as previously described [28]. At 7 days after the second course of sensitization (Day 38), mice were individually placed in a horizontal cylindrical chamber and challenged via the airways with 1 % TDI dissolved in ethyl acetate:olive oil (1:4) by ultrasonic nebulization (NE-U12; Omron, Tokyo, Japan). As a control, mice were sensitized and challenged using the same protocol but using only the solvent, ethyl acetate:olive oil (1:4).

BAL was performed at 48 h after the challenge. At the time of lavage, mice were sacrificed by ether inhalation (Junsei Chemical Co., Ltd, Tokyo, Japan). Chest cavity was exposed to allow for expansion, after which the trachea

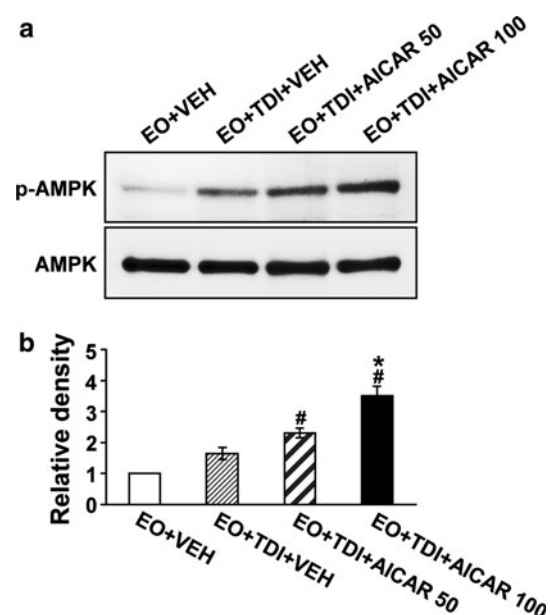


Fig. 1 Effect of AICAR on AMPK phosphorylation in lung tissues of TDI-sensitized and -challenged mice. Sampling was performed at 48 h after the challenge in ethyl acetate plus olive oil (EO)-inhaled control mice treated with drug vehicle (PBS) (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). **a** Representative western blotting of p-AMPK and AMPK protein. **b** Densitometric analyses are presented as the relative ratio of p-AMPK to AMPK. The relative ratio of p-AMPK in the lung tissues of EO + VEH is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. # p < 0.05 versus EO + VEH, * p < 0.05 versus EO + TDI + VEH

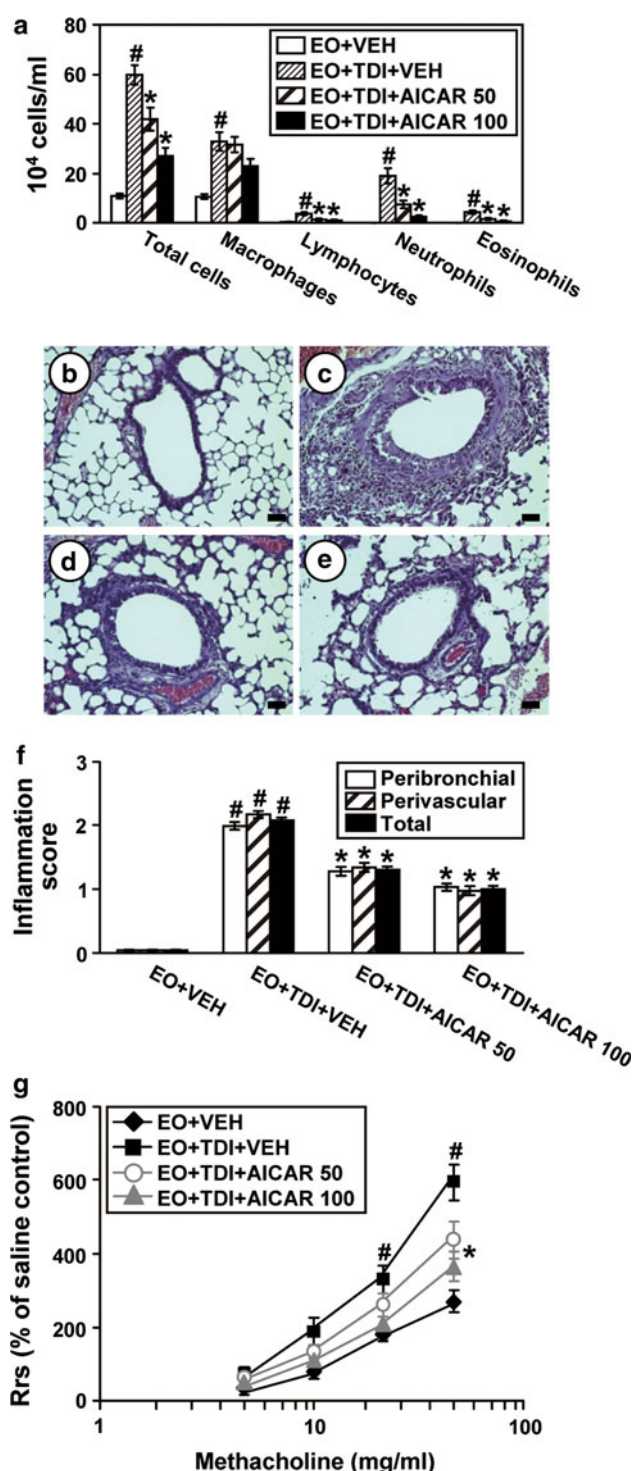


Fig. 2 Effect of AICAR on total and differential cell counts in BAL fluids (**a**), pathological changes in lung tissues (**b–f**), and airway responsiveness (**g**) of TDI-sensitized and -challenged mice. **a** Differential cell counts in BAL fluids from control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). Representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed at 48 h after the challenge in EO + VEH (**b**), EO + TDI + VEH (**c**), EO + TDI + AICAR 50 (**d**), and EO + TDI + AICAR 100 (**e**). Bars indicate scale of 50 μ m. **f** Scores of peribronchial, perivascular, and total lung inflammation. **g** Airway responsiveness. Data represent mean $\pm$ SEM from seven mice per group. # p < 0.05 versus EO + VEH, * p < 0.05 versus EO + TDI + VEH

cytopsin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc., Aguada, Puerto Rico) to determine differential cell counts. Two independent, blinded investigators counted the cells under a microscope. Approximately 400 cells were counted in each of four different random locations. Inter-investigator variation was less than 5 %. Numbers counted by two investigators were averaged and these values were used to calculate differential cell counts.

Administration of AICAR, 2-methoxyestradiol (2ME2), or CBO-P11

AICAR (50 or 100 mg/kg; Sigma-Aldrich, St. Louis, MO) diluted with phosphate buffered saline (PBS) was administered intraperitoneally two times at a 24-h interval, beginning 1 h after the challenge with TDI. 2ME2 (100 mg/kg body weight/day; Calbiochem-Novobiochem Corp., San Diego, CA) was suspended in 0.5 % carboxymethylcellulose (CMC) (Sigma-Aldrich) and administered by oral gavage four times at 24-h intervals, beginning 2 days before the TDI challenge. Cyclopeptidic vascular endothelial growth inhibitor, CBO-P11 (*D*-Phe-Pro (79-93); Calbiochem-Novobiochem Corp.) diluted with dimethylsulfoxide (DMSO) was used to inhibit VEGFA activity. CBO-P11 (2 mg/kg body weight/day) was administered intraperitoneally two times at a 24-h interval, beginning 1 h before the TDI inhalation.

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of proteins. Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA). Samples were loaded on a SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont,

was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9 % NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then stored at 4 $^{\circ}$ C. A part of each pool was then centrifuged, and the supernatant was kept at -70° C until use. Total cell numbers were counted using a hemocytometer. Smears of BAL cells were prepared with a

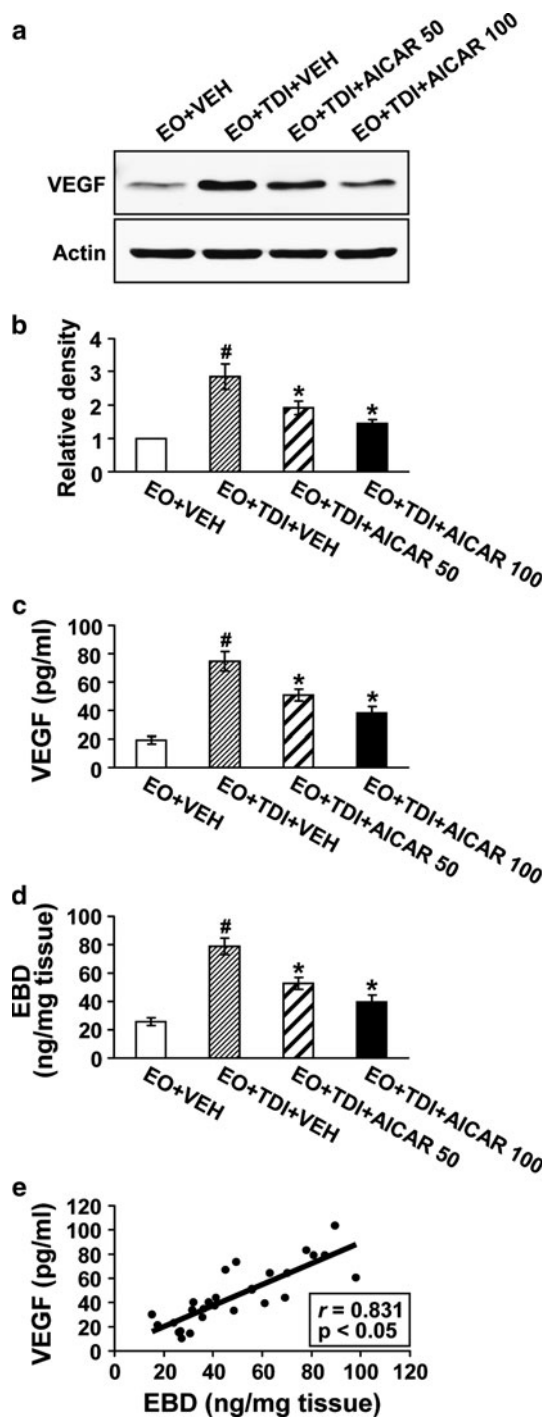


Fig. 3 Effect of AICAR on VEGFA expression in lung tissues and in BAL fluids and plasma exudation. Sampling was performed at 48 h after the challenge in control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). **a** Representative western blotting of VEGFA in lung tissues. **b** Densitometric analyses are presented as the relative ratio of VEGFA to actin. The relative ratio of VEGFA in the lung tissues of EO + VEH is arbitrarily presented as 1. **c** Enzyme immunoassay of VEGFA in BAL fluids. **d** Measurement of plasma exudation using EBD. **e** Correlation between levels of VEGFA protein in BAL fluids and plasma exudation. Data represent mean $\pm$ SEM from seven mice per group. # $p < 0.05$ versus EO + VEH, * $p < 0.05$ versus EO + TDI + VEH

Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-4 Ab (1:800; Serotec Ltd, Oxford, UK), anti-IL-5 Ab (1:1,000; Santa Cruz Biotechnology), anti-IL-13 Ab (1:1,000; R&D Systems, Inc., Minneapolis, MN), or anti-IL-10 Ab (1:800; Abcam, Cambridge, MA) overnight at 4 °C. Anti-mouse or anti-rabbit horseradish peroxidase conjugated IgG (1:2,500) was used to detect binding of the Abs. The membranes were stripped and reblotted with anti-actin Ab (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific Abs was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

Nuclear protein extractions for analyses of HIF-1 α , HIF-1 β , and HIF-2 α

Lungs were removed and homogenized in eight volumes of a lysis buffer containing 1.3 M sucrose, 1.0 mM MgCl₂, and 10 mM potassium phosphate buffer (pH 7.2). The homogenate was filtered through four layers of gauze and centrifuged at 1,000 $\times g$ for 15 min. The resulting pellets were carefully harvested and resuspended in 10 mM potassium phosphate buffer (pH 7.2) containing 2.4 M sucrose and 1.0 mM MgCl₂ to maintain a final 2.2 M sucrose concentration and centrifuged at 100,000 $\times g$ for 1 h. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM MgCl₂, and 20 mM Tris-HCl, pH 7.2, and centrifuged at 1,000 $\times g$ for 10 min. The pellets were solubilized with a solution containing 50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM ethylene diamine tetraacetic acid, 20 % glycerol, 2 % Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 2 h with gentle stirring and centrifuged at 12,000 $\times g$ for 30 min. The resulting supernatant was used as soluble nuclear proteins for determination of levels of HIF-1 α , HIF-1 β , and HIF-2 α . The levels of these proteins were analyzed by Western blotting using Ab against

Buckinghamshire, UK) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5 % non-fat dry milk in Tris-buffered saline containing Tween 20 (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1 % Tween 20) for 1 h, and the blots were then incubated with an anti-AMPK antibody (Ab) (1:700; Cell Signaling Technology, Beverly, MA), anti-phosphorylated AMPK (p-AMPK) Ab (1:700; Cell Signaling Technology), anti-VEGFA Ab (1:500;

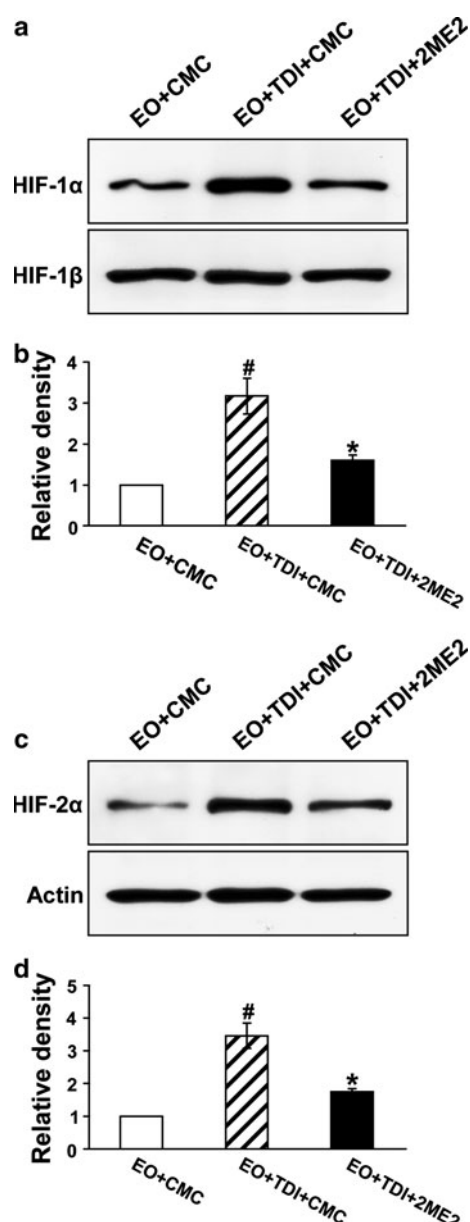


Fig. 4 Effect of 2ME2 on levels of HIF-1 α , HIF-1 β , and HIF-2 α in nuclear protein extracts from lung tissues. Sampling was performed at 48 h after the challenge in control mice treated with CMC (EO + CMC), TDI-inhaled mice treated with CMC (EO + TDI + CMC), and TDI-inhaled mice treated with 2ME2 (EO + TDI + 2ME2). **a** Representative western blotting of HIF-1 α and HIF-1 β . **b** Densitometric analyses are presented as the relative ratio of HIF-1 α to HIF-1 β . The relative ratio of HIF-1 α in the lung tissues of EO + CMC is arbitrarily presented as 1. **c** Representative western blotting of HIF-2 α . **d** Densitometric analyses are presented as the relative ratio of HIF-2 α to actin. The relative ratio of HIF-2 α in the lung tissues of EO + CMC is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. # p < 0.05 versus EO + CMC, * p < 0.05 versus EO + TDI + CMC

HIF-1 α (1:600; R&D Systems, Inc.), HIF-1 β (1:800; Cell Signaling Technology), or HIF-2 α (1:800; Novus Biologicals, Inc., Littleton, CO) as described above.

Measurement of VEGFA and cytokines

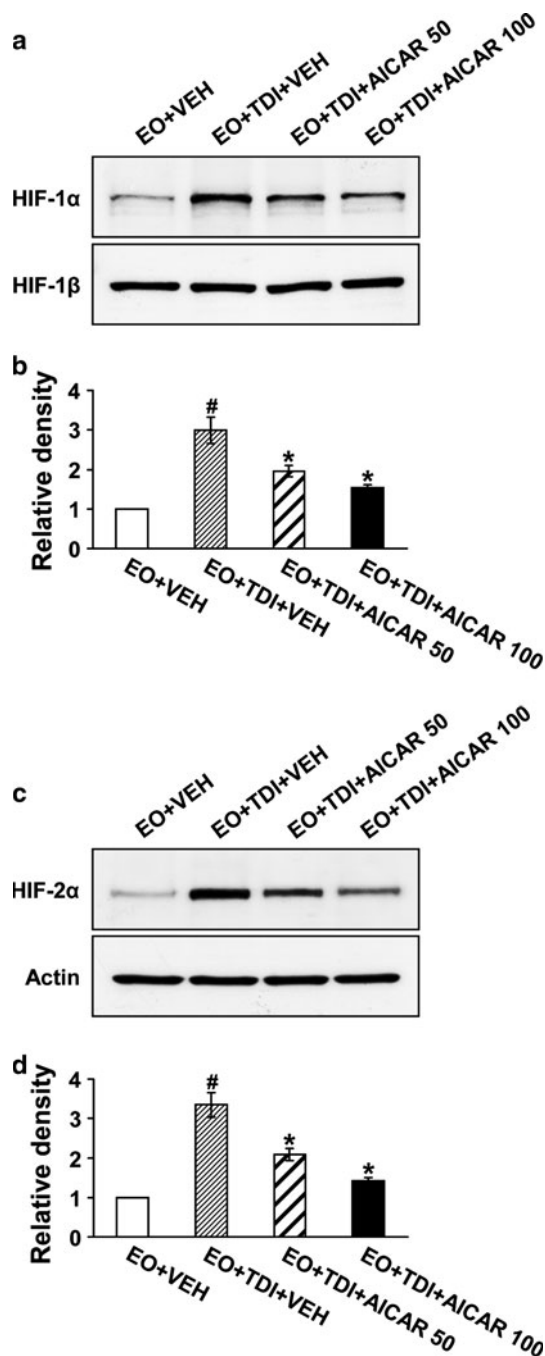
Levels of VEGFA, IL-4, IL-5, IL-13, and IL-10 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (VEGFA; R&D Systems, Inc.; IL-4, IL-5, and IL-10; Invitrogen, Carlsbad, CA; IL-13; Bender MedSystems, Vienna, Austria). Sensitivities for VEGFA, IL-4, IL-5, IL-13, and IL-10 assays were 3, 5, 3, 2.8, and 13 pg/ml, respectively.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9 % saline at a final concentration of 5 mg/ml. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 min, the animals were killed and their chests were opened. Normal saline containing 5 mM ethylene diamine tetraacetic acid was perfused through the aorta until all venous fluid returning to the opened right atrium was clear. Lungs were removed and weighed wet. EBD was extracted in 2 ml formamide kept in a water bath at 60 °C for 3 h and the absorption of light at 620 nm was measured using a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01–10 μ g/ml and is expressed as ng of dye/mg of wet lung.

Histology

At 48 h after the last challenge, mice were euthanized for histological assessment. The lungs and trachea of mice were filled with 10 % (volume/volume) neutral buffered formalin intratracheally and then were removed from the mice. For fixation, the neutral buffered formalin was also used. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). Stained slides were analyzed under identical light microscope (Axio Imager M1, Karl Zeiss, Germany) conditions, including magnification (20 $\times$), gain, camera position, and background illumination. Inflammation score was graded by three independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere [39]. A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of



inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere [40]. Anesthesia was achieved with 45 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through

◀ **Fig. 5** Effect of AICAR on levels of HIF-1α, HIF-1β, and HIF-2α in nuclear protein extracts from lung tissues. Sampling was performed at 48 h after the challenge in control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). **a** Representative western blotting of HIF-1α and HIF-1β. **b** Densitometric analyses are presented as the relative ratio of HIF-1α to HIF-1β. The relative ratio of HIF-1α in the lung tissues of EO + VEH is arbitrarily presented as 1. **c** Representative western blotting of HIF-2α. **d** Densitometric analyses are presented as the relative ratio of HIF-2α to actin. The relative ratio of HIF-2α in the lung tissues of EO + VEH is arbitrarily presented as 1. Data represent mean ± SEM from seven mice per group. #*p* < 0.05 versus EO + VEH; **p* < 0.05 versus EO + TDI + VEH

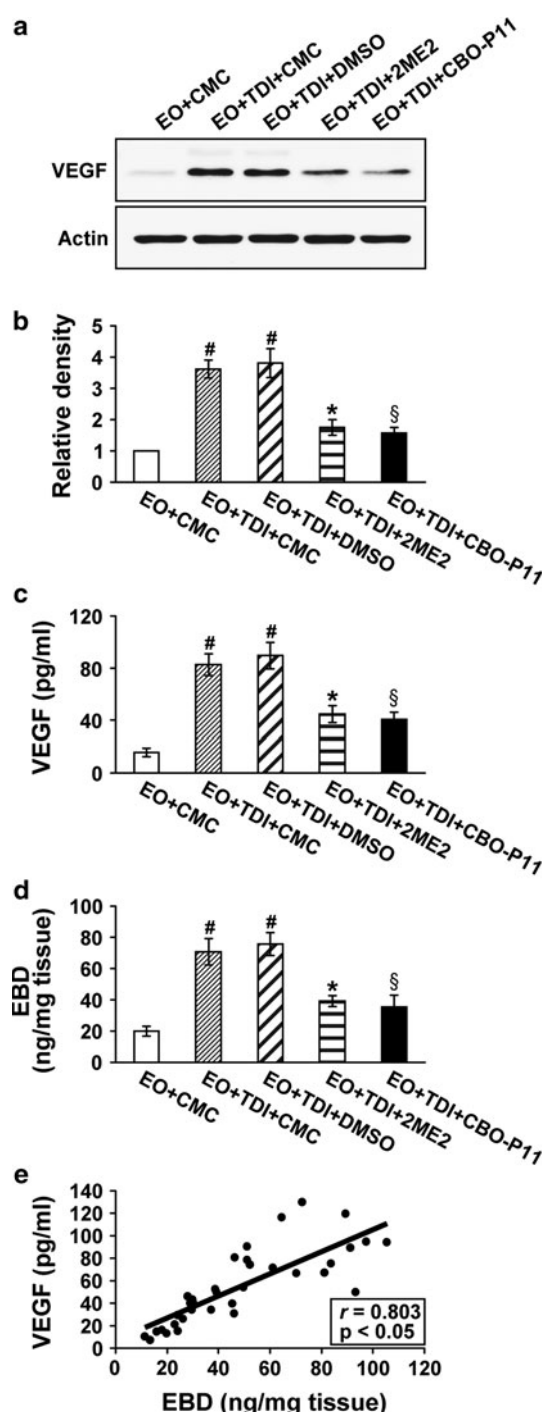
midcervical incision, tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (5–50 mg/ml in saline). After each methacholine challenge, the data of respiratory system resistance (Rrs) was continuously collected. Maximum values of Rrs were selected to express changes in airway function, which was represented as a percentage change from baseline after saline aerosol.

Measurement of intracellular ROS

ROS were measured by a method previously described [41]. BAL cells were washed with PBS. To measure intracellular ROS, cells were incubated for 10 min at room temperature with PBS containing 3.3 μM 2',7'-dichlorofluorescein (DCF) diacetate (Molecular probes, Eugene, OR), to label intracellular ROS. We performed fluorescence-activated cell sorting analysis with DCF stained cells (1 × 10⁴ cells) in BAL fluids to measure ROS levels using a FACSCalibur instrument (BD Biosciences, San Jose, CA). The data were analyzed with a CellQuest Pro program (BD Biosciences).

Determination of oxidative damage

The degree of oxidative damage was assessed by measurement of malondialdehyde (MDA) levels in homogenized



lung tissues using the OxiSelect thiobarbituric acid-reactive substances assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's protocol. The MDA values were expressed as $\mu\text{M}/\text{mg}$ lung tissue protein.

Densitometric analyses and statistics

All immunoreactive and phosphorylated signals were analyzed by densitometric scanning (Gel Doc XR, Bio-Rad

Fig. 6 Effect of 2ME2 or CBO-P11 on VEGFA expression and plasma exudation in lung of TDI-sensitized and -challenged mice. Sampling was performed at 48 h after the challenge in control mice treated with CMC (EO + CMC), TDI-inhaled mice treated with CMC (EO + TDI + CMC), TDI-inhaled mice treated with DMSO (EO + TDI + DMSO), TDI-inhaled mice treated with 2ME2 (EO + TDI + 2ME2), and TDI-inhaled mice treated with CBO-P11 (EO + TDI + CBO-P11). **a** Representative western blotting of VEGFA in lung tissues. **b** Densitometric analyses are presented as the relative ratio of VEGFA to actin. The relative ratio of VEGFA in the lung tissues of EO + CMC is arbitrarily presented as 1. **c** Enzyme immunoassay of VEGFA in BAL fluids. **d** Measurement of plasma exudation using EBD. **e** Correlation between levels of VEGFA protein in BAL fluids and plasma exudation. Data represent mean $\pm$ SEM from seven mice per group. # $p < 0.05$ versus EO + CMC, * $p < 0.05$ versus EO + TDI + CMC, § $p < 0.05$ versus EO + TDI + DMSO

Laboratories Inc.). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by Scheffe's test. Pearson's correlation was calculated to assess the correlation between data. Statistical significance was set at $p < 0.05$.

Results

AICAR activates AMPK in the lung of TDI-sensitized and -challenged mice

To confirm the ability of AICAR to activate AMPK in the lung, we determined AMPK phosphorylation in lung tissues of TDI-inhaled mice treated with AICAR. Western blot analysis revealed that AICAR at 50 and 100 mg/kg increased p-AMPK protein levels in lung tissues approximately 2.3- and 3.5-fold, respectively, as compared with the levels in the control mice treated with drug vehicle (PBS) (Fig. 1). However, no significant changes in total AMPK protein levels were observed in any of the groups tested.

AICAR attenuates TDI-induced airway inflammation

As the hallmark of TDI-induced asthma is the infiltration of inflammatory cells into the airways, we investigated the effect of AICAR on numbers of inflammatory cells in BAL fluids. The numbers of total cells, macrophages, lymphocytes, neutrophils, and eosinophils in BAL fluids were dramatically increased at 48 h after TDI inhalation compared with the numbers in the control mice (Fig. 2a). Treatment with AICAR significantly decreased the numbers of total cells, lymphocytes, neutrophils, and eosinophils.

Histological analyses revealed typical pathological features of asthma in the TDI-exposed mice. Numerous inflammatory cells infiltrated around the bronchioles and mucus and debris accumulated in the lumens of bronchioles

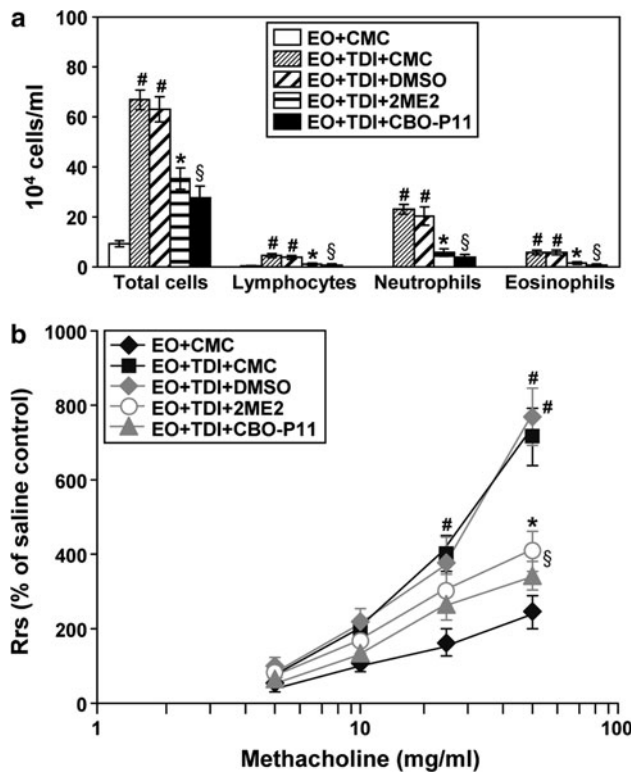


Fig. 7 Effect of 2ME2 or CBO-P11 on total and differential cell counts in BAL fluids (**a**) and airway responsiveness (**b**) of TDI-sensitized and -challenged mice. **a** Differential cell counts in BAL fluids from control mice treated with CMC (EO + CMC), TDI-inhaled mice treated with CMC (EO + TDI + CMC), TDI-inhaled mice treated with DMSO (EO + TDI + DMSO), TDI-inhaled mice treated with 2ME2 (EO + TDI + 2ME2), and TDI-inhaled mice treated with CBO-P11 (EO + TDI + CBO-P11). **b** Airway responsiveness. Data represent mean $\pm$ SEM from seven mice per group. #*p* < 0.05 versus EO + CMC, **p* < 0.05 versus EO + TDI + CMC, §*p* < 0.05 versus EO + TDI + DMSO

(Fig. 2c) as compared with the control mice (Fig. 2b). Mice treated with AICAR (Fig. 2d, e) showed marked reductions in the infiltration of inflammatory cells in the peribroncholar region and in the amount of debris in the airway lumens. In addition, the scores of peribronchial, perivascular, and total lung inflammation were significantly increased after TDI inhalation compared with the scores in the control mice (Fig. 2f). AICAR treatment was efficacious in decreasing the scores of lung inflammation.

AICAR reduces TDI-induced airway hyperresponsiveness (AHR)

To determine the attenuating effect of AICAR on AHR, airway responsiveness was assessed as a percent increase of Rrs in response to increasing doses of methacholine. In TDI-sensitized and -challenged mice, the dose-response curve of percent Rrs shifted to the left compared with that

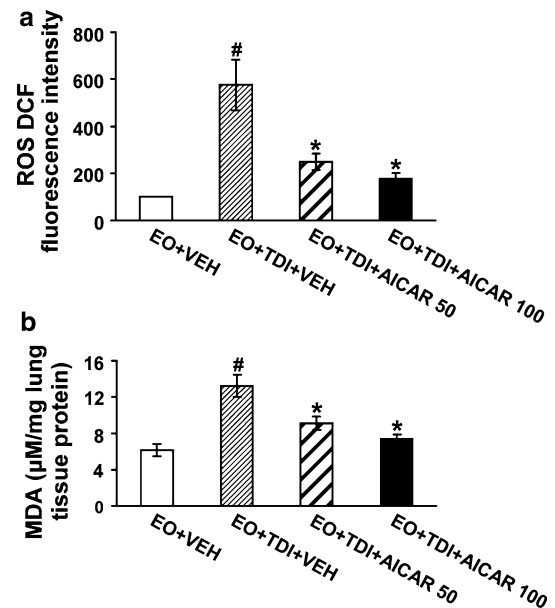


Fig. 8 Effect of AICAR on ROS levels in BAL cells and MDA levels in lung tissues of TDI-sensitized and -challenged mice. Sampling was performed at 48 h after the challenge in control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). **a** DCF fluorescence intensity is presented as the relative ratio of ROS levels in EO + TDI + VEH, EO + TDI + AICAR 50, or EO + TDI + AICAR 100 to those in EO + VEH. The ROS levels in the BAL fluids of EO + VEH is arbitrarily presented as 100. **b** MDA levels in lung tissues. Data represent mean $\pm$ SEM from seven mice per group. #*p* < 0.05 versus EO + VEH, **p* < 0.05 versus EO + TDI + VEH

of the control mice (Fig. 2g). In addition, the percent Rrs produced by administration of methacholine (25 and 50 mg/ml) was significantly increased in the TDI-inhaled mice compared with the control mice. TDI-sensitized and -challenged mice treated with AICAR showed the dose-response curve of percent Rrs that shifted to the right. TDI-inhaled mice treated with 100 mg/kg of AICAR showed a significant reduction in percent Rrs produced by methacholine at 50 mg/ml compared with that of mice treated with drug vehicle only. These results indicate that AICAR reduces TDI-induced AHR.

AICAR down-regulates VEGFA expression and plasma extravasation

Given the fundamental role of VEGFA in pathophysiological changes of asthma [27, 28], we examined the effect of AMPK activation on VEGFA expression and vascular permeability in TDI-induced airway inflammation. Western blot analysis showed that levels of VEGFA protein in

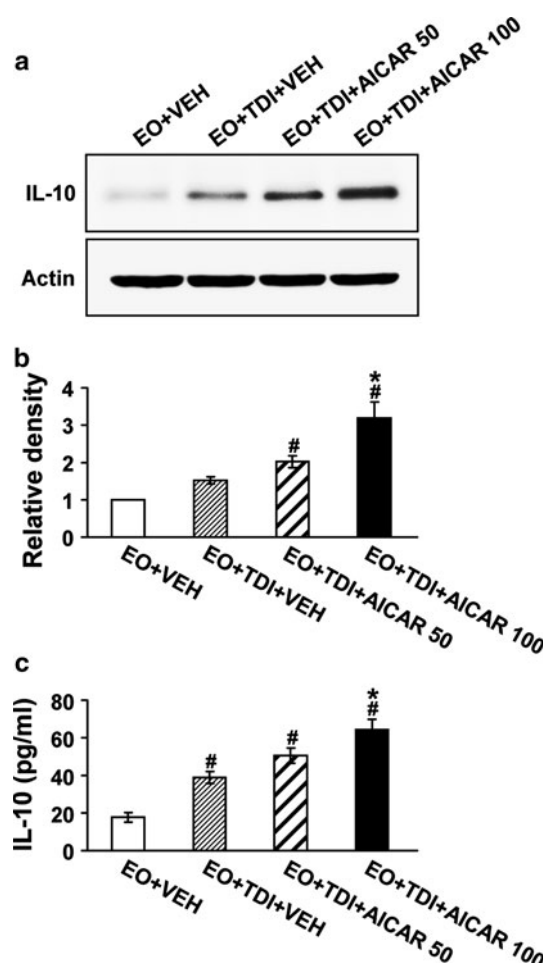


Fig. 9 Effect of AICAR on IL-10 protein levels in lung tissues and in BAL fluids. Sampling was performed at 48 h after the challenge in control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). **a** Representative western blotting of IL-10 in lung tissues. **b** Densitometric analyses are presented as the relative ratio of IL-10 to actin. The relative ratio of IL-10 in the lung tissues of EO + VEH is arbitrarily presented as 1. **c** Enzyme immunoassay of IL-10 in BAL fluids. Data represent mean $\pm$ SEM from seven mice per group. # $p < 0.05$ versus EO + VEH, * $p < 0.05$ versus EO + TDI + VEH

lung tissues were dramatically increased at 48 h after TDI inhalation compared with the levels in the control mice (Fig. 3a, b). Treatment of mice with AICAR resulted in a significant decrease in VEGFA levels in lung tissues. Consistent with the results obtained from the Western blot analysis, enzyme immunoassay revealed that levels of VEGFA in BAL fluids were significantly higher in TDI-inhaled mice than in the control mice and that treatment with AICAR remarkably reduced the TDI-induced increase in VEGFA levels (Fig. 3c).

EBD assay showed that plasma extravasation was substantially increased after TDI inhalation compared with

that in the control mice (Fig. 3d). The increase in plasma extravasation was dramatically decreased by AICAR treatment. To demonstrate the relationship of vascular permeability with VEGFA expression in TDI-inhaled mice, we evaluated the correlation between VEGFA protein in BAL fluids and plasma exudation. The levels of VEGFA protein in BAL fluids were significantly correlated with the levels of plasma extravasation ($r = 0.831$; $p < 0.05$) (Fig. 3e).

HIF-1 α and HIF-2 α are activated in lung tissues of TDI-inhaled mice

To evaluate whether HIF-1 α and HIF-2 α are activated in TDI-induced occupational asthma, we measured HIF-1 α and HIF-2 α protein levels in nuclear protein extracts from lung tissues using Western blotting. Both isoforms of HIF- α subunit were greatly increased after TDI inhalation compared with the levels in the control mice (Fig. 4). As expected, the increased levels of HIF-1 α and HIF-2 α in nuclear extracts from lung tissues of TDI-inhaled mice were greatly reduced by treatment with 2ME2, a non-specific inhibitor of HIF.

AICAR negatively modulates TDI-induced activation of HIF-1 α and HIF-2 α

We next determined whether AMPK influences the activation of HIF-1 α and HIF-2 α in TDI-induced asthma. Treatment of mice with AICAR significantly lowered the increases in two HIF- α isoform levels in nuclear protein extracts from lung tissues of TDI-inhaled mice (Fig. 5).

2ME2 and CBO-P11 suppress VEGFA expression, vascular permeability, airway inflammation, and AHR induced by TDI

In order to support the notion that the therapeutic effect of AMPK activator on TDI-induced asthma is mediated by down-regulation of HIF/VEGFA signaling, we sought to explore whether the blockade of HIF/VEGFA pathway can affect TDI-induced airway responses including vascular permeability using 2ME2 and CBO-P11, an inhibitor of VEGFA activity. Both 2ME2 and CBO-P11 significantly suppressed VEGFA expression in lung tissues and BAL fluids (Fig. 6a–c). The increased plasma extravasation after TDI inhalation was also reduced by administration of 2ME2 and CBO-P11 (Fig. 6d). Moreover, the levels of VEGFA in BAL fluids showed a significant positive correlation with the levels of plasma extravasation ($r = 0.803$; $p < 0.05$) (Fig. 6e). As shown in Fig. 7a, 2ME2 and CBO-P11 dramatically decreased influx of inflammatory cells

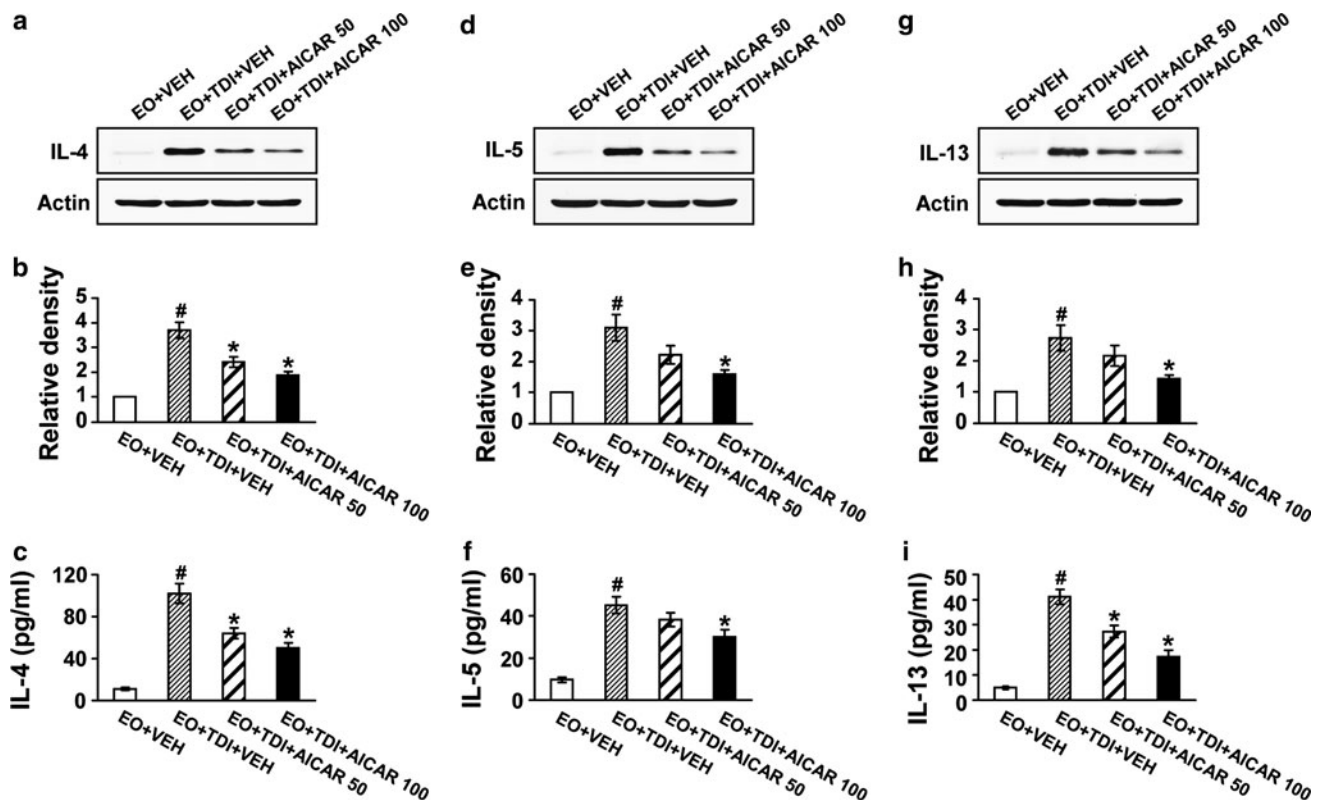


Fig. 10 Effect of AICAR on IL-4 (a–c), IL-5 (d–f), and IL-13 (g–i) protein levels in lung tissues and BAL fluids. Sampling was performed at 48 h after the challenge in control mice treated with drug vehicle (EO + VEH), TDI-inhaled mice treated with drug vehicle (EO + TDI + VEH), TDI-inhaled mice treated with 50 mg/kg of AICAR (EO + TDI + AICAR 50), and TDI-inhaled mice treated with 100 mg/kg of AICAR (EO + TDI + AICAR 100). The expression of IL-4 (a), IL-5 (d), and IL-13 (g) in lung tissues was

identified by western blotting. Densitometric analyses are presented as the relative ratio of IL-4 (b), IL-5 (e), and IL-13 (h) to actin. The relative ratio of each molecule in the lung tissues of EO + VEH is arbitrarily presented as 1. The levels of IL-4 (c), IL-5 (f), and IL-13 (i) were measured in BAL fluids by enzyme immunoassay. Data represent mean $\pm$ SEM from seven mice per group. # p < 0.05 versus EO + VEH, * p < 0.05 versus EO + TDI + VEH

into the airway lumen. Moreover, AHR induced by TDI was improved by treatment with 2ME2 and CBO-P11 (Fig. 7b).

AICAR reduces oxidative stress in TDI-sensitized and -challenged mice

Oxidative stress is recognized as a key component of airway inflammation in TDI-induced occupational asthma [42]. A previous study has reported that simulators of AMPK reduce ROS release in neutrophils [13]. To investigate effect of AMPK on TDI-induced ROS production, ROS levels in BAL cells were measured. The ROS levels in BAL cells were considerably higher in TDI-inhaled mice than in the control mice (Fig. 8a). The increased ROS generation was substantially reduced by activation of AMPK with AICAR.

We assessed oxidative damage by measuring MDA in lung tissues. The MDA levels in lung tissues were increased after TDI challenge and markedly decreased by treatment with AICAR (Fig. 8b).

AICAR enhances TDI-induced expression of IL-10 in the lung

Since IL-10 is an immune regulatory cytokine that suppresses inflammatory reactions [43], we tested the effect of AMPK activation on IL-10 expression in the lung. TDI challenge induced an increase in levels of IL-10 protein detected by Western blotting in lung tissues, as compared with levels in the control mice (Fig. 9a, b). The increased IL-10 levels in lung tissues were further increased by administration of AICAR. Enzyme immunoassay also showed that levels of IL-10 in BAL fluids were increased after TDI inhalation and that AICAR treatment further enhanced the increase in IL-10 levels of BAL fluids (Fig. 9c).

AICAR down-regulates TDI-induced expression of Th2 cytokines in the lung

To ascertain the effect of AMPK activation on Th2 cytokine expression in lungs, we measured the levels of IL-4,

IL-5, and IL-13 in lung tissues or BAL fluids from AICAR-treated and untreated mice. Levels of IL-4, IL-5, and IL-13 detected by Western blotting in lung tissues as well as by ELISA in BAL fluids were significantly increased at 48 h after TDI inhalation compared with the levels in the control mice (Fig. 10). AICAR treatment was associated with apparent reductions of these cytokine levels in both lung tissues and BAL fluids.

Discussion

To our knowledge, the present study is the first to document the beneficial effect of AMPK pathway on TDI-induced airway inflammatory responses, especially on vascular permeability and oxidative stress. Although the pathogenesis of occupational asthma caused by low-molecular-weight compounds such as TDI remains largely uncertain, TDI-induced asthma is characterized by airway inflammation and AHR associated with increased microvascular permeability. In this study, AMPK activator ameliorated airway inflammation and AHR and also decreased levels of HIF-1 α , HIF-2 α , VEGFA, and plasma exudation in a murine model of TDI-induced asthma. In addition, inhibition of HIF and VEGFA activity was dramatically effective in decreasing vascular exudation and reversing pathophysiologic features of TDI-induced asthma. We also found that AMPK activator reduced ROS generation and Th2 cytokine levels but enhanced the expression of IL-10 in the lung. These results suggest that AMPK activation exerts the therapeutic effect in TDI-induced asthma by reducing vascular permeability via HIF/VEGFA pathway as well as by inhibiting ROS production.

AMPK pathway has been suggested to be a novel signaling molecule modulating inflammatory and immune responses [4, 5]. In animal models of several inflammatory diseases, AMPK activator can prevent the progression of inflammatory responses [4, 10, 12]. Consistent with these results obtained from other diseases, we showed that AMPK activation notably alleviated airway inflammation and AHR in the murine model of TDI-induced asthma. In the search for underlying mechanisms by which AMPK controls TDI-induced asthma, we found that AMPK activator suppressed not only HIF-1 α , HIF-2 α , and VEGFA activity but also plasma extravasation enhanced after TDI inhalation. VEGFA increases microvascular permeability so that plasma proteins and inflammatory cells can leak into the extravascular space, which contributes to edema and inflammation of the airway [28]. In this study, VEGFA expression and plasma extravasation were remarkably increased in response to TDI challenge, and VEGFA levels in BAL fluids were significantly correlated with the levels of plasma extravasation. HIF pathway activates

transcription of the gene encoding VEGFA and thus is expected to be implicated in TDI-induced airway inflammation. Indeed, HIF-1 α and HIF-2 α protein levels in nuclear protein extracts from lung tissues were increased after TDI inhalation in our murine model. We also found that VEGFA expression was remarkably reduced by inhibition of HIF-1 α and HIF-2 α with 2ME2 and that VEGFA inhibitor, CBO-P11 as well as 2ME2 significantly suppressed vascular permeability, airway infiltrations of inflammatory cells, and AHR induced by TDI. These observations support the notion that HIF-mediated VEGFA expression contributes to TDI-induced airway responses through enhancing vascular permeability. Importantly, in the current study the increased levels of HIF-1 α , HIF-2 α , and VEGFA after TDI inhalation were remarkably decreased by administration of AMPK activator in association with lower plasma exudation. An inhibitory effect of AICAR on HIF-1 α expression has been observed in retinal epithelial cells, which is consistent with our *in vivo* results [44]. Based on these findings, we suggest that AMPK activation reduces vascular permeability by down-regulating HIF/VEGFA pathway in TDI-induced asthma.

It is noteworthy that we found the suppressive action of AMPK activation on oxidative stress in airway inflammation. Oxidative stress is associated with the pathogenesis of TDI-induced occupational asthma [42]. Activated inflammatory cells in the airway produce large amount of ROS. Beside generation of ROS via cellular pathways, formation of ROS in the lung can also take place by exogenous sources like ozone, ionizing radiation, chemicals, and dust particles [14]. A recent *in vitro* study has demonstrated that TDI exposure induces ROS generation in airway epithelial cells [42]. In agreement with these observations, the levels of ROS production in cells from BAL fluids were substantially elevated after TDI inhalation in our murine model. Increased ROS levels lead to mucus hypersecretion, smooth muscle contraction, and epithelial shedding [14]. In addition, oxidative stress results in activation of redox-sensitive transcription factor and consequent induction of many inflammatory genes that are abnormally expressed in airway inflammation [45]. Previous studies have reported that natural products, which act as a ROS scavenger, potentially protect TDI-induced asthma by down-regulating various proinflammatory mediators including Th2 cytokines [46, 47]. Hence, enhancement of antioxidant capacity can be an attractive approach to treat TDI-induced asthma. Recently, activation of AMPK has been reported to have antioxidant action capable of regulating intracellular ROS in neutrophils and endothelial cells [13, 48]. Supporting *in vitro* results, we found that AICAR treatment profoundly decreased ROS levels in BAL cells of TDI-induced asthma. Our results also showed that AICAR was effective in ameliorating oxidative damage, quantified by the MDA

levels. Moreover, ROS increase vascular permeability by inducing VEGFA expression through up-regulation of HIF-1 α activation [49–51]. Therefore, AMPK activation may negatively modulate vascular permeability partly by inhibiting ROS–HIF–VEGFA axis. Taken together, these findings suggest that suppression of oxidative stress is one of important beneficial roles of AMPK in TDI-induced asthma.

This study provides evidence for an immune regulatory function of AMPK that is regulation of IL-10 in airway inflammatory diseases. IL-10 has emerged as an anti-inflammatory cytokine relevant to the inhibition of asthma pathology [52]. A recent study has demonstrated that activation of AMPK regulates T cell-mediated immune responses by inducing the generation of IL-10 in spleen cells [53]. Previously, we have demonstrated that reduction in oxidative stress by treatment with an antioxidant increases levels of IL-10 in lung tissues and in serum of a murine model of asthma, suggesting that IL-10 is regulated through ROS/antioxidant balance [54]. The present results showed that AICAR increased IL-10 expression with decreasing ROS production. Thus, AMPK signaling pathway seems to modulate IL-10 expression, at least in part, via inhibition of ROS generation in TDI-induced asthma.

Animal models of asthma have been extensively used to examine mechanisms of the disease, to evaluate the activity of a variety of genes and cellular pathways, and to predict the safety of new drugs or chemicals before being used in clinical studies [55]. Among various animal models of asthma, mouse is usually chosen by many researchers thanks to its numerous advantages compared to the use of other animals, including IgE production as the primary allergic antibody, availability of numerous immunological reagents, various inbred strains, easy breeding, short gestational period, and small and relatively inexpensive cost [56]. However, mouse like other species has several limitations: mouse does not display spontaneous symptoms or long-lasting bronchoconstriction as seen in asthmatics, its lung is more fully developed at birth so environmental influences have different effects, the structure of mouse lung is very different from human, and mouse is an obligate nasal breather and is limited in chronic asthma. Therefore, it is clear that the results from studies with the mouse model of asthma are not easily translated to humans, and therapeutic initiatives successful in this animal model have generally been of limited success in the clinic [57].

In conclusion, our results have demonstrated that AMPK activation attenuates TDI-induced airway inflammation and hyperresponsiveness. AMPK activation by AICAR also decreases vascular permeability by modulating HIF activation and VEGFA expression in TDI-induced asthma. Moreover, administration of AICAR reduces ROS production, while enhancing the expression of an anti-inflammatory cytokine

IL-10. Our data suggest novel anti-inflammatory mechanisms of AMPK activation, that is, down-regulation of HIF/VEGFA pathway and inhibition of ROS generation with inducing IL-10 expression. These findings point to the considerable potential of AMPK activation in the treatment of occupational asthma and other airway inflammatory diseases, although extrapolation of these findings to humans needs to be judicious.

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Conflict of interest The authors declared that they have no conflict of interest.

References

- Gautrin D, Newman-Taylor AJ, Nordman H, Malo JL. Controversies in epidemiology of occupational asthma. *Eur Respir J*. 2003;22:551–9.
- Maestrelli P, Boschetto P, Fabbri LM, Mapp CE. Mechanisms of occupational asthma. *J Allergy Clin Immunol*. 2009;123:531–42.
- Carling D. AMP-activated protein kinase: balancing the scales. *Biochimie*. 2005;87:87–91.
- Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. *J Immunol*. 2005;175:566–74.
- Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S, et al. Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. *Clin Exp Immunol*. 2009;156:542–51.
- Wong AK, Howie J, Petrie JR, Lang CC. AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. *Clin Sci (Lond)*. 2009;116:607–20.
- Kim AS, Miller EJ, Young LH. AMP-activated protein kinase: a core signalling pathway in the heart. *Acta Physiol (Oxf)*. 2009;196:37–53.
- Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem*. 1995;229:558–65.
- Hardie DG, Hawley SA. AMP-activated protein kinase: the energy charge hypothesis revisited. *BioEssays*. 2001;23:1112–9.
- Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2008;295:L497–504.
- Sag D, Carling D, Stout RD, Suttles J. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol*. 2008;181:8633–41.
- Kim TB, Kim SY, Moon KA, Park CS, Jang MK, Yun ES, et al. Five-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates poly (I:C)-induced airway inflammation in a murine model of asthma. *Clin Exp Allergy*. 2007;37:1709–19.
- Alba G, El Bekay R, Alvarez-Maqueda M, Chacón P, Vega A, Monteseirín J, et al. Stimulators of AMP-activated protein kinase

- inhibit the respiratory burst in human neutrophils. *FEBS Lett.* 2004;573:219–25.
14. Park SJ, Lee YC. Antioxidants as novel agents for asthma. *Mini Rev Med Chem.* 2006;6:235–40.
 15. Barrett N, Austen KF. Airway inflammation in asthma: Th2 cell immunity and beyond. *Immunity.* 2009;31:425–37.
 16. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med.* 1995;332:133–8.
 17. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, et al. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med.* 2007;176:858–64.
 18. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol.* 2010;63:601–10.
 19. Toldi G, Molvarec A, Stenczer B, Müller V, Eszes N, Bohács A, et al. Peripheral T(h)1/T(h)2/T(h)17/regulatory T-cell balance in asthmatic pregnancy. *I. Int Immunol.* 2011;23:669–77.
 20. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in immune regulation of allergic diseases. *Eur J Immunol.* 2010;40:1232–40.
 21. Xue K, Zhou Y, Xiong S, Xiong W, Tang T. Analysis of CD4+ CD25+ regulatory T cells and Foxp3 mRNA in the peripheral blood of patients with asthma. *J Huazhong Univ Sci Technolog Med Sci.* 2007;27:31–3.
 22. Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol.* 2010;151:297–307.
 23. O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev.* 2008;223:114–31.
 24. John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, et al. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1 alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med.* 1998;157:256–62.
 25. Lim S, Crawley E, Woo P, Barnes PJ. Haplotype associated with low interleukin-10 production in patients with severe asthma. *Lancet.* 1998;352:113.
 26. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med.* 2004;199:1567–75.
 27. Ribatti D, Puxeddu I, Crivellato E, Nico B, Vacca A, Levi-Schaffer F. Angiogenesis in asthma. *Clin Exp Allergy.* 2009;39:1815–21.
 28. Lee YC, Kwak YG, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol.* 2002;168:3595–600.
 29. Kanazawa H, Nomura S, Yoshikawa J. Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med.* 2004;169:1125–30.
 30. Knox AJ, Corbett L, Stocks J, Holland E, Zhu YM, Pang L. Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoïd-dependent mechanism. *FASEB J.* 2001;15:2480–8.
 31. Wen FQ, Liu X, Manda W, Terasaki Y, Kobayashi T, Abe S, et al. TH2 cytokine-enhanced and TGF beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-gamma and corticosteroids. *J Allergy Clin Immunol.* 2003;111:1307–18.
 32. Kazi AS, Lotfi S, Goncharova EA, Tliba O, Amrani Y, Krymskaya VP, et al. Vascular endothelial growth factor-induced secretion of fibronectin is ERK dependent. *Am J Physiol Lung Cell Mol Physiol.* 2004;286:L539–45.
 33. Faffe DS, Flynt L, Mellema M, Moore PE, Silverman ES, Subramaniam V, et al. Oncostatin M causes eotaxin-1 release from airway smooth muscle: synergy with IL-4 and IL-13. *J Allergy Clin Immunol.* 2005;115:514–20.
 34. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med.* 2004;10:1095–103.
 35. Loboda A, Jozkowicz A, Dulak J. HIF-1 and HIF-2 transcription factors—similar but not identical. *Mol Cells.* 2010;29:435–42.
 36. Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci USA.* 1997;94:4273–8.
 37. Wiesener MS, Jürgensen JS, Rosenberger C, Scholze CK, Hörstrup JH, Warnecke C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J.* 2003;17:271–3.
 38. Lee SY, Kwon S, Kim KH, Moon HS, Song JS, Park SH, et al. Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients. *Ann Allergy Asthma Immunol.* 2006;97:794–9.
 39. Tournay KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy.* 2000;30:79–85.
 40. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med.* 1997;186:449–54.
 41. Lee KS, Kim SR, Park SJ, Park HS, Min KH, Jin SM, et al. Peroxisome proliferator activated receptor-gamma modulates reactive oxygen species generation and activation of nuclear factor-kappaB and hypoxia-inducible factor 1alpha in allergic airway disease of mice. *J Allergy Clin Immunol.* 2006;118:120–7.
 42. Hur GY, Kim SH, Park SM, Ye YM, Kim CW, Jang AS, et al. Tissue transglutaminase can be involved in airway inflammation of toluene diisocyanate-induced occupational asthma. *J Clin Immunol.* 2009;29:786–94.
 43. Robinson DS. Regulatory T cells and asthma. *Clin Exp Allergy.* 2009;39:1314–23.
 44. Treins C, Murdaca J, Van Obberghen E, Giorgetti-Peraldi S. AMPK activation inhibits the expression of HIF-1alpha induced by insulin and IGF-1. *Biochem Biophys Res Commun.* 2006;342:1197–202.
 45. Henderson WR Jr, Chi EY, Teo JL, Nguyen C, Kahn M. A small molecule inhibitor of redox-regulated NF-kappa B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. *J Immunol.* 2002;169:5294–9.
 46. Mahajan SG, Mali RG, Mehta AA. Effect of *Moringa oleifera* Lam. seed extract on toluene diisocyanate-induced immune-mediated inflammatory responses in rats. *J Immunotoxicol.* 2007;4:85–96.
 47. Kim SH, Park HJ, Lee CM, Choi IW, Moon DO, Roh HJ, et al. Epigallocatechin-3-gallate protects toluene diisocyanate-induced airway inflammation in a murine model of asthma. *FEBS Lett.* 2006;580:1883–90.
 48. Kim JE, Kim YW, Lee IK, Kim JY, Kang YJ, Park SY. AMP-activated protein kinase activation by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) inhibits palmitate-induced endothelial cell apoptosis through reactive oxygen species suppression. *J Pharmacol Sci.* 2008;106:394–403.

49. Biswas S, Gupta MK, Chattopadhyay D, Mukhopadhyay CK. Insulin-induced activation of hypoxia-inducible factor-1 requires generation of reactive oxygen species by NADPH oxidase. *Am J Physiol Heart Circ Physiol*. 2007;292:H758–66.
50. Lee KS, Kim SR, Park HS, Park SJ, Min KH, Lee KY, et al. A novel thiol compound, *N*-acetylcysteine amide, attenuates allergic airway disease by regulating activation of NF-kappaB and hypoxia-inducible factor-1alpha. *Exp Mol Med*. 2007;39:756–68.
51. Irwin DC, McCord JM, Nozik-Grayck E, Beckly G, Foreman B, Sullivan T, et al. A potential role for reactive oxygen species and the HIF-1alpha-VEGF pathway in hypoxia-induced pulmonary vascular leak. *Free Radic Biol Med*. 2009;47:55–61.
52. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity*. 2009;31:438–49.
53. Nath N, Khan M, Paintlia MK, Singh I, Hoda MN, Giri S. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. *J Immunol*. 2009;182:8005–14.
54. Kim SR, Lee KS, Park SJ, Min KH, Lee MH, Lee KA, et al. A novel dithiol amide CB3 attenuates allergic airway disease through negative regulation of p38 MAPK. *Am J Respir Crit Care Med*. 2011;183:1015–24.
55. Karol MH. Animal models of occupational asthma. *Eur Respir J*. 1994;7:555–68.
56. Shin YS, Takeda K, Gelfand EW. Understanding asthma using animal models. *Allergy Asthma Immunol Res*. 2009;1:10–8.
57. Persson CG, Erjefalt JS, Korsgren M, Sundler F. The mouse trap. *Trends Pharmacol Sci*. 1997;18:465–7.