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Therapy with resveratrol attenuates obesity-associated allergic airway inflammation in mice

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ABSTRACT

Obesity and insulin resistance have been associated with deterioration in asthma outcomes. High oxidative stress and deficient activation of AMP-activated protein kinase (AMPK) have emerged as important regulators linking insulin resistance and inflammation. This study aimed to evaluate the effects of resveratrol on obesity-associated allergic pulmonary inflammation. Male C57/Bl6 mice fed with high-fat diet to induce obesity (obese group) or standard-chow diet (lean group) were treated or not with resveratrol (100 mg/kg/day, two weeks). Mice were sensitized and challenged with ovalbumin (OVA). At 48 h thereafter, bronchoalveolar lavage fluid was performed, and lungs collected for morphological studies and Western blot analysis. Treatment of obese mice with resveratrol significantly reduced hyperglycemia and insulin resistance, as well as the body measures (body mass, fat mass, % fat, and body area). OVA-challenge promoted a higher increase in pulmonary eosinophil infiltration in obese compared with lean mice, which was nearly abrogated by resveratrol treatment. Resveratrol markedly increased the phosphorylated AMPK expression in lung tissues of obese compared with lean mice. Resveratrol reduced the p47phox expression and reactive-oxygen species (ROS) production, and elevated the superoxide dismutase (SOD) levels in lung tissues of obese mice. The increased pulmonary levels of TNF-α and inducible nitric oxide synthase (iNOS) in obese mice were also normalized after resveratrol treatment. In lean mice, resveratrol failed to affect the levels of fasting glucose, p47phox, ROS levels, TNF-α, iNOS and phosphorylated AMPK. Resveratrol exhibits protective effects in obesity-associated lung inflammation that is accompanied by local AMPK activation and antioxidant property.

1. Introduction

Asthma is a disorder of the conducting airways leading to variable airflow obstruction in association with airway hyperresponsiveness [1]. Airway eosinophilia is commonly associated with increased risk for asthma exacerbation, severity, and poor prognosis [2]. Eosinophils have been classically considered effector cells with pro-inflammatory actions via the synthesis and release of multiple substances such as the highly basic and cytotoxic granule proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EPX) [1]. Eosinophils are also capable of undergoing de novo synthesis of nitric oxide (NO), and measurement of exhaled NO level has been employed as a biomarker of airway inflammation [3]. The enzyme inducible NO synthase (iNOS) is highly expressed in lung tissues and eosinophils of asthmatic patients and animals, generating high levels of NO in the exhaled air [4]. Activation of NF-κB by TNF-α increases iNOS transcription [5], leading subsequently to NO overproduction, which in turn modulates the eosinophil recruitment into the pulmonary tissue [6,7]. Moreover, high levels of NO-reacting oxidants such as superoxide anion, hydrogen peroxide, and hydroxyl radicals have been implicated in allergic lung diseases, which may be produced by resident and non-resident cells infiltrating the airways of asthmatic individuals [8]. Increased oxidative stress leads to inactivation of superoxide dismutase (SOD) in asthmatic individuals aggravating airway obstruction [9].

Obese individuals with asthma exhibit worse asthma-related quality of life, worse asthma control, and more asthma-related hospitalizations in comparison with those with asthmatics with normal body mass index [10,11]. Studies indicate a high prevalence of insulin resistance (IR) in obese and asthmatic patients versus obese non-asthmatics, suggesting that insulin resistance may be a causal link between asthma and obesity [12–15]. The exacerbation of the allergic eosinophilic inflammation in obese mice is normalized by suppression of insulin resistance, which is accompanied by reductions of the levels of pro-inflammatory markers such as TNF-α and NO metabolites [16,17].

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Resveratrol (*trans*-resveratrol) is a natural polyphenolic compound found in the skin of red grapes. In rodents and humans, resveratrol promotes cardio-neuroprotective, anti-diabetic and anti-cancer effects [18]. Resveratrol therapy can also prevent the hypertensive response in rats fed a high-fat diet [19]. Recently, resveratrol was shown to alleviate thermal hyperalgesia and mechanical allodynia in a neuropathic mice model [20]. Resveratrol activates AMP-activated protein kinase (AMPK) [21,22], and AMPK improvement in turn improves high-fat diet-induced insulin resistance [17,23]. In a mouse model of asthma, resveratrol significantly reduced the levels of the Th2 cytokines, airway hyperresponsiveness, eosinophilia, and mucus hypersecretion [24]. In the same animal model, the protective effects of resveratrol was associated with restored mitochondrial function and inositol polyphosphate-4-phosphatase A expression in the lungs, as well as with decreased PI3K–Akt signaling [25]. There are growing evidences suggesting that the obesity-related asthma phenotype does not necessarily involve the classical Th2-dependent inflammatory process [26]. In the present study we tested the hypothesis that resveratrol attenuates the pulmonary allergic inflammation in insulin-resistant obese mice. Therefore, we treated high-fat-fed obese mice with resveratrol, and evaluated the pulmonary eosinophilic inflammation in response to ovalbumin (OVA) challenge. The levels of oxidant and antioxidant biomarkers in the lung tissue, as well as the protein expressions of iNOS and phosphorylated AMPK were evaluated in this study.

2. Materials and methods

2.1. Animals and diet

All animal procedures and experimental protocols are in accordance with and were approved by the Ethics Committee in Animal Use, State University of Campinas (CEUA-UNICAMP), protocol 2012/2709-1. Animals were housed on a 12-h light–dark cycle and fed for 12 weeks with either a standard chow diet (70% carbohydrate, 20% protein, 10% fat) or a high-fat diet that induces obesity (29% carbohydrate, 16% protein, 55% fat) [16]. Lean and obese mice were treated with vehicle (water) or resveratrol (100 mg/kg/day) by gavage for two weeks [27].

2.2. Mouse densitometry

Mice were weighed, anesthetized with intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (90 mg/kg), and then subjected to a dual-energy X-ray absorptiometry scan (DEXA; QDR Series, Hologic Apex Software Inc., Bedford, MA, USA). Body scan measurements allowed the following parameters: total mass, fat mass, % fat, body area and bone mineral composition (BMC). Mouse body scans were obtained for each animal.

2.3. Insulin tolerance test (ITT)

After 6-h fasting, systemic insulin sensitivity was analyzed by the Insulin Tolerance Test (ITT). Briefly, tail blood samples were collected before (0 min) and at 5, 10, 15, 20, 25 and 30 min after an intraperitoneal injection of 1.00 U/kg of regular insulin (Novolin R, NovoNordisk, Bagsvaerd, Denmark). Glucose concentrations were measured using a glucometer (ACCUCHEK Performa; Roche Diagnostics, Indianapolis, IN, USA) and the values were used to calculate the constant rate for blood glucose disappearance (K_{ITT}), which is based on the linear regression of the Neperian logarithm of glucose concentrations obtained from 0 to 30 min of the test. K_{ITT} was calculated using the formula $0.693 / (t1 / 2) \times -1 \times 100$ [28].

2.4. Sensitization procedure and OVA challenge

Lean and obese mice were actively sensitized with a subcutaneous injection (0.4 ml) of 100 μg of OVA (grade V; Sigma-Aldrich Co., St. Louis, MO) mixed with 1.6 mg $Al(OH)_3$ in 0.9% NaCl (day zero). One week later (day 7), mice received a second subcutaneous injection of 100 μg OVA (0.4 ml). On days 14 and 15, mice were intranasally challenged with OVA (10 μg/50 μl) twice a day. At 48 h after the first challenge, the BAL fluid was performed, and lungs were also collected for morphological studies and Western blot. The lungs were washed by flushing phosphate-buffered saline (PBS). The PBS was instilled through the tracheal cannula in 5-aliquots of 300 μl. The fluid recovered after each instillation was centrifuged (500 ×*g*, 10 min, 4 °C), and BAL fluid supernatant stored at −80 °C. The cell pellet was resuspended in 200 μl of PBS and total (Neubauer) and differential (Diff-Quick stain) cell counts were done. Fig. 1 depicts the experimental protocols for airway sensitization and challenge with ovalbumin (OVA) in mice treated or not with resveratrol.

2.5. Morphometrical lung analysis

Lungs were perfused via the right ventricle with 10 ml PBS to remove residual blood, immersed in 10% phosphate buffered formalin for 24 h and then kept in 70% ethanol until embedding in paraffin. Tissues were sliced (5 μm sections) and stained with hematoxylin/eosin for light microscopy examination. Morphometrical analysis was performed using a Leica DM 5000B digital camera, and Leica Q Win Image Processing and Analysis Software. For each different staining, the area of positivity was measured in mm^2 for 5 bronchioles per slide.

2.6. Measurements of TNF-α, SOD and glutathione (GSH)

TNF-α in BAL fluid was measured using commercially available DuoSet ELISA kits, following the instructions of the manufacturer (R & D, Minneapolis, USA). SOD and GSH concentrations in lung tissue were determined using commercially available kits (Cayman Chemical, Ann Arbor, MI, USA).

2.7. Western blotting for iNOS, p47phox, PDE4, and phosphorylated AMPK

Lung tissues were homogenized in SDS lysis buffer with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Inc., Westbury, NY) and centrifuged. Protein concentrations in supernatants were determined by the Bradford assay, and an equal amount of protein from each sample (50 μg) was treated with Laemmli buffer containing dithiothreitol (100 mM). Samples were heated in a boiling water bath for 10 min and resolved by SDS-PAGE. Electrotransfer of proteins to a nitrocellulose membrane was performed for 60 min at 15 V (constant) in a semi-dry device (Bio-Rad, Hercules, CA, USA). Nonspecific protein binding to nitrocellulose was reduced by pre-incubating the membrane overnight at 4 °C in blocking buffer (0.5% non-fat dried milk, 10 mM Tris, 100 mM NaCl, and 0.02% Tween 20). Specific antibodies such as anti-

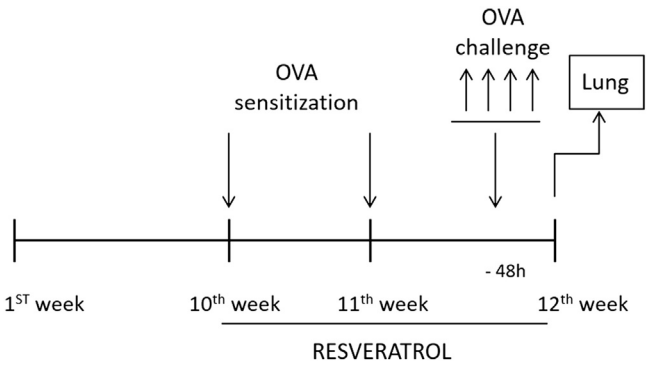

Fig. 1. Protocol for sensitization and challenge with ovalbumin (OVA) in high-fat obese and standard-chow-fed lean mice, treated or not with resveratrol (100 mg/kg, 2 weeks). BAL, bronchoalveolar lavage fluid.