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Benzylamine and methylamine, substrates of semicarbazide-sensitive amine oxidase, attenuate inflammatory response induced by lipopolysaccharide

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ABSTRACT

Current evidence indicates that semicarbazide-sensitive amine oxidase (SSAO) substrates possess insulin-mimic effect, which was thought to play an anti-inflammatory role. The purpose of the present study was to determine whether SSAO substrates benzylamine (BZA) and methylamine (MA) attenuate inflammatory response induced by lipopolysaccharide (LPS). BALB/c mice peritoneal macrophages (PMs) that express SSAO and RAW264.7 mouse macrophages that do not express SSAO were used in vitro studies. Experimental mice were given BZA or MA through intraperitoneal injection before LPS challenge. The results showed that BZA or MA treatment significantly reduced LPS-induced pro-inflammatory mediators (nitric oxide, TNF-α) production, the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and glucose consumption in murine PMs, but not in RAW264.7 cell line. The metabolites of BZA or MA catalyzed by SSAO, hydrogen peroxide, formaldehyde, and benzaldehyde could also significantly decrease LPS-induced nitric oxide and TNF-α production, iNOS and COX-2 expression, and glucose consumption in vitro. In addition, BZA or MA administration could significantly decrease plasma pro-inflammatory mediators and the expression of iNOS and COX-2 in liver and lung, and could also attenuate LPS-induced transient hyperglycemia and chronic hypoglycemia. These findings indicated that substrates of SSAO might be involved in the anti-inflammatory effects. The metabolites of BZA and MA catalyzed by SSAO might be responsible for the anti-inflammatory effects. Moreover, BZA or MA administration could be useful for normalization of glucose disposal during endotoxemia.

1. Introduction

Lipopolysaccharide (LPS) is the major inducer of inflammatory host responses in Gram-negative bacterial infection [1]. During infection, LPS is released in the circulation, where it stimulates the toll-like receptor-4 (TLR4)-MD2-CD14 receptor complex on inflammatory cells, which release massive amounts of pro-inflammatory mediators such as TNF-α, nitric oxide (NO), and arachidonic acid [2,3]. Although activation of the innate immune response during host-pathogen interaction is initially adaptive, the over production of inflammatory mediators can generate systemic activation, which disturbs normal cellular functions, induces metabolic changes, and leads to multiple organ dysfunction syndromes (MODS) or lethal septic shock [2]. Despite significant progress in understanding its pathophysiology, systemic inflammatory response syndrome (SIRS)/sepsis continues to be the most common cause of death in intensive care units for lack of effective treatment [4].

Semicarbazide-sensitive amine oxidase (SSAO, EC; 1.4.3.6) is a group of enzymes containing copper and quinone and sensitive to semicarbazide [5]. It is capable of deaminating short-chain primary amines, and producing corresponding aldehyde, hydrogen peroxide (H_2O_2), and ammonia. This enzyme is present either as membrane form located in the vascular system and adipocytes or as soluble form in blood. The membrane form is identical with vascular adhesion protein-1, and is the source of the soluble form [6]. This enzyme has been widely studied during the past decades, and thought to play a role in lymphocyte trafficking and contribute to the process of inflammation [5]. Recent studies showed that SSAO-mediated deamination was involved in glucose transport in the adipocytes and smooth muscle cells [7,8]. Based on the functions of SSAO, its substrates and inhibitors were explored as promising drugs for diabetes or inflammatory diseases in animal experiments [9–12]. Low-molecular-weight SSAO inhibitors were showed to attenuate inflammation through reducing leukocyte adhesion and plasma pro-inflammatory mediators [9,10]. SSAO substrates, however, possess insulin-mimic effect [11,12]. Oral administration of benzylamine (BZA), one of the SSAO substrates, could improve glucose tolerance and lower fasting blood glucose in high-fat diet mice [13]. In recent years, insulin administration was proven valid in reducing mortality of critical illness or sepsis [14]. The dose of insulin used that

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maintained blood glucose level lower than 110 mg/dL could prevent the incidence of MODS [14]. Lowering blood glucose and direct regulation of inflammatory activation process were thought to be involved in insulin-induced anti-inflammation effects [14,15]. Since SSAO substrates can mimic insulin effect, and BZA was recently reported to inhibit inducible nitric oxide synthase (iNOS) expression [16], we addressed the question of whether SSAO substrates BZA or methylamine (MA) would interfere with sepsis pathology induced by LPS. Therefore, the effects of BZA and MA on LPS-induced inflammatory response were examined both in vitro and in vivo. We demonstrated that BZA and MA were capable of inhibiting LPS-induced activation of murine peritoneal macrophages (PMs). Administration of BZA or MA mitigated LPS-induced over production of pro-inflammatory mediators and glucose metabolic disorders in vivo. These effects might be resulted from the metabolites of BZA or MA mediated by SSAO catalyzation.

2. Materials and methods

2.1. Materials

LPS from *Escherichia coli* (serotype 055:B5), benzylamine, methylamine, semicarbazide (SEM), clorgiline, 2,4-dinitro-phenyl-hydrazine (DNPH), hydrogen peroxide (3%), formaldehyde (FA) (37%), and benzaldehyde (BZ) were purchased from Sigma Chemical Company (St. Louis, Mo). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein (DCFH)–diacetate (DA), and BCA Protein Assay Reagent Kit were purchased from Beyotime Institute of Biotechnology (Shanhai, China). Rabbit polyclonal anti-SSAO antibodies and rabbit polyclonal anti-iNOS monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cyclooxygenase-2 (COX-2) monoclonal antibodies were from Cayman Chemical (USA). Rabbit anti-β-actin antibodies were from Biosynthesis Biotechnology (Beijing, China). Cy3-conjugated anti-rabbit IgG was from Beyotime Institute of Biotechnology. Goat anti-rabbit IgG secondary antibody linked to horseradish peroxidase was purchased from Jakson Immuno Research Laboratories (Dianova, Hamburg, Germany). Protease inhibitor cocktail and dimethyl sulfoxide (DMSO) were purchased from Sangon Biological (Shanghai, China). DMEM/F12 (1:1) and new born calf serum (NCS) were from Invitrogen (USA). Nitric Oxide Detection Kit and Glucose Detection Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse TNF-α Enzyme Immunoassay Kit was obtained from Boster Biological Technology (Wuhan, China). All other chemicals in this study were of analytical grade.

2.2. Cell culture and animals

Macrophages were isolated from the peritoneal cavity of BALB/c mice (22–25 g), as described previously [17]. Briefly, the cells were recovered by centrifugation at 200×*g* for 10 min, then were stored in freezing medium (DMEM/F12 (1:1, v/v), 20% NCS, and 10% DMSO) in liquid nitrogen. For further experiments, the cells were thawed and cultured in DMEM/F12 (1:1, v/v) with 10% NCS. After incubation for 1 h at 37 °C in a 5% CO_2 atmosphere, nonadherent cells were removed by extensive washing with PBS. The purity of the macrophages was assessed by Giemsa staining (>95%). Experiments were performed in the same medium without serum. RAW264.7 macrophage cell line was maintained in DMEM/F12 medium supplemented with 10% NCS.

The BALB/c mice (weight of 22–25 g) were obtained from Sino British SIPPR/BK Lab Animal Ltd. (Shanghai), and placed in cages and housed in a temperature-controlled room with a 12-h light, 12-h dark cycle. All the research was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, and was approved by the Medical Animal Care and Welfare Committee of Shantou University Medical College.

2.3. Immunofluorescence microscopy analysis of SSAO protein expression and HPLC analysis of SSAO activity

The presence of SSAO proteins was assessed by immunofluorescence cell staining: The cells were cultured on sterile glass cover slides, and then fixed at room temperature with 4% paraformaldehyde for 20 min. After washing with phosphate-buffered saline (PBS), the cells were permeabilized with 0.2% Triton X-100 for 10 min. Then unspecific binding was blocked with PBS containing 10% goat serum. The cells were then incubated with rabbit anti-SSAO antibodies at a 1:100 dilution overnight at 4 °C, washed extensively, and followed by staining with Cy3-conjugated anti-rabbit IgG at a 1:200 dilution for 1 h at room temperature. After further staining with 33258 Hoechst and final washing, the slides were mounted using 60% glycerol in PBS. Immunostained cells were observed and photographed using a Carl Zeiss microscope.

SSAO activity was determined using the high performance liquid chromatographic (HPLC) system as previously described [18]. Briefly, the cells were harvested, washed with PBS, and homogenized using an Ultrasonic Homogenizer (Sonics, USA). A 0.2-ml aliquot of homogenates was preincubated with clorgiline at room temperature for 30 min to inhibit the activity of monoamine oxidase (MAO), followed by incubation with benzylamine at 37 °C for 30 min. Benzaldehyde, the enzymatic reaction product, was derivatized with 2,4-dinitrophenyl-hydrazine (DNPH), and analyzed with HPLC and ultra-violet detection. SSAO activity was defined as benzaldehyde (nmol) formed per mg protein per hour.

2.4. MTT assay

BALB/c mice peritoneal macrophages (PMs) were seeded equivalently into 96-well microplates and incubated for 24 h. Then the cells were treated with 200 μl serum-free culture medium containing either BZA (0, 50, 100, 200, and 500 μM) or equal molar concentration of MA, or H_2O_2 (0, 2, 10, 20, and 50 μM), or equal molar concentration of FA or BZ, for 2, 6, and 12 h. Duplications were set up at every concentration and time points. After exposure, the cells were incubated with 0.5 mg/ml of MTT for 4 h. Following incubation, the solution was aspirated and 200 μl of DMSO was added, gently shaking for 10 min. Absorbance was recorded at 570 nm using a microplate reader (Thermo, USA). The cell viability was determined using the formula: cell viability (%) = [Absorbance(sample)/Absorbance(control)] × 100%, where Absorbance (sample) represented the reading from the treated cells and Absorbance (control) from the control.

2.5. Measurement of H_2O_2 levels

DCFH-DA was used as an indicator of intracellular or medium H_2O_2 levels [19]. Briefly, murine PMs were seeded at 2×10^6/well into 24-well tissue-culture plates beforehand. Then the cells were incubated for 1 h at dark in the presence of 15 μM DCFH-DA, with or without BZA, MA (0, 200, and 500 μM) and selective SSAO inhibitor semicarbazide. After incubation, the supernatant was collected, and the cells were then rinsed twice with PBS, and lysed in 0.1% v/v Triton X-100 in PBS. Fluorescence of culture medium and lysation at 485 nm excitation/530 nm emission wavelengths was recorded. Results were expressed as the percentage of fluorescence from control.

2.6. Cell treatments

Murine PMs or RAW264.7 cells were seeded at a density of 5.5×10^5/ml into 24-well tissue-culture plates, and allowed to attach to the bottom of the plates overnight and then serum-starved for 2 h,