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The therapeutic efficacy of glutamine for rats with smoking inhalation injury

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ABSTRACT

Smoke inhalation injury represents a major cause of mortality in burn patients and is associated with a high incidence of pulmonary complications. Glutamine (GLN) is considered a conditionally essential amino acid during critical illness and injury. However, whether GLN could attenuate lung injury caused by smoke inhalation is still unknown. The purpose of this study is to investigate whether GLN has a beneficial effect on smoke inhalation induced lung injury. In our present work, rats were equally randomized into three groups: Sham group (ambient air inhalation plus GLN treatment), Control group (smoke inhalation plus physiological saline) and GLN treatment group (smoke inhalation injury plus GLN treatment). At sampling, bronchoalveolar lavage fluid was performed to determine total protein concentration and pro-inflammatory cytokine levels. Lung tissues were collected for wet/dry ratio, histopathology, hydroxyproline and Western blotting measurement. Our results exhibited that GLN attenuated the lung histopathological alterations, improved pulmonary oxygenation, and mitigated pulmonary edema. At 28 days post-injury, GLN mitigated smoke inhalation-induced excessive collagen deposition as evidence by Masson–Goldner trichrome staining and hydroxyproline content. GLN mitigated smoke inhalation-induced lung inflammatory response, and further prevented the activity of NF-kappa-B. More importantly, results from Western blotting and Immunohistochemistry exhibited that GLN enhanced the expression of HSF-1, HSP-70 and HO-1 in lung tissues. Our data demonstrated that GLN protected rats against smoke inhalation-induced lung injury and its protective mechanism seems to involve in inhibition inflammatory response and enhancing HSP expression.

1. Introduction

Smoke inhalation injury is initiated by the uninhibited absorption of inhaled smoke and toxicants in the respiratory system, and is the leading cause of mortality from structural fires, as a result of complications such as, acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS) and chronic obstructive pulmonary disease (COPD) [1,2]. Smoke inhalation increases burn-related mortality by 20%, making the mortality of combined smoke/burn injury 30%–90% [2–5]. Over the past years, major advances have been made in the treatment of burns, but advances in smoke inhalation injury were limited [1,6].

Glutamine (GLN) is the most abundant amino acid in the body and considered a conditionally essential amino acid during critical illness and injury. GLN depletion occurs early during stays in the intensive care unit, and is a hallmark of critical illness [7,8]. Besides nutritional support, a surge of data revealed that GLN has the ability to regulate cellular immune functions and had a positive impact on clinical outcome of critically ill patients [9]. Furthermore, increasing experimental work exhibited that GLN supplementation was helpful for treatment of various diseases, including acute lung injury (ALI) induced by IR (ischemia reperfusion), endotoxin, hyperoxia and sepsis in animal models, but its effects on smoke inhalation-induced lung injury have not yet been studied [10–15].

Heat shock proteins (HSPs) are a group of self-protective proteins that contribute to cell survival after various forms of stress, including hyperthermia, oxidative stress, and toxin exposure [16,17]. The most abundant and well characterized of the inducible heat shock proteins is the 70-kDa family of HSP (HSP-70) [18]. GLN is known as a safe enhancer of HSP-70 [19]. In addition, once HSP-70 gene was knocked out, the protective effect of GLN disappeared [20]. In the present work, we made a hypothesis that GLN supplement could attenuate smoke inhalation-induced lung injury. Therefore, we built a rat model of smoke inhalation to investigate its protective effect and the underlying mechanism.

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2. Materials and methods

2.1. Animals and regents

Male Sprague–Dawley rats, weighing approximately 180 to 200 g, were purchased from Experimental Animal Center, Second Military Medical University (Shanghai, China). Those rats were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle and free access to food and water. Rats were acclimated for 3 days before the experiment. All animal procedures were processed in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication no. 96-01).

GLN and pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in physiological saline. Enzyme-linked immunosorbent assay (ELISA) kits of IL-8 were obtained from R&D Systems (Minneapolis, MN, USA). Total protein extraction kit was purchased from Cell Signaling Technology (Beverly, MA, USA). The following primary antibodies were used: anti-P-heat shock factor-1(HSF-1, Stressgen Biotechnologies, Victoria, BC, Canada), anti-HSP-70 (Cell Signaling Technology), anti-hemeoxygenase-1(HO-1, Cell Signaling Technology), anti-p-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IκB-β (Santa Cruz Biotechnology) and anti-β-actin (Sigma-Aldrich). The anti-rabbit and mouse secondary antibody was provided by Shanghai Sunteam Biotech, Inc. The ECL Chemiluminescence kit and bicinchoninic acid (BCA) protein assay were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). FragEL™ DNA Fragmentation Detection Kit was purchased from the Merck & Co. (Merck KGaA, Darmstadt, Germany).

2.2. Rat model of smoke inhalation

A rat model of smoke inhalation has been established by our department and smoke inhalation induced-lung injury was performed as previously described. Briefly, in our experiment, there were six rats, housed in a highly permeable metal cage, and were exposed each time. Smoke was generated by slowly smoldering wood shavings (120 g/kg body weight). The data presented in our manuscript are derived from rats exposed to successive 9 min periods of smoke separated into three times by 30 s exposures to ambient air [21].

2.3. Experimental design

In our present work, fifty-four rats were randomized into the following three groups (n = 18 in each group): (1) Sham group (ambient air inhalation plus GLN treatment, S group); (2) Control group (smoke inhalation plus physiological saline, C group); and (3) GLN treatment group (smoke inhalation plus GLN treatment, G group). GLN (750 mg/kg body weight) was administered via tail vein at 30 min post-smoke inhalation as well as subsequently every day before sacrifice. The dose of GLN used in the present study was selected on the basis of previous publications [12,13]. Rats in the Control group were treated with the same volume of physiological saline. In all of the three groups, rats were killed by an overdose of pentobarbital sodium (100 mg/body weight) to collect the lung and blood tissues at 12 h, 24 h and 28 days following smoke inhalation.

2.4. Arterial blood analysis

At 12 h post-smoke inhalation, about 1 ml arterial blood was obtained from the left carotid artery. Arterial partial pressure of oxygen (PaO_2), arterial partial pressure of carbon dioxide ($PaCO_2$) and pH were measured by a Blood Gas Analyzer (Premier 3000, Instrumentation Laboratory, USA).

2.5. BALF analysis

The separated left lung was slowly administered three sequential 1 ml of ice cold physiological saline via intra-tracheal route. About 2.7 ml of BALF was collected and centrifuged at 2000 rpm at 4 °C for 10 min and then, the supernatant was collected for total protein concentration and IL-8 measurement. IL-8 in the BALF was measured by commercially available ELISA kit according to the manufacturer's protocol. Protein concentration in the BALF was measured by BCA method to evaluate vascular permeability in the airways.

2.6. Lung wet-to-dry weight (W/D) ratio

The separated right upper lobe was collected to assess the lung W/D ratio. Each lung was blotted dry, weighed, and then dried in an oven at 60 °C for 48 h to obtain the "dry" weight. The lung W/D weight ratio was calculated to assess tissue edema.

2.7. Western blotting analysis

About 200 mg right middle lobe lung tissues were homogenized in 1 ml tissue protein extraction reagent. Homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatants were collected. Protein concentration of the supernatants was determined by BCA protein assay kit. Protein extracts (60 μg per lane) were run on 10% SDS-PAGE gels, and then transferred to polyvinylidene fluoride membranes and probed with primary antibodies against p-HSF-1, HSP-70, HO-1, p-NF-κB p65, IκB-β and β-actin, respectively. Membranes were then incubated with the horseradish peroxidase-tagged anti-rabbit or mouse secondary antibody and visualized with the enhanced chemiluminescence reagent followed by autoradiography. All blots were normalized against β-actin to control for protein loading.

2.8. Lung histological assessment

The right lower lobe lung samples were fixed by complete immersion in 4% polyformalin for 24 h, embedded in paraffin, sectioned to 6 μm thick, and stained with hematoxylin and eosin (HE) or Masson–Goldner trichrome. Lung tissue pathological changes were observed under a light microscope and also evaluated morphologically by scoring histology specimens in a blinded fashion. Hyperemia, atelectasis and neutrophil infiltration were scored as: 0 = minimal; 1 = mild; 2 = moderate; 3 = severe; and 4 = maximal. Intra-alveolar edema was scored as: 0 = absent; and 1 = present.

2.9. Immunohistochemistry for HSP-70 and HO-1

Immunohistochemical analysis was carried out as previously described [22]. Briefly, the sections of lung tissues were deparaffinized and incubated with anti-rat-HSP-70 and HO-1 antibody overnight at 4 °C. The sections were washed with phosphate buffered saline (pH = 7.4), and then incubated with anti-rabbit-IgG. Secondary labeling was achieved by using biotinylated rabbit anti-rat antibody. Horseradish peroxidase-conjugated avidin and brown-colored diaminobenzidine were used to visualize the labeling. Finally, the slides were counterstained with hematoxylin and reviewed by pathologists blinded to the treatment groups.

2.10. Hydroxyproline measurement

Hydroxyproline was determined using the method described by Inayama et al. [23]. Briefly, tissue samples were homogenized in 0.9% physiological saline. The assay was performed using 20 mg of the lyophilized sample subjected to alkaline hydrolysis in 300 μl of H_2O plus 75 μl of 10 mmol/l NaOH at 120 °C for 20 min. An aliquot of 50 μl of the hydrolyzed tissue was added to 450 μl of chloramine