Biomedicine & Pharmacotherapy 84 (2016) 51–59

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Anti-fatigue effect by active dipeptides of fermented porcine placenta through inhibiting the inflammatory and oxidative reactions

Sun-Young Nam[a], Hyung-Min Kim[a,*], Hyun-Ja Jeong[b,*]

[a] Department of Pharmacology, College of Korean Medicine, Kyung Hee University, Seoul, 02447, Republic of Korea
[b] Department of Food Technology and Inflammatory Disease Research Center, Hoseo University, Asan, Chungnam, 31499, Republic of Korea

ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 26 August 2016
Accepted 6 September 2016

Keywords:
Glycine-leucine
Inflammatory cytokine
Superoxide dismutase
Nitric oxide
Forced swimming test

ABSTRACT

Background: Recently, we reported an anti-fatigue effect of fermented porcine placenta (FPP). Glycine-leucine (GL) and leucine-glycine (LG) dipeptides are main peptides of FPP. However, the therapeutic effects and underlying mechanisms of GL and LG dipeptides on fatigue are still unclear.
Methods: Herein, we examined the anti-fatigue properties of GL and LG dipeptides using RAW264.7 macrophages and forced swimming test (FST) animal model.
Results: Our data revealed that lipopolysaccharide (LPS)-induced interleukin (IL)-1β, tumor necrosis factor-α, and IL-6 productions were markedly inhibited by GL or LG in RAW264.7 macrophages without inducing cytotoxicity. LPS-enhanced nitric oxide (NO) production and inducible nitric oxide synthase expression were inhibited by GL or LG, whereas superoxide dismutase (SOD) activities were significantly enhanced by GL or LG in LPS-stimulated RAW264.7 macrophages. The present study also figured out that these effects of GL and LG were mediated by blockade of caspase-1 and nuclear factor-κB activation. In FST-induced fatigue mouse model, the mice which received the GL or LG for 21 days showed significant decreases of IL-1β, IL-6, and NO serum levels. Treatment with GL or LG significantly enhanced levels of SOD and glycogen and significantly lowered levels of lactate dehydrogenase, aspartate transaminase, and alanine transaminase.
Conclusion: Taken together, our results indicated that GL and LG dipeptides, active components of FPP, should be considered as candidate of anti-fatigue agents.

1. Introduction

Fatigue is a ubiquitous physiological process, which involves a significant decline in ability for work, vitality, and activities. Fatigue is an acquired multisystem disease occurred from widespread inflammation, neuropathology, and immune system dysfunction [1,2]. Therefore, fatigue is often associated with inflammatory diseases such as cancer, anemia, and chronic fatigue syndrome (CFS) [3,4]. The forced swimming test (FST) has been used to investigate whether certain agents have anti-fatigue effect in various fatigue models, including exercise-induced fatigue and CFS [5,6].

The etiology of fatigue remains to be elucidated; however, diverse proinflammatory cytokines are known to be associated with the severity of fatigue [7]. Earlier studies have found a number of proinflammatory cytokines in patients with CFS [8]. There is also evidence that interleukin (IL)-6 contribute to the symptoms of fatigue in healthy volunteers and patients diagnosed with CFS [9].

Another potential cause of fatigue is oxidative stress which is generated by an imbalance the activities of reactive oxygen species (ROS) and antioxidant molecules. Excessive ROS production results in the oxidative damage to a variety range of biological molecules including proteins, nucleic acid, lipids and the diverse pathological states [10]. CFS patients display an increased oxidative stress, coupled to increased secretion of ROS, as well as lipid peroxidation and glutathione oxidation [11,12]. Raised oxidative stress levels were observed following exercise in patients with CFS [13]. Oxidative imbalance in skeletal muscle resulted in increased muscle fatigability [14]. Thus, antioxidants can be used to alleviate fatigue by counteracting oxidative stress. Superoxide dismutase (SOD) is a powerful antioxidant enzyme and plays a significant role in cleaning ROS. The presence of a large amount of SOD in the body results in the reduction free radical damage to cells, delay fatigue, and accelerate recovery after a fatigue stage [15].

Macrophages are a main source of inflammatory mediators. Excessive secretion of these mediators contributes to the

* Corresponding authors.
E-mail addresses: hmkim@khu.ac.kr (H.-M. Kim), hjjeong@hoseo.edu (H.-J. Jeong).

http://dx.doi.org/10.1016/j.biopha.2016.09.012
0753-3322/

pathogenesis of multiple chronic diseases including fatigue, inflammatory arthritis, asthma, endotoxin shock, cancer, and atherosclerosis [16,17]. In addition, macrophages-derived inflammatory mediators have been related to sleep disorders including sleep apnea syndrome, narcolepsy, idiopathic hypersomnia, and chronic fatigue syndrome [16]. A study by Carmichael et al. reported that brain macrophages depletion via clodronate-filled liposomes attenuated the elevated brain IL-1β response and delayed fatigue [18].

Dipeptides are comprised of two amino acids. Anti-inflammatory activity of neuropeptide FF has been reported in numerous studies [19]. Furthermore, previous studies have shown that carnosine (alanine and histidine dipeptide) can help to delay the fatigue via their antioxidant activity [20,21]. However, there is only limited available information regarding the detailed mechanisms by which dipeptides exert anti-fatigue properties. Recently, we reported an anti-fatigue effect of fermented porcine placenta (FPP). Glycine-leucine (GL) and leucine-glycine (LG) dipeptides are the main peptides in FPP [22].

Inflammatory mediators are tightly connected with fatigue [7]. Therefore, biological agents targeting inflammatory mediators have potential therapeutic advantages in treating fatigue. Thus, we investigated the anti-fatigue effects of GL and LG dipeptides on the levels of inflammatory mediators using RAW264.7 macrophages *in vitro* and FST animal *in vivo* model.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and sodium nitrite were purchased from Sigma Chemical Co. (St Louis, MO, USA). Murine recombinant (r)TNF-α, rIL-1β, and rIL-6, anti-mouse TNF-α, IL-1β, and IL-6, and biotinylated mouse TNF-α, IL-1β, and IL-6, and were purchased from R&D Systems (Minneapolis, Minnesota, USA). Antibodies of caspase-1, inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB), phosphorylated (p)IκB-α, and GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, New York, USA).

2.2. Dipeptides analysis

FPP (Fermented Placenta Extract Powder A (K)) was purchased from HORUS co., Ltd. (Japan). Dipeptides were analyzed by using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). FPP contains GL (240–430 ppm), LG (110–240 ppm), and a lot of amino acids. We investigated the anti-fatigue effect of FPP and its main dipeptides, GL and LG. LG and GL were purchased from Bachem AG (Bubendorf, Swizerland). FPP, GL, and LG was dissolved in distilled water (DW) and prepared at a dose of 10 μg/ml according to previous report [23].

2.3. Cell culture

RAW264.7 macrophages were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat inactivated FBS at 37 °C, 5% CO_2 and 95% humidity. FPP, GL, and LG were diluted with DMEM containing 10% FBS. Cells were pretreated with GL (0.1, 1, and 10 μg/ml), LG (0.1, 1, and 10 μg/ml), or FPP (10 μg/ml) for 1 h prior to LPS stimulation.

2.4. Assay of cytokine release

RAW264.7 macrophages (1×10^5 cells/well) were pretreated with GL (0.1, 1, and 10 μg/ml), LG (0.1, 1, and 10 μg/ml), or FPP (10 μg/ml) for 1 h and then stimulated with LPS (10 μg/ml) for 24 h. The amounts of TNF-α, IL-1β, and IL-6 secreted from RAW264.7 macrophages were measured by a modified enzyme-linked immunosorbent assay (ELISA). The ELISA was devised by coating 96-well plates of murine monoclonal antibodies with specificity for TNF-α, IL-1β, and IL-6. Before use and between subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. The r TNF-α, rIL-1β, and rIL-6 were diluted and used as a standard. Serial dilutions starting from 10 ng/ml were used to establish the standard curve. After 2 h incubation at 37 °C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-mouse TNF-α, IL-1β, and IL-6 were added and the plates were incubated at 37 °C for 2 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 30 min at 37 °C. Wells were again washed and substrate solution was added. Color development was measured at 405 nm using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA).

2.5. MTT assay

RAW264.7 macrophages (1×10^5 cells/well) were cultured for 24 h with GL (0.1, 1, and 10 μg/ml), LG (0.1, 1, and 10 μg/ml), or FPP (10 μg/ml). Cell aliquots were incubated with 20 μl of a MTT solution (5 mg/ml) for 4 h at 37 °C under 5% CO_2 and 95% air. Consecutively, 250 μl of DMSO was added to extract the MTT formazan and the absorbance of each well was read using an ELISA reader at 540 nm (Molecular Devices Corp., Sunnyvale, California, USA).

2.6. Measurement of nitrite concentration

RAW264.7 macrophages (1×10^5 cells/well) were pretreated with GL (0.1, 1, and 10 μg/ml), LG (0.1, 1, and 10 μg/ml), or FPP (10 μg/ml) for 1 h and then stimulated with LPS (10 μg/ml) for 48 h. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined using an ELISA reader (Molecular Devices Corp., Sunnyvale, California, USA). NO_2^- was determined by using sodium nitrite as a standard.

2.7. Preparation of nuclear and cytosolic extracts

Briefly, after activating the cells for the times indicated, we washed cells in ice-cold phosphate-buffered saline and centrifuged them at 15,000 × *g* for 1 min. We then resuspended them in 40 μl of a cold hypotonic buffer (10 mM Hepes/KOH, 2 mM MgCl2, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). Next, we allowed the cells to swell on ice for 15 min; we lysed them gently with 2.5 μl of 10% Nonidet P-40, and we centrifuged them at 15,000 × *g* for 3 min at 4 °C. The supernatant was aliquots (cytosolic protein) and the pellets were gently resuspended in 40 μl of cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF pH 7.9) and then left on ice for 20 min. After conducting the centrifugation (15,000 × *g* for 15 min at 4 °C), we froze the aliquots of supernatant containing the nuclear proteins in liquid nitrogen and stored them at −80 °C until ready for analysis. Finally, we used