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Pilose antler peptide attenuates LPS-induced inflammatory reaction

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ABSTRACT

The present study was designed to study the effects of pilose antler peptide (PAP) on primary culture of nucleus pulposus cells in intervertebral disc. We demonstrated that PAP significantly inhibited lipopolysaccharides (LPS) induced over-production of inflammatory factors including interleukin-1β (IL-1β), tumor necrosis Factor-α (TNF-α) and interleukin-6 (IL-6) in nucleus pulposus cells. PAP also attenuated increase of malondialdehyde (MDA) and decrease of superoxide dismutase (SOD) induced by LPS challenge in a concentration-dependent manner. Moreover, the expression of the protein levels of mitogen-activated protein kinase (MAPK)/nuclear transcription factor-κB(NF-κB) were increased accompanying with the LPS challenge, which were significantly reversed after PAP treatment. Our results demonstrated the ability of PAP to antagonize LPS-mediated inflammation in primary culture of nucleus pulposus in intervertebral disc, suggesting a beneficial potential for its clinical application.

1. Introduction

Intervertebral disc is consisted of nucleus pulposus, annulus fibrosus and cartilage endplates, which is the main structure to provide stability and flexibility to the spinal column [1,2]. However, intervertebral disc degeneration, when comes to the condition of cell viability decrease, attenuation of type II collagen and proteoglycan synthesis, and dehydration of nucleus pulposus, has been considered as an irreversible process [3]. Also, intervertebral disc degeneration is a significant contributor to the development of low back pain and other spinal degenerative diseases [4]. Although the etiology of intervertebral disc degeneration is still obscure, a genetic component and abnormalities of inflammation and oxidative stress are strongly suggested.

Inflammation has been suggested to be closely involved with the pathophysiological process of intervertebral disc degeneration [5]. The inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), play an important role in intervertebral disc degeneration [6]. The elevated inflammatory factors are a stimulant of the inflammatory cascade. The inflammatory factors, instead of directly degrading the intervertebral disc, promote the production of inflammatory substances by the disc cells and accelerate intervertebral disc degeneration [7]. Suppression of inflammatory cytokines showed beneficial effects in preventing the intervertebral disc degeneration [8]. Restriction of interleukin-1β (IL-1β) was found to inhibit NF-κB signaling or deacetylate the transcription factors, which promotes extracellular matrix repair and defends against disc regeneration. [9]

Oxidation and reduction processes are also closely associated with the progression of intervertebral disc degeneration. Oxidative stress has been suggested to inhibit the proliferation, stimulate premature senescence and lead to a catabolic phenotype in human nucleus pulposus intervertebral disc cells [10]. The antioxidative nanofullerol was reported to prevent intervertebral disc degeneration [11,12].

Deer antlers, namely“lu rong” in China, “nokyong” in Korea or “tokujo” in Japan, were widely used folk medicines in Asia. These soft growing tissues were applied in traditional Chinese medicine for strengthening kidney, nursing the blood treating neurosis and prolonging life. Deer antlers have been reported to exert a variety of properties, such as anti-inflammatory, anti-stress and anti-aging effects in previous research. Pilose antler peptide (PAP: MW: 7200; amino acid residue: 68) is isolated from the deer antlers and has been shown beneficial effects on chronic inflammatory and oxidative damages [13–15]. However, the role of PAP during intervertebral disc degeneration have not been investigated before. The

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underlying mechanisms of its actions are still obscure. The present study was designed to study the effects of PAP on LPS-induced primary culture of nucleus pulposus in intervertebral disc and explore the underlying mechanisms.

2. Materials and methods

2.1. Reagents and kits

LPS was purchased from Sigma (St. Louis, MO, USA) and applied as an inflammatory stimulant. The drug, PAP was obtained from Changchun University of Chinese Medicine. Enzyme-linked immunosorbent assay Enzyme-linked immunosorbent assay (ELISA) kits of cytokines IL-1β, IL-6 and TNF-α were purchased from eBioscience. CO., LTD. Primary antibodies a were got from Cell Signaling Technology (Danvers, USA).

2.2. Nnucleus pulposus cells isolation and culture

The nucleus pulposus cells were extracted and isolated from the lumbar spines of Sprague Dawley rats (6–8 weeks). The spines was separated between the lumbar discs, and the nucleus pulposus were totally separated by a sterile scalpel blade. Next, the nucleus pulposus was washed with PBS and digested with trypsin and collagenase. The culture of nucleus pulposus cells were incubated in complete medium, consisting of DMEM/F12 (Life Technologies, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS; Life Technologies) with penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 °C and 5% CO_2.

2.3. Cell viability assay

To evaluate the potential cytotoxic effects of PAP, cell viability assay was performed by cell counting kit-8 (CCK-8). The nucleus pulposus cells were plated in 96-well plates with the density at 5×10^3 per well. The nucleus pulposus cells were incubated with moracin at concentrations (1, 4, 8, 16, 32, 64, 128 uM) for 24 h, then 10 ml CCK-8 solution was added for incubation another 2 h. The optical density was measured at 450 nm with the culture medium as a blank.

2.4. Cell culture and treatments

The nucleus pulposus cells were randomly divided into the following groups: (1) control group; (2) LPS(5 mg/kg); (3) LPS group treated with 4 μM PAP; (4) LPS group treated with 8 μM PAP; (5) LPS group treated with 16 μM PAP; (6) LPS group treated with 16 μM PAP and the specific p38 MAPK(SB203580 (10.0 μM). for investigating the roles of the p38 MAPK pathway in LPS-induced injury rescued by PAP, the specific p38 MAPK, SB203580 (10.0 μM), was added for 30 min prior to treatment with PAP and LPS stimulation.

2.5. Determination of inflammatory cytokines in cell supernatant

The concentrations of inflammatory cytokines IL-1β, IL-6, TNF-α in cell supernatant were recorded by an enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions (Beyotime Biotechnology). The results were expressed as pictograms per milligram protein.

2.6. Determination of SOD and MDA in cell supernatant

The level of superoxide dismutase (SOD) and malondialdehyde (MDA) in cell supernatant were recorded using the commercial kits on the basis of the manufacturer's instruction (Beyotime Biotechnology).

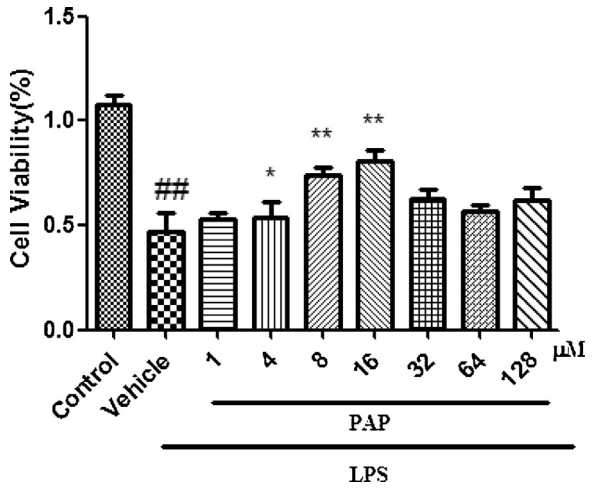

Fig. 1. Effects of PAP on cell viability.
*The values are presented as the mean ± standard deviation. *$P < 0.05$compared to the LPS group; **$P < 0.01$ compared to the LPS group; ##$P < 0.01$ compared to the control group.

2.7. Western blot

The primary culture of nucleus pulposus in intervertebral disc were homogenized in RIPA buffer with 0.1% phenylmethylsulfonyl fluoride. The total protein content was quantified and equal amounts of protein were loaded on 8% to 12% SDS-polyacrylamide gel electrophoresis. The transferred PVDF membranes from SDS-polyacrylamide gel electrophoresis were blocked in skim milk then incubated with the appropriate concentration of specific antibodies overnight. On the second day, PVDF membranes were incubated with second antibody at room temperature for 1 h after washing three times by TBST. The immunoreactive bands were interacted with an enhanced chemiluminescence (ECL) kit and visualized on a gel imaging system (Tanon Science & Technology Co., Ltd., China).

2.8. Statistical analyses

Results were presented as mean ± S.E.M. Multiple comparisons were performed by analysis of variance (ANOVA) with Newman–Keuls post hoc test by SPSS 17.0 (SPSS Inc., USA). A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of PAP on cell viability

To study the cell viability of PAP on LPS induced primary nucleus pulposus cells, we cultured nucleus pulposus cells with different concentrations of PAP (1, 4, 8, 16, 32, 64, 128 μM) for 24 h with or without LPS by CCK-8 assay. As shown in Fig. 1, PAP increased cell viability at concentrations of 4–16 μM. Thus, we chose PAP at 4, 8, 16 μM for the following experiments (Fig. 1).

Effects of PAP on inflammatory cytokines in LPS-induced primary nucleus pulposus cells

The nucleus pulposus cell cultures were incubated with moracin (4, 8, 16 μM) and stimulated with 10 mg/ml LPS, followed by an ELISA to measure the protein levels of IL-1β, IL-6 and TNF-α. As shown in our results, in the supernatant of primary cell cultures, the levels of inflammatory cytokines were significantly increased in