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Ghrelin prevents articular cartilage matrix destruction in human chondrocytes

Jie liu[a,*,1], Lie Cao[b,1], Xueming Gao[a], Zhixin Chen[a], Shifang Guo[a], Zongru He[a], Yaowen Qian[a], Yongzhi Yu[a], Gang Wang[c]

[a] Orthopaedic Department, People's Hospital of Gansu Provincial, Lanzhou, 730000, China
[b] Pediatrical Department of Gansu Provincial Maternity and Child-Care Hospital, Lanzhou, 730000, China
[c] Traditional Chinese Hospital of Gansu Gaotai County, Lanzhou, 730000, China

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis worldwide. Excessive production of pro-inflammatory cytokines such as interleukin-1β (IL-1β) plays a key role in the pathogenesis of OA. OA is generally characterized by degradation of extracellular matrixes such as type II collagen and aggrecans mediated by matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Ghrelin is a secreted peptide hormone regulating appetite and the distribution and rate of use of energy. However, the physiological and pharmacological roles of Ghrelin on the pathological progression of OA haven't been reported before. In the current study, our results indicate that Ghrelin reduced IL-1β-induced expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in a concentration-dependent manner. Notably, Ghrelin ameliorated IL-1β-induced degradation of type II collagen and aggrecan. Mechanistically, Ghrelin is able to inhibit the expression of IRF-1 mediated by inactivating the JAK2/STAT3 pathway. However, Ghrelin didn't have any impact on IL-1β induced activation of p38. Taken together, our findings identify a novel function of Ghrelin on inhibiting the degradation of type II collagen and aggrecan.

1. Introduction

As the most common form of arthritis worldwide, osteoarthritis (OA) is a major cause of pain and disability in elderly people [1]. Several studies aimed to identify the mechanisms underlying articular cartilage changes associated with OA, however, the pathogenesis of OA remains unclear [2]. Activation of degradative enzymes leads to the loss and degradation of proteoglycans and collagen in articular cartilage [3]. Increasing evidence has revealed that the matrix metalloproteinases (MMPs) play a pivotal role in the pathological development of OA [4,5]. Among the various MMPs, MMP-13 is the collagenase, which directly cleaves type II collagen, and MMP-3 is a stromelysin which degrades proteoglycans and activates procollagenase in articular cartilage [6,7]. Both MMP-3 and MMP-13 have been reported to be up-regulated in the synovium and the surface of cartilage in OA patients [8,4]. The disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family are essential aggrecanases contribute to proteoglycan/aggrecan depletion and are associated with cartilage degradation during OA. Among them, ADAMTS4 and 5 were identified as the major aggrecanases during OA development [9,10]. The development of therapies targeting these enzymes has been considered as a strategy to decelerate articular cartilage degradation.

Ghrelin, a 28 amino-acid secreted peptide hormone [11], provides a neuroendocrine link between the gut and the brain, modulating metabolism in response to nutrient availability [12,13]. Increasing evidence has shown that ghrelin has a multiplicity of physiological functions [14]. Ghrelin has displayed its anti-oxidant and anti-inflammatory property in different types of tissues and cells. A recent study demonstrate that ghrelin inhibits the oxidized low-density lipoprotein (oxLDL)-induced inflammatory response and decreases reactive oxygen species (ROS) generation through the accumulation of uncoupling protein 2 (UCP2) in human umbilical vein endothelial cells (HUVECs) [15]. In addition, ghrelin suppresses TNF-α-activated endothelial inflammation and monocyte adhesion through regulating the AMPK/NF-κB signaling pathway [16]. Ghrelin knockout mice showed suppression of the cholinergic anti-inflammatory pathway, as indicated by reduced parasympathetic nerve activity and higher plasma interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels, suggesting that endogenous ghrelin

* Corresponding author at: No. 224 West road, Lanzhou, Orthopaedic Department, People's Hospital of Gansu Provincial, Lanzhou, Gansu Province, 730000, China.
E-mail address: liujie1698@yeah.net (J. liu).
[1] Equal contribution.

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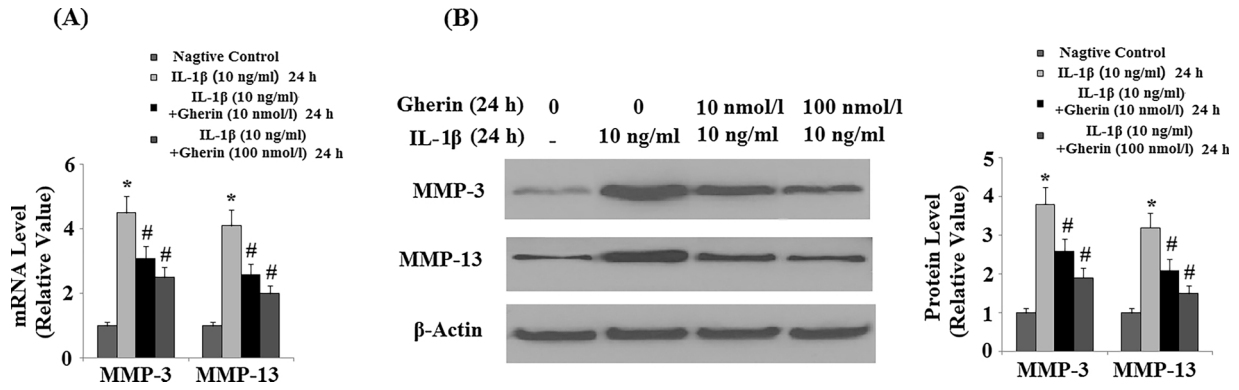

Fig. 1. Ghrelin suppresses the expression of MMP-3 and MMP-13 induced by IL-1β (10 ng/ml). Human primary chondrocytes were treated with IL-1β (10 ng/ml) in the absence or presence of Ghrelin (10 nmol/l and 100 nmol/l) for 24 h. (A), mRNA levels of MMP-3 and MMP-13 were determined by real-time PCR; (B), Protein levels of MMP-3 and MMP-13 were measured by western blot analysis (*, P < .001 vs. non-treatment group; #, P < .001 vs. IL-1β treatment group, ANOVA, n = 5–6).

has been shown to exert a beneficial effect on cardiac dysfunction and postinfarction remodeling [17]. These findings of anti-inflammatory properties suggest that ghrelin might have a potential therapeutic effect in OA.

2. Materials and methods

2.1. Cell culture

Human primary chondrocytes obtained from Lonza (Walkersville, USA) were cultured in CGM™ chondrocyte growth medium with SingleQuot Kit diluted according to manufacturer's instructions. 90% confluence cells were used for all experiments unless special noted. Cells were treated with 10 ng/ml IL-1β [18–20] with or without Ghrelin at different concentrations (10 nmol/l and 100 nmol/l) for 24 h. Primary chondrocytes were treated with the JAK2 inhibitor AG490 (10 μM) diluted in dimethyl sulphoxide (DMSO) and same volume DMSO was used in control group without AG490.

2.2. RNA isolation and real-time PCR

After necessary treatment, human primary chondrocytes were washed with DPBS for twice briefly before RNA isolation. Total RNA from human primary chondrocytes was isolated by using Trizol (Thermo Fisher, USA) in accordance with the protocol from the manufactory. RNA quantity was measured by using NanoDrop (Thermo Fisher, USA). 2 μg of total RNA was used to synthesize cDNA by using a reverse transcription PCR kit (Bio-rad, USA). Expression levels of genes were quantified by real-time PCR with a high-performance real-time PCR on LightCycler systems (Roche, Switzerland). The relative level of target mRNA level was normalized to the level of GAPDH using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blot analysis

Protein was isolated from human primary chondrocytes by using RIPA lysis and extraction buffer (Thermo Fisher, USA). Equal amount of total protein (20 μg) was loaded per sample on 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad, USA). Separated proteins were transferred onto high quality polyvinylidene difluoride (PVDF) membranes (Life technologies, USA). The membranes were then washed twice with TBST followed by being blocked for 1 h at room temperature using 10% fat-free dry milk diluted in TBST buffer. The membranes were sequentially incubated with specific primary antibodies overnight in cold room and appropriate peroxidase-conjugated secondary antibodies for 1 h at RT. Blots were developed with an ECL chemiluminescence system according to the manufacturer's instructions.

2.4. Immunofluorescence staining

Human primary chondrocytes were cultured on sterile glasses with necessary treatment. Cells were washed briefly with PBS twice before fixing with 10% formalin in PBS for 15 min. Cells were then blocked with 2.5% BSA diluted in TBST for 1 h at room temperature and incubated with anti-IRF1 antibody (1:500, Abcam, USA) diluted in TBST buffer with 2.5% BSA, 0.5% triton X-100 overnight at 4 °C. After washing twice with TBST, the cells were then incubated with AlexaFluor®594 Goat anti-mouse secondary antibody (1:200, Abcam, USA) for 1 h in room temperature. Images were captured by using a fluorescent microscope (Leica, Germany) and analyzed using the Image J software (National Institute of Health, USA).

2.5. Statistical analysis

All experimental data are presented as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences between means were considered significant at $p < .05$.

3. Result

Degradation of cartilage matrix induced by proinflammatory cytokines such as IL-1β is mediated by MMPs, aggrecanases, and other catabolic enzymes. Among all MMPs, MMP-3 and MMP-13 are the two most important enzymes, which directly and indirectly degrade extracellular matrix. Therefore, we first intended to find out whether Ghrelin regulated the expression of MMP-3 and MMP-13. Human primary chondrocytes were treated with IL-1β (10 ng/ml) in the absence or presence of Ghrelin infusion at 10 nmol/l and 100 nmol/l for 24 h. As expected, our results indicate that Ghrelin inhibited the increase in expression of MMP-3 and MMP-13 induced by IL-1β at both the mRNA level measured by real time PCR (Fig. 1A) and the protein level measured by western blot analysis (Fig. 1B).

As we all known, degradation of aggrecan is another important pathological process in OA and is mainly regulated ADAMTS-4 and ADAMTS-5. Hence, we next evaluated the effect of Ghrelin on the expression of ADAMTS-4 and ADAMTS-5. Interestingly, our results demonstrate that Ghrelin also inhibited the expression of ADAMTS-4 and ADAMTS-5 at both the mRNA level (Fig. 2A) and the protein level (Fig. 2B) induced by IL-1β.

The effect of Ghrelin on the expression of type II collagen and aggrecan is unknown, so we investigated the expression level of type II collagen and aggrecan. Our results indicate that Ghrelin ameliorated IL-1β-induced degradation of both type II collagen and aggrecan measured by western blot analysis (Fig. 3).

Increasing evidences have revealed that transcription of MMP-3 and