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Ghrelin promotes human non-small cell lung cancer A549 cell proliferation through PI3K/Akt/mTOR/P70S6K and ERK signaling pathways

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ABSTRACT

Ghrelin is a gastric acyl-peptide that plays an important role in cell proliferation. In the present study, we explored the role of ghrelin in A549 cell proliferation and the possible molecular mechanisms. We found that ghrelin promotes A549 cell proliferation, knockdown of the growth hormone secretagogue receptor (GHSR) attenuated A549 cell proliferation caused by ghrelin. Ghrelin induced the rapid phosphorylation of phosphatidylinositol 3-kinase (PI3K), Akt, ERK, mammalian target of rapamycin (mTOR) and P70S6K. PI3K inhibitor (LY 294002), ERK inhibitor (PD98059) and mTOR inhibitor (Rapamycin) inhibited ghrelin-induced A549 cell proliferation. Moreover, GHSR siRNA inhibited phosphorylation of PI3K, Akt, ERK, mTOR and P70S6K induced by ghrelin. Akt and mTOR/P70S6K phosphorylation was inhibited by LY 294002 but not by PD98059. These results indicate that ghrelin promotes A549 cell proliferation *via* GHSR-dependent PI3K/Akt/mTOR/P70S6K and ERK signaling pathways.

1. Introduction

Ghrelin is a 28 amino acid peptide which has multiple physiological functions including hormonal secretion, regulation of energy homeostasis and modulation [1]. Recent studies have demonstrated that ghrelin participates in many physiological processes related to tumor progression that include proliferation, apoptosis, metastasis [2–4]. Many cancer studies have reported expression of ghrelin and its receptor (GHSR) in various tumors. These findings suggest that ghrelin might play a critical role in cancer progression.

Emerging studies have investigated the potential role of ghrelin in tumor cell proliferation. Ghrelin has different effects on cancer cell proliferation or anti-apoptosis among various cancer cell types. It has been reported that ghrelin promotes colon cancer cell proliferation [5], other researches reported that ghrelin participates in inducing pancreatic adenocarcinoma cells and human colon cancer cell proliferation [6–8]. Ghrelin protects against A549 cell apoptosis induced by LPS through activating the PI3K/Akt and ERK pathways [9].

However, studies on the functional role of the ghrelin in the regulation of cancer cell proliferation are controversial. Several studies have reported that ghrelin may inhibit cancer cell proliferation. Ghrelin inhibited PC-3 cells proliferation through regulating the expression of T-type $Ca^{(2+)}$ channel [10]. It also reported that ghrelin induced a dose-dependent inhibition on the H345 cell proliferation and increased apoptosis [11]. A study has reported that ghrelin promotes cell proliferation in a low dose treatment, whereas high dose treatment of ghrelin leads to a decrease in cell growth [12].

Ghrelin activates multiple signaling pathways, a previous study reported that ghrelin induced the IEC-6 cells proliferation through ERK1/2 phosphorylation [13]. Ghrelin has been reported to activate EGFR and PI3K/Akt signaling pathway through binding to GHSR in Caco-2 cells [8]. The molecular mechanisms underlying the effects of ghrelin on tumor cell proliferation is still unclear. In the present study, we investigate the effects of ghrelin on the proliferation of human non-small cell lung cancer A549 cell and the involved mechanism.

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2. Materials and methods

2.1. Materials

Ghrelin was purchased from Phoenix Pharmaceutical (USA). A BrdU assay kit was obtained from Merck (Darmstadt, Germany). Cell culture reagents and a transfection kit were acquired from Gibco (Invitrogen, NY, USA). Rapamycin and LY 294002 and anti-rabbit IgG peroxidase were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies of PI3K, phospho-PI3K Akt, phospho-Akt, mTOR, phospho-mTOR, p70S6K, phospho-p70S6K, ERK, phospho-ERK and PD98059 were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

The lung adenocarcinoma cell line A549 cell was purchased from the China Center for Type Culture Collection (Wuhan, China). A549 cells were cultured in flasks in RPMI growth medium supplemented with 5% FBS, 100 U/ml of penicillin, and 100 pg/ml of streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. BrdU cell proliferation analysis

A549 cells were seeded into 96-well plates at density of 5×10^3 cells per well and were serum starved for 24 h before ghrelin treatment. In order to investigate the mechanism by which ghrelin influenced proliferation, cells were transfected with GHSR siRNA or control si RNA, or cells were treated with different specific inhibitors of PI3K, ERK or mTOR as indicated below. After culture with ghrelin for 48 h, 10 mM BrdU was added to the culture medium for incorporation into the DNA of replicating cells. The integrated BrdU was detected by a BrdU assay kit according to the manufacturer's instructions.

2.4. Cell transfection

For downregulation of GHSR, A549 cells were transduced with GHSR siRNA, or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol.

2.5. Western blot analysis

Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 mg/ml aprotinin. Cell lysates were separated by 8%–12% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in 5% nonfat dry milk for 1 h and incubated overnight at 4 °C with the primary antibodies. Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology). The bands were visualized using a ChemicDoc XRS system (Bio-Rad) and quantified using Quantity One imaging software (Bio-Rad).

2.6. Statistical analyses

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's *t*-test. The difference was significant if the p value was <0.05.

3. Results

3.1. Ghrelin promotes A549 cell proliferation

Ghrelin was reported to participate in promoting the proliferation of tumor cell. In this study, we found that ghrelin promoted A549 cell proliferation in dose-dependent manner, with a maximal response after stimulation with 5 nM ghrelin (Fig. 1A). As formerly mentioned, ghrelin exerts it's biological effects through binding to GHSR. To investigate whether GHSR is implicated in ghrelin-induced A549 cell proliferation, we down-regulated GHSR expression with GHSR siRNA. Western blot analysis showed that the protein level of GHSR was suppressed by GHSR siRNA (Fig. 1B). Knockdown of GHSR attenuated the proliferation of A549 cell induced by ghrelin (Fig. 1C). These results indicate that GHSR participates in ghrelin-induced A549 cell proliferation.

3.2. PI3K/Akt and ERK pathways are involved in ghrelin-induced A549 cell proliferation

Ghrelin activates multiple signal transduction pathways, including ERK1/2, PI3K/Akt, and Jak2/STAT3 pathways [5,8,14]. We investigated the phosphorylation of PI3K/Akt and ERK by ghrelin treatment in A549 cell. Western blot analysis showed that ghrelin

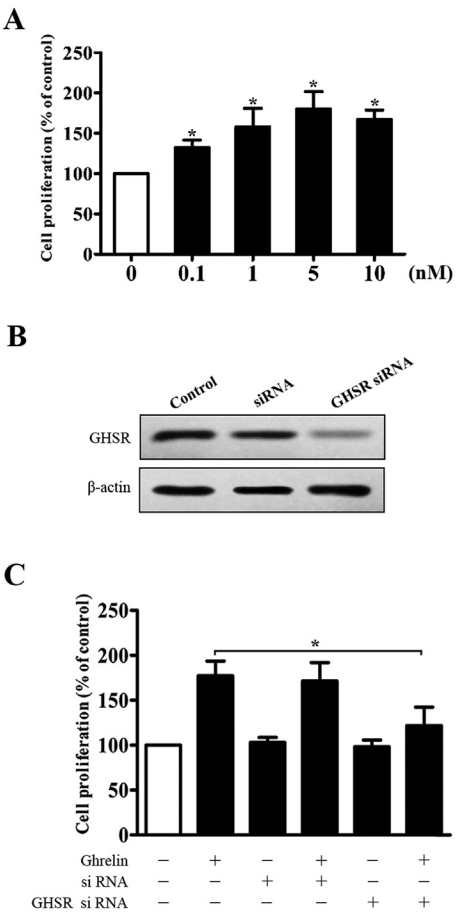

Fig. 1. Ghrelin induces A549 cells proliferation. (A) Treatment of A549 cells with ghrelin for 48 h. Cell proliferation was measured by BrdU assay. (B) A549 cells were transfected with GHSR si RNA or control si RNA, the protein level of GHSR was measured by Western blot. (C) GHSR knockdown A549 cells were treated with ghrelin (5 nM) for 48 h. Cell proliferation was measured by BrdU assay. Results as given as the mean ± S.E.M. of three experiments. *P < 0.05, compared to the control group without ghrelin treatment or the group with ghrelin treatment.

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