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ORIGINAL ARTICLE

Nitric oxide inhibits ghrelin-induced cell proliferation and ERK1/2 activation in GH3 cells

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Abstract Ghrelin stimulates growth hormone release and cell proliferation, which strongly supports a significant role for this peptide in the control of growth hormone-releasing adenomas function and growth. Nitric oxide can influence the stimulatory effects of ghrelin on growth hormone secretion in growth hormone-releasing adenomas. However, the effect of nitric oxide (NO) on ghrelin-induced cell proliferation and the mechanism of this effect in the adenoma were not clarified. In this study, we observed that ghrelin, at a concentration of 10^{-9} to 10^{-6} M, significantly increased BrdU incorporation into rat GH3 cells. A NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), blunted basal, and ghrelin-induced cell proliferation. A blocker of NO synthase, Nw-nitro-L-arginine methyl ester hydrochloride (NAME), had no influence on these actions. The activation of extracellular signal-regulated kinase (ERK) 1/2 was examined by western blotting. The results showed that SNAP reduced ghrelin-stimulated ERK1/2 activation but NAME had no influence on this activation. Together, this study indicates that NO inhibited ghrelin-induced cell proliferation by blocking ERK1/2 activation in GH3 cells.

Keywords Nitric oxide · Ghrelin · Cell proliferation · Extracellular signal-regulated kinase

Introduction

Ghrelin, a 28 amino acid peptide, is originally isolated and cloned from rat stomach [1]. Ghrelin has been found to stimulate growth hormone (GH) release in vivo and in vitro in a number of species [2, 3] by binding to GH secretagogue receptor (GHS-R) [4]. On the other hand, in GH3 cells [5], a clonal strain derived from a radiation-induced rat pituitary tumor cells that synthesize and secrete both prolactin and GH [6], and cell lines expressing the GHS-R mRNA [7], ghrelin exerts a proliferative effect via the extracellular signal-regulated kinase 1/2 (ERK 1/2) activation.

Nitric oxide (NO) is a readily diffusible, short-lived molecule produced by the action of NO synthase (NOS) on L-arginine. NO is a neurotransmitter in the central and peripheral nervous system [8]. NO can act both intracellularly as a second messenger and extracellularly as a conveyor of information between cells [9] and it also acts as a neuromodulator in the endocrine system [10], including the somatotrophic axis [11]. Recently, an increasing body of evidence confirms that NO is involved in the regulation of GH secretion both in somatotrophs [12, 13] and GH-releasing adenomas [14, 15]. Simultaneously, NO may restrain rather than mediate the apoptotic effect of tumor necrosis factor (TNF)-alpha in rat anterior pituitary cells [16]. However, the effects of NO on ghrelin-induced cell proliferation in GH-releasing adenomas and the

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mechanism of the effect have not been clarified. In this study, the regulatory effects of NO on cell proliferation and the intracellular signaling triggered by ghrelin in rat GH3 cells were investigated.

Results

Ghrelin-stimulated proliferation of GH3 cells

Firstly, we found that ghrelin-induced the proliferation of GH3 cells in a dose-dependent manner after incubation for 48 h. BrdU assay is an accurate method to determine the proliferation of cells, and we used this method to determine the proliferation of GH3 cells. The results showed that ghrelin increased the incorporation of BrdU into GH3 cells dose-dependently with significant responses from 10^{-9} M to 10^{-6} M after incubation for 48 h (Fig. 1a).

Effects of NO on cell proliferation

We observed the effect of NO on cell proliferation in rat GH3 cells. The cells were treated with SNAP to donate NO and NAME to inhibit endogenous NO synthesis. As shown in Fig. 1b and c, SNAP suppressed both basal cells proliferation from 5 to 10 mM, which release NO (nitrite level in medium; Fig. 1f), after incubation for 48 h and ghrelin-induced cell proliferation from 1 to 10 mM. As showing in Fig. 1d and e, SNAP (10 mM) inhibited both basal and ghrelin-induced cell proliferation from day 2. Nevertheless, NAME had no influence on cell proliferation.

NO inhibited ghrelin-stimulated ERK1/2 activation

According to the evidence provided by previous studies [5], ghrelin stimulates cell proliferative effect via the ERK1/2 activation in GH3 cells, so we measured the

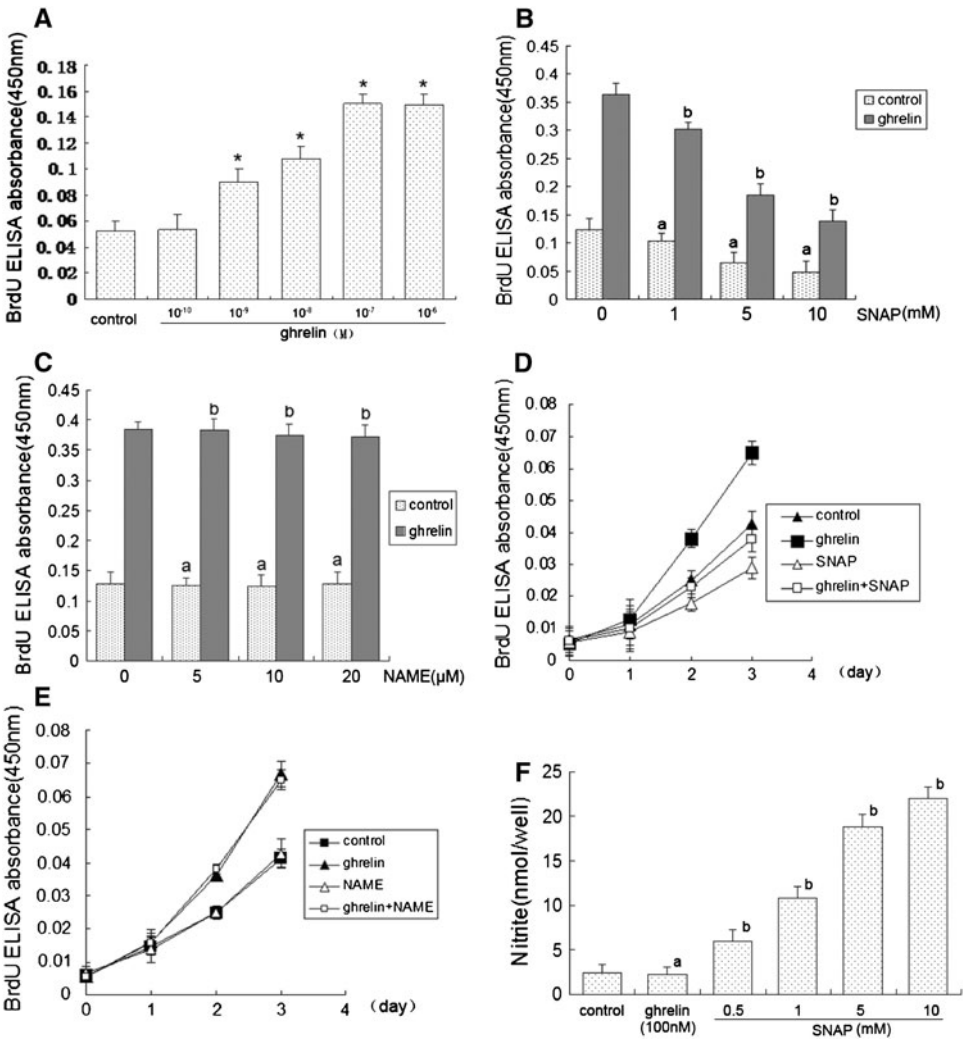

Fig. 1 **a** Ghrelin-induced cell proliferation. The BrdU incorporation assay showed that ghrelin-stimulated proliferation of GH3 cells (* $P < 0.01$ vs control group). The data were presented as mean ± SEM, $n = 12$; NO inhibited basal and ghrelin-stimulated cell proliferation. SNAP, a NO donor, from 5 mM inhibited the basal (**a**: [a] $P < 0.05$ vs control) and ghrelin-stimulated cell proliferation (**b**: [b] $P < 0.01$ vs control) after incubation for 48 h; NAME had no influence on the cell proliferation after incubation for 48 h (**c**: [a] $P > 0.05$ vs control; [b] $P > 0.05$ vs control). **d**, **e** From day 2, cell proliferation was inhibited by SNAP (10 mM) significantly. NAME (20 μM) had no influence on the cell proliferation. **f** Nitrite levels determinations in medium of control, ghrelin- and SNAP-treated wells. Serum-free defined medium was incubated at 37°C for 4 h in the presence of ghrelin (10^{-7} M) or SNAP (0.5–10 mM) ([a] $P > 0.05$ vs control; [b] $P < 0.05$ vs control). The data were presented as mean ± SEM, $n = 10$

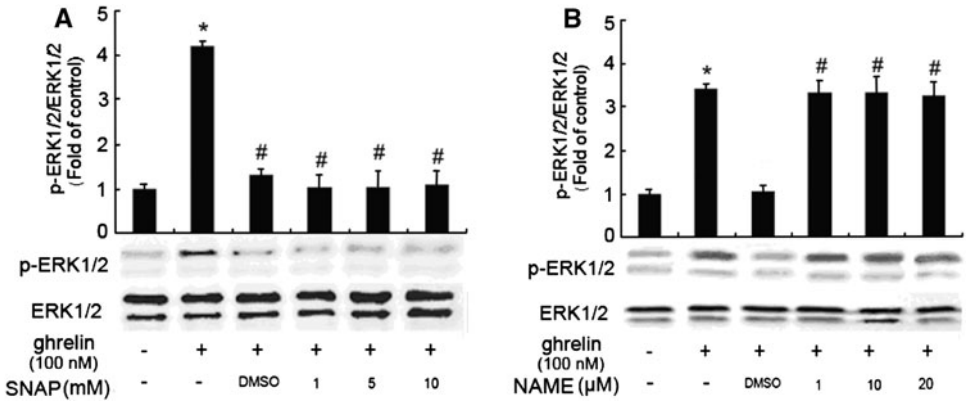

Fig. 2 NO inhibited ghrelin-stimulated ERK1/2 activation. **a** SNAP from 1 mM reduced ghrelin-induced ERK1/2 activation (* $P < 0.01$ vs control; # $P < 0.01$ vs ghrelin-treated group). **b** NAME had no influence on ERK1/2 activation (* $P < 0.01$ vs control; # $P > 0.05$ vs ghrelin-treated group). DMSO concentration (0.2%) was used. Equal amount of cells protein was subjected to Western blotting and ERK1/2 was measured to show equal protein loading. Data are expressed as the mean ± SEM of six and four independent experiments, respectively

ERK1/2 activation to explore the regulatory effect of NO on this enzyme. It was found that SNAP reduced ghrelin-stimulated ERK1/2 activation from 1 to 10 mM and NAME had no influence on this activation in GH3 cells (Fig. 2).

Discussion

Ghrelin, a nature ligand of GHS-R, stimulates GH secretion and cell growth in somatotrophes and GH-releasing adenomas. Ghrelin is a significant factor in the function and growth of the adenomas over expressing GHS-R [17]. Tsumori et al. [18] reported that NO played an inhibitory role in basal GH release and TRH-stimulated hormone release in an autocrine or paracrine fashion through a cGMP-dependent pathway in GH3 cells. However, some studies show that NO enhances the basal and ghrelin-induced GH secretion in cultured somatotrophs [19] and cultured human GH-secreting adenomas [20]. A study on human osteoblastic TE85 cells [21] revealed that ghrelin-stimulated proliferation via intracellular NO/cGMP signaling pathway and NO played a stimulatory role in ghrelin-induced proliferation. In this study, a NO donor was used to explore the effects of generated NO on the proliferation of GH3 cells. The results indicated that NO played an inhibitory role in basal and ghrelin-stimulated cell proliferation, but blocking NO generation could not affect these responses by using NAME from 5 to 20 μM.

Moreover, it is reported that ghrelin exerts a proliferative effect via ERK1/2 signaling pathway on GH3 cells [5] and 3T3-L1 adipocytes [22] expressing GHS-R mRNA. ERK1/2 is a protein-serine/threonine kinase mainly involved in the activation of nuclear transcription factors controlling cell proliferation, differentiation and death [23]. Maekawa et al. [24] recently demonstrated that peritoneal macrophages activated by LPS and IL-2 suppressed the proliferation of ascites hepatoma AH-130 cells via the production of TNF-alpha and NO. It has been shown that eNOS-overexpressing endothelial cells significantly inhibited smooth muscle cells proliferation in vitro [25]. However, in the guinea pig fetus, NO mediates the inhibition of MAPK phosphatases and leads to increased activation of p38, ERK, and JNK [26]. In this study, it was first evidenced that NO could attenuate the ERK1/2 activation stimulated by ghrelin.

One reason of these different results is contributed to the different intracellular levels of NO. NO donors show an inhibitory effect on GH secretion, probably because they release high nitrite levels [14]. In addition, NO inhibition may act by increasing the hypothalamic somatostatinergic tone and blunting endogenous GHRH secretion [27]. Hall and Garthwaite [28] reported that physiological NO concentration ranged to be 100 pM (or below) up to approximately 5 nM. Lower NO concentrations promote cell survival and proliferation, while higher levels favor cell cycle arrest, apoptosis and senescence [29]. At low nanomolar concentrations, activation of sGC is the major event initiated by NO; at micromolar concentrations, NO reacts with superoxide anion to form reactive peroxynitrite, thereby leading to the oxidation of important cellular proteins [30]. The concentration of NO will determine its chemistry, the distance it diffuses and the type of signaling targets it interacts with [29].

In conclusion, we used GH3 cells as a model of GH-releasing adenoma in vitro. We provided the first evidence that NO suppressed basal and ghrelin-induced cell proliferation and ERK1/2 activation, which offered novel evidences to search for drug treatment of GH-releasing adenomas. Maybe we can weaken the function and growth

of GH-releasing adenomas through regulating the generation level of NO by taking medicines.

Materials and methods

Reagents

Chemical products were purchased from Sigma Chemical Co. (St Louis, MO, USA). Accordingly, all drugs used were directly dissolved in Dulbecco's minimal essential medium (DMEM; Hyclone Co., South Logan, UT, USA) with the exception of SNAP which was previously dissolved in water, respectively, to make a stock solution. Aliquots of concentrated stock solutions were stored at −20°C until use, when they were diluted to final concentrations in DMEM. 5-Bromo-2′-deoxyuridine (BrdU) ELISA kits were purchased from Roche (Sandhofer, Mannheim, Germany).

Cell culture

GH3 cells (from the cell collection of Chinese Academy of Sciences, Sanlihe Rd., Beijing, China) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; Hyclone Co., South Logan, UT, USA). Cultures were maintained at 37°C in a humidified atmosphere (95% air; 5% CO_2). The medium was changed every 2–3 days.

Experimental design

GH3 cells were plated into 96-well plates (Costar Co., Cambridge, MA, USA) at a density of 2×10^3 cells/well. After culture for 24 h, (i) cells were incubated with ghrelin in different doses (0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) for 48 h; (ii) before treatment with ghrelin (10^{-7} M) for 48 h, cells were incubated with the following substances: [1] SNAP (0, 1, 5, 10 mM); [2] NAME (0, 5, 10, 20 μM) for 2 h; (iii) cells were incubated with SNAP (10 μM) or NAME (20 μM) for 2 h before treatment with ghrelin (10^{-7} M) for 1, 2, and 3 days, respectively.

BrdU assay

BrdU ELISA is based on the fixation and denaturation of cells within the plates where they were grown, followed by the detection of the immobilized antigen with the peroxidase-coupled antibody and the reaction with soluble peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB). At the end of the incubation periods, BrdU was added into the medium for 40 min at 37°C before incubation with 100 μl FixDenat for 30 min at room temperature. After aspiration of FixDenat, the cells were washed with 1 ml blocking buffer (3% BSA in PBS) and then incubated for 90 min with 100 μl the anti-BrdU peroxidase-conjugated antibody. The incubation with anti-BrdU peroxidase-conjugated antibody was terminated by washes with blocking buffer for three times, and then 100 μl TMB substrates were added into the wells. After 5 min incubation with TMB, 25 μl 2 M H_2SO_4 was added to stop the reaction. The absorbance at a wavelength of 450 nm was measured with Multiskan Ascent (Thermo Scientific). All the measurements were performed in triplicate. Results were calculated as the percentages of proliferation with respect to vehicle-treated cells.

Protein extraction and western blotting

GH3 cells were plated into six-well plates (Costar Co., Cambridge, MA, USA) at a density of 3×10^5 cells/well in 1.5 ml of DMEM supplemented with 10% FBS. Cultures were maintained at 37°C in a humidified atmosphere (95% air; 5% CO_2) before treatment. After culture for 3 days, a 6-h preincubation in FBS-free medium was used for stabilization. Then, cells were incubated with DMEM alone (control group) or DMEM containing one of the following substances: (i) SNAP (0, 1, 5, 10 mM); (ii) NAME (0, 5, 10, 20 μM) for 2 h. Thereafter, cells were challenged for 3 h at 37°C with ghrelin (10^{-7} M) in the presence or absence of the corresponding SNAP or NAME. Cells proteins were extracted by RIPA buffer (Beyotime Co., Beijing, China). Then, cell lysates were prepared for western blotting. An alternative assessment of proliferation was afforded by the detection of GH3 cell ERK1/2 activation. Lysates were briefly sonicated and cleared by centrifugation. Protein concentrations were determined by bicinchoninic acid assay (Beyotime Co., Beijing, China). Equal amounts of cell extracts were separated by SDS-PAGE (10%), and proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 5% nonfat, dried milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) at room temperature for 1 h and then incubated with antibodies at 37°C for 2 h or 4°C overnight. Blots were then washed and incubated with horseradish peroxidase-labeled secondary antibodies at 37°C for 1 h. Peroxidase activities on the blots were detected by Super-Signal chemiluminescent substrate (Pierce Co., Rockford, IL, USA).

Nitrite measurement

Nitrite levels determinations in medium of control, ghrelin- and SNAP-treated wells. Cells were incubated at 37°C for 4 h with serum-free defined medium in the present of ghrelin (100 nM) or SNAP (0.5–10 mM). Then, equal

volumes of samples and Greiss reagent (Sigma) were mixed. The mixtures were incubated at room temperature for 10 min and quantified spectrophotometrically at 550 nm [31]. Nitrite levels were determined by a standard curve of increasing levels (0–40 μM) of sodium nitrite (Sigma).

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