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Ghrelin Induces Cell Migration Through GHS-R, CaMKII, AMPK, and NF-κB Signaling Pathway in Glioma Cells

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ABSTRACT

Ghrelin is a newly discovered gastric peptide which stimulates food intake, energy balance, and growth hormone release. Recent reports have also shown that circulating ghrelin can efficiently reach the brain. However, the molecular mechanisms and pathophysiologic roles underlying ghrelin-induced glioma migration remain unclear. Glioma is the most common primary adult brain tumor with poor prognosis because of the spreading of tumor cell to the other regions of brain easily. In present study, we found that application of recombinant human ghrelin enhances the glioma cell migration in both rat C6 and human U251 cells. Ghrelin and its receptor GHS-R (growth hormone secretagogue receptor) are expressed in a wide variety of tissues and cell types, including various cancer cells. However, little is known about the expression of ghrelin or GHS-R in brain tumors. Here, we found that ghrelin increased GHS-R receptor up-regulation, and the enhancement of ghrelin-induced glioma cell motility markedly inhibited by a GHS-R antagonist. In addition, ghrelin-mediated migration was attenuated by treatment of CaMKII inhibitor, and AMPK inhibitors and pre-transfection with AMPK siRNA. Moreover, ghrelin stimulation also increased the phosphorylation of CaMKII and AMPK. Treatment with three different types of NF-κB inhibitors or pre-transfection with KM-IKKα, or KM-IKKβ also reduced ghrelin-induced glioma cell migration. Moreover, treatment of ghrelin also induced IKKα/β activation, IκBα phosphorylation, p65 phosphorylation at Ser^{536}, and increased NF-κB-DNA binding activity and κB-transcriptional activity. These results indicate that ghrelin enhances migration of glioma cells is mainly regulated by the GHS-R, CaMKII, AMPK, and NF-κB pathway. J. Cell. Biochem. 112: 2931–2941, 2011.

KEY WORDS: GHRELIN; MIGRATION; CaMKII; AMPK; NF-κB

Ghrelin is a newly discovered hormone from the stomach, which stimulates appetite and food intake, energy balance, and weight gain [De Vriese and Delporte, 2008]. In addition, ghrelin also increases myocardial contractility and remodeling, and regulates vasodilation [Locatelli et al., 1999; Shimizu et al., 2003; Shinde et al., 2005; Xu et al., 2003]. Furthermore, low ghrelin concentration has result in several metabolic syndromes including obesity, insulin resistance, hypertension, and type II diabetes

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[Tschop et al., 2001; Poykko et al., 2003; Yildiz et al., 2004]. It has been reported that administration of ghrelin in patients with metabolic syndrome improves endothelial dysfunction [Tesauro et al., 2005]. Recent studies have also shown that ghrelin is capable of inhibiting inflammatory cytokine expression [Dixit et al., 2004; Li et al., 2004], promoting neurogenesis [Zhang et al., 2004], and to exerting prosurvival effects on cardiomyocytes [Baldanzi et al., 2002].

Given the association between obesity and the susceptibility to develop various cancers, it seems feasible that ghrelin/GHS-R interactions may play a pathophysiological role in these complex conditions [Calle and Kaaks, 2004]. Ghrelin binds to the transmembrane domain of the G protein-coupled receptor known as the growth hormone secretagogue receptor (GHS-R) [Smith et al., 1999]. A recent report has hypothesized that the ghrelin/GHS-R axis may operate through a similar autocrine/paracrine role in cancer biology [Jeffery et al., 2003]. Ghrelin and GHS-R have been reported to be expressed in variety of cancers, including breast carcinomas, prostate cancer cells, hepatomas, and gastrointestinal cancer [Papotti et al., 2000; Murata et al., 2002; Corbetta et al., 2003; Cassoni et al., 2004]. However, little is known about the expression of ghrelin or GHS-R in cancers of central nervous system.

Circulating ghrelin can reach the brain by crossing the BBB or via circumventricular organs [Banks et al., 2002; Pan et al., 2006]. Interestingly, it has been reported that ghrelin/GHS-R constitute an autocrine pathway in astrocytoma motility [Dixit et al., 2006]. Glioblastoma is one of the most common primary central nervous system tumors, and its biology reveals significant problems for successful treatment. Chief among these hurdles is the aggressive local invasion of malignant cells from the original tumor. Invasion into the surrounding normal brain renders complete surgical resection impossible. Similarly, chemotherapy and ionizing radiation alone or in combination have produced only a modest increase in median survival due to problems both with the effective targeting of the invading cells and their innate resistance to conventional radiotherapy and chemotherapy [Stupp et al., 2005; Koul et al., 2006]. Despite commonly used treatment procedures, such as surgery, radiation, and chemotherapy [Boiardi et al., 1991], the survival of patients with such tumors has not been improved [Kondo et al., 2004]. Effective treatment will ultimately require a more thorough understanding of the signaling pathways that drive glioma invasion as well as the identification and specific targeting of the critical signaling effectors.

In the present study, we investigated the ghrelin-induced increase cell migration and its underlying signaling cascade in glioma cells. Our data showed that ghrelin interacts with GHS-R and activates the CaMKII, AMPK, and NF-κB pathways resulting in tumor migration.

MATERIALS AND METHODS

MATERIALS

Ghrelin was purchased from PeproTech (Rocky Hill, NJ) and dissolved in phosphate-buffered saline (PBS). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM and Lipofectamine™ 2000 (LF2000) were purchased from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG, primary antibodies against β-actin, IκBα, IKKα/β, p65, and p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against IKKα/β phosphorylated at $Ser^{180/181}$ and p65 phosphorylated at Ser^{573} and AMPK phosphorylated at Thr^{172} were purchased from Cell Signaling and Neuroscience (Danvers, MA). Rabbit polyclonal antibody against AMPK phosphorylated at Ser^{485} was purchased from Abcam, Inc. (Cambridge, MA). The electrophoretic mobility shift assay (EMSA) gel shift kit was purchased from Panomics (Redwood City, CA). TPCK, PDTC, and Compound C were purchased from Calbiochem (San Diego, CA). NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). The dominant-negative kinase inactive mutant (KM) of IKKα and IKKβ, and the phosphorylation site mutant of FAK (Y397F) were gifts from Dr. C.-H. Tang (China Medical University, Taichung, Taiwan). TPCK, PDTC, and Compound C were purchased from Calbiochem. 9-β-D-arabinofuranoside, Bay 11-7082, D-Lys3-GHRP-6, and KN-93 were obtained from Sigma-Aldrich (St. Louis, MO). D-Lys3-GHRP-6 was dissolved in H_2O, all other inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in experimental media was less than 0.1%.

CELL CULTURES

C6 and U251 cells were originated from a rat and a human brain glioma, respectively. Both cell lines were purchased from the American Type Culture Collection (Manassas, VA). C6 cells was maintained in F12 medium (Invitrogen Life Technologies), while U251 cells was maintained in 75 cm^2 flasks with DMEM. All cells were cultured in medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air.

TRANSFECTION

C6 cells were transiently transfected with 0.8 μg KM-IKKα, KM-IKKβ or pcDNA3 empty vectors, or 100 nM AMPKα or control siRNA. The siRNAs against human GHS-R1, FAK, and Control siRNA (at a final concentration of 100 nM) were purchased commercially from Santa Cruz Biotechnology. Plasmid DNA or siRNA were premixed with LF2000 in OPTI medium (Invitrogen Life Technologies) for 20 min and applied to the cells. Medium containing 20% FBS (volume equals to original LF2000-containing medium) was added 6 h later. After transfection for 24 h, LF2000-containing medium was replaced with fresh serum-free medium and treated with ghrelin for another 24 h.

REPORTER GENE ASSAY

Cells were co-transfected with 0.8 μg pκB-luciferase plasmid, 0.4 μg β-galactosidase expression vector and either control vector (pcDNA3.1), KM-IKKα or KM-IKKβ (0.4 μg). The cells were grown to 80% confluence in 6-well plates and were transfected with LF2000 on the following day. After 24 h transfection, medium containing LF2000 was replaced with fresh serum-free DMEM medium and treated with ghrelin for another 24 h. To prepare lysates, 100 μl of reporter lysis buffer (Promega) was added to each

well, and cells were scraped from plates. The supernatants were collected after centrifugation at 10,000*g* for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (30 μg) were placed into wells of an opaque white 96-well microplate. The luciferase activity was determined by a dual-luciferase reporter assay system (Promega), and activity value was normalized by a β-galactosidase expression vector.

WESTERN BLOT ANALYSIS

The whole cell extracts were prepared as described previously [Lu et al., 2010b]. Briefly, cells were treated with ghrelin for various time periods and washed with cold PBS, lysed for 30 min on ice with a radioimmunoprecipitation assay buffer. The supernatants containing whole cell lysated proteins were collected by centrifugation at 13,000*g* for 20 min.

The nuclear extracts were prepared as described previously [Lin et al., 2010; Lu et al., 2010a]. Cells were rinsed with PBS and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. The lysates were separated into cytosolic and nuclear fractions by centrifugation at 12,000*g* for 10 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclear fraction was resuspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 13,000*g* for 20 min and stored at −80°C.

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then probed overnight with primary antibody at 4°C. After undergoing three times PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). The blots were subsequently stripped by incubation in a stripping buffer and reprobed for β-actin as a loading control. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

MIGRATION ASSAY

In vitro migration assays were performed using Costar Transwell inserts (Costar, NY; pore size, 8 μm) in 24-well plates. Before performing the migration assay, cells were pre-treated for 30 min with different concentrations of inhibitors, or transfected with various mutants or siRNA for 24 h. According to cell viability assay, various concentrations of inhibitors used did not affect glioma cell death (data not shown). Approximately 1×10^4 cells in 100 μl of serum-free medium was placed in the upper chamber, and 400 μl of the same medium containing ghrelin was placed in the lower chamber. The plates were incubated for 24 h at 37°C in 5% CO_2, then the cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper sides of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the undersides of the filters were examined and counted under microscope. Each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of ghrelin treatment (corrected migration cell number = counted migration cell number/percentage of viable cells). Images of migratory cells were observed and acquired at 24 h with digital camera and light microscope.

REVERSE TRANSCRIPTASE-PCR (RT-PCR)

Total RNA was extracted from cells using TRIzol kit (MDBio, Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using the oligo(dT) primer, then amplified using oligonucleotide primers:

GHS-R1a: 5′-GCGCTCTTCGTGGTGGGCATCT-3′ and 5′-GTGGCGC-GGCATTCGTTGGT-3′;
GHS-R1b: 5′-TGTCCAGCGTCTTCTTCTTTCTAC-3′ and 5′-TCAGC-GGGTGCCAGGACTC-3′;
GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCC-TGTTGCTGTA-3′.

Each PCR cycle was carried out for 30 s at 95°C, for 30 s at 55°C, and for 1 min at 68°C. PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. GAPDH was used as an internal control. Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics). GHS-R1a and GHS-R1b mRNA levels were normalized to GAPDH mRNA levels and expressed relative to control group.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The EMSA gel shift kit was performed according to the manufacturer's protocol. Nuclear extract (1 μg) of C6 glioma was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes followed by incubation at room temperature for 30 min. After electrophoresis on an 8% polyacrylamide gel, the samples on gel were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA). The membrane was cross-linked in an oven for 3 min and then developed with the addition of the blocking buffer and streptavidin–horseradish peroxidase conjugate, before being subject to Western blot analysis.

MEASUREMENT OF CELL VIABILITY

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells cultured in 24-well plates were treated with various concentrations of ghrelin for 24 h. After treatment, MTT (0.5 mg/ml) was incubated for 60 min at 37°C, the culture medium was then removed, and cells were dissolved in DMSO and shaken for 10 min. OD values at 550 nm were measured by using microplate reader. The absorbance indicates the enzymatic activity of mitochondria and provides information on cell viability.

STATISTICAL ANALYSIS

The values given are means ± SEM. The significance of difference between the experimental group and control groups was assessed by the Student's *t*-test. The difference was significant if the *P* value was <0.05.

RESULTS

GHRELIN/GHS-R ENHANCES THE MIGRATION OF GLIOMA CELLS

Numerous reports showed that ghrelin expresses in various cancer cells [Papotti et al., 2000; Murata et al., 2002; Corbetta et al., 2003; Cassoni et al., 2004]. It has also been shown that ghrelin plays an important role in astrocytoma motility through an autocrine activity [Dixit et al., 2006]. However, whether ghrelin affects glioma cell migration and the involved signaling pathways remain unclear. Here, ghrelin-regulated glioma cell migration was examined using the Transwell assay with correction of ghrelin-induced proliferation effects [Lu et al., 2010a,b]. As shown in Figure 1A, ghrelin enhanced migration of both rat and human malignant glioma cells (C6 and U251 cells, respectively) in a concentration-dependent manner. Furthermore, the pictures of the migration cells were also showed in Figure 1B. Stimulation of cells with ghrelin up to 30 nM did not affect cell viability assessed by the MTT assay (data not shown). Ghrelin and GHS-R (ghrelin receptor) have been reported to be expressed in variety of cancers [Papotti et al., 2000; Murata et al., 2002; Corbetta et al., 2003; Cassoni et al., 2004]. To investigate the role of GHS-R in the ghrelin-mediated increase of cell migration, the expression of GHS-R after ghrelin stimulation in C6 cells was determined. The protein level of GHS-R was relatively lower, but the protein (Fig. 2A) and mRNA (Fig. 2B) expression of ghrelin receptors significantly increased in response to ghrelin treatment. We then examined whether ghrelin/GHS-R interaction is involved in the signal transduction pathways leading to glioma cells migration. Ghrelin-induced C6 glioma cell migration was effectively antagonized by treatment with D-Lys3-GHRP-6 (50 μM), a GHS-R antagonist (Fig. 2C). Moreover, pre-transfected with siRNA against GHS-R1 also effectively reduced ghrelin-induced glioma cell migration (Fig. 2D). These data suggest that ghrelin-induced glioma cell migration may occur via ghrelin receptor.

CaMKII AND AMPK SIGNALING PATHWAYS ARE INVOLVED IN THE GHRELIN-MEDIATED GLIOMA CELL MIGRATION

Ghrelin is a brain-gut peptide that stimulates appetite and regulates the energy balance [Gnanapavan et al., 2002; Higgins et al., 2007]. AMPK is a key enzyme regulator of energy homeostasis both centrally and peripherally [Kola et al., 2006; Xue and Kahn, 2006], and also is a mediator of several appetite-regulating hormones [Kola and Korbonits, 2009]. Next, we examined the effects of AMPK in ghrelin-induced glioma cell migration. As shown in Figure 3A, ghrelin-induced glioma cells migration were greatly reduced by treatment with AMPK inhibitors 9-β-D-arabinofuranoside (ara-A) or Compound C (Fig. 3A). Moreover, pre-transfection of cells with AMPK siRNA also reduced ghrelin-induced glioma cell migration (Fig. 3B). Furthermore, stimulation of C6 cells with ghrelin increased AMPK phosphorylation at Thr^{172} and Ser^{485} sites (Fig. 3C). AMPK is catalyzed by upstream kinases such as LKB1 and CaMKII [Hawley et al., 2005; Woods et al., 2005; Lu et al., 2010c]. Ghrelin also increased phosphorylation of AMPK upstream molecular regulators LKB1 (Ser^{428}) and CaMKII (Thr^{286}) within a transient period from 5 to 60 min (Fig. 3D). Treatment of CaMKII inhibitor KN-93 also inhibited ghrelin-induced glioma cell migration (Fig. 3E). These results demonstrate that ghrelin-induced glioma cell migration is regulated by CaMKII/AMPK signaling pathway.

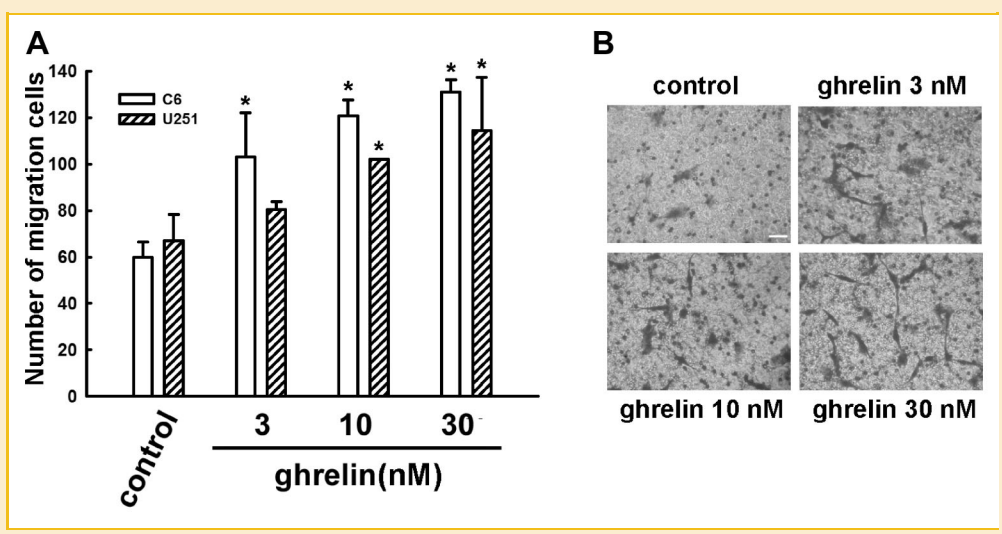

Fig. 1. Ghrelin induces the migration activity of glioma cells. A: C6 and U251 cells were incubated with various concentrations of ghrelin, and in vitro migration activities assayed by using Transwell after 24 h showed that migrated cells were increased. Results are expressed as the means ± SEM of at least four independent experiments. The control group was added the equal volume of vehicle PBS buffer. B: Cells were treated with various concentrations of ghrelin for 24 h and in vitro migration activities were measured with the Transwell after 24 h. The migration cells were visualized by phase-contract imaging. Scale bar: 10 μm.

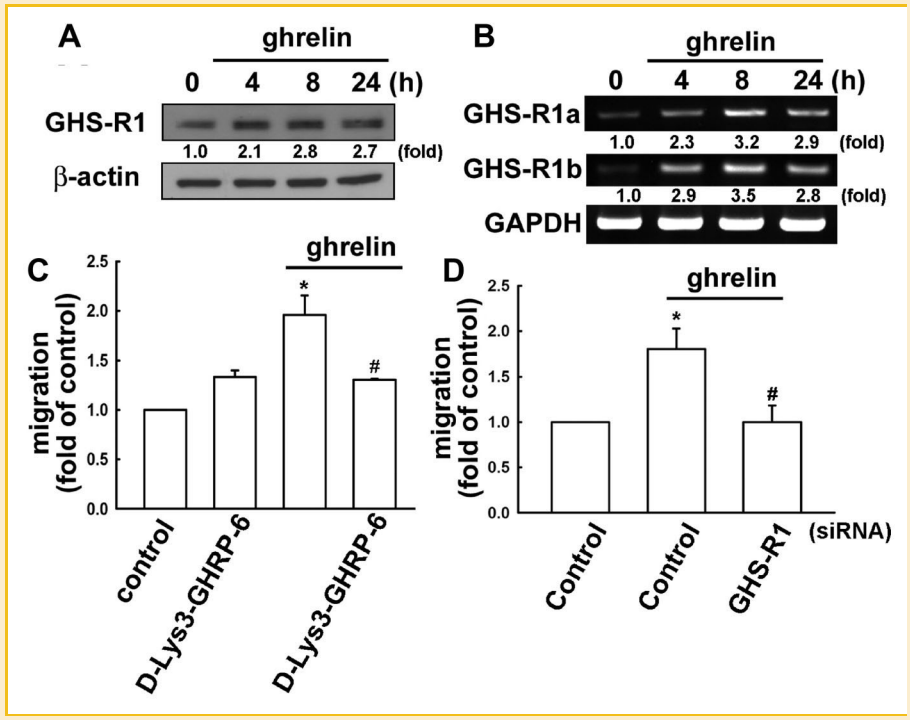

Fig. 2. GHS-R receptor is involved in ghrelin-directed migration of C6 glioma cells. A: C6 cells were treated with ghrelin for indicated time periods, and the GHS-R1 protein level was quantified by Western blot. Relative value of each protein expression of GHS-R1 was normalized by β-actin. B: Cells were treated with ghrelin for indicated time periods, and the GHS-R1a and GHS-R1b mRNA level were quantified by RT-PCR. Relative value of each mRNA expression of GHS-R1a and GHS-R1b were normalized by β-actin. Cells were pre-treated with ghrelin receptor antagonist (D-Lys3-GHRP-6) for 30 min (C) or transfected with siRNA against GHS-R1 or Control siRNA for 24 h (D) followed by treatment with ghrelin for another 24 h, and in vitro migration activities were measured with the Transwell after 24 h. The control group was added equal volume of vehicle. Results are expressed as the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the ghrelin treatment group.

INVOLVEMENT OF IKKα/β PHOSPHORYLATION AND NF-κB ACTIVATION IN GHRELIN-INDUCED CELL MIGRATION

As previously mentioned, NF-κB activation is a necessary transcription factor in ghrelin-regulated biological effects [Zhao et al., 2006; Hou et al., 2009; Liu et al., 2010]. To examine whether NF-κB activation is involved in ghrelin-induced glioma cell migration, three different NF-κB inhibitors were used. Our results showed that pyrrolidine dithiocarbamate (PDTC), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) or Bay 117082 inhibited ghrelin-induced glioma cell migration (Fig. 4A). However, treatment of cells with these inhibitors at the concentrations did not affect cell viability, which was assessed by MTT assay (data not shown). Pre-transfection of KM-IKKα and KM-IKKβ mutants also reduced ghrelin-enhanced migration in glioma cells (Fig. 4B). We then further examined the upstream molecules involved in ghrelin-induced NF-κB activation. Treatment of C6 glioma cells with ghrelin increased IKKα/β phosphorylation (Fig. 4C) and IκBα phosphorylation (Fig. 4D) in a time-dependent manner. Moreover, treatment of C6 cells with ghrelin also induced p65 phosphorylation at Ser^{536} in a time-dependent manner (Fig. 4E). In addition, treatment of C6 glioma cells with ghrelin resulted in a marked accumulation of p65 and p50 NF-κB in nucleus (Fig. 5A). We then assessed whether ghrelin affects the binding of transcription factor to the DNA binding site. Ghrelin stimulation significantly increased the DNA binding activity of NF-κB in a time-dependent manner, as determined by EMSA (Fig. 5B). The p50 and p65 NF-κB antibodies reduced ghrelin-enhanced DNA binding activity (Fig. 5B). Treatment of CaMKII and GHS-R inhibitors also antagonized ghrelin-induced accumulation of p50 and p65 NF-κB in nucleus (Fig. 5C), and the increase of NF-κB-DNA binding activity (Fig. 5D). In addition, treatment of AMPK inhibitors also reduced ghrelin-induced IKKα/β phosphorylation (Fig. 6A) and p65 Ser^{536} phosphorylation (Fig. 6B). Treatment of AMPK inhibitor Compound C also reduced ghrelin-increased NF-κB-DNA binding activity (Fig. 6C). To directly determine NF-κB activation after ghrelin treatment, cells were transiently transfected with κB-luciferase plasmid as an indicator of NF-κB activation. As shown in Figure 6D, treatment of ghrelin increased κB-luciferase activity in C6 glioma cells. Furthermore, treatment of AMPK inhibitors or co-transfection with KM-IKKα or KM-IKKβ mutant effectively reduced ghrelin-induced κB-luciferase activity. Taken together, these results suggest that the NF-κB activation is regulated by CaMKII/AMPK signal pathway, and NF-κB plays an important role in ghrelin-induced glioma cell migration.

DISCUSSION

Glioma is the most common primary malignant cancer affecting the central nervous system. However, the median survival of patients

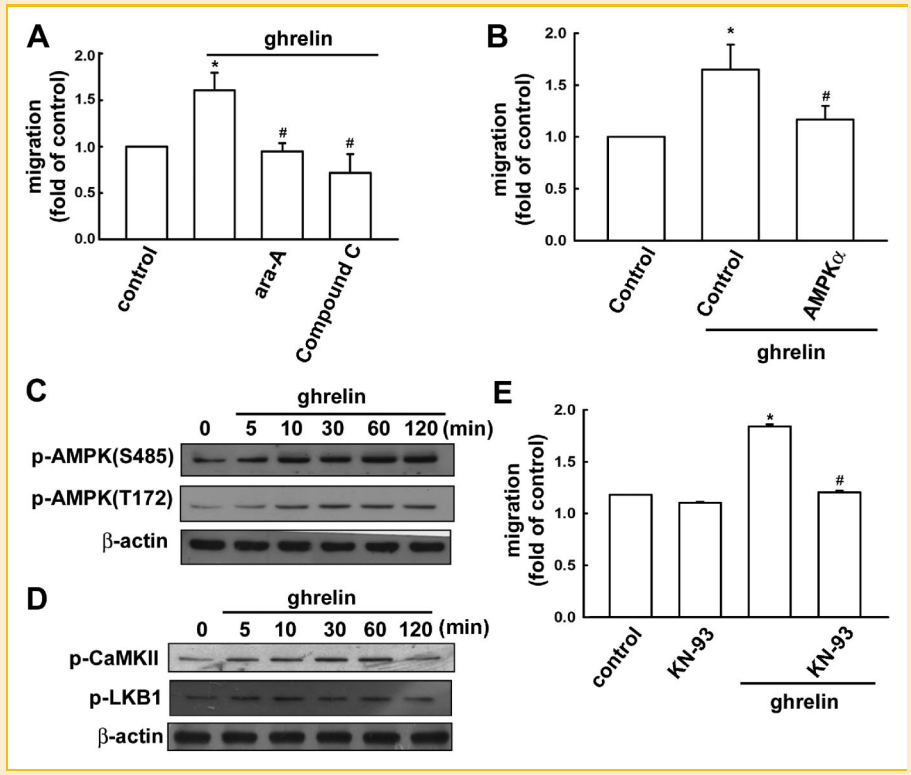

Fig. 3. The CaMKII/AMPK signaling pathways are involved in ghrelin-induced cell migration. C6 cells were pre-treated with AMPK inhibitors 9-β-D-arabinofuranoside (Ara A, 20 μM) or Compound C (10 μM) for 30 min (A), or pre-transfected with AMPKα siRNA for 24 h (B) followed by stimulation with ghrelin, and the in vitro migration were measured with the Transwell after 24 h ghrelin treatment. Results are expressed as the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with ghrelin-treated group. Cells were incubated with ghrelin for indicated time periods, and the phosphorylated of AMPKa Ser^{485} and AMPKa Thr^{172} (C), or LKB1 and CaMKII (D) were determined by Western blot analysis. Note that ghrelin activated the AMPK and CaMKII pathways. Similar results are expressed of three independent experiments. E: Cells were pre-treated with CaMKII inhibitor KN-93 for 30 min followed by stimulation with ghrelin, and the in vitro migration were measured with the Transwell after 24 h ghrelin treatment. The control group was added equal volume of vehicle. Results are expressed as the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the ghrelin-treated group.

diagnosed with a high-grade glioma is less than 1 year [Westermarck and Kahari, 1999]. The pathologic characteristic of their insidious infiltration of the brain leads to the poor prognosis after surgery and/or radiation therapy. Invasion of glioma cells into adjacent brain structures occurs through the activation of multigenic moleculars, including matrix metalloproteinases (MMPs), which play important roles in tumor invasion because of their ability to degrade many extracellular matrix components and enabling tumor cells to invade the surrounding stroma. We have previously reported that leptin [Yeh et al., 2009] and GDNF [Lu et al., 2010a] enhance migration and invasion of glioma cells by increasing MMP production. Previous reports also showed that adiponectin, an adipokine, activates AMPK activation and up-regulates MMP expression [Handy et al., 2010; Kang et al., 2010; Tong et al., 2011]. Here we also found that ghrelin affects the expression of MMP in human glioma cells (Suppl. Fig. S1). The elucidation of the molecular biology of cancer cells in recent years has identified alterations in various molecular signaling pathways in different cancers. This information is currently being exploited to develop potential therapeutic targets. It has been reported that ghrelin stimulates angiogenesis in human microvascular endothelial cells [Li et al., 2007]. Previous reports also demonstrated that endogenously produced ghrelin enhances colorectal cancer [Waseem et al., 2008] and pancreatic adenocarcinoma [Duxbury et al., 2003] cell motility. Interestingly, it has been reported that ghrelin is efficient transport across the blood–brain barrier [Banks et al., 2002; Pan et al., 2006]. It has also been reported that ghrelin and its receptor (GHS-R) increases astrocytoma cell growth and invasion [Dixit et al., 2006]. Recently, several studies have reported the biological functions of ghrelin in the nervous system. However, the molecular mechanism and pathophysiologic role underlying ghrelin-induced glioma migration remain unclear. Here we found that exogenous application of ghrelin enhanced the migration ability of human U251 and rat C6 glioma cells.

GHS-R is a transmembrane G protein-coupled receptor for ghrelin and presents in many brain regions including the hypothalamus, hippocampus, thalamus, and ventral tegmental area [Guan et al., 1997; Zigman et al., 2005], and stimulation of GHS-R with ghrelin leads to activation of multiple downstream signalings [Kojima et al., 1999; Nakazato et al., 2001]. GHS-Rs have been cloned for two different isoforms that were named GHS-R1a and GHS-R1b [Howard et al., 1996]. GHS-R1a predominantly

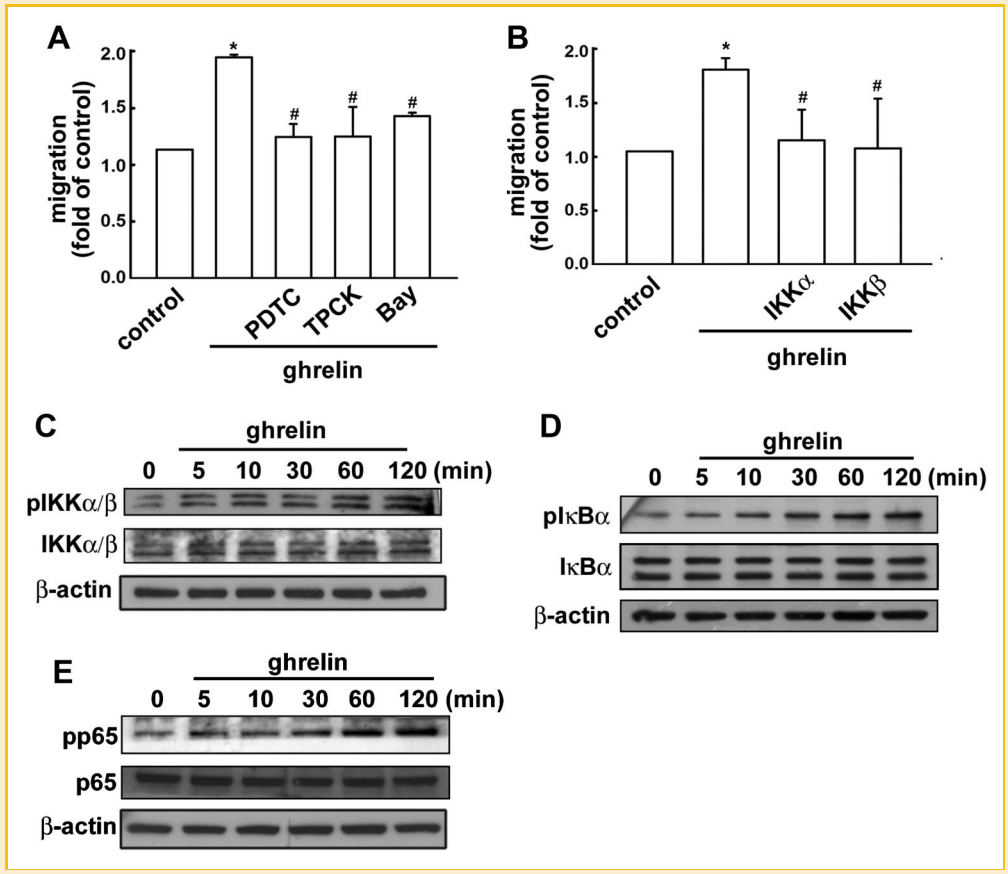

Fig. 4. Ghrelin induces cell migration through NF-κB in C6 glioma. Cells were pre-treated with three different NF-κB inhibitors PDTC, TPCK and Bay 11-7082 for 30 min (A), or pre-transfected with KM-IKKα or KM-IKKβ for 24 h (B) followed by stimulation with ghrelin, and the in vitro migration were measured with the Transwell after ghrelin treatment for 24 h. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the ghrelin-treated group. C: Cells were incubated with ghrelin (10 μg/ml) for the indicated time intervals, and cell lysates were separated by SDS-PAGE. Note that ghrelin induces IKKα/β phosphorylation (C), IκBα phosphorylation (D), and p65 Ser^{536} phosphorylation (E). Results are the representatives of three independent experiments. The control group was added equal volume of vehicle.

expresses in the pituitary and encodes for the full-length biologically active receptor. In contrast, GHS-R1b encodes for a truncated isoform and expresses widespread in all tissues studied. Ghrelin and GHS-R have been reported to be expressed in variety of cancers [Papotti et al., 2000; Murata et al., 2002; Corbetta et al., 2003; Cassoni et al., 2004]. It has been reported that ghrelin and GHS-R over-express in malignant intestinal epithelial cells and leading to their enhanced proliferative and invasive behavior [Waseem et al., 2008]. In the present study, stimulation of ghrelin increases the protein expression of GHS-R1 (Fig. 2A), and increases mRNA expression of GHS-R1a and GHS-R1b (Fig. 2B). We also used GHS-R inhibitor and siRNA against GHSR1 to examine the role of GHS-R in ghrelin-mediated glioma migration. Transfection of siRNA against GHS-R1 effectively inhibits ghrelin-induced glioma migration (Fig. 2D). We also compared the expression of GHS-R and migration ability between two different glioma cells. Human U251 glioma cells express higher level of GHS-R than C6 glioma cells (Suppl. Fig. S2A). In parallel, U251 glioma cells also exhibit higher migration ability (Suppl. Fig. S2B). Furthermore, treatment of GHS-R inhibitor significantly attenuates the enhancement effects of ghrelin on glioma migration (Fig. 2C), NF-κB translocation (Fig. 5C) and DNA binding activity (Fig. 5D). These results indicate the involvement of ghrelin-GHS-R1 interaction in ghrelin-mediated effects in glioma. Our results also reveal that ghrelin increases the ghrelin receptor expression in a time-dependent manner (Fig. 2A,B), indicating that the ligand may enhance the expression of its receptor.

Orexigenic agents such as ghrelin stimulate hypothalamic AMPK leading to increase in appetite. AMPK has been shown to play a critical role in the regulation of energy metabolism under both physiological and pathological conditions. It has been reported that ghrelin activates AMPK in skeletal muscles [Barazzoni et al., 2005], beta-cells [Wang et al., 2010], endothelial cells [Xu et al., 2008], and neurons [Kohno et al., 2008; Sangiao-Alvarellos et al., 2010], which regulates many physiologic functions in these tissues. Here, we demonstrated that treatment of AMPK inhibitors ara A or Compounds C, and pre-transfection of AMPKα siRNA antagonize the ghrelin-mediated glioma migration, suggesting that AMPK activation is an obligatory event in ghrelin-induced glioma migration. The key step in the activation of AMPK is mediated

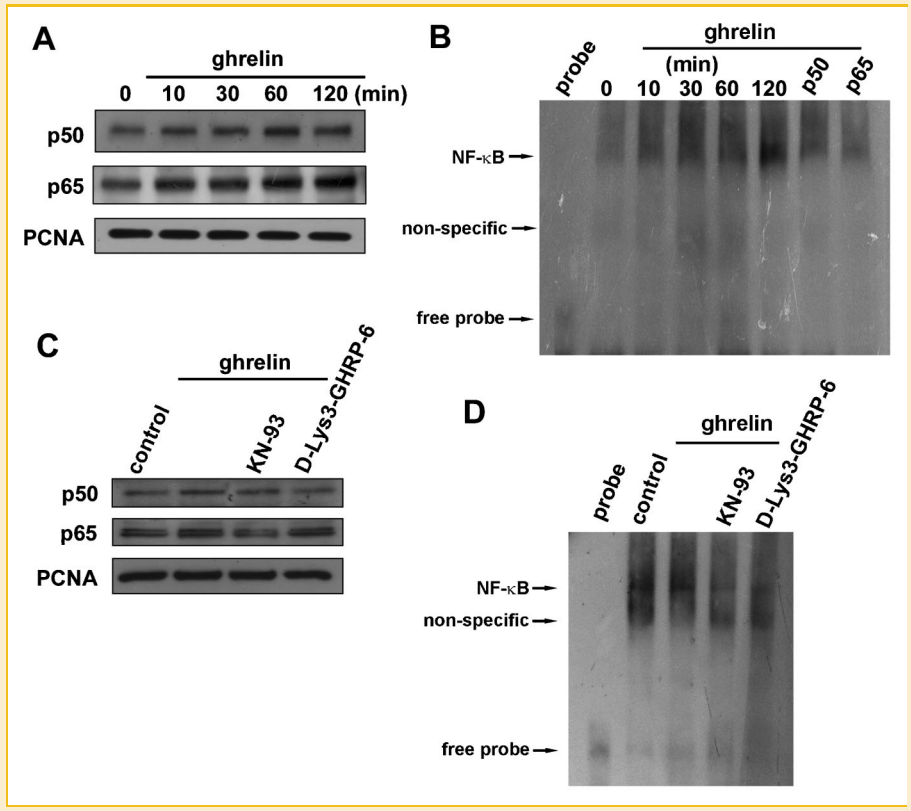

Fig. 5. Ghrelin induces NF-κB translocation and increases NF-κB-DNA binding activities in C6 glioma. A: Cells were treated with ghrelin for indicated time periods, and the levels of nuclear p50 and p65 were determined by immunoblotting with p50- and p65-specific antibody, respectively. B: Nuclear extracts were obtained from cells treated with ghrelin for various time periods, or treated with ghrelin for 120 min and premixed with anti-p50 or anti-p65-specific antibody. Nuclear extracts were incubated with a NF-κB probe and then analysis by EMSA. The NF-κB-specific complex is indicated by an arrow. C: Cells were treated with KN-93 or D-Lys3-GHRP-6 followed by stimulation with ghrelin for 60 min, the levels of nuclear p50 and p65 were determined. D: Nuclear extracts were obtained from cells treated with KN-93 or D-Lys3-GHRP-6 followed by stimulation with ghrelin for 120 min. The nuclear extracts were incubated with a NF-κB probe and then analysis by EMSA. The control group was added equal volume of vehicle. Results are the representatives of three independent experiments.

through its major regulatory phosphorylation site, threonine 172 (Thr^{172}) [Hardie, 2003]. Recent studies have identified LKB1 and CaMKII as the upstream kinases that phosphorylate AMPK Thr^{172} [Hawley et al., 2005; Woods et al., 2005; Carling et al., 2008]. Our results exhibited that ghrelin promotes the phosphorylation of CaMKII (Thr^{286}) and LKB1 (Ser^{428}) in human glioma cells. On the other hand, AMPK Ser^{485} in the α-subunit is an auto-phosphorylation site as well as a target site for AKT [Horman et al., 2006]. Phosphorylation of Ser^{485} by AKT also regulates the AMPK activity. Our recent report has also shown that AICAR (5-aminoimidazole-4-carboxamide-1-β-ribofuranoside), an AMPK activator, stimulates AMPKα Thr^{172} and Ser485 phosphorylation [Lu et al., 2010c]. We thus evaluated Ser^{485} phosphorylation in response to the effects of ghrelin. Here, we demonstrated that ghrelin increases AMPKα Thr^{172} and Ser^{485} phosphorylation, and may through activated LKB1 (Ser^{428}) and CaMKII (Thr^{286}). We further showed that inhibiting CaMKII by the pharmacological inhibitor KN93 effectively reduces ghrelin-induced glioma migration, NF-κB translocation and NF-κB-DNA binding activity, suggesting that CaMKII is a convergent point to mediate AMPK activation in ghrelin-mediated glioma migration.

The NF-κB signaling pathway regulates several physiological and pathophysiological processes. Recently, our and other reports have shown that NF-κB signaling pathway play a key role of cancer cell motility in response to growth factors and adipokines [Huang et al., 2007; Su et al., 2009; Tan et al., 2009; Tang and Lu, 2009; Lu et al., 2010a,b]. The results of this study showed that NF-κB activation contributes to ghrelin-induced glioma cell migration, and that the inhibitors of the NF-κB dependent signaling pathway. It has also been reported that FAK is a potential signaling molecule regulates NF-κB and is capable of regulating glioma cell migration [Kim et al., 2005; Park et al., 2008]. Our supplementary results demonstrated that ghrelin increases phosphorylation of tyrosine 397 of FAK (Suppl. Fig. S3A). Furthermore, the FAK (Y397F) mutant and FAK siRNA antagonize the ghrelin-mediated potentiation of migration activity (Suppl. Fig. S3B), suggesting that FAK activation is involved in ghrelin-induced glioma cell migration. In the present study, we also found that treatment of human glioma cells with ghrelin results in increase IKKα/β phosphorylation, IκBα phosphorylation, p65 and p50 accumulation in the nucleus, and the binding of p65 and p50 to NF-κB element. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that ghrelin increases

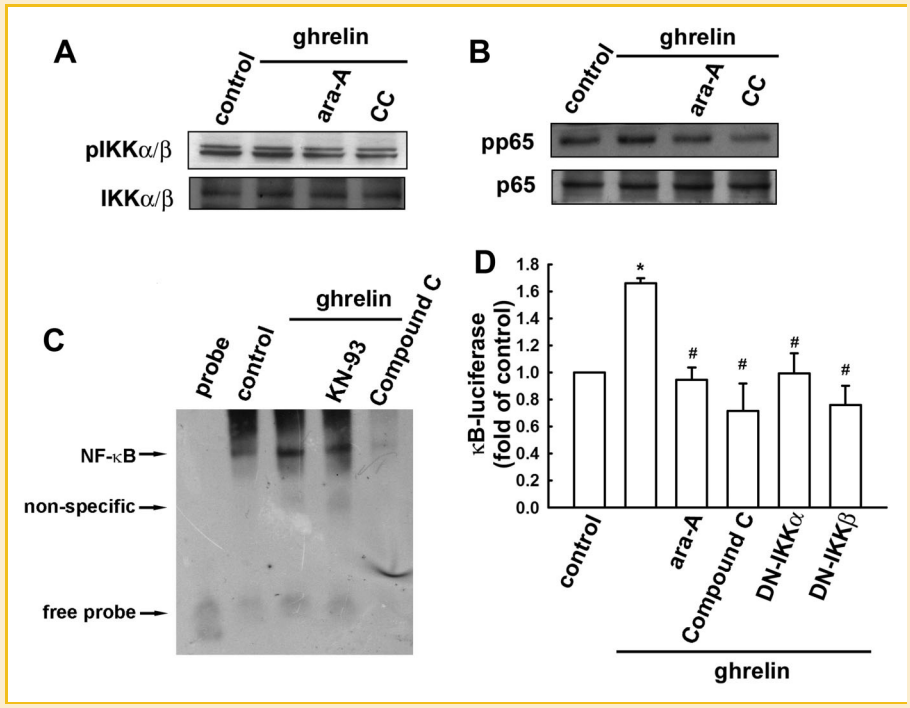

Fig. 6. Ghrelin-induced NF-κB activation is regulated by CaMKII/AMPK signaling pathway. C6 cells were pre-treatment with 9-β-D-arabinofuranoside (ara A, 20 μM) or Compound C (CC, 10 μM) for 30 min followed by stimulation with ghrelin for 60 min. The cell lysates were then evaluated using immunoblotting with antibody specific for phosphor-IKKα/β (A), and phospho-p65 Ser^{536} (B). C: Nuclear extracts were obtained from cells treated with KN-93 or Compound C followed by stimulation with ghrelin for 120 min. The nuclear extracts were incubated with a NF-κB probe and then analysis by EMSA. Results are the representatives of three independent experiments. D: κB-luciferase was assayed by pre-treatment with 9-β-D-arabinofuranoside (ara A, 20 μM) or Compound C (10 μM) for 30 min, or co-transfection with IKKα or IKKβ dominant-negative mutant for 24 h followed by stimulation with ghrelin for another 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. The control group was added equal volume of vehicle. Results are expressed as the means ± SEM of four independent experiments. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the ghrelin-treated group.

NF-κB transcriptional activity. These results suggest that ghrelin increases glioma cell migration through the GHS-R receptor and activation of CaMKII, AMPK, and IKKα/β, which enhances binding of p65 and p50 to the NF-κB site, resulting in the tumor migration.

It has been reported that the circulating ghrelin concentration is less than 1 nM in healthy human plasma [Kojima et al., 1999; Kitamura et al., 2003]. The concentration of ghrelin expression is up-regulated about two- to threefold upon fasting [Tschop et al., 2000; Dornonville de la Cour et al., 2001; Toshinai et al., 2001]. Importantly, infusion of ghrelin up to 30 nM has been used in clinical studies [Vestergaard et al., 2007; Strasser et al., 2008]. It has also been reported that administration of ghrelin decreases memory [Carlini et al., 2008] and increases anxiety-like behavior [Carlini et al., 2002] in animal model. Here, we showed that ghrelin at concentration up to 30 nM increases glioma cell migration. Our results and previous reports provide a difference concern that the use of ghrelin in clinical trials should be more careful.

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