EXPERIMENTAL STUDY

Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway

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Abstract

Objectives: Ghrelin is a brain-gut peptide with GH-releasing and appetite-inducing activities and a widespread tissue distribution. Ghrelin is the endogenous ligand of the GH secretagogue receptor type 1a (GHS-R1a), and both ghrelin and the GHS-R1a are expressed in the pituitary. There are conflicting data regarding the effects of ghrelin on cell proliferation. A positive effect on proliferation and activation of the mitogen-activated protein kinase (MAPK) pathway has been found in hepatoma, adipose, cardiomyocyte and prostate cell lines. However, ghrelin has also been shown to have anti-proliferative effects on breast, lung and thyroid cell lines. We therefore examined the effect of ghrelin on the rat pituitary cell line GH3.

Methods: RT-PCR was used for the detection of GHS-R1a and pre-proghrelin mRNA expression in GH3 cells. The effect of ghrelin on cell proliferation was studied using [3H]thymidine incorporation; cell counting and the activation of the MAPK pathway were studied using immunoblotting and inhibitors of the extracellular signal-regulated kinase 1 and 2 (ERK 1/2), protein kinase C (PKC) and tyrosine phosphatase pathways.

Results: GHS-R1a and ghrelin mRNA expression were detected in GH3 cells. Ghrelin, at 10^{-10} to 10^{-6} M concentrations, significantly increased [3H]thymidine incorporation (at 10^{-9}M, 183±13% (means±S.E.M.) compared with untreated controls), while 1,2-phorbol 1,3-myristate acetate (PMA) at 10^{-7} M (used as a positive control) caused a 212±14% increase. A reproducible stimulatory effect of desoctanoyl ghrelin was also observed on [3H]thymidine incorporation (135±5%; P < 0.01 at 10^{-9} M compared with control), as well as on the cell count (control 6.8×10^4±8.7×10^3 cells/ml vs desoctanoyl ghrelin (10^{-9} M) 1.04×10^5±7.5×10^3 cells/ml; P < 0.01). Ghrelin caused a significant increase in phosphorylated ERK 1/2 in immunoblotting, while desoctanoyl ghrelin showed a smaller but also significant stimulatory effect. The positive effect of ghrelin and desoctanoyl ghrelin on [3H]thymidine incorporation was abolished by the MAPK kinase inhibitor U0126, the PKC inhibitor GF109203X and the tyrosine kinase inhibitor tyrphostin 23, suggesting that the ghrelin-induced cell proliferation of GH3 cells is mediated both via a PKC–MAPK-dependent pathway and via a tyrosine kinase-dependent pathway. This could also be clearly demonstrated by Western blot analysis, where a transient increase in ERK 1/2 phosphorylation by ghrelin was attenuated by all three inhibitors.

Conclusion: We have shown a novel role for ghrelin in stimulating the proliferation of a somatotroph pituitary tumour cell line, suggesting that ERK activation is involved in mediating the effects of ghrelin on cell proliferation. Desoctanoyl ghrelin showed a similar effect. As ghrelin has been shown to be expressed in both normal and adenomatous pituitary tissue, locally produced ghrelin may play a role in pituitary tumorigenesis via an autocrine/paracrine pathway.

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Introduction

Ghrelin is a brain-gut peptide with various activities including growth hormone (GH) release via hypothalamic as well as direct pituitary pathways, causing an increase in appetite and direct effects on the exocrine and endocrine pancreatic function, on carbohydrate metabolism, on the cardiovascular system, on gastric acid secretion and on stomach motility, as well as on sleep (1). Ghrelin was identified as the endogenous ligand of the GH secretagogue receptor type 1a (GHS-R1a), but probably other, currently unidentified, receptors also play a role in its diverse effects (2, 3). The 28 amino acid peptide has several variants with similar biological activity, all of these analogues being subject to a unique post-translational modification:
the hydroxyl group of the third residue serine is esteri-
6fied by octanoic acid (4, 5). Ghrelin is the first natural
peptide reported to have this modified modification
which is essential for its GH-releasing activity (2). We
have previously demonstrated the widespread tissue
distribution of ghrelin mRNA in the human, while
the expression of the GHS-R1a is much more limited,
and it has been suggested that ghrelin may have an
even wider role than previously thought with some of
these effects probably mediated via a receptor different
from the cloned GHS-R1a (6).

There are conflicting reports on the effect of ghrelin
on cell proliferation. It has been shown to have an
anti-proliferative effect on thyroid, breast and lung
cancer cell lines (7–10) whilst other studies have
shown a (pro-)proliferative effect on prostate, liver,
adrenal, cardiac and adipose cells (11–16). Ghrelin
and the GHS-R1a are both present in the pituitary
(17), but possible effects on pituitary cell proliferation
have not previously been reported.

In this study we have therefore examined whether
ghrelin affects proliferation of a somatotroph pituitary
tumour cell line, and whether any such effects involve
the extracellular signal-related kinase (ERK) family of
mitogen-activated protein kinase (MAPK) proteins.

Materials and methods

Cell culture

The rat somatotroph cell line GH3 cells were obtained
from ATCC (LGC Promochem, Teddington, London,
UK) and were grown in monolayer in culture medium
containing Dulbecco’s modified Eagle’s medium sup-
plemented with high glucose (4500 mg/l) containing
10% (v/v) fetal calf serum (FCS), penicillin
and streptomycin (100 µg/ml), and fungizone
(125 µg/l) (Life Technologies, Paisley, Strathclyde,
UK). Cells were cultured at 37 ºC in a humidified atmos-
phere of 95% air and 5% CO2. Cells were plated at a
density of 1 × 10⁶ cells/well in six-well plates for
Western blotting for ERK, and 5 × 10⁴ cells/well in
24-well plates for the proliferation studies.

RT-PCR

Total RNA was prepared from 10⁶ cultured GH3 cells
using the SV total RNA isolation system (Promega,
Southampton, Hants, UK), which includes a deoxyribonu-
cl ease step, according to the manufacturer’s instruc-
tions, and cDNA was synthesized as described previously (18).
Omission of reverse transcriptase and water replacing the ribonucleic acid template were used
as a negative controls. The PCR was performed using
intron-spanning rat GH3-R1a primers (5’-CTCATCAGGG-
AGAAGCTATG-3’ sense and 5’-CAGTTGCAGTACTG-
GCTGA-3’ antisense; GenBank number U94321) and
ghrelin primers (5’-TGGAGCCAGAGCACCAGAMA-3’
sense and 5’-CTGAGCTCTTGACAGCTTGA-3’ antisense;
GenBank number AB029433). The PCR mixture con-
sisted of 5 µl cDNA, 1 µl 20 mM rat GHS-R1a primers
or rat ghrelin primers, 2.5 µl 10 × Taq buffer, 1.5 µl
MgCl₂ (1.5 µM), 0.25 µl dNTP (20 mM), 0.125 µl Taq
enzyme (Promega) and 13.625 µl water to a final
volume of 25 µl. The reaction mixture was subjected to
40 cycles of PCR amplification consisting of denaturation
for 60 s at 94 ºC, annealing for 60 s at 55 ºC and exten-
sion for 60 s at 72 ºC. after an initial denaturation for
5 min at 95 ºC. A final extension cycle of 10 min at
72 ºC was used. The oligonucleotide primers allowed
amplification of a 216 base pair GHS-R1a and a 188
base pair ghrelin product, which were verified by restric-
tion enzyme analysis and sequencing.

[³²P]thymidine incorporation

GH3 cells were plated at 5 × 10⁴ cells/well in media
containing 2% charcoal-stripped FCS and various con-
centrations of ghrelin, desoctanoylated ghrelin and
12-phorbol 13-myristate acetate (PMA; 10⁻⁷ M) or epi-
dermal growth factor (EGF; 10⁻⁸ M; positive control;
CN Biosciences, Nottingham, Notts, UK) for 72 h with
the addition of 2 µCi/well [³²P]thymidine (Amersham
International plc, Amersham, Bucks, UK) for a further
6 h. A time-course of 24 h, 48 h and 72 h was per-
formed for ghrelin stimulation and 72 h was selected for
further experiments. Studies were also performed to
investigate the effect of rat ghrelin or desoctanoyl
ghrelin-induced (a kind gift from M Kojima, Kurume
University, Fukuoka, Japan) proliferation and the
effect of U0126 (an MAPK kinase (MEK) inhibitor),
GF109203X (a protein kinase C (PKC) inhibitor; both
from CN Biosciences), tyrphostin 23 (a tyrosine
kinase inhibitor; Sigma, Poole, Dorset, UK), wortma-
nin (a phosphatidylinositol 3-kinase (PI(3)K) inhibitor;
CN Biosciences) and H-89 (a protein kinase A (PKA)
inhibitor; Sigma) upon ghrelin-induced MAPK stimu-
ation. In these experiments U0126 was used at
10⁻⁴ M, a concentration previously reported to block
half-maximal ERK activity (19). GF109203X at
10⁻⁶ M (20) and 10⁻⁸ M, tyrphostin at 10⁻⁵ M (14),
wortmannin at 5 × 10⁻⁹ M (21) and H89 at 10⁻⁷ M
(22). Inhibitors were added 30 min before each treat-
ment. Cells were harvested before counting in the pre-
sence of scintillation fluid using a Microbeta 1450
counter (Wallac, Turku, Finland). Experiments were
repeated at least three times.

Cell count

GH3 cells were plated at 5 × 10⁴ cells/well in media
containing 2% charcoal-stripped FCS and were treated
with stimulators and inhibitors as above. Cell number
was counted 24 h, 48 h and 72 h after treatment.
Protein extractions and measurement of ERK

Serum-starved GH3 cells were treated with $10^{-6}$ M to $10^{-10}$ M ghrelin, $10^{-9}$ M desoctanoylated ghrelin or $10^{-7}$ M PMA as a positive control. In the inhibitory studies, $10^{-6}$ M U0126, $10^{-6}$ M and $10^{-8}$ M GF109203X or $10^{-5}$ M tyrphostin 23 were added 30 min before treatment. After stimulation for 5 or 15 min, the cells were washed briefly with ice-cold phosphate-buffered saline (PBS) before treatment with Cytobuster protein extraction reagent (Novagen, CN Biosciences) and a phosphatase inhibitor, phosphatase cocktail I and II (Sigma). Cells were incubated on ice for a further 5 min. The protein concentration of the lysates was normalized using the Bradford assay (Bio-Rad, Hemel Hempstead, Middx, UK). Typically, 7–30 µg samples were subjected to 10% SDS-PAGE separation, with protein transfer to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk for 90 min and incubated overnight at 4°C using anti-phosphorylated ERK 1/2 or anti-total ERK 1/2 (both at 1:10000) as the primary antibodies (Promega). The membrane was washed three times with PBS containing 0.05% Tween and subsequently incubated with horseradish peroxidase-conjugated anti-rabbit antibody as the secondary antibody (1:10 000; Dako, Glostrup, Denmark) for 2 h at room temperature. A chemiluminescent peroxidase substrate, ECL Plus (Amersham-Pharmacia, Amer- sham, Bucks, UK), was applied according to the manufacturer’s instructions, and the membranes were exposed briefly to X-ray film. Total ERK 1 and 2 (ERK 1/2) was determined by stripping the PVDF membrane with 0.985 g Tris–hydrochloride, 2 g SDS, 781 µl 2-mercaptoethanol (pH 4), and re-probing for the total with anti-total ERK 1/2 antibody. Densitometric readings of the resulting bands were evaluated using the Scientific Imaging system (Kodak, New Haven, CT, USA). Experiments were repeated at least three times.

Data presentation and analysis

The data were analysed using the StatsDirect computer software program (I Buchan, Cambridge, Cambs, UK). The data were not normally distributed, as assessed by the Shapiro–Wilk test. The Kruskal–Wallis test was therefore used followed by the Conover–Inman test. The data were calculated from at least three separate experiments and are shown as means±S.E.M. Significance was taken at $P < 0.05$.

Results

GHS-R1a and pre-proghrelin mRNAs were shown to be expressed by the GH3 pituitary somatotroph cell line using RT-PCR (Fig. 1). PMA caused the expected increase in [3H]thymidine incorporation of GH3 cells after 72 h of treatment (212±14% above untreated controls; $P < 0.001$; Fig. 2). Ghrelin significantly increased proliferation at $10^{-10}$ to $10^{-6}$ M concentrations ($10^{-10}$ M 134±8% ($P < 0.01$), $10^{-9}$ M 183±13% ($P < 0.001$), $10^{-7}$ M 134±5% ($P < 0.01$), $10^{-6}$ M 153±12% ($P < 0.001$)) above untreated controls, suggesting increased DNA synthesis of GH3 cells. A dose–response curve with desoctanoylated ghrelin at $10^{-6}$ to $10^{-12}$ M also showed a significant increase in proliferation at $10^{-10}$ to $10^{-6}$ M concentrations ($10^{-10}$ M 149±18% ($P < 0.01$), $10^{-9}$ M 149±8% ($P < 0.01$), $10^{-8}$ M 139±3% ($P < 0.01$), $10^{-6}$ M 151±16% ($P < 0.01$); Fig. 2).

We next studied the effect of the inhibitors $10^{-6}$ M U0126, $10^{-6}$ M and $10^{-8}$ M GF109203X and $10^{-7}$ M tyrphostin 23, on ghrelin-activated cell proliferation. The effect of these inhibitors on their appropriate pathways are shown in Fig. 3: $10^{-7}$ M PMA (known to activate the PKC–MAPK pathway) and $10^{-8}$ M EGF (which activates the tyrosine kinase–MAPK pathway) and inhibitors for both pathways were used. The PKC inhibitor GF109203X inhibited the effect of PMA, but
not EGF, while the tyrosine kinase inhibitor tyrphostin 23 inhibited the effect of EGF, but not PMA, as expected (Fig. 3). $10^{-5}$ M and $10^{-6}$ M ghrelin-induced proliferation ($183 \pm 13\%$ and $153 \pm 12\%$) was significantly attenuated by $10^{-6}$ M U0126 ($96 \pm 11\%$ and $90 \pm 9\%$ respectively; $P < 0.001$ for both compared with ghrelin at $10^{-5}$ M and $10^{-6}$ M alone; Fig. 4A and B). GF109203X showed inhibition at both concentrations used; GF109203X at $10^{-8}$ M compared with ghrelin at $10^{-9}$ M and ghrelin at $10^{-6}$ M ($78 \pm 10\%$ ($P < 0.0001$) and $71 \pm 8\%$ ($P < 0.01$), and GF109203X at $10^{-6}$ M $44 \pm 3\%$ and $52 \pm 1\%$ (both $P < 0.0001$). Furthermore, $10^{-5}$ M tyrphostin 23 caused significant inhibition of the cell proliferation caused by $10^{-9}$ M and $10^{-6}$ M ghrelin ($86 \pm 4\%$ and $81 \pm 4\%; P < 0.001$ for both; Fig. 4A and B). The inhibitors on their own all significantly inhibited cell proliferation compared with the control: $10^{-6}$ M U0126 $74 \pm 5\%$ ($P < 0.05$), $10^{-5}$ M GF109203X $65 \pm 12\%$ ($P < 0.01$), $10^{-6}$ M GF109203X $48 \pm 3\%$ ($P < 0.0001$), $10^{-5}$ M tyrphostin 23 $75 \pm 2\%$ ($P < 0.01$; Fig. 4C). The inhibition caused by the inhibitors was much greater for the ghrelin-stimulated response than the inhibition of the unstimulated proliferation; for example, the proliferation induced by $10^{-9}$ M ghrelin was reduced by U0126 by $87\%$ while the change for basal proliferation was inhibited only by $26\%$. Similar to ghrelin, the effect of desoctanoyl ghrelin on thymidine incorporation was inhibited by all three inhibitors (U0126 $62\%$, $10^{-8}$ M GF109203X $84\%$ and tyrphostin 23 $34\%; P < 0.01$ for each). The treatment of cells with $10^{-9}$ M and $10^{-6}$ M ghrelin combined with EGF or PMA showed no inhibition or synergism of the proliferative effect (data not shown).

Desoctanoyl ghrelin significantly increased cell numbers compared with normals at 48 and 72 h of incubation (at 72 h, control $6.8 \times 10^{\pm 8.7} \times 10^3$ cells/ml vs $1.04 \times 10^{\pm 7.5} \times 10^3$ cells/ml; $P < 0.01$), while the effect was significantly inhibited by U0126 ($2.38 \times 10^{\pm 5.5} \times 10^3$ cells/ml; $P < 0.001$) (Fig. 5).

The effect of ghrelin on phosphorylated ERK 1/2 was evaluated with immunoblotting with PMA as positive control. Stimulation of GH3 cells with ghrelin ($10^{-7}$ M) failed to inhibit ghrelin-induced proliferation (data not shown).

The PI(3)K inhibitor wortmannin (used at a concentration of $5 \times 10^{-9}$ M) and the PKA inhibitor H-89 (used at $10^{-7}$ M) failed to inhibit ghrelin-induced proliferation (data not shown).

The effect of ghrelin on phosphorylated ERK 1/2 was evaluated with immunoblotting with PMA as positive control. Stimulation of GH3 cells with ghrelin ($10^{-7}$ M to $10^{-10}$ M) led to an increase in the amount of phosphorylated ERK 1/2 (Fig. 6A). Maximal
phosphorylation was reached at 5 min, with a slight attenuation at 15 min. Similar to the proliferation studies, desoctanoylated ghrelin also showed increased activation of ERK 1/2 compared with control (P < 0.01; Fig. 6B).

To assess the effect of signalling pathway inhibitors on ERK 1/2 phosphorylation, cells were pretreated with the MEK inhibitor U0126, the tyrosine kinase inhibitor tyrphostin 23 and the PKC inhibitor GF109203X at the concentrations described above. Similar to the proliferation studies, GF109203X inhibited the action of PMA while tyrphostin 23 inhibited the effect of EGF, but not vice versa (data not shown). All three inhibitors blocked the ghrelin-stimulated increase in ERK 1/2 phosphorylation (Fig. 7). These results suggested that the signalling cascade from ghrelin to the ERK is mediated through PKC as well as transactivation via the tyrosine kinase pathways.

**Discussion**

These studies provide novel evidence that ghrelin stimulates proliferation of the GH3 pituitary somatotroph tumour cell line. It also suggests that ERK activation mediates this effect. Both these effects may be mediated through the GHS-R1a, shown to be expressed in these cells. Our finding may link circulating or locally produced ghrelin to pituitary tumorigenesis via an autocrine/paracrine pathway. Indeed, ghrelin mRNA is expressed in pituitary tumours with the highest levels in non-functioning pituitary adenomas; however, the level of ghrelin mRNA expression did not correlate with tumour size or with the grading of the tumours (17, 23). It has been demonstrated that GHS-R1a mRNA expression is increased in some somatotroph tumours, suggesting that the receptor may be a promoting factor in tumorigenesis (17, 24). A study in prostate cancer cell lines (11) has shown co-expression of the GHS-R1a and ghrelin, as in pituitary cells, and demonstrated increased cell proliferation in vitro in response to ghrelin, implying a potential promoting role for ghrelin in this tissue. Similarly, cultured adrenal zona glomerulosa cells also showed an increased rate of proliferation (14). Ghrelin also activates the MAPK pathway and prevents apoptosis in cultured primary or H9c2 cardiomyocytes (16). Baldanzi et al. suggest...
that ghrelin inhibits cell death by activating at least two survival signalling pathways conveyed by ERK 1/2 and PI(3)K/Akt (16). Ghrelin-stimulated cell proliferation seen in this study may occur directly via the MAPK pathway involving the GHS-R1a (Fig. 8). The MAPK pathway consists of a cascade of signalling molecules that sequentially activate each other by phosphorylation, the final messenger being able to migrate to the nucleus to phosphorylate transcription factors involved in gene transcription. Mammalian cells contain three major classes of MAPK, the extracellular signal-related kinases, c-Jun N-terminal kinase/stress-activated protein kinases and p38 MAPK (25). However, the MAPKs ERK 1/2 are thought to be key signalling molecules in the control of gene transcription events that lead to cell proliferation. It is now well established that not just cytokine and growth factor receptors but also G protein-coupled receptors can stimulate the MAPK pathway (26). We have, for example, shown that pituitary adenylate cyclase-activating polypeptide can stimulate ERK 1/2 via its G protein-coupled receptor (GPCR), stimulating phospholipase C and PKC (27), and similar effects have been demonstrated for other Gq-coupled receptors such as gonadotrophin-releasing hormone and thyrotrophin-releasing hormone (28, 29). Interestingly, it has recently been demonstrated that peptides with receptors primarily working via the cAMP pathway can also activate the PKC pathway and cause activation of MAPK (30). The transactivation of the tyrosine kinase pathway by GPCRs has been well established, probably acting via the β and γ subunits of the G protein (31, 32). The GHS-R1a belongs to the family of receptors operating via the Gq-phospholipase C (PLC) pathway (33). Receptors operating via this specific pathway have been shown to transactivate tyrosine kinase receptors (34, 35). It has been suggested that in adrenal cells the proliferative effect of ghrelin is via the tyrosine kinase pathway and independent of PKC, as a PKC inhibitor did not attenuate the effect of ghrelin while the tyrosine kinase inhibitor did (14). Our studies suggest a slightly different mechanism in the pituitary as an inhibitory effect of both a tyrosine kinase inhibitor as well as a PKC inhibitor was observed (Figs 4, 7 and 8). The mechanism of the effect of ghrelin on MAPK activation could include the activation of the GHS-R1a receptor, although some data point to the involvement of alternative pathways. Via the α subunit of this receptor the PLC–PKC pathway is activated and Raf–MEK–MAPK activation occurs. Activation of the GHS-R1a also leads to transactivation of a tyrosine kinase receptor via the β and γ subunits, which activates MAPK via the Ras–Raf–MEK pathway. We found no effect of PKA inhibitor H89 and PI(3)K inhibitor wortmannin on the ghrelin-induced cell proliferation. The cytoprotective effect of ghrelin on apoptosis in cardiomyocytes was abolished by wortmannin, but higher doses (10−7 M) were used in that study (16). The presence of multiple intracellular mechanisms has been previously demonstrated in other systems (36). The different effects of ghrelin or its analogues on cell proliferation in different cell types is intriguing, but could be explained by differences in the specific array of second messengers and transcription factors available in a specific tissue (37). Parallel demonstration of these opposite effects using the same experimental set up and conditions would be of considerable interest. We have shown earlier that Chinese hamster ovary (CHO) cells do not contain functional GHS receptors (38).
and currently we are studying the effect of ghrelin and des-octanoyl ghrelin on the proliferation of CHO cells with and without transfection with the human GHS-R. Ghrelin in its active, octanoylated form stimulates calcium release and GH release both in vitro and in vivo, while the desoctanoyl form, which is present at a much higher concentration in the circulation as well as in tissues, is completely inactive regarding these effects (2, 17). The majority of the studies investigating the various effects of ghrelin have not studied the effect of desoctanoyl ghrelin. However, desoctanoyl ghrelin has been shown to have an anti-proliferative effect in breast cancer lines (9) and it can prevent apoptosis in cardiomyocytes via the MAPK pathway (16). Our results, showing a significant effect of desoctanoyl ghrelin on cell proliferation, which can be inhibited by all the three inhibitors used, and its effect on phosphorylated ERK 1/2 activation in GH3 cells, are therefore not completely unexpected. Despite the presence of GHS-R1a mRNA in GH3 cells (39), similar to GH-releasing hormone, GHSs do not release GH from this cell line (40). Future studies are necessary to investigate the effects of desoctanoyl ghrelin and identify whether it acts via the classical GHS-R1a receptor or via alternative mechanisms.

In summary, we have shown that ghrelin as well as desoctanoyl ghrelin causes stimulation of GH3 cell proliferation via activation of the MAPK pathway.

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