Ghrelin, a 28-amino-acid n-octanoylated peptide, acts via the growth hormone secretagogue receptor (GHS-R) to stimulate growth hormone release (1, 2) and has a range of other biological actions including stimulation of food intake, control of energy expenditure, modulation of insulin signaling and cardiovascular effects (3–7). The finding that ghrelin has a proliferative effect was first described in the HepG2 hepatoma cell line (6) and prostate cancer cell lines (8). Subsequently, it has been shown that growth of other cell types is enhanced by ghrelin (9–18). We have recently shown that ghrelin also stimulates proliferation in several breast cancer cell lines (19) in contrast to earlier studies (20).

Both ghrelin and the GHS-R (a G protein–coupled receptor) are widely expressed in normal tissues (1, 21–23) as well as in various tumors, including human pituitary adenomas and various endocrine neoplasms of the lung, stomach, and pancreas (24–28). We have previously shown that components of the ghrelin/GHS-R axis, including an apparent human exon 3–deleted preproghrelin mRNA variant, are expressed in prostate cancer cell lines (8). The exclusion of the third exon from the preproghrelin transcript causes a translational frameshift which results in expression of a unique truncated proghrelin C-peptide, as well as mature ghrelin, in human and mouse tissues (29, 30).

Few studies have investigated the signaling mechanism of the endogenous ligand ghrelin through the GHS-R. In the pituitary, ghrelin activates the GHS-R, triggering activation of protein kinase C and a transient increase in intracellular calcium levels, which in turn induces growth hormone secretion (11). Recent reports have indicated that ghrelin can also activate mitogen-activated protein kinase (MAPK) cascades in some tissues (11, 12, 14, 16, 17, 31, 32). The MAPK signaling pathways play...
an important role in mammalian cells where they modulate many cellular events including cell proliferation, differentiation, and development (33). The GHS-R can cross-talk with the MAPK pathway to promote cell proliferation in several cancer cell lines, including HepG2 hepatoma cells, pancreatic adenocarcinoma cells, and GH3 rat pituitary somatotroph cells (6, 10, 12). Similarly, activation of MAPK pathways could be involved in the increased proliferation observed in prostate cancer cells after ghrelin treatment (8).

The data presented here provide the first evidence that ghrelin and exon 3–deleted preproghrelin are highly expressed in prostate carcinoma specimens compared with normal prostate tissues and that the unique COOH-terminal peptide derived from exon 3–deleted preproghrelin is cleaved in the LNCaP prostate cancer cell line. We also show that the PC3 and LNCaP prostate cancer cell lines secrete mature ghrelin and that ghrelin initiates cross-talk with the MAPK signaling cascade to promote cell proliferation in prostate cancer cell lines. These findings suggest that the ghrelin/GHS-R axis could play an important role in prostate cancer.

Materials and Methods

Cell culture. Androgen-independent PC3 and DU-145 and androgen-dependent LNCaP and ALVA-41 human prostate cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cells were cultivated in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen), penicillin G (50 units/mL), and streptomycin sulfate (50 µg/mL; Invitrogen) in 80-cm² tissue culture flask (Nagle Nunc International, Roskilde, Denmark) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Cells were tested to be free from Mycoplasma contamination (American Type Culture Collection Mycoplasma detection kit).

Reverse transcriptase-PCR. Reverse transcription of prostate cancer cell line mRNA and normal prostate mRNA (Clontech, Palo Alto, CA) using Superscript II reverse transcriptase (Invitrogen) was done as previously described (8). Reverse transcription-PCR to detect full-length preproghrelin and exon 3–deleted preproghrelin was done in 50-µL reactions containing 1 unit of Red Hot Polymerase (Integrated Sciences, Melbourne, Australia), 10 × PCR buffer (Integrated Sciences), 100 µmol/L deoxynucleotide triphosphates (Roche, Basel, Switzerland), 100 pmol/L sense (5'-gccacctgctgcaacct-3') and antisense primers (5'-gagaccttggctctcg-3', Proligo, Armidale, Australia) from ghrelin exons 2 and 5, respectively, and 2 µL of CDNA or sterile distilled water (no template negative control). Thermal cycling consisted of 5 minutes of denaturation at 95°C, 40 cycles of 30 seconds at 95°C, 30 seconds at annealing temperature (50°C), 1-minute extension at 72°C, followed by a final 10-minute extension at 72°C on a PTC-200 Thermal cycler (MJ Research, Watertown, MA). DNA sequencing of the purified PCR product was carried out at the Australia Genome Research Facility (Brisbane, Australia) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit protocol (Applied Biosystems, Foster City, CA). Sequences were identified using BLAST software (Entrez).

Immunohistochemistry for ghrelin and exon 3–deleted preproghrelin expression in prostate tissue sections. Protein expression and localization of ghrelin and the exon 3–deleted preproghrelin variant were detected using immunohistochemistry on paraffin-embedded prostate tissue sections. Twenty-six prostate cancer and 11 benign prostatic hypertrophy tissue specimens were obtained with ethical approval (Queensland University of Technology ethics approval 1992H) and normal prostate sections were purchased from Peterborough Hospital Tissue Bank (Peterborough, United Kingdom). Tissue sections were microwaved in citric acid buffer for 10 minutes (pH 6) to enhance antigen retrieval, then incubated overnight with decreasing concentrations of primary antibody diluted in 0.01 mol/L PBS containing 1% bovine serum albumin (BSA; Sigma, St Louis, MO). Immunodetection was done using the Envision plus diaminobenzene staining kit (DAKO, Kyoto, Japan) according to the instructions of the manufacturer. Polyclonal anti-ghrelin antibodies were raised in rabbits (Institute for Medical and Veterinary Sciences, Adelaide, Australia) against the mature Ser-3 n-octanoylated human ghrelin peptide (GSSFLSPEHQRVQKRSEKPKPAKLIQPR) conjugated to diptheria toxin (Mimotopes, Melbourne, Australia) and affinity purified as previously described (8). Antibodies were also raised against the putative 16-amino-acid COOH-terminal peptide encoded by the exon 3–deleted preproghrelin variant (COOH-terminal Δ3 peptide; RPQPTSDRPQALLTSL; Mimotopes) and affinity purified. For both antibodies, negative controls included omission of primary antibody and preabsorption of antibody with excess peptide (1 mg/mL) to which the antibody was raised.

Western blot analysis of prostate cancer cell line lysate and conditioned media. To establish if PC3 and LNCaP cell lines secrete ghrelin into conditioned media, Western blot analysis was undertaken. PC3 or LNCaP cells (3 × 10⁶ seeded into 80-cm² tissue culture flasks) were allowed to attach overnight at 37°C. They were incubated in serum-free RPMI 1640, 0.1% BSA, and 20 mmol/L 4-(2-aminoethyl)benzenesulfon fluoride hydrochloride (Sigma) for 24 hours and conditioned medium was collected and frozen at –80°C. Medium was concentrated using Centriprep Centrifugal filter units with Ultracel-YM membranes and a 3-kDa nominal molecular weight cutoff (Millipore, Bedford, MA) according to the instructions of the manufacturer. Concentrated medium was boiled for 5 minutes at 100°C in 2× Tricine sample buffer [200 mmol/L Tris-HCl (pH 6.8), 2% SDS, 40% glycerol, 0.04% Coomassie blue G-250, and 350 mmol/L DTT] and electrophoresed through a 10% to 20% Tricine gradient gel (Bio-Rad, Hercules, CA). The protein was electrotransferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 1 hour at 4°C at 200 mA in transfer buffer (10 mmol/L NaHCO₃, 3 mmol/L Na₂CO₃, 20% methanol, pH 9.9). After the membrane was blocked overnight with 1% BSA in PBS, it was incubated with the polyclonal anti-ghrelin antibody diluted in TBS-0.05% Tween 20 containing 1% BSA for 1 hour at room temperature. After several brief washes in TBS-0.05% Tween 20, the membrane was incubated with horseradish peroxidase–conjugated antirabbit secondary antibody (1:100,000 dilution; Jackson Immunoresearch, West Grove, PA) at room temperature for 1 hour. After several brief washes, chemiluminescent SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) was applied and the membrane was exposed to X-ray film and developed (Agfa-Gaert, Morstel, Belgium). Ghrelin peptide and fresh serum-free RPMI 1640 were electrophoresed concurrently with the samples as positive and negative controls. Western blot analysis on LNCaP prostate cancer cell line, human stomach carcinoma, and normal human stomach lysates (Pierce) was also done as previously described (8) to determine if COOH-terminal Δ3 peptide is cleaved from the rest of the exon 3–deleted preproghrelin peptide and to ensure the specificity of the anti–COOH-terminal Δ3 peptide antibody.

Mitogen-activated protein kinase activation assays. PC3 and LNCaP cells were plated in a six-well plate at a seeding density of 6 × 10⁵ per well, grown to 70% to 80% confluency, and then serum deprived overnight at 37°C. Mature n-octanoylated ghrelin peptide (or COOH-terminal Δ3 peptide) was dissolved in 1× PBS, 0.05% BSA and added to RPMI 1640 with antibiotics, 0.1% BSA, and 20 mmol/L 4-(2-aminoethyl)benzenesulfon fluoride hydrochloride. Cells were treated with various concentrations of ghrelin or the COOH-terminal Δ3 peptide (0, 1, 10, 100, and 1,000 nmol/L) for 5, 15, 30, and 60 minutes at 37°C. As a positive control, PC3 cells were treated with 1 µg/mL anisomycin, a potent MAPK pathway stimulating agent (34). All cells were harvested in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₂VO₄, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L β-glycerophosphate] and 1 Complete
EDTA-free protease inhibitor buffer in 20-mL buffer (Roche). The cell lysates were kept on ice for 30 minutes and cleared by centrifugation (14,000 × g) for 20 minutes at 4°C. Equal amounts of protein (10 μg/lane) were prepared for each sample and denatured with 4× sample loading buffer [250 mmol/L Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 20 mmol/L DTT, 0.01% bromophenol blue]. Samples were then boiled at 100°C for 5 minutes and separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to a Protran nitrocellulose membrane as previously described (8). To detect MAPK activation, the membrane was probed (overnight) with a primary polyclonal rabbit antibody (diluted 1:5,000 in 1% BSA/TBS-0.05% Tween 20) raised against the activated (phosphorylated) p44/42 [extracellular signal-regulated kinase (ERK) 1/2] MAPKs (Promega, Madison, WI). Total ERK1/2 (both active and inactive forms) was detected using a rabbit anti-ERK antibody (1:5,000; Promega) as a loading control to allow normalization of active ERK1/2 levels. A mouse anti-β-tubulin monoclonal antibody (1:2,000; Upstate Biotechnology, Inc., Lake Placid, NY) was also used as a loading control. The c-Jun NH2-terminal kinase (JNK) and p38 MAPK pathways were interrogated using rabbit anti–active JNK (1:3,000) and p38 monoclonal antibodies (1:2,000; Promega). The membrane was washed five times in TBS-0.05% Tween 20 and incubated with horseradish peroxidase–linked antirabbit immunoglobulin G antibody (1:100,000 dilution; Jackson ImmunoResearch) or with anti-mouse immunoglobulin G (1:5,000; Jackson ImmunoResearch) to detect the β-tubulin antibody. The membrane was then treated with chemiluminescent substrate and signal was detected as described above. Activation (phosphorylation) levels of ERK were assessed using ImageJ software (National Center for Biotechnology Information) under conditions that yielded a linear response, with normalization to total ERK.

To investigate whether ghrelin-induced MAPK activation (phosphorylation) can be suppressed by blocking MAPK kinase 1 and 2 (responsible for activating ERK1/2), the MAPK inhibitors PD98059 and U0126 were employed. The MAPK activation assay and Western blot analysis described above were done after the cells were treated with either 30 μmol/L PD98059 or 10 μmol/L U0126 for 30 minutes before treatment with 10 nmol/L ghrelin for 15 minutes. Previous studies had shown that the chosen concentrations for PD98059 and U0126 were sufficient to completely suppress MAPK activation (35, 36). Control incubations included incubation with 10 nmol/L ghrelin alone, 30 μmol/L PD98059 or 10 μmol/L U0126 alone, no treatment, or vehicle alone (DMSO, ICN Biomedicals, Cleveland, OH) for 15 minutes. The maximum concentration of DMSO in any experiment was 0.1% (v/v), which does not affect the activation of any MAPKs examined (37).

Cell proliferation and apoptosis assays. Cell proliferation assays were carried out using the Sigma 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay protocol. LNCaP cells were resuspended in RPMI 1640 containing 10% fetal bovine serum and seeded into 96-well plates at 10,000 per well and cultured for 72 hours in the presence or absence of synthetic n-octanoylated ghrelin or COOH-terminal Δ3 peptide at concentrations ranging from 0 to 1,000 nmol/L. Medium was replaced every 24 hours for both treated and untreated cells. After 72 hours, the medium was aspirated and the cells incubated in 0.5 μg/mL MTT diluted in phenol red–free RPMI 1640 for 2 hours. A multwell plate reader (Beckman Biomek Plate Reader, Brisbane, Australia) was used to measure absorbances at 550 nm with a reference wavelength of 650 nm.

To investigate whether ghrelin protects prostate cancer cell lines from chemically induced apoptosis, LNCaP cells were seeded in 96-well plates at a density of 10,000 per well and preincubated with 0, 1, 10, 100, or 1,000 nmol/L n-octanoylated ghrelin for 15 minutes. Cells were then coincubated for 24 hours with fresh media containing ghrelin and 1 μg/mL actinomycin D (Sigma), a potent apoptotic agent in cancer cell lines (38). Medium was aspirated and cell viability was quantified using the MTT dye technique as described above. Apoptosis assays were also repeated in the same manner using COOH-terminal Δ3 peptide instead of ghrelin.

Additionally, cell proliferation assays were done in the presence of the potent MAPK kinase inhibitors PD98059 and U0126. Briefly, 72 hours before the assay, cells were seeded into 96-well plates (5,000 per well for PC3 cells; 10,000 per well for LNCaP cells) and allowed to attach overnight. After 24 hours, the medium was replaced with serum-free RPMI 1640 with 10% fetal bovine serum, antibiotics, 0.1% BSA, and 20 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride with or without ghrelin (10 nmol/L) ± either PD98059 (30 μmol/L), U0126 (10 μmol/L), or vehicle (0.1% DMSO). The MTT assay was then carried out to measure cell proliferation. For all of the functional assays, three independent experiments were done with n = 16 for each treatment.

Statistical analysis. The data were expressed as mean ± SE unless otherwise stated. The statistical significance was tested using one-way ANOVA followed by Tukey’s post hoc comparisons. P < 0.05 was considered statistically significant.

Results

Full-length preproghrelin and exon 3–deleted preproghrelin expression in prostate cancer cell lines and tissues. Full-length preproghrelin mRNA transcripts of the predicted size (501 bp) were detected in ALVA-41, DU-145, LNCaP, and PC3 prostate cancer cell lines and normal prostate tissue cDNA by nonquantitative reverse transcription-PCR (Fig. 1A). A smaller band (392 bp) representing exon 3–deleted preproghrelin (confirmed by cDNA sequencing) was also identified in the cell lines and normal tissue (Fig. 1A). Coding mRNA for exon 3–deleted preproghrelin is predicted to produce a 91-amino-acid protein with a unique COOH-terminal peptide sequence (COOH-terminal Δ3 peptide; Fig. 1B).

Both the mature ghrelin peptide and the COOH-terminal Δ3 peptide are expressed in normal prostate and prostate cancer tissue as indicated by immunohistochemical analysis (Fig. 1C). The staining intensity for each antibody was quantified by a 0 to 3+ scale, with 0 representing no staining and 3+ signifying the most intense immunoreactivity. In the representative tissues shown here, ghrelin expression is mostly confined to the glandular epithelial cell cytoplasm with staining intensity lower in the normal glandular tissue (Fig. 1C-iv) compared with malignant prostate epithelium (Fig. 1C-iii). Lower levels of expression of the COOH-terminal Δ3 peptide were also evident in normal prostate tissue (Fig. 1C-iv) compared with prostate cancer tissue (Fig. 1C-v). Negative controls were nonimmunoreactive (Fig. 1C-i). Multiple fields from each slide were examined. There was a consistent increase in staining for both ghrelin and the COOH-terminal Δ3 peptide in 100% of the 26 prostate cancer specimens examined compared with normal and benign prostatic hyperplasia tissues, with all cancer specimens scored as 2+ or 3 for ghrelin and 3+ for the COOH-terminal Δ3 peptide. None of the benign glands exhibited staining of ≥1 for either peptide (Table 1).

Secretion of ghrelin into conditioned medium by prostate cancer cells and expression of COOH-terminal Δ3 in the LNCaP cell line. Western blot analysis of conditioned medium, which had been collected from a 24-hour culture of the PC3 cell line, detected protein of ~3 kDa, which was the same size as a concurrently electrophoresed sample of synthetic n-octanoylated mature (28-amino-acid) ghrelin peptide (Fig. 2A). A similar protein band was detected in LNCaP conditioned medium (data not shown) showing that mature
Ghrelin is secreted from both prostate cancer cell lines. The COOH-terminal Δ3 peptide from exon 3–deleted preproghrelin was detected in the LNCaP prostate cancer cell line lysate, as well as in human stomach carcinoma and normal stomach tissue lysates, by Western blot analysis (Fig. 2B). The endogenous peptide ran at approximately the same size (2 kDa) as the synthetic 16-amino-acid peptide (Mimotopes; Fig. 2B). This shows that the COOH-terminal Δ3 peptide is cleaved from the remainder of the exon 3–deleted preproghrelin peptide and validates the use of the peptide in functional assays.

Ghrelin-induced activation of ERK1/2 in PC3 and LNCaP prostate cancer cell lines. To determine if ghrelin or COOH-terminal Δ3 peptide treatment stimulated ERK1/2 activation, PC3 and LNCaP cells were initially treated with varying concentrations of ghrelin or COOH-terminal Δ3 peptide (0-1,000 nmol/L) for 5, 15, 30, and 60 minutes and the level of ERK1/2 activation was analyzed by Western blot. An anti–active MAPK antibody that specifically recognizes dually phosphorylated MAPK (phosphorylated ERK1/2) was used. Ghrelin treatment at various doses activated ERK1/2 within

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<td>33</td>
<td>COOH-terminal Δ3 peptide</td>
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NOTE: Staining intensity is indicated by a 0 to 3+ scale.
5 minutes and maximum activation was reached by 15 minutes in PC3 cells (Fig. 3A and B), returning to basal levels after 60 minutes (Fig. 3C). Ghrelin induced potent MAPK pathway activation within 5 minutes in LNCaP cells (Fig. 3D); however, this decreased to almost undetectable levels by 15 minutes after treatment (Fig. 3E). There was no ERK1/2 activation in the LNCaP cell line at 30 or 60 minutes after ghrelin treatment (data not shown). As expected, anisomycin-treated cells activated ERK1/2 at all time points in both prostate cancer cell lines. None of the treatments altered the levels of total (active and inactive) ERK1/2 or β-tubulin protein expression. Treatment with COOH-terminal Δ3 peptide failed to induce phosphorylation of ERK1/2 in either cell line (data not shown).

c-Jun NH2-terminal kinase and p38 activation. To determine whether ghrelin stimulates the activation of the alternative JNK and p38 MAPK pathways, the MAPK assay and Western blot analysis were done on the PC3 prostate cancer cell line using JNK and p38 antibodies that specifically recognize dually phosphorylated (activated) forms. Ghrelin treatment at various concentrations did not activate p38 kinase at 5, 15, or 30 minutes after treatment (data not shown) but p38 kinase was activated by 60 minutes (Fig. 4). JNK kinase activation was not stimulated by ghrelin treatment at 5, 10, 15, and 30 minutes after treatment (data not shown). The positive control treatment, anisomycin, induced JNK and p38 kinase activation at all time points in both prostate cancer cell lines.

Mitogen-activated protein kinase inhibition studies. To confirm the specificity of MAPK activation, the specific MAPK kinase inhibitors PD98059 and U0126 were employed. Using Western blot analysis, no ERK1/2 activation was observed in either PC3 or LNCaP prostate cancer cell lines in the absence of any treatment, when cells were treated with the vehicle alone, or when cells were treated with PD98059 or U0126 alone (Fig. 5). Ghrelin treatment markedly increased ERK1/2 activation in LNCaP cells (Fig. 5) and PC3 cells (data not shown) at 15 minutes posttreatment, and this was completely suppressed by coinubcation with either U0126 or PD98059 (Fig. 5).

Proliferation and apoptosis assays. LNCaP cells incubated with octanoylated ghrelin for 72 hours showed an increase in the rate of cell proliferation compared with untreated controls (Fig. 6A). This dose-related increase peaked at 10 nmol/L ghrelin with viable cell numbers increasing to 45.0% above untreated controls (P < 0.01). Significant increases in cell proliferation (22.0% ± 4.0% above untreated controls, P < 0.05) were also observed at the lower dose of 1 nmol/L ghrelin (Fig. 6A). We have previously reported a similar response to 5 and 10 nmol/L ghrelin treatments in PC3 cells (8). Treatment of LNCaP and PC3 cell lines with COOH-terminal Δ3 peptide did not affect cell proliferation (data not shown).

To determine if ghrelin protects prostate cancer cell lines from experimentally induced apoptosis, LNCaP cells were preincubated with ghrelin for 15 minutes and then treated with actinomycin D for 24 hours. Treatment with actinomycin D significantly increased LNCaP cell death by 19% (P < 0.001) compared with untreated controls. However, ghrelin treatment (0-1,000 nmol/L) did not inhibit actinomycin D–induced apoptosis (Fig. 6B). Similarly, pretreatment with COOH-terminal Δ3 peptide also failed to protect LNCaP cells from actinomycin-induced apoptosis (data not shown).

To investigate whether the ghrelin-induced MAPK pathway is responsible for ghrelin-induced cell proliferation, cell proliferation assays were done in the presence of ghrelin ± PD98059 or U0126. Ghrelin treatment alone stimulated cell growth (40% above control) over 72 hours. Treatment with U0126, PD98059, or vehicle alone (0.1% DMSO) had no significant effect but both U0126 and PD98059 treatment completely prevented the stimulation of cell proliferation by ghrelin (10 nmol/L) in both LNCaP cells (Fig. 6C) and PC3 cells (Fig. 6D). These data suggest that MAPK pathway activation is essential for ghrelin-induced cell proliferation in LNCaP and PC3 cell lines.

Discussion

Prostate cancer is the most common noncutaneous cancer in men in the Western world and the second leading cause of cancer-related death, and this is mainly attributed to androgen-independent prostate cancer (39). Growth factors may be instrumental in the emergence of this hormone-refractory phenotype (40). This study shows for the first time that prostate cancer cell lines secrete ghrelin in vitro. This study has also shown that the mechanism underlying the effect of ghrelin on cell proliferation involves activation of the ERK1/2 MAPK pathway. Although ghrelin did not stimulate the JNK pathway, the alternative p38 MAPK was phosphorylated in PC3 prostate cancer cell lines after 60 minutes of treatment with ghrelin, suggesting that ghrelin may also signal through this MAPK cascade. The p38 MAPK pathway is less well characterized than the ERK1/2 pathway but is known to be strongly activated by cellular stress and enhances proliferation of normal prostate cells (41).

Functional assays done here showed that ghrelin had a dose-dependent proliferative effect on the LNCaP cell line, with the response peaking at 10 nmol/L ghrelin. Similarly, stimulation of proliferation in the PC3 prostate cancer cell line, HepG2 hepatoma cell line (6), GH3 rat pituitary somatotroph tumor cells (12), pancreatic adenocarcinoma cell lines (10), and...
the breast cancer cell lines MDA-MB-435 and MDA-MB-231 (19) has been shown in response to ghrelin treatment. Conflicting studies, however, have reported that high concentrations of ghrelin inhibit the proliferation of several adenocarcinoma cell lines in vitro (20, 42, 43), indicating that the proliferative effect of ghrelin may be both highly dose dependent and cell type specific.

This study also reports that varying concentrations of ghrelin can rapidly stimulate MAPK pathway (ERK1 and ERK2) activation in both PC3 and LNCaP prostate cancer cell lines and that this pathway underpins the proliferative effect of ghrelin in the prostate (Fig. 6A; ref. 8). It should be noted that the minimum concentration of ghrelin required to activate ERK1/2 (0.1 nmol/L) is 10-fold less than the concentration seen to significantly increase cell proliferation of prostate cancer cell lines in vitro. This may reflect a relative insensitivity of the proliferation assays used here. This also shows, however, that ghrelin can signal through MAPK pathways at physiologically relevant concentrations, as ghrelin circulates in human serum at a concentration of ~0.1 nmol/L (1, 44). Locally secreted ghrelin may, however, reach significantly higher concentrations. Although ghrelin potently activates the MAPK pathway in both PC3 and LNCaP cells within 5 minutes, ghrelin stimulation resulted in noticeably higher and sustained MAPK activation in the PC3 cells than in LNCaP cells. A similar observation was also seen in epidermal growth factor–induced MAPK activation in androgen-independent DU-145 cells and androgen-dependent LNCaP cells (45) and raises the possibility that endogenous androgen receptor expression in LNCaP cells modulates ghrelin-induced MAPK activation, as the androgen receptor has been shown to cross-talk with MAPK pathways in prostate cancer (46).

Activation of the ERK1/2 MAPK pathway has been correlated with disease progression in several cancer models (47). Histopathologic studies have detected high levels of ERK1/2 activation in more advanced, metastatic and androgen-independent prostate cancers, suggesting a potential

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Fig. 3. Western blot analysis of the activation of ERK1 and ERK2 by ghrelin in PC3 and LNCaP prostate cancer cells. Serum-starved PC3 cells (A–C) and LNCaP cells (D and E) were treated with various concentrations of ghrelin (0, 0.1, 1, 10, 100, and 1,000 nmol/L) for 5, 15, 30, or 60 minutes. Cell protein extract (10 μg) was analyzed by Western blot using an anti–active ERK1/2 antibody (Promega). Total ERK expression (active and inactive ERK1/2) was determined and total protein loading was also assessed by blotting with an anti-β-tubulin antibody. Each blot is representative of three similar experiments and the densitometry data are expressed using histograms (columns, mean; bars, SE) corrected for total ERK1/2 present in each protein extract. *, P < 0.05, versus control; **, P < 0.01, versus control. Intermediate time points where no ERK stimulation was detected are not shown. Densitometries for (C) (PC3 cells at 60 minutes) and (E) (LNCaP cells at 15 minutes) were not meaningful and therefore are not shown. +, positive control sample for ERK1/2 activation (anisomycin-stimulated PC3 or LNCaP cell extract).
contribution of this cascade to prostate cancer progression (48). Elevated levels of MAPK activity have also been shown in androgen-independent prostate cancer cells (49) and this is consistent with the observation that ghrelin may be able to induce higher and sustained levels of MAPK activation in the PC3 prostate cancer cell line compared with the LNCaP cell line. Although the precise role of the ERK1/2 MAPK cascade in prostate cancer requires elucidation, it is well documented that growth factor receptor signaling plays a crucial role in the control of prostate cancer cell proliferation, particularly in advanced stages of the disease. Hence, the MAPK pathway potentially represents a promising target for signal transduction–based cancer chemotherapy for prostate cancer patients (50).

Ghrelin-induced activation of MAPK pathways also accounts for the antiapoptotic effect of ghrelin treatment reported in cardiomyocytes and endothelial cells (31), 3T3-L1 adipocytes (16) and adrenal cells (17). Assays done on LNCaP and PC3 prostate cancer cell lines in this study show no effect of ghrelin treatment on apoptosis, concurring with reports in other cell lines (11, 51). Ghrelin may even enhance apoptosis in some tumor cell lines (51). The complexity underlying MAPK signal transduction is highlighted by the fact that activation of ERK1/2 can reduce or, conversely, increase apoptosis in prostate cancer cell lines, depending on the activating agent (40). The present study clarifies that ghrelin promotes cell growth via ERK1/2 phosphorylation, without inhibiting apoptosis, at least in prostate cancer cell lines.

Exon 3–deleted preproghrelin, a recently identified preproghrelin variant in humans and the mouse, retains coding sequence for mature ghrelin. Due to a translational frameshift, it also produces a unique 16-amino-acid COOH-terminal peptide sequence (19), which begins with a potential dibasic proteolytic cleavage site (Arg-Arg). It remains to be investigated whether the alternative C-peptide or its potential proteolytic products from exon 3–deleted preproghrelin circulate in human serum, as does the C-peptide derived from wild-type (full-length) proghrelin (44). The preliminary studies reported here indicate that, despite being cleaved from the exon 3–deleted preproghrelin protein, the unique COOH-terminal Δ3 peptide (RPQPTSDRPQALLTSL) did not activate the MAPK pathway, influence cell proliferation, or have an effect on apoptosis when incubated with the PC3 or LNCaP prostate cancer cell lines. Our recent demonstration that exon 3–deleted preproghrelin mRNA is up-regulated in metastatic breast cancer cell lines when compared with a benign breast epithelial cell line (19), however, indicates that the novel isoform may be physiologically significant. The present study is the first to show that ghrelin and exon 3–deleted preproghrelin are also highly expressed at the protein level in prostate cancer when compared with normal prostate tissue. As the exon 3–deleted preproghrelin isoform also encodes mature ghrelin, an increase in its expression in cancer may augment autocrine/paracrine levels of ghrelin in prostate tumors, thereby further enhancing MAPK activation and cell proliferation. The increased level of production of this isoform may be driven by cancer-specific alternative splicing mechanisms (52). In any case, increased expression of the isoform in malignant tissues compared with benign tissues indicates that it has potential as a diagnostic or prognostic marker for cancer. A recent study reporting that 15% of a cohort of men with normal serum prostate-specific antigen (PSA) levels (0-4 ng/mL) were actually harboring subclinical prostate neoplasms (53) highlights the need for more sensitive, as well as adjunctive, markers for prostate cancer.

The present study provides new insights into the role of ghrelin in stimulating prostate cancer cell proliferation and the signaling mechanisms involved. In light of our findings, the application of specific inhibitors that block ghrelin production or signaling may provide new avenues for the design of better adjunctive therapies for prostate cancer. Indeed, recent research has shown that neutralizing antibodies directed against autocrine ghrelin attenuate cell proliferation in the human erythroleukemic HEL cell line (13). Given that the present study shows that prostate cancer cells also secrete ghrelin, a similar approach could be taken with prostate cancer.

**Fig. 4.** Western blot analysis showing the activation of the p38 MAPK pathway by ghrelin in the PC3 prostate cancer cell line. Serum-starved PC3 cells were treated with increasing doses of ghrelin (0-1,000 nmol/L) and cell extract (10 μg) was analyzed by Western blotting using anti–active p38 antibodies specific for dually phosphorylated p38 kinase (Promega). After 60-minute treatment with ghrelin (but not after 5, 15, or 30 minutes), p38 kinase was activated in the PC3 cell line at all ghrelin concentrations. Total protein loading was assessed by blotting with an anti–β-tubulin antibody. Each blot is representative of three similar experiments. +, positive control for activated and phosphorylated p38 kinases (anisomycin-stimulated PC3 cell extract).

**Fig. 5.** Western blot analysis of ghrelin-induced ERK1/2 activation in the LNCaP prostate cancer cell line in the presence of the MAPK inhibitors PD98059 and U0126. Phosphorylation of ERK1/2 in LNCaP cells was examined using an anti–active ERK1/2 antibody (Promega) after pretreatment with 30 μmol/L PD98059 or 10 μmol/L U0126 for 30 minutes, followed by 10 μmol/L ghrelin treatment for 15 minutes. No treatment (lane 7), vehicle (0.1% DMSO; lane 2), and 30 μmol/L PD98059 or 10 μmol/L U0126 alone (lane 5, left and right, respectively) represent negative controls. Treatment with 10 nmol/L ghrelin for 15 minutes (lane 3) was done as a positive control. Total ERK (active and inactive ERK1/2) was detected using an anti–ERK antibody (Promega). Each blot is representative of three similar experiments.
In conclusion, this study provides substantial evidence that an autocrine/paracrine pathway involving ghrelin is capable of stimulating growth of prostate cancer cells in vitro through the activation of the MAPK cascade and that expression of ghrelin and exon 3–deleted preproghrelin is up-regulated in prostate cancer compared with normal prostate tissue. Further in vitro and in vivo studies are required to explore the nature of the cross-talk between the G protein–coupled GHS-R and MAPK pathways. Inhibition of the ghrelin axis as a mechanism for modulating prostate cancer cell growth may potentially unearth a novel adjucitve therapy for prostate cancer. The evaluation of ghrelin and exon 3–deleted preproghrelin expression as a potential prognostic marker for prostate cancer is also justified.

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References


Fig. 6. MTT assays on LNCaP and PC3 prostate cancer cells to measure the effect of exogenous ghrelin (± MAPK inhibitors) on cell proliferation (A, C, and D) and actinomycin D–induced apoptosis (B). A, ghrelin (0–1000 nmol/L) was incubated with LNCaP cells for 72 hours (with medium change every 24 hours). Cell proliferation was determined by the MTT assay as described in Materials and Methods. B, actinomycin D (1 μg/mL) was added to LNCaP cells for 24 hours with or without ghrelin cotreatment (0–1,000 nmol/L). Data were expressed as % cell death (i.e., % decrease in MTT absorbance reading) compared with the control. The effect of the MAPK pathway inhibitors PD98059 and U0126 on ghrelin-induced cell proliferation is shown in LNCaP (C) and PC3 (D) cells. Cells were treated with ghrelin (10 nmol/L) ± 30 μmol/L PD98059 (G+P) or 10 μmol/L U0126 (G+U). No ghrelin treatment (C), vehicle (0.1% DMSO; V), 30 μmol/L PD98059 (P), and 10 μmol/L U0126 (U) alone were negative controls. In all cases (A–D), absorbance readings for each treatment were converted to % above control (columns, mean; bars, S.E.). Analysis was made on combined data from three identical experiments, each with n = 16 for each ghrelin concentration. *, P < 0.05; **, P < 0.01, compared with controls (A and B) or all other treatments (C and D; one-way ANOVA followed by Tukey’s post hoc comparisons).
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Ghrelin and a Novel Preproghrelin Isoform Are Highly Expressed in Prostate Cancer and Ghrelin Activates Mitogen-Activated Protein Kinase in Prostate Cancer

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