Ghrelin Modulates the Downstream Molecules of Insulin Signaling in Hepatoma Cells*

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Masahiro Murata‡, Yasuyoshi Okimura§, Keiji Iida‡, Michihiro Matsumoto‡, Hideaki Sowa‡, Hidesuke Kaji‡**, Masayasu Kojima‡, Kenji Kangawa‡, and Kazuo Chihara‡

From the ‡Third Division and §Second Division, Department of Medicine and the ‡Department of Basic Allied Medicine, Kobe University School of Medicine, Kobe, 650-0017, the **College of Nursing Art and Science, Hyogo, Akashi 673-8588, and the ‡‡Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Osaka 656-8565, Japan

Ghrelin was identified in the stomach as an endogenous ligand specific for the growth hormone secretagogue receptor (GHS-R). GHS-R is found in various tissues, but its function is unknown. Here we show that GHS-R is found in hepatoma cells. Exposure of these cells to ghrelin caused up-regulation of several insulin-induced activities including tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, mitogen-activated protein kinase activity, and cell proliferation. Unlike insulin, ghrelin inhibited Akt kinase activity as well as up-regulated gluconeogenesis. These findings raise the possibility that ghrelin modulates insulin activities in humans.

Growth hormone (GH) is synthesized and secreted from the anterior pituitary under complex regulation mechanisms. The two hypothalamic peptides, GH-releasing hormone and somatostatin, coordinately exert the positive and negative control of GH release, respectively (1). On the other hand, GH secretagogues (GHSs) were discovered as a series of small peptide derivatives of pentapeptides Leu- and Met-enkephaline, which selectively stimulate GH secretion from pituitary cells. The prototype of this class of GHSs, GHRP-6, was found to be extremely potent and specific in mammals and particularly in humans (2). Non-peptidyl GHSs, L-692,429 (3) and L-163,191 (4), were also manufactured to improve oral bioavailability. The GHS receptor (GHS-R) was cloned by the robust expression system that pig pituitary poly(A) RNA was coinjected into Xenopus oocytes together with cDNA encoding Gαs (5) and subsequently in rats (6). GHS-R was prominently expressed in several hypothalamic nuclei and also in the dentate gyrus and CA2 layers of the hippocampus (5). In searching an endogenous ligand for GHS-R, however, all efforts to use the brain extracts proved fruitless.

Recently, an endogenous ligand for GHS-R, named ghrelin, was purified from the extracts of the stomach and found to be abundant exclusively in the stomach (7). GHS-R mRNA is expressed not only in the pituitary and brain but also in other tissues such as pancreas (8), suggesting that ghrelin may have other physiological functions in addition to the regulation of GH release. Furthermore, a very recent report showed that ghrelin caused hyperphagia and obesity (9). These findings let us to explore the possibility that ghrelin may play some role in glucose homeostasis and metabolism and modulate insulin action.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials used in this study were as follows. Horseradish peroxidase-conjugated monoclonal antiphosphotyrosine antibody (RC20) was purchased from Transduction Laboratories. Anti-insulin receptor substrate-1 (IRS-1, anti-phosphatidylinositol 3-kinase (PI3K)p85α, anti-adaptor molecule growth factor receptor-bound protein 2 (GRB2), anti-insulin receptor β (IRβ), and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IRS-2 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PD98059, p44/42 mitogen-activated protein kinase (MAPK) assay kit, and Akt kinase assay kit were purchased from New England Biolabs. CellTiter 96 AQueous One Solution Cell Proliferation Assay was purchased from Promega. Phosphatidylinositol (PtdIns), and 8-CPT-cAMP were purchased from Sigma. Gene Images random prime labeling module, Gene Images CDP-star detection module and γ-33-PiATP were purchased from Amersham Biosciences Inc. TLC plates were purchased from Merck.

Cell Culture—HepG2 cells, human hepatocarcinoma cell line, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (200 units/ml), and streptomycin (50 μg/ml). All hormone treatments were started after maintaining the cells for 12 h in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (BSA). Then the cells cultured in Dulbecco's modified Eagle's medium with 0.1% BSA and varying concentrations of test substances were collected for Western blot analysis, MAPK assay, and Akt kinase assay. H4-II-E cells, rat hepatoma cell line, were maintained in α-modified Eagle's medium containing 10% fetal bovine serum, penicillin (200 units/ml), and streptomycin (50 μg/ml). All treatments were started after maintaining the cells for 12 h in α-modified Eagle's medium with 0.1% BSA. Cells were pretreated first with 8-CPT-cAMP for 3 h and then with insulin, ghrelin, or both insulin and ghrelin and collected for Northern blot analysis.

Reverse Transcriptase (RT)-PCR—Total RNA was prepared from cultured HepG2 cells and H4-II-E cells using Trizol according to the manufacturer's instructions (Invitrogen). One μg of total RNA was reverse-transcribed for 1 h at 37 °C in 20 μl of reaction medium made up of 10 mmol/l Tris-Cl (pH 8.3), 50 mmol/l KCl, 0.5 mmol/l dithiothreitol, 0.5 unit/ml RNAsin, 2.5 mmol/l dNTPs, 250 μmol/l dNTPs, 1 μg/ml random primers, 500 μg/ml RNase-free BSA, and 200 U/ml M-MLV reverse transcriptase (Promega).
up of 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, pH 8.3, 1 mM dNTPs, 15 pmol of antisense primer, and 200 units of Moloney murine leukemia virus RT (M-MLV, Promega). The PCR amplifications were performed in 50 μl of medium containing 50 mM Tris-HCl, 50 mM KCl, 1 mM MgCl₂, pH 9.0, 0.2 mM dNTPs, 15 pmol of sense and antisense primers, and 1 U of Taq polymerase (Promega). The reaction mixtures were subjected to 40 cycles of PCR amplification of GHS-R cDNA consisting of denaturation for 60 s at 94°C, annealing for 60 s at 45°C, and elongation for 90 s at 72°C. The oligonucleotide primers (5'-CTCTGGATCCCGGCGCTAGTAC-3' sense and 5'-CTCGGATCCCTCGGAGGTCG-3' antisense) allowed amplification of a 629-bp GHS-R cDNA fragment, and the products were verified with DNA sequencing (model 310, PerkinElmer Life Sciences). The reaction mixtures were subjected to 40 cycles of PCR amplification of PEPCK cDNA consisting of denaturation for 60 s at 94°C, annealing for 60 s at 56°C, and elongation for 120 s at 72°C. The oligonucleotide primers (5'-AGCTTGCAAGCTTGGCAGCG-3' sense and 5'-CCAGGTTTGACCAAGGTTT-3' antisense) allowed amplification of a 575-bp PEPCK cDNA product (10), and the product was verified with DNA sequencing. The β-actin sequence of the upstream primer was 5'-TGACCCAGATCATGTTGAGACG-3' and of the downstream primer was 5'-CCATACCCCAAGAAGGAAAAG-3'. After an initial denaturation for 30 s at 94°C, PCR was performed for 40 cycles. The conditions for PCR were 94°C for 60 s, 60°C for 60 s, 72°C for 60 s. The β-actin-coding region was obtained by RT-PCR and verified with DNA sequencing.

Western Blot Analysis—Cells were lysed in 20 mM Tris-HCL, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM Na₂VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin for 30 min on ice and centrifuged at 10000 g for 30 min at 4°C. The supernatant was incubated with antibodies specific for IRS-1, IR-β, Insulin, and GSK-3 fusion protein for 30 min at 4°C. The cells were washed once with ice-cold saline buffer and then loaded onto a 1.2% agarose/formaldehyde gel made up in 1× TBE buffer. The supernatant was incubated with immobilized Akt antibody for 2 h at 4°C, and then washed with polyvinylidene difluoride membrane for 30 min. Immunoblot was blocked by incubation in TBST containing 5% skim milk at room temperature for 1 h and then probed with 1:1000 dilution of anti-phospho-Elk-1 antibody. The membranes were washed three times in TBST, incubated with a 1:1000 dilution of horseradish peroxidase-linked anti-rabbit IgG antibody, and then reacted with LumiGLO reagent according to the manufacturer's instructions. The membranes were exposed briefly to x-ray film.

Phosphatidylinositol 3-Kinase Activity—In vitro phosphorylation of PtdIns was carried out in the immune complexes as described previously (11). Subconfluent HepG2 cells grown in 100-mm dishes were made quiescent by overnight incubation in Dulbecco's modified Eagle's medium with 0.1% BSA. The quiescent cells were incubated with ghrelin for 20 min, insulin for 3 min, or ghrelin for 20 min and then insulin for 3 min, and washed once with ice-cold phosphate-buffered saline and twice with 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 100 μM Na₂VO₄ (buffer A). The samples were treated with a 1:1000 dilution of anti-IRS-1 antibody for 4 h at 4°C, and immune complexes were precipitated from the supernatant with protein A-Sepharose and washed successively in PBS containing 1% Nonidet P-40 and 100 mM Na₂VO₄ (three times); 100 mM Tris-HCl, pH 7.5, containing 500 mM LiCl and 100 mM Na₂VO₄ (three times); and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 mM Na₂VO₄ (two times). Isolated pellets were resuspended in 50 μl of 1× TKM and incubated at 30°C for 30 min at 72°C. The pellets were incubated with anti-IRS-1 antibody for 4 h at 4°C, and immune complexes were precipitated from the supernatant with protein A-Sepharose and washed successively in PBS containing 1% Nonidet P-40 and 100 mM Na₂VO₄ (three times); 100 mM Tris-HCl, pH 7.5, containing 500 mM LiCl and 100 mM Na₂VO₄ (three times); and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA and combined with 10 μl of 100 mM MnCl₂ and 10 μl of 2 μg/ml PtdIns sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The phosphorylation reaction was started by adding 10 μl of 440 μM ATP containing 30 μCi of [γ-³²P]ATP. After 10 min at 22°C, the reaction was stopped with 20 μl of 8% HCl and 160 μl of CHCl₃:methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate that had been coated with 1% potassium oxalate. TLC plates were developed in CHCl₃:CH₃OH:H₂O:NH₃:OH (60:47:11.3:2), dried, and visualized by autoradiography.

Northern Blot Analysis—Total RNA was prepared from cultured H-4-II-E cells using Trizol. Twenty μg of total RNA was heat-denatured for 5 min at 70°C in buffer containing 50% formamide, 2 μM formaldehyde, 1× 0.02 MOPS, 50 mM sodium acetate, and 10 mM Na₃EDTA and then loaded onto a 1.2% agaroseformaldehyde gel made up in 1× MOPS, pH 7.4, at 50 V for 3–4 h. After electrophoresis, RNA was transferred onto a Hybond N membrane by capillary action and fixed onto the filters by ultraviolet light cross-linking. The blot was probed with either a rat PEPCK or a rat β-actin probe labeled by a Gene Images random prime labeling module and detected by a Gene Images CDP-star detection module according to the manufacturer's instructions, and the membranes were exposed to x-ray film for 30–60 min.

Cell Proliferation Assays—Cell proliferation assays were performed by CellTitier 96 AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's instructions (Invitrogen). Briefly, cells were washed with phosphate-buffered saline, lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% BSA) and then loaded onto a 1.2% agarose/formaldehyde gel made up in 1× MOPS, pH 7.4, at 50 V for 3–4 h. After electrophoresis, RNA was transferred onto a Hybond N membrane by capillary action and fixed onto the filters by ultraviolet light cross-linking. The blot was probed with either a rat PEPCK or a rat β-actin probe labeled by a Gene Images random prime labeling module and detected by a Gene Images CDP-star detection module according to the manufacturer's instructions, and the membranes were exposed to x-ray film for 30–60 min.

Akt in Vitro Kinase Assays—Akt kinase activity of HepG2 cells was measured by using Akt kinase assay kit according to the manufacturer's instructions. In short, the cells were washed with phosphate-buffered saline, lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₄, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% BSA) and then loaded onto a 1.2% agarose/formaldehyde gel made up in 1× MOPS, pH 7.4, at 50 V for 3–4 h. After electrophoresis, RNA was transferred onto a Hybond N membrane by capillary action and fixed onto the filters by ultraviolet light cross-linking. The blot was probed with either a rat PEPCK or a rat β-actin probe labeled by a Gene Images random prime labeling module and detected by a Gene Images CDP-star detection module according to the manufacturer's instructions, and the membranes were exposed to x-ray film for 30–60 min.
was added to the culture medium. The methanethiosulfonate tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. After incubating the plate in a CO₂ incubator, the absorbance at 490 nm was recorded with a 96-well plate reader. All samples were assayed in duplicate, and each experiment was repeated at least three times.

**Statistical Analysis**—The data were expressed as the mean ± S.E. unless noted otherwise. Statistics were analyzed by one-way repeated measures analysis of variance with a significance level of 0.05.

**RESULTS**

To investigate the possible effects of ghrelin on insulin-regulated responses, we looked for cell lines expressing a GHS-R. Various cell lines derived from the liver, adipose tissue, and muscle were screened by RT-PCR with oligonucleotides that have been reported previously (12). A human hepatocellular carcinoma cell line, HepG2 cells, provided one PCR product, for which identity with human GHS-R mRNA was confirmed by DNA sequencing. The same product was obtained by RT-PCR from a human pituitary cDNA library, a human liver cDNA library, and a rat hepatoma cell line, H4-II-E cells (Fig. 1A). We next investigated the effect of ghrelin on the profile of tyrosine-phosphorylated cellular proteins. HepG2 cells were treated with 0.1–100 nM ghrelin for 20 min. Control cells gave 96 ± 6 DU. Cells treated with 0.1, 1, 10, and 100 nM ghrelin gave 102 ± 7, 104 ± 8, 215 ± 20, and 324 ± 16 DU, respectively. The representative result is shown in the upper panel. In the lower panel, all values are the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks: *p < 0.01 versus vehicle control. C, dose-dependent increase by ghrelin of tyrosine phosphorylation of IRS-1. HepG2 cells were treated with 0.1–100 nM ghrelin for 20 min. Control cells gave 96 ± 6 DU. Cells treated with 0.1, 1, 10, and 100 nM ghrelin gave 102 ± 7, 104 ± 8, 215 ± 20, and 324 ± 16 DU, respectively. The representative result is shown in the upper panel. In the lower panel, all values are the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks: *p < 0.01 versus vehicle control. D, HepG2 cells were treated for 20 min with vehicle alone (103 ± 10 DU), 100 nM ghrelin (178 ± 15 DU), 100 nM ghrelin + 25 μM [D-Lys-3]GHRP-6 (97 ± 10 DU), and 25 μM [D-Lys-3]GHRP-6 alone (97 ± 6 DU). The representative result is shown in the upper panel. In the lower panel, all values are the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks: *p < 0.01 versus vehicle control. The amount of IRS-1, determined in the same blot by anti-IRS-1, was not changed in panels B–D.
phototyrosine (anti-Tyr(P)). Ghrelin treatment for 10–20 min caused a significant increase in the amount of tyrosine-phosphorylated IRS-1 in HepG2 cells compared with those without ghrelin treatment (354 ± 42 versus 100 ± 5% basal level at 10 min, p < 0.01; 364 ± 47 versus 106 ± 5% basal level at 20 min, p < 0.01) (Fig. 1B). When we cultured HepG2 cells for 20 min with varying concentrations of ghrelin, 10–100 nM ghrelin caused a significant and dose-dependent increase in the amount of tyrosine-phosphorylated IRS-1 as shown in Fig. 1C. Furthermore, ghrelin-induced tyrosine phosphorylation of
Ghrelin up-regulates the association of GRB2 with IRS-1 and the association of PI3K with IRS-1. Serum-starved HepG2 cells were treated with 100 nM ghrelin for 20 min followed by 100 nM insulin for 3 min. A, cell extracts were immunoprecipitated (IP) with polyclonal anti-GRB2 and immunoblotted with anti-IRS-1. The basal amount of GRB2 associated with IRS-1 was increased both by ghrelin and by insulin and was further increased by a combined treatment with both ghrelin and insulin. The representative result is shown in the upper panel. In the lower panel, all values are the mean ± S.E. of the amount of GRB2-associated IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks: *, p < 0.01 versus control; **, p < 0.01 versus insulin-treated cells. The amount of GRB2, determined in the same blot by anti-GRB2, was not changed. B, cell extracts were immunoprecipitated with polyclonal anti-PI3K and immunoblotted with anti-IRS-1. The basal amount of PI3K associated with IRS-1 was increased both by ghrelin and by insulin as well as by a combined treatment with both ghrelin and insulin. The representative result is shown in the upper panel. In the lower panel, all values are the mean ± S.E. of the amount of PI3K-associated IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks: *, p < 0.01 versus control. The amount of PI3K, determined in the same blot by anti-PI3K, was not changed.

IRS-1 was canceled by an antagonist for GHS-R, [α-Lys-3]GHRP-6 (Fig. 1D).

HepG2 cells were treated with 100 nM ghrelin for 20 min followed by stimulation with 100 nM insulin for 1 min, and cellular proteins were analyzed by immunoblot analysis with anti-Tyr(P). Ghrelin and insulin significantly increased tyrosine-phosphorylated IRS-1 levels up to 172 ± 10 and 262 ± 15 densitometric units (DU), respectively, compared with vehicle alone (99 ± 9 DU). Combined stimulation with ghrelin and insulin resulted in an additive increase of tyrosine-phosphorylated IRS-1 levels up to 442 ± 23 DU (p < 0.01, n = 5) (Fig. 2A). The tyrosine phosphorylation of IRβ chain was not stimulated by the presence of ghrelin (Fig. 2B). Ghrelin also increased tyrosine phosphorylation of IRS-1 in H4-II-E cells (Fig. 2D). To investigate whether the effect of ghrelin on tyrosine phosphorylation is specific for IRS-1, we tested the effect of ghrelin on tyrosine phosphorylation of IRS-2. HepG2 cells were treated with 100 nM ghrelin for 20 min followed by stimulation with 100 nM insulin for 1 min. Ghrelin did not increase tyrosine phosphorylation of IRS-2 (Fig. 2C).

Downstream signaling of IRS-1 is mediated by several associated proteins including GRB2 and PI3K (13). We therefore tested the effect of ghrelin on the interaction of GRB2 or PI3K with IRS-1. When HepG2 cells were pretreated with 100 nM ghrelin for 20 min followed by stimulation with 100 nM insulin for 3 min, the amount of GRB2-associated IRS-1 (basal, 114 ± 10 DU) was increased either by ghrelin (258 ± 34 DU, p < 0.01) or by insulin (288 ± 33 DU, p < 0.01). Combined treatment with ghrelin and insulin resulted in an additive increase (418 ± 44 DU, p < 0.01, n = 5) (Fig. 3A). Similarly treated cells were analyzed to examine the association of PI3K with IRS-1. PI3K-associated IRS-1 in vehicle-treated cells (114 ± 10 DU) was increased either by ghrelin (220 ± 28 DU, p < 0.01) or by insulin (275 ± 32 DU, p < 0.01). However, no additive increase in PI3K associated with IRS-1 was found by a combined treatment with ghrelin and insulin (211 ± 24 DU, p < 0.01, n = 5) (Fig. 3B).

Furthermore, because MAPKs and Akt are the downstream substrates for GRB2 and PI3K, respectively, we tested whether MAPKs and Akt are involved in cellular responses to ghrelin. Phosphorylated active MAPK was collected from cell lysates using anti-phospho-MAPK antibody, and its enzyme activity was determined by the amount of phosphorylated Elk-1 fusion protein. HepG2 cells were pretreated with 100 nM ghrelin for 20 min followed by stimulation with 100 nM insulin for 3 min and were then analyzed for MAPK activity. MAPK activity in vehicle-treated cells (75 ± 14 DU) was increased either by ghrelin (246 ± 24 DU, p < 0.01) or by insulin (389 ± 36 DU, p < 0.01). Combined treatment with ghrelin and insulin caused an additive increase (546 ± 58 DU, p < 0.01, n = 5) (Fig. 4A). These response patterns of MAPK activity to either insulin, ghrelin, or the combination of both were compatible with those of GRB2-associated IRS-1 (Fig. 3A). We also measured the Akt kinase activity determined by the amount of phosphorylated glycogen synthase kinase-3 (GSK-3) fusion protein. Similarly treated cells were analyzed for Akt kinase activity. Akt kinase activity in vehicle-treated cells (154 ± 24 DU) was increased by insulin (524 ± 126 DU, p < 0.01) and, in contrast, decreased by ghrelin (29 ± 8 DU, p < 0.01) as well as by a combined treatment with ghrelin and insulin (82 ± 17 DU, p < 0.01, n = 5) (Fig. 4B). These findings were not correlated with the association of PI3K with IRS-1. We therefore measured the amount of PI3K activity in IRS-1 immunoprecipitates. IRS-1-associated PI3K activity in vehicle-treated cells (64 ± 10 DU)
FIG. 4. Ghrelin up-regulates MAPK activity and down-regulates Akt kinase activity, whereas it up-regulates IRS-1-associated PI3K activity. HepG2 cells were treated as described in the legend for Fig. 3A. A, the basal level of MAPK activity, as determined by the amount of phosphorylated Elk-1, was increased both by ghrelin and by insulin and was further increased by a combined treatment with both ghrelin and insulin. Statistical significance is shown by asterisks: * , p < 0.01 versus control; ** , p < 0.01 versus insulin-treated cells. B, the basal amount of Akt kinase activity, as determined by the amount of phosphorylated GSK-3, was increased by insulin and, in contrast, was decreased both by ghrelin and by a combined treatment with ghrelin and insulin. Statistical significance is shown by asterisks: * , p < 0.01 versus control; ** , p < 0.01 versus insulin-treated cells. C, phosphorylation of PtdIns was carried out in the immune complexes as described under “Experimental Procedures.” The basal amount of IRS-1-associated PI3K activity was increased both by ghrelin and by insulin as well as by a combined treatment with both ghrelin and insulin. The representative result is shown in the left panel. In the right panel, all values are the mean ± S.E. of the amount of IRS-1-associated PI3K activity after densitometric analysis (n = 4). Statistical significance is shown by asterisks: * , p < 0.01 versus control.

We also investigated whether ghrelin affects glucose homeostasis in cell culture. Hepatic and renal gluconeogenesis is crucially important in maintaining glucose homeostasis. The rate-limiting enzyme of gluconeogenesis is phosphoenolpyruvate carboxykinase (PEPCK). This enzyme has no known allosteric control and is down-regulated by insulin at the transcriptional level. The rat hepatoma cell line, H4-II-E cells, has been used successfully to study the regulation of PEPCK expression, whereas HepG2 cells do not express PEPCK efficiently (14–16). The amount of PEPCK mRNA in H4-II-E cells treated first with 8-CPT-cAMP and then with insulin was reduced compared with cells treated with 8-CPT-cAMP alone. Surprisingly, incubation of the cells with ghrelin for 1 to 2 h before adding insulin partially reversed the down-regulating effect of insulin on PEPCK mRNA levels (Fig. 5). Another main regulator of PEPCK gene expression is cAMP. Hence, we investigated the intracellular cAMP using the enzyme immunoassay for cAMP. Ghrelin did not increase the intracellular cAMP in H4-II-E cells (data not shown).

Next we tested the effects of ghrelin on cell proliferation. On the basis of the analogous data obtained with ghrelin and insulin with regard to the stimulation of MAPK activity in HepG2 cells, we hypothesized that ghrelin causes cell proliferation in HepG2 cells via MAPK pathway. Ghrelin stimulated proliferation of HepG2 cells, and a MAPK kinase-1-specific inhibitor, PD98059, completely blocked both ghrelin- and insulin-induced cell proliferation (Fig. 6).

DISCUSSION

We have demonstrated for the first time that ghrelin treatment causes stimulation of the IRS-1-GRB2-MAPK pathway as well as cell proliferation and that ghrelin inhibits Akt kinase activity as well as up-regulates PEPCK gene expression.

The effect of ghrelin on IRS-1 phosphorylation is unlikely to be mediated via IR, because tyrosine phosphorylation of the IRβ chain was not stimulated by the presence of ghrelin. However, it would be exerted independently by an activation of a GHS-R signaling cascade. We found that ghrelin-induced tyrosine phosphorylation of IRS-1 was blunted by an antagonist for GHS-R, [D-Lys-3]GHRP-6, and that other GHSs, GHRP-6 and GHRP-2, induced a significant increase in the amount of tyrosine-phosphorylated IRS-1 in HepG2 cells (data not shown). Therefore, the downstream molecules of GHS-R signaling can cross-talk with the insulin-signaling pathway. Indeed, it has been reported that IRS-1 is phosphorylated by growth factors and cytokines, including insulin-like growth factor-I, interferon-α, interleukin-4, and interleukin-9 as well (11, 17–20). It is unique, however, that GHS-R is the G protein-coupled receptor that cross-talks with the insulin-signaling pathway. Hence, it remains to be elucidated what molecules in the GHS-R signaling pathway affect the tyrosine phosphorylation of IRS-1 (Fig. 7).
IRS-1 is characterized to possess the 20–22 potential tyrosine phosphorylation sites that are conserved between IRS-1 homologs. The surrounding amino acid residues are also highly conserved, and several of these represent potential binding sites for proteins that contain Src homology 2 (SH2) domains (21, 22). IRS-1 interacts with many SH2 proteins with diverse phosphotyrosine motif requirements including PI3K and GRB2 (13, 21). In the present study, ghrelin increased the tyrosine phosphorylation of IRS-1, association of GRB2 with IRS-1, and MAPK activity, indicating up-regulation of the IRS-1-GRB2-MAPK pathway. Furthermore, ghrelin-stimulated proliferation of HepG2 cells and PD98059 completely blocked ghrelin-induced cell proliferation, indicating that MAPKs were essential in HepG2 cell proliferation caused by ghrelin. However, ghrelin suppressed Akt kinase activity, despite of the presence of insulin, as well as up-regulating the amount of PEPCK mRNA expression, although it increased not only the association of PI3K with IRS-1 but also IRS-1-associated PI3K activity.

The phospholipid kinase PI3K is activated by virtually all receptor tyrosine kinases. Activated PI3K phosphorylates PtdIns(4)P and PtdIns(4,5)P2 to generate the membrane-embedded second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3. These lipids play a crucial role in the activation of Akt. PtdIns(3,4,5)P3 mediated membrane translocation of a variety of signaling proteins, including the protein-serine/threonine kinases, 3'-phosphoinositide-dependent kinase-1 (PDK-1), and Akt. Akt is phosphorylated by PDK-1 on Thr-308 in its activation loop. Phosphorylation of Thr-308 is a prerequisite for kinase activation, but phosphorylation of the C-terminal hydrophobic residue is required as well for full activation of Akt kinase. The Akt Ser-473 kinase (hypothetical PDK-2) remains to be identified (23–25).

Furthermore, the activity of effector proteins that are dependent on PI3K activation can be negatively regulated by PTEN (phosphate and tensin homolog deleted on chromosome 10).
ten) and SHIP (SH2-containing inositol 5’-phosphatase), two phosphoinositide-specific phosphatases that dephosphorylate the 3’ and 5’ positions of the inositol ring of phosphoinositides, respectively, leading to inhibition of cellular responses mediated by PI3K products (26, 27). In the present study, the dissociation of the downstream molecules in the IRS-1-PI3K-Akt pathway remains difficult to explain, but the presence of a potent inhibiting mechanism of Akt kinase activity by ghrelin, even under full activation by the PI3K-IRS-1 association, is likely. It is possible that these enzymes, such as PDK-1, PDK-2, SHIP, and PTEN, may be affected by GHS-R-mediated signaling molecules. Hence, it remains to be elucidated what molecules in the GHS-R signaling pathway affect the IRS-1-PI3K-Akt pathway (Fig. 7).

The repression of the PEPCK gene by insulin has been studied in detail. There is conflicting evidence as to which signaling pathways may be involved in insulin repression of PEPCK gene expression, the activation of the PI3K pathway, or the activation of the MAPK pathway in which GRB2 engages IRS-1, IRS-2, Shc, or SHP2 (28–30). Recently, however, accumulating evidence suggests that the signaling pathways in insulin repression of PEPCK gene expression may involve the activation of PI3K pathway but not the MAPK pathway (30–32). Our findings that ghrelin inhibits Akt activity may be in agreement with the recent data that PI3K pathway is involved in the insulin-induced repression mechanism of PEPCK expression. Another main regulator of PEPCK gene expression is cAMP. However, ghrelin did not increase the intracellular cAMP in H4-II-E cells (data not shown). Therefore, the signaling pathway by which ghrelin stimulates PEPCK gene expression remains as yet unknown. We propose that ghrelin can affect gluconeogenesis, at least in the H4-II-E cells, by attenuating the effect of insulin on the expression of PEPCK. Considering the effect of ghrelin on glucose homeostasis, it is of note that GHSs are diabetogenic in rats (33). Physiological significance of ghrelin in vivo animals is to be clarified.

In conclusion, we found two novel actions of ghrelin in addition to its GH-releasing action: one is insulin-like action stimulating the IRS-1-GRB2-MAPK pathway, which in turn activates cell proliferation; and the other is anti-insulin action suppressing Akt activity and up-regulation of gluconeogenesis. Although the mechanism by which ghrelin affects insulin signaling pathways remains not fully understood, our findings obtained in hepatoma cells strongly implicate the peripheral actions of ghrelin in glucose homeostasis and in mitogenic processes in humans.

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