Obestatin Promotes Survival of Pancreatic β-Cells and Human Islets and Induces Expression of Genes Involved in the Regulation of β-Cell Mass and Function

Riccarda Granata,[1,2] Fabio Settanni,[1,2] Davide Gallo,[1,2] Letizia Trovato,[1,2] Luigi Biancone,[2] Vincenzo Cantaluppi,[2] Rita Nano,[3] Marta Annunziata,[1,2] Pietro Campiglia,[4] Elisa Arnoletti,[5] Corrado Ghè,[5] Marco Volante,[6] Mauro Papotti,[6] Giampiero Muccioli,[5] and Ezio Ghigo[2]

OBJECTIVE—Obestatin is a newly discovered peptide encoded by the ghrelin gene whose biological functions are poorly understood. We investigated obestatin effect on survival of β-cells and human pancreatic islets and the underlying signaling pathways.

RESEARCH DESIGN AND METHODS—β-Cells and human islets were used to assess obestatin effect on cell proliferation, survival, apoptosis, intracellular signaling, and gene expression.

RESULTS—Obestatin showed specific binding on HIT-T15 and INS-1E β-cells, bound to glucagon-like peptide-1 receptor (GLP-1R), and recognized ghrelin binding sites. Obestatin exerted proliferative, survival, and antiapoptotic effects under serum-deprived conditions and interferon-γ/tumor necrosis factor-α/interleukin-1β treatment, particularly at pharmacological concentrations. Ghrelin receptor antagonist [D-Lys^3]-growth hormone releasing peptide-6 and anti-ghrelin antibody prevented obestatin-induced survival in β-cells and human islets. β-Cells and islet cells released obestatin, and addition of anti-obestatin antibody reduced their viability. Obestatin increased β-cell cAMP and activated extracellular signal–related kinase 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI 3-kinase)/Akt; its antiapoptotic effect was blocked by inhibition of adenylyl cyclase/cAMP/protein kinase A (PKA), PI 3-kinase/Akt, and ERK1/2 signaling. Moreover, obestatin upregulated GLP-1R mRNA and insulin receptor substrate-2 (IRS-2) expression and phosphorylation. The GLP-1R antagonist exendin-(9-39) reduced obestatin effect on β-cell survival. In human islets, obestatin, whose immunoreactivity colocalized with that of ghrelin, promoted cell survival and blocked cytokine-induced apoptosis through cAMP increase and involvement of adenylyl cyclase/cAMP/PKA signaling. Moreover, obestatin *1*) induced PI 3-kinase/Akt, ERK1/2, and also cAMP response element–binding protein phosphorylation; *2*) stimulated insulin secretion and gene expression; and *3*) upregulated GLP-1R, IRS-2, pancreatic and duodenal homeobox-1, and glucokinase mRNA.

CONCLUSIONS—These results indicate that obestatin promotes β-cell and human islet cell survival and stimulates the expression of main regulatory β-cell genes, identifying a new role for this peptide within the endocrine pancreas. ***Diabetes*** **57: 967–979, 2008**

Obestatin is a 23–amino acid amidated peptide, recently identified as a product of the ghrelin gene (1). It was originally reported to be the ligand for the orphan receptor G-protein–coupled receptor 39 (GPR39); however, several groups were unable to confirm that obestatin has agonist properties on GPR39 or activates specific GPR39 signaling (2–6). Therefore, to date, the receptor for obestatin remains unknown.

Obestatin has been reported to reduce food intake, body weight gain, gastric emptying, and jejunal motility (1,7,8). Moreover, it was found to counteract ghrelin stimulatory effects on these end points (1,9) and to inhibit ghrelin-induced growth hormone secretion in vivo (9) but not in vitro (10), suggesting that it would serve as a physiological opponent of ghrelin. However, a number of studies failed to confirm obestatin anorexigenic effects (11–14), and besides not being the cognate ligand for GPR39, its biological actions seem to be a controversial issue.

Obestatin has been reported to inhibit thirst and to influence memory, anxiety, and sleep via central activities (10,15,16). At the cellular level, it stimulates proliferation of human retinal cells (17) but has no effect on murine cardiomyocyte and condrocyte viability (18,19).

Recently, we have demonstrated that ghrelin, both acylated or unacylated (UAG), stimulates proliferation and prevents apoptosis of pancreatic β-cells and human islets (20,21). In addition, ghrelin increased glucose-induced β-cell insulin secretion, indicating a role in regulating β-cell survival and function.

Decreased β-cell mass due to apoptosis is an important hallmark of both type 1 and type 2 diabetes (22). Identification of molecules that promote both β-cell survival and insulin secretion and regulate pancreatic gene expression would be important for understanding the pathophysiol-

From the [1]Laboratory of Molecular and Cellular Endocrinology, Department of Internal Medicine, University of Turin, Turin, Italy; the [2]Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Turin, Turin, Italy; the [3]Department of Medicine, Transplant Unit, Scientific Institute San Raffaele, Vita-Salute University, Milan, Italy; the [4]Department of Pharmaceutical Sciences, University of Salerno, Fisciano (Salerno), Italy; the [5]Department of Anatomy, Pharmacology, and Forensic Medicine, University of Turin, Turin, Italy; and the [6]Department of Clinical and Biological Sciences and San Luigi Hospital, University of Turin, Turin, Italy.

Address correspondence and reprint requests to Riccarda Granata, PhD, Laboratory of Molecular and Cellular Endocrinology, Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Turin, Corso Dogliotti, 14-10126 Turin, Italy. E-mail: riccarda.granata@unito.it.

Received for publication 15 August 2007 and accepted in revised form 13 December 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 9 January 2008. DOI: 10.2337/db07-1104.

BrdU, 5-bromo-2-deoxyuridine; CREB, cAMP response element–binding protein; [D-Lys^3]-GHRP-6, [D-Lys^3]-growth hormone releasing peptide-6; EIA, enzyme immunoassay; ERK, extracellular signal–related kinase; Ex-4, exendin-4; Ex-9, exendin-(9-39); FBS, fetal bovine serum; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GPR39, G-protein–coupled receptor 39; GRLN-R, ghrelin receptor; IBMX, 3-isobutyl-1-methylxanthine; IL-1β, interleukin-1β; IFN-γ, interferon-γ; IRS-2, insulin receptor substrate-2; KRBH, Krebs-Ringer bicarbonate HEPES buffer; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PDX-1, pancreatic and duodenal homeobox-1; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; RIA, radioimmunoassay; TNF-α, tumor necrosis factor-α; UAG, unacylated ghrelin; TUNEL, TdT-mediated dUTP nick-end labeling.

ogy of diabetes, leading to new therapeutic strategies aimed at improving β-cell function and increasing the amount of islets for islet transplantation (22,23).

Obestatin expression was found in fetal and neonatal rat pancreas and stomach, and its immunoreactivity positively correlated with insulin secretion, suggesting that pancreatic obestatin would contribute to β-cell function (24); however, its effect on insulin secretion is controversial (7,25). In all, obestatin actions within the endocrine pancreas are still largely unknown.

Given that it is encoded by the ghrelin gene, we sought to investigate whether obestatin would display survival effects in pancreatic β-cells and human islets. In addition, we evaluated whether obestatin would influence the expression of the major genes that regulate β-cell differentiation, survival, and function.

RESEARCH DESIGN AND METHODS

Reagents. [D-Lys^3]-growth hormone releasing peptide-6 ([D-Lys^3]-GHRP-6), Exendin-4 (Ex-4), ghrelin, and obestatin rat and human polyclonal antibodies were from Phoenix Pharmaceuticals (Belmont, CA). Acylated ghrelin, UAG, and glucagon-like peptide-1 (GLP-1) were from NeoMPS (Strasbourg, France); Exendin-(9-39) (Ex-9) from Bachem (Weil am Rhein, Germany). NF-449, PD98059, MDL-12330A, Hoechst 33258, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and wortmannin were from Sigma-Aldrich (Milano, Italy). Cytokines and anti-P-Src[pY^{318}] antibody were from Biosource (Invitrogen, S. Giuliano Milanese, Italy). KT-5720 was from Biomol Research Laboratory (DBA, Milan, Italy). Cell culture reagents were from Invitrogen. P–Extracellular signal–related kinase 1/2 (ERK1/2), P-Akt (Ser473), P–cAMP response element–binding protein (P-CREB) (Ser133), and CREB antibodies were from Cell Signaling Technology (Celbio, Milano, Italy). Total antibodies were from Santa Cruz Biotechnology (DBA). Anti–phosphotyrosine-PY20 was from BD Transduction Laboratories (Milano, Italy). Real-Time PCR reagents were from Applied Biosystems (Monza, Italy).

Chemistry. Human amidated obestatin was synthesized on a solid phase, using standard Fmoc/tBu chemistry, as previously described (26).

Cell culture. Hamster HIT-T15 insulin-secreting β-cells were obtained and cultured as described previously (20,21). INS-1E rat β-cells were provided by Prof. Claes B. Wollheim (University Medical Center, Geneva, Switzerland) and cultured as described previously (20).

Human islet isolation. Human islets were obtained from pancreata of multiorgan donors as described previously (20). Islet preparations with purity >70%, not suitable for transplantation, were used after approval by the local ethical committee. Islets (10,000) were cultured in CMRL (Invitrogen) with 10% fetal bovine serum (FBS) (20).

Receptor binding assay. Obestatin binding was assayed on HIT-T15 and INS-1E cell membranes (30,000*g* pellet) using ^{125}I-labeled obestatin. Human obestatin (NeoMPS) was radioiodinated with activity of 1,800–2,100 Ci/mmol (GE Healthcare, Milano, Italy). For saturation studies, 100 μg cell membranes was incubated with 0.035–4 nmol/l ^{125}I-obestatin, and data were analyzed as previously described (20). Competition studies were performed by incubating 150 μg β-cell membranes with 1 nmol/l ^{125}I-obestatin with or without human ghrelin, porcine motilin, GLP-1, exendin-9, and D-[Lys^3]-GHRP-6 (0.01 nmol/l-1 μmol/l) (NeoMPS), as previously described (20). Competition studies were performed by incubating human islets (100 μg) with 1 nmol/l ^{125}I-obestatin, with or without 1 μmol/l unlabeled obestatin. Binding specificity was also tested with 1 μmol/l acylated ghrelin, motilin, or GLP-1. GLP-1 and ghrelin binding were assayed using ^{125}I-labeled GLP-1 (GE Healthcare) and ^{125}I-labeled Tyr^4–acylated ghrelin, respectively (20,27).

Cell proliferation. β-Cell proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation ELISA (Roche), as described previously (20). Cells were seeded on 96-well plates at 5×10^3 cells/well in serum-containing medium until 60–70% confluence and serum-starved for 24 h (HIT-T15) or 12 h (INS-1E) before 24-h treatments.

Cell survival. Cell survival was assessed by Trypan blue exclusion and MTT. β-Cells were stained with Trypan blue dye (Sigma) (0.04% [w/v]). Viable (unstained) and nonviable (blue stained) cells (300 cells/dish) were counted on a hemocytometer by light microscopy in 10 random fields. MTT was performed in serum-starved β-cells and islets that were seeded on 96-well plates (5×10^3 cells/well, 25 islets/well, respectively), as previously described (20).

Hoechst staining. Morphological changes in the nuclei of apoptotic cells were detected by Hoechst 33258 as described previously (20). Five hundred stained nuclei were double counted under a fluorescence microscope (DAPI filter).

Caspase 3 activity. Caspase 3 activity was assessed by Caspase-3 Colorimetric kit (Assay Designs, Bologna, Italy) in cell lysates, according to the manufacturer's instruction.

TdT-mediated dUTP nick-end labeling. Formalin-fixed human islet preparations were analyzed by double immunohistochemical staining with insulin and TdT-mediated dUTP nick-end labeling (TUNEL), using the In Situ Cell Death Detection kit POD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations.

Hormone secretion. Obestatin, ghrelin, and GLP-1 secretion from β-cell or human islet concentrated conditioned medium (~18-fold) was assessed using rat Obestatin enzyme immunoassay (EIA) kit and human Obestatin radioimmunoassay (RIA) kit, Ghrelin RIA kit, and GLP-1 EIA kit (Phoenix), respectively, following the manufacturer's instruction.

cAMP assays. Starved 8×10^5 INS-1E β-cells and 1×10^3 human islets were seeded in 100-mm dishes. After incubations, in the presence of 100 μmol/l 3-isobutyl-1-methylxanthine (IBMX), cAMP was measured from lysates using the Direct Cyclic AMP EIA kit (Assay Designs) according to the manufacturer's instructions.

Insulin receptor substrate-2 immunoprecipitation. INS-1E cells were scraped in ice-cold lysis buffer (Mammalian Cell Lysis kit; Sigma); 300 μg proteins was incubated overnight at 4°C with insulin receptor substrate-2 (IRS-2) antibody (1:100 dilution), followed by binding to protein A-Sepharose (Sigma-Aldrich). The pellet was resuspended in 40 μl Laemmli buffer and proteins separated by 8% SDS-PAGE.

Western blotting. Forty micrograms of protein for P-ERK, P-Akt, P-Src, and P-CREB were resolved in 12% SDS-PAGE. All proteins, including IRS-2 immunoprecipitates, were treated as described previously (20) and incubated with the specific antibody (P-tyrosine for IRS-2) (1:500 for P-tyrosine; 1:1,000 for others). Blots were reprobed with antibodies against IRS-2, ERK1/2, Akt, Src, and CREB for normalization. Immunoreactive proteins were visualized using horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibody (1:3,000 and 1:1,000, respectively) by ECL (Perkin Elmer Life Sciences).

Real-time PCR. RNA isolation and reverse transcription from 3 μg RNA were performed as described previously (20). Real-time PCR was conducted using ABI-Prism 7300 (Applied Biosystems) in 25-μl volume containing 2 μl cDNA under the conditions recommended by the supplier. The following TaqMan Gene Expression Assays were used: rat GLP-1R (Rn00562406_m1), rat IRS-2 (Rn01482605_s1), human GLP-1R (Hs00157705_m1), human glucokinase (Hs00277220_m1), human pancreatic and duodenal homeobox-1 (PDX-1) (Hs00236830_m1), human insulin (Hs02741908_m1), human IRS-2 (Hs00275843_s1), and eukaryotic 18S rRNA (Hs99999901_s1). Results were normalized to 18s rRNA, and relative quantification analysis was performed with ABI Prism 7300 SDS software, using the comparative cycle threshold (Ct) ($2-\Delta\Delta Ct$) method. mRNA was expressed as fold induction over control (vehicle).

Insulin secretion. Human islets ($n = 3$) were incubated for 1 h at 37°C in Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 0.5% BSA alone or with 2 mmol/l glucose, either in absence or presence of 100 nmol/l obestatin. The 2 mmol/l glucose pretreated islets were incubated for 1 h in KRBH/0.5% BSA containing 2, 7.5, 15, or 25 mmol/l glucose, with or without 100 nmol/l obestatin. Insulin secretion was assessed by a human RIA kit (DiaSorin, Saluggia, Italy), according to the manufacturer's instructions.

Immunohistochemical staining. Human pancreas fragments were obtained from five surgical pancreatic resections for non-neoplastic conditions and fixed in 4% buffered formalin. Fetal pancreas was from a spontaneous abortion at the 15th week of gestation. Obestatin was localized by means of an anti-human antibody (1/300; Phoenix Pharmaceuticals) preceded by antigen retrieval using a biotin-free system (EnVision-HRP; DakoCytomation, Glostrup, Denmark) and diaminobenzidine as chromogen. For double immunohistochemical procedure, obestatin was first visualized using aminoethylcarbazole as a red chromogen (Dako AEC Substrate-Chromogen System), and subsequently, the slides were incubated with ghrelin (polyclonal, 1/400; Phoenix), somatostatin (polyclonal, 1/800; Dako), glucagon (polyclonal; prediluted; Dako), or insulin (monoclonal HB125, 1/200; Biogenex) antibodies, followed by immunoalkaline phosphatase (Envision-AP; Dako) incubation with Vector blue alkaline phosphatase substrate kit III (Vector Laboratories) as chromogen.

Statistical analysis. Results are expressed as means ± SE. Statistical analyses were performed using Student's t test or one-way ANOVA. Significance was established when $P < 0.05$.

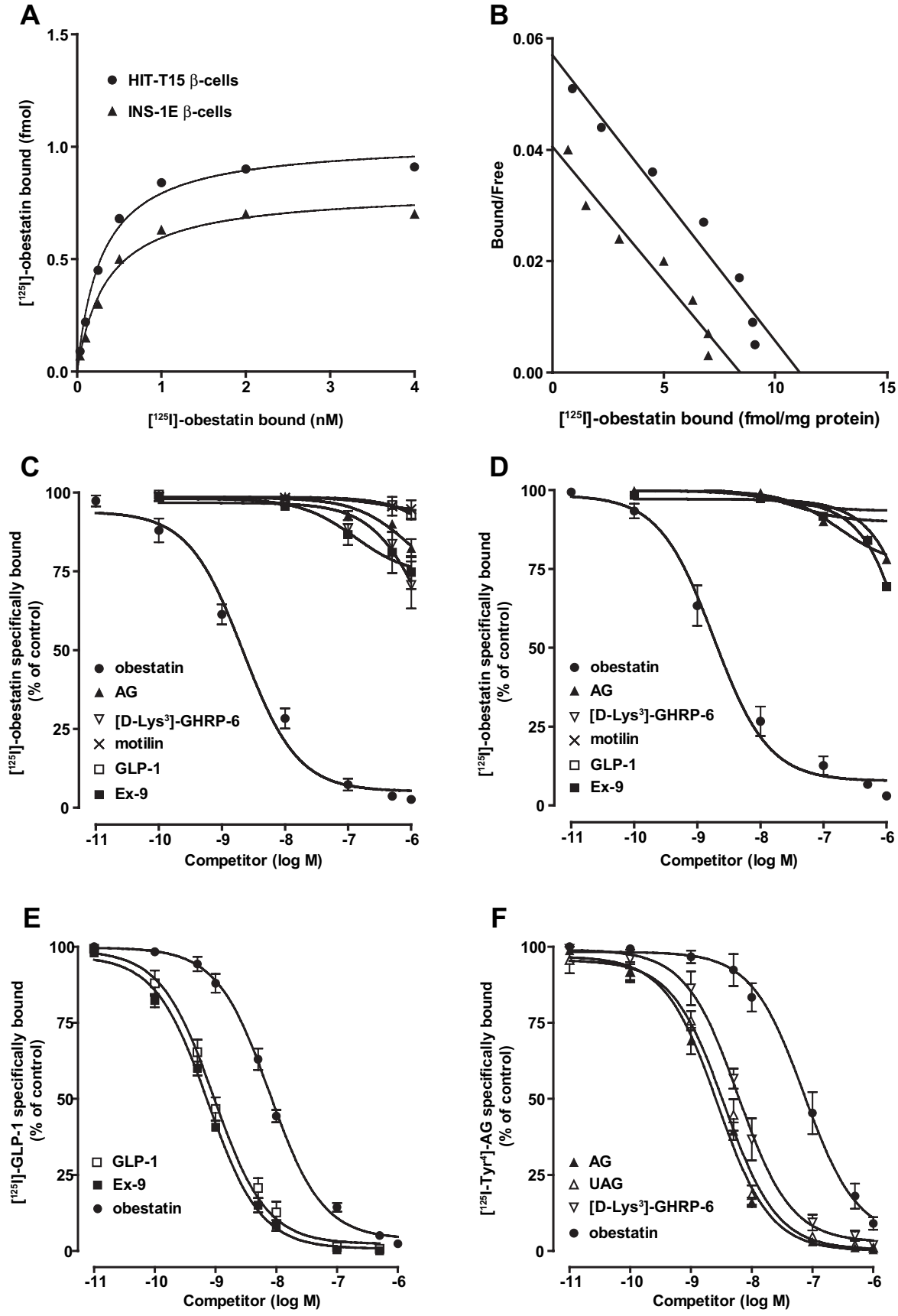

FIG. 1. Obestatin binding analysis in HIT-T15 and INS-1E pancreatic β-cell lines. *A*: Representative saturation isotherms of ^{125}I-obestatin specific binding to HIT-T15 and INS-1E membranes. *B*: Scatchard analysis of the saturation binding curves to calculate the maximum binding capacity (B_{max}) and the dissociation constant (K_d) values. *C* and *D*: Competition for ^{125}I-obestatin to HIT-T15 (*C*) and INS-1E (*D*) cell membranes by the indicated competitors. *E* and *F*: Competition for ^{125}I–GLP-1 (*E*) and ^{125}I-Tyr^4–acylated ghrelin (AG) (*F*) to HIT-T15 cell membranes by the indicated competitors. Data represent the means ± SE of three independent experiments.

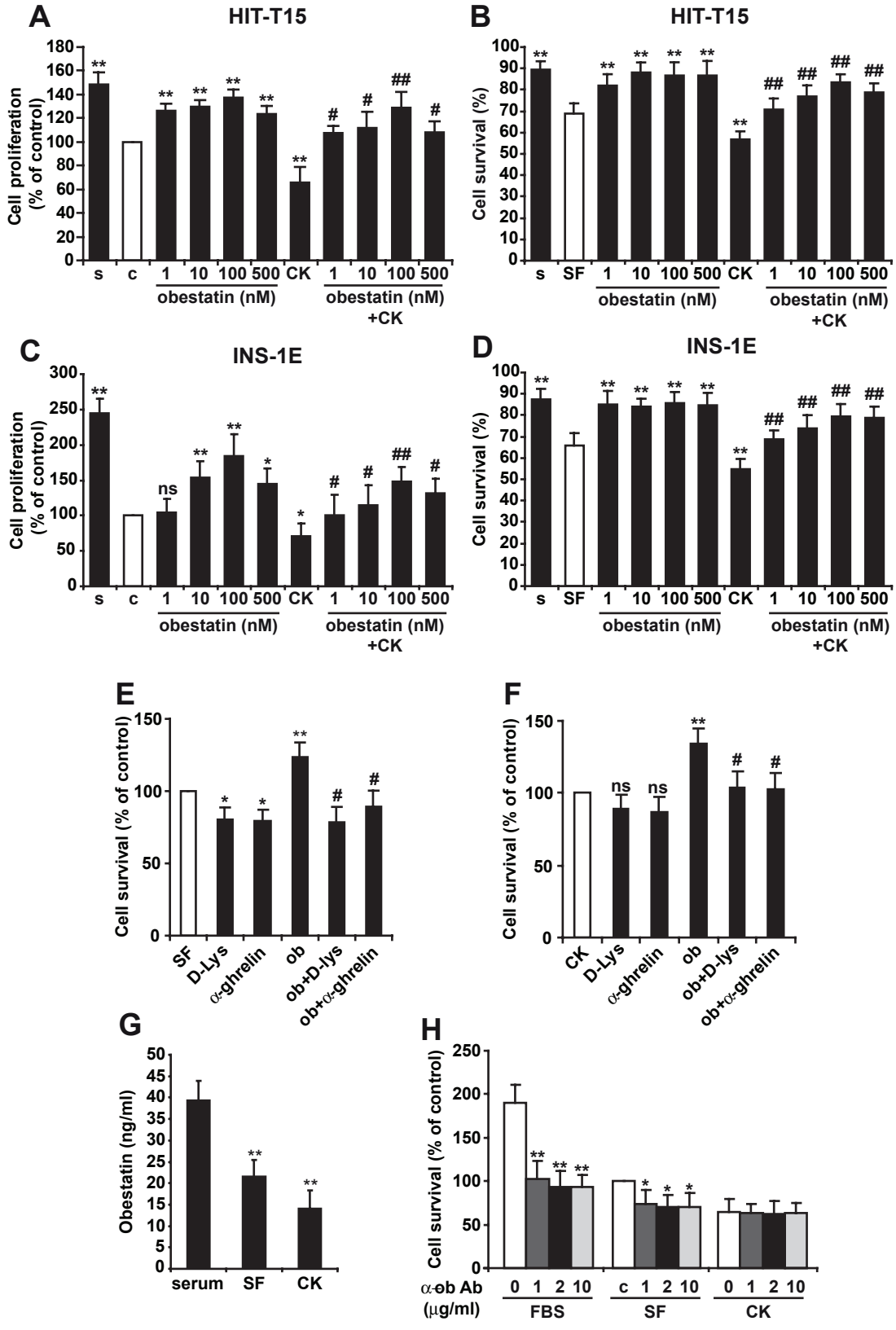

FIG. 2. Obestatin promotes proliferation and survival of HIT-T15 and INS-1E β-cells. Cells were cultured in either the presence of serum (s) or serum starved for 24 h (HIT-T15) or 12 h (INS-1E) and subsequently incubated in serum-free medium for further 24 h with or without either obestatin at the concentrations indicated or cytokines (CK) (100 ng/ml IFN-γ, 200 ng/ml TNF-α, and 10 ng/ml IL-1β for HIT-T15 cells; 50 ng/ml IFN-γ,100 ng/ml TNF-α, and 5 ng/ml IL-1β for INS-1E cells). *A* and *C*: Cell proliferation, measured by BrdU incorporation, of HIT-T15 (*A*) and INS-1E cells (*C*). Values are expressed as percent relative to control cells in serum-free medium (c) and are means ± SE of three independent experiments, each performed in quadruplicate (P* < 0.05, ***P* < 0.01 vs. c; #*P* < 0.05, ##*P* < 0.01 vs. CK alone). *B* and *D*: Cell survival assessed by Trypan blue exclusion in HIT-T15 (*B*) and INS-1E cells (*D*) by counting, under a light microscope, living cells that exclude the blue dye. SF, serum-free medium. Data are expressed as percentage of living cells calculated on the total number of cells and are the means ± SE of three**

RESULTS

Obestatin binding on HIT-T15 and INS-1E β-cells. HIT-T15 cells showed saturable specific obestatin binding (Fig. 1*A*), with maximum affinity at 2 nmol/l, consistent with obestatin physiological circulating levels (600–800 pg/ml) (7). Scatchard analysis (Fig. 1*B*) and Hill equation, with a slope close to 1, suggested the existence of a single class of binding sites (K_d 0.32 ± 0.03 nmol/l; B_{max} 11.8 ± 0.6 fmol/mg protein) ($n = 4$). Similar results were found in INS-1E cells (K_d 0.35 ± 0.03 nmol/l; B_{max} 7.8 ± 0.5 fmol/mg protein) ($n = 4$) (Fig. 1*A* and *B*).

Unlabeled obestatin dose dependently competed with ^{125}I-obestatin for HIT-T15 binding sites (Fig. 1*C*). Obestatin concentrations for inhibiting radiotracer binding by 50% (IC_{50}) were 2.2 ± 0.3 nmol/l ($n = 3$). Similar results were observed in INS-1E cells (Fig. 1*D*) (IC_{50} 1.9 ± 0.2 nmol/l) ($n = 3$). ^{125}I-obestatin binding to HIT-T15 (Fig. 1*C*) or INS-1E (Fig. 1*D*) cells was specific and slightly inhibited by motilin (5–6%), GLP-1 (7–11%), acylated ghrelin (18–22%), [D-Lys^3]-GHRP-6 (18–22%), and Ex-9 (25–30%) at the maximal concentration tested.

Next, we evaluated the ability of obestatin to inhibit ^{125}I–GLP-1 and ^{125}I-Tyr^4–acylated ghrelin binding to β-cell membranes. ^{125}I–GLP-1 binding to HIT-T15 membranes was dose dependently inhibited by unlabeled GLP-1 and Ex-9 and, although with lower affinity, by obestatin (Fig. 1*E*) (IC_{50} [$n = 3$] for GLP-1, 0.85 ± 0.09 nmol/l; for Ex-9, 0.67 ± 0.07 nmol/l; and for obestatin, 8.8 ± 1.5 nmol/l). Obestatin (10 nmol/l to 1 μmol/l), although less than acylated ghrelin, UAG, or [D-Lys^3]-GHRP-6, dose dependently inhibited ^{125}I-Tyr^4–acylated ghrelin binding on HIT-T15 cells (Fig. 1*F*) (IC_{50} [$n = 3$] for acylated ghrelin, 2.8 ± 0.2 nmol/l; for UAG, 3.9 ± 0.5; for [D-Lys^3]-GHRP-6, 7.3 ± 0.5 nmol/l; and for obestatin, 75.0 ± 7.5 nmol/l. Similar results were obtained on INS-1E cells (data not shown). These findings indicate that obestatin, besides recognizing specific binding sites in β-cells, interacts with GLP-1R and acylated ghrelin/UAG binding sites.

Obestatin promotes β-cell proliferation and survival. Cell proliferation and survival were assessed in HIT-T15 and INS-1E β-cells incubated with obestatin (1–500 nmol/l) in serum-deprived medium, either alone or with interferon-γ (IFN-γ)/tumor necrosis factor-α (TNF-α)/interleukin-1β (IL-1β), whose synergism is involved β-cell death in both type 1 and type 2 diabetes (28). BrdU and Trypan blue results showed that obestatin promoted cell proliferation (Fig. 2*A* and *C*) and survival (Fig. 2*B* and *D*), respectively. These effects were significant at 1 and 10 nmol/l and maintained at the highest concentrations (100–500 nmol/l), although reduced for cell proliferation. Similar results were obtained by performing MTT assays (data not shown).

Next, obestatin survival action was determined with respect to that of ghrelin, which exerts similar effects (20). Differently from HIT-T15, INS-1E cells express the ghrelin receptor (GRLN-R) or growth hormone secretagogue receptor 1a (29–32). As expected, INS-1E cell viability (assessed by MTT) in serum-free medium but not under cytokine synergism was reduced by blockade of the GRLN-R with its antagonist [D-Lys^3]-GHRP-6 and, consistently, by immunoneutralization of endogenous ghrelin with anti-ghrelin antibody, which recognizes both acylated ghrelin and UAG. Either [D-Lys^3]-GHRP-6 or anti-ghrelin antibody blocked obestatin-induced cell survival in both experimental conditions (Fig. 2*E* and *F*). Similarly to HIT-T15 cells (20), ghrelin was released by INS-1E cells and its secretion was reduced by serum starvation and cytokines (data not shown).

Obestatin secretion in INS-1E cell medium (39.4 ng/ml) was reduced by serum starvation (21.5 ng/ml) and cytokines (14 ng/ml) (Fig. 2*G*), consistent with increased β-cell death. Anti-obestatin antibody decreased cell survival in all conditions except under cytokine treatment, where cell viability was likely too low to be further reduced (Fig. 2*H*). Similar results were obtained in HIT-T15 β-cells (data not shown).

These results indicate that exogenous obestatin promotes β-cell proliferation and survival and suggest that endogenous obestatin exerts similar effects. Furthermore, ghrelin, at least partly through GRLN-R, is involved in obestatin-induced β-cell survival.

Obestatin prevents β-cell apoptosis. HIT-T15 and INS-1E cells were cultured for 24 h in serum-free medium alone or with IFN-γ/TNF-α/IL-1β. In both cell lines, apoptosis, assessed by Hoechst staining of apoptotic nuclei and by caspase 3 activation, increased under serum starvation and even more in the presence of cytokines. Obestatin, at 100 nmol/l, reduced apoptosis in both experimental conditions (Fig. 3*A*–*E*). This effect was dose dependent, with 1 nmol/l being the lowest significant concentration (data not shown), in agreement with the previous results on β-cell proliferation and survival.

Obestatin antiapoptotic effect in β-cells involves adenylyl cyclase/cAMP/protein kinase A, phosphatidylinositol 3-kinase/Akt, and ERK1/2. Intracellular cAMP levels, investigated in INS-1E cells in response to obestatin, increased after 5–30 min and decreased thereafter, although being significantly above control (Fig. 3*F*). NF449, a specific antagonist of the $G\alpha_s$ protein (33), whose engagement results in adenylyl cyclase activation and cAMP production (34), blocked obestatin antiapoptotic effect in serum-starved and cytokine-treated cells. Similar results were obtained by inhibiting adenylyl cyclase and protein kinase A (PKA) with MDL12330A and KT5720, respectively (20) (Fig. 3*G*).

Obestatin rapidly promoted ERK1/2 and Akt phosphorylation but had no effect on Src kinase, which has been associated with Ca^{2+}-dependent insulin secretion in INS-1E cells (35) (Fig. 3*H*). Consistently, ERK1/2 and Akt and inhibitors (PD98059 and wortmannin, respectively), but not the Src inhibitor PP2 (20,35), blocked obestatin effect against both serum starvation– and cytokine-induced apoptosis (Fig. 3*I*). These findings suggest that obestatin prevents β-cell apoptosis through adenylyl cy-

experiments ($n = 5$) ($P < 0.01$ vs. SF; ##$P < 0.01$ vs. CK alone). *E* and *F*: Cell survival (MTT) of INS-1E cells cultured for 24 h in serum-free medium (SF) alone (*E*) or with cytokines (*F*) in the presence or absence of obestatin (100 nmol/l), with or without either 0.5 μmol/l [D-Lys^3]-GHRP-6 or 10 μg/ml anti-ghrelin antibody (α-ghrelin), which were added to cell culture medium 30 min before obestatin. Results are expressed as percentage of control (SF for *E*; CK for *F*) and are the means ± SE of eight replicates ($n = 3$) (*$P < 0.05$, **$P < 0.01$ vs. SF (E) or CK (F); #$P < 0.01$ vs. CK in both graphs; ns, not significant). *G*: Obestatin secretion in INS-1E conditioned medium cultured for 24 h in either the presence of serum (10% FCS) or in serum-free medium (SF) ± cytokines (CK). Results are the means ± SE of three independent experiments, each performed in quadruplicate (**$P < 0.01$). *H*: INS-1E cell survival (by MTT) assessed after 24 h in INS-1E cultured in serum-containing medium (FBS) or in serum-free (SF) medium alone or with cytokines (CK), either in presence or absence of an anti-obestatin antibody (α-ob Ab), at the concentrations indicated. Results are expressed as the percentage of control. *$P < 0.05$, **$P < 0.01$ vs. 0 μg/ml α-ob Ab at each condition.**

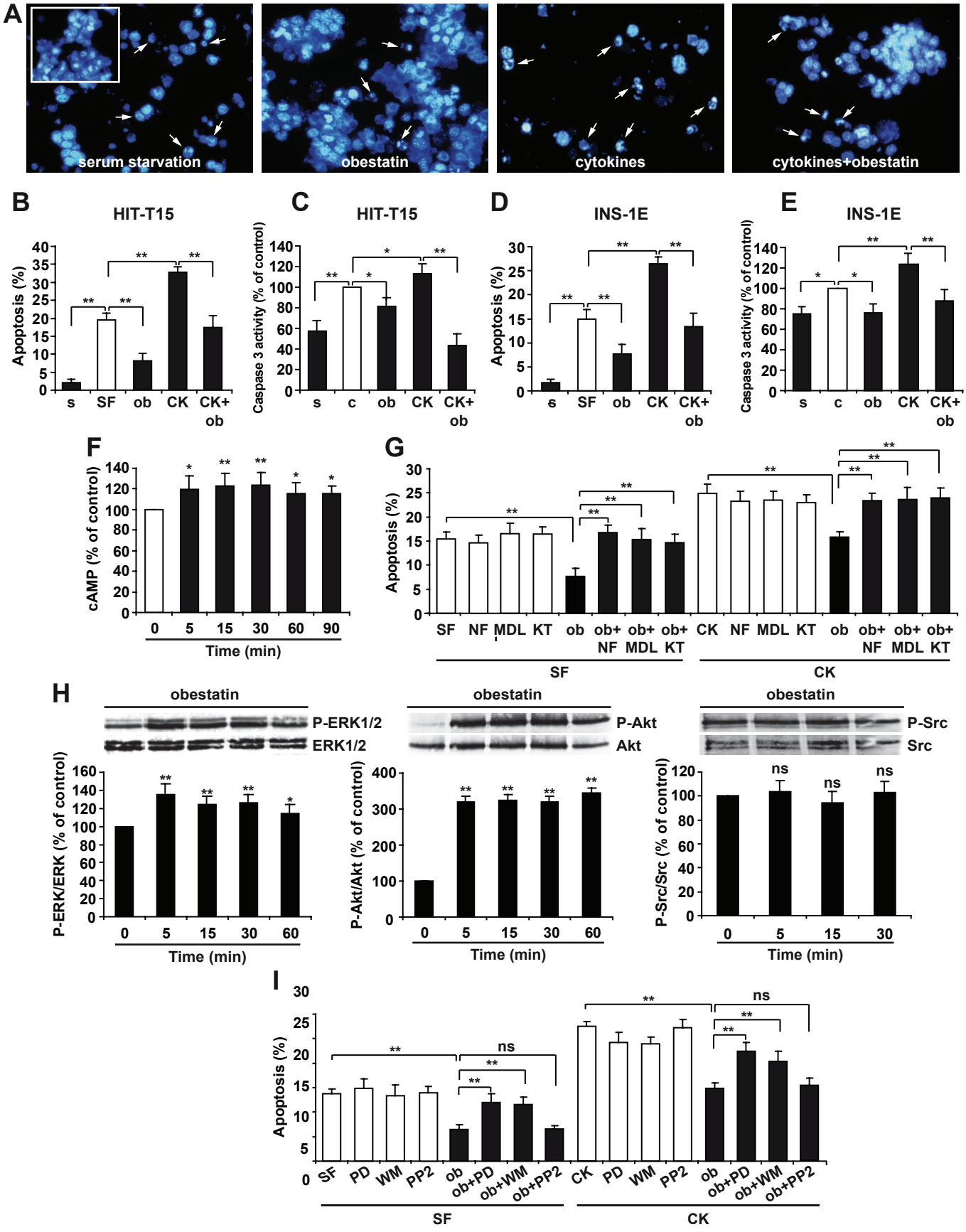

FIG. 3. Obestatin inhibits β-cell apoptosis induced by serum starvation and cytokine synergism. HIT-T15 and INS-1E cells were cultured in the presence of serum or in serum-free medium (SF) for 24 or 12 h respectively and then incubated in serum-deprived medium for 24 h further with either 100 nmol/l obestatin or cytokines (CK) (100 ng/ml IFN-γ, 200 ng/ml TNF-α, and 10 ng/ml IL-1β for HIT-T15 cells; 50 ng/ml IFN-γ,100 ng/ml TNF-α, and 5 ng/ml IL-1β for INS-1E cells). *A*: Hoechst 33258 nuclear immunofluorescence staining (original magnification ×100) of INS-1E cells cultured

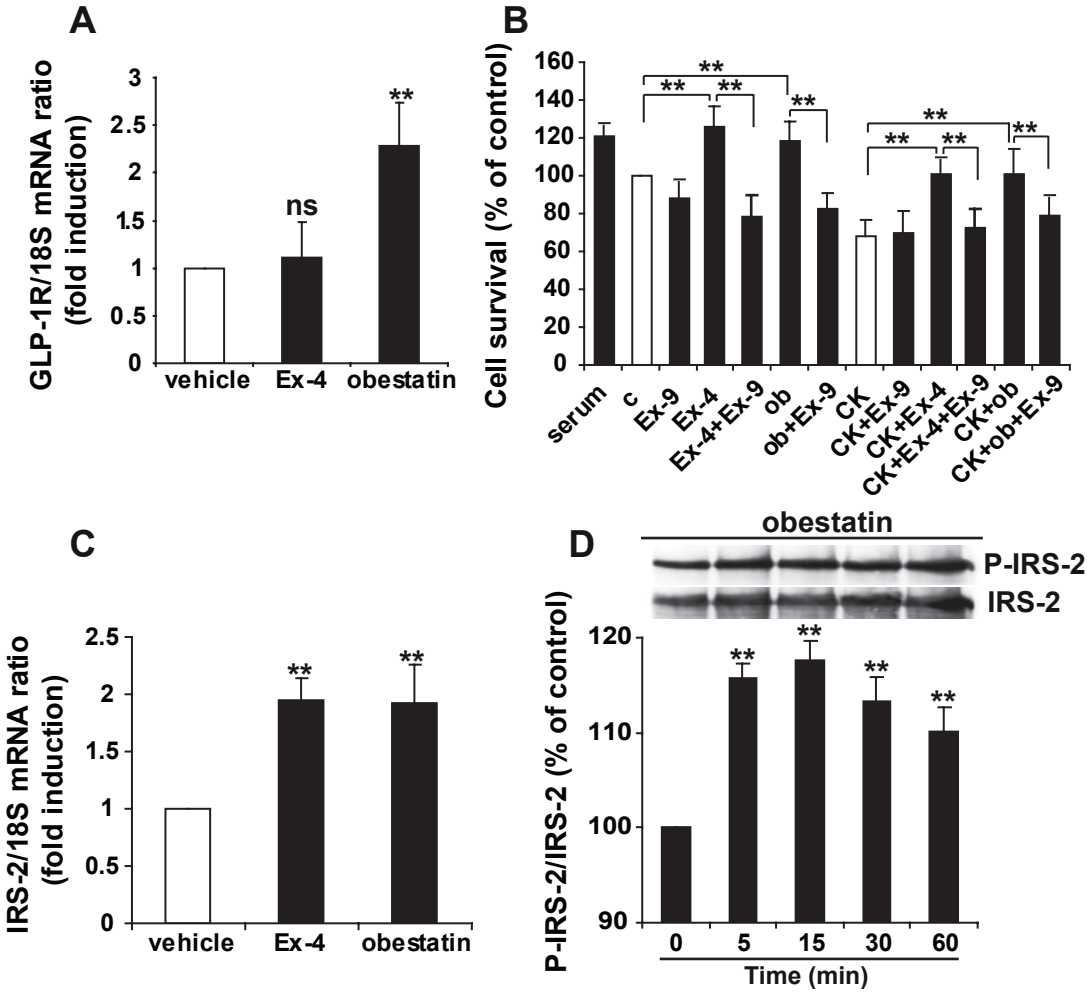

FIG. 4. Obestatin effects in INS-1E β-cells involve GLP-1R and IRS-2. *A*: GLP-1R mRNA expression, assessed by real-time PCR, in cells cultured in serum-free medium for 24 h in the presence or absence of 100 nmol/l Ex-4 or 100 nmol/l obestatin. Data are expressed as fold increase over untreated cells (vehicle) and are normalized to 18S rRNA transcript level. Results are the means ± SE of three independent experiments (P < 0.01 vs. vehicle; ns, not significant). *B*: Cell survival (MTT) of INS-1E cells cultured for 24 h in the presence of serum or in serum-free medium alone (c) or with cytokines (CK) (50 ng/ml IFN-γ, 100 ng/ml TNF-α, and 5 ng/ml IL-1β), 500 nmol/l Ex-9, 100 nmol/l Ex-4, or 100 nmol/l obestatin (ob). Data are expressed as percentage of control (c) and are means ± SE of eight replicates (n = 3) (**P < 0.01). *C*: IRS-2 mRNA assessed by real-time PCR in INS-1E cells cultured for 24 h in serum-deprived medium with either 100 nmol/l Ex-4 or 100 nmol/l obestatin. Results, normalized to 18S rRNA transcript level, are expressed as fold increase over untreated cells (vehicle) and are the means ± SE of three independent experiments (**P < 0.01 vs. vehicle). *D*: IRS-2 phosphorylation measured by specific immunoblotting in IRS-2 immunoprecipitates from INS-1E cells treated with 100 nmol/l obestatin for the indicated times (*top panel*). Equal protein loading was determined by reprobing with antibody to IRS-2 (*bottom panel*). Shown is a representative blot from three independent experiments. Graph represents the densitometric analysis of P-IRS-2 normalized to IRS-2 and reported as percent untreated (time 0). Results are means ± SE from three experiments (**P < 0.01).**

clase/cAMP/PKA, phosphatidylinositol 3-kinase (PI 3-kinase)/Akt, and ERK1/2.

Obestatin-induced β-cell survival involves GLP-1R. GLP-1R signaling, activated by GLP-1 and by agonists such as Ex-4, promotes β-cell growth and survival and insulin secretion (34,36,37).

Real-time PCR results showed that in INS-1E cells, obestatin increased GLP-1R transcript over twofold, after 24-h incubation, whereas Ex-4 had no effect (Fig. 4*A*). Ex-9, a specific GLP-1R antagonist (27), besides inhibiting Ex-4 survival action as expected, prevented obestatin-induced cell survival in either serum-starved or cytokine-

in serum-free medium either alone or in the presence of cytokines, with or without obestatin. *Inset*, cells cultured with serum; arrows, apoptotic cells. *B* and *D*, Apoptosis measured by counting fragmented/condensed Hoechst-stained nuclei. Values are expressed as percentage of apoptotic cells and are the means ± SE of duplicate determinations (500 cells each, n = 3). **P < 0.01. *C* and *E*: Caspase 3 activation expressed as percentage of control cells (c) in serum-free medium. Values are the means ± SE of three independent experiments. *P < 0.05; **P < 0.01. *F*: Obestatin-induced cAMP increase. Serum-starved INS-1E cells were cultured for the indicated times with 100 nmol/l obestatin, in the presence of 100 μmol/l IBMX, which was added to the culture medium 30 min before stimulation. Results are the means ± SE of three independent experiments performed in triplicate (*P < 0.05, **P < 0.01 vs. basal time point). *G*: Apoptosis, assessed at 24 h by Hoechst 33258 staining, in serum-starved or cytokine-treated INS-1E cells incubated with 10 μmol/l NF449, 100 nmol/l MDL12330A, or 5 μmol/l KT-5720 30 min before addition of obestatin. **P < 0.01. *H*: ERK1/2, Akt, and Src phosphorylation evaluated by Western blot on whole lysates from INS-1E cells stimulated with 100 nmol/l obestatin for the indicated times (*top panels*). Equal protein loading was determined by reprobing with antibodies to the respective total proteins (*bottom panels*). Shown are representative blots from three independent experiments. Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percentage of basal (*P < 0.05, **P < 0.01). *I*: Apoptosis in INS-1E cells by Hoechst staining, in the presence of obestatin, with or without 40 μmol/l PD98059, 100 nmol/l wortmannin (WM), or 10 μmol/l PP2. Data are the means ± SE from three independent experiments (P < 0.01; ns, not significant).**

treated cells (Fig. 4*B*). Obestatin and Ex-4 showed equal effect on β-cell protection. Moreover, GLP-1 secretion by INS-1E cells (~85 ng/ml) was not affected by obestatin (data not shown). Consistent with the results showing obestatin binding to GLP-1R, these findings suggest that GLP-1R signaling is involved in obestatin survival effects.

Obestatin induces expression and activation of IRS-2 in β-cells. IRS-2 is essential for β-cell growth, function, and survival and is involved in GLP-1R–mediated effects through cAMP signaling (36–39).

By real-time PCR, we found that similarly to Ex-4, obestatin induced IRS-2 mRNA increase (1.8-fold) after 24-h incubation in INS-1E cells (Fig. 4*C*). Furthermore, immunoblot analysis showed that obestatin promoted IRS-2 phosphorylation at 5 and 15 min and declined toward basal level thereafter (Fig. 4*D*). These results strengthen the hypothesis of common signaling pathways for GLP-1R agonists and obestatin.

Obestatin binding and immunoreactivity in human islets. Specific obestatin binding greater than in HIT-T15 β-cells was observed in human islets (19.2 fmol/mg) (data not shown). Unlabeled obestatin, but not ghrelin, motilin, or GLP-1, competed with ^{125}I-obestatin for binding on islet membranes (Fig. 5*A*).

Obestatin release by human islets was decreased by serum starvation and by cytokine synergism (Fig. 5*B*). Anti-obestatin antibody reduced islet cell viability in the presence of serum and in serum-free conditions but not after addition of cytokines (Fig. 5*C*). Consistent with β-cell results, either [D-Lys^3]-GHRP-6 or anti-ghrelin antibody reduced obestatin-induced cell survival in all experimental conditions (data not shown).

Obestatin protein was detected in cytoplasms of fetal and adult pancreatic islets. Double immunohistochemical staining evidenced that obestatin colocalized with ghrelin in scattered cells located at the periphery of the islets, likely in the so-called ghrelin-producing ε-cells (40). Neither somatostatin, glucagon, nor insulin colocalized with obestatin, indicating that δ-, α-, and β-cells, respectively, do not produce obestatin (Fig. 5*D*).

Obestatin promotes human islet cell survival through adenylyl cyclase/cAMP/PKA signaling and activation of PI 3-kinase/Akt, ERK1/2, and CREB. Hoechst staining of islet cells exposed to either serum starvation or IFN-γ/TNF-α/IL-1β showed reduced cell density and increased apoptosis, particularly in the presence of cytokines. Obestatin strongly enhanced islet cell mass and reduced the number of apoptotic nuclei (Fig. 6*A*). Furthermore, it increased cell survival and counteracted caspase 3 activation (Fig. 6*B* and *C*).

cAMP levels were increased by obestatin at 5 min and decreased thereafter (Fig. 6*D*). Obestatin protection against serum starvation– and cytokine-induced cell death was prevented by blockade of adenylyl cyclase and PKA with MDL12330A and KT5720, respectively, but not by the Src inhibitor PP2 (Fig. 6*E*). Obestatin promoted ERK1/2 and Akt phosphorylation at 5 min, decreasing thereafter and being maintained up to 60 min, respectively (Fig. 6*F* and *G*).

Next, we investigated whether obestatin activated CREB, which plays an important role in β-cell survival and function (38,41). Obestatin-induced CREB phosphorylation peaked at 5 min, consistent with maximum cAMP induction and ERK activation (Fig. 6*H*). Moreover, differently from β-cells, in human islets, obestatin decreased Src phosphorylation (Fig. 6*I*). Obestatin antiapoptotic effect was also demonstrated in human islet primary β-cells by counting double-stained insulin- and TUNEL-positive cells (Fig. 7*A* and *B*).

These findings demonstrate that in human islets, obestatin promotes cell survival and prevents apoptosis through mechanisms involving adenylyl cyclase/cAMP/PKA signaling and activation of ERK1/2, PI 3-kinase/Akt, and CREB.

Obestatin promotes insulin secretion and gene expression in human islets. Based on the results showing obestatin binding to GLP-1R and the similar actions displayed by obestatin and GLP-1R agonists, we investigated obestatin effect on human islet insulin secretion. Obestatin increased insulin secretion in both absence and presence of glucose (2–25 mmol/l) (Fig. 7*C*). Moreover, insulin mRNA was increased by obestatin at 24 h (Fig. 7*D*), decreasing thereafter (data not shown). These results indicate that obestatin promotes insulin release and synthesis in human islets.

Obestatin stimulates GLP-1R, IRS-2, PDX-1, and glucokinase mRNA in human islets. In keeping with β-cell findings, real-time PCR results showed that in human islets, obestatin induced both GLP-1R and IRS-2 mRNA at 24 h (1.8- and 1.6- fold, respectively) (Fig. 8*A* and *B*).

The transcription factor PDX-1 is essential for pancreatic development, maintenance of β-cell mass, and insulin transcription (37,42,43). Glucokinase, a glucose sensor for insulin secretion, regulates β-cell mass and function (44,45). Real-time PCR experiments demonstrated that obestatin increased PDX-1 and glucokinase mRNA (more than three- and twofold, respectively) (Fig. 8*C* and *D*). These findings indicate that obestatin promotes expression of genes that are essential for β-cell function, survival, and glucose sensing.

DISCUSSION

This study shows that obestatin promotes survival of β-cells and human pancreatic islets. Moreover, obestatin activates signaling pathways and induces expression of genes that regulate β-cell growth, differentiation, insulin biosynthesis, and glucose metabolism.

β-Cell loss, partially caused by inflammatory cytokines, is well known in type 1 diabetes (22), and autopsy studies demonstrated reduced β-cell mass in humans with type 2 diabetes (46). One way to preserve β-cell viability and function is to reduce apoptosis. Here, we show that obestatin potently stimulated proliferation, increased cell survival, and reduced apoptosis in β-cells and human islets cultured in either absence of serum or presence of cytokines, both conditions known to induce apoptosis (20,22, 27,28,47). Obestatin antiapoptotic effect was confirmed by reduced activation of caspase 3, a major mediator of apoptosis (48), and was also demonstrated in human islet β-cells.

Although its receptor is unknown, obestatin recognized high-affinity binding sites on β-cell and islet cell membranes; moreover, although with low affinity, it interacted with acylated ghrelin and UAG binding sites, suggesting a functional interplay between obestatin and ghrelin. This assumption is sustained by evidence that [D-Lys^3]-GHRP-6 reduced obestatin survival action, likely by preventing obestatin binding to GRLN-R or/and by reducing the autocrine/paracrine effect of endogenous acylated ghrelin, as previously reported (20). Consistently, anti-ghrelin antibody reduced obestatin survival effect, possibly by sequestering endogenous ghrelin released from β-cells and

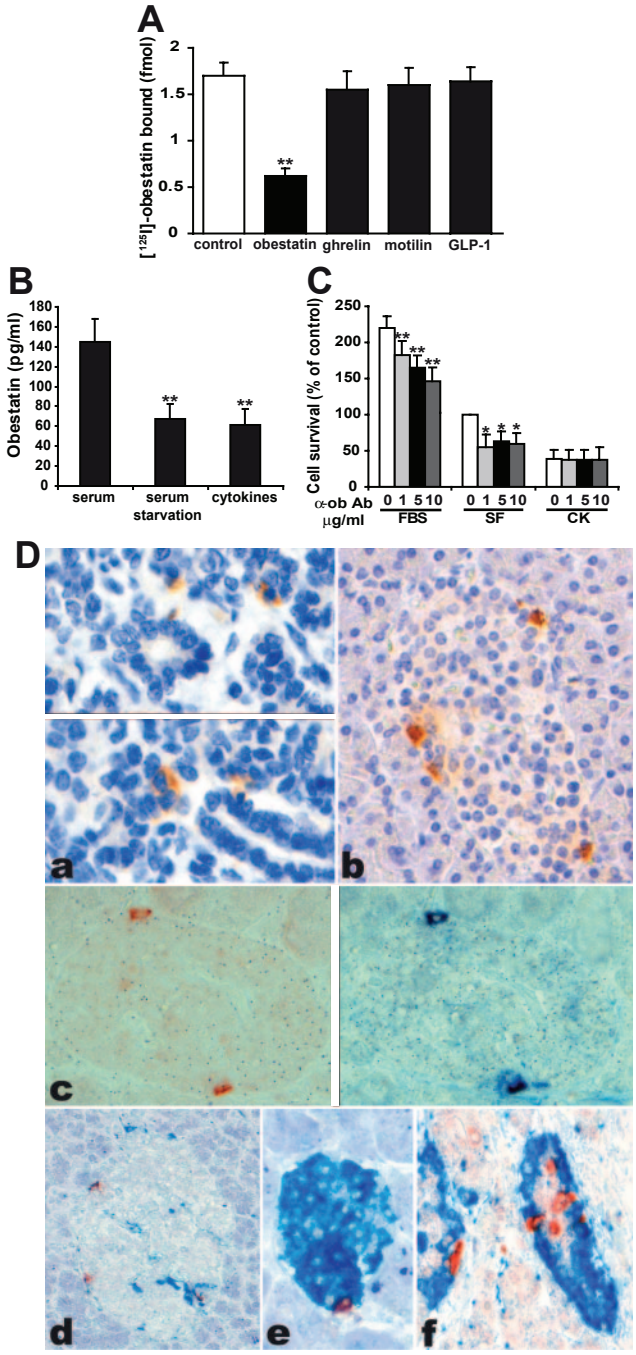

FIG. 5. Binding studies and obestatin secretion and immunoreactivity in human pancreatic islets. *A*: Competition for ^{125}I-obestatin in human islet membranes by a single concentration of the indicated competitors. Binding assay was performed by incubating a fixed amount of membrane protein (100 μg/tube) with 1 nmol/l ^{125}I-obestatin alone (control) or with 1 μmol/l indicated competitors. Data are the means ± SE from three independent experiments ($P < 0.01$ vs. control). *B*: Obestatin secretion from human islets cultured for 72 h in the presence of serum (10% FBS) or in serum-starved medium, either alone or with cytokines. Results are the means ± SE of three independent experiments, each performed in quadruplicate (**$P < 0.01$). *C*: Islet cell survival assessed by MTT after 72-h incubation in serum-containing medium (FBS) or in serum-free medium (SF) alone or with cytokines (CK) and either absence or presence of anti-obestatin antibody (α-ob Ab), at the concentrations indicated. Results are expressed as the percentage of control and are means ± SE of three independent experiments (*$P < 0.05$, **$P < 0.01$ vs. 0 μg/ml α-ob Ab at each condition). *D*: Obestatin protein localization in the human pancreas. Obestatin was detected in single cells of the fetal (*a*) and adult (*b*) human pancreas. Double immunohistochemical procedure of the same human pancreatic islet (*c*) for obestatin (*left*, red) and ghrelin (*right*, blue; subsequent incubation in the same section) demonstrated colocalization of the two hormones in the same cell; by contrast, no colocalization of obestatin (red) was found with somatostatin-producing (*d*), insulin-producing (*e*), and glucagon-producing (*f*) cells (all visualized in blue). *a* and *b*: Obestatin, brown color, immunoperoxidase with diaminobenzidine. *c* (*left*) to *f*: Obestatin, red color, immunoperoxidase with aminoethylcarbazole. *c* (*right*) to *f*: Ghrelin, somatostatin, insulin, glucagon, respectively, all blue color, immunoalkaline phosphatase with Vector blue alkaline phosphatase substrate kit III (original magnifications ×600 in *a*; ×400 in *b–f*. Results are representative of at least three independent experiments.**

human islets. Moreover, like ghrelin, obestatin is secreted by β-cells and human islets and anti-obestatin antibody reduced β-cell and islet-cell survival in both presence and absence of serum but not under cytokine synergism, where cell death was increased and obestatin secretion reduced. These results suggest that obestatin promotes survival either directly, through a specific receptor, or indirectly, by engagement of the ghrelin system. Furthermore, together with ghrelin, endogenous obestatin would regulate cell survival via autocrine/paracrine mechanisms.

At variance with pancreatic islets, comprising cell types with specific function and expression profile, INS-1E are tumor β-cells expressing multiple hormones (insulin, ghrelin, and obestatin), as demonstrated by our findings.

In human islets, obestatin, besides being strongly expressed in fetal and adult pancreas, colocalized with ghrelin, likely in ε-cells (40), but neither with insulin nor with somatostatin or glucagon. Obestatin and ghrelin coexpression in ε-cells, suggest that these hormones, products of the same gene, act in concert as local regulators of β-cell fate and function.

cAMP, a main regulator of β-cell growth and survival, promotes insulin secretion and expression of numerous β-cell genes (34,37,38,41). Obestatin upregulated β-cell and human islet cAMP; moreover, adenylyl cyclase and PKA inhibitors prevented obestatin antiapoptotic action, which was also blocked by a $G\alpha_s$ antagonist, strongly suggesting the involvement of a GPCR. Consistently, obestatin was previously shown to increase cAMP in different cells, although via GPR39, the recently denied obestatin receptor (1,5).

Cross-talk between cAMP/PKA and ERK1/2 has been reported in β-cells (49). ERK1/2 has been shown to control phosphorylation and protein level of CREB (41), which is required in glucose homeostasis and β-cell survival by regulating IRS-2 expression (38). Obestatin stimulated ERK1/2 phosphorylation in both β-cells and human islets and increased islet CREB phosphorylation on serine 133, the site initiating gene transcription (38).

Obestatin also stimulated Akt phosphorylation. PI 3-kinase/Akt regulates β-cell growth, survival, and metabolism; furthermore, Akt is a downstream target of IRS-2 which, via cAMP controls β-cell fate and function (36–38,50). Obestatin antiapoptotic effect was blocked by both ERK1/2 and PI 3-kinase/Akt inhibitors, consistent with studies showing ERK1/2 and PI 3-kinase/Akt involvement in obestatin-induced retinal cell proliferation (17). Interestingly, in human islets obestatin decreased the phosphorylation of Src, whose expression in INS-1E β-cells has been related with its inhibitory role on Ca^{2+}-dependent insulin secretion (35).

GLP-1 and GLP-1R agonists stimulate insulin secretion, decrease plasma glucose in type 2 diabetes and promote β-cell growth and survival through cAMP/PKA, ERK1/2, and PI 3-kinase/Akt (36–38,43). Obestatin upregulated GLP-1R mRNA; furthermore, besides blocking Ex-4 effect,

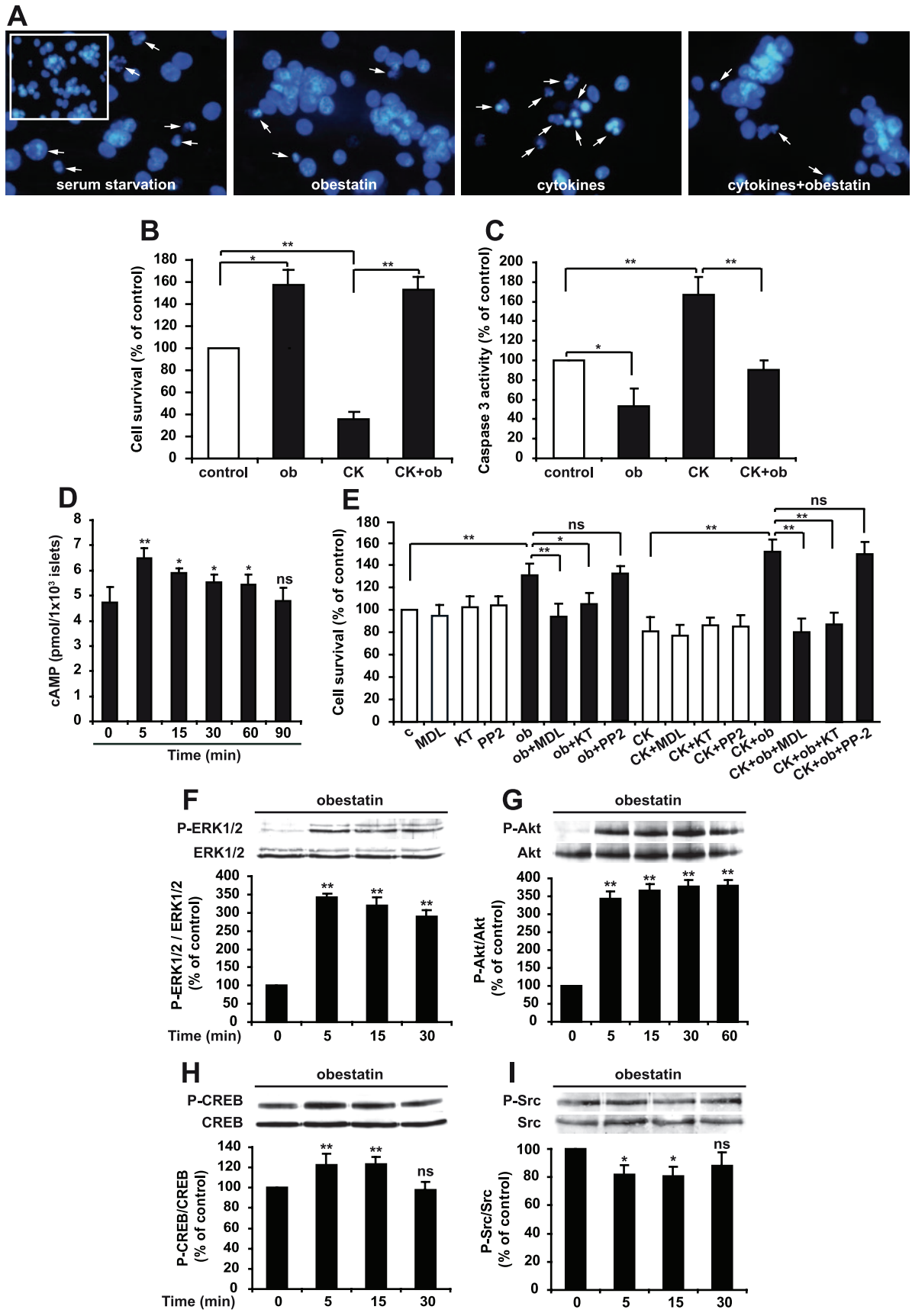

FIG. 6. Obestatin exerts survival and antiapoptotic effects in human islets. Islet cells were incubated for 72 h in the presence of serum or in serum-free medium (control) either alone or with IFN-γ/TNF-α/IL-1β (CK) (5 ng/ml each) and 100 nmol/l obestatin (ob). *A*: Hoechst 33258 nuclear immunofluorescence staining (magnification ×200) of dispersed islet cells (using Acutase, Sigma). *Inset*, cells cultured with serum; arrows, apoptotic cells. *B*: Cell viability assessed by MTT. Results are expressed as percentage of control and are means ± SE of three independent experiments (*$P < 0.05$, **$P < 0.01$). *C*: Apoptosis determined by caspase 3 activity (ELISA). Data are the means ± SE of three

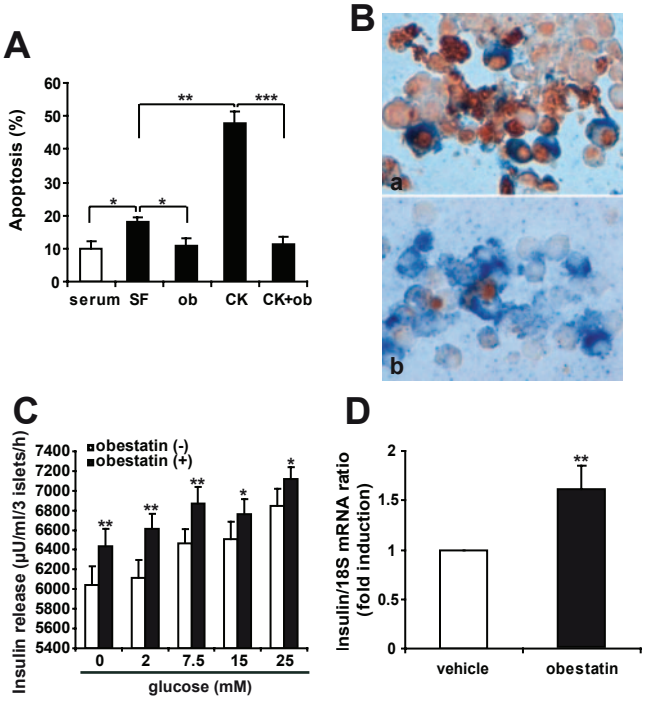

FIG. 7. Apoptosis and insulin secretion in human islet β-cells. *A*: Human islets were treated with or without serum (SF, serum-free) and in either presence or absence of 100 nmol/l obestatin (ob) and cytokines (CK) (IFN-γ/TNF-α/IL-1β, 5 ng/ml each) for 72 h. β-Cell apoptosis was assessed by counting double-stained insulin- and TUNEL-positive cells. Results are expressed as percent of total insulin-positive cells (n = 10 fields, *P < 0.05; **P < 0.01; ***P < 0.001). *B*: Representative micrographs of double stained (insulin and TUNEL) β-cells in human islets treated with cytokines, either alone (*a*) or with obestatin (*b*). Magnification ×400. *C*: Insulin release, detected by RIA in conditioned medium from serum-starved islets (n = 3) incubated alone or with 2 mmol/l glucose for 1 h and then for a further 1 h with the indicated concentrations of glucose, either in the presence or absence of 100 nmol/l obestatin. Values are the means ± SE of triplicate determinations from at least three independent experiments (*P < 0.05, **P < 0.01 vs. untreated at each glucose concentration). *D*: Human islets were cultured for 24 h in serum-starved medium either alone or with 100 nmol/l obestatin. Insulin mRNA levels were measured by real-time PCR. Results, normalized to 18S rRNA transcript level, are expressed as fold increase over untreated islets (vehicle) and are the means ± SE of three independent experiments (P < 0.01).**

Ex-9 reduced obestatin-induced survival. Obestatin, however, did not influence GLP-1 secretion, suggesting that its survival action is not dependent on GLP-1. Together with the results showing that obestatin binds to GLP-1R and that its effects are similar to those of GLP-1R agonists, these data indicate that obestatin survival action involves GLP-1R signaling. Accordingly, similarly to Ex-4, obestatin induced IRS-2 mRNA and increased IRS-2 phosphorylation, in agreement with data showing that Ex-4 promotes IRS-2 expression and activation through cAMP/CREB-mediated mechanisms (38). Obestatin even stimulated human islet insulin secretion and gene expression and increased PDX-1 mRNA, which, besides being involved in

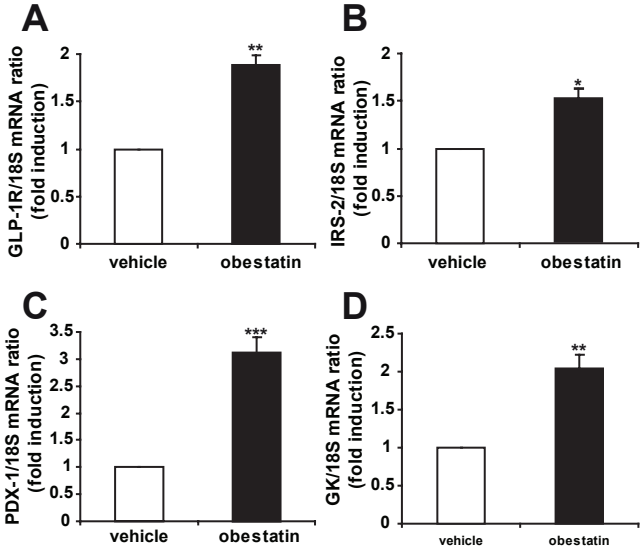

FIG. 8. Obestatin-induced GLP-1R, IRS-2, PDX-1, and glucokinase mRNA in human islets. Islets were cultured for 24 h in serum-free medium alone or with 100 nmol/l obestatin. mRNA levels for GLP-1, IRS-2, PDX-1, and glucokinase were determined by real-time PCR (*A–D*). Results, normalized to 18S rRNA transcript, are expressed as fold increase over untreated cells (vehicle) and are the means ± SE of three independent experiments (*P < 0.05, **P < 0.01).

GLP-1R–mediated pathways, is essential for pancreatic development and insulin transcription (37,42,43). Similarly, obestatin upregulated glucokinase expression, a target of PDX-1, an important glucose sensor for insulin secretion and regulator of β-cell mass and function (44,45).

Although we showed obestatin-induced survival and proliferation also at low concentrations, this study was conducted using pharmacological rather than physiological doses. Similarly, the beneficial effects of GLP-1 analogs have been previously demonstrated using mainly pharmacological concentrations (37). Future studies will address obestatin effects at more physiological levels.

In summary, we demonstrate that obestatin promotes survival and prevents apoptosis in both β-cells and human islets. Moreover, obestatin induces expression of genes that regulate β-cell fate, insulin biosynthesis, and glucose sensing. Obestatin actions involve multiple molecular mechanisms, suggesting functional interplay with ghrelin and GLP-1R signaling. Future studies will clarify whether obestatin represents a new and promising peptide for therapeutic strategies aimed at improving β-cell mass and function.

ACKNOWLEDGMENTS

G.M. has received funding from Ricerca Scientifica Applicata, Regione Piemonte, 2004; Ministero dell'Università e della Ricerca, 2005; and Finanziamento Ex-60% 2006 and 2007. E.G. has received funding from Sixth EC Program LSHM-CT-2003-503041, CIPE 2004, MIUR 2005, and FIN-

independent experiments (*P < 0.05, **P < 0.01). *D*: cAMP levels in serum-starved cells incubated, for the indicated times, with 100 nmol/l obestatin in the presence of 100 μmol/l IBMX, which was added to the culture medium 30 min before stimulation. Results are the means ± SE of three independent experiments performed in triplicate. *P < 0.05, **P < 0.01. vs. basal time point; ns, not significant. *E*: Survival (MTT) of islet cells cultured either alone or in the presence of cytokines, with or without obestatin (ob) and the inhibitors 100 nmol/l MDL12330A, 5 μmol/l KT-5720, and 10 μmol/l PP2, which were added 30 min before obestatin. Data are means ± SE from three independent experiments (*P < 0.05, **P < 0.01). *F–I*: Phosphorylation of ERK1/2 (*F*), Akt (*G*), CREB on serine 133 (*H*), and Src (*I*) evaluated by Western blot on whole lysates from human islet cells stimulated with 100 nmol/l obestatin for the indicated times (*top panels*). Equal protein loading was determined by reprobing with antibodies to the respective total proteins (*bottom panels*). Shown are representative blots from three independent experiments. Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percentage of basal (*P < 0.05, **P < 0.01; ns, not significant).

60% 2006 and 2007. This work has received funding from the SMEM Foundation, Turin, Italy and the Juvenile Diabetes Research Fund (award no. 6-2005-1178).

We thank Dr. Silvia Destefanis, Dr. Ele Ferrannini, Dr. Eleonora Santini, Dr. Antonio Secchi, Marina Taliano, and Dr. Matthias Tschöp for their contribution to the study. We also thank the European Consortium for Islet Transplantation "Islets for Research Distribution Program," Transplant Unit, Scientific Institute San Raffaele, Vita-Salute University (Milan, Italy) for providing human islets.

REFERENCES

1. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ: Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 310:996–999, 2005
2. Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W: Obestatin does not activate orphan G protein-coupled receptor GPR39. *Biochem Biophys Res Commun* 351:21–25, 2006
3. Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, Storjohann L, Stidsen CE, Jones R, Beck-Sickinger AG, Schwartz TW: GPR39 signaling is stimulated by zinc ions but not by obestatin. *Endocrinology* 148:13–20, 2007
4. Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Audry H, Llorens-Cortes C: Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". *Science* 315: 766, 2007
5. Zhang JV, Klein C, Ren P-G, Kass S, Ver Donck L, Moechars D, Hsueh AJW: Response to comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake." *Science* 315: 766, 2007
6. Tremblay F, Perreault M, Klaman LD, Tobin JF, Smith E, Gimeno RE: Normal food intake and body weight in mice lacking the G protein-coupled receptor GPR39. *Endocrinology* 148: 501–506, 2007
7. Green BD, Irwin N, Flatt PR: Direct and indirect effects of obestatin peptides on food intake and the regulation of glucose homeostasis and insulin secretion in mice. *Peptides* 28: 981–987, 2007
8. Lagaud GJ, Young A, Acena A, Morton MF, Barrett TD, Shankley NP: Obestatin reduces food intake and suppresses body weight gain in rodents. *Biochem Biophys Res Commun* 357:264–269, 2007
9. Zizzari P, Longchamps R, Epelbaum J, Bluet-Pajot MT: Obestatin partially affects ghrelin stimulation of food intake and GH secretion in rodents. *Endocrinology* 148:1648–1653, 2007
10. Samson WK, White MM, Price C, Ferguson AV: Obestatin acts in brain to inhibit thirst. *Am J Physiol Regul Integr Comp Physiol* 292:R637–R643, 2007
11. de Smet B, Thijs T, Peeters TL, Depoortere I: Effect of peripheral obestatin on gastric emptying and intestinal contractility in rodents. *Neurogastroenterol Motil* 19:211–217, 2007
12. Bassil AK, Haglund Y, Brown J, Rudholm T, Hellstrom PM, Naslund E, Lee K, Sanger GJ: Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *Br J Pharmacol* 150:58–64, 2007
13. Gourcerol G, St-Pierre DH, Tache Y: Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? *Regul Pept* 141:1–7, 2007
14. Sibilia V, Bresciani E, Lattuada N, Rapetti D, Locatelli V, De Luca V, Dona F, Netti C, Torsello A, Guidobono F: Intracerebroventricular acute and chronic administration of obestatin minimally affect food intake but not weight gain in the rat. *J Endocrinol Invest* 29:RC31–RC34, 2006
15. Carlini VP, Schioth HB, Debarioglio SR: Obestatin improves memory performance and causes anxiolytic effects in rats. *Biochem Biophys Res Commun* 352:907–912, 2007
16. Szentirmai E, Krueger JM: Obestatin alters sleep in rats. *Neurosci Lett* 404:222–226, 2006
17. Camina JP, Campos JF, Caminos JE, Dieguez C, Casanueva FF: Obestatin-mediated proliferation of human retinal pigment epithelial cells: regulatory mechanisms. *J Cell Physiol* 211:1–9, 2007
18. Iglesias MJ, Salgado A, Pineiro R, Rodino BK, Otero MF, Grigorian L, Gallego R, Dieguez C, Gualillo O, Gonzalez-Juanatey JR, Lago F: Lack of effect of the ghrelin gene-derived peptide obestatin on cardiomyocyte viability and metabolism. *J Endocrinol Invest* 30:470–476, 2007
19. Lago R, Gomez R, Dieguez C, Gomez-Reino JJ, Lago F, Gualillo O: Unlike ghrelin, obestatin does not exert any relevant activity in chondrocytes. *Ann Rheum Dis* 66:1399–1400, 2007
20. Granata R, Settanni F, Biancone L, Trovato L, Nano R, Bertuzzi F, Destefanis S, Annunziata M, Martinetti M, Catapano F, Ghe C, Isgaard J, Papotti M, Ghigo E, Muccioli G: Acylated and unacylated ghrelin promote proliferation and inhibit apoptosis of pancreatic beta-cells and human islets: involvement of 3′,5′-cyclic adenosine monophosphate/protein kinase A, extracellular signal-regulated kinase 1/2, and phosphatidyl inositol 3-kinase/Akt signaling. *Endocrinology* 148:512–529, 2007
21. Granata R, Settanni F, Trovato L, Destefanis S, Gallo D, Martinetti M, Ghigo E, Muccioli G: Unacylated as well as acylated ghrelin promotes cell survival and inhibit apoptosis in HIT-T15 pancreatic beta-cells. *J Endocrinol Invest.* 29:RC19–RC22, 2006
22. Donath MY, Halban PA: Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia* 47:581–589, 2004
23. Emamaullee JA, Stanton L, Schur C, Shapiro AM: Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long term function in islet transplantation. *Diabetes* 56:1289–1298, 2007
24. Chanoine JP, Wong AC, Barrios V: Obestatin, acylated and total ghrelin concentrations in the perinatal rat pancreas. *Horm Res* 66:81–88, 2006
25. Qader SS, Håkanson R, Rehfeld JF, Lundquist I, Salehi A: Proghrelin-derived peptides influence the secretion of insulin, glucagon, pancreatic polypeptide and somatostatin: a study on isolated islets from mouse and rat pancreas. *Regul Pept* 146:230–237, 2008
26. Scrima M, Campiglia P, Esposito C, Gomez-Monterrey I, Novellino E, D'Ursi AM: Obestatin conformational features: a strategy to unveil obestatin's biological role? *Biochem Biophys Res Commun* 363:500–505, 2007
27. Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B: Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J Biol Chem* 268:19650–19655, 1993
28. Mandrup-Poulsen T: β-Cell apoptosis: stimuli and signaling. *Diabetes* 50:S58–S63, 2001
29. Gauna C, Delhanty PJ, van Aken MO, Janssen JA, Themmen AP, Hofland LJ, Culler M, Broglio F, Ghigo E, van der Lely AJ: Unacylated ghrelin is active on the INS-1E rat insulinoma cell line independently of the growth hormone secretagogue receptor type 1a and the corticotropin releasing factor 2 receptor. *Mol Cell Endocrinol* 251:103–111, 2006
30. Davenport AP, Bonner TI, Foord SM, Harmar AJ, Neubig RR, Pin JP, Spedding M, Kojima M, Kangawa K: International Union of Pharmacology: LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol Rev* 57:541–546, 2005
31. Muccioli G, Baragli A, Granata R, Papotti M, Ghigo E: Heterogeneity of ghrelin/growth hormone secretagogue receptors: toward the understanding of the molecular identity of novel ghrelin/GHS receptors. *Neuroendocrinology* 86:147–164, 2007
32. van der Lely AJ, Tschop M, Heiman ML, Ghigo E: Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* 25:426–457, 2004
33. Hohenegger M, Waldhoer M, Beindl W, Boing B, Kreimeyer A, Nickel P, Nanoff C, Freissmuth M: Gsalpha-selective G protein antagonists. *Proc Natl Acad Sci U S A* 95:346–351, 1998
34. Hui H, Nourparvar A, Zhao X, Perfetti R: Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5′-adenosine monophosphate-dependent protein kinase A- and a phosphatidylinositol 3-kinase-dependent pathway. *Endocrinology* 144:1444–1455, 2003
35. Cheng H, Straub SG, Sharp GW: Inhibitory role of Src family tyrosine kinases on Ca2+-dependent insulin release. *Am J Physiol Endocrinol Metab* 292:E845–E852, 2007
36. Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G, White MF: Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. *J Biol Chem* 281:1159–1168, 2006
37. Doyle ME, Egan JM: Mechanisms of action of glucagon-like peptide 1 in the pancreas. *Pharmacol Ther* 113:546–593, 2007
38. Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walzer J, Lin X, White M, Montminy M: cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev* 17: 1575–1580, 2003
39. Hennige AM, Burks DJ, Ozcan U, Kulkarni RN, Ye J, Park S, Schubert M, Fisher TL, Dow MA, Leshan R, Zakaria M, Mossa-Basha M, White MF: Upregulation of insulin receptor substrate-2 in pancreatic beta cells prevents diabetes. *J Clin Invest* 112:1521–1532, 2003
40. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* 101:2924–2929, 2004
41. Costes S, Broca C, Bertrand G, Lajoix AD, Bataille D, Bockaert J, Dalle S: ERK1/2 control phosphorylation and protein level of cAMP-responsive element-binding protein: a key role in glucose-mediated pancreatic β-cell survival. *Diabetes* 55:2220–3220, 2006

42. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
43. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ: β-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. *Diabetes* 54:482–491, 2005
44. Matschinsky FM: Banting Lecture 1995: A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223–241, 1996
45. Terauchi Y, Takamoto I, Kubota N, Matsui J, Suzuki R, Komeda K, Hara A, Toyoda Y, Miwa I, Aizawa S, Tsutsumi S, Tsubamoto Y, Hashimoto S, Eto K, Nakamura A, Noda M, Tobe K, Aburatani H, Nagai R, Kadowaki T: Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. *J Clin Invest* 117:246–257, 2007
46. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102–110, 2003
47. Yermen B, Tomas A, Halban PA: Pro-survival role of gelsolin in mouse β-cells. *Diabetes* 56:80–87, 2007
48. Mathis D, Vence L, Benoist C: Beta-cell death during progression to diabetes. *Nature* 414:792–798, 2001
49. Briaud I, Lingohr MK, Dickson LM, Wrede CE, Rhodes CJ: Differential activation mechanisms of Erk-1/2 and p70(S6K) by glucose in pancreatic β-cells. *Diabetes* 52:974–983, 2003
50. Mohanty S, Spinas GA, Maedler K, Zuellig RA, Lehmann R, Donath MY, Trub T, Niessen M: Overexpression of IRS2 in isolated pancreatic islets causes proliferation and protects human beta-cells from hyperglycemia-induced apoptosis. *Exp Cell Res* 303:68–78, 2005