Hepatocyte growth factor protects rat RINm5F cell line against free fatty acid-induced apoptosis by counteracting oxidative stress

Carmela Santangelo, Paola Matarrese1, Roberta Masella, Maria Chiara Di Carlo2, Angela Di Lillo2, Beatrice Scazzocchio, Elio Vecchi2, Walter Malorni1, Riccardo Perfetti3 and Emanuela Anastasi2

National Centre for Food Quality and Risk Assessment, Section of Nutrition, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
1Department of Drug Research and Evaluation, Section of Cell Aging and Degeneration, Istituto Superiore di Sanità, Rome, Italy
2Department of Clinical Sciences University ‘La Sapienza’, Rome, Italy
3Division of Endocrinology and Metabolism Cedars-Sinai Medical Center, University of California, Los Angeles, California, USA

(Requests for offprints should be addressed to C Santangelo; Email: c.santan@iss.it)

Abstract

Type 2 diabetes is characterized by peripheral insulin resistance, pancreatic β-cells dysfunction, and decreased β-cell mass with increased rate of apoptosis. Chronic exposure to high levels of free fatty acids (FFAs) has detrimental effects on β-cell function and survival. FFAs have adverse effects on mitochondrial function, with a consequent increase in the production of reactive oxygen species. Hepatocyte growth factor (HGF) plays a critical role in promoting β-cell survival. In the present study, we investigated whether HGF was capable of protecting β-cells from death induced by prolonged exposure to FFAs. RINm5F cell line was cultured in the presence of FFAs (oleate:palmitate 2:1) for 72 h in order to induce apoptosis. Simultaneous administration of HGF and FFAs significantly suppressed the impaired insulin secretion and FFA-induced apoptosis. Specifically, HGF exerted its protective effect by counteracting: (i) the overproduction of either hydrogen peroxide and superoxide anion, (ii) the reduction of intracellular FFA-induced apoptosis. Hepatocyte growth factor (HGF) plays a critical role in promoting β-cell survival. In

Introduction

The correct balance between apoptosis and cell proliferation is a crucial factor in maintaining an appropriate mass of completely functional β-cells within the pancreatic islets. Type 2 diabetes occurs when the insulin secretory activity no longer meets the increase in demand due to presence of insulin resistance. Both a dysfunction in the glucose-dependent modality of secreting insulin and an insufficient β-cell mass have been considered as potential pathogenetic mechanisms leading to cell failure (Dickson & Rhodes 2004, Rhodes 2005). In the most recent years, concurrent with the obesity epidemic, the incidence of type 2 diabetes has shown a dramatic increase. In the setting of obesity, chronic hyperglycemia and hyperlipidemia contribute to β-cells dysfunction and a decrease of β-cell mass (Dickson & Rhodes 2004).

Free fatty acids (FFAs), at physiological concentrations, modulate the process of basal and glucose-induced insulin secretion in pancreatic β-cells, and chronic elevation of plasma fatty acid levels has been shown to be associated with insulin resistance and β-cell dysfunction (Lee et al. 1994, Evans et al. 2003).

It has been demonstrated that prolonged exposure of cultured human islets to FFAs has detrimental effects for β-cell function, including impairment of glucose-induced insulin release, suppression of proinsulin biosynthesis, and β-cell loss by apoptosis (Zhou & Grill 1995, Carpentier et al. 1999, Lupi et al. 2002). These findings are consistent with studies on the Zucker diabetic fatty rat showing that lipid accumulation in β-cells causes a substantial reduction of β-cell mass through apoptosis, and is associated with the development of diabetes (Shimabukuro et al. 1998a). In vitro studies indicate that long-term exposure of β-cell to FFAs induces adverse effects on mitochondrial function, with elevated production of reactive oxygen species (ROS) and, consequently, increased oxidative stress leading to β-cell death.
proliferation and differentiation (Otonkoski et al. 2002). Oxidative stress refers to the imbalance between the production of ROS and ability of the cells to defend against them. The concentration of ROS and the cellular microenvironment appear to be important in determining the mode of cell death. Recent reports suggest that bcl-2 might prevent apoptosis by regulating the cellular antioxidant defense mechanisms, thus acting as free radical scavenger (Jang & Surth 2003). Indeed, bcl-2-overexpressing cells exhibit elevated expression of antioxidant enzymes and γ-glutamylcysteinylglycine (GSH); conversely, downregulation of bcl-2 expression is associated with GSH depletion (Voehringer 1999). Therapeutic approaches designed to arrest β-cell apoptosis by altering the balance between offending and defending mechanism(s), may be of importance in the management of type 2 diabetes.

Hepatocyte growth factor (HGF) is a mesenchyme-derived multifunctional protein that plays a critical role in cell survival, proliferation, migration, and differentiation. Earlier studies demonstrated that HGF and its specific receptor, a transmembrane tyrosine kinase encoded by c-met protooncogene, are highly expressed during pancreas development. HGF has also been shown to have insulinotropic properties and to promote β-cell proliferation and differentiation (Otonkoski et al. 1994). HGF is also an important modulator of cell death; it protects cardiomyocytes, endothelial cells, and neurons from apoptosis (Zhang et al. 2000, Kitta et al. 2001, Nakagami et al. 2002). A potential role for HGF in the pancreatic β-cell has been recently explored; the authors have demonstrated that the administration of HGF in transgenic mouse induced an increase of pancreatic β-cell and islet number, islet size, and overall islet mass (Garcia-Ocana et al. 2003). Recently, the same authors have demonstrated that by using adenosine, the overexpression of HGF in murine islets markedly improved the function and survival of islets transplanted into diabetic mice; the protective effects of HGF have been observed even in Lewis rat in which islets were delivered intraperitoneally and accompanied by immunosuppressant therapy (Lopez-Talavera et al. 2004).

The importance of identifying antiapoptotic agents to protect β-cell from apoptosis and the ability of HGF to enhance β-cell survival in different experimental settings, led us to investigate, in insulin-producing RINm5F cells, the effect of HGF on cell death induced by prolonged exposure to FFAs.

Materials and methods

Cell line culture: FFA exposure and HGF treatment

The insulin-producing cells, RINm5F, were cultured in 75 ml flasks in the presence of RPMI 1640 medium with 10% fetal calf serum (FCS; Gibco, BRL) and 100 μg/ml penicillin, and 50 μg/ml streptomycin, at 37 °C under a humidified condition of 95% air and 5% CO2. On reaching 80% confluence, the cultures were washed twice with RPMI 1640 (without FBS) and kept in serum-free medium for 6 h before the induction of cell apoptosis. This was obtained by culturing cells in the presence of high levels of FFAs for 72 h. FFAs (2 mmol/l long-chain fatty acid oleate:palmitate 2:1) were prepared as previously described (Lee et al. 1994, Piro et al. 2002). Briefly, we first prepared a 2% fatty acid-free BSA solution in RPMI 1640, and then mixed oleate and palmitate (2:1) to obtain a 22 mmol/l stock solution. At each experiment, aliquots of stock solution were dissolved in the culture medium RPMI (Roswell Park Memorial Institute) 1640. For control experiments, BSA in the absence of fatty acids was prepared as described above. The unbound FFA concentrations were 180.6 and 20.3 nmol/l respectively for oleic and palmitic acids. Unbound concentration was calculated using the FFA-albumin association constants for the first six binding sites of albumin (Richieri et al. 1993, Cnop et al. 2001). At the end of the incubation, after a washout of the cell layer with PBS, untreated and FFA-treated adherent cells were scraped off the culture dishes, collected together with detached cells floating in the medium, and spun at 12000g for 30 s. The protective effect of HGF was studied by incubating FFA-treated cells with 50 ng/ml HGF (R&D System, Minneapolis MN, USA) for 72 h; fresh aliquots of HGF were added every 8 h. Control cultures were grown under the same conditions as treated cells but in the absence of the drugs (FFAs and/or HGF). LY294002 (Calbiochem Co, Darmstadt, Germany), a selective inhibitor of protein kinase activity PI3-K, was used at 50 μM concentration, previously shown to be effective in blocking PI3-K activity (Hui et al. 2003). Depending on the specific assay for which the cell culture was prepared, the pellets were either stored at −70 °C or immediately used for the experiment.

Morphological determination of apoptosis

For morphological studies, the cells were grown in chamber slides and treated with HGF and FFA as described above. They were then washed in PBS (pH 7.4) and fixed for 20 min in 2% paraformaldehyde in PBS (pH 7.4) at room temperature (RT). After a wash in PBS, the cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, rinsed twice in PBS, and stained with the karyophilic dye Hoechst 33342 (10 μg/ml) for 5 min at RT. After a final wash in PBS, the cells were mounted in Fluoromount G and visualized under u.v. light with an Axiosphoto microscope (Carl Zeiss, New York, NY, USA).
Analysis of insulin secretion

After incubation with FFAs, in the presence or absence of HGF, the insulin secretion was assessed as previously described (Anastasi et al. 2005). Cells were kept in 5.5 mM glucose until the day of the experiment, then challenged with either 20 mM glucose or re-exposed to 5.5 mM glucose for 60 min. The level of insulin in the culture medium was measured by RIA (Linco Research, Inc. St. Charles, MO, USA) and normalized for the total cellular protein content detected in the pellet of each individual culture according to Bradford method (Bio-Rad Laboratories, Inc.).

Annexin V assay

Quantitative evaluation of apoptosis was performed by flow cytometry after double staining with annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Eppendorf s.r.l., Milan, Italy), which allows discrimination among early apoptotic (single annexin V positive), late apoptotic (double annexin V/propidium iodide (PI) positive), and necrotic cells (single PI positive) which enables to differentiate cells that have lost membrane integrity (necrotic cells) from living cells by means of red staining of their nuclei with PI.

Analysis of the redox balance

For ROS production evaluation, control and treated cells (5×10^5 cells) were incubated in 495 μl Hanks’ balanced salt solution (pH 7-4) containing 10 μM dihydrorhodamine 123 (Molecular Probes, Eugene, OR, USA) or 1 μM dihydroethidium (Molecular Probes) in polypropylene test tubes for 15 min at 37°C. After this time, cells were washed in ice-cold PBS and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488 argon laser. Monochlorobimane (Molecular Probes) staining was performed for GSH as previously described (Sahaf et al. 2003). Cells exposed to the GSH depleting drug L-buthionine-[S,R]-sulfoximine 7.5 mM (Sigma) for 16 h were considered as negative controls (data not shown). After washings, samples were analyzed by a LRS II cytometer (Becton and Dickinson, San Jose’, CA, USA) equipped with a UVB laser. The median values of fluorescence intensity histograms were used to provide semiquantitative assessment of GSH content and ROS production.

Mitochondrial membrane potential (MMP)

The MMP (Δψ) of control and treated cells was studied by flow cytometry using 10 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carboxyamine iodide (JC-1; Molecular Probes). JC-1 is a metachromatic probe able to selectively enter the mitochondria. It exists in a monomeric form (in the green channel, FL1) but, depending on the membrane potential, it can form J-aggregates associated with a large shift in the emission range (in the orange channel, FL2). Analysis by JC-1 can be both qualitative (considering shift from green to orange) and quantitative (considering the ‘pure’ fluorescence intensity). As a methodological control (not shown), cells were also treated with increasing concentrations (from 0.1 to 10 μg/ml) of K⁺ ionophore valinomycin (Sigma) which dissipates MMP but not pH gradient (Cosarizza et al. 1993). One additional probe, tetramethylrhodamine ester (TMRM; 1 μM, Molecular Probes, red fluorescence), was also used to confirm the data obtained by JC-1.

RT-PCR analysis of Bax and bcl-2 mRNA expression

The expression of bcl-2 and Bax genes, in RINm5F cell lines, was evaluated by RT-PCR in all of the experimental conditions. Total RNA was extracted from each sample with Trizol (Invitrogen-Life Technologies) according to manufacturer’s instructions. First-strand cDNA synthesis was performed in a total volume of 20 μl using 2 μg of each RNA sample primed with random hexamers with 200 U Superscript II (Invitrogen-Life Technologies); cDNA aliquots corresponding to 200 ng RNA were subsequently amplified in 25 μl reaction volume containing 20 pmol of sense and antisense-specific primers, and 2.5 U Taq DNA polymerase (Invitrogen-Life Technologies). The following primers were used for bcl-2 (Accession no. U34964), sense: 5’ GGA GGA TTG TGG CCT TCT TTG AG 3’ (270 bp product); antisense: 5’ TAT GCA CCC AGA GTG ATG CAG GC 3’; and Bax (Accession no. U32098), sense: 5’ TGA ACT GGA CAA CAA CAT GGA GC 3’; antisense: 5’ GGT CTT GGA TCC AGA CAA ACA GC 3’ (259 bp product). Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as RNA control was analyzed employing the following primers, sense: 5’ GGA GCC AAA AGG GTC.ATC.ATC 3’; and antisense: 5’ AGA GGC AGG GAT CAT GTT CTG 3’ (342 bp product). Reaction conditions were standardized in order to observe a linear amplification of PCR products. All PCR products were electrophoresed on 1.5%-5% agarose gel and the bands visualized by ethidium bromide staining. Semi-quantitative analysis was performed by densitometric gel scanning using the ‘Gel Doc 2000’ video image system (Bio-Rad Laboratories). Results have been expressed as the ratio between the molecule of interest and GAPDH in each sample analyzed.

Western blot analysis

For immunblotting determination of CuZnSOD, MnSOD (SOD; superoxide dismutase), catalase
(CAT), bcl-2, and bax molecules, cells were washed twice with ice-cold PBS and whole cell extract was prepared by treating cells with 50 μl Triton X 1% (Sigma), 5 μl of a mixture of protease inhibitors (Sigma), and incubated on ice for 20 min. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad). The cell lysates (15–50 μg/sample) were separated by 12–15% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes, blocked with PBS containing 5% dry milk and 0.1% Tween 20, were treated with antibodies that recognize Cu/ZnSOD (Upstate Biotechnology, Lake Placid, NY, USA), MnSOD (Stressgen, Ann Arbor, MI, USA), CAT (Sigma), bax, bcl-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), and β-tubulin (Santa Cruz Biotechnology Inc). The blots were treated with appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc) followed by ECL detection (Amersham Biosciences). Densitometric analysis was performed with a molecular imager FX (Bio-Rad).

Data and statistical analysis

Data

For the flow cytometry studies, all samples were analyzed with a FACScan flow cytometer (Becton Dickinson) equipped with a 488 argon laser. At least 20,000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software or PC computer using DIVA Software (for GSH analysis; both by Becton and Dickinson). Data are reported as mean values of at least four separate experiments ± s.d.

Statistical analysis

Statistical analysis of apoptosis data was performed by non-parametric ANOVA test. Statistical significance of flow cytometry studies was calculated by using the parametric Kolmogorov–Smirnov (K/S) test. As a general rule, only P values of less than 0.01 were considered as significant. As for RT-PCR and western blotting analysis, data are expressed as means ± s.d. Statistical analysis was performed by ANOVA test. Differences were considered statistically significant when P<0.05.

Results

HGF protects insulin-secreting RINm5F cells from FFA-induced apoptosis

To examine whether HGF could protect against FFA-induced cell death, we cultured RINm5F cells, in vitro, for 72 h with or without 2 mmol/l long-chain fatty acid mixture (2:1 oleate to palmitate) in the presence or absence of 50 ng/ml of HGF. Our results showed that prolonged exposure to FFAs induced apoptotic cell death in rat RINm5F cells, and more importantly that the administration of HGF prevented the effect of FFAs on cell viability. Cytotoxicity was assayed by the morphological features of cell nuclei, demonstrating that, whereas the exposure of cells to FFAs promoted changes in the nuclear morphology characteristic, condensed and fragmented of apoptotic cells, the treatment with HGF in association with FFAs was capable of preventing nuclear fragmentation and inhibiting cell apoptosis (Fig. 1A).

To assess functional modifications, glucose-dependent secretion of insulin was evaluated in RINm5F cells exposed to FFA mixture in the presence or absence of HGF. Insulin release from cells exposed only to FFAs mixture was markedly impaired in absence of HGF (FFAs versus control; *P<0.01). The presence of HGF did not modify basal insulin release (at 5·5 mmol/l glucose) versus HGF untreated cells. When challenged with 20·0 mmol/l glucose, HGF + FFAs-treated cells showed a significant increase of insulin release when compared with FFA-treated cells. (*P<0.01; Table 1). To quantify the degree of cell apoptosis in the various culture conditions, we performed a biparametric cytofluorimetric analysis by using double staining with annexin V-FITC/PI. The annexin V/propidium iodide double staining allows discrimination among three different cell populations: (i) cells in the early phases of apoptosis (single annexin V positive), (ii) cells in the late apoptotic phases (double annexin V/PI positive), and (iii) necrotic cells (single PI positive) We found that exposure to FFAs was able to induce apoptosis in a significant percentage of cells (about 40%, *P<0.01 versus control; Fig. 1B). To note, in our experimental condition, only a small percentage of cells (<10%) underwent secondary necrosis after FFAs administration. Importantly, HGF (50 ng/ml), which did not
Protective action of HGF on RIN cells

A

FFA

FFA+HGF

B

CTRL

FFA

HGF

HGF+FFA

% of apoptotic cells

CTRL     FFA     HGF     FFA+HGF

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influence *per se* apoptosis in β-cells (*P*> 0.05 versus control), counteracted the effects of FFAs. Indeed, simultaneous administration of HGF and FFAs significantly reduced the occurrence of apoptosis (about 69%; *P*< 0.01 versus FFAs; Fig. 1B).

### HGF inhibits mitochondrial damage and ROS production

To evaluate the protective activity of HGF against FFA-induced apoptosis, we analyzed ROS production in the experimental conditions, above described, by a semiquantitative flow cytometry method. Our results confirmed that long-term FFAs exposure resulted in a significant overproduction of either hydrogen peroxide, \( \text{H}_2\text{O}_2 \), (Fig. 2A) or superoxide anion, \( \text{O}_2^- \), (Fig. 2B) with respect to cells cultured in the absence of FFAs. Importantly, HGF treatment was capable of preventing FFA-induced oxidative stress by significantly decreasing ROS production (*P*< 0.01 versus FFA).

We next investigated the cellular antioxidant defenses by analyzing GSH content by semiquantitative flow cytometry method and protein expression of MnSOD, CuZnSOD, and CAT enzymes by western blotting. We observed a significant decrease of intracellular GSH (Fig. 2C) content in FFA-treated cells whereas levels of MnSOD, CuZnSOD, and CAT proteins were unchanged (data not shown). When HGF was added to FFA-treated cells, the decrease of reduced GSH was strongly attenuated (*P*< 0.01 versus FFA; Fig. 2C) and no differences were observed in the expression of antioxidant enzymes tested (data not shown).

To further investigate the FFA-induced cell death, we analyzed the MMP in living cells by using JC-1 probe. This experiment demonstrated an involvement of mitochondrial transition pore (Fig. 2D), indicating that the treatment with FFAs induced the depolarization of the mitochondrial membrane in a significant percentage of insulin-producing cells (≥ 45%) when compared with control cells (about 5%; *P*< 0.01). On the other hand, the administration of HGF was able to significantly (*P*< 0.01) prevent this depolarization of mitochondrial membrane.

### FFA and HGF modulate \( \text{bcl-2} \) expression

Our study found that FFAs induced a significant reduction of \( \text{bcl-2} \) expression, both at the mRNA (Fig. 3A and B; *P*< 0.01) and protein level (Fig. 3C and D; *P*< 0.01) in RINm5F cells line. On the other hand, no significant change in bax expression was detected both at the mRNA (Fig. 3A and B) and at the protein (Fig. 3E and F) level. However, HGF treatment alone did not affect the expression of \( \text{bcl-2} \) molecule in untreated control cells, but it was able to prevent the decrease of \( \text{bcl-2} \) mRNA (*P*< 0.05) and FFA-induced protein (*P*< 0.01) (Fig. 3A–D).

### HGF acts via PI3-K

The involvement of PI3-kinase in the survival effect of HGF was investigated by pre-treatment of RIN cells with LY294002 (50 μM), a synthetic specific inhibitor of PI-3-kinase, 30 min before FFAs + HGF stimulation. Data obtained clearly show that LY294002 abolishes the protective effect of HGF (Fig. 4). Inhibition of PI-3-kinase led to an increase in the number of apoptotic cells, similarly to that observed in FFA-treated cells (Fig. 4A). Accordingly, pre-treatment with LY294002 also abrogated the protective effect exerted by HGF on the mitochondrial-membrane polarization state (Fig. 4B). Furthermore, the production of \( \text{H}_2\text{O}_2 \) (Fig. 4C) and \( \text{O}_2^- \) (Fig. 4D) induced by FFAs was not prevented by HGF when LY294002 was present in the culture medium. Moreover, the pre-treatment with LY294002 completely prevented the increase of reduced GSH induced by HGF (Fig. 4E). Finally, the decrease of \( \text{bcl-2} \) protein prevented by HGF in the presence of FFA, was completely counteracted by LY294002 (Fig. 4F and G).

### Discussion

The present study was undertaken to investigate whether HGF exerted protective activity against cell death induced by a prolonged exposure to FFAs in insulin-producing RINm5F cell line. In addition, we provided an early elucidation of the mechanism by which HGF improves function and viability of insulin secreting cells. Our study found that HGF was able to inhibit, in RINm5F cells, the apoptosis and to prevent metabolic dysfunction induced by the exposure to FFAs. Furthermore, HGF inhibited oxidative stress induced by FFAs, thus exhibiting a ROS scavenging property. Finally, we showed that
Figure 2 HGF inhibits mitochondrial damage and ROS production. Semiquantitative cytofluorimetric analysis of: (A) hydrogen peroxide production, H$_2$O$_2$; (B) superoxide anion production, O$_2^-$; and (C) intracellular GSH content after long-term exposure of RINm5F cells to FFAs. In the upper panels results obtained in a representative FACS experiment are shown. In the lower panels the mean of data obtained from four independent experiments ± S.D. is reported. $P<0.01$. (D) Biparametric flow cytometry analysis after staining of living cells with JC-1. In the area under the dashed line, the numbers indicate the percentage of cells with depolarized mitochondria. Results obtained in a representative experiment (upper panel) and the average of data obtained in four independent experiments (lower panel, mean ± S.D.; $^*P<0.01$) are reported. CTRL, control.
HGF might regulate the expression of antiapoptotic protein bcl-2 likely via PI3-K/Akt-dependent pathway. Previous reports have demonstrated that HGF is a survival factor with antioxidant properties for various cell types. For example, HGF protects adult cardiac myocytes against apoptosis induced by hydrogen peroxide, serum deprivation, and chemotherapeutic agents inducing oxidative stress (Kitta et al. 2001). In addition, HGF was found to protect cerebellar granule neurons against apoptotic death induced by low concentration of potassium (Zhang et al. 2000), and to rescue SAEC cell line from apoptosis induced by the treatment with tumour necrosis factor-α or hydrogen peroxide (Okada et al. 2004). More importantly, it has been shown that HGF is a useful tool to preserve and enhance islet function and survival (Garcia-Ocana et al. 2003, Lopez-Talavera et al. 2004). Our results showed that HGF is able to improve function and viability of RIN5mF cells in accordance with a recent report indicating that HGF/c-met has an essential role in the maintenance of normal glucose-dependent insulin secretion, and that genetic alterations in the HGF/c-met receptor/system, combined with environmental factors, may be implicated in the predisposition to develop type 2 diabetes (Roccisana et al. 2005). Oxidative stress is considered an important mediator of cellular damage following a prolonged exposure of pancreatic β-cell to elevated levels of FFAs (Lupi et al. 2002, Evans et al. 2003). Our observations, consistent with previous reports (Tiedge et al. 1997, Carlsson et al. 1999), demonstrated that long-term exposure to FFAs resulted in a significant overproduction of either hydrogen peroxide (H₂O₂) or superoxide anion O₂⁻ in RINm5F cells when compared with untreated cells. Moreover, these phenomena were accompanied by depolarization of mitochondrial membrane in a significant percentage of FFAs-treated cells.

Figure 3 HGF modulates bcl-2 expression. Total RNA was isolated from RINm5F cells incubated for 72 h without (control; CTRL) or with free fatty acids (FFA) and in the presence of HGF with (FFA + HGF) or without FFA (HGF). (A) Expression of bcl-2, bax, and endogenous control GAPDH was evaluated by RT-PCR. PCR products were electrophoresed on 1.5% agarose gel and analyzed by densitometric gel scanning. A representative experiment of three is shown. (B) Densitometric analysis of the bands is shown and results are expressed as means ± s.d. of the different experiments. *P<0.01 versus control; †P<0.05 versus FFAs. (C) Bcl-2 and bax expression evaluated by western blot analysis. A representative experiment of three is shown. (D) Densitometric analyses are reported as means ± s.d. of the three different experiments. *P<0.01 versus FFAs.
Figure 4 The antiapoptotic action of HGF requires the activation of a PI3K-dependent signaling pathway. HGF antiapototic activity was tested in the presence of the PI3K inhibitor LY294002. (A) Quantitative flow cytometry analysis of apoptosis after staining with annexin V and (B) mitochondrial membrane potential after staining of living cells with JC-1. Results are expressed as the percentage of annexin V-positive cells and percentage of cells with depolarized mitochondria membrane respectively. (C) Semi-quantitative flow cytometry analysis of hydrogen peroxide production \( \text{H}_2\text{O}_2 \), (D) superoxide anion production \( \text{O}_2^- \), and (E) intracellular GSH content. (F) Bcl-2 and bax protein expression evaluated by western blot and (G) densitometric analyses are reported as means ± S.D. of the four different experiments conducted in the following experimental conditions: LY294002, FFA, FFA + HGF, and FFA + HGF + LY294002. *\( P < 0.01 \) versus FFAs. Dashed lines in the histograms represent the values found in control untreated cells for each specific parameter. Results reported in (A–G) are the means ± S.D. from four independent experiments conducted in the following experimental conditions: LY294002, FFA, FFA + HGF, and FFA + HGF + LY294002. *\( P < 0.01 \).
It was noteworthy that by reducing $H_2O_2$ and $O_2^-$ production, HGF prevented FFA-induced oxidative stress, thus protecting mitochondrial function and rescuing ability to glucose response. It has been established that mitochondrial function is essential for glucose-stimulated secretion, and glucose-sensing defect observed in type 2 diabetes is caused by $\beta$-cell mitochondrial dysfunction (Lowell & Shulman 2005). Long-term exposure to FFAs determines an increase in energy flux through the electron transport chain, which leads to an increased ROS production and a proton leak that impairs glucose-stimulated insulin secretion, resulting in $\beta$-cell dysfunction. (Barbu et al. 2002, Lowell & Shulman 2005). In addition, the detrimental effect of increased oxidative stress might be due, in part, to the low levels of antioxidant enzyme defense system (SOD, CAT, glutathion peroxidase) present in $\beta$-cells when compared with other tissues (Lenzen et al. 1996, Tiedge et al. 1997). We showed that HGF was able to strongly attenuate depletion of reduced GSH levels, induced by FFA treatment in RIN5mF cells, whereas it did not modify protein expression of MnSOD, CuZnSOD, and CAT antioxidant enzymes.

Our data suggest that HGF exerts its protective effect by strengthening the intrinsic antioxidant defenses of RINmF5 cells through modulation of GSH intracellular and thus protecting them by acting on the redox status. This hypothesis is supported by recent findings showing that in type 2 diabetes, the human $\beta$-cell has functional defects associated with multiple alterations and increased oxidative stress and that 24-h exposure to glutathione improves glucose-stimulated insulin release, suggesting that it should be possible to reverse the functional impairment of type 2 diabetic islets by reducing oxidative stress in islet cells (Del Guerra et al. 2005).

Cells susceptibility to apoptosis is regulated by a balanced expression between apoptosis-suppressing and -inducing proteins. In humans and rodents islets, FFAs-induced apoptosis is associated with the downregulation of bcl-2/bax ratio (Shimabukuro et al. 1998b, Lupi et al. 2002). It has been reported that overexpression of bcl-2 proteins enhances islet viability (Rabinovitch et al. 1999). Our results demonstrated that HGF was capable of maintaining, in RINm5F cells, the bcl-2/bax ratio observed in control untreated cells, by counteracting the downregulation of the expression of bcl-2 induced by FFA-incubation. Several lines of evidence suggest that bcl-2 family members contribute to redox regulation in mammalian cells; bcl-2 deficiency mice show a chronic oxidative stress phenotype (Veis et al. 1993) and bcl-2 raises intracellular level of glutathione and other thiols in fibroblasts and other cells types (Papadopoulos et al. 1998, Rudin et al. 2003). Moreover, bcl-2 appears to be a potent antioxidant and survival factor able to decrease ROS levels through regulation of cellular antioxidant enzymes (i.e., SOD and CAT; Deng et al. 2003). The role of bcl-2 in regulation of ROS levels is consistent with its mitochondrial localization, since the majority of ROS is produced in this organelle. However, the mechanism by which bcl-2 prevents ROS-induced apoptosis is unknown. HGF has multifunctional activities and exerts its prosurvival action by different mechanisms depending on the apoptotic stimuli. In renal epithelial cells, apoptosis induced by serum withdrawal is prevented by HGF overexpression through rapid phosphorylation of Akt, followed by phosphorylation/inactivation of Bad and simultaneously induced a dramatic expression of bcl-xL (Liu 1999). On the other hand, in human endothelial cells, hypoxia-induced apoptosis was attenuated by HGF and it was accompanied by a significant increase of bcl-2 molecule (Yamamoto et al. 2001). The survival effects of HGF in $\beta$-cells appear to involve the PI3-K and Akt pathway (Holst et al. 1998, Garcia-Ocana et al. 2003). Moreover, adenoviral-mediated expression of constitutively active Akt completely protected mouse INS-1 $\beta$-cell line (insulinomine $\beta$-cell line) from FFA-induced apoptosis (Wrede et al. 2002). Our data demonstrated that the rescue effect of HGF on the FFAs-induced apoptosis involves the PI3-kinase/Akt pathway. Indeed, pre-treatment with LY294002, a synthetic specific inhibitor of PI3-kinase, abolished the protective effect of HGF, resulting in the overproduction of $H_2O_2$ and $O_2^-$, in the decrease of reduced GSH as well as in the decrease of bcl-2 molecule. The above results provide evidences that PI3-K is an essential mediator through which HGF inhibits FFA-induced oxidative stress and apoptosis in RINm5F cells possibly bcl-2 regulated. Because of its strong prosurvival and antioxidant action, HGF treatment appears to be a useful approach to preserve $\beta$-cell function and survival in the lipotoxicity setting.

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