Antiapoptotic effects of GLP-1 in murine HL-1 cardiomyocytes

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1Division of Cardiovascular Sciences, Center for Applied Medical Research and 3Department of Cardiology and Cardiovascular Surgery, University Clinic, School of Medicine, University of Navarra, Pamplona, Spain; and 2MSD A/S, Glostrup, Denmark

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Ravassa S, Zudaire A, Carr RD, Diez J. Antiapoptotic effects of GLP-1 in murine HL-1 cardiomyocytes. Am J Physiol Heart Circ Physiol 300: H1361–H1372, 2011. First published January 28, 2011; doi:10.1152/ajpheart.00885.2010.—Activation of apoptosis contributes to cardiomyocyte dysfunction and death in diabetic cardiomyopathy. The peptide glucagon-like peptide-1 (GLP-1), a hormone that is the basis of emerging therapy for type 2 diabetic patients, has cytoprotective actions in different cellular models. We investigated whether GLP-1 inhibits apoptosis in HL-1 cardiomyocytes stimulated with staurosporine, palmitate, and ceramide. Studies were performed in HL-1 cardiomyocytes. Apoptosis was induced by incubating HL-1 cells with staurosporine (175 nM), palmitate (135 μM), or ceramide (15 μM) for 24 h. In staurosporine-stimulated HL-1 cardiomyocytes, phosphorylidyloserin exposure, Bax-to-Bcl-2 ratio, Bad phosphorylation (Ser136), BNIP3 expression, mitochondrial membrane depolarization, cytochrome c release, caspase-3 activation, DNA fragmentation, and mammalian target of rapamycin (mTOR)/p70S6K phosphorylation were assessed. Apoptotic hallmarks were also assessed in the absence of some of the above treatments. In addition, phosphorylidyloserin exposure and DNA fragmentation were analyzed in palmitate- and ceramide-stimulated cells. Staurosporine increased apoptosis in HL-1 cardiomyocytes. GLP-1 (100 nM) partially inhibited staurosporine-induced mitochondrial membrane depolarization and completely blocked the rest of the staurosporine-induced apoptotic changes. This cytoprotective effect was mediated by phosphorylidyloserin 3-kinase (PI3K) and partially dependent on ERK1/2. Increasing concentrations of glucose did not influence GLP-1-induced protection against staurosporine. Furthermore, GLP-1 inhibited palmitate- and ceramide-induced phosphorylidyloserin exposure and DNA fragmentation. Incretin GLP-1 protects HL-1 cardiomyocytes against activation of apoptosis. This cytoprotective ability is mediated mainly by the PI3K pathway and partially by the ERK1/2 pathway and seems to be glucose independent. It is proposed that therapies based on GLP-1 may contribute to prevent cardiomyocyte apoptosis.

Diabetic cardiomyopathy is a distinct primary disease process that leads to left ventricular dysfunction and heart failure in diabetic patients. Mechanistically, the complexity of diabetic cardiomyopathy is attributed to abnormal cellular metabolism and defects in organelles such as mitochondria, sarcosomes, and endoplasmic reticulum, all of which may lead to activation of apoptosis, a process that contributes to cardiac dysfunction and failure via cardiomyocyte loss and functional derangements of cellular organelles in viable cardiomyocytes. Of interest, it has been reported that diabetic patients with dilated cardiomyopathy had significantly more apoptotic cardiomyocytes compared with nondiabetic patients with dilated cardiomyopathy. Furthermore, different experimental studies have shown that blockade of myocardial apoptosis results in significant prevention of diabetes-induced cardiac dysfunction.

The glucagon like peptide-1 (GLP-1) is an incretin rapidly released after meal intake that stimulates insulin output from pancreatic β-cells in a glucose-dependent manner. Notably, patients with type 2 diabetes mellitus are partially deficient in GLP-1 secretion, although this conjecture remains controversial. Cardioprotective actions of GLP-1 have been described in several ischemia/reperfusion models, accompanied by cardiac function improvement and enhanced cell survival. Also, GLP-1 has been reported to improve left ventricular function and remodeling and prolong survival in rats with chronic heart failure. Specifically, GLP-1 increases myocardial insulin activity and glucose uptake and activates kinases of the so-called reperfusion injury salvage kinase pathway (RISK pathway). Of interest, GLP-1 has been described as an antiapoptotic factor in different cell types, including cholangiocytes, neuronal cells, and pancreatic β-cells.

Therefore, we aimed to investigate whether GLP-1 may also protect against activation of the apoptotic process in HL-1 cardiomyocytes. Given that the cardiac cytoprotective actions of GLP-1 are mostly dependent on activation of kinases, we have mimicked a situation of protein kinase inhibition in HL-1 cardiomyocytes. To that effect, we have used the classical proapoptotic stimuli staurosporine, an antibiotic whose main biological activity is the inhibition of protein kinases through prevention of ATP binding to the kinase. We have studied the involvement of the RISK pathway and the influence of extracellular glucose concentration on the GLP-1 potential cytoprotective actions in staurosporine-stimulated HL-1 cardiomyocytes. Finally, additional experiments were performed to investigate the antiapoptotic effects of GLP-1 in the presence of lipotoxic agents known to play a role in the development of diabetic cardiomyopathy.

EXPERIMENTAL STUDIES

HL-1 Cell Culture

HL-1 murine cells were a gift from Dr. William C. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). They were cultured in Claycomb medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% norepinephrine, and 1% L-glutamine, in a 5% CO₂ humidified atmosphere at 37°C. The HL-1 cells were used for experimentation after reaching ~80% confluence.

To perform the experiments, HL-1 cells were incubated with Claycomb culture medium supplemented with 0.1% FBS, 1% penicillin/streptomycin, 1% norepinephrine, 1% L-glutamine, and validate...
phosphatidylinositol 3-kinase (PI3K) pathway, wortmannin (10 μM, 100 nM stock solution) was used to control the osmotic effect of the high glucose concentration. Staurosporine (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) (100 mM stock solution) and a 1:10 dilution was performed to reach the working concentration. Experiments previously done demonstrated that DMSO dilutions higher than 1% do not influence apoptosis and viability in HL-1 cardiomyocytes. However, because palmitate stock solution (10 mM) was performed in Dulbecco’s phosphate-buffered saline (Invitrogen), including BSA (Sigma) at 3.3 mM, an equivalent amount of BSA was dissolved in basal Claycomb medium to control BSA effects in palmitate-stimulated HL-1 cardiomyocytes.

Protein Extraction and Subcellular Fractioning

Total protein from human cardiac biopsies and HL-1 cells was obtained by lysis in urea/thiourea buffer (7 M urea, 2 M thiourea, 4% Chaps, 1% dithiothreitol).

The cytosol-enriched fraction was obtained as previously described, with some modifications (47). Briefly, 1.2 × 10^9 HL-1 cells were lysed in 25 μL of protease inhibitor cocktail (Sigma) and 150 μL of ice-cold homogenization buffer [20 mM Tris·HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, and 6 mM β-mercaptoethanol] by five freeze-thaw steps and centrifuged at 40,000 g for 1 h. The supernatant was considered the cytosol-enriched fraction. The mitochondrial-enriched fraction was obtained as previously described (30) with some modifications. Briefly, 6 × 10^9 HL-1 cells were homogenized in 10 mM Tris, pH 7.0, 10% sucrose, and 0.1 mM EDTA (buffer 1) by five freeze-thaw steps, and centrifuged at 1,000 g for 10 min. The supernatant was named S1. The pellet was resuspended in buffer 1 and centrifuged for 10 min at 1,000 g. The supernatant of the second centrifugation (S2) was mixed with S1 and centrifuged for 10 min at 5,200 g. The pellet was resuspended in buffer 1 and recentrifuged at 17,500 g for 20 min. The supernatant was discarded, and the pellet was resuspended in urea/thiourea buffer (mitochondrial-enriched fraction).

Analysis of Apoptotic Hallmarks

Phosphatidylserine externalization. To analyze phosphatidylserine (PS) exposition, the “Annexin-V-FITC” Kit was employed (Pharmatarget). Cellular fluorescence was determined by flow cytometry (FAC Scan; Becton-Dickinson) using the Cell Quest program. Data were expressed as the percentage of Annexin-V-positive/PI-negative cells, from the total number of HL-1 cells.

Bax-to-Bcl-2 ratio. To analyze the expression of Bax and Bcl-2, 10 μg of total protein were loaded on 12% polyacrylamide gel, electrophoresis was performed, and proteins were transferred to nitrocellulose membranes. A Bax-specific rabbit polyclonal antibody and a Bcl-2-specific mouse polyclonal antibody (Santa Cruz Biotecnology) were used at 1:500. The blots were reprobed with a monoclonal β-actin antibody (Sigma) as loading control. The bands were visualized and analyzed as previously described (42).

Phosphorylated Bad-to-total Bad ratio. To analyze the phosphorylation status of the proapoptotic protein Bad, 10 μg of total protein were loaded on 12% polyacrylamide gel, electrophoresis was performed, and proteins were transferred to nitrocellulose membranes. A Bad-specific rabbit monoclonal antibody and a phosphorylated (Ser136) Bad-specific rabbit polyclonal antibody (Cell Signaling) were used at 1:2,000. The blots were reprobed with a monoclonal β-actin antibody (Sigma) as loading control. The bands were visualized and analyzed as previously described.

BNIP3 expression. To analyze the expression of BNIP3, 10 μg of total protein were loaded on 12% polyacrylamide gel, electrophoresis was performed, and proteins were transferred onto nitrocellulose membranes. A BNIP3-specific rabbit polyclonal antibody (Cell Signaling) was used at 1:1,000. The blots were reprobed with a monoclonal β-actin antibody (Sigma) as loading control. The bands were visualized and analyzed as previously described.

Depolarization of the mitochondrial membrane. To analyze mitochondrial membrane potential, we used the JC-1 dye (Molecular Probes, Invitrogen), which shifts the fluorescence emission from red (~590 nm) to green (~527 nm) as mitochondrial membrane is depolarized. HL-1 cells were incubated with JC-1 for 30 min at 37°C, and cellular fluorescence was determined by flow cytometry analysis. Data are expressed as the percentage of cells with mitochondrial membrane depolarization from the total number of HL-1 cells.

Cytochrome c quantification. Ten micrograms of protein from mitochondrial- and cytosol-enriched fractions were separated on 12% polyacrylamide gel and transferred onto nitrocellulose membranes as previously described. A polyclonal antibody against cytochrome c was used at a dilution of 1:1,000 (Cell Signaling) was used. The blots were reprobed with the monoclonal β-actin antibody (Sigma) and cyclooxygenase IV (Abcam) antibodies for the cytosolic and mitochondrial fractions, respectively, as loading controls. The bands were visualized and analyzed as previously described.

Caspase-3 activation. To analyze the activation of caspase-3, 10 μg of total protein were separated on 12% polyacrylamide gel and transferred onto nitrocellulose membranes. A caspase-3-specific rabbit polyclonal antibody (Cell Signaling) was used at a dilution of 1:1,000 was used to analyze the 35-kDa inactive procaspase and the 17-kDa active fragment. The blots were reprobed with a monoclonal β-actin antibody (Sigma) as a loading control. The bands were visualized and analyzed as previously described.

DNA fragmentation. DNA fragmentation in HL-1 cells was detected by the terminal deoxynucleotidyl transferase reaction using a detection kit (Roche Diagnostics, Mannheim, Germany). The fluorescence changes were analyzed by flow cytometry as previously described. Data are expressed as the percentage of HL-1 cells with fragmented DNA from the total number of HL-1 cells.

Analysis of Survival Pathways

Mammalian target of rapamycin/p70S6K survival kinases activation. To analyze the phosphorylation status of the mammalian target of rapamycin (mTOR) and p70S6K survival kinases, 10 μg of total protein were loaded on 6% polyacrylamide gel, electrophoresis was performed, and proteins were transferred onto nitrocellulose membranes. A mTOR-specific rabbit polyclonal antibody (1:2,000), a phosphorylated (Ser2448) mTOR-specific rabbit polyclonal antibody (1:2,000), a p70S6K-specific rabbit monoclonal antibody (1:2,000), and a phosphorylated (Thr89) p70S6K-specific rabbit polyclonal antibody (1:2,000) were used. The blots were reprobed with a monoclonal β-tubulin antibody (Sigma) as a loading control. The bands were visualized and analyzed as previously described.

Statistical Analysis

Variables are expressed as means ± SE. Differences among different conditions were tested by one-way ANOVA followed by a Student-Newman-Keuls test once normality was checked (Shapiro-Wilks test); otherwise, the nonparametric Kruskal-Wallis test followed by a Mann-Whitney U-test (adjusting the α-level by Bonferroni inequality) was used. Statistical significance was defined as two-sided P < 0.05. The analyses were performed using the program SPSS (15.0 version).
RESULTS

Studies Performed in Staurosporine-stimulated HL-1 Cardiomyocytes

Staurosporine and GLP-1 dose-response stimulation curves. Dose-response curves were performed to establish the EC_{50} value of staurosporine for induction and execution of the apoptotic process. As shown in Fig. 1, there was a dose-dependent increase in both PS exposure (Fig. 1A) and DNA fragmentation (Fig. 1B) in response to staurosporine incubated for 24 h. EC_{50} values of 175 nM were obtained.

In additional experiments, HL-1 cells were incubated with 175 nM staurosporine for 24 h in the presence of different concentrations of GLP-1 (50–200 nM). As shown in Fig. 1, GLP-1 inhibited in a dose-dependent manner the increase in PS exposure (Fig. 1C) and DNA fragmentation (Fig. 1D) induced by staurosporine in HL-1 cardiomyocytes. IC_{50} values for GLP-1 inhibition of DNA fragmentation were close to 100 nM, which was the chosen concentration to further investigate the mechanisms by which GLP-1 exerts its cytoprotective actions.

Effects of GLP-1 on Apoptotic Hallmarks

PS exposure. Compared with control cells, staurosporine induced an increase in PS exposure in HL-1 cardiomyocytes that was prevented in the presence of GLP-1 (Fig. 2A). Coincubation of GLP-1 with U-0126 and Rp-cAMP did not modify the inhibitory effects of the peptide on staurosporine-induced PS exposure. However, when HL-1 cells were incubated with staurosporine and GLP-1 in the presence of wortmannin, the peptide did not prevent the increase in PS exposure (Fig. 2A).

Bax-to-Bcl-2 ratio. As shown in Fig. 2B, the increase in the Bax-to-Bcl-2 ratio induced by staurosporine in HL-1 cardiomyocytes was prevented in the presence of GLP-1. When cells were incubated with staurosporine and GLP-1 in the presence of U-0126, the Bax-to-Bcl-2 ratio did tend to decrease compared with cells incubated with staurosporine alone, but the difference did not reach statistical significance. Rp-cAMP did not influence the GLP-1 inhibitory effect on staurosporine-induced Bax-to-Bcl-2 ratio increment. However, when cells were incubated with staurosporine and GLP-1 in the presence of wortmannin, the incretin failed to prevent the increase in the Bax-to-Bcl-2 ratio induced by staurosporine (Fig. 2B).

Phosphorylated Bad-to-total Bad ratio. As shown in Fig. 3A, cells incubated with staurosporine exhibited a significant decrease in Bad inactivation by phosphorylation at Ser^{136} compared with control cells. This decrement was blunted when cells were incubated with staurosporine and GLP-1. However,
cells incubated with staurosporine and GLP-1 in the presence of U-0126, Rp-cAMP, or wortmannin showed that a phosphorylated Bad state significantly decreased compared with control cells (Fig. 3A).

BNIP3 expression. As shown in Fig. 3B, the increase in BNIP3 expression induced by staurosporine in HL-1 cardiomyocytes was prevented in the presence of GLP-1. Coincubation of GLP-1 with U-0126 and Rp-cAMP did not modify the inhibitory effects of the peptide on staurosporine-induced BNIP3 overexpression. However, when HL-1 cells were incubated with staurosporine and GLP-1 in the presence of wortmannin, the peptide did not prevent the increase in BNIP3 expression (Fig. 3B).

Depolarization of the mitochondrial membrane. As shown in Fig. 4A, staurosporine induced mitochondrial membrane depolarization in HL-1 cardiomyocytes compared with control cells. This alteration was partially inhibited in the presence of GLP-1 (Fig. 4A). Neither U-0126 nor Rp-cAMP modified this inhibitory effect of GLP-1. Nevertheless, HL-1 cells coincubated with staurosporine and GLP-1 in the presence of wortmannin exhibited similar mitochondrial membrane depolarization compared with staurosporine-stimulated cells (Fig. 4A).

Cytochrome c release from the mitochondria. Figure 4B shows that, compared with control cells, staurosporine-stimulated HL-1 cells exhibited increased cytochrome c release from mitochondria that was prevented when GLP-1 was present in the culture medium. Coincubation with U-0126 and Rp-cAMP did not modify the GLP-1 inhibitory effect on staurosporine. However, when HL-1 cells were coincubated with staurosporine and GLP-1 in the presence of wortmannin, the staurosporine-induced cytochrome c release from mitochondria was not prevented by GLP-1 (Fig. 4B).

Caspase-3 activation. As shown in Fig. 5A, cells incubated with staurosporine exhibited a significant increase in caspase-3 activation compared with control cells. This increment was
blunted when cells were incubated with staurosporine and GLP-1. Cells incubated with staurosporine and GLP-1 in the presence of U-0126 exhibited a tendency to decrease caspase-3 activation compared with cells incubated with staurosporine alone, but the difference was not statistically significant. Rp-cAMP did not modify GLP-1 effects on caspase-3 activation. Nevertheless, when HL-1 cells were coincubated with staurosporine and GLP-1 in the presence of wortmannin, caspase-3 activation was still significantly increased compared with control cells (Fig. 5A).

**DNA fragmentation.** Figure 5B shows that DNA fragmentation was increased in HL-1 cells incubated with staurosporine compared with control cells. This increment was blunted when staurosporine was incubated in the presence of GLP-1 (Fig. 5B). When HL-1 cells were coincubated with staurosporine and GLP-1 in the presence of U-0126, DNA fragmentation did tend to decrease compared with cells incubated with staurosporine alone, the value being not significantly different from the one measured in control cells. Coincubation of staurosporine with GLP-1 in the presence of Rp-cAMP did not modify the GLP-1 inhibitory effect on staurosporine-induced DNA fragmentation in HL-1 cardiomyocytes. However, when cells were coincubated with staurosporine and GLP-1 in the presence of wortmannin, DNA fragmentation remained significantly increased compared with control cells (Fig. 5B).

**Effects of GLP-1 on Survival Pathways**

**mTOR activation.** As shown in Fig. 6A, cells incubated with staurosporine exhibited a significant decrease in mTOR activation by phosphorylation at Ser2448 compared with control cells. This decrement was inhibited when cells were incubated with staurosporine and GLP-1. Cells incubated with staurosporine and GLP-1 in the presence of U-0126 and wortmannin showed lower mTOR phosphorylation that, although presenting no differences compared with GLP-1-incubated cells, were significantly decreased compared with control cells. Incubation of HL-1 cells with GLP-1 in the presence of Rp-cAMPs resulted in mTOR phosphorylation no different from any of the other groups (Fig. 6A).

**p70S6K activation.** As shown in Fig. 6B, cells incubated with staurosporine exhibited a significant decrease in p70S6K activation by phosphorylation at Thr389 compared with control cells. This decrement was partially inhibited when cells were incubated with staurosporine and GLP-1. Cells incubated with staurosporine and GLP-1 in the presence of U-0126 and Rp-cAMP
showed lower mTOR phosphorylation that, although presenting no differences compared with GLP-1-incubated cells, were significantly decreased compared with control cells. However, when cells were coincubated with staurosporine and GLP-1 in the presence of wortmannin, p70S6K activation remained significantly inhibited compared with control and GLP-1-incubated cells (Fig. 6B).

Influence of Glucose Concentration on GLP-1 Cytoprotective Actions

All the above data indicate that GLP-1 inhibits early and late steps of the apoptotic cascade activated by staurosporine in HL-1 cardiomyocytes incubated in the absence of glucose. To investigate whether glucose influences the cytoprotective actions of GLP-1, we incubated HL-1 cardiomyocytes with staurosporine and GLP-1 in Claycomb culture medium supplemented without or with low (5 mM) or high (10 mM) concentrations of glucose, equivalent to physiological (90 mg/dl) and supraphysiological (180 mg/dl) concentrations of blood glucose, respectively. After analyzing all apoptotic hallmarks, we observed that the inhibitory effects of GLP-1 were maintained also in HL-1 cells stimulated with staurosporine in the presence of the two concentrations of glucose (Table 1). Of interest, GLP-1 inhibition of staurosporine-induced mitochondrial membrane depolarization was more pronounced in HL-1 cardiomyocytes incubated in the presence of 10 mM glucose than in cells incubated in the other two conditions (Table 1).

Studies Performed in Palmitate- and Ceramide-Stimulated HL-1 Cardiomyocytes

Palmitate and ceramide are known to contribute to the development of diabetic cardiomyopathy by inducing cardiomyocyte apoptosis among other effects (10, 17). We have tested GLP-1 cytoprotective effects in HL-1 cardiomyocytes incubated with these lipotoxic agents. The experiments have been performed in the absence of glucose, since the data collected in the staurosporine experiments suggest that GLP-1 antiapoptotic actions are independent of glucose concentration.
Palmitate and GLP-1 Dose-Response Stimulation Curves

Dose-response curves were performed to establish the EC50 value of palmitate for induction and execution of the apoptotic process. As shown in Fig. 7, there was a dose-dependent increase in both PS exposure (Fig. 7A) and DNA fragmentation (Fig. 7B) in response to palmitate incubated for 24 h. EC50 values of 135 μM were obtained.

In additional experiments, HL-1 cells were incubated with 135 μM palmitate for 24 h in the presence of different concentrations of GLP-1 (50–200 nM). As shown in Fig. 7, GLP-1 inhibited in a dose-dependent manner the increase in PS exposure (Fig. 7C) and DNA fragmentation (Fig. 7D) induced by palmitate in HL-1 cardiomyocytes. At concentration values of 150–200 nM, GLP-1 inhibited the activation of both apoptotic hallmarks.

Ceramide and GLP-1 Dose-Response Stimulation Curves

Dose-response curves were performed to establish the EC50 value of ceramide for induction and execution of the apoptotic process. As shown in Fig. 8, there was a dose-dependent increase in both PS exposure (Fig. 8A) and DNA fragmentation (Fig. 8B) in response to ceramide incubated for 24 h.

To perform GLP-1 dose-response inhibition curves, HL-1 cardiomyocytes were incubated with 15 μM ceramide for 24 h in the presence of different concentrations of GLP-1 (50–200 nM). As shown in Fig. 8, GLP-1 inhibited in a dose-dependent manner the increase in PS exposure (Fig. 8C) and DNA fragmentation (Fig. 8D) induced by ceramide in HL-1 cardiomyocytes. At concentration values of 150–200 nM, GLP-1 inhibited the activation of both apoptotic hallmarks.

DISCUSSION

The main findings of this study are as follows: 1) GLP-1 prevents staurosporine-induced apoptosis and survival pathway inactivation in HL-1 cardiomyocytes; 2) these cytoprotective effects of GLP-1 are mediated via activation of the PI3K and partially through ERK1/2 pathways, and are maintained in the presence of supraphysiological concentrations of glucose; and 3) GLP-1 cytoprotective effects are also present in palmitate- and ceramide-stimulated HL-1 cardiomyocytes.
GLP-1 has already been described as an antiapoptotic factor in cardiac ischemia-reperfusion (7, 46) and heart failure (38) in vivo models. However, only one to three apoptotic hallmarks were included in these studies. More detailed in vitro studies of the antiapoptotic mechanisms of GLP-1 have already been performed in several cellular models. For instance, in cholangiocytes, GLP-1 induces Bax downregulation and inhibits cytochrome c release and DNA fragmentation (29). In addition, GLP-1 inhibits Bax expression (39), upregulates Bcl-2 (27), and inhibits depolarization of the mitochondrial membrane (23) in neuronal cells. Finally, in pancreatic β-cells, GLP-1 induces upregulation of Bcl-2 (12), Bad phosphorylation, and inactivation (41) and reduces caspase-3 activity (45) and DNA fragmentation (6, 26). Remarkably, there is not available data concerning GLP-1 and BNIP3 interactions. BNIP3 is a proapoptotic protein known to induce mitochondrial damage by provoking mitochondrial swelling and release of cytochrome c in heart mitochondria (13, 40). Notably, this is the first time that an inhibitory effect of GLP-1 on the mitochondrial-damaging proapoptotic protein BNIP3 is described. In addition, we have confirmed and expanded these data by demonstrating that GLP-1 prevents the exposure of PS, the increase of the Bax-to-Bcl-2 ratio, Bad activation, mitochondrial membrane depolarization, the release of cytochrome c, caspase-3 activation, and DNA fragmentation induced by staurosporine in HL-1 cardiomyocytes. It is important to remark that mitochondrial membrane depolarization is the only alteration not completely recovered in the presence of GLP-1. In this regard, several studies have demonstrated that the maneuvers that inhibit the apoptotic process not necessarily are accompanied by the full recovery of the mitochondrial membrane potential (1, 20). In addition, we have found that GLP-1 prevents staurosporine inhibition of the mTOR/p70S6K survival pathway. These results support previous observations demonstrating that GLP-1 actions may be dependent on this survival pathway as it has been reported in PC12 cells (23) and in isolated rat hearts (9).

We have found that GLP-1 requires activation of the PI3K pathway to prevent staurosporine-induced apoptosis in HL-1 cells. In this sense, findings from previous studies involve PI3K-dependent pathways as the mechanism by which GLP-1 exerts its antiapoptotic actions in other cell types (23, 27, 49). In addition, GLP-1 has also been described to inhibit apoptosis in pancreatic β-cells via the ERK1/2 pathway (6, 41). In this regard, because we find that GLP-1 actions on the increase of the Bax-to-Bcl-2 ratio, Bad phosphorylation, caspase-3 activa-
tion, DNA fragmentation, and mTOR/p70S6K activation are interfered in cells in which the ERK1/2 pathway has been blocked, the possibility exists that this pathway may mediate in part the anti-apoptotic actions of GLP-1 in HL-1 cells.

It has been reported that, in freshly isolated rat islets, GLP-1 was more effective in stimulating insulin secretion in the presence of high glucose concentrations (16). Furthermore, it has been reported that GLP-1 increased ERK activation in a

Table 1. Effects of GLP-1 on staurosporine-induced apoptosis in the absence or presence of physiological or supraphysiological concentrations of glucose

<table>
<thead>
<tr>
<th>Apoptotic Hallmarks</th>
<th>0 mM</th>
<th>ST</th>
<th>Physiological (5 mM)</th>
<th>ST</th>
<th>GLP-1</th>
<th>Supraphysiological (10 mM)</th>
<th>ST</th>
<th>GLP-1</th>
</tr>
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<tbody>
<tr>
<td>PS externalization, cells/100 cells</td>
<td>6.4 ± 0.5</td>
<td>21.6 ± 5.6*</td>
<td>9.7 ± 0.8</td>
<td>5.2 ± 0.1</td>
<td>18.5 ± 2.9*</td>
<td>6.8 ± 0.4</td>
<td>5 ± 0.2</td>
<td>21 ± 1.2‡</td>
</tr>
<tr>
<td>ΔΨ&lt;sub&gt;m&lt;/sub&gt; depolarization (Abs 527/Abs 590 nm)</td>
<td>0.4 ± 0.2</td>
<td>4.5 ± 0.3‡</td>
<td>2.4 ± 0.4§</td>
<td>0.4 ± 0.01</td>
<td>2.5 ± 0.7*</td>
<td>1.4 ± 0.2§</td>
<td>0.5 ± 0.01</td>
<td>2.9 ± 0.2‡</td>
</tr>
<tr>
<td>Bax-to-Bcl-2 ratio</td>
<td>1.5 ± 0.04</td>
<td>4.6 ± 0.8*</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.04</td>
<td>3 ± 0.4*</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.04</td>
<td>4 ± 0.5*</td>
</tr>
<tr>
<td>Cytochrome c (cytosol/mitochondrial ratio)</td>
<td>1.2 ± 0.1</td>
<td>6.4 ± 1.1*</td>
<td>1.6 ± 0.3</td>
<td>1.3 ± 0.04</td>
<td>6.1 ± 0.8*</td>
<td>2.2 ± 0.6</td>
<td>0.9 ± 0.02</td>
<td>5.8 ± 1.3*</td>
</tr>
<tr>
<td>Caspase-3 (17/35 kDa ratio)</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.05†</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.003</td>
<td>0.7 ± 0.1†</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.02</td>
<td>0.7 ± 0.1†</td>
</tr>
<tr>
<td>DNA fragmentation, cells/100 cells</td>
<td>7.8 ± 0.3</td>
<td>15.1 ± 1.4†</td>
<td>9.8 ± 0.6</td>
<td>10.6 ± 0.4</td>
<td>18.8 ± 2.4*</td>
<td>14.5 ± 1.1</td>
<td>9.6 ± 0.2</td>
<td>16.7 ± 1.9*</td>
</tr>
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Values are expressed as means ± SE. ST, staurosporine; GLP-1, glucagon-like peptide-1; PS, phosphatidylserine; ΔΨ<sub>m</sub>, mitochondrial membrane potential; Abs, absorbance. *P < 0.05 vs. control and ST + GLP-1. †P < 0.01 vs. control and ST + GLP-1. ‡P < 0.001 vs. control and ST + GLP-1. §P < 0.05 vs. control.
...glucose-dependent manner in pancreatic β-cells (14). In these cells, the presence of glucose was found to be required for GLP-1 interactions with mitochondrial-related processes such as membrane depolarization and ATP production (18). Interestingly, when HL-1 cardiomyocytes are incubated in the presence of concentrations of glucose that may correspond to either normoglycemia or the hyperglycemia found in diabetic patients, GLP-1 maintains its cytoprotective ability, being able to completely prevent staurosporine-induced apoptosis, including complete inhibition of mitochondrial membrane depolarization. It thus appears that the anti-apoptotic actions of GLP-1 in this cellular model are not inhibited, but rather potentiated, by glucose. These data underline the importance of controlling for glucose concentration in studies of cardioprotective mechanisms.

Interestingly, we have confirmed that GLP-1 exerts cytoprotective actions in HL-1 cardiomyocytes incubated with apoptotic stimuli more related to diabetes such as the lipotoxic agents palmitate and ceramide. In this regard, GLP-1 analogs have been described to protect pancreatic β-cells against palmitate-induced apoptosis (12). However, no data concerning GLP-1 effects on ceramide-induced apoptosis are available in the literature. Therefore, this is the first study demonstrating that GLP-1 inhibits palmitate- and ceramide-induced apoptosis in a cardiac cell line.

**Limitations.** The in vitro experiments have been performed in HL-1 cardiomyocytes. Although this is a cardiac muscle cell line derived from a mouse atrial cardiomyocyte tumor lineage, these cells maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties characteristic of adult cardiomyocytes (11, 48). It must be also noted that 100 nM GLP-1 is a supraphysiological concentration according to levels of GLP-1 usually reported in humans (0.01–0.1 nM) (33). However, it was considered to be appropriate since higher concentrations of analogs of GLP-1 resistant to endopeptidases have been used in some clinical studies. For example, to improve glycemic control in patients with type 2 diabetes, liraglutide, a GLP-1 analog, is used at 0.6–1.8 mg/day, which corresponds to 160–480 nM/day (34). The experiments with palmitate and ceramide were performed in the absence of glucose, since GLP-1 ant apoptotic effects seemed to be independent of glucose, as observed in staurosporine-stimulated HL-1 cardiomyocytes. However, it would be very interesting to study how glucose may affect ceramide- and palmitate-induced apoptosis and whether GLP-1 is influenced by glucose in these conditions. Furthermore, only phosphatidylserine exposure and DNA fragmentation were measured to evaluate GLP-1 cytoprotective effects against palmitate and ceramide. In this re...

Fig. 8. Dose-dependent increase in phosphatidylserine exposure (A) and DNA fragmentation (B) in HL-1 cardiomyocytes incubated with ceramide for 24 h. C and D: GLP-1-induced dose-dependent inhibition of phosphatidylserine exposure and DNA fragmentation, respectively, in HL-1 cardiomyocytes incubated with 15 μM ceramide for 24 h. Values represent means ± SE (n = 3). * P < 0.05 vs. control.
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regard, it would be very interesting to evaluate GLP-1 cytoprotective effects by analyzing the rest of the apoptotic parameters in HL-1 cells stimulated with these stimuli. Finally, we performed additional experiments to study whether GLP-1 anti-apoptotic effects were mediated by the GLP-1 receptor by using the GLP-1 receptor antagonist exendin-(9−39) amide. However, inconclusive data were obtained. In this regard, it is worth notice that exendin-(9−39) has been reported to activate PI3K and ERK pathways (15, 44); therefore, we have considered that exendin-(9−39) may not function as a proper GLP-1 receptor antagonist in our model.

In summary, we have demonstrated that GLP-1 protects cardiomyocytes against staurosporine-induced apoptosis blocking all the steps of the apoptotic process via activation of the PI3K pathway. Because these cytoprotective actions of GLP-1 are maintained in hyperglycemic conditions and extended to other lipotoxic stimuli known to induce cellular damage in the context of diabetic cardiomyopathy, our findings support the notion that agents based upon GLP-1 may exert favourable cardiac effects in diabetic patients via preservation of cardiomyocyte mass and function.

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DISCLOSURES

None declared.

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