Glucagon-Like Peptide-1 Counteracts Oxidative Stress-Dependent Apoptosis of Human Cardiac Progenitor Cells by Inhibiting the Activation of the c-Jun N-terminal Protein Kinase Signaling Pathway


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Increased apoptosis of cardiac progenitor cells (CPCs) has been proposed as a mechanism of myocardial damage and dysfunction. Glucagon-like peptide-1 (GLP-1) has been shown to improve heart recovery and function after ischemia and to promote cell survival. The protective effects of GLP-1 on oxidative stress-induced apoptosis were investigated in human CPCs isolated from human heart biopsies. Mesenchymal-type cells were isolated from human heart biopsies, exhibited the marker profile of CPCs, differentiated toward the myocardiocyte, adipocyte, chondrocyte, and osteocyte lineages under appropriate culture conditions, and expressed functional GLP-1 receptors. CPCs were incubated with GLP-1 with or without hydrogen peroxide (H2O2). Phospho- and total proteins were detected by immunoblotting and immunofluorescence analysis. Gene expression was evaluated by quantitative RT-PCR. The role of the canonical GLP-1 receptor was assessed by using the receptor antagonist exendin(9–39) and receptor-specific siRNA small interfering RNAs. Cell apoptosis was quantified by an ELISA assay and by flow cytometry-detected Annexin V. Exposure of CPCs to H2O2 induced a 2-fold increase in cell apoptosis, mediated by activation of the c-Jun N-terminal protein kinase (JNK) pathway. Preincubation of CPCs with GLP-1 avoided H2O2-triggered JNK phosphorylation and nuclear localization, and protected CPCs from apoptosis. The GLP-1 effects were markedly reduced by coincubation with the receptor antagonist exendin(9–39), small interfering RNA-mediated silencing of the GLP-1 receptor, and pretreatment with the protein kinase A inhibitor H89. In conclusion, activation of GLP-1 receptors prevents oxidative stress-mediated apoptosis in human CPCs by interfering with JNK activation and may represent an important mechanism for the cardioprotective effects of GLP-1. (Endocrinology 153: 5770–5781, 2012)

Multipotent progenitor cells, capable of differentiating into cardiomyocytes, endothelial cells, and smooth muscle cells in vitro (1) and in vivo (1–4), have been identified in the adult heart. The viability of cardiac progenitor cells (CPCs) is thought to be essential for constant tissue repair and renewal in the adult heart (1, 3, 5, 6). Conversely, early senescence, growth limitation, and enhanced apoptotic death of CPCs contributing to the loss of functional myocardial tissue have been described in aging-associated cardiomyopathy in humans (7) and experimental diabetes in rodents (8). Upon ischemia and metabolic impairment, generation of reactive oxygen species and enhanced intracellular oxidative stress represent major events leading to CPCs dysfunction and death (9, 10). Hence, therapies that can directly inhibit oxidative stress-mediated apoptosis of the CPCs, leading to en-

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Abbreviations: CPC, Cardiac progenitor cells; CREB, cAMP-response-element-binding protein; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLP-1, glucagon-like peptide-1; GLP-1r, glucagon-like peptide-1 receptor; JNK, c-Jun N-terminal kinase; PKA, protein kinase A; MKK, mitogen-activated kinase kinase; siRNA, small interfering RNA.
hanced CPCs survival (11), hold a great cardioprotective potential.

Glucagon-like peptide-1 (GLP-1)-based therapies represent a recent treatment option for type 2 diabetes (12). GLP-1 receptors (GLP-1R) have been demonstrated in the heart and vasculature of both rodents and humans (13, 14) and mediate regulatory effects on heart rate, blood pressure, vascular tone, and myocardial contractility (15, 16). GLP-1R agonists were also shown to exhibit cardioprotective effects in experimental models of dilated cardiomyopathy (17), hypertensive heart failure (18), and myocardial infarction (19–21). GLP-1 infusion may also improve cardiac contractile function in chronic heart failure patients, with or without diabetes (22, 23), and in myocardial infarction patients after successful angioplasty (24). This is of interest, in light of the ongoing debate on the cardiovascular safety of specific hypoglycemic drugs and the unmet need to pharmacologically address both the metabolic and cardiovascular abnormalities in type 2 diabetes.

The cellular targets and molecular mechanisms underlying the cardioprotective effects of GLP-1 are still poorly understood. At a cellular level, GLP-1 and GLP-1 mimetics antagonize apoptosis in various cell types and, specifically, prevent TNFα-induced apoptosis of β-cells by interfering with the activation of the stress kinase c-Jun N terminal protein kinase (JNK) (25). Whether GLP-1 exerts direct prosurvival effects on the CPCs compartment, which may be relevant to its ability to improve cardiac survival and function in humans, has not been investigated. The aim of this study was to isolate CPCs from human heart biopsies, and to investigate the ability of GLP-1 to prevent hydrogen peroxide (H₂O₂)-induced apoptosis in these cells.

Materials and Methods

Human heart biopsies

Cardiac biotic samples were obtained from the right atrial appendage of 10 subjects, undergoing major open heart surgery (Supplemental Table 1 published on The Endocrine Society’s Journals web site at http://endo.endojournals.org). The study protocol was approved by the independent Ethics Committee of the Azienda Ospedaliero-Universitaria of the University of Bari, Bari, Italy.

Isolation and culture of CPCs

The cardiac specimens (~50 mg) were transported to the laboratory in Hank’s balanced salt solution (pH 7.4), cut into fragments of 1–2 mm³, washed with Ca²⁺-Mg²⁺-free PBS, incubated in Hank’s balanced salt solution supplemented with 0.5 g/liter collagenase and 40 g/liter BSA, fraction V, for 30 min at 37 C, and then seeded on porcine gelatin 1%-coated dishes in F-12 Nutrient Mixture medium (Ham) (Life Technologies, Inc., Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 8 mmol/liter glucose. Dishes were incubated in a humidified (95%) atmosphere with 5% CO₂ at 37 C. After 7–9 d, a layer of mesenchymal stromal-like cells arose from the adherent explants. Cells were grown in six-well plates to 80–85% confluence and then pretreated with or without GLP-1 or exendin-4 (Eli Lilly and Co., Indianapolis, IN) or GLP-1(9–36) amide (Bachem, Torrance, CA), followed by challenge with 0.1–0.5 mmol/liter H₂O₂, as indicated.

Flow cytometry

Cells were washed in PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with PBS supplemented with 5% BSA and 0.1% Triton X-100 for 20 min, incubated with the appropriate monoclonal antibodies [c-Kit-PE, CD133-PE, CD34-fluorescein isothiocyanate (FITC), and CD45-PerCP (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), CD105-APC (Biolegend, San Diego, CA), and CD31-FITC (BD Biosciences, San Jose, CA)] for 1 h, and analyzed with a FACSCount flow cytometer (Becton Dickinson, Mountain View, CA), at room temperature using the FACS DIVA Software. To evaluate MEF2C and Nkx 2.5, the cells were washed in PBS, fixed with 4% paraformaldehyde, permeabilized with PBS supplemented with 0.1% Triton X-100 and 0.1% sodium citrate, and incubated with blocking solution containing 5% rabbit serum in PBS. The cells were then incubated with antirabbit donkey antibody, and finally with polyclonal MEF2c and Nkx 2.5 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS overnight at 4 C (1:50). Cells were then washed in PBS and incubated with antigo antibody conjugated with FITC in PBS (1:50) for 1 h at room temperature.

Real-time RT-PCR (qPCR)

Total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was removed by DNase digestion, and 500 ng of total RNA were used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit and primers designed with Primer Express 3.0 (Applied Biosystems, Weiterstadt, Germany) (Supplemental Table 2). PCRs were carried out in an ABI PRISM 7500 System (Applied Biosystems) under the following conditions: 50 C for 2 min, 95 C for 10 min, 40 cycles at 95 C for 15 sec, and 60 C for 1 min. The mRNA level of each gene was normalized using 18S as internal control.

Identification of GLP-1R mRNA by direct sequencing

Total RNA was extracted from CPCs and human atrial tissue, and cDNA was synthesized. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and then sequenced. Primers used for PCR were: forward, 5’-GCGCCACGAGCTTGTGCTGCAA-3’; and reverse, 5’-CTTGGCACAAGTCTGATTTGA-3’. The expected PCR product was 885 bp. Sequencing was performed by PRIMM (Milan, Italy).

cAMP assay

Cells were lysed in 0.1 M HCl for 10 min and centrifuged at 1300 × g for 10 min at room temperature. cAMP concentrations in the supernatants were determined with a direct enzyme im-
munoassay kit (cAMP enzyme immunoassay kit, Direct; Sigma-Aldrich, St. Louis, MO).

**Multilineage differentiation**

CPCs were cultured in MEM supplemented with 10 μM 5-azacytidine to kill dividing cells, 2% (vol/vol) equine serum, 20 nmol/liter insulin (Eli Lilly, Indianapolis, IN), and 20 nmol/liter triiodothyronine. After 24 h, cells were washed twice with PBS, and the medium was changed to complete medium without 5-azacytidine. mRNA levels of the cardiac-specific marker troponin-T were analyzed after 4 wk by qPCR. To promote *in vitro* mineralization, the CPCs were cultured in MEM supplemented with glutamine (292 mg/liter), 10% fetal calf serum, 10 mm β-glycerophosphate, 0.1 μM l-ascorbic acid-2-phosphate, and 100 nmol/liter dexamethasone for 3 wk, and then stained with Alizarin red staining; cells were cultured in osteogenic medium (β-glycerophosphate, dexamethasone, and ascorbic acid), and within 3 wk the cell monolayer was extensively covered by Alizarin red-positive calcified extracellular matrix. E4–E6. Chondrogenic differentiation, confirmed by Alcian Blue staining; cells were seeded at high density in micromass culture and treated with chondrogenic medium StemPRO (Life Technologies, Inc., Invitrogen) that induced the formation of three-dimensional chondrogenic cell aggregates. Treated micromass stained positive for Alcian blue within 2 wk, indicating the presence of sulfated proteoglycans. E7–E9. Adipogenic differentiation, demonstrated by Oil Red O staining; after the induction with adipocyte differentiation medium (insulin, dexamethasone, triiodothyronine, rosiglitazone, and isobutylmethylxanthine), fat globules were noticed within 2–3 wk that stained positive with Oil Red O.

**Small interfering RNA (siRNA)-mediated GLP-1R silencing**

Three primer pair sequences for siRNA-mediated silencing of the human GLP-1R (Supplemental Table 3) were purchased from Invitrogen. A nonsilencing fluorescently labeled siRNA was used as negative control (Qiagen). CPCs were transfected using Lipofectamine 2000 (Invitrogen). Cells were plated at 50% confluency and transfected with the three siRNA sequences at 25 nmol/liter for 48 h. GLP-1R transcript levels were analyzed by qPCR (Supplemental Fig. 1).

**Apoptosis**

Cellular apoptosis was measured by 1) evaluating cytoplasmic oligonucleosomes using the Cell Death Detection ELISA PLUS kit (Roche Biochemicals Indianapolis, IN); 2) immunoblotting cellular lysates with cleaved caspase-3 (Asp (175)) antibodies; and 3) detecting Annexin V by the Annexin V-FITC kit (Beckman Coulter Co., Marseille, France) followed by flow cytometry.

**Immunofluorescence analyses**

Cells were seeded on coverslips, cultured for 24 h in complete medium, fixed in ice-cold 4% paraformaldehyde for 20 min, and
permeabilized in Tris-buffered saline/0.2% Triton X-100 for 10 min. For JNK detection, samples were incubated with a primary polyclonal antibody against phosphorylated JNK (1:100) in Tris-buffered saline/3% BSA overnight at 4°C, and then with a secondary Alexa (488) Fluor anti-rabbit goat antibody (1:2000, Molecular Probes, Eugene, OR) for 1 h at 25°C. Nuclei were stained with TO-PRO-3 (1:3000, Molecular Probes). Pictures were acquired on a Leica TCS SP2 laser scanning spectral confocal microscope (Leica Microsystems, Heerbrugg, Switzerland); all images were taken at the same magnification.

Immunoblotting

CPCs were mechanically detached in ice-cold lysis buffer containing 50 mmol/liter HEPES (pH 7.5), 150 mmol/liter NaCl, 1 mmol/liter MgCl₂, 1 mmol/liter CaCl₂, 4 mmol/liter EDTA, 1% Triton X-100, 10% glycerol, 50 mmol/liter NaF, and 10 mmol/liter Na₃VO₄, supplemented with 100 μmol/liter phenylmethylsulfonyl fluoride, 5 ng/ml leupeptin, and 2 mmol/liter Na₃VO₄. After 15 min at 4°C, the preparation was centrifuged at 12,000 g for 10 min at 4°C. Equal protein samples were subjected to SDS-PAGE and immunoblotting, as previously described (25).

In vitro caspase-3 activity assay

Caspase-3 activity was detected using the Caspase-3 Colorimetric Assay Kit (Millipore, Corp., Boston, MA), according to the manufacturer’s instructions. Assays were performed in 96-well plates by incubating 80 μg of cell lysates in 100 μl reaction buffer containing the caspase-3 substrate Ac-DEVD-pNA. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a microtiter plate reader (Beckman Coulter Co.).

Antibodies and specialized reagents

Polyclonal anti-cAMP-response-element-binding protein (CREB), anti-phospho-CREB (Tyr), anti-mitogen-activated kinase kinase (MKK4), anti-phospho-MKK4 [Ser (257)/Thr (261)], anti-MKK7, anti-phospho-MKK7 [Ser (271)/Thr (275)], anti-stress-activated protein kinase/JNK, antiphospho-stress-activated protein kinase/JNK [Thr (183)/Tyr (185)], anti-c-Jun, anti-phospho-c-Jun [Ser (63)], and cleaved caspase-3 [Asp (175)] were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Polyclonal anti-glycerinaldehyde 3-phosphate dehydroge-
nase (GAPDH), anti-β-actin, and anti-GLP-1R were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). GLP-1 (7–36), exendin-4, and exendin(9–39) were from Sigma-Aldrich. SP600125 and H89 were from Calbiochem (San Diego, CA). The JNK inhibitor D-TAT-JNK-I peptide (28), linked to a 5(6)-carboxyfluorescein fluorochrome was from PRIMM. Fluorochrome was used to visualize cell entry and accumulation (data not shown). D-JNK-I selectively blocks the access of JNK to c-Jun and other substrates by a competitive mechanism, without affecting JNK phosphorylation (28). Unless otherwise specified, all chemical reagents were purchased from Sigma-Aldrich.

Statistical analysis

All data are presented as means ± SEM of at least three independent experiments. Data are expressed as percentage of control or basal values, as appropriate. Statistical analysis was performed by one-way ANOVA and Student’s t test. Significance was assumed at a P < 0.05. The Minitab 15.0 software (Minitab Inc., State College, PA) was used for statistical analyses.

Results

Isolation of human CPCs

Human heart bioptic samples were obtained in the course of open surgery, minced, and plated. Approximately 1 wk after plating, fibroblast-like cells emerged from tissue fragments and reached confluence within 12–15 d (Fig. 1A). qPCR analysis identified mRNA expression of typical stem cell markers, including c-kit, MDR-1, and CD105, early myogenic transcriptional factors, including GATA-4 and MEF2C, and the endothelial cell marker CD31 (Fig. 1B). Conversely, cardiac-derived cells were negative for markers of the mature cardiomyocyte, including MHCα and MHCβ, and for the selective macrophage marker EMR-1 (Fig. 1B). Flow-cytometry analysis demonstrated that more than 80% of the isolated cells were CD105+ve and expressed the CPC markers MEF2C and Nkx2.5 (Fig. 1C and Supplemental Fig. 2A). This analysis also identified cell...
subpopulations expressing c-kit (∼2–3%), CD34 (3–4%), and CD31 (∼10%), whereas CD133 and CD45 were essentially undetectable. Finally, a significant fraction (8%) of the isolated cells showed the marker profile of mature endothelial cells (CD31+ve and CD133−ve) (Fig. 1C and Supplemental Fig. 2A). Thus, the majority of the isolated cells show the typical phenotype of human CPCs (29, 30).

The isolated human CPCs retained their morphological appearance and marker profile up to 15 passages in culture (data not shown). However, the exposure of cells to 10 μM 5-azacytidine for 24 h resulted in a significant decline in the number of cells expressing endothelial markers (data not shown), and an increase in troponin-T gene expression (Fig. 1D), a specific marker of mature cardiomyocytes.

Multipotency of human CPCs

After incubation with the appropriate media, CPCs acquired the adipocyte, osteocyte, or chondrocyte phenotypes, showing accumulation of triglyceride, calcified matrix, or glycosaminoglycans, respectively (Fig. 1E), as well as expression of phenotype-specific gene markers (data not shown).

The GLP-1R in human CPCs

GLP-1R mRNA was readily detectable at relatively high levels in human pancreatic islets (Fig. 2A); although at lower levels, GLP-1R expression could be also found in the human CPCs, in which GLP-1R mRNA levels were similar to those found in human umbilical vein endothelial cells (HUVECs) and in human bone marrow stem cells (Fig. 2A). Conversely, GLP-1R levels were low to undetectable in human renal tubular stem cells and human adipose tissue-derived stem cells (27) (Fig. 2A). GLP-1R was also readily detected in human heart muscle, but not in human skeletal muscle and adipose tissue (Supplemental Fig. 2B). Amplicons obtained by RT-PCR showed the expected size (Fig. 2B) and aligned with the human GLP-1R sequence (data not shown).

Exposure of CPCs to GLP-1 resulted in a time-dependent increase in intracellular cAMP levels (Fig. 2D and data not shown), as well as in a dose- and time-dependent phosphorylation of the transcription factor CREB, which is activated by GLP-1 through the cAMP/protein kinase A (PKA) pathway (31) (Fig. 2C and Supplemental Fig. 2C). The GLP-1-induced increase in cAMP in CPCs was abolished when cells were incubated in the presence of the classical GLP-1R antagonist exendin(9–39) (P < 0.05 vs. control; Fig. 2D). The ability of GLP-1 to induce CREB phosphorylation was also abolished in the presence of exendin(9–39) (Fig. 2E). Targeting the canonical GLP-1R with specific siRNA sequences also abrogated the ability of GLP-1 to activate CREB (Fig. 2E). Thus, the effects of
GLP-1 on human CPCs are mediated by the known GLP-1R.

**Induction of apoptosis in human CPCs and protective effects of GLP-1**

To evaluate the effects of oxidative stress on CPC apoptosis, cells were incubated in the presence of 0.1–0.5 mmol/liter hydrogen peroxide for various times, and cell apoptosis was evaluated by measuring the release of cytoplasmic oligosomes. At both shorter time points (up to 20 min), and longer times (16 h), H$_2$O$_2$ induced a significant increase in apoptosis (*P < 0.05 vs. untreated cells; Fig. 3, A and B, respectively, and Supplemental Fig. 3). Treatment with 20 mmol/liter GLP-1 or the longer-acting GLP-1R agonist exendin-4 markedly attenuated the effects of H$_2$O$_2$ on CPC apoptosis (Fig. 3, A and B).

Treatment with GLP-1 prevented the H$_2$O$_2$-mediated increase in the proportion of early and late apoptotic CPCs, respectively, with minimal effects on necrotic cells, while increasing the proportion of vital cells, which was diminished after H$_2$O$_2$ incubation (Fig. 3C). GLP-1 also reduced the H$_2$O$_2$-induced caspase-3 cleavage (*P < 0.05 vs. control; Fig. 3D). The ability of GLP-1 to prevent the H$_2$O$_2$-induced apoptosis of the CPCs was abolished when cells were preincubated in the presence of the GLP-1R antagonist exendin(9–39), the siRNA sequences specific to the human GLP-1R, or the PKA inhibitor H89 (Fig. 3E).

The signaling mechanisms involved in the H$_2$O$_2$-induced apoptosis and protective effects of GLP-1, respectively, were investigated next. Time-course studies showed that exposure to H$_2$O$_2$ resulted in a rapid time-dependent increase in cytoplasmic oligonucleosomes (*P < 0.05 vs. untreated cells; Fig. 4A); significant increases in both cleaved caspase-3 protein levels and caspase activity were also evident after 20 min (*P < 0.05 vs. untreated cells; Fig. 4B). The ability of H$_2$O$_2$ to induce JNK activation in the human CPCs was similarly rapid, because the phosphorylation levels of both the 46-kDa and 54-kDa JNK isoforms were increased in a time-dependent manner within minutes of exposure to H$_2$O$_2$ (2.5-fold vs. untreated cells; Fig. 4C and Supplemental Fig. 4C). This was associated with a coordinate increase in the phosphorylation levels of MKK7, which was already evident at 5 min and was augmented up to 20 min (*P < 0.05 vs. untreated cells; Fig. 4C and Supplemental Fig. 4B), and MKK7, which was also markedly enhanced by H$_2$O$_2$ (*P < 0.05 vs. untreated cells; Fig. 4C and Supplemental Fig. 4A). Finally, phosphorylation of the JNK substrate c-Jun was similarly increased 1.5- to 2.0-fold in response to H$_2$O$_2$ (*P < 0.05 vs. untreated cells; Fig. 4C and Supplemental Fig. 4D).

When the CPCs were preincubated with SP600125, a specific JNK inhibitor, H$_2$O$_2$-induced apoptosis was de-
creased by 90% (P < 0.05 vs. control; Fig. 4D). In parallel, 
H$_2$O$_2$-stimulated phosphorylation of both JNK and c-jun
were almost completely prevented (83% and 95% reduc-
tion vs. control, respectively, P < 0.05; Supplemental Fig.
5A). By contrast, the phosphorylation of MKK4, which
is not a target for SP600125, was unaffected (P = 0.812
vs. control; Supplemental Fig. 5A). Incubation of H$_2$O$_2$-
treated CPCs with the JNK inhibitor peptide D-JNK-I,
which blocks JNK activity without affecting JNK phos-
phorylation, resulted in a 78% reduction of c-jun
phosphorylation, in the presence of unaltered phospho-JNK
levels (Supplemental Fig. 5B), and similarly reduced
H$_2$O$_2$-induced CPCs apoptosis (P < 0.05 vs. control;
Fig. 4D).

Treatment of the CPCs with GLP-1 for 16 h markedly
reduced H$_2$O$_2$-dependent phosphorylation of the p46 and
p54 JNK isoforms (P < 0.05 vs. control H$_2$O$_2$-treated
cells), which was similar to the unstimulated condition
(P = 0.7 vs. basal cells; Fig. 5C). Similarly, GLP-1 reduced
the increased levels of MKK7, MKK4, and c-jun
phosphorylation observed in response to H$_2$O$_2$ (P < 0.05 vs.
control; Fig. 5, A, B, and D). However, the total cellular
content of each of these signaling proteins was not mod-
ified (Supplemental Fig. 6). The effects of GLP-1 on JNK
activation were also investigated by evaluating the cellular
localization of the phosphorylated kinase by immunoflu-
orescence. Exposure to H$_2$O$_2$ induced rapid nuclear and
perinuclear accumulation of phosphorylated JNK (Fig. 6),
and this was almost completely abrogated in the human CPCs treated with
GLP-1 (Fig. 6).

**Discussion**

Multipotent cells resident in the heart
have been identified on the basis of their
expression of surface stem cell markers,
such as c-kit, MDR-1, or Sca-1 (29, 32,
33). Recently, CPCs have been also ob-
tained from fragments of human heart
tissue, particularly from the auricle, an
anatomical appendix of the atrium gen-
erally removed during heart surgery (4,
30, 33, 34). The auricle-derived CPCs
can be transplanted into infarcted myo-
cardium of rats and are able to generate
myocardial grafts in an apparently
identical manner compared with CPCs
from other cardiac sites (33). The hu-
man CPCs express markers typical of
myocardial progenitors, with a signifi-
cant fraction of cells expressing stem cell and endothelial
cell markers (29, 30), and maintain their phenotypic pro-
file in culture. In this study, the vast majority of cells iso-
lated from human heart auricle were found to be positive
for CD105, which is a general mesenchymal cell marker
(35), and for myogenic transcription factors (MEF2C and
Nkx 2.5), which are typical of the myocardial progenitor
stage (36, 37). By contrast, markers of the mature card-
omyocyte, including MHC$_\alpha$ and MHC$_\beta$, were not
detected. Thus, the population of proliferating auricle-de-
rived cells used in this study appears to be enriched in
precursor cells with a lineage restriction toward the car-
diomyocyte phenotype, in line with previous observations
(4, 30, 33). Approximately 2% of the isolated cells were
found to express endothelial cell markers (CD34 and
CD31), likely representing contaminating cells of the en-
dotheial lineage, whereas expression of CD133 and
CD45 was not identified. Albeit oriented toward the car-
diomyocyte differentiation program, these human CPCs
may acquire phenotypes of other lineages, including the
osteoblast, chondroblast, and adipose lineages, under ap-
propriate culture conditions (Ref. 33 and Fig. 1E), like
multipotent mesenchymal stromal cells (35). The reacti-
vation of the cardiomyocyte differentiation program in the
CPCs has also been described, using the DNA-demethy-
latating agent 5-azacytidine, oxytocin, multiple growth fac-
tors, or coculture with rat neonatal cardiomyocytes (38).
In this study, a significant increase in the expression of
troponin-T, a cardiomyocyte marker, was observed when CPCs were treated with 5-azacytidine (Fig. 1D). Adding GLP-1 or the GLP-1 receptor agonist exendin-4 to 5-azacytidine up to 4 wk did not modify the expression of troponin-T in the CPCs (data not shown). The full differentiation of human precursors into mature, spontaneously beating cardiomyocytes has been obtained by coculturing human CPCs with neonatal rat cardiomyocytes (30, 34), using hyperpolarization protocols (39), or combining treatment with both 5-azacytidine and TGF-β (40).

In addition to the effects on the endocrine pancreas, GLP-1 and its analogs behaving as GLP-1R agonists have been shown to exert wide-ranging cardiovascular effects, such as modulation of cardiac structure and function, blood pressure, and vascular tone (41, 42). Treatment with GLP-1 and GLP-1R agonists has been shown to exert beneficial effects in the setting of ischemic damage, in isolated rodent hearts ex vivo (19, 43), and experimental rat and porcine models in vivo (19, 20), and when experimental diabetes is induced (21). Favorable functional effects of GLP-1 have also been described in experimental heart failure, both in dogs and rodents (17, 18). Finally, initial clinical observations suggest that GLP-1 may improve contractile function in patients with coronary heart disease (24) or chronic heart failure (17, 45). However, the cellular and molecular basis for the GLP-1-mediated enhanced resistance of the cardiac tissue against conditions of ischemia and/or metabolic impairment is still incompletely defined. Inhibition of CPCs apoptosis and promotion of their survival by GLP-1 may augment the CPCs compartment and favor the cardiomyocyte-regenerative potential under specific disease states.

Recent studies have demonstrated that some stem cells express the GLP-1R. Specifically, GLP-1R mRNA was detected in mouse embryonic stem cells and human bone marrow-derived mesenchymal stem cells, and GLP-1 displayed antiapoptotic and prosurvival actions in both types of stem cells (46, 47). In this study, we show, for the first time, that human CPCs express a functional GLP-1R, whereas GLP-1R mRNA is very low in human adipose tissue and renal tubular stem cells. Thus, GLP-1R expression is not common to all cells displaying a stem cell phenotype, implying a tissue-specific control of the GLP-1R gene promoter that dictates in which progenitor cells GLP-1 may act as a prosurvival factor. Experiments in isolated mouse cardiomyocytes lacking the known GLP-1R suggest that some cardioprotective actions of GLP-1 may be independent of this receptor and could be also mediated by its metabolite GLP-1(9–36), resulting from dipeptidyl peptidase-4-mediated cleavage of native GLP-1 (48). It is possible that GLP-1(9–36) may contribute to the antiapoptotic effects observed in the human CPCs, because dipeptidyl peptidase-4 is expressed by these cells (Leonardini A., and F. Giorgino, unpublished data). Indeed, preincubation of CPCs with GLP-1(9–36) resulted in inhibition of H2O2-induced apoptosis (Supplemental Fig. 7). The prosurvival effects of GLP-1 on CPCs may be potentially mediated by an additional GLP-1 receptor, which has been postulated but has not been yet identified. GLP-1 binding to this receptor was shown to result in activation of the cAMP/PKA pathway and to be inhibited by the GLP-1(9–39) antagonist (48).

In CPCs treated with a mixture of three distinct GLP-1R-specific siRNAs, GLP-1 failed to induce CREB phosphorylation (Fig. 2E) and to prevent H2O2-induced apoptosis (Fig. 3E). The protective effect of GLP-1(9–36) on H2O2-induced apoptosis was similarly abrogated in the presence of the GLP-1R-specific siRNAs (Supplemental Fig. 7). These results suggest that both GLP-1 and GLP-1(9–36) preferentially signal via the known GLP-1R in the human CPCs.

Inhibition of the JNK-signaling pathway appears to represent an important mechanisms for the ability of GLP-1 to prevent apoptosis and enhance cell survival. In both human and rodent insulin-secreting cells, the GLP-1 analog exendin-4 was recently shown to protect the cells from cytokine-induced apoptosis by inhibiting the JNK pathway (25, 49). In this study, the ability of...
GLP-1 to prevent oxidative stress-induced apoptosis of human cardiac precursors is reported for the first time, this effect being also mediated by inhibition of JNK activation (Figs. 5 and 6). The JNK kinase is activated in response to various cellular stressors, including DNA-damaging agents, heat shock, proinflammatory cytokines, free fatty acids, and oxidative stress, and this follows the phosphorylation of JNK threonine and tyrosine residues by MKK7 and/or MKK4. In the rat heart, JNK1 translocates to the cell nucleus from the cytoplasm in response to ischemia and is activated in the nucleus during postischemic reperfusion, leading to phosphorylation of c-jun (44). In the human CPCs, exposure to H2O2 was followed by rapid phosphorylation of MKK4, MKK7, JNK, and c-jun (Fig. 4C), as well as by nuclear accumulation of phosphorylated JNK (Fig. 6). Conversely, pretreatment with GLP-1 prevented both phosphorylation and nuclear localization of phosphorylated JNK (Figs. 5C and 6). The inhibitory effect of GLP-1 on JNK appears to require inhibition of the MKK7/MKK4-signaling cascade (Fig. 5, A and B), suggesting a predominant regulation at levels upstream of JNK phosphorylation, as reported also in β-cells (25). Remarkably, this effect requires treatment of cells with the GLP-1R agonists for at least 8 h (Ref. 25 and data not shown).

In conclusion, exposure to GLP-1 prevents oxidative stress-induced apoptosis in cardiac precursors isolated from human heart biopsies via inhibition of the JNK pathway, and this occurs through the canonical GLP-1 receptor. These results may contribute to explain the reported ability of GLP-1 and its analogs to produce prosurvival effects on the human heart (Fig. 7).

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