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

L. Laviola,* A. Leonardini,* M. Melchiorre, M. R. Orlando, A. Peschechera, A. Bortone, D. Paparella, A. Natalicchio, S. Perrini, and F. Giorgino

Department of Emergency and Organ Transplantation, Section of Internal Medicine Endocrinology, Andrology and Metabolic Diseases (L.L., A.L., M.M., M.R.O., A.P., A.N., S.P., F.G.), and Section of Cardiac Surgery (A.B., D.P.), University of Bari Aldo Moro, I-70124 Bari, Italy

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 (H_2O_2). 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 silencer small interfering RNAs. Cell apoptosis was quantified by an ELISA assay and by flow cytometry-detected Annexin V. Exposure of CPCs to H_2O_2 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 H_2O_2-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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* L.L. and A.L. contributed equally to this paper.

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 (H_2O_2)-induced apoptosis in these cells.

Materials and Methods

Human heart biopsies

Cardiac bioptic 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 mm^3, washed with Ca^{2+}-Mg^{2+}-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% CO_2 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 H_2O_2, 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 FACSCanto flowcytometer (Becton Dickinson, Mountain View, CA), at room temperature using the FACSDiva 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 antigoat 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′-GCCACAGACTTGTTCTGCAA-3′; and reverse, 5′-CTTGGCAAGTCTGCATTTGA-3′; the expected PCR product is 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-

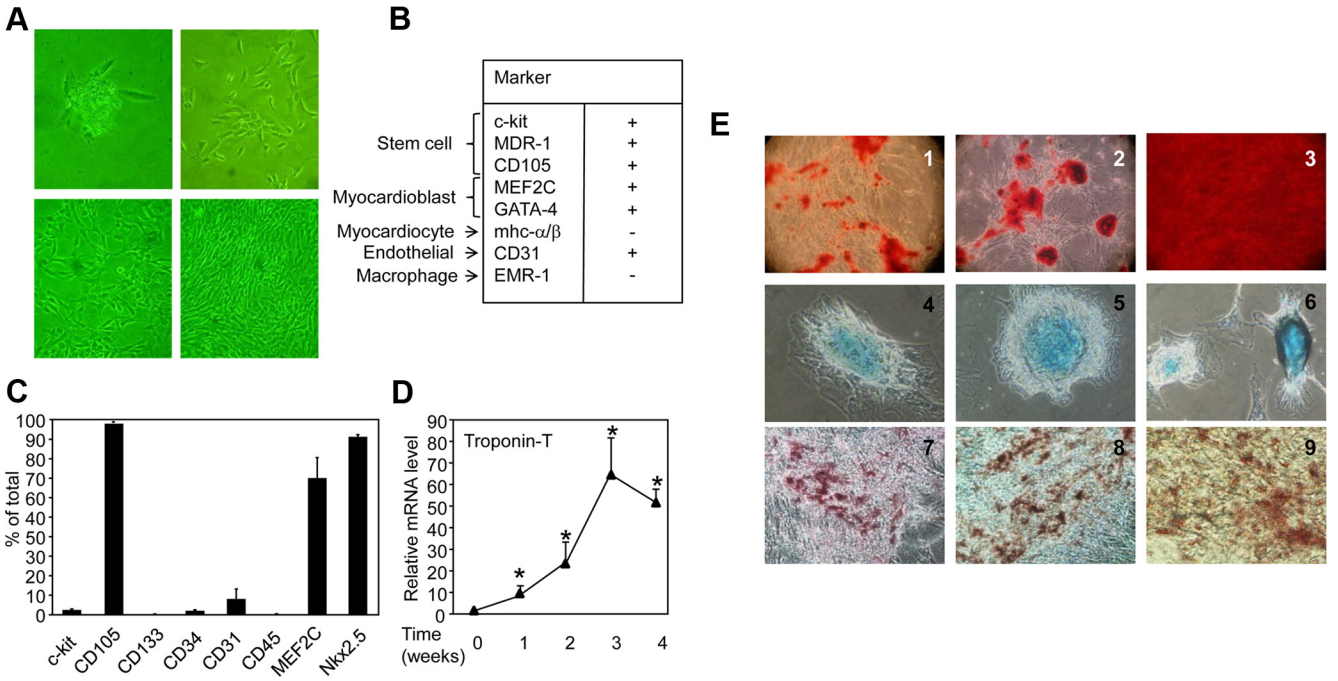

FIG. 1. Isolation and characterization of human CPCs. A, Representative primary culture of human CPCs under optical microscopy. B, Marker expression profile of human CPCs assessed by qPCR; +, detected; −, not detected. C, Flow-cytometry analysis of human CPCs. Cells were incubated with antibodies specific for stem cell markers (c-*kit*, CD105), cardiomyogenic transcription factors (Nkx2.5, MEF2C), endothelial progenitor (CD133), hematopoietic progenitor (CD34), mature hematopoietic (CD45), and mature endothelial (CD31) cell markers. D, Expression of troponin-T in human CPCs. Cells were exposed to 10 μM 5-azacytidine for 24 h and then maintained in culture medium containing 2% fetal bovine serum for 4 wk. Troponin-T gene expression was evaluated by qPCR. E, Three-lineage differentiation potential of human CPCs. Cells were exposed to adipogenic, osteogenic, or chondrogenic culture conditions, respectively. E1–E3, Osteogenic differentiation, demonstrated by 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.

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 to detect calcified extracellular matrix deposits, as previously reported (26). Adipogenesis was induced as previously described, and intracellular triglycerides were visualized by Oil red O staining (27). Chondrogenic differentiation was induced using the micromass culture technique; after 2 wk, the micromasses were stained with 1% (wt/vol) Alcian Blue in 0.1 N HCl (pH 1.0) for 30 min to detect the presence of sulfated proteoglycan-rich matrix.

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

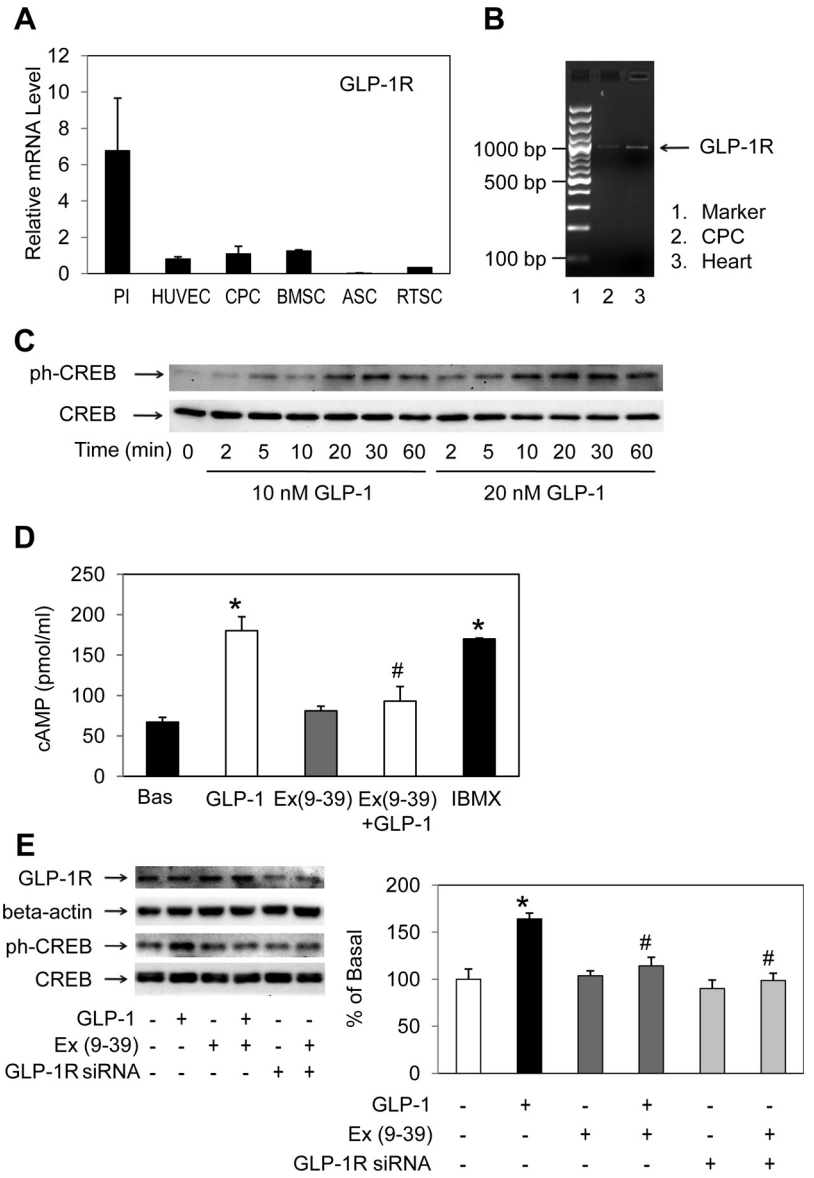

FIG. 2. Identification of GLP-1R in human CPCs. A, GLP-1R mRNA levels assessed by qPCR in human pancreatic islets (PI), the endothelial cell line human umbilical vein endothelial cells (HUVECs), human CPC, human bone marrow stem cells (BMSC), human sc adipose tissue-derived stem cells (ASC), and human renal tubular stem cells (RTSC). B, RT-PCR analysis of GLP-1R in lysates of human CPCs and human heart tissue. C, GLP-1-induced CREB phosphorylation in human CPCs. Cells were treated with 10 nmol/liter or 20 nmol/liter GLP-1 for different times, as indicated, and both phospho-CREB and CREB were visualized by immunoblotting with specific antibodies. D, GLP-1-regulated cAMP levels in human CPCs. Cells were incubated in the presence of 20 nmol/liter GLP-1 for 10 min or left untreated (Bas), and intracellular cAMP levels were detected by an ELISA assay. The effects of the GLP-1R antagonist exendin(9–39) [Ex (9–39), 20 nmol/liter] on the ability of GLP-1 (20 nmol/liter) to increase cAMP levels in human CPCs are also shown. Isobutylmethylxanthine was used as a positive control. *. $P < 0.05$ *vs.* basal; #, $P < 0.05$ *vs.* GLP-1 alone. Data represent the mean ± SE of at least three experiments. E, Ability of Ex(9–39) and siRNAs specific to the human GLP-1R to prevent GLP-1-induced CREB phosphorylation. CPCs were treated with Ex(9–39) or transfected with three GLP-1R siRNAs for 48 h and then stimulated with 20 nmol/liter GLP-1 for 10 min. Protein lysates (25 μg) were then subjected to 10% SDS-PAGE and immunoblotting with anti-GLP-1R and anti-phospho-CREB antibodies. β-actin and GAPDH content in total cell lysates is shown as a loading control. IBMX, Isobutylmethylxanthine.

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 $MgCl_2$, 1 mmol/liter $CaCl_2$, 4 mmol/liter EDTA, 1% Triton X-100, 10% glycerol, 50 mmol/liter NaF, and 10 mmol/liter NaPP, supplemented with 100 μmol/liter phenylmethylsulfonyl fluoride, 5 ng/ml leupeptin, 1 μg/ml aprotinin, and 2 mmol/liter Na_3VO_4. 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-glyceraldehyde 3-phosphate dehydroge-

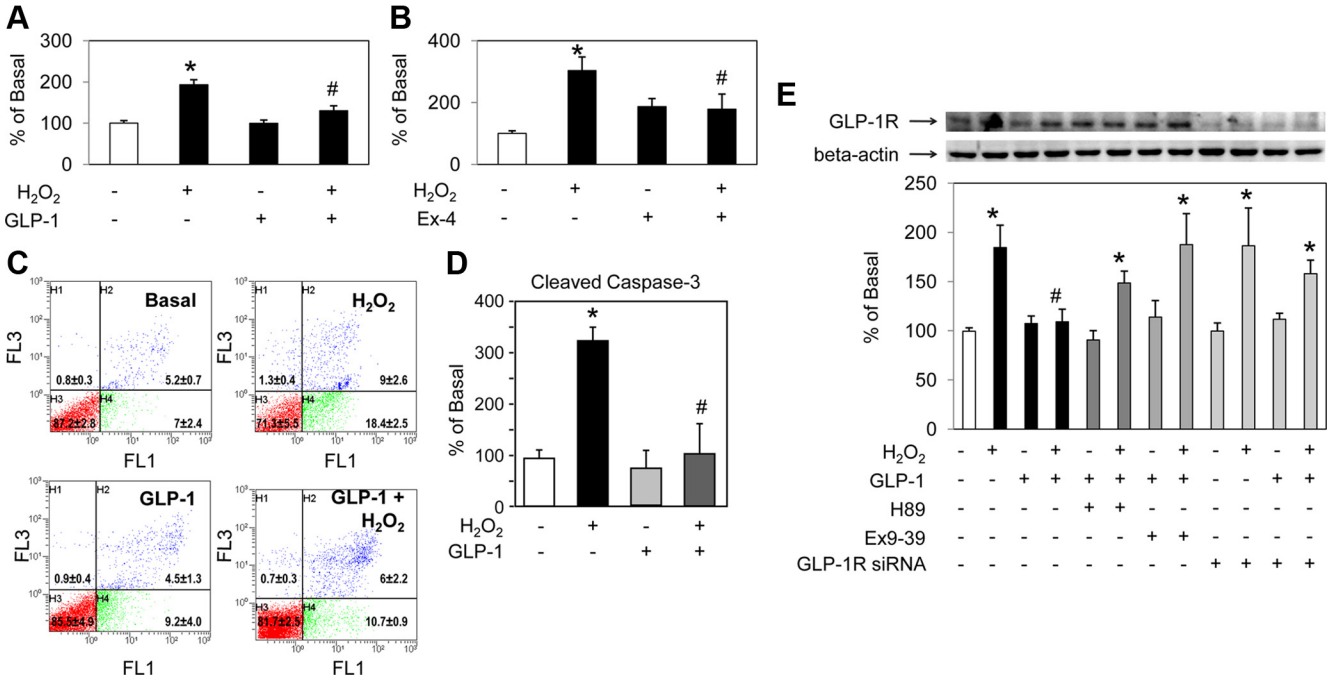

FIG. 3. Induction of apoptosis in human CPCs and protective effects of GLP-1. A, Human CPCs were incubated in the presence or absence of 20 nmol/liter GLP-1 before challenge with 0.5 mM H_2O_2 for 20 min. Apoptosis was evaluated by an ELISA of cytoplasmic oligonucleosomes. B, Human CPCs were incubated in the presence or absence of 20 nmol/liter exendin-4 (Ex-4), a relatively long-acting GLP-1 analog, before exposure to 0.2 mmol/liter H_2O_2 for 16 h. Apoptosis was evaluated by an ELISA of cytoplasmic oligonucleosomes. C, Assessment of cell apoptosis and survival by annexin V-FITC/propidium iodide (PI) staining. Cells were collected by centrifugation at 1000 × *g* for 5 min at room temperature, washed twice in cold PBS, treated with 1 μl Annexin V-FITC and 10 μl propidium iodide, and incubated in the dark for 15 min at room temperature; the stained samples were processed by flow cytometry. Cells were incubated in the presence or absence of 20 nmol/liter GLP-1 before challenge with H_2O_2 for 20 min. In each panel, H1, H2, H3, and H4 represent necrotic, late apoptotic, viable, and early apoptotic cells, respectively. D, Quantification of cleaved caspase-3, assessed by immunoblotting with specific antibodies, in cells pretreated with 20 nmol/liter GLP-1 or left untreated, and then exposed to 0.5 mmol/liter H_2O_2 for 20 min. E, Effects of GLP-1 on H_2O_2-induced apoptosis 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. Cells were pretreated with 20 nmol/liter exendin(9–39) for 8 h or 5 μmol/liter H89 for 2 h, transfected with the 25 nmol/liter each of the three siRNA sequences for 48 h, or left untreated, and then incubated with or without 20 nmol/liter GLP-1 for 16 h before challenge with 0.5 mmol/liter H_2O_2. Apoptosis was evaluated by ELISA assay of cytoplasmic oligonucleosomes. Protein lysates (25 μg) were subjected to 10% SDS-PAGE and immunoblotting with anti-GLP-1R and β-actin antibodies, respectively. All data are presented as mean ± SE; *, $P < 0.05$ *vs.* no H_2O_2; #, $P < 0.05$ *vs.* H_2O_2 alone.

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

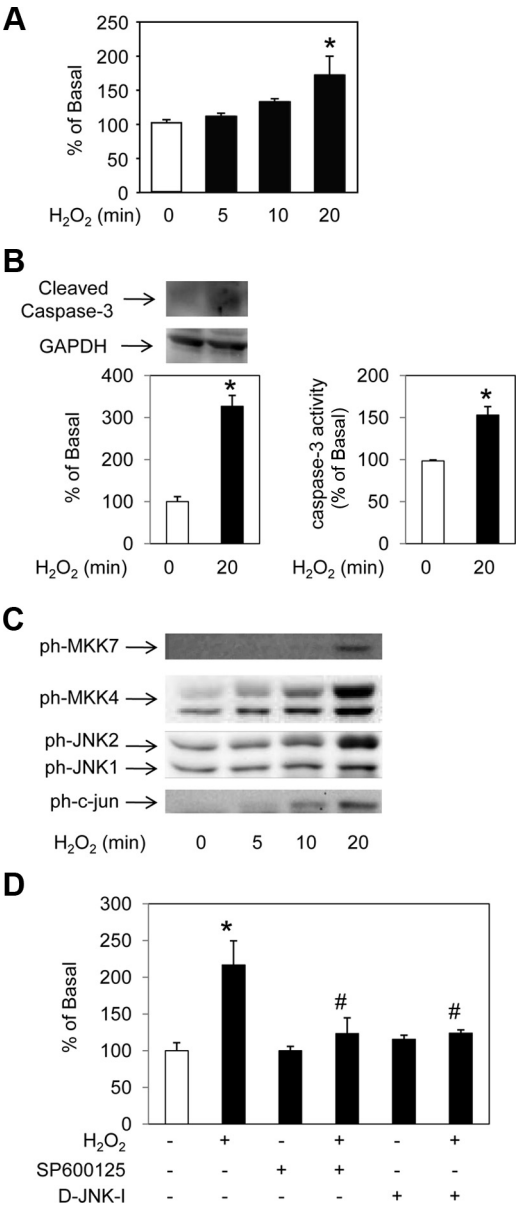

FIG. 4. Signaling mechanisms involved in the proapoptotic action of H_2O_2 in human CPCs. Cells were incubated with 0.5 mmol/liter H_2O_2 for the indicated times or left untreated. A, Apoptosis assessed by an ELISA of cytoplasmic oligosomes. B, Quantifications of cleaved caspase-3, assessed by immunoblotting, and of caspase-3 activity by Caspase-3 Colorimetric Assay Kit. C, MKK7, MKK4, JNK, and c-*jun* phosphorylation, assessed by immunoblotting with specific antibodies. D, Effects of JNK inhibition with SP600125 or the JNK inhibitor peptide D-JNK-I on H_2O_2-induced apoptosis and JNK signaling. Human CPCs were pretreated with 10 μmol/liter SP600125 for 1 h or 10 μg/ml D-JNK-I overnight or left untreated, and then exposed to 0.5 mmol/liter H_2O_2 for 20 min. Apoptosis was evaluated by an ELISA assay for cytoplasmic oligonucleosomes. All data are presented as mean ± SE of at least n = 3 experiments. *, $P < 0.05$ *vs.* basal (no H_2O_2); #, $P < 0.05$ *vs.* H_2O_2 alone. ph-, Phosphorylated.

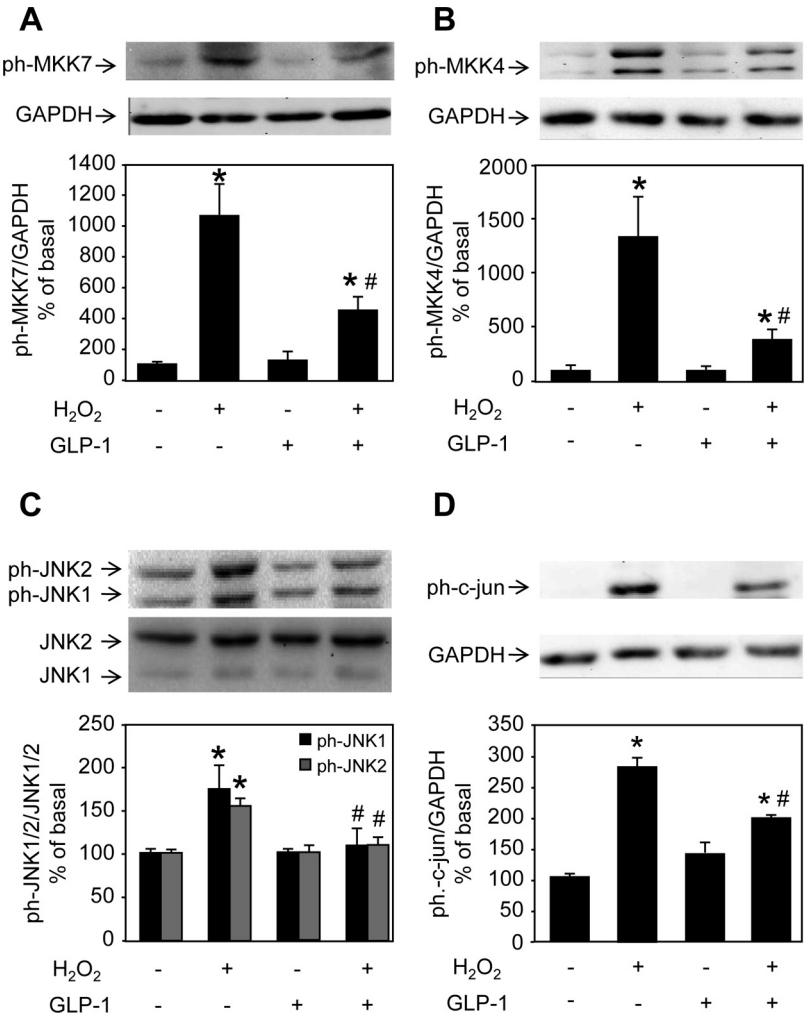

FIG. 5. Effects of GLP-1 on H_2O_2-induced activation of the JNK signaling pathway. Human CPCs were incubated in the presence or absence of 20 nmol/liter GLP-1 and then exposed to 0.5 mmol/liter H_2O_2 for 20 min. Each panel shows a representative immunoblot (*top*) and the quantitation of n = 3 independent experiments (*bottom*). A, Phosphorylation of MKK7. B, Phosphorylation of MKK4. C, Phosphorylation and protein content of JNK. The JNK1 and JNK2 protein isoforms were quantitated separately, and results are expressed as ratio of phosphorylated to total protein. D, Phosphorylation of c-*jun*. In panels A, B, and D, GAPDH was used as loading control. All data are presented as mean ± SE. *, $P < 0.05$ *vs.* basal (no H_2O_2); #, $P < 0.05$ *vs.* control cells exposed to H_2O_2 alone. ph-, Phosphorylated.

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_2O_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 nmol/liter GLP-1 or the longer-acting GLP-1R agonist exendin-4 markedly attenuated the effects of H_2O_2 on CPC apoptosis (Fig. 3, A and B).

Treatment with GLP-1 prevented the H_2O_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_2O_2 incubation (Fig. 3C). GLP-1 also reduced the H_2O_2-induced caspase-3 cleavage ($P < 0.05$ *vs.* control; Fig. 3D). The ability of GLP-1 to prevent the H_2O_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_2O_2-induced apoptosis and protective effects of GLP-1, respectively, were investigated next. Time-course studies showed that exposure to H_2O_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_2O_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_2O_2 (2.5-fold *vs.* untreated cells, $P < 0.05$; Fig. 4C and Supplemental Fig. 4C). This was associated with a coordinate increase in the phosphorylation levels of MKK4, 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_2O_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_2O_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_2O_2-induced apoptosis was de-

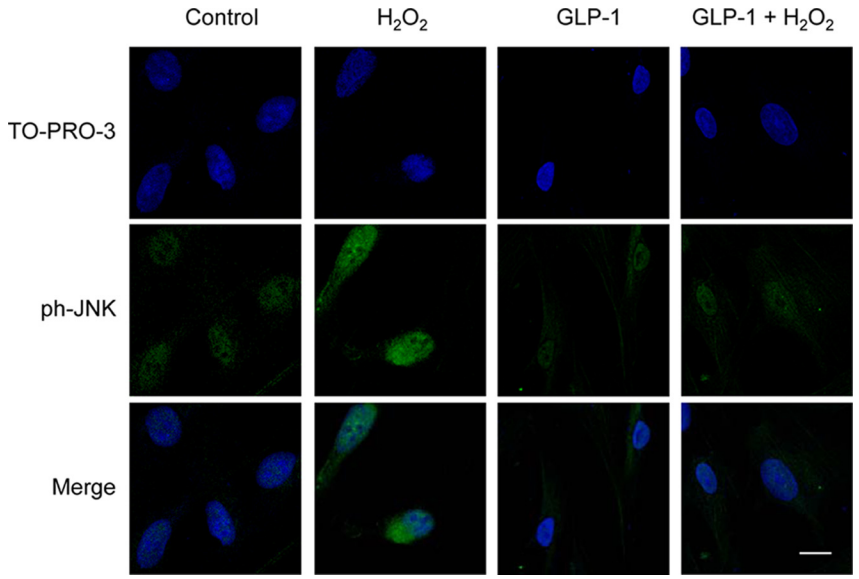

FIG. 6. Effects of GLP-1 on the cellular localization of phosphorylated JNK in response to stimulation with H_2O_2. Human CPCs were preincubated with 20 nmol/liter GLP-1 before challenge with 0.5 mmol/liter H_2O_2 for 20 min. Then, cells were fixed and incubated with TO-PRO-3 (*blue*), and phospho-JNK1/2 antibody followed by Alexa Fluor (488) antirabbit antibody (*green*), as described in *Materials and Methods*. *Scale bar*, 15 μm. ph-, Phosphorylated.

creased by 90% ($P < 0.05$ *vs.* control; Fig. 4D). In parallel, H_2O_2-stimulated phosphorylation of both JNK and c-*jun* were almost completely prevented (83% and 95% reduction *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_2O_2-treated CPCs with the JNK inhibitor peptide D-JNK-I, which blocks JNK activity without affecting JNK phosphorylation, resulted in a 78% reduction of c-*jun* phosphorylation, in the presence of unaltered phospho-JNK levels (Supplemental Fig. 5B), and similarly reduced H_2O_2-induced CPCs apoptosis ($P < 0.05$ *vs.* control; Fig. 4D).

Treatment of the CPCs with GLP-1 for 16 h markedly reduced H_2O_2-dependent phosphorylation of the p46 and p54 JNK isoforms ($P < 0.05$ *vs.* control H_2O_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_2O_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 modified (Supplemental Fig. 6). The effects of GLP-1 on JNK activation were also investigated by evaluating the cellular localization of the phosphorylated kinase by immunofluorescence. Exposure to H_2O_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 obtained from fragments of human heart tissue, particularly from the auricle, an anatomical appendix of the atrium generally removed during heart surgery (4, 30, 33, 34). The auricle-derived CPCs can be transplanted into infarcted myocardium of rats and are able to generate myocardial grafts in an apparently identical manner compared with CPCs from other cardiac sites (33). The human CPCs express markers typical of myocardial progenitors, with a significant fraction of cells expressing stem cell and endothelial cell markers (29, 30), and maintain their phenotypic profile in culture. In this study, the vast majority of cells isolated 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 cardiomyocyte, including MHCα and MHCβ, were not detected. Thus, the population of proliferating auricle-derived cells used in this study appears to be enriched in precursor cells with a lineage restriction toward the cardiomyocyte 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 endothelial lineage, whereas expression of CD133 and CD45 was not identified. Albeit oriented toward the cardiomyocyte differentiation program, these human CPCs may acquire phenotypes of other lineages, including the osteoblast, chondroblast, and adipose lineages, under appropriate culture conditions (Ref. 33 and Fig. 1E), like multipotent mesenchymal stromal cells (35). The reactivation of the cardiomyocyte differentiation program in the CPCs has also been described, using the DNA-demethylating agent 5-azacytidine, oxytocin, multiple growth factors, 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 H_2O_2-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 H_2O_2-induced apoptosis (Fig. 3E). The protective effect of GLP-1(9–36) on H_2O_2-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

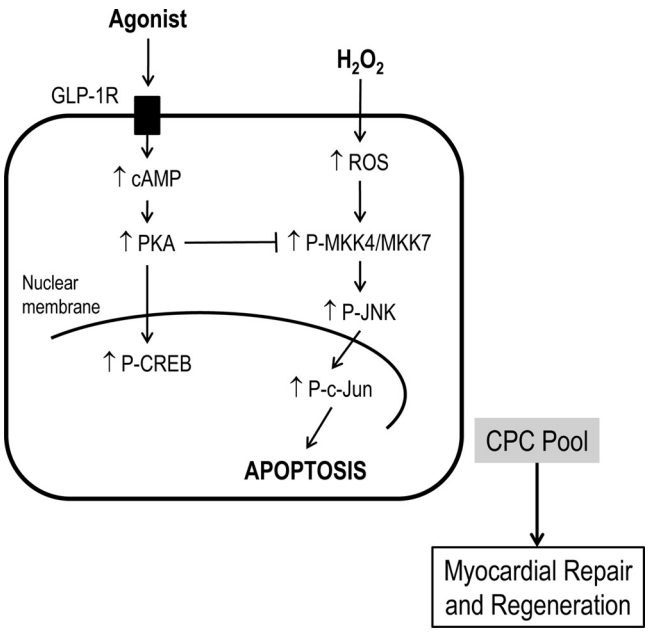

FIG. 7. Mechanism by which GLP-1 receptor activation may exert prosurvival effects in the human CPCs, leading to an increase in the CPC pool responsible for myocardial repair and regeneration. GLP-1 receptor agonists induce the activation of PKA, and this results in inhibition of the H_2O_2-induced activation of the MKK4/MKK7/JNK signaling cascade that mediates CPC apoptosis. ROS, Reactive oxygen species.

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 H_2O_2 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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Address all correspondence and requests for reprints to: Francesco Giorgino, M.D., Ph.D., Department of Emergency and Organ Transplantation, Section of Internal Medicine, Endocrinology, Andrology and Metabolic Diseases, University of Bari Aldo Moro, Piazza Giulio Cesare, 11, I-70124 Bari, Italy. E-mail: francesco.giorgino@uniba.it.

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