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Glucagon-Like Peptide (GLP)-1(9-36)Amide-Mediated Cytoprotection Is Blocked by Exendin(9-39) Yet Does Not Require the Known GLP-1 Receptor

Kiwon Ban, Kyoung-Han Kim, Chan-Kyung Cho, Meghan Sauvé, Eleftherios P. Diamandis, Peter H. Backx, Daniel J. Drucker,* and Mansoor Husain*

Departments of Physiology (K.B., K.-H.K., P.H.B., M.H.), Laboratory Medicine and Pathobiology (C.-K.C., E.P.D., D.J.D., M.H.), and Medicine (P.H.B., D.J.D., M.H.), Samuel Lunenfeld Research Institute (M.S., D.J.D.), Toronto General Hospital Research Institute (M.H.), Banting and Best Diabetes Centre (M.S., D.J.D.), and Heart and Stroke Richard Lewar Centre of Excellence in Cardiovascular Research (K.B., K.-H.K., P.H.B., M.H.), University of Toronto, Toronto, Ontario, Canada M5G 2C4

The widely expressed dipeptidyl peptidase-4 enzyme rapidly cleaves the gut hormone glucagon-like peptide-1 [GLP-1(7-36)amide] at the N terminus to generate GLP-1(9-36)amide. Both intact GLP-1(7-36)amide and GLP-1(9-36)amide exert cardioprotective actions in rodent hearts; however, the mechanisms underlying the actions of GLP-1(9-36)amide remain poorly understood. We used mass spectrometry of coronary effluents to demonstrate that isolated mouse hearts rapidly convert infused GLP-1(7-36)amide to GLP-1(9-36)amide. After ischemia-reperfusion (I/R) injury of isolated mouse hearts, administration of GLP-1(9-36)amide or exendin-4 improved functional recovery and reduced infarct size. The direct actions of these peptides were studied in cultured neonatal mouse cardiomyocytes. Both GLP-1(9-36)amide and exendin-4 increased levels of cAMP and phosphorylation of ERK1/2 and the phosphoinositide 3-kinase target protein kinase B/Akt. In I/R injury models *in vitro*, both peptides improved mouse cardiomyocyte viability and reduced lactate dehydrogenase release and caspase-3 activation. These effects were attenuated by inhibitors of ERK1/2 and phosphoinositide 3-kinase. Unexpectedly, the cardioprotective actions of GLP-1(9-36)amide were blocked by exendin(9-39) yet preserved in Glp1r$^{-/-}$ cardiomyocytes. Furthermore, GLP-1(9-36)amide, but not exendin-4, improved the survival of human aortic endothelial cells undergoing I/R injury, actions sensitive to the nitric oxide synthase inhibitor, *N*(G)-nitro-L-arginine methyl ester (L-NAME). In summary, our findings demonstrate separate actions for GLP-1(9-36)amide *vs.* the GLP-1R agonist exendin-4 and reveal the existence of a GLP-1(9-36)amide-responsive, exendin(9-39)-sensitive, cardioprotective signaling pathway distinct from that associated with the classical GLP-1 receptor. **(*Endocrinology* 151: 0000–0000, 2010)**

Glucagon like peptide-1 [GLP-1(7-36)amide], also known as GLP-1, is released from gut endocrine cells in response to nutrient ingestion, stimulating glucose-dependent insulin secretion by activating a G protein-coupled GLP-1 receptor (GLP-1R) expressed on pancreatic islet cells (1–3). However, the half-life of intact GLP-1 is extremely short (<2 min), in part due to renal clearance. GLP-1 is also rapidly metabolized to GLP-1(9-36)amide by the enzyme dipeptidyl peptidase-4 (DPP-4), which is abundantly expressed in many cell types with primary ex-

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* D.J.D. and M.H. contributed equally to this work.

Abbreviations: AU, Arbitrary unit; CCF, constant coronary flow; CCPP, constant coronary perfusion pressure; CM, cardiomyocyte; CREB, cAMP response element binding; DPP-4, dipeptidyl peptidase-4; EC, endothelial cell; Ex-4, exendin-4; Ex(9-39), exendin(9-39); GLP, glucagon like peptide; GLP-1R, GLP-1 receptor; HAEC, human aortic endothelial cell; H/R, hypoxia-reoxygenation; I/R, ischemia-reperfusion; LDH, lactate dehydrogenase; L-NAME, *N*(G)-nitro-L-arginine methyl ester; LVDP, left ventricular developed pressure; MS, mass spectrometry; MTT, 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazodium bromide; NOS, nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; TTC, 2,3,5-triphenyl tetrazolium chloride; WT, wild type.

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pression in endothelial cells (ECs) (4-6). The DPP-4-generated metabolite of GLP-1, GLP-1(9-36)amide, was initially believed to be either biologically inactive or a weak antagonist of the canonical GLP-1R (7, 8). Although a study in pigs suggested that an iv infusion of GLP-1(9-36)amide enhanced glucose disposal independent of incretin action (9), GLP-1(9-36)amide administration to healthy humans had no effect on glucose clearance or insulin secretion (10). Notably, levels of GLP-1(9-36)amide after GLP-1 infusion did not correlate with changes in plasma glucose in diabetic subjects (11).

In contrast, multiple lines of evidence support biological actions of GLP-1(9-36)amide in the cardiovascular system. GLP-1(9-36)amide increased myocardial glucose uptake and improved left ventricular performance in dogs with dilated cardiomyopathy (12), and administration of GLP-1(9-36)amide after global ischemia in rats significantly improved left ventricular pressure, although it failed to reduce infarct size (13). We recently showed that GLP-1(9-36)amide was a cGMP-producing vasodilatory molecule capable of cardioprotective effects in hearts isolated from GLP-1R knockout mice ($Glp1r^{-/-}$) (14). Furthermore, suppression of the cardioprotective and vasodilatory effects of GLP-1 by the DPP-4 inhibitor sitagliptin suggested that GLP-1(9-36)amide may function as a key intermediary in a subset of the cardiovascular effects of GLP-1 (14). A vasodilatory effect of GLP-1(9-36)amide concentrations that cover the physiological range *in vivo* was recently demonstrated in rat femoral artery preparations (15, 16).

Although DPP-4-resistant GLP-1R agonists (exendin-4, liraglutide) and DPP-4 inhibitors (sitagliptin, vildagliptin, saxagliptin) (17) are likely to produce different effects on circulating levels of GLP-1(9-36)amide *in vivo*, the mechanisms mediating the cardiovascular actions of GLP-1(9-36)amide are poorly understood. We now show that the isolated mouse heart rapidly converts GLP-1 to GLP-1(9-36)amide, and that the latter peptide is capable of reducing infarct size in an *ex vivo* model of ischemia-reperfusion (I/R). Using a constant flow model of coronary perfusion, we show that the protection against I/R injury afforded by GLP-1(9-36)amide does not entirely depend on its vasodilatory effects. In addition, we demonstrate that GLP-1(9-36)amide exerts direct cytoprotective actions on isolated mouse cardiomyocytes (CMs) via phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt- and ERK1/2-dependent mechanisms. Finally, we found that GLP-1(9-36)amide, but not the classical GLP-1R agonist exendin-4, protects cardiomyocytes from $Glp1r^{-/-}$ mice and human aortic endothelial cells (HAECs) against ischemic- and oxidative stress-induced injury models. Surprisingly, the actions of GLP-1(9-36)amide are blocked by exendin(9-39), a widely used GLP-1R antagonist. Together these novel data further define the selectivity of GLP-1R-dependent and -independent cardioprotective pathways and implicate the existence of an alternative receptor for GLP-1(9-36)amide in both CMs and ECs.

Materials and Methods

Animals

All protocols were approved by Toronto General Hospital and conformed to guidelines of the Canadian Council on Animal Care. Experiments were performed in 12-wk-old male C57BL/6 wild-type and $Glp1r^{-/-}$ mice. C57BL/6 mice were purchased from Charles River (Montréal, Canada) and housed for at least 2 wk before experiments. Creation, characterization, and genotyping of $Glp1r^{-/-}$ mice are described elsewhere (18).

Reagents

GLP-1 [GLP-1(7-36)amide] and GLP-1(9-36)amide were synthesized by Bachem (Torrance, CA) and exendin-4 (Ex-4) and the GLP-1R antagonist exendin(9-39) [Ex(9-39)] were purchased from California Peptide (Napa, CA). Stock solutions were made initially in water and then diluted in either Krebs-Hensleit or PBS solutions to achieve the final concentrations employed. All other chemicals used were of reagent-grade (Sigma, Mississauga, Ontario, Canada).

Isolated heart preparations

Isolated mouse hearts were prepared as described (14, 19, 20). The glucose concentration in perfusion buffers was 11 mM. Only data derived from hearts demonstrating intrinsic heart rates greater than 350 beats/min were used.

Mass spectrometry (MS) analysis

Coronary effluent samples were serially collected from isolated mouse hearts infused on Langendorff preparations with GLP-1 or GLP-1(9-36)amide. Samples were analyzed with a LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA) with a nanoelectrospray ionization source to identify specific signatures of GLP-1 and GLP-1(9-36)amide. Detailed methodology for this MS approach is provided in the Supplemental Materials and Methods published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Myocardial I/R injury *ex vivo*

Constant coronary perfusion pressure model

Isolated mouse hearts were mounted on a Langendorff apparatus and perfused with Krebs-Hensleit buffer under a constant coronary perfusion pressure (CCPP) of 80 mm Hg. After a 20-min equilibration phase, hearts were subjected to 30 min of global (no flow) ischemia and 120 min of reperfusion. Either GLP-1(9-36)amide (0.3 nM) or Ex-4 (3 nM) was added to the perfusion buffer for the first 60 min of the reperfusion period. The heart rate (beats per minute) and left ventricular developed pressure (LVDP; mm of mercury) were continuously monitored. Recovery of LVDP was measured at the end of reperfusion and

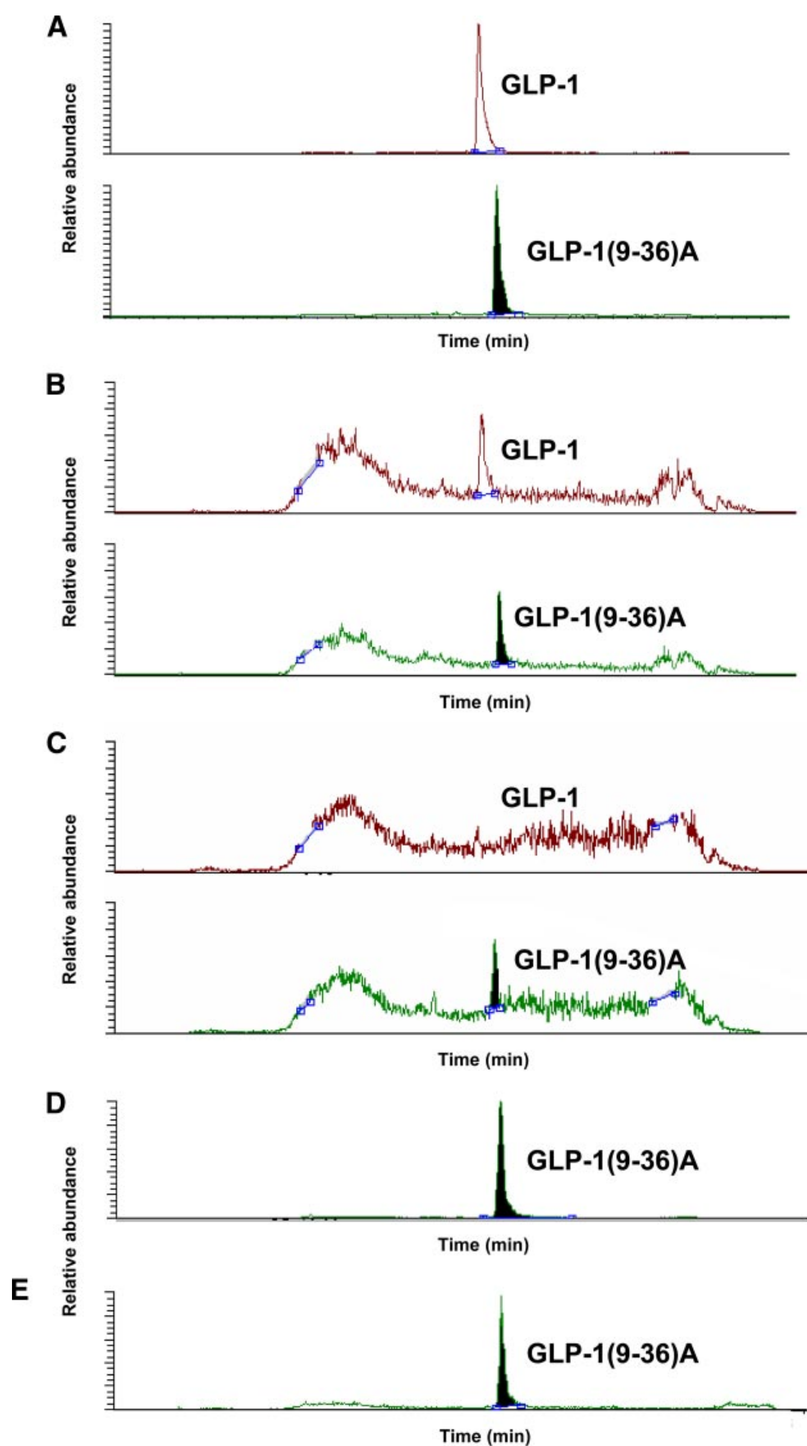

FIG. 1. GLP-1 is rapidly degraded to GLP-1(9-36)amide in the isolated mouse heart. A, Specific peaks generated by pure GLP-1 [GLP-1(7-36)amide (*upper panel*)] and pure GLP-1(9-36)amide (*lower panel*) peptides. B, Both GLP-1 (*upper panel*) and GLP-1(9-36)amide (*lower panel*) were detected in coronary effluent samples collected 15 min after initiation of a GLP-1 (0.3 nM) infusion. C, Little GLP-1 (*upper panel*) but abundant GLP-1(9-36)amide (*lower panel*) was detected in coronary effluent samples collected 30 min after initiation of the GLP-1 infusion. D, GLP-1(9-36)amide was found in coronary effluents collected either 15 min, or 30 min (E) after initiation of a GLP-1(9-36)amide (0.3 nM) infusion. The panels shown are representative of data obtained from four individual hearts in each experimental group.

expressed as a percentage of the LVDP at the end of perfusion (*i.e.* before ischemia). Infarct size was measured by staining hearts with 2,3,5-triphenyl tetrazolium chloride (TTC) after I/R as previously described (20). Digital images of TTC-stained sections were acquired on a DMLB microscope (Leica Microsystems, Wetzlar, Germany) and analyzed with image J software (NIH Image; National Institutes of Health, Bethesda, MD).

Constant coronary flow (CCF) model

By modifying our Langendorff apparatus to deliver CCF rather than CCPP, we attempted to limit putative vasodilatory effects of GLP-1(9-36)amide or Ex-4 on LVDP and infarct size. During the initial stabilization period, the CCF rate (milliliters per minute) was fixed at the value achieved with a resting CCPP of 80 mm Hg and maintained throughout subsequent reperfusion. All other experimental steps and analyses were identical with those under the CCPP mode.

Cellular I/R injury *in vitro*

Flow-independent cytoprotective effects of our GLP-1-like peptides were also examined on freshly isolated neonatal mouse ventricular CMs or HAECs undergoing simulated I/R injury models in culture. CMs were isolated from 1-d-old wild-type (WT) or Glp1r$^{-/-}$ mice as previously described (21, 22), with details provided in the Supplemental Materials and Methods. After serum deprivation for 24 h, CMs were exposed to either hypoxia-reoxygenation (H/R) (23–27) or H_2O_2 (100 μM) (28–32). In the H/R injury model, CMs were transferred to a sealed hypoxia chamber (Billup-Rothenberg, Inc., Del Mar, CA) in which 5% CO_2 and 95% N_2 gas were continuously infused for 48 h. Subsequently reoxygenation was initiated by transferring the CMs to an incubator under normal gas conditions (5% CO_2-95% air) and maintained for 7 h (23–27). Peptides of interest were added at the onset of reoxygenation. Same volume (1 μl) of PBS was added into control groups. In the H_2O_2 model, designed to mimic the oxidative stress of I/R, CMs were exposed to H_2O_2 (100 μM) for 7 h after preincubation with GLP-1(9-36)amide (0.3 nM) or Ex-4 (3 nM) for 20 min (28–32). HAECs were purchased (Cascade Biologics, Portland, OR) and cultured as per the vendor's instructions. Briefly, HAECs were plated in 24-well plates and cultured in EGM2 media (Invitrogen Canada Inc., Burlington, Ontario, Canada) containing 4 mmol/liter glucose and 2% fetal bovine serum plus growth factor additives. Cells were then serum deprived (0.2% fetal bovine serum) for 24 h. To simulate I/R injury, cells were transferred to an ischemic buffer (mmol/liter: 137, NaCl; 3.8 KCl; 0.49 $MgCl_2$; 0.9 $CaCl_2$.2 H_2O; 4 HEPES; 10 deoxyglucose; 0.75 sodium dithionate; 12 KCl; 20 lactate, pH 6.5) and incubated for 4 h (33–35). Reperfusion was achieved by replacing the buffer with normal EGM2 media for 7 h. At the onset of reperfusion, either GLP-1(9-36)amide (0.3 nM) or Ex-4 (3 nM) was added to the media.

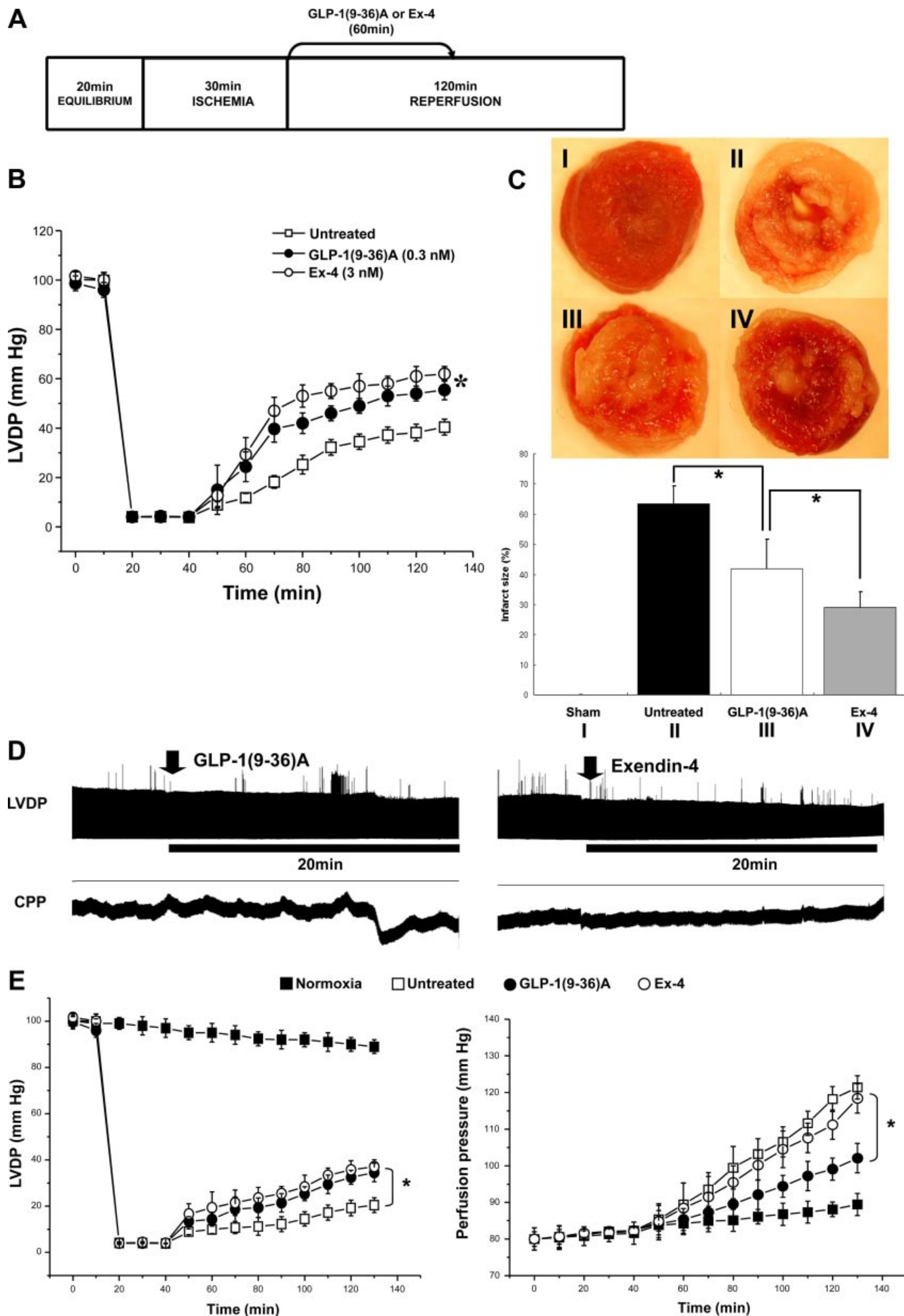

FIG. 2. Treatment with GLP-1(9-36)amide or Ex-4 improved functional recovery and limited infarct size after I/R of mouse hearts *ex vivo*. A, Experimental protocol demonstrating sequence and duration of peptide infusions, ischemia, and reperfusion. B, Functional recovery of control (untreated; n = 12), GLP-1(9-36)amide (0.3 nM; n = 8), and Ex-4 (3 nM; n = 7) treatments on isolated hearts undergoing I/R in CCPP mode ($P < 0.001$). C, Infarct size of control (untreated; n = 6), GLP-1(9-36)amide (0.3 nM; n = 6), and Ex-4 (3 nM; n = 6) treatments as determined by TTC staining on isolated hearts undergoing I/R in the CCPP mode ($P < 0.001$). D, Representative LVDP and coronary perfusion pressure (CPP) recordings from normoxic hearts after GLP-1(9-36)amide or Ex-4 treatment in CCF mode. E, Functional recovery (*left panel*; $P = 0.003$) and CPP (*right panel*; $P = 0.002$) of control (untreated; n = 10), GLP-1(9-36)amide (0.3 nM; n = 6), and Ex-4 (3 nM; n = 6) treatments on isolated hearts undergoing normoxia or I/R in CCF mode. Each *data point* represents mean ± SE. *, $P < 0.05$ compared with untreated control group by one-way ANOVA at the 130-min time point.

In the H_2O_2 model, HAECs were exposed to H_2O_2 (700 μM) for 7 h after preincubation with GLP-1(9-36)amide or Ex-4 for 20 min (28–32, 35).

Pharmacological inhibitors

To explore mechanisms activated by GLP-1(9-36)amide or Ex-4, antagonists of the GLP-1R [Ex(9-39); 5–50 nM], PI3K (LY294002; 10 μM), ERK1/2 (PD98059; 10 μM), and nitric oxide synthase (NOS) [SN (G)-nitro-L-arginine methyl ester (L-NAME); 100 μM] were examined in CMs and HAECs undergoing simulated I/R injury. Each agent was used for 30 min before the addition of GLP-1(9-36)amide or Ex-4.

Cytoprotection assays

The 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazodium bromide (MTT), extracellular lactate dehydrogenase (LDH) release, and caspase-3 activation assays used have been previously described (36–41). All experiments were repeated at least three times, and each condition tested was performed in quadruplicate in each experiment.

Western blot

Protein extracts from CMs were prepared as described (19, 21). Western blot was performed with rabbit monoclonal antibodies for PKB (Akt), phospho-PKB/Akt (Ser473), ERK1/2, phospho-ERK1/2 (Ser9), and cAMP response element binding (CREB) and phospho-CREB as per the manufacturers' instructions. All antibodies were purchased from Cell Signaling (Danvers, MA). Briefly, equal amounts of protein loaded on 12% percentages of sodium dodecyl sulfate-polyacrylamide gels were transferred electrophoretically to polyvinyl difluoride membranes. The membrane were blocked with 5% nonfat milk, incubated in various concentrations of primary specific antibody overnight at 4 C, and then incubated in horseradish peroxidase-conjugated secondary antibody. Finally, the immunoreactive bands were visualized by a chemiluminescence reagent (ECL Plus; GE Healthcare, Indianapolis, IN) and quantified by densitometry (Bio-Rad, Hercules, CA).

cAMP assay

After 24 h of serum deprivation, CMs were preincubated with 3-isobutyl-*1*-methylxanthine (250 μM; Sigma) for 30 min. Then CMs were treated with varying doses (0.03–30 nM) of either GLP-1(9-36)amide or Ex-4 for 20 min. Samples were then collected and analyzed using a cAMP RIA kit (Amersham, Little Chalfont, UK). All experiments were performed in quadruplicate.

Statistical analyses

All data are presented as mean ± SE. A one-way ANOVA was used to evaluate the differences among groups. If the ANOVA was significant, the Student-Newman-Keuls (Student-Nweman-Keuls) *post hoc* test was used for pair-wise multiple comparisons. Significance was defined as $P < 0.05$.

Results

GLP-1(9-36)amide is generated in the coronary circulation of hearts infused with GLP-1

To determine whether GLP-1(9-36)amide can be generated from the native GLP-1 (GLP-1(7-36)amide) peptide by the coronary circulation, we used MS to analyze coronary effluents of isolated mouse hearts infused with GLP-1 or GLP-1(9-36)amide. MS/MS spectra were obtained by injecting pure GLP-1 or GLP-1(9-36)amide peptides, and their identity was verified by matching experimental with theoretical spectra (Fig. 1A). Based on this analysis, parent ions of m/z 1100.37 (3+) and m/z 1030.98 (3+) were chosen to detect GLP-1 and GLP-1(9-36)amide, respectively, in the coronary effluent samples. Next, we generated extracted ion chromatograms (Fig. 1, B–E) of each peptide from coronary effluent samples using prominent charge states that were empirically determined for the peptides ($[M+^3H]^{+3}$ for both GLP-1 and GLP-1(9-36)amide). The presence of GLP-1 and GLP-1(9-36)amide in the coronary effluent was verified based on both the retention time values for peptide ions and the MS/MS spectra result. In this manner, we demonstrated significant amounts of both GLP-1 and GLP-1(9-36)amide in coronary effluent samples collected 15 min after initiation of a GLP-1 infusion, indicating that GLP-1 is rapidly converted to GLP-1(9-36)amide in the isolated heart *ex vivo* (Fig. 1B). Of interest, after 30 min of a continuous GLP-1 infusion, the vast majority of peptide collected was GLP-1(9-36)amide, with only minimal amounts of GLP-1 being detected (Fig. 1C). In contrast, GLP-1(9-36)amide levels were stable in coronary effluents samples collected 15 or 30 min after initiation of a continuous infusion of GLP-1(9-36)amide (Fig. 1, D and E).

GLP-1(9-36)amide and Ex-4 reduce infarct size in isolated mouse hearts *ex vivo*

Because both GLP-1(9-36)amide and Ex-4 improved LVDP after I/R in rodent hearts (13, 14), we tested the ability of either agent to reduce infarct size because GLP-1(9-36)amide did not reduce infarct size in a rat model of I/R (13). As GLP-1(9-36)amide also exhibits vasodilatory properties (14), we treated isolated mouse hearts with GLP-1(9-36)amide (0.3 nM) or Ex-4 (3 nM) for the first 60 min of a 120-min reperfusion period after 30 min of global ischemia (Fig. 2A) and compared results obtained from a CCPP model with those from a CCF model. Both GLP-1(9-36)amide and Ex-4 improved recovery of LVDP after I/R injury in the CCPP model (GLP-1(9-36)amide: 58.1 ± 4.6%, n = 8; Ex-4: 63.1 ± 4.7%, n = 7 *vs.* untreated controls: 38.4 ± 2.7%, n = 12; $P < 0.05$; Fig. 2B). Treatment with GLP-1(9-36)amide or Ex-4 also reduced infarct size after I/R, as determined by TTC staining in the CCPP model [GLP-1(9-36)amide: 42.8 ± 7.2%, n = 6; Ex-4: 29.8 ± 4.8%, n = 6 *vs.* untreated controls: 63.4 ± 5.1%, n = 6; $P < 0.05$; Fig. 2C]. In CCF mode, GLP-1(9-36)amide, but not Ex-4, reduced coronary perfusion pressure during normoxia (Fig. 2D). GLP-1(9-36)amide again increased recovery of

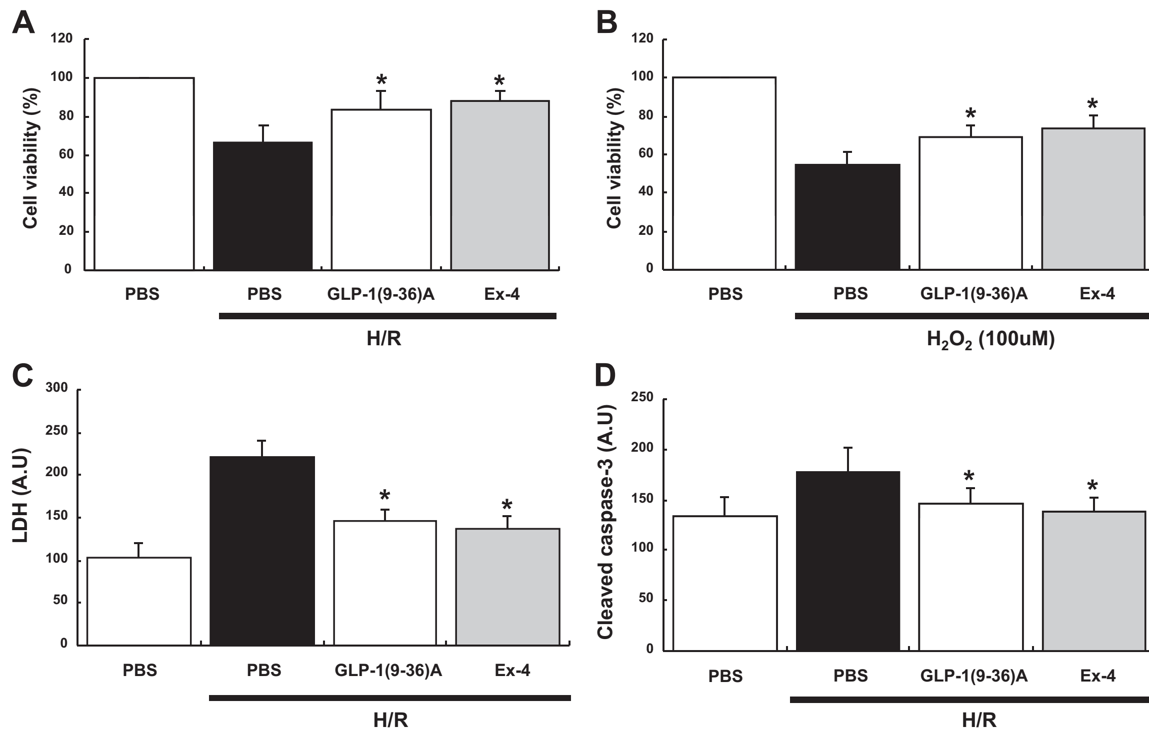

FIG. 3. Direct cytoprotective effects of GLP-1(9-36)amide and Ex-4 on CMs undergoing simulated I/R injury. GLP-1(9-36)amide and Ex-4 increased survival of cultured mouse neonatal CMs after either H/R ($P = 0.002$) (A) or H_2O_2 treatment ($P < 0.001$) (B), as determined by the MTT assay. Cell viability was also measured by assaying extracellular release of LDH ($P < 0.001$) (C) and activation of caspase-3 ($P = 0.048$) (D). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS controls.

LVDP (39.7 ± 3.5%, n = 6) *vs.* untreated controls (21.2 ± 2.1%, n = 10, $P < 0.05$) and did so with a lower mean coronary perfusion pressure during reperfusion (102.1 ± 4.4 *vs.* 121.4 ± 5.6 mm Hg, $P < 0.05$). Ex-4 provided a similar degree of LVDP recovery (42.5.2 ± 4.8%, n = 6, $P < 0.05$ *vs.* untreated controls) but did so without lowering coronary perfusion pressure (118.4 ± 5.6 mm Hg, P = NS *vs.* untreated controls) (Fig. 2E). However, under the admittedly nonphysiological conditions of CCF mode, neither GLP-1(9-36)amide nor Ex-4 reduced infarct size compared with untreated controls (P = NS).

GLP-1(9-36)amide and Ex-4 protect cardiomyocytes from I/R injury models via PI3K and ERK1/2

We next examined whether the actions of GLP-1(9-36)amide or Ex-4 were direct or indirect by examining the effects of these peptides on isolated mouse neonatal ventricular CMs. In CMs exposed to simulated I/R injury models *in vitro*, administration of GLP-1(9-36)amide (0.3 nM) or Ex-4 (3 nM) significantly improved CM viability as determined by the MTT assay. These findings were consistent whether CMs were exposed to H/R [GLP-1(9-36)amide: 83.7 ± 9.3%; Ex-4: 87.8 ± 5.1%; *vs.* PBS controls: 66.1 ± 9.4%; $P < 0.05$; Fig. 3A] or oxidative stress induced by H_2O_2 [GLP-1(9-36)amide: 69.1 ± 7.5%; Ex-4: 73.7 ± 7.9% *vs.* PBS controls: 54.5 ± 6.7%; $P < 0.05$; Fig. 3B]. In CMs exposed to H/R, both peptides lowered LDH release [GLP-1(9-36)amide: 145.7 ± 12.8 arbitrary units (AUs); Ex-4: 137.7 ± 13.3 AU *vs.* PBS controls: 221.8 ± 17.4 AU; $P < 0.05$; Fig. 3C] and caspase-3 activation [GLP-1(9-36)amide: 146.8 ± 26.1 AU; Ex-4: 138.9 ± 27.5 AU *vs.* PBS control: 177.2 ± 31.9 AU; $P < 0.05$; Fig. 3D].

To dissect molecular mechanism(s) underlying these effects, we examined the effects of GLP-1(9-36)amide and Ex-4 on known prosurvival proteins, including PKB/Akt, ERK1/2, and CREB. In normoxic CM cultures, both Ex-4 and GLP-1(9-36)amide increased phosphorylation of PKB/Akt, ERK1/2, and CREB (Fig. 4A) and increased cAMP formation in CMs in a dose-dependent manner (Fig. 4B). The prosurvival effects of both agents were significantly attenuated after cotreatment with inhibitors of PI3K (LY294002), ERK1/2 (PD98059), but not NOS (L-NAME) (Fig. 4C). To compare the minimum concentrations of GLP-1(9-36)amide and Ex-4 capable of cytoprotective action, we undertook a dose-response study covering six log doses of each agent (0.003–300 nM) in CM cultures undergoing simulated I/R injury with H_2O_2. A minimum cytoprotective concentration of 0.3 nM was identified for both peptides (Fig. 4D).

The actions of GLP-1(9-36)amide are attenuated by the GLP-1R antagonist Ex(9-39) but preserved in cardiomyocytes from Glp1r$^{-/-}$ mice

To investigate whether the CM actions of the structurally distinct peptides GLP-1(9-36)amide and Ex-4 were

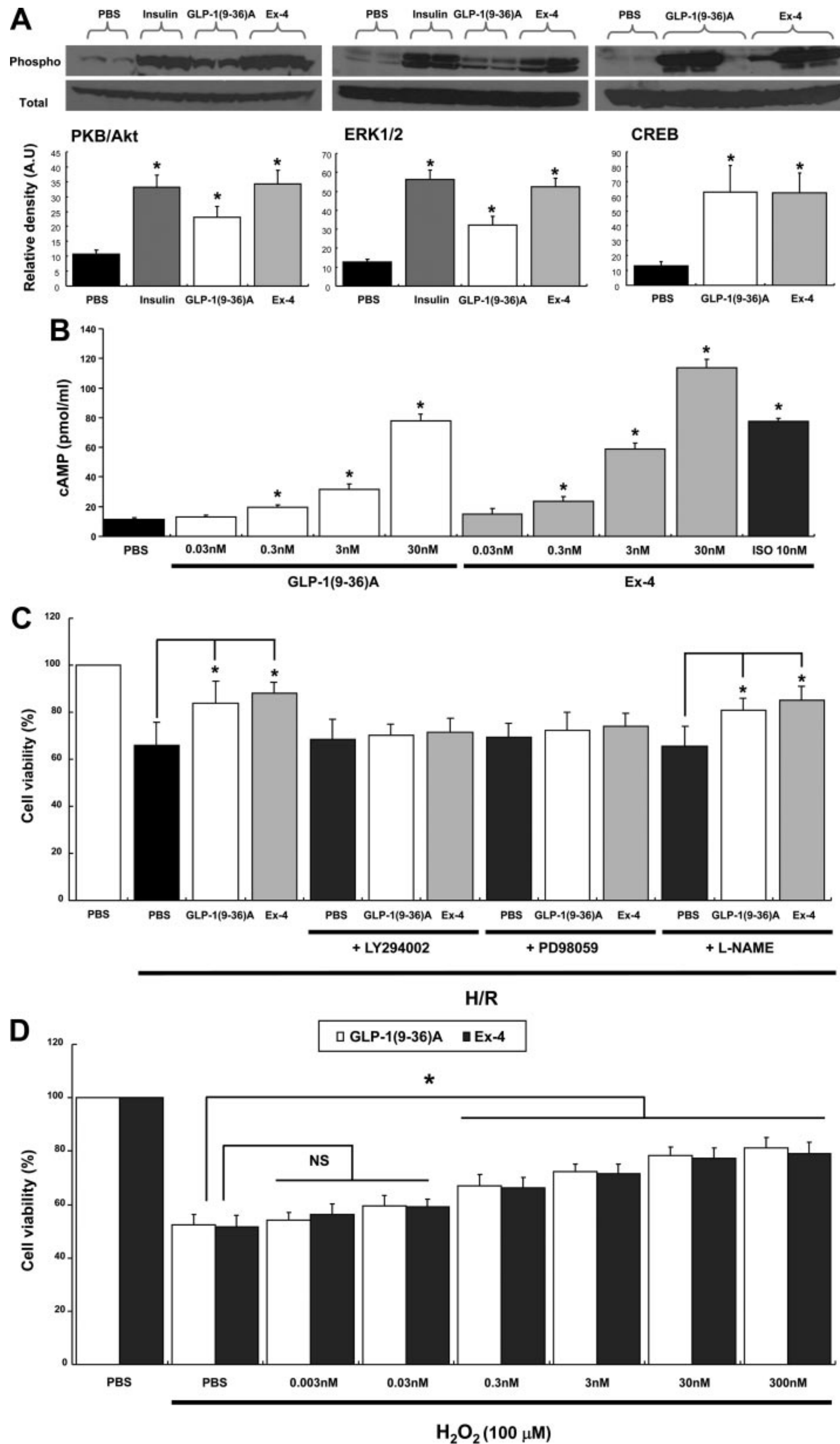

FIG. 4. GLP-1(9-36)amide and Ex-4 activate cytoprotective signaling pathways in CMs. A, Both GLP-1(9-36)amide and Ex-4 treatment of cultured mouse neonatal CMs maintained in normoxic condition increased phosphorylation of prosurvival kinases, PKB/Akt ($P < 0.001$), ERK1/2 ($P < 0.001$), and CREB ($P = 0.031$), as indicated by representative Western blots and corresponding densitometric quantification (n = 4 or 5/group). B, Treatments with GLP-1(9-36)amide and Ex-4 increased cAMP formation in a dose-dependent manner in cultured mouse neonatal CMs (n = 4/dose; $P < 0.001$). C, Cytoprotective effects of GLP-1(9-36)amide and Ex-4 were attenuated by pretreatment with LY294002 (10 μM), or PD98059 (10 μM) but not L-NAME (100 μM) ($P = 0.002$). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS controls in each group. D, Treatment with GLP-1(9-36) amide and Ex-4 increased survival in a dose-dependent manner in cultured mouse neonatal CMs after H_2O_2 treatment as determined by the MTT assay ($P < 0.001$). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS controls.

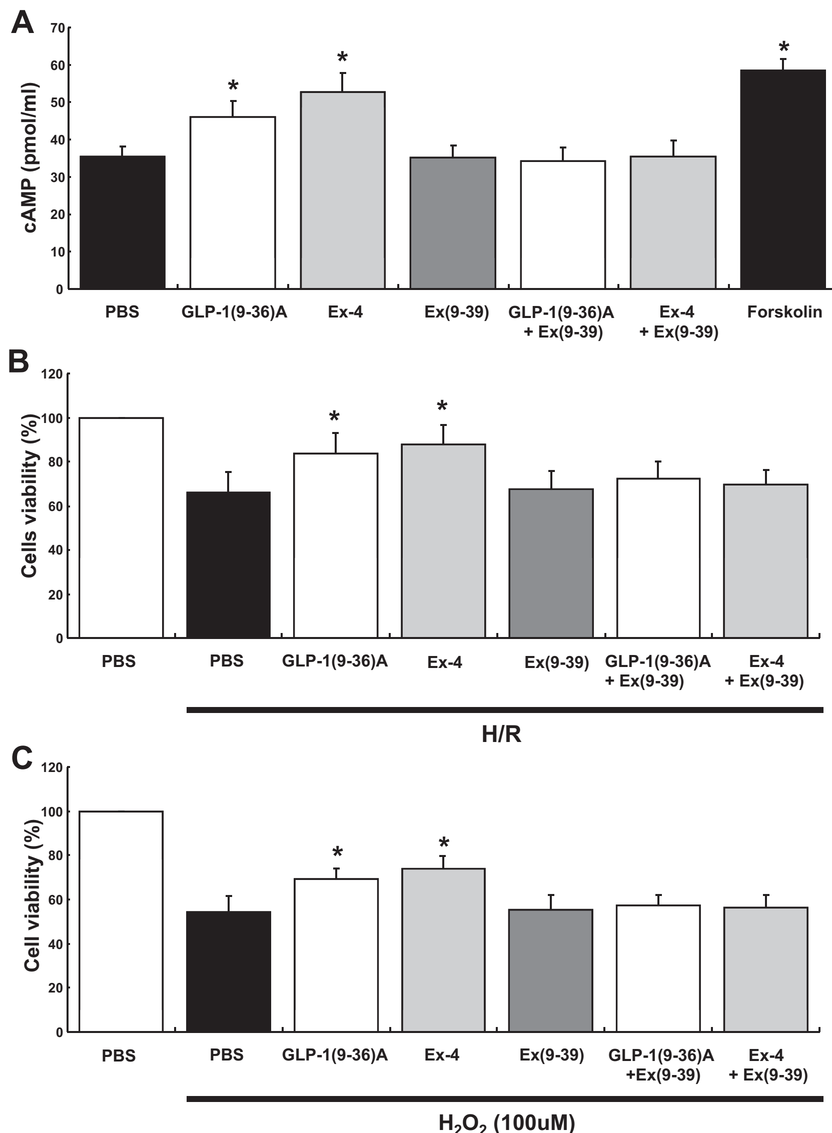

FIG. 5. Actions of GLP-1(9-36)amide and Ex-4 in CMs were attenuated by pretreatment with Ex(9-39). A, Pretreatment with the GLP-1R antagonist Ex(9-39) (5 or 50 nM) blocked the ability of GLP-1(9-36)amide (0.5 nM) and Ex-4 (3 nM) to increase cAMP formation in cultured mouse neonatal CMs ($P < 0.001$). B and C, Cytoprotective effects of both GLP-1 and GLP-1(9-36)amide in CMs undergoing H/R ($P = 0.033$) or H_2O_2 (100 μM) ($P = 0.009$) injury were abolished by pretreatment with Ex(9-39) (5 or 50 nM). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS controls in each group.

mediated via GLP-1R signaling, we used the GLP-1R antagonist Ex(9-39) and CMs isolated from mice with genetic disruption of a functional GLP-1R (Glp1r$^{-/-}$). The increase in cAMP in WT CMs after treatment with Ex-4 was completely abolished by cotreatment with the classical GLP-1R antagonist Ex(9-39; 50 nM) (Fig. 5A). Unexpectedly, Ex(9-39; 5 nM) also blocked the stimulatory actions of GLP-1(9-36)amide on cAMP formation (Fig. 5A). Consistent with these observations, the salutary effects of both peptides on CMs exposed to either I/R or H_2O_2 were diminished when coincubated with Ex(9-39) (5–50 nM) (Fig. 5, B and C). In contrast to the data obtained using the peptide antagonist Ex(9-39), the cAMP stimulation and prosurvival effects of GLP-1(9-36)amide, but not Ex-4, remained evident in Glp1r$^{-/-}$ CMs (Fig. 6, A and B). Also noteworthy was the finding that Ex(9-39) (5 nM) attenuated the effects of GLP-1(9-36)amide in CMs lacking a functional GLP-1R (Fig. 6C), suggesting that Ex(9-39) is also a functional GLP-1(9-36)amide antagonist.

GLP-1(9-36)amide protects human endothelial cells from I/R injury models

Given the ability of GLP-1(9-36)amide to vasodilate coronary, femoral, and mesenteric arteries (14, 16), we sought to examine whether GLP-1(9-36)amide exerted cytoprotective effects in ECs. GLP-1(9-36)amide but not Ex-4 improved survival of HAECs after exposure to H/R or H_2O_2 (Fig. 7, A and B). These salutary effects of GLP-1(9-36)amide were abrogated by cotreatment with the NOS inhibitor L-NAME (Fig. 7C).

Discussion

Consistent with previous observations of GLP-1 degradation in the systemic circulation, we now demonstrate that the isolated mouse heart rapidly converts GLP-1 to GLP-1(9-36)amide. We also show that the metabolite GLP-1(9-36)amide exerts cardioprotective actions in the mouse heart *ex vivo*, limiting infarct size after I/R injury. Consistent with coronary flow-independent cytoprotection, GLP-1(9-36)amide directly activates PKB/Akt, ERK1/2, and CREB and directly protects CMs from simulated I/R injury via PI3K- and ERK1/2-dependent mechanisms. Our finding that GLP-1(9-36)amide but not Ex-4 also promotes cell survival in CMs from Glp1r$^{-/-}$ mice and in HAECs strongly supports the existence of an alternative receptor for GLP-1(9-36)amide, distinct from the classical GLP-1R, in both CMs and ECs. Together these results extend our understanding of the cell physiology and molecular mechanisms of action of GLP-1(9-36)amide and shed new insights on the potential cardiovascular effects of incretin-targeted therapeutics.

In a previous report, we suggested a novel two-pathway schema for cardiovascular actions of GLP-1: one depend-

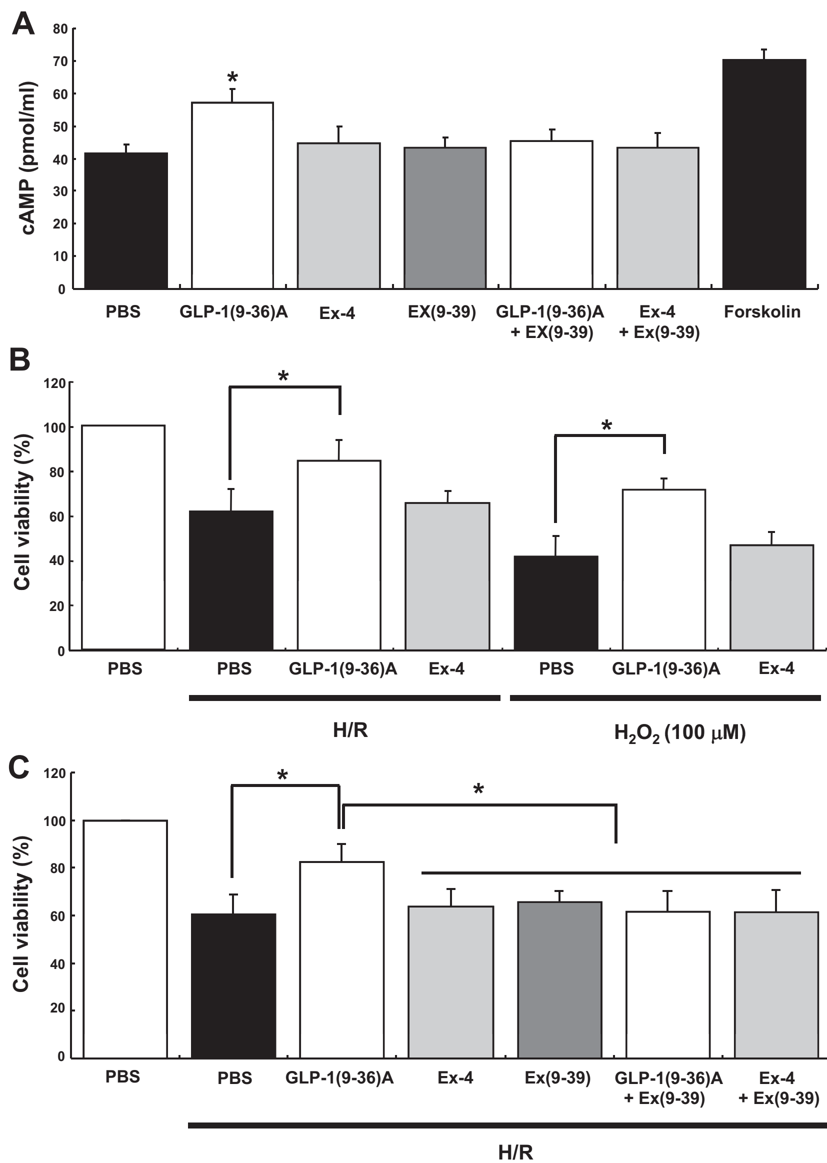

FIG. 6. Actions of GLP-1(9-36)amide were maintained in CMs from Glp1r$^{-/-}$ mice but still abolished by Ex(9-39). A, GLP-1(9-36)amide (0.3 nM) but not Ex-4 (3 nM) augmented cAMP formation in cultured neonatal CMs from Glp1r$^{-/-}$ mice, an effect blocked by pretreatment with Ex(9-39) (5 nM) ($P < 0.001$). B and C, Cytoprotective actions of Ex-4, but not GLP-1(9-36)amide, were lost in CMs from Glp1r$^{-/-}$ mice ($P < 0.001$). The cytoprotective effects of GLP-1(9-36)amide in Glp1r$^{-/-}$ CMs were abolished by pretreatment with Ex(9-39) (5 nM) ($P < 0.001$). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS control group.

ing on the canonical GLP-1R found on ECs, vascular smooth muscle cells, and CMs (14) and a second depending on rapid metabolism of GLP-1 to GLP-1(9-36)amide, the latter having GLP-1R-independent effects. In this regard, we developed a MS approach to qualitatively detect the presence of GLP-1 and GLP-1(9-36)amide in coronary effluents. MS has emerged as a powerful analytical technique for protein identification and can also be applied to peptide metabolism due to its high sensitivity and resolution (42). We were able to detect GLP-1(9-36)amide within 15 min of the initiation of a GLP-1 infusion to isolated mouse hearts *ex vivo*. The fact that only trace amounts of intact GLP-1 remained in coronary effluents after 30 min of continuous GLP-1 infusion supports the notion that GLP-1(9-36)amide may function as a critical intermediary in GLP-1-induced cardiovascular biology.

Consistent with previous studies, administration of GLP-1(9-36)amide or the degradation resistant GLP-1R agonist Ex-4 significantly improved functional recovery and reduced infarct size. However, unlike the study by Sonne *et al.* (13) in which Ex-4 but not GLP-1(9-36)amide reduced infarct size in a rat model of I/R, the current study found that both Ex-4 and GLP-1(9-36)amide are capable of limiting infarct size in a mouse model of I/R using the CCPP mode of myocardial reperfusion. Although isolated hearts in CCF mode allowed us to examine vascular effects, this preparation has the disadvantage of not resembling *in vivo* conditions. Autoregulation of the myocardial vasculature is lost in this model, and the results obtained with respect to infarct size differed considerably from those obtained in CCPP mode. Whereas the absence of an infarct-sparing effect of GLP-1(9-36)amide in CCF mode suggests that vasodilatory actions are essential for cardioprotection, data from our *in vitro* studies examining isolated cell types support the notion that vasodilatory effects of GLP-1(9-36)amide are not the only mechanism underlying its cardioprotective action.

Whereas the magnitude of the cardioprotective effects of Ex-4 appeared to be greater than that of GLP-1(9-36)amide, it is important to highlight that the dose of Ex-4 used (3 nM) was an order of magnitude greater than that of GLP-1(9-36)amide (0.3 nM). Indeed, in isolated CMs undergoing simulated I/R injury, the dose-response relationship for cytoprotective effects of both agents was essentially identical.

Our finding that GLP-1(9-36)amide but not Ex-4 was capable of lowering coronary perfusion pressure during the reperfusion phase provides further support for the notion that GLP-1(9-36)amide has vasodilatory effects (even when coronary flow was kept constant) beyond its direct cardioprotective actions (14). We have extended these observations by now demonstrating that GLP-1(9-36)amide

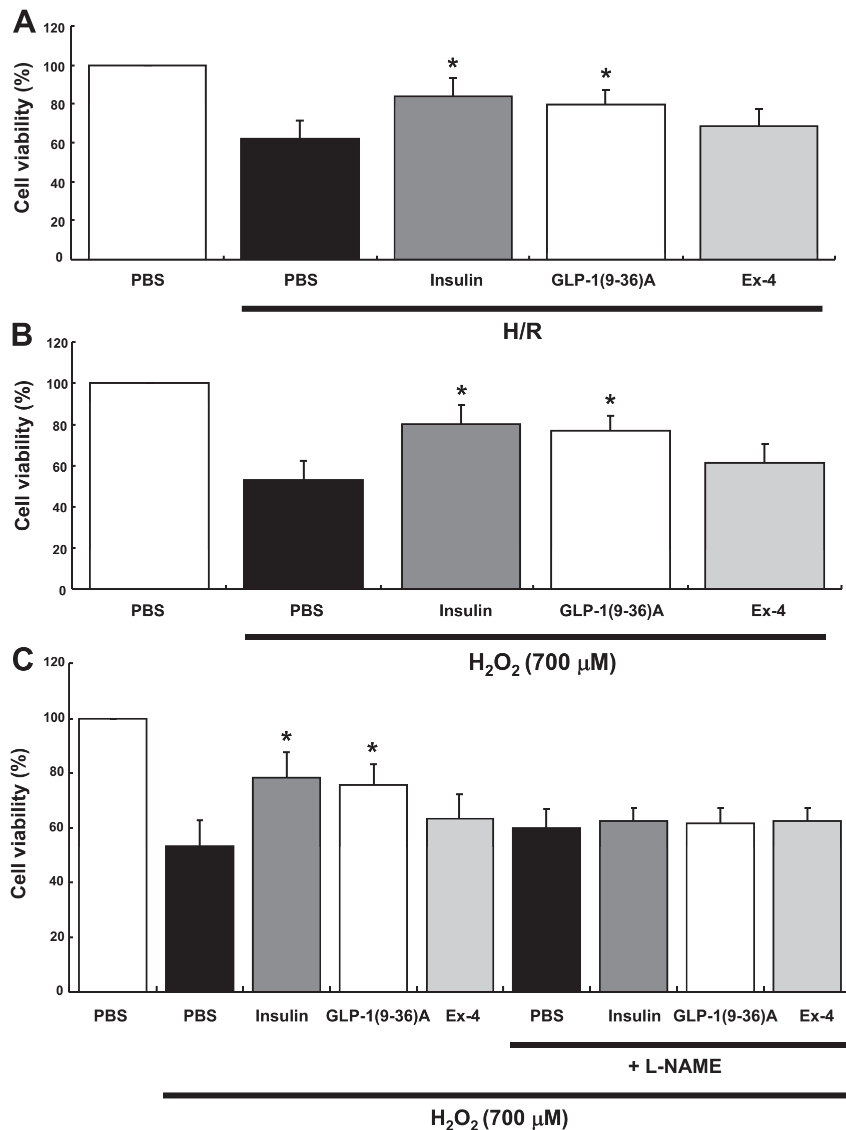

FIG. 7. GLP-1(9-36)amide but not Ex-4 exerts cytoprotective effects in human ECs. A, GLP-1(9-36)amide but not Ex-4 improved survival of HAECs undergoing simulated I/R injury (H/R: $P = 0.033$; H_2O_2: $P = 0.014$). B, Salutary effects of GLP-1(9-36)amide in HAECs were abolished by pretreatment with L-NAME (150 μM) ($P = 0.009$). Bar graphs show mean ± SE. *, $P < 0.05$ compared with PBS controls.

exerts direct effects on two cardiovascular cell types, namely CMs and ECs. In cultured neonatal CMs from WT mice, I/R injury was induced by either H/R or H_2O_2 treatment protocols, both well-recognized models for simulating I/R injury *in vitro* (23–27). Treatment of CMs with GLP-1(9-36)amide or Ex-4 resulted in improved cell viability in response to these I/R injury models, as determined by the MTT assay, LDH release, and caspase-3 activation.

Previous studies demonstrated that multiple downstream signaling pathways are involved in mediating the protective effects of GLP-1 (43). Similar to findings made with whole hearts studied *ex vivo*, we observed that both GLP-1(9-36)amide and Ex-4 significantly increased cAMP levels and phosphorylation of PI3K targets PKB/Akt and ERK1/2 and CREB in CMs maintained under normoxic conditions *in vitro*. It is important to note that each of these signaling pathways has been reported to play a critical role in cardioprotection against I/R injury (44–49). Of these key candidates, the cytoprotective effects of both GLP-1(9-36)amide and Ex-4 were attenuated by inhibitors of PI3K and ERK1/2. Together these data provide strong evidence that both GLP-1(9-36)amide and Ex-4 exert cytoprotective effects through PI3K-PKB- and MAPK-ERK1/2-dependent signaling pathways.

Consistent with our hypothesis that a GLP-1R-independent (*i.e.* alternate receptor) signaling mechanism exists for GLP-1(9-36)amide, the actions of GLP-1(9-36)amide, but not Ex-4, were maintained in CMs isolated from $Glp1r^{-/-}$ mice. Moreover, only GLP-1(9-36)amide, and not Ex-4, improved the survival of HAECs undergoing simulated I/R injury. Unexpectedly, the actions of GLP-1(9-36)amide in CMs isolated from both WT and $Glp1r^{-/-}$ mice were blunted by cotreatment with Ex(9-39). This intriguing finding suggests that Ex(9-39) is capable of blocking not only GLP-1R-dependent mechanisms but also GLP-1R-independent actions mediated by GLP-1(9-36)amide. To date, it has been widely assumed that the actions of Ex(9-39) are relatively specific for the classical GLP-1R. However, our current results strongly suggest Ex(9-39) also functions as a GLP-1(9-36)amide antagonist. In retrospect, there have been many previous reports on nonspecific effects of Ex(9-39). For example, Ex(9-39) antagonized cAMP production and insulin release by glucose-dependent insulinotropic polypeptide (50, 51), and Ex(9-39) alone can cause concentration-dependent vasorelaxation (52). Hence, our current findings should prompt a reassessment of the specificity of Ex(9-39) when used as a putative GLP-1R antagonist.

Finally, it is noteworthy that although the concentrations of GLP-1(9-36)amide capable of eliciting vasodilatory responses in the rat femoral artery (0.001–10 nM) (16) overlap the range of GLP-1(9-36)amide concentrations measured in man (0.025–0.1 nM) (15), our study identi-

fied the minimum cytoprotective concentration of GLP-1(9-36)amide (or Ex-4) in isolated CMs to be 0.3 nM, a value admittedly more pharmacological than physiological. Nevertheless, local tissue concentrations of GLP-1(9-36)amide have not been well studied and the minimum concentration of GLP-1(9-36)amide capable of eliciting cardioprotective actions *in vivo* has yet to be established.

In conclusion, we demonstrate that GLP-1(9-36)amide exhibits many of the beneficial cardiovascular effects attributed to GLP-1 but mediates these effects through a GLP-1 receptor-independent, Ex(9-39)-sensitive mechanism. Identification and characterization of the putative GLP-1(9-36)amide receptor and further elucidation of the relevance of GLP-1(9-36)amide action in humans may lead to enhanced understanding of the cardiovascular biology of GLP-1 and related peptides.

Acknowledgments

Address all correspondence and requests for reprints to: Mansoor Husain, TMDT 3-904, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4. E-mail: mansoor.husain@utoronto.ca.

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References

1. **Elrick H, Stimmler L, Hlad Jr CJ, Arai Y** 1964 Plasma insulin responses to oral and intravenous glucose administration. J Clin Endocrinol Metab 24:1076–1082
2. **McIntyre N, Holdsworth CD, Turner DS** 1965 Intestinal factors in the control of insulin secretion. J Clin Endocrinol Metab 25:1317–1324
3. **Baggio LL, Drucker DJ** 2007 Biology of incretins: GLP-1 and GIP. Gastroenterology 132:2131–2157
4. **Kieffer TJ, McIntosh CH, Pederson RA** 1995 Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 *in vitro* and *in vivo* by dipeptidyl peptidase IV. Endocrinology 136:3585–3596
5. **Mentlein R, Gallwitz B, Schmidt WE** 1993 Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem 214:829–835
6. **Vilsbøll T, Agersø H, Krarup T, Holst JJ** 2003 Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects. J Clin Endocrinol Metab 88:220–224
7. **Deacon CF, Johnsen AH, Holst JJ** 1995 Degradation of glucagon-like peptide-1 by human plasma *in vitro* yields an N-terminally truncated peptide that is a major endogenous metabolite *in vivo*. J Clin Endocrinol Metab 80:952–957
8. **Knudsen LB, Pridal L** 1996 Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36) amide after *in vivo* administration to dogs, and it acts as an antagonist on the pancreatic receptor. Eur J Pharmacol 318:429–435
9. **Deacon CF, Plamboeck A, Møller S, Holst JJ** 2002 GLP-1-(9-36) amide reduces blood glucose in anesthetized pigs by a mechanism that does not involve insulin secretion. Am J Physiol 282:E873–E879
10. **Vahl TP, Paty BW, Fuller BD, Prigeon RL, D'Alessio DA** 2003 Effects of GLP-1-(7-36)NH2, GLP-1-(7-37), and GLP-1-(9-36)NH2 on intravenous glucose tolerance and glucose-induced insulin secretion in healthy humans. J Clin Endocrinol Metab 88:1772–1779
11. **Zander M, Madsbad S, Deacon CF, Holst JJ** 2006 The metabolite generated by dipeptidyl-peptidase 4 metabolism of glucagon-like peptide-1 has no influence on plasma glucose levels in patients with type 2 diabetes. Diabetologia 49:369–374
12. **Nikolaidis LA, Elahi D, Shen YT, Shannon RP** 2005 Active metabolite of GLP-1 mediates myocardial glucose uptake and improves left ventricular performance in conscious dogs with dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 289:H2401–H2408
13. **Sonne DP, Engstrøm T, Treiman M** 2008 Protective effects of GLP-1 analogues exendin-4 and GLP-1(9-36) amide against ischemia-reperfusion injury in rat heart. Regul Pept 146:243–249
14. **Ban K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M** 2008 Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation 117:2340–2350
15. **Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, Gaeckler D, Schmidt WE, Gallwitz B** 2004 Secretion, degradation, and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects. Diabetes 53:654–662
16. **Nathanson D, Erdogdu O, Pernow J, Zhang Q, Nyström T** 2009 Endothelial dysfunction induced by triglycerides is not restored by exenatide in rat conduit arteries *ex vivo*. Regul Peptides 157:8–13
17. **Drucker DJ, Nauck MA** 2006 The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368:1696–1705
18. **Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ** 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. Nat Med 2:1254–1258
19. **Ban K, Cooper AJ, Samuel S, Bhatti A, Patel M, Izumo S, Penninger JM, Backx PH, Oudit GY, Tsushima RG** 2008 Phosphatidylinositol 3-kinase γ is a critical mediator of myocardial ischemic and adenosine-mediated preconditioning. Circ Res 103:643–653
20. **Noyan-Ashraf MH, Momen MA, Ban K, Sadi AM, Zhou YQ, Riazi AM, Baggio LL, Henkelman RM, Husain M, Drucker DJ** 2009 GLP-1R agonist liraglutide activates cytoprotective pathways and improves outcomes after experimental myocardial infarction in mice. Diabetes 58:975–983
21. **Kim KH, Oudit GY, Backx PH** 2008 Erythropoietin protects against doxorubicin-induced cardiomyopathy via a phosphatidylinositol 3-kinase-dependent pathway. J Pharmacol Exp Ther 324:160–169

22. **Costantini DL, Arruda EP, Agarwal P, Kim KH, Zhu Y, Zhu W, Lebel M, Cheng CW, Park CY, Pierce SA, Guerchicoff A, Pollevick GD, Chan TY, Kabir MG, Cheng SH, Husain M, Antzelevitch C, Srivastava D, Gross GJ, Hui CC, Backx PH, Bruneau BG** 2005 The homeodomain transcription factor Irx5 establishes the mouse cardiac ventricular repolarization gradient. Cell 123:347–358
23. **Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K** 2000 Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in mouse heart. Circulation 101:660–667
24. **Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL** 2002 Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 105: 1497–1502
25. **Morisco C, Marrone C, Trimarco V, Crispo S, Monti MG, Sadoshima J, Trimarco B** 2007 Insulin resistance affects the cytoprotective effect of insulin in cardiomyocytes through an impairment of MAPK phosphatase-1 expression. Cardiovasc Res 76:453–464
26. **Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, Ma XL** 2007 Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. Circulation 115:1408–1416
27. **Oshima Y, Ouchi N, Sato K, Izumiya Y, Pimentel DR, Walsh K** 2008 Follistatin-like 1 is an Akt-regulated cardioprotective factor that is secreted by the heart. Circulation 117:3099–3108
28. **Ohori K, Miura T, Tanno M, Miki T, Sato T, Ishikawa S, Horio Y, Shimamoto K** 2008 Ser9 phosphorylation of mitochondrial GSK-3β is a primary mechanism of cardiomyocyte protection by erythropoietin against oxidant-induced apoptosis. Am J Physiol Heart Circ Physiol 295:H2079–H2086
29. **Eguchi M, Liu Y, Shin EJ, Sweeney G** 2008 Leptin protects H9c2 rat cardiomyocytes from H2O2-induced apoptosis. FEBS J 275: 3136–3144
30. **Han H, Long H, Wang H, Wang J, Zhang Y, Wang Z** 2004 Progressive apoptotic cell death triggered by transient oxidative insult in H9c2 rat ventricular cells: a novel pattern of apoptosis and the mechanisms. Am J Physiol Heart Circ Physiol 286:H2169–H2182
31. **Murata H, Ihara Y, Nakamura H, Yodoi J, Sumikawa K, Kondo T** 2003 Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt. J Biol Chem 278:50226–50233
32. **Yasuoka C, Ihara Y, Ikeda S, Miyahara Y, Kondo T, Kohno S** 2004 Antiapoptotic activity of Akt is down-regulated by Ca2+ in myocardiac H9c2 cells: evidence of Ca2+-dependent regulation of protein phosphatase 2Ac. J Biol Chem 279:51182–51192
33. **Emanueli C, Bonaria Salis M, Stacca T, Pintus G, Kirchmair R, Isner JM, Pinna A, Gaspa L, Regoli D, Cayla C, Pesquero JB, Bader M, Madeddu P** 2002 Targeting kinin B1 receptor for therapeutic neovascularization. Circulation 105:360–366
34. **Esumi K, Nishida M, Shaw D, Smith TW, Marsh JD** 1991 NADH measurements in adult rat myocytes during simulated ischemia. Am J Physiol Heart Circ Physiol 260:H1743–H1752
35. **Ma H, Zhang HF, Yu L, Zhang QJ, Li J, Huo JH, Li X, Guo WY, Wang HC, Gao F** 2006 Vasculoprotective effect of insulin in the ischemic/reperfused canine heart: role of Akt-stimulated NO production. Cardiovasc Res 69:57–65
36. **Bergmann MW, Rechner C, Freund C, Baurand A, El Jamali A, Dietz R** 2004 Statins inhibit reoxygenation-induced cardiomyocyte apoptosis: role for glycogen synthase kinase 3β and transcription factor β-catenin. J Mol Cell Cardiol 37:681–690
37. **Dhanasekaran A, Gruenloh SK, Buonaccorsi JN, Zhang R, Gross GJ, Falck JR, Patel PK, Jacobs ER, Medhora M** 2008 Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia. Am J Physiol Heart Circ Physiol 294:H724–H735
38. **Germack R, Dickenson JM** 2005 Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. J Mol Cell Cardiol 39:429–442
39. **Gomez LA, Alekseev AE, Aleksandrova LA, Brady PA, Terzic A** 1997 Use of the MTT assay in adult ventricular cardiomyocytes to assess viability: effects of adenosine and potassium on cellular survival. J Mol Cell Cardiol 29:1255–1266
40. **Thuerauf DJ, Marcinko M, Gude N, Rubio M, Sussman MA, Glembotski CC** 2006 Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes. Circ Res 99:275–282
41. **Woo AY, Cheng CH, Waye MM** 2005 Baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism. Cardiovasc Res 65:244–253
42. **Kulasingam V, Smith CR, Batruch I, Buckler A, Jeffery DA, Diamandis EP** 2008 "Product ion monitoring" assay for prostate-specific antigen in serum using a linear ion-trap. J Proteome Res 7:640–647
43. **Bose AK, Mocanu MM, Carr RD, Brand CL, Yellon DM** 2005 Glucagon-like peptide-1 (GLP-1) can directly protect the heart against ischemia/reperfusion injury. Diabetes 54:146–151
44. **Das S, Tosaki A, Bagchi D, Maulik N, Das DK** 2005 Resveratrol-mediated activation of cAMP response element-binding protein through adenosine A3 receptor by Akt-dependent and -independent pathways. J Pharmacol Exp Ther 314:762–769
45. **Marais E, Genade S, Lochner A** 2008 CREB activation and ischaemic preconditioning. Cardiovasc Drugs Ther 22:3–17
46. **Markou T, Hadzopoulou-Cladaras M, Lazou A** 2004 Phenylephrine induces activation of CREB in adult rat cardiac myocytes through MSK1 and PKA signaling pathways. J Mol Cell Cardiol 37:1001–1011
47. **Matus M, Lewin G, Stümpel F, Buchwalow IB, Schneider MD, Schütz G, Schmitz W, Müller FU** 2007 Cardiomyocyte-specific inactivation of transcription factor CREB in mice. FASEB J 21:1884–1892
48. **Mehrhof FB, Müller FU, Bergmann MW, Li P, Wang Y, Schmitz W, Dietz R, von Harsdorf R** 2001 In cardiomyocyte hypoxia, insulin-like growth factor-I-induced antiapoptotic signaling requires phosphatidylinositol-3-OH-kinase-dependent and mitogen-activated protein kinase-dependent activation of the transcription factor cAMP response element-binding protein. Circulation 104:2088–2094
49. **Nagy N, Shiroto K, Malik G, Huang CK, Gaestel M, Abdellatif M, Tosaki A, Maulik N, Das DK** 2007 Ischemic preconditioning involves dual cardio-protective axes with p38MAPK as upstream target. J Mol Cell Cardiol 42:981–990
50. **Gault VA, O'Harte FP, Harriott P, Mooney MH, Green BD, Flatt PR** 2003 Effects of the novel (Pro3)GIP antagonist and exendin(9-39)amide on GIP- and GLP-1-induced cyclic AMP generation, insulin secretion and postprandial insulin release in obese diabetic (ob/ob) mice: evidence that GIP is the major physiological incretin. Diabetologia 46:222–230
51. **Wheeler MB, Gelling RW, McIntosh CH, Georgiou J, Brown JC, Pederson RA** 1995 Functional expression of the rat pancreatic islet glucose-dependent insulinotropic polypeptide receptor: ligand binding and intracellular signaling properties. Endocrinology 136:4629–4639
52. **Green BD, Hand KV, Dougan JE, McDonnell BM, Cassidy RS, Grieve DJ** 2008 GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving KATP and cAMP. Arch Biochem Biophys 478:136–142