The GLP-1R Agonist Liraglutide Activates Cytoprotective Pathways and Improves Outcomes Following Experimental Myocardial Infarction in Mice.

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ABSTRACT

Objective: Glucagon-like peptide-1 receptor (GLP-1R) agonists are used to treat type 2 diabetes and transient GLP-1 administration improved cardiac function in humans following acute myocardial infarction (MI) and percutaneous revascularization. However, the consequences of GLP-1R activation prior to ischemic myocardial injury remain unclear.

Research Design and Methods: We assessed the pathophysiology and outcome of coronary artery occlusion in normal and diabetic mice pre-treated with the GLP-1R agonist liraglutide.

Results: Male C57BL/6 mice were treated twice daily for 7 d with liraglutide or saline followed by induction of MI. Survival was significantly higher in liraglutide-treated mice. Liraglutide reduced cardiac rupture (12/60 vs. 46/60; P=0.0001), and infarct size (21±2% vs. 29±3%, P=0.02), and improved cardiac output (12.4±0.6 vs. 9.7±0.6; ml/min; P=0.002). Liraglutide also modulated the expression and activity of cardioprotective genes in the mouse heart including Akt, GSK3β, PPARβ−δ, Nrf-2, and HO-1. The effects of liraglutide on survival were independent of weight loss. Moreover, liraglutide conferred cardioprotection and survival advantages over metformin, despite equivalent glycemic control, in diabetic mice with experimental MI. The cardioprotective effects of liraglutide remained detectable 4 days following cessation of therapy and may be partly direct, as liraglutide increased cyclic AMP formation and reduced the extent of caspase-3 activation in cardiomyocytes in a GLP-1R-dependent manner in vitro.

Conclusions: These findings demonstrate that GLP-1R activation engages pro-survival pathways in the normal and diabetic mouse heart, leading to improved outcomes and enhanced survival following MI in vivo.
The protection against ischemic damage provided by cycling periods of ischemia and reperfusion, i.e. ischemic pre-conditioning (IP), may last for hours or even days (1). Although the molecular basis of ischemic preconditioning is complex and incompletely understood, there is active interest in the development of therapeutic interventions that protect the myocardium against ischemic injury.

Glucagon-like peptide-1 (GLP-1), a member of the proglucagon-derived peptide family (2), exerts favorable actions on cardiovascular function in both pre-clinical and clinical studies. A functional GLP-1 receptor (GLP-1R) is expressed in the heart (3) and GLP-1R agonists directly activate cardiomyocyte signaling pathways (4). As the GLP-1R is also expressed in the endocrine pancreas, GLP-1 may regulate cardiac function indirectly through metabolic control of glucose, insulin, glucagon and free fatty acids. Furthermore, GLP-1R agonists activate the peripheral and central nervous system, including regions of the CNS important for control of cardiovascular function (5-7). Hence, the mechanisms through which GLP-1 can modulate cardiac function are complex and incompletely understood.

Transient GLP-1 administration improves outcomes in experimental models of cardiac injury such as pacing-induced heart failure (8) and experimental ischemia induced by coronary artery ligation (9-13). Moreover, a pilot study of GLP-1 administration for 72 h in human subjects with left ventricular dysfunction following myocardial injury and angioplasty demonstrated reduced hospital stay, and improved global and regional left ventricular wall motion scores, benefits which remained detectable even several weeks following hospital discharge (14). Similarly, a 5 week course of GLP-1 infusion improved parameters of left ventricular function, functional status and quality of life in both diabetic and non-diabetic subjects with congestive heart failure (15).

Although the glucoregulatory actions of the first clinically approved GLP-1R agonist, exenatide, have been extensively studied in the clinic (2), there is limited information available about the effects of exenatide on the normal or ischemic heart (16). A second GLP-1R agonist liraglutide is a human dipeptidyl peptidase-4 (DPP-4)-resistant GLP-1 analogue that exhibits a prolonged pharmacokinetic profile, relative to native GLP-1, due to non-covalent association with albumin (2; 17; 18), and has completed phase 3 clinical trials in human subjects with type 2 diabetes. Although liraglutide appears to be a promising anti-diabetic agent (19), the effects of liraglutide on the cardiovascular system have not been examined.

We have now assessed whether liraglutide exerts cardioprotective actions in a preclinical murine model of experimental ischemia following coronary artery occlusion. We show that treatment with liraglutide prior to induction of ischemia leads to activation of pro-survival kinases and cytoprotective genes in the heart, and limits infarct size, expansion and cardiac rupture in the normal and diabetic heart. Moreover, liraglutide increases cAMP and reduces apoptosis in a GLP-1R-dependent manner in murine cardiomyocytes cultured in vitro. These findings extend our understanding of the cardioprotective actions of GLP-1R agonists and provide testable hypotheses for examining the cardiovascular effects of GLP-1R agonists in human subjects with type 2 diabetes.

METHODS
Animals: Protocols were approved by the Animal Care Committee of the Toronto General Hospital in accordance with guidelines of the Canadian Council for Animal Care. Male 10-12 wk old C57BL/6 mice were obtained from Charles River...
(Montreal, Quebec, Canada) and housed for at least 2 wk before experimentation.

**Drug treatments:** The volume of individual i.p. injections was 100 μl. The experimental protocols are summarized in Supplementary Figure 1. One group of animals (n=75) was injected with the GLP-1R agonist liraglutide (Novo Nordisk, Novo Alle, Bagsvaerd, Denmark) at a previously utilized dose of 200 μg/kg, i.p. twice daily (18) for 7 d prior to permanent surgical ligation of the left anterior descending (LAD) artery as previously detailed (20). A parallel group of control animals (n=75) was injected with an equivalent volume of the vehicle, phosphate-buffered saline (PBS). After this treatment period, some animals (n=10 per group) were euthanized just prior to planned LAD ligation, with hearts dissected immediately, weighed, and frozen. A second group of mice received PBS or liraglutide (n=10 each) for 7 d and were subjected to sham surgery without LAD occlusion. Since a regimen of liraglutide 200 μg/kg i.p twice daily (LIR 200) induced weight loss in mice, we also studied the effects of LAD ligation in separate groups of mice after (a) administration of a lower dose of liraglutide (75 μg/kg i.p. twice daily: LIR 75) that did not produce significant weight loss or (b) pair-feeding to induce weight loss in control animals comparable to that seen in mice treated with LIR 200. Separate groups of PBS- and liraglutide-treated mice (n=13 per group), were euthanized on d4 post-MI for biochemical and histological analyses. Left ventricular tissues from infarct and peri-infarct zones and remote (non-infarcted) area were separated, snap-frozen and stored at -80°C. The remaining mice were monitored for 28 d post-MI for survival analysis and histomorphometry for infarct size. In separate studies, mice were maintained on a high fat diet (HFD: 45% Kcal from fat, D-12451, Research Diets) for one month, following which diabetes was induced by treatment with streptozotocin (STZ, 90 mg/kg i.p.). After stratification by degree of hyperglycemia at 3 wks following STZ injection, mice were randomized to receive treatment for 7 d with placebo (PBS, 100 μl, i.p. twice daily), metformin in chow (6.76 g/kg of mouse diet) or liraglutide 75 μg twice daily (LIR 75) prior to sham or LAD-ligation surgery [n=23/group: 5 sham; 18 LAD-ligation]. Diabetic mice were maintained on the HFD until euthanasia on d28 post-surgery. To investigate the role of the known GLP-1R in the cardiac actions of liraglutide, male Glp1r−/− mice, 10-12 weeks of age in the C57BL/6 background and their littermate (Glp1r+/+) controls (n=6/group) were injected with either PBS or liraglutide 75 μg twice daily for one week, then euthanized and their hearts were used for Western blot assessment of pro-survival kinases.

**Necropsy:** Cardiac examinations were performed on deceased mice post-MI. The presence of a large amount of blood or clot around the heart and in the thoracic cavity, as well as a perforation of the infarct or peri-infarct area was taken to indicate cardiac rupture.

**Blood glucose:** Prior to anesthesia, non-fasting blood glucose measurements were obtained via a tail nick, using a hand-held glucometer and One-Touch glucometer strips (LifeScan Canada, Ltd., Burnaby, British Columbia, Canada).

**Cardiac hypertrophy:** Heart-to-body weight ratios were calculated for each animal at the time of terminal sacrifice.

**Infarct size:** Demarcation of the infarct area was performed in separate groups of PBS- and liraglutide-treated mice using either 2,3,5-triphenyl tetrazolium chloride (TTC) or hematoxylin and eosin (H&E) staining at 2 and 28 d post-MI respectively, as described (20; 21). Briefly, sections from three levels of each heart (5 μm: apical, mid-ventricular, and basal) were stained with H&E, scanned and the circumference of the fibrotic infract area and entire left ventricle was measured with
Image J software (NIH). The mean of measurements of % infarct size (circumference) was then calculated (infarct circumference/total LV circumference x 100%).

For analysis after TTC staining, hearts were sectioned perpendicular to the long axis with a thickness of ~2 mm from the apical, mid-ventricular and basal (immediately below the ligation point) regions and incubated in the pre-warmed (30°C) fresh TTC solution for 15 min. They were then weighed and transferred to a 4% para-formaldehyde solution for 1 h before image acquisition with a digital camera. Infarct and total left ventricle areas were then measured by Image J for determination of % infarct size (area) by calculation (infarct area/total LV area x 100%).

Zymography: MMP-9 was assessed in the infarct zone 4 days post-MI as described (22).

Cell culture, cAMP assay & TNF-α-induced apoptosis: Cardiomyocytes from newborn mice were prepared using a modified protocol (23). For cAMP assay, neonatal mouse cardiac myocytes were pre-incubated with IBMX (250 μM; Sigma) for 30 min to inhibit cAMP degradation followed by subsequent treatment periods of: 20 min for liraglutide (100nM), 30 min for the GLP-1R antagonist exendin (Ex) (9-39) (1 μM); and 15 min for forskolin (100 nM). For the liraglutide + Ex(9-39) treatment, cells were exposed to Ex(9-39) for 30 min prior to co-incubation with fresh Ex(9-39) and liraglutide for an additional 20 min. All experiments were performed in quadruplicate. Samples were collected and analyzed using a cAMP radioimmunnoassay kit (Amersham, Little Chalfont, UK). For TNF-α experiments, cells were grown on 6-well dishes, serum deprived for 24 h, incubated for 1 h with 10, 100 and 1000 nM doses of liraglutide with or without 10 μM Ex(9-39) followed by co-incubation with liraglutide and TNF-α (100 ng/ml, Sigma) for another 24 h to induce apoptosis (24). As a positive control for the induction of apoptosis, another group of cardiomyocytes was exposed to 0.5 μM H2O2 as described (25). All experiments were performed in triplicates. Western blot analysis using whole cell extracts was employed to quantify levels of cleaved caspase 3.

Western blot: Extracts from cells, whole hearts and infarct regions were prepared as described (26). Rabbit polyclonal primary antibodies against GLP-1R (LS-A1205, MBL International), Nrf2 (C20, Santa Cruz Biotechnology), Heme-oxygenase-1 (HO-1; Stressgen), PPAR-β/δ (H-74, Santa Cruz), and monoclonal antibodies against Akt, phospho-Akt (Ser473), GSK-3β, phospho-GSK-3beta (Ser9), and cleaved-caspase 3 (all from Cell Signaling), as well as goat-polyclonal anti-ANP antibodies (Santa Cruz) were used as per manufacturer’s instructions. Mouse monoclonal anti-β-actin (Sigma) and anti-TFIID (Santa Cruz) antibodies and a rabbit polyclonal anti-GAPDH (Santa Cruz) were used to evaluate the amount of protein loaded in each sample.

RT-PCR: Total RNA was isolated from hearts using TRIZOL (Invitrogen), quantified (2 μg) and treated with DNase-I. cDNA synthesis was performed by Superscript III reverse transcriptase (Invitrogen), and specific primers for HO-1 (F: GCC-CTT-CTG-GTA-TGG-GCC-TCA-CTG-G; R: GCC-TCT-GAC-GAA-GTG-ACG-CCA-TCT-G); Nrf2 (F: TCT-CCT-CGC-TGG-AAA-AAG-AA; R: AAT-GTG-CTG-GCT-GTG-CTT-TA). PCR products were visualized on 1.5% agarose gels with ethidium bromide. GAPDH reaction product served as a loading and RT efficiency control.

Ischemia-reperfusion: Isolated hearts were prepared as previously described (27). Isolated hearts underwent a 20 min equilibration phase, followed by a 40 min perfusion phase during which left ventricular developed pressure (LVDP) was continuously recorded. Then, 30 min of sustained global ischemia was generated by clamping inflow to
the heart followed by reperfusion for 40 min. In some experiments liraglutide was added to the buffer during the final 20 min of the perfusion phase (i.e. pre-ischemia), in others it was added to the buffer only during reperfusion (i.e. post-ischemia). Recovery of LVDP was measured at the end of reperfusion. These experiments were also performed using hearts isolated from mice following 1 d (acute) or 7 d (chronic) of twice daily liraglutide (200 μg/kg i.p.).

Ultrasound biomicroscopy. Image acquisition and data analysis were carried out as described (28; 29). Three groups of non-diabetic mice, including 8 control mice not subjected to LAD occlusion, and 15 liraglutide-treated and 15 saline-treated mice were studied on d28 post-MI using high frequency ultrasound imaging. Identical procedures were used to image diabetic mice [sham (n=5/group), LAD-ligated (n=10-11/group)].

Statistical analysis: All data are expressed as mean ± SE. Survival analysis was done by the Kaplan-Meier method (Figure 1A-B). Student’s t-test was used to compare two groups for data shown in Figures 2-4. For analysis of echocardiographic data (Table 1) and specific time points in ischemia-reperfusion experiments (Figure 5) and cAMP and TNF-α studies (Figure 6), a one-way analysis of variance (ANOVA) was used to evaluate the difference among groups. If the ANOVA was significant, the Student-Newman-Keuls (SNK) post hoc test was used for pair-wise multiple comparisons. Significance was defined as P<0.05.

Author’s declaration: The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the contents of the manuscript as written.

RESULTS
Liraglutide increased survival in mice post-MI: Immediate peri-operative mortality (within 24 h of LAD ligation) was 8.3% in PBS- (n=85) and 5% in LIR 200-treated (n=60) mice (P=0.43) with no deaths in the sham-operated animals (n=20). By d28 post-MI, LIR 200-treated mice exhibited reduced mortality compared to PBS-treated controls (12/60 vs. 46/60; P=0.0001) (Fig 1A). As mice treated with LIR 200 also exhibited weight loss compared to PBS-treated controls (-1.65 ± 0.05 vs. +0.72 ± 0.02; g; P =0.0001), we carried out additional experiments to determine whether the effects of LIR on survival post-MI were dependent on weight loss. A separate group of mice was pair-fed to achieve comparable weight loss to animals receiving LIR 200 for 7 d; no difference in survival was noted between mice treated with LIR 200 vs. pair-fed controls (Fig. 1A). We next assessed a range of liraglutide doses on food intake and body weight and then determined the effect of pre-treatment with a weight-neutral dose, LIR 75, on post-MI survival. Mice treated with LIR 75 for 7 d did not experience significant weight loss (0.30 ± 0.06 g; P=0.85) but exhibited a marked improvement in survival following LAD ligation (Fig. 1A, P=0.0001). Similarly, pre-treatment of diabetic mice for 1 wk with LIR 75 also reduced mortality following LAD occlusion (Fig 1B, P=0.04); in contrast, comparable treatment with metformin that produced equivalent reduction in glycemia (Supplementary Table 1) did not improve survival after MI (P=0.5, NS). Together, these data suggest that pre-treatment with liraglutide enhances survival following MI independent of effects on body weight or blood glucose.

Liraglutide prevented cardiac rupture post-MI: To understand the mechanisms by which LIR improves outcomes following MI in mice, we performed post-mortem examinations of all animals. Excluding d1, post-mortem analysis in non-diabetic mice revealed that all spontaneous death events within 10 d post-MI were associated with
evidence of cardiac rupture. Timing and incidence of cardiac rupture differed among the groups, occurring as early as d3 post-MI and peaking in incidence at d5 post-MI in PBS-treated mice (Fig. 1C). By contrast, mice pre-treated with LIR 200, and to an even greater extent LIR 75, exhibited fewer and later cardiac rupture events post-MI (Fig 1C).

**Liraglutide reduced infarct expansion post-MI:** Cardiac rupture is known to occur more frequently with larger infarcts (30) and is believed to manifest an imbalance between inflammatory and fibrotic responses, as well hemodynamic forces acting on the infarct (31; 32). To explore these possibilities in more detail we first analyzed H&E-defined infarct size at d28 post-MI in non-diabetic mice. Liraglutide (LIR 200) significantly reduced infarct size compared to PBS-treated mice (20.9 ± 1.7 vs. 28.8 ± 3.3; % total LV circumference, P=0.02) (Fig 2). Furthermore, cardiac hypertrophy, as manifest by HW/BW ratio, was significantly reduced in LIR 200-treated mice (Fig 2). To determine if these results partly reflected survivor bias, we also examined TTC-defined infarct size at d2 post-MI. Intriguingly, this analysis revealed no significant difference in infarct size between LIR 200- (n=14) and PBS- (n=15) treated groups (40.2 ± 4.2 vs. 39.3 ± 3.8; P=0.86). Also, despite significantly higher survival in liraglutide-treated diabetic mice (Fig 1B), no differences were observed in either infarct size or HW/BW ratios in diabetic animals at d28 post-MI (data not shown).

**Liraglutide activates cardioprotective signaling pathways in the heart:** To determine the mechanisms underlying liraglutide-induced improvements in survival and infarct remodeling post-MI, we studied the expression of selected genes and proteins known to modulate cardiomyocyte survival. LIR 200 administered twice daily for 7 d to normal healthy mice (without MI) increased phosphorylation of the pro-survival kinase Akt (pAkt/Akt: 2.10 ± 0.01 fold over control, P=0.002, Fig. 3A). Similarly, liraglutide increased phosphorylation and thereby reduced the activity of GSK3β, a known Akt substrate and increased levels of the nuclear receptor PPARβ/δ, and the redox-sensitive basic leucine zipper transcription factor nuclear factor erythroid-2 related factor-2 (Nrf2). LIR also induced mRNA and protein levels of heme oxygenase-1 (HO-1) (Fig 3A), a protein strongly implicated in cardioprotection in response to ischemic injury (33). To assess the importance of the known GLP-1R for the actions of liraglutide, we administered LIR 75 for an identical 7 day treatment period in mice with genetic absence of a functional GLP-1R and littermate controls. Liraglutide increased phosphorylation of Akt and GSK3β in both wild-type (Fig 3B) and Glp1r+/+ littermate controls (Fig 3C), but not in Glp1r-/- mice (Fig 3D).

To assess whether the effects of liraglutide remained detectable after cessation of liraglutide therapy and prior to the peak incidence of mortality post-MI (which occurred on d5 - Fig. 1A), we examined the hearts of LIR- and PBS-treated controls 4 d post-MI (i.e. 4 d after the final dose of LIR or PBS). The HW/BW ratio was reduced in LIR-treated mice (5.53 ± 0.14 vs. 5.83 ± 0.15; mg/g; P=0.03) at d4 and levels of phosphorylated Akt were significantly higher in the infarct area of hearts from LIR-treated mice (Fig 4). Similarly, LIR pre-treatment was associated with increased GSK phosphorylation at Ser 9 and a significant reduction in levels of cleaved caspase-3 and ANP (Fig. 4). Moreover, quantitative gelatin zymography showed a significant decrease in MMP-9 activity in hearts from LIR-treated mice (Fig. 4). Together, these data demonstrate a persistent effect of LIR pre-treatment on cardiac pro-survival and remodeling pathways detectable both before (Fig 3) and after (Fig 4) induction of ischemic injury.
Liraglutide-treated mice exhibit improved cardiac performance: The results of cardiac ultrasound biomicroscopy performed 4 wks after LAD ligation or sham surgery in non-diabetic mice are shown in Table 1. Following experimental MI, measures of systolic function such as cardiac output (CO) and stroke volume (SV) were significantly increased in liraglutide-treated mice. Measures of diastolic function, such as mitral inflow velocities (E/A ratio) were also improved in liraglutide-treated mice and left ventricular dilatation in the LIR-treated group was less severe than in PBS-treated control mice (Table 1). In contrast, although liraglutide-treated diabetic mice exhibited a significant increase in cardiac output in the absence of experimental MI, no consistent differences in echocardiographic parameters were detected in diabetic mice treated with liraglutide vs. metformin at d28 post MI (data not shown).

Liraglutide treatment in vivo prevented ischemia-reperfusion injury of isolated hearts ex vivo: To determine whether liraglutide exhibits rapid cardioprotective actions in the isolated mouse heart ex vivo, we examined the effects of acute liraglutide administration in the isolated perfused mouse heart. Direct infusions of liraglutide immediately prior to and post-ischemia did not improve functional recovery following ischemia-reperfusion (I/R) injury in the isolated mouse heart (Fig 5A). In contrast, pre-treatment of normal healthy mice with LIR (200 μg/kg, b.i.d.) in vivo for 1 or 7 d prior to heart isolation enhanced recovery of left ventricular developed pressure (LVDP) following I/R relative to untreated controls (1 d: 41.54 ± 1.5, n = 4; 7 d: 38.31±0.6 mmHg, n = 5; untreated: 28.24 ± 1.8 mmHg, n=5; P<0.01, Fig 5B). These results imply that LIR-induced cardioprotection may require a minimum dose or pre-treatment period for the activation of a cardiac gene and protein expression profile, and/or may be indirect, reflecting the consequences of GLP-1R activation in non-cardiac tissues.

Liraglutide induces cAMP and reduces apoptosis in neonatal mouse cardiomyocytes. We next examined whether liraglutide exerts direct actions on mouse cardiomyocytes. Liraglutide (100 nM) significantly increased cAMP formation in mouse cardiomyocytes (P<0.0001) in a GLP-1R-dependent manner, as cAMP stimulation was abolished by the GLP-1R antagonist Ex (9-39) (Fig 6A). To determine whether liraglutide directly modulates cardiomyocyte survival, we examined the effects of liraglutide in TNF-α-treated neonatal cardiomyocytes. Liraglutide dose-dependently reduced TNF-α-induced activation of caspase 3 in cardiomyocytes in vitro. Furthermore, the protective effect of liraglutide was completely abolished following co-incubation of cells with Ex (9-39) (Fig 6B).

DISCUSSION
In the present study we demonstrate that liraglutide administration induced changes in the expression of cardioprotective proteins in the normal non-atherosclerotic murine heart characterized by phosphorylation of Akt and GSK3β and increased expression of Nrf2, PPAR-β/δ and HO-1. Furthermore, these changes were associated with improved survival of mice after experimental ischemia despite cessation of liraglutide therapy. These findings, taken together with the results of echocardiography, demonstrate that a brief 7 d period of liraglutide pretreatment leads to improvements in cardiomyocyte survival and sustained improvement in cardiac function that remain detectable even 4 wks after cessation of liraglutide and induction of experimental MI.

Importantly, the beneficial effects of liraglutide were independent of weight loss, as a lower dose of liraglutide (75 μg/kg) that did not produce weight loss also protected the heart and increased survival to a greater
extent than that observed with the higher LIR 200 μg/kg dose. Hence, the weight loss observed with higher pharmacological doses of GLP-1R agonists is not required for the beneficial effects of these agents on cardiovascular function (2).

Although the mechanisms underlying the reduced cardiac rupture and improved survival remain incompletely understood, notable findings from our studies include the modulation of putative mediators (Akt, GSK3β, HO-1, PPAR-β/δ and Nrf2) known to be important for cardiomyocyte survival (34-38). Furthermore, we observed reduced levels of MMP-9 and cleaved caspase 3 in the infarct region of liraglutide-treated mice at day 4 post-MI. These findings are reminiscent of observations made in studies of the effects of GLP-1 on survival of β-cells and neurons, where GLP-1R activation leads to inhibition of caspase activation and increased cytoprotection (39; 40).

Remarkably, functional assessment with echocardiography showed that left ventricular systolic function remained improved even 4 wks after the last dose of liraglutide. Furthermore, we observed significantly less dilatation of the left ventricle of liraglutide-treated mice. These findings are consistent with pre-clinical data demonstrating that GLP-1R agonists improve left ventricular function in rodent and canine models of experimental cardiac dysfunction (8; 9; 11-13; 41). Moreover, the sustained benefits of transient liraglutide therapy that persisted for weeks following cessation of therapy is consistent with studies demonstrating that a 72 h infusion of GLP-1 in human subjects following MI produces sustained improvements in cardiovascular function that remained detectable even months after cessation of GLP-1 administration (14).

An unexpected finding was the observation that liraglutide, unlike native GLP-1 or GLP-1(9-36) (3), was not effective in directly improving LV pressure, or recovery of LV pressure after I/R injury, when administered either immediately prior to induction of ischemia or during the reperfusion phase to isolated hearts ex vivo. In contrast, administration of liraglutide in vivo for as little as 1 d (2 injections) prior to ischemia was sufficient to confer a cardioprotective benefit in subsequent I/R experiments. Hence, our findings raise the possibility that the cardioprotective actions of liraglutide are complex and may require a specific minimum dose or defined time period for induction of a protective gene/protein profile that promotes resistance to cardiomyocyte injury. Alternatively, the cardioprotective actions of liraglutide observed in vivo may be partly indirect, mediated perhaps through neural, hormonal, or metabolic factors. This latter possibility contrasts with findings demonstrating direct protection by native GLP-1 in I/R studies (9; 10; 42).

Recent studies have implicated a role for the metabolite GLP-1(9-36) as a cardioactive peptide with inotropic effects in dogs with heart failure (41). The GLP-1 metabolite, GLP-1(9-36), also exerts cardioprotective actions when administered post-ischemia (3; 16) further highlighting the complexity of different GLP-1-related peptides on the ischemic heart. Liraglutide is relatively resistant to cleavage by DPP-4, and contains two amino acid modifications, a substitution and an addition, together with a fatty acid side chain. Hence, whether liraglutide is metabolized to a peptide with GLP-1(9-36)-like activity remains unclear. Nevertheless, the observation that liraglutide fails to induce the phosphorylation of Akt and Gsk3β in the Glp1r-/- heart, together with the demonstration that liraglutide increased cAMP and reduced apoptosis in cardiomyocyte cultures in a GLP-1R-dependent manner, strongly implicates the known GLP-1R as a critical mediator of liraglutide action in the murine heart.
Currently, drugs acting on the GLP-1R axis have been approved for the treatment of diabetes and have the added benefits of reducing appetite and body weight, and in some instances, blood pressure, leading to improvement of cardiovascular risk factors (43; 44). Given the experimental data presented here and elsewhere (8; 9; 13; 16), the possibility that these agents may have beneficial effects on cardiovascular outcomes of patients with diabetes, independent of improvements in blood pressure and blood glucose requires further study.

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DISCLOSURES
Dr. Husain has served as a consultant within the past 12 months to Sanofi-Aventis and Merck & Co. Inc. Dr. Drucker has served as an advisor or consultant within the past 12 months to Amylin Pharmaceuticals, Arisaph Pharmaceuticals Inc., Eli Lilly Inc, Emisphere Technologies Inc., Glaxo Smith Kline, Glenmark Pharmaceuticals, Hoffman LaRoche Inc., Isis Pharmaceuticals Inc., Merck Research Laboratories, Novartis Pharmaceuticals, Novo Nordisk Inc., Phenomix Inc, Takeda, and Transition Pharmaceuticals Inc. Neither Dr. Drucker, Dr. Husain or their family members hold stock directly or indirectly in any of these companies. None of the other authors have any conflicts or duality of interest to disclose.
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Figure 1. Liraglutide pre-treatment improves outcomes following myocardial infarction in mice. Kaplan-Meier survival curves show survival after MI in A: non diabetic mice: sham (n=20), liraglutide-MI (75 μg/kg, n=35 or 200 μg/kg, n=60), PBS-MI (n=60), and pair-fed mice (n=25), P=0.0001 for LIR 75 & 200 vs. PBS; B: diabetic mice: sham (n=15, 5/treatment group), PBS-MI (n=18), metformin-MI (n=18), liraglutide-MI (75 μg/kg, n=18), P=0.04 for LIR 75 vs. PBS. C: Frequency and timing of cardiac rupture in non-diabetic mice is shown as a percentage of total group. One wk pre-treatment with liraglutide (200 or 75 μg/kg i.p. twice daily) had no significant effects on random blood glucose levels in adult non-diabetic mice (PBS: 7.6 ± 4.0 vs. LIR 200: 6.3 ± 0.6 vs. LIR 75: 6.1 ± 0.4; mmol/L; P=0.72). Blood glucose levels in diabetic mice are shown in Supplementary Table I.
Figure 2. Effects of liraglutide pre-treatment on infarct size and heart weight. A: Representative photomicrographs of H&E-stained hearts 28d post-MI depict decreased infarct size (arrows) in liraglutide- (n=36) vs. PBS-treated mice (n=21) as confirmed by morphometric quantification of % total LV circumference (*P = 0.025). B: Heart:body weight ratio was reduced in liraglutide- vs. PBS-treated mice 28d post-MI (N=36 and 21 respectively, **P = 0.001). Data shown are mean ± SE.
Figure 3. Effects of liraglutide pre-treatment on levels of cardiac genes and proteins. The expression of genes/proteins prior to LAD ligation is analyzed in liraglutide-treated hearts. A: Representative Western blots for wild-type mice treated with LIR 200 as outlined in Supplementary Figure 1. Corresponding densitometric quantification (n=6/group) of fold changes in phosphorylation of prosurvival kinases Akt and GSK3β, and in expression of PPAR-β/δ, Nrf2 and HO-1 are shown in top 5 panels. Bottom panel depicts a representative agarose gel (n=6/group) showing HO-1-specific mRNA levels by RT-PCR. Data shown are mean ± SE; *P<0.05. B-D: Representative Western blot analysis of cardiac prosurvival kinases in LIR 75-treated wild-type (B), Glp1r+/+ littermate controls (C) and Glp1r−/− (D) mice, respectively.
Figure 4. Effects of liraglutide pre-treatment on cardioprotective signaling pathways after MI in mice. Liraglutide pre-treatment for 7 d has persistent cardioprotective effects detectable 4 d post-MI as indicated by representative Western blots and corresponding densitometric quantification (n=6/group) of fold changes in phosphorylation of Akt and GSK3β, cleavage of caspase-3, and expression of ANP, and activity of MMP-9 activity by zymography in liraglutide-vs. PBS-treated mice. Data shown are mean ± S.E.; *P<0.05.
Figure 5. Effects of liraglutide on recovery of left ventricular function after ischemia-reperfusion injury in isolated hearts. A: Effect of direct infusions of liraglutide- (0.3, 3, and 30 nM, n=5/group) or no treatment (vehicle, n=21) either pre- or post-ischemia in isolated murine hearts subjected to experimental ischemia-reperfusion (I/R) ex vivo. B: Two additional groups of mice (n=5/group) received intra-peritoneal injections of liraglutide b.i.d. for 1 or 7 d before ex-vivo experiments. Data shown are mean ± SE. *P<0.01 compared to untreated controls.
Figure 6. Liraglutide induces cAMP formation and reduces caspase-3 activation in murine cardiomyocytes \textit{in vitro}. A. Liraglutide (100 nM) increases cAMP formation in cultured neonatal cardiomyocytes. The actions of liraglutide were abolished by the GLP-1R antagonist exendin(9-39). B. Liraglutide (L: 10-1000 nM) reduced TNF-\(\alpha\)-induced activation of caspase-3 in a dose-dependent manner in cultured neonatal mouse cardiomyocytes. Co-treatment with Exendin (9-39) (Ex: 10 \(\mu\)M) abolished the protective effects of liraglutide. Positive control represents treatment of cells with the potent apoptosis-inducing agent H\(_2\)O\(_2\). Data shown are mean \pm SE; *P < 0.01, and **P<0.001 vs. cultures only treated with TNF\(\alpha\).
Table 1. Ultrasound biomicroscopy-defined cardiac dimensional, functional and hemodynamic parameters in mice on d28 post-op.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n=8)</th>
<th>MI-Placebo (n=15)</th>
<th>MI-Liraglutide (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.9 ± 0.3</td>
<td>29.5 ± 0.4</td>
<td>29.3 ± 0.5</td>
</tr>
<tr>
<td>Aortic flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>393 ± 12</td>
<td>451 ± 18</td>
<td>435 ± 12</td>
</tr>
<tr>
<td>Peak velocity (cm/s)</td>
<td>86.3 ± 2.2</td>
<td>68.7 ± 2.7(^a)</td>
<td>80.3 ± 3.2(^b)</td>
</tr>
<tr>
<td>VTI (cm)</td>
<td>3.03 ± 0.08</td>
<td>2.09 ± 0.13(^a)</td>
<td>2.61 ± 0.12(^a, b)</td>
</tr>
<tr>
<td>AO diameter (mm)</td>
<td>1.15 ± 0.02</td>
<td>1.15 ± 0.02</td>
<td>1.19 ± 0.01</td>
</tr>
<tr>
<td>LV SV (µl)</td>
<td>31.3 ± 0.9</td>
<td>22.1 ± 1.8(^a)</td>
<td>28.8 ± 1.4(^b)</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>12.3 ± 0.5</td>
<td>9.7 ± 0.6(^a)</td>
<td>12.4 ± 0.6(^b)</td>
</tr>
<tr>
<td>Mitral flow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>413 ± 11</td>
<td>433 ± 14</td>
<td>424 ± 9</td>
</tr>
<tr>
<td>Peak E velocity (cm/s)</td>
<td>71.8 ± 1.9</td>
<td>59.0 ± 3.6(^a)</td>
<td>59.9 ± 2.2(^a)</td>
</tr>
<tr>
<td>Peak A velocity (cm/s)</td>
<td>47.0 ± 1.5</td>
<td>30.8 ± 3.5(^a)</td>
<td>43.6 ± 2.0(^b)</td>
</tr>
<tr>
<td>Peak E/A ratio</td>
<td>1.54 ± 0.04</td>
<td>2.54 ± 0.44(^a)</td>
<td>1.40 ± 0.06(^b)</td>
</tr>
<tr>
<td>Left ventricular chamber dimensions by M mode</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>417 ± 10</td>
<td>442 ± 17</td>
<td>441 ± 15</td>
</tr>
<tr>
<td>LV EDD (mm)</td>
<td>4.33 ± 0.05</td>
<td>6.12 ± 0.23(^a)</td>
<td>5.52 ± 0.09(^a, b)</td>
</tr>
<tr>
<td>LV ESD (mm)</td>
<td>3.33 ± 0.08</td>
<td>5.73 ± 0.30(^a)</td>
<td>3.02 ± 0.14(^a, b)</td>
</tr>
<tr>
<td>FS (%)</td>
<td>23.0 ± 1.2</td>
<td>7.0 ± 1.5(^a)</td>
<td>9.2 ± 1.3(^a)</td>
</tr>
</tbody>
</table>

AO: aortic orifice; CO: cardiac output; FS: left ventricular fractional shortening; HR: heart rate; LV SV: left ventricular stroke volume; LV EDD: left ventricular end-diastolic diameter; LV ESD: left ventricular end-systolic diameter; VTI: velocity-time integral of Doppler flow waveform. In mitral inflow, the peak E velocity represents the maximal velocity of the early diastolic wave caused by active left ventricular relaxation. The peak A velocity represents the maximal velocity caused by left atrial contraction in late diastole. Data are expressed as mean ± SE. Superscript “\(^a\)” denotes difference (P<0.05) with the corresponding value in the sham controls. Superscript “\(^b\)” denotes difference (p<0.05) with the corresponding value in the placebo (PBS)-treated controls.