Inactivation of the cardiomyocyte glucagon-like peptide-1 receptor (GLP-1R) unmasks cardiomyocyte-independent GLP-1R-mediated cardioprotectiona,b

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ABSTRACT

GLP-1R agonists improve outcomes in ischemic heart disease. Here we studied GLP-1R-dependent adaptive and cardioprotective responses to ventricular injury. Glp1r+/− hearts exhibited chamber-specific differences in gene expression, but normal mortality and left ventricular (LV) remodeling after myocardial infarction (MI) or experimental doxorubicin-induced cardiomyopathy. Selective disruption of the cardiomyocyte GLP-1R in Glp1rPM−/− mice produced no differences in survival or LV remodeling following LAD coronary artery occlusion. Unexpectedly, the GLP-1R agonist tiraglutide still produced robust cardioprotection and increased survival in Glp1rPM−/− mice following LAD coronary artery occlusion. Although tiraglutide increased heart rate (HR) in Glp1rPM−/− mice, basal HR was significantly lower in Glp1rPM−/− mice. Hence, endogenous cardiomyocyte GLP-1R activity is not required for adaptive responses to ischemic or cardiomyopathic injury, and is dispensable for GLP-1R agonist-induced cardioprotection or enhanced chronotropic activity. However the cardiomyocyte GLP-1R is essential for the control of HR in mice.

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1. INTRODUCTION

Type 2 diabetes mellitus is treated using pharmacotherapy with agents acting through distinct anti-diabetic mechanisms that may be associated with unexpected adverse effects on cardiovascular outcomes, independent of glycemic control [1]. For example, thiazolidinediones increase fluid retention and peripheral edema in diabetic subjects with heart failure [2] whereas some dipeptidyl peptidase 4 (DPP-4) inhibitors increase the rate of hospitalization for heart failure[3]. The physiological importance of the endogenous GLP-1R for the response to cardiac injury has not been elucidated. Furthermore, the surprising demonstration that ventricular cardiomyocytes do not express the GLP-1R [10] raises important questions about mechanisms linking GLP-1R signaling to ventricular function and cardioprotection. We have now examined the physiological importance of endogenous GLP-1R signaling for the response to ischemic injury or doxorubicin-induced cardiomyopathy in Glp1r−/− mice and in newly generated Glp1rPM−/− mice with cardiomyocyte-specific inactivation of the Glp1r. Surprisingly, global or cardiomyocyte-specific disruption of GLP-1R signaling in mice does not influence index and reduced infarct size relative to the ischemic area at risk in human subjects with acute myocardial infarction (MI) [7].

Although pre-clinical studies demonstrate that GLP-1R agonists preserve ventricular function and reduce infarct size [8,9], the physiological importance of the endogenous GLP-1R for the response to cardiac injury has not been elucidated. Furthermore, the surprising demonstration that ventricular cardiomyocytes do not express the GLP-1R [10] raises important questions about mechanisms linking GLP-1R signaling to ventricular function and cardioprotection. We have now examined the physiological importance of endogenous GLP-1R signaling for the response to ischemic injury or doxorubicin-induced cardiomyopathy in Glp1r−/− mice and in newly generated Glp1rPM−/− mice with cardiomyocyte-specific inactivation in all future studies suggest that native GLP-1 or degradation-resistant GLP-1 agonists are required in experiments with subjects with ischemic cardiac injury or heart failure [4–6]. The largest randomized controlled trial demonstrated that a 6 h infusion of exenatide significantly improved the myocardial salvage
the extent of injury or survival after MI or experimental cardiomyopathy.

As GLP-1R agonists are administered to humans prior to or following the development of ischemic myocardial injury, we also studied the actions of GLP-1R agonists in mice when administered before or after coronary artery ligation. Surprisingly, administration of exendin-4 after the onset of MI did not modify infarct size or survival. Unexpectedly, the GLP-1R agonist liraglutide continued to produce robust cardioprotection in Glp1r−/− mice. Although the cardiomyocyte GLP-1R was not required for liraglutide-mediated increases in heart rate (HR), basal HR was significantly lower in Glp1r−/− mice. Taken together, these findings demonstrate that the cardiomyocyte GLP-1R is not essential for i) the endogenous physiological response to ischemic or cardiomyopathic injury, ii) GLP-1R-dependent cardioprotection or iii) the pharmacological GLP-1R-dependent increase in HR. In contrast, basal signaling through the atrial cardiomyocyte GLP-1R is essential for control of HR in mice.

2. METHODS

2.1. Animal care
Animal experiments were carried out using protocols approved by Mt. Sinai Hospital and The Toronto Centre for Phenogenomics (TCP; Toronto, ON, Canada). Mice were housed under a 12-h light/dark cycle in the TCP animal facility with free access to standard rodent diet (2018, 18% kcal from fat; Harlan Teklad, Mississauga, ON, Canada) and water, unless otherwise noted. Experiments were carried out in male mice acclimatized to handling. Glp1r−/− mice have been described [11]. To generate Glp1r+/− mice, Mer-CreMer transgenic mice expressing tamoxifen-inducible Cre driven by the α-myosin heavy chain (αMHC) promoter were bred with floxed Glp1r mice [12]. Cre-induced inactivation of the Glp1r gene was carried out via 6 intraperitoneal (i.p.) injections of tamoxifen (50 mg/kg) over 8 days (Supplementary Figure 1). As induction of Cre in cardiac myocytes induces a transient, reversible cardiomyopathy [13], mice were allowed 5 weeks to recover before experimentation.

2.2. Permanent left anterior descending (LAD) coronary artery occlusion
Experimental MI was induced via permanent ligation of the LAD coronary artery in 10–12-week-old male Glp1r+/− mice and Glp1r+/+ littermates, or 16–20-week-old Glp1r−/− mice and their αMHC-Cre littermates as described [14]. Cardiac examinations were performed on all deceased mice. The presence of a large amount of blood or clot around the heart and in the thoracic cavity, in addition to a perforation of the infarct or peri-infarct area was indicative of cardiac rupture.

2.3. Experimental cardiomyopathy
Experimental cardiomyopathy was induced via single i.p. injection of doxorubicin (20 mg/kg) in Glp1r+/− mice and Glp1r+/+ littermates, or in C57BL/6J mice [15]. Mice were followed for 10 days and hearts from surviving mice underwent histological assessment, or analysis of gene and protein expression.

2.4. Treatment with GLP-1R agonists
In subsets of experiments involving experimental MI or cardiomyopathy, groups of mice were treated with either liraglutide (30 μg/kg i.p. twice daily, Novo Nordisk), the GLP-1R agonist, exendin-4 (5 nmol/kg i.p. twice daily, CHI Scientific), or saline. All injections took place between 7:00—8:00 am and 4:00—5:00 pm. To assess consequences of GLP-1R activation before induction of ischemia, liraglutide was administered twice daily for 1 week before MI [14]. To assess the effects of activating the GLP-1R following induction of ischemia or cardiomyopathy, exendin-4 injections were initiated concurrent with induction of MI or cardiomyopathy.

2.5. Histology and assessment of left ventricle (LV) infarct scar formation
Animals were anesthetized using avertin (250 mg/kg i.p. injection). The chest was opened and an apical injection of 1 M KCl arrested the heart in diastole. Hearts were perfusion-fixed with 4% buffered formalin at physiological pressure, post-fixed in formalin, embedded in paraffin, sectioned at 6 μm, and stained with Masson’s Trichrome or hematoxylin and eosin (H&E). Cardiac morphometry was performed with Aperio ImageScope Viewer software (Aperio Technologies) using digital planimetry [14,16]. Infarcted/scarred LV area was calculated as a % of total LV area. Cardiac hypertrophy was quantified as the heart weight-to- or ventricular weight-to-body weight or tibia length ratio. LV atrial natriuretic peptide (ANP) expression was determined via immunohistochemistry utilizing anti-ANP (Santa Cruz) antibody.

2.6. Assessment of heart rate (HR) via telemetry
HR was assessed in conscious, freely moving mice via implantation of radiotelemetry devices (PA-C10 from DSI) as previously described [10]. Mice were allowed 1 week to recover following device implantation prior to data collection. In some experiments, mice were injected with liraglutide (30 μg/kg i.p.) or saline, or subjected to fasting—refeeding.

2.7. Determination of plasma ANP
Plasma ANP levels were quantified using commercially available enzyme-linked immunosorbent assays (ELISA) (Ray Bio, USA) [10].

2.8. Glucose tolerance
Oral and i.p. glucose tolerance tests were performed in fasted mice using a glucose dose of 2 g/kg. During i.p. glucose tolerance testing, mice were injected with either exendin-4 (24 nmol/kg), or saline, 15 min prior to glucose injection.

2.9. Plasma insulin
Plasma was collected from mice via tail bleed at 15 min post-glucose administration during both oral and i.p. glucose tolerance, and plasma insulin levels were determined by ELISA (Alpco Diagnostics).

2.10. Real time quantitative PCR
First-strand cDNA was synthesized from total RNA using the SuperScript III synthesis system (Invitrogen, Carlsbad, CA). Real time PCR was carried out with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative mRNA transcript levels were quantified with the 2−ΔΔCt method [17] using cyclophilin as an internal control. PCR primers are shown in Supplementary Table 1.

2.11. Immunoblot analysis
Frozen powdered ventricular tissue (20 mg) was homogenized as described [18] and following gel electrophoresis, immunoblotting was carried out using antibodies listed in Supplementary Table 2. Immuno- blots were visualized with the enhanced chemiluminescence Western blot detection kit (Perkin Elmer) and quantified with Carestream Molecular Imaging Software (Kodak).
2.12. Statistical analysis
All values are presented as mean ± SE (n observations). The significance of differences was determined by a Kaplan Meier survival analysis, an unpaired 2-tailed Student’s t-test, a two-way analysis of variance (ANOVA), or a one-way ANOVA followed by a Bonferroni post-hoc analysis where appropriate. Differences were considered significant when \( P < 0.05 \).

3. RESULTS

3.1. Glp1r\(^{-/-}\) mice do not exhibit enhanced susceptibility to ischemia-induced mortality or experimental cardiomyopathy
Although the cardiovascular consequences of pharmacological activation of the GLP-1R have been extensively studied [8], little is known about the endogenous physiological importance of basal GLP-1R signaling for the response to ventricular injury. We first backcrossed Glp1r\(^{-/-}\) mice, originally on a CD1 background [11,19], for 6 generations to the C57BL/6 background, and observed normal cardiac structure in C57BL/6 Glp1r\(^{-/-}\) mice (Supplementary Figure 2). To determine whether loss of basal GLP-1R signaling impairs the response to cardiac injury, Glp1r\(^{-/-}\) and littermate Glp1r\(^{+/+}\) mice were subjected to permanent occlusion of the LAD coronary artery. Although results with Glp1r\(^{-/-}\) mice trended towards increased mortality, these differences were not statistically significant (Figure 1A). Levels of Tnfr and Ccl2 mRNA transcripts were reduced, whereas Gdf5 expression was increased in ventricular RNA from Glp1r\(^{-/-}\) mice (Supplementary Figure 3), however no differences in LV infarct scar formation or cardiac hypertrophy were detected (Figure 1B and C).

As GLP-1R agonists ameliorate the severity of experimental and clinical ventricular dysfunction [4,20,21], we assessed whether loss of basal GLP-1R signaling modified outcomes in mice with doxorubicin-induced cardiomyopathy. Glp1r\(^{-/-}\) mice exhibited no differences in survival following doxorubicin administration (Figure 1D). Although the extent of cardiac atrophy was attenuated (Figure 1E) and expression of inflammation-associated genes such as Tnfr, Ccl2, Hmox1, and Tgf\(\beta\)2 was reduced in ventricles from Glp1r\(^{-/-}\) mice (Supplementary Figure 4), plasma levels of ANP (Figure 1F), LV ANP expression (Figure 1G), and levels of Nppa and Nppb mRNA (Figure 1H) were similar in Glp1r\(^{-/-}\) vs. Glp1r\(^{+/+}\) mice. Hence, whole body deletion of the Glp1r does not impair the adaptive response to ischemic or cardiomyopathic ventricular injury.

3.2. Systemic GLP-1R activation with exendin-4 does not modify cardiovascular outcomes following induction of ischemia or experimental cardiomyopathy
Treatment of rodents with GLP-1R agonists prior to induction of ischemia produces robust cardioprotection [14], however it remains unclear whether GLP-1R activation commenced after induction of the injury affected the outcomes.

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**Figure 1**: Whole body GLP-1R deficiency does not influence cardiovascular outcomes following LAD-ligation induced MI and Doxorubicin-induced cardiomyopathy. A: Survival in Glp1r\(^{-/-}\) mice and their Glp1r\(^{+/+}\) littermates over 9 days following permanent LAD coronary artery occlusion (n = 9). B: % LV infarct scar formation from Masson’s Trichrome-stained heart sections from Glp1r\(^{-/-}\) and wild-type littermates at day 9 post-LAD ligation (n = 6). C: Cardiac hypertrophy at day 9 post-LAD ligation (n = 6–8). D: Survival in Glp1r\(^{-/-}\) and Glp1r\(^{+/+}\) littermates following treatment with a single dose of doxorubicin (20 mg/kg, i.p. injection) (n = 14–15). E: Heart weight/tibia length ratios at 10 days post-doxorubicin (n = 4–5). F: Plasma ANP levels 4 days post-doxorubicin treatment (Sham, n = 3, Doxorubicin, n = 5–4). G: Histology for ANP expression in LV from sham and doxorubicin-treated mice: H: Nppa and Nppb mRNA expression (normalized to PpiA) in ventricles (n = 3–5). Values represent mean ± SE. The significance of differences was determined by a two-way ANOVA followed by a Bonferroni post-hoc analysis.*significantly different from sham counterpart.
ischemic injury is similarly beneficial in rodents in vivo [8]. Importantly, exendin-4 (exenatide) has been administered to human subjects after the onset of ischemic injury or MI, with promising or indeterminate results [5,7,23]. Hence, we asked whether administration of exendin-4 to mice with ischemic cardiac injury might be similarly cardioprotective. Treatment of C57BL/6J mice with exendin-4 for 1 week starting after LAD coronary artery ligation (5 nmol/kg i.p. twice daily), a dose that improved glucose tolerance in separate groups of mice (data not shown), but did not perturb body weight or random fed glycemia, (Supplementary Figure 5) did not improve survival (Figure 2A), nor improve MI-induced LV remodeling, as evidenced by similar LV infarct scar formation and MI-induced cardiac hypertrophy (Figure 2B and C).

As both native GLP-1 and GLP-1R agonists such as exendin-4 have yielded variable outcomes when administered in experimental models and clinical trials of heart failure [6,21,23,24], we asked whether exendin-4 would improve outcomes in mice with experimental cardiomyopathy. Treatment of C57BL/6J mice with exendin-4 for 1 week did not improve survival following doxorubicin-induced cardiomyopathy (Figure 2D), nor the extent of cardiac atrophy (Figure 2E). In contrast, the increase in LV Nppa mRNA transcripts in saline-treated mice was absent in exendin-4-treated mice (Figure 2F). Systemic exendin-4 treatment also reduced ventricular inflammatory gene expression (Il1β and Hmox1) 48 h post-LAD coronary artery occlusion (Supplementary Figure 6A), whereas exendin-4 had less robust effects on ventricular or atrial inflammatory gene expression profiles in the setting of experimental cardiomyopathy (Supplementary Figure 7A). Despite localization of Gip1r expression to cardiac atria [10], exendin-4 did not produce major changes in levels of atrial mRNA transcripts after LAD coronary artery occlusion (Supplementary Figure 6B), or following doxorubicin administration (Supplementary Figure 7B). Hence, systemic GLP-1R activation produces modest changes in cardiac gene expression but does not modify outcomes after ischemic or cardiomyopathic ventricular injury in WT mice.

### 3.3. Mice with cardiomyocyte-specific inactivation of the Gpl1r (Gip1r<sup>CM</sup><sup>−/−</sup>) have normal cardiac structure

As Gip1r<sup>−/−</sup> mice may exhibit developmental or compensatory adaptive metabolic changes that could indirectly influence their response to cardiac injury [25–27], we generated mice with inducible inactivation of the Gip1r in cardiac myocytes (Gip1r<sup>CM</sup><sup>−/−</sup>; Supplementary Figure 1). Tamoxifen-induction of Cre expression resulted in ~90% reduction in atrial Gip1r mRNA expression with no change in lung or pancreas Gip1r mRNA expression in Gip1r<sup>CM</sup><sup>−/−</sup> mice (Figure 3A). Oral glucose tolerance (Figure 3B) was normal and the glucoregulatory actions of exendin-4 (Figure 3C and D) were preserved in Gip1r<sup>CM</sup><sup>−/−</sup> mice, consistent with selective inactivation of the Gip1r in cardiomyocytes. Cardiac structure (5 weeks after the last tamoxifen injection) was normal (Figure 3E–G) indicating that selective reduction of Gip1r expression in adult cardiomyocytes does not produce unexpected changes in cardiac chamber development or glucose homeostasis.

### 3.4. Gip1r<sup>CM</sup><sup>−/−</sup> mice do not exhibit enhanced mortality or adverse LV remodeling after MI

To determine whether selective loss of the cardiomyocyte GLP-1R impaired cardiomyocyte gene or protein expression or the response to ischemic injury, we induced experimental MI by coronary artery ligation in Gip1r<sup>CM</sup><sup>−/−</sup> mice. Viable ventricular myocardium adjacent to the site of infarct from Gip1r<sup>CM</sup><sup>−/−</sup> mice 48 h post-LAD coronary artery occlusion revealed few differences in expression of proteins previously implicated in GLP-1R-dependent cardioprotection, with the exception of heme-oxygenase 1 expression and S' AMP activated protein kinase phosphorylation, which were lower in Gip1r<sup>CM</sup><sup>−/−</sup> mice (Figure 4).

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**Figure 2:** Systemic GLP-1R activation does not influence cardiovascular outcomes following LAD-ligation induced MI and Doxorubicin-induced cardiomyopathy. A: Survival in C57BL/6J mice treated with saline (PBS) or exendin-4 (5 nmol/kg BW i.p., twice daily) for 9 days following permanent LAD coronary artery occlusion (n = 9–11). B: % LV infarct scar formation in heart sections analyzed at day 9 post-LAD ligation (n = 6–7). C: Cardiac hypertrophy analyzed at day 9 post-LAD ligation (n = 8–9). D: Survival following treatment with a single dose of doxorubicin (20 mg/kg i.p. injection) (n = 10–20). Mice were injected for 7 days with saline (PBS) or exendin-4 (5 nmol/kg BW i.p. injection twice daily) with the first injection taking place concurrently with the doxorubicin treatment. E: Heart weight/tibia length ratios at 10 days post-doxorubicin (n = 4–6). F: Nppa and Nppb mRNA expression (normalized to P0sd in ventricles from saline- and exendin-4 treated C57BL/6J mice at 10 days post-doxorubicin treatment (n = 4–5). Values represent mean ± SE. The significance of differences was determined by a two-way ANOVA followed by a Bonferroni post-hoc analysis. *significantly different from sham counterpart.
Similarly, RNA analyses did not reveal major differences in ventricular expression of key genes involved in inflammation, extracellular matrix remodeling, or natriuresis in Glp1r<sup>CM-/-</sup> mice, other than an exacerbation of the MI-induced increase in Ccl2, Mmp9, and Nppb mRNA expression (Supplementary Figure 8A–C). In contrast, we observed significant differential expression of genes important for inflammation (Il-6), extracellular matrix remodeling (Mmp9, Timp1), and natriuresis (Npnb), and attenuation of MI-induced increases in Il1b, Ccl2, Hmox1, and Gdf15 (Supplementary Figure 8D–F) in atrial RNA from Glp1r<sup>CM-/-</sup> hearts 48 h post-LAD coronary artery occlusion. Despite multiple chamber-specific differences in mRNA and protein expression, Glp1r<sup>CM-/-</sup> mice exhibited no alterations in survival following LAD coronary artery occlusion (Figure 5A). Ventricular ANP expression, an indirect indicator of heart failure was not dysregulated (Figure 5B), and LV remodeling, assessed by cardiac hypertrophy (ventricular weight/body weight ratios), LV infarct scar formation, LV chamber diameter, and LV wall thinning (Figure 5C–G) were also similar in Glp1r<sup>CM-/-</sup> vs. αMHC-Cre control littermate hearts. Hence selective loss of the cardiomyocyte GLP-1R results in significant changes in cardiac gene expression but has minimal impact on outcomes and the adaptive remodeling response after experimental MI.

3.5. The cardiomyocyte GLP-1R is not required for the cardioprotective actions of liraglutide

We previously demonstrated that GLP-1R activation with liraglutide prior to MI produced robust cardioprotection in wild-type mice [14]. To determine whether cardioprotective effects of systemic GLP-1R agonists are mediated through the cardiomyocyte GLP-1R, we treated Glp1r<sup>CM-/-</sup> mice and their αMHC-Cre littermates with liraglutide for 1 week (Figure 6A) with a dose (30 μg/kg i.p. twice daily) that does not induce weight loss [14] (Supplementary Figure 9). Unexpectedly, cardioprotection with liraglutide was as potent in Glp1r<sup>CM-/-</sup> mice as in αMHC-Cre littermates (Figure 6B). Furthermore, liraglutide improved cardiac hypertrophy (ventricular weight/body weight ratios), and reduced LV infarct scar formation and LV wall thinning to a similar extent in Glp1r<sup>CM-/-</sup> vs. αMHC-Cre mice (Figure 6C–G). These findings demonstrate that cardiomyocyte GLP-1R activity is not essential for physiological or pharmacological cardioprotective responses engaged by GLP-1R signaling.
Figure 4: Analysis of candidate cardioprotective proteins in hearts from Glp1r<sup>Cμ/Cμ</sup> mice following MI. A: Akt phosphorylation, B: Glycogen synthase kinase 3β (GSK3β) phosphorylation, C: 5′ AMP activated protein kinase (AMPK) phosphorylation, D: Heme oxygenase-1 (HO-1) expression, E: Nuclear respiratory factor 2 (Nrf2) expression, and F: Peroxisome proliferator activated receptor β (PPARβ) expression in ventricles (viable myocardium) extracted from Glp1r<sup>Cμ/Cμ</sup> mice and their αMHC-Cre littermates 48 h post-LAD ligation (n = 3—4). Values represent mean ± SE. The significance of differences was determined by a two-way ANOVA followed by a Bonferroni post-hoc analysis. *significantly different from sham counterpart. #significantly different from corresponding αMHC-Cre littermates.

Figure 5: Cardiovascular outcomes following ischemic injury in Glp1r<sup>Cμ/Cμ</sup> mice. A: Survival in Glp1r<sup>Cμ/Cμ</sup> mice and control littermates following permanent LAD coronary artery occlusion (n = 14). B: Histology of LV cross sections for ANP expression in Glp1r<sup>Cμ/Cμ</sup> mice and their αMHC-Cre littermates at day 28 post-LAD coronary artery occlusion. C: Cardiac hypertrophy (ventricular weight/body weight) in Glp1r<sup>Cμ/Cμ</sup> mice and control littermates at day 28 post-LAD coronary artery occlusion (n = 4—9). D: Representative Masson’s Trichrome stained heart sections depicting, E: % LV infarct scar formation, F: LV internal diameter (LVID), and G: LV infarct wall thickness in Glp1r<sup>Cμ/Cμ</sup> mice and their αMHC-Cre littermates at day 28 post-LAD coronary artery occlusion (n = 4—5). Values represent mean ± SE.
3.6. The cardiomyocyte GLP-1R controls basal HR

As the GLP-1R is predominantly expressed in the atria, and not the ventricle, of rodents and primates [10,28,29], we asked whether atrial GLP-1R expression, perhaps in a subset of pacemaker cells [29], might be important for the pharmacological or physiological control of HR. As GLP-1R agonists increase HR in rodents and humans [8], we assessed HR after acute administration of liraglutide. Despite marked reduction in atrial Glp1r expression, Glp1r−/− mice remained equally sensitive to acute liraglutide-induced increases in HR, compared to responses measured in αMHC-Cre control littermates (Figure 7A–D). As levels of GLP-1 rise in the postprandial state, and HR increases following food ingestion [30], we asked whether cardiomyocyte GLP-1R signaling transduces a component of the meal-stimulated increase in HR. Although HR increased briskly in control mice after refeeding, the HR response to refeeding was similar in Glp1r−/− mice (Figure 7E). Finally, we asked whether loss of cardiomyocyte Glp1r expression might affect basal HR. Assessment of HR in freely moving conscious mice via radiotelemetry revealed a significant reduction in basal HR in Glp1r−/− mice (Figure 7F). Hence, while the atrial GLP-1R is not required for the acute chronotropic response to liraglutide or refeeding, selective loss of GLP-1R signaling in cardiomyocytes disrupts the normal control of HR in mice.

4. DISCUSSION

Our results provide multiple new insights that redefine the pharmacology and physiology of GLP-1R-dependent actions in the cardiovascular system. First, complete loss of GLP-1R activity in Glp1r−/− mice has no impact on cardiovascular outcomes after experimental MI or cardiomyopathy. Second, selective disruption of the cardiomyocyte GLP-1R produces alterations in atrial gene expression after experimental MI, however cardiac structure, left ventricular remodeling, infarct size, and survival are not perturbed in Glp1r−/− mice. Third, although clinical studies suggest GLP-1R agonists such as exenatide may ameliorate ischemic cardiac injury when administered after the onset of ischemia, we did not observe improvement in outcomes following exendin-4 administration to mice after LAD coronary artery ligation. Fourth, despite putative benefits of GLP-1R agonists in heart failure [8], whole body loss of Glp1r expression does not modify outcomes after induction of cardiomyopathy with doxorubicin, and activating GLP-1R signaling produced no improvement in outcomes in doxorubicin-treated mice. Fifth, although the cardioprotective actions of liraglutide in mice require the GLP-1R [10,14], cardioprotection with liraglutide in mice with experimental MI is independent of the cardiomyocyte GLP-1R. Sixth, whereas the cardiomyocyte GLP-1R is not required for the increase in HR following a) refeeding and b) liraglutide administration, basal HR is significantly lower in Glp1r−/− mice.
eliminate the possibility that germline inactivation of the Glp1r is associated with subtle defects due to developmental compensation, we defined key cardiovascular phenotypes in Glp1r^-/- mice with conditional inactivation of the Glp1r in adult mice. Notably, the response to coronary artery occlusion was similar in Glp1r^-/- mice and their αMHC-Cre littermate control mice. Hence, the highly concordant data from Glp1r^-/- and Glp1r^-/- mice clearly demonstrate that loss of the cardiomyocyte GLP-1R does not modify the susceptibility to experimental cardiac injury.

The lack of physiological importance of the endogenous cardiomyocyte GLP-1R in the setting of ischemia or doxorubicin-induced cardiomyopathy was surprising given evidence demonstrating robust cardioprotective properties of degradation-resistant GLP-1R agonists [14,31–34]. Greatly complicating interpretation of the existing literature are reports illustrating cardioprotective actions of native GLP-1, which may act through the known GLP-1 receptor, or through GLP-1R-independent pathways via generation of GLP-1(9–36) or GLP-1(28–36) [4,32,35,36]. Importantly, data generated using native GLP-1 or GLP-1(9–36) in the cardiovascular system cannot be inferred to be relevant to mechanisms activated by degradation-resistant GLP-1R agonists [8]. Indeed the available genetic evidence using Glp1r^-/- mice demonstrates that the key metabolic and cardiovascular actions of exenin-4 and liraglutide are mediated through the known GLP-1R [10,11,14,37].

A unifying explanation for our results and published literature may lie in the demonstration [10,28,29,38], that the cardiac GLP-1R is localized through the known GLP-1R [10,11,14,37]. The demonstration [10,28,29,38], that the cardiac GLP-1R is localized through the known GLP-1R [10,11,14,37].
defective ANP responses following coronary artery occlusion or doxorubicin-induced cardiomyopathy. Thus, while activation of atrial GLP-1R signaling induces ANP secretion in hypertensive mice, the increase in ANP levels during progression of MI and heart failure is normal in the absence of a functional GLP-1R.

Multiple studies demonstrate that systemic administration of native GLP-1 or exenatide [5,7,39] or GLP-1 infusion directly into the coronary circulation produces cardioprotection [32,40]. Under some scenarios, ANP itself exerts cardioprotective actions [41]. Surprisingly however, the GLP-1R agonist liraglutide continues to exhibit robust cardioprotection following coronary artery occlusion in Glp1r<sup>+/−</sup> mice, indicating that the atrial GLP-1R is not required for GLP-1R agonist-mediated cardioprotection in vivo.

Our current data necessitates reassessment of how GLP-1R agonists exert their cardioprotective actions [8,9,36]. It seems likely that GLP-1R-dependent cardioprotection in vivo arises through indirect mechanisms, perhaps through effects on metabolism, or changes in neural transmission or blood flow. Evidence for the possible importance of indirect metabolic changes arises from studies in rats treated with albiglutide and subjected to ischemia-reperfusion injury; these hearts exhibited increased myocardial carbohydrate oxidation and decreased fatty acid oxidation [42], a metabolic profile associated with improved efficiency of contractile function and consistent with indirect mechanisms transduced through elevations in plasma insulin and activation of the cardiac insulin receptor. Thus, we focused our initial studies in normoglycemic non-diabetic Glp1r<sup>−/−</sup> mice for several reasons. First, the development of hyperglycemia is associated with multiple metabolic and cardiovascular abnormalities [43], which may be partially corrected by administration of GLP-1R agonists, confounding attribution of direct vs. indirect mechanisms. Second, the cardioprotective actions of GLP-1R agonists are preserved in normoglycemic and diabetic mice and humans [14,44]. Third, GLP-1R agonists markedly increase insulin secretion under conditions of hyperglycemia, which may indirectly activate myocardial signaling pathways [43]. Indeed, we observed a significant increase in Akt/GSK3β phosphorylation in ventricular extracts from Glp1r<sup>+/−</sup> mice treated with a much higher 200 μg/kg dose of liraglutide, consistent with increased insulin secretion and activation of myocardial insulin signaling pathways (Supplementary Figure 10). Notably, these effects were absent in Glp1r<sup>+/−</sup> mice and restored in Pdx1-hGLP1R:Glp1r<sup>−/−</sup> mice (Supplementary Figure 10) previously shown to exhibit selective restitution of GLP-1R agonist-induced insulin secretion [45].

Our studies in normoglycemic animals raise important questions as to whether systemic GLP-1R activation will similarly confer protection against ischemic injury in obese, hyperglycemic and hyperinsulinemic Glp1r<sup>−/−</sup> mice. Although actions of GLP-1R agonists to increase insulin and enhance cardiac glucose uptake, or engage neural circuits regulating cardiovascular function are important considerations [8,46], these mechanisms would not explain the direct cardioprotective actions ascribed to GLP-1R agonists in ischemia-reperfusion studies ex vivo [8]. Hence, we hypothesize that GLP-1R signaling in cardiac blood vessels, perhaps in endothelial cells or smooth muscle cells, may also contribute in part to modulation of blood flow and cardioprotection. Indeed, Richards et al. used expression of a fluorescent reporter protein under control of endogenous Glp1r regulatory sequences to localize reporter expression within ventricular blood vessels in cells that also co-expressed smooth muscle actin [38]. A vascular target for GLP-1 action in the heart would also be consistent with studies demonstrating that GLP-1 increases microvascular blood volume and microvascular muscle blood flow in rats [47] and recruits cardiac muscle microvasculature in healthy humans [48]. Furthermore, GLP-1 enhances both mesenteric and coronary blood flow [32,49]. Although the actions of native GLP-1 on blood vessels may potentially be ascribed to GLP-1(9–36), the degradation-resistant GLP-1R agonist exenatide robustly increased myocardial blood flow in human subjects with T2D [50]. Hence it seems likely that clinically utilized degradation-resistant agonists such as exenatide, liraglutide, and lixisenatide, may modulate myocardial blood flow through the known GLP-1R, however whether such increases in blood flow arise independent of changes in heart rate is difficult to ascertain.

The localization of the GLP-1R to sinoatrial nodal cells in primates [29], raises the possibility that GLP-1R agonists increase HR through direct activation of atrial pacemaker cells. Nevertheless, we did not observe differences in the extent of HR elevation following liraglutide administration in control vs. Glp1r<sup>+/−</sup> mice. The mechanisms underlying GLP-1R-dependent increases in HR in rodents are complex, and involve integration of neural signals from both the sympathetic and parasympathetic nervous system [51,52]. Hence, it may not be surprising that GLP-1R agonists exemplified by liraglutide remain capable of increasing HR in mice despite marked attenuation of atrial cardiomyocyte GLP-1R signaling. Nevertheless, our findings reveal an important role for basal cardiomyocyte GLP-1R signaling in regulation of chronotropic activity as we observed a significant reduction in baseline HR in Glp1r<sup>+/−</sup> mice. Hence future studies should aim to elucidate how atrial GLP-1R activity provides signals that integrate with neural and cardiac mechanisms linked to overall control of HR in vivo.

In summary, although GLP-1R agonists produce multiple indirect and direct cardioprotective actions in the cardiovascular system, complete whole body inactivation of the GLP-1R in Glp1r<sup>+/−</sup> mice, or selective reduction of cardiac GLP-1R expression in Glp1r<sup>+/−</sup> mice does not impair the physiological response to experimental cardiac injury. Moreover, GLP-1R agonists still induce potent cardioprotection despite ablation of cardiomyocyte GLP-1R activity. Furthermore, although the cardiomyocyte GLP-1R is not required for increases in HR following systemic GLP-1R activation, basal HR is significantly reduced in Glp1r<sup>+/−</sup> mice. Our findings reorient the field towards future studies of a) indirect mechanisms linking GLP-1R activation in non-cardiomyocyte cell types to robust cardioprotection and enhancement of ventricular function and b) the importance of atrial GLP-1R signaling for control of HR, in both pre-clinical and clinical studies.

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**Author details**

JRU, LLB, JEC, EEM, MK, MGK, XC, JB carried out experiments, analyzed data and contributed to writing of manuscript. DAS, RJS, and DJD planned experiments, analyzed data and contributed to writing of manuscript. Daniel J. Drucker is the primary person (guarantor) taking responsibility for the contents of the article.
CONFLICT OF INTEREST

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molmet.2014.04.009.

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