Exendin-4 attenuates high glucose-induced cardiomyocyte apoptosis via inhibition of endoplasmic reticulum stress and activation of SERCA2a

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Younce CW, Burmeister MA, Ayala JE. Exendin-4 attenuates high glucose-induced cardiomyocyte apoptosis via inhibition of endoplasmic reticulum stress and activation of SERCA2a. Am J Physiol Cell Physiol 304: C508–C518, 2013. First published January 9, 2013; doi:10.1152/ajpcell.00248.2012.—Hyperglycemia-induced cardiomyocyte apoptosis contributes to diabetic cardiomyopathy. Glucagon-like peptide-1 (Glp1) receptor (Glp1r) agonists improve cardiac function and survival in response to ischemia-reperfusion and myocardial infarction. The present studies assessed whether Glp1r activation exerts direct cardioprotective effects in response to hyperglycemia. Treatment with the Glp1r agonist Exendin-4 attenuated apoptosis in neonatal rat ventricular cardiomyocytes cultured in high (33 mM) glucose. This protective effect was mimicked by the cAMP inducer forskolin. The Exendin-4 protective effect was blocked by the Glp1r antagonist Exendin(9-39) or the PKA antagonist H-89. Exendin-4 also protected cardiomyocytes from hydrogen peroxide (H2O2)-induced cell death. Cardiomyocyte protection by Exendin-4 was not due to reduced reactive oxygen species levels. Instead, Exendin-4 treatment reduced endoplasmic reticulum (ER) stress, demonstrated by decreased expression of glucose-regulated protein-78 (GRP78) and CCAT/enhancer-binding homologous protein (CHOP). Reduced ER stress was not due to activation of the unfolded protein response, indicating that Exendin-4 directly prevents ER stress. Exendin-4 treatment selectively protected cardiomyocytes from thapsigargin- but not tunicamycin-induced death. This suggests that Exendin-4 attenuates thapsigargin-mediated inhibition of the sarco/endoplasmic reticulum Ca2+ ATPase-2a (SERCA2a). High glucose attenuates SERCA2a function by reducing SERCA2a mRNA and protein levels, but Exendin-4 treatment prevented this reduction. Exendin-4 treatment also enhanced phosphorylation of the SERCA2a regulator phospholamban (PLN), which would be expected to stimulate SERCA2a activity. In sum, Glp1r activation attenuates high glucose-induced cardiomyocyte apoptosis in association with decreased ER stress and markers of enhanced SERCA2a activity. These findings identify a novel mechanism whereby Glp1-based therapies could be used as treatments for diabetic cardiomyopathy.

glucagon-like peptide-1; hyperglycemia; heart

DIABETIC CARDIOMYOPATHY, a unique pathology occurring independently of coronary artery disease and hypertension, is a major complication that increases morbidity and mortality in diabetic individuals (52, 56). Currently, there is no single effective treatment for diabetic cardiomyopathy (56). An understanding of the molecular mechanisms that lead to diabetic cardiomyopathy will uncover novel therapeutic interventions for heart failure in diabetic patients.

Glucagon-like peptide-1 (Glp1) is a gut-secreted hormone identified for its ability to stimulate insulin secretion in response to oral nutrient intake (9). This incretin hormone also exerts cardioprotective effects in response to myocardial infarction (MI) and ischemia-reperfusion injury (1, 33, 35, 46, 55). These effects are mediated at least in part by a Glp1 receptor (Glp1r) expressed in cardiomyocytes (1). Several observations support a pleiotropic role for Glp1r agonists on the cardiovascular system (35). The Glp1r agonist Exendin-4 reduces infarct area and myocardial stiffness in pigs undergoing left circumflex coronary artery occlusion followed by 3 days of reperfusion (55). Liraglutide, another Glp1r agonist, reduces infarct size and improves cardiac output in mice undergoing left anterior descending artery ligation (33). In a canine model of dilated cardiomyopathy, Glp1 infusion improved left ventricular ejection fraction (LVEF) (32). LVEF is improved in patients with chronic heart failure receiving Glp1 compared with standard therapy (45). Whether activation of the cardiomyocyte Glp1r can attenuate cardiac dysfunction specifically associated with diabetic cardiomyopathy is unknown.

Fatty acid-induced and hyperglycemia-induced cardiomyocyte loss, whether by apoptosis or necrosis, contributes to the pathophysiological progression of the diabetic heart (10, 14, 26, 44, 49, 59). Glp1r activation attenuates palmitate and ceramide-induced apoptosis in HL-1 atrial cardiomyocytes (39). It is unclear whether this effect extends to hyperglycemia-induced cardiomyocyte apoptosis. Hyperglycemia-induced cardiomyocyte apoptosis is associated with increased reactive oxygen species (ROS) production and subsequent endoplasmic reticulum (ER) stress (27, 59). ER stress activates the unfolded protein response (UPR), a set of signaling pathways that halt cellular processes such as protein translation and that upregulate expression of chaperone proteins to restore normal ER function. However, when chronically activated, some UPR pathways can lead to autophagy and cell death (16, 40, 58, 59). Glp1r activation decreases ER stress in pancreatic β-cells in response to both hyperglycemia and hyperlipidemic challenge (6, 60) and prevents ER stress-induced cell death in pancreatic microendothelial cells (13). This raises the possibility that Glp1r activation could protect cardiomyocytes from hyperglycemia-induced apoptosis via a reduction in ER stress.

Here we provide evidence that Glp1r activation in cardiomyocytes attenuates high glucose-induced cardiomyocyte death by preventing ER stress. This was not associated with enhanced activation of the UPR. Surprisingly, Glp1r activation also did not reduce ROS levels. These results suggest that Glp1r activation attenuates high glucose-induced apoptosis by directly preventing ER stress. Studies using chemical ER stressors demonstrated that cardiomyocyte protection by Glp1r activation is associated with markers of enhanced sarco/endoplasmic reticulum Ca2+ ATPase-2a (SERCA2a) activity. The present study uncovers a novel mecha-
nism for the cardioprotective effects mediated by the Glp1r in response to hyperglycemic stress.¹

MATERIALS AND METHODS

Neonatal cardiomyocyte isolation. The experimental procedures were approved by the Animal Care and Use Committee of the Sanford-Burnham Medical Research Institute at Lake Nona and conformed to the regulations in Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). Neonatal rat ventricular myocytes (NRVM) were isolated from hearts of 2- to 3-day-old CD rats (Charles River) using trypsin and collagenase digestive enzymes. Rat pups were euthanized by decapitation, hearts were removed surgically, and ventricular cardiomyocytes were prepared using a neonatal cardiomyocyte isolation kit (Warnington). Cardiomyocytes were preplated for 2 h in DMEM supplemented with 15% FBS containing appropriate antibiotics to reduce nonmyocyte contamination and then plated (1.5 × 10⁶ cells) in six-well plates incubated at 37°C and 5% CO₂ in humidified atmosphere.

Cell culture and treatments. NRVM were grown in modified DMEM (10% FBS, 1% penicillin, and 1% streptomycin) in 5 mmol/l D-glucose for 48 h following isolation. Cells were then incubated in either low glucose (5 mmol/l D-glucose) or high glucose (33 mmol/l D-glucose) in the presence or absence of either Exendin-4 (30 nmol/l) or forskolin (1 μmol/l) or with mannitol (33 mmol/l) in the presence or absence of Exendin-4 (30 mmol/l) for 24–48 h. In some experiments, cells were treated with 300 mmol/l of the Glp1r antagonist Exendin(9-39) or the cAMP-dependent protein kinase (PKA) inhibitor H-89 (10 μmol/l) for 30 min before incubation with Exendin-4. In other experiments, cardiomyocytes were treated under low-glucose conditions with 250 μmol/l hydrogen peroxide (H₂O₂) to induce oxidative stress or 300 mmol/l thapsigargin (Sigma) or 1 μg/ml tunicamycin (Sigma) to directly induce ER stress.

Cell death assessment. Cell viability and death were measured by Trypan Blue (Hyclone) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Promega) assays using manufacturer instructions. For Trypan Blue, 1 × 10⁵ cells were collected and treated with a 1:1 dilution of Trypan Blue. After 2 min, cells were counted on a hemocytometer. TUNEL experiments were performed in four-well chamber slides, and cells were seeded at 0.5 × 10⁶ cells/well before experimentation. Cells were then assessed macroscopically.

ROS measurements. ROS production was evaluated fluorometrically using dihydrorhodamine 123 (DHR123). Cells were treated with 10 μmol/l DHR123 for 15 min at 37°C and 5% CO₂. Cells were washed 3× with 1× PBS. Cells (5 × 10⁵) were then subjected to fluorometric analysis (excitation: 488 nm; emission: 535 nm).

RT-PCR. Total RNA was isolated using High Pure RNA isolation kit (Roche). First-strand cDNA was synthesized using 1 μg of total RNA (DNase treated) using a RT cDNA synthesis kit (High Capacity, kit (Roche). First-strand cDNA was synthesized using 1

¹This article is the topic of an Editorial Focus by Yumei Ye and Yochai Birnbaum (57a).

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>C36B4</td>
<td>5'-CACCTTCCCATCCGTTGAA-3'</td>
<td>5'-TGCTGCCAGTTTCTGTGTC-3'</td>
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<tr>
<td>CHOP</td>
<td>5'-TCTTGCCTTCCCTTCGAGACA-3'</td>
<td>5'-TGCAGGGTGCAAGTATGGACTG-3'</td>
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<td>GRP78</td>
<td>5'-ATATCGGAGGTGGGCAAACCAAGA-3'</td>
<td>5'-TGTTAAGTCCAGCAATCTGATGTG-3'</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>5'-ATGTGCTATTCTGCTGAGTTG-3'</td>
<td>5'-CATAGGGTTAGTTCCAGTAAA-3'</td>
</tr>
<tr>
<td>XBP-1</td>
<td>5'-TTACGAGAGAAATCCTATGCGG-3'</td>
<td>5'-GGGTGCAGATTGCTCCAGATGGC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GGGTCCAATGTTGCTCCAGAATGC-3'</td>
<td>5'-GGGTGAAGTCCAGATGGC-3'</td>
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Real-time PCR was performed using SsoFast Evagreen Supermix (Bio-Rad). For analysis of X-box binding protein-1 (XBP-1), PCR was performed with Tera PCR Direct Red Dye Premix (Clontech). PCR products were run on a 2.5% agarose gel to increase band separation. Primers for all PCR protocols are listed in Table 1.

Immunoblot analysis. NRVM and left ventricles were treated with cell lysis buffer [0.5% NP40, 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, 10 μl/ml protease inhibitor cocktail (Sigma P8340), and 10 μl/ml phosphatase inhibitor cocktail (Sigma)], and protein samples were collected and subjected to immunoblot analysis using polyclonal antibodies specific for glucose-regulated protein 78 (GRP78) (1:1,000, Cell Signaling), CCAT/enhancer-binding homologous protein (CHOP; 1:250, Cell Signaling), growth arrest and DNA damage-inducible protein 34 (GADD34) (1:1,000, Santa Cruz), phospho-eukaryotic initiation factor 2α (elF2α) (1:1,000, Cell Signaling), elF2α (1:1,000, Cell Signaling), SERCA2a (1:1,000, Cell Signaling), phospho-lamban (PLN) (1:1,000, Cell Signaling), phospho-PLN (S16/T17; 1:500, Cell Signaling), cysteine-aspartic protease 3 (caspase 3; 1:1,000, Cell Signaling), and β-actin (1:2,000, Cell Signaling). Immunoblots were normalized to β-actin expression.

Statistical analysis. Data were analyzed using Graphpad statistical software (SPSS) and are presented as means ± SE. Groups were compared using ANOVA analysis followed by either a Bonferroni or a Tukey post test. Differences were considered significant at P < 0.05.

RESULTS

Exendin-4 attenuates high glucose-induced cardiomyocyte apoptosis via the Glp1r and activation of PKA. To test the hypothesis that Glp1r activation directly attenuates high glucose-induced cardiomyocyte death, NRVM were incubated in high glucose (33 mmol/l) in the presence or absence of Exendin-4 (10 or 30 mmol/l). Exendin-4 treatment attenuated high glucose-induced apoptosis assessed by Trypan Blue staining and TUNEL assay (Fig. 1A, B). This was blocked by pretreatment with the Glp1r antagonist Exendin(9-39), demonstrating that the antiapoptotic effect of Exendin-4 is mediated via the Glp1r (Fig. 1A). Exendin-4 had no significant effect on the basal level of cell death observed under control (5 mmol/l glucose) conditions (Fig. 1A). Cells were also incubated in 33 mmol/l mannitol to account for osmolarity effects on cell viability. Exendin-4 had no significant effect on mannitol-induced death (Fig. 1A). These results demonstrate that the antiapoptotic effects of Exendin-4 directly target glucose toxicity. Exendin-4 inhibited high glucose-induced activation of proapoptotic caspase 3 as indicated by a decrease in cleaved caspase 3 products at 6, 12, and 24 h, respectively (Fig. 1C). Interestingly, Exendin-4 reduced high glucose-induced protein levels of cleaved caspase 3 below control levels at 6 and 24 h (Fig. 1C). However, Exendin-4 did not affect cleavage of caspase 3 under low-glucose conditions (Fig. 1D). Taken together, these results demonstrate that Glp1r activation attenuates high glucose-induced cardiomyocyte apoptosis.
Glp1r activation stimulates cAMP formation and subsequently induces PKA activity in cardiomyocytes (57). To determine whether increasing cAMP levels attenuates high glucose-induced cardiomyocyte death, NRVM were incubated in high glucose in the presence or absence of the adenylyl cyclase activator forskolin. Like Exendin-4, forskolin treatment attenuated high glucose-induced apoptosis (Fig. 1E).

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verse the cardioprotective effects of Exendin-4. H-89 blunted the attenuation of cardiomyocyte death by Exendin-4 in response to high glucose (Fig. 1E), suggesting that the protective effects of Glp1r activation on high glucose-induced apoptosis are mediated at least in part via PKA activation.

Exendin-4 attenuates cardiomyocyte cell death without reducing ROS levels. In cardiomyocytes, hyperglycemia induces oxidative stress, a trigger for apoptosis (44). To test the hypothesis that Glp1r activation attenuates high glucose-induced cardiomyocyte apoptosis by reducing oxidative stress, ROS levels were measured under low glucose and high glucose conditions in the presence or absence of Exendin-4. ROS levels increased in cardiomyocytes cultured in high glucose and peaked at 12 h posttreatment (Fig. 2A). Contrary to our hypothesis, Exendin-4 did not reduce ROS levels in cells incubated in high glucose. This suggests that attenuation of high glucose-induced apoptosis by Exendin-4 occurs downstream of oxidative stress or via an alternative pathway.

To confirm that Glp1r activation exerts cardioprotective effects independent of a reduction in oxidative stress, cardiomyocytes were incubated under low-glucose conditions in the presence of the direct oxidative stressor H2O2. Cell death was increased by 250 μmol/l H2O2 to a degree similar to that observed in response to high glucose, albeit within a shorter time frame (4 h) compared with high glucose (48 h; Fig. 2B). Exendin-4 also reduced cell death in response to H2O2 but did not reduce ROS levels (Fig. 2C). This further supports the hypothesis that Glp1r activation attenuates cardiomyocyte apoptosis independently of reduced oxidative stress.

Exendin-4 attenuates high glucose-induced ER stress without reducing oxidative stress. Oxidative stress induces ER stress (31), and hyperglycemia-induced ER stress in cardiomyocytes results from increased oxidative stress (44, 59). Prolonged ER stress stimulates apoptosis (61). Thus, Exendin-4 could attenuate high glucose-induced cell death by reducing ER stress. To test this, levels of the proapoptotic ER stress marker CHOP were measured in NRVM incubated in high glucose in the presence or absence of Exendin-4. CHOP transcript levels increased dramatically within 1 h of incubation in high glucose, but Exendin-4 reduced this effect (Fig. 3A). CHOP protein levels were significantly elevated after 24 h of high glucose treatment, but this was also attenuated by Exendin-4 (Fig. 3B). Protein levels of the ER stress-induced chaperone GRP78 were also elevated in high-glucose-treated cardiomyocytes, but Exendin-4 reduced this effect (Fig. 3B). Pretreatment with the Glp1r antagonist Exendin(9-39) reversed the effect of Exendin-4 on high glucose-induced GRP78 protein levels (Fig. 3C), demonstrating that suppression of ER stress by Exendin-4 is mediated via the Glp1r.

To verify the hypothesis that Glp1r activation attenuates ER stress downstream of oxidative stress, ER stress markers were measured in response to H2O2 treatment in the presence of Exendin-4. As shown in Fig. 3D, Exendin-4 reduced the expression of ER stress markers GRP78 and PDI. Given our observation that Exendin-4 failed to reduce ROS levels in response to either high glucose or H2O2, the reduction of ER stress markers by Exendin-4 supports the hypothesis that cardioprotective effects of Glp1r activation are mediated by attenuation of ER stress downstream of oxidative stress.

Exendin-4 does not enhance activation of the UPR. ER stress activates three branches of the UPR—protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1α (IRE1α)—to resolve the source of ER stress (61). We tested the hypothesis that Exendin-4-induced Glp1r activation reduces ER stress markers (Fig. 3) by enhancing activation of the UPR. PERK phosphorylates eIF2α, halting most protein translation. PERK also activates activating transcription factor 4 (ATF4), which induces CHOP expression. CHOP stimulates expression of the eIF2α phosphatase GADD34, thus completing a feedback mechanism utilized by PERK to regulate eIF2α and, therefore, protein translation. If the ER stress is not sufficiently resolved, then sustained CHOP expression promotes apoptosis. Incubating cardiomyocytes in high glucose increased phosphorylation of eIF2α and expression of GADD34 at 0.5 and 1 h, indicating a rapid but transient activation of the PERK arm of the UPR (Fig. 4, A and B). Contrary to our hypothesis, Exendin-4 prevented the activation of the PERK arm in high-glucose condi-

Fig. 2. Glp1r activation attenuates high glucose- and H2O2-induced cardiomyocyte cell death without reducing oxidative stress. A: NRVM were incubated in 5 mmol/l glucose or 33 mmol/l glucose in the presence or absence of 30 nmol/l Exendin-4. At 1, 2, 6, 12, and 24 h after treatment, cellular reactive oxygen species (ROS) production was assessed via fluorometric analysis using dihydrorhodamine 123 (DHR123: excitation: 488 nm; emission: 535 nm). *P < 0.05 vs. LG. B: NRVM were incubated in 5 mmol/l glucose with and without 250 μmol/l H2O2 in the presence or absence of 30 nmol/l Exendin-4. After 4 h, cells were microscopically assessed for death using Trypan Blue. *P < 0.05 vs. control (no H2O2 or Ex4); #P < 0.05 vs. H2O2. C: cellular ROS production was assessed in NRVM incubated in 5 mmol/l glucose with and without 250 μmol/l H2O2 in the presence or absence of 30 nmol/l Exendin-4 at 0, 0.5, 1, 2, and 4 h after treatment. *P < 0.05 vs. control (no H2O2 or Ex4). Data are presented as means ± SE for 3 separate experiments.
Treatment with Exendin-4 did not attenuate this effect. These results indicate that neither high glucose nor Glp1r activation alters ATF6 or IRE1α activation in cardiomyocytes. Taken together, our results indicate that Glp1r activation does not attenuate high glucose-induced cardiomyocyte apoptosis by enhancing the induction of the UPR.

**Glp1r activation protects cardiomyocytes from thapsigargin-induced cell death but not tunicamycin-induced cell death.** Results obtained thus far suggest that Glp1r activation protects cardiomyocytes from apoptosis at a step downstream of oxidative stress but upstream of the UPR. This raises the possibility that Glp1r activation directly prevents ER stress, thus negating the need to activate the UPR. To test this, ER stress was directly induced in cardiomyocytes with thapsigargin, a SERCA2a inhibitor, and tunicamycin, an N-linked glycosylation inhibitor (30, 47). In response to thapsigargin, Exendin-4 attenuated cardiomyocyte apoptosis (Fig. 6A, left). Similar to the effects observed in response to high glucose (Fig. 3),...
Exendin-4 attenuated the thapsigargin-induced increase in the ER stress markers CHOP and GRP78 (Fig. 6, B–D). Contrasting the results obtained with thapsigargin, Exendin-4 did not protect cardiomyocytes from tunicamycin-induced cell death (Fig. 6A, right). These results suggest that Exendin-4 protects cardiomyocytes from apoptosis by directly blocking ER stress. Furthermore, the selective protection against thapsigargin-induced apoptosis suggests that Glp1r activation prevents ER stress associated with impaired SERCA2a function.

Glp1r activation prevents high-glucose impairment of SERCA2a function in cardiomyocytes. The fact that Exendin-4 attenuates cell death in response to the chemical SERCA2a inhibitor thapsigargin is significant to the present studies since high glucose also impairs cardiomyocyte SERCA2a function (3, 5, 12, 50). This suggests that Exendin-4 could also protect cardiomyocytes from high glucose-induced apoptosis by enhancing SERCA2a activity. SERCA2a function is dictated in part via its association with the sarco/endoplasmic reticulum membrane protein PLN. Phosphorylation of PLN at Ser\(^1\) enhances SERCA2a activity and is necessary for a second activating phosphorylation at Thr\(^1\) by Ca\(^2\+)/calmodulin-dependent protein kinase II (CAMKII; 24). We tested the hypothesis that Exendin-4 stimulates PLN phosphorylation. Exendin-4 treatment transiently enhanced levels of phosphorylated PLN (normalized to total PLN) in cardiomyocytes cultured in high glucose compared with untreated cells cultured in either high or low glucose (Fig. 7A). Interestingly, high glucose alone briefly stimulated PLN phosphorylation (Fig. 7A). SERCA2a function is also regulated by its expression levels (24). We show that SERCA2a mRNA (Fig. 7B) and protein levels (Fig. 7C) are decreased in cardiomyocytes cultured in high glucose, but this effect is prevented by Exendin-4. Taken together, these results suggest that cardiomyocyte Glp1r activation may protect cardiomyocytes from high glucose-induced cell death by enhancing SERCA2a activity.

**DISCUSSION**

The present studies show that Glp1r activation exerts cytoprotective effects against high glucose-induced apoptosis in cultured cardiomyocytes. Hyperglycemia-induced cardiomyocyte apoptosis is associated with the development of diabetic cardiomyopathy (26, 44), and attenuation of hyperglycemia-induced cardiomyocyte cell death prevents the progression of diabetes-associated cardiac complications (4). Although the glucose-lowering properties of Glp1r agonists provide beneficial effects against hyperglycemia-induced cardiac damage, the present findings demonstrate that cardiomyocyte Glp1r activa-
Exendin-4 attenuates glucose-induced cardiomyocyte apoptosis

Fig. 5. Exendin-4 reduces high glucose-induced activation of the activating transcription factor 6 (ATF6) branch of the UPR. NRVM were treated with 5 mmol/l glucose, 33 mmol/l glucose, or 33 mmol/l glucose with 30 nmol/l Exendin-4. RNA was isolated at 1, 6, and 12 h following treatment and assessed using a RT-PCR protocol that can detect unspliced X-box binding protein-1 (uXBP-1) and spliced XBP-1 (sXBP-1). Data are presented as fold change in uXBP-1 and sXBP-1 normalized to β-actin from 1 h. *P < 0.05 vs. LG; †P < 0.05 vs. HG; †P < 0.05 vs. HG + Ex4. Data are presented as means ± SE for 6 separate experiments.

Exendin-4 reduces high glucose-induced activation of the activating transcription factor 6 (ATF6) branch of the UPR. NRVM were treated with 5 mmol/l glucose, 33 mmol/l glucose, or 33 mmol/l glucose with 30 nmol/l Exendin-4. RNA was isolated at 1, 6, and 12 h following treatment and assessed using a RT-PCR protocol that can detect unspliced X-box binding protein-1 (uXBP-1) and spliced XBP-1 (sXBP-1). Data are presented as fold change in uXBP-1 and sXBP-1 normalized to β-actin from 1 h. *P < 0.05 vs. LG; †P < 0.05 vs. HG; †P < 0.05 vs. HG + Ex4. Data are presented as means ± SE for 6 separate experiments.

Induction of ER stress is a downstream mechanism by which hyperglycemia-induced oxidative stress results in cardiomyocyte death (59). We show that Glp1r activation attenuates the expression of ER stress markers in response to high glucose and H2O2. This suggests that the cardioprotective effects of Glp1r activation are mediated via attenuation of deleterious effects downstream of oxidative stress, including ER stress. Since Exendin-4 did not ameliorate an inducer of ER stress (i.e., oxidative stress), we tested the hypothesis that Exendin-4 enhances the activation of mechanisms that relieve ER stress. The UPR is a set of signaling pathways activated as a means to resolve the initial cause of ER stress (48, 61). While acute activation of the UPR is cytoprotective, prolonged activation of the UPR initiates a proapoptotic pathway (48). High glucose activated the PERK arm of the UPR, but surprisingly, Glp1r activation prevented this activation. Indeed, the pattern of UPR protein expression in cardiomyocytes cultured in high glucose with Exendin-4 was equivalent to that observed in cardiomyocytes cultured under normoglycemic conditions. High glucose-induced phosphorylation of eIF2α and increased CHOP and GADD34 expression were all prevented by Exendin-4. This contrasts a previous study in pancreatic β-cells where Glp1r activation reduced eIF2α phosphorylation as a result of an increased expression of CHOP and GADD34 (60). Our results suggest that in cardiomyocytes, unlike β-cells, Glp1r activation may circumvent the need to activate the UPR altogether. This is consistent with a previous study demonstrating that, in human umbilical vein endothelial cells, activation of Glp1r can prevent high glucose-induced protein expression of the UPR markers GRP78, IRE1α, and PDI (43). In contrast, another study showed that a 1-wk treatment with the Glp1r agonist Liraglutide induces the UPR in the hearts of mice fed a high-fat diet for 32 wk (34). In this latter study, it is possible that activation of the UPR occurred in vascular endothelial cells and not in cardiomyocytes. Alternatively, Glp1r activation may have different effects on UPR activation depending on the manner in which ER stress is induced (i.e., fatty acids vs. glucose).

There was an insignificant tendency for elevated glucose to induce transcript levels of unspliced XBP-1 mRNA, and Glp1r activation prevented this effect. However, there were no significant differences in splicing of XBP-1 mRNA, suggesting that IRE1α activity was not induced under high-glucose conditions. However, a previous study showed that hyperglycemia-induced cardiomyocyte cell death occurs via IRE1α activation of c-Jun NH2-terminal kinase (JNK) (59). Thus we cannot exclude the possibility that IRE1α is still active under hyperglycemic conditions but has a preference for JNK over XBP-1. Under this scenario it is plausible that Glp1r activation prevents JNK activation. This idea is supported by a recent study showing that Glp1 activation attenuates H2O2 induction...
of JNK phosphorylation and nuclear localization in HCP cells (25). In sum, our data demonstrate that Exendin-4 prevents the activation of the UPR in response to high glucose in cardiomyocytes. Taken together with our results showing no decrease in ROS levels, these data suggest that Glp1r activation protects cardiomyocytes from cell death downstream of oxidative stress but upstream of the UPR. One likely scenario is that Glp1r activation directly prevents ER stress itself, thereby rendering activation of the UPR unnecessary.

To test whether Glp1r activation directly blocks ER stress, we assessed cell death in response to the ER stressors thapsigargin and tunicamycin. Exendin-4 attenuated thapsigargin-induced cardiomyocyte death. This was associated with reduced levels of GRP78 and CHOP. Interestingly, it has been reported that hyperglycemia-induced cardiomyocyte apoptosis is due in part to impaired SERCA function (11), the same mechanism of action of thapsigargin.

Rodent models of both type 1 and type 2 diabetes display reduced SERCA2a function, lengthened relaxation times, and impaired contractility (2, 3, 5, 22, 53, 62). In cardiomyocytes, SERCA2a controls the removal of cytosolic Ca$^{2+}$ and the storage of Ca$^{2+}$ in the sarco/endoplasmic reticulum (24). Replenishment of sarco/endoplasmic reticulum Ca$^{2+}$ stores is critical for cardiomyocyte contraction. As SERCA2a activity decreases, cardiomyocyte relaxation becomes slower and subsequent contractions are impaired. SERCA2a activity is determined by its expression levels and also via its association with the sarco/endoplasmic membrane protein PLN, a protein whose phosphorylation enhances SERCA2a function (24). Here we demonstrate that Exendin-4 prevents the decrease in SERCA2a mRNA and protein levels observed in NRVM cultured in high glucose. Furthermore, we show that Exendin-4 stimulates the activating phosphorylation of PLN, an event that would be expected to enhance SERCA2a function. These results propose a novel mechanism by which Glp1r activation enhances cardiomyocyte viability and function via stimulation of SERCA2a. This could potentially counter the deleterious effects of diabetes and its associated hyperglycemia on cardiomyocytes from cell death downstream of oxidative stress but upstream of the UPR. One likely scenario is that Glp1r activation directly prevents ER stress itself, thereby rendering activation of the UPR unnecessary.

Fig. 6. Glp1r activation attenuates thapsigargin (TG)-induced ER stress and cardiomyocyte death but does not prevent tunicamycin (TM)-induced cardiomyocyte death. A: NRVM were incubated in 5 mmol/l glucose and treated with or without the ER stress inducers thapsigargin (300 mmol/l) or tunicamycin (1 μg/ml) in the presence or absence of 30 mmol/l Exendin-4. At 24 h, cells were microscopically assessed for death using Trypan Blue. *P < 0.05 vs. control (no ER stress inducer and no Ex4); #P < 0.05 vs. TG. B-D: protein levels of ER stress markers GRP78 and CHOP were measured at 15, 30, and 60 min following treatment. Data were normalized to β-actin. *P < 0.05 vs. control; #P < 0.05 vs. TG. Data are presented as means ± SE for 3 separate experiments.
Fig. 7. Glp1r activation prevents impairment of sarco(endoplasmic reticulum Ca\(^{2+}\) ATPase-2a (SERCA2a) function by high glucose. NRVM were treated with 5 mmol/l glucose, 33 mmol/l glucose, or 33 mmol/l glucose with 30 nmol/l Exendin-4. A: protein levels of phosphorylated (S16/T17) and total phospholamban (PLN) were measured from cell lysates collected at 1, 2, and 3 h following treatment. Data were normalized to total PLN. B: at 0.5 and 1 h, RNA was isolated and assessed using qRT-PCR for SERCA2a. Data were normalized to C36B4. C: protein levels of SERCA2a were measured from cell lysates collected at 1, 2, and 3 h following treatment. Data were normalized to β-actin. *P < 0.05 vs. LG; #P < 0.05 vs. HG.

Cardiomyocyte SERCA2a function. This effect of Glp1r activation is consistent with a recent study showing that Exendin-4 restores SERCA2a function in insulin-resistant macrophages (28). Further supporting a potential link between Glp1r activation and SERCA2a activity, mice with a functional disruption of the Glp1r exhibit impaired cardiac contractility (17). Impaired SERCA function in response to hyperglycemia is attributed to decreased PKA activity (11). Glp1r activation

Fig. 8. Proposed mechanism by which Glp1r activation attenuates hyperglycemia-induced apoptosis in cardiomyocytes by alleviating ER stress. Left: hyperglycemia leads to decreased SERCA2a functionality that is associated with decreased PKA activity. The ensuing dysregulation of Ca\(^{2+}\) homeostasis leads to ER stress. Subsequently, the UPR is activated to correct the increased load of misfolded proteins. If prolonged, the UPR will initiate signaling events that commit the cardiomyocyte to apoptosis. Right: Glp1r activation with the agonist Exendin-4 attenuates hyperglycemia-induced apoptosis in a cAMP-PKA-dependent manner. Glp1r activation results in increased protein expression of SERCA2a and phosphorylation of PLN, suggesting improved SERCA2a functionality. Activation of Glp1r decreases ER stress and prevents the UPR from being activated. We propose a model whereby Glp1r activation attenuates hyperglycemia-induced cardiomyocyte apoptosis in a cAMP-PKA-dependent manner by improving SERCA2a function thus increasing Ca\(^{2+}\) homeostasis and concomitantly relieving ER stress.
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stimulates cardiomyocyte cAMP levels and PKA activity (57). This provides a potential mechanism by which Exendin-4 stimulates SERCA2a function and subsequently attenuates ER stress-induced apoptosis in high glucose-treated cardiomyocytes. Supporting this, the antiapoptotic effects of Exendin-4 are blocked by the PKA inhibitor H-89. We also show that the adenylyl cyclase activator forskolin mimics the cardioprotective effects of Exendin-4 under high-glucose conditions. However, we cannot exclude the possibility that the protective effects of Exendin-4 are mediated by other signaling proteins since H-89 inhibits protein kinases other than PKA (29). Furthermore, elevations in Glp1r-mediated increases in cAMP also activate cAMP-regulated guanine nucleotide exchange factors such as Epac that also play a role in regulating Ca^{2+} homeostasis (19).

The current studies expand the cardioprotective role of Glp1r activation to include a beneficial effect in response to hyperglycemic damage. Our data propose that Glp1r activation attenuates hyperglycemia-induced cardiomyocyte apoptosis by reducing oxidative stress-induced ER stress. Furthermore, we identify SERCA2a as a novel target that may play an important role in the cardioprotective effects mediated by Glp1r agonists (Fig. 8). There is currently no single effective treatment available for the management of diabetic cardiomyopathy. Our findings expand the potential benefits of Glp1r-based drugs in the context of diabetes-associated heart disease and suggest that Glp1r agonists may provide salutary effects on cardiac function beyond glycemic control.

Limitations. Although isolated NRVM are commonly used for studying cardiac dysfunction, their neonatal properties may not represent physiological responses observed in adult cardiomyocytes. The advantage of NRVM is that they require a less demanding isolation procedure and provide greater yields than adult cardiomyocytes (42). Nevertheless, future experiments in isolated adult cardiomyocytes and in vivo will be required to provide further evidence of the mechanisms mediating the cardioprotective effects of Glp1r activation in diabetic cardiomyopathy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
C.Y., M.A.B., and J.E.A. conception and design of the research; C.Y. and M.A.B. performed the experiments; C.Y., M.A.B., and J.E.A. analyzed the data; C.Y., M.A.B., and J.E.A. interpreted the results of the experiments; C.Y. prepared the figures; C.Y., M.A.B., and J.E.A. edited and revised the manuscript; C.Y., M.A.B., and J.E.A. approved the final version of the manuscript.

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