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Protection of pancreatic β-cells against glucotoxicity by short-term treatment with GLP-1

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) reduces pancreatic β-cell apoptosis in type 2 diabetes. Glucotoxiciy is a main cause of β-cell apoptosis in type 2 diabetes. The aims of this study were to investigate the anti-apoptotic mechanisms of GLP-1 against glucotoxicity and whether physiological short-term treatment with GLP-1 can protect β-cells from glucotoxicity-induced apoptosis. GLP-1 treatment for only 30 min alleviated high glucose-induced β-cell apoptosis. The effect of GLP-1 was related with phosphoinositide 3-kinase (PI3K)/AKT-S473 phosphorylation. The increase in pAKT-S473 led to suppression of FoxO-1. GLP-1-induced AKT-S473 activation and FoxO-1 suppression were abolished by the selective inactivation of mTOR complex (mTORC) 2 using small interfering RNA directed towards the rapamycin-insensitive companion of mTOR. The protective effect of GLP-1 on β-cell apoptosis was also abolished by the selective inactivation of mTORC2. Hence, the protective effect of GLP-1 against glucotoxicity may be mediated by FoxO-1 suppression through the PI3K/mTORC2/AKT-S473 phosphorylation. This report provides evidence that short-term treatment with GLP-1 is beneficial to protect against glucotoxicity-induced β-cell apoptosis.

1. Introduction

Deficient pancreatic β-cell mass has been proposed as a factor contributing to the insulin secretory defect in type 2 diabetes. Animal and human studies have found that the decrease in β-cell mass is mainly via apoptosis [1–3]. A considerable body of evidence has suggested that glucose, the main regulator of insulin biosynthesis and insulin secretion, exerts negative effects on β-cell viability as well as β-cell function when present in excessive amounts over a prolonged period. This effect is termed glucotoxicity [4–6].

Glucagon-like peptide-1 (GLP-1) is a potent incretin hormone secreted by intestinal L-cells in response to meals [7]. The physiological importance of GLP-1 in glucose homeostasis via potentiation of glucose-stimulated insulin secretion has been elucidated [8]. In addition, animal and human studies have shown that GLP-1 plays a central role in the homeostasis of pancreatic β-cell mass, which is also critical in the adjustment to high blood glucose levels [9]. GLP-1 secretion is decreased in type 2 diabetes and externally-applied GLP-1 prevents β-cell apoptosis in animal models of diabetes [10,11] and glucolipotoxicity [12–14]. The native form of GLP-1 has a biological half-life of only a few minutes (<2 min) due to its very rapid degradation in plasma by dipeptidyl peptidase IV (DPPIV), although significant GLP-1 levels are sustained for 30 min in plasma [15]. Nevertheless, the transient life time of GLP-1 has been debate on its use for the treatment of type 2 diabetes. Numerous studies have suggested that treatment of normal and diabetic rodents with GLP-1 stimulates β-cell proliferation and neogenesis and decreases β-cell apoptosis, which all encourage expansion of β-cell mass [9]. However, the appropriate minimal treatment time and molecular mechanism of GLP-1 to protect β-cells against glucotoxicity via abrogation of apoptosis remains unclear.

Abbreviations: ATF, activating transcription factor; CHOP, C/EBP homologous protein; Epac, exchange protein activated by cAMP; FoxO-1, forkhead transcription factors of the O subclass 1; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GRP, glucose-regulated protein; mTORC, multiprotein complex termed mammalian target of rapamycin complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide; PI3K, phosphoinositide 3-kinase.

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This study investigated whether the physiological 30-min treatment with GLP-1 reduces glucotoxicity-induced pancreatic β-cell apoptosis. The results indicate that GLP-1-induced FoxO-1 suppression, which is essential for reducing glucotoxicity-induced apoptosis, is mediated by activation of mTORC2/protein kinase B (pAKT S473).

2. Materials and methods

2.1. Experimental animals

C57BL/6 (wild-type) mice were purchased from Jung-Ang Experimental Animals (Seoul, Korea). All animal experiments were approved by the Keimyung University Institutional Ethics Committee, Daegu, Korea (KM-2013-35).

2.2. Cell culture condition and isolation of islets

INS-1 cells (passage 20–40) were cultured at 37 °C in a humidified incubator containing 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 11 mM glucose and 50 μM β-mercaptoethanol. Mouse pancreatic islets were isolated by collagenase digestion. To collect dispersed islet cells, the islets were incubated in 1 ml Accutase solution (Innovative Cell Technologies, San Diego, CA, USA) for 15 min at 37 °C. The cells were gently pipetted up and down 15-20 times to promote dissociation. The dispersed islet cells were then cultured in poly-L-lysine coated coverslips in RPMI-1640 medium containing 11.1 mM glucose for 2–3 days for analyses.

2.3. Induction of glucotoxicity

Fluctuation from hypoglycemia to hyperglycemia is a common and important phenomenon in diabetic patients, in whom the fluctuations are more severe than those in healthy individuals [16]. To induce glucotoxicity by fluctuating glucose levels, INS-1 cells and dispersed islet cells were exposed to fluctuating glucose concentrations from 2.5 mM (low glucose) for 1 h to 17 mM (high glucose) for 48 h, and from 5 mM (low glucose) for 1 h to 25 mM (high glucose) for 48 h, respectively. The fluctuation medium was RPMI-1640 containing 1% FBS and 50 μM β-mercaptoethanol at 37 °C. To explore the effect of GLP-1 on glucotoxicity, GLP-1 was treated simultaneously with high glucose. After a 30-min treatment with GLP-1, cells were washed to remove GLP-1 and then exposed to elevated glucose without GLP-1 for 48 h. Cell viability and apoptosis were then analyzed.

2.4. Cell viability assay

Cell confluence was measured using a JuLI™ Br live cell movie analyzer (NanoEnTek, Seoul, Korea) at 0, 6, 12, 24 and 48 h after the high glucose exposure. Cell viability was independently measured using an established *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide* (MTT) assay after 48-h high glucose exposure. INS cells were labeled with MTT solution for 4 h, and the resulting formazan was solubilized with dimethyl sulfoxide. The absorption was measured at 570 nm (620 nm as a reference) using a microplate reader (Bio-Rad, Hercules, CA, USA). Finally, the percentage of viable cells [{(total cells − dead cells)/total cells} × 100] was calculated.

2.5. Fluorescence activated cell sorting (FACS) analysis

After the 48-h exposure to elevated glucose, cells were fixed in 80% ethanol at −20 °C for flow cytometric analysis of DNA content. Ethanol-fixed cells were stained with a propidium iodide (PI) staining solution (50 μg/ml PI, 100 μg/ml RNase A, 0.1% w/v sodium citrate and 0.1% v/v NP-40) for 30 min. Cytometric analysis was performed with a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA) and Cell Quest software.

2.6. Western blot analysis

Cells were treated with high glucose in the presence or absence of GLP-1 for 30 min. Crude cell extracts were obtained as previously described [17]. The protein samples were resolved using 10–12% SDS-PAGE. Antibodies to pAKT S473, pAKT T308, AKT, pFoxO-1 S256, FoxO-1, regulatory-associated protein of mTOR (Raptor), rapamycin-insensitive companion of mTOR (Rictor), p-p70S6kinase (Thr389), p70S6kinase, phosphorylated protein kinase C (pPKC) T638/641, PKC and cleaved caspase 3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Small interfering (si) Raptor and siRictor used to suppress the expression of Raptor and Rictor were obtained from Santa Cruz Biotechnology for the siRNA of each gene. INS-1 cells were transfected with 20 nM siRNA for each gene using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), and further experiments were performed after a 48-h transfection. Scrambled siRNA (Invitrogen) was used for negative controls. The relative band intensities were compared via band scanning using a Gel Doc® XR (Bio-Rad) with Quantity One software, version 4.5.2.

2.7. Quantitative real-time PCR analysis

After the indicated time, total cellular RNA was extracted from INS-1 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was eluted with Rnase-free water. Reverse transcription was performed using the first strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's protocols. Real-time PCR amplification was performed using the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) and the Prism 7500 real-time PCR detection system. Relative amounts of mRNA were normalized by use of the gene encoding glyceraldehyde 3-phosphate dehydrogenase and calculated using the ΔΔCT (cycle threshold) method. The specific primers for activating transcription factor (ATF) 4 were 5′-GTT GGT CAG TGC CTC AGA CA-3′ (forward) and 5′-CAT TCG AAA CAG AGC ATC GA-3′ (reverse). The primers for C/EBP homologous protein (CHOP) were 5′-CCA GCA GAG GTC ACA AGC AC-3′ (forward) and 5′-CGC ACT GAC CAC TCT GTT TC-3′ (reverse). The primers for 78-kDa glucose-regulated protein (GRP78) were 5′-AAC CCA GAT GAG GCT GTA GCA-3′ (forward) and 5′-ACA TCA AGC AGA ACC AGG TCA C-3′ (reverse).

2.8. Statistical analyses

The results are expressed as the mean ± SEM. SPSS version 20.0 (SPSS, Chicago, IL, USA) was used for the statistical analyses. Comparisons between the two groups were performed using a Student's two-tailed *t*-test. For the comparisons of more than two groups, the significance was tested using ANOVA with Bonferroni correction. A p value <0.05 was considered significant.

3. Results

3.1. Short-term treatment with GLP-1 protects β-cells from glucotoxicity-induced apoptosis

To induce glucotoxicity by fluctuating glucose levels, INS-1 cells were pre-exposed to 2.5 mM glucose for 1 h and then exposed to

17 mM glucose (or 11 mM glucose for control) with or without 20 nM GLP-1 for 30 min. GLP-1 was removed by washing and cells exposed to 17 mM glucose for 48 h in the absence of GLP-1 (Fig. 1A). After exposure to high glucose levels for 48 h, cell survival was inhibited by approximately 40% (Fig. 1B and C). Additionally, the high-glucose-induced inhibition of cell survival was significantly alleviated by GLP-1 or the GLP-1R agonist exendin-4 treatment for 30 min, but not insulin or the GLP-1R antagonist exendin-9 (Fig. 1B). The exposure of INS-1 cells to high glucose levels for 48 h resulted in an increased cell population at the sub-G1 phase of the cell cycle; short-term treatment with GLP-1 significantly alleviated this effect (Fig. 1D). Accordingly, glucotoxicity significantly

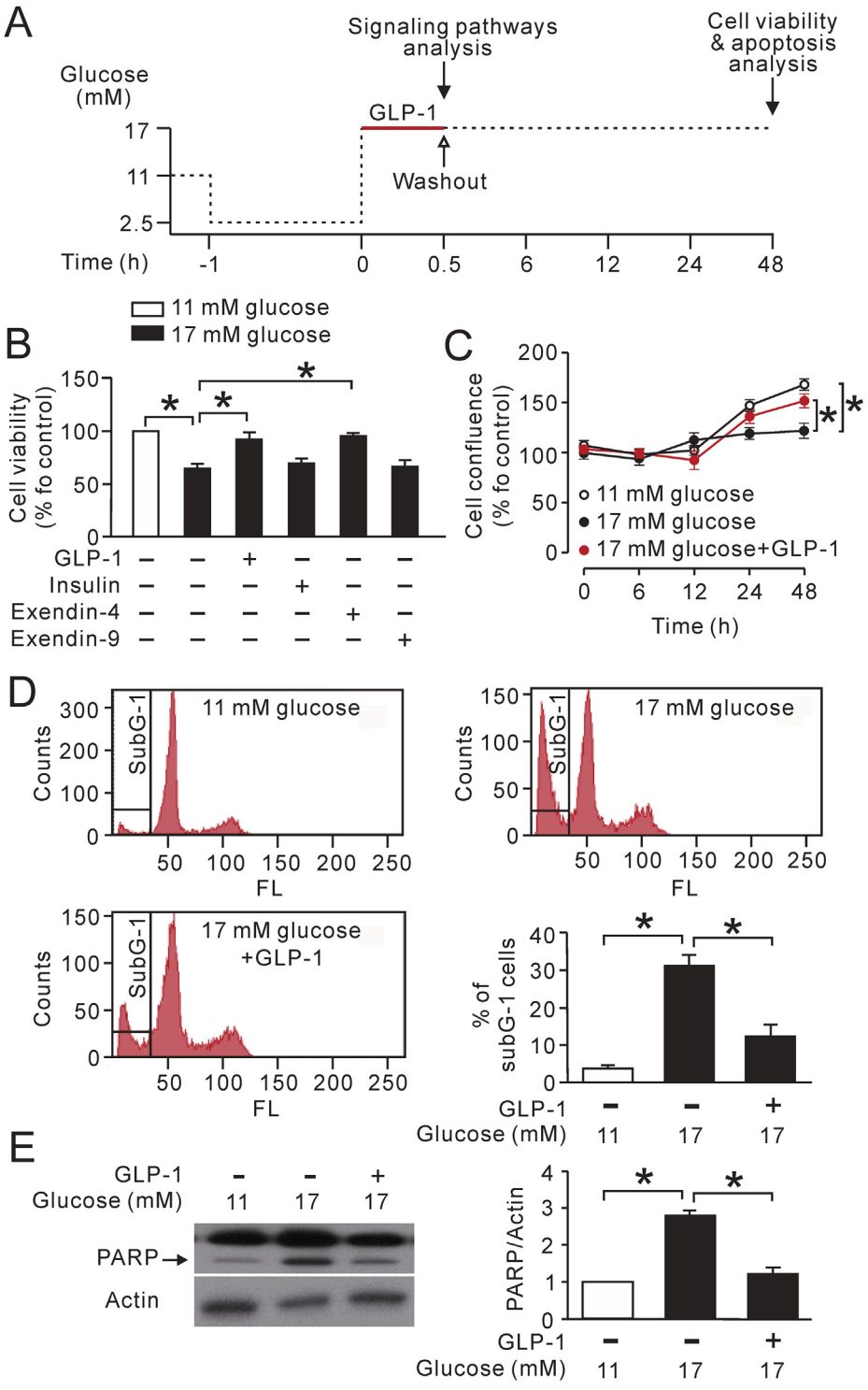

Fig. 1. Effect of short-term treatment with GLP-1 on glucotoxicity-induced apoptosis in pancreatic β-cells. (A) Schematic diagram of the experimental protocols used for the short-term treatment with GLP-1 (20 nM) in INS-1 cells during high glucose exposure. Cell viability was determined using the MTT assay (B) and a live-cell confluence analyzer (C). $n = 8$. (D) The cell cycle distribution was determined using a flow cytometric analysis of DNA content after staining with propidium iodide. $n = 6$. (E) The expression of cleaved PARP was detected by Western blot analysis of whole cell lysates. $n = 3$. $^{*}p < 0.05$.

enhanced the levels of the cleaved form of poly ADP ribose polymerase (PARP) compared with the control group, which was indicative of increased apoptosis. Short-term treatment with GLP-1 also blocked high-glucose-induced cleavage of PARP (Fig. 1E). These results suggest that fluctuating elevated levels of glucose induce β-cell apoptosis, which is attenuated by the short-term treatment with GLP-1.

3.2. Short-term treatment with GLP-1 activates the PI3K/AKT S473/FoxO-1 pathway

Short-term treatment with GLP-1 increased AKT S473 phosphorylation in INS-1 cells. Phosphorylation of AKT T308, another AKT phosphorylation site, was not increased by GLP-1 (Fig. 2A). The effect of GLP-1 on AKT S473 phosphorylation was replicated by exendin-4, but not by exendin-9 (Fig. 2B and C). GLP-1 induced a time-dependent increase in AKT S473 phosphorylation from 5 min after GLP-1 treatment (Fig. 2D). Because both GLP-1 and insulin are well-known activators of phosphoinositide-3-kinase (PI3K) and AKT [19,20], we attempted to determine which was more related to the increase in AKT S473 phosphorylation. The phosphorylation of AKT T308 was significantly increased by insulin, but the phosphorylation of AKT S473 was not changed (Fig. 2E). To confirm the involvement of the PI3K/AKT signaling pathway in the short-term treatment with GLP-1, the phosphorylation of AKT S473 was measured in the presence of various GLP-1/cAMP signaling pathway inhibitors. Pretreatment with the PI3K inhibitor LY294002 blocked the effect of GLP-1 on the phosphorylation of AKT S473, while pretreatment with the non-selective protein kinase A (PKA) inhibitor H-89, adenylate cyclase inhibitor MDL-12330A, epidermal growth factor receptor (EGFR) inhibitor AG1478 or c-Src inhibitor PP-1 did not affect (Fig. 2F–H). Next, to investigate the downstream signaling mechanism following the GLP1-induced PI3K/AKT S473 activation, FoxO-1, p70S6K or AMPK activity was investigated. Short-term treatment with GLP-1 increased FoxO-1 phosphorylation (Fig. 2I), while the phosphorylation of p70S6K or AMPK was

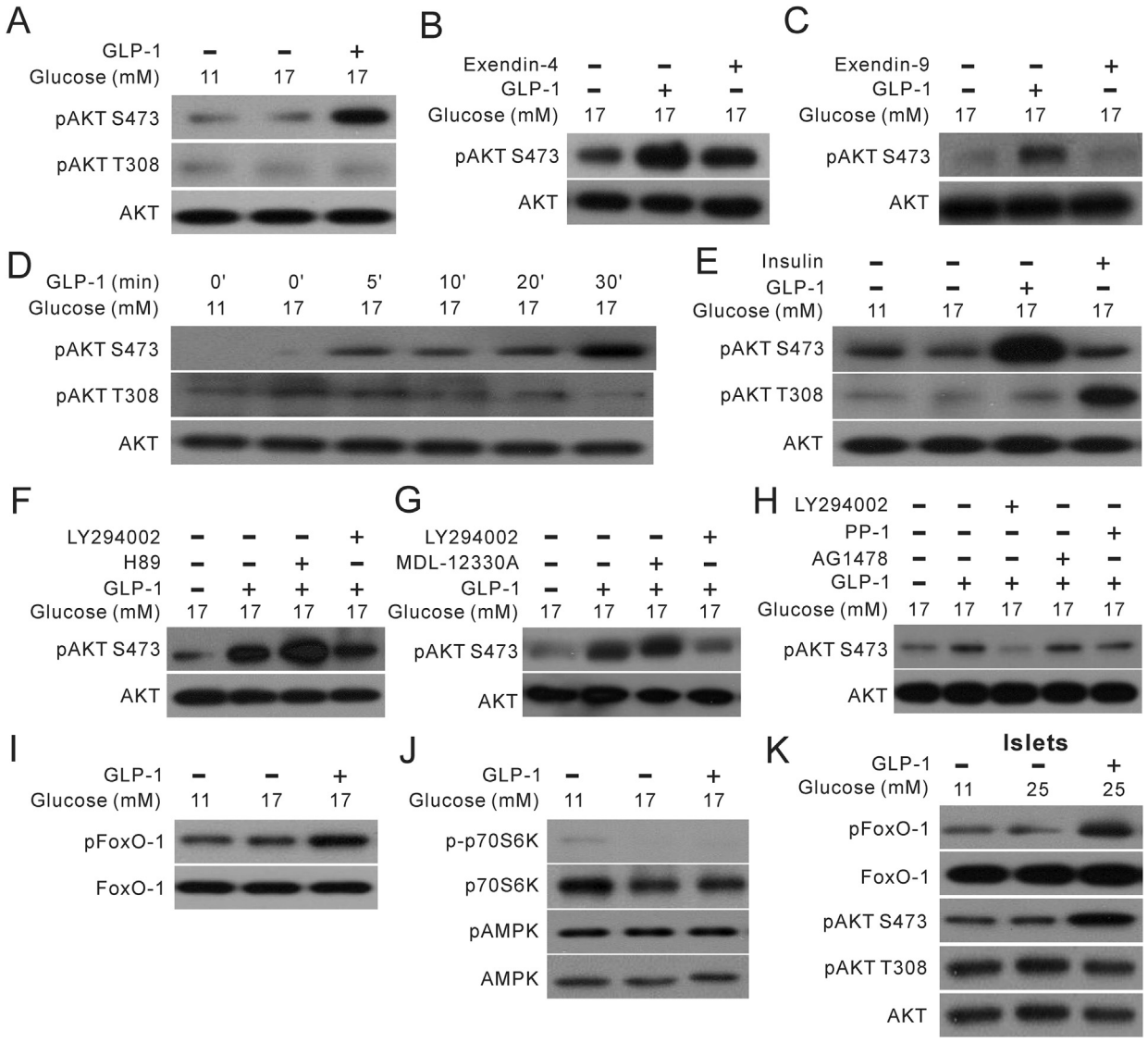

Fig. 2. Involvement of short-term treatment with GLP-1 in the PI3K/AKT S473/FoxO-1 signaling pathway. Cells were treated with high glucose in the presence of GLP-1 (20 nM), exendin-4 (20 nM), exendin-9 (20 nM) or insulin (20 nM) for 30 min. Phosphorylation levels of AKT S473 (A–H), FoxO-1 (I), p70S6K and AMPK (J) were detected by Western blot analysis of INS-1 cell lysates. (K) Phosphorylation levels of AKT S473 and FoxO-1 were detected by Western blot analysis of mouse islet cell lysates. Cells were preincubated for 30 min with the following inhibitors: LY294002 (50 μM); H-89 (5 μmol/l); MDL-12330A (10 μM); PP-1 (300 nM); AG1478 (150 nM).

not changed by GLP-1 (Fig. 2J). These results indicated that the short-term treatment with GLP-1 activates the PI3K/AKT S473/FoxO-1 pathway in INS-1 cells and pancreatic islets (Fig. 2K).

3.3. mTORC2 is required for the increase in AKT S473/FoxO-1 phosphorylation by GLP-1

To examine which mTORC is involved in GLP-1-induced AKT S473/FoxO-1 phosphorylation, we tested the effect of gene knockdown for endogenous mTORC1 or mTORC2 in INS-1 cells (Fig. 3). In INS-1 cells transfected with siRictor for mTORC2 activity inhibition, the increase in AKT S473/FoxO-1 phosphorylation by GLP-1 was significantly reduced (Fig. 3B and D) compared with those transfected with siRaptor for mTORC1 activity inhibition (Fig. 3A and C). Moreover, GLP-1 increased the phosphorylation of PKCα, which is mediated by mTORC2 [21], in siRaptor-transfected INS-1 cells, but the effect of GLP-1 was abolished by siRictor-transfected INS-1 cells (Fig. 3E). To understand the molecular mechanism by which GLP-1 protects glucotoxicity-induced apoptosis, we investigated the relationship between the apoptosis and mTORC2 signaling. The decrease in the cleaved form of caspase 3, a cellular marker of apoptosis, by GLP-1 was significantly inhibited in siRictor-transfected cells compared with siRaptor-transfected INS-1 cells (Fig. 3F and G). These results suggest that mTORC2 activation by GLP-1 plays a key role in alleviating the glucotoxicity-induced apoptosis.

3.4. Prolonged treatment with GLP-1 in high glucose condition diminishes cell viability probably through endoplasmic reticulum (ER) stress

We next examined effects of non-physiological, long-term treatment with GLP-1 against glucotoxicity-induced apoptosis. INS-1 cells were exposed to 17 mM glucose with or without 20 nM GLP-1 for 48 h (Fig. 4A). As expected, high-glucose-induced inhibition of cell survival was further increased by 48-h treatment with GLP-1 (Fig. 4B). It also much potentiated CHOP, ATF4 and GRP78, which were alleviated by the short-term treatment with GLP-1 (Fig. 4C). These results suggest that prolonged treatment with GLP-1 may accelerate β-cell apoptosis by inducing ER stress.

4. Discussion

It has been known that GLP-1 protects human islet cells against glucolipotoxicity through PI3K/AKT/NF-kB pathways [12]. In addition, the involvement of pAKT S473 in GLP-1R-mediated protection from cytokine-induced apoptosis has been demonstrated in INS-1 cells [22]. However, the mechanism of GLP-1 in the reduction of glucotoxicity-induced apoptosis is not well established. In the present study, we demonstrate that the short-term treatment with GLP-1 reduces glucotoxicity-induced apoptosis by suppressing FoxO-1 due to PI3K/mTORC2/pAKT S473 activation in pancreatic β-cells (Fig. 4D). Consistent with our data, FoxO-1/3a is phosphorylated only by mTORC2-dependent AKT S473 phosphorylation [23,24]. Moreover, mTORC2 inactivation in rat islets increases apoptosis to an extent similar to that induced by rapamycin, whereas inactivation of mTORC1 has no significant effect on cell viability [25]. We did not directly examine the activation mechanism that links GLP-1 receptor (GLP-1R) to PI3K, although GLP-1-induced pAKT S473 activation was abolished by LY294002. GLP-1R activation induces PKA- and EGFR-dependent PI3K activation in pancreatic β-cells [26,27]. In contrast, we showed that PKA and EGFR signaling were not related to GLP-1-induced pAKT S473 activation. Given that GLP-1R signaling comprises multiple pathways that involve complicated molecular mechanisms, further studies are required to clarify the molecular mechanisms regulating PI3K activation.

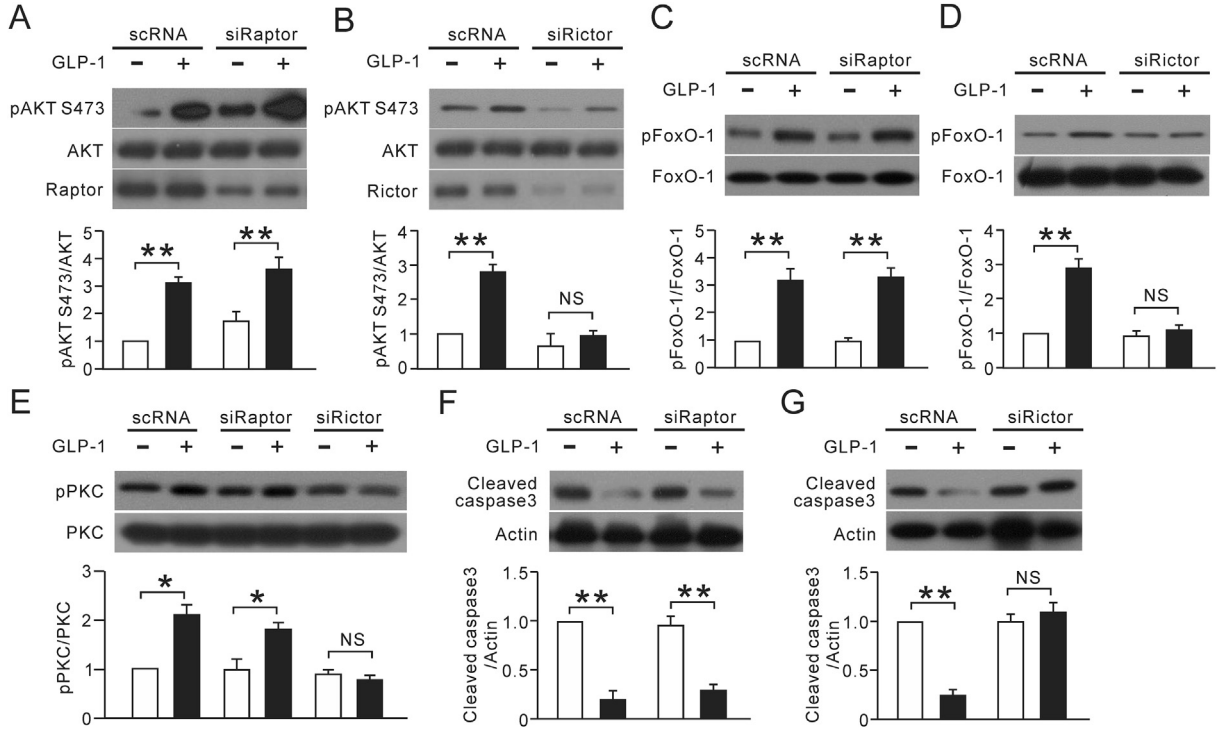

Fig. 3. GLP-1-induced pAKT S473 is related to mTORC2 activation. INS-1 cells were transfected with scrambled siRNA (scRNA), siRaptor or siRictor for 48 h and then treated with high glucose in the presence of GLP-1 (20 nM) for 30 min. The relative ratios of pAKT S473 (A, B), pFoxO-1 (C, D), pPKC (E) and cleaved caspase 3 (F, G) were plotted based on the quantification of band intensity. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$. NS, not significant.

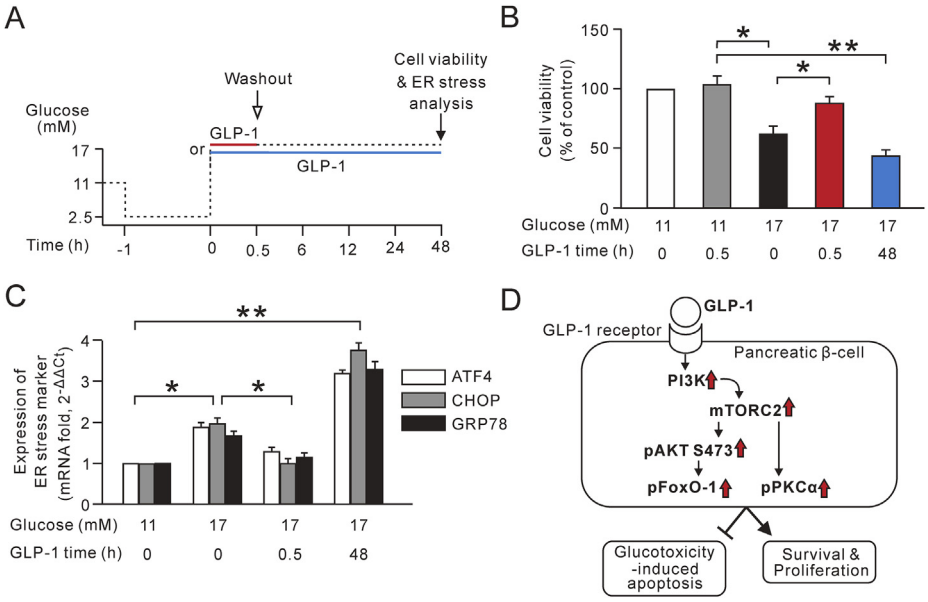

Fig. 4. Effect of prolonged treatment with GLP-1 on glucotoxicity-induced apoptosis in pancreatic β-cells. (A) Schematic diagram of the experimental protocols used for the treatment with GLP-1 (20 nM) in INS-1 cells during high glucose exposure. (B) Cell viability was determined using the MTT assay. (C) The expression of ATF4, CHOP and GRP78 was determined using quantitative real-time PCR analysis in INS-1 cells treated with GLP-1 for 30 min or 48 h. $n = 8$. $^{*}p < 0.05$, $^{**}p < 0.01$; NS, not significant. (D) Proposed mechanism underlying the protective effects of short-term treatment with GLP-1 against glucotoxicity-induced apoptosis in pancreatic β-cells.

The main actions of GLP-1 are to stimulate insulin secretion and to inhibit glucagon secretion, thereby contributing to limitation of postprandial glucose excursions. As well as effects of enhancing insulin secretion, GLP-1R activation also increases insulin synthesis, β-cell proliferation and the protective effects against glucotoxicity [9]. However, concerns have recently been raised about the potential adverse effects of prolonged GLP-1R activation [28,29]. It could be associated with an increased risk of pancreatitis in type 2 diabetics [30]. Moreover, long-term treatment with GLP-1 receptor activators might lead to an increased risk of pancreatic cancer [31,32]. Although GLP-1 has a biological half-life of only a few minutes, plasma GLP-1 remains significantly elevated for 30 min [15,18]. Therefore, we investigated the effect of a 30-min GLP-1 treatment time. Quantitative analysis of glucotoxicity-induced apoptosis confirmed the acute effects of GLP-1 application, which reduced pancreatic β-cell apoptosis under the glucotoxic condition. We further demonstrated that prolonged treatment with GLP-1 for 48 h markedly increased glucotoxicity-induced β-cell apoptosis by increasing ER stress in contrast to short-term treatment with GLP-1.

These results may point the way to an effective treatment strategy for type 2 diabetes that reduces the adverse effects of long-term GLP-1 treatment. The data reveal a crucial role of GLP-1 in anti-apoptosis and its signaling mechanism to protect β-cells from glucotoxicity-induced apoptosis.

Conflict of interest

None.

Acknowledgments

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