

CHOP Deficiency Ameliorates ERK5 Inhibition-Mediated Exacerbation of Streptozotocin-Induced Hyperglycemia and Pancreatic β -Cell Apoptosis

Dae-Hwan Nam^{1,2}, Jung-Hwa Han¹, Jae Hyang Lim³, Kwon Moo Park⁴, and Chang-Hoon Woo^{1,*}

¹Department of Pharmacology and Smart-aging Convergence Research Center, Yeungnam University College of Medicine, Daegu 42415, Korea, ²Predictive Model Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon 34114, Korea, ³Department of Microbiology, Ewha Womans University School of Medicine, Seoul 03760, Korea, ⁴Department of Anatomy, Kyungpook National University School of Medicine, Daegu 41566, Korea

*Correspondence: changhoon_woo@yu.ac.kr

<http://dx.doi.org/10.14348/molcells.2017.2296>

www.molcells.org

Streptozotocin (STZ)-induced murine models of type 1 diabetes have been used to examine ER stress during pancreatic β -cell apoptosis, as this ER stress plays important roles in the pathogenesis and development of the disease. However, the mechanisms linking type 1 diabetes to the ER stress-modulating anti-diabetic signaling pathway remain to be addressed, though it was recently established that ERK5 (Extracellular-signal-regulated kinase 5) contributes to the pathogenesis of diabetic complications. This study was undertaken to explore the mechanism whereby ERK5 inhibition instigates pancreatic β -cell apoptosis via an ER stress-dependent signaling pathway. STZ-induced diabetic WT and CHOP deficient mice were i.p. injected every 2 days for 6 days under BIX02189 (a specific ERK5 inhibitor) treatment in order to evaluate the role of ERK5. Hyperglycemia was exacerbated by co-treating C57BL/6J mice with STZ and BIX02189 as compared with mice administered with STZ alone. In addition, immunoblotting data revealed that ERK5 inhibition activated the unfolded protein response pathway accompanying apoptotic events, such as, PARP-1 and caspase-3 cleavage. Interestingly, ERK5 inhibition-induced exacerbation of pancreatic β -cell apoptosis was inhibited in CHOP deficient mice. Moreover, transduction of adenovirus encoding an active mutant

form of MEK5 α , an upstream kinase of ERK5, inhibited STZ-induced unfolded protein responses and β -cell apoptosis. These results suggest that ERK5 protects against STZ-induced pancreatic β -cell apoptosis and hyperglycemia by interrupting the ER stress-mediated apoptotic pathway.

Keywords: apoptosis, β -cell, CHOP, ER stress, ERK5

INTRODUCTION

The prevalence of diabetes is correlated with that of obesity worldwide and has reached epidemic proportions. Type 1 diabetes (T1D) is a metabolic disease that occurs when the body is unable to produce enough of the hormone insulin, due to the destruction of insulin-producing β -cells in pancreatic islets (Apostolou et al., 2003). Streptozotocin (STZ) is widely used to induce experimental T1D in rodent models (Szkudelski, 2001) and does so by inducing pancreatic β -cell apoptosis (Hayashi et al., 2006; Le May et al., 2006). Also, STZ-induced hyperglycemia has been reported to accelerate endoplasmic reticulum (ER) stress-mediated pancreatic β -cell apoptosis in a murine model (Ahn et al.,

Received 12 September, 2016; revised 14 May, 2017; accepted 6 June, 2017; published online 6 July, 2017

eISSN: 0219-1032

© The Korean Society for Molecular and Cellular Biology. All rights reserved.

© This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>.

2015). In addition, recent studies indicate ER stress plays a crucial role in pancreatic β -cell apoptosis, and have established that ER stress-mediated apoptosis is responsible for initiating β -cell apoptosis in T1D (Eizirik et al., 2008; Oyadomari et al., 2002).

ER stress response is mediated by three sensors, that is inositol-requiring protein-1 α (IRE-1 α), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK), located on the ER membrane. ER stress is transduced by unfolded protein responses (UPRs) initiated by the dissociation of Grp78 from these three ER membrane sensors (Oyadomari and Mori, 2004; Sano and Reed, 2013; Szegezdi et al., 2006). First, activation of IRE1 α causes the recruitments of TRAF2 and ASK1, which lead to the downstream activation of JNK. Second, activation of ATF6 causes its translocation to Golgi where its cytosolic domain is sequentially cleaved by two proteases, S1P and S2P, to produce the active form (Sano and Reed, 2013). Third, PERK activation leads to the phosphorylation of eukaryotic initiation factor 2 (eIF2 α), which inhibits protein synthesis. Phosphorylation of eIF2 α also enables the preferential translations of UPR-dependent genes, such as, activating transcriptional factor 4 (ATF4). The next phase of ATF4-mediated C/EBP homologous protein (CHOP) activation can also induce apoptosis via a transcriptional route (Oyadomari and Mori, 2004; Szegezdi et al., 2006). In particular, chronic and persistent ER stress induces the degenerative ER stress pathway and leads to apoptosis, and a growing number of authors have suggested CHOP is a key regulator of this degenerative pathway. In addition, ER stress is considered to promote the transcription of CHOP (Oyadomari and Mori, 2004; Szegezdi et al., 2006), an important regulator of ER stress-mediated apoptosis and metabolic gene expression (Oyadomari and Mori, 2004). Interestingly, CHOP knock-out mice have been reported to be protected from renal (Lakshmanan et al., 2011), pancreatic (Song et al., 2008), heart (Nam et al., 2015), and liver (Tamaki et al., 2008) dysfunction in murine models of metabolic disease, indicating CHOP deficiency improves pancreatic β -cell survival and ameliorates diabetic complications.

Extracellular-signal-regulated kinase 5 (ERK5; also called big MAP kinase 1) is a member of the MAPK family and is activated by MEK5 upstream kinase via phosphorylation of TEY motif and C-terminal transcriptional domain. In a previous study, we reported diabetic stimuli reduced the ERK5 transcriptional activity through ERK5-SUMOylation in endothelial cells, and that ERK5-SUMOylation was increased in a mouse model of STZ-induced diabetic cardiac dysfunction induced by myocyte apoptosis (Le et al., 2012; Woo et al., 2008). Recently, it was reported that high fat diet Adipo-ERK5 deficient mice exhibited increased body weight as compared with control littermates, thus suggesting ERK5 plays a protective role of ERK5 in metabolic disease (Shishido et al., 2008; Zhu et al., 2014). However, the role played by ERK5 in the diabetic pancreas has not been addressed. In the present study, we examined the mechanisms underlying the ERK5-mediated anti-diabetic effects of pancreatic β -cells in a mouse model of type 1 diabetes.

MATERIALS AND METHODS

Reagents and antibodies

Chemicals and materials were obtained from commercial sources as follows: streptozotocin (Sigma, USA) and BIX02189 (a specific inhibitor of MEK5 and ERK5 (Tatake et al., 2008) were purchased from Selleck Chemicals (USA). Antibodies were purchased from the following vendors: KDEL (Enzo Life Sciences, Lörrach, Germany), GADD, p-PERK, PERK, ATF4, and HA-probe (Santa Cruz, USA), p-eIF2 α , eIF2 α , p-ERK5, ERK5, caspase-3, and PARP-1 (Cell Life Sciences, USA) and α -tubulin (Sigma, USA).

Cell culture

INS-1 cells (a rat insulinoma cell line) from passage 15 to 30 were seeded at equal densities and cultured in RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 50 μ M β -mercaptoethanol. Cells were incubated in a humidified 5% CO₂/95% air atmosphere at 37°C and grown until 80% confluent.

Protein extraction and Western blot analysis

Cells were lysed in RIPA buffer (pH 7.4) supplemented with 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 0.01 mM PIC (protease inhibitor cocktail). For Western blotting, proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, which were immunoblotted with indicated primary antibodies and then with corresponding secondary antibodies. Signals were visualized using chemiluminescence detection reagents (Millipore, USA), according to the manufacturer's instructions.

Quantitative real time RT-PCR

The mRNA levels were determined by quantitative real time RT-PCR (qRT-PCR). Briefly, total RNA was isolated using TRIzol® Reagent (Invitrogen, USA), and reverse transcription reaction was conducted using TaqMan reverse transcription reagents (Applied Biosystems, USA), according to the manufacturer's instructions. qRT-PCR was conducted with 1 μ l of template cDNA and Power SYBR Green (Applied Biosystems) in an ABI PRISM 7500 unit (Applied Biosystems). Quantification was carried out using the efficiency-corrected $\Delta\Delta$ Cq method. The primers used to amplify DNA sequences were as follows: rat CHOP, forward 5'-AGCTGAGTCTCTGCCTTCG-3' and reverse 5'-AATCCGCCCTCTCTTTAGAG-3'; rat GAPDH, forward 5'-TGGCACCCAGCACAATGAA-3' and reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (Liu et al., 2013).

Small interfering RNA (siRNA) and Adenovirus vector

Cells were transiently transfected with control siRNA or siRNA against ERK5 or CHOP using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. Rat ERK5 siRNA and rat CHOP siRNA were purchased from Bioneer (Korea). The targeting sequences of the siRNAs were as follows: rat CHOP siRNA, sense, 5'-CUGGGAAACA GCGCAUGAA-3'; antisense, 5'-UUCAUGCUGUUUCCAG-3' (He et al., 2012) and rat ERK5 siRNA, 5'-GAAAGGGTGC GAGCCTATAUU-3' and human ERK5 siRNA, 5'-GGGCTAT ATCCAGAGCUU-3'³⁵. Non-specific control siRNA (Bioneer)

was used as a negative control. The constitutively active form of MEK5 α (CA-MEK5 α) was cloned as previously described (Le et al., 2013).

Animal experiments

Male C57BL/6J mice (WT, 8 week old, Jackson Laboratories, USA) and male CHOP^{-/-} mice (C57BL/6J background, 8 week old, Jackson Laboratories) were injected IP with 150 mg/kg body weight STZ. To determine the effect of ERK5 inhibitor, 10 mg/kg body weight of BIX02189 in 100 μ L saline was injected IP every 2 days for 6 days. To measure blood glucose levels, mice were fasted for 12 h with free access to water. Blood samples were obtained by tail tip puncture using a glucometer (Accu-Chek; Roche, USA). All animal experiments were handled in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Yeungnam University College of Medicine, Daegu, Republic of Korea (The approved number of IACUC: YUMC-AEC2015-030).

Immunostaining and TUNEL assay

For immunohistochemical examinations, we used 4% paraformaldehyde fixed, paraffin-embedded pancreas tissue sections. Sections were stained with a rabbit anti-insulin polyclonal antibody (Santa Cruz, USA) or a mouse anti-Chop monoclonal antibody (Santa Cruz, USA) as the primary antibody; Cy3-labeled anti-rabbit IgG (Amersham Pharmacia Biotech, USA) or Alexa Fluor 488-labeled anti-mouse IgG

(Molecular Probes Inc., USA) were used as the secondary antibody. Apoptosis was measured by TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) staining, which targets *in situ* DNA fragmentation, using the *In Situ* Cell Death Detection Kit (Roche, USA) as described previously (Nam et al., 2015).

Statistical analysis

Results in the bar graph are expressed as means \pm SD. Statistical evaluations were performed using ANOVA and Student *t*-tests wherever indicated. All results are presented as the means of at least three independent experiments, and probability values (*p* values) of < 0.05 were considered significant.

RESULTS

ERK5 inhibition exacerbated STZ-induced hyperglycemia and pancreatic apoptosis.

Recent reports suggest that ERK5 activation protects against diabetic stress-induced endothelial inflammatory responses and diabetic cardiac dysfunction after myocardial infarction (Le et al., 2012; Shishido et al., 2008). However, the mechanism by which ERK5 affects hyperglycemia and pancreatic β -cell apoptosis has not been addressed. To assess the role of ERK5 in diabetes, we used BIX02189, a specific ERK5 inhibitor, in a STZ-induced mouse model of type 1 diabetes. Fasting blood glucose levels were found to progressively increase

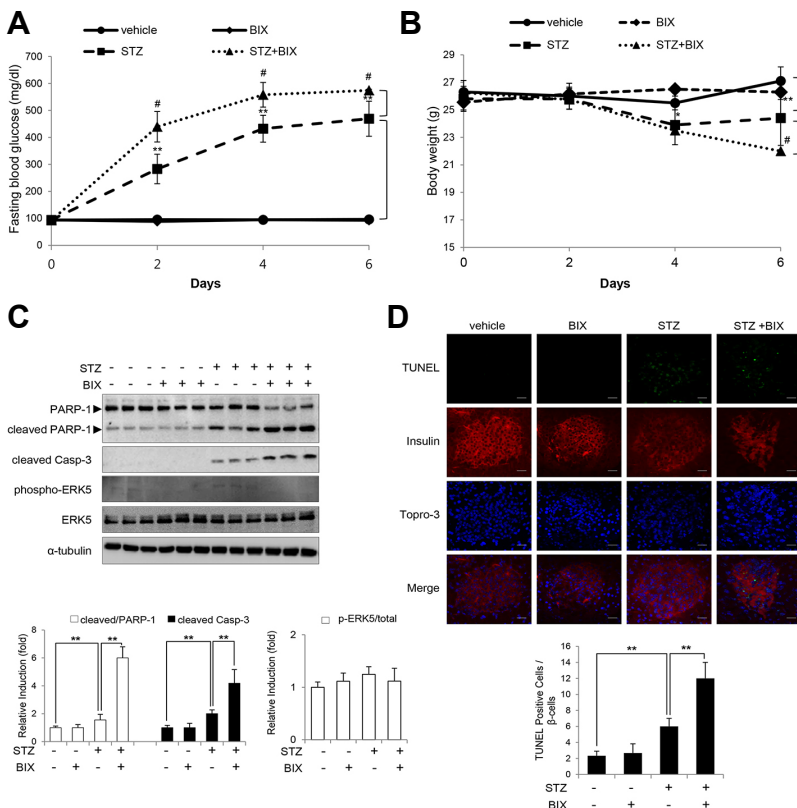


Fig. 1. ERK5 inhibition enhanced STZ-induced hyperglycemia and pancreatic cell death. Mice received a single i.p. injection of streptozotocin (STZ, 150 mg/kg body weight) and BIX02189 (BIX, 10 mg/kg body weight) every 2 days for 6 days, whereas vehicles were administered citrate buffer. (A) Fasting blood glucose levels were determined using a glucometer and blood obtained by tail tip puncture. Results are presented as means \pm SDs ($n = 10$ mice). ANOVA: ** $p < 0.01$ vehicle vs. STZ; # $p < 0.05$ STZ vs. STZ+BIX. (B) Body weights were measured. Results are presented as means \pm SDs ($n = 10$ mice). ANOVA: * $p < 0.05$ vehicle vs. STZ; ** $p < 0.01$ vehicle vs. STZ; # $p < 0.05$ STZ vs. STZ+BIX. (C) Protein levels were analyzed by immunoblotting with specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: ** $p < 0.01$. (D) Pancreas tissue sections were subjected to TUNEL staining. Representative photomicrographs showing TUNEL (apoptotic, green), insulin (pancreatic β -cells, red), and Topro-3 (nuclei, blue) signals and merged images (original magnification $\times 400$, Scale bars: 50 μ m). Arrowheads indicate TUNEL positive nuclei. Bar graphs present percentages of numbers of TUNEL positive cells among total numbers of pancreatic β -cells counted. ANOVA: ** $p < 0.01$.

in STZ treated mice (Fig. 1A). In STZ treated mice co-treated with BIX02189, a significant increase in blood glucose level was observed at only 2 days post-treatment, indicating ERK5 inhibition exacerbates STZ-induced hyperglycemia. During the 6-day experimental period, the body weights of STZ treated mice were lower than those of treatment naïve controls, and body weight losses by STZ plus BIX02189 treated mice were significantly greater than those of STZ treated mice, suggesting ERK5 inhibition exacerbated hyperglycemia and body weight loss in our STZ-induced model (Fig. 1B).

We next investigated whether ERK5 inhibition increases β -cell apoptosis induced by STZ. Immunoblotting data showed that cleaved PARP-1 and caspase-3 levels were slightly higher in STZ treated mice than in treatment naïve controls and that these levels were higher in STZ plus BIX02189 treated co-treated mice than in STZ treated mice (Fig. 1C). Consistent with our immunoblotting observations, numbers of TUNEL-positive cells in pancreatic islets were significant higher in STZ plus BIX02189 co-treated mice than STZ treated mice (Fig. 1D). These results suggest that ERK5 inhibition augments STZ-induced β -cell apoptosis, hyperglycemia, and body weight loss.

ER stress was involved in the ERK5 inhibition-mediated exacerbation of STZ-induced pancreatic β -cell apoptosis

It has been established that ER stress contributes to the pathogenesis of diabetes and β -cell apoptosis (Oyadomari et al., 2002; Papa, 2012), and thus we evaluated UPRs in pancreas tissues from mice treated with STZ or STZ plus BIX02189. As shown in Fig. 2A, protein expression levels of GRP78/94, CHOP, ATF4, phospho-PERK, and phospho-eIF2 α were significantly higher in STZ treated mice than in treatment naïve controls, and significantly higher in STZ plus BIX02189 co-treated mice than in STZ treated mice.

Immunohistochemical analysis of pancreatic tissues showed that CHOP was expressed in insulin-producing cells (Fig. 2B). CHOP expression was moderate in islets from STZ

treated mice, but was significantly higher than this in STZ plus BIX02189 co-treated mice. These results suggested that ERK5 inhibition-induced β -cell apoptosis involved the participation of CHOP.

ERK5 regulates STZ-induced apoptosis in a UPR-dependent manner

To confirm the role of ERK5 in the regulation of UPRs and apoptosis in pancreatic β -cells, we examined the effect of ERK5 inhibitor and the siRNA knockdown of ERK5 in INS-1 cells. It has been reported that STZ causes apoptosis at low doses but necrosis at high doses in INS-1 cells (Saini et al., 1996) and thus, we selected a STZ concentration that induced INS-1 cell apoptosis (data not shown). Western blot analysis revealed that STZ inductions of the cleaved forms of PARP-1 and caspase-3 were significantly increased in the presence of BIX02189 (Fig. 3A). In addition, STZ-induced apoptosis was also enhanced by transfecting cells with ERK5 siRNA (Fig. 3B). Furthermore, STZ plus BIX02189 co-treatment increased the expressions of proteins associated with UPRs, that is, of GRP 94/78, CHOP, ATF4, phospho-PERK, and phospho-eIF2 α as compared with STZ alone (Fig. 3A). Transfection with ERK5 siRNA had the same effects as BIX02189 on these UPR associated proteins, suggesting that ERK5 regulates their expressions. These observations suggested that ERK5 exerts protective effects by ameliorating the inductions of UPR-associated proteins and apoptosis in pancreatic β -cells.

It has been established that ER stress-mediated apoptosis plays an important role in the destruction of INS-1 cells (Oyadomari et al., 2002; Yusta et al., 2006). The protective effect of ERK5 activation was investigated by transducing INS-1 cells with adenovirus encoding a constitutive active form of MEK5 α (CA-MEK5 α). ERK5 activation resulted in a significant decrease in the expressions of apoptotic marker proteins and UPR-associated proteins induced by STZ (Fig. 3C). In addition, the overexpression of CA-MEK5 α prevented

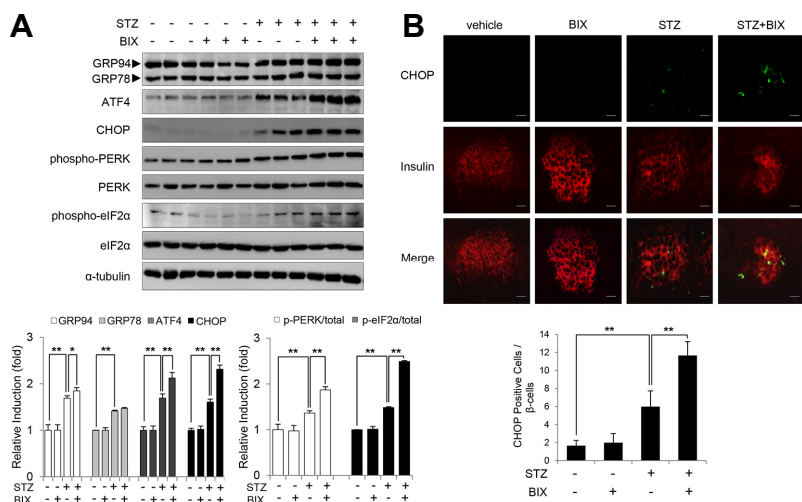


Fig. 2. Effects of ERK5 inhibitor on UPR in diabetic pancreas. (A) Pancreas tissue lysates of vehicle, streptozotocin (STZ), and STZ+BIX02189 (BIX) treated mice were immunoblotted. Protein levels were analyzed by immunoblotting with specific antibodies. Bar graphs present the densitometric quantifications of western blot bands. ANOVA: * $p < 0.05$; ** $p < 0.01$. (B) Pancreatic sections from vehicle, STZ, and STZ+BIX were immunostained for CHOP and insulin. Immunofluorescence analysis of CHOP (green), insulin (red), Topro-3 (blue) and merged images (original magnification $\times 400$) in mouse islets. Bar graphs present percentages of numbers of CHOP positive cells among total numbers of pancreatic β -cells counted. Results are expressed as means \pm SDs and are representative of three independent experiments. ANOVA: ** $p < 0.01$ ($n = 3$).

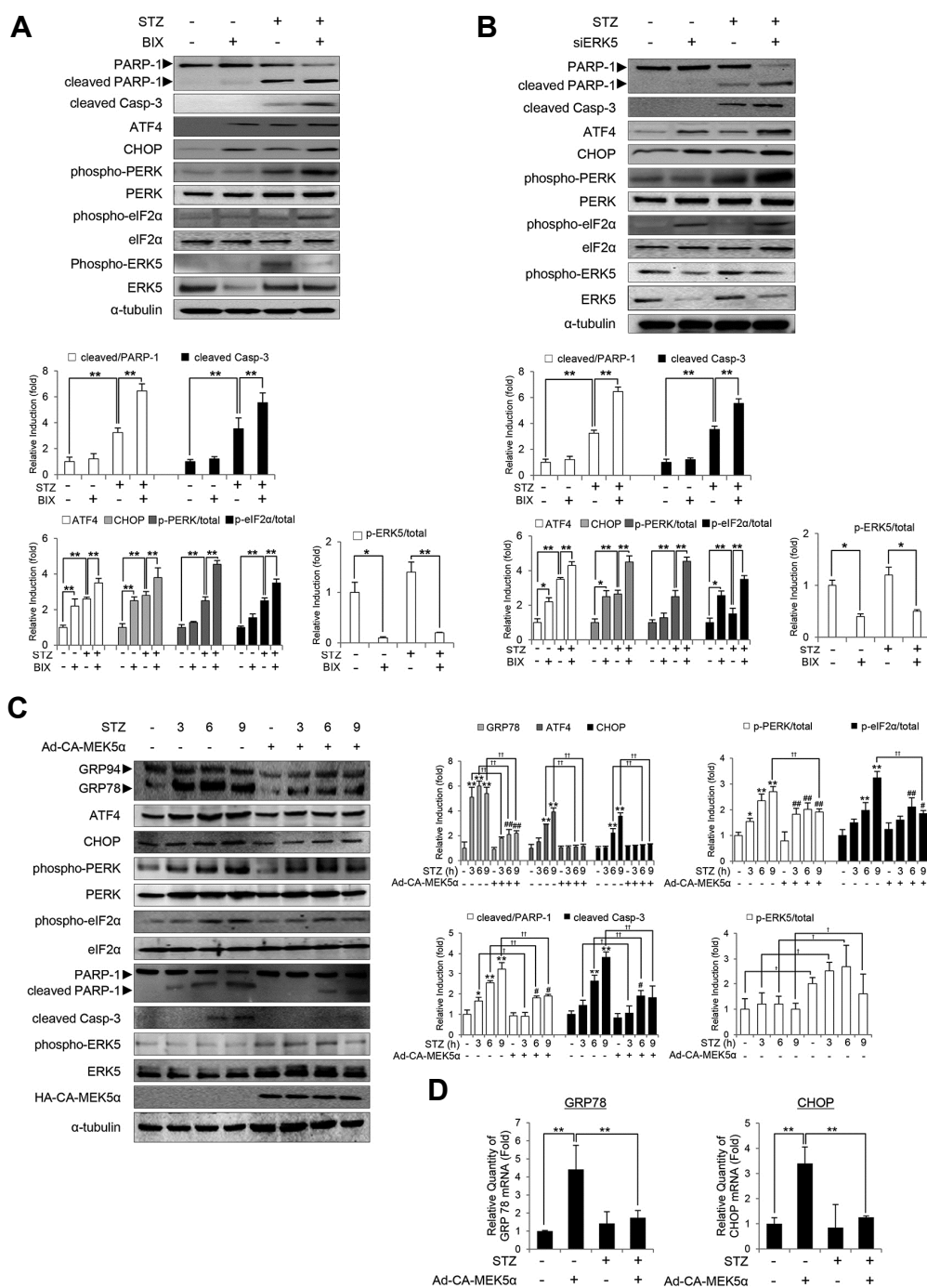


Fig. 3. Roles of ERK5 on STZ-induced UPR and apoptosis in INS-1 cells. (A) INS-1 cells were pretreated with BIX02189 (BIX, 2 μ M) for 1 h and then incubated with streptozotocin (STZ, 10 mM) for 9 h. Protein levels were analyzed by immunoblotting using specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: * $p < 0.05$; ** $p < 0.01$. (B) INS-1 cells were transfected with control or ERK5 siRNA (100 pM) for 48 h, and then treated with STZ (10 mM) for 9 h. Protein levels were analyzed by immunoblotting with specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: * $p < 0.05$; ** $p < 0.01$. (C) INS-1 cells were transduced with Ad-CA-MEK5 α to activate ERK5. After infection at 10 MOI with Ad-LacZ or Ad-CA-MEK5 α for 24 h, cells were treated with STZ (10 mM) for 3, 6, or 9 h. Protein levels were analyzed by immunoblotting using specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; #, $p < 0.05$ vs. Ad-CA-MEK5 α ; ##, $p < 0.01$ vs. Ad-CA-MEK5 α ; †, $p < 0.05$; ††, $p < 0.01$. (D) INS-1 cells were transduced with Ad-CA-MEK5 α to activate ERK5. After infection at 10 MOI with Ad-LacZ or Ad-CA-MEK5 α for 24 h, cells were treated with STZ (10 mM) for 6 h. Relative mRNA expression levels of GRP78 and CHOP were determined by qRT-PCR using GAPDH as the internal control. Results are expressed as means \pm SDs and are representative of three independent experiments. ANOVA: ** $p < 0.01$.

mRNA induction of GRP78 and CHOP by STZ (Fig. 3D). These results suggest that ERK5 protected INS-1 cells from ER stress.

CHOP deficiency ameliorated the ERK5 inhibition-mediated exacerbation of STZ-induced hyperglycemia and pancreatic cell death

Previous studies have suggested that CHOP may be involved in STZ-induced apoptosis mediated by ER stress pathway (Araki et al., 2013; Zhang et al., 2011). To assess the potential mediatory role of CHOP in pancreatic beta cell death, INS-1 cells were transfected with CHOP siRNA or control siRNA. As shown in Fig. 4A, CHOP knockdown significantly reduced the induction of apoptosis by STZ plus BIX02189, which suggested that CHOP induction is responsible for the ERK5 inhibition-mediated exacerbation of STZ-induced β -cell apoptosis.

To clarify the physiological relevance of the CHOP-mediated degenerative pathway in ERK5 inhibition-mediated exacerbation of STZ-induced hyperglycemia and body weight loss, we used CHOP deficient mice. It was found the number of days required to reach the diabetic state and average glucose levels after STZ administration differed significantly in CHOP deficient and control C57BL/6J mice. As was expected, fasting blood glucose concentrations showed that ERK5 inhibition-induced hyperglycemia was significantly suppressed in CHOP deficient mice (Fig. 4B). In addition, C57BL/6J control mice had significantly lower body weights than CHOP deficient mice (Fig. 4C). Moreover, immunoblotting data showed that STZ plus BIX02189 co-treatment induced a dramatic increase in apoptotic markers and TUNEL-positive cell numbers, and that these changes were attenuated in CHOP deficient mice (Figs. 4D and 4E), suggesting the protective effects of ERK5 on STZ-induced pancreatic β -cell damage are mediated by interrupting a CHOP-dependent degenerative pathway. Interestingly, total amounts of ATF4 were significantly upregulated by the combination of STZ and BIX02189 in littermate control mice, but these inductions were not inhibited in CHOP KO. Since CHOP is a downstream molecule in PERK-eIF2 α -ATF4-CHOP signaling pathway, CHOP deficiency might not affect the upstream signaling pathway. These results suggest that CHOP deficiency reduced the pancreatic apoptosis induced by the combination of STZ and BIX02189 without affecting upstream signaling pathway

DISCUSSION

In the present study, we investigated the involvement of ERK5 in STZ-induced apoptosis (Figs. 1C and 1D) and ER stress (Fig. 2) in pancreatic islets. Our findings indicate that ERK5 protects against STZ-induced β -cell apoptosis and hyperglycemia by interrupting the ER stress-mediated apoptotic pathway (Fig. 3), and suggest ERK5 activation has therapeutic potential for the management of ER stress-dependent diabetes and its complications.

STZ has been widely used to produce animal models of diabetes (Szkudelski, 2001) and published observations suggest that ER stress-associated apoptosis underlies STZ-

induced hyperglycemia (Saini et al., 1996) and that ER stress induces pancreatic β -cell apoptosis and peripheral insulin resistance (Araki et al., 2003). Recently, ERK5-SUMOylation was detected in a murine model of diabetic cardiac dysfunction induced by STZ following myocardial infarction (Shishido et al., 2008; Woo et al., 2008). In addition, the authors showed that CA-MEK5 α overexpression could activate ERK5 by inhibiting its SUMOylation (Shishido et al., 2008). Furthermore, in another study, blood glucose concentrations were found to be elevated in adipocyte specific ERK5 deficient mice and insulin sensitivity to be attenuated as compared with wild-type mice (Zhu et al., 2014).

STZ-induced apoptosis in pancreas islets was observed as early as 3 days after the administration of STZ plus BIX02189 in control mice (Fig. 1D). Morphological observations in the mice administered STZ plus BIX02189 revealed marked involution of pancreatic islets and marked increases in the number of CHOP protein expression from 2 days after administration (Fig. 2B). We also found that STZ plus BIX02189-induced ER stress and apoptosis were prevented in CHOP deficient mice but not in littermate controls (Fig. 4). Collectively, these results show that the islets of STZ-induced diabetic mice are under ER stress and that the protection afforded by ERK5 in our diabetic model is in part derived from the inhibition of CHOP-mediated apoptosis.

Although STZ-induced pre-prandial glucose levels were dramatically blocked in CHOP KO compared to littermate controls, decent amount of apoptosis was still found in STZ-treated islet in CHOP KO. The sum of our findings support the idea that CHOP is a fundamental factor causing β -cell dysfunction and apoptosis in response to the chronic ER stress that coincides with β -cell compensation for insulin resistance. Our findings suggest that in the absence of a death signal, UPR signaling can improve protein secretory capacity and preserve the functional integrity of the ER in β -cells. It remains to be examined whether β -cell failure and loss of glucose homeostasis *in vivo* result from an inadequate UPR adaptive response or activation of an apoptotic response due to chronic, unresolved ER stress.

To determine whether ERK5 is directly involved in STZ-induced ER stress, we transduced INS-1 cells with Ad-CA-MEK5 α to activate ERK5 (Fig. 3C). A previous report suggested that CHOP is induced by PERK/eIF2 α /ATF4 and activates the expressions of proapoptotic genes (Marciniak et al., 2004). We found ERK5 activation inhibited STZ-induced PERK pathway regulators and apoptosis in INS-1 cells, and that the STZ-induced mRNA expressions of GRP78 and CHOP were significantly diminished by ERK5 activation. These results suggest ERK5-dependent suppression of CHOP expression is regulated in a transcriptional level, but not post-translational modification. However, it is not clear whether ERK5 activation regulates mRNA expression of CHOP through promoter regulation or not.

ERK5 activation also inhibited the STZ-induced protein expressions of GRP78 and GRP94 and of GRP78 at the mRNA level. These results suggest that ERK5 activation inhibits not only PERK pathway but also GRP78-dependent pathway. It was previously shown ERK5 regulates Akt activation, (Roberts et al., 2010) and that the PI3K/Akt pathway protects

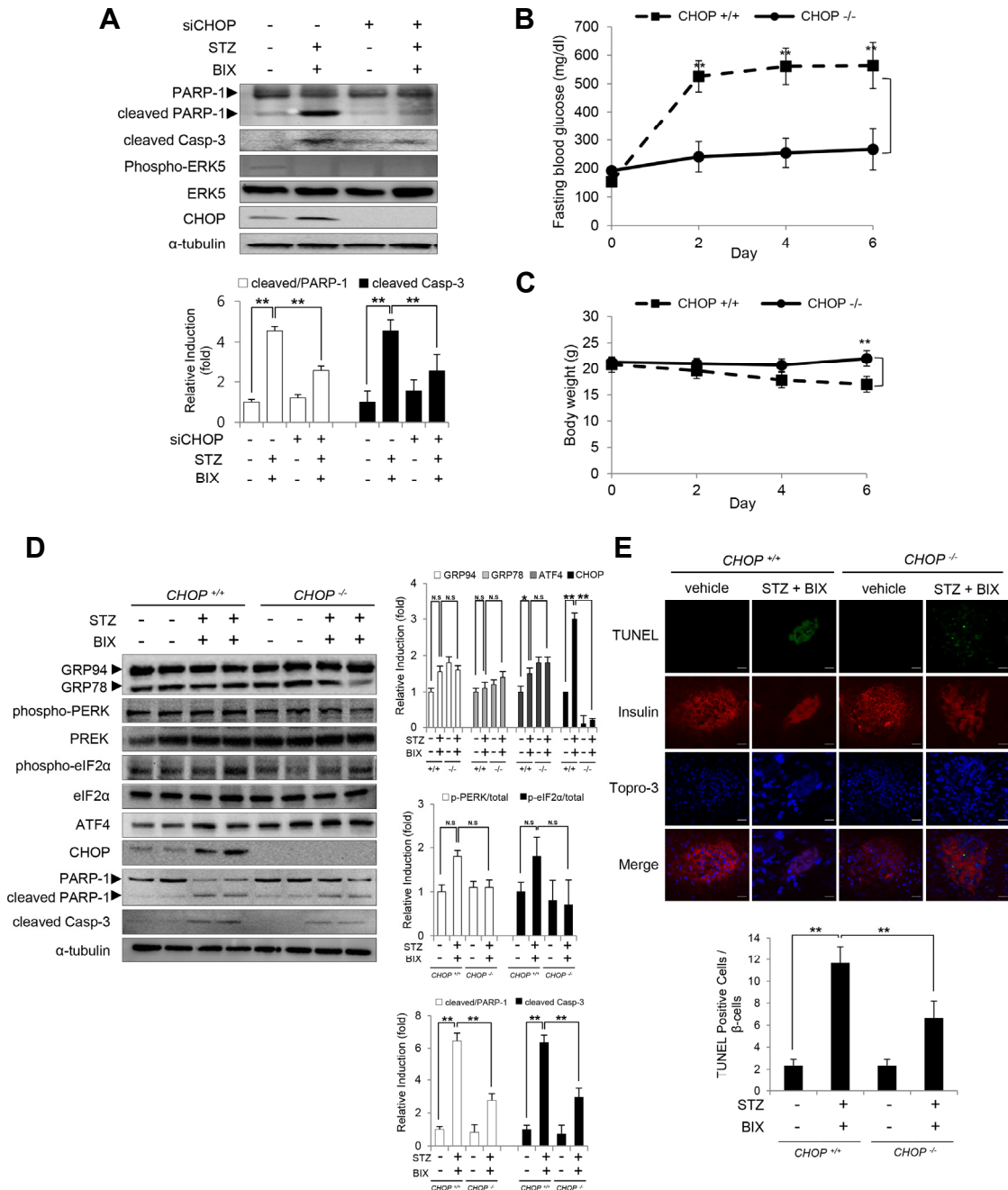


Fig. 4. CHOP deficiency ameliorated ERK5 inhibition-mediated exacerbation of STZ-induced hyperglycemia and pancreatic cell death. (A) INS-1 cells were transfected with control or CHOP siRNA (100 pM) for 48 h, and then treated with streptozotocin (STZ, 10 mM) in the presence or absence of BIX02189 (BIX, 2 μ M) for 9 h. Protein levels were analyzed by immunoblotting using specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: ** p < 0.01. (B-E) Littermate *CHOP*^{+/+} mice or *CHOP*^{-/-} mice received single i.p. injections of STZ (150 mg/kg body weight) or STZ+BIX (10 mg/kg body weight) every 2 days for 6 days; vehicles were administered citrate buffer. (B) Fasting blood glucose levels were measured in blood obtained by tail tip puncture using a glucometer. Results are presented as means \pm SDs (n = 10 mice). ANOVA: ** p < 0.05. (C) Body weights were measured. Results are presented as means \pm SDs. ANOVA: * p < 0.05 STZ vs. control; ** p < 0.01 STZ+BIX02189 vs STZ. (D) Pancreas tissue lysates of control and STZ+BIX treated *CHOP*^{+/+} or *CHOP*^{-/-} mice were immunoblotted. Protein levels were analyzed by immunoblotting with specific antibodies. Bar graphs present the densitometric quantifications of Western blot bands. ANOVA: * p < 0.05; ** p < 0.01; N.S (Not significant). (E) Pancreas tissue sections from *CHOP*^{+/+} or *CHOP*^{-/-} mice were subjected to TUNEL staining. Representative photomicrographs showing TUNEL (apoptotic, green), insulin (pancreatic β -cells, red), Topro-3 (nuclei, blue) signals and their merged images (original magnification \times 400, Scale bars: 50 μ m). Bar graphs present percentages of numbers of TUNEL positive cells among numbers of total pancreatic β -cells counted. Results are expressed as means \pm SDs and are representative of three independent experiments. ANOVA: ** p < 0.01 (n = 3).

against ER stress-induced apoptosis via GRP78 regulation (Dai et al., 2010). Together these results suggest MEK5-ERK5 signaling modulates ER stress-mediated apoptosis via the PI3-kinase-Akt pathway.

The *in vivo* data from a murine model in the present study showed the role of ERK5-dependent suppression of CHOP expression in STZ-induced pancreatic β -cell dysfunction. Since CHOP deficiency rescued pancreatic β -cell dysfunction and hyperglycemia in Akita mouse model of diabetes which has genetic mutation of pro-insulin (Oyadomari et al., 2002). This mutation induced ER stress and CHOP induction. We thus believe that ERK5-dependent suppression of CHOP might act as a therapeutic target for ER stress-dependent diabetes. It remains to be determined in our further study whether ERK5 has similar mechanistic pathway in autoimmune response during the progression on type 1 diabetes.

In conclusion, the results of the present study demonstrate that ERK5 protects against STZ-induced hyperglycemia by regulating pancreatic β -cell apoptosis. In addition, they show that ERK5 inhibition exacerbated ER stress and β -cell apoptosis in STZ-induced murine model of diabetes and that a genetic deficiency in CHOP reduces pancreatic β -cell apoptosis in the co-treatment of STZ and an ERK5 inhibitor. Our observations support the proposition that ERK5 activation-mediated inhibitions of ER stress and apoptosis should be considered a potent therapeutic target for the treatment of diabetes and its complications.

ACKNOWLEDGEMENTS

This study was supported by the Medical Research Center Program (2015R1A5A2009124) and Basic Science Research Program (2015R1D1A1A01059398) through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning. Additional support was provided by the Health Fellowship Foundation grants (Dae-Hwan Nam).

REFERENCES

- Ahn, C., An, B.S., and Jeung, E.B. (2015). Streptozotocin induces endoplasmic reticulum stress and apoptosis via disruption of calcium homeostasis in mouse pancreas. *Mol. Cell. Endocrinol.* *412*, 302-308.
- Apostolou, I., Hao, Z., Rajewsky, K., and von Boehmer, H. (2003). Effective destruction of Fas-deficient insulin-producing beta cells in type 1 diabetes. *J. Exp. Med.* *198*, 1103-1106.
- Araki, E., Oyadomari, S., and Mori, M. (2003). Impact of endoplasmic reticulum stress pathway on pancreatic beta-cells and diabetes mellitus. *Exp. Biol. Med.* *228*, 1213-1217.
- Dai, R.Y., Chen, S.K., Yan, D.M., Chen, R., Lui, Y.P., Duan, C.Y., Li, J., He, T., and Li, H. (2010). PI3K/Akt promotes GRP78 accumulation and inhibits endoplasmic reticulum stress-induced apoptosis in HEK293 cells. *Folia Biol.* *56*, 37-46.
- Eizirik, D.L., Cardozo, A.K., and Cnop, M. (2008). The role for endoplasmic reticulum stress in diabetes mellitus. *Endocrine Rev.* *29*, 42-61.
- Hayashi, K., Kojima, R., and Ito, M. (2006). Strain differences in the diabetogenic activity of streptozotocin in mice. *Biol. Pharm. Bull.* *29*, 1110-1119.
- He, Z., Ostrowski, R.P., Sun, X., Ma, Q., Tang, J., and Zhang, J.H.

(2012). Targeting C/EBP homologous protein with siRNA attenuates cerebral vasospasm after experimental subarachnoid hemorrhage. *Exp. Neurol.* *238*, 218-224.

Lakshmanan, A.P., Thandavarayan, R.A., Palaniyandi, S.S., Sari, F.R., Meilei, H., Giridharan, V.V., Soetikno, V., Suzuki, K., Kodama, M., and Watanabe, K. (2011). Modulation of AT-1R/CHOP-JNK-Caspase12 pathway by olmesartan treatment attenuates ER stress-induced renal apoptosis in streptozotocin-induced diabetic mice. *Eur. J. Pharm. Sci.* *44*, 627-634.

Le, N.T., Takei, Y., Shishido, T., Woo, C.H., Chang, E., Heo, K.S., Lee, H., Lu, Y., Morrell, C., Oikawa, M., et al. (2012). p90RSK targets the ERK5-CHIP ubiquitin E3 ligase activity in diabetic hearts and promotes cardiac apoptosis and dysfunction. *Circ. Res.* *110*, 536-550.

Le, N.T., Heo, K.S., Takei, Y., Lee, H., Woo, C.H., Chang, E., McClain, C., Hurley, C., Wang, X., Li, F., et al. (2013). A crucial role for p90RSK-mediated reduction of ERK5 transcriptional activity in endothelial dysfunction and atherosclerosis. *Circulation* *127*, 486-499.

Le May, C., Chu, K., Hu, M., Ortega, C.S., Simpson, E.R., Korach, K.S., Tsai, M.J., and Mauvais-Jarvis, F. (2006). Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proc. Natl. Acad. Sci. USA* *103*, 9232-9237.

Liu, Z.W., Zhu, H.T., Chen, K.L., Dong, X., Wei, J., Qiu, C., and Xue, J.H. (2013). Protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling pathway plays a major role in reactive oxygen species (ROS)-mediated endoplasmic reticulum stress-induced apoptosis in diabetic cardiomyopathy. *Cardiovasc. Diabetol.* *12*, 158.

Marciniak, S.J., Yun, C.Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H.P., and Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev.* *18*, 3066-3077.

Nam, D.H., Han, J.H., Lee, T.J., Shishido, T., Lim, J.H., Kim, G.Y., and Woo, C.H. (2015). CHOP deficiency prevents methylglyoxal-induced myocyte apoptosis and cardiac dysfunction. *J. Mol. Cell. Cardiol.* *85*, 168-177.

Oyadomari, S., and Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* *11*, 381-389.

Oyadomari, S., Araki, E., and Mori, M. (2002). Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis* *7*, 335-345.

Oyadomari, S., Koizumi, A., Takeda, K., Gotoch, T., Akira, S., Araki, E., and Mori, M. (2002). Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J. Clin. Invest.* *109*, 525-532.

Papa, F.R. (2012). Endoplasmic reticulum stress, pancreatic beta-cell degeneration, and diabetes. *Cold Spring Harb. Perspect. Med.* *2*, a007666.

Roberts, O.L., Holmes, K., Müller, J., Cross, D.A.E., and Cross, M.J. (2010). ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells. *J. Cell Sci.* *123*, 3189-3200.

Saini, K.S., Thompson, C., Winterford, C.M., Walker, N.I., and Cameron, D.P. (1996). Streptozotocin at low doses induces apoptosis and at high doses causes necrosis in a murine pancreatic beta cell line, INS-1. *Biochem. Mol. Biol. Int.* *39*, 1229-1236.

Sano, R., and Reed, J.C. (2013). ER stress-induced cell death mechanisms. *Biochim. Biophys. Acta* *1833*, 3460-3470.

Shishido, T., Woo, C.H., Ding, B., McClain, C., Molina, C.A., Yan, C., Yang, J., and Abe, J. (2008). Effects of MEK5/ERK5 association on small ubiquitin-related modification of ERK5: implications for diabetic ventricular dysfunction after myocardial infarction. *Circ. Res.* *102*, 1416-1425.

- Song, B., Scheuner, D., Ron, D., Pennathur, S., and Kaufman, R.J. (2008). Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J. Clin. Invest.* *118*, 3378-3389.
- Szegezdi, E., Logue, S.E., Gorman, A.M., and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* *7*, 880-885.
- Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol. Res.* *50*, 537-546.
- Tamaki, N., Hatano, E., Taura, K., Tada, M., Kodama, Y., Nitta, T., Iwaisako, K., Seo, S., Nakajima, A., Ikai, I., et al. (2008). CHOP deficiency attenuates cholestasis-induced liver fibrosis by reduction of hepatocyte injury. *Am. J. Physiol.* *294*, G498-505.
- Tatake, R.J., O'Neill, M.M., Kennedy, C.A., Wayne, A.L., Jakes, S., Wu, D., Kugler, S.Z., Jr., Kashem, M.A., Kaplita, P. and Snow, R.J. (2008). Identification of pharmacological inhibitors of the MEK5/ERK5 pathway. *Biochem. Biophys. Res. Commun.* *377*, 120-125.
- Woo, C.H., Shishido, T., McClain, C., Lim, J.H., Li, J.D., Yang, J., Yan, C. and Abe, J. (2008). Extracellular signal-regulated kinase 5 SUMOylation antagonizes shear stress-induced antiinflammatory response and endothelial nitric oxide synthase expression in endothelial cells. *Circulation Res.* *102*, 538-545.
- Yusta, B., Baggio, L.L., Estall, J.L., Koehler, J.A., Holland, D.P., Li, H., Pipeleers, D., Ling, Z. and Drucker, D.J. (2006). GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. *Cell Metabol.* *4*, 391-406.
- Zhang, R., Kim, J.S., Kang, K.A., Piao, M.J., Kim, K.C., and Hyun, J.W. (2011). Protective Mechanism of KIOM-4 in streptozotocin-induced pancreatic beta-cells damage is involved in the inhibition of endoplasmic reticulum stress. *Evid. Based Complement. Alternat. Med.* Article ID 231938.
- Zhu, H., Guariglia, S., Li, W., Brancho, D., Wang, Z.V., Scherer, P.E., and Chow, C.W. (2014). Role of extracellular signal-regulated kinase 5 in adipocyte signaling. *J. Biol. Chem.* *289*, 6311-6322.