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Dipeptidyl petidase-IV inhibitor (gemigliptin) inhibits tunicamycin-induced endoplasmic reticulum stress, apoptosis and inflammation in H9c2 cardiomyocytes

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The direct effects of dipeptidyl peptidase-IV (DPP-IV) inhibitors on endoplasmic reticulum (ER) stress-induced apoptosis and inflammation in cardiomyocytes have not been elucidated. H9c2 cell viability, which was reduced by tunicamycin, was increased after DPP-IV inhibitor gemigliptin treatment. Gemigliptin significantly decreased the tunicamycin-mediated increase in glucose regulated protein 78 (GRP78) expression and ER stress-mediated signaling molecules such as protein kinase RNA-like endoplasmic reticulum kinase (PERK)/C-EBP homologous protein (CHOP) and inositol-requiring enzyme 1α (IRE1α)/c-Jun N-terminal kinase (JNK)-p38. Furthermore, gemigliptin effectively induced A*kt* phosphorylation in a dose-dependent manner. Using flow cytometry and Hoechst staining, we showed that treatment with A*kt* inhibitor significantly blocked the anti-apoptotic effects mediated by gemigliptin. The reduction in tunicamycin-induced GRP78 level and PERK/CHOP pathway activity by gemigliptin was reversed after treatment with A*kt* inhibitor. In conclusion, gemigliptin effectively inhibited ER stress-induced apoptosis and inflammation in cardiomyocytes via A*kt*/PERK/CHOP and IRE1α/JNK-p38 pathways, suggesting its direct protective role in cardiovascular diseases.

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1. Introduction

Cardiovascular disease is the leading cause of mortality in patients with type 2 diabetes. Hyperglycemia increases the production of intracellular reactive oxygen species (ROS), subsequently inducing apoptotic cell death and inflammation in vascular cells (Folli et al., 2011). Excessive oxidative stress plays a pivotal role in the onset and development of diabetic vascular complications (Giacco and Brownlee, 2010). However, there are no effective therapeutic strategies for the reduction of oxidative stress to prevent diabetic complications.

Glucagon-like peptide-1 (GLP-1), which is synthesized and secreted by intestinal L-cells in response to nutrient ingestion, enhances pancreatic insulin secretion and is rapidly degraded by dipeptidyl peptidase-IV (DPP-IV). Currently, GLP-1 agonists or DPP-IV inhibitors are the major treatment options for patients with type 2 diabetes. Although GLP-1 and DPP-IV inhibitors primarily regulate blood glucose, the wide distribution of GLP-1 receptor and DPP-IV in the cardiovascular system, including the endothelium, cardiomyocytes, and coronary smooth muscle cells, suggests a potential role in cardiovascular diseases (Zhong et al., 2013). Recently, Wang et al. (2013) showed that GLP-1 decreases the induction of ROS and enhancement of the apoptotic index by high glucose in cardiac microvascular endothelial cells. GLP-1 receptor activation leads to activation of extracellular signal-regulated kinases (Erk) 1/2, protein kinase C, and phosphatidylinositol-3-kinase activation, which are all involved in protection against ischemia/reperfusion injury (Hocher et al., 2013). Furthermore, administration of GLP-1 at the time of reperfusion is effective in

Abbreviations: ROS, reactive oxygen species; GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase-IV; Erk, extracellular signal-regulated kinases; ER, endoplasmic reticulum; IL-6, interlukin-6; TNF-α, tumor necrosis factor-α; BNP, brain natriuretic peptide; SDF-1α, stromal derived factor-1α; GRP78, glucose regulated protein 78; PERK, protein kinase RNA-like endoplasmic reticulum kinase; IRE1α, inositol-requiring enzyme 1α; JNK, c-Jun N-terminal kinase; CHOP, C-EBP homologous protein; eIF2α, eukaryotic translational initiation factor 2α; NK, natural killer; UPR, unfolded protein response; ATF-6, activating transcription factor-6; EPC, endothelial progenitor cells.

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decreasing myocardial infarct size in patients with acute myocardial infarction (Lonborg et al., 2012).

Similarly, there have been reports of the cardioprotective and anti-apoptotic effects of DPP-IV inhibitors. Picatoste et al. (2013) demonstrated that DPP-IV inhibitors such as sitagliptin reduce cardiac pro-apoptotic/necrotic and fibrotic expression in diabetic rats. Another DPP-IV inhibitor, linagliptin, is associated with reduced formation of cardiac, vascular and blood ROS in addition to attenuated aortic infiltration with inflammatory cells (Kröller-Schön et al., 2012). However, the cardioprotective effects of DPP-IV inhibitors have mostly been attributed to indirect effects through increased GLP-1 bioavailability. To the best of our knowledge, there has been no study focusing on the direct effects of DPP-IV inhibitors on the oxidative stress in cardiomyocytes. In addition to its enzymatic activity, DPP-IV plays a critical role as an inflammatory mediator by providing co-stimulatory signals to T cells (Tanaka et al., 1993). Thus, if DPP-IV is pro-inflammatory, its inhibitors could potentially be anti-inflammatory and possibly anti-apoptotic.

Therefore, this study was designed to determine the anti-apoptotic and anti-inflammatory effects of DPP-IV inhibitior in cardiomyocytes and to characterize the underlying molecular mechanism. For this purpose, we examined whether gemigliptin, a novel DPP-IV inhibitor developed in Korea (Rhee et al., 2013), reduces tunicamycin-induced endoplasmic reticulum (ER) stress and apoptosis signaling molecules in cardiomyocytes. Tunicamycin is a representative pharmacological inducer of ER-stress by means of inhibiting the action of DPAGT1, which has a role for N-glycosylation of proteins in ER (Bassik and Kampmann, 2011). Next, we explored whether gemigliptin activates A*kt* phosphorylation and whether the anti-apoptotic effect of gemigliptin is A*kt*-dependent. Finally, we determined whether gemigliptin influences the expression of inflammatory cytokines and other substrates such as interlukin-6 (IL-6), tumor necrosis factor-α (TNF-α), brain natriuretic peptide (BNP), and stromal derived factor-1α (SDF-1α) in cardiomyocytes.

2. Materials and methods

2.1. Cell culture

H9c2 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, South Korea), and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 4.5 g/L D-glucose, 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 50 U/ml penicillin and 50 g/ml streptomycin (Invitrogen) at 37 °C with 5% CO_2.

2.2. Cell viability measurement

H9c2 cells were seeded into 96-well plates (2×10^4 cells per well). After 24 h, the cells were starved in serum-free DMEM for 3 h. Cells were pretreated with gemigliptin (250 μM; LG Life Sciences Ltd., Seoul, Korea) or gemigliptin and Akti-1/2 trifluoroacetate salt hydrate (2 μM; A*kt* inhibitor VIII; Sigma Aldrich, St. Louis, MO, USA) for 30 min, and then treated with tunicamycin (10 μM; Sigma Aldrich) for 16 h. Cell viability was measured using an EZ-CYTOX kit (DAEILAP, Seoul, Korea).

2.3. Western blot analysis

Proteins were separated using 10–12% acrylamide gels and transferred to nitrocellulose membranes (Amersham Bioscience, Westborough, MA, USA). The membranes were blocked with 5% non-fat dry milk or 5% BSA for 1 h at room temperature, and incubated with primary antibody (1:500–2000 dilution) for 16 h at 4 °C. After washing with 0.05% TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience, 1:5000 dilution) for 1 h at room temperature, and developed using chemiluminescence solution (Bio-Rad Laboratories, CA, USA).

Sources for antibodies used in this study were as follows; polyclonal anti-phosphorylated IRE1α (1:500 dilution; Thermo scientific, MA, USA), polyclonal anti-total IRE1α (1:1000 dilution), polyclonal anti-phosphorylated eIF2α (1:500 dilution), polyclonal anti-total eIF2α (1:1000 dilution), polyclonal anti-GRP78 (1:500 dilution), monoclonal anti-beta actin (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-phosphorylated PERK (1:1000 dilution), monoclonal anti-total PERK (1:1000 dilution), monoclonal anti-CHOP (1:1000 dilution), polyclonal anti-phosphorylated JNK (1:1000 dilution), monoclonal anti-total JNK (1:2000 dilution), monoclonal anti-phosphorylated p38 (1:1000 dilution), polyclonal anti-total p38 (1:2000 dilution), polyclonal anti-phosphorylated A*kt* (1:1000 dilution), polyclonal anti-total A*kt* (1:2000 dilution; Cell Signaling Technology, Boston, MA, USA).

2.4. Flow cytometry

H9c2 cells were pre-incubated with gemigliptin (250 μM) or gemigliptin and A*kt* inhibitor (2 μM) for 30 min, and then treated with tunicamycin for 16 h. Stimulated cells were fixed with 1% formaldehyde and washed twice with PBS. The fixed cells were stained by FITC-conjugated Annexin V and PI using an Apoptosis Detection Kit (Cell Signaling Technology) and analyzed with a Cytomic FC500 (Beckman Coulter, CA, USA).

2.5. Hoechst staining

After treatment, the cells were fixed with pre-chilled PBS containing 1% formaldehyde for 30 min and then washed with PBS. The fixed cells were stained with Hoechst dye (1 μg/ml; Sigma Aldrich) for 15 min in the dark. The stained cells were washed and analyzed under a fluorescence microscope.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

To analyze gene expression in H9c2 cells, qRT-PCR was performed after stimulation with tunicamycin or tunicamycin and gemigliptin using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sets for qPCR were as follows: TNF-α, 5′-ACA TGG GCT CAT ACC AGG GC-3′ (forward) and 5′-GCC TCC TCT CTG CA TCA AG-3′ (reverse); IL-6, 5′-TGC TTT CAA GAT GAG TTG GAT GG-3′ (forward) and 5′-CCC AAC TTC CAA TGC TCT CC-3′ (reverse); IL-1β, 5′-ACA CGG GTT CCA TGG TGA AG-3′ (forward) and 5′-TCA GCA CCT CTC AAG CAG AGC-3′ (reverse); BNP, 5′-TGG GCA GAA GAT AGA CCG GA-3′ (forward) and 5′-ACA ACC TCA GCC CGT CAC AG-3′ (reverse) (Johns et al., 2004); SDF-1, 5′-CCA GAG CCA ACG TCA AAC ATC-3′ (forward) and 5′-GGG TCA ATG CAC ACT TGT CTG-3′ (reverse); and β-actin, 5′-GCT GAC AGG ATG CAG AAG GAG-3′ (forward) and 5′-GAG CCA CCA ATC CAC ACA GAG-3′ (reverse). The thermal profile conditions were as follows: pre-incubation at 95 °C for 10 min followed by 40 cycles of amplification at 95 °C for 10 s and 58 °C for 20 s. Gene transcript levels were calculated by the $2-\Delta\Delta CT$ method.

2.7. Statistical analysis

ANOVA was used for statistical comparisons. All statistical analyses were evaluated using SPSS program, and results are expressed as mean ± SD. All statistical data were calculated from a minimum

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