Emulsified isoflurane alleviates rat islet beta cell RIN-m5F apoptosis induced by glucose via inhibiting endoplasmic reticulum stress

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Abstract

Background Diabetes mellitus (DM) is a critical disease that considered a detriment to the health of people all over the world. Endoplasmic reticulum stress (ERS) is the response cause by endoplasmic reticulum misfolded and unfolded protein aggregation, which induces cell apoptosis. Our previous work showed that Elso could alleviate ERS in lung reperfusion injury. This study aimed to elucidate whether Emulsified isoflurane (Elso) could alleviate apoptosis induced by glucose in rat islet beta cell RIN-m5F via inhibiting ERS. **Methods** RIN-m5F cells were divided into five groups: Control group, cultured in 0.1M glucose for 24h (0.1G group), culture in 0.3M glucose for 24h (0.3G group), culture in 0.3M glucose with 57uM Elso for 24h (0.3G+57E group), and culture in 0.3M glucose with 76uM Elso for 24h (0.3G+76E group). First, the cellular proliferation was measured by MTT assay, and the level of insulin secretion was measured with ELISA kit. Second, the expression of Bax and Bcl-2 were detected by Western blotting. The level of caspase-3 activity was assessed by colorimetric method. Finally, the CHOP and GRP78 expression were detected by Western blotting. The level of ATF6, Xbp1 and eIF2 α mRNA were assessed by qRT-PCR after treated with Elso for 24h. **Results** High glucose induced significant loss of RIN-m5F cell viability, and stimulated the secretion of insulin; Elso improved the survival and protected the function of RIN-m5F. Compare to 0.3G group, treatment with Elso inhibited the activity of caspase-3, decreased the expression of Bax and increased the expression of Bcl-2. The expression of CHOP and GRP78 were inhibited by Elso at 24 h after treatment, and decrement of CHOP and GRP78 expression were correlated with Elso concentration. The level of ATF6, Xbp1 and eIF2 α mRNA of RIN-m5F were enhanced culture with high glucose, but only eIF2 α mRNA was decreased by Elso treatment. **Conclusion** High glucose induces rat islet beta cell RIN-m5F apoptosis and aggravates the function of beta cells. Elso protects beta cells from glucose-induced apoptosis, and anti-apoptosis is mediated, at least in part, by inhibiting ERS.

Background

Diabetes mellitus (DM) are series of diseases feature with hyperglycemia; the most common forms of DM are type 1 DM (T1DM) and type 2 DM (T2DM). In China in 2013, the estimated overall prevalence of DM among adults was 10.9% and that of prediabetes was 35.7% [1]. Seriously, result of obesity, unhealthy diet, sedentary lifestyle and genetic factors, the prevalence of T2DM in adolescents and young adults has been dramatically increasing[2].

In T2DM patients, during insulin resistance and impending diabetes, the pancreatic beta cell capable to synthesize and secrete sufficiently insulin for glucostasis[3]. Regretfully, excessive synthesis of insulin trigger multiple pathological reactions[4-6], which cause progressive loss of beta cell function, insulin deficiency and overt T2DM if there are no effective interventions. Endoplasmic reticulum stress (ERS) is a adaptive response during stress, in T2DM patients it can be triggered by an imbalance between the folding capacity of the Endoplasmic reticulum (ER) and irreparably unfolded and misfolded proteins aggregate in lumen cause by excessive pro-insulin synthesis[7]. Recent studies authenticated that ERS involved in the development of insulin resistance and progression to T2DM, and inhibit ERS would protect the function of beta cells[7, 8].

Emulsified isoflurane (Elso), emulsified preparation of isoflurane, has marked anesthetic potency and comparable safety index and certain safety factor as propofol in patients and animals[9, 10]. Furthermore, researches demonstrated that Elso had extensive protection in organs, including liver, lung and kidney [11, 12]. Whether Elso could protect pancreas remains unknown. Our previous study showed that Elso administered before ischemia could protect lung against reperfusion injury through inhibiting ERS pathway (published in Chinese). Therefore, we hypothesized that Elso could contribute to increasing beta cell mass by inhibiting ERS.

Methods

Cell Culture and treatment

Rat islet beta cell line (RIN-m5F) was purchased from China Center for Type Culture Collection (CCTCC). The cells were cultured in RPMI-1640 (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37°C and a 5% CO₂ atmosphere. RIN-m5F cells were then randomly divided into five doses of glucose and Elso for different culture. Elso (8%) was obtained from the Laboratory of Anesthesiology and Critical Care Medicine, West China Hospital of Sichuan University (Chengdu, China). On attaining 70-80% confluency, cells were seeded into 6-wellplate at a density of 2×10^6 and grown over night. We choose glucose with final concentrations of 0.1M (0.1G group) and 0.3M (0.3G group) to research the influence of high glucose to RIN-m5F cells. We use 57uM (0.3G+57E group) and 76uM (0.3G+76E group) Elso to study the Elso-protection based on preliminary experiments.

Cell viability assay

Indicated cells were cultured in 96-well plates, followed by drug exposure for 24h. The rate of cell apoptosis was examined by MTT assay. Briefly, after exposure to glucose and Elso, 200ul of MTT solution (0.5mg/ml, Sangon Biotech, Shanghai, China) was subsequently added. After 4h, 100 ul of dimethyl sulfoxide was added and mix gently for 10minutes. Then, measure the absorbance at 570 nm using microplate reader.

Insulin level assay

Cells were seeded overnight in 6-well plates and treated as indicated. Insulin levels in culture medium were quantified using an insulin rat ELISA kit ((Beyotime Biotechnology, Nantong, China) according to the manufacturer's instructions.

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase-3 Activity Assay kit (Beyotime Biotechnology, Nantong, China) following the manufacturer's instructions. In brief, the protein samples from cells were obtained and 100 µg protein was added to a reaction buffer containing Ac-DEVD-pNA (2mM), incubated at 37°C for 2 h. the absorbance of yellow pNA (the cleavage product) was measured with microplate reader at 405nm. The caspase-3 activity was recorded as the ratio to that of the control group.

Western blotting analysis

After treatment, cellular protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Nantong, China) containing 1mM PMSF for 30 minutes on ice. The supernatant containing soluble total protein was collected after centrifuge at 12,000rpm for 10min at 4°C. The BCA Protein Assay Kit (Sangon Biotech, Shanghai, China) was used to evaluate the protein concentrations. Approximately 50ug of protein was separated by electrophoresis in 12% SDS-polyacrylamide gels, and were subsequently transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk powder in 0.01M PBS (pH 7.4) and 0.05% Tween-20 at room temperature for 1h. Subsequently, they were incubated with primary antibodies against Bax, Bcl-2, GPR78, CHOP (Absin Bioscience, Shanghai, China, 1:1000) and GAPDH (Santa Cruz, USA, 1:1000) overnight at 4°C. Membranes were washed five times for 5min with PBST and then incubated with the appropriate HRP-conjugated secondary antibody at room temperature for 2h. The expression was detected by enhanced chemiluminescence (ECL) Detection Kit (Absin Bioscience, Shanghai, China) using G:BOX F3 Gel Documentation System (Syngene, United Kingdom). Quantification of the band intensities was performed with ImageJ.

Quantitative real-time polymerase chain reaction

The expression of mRNA was detected by Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from cells after treatment with Trizol reagents (Invitrogen, USA) according to the manufacturer's protocol. The concentration of total RNA was measured by spectrophotometer (thermoscientific, USA). cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Japan). Then cDNA was used as template for qPCR with Premix Ex Taq $\square$ (Takara, Japan) on Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, CA). The mRNA levels were normalized to GAPDH. Relative quantification was achieved by the comparative $2^{-\Delta\Delta Ct}$ method. The nucleotide sequences of the PCR primers were as follows (5' to 3'): GRPDH forward-AGTGCCAGCCTCGTCTCATA, GRPDH reverse-TACGGCCAAATCCGTTTACACA, ATF6 forward-GAGTCGCCTTTTAGTCCGGT, ATF6 reverse-ACTCCCAGTCTTCACCTGGTC, Xbp1 forward-GTCCGCAGCACTCAGACTAC, Xbp1 reverse-ATCTGAAGAGGCAACAGCGT, Eif2 α forward-GCAATGGAGAAAATTTGCCTTGA, Eif2 α reverse-TCATCTGACCAGGAAGGACAC (Sangon Biotech, Shanghai, China).

Statistical analysis

Data were analyzed using SPSS 19.0 (SPSS Inc.,Chicago,USA). Data are presented as mean $\pm$ standard deviation. Different experimental groups were compared by Student's t test. Two-tailed values value of $P<0.05$ was considered statistically significant.

Results

Elso improves the survival and protects the function of RIN-m5F

We evaluated the influence of high glucose on the morphology in RIN-m5F cells, after exposed to 0.1M and 0.3M glucose for 24 hours, cell shrinkage appeared, quantity and rate of cellular attachment were reduced. Cells shrinkage and rounded off were significantly decreased, quantity and rate of cellular attachment were significantly increased when 57uM or 76uM Elso was added (Fig.1A).

In addition, increasing of glucose in the culture media induced significant loss of cell viability, as estimated by the MTT assay, and in a concentration-dependent manner. Compare to control group, the viability significantly decreased in 0.1G group (0.9603 ± 0.052 , $P<0.05$). Viability of 0.3G group also markedly decreased compare to control group (0.5061 ± 0.0140 , $P<0.05$). Compare to 0.3G group, 57uM Elso (1.2717 ± 0.0392 , $P<0.05$) improved the survival of RIN-m5F, and 76uM Elso (1.4935 ± 0.0262 , $P<0.05$) also improved the survival (Fig.1B). Indicating Elso improves the survival of RIN-m5F cells.

Besides, we assessed the impact of Elso on insulin secretion of RIN-m5F cells, we measured the insulin concentration in the culture medium after 24h treatment. The results suggested that the insulin secretion was significantly enhanced after 24h cultivation with 0.1M (17.76 ± 2.20 , $P<0.05$) and 0.3M (20.88 ± 1.72 , $P<0.05$) glucose compare to control group (12.98 ± 1.65). In contrast, cells with 57uM (18.12 ± 2.19 , $P<0.05$) and 76uM (15.92 ± 1.66 , $P<0.05$) Elso have maintained the insulin secretion capacity compare to 0.3G group (Fig.1C).

Elso protects RIN-m5F from glucose-induced apoptosis

Above results showed high glucose diminished the viability of RIN-m5F, and the reduction was reversed by treatment with Elso. Then, we investigated the mechanism of high glucose damage. First, Ac-DEVD-pNA was used as the catalytic substrate for caspase-3 activity detection. Compared to control group, caspase-3 activity increased in 0.1G group (1.72 ± 0.10 , $P<0.05$), and reached a maximum value (1.97 ± 0.16 , $P<0.05$) in 0.3G group. After Elso treatment, compared to 0.3G group, caspase-3 activity decreased in 0.3G+57E group (0.91 ± 0.02 , $P<0.05$), and marked decreased in 0.3G+76E group (0.70 ± 0.15 , $P<0.05$)(Fig.2A).

Besides, we detected the expression of biomarkers of apoptosis: Bcl-2 and Bax. Results showed in 0.1G group (1.54 ± 0.11 , $P<0.05$) the high glucose environment induced Bax expression elevated than control

group (1.27 ± 0.17), and significant increased in 0.3G group (1.76 ± 0.06 , $P < 0.05$) relative to the control group. In contrast, Bax expression was inhibited by treatment with 57uM (1.53 ± 0.11 , $P < 0.05$) and 76uM (1.44 ± 0.12 , $P < 0.05$) Also for 24h compared to 0.3G group. Compare to control group, high glucose environment induced Bcl-2 expression decrease, and in a concentration-dependent manner (0.86 ± 0.06 vs. 0.64 ± 0.11 , 0.86 ± 0.06 vs. 0.61 ± 0.08 , $P < 0.05$). But, there no difference was detected between 0.3G and Elso treatment group (Fig.2B).

Elso alleviates apoptosis of RIN-m5F by inhibiting ERS

To verify the hypothesis that ERS involves in the protection of Elso, we detected the expression of GRP78 and CHOP. Compared to control group, 0.1M glucose in the culture media enhanced the expression of CHOP (0.37 ± 0.06 vs. 0.61 ± 0.07 , $P < 0.05$), also enhanced in 0.3G group (0.37 ± 0.06 vs. 0.87 ± 0.07 , $P < 0.05$). But expression of GRP78 had no difference between control group and 0.1G group (0.60 ± 0.06 vs. 0.70 ± 0.08 , $P > 0.05$), increase of GRP78 expression appeared in 0.3G group compared to control group (0.60 ± 0.06 vs. 0.83 ± 0.05 , $P < 0.05$). To our excited, all the expression of GRP78 and CHOP were inhibited with Elso treatment for 24h. Respectively, Compare to 0.3G group, GRP78 expression significantly decreased in 57uM (0.83 ± 0.05 vs. 0.66 ± 0.06 , $P < 0.05$) and 76uM (0.83 ± 0.05 vs. 0.45 ± 0.06 , $P < 0.05$) Elso, the CHOP expression also decreased in 57uM (0.87 ± 0.07 vs. 0.46 ± 0.10 , $P < 0.05$) and 76uM (0.87 ± 0.07 vs. 0.31 ± 0.10 , $P < 0.06$) Elso (Fig.3A).

Finally, we detected the expression of element of ERS canonical signaling pathway: ATF6, Xbp1 and eIF2 α . Results showed that high glucose increase the mRNA expression of ATF6, Xbp1 and eIF2 α . Respectively, Compare to control group, mRNA expression of ATF6 increased in 0.1G (1.53 ± 0.15 , $P < 0.05$) and in 0.3G (1.64 ± 0.16 , $P < 0.05$), mRNA expression of Xbp1 increased in 0.1G (1.62 ± 0.24 , $P < 0.05$) and in 0.3G (1.77 ± 0.13 , $P < 0.05$), mRNA expression of eIF2 α increased in 0.1G (2.94 ± 0.44 , $P < 0.05$) and in 0.3G (6.71 ± 0.43 , $P < 0.05$). After treated with Elso for 24h, the expression of eIF2 α mRNA was inhibited, and in a concentration-dependent manner. Compare to 0.3G group, expression of eIF2 α mRNA were significantly decreased by 57uM Elso (6.71 ± 0.43 vs. 5.61 ± 0.36 , $P < 0.05$) and 76uM (6.71 ± 0.43 vs. 3.81 ± 0.41 , $P < 0.05$) Elso. But, there no difference was detected between 0.3G and Elso treatment group in the expression of ATF6 mRNA, Xbp1 mRNA (Fig.3B).

Discussion

Glucotoxicity, which refers to irreversible damage of islet beta cells, plays an important role in the development of DM[13]. There are lots of articles involved in glucose induced apoptosis about rodent primary islet cells, INS-1 cells and MIN6 cells[14, 15]. But few articles pay attention to rat islet beta cell RIN-m5F. Thus, in this study, we validated the damage of glucose on RIN-m5F. Our results showed that 0.1M and 0.3M glucose stimulate the secretion of insulin, alter the morphology and decrease the viability within 24h treatment. Kornelius et al. also revealed that exposure of RIN-m5F cells to high glucose and

FFA levels increase apoptosis[16]. The effect of glucotoxicity on RIN-m5F was explicit, we next sought the protection against glucotoxicity.

Isoflurane is an isomer of enflurane, which was commonly used in inhalation anesthesia. But, isoflurane can induce neurogenetic damage and neurocognitive disorder, even accelerate the process of Alzheimer's disease[17-19]. With a view to the adverse effects of inhalational anesthetic compared to intravenous anesthetic, Elso was manufactured by West China Hospital of Sichuan University, which liquid isoflurane was mixed with intralipid at the final concentration of 8% (v/v). Except marked anesthetic potency and safety, Hu et al. demonstrated that Elso pretreatment protects the myocardial ischemia and reperfusion injury in rats, which may be mediated by the inhibition of apoptosis and cell damage [20]. In this study, we found that Elso protects the morphology and enhances the viability of RIN-m5F and restores the synthesis of insulin in 0.1M and 0.3M glucose treatment. Besides, our results showed Elso inhibits the activity of caspase-3. Caspase-3 is a predominant player in the execution of apoptosis, which can target key structural and regulatory proteins to bring about apoptotic cell death[21]. Cao et al. indicated that Caspase-3 is critical for the induction of MIN6 cells apoptosis and its activation is further confirmed to be related to the NF- κ B-mediated Bcl-2 down-regulation[22]. B cell leukemia/lymphoma 2 (Bcl-2) family proteins are the key to the regulation and execution of apoptosis, among them, Bcl-2 is a pro-survival protein, and Bcl2 associated X (Bax) is a pro-apoptotic protein[23]. In this study, we found that high glucose induced Bcl-2 expression decrease and increase Bax expression, but Elso down-regulated the expression of Bax and up-regulated the expression of Bcl-2. It seems Elso protected RIN-m5F against glucotoxicity may be relevant to apoptosis.

The mechanisms of apoptosis are highly complex, involving a cascade of molecular events. Increased protein synthesis, imbalance of ER calcium levels, glucose and energy deprivation that trigger ERS can induce beta cell failure by UPR[24]. Glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) are the best studied inducers of ERS. GRP78 is an important sensor of misfolded proteins in ER lumen. When misfolded proteins accumulated, GRP78 was competed binding by misfolded proteins with EIF2AK3, which resulted in protein kinase RNA (PKR)-like ER kinase (PERK) activation and phosphorylation of eukaryotic translation initiation factor-2 (eIF2 α)[25]. A increased availability of GRP78 may re-establish homeostasis of endoplasmic reticulum by attenuating UPR[26]. UPR protects cells from stress and contributes to the re-establishment of endoplasmic reticulum homeostasis; however, prolonged UPR promotes CHOP expression. CHOP induces the expression of growth arrest and DNA damage 34 (GADD34), activates death receptor 5 (DR5) and decreases Bcl-2 anti-apoptotic protein[27]. In this study, we demonstrated that Elso increased the expression of GRP78 both in the concentration of 57 μ M and 76 μ M with 24h treatment. In addition, Elso decreased CHOP expression in 57 μ M and 76 μ M. ERS trigger downstream response mediated by three canonical signaling sensors: protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 α (IRE1 α), and activating transcription factor-6 (ATF6)[28]. Next, we detected the mRNA expression of ATF6, X-box-binding protein 1 (Xbp1) and eIF2 α . Xbp1 is a molecule that mediated with the activation of IRE1 α . Our results show that high glucose increased the expression of ATF6, Xbp1 and eIF2 α , and eIF2 α mRNA expression decreased after treated

with 57uM and 76uM Elso. These results provide the evidence that Elso protects RIN-m5F may via, at least in part, inhibiting ERS.

Conclusion

The current study demonstrated ERS-mediated apoptosis was induced by high glucose in rat islet beta cell RIN-m5F. Elso promoted RIN-m5F function and anti-apoptosis by inhibiting ERS. The application of Elso during operation of DM patients may protect the function of pancreas and impede the stress hyperglycemia.

Abbreviations

DM: Diabetes mellitus; ERS: Endoplasmic reticulum stress; Elso: Emulsified isoflurane; UPR: unfolded protein response; Bcl-2: B cell leukemia/lymphoma 2; Bax: BCL2 associated X; GRP78: Glucose regulated protein 78; CHOP: C/EBP homologous protein; PERK: protein kinase RNA (PKR)-like ER kinase; IRE1 α : inositol-requiring enzyme 1 α ; ATF6: activating transcription factor-6; Xbp1: X-box-binding protein 1; eIF2 α : eukaryotic translation initiation factor-2.

Declarations

Ethics and Consent to Participate

Not Applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WD and SW designed the research; WD and ZY did the most part of the experiments; JZ analyzed experimental results and interpreted data; JS and MY helped in molecular experiments. All authors read and approved the final manuscript.

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Not applicable.

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Figures

Figure 1

Viability and function of RIN-m5F after cultured with different concentrations of glucose or glucose+Elso for 24 hours. (A) Microscopic images of cells taken after 24 hours of treatment (10X). (B) Viability analysis with MTT assay of RIN-m5F after treatment. (C) Insulin secretion of RIN-m5F after treatment. *P < 0.05 vs. control group; #P < 0.05 vs. 0.3G group.

Figure 2

Apoptosis analysis of RIN-m5F with different concentrations of glucose and glucose+Elso for 24 hours. (A) Caspase-3 activity analysis of RIN-m5F after glucose and glucose+Elso treatment for 24 hours. (B) Bax and Bcl-2 expression of RIN-m5F after glucose and glucose+Elso treatment for 24 hours. *P < 0.05 vs. control group; #P < 0.05 vs. 0.3G group.

Figure 3

analysis of ERS canonical signaling pathway of RIN-m5F with different concentrations of glucose and glucose+Elso treatment for 24 hours. (A) Detection of GRP78 and CHOP expression of RIN-m5F after treatment for 24 hours. (B) mRNA level analysis of ATF6, eIF2α and Xbp1 of RIN-m5F after glucose and glucose+Elso treatment for 24 hours. *P < 0.05 vs. control group; #P < 0.05 vs. 0.3G group.