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Original Article

DR5 but not miRNA-181 or miRNA-211 is involved in ER stress-mediated apoptosis induced by palmitate in islet β cells

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Abstract: Free fatty acid (FFA)-induced apoptosis and endoplasmic reticulum (ER) stress of pancreatic β-cells contribute to the development of type 2 diabetes; however, the molecular mechanisms involved are not explicated clearly. In this study we have investigated the role of miRNA-181 and miRNA-211 and DR5 in saturated FFA-induced apoptosis of the rat pancreatic β-cells INS-1. Rat pancreatic β-cells INS-1 were treated with or without palmitate for 24 h. CCK-8 was used for cell viability detection. The miRNA-181 and -211 and mRNA expression of GRP78, ATF4, CHOP and DR5 were explored by quantitative real-time PCR. We found that palmitate at apoptosis-inducing concentration activated ER stress signaling pathways and the expressions of GRP78, ATF4 and CHOP were significantly increased after stimulation in INS-1 cells. In addition, mRNA level of DR5 was also upregulated in palmitate treated group. But the expressions of miRNA-181 and -211 did not significantly changed during lipoapoptosis by palmitate in INS-1 cells. We have demonstrated that miRNA-181 and miRNA-211 are not key players in ER stress-related apoptosis induced by saturated FFA in rat pancreatic β cells INS-1. However, DR5 appears to be involved in the modulation of saturated FFA-induced apoptosis of INS-1 cells, probably by a mechanism dependent on ER stress signaling.

Keywords: Lipotoxicity, β cell, ER stress, death receptor 5, miRNA-181, miRNA-211

Introduction

Type 2 diabetes (T2D) is a chronic metabolic disorder that results from defects in both insulin secretion and insulin action [1]. The major defect leading to a decrease in islet β cell mass with insufficient insulin secretion in type 2 diabetes is increased apoptosis [2]. It has been reported that fatty acid-induced islet β cell apoptosis is a link between obesity and T2D [3]. The prevalence of obesity and T2D is rising dramatically recent years, leading to heavy medical and economic burden associated with these disorders and their related complications [4]. Revealing the specific mechanism of fatty acid-induced islet β cell apoptosis helps to develop adaptive strategies for the prevention and therapy of T2D. The deleterious effects of free fatty acids (FFA) on islet β cells is known as lipotoxicity and FFA-induced endoplasmic reticulum (ER) stress contributes to β cell apoptosis of T2D [5, 6]. Sustained FFA exposure induced irreversibly fatal ER stress by activating activated transcription factor 4 (ATF4)/*C/EBP* homologous protein (CHOP) pathway [7, 8].

MicroRNAs (miRNAs) are ~22 nucleotide-long endogenous, non-coding RNAs that generally silence gene expression post-transcriptionally. MiRNAs play a key role in the regulation of gene expression in vital biological events such as cell proliferation, differentiation, death and malignant transformation through translational repression and/or mRNA destabilization [9]. MiRNAs are regulators of β cell differentiation and function, inappropriate islet miRNAs expression acts as a potential cause of T2D [10]. Previous studies suggest that part of the pro-apoptotic effects of palmitate on β cells is caused by the expression change of miRNA-34a, miRNA-146 [11] and miRNA-375, which is reported to enhance palmitate-induced lipo-

apoptosis in insulin-secreting NIT-1 cells [12]. These studies make miRNAs as novel therapeutic targets with the potential effects of protecting the β cells. Besides, miRNAs have diversified regulatory effects on ER stress response [13]. They regulate ER stress through the trafficking machinery, the calcium homeostasis machinery, the folding and quality control machinery or targeting the transducers of ER stress pathways [14-19]. Thereinto, increased levels of miRNA-181 repress glucose-regulated protein 78 (GRP78), a resident chaperone assisting protein folding and inactivating ER stress transducers through combining with them in ER, by translational arrest [20]. Proximal CHOP promoter is the directly target of miRNA-211 and the expression of CHOP is negatively regulated by miRNA-211 [21].

Death receptor 5 (DR5) is a receptor of cytotoxic ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and trigger the extrinsic apoptotic pathway via TRAIL-DR5 complex [22]. But it has been demonstrated that persistent ER stress induces DR5 transcription via the UPR mediator CHOP, driving ligand-independent DR5 activation and apoptosis engagement [23]. In addition, ER stress-induced CHOP activation by palmitate transcriptionally up-regulates DR5, likely resulting in ligand-independent hepatocyte lipoapoptosis by this death receptor [24].

In this study, we explored the potential contribution of miRNA-181 and -211 which targeted at ER stress signaling molecules GRP78 and CHOP respectively during lipoapoptosis by saturated FFA (palmitate) and whether DR5 was involved in this lipoapoptosis in rat pancreatic β cell line INS-1.

Methods

Reagents

RPMI 1640 medium and fetal bovine serum were products of Gibco (USA). Free fatty acid (FFA)-free bovine serum albumin (BSA) was purchased from Equitech Bio (USA). Sodium palmitate, sodium pyruvate, glutamine, HEPES and β-mercaptoethanol were got from Sigma (USA). Penicillin-streptomycin solution was obtained from Beyotime (China). Transcriptor First Strand cDNA Synthesis Kit and Faststart Universal SYBR Green Master (ROX) were taken from Roche (Germany). Cell Counting Kit-8 (CCK-8) was bought from Dojindo (Japan). E.Z.N.Z. miRNA Kit was purchased from OMEGA (USA).

Preparation of palmitate

Palmitate-albumin mixture was prepared before adding into INS-1 cells [25]. Briefly, a 5 mM palmitate/5% BSA (5 mM PA) stock solution was prepared by dissoluting 0.5 mmol sodium palmitate with 100 ml 5% FFA-free BSA solution in a 55°C water bath for complete dissolution.

The stock solutions could stored in -20°C after sterile filtration for 3-4 weeks stably. During experiment, 5 mM PA was diluted in culture medium.

Cell culture

The rat insulinoma cell line INS-1 was purchased from basic medical institution of Chinese Academy of Medical Science, China. Cells were cultured in RPMI 1640 medium containing 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 50 μmol/L beta-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 11 mmol/L glucose at 37°C in an atmosphere of 5% CO_2 [26]. For different experiments, cells were cultured with FBS-containing medium in 6-well plates or 96-well plates until reaching 80% confluence. Then they were divided into two groups, the control group and the PA group. In the control group, cells were cultured with RPMI 1640 containing 0.25% BSA for 24 h; in the PA group, cells were cultured with RPMI 1640 containing 0.25 mM/0.25% BSA for 24 h.

Detection of cell viability

INS-1 cells were seeded in 96-well and treated with or without PA for 24 h. Cell viability was detected by CCK-8 as it was discribed in the manufacture's protocols. Absorbance of 96-well plates was measured on 450 nm using microplate reader.

Isolation and analysis of RNA

MicroRNAs and mRNAs were isolated from INS-1 cells using E.Z.N.Z. miRNA Kit (OMEGA, USA). Transcriptor First Strand cDNA Synthesis Kit was used for miRNA reverse transcription

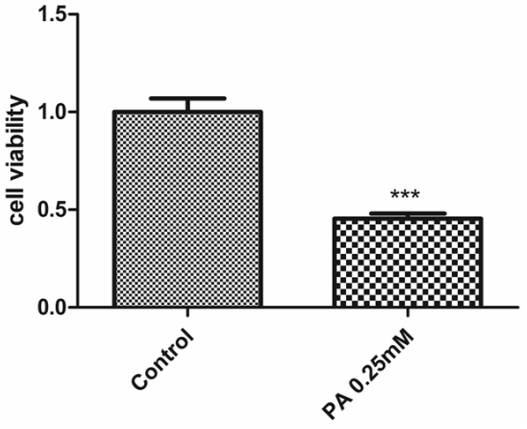

Figure 1. The viability of INS-1 cells was decreased under lipotoxicity. Cell viability of INS-1 was tested by Cell Counting Kit-8 under lipotoxicity. INS-1 cells were stimulated by 0.25 mmol/L palmitate (PA) for 24 hours. And results showed that PA treated group had significant lower cell survival rate than the control group. ***P<0.001.

by miRNA-special stem-loop reverse transcription primer and for mRNA reverse transcription by anchored- oligo$(dT)_{18}$ primer. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on Bio-Red CFX96 Touch™ Real-Time PCR Detection System with the following cycling parameters: polymerase activation (10 min at 95°C) and amplification (40 cycles of 15 s at 95°C and then 1 min at 60°C). Faststart Universal SYBR Green Master (ROX) was used for the detection and quantification of genes that were expressed as mRNA or miRNA, and the level was normalized to β-actin or U6 respectively using ΔΔCt method.

Primer sequences for qRT-PCR

DR5: Forward 5'-TCTCATGCGGCATATGGGTC-3', Reverse 5'-CCCGTTTG GTAGAGCCACTT-3'; CHOP: Forward 5'-TCACAAGCACCTCCCAAAG-3', Reverse 5'-CGCACTGACCACTCTGTTTC-3'; GRP78: Forward 5'-CTCCGGC GTGAGGTAGAAAA-3', Reverse 5'-AGAGCGGAACAGGTCCATGT-3'; ATF4: Forward 5'-GTTGGTCAGTGCCTCAGACA-3', Reverse 5'-CATTCGAAACA GAGCATCGA-3'. β-actin primers were purchased from Sangon Biotech (Shanghai, China; Order No. B661201). U6: Forward 5'-ATTGGAACGATACAGAGAAGATT-3', Reverse 5'-GGAA CGCTTCACGAATTTG-3'; miRNA 181: Forward 5'-TGCCGAACATTCAAC GC T-3', Reverse 5'-CAGAGCAGGGTCCGAGGTA-3'; miRNA 211: Forward 5'-ATT GGAACGATACAGAGAAGATT-3'; Reverse 5'-GGAACGCTTCACGAATTG-3'.

Statistical analyses

Statistical analyses were performed with SPSS 20.0 for Windows. Data was present as mean ± standard error (SE) and were evaluated by unpaired, two-tailed Student's t test for differences between two groups. P<0.05 was considered statistically significant.

Results

The viability of INS-1 cells was decreased under lipotoxicity

In the study, the viability of INS-1 cells treated with or without 0.25 mmol/L PA for 24 h was detected by CCK-8. It was showed that cell survival rate was obvious decreased in the 0.25 mmol/L PA treated group compared with the control group (cell survivl rate in PA treated group was lower than 50% of the control group; ***P<0.001, **Figure 1**). This result indicated that INS-1 cells had increased apoptosis and decreased viability under lipotoxicity of PA, a saturated FFA.

ER stress was induced in INS-1 cells under lipotoxicity

We investigated the effects of PA on ER stress in INS-1 cells. The mRNA levels of genes related to ER stress (GRP78, ATF4 and CHOP) were quantified in PA treated group and control group by qRT-PCR. As shown in **Figure 2**, compared with the control group, after 0.25 mmol/L PA treated for 24 h, mRNA levels of GRP78, ATF4, CHOP were increased in INS-1 (GRP78 mRNA inseased nearly 3.3-fold, **P=0.001<0.05; ATF4 mRNA increased nearly 0.9-fold, *P= 0.02<0.05; CHOP mRNA increased nearly 3.3-fold, *P=0.02<0.05). These data showed that ER stress was induced in INS-1 cells uner lipotoxicity of PA.

miRNA-181 and -211 were not involved in ER stress response under lipotoxicity

To identify whether miRNA-181 and-211 were involved in the regulation of ER stress in β cells under lipotoxicity, we deteced the expressions of miRNA-181 and -211 in 0.25 mmol/L PA treated group and control group. But RNA levels

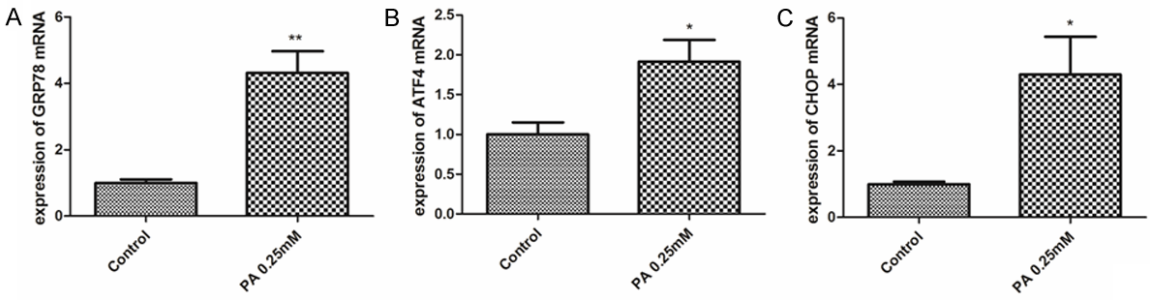

Figure 2. ER stress was induced in INS-1 cells under lipotoxicity. After 0.25 mmol/L PA treated for 24 h, mRNA expression levels of GRP78, ATF4 and CHOP (genes of endoplasmic reticulum (ER) stress signalings), were increased. A: The mRNA expression level of GRP78, **P=0.001<0.05 versus control group. B: The mRNA expression level of ATF4, *P=0.02<0.05 versus control group. C: The mRNA expression level of CHOP, *P=0.02<0.05 versus control group.

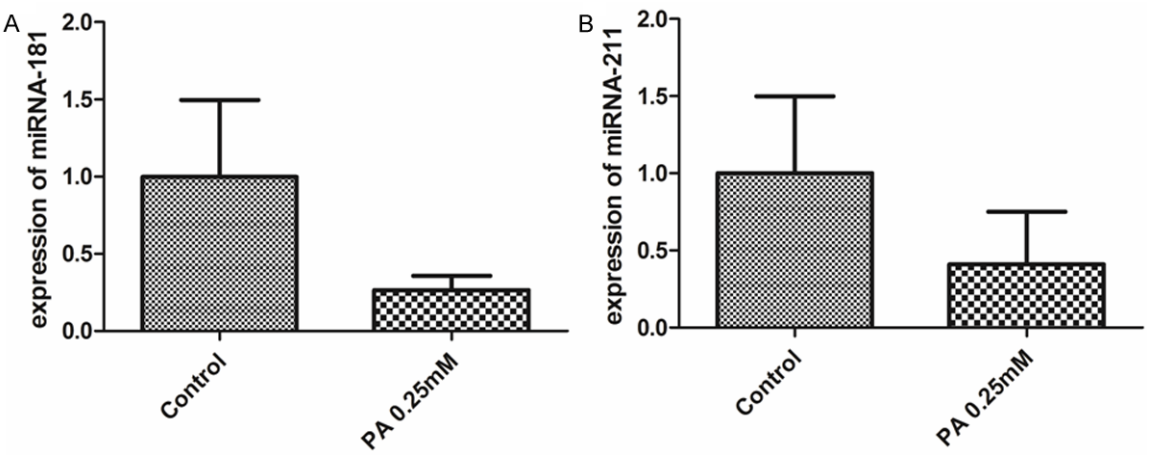

Figure 3. miRNA-181 and -211 were not involved in ER stress response under lipotoxicity. After 0.25 mmol/L PA treated for 24 h, expression levels of miRNA-181 and miRNA-211 were not significantly changed. A: The expression level of miRNA-181, P=0.219>0.05 versus control group. B: The mRNA expression level of miRNA-211, P=0.385>0.05 versus control group.

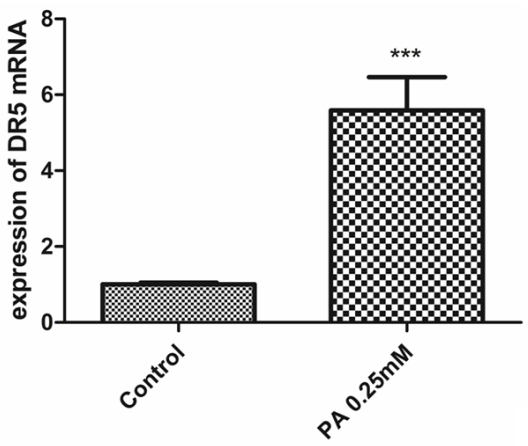

Figure 4. mRNA level of DR5 was increased under lipotoxicity. After 0.25 mmol/L PA treated for 24 h, mRNA expression levels of DR5 was increased, ***P=0.001<0.05 versus control group.

of miR-181 and miR-211 had no significant changes (P=0.219>0.05 and P=0.385>0.05 respectively; **Figure 3**) corresponding to their prospective targets GRP78 and CHOP. These data approved that miRNA-181 and -211 did not participate in the regulation of ER stress response in INS-1 cells under lipotoxicity of PA.

mRNA level of DR5 was increased under lipotoxicity

For further unveiling the regulatory mechanism of ER stress-induced apoptosis in β cells, we studied the mRNA expression of DR5 in INS-1 cells with or without PA treated. Interestingly, mRNA expression of DR5 in PA treated group was nearly 4.6-fold higher than the control group (***P=0.001<0.05; **Figure 4**). This result disclosed that DR5 may be involved in the re-

gulation of ER stress-induced apoptosis in β cells.

Discussion

ER stress-mediated apoptosis of β cell by saturated FFAs makes great contribution to the development of T2D, while unsaturated FFAs (e.g. oleate) can rescue β cells from palmitate-induced apoptosis by preventing the activtion of UPR and activating pro-survival pathways of the ER stress response [26, 27]. In this study, we identified the ER stress-mediated lipoapoptisis effect of saturated FFA (palmite) in rat pancreatic β cells INS-1. Previous researches have found that prolonged exposure to elevated saturated FFAs contributes to the development and progression of multiple complications of diabetes via ER stress pathways [28, 29] and mitigating ER stress by chemical chaperones or other drugs is favourable to restoring the impaired functions and attenuating apoptosis by FFAs in both β cells [30] and other cells [31, 32]. Hence, obese population with abmormal lipid metabolism and high risk of diabtes are suggested to lose weight, and control lipid with drugs if necesssary.

It is well known that miRNAs are key regulatory factors in β cell development, identity and disease [33]. Dysregulation of specific miRNAs is related to apoptosis of β cells and/or hyperglycemia. In our research, we did not prove miRNA-181 and miRNA-211 were regulators of ER stress-mediated apoptosis induction by palmitate in INS-1 cells. But anti-miRNA oligonucleotides (AMO) have presented impressive potential in human diseases and diabetes therapy [34, 35]. Besides, serum miRNAs are promising biomarkers for diabetes diagnosis [36, 37]. From this point of view, further studies should be conducted on miRNA regulatory network in β cells.

The 'activation of death receptors' mechanism of increased β cell apoptosis in type 1 diabetes (T1D) has been systematically reviewed by Johnson and Luciani [38]. The activation of death receptors was ligand-dependent in T1D. Recent studies on death receptors are mainly focused on cancer therapy [39]. But Cazanave and collegues' research showed that DR5 signaling was ligand-independently activated by ER stress-induced CHOP activation and promoted hepatocyte lipoapoptosis progress [24]. This finding provided new insight into lipoapoptosis. In our research, we found the DR5 was transcriptionally upregulated in ER stress-mediated apoptosis induction by palmitate in INS-1 cells. Activation of death receptors may also contribute to T2D, unlike in T1D, it would be ligand-independent but ER stress mediator CHOP-dependent.

In conclusion, we have demonstrated that miRNA-181 and miRNA-211 are not key players in ER stress-related apoptosis induction by saturated FFA in rat pancreatic β cells INS-1. However, the controlled expression of DR5 downstream of ER stress and its impact on β cells lipoapoptosis could constitute a novel signaling circuit whose regulation could be involved in the development of T2D.

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Disclosure of conflict of interest

None.

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