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Pioglitazone ameliorates palmitate induced impairment of mitochondrial morphology and function and restores insulin level in β cells

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This study aimed to investigate the effect of pioglitazone (PIO) on insulin secretion and mitochondrial ultrastructure and function in β cells. HIT-T15 cells were treated with control or palmitate (free fat acids, FFA) or/and PIO and divided into 7 groups: Control group; 0.5 mmol/l FFA (LF); 0.5 mmol/l FFA plus 10-7 mol/l PIO (LFLP); 0.5 mmol/l FFA plus 10-5 mol/l PIO (LFHP); 1.0 mmol/l FFA (HF); 1.0 mmol/l FFA plus 10-7 mol/l PIO (HFLP); 1.0 mmol/l FFA plus 10-5 mol/l PIO (HFHP). Apoptotic peaks, mitochondrial ultrastructure, ATP/ADP, mRNA levels of peroxisome proliferater activated receptor gamma coactivator-1 (PGC-1) and nucleus respiratory factor-1 (NRF-1) as well as insulin secretion were measured. The results showed that palmitate impaired mitochondrion structure, which could be alleviated by PIO. Palmitate could increase apoptotic peaks, decrease ATP/ADP ratio, enhance the expression of PGC-1mRNA and NRF-1mRNA, and decrease glucose stimulated insulin secretion (GSIS). In contrast, PIO could decrease apoptotic peaks, restore partly ATP/ADP ratio, decrease the expression of PGC-1 mRNA and NRF-1 mRNA, and increase GSIS level. These results demonstrate that PIO could ameliorate palmitate induced damage to mitochondrion ultrastructure and function and restore GSIS, accompanied by the modulation of PGC-1 and NRF-1 expression. These findings provide new insight into the hypoglycemic effects of PIO and help develop new agents for diabetes therapy.

1. Introduction

Pancreatic β-cell dysfunction is an important pathogenic hallmark of type 2 diabetes mellitus. The deficiency of β-cell secretion is highly related to mitochondrial dysfunction (Maechler et al. 1997). Thiazolidinediones (TZD) are compounds that act as the ligands of peroxisome proliferator–activated receptor gamma (PPARγ) and exhibit beneficial effects on glucose homeostasis via enhancement of insulin sensitivity and preservation of β-cell function. Pioglitazone (PIO) is one of TZDs currently used in the clinical and could improve insulin secretion in diabetic model mice and type 2 diabetes patients (Kutoh 2010).

It is known that increased levels of plasma free fatty acids (FFAs) in obese subjects are associated with insulin resistance and pancreatic β-cell dysfunction. Palmitate, a saturated FFA, exhibits cytotoxicity and induces the apoptosis of β-cells (Morgan 2009). In this study, we employed a well established hamster β-cell line HIT-T15 as the experimental model to investigate the effect of PIO on insulin secretion and mitochondrial ultrastructure and function in HIT-T15 cells treated with palmitate. In addition, we examined the level of ATP/ADP and the expression of peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1) and nuclear respiratory factor-1 (NRF-1) in HIT-T15 cells to explore the possible mechanism underlying the action of PIO.

2. Investigations and results

2.1. *PIO prevents palmitate induced apoptosis of HIT-T15 cells*

Palmitate is known to induce the apoptosis of β-cells (Morgan 2009). First we examined the effect of PIO on palmitate induced apoptosis of HIT-T15 cells. By flow cytometry and TUNEL staining, we observed that palmitate increased the apoptotic ratio compared with the control group (0.1% DMSO) ($P < 0.05$), and PIO lowered the apoptotic ratio induced by palmitate in a dose dependent manner ($P < 0.05$). The apoptotic ratios of all groups are shown in Table 1. Representative TUNEL staining results were shown with apoptotic cells stained in brown and non-apoptotic cells in blue (Fig. 1).

Next we examined the apoptotic morphology of HIT-T15 cells by electron microscopy. The results showed that compared with control group (Fig. 2A), the mitochondria in the cells of LF group were swollen and their ultrastructure was damaged (Fig. 2B). In addition, typical apoptotic morphology such as

Table 1: Effects of palmitate and PIO on apoptosis of HIT-T15 cells

Groups	Apoptotic ratio by FCM	Apoptotic ratio by TUNEL
Control	7.33 ± 0.58	5.65 ± 0.32
FFA 0.5 mmol/l	9.73 ± 0.38*	8.31 ± 0.32*
FFA 0.5 mmol/l + PIO10^{-7} mol/l	7.80 ± 0.35†	7.01 ± 0.41†
FFA 0.5 mmol/l + PIO10^{-5} mol/l	6.10 ± 0.17†	6.79 ± 0.45†
FFA 1.0 mmol/l	12.97 ± 0.73*†	10.80 ± 0.27*
FFA 1.0 mmol/l + PIO10^{-7} mol/l	10.20 ± 0.06‡	7.70 ± 0.23*‡
FFA 1.0 mmol/l + PIO10^{-5} mol/l	7.35 ± 0.26‡	6.59 ± 0.21‡

* $P < 0.05$ vs. control group (0.1% DMSO); †: $P < 0.05$ vs. FFA 0.5 mmol/l group; ‡: $P < 0.05$ vs. FFA 1.0 mmol/l group.

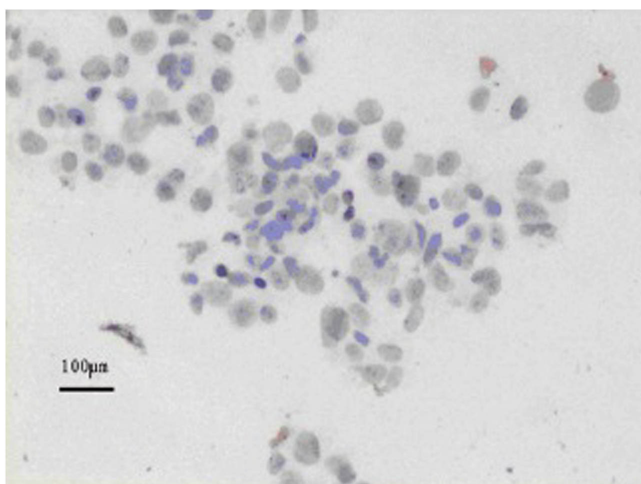

Fig. 1: TUNEL staining of HIT-T15 cells treated with FFA 0.5 mmol/l + PIO 10^{-7} mol/l. Apoptotic cells were stained in brown and non-apoptotic cells in blue. Magnification: X 100

nuclear shrinkage, chromatin condensation and assemble under karyotheca was observed in the cells of LF group (Fig. 2C). However, swollen mitochondria and apoptotic cells were much less in LFLP group (Fig. 2D) and LFHP group (Fig. 2E). These results are consistent with the results of flow cytometry and TUNEL analysis and together suggest that PIO prevents palmitate induced apoptosis of HIT-T15 cells.

2.2. PIO relieves palmitate induced decrease of ATP/ADP ratio in HIT-T15 cells

Compared with control group, ATP/ADP ratio in the cells of LF and HF groups decreased ($P < 0.05$). However, when the cells were treated with PIO, ATP/ADP ratio was elevated significantly compared to that of LF and HF groups ($P > 0.05$). The ATP/ADP ratios in the cells of all groups are shown in Table 2.

2.3. PIO restores insulin secretion of HIT-T15 cells treated by palmitate

After HIT-T15 cells were cultured for 48 h, total insulin levels in all treated groups increased compared with control group ($P < 0.05$), but GSIS declined in LF and HF groups compared with control group ($P < 0.05$). When the cells were treated with PIO, GSIS increased dose dependently in LFHP and HFHP groups compared with LF and HF groups, respectively ($P < 0.05$). Insulin secretion of HIT-T15 cells of all groups is shown in Table 3.

Table 2: Effects of palmitate and PIO on ATP/ADP ratio in HIT-T15 cells

Group	ATP/ADP ratio
Control	3.15 ± 1.14
FFA 0.5 mmol/l	0.70 ± 0.14*
FFA 0.5 mmol/l + PIO10^{-7} mol/l	1.71 ± 0.12†
FFA 0.5 mmol/l + PIO10^{-5} mol/l	1.58 ± 0.07†
FFA 1.0 mmol/l	0.41 ± 0.08*
FFA 1.0 mmol/l + PIO10^{-7} mol/l	1.80 ± 0.36‡
FFA 1.0 mmol/l + PIO10^{-5} mol/l	1.94 ± 0.61‡

*: $P < 0.05$ vs. control group (0.1% DMSO); †: $P < 0.05$ vs. FFA 0.5 mmol/l group; ‡: $P < 0.05$ vs. FFA 1.0 mmol/l group.

Table 3: Effects of palmitate and PIO on insulin secretion in HIT-T15 cells

Group	total insulin secretion (ng/ml)	GSIS(ng/ml)
Control	1.01 ± 0.10	1.03 ± 0.20
FFA 0.5 mmol/l	1.16 ± 0.20	0.35 ± 0.05*
FFA 0.5 mmol/l + PIO10^{-7} mol/l	1.20 ± 0.32	0.34 ± 0.07*
FFA 0.5 mmol/l + PIO10^{-5} mol/l	1.14 ± 0.01*	0.81 ± 0.3†
FFA 1.0 mmol/l	1.22 ± 0.20*	0.07 ± 0.00*
FFA 1.0 mmol/l + PIO10^{-7} mol/l	1.20 ± 0.00*	0.19 ± 0.09
FFA 1.0 mmol/l + PIO10^{-5} mol/l	1.16 ± 0.32*	0.67 ± 0.20‡

*: $P < 0.05$ vs. control; †: $P < 0.05$ vs. FFA 0.5 mmol/l group; ‡: $P < 0.05$ vs. FFA1.0 mmol/l group.

2.4. PIO inhibits palmitate induced upregulation of NRF-1 and PGC-1 expression in HIT-T15 cells

To explore the potential mechanism responsible for the effects of PIO we observed above, we examined the expression of NRF-1 and PGC-1 by real-time PCR. The relative copy numbers of NRF-1 mRNA and PGC-1 mRNA in different groups of HIT-T15 cells are shown in Table 4. It was obvious that palmitate could upregulate the expression of NRF-1 and PGC-1 at mRNA level in a dose dependent manner ($P < 0.05$ compared to control). However, when the cells were treated with PIO, the expression of NRF-1 mRNA and PGC-1 mRNA was downregulated, especially significantly decreased in HFHP group compared to HF group ($P < 0.05$). These data indicate that PIO inhibits palmitate induced upregulation of NRF-1 and PGC-1 expression in HIT-T15 cells.

3. Discussion

PIO, a TZD drug, has been shown to regulate gene expression by acting on PPARγ (Kawai et al. 2002; Lupi et al. 2004).

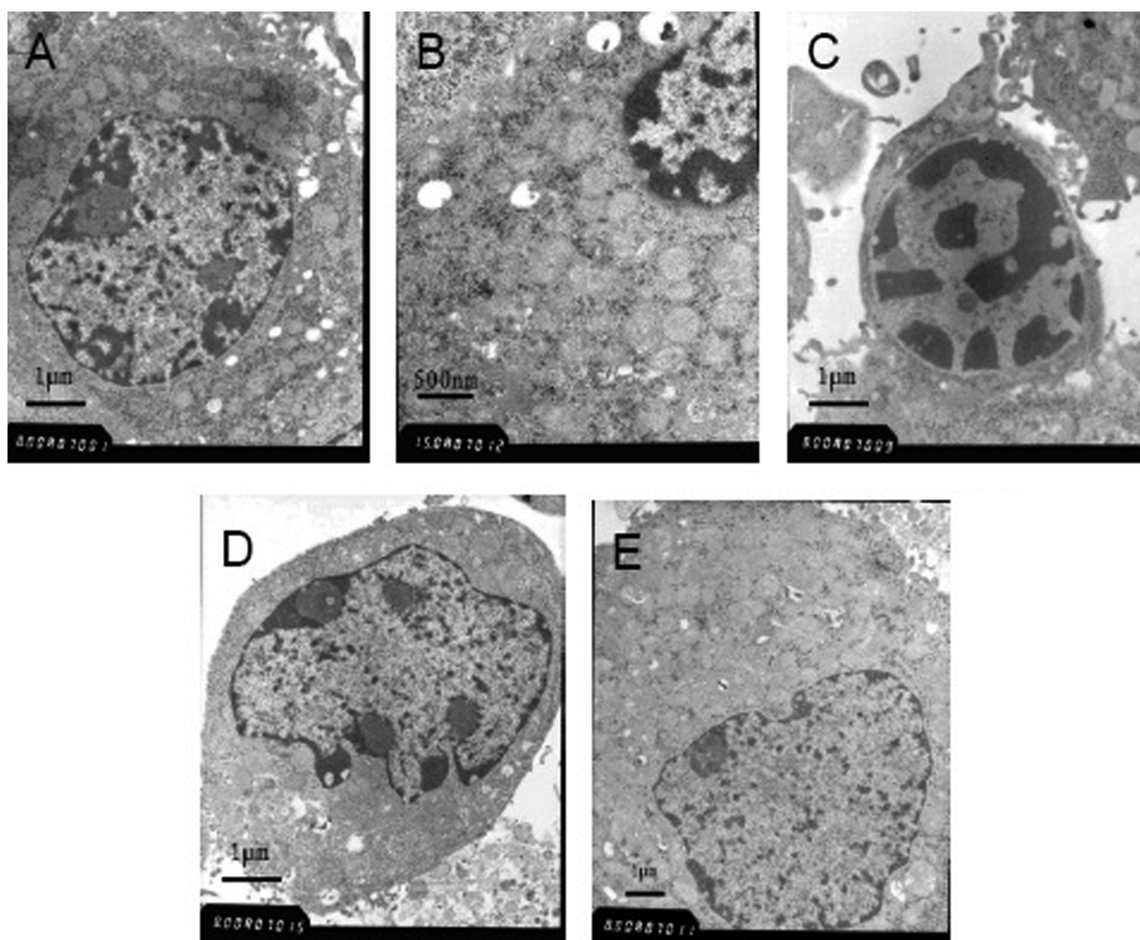

Fig. 2: Electron microscopy of the apoptotic morphology of HIT-T15 cells treated with FFA and PIO. A: control group (0.1% DMSO); B and C: LF group (FFA 0.5 mmol/l); D: LFLP group (FFA 0.5 mmol/l + PIO 10^{-7} mol/l); E: LFHP group (FFA 0.5 mmol/l + PIO 10^{-5} mol/l)

PGC-1 and NRF-1 are tightly related to mitochondrial function. PGC-1 could induce mitochondrial biogenesis and has been proposed as a new target for pharmaceutical intervention in diabetes (Wu and Boss 2007). NRF-1 is one of the nuclear transcription factors that regulate the synthesis of mitochondrial respiratory chain subunits by interacting with mitochondrial and nuclear genomes. Both PGC-1 and NRF-1 are implicated in the regulation of insulin secretion. For example, Yoon et al. (2003) reported that PGC-1 expression at both protein and mRNA levels was elevated in diabetic mice islets, and PGC-1 could inhibit GSIS. In addition, cold induced expression of PGC-1 could decrease insulin secretion, and inhibition of islet PGC-1 expression could partially restore insulin secretion (De Souza et al. 2003).

Table 4: Effects of palmitate and PIO on NRF-1 and PGC-1 mRNA levels in HIT-T15 cells

Group	NRF-1 mRNA	PGC-1 mRNA
Control	0.83 ± 0.28	0.81 ± 0.16
FFA 0.5 mmol/l	1.94 ± 0.91	2.36 ± 0.81
FFA 0.5 mmol/l + PIO10^{-7} mol/l	0.38 ± 0.02	1.21 ± 0.29
FFA 0.5 mmol/l + PIO10^{-5} mol/l	0.49 ± 0.23	1.63 ± 0.20
FFA 1.0 mmol/l	$2.94 \pm 1.00^{*}$	$4.16 \pm 1.42^{*}$
FFA 1.0 mmol/l + PIO10^{-7} mol/l	1.67 ± 0.58	1.89 ± 0.81
FFA 1.0 mmol/l + PIO10^{-5} mol/l	$0.45 \pm 0.28^{\dagger}$	$0.89 \pm 0.34^{\dagger}$

* $P < 0.05$ vs. control group, †: $P < 0.05$ vs. FFA 1.0 mmol/l group.

Consistent with these previous reports, in the present study we observed that palmitate could increase the expression of PGC-1 mRNA and NRF-1 mRNA and decrease GSIS in HIT-T15 cells. More importantly, we found that theses effects of palmitate were partly antagonized by PIO. This is in agreement with a study showing that rosiglitazone, another PPARγ agonist, could prevent the inhibition of GSIS in human islet cells induced by FFA (Lupi et al. 2004). PGC-1 could interact with many transcription factors including PPARγ and NRF-1 (Berger and Moller 2002). The docking of PGC-1 to PPARγ results in a large increase in transcriptional activity and PGC-1 can stimulate a powerful induction of NRF-1 and NRF-2 expression, which is tightly related with the production of ATP. Based on these data we speculate that PGC-1 plays a crucial role in mediating the effects of PPARγ agonists such as PIO. The change of GSIS level induced by FFA and alleviated by PIO may be regulated by the expression of PGC-1 and NRF-1, which cause the change of ATP/ADP ratio in β-cells.

In addition, by flow cytometry, TUNEL and electron microscopy analysis we demonstrated that palmitate could damage mitochondrial ultrastructure and induce mitochondrial swelling and cellular apoptosis, whereas PIO could alleviate these changes. This is consistent with previous studies showing the protective effects of PIO on fatty acid-induced oxidative stress (Gumieniczek et al. 2008; Saitoh et al. 2008). In conclusion, our experiments suggest that FFA can damage mitochondrial ultrastructure and lower GSIS of β-cells, and PIO can improve those changes, which may be mediated by the expression of PGC-1 and NRF-1. These findings provide new insight into the

hypoglycemic effects of PIO and help develop new agents for diabetes therapy.

4. Experimental

4.1. Reagents

Palmitate (Sigma, USA, 51.284 mg) was dissolved in 2 ml 0.2 mmol NaOH in a water bath at 70 °C, at the same time 1.8 g bovine serum albumin (BSA) was dissolved in 18 ml H_2O in a water bath at 55 °C. Next the above two solutions were mixed, filtrated and stored at −20 °C as stock solution of palmitic acid (10 mmol/L). PIO (Anketuo, Sichuan Dikang Company, China) was dissolved in dimethyl sulphoxide (DMSO), and the ultimate concentration of DMSO was not more than 0.1%.

4.2. Cell culture and treatment

HIT-T15 cell line was obtained from American Type Culture Collection (USA) and cultured in DMEM medium (Gibco, USA) at 37 °C with 5% CO_2 and 95% air. The cells were divided into 7 groups for different treatment and cultured for 48 h: 1). Control group treated with DMSO; (2). 0.5 mmol/l FFA (LF); (3) 0.5 mmol/l FFA+10^{-7} mol/l PIO (LFLP); (4) 0.5 mmol/l FFA+10^{-5} mol/l PIO (LFHP); (5) 1.0 mmol/l FFA (HF); (6) 1.0 mmol/l FFA+10^{-7} mol/l PIO (HFLP); (7) 1.0 mmol/l FFA+10^{-5} mol/l PIO (HFHP).

4.3. Detection of apoptosis by flow cytometry

HIT-T15 cells were treated with different concentrations of palmitate and PIO for 48 h at 37 °C in a 5% CO_2 atmosphere. A DMSO group was added as a control group. Cells were digested by pentazyme and fixed as cell suspension in ice-cold 70% ethanol. The samples were analyzed by flow cytometry (EPICS XL, Coulter Company, USA) with 488-nm laser line and analyzed using Cell Quest software.

4.4. TUNEL staining

HIT-T15 cells were plated on 20-mm coverslips at 4×10^4 cells/slip and incubated at 37 °C and 5% CO_2 for 48 h. Cells were fixed by polyformaldehyde and treated by 0.1% Triton x100 at 37 °C for 10 min. Then the cells were stained by TUNEL kit (Roche, Switzerland) for 1 h following the manufacturer's protocol. Finally the samples were stained by DAB and haematoxylin and apoptotic cells were stained in brown and non-apoptotic ones in blue.

4.5. Electron microscopy

HIT-T15 cells of the different groups were collected after trypsin digestion and fixed in 0.3% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min at 4 °C. Next the cells were postfixed in 1% osmium tetroxide in PBS for 1 h at 4 °C, dehydrated in a graded acetone series and embedded in epoxy resin. Then the ultrathin sections were cut and placed on copper and nickel grids. The sections were stained by uranium acetate and lead citrate and the mitochondrial ultrastructure was examined by electron microscope (Hitachi, Japan) at 80 kv.

4.6. Assay of ATP/ADP levels by hyperperformance liquid chromatography (HPLC)

HIT-T15 cells of the different groups were collected and lysed in 2 ml 10% $HClO_4$ at 4 °C and the pH was adjusted to 7 by K_2CO_3. Supernatants were collected after centrifugation at 4 °C. ATP and ADP levels were detected by HPLC (ZOBAX○R, HP, USA). Detection conditions were as follows: chromatogram column Phenomenex○RLuna 250 mm × 4.6 mm, velocity of flow 0.81 ml/min, detected- wavelength 254 nm, and column temperature 20 °C.

4.7. Real-time PCR

Total RNA was isolated from HIT-T15 cells by using TRIZOL reagent. cDNA was synthesized by using reverse transcription reagent. RT-PCR was performed using cDNA as the template and the following primers: NRF-1 Forward 5'-CACCGTGTCGCTCATCCA-3', Reverse 5'-CAATTT-TGTTCCACCTCTCCAT-3'; Taqman probe: 5'- FAM- AGCATCAGCCA-ATGTGGCTAC-TAMRA-3'; PGC-1 Forward 5'- GCCAAACCAACAAC-TTTATCT-3', Reverse 5'- GTTAGGCCTGCAGTTCCAGA-3'; Taqman probe: ATM: 5'-FAM- CCAAATGACCCCAAGGGTTCC-TAMRA-3'; GAPDH Forward 5'-TGGGTGTGAACCACGAGAA-3', Reverse 5'-GGCATGGACTGTGGTCATGA-3'; Taqman probe: GAPDHTM: 5'-CTGCACCACCAACTGCTTAGC-3' (Shanghai Biological Limited Company, China).
Amplification conditions were as follows: 5 min at 95 °C (one cycle); 10 s at 94 °C; 30 s at 56 °C and 1 min at 72 °C (45 cycles); and 72 °C for 5 min (one cycle). RT-PCR was performed three times in triplicate. The relative mRNA levels of NRF-1 and PGC-1 were compared to that of GAPDH and calculated by the $2^{-\Delta\Delta Ct}$ method. Each Ct value used for these calculations was the mean of the triplicate for each reaction.

4.8. Assay of insulin level

HIT-T15 cells of the different groups were collected after treatment and washed by PBS, then the cells were cultured in media containing 22 mmol/l glucose for 1 h. The culture media were collected and the insulin level in the media was evaluated by radioimmune assay (Linco Company, USA) following the manufacturer's protocol.

4.9. Statistical analysis

All data were expressed as mean±SD. One-way ANOVA analysis was performed using SPSS 12.0 software to compare statistical significance between the groups. If an overall difference was shown by the ANOVA, post hoc contrasts were performed with S-N-K for two-group comparison. $P < 0.05$ was considered to be significant.

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