Roles of Inhibitors of Poly(ADP-ribose) Polymerase in Protecting Rat RINm5F Cell Line against Free Fatty Acid-induced Apoptosis

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Key words
- type 2 diabetes
- FFA
- inhibitors of poly(ADP-ribose) polymerase
- RINm5F cell line

Abstract

Background: Chronic exposure to high levels of free fatty acid (FFA) has adverse effects on the function of pancreatic beta-cell, with a consequent increase in the production of reactive oxygen species. Poly (ADP-Ribose) polymerase (PARP-1) overactivation leads to massive NAD^+ consumption and ATP depletion with induction of cellular necrosis under high reactive oxygen species. In the present study, we investigated whether inhibitors of poly(ADP-ribose) polymerase were capable of protecting beta-cells from death induced by extended exposure to FFA.

Materials and Methods: RINm5F cell line was cultured in the presence of FFA (palmitate) in order to induce apoptosis, and then cells were treated with low-potency poly(ADP-ribose) polymerase inhibitor (3-aminobenzamide) or potent poly(ADP-ribose) polymerase inhibitor (PJ34). In order to explore whether poly(ADP-ribose) polymerase inhibitors could inhibit the apoptosis induced by FFA, expression of PARP-1 was measured by RT-qPCR and Western blot, while the apoptosis of RINm5F cells were analyzed by flow cytometry and Tdt-mediated dUTP Nick-End Labeling(TUNEL).

Results: Low-potency poly(ADP-ribose) polymerase inhibitor (3-aminobenzamide) significantly suppressed the impaired insulin secretion and FFA-induced apoptosis ($P<0.01$). However, potent poly(ADP-ribose) polymerase inhibitor (PJ34) had no significant effects on FFA-induced apoptosis ($P>0.05$). Moreover, low-potency inhibitors of PARP-1 increased PDX-1 expression down-regulated by FFA-treatment.

Conclusions: These findings suggested that low-potency inhibitors of poly(ADP-ribose) polymerases could protect rat RINm5F cell line against free fatty acid-induced apoptosis, and it was through regulatory pathway of regulating PDX-1 expression.

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Introduction

At present, the incidence of type 2 diabetes (T2D) has shown a dramatic increase concurrent with the obesity epidemic. Reduced insulin sensitivity, often termed insulin resistance, and impaired pancreatic islet beta-cell function are the two key components in the pathogenesis of type 2 diabetes (Chiasson et al., 2004; Steppel et al., 2004; Rhodes, 2005). Importantly, recent research indicates that a loss of pancreatic beta-cells function has been noticed and a loss of beta-cell mass has occurred in T2D. (Maedler et al., 2004). In the population of obesity, chronic hyperglycemia and hyperlipidemia contribute to beta-cell dysfunction and a decrease of beta-cell mass, which increase the risk of diabetes (Dickson et al., 2004). Free fatty acid (FFA), at physiological concentrations, has detrimental effects on the function of beta-cell, including impairment of glucose-induced insulin release, suppression of proinsulin biosynthesis, and beta-cell loss by apoptosis (Lupi et al., 2002). The studies on the Zucker diabetic fatty rat have shown that lipid accumulation in beta-cell can cause a substantial reduction of beta-cell mass through apoptosis, and is found to be associated with the development of diabetes (Shimabukuro et al., 1998). In vitro studies have indicated that long-term exposure of beta-cell to FFA induced adverse effects on mitochondrial function, with elevated production of reactive oxygen species (ROS), consequently, increased oxidative stress leading to beta-cell death (Lupi et al., 2002; Wang et al., 2004).

Oxidative stress refers to the imbalance between the production of ROS and ability of the cells to defend against ROS. Excessive production of ROS

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induces mass nuclear fragmentation, which activates poly(ADP-ribose) polymerase(PARP) and reduces the cellular level of NAD^+. The enzyme inhibitors can prevent this drop in NAD^+ level (Shall, 1984). This protection of the NAD^+ level after DNA damage and PARP activation has become the basis of a veritable industry in the study of ischaemia-reperfusion injury (Pieper et al., 1999). The drop in cellular NAD^+ is spontaneously reversible in general if the decrease does not exceed about 75% of the initial NAD^+ level (Shall, 1984)

3-Aminobenzamide is a PARP inhibitor which is currently being explored in clinical trials as a means to prevent the onset of insulin dependent type 1 diabetes in children who are at risk of developing the disease. Chemical inhibitors of PARP, as well as PARP gene disruption in mice, have been shown to counteract hyperglycemia and destruction of the insulin-producing beta-cell induced by certain beta-cell toxins such as streptozotocin (Mabley et al., 2001). Consistent with this possibility, the PARP inhibitor 3-aminobenzamide increases beta-cell regeneration and prevents diabetes in partially pancreatectomized rats (Yonemura et al., 1984).

PDX-1 played a central role in pancreatic beta-cell differentiation and insulin secretion (Offield et al., 1996; Jonsson et al., 1994; Fernandes et al., 1997). It controlled the expression of insulin and other beta-cell-specific genes (Marshak et al., 1996). Gremlich and her colleagues (Gremlich et al., 1997) reported that palmitate treatment of isolated pancreatic islets induced a marked decrease in PDX-1 mRNA and protein expression, as well as in PDX-1 binding activity with insulin and GLUT2 genes, which was associated with a decline in insulin secretion. In the present study we also tested the expression of PDX-1 in the insulin-producing RIN5mF cell line treated with FFA.

RIN lines were derived from a transplantable islet-cell tumor induced by high-dose irradiation in an inbred NEDH (New England Deaconess Hospital) rat strain (Chick et al., 1977). Two lines were derived from this initial tumor. One of the lines, named RIN-r, was derived from tumors maintained in NEDH rats. The other line, named RIN-m, was derived from a tumor that had been maintained in nude mice (Gazdar et al., 1980). Further research on these early cell lines led to various subclones originating from the RIN-m line (Bhathena et al., 1982). The RIN-m5F subclone, derived from the RIN-m line, was selected for our studies based on its high insulin secretion rate.

Nevertheless, identifying anti-apoptotic agents to protect beta-cell from apoptosis was important; therefore, the study was designed to investigate the effect of inhibitors of poly (ADP-Ribose) polymerase on cell death induced by prolonged exposure to FFA in insulin-producing RIN5mF cell line.

Materials and Methods

Reagents

We purchased rabbit polyclonal antibodies against PARP from Cell Signaling Technology (Madision, USA) and rabbit polyclonal antibodies against PDX-1 from Santa Cruz Biotechnology (CA, USA). DNase I (RNase Free) and PrimeScript™ RT reagent Kit Perfect Real Time were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Real-time PCR Master Mix was purchased from Toyobo Co., Ltd. (Osaka, Japan). Insulin Radioimmunoassay Kit was purchased from CHEMCLIN (Beijing, China). Annexin-V fluorescein isothyocyanate (FITC) Apoptosis Assay Kit was purchased from Invitrogen Life Technologies (California, USA).

Cell culture

The insulin-producing RINm5F cell line was kindly provided by Dr. Xiao H (Nanjing Medical University, Nanjing, China). The cells were cultured in the presence of RPMI 1640 medium (Hyclone, UT, USA) with 10% fetal bovine serum (FBS, Hyclone, UT, USA) and 100 mg/ml penicillin, and 50 mg/ml streptomycin, at 37 °C under a humidified condition of 95% air and 5% CO_2. On reaching 80% confluence, the cultures were washed twice with RPMI 1640 (without FBS) and kept in serum free medium for 2 h before the induction of cell apoptosis, which was obtained by culturing cells in the presence of high levels of FFA for each time. Palmitate (PA, Sigma Aldrich, St Louis, MO, USA) was prepared as previously described (Piro et al., 2002). Briefly, we first mixed palmitate with ethanol to obtain a 100 mM stock solution, and prepared a 1% fatty acid free BSA solution in RPMI 1640, and then aliquots of stock solution were dissolved in the RPMI 1640 containing 1% fatty acid free BSA. For the control experiments, BSA in the absence of fatty acids was prepared as described above. The PA concentration was 0.25 mM for each time. At the end of the incubation, cells were scraped off from the culture dishes after a washout of the cell layer with PBS, collected together with detached cells floating in the medium. The protective effects of inhibitors of poly(ADP-ribose) polymerase were studied by incubating FFA treated cells with or without PJ34 (0.1 µM to 10 µM) (Calbiochem, Darmstadt, Germany) and 3-aminobenzamide(3-AB, 0.45 mM to 18.0 mM) (Calbiochem, Darmstadt, Germany); Control cultures were grown under the same conditions as treated cells but in the absence of the drugs (PA and/or PJ34 and/or 3-AB).

MTT assay

The assay was based on the ability of viable cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, St Louis, MO, USA) to insoluble colored formazan crystals. For MTT assay, the cells were cultured in a 96-well plate and treated with FFA and/or 3-AB/PJ34 as described above, and then MTT (5 mg/ml) was added to each well. The cells were incubated at 37 °C for 4 h. The medium was removed, and then the crystals were lysed with 150 µl of DMSO (Sigma, St. Louis, MO, USA). The optical density was read at 492 nm. Graphs were representative of three separate experiments.

Real-time quantitative PCR analysis

The expression of PARP-1 genes in RINm5F cell line was evaluated by Real-time quantitative PCR (RT-qPCR). Total RNA was extracted from each sample with Trizol (Invitrogen-Life Technologies, California, USA) according to manufacturer's instructions. Following treatment with DNase I (TaKaRa Biotechnology Co., Ltd.) at 37 °C for 30 min, RNA quantification was performed using spectrophotometry. The RNA(1 µg) was subsequently incubated with 1 µl of Oligo dT primer (50 µM), 1 µl of Random 6 mers (100 µM), 1 µl of PrimeScript™ RT Enzyme Mix I, 4 µl of 5 × PrimeScript™ Buffer and RNase Free dH_2O, and first-strand cDNA synthesis was performed in a total volume of 20 µl.

The primers were used for PARP-1and β-actin (as shown in **Table 1**). The PCR reactions were performed in a LightCycler apparatus using Real-time PCR Master Mix SYBR Green I (Osaka, Japan). Thermocycling was done in a final volume of 20 µl containing 2 µl of cDNA sample, 0.4 µl of the up-primer, 0.4 µl down-primer, 10 µl of SYBR Green Realtime PCR Master Mix, and 7.2 µl of dH_2O. After 30 s at 95 °C to denature the cDNA and to activate the Taq DNA polymerase, the cycling conditions were as follows:

Table 1 The primers of PARP-1 and β-actin.

	Primers	Amplified fragment
PARP-1	Sense: 5′-CACTCCTGAACAACGCAGACA- 3′ Antisense: 5′- GTGCTTGCCCTTGGGTAACTT- 3′	590 bp
β-actin	Sense: 5′-CATCTCTTGCTCGAAGTCCA-3′ Antisense: 5′-ATCATGTTTGAGACC TTCAACA-3′	308 bp

40 cycles consisting of denaturation at 95 °C for 5 s, annealing at 54 °C for 5 s, and extension at 72 °C for 30 s.

The Ct used in the real-time PCR quantification was defined as the PCR cycle number that crossed an arbitrarily chosen signal threshold in the log phase of the amplification curve. To verify the fold change of PARP-1 gene expression, calculated Ct values were normalized to Ct values of beta-actin amplified from the same sample (ΔCt = Ct(PARP-1) – Ct(β-actin)), and the $2^{-\Delta\Delta Ct}$ method was used to calculate fold change (Livak and Schmittgen, 2001). Each sample had triplicates and all reactions were triplicated independently to ensure the reproducibility of the results.

Western blot analysis

Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 10 % glycerol, 1 % Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. Lysates were centrifugated (15 000 × g) at 4 °C for 10 min and the supernatants were transferred to clean microcentrifuge tubes. Equal amounts of the soluble protein were denatured in SDS, resolved on a 12 % SDS-polyacrylamide gel, and transferred to a PVDF membrane. The immunoblotting was performed as described (Adler et al., 1999). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies were used against primary antibody. The proteins were visualized using Lumi-Light Western Blotting Substrate (Roche Molecular Biochemicals).

Analysis of insulin secretion

After incubation with PA, in the presence or absence of 3-AB/PJ34, the insulin secretion was assessed as previously described (Anastasi et al., 2005). Cells were kept in 3.3 mM glucose for 60 min, then challenged with 22.2 mM glucose for 60 min, and collected supernatant. The level of insulin in the culture medium was measured by RIA, and normalized for the total cellular protein content detected in the pellet of each individual culture according to Bradford method (Bio-Rad Laboratories, Inc. USA).

Annexin V assay

Quantitative evaluation of apoptosis was performed by flow cytometry after double staining with annexin V fluorescein isothyocyanate (FITC) apoptosis detection kit, which allowed discrimination among early apoptotic (single annexin V positive), and necrotic cells (double annexin V/propidium iodide (PI) positive), which could differentiate cells that had lost membrane integrity (necrotic cells) from living cells by means of red staining of their nuclei with PI.

RINm5F cells (1×10^6 cells per well) were cultured in six-well dishes and treated without (control) or with PA and in the presence of inhibitors of PARP-1(PA + PJ34 or PA + 3-AB) for 24 h. The cells of each well were then harvested and washed with PBS, then discarded the supernatants and resuspended the cells in annexin-binding buffer. Cells were stained with 5 μl of annexin V and 1 μl of 100 μg/ml PI working solution. Then analysis was made of the stained cells by flow cytometry, measuring the fluorescence emission at 530 nm and > 575 nm. The apoptosis of cells was investigated using flow cytometry analysis as described in materials and methods. Graphs were representative of three separate experiments.

TUNEL assay

An in situ Cell Death Detection Kit (KeyGEN, Nanjing, China) was used to detect apoptotic cells. With this method, terminal deoxynucleotidyltransferase (TdT) was used to catalyze the polymerization of fluorescein-labeled nucleotides to free 3-OH termini of DNA strand breaks. After incubation with PA, in the presence or absence of 3-AB/PJ34 for 48 h, RINm5F cells were washed twice with cold PBS. Cells were then fixed with 200 μl of 4 % paraformaldehyde and incubated for 10 min at room temperature. After being rinsed with PBS, cells were impacted with 200 μl of impacting medium (3 % H_2O_2 in methanol). Then also rinsed with PBS, cells were permeated with 250 μl of permeabilization solution (0.1 % Triton X-100 in 0.1 % sodium citrate) and incubated on ice for 2 min. Then, 50 μl of TUNEL reaction mixture (1 μl Biotin and 4 μl TdT Enzyme in 45 μl Equilibration Buffer) was added to the samples and the positive controls, (50 μl of label solution only was added to the negative controls), and cells were incubated at 37 °C for 1 h in dark. Rinsed with PBS, 50 μl of Streptavidin-HRP solution (0.5 μl Streptavidin-HRP in 99.5 μl PBS) was added. Then rinsed with PBS, 50 μl of DAB was added for coloration. The percentage of positive cells was determined on the basis of the evaluation of cells treated with TUNEL reagents lacking TdT by using a cursor setting that yielded fewer than 2 % positive cells. Graphs are representative of three separate experiments.

Statistical analysis

Statistical analysis of apoptosis data was performed by non-parametric one-way analysis of variance (ANOVA) test. As a general rule, only P values of less than 0.05 were considered to be significant. As for RT-qPCR and western blotting analysis, data was expressed as means ± S.D. Statistical analysis was performed by ANOVA test. Differences were considered statistically significant when $P < 0.05$.

Results

Effects of PARP-1 inhibitors on cell viability of RINm5F cell line in primary culture

After treating RINm5F cells with the potent inhibitor of PARP-1 PJ34 (0.1 μM to 10 μM) or the low-potency inhibitor of PARP-1 3-AB (0.45 mM to 18.0 mM) for 16 h, MTT assays were performed to evaluate cell viability. We found that treatment of the cells with high concentrations of PJ34 (1 μM to 10 μM) or 3-AB (4.5 mM to18 mM) for 16 h could suppress the viability of the RINm5F cells significantly along with the increase of concentration ($P < 0.05$, $P < 0.01$; **Fig. 1**). However, treatment of the cells with low concentrations of PJ34 (0.1 μM or 0.5 μM) or 3-AB (0.45 mM or 0.9 mM) for 16 h had no effect on the cell viability (**Fig. 1**). In order to inhibit the basal ADP-ribosylation activities effectively, we selected the suitable concentration of 3-AB (0.9 mM)

and PJ34 (0.5 µM) to do the following experiments, which had no effect on the survival of cells.

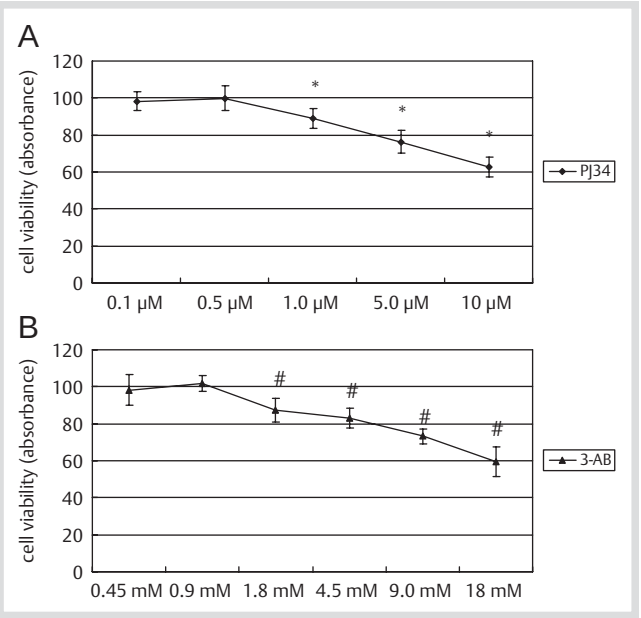

Fig. 1 Effects of PARP-1 inhibitors on cell viability of RINm5F cell line. (**A**) The cell viability of RINm5F cells was detected by MTT assays when treated with the potent inhibitor of PARP-1 PJ34 (0.1 µM to 10 µM) for 16 h. The data shown were means ± SD of three separate experiments. Treatment of the cells with higher concentrations of PJ34 (1 µM, 5 µM, 10 µM) suppressed the viability of the RINm5F cells (*P < 0.01), and the lower concentrations of PJ34 (0.1 µM or 0.5 µM) had no effect on the cell viability (P > 0.05). (**B**) The cell viability of RINm5F cells was detected by MTT assays when treated with the low-potency inhibitor of PARP-1 3-AB (0.45 mM to 18 mM) for 16 h. The data shown were means ± SD of three separate experiments. Treatment of the cells with higher concentrations of 3-AB (4.5 mM, 9 mM, 18 mM) also suppressed the viability of the RINm5F cells (#P < 0.01), and lower concentrations of 3-AB (0.45 mM or 0.9 mM) had no effect on the cell viability (P > 0.05).

PA modulates PARP gene expression and protein production in RINm5F cells

The present study demonstrated that treating RINm5F cells with PA (0.25 mM) for 12 h led to a 1.87-fold increase in PARP-1 mRNA expression (*P < 0.01 versus 0 h; ◉ **Fig. 2**), and led to 2.48-fold increase for 24 h (**P < 0.01 versus 0 h). We also found that treating the cells with PA (0.25 mM) for 24 h and 36 h led to a dramatic increase in PARP-1 protein expression (*P < 0.05 versus 0 h, **P < 0.01 versus 0 h; ◉ **Fig. 3**).

Inhibitors of PARP-1 modulate the PDX-1 protein expression

Our study indicated that low-potency inhibitor of poly(ADP-ribose) polymerase (0.9 mM 3-AB) significantly increased PDX-1 expression at 24 h, 36 h and 48 h (P < 0.01 versus PA 24 h; P < 0.01 versus PA 36 h; P < 0.05 versus PA 48 h, ◉ **Fig. 4**) which was down-regulated by PA. Thus potent inhibitor of poly(ADP-ribose) polymerase (0.5 µM PJ34) had no significant effects on the PDX-1expression at 24 h, 36 h and 48 h which was down-regulated by PA(P > 0.05 versus PA 24 h; P > 0.05 versus PA 36 h; P > 0.05 versus PA 48 h, ◉ **Fig. 4**).

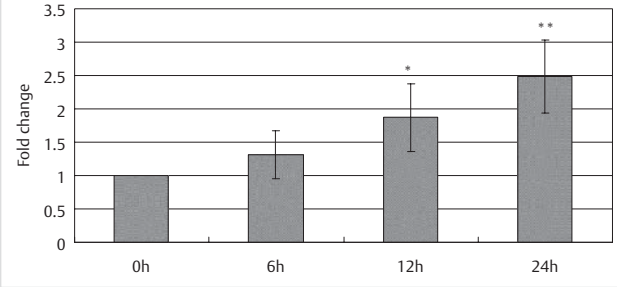

Fig. 2 PA modulated PARP-1 mRNA expression (RT-qPCR). Expression of PARP-1 and an internal control β-actin was evaluated by RT-qPCR. The bars showed the expression in relationship to 0 h (set to 1) as means ± S.D. of three separate experiments. PARP-1 mRNA expression increased 1.87-fold when RINm5F cells were treated with PA (0.25 mM) for 12hrs (*P < 0.01 versus 0 h), and increased 2.48-fold for 24hrs (**P < 0.01 versus 0 h).

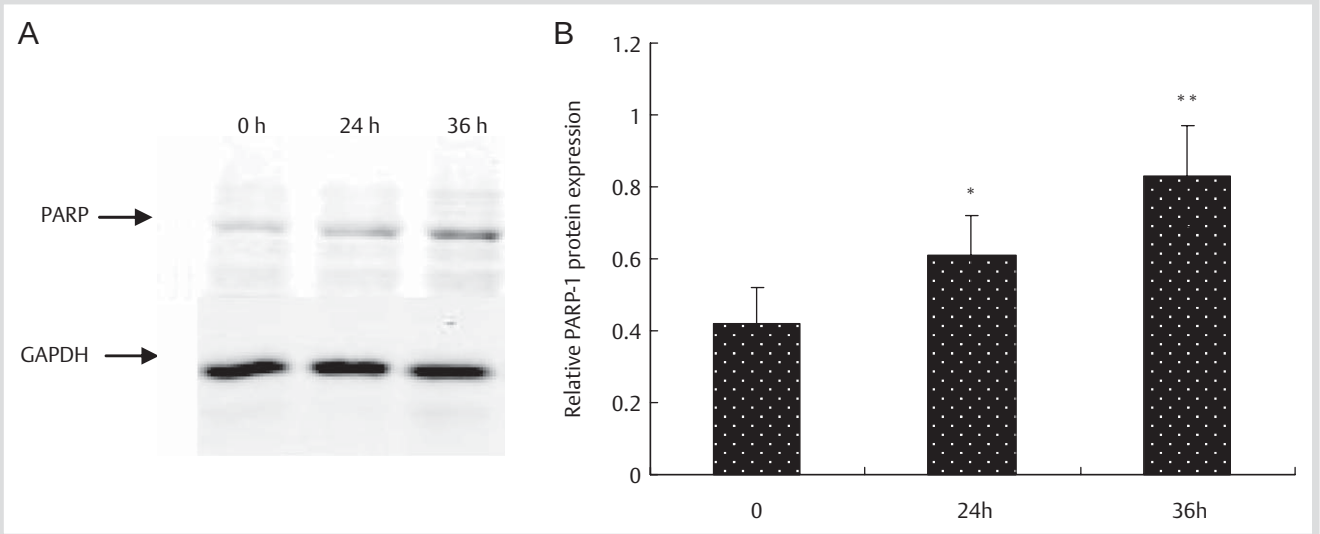

Fig. 3 PA modulated PARP-1 protein expression. (**A**) Expression of PARP-1 and an internal control β-actin was evaluated by western blot analysis. A representative experiment of three was shown. (**B**) Densitometric analyses were reported as means ± S.D. of the three separate experiments. *P < 0.05 versus 0h; **P < 0.01 versus 0 h.

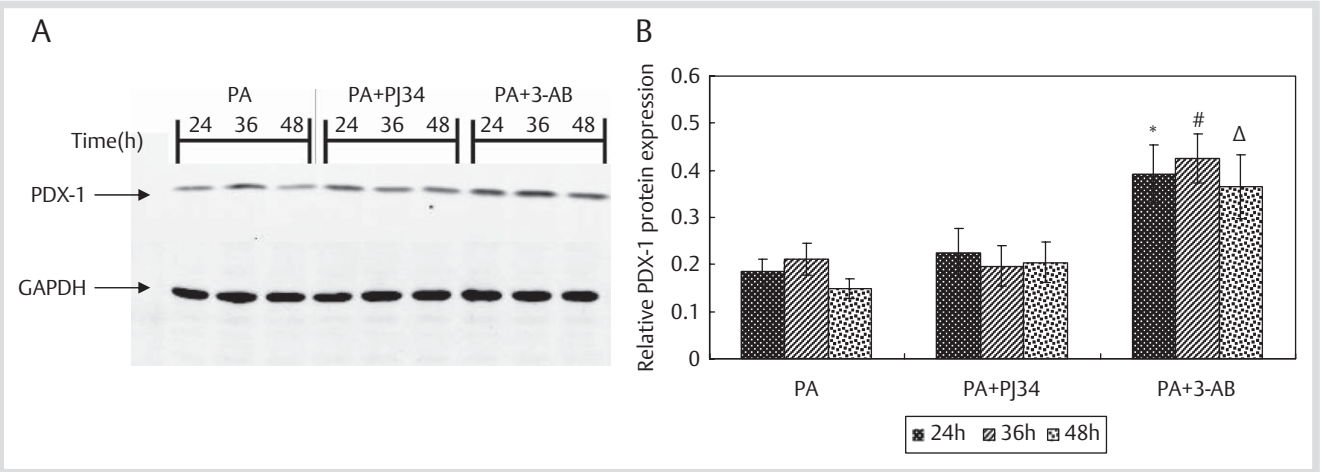

Fig. 4 Analysis of relative PDX-1 protein expression. (**A**) PDX-1 expression was evaluated by western blot analysis. A representative experiment of three was shown. (**B**) Densitometric analyses were reported as means ± S.D. of the three separate experiments. *P < 0.01 versus PA 24h; #P < 0.01 versus PA 36h; ΔP < 0.05 versus PA 48 h.

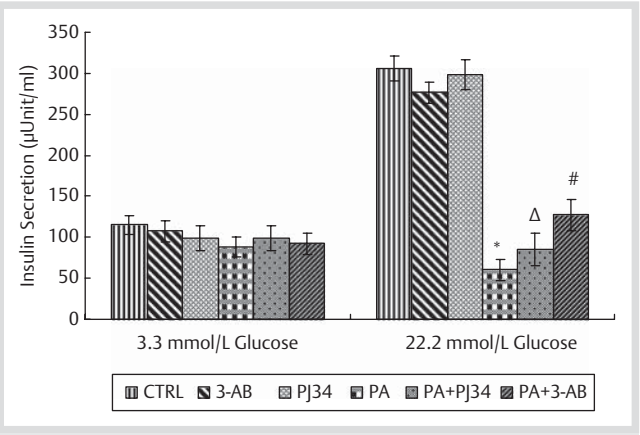

Fig. 5 Analysis of insulin secretion from RINm5F cells. The level of insulin secretion in response to glucose (3.3 mM and 22.2 mM) concentration after a 24-h incubation with control medium (CTRL), medium containing free fatty acids (PA), plus PJ34 or 3-AB (PA + PJ34, PA + 3-AB), and medium containing PJ34 or 3-AB alone (PJ34, 3-AB) was evaluated by RIA. Data were means ± S.D. of three separate experiments. *P < 0.01 versus control; #P < 0.01versus PA; ΔP > 0.05 versus PA.

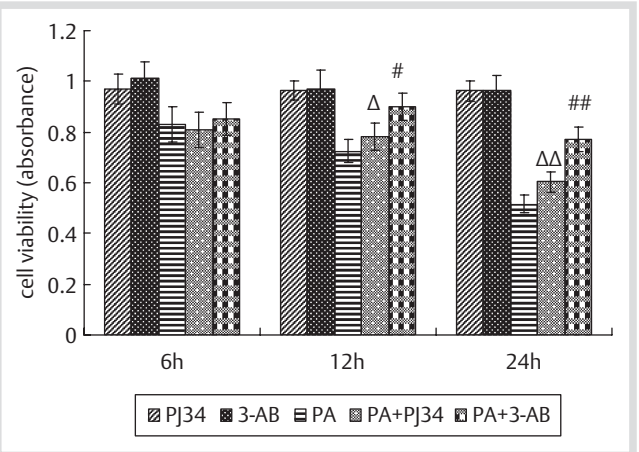

Fig. 6 Effects of inhibitors of PARP-1 on RINm5F cell viability. After treating RINm5F cells without (control) or with free fatty acids (PA) and in the presence of inhibitors of PARP-1(PA + PJ34 or PA + 3-AB) for 6 h,12 h and 24 h, MTT assays were performed to evaluate cell viability. The data shown were means ± SD of three separate experiments. PA decreased cell viability, and the administration of 3-AB for 12 h and 24 h inhibited the effect of PA on cell viability (#P < 0.01;##P < 0.01), but PJ34 had no significant effect on cell viability decreased by PA (ΔP > 0.05; ΔΔP > 0.05). Importantly, low-potency PARP-1inhibitor (0.9 mM 3-AB) did not influence per se apoptosis in beta-cells.

Effects of poly (ADP-ribose) polymerase inhibitors on insulin-secretion of RINm5F cell line

To assess functional modifications, glucose-dependent secretion of insulin was evaluated in RINm5F cells exposed to FFA (0.25 mM PA) mixture in the presence or absence of PARP-1 inhibitors (3-AB or PJ34). Insulin released from cells exposed only to PA mixture was markedly impaired in the absence of inhibitor of PARP-1 (*P < 0.01; PA versus CTRL; ▶ **Fig. 5**). The presence of PARP-1 inhibitors caused no modification of basal insulin release (at 3.3 mM glucose) versus PARP-1 inhibitors untreated cells. When challenged with 22.2 mM glucose, 3-AB + PA-treated cells showed a significant increase of insulin release when compared with PA-treated cells (verses PA treatment alone, #P < 0.01; ▶ **Fig. 5**). However, the protective effect of PJ34 was not significant (verses PA treatment alone, ΔP > 0.05; ▶ **Fig. 5**). Taken together, these results suggested that the low-potency inhibitor of poly(ADP-ribose) polymerase played a central role in the inhibitory effects of PA on beta-cells.

Effects of poly(ADP-ribose) polymerase inhibitors on PA-induced apoptosis of RINm5F cells

To examine whether inhibitors of poly(ADP-ribose) polymerase could protect against FAA induced cell death, we cultured RINm5F cells in vitro for 6 h,12 h and 24 h with or without PA (0.25 mM) in the presence or absence of PARP-1 inhibitors. Our results showed that prolonged exposure to PA decreased cell viability in rat RINm5F cells, and more important was that the administration of 3-AB for 12 h and 24 h inhibited the effect of PA on cell viability (#P < 0.01; ##P < 0.01), but PJ34 had no significant effect on cell viability decreased by PA (ΔP > 0.05, ΔΔP > 0.05). Importantly, low-potency PARP-1 inhibitor (0.9 mM 3-AB) which had no influence on per se apoptosis in beta-cells, probably counteracted the effects of FFA (▶ **Fig. 6**).

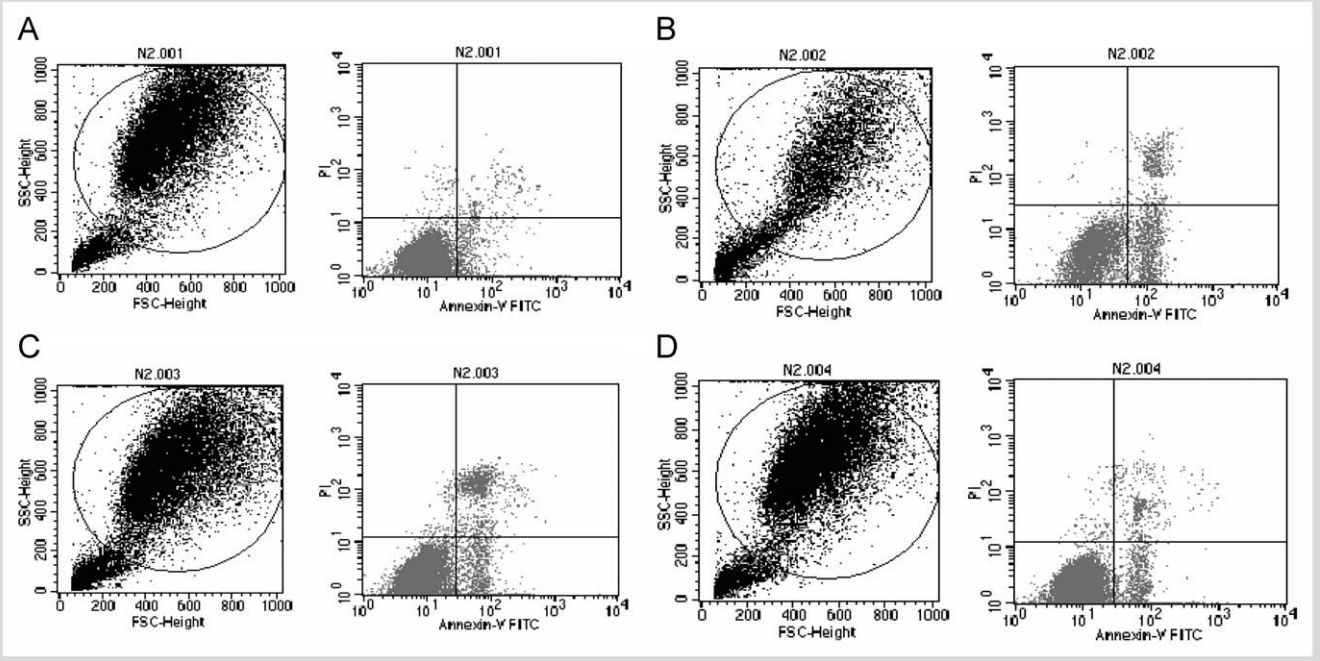

Fig. 7 Effects of inhibitors of PARP-1 on RINm5F cell apoptosis. RINm5F cells (1×10^6 cells per well) were cultured in six-well dishes and treated without (Control. A) or with free fatty acids (PA. B), and in the presence of inhibitors of PARP-1(PA + PJ34.C or PA + 3-AB.D) for 24 h. The data shown were means ± SD of three separate experiments. PA was able to induce apoptosis in a remarkable percentage of cells (about 20 %; $^*P < 0.01$versus control). Indeed, simultaneous administration of 3-AB and PA significantly reduced the occurrence of apoptosis (about 8 %; $^{\#}P < 0.01$ versus PA), but simultaneous administration of PJ34 and PA did not reduce the apoptosis (about 16 %; $^{\triangle}P > 0.05$ versus PA).

To quantify the degree of cell apoptosis in the various culture conditions, we performed a flow cytometry analysis by using double staining with annexin V-FITC/PI. The annexin V/propidium iodide double staining allowed discrimination among three separate cell populations: (i) cells in the early phases of apoptosis (single annexin V positive), (ii) cells in the late apoptotic phases (double annexin V/PI positive), and (iii) necrotic cells (single PI positive). We found that exposure to FFA for 12 h was able to induce apoptosis in a significant percentage of cells (about 20 %, $^*P < 0.01$versus control; **Fig. 7B**). Indeed, simultaneous administration of 3-AB and PA significantly reduced the occurrence of apoptosis (about 8 %; $^{\#}P < 0.01$ versus PA; **Fig. 7D**), but simultaneous administration of PJ34 and PA reduced no apoptosis (about 16 %; $^{\triangle}P > 0.05$ versus PA; **Fig. 7C**).

In TUNEL assay, we found that exposure to PA was able to induce apoptosis in a large percentage of the cells (about 40 %, $^*P < 0.01$versus control; **Fig. 8B**). Indeed, simultaneous administration of 3-AB and PA significantly reduced the occurrence of nuclear fragmentation and inhibited cell apoptosis (about 19 %; $^{\#}P < 0.01$ versus PA; **Fig. 8D**), but simultaneous administration of PJ34 and PA reduced no apoptosis (about 30 %; $^{\triangle}P > 0.05$ versus PA; **Fig. 8**. C). Results suggested that the exposure to PA promoted the condensation and fragmentation of apoptotic cells, and that the treatment with 3-AB in association with PA was capable of preventing nuclear fragmentation and inhibiting cell apoptosis.

Discussion

Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme, which is activated by DNA strand breaks. PARP comprises a 42 kDa N-terminal DNA binding domain (DBD) which is separated from a 54 kDa C-terminal catalytic NAD^+ binding domain by a 22 kDa polypeptide fragment containing automodification sites(Murcia et al., 1994). Apart from ribosylating itself, the enzyme also ribosylates other proteins, and some of which have been recognized as histones and nuclear enzymes involved in DNA repair and replication (Simbulan-Rosenthal et al., 1996). The important effects of PARP have been demonstrated by gene knockout techniques in the PARP null mouse exhibit multiple phenotypic defects such as increased chromosomal aberrations including breaks, fusions and telomere shortening in embryonic fibroblasts (d'Adda di Fagagna et al., 1999; Le Rhun et al., 1998). PARP has been shown to be enzymatically cleaved by caspase-3 during early apoptosis (Nicholson et al., 1995).

Our observations demonstrated that long-term exposure to FFA resulted in a significant overproduction of PARP-1 in RINm5F cells when compared with untreated cells. Moreover, these phenomena were accompanied with impairment of glucose-induced insulin release and cell viability, decrease of the PDX-1 protein expression and increase of cell apoptosis in FFA-treated cells. Previous studies confirmed that oxidative stress was viewed as an important mediator of cellular damage following a prolonged exposure of pancreatic beta-cell to elevated levels of FFA (Lupi et al., 2002; Evans et al., 2003). Interestingly, Gremlich (Gremlich et al., 1997) and Yoshikawa (Yoshikawa et al., 2001) observed that palmitate decreased overall cellular expression of PDX-1. Those reports were in line with our results. But Kharroubi (Kharroubi et al., 2004) failed to observe a decrease in PDX-1 expression in both INS-1 cells and rat islets after 24 h of treatment with palmitate.

Overactive PARP-1 induced a rapid depletion of the NAD^+ in islet cells. In type 1 diabetes model, streptozotocin (STZ) induced islet DNA strand breaks, as well as induced a rapid depletion of the NAD^+ in islet cells similarly (Ho et al., 1972; Schein et al., 1973).

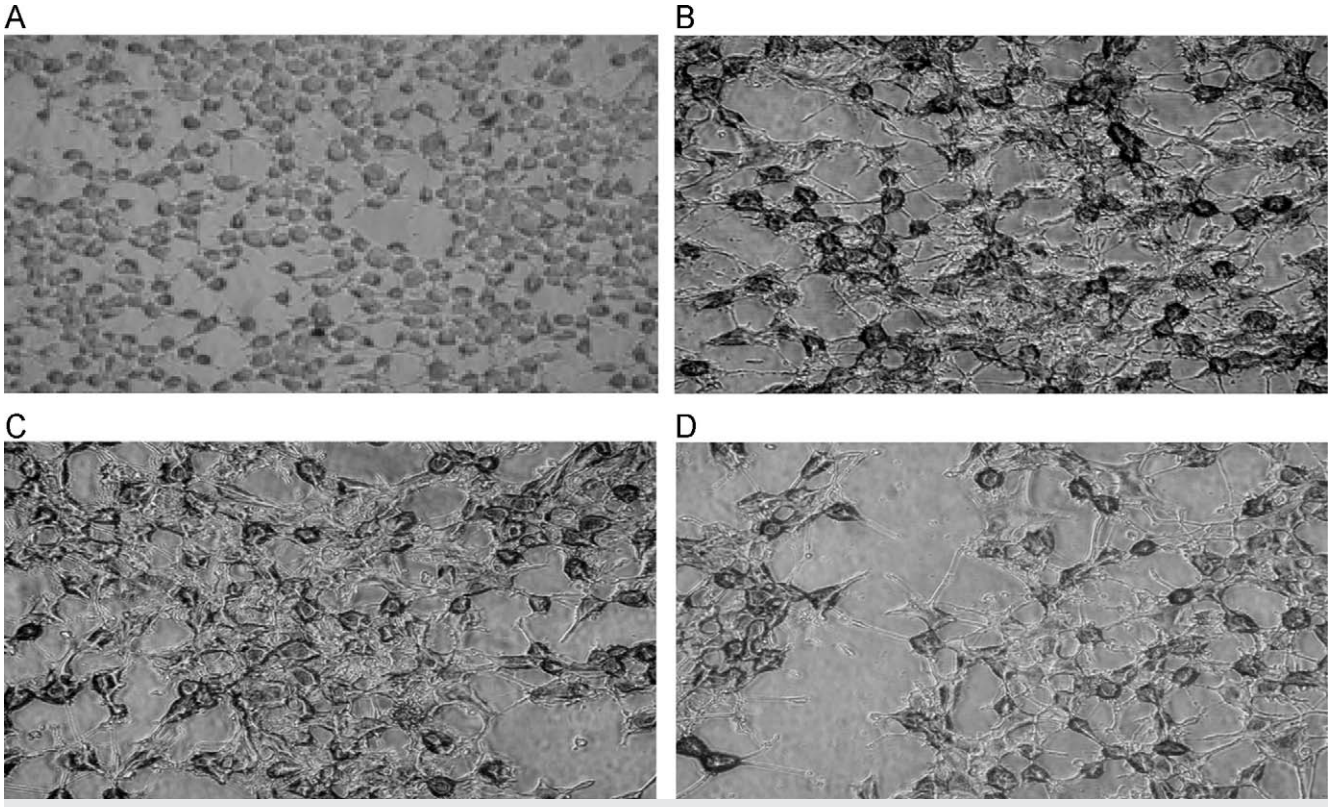

Fig. 8 Detection of apoptosis of RINm5F cells for 48 h by TUNEL. RINm5F cells (1×10^6 cells per well) were cultured in 24-well dishes and treated without (Control. A) or with free fatty acids (PA. B) and in the presence of inhibitors of PARP-1 (PA + PJ34.C or PA + 3-AB.D) for 48 h. Data were means ± S.D. of three separate experiments. PA was able to induce apoptosis in a substantial percentage of cells (about 40 %; $^*P < 0.01$ versus control). Indeed, simultaneous administration of 3-AB and PA significantly reduced the occurrence of nuclear fragmentation and inhibited cell apoptosis (about 19 %; $^{\#}P < 0.01$ versus PA), but simultaneous administration of PJ34 and PA did not reduce the apoptosis (about 30 %; $^{\Delta}P > 0.05$ versus PA).

This drop in the NAD^+ level could be prevented by the administration of 3-aminobenzamide or nicotinamide (Yamamoto et al., 1980; Okamoto, 1988; Uchigata et al., 1982). This inhibition of PARP-1 prevented the development of diabetes, in exactly the same way that diabetes was prevented in the PARP-1 knockout animals. It should be stressed, however, that when diabetes was prevented by inhibiting PARP-1, the animals did not develop diabetes because the beta-cells of the islets of the pancreas were saved from destruction. But the outcome for those animals was a very high incidence of pancreatic islet beta-cell tumor (Yamagami et al., 1985). Our results were coincident with previously description that low-potency PARP-1 inhibitors (3-aminobenzamide) were able to inhibit the dysfunction and apoptosis in RINm5F cells treated with FFA. Potent PARP-1 inhibitors (PJ34) were also tested for their ability to modulate insulin secretion and cell apoptosis, which was ineffective at attenuating insulin secretion and increasing cell apoptosis treated with FFA.

In a previous study on insulin producing cells, Ye DZ et al. found that incubation for 16 h of 10 mM 3-aminobenzamide or 5 µM PJ34 was required for complete inhibition of basal ADP-ribosylation activities in intact cells (Ye et al., 2006). Moreover, studies have observed that the same concentrations of these PARP-inhibitors induced cell death rapidly in insulin-producing cells (Saldeen et al., 1998), and our observations demonstrated that concentrations of low-potency PARP-1 inhibitors 3-aminobenzamide (4.5 mM, 9 mM, 18 mM) and potent PARP-1 inhibitors PJ34 (1 µM, 5 µM, 10 µM) were cytotoxic. Differences in the ability of low-potency PARP inhibitors and potent PARP-1 inhibitors to modulate insulin secretion and cell apoptosis induced by FFA were possibly caused by their chemical structures and concentrations used in RINm5F cells. Most PARP inhibitors were monocyclic carboxamides (nicotinamide and 3-aminobenzamide), bicyclic lactams (PD128763), or polycyclic lactams (PJ34) (Li et al., 2001). The amide group of nicotinamide or 3-aminobenzamide could rotate relative to the plane of the aromatic ring, making them less potent for PARP inhibition. Constraining mono-aryl carboxamides into bicyclic lactams increased potency. Three-ring structures further increased the potency, thus making PJ34 more potent than PD128763 (Okazaki et al., 1999).

Low-potency inhibitors of PARP-1 (3-aminobenzamide and nicotinamide) could function as antioxidants (Czapski et al., 2004; Kamat et al., 1999), suggesting that the low-potency PARP inhibitors might attenuate FFA-mediated suppression of insulin secretion and increase of cell apoptosis by reducing oxidative stress. This was an attractive possibility because beta-cells had low intrinsic antioxidant defense mechanisms, making them particularly susceptible to damage by oxidative stress (Robertson, 2004). This hypothesis was supported by recent findings that in type 2 diabetes, the human beta-cell had functional defects associated with multiple alterations and increased oxidative stress and that 24 h exposure to glutathione improves glucose-stimulated insulin release, suggesting that it was possible to reverse the functional impairment of type 2 diabetic islets by reducing oxidative stress in islet cells (Del Guerra et al., 2005).

Our results demonstrated that low-potency PARP-1 inhibitor (3-aminobenzamide) was capable of maintaining the PDX-1 expression observed in RINm5F cell line, by increasing the down-regulation of the expression of PDX-1 induced by PA-incubation. These observations suggested that 3-aminobenzamide had the ability to protect beta-cell from dysfunction and apoptosis via regulation of the PDX-1 expression, because PDX-1 played crucial roles in islet development, growth, and function.

In conclusion, 3-aminobenzamide could inhibit not only the suppression of insulin secretion induced by PA, but also the increase of apoptosis of RIN5mF cells induced by PA, and the inhibition effect was positively related to the PDX-1 expression. The finding indicated that it was through regulatory pathway of regulating PDX-1 expression that 3-aminobenzamide protected rat RINm5F cell line against free fatty acid-induced apoptosis. Because of its strong pro-survival and anti-apoptosis role, the low-potency PARP inhibitor (3-aminobenzamide) treatment appeared to be a useful approach to preserving beta-cell function and survival in the lipotoxicity setting.

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