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The Role of Parkin in Rat Pancreatic Beta Cells Fate

Hasibe Verdi[1*], Hatice Pinar Baysan Cebi[1], Yaprak Yilmaz Yalcin[1], Tulin Ozkan[2], Mehtap Akcil Ok[3], Asuman Sunguroglu[2], Fatma Belgin Atac[1]

[1] Baskent University, Department of Medical Biology, Ankara, Turkey
[2] Ankara University, Department of Medical Biology, Ankara, Turkey
[3] Baskent University, Department of Nutrition and Dietetics, Ankara, Turkey

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ABSTRACT

Parkin is a member of the mitochondrial quality control system that plays a major role in mitophagy. Although the loss of function mutations in the Parkin gene has been associated with the Familial Parkinson's phenotype, research in recent years points out that Parkin's function is not limited to neurodegenerative diseases. Parkin's function impressing key cellular quality control mechanisms, including the ubiquitin-proteasome and autophagy-lysosome systems, makes it an important player in the maintenance of cellular homeostasis. In this study, we investigated whether Parkin affects cell viability and ER stress responses under lipotoxic conditions in INS-1E cells. Our results may suggest that silencing Parkin may affect autophagy in addition to apoptosis. We also showed that Parkin may have a protective effect against lipo-toxic effects in INS-1E cells. Consistent with previous studies, we observed that stress responses were different for high and low palmitic acid doses. The Parkin being inhibited under high-dose PA treatment and active under low-dose PA treatment indicate that regulation of stress responses is controlled by environmental conditions. Our preliminary findings may suggest that in low lipotoxic conditions, Parkin affects the ER stress response by modulating Chop activity and Ca^{2+} release from the ER to the cytoplasm.

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Introduction

In type 2 diabetes (T2D), research indicates that a progressive decrease in pancreatic beta cell (β-cell) mass and impairment of function are features of the diseas (1,2). Free fatty acids (FFA), especially saturated fatty acids have been identified as one of the most important causal factors in the development of T2D (1,3,4). Palmitic acid (PA), an abundant saturated fatty acid existing in the human body, is closely linked to metabolic diseases (5,6). Acute PA overload in the pancreatic β-cells amplifies insulin secretion, but chronic exposure to PA leads to β-cells dysfunction, causing a decrease in insulin secretion, inhibition of necessary genes for cell differentiation, and triggering apoptosis (2,7). This entire process has been named lipotoxicity. The cell's reactions to lipotoxicity are formed by the distinctive responses of each organelle and by crosstalk with each other.

Protein homeostasis is composed of protein synthesis, folding and degradation (8). The folding of proteins into their specific three-dimensional conformation has a vital impact on cellular homeostasis. In addition to proteins synthesized on free ribosomes, the rough endoplasmic reticulum is another site where most membrane and secretory proteins are synthesized. Endoplasmic reticulum (ER) resident molecular chaperones and lectins ensure proper folding of proteins. ER is an organelle that controls phospholipid, cholesterol, steroid biosynthesis, glycogen degradation, detoxification reactions, and intracellular calcium ion (Ca^{+2}) homeostasis, as well as protein folding, maturation, quality control, and traffic (9). Perturbations in any of these functions may result in aggregation of misfolded proteins. Cells have a well-organized system of unfolded protein response (UPR), ER-associated degradation (ERAD), autophagy, and mitochondrial biogenesis to restore normal ER function and cell homeostasis (10). The combined activity of all these processes has a major impact on the decision of cell survival by reducing the stress on ER or activating cell death programs (11).

Autophagy is a lysosomal degradation of the excess/damaged cytoplasmic proteins or organelles of the cell to provide energy and macromolecular precursors under stress conditions. As a self-renewal process, it is functional in promoting cellular homeostasis and survival (12). The association of elevated or impaired levels of autophagy has been reported in several diseases; cancer, neurodegenerative diseases, obesity, and type 2 diabetes (13–16). Due to the intensive insulin synthesis and secretion, β-cells are particularly sensitive to ER stress and the subsequent accumulation of UPR. Factors contributing to ER stress may lead to unfolded protein accumulation and cause β-cells dysfunction. However, under extended stress conditions, the loss of β-cells is inevitable. Prolonged exposure to FFA, particularly PA triggers ER stress, activates UPR and induces pancreatic β-cells dysfunction (2,4,17–19).

Mitochondria has a major impact on cellular surveillance. The evident molecular data strongly improves that not only the dysregulation of insulin production, but glucose-stimulated insulin secretion (GSIS) may associate with mitochondrial dysfunction in β-cells. The Mito-

* Corresponding author. Email: hasibetez@gmail.com

chondrial Unfolded Protein Response (Mt-UPR)" (20) and mitochondrial quality control (QC) (21) are the two well-known systems functional for mitochondrial restoration.

Mitophagy is one of the main contributors to mitochondrial quality control (QC) and differs from macroautophagy by the selective degradation of damaged mitochondria (21). PTEN-induced kinase 1 (PINK1) is a mitochondrial serine/threonine-protein kinase that protects cells from stress-induced mitochondrial dysfunction. As a result of the mitochondrial impairment, PINK1 accumulates on the outer mitochondrial membrane. An E3 ubiquitin ligase named Parkin is activated by following the colocalization with PINK-1 on the damaged mitochondria. PINK1 phosphorylates the Parkin Ub1 domain and increased its ubiquitin ligase activity (22). By several molecular analyses, it was demonstrated that the loss of function mutations in *PINK1* and *PARK2* genes encoding PINK1, and Parkin proteins respectively are associated with autosomal-recessive juvenile Parkinsonism (AR-JP) and display an important role of PINK1/Parkin-mediated mitophagy in neuronal degeneration (22–24). Although most of the studies have focused on the role of Parkin in neurodegenerative diseases, particularly PD, and associated with mitophagy, recent findings indicate that Parkin may have different functions. Parkin has been shown to suppress ER stress-induced cell death induced by chemical agents in SH-SY5Y and 293T cells and the response of Parkin induced by ER stress has been shown to be cell-type specific (25,26). It has been reported that the transcription factor ATF4, which is translocated to the nucleus by increasing ER stress, increases Parkin expression, and Parkin protects cells from stress-induced cell death independent of ubiquitin-proteasome activity (26). In cardiac cells, Parkin has been identified as a novel post-translational regulatory moderator of CHOP stability which regulates apoptotic cell death against chronic ER stress (27). Moreover, to neuron and cardiac cells, the role of Parkin in β cells has also been investigated. Two independent linkages and genome-wide association (GWA) studies in Korean and UK populations suggested *PARK2* gene was associated with T2D, and Parkin is required in the production and secretion of insulin (28,29) In addition, it has been demonstrated that in hyperglycemic conditions FFA overload, the Parkin protein expression in β cells has been reduced (30). But Corsa et al indicated that Parkin is decreased in the substantia nigra (SN) of obese and diabetic mice but increased in their vascular walls or adipose tissues and knocking out parkin in diet-induced obese mice did not affect insulin secretion, β cell formation, and islet structure (31).

By lagging apoptosis, autophagy may induce an adaptive response by reducing the effects of ER stress and supporting the surveillance of β cells (12). The high-fat diet and metabolic stress are known to upregulate β-cells autophagy as a protective mechanism, therefore the impairment of β-cells autophagy may associate with harmful metabolic effects (32,33). Parkin monitors physiological deterioration and regulates homeostasis, and thus is an important quality control protein. So, the identification of the molecular mechanisms enrolled in the regulation of β-cells autophagy under stress conditions and the effect of Parkin is not only essential for a better understanding of β-cells survival mechanism but developing β-cells targeted therapies (12).

Parkin protein may have a major impact on β-cells physiology, so understanding how the Parkin protein behaves in lipotoxic conditions may provide valuable insights into T2D treatment strategies. The study aimed to investigate whether Parkin, plays a role in cell viability/death and organelle stress under lipotoxic conditions in rat pancreatic β-cell line (INS-1E). Our hypothesis is to test that Parkin plays a role in cell survival by increasing autophagy activation in lipotoxic conditions.

Materials and Methods

INS-1E Cell Culture

The rat insulinoma (INS-1E) cells (a kind gift of P. Maechler, University Medical Center, Geneva, Switzerland) were cultured at 37°C in a humidified atmosphere (5% CO2) in RPMI-1640 medium (Sigma-Aldrich, R0883), which 1mM sodium pyruvate (Sigma-Aldrich S8636), 50 µM 2-mercaptoethanol (Sigma-Aldrich, M3148) 2mM L-glutamine (Sigma- Aldrich, G6392), 10mM HEPES (pH 7,3) (Sigma- Aldrich, H3784). 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma- Aldrich, P4333) (34). Following gentle trypsinization cells were seeded at a density of 4×10^4 cells/cm^2 in 25-cm^2 Falcon bottles (Orange Scientific, 4420100) with an 8-mL complete medium for maintenance culture once a week.

Palmitic Acid (PA) Preparation

PA was used to create lipotoxic running (35). The saturated PA (Sigma- Aldrich, P0500) was prepared by bovine serum albumin (BSA) using a slight modification that was described previously (36). PA was dissolved in ethanol at 200 mmol/L and then mixed with 10% FFA-free BSA (Sigma A9418), for a final concentration of 5 mmol/L. All filter-sterilized solutions with a pH of 7.5, were kept at -20°C until usage. Control solutions containing ethanol and BSA were prepared similarly. Different doses of PA (0.3 mM and 0.5 mM) for 12, 24 h. were used for the treatment of cultured cells.

Cell Viability and Cytotoxicity

For the quantitative determine the cellular proliferation MTT assay was used. The experiments were carried out in 96-well plates (Orange Scientific, 4430100) in compliance with the cell proliferation kit protocol (Cell proliferation kit I (MTT), 11465007001, Roche). An automated microplate reader (Epoch 2.0) was used for the measurement of the absorbance at 550 and 690 nm. BioTek Gen5 microplate reader software was used for analyzing the results.

Cells with a concentration of 4×10^4 cells/well were seeded in 96-well plates with a 100 µL medium incubated for confluency at 37 °C and CO_2 (%5). Following 72 h of incubation, PA was added to each plate and incubated at unique durations of 0 (for control), 12, and 24 h. All cell viability experiments were carried out in triplicate.

RNA Isolation and Gene Expression

INS-1E cells seeded at a concentration of 1×10^6 cells/well in 6-well plates (Orange Scientific, 4430500) with a 1mL medium, were incubated for the confluency at 37°C in a humidified atmosphere (%5 CO_2). After 72 h incubation, a fresh medium containing 0.3 mM PA and 0.5 mM PA for 0 (control), 12, and 24 h were added. At the end of the PA incubation period, by using Trypsin-EDTA, the cells were detached and used for RNA isolation (Qiagen,

Table 1. The real-time ready probes and accession numbers used in qRT-PCR.

Genes	Gene (probe) name	Assay ID	Accession No.
ER stress genes	*Grp78 (Hspa5)*	505446	NM_013083.2
	Atf6	500841	NM_012966.1
	Perk (Eif2ak3)	505460	NM_031599.2
	Chop (Ddit3)	502820	NM_001109986.1
Mitochondrial stress genes	*Hsp10 (Hspe1)*	503340	NM_012966.1
	Clpp	506366	XM_001055676.6
	Park2	506368	NM_020093.1
MAM genes	*Vdac (Vdac1)*	506371	NC_005109.4
	Ip3r (Itpr1)	500259	NM_001007235.2
Autophagy genes	*Lc3-I (Map1lc3a)*	505459	NM_199500.2
	Ulk (Ulk1)	506369	NM_001108341.1
	Beclin1 (Becn1)	504693	NM_001034117.1
Apoptotic genes	*Bax*	500244	NM_017059.2
	Bcl2 (Bcl-2)	502821	NM_016993.1
Reference genes	*Actb*	500152	NM_031144.3
	G6pd	502302	NM_017006.2

RNeasy Mini Kit 74104). The quality and quantity of the isolated RNAs were evaluated by Epoch 2.0. and all-in-one reader software BioTek Gen 5. Next, mRNAs were converted into cDNAs (Roche Transcriptor First Strand cDNA Synthesis Kit 05081963001). Quantitative Real-Time PCR (qRT-PCR) was carried out using the LightCycler 480 master probe kit (Roche LightCycler 480 master probe kit 04707494001). The real-time ready probes and accession numbers are shown in Table 1. *Actb* and *G6pd* were the reference genes that served as internal control. All samples were assayed thrice.

Cytosolic Ca^{2+}Analysis

INS-1E cells seeded at a concentration of 4×10^4 cells/well were seeded in 96-well plates with a 100 µL medium and incubated for the confluency at 37 °C in (CO_2 %5). After 72 h of incubation, PA was added to each plate. The plates have been incubated at unique durations of 0, 12, and 24 h, and this whole process has been replicated in triplicate. To detect intracellular Ca^{2+} level, Thermo Scientific Fluo 4 NW Calcium Assay Kit (Invitrogen, F36206) was used according to the manufacturer's instruction. All measurements were done using Epoc 2.0, and the Biotek Gen5an Reader software was used. Changes in relative fluorescence units (ΔRFU) from the Fluo-4-NW-dye quantify alterations in free cytosolic Ca^{2+} concentrations after PA treatment. The excitation and emission wavelengths were 494 and 516nm respectively.

Annexin V Apoptosis Detection by Flow Cytometry

INS1E cells (1×10^6) were seeded in 6-well plates and cultured for 72 h under standard conditions. Following a 12 h of incubation with a culture medium containing PA cells were labeled with Annexin V-PE and 7-AAD, and flow cytometry analysis was performed using the Accuri C6 device to detect apoptosis (BD Bioscience, PE Annexin V Apoptosis Detection Kit 559763). Cells that are considered viable with intact membranes are PE Annexin V and 7-AAD negative; cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative; cells that are in late apoptosis are PE Annexin V negative and 7-AAD positive; and cells that are already dead are both PE Annexin V and 7-AAD positive.

Caspase-3/7 Activity Assay

Cells were seeded in plates and cultured on confluency under standard conditions. After 12 and 24 h of PA incubation, caspase3/7 enzymatic activity was measured by using the Cell Event Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific/USA) as was previously described by Pawlak (37).

Detection of Reactive Oxygen Species (ROS)

1×10^6 INS-1E cells/well were seeded and incubated in 6-well plates. On 72 h of incubation, PA and siRNA treatments were accomplished at proper times and doses to each plate. The plates have been incubated at unique durations of 0, 12, and 24 h, and this whole process has been replicated in triplicate. Total ROS/Superoxide Detection kit (Enzo Life Sciences, ENZ-51010) was used in order to detect the superoxide production according to the manufacturer's instructions. The stained cells are analyzed by using Olympos IX73 fluorescence microscope equipped with standard green (ROS, 490/525 nm) and orange (superoxide 550/620 nm) filter sets. Images obtained from the fluorescent microscope were analyzed using the ImageJ software (National Institutes of Health).

Western Blotting

After lysing INS-1E cells with a commercial kit (cOmplete™ Lysis-M, Roche, Merck) the cellular debris was removed after 10 min. 14000 g centrifugation at room temperature. For protein concentration of the extracts, Bradford protein assay was used. Samples (30-50µg) were mixed with a 1×SDS sample buffer (50mM Tris pH6.8, 2% SDS, 10% glycerol, 50mM DTT, and 0.01% bromophenol blue) and were analyzed by using SDS-PAGE on 10% polyacrylamide gels. Then proteins were transferred onto a PVDF membrane (Millipore). After 12 h of incubation with primary antibodies, excess primary antibodies were washed, then, proteins were incubated for an hour at room temperature with secondary antibodies. Finally, membranes were developed via a chemiluminescent detection kit (WBLUF0100, Luminata Millipore).

Developed protein bands were measured by LI-COR. Color Prestained Protein Standard (NEB, USA) was used as a protein marker. The following antibodies were used: Ulk; Abcam; Anti-ULK1 antibody (ab167139), Beclin; CST; Beclin-1 (D40C5) mAb #3495, LC3-II; CST; LC3B Antibody #2775, Parkin; SİGMA; Anti-Parkin antibody, Mouse monoclonal P6248, and Pan-Actin; CST; Pan-Actin (D18C11) Rabbit mAb #8456. The following secondary antibodies were used: BioLegend; HRP Donkey anti-rabbit IgG 406401 BioLegend; HRP Goat anti-mouse IgG 405306.

Quantification of Glucose-Stimulated Insulin Secretion (GSIS)

Insulin secretion assay was conducted as was previously described (38). INS-1E cells were seeded in 24-well plates at a density of 2x105 and cultured for 48 h. and reached confluency. After treatment with PA or/and siRNA (Park2 or Nc siRNA), the cells were washed twice with phosphate-buffered saline (PBS). After 30 min. Incubation with Krebs-Ringer buffer (KRB: 120 mM NaCl, 5 mM NaHCO3, 5 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 10 mM HEPES, and 0.2% BSA, pH 7.2) the cells were incubated with KRB containing 2.8 or/and 16.7 mM glucose for 60 min. Finally, the collected supernatants were used for insulin analysis by using an ELISA kit according to the manufacturer's instructions (Rat/Mouse Insulin ELISA, EZRMI-13K Sigma-Aldrich-Merck) and analysis was done using Epoc 2.0, and the BioTek Gen5an Reader software was used.

siRNA Transfection

INS-1E cells incubated with 0.3mM PA had the highest *Park2* gene expression at the 24th hour. Therefore, this hour was assigned for the siRNA silencing. The cells were transfected with 3 different siRNA pools (Qiagen, Germany, FlexiTube siRNA, SI1957627 Park2_1, SI01957634 Park2_2, SI01957648 Park2_4). Cells were transfected with siRNAs and Lipofectamine-2000 (Thermo Fisher Scientific, Transfection Reagent) in compliance with the manufacturer's instructions. siRNA without any sequence match to known mRNA sequence in the mammalian genome was the negative control (Qiagen, Allstars Neg. Control siRNA 1027281).

For the determination of the optimum concentration of siRNA for silencing *Park2* siRNAs, a series of different concentrations of 50 pmol, 100 pmol, and 150 pmol was added to the 6-well plate containing 2mL medium without antibiotics. After 72 h of incubation, cells were transfected with the siRNA buffer containing 5µL Lipofectamine-2000 in 245µL serum-free OPTI MEM medium (ThermoFisher Scientific,31985062) mix shortly, and incubated at room temperature for 5 min to form a complex. 6 h after the transfection, the medium was replaced with fresh Opti-MEM. 30 h after transfection, cells were incubated with 0.3mM PA. 24 h after incubation, cells were collected for later analysis.

Statistical Analysis

Experiments were performed in triplicate and all the quantitative data were presented as mean ± SD. For the statistical evaolution of the data SPSS version 22.0 was used. Data were evaluated with a two-tailed, unpaired Student's t-test or compared by one-way analysis of variance. A value of $p< 0.05$ was considered statistically significant.

Results

Parkin expression is altered in low-dose and high-dose lipotoxic conditions

Palmitate-induced lipotoxicity is one of the most significant causes of β-cells dysfunction, which results in insulin resistance, glucose intolerance, and T2D (12,18). The induction of ER stress by PA in INS-1E cells has been reported previously (3,4). The function of Parkin in maintaining β-cells activity and its stress response is still a subject to be elucidated (28,30,31). A study performed in human aortic endothelial cells strongly implies dose-dependent effects of PA in Parkin regulation (39). In light of this finding, we try to determine whether Parkin functions in lipotoxic conditions, we created a lipotoxic effect with two different concentrations of PA (0.5mM and 0.3mM) in INS-1E cells.

Investigating whether the *Park2* gene expression is affected by lipotoxic conditions was the first step of this study. Our results have revealed that the increase in the *Park2* gene mRNA and the Parkin protein expression was statistically significant at the 24th hour in incubation with 0.3mM PA, and there was a statistically significant decrease at the 24th hour in incubation with 0.5mM PA was determined. To demonstrate the effect of *Park2* gene in ER-UPR under lipotoxic conditions, *Park2* gene was silenced with siRNA in INS-1E cells and treated with 0.3 mM PA for 24 hours which has the highest mRNA expression. Administration of 0.3mM PA with *Park2* siRNA to INS-1E cells for 24 hours reduced *Park2* mRNA levels by 86%. (Figure 1 A-D).

PA in Different Concentrations Affects INS-1E Cell Viability

There was a decrease in cell viability depending on the

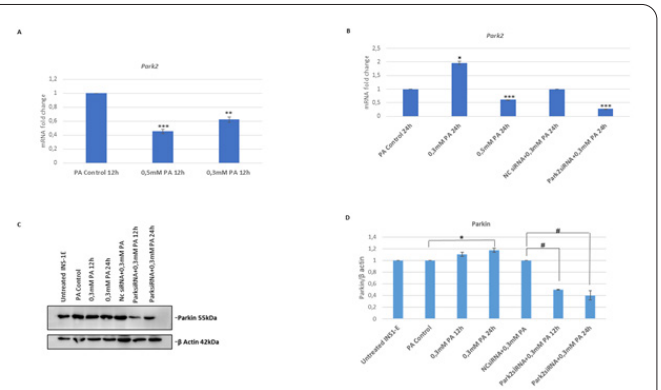

Figure 1. Effects of the 0.3mM PA, 0.5mM PA and Park2siRNA+0,3mM PA treatments on Parkin mRNA and protein level (**A)-(B)** mRNA levels at the 12 and 24th hour in INS-1E cells respectively. Gene-specific real-time ready probes were used for qRT-PCRanalaysis and Gapdh was for both relative control and normalizing concentration. The error bars represent mean ± SEM from three independent experiments (n = 3). *p <0.05; **p <0.01; ***p <0.001 vs control (**C)** Western blot analaysis of Parkin. Alterations of Parkin protein level in response to 0,3mM PA, 0,5mM PA and Park2siRNA+0.3mM PA 12h and 24h. (**D)** Statistical analysis of the expression of Parkin protein detected by western blot in cells as described in Figure c. The error bars represent mean ± SEM from three independent experiments (n = 3), *p <0.05 vs PA control, # p <0.05 vs NcsiRNA+0,3mM PA.

PA dose. 0.5 mM PA was applied to INS-1e cells for 24 hours a significantly decreased cell viability (49%) was observed, this value was 89% in 0.3mM PA application. The next step was to investigate the effect of silencing the *Park2* gene expression on the viability of INS-1E cells by the administration of 0.3 mM PA. Cell viability was found to be 66% and %85 for the 24th hour Park 2 siRNA+0.3 mM PA application and siRNA+0.3mM PA (control) application respectively. However, the difference between 0.5 mM PA treatment and *Park2* siRNA+0.3 mM PA treatment was statistically insignificant. It was found that the viability decreased significantly due to the silencing of the *Park2* gene. These results indicate that the knockdown of *Park2* in INS-1E cells affects cell viability (Figure 2A).

Parkin Affects Cell Death in PA-Treated INS-1E Cells

In order to demonstrate the mode of cell death induced by PA in INS-1E cells, in addition to dual staining with Annexin V and 7AAD, cells were also analyzed by flow cytometry. Annexin V/7AAD staining showed that the apoptotic cell rate (sum of early and late apoptosis) was considerably increased after 24 h of application with 0.5mM PA (52.6%) compared to control cells (15,10%). Apoptosis was also induced (23.3%) with 0.3 mM PA incubation. The results show that 0.5 mM PA treatment significantly increases the percentage of apoptotic cells over 0.3 mM PA treatment The difference between the two applications was found to be statistically significant (Fig 3A and 3B). Next, it was investigated whether the modulation of *Park2* gene expression could affect apoptotic cell death against the lipotoxic effect. Measurements at the 24th hour for cells incubated with *Park2*siRNA+0.3mM PA showed the apoptotic cell rate was 32,9 % ($p<0.001$ vs control group and $p<0.05$ vs Nc siRNA+0,3mM PA 24h). Although silencing the *Park2* gene increases the apoptosis rate to, however, this increase was not as high as in 0,5mM PA treatment (Figure 2B, 2C).

Caspase 3/7 activities were investigated to determine the effect of PA on mitochondria initiating the apoptotic process in rat INS-1E cells. The results have revealed a significant increase in caspase 3/7 activity compared to the control at 12 hours and 24 hours after 0.5 mM PA administration (Figure 2D, 2E). The caspase 3/7 activity of the 0.3mM PA-treated cells was lower compared to the 0.5mM PA-treated cells at 12h and 24h. *Park2*siRNA application resulted in a significantly higher caspase 3/7 activity compared to the 0.5mM PA application at the 24th hour (Figure 2E). Data from cell viability, Annexin V/AD and caspase 3/7 measurements appear to be in agreement with each other, providing convincing evidence that Parkin affects INS-1E cell survival versus PA effect. Moreover, our results demonstrate that PA-induced cell death was further increased by silencing *Park2*. (Figure 2E).

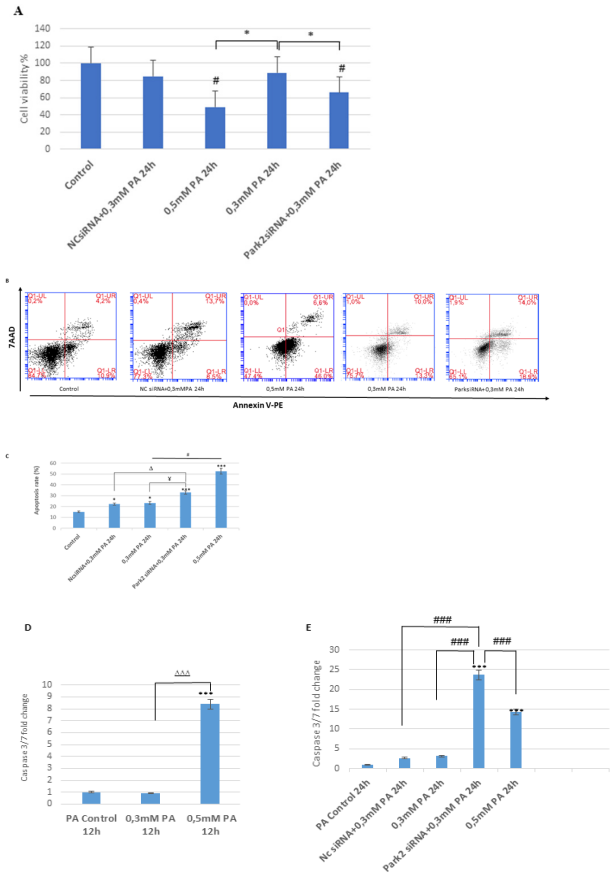

Figure 2. PA and Park2 siRNA effect on cell viability and apoptosis in INS-1E cells. Cells treated with 0,5 mM PA 24 h, 0,3mM PA 24h, Park2siRNA+0,3mM PA and NcsiRNA+0,3mM PA 24 h. **(A)** MTT test was used to determine cell viability %. #$p<0.05$ vs control group, *$p<0.05$ **(B)** Annexin V-PE/7-AAD apoptosis detection of INS-1E cells **(C)** Apoptosis rate was analyzed. The apoptosis rate refers to the sum of early and late apoptosis (Q1-LL, intact cells; Q1-LR, early apoptotic cells; Q1-UR, late apoptotic cells; Q1-UL, necrotic cells). The bars denote the apoptosis rate (Q1-LR + Q1-UR). *$p<0.01$, ***$p<0.0001$ vs control group, #$p<0.0001$ 0,3mM PA 24h vs 0,5mM PA24h, $^{\Delta}p<0.05$ Nc siRNA+0,3mM PA 24h vs ParksiRNA+0,3mM PA 24h, $^{¥}p<0.01$ 0,3mM PA24h vs ParksiRNA+ 0,3mM PA 24h. **(D)** Analysis of apoptosis using Caspase 3/7 activity in PA-induced INS-1E cells. Cells were treated with 0.3mM PA 12h and 0.5mM PA 12h. ***$p<0,0001$ vs control group, $\Delta\Delta\Delta p<0,0001$ 0,3mM PA 12h vs 0,5mM PA12 h, **(E)** Cells were treated with NcsiRNA+0.3mM PA 24 h, 0,3mM PA 24h, Park2siRNA+0,3mM PA 24h. and 0.5mM PA 24h. ***$p<0,0001$ vs control group, ###$p<0,001$ vs ParksiRNA+0,3mM PA 24h The error bars represent mean ± SEM from three independent experiments (n = 3).

***Park2* Silencing Affects Apoptosis and Autophagy**

Administration of 0.5 mM PA and silencing of the *Park2* gene increased the rate of apoptosis in INS-1E cells and was confirmed by annexin V and caspase analyses. The *Bax/Bcl2* mRNA results at 12 and 24 h are consistent with these results as well. *Bax/Bcl2* mRNA level obtained with 0.5 mM PA application at the 12th hour was statistically significantly higher than 0.3 mM PA application (Figure 3A). In addition, silencing *Park2* at 24 h increased this rate was observed (Figure 3B). Consistently, *Park2*siRNA+0.3mM PA treatment also increased mRNA expression of CHOP, the apoptosis-associated transcription factor of the ER-UPR (Figure 3, Figure 5D). With these data, the lack of *Park2* under low-dose lipotoxic conditions triggers apoptotic cell death is demonstrated.

Either the loss of mitochondrial membrane potential or disturbance in mitochondrial can activate PINK-1-Parkin-dependent mitophagy (40,41). Additionally, it was reported that various molecular markers of autophagy as *Beclin1, Atg5, Atg7, and Lc3-1* as can be triggered by Parkin expression in β amyloid protein clearance in neurodegeneration models (42,43). However, such a relationship between *Beclin-1, Lc3-1 and Ulk1* and *Park2* in pancreatic

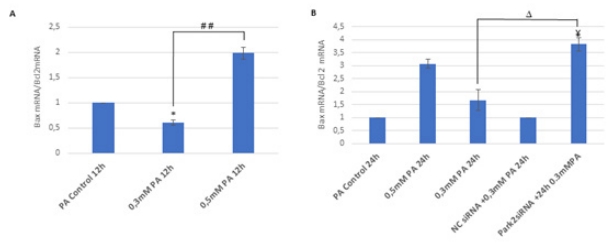

Figure 3. The Bax/Bcl2 mRNA expression ratio at PA and Park2 siRNA+0,3mM PA treatments. **(A)** 0.3mM PA 12h and 0,5mM PA 12h treatments and Bax mRNA/Bcl2 mRNA ratio *p <0.05 vs control; ##p <0.01 0,3mM PA 12h vs 0,5mM PA 12h at the 12 and 24th hour in INS-1E cells. **(B)** 0.3mM PA 24h, 0,5mM PA 24h, Park2siRNA+0.3mM PA 24h treatments and Bax mRNA/Bcl2 mRNA ratio ¥p <0.05 vs NcsiRNA+0,3mM PA; Δ p <0.05 0,3mM PA 24h vs Park2siRNA+0.3mM PA 24h. Gene-specific real-time ready probes were used for qRT-PCRanalaysis and Gapdh was for both relative control and normalizing concentration. The error bars represent mean ± SEM from three independent experiments (n = 3).

β-cells has not been previously defined. Our results show that there was a decrease in the mRNA and protein levels of the autophagy markers *Beclin1, Lc3-I,* and *Ulk1* under the 0.5 mM PA treatment compared to the reference gene at the 12th and 24th hours. ULK1, which plays an important role in the initiation of autophagy, has been shown to be degraded in hepatocytes in the presence of 0.5mM PA (44). Similarly, Ulk1 can be degraded in INS-1E cells after 24 h of incubation of high-dose PA. Additionally, the findings obtained in 0.3 mM PA treatment were different. In our study, Ulk1 and Lc3-1 mRNA and protein levels increased at 12 and 24 hours compared to the reference gene for 0.3mM PA incubation. Despite the increase in *Ulk1* and *Lc3-I* mRNAs in 0.3mM PA 12h treatment, we cannot explain the statistically significant decrease in *Beclin1* mRNA. However, the presence of Beclin 1 expression was demonstrated in 0.3mM PA 12h treatment by western blot analysis. These molecular data show that autophagy is active with the incubation of 0.3 mM PA, while incubation with high-dose PA inhibits autophagy. As a next step in this study *Park2* gene was silenced to test whether it has a possible effect on autophagy flux at 0.3mM PA 24h conditions. According to the results of the western blot, we observed that *Ulk1, Beclin1, and Lc3-II* decreased after silencing the *Park2* gene (Figure 4). This finding strongly points to a possible function of Parkin in autophagy flux in addition to its role in mitophagy and should be investigated in further studies.

Silencing *Park2* Only Affects *Chop*

The ER stress markers *Grp78, Perk, Atf6,* and *Chop* have been shown to be transcriptionally upregulated by PA (7,17,18). It has been confirmed in our study that mRNA expression levels for these markers vary depending on the time and dose of PA administration in INS-1E cells. We determined that there was a statistically significant increase in Grp78 mRNA levels alone in the 0.5 mM PA 12h and 0.3 mM PA 12h treatment compared to the control mRNA. Although *Perk* mRNA (3.8-fold) increased in 0.3mM PA 12h, *Atf6* mRNA (1.5-fold) in 0.5mM PA 12h, and *Chop* mRNA (1.5-fold) in 0.5mM PA 12h., however, these increases were statistically insignificant (Figure 5A). In 0.5 mM PA 24-hour treatment, *Grp78* and *Chop* mRNA levels were found to be statistically significantly increased compared to control mRNA. There was a different mRNA expression pattern in the low-dose PA administration. While the increase in *Atf6* and *Chop* mRNAs in 0.3mM PA 24h treatment was statistically significant compared to the control, the increase in *Grp78* mRNA level was not. Furthermore, it was determined that *Perk* mRNA was decreased compared to the control (Figure 5B-E). These findings suggest that the regulation of ER-UPR genes depends on not only the effect of the stress factor but the duration of the stress as well. Repression of the *Park2* gene by specific siRNA increased basal *Chop* and *Grp78* mRNA levels and decreased *Perk* and *Atf6* mRNA levels, but the change in *Chop* only was statistically significant. Although these findings are insufficient to say that Parkin has an effect on the ER-UPR activated under lipotoxic conditions, it can be speculated that a possible effect of Parkin may be on Chop.

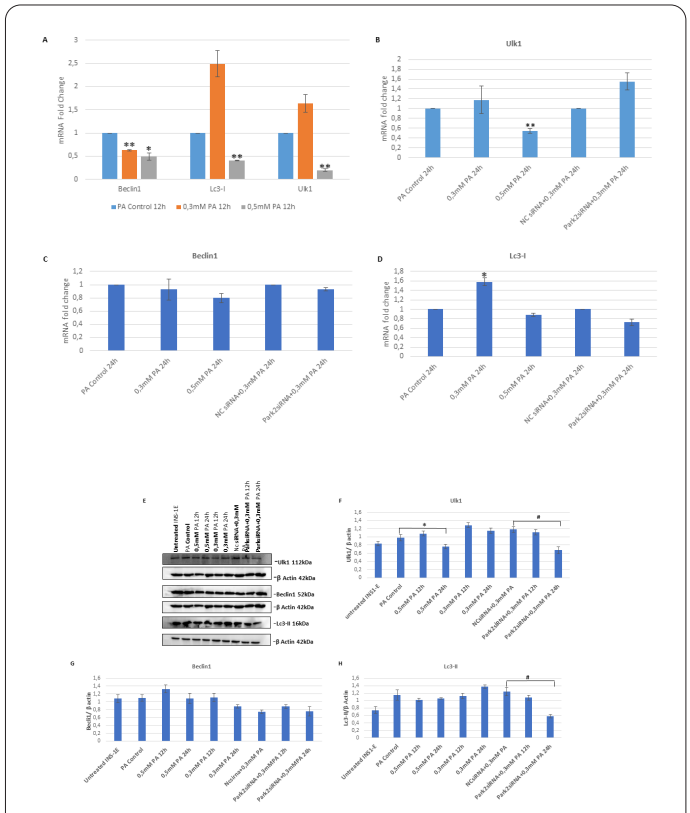

Figure 4. Effects of the PA and Park2 siRNA treatments on autophagy genes in INS-1E cells. **(A)** 0.3mM PA 12h and 0,5mM PA 12h treatments and Ulk1, Beclin1, and Lc3-1 mRNA expression levels change. Gene-specific real-time ready probes were used for qRT-PCRanalaysis and Gapdh was for both relative control and normalizing concentration. **(B-D)** 0.3mM PA 24h, 0,5mM PA 24h, Park2 siRNA+0.3mM PA, NcsiRNA+0,3mM PA 24h treatments and changes in mRNA expression levels of **(B)**Ulk1, **(C)** Beclin1 and **(D)** Lc3-1 genes. The error bars represent mean ± SEM from three independent experiments (n = 3). *p <0.05; **p <0.01 vs control. **E** Alterations of Ulk1, Beclin1, and Lc3-II protein levels in response to PA and Park2siRNA+0,3mM PA 24h and NcsiRNA+0,3mM PA 24h analysed by western blotting **F-H** Statistical analysis of the expression of **(F)**Ulk, **(G)** Beclin1 and **(H)** Lc3-II proteins detected by western blot in cells as described in E. The error bars represent mean ± SEM from three independent experiments (n = 3). *p <0.05vs PA control, #p <0.05 vs NcsiRNA+0,3mM PA 24h.

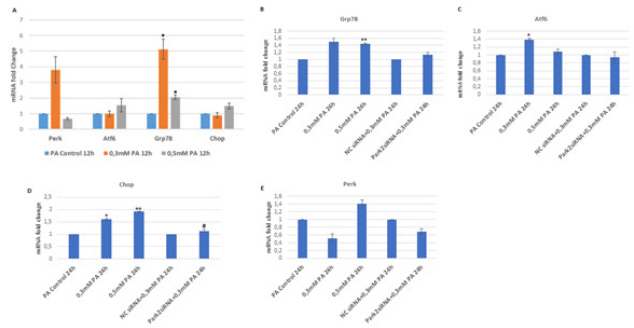

Figure 5. Effects of the PA and Park2 siRNA+0,3mM PA treatments on ER-UPR genes**(A)** 0.3mM PA 12h and 0.5mM PA 12h treatments and change in Grp78, Atf6, Perk and Chop mRNA expression levels **(B-E)** 0.3mM PA 24h, 0,5mM PA 24h, Park2 siRNA+0.3mM PA 24h, NcsiRNA+0,3mM PA treatments and change in **(B)** Grp78, **(C)** Atf6, **(D)** Chop and **(E)** Perk mRNA expression levels. Gene-specific real-time ready probes were used for qRT-PCR analysis and Gapdh was for both relative control and normalizing concentration. The error bars represent mean ± SEM from three independent experiments (n = 3). *p <0.05; **p <0.01 vs control, #p<0,05 vs NcsiRNA+0,3mM PA 24h.

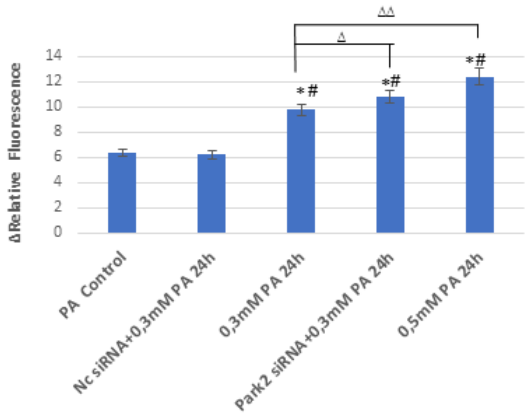

Figure 6. Effect of PA and ParksiRNA on cytosolic Ca^{2+} concentration. Cytosolic Ca^{+2} concentration changes in PA and Park2siRNA+0.3mM PA 24h treated INS-1E cells. *p<0.0001 vs Control group, #p<0.0001 vs Ncsirna+0,3mM PA 24h group, $^{\Delta\Delta}$p<0.01 0.3mM PA 24h vs 0.5mM PA 24h, $^{¥}$p<0.05 0.3mM PA 24h and Park2siRNA+0.3mM PA 24h. Values are means±SEM. Data are representative of at least three independent experiments.

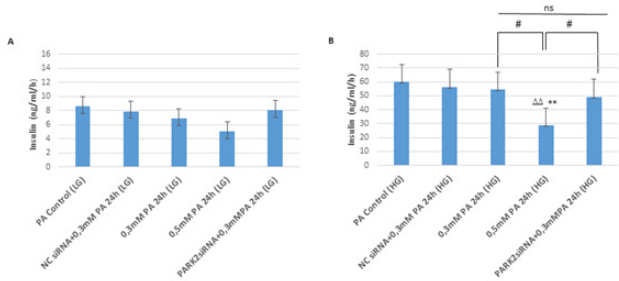

Figure 7. PA and Park2siRNA+0,3mM PA-induced impairment of GSIS in INS-1E cells. The cells were stimulated with **(A)** low dose (2.8mM) and **(B)** high dose (16.7mM) glucose and insulin secretion was determined as described in materials and method. **p<0.001 vs PA control (HG), $^{\Delta\Delta}$p<0.001 vs NcsiRNA+0,3mM PA, #p<0.01 vs 0,5mM PA 24h, ns; not significant. Values are means±SEM. Data are representative of at least three independent experiments.

Parkin Plays a Role in Ca^{+2} Release From the ER

PA-induced ER stress occasioned a decrease in the ER Ca^{2+} pool and an increase in cytosolic Ca^{2+} (45). At this point, we investigated the intracellular Ca^{+2} change using the Ca $^{+2}$ indicator Fluo-4. According to ΔRelative Fluorescence Units (ΔRFU) results, the highest concentration was observed under the 0.5 mM PA 24h treatment and compared with the control was also found to be statistically significant. In addition, the difference between the cytosolic Ca^{+2} concentration for the 0.5mM PA 24h and 0.3mM PA 24h treatments was found to be statistically significant. Reduced *Park2* gene expression significantly affected the cytosolic Ca^{+2} concentration in INS-1E cells treated with 0.3 mM PA. No significant change was found between the 0.5 mM PA 24h treated cells and the *Park2*siRNA+0,3mM PA 24h treated cells. Comparing the results of *Park2* siRNA+0.3mM PA 24h treatment and 0.3mM PA 24h treatment showed that the value of *Park2* siRNA+0.3mM PA 24h treatment was higher, and the difference was statistically significant. The results of *Park2*siRNA+0,3mM PA 24h and NcsiRNA+0,3mM PA 24h treatments were also found to be statistically different (Figure 6). Our results suggest that Parkin may play a role in Ca^{+2} release from the ER.

PA Treatment with *Park2* Silencing Not Affect Glucose-Stimulated Insulin Secretion (GSIS) and ROS Production

PA causes increased ROS production and impaired insulin secretory capacity in INS-1E cells (46,47). The relationship between ROS produced due to mitochondrial dysfunction and GSIS defect caused by palmitic acid has been demonstrated in a previous study (47). Also, a study showed that Parkin is expressed in β-cells and is involved in the production of insulin and ROS in INS-1E β-cells (28). However, another research group has reported that Parkin did not control the function of β- cells (31). The effect of Parkin on β-cells function is still not fully elucidated. Due to conflicting reports on Parkin β-cells function, here, we investigated how GSIS and ROS production were affected when the *Park2* gene was silenced under lipotoxic conditions. When the 0.3mM PA, 0.5mMPA, and *Park2*siRNA+0,3mM PA treatments were compared in terms of GSIS levels, the change in the 0.3mM PA and *Park2*siRNA+0,3mM PA groups at the 24th hour was not statistically significant compared to the control group and NcsiRNA+0,3mM PA group. The highest insulin level was observed under 0.3mM PA 24h treatment and the lowest insulin level was observed under 0.5mM PA 24h treatment. The decrease in 0.5mM PA 24h treatment was statistically significant compared to the control and NcsiRNA+0.3mM PA 24h treatment. Our data suggest that Parkin deficiency is not crucial for insulin production (Figure 7). Consistent with the GSIS results, our experiments confirmed that the exposure of 0.5 mM PA 24h induces ROS/superoxide production and 0.3 mM PA 24h treatment does not. In addition, there was no statistically significant difference between 0.3mM PA 24h and *Park2* siRNA+0.3mM PA 24h treatments. Contrary to our expectations, silencing *Park2* did not increase ROS or superoxide production (Figure 8).

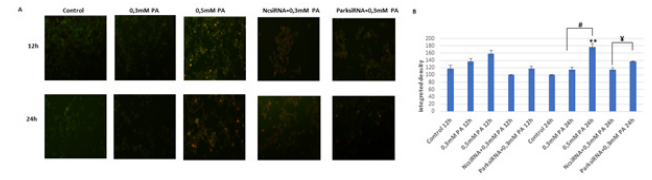

Figure 8. ROS/Superoxide detection after PA and ParksiRNA+0,3mM PA treatment **p < 0.01 vs control, #p<0,05 0,5mM PA 24h vs 0,3mM PA 24h, ¥ p<0,05 Park2siRNA+0,3mM PA vs NcsiRNA+0,3mM PA. **(A)** 0.3 mM PA and 0.5 mM PA, Park2siRNA+0,3mM PA and Nc siRNA+0,3mM PA treatments at the 12- 24th-hour fluorescent microscope view of ROS / Superoxide formation. After PA treatment, immunofluorescence staining images of cells were detected by ROS/ Superoxide detection solution and pyocyanin. The oxidative stress level in cells was determined by a green channel (490-525 nm) and superoxide formation was determined by an orange channel (550 / 620nm) by a fluorescence microscope (20 X) Images are representative of three independent experiments **(B)** The integrated intensity of ROS/superoxide generation in cells treated with 0.3mm PA, 0.5mM PA, NcsiRNA+0.3mm PA, and ParksiRNA+0.3mM PA at 12 and 24 hours. Values are means ± SEM. Data are representative of at least three independent experiments.

Discussion

This study aimed to show whether Parkin, whose role has been studied mostly in Parkinson's disease, plays a role in cell viability/death and organelle stress at lipotoxic conditions in rat pancreatic β-cell line (INS-1E).

Parkin is a 52 kDa protein encoded by the *PARK2* gene. It is a member of the E3 ubiquitin ligase family and tags ubiquitin to the target protein to be destroyed by the ubiquitin-proteasome system (UPS). In addition to proteasomal degradation; Parkin is also functional in the selective clearance of damaged and dysfunctional mitochondria by PINK1/Parkin-mediated mitophagy (21,22). Loss-of-function mutations in the *PARK2* and *PINK1* genes are known to account for most of the cases of familial forms of Parkinson's disease (23).

Due to the increased energetic demands post-mitotic neuronal cells are more susceptible to mitochondrial damage that's why most of the reports are focused on the involvement of mitochondrial damage and neurodegenerative disorders, however, Jin et al have reported that Parkin has a major impact both in the regulation of insulin production and GSIS caused by mitochondrial alterations in β-cells.(28) Silencing the *Park2* gene in INS-1E cells for 48 hours does not result in a significant difference in cell viability compared to the control group. However, when *Park2* gene expression is inhibited, β-cells function is impaired due to impaired mitochondrial quality control (28). The response of the Parkin protein to PA has been previously demonstrated in endothelial cells (39). However, this study is mainly focused on the relationship between PA-Parkin-mitophagy. It is not yet known whether Parkin responds to PA-induced lipotoxic stress in pancreatic β-cells.

Although it has been shown that downregulation of *Park2* by siRNA under non-stress conditions does not affect cell viability, (28) in our study, we determined that the viability rate of 0.3mM PA-treated INS-1E cells and the viability rate of the cells treated with *Park2* siRNA+ 0.3mM PA varied significantly from each other. The fact that Parkin is not overexpressed in 0.5 mM PA treatment and is active for 0.3 mM PA treatment indicates that the stress responses in which Parkin plays a role are controlled by ambient conditions. Any pathway has not been identified that could explain the differential expression of Parkin in high and low lipotoxic conditions yet. However, there is a strong possibility that it may be regulated by RNA interference. A second possibility is when the stress is too severe, both the protective and survival mechanisms may shut down by UPR to inhibit the Parkin expression.

Autophagy is one of the survival mechanisms of the cell and supports cell survival under apoptosis-inducing stress conditions such as lipotoxicity, ER stress, and oxidative stress due to mitochondrial damage (4,12,48). Chronic exposure to glucose and fatty acids causes cellular stress and can not only trigger the development of diabetes but also inhibit the cellular adaptive and survival mechanisms by which cells protect themselves from this stress (11). In particular, PA impairs autophagic flux by activating mTORC1 and induces β-cells death by increasing ER stress (12,49). Although autophagy is important in maintaining the function and viability of pancreatic β-cells, it also plays a role in cell damage depending on the type of stress and exposure duration (1,2,49). However, these mechanisms still await clarification.

It has been shown that depending on environmental conditions mono or poly-ubiquitination of the target protein by Parkin determines the choice of apoptosis or autophagy (50). It has been reported in previous studies that Bcl2, (51) Bak, (52) and Bax, (52–54) which are mitochondrial outer membrane proteins, are the substrate of Parkin. Based on these findings, it can be suggested that Parkin plays an important role in the decision between apoptosis or autophagy by regulating the relationship between pro-anti-apoptotic proteins. On silencing the *Park2*, a statistically significant increase in the *Bax* mRNA/*Bcl* 2 mRNA level was determined in our study. This result is also consistent with caspase 3/7 findings. Our data strongly suggest that the crosstalk between autophagy and apoptosis is impaired in low lipotoxic conditions and the absence of *Park2.*

In our experiment, the lowest cell viability rate was 49% at 24 hours under 0.5mM PA treatment. Furthermore, analysis of Annexin V and Caspase 3/7 reveals that cell death is via apoptosis under these experimental conditions. Furthermore, the findings of the Annexin V and Caspase 3/7 studies show that the apoptotic activity in cells treated with *Park2*siRNA 0.3mM PA was significantly higher than in cells treated with 0.3mM PA alone. In our study, western blot results indicated that autophagic activity in cells treated with 0.3mM PA was higher than with 0.5mM PA. Additionally, the expression of autophagic markers Ulk1 and Lc3-II was found statistically significantly decreased in *Park* siRNA+0.3mM PA conditions compared to the control. In light of all these results, we think that silencing of the *Park2* gene causes downregulation of autophagy. Although there are limitations in this study, it can be mentioned that Parkin affects the autophagic flux under low lipotoxic conditions.

ER stress due to lipotoxicity in many multifactorial diseases such as T2D, metabolic syndromes, neurodegenerative, cardiovascular, liver, lung and kidney diseases as well as cancer has been extensively researched (6,39,55–59). The UPR activation depends not only on the type of cell

applied but also on the type and duration of the stressor. Our results were compatible with previous reports (3,17,60) in that PA-impaired UPR balance and triggered ER stress in INS-1E cells depending on time and dose. Shinjo et al. reported an increase in Perk and Chop expression, but no alteration in mRNA and protein levels in Grp78, Atf6 and Xbp1 under 0.3 mM PA treatment in HepG2 cells (61). In our study, only the increase in *Grp78* mRNA was statistically significant in 0.3 mM PA 12h and 0.5 mM PA 12h treatments. The increase detected in the mRNA of other UPR elements in low and high-dose PA treatments at 12h was statistically insignificant. Expression patterns of ER-UPR elements also changed at 24-hour treatments.

We found a statistically significant increase only in the mRNA level of *Chop* due to silencing the *Park2*. Although our findings are at the mRNA level, it cannot be said that there is a significant connection between Parkin and ER-UPR. However, silencing *Park2* may affect *Chop*. Chop is an important transcription factor that plays an active role in determining cell fate under stress conditions (62). The connection between the silencing of *Park2* and decreased cell viability may be associated with the activity of Chop. Parkin acts as a post-translational regulatory moderator, ensuring the stability of Chop in cardiac cells (27). Parkin may have a similar function in rat pancreatic β cells. The available data are still insufficient to explain exactly how the mechanisms work.

Ca^{+2} is an important intracellular signaling molecule that plays a role in important metabolic pathways for the cell such as energy metabolism and protein synthesis. The cytoplasmic Ca^{+2} uptakes to the ER lumen by Sarco/Endoplasmic Reticulum Ca^{2+}-ATPases, (SERCA) pump and are released into the cytosol in response to diverse physiological triggers. It has been reported that PA inhibits SERCA activity (63) and deterioration of Ca^{2+} concentration in the ER due to lipotoxicity affects the working capacity of chaperones, the folding process of proteins, and actives UPR, prolonged activation of the UPR causes apoptotic β-cells death (3,11). PA induces Ca^{2+} influx not only from the ER to the cytoplasm but also from the MAM region to the mitochondria. Increased Ca^{2+} load in mitochondria causes ROS production and β cell dysfunction (64). Our findings show the highest cytosolic Ca^{2+} level in the 24 h treatment of 0.5mM PA. Silencing Park2 in 0.3mM PA 24h treatment affected Ca^{2+} influx to the cytosol. It was observed that there was a significant change between 0,3mM PA treatment and Park2siRNA+0,3mM PA. Our data is also consistent with our findings of cell viability and apoptosis. In conclusion, the absence of Parkin seems to cause an increase in Ca^{2+} output from the ER to the cytosol. We think that the interaction of Parkin with IP3R and/or RyR, which is known to function in Ca^{2+} exit from the ER (65), may play a role in this mechanism.

The mitochondrion is a dynamic organelle responsible for the cell's metabolite flow, energy production, and the maintenance of the redox state. The *Park*2 silenced β-cells also exhibited increased mitochondrial fragmentation and ROS production and decreased mitochondrial membrane potential (28). Insulin production and excretion from β-cells are strongly dependent on healthy mitochondrial functions like efficient ATP production (66). Increased ROS production activates Uncoupling Protein 2 (UCP2), resulting in decreased ATP production and reduced insulin production (67). In concordance with the previously published data, we found that 0.5 mM PA treatment increased ROS production in β-cells and impaired insulin synthesis capacity (47,68). Moreover, we observed that ROS production and insulin synthesis was not affected in the 0.3 mM PA treatment. However, silencing *Park2* did not cause an increase in ROS/superoxide production.

In this study, we investigated whether Parkin, an E3 ubiquitin ligase with a well-established role in mitophagy, affects cell viability and ER stress responses under lipotoxic conditions in INS-1E cells. Our results may suggest that silencing Parkin affects autophagy in addition to apoptosis. In addition, this study showed for the first time that Parkin may have a protective effect against lipo-toxic effects in INS-1E cells. Consistent with previous studies, we observed that stress responses were different for high and low PA doses. The Parkin being inhibited under high-dose PA treatment and active under low-dose PA treatment indicate that regulation of stress responses is controlled by environmental conditions. Our preliminary findings suggest that in low lipotoxic conditions, Parkin affects the ER stress response by modulating Chop activity. Silencing *Park2* increased Ca^{+2} release from the ER to cytosol and alterations in intracellular Ca^{2+} homeostasis ultimately induce apoptosis. Although detailed knowledge has been obtained about the role of Parkin in mitochondrial quality control and mitophagy, it has not yet been clarified how it behaves in tissue-specific cellular stress.

Conclusion

Our findings motivate understanding of the full extent of the functions of the Parkin, which plays a not fully clarified role in the normal physiological functioning of rat pancreatic β-cells, as this will understand the etiopathogenesis of diseases such as T2D, obesity, and metabolic syndrome. Furthermore, revealing the comprehensive role of the Parkin protein in ER –mitochondrial interaction will be important in understanding disease-related pathogenesis.

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Compliance with ethical standards

Disclosure statement

The authors declare that they have no conflict of interest.

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