IGF-1 inhibits palmitic acid-induced mitochondrial apoptosis in macrophages

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Abstract. Insulin growth factor-1 (IGF-1) is an endocrine regulator that plays an important role in normal growth and development. IGF-1 mediated effects may result in protecting macrophages from immunometabolic response. However, it is unclear whether IGF-1 has a protective effect on fatty acid-induced macrophages damage. In the present study, THP-1 cells were differentiated into macrophages and stimulated with palmitic acid (PA) in the absence or presence of IGF-1. Macrophages apoptosis was measured by Cell Counting Kit-8 assay, flow cytometry, Hoechst 33342 staining and western blotting. The mitochondrial damage was evaluated using JC-1 staining and mitochondrial reactive oxygen species detection. The activation of mitophagy was assessed using immunofluorescence and western blotting. As a result, IGF-1 significantly restored the survival rate in macrophages, while the apoptosis was inhibited through mitochondrial pathway. In addition, IGF-1 protected the mitochondrial damage induced by PA. Furthermore, PA induced mitophagy via phosphatase and tensin homolog-induced putative kinase protein 1/Parkin, which was reversed by IGF-1. Taken together, the present study demonstrated the protective effect of IGF-1 on PA-induced mitochondrial apoptosis in macrophages, which might provide a potential therapeutic strategy for treatment of lipotoxicity.

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Introduction

During the last decades, the effects of dietary fat intake has provided more insight into the induction of metabolic syndrome, such as obesity, type 2 diabetes mellitus and cardiovascular disease (1,2). Evidence is emerging that elevated levels of lipid substances evoke lipotoxicity and cell death pathways, thereby contributing to the pathological process in the body (3). In a variety of lipid substances, saturated free fatty acid has the highest lipotoxicity, whereas diary western diet often contains a great amount of free fatty acid (4). Palmitic acid (PA) is one of the most common free fatty acid, which stimulates apoptosis in experimental systems (5,6). However, the exact mechanism of PA has not been established and may vary by cell type (7-9).

Macrophages are essential to the maintenance of the organism homeostasis, immunological control, and pathogen defense (10). Previous studies have confirmed that macrophages apoptosis is a risk factor for lipid metabolism diseases by controlling proliferation (11), lipoprotein efferocytosis and the release of proinflammatory cytokines (12,13). Oxidized low-density lipoprotein, cholesterol and fatty acid can all induce apoptosis of macrophages, leading to the acceleration of immunometabolism response. Strategies to protect macrophages from lipotoxicity may provide a new therapeutic approach.

Insulin growth factor-1 (IGF-1), an endogenous growth factor, has been extensively studied for its role in physiological and pathological processes. IGF-1 and its receptor are expressed

in most cells in body, which are inversely related to the risk of many metabolic disease (14,15). For example, elevated IGF-1 levels reduce the progression of cardiovascular disease (16,17). IGF-1 also contributes to the immune homeostasis during metabolic stresses (18). Notably, overexpression of IGF-1 improves the prognosis in the mice fed with high diet food by reducing apoptosis of macrophages. Evidence is emerging that these effects depend on the protective role of IGF-1 on macrophages from external stimuli or internal signaling imbalance (19-21). However, as an important contributor of lipotoxicity, the influence of IGF-1 on PA-induced macrophages remains to be elucidated.

Therefore, the present study investigated the effect of IGF-1 on PA-induced macrophage apoptosis with the aim of providing a scientific basis for further understanding of IGF-1 in dyslipidemia diseases.

Materials and methods

Materials. THP-1 was obtained from the Cell Bank of the Typical Culture Collection Committee of the Chinese Academy of Sciences; Fetal bovine serum, 1640 medium from Gibco (Thermo Fisher Scientific, Inc.); PBS buffer, trypsin–EDTA and penicillin from Guangzhou Xinhe Technology Co., Ltd. IGF-1 and phorbol 12-myristate 13-acetate (PMA), Cell Counting Kit-8, mitochondrial membrane potential assay kit, ECL chemiluminescence reagent were purchased from MedChemExpress; GAPDH antibody, cytochrome c antibody, phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1) antibody and Parkin antibody was purchased from Affinity Biosciences; Bax antibody was purchased from Cell Signaling Technology, Inc.; caspase-3 antibody and Bcl-2 antibody were purchased from Abcam; bovine serum albumin, DAPI, Hoechst 33342 and Normal Goat Serum were purchased from Beijing Solarbio Science & Technology Co., Ltd.; Annexin V-FITC/PI apoptosis assay kit was purchased from Elabscience; BCA kit, caspase-3 activity assay kit, mitochondrial isolation kit and horseradish peroxidase-labelled goat anti-rabbit IgG were purchased from Hangzhou Biyuntian Biotechnology Co., Ltd.

Cell culture. Human-derived THP-1 cells were grown and incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% inactivated fetal bovine serum and 1% penicillin/streptomycin at 37˚C and 5% CO_2. THP-1 cells were treated for 48 h with 100 nmol/ml PMA to induce macrophages differentiation, in which the cells transition from suspended to adherent growth and from round to irregular. Then the cells were treated with different doses of PA (0-800 μmol/l) dissolved in bovine serum albumin.

Cell viability assay. The effect of IGF-1 on the activity of THP-1 macrophages was assessed by the Cell Counting Kit-8 assay (cat. no. HY-K0301; MedChemExpress). THP-1 macrophages were co-treated with IGF-1 with PA (cat. no. P0500; MilliporeSigma) at different concentrations (1.0, 1.5, 2, 2.5 ng/ml) for 24 h. Then 10 μl of cell counting kit-8 reagent was added to each well, incubated for 4 h and the OD value measured at 450 nm and plot the standard curve.

Western blotting. Total protein was extracted using lysis buffer including protease inhibitors (cat. no. HY-K1001; MedChemExpress). Macrophages were washed with PBS after being lysed in an ice bath for 30 min in cell lysis buffer. Collect the cells in a centrifuge tube and centrifuge at 12,000 x g for 30 min at 4˚C. The supernatant is the total cell protein. Protein concentration was determined using a BCA protein assay kit (cat. no. P0011; Hangzhou Biyuntian Biotechnology Co., Ltd.). Using 10-15% SDS-PAGE, equal amounts of protein (20 or 30 μg) were resolved and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk for 1 h at room temperature followed by incubation with the appropriate primary antibody: Rabbit anti-Bax (1:1,000; cat. no. 41162S; Cell Signaling Technology, Inc.), rabbit anti-Bcl-2 (1:1,000; cat. no. ab32124; Abcam), rabbit anti-caspase-3 (1:1,000; cat. no. ab32042; Abcam), rabbit anti-GAPDH (1:1,000; cat. no. AF7021; Affinity Biosciences), rabbit anti-cytochrome c (1:1,000; cat. no. AF0146; Affinity Biosciences), rabbit anti-PINK1 (1:1,000; cat. no. DF7742; Affinity Biosciences) and rabbit anti-Parkin (1:1,000; cat. no. AF0235; Affinity Biosciences) overnight at 4˚C. Following that, secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG) were incubated for 1 h at room temperature. Finally, the Bio-Rad gel detection system (Bio-Rad Laboratories, Inc.) was used to observe the fluorescence intensity detection bands and ImageJ software (version 1.8.0; National Institutes of Health) used to quantify their density, and the ratio of the target protein to the internal reference protein was used as expression level of the target protein.

Flow cytometry. Annexin V-FITC/PI cell apoptosis detection kit (cat. no. E-CK-A211; Elabscience) was used for the analysis of cell apoptosis (early + late apoptotic cells) in accordance with the instructions. The treated cells were digested with trypsin solution without EDTA, harvested, washed with PBS, resuspended in 500 μl of 1X Annexin V Binding Buffer; 5 μl of Annexin V-FITC and PI was added and incubated in the dark for 15 min at room temperature. BD Accuri C6 Plus cytometer (BD Biosciences) was used for data collection. Flow.JoX (version 10.0.7; flowjo.com) software was used for data analysis.

Caspase-3 activity assay. The activity of caspase-3 was assessed using the caspase-3 kit assay (cat. no. C1115; Beyotime Institute of Biotechnology). The cells were lysed after treatment and Ac-DEVD-pNA was used as a substrate for caspase-3. Caspase-3 activity and absorbance were measured at OD 405.

Hoechst 33342 staining. Cells were stained with Hoechst 33342 (cat. no. C0031; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at 37˚C and then detected by fluorescence microscopy. When apoptosis occurs in cells, the nuclei of the apoptotic cells can be seen to be densely stained, or fragmented and densely stained.

Mitochondrial and cytoplasmic protein isolation. Mitochondria were isolated from THP-1 macrophages according to the instructions of the Cellular Mitochondrial

Isolation Kit (cat. no. C3601; Beyotime Institute of Biotechnology). THP-1 macrophages were digested with trypsin, harvested, resuspended in wash buffer, and centrifuged at 200 x g for 5 min at 4˚C to collect the cellular sediment. Cells were resuspended in mitochondrial isolation reagent and incubated in an ice bath for 15 min, after which a glass homogenizer was used to obtain cell homogenates. The mitochondrial precipitates were separated by gradient centrifugation for 10 min at 1,000 x g and 4˚C, then gently transfer the supernatant to another centrifuge tube. The supernatant was 3,500 x g again, centrifuged at 4˚C for 10 min, and then transferred to a centrifuge tube. The collected supernatant was again centrifuged at 12,000 x g for 10 min at a 4˚C. The cytoplasmic protein concentration was determined by the BCA method (Hangzhou Biyuntian Biotechnology Co., Ltd.).

Detection of mitochondrial membrane potential by JC-1. The mitochondrial membrane potential was measured using the fluorescent dye JC-1 (cat. no. HY-K0601; MedChemExpress). THP-1 cells were seeded in a small confocal dish, the number of seeded cells being approximately $2x10^5$. They were cultured in an incubator at 37˚C for 48 h to induce differentiation into macrophages, then treated with drugs for 24 h, stained with JC-1 for 20 min at 37˚C, stained with DAPI (cat. no. C0065; Beijing Solarbio Science & Technology Co., Ltd.)-labelled nuclei for 10 min at 37˚C and 5% CO_2, washed with PBS and 500 μl of 10% FBS RPMI-1640 medium added. The changes in cell membrane potential were observed under a laser confocal microscope (Carl Zeiss AG).

Detection of mitochondrial reactive oxygen species (ROS) generation. Mitochondrial ROS production was detected according to the mitochondrial ROS kit (cat. no. BB-46091, BestBio). The treated cells were digested with trypsin solution without EDTA, washed with PBS and incubated with mitochondrial reactive oxygen species staining solution at 37˚C in the dark for 20 min. Pre-cooled 500 μl 1X PBS was added to the centrifuge tubes to resuspend the cells and immediately detected with a BD Accuri C6 Plus cytometer (version 1.0.23.1; BD Biosciences). Flow.JoX (version 10.0.7; flowjo.com) software was used for data analysis.

Immunofluorescence staining. Treated cells were stained with green fluorescent MitoTracker Green FM (200 nM; cat. no. HY-135056; MedChemExpress) for 30 min, then fixed with pre-cooled methanol for 30 min, permeabilized with 0.1% Triton X-100 for 1 min and incubated blocked with 10% goat serum for 2 h at room temperature. Cells were incubated with LC3 primary antibody (1:1,000; cat. no. 3868; Cell Signaling Technology, Inc.) overnight at 4˚C. After three washes with PBS, cells were incubated with secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (1:500; cat. no. S0006; Affinity Biosciences) for 1 h. After staining with DAPI for 10 min at 37˚C, cells were observed using a confocal microscope (Carl Zeiss AG). Colocalization was assessed by line scanning using ImageJ software (version 1.8.0; National Institutes of Health) and line plots were generated using GraphPad (version 8.4.3; Dotmatics).

Mitochondrial-lysosome colocalization analysis. Mitophagy was detected by co-localization of mitochondria with lysosome. Cells were incubated with LysoTracker (100 nM; cat. no. L7528; Beijing Solarbio Science & Technology Co., Ltd.) and MitoTracker (300 nM; cat. no. 8778; Cell Signaling Technology, Inc.) for 30 min at 37˚C and 5% CO_2. The cells were then washed with PBS. Cells were fixed with pre-cooled methanol in the dark and incubated in an ice bath for 30 min. Bright green fluorescence represented mitochondria and bright red fluorescence represented lysosomes. Cell images were acquired using a confocal microscope (Carl Zeiss AG).

Statistical analysis. SPSS 16.0 software (SPSS, Inc.) was used for statistical analysis of the data. Data normality was tested using the Kolmogorov-Smirnov test. Normally distributed quantitative variables are described as mean ± standard deviation. Normally distributed continuous variables were compared between groups using one-way analysis of variance. The LSD method was used for homogeneity of variance, and Games-Howell method was used for non-homogeneity of variance. While non-normally distributed variables were compared using the rank sum test. Qualitative variables were described by frequency (percentage) and χ^2 test was used for comparison between groups. Pearson's product-moment correlation coefficient was used to analyze the correlation between the indicators following normal distribution, and Spearman's rank correlation coefficient was used to analyze the correlation between the indicators not following normal distribution. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IGF-1 increases survival in PA-stimulated macrophages. To assess the effect of IGF-1 on cell viability, Cell Counting Kit-8 assay was performed. Cell viability was examined at different PA concentrations (0-800 μmol/l) and 400 μmol/l was selected for subsequent experiments (Fig. 1A). Co-treatment of IGF-1 with PA at different concentrations (1.0, 1.5, 2, 2.5 ng/ml) for 24 h increased cell viability in a dose-dependent manner (Fig. 1B). Based on the results, 2.5 ng/ml IGF-1 was selected as the working concentration for subsequent experiments.

IGF-1 attenuates apoptosis in PA-stimulated macrophages. An increasing body of research suggests that PA triggers a number of relatively distinct mechanisms underlying apoptosis, including endoplasmic reticulum stress, ceramide and mitochondrial malfunction (22,23). However, the mechanism by which PA induces apoptosis in macrophages has not been fully elucidated. The present study investigated PA-induced apoptosis in macrophages. Apoptosis level was observed when cells were treated with PA at a concentration of 400 μmol/l. However, following co-treatment with IGF-1, PA-induced apoptosis was inhibited (Fig. 2). The results of Hoechst 33342 staining showed that compared with the control group, macrophages nuclei were deformed in the PA group and the apoptosis rate was obviously increased. Compared with the PA group, the IGF-1 and PA co-treated group reduced the apoptosis of macrophages (Fig. 2A and B). Flow cytometry analysis using Annexin V/PI double staining also confirmed that apoptosis

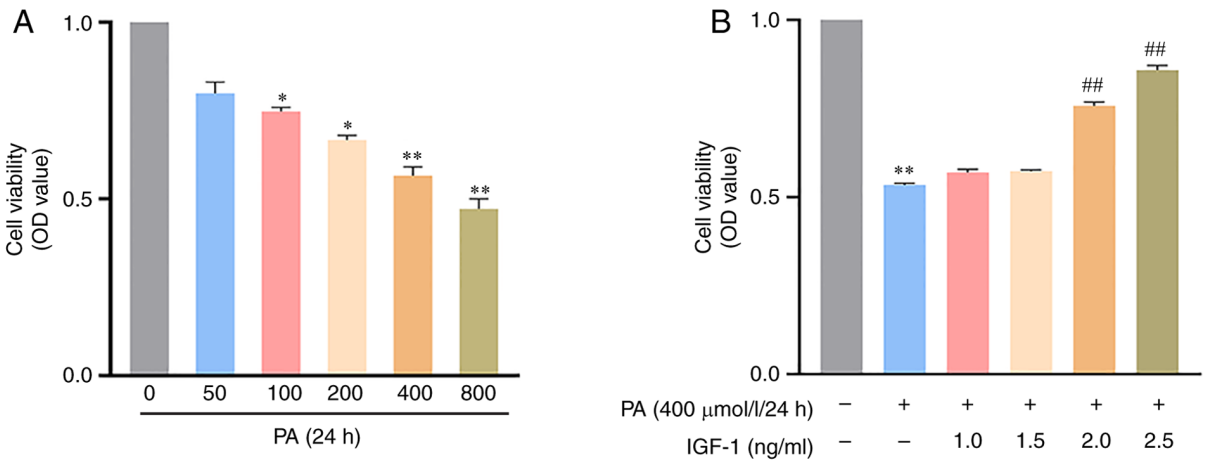

Figure 1. CCK8 activity assay. (A) Dose-dependent effect of PA on cell viability. (B) Effect of IGF-1 on PA-induced macrophage survival. Mean ± SD. n=3. *P<0. 05, **P<0. 01 vs. control; ##P<0. 01 vs. PA group. PA, palmitic acid; IGF-1, insulin growth factor-1.

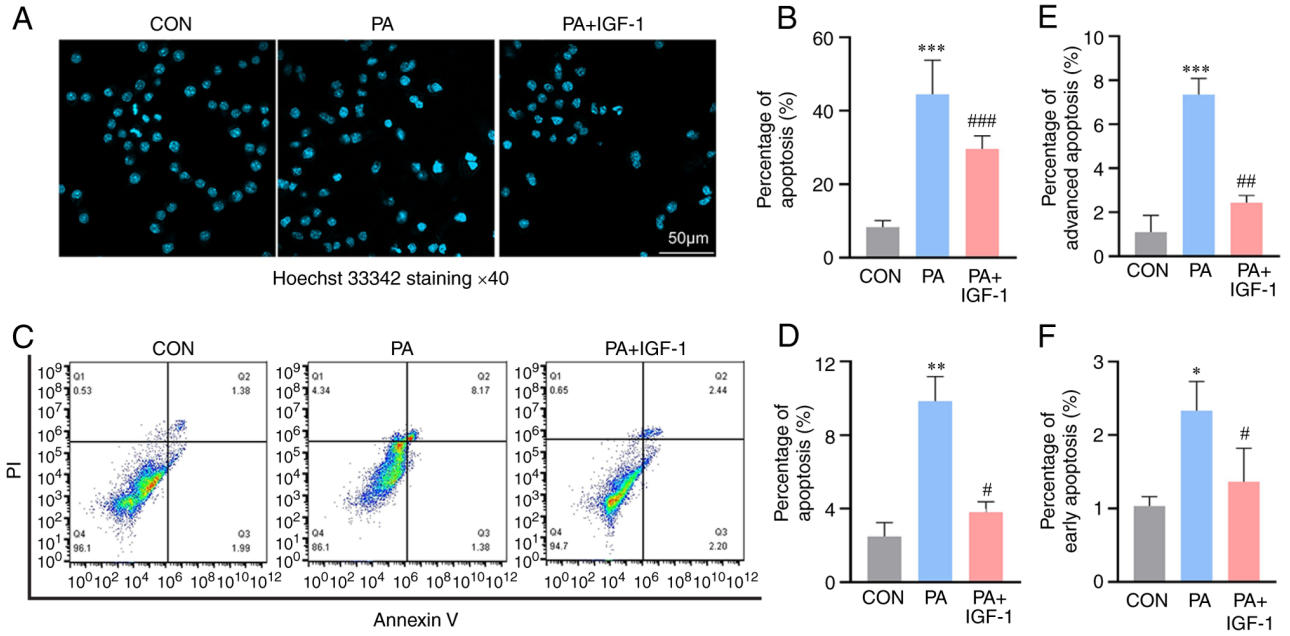

Figure 2. Effect of IGF-1 on PA-induced apoptosis of macrophages. (A) Hoechst 33342 staining was performed to observe apoptotic cells. (B) Quantitative analysis of Hoechst 33342 staining (C) Annexin V/PI staining to detect cell apoptosis. (D) Quantitative analysis of Annexin V/PI staining for total apoptosis (E) Quantitative analysis of Annexin V/PI staining for advanced apoptosis. (F) Quantitative analysis of Annexin V/PI staining for early apoptosis. Mean ± SD. n=3. *P<0. 05, **P<0. 01, ***P<0. 001 vs. control group; #P<0. 05, ##P<0. 01, ###P<0. 001 vs. PA group. IGF-1, insulin growth factor-1; PA, palmitic acid.

was markedly increased in the PA group, while apoptosis was significantly reduced by co-treatment with IGF-1 (Fig. 2C-F). The above results suggested that IGF-1 attenuates PA damage to macrophages.

IGF-1 reduces caspase-3 expression in PA-stimulated macrophages. Caspase-3 is an indication of apoptosis in the late process. For the purpose of determining how IGF-1 affected the expression of caspase-3 in macrophages, a caspase-3 activity kit and western blotting was employed. The findings demonstrated that macrophages in the PA group had considerably higher caspase-3 expression (Fig. 3A and B). Caspase-3 activity and protein levels were considerably reduced with the co-treatment with IGF-1 compared with the PA group (Fig. 3C and D).

Effect of IGF-1 on PA-induced expression of Bcl-2, Bax in macrophages. Bcl-2 and Bax genes are among the main genes involved in apoptosis. Studies have shown that Bcl-2 has a direct effect on mitochondrial membrane proteins and can directly prevent the opening of channels in the outer mitochondrial membrane, thus preventing the release of cytochrome c and achieving inhibition of apoptosis (24-26). Western blotting results showed that IGF-1 upregulated the expression of anti-apoptotic protein Bcl-2 and downregulated the expression of pro-apoptotic protein Bax compared with the PA group, while the Bcl-2/Bax ratio was significantly higher (Fig. 4).

IGF-1 attenuates PA-induced mitochondrial apoptosis pathway in macrophages. The ability of IGF-1 ability to inhibit macrophages apoptosis was further studied. Previous

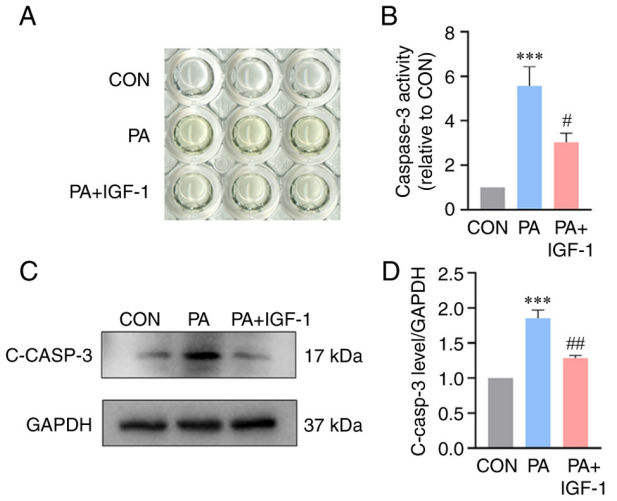

Figure 3. Effect of IGF-1 on PA-induced expression of caspase-3 in macrophages. (A) Caspase-3 activity detection. (B) Quantitative analysis of caspase-3 activity. (C) Western blotting detection of c-caspase-3 protein expression. (D) Quantitative analysis of caspase-3 protein expression. Mean ± SD. n=3. ***P<0. 001 vs. control group; $^{\#}$P<0. 05, $^{\#\#}$P<0. 01 vs. PA group. IGF-1, insulin growth factor-1; PA, palmitic acid.

research findings revealed that IGF-1 might regulate mitochondrial activity, which is crucial for cell survival (27,28). The current study examined the levels of mitochondrial apoptosis. According to the flow cytometry findings, macrophages in the IGF-1 group had considerably lower levels of mitochondrial ROS than those in the PA group (Fig. 5C and D). Changes in the potential of the mitochondrial membrane are related to ROS levels. Longer mitochondrial permeability transition pore openings might result in ROS bursts that disturb mitochondria at higher ROS levels (29). JC-1 staining demonstrated that PA treatment reduced the mitochondrial membrane potential of macrophages, but IGF-1 administration increased the membrane potential (Fig. 5A and B). Furthermore, the concentration of cytochrome c protein in the cytoplasm increased in the PA group at the same time and IGF-1 prevented the release of cytochrome c into the cytoplasm (Fig. 5E and F). According to the findings, IGF-1 prevented macrophages apoptosis via the mitochondrial pathway.

IGF-1 diminishes PA-induced mitophagy in macrophages. Previous studies have suggested that excessive mitophagy might result in mitochondrial damage (30-32). To evaluate whether IGF-1 protect mitochondrial damage based on mitophagy, the present study initially measured the engulfing mitochondria using co-localization of autophagosomes and mitochondria. The combined fluorescence signal of LC3 and MitoTracker showed that PA significantly enhanced the colocalization of autophagosomes with mitochondria (Fig. 6A and B). Under confocal microscopy, mitophagy and lysosome fusion was observed in the PA group. IGF-1 co-treatment significantly reduced mitochondrial lysosomal fusion, implying that IGF-1 inhibited mitophagy (Fig. 6C and D). The PINK1/Parkin pathway has recently been recognized as a crucial signaling pathway driving mitophagy in mammalian cells (33-35). Thus, PINK1 and Parkin expression levels were evaluated. Treatment with PA increased PINK1 and Parkin in the macrophages and the co-treatment with IGF-1 decreased the expression of both proteins (Fig. 6E-G). Taken together, these findings suggested that IGF-1 diminished PA-induced mitophagy.

Discussion

Lipotoxicity is the term for excessive lipid accumulation in non-adipose tissue, which can lead to cell death and altered immune responses, particularly in macrophages (36,37). There is increasing evidence that lipid disorders can have a direct or indirect effect on the immune cells. Data from the present study showed that treatment of macrophages with PA significantly reduced cell viability and induction of apoptosis.

IGF-1 is an endocrine and autocrine/paracrine growth factor widely expressed in human tissues and organs (38,39). Early research has shown that IGF-1 is associated with the protective role on immune cells. For example, IGF-1 may improve cell metabolism and survival by controlling the expression of genes via apoptotic pathways (40,41). A recent study found that overexpression of IGF-1 in macrophages reduced high-fat diet-induced macrophages apoptosis in a murine model (42). The effect of IGF-1 on PA-induced macrophages apoptosis in dyslipidemia pathophysiology is still an unexplored field. The present study observed that IGF-1 restored PA-induced nuclear deformation by Hoechst 33342 staining and IGF-1 inhibited PA-induced macrophages apoptosis by Annexin V/PI staining. As a downstream signal of apoptosis, the expression of caspase-3 was positively correlated with cell apoptosis. According to the analysis of caspase-3 activity, PA treatment-induced caspase-3 activity and protein expression were markedly reduced by IGF-1 treatment. These results suggested that IGF-1 attenuated macrophages apoptosis.

Apoptosis is a key intracellular homeostasis regulatory process. The endogenous mitochondrial pathway, the endoplasmic reticulum pathway and exogenous death receptor pathway are considered the basic pathways of apoptosis (43). The endogenous mitochondrial pathway is activated by permeabilization of the outer mitochondrial membrane (44). The Bcl-2 protein family is found in mitochondria and regulates the permeabilization of the outer mitochondrial membrane (25,45,46). When several apoptotic factors (for example, cytochrome c) are released from the mitochondrial intermembrane space, they induce the formation of the apoptosome together with caspase-9, which then activates caspase-3, thereby activating the common pathway of apoptosis (47,48). When the endoplasmic reticulum stress is excessive or lasts too long, the unfolded protein response fails to hydrolyze unfolded or misfolded proteins in time, then the apoptotic-signaling molecules are activated, causing apoptosis (49). In the death receptor pathway, tumor necrosis factor-related apoptosis-inducing ligand combines with cell surface death receptors such as death receptor-4 and death receptor-5 to form a death-inducing signaling complex, leading to the recruitment of caspase-8 ultimately leading to apoptosis (50). Studies have reported that IGF-I has strong anti-apoptotic activity in a variety of cell types and can protect cells through different apoptotic mechanisms (51-53). IGF-1 regulates PI3K/AKT/Forkhead box O signaling to reduce cytochrome c levels in the cytoplasm, thereby suppressing

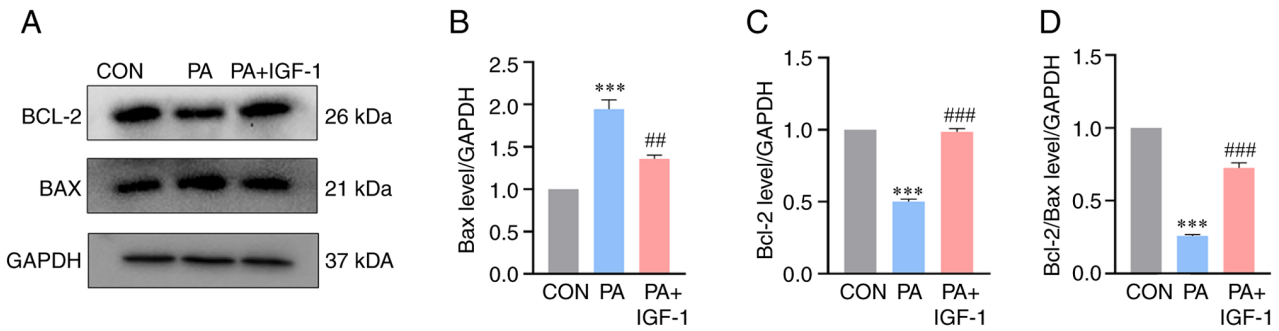

Figure 4. The effect of IGF-1 on PA-induced expression of Bcl-2 and Bax in macrophages. (A) Western blotting detection of Bcl-2, Bax protein expression. (B-D) Quantitative analysis of Bax, Bcl-2, and Bcl-2/Bax ratio. Mean ± SD. n=3. ***P<0.001 vs. control group; ##P<0. 01, ###P<0. 001 vs. PA group. IGF-1, insulin growth factor-1; PA, palmitic acid.

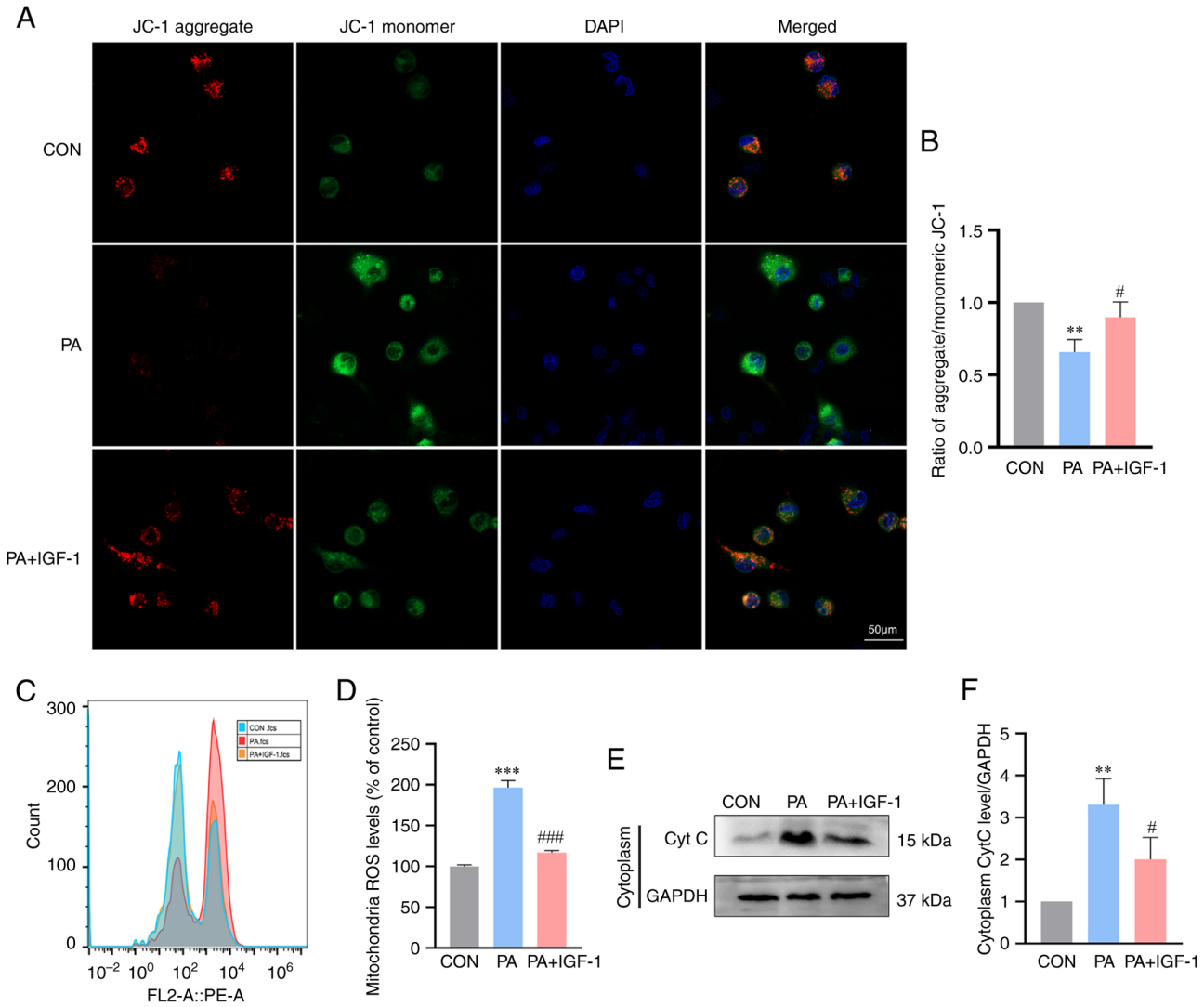

Figure 5. Effect of IGF-1 on PA-induced mitochondrial function in macrophages. (A) JC-1 staining to show changes in mitochondrial membrane potential. (B) Quantitative analysis of JC-1 staining. (C) Fluorescence intensity of mitochondrial ROS observed by flow cytometry. (D) Quantitative analysis of mitochondrial ROS. (E) Western blotting detection of protein expression of cytochrome c. (F) Quantitative analysis of cytochrome c protein expression. Mean ± SD. n=3. **P<0. 01, ***P<0. 001 vs. control group; #P<0. 05, ###P<0. 001 vs. PA. IGF-1, insulin growth factor-1; PA, palmitic acid; ROS, reactive oxygen species.

cleaved caspase-3 formation and apoptosis (41,54,55). Another study demonstrated that IGF-1 can reduce lipopolysaccharide-induced neuronal apoptosis through the mitochondrial pathway (41). Kurshan *et al* (56) found that IGF-1 protects cells from endoplasmic reticulum stress-induced apoptosis via enhancement of the adaptive capacity of endoplasmic reticulum. In addition, inhibition of the IGF-1 receptor increases death receptor-mediated apoptosis in colon cancer cells (57).

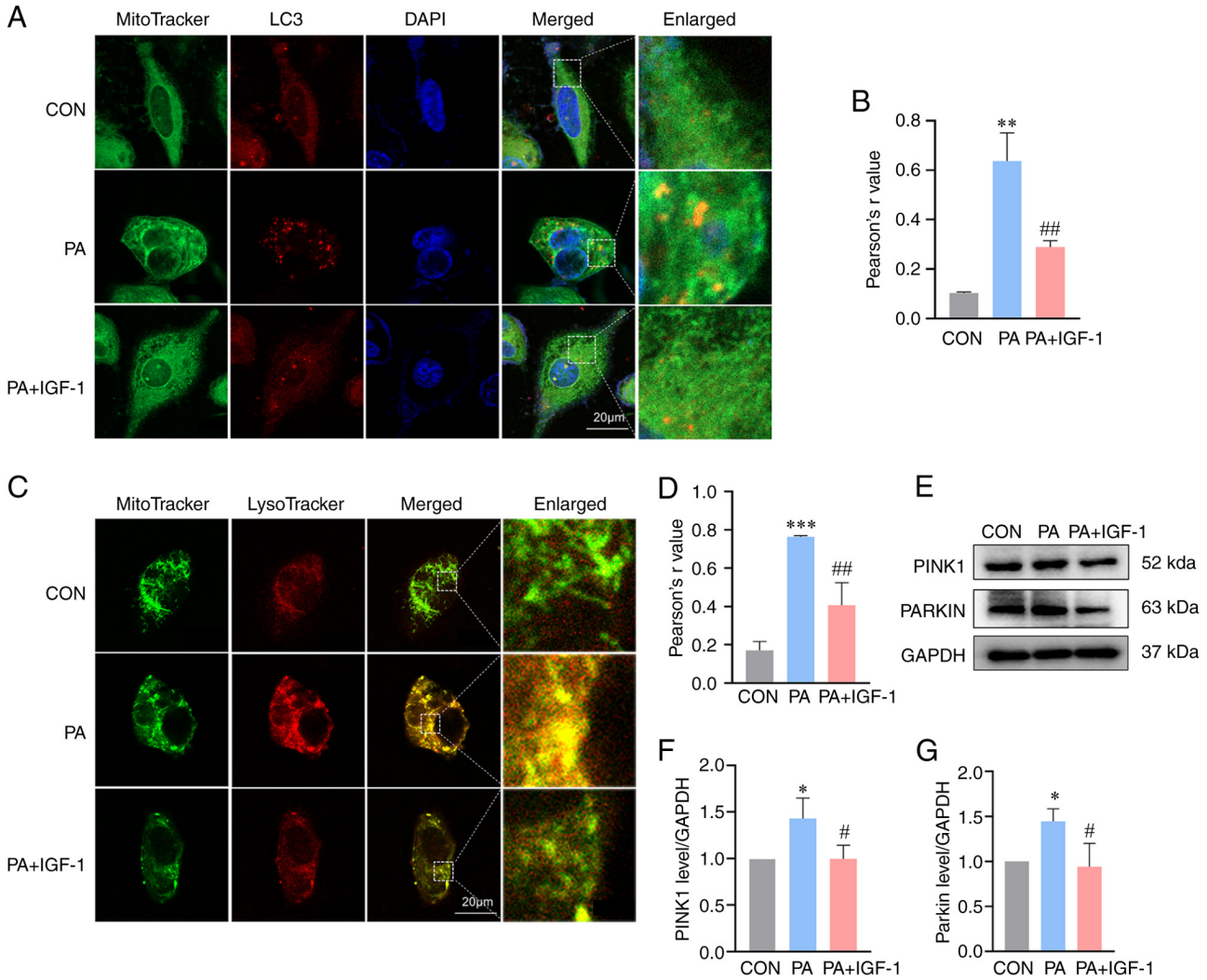

Figure 6. IGF-1 diminished PA-induced mitophagy in macrophages. (A) Colocalization of mitochondria and LC3. (B) The colocalization of the mitochondria and LC3 was quantitatively analyzed using the Pearson correlation coefficient. (C) Colocalization of mitochondria and lysosomes. (D) The colocalization of the mitochondria and lysosomes was quantitatively analyzed using the Pearson correlation coefficient. (E) Western blotting analysis of mitophagy-related proteins. (F and G) Quantitative analysis of PINK1 and Parkin protein expression. Mean ± SD. n=3. *P<0. 05, **P<0. 01, ***P<0. 001 vs. control group; $^{\#}$P<0. 05, $^{\#\#}$P<0. 01 vs. PA. IGF-1, insulin growth factor-1; PA, palmitic acid.

The present study demonstrated that IGF-1 can regulate mitochondrial apoptosis induced by PA in macrophages, although the other apoptotic mechanisms cannot be excluded. The precise mechanisms need to be investigated further.

The Bcl-2 family is involved in the transmission and reception of apoptotic signals in the mitochondrial apoptotic pathway, as well as modulating apoptosis upstream to regulate caspase-3 expression levels (48). Bcl-2 and Bax are essential proteins in this family that play opposing and complementary roles in the mitochondrial apoptotic pathway of cells. Bax proteins play a major role in facilitating the evolution of apoptosis by heterodimerizing with the anti-apoptotic protein Bcl-2 upon activation and altering the permeability of the cellular mitochondrial membrane, resulting in a homeostatic imbalance that promotes apoptosis (45,58). The Bcl-2/Bax ratio is an important indicator of cell apoptotic susceptibility (59) and directly determines the degree of opening of various channels in the outer mitochondrial membrane (60,61). The present study demonstrated that IGF-1 increased the expression of Bcl-2 protein, inhibited the expression of Bax protein and elevated the Bcl-2/Bax ratio. These results suggested that IGF-1 might improve the mitochondrial apoptosis by regulating the proportion of Bcl-2 family members and mitochondrial outer membrane channels.

Mitochondria are key organelles of eukaryotic cells that undertake important processes, such as cellular metabolite conversion and oxidative phosphorylation, as well as ATP synthesis (62). Mitochondria are organelles that produce ROS, a major factor in the induction of apoptosis. When the level of ROS reaches a certain point, the accumulated ROS will activate the opening of the mitochondrial permeability transition pore, leading to a decrease in mitochondrial membrane potential (63). This allows cytochrome c, located in the lumen between the inner and outer mitochondrial membranes, to be released from the mitochondria into the cytoplasm, which in turn mediates the onset of apoptosis via the caspase-3 (64). PA can induce mitochondrial apoptosis in macrophages, which was confirmed by the present study. Furthermore, it was found that PA treatment increased mitochondrial ROS production, decreased mitochondrial membrane potential, and increased

cytoplasmic cytochrome c protein aggregation, whereas IGF-1 reversed these effect. This indicated that IGF-1 can reverse PA-induced mitochondrial apoptosis.

Mitophagy is an important process that regulates mitochondrial dynamics. A major cause of mitochondrial dysfunction is dysregulation of mitophagy (32). When mitochondria are degraded in significant amounts due to over-activated mitophagy, it results in mitochondrial dysfunction and continuing production of ROS (65). Hence, it was hypothesized that a critical molecular mechanism by which IGF-1 prevents macrophages apoptosis involves the reduction of mitophagy. Confocal microscopy showed that colocalization of lysosomes and mitochondria was significantly downregulated by IGF-1 compared with PA treated group. This suggested that IGF-1 might partly prevent autophagosome from fusion with lysosomes. The PINK1/Parkin pathway is a classic signaling pathway during mitophagy (33,34). When mitochondria are depolarized, PINK1 is prevented from entering the inner membrane, resulting in the accumulation of PINK1 on the surface of damaged mitochondria. At this stage, PINK1 on the mitochondrial surface recruits Parkin from the cytoplasm to the damaged mitochondria, inducing and promoting mitophagy (35). In the present study, expression of PINK1 and Parkin was inhibited when IGF-1 treatment *in vitro*. The above results suggested that IGF-1 inhibited excessive mitophagy induced by PA via the PINK1/Parkin pathway.

There are some limitations to the current study. It demonstrated that IGF-1 had a significant inhibitory effect on PA-induced macrophages apoptosis (Hoechst 33342 staining and flow cytometry) and mitochondrial apoptosis (Bcl-2/Bax ratio). Although the other apoptotic pathways could not be excluded, these results indicated that mitochondrial apoptosis is involved in the action of IGF-1. It is worth noting that the effect of PA 400 μmol/l appeared to be partly blocked by IGF-1 2.5 ng/ml, suggesting that other apoptotic pathways may also be involved in the pro-apoptotic effect of PA. Moreover, the inhibition of mitochondrial apoptosis by IGF-1 was not complete, which might partially explain the inability of IGF-1 to completely inhibit the effect of PA. Another limitation was the use of only one cell line, THP-1. Therefore, the mechanism of IGF-1 anti-apoptotic effect and its effect on macrophages apoptosis in other cell lines remain to be further studied.

In summary, the present study demonstrated that IGF-1 can partly inhibit macrophages apoptosis by protecting mitochondria. The present study provided a further scientific basis for understanding the therapeutic effects of IGF-1 on PA-induced dyslipidemia macrophages apoptosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WT, MZ, YW, LJ, YO and WJ were involved in conception and design and manuscript writing. DM, MH, HL, YZ, GZ, PH and YO performed experiments, data analysis and interpretation. LJ, YO and WJ wrote the manuscript or revising it critically for important intellectual content. GH, PH and YO given final approval of the version to be published. WT and MZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Sixth Affiliated Hospital of Guangzhou Medical University waives the requirement for authors to obtain ethical approval for the use of commercially available cells.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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