

Role of SIRT1/PGC-1 α in mitochondrial oxidative stress in autistic spectrum disorder

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Abstract: Autistic spectrum disorder (ASD) is a neurodevelopmental disorder and has a high prevalence in children. Recently, mitochondrial oxidative stress has been proposed to be associated with ASD. Besides, SIRT1/PGC-1 α signaling plays an important role in combating oxidative stress. In this study, we sought to determine the role of SIRT1/PGC-1 α signaling in the ASD lymphoblastoid cell lines (LCLs). In this study, the mRNA and protein expressions of SIRT1/PGC-1 α axis genes were assessed in 35 children with ASD and 35 healthy controls (matched for age, gender, and IQ). An immortalized LCL was established by transforming lymphocytes with Epstein–Barr virus. Next, we used ASD LCLs and control LCLs to detect SIRT1/PGC-1 α axis genes expression and oxidative damage. Finally, the effect of overexpression of PGC-1 α on oxidative injury in the ASD LCLs was determined. SIRT1/PGC-1 α axis genes expression was downregulated at RNA and protein levels in ASD patients and LCLs. Besides, the translocation of cytochrome *c* and DIABLO from mitochondria to the cytosol was found in the ASD LCLs. Moreover, the intracellular reactive oxygen species (ROS) and mitochondrial ROS and cell apoptosis were increased in the ASD LCLs. However, overexpression of PGC-1 α upregulated the SIRT1/PGC-1 α axis genes expression and reduced cytochrome *c* and DIABLO release in the ASD LCLs. Also, overexpression of PGC-1 α reduced the ROS generation and cell apoptosis in the ASD LCLs. Overexpression of PGC-1 α could reduce the oxidative injury in the ASD LCLs, and PGC-1 α may act as a target for treatment.

Keywords: autistic spectrum disorder, PGC-1 α , SIRT1, mitochondria, oxidative stress

Introduction

Autistic spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by stereotypical behaviors and by impairments in social and language skills.¹ Furthermore, children with ASD have a very high prevalence of comorbid mental health conditions, such as learning disabilities, attention-deficit hyperactivity disorder, emotional disorders, and chronic tic disorder.^{2,3} Besides, the prevalence of the ASD is 1 in 88 children in population.⁴ In addition, the prevalence of the ASD in male population is almost five times more than that in female population.⁵ ASD has a genetic component⁶ and may be exacerbated by environmental factors, such as diet quality⁷ and environmental chemicals.⁸ Nowadays, ASD has become a major public health issue, and many researches focus on it. However, the underlying etiology of ASD is still unknown.

Oxidative stress is defined as a homeostatic imbalance between an increase in reactive oxygen species (ROS) and a decrease in the ability of the endogenous antioxidant defense systems to scavenge free radicals.⁹ Oxidative stress induces the secretion of many vasoactive and proinflammatory molecules,¹⁰ which leads to neuroinflammation.¹¹ Moreover, oxidative stress has been implicated in some psychiatric disorders, such as

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schizophrenia¹² and Alzheimer's disease.¹³ Oxidative stress has also been associated with some individuals with ASD.^{14,15}

Mitochondria are a major source of intracellular ROS production, and ROS have been considered as second messengers that participate in physiological processes such as apoptosis.¹⁶ During apoptosis a variety of cellular signals from the mitochondria, membrane, and cytosol are activated by stimuli.¹⁷ Additionally, mitochondria may sense these cellular signals and then lead to cell apoptosis by releasing proapoptotic factors into the cytosol, such as Smac-DIABLO and cytochrome *c* (cyto *c*).¹⁸ Moreover, the apoptosis induced by mitochondrial oxidative stress has been proposed to be associated with ASD.¹⁹

Peroxisome proliferator-activated receptor- γ co-activator 1 α (PGC-1 α) is a major regulator for mitochondrial function in many tissues, including the nervous system.²⁰ And the activity of PGC-1 α can be modulated by NAD⁺-dependent deacetylation through the functionally interactive metabolic biosensors sirtuin-1 (SIRT1).²¹ In addition, PGC-1 α activates transcriptional factors such as nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), which induce the respiration proteins of mitochondria.²² However, the role of SIRT1/PGC-1 α in mitochondrial oxidative stress in ASD has not been investigated.

In this study, we sought to determine the role of SIRT1/PGC-1 α signaling in the ASD lymphoblastoid cell lines (LCLs). First, we examined the expression of SIRT1–PGC-1 α axis genes in ASD patients and LCLs. Then, we measured the intracellular ROS and mitochondrial ROS generation and cell apoptosis in the ASD LCLs. Finally, we detected the effect of PGC-1 α on the ROS generation and cell apoptosis in the ASD LCLs. For the first time, we demonstrated that overexpression of PGC-1 α could reduce the oxidative injury in the ASD LCLs and that PGC-1 α may act as a target for treatment.

Materials and methods

Participants

Two groups, each consisting of 35 Chinese Han male children between the ages of 6 and 12 years, participated in the study. For the autism group, patients were diagnosed with ASD according to international standard diagnostic tests, which consist of meeting criteria on the social and communication domains and algorithm total of the Autism Diagnostic Observation Schedule-Generic (ADOS-G)²³ and on two of three algorithm domains of the Autism Diagnostic Interview Revised.²⁴ For the control group, the healthy controls were normally developing, healthy children unrelated to the autistic subjects and without mental (eg, schizophrenia) or genetic disorders (eg, fragile X syndrome). IQ was evaluated with the standardized Raven test²⁵ for the two groups (mean IQ: ASD, $M = 103.4$, standard deviation [SD] = 12.6; healthy control, $M = 109.3$, SD = 14.5). Written informed consent was obtained from all participants or their legal guardians. The study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. Five milliliters of the whole blood was taken from all participants for further studies. The characteristics of participants ($n=35$ in each group) who volunteered for the study are listed in Table 1.

Lymphoblastoid cell lines and cell culture

Ten ASD LCLs were derived from 35 ASD patients, and ten control LCLs were derived from 35 healthy controls. Immortalized LCLs were established by transforming lymphocytes with Epstein–Barr virus (EBV) following previously described procedures.²⁶ In brief, 5 mL whole blood was drawn from ten ASD patients and ten healthy controls. After centrifugation at 3,000 rpm for 15 minutes, the Buffy coat layer was drawn and diluted with HBSS (Hanks-balanced salt solution). Then, the lymphocytes were isolated by using Ficoll gradient centrifugation. After washing with HBSS, the lymphocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 culture

Table 1 Characteristics of participants ($n=35$ in each group) who volunteered for the study

Characteristics	Control group ($n=35$)	ASD group ($n=35$)
Age, years	9.30 $\pm$ 1.60	8.50 $\pm$ 3.20
IQ	109.30 $\pm$ 14.50	103.40 $\pm$ 12.60
Gender	Male	Male
Autism Diagnostic Observation Schedule score		
Communication	1.21 $\pm$ 0.76	6.83 $\pm$ 1.94
Social interaction	0.22 $\pm$ 0.41	10.31 $\pm$ 2.05
Communication + social interaction	1.43 $\pm$ 1.33	17.14 $\pm$ 4.52
Autism Diagnostic Interview Revised		
Social interaction domain	1.52 $\pm$ 0.25	21.20 $\pm$ 5.68
Communication domain	0.86 $\pm$ 0.37	11.42 $\pm$ 3.22
Social interaction domain + communication domain	2.38 $\pm$ 0.71	32.62 $\pm$ 9.56

Note: Data presented as mean $\pm$ SD.

Abbreviations: ASD, autistic spectrum disorder; SD, standard deviation.

medium (Gibco, BRL, Thermo Fisher Scientific, Waltham, MA, USA) with phytohemagglutinin and then transformed with EBV suspension. The cells were studied at passage 12 on average. Because at this low passage number, genomic stability is very high.²⁷ Cells were incubated in RPMI 1640 culture medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO₂ in a humidified chamber.

Cell transfection

Three ASD LCLs randomly selected from ten ASD LCLs were used for cell transfection. A PGC-1 α overexpression plasmid, pCIG-PGC-1 α , was commercially constructed by GenePharma (Suzhou, People's Republic of China), and the empty pCIG vector was used as the control. These two plasmids were transfected into ASD LCLs using the Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's instructions. Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed in an Agilent Technologies (Santa Clara, CA, USA) Bioanalyzer by using SYBR Green PCR Master Mix (Roche, Pleasanton, CA, USA). Relative mRNA levels were calculated using the 2^{- $\Delta\Delta C_t$} method. GAPDH was used as an internal control. A complete list of primer sets is given below:

PGC-1 α Forward: 5'-CACCAGCCAACACTCAGCTA-3'

PGC-1 α Reverse: 5'-GTGTGAGGAGGGTCATCGTT-3'

SIRT1 Forward: 5'-GCAACAGCATCTTGCCTGAT-3'

SIRT1 Reverse: 5'-GTGCTACTGGTCTCACTT-3'

NRF1 Forward: 5'-GGTGCCTAGTGAGAGTGA GTCCCCC-3'

NRF1 Reverse: 5'-TCGGGGCTGAAGAGGGGAG AAGTC-3'

TFAM Forward: 5'-GGAGGCAAAGGATGATTCGG-3'

TFAM Reverse: 5'-TCGTCCAACCTCAGCCATCT-3'

GAPDH Forward: 5'-TCCCAGCACACTTA ACTTAGC-3'

GAPDH Reverse: 5'-AGCCACAAGAAACAC TCAGG-3'

Mitochondria-free cytosolic fractionation

Mitochondria-free cytosolic protein fractions were prepared as described previously.²⁸ Briefly, cells were lysed

in ice-cold extraction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene diamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, and 1 mM dithiothreitol, pH 7.4) supplemented with protease inhibitor cocktail. After homogenization in a small glass homogenizer with Teflon pestle, the homogenates were first centrifuged at 800 $\times g$ for 10 minutes at 4°C to remove the nuclei and cell debris. Then, the supernatants were centrifuged twice at 16,000 $\times g$ for 20 minutes at 4°C to remove the mitochondria. The final supernatants were collected as mitochondria-free cytosolic protein fractions.

Western blot analysis

The total protein extracts were prepared as described previously.²⁹ In brief, cells were lysed in cold radioimmunoprecipitation assay buffer with protease and phosphatase inhibitor cocktail (Roche). Equal amounts of protein samples were separated on SDS-PAGE, and then transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Then, membranes were probed with anti-PGC-1 α (1:2,000; Santa Cruz Biotechnology, Dallas, TX, USA), anti-TFAM (1:16,000; Novus Bio, Littleton, CO, USA), anti-NRF1 (1:1,000; Santa Cruz Biotechnology), anti-SIRT1 (1:2,000; Sigma-Aldrich, St Louis, MO, USA), anti-cyto *c* (1:2,000; eBioscience, San Diego, CA, USA), anti-DIABLO (1:1,000; Cell Signaling Technologies, Danvers, MA, USA), and anti-GAPDH antibodies (1:1,000; Cell Signaling Technologies). The blots were then analyzed by chemiluminescence detection (ECL, Amersham, GE Healthcare Life Sciences, Chicago, IL, USA).

Mitochondrial transmembrane potential ($\Delta\Psi_m$) assay

JC-1 is a fluorescent probe that is sensitive to mitochondrial membrane potential ($\Delta\Psi_m$).³⁰ When the mitochondrial membrane potential is low, JC-1 cannot concentrate in the mitochondria matrix, the JC-1 monomer emits green fluorescence. And when the mitochondrial membrane potential is high, JC-1 concentrates in the mitochondrial matrix, which forms J-aggregates to produce red fluorescence. A decrease in red/green ratio indicates apoptosis.³¹ LCLs were seeded into 12-well plates (10⁵ cells per well). Change in $\Delta\Psi_m$ was detected using a MitoProbe JC-1 Assay Kit (Invitrogen) according to the manufacturer's instructions. The images were then taken using a confocal laser scanning microscopy (FV1200; Olympus, Tokyo, Japan). Fluorescence was measured at 530 nm excitation/590 nm emission. Finally, the ratio of red/

green fluorescence was examined by using imaging software ImageJ.

Intracellular ROS and mitochondrial ROS generation assay

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) can be cleaved intracellularly by nonspecific esterase and then oxidized by ROS to turn to high fluorescence.³² Intracellular ROS levels were determined with fluorescent DCFH-DA. Briefly, cells at a density of $10^5/\text{mL}$ were plated in each 35-mm culture plate (Thermo Fisher Scientific). Then, they were incubated in DCFH-DA ($10\text{ }\mu\text{mol/L}$; Sigma-Aldrich). The DCFH-DA fluorescence intensities were determined using a confocal laser scanning microscopy (FV1200; Olympus). Fluorescence was measured at 490 nm excitation/520 nm emission. The average fluorescence intensities of DCFH-DA in the cells were analyzed using imaging software ImageJ.

Mitochondrial ROS generation was evaluated by the use of the probe MitoSOX Red. Cells were seeded at a density of $10^5/\text{mL}$ in glass bottom culture dishes (NEST, 801001). Then, they were incubated with $2.5\text{ }\mu\text{M}$ MitoSOX Red for 30 minutes at 37°C . After that, cells were treated with 200 nM MitoTracker green for 15 minutes to stain mitochondria. Subsequently, cells were examined under a confocal laser scanning microscopy (FV1200; Olympus). Fluorescence was measured at 510 nm excitation/585 nm emission. ImageJ software was used to analyze the image to determine the average optical density.

Complex activity assay

Mitochondrial extracts were obtained by using a proprietary mitochondria isolation kit (BioChain Institute, Newark, CA, USA) according to the manufacturer's instructions. To measure mitochondrial complex I activity, an NADH oxidation assay was used.³³ The buffer used for complex I assay was 2 mM KCN, 35 mM potassium phosphate, pH 7.2, 5 mM MgCl_2 , 2.5 mg/mL free fatty acid-bovine serum albumin, and $2\text{ }\mu\text{g/mL}$ antimycin. Then, $10\text{ }\mu\text{mol}$ NADH was made up freshly in this buffer, and 15 mM coenzyme Q_1 was prepared in 100% ethanol. After that, $10\text{ }\mu\text{L}$ NADH and $4\text{ }\mu\text{L}$ coenzyme Q_1 were added to the reaction mixture. The reaction was started with $20\text{ }\mu\text{g}$ of mitochondrial extracts. Then, complex I activity was measured at 450 nm .

Mitochondrial complex III activity was measured by monitoring the reduction of cyto *c* by ubiquinol as described previously.³⁴ The reaction mixture contained 1 mM KCN, 35 mM potassium phosphate buffer pH 7.2, 5 mM MgCl_2 , 1 mM ethylene diamine tetraacetic acid, 15 mM cyto *c*, 5 mM rotenone, and $20\text{ }\mu\text{g}$ of mitochondrial extracts. The

reaction was initiated by $15\text{ }\mu\text{M}$ ubiquinol. The activity of complex III due to the reduction of cyto *c* was measured by the increase in absorbance at 550 nm .

Flow cytometry analysis

Cells apoptosis were analyzed using an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Viable (annexin⁻/PI⁻), necrotic nonviable (annexin⁻/PI⁺), apoptotic (annexin⁺/PI⁻), and late apoptotic/necrotic (annexin⁺/PI⁺) cells were characterized based on the methods described previously.³⁵

Immunophenotyping analysis

All cell lines were tested for cell surface antigen expression by direct immunofluorescence and flow cytometric analysis. The cells were stained with CD19-PE and CD20-FITC in the dark for 30 minutes. IgG1-FITC and IgG1-PE were used as isotype-matched controls. Cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Statistics

Data were expressed as means $\pm$ SD. Statistical significance of the data were performed by Student's *t*-test. Values were considered to be statistically significant at $P < 0.05$. All experiments were repeated at least for three times.

Results

Cell surface immunophenotyping

The lymphotropic virus of EBV is routinely used in the laboratory to transform B lymphocytes and generate B LCLs.³⁶ The EBV-LCLs of ASD patients and healthy controls were characterized for cell surface markers by direct immunostaining and fluorescent-activated cell sorting (FACS) analysis. Figure 1 showed both EBV-LCLs were positive for B cell markers CD19 and CD20, indicating EBV-LCLs was established successfully.

Expression of *SIRT1*–*PGC-1 α* axis genes in ASD patients

To determine the expression of *SIRT1*, *PGC-1 α* , *NRF1*, and *TFAM* in the ASD patients, qRT-PCR and Western blot assays were used. Our data found that the *SIRT1*, *PGC-1 α* , *NRF1*, and *TFAM* mRNA were significantly decreased in the ASD patients compared with that in the healthy controls (Figure 2A–D). The same results were also observed for the protein expressions (Figure 2E). Thus, our results indicated that *SIRT1/PGC-1 α* axis genes expression was downregulated at RNA and protein levels in ASD patients.

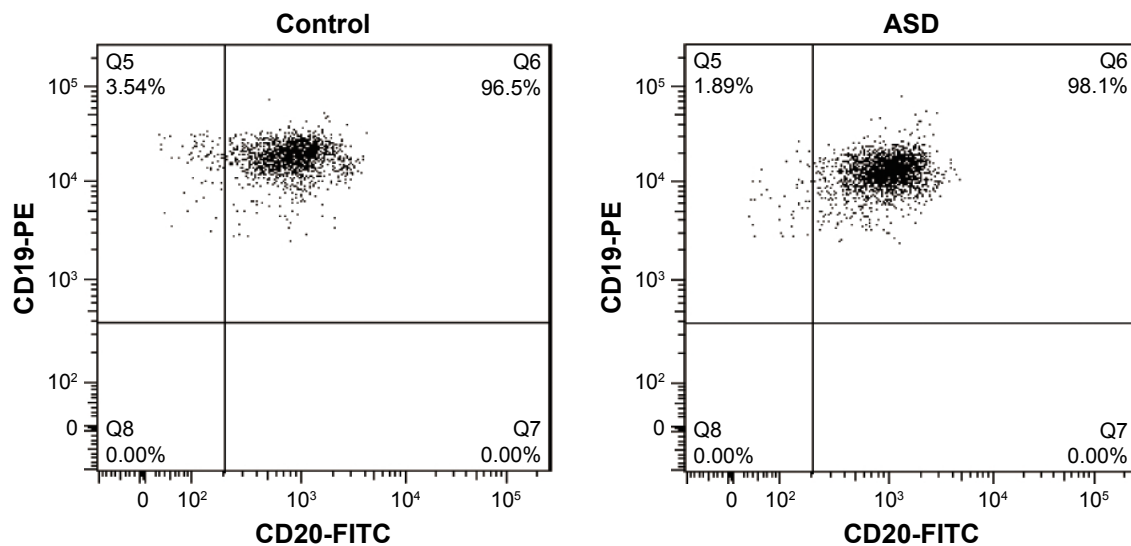


Figure 1 Immunophenotyping by FACS.

Note: FACS analyses for CD19 and CD20.

Abbreviation: FACS, fluorescent-activated cell sorting.

Expression of SIRT1–PGC-1 α axis genes and translocation of apoptogenic proteins in the ASD LCLs

Next, the expression of SIRT1, PGC-1 α , NRF1, and TFAM in the ASD LCLs were measured. As shown in Figure 3A–E, the expression of SIRT1, PGC-1 α , NRF1, and TFAM in the ASD LCLs was reduced at both RNA and protein levels.

During mitochondria-mediated apoptosis, the outer mitochondrial membrane becomes permeable, which leads to the release of apoptogenic protein.³⁷ cyto *c* and DIABLO proteins are released to cytosol from mitochondria in response to apoptosis.³⁸ As shown in Figure 3F, a significant increase in the expression levels of cyto *c* and DIABLO proteins, which were detected from mitochondria-free cytosolic proteins, was observed in the ASD LCLs. These results demonstrated that the expression of genes in the SIRT1/PGC-1 α axis was downregulated as was cyto *c* and DIABLO released from mitochondria to the cytosol in the ASD LCLs.

Cell apoptosis in the ASD LCLs

To elucidate cell apoptosis in the ASD LCLs, we used JC-1 staining to evaluate the loss of $\Delta\Psi_m$. In the ASD LCLs, JC-1 red/green fluorescence ratio decreased significantly compared with that in the control LCLs (Figure 4A). And, the feeble mitochondrial membrane potential indicated the primary change of cell apoptosis in the ASD LCLs.

Subsequently, apoptosis of ASD LCLs was further confirmed by using Annexin V-FITC staining and FACS analysis. A representative FACS analysis plot is depicted in Figure 4D. In comparison with control LCLs, ASD LCLs

displayed a significant increase in apoptosis (annexin⁺/PI⁺). These results revealed that ASD induced cell apoptosis in the LCLs.

Intracellular ROS and mitochondrial ROS generation in the ASD LCLs

Increased ROS generation is involved in the induction of apoptosis.³⁹ To examine the intracellular ROS and mitochondrial ROS in the ASD LCLs, DCFH-DA and MitoSOX Red staining were used. The DCFH-DA is a H₂O₂-sensitive fluorescence probe and can be applied to assess the intracellular H₂O₂.⁴⁰ MitoSOX Red is a live-cell permeant dye that can selectively and rapidly target mitochondria and is used to detect mitochondrial O₂.⁴¹ As shown in Figure 4B, cells stained with DCFH-DA demonstrated a significant increase in intracellular ROS production in the ASD LCLs compared with that in the control LCLs. In addition, to evaluate mitochondrial superoxide, we used MitoSOX Red probe. The results demonstrated that the mitochondrial ROS generation also increased in the ASD LCLs (Figure 4C).

Activities of mitochondrial respiratory complexes in the ASD LCLs

As excessive production of ROS impairs the activities of respiratory chain complexes,⁴² we next measured the activities of respiratory chain complexes, complexes I and III. In Figure 4E, the results showed the activities of complexes I and III were significantly decreased in the ASD LCLs than that in the control LCLs. Thus, the results demonstrated that

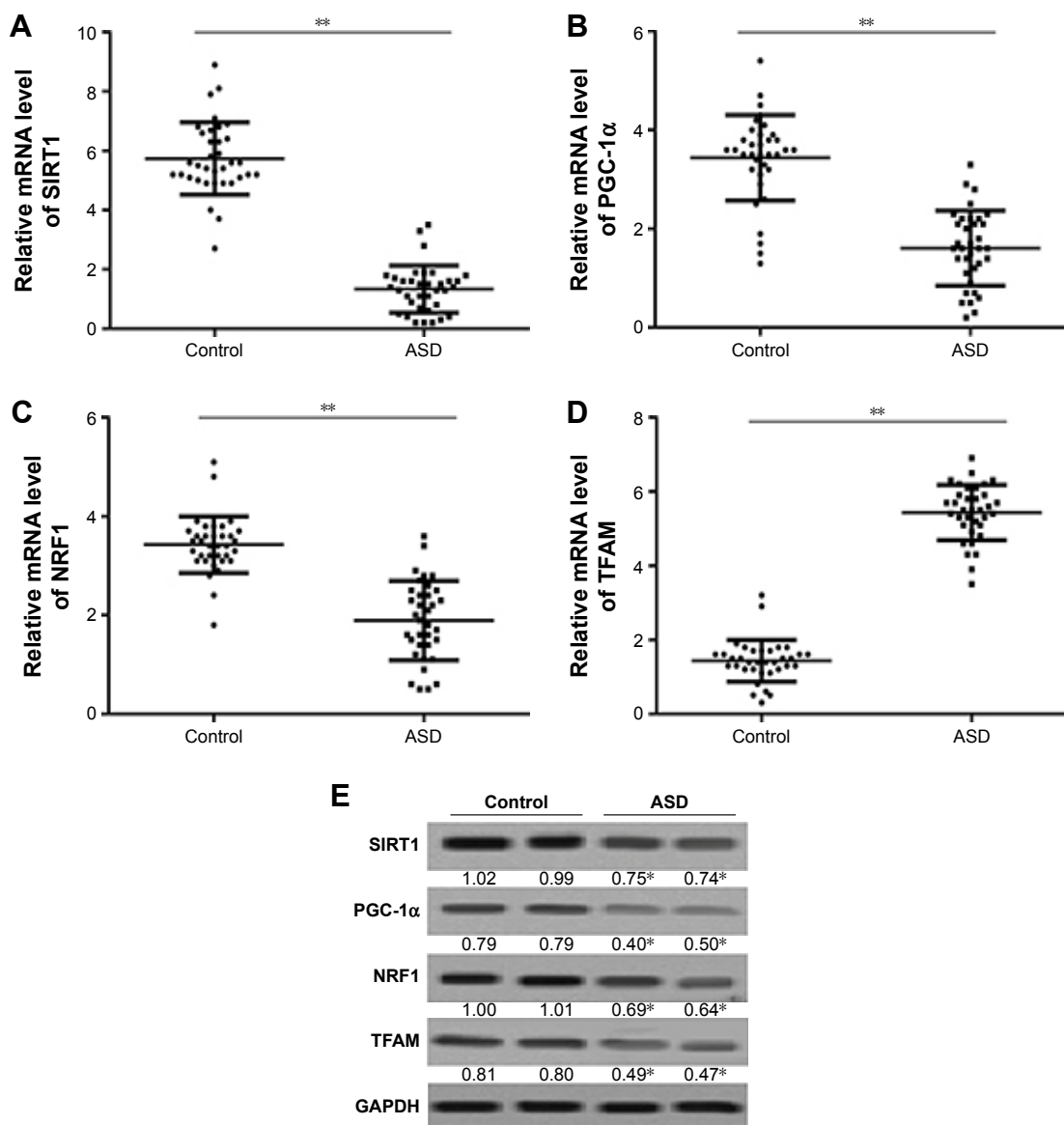


Figure 2 Relative mRNA and protein expression levels of SIRT1, PGC-1α, NRF1, and TFAM in ASD patients and healthy controls.

Notes: (A) Relative mRNA expression levels of SIRT1 (n=35). (B) Relative mRNA expression levels of PGC-1α (n=35). (C) Relative mRNA expression levels of NRF1 (n=35). (D) Relative mRNA expression levels of TFAM (n=35). (E) SIRT1, PGC-1α, NRF1, and TFAM protein expressions detected by Western blot assays. Representative gels were from two different samples out of 35 samples. * $P < 0.05$, ** $P < 0.01$ compared with control group.

Abbreviation: ASD, autistic spectrum disorder.

ROS induced damage to the mitochondrial respiratory chain in the ASD LCLs.

Effect of PGC-1α on the expression of SIRT1–PGC-1α axis genes and translocation of apoptogenic proteins in the ASD LCLs

To investigate the effect of PGC-1α on the expression of SIRT1, PGC-1α, NRF1, and TFAM in the ASD LCLs, the ASD LCLs were transfected with PGC-1α overexpression vector and empty vector. As shown in Figure 5A–E, overexpression of PGC-1α increased the expression of SIRT1,

PGC-1α, NRF1, and TFAM in the ASD LCLs at both RNA and protein levels. Moreover, overexpression of PGC-1α decreased the expression levels of cyto *c* and DIABLO proteins in the ASD LCLs (Figure 5F). These results revealed that overexpression of PGC-1α upregulated the SIRT1/PGC-1α axis genes expression and reduced cyto *c* and DIABLO release in the ASD LCLs.

Effect of PGC-1α on the cell apoptosis in the ASD LCLs

To detect the effect of PGC-1α on cell apoptosis in the ASD LCLs, JC-1 staining was used to evaluate the loss of $\Delta\Psi_m$.

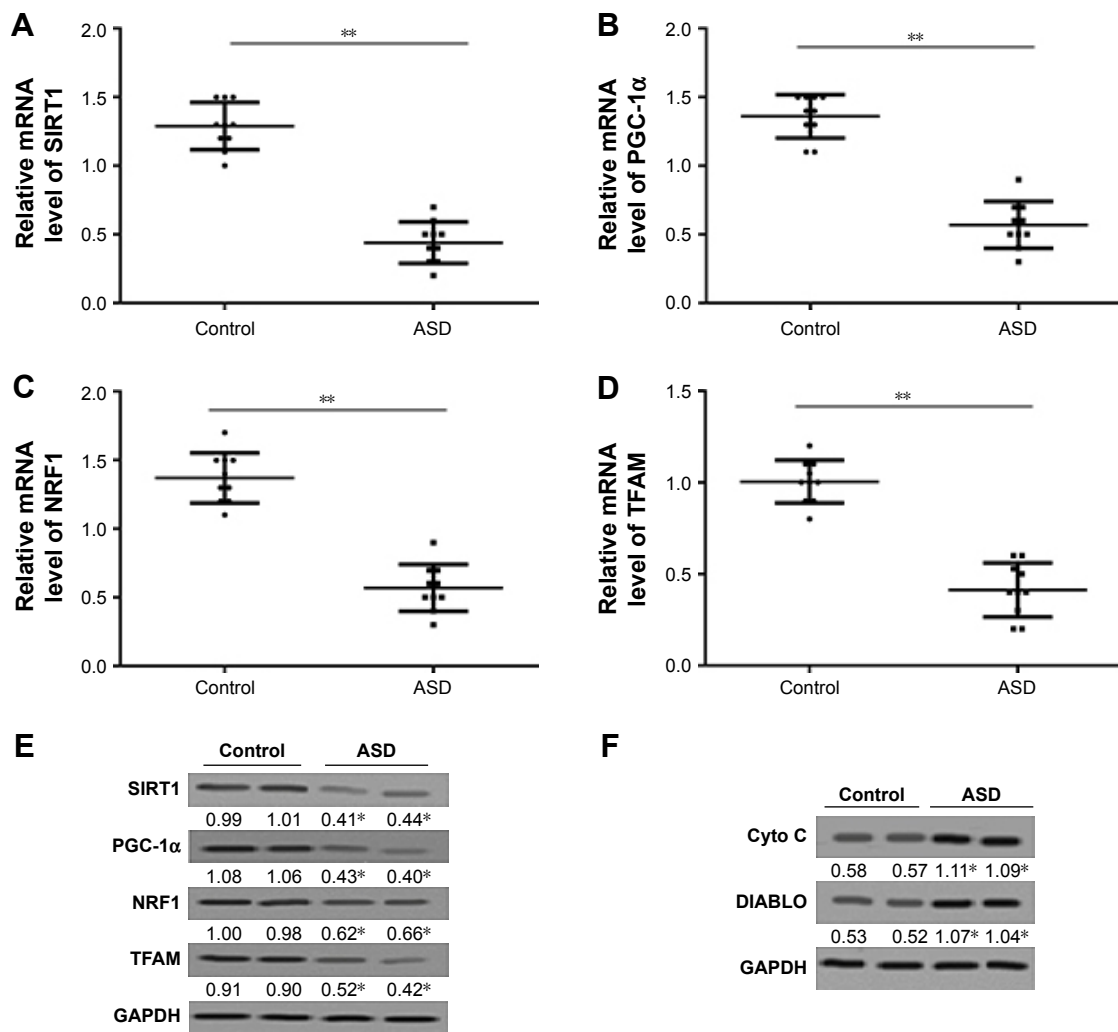


Figure 3 Relative mRNA and protein expression levels of SIRT1, PGC-1 α , NRF1, and TFAM and cyto c and DIABLO released from mitochondria to the cytosol in the ASD and control LCLs.

Notes: (A) Relative mRNA expression levels of SIRT1 (n=10). (B) Relative mRNA expression levels of PGC-1 α (n=10). (C) Relative mRNA expression levels of NRF1 (n=10). (D) Relative mRNA expression levels of TFAM (n=10). (E) SIRT1, PGC-1 α , NRF1, and TFAM protein expression levels detected by Western blot assays. (F) cyto c and DIABLO protein expression levels detected by Western blot assays. Representative gels were from two different samples out of 10 samples. * $P < 0.05$, ** $P < 0.01$ compared with control group.

Abbreviations: ASD, autistic spectrum disorder; LCL, lymphoblastoid cell line.

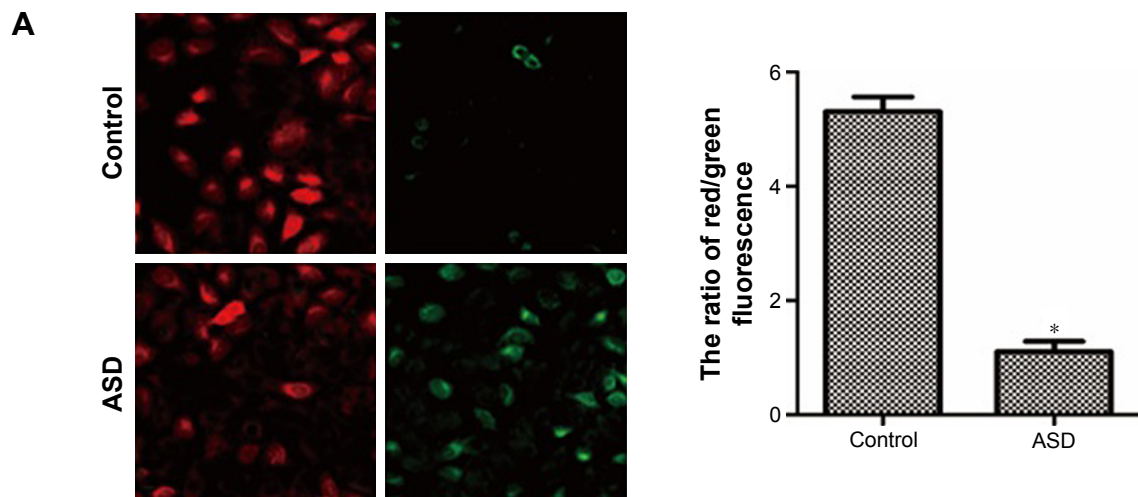


Figure 4 (Continued)

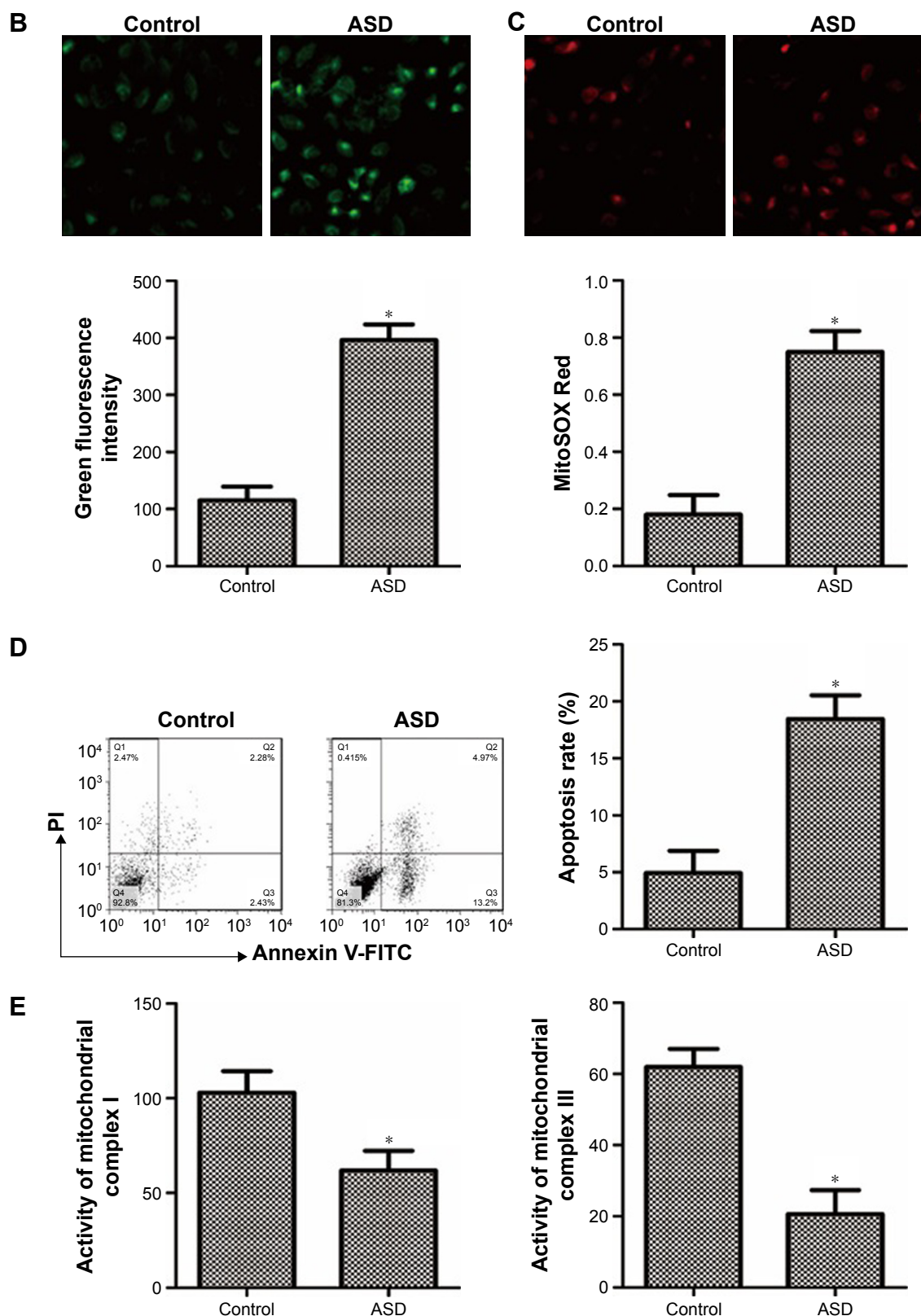


Figure 4 ASD induced upregulation of intracellular ROS and mitochondrial ROS generation and apoptosis of LCLs.

Notes: (A) The representative images showed changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) in the control and ASD LCLs. Magnification, 100 $\times$. The graph represents the ratio of red/green fluorescence (n=3). (B) The representative images showed intracellular ROS detected by DCFH-DA staining. Magnification, 100 $\times$. The graph represents the intensity of green fluorescence (n=3). (C) The representative images showed mitochondria ROS production detected by MitoSOX staining. Magnification, 100 $\times$. The MitoSOX fluorescence intensity in the control and ASD LCLs (n=3). (D) Cells were double stained with Annexin V-FITC and PI and analyzed by flow cytometry. And percentage of apoptotic cells is shown in the histogram (n=3). (E) Enzymatic activity of mitochondrial complex I and complex III in the control and ASD LCLs (n=3). * $P < 0.05$ compared with the control LCLs.

Abbreviations: ASD, autistic spectrum disorder; LCL, lymphoblastoid cell line; ROS, reactive oxygen species.

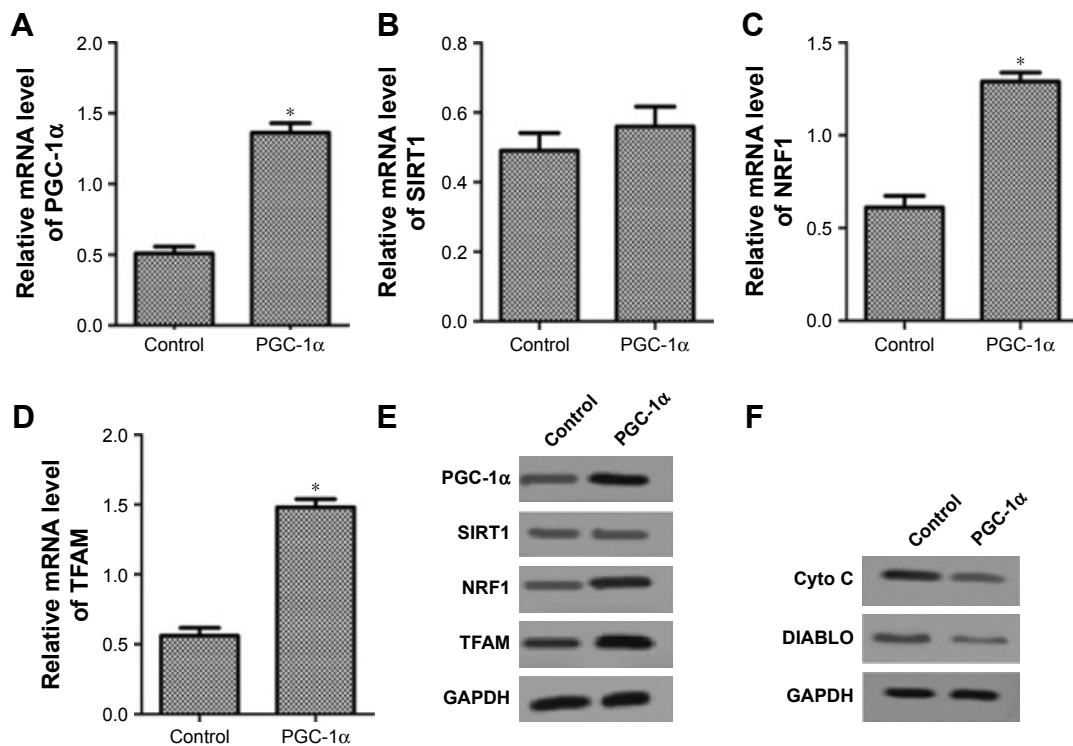


Figure 5 Relative mRNA and protein expression levels of SIRT1, PGC-1 α , NRF1, and TFAM and cyto c and DIABLO released from mitochondria to the cytosol in the ASD LCLs transfected with PGC-1 α overexpression vector and empty vector (control).

Notes: (A) Relative mRNA expression levels of PGC-1 α (n=3). (B) Relative mRNA expression levels of SIRT1 (n=3). (C) Relative mRNA expression levels of NRF1 (n=3). (D) Relative mRNA expression levels of TFAM (n=3). (E) SIRT1, PGC-1 α , NRF1, and TFAM protein expression levels detected by Western blot assays. (F) cyto c and DIABLO protein expression levels detected by Western blot assays. Representative gels were from one sample out of three samples. * $P < 0.05$ compared with ASD LCLs transfected with empty vector.

Abbreviations: ASD, autistic spectrum disorder; LCL, lymphoblastoid cell line.

The mitochondrial membrane potential was significantly higher in PGC-1 α overexpression group than that in the control group (Figure 6A). Thus, overexpression of PGC-1 α inhibited cell apoptosis in the ASD LCLs. In addition, identical results were showed by Annexin V/PI staining (Figure 6D).

Effect of PGC-1 α on intracellular ROS and mitochondrial ROS generation in the ASD LCLs

To examine the effect of PGC-1 α on intracellular ROS and mitochondrial ROS in the ASD LCLs, DCFH-DA and Mito-SOX Red staining were used. As shown in Figure 6B and C,

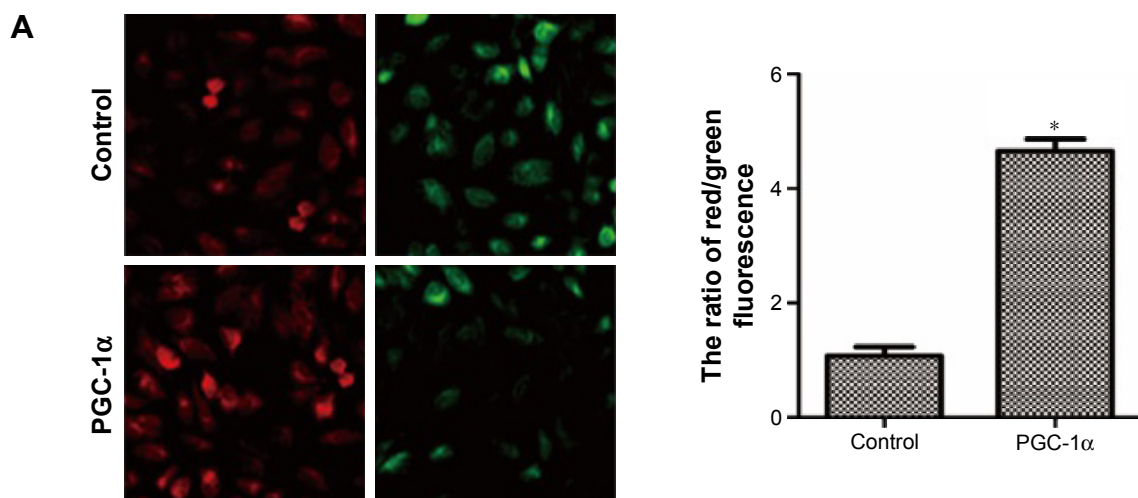


Figure 6 (Continued)

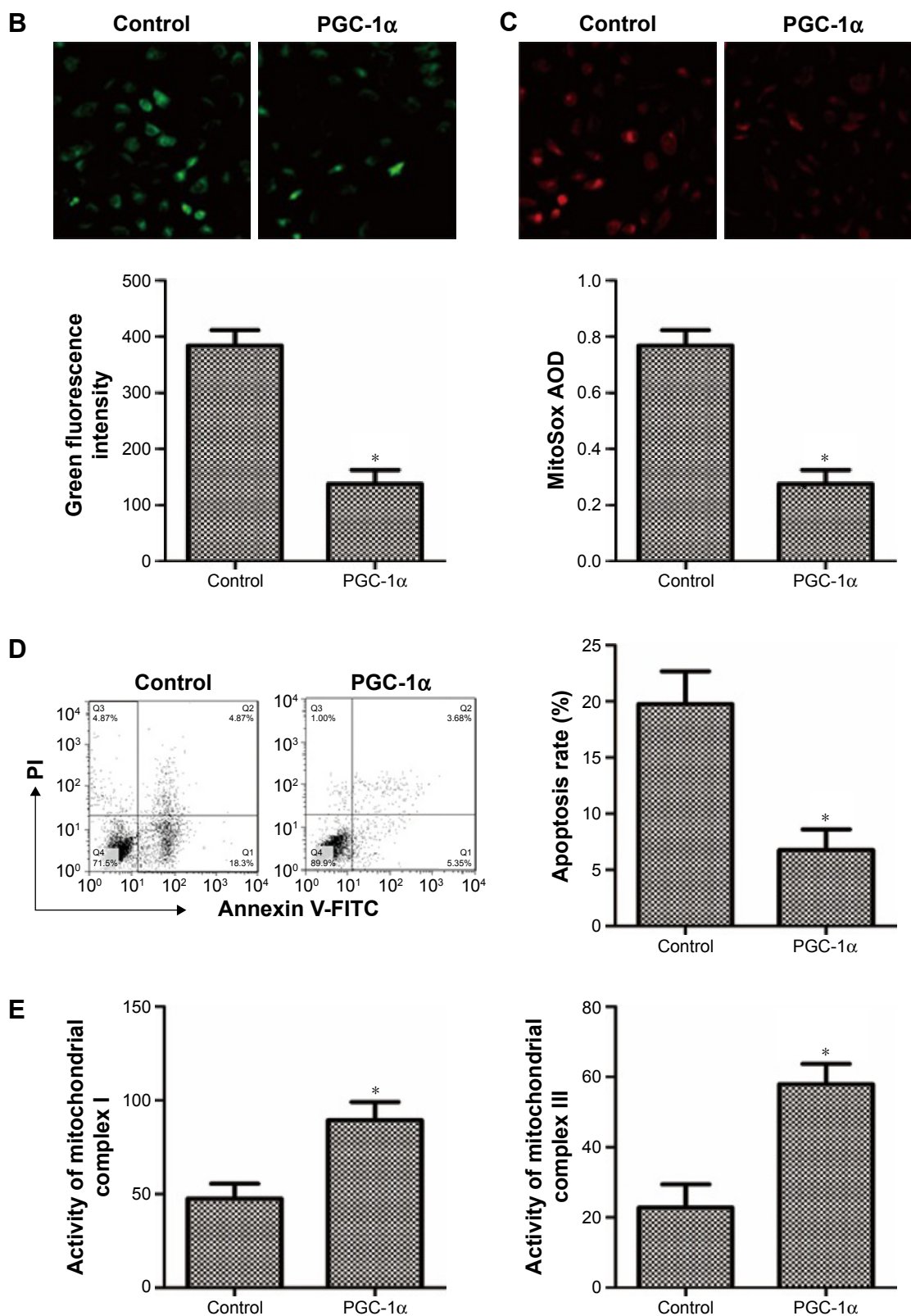


Figure 6 Effect of PGC-1 α on intracellular ROS and mitochondrial ROS generation and apoptosis in the ASD LCLs.

Notes: (A) The representative images show changes in $\Delta\Psi_m$ in the ASD LCLs transfected with PGC-1 α overexpression vector and empty vector. Magnification, 100 $\times$. The graph represents the ratio of red/green fluorescence ($n=3$). (B) The representative images show intracellular ROS detected by DCFH-DA staining. Magnification, 100 $\times$. The graph represents the intensity of green fluorescence ($n=3$). (C) The representative images show mitochondria ROS production detected by MitoSOX staining. Magnification, 100 $\times$. (D) Cells were double stained with Annexin V-FITC and PI and analyzed by flow cytometry. And the percentage of apoptotic cells is shown in the histogram ($n=3$). (E) Enzymatic activity of mitochondrial complex I and complex III ($n=3$). * $P<0.05$ compared with ASD LCLs transfected with empty vector.

Abbreviations: ASD, autistic spectrum disorder; DCFH-DA, dichloro-dihydro-fluorescein diacetate; LCL, lymphoblastoid cell line; ROS, reactive oxygen species.

a significant decrease in intracellular ROS and mitochondrial ROS in the ASD LCLs transfected with PGC-1 α overexpression vector was observed compared with that in control group.

Effect of PGC-1 α on activities of mitochondrial respiratory complexes in the ASD LCLs

Subsequently, we evaluated the effect of PGC-1 α on the activities of respiratory chain complexes, complexes I and III. The results showed that overexpression of PGC-1 α dramatically increased the activities of complexes I and III in the ASD LCLs compared with the control group (Figure 6E). These results demonstrated overexpression of PGC-1 α could repress ROS-induced damage to the mitochondrial respiratory chain in the ASD LCLs.

Discussion

ASD is a complex neurodevelopmental disorder that is thought to occur as a result of interaction between epigenetic effects, variable susceptibility genes, and environmental factors.^{43–45} And previous studies indicated that oxidative stress might contribute to the pathogenesis of ASD.⁴⁶ Tang et al⁴⁷ found elevated levels of biomarkers of oxidative stress in the temporal cortex of the ASD group and in the same brain region where mitochondrial electron transport chain (ETC) complex deficiencies exist. Chauhan and Chauhan⁴⁸ demonstrated the presence of markers of oxidative stress in urine and blood in the individuals with ASD. However, the studies associated with the molecular mechanisms of oxidative stress in ASD are limited. Therefore, our study aimed to find the damage of mitochondrial oxidative stress and the effect of SIRT1/PGC-1 α signaling on oxidative stress in ASD.

Increasing evidence has shown that mitochondrial dysfunction occurs in many neurodegenerative diseases, such as Parkinson's disease,⁴⁹ schizophrenia,⁵⁰ and Alzheimer's disease.⁵¹ Recent researches have indicated that mitochondrial abnormalities may also affect the developing of brain and trigger a cascade of events, resulting in neurodevelopmental disorders like autism.^{52,53} ROS can activate immune cells, which leads to mitochondrial dysfunction.⁵⁴ Thus, the mitochondrial oxidative stress may be one of the potential etiologic factors in ASD.

Previous studies demonstrated that LCLs derived from children with autistic disorder had higher levels of ROS and exhibited a significant decrease in both intracellular and mitochondrial glutathione redox capacity than that in control LCLs.⁵⁵ Besides, children with autism are more sensitive to

necrosis caused by oxidative stress than their nonautistic siblings.⁵⁶ In the present study, we found that intracellular ROS (mainly hydrogen peroxide and hydroxyl radicals) and mitochondrial ROS, mainly superoxide anion, were accumulated at higher levels in the ASD LCLs compared to that in the control LCLs. As a result, oxidative stress existed in the ASD LCLs.

The mitochondrial respiratory chain is an important structural and functional part of mitochondria and is located in the inner mitochondrial membrane.⁵⁷ Moreover, the production of mitochondrial superoxide radicals occurs mainly at two discrete points in the ETC, namely, at complex I and complex III.⁵⁸ Indeed, mitochondria are a major source of ROS as well as the target for ROS-mediated damage.⁵⁹ The ETC complex I dysfunction can produce high levels of ROS.⁶⁰ In turn, ROS can suppress the function of ETC, such as complex I activity,⁶¹ leading to mitochondrial dysfunction. Our results showed the activities of complexes I and III were significantly decreased in the ASD LCLs than in the control LCLs, suggesting oxidative stress impaired mitochondrial respiratory chain in the ASD LCLs.

In addition, ROS are considered to participate in various cellular functions, including cell differentiation, proliferation, and apoptosis.⁶² Additionally, the increase of ROS production can directly and indirectly lead to impairments in mitochondrial respiratory chain⁶³ and activate mitochondrial and non-mitochondrial-dependent biochemical cascades that lead to cell apoptosis.⁶⁴ JC-1 is widely used in detection of $\Delta\Psi_m$ in various cells or tissues.⁶⁵ Besides, changes in fluorescence from red to green induced by JC-1 indicated the decline of the membrane potential, and a decrease in $\Delta\Psi_m$ implied early apoptosis.⁶⁶ Our study showed that the red fluorescence intensity was decreased, whereas the green fluorescence intensity was increased. The results indicated that the $\Delta\Psi_m$ declined and that apoptosis was activated in the ASD LCLs.

It has been reported that SIRT1/PGC-1 α signaling plays an important role in neuronal survival based on the fact that transgenic mice in which the expression of PGC-1 α is silenced have neurodegenerative lesions.⁶⁷ PGC-1 α is a master regulator of cell metabolism, oxidative stress, and mitochondrial biogenesis.²⁰ Recently, an important function of PGC-1 α in dopaminergic neurons to defend against oxidative stress and increase neuronal viability in Parkinson's disease has been discovered.⁶⁸ Furthermore, lack of PGC-1 α increased the sensitivity of brain neurons against oxidative stress.²⁰ In this study, we showed that overexpressing of PGC-1 α protected against ROS generation and inhibited cell

apoptosis in the ASD LCLs, which suggested a key role of SIRT1/PGC-1 α signaling in the ASD LCLs.

In conclusion, we demonstrated mitochondrial oxidative stress may affect a significant subgroup of ASD children and that the SIRT1/PGC-1 α signaling pathway may be a promising medical treatment for ASD.

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Disclosure

The authors report no conflicts of interest in this work.

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