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Original Article

SIRT2 plays a significant role in maintaining the survival and energy metabolism of PIEC endothelial cells

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Abstract: SIRT2, a member of the sirtuin (SIRT1-7) family, is a tubulin deacetylase. It has been reported that SIRT2 mediates cellular stress responses and is highly expressed in vascular endothelial cells, while its roles in cell survival and energy metabolism of endothelial cells remain unknown. In the current study, we tested our hypothesis that SIRT2 plays an important role in the cell survival and energy metabolism of endothelial cells, using a porcine vascular endothelial cell line (PIEC) as a cellular model. Our study showed that both SIRT2 inhibitor AGK2 and SIRT2 siRNA led to a significant reduction of the cell survival of PIEC cells. Our FACS-based Annexin V/7-AAD assay and Hoechst staining showed that both SIRT2 inhibitor and SIRT2 siRNA led to a significant increase in apoptosis and necrosis of the cells. Moreover, the SIRT2 inhibition led to both mitochondrial depolarization and decreases in the intracellular ATP level of the cells. Collectively, our study has provided the first evidence suggesting that SIRT2 plays a significant role in maintaining both the survival and the mitochondrial membrane potential of PIEC cells, which may account for the major effects of SIRT2 on the intracellular ATP level of the cells.

Keywords: Apoptosis, intracellular ATP, mitochondrial membrane potential, SIRT2, vascular endothelial cells

Introduction

Mammalian sirtuin family proteins (SIRT1-SIRT7) are NAD^+-dependent deacetylases [1], which play important roles in various biological processes including aging and inflammation [2]. Accumulating evidence has suggested that sirtuins are also involved in cardiovascular physiology and diseases [3, 4]. For examples, SIRT1 controls endothelial angiogenic functions during vascular growth [3]; SIRT1 and SIRT3 can promote cardiomyocyte survival and prevent cardiac remodeling in response to different stress stimuli [5, 6]; and in blood vessels, activation of SIRT1, SIRT4 and SIRT6 can produce various beneficial effects such as suppressing inflammation [7-9]. Moreover, SIRT1, SIRT3 and SIRT6 may also exert protective effects on cardiovascular system by improving glucose and lipid metabolism [10, 11].

Endothelial cells play pivotal role in maintaining vascular homeostasis. SIRT1 has been shown to mediate a number of endothelial cell functions [4, 12, 13]. For example, SIRT1 binds to and activate endothelial nitric oxide synthase (eNOS), thus increasing vasorelaxation [13]. In contrast, there has been no information regarding the roles of SIRT2 in endothelial cells. Since there are multiple common factors in SIRT1- and SIRT2-initiated downstream events, including FOXO1, FOXO3, and NF-κB, we hypothesized that SIRT2 may also play significant roles in the biological properties of endothelial cells.

Predominantly localized in cytosol, SIRT2 can deacetylate tubulin, partitioning defective 3 homologue (PAR3), phosphoenolpyruvate carboxykinase (PEPCK), and FOXO1 [14]. Increasing evidence has indicated that the roles of SIRT2 in cell survival appear to be cell type- and context-specific. For example, in cellular and Drosophila models of Parkinson's disease, SIRT2 inhibition was shown to increase cell survival [15]. However, SIRT2 inhibition was shown to induce cell death in glioma cells, microglia and PC12 cells [16-18]. Because SIRT2 has been shown to play significant roles in the survival of

multiple types of cells [19-21], we hypothesized that SIRT2 may play important roles in the survival and cellular functions of endothelial cells.

It has been reported that SIRT2 inhibition led to a decrease in the intracellular ATP level in PC12 cells [16], while the underlying mechanisms are poorly understood. In our current study, we used PIEC cells as an endothelial cell model to test our hypothesis that SIRT2 plays significant roles in the cell survival and energy metabolism of endothelial cells. Our study has provided evidence supporting this hypothesis.

Methods and materials

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted.

Cell cultures

PIEC cells were purchased from the Cell Resource Center of Shanghai Institute of Biological Science, Chinese Academy of Sciences. The cells were plated into 24-well cell culture plates at the initial density of 1×10^5 cells/mL in Dulbecco's Modified Eagle Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum (PAA, Linz, Australia). The cells were maintained at 37°C in a 5% CO_2 incubator.

SIRT2 silencing

PIEC cells were transfected with either SIRT2 siRNA oligonucleotides (5' GGCAUGGACUUCGAUUCAATT 3') against pig SIRT2 (Genepharma, Shanghai, China) or Control siRNA oligonucleotides (5' UUCUCCGAACGUGUCACGUTT 3') (Genepharma, Shanghai, China), when the cells were approximately 40% confluent. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection according to the manufacturer's instructions. For each well of a 24-well plate, 100 µl Opti-MEM containing 0.06 nmol of the siRNA oligonucleotides and 2 µl lipofectamine 2000 was added into 500 µl culture media of the cells. The SIRT2 level of PIEC cells was determined by Western Blot 48 hrs after the transfection.

Intracellular and extracellular lactate dehydrogenase (LDH) assay

Cell death was determined by extracellular LDH assay. Briefly, 100 µl of extracellular media was mixed with 150 µl reaction buffer A (500 mM potassium phosphate buffer, pH 7.5, containing 0.3 mM NADH and 2.5 mM sodium pyruvate). Subsequently changes of the A340 nm of the samples were monitored over 90 sec by a plate reader.

Intracellular LDH assay was used to quantify cell survival. Briefly, after extracellular media was removed, the cells were lysed for 20 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.01% bovine serum albumin (pH 7.5). 50 µl cell lysates were mixed with 150 µL reaction buffer A . The A_{340nm} change was monitored over 90 sec by a plate reader. Percentage of cell survival was calculated by normalizing the LDH values of the samples to the LDH activity of the controls.

Flow cytometry-based annexin V/7-AAD assay

The flow cytometry assay was performed to determine the levels of early-stage apoptosis, late-stage apoptosis, and necrosis using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. Briefly, PIEC cells were harvested by 0.25% trypsin-EDTA, washed by cold PBS once and resuspended in cold binding buffer at concentrations between 1×10^6 and 1×10^7 cells/ml. 5 µL of labeled Annexin V was added into 100 µL of the cell suspensions. After incubation on ice for 15 min, 200 µL binding buffer and 5 µL 7-AAD solution were added into the cell suspensions. The number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

Flow cytometry-based JC-1 assay

Mitochondrial membrane potential ($\Delta\psi_m$) was measured by flow cytometry-based JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) assay. PIEC cells were harvested by 0.25% trypsin-EDTA, and incubated in cell media containing 10 µg/mL JC-1 (Enzo Life Sciences, Farmingdale, NY, USA) for 20 min in 37°C. PIEC cells were washed once with PBS and analyzed by a flow cytome-

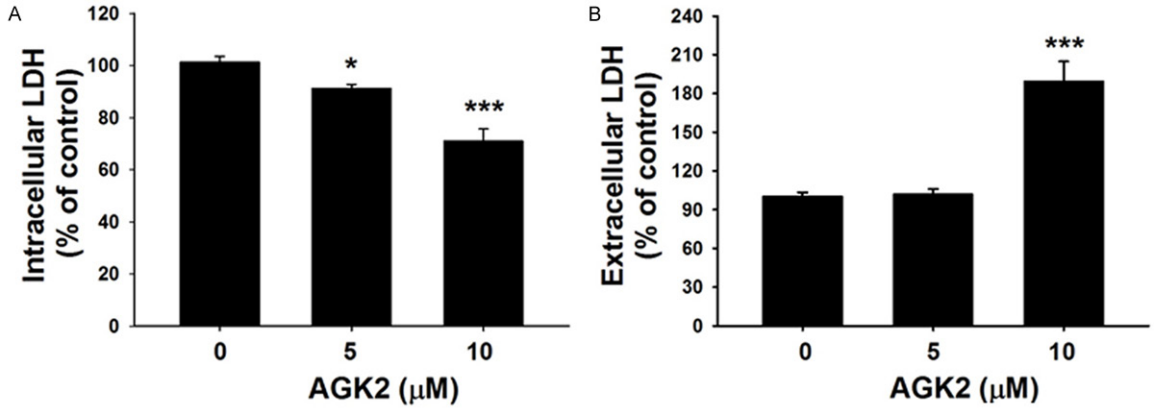

Figure 1. SIRT2 inhibition dose-dependently led to a decrease in the intracellular LDH activity and a significant increase in the extracellular LDH activity of PIEC cells. A. AGK2 treatment dose-dependently decreased the intracellular LDH activity of the cells, assessed 24 hrs after the AGK2 treatment. $N = 12$. Data were collected from three independent experiments. ***, $p < 0.001$. B. AGK2 treatment led to a significant increase in the extracellular LDH activity of the cells, assessed 24 hrs after the AGK2 treatment. $N = 12$. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

ter (FACSAria II, BD Biosciences), which detected emission fluorescence at the wavelength of 575 nm (PE-A) and the wavelength of 518 nm (FITC-A) with excitation wavelength of 488 nm. The $\Delta\psi_m$ of each cell was calculated by the ratio of the fluorescence at wavelength of 575 nm/ the fluorescence at 518 nm.

Western blot

PIEC cells were harvested and lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM PMSF. Cell lysates were centrifuged at 12,000 g for 20 min at 4°C. After quantifications of the samples using BCA Protein Assay Kit (Pierce Biotechonology, Rockford, IL, USA), 30 μg of total protein was electrophoresed through a 10% SDS-polyacrylamide gel, and then transferred to 0.45 μm nitrocellulose membranes. The blots were incubated overnight at 4°C with a rabbit polyclonal anti-SIRT2 antibody (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then incubated with HRP-conjugated secondary antibody (Epitomics, Hangzhou, Zhejiang Province, China). Protein signals were detected using the ECL detection system (Pierce Biotechonology, Rockford, IL, USA). An anti-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to normalize the sample loading and transfer. The intensities of the bands were quantified by densitometry using Gel-Pro Analyzer.

ATP assay

Intracellular ATP levels were quantified using the Roche ATP Bioluminescence Assay Kit (HS II), according to the protocol provided by the vendor. Briefly, cells were washed once with cold PBS, and lysed for 15 min with the Cell Lysis Reagent. Then 50 μL of the lysates was mixed with 50 μL of the Luciferase Reagent. A plate reader (Biotek Synergy 2) was used to detect the chemiluminescence of the samples. The protein concentrations were determined by BCA assay. The ATP concentrations of the samples were calculated using an ATP standard, and normalized to the protein concentrations of the samples.

Hoechst staining

The nuclear size of cells was measured by Hoechst staining [22]. Briefly, cells were treated with 10 μg/mL bisbenzimide (Hoechst 33342; Sigma) in cell medium for 20 min. The stained nuclei were photographed under a fluorescence microscope. Randomly picked 5 fields in each well were photographed. The nuclear sizes were quantified using Image-Pro Plus 6.

Statistical analyses

All data are presented as mean ± SE. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. *P* values less than 0.05 were considered statistically significant.

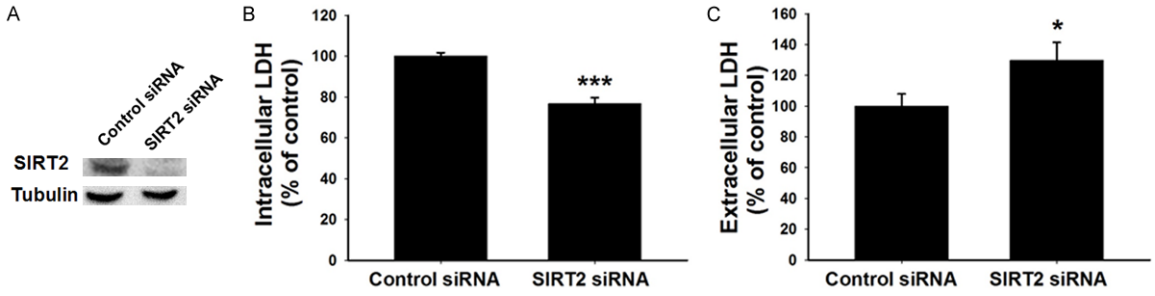

Figure 2. Treatment with SIRT2 siRNA led to a decrease in the intracellular LDH activity and a significant increase in the extracellular LDH activity of PIEC cells. (A) Western blot assays on SIRT2 showed that the SIRT2 siRNA reduced the expression of SIRT2. SIRT2 siRNA treatment led to a significant decrease the intracellular LDH activity (B) and a significant increase in the extracellular LDH activity (C) of the cells, assessed 48 hrs after the SIRT2 siRNA treatment. N = 12. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

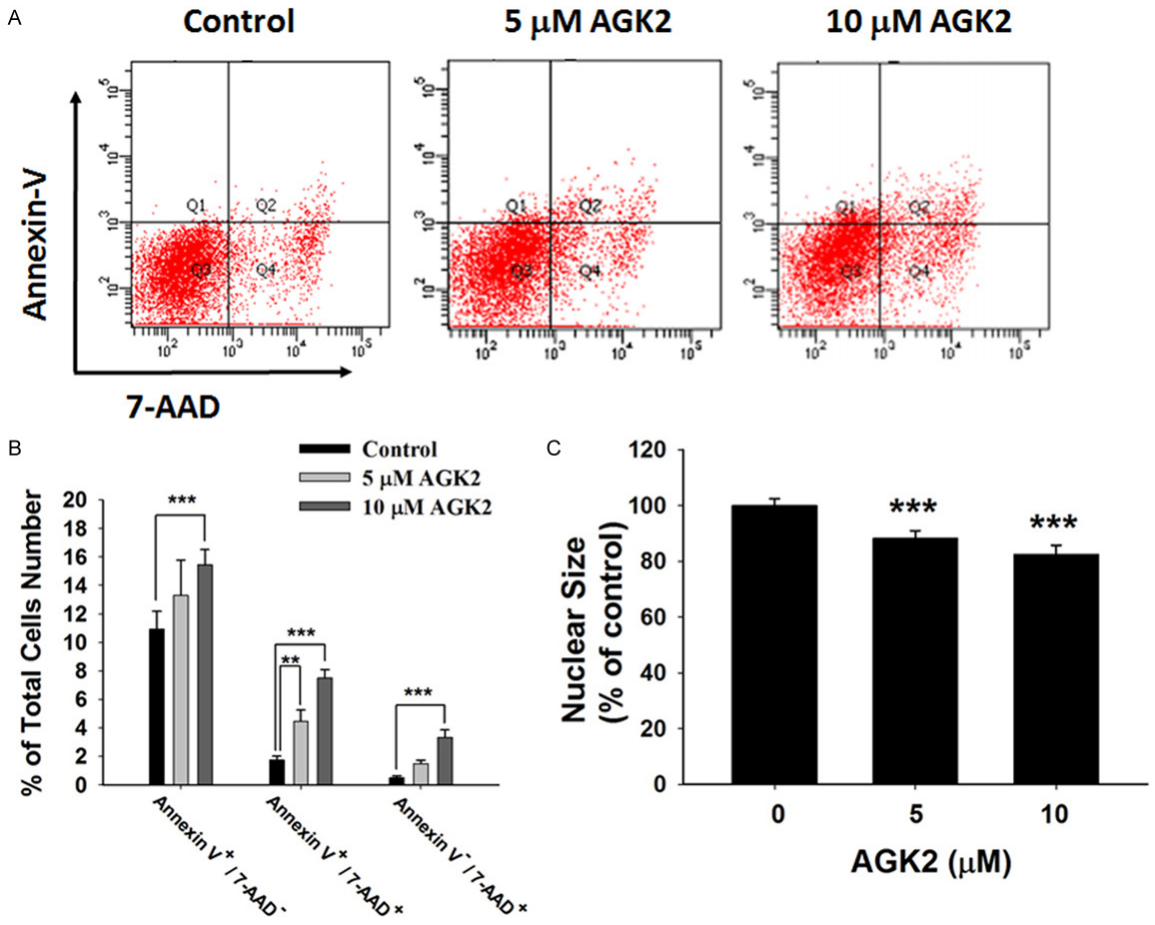

Figure 3. SIRT2 inhibition led to significant increases in both apoptosis and necrosis of the cells. A. FACS-based Annexin V/7-AAD assay showed that SIRT2 inhibition induced increases in the number of early-stage apoptosis cells (Annexin $V^+/7\text{-}AAD^-$), late-stage apoptosis cells (Annexin $V^+/7\text{-}AAD^+$) and necrotic cells (Annexin $V^-/7\text{-}AAD^+$), assessed 24 hrs after treatment. B. Quantifications of the results from the FACS-based Annexin V/7-AAD assay showed that the SIRT2 inhibition induced significant increases in the number of both apoptosis and necrosis of the cells. C. Hoechst staining assay showed that AKG2 treatment induced a decrease in nuclear size of PIEC cells. *N* = 10. Data were collected from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

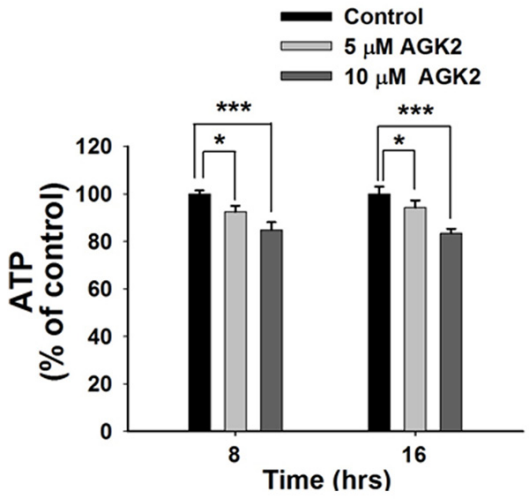

Figure 4. SIRT2 inhibition decreased intracellular ATP levels of PIEC cells. ATP assay showed that after AGK2 treatment for 8hrs or 16hrs, intracellular ATP levels were significantly decreased in PIEC cells. N = 12. Data were collected from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Both SIRT2 inhibitor and SIRT2 siRNA led to decreased survival of PIEC cells

To study the roles of SIRT2 in the survival of PIEC cells, we treated PIEC cells with AGK2, a specific SIRT2 inhibitor [23], for 24 hrs. By assessing intracellular LDH activity and extracellular LDH activity, we determined the effects of the decreased SIRT2 activity on the survival and death of the cells, respectively. Intracellular LDH assays showed that the SIRT2 inhibition dose-dependently decreased the intracellular LDH activity of the cells (**Figure 1A**), suggesting that decreased SIRT2 led to a reduction of the cell survival. Consistent with our intracellular LDH assays, our extracellular LDH assays showed that the SIRT2 inhibition dose-dependently increased the extracellular LDH activity of the cells (**Figure 1B**), suggesting that decreased SIRT2 activity can induce cell death.

We also used SIRT2 siRNA to decrease the SIRT2 level in PIEC cells. Our Western blot assay showed that the SIRT2 siRNA was capable of producing a significant decrease in the SIRT2 level of the cells (**Figure 2A**). By assessing intracellular and extracellular LDH activity, we determined the effects of decreased SIRT2 on the survival and death of PIEC cells, respectively. Our LDH assay showed that the SIRT2 siRNA led to a significant reduction of the intracellular LDH activity (**Figure 2B**) and a significant increase in the extracellular LDH activity of the cells (**Figure 2C**).

SIRT2 inhibition led to both apoptosis and necrosis of PIEC cells

FACS-based annexin V/7-AAD staining assay showed that treatment of the cells with AGK2 led to increases in early-stage apoptosis (Annexin $V^+/7\text{-}AAD^-$) cells, late-stage apoptosis cells (Annexin $V^+/7\text{-}AAD^+$) and necrotic cells (Annexin $V^-/7\text{-}AAD^+$) (**Figure 3A**, **3B**).

Since nuclear condensation is one of the hallmarks of apoptosis, we applied Hoechst staining to determine the nuclear size of PIEC cells. Consistent with our V/7-AAD staining assay, our Hoechst staining assay showed that AGK2 treatment induced a significant decrease in the nuclear size of PIEC cells (**Figure 3C**), which further suggested that SIRT2 inhibition led to apoptosis of PIEC cells.

SIRT2 inhibition led to decreases in both $\Delta\psi_m$ and the intracellular ATP level of PIEC cells

We also found that the SIRT2 inhibition also led to a significant decrease in the intracellular ATP level of the cells (**Figure 4**), assessed at either 8 hrs or 16 hrs after the AGK2 treatment. We determined the effect of SIRT2 inhibition on the $\Delta\psi_m$ of the cells by FACS-based JC-1 assay. Sixteen hrs after the AGK2 treatment, the $\Delta\psi_m$ of the PIEC endothelial cells were significantly decreased (**Figure 5A**, **5B**), suggesting that the AGK2 inhibition was capable of decreasing the $\Delta\psi_m$ of the cells.

Discussion

The major findings of our study include: First, both SIRT2 inhibition and SIRT2 siRNA treatment led to a significant decrease in the survival of PIEC endothelial cells; second, SIRT2 inhibition led to significant increases in both apoptosis and necrosis of the cells; third, SIRT2 inhibition led to a significant decrease in the intracellular ATP level of the cells; and fourth, the SIRT2 inhibition led to mitochondrial depolarization of the cells. In summary, our study has provided the first evidence suggesting that SIRT2 plays a significant role in the cell survival in PIEC endothelial cells. Our study has also suggested that SIRT2 plays significant roles in

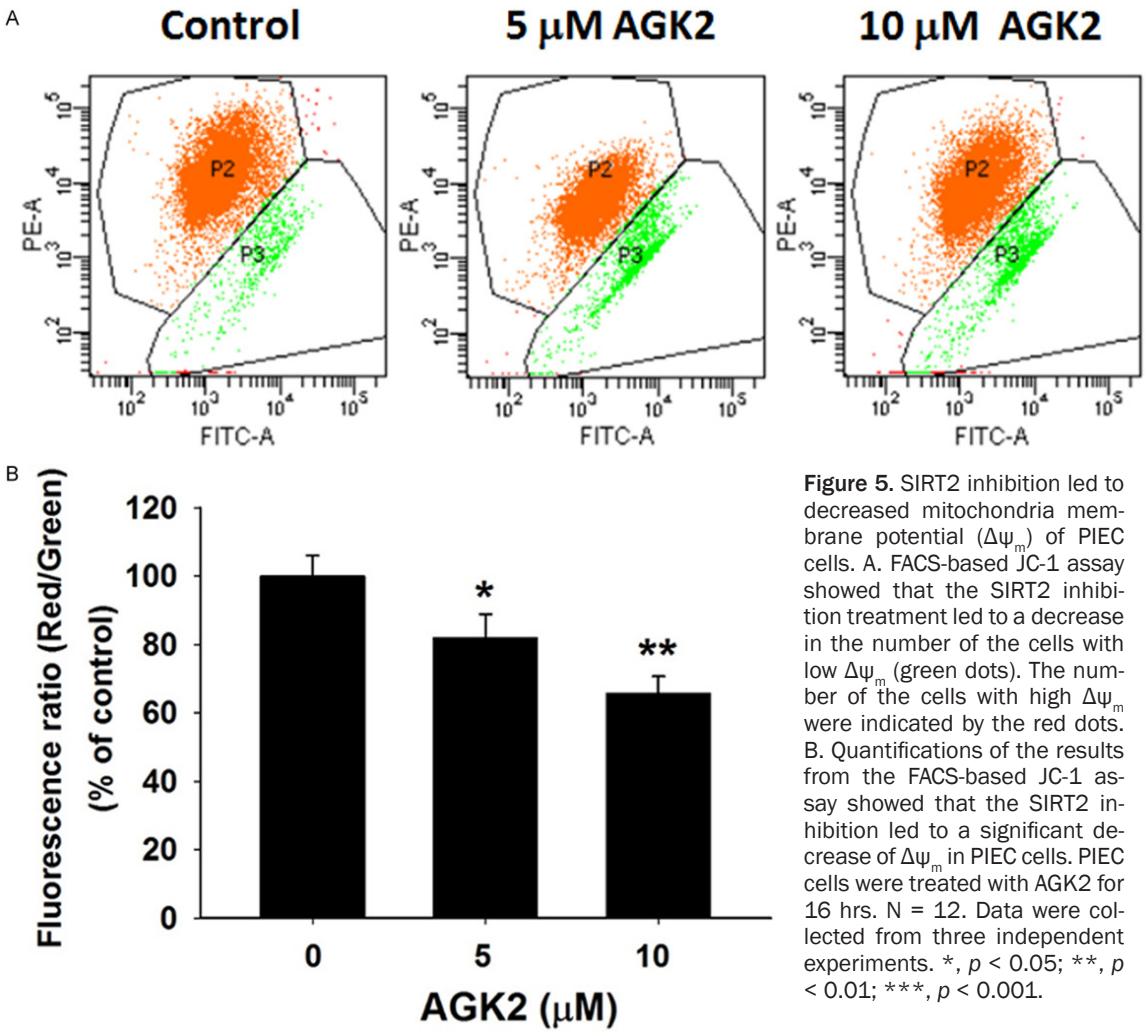

Figure 5. SIRT2 inhibition led to decreased mitochondria membrane potential ($\Delta\psi_m$) of PIEC cells. A. FACS-based JC-1 assay showed that the SIRT2 inhibition treatment led to a decrease in the number of the cells with low $\Delta\psi_m$ (green dots). The number of the cells with high $\Delta\psi_m$ were indicated by the red dots. B. Quantifications of the results from the FACS-based JC-1 assay showed that the SIRT2 inhibition led to a significant decrease of $\Delta\psi_m$ in PIEC cells. PIEC cells were treated with AGK2 for 16 hrs. N = 12. Data were collected from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

maintaining both the mitochondrial membrane potential and the intracellular ATP level of endothelial cells.

SIRT2, a NAD^+-dependent tubulin deacetylase, plays important role in various biological processes, including cell cycle, cell motility and redox homeostasis [19, 24, 25]. Several studies have suggested a cell-type and context-dependent role of SIRT2 in cell viability. Our study has provided the following evidence suggesting that SIRT2 plays a significant role in maintaining the basal survival of PIEC endothelial cells: First, both SIRT2 siRNA and SIRT2 inhibitor AGK2 led to decreased intracellular LDH activity and increased extracellular LDH activity; and second, AGK2 led to significant increases in both apoptosis and necrosis of PIEC endothelial cells. This study is consistent with our previous observation suggesting that SIRT2 is required for the basal survival of C6 glioma cells, PC12 cells and BV2 microglial cells [16-18].

A previous study has shown that SIRT2 inhibition decreased H_2O_2-induced endothelial cell death. In their study, they did not observe toxic effects of SIRT2 inhibition on the basal survival of human umbilical vein endothelial cells (HUVECs) [26]. However, our study showed that both SIRT2 inhibition and SIRT2 siRNA led to decreased endothelial cell survival. Essentially, our finding is not contradictory to their observation due to major differences between our experimental conditions: in their study, HUVECs were treated with 10 μM AGK2 for only 2 hrs, while in our study the PIEC cells were treated with 10 μM AGK2 for 24 hrs.

Our study also showed that AGK2 treatment of the cells for 8 hrs was sufficient to decrease the intracellular ATP levels of PIEC endothelial

cells. This result is consistent with our report regarding the capacity of SIRT2 inhibition to decrease intracellular ATP levels of PC12 cells [16]. It is important to investigate the mechanisms underlying the effect of SIRT2 on the intracellular ATP concentrations. Our previous study has shown that AGK2 can lead to decreased intracellular ATP levels by activating PARP [18]. Other labs have reported controversial results regarding the effects of SIRT2 on the activity of phosphoglycerate mutase-an enzyme in glycolysis [27, 28]. However, there has been no study regarding the relationships between SIRT2 and mitochondrial membrane potential-a key index for the capacity of mitochondrial ATP production. Our current study used JC-1 assay to determine the effects of SIRT2 inhibition on the mitochondrial membrane potential of PIEC cells, showing that the SIRT2 inhibitor can produce mitochondrial depolarization of the cells. To our knowledge, it is the first evidence suggesting that SIRT2 plays a significant role in maintaining mitochondrial membrane potential. Because mitochondrial depolarization plays a key role in apoptosis and ATP production, our observation has suggested that SIRT2 inhibition may induce cell apoptosis and decreased intracellular ATP levels by producing mitochondrial depolarization. Future studies are warranted to investigate the mechanisms underlying the effects of SIRT2 inhibition on mitochondrial membrane potential.

In summary, our current observations have indicated that SIRT2 activity is required for maintaining the cell survival and intracellular ATP level of PIEC endothelial cells. Our study has also provided the first evidence suggesting that SIRT2 activity is required for maintaining the mitochondrial membrane potential of PIEC endothelial cells, which may underlie the effects of SIRT2 on the cell survival and intracellular ATP level of the cells.

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Disclosure of conflict of interest

None.

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