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Resveratrol induces Sirt1-dependent apoptosis in 3T3-L1 preadipocytes by activating AMPK and suppressing AKT activity and survivin expression☆

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Abstract

Resveratrol is a natural polyphenolic compound with anti-inflammatory, antioxidant and neuroprotective properties, and it serves as a chemopreventive and chemotherapeutic agent. However, only very limited data have been obtained regarding the effects of resveratrol on preadipocytes, and the mechanisms of these effects remain largely unknown. In this study, murine 3T3-L1 preadipocytes were incubated with resveratrol, and cell apoptosis was investigated. Resveratrol caused S-phase arrest to inhibit cell proliferation and significantly increased the lactate dehydrogenase leaking ratio. Hoechst 33258 staining and transmission electron microscopy revealed the ultrastructural changes in nuclear chromatins of apoptotic cells. Furthermore, resveratrol activated the mitochondrial signaling with decreases in the mitochondrial membrane potential, cytochrome *c* release and the activation of caspase 9 and caspase 3. Resveratrol treatment also increased the protein level of Sirt1. By using small interfering RNAs of Sirt1, adenosine-monophosphate-activated protein kinase (AMPK) α, survivin and the AMPK agonist (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) and specific inhibitors for protein kinase B (AKT) or caspases, it was demonstrated that activation of Sirt1 inhibited AKT activation and further decreased the expression of survivin. It could also increase AMPK activation. Both signaling pathways activated mitochondrion-mediated pathway. Our findings clarified the apoptotic effects of resveratrol in 3T3-L1 preadipocytes and revealed the involved pathway including AMPK, AKT and survivin, suggesting its potential therapeutic application in the treatment or prevention of obesity and related metabolic symptoms.

Keywords: Resveratrol; Silent information regulator 1; AMP-activated protein kinase α; Protein kinase B; Survivin; Apoptosis

1. Introduction

Obesity is considered an epidemic of modern society. Its prevalence keeps increasing in most countries due to high-calorie diets and lack of physical activity, which causes the excessive energy to be stored in the form of triacylglycerols in adipocytes. Adipose tissue plays an important role in energy homeostasis. However, excess adipose tissue leads to insulin resistance, the "common soil" of metabolic disorders such as type 2 diabetes, hyperlipidemia, atherosclerosis and hypertension [1]. The fat mass is determined by the average size of fat cells and/or the number of adipocytes. Increased storage of triacylglycerols in fully differentiated adipocytes results in enlarged volume of fat cells, while the number of adipocyte increases due to increased proliferation and differentiation of preadipocytes [2]. Therefore, decreased fat mass may involve the loss of lipids (through lipolysis and lipogenesis), the inhibition of adipogenesis or the apoptosis of preadipocytes, which results in the loss of mature fat cells. Apoptosis is critical for many physiological processes, including energy homeostasis, tissue differentiation and the elimination of tumorigenic or mutated cells [3]. Therefore, the strategy of up-regulating preadipocytes' apoptosis to reduce the body fat content has begun to attract widespread attention.

Previous reports have revealed the link between obesity and the bioactive components in plant foods, and phytochemicals are being used for the treatment of obesity in both cultured cells and animal models [4,5]. Resveratrol (3,5,4′-trihydroxy-trans-stilbene, Res) is one of the widely studied phytochemicals, which has potent pharmacological effects and presents in grapes, peanuts, wine (especially in red wine) and a wide variety of other food sources [6]. Res acts as a cancer chemopreventive and chemotherapeutic agent and has anti-

Abbreviations: Cyt C (Cyto), cytochrome *c* (cytoplasm); AMP, adenosine monophosphate; PI3K, phosphatidylinositol 3-hydroxy kinase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

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inflammatory, antioxidant, antileukemic and neuroprotective properties [7]. Res was found to improve dyslipidemia, hyperinsulinemia and hypertension in obese Zucker rats and to produce anti-inflammatory effects in visceral adipose tissue [8]. The antiproliferative and apoptosis-inducing effects of Res cause cell cycle arrest and apoptosis in different cancer cell lines [9]. Res was also found to inhibit adipogenesis and induce apoptosis in mature adipocytes [10,11]. In preadipocytes, Res has been reported to suppress cell proliferation, yet the mechanisms remain unknown [12]. More recently, Res was found to sensitize human SGBS preadipocytes to TRAIL-induced apoptosis in a Sirt1-independent pathway [13]. However, little is known about the effects and mechanisms of Res on apoptosis in murine 3T3-L1 preadipocytes.

Sirt1, a homolog of the yeast protein silent information regulator 2 (Sir2), may play a critical role in the regulation of cell apoptosis, cell cycle, gene transcription and other cell processes to extend life span [14]. Recently, Res has attracted much attention for its ability of enhancing the deacetylase activity of Sirt1 [15]. Res was found to inhibit cell proliferation and differentiation of pig preadipocytes by increasing the expression of Sirt1 mRNA [16]. Adenosine-monophosphate-activated protein kinase (AMPK) is a eukaryotic heterotrimeric serine/threonine kinase and a metabolic master switch that senses nutritional or environmental stress. AMPK is also implicated in cancer development and is considered as a potential antitumor target molecule [17]. It has been suggested that the activation of AKT and the high-level expression of survivin in tumor cells are important for neoplasia. AKT plays a critical role in apoptosis and tumorigenesis as an oncogene [18]. Survivin is a member of the protein family that inhibits cell apoptosis by blocking the activation of caspases [19]. Interestingly, the activation of AMPK reduced cell survival by inhibiting survivin expression and without changing the activity of AKT activity in prostate cancer cells [20]. However, the functions of Sirt1, AMPK, AKT or survivin in preadipocytes are far less clear than in certain tumor cell lines. Indeed, mitochondrial signaling system also participates in cell apoptosis. For example, the release of cytochrome *c* (Cyt C) from mitochondria activates the proteolytic caspase cascades and causes apoptosis [21].

In this study, we found that Res had novel effects on the proliferation and apoptosis of murine 3T3-L1 preadipocytes. To clarify the mechanisms, we examined the effects of Res on (a) Sirt1 expression, (b) AMPK and AKT activation, (c) survivin expression and (d) mitochondrial levels of Cyt C and mitochondrial membrane potential (MMP) and activation of caspase 9 and caspase 3. Furthermore, we used small interfering RNA (siRNA) (Sirt1, AMPKα, survivin), activator [5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR)] or specific inhibitors (wortmannin, Z-LEHD-FMK, Z-DEVD-FMK) to investigate the apoptotic signaling pathways induced by Res in 3T3-L1 preadipocytes.

2. Materials and methods

2.1. Reagents

Res was purchased from Sigma-Aldrich (St. Louis, MO, USA). Res (80 mM) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) stored as small aliquots at −20°C for the cell culture experiments. DMSO was used as vehicle control. Penicillin, streptomycin, Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Antibodies against AMPKα, phospho-AMPKα (Thr172), AKT, phospho-AKT (Ser473), survivin, Cyt C, cleaved caspase 9, caspase 3 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against Sirt1 was purchased from Abcam (Cambridge, UK). The goat anti-rabbit secondary antibody with green fluorescence and the goat anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies fluorescence were purchased from Invitrogen (Grand Island, NY, USA). The ready-to-use solution of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was purchased from Roche (Basel, CH). Hoechst 33258 and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich and were prepared in phosphate-buffered saline (PBS) and DMSO, respectively. The detection kit for lactate dehydrogenase (LDH) release assay was purchased from Promega (Madison, WI, USA). The detection kits for cell apoptosis, cell cycle and MMP were purchased from BD Biosciences (San Jose, CA, USA). The siRNA oligos of Sirt1, AMPKα and survivin and the negative and positive controls were synthesized by Invitrogen. AICAR solution (250 mM in water) was purchased from Cell Signaling Technology. Wortmannin [a specific inhibitor of phosphatidylinositol 3-hydroxy kinase (PI3K)] was purchased from Axxora (San Diego, CA, USA). Z-LEHD-FMK (caspase 9 inhibitor) and Z-DEVD-FMK (caspase 3 inhibitor) were purchased from R&D Systems (Minneapolis, MN, USA). Among them, wortmannin, Z-LEHD-FMK and Z-DEVD-FMK were prepared in DMSO as stock solutions and were diluted when needed.

2.2. Cell culture

The cell line of murine 3T3-L1 preadipocyte was obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The passage numbers of the cells were from 5 to 20. Cells were cultured in DMEM with 10% FBS and supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C with 5% of CO_2.

2.3. Cell viability assay

A seeding density of 4000 cells/well was used for cell culture in 96-well plate, and cells were cultured with 100 μl DMEM overnight before Res treatment. Cells were incubated with either DMSO or the appropriate agents. The final concentration of solvent was less than 0.1% in cell culture medium. Ten microliters of WST-1 solution was added to each well. Cells were incubated for 3 h at 37°C and then measured spectrophotometrically in a microplate reader (Bio-Rad, USA) at 490 nm, which was proportional to the number of live cells. Inhibition (%) was expressed as the percentage of cell growth compared with the control.

2.4. LDH release assay

LDH release was determined using a CytoTox 96 nonradioactive cytotoxicity assay kit. Cells (2×10^4 cells per well) were plated in 24-well plate with 500 μl of DMEM overnight before Res treatment. After different doses of Res treatment for 24, 48, 72 or 96 h, the medium from each well was collected to measure the amount of released LDH. Cells in adjacent wells were exposed to lysis buffer (2% Triton-X100), and the medium was collected to measure the amount of total cellular LDH. The amount of LDH from each sample was measured at 490 nm by the microplate reader. The percentage of released LDH vs. total cellular LDH was calculated as the LDH leaking ratio.

2.5. Hoechst 33258 staining

Cells (1×10^5 cells per well) were plated in six-well plate with 2 ml DMEM overnight before Res treatment. After different doses of Res treatment for 48 h, the medium was removed, and cells were rinsed once with cold PBS and then fixed with 4% formaldehyde in PBS for 15 min at 37°C. The cells were washed twice with PBS, and then the nuclei were stained with Hoechst 33258 (10 μg/ml) for 5 min before being washed twice with PBS and dried.

2.6. Electron microscopy

Cells were incubated with different doses of Res for 48 h and examined by transmission electron microscopy. After being washed, the cells were fixed in 2.5% glutaraldehyde, dehydrated in graded alcohols and embedded in epoxy resin. The sections were stained with uranyl acetate and lead citrate and then viewed in the electron microscope (Philips, the Netherlands).

2.7. Apoptosis assay

Cell apoptosis was determined by flow cytometry. In brief, 1×10^5 cells were plated in six-well plate overnight and exposed to the appropriate agents. Cells were harvested, washed twice with cold PBS and resuspended in binding buffer to reach 1×10^5 cells/ml. A suspension of 100 μl was taken and incubated with 5 μl of Annexin V and 5 μl of 7-amino-actinomycin D (7-AAD) in the dark for 20 min at room temperature. Finally, 400 μl of binding buffer was added to each sample, and the stained cells were analyzed by flow cytometry in 1 h. The percentages of distribution of normal (Annexin V−/7-AAD−), early apoptotic (Annexin V+/7-AAD−), late apoptotic (Annexin V +/7-AAD+) and necrotic cells (Annexin V−/7-AAD+) were calculated by Cell Quest software.

2.8. Cell cycle arrest assay

Cell cycle was assayed according to the manual instructions of the detection kit. Briefly, cells were exposed to different doses of Res for 24 h. Adherent cells were trypsinized, washed and then resuspended in 200 μl PBS. Cells were fixed by the addition of 2 ml ice-cold 70% ethanol while vortexing vigorously and incubated at 4°C for 2 h. Cells were pelleted (600*g* for 10 min), resuspended in 800 μl PBS and then treated with RNase (room temperature, 20 min) and proteinase (room temperature, 20

min). Propidium iodine (PI) was added to reach the final concentration of 2 μg/ml on ice for 30 min. The DNA contents of cells were analyzed using the Becton Dickinson FACScan and Cell Quest software. Subsequent data analysis was conducted using Modfit LT software.

2.9. MMP detection

MMP was determined using the MitoScreen Detection Kit. Cells were seeded in six-well plate overnight and treated with the appropriate agents for 6 h. Cells were washed with 2 ml PBS, digested with 0.25% trypsin and transferred to Eppendorf tubes. After centrifugation (600*g* for 5 min), cells were incubated with 10 μg/ml JC-1 at 37°C for 15 min in a humidified incubator with 5% CO_2. The cells were washed with assay buffer, resuspended and analyzed with flow cytometry.

2.10. Western blot analysis

After various treatments for indicated time, cells were lysed in 0.2 ml ice-cold lysis buffer [20 mM Tris-HCl, pH=7.4, 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 mM NaF, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride]. Cytoplasmic extracts for the detection of Cyt C (Cyto) were prepared by using Cytoplasm Extract Kit (Beyotime, China). The proteins were separated on 12% SDS polyacrylamide gels and transferred electrophoretically (Bio-Rad) onto polyvinylidene fluoride membranes (Millipore, USA). Blots were blocked for

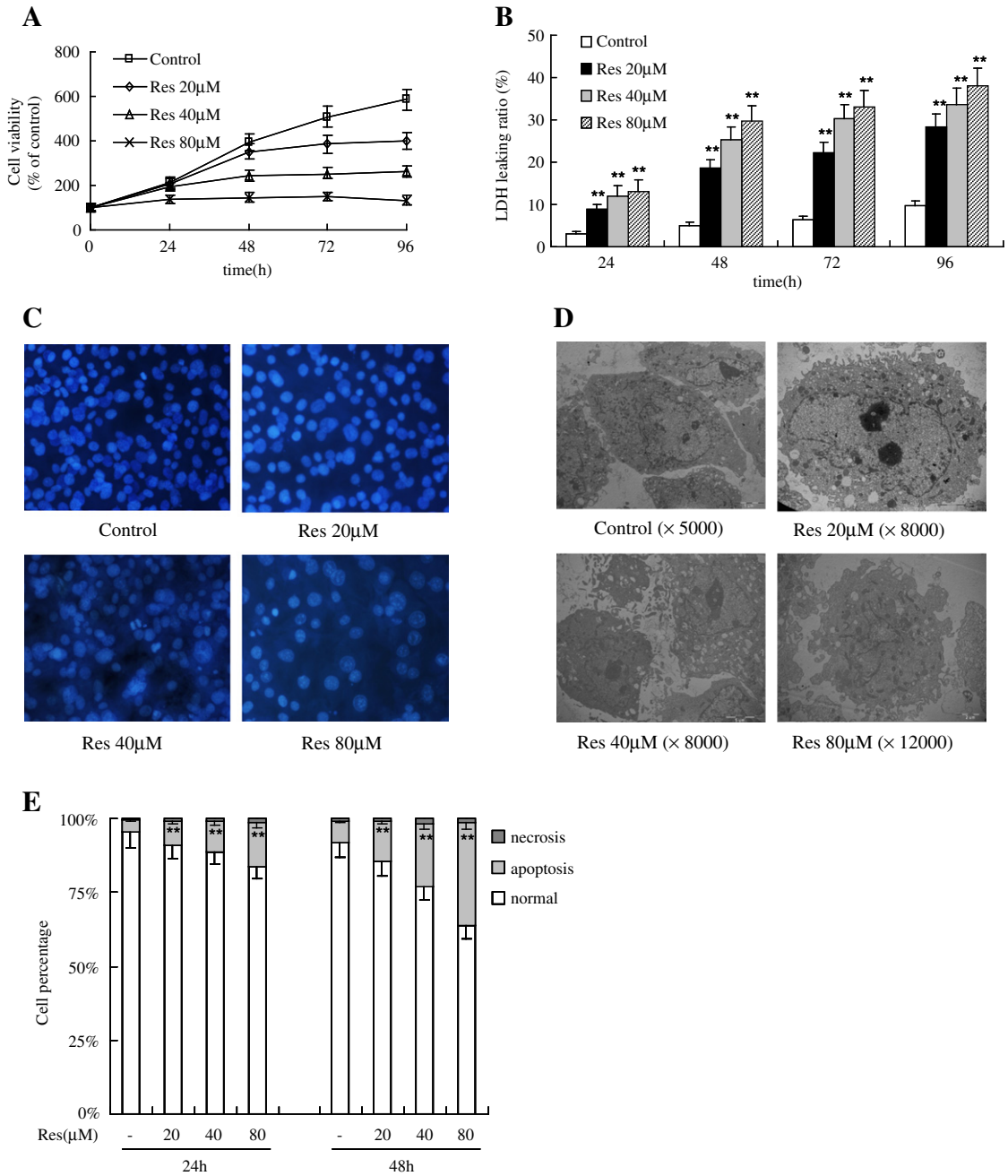


Fig. 1. Res inhibits cell proliferation and promotes cell apoptosis. (A) Cell proliferation. 3T3-L1 preadipocytes were treated with different doses of Res for 24, 48, 72 and 96 h. Cell viability was determined by the WST-1 colorimetric assay. The curve represents the cell viability at different time points from three independent experiments; error bar is the S.D., $n=9$. (B) LDH release assay. The percentage of released LDH vs. the total intracellular LDH was calculated as the LDH leaking ratio. $^{**}P<.01$, vs. respective control. $n=6$. (C) Cell nucleus morphological transformation. Cells were incubated with different doses of Res for 48 h. Chromatin condensation was visualized by Hoechst 33258. Magnification, ×200. (D) Transmission electron microscopy. Cells were incubated with Res for 48 h. The magnification is showed in the figure. (E) Percentage of apoptotic cells. Cells were treated with different doses of Res for 24 or 48 h and then stained by Annexin V and 7-AAD. The necrotic, apoptotic and normal cells were assayed with flow cytometry. $^{**}P<.01$, vs. respective control. $n=6$.

2 h in blocking buffer (5% nonfat dry milk in PBST [PBS+0.05% Tween-20] buffer) and incubated with primary antibodies (1:1000 dilution) overnight at 4°C and with GAPDH antibody (1:1000 dilution) as a internal control. After being washed with PBST, the blots were incubated with appropriate HRP-conjugated secondary antibodies (1:5000) at 37°C for 1 h and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia, UK).

2.11. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 20 min at 37°C and permeabilized with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 15 min. After blocking with 3% BSA for 30 min, cells were incubated with primary antibodies, rabbit anti-Sirt1 antibody (1:1000) or rabbit anti-survivin antibody (1:1000), followed by secondary antibody incubation (marked with green fluorescence, 1:500). The cells were then stained with DAPI (0.5 mg/ml, dissolved in PBS) and observed under a confocal fluorescence microscope (Carl Zeiss, Germany).

2.12. siRNA transfection and Res treatment

Cells were transfected with siRNA oligos using Lipofectamine2000 transfection reagent (Invitrogen), following the manufacturer's manual. The cells were 50% confluent during transfection. The lipofectamine/siRNA complexes were prepared in Opti-MEMI serum-free media and added drop-wise to the cells. Each siRNA transfection was done in triplicate with negative and positive controls (Invitrogen). The siRNA sequences were 5′-CCA AAC UUU GUU GUA ACC CUG UAA A-3′ (Sirt1), 5′-ACC GAG CUA UGA AGC AGC UGG GUU U-3′ (AMPKα) and 5′-AAG GAC CAC CGC AUC UCU ACA-3′ (survivin). Twenty-four hours after transfection, cells were incubated with different concentrations of Res for the indicated times and processed using detection kits.

2.13. Cells treated with AICAR or specific inhibitors and Res

Cells were trypsinized and adjusted to the appropriate cell density (5×10^4/ml), plated to six-well plate and cultured overnight. AICAR (the activator of AMPK), wortmannin (a specific inhibitor of PI3K), Z-LEHD-FMK (an inhibitor of caspase 9) or Z-DEVD-FMK (an inhibitor of caspase 3) was added with 40 μM Res to treat the cells for 48 h.

2.14. Statistical analysis

Data were expressed as mean±standard deviation, and SPSS 11.0 software was used for statistical analysis. Analyses were performed using analysis of variance with Bonferroni's test. The significance level was set at $P<.05$ or $P<.01$. Each experiment was repeated for three times.

3. Results

3.1. Res inhibits cell proliferation and promotes apoptosis

We first examined the effects of Res on the viability of 3T3-L1 preadipocytes. Cells were treated with different concentrations (0, 20, 40, 80 μM) of Res for 24, 48, 72 and 96 h. After the treatments, the number of live cells was determined by WST-1 assay. As shown in Fig. 1A, Res reduced cell viability in a time- and dose-dependent manner, indicating an inhibition of population growth or a promotion of cell apoptosis by Res treatment. To confirm this observation, LDH

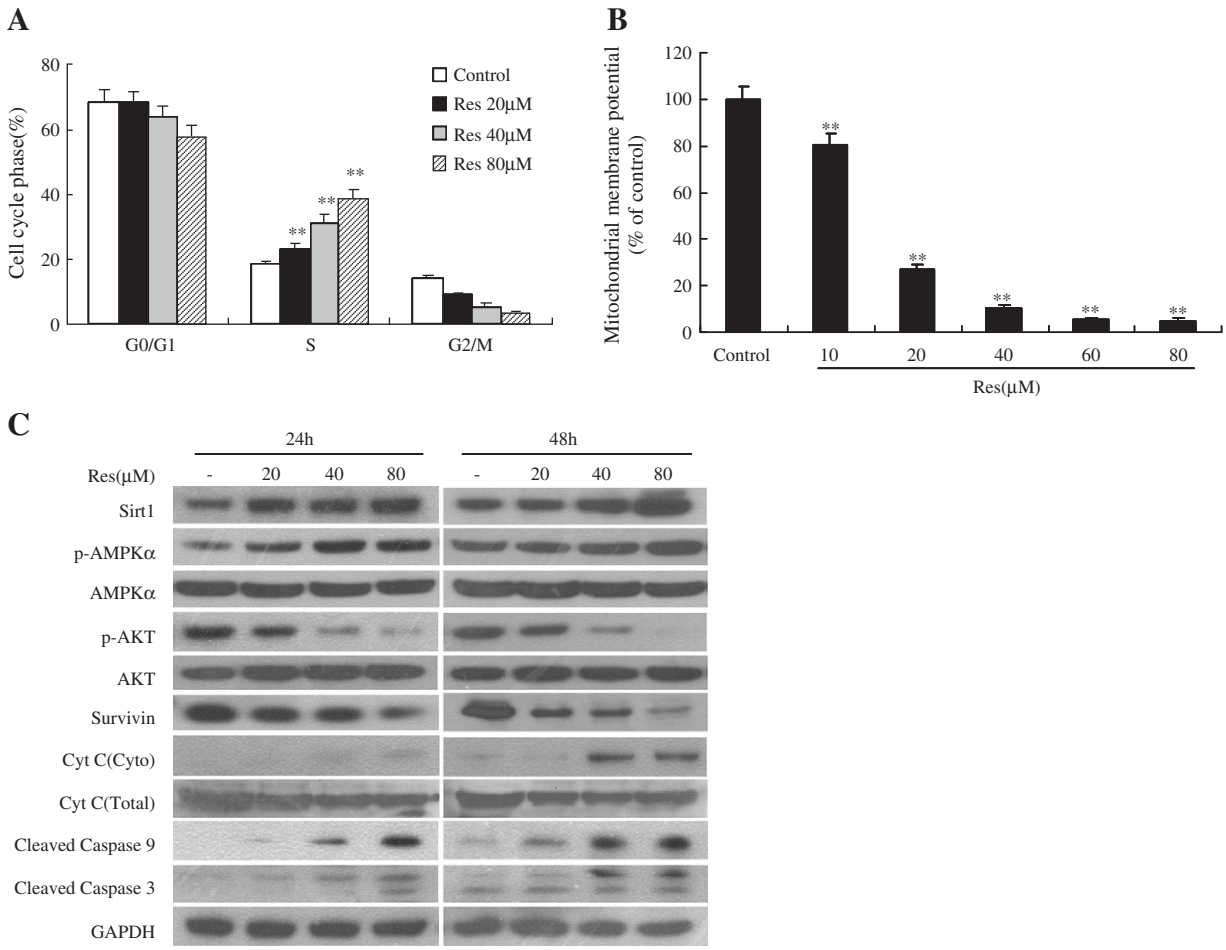


Fig. 2. Effects of Res on cell cycle, MMP and protein expression. (A) Res mediates S phase arrest in a dose-dependent manner. Cells were treated with different doses of Res for 24 h and stained with PI, then analyzed by flow cytometry. **$P<.01$, vs. control. $n=6$. (B) Res down-regulates MMP. Cells were treated with Res for 6 h and stained with JC-1, then analyzed by flow cytometry. **$P<.01$, vs. control. $n=6$. (C) Res changes protein expression in 3T3-L1 preadipocytes. Cells were incubated with Res for 24 and 48 h. Protein levels of Sirt1, p-AMPKα (Thr172), AMPKα, p-AKT (Ser473), AKT, survivin, Cyt C, cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

release assay was used to detect cell apoptosis. As shown in Fig. 1B, 24-, 48-, 72- or 96-h treatment of Res all significantly increased the LDH leaking ratio compared to the respective controls ($P<.01$). The apoptotic cells showed signs of chromatin condensation. The presence of typical apoptotic nuclei was demonstrated by Hoechst 33258. The nuclei appeared to be slightly smaller and fragmented and had a brighter fluorescence than the control (Fig. 1C). Transmission electron microscopy was used to determine whether the treated cells had ultrastructural changes consistent with the apoptosis. As shown in Fig. 1D, the apoptotic cells had a small, condensed and

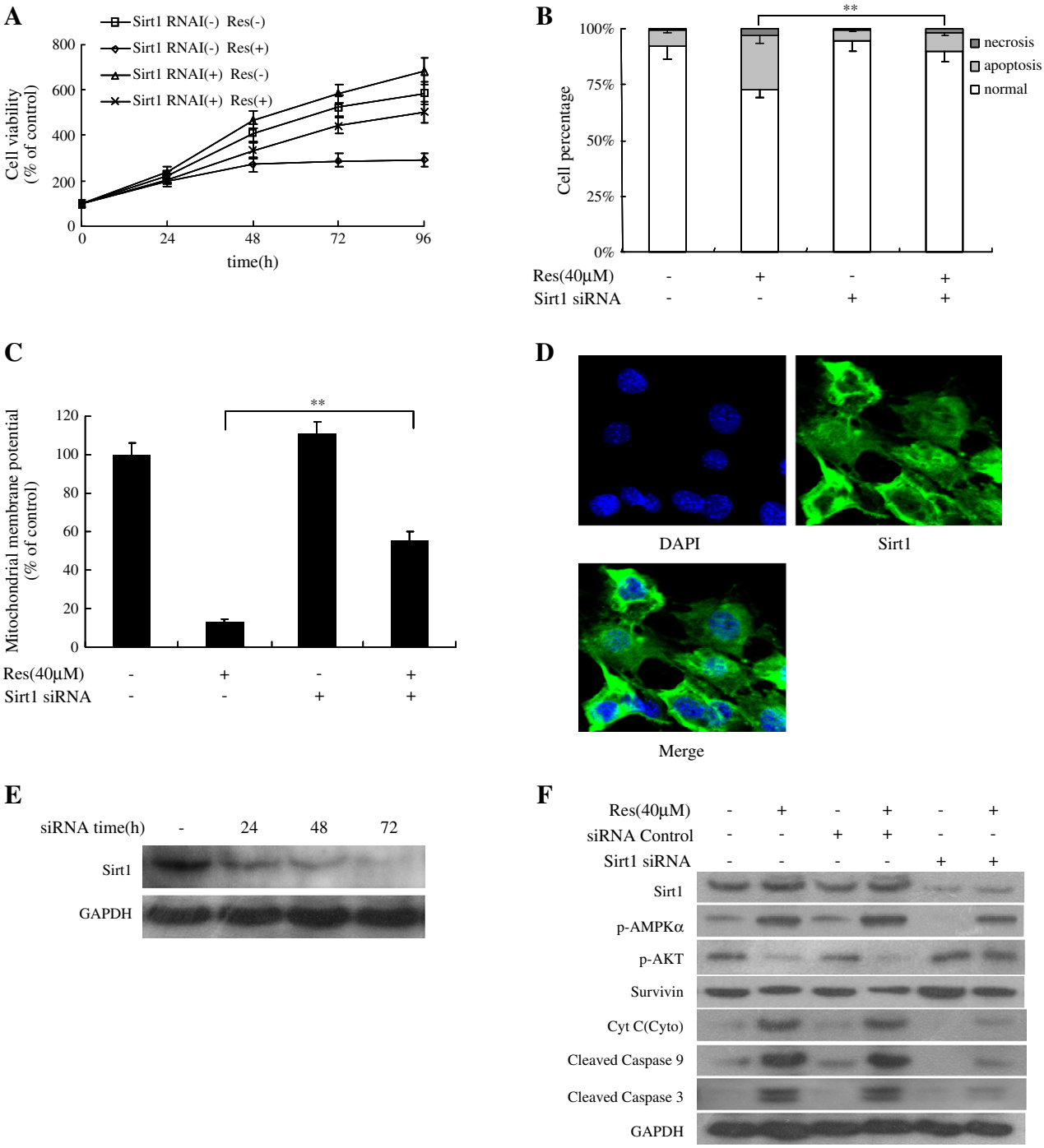


Fig. 3. Res induces cell apoptosis by activating Sirt1. (A) Effect of Sirt1 siRNA on cell proliferation. Cells were transfected with Sirt1 siRNA for 24 h and then treated with vehicle or 40 μM Res for 24, 48, 72 and 96 h. Cell viability was determined by the WST-1 colorimetric assay. (B) Effects of Sirt1 siRNA on cell apoptosis. Cells were transfected with Sirt1 siRNA for 24 h and then treated with 40 μM Res for 48 h. The necrotic, apoptotic and normal cells were assayed with flow cytometry. **$P<.01$, vs. control. $n=6$. (C) Effects of Sirt1 siRNA on MMP. Cells were transfected with Sirt1 siRNA for 24 h and then treated with 40 μM Res for 6 h. **$P<.01$, vs. control. $n=6$. (D) The localization of Sirt1 in 3T3-L1 preadipocytes. Cells were stained with anti-Sirt1 antibody (green fluorescence), and the nuclei were stained with DAPI (blue fluorescence). Magnification, ×800. (E) The time-dependent effects of Sirt1 siRNA on Sirt1 expression. Cells were treated with Sirt1 siRNA for 24, 48 or 72 h, and protein levels of Sirt1 and GAPDH were detected by Western blot. (F) Effects of Sirt1 siRNA on downstream protein expression. Cells were incubated with Sirt1 siRNA for 24 h and then treated with 40 μM Res for 48 h. Protein levels of Sirt1, p-AMPKα (Thr172), p-AKT (Ser473), survivin, Cyt C (Cyto), cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

approximately round-shaped appearance. The nuclei were irregular with patches of condensed nuclear chromatin. A large number of vacuoles and some mitochondria and organelle breakdown were observed in cytoplasm (80 μM Res). To detect the number of apoptotic cells, the treated cells were stained by Annexin V and 7-AAD, and the necrotic, apoptotic or normal cells were assayed with flow cytometry. As shown in Fig. 1E, Res treatment decreased the number of normal cells in a time- and dose-dependent manner. The numbers of both early and late apoptotic cells were significantly increased in a time- and dose-dependent manner ($P<.01$). The percentage of apoptotic cells reached 15.1% or 34.9% with Res treatment (80 μM) for 24 or 48 h, compared to the control's 4.2% or 7.2%.

We next investigated the effects of Res on cell cycle and MMP by fluorescence activating cell sorter analysis. As shown in Fig. 2A, treatment of Res for 24 h resulted in an accumulation of S-phase cells in a dose-dependent manner ($P<.01$). It may be caused by changed checkpoint in S-phase that slowed down the cell cycle progression and promoted the apoptosis (Fig. 1E). Fig. 2B showed the reduction of fluorescence intensity with the increased concentrations of Res, indicating the collapse of MMP after the exposure to Res for 6 h. Therefore, Res could significantly decrease MMP before cell apoptosis ($P<.01$). Western blot was used to detect the changes in protein activity and expression with Res treatment. A time- and dose-dependent increase in Sirt1 expression level was observed with Res treatment. The constitutively phosphorylated AMPKα (Thr172) was increased and the constitutively phosphorylated AKT (Ser473) and the survivin protein expressions were suppressed with Res treatment in a time- and dose-dependent manner. However, the levels of total proteins of AMPKα and AKT did not change, which indicates that Res-induced apoptosis is mediated by the phosphorylation of AMPKα or AKT. Res treatment also increased the protein levels of Cyt C (Cyto), cleaved caspase 9 and cleaved caspase 3, without changes in the total Cyt C protein (Fig. 2C).

3.2. Res induces cell apoptosis by up-regulating protein level of Sirt1

To elucidate the functions of Sirt1 in apoptosis of 3T3-L1 preadipocytes, cells were transfected with Sirt1 siRNA for 24 h and then treated with 40 μM Res. Fig. 3A showed that pretreatment of Sirt1 siRNA increased cell viability time dependently compared to the control. In addition, the transfection of Sirt1 siRNA significantly decreased the number of apoptotic cells ($P<.01$, Fig. 3B), which indicates that Sirt1 mediates Res-induced apoptosis in preadipocytes. Fig. 3C showed that Sirt1 siRNA significantly increased MMP in Res-treated cells ($P<.01$). Immunofluorescence assay was used to locate Sirt1 protein. As shown in Fig. 3D, Sirt1 is localized in the cytoplasm and nucleus in 3T3-L1 preadipocytes. Western blot was used to detect the activity and level changes of apoptosis-related proteins in Sirt1 siRNA cells. Fig. 3E showed that the transfection of Sirt1 siRNA suppressed protein level of Sirt1 time dependently. As shown in Fig. 3F, the Sirt1 siRNA cells had partially suppressed phosphorylation of AMPKα (Thr172), increased phosphorylation of AKT (Ser473) and increased protein level of survivin. Furthermore, the protein levels of Cyt C (Cyto), cleaved caspase 9 and cleaved caspase 3 were also largely suppressed. Therefore, Sirt1 may up-regulate the activity of AMPKα and down-regulate the activity of AKT, which may play a critical role in Res-induced cell apoptosis.

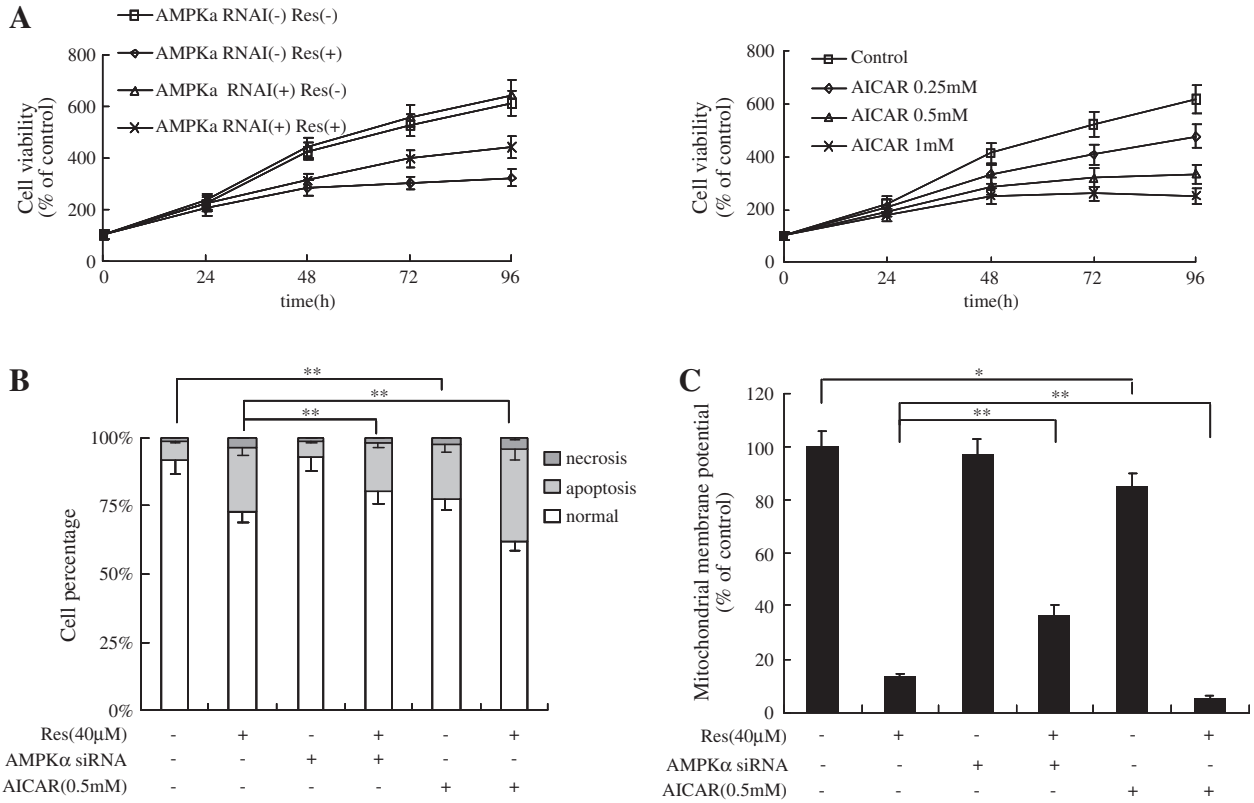
3.3. Phosphorylation of AMPKα mediates Res-induced apoptosis

To determine whether increased phosphorylation of AMPKα contributes to Res-induced apoptosis, the cells were given two types of treatments: AMPK agonist (AICAR) with Res for 48 h and siRNA transfection for 24 h then Res treatment. As shown in Fig. 4A, pretreatment of AMPKα siRNA increased cell viability time dependently (left), and AICAR itself reduced cell viability both time and dose dependently (right). Pretreatment of AMPKα siRNA also decreased the number of apoptotic cells ($P<.01$); AICAR treatment (0.5mM), however, increased the number of apoptotic cells ($P<.01$) (Fig. 4B). These results indicate that activation of AMPKα at least partially mediates the Res-induced apoptosis. Fig. 4C showed that AMPKα siRNA significantly increased the MMP in Res-treated cells ($P<.01$), while AICAR decreased the MMP with or without Res treatment ($P<.05$ or $P<.01$). We then used Western blot assay to detect the protein activity and level changes related to apoptosis with AMPKα siRNA or AICAR treatment. AMPKα siRNA suppressed the phosphorylation and the level of AMPKα protein in a time-dependent manner (Fig. 4D, upper). Pretreatment of AMPKα siRNA before Res treatment partially suppressed the protein levels of Cyt C (Cyto), cleaved caspase 9 and cleaved caspase 3. However, the phosphorylation of AKT (Ser473) and the protein levels of Sirt1 and survivin were not affected (Fig. 4D, lower). In a dose-dependent manner, AICAR treatment activated AMPKα (Thr172) and increased the protein levels of Cyt C (Cyto), cleaved caspase 9 and cleaved caspase 3, while the protein levels of AMPKα, Sirt1 and survivin did not change. Interestingly, AICAR treatment not only increased the phosphorylation of AKT (Ser473) dose dependently, but also induced cell apoptosis. We speculated that cellular stress promoted the phosphorylation of AKT in AICAR-induced apoptosis (Fig. 4E). Fig. 4F showed similar changes of protein levels with the treatment of AICAR and Res. All of the results above suggest that the activation of AMPKα up-regulates the apoptosis-related proteins (Cyt C, caspase 9, caspase 3) and triggers apoptosis in preadipocytes.

3.4. Inhibition of phospho-AKT suppresses survivin expression and mediates Res-induced apoptosis

Based on the observation that AKT (Ser473) phosphorylation was inhibited in Res-induced apoptosis, we hypothesized that the activation of AKT function as a brake to slow down apoptosis in 3T3-L1 preadipocytes and inhibition of AKT activation sensitizes cells to apoptosis. Cells were treated with the PI3K inhibitor wortmannin with or without Res. As shown in Fig. 5A, wortmannin (5 μM) treatment decreased cell viability time dependently. After 48 h of wortmannin (5 μM) treatment, the number of apoptotic cells was increased significantly ($P<.01$). Fig. 5C showed that wortmannin significantly decreased the MMP with or without Res treatment ($P<.01$). Forty-eight-hour treatment of wortmannin suppressed survivin expression via the inhibition of phospho-AKT dose dependently. Consequently, the apoptosis-related proteins (Cyt C, caspase

Fig. 4. Phosphorylation of AMPKα mediates apoptosis induced by Res. (A) Effects of AMPKα siRNA or AICAR on cell proliferation. Cells were transfected with AMPKα siRNA for 24 h and then treated with 40 μM Res (left) or incubated with different doses of AICAR (right) for 24, 48, 72 and 96 h. Cell viability was determined by the WST-1 colorimetric assay. (B) Effects of AMPKα siRNA or AICAR on cell apoptosis. Cells were transfected with AMPKα siRNA for 24 h and then treated with 40 μM Res for 48 h or incubated with 0.5 mM AICAR and 40 μM Res for 48 h. Cells were assayed with flow cytometry. $^{**}P<.01$, vs. respective control. $n=6$. (C) Effects of AMPKα siRNA or AICAR on MMP. Cells were transfected with AMPKα siRNA for 24 h and then treated with 40 μM Res or 0.5 mM AICAR for 6 h. $^{*}P<.05$, $^{**}P<.01$, vs. control. $n=6$. The time-dependent effects of AMPKα siRNA on the expression of AMPKα and p-AMPKα (D, upper). Cells were transfected with AMPKα siRNA for 24, 48 or 72 h, and protein levels of AMPKα, p-AMPKα (Thr172) and GAPDH were detected by Western blot. Cells were transfected with AMPKα siRNA for 24 h and then treated with 40 μM Res for 48 h (D, lower), incubated with different doses of AICAR for 48 h (E), or treated with 0.5 mM AICAR and 40 μM Res for 48 h (F). Protein levels of Sirt1, p-AMPKα (Thr172), p-AKT (Ser473), survivin, Cyt C (Cyto), cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.


A
AMPKa RNAI(-) Res(-)
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Cell viability (% of control)
time(h)
Control
AICAR 0.25mM
AICAR 0.5mM
AICAR 1mM
B
Cell percentage
necrosis
apoptosis
normal
Res(40μM)
AMPKα siRNA
AICAR(0.5mM)
C
Mitochondrial membrane potential (% of control)
Res(40μM)
AMPKα siRNA
AICAR(0.5mM)
D
siRNA time(h)
p-AMPKα
AMPKα
GAPDH
Res(40μM)
siRNA Control
AMPKα siRNA
Sirt1
p-AMPKα
p-AKT
Survivin
Cyt C(Cyto)
Cleaved Caspase 9
Cleaved Caspase 3
GAPDH
E
AICAR(mM)
Sirt1
p-AMPKα
AMPKα
p-AKT
Survivin
Cyt C(Cyto)
Cleaved Caspase 9
Cleaved Caspase 3
GAPDH
F
Res(40μM)
AICAR(0.5mM)
Sirt1
p-AMPKα
p-AKT
Survivin
Cyt C(Cyto)
Cleaved Caspase 9
Cleaved Caspase 3
GAPDH

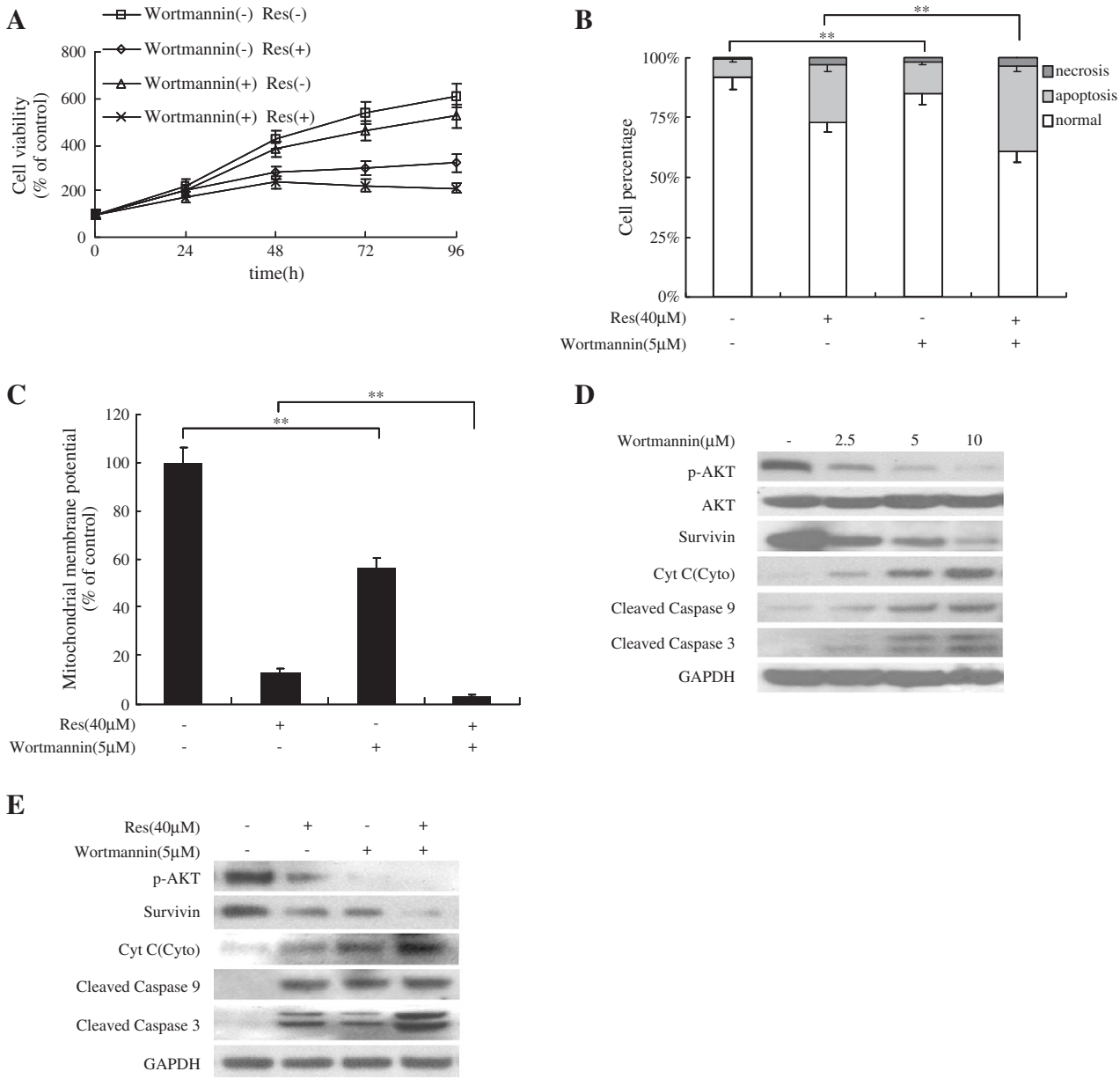

Fig. 5. Phosphorylation of AKT mediates apoptosis induced by Res. (A) Effects of wortmannin on cell proliferation. Cells were incubated with 5 μM wortmannin and 40 μM Res for 24, 48, 72 and 96h. Cell viability was determined by the WST-1 colorimetric assay. (B) Effects of wortmannin on cell apoptosis. Cells were treated with 5 μM wortmannin and 40 μM Res for 48 h, and the necrotic, apoptotic and normal cells were assayed with flow cytometry. $^{**}P<.01$, vs. respective control. $n=6$. (C) Effects of wortmannin on MMP. Cells were incubated with 5 μM wortmannin and 40 μM Res for 6 h. $^{**}P<.01$, vs. control. $n=6$. Cells were incubated with different doses of wortmannin (D) or 5 μM wortmannin and 40 μM Res (E) for 48 h. Protein levels of p-AKT (Ser473), survivin, Cyt C (Cyto), cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

9, caspase 3) were increased with wortmannin treatment (Fig. 5D). Similar protein level changes were observed with Res combination treatment (Fig. 5E). These results indicate that phospho-AKT plays a critical role in cell apoptosis.

To determine whether Res-induced apoptosis is mediated through the inhibition of survivin expression, cells were pretreated with survivin siRNA for 24 h before the Res treatment. Fig. 6A showed that pretreatment of survivin siRNA decreased cell viability in a time-dependent manner with or without Res treatment. In addition, survivin siRNA significantly increased the number of apoptotic cells ($P<.01$, Fig. 6B). Fig. 6C showed that survivin siRNA significantly decreased the MMP with or without Res treatment ($P<.05$ or $P<.01$). Immunofluorescence assay was used to locate the survivin protein. As shown in Fig. 6D, survivin was localized in the cytoplasm in 3T3-L1 preadipocytes. Fig. 6E showed that the transfection of survivin siRNA suppressed the protein level of survivin time dependently. As shown in Fig. 6F, survivin siRNA increased the protein levels of Cyt C (Cyto), cleaved caspase 9 and cleaved caspase 3. These findings suggest that Res-induced apoptosis, at least in part, is through the regulation of survivin expression. The AKT-survivin pathway may mediate Res-induced apoptosis in preadipocytes.

3.5. Caspase 9 and caspase 3 mediate Res-induced apoptosis

The observation of Cyt C release from mitochondria and caspase activation in Res-induced apoptosis prompted us to determine whether the inhibition of caspase activity affects cell apoptosis. Cells were incubated with 40 μM Res and caspase 9 inhibitor (20 μM

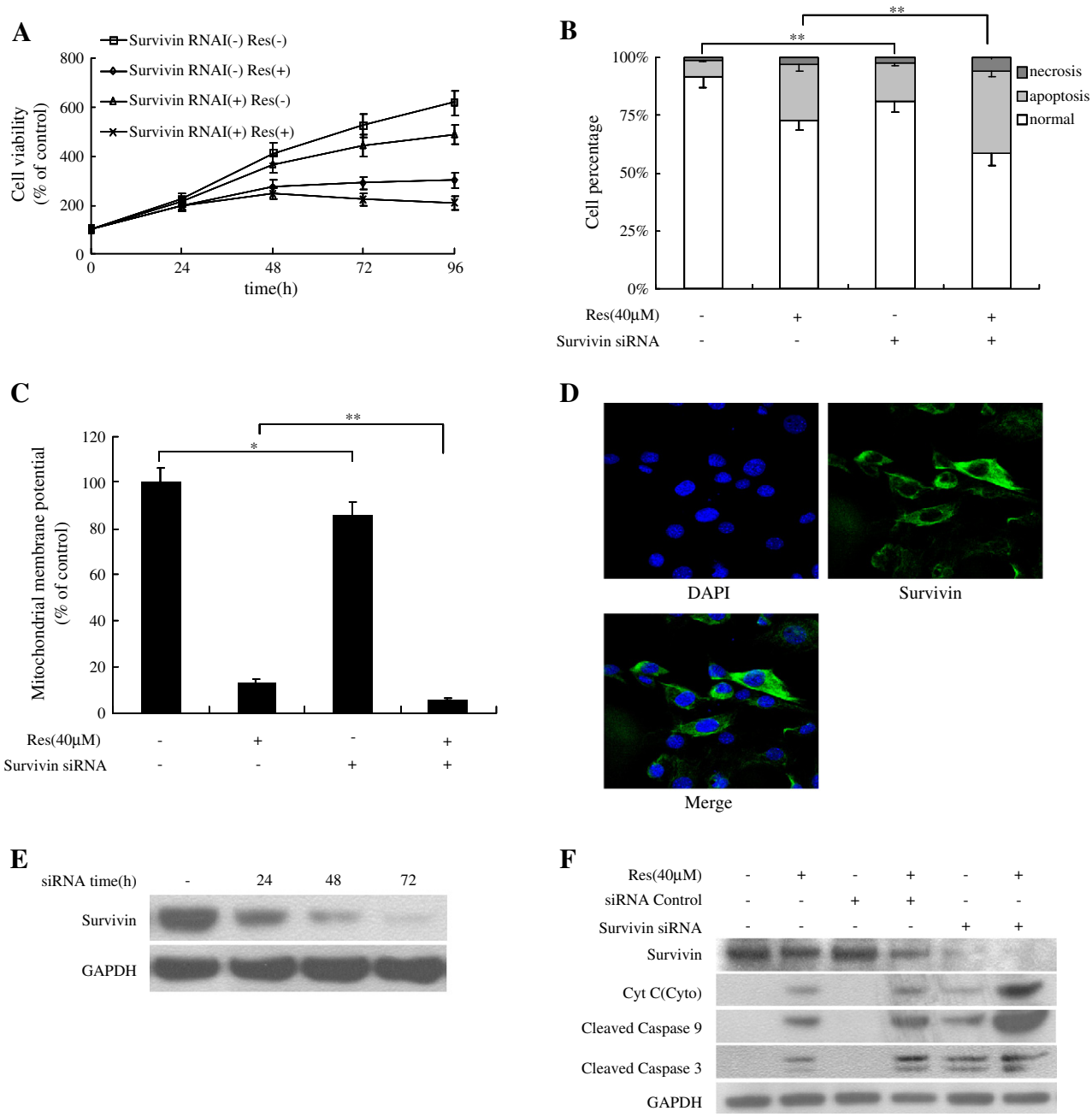

Fig. 6. Survivin mediates apoptosis induced by Res. (A) Effects of survivin siRNA on cell proliferation. Cells were transfected with survivin siRNA for 24 h and then treated with 40 μM Res for 2h, 48, 72 and 96 h. Cell viability was determined by the WST-1 colorimetric assay. (B) Effects of survivin siRNA on cell apoptosis. Cells were transfected with survivin siRNA for 24 h and then treated with 40 μM Res for 48 h. Cells were assayed with flow cytometry. $^{**}P<.01$, vs. respective control. $n=6$. (C) Effects of survivin siRNA on MMP. Cells were incubated with survivin siRNA for 24 h and then treated with 40 μM Res for 6 h. $^{*}P<.05$, $^{**}P<.01$, vs. control. $n=6$. (D) The localization of survivin in 3T3-L1 preadipocytes. Cells were stained with anti-survivin antibody (green fluorescence), and the nuclei were stained with DAPI (blue fluorescence). Magnification, ×400. (E) The time-dependent effects of survivin siRNA on survivin expression. Cells were transfected with survivin siRNA for 24, 48 or 72 h, and protein levels of survivin and GAPDH were detected by Western blot. (F) Effects of survivin siRNA on protein expression. Cells were transfected with survivin siRNA for 24 h and then treated with 40 μM Res for 48 h. Protein levels of survivin, Cyt C (Cyto), cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

Z-LEHD-FMK) or caspase 3 inhibitor (20 μM Z-DEVD-FMK). Fig. 7A showed that the treatment of inhibitors and Res significantly increased cell viability in a time-dependent manner. Cells were treated with Res and inhibitors for 48 h. As shown in Fig. 7B, Z-LEHD-FMK or Z-DEVD-FMK treatment significantly decreased the number of apoptotic cells ($P<.01$). Fig. 7C showed that caspase 9 or caspase 3 inhibitor did not significantly affect the MMP with or without Res treatment ($P>0.05$). Fig. 7D showed that Z-LEHD-FMK treatment mostly suppressed the production of cleaved caspase 9 and partially inhibited the production of cleaved caspase 3. However, Z-DEVD-FMK treatment suppressed the production of cleaved caspase 3, but cleaved caspase 9 was unaffected. These results indicate that inhibition of caspases activity attenuates Res-induced cell apoptosis.

4. Discussion

The polyphenolic phytoalexin Res has been proved to inhibit cell proliferation and promote cell apoptosis in many different cancer cell

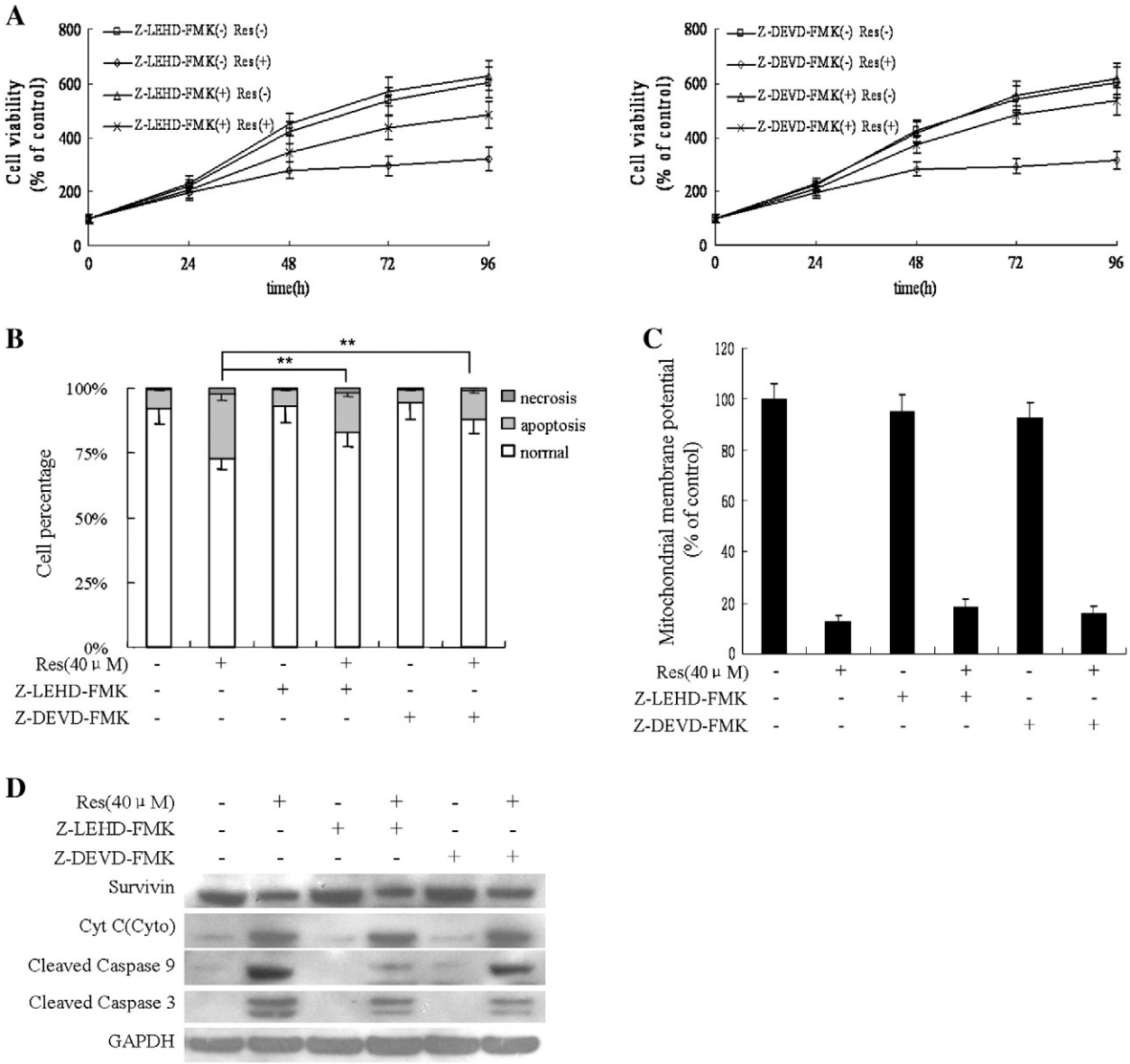

Fig. 7. Caspase 9 and caspase 3 mediate apoptosis induced by Res. (A) Effects of specific inhibitors on cell proliferation. Cells were incubated with 40μM Res and caspase 9 inhibitor (20 μM Z-LEHD-FMK, left) or caspase 3 inhibitor (20 μM Z-DEVD-FMK, right) for 24, 48, 72 and 96 h. Cell viability was determined by the WST-1 colorimetric assay. (B) Effects of specific inhibitors on cell apoptosis. Cells were incubated with specific inhibitors and 40 μM Res for 48 h. Cells were assayed with flow cytometry. $^{**}P<.01$, vs. control. $n=6$. (C) Effects of specific inhibitors on MMP. Cells were incubated with specific inhibitors together with 40 μM Res for 6 h. $P>0.05$, vs. control. $n=6$. (D) Effects of specific inhibitors on protein expression. Cells were incubated with specific inhibitors together with 40 μM Res for 48 h. Protein levels of survivin, Cyt C (Cyto), cleaved caspase 9, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

lines, such as HL60 leukemia cells, T47D breast carcinoma cells and Yoshida AH-130 rat hepatoma cells [22]. In human adipocytes, Res was reported to inhibit preadipocyte proliferation and adipogenic differentiation in a Sirt1-dependent manner and to stimulate the basal and insulin-stimulated glucose transport to attenuate insulin resistance [23,24]. Furthermore, Res can prevent obesity by down-regulating adipogenic and inflammatory processes or by promoting fat mobilization [23,25] . In this study, we found that Res treatment reduced cell viability both time and dose dependently. Res treatment also significantly increased the LDH leaking ratio, which indicated that the cell membrane integrity was injured. Furthermore, Hoechst 33258 staining and transmission electron microscopy revealed the ultrastructural changes in nuclear chromatins, which were consistent with the apoptosis. Flow cytometry assay showed that Res treatment increased the number of apoptotic cells in a time- and dose-dependent manner. The inhibition of cell proliferation and induction of cell apoptosis may be caused by cell cycle arrest, such as G0/G1-phase arrest or S-phase arrest [26]. Our results indicate that Res treatment could affect the checkpoints in S-phase (when the DNA synthesis occurs), which would result in decelerated cell cycle progression. Mitochondrial membrane potential event was detected and indicated the initiation of cellular apoptosis. We found that Res treatment increased the loss of mitochondria membrane potential in a dose-dependent manner. These results suggest that Res has novel effects on the proliferation and apoptosis of murine 3T3-L1 preadipocytes.

Sirt1, a mammalian homolog of Sir2, functions as a nicotinamide adenine nucleotide (NAD+)-dependent histone deacetylase. It deacetylates the lysine residues of both histone proteins and some apoptosis-inducible nuclear proteins, such as p53, forkhead family

proteins, NF-κB and FOXO family proteins, to promote cell survival in tumors [27,28]. Sirt1 is localized in nuclei in most cancer cells. In response to apoptotic stimuli, p53 and FOXO3 are acetylated and activated to promote apoptosis, while Sirt1 deacetylates acetylated p53 and FOXO and prevents apoptosis [29]. However, Sirt1 is partially localized in cytoplasm in certain cell lines, and the cytoplasm-localized Sirt1 was associated with apoptosis leading to increased sensitivity to apoptosis. The apoptosis enhanced by cytoplasm-localized Sirt1 is dependent on caspases [30]. In this study, we found that Sirt1 is localized in both cytoplasm and nucleus in 3T3-L1 preadipocytes, and the protein level of Sirt1 was increased in Res-induced apoptotic cells. Pretreatment of Sirt1 siRNA increased cell viability and MMP and decreased the number of apoptotic cells. In addition, the protein levels of Cyt C (Cyto) and cleaved caspases (caspase 9 and caspase 3) were suppressed with Sirt1 siRNA, indicating that Sirt1-enhanced apoptosis may depend on the regulation of caspases activity. Recently, it was reported that Res sensitized human SGBS preadipocytes to death-receptor (TRAIL)-induced apoptosis. Silencing of Sirt1 did not interfere with Res- and TRAIL-induced apoptosis, as determined by analysis of DNA fragmentation. However, the treatment of Res and TRAIL increased the expression of cleaved caspase 8 [13]. These findings indicate another mechanism of Res-induced apoptosis in human preadipocytes, which means that the synergistic effects of Res and TRAIL on cell apoptosis are in a Sirt1-independent manner. The results seem to be contrary to our study and might be related to the concentration effects and differences in cell types or species. However, a recent study showed that Res caused cell apoptosis in porcine primary preadipocytes via activating the expression Sirt1 [31], and it was found that Sirt1 siRNA transfection blocked 1-methyl-4-phenylpyridinium ion (MPP^+)-induced apoptosis in SH-SY5Y cells [32].

AMPK is a eukaryotic heterotrimeric serine/threonine kinase and a central sensor of cellular energy. AMPK activation has been shown to induce apoptosis in human gastric cancer cells, lung cancer cells, prostate cancer cells, pancreatic cells and hepatic carcinoma cells [33,34]. It can also enhance the oxidative-stress-induced apoptosis in mouse neuroblastoma cells [35]. In a recent study, a combinatorial treatment of Res and etoposide induced apoptosis in etoposide-resistant colon cancer cells by activating AMPK signaling cascade [36]. In this study, we identified a novel proapoptotic function of AMPK in preadipocytes. We found that pretreatment of AMPKα siRNA attenuated Res-induced apoptosis, while AICAR showed opposite effects. Moreover, we observed that pretreatment of Sirt1 siRNA inhibited the robust activation of AMPKα, suggesting that the effects of Res on the activation of AMPK were dependent on Sirt1. This is consistent with the discovery that Res mediates tumor cell proliferation and mammalian target of rapamycin (mTOR) protein translation via Sirt1-dependent AMPK activation [37]. However, its mechanism requires further investigation.

AKT belongs to a family of serine/threonine protein kinases that are activated by different stimuli, including growth factor stimulation, stress or protein phosphatase inhibitors, in a PI3K-dependent manner [38]. Previous studies suggest that AKT plays a critical role in tumorigenesis. AKT is amplified and overexpressed in gastric adenoma, ductal breast carcinoma and ovarian cancer [18]. Furthermore, the activity of AKT is elevated in lung, breast and prostate cancers [39,40]. It has been reported that Res inhibits skin tumorigenesis through PI3K and AKT proteins that are implicated in cancer development and progression [41]. In 3T3-L1 preadipocytes, we found that Res time and dose dependently inhibited the constitutively activated AKT, and the inhibition of PI3K by wortmannin sensitized cells to Res-induced apoptosis. It suggests that Res inhibits cell growth and induced apoptosis at least partially through the down-regulation of AKT signaling. To know whether Sirt1 and AKT interact, we treated cells with Sirt1 siRNA and found that AKT activity was significantly increased, suggesting that the effects of Res on AKT depend on the expression of Sirt1. A previous study showed that nicotinamide inhibits Sirt1 activity through AKT activation and the downstream modulation of MMP, and Cyt C release to prevent genomic DNA degradation during anoxia in neuronal cells [42], which is consistent with our results. A recent study demonstrated that AMPK activation by AICAR resulted in growth arrest in S-phase by inhibiting PI3K-AKT pathway in cancer cells [43]. However, our results showed that AICAR treatment increased the phosphorylation of AKT dose dependently, although it did induce apoptosis. We speculated that cellular stresses activated AKT during the AICAR-stimulated apoptosis. A previous study also found that AKT was activated with AICAR treatment in the childhood acute lymphoblastic leukemia cells, while AICAR itself caused cell apoptosis and inhibition of the PI3K-AKT pathway enhanced the cytotoxic activity of AICAR [44].

Survivin is one of the major regulators of cell division and apoptosis. Both *in vitro* and *in vivo* studies have demonstrated that survivin inhibits cell death especially apoptosis and is up-regulated in the majority of cancers. In terminally differentiated normal tissues, however, survivin is barely detectable [19]. To our best knowledge, our study is the first one to confirm the expression of survivin in 3T3-L1 preadipocytes. Survivin is localized in cytoplasm, and the expression of survivin was inhibited in Res-treated preadipocytes. Pretreatment of survivin siRNA sensitized cells to apoptosis, indicating that survivin mediates Res-induced apoptosis. It was found that up-regulation of survivin follows the AKT activation. AKT was identified as a positive regulator of survivin expression in endothelial cells, and succedent studies claimed AKT/survivin signaling as an anti-apoptotic pathway in prostate cancer, lung cancer, myeloma and leukemia [45]. Indeed, when AKT activity was blocked by wortmannin, the expression of survivin was attenuated, which is consistent with previous investigation. It was found that inactivation of AMPK by compound C or AMPKα siRNA restored the activities of AKT and mTOR and the expression of survivin in deguelin-treated malignant human bronchial epithelial cells [46]. However, in our study, the treatment with AMPK siRNA or AICAR did not significantly affect the expression of survivin expression. Based on these results, we propose that Res inhibits survivin expression via AKT pathway but independent of AMPK activation.

Many proapoptotic signals engage the apoptotic machinery via the mitochondrion-mediated pathway to stimulate the release of Cyt C from mitochondria. In the cytosol, Cyt C promotes the oligomerization of apoptotic peptidase activating factor-1, leading to the recruitment and activation of caspase 9 in a large complex called the apoptosome. Once activated, caspase 9 cleaves and activates the downstream caspase 3 [47]. We observed increased Cyt C (Cyto) expression and caspases activation with Res treatment. The treatment with caspases inhibitor blocked Res-induced apoptosis, indicating an important role of caspase 9 and caspase 3 in this process. With the treatment of AICAR or survivin siRNA, the Cyt C (Cyto) expression and caspases activation were reduced. These findings also suggest that Res-induced AMPK activation and AKT inactivation (suppresses survivin expression) promote effective release of Cyt C from mitochondria, activation of caspases and eventually the apoptosis in 3T3-L1 preadipocytes.

In summary, our current findings suggest that Res could inhibit proliferation and induce apoptosis in murine 3T3-L1 preadipocytes. We thus propose that the Res-induced apoptosis and Res-inhibited proliferation are caused by the up-regulated Sirt1, which activates AMPK, inhibits AKT and suppresses survivin expression, and by the subsequently activated mitochondrion-mediated pathway including MMP descendent, Cyt C release and caspases activation (Fig. 8). This study firstly clarifies a novel mechanism of Res-induced apoptosis in 3T3-L1 preadipocytes, which might provide a new strategy to treat or prevent obesity and help overcome the metabolic stresses and symptoms.

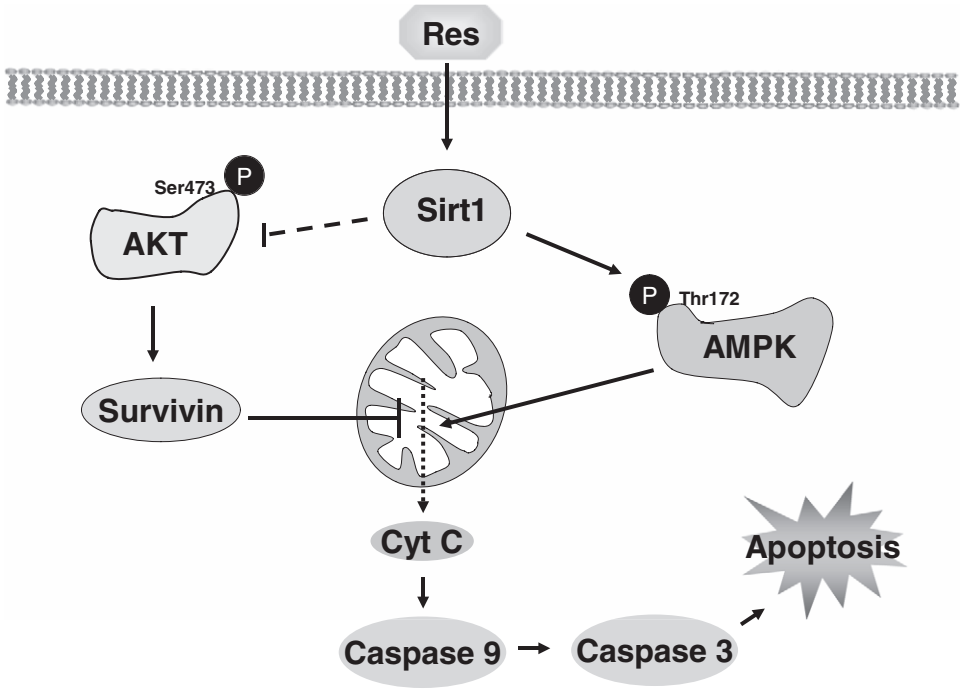

Fig. 8. Schematic illustration of the apoptotic signaling of 3T3-L1 preadipocytes induced by Res. Res induces cell apoptosis by increasing the expression of Sirt1, which might inhibit AKT activity and further decrease the expression of survivin. The Res-activated Sirt1 could also increase AMPK activity. Both AKT and AMPK pathways promote the release of Cyt C from mitochondria and the activation of caspase 9 and caspase 3, which would finally induce apoptosis in 3T3-L1 preadipocytes.

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