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Resveratrol induces cell apoptosis in adipocytes via AMPK activation

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ABSTRACT

Resveratrol is identified as polyphenolic compound with anti-inflammatory, antioxidant, anti-insulin resistance characteristics. Moreover, resveratrol exerts pro-apoptotic effects in varieties of cancer cell lines. However, effects and mechanisms of resveratrol on the regulation of adipocytes apoptosis remain largely unknown. In this study, we found that resveratrol treatment could induce cell apoptosis in murine 3T3-L1 adipocytes. Furthermore, resveratrol activated the mitochondrial apoptotic signaling pathway with the decrease in the mitochondrial membrane potential (MMP), and the activation of caspase 3. Mechanistically, we found that phosphorylation level of AMP-activated protein kinase α (AMPKα) was elevated, accompany with reduced level of phosphorylation of protein kinase B (AKT) when cells were exposed to resveratrol. By using small interfering RNAs of AMPKα and specific inhibitor for p-AKT, it was shown that activation of AMPKα could inhibit downstream of p-AKT, consequently activating mitochondrion-mediated apoptotic pathway. Additionally, we observed similar pro-apoptotic effects of Res on mouse primary adipocytes. Our findings clarified the apoptotic effects and underlying mechanisms of resveratrol in adipocytes, suggesting its potential therapeutic application in the treatment or prevention of obesity and related metabolic symptoms.

1. Introduction

Obesity is considered a major risk factor for metabolic diseases, such as type 2 diabetes, liver steatosis, hyperlipidemia, atherosclerosis, etc. Excess energy intake, and (or) lacking of physical activity, leading to excess triacylglycerols stored in adipocytes, eventually impair energy homeostasis [1]. The fat mass is determined by the size of fat cells and/or the number of adipocytes. Therefore, the strategy to reduce fat mass may involve the direct loss of lipids (through lipolysis), the inhibition of adipogenesis or the apoptosis of adipocytes. Among these, the reduction of adipocyte number, for example, via induction of cell apoptosis, becomes attractive and receives widespread attention as a promising way to attenuate obesity.

Resveratrol (Res) is one of the phytochemicals which is enriched in grapes, peanuts, wine, and other food sources [2]. Res exerts biological functions as a cancer chemopreventive and chemotherapeutic agent and has anti-inflammatory, antioxidant, and neuroprotective properties [3]. The anti-proliferative and apoptosis-inducing effects of Res cause cell cycle arrest and apoptosis in different cancer cell lines [4]. In addition, Res was found to improve dyslipidemia, hyperinsulinemia and hypertension in animal models [5–7]. Despite the beneficial effects of Res on maintenance of energy homeostasis, Res directly affects lipid synthesis in fat cells by regulating adipogenesis and cell apoptosis [8,9]. In the previous research, we found out Res could inhibit cell differentiation in 3T3-L1 adipocytes via activation of AMPK [10]. Moreover, we reported that Res could induce cell apoptosis in 3T3-L1 pre-adipocytes in SIRT1-dependent manner [11], suggesting the important role of Res involved in determination of fat cell fates. Up to date, it was reported that Res could induce cell apoptosis in 3T3-L1 adipocytes [8,9], however, the underlying mechanisms remain unclear.

AMPK is a serine/threonine protein kinase, which is activated by cellular stress when ATP is depleted. AMPK is also implicated in cancer development and is considered as a potential anti-tumor target molecule [12]. Interestingly, in the previous study, we showed Res could stimulate cell apoptosis in pre-adipocytes by activation of SIRT1, which consequently inhibited AKT activation and further decreased the expression of survivin, meanwhile,

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AMPK phosphorylation was also elevated. Therefore, both signaling cascades activated mitochondrion-mediated apoptotic pathway [11]. Nevertheless, the regulatory role of AMPK in regulation of cell fate in adipocytes remains still unknown.

In this study, we focus on the regulatory role of Res on cell apoptosis in murine adipocytes. We furthermore investigate the novel underlying mechanisms and clarify the critical role of AMPK implicated in regulation of these processes.

2. Materials and methods

2.1. Reagents

Res was purchased from Sigma–Aldrich (St Louis, MO, USA). Penicillin, streptomycin, Dulbecco modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). Primary antibodies against AMPKα, phospho-AMPKα (Thr172), AKT, phospho-AKT (Ser473), cleaved caspase 3 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated secondary antibody was purchased from Invitrogen (Grand Island, NY, USA). WST-1 was purchased from Roche (Basel, CH). The kit for lactate dehydrogenase (LDH) release assay was purchased from Promega (Madison, WI, USA). The detection kits for cell apoptosis and mitochondrial membrane potential were purchased from BD Biosciences (San Jose, CA, USA). The siRNA oligos of AMPKα were purchased by Invitrogen. AICAR was from Cell Signaling Technology. Wortmannin was purchased from Axxora (San Diego, CA, USA).

2.2. Cell culture

Murine 3T3-L1 pre-adipocyte was obtained from the Type Culture Collection of the Chinese Academy of Science (Shanghai, CHN). 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. Two days after cell confluence, adipocyte differentiation was induced with a mixture of IBMX (0.5 mmol/L), dexamethasone (1 μmol/L), and insulin (10 mg/L), in DMEM containing 10% FBS. After 48 h, the induction medium was replaced with DMEM containing only insulin (10 mg/L). Fresh culture medium was replaced after 2 d and, subsequently, every other day for 4 d or until >90% of cells attained adipocyte morphology. Mouse primary adipocytes were isolated according to the protocol described previously [13]. Cells were cultured in normal DMEM and applied for corresponding Res treatments.

2.3. LDH release assay

After Res treatment, the medium from each well was collected to measure the amount of released LDH. Cells in adjacent wells were subjected to lysis buffer (2% Triton X-100) and collected to measure total cellular LDH. The release assay was determined by using cytotoxicity assay kit. The amount of LDH from each sample was measured at 490 nm by microplate reader. The LDH leaking ratio was calculated as the percentage of released LDH vs. total amount.

2.4. Apoptosis assay and mitochondrial membrane potential (MMP) detection

Cell apoptosis was determined by flow cytometry analysis according to the instruction from the kit (BD Biosciences, USA). Basically, Res-treated cells were harvested and incubated with Annexin V and 7-amino-actinomycin D (7-AAD) and the stained cells were analyzed by flow cytometry. 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. MMP was determined using the kit according to the instruction from the BD biosciences company. After Res treatment, cells were harvested and incubated with JC-1. The cells were washed with assay buffer, resuspended and analyzed with flow cytometry.

2.5. Western blot analysis

After exposed to corresponding treatments, cells were lysed in proper volume of ice-cold lysis buffer (20 mM Tris-HCl, pH = 7.4, 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/ml leupeptin and 1 mM PMSF). The proteins were exacted and separated on 10% SDS-polyacrylamide gels and transferred electrophoretically (Bio Rad) onto PVDF membranes (Millipore, USA). Blots were blocked for 1 h in blocking buffer (5% non-fat dry milk in PBST buffer) and incubated with different primary antibodies (1:1000 dilution) overnight at 4 °C, and with GAPDH antibody (1:1000 dilution) as an internal control. After being washed with PBST for twice, 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).

2.6. siRNA transfection and Res treatment

Cells were transfected with siRNA oligo using Lipofectamine™ 2000 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 dropwise to the cells. The siRNA sequence was: 5′-ACC GAG CUA UGA AGC AGC UGG GUU U-3′ (AMPKα). 30 h after transfection, cells were incubated with different concentrations of Res for the indicated times and processed using detection kits.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation, and SPSS 13.0 software was used for statistical analysis. Analyses of variance were performed using ANOVA with Bonferroni's test. The significance level was set at $P < 0.05$ or $P < 0.01$. Each experiment was repeated for at least three times.

3. Results

3.1. Res promotes cell apoptosis in 3T3-L1 adipocytes

We first investigated the effect of Res on LDH leaking of 3T3-L1 adipocytes. After different concentrations of Res treatment (25, 50, 100 μM), as shown in Fig. 1A, the LDH leaking ratio was dose-dependently increased, suggesting Res may induce cell apoptosis. Moreover, as shown in Fig. 1B, compared with untreated group, the mitochondrial membrane potential (MMP) level was impaired in a dosage dependent manner. Finally, the flow cytometry assay showed that after Res treatment, the apoptotic cells ratio was increased significantly from 5.8% to 39.6% (Fig. 1C).

We detected the expression levels of proteins which might be involved in the regulation of apoptosis. As shown in Fig. 1D, after Res treatment, the phosphorylation level of AMPKα was elevated, while p-AKT showed opposite pattern. More importantly, the apoptosis marker, cleaved caspase 3 protein level was up-regulated,

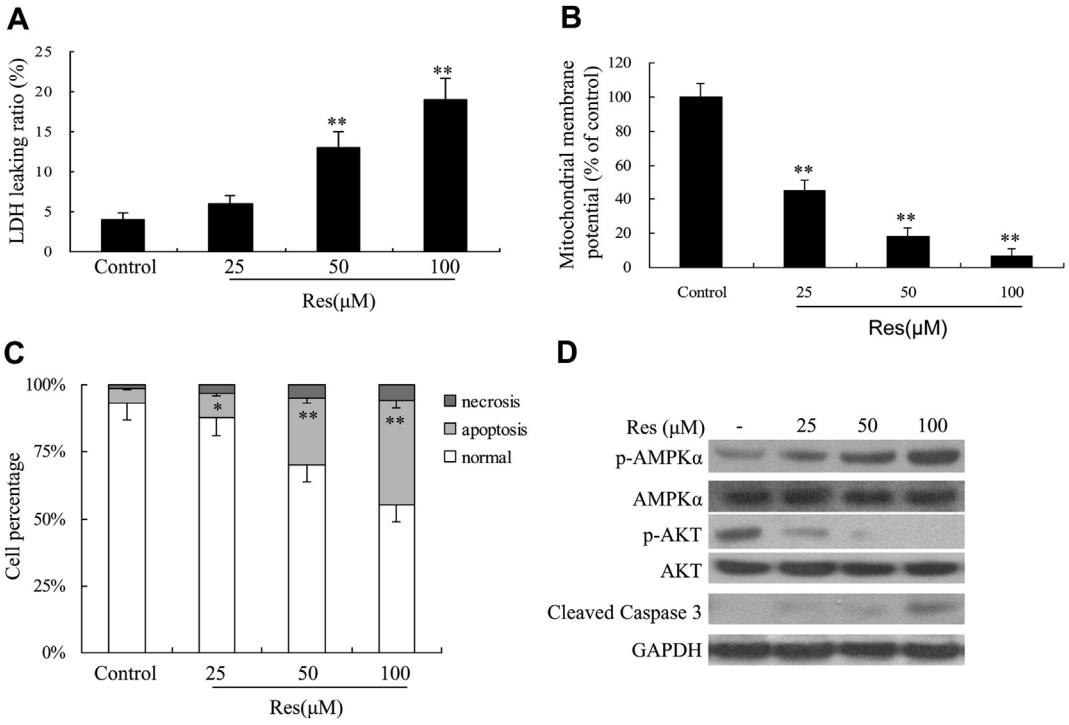

Fig. 1. Res induces cell apoptosis in 3T3-L1 adipocytes. (A) LDH release assay. Cells were treated with different dosages of Res for 24 h, the percentage of released LDH vs. the total intracellular LDH was calculated as the LDH leaking ratio. $^{**}P < 0.01$, vs. control. $n = 3$. (B) Mitochondrial membrane potential level. Cells were treated with Res for 12 h and stained with JC-1, then analyzed by flow cytometry. $^{**}P < 0.01$, vs. control. $n = 3$. (C) Percentage of apoptotic cells. Cells were treated with Res for 24 h and then stained by Annexin V and 7-AAD. The necrotic, apoptotic and normal cells were assayed with flow cytometry. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. control. $n = 3$. (D) Protein expression levels. Cells were incubated with Res for 24 h. Protein levels of p-AMPKα (Thr172), AMPKα, p-AKT (Ser473), AKT, cleaved caspase 3 and GAPDH (loading control) were detected by Western blot. Each panel represents at least three independent experiments.

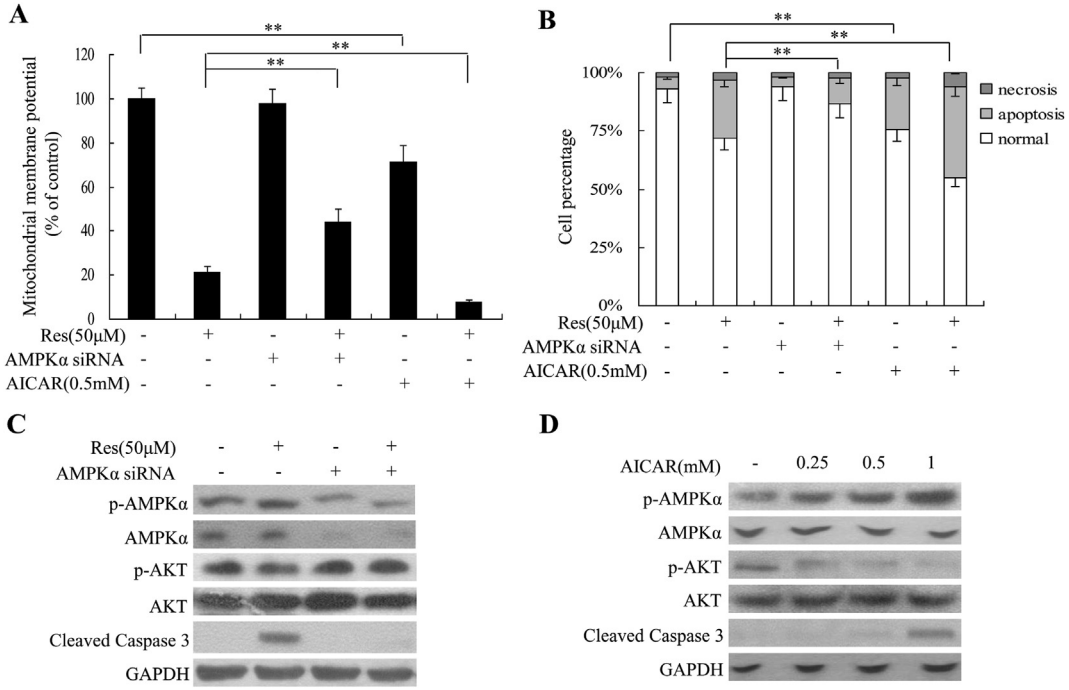

Fig. 2. Phosphorylation of AMPKα mediates apoptosis induced by Res. (A) Effect of AMPKα siRNA or AICAR on MMP level. Cells were transfected with AMPKα siRNA and then treated with 50 μM Res or 0.5 mM AICAR for 12 h $^{**}P < 0.01$, vs. control. $n = 3$. (B) Effect of AMPKα siRNA or AICAR on cell apoptosis. Cells were treated as described before for 24 h, and then assayed with flow cytometry. $^{**}P < 0.01$, vs. respective control. $n = 3$. Cells were transfected with AMPKα siRNA for 30 h and then treated with 50 μM Res for 24 h (C), or incubated with different doses of AICAR for 24 h (D). Protein levels of p-AMPKα (Thr172), AMPKα, p-AKT (Ser473), AKT, cleaved caspase 3 and GAPDH were detected by Western blot. Each picture represents at least three independent experiments.

suggesting Res caused cell apoptosis in 3T3-L1 cells via mitochondrial apoptotic pathway.

3.2. p-AMPKα mediates cell apoptosis induced by Res

As shown in Fig. 2A, cells were pre-treated with AMPKα siRNA, which could significantly rescue the reduced level of MMP caused by Res. However, addition of AICAR (activator of p-AMPKα), could synergistically reduce MMP level with Res treatment. In line with these observations, it was appreciable that AMPKα siRNA treatment reduced apoptotic cell number induced by Res, while AICAR showed opposite pattern (Fig. 2B).

Proteins expression levels were analyzed by Western blot. As shown in Fig. 2C and D, phosphorylation level of AMPKα was substantially reduced upon siRNA treatment, while AICAR could strongly stimulate p-AMPKα level. Importantly, after AMPKα siRNA treatment, p-AKT level was elevated, and cleaved caspase 3 was significantly diminished. In support of these data, AICAR treatment could reduce p-AKT level and increase cleaved caspase 3 protein level. Taken together, these data suggest that p-AMPKα mediates the apoptotic effect of Res on 3T3-L1 adipocytes, and probably AMPK is the upstream of AKT in signaling pathway involved in this process.

3.3. p-AKT mediates cell apoptosis induced by Res

To further clarify the role of AKT in regulation of apoptosis, we used the wortmannin (inhibitor of PI3K-AKT pathway) to treat adipocytes. As shown in Fig. 3A and B, wortmannin treatment further decreased MMP level while apoptotic cell number was significantly increased. Mechanistically, phosphorylation level of AKT was impaired after wortmannin treatment. Supportively, cleaved caspase 3 level was up-regulated (Fig. 3C).

3.4. Res induces cell apoptosis in mouse primary adipocytes

To further confirm the apoptotic effects and mechanisms of Res on adipocytes, we treated mouse primary adipocytes with different doses of Res. As shown in Fig. 4A, Res treatment could significantly increase LDH leaking. Consistently, apoptotic cell number was increased from 9.6% to 36.7% after exposed to 100 μM of Res (Fig. 4B). Moreover, p-AMPKα level was elevated while p-AKT level was reduced. Therefore, mitochondrial apoptotic pathway was activated and cleaved caspase 3 level was up-regulated (Fig. 4C). Taken together, these data suggest Res induces cell apoptosis in primary adipocytes as well.

4. Discussion

Res was reported to cause cell apoptosis in various kinds of cancer cell lines, such as leukemia cells, breast carcinoma cells and hepatoma cells [14,15]. Moreover, it has been reported that Res induces cell apoptosis in pre-adipocytes and mature adipocytes [8,9,12], although the mechanisms are poorly understood. In this study, we demonstrate Res induces cell apoptosis in 3T3-L1 adipocytes, as well as in murine primary adipocytes. After exposed to Res treatment, based on the reduced MMP level and the elevated cleaved caspase 3, we propose that Res promotes cell apoptosis in adipocytes via activating mitochondrial apoptotic pathway, which is consistent with the previous observation that Res could induce apoptosis in pre-adipocytes via SIRT1-dependent activation of mitochondrial signaling pathway [12].

We further clarify the novel regulatory role of AMPK in adipocytes apoptosis. AMPK activation has been widely shown to cause apoptosis in different kinds of cells, i.e., lung cancer cells, pancreatic cells, hepatic carcinoma cells, and endothelial cells [16–18]. Here we identify a novel pro-apoptotic function of AMPK in adipocytes. We discover that pretreatment of AMPKα siRNA attenuated Res-induced apoptosis, however, AICAR treatment exerted opposite effects, suggesting AMPK phosphorylation mediates adipocytes apoptosis. Moreover, our data showed p-AKT level was negatively regulated by AMPK. Given that PI3K-AKT signaling pathway is widely involved in regulation of cell proliferation, metabolism, and apoptosis [19,20]. It is reasonable to speculate that AMPK could regulate PI3K-AKT cascades for downstream events, however, whether AMPK could directly affect the phosphorylation level of

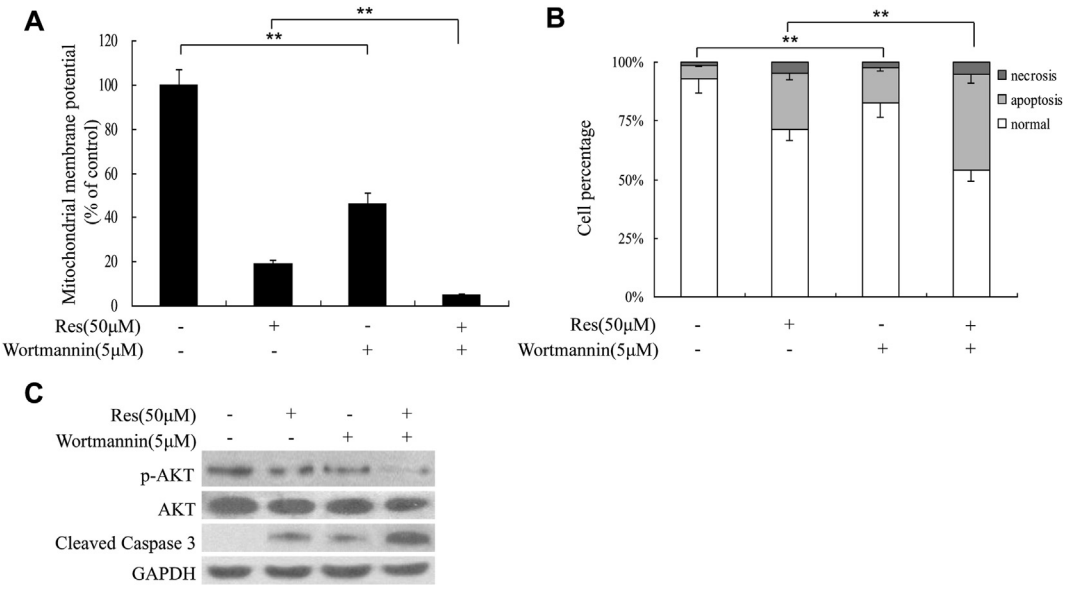

Fig. 3. Phosphorylation of AKT mediates apoptosis induced by Res. (A) Effect of wortmannin treatment on MMP level. Cells were incubated with 5 μM wortmannin and 50 μM Res for 12 h $^{**}P < 0.01$, vs. corresponding control. $n = 3$. (B) Effects of wortmannin on cell apoptosis. Cells were treated as described before for 24 h, and then assayed with flow cytometry. $^{**}P < 0.01$, vs. respective control. $n = 3$. (C) After cells were treated, cell lysates were analyzed by Western blot. Protein levels of p-AKT (Ser473), AKT, cleaved caspase 3 and GAPDH were detected. Each picture represents at least three independent experiments.

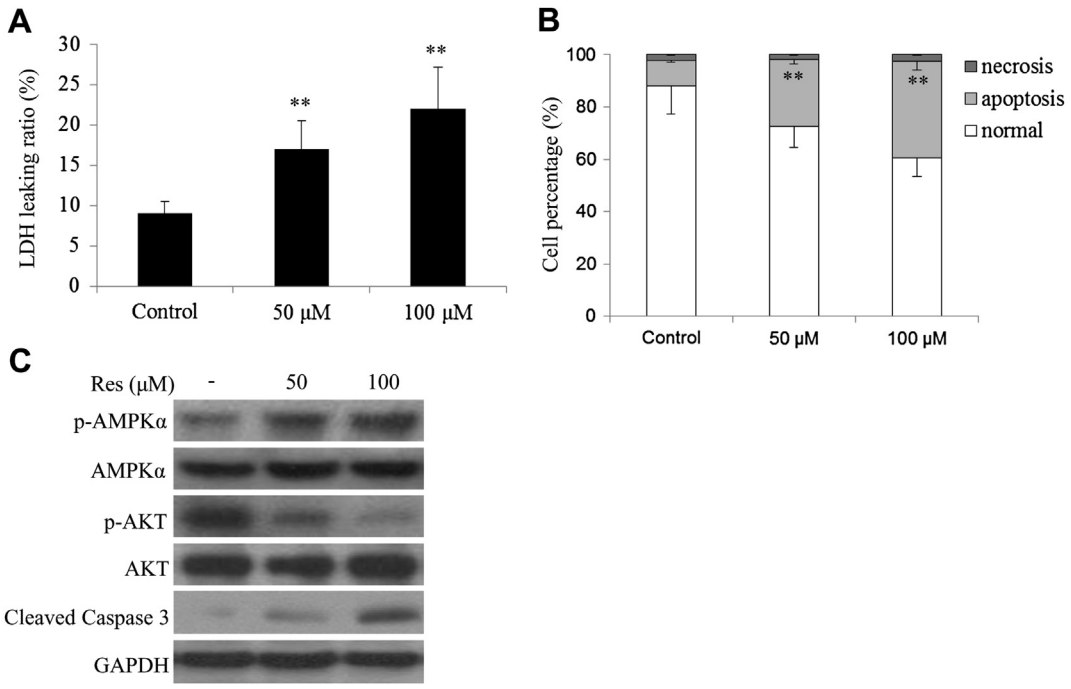

Fig. 4. Res induces cell apoptosis in mouse primary adipocytes. (A) LDH release assay. Mouse primary adipocytes were treated with Res (50, 100 μM) for 24 h, and LDH leaking ratio was calculated as described before. $^{**}P < 0.01$, vs. control. $n = 3$. (B) Percentage of apoptotic cells. Cells were treated with Res for 24 h and then stained by Annexin V and 7-AAD. The necrotic, apoptotic and normal cells were assayed with flow cytometry. $^{**}P < 0.01$, vs. control. $n = 3$. (C) Protein expression levels. Cells were incubated with Res for 24 h. Protein levels of p-AMPKα (Thr172), AMPKα, p-AKT (Ser473), AKT, cleaved caspase 3 and GAPDH were detected by Western blot. Each panel represents at least three independent experiments.

PI3K or AKT remains mysterious and requires further investigation. AKT, a family of serine/threonine protein kinases, is activated by different stimuli, such as growth factors, stress, or protein phosphatase inhibitors [21]. In 3T3-L1 adipocytes, we found Res inhibited the phosphorylation of AKT, and inhibition of PI3K by wortmannin treatment sensitized cells to Res-induced apoptosis, indicating that p-AKT is playing a central role of regulation apoptosis. Most interestingly, we showed similar phenotypes of Res on induction of cell apoptosis in primary adipocytes, furthermore, p-AMPK and p-AKT were up- or down-regulated accordingly, suggesting the similar regulatory roles of AMPK and AKT in this process.

In summary, our data suggest that Res could induce apoptosis in murine adipocytes. We propose that the Res-induced apoptosis is caused by activation of AMPK, sequential diminished of p-AKT, and consequent activated mitochondrion-mediated pathway including caspase 3 activation. This study firstly demonstrates a novel mechanism of Res-induced apoptosis in adipocytes, which would be probably beneficial for the application of Res to treat or prevent obesity and other metabolic diseases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Transparency document

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