

Resveratrol induces human keratinocyte damage via the activation of class III histone deacetylase, Sirt1

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Abstract. Human skin diseases are various and induce chronic inflammatory disorders, including psoriasis, atopic dermatitis and certain forms of ichthyosis. Psoriasis is a chronic inflammatory skin disease characterized by circumscribed, red, thickened plaques. Regulation of the balance between growth, differentiation and death is critical to keratinocytes; when altered, epidermal keratinocytes undergo hyperproliferation, abnormal differentiation and inflammatory infiltration. In the present study, we focused on the effects of resveratrol, found in red wine and peanuts, on the cell death of keratinocytes. We additionally studied the mechanism of resveratrol on Sirt1, a class III histone deacetylase, and Akt phosphorylation. Resveratrol caused apoptosis and increased Sirt1 expression in human HaCaT keratinocytes, following a decrease in the p62 protein level. Inhibition of Sirt1 by Sirt1 inhibitor restored cell viability and protein levels. Furthermore, we showed that resveratrol-induced Sirt1 blocked Akt phosphorylation. The present results indicated that resveratrol inhibited the Akt pathways by inducing Sirt1, thus leading to cell death. These data suggest that resveratrol-mediated activation of Sirt1 histone deacetylase may be a potential therapeutic target for skin diseases including psoriasis.

Introduction

Psoriasis is a common chronic skin disease that is characterized by hyperproliferation and disrupted differentiation of keratinocytes (1-3). Psoriasis leads to a decrease in apoptotic cell death in keratinocytes and an increased resistance of intra-lesional keratinocytes to apoptosis (4). We studied the inhibition of keratinocyte proliferation and the regulation of keratinocyte differentiation by induced apoptosis for the treatment of psoriasis.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a natural polyphenol, mainly found in grapes, berries and red wine (5). There are several studies on the bioactivities of resveratrol including anti-inflammatory, antioxidant, antimicrobial and neuroprotective effects (6).

Additionally, resveratrol has strong chemopreventive effects against skin, breast, prostate and lung tumors (7). The cancer preventive effects of resveratrol were demonstrated in the prevention of tumor growth in an animal model (8). Resveratrol is reportedly an activator of Sirt1 both *in vivo* and *in vitro* (9,10).

Silent information regulator 2 (Sir2) homolog 1 (Sirt1) is a member of the conserved family of NAD-dependent deacetylases, the sirtuin family, that function in enzymatic cleavage of NAD to the deacetylation of protein substrates. Sirt1 plays a critical role in the regulation of numerous cellular processes, including cell cycle progression, nutrient metabolism, cellular ageing (11) and cell apoptosis (12). Sirt1 has nuclear substrates such as p53, and the DNA repair proteins APE/Ref1 and PARP1 (13-15). Resveratrol was found to inhibit cell proliferation and differentiation by increasing the expression of Sirt1 mRNA (16).

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a downstream effector of phosphatidylinositol 3-kinase (PI3K), activated by several stimuli, such as growth factor stimulation, stress or protein phosphatase inhibitors, in a PI3K-dependent manner (17). AKT is involved in multiple cellular signaling pathways and functions as a transducer of many actions initiated by growth factors and other receptors that activate PI3K (18-20). One of the major roles of Akt is to regulate cell survival (18,21), including apoptosis and tumorigenesis as an oncogene (22). Recent studies have shown that Sirt1 inactivates Akt and suppresses survivin expression and the subsequently activated mitochondrion-mediated pathway, including MMP activity, cytochrome *c* release and caspase activation (23).

The novel finding of the present study was that resveratrol induced HaCaT keratinocyte cell death. We investigated the role of resveratrol on the apoptosis of human HaCaT keratinocytes and clarified the mechanisms. Resveratrol was associated with increased expression and activation of Sirt1, by enhancing the deacetylation of p53, which downregulated Akt phosphorylation. The results indicated that resveratrol-induced HaCaT keratinocyte cell death may be regulated by a Sirt1/phospho-Akt-dependent signaling pathway.

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Materials and methods

Cell culture. HaCaT, an immortalized, non-tumorigenic human keratinocyte cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics under standard conditions (37°C, 5% CO₂, in a humidified incubator). HaCaT cells were seeded in 60-mm culture dishes in standard medium, or in the presence of different concentrations of resveratrol (25 and 100 μM) for the proliferation studies. Triplicate dishes were trypsinized at appropriate intervals, and the cell number was determined by counting cell suspension in a Neubauer hemacytometer. The values reported represent the mean ± SEM of 3 independent samples per each experimental point.

Crystal violet assay. Cell viability was evaluated by crystal violet staining. Briefly, the cells were stained with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde) for 10 min at room temperature (RT) and washed 4 times with water. The stained cells were lysed with 1% sodium dodecyl sulfate (SDS), and the absorbance was measured at 550 nm. Cell viability was calculated based on the relative dye intensity compared with the controls.

Lactate dehydrogenase (LDH) assay. Cytotoxicity was assessed in the supernatants using the LDH cytotoxicity detection kit (Takara Bio, Tokyo, Japan) according to the manufacturer's protocol. LDH activity was determined by measuring the absorbance at 490 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Annexin V assay. Apoptosis was assessed in detached cells with an Annexin V assay kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer's protocol. Annexin V levels were determined by measuring fluorescence at a 488-nm excitation and a 525/30 emission using the Guava easyCyte HT System (Millipore, Bedford, MA, USA).

Western blot analysis. HaCaT cells were lysed in buffer [25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM DTT (dithiothreitol) and a protease inhibitor mixture at pH 7.4]. Proteins were electrophoretically resolved by 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubations with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. The antibodies used for immunoblotting were Sirt1 (Santa Cruz Biotechnology), phospho-Akt, acetyl-p53 (both from Epitomics, Burlingame, CA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). Images were examined using a Fusion-FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France).

Construction of recombinant adenoviruses. The Sirt1-overexpressing adenovirus (Ad-Sirt1) was provided by Professor Byung-Hyun Park of Chonbuk National University (Jeonju, Jeonbuk, Korea). The lacZ-bearing adenovirus (Ad-lacZ) was used as the control. Recombinant adenoviruses were amplified in human embryonic kidney (HEK)-293 cells

and purified using the Vivapure AdenoPACK kit (Sartorius AG, Göttingen, Germany) according to the manufacturer's instructions.

Statistical evaluation. All data are expressed as mean ± standard deviation and were compared by Student's t-test, analysis of variance and Duncan's test using SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). Results were considered significant at $p < 0.05$, $p < 0.001$ or $p < 0.01$, as appropriate.

Results

Resveratrol induces cell damage in HaCaT keratinocytes. We first examined the influence of resveratrol on the cell viability of human HaCaT cells to determine whether resveratrol induces cell death or promotes cell proliferation and cell survival. Cells were exposed to different concentrations (5, 10, 20 and 50 μM) of resveratrol for 12 h. After resveratrol treatment, based on the cell morphology and the number of cells by microscopy and crystal violet assay, we determined that resveratrol induced cell death (Fig. 1A and B). We next performed the LDH assay for detecting cell death. As shown in Fig. 1C, resveratrol treatment significantly increased LDH release. The Annexin V-positive cell population was also increased following resveratrol treatment, compared to that of the control (Fig. 1D). These data indicated that resveratrol induced HaCaT cell death.

Resveratrol induces cell death through the Sirt1 pathway. We examined the Sirt1 protein levels in the HaCaT keratinocytes treated with resveratrol to understand the molecular mechanism of resveratrol in human keratinocytes. Resveratrol increases the deacetylase activities of Sirt1. Hence, we investigated whether resveratrol induced cell death by increasing Sirt1. First, we evaluated Sirt1 expression following resveratrol treatment at concentrations of 5, 10, 20 and 50 μM. Sirt1 protein levels dose-dependently increased in the resveratrol-treated group as compared to the control group (Fig. 2A). We next determined whether resveratrol-induced Sirt1 had deacetylase activity by measuring the acetylated p53 protein levels. Acetyl-p53 protein levels were gradually decreased (Fig. 2A). Moreover, sirtinol, Sirt1 inhibitor, inhibited the resveratrol-induced increase in Sirt1 protein and decrease in acetyl-p53 protein (Fig. 2B).

Next, to determine whether resveratrol-induced Sirt1 activation is related to cell death, HaCaT keratinocytes were treated with resveratrol with or without sirtinol. Crystal violet assay showed that resveratrol induced cell death and sirtinol restored cell viability (Fig. 2C). Consistent with these results, resveratrol-treated cells exhibited increased LDH release, while treatment with sirtinol blocked the effects of resveratrol (Fig. 2D). The influence of sirtinol on resveratrol-induced apoptosis in HaCaT keratinocytes was determined by the Annexin V assay (Fig. 2E). The resveratrol-mediated increase in Annexin V-positive cells was decreased by treatment with sirtinol. These results demonstrated that resveratrol induced cell death via Sirt1.

Resveratrol-mediated cell death is associated with Akt phosphorylation. The effect of resveratrol on apoptotic proteins

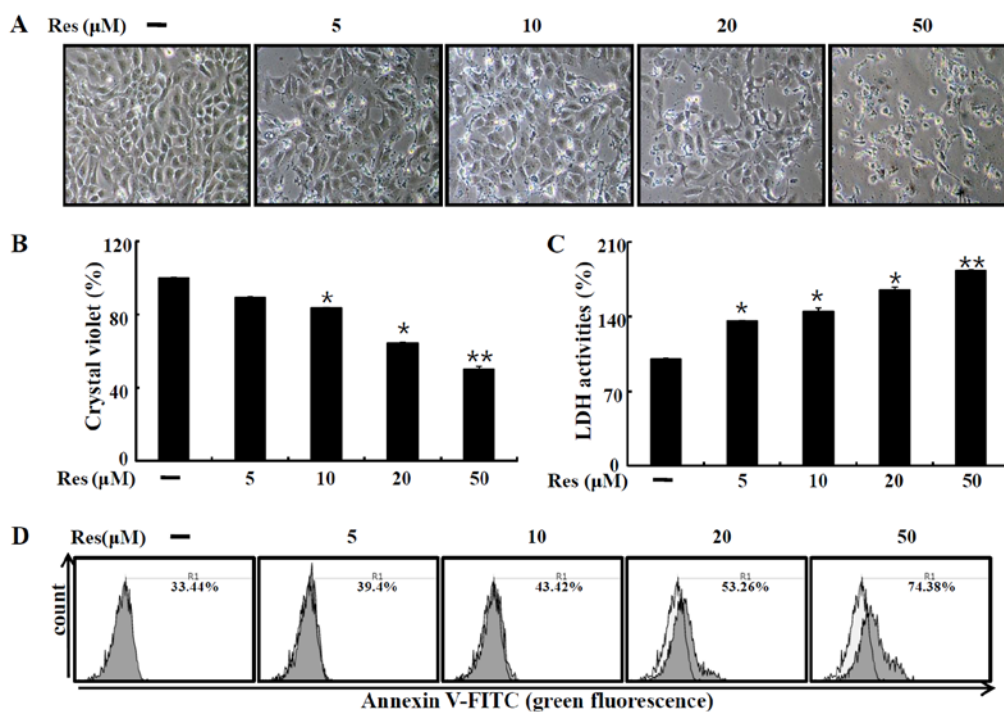


Figure 1. Resveratrol causes cell death in HaCaT keratinocytes. HaCaT keratinocytes were treated with resveratrol at increasing concentrations (5, 10, 20 and 50 μ M) for 12 h. (A) The treated cells were photographed using light microscopy. (B) Cell viability was measured by crystal violet staining. Viability of the control cells was set at 100%, and viability was measured relative to the control. Bar graph indicates the mean \pm SEM (n=3). (C) Lactate dehydrogenase (LDH) assay was used to quantify LDH release into the medium. Bar graph indicates the mean \pm SEM (n=3). *p<0.05, **p<0.01, significant differences between the control and each treatment group. (D) Cell viability was measured by the Annexin V assay. Res, resveratrol.

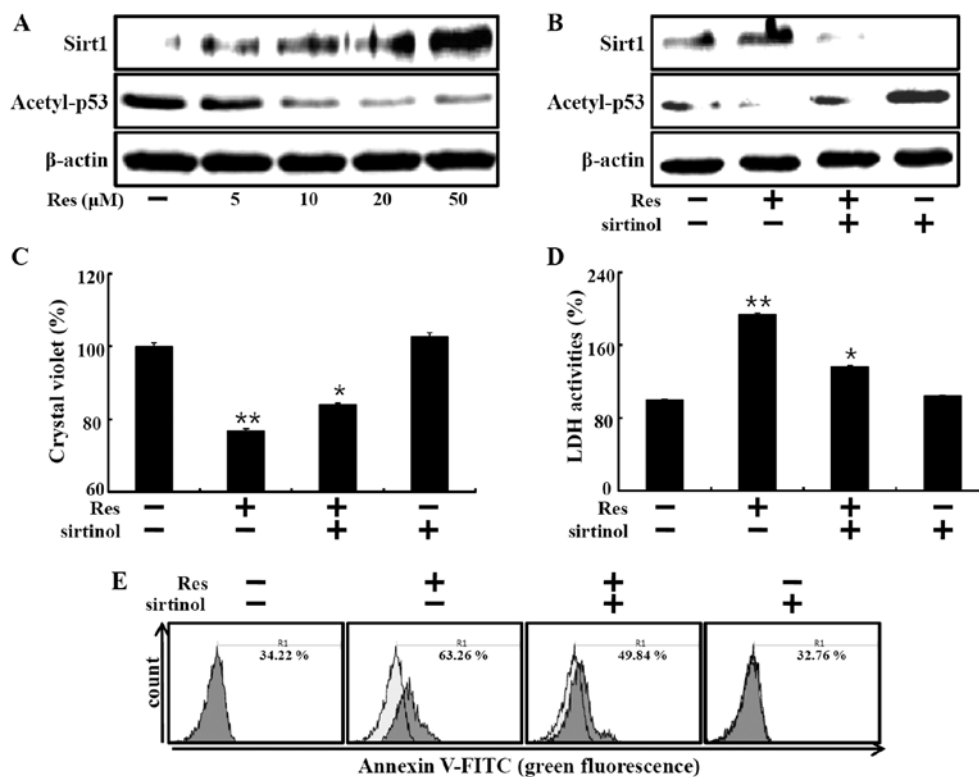


Figure 2. Resveratrol regulates Sirt1 and acetyl-p53 protein levels in HaCaT keratinocytes. (A) HaCaT keratinocytes were incubated with the indicated concentrations of resveratrol (5, 10, 20 and 50 μ M) for 12 h. The treated cells were assessed for Sirt1 and acetyl-p53 by western blot analysis. Results were normalized to β -actin. (B) HaCaT keratinocytes were treated with 50 μ M of resveratrol in the presence of Sirt1 inhibitor (sirtinol) for 12 h. The treated cells were assessed for Sirt1 and acetyl-p53 by western blot analysis. (C) Cell viability was measured by crystal violet staining. Viability of the control cells was set at 100% and viability relative to the control was measured. (D) LDH assay was used to quantify LDH release into the medium. Bar graphs indicate the mean \pm SEM (n=3). *p<0.05, **p<0.01, significant differences between the control and each treatment group. (E) Cell viability was measured by the Annexin V assay. Res, resveratrol.

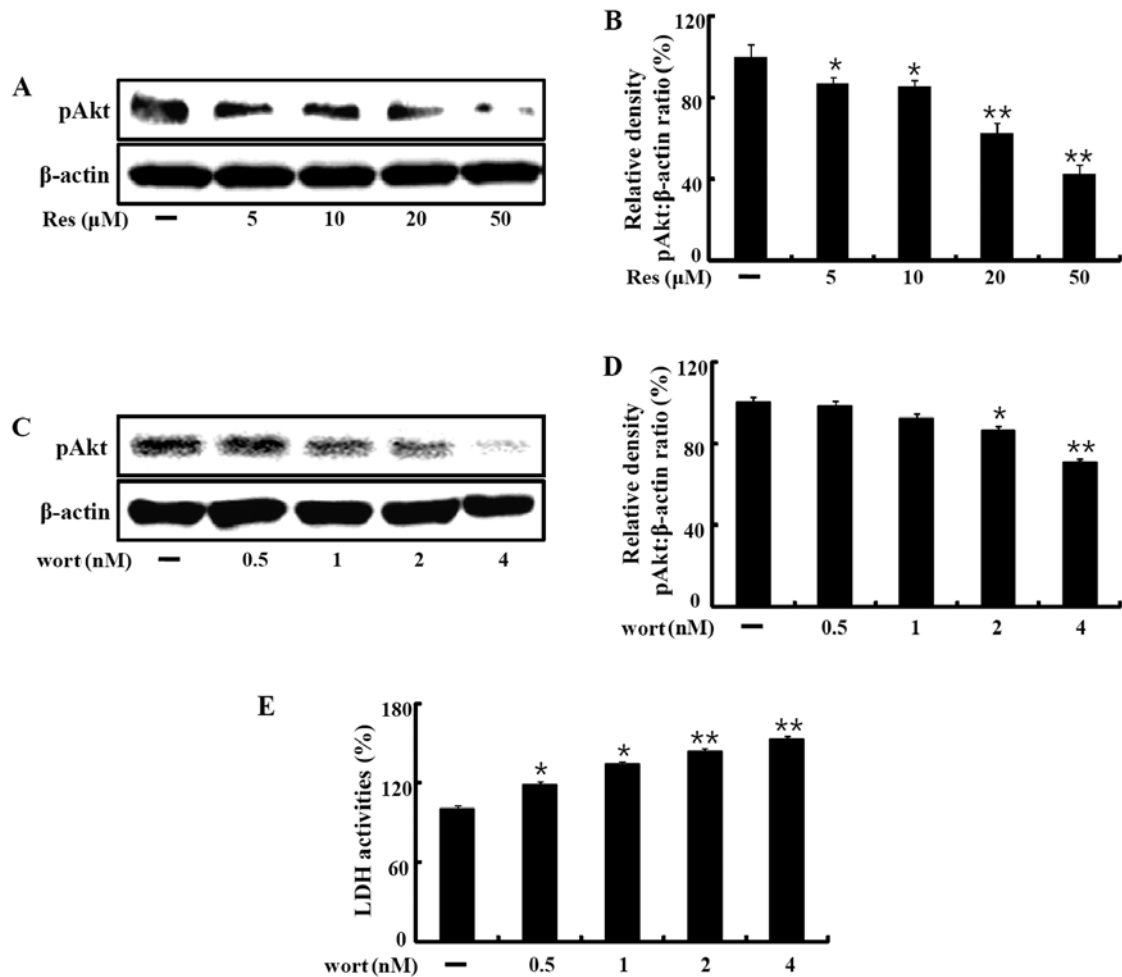


Figure 3. Resveratrol induces cell death through inhibition of Akt phosphorylation. (A and B) HaCaT keratinocytes were incubated with the indicated concentrations of resveratrol (5, 10, 20 and 50 μ M) for 12 h. The treated cells were assessed for phospho-Akt by western blot analysis. (C and D) HaCaT keratinocytes were incubated with wortmannin, an Akt inhibitor, for 12 h at increasing concentrations (5, 10, 20 and 50 μ M). Western blot analyses for phospho-Akt were performed on HaCaT keratinocytes. β -actin was used as the loading control. (E) LDH assay was used to quantify LDH release into the medium. Bar graphs indicates the mean \pm SEM (n=3). *p<0.05, **p<0.01, significant differences between the control and each treatment group. Res, resveratrol; Wort, wortmannin.

and survival signals was investigated by measuring the expression of Akt phosphorylation by western blot analysis. The results showed that resveratrol inhibited Akt activation dose-dependently (Fig. 3A and B). Akt inhibition by exposure to different concentrations of wortmannin (0.5, 1, 2 and 4 μ M) also decreased the phospho-Akt protein levels (Fig. 3C and D). We evaluated the released LDH activity in the media of the HaCaT keratinocytes treated with wortmannin alone. The data showed that wortmannin dose-dependently increased LDH release (Fig. 3E).

Resveratrol-induced Sirt1 increase leads to cell death through Akt phosphorylation. Next, we investigated whether resveratrol-induced Sirt1 activation is involved in Akt phosphorylation. HaCaT keratinocytes were treated with resveratrol with or without sirtinol. Resveratrol decreased phospho-Akt protein expression at 50 μ M, and this effect was restored by the Sirt1 inhibitor, sirtinol (Fig. 4A and B). We next treated the cells with an adenovirus of Sirt1 and found that AKT phosphorylation was decreased dose-dependently. Cells were transfected with adenoviruses expressing Sirt1 at a multiplicity of infection

(MOI) of 100, 200, 500 and 1,000 pfu/cell to directly evaluate the role of Sirt1 in HaCaT keratinocytes. Sirt1-overexpressing cells exhibited increased Sirt1 protein levels and a markedly decrease in acetyl-p53 protein. Overexpression of Sirt1 led to a decrease in phospho-Akt protein levels (Fig. 4C-E). Sirt1, acetyl-p53 and phospho-Akt expression in Ad-LacZ-infected cells was not changed when compared to the levels in the control cells. These results showed that resveratrol-induced Sirt1 upregulation led to cell death of HaCaT keratinocytes through phospho-Akt.

Discussion

Psoriasis is a chronic inflammatory skin disease involving hyperproliferation and abnormal differentiation of epidermal keratinocytes. Several therapies for mild psoriasis are currently available (24). However, due to the complex nature of psoriasis, most therapies have adverse side-effects. Apoptosis is a physiological process in the skin to regulate keratinocyte proliferation and epidermal growth. Lack of apoptosis leads to increase in anti-apoptotic proteins in the skin that cause

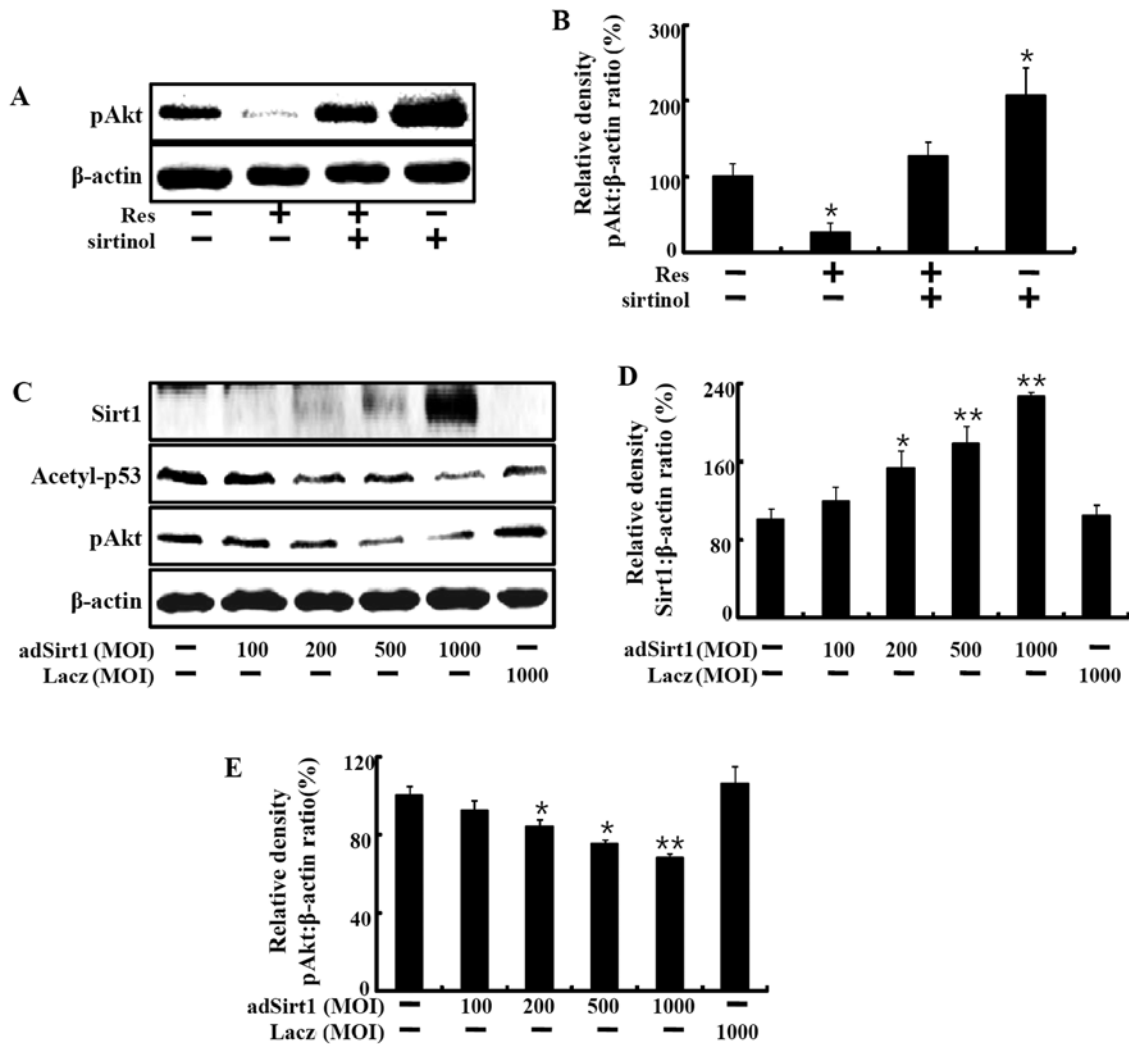


Figure 4. Resveratrol-mediated Sirt1 leads to phospho-Akt inactivation. (A and B) HaCaT keratinocytes were treated with 50 μ M of resveratrol in the presence of sirtinol (10 μ M) for 12 h. Phospho-Akt was analyzed by western blotting in the HaCaT keratinocytes. β -actin was used as the loading control. (C-E) HaCaT keratinocytes were transfected with Sirt1-overexpressing adenovirus (adSirt1) or Lacz-bearing adenovirus (Lacz). Western blotting for Sirt1, acetyl-p53 and phospho-Akt proteins was conducted in the HaCaT keratinocytes. Bar graphs indicate the mean \pm SEM (n=3). *p<0.05, **p<0.01, significant differences between the control and each treatment group. Res, resveratrol; MOI, multiplicity of infection.

psoriasis (24). We investigated an effective therapy to inhibit hyperproliferation and induce apoptosis in keratinocytes for psoriasis treatment.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a natural phenol known as a stilbenoid (25), was found to prevent inflammation and apoptosis of human and murine primary keratinocytes against UVB irradiation or H₂O₂ (26-28). However, resveratrol exhibited differential responses in HaCaT and primary human keratinocytes. Resveratrol sensitized UV-induced apoptosis in HaCaT keratinocytes (29). Cellular apoptosis is a main mechanism for blocking proliferation of cultured cells, hence we investigated whether resveratrol alone promoted apoptosis in HaCaT keratinocytes.

Resveratrol has attracted much attention for its ability to enhance the deacetylase activity of Sirt1 (30). Sirt1 is an NAD-dependent histone deacetylase that is a major regulator of multiple biological processes, including the stress response, apoptosis, the regulation of gene transcription and the cell cycle (12,31). Although Sirt1 mainly has protective effects against apoptosis in many cells, some cells undergo cell death

by Sirt1 expression and activation (23,32). We showed that the resveratrol-mediated increase in Sirt1 led to cell death in HaCaT keratinocytes.

Numerous studies suggest that Akt plays a critical role in tumorigenesis and cell survival. Resveratrol reportedly inhibits skin tumorigenesis through PI3K and Akt, proteins that are implicated in cancer development and progression (33). Akt phosphorylation involved in the apoptotic process raised the possibility that Akt regulates cell survival by directly phosphorylating components of the cell death pathway (21). We found that resveratrol inhibited phospho-Akt and the inhibition of PI3K by wortmannin-mediated resveratrol-induced apoptosis in HaCaT keratinocytes. Our data indicated that resveratrol inhibited cell proliferation and induced apoptosis through the inactivation of Akt signaling. Collectively, the present study demonstrated that resveratrol regulated cell death in HaCaT keratinocytes via Sirt1/phospho-Akt-mediated signaling pathways. Additionally, these findings suggested that resveratrol may be used as a potential therapeutic agent for psoriasis.

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