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Resveratrol alleviates LPS-induced injury in human keratinocyte cell line HaCaT by up-regulation of miR-17

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ABSTRACT

Background: Resveratrol (RSV), an edible polyphenolic phytoalexin, plays an important role in ameliorating inflammation, including skin inflammation after burn injury. However, the specific molecular mechanism underlying its anti-inflammation effect is still unclear. Herein, the effect and the mechanism underlying the protection of HaCaT cells by RSV against inflammation were examined.

Methods: Lipopolysaccharide (LPS)-induced inflammation and the cytoprotection of RSV were evaluated by detecting viability, apoptosis, expressions of apoptosis-associated proteins and the productions of pro-inflammatory factors by CCK-8 assay, flow cytometer, Western blot, and qRT-PCR. miR-17 expression in RSV-treated HaCaT cells was determined by qRT-PCR. The role of miR-17 in protective effect of RSV was investigated after altering its expression using transfection assay. The main ingredients in PTEN/PI3K/AKT and mTOR pathways were quantified by Western blot.

Results: LPS-induced HaCaT cell injury was inhibited by RSV administration. RSV promoted viability, inhibited apoptotic cell rate, increased Bcl-2 expression, decreased Bax, cleaved-Caspase-3, and cleaved-Caspase-9 expressions. RSV also inhibited inflammation injury of HaCaT cells by reducing productions of IL-6, IL-8, and TNF-α. miR-17 was up-regulated in LPS and RSV-co-treated cells. The protective effect of RSV might contribute to overexpression of miR-17. In the mechanism study, RSV-miR-17 axis was found to activate PTEN/PI3K/AKT and mTOR pathways in LPS-treated cells.

Conclusion: RSV alleviated LPS-induced injury in human keratinocyte cell line HaCaT through activations of PTEN/PI3K/AKT and mTOR pathways, which were modulated by miR-17.

1. Introduction

Burn injury was usually induced by hot liquid, steam, high temperature gas, flame, hot metal liquid or solid and etc., accompanied with histologic lesion, mainly including skin burn and mucosa damage, and the major burn may induce the activation of the inflammatory cascade leading to local tissue damage to result in the subsequent immunosuppression development and then further increase the patients' susceptibility to sepsis and other deleterious systemic in all organs and systems [1,2]. Skin inflammation caused by burn injury usually induces skin infection [3]. The natural protective function and barrier role of skin was impaired when it was burned and suffered improper disposal of burned surface, which accelerates water loss and makes burned skin be a portal for bacterial invasion [4]. Therefore, it was important to inhibit skin inflammation.

Many traditional and natural medicines are widely used to inhibit wound infection and inflammation after burn injury [5]. For example, moist exposed burn ointment (MEBO, containing β-sitosterol, berberine, sesame oil, and other plant ingredients), developed from traditional Chinese medicine, has long been known to be an anti-inflammatory compound [6]. Resveratrol (RSV, 3,4′,5-trihydroxystilbene) is a natural and edible polyphenol phytoalexin produced from plants in response to environmental stress. RSV is present in some plant origin food, most notably grapes and red wine [7]. RSV provides protective effects against numerous diseases, exhibiting anti-inflammatory, anti-apoptotic, anti-oxidant, anti-fibrotic, anti-hypertensive, and anti-cancer activities [8]. RSV was shown to protect hippocampal neurons from cerebral ischemia-reperfusion injury in rats [9]. RSV might be a potential homeostatic regulator in Alzheimer disease and exerted the neuroprotective effects on Alzheimer disease [10,11]. Additionally, RSV

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promoted cutaneous wound healing when it was applied to polymeric surgical dressings [12]. Meanwhile, growing evidences indicated that RSV played a protective role in respiratory diseases [8].

The effects of RSV on the treatment of burn-induced inflammatory response were studied by a few studies. Tao et al. reported that RSV inhibited the inflammation cascade reaction and increased the survival rate of severe burn [13]. RSV attenuated inflammation and reduced stricture formation in the caustic esophageal burns [14]. Besides, RSV produced analgesic effects in rats with burn injury pain [15].

Although, the anti-inflammatory effect of RSV was researched in several studies, but the specific action mechanism was not clear so far. In this study, HaCaT cells were treated with lipopolysaccharide (LPS), which was used to mimic the inflammation in burn injury. Then, the effects of RSV on inflammatory response and its possible action mechanism were investigated.

2. Materials and methods

2.1. Cell culture and treatment

HaCaT cell line, purchased from the Cell Lines Service (Heidelberg, Germany) were cultured in DMEM and Ham F12-medium (DMEM:F12, 1:1 mixture) supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2 and 95% air. HaCaT cells were incubated with LPS (from *Escherichia coli* O127:B8, Sigma-Aldrich, St. Louis, Missouri, USA) for 12 h to induce inflammatory injury. RSV (purity ≥ 99%, Sigma-Aldrich) was dissolved in DMSO obtaining concentration of 100 mM and was diluted to 10, 20, 30, 40, and 50 μm to treat cells for 24 h.

2.2. CCK-8 assay

The effects of LPS and RSV on viability of HaCaT cells were analyzed by CCK-8 kit (Beyotime, Shanghai, China). HaCaT cells were seeded in 96-well plate at 5000 cells/well. After different treatments, 10 μL CCK-8 solution was added in the 90 μL culture media per well and cells were then incubated for 2 h at 37 °C. The absorbance of wells was measured using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.3. Apoptosis assay

The effects of LPS and RSV on apoptosis of HaCaT cells were analyzed using PI and FITC-conjugated Annexin V staining method and then evaluated with flow cytometry analysis. After treated with LPS and RSV and washed with PBS, HaCaT cells were suspended in 1 × binding buffer with density of 10^6 cells/mL. 100 μL of cell suspension was added in the tube supplemented with 5 μL Annexin V-FITC and 5 μL PI. Afterwards, cells were incubated in the PI/Annexin V-FITC solution for 15 min. Finally, the tube was added with 300 μL 1 × binding buffer and apoptotic cell rate was analyzed by flow cytometry.

2.4. Transfection assay

miR-17 inhibitor and its negative control (NC), synthesized by GenePharma Co. (Shanghai, China) were transfected into HaCaT cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.5. qRT-PCR analysis

All RNAs were extracted from HaCaT cells by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). For miR-17 analysis, Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) using probe for miR-17 were respectively used for synthesis of cDNA and PCR analysis. The expression of miR-17 was normalized to U6 expression. For analyses of IL-6, IL-8, and TNF-α, Reverse Transcription Kit (Takara, Dalian, China) and SYBR Premix ExTaq II kit (Takara) were respectively used to reverse transcription and PCR analyses. The expressions of IL-6, IL-8, and TNF-α were normalized to GAPDH expression. The relative expressions of miR-17, IL-6, IL-8, and TNF-α were calculated using ($2^{-\Delta\Delta Ct}$) method.

2.6. Western blot

After treatment, harvested HaCaT cells were lysed in Triton X-100 lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich), Tris-HCl (pH 7.5, 25 mM), NaCl (137 mM), KCl (2.7 mM), and Triton X-100 (1%). After quantified using BCA™ Protein Assay Kit (Beyotime), proteins were resolved and separated over 12% SDS-PAGE and then transferred to PVDF membranes. After blocking in 5% non-fat milk, PVDF membranes carrying proteins were successively incubated with primary antibodies and secondary antibodies. The primary antibodies against Bax (ab32503), Bcl-2 (ab32124), pro-Caspase-3 (ab2302), cleaved-Caspase-3 (ab2302), pro-Caspase-9 (ab32539), cleaved-Caspase-9 (ab32539), IL-6 (ab6672), IL-8 (ab18672), TNF-α (ab6671), PTEN (ab32199), t-PI3K (ab180967), p-PI3K (ab182651), t-AKT (ab179463), *p*-AKT (ab131443), t-mTOR (ab2732), *p*-mTOR (ab63552), t-p70S6K (ab32529), p-p70S6K (ab2571), and β-actin (ab8226), as well as the secondary antibodies including goat anti-rabbit IgG (ab205718) and goat anti-mouse IgG (ab6789) marked by horseradish peroxidase were purchased from Abcam (Cambridge, MA, USA). After incubation with the corresponding secondary antibody, immunoblots were stripped by Restore Western Blot Stripping Buffer for 15 min and chemiluminiscence detection was performed using ECL kit. Densitometry analyses were conducted by Scion Image software package.

2.7. Statistical analysis

All assays were repeated three times. Data were expressed as mean ± SD and analyzed using Graph Pad Prism version 6.0 software (Graph Pad Software, San Diego California, USA). One-way analysis of variance (ANOVA) and a Bonferroni correction for multiple comparisons were used to analyze the data. When $P < 0.05$, effects were indicated to be statistically significant.

3. Results

3.1. LPS induced HaCaT cell injury

HaCaT cells were treated with different concentrations of LPS to induce different degrees of inflammatory injury. As shown in Fig. 1A, viability of HaCaT cells was decreased in a dose-dependent manner. The middle concentration of LPS with 7.5 μg/mL was applied for further studies. LPS treatment significantly enhanced apoptotic cell rate ($P < 0.001$, Fig. 1B), decreased Bcl-2 expression, increased Bax expression, and promoted activations of Casapse-3 and Caspase-9 (Fig. 1C). Next, the inflammatory response induced by LPS was evaluated by detecting productions of IL-6, IL-8, and TNF-α and results showed that their mRNA and protein levels were all augmented by LPS (Fig. 1D and E). These data suggest that LPS induced inflammatory injury in HaCaT cells by suppressing viability, promoting apoptosis, and increasing productions of some pro-inflammatory cytokines.

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