Resveratrol Inhibits Hydrogen Peroxide-Induced Apoptosis in Endothelial Cells via the Activation of PI3K/Akt by miR-126

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Aim: Resveratrol (RSV) is an edible polyphenolic phytoalexin present in different plant species that plays an important role in improving endothelial dysfunction. However, the molecular mechanisms underlying these effects are unknown. In the present study, the mechanism underlying the protection of CRL-1730 cells by RSV against oxidative stress was examined.

Methods: We first assessed the effects of RSV on the cell viability and apoptosis of CRL-1730 cells exposed to hydrogen peroxide (H₂O₂). Real-time PCR was used to determine the microRNA-126 (miR-126) expression in cells treated with RSV and/or H₂O₂. We also evaluated the PI3K/Akt signaling pathway in CRL-1730 cells following upregulation of the miR-126 expression. Finally, we determined the effects of miR-126 on RSV against oxidative injury using an miR-126 inhibitor.

Results: Treatment with RSV resulted in a significant increase in survival and a decrease in the apoptosis of CRL-1730 cells exposed to H₂O₂. We also found that H₂O₂ significantly suppressed the expression of miR-126, which was reversed by RSV in a dose-dependent manner. The overexpression of miR-126 decreased PIK3R2 (p85-β) and enhanced Akt phosphorylation, which resulted in an increase in the survival of CRL-1730 cells exposed to H₂O₂. More importantly, the downregulation of the miR-126 expression reversed the effects of RSV on the survival and apoptosis of CRL-1730 cells exposed to H₂O₂. In addition, the knockdown of Ets-1 reversed the effects of RSV on the miR-126 expression in CRL-1730 cells exposed to H₂O₂.

Conclusions: In this study, we demonstrated that the protection of endothelial cells by RSV against oxidative injury is due to the activation of PI3K/Akt by miR-126.


Key words: Endothelial cells, Oxidative stress, Resveratrol, miR-126, PI3K/Akt
the 3’-untranslated region (3’-UTR) of target mRNAs. Research indicates that endothelial miRNAs have important functions in both proangiogenesis and antiangiogenesis. Among endothelial miRNAs, miR-126 is one of the most enriched miRNAs in human endothelial cells and regulates many aspects of endothelial cell biology, such as cell migration and cell survival. Meng et al. reported that the signal pathway of miR-126 affecting endothelial cells is partially mediated via PI3K/Akt/eNOS regulation.

Akt (protein kinase B) is a critical component in the phosphatidyl inositol 3-kinase (PI3K) pathway that plays a pivotal role in the apoptosis and survival of endothelial cells. Although H$_2$O$_2$ stimulates Akt phosphorylation, the inhibition of Akt activation significantly enhances the apoptosis of epithelial cells treated with H$_2$O$_2$. As a result, Akt activation confers protection against oxidative stress-induced apoptosis. The specific aim of this study was to evaluate whether miR-126, which regulates the PI3K/Akt activity, is involved in the protection of endothelial cells by RSV from oxidative stress.

**Materials and Methods**

**Chemicals and Reagents**

RPMI-1640 was obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Newborn calf serum (NCS) was obtained from PAA Laboratories (GmbH, Linz, Austria). Resveratrol, H$_2$O$_2$, wortmannin and dimethylthiazoly-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. The Annexin V-EGFP/PI Apoptosis Detection Kit was obtained from KeyGen (Nanjing, China). The caspase-3 activity assay kit was obtained from Beyotime (Nantong, China). Rabbit polyclonal antibodies against phosphorylated Akt and total Akt were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA). Polyclonal antibodies against p85-β were obtained from Abcam (Cambridge, England). Antibodies against Ets-1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Detergent Compatible (DC) Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

**Cell Culture**

The human umbilical vein endothelial cell line (CRL-1730) was purchased from ATCC (Manassas, VA, USA). The endothelial cells were cultured in RPMI-1640 medium supplemented with 10% NCS, 1% sodium bicarbonate and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO$_2$. The cultured cells were treated with resveratrol (dissolved in Dimethyl sulfoxide (DMSO)) in complete 1640 medium. To obtain reliable results, the final concentration of DMSO in the culture medium was maintained at less than 0.1%.

**MTT Assay**

Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays. Briefly, CRL-1730 cells were seeded in 96-well dishes at $1 \times 10^4$ cells per well and treated with H$_2$O$_2$ and resveratrol. Then, each well was supplemented with 10 μL of MTT and incubated for four hours at 37°C. The medium was then removed, and 150 μL of DMSO was added to solubilize the MTT formazan. The optical density was read at 570 nm.

**Flow Cytometry Analysis**

This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in early apoptotic cells. Briefly, the cells were resuspended in a binding buffer. Then, annexin V-EGFP and PI were added, and the solution was incubated at room temperature for 15 minutes in the dark, after which an assay was performed using the FACScan (Becton Dickinson). The percentage of apoptosis was computed using the CellQuest software program (Becton Dickinson).

**Caspase-3 Activity Assay**

The caspase-3 activity was analyzed using the caspase-3 activity assay kit according to the manufacturer’s protocol. The cells were lysed, and the total cellular protein extracts were quantified using a protein assay kit. Next, an equal amount of total protein extract was incubated at 37°C overnight with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for the caspase-3 assay. The amount of pNA released was estimated by determining the absorbance at 405 nm on a microplate ELISA reader (Bio-Rad Laboratories). The relative activity of caspase-3 was calculated as follows: caspase-3 activity = (mean experimental absorbance / mean control absorbance) x 100 (%).

**Quantitative Real-Time PCR (Q-PCR) Analysis of the miRNA Expression**

Approximately $5 \times 10^6$ cells were treated with H$_2$O$_2$ and/or RSV. miRNAs were isolated and purified using Trizol reagent (Invitrogen, USA), according to the manufacturer’s protocol. The miR-126 level was quantified using real-time PCR with the TransStartTM SYBR Green qPCR Supermix (TransGen Biotech, USA).
Beijing, China) and U6 small nuclear RNA as an internal normalized reference. For miR-126, the primers were as follows: forward, 5′-TATAAGATCTGGAGATTAGTTGCCCGAGAAGCT-3′ and reverse, 5′-ATATGATCTCCTCAGGGCTATGCGCCCTAAGTAC-3′. For U6, the primers were as follows: forward, 5′-CTCGCTTCAGGGCAACATTTGC-3′ and reverse, 5′-AACGCTTCAGGAATTTGTGCTA-3′.

**Western Blot Analysis**

CRL-1730 cells were lysed with ice-cold lysis buffer containing the following: 50 mmol/L of Tris-HCl, pH 7.4; 1% NP-40; 150 mmol/L of NaCl; 1 mmol/L of EDTA; 1 mmol/L of phenylmethylsulfonyl fluoride; and a complete proteinase inhibitor mixture (one tablet per 10 mL; Roche Molecular Biochemicals, Indianapolis, IN, USA). The protein concentrations in the cell lysates were quantified using the DC protein assay kit (Bio-Rad), after which a Western blot analysis was performed.

**Transfection Procedures**

miR-126 was knocked down or overexpressed using transfection with a miRNA inhibitor or miRNA mimic using siPort Neo-FX (Ambion) according to the manufacturer’s recommendations. The miR-126 mimic (5′-UCGUACCUGAGAUAAUAUGCG-3′), miR-126 inhibitor (5′-CGCAUAAUACUGACGUAAGA-3′) and negative control (5′-CAGUACUUUUGUGUAAGAA-3′) were synthesized by RIBOBIO (Ribobio Co. Ltd, Guangzhou, China). All of the oligonucleotides were transfected at a final concentration of 100 nM. CRL-1730 cells were transfected with the miR-126 inhibitor or mimic using siPort Neo-FX (Ambion) according to the manufacturer’s recommendations.

**Knockdown of PIK3R2 (p85-β) and Ets-1 Using RNA Interference**

p85-β-specific small interfering RNA (siRNA), Ets-1 siRNA and control siRNA were synthesized by RIBOBIO (Ribobio Co. Ltd, Guangzhou, China). The sequences of the p85-β siRNAs and Ets-1 siRNA were as follows: 5′-UUGUCGAUCUCUGU- GUCCGAGG-3′ (p85-β siRNAs); 5′-GGACAAACG-CUGUCAUUCC-3′ (Ets-1 siRNA). CRL-1730 cells were transiently transfected with siRNA using the Lipofectamine 2000 reagent according to the manufacturer’s instructions.

**Statistical Analysis**

The statistical analysis was performed using the statistical analysis SPSS 13.0 software program. The statistical analyses were performed using either an analysis of variance (ANOVA) or Student’s t-test. Each experiment was performed in at least triplicate. The results are presented as the mean ± SD. A p value of <0.05 was deemed to be significant.

**Results**

**Effects of RSV on the Survival of HUVECs Exposed to H$_2$O$_2$**

The effects of RSV on the survival of HUVECs exposed to H$_2$O$_2$ were evaluated using MTT assays. The results in Fig.1A show that incubation of CRL-1730 cells with different concentrations of H$_2$O$_2$ (10, 50, 100 and 200 μM) for 24 hours decreased the viability of the cells in a dose-dependent manner. When the CRL-1730 cells were treated with 10, 50, 100 and 200 μM of H$_2$O$_2$, the cell viability was decreased to 87.7 ± 4.92%, 59.4 ± 4.32%, 49.4 ± 3.83% and 32.3 ± 2.97%, respectively. Treatment of CRL-1730 cells with different concentrations of RSV (10, 30 and 50 μM) reversed the decrease in viability induced by H$_2$O$_2$ (100 μM) in a dose-dependent manner (Fig. 1B). The rates of viability associated with 10, 30 and 50 μM of RSV were 63.1 ± 3.6%, 82.0 ± 3.75% and 86.8 ± 3.19%, respectively. RSV alone (50 μM) did not affect viability compared to the control.

**Effects of RSV on the Apoptosis of HUVECs Exposed to H$_2$O$_2$**

To further confirm the protective effects of RSV on HUVEC survival, we pretreated the cells with RSV (30 μM) for two hours, then added H$_2$O$_2$ (100 μM) for 24 hours. The pro-apoptotic effect was quantified using annexin V-FITC/PI double-staining and a flow cytometric analysis. The percentages of cell populations at various stages of apoptosis are shown in Fig.2A. After H$_2$O$_2$ treatment, the number of cells that underwent apoptosis (early apoptosis, annexinV$^+$/PI$^-$ or late apoptosis annexinV$^+$/PI$^+$) significantly increased. Treatment of the cells with RSV reduced the percentage of apoptotic cells associated with H$_2$O$_2$ exposure.

Activation of caspases plays an important role in the execution of apoptosis [5]. To determine whether RSV alters the caspase activity in endothelial cells exposed to H$_2$O$_2$, we assessed the caspase-3 activity using the caspase-3 activity assay. As shown in Fig.2B, caspases-3 was activated following exposure to H$_2$O$_2$ compared to that observed in the control group. This increase was suppressed by pretreatment with RSV.
Effects of RSV on the miR-126 Expression in HUVECs Exposed to H₂O₂

To identify the mechanisms underlying the effects of RSV on the survival of HUVECs exposed to H₂O₂, we performed real-time PCR to detect the miR-126 expression. Exposure of the cells to different concentrations of H₂O₂ resulted in a dose-dependent decrease in the expression of miR-126 (Fig. 3A). Treatment with RSV increased the H₂O₂-induced decrease in the miR-126 expression in a dose-dependent fashion (Fig. 3B). RSV alone did not affect the miR-126 expression.

Effects of miR-126 on the p85-β Expression and the Phosphorylation of Akt in HUVECs

It has been reported that p85-β is a target for miR-126 in the activation of PI3K/Akt in umbilical vascular endothelial cells. To determine whether miR-126 regulates the PI3K/Akt signaling pathway in human umbilical vein endothelial cells, we investigated the levels of p85-β, phospho-Akt and total Akt in cells transfected with the miR-126 mimic. The results of real-time PCR revealed that the miR-126 mimic significantly increased the expression of miR-126 in the CRL-1730 cells (p < 0.01) (Fig. 4A), suggesting that the miR-126 mimic was efficiently introduced into the cells and acted to upregulate the miR-126 expression.

The results of a Western blot analysis showed that the miR-126 mimic inhibited the p85-β expression and stimulated the phosphorylation of Akt proteins, while the total Akt protein levels remained constant in the CRL-1730 cells (Fig. 4B).

Effects of miR-126 and siRNA-p85-β on the Survival of HUVECs Exposed to H₂O₂

The viability of the CRL-1730 cells was significantly increased in the group treated with transfection of the miR-126 mimic compared to that observed in the control group. Meanwhile, H₂O₂ exposure significantly reduced the cell viability compared to that observed in the control group. Transfection of the miR-126 mimic enhanced the viability of the CRL-1730 cells exposed to H₂O₂. This effect was partially inhibited by wortmannin (an inhibitor of PI3K) treatment (Fig. 5A). Furthermore, wortmannin markedly inhibited miR-126-induced Akt phosphorylation, as shown in Fig. 5C.

To investigate whether p85-β is involved in the decrease in the viability of HUVECs exposed to H₂O₂, we used siRNA-p85-β to downregulate the p85-β expression. As shown in Fig. 5D, siRNA-p85-β effectively silenced the p85-β expression. In addition, siRNA-p85-β greatly enhanced Akt phosphorylation in the CRL-1730 cells. Similar to the miR-126 mimic, siRNA-p85-β increased the viability of CRL-1730 cells exposed to H₂O₂ (Fig. 5B).

Downregulation of the miR-126 Expression Reverses the Effects of RSV

To evaluate the role of miR-126 in the effects of RSV on the survival of CRL-1730 cells exposed to H₂O₂, we treated cells with the miR-126 inhibitor. As shown in Fig. 6A, the miR-126 inhibitor significantly
Ets-1 Mediated the Induction of the miR-126 Expression by RSV

It has been reported that the transcription factors Ets-1 and Ets-2 regulate the miR-126 expression in endothelial cells. To investigate whether Ets-1 and Ets-2 are involved in the regulation of the miR-126 expression in HUVECs exposed to H2O2 and/or RSV, we measured the Ets-1 and Ets-2 protein levels using a Western blot analysis. We found that H2O2 decreased the Ets-1 expression, which was reversed by RSV. However, H2O2 and/or RSV had no effect on the decreased the expression of miR-126 in the CRL-1730 cells.

Treatment with RSV (50 μM) enhanced the viability of CRL-1730 cells exposed to H2O2, which was partially attenuated by the miR-126 inhibitor. In addition, RSV treatment significantly decreased the caspase-3 activity in cells exposed to H2O2, which was reversed by the miR-126 inhibitor.

Fig. 2. RSV decreased the apoptosis of CRL-1730 cells exposed to H2O2. Following pretreatment with RSV (30 μM) for two hours, the CRL-1730 cells were treated with H2O2 (100 μM) for an additional 24 hours, and apoptosis was detected using a flow cytometry analysis (A) and caspase-3 activity assays. *p<0.05, indicates a significant difference from the control group. #p<0.05, indicates a significant difference from the H2O2-treated group.

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Resveratrol Protects the Endothelium

The molecular mechanisms underlying the protection of human umbilical vein endothelial cells by RSV against oxidative stress damage. The major finding of the present study is that treatment with RSV significantly increased the H2O2-induced decrease in the miR-126 expression in a dose-dependent fashion. The inhibition of the miR-126 expression attenuated the effects of RSV on the survival and apoptosis of CRL-1730 cells exposed to H2O2.

RSV is an important antioxidant that plays a key role in decreasing endothelial oxidative stress. Kao et al. demonstrated that RSV reduces reactive oxygen species (ROS) production in human endothelium exposed to H2O2. We also found that RSV inhibited ROS production.

expression of Ets-2 (Fig. 7A). Next, we used siRNA-Ets-1 to downregulate the Ets-1 expression. As shown in Fig. 7B, siRNA-Ets-1 effectively silenced the Ets-1 expression. Knockdown of Ets-1 reversed the effects of RSV on the miR-126 expression in CRL-1730 cells exposed to H2O2 (Fig. 7C).

Discussion

This study was conducted in an attempt to explore
followed by continuous incubation with H₂O₂ for 24 hours. It has been reported that a high concentration of RSV is slightly cytotoxic to hepatocytes\(^{21}\). In our study, RSV demonstrated a protective effect at all concentrations used (10, 30 and 50 \(\mu\)M), and RSV alone production induced by H₂O₂ in CRL-1730 cells (data not shown). In the present study, we showed that RSV has a protective effect on endothelium facing H₂O₂-induced cell injury. These protective effects were observed in cells pretreated with RSV for two hours followed by continuous incubation with H₂O₂ for 24 hours. It has been reported that a high concentration of RSV is slightly cytotoxic to hepatocytes\(^{21}\). In our study, RSV demonstrated a protective effect at all concentrations used (10, 30 and 50 \(\mu\)M), and RSV alone

**Fig. 5.** An overexpression of miR-126 and knockdown of the p85-\(\beta\) expression increased the survival of CRL-1730 cells exposed to H₂O₂ in an Akt-dependent manner. The cells were transfected with the miR-126 mimic, then treated with H₂O₂ (100 \(\mu\)M) with or without wortmannin for 24 hours. The cell viability was determined using MTT assays (A). The cells were transfected with siRNA-p85-\(\beta\) for 12 hours, then treated with H₂O₂ (100 \(\mu\)M) with or without wortmannin for an additional 24 hours. The cell viability was determined using MTT assays (B). CRL-1730 cells were treated with wortmannin (300 nM) for 24 hours, then harvested for Western blotting (C). CRL-1730 cells were transfected with siRNA-p85-\(\beta\) (100 nM) for 24 hours, then harvested for Western blotting (D). \(^*p<0.05\), indicates a significant difference from the respective control group.
Resveratrol Protects the Endothelium

Noratto et al. reported that miR-126 is involved in the effects of polyphenolics in protecting HUVECs from inflammation. In this study, we found that H$_2$O$_2$ inhibited the expression of miR-126 in human HUVECs, which indicates that miR-126 may be involved in preventing endothelial oxidative stress. A further analysis showed that treatment with RSV increased the miR-126 expression in HUVECs exposed to H$_2$O$_2$. When the miR-126 inhibitor was transfected in the cells, the effects of RSV in protecting HUVECs from H$_2$O$_2$-induced oxidative stress had no effect on the viability or apoptosis of the CRL-1730 cells, which suggests that RSV has beneficial effects on the endothelium without any negative side effects.

To further clarify the mechanisms involved in the protection of endothelial cells by RSV against oxidative stress damage, we investigated the effects of RSV on the expression of miR-126. As one of the most enriched microRNAs in the human endothelium, miR-126 has been demonstrated to play a critical role in the regulation of angiogenic signaling and cell survival. Noratto et al. reported that miR-126 is involved in the effects of polyphenolics in protecting HUVECs from inflammation. In this study, we found that H$_2$O$_2$ inhibited the expression of miR-126 in human HUVECs, which indicates that miR-126 may be involved in preventing endothelial oxidative stress. A further analysis showed that treatment with RSV increased the miR-126 expression in HUVECs exposed to H$_2$O$_2$. When the miR-126 inhibitor was transfected in the cells, the effects of RSV in protecting HUVECs from H$_2$O$_2$-induced oxidative stress had no effect on the viability or apoptosis of the CRL-1730 cells.

![Graph A](image1.png)

**Graph A.** Relative miR-126 level in HUVECs treated with RSV and H$_2$O$_2$.

![Graph B](image2.png)

**Graph B.** Cell viability of CRL-1730 cells after treatment with RSV and H$_2$O$_2$.

![Graph C](image3.png)

**Graph C.** Caspase-3 activity of CRL-1730 cells after treatment with RSV and H$_2$O$_2$.

* $p<0.05$, indicates a significant difference from the respective control group.

**Fig. 6.** The miR-126 inhibitor reversed the effects of RSV in CRL-1730 cells. CRL-1730 cells were transfected with the miR-126 inhibitor (100 nM) for 24 hours, then harvested for a real-time PCR analysis. The cells were then treated with H$_2$O$_2$ (100 μM) with or without RSV for an additional 24 hours. The cell viability and degree of apoptosis were determined using MTT assays and caspase-3 activity assays, respectively. $^*p<0.05$, indicates a significant difference from the respective control group.
apoptosis were reversed (Fig. 6C). These results suggest that miR-126 is involved in the effects of RSV in protecting human HUVECs against oxidative injury.

In non-small cell lung cancer, miR-126 has been shown to decrease tumor growth as a tumor suppressor by inhibiting the PI3K-Akt activity. However, in endothelial progenitor cells, the inhibition of apoptosis by miR-126 is partially mediated via the PI3K/Akt/eNOS signaling pathway. The present study also showed that the overexpression of miR-126 decreased the p85-β expression and increased Akt phosphorylation (Fig. 4B). It has been shown that p85-β negatively regulates the activity of PI3 kinase. Our data also showed that knockdown of the p85-β expression results in Akt activation. Akt is a critical component in the PI3K/Akt pathway and plays a pivotal role in the apoptosis and survival of endothelial cells.
increased apoptosis induced by H$_2$O$_2$\textsuperscript{28}. We found that Akt phosphorylation was reduced when HUVECs were treated with H$_2$O$_2$ for 24 hours, consistent with the findings of a previous report\textsuperscript{29}. A further analysis showed that the overexpression of miR-126 attenuated the H$_2$O$_2$-induced decrease in viability. This effect was partially inhibited by wortmannin treatment (Fig. 5A). Therefore, Akt activation stimulated by miR-126 confers protection against oxidative stress-induced apoptosis.

It has been reported that the transcription factor Ets-1 regulates the miR-126 expression in endothelial cells\textsuperscript{19}. Our data also showed that the knockdown of Ets-1 alone inhibited the miR-126 expression in HUVECs (Fig. 7C). A further analysis showed that the knockdown of Ets-1 reversed the effects of RSV on the miR-126 expression in CRL-1730 cells exposed to H$_2$O$_2$. As a result, we chose to test the hypothesis that Ets-1 is involved in the upregulation of miR-126 by RSV.

In summary, our results demonstrated that RSV exerts protective effects against H$_2$O$_2$-induced apoptosis in human umbilical vein endothelial cells in vitro. Our results also suggest that the antiapoptotic effects associated with RSV treatment are due to increases in the miR-126 expression that result in the activation of the PI3K/Akt signaling pathway. Ets-1 is involved in the induction of the miR-126 expression by RSV. The findings presented herein provide an important basis for further investigations to better understand the actions of RSV in HUVECs and the possible beneficial effects of RSV treatment on the prevention of cardiovascular disease.

**Conflicts of Interest**

None to declare.

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