Profound negative regulatory effects by resveratrol on vascular smooth muscle cells: a role of p53–p21WAF1/CIP1 pathway

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Abstract

We investigated the role of resveratrol, a polyphenol rich in red wine, in cell cycle progression and apoptosis of vascular smooth muscle cells (VSMCs). Resveratrol inhibited the growth of human aortic VSMCs at concentrations as low as 1 \textmu M. This was due to the profound dose-dependent inhibition of DNA synthesis by resveratrol. DNA synthesis was more effectively inhibited when cells were pretreated with resveratrol. Resveratrol caused a dose-dependent increase in intracellular p53 and p21WAF1/CIP1 levels. At lower concentrations (6.25–12.5 \textmu M), resveratrol effectively blocked cell cycle progression of serum-stimulated VSMCs without inducing apoptosis, while the higher concentration of resveratrol (25 \textmu M) selectively induced apoptosis in the same VSMCs. Intriguingly, however, the same high concentration of resveratrol could not induce apoptosis in quiescent VSMCs. These differential biological effects of resveratrol on quiescent and proliferating VSMCs suggest that resveratrol may be capable of selectively eliminating abnormally proliferating VSMCs of the arterial walls in vivo.

Keywords: Resveratrol; Atherosclerosis; Smooth muscle cells; French Paradox; Restenosis

Resveratrol, a polyphenolic phytoalexin, is produced by grapes and other plants in response to infection or injury (Fig. 1) [1]. It is abundant in red wine (up to \textasciitilde 75 \textmu M) but essentially nonexistent in beer, spirits, and white wine (less than 1 \textmu M) [2–4]. Because of this, resveratrol has been considered to be one of the candidate molecules to explain the French Paradox, a phenomenon that the French have relatively low rates of ischemic heart diseases despite a large consumption of saturated fats and high serum cholesterol levels [5,6].

Although the biological properties of resveratrol have been investigated in tumor cell lines, they have been relatively poorly studied in vascular smooth muscle cells (VSMCs), whose proliferation plays an important role in the pathogenesis of proliferative cardiovascular disorders (PCVDS), namely atherosclerosis [7–12] and postangioplasty restenosis [13–15].

We investigated the biological role of resveratrol in VSMC growth. The data presented here suggest that resveratrol is a potent growth inhibitor of VSMCs whose action is at least partly mediated through the activation of p53–p21WAF1/CIP1 pathway. The resveratrol-induced upregulation of total p53 protein in VSMCs is a new finding. In the VSMCs, the activation of p53–p21WAF1/CIP1 pathway by resveratrol has not been reported. Importantly resveratrol exhibited differential biological effects on VSMCs, depending on the state of proliferation and on the concentration of resveratrol: In quiescent VSMCs, both at low and high concentrations, resveratrol arrested the cell cycle progression, without inducing apoptosis. In proliferating VSMCs, resveratrol arrested cell cycle arrest at lower concentrations while it induced apoptosis in higher concentrations. These differential biological effects of resveratrol on VSMCs have not been reported in the literature. We propose that cardioprotective effects of resveratrol are at least partly due to its...
negative regulatory effects on VSMC proliferation and survival.

Materials and methods

Materials. Resveratrol (Sigma, St. Louis, MO) was dissolved in 100% ethanol at a concentration of 50 mM and stored at −80 °C in stoppered glass vials until used.

Tissue culture. HAVSMCs (Clonetics, Walkersville, MD) were maintained according to the manufacturer’s instructions in Media 231 (Cascade Biologics, Portland, OR) with 1% penicillin-streptomycin and serum supplements.

Cell growth assay. To assess cell growth under the various concentrations of resveratrol, 2 × 10^5 HAVSMCs were seeded in duplicate in 6-well plates. Cells were serum-starved for 24 h, preincubated with resveratrol for 24 h without serum, and then serum-stimulated for 120 h in the presence of various concentrations of resveratrol, ranging from 0 (vehicle only, 100% ethanol) to 25 μM. At the end of the stimulation period, cells were washed twice with phosphate-buffered saline (PBS), harvested by trypsinization, and counted using a hemocytometer.

Thymidine uptake assay. The effect of resveratrol on thymidine uptake was assayed in HAVSMCs stimulated by indicated concentrations of serum (0%, 1.25%, 2.5%, or 5%). HAVSMCs were seeded in triplicate in 24-well plates at a density of 2 × 10^4/well. Cells were serum-starved for 24 h, preincubated with resveratrol normally for 24 h but, when appropriate, for periods ranging from 0 to 12 h, and serum-stimulated for 24 h in the presence of 1 μCi/ml [methyl-^3H]thymidine (Amersham Biosciences, Piscataway, NJ) and resveratrol (0–100 μM). Cells were then washed twice with PBS and harvested into RIPA buffer (50 mM Tris-Cl, pH 7.2, 1% NP-40, 150 mM NaCl, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Protein concentrations of lysates were measured using a Micro BCA Protein Assay kit (Pierce, Rockford, IL), according to the manufacturer’s instructions. Lysates were then mixed with 2 μl of scintillation fluid (Universol; ICN, Irvine, Rockford, IL), according to the manufacturer’s instructions. Lysates were then mixed with 2 ml of scintillation fluid (Universol; ICN, Irvine, Rockford, IL), according to the manufacturer’s instructions.

Western blot analysis of vascular smooth muscle cells. To evaluate the role of p53 and p21^WAF1/CIP1 in the inhibition of growth and DNA synthesis by resveratrol, lysates of serum-stimulated HAVSMCs treated with varying concentrations of resveratrol (0–100 μM) were subjected to Western blot analysis. Approximately 1 × 10^6 HAVSMCs each were plated in 10-cm tissue culture dishes and serum-starved for 24 h in Media 231 (Cascade Biologics) containing no serum. Cells were then preincubated in Media 231 containing various concentrations of resveratrol (or vehicle) and no serum. Finally, cells were serum-stimulated in Media 231 containing various concentrations of resveratrol (or vehicle) and harvested after 24 h. Cells were directly harvested into SDS gel loading buffer (5 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) in order to minimize the degradation of proteins. Western blot analysis was performed as previously described [16,17], using anti-p53, anti-p21^WAF1/CIP1, and anti-α-actin antibodies (Chemicon, Temecula, CA) as probes.

Statistical analysis. Statistical analysis was performed using Minitab statistical software (Minitab, State College, PA). Group data are expressed as means ± standard deviation (SD). For comparison of multiple values from two different treatment groups, Student’s t test was employed (p < 0.05). For comparison of multiple values from more than two treatment groups, analysis of variance (ANOVA) using Fisher’s pairwise comparison was performed (confidence interval, 95%).

Results and discussion

Resveratrol inhibits HAVSMC growth at a very low concentration

In order to evaluate the effect of resveratrol on the growth of HAVSMCs, cells were incubated for 120 h in
the presence of various concentrations of resveratrol (0–25 μM). At the end of the incubation, cells were counted and the numbers of cells, expressed as the percentage of the number of cells incubated without resveratrol (control, 100% ethanol), were plotted against concentrations of resveratrol (Fig. 2). Resveratrol inhibited the growth of serum-stimulated HAVSMCs (Fig. 2). Moreover, the inhibition by resveratrol of HAVSMC growth was dose-dependent (Fig. 2). Notably, as low as 1.1 μM of resveratrol significantly inhibited the growth of HAVSMCs, in comparison with vehicle (100% ethanol, control) (94.5 ± 5.1% at 120 h, \( P < 0.05 \)) (Fig. 2).

**Resveratrol inhibits HAVSMC DNA synthesis in a dose-dependent fashion**

Next, in order to evaluate the effect of resveratrol on DNA synthesis, thymidine uptake assay was performed using HAVSMCs. In the absence of serum, HAVSMCs took up very little thymidine, regardless of the presence of resveratrol (Fig. 3A, 1st and 2nd columns). Upon serum stimulation, the uptake of thymidine by HAVSMCs became robust, increasing more than 6-fold above that by unstimulated cells (1586 ± 64.2 vs. 10,055 ± 1600.7 in the absence and presence of serum, respectively; \( P < 0.05 \)) (Fig. 3A, 1st and 3rd columns). Resveratrol significantly inhibited thymidine uptake by serum-stimulated HAVSMCs in a dose-dependent fashion (\( P < 0.001 \), ANOVA) (Fig. 3A, 3rd–9th columns). In comparison with untreated HAVSMCs, HAVSMCs treated with resveratrol at concentrations of 6.25 μM or higher showed significant inhibition of thymidine uptake (\( P < 0.05 \), Fisher’s pairwise comparisons) (Fig. 3A, 3rd and 5th columns).

Next, the effect of resveratrol on DNA synthesis in the presence of various degrees of growth stimuli was evaluated. As expected, resveratrol (12.5 μM) significantly inhibited thymidine incorporation at all serum concentrations tested (1.25%, 2.5%, and 5%). The degree of inhibition was more pronounced at the lowest of these three serum concentrations (Fig. 3B). (91.6, 73.5, and 74.9% inhibition for 1.25, 2.5, and 5% sera, respectively; \( P < 0.005 \)).

The preincubation with resveratrol enhances its growth inhibitory activity on HAVSMCs

Importance of the presence of resveratrol in HAVSCs at the time of serum stimulation was then evaluated. In the absence of resveratrol, serum stimulation resulted in a robust 5-fold increase in thymidine uptake (Fig. 3C; “Serum +, Resveratrol −, Preincubation 0 h” vs. “Serum −, Resveratrol −, Preincubation 0 h”). Even without preincubation, resveratrol (12.5 μM) significantly reduced thymidine uptake (100 ± 3.3 vs. 75.7 ± 6.5%, \( P < 0.05 \)) (Fig. 3C; “Serum +, Resveratrol −, Preincubation 0 h” vs. “Serum +, Resveratrol +, Preincubation 0 h”). Nevertheless, resveratrol’s inhibitory effect on thymidine uptake was significantly greater with
longer preincubation ($P < 0.001$, ANOVA) (Fig. 3C, Preincubation 0–24 h). Notably, the inhibition of thymidine uptake by serum-stimulated HAVSMC was substantially greater after 24 h of preincubation (74.2% vs. 40.6% after 24 vs. 12 h; $P < 0.001$) (Fig. 3C; Preincubation 12 vs. 24 h).

**Resveratrol increases intracellular p53 level in a dose-dependent fashion**

The role of p53 in resveratrol-induced inhibition of growth and DNA synthesis in HAVSMCs was then evaluated. In the absence of serum, p53 signal was readily detectable by Western blot analysis (Fig. 4A, 1st lane). After 24 h of serum stimulation in the absence of resveratrol, p53 signal was barely detectable (Fig. 4A, 2nd lane). Strikingly, the presence of resveratrol at concentrations as low as 6.25 μM was associated with distinctly increased levels of intracellular p53 (Fig. 4A, 3rd lane). Moreover, higher concentrations of resveratrol were associated with higher levels of p53 in serum-stimulated HAVMSCs (Fig. 4A, 2nd–6th lanes). Consistently, in the absence of resveratrol, no p53 signals were detected by immuno-cytochemistry (Fig. 4B, Vehicle). In the presence of 6.25 μM resveratrol, however, there were discrete increases in p53 signal in both the nucleus and cytoplasm (Fig. 4B, 6.25 μM). The number of cells positive for nuclear p53 increased as resveratrol concentration increased from 6.25 to 50 μM (Fig. 4B, 6.25, 12.5, and 50 μM).

The mechanism of p53 accumulation in resveratrol-treated VSMCs is most likely due to the increase in p53 acetylation [18]. The acetylation of p53 has been shown to enhance the activity and stability of p53, while deacetylation, predominantly caused by SIRT1, destabilizes and facilitates the degradation of p53 [19–21]. Intriguingly, Howitz and co-workers showed that resveratrol, at very low concentration (0.5 μM), activated SIRT1 while it inhibited SIRT1 at higher concentrations [18]. The increased intracellular p53 in the present study indicates that at the concentration tested (6.25–50 μM), resveratrol may block SIRT1-mediated p53 deacetylation.

Tumor suppressor protein p53 has been shown to play an important role in PCVDs. To study the role of p53 in atherosclerosis, Guevara et al. cross-bred p53−/− mice [22] with apoE−/− mice [23] and produced p53+/+/apoE−/−, p53−/−/apoE−/−, p53−/−/apoE+/+ or p53+/+/apoE+/+ strains [24]. In comparison with p53+/+/apoE−/− mice, p53−/−/apoE−/− mice developed accelerated aortic atherosclerosis in the presence of similarly elevated serum cholesterol, thus supporting the notion that p53 protects against atherosclerosis [24]. Meanwhile, Spiers and coworkers demonstrated the frequent presence in restenotic plaques of IE84, a human cytomegalovirus (hCMV) protein, that physically interacts with and blocks the transcriptional activity of p53.

![Fig. 4](image-url)
In order to test the role of p21WAF1/CIP1, a negative cell cycle regulator induced by p53, in the growth inhibition of HAVSMCs treated with resveratrol, Western blot analyses of p21WAF1/CIP1 were performed. Consistent with previous reports [28], p21WAF1/CIP1 was readily detectable in serum-starved HAVSMCs by Western blot analysis (Fig. 4C, 1st lane). Upon serum stimulation in the absence of resveratrol, however, p21WAF1/CIP1 became undetectable (Fig. 4C, 2nd lane). The presence of resveratrol in the media was associated with increased p21WAF1/CIP1 levels and the increase was more obvious with higher concentrations of resveratrol (Fig. 4C, 2nd–6th lanes).

In cancerous cell lines, resveratrol (>2.5–10 μM) has been shown to increase the intracellular p53 concentration [29–31]. In addition, Narayanan et al. [32] showed in prostate cancer cells that resveratrol treatment was associated with the increase in the transcripts of p53-inducible genes, including p21WAF1/CIP1, p53-inducible gene-7 (PIG7), PIG8, and PIG10. Despite these reports in transformed cells, the role of resveratrol in the p53-pathway of primary, nontransformed VSMCs has not been clearly elucidated. Recently, Haider et al. [33] reported that resveratrol at the concentration of 100 μM increased Ser15-phosphorylated p53 without changing the total amount of p53. Interestingly, in their work, the upregulation of Ser15-phosphorylated p53 was not associated with increase in p21WAF1/CIP1 [33]. Although resveratrol has been shown to increase both total p53 [29–31] and p21WAF1/CIP1 [30,32,34] in cancerous cells, the current work is the first to show that resveratrol induces, even at very low concentrations (6.25–12.5 μM), total p53 and its transcriptional target p21WAF1/CIP1 in VSMCs (Fig. 4).

Resveratrol does not cause apoptosis in quiescent HAVSMCs

Whether resveratrol causes HAVSMC apoptosis at nutritionally attainable concentrations (0–25 μM) was then evaluated. Under low-serum conditions where cell cycle progression assessed by thymidine uptake was minimal (Fig. 5A, 0.5% serum, closed columns), the resveratrol did not cause more apoptosis than did the control vehicle at any concentrations tested (0, 6.25, 12.5, and 25 μM; Fig. 5B, open columns).

Resveratrol induces apoptosis in proliferating HAVSMCs at higher concentrations

Under growth-promoting conditions (5% serum) where vigorous cell cycle progression exists as evidenced by robust thymidine uptake in the absence of resveratrol (Fig. 5A, 5% serum, the closed columns; 486.6 ± 42.9 vs. 5928 ± 145 for 0.5 vs. 5% sera, respectively, P < 0.005), there was no increase in apoptosis at concentrations of resveratrol up to 12.5 μM (Fig. 5B, first 3 closed columns), but there were drastic increases in apoptosis at 25 μM (Fig. 5B, last closed column; 1.69 ± 0.12 vs. 0.94 ± 0.02 for 25 and 0 μM resveratrol, respectively;
Therefore, a high concentration of resveratrol (25 μM) selectively induced apoptosis in HAVSMCs which were actively proliferating, but not in HAVSMCs which were quiescent, while low concentrations of resveratrol (0–12.5 μM) did not induce apoptosis in HAVSMCs regardless of their proliferation status.

As a chemopreventive agent against atherosclerosis, the property of resveratrol demonstrated above that it blocks cell cycle progression without inducing apoptosis at low concentrations (6.25–12.5 μM) is important. The adenovirus-mediated overexpression of p53 has been shown to be highly cytotoxic to VSMCs presumably because of the p53-mediated activation of apoptosis pathway [35]. It is likely, therefore, that the overexpression of p53 in the arterial wall by gene transfer methods would be associated with severe vascular damage due to apoptosis. On the other hand, resveratrol could be safely used in vivo as a naturally occurring “p53 inducer” that induces p53 protein in VSMCs of the vascular wall, without causing significant apoptosis or cytotoxicity when administered to achieve certain concentrations (≤12.5 μM).

That higher concentration (25 μM) resveratrol differentially induced apoptosis in proliferating VSMCs but not in quiescent VSMCs (Fig. 5) is also significant because resveratrol will be able to selectively kill VSMCs that are actively proliferating, when administered at higher concentrations. In other words, resveratrol will be well tolerated by quiescent VSMCs at higher concentrations (≥25 μM), while activated VSMCs will be selectively eliminated by resveratrol at these concentrations. It opens the possibility of the clinical use of resveratrol at higher concentrations (≥25 μM) to actively “delete” unwanted, proliferating VSMCs in certain pathological conditions, such as postangioplasty restenosis.

Finally, resveratrol has been shown to have multiple vascular biological activities other than the activation of p53–p21WAF1/CIP1 pathway. First, resveratrol shares some structural similarities with synthetic estrogen diethylstilbestrol (DES; 4,4′-dihydroxy-trans-α,β-diethylstibene) and binds estrogen receptor and activated estrogen-receptor-dependent genes in vitro [36]. Second, resveratrol inhibits TNF-α-induced expression of tissue factor in vascular endothelial cells, which suggests that resveratrol can inhibit thrombogenesis [37]. Third, resveratrol enhances expression and activity of nitric oxide synthase in endothelial cells [2]. Further investigation is called for to determine the relative contributions of these pathways in vascular tissue.

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**References**


