

## Profound negative regulatory effects by resveratrol on vascular smooth muscle cells: a role of p53–p21<sup>WAF1/CIP1</sup> pathway

Zakar H. Mnjoyan<sup>a</sup> and Ken Fujise<sup>a,b,\*</sup>

<sup>a</sup> *Research Center for Cardiovascular Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, The University of Texas Health Science Center at Houston, Houston, TX, USA*

<sup>b</sup> *Division of Cardiology, Department of Internal Medicine, Medical School, The University of Texas Health Science Center at Houston, Houston, TX, USA*

Received 23 September 2003

### 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  $\mu$ 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 p21<sup>WAF1/CIP1</sup> levels. At lower concentrations (6.25–12.5  $\mu$ M), resveratrol effectively blocked cell cycle progression of serum-stimulated VSMCs without inducing apoptosis, while the higher concentration of resveratrol (25  $\mu$ 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.

© 2003 Elsevier Inc. All rights reserved.

*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  $\sim$ 75  $\mu$ M) but essentially nonexistent in beer, spirits, and white wine (less than 1  $\mu$ 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–p21<sup>WAF1/CIP1</sup> pathway. The resveratrol-induced upregulation of total p53 protein in VSMCs is a new finding. In the VSMCs, the activation of p53–p21<sup>WAF1/CIP1</sup> 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 caused 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

\* Corresponding author. Fax: +713-500-6556.

E-mail address: [Kenichi.Fujise@uth.tmc.edu](mailto:Kenichi.Fujise@uth.tmc.edu) (K. Fujise).

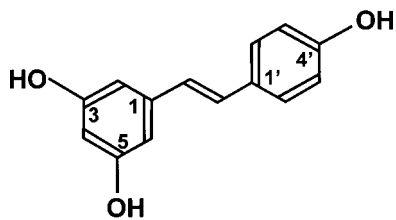


Fig. 1. Chemical structure of resveratrol. Resveratrol (3,4,5-trihydroxystilbene, mol. wt. 228) a polyphenolic phytoalexin having three phenolic hydroxyl groups, a stilbenic double bond, and connecting aromatic rings in the *trans*-conformation.

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 50mM and stored at  $-80^{\circ}\text{C}$  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,  $2 \times 10^5$  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  $\mu\text{M}$ . 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  $2 \times 10^4$ /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  $\mu\text{Ci}/\text{ml}$  [methyl- $^3\text{H}$ ]thymidine (Amersham Biosciences, Piscataway, NJ) and resveratrol (0–100  $\mu\text{M}$ ). Cells were then washed twice with PBS and harvested into RIPA buffer (50mM Tris–Cl, pH 7.2, 1% NP-40, 150mM NaCl, 1% sodium dodecyl sulfate, 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 ml of scintillation fluid (Universol; ICN, Irvine, CA) and subjected to scintillation counting to determine tritium content, using a LS6500 Multipurpose Scintillation Counter (Beckman; Fullerton, CA). The thymidine uptake index was calculated as (total counts [dpm])/(total protein amount [ $\mu\text{g}$ ]).

**Western blot analysis of vascular smooth muscle cells.** To evaluate the role of p53 and p21<sup>WAF1/CIP1</sup> in the inhibition of growth and DNA synthesis by resveratrol, lysates of serum-stimulated HAVSMCs treated with varying concentrations of resveratrol (0–100  $\mu\text{M}$ ) were subjected to Western blot analysis. Approximately  $1 \times 10^6$  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<sup>WAF1/CIP1</sup>, and anti- $\alpha$ -actin antibodies (Chemicon, Temecula, CA) as probes.

**Immunocytochemistry.** To determine the intracellular concentration and localization of p53,  $5 \times 10^4$ /well HAVSMCs were seeded in 4-well Lab-Teck chamber slides (Nalge Nunc International, Rochester, NY), serum-starved, preincubated with various concentrations (0, 6.25, 12.5, and 50  $\mu\text{M}$ ) of resveratrol, stimulated with serum as described above, and subjected to immunocytochemical staining as described previously [16,17]. Briefly, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized at  $-20^{\circ}\text{C}$  with acetone–methanol solution (v/v 1:1), blocked with 10% normal goat serum, and probed with anti-p53 (Santa Cruz Biotechnology). Bound primary antibodies were detected with goat secondary antibody conjugated to Rhodamine Red X (Jackson ImmunoResearch Laboratories, West Grove, PA). The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma). Slides were examined under a Zeiss Axioskop fluorescent microscope (Carl Zeiss, Hertsfordshire, UK) equipped with a Zeiss image processing system using appropriate filter sets.

**TUNEL staining.** To determine whether concentrations of resveratrol that significantly inhibited thymidine uptake would induce apoptosis, HAVSMCs were treated with various concentrations of resveratrol (0–25  $\mu\text{M}$ ) in the presence of either high (5%) or low (0.5%) concentration of serum and then subjected to TUNEL staining assay to determine apoptotic indices. Terminal deoxynucleotidyl transferase (TdT)-deoxyuridine nick-end labeling (TUNEL) staining, originally described by Surh et al. [38] was performed using a FragEL DNA Fragmentation Detection Kit (Oncogene Research Products; Boston, MA) according to the manufacturer's instructions, with the following modifications. Approximately  $5 \times 10^4$ /well HAVSMCs were seeded in duplicate in 8-well Lab-Teck chamber slides (Nalge Nunc International). Cells were serum-starved for 24 h, preincubated with various concentrations (0, 6.25, 12.5, and 25  $\mu\text{M}$ ) of resveratrol, serum-stimulated for 24 h, harvested in  $-20^{\circ}\text{C}$  acetone solution for 10 s and allowed to dry overnight. Next, cells were treated with 1% paraformaldehyde in PBS and then with 0.1% peroxidase in PBS, labeled with deoxynucleotides conjugated to biotin by the TdT enzyme, and incubated with horseradish peroxidase (HRP) conjugated to streptavidin. After extensive washing with PBS, bound HRP was detected by 3,3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide. Finally, cells were counterstained lightly with hematoxylin and eosin. Cells with brown (DAB-positive) nuclei were considered to be TUNEL positive. The apoptotic index, defined as the number of cells with DAB-positive nuclei divided by the total number of cells counted and expressed as a percentage, was then calculated. At least 500 cells were counted per chamber. Experiments were performed twice.

**Statistical analysis.** Statistical analysis was performed using Minitab statistical software (Minitab, State College, PA). Group data are expressed as means  $\pm$  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

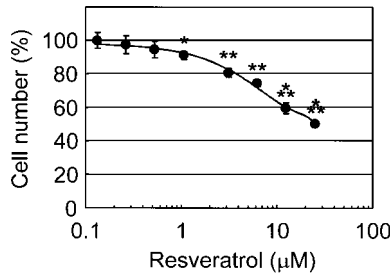


Fig. 2. Growth inhibition of HAVSMCs by resveratrol. Growth inhibition of HAVSMCs was observed at a resveratrol concentration of 1.1  $\mu\text{M}$ . Asterisks indicate significant growth inhibition: \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$  ( $n = 2$ ). Experiments were repeated at least twice with identical results.

the presence of various concentrations of resveratrol (0–25  $\mu\text{M}$ ). 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  $\mu\text{M}$  of resveratrol significantly inhibited the growth of HAVSMCs, in comparison with vehicle (100% ethanol, control) ( $94.5 \pm 5.1\%$  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 ( $1586 \pm 64.2$  vs.  $10,055 \pm 1600.7$  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  $\mu\text{M}$  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  $\mu\text{M}$ ) 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

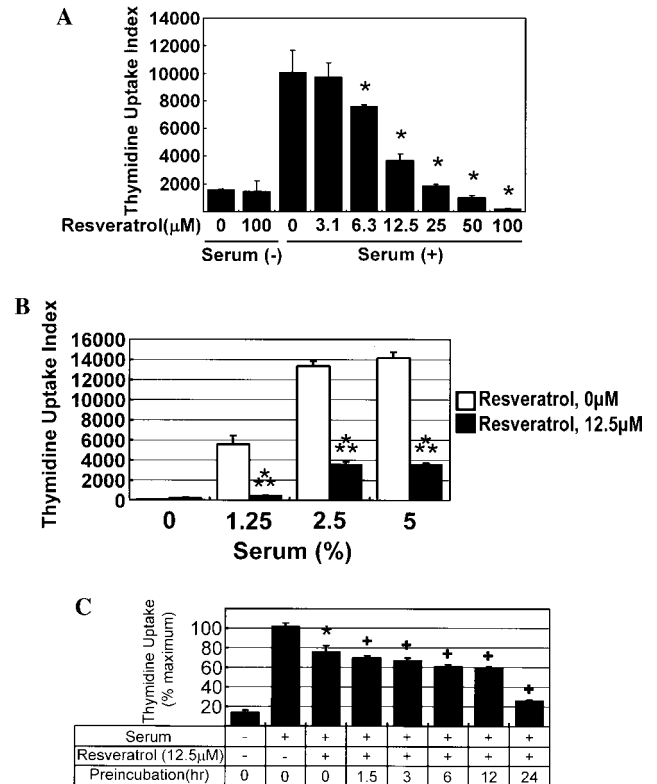


Fig. 3. Inhibition of HAVSMC thymidine uptake by resveratrol. (A) Dose-dependent inhibition of thymidine uptake by resveratrol ( $n = 4$ ). Asterisk (\*) indicates significant inhibition in resveratrol-treated cells versus vehicle-treated, serum-stimulated cells (confidence interval, 0.05; ANOVA and Fisher's pairwise comparison). (B) Inhibition by resveratrol (12.5  $\mu\text{M}$ ) of thymidine uptake in cells serum-stimulated in various degrees (0–5%). Open columns, vehicle-treated cells; closed columns, resveratrol-treated cells. Asterisks (\*\*\*) indicate significant inhibition ( $P < 0.005$ ) ( $n = 4$ ). (C) Augmented inhibition of HAVSMC thymidine uptake by preincubation with resveratrol. Thymidine uptake is expressed as a percentage of the maximum thymidine incorporation in vehicle-treated, serum-stimulated cells ( $n = 4$ ). Asterisk (\*) and cross (+) indicate significant inhibition (\* $P < 0.05$ ; +  $P < 0.001$ ).

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 HAVSMCs 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 0h" vs. "Serum -, Resveratrol -, Preincubation 0h"). Even without preincubation, resveratrol (12.5  $\mu\text{M}$ ) significantly reduced thymidine uptake ( $100 \pm 3.3$  vs.  $75.7 \pm 6.5\%$ ,  $P < 0.05$ ) (Fig. 3C; "Serum +, Resveratrol -, Preincubation 0h" vs. "Serum +, Resveratrol +, Preincubation 0h"). 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  $\mu\text{M}$  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 HAVSMCs (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  $\mu\text{M}$  resveratrol, however, there were discrete increases in p53 signal in both the nucleus and cytoplasm (Fig. 4B, 6.25  $\mu\text{M}$ ). The number of cells positive for nuclear p53 increased as

resveratrol concentration increased from 6.25 to 50  $\mu\text{M}$  (Fig. 4B, 6.25, 12.5, and 50  $\mu\text{M}$ ).

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  $\mu\text{M}$ ), 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  $\mu\text{M}$ ), 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<sup>-/-</sup> mice [22] with apoE<sup>-/-</sup> mice [23] and produced p53<sup>+/+</sup>/apoE<sup>-/-</sup>, p53<sup>-/-</sup>/apoE<sup>-/-</sup>, p53<sup>-/-</sup>/apoE<sup>+/+</sup> or p53<sup>+/+</sup>/apoE<sup>+/+</sup> strains [24]. In comparison with p53<sup>+/+</sup>/apoE<sup>-/-</sup> mice, p53<sup>-/-</sup>/apoE<sup>-/-</sup> 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

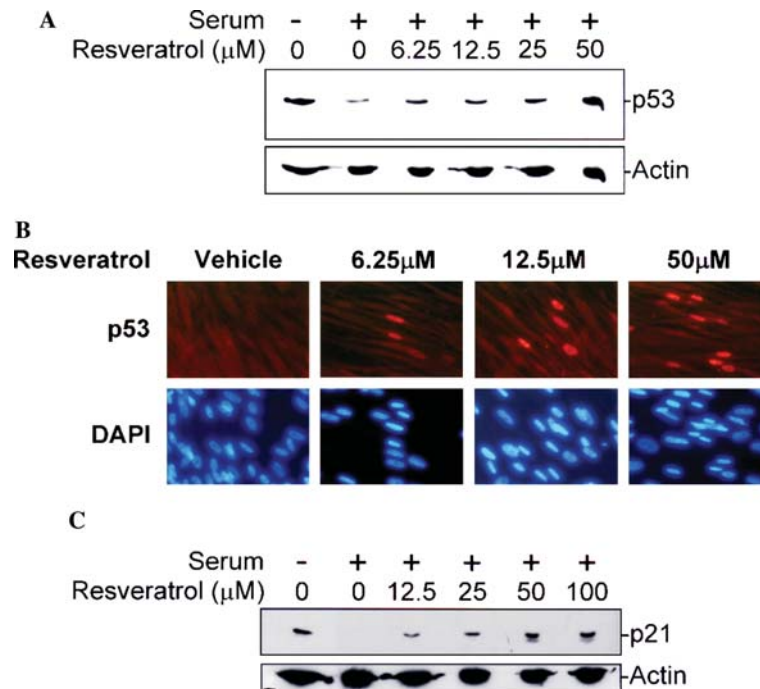


Fig. 4. Dose-dependent upregulation of p53 and its target gene by resveratrol. (A) Induction of p53 by resveratrol in HAVSMCs. Standard Western blot analysis using anti-p53 antibody (DO-1) was performed as described in Materials and methods (top). Loading conditions were evaluated by probing the same membrane with anti-actin antibody (bottom). (B) Induction and nuclear localization of p53 by resveratrol in HAVSMCs. (C) Induction of p21<sup>WAF1/CIP1</sup> by resveratrol in HAVSMCs. Standard Western blot analysis using anti-p21<sup>WAF1/CIP1</sup> antibody was performed as described in Materials and methods (top). Loading conditions were evaluated by probing the same membrane with anti-actin antibody (bottom). All experiments were performed at least three times with identical results.

[25,26], suggesting that the dysfunction of p53 caused by the presence of IE84 might lead to overgrowth of neointima and to postangioplasty restenosis [25]. Furthermore, our laboratory recently reported that VSMCs, when stimulated by a mitogen, upregulated p53 at the same time they proliferated, suggesting that p53 may be a built-in negative regulator of VSMC growth [27]. Taken together with these observations, our results suggest that the upregulation of p53 by a moderate and chronic consumption of red wine rich in resveratrol may be one of the mechanisms by which resveratrol protects the cardiovascular system and contributes to the French Paradox.

*Resveratrol-induced upregulation of intracellular p53 is associated with the upregulation of p21<sup>WAF1/CIP1</sup>*

In order to test the role of p21<sup>WAF1/CIP1</sup>, a negative cell cycle regulator induced by p53, in the growth inhibition of HAVSMCs treated with resveratrol, Western blot analyses of p21<sup>WAF1/CIP1</sup> were performed. Consistent with previous reports [28], p21<sup>WAF1/CIP1</sup> was readily detectable in serum-starved HAVSMCs by Western blot analysis (Fig. 4C, 1st lane). Upon serum stimulation in the absence of resveratrol, however, p21<sup>WAF1/CIP1</sup> became undetectable (Fig. 4C, 2nd lane). The presence of resveratrol in the media was associated with increased p21<sup>WAF1/CIP1</sup> 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  $\mu\text{M}$ ) 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 p21<sup>WAF1/CIP1</sup>, 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  $\mu\text{M}$  increased Ser<sup>15</sup>-phosphorylated p53 without changing the total amount of p53. Interestingly, in their work, the upregulation of Ser<sup>15</sup>-phosphorylated p53 was not associated with increase in p21<sup>WAF1/CIP1</sup> [33]. Although resveratrol has been shown to increase both total p53 [29–31] and p21<sup>WAF1/CIP1</sup> [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  $\mu\text{M}$ ), total p53 and its transcriptional target p21<sup>WAF1/CIP1</sup> in VSMCs (Fig. 4).

*Resveratrol does not cause apoptosis in quiescent HAVSMCs*

Whether resveratrol causes HAVSMC apoptosis at nutritionally attainable concentrations (0–25  $\mu\text{M}$ ) was

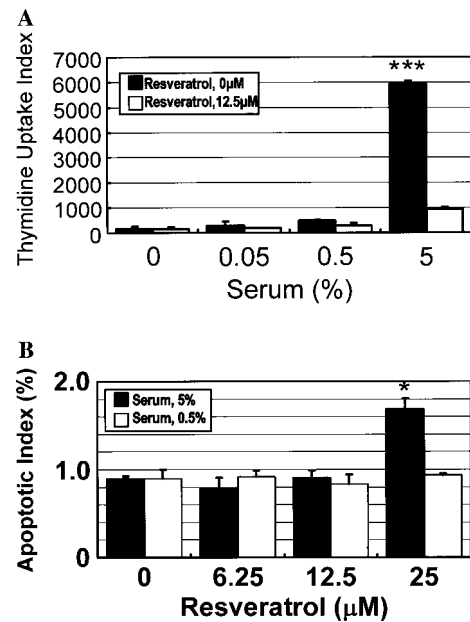


Fig. 5. Differential induction of apoptosis by resveratrol in proliferating HAVSMCs. (A) Minimal thymidine uptake in low ( $\leq 0.5\%$ ) serum conditions. Closed columns: vehicle; open columns: 12.5  $\mu\text{M}$  resveratrol. Vehicle-treated cells stimulated by 5% serum had significantly higher thymidine uptake than did resveratrol-treated cells stimulated by 5% serum (6-fold difference,  $P = 0.001$ ) or vehicle-/resveratrol-treated cells stimulated by low ( $\leq 0.5\%$ ) serum ( $>10$ -fold difference,  $P < 0.001$ ) ( $n = 4$ ). (B) Differential killing effect of resveratrol on VSMCs at high (25  $\mu\text{M}$ ) concentration. TUNEL staining was performed on HAVSMCs stimulated with either standard (5%) serum (closed columns) or low (0.5%) serum (open columns) as described in Materials and methods ( $n = 2$ ). Asterisk (\*) indicates a significantly higher apoptotic index in resveratrol-treated cells stimulated with a high concentration of serum than in resveratrol-treated cells stimulated with a low concentration of serum where cell did not uptake a significant amount of thymidine (see A) ( $P < 0.05$ ). All experiments were repeated at least twice with identical results.

then evaluated. Under low-serum conditions where cell cycle progression assessed by thymidine uptake was minimal (Fig. 5A, 0.5% serum, closed column), the resveratrol did not cause more apoptosis than did the control vehicle at any concentrations tested (0, 6.25, 12.5, and 25  $\mu\text{M}$ ; 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;  $486.6 \pm 42.9$  vs.  $5928 \pm 145$  for 0.5 vs. 5% sera, respectively,  $P < 0.005$ ), there was no increase in apoptosis at concentrations of resveratrol up to 12.5  $\mu\text{M}$  (Fig. 5B, first 3 closed columns), but there were drastic increases in apoptosis at 25  $\mu\text{M}$  (Fig. 5B, last closed column;  $1.69 \pm 0.12$  vs.  $0.94 \pm 0.02$  for 25 and 0  $\mu\text{M}$  resveratrol, respectively;

$P < 0.05$ ). Therefore, a high concentration of resveratrol (25  $\mu\text{M}$ ) selectively induced apoptosis in HAVSMCs which were actively proliferating, but not in HAVSMCs which were quiescent, while low concentrations of resveratrol (0–12.5  $\mu\text{M}$ ) 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  $\mu\text{M}$ ) 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 ( $\leq 12.5 \mu\text{M}$ ).

That higher concentration (25  $\mu\text{M}$ ) 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 ( $\geq 25 \mu\text{M}$ ), 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 ( $\geq 25 \mu\text{M}$ ) 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–p21<sup>WAF1/CIP1</sup> pathway. First, resveratrol shares some structural similarities with synthetic estrogen diethylstilbestrol (DES; 4,4'-dihydroxy-*trans*- $\alpha,\beta$ -diethylstilbene) and binds estrogen receptor and activated estrogen-responsive genes in vitro [36]. Second, resveratrol inhibits TNF- $\alpha$ -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.

## Acknowledgments

This study was supported in part by grants from the National Heart, Lung, and Blood Institute (HL04015 and HL68024) and a McDonald General Research Grant at St. Luke's Episcopal Hospital, Houston, TX (to K.F.).

## References

- [1] Y. Schneider, B. Duranton, F. Gosse, R. Schleiffer, N. Seiler, F. Raul, Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis, *Nutr. Cancer* 39 (2001) 102–107.
- [2] T. Wallerath, G. Deckert, T. Ternes, H. Anderson, H. Li, K. Witte, U. Forstermann, Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase, *Circulation* 106 (2002) 1652–1658.
- [3] X. Gu, L. Creasy, A. Kester, M. Zeece, Capillary electrophoretic determination of resveratrol in wines, *J. Agric. Food Chem.* 47 (1999) 3223–3227.
- [4] J.M. Wu, Z.R. Wang, T.C. Hsieh, J.L. Bruder, J.G. Zou, Y.Z. Huang, Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review), *Int. J. Mol. Med.* 8 (2001) 3–17.
- [5] S. Renaud, M. de Lorgeril, Wine, alcohol, platelets, and the French Paradox for coronary heart disease, *Lancet* 339 (1992) 1523–1526.
- [6] M.H. Criqui, B.L. Ringel, Does diet or alcohol explain the French Paradox?, *Lancet* 344 (1994) 1719–1723.
- [7] G.E. Austin, N.B. Ratliff, J. Hollman, S. Tabei, D.F. Phillips, Intimal proliferation of smooth muscle cells as an explanation for recurrent coronary artery stenosis after percutaneous transluminal coronary angioplasty, *J. Am. Coll. Cardiol.* 6 (1985) 369–375.
- [8] K.N. Garratt, W.D. Edwards, U.P. Kaufmann, R.E. Vlietstra, D.R. Holmes Jr., Differential histopathology of primary atherosclerotic and restenotic lesions in coronary arteries and saphenous vein bypass grafts: analysis of tissue obtained from 73 patients by directional atherectomy, *J. Am. Coll. Cardiol.* 17 (1991) 442–448.
- [9] M. Kearney, A. Pieczek, L. Haley, D.W. Losordo, V. Andres, R. Schainfeld, K. Rosenfield, J.M. Isner, Histopathology of in-stent restenosis in patients with peripheral artery disease, *Circulation* 95 (1997) 1998–2002.
- [10] J. Masuda, R. Ross, Atherogenesis during low level hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque, *Arteriosclerosis* 10 (1990) 178–187.
- [11] J.G. Pickering, L. Weir, J. Jekanowski, M.A. Kearney, J.M. Isner, Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization, *J. Clin. Invest.* 91 (1993) 1469–1480.
- [12] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, *Nature* 362 (1993) 801–809.
- [13] S. Morimoto, Y. Mizuno, S. Hiramitsu, K. Yamada, N. Kubo, M. Nomura, T. Yamaguchi, H. Kitazume, K. Kodama, H. Kurogane, Restenosis after percutaneous transluminal coronary angioplasty—a histopathological study using autopsied hearts, *Jpn. Circ. J.* 54 (1990) 43–56.
- [14] M. Nobuyoshi, T. Kimura, H. Ohishi, H. Horiuchi, H. Nosaka, N. Hamasaki, H. Yokoi, K. Kim, Restenosis after percutaneous transluminal coronary angioplasty: pathologic observations in 20 patients, *J. Am. Coll. Cardiol.* 17 (1991) 433–439.
- [15] K.R. Karsch, K.K. Haase, M. Wehrmann, S. Hassenstein, H. Hanke, Smooth muscle cell proliferation and restenosis after stand alone coronary excimer laser angioplasty, *J. Am. Coll. Cardiol.* 17 (1991) 991–994.
- [16] K. Fujise, D. Zhang, J. Liu, E. Yeh, Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen, *J. Biol. Chem.* 275 (2000) 39458–39465.
- [17] F. Li, D. Zhang, K. Fujise, Characterization of fortilin, a novel anti-apoptotic protein, *J. Biol. Chem.* 276 (2001) 47542–47549.
- [18] K.T. Howitz, K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu, J.G. Wood, R.E. Zipkin, P. Chung, A. Kisielewski, L.L. Zhang, B. Scherer, D.A. Sinclair, Small molecule activators of

- sirtuins extend *Saccharomyces cerevisiae* lifespan, *Nature* 425 (2003) 191–196.
- [19] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, *Cell* 107 (2001) 149–159.
- [20] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu, Negative control of p53 by Sir2alpha promotes cell survival under stress, *Cell* 107 (2001) 137–148.
- [21] E. Langley, M. Pearson, M. Faretta, U.M. Bauer, R.A. Frye, S. Minucci, P.G. Pelicci, T. Kouzarides, Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence, *EMBO J.* 21 (2002) 2383–2396.
- [22] L.A. Donehower, M. Harvey, B.L. Slagle, M.J. McArthur, C.A.J. Montgomery, J.S. Butel, A. Bradley, Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours, *Nature* 356 (1992) 215–221.
- [23] R.L. Reddick, S.H. Zhang, N. Maeda, Atherosclerosis in mice lacking apo E. Evaluation of lesion development and progression, *Arterioscler. Thromb.* 14 (1994) 141–147.
- [24] N.V. Guevara, H.S. Kim, E.I. Antonova, L. Chan, The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo, *Nat. Med.* 5 (1999) 335–339.
- [25] E. Speir, R. Modali, E.S. Huang, M.B. Leon, F. Shawl, T. Finkel, S.E. Epstein, Potential role of human cytomegalovirus and p53 interaction in coronary restenosis, *Science* 265 (1994) 391–394.
- [26] Y.F. Zhou, M.B. Leon, M.A. Waclawiw, J.J. Popma, Z.X. Yu, T. Finkel, S.E. Epstein, Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy, *N. Engl. J. Med.* 335 (1996) 624–630.
- [27] Z.H. Mnjoyan, R. Dutta, D. Zhang, B.B. Teng, K. Fujise, Paradoxical upregulation of tumor suppressor protein p53 in serum-stimulated vascular smooth muscle cells: a novel negative-feedback regulatory mechanism, *Circulation* 108 (2003) 464–471.
- [28] S. Waga, G.J. Hannon, D. Beach, B. Stillman, The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA, *Nature* 369 (1994) 574–578.
- [29] C. Huang, W.Y. Ma, A. Goranson, Z. Dong, Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway, *Carcinogenesis* 20 (1999) 237–242.
- [30] A. Shih, F.B. Davis, H.Y. Lin, P.J. Davis, Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism, *J. Clin. Endocrinol. Metab.* 87 (2002) 1223–1232.
- [31] J. Lu, C.H. Ho, G. Ghai, K.Y. Chen, Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts, *Carcinogenesis* 22 (2001) 321–328.
- [32] B.A. Narayanan, N.K. Narayanan, G.G. Re, D.W. Nixon, Differential expression of genes induced by resveratrol in LNCaP cells: P53-mediated molecular targets, *Int. J. Cancer* 104 (2003) 204–212.
- [33] U.G. Haider, D. Sorescu, K.K. Griendling, A.M. Vollmar, V.M. Dirsch, Resveratrol increases serine 15-phosphorylated but transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells, *Mol. Pharmacol.* 63 (2003) 925–932.
- [34] P.L. Kuo, L.C. Chiang, C.C. Lin, Resveratrol-induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells, *Life Sci.* 72 (2002) 23–34.
- [35] D. Katayose, R. Wersto, K. Cowan, P. Seth, Consequences of p53 gene expression by adenovirus vector on cell cycle arrest and apoptosis in human aortic vascular smooth muscle cells, *Biochem. Biophys. Res. Commun.* 215 (1995) 446–451.
- [36] B.D. Gehm, J.M. McAndrews, P.Y. Chien, J.L. Jameson, Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor, *Proc. Natl. Acad. Sci. USA* 94 (1997) 14138–14143.
- [37] U.R. Pendurthi, J.T. Williams, L.V. Rao, Resveratrol, a polyphenolic compound found in wine, inhibits tissue factor expression in vascular cells: a possible mechanism for the cardiovascular benefits associated with moderate consumption of wine, *Arterioscler. Thromb. Vasc. Biol.* 19 (1999) 419–426.
- [38] C.D. Surh, J. Sprent, T-cell apoptosis detected in situ during positive and negative selection in the thymus, *Nature* 372 (1994) 100–103.