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Resveratrol Increases Nitric Oxide Synthase, Induces Accumulation of p53 and $p21^{WAF1/CIP1}$, and Suppresses Cultured Bovine Pulmonary Artery Endothelial Cell Proliferation by Perturbing Progression through S and G_2[1]

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ABSTRACT

Epidemiological studies have shown that the regular consumption of red wine may in part account for the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis. This phenomenon, commonly referred to as the French paradox, may be associated with red wine constituents that exhibit tumor-preventive properties as well as inhibit reactions that increase the risk of coronary heart disease. Here we show that resveratrol, a polyphenol in red wine, induces nitric oxide synthase, the enzyme responsible for the biosynthesis of NO, in cultured pulmonary artery endothelial cells, suggesting that resveratrol could afford cardioprotection by affecting the expression of nitric oxide synthase. We also show that resveratrol inhibits the proliferation of pulmonary artery endothelial cells, which, based on flow cytometric analysis, correlates with the suppression of cell progression through S and G_2 phases of the cell cycle. Western blot analysis and immunocytochemical protein detection combined with multiparameter flow cytometry further demonstrate that the perturbed progression through S and G_2 phases is accompanied by an increase in the expression of tumor suppressor gene protein p53 and elevation of the level of cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$. All of the observed effects of resveratrol, including induction of apoptosis at its higher concentration, are also compatible with its putative chemopreventive and/or antitumor activity.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystibene) is a phytoalexin present in plants and various human foods (1). A primary impetus for research on resveratrol has come from the paradoxical observation that a low incidence of CHD[3] may coexist with intake of a high fat diet, a phenomenon known as the French paradox. Because of the French population's preference for red wine, it has been suggested that red wine ingredients, including polyphenolic compounds such as resveratrol, may contribute to the observed congruence of the "French paradox" (1, 2). The exact mechanism by which resveratrol acts to mitigate a high fat diet from increasing the risk for CHD has not been totally elucidated but may relate to its ability to inhibit copper ion-catalyzed oxidation of low-density lipoprotein (3, 4), ribonucleotide reductase (5), DNA polymerase (6), suppressor of cell growth in general (7, 8), and other as yet undiscovered functions. Jang *et al.* (9) reported recently, using model assay systems, that resveratrol acts as a pleiotropic biological effector to regulate initiation, promotion, and progression that underlie malignant transformation. These studies add a new dimension to the expanding role of resveratrol as a potential cancer chemopreventive agent, because it possesses the three basic mechanisms envisaged for such agents, *i.e.*, suppression of cell replication, induction of apoptosis, and restoration of differentiation (or reverse or abnormal differentiation; Ref. 10).

NO is a signal-transducing molecule discovered originally on the basis of its vasodilatory properties (11). It is synthesized by NOS, which has three distinguishable isoforms, NOS-1 (ncNOS), NOS-2, and NOS-3 (ecNOS; Refs. 12 and 13). The last form, constitutively expressed in endothelial cells, may inhibit contractile tone and vascular smooth muscle cell proliferation through paracrinally produced NO (14). Other biological functions ascribed to NO include inhibition of platelet adhesion and aggregation (15–17), reduction of expression of adhesion molecule and chemokines (18–21), and suppression of cell growth and migration (22, 23). These emerging roles of NO play an integral part in the prevention of initiation, progression, and complications of atherosclerosis (23–25) For example, impairment of NO synthesis, or increased inactivation of NO by superoxide radicals, may account for the increased peripheral vascular tone associated with hypertension and may contribute to its clinical consequence (26). Inhibition of NO production in bovine endothelial cells by an L-arginine antagonist reportedly induced DNA replication, promoted cellular transition from prereplicative to replicative phases, and increased c-*myc* and c-*fos* oncogene expression (27).

Endothelial cells are known to play an integral role in maintaining the integrity and functioning of the vascular endothelium. Homeostasis of the vascular endothelium, both in terms of metabolic and physiological activities, is subject to fine tuning by individual nutrients or nutrient derivatives. We therefore investigated the effects of resveratrol on growth and specific gene expression of cultured BPAE cells. We found that resveratrol induced NOS, reduced endothelial cell proliferation, and most interestingly, perturbed progression through the cell cycle, particularly through late S and G_2. Furthermore, the suppression of cell cycle progression upon treatment with resveratrol was accompanied by the accumulation of the tumor suppressor p53 concomitant with the cyclin-dependent kinases inhibitor $p21^{WAF1/CIP1}$.

MATERIALS AND METHODS

Stock solution (12.5 mM) of resveratrol (Sigma Chemical Co.) was prepared in DMSO and stored at −20°C. For treatment, the resveratrol was diluted in RPMI 1640 and added to cultures to give the desired final concentrations. Untreated cultures received the same amount of the carrier solvent (0.2% DMSO).

Cell Culture and Treatment with Resveratrol. The BPAE cells isolated from the distal main intrapulmonary artery of calf lungs were generously provided by Dr. Susan C. Olson of this department. Relative homogeneity of the cell preparation and their identification as endothelial cells by fluorescent staining for diacylated low-density lipoprotein were based on procedures detailed in an earlier publication (28). Cells were routinely maintained in MEM supplemented with 15% fetal bovine serum and containing D-valine instead of L-valine to suppress fibroblast growth, as described (28, 29). Cells seeded at an initial density of 1×10^5 cells/ml were treated with 10, 50, and 100×10^{-6} M resveratrol and maintained for up to 3 days. Cells were harvested by trypsinization, and the cell numbers were determined using a hemocytometer. Cell viability was checked by trypan blue exclusion.

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[3] The abbreviations used are: CHD, coronary heart disease; NOS, NO synthase; ecNOS, NOS-3; BPAE, bovine pulmonary artery endothelial.

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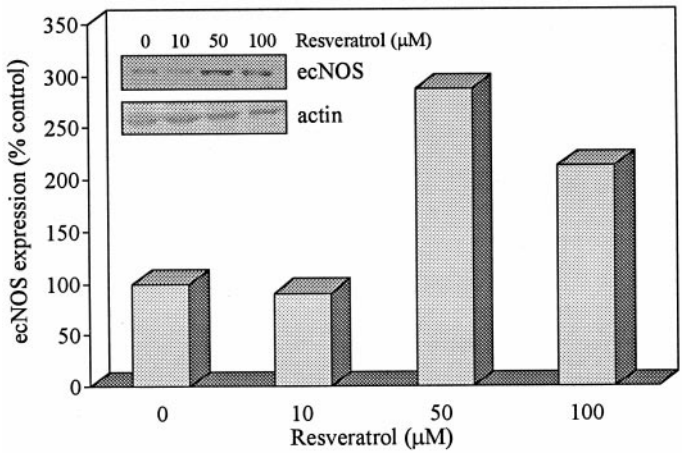

Fig. 1. Effects of resveratrol on expression of ecNOS. Cell extracts from control and day 3 resveratrol-treated cells were electrophoresed using 10% SDS PAGE. The detection of ecNOS, and reprobing of the blots for actin, by Western blot analysis, was as described in "Materials and Methods." The blots were scanned, and the intensity of the bands corresponding to the proteins being assayed was quantified and expressed as normalized arbitrary units. The data were averaged from two separate experiments, each assayed in triplicate.

Measurement of Cell Cycle Progression. The effects of resveratrol on cell progression through the cell cycle were determined as described previously (30–34).

Immunocytochemical Detection of p53. The cells were harvested, washed with PBS, and fixed in suspension in ice-cold 80% ethanol for up to 24 h. After fixation, the cells were washed twice with PBS and then suspended in 1 ml of 0.25% Triton X-100 in PBS on ice for 5 min. The cells were then centrifuged ($300 \times g$ for 5 min); the cell pellet was suspended in 100 μl of PBS containing 0.5 μg of the FITC-conjugated anti-p53 monoclonal antibody (clone DO-7; PharMingen, San Diego, CA) and 1% BSA and incubated for 2 h at room temperature. Parallel cell samples were incubated with anti-$p21^{WAF1/CIP1}$ monoclonal antibody (clone SX118; PharMingen), rinsed with PBS containing 1% BSA, and incubated with FITC-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR), as described (30–34). The cells were then rinsed with PBS containing 1% BSA and resuspended in 5 μg/ml of propidium iodide (Molecular Probes) and 0.1% RNase A (Sigma) in PBS and incubated at room temperature for 20 min before measurement. Control cells were treated identically, except instead of using anti-p53 antibody, the cells were incubated with the isotypic antibody (IgG2b), at the same titer.

Cellular fluorescence was measured with the ELITE ESP flow cytometer/cell sorter (Coulter, Miami, FL) using the argon ion laser (emission at 488 nm). Fluorescence signals were collected using the standard configuration of the flow cytometer (green fluorescence for p53 and red fluorescence for propidium iodide); details of this analysis are presented elsewhere (30–34).

Western Blot Analysis. Control and treated cells were lysed by repeated freeze-thaw cycles with buffer containing 10 mM HEPES (pH 7.5), 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5% NP40, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of the protease inhibitors aprotinin, pepstatin, and leupeptin. Cell-free extracts were obtained by centrifugation in a microcentrifuge. Lysates (7–10 μg) from control and treated cells were separated on 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes, and the membranes were incubated with the respective primary and secondary antibodies. Specific immunoreactive bands were identified by enhanced chemiluminescence or color reaction, as described previously (35–37).

RESULTS

Induction of ecNOS by Resveratrol. Existence of the "French paradox" (1), as well as the large body of epidemiological data pointing to protection by fruits and vegetables against CHD (38), suggest that resveratrol could exert cardiovascular protective functions by effecting specific gene changes in endothelial cells, including those integrally involved in the biogenesis of NO. Because NO is a short-lived gas and virtually impossible to visualize directly, we tested the effects of resveratrol by assaying changes in ecNOS, an isoform of NOS specifically and constitutively expressed in endothelial cells. Western blot analysis showed that treatment of subconfluent BPAE cells with 50–100 μM resveratrol resulted in a 3-fold increase in ecNOS, whereas 10 μM resveratrol had no effect (Fig. 1).

Effects of Resveratrol on Growth of BPAE Cells. To test whether resveratrol affected the growth of BPAE cells, asynchronous cells were incubated with various amounts of resveratrol, and the change in cell number over time was determined. Resveratrol inhibited cell proliferation in a time- and concentration-dependent manner, with 10 μM reducing growth by 30%, and 50–100 μM completely preventing cell proliferation after a 3-day treatment with the polyphenol (Fig. 2). When examined by phase contrast microscopy, control cells displayed the characteristic cobblestone-like growth patterns typical of these cells maintained in culture (28, 29). In contrast, cells treated with 50–100 μM resveratrol assumed a long, spindle-shaped morphology in the tissue culture flasks, which, following trypsinization and resuspension in PBS, appeared under the microscope predominately as giant cells, compared with the controls (data not shown).

Cell Cycle Effects of Resveratrol. Exponentially growing untreated and resveratrol-treated BPAE cells were subjected to flow cytometric analysis after staining their DNA and immunocytochemical detection of either p53 or $p21^{WAF1/CIP1}$ (Fig. 3). The DNA content frequency histograms (Fig. 3, *insets*) clearly indicate that in the presence of resveratrol the cells accumulate in S and G_2-M phases of the cycle. An increase in proportion of S phase cells, from 8 to 14% and of G_2 cells from 4 to 9% was observed at 10 μM concentration of resveratrol.

A much more dramatic effect was evident at 50 μM concentration, where the proportion of S phase was increased to 35% and of G_2 to 42%, concomitant with a drop in proportion of G_1 cells from 82%, in control to 16% (Fig. 3*A*). Few cells altogether were present in the cultures treated with 100 μM resveratrol, but there was predominance of S and G_2 cells in this culture as well (Fig. 3*D*). It is evident from the DNA content frequency histogram as well as from the p53 *versus* DNA, or p21 *versus* DNA scatterplots representing these cultures, that most cells were arrested in the late portion of the S phase. That is, the proportion of cells with DNA index 1.4–1.9 was about 4-fold greater than of the cells with DNA index between 1.1 and 1.3.

The presence of cells with fractional DNA content, which is a

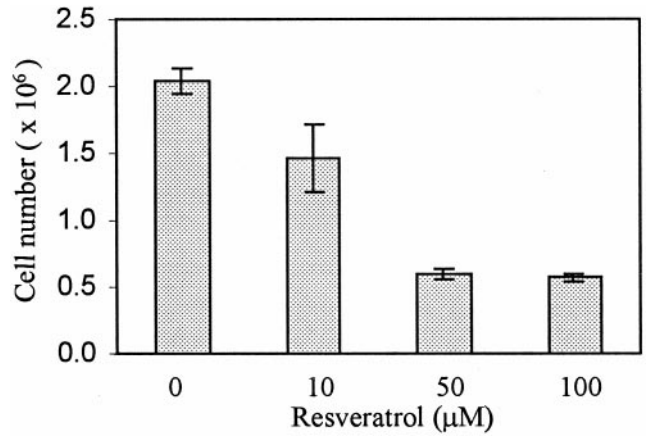

Fig. 2. Effects of resveratrol on growth of BPAE cells. Cell growth and viability, using cells treated with various concentrations of resveratrol for 72 h, was measured as described in "Materials and Methods." Values are the means of two experiments, each assayed in triplicate, with a SE (*bars*) of 15%.

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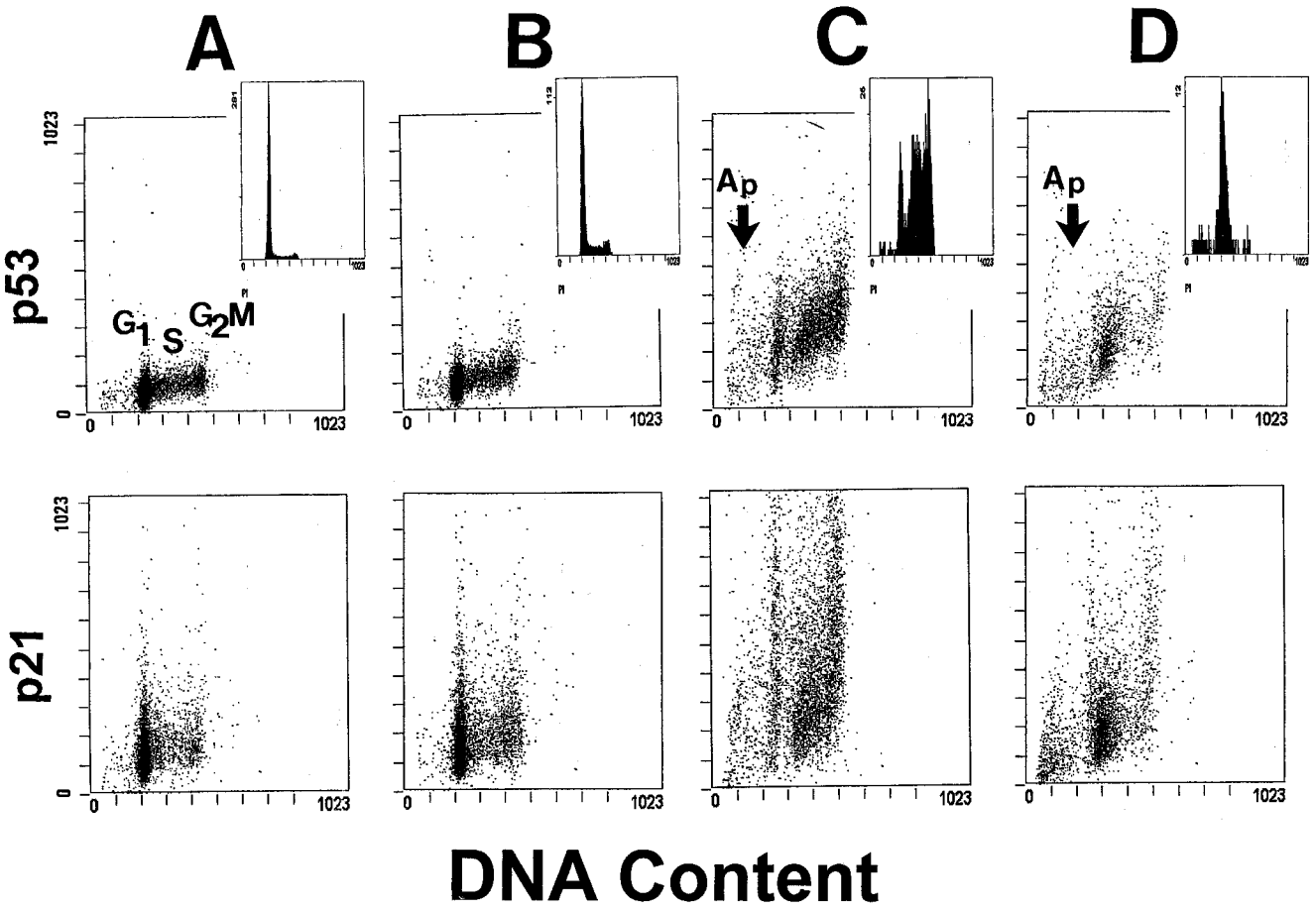

Fig. 3. Effect of different concentrations of resveratrol on cell cycle and expression of p53 and p21$^{WAF1/CIP1}$ of BPAE cells, measured by flow cytometry. Cells were treated with resveratrol for 72 h, and cycle analysis was measured in two separate experiments, with similar results. The scatterplots represent bivariate distributions of DNA content *versus* p53 (*top panels*) or DNA *versus* p21$^{WAF1/CIP1}$ (*bottom*). The cells were untreated (*A*, control) or treated with 10, 50, and 100 μM resveratrol (*B*, *C*, and *D*, respectively) for 72 h. The *insets* represent cellular DNA content frequency histograms that reveal the cell cycle distribution in the respective cultures. On the basis of DNA content, one can distinguish cells in G_1 from S and G_2-M, as shown. Note accumulation of cells in the late portion of S phase (and also in G_2-M) at 50 μM and at the very early portion of S at 100 μM concentration of resveratrol. Apoptotic cells (*Ap*) can be identified on the basis of their fractional DNA content.

characteristic feature of apoptosis (39), was also apparent in the resveratrol-treated cultures. Their frequency was increasing with the increase in concentration of resveratrol, from about 2% in control to 4, 7, and 20% at 10, 50, and 100 μM resveratrol, respectively.

Effect of Resveratrol on p53 and p21$^{WAF1/CIP1}$ Expression. Expression of p53, detected immunocytochemically, was increased in BPAE cells grown in the presence of resveratrol (Figs. 3 and 4). Minor increase, by ~50%, and only in G_2-M cells, was observed at 10 μM resveratrol concentration. The increase, particularly for S and G_2-M cells, was more pronounced at 50 and 100 μM resveratrol concentration (Figs. 3 and 4). Thus, at 50 μM concentration, whereas p53 level in G_1 cells increased by 75%, it nearly quadrupled in S and G_2-M cells. A similar trend was apparent at 100 μM concentration. Interestingly, the expression of p53 was greatly elevated in some cells with fractional DNA content.

The resveratrol-induced increase in expression of p53 in BPAE cells was paralleled by the elevated level of p21$^{WAF1/CIP1}$ (Figs. 3 and 4). As in the case of p53, the rise in p21$^{WAF1/CIP1}$, when presented in the mean values (Fig. 4), was minimal at 10 μM concentration. However, because of intercellular variability in expression of p21$^{WAF1/CIP1}$, even in the absence of significant changes in the mean value (Fig. 4), individual cells showed an elevated level of this protein at 10 μM resveratrol concentration (Fig. 3). The effect was much more pronounced at 50 and 100 μM resveratrol concentration and was observed at all phases of the cell cycle (Figs. 3 and 4).

Analysis of p53 levels by Western blotting confirmed findings by flow cytometry (Fig. 5). Thus, the expression of p53 was up-regulated in a dose-dependent manner, with 50–100 μM resveratrol resulting in a 60 and 72% increase, respectively, of p53. Expression of actin, however, was unaffected and remained comparable with control. Attempts to detect changes in the expression of p21$^{WAF1/CIP1}$ by Western blot analysis, using two commercial antibodies (clones 18A10-H5–63.1 and SX118; PharMingen), failed to detect the presence of immunoreactive bands.

DISCUSSION

The present study was designed to test whether resveratrol, as a polyphenol present relatively abundantly in red wine, may exert cardioprotective effects and, as such, provide part of the mechanism underlying the French paradox. Because endothelial injury is commonly considered by many as playing a pivotal role in initiating atherosclerosis (40), which accounts for the majority of newly diagnosed CHD cases (40, 41), we focused on studying the effects of resveratrol in BPAE. Our data show that the growth of BPAE cells in the presence of resveratrol led to several cellular changes, notably the induction of ecNOS, p53, and p21$^{WAF1/CIP1}$ and suppression of cell proliferation. These data not only provide evidence supporting the hypothesis that resveratrol regulates endothelial cell growth but also for the first time indicate a role for the induction of ecNOS and p53

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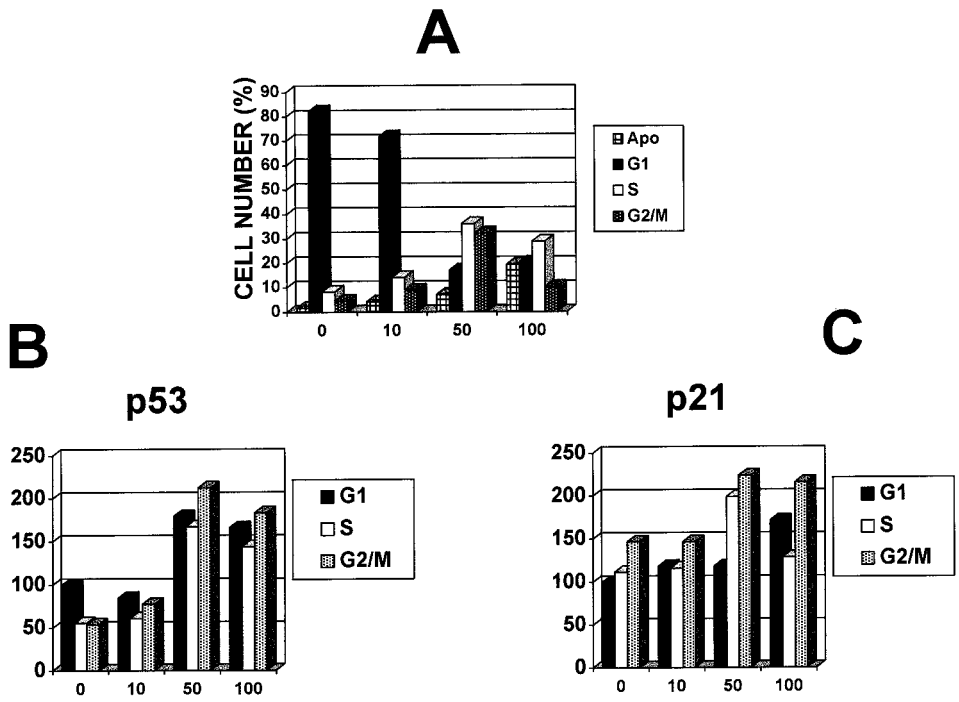

Fig. 4. Resveratrol-induced changes in cell cycle distribution (*A*) and expression of p53 (*B*) and $p21^{WAF1/CIP1}$ (*C*) of BPAE cells treated for 3 days with different concentrations of resveratrol (0–100 μM). The raw data, shown in Fig. 3, were transformed, using the DNA histograms deconvolution program to estimate the percentage of apoptotic cells (*Ap*) and cells in G_1, S and G_2-M phases of the cycle (*A*). The mean values of p53-associated (*B*) or $p21^{WAF1/CIP1}$-associated (*C*) immmunofluorescence were estimated separately for cells in G_1, S, and G_2-M phases of the cycle by gating analysis. These means were then corrected for the component of nonspecific fluorescence, by subtraction of the mean value of the cells stained with isotypic IgG, and normalized to be comparable with the mean value of G_1 cell population of the cells from the untreated culture (= 100).

expression by this polyphenol. It is difficult to assess, at present, to what extent the induction of ecNOS is interrelated to the other changes. It is quite likely, however (as it will be discussed later), that inhibition of cell proliferation is causally associated with the observed up-regulation of p53 and induction of $p21^{WAF1/CIP1}$. Taken together, these results give credence to the notion that resveratrol, through overexpression of ecNOS and p53, may reduce damage to the vascular endothelium and, in turn, the development of disease states such as atherosclerosis and thrombosis.

Regulation of Endothelial Cell Growth and ecNOS by Resveratrol. The ability of resveratrol to modulate endothelial cell proliferation and specific gene expression changes may be significant in several ways. The endothelium, with its endothelial cell lining, presents an extremely critical and vulnerable site for oxidant injury, the results of which are losses of both microvascular metabolic function and barrier properties (25). Vascular injury, due to excess production of reactive oxygen species, is known to occur secondary to diverse phenomena that include trauma, acute inflammation, sepsis, tissue ischemia-reperfusion, oxygen toxicity, and exposure to xenobiotics capable of redox recycling (25). To minimize development of oxidant stress, the endothelial cells rely upon the presence of multiple overlapping defense mechanisms within its cellular milieu, including NO, that collectively serve to protect intracellular sites at risk from oxidant stress, including inhibition of the formation of reactive oxygen intermediates (25, 42). Our studies show that resveratrol, at concentrations comparable with those found in wines and grapes (1, 9), effectively suppresses endothelial cell proliferation and induces ecNOS (Fig. 1). Suppression of cell growth accompanied by their accumulation in the S and G_2 phases of the cell cycle (Figs. 3 and 4) increases the likelihood that any damage, episodic or pervasive, sustained by the endothelial cells could be repaired in an orderly and timely fashion. The cumulative effect of such an inhibition could be decreased propensity for development of endothelial injury, which is often regarded as the triggering event for development of both the fatty streaks and the formation of atherosclerotic plaques (40, 41). Another implication of these findings is that resveratrol, taken in food or beverages, could provide a gradual yet sustained increase in NO production. The NO concentrations reached may be sufficient to tip the balance between prooxidant and antioxidant states, favoring the latter and providing an additional safeguard against endothelial cell damage.

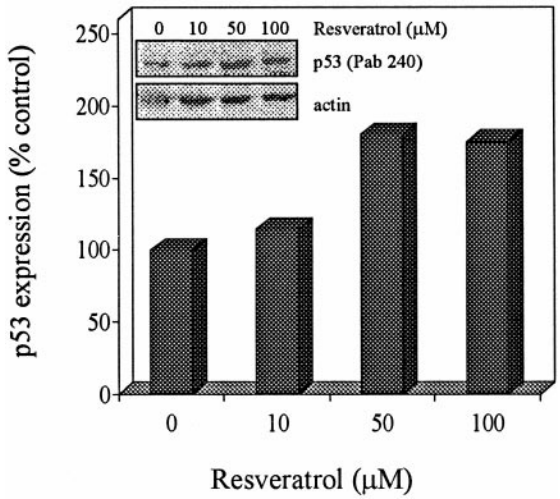

Fig. 5. Effects of resveratrol on expression of p53. Conditions of treatment and Western blot analyses were performed as described in the legend to Fig. 1.

Effect of Resveratrol on Cell Cycle Progression and Expression of p53 and $p21^{WAF1/CIP1}$. As revealed by flow cytometry, the cells accumulated in S and G_2-M phase of the cycle. Because there was no evidence of the increased percentage of mitotic cells in the resveratrol-treated cultures, when examined by microscopy, the observed accumulation in G_2-M indicates cell arrest in G_2 rather than in mitosis. At higher resveratrol concentration and longer exposure times, apoptosis was detected; the apoptotic cells were characterized by fractional DNA content (Fig. 3).

It is plausible that the observed cell cycle effects induced by resveratrol were causally related to the up-regulation of p53 and $p21^{WAF1/CIP1}$. That is, the present findings, using a combination of

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flow cytometry and Western blot analysis, demonstrated that the slowdown in cell progression through the cycle, which manifested in accumulation of cells in S and G_2 phases of the cell cycle, was closely paralleled by the elevated levels of p53 and $p21^{WAF1/CIP1}$. Thus, the cell arrest in S and G_2-M, the increase in p53 and in $p21^{WAF1/CIP1}$, all were observed at 50 and 100 μM resveratrol concentrations. However, although the absolute increase in p53 was most pronounced for cells in S and G_2-M phase (Fig. 3), the increase was of similar magnitude when recalculated per unit of DNA, regardless of the cell cycle position (Fig. 4*B*). The up-regulation of p53 is, most likely, responsible for transcriptional induction of $p21^{WAF1/CIP1}$. The latter is the key inhibitor of the cell cycle progression machinery arresting the cells at check-points, including the G_2 checkpoint (43) to allow for repair of the damage, primarily to DNA. Its up-regulation in BPAE cells by resveratrol, as seen presently, is in all probability directly responsible for inhibiting the cyclin-dependent kinase complexes operated by Cdk2 and Cdc2 (Cdk1), and thereby for suppression of cell transit through S and G_2.

Wild-type p53 is up-regulated in the cell by its increased half-life through inhibition of its degradation (44, 45), as well as modulation of its stability by posttranslational events such as phosphorylation and acetylation (46–50). It is likely, therefore, that its up-regulation in BPAE cells by resveratrol occurs by similar types of mechanism. The role of p53, in addition to induction of $p21^{WAF1/CIP1}$, is also in protection of the genome integrity via physical interaction with DNA, as well as in regulation of cell propensity to apoptosis. The latter function was shown to involve the induction of expression of the apoptosis-promoting gene *bax* (51). High level of p53 expression in the cells with fractional DNA content (Fig. 3, *C* and *D*), *i.e.*, in apoptotic cells, strongly suggests that their apoptosis may be associated with up-regulation of p53.

The resveratrol-induced suppression of BPAE cell proliferation, as observed presently in cultures, if it does occur *in vivo*, *e.g.*, as a result of consumption of this agent, may have several consequences, that is, it is known that cell proliferation, in particular proliferation of vascular smooth muscle cells, plays the key role in pathogenesis of atherosclerosis (22–25, 40, 41). It is likely, therefore, that similar to BPAE, proliferation of vascular smooth muscle cells also is inhibited by resveratrol. If indeed resveratrol consumed as a constituent of red wine is responsible for lowering the incidence of atherosclerosis, this may be one of its possible mechanisms of action.

As mentioned in the "Introduction," there is evidence that resveratrol may have cancer chemopreventive activity. The effects of resveratrol on BPAE cells observed in the present study, *i.e.*, induction of p53 and $p21^{WAF1/CIP1}$, suppression of cell proliferation, cell cycle arrest at specific points in S and G_2, and induction of apoptosis, all are compatible with its putative chemopreventive and/or antitumor activity.

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