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Resveratrol modulates gene expression associated with apoptosis, proliferation and cell cycle in cells with mutated human c-Ha-Ras, but does not alter c-Ha-Ras mRNA or protein expression☆

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Abstract

An accumulating body of evidence suggests that resveratrol can inhibit carcinogenesis through antiproliferative and apoptotic effects. One proposed mechanism for this is the modulation of genes, for example, Ras and p53, frequently associated with human cancer. To test the effect of resveratrol on gene expression, we used the WR-21 cell line because it contains a mutated human c-Ha-ras gene. Cells at ≥70% confluency were incubated with media alone or with increasing concentrations of trans-resveratrol (0.1–1000 μM) for 24 h. Resveratrol (30–100 μM) decreased cellular proliferation by 80% (bromodeoxyuridine incorporation) and increased apoptosis by 60% (TUNEL). Cells were then treated with media alone or with 50-μM resveratrol for 24 h. RNA was isolated for nylon-based macroarray analyses and protein for immunoblotting. Resveratrol increased (+) and decreased (−) gene expression associated with apoptosis (Birc5+, Cash+, Mcl-1+, Mdm2+, Rpa-like+), cellular proliferation (Ctsd+, Mdm2+, Egr1+, ODC+) and cell cycle (cyclin D+, cyclin g+, Gadd45a−, Mad2l−, Mdm2+). Resveratrol consistently increased by ≥6-fold Mdm2 expression and other downstream p53 effectors, but not p53 itself at 24 h. Subsequent cell cycle analysis indicated a significant accumulation of cells in G2/M, and a decrease in G1/G0 suggesting a G2/M blockade. Further RT-PCR and Western blot analyses indicated no differential changes in Ras mRNA expression or $p21^{ras}$ protein levels, respectively. These results suggest that resveratrol potently inhibits cellular proliferation, increases apoptosis, alters cell cycle dynamics and modulates associated gene expression. Furthermore, these effects appear mediated, in part, by p53 without direct modulation of mutant c-Ha-ras expression.

Keywords: Apoptosis; Cell cycle; Proliferation; Ha-ras; p53

1. Introduction

An increasing number of studies clearly suggests that individuals who consume three or more servings of fruits and vegetables per day have a lower risk for cancer [1,2]. This inverse association has been recognized and attributed, in part, to the antioxidant potential of individual components. The mechanisms contributing to this protective effect are unclear but likely involve the interaction of dietary molecules with potentially deleterious reactive oxygen species (ROS).

One dietary antioxidant that has received increasing attention for its potential contribution as a dietary chemopreventive agent is the phytoalexin resveratrol [3–7]. This is based on striking inhibitory effects on cellular events associated with cancer initiation, promotion and progression [8,9]. trans-Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a naturally occurring phytochemical found in grapes, wine, peanuts and cranberries and is produced in response to environmental stress where it functions as a naturally occurring plant antibiotic. The initial interest in resveratrol was for prevention of cardiovascular disease (CVD) because it is found in high concentrations in grape skins and red wines [10]. Consumption of the latter is often inversely

Abbreviations: BrdU, Bromodeoxyuridine; DMSO, Dimethylsulfoxide; GAPDH, Glyceraldehyde phosphate dehydrogenase; LDH, Lactate dehydrogenase; PI, Propidium iodide; ROS, Reactive oxygen species; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TUNEL, Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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associated with CVD. In 1997, a landmark paper clearly suggested the potent chemopreventive effects of resveratrol against the three stages of carcinogenesis [9]. Since then, resveratrol has received increasing attention for its potential contribution as a dietary chemopreventive agent [3–7]. Studies thus far demonstrate that resveratrol inhibits initiation and promotion of carcinogen-induced skin cancer in mice and subsequent malignant progression due, in part, to modulation of metabolism of polyaromatic hydrocarbons, that is, benzo(*a*)pyrene [8,9,11]. The protective mechanism(s) of resveratrol are unclear and merit further investigation particularly regarding in vivo models.

The Ras protooncogene is the most frequently mutated oncogene in human cancer, occurring in ~30% of all human cancers and up to 97% in some cancers [12]. The functional spectrum of the Ras G-protein superfamily encompasses almost all cellular processes due to expansive and functional connectivity of effector pathways [13]. However, any one of many single genetic mutations, and specifically at codons 12, 13, 59 and 61, can foster expression of a highly oncogenic $p21^{ras}$ kilodalton protein locking the cell into an actively proliferating state through continuous activation of kinase signaling pathways [14]. The Ras GTPases function as molecular switches that link external, extracellular stimuli with a diverse range of intracellular outcomes [15]. As a result, a major aspect of the choice between life and death in a cell depends largely on Ras expression. Recently, $p21^{ras}$ has been identified as a common signaling target linking free radical production, alterations in cellular redox status and cell signaling [16]. Moreover, ras is both stimulated by and produces ROS during cell signaling, further supporting the emerging role of low-level ROS in cell signaling pathways and potential disruption by supplemental antioxidants [17]. Since Ras is overexpressed in a large number of cancers and is pivotal in multiple signal transduction cascades, inhibition of Ras signaling has become an emerging therapeutic target.

Mutations of the p53 tumor suppressor gene and the ras protooncogene are the two most prevalent genetic alterations in human cancers [12,18]. Upon DNA damage, p53 halts the cell cycle at the G1 checkpoint and directs mutated, irreparable cells to undergo apoptosis before mutations can become permanent or fixed [19]. The functions of protooncogenic Ras and p53 tumor suppressor proteins are often considered opposing processes in maintaining cell number balance [20]. However, Ras is purported to regulate the p53 pathway through at least three specific pathways, as well as function through p53-independent mechanisms [21]. Moreover, $p21^{ras}$, typically a proliferative protein, may paradoxically induce cell cycle arrest, induce p53 and its target genes to arrest proliferation and induce senescence suggesting potential interaction of p53 and Ras.

The increasing use of individual, purified antioxidants as dietary supplements, often in high doses, has stimulated growing concern due to the lack of scientific data regarding the potential adverse effects on cells. As a result, it is vital to study the effects of antioxidant supplementation at the cellular and molecular levels particularly in the context of neoplasia. The use of transgenic mice designed to overexpress activated forms of oncogenes such as Ras represents a novel approach in understanding the complex multistage nature of carcinogenesis and has permitted linkage of genes with specific tumor processes. One useful novel in vitro model derived from a transgenic mouse is the WR-21 cell line [22]. This cell line was derived from a submandibular salivary adenocarcinoma and induces aggressive highly anaplastic solid tumors when injected into athymic nude mice. Cells from this transgenic mouse express an activated human c-Ha-Ras transgene (mutated at codon 12) and express mutant (Asp 12) human 21-kDa Ras protein as well as p53.

In the current study, we used the WR-21 cell line to explore the potential effect of resveratrol on oncogenic ras expression and subsequent changes in cellular proliferation, apoptosis, cell cycle and associated gene expression. After conducting a dose response study, we selected a single concentration of resveratrol to test at a single commonly used time point to determine any potential effects on gene expression and ultimate cell number balance.

2. Materials and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), resveratrol (3,4′,5-trihydroxy-trans-stilbene) and other cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). Plasticware, including multiwell plates and flasks, was purchased from Corning (Corning, NY).

2.2. Cell culture

WR-21 murine salivary tumor cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in flasks at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown in DMEM containing 4.5 g/L glucose and supplemented with 10% FBS, 1% penicillin/streptomycin solution and 1% amphotericin B solution. Stock flasks were grown to approximately 70% confluency and subcultured routinely. Medium renewal was twice weekly.

For experiments, cells were grown in T25 and T75 flasks or multiwell plates. At approximately 70% confluency, medium was removed and cells were treated with either medium alone containing 0.2% DMSO vehicle control or increasing concentrations of trans-resveratrol (0.1–1000 μmol/L). After a 24-h incubation, medium was removed, cells were washed twice with PBS and harvested according to the specific assay.

2.3. Bromodeoxyuridine incorporation assay

Cellular proliferation was assessed using a bromodeoxyuridine (BrdU) incorporation assay. Control and treated

cells were pulsed with BrdU label (1:2000) at the time of resveratrol addition and incubated for 24 h. After washing the monolayers with HBSS, cells were harvested and BrdU incorporation into DNA determined by ELISA as described by the manufacturer (Trevigen, Gaithersburg, MD). After the addition of stop solution, samples were analyzed at dual wavelengths of 450–540 nm on a SpectraFluor Plus multiwell plate reader (Tecan, Research Triangle Park, NC).

2.4. TUNEL assay

Late apoptosis was analyzed by the TUNEL method using a colorimetric kit as described by the manufacturer (Trevigen). Control and treated cells were washed with HBSS, fixed with buffered formaldehyde, permeabilized with methanol and incubated with biotinylated nucleotide (1 h at 37°C with reaction mix). After incubation, streptavidin–HRP was added followed by stop solution (2 N HCl), and samples were analyzed at 450 nm.

2.5. Agarose gel electrophoresis

Late apoptosis was also analyzed by DNA fragmentation, or DNA banding, using agarose gel electrophoresis. DNA was extracted from control and treated cells using a DNA mini-prep kit (Qiagen, Valencia, CA). DNA (10 μg/well) was loaded onto a 1.2% agarose gel and electrophoresed for 1 h at 85 V using TBE running buffer (89 mM boric acid, 89 mM Tris base, 2 mM EDTA). DNA was visualized by ethidium bromide intercalation into DNA and photodocumented.

2.6. Lactate dehydrogenase release

To determine disruption of the plasma membrane as an indicator of necrosis, we analyzed the release of cytoplasmic lactate dehydrogenase (LDH) into the medium as previously described [23]. An aliquot of media was removed from wells of control and treated cells and centrifuged to remove any nonadherent debris. Monolayers were then washed with HBSS, trypsinized and centrifuged (1000×*g*, 10 min at 25°C). Harvest solution (2 ml of 1% Triton X-100) was added to each pellet, sonicated for 5 s (output control, 2; duty cycle, 20%) using a Branson sonifier (Branson, Golden, CO), and incubated on ice for 1 h. Both conditioned medium and supernatant from the cell suspensions were analyzed. To each sample, reaction mixture (150 μl) containing 85-μM NADH and 23 mM pyruvate in 0.1 M phosphate buffer (pH 7.5) was added to initiate the enzymatic reaction, and the rate of change in the absorption at 340 nm was monitored for 10 min (Beckman DU-640 UV/vis spectrophotometer, Fullerton, CA). The amount of cellular LDH released to the medium was calculated from the maximal rate of change in absorbance and the extinction coefficient 6.3×10^3 L/(mol cm). Data are expressed as nanomole of NADH oxidized per minute per milligram protein.

2.7. Macroarray analysis of gene expression

Differential gene expression was analyzed using three independent pathway-specific nylon-based macroarrays (125 genes per array) containing multiple transcription factors and genes associated with apoptosis, cell cycle or signal transduction. To perform gene array analysis, total RNA was extracted from cell samples using an RNA Midi kit (Qiagen) and quantitated by UV spectroscopy using a Beckman Coulter DU 640 spectrophotometer. Quality of RNA samples was routinely determined by formaldehyde gel electrophoresis run for 1 h at 70 V using MOPS running buffer (200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA). After quantitation, total RNA (5 μg) from either control or resveratrol treated cells was reverse-transcribed to the corresponding cDNA using a proprietary primer mix according to manufacturer's directions (SuperArray, Frederick, MD). The synthesized cDNA probes for control and treated samples were independently hybridized to gene-specific cDNA fragments of specific pathways spotted on nylon membranes. After addition of CDP-Star chemiluminescent substrate (Applera, Norwalk, CT), the macroarrays were exposed to film and quantitated by densitometry using ScanAlyze and GEArray software programs (SuperArray). All arrays were repeated in four to six independent experiments.

2.8. Quantitation of macroarray gene expression

Expression differences between control and treated were calculated by the ratio of control membrane intensity (of gene spot) to the internal control (β-actin) for that membrane divided by the ratio of the treated membrane intensity (same gene spot) to the respective membrane internal control. The use of glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin to calculate response ratios allowed comparison of all independent experiments. A ratio greater than 1 indicated a treatment-related gene induction and ratio less than 1 indicated a treatment-related suppression of gene expression.

2.9. Cell cycle kinetics by flow cytometry analysis

The DNA content of fixed cells was analyzed with propidium iodide (PI) to determine whether resveratrol affected cell cycle kinetics. After treatment with DMSO vehicle or 50-μM resveratrol in T25 flasks for 24 h, media was removed and cells were rinsed with PBS. Cells were trypsinized and collected, and 1×10^6 cells were suspended in PBS. After brief centrifugation (5 min at 1000×*g*), cells were resuspended in 0.5 ml of PBS, and 4.5 ml of 70% ethanol was added to fix and permeabilize the cells.

Cells were centrifuged for 5 min at 1000×*g* and the supernatant was decanted. The cell pellet was washed with PBS and resuspended in 1 ml of PI (100 μg/ml)/0.1% Triton X-100 solution containing RNaseA. After incubation for 15 min at 37°C, the DNA content was analyzed on a Coulter

XL MCL cytometer to determine the relative percentage of cells in each phase of the cell cycle.

2.10. Western analysis for $p21^{ras}$ expression

After 24 h, control and treated monolayers were washed with PBS and lysis buffer (1% Triton X-100, 25 mM Tris HCl, 150 mM NaCl, 5 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany) was added. Cells were mechanically disrupted, transferred to Eppendorf tubes and incubated at 4°C with agitation for 1 h. After incubation, lysed cells were centrifuged for 10 min at 16,000×*g* to precipitate cellular debris. An aliquot of supernatant was used to determine protein concentration by the bicinchoninic acid assay (Pierce, Rockford, IL).

Samples containing 25 μg of protein was separated by SDS-PAGE (4% stacking gel, 12% separating gel), transblotted to a nitrocellulose membrane and probed using a monoclonal rat anti-v-H-ras antibody and an HRP-conjugated IgG secondary antibody (Zymed, San Francisco, CA). A purified Ha-ras standard (Panvera, Carlsbad, CA) was used as a positive control. Membranes were incubated with SuperSignal West Pico chemiluminescent substrate as described by the manufacturer (Pierce), exposed to film and quantitated by densitometry (Scion Image, Frederick, MD).

2.11. Quantitative real time RT-PCR for c-Ha-ras mRNA expression

RNA for the RT-PCR reaction was extracted as described before (Qiagen). Quantitative RT-PCR for c-Ha-ras was performed using the TaqMan procedure and a Perkin-Elmer/Applied Biosystems Division 7700 sequence detector. The forward primer sequence was CTACGGCATCCCCTACATCG and reverse primer sequence was TGTAGAAGGCATCCTCCACTCC. The sequence of the probe was ACCTCGGCCAAGACCCGGCA.

3. Results

3.1. Resveratrol inhibits cellular proliferation and induces apoptosis and necrosis

Preincubation of WR-21 cells with increasing concentrations of resveratrol (1–1000 μM) significantly reduced cellular proliferation as indicated by BrdU incorporation into DNA by 80% at concentrations greater than 30 μM (Fig. 1). We noted a concomitant increase in apoptosis by 60% from 10 to 100 μM followed by a decline of both parameters at higher concentrations. For all subsequent experiments, we selected a resveratrol concentration of 50 μM, which occurred on the linear portions of the curves and was within the concentration range routinely used by others. We corroborated apoptosis by agarose gel electrophoresis (Fig. 2A). DNA fragmentation, characteristic of apoptosis, was evident in resveratrol-treated cells but not in control cultures. In addition to apoptosis, we observed increased necrosis as an alternate form of cell death as determined by the cytoplasmic release of LDH to medium characteristic of plasma membrane disruption. Lactate dehydrogenase release in resveratrol-treated cells (4.5%) was significantly increased by threefold compared to controls (1.5%) (Fig. 2B). There were no differences between control cultures or cells treated with the DMSO vehicle control.

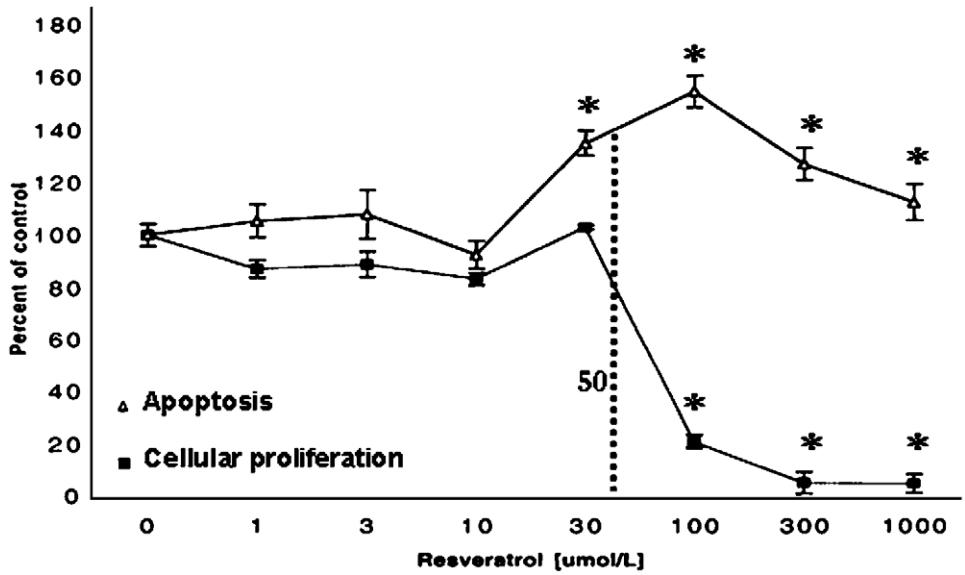


Fig. 1. Apoptosis and cellular proliferation are modulated in WR-21 cells containing a mutated human Ras oncogene incubated overnight with or without increasing concentrations of resveratrol for 24 h. For BrdU incorporation (closed squares), cultures were pulsed for 24 h with BrdU prior to harvest, fixation and analysis by ELISA. For apoptosis (open triangles), cultures were treated for 24 h, harvested, fixed and analyzed by the TUNEL method. Data are expressed as percent response compared to respective control and are means±S.E. from three to five independent experiments.

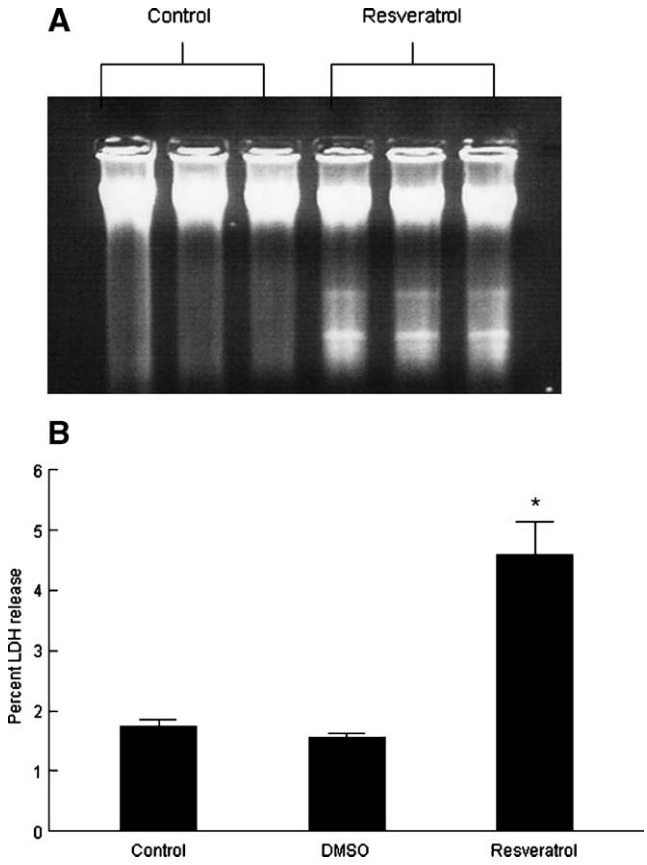


Fig. 2. Resveratrol induces apoptosis and necrosis. Agarose gel electrophoresis of DNA extracted from cells incubated with medium alone or 50-μM resveratrol. DNA banding indicates apoptosis (panel A). Lactate dehydrogenase release by cells incubated in medium alone or media with DMSO vehicle (0.2%) or 50-μM resveratrol. Data indicate cytoplasmic LDH release as a percentage of total LDH activity (media + cellular), and are means±S.E. from three independent experiments ($n=5$) (panel B).

3.2. Resveratrol modulates gene expression associated with proliferative, apoptotic and cell cycle pathways

Resveratrol modulated gene expression of major pathways of cellular proliferation, apoptosis and cell cycle as determined by independent macroarray gene expression arrays. We selected genes of interest based on three criteria including (1) reproducibility between independent experiments, (2) uniformity of responses in the same direction and (3) observation of responses with similar magnitudes (within an order of magnitude).

Resveratrol up-regulated numerous genes associated with signal transduction pathways (Fig. 3). Genes that were notably up-regulated were cathepsin D (Ctsd), mouse 3T3 cell double minute 2 (Mdm2), ornithine decarboxylase (ODC) and early growth response 1 (Egr1), which were increased four-, five-, two-, and threefold, respectively. We did not observe resveratrol-induced suppression of any of the genes associated with this macroarray.

Resveratrol also up-regulated genes associated with apoptotic pathways (Fig. 4). Birc5, Cash, and Mcl-1 are inhibitors of apoptosis and were up-regulated up to sevenfold after resveratrol treatment. Mdm2 appeared on the apoptosis arrays and induction was corroborated since expression increased ninefold. RPA-like, a p53 responsive gene, was also up-regulated by fourfold.

Resveratrol increased the expression of genes associated with cell cycle (Fig. 5). The most notable increases in gene expression were cyclin D (Ccnd1) and cyclin G (Ccng), which both increased by approximately fourfold. Given the pivotal importance of Mdm2, it appeared on this third set of independent arrays and was increased by sixfold, but neither p53 nor Gadd45a, a p53-dependent gene, were differentially expressed at the 24-h time point. Gene expression of Mad2l was decreased by threefold.

3.3. Resveratrol induces G2/M cell cycle arrest

Since genes associated with cell cycle were altered, we next analyzed cells by flow cytometry to determine whether resveratrol had any effects on cell cycle kinetics or distribution of cells in the cell cycle. The results indicated a clear 50% reduction of cells in G1/G0 and a marked 400% increase in cells accumulating in the G2/M phase. Cell numbers in S-phase were not different between control and resveratrol-treated cultures (Fig. 6).

3.4. Resveratrol does not alter Ras protein or mRNA expression after 24 h incubation

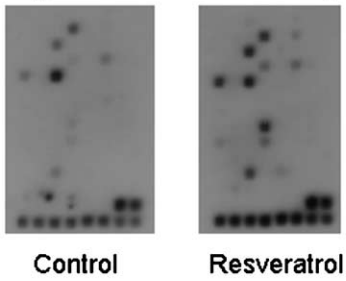
In view of accumulating data, the next question centered on whether Ras expression at either the mRNA or protein levels would be altered at the 24 h time point. Western blot analysis indicated no differential changes in expression of $p21^{ras}$ protein (Fig. 7A) between groups. Data were normalized to the internal housekeeping gene β-actin (Fig. 7B). At the mRNA level, we analyzed samples by quantitative real time RT-PCR and found no resveratrol-related effect on Ras oncogene expression (Fig. 7C).

4. Discussion

The current project was designed to reveal the effect of resveratrol on ras-mediated gene expression in the context of neoplasia. In this study, we noted clear induction of apoptosis and concomitant inhibition of cellular proliferation in cells incubated with resveratrol with some evidence of cytotoxicity. Cells also accumulated in the G2/M phase of the cell cycle at 24 h with clear induction of genes associated with a p53-mediated effect. However, oncogenic $p21^{ras}$ mRNA and protein were similarly expressed in control and resveratrol-treated cells at 24 h. We also observed altered gene expression associated with apoptotic, cell signaling and cell cycle pathways.

Resveratrol potently inhibited cellular proliferation and induced apoptosis in novel WR-21 cells in agreement with the results of others. For example, resveratrol inhibited cellular proliferation and apoptosis in promyelocyte leukemia cells (HL60), MCF-7 human breast cancer cells, Caco-2

A



B

Birc2	Birc3	Bmp2	Bmp4	Brca1	Ccnd1	Cd5	Cdk2
Cdkn1a	Cdkn1b	Cdkn1c	Cdkn2a	Cdkn2b	Cdkn2c	Cdkn2d	Cdx1
Cebpb	Csf2	Csnb	Ctsd	Cyp19	Egfr	Egr1	Ei24
En1	Fasn	Fn1	Fos	Foxa2	Gadd45a	Gys1	Hip
Hk2	Hoxa1	Hoxb1	Hsf1	Hsp25	Hsp90	Icam1	Igfbp3
Il2	Il2ra	Il4	IL4R	Irf1	Jun	Junb	Klk3
Klk6	Lep	Lta	Mdm2	Mmp10	Mmp7	Myc	N4wbp4-pend
Nfkb1	Nfkbia	Nos2	Odc	Pgr	Pkca	Prkce	Ptch
Ptch2	Pten	Ptgs2	Rbp1	Rbp2	Scya2	Sycb9	Sele
Selp	Stra6	Stra8	Tnfa	Tnfrsf10b	Tnfrsf6	Tnfsf6	Trfr
Trim25	Trp53	Vcam1	Wisp1	Wisp2	Wnt1	Wnt2	Wsb1
PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapd
Ppia	Ppia	Ppia	Ppia	Rpl13a	Rpl13a	Actb	Actb

C

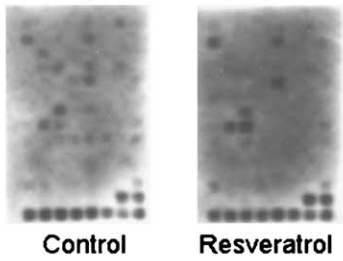
Gene Name	Fold change
Ctsd	+ 3.4
Mdm2	+ 7.1
ODC	+ 3.1
Egr1	+ 1.9

Fig. 3. Resveratrol up-regulates genes associated with pathways of signal transduction. Cells were incubated with media (0.2% DMSO vehicle) alone or with 50-μM resveratrol for 24 h. After harvest, RNA was extracted and quantitated to generate separate probes by PCR using reverse transcriptase. Each membrane was independently incubated with probe overnight, washed and exposed to film before densitometric quantitation (panel A). A key, indicating the location of each gene on the macroarray, is pictured in panel B. Expression differences were calculated by the ratio of resveratrol-treated membrane intensity (of specific gene spot) to its internal housekeeping gene and divided by the ratio of the control membrane intensity (same gene spot) to its internal housekeeping gene. GAPDH and β-actin were used to calculate response ratios (RRs). RR < 1 indicates resveratrol inhibition of gene expression and RR > 1 indicates resveratrol induction of gene expression (panel C).

enterocytes and two human prostatic cell lines (pPANC-1 and AsPC-1) over identical times and concentrations [24–27]. Inhibitory concentrations (IC50) have been reported over a range of 5–150 μM in various cell types and overlap with the concentration of resveratrol used in our study [27–30]. We also noted cytotoxicity at 50 μM as revealed by increased LDH release in agreement with others using keratinocytes, HL-60 cells and prostate cells over an identical concentration range [24]. Thus, resveratrol induced both apoptosis and necrosis, which is consistent with the notion that the processes typically occur simultaneously to some extent.

Our results indicated a clear G2/M arrest in WR-21 cells. In human leukemia and HT-29 colon cancer cells, resveratrol inhibited proliferation and induced apoptosis that was preceded by dose-dependent cell cycle arrest in the G2/M phase correlating with increased expression of cyclins A and B, although in another study progression from S to G2 phase in Caco-2 cells, was perturbed at 50 μM resveratrol [27,28,31]. In other cell lines of various histogenetic origin including fibroblasts, mouse mammary epithelial cells, human breast, colon, and prostate cells, resveratrol (IC50 20–100 μM) reduced the percentage of cells in G2/M phase and increased cells in S phase often with decreases in cyclin

A



B

Apaf1	April	Arc	Asc-pend	Atm	Bad	Bak	Bar-like
Bax	Bcl10	Bcl2	Bcl2a1d	Bcl2l	Bcl2l10	Bcl2l2	Bid
Bid3	Biklk	Bim	Birc1a	Birc1b	Birc1e	Birc2	Birc3
Birc4	Birc5	Birc6	Blk	Bnip3	Bokl-pend	Cash	Casp1
Casp11	Casp12	Casp14	Casp2	Casp3	Casp6	Casp7	Casp8
Casp8ap2	Casp9	Cdkn1a	Chek1	Cidea	Cideb	Cradd	Dapf2
Dffa	Dffb	DR6	Fadd	Gadd45a	Hus1	Lta	Ltb
Ltbr	Mcl1	Mdm2	Myd88	Nop30-like	Rad53	Ripk1	Rpa-like
Tank	Tnf	Tnfrsf10b	Tnfrsf11a	Tnfrsf11b	Tnfrsf12	Tnfrsf1a	Tnfrsf1b
Tnfrsf4	Tnfrsf5	Tnfrsf6	Tnfrsf7	Tnfrsf8	Tnfrsf9	Tnfsf10	Tnfsf11
Tnfrsf12	Tnfrsf14	Tnfsf4	Tnfsf5	Tnfsf6	Tnfsf7	Tnfsf8	Tnfsf9
Traf1	Traf2	Traf3	Traf4	Traf5	Traf6	Traip	Trp53
PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapd
Ppia	Ppia	Ppia	Ppia	Rpl13a	Rpl13a	Actb	Actb

C

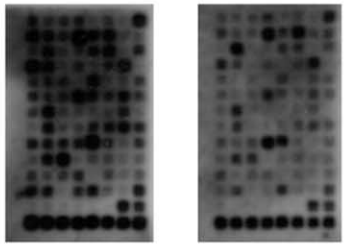
Gene name	Fold change
Birc 5	+ 3.7
Cash	+ 2.6
Mcl1	+ 5.7
Mdm2	+ 9.7
Rpa-like	+ 3.7

Fig. 4. Resveratrol up-regulates genes associated with apoptotic pathways. Samples were processed as described in Fig. 3 legend.

D1 expression [29,30]. In HL-60 promyelocytes, resveratrol (30 μM) arrested cellular proliferation in the S/G2 phase transition with the absence of cells in G2/M. Instead, cells accumulated in G1 and S phases after 24 h with increases in expression of cyclins A and E [32]. Our results uniquely demonstrate increases in cyclins D and G, but not A and E, and arrest in G2/M, and not S, as demonstrated by others suggesting a cell-specific, resveratrol-dependent or interactive effect. Our results also suggest, in accordance with numerous reports, that p53 may function at the G2/M checkpoint in a cell type-specific manner [33].

The putative anticarcinogenic properties of wine polyphenols such as resveratrol occur largely through p53-dependent apoptosis and cell cycle arrest [34]. We noted clear induction of downstream effectors of p53 expression, but not p53 itself, at 24 h strongly supporting the involvement of p53 signaling. In HepG2 and Hep3B, two human liver cancer lines, resveratrol induced apoptotic death through a p53-mediated pathway [35–37]. Additionally, resveratrol suppressed cell transformation and induced apoptosis through a p53-dependent pathway in fibroblasts and murine epidermal cells [38]. In another study, resveratrol induced apoptosis via a p53-mediated effect in fibroblasts after the induced expression of oncogenic H-ras [39]. Ras functions via a MAPK signal transduction pathway to increase p53 expression, serine-15 phosphory-

A



B

Abl1	Apaf1	Atm	Bax	Bcl2	Brca1	Ccna1	Ccna2
Ccnb1-rs1	Ccnb2	Ccnc	Ccnd1	Ccnd2	Ccnd3	Ccne	Ccne2
Ccnf	Ccng	Ccng2	Ccnh	Cdc16	Cdc20	Cdc25a	Cdc25b
Cdc2a	Cdc37	Cdc45l	Cdc6	Cdc7l1	Cdk2	Cdk4	Cdk5r
Cdk6	Cdk7	Cdk8	Cdkn1a	Cdkn1b	Cdkn1c	Cdkn2a	Cdkn2b
Cdkn2c	Cdkn2d	Chek1	Cks1	Cul1	Cul2	Cul3	Cul4a
Cul4b	Dp1	Dp2	E2f1	E2f2	E2f3	E2f4	E2f5
E2f6	Foxm1	Gadd45a	Hus1	Mad2l1	Mcmd	Mcmd2	Mcmd4
Mcmd5	Mcmd6	Mcmd7	Mdm2	Mki67	Mre11a	Nbn	Nedd8
Nfkbia	Pcna	Prc1	Rad17	Rad50	Rad51	Rad53	Rad9
Rb1	Rbl1	Rbl2	Rbx1	Roc2-pend	Rpa	Skp2	Tceb1l
Timp3	Trp53	Trp63	Ubc	Ube1x	Ube3a	Ubl1	Ywhae
PUC18	PUC18	PUC18	Blank	Blank	Blank	Gapd	Gapd
Ppia	Ppia	Ppia	Ppia	Rpl13a	Rpl13a	Actb	Actb

C

Gene name	Fold change
Ccnd1	+ 3.7
Ccng	+ 4.3
Gadd45a	- 2.8
Mad2l	- 5.3
Mdm2	+ 6.3
p53	- 2.4

Fig. 5. Resveratrol modulates genes involved in cell cycle progression. Samples were processed as described in Fig. 3 legend.

lation, p53-DNA binding and p53-dependent apoptosis in papillary thyroid, follicular thyroid carcinoma cell lines and DU145 cells [40,41]. Although we did not observe a difference in ras expression, interaction of resveratrol with constitutive oncogenic Ras expression may have modified and contributed to the observed response.

To examine gene expression, we analyzed differences using nylon-based membrane arrays targeting specifically apoptosis, cell cycle and signaling pathways. We noted up-regulation of several genes associated with p53-mediated apoptosis and cell cycle arrest. Birc5, or survivin, typically decreases in apoptosis, but we noted a ~2.5-fold increase. It is noteworthy that Birc5 regulates cellular division and functions as a chromosomal passenger protein required during mitosis in the G2/M phase suggesting involvement in cell cycle arrest [42]. The role of survivin in apoptosis is unclear and studies have defined a novel p53-survivin signaling pathway activated by DNA damage that results in decreased survivin, cell cycle arrest and apoptosis involving Mdm2 cleavage [43,44]. We also noted three- to fourfold increases in CASH, also known as usurpin, and Mcl-1, a bcl-2 family protein. Mcl-1 is cleaved during apoptosis

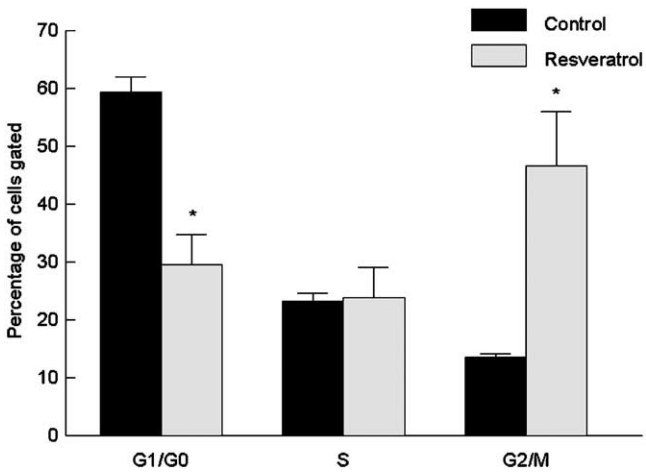


Fig. 6. Resveratrol alters cell cycle dynamics of WR-21 cells. Cells were incubated 24 h with or without resveratrol and harvested as described previously. Cells were stained with PI and analyzed by flow cytometry. Fig. 6 displays quantitation of data expressed as percent of cells in each phase of cell cycle.

producing a proapoptotic molecule induced by survival and differentiation signals such as cytokines and growth factors, that is, Ras [45]. Evidence also exists that MCl1 binds PCNA causing cell cycle arrest. Lastly, RPA-like, replication protein A was up-regulated by fourfold and is a major eukaryote single-stranded DNA binding protein required for DNA replication, repair (nucleotide excision) and recombination-DNA metabolism [46]. Moreover, RPA-like may function in DNA damage responses through p53 up-regulation, p53 inactivation and decreased binding of p53 protein [47]. Collectively, numerous downstream effectors of p53 were modulated supporting a p53-mediated effect.

We next analyzed genes associated with cell signaling pathways. Cathepsin D, a protease, was up-regulated by fivefold and is often associated with metastases. However, resveratrol can up-regulate expression of cathepsin D by 50–100% in numerous cell lines, although there appears to be no correlation between Ha-ras expression and cathepsin activity [48,49]. Endocytosis and exocytosis of cathepsin B and targeting of lysosomal proteases may be regulated by ras and ras-related proteins in multiple cell types including fibroblasts and epithelial cells [50,51]. Ornithine decarboxylase activity was up-regulated 2.5-fold after resveratrol exposure in contrast to reports indicating up to twofold reductions in Caco-2 cells [52]. Ras can induce ODC activity, and in fact, oncogene overexpression in ras12V mutant NIH3T3 cells increases ODC activity by 20-fold [53]. Moreover, ODC is induced by growth factors, estrogens and potentially phytoestrogenic resveratrol promoting G1-S phase progression. Although purportedly non-DNA damaging, resveratrol can interfere with DNA replication and cell division and activate Egr1 transcription supporting cell cycle arrest rather than DNA damage as an inducer [54,55]. Egr1 expression increased fourfold in this report. Resveratrol (50 μM) induced Egr1 expression >3-fold in human embryonic kidney cells and induced apoptosis by stimulating p53 synthesis [56,57]. Although Egr1 is growth promoting, Egr1 can be proapoptotic due to increased p53 synthesis by direct activation of the p53 promoter, enhanced binding of the transcription factor c-jun, and transactivation of the PTEN gene [56].

Cell cycle progression is regulated by regulatory cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors [58]. In this study, cyclin D was unexpectedly increased fourfold. Overexpression is a common observation associated with human tumorigenesis and metastases. Cyclin D couples extracellular signals to cell cycle and, upon

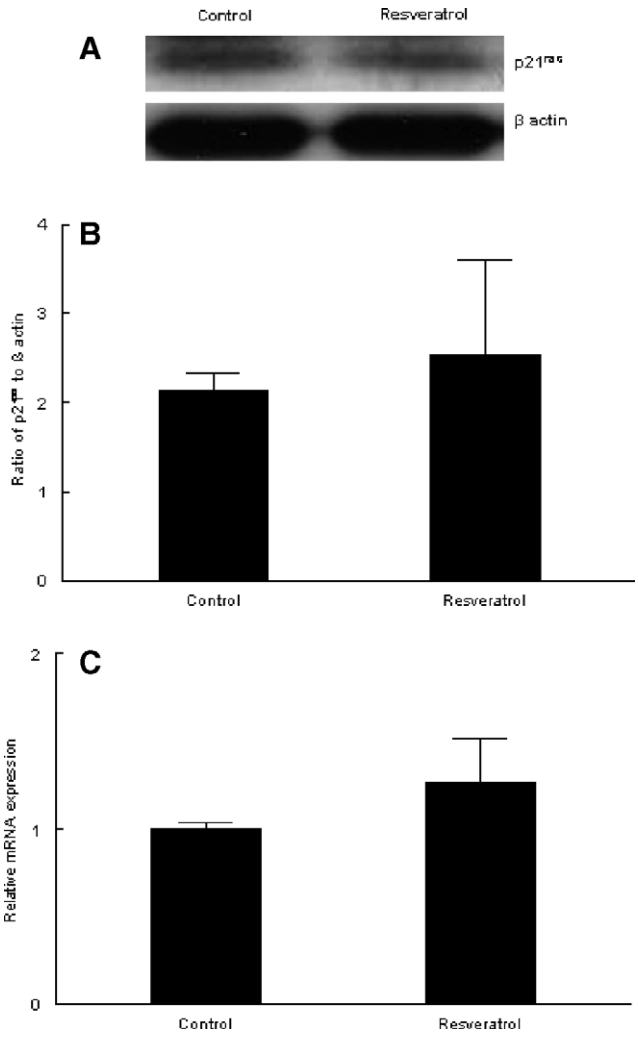


Fig. 7. Resveratrol treatment does not alter $p21^{ras}$ protein or mRNA expression. Cells were analyzed by Western blotting to detect c-Ha-ras oncoprotein expression as described in Materials and Methods. The autoradiograph shows representative samples from control (0.2% DMSO vehicle) and resveratrol-treated WR-21 cells (panel A). Data were quantitated by densitometry using Scion Image software and are normalized to β-actin expression. Data are means±S.E. each conducted in six independent experiments (panel B). Ras mRNA expression was analyzed by real-time RT-PCR using ribosomal 18S as an internal control. Representative results from three independent experiments are shown (panel C).

mitogenic stimulation, for example, Ras, is activated, and the cells progress from G0 to G1. Thus, resveratrol, which typically decreases cyclin D expression, may be facilitating increased ras-mediated cyclin D expression [27].

In this study, we noted a clear decrease in cells at the G0/G1 phase, a concomitant shift of cells to G2/M and no difference in S phase suggesting a cell cycle block at G2/M. In other studies, resveratrol strongly inhibited cell growth in a dose- and time-dependent manner through increased expression of cyclins A, E and B1, and accumulation in G0/G1, G2/M and S phases in human melanoma cells [59]. Resveratrol also induced arrest in S phase in several cancer cell lines including MCF-7, HL60 and SW480 [30]. Similar to our data, resveratrol inhibited proliferation and disrupted cell division in HT29 colon cancer cells with accumulation of cells in G2 phase [31]. Resveratrol and other stilbenes have been shown to induce G2/M arrest as clearly demonstrated in this study [60].

Our data indicate increased cyclin G expression by fivefold, which functions in G2/M transition control and increases apoptosis [61]. Cyclin G has also been identified as a target gene of p53, is Mdm2 dependent and expression is increased after p53 induction [62,63]. Specifically, cyclin G is a regulatory component of the PP2A holoenzyme, which activates Mdm2 through dephosphorylation, and subsequently inhibits p53 expression [64]. Overexpression of cyclin G also increases apoptosis. Mdm2 is up-regulated by p53 and is an autoregulatory inhibitor of p53 targeting its destruction [65]. Resveratrol consistently increased by >4-fold Mdm2 expression and cyclin G expression in WR-21 cells, but not p53 or $p21^{cip/waf}$, at 24 h. p53 activation typically induces a G1 arrest through induction of $p21^{cip/waf}$ and concurrent inhibition of cyclin D. The absence of p53 and $p21^{cip/waf}$ expression is likely due to preferential G2/M arrest as noted here, instead of G1 arrest, the temporal nature of protein expression, and selected time point.

Numerous studies demonstrate that p53 and cyclin G function at the G2/M checkpoint [61]. This checkpoint is activated when DNA synthesis is blocked and prevents segregation of damaged or incompletely synthesized DNA. Resveratrol effectively inhibits ribonucleotide reductase, which catalyzes reduction of ribonucleotides into corresponding deoxyribonucleotides, and prevents DNA synthesis and cellular proliferation [66]. p53 is also involved in spindle checkpoint that blocks rereplication of DNA when the mitotic spindle has been damaged by inhibiting entry into the S phase. Our results indicate no difference in DNA synthesis, although a potent inhibition of DNA replication was observed when cells were incubated with resveratrol. Although p53 was not up-regulated by resveratrol at 24 h, we did observe a marked, increased expression of cyclin G, which has not been reported before. This suggests an interaction among cyclin G, p53 and resveratrol.

Activation of Ras during normal cell signaling or through mutation in neoplasia can suppress p53, and as a result, facilitate cellular proliferation and survival. Subsequently, Ras-induced Mdm2 expression may block p53 from inducing apoptosis or growth arrest in the early stages of tumor development allowing coexistence of Ras mutations and wild-type p53. Elevated mdm2 protein levels induced by Ras activation may bind mutated forms of p53 and abolish any remaining p53 function [20]. The clear expression of oncogenic $p21^{ras}$ suggests that Ras may be involved in the observed protective effects through, perhaps, interaction with resveratrol. Thus, although no changes occurred in $p21^{ras}$ protein or mRNA levels at 24 h, alterations and interactions at the functional level are possible. It will be critical to determine alterations in posttranslational modification of the $p21^{ras}$ protein, alterations in intracellular mobilization and interactions with resveratrol as well as the temporal expression of proteins associated with apoptosis, cellular proliferation and cell cycle.

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