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Time-dependent resveratrol-mediated mRNA and protein expression associated with cell cycle in WR-21 cells containing mutated human c-Ha-Ras

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Cancer results from an undesirable imbalance between cellular proliferation and apoptosis. Both processes may be modulated at the level of gene expression, viz., p53 and c-Ha-ras, by dietary bioactive components such as resveratrol. We tested the time-dependent effect of resveratrol on gene and protein expression in WR-21 cells containing a mutated human c-Ha-ras oncogene. We demonstrate cyclic resveratrol-mediated expression of p53, mdm2, $p21^{cip/waf}$, Rb, and cyclin G at both the RNA and the protein level at < 8 h. However, ras was not differentially expressed at either the RNA or the protein level. p53 was upregulated followed by $p21^{cip/waf}$, then mdm2, and cyclin G, all downstream p53-activated targets. RNA transcription increased at > 8 h for all genes except p53, but protein levels did not suggest uncoupling of transcription and translation. At 24 h, both p53 and Rb expression returned to baseline, suggesting collapse of DNA structure and spindle assembly checkpoints characteristic of mitotic catastrophe. In summary, resveratrol at < 8 h induced p53-mediated effects, including apoptosis and cell-cycle arrest (G2/M). However, later, it induced cell-cycle checkpoint dysfunction, indicative of mitotic catastrophe. Thus, future studies should better elucidate the temporal mechanism of the dietary bioactive agent resveratrol on cancer cells.

Keywords: c-Ha-ras / mRNA expression / p53 / Protein expression / Temporal

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1 Introduction

Cancer is a leading cause of death in the United States. Accumulating evidence suggests that bioactive dietary compounds such as resveratrol may protect against processes associated with carcinogenesis. Indeed, numerous dietary compounds may modulate gene expression and signal transduction pathways associated with neoplasia, including proliferative, apoptotic, and cell-cycle pathways [1–3]. Two pathways of particular interest because they are the most frequently mutated in cancer, are the c-Ha-Ras oncogenic proliferative pathway and the p53 tumor suppressor protein-mediated apoptosis and cell-cycle arrest pathways [4–6]. The potential modulation of these pathways by bioactive agents represents a promising chemoprevention strategy. However, more information is needed regarding the temporal influences of dietary exposure of these bioactive agents on gene and protein expression.

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The Ras proto-oncogene is the most frequently mutated oncogene in human cancer, occurring in ~30% of all human cancers and up to 97% in some cancers [7]. The functional spectrum of the Ras G-protein superfamily encompasses almost all cellular processes due to crosstalk of multiple effector pathways [8]. However, any one of many single genetic mutations, and specifically at codons 12, 13, 59, and 61, can foster expression of a highly oncogenic $p21^{ras}$-kDa protein, locking the cell into an actively proliferating state through continuous activation of kinase signaling pathways [9]. As a result, a major aspect of the choice between life and death in a cell depends largely on Ras expression. As Ras is overexpressed in a large number of cancers and is pivotal in multiple signal transduction cascades, inhibition of Ras signaling, particularly by dietary bioactive agents, has become an emerging therapeutic target.

Mutation and/or loss of the p53 tumor suppressor gene are some of the most frequent mutations in human cancers with an average incidence of 70 and up to 95% in some lesions [10, 11]. Upon DNA damage, this transcription factor halts the cell cycle at the G1 checkpoint primarily and directs the mutated, irreparable cells to undergo apoptosis before

mutations can become permanent or fixed. Numerous downstream effectors of p53 link it to co-regulation of apoptosis, cell cycle, and DNA repair. Thus, modulation of p53-mediated events is also a critical therapeutic target.

Mutations of the p53 tumor suppressor gene and the Ras proto-oncogene are the two most prevalent genetic alterations in human cancers [7, 10]. The functions of Ras and p53 proteins are often considered opposing processes in maintaining cell number balance [12]. However, Ras may regulate the p53 pathway through at least three specific pathways, as well as function through p53-independent mechanisms [13]. 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. Oncogenes can also induce proliferation and promote cellular survival, as well as induce apoptosis and growth arrest via the simultaneous production of proliferative and antiproliferative signals. Thus, it is essential to better understand the interaction of such pathways in the cancer process as well as the temporal effects of dietary compounds in order to develop effective strategies for cancer therapies [14 – 16]. However, little information is available regarding time-dependent effects on specific relevant gene combinations such as p53 and c-Ha-ras.

One dietary bioactive agent that has received increasing attention for its potential contribution as a chemopreventive agent is the phytoalexin resveratrol, particularly regarding its ability to modulate p53 gene expression [4, 17, 18]. Trans-resveratrol (3, 4′,5-trihydroxy-trans-stilbene) is a naturally occurring dietary phytochemical found in grapes, wines, peanuts, and cranberries. It is produced in response to environmental stress where it functions as a naturally occurring plant antibiotic. Of particular interest is that resveratrol has been shown to inhibit cellular proliferation in numerous diverse human cell lines [19 – 21]. Moreover, resveratrol has been shown to induce p53-dependent apoptosis in human breast cancer cells, human neuroblastoma cells, and human promyelocytic leukemia cells as examples, suggesting efficacy in other cancer models [20 – 23]. It has also been shown that resveratrol can induce cell-cycle arrest and apoptosis via p53-dependent mechanisms [23 – 25]. We have demonstrated using WR-21 salivary adenocarcinoma cells with mutated oncogenic ras, that resveratrol can induce apoptosis, inhibit cellular proliferation, and induce cell-cycle arrest at a single time point [26]. As a result, although resveratrol can be protective in carcinogenesis, data are unclear regarding the ability of resveratrol to affect positively or negatively cell-cycle arrest, apoptosis, or mitogenesis and may reflect the dependence of duration or timing of dietary exposure. The temporal relationship of expression of key genes and proteins associated with these processes is lacking, particularly regarding mechanistic action.

In the current study, we used the WR21 cell line to explore the effects of resveratrol, in combination with oncogenic ras, on the temporal expression of six pivotal genes and proteins associated with p53 and ultimately cell cycle and apoptosis. Since these genes and gene products are involved in pathways that interact, it is critical to determine the temporal expression of mRNA and protein associated with apoptosis, cellular proliferation and cell cycle to better discern and identify potential chemoprotective targets. To that end, we have selected for further study p53, Mdm2, $p21^{cip/waf}$, cyclin G, Ras, and Rb.

2 Materials and methods

2.1 Reagents and cell culture

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

WR21 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. At approximately 70% confluency, medium was removed and cells were treated with either medium alone containing 0.2% DMSO vehicle control or trans-resveratrol (50 μM). Our pilot studies have indicated an effective concentration range of 30 – 100 μmol/L in WR21 cells. Thus, we selected 50 μmol/L because it occurs on the linear part of the curve [26]. After medium was removed, cells were washed twice with PBS, and harvested according to the respective protocol for each experimental assay at the following time intervals: 0, 2, 4, 6, 8, 12, 18, and 24 h. Experiments were repeated independently two-to-three times and samples for each time point were pooled for either Western blot analysis or RT-PCR. The rationale for pooling samples was to circumvent the intrinsic variability that often accompanies these techniques.

2.2 Western analysis for protein expression

After each selected time point (0, 2, 4, 6, 8, 12, and 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 for 1 h with agitation. After incubation, lysed cells were centrifuged for 10 min at $16000 \times g$ to precipitate cellular debris. An aliquot of supernatant was used to determine protein concentration by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Samples containing 25 µg of protein were separated by SDS-PAGE (4% stacking gel, 12% separating gel), transblotted to an NC membrane, and probed independently using polyclonal rabbit anti-p53, -mdm2, -p21$^{cip/waf}$, -c-Ha-ras, -cyclin G, and -pRb antibodies with subsequent addition of HRP-conjugated IgG secondary antibody (Zymed, San Francisco, CA). 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). The NC membrane was then probed with an HRP-conjugated anti-β actin antibody, incubated with chemiluminescent substrate, and exposed to film to ensure that each lane was loaded with an equal amount of protein and to permit normalization.

2.3 Quantitative real time RT-PCR for p53, mdm2, p21$^{cip/waf}$, c-Ha-ras, cyclin G and pRb mRNA expression

RNA for the RT-PCR reaction was extracted as described before using an RNeasy midi preparatory kit (Qiagen, Valencia, CA). Quantitative RT-PCR for p53, mdm2, p21$^{cip/waf}$, c-Ha-ras, cyclin G and pRb was performed using the TaqMan procedure and a Perkin-Elmer/Applied Biosystems Division 7700 sequence detector with the assistance of the Nucleic Acids Core at the Pennsylvania State University. For all samples, forty cycles of reverse transcription were conducted and the forward and reverse primers and probe sequences are listed in Table 1.

2.4 Statistical analysis

Samples from each independent experiment at each time point were pooled for subsequent Western blotting and RT-PCR, generating a single homogeneous mixture for each.

3 Results

In a previous report, we noted no differential expression of p53 after incubation with resveratrol for 24 h [26]. Thus, our first aim was to determine if p53 expression was detectable at earlier time points. Our results indicate a suppression of p53 protein expression in the treatment group at ≤ 8 h when compared to control, and, while elevated at later time points, the treated group at 12 h was suppressed (Fig. 1A). Although p53 expression increased, resveratrol-mediated suppression was present at all time points (Fig. 1B). RNA expression was increased 1.8-fold at the zero time point and more than 3-fold at 6 h (Fig. 1C). At 24 h, there was no differential expression of RNA. Cycling of the protein and mRNA levels was noted primarily at time points ≤ 8 h. At 24 h, both protein and mRNA levels in control and treatment groups were similar, corroborating previous experiments indicating no differential expression [26].

Table 1. Sequences of primers and probes for quantitative RT-PCR of the six pivotal genes selected for further study

Gene Name		Sequences
p53	fp[a)]	GGA GAG ACC GCC GTA CAG AAG
	rp[b)]	GCT TGT GCA GGT GGG CA
	probe	CCC AGG GAG CGC AAA GAG AGC G
Mdm2	fp	TCC ACA GAG ACG CCC TCG
	rp	TGA TCC AGG CAA TCA CCA GA
	probe	AGG ATC TTG ACG ATG GCG TAA GTG AGC A
p21cip/waf	fp	AGC GGC CTG AAG ATT CCC
	rp	AAA TCT GTC AGG CTG GTC TGC
	probe	CGG AAC ATC TCA GGG CCG AAA ACG
c-Ha-ras	fp	CTA CGG CAT CCC CTA CAT CG
	rp	TGT AGA AGG CAT CCT CCA CTC C
	probe	ACC TCG GCC AAG ACC CGG CA
Cyclin G	fp	GCG ACT GAA GAG GAA AGG AAT G
	rp	TCT GAA ACC GTG AAC CTA TAC TGA CT
	probe	CCC ACT GGC GAC TGA TTT GAT CCG
Rb	fp	TGT ACC GTC TAG CAT ATC TCC GAC
	rp	ATG ATG TGC TCT AGC TCT GGG TG
	probe	AAA TAC ACT CTG TGC ACG CCT TCT GTC TGA

a) fp, Forward primer.
b) rp, Reverse primer.

We next analyzed expression of the p53 autoregulatory inhibitor, Mdm2. Mdm2 protein and mRNA levels increased in a time-dependent manner after 6 h. At 24 h, both protein and mRNA levels were upregulated. Mdm2 protein levels were notably increased in control cells at ≤ 4 h, but less so at later time points (Fig: 2A). In contrast, Mdm2 mRNA was expressed at the earlier time points with a peak at 8 h after resveratrol treatment and robustly and linearly expressed at later time points as well. There was a maximum suppression of protein levels by resveratrol at 4 h (Fig. 2B). RNA expression was not suppressed at any time point and increased linearly up to 15-fold beginning at the 8-h time point (Fig. 2C).

An immediate downstream effector of p53 is the p21$^{cip/waf}$ cyclin-dependent kinase inhibitor. The p21$^{cip/waf}$ protein appears to be modulated in a manner similar to p53 protein, due in part to p21$^{cip/waf}$ functioning as a downstream effector of p53. The p53 upregulates p21$^{cip/waf}$, which inhibits the cdk/cyclin D interaction during cell cycle. In control cells, p21$^{cip/waf}$ was expressed at all time points and appeared to

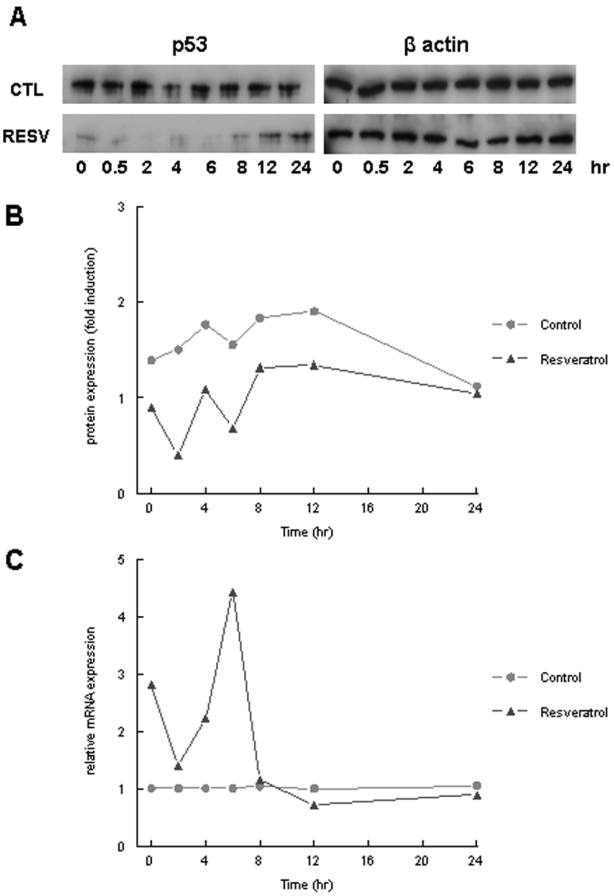

Figure 1. (A) Western blot analysis of p53 protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0–24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of p53 protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data, demonstrating the effect of resveratrol on mRNA expression of p53.

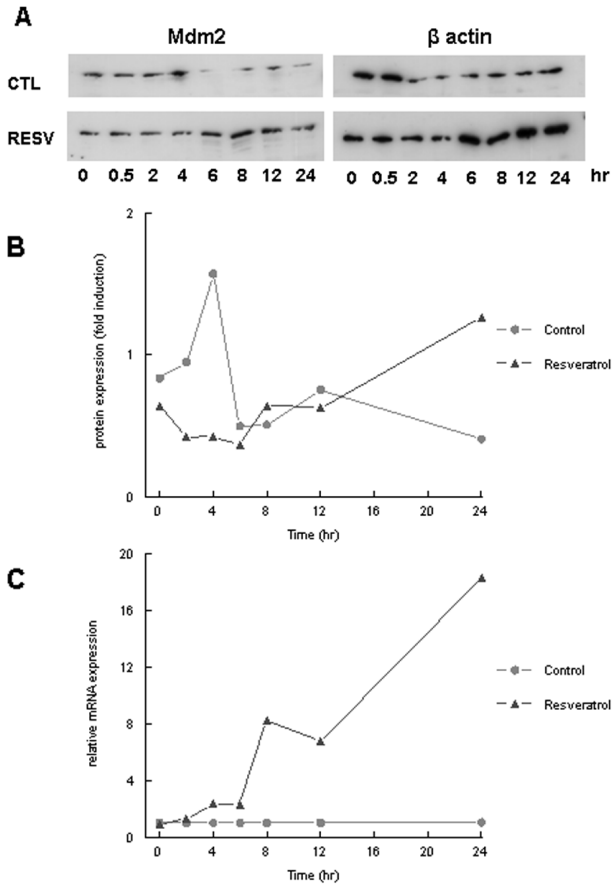

Figure 2. (A) Western blot analysis of Mdm2 protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0–24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of Mdm2 protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data, demonstrating the effect of resveratrol on mRNA expression of Mdm2.

increase linearly to a maximum at 24 h (Fig. 3A). In resveratrol-treated cells, $p21^{cip/waf}$ was expressed at all time points as well and expression was further increased at 24 h. The $p21^{cip/waf}$ protein expression was upregulated at the zero time point by 80%, suppressed by 30% at 2 h, and once again upregulated by 50% after 4 h (Fig. 3B). After resveratrol-mediated cycling, $p21^{cip/waf}$ expression was suppressed at times > 8 h with a minimum at 24 h. In contrast, RNA expression was notably increased by resveratrol at all time points and exhibited a robust increase to sixfold after 24 h (Fig. 3C).

We next analyzed $p21^{ras}$ oncogenic expression. Ras protein expression appeared to increase in control cells beginning at 6 and up to 24 h (Fig. 4A). In resveratrol-treated cells, there was clear expression at all time points that appeared more intense than seen in control cells. Resveratrol marginally increased Ras expression 1.5-fold after 6 h, but returned to baseline at later time points (Fig. 4B). RNA expression was suppressed by approximately 50% from 2–12 h but exhibited an increase, although insignificant, in expression to 6-fold after 2 h (Fig. 4C).

We noted in a previous study that cyclin G mRNA expression was stimulated by treatment with resveratrol [26]. Thus, we determined the temporal pattern of cyclin G expression. In control cells, we noted a clear, but not different, expression of cyclin G at all time points (Fig. 5A). In resveratrol-treated cells, we also noted expression at all time points, but clearly, there were maxima at 6 and 24 h. We noted increased resveratrol-mediated expression at 0 and 6 h (Fig. 5B). There was marginal suppression at 2 h but more notable inhibition at 6–12 h. By 24 h, expression was not different between control and treated cells. RNA levels also cycled marginally at early time points (< 6 h),

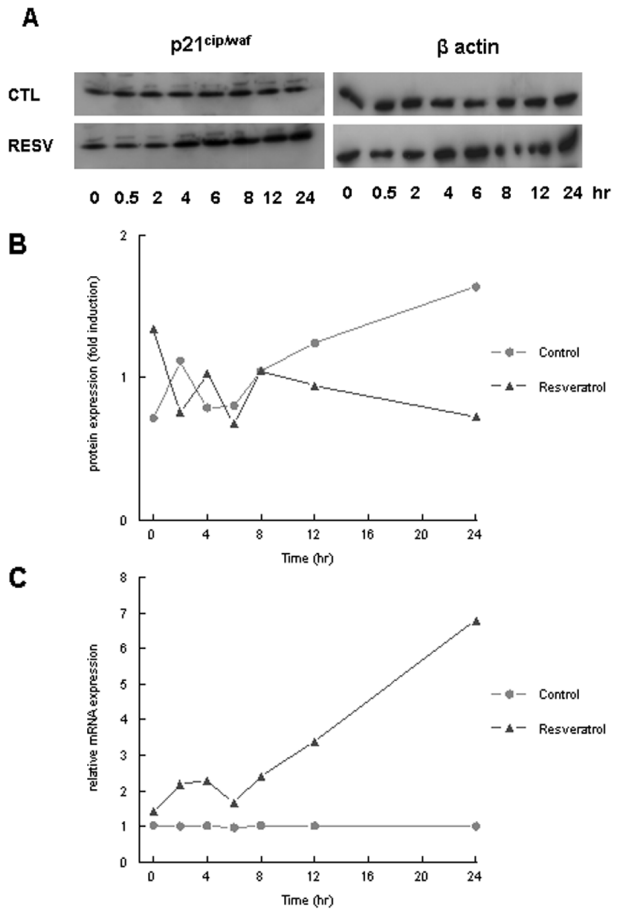

Figure 3. (A) Western blot analysis of p21$^{cip/waf}$ protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0–24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of p21$^{cip/waf}$ protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data demonstrating the effect of resveratrol on mRNA expression of p21$^{cip/waf}$.

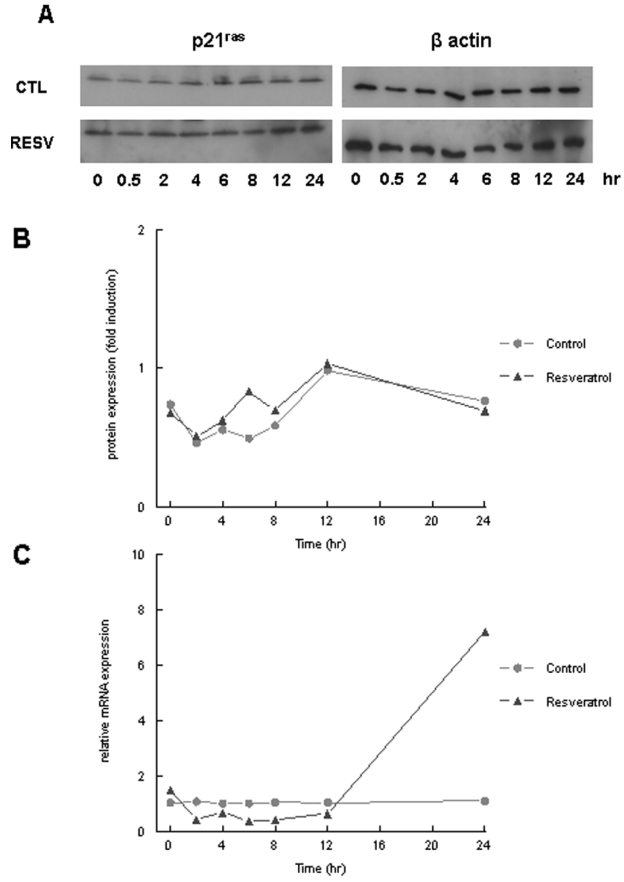

Figure 4. (A) Western blot analysis of p21^{ras} protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0–24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of p21^{ras} protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data demonstrating the effect of resveratrol on mRNA expression of p21^{ras}.

but exhibited a linear, robust response at ≥ 8 h and up to 6-fold at 24 h (Fig. 5C).

Although p53 is considered to be the gatekeeper of the genome, Rb is a critical and important restriction point in the cell cycle. Protein expression of Rb was noted at the earliest time points in control cells with a subsequent decline at later times. After resveratrol treatment, Rb protein was increased marginally at times < 12 h (Fig. 6A). When normalized to beta actin, expression in resveratrol-treated cells was more notable at earlier time points (Fig. 6B). RNA levels appeared slightly suppressed from 2–12 h but increased linearly, but not significantly, up to twofold by 24 h (Fig. 6C).

4 Discussion

Our previous results indicated a clear induction of apoptosis and inhibition of cellular proliferation in the WR21 cell line, a transgenic cell line containing a mutated human c-Ha-ras gene, when incubated with resveratrol (50 μM) for 24 h [26]. Resveratrol also induced G_2/M arrest, and induced gene expression associated with a p53-mediated effect, including apoptosis, cell signaling, and cell cycle. Surprisingly, neither c-Ha-Ras nor p53 were modulated at the RNA or protein level at 24 h. Thus, we conducted a time course of mRNA and protein expression of Ras and p53, as well as other pivotal downstream effectors to establish expression profiles of molecules potentially modulated by resveratrol. We have demonstrated the cyclic expression of

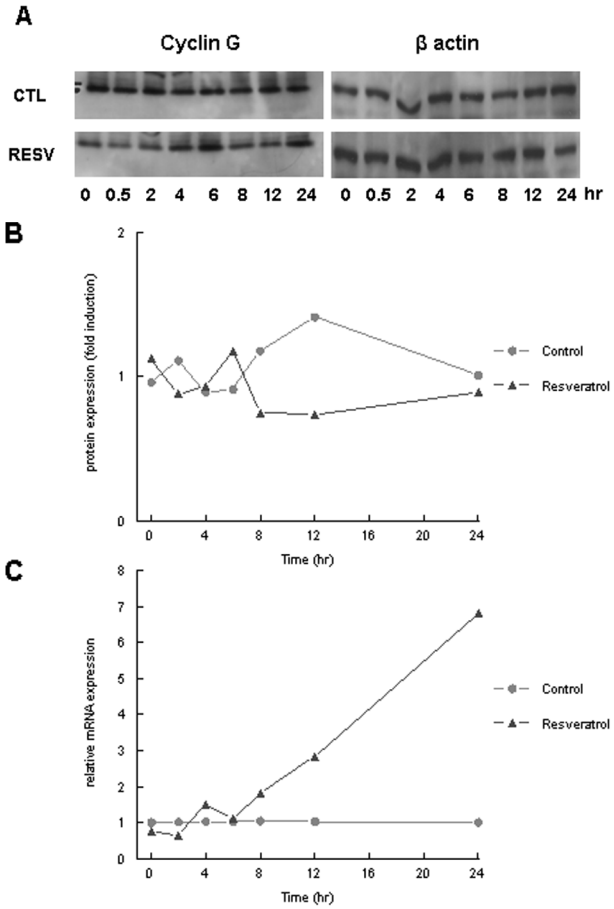

Figure 5. (A) Western blot analysis of cyclin G protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0 – 24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of cyclin G protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data demonstrating the effect of resveratrol on mRNA expression of cyclin G.

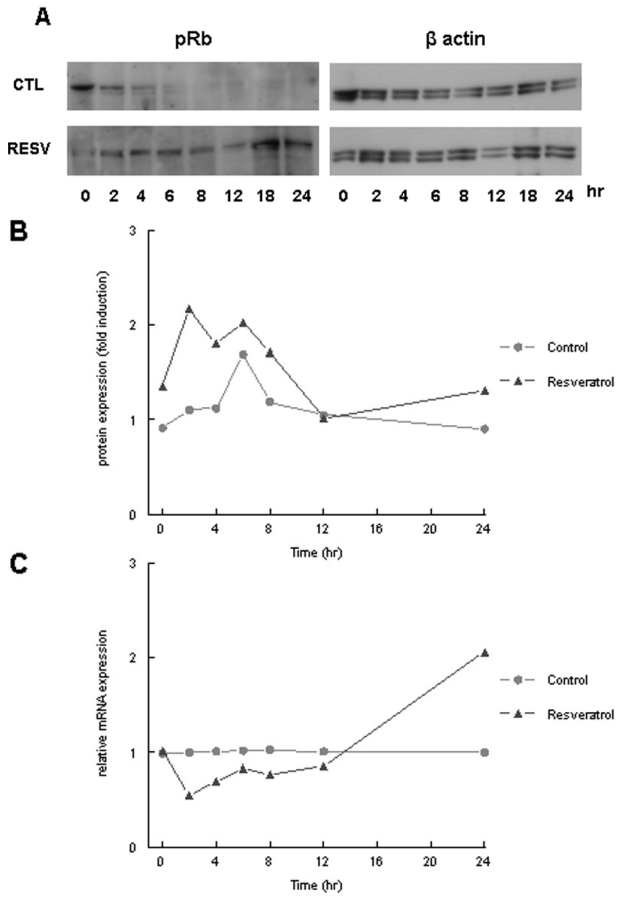

Figure 6. (A) Western blot analysis of Rb protein. The autoradiograph is representative of two-to-six pooled samples from independent experiments and shows data from control and resveratrol-treated WR21 cells after 0 – 24 h post-treatment with media alone or containing 50 μM resveratrol. (B) Quantitation of Rb protein levels after normalization to beta actin expression. (C) Quantitative RT-PCR data demonstrating the effect of resveratrol on mRNA expression of Rb.

p53, mdm2, $p21^{cip/waf}$, Rb, and cyclin G at both the RNA and protein level at earlier time points. We also demonstrate at times > 8 h increasing asynchrony and apparent uncoupling of transcription and translation after resveratrol, suggesting induction of mitotic catastrophe, a form of apoptosis occurring during mitosis.

Our initial question was whether resveratrol-mediated p53 expression would be different at earlier time points compared to the 24-h time point. We noted that resveratrol suppressed p53 protein expression at time points < 24 h, but there was time-dependent cycling of protein levels at < 8 h, suggesting altered relative levels within the cell, which may have contributed to our previous results. This is in contrast to numerous reports demonstrating resveratrol-mediated increases in p53 protein levels in human lung cells, HepG2 human liver cancer cells, vascular smooth muscle cells, lymphoid and myeloid cells, and gastric adenocarcinoma cells [1, 25, 27, 28]. There are, however, reports of resveratrol-mediated reductions of p53 expression. Specifically, MCF-7 cells, a breast cancer cell line with wild-type p53, responded to resveratrol with marked suppression of p53 to 10% that of controls using 100 μM resveratrol [29]. In COLO 320 HSR human colorectal cells, resveratrol also markedly decreased p53 expression [30]. Together, these data suggest resveratrol-dependent effects on p53 expression and cell type-specific sensitivity.

We have noted previously induction of apoptosis after resveratrol with upregulation of p53-associated genes. Resveratrol-induced apoptosis via a p53-dependent mechanism has been well documented [3, 31, 32]. In fact, apoptosis induced by resveratrol only occurs in cells with functional wild-type p53 but not in mutant p53-expressing

cells [33]. In a p53-knockout cell line, NH32 cells overrode the G2/M checkpoint where TKE cells with normal p53 accumulated in G2/M in agreement with our previous reports [26, 34]. Mutant p53 is associated with absent or decreased expression of $p21^{cip/waf}$ levels and increased cellular proliferation [1]. We have noted marginal changes in p53 and $p21^{cip/waf}$ expression but potent inhibition of cellular proliferation. In fact, wild-type p53 is normally undetectable in normal cells due to its short half-life. However, in tumor cells with mutated p53 there can be 100-fold induction of p53, which is detectable due to prolonged half-life and stability [30]. This supports the presence of wild-type p53 in our study. However, the apparent complete loss of p53 transcription and translation at 24 h suggests loss of this key cell-cycle checkpoint.

The cyclin-dependent kinase inhibitor $p21^{cip/waf}$ is an immediate downstream effector of p53 and is upregulated subsequent to p53 induction. Our results indicate a resveratrol-mediated increase in $p21^{cip/waf}$ from 4 to 24 h. Moreover, the pattern of protein expression superimposed with p53 expression at < 8 h, as expected. However, expression in control cultures was asynchronous with resveratrol-treated cells. The p53 RNA levels returned to baseline at 8 h, but $p21^{cip/waf}$ RNA expression continued to increase after 8 h, suggesting an uncoupling of the two. Resveratrol induces both p53 and $p21^{cip/waf}$ protein and RNA in numerous cell models, including A549 human lung cancer cells, papillary thyroid cancer cells, and human gastric adenocarcinoma cells when incubated over 24–72 h with 5–100 μM resveratrol [1, 3, 35]. Resveratrol also decreased growth of vascular smooth muscle cells at concentrations as low as 1 μM with dose-dependent increases in both p53 and $p21^{cip/waf}$ [28]. Other reports have also shown rapid robust upregulation of $p21^{cip/waf}$ subsequent to p53 upregulation after resveratrol [1, 4, 27, 35].

As p53 expression is normally tightly regulated, we next analyzed its autoregulatory inhibitor Mdm2, which induces p53 destruction [36]. Previous work showed that resveratrol consistently increased by > fourfold Mdm2 mRNA expression [26]. In this study, resveratrol suppressed protein expression of Mdm2 at earlier time points, but both RNA and protein increased markedly after 8–24 h, suggesting a role in p53 inhibition. At earlier time points, Mdm2 mRNA expression followed increased p53 protein expression as expected. Interestingly, Mdm2 is the only gene whose RNA and protein products increased concomitantly at later time points. Together, the data indicate that the p53-$p21^{cip/waf}$ axis remained functional at < 8 h, but rapidly became non-functional at later times.

It appears that at time points < 8 h, there is a time-dependent cycling of protein expression, but at 8 h protein and RNA expression become increasingly uncoordinated. Moreover, there is little correlation between RNA and protein expression for many of the genes analyzed at later time points. Resveratrol potently induced RNA expression via increased transcription or RNA stability for all genes except p53 at >8 h but decreased protein levels via decreased translation or protein stability with the exception of Mdm2. Resveratrol has been shown to exhibit transcriptional, post-transcriptional, and translational control of genes [37]. The mechanisms for these effects are unclear but translational regulation may be occurring in this model.

Interestingly, Rb, a tumor suppressor at the G1 checkpoint, expressed in a pattern similar to Mdm2 at = 8 h [38, 39]. Although Rb exhibits a similar, marked increase at later times, expression of both mRNA and protein return to baseline at 24 h similar to p53, suggesting that both tumor suppressor checkpoint proteins are non-functional, which is consistent with mitotic catastrophe.

Cyclin G has been identified as a downstream target gene of p53, is Mdm2 dependent, and its expression is increased after p53 induction [40, 41]. Moreover, cyclin G, as well as p53, controls, in part, the G2/M transition, induces apoptosis, and is a negative feedback regulator of p53 [42]. We noted oscillation of protein expression of cyclin G that coincided with other gene expression, *i.e.* Ras, but was clearly asynchronous with control cells, suggesting a resveratrol-mediated effect. In previous results, we have shown a potent resveratrol-induced G_2/M arrest consistent with a lack of p53 protein [26]. In cyclin $G^{-/-}$ cells, there is p53 accumulation after 24 h compared to wild-type and re-introduction of cyclin G restored activity [43].

Previously, under identical experimental conditions we noted no significant differential expression of Ras at 24 h [26]. In this study, Ras protein was not significantly modulated at any time point although a marginal increase of 1.5-fold occurred at 6 h. Ras RNA expression increased at later time points similarly to mdm2, $p21^{cip/waf}$, and cyclin G, suggesting a nonspecific induction of transcription. Interestingly, resveratrol functions via a Ras-MAPK kinase-MAPK signal transduction pathway to increase p53 expression, serine phosphorylation of p53, and p53-dependent apoptosis in several cell lines. Moreover, introduction of antisense Ras into two papillary thyroid carcinoma cell lines and two follicular thyroid carcinoma cell lines treated with resveratrol at 1–10 μM prevented resveratrol-mediated effects on apoptosis [3]. However, the lack of differential Ras expression in the WR21 cell line suggests little involvement of oncogenic Ras. Collectively, these data indicate clear time-dependent cycling of protein and mRNA expression in cells containing mutated c-Ha-Ras and exposed to resveratrol. At later time points, there appears to be asynchrony of cell-cycle control with increased RNA expression. In contrast, significant changes in protein levels did not occur and both tumor suppressor proteins appear non-functional, suggest-

ing DNA structure and spindle assembly cell-cycle checkpoint failure characteristic of mitotic catastrophe [44, 45].

The increasing use of individual bioactive agents, often in high doses, has stimulated growing concern due to the potential adverse effects on cells. Thus, it is vital to study the actions of bioactive components at the cellular and molecular levels particularly in the context of neoplasia. Moreover, the timing and duration of administration of bioactive agents is critical and likely is responsible for the large variability in reported results. Our results suggest that oncogenic Ras may not be involved in the chemoprotective effects of resveratrol in this model, which may be applicable to other cells with mutated ras. Instead, it appears that a p53-mediated effect is activated by resveratrol as well as induction of several cell-cycle genes at early time points. At later time points, the p53-mediated effect dissipates, as well as G1 checkpoint control. A better understanding of mechanistic actions of bioactive agents will permit the design of more effective chemopreventive agents and, perhaps, the advocacy of particularly beneficial diets, especially those containing resveratrol-rich food sources.

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5 References

[1] Atten, M. J., Godoy-Romero, E., Attar, B. M., Milson, T., Zopel, M., Holian, O., *Invest. New Drugs* 2005, *23*, 111 – 119.

[2] Dong, Z., Nomura, M., Huang, C., Ma, W. Y., *Adv. Exp. Med. Biol.* 2001, *492*, 55 – 67.

[3] Shih, A., Davis, F. B., Lin, H. Y., Davis, P. J., *J. Clin. Endocrinol. Metab.* 2002, *87*, 1223 – 1232.

[4] Dong, Z., *Mutat. Res.* 2003, *523 – 524*, 145 – 150.

[5] McMahon, M., Woods, D., *Biochim. Biophys. Acta* 2001, *1471*, M63 – M71.

[6] Burzynski, S. R., *Med. Hypotheses* 2005, *64*, 201 – 208.

[7] Ayllon, V., Rebollo, A., *Oncogene* 1998, *17*, 1395 – 1413.

[8] Campbell, S., Khosravi-Far, R., Rossman, K., Clark, G., Der, C., *Oncogene* 1998, *17*, 1395 – 1413.

[9] Macar, I., Lounsbury, K., Richards, S., McKiernan, C., Bar-Sagi, D., *FASEB J.* 1996, *10*, 625 – 630.

[10] Levine, A. J., *Cell* 1997, *88*, 323 – 331.

[11] Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A., *Nature* 1992, *356*, 215 – 221.

[12] Ries, S., Biederer, C., Woods, D., Shigman, O., Shirasawa, S., Sasazuki, T., McMahon, M. *et al.*, *Cell* 1999, *1*, 321 – 330.

[13] Soleas, G., Goldberg, D., Grass, L., Leveesque, M., Diamandis, E., *Clin. Biochem.* 2001, *34*, 415 – 420.

[14] Gusman, J., Malonne, H., Atassi, G., *Carcinogenesis* 2001, *22*, 1111 – 1117.

[15] Savouret, J. F., Quesne, M., *Biomed. Pharmacother.* 2002, *56*, 84 – 87.

[16] Roberti, M., Pizzirani, D., Simoni, D., Rondanin, R., Baruchello, R., Bonora, C., Buscemi, F. *et al.*, *J. Med. Chem*. 2003, *46,* 3546 – 3554.

[17] Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H. *et al.*, *Science* 1997, *275*, 218 – 220.

[18] Holmes-McNary, M., Baldwin, A. S., Jr., *Cancer Res.* 2000, *60*, 3477 – 3483.

[19] Kim, Y. A., Choi, B. T., Lee, Y. T., Park, D. I., Rhee, S. H., Park, K. Y., Choi, Y. H., *Oncol. Rep.* 2004, *11*, 441 – 446.

[20] Ding, X. Z., Adrian, T. E., *Pancreas* 2002, *25*, e71 – 76.

[21] Ferry-Dumazet, H., Garnier, O., Mamani-Matsuda, M., Vercauteren, J., Belloc, F., Billiard, C., Dupouy, M. *et al.*, *Carcinogenesis* 2002, *23*, 1327 – 1333.

[22] Kang, J. H., Park, Y. H., Choi, S. W., Yang, E. K., Lee, W. J., *Exp. Mol. Med.* 2003, *35*, 467 – 474.

[23] Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D., Weinstein, I. B., *Clin. Cancer Res.* 2002, *8*, 893 – 903.

[24] Wolter, F., Akoglu, B., Clausnitzer, A., Stein, J., *J. Nutr.* 2001, *131*, 2197 – 2203.

[25] Kuo, P. L., Chiang, L. C., Lin, C. C., *Life Sci.* 2002, *72*, 23 – 34.

[26] Young, L. F., Hantz, H. L., Martin, K. R., *J. Nutr. Biochem.* 2005, *16*, 663 – 674.

[27] Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P., Shishodia, S., Takada, Y., *Anticancer Res.* 2004, *24*, 2783 – 2840.

[28] Mnjoyan, Z. H., Fujise, K., *Biochem. Biophys. Res. Commun.* 2003, *311*, 546 – 552.

[29] Avila, M. A., Velasco, J. A., Cansado, J., Notario, V., *Cancer Res.* 1994, *54*, 2424 – 2428.

[30] Soleas, G. J., Goldberg, D. M., Grass, L., Levesque, M., Diamandis, E. P., *Clin. Biochem.* 2001, *34*, 415 – 420.

[31] Huang, C., Ma, W. Y., Goranson, A., Dong, Z., *Carcinogenesis* 1999, *20*, 237 – 242.

[32] Liontas, A., Yeger, H., *Anticancer Res.* 2004, *24*, 987 – 998.

[33] Laux, M. T., Aregullin, M., Berry, J. P., Flanders, J. A., Rodriguez, E., *J. Altern. Complement Med.* 2004, *10*, 235 – 239.

[34] Schneider, Y., Fischer, B., Coelho, D., Roussi, S., Gosse, F., Bischoff, P., Raul, F., *Cancer Lett.* 2004, *211*, 155 – 161.

[35] Lee, E. J., Min, H. Y., Joo Park, H., Chung, H. J., Kim, S., Nam Han, Y., Lee, S. K., *Life Sci.* 2004, *75*, 2829 – 2839.

[36] Tao, W., Levine, A. J., *Proc. Natl. Acad. Sci. USA* 1999, *96*, 6937 – 6941.

[37] Juan, S. H., Cheng, T. H., Lin, H. C., Chu, Y. L., Lee, W. S., *Biochem. Pharmacol.* 2005, *69*, 41 – 48.

[38] Kim, Y. A., Lee, W. H., Choi, T. H., Rhee, S. H., Park, K. Y., Choi, Y. H., *Int. J. Oncol.* 2003, *23*, 1143 – 1149.

[39] Adhami, V. M., Afaq, F., Ahmad, N., *Biochem. Biophys. Res. Commun.* 2001, *288*, 579 – 585.

[40] Okamoto, K., Prives, C., *Oncogene* 1999, *18*, 4606 – 4615.

[41] Ohtsuka, T., Jensen, M., Kim, H., Kim, H., Lee, S., *Oncogene* 2004, *22*, 1678 – 1687.

[42] Shimizu, A., Nishida, J., Ueoka, Y., Kato, K., Hachiya, T., Kuriaki, Y., Wake, N., *Biochem. Biophys. Res. Commun.* 1998, *242*, 529 – 533.

[43] Kimura, S. H., Nojima, H., *Genes Cells* 2002, *7*, 869 – 880.

[44] Castedo, M., Perfettini, J. L., Roumier, T., Andreau, K., Medema, R., Kroemer, G., *Oncogene* 2004, *23*, 2825 – 2837.

[45] Nitta, M., Kobayashi, O., Honda, S., Hirota, T., Kuninaka, S., Marumoto, T., Ushio, Y., Saya, H., *Oncogene* 2004, *23*, 6548 – 6458.

