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Resveratrol Increases Serine^{15}-Phosphorylated but Transcriptionally Impaired p53 and Induces a Reversible DNA Replication Block in Serum-Activated Vascular Smooth Muscle Cells

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ABSTRACT

Resveratrol (RV), a polyphenolic stilbene derivative, has been proposed to exert a plethora of beneficial cardiovascular effects. Of these, in particular, inhibition of vascular smooth muscle cell (VSMC) proliferation shows great promise for preventing cardiovascular disease. In the present study, we show that RV leads to a reversible arrest in early S phase of the VSMC cycle, accompanied by an accumulation of hyperphosphorylated retinoblastoma protein. In contrast to studies with other cell systems, RV decreases cellular levels of the cyclin-dependent kinase inhibitors p21^{Cip1} and p27^{Kip1}. This is of particular interest because phosphorylated p53 protein (serine^{15}) is strongly enhanced by this substance. We further found that RV only slightly inhibits phosphorylation of Erk 1/2, protein kinase B/Akt, and p70^{S6} kinase upon serum stimulation. Thus, inhibition of these kinases is not likely to contribute to the cell cycle effect of RV. Importantly, the observed S phase arrest is not linked to an increase in apoptotic cell death: there was no detectable increase in apoptotic nuclei and in levels of the proapoptotic protein Bax. This is the first study elucidating the molecular pathways mediating the antiproliferative properties of RV in VSMCs.

Vascular smooth muscle cells (VSMCs) in the arterial media are fully differentiated to play their physiological roles as regulators of vascular wall tension. They proliferate at low indices or persist in the G_0 phase of the cell cycle. Entry into and progression through the cell cycle by vascular cells is considered a key event in vascular proliferative diseases such as primary atherosclerosis and post-angioplasty restenosis (Jackson and Schwartz, 1992; Braun-Dullaeus et al., 1998). Thus, the VSMC cycle provides a potent therapeutic target, as demonstrated by studies successfully preventing restenosis after angioplasty by using antisense oligodeoxynucleotides against cell cycle-regulatory genes or gene transfer of nonphosphorylated retinoblastoma protein (Rb) (Morishita et al., 1993; Chang et al., 1995).

trans-Resveratrol (RV, *trans*-3,5,4′-trihydroxystilbene) is a polyphenolic stilbene derivative found in a narrow range of spermatophytes, of which vines, peanuts, and pines are the prime representatives. As a major constituent of red wine RV has been proposed to account, at least in part, for the beneficial effects attributed to this beverage in cardiovascular diseases (Soleas et al., 2001; Wu et al., 2001). In vivo studies, for example, suggested that RV can protect against intimal hyperplasia after endothelial denudation in an experimental rabbit model (Zou et al., 2000). More recent data imply that RV decreases NAD(P)H oxidase activity in rat aortic homogenates (Orallo et al., 2002). Our own studies showed that RV suppresses angiotensin II-induced VSMC hypertrophy (Haider et al., 2002). An antiproliferative property presumably contributes to cardio- and vasoprotective effects of this natural compound (Zou et al., 1999; Mizutani et al., 2000). Although this property of RV is documented for various cell types, including VSMCs, it seems that, depending on the cell type investigated, there are considerable mechanistic differences in the way the cell cycle is altered (Hsieh et al., 1999; Zou et al., 1999; Adhami et al., 2001; Sgambato et al., 2001). For VSMCs in particular, the underlying mechanism has never been thoroughly investigated. However, deeper knowledge of the molecular basis of RV effects is crucial to validate the potential of this substance as a beneficial agent in cardiovascular disease. Therefore, this study was aimed to gain further insight into the pathways mediating inhibition of VSMC proliferation by RV.

ABBREVIATIONS: VSMC, vascular smooth muscle cell; RV, *trans*-resveratrol; MAPK, mitogen-activated protein kinase; Rb, retinoblastoma protein; BrdU, bromodeoxyuridine; PI, propidium iodide; Aph, aphidicolin; PKB, protein kinase B; IR, γ-irradiation; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; Erk, extracellular signal-regulated kinase; CDK, cyclin-dependent kinase; FACS, fluorescence-activated cell sorting.

Materials and Methods

Reagents. Materials were obtained from the following suppliers: antibodies against phospho-Akt (Ser^{473}), phospho-$p70^{S6k}$ (Thr^{421}/Ser^{424}), phospho-Erk 1/2 MAPK (Thr^{202}/Tyr^{204}), phospho-p38 MAPK (Thr^{180}/Tyr^{182}), phospho-p53 (Ser^{15}) as well as Akt, Erk1/2, $p70^{S6k}$, and p53 (mouse monoclonal 1C12) were from Cell Signaling Technology (Frankfurt, Germany). Antibodies against Bax, p27, p21, and retinoblastoma protein (Rb) as well as the BrdU flow cytometry kit were purchased from BD Biosciences (Heidelberg, Germany). Hoechst 33342, RV, and propidium iodide (PI) were from Sigma (Taufkirchen, Germany). Cell death detection $ELISA^{PLUS}$ was from Roche (Mannheim, Germany). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Dianova (Hamburg, Germany). Serotec (Eching, Germany) provided horseradish peroxidase-conjugated goat anti-mouse antibody. Aphidicolin (Aph) was from Calbiochem (Schwalbach, Germany). Calf serum was from Invitrogen (Karlsruhe, Germany). Phenol red-free Dulbecco's modified Eagle's medium was obtained from Pan Biotech GmbH (Aidenbach, Germany).

Cell Culture. VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously (Griendling et al., 1991). Cells were grown in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (growth medium) and passaged twice a week by harvesting with trypsin/EDTA and seeding into 75-cm^2 flasks. For experiments, cells between passage 7 and 15 were used at 70 to 95% confluence.

Western Blotting. VSMCs in 60-mm dishes at 70 to 95% confluence were made quiescent in serum-free medium overnight if not otherwise indicated. Cells were preincubated with RV, Aph, or vehicle (dimethyl sulfoxide) for 30 min before stimulation with 10% calf serum for the indicated times. After treatment, cells were harvested on ice, and Western blot was performed as described previously (Ushio-Fukai et al., 1999). Briefly, equal amounts of proteins (60 μg for Rb, 20 μg for all other targets) were separated by SDS-polyacrylamide gel electrophoresis (7.5% for Rb, 10% for MAPK, $p70^{S6}$ kinase, protein kinase B (PKB)/Akt, and p53, 15% for Bax, $p21^{Cip1}$, and $p27^{Kip1}$) and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Freiburg, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked in 5% dry milk powder in Tris-buffered saline containing 1% Tween 20 (1 h) and incubated overnight at 4°C with the specified antibody. Proteins were visualized by secondary antibodies conjugated to horseradish peroxidase and the Renaissance Plus reagent (PerkinElmer Life Science, Köln, Germany) and quantified with a Kodak Digital Science image station 440 cf (PerkinElmer Life Science, Köln, Germany). For γ-irradiation (IR) experiments, cells were treated with 10 Gy for 12 h before harvesting.

Cell Cycle Analysis. Cells were seeded at a density of 50,000 cells/well in six-well plates and kept in growth medium for 24 h. After serum starvation for 4 days, cells were pretreated with or without RV for 30 min using the indicated concentrations and subsequently stimulated with 10% calf serum. Twenty-two hours later cells were trypsinized, washed once with phosphate-buffered saline (PBS), and resuspended in a hypotonic PI solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/ml PI. After incubation at 4°C overnight, PI-stained nuclei were analyzed by flow cytometry (FACScalibur; BD Biosciences, Germany).

Identification of Actively Cycling Cells with BrdU/7-Amino-actinomycin Costaining. Cells were seeded at a density of 80,000 cells/well in six-well plates and made quiescent by serum withdrawal for 4 days. Cells were stimulated by addition of 10% calf serum. After 4 h, 1 μM Aph was added and cells were grown for another 15 h in the presence of Aph and 10% calf serum. Subsequently, Aph was removed by washing cells once with PBS before adding fresh growth medium containing 2 μM Aph, 100 μM RV, or vehicle (dimethyl sulfoxide) for another 4 h. The cells were then pulse-labeled for 30 min with 10 μM BrdU, harvested, and processed as described by the manufacturer. This two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (defined by 7-Amino-actinomycinstaining intensities). Analysis of stained cells was performed by flow cytometry (FACScalibur).

To determine whether RV-induced cell cycle block is reversible, cells were seeded at a density of 80,000 cells/well in six-well plates. After serum starvation for 2 days, cell cycle arrest was induced by treatment with 100 μM RV in growth medium for 19 h. RV was removed by washing cells once with PBS. Afterward, fresh growth medium with or without 100 μM RV was added and cells were grown for another 4 h. During the last 30 min, cells were pulse labeled with 10 μM BrdU and processed as described above.

Cytotoxicity. Cells were grown to 80% confluence, serum-starved overnight, and subsequently stimulated with 10% calf serum with or without RV for 22 h, trypsinized, washed once with PBS, stained with 2.5 μg/ml PI for 5 min, and analyzed by flow cytometry (FACScalibur). Cells unable to exclude PI (PI-stained nuclei) were considered dead.

Apoptosis. Apoptotic cell death was excluded by 1) nucleosome ELISA using the cell detection $ELISA^{PLUS}$ kit from Roche (Mannheim, Germany) according to the manufacturers instruction; 2) staining of apoptotic nuclei with Hoechst 33342 by subsequent fluorescence microscopy (Axiovert 25; Carl Zeiss, Jena, Germany), and 3) cell cycle analysis (lack of a sub-G_1-peak). In all cases, cells were seeded, grown for 24 h, serum-starved overnight, and subsequently treated with up to 100 μM RV or vehicle for 24 h.

Statistical Analysis. All experiments were performed at least three times. Results are expressed as mean ± S.E. Statistical analysis was performed by analysis of variance followed by a Dunnett multiple comparison test or by a paired two-tailed student's t test using Prism version 3.00 for Windows (GraphPad Software). P values <0.05 were considered significant.

Results

Resveratrol Reversibly Inhibits Cell Cycle Progression in VSMCs. Previous studies demonstrate an antiproliferative effect of RV in various cancer cell lines (Adhami et al., 2001; Sgambato et al., 2001) as well as in cardiovascular cells (Hsieh et al., 1999; Zou et al., 1999); Consistent with these results, we observed a significant dose-dependent reduction of serum-induced VSMC proliferation in cells treated with 10 to 100 μM RV (data not shown). To elucidate the exact mechanism underlying the antimitogenic effect of RV in VSMCs, we performed cell cycle analysis of cells treated with various concentrations of RV for 22 h (Fig. 1A). These data suggest a dose-dependent accumulation of RV-treated cells at the G_1/S-interphase, with 100 μM RV leading to a complete block in cell cycle progression. The observed DNA replication block is, however, reversible, because cells arrested by RV treatment are still able to cycle through S phase when RV is removed (Fig. 1B). Neither Hoechst, PI staining, a nucleosome ELISA, nor cell cycle analysis revealed cytotoxic effects at this concentration (data not shown and Fig. 1A, f); therefore, 100 μM RV was used for all other cell cycle experiments.

Resveratrol Only Weakly Affects Phosphorylation of PKB/Akt, Erk 1/2, and $p70^{S6}$ Kinase. It is widely accepted that mitogens promote cell cycle progression of quiescent cells via the PKB/Akt and the Erk 1/2 signaling cascades, both critically involved in accumulation of D-type cyclins in the G_1 phase (Sherr and Roberts, 1999). $p70^{S6}$ kinase is activated downstream of PKB/Akt and Erk 1/2 in VSMCs

(Eguchi et al., 1999) and has been implicated in cell proliferation (Vinals et al., 1999). We therefore hypothesized that inhibition of these kinases might contribute to the observed antimitogenic effect of RV. As shown in Fig. 2A, serum stimulation of VSMCs led to a rapid increase in PKB/Akt, Erk 1/2, and $p70^{S6}$ kinase phosphorylation that was sustained for several hours. We were unable to detect an induction of p38 MAPK phosphorylation by serum in VSMCs. To our surprise, RV only weakly inhibited PKB/Akt and $p70^{S6}$ kinase phosphorylation and failed to significantly reduce Erk 1/2 phosphorylation, as assayed 30 min after serum stimulation (Fig. 2B). Because RV affected cell cycle progression in concentrations as low as 10 μM and arrest was complete at 100 μM RV, inhibition of these kinases is unlikely to primarily account for this effect.

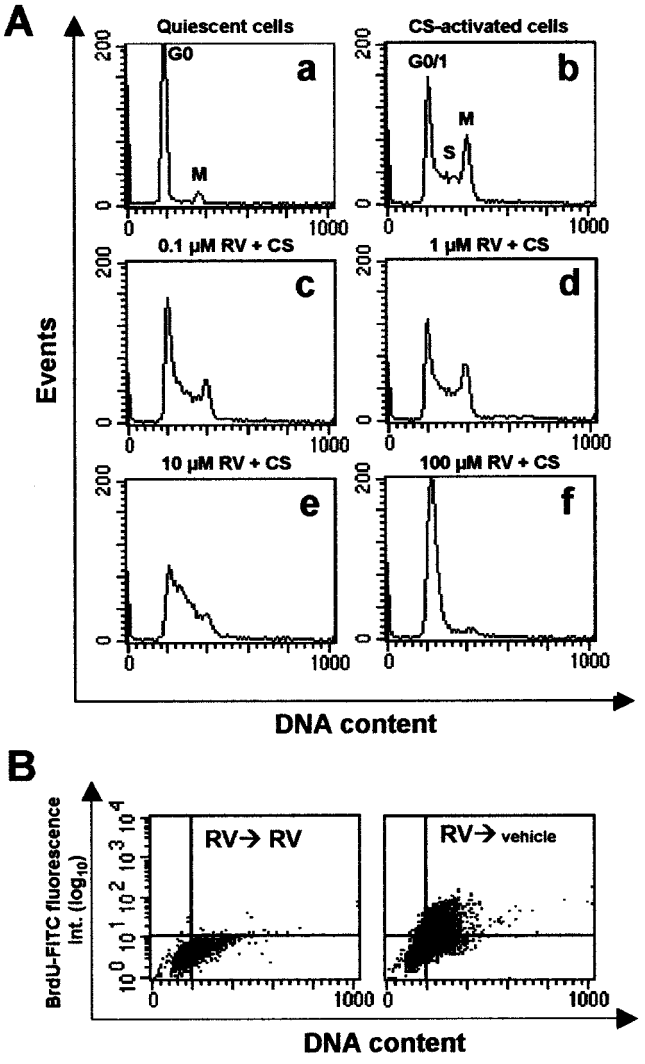

Fig. 1. A, RV-induced changes in cell cycle distribution of serum-stimulated cells. Quiescent cells were preincubated with or without various concentrations of RV for 30 min (a, b, 0 μM; c, 0.1 μM; d, 1 μM; e, 10 μM; and f, 100 μM) and subsequently stimulated with (b–f) or without (a) 10% calf serum (CS) for 22 h. Cells were harvested and nuclei stained with propidium iodide as described under *Materials and Methods*. Data show one representative experiment of three. B, RV-induced cell cycle arrest is reversible. Quiescent VSMCs were treated with 100 μM RV in growth medium for 19 h. Medium was changed and cells were treated for 4 h with or without 100 μM RV in the presence of 10% calf serum. During the last 30 min, cells were pulse labeled with BrdU and subsequently processed as described under *Materials and Methods*. Images show one representative experiment of three.

Resveratrol Increases Retinoblastoma Protein Phosphorylation. In late G_1 phase, cells reach the so-called restriction point. Beyond this point, cells are committed to DNA replication, and further cell cycle progression proceeds independently of growth factor stimulation (Sherr, 1996). Rb is a key component of the molecular network controlling the restriction point. Hypophosphorylated Rb binds the E2F family of transcription factors, and thus inhibits transcription of E2F-responsive genes necessary for cell cycle progression. Hyperphosphorylation of Rb by cyclin-dependent kinases leads to dissociation of Rb from promotor-bound E2F, allowing transcription of E2F-regulated genes (Sherr, 1996; DePinho, 1998). Antimitogenic agents affecting the G_1 phase, such as inhibitors of the phosphoinositol 3-kinase and the $p70^{S6}$ kinase pathway, such as wortmannin or rapamycin, keep Rb in the hypophosphorylated, active state (data not shown; Vinals et al., 1999). Therefore, we investigated the impact of RV on Rb hyperphosphorylation. As shown in Fig. 3A, serum alone led to a continuous increase of Rb hyperphosphorylation over 22 h, first detectable after 6 to 8 h. Interestingly, pretreatment of cells with RV (100 μM) did not prevent Rb hyperphosphorylation; rather, it resulted in a dramatic increase (1.7-fold at 22 h) in hyperphosphorylated Rb (Fig. 3B).

Resveratrol Does Not Enhance $p21^{Cip1}$ and $p27^{Kip1}$ Protein Expression. Because several studies have linked RV with an up-regulation of $p21^{Cip1}$ (Ahmad et al., 2001; Hsieh et al., 1999), we speculated that RV may induce cell cycle arrest through up-regulation of the cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Even though Cip/Kip proteins are potent inhibitors of cyclin E- and A-dependent kinases, which are rate-limiting and essential for DNA replication, they act as positive regulators of cyclin D-dependent kinases that phosphorylate Rb (Sherr and Roberts, 1999). Figure 4A shows time courses of $p21^{Cip1}$ and $p27^{Kip1}$ protein levels in calf serum-stimulated VSMCs. As expected, $p21^{Cip1}$ levels were low in quiescent cells but up-regulated in late G_1 phase in response to serum. In contrast, $p27^{Kip1}$ levels decreased over time upon stimulation with serum as reported before (Sherr and Roberts, 1999). In striking contrast to results from other groups using different cell systems, in VSMCs, RV induced neither $p21^{Cip1}$ nor $p27^{Kip1}$ protein expression. Quite to the contrary, RV substantially reduced protein levels of these CDK inhibitors (Fig. 4B). Together, these results suggest that RV does not affect cell cycle progression in G_1 phase.

Resveratrol Leads to Cell Cycle Arrest in Early S Phase. Cell cycle analysis revealed that these cells require about 10 to 12 h to reach S phase and 22 to 24 h to complete one cell cycle (data not shown). Therefore, we assessed hyperphosphorylated Rb levels after 12 and 22 h of serum stimulation in the presence of Aph (1 μM), a well characterized inhibitor of DNA polymerase α and δ, or RV (100 μM). Neither Aph nor RV altered the Rb phosphorylation state until 12 h, consistent with the idea that both compounds do not affect G_1 phase. However, after 22 h, both substances clearly increased hyperphosphorylated forms of Rb. Similarly, the expression of $p21^{Cip1}$ and $p27^{Kip1}$ was not changed until 12 h of treatment with Aph and RV, respectively, and after 22 h, $p27^{Kip1}$ levels seemed lower in response to both stimuli. Interestingly, unlike RV, Aph did not lead to a de-

crease of p21^{Cip1} protein levels after 22 h (Fig. 5A). Thus, RV seems to act similarly, although not identically, to the DNA polymerase inhibitor Aph.

To corroborate that RV causes a DNA replication block in S phase, we determined whether RV is still able to inhibit cell cycle progression when cells are already in early S phase. Therefore, we accumulated cells in early S phase by employing Aph. Subsequent treatment of released cells with RV (or

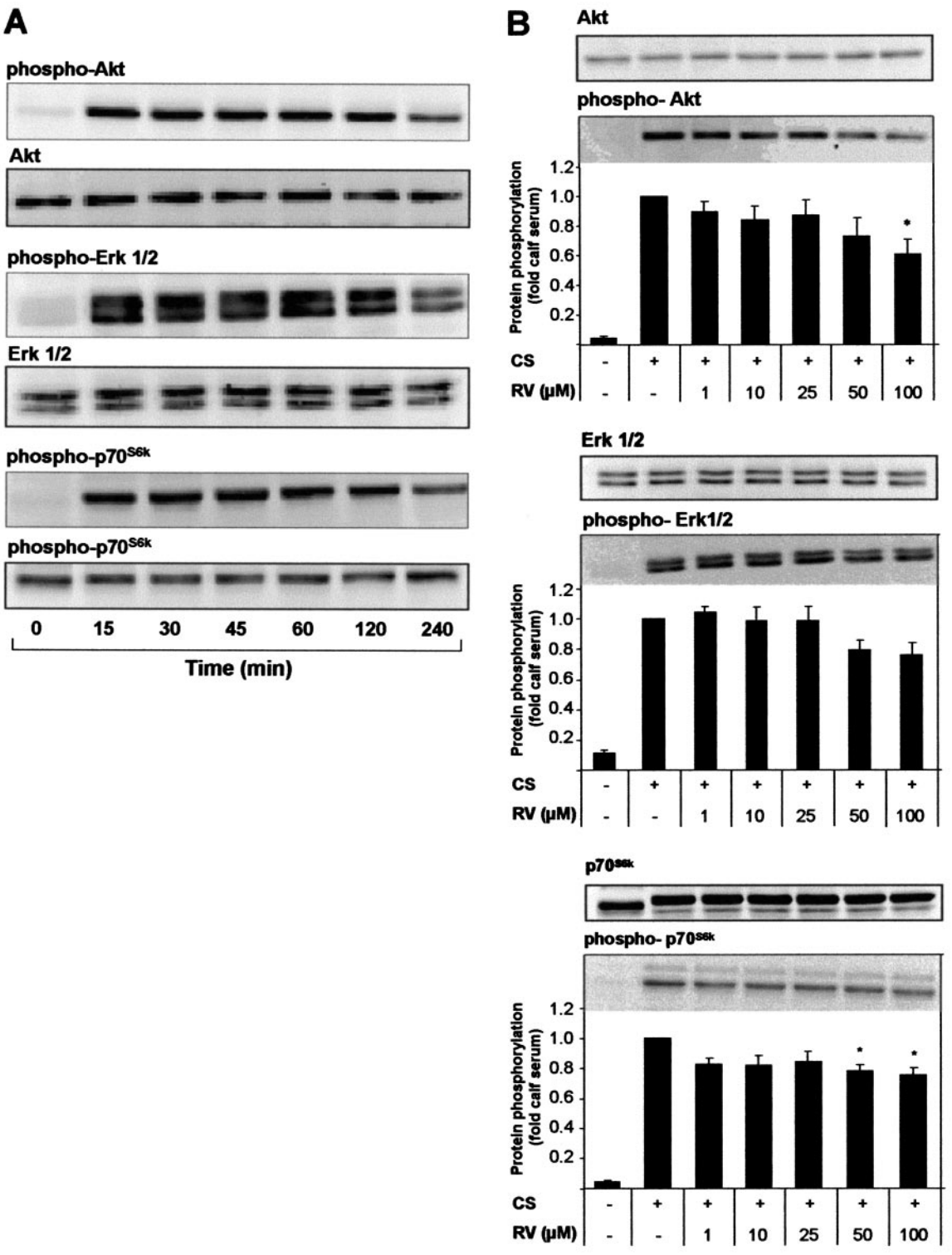

Fig. 2. A, effect of serum stimulation on phosphorylation of PKB/Akt, Erk 1/2, and p70^{S6} kinase. Quiescent cells were treated with 10% calf serum for the indicated times. Top, a representative Western blot for phosphorylated kinases. Bottom, total kinase levels as control. B, effect of RV on serum-induced phosphorylation of PKB/Akt, Erk 1/2, and p70^{S6} kinase. Cells were preincubated with RV in the indicated concentrations or vehicle only for 30 min and subsequently stimulated with (+) or without (−) 10% calf serum (CS) for 30 min. Bottom, a representative Western blot for phosphorylated kinases. Top, total kinase levels as control. Graphs at the bottom represent averaged data, quantified by densitometry and normalized to values of CS-stimulated cells. Values are mean ± S.E. of three independent experiments. *, $P < 0.05$; (analysis of variance/Dunnett).

Aph, as a positive control) still prevented cells from synthesizing DNA, as shown by the complete absence of BrdU incorporation (Fig. 5B). Thus, RV is indeed able to arrest VSMC in early S phase.

Resveratrol Enhances Serine^{15} Phosphorylation of p53. Gottifredi et al. (2001) have recently shown that blocked DNA replication induces increased levels of serine^{15}-phosphorylated p53; however, p53 is functionally impaired because serine^{15}-phosphorylated p53 is not accompanied by p21 accumulation. We therefore examined whether RV leads to increased levels of serine^{15}-phosphorylated p53. Indeed, Western blots depicted in Fig. 6A show a dramatic increase in phospho-p53 levels after RV as well as Aph treatment. Consistent with the idea that RV blocks DNA synthesis only transiently, the increase in phosphorylated p53 was not accompanied by an increase in p21^{Cip1} (Fig. 5A) and Bax levels (Fig. 6A), both genes known to be regulated by p53 (el Deiry et al., 1993; Miyashita and Reed, 1995).

γ-Irradiation Can Rescue p53 from Aph- and RV-Induced Transcriptional Block. Little is known about regulation of p53 in VSMCs. Therefore, to further elucidate why enhanced p53 serine^{15} phosphorylation by RV and Aph is not accompanied by increased expression of target genes such as p21 in these cells, we performed additional experiments using IR (Fig. 6B). As expected, IR alone and in combination with RV or Aph lead to a strong increase in serine^{15}-phosphorylated p53 levels. However, in contrast to findings by Gottifredi et al. (2001) in RKO cells, neither RV nor Aph could prevent the augmentation of p21 levels after IR. Interestingly, although RV, Aph, and IR increase p53 serine^{15} phosphorylation in VSMCs and IR also increases p53 transactivation, total levels of p53 are unaltered.

Discussion

The present study provides valuable insight into the molecular mechanism used by RV to mediate its antiproliferative effect in VSMCs. We found that, in contrast to earlier findings (Zou et al., 1999), RV-treated cells do not accumulate in G_1 phase. Rather, they undergo a reversible DNA replication block in early S phase, as supported by the following observations: 1) RV does not primarily act via the MAPK or PKB/Akt signaling cascades that link mitogenic stimuli via the G_1 phase to the cell cycle machinery (Sherr and Roberts, 1999); 2) RV does not prevent Rb hyperphosphorylation, as shown for antimitogenic substances affecting G_1 phase (Vinals et al., 1999); 3) RV does not increase p21^{Cip1} and p27^{Kip1} protein levels, which may inhibit cyclin E- and A-dependent kinases; 4) instead, RV clearly prevents further cell cycle progression of VSMCs that are released from an arrest in early S phase induced by Aph treatment; 5) RV induces a reversible S phase arrest because cell cycle progression is restored after RV displacement. Most interestingly, we demonstrated that RV increases serine^{15}-phosphorylated but transcriptionally impaired p53, in a manner similar to that

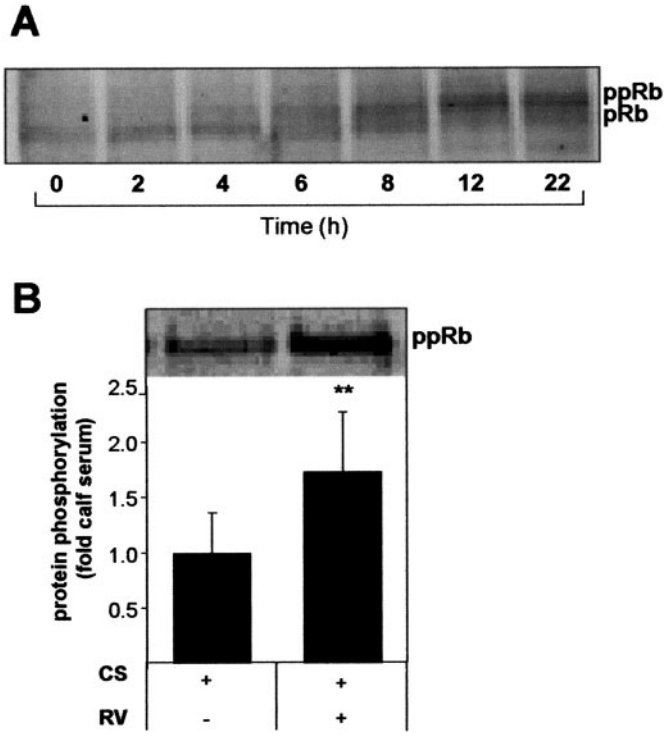

Fig. 3. Representative Western blots from among three (A) and five (B) blots of Rb hyperphosphorylation. A, time course of Rb hyperphosphorylation in response to calf serum. Quiescent VSMCs were stimulated with 10% calf serum for the indicated times. B, effect of RV (100 μM) on Rb hyperphosphorylation (22 h). RV (100 μM) was added to quiescent VSMCs 30 min before adding 10% calf serum (CS). After 22 h, cells were harvested and western blot was performed as described under *Materials and Methods*. B, averaged data quantified by densitometry of immunoblots. Values are mean ± S.E. of five independent experiments. **, $P <$ 0.01 (paired t test).

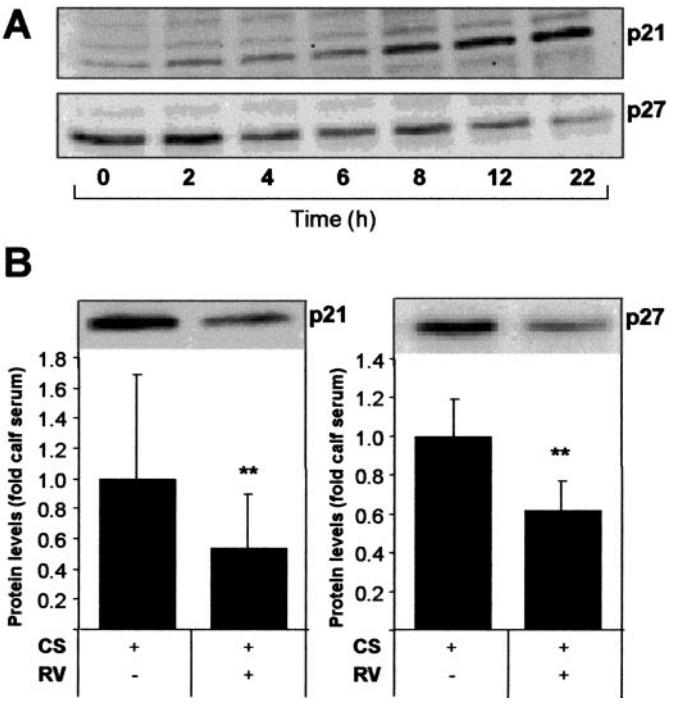

Fig. 4. Western blot analysis of p21^{Cip1} and p27^{Kip1} protein expression. A, time course of p21^{Cip1} and p27^{Kip1} expression in VSMC in response to calf serum. One Western blot that is representative of three is shown. B, effect of RV (100 μM) on p21^{Cip1} and p27^{Kip1} expression in calf serum stimulated cells (22 h). Quiescent cells were preincubated with RV (100 μM) for 30 min before adding 10% calf serum (CS). After 22 h, cells were harvested and Western blot analysis was performed as described under *Materials and Methods*. One Western blot that is representative of four is shown. The graph shows averaged data quantified by densitometry of immunoblots. Values are ± S.E. of four independent experiments. **, $P <$ 0.01 (paired t test).

recently shown to occur in response to substances that induce a reversible DNA replication block (Gottifredi et al., 2001).

When quiescent cells are exposed to mitogens, cyclin D is the first cyclin to be induced when cells progress through G_1 phase. The Erk 1/2 signaling cascade as well as PKB/Akt governed pathways contribute to accumulation of cyclin D and its assembly with cyclin-dependent kinases (CDKs) 4 and 6 (Sherr and Roberts, 1999). Thus, both pathways play a critical role in linking mitogenic cues to the G_1 phase of the cell cycle. $p70^{S6}$ kinase, downstream of PKB/Akt and Erk 1/2 (Eguchi et al., 1999), is thought to play a role in cell cycle progression because of its capacity to phosphorylate the ribosomal protein S6 in response to mitogens. Although cell cycle analysis (Fig. 1) and previously published data (Zou et al., 1999) suggest that RV might target G_1 phase, we detected only slight inhibition of PKB/Akt and $p70^{S6}$ kinase phosphorylation. Erk 1/2 phosphorylation is not significantly altered at all, suggesting that factors other than these kinases mediate the antiproliferative effect of RV. These results contrast with recent findings from our group revealing that RV strongly inhibits phosphorylation of PKB/Akt, Erk 1/2, and $p70^{S6}$ kinase upon angiotensin II stimulation in VSMCs (Haider et al., 2002). Furthermore, El-Mowafy and White (1999) have also shown an inhibition of Erk 1/2 phosphorylation in endothelin-1–stimulated porcine coronary arteries pretreated with RV. Thus, the effect of RV on MAPK, PKB/Akt, and $p70^{S6}$ kinase activation seems to be highly stimulus-dependent, suggesting that RV affects neither of these kinases directly.

Basically, all signals relevant for G_1 progression into S phase finally culminate in Rb hyperphosphorylation. Rb senses and integrates a multitude of proliferative and antiproliferative signals by interacting with members of the E2F family of transcription factors (Weinberg, 1995). In its hypophosphorylated, active state, Rb forms a complex with E2F, thereby blocking its transcriptional activation, and actively represses transcription of cell cycle genes (DePinho, 1998). Hyperphosphorylation of Rb, which is accomplished first by cyclin D-dependent kinases in mid-G_1 phase and then completed by cyclin E-CDK2 leads to its dissociation from promotor-bound E2F, thus allowing transcription of E2F-regulated genes (Sherr, 1996; DePinho, 1998). Antimitogenic substances acting in G_1 phase consequently prevent Rb hyperphosphorylation (Vinals et al., 1999). In contrast to recent findings showing that RV decreases the hyperphosporylated form of Rb in human epidermoid carcinoma (A431) cells (Adhami et al., 2001), we found that in serum-activated VSMCs, RV strongly increases Rb hyperphosphorylation.

This result led us to the hypothesis that RV might up-regulate proteins of the Cip/Kip family of CDK inhibitors, such as $p21^{Cip1}$ and $p27^{Kip1}$. Cip/Kip family members alter the activities of cyclin D-, E-, and A-dependent kinases. Initially, the effect of $p21^{Cip1}$ and $p27^{Kip1}$ was thought to be exclusively inhibitory, but more recent work has revealed that Cip/Kip proteins, although potent inhibitors of cyclin E- and A-dependent CDK2, act as positive regulators of cyclin D-dependent kinase (Sherr and Roberts, 1999). Consequently, we speculated that an up-regulation of $p21^{Cip1}$ or $p27^{Kip1}$ may lead to an increase in Rb hyperphosphorylation through activation of CDK4/6, concurrently preventing cell cycle progression by inhibiting CDK2, a kinase indispensable for cell cycle progression. In contrast to the majority of results obtained in different cell culture models (Hsieh et al., 1999; Ahmad et al., 2001; Shih et al., 2002), we observed a decrease of $p21^{Cip1}$ and $p27^{Kip1}$ levels after 22 h of treatment. Therefore, an induction of Cip/Kip proteins can be excluded as a mechanistic reason for RV's antimitogenic action in VSMCs.

So far all results pointed more to the possibility of a RV-mediated putative DNA replication block in early S phase instead of an interference in G_1. To investigate whether RV arrests VSMCs in early S phase, we determined levels of Rb hyperphosphorylation caused by RV and, as a positive control, by the DNA polymerase inhibitor Aph, after 12 and 22 h of serum stimulation. As expected, neither RV nor Aph increases levels of Rb hyperphosphorylation until 12 h. At this time point, cells are still at the G_1/S-interphase and are thus not affected by a putative S phase block. After 22 h, however,

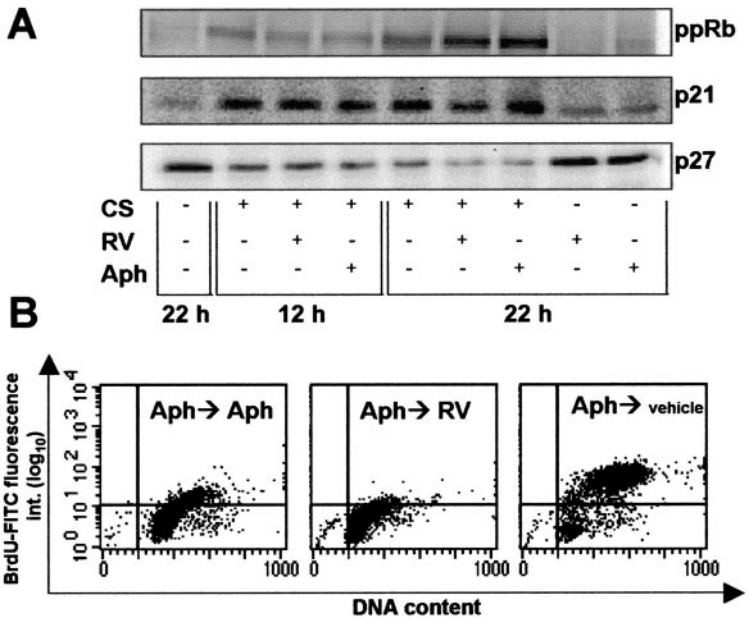

Fig. 5. RV arrests cell cycle in early S phase. A, Western blot analysis of retinoblastoma protein hyperphosphorylation and $p21^{Cip1}$ and $p27^{Kip1}$ protein expression at two different time points (12 and 22 h) using Aph as a positive control. Cells were treated with RV (100 μM), Aph (1 μM), or vehicle for 30 min before stimulation with (+) or without (−) 10% calf serum (CS) for the indicated times. One Western blot that is representative of three is shown. B, RV inhibits S phase progression of VSMCs at the G_1/S interphase. Quiescent VSMCs were synchronized in early S phase by Aph as described under *Materials and Methods*. After Aph release, cells were grown in fresh growth medium (10% calf serum) supplemented with 2 μM Aph, 100 μM RV, or vehicle for another 4 h. During the last 30 min, cells were pulse-labeled with BrdU and subsequently processed as described under *Materials and Methods*. Images show one experiment representative of three.

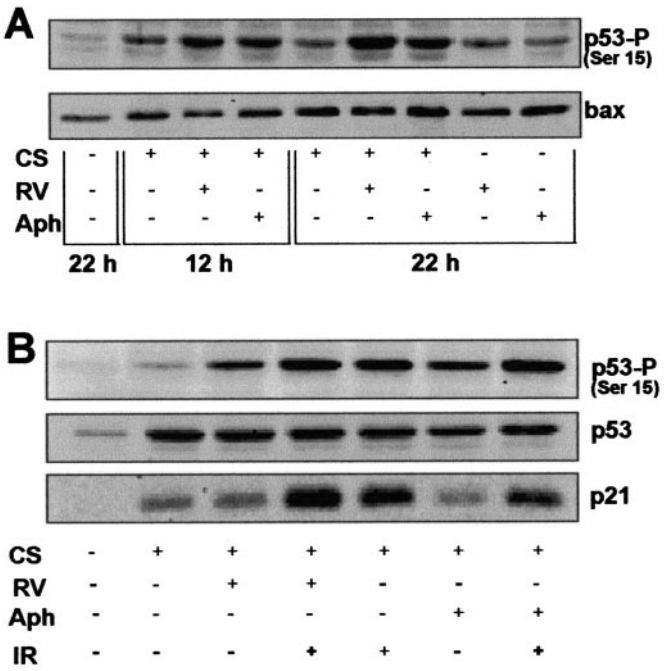

Fig. 6. Effects of RV, Aph and γ-irradiation on p53 serine[15] phosphorylation, Bax, p53, and p21 levels. A, Western blot analysis of p53 phosphorylation (serine[15]) as well as Bax protein expression at two different time points (12 and 22 h) using Aph (1 μM) as a positive control. Cells were treated with RV (100 μM), Aph (1 μM) or vehicle for 30 min before stimulation with (+) or without (−) 10% calf serum (CS) for the indicated times. One Western blot that is representative of three is shown. B, Western blot analysis of p53 phosphorylation (serine[15]) and total p53 levels and p21 levels in RV-treated and γ-irradiated cells using Aph as a control. Serum-starved cells were treated with growth medium containing RV (100 μM), Aph (1 μM), or vehicle for 12 h before IR (10 Gy). Cells were harvested 12 h after IR. One Western blot that is representative of four is shown.

accumulation of RV- or Aph-treated cells in S phase leads to a concomitant accumulation of hyperphosphorylated Rb compared with normally progressing control cells. Consistently, $p21^{Cip1}$ and $p27^{Kip1}$ levels also seem to be altered only after prolonged treatment, corroborating that G_1 is not affected by RV and also not, as already known, by Aph. Interestingly, in contrast to RV, Aph treatment does not down-regulate $p21^{Cip1}$ protein levels, arguing against an identical mechanism of these two substances. Thus, RV seems to block DNA replication in VSMCs in a manner similar to that of the DNA polymerase inhibitor Aph but without using its exact molecular mechanism of action.

Interestingly, cell-free in vitro studies have provided evidence that RV is an inhibitor of DNA polymerase α and δ (Stivala et al., 2001) and of the ribonucleotide reductase (Fontecave et al., 1998). Experiments performed in a fibroblast cell line revealed that the ribonucleotide reductase inhibitor hydroxyurea also leads to accumulation of cells in early S phase, accompanied by an increase in hyperphosphorylated Rb (Linke et al., 1996). Two distinct studies point to similarities between RV and hydroxyurea in terms of ribonucleotide reductase inhibition (Fontecave et al., 1998; Rodrigue et al., 2001). We showed, however, that RV completely prevents further S phase progression in cells released from Aph block. This suggests that a preceding depletion of the deoxyribonucleotide pools is not a prerequisite for RV-induced cell cycle arrest in VSMCs.

The tumor-suppressor protein p53 is critically involved in cell cycle control and seems to play a key role in VSMC proliferation after vascular injury. In this context, loss of p53 activity has been implicated in the pathogenesis of human restenosis (Speir et al., 1994), and transfer of wild-type p53 gene has been shown to inhibit VSMC proliferation in vivo and in vitro (Yonemitsu et al., 1998). In normally proliferating cells, p53 is maintained at low levels. This is a consequence of the interaction with the Mdm2 protein, which targets p53 for ubiquitination and degradation. Stress signals such as γ-irradiation trigger p53 phosphorylation at a number of N-terminal sites, which affects its affinity for Mdm2, leading to p53 stabilization (Oren, 1999). Phosphorylation at serine[15], in particular, has been implicated in this process (Shieh et al., 1997; Oren, 1999). However, more recent data suggest that serine[15] phosphorylation does not alter p53 interaction with Mdm2. Instead, it is proposed to be responsible for p53 transactivation by promoting interaction with cAMP response element-binding protein binding protein/p300 (Lambert et al., 1998; Dumaz and Meek, 1999; Schon et al., 2002). This seems to be the case in VSMCs, because IR, RV, and Aph all cause enhanced serine[15] phosphorylation but fail to increase total levels of p53.

Our results show that a reversible block of DNA replication triggered by substances such as Aph or RV leads to an increase of serine[15] phosphorylation of p53. However, in striking contrast to elevated levels of serine[15]-phosphorylated p53 caused by IR, DNA replication block does not lead to enhanced levels of p21, a p53-regulated protein. This is consistent with results recently reported by Gottifredi et al. (2001). Therefore, serine[15]-phosphorylated but transcriptionally impaired p53 may be considered a marker for reversible S phase arrest.

However, there seem to be considerable mechanistic differences between p53 regulation by Aph in different cell lines: in RKO cells used by Gottifredi et al. (2001), hydroxyurea and Aph were able to suppress induction of p21 by IR, suggesting that p53 is held in a state of active repression. This does not seem to be the case in VSMCs because IR increases p21 levels despite the presence of Aph or RV, respectively (Fig. 6B). Therefore, it may be speculated that the molecular mechanism of action of RV and Aph is different in VSMCs compared with RKO cells: for VSMCs, active repression of p53 by Aph and RV seems less likely; instead, it seems probable that the impaired response to p53 after RV and Aph treatment may be because of the lack of critical modifications or cofactors of the p53 protein. That way, p53 can be considered as partially latent, with the full transcriptional activity being restored after IR, a stimulus known to fully activate the protein.

The induction pattern for p53 by RV and Aph was similar in our cells. We therefore conclude that our results corroborate the hypothesis that RV induces a reversible DNA replication block comparable, but not identical, with that of Aph in VSMCs.

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