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The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle☆

Eulalia Pozo-Guisado[a], Alberto Alvarez-Barrientos[b], Sonia Mulero-Navarro[a], Belen Santiago-Josefat[a], Pedro M. Fernandez-Salguero[a,*]

[a]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, Avenida de Elvas s/n, 06071 Badajoz, Spain*

[b]*Centro de Citometría de Flujo y Microscopía Confocal, Facultad de Farmacia, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*

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Abstract

Resveratrol, a natural phytoalexin, has gained much interest on the basis of its potential chemopreventive activity against human cancer. In this work, using the human breast cancer cell lines MCF-7 and MDA-MB-231, we have analyzed a possible mechanism by which resveratrol could interfere with cell cycle control and induce cell death. Our results show that although resveratrol inhibited cell proliferation and viability in both cell lines, apoptosis was induced in a concentration- and cell-specific manner. In MDA-MB-231, resveratrol (up to 200 μM) lowered the expression and kinase activities of positive G1/S and G2/M cell cycle regulators and inhibited ribonucleotide reductase activity in a concentration dependent manner, without a significant effect on the low expression of tumor suppressors p21, p27, and p53. These cells died by a non-apoptotic process in the absence of a significant change in cell cycle distribution. In MCF-7, resveratrol produced a significant and transient (<50 μM) increase in the expression and kinase activities of positive G1/S and G2/M regulators. Simultaneously, p21 expression was markedly induced in presence of high levels of p27 and p53. These opposing effects resulted in cell cycle blockade at the S-phase and apoptosis induction in MCF-7 cells. Thus, the antiproliferative activity of resveratrol could take place through the differential regulation of the cell cycle leading to apoptosis or necrosis. This could be influenced, among other factors, by the concentration of this molecule and by the characteristics of the target cell.

Keywords: Resveratrol; Apoptosis; Cell cycle blockade; Ribonucleotide reductase; Breast cancer cells; Chemoprevention

1. Introduction

The *trans*-isomer of resveratrol (3,4′,5-trihydroxystilbene) is a natural phytoalexin that certain plant species synthesize in response to fungal infections. Resveratrol and its derivatives are present in significant amounts in grape skin and, as a consequence, in many different red wines [1–4]. Population studies have suggested that regular and moderate red wine consumption could have beneficial effects in human health [5,6]. These protective effects of resveratrol have been related to its potential to modulate a wide variety of cellular functions. Thus, this molecule inhibits polymorphonuclear leukocyte function in coronary heart disease [7], partially prevents platelet aggregation [8,9], ameliorates certain forms of oxidative stress [10,11], inhibits 5-lipoxygenase activity and prostaglandin synthesis [12,13], and has antiestrogenic activity [14,15].

Several reports have shown an association between cell proliferation, cancer, and apoptosis. Increasing experimental data suggest that cell cycle control, particularly at the

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* Corresponding author. Tel.: +34-924-289300x6867; fax: +34-924-289419.

E-mail address: pmfersal@unex.es (P.M. Fernandez-Salguero).

Abbreviations: 5′-CDP, cytidine 5′-diphosphate; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GST-pRb, gluthathione-S-transferase-retinoblastoma protein; PBS, phosphate buffered saline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; D-MEM, Dulbecco's-modified Eagle's medium.

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G1/S and G2/M transitions, represent a major task for the cell to ensure an accurate cell division. Carcinogenic processes often affect progression through the S phase by inducing changes in expression and/or activity of cell cycle regulators [16], thus offering potential targets for chemoprevention and cancer treatment in humans [17–19].

Molecules with the potential to alter cell growth in tumor cells, without significantly affecting the viability of normal cells, represent useful anticancer agents. During the last few years, activation of apoptosis has been proposed as a potential mechanism for a chemotherapeutic agent to induce the elimination of cancerous cells [20,21]. Recent data have shown that resveratrol could inhibit tumor development in a mouse skin carcinogenesis study [22] and cellular proliferation in cancer-derived cell lines [23–25]. This antiproliferative activity of resveratrol has also been shown to involve apoptosis induction in cancer cells [24–26]. However, the mechanisms through which resveratrol promotes changes in cell cycle and their relevance in apoptotic vs. non-apoptotic cell death deserves further investigation.

Tumors are usually formed by cells with different phenotypes and metastatic potential. Antiproliferative molecules could exert their effects by inducing cell-specific alterations in the expression and/or activity of cell cycle regulators. In this context, it could be of interest to analyze the effects of resveratrol on cell cycle control, eventually leading to apoptotic or non-apoptotic cell death in a cell-specific manner. In this work, using two cell lines derived from human breast tumors such as MCF-7 and MDA-MB-231, differing in their proliferative potential, we have analyzed the activation by resveratrol of cell-specific mechanisms altering cell cycle control and inducing apoptotic vs. non-apoptotic death. We have found that although resveratrol decreased cell viability and proliferation in both cell lines, apoptotic cell death was only induced in MCF-7. Further, apoptosis induction was related to cell type-specific regulation of the cell cycle at the G1/S and G2/M transitions. Our results suggest that the properties of the target cancer cell and the concentration of resveratrol could significantly influence the mechanism of action of this antiproliferative molecule, the cellular pathway leading to tumor regression and its chemopreventive potential.

2. Materials and methods

2.1. Chemicals and reagents

Resveratrol, DAPI, ATP, DTT, CDP, 2′-deoxycytidine 5′-diphosphate, and the human antibody for β-actin were from Sigma–Aldrich Chemical. [5-^{3}H]-CDP was purchased from Amersham Biosciences. Affi-gel 601 (boronate gel) was from Bio-Rad Laboratories. Dulbecco's-modified Eagle's medium (D-MEM) and fetal bovine serum were purchased from Life Technologies. Histone H1 protein was obtained from Stress-Gen. Antibodies against cyclin B1 (sc-245), cyclin D1 (sc-717), cyclin E (sc-481), CDK4 (sc-260), p21 (sc-397), p27 (sc-527), p53 (sc-100) and cdc2 (sc-54), protein A/G plus agarose and gluthathione-S-transferase-retinoblastoma protein (GST-pRB) were from Santa Cruz Biotechnology.

2.2. Cell culture

The human breast epithelial cell lines MCF-7, an estrogen receptor-positive cell line derived from an *in situ* carcinoma, and MDA-MB-231, an estrogen receptor-negative cell line derived from a metastatic carcinoma, were used in this study. Both were grown in D-MEM medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B. Cells were routinely propagated and cultured at 37° in a 5% CO_2 atmosphere. For treatment with resveratrol, cells were counted and plated at the same initial density. After 24 hr, when cultures reached 70–80% confluence, cells were treated with the indicated concentrations of *trans*-resveratrol dissolved in sterile DMSO. Treatments were done for lengths of time ranging from 12 to 60 hr, with the addition of fresh resveratrol and culture medium each day.

2.3. Cell viability and proliferation

Cell viability was determined by the MTT reduction assay as described [27]. Briefly, D-MEM culture medium was removed and cells incubated at 37° for 20 min in Locke's solution (10 mM HEPES pH 7.4, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 134 mM NaCl, 4 mM $NaHCO_3$, 5 mM glucose) containing 0.35 mM MTT. This solution was carefully aspirated and the formazan produced by the mitochondrial dehydrogenase activity dissolved in DMSO. Cell viability was measured as the difference in absorbance between 490 and 650 nm (baseline). Experiments were done by varying the concentration of resveratrol and the length of treatment. From these experiments, we selected treatments of 36 and 48 hr for MCF-7 and MDA-MB-231 cells, respectively. Untreated, control cells, were incubated under identical conditions with the same volume of DMSO. All the experiments were done in duplicate in at least three cultures from each cell line.

Cell proliferation was determined by measuring the rate of DNA synthesis. Cultures growing in 24-well plates were treated with resveratrol and, at the indicated times, 1 μCi [methyl-^{3}H]-thymidine (sp. act., 7 Ci/mmol) was added for 2 hr. Labelling media was removed and cells fixed for 2 hr at room temperature in 1 mL methanol/acetic acid (1:1). Fixed cells were washed with 80% ethanol and incubated with 0.05% trypsin–EDTA for 30 min at 37°. Cells were then lysed for 5 min at room temperature by the addition of 1% (w/v) SDS and incorporated thymidine quantitated in a Beckman LS 3801 liquid scintillation counter.

2.4. Nuclear staining with DAPI

After treatment, cells were quickly washed in ice-cold PBS, fixed in methanol at −20° for 5 min, air dried and incubated for 5 min with the DNA-specific fluorochrome DAPI. The excess of DAPI was removed and cells were observed and photographed using a Zeiss fluorescence microscope.

2.5. Polyacrylamide gel electrophoresis and Western blot

After treatment with resveratrol, cells were washed twice with PBS and lysed in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 1 mM phenyl-methyl sulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 10 mM β-glycerophosphate, and 4 μg/μL complete protease inhibitor cocktail). Lysates were centrifuged at 15,000 *g* for 30 min at 4° and protein concentration determined in the supernatants as indicated below. Ten micrograms of protein were mixed with SDS-sample buffer, denatured, and electrophoresed using 10 or 12% SDS–PAGE gels. After electrophoresis, gels were transferred to nitrocellulose membranes by electroblotting and membranes blocked for 2 hr at room temperature in TBS-T (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) containing 5% (w/v) non-fat milk. Membranes were then incubated for 1 hr at room temperature with the corresponding primary antibodies diluted 1:1000 in blocking solution. After extensive washing in TBS-T, the corresponding secondary antibody was added and incubation continued for 1 hr at room temperature. After extensive washing in TBS-T, Super-signal luminol substrate (Pierce) was added and membranes exposed for 30 min to a chemiluminescence imaging screen (Bio-Rad Laboratories). The screen was scanned using a Molecular Imager FX System from Bio-Rad Laboratories.

2.6. Flow cytometry analysis

For flow cytometry analysis, cultures were synchronized by serum deprivation. Briefly, at the appropriate cell density, complete medium was replaced by serum-free medium for 40 hr. After this time, serum concentration was restored to 10% and resveratrol treatments performed during 36 or 48 hr for MCF-7 or MDA-MB-231, respectively. By using this method, between 85 and 90% of the MCF-7 and around 80% of the MDA-MB-231 were synchronized at G1/G0 as determined by flow cytometry analysis. Cell cycle distribution and ploidy status of MCF-7 and MDA-MB-231 cells, after treatment with resveratrol, was determined by flow cytometry DNA analysis. At each concentration of resveratrol, cells were detached from the plates by the addition of 0.25% trypsin, washed in PBS, fixed in 70% ethanol at 4° and treated with RNase (10 mg/mL) for 30 min at 37°. The DNA content per cell was evaluated in a FACScan flow cytometer (Becton–Dickinson) after staining the cells with propidium iodide (50 μg/mL) for 15 min at room temperature in the dark. For cell cycle analysis, only signals from single cells were considered (10,000 cells/sample).

2.7. Ribonucleotide reductase activity assay

Ribonucleotide reductase activity was measured using [5-^{3}H]-CDP as substrate. After treatment with resveratrol, cells were trypsinized, sequentially washed in PBS and ice-cold HEPES buffer (100 mM HEPES pH 7.6, 15 mM magnesium acetate, 10 mM DTT) and centrifuged. Cell pellets were gently homogenized in HEPES buffer at 4° with 25 strokes of a loose-fitting Dounce homogenizer and centrifuged at 15,000 *g* for 60 min at 4°. Protein concentration was determined in the supernatants as described below. The following reagents were incubated in HEPES buffer at 30° for 50 min in a final volume of 100 μL: 5 mM ATP, 50 μM non-labeled CDP, 1 μCi [5-^{3}H]-CDP (sp. act., 22 Ci/mmol), and 75–100 μg cell extract. The reaction was stopped by heating at 90° for 2 min. In order to separate substrate (cytidine 5′-diphosphate) from product (2′-deoxycytidine 5′-diphosphate), samples were subjected to boronate gel affinity chromatography as described [28]. The amount of tritiated product was quantitated by liquid scintillation counting. High performance liquid chromatography was used to confirm that the boronate gel retained at least 95% of the substrate whereas eluted more than 95% of the product (results not shown).

2.8. Immunoprecipitation and kinase activity

Cultures were grown and treated with solvent (DMSO), 10, 50, 100, or 150 μM resveratrol for the indicated times. Cells were then lysed on ice for 10 min with 500 μL IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40) containing 1 mM phenyl-methyl sulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 mM β-glycerolphosphate, 1 mM DTT, and 4 mg/mL complete protease inhibitor cocktail. After brief mixing, lysates were centrifuged at 15,000 *g* for 5 min at 4°. Pellets were discarded and protein concentration determined in the supernatants as indicated below. An amount of 400 μg protein was used for each immunoprecipitation reaction. To eliminate non-specific binding, lysates were pre-incubated for 1 hr at 4° with 20 μL protein A/G Plus-agarose beads. After removing the beads by centrifugation, cell lysates were rotated overnight at 4° with 1-μg anti-cyclin D1 or anti-cdc2 polyclonal antibodies. Extracts were then incubated at 4° for an additional 60 min with 30 μL protein A/G Plus-agarose beads. Beads were washed three times in IP buffer and two times in kinase buffer (20 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) and finally resuspended in a total volume of 10 μL kinase buffer. Kinase activity was assayed at 30° for 30 min in

25 μL kinase buffer containing 10 μL protein A/G Plus-agarose beads, 1 mM NaF, 1 mM sodium-orthovanadate, 1 μg GST-Rb or 5 μg Histone H1 target proteins, 10 μCi ^{32}P-γ-ATP (sp. act., 3000 Ci/mmol), and 1 mM non-labeled ATP. After the reaction was completed, SDS-sample buffer was added and samples boiled for 5 min. The levels of phosphorylated GST-Rb or Histone H1 were determined by SDS–PAGE electrophoresis and autoradiography using phosphor-screens on a Molecular Imager FX-System (Bio-Rad Laboratories).

2.9. Protein concentration and statistical significance of the results

Protein concentration was determined using Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as standard. Statistical analysis were done using ANOVA on the Instat software program (GraphPAD Sotfware). Data shown are mean ± SD.

3. Results

3.1. Resveratrol inhibited cell viability in MCF-7 and MDA-MB-231

Treatment with resveratrol produced an inhibition in the time-dependent kinetic of cell growth in both MCF-7 and MDA-MB-231 (Fig. 1). Although growth inhibition was concentration-dependent in either cell line, it was more pronounced at shorter times in MCF-7 (Fig. 1A) than in MDA-MB-231 (Fig. 1B). In fact, MDA-MB-231 cells were almost insensitive to any of the concentrations of resveratrol used for treatments shorter than 36 hr. The shape of the growth curves for these cell lines were differentially affected by increasing concentrations of resveratrol, suggesting a different sensitivity to this molecule between both cell types. From these results, we selected treatments with resveratrol for 36 hr in MCF-7 or 48 hr in MDA-MB-231.

3.2. Resveratrol decreased cell proliferation and cell numbers in MCF-7 and MDA-MB-231

To analyze if the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in presence of resveratrol (Fig. 2A). Resveratrol inhibited thymidine incorporation in both cell lines in a concentration-dependent manner. Furthermore, the profile of DNA synthesis obtained was similar between MCF-7 and MDA-MB-231. To determine if the observed inhibition in cell viability and proliferation were associated to the induction of cell death, we determined cell numbers in both cultures after resveratrol treatment (Fig. 2B). It can be observed that increasing resveratrol concentration resulted in decreased cell numbers in MCF-7 and MDA-MB-231. At high concentrations of the molecule (e.g. above 100 μM) cell death was similar in both cell lines. Interestingly, at concentrations around 50 μM, MCF-7 appeared to be significantly less sensitive to cell death than MDA-MB-231 (Fig. 2B).

Taken together, these data suggested that for both cell lines, decreased cell viability and proliferation correlated to cell death at high concentrations of resveratrol. However, at concentration below 50 μM, diminished cell viability and proliferation was followed by a corresponding increase in cell death in MDA-MB-231 but not in MCF-7. These differential effects of resveratrol, particularly at low concentrations, could be reflecting a cell-specific alteration of the cell cycle, and eventually, the activation of different mechanisms of cell death.

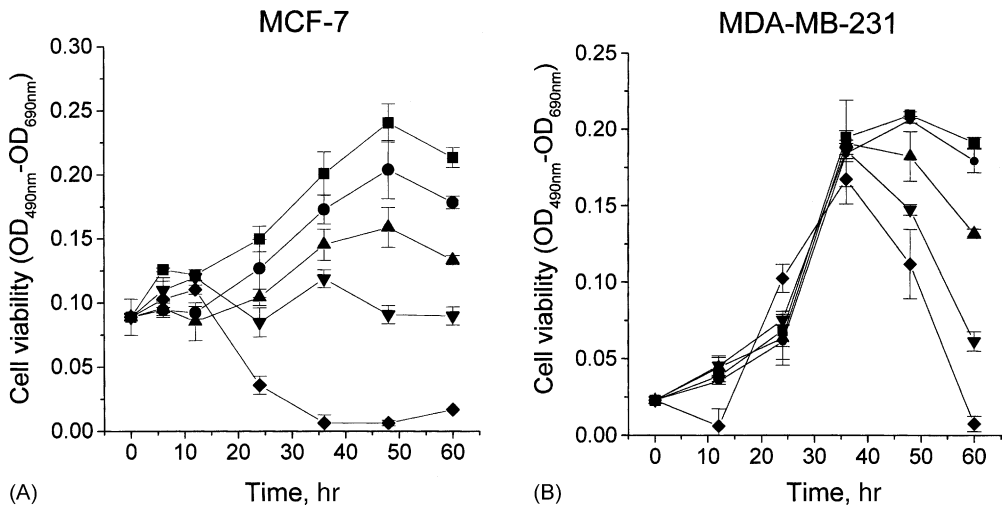

Fig. 1. Resveratrol decreases cell viability in MCF-7 and MDA-MB-231 human breast cancer cells. Cells were treated with different concentrations of resveratrol and viability determined with time by the MTT reduction assay. (A) Cell viability in MCF-7 and (B) cell viability in MDA-MB-231. The following concentrations of resveratrol were used: none (■), 10 μM (●), 50 μM (▲), 100 μM (▼) and 200 μM (◆). Measurements were done in duplicate in three different cultures.

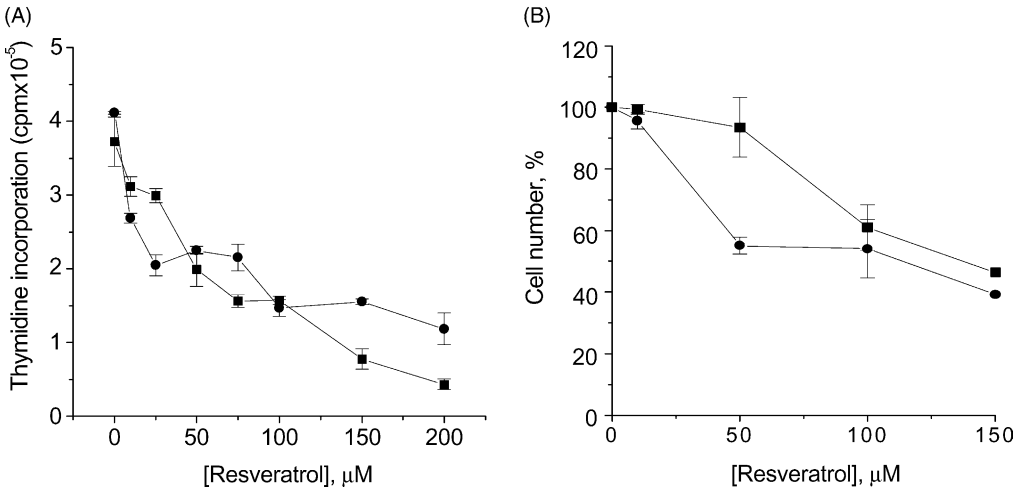

Fig. 2. Resveratrol inhibits cell proliferation and decreases cell numbers in MCF-7 and MDA-MB-231. (A) Cells were treated with increasing concentrations of resveratrol for 36 hr (MCF-7) or 48 hr (MDA-MB-231) and cell proliferation determined by measuring the rate of [^{3}H]-thymidine incorporation during DNA synthesis. (B) Cell numbers were determined after resveratrol treatment in MCF-7 and MDA-MB-231 by trypsin digestion and cell counting using a hematocytometer. Symbols correspond to MCF-7 (■) and MDA-MB-231 (●). The experiment was done in duplicate in at least three different cultures.

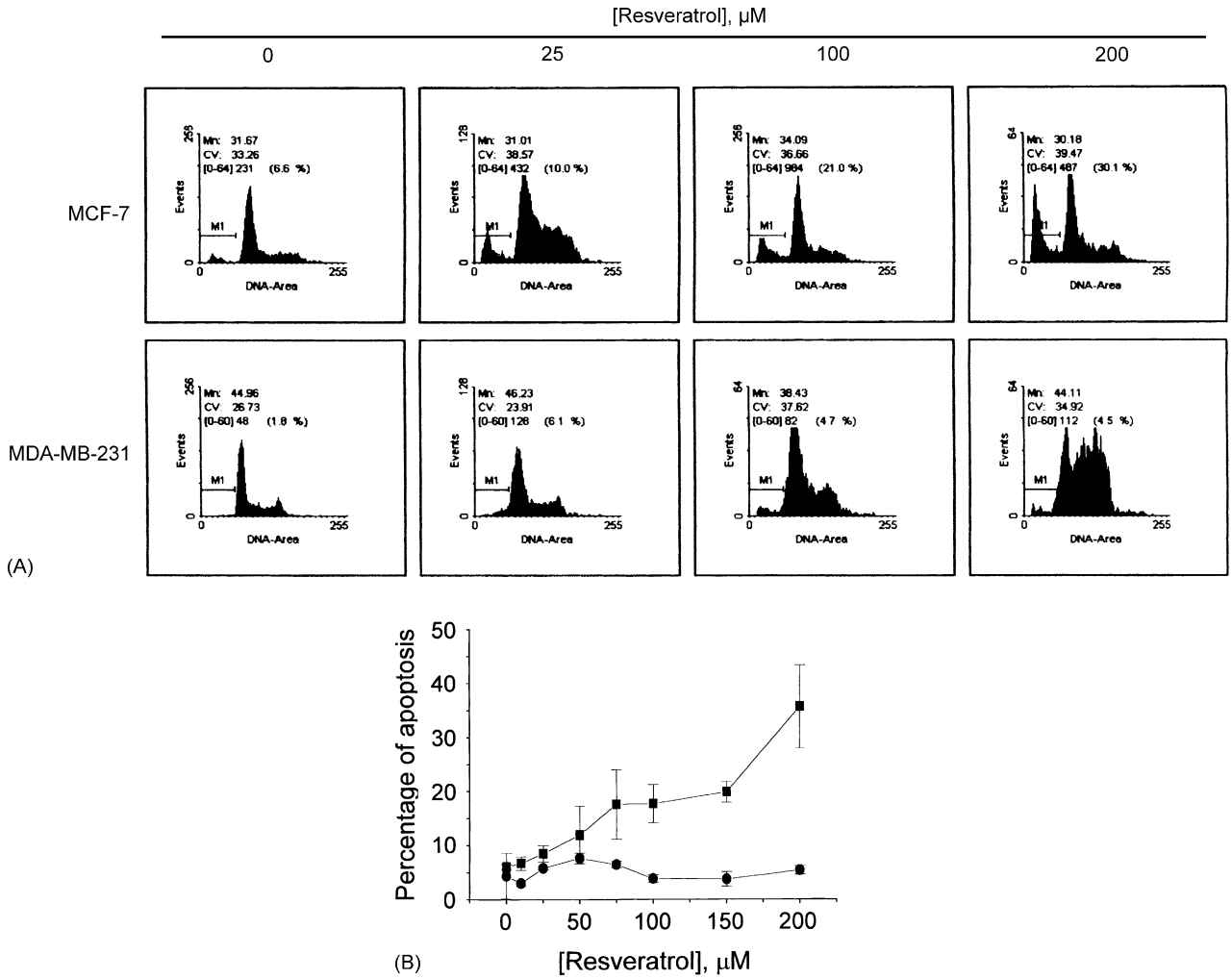

Fig. 3. Resveratrol induces apoptosis in MCF-7 but not in MDA-MB-231 cells. MCF-7 (■) and MDA-MB-231 (●) cells were treated with resveratrol at the indicated concentrations for 36 or 48 hr, respectively. Apoptosis was analyzed by flow cytometry after staining the cells with propidium iodide. (A) Representative profiles of cell cycle status for both cell lines. The area of the peak corresponding to hypodiploid DNA is indicated as M1. (B) Quantitative analysis of apoptosis was done using profiles as those shown in panel A. Note the absence of hypodiploid DNA in MDA-MB-231 and its increase with the concentration of resveratrol in MCF-7. Data shown are mean ± SD from triplicate determinations. The experiment was done two times with similar results.

3.3. Resveratrol induced apoptosis in MCF-7 but not in MDA-MB-231

To analyze the contribution of apoptosis to this process, we determined the ploidy status of MCF-7 and MDA-MB-231 by flow cytometry in presence of resveratrol (Fig. 3). We found that resveratrol increased the area of the peak corresponding to a hypodiploid (apoptotic) DNA content (M1 peak in the representative profiles shown in Fig. 3A) in MCF-7 but not in MDA-MB-231. A quantitative analysis of apoptosis is presented in Fig. 3B. It can be observed that resveratrol induced apoptosis in MCF-7 in a concentration-dependent manner, reaching values close to 40% at 200 μM. On the contrary, no hypodiploid DNA was found in MDA-MB-231 in presence of resveratrol (Fig. 3). These results indicated that: (i) resveratrol induced cell death by apoptosis in MCF-7 but not in MDA-MB-231; (ii) at concentrations of resveratol around 50 μM, apoptosis and cell death were low and of similar magnitude in MCF-7; and (iii) at low concentrations, apoptosis numbers in MCF-7 were significantly lower than the decrease in cell proliferation. From these results, it appeared that in MCF-7 but not in MDA-MB-231, low concentrations of resveratrol could be inducing cell accumulation along the cell cycle with a minimal effect on cell death.

To further characterize this cell-specific apoptotic process in MCF-7 cells, we analyzed the presence of chromatin condensation and nuclear fragmentation by fluorescence microscopy using the DNA-binding fluorescent dye DAPI (Fig. 4). In absence of resveratrol, cultures from both cell lines presented nuclei with homogeneous chromatin distribution (Fig. 4A and C). In presence of 50 μM resveratrol, no significant change in chromatin distribution was observed in the nuclei of MCF-7 cells (Fig. 4D). However, in this same cell line, concentrations of resveratrol of 100 μM (Fig. 4E) or 150 μM (Fig. 4F) induced chromatin condensation and nuclear fragmentation (arrows), that suggested the presence of apoptotic cells. In agreement to the data shown above, MDA-MB-231 cells, treated with 150 μM resveratrol, did not show an apoptotic phenotype (Fig. 4B). Interestingly, the mitotic features present in untreated cultures from both cell lines

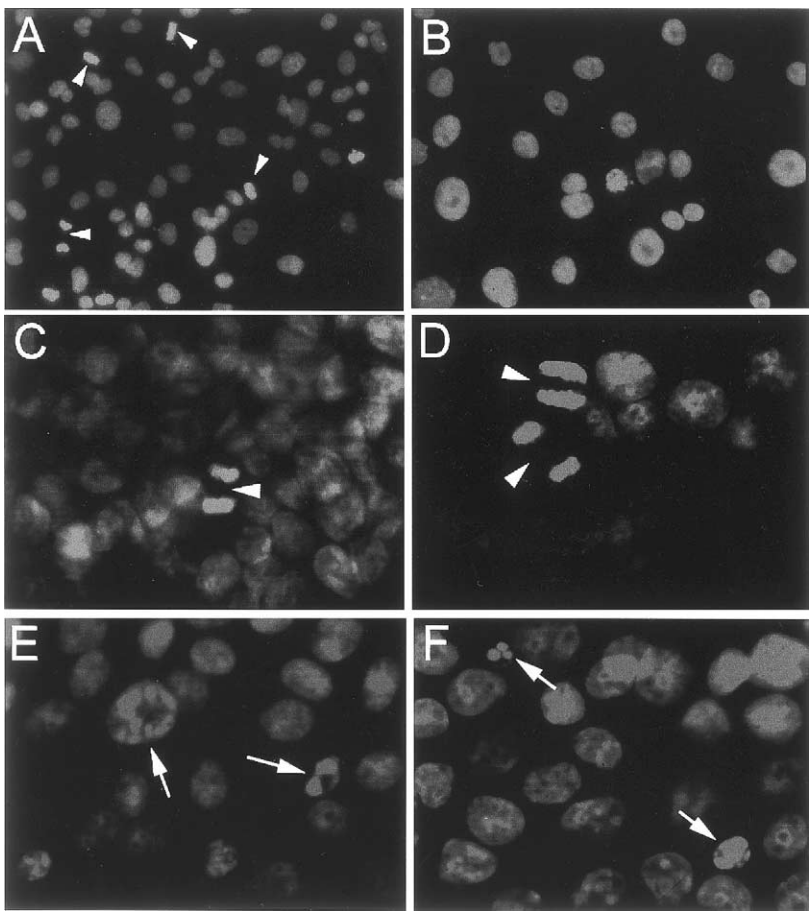

Fig. 4. DAPI immunofluorescence shows apoptosis induction in MCF-7 cells. Apoptosis induction by resveratrol in MCF-7 (C–F) and MDA-MB-231 (A and B) was analyzed by immunofluorescence after nuclear staining with DAPI. Cells were treated with DMSO (A and C), 50 μM (D), 100 μM (B and E), or 150 μM (F) resveratrol for 36 hr (MCF-7) or 48 hr (MDA-MB-231). Mitotic features including cells in metaphase and anaphase are indicated by arrowheads. Cells undergoing apoptosis and presenting chromatin condensation and nuclear fragmentation are indicated by arrows. Magnification is 400×. Representative images from three different experiments are shown.

(Fig. 4A and C, arrowheads), were completely absent after treatment with concentrations of resveratrol of 100 μM (Fig. 4E) or 150 μM (Fig. 4B and F). Cells in mitosis could still be found in MCF-7 cells treated with 50 μM resveratrol for 36 hr (Fig. 4D, arrowheads). Thus, in addition to the cell-specific induction of apoptosis in MCF-7, resveratrol also appeared to inhibit mitosis in both MCF-7 and MDA-MB-231 cells.

3.4. Effect of resveratrol on cell cycle distribution

Considering that resveratrol decreased cell proliferation and induced cell death, we analyzed the effect of this molecule on cell cycle distribution by flow cytometry (Fig. 5). Cultures were synchronized by serum deprivation for 40 hr and this resulted in cell cycle arrest at G0/G1 for close to 90% of the MCF-7 and 80% of the MDA-MB-231. Serum deprivation was more effective in synchronizing MCF-7 cells, in agreement with their lower proliferation rates (see below). In MCF-7, 50 μM resveratrol induced accumulation of cells in S phase, concomitantly to a significant decrease in G0/G1 cells and, to a much lesser extent, to G2/M cells. Increasing the concentration to 150 μM restored the G0/G1 population to close to initial control levels. In MDA-MB-231, resveratrol only induced

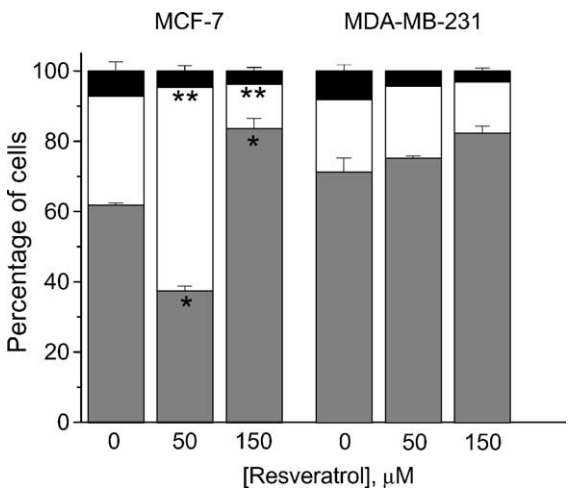

Fig. 5. Effect of resveratrol on cell cycle distribution in MCF-7 and MDA-MB-231. Cells were synchronized by serum deprivation as indicated in Section 2. Then, serum was restored to 10% and cells were left untreated (0) or treated with the indicated concentrations of resveratrol for 36 hr (MCF-7) or 48 hr (MDA-MB-231). Cell cycle distribution in synchronized cultures, before the addition of serum and/or resveratrol is also shown (SIN). Cells were then stained with propidium iodide and their DNA content determined by flow cytometry as indicated in Section 2. Gray bars correspond to G0/G1, clear bars to S phase, and black bars to G2/M. Data shown are mean ± SD from triplicate determinations. The experiment was repeated twice with similar results. The level of significance for the different cell populations after resveratrol treatment has been calculated by ANOVA with respect to the corresponding controls in absence of resveratrol: (*) the changes in the numbers of cells at the G0/G1 phase are significant at $P < 0.001$; (**) the changes in cell numbers at the S phase are significant at $P < 0.002$.

and slight accumulation of G0/G1 cells. Thus, resveratrol differentially affected cell cycle distribution between both cell lines and this could be potentially related to the selective induction of apoptosis. Cell accumulation at the S phase in MCF-7 cells treated with 50 μM resveratrol could account for the low rates of cell death in presence of a significant decrease in cell proliferation. Additionally, the reduction in the number of cells at the G2/M phase agreed with the absence of mitotic features in either cell line at high concentrations of resveratrol (see Fig. 4).

3.5. Resveratrol induced changes in the expression and activity of cell cycle regulators

Based on the cell-specific effects of resveratrol on cell cycle distribution and apoptosis in MCF-7 with respect to MDA-MB-231, we have analyzed the induction of differential changes in cell cycle regulatory proteins (Fig. 6). With respect to MDA-MB-231, resveratrol induced a concentration-dependent inhibition in the expression of regulators of the G1/S transition of the cell cycle such as cyclin D1, CDK4, and cyclin E, reaching very low levels at 150 μM (Fig. 6A). In contrast, MCF-7 cells exhibited a transient increase in the expression of these proteins that peaked at 50 μM resveratrol (Fig. 6A). This increase was more significant for cyclin D1 and cyclin E, both major regulators of the G1/S transition that promote entry into the S phase of the cell cycle. At concentrations of resveratrol higher than 50 μM, the expression of these proteins returned to close to control levels, with a pattern of decay similar to that observed in MDA-MD-231 cells (Fig. 6A). By following cell growth with time, we have determined that duplication times were 16 and 33 hr for MDA-MB-231 and MCF-7, respectively. In agreement to this observation, basal levels of positive regulators were higher in MDA-MB-231 than in MCF-7. Since cell cycle progression depends on the ratio between positive and negative regulators, we also analyzed the expression of cell cycle tumor suppressors p21, p27, and p53. These proteins were almost undetectable in MDA-MB-231 cells, whereas were present at detectable levels in MCF-7 (Fig. 6B). Although in MCF-7 resveratrol did not essentially affect p53 and p27 levels, it induced a marked and transient increase in p21 expression. Further, the profile of p21 expression in presence of resveratrol was similar to that observed for cyclin D1, CDK4, and cyclin E (Fig. 6A). Expression of these tumor suppressors at concentrations of resveratrol around 150 μM remained significant in MCF-7 but not in MDA-MB-231. Since resveratrol affected cell cycle distribution at the G2/M phases (see Fig. 5), we studied changes in the expression of regulatory proteins involved in the control of the G2/M transition. As shown in Fig. 7, and in a similar fashion to the effects reported for the G1/S transition, resveratrol inhibited the expression of the G2/M regulators cdc2 and cyclin B1 in a concentration-dependent manner in MDA-MB-231 cells. In MCF-7 cells, a

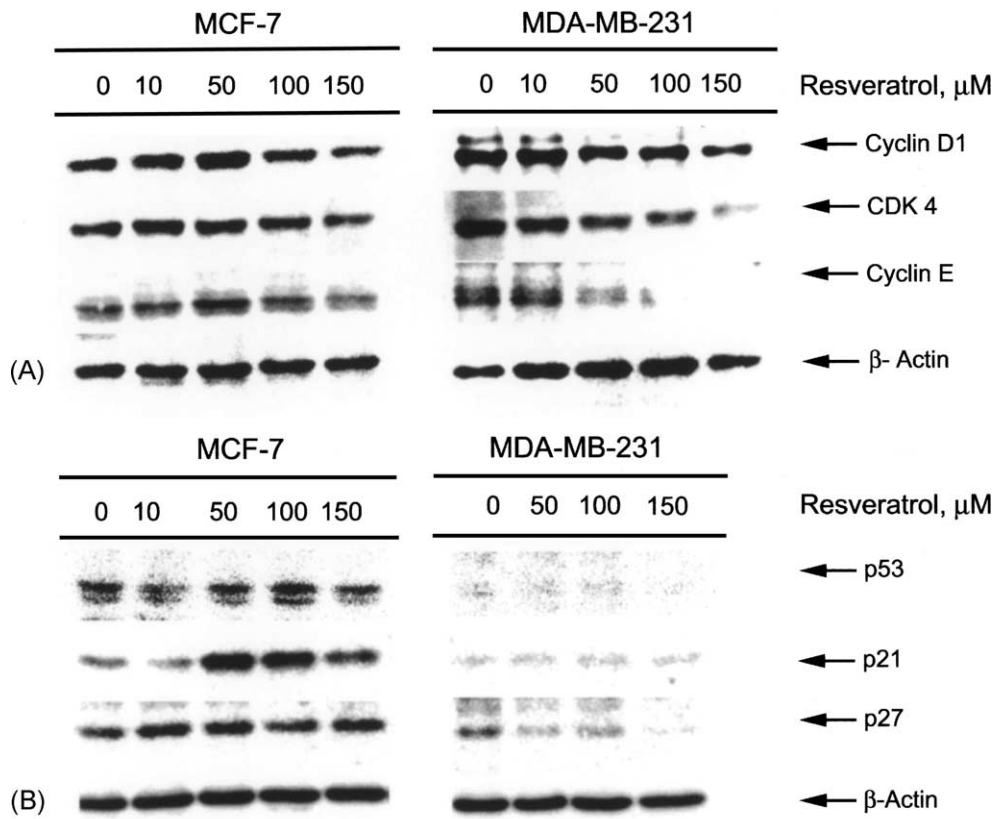

Fig. 6. Resveratrol modulates the expression of cell cycle regulators of the G1/S transition. Cells were treated with the indicated concentrations of resveratrol and cellular extracts analyzed for protein expression by Western blot. (A) The expression of positive regulators such as cyclin D1, CDK4, and cyclin E was analyzed in MCF-7 (left panel) and MDA-MB-231 (right panel). Note the transient increase in the expression of cyclin E, a gene regulated by cyclin D1/CDK4 kinase activity. As expected, the basal level of positive regulators in MDA-MB-231 cells was higher than in MCF-7. (B) The expression of tumor suppressors p53, p27, and p21 was also analyzed. Note the marked and transient increase in p21 expression in MCF-7 cells. The basal level of negative regulators in MDA-MB-231 cells was significantly lower than in MCF-7. The expression of human β-actin was analyzed to confirm sample integrity and gel loading. Ten micrograms protein were used for each experimental condition. The experiment was repeated at least twice with similar results.

transient increase in cdc2 and cyclin B1 expression was observed that reached a maximum at 50 μM resveratrol, to decrease to very low levels at 150 μM (Fig. 7). Thus, resveratrol differentially affected the expression of G1/S and G2/M regulators between MCF-7 and MDA-MB-231, particularly at low concentrations. Nevertheless, within each cell type, resveratrol influenced the expression of G1/S and G2/M regulators with similar pattern. Since kinase activity, rather than just protein expression, is generally considered a major factor controlling cell cycle progression, we measured the activity of cyclin D1/CDK4 (using GST-pRB as target) and cyclin B1/cdc2 (using histone H1 as target) complexes

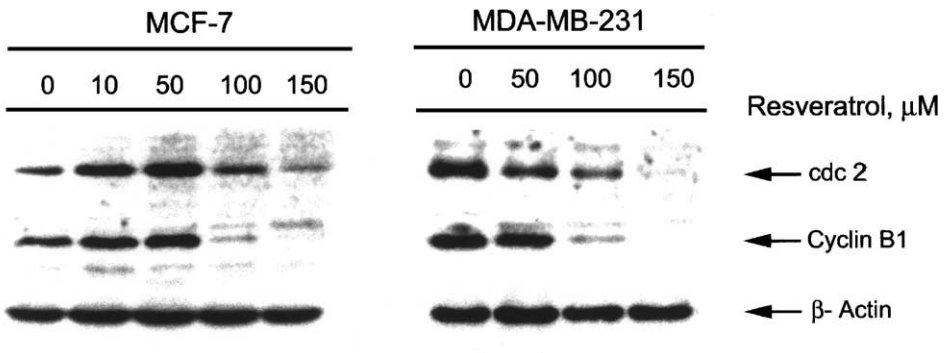

Fig. 7. Resveratrol modulates the expression of cell cycle regulators of the G2/M transition of the cell cycle. After treatment with the indicated concentrations of resveratrol, cellular extracts were analyzed by Western blot for the expression of positive regulators of the G2/M transition. Protein levels of cdc2 and cyclin B1 were determined in MCF-7 (left panel) and MDA-MB-231 (right panel). As expected, basal expression of both proteins were higher in MDA-MB-231 than in MCF-7. The expression of human β-actin was measured to confirm sample integrity and gel loading. Ten micrograms protein were used for each experimental condition. The experiment was repeated at least twice with similar results.

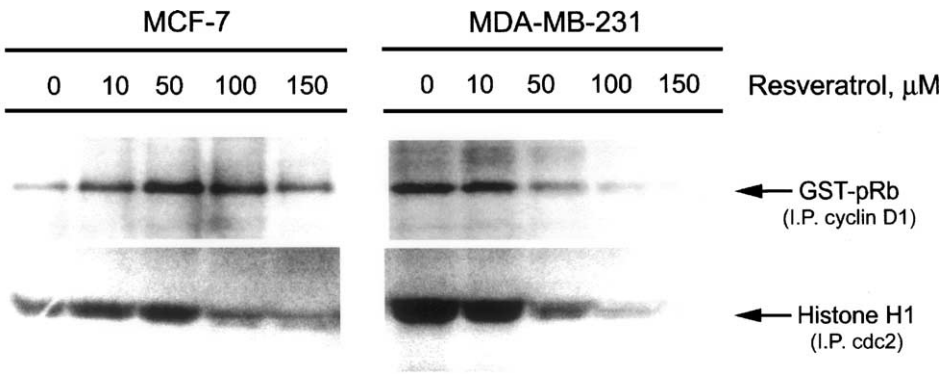

Fig. 8. Kinase activity of G1/S and G2/M regulators are altered in presence of resveratrol. MCF-7 (left panel) and MDA-MB-231 (right panel) were treated with resveratrol and cellular extracts immunoprecipitated with anti-cyclin D1 (upper panels) or anti-cdc2 (lower panels) antibodies. Immunoprecipitates were used to measure kinase activities associated to cyclin D1/CDK4 (G1/S) or cyclin B1/cdc2 (G2/M) using GST-pRB or Histone H1 as target proteins. Note the transient increase in kinase activity at 50 μM resveratrol in MCF-7 cells. The experiment was repeated three times with similar results. Basal kinase activity for cyclin D1/CDK4 and cyclin B1/cdc2 was higher in MDA-MB-231 than in MCF-7.

in resveratrol-treated MCF-7 and MDA-MB-231 cultures (Fig. 8). In MCF-7 cells, resveratrol induced a transient increase in kinase activity of cyclin D1/CDK4 and cyclin B1/cdc2 complexes with a pattern that, after reaching a maximum at 50 μM, decreased to close to control levels by 150 μM (Fig. 8, left panel). On the contrary, in MDA-MB-231 cells, an increase in the concentration of resveratrol induced a progressive decrease in the kinase activity of both types of complexes (Fig. 8, right panel). Thus, resveratrol affected the expression and kinase activity of cell cycle regulators depending on the cell type. Additionally, the differential patterns of kinase activities between MCF-7 and MDA-MB-231, closely followed the differences in protein expression and cell cycle distribution between both cell lines.

3.6. Resveratrol inhibited ribonucleotide reductase activity in MCF-7 and MDA-MB-231

Ribonucleotide reductases are complex enzymatic systems that catalyze the reduction of ribonucleotides to deoxyribonucleotides. This enzymatic conversion provides cells with the appropriate nucleotide pools that will be required for DNA synthesis early during the S phase of the cell cycle. Since resveratrol has been shown to inhibit ribonucleotide reductase *in vitro* [29], we have analyzed if this activity was also affected by resveratrol in our experimental conditions. Resveratrol inhibited ribonucleotide reductase activity in MCF-7 (Fig. 9A) as well as in MDA-MB-231 (Fig. 9B) in a concentration-dependent manner. The inhibition in MCF-7 was around 20% at

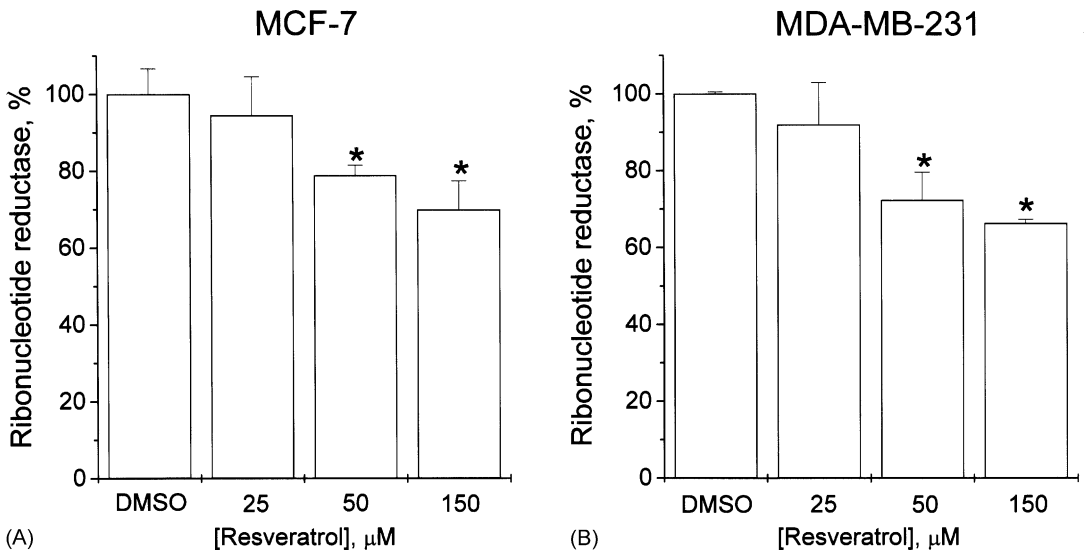

Fig. 9. Resveratrol inhibits ribonucleotide reductase activity in MCF-7 and MDA-MB-231. After treatment with the indicated concentrations of resveratrol, cell extracts were obtained and assayed for ribonucleotide reductase activity using [^{3}H]-CDP as indicated in Section 2. Determinations were done using 100 μg (MCF-7) or 75 μg (MDA-MB-231) protein. The level of significance for the different experimental conditions has been calculated by ANOVA with respect to the corresponding controls in absence of resveratrol (DMSO). (∗) The change in ribonucleotide reductase activity is significant at $P < 0.05$. Determinations were done in triplicate and the experiment repeated two times.

50 μM and close to 30% at 150 μM. Ribonucleotide reductase activity in MDA-MB-231 was also inhibited by 25% at 50 μM resveratrol and by 35% at 150 μM. Thus, decreased ribonucleotide reductase activity could contribute to the antiproliferative activity of resveratrol by partially inhibiting cell cycle progression through the S phase. However, this enzymatic activity did not seem to have a major contribution to the differential effects induced by resveratrol in MCF-7 with respect to MDA-MB-231.

4. Discussion

Since tumors are often constituted by different cell types, the phenotype of cancer cells is generally considered as a relevant parameter to their degree of malignancy, and as a consequence, to the response that could be expected from a chemopreventive treatment [30,31]. In this study, using two human breast cancer cell lines with different proliferative potentials, we have analyzed the effect of resveratrol on cell proliferation, cell cycle, and on the induction of apoptosis in a cell-specific manner. In agreement to previous data [23], we have found that resveratrol inhibited cell viability in MCF-7 and MDA-MB-231 in a concentration- and time-dependent manner (Fig. 1). We also found that, in both cell lines, decreased viability was related to an inhibition in cell proliferation as measured by the rate of DNA synthesis (Fig. 2A). By measuring cell numbers in presence of resveratrol, we could determine that the decrease in proliferation resulted in increased rates of cell death (Fig. 2B). At concentrations of resveratrol close to 50 μM, although a significant decrease in cell proliferation was found in either cell line, cell death was relevant in MDA-MB-231 but not in MCF-7. These results suggest that treatment of MCF-7 with low concentrations of resveratrol decreased proliferation by blocking cell cycle rather than by inducing cell death. On the contrary, at high concentrations of resveratrol, proliferation decreased and cell death increased in both MCF-7 and MDA-MB-231, suggesting that the cell cycle could be similarly affected in the two cell lines. These data, however, could not distinguish between different mechanisms of cell death.

Resveratrol, through different regulatory mechanisms, has been shown to induce apoptosis in tumor cell lines from different origins [24,26]. By using flow cytometry and DAPI immunofluorescence (Figs. 3 and 4), we have found that resveratrol induced apoptosis in MCF-7 but not in MDA-MB-231. The magnitude of apoptosis at low concentrations of resveratrol in MCF-7 was similar to the extent of cell death and both were below the inhibition in cell proliferation, further suggesting a blockade along the cell cycle. At high concentrations of resveratrol, the decrease in proliferation was similar to the increase in cell death for both cell lines, suggesting a similar effect on cell cycle. Therefore, the characteristics of the target cell could determine not only the effects of varying concentrations of resveratrol on cell proliferation but also the activation of mechanisms leading to apoptotic or non-apoptotic death.

The analysis of cell cycle distribution further supported such a differential regulatory mechanism between MCF-7 and MDA-MB-231 when treated at low concentrations of the molecule (Fig. 5). Treatment of MCF-7 cells with 50 μM resveratrol induced a marked increase in S phase cells, supporting that this degree of cell accumulation could prevent from a high level of apoptosis. Concentrations around 150 μM did not significantly alter cell cycle distribution in this cell line, suggesting that apoptosis was taking place without cells been accumulated at any phase of the cycle. MDA-MB-231 cells, in which decreased proliferation was coincident with increased non-apoptotic cell death, did not show cell cycle blockade at the S phase of the cell cycle at any of the concentrations of resveratrol used. In agreement to our data, the effect of resveratrol on cell cycle distribution also varies among different cell lines. Thus, whereas in the human breast cancer cell line MDA-MB-435, cells accumulated at the S phase after 72 hr treatment with 25 μM resveratrol [32], G1 population increased in the human epidermoid cell line A431 treated with 50 μM resveratrol for 24 hr [33]. In addition, recent data have shown that apoptosis increased in MCF-7 cells treated with high concentrations of resveratrol (300 μM) and that this could be associated to S phase arrest [34]. Therefore, these data suggest that resveratrol could be altering cell cycle in a concentration and cell-specific manner and that this could account for the observed correlation between inhibition of proliferation, cell cycle blockade, and cell death.

The differences in expression and activity of cell cycle regulators of the G1/S and G2/M transitions, further supported a concentration and cell-specific effects of resveratrol on cell proliferation and apoptosis (Figs. 6–8). In MCF-7, concentrations of resveratrol around 50 μM induced a marked increase in the expression and kinase activity of positive G1/S regulators, simultaneously that also induced the expression of the tumor suppressor p21 and inhibited ribonucleotide reductase activity. On the contrary, MDA-MB-231 exhibited a concentration-dependent inhibition in expression and activity of cell cycle regulators and ribonucleotide reductase activity, in parallel to an undetectable expression of tumor suppressors p21, p27, and p53.

Considering that p21 has been recently shown to be involved in cell cycle arrest by resveratrol in A431 cells [26], we suggest that in MCF-7 cells, the activation of the G1/S transition by increased kinase activity, simultaneously to p21 induction, in presence of significant levels of p27 and p53 and with a partially inhibited ribonucleotide reductase, could result in cell accumulation and blockade at the S phase of the cell cycle. Taking into account the rate of cell death in these conditions, cell cycle analysis further

support that resveratrol preferentially induced cell accumulation at the S phase rather than a large proportion of cell death. This could finally result in low levels of apoptosis in MCF-7 at concentrations of resveratrol below 50 μM. Thus, resveratrol-induced cell cycle arrest and apoptosis could be the result of opposing stimulus promoting and blocking the G1/S transition of the cell cycle. In MDA-MB-231, in which cell cycle activation was not taking place, the expression of tumor suppressors p21, p27, and p53 was almost undetectable and ribonucleotide reductase activity was partially inhibited, cell death could be mediated by non-apoptotic mechanisms without a significant alteration in cell cycle distribution. Therefore, although different pathways could contribute to apoptosis induction by resveratrol in MCF-7 cells, our data suggest a mechanism resembling the one proposed for post-mitotic neurons, in which potassium deprivation induced G1/S transition in the absence of adequate levels of transcription factors required for DNA synthesis and cell cycle progression [35,36]. In either cell line, the effects of resveratrol on the G2/M transition closely followed the pattern observed for G1/S. However, since only a minor proportion of cells were present in G2/M, it is reasonable to assume that they contributed to a lesser extent to cell death.

Ribonucleotide reductase has been shown to be inhibited by resveratrol *in vitro* [29]. This enzymatic activity was inhibited by resveratrol to a similar extent in MCF-7 and MDA-MB-231 (Fig. 9). Although decreased deoxynucleotide pools could contribute to blockade at the S phase of the cell cycle, most probably they do not have a major role in the cell-specific effects of resveratrol. Thus, the cell-specific and concentration-dependent effects of resveratrol on cell cycle regulators, could represent relevant parameters controlling the induction of apoptotic vs. non-apoptotic cell death in human breast cancer cells. Concentrations of resveratrol above 50 μM increased apoptosis in MCF-7 cells, possibly as the result of lowered cyclin D1/CKD4-dependent kinase activity in presence of still high levels of tumor suppressors. Under this balance of cell cycle regulators, cells would not be driven to nor accumulate at the S phase. This could result in a reversal of the S phase arrest and increased apoptosis from G1. In this context, S-phase accumulation at concentrations of resveratrol below 50 μM and reversal of this arrest at higher concentrations has also been observed in Caco-2 cells [37]. In agreement to our data, the release from S phase arrest in Caco-2 cells was followed by pRB hypophosphorylation. Therefore, cell cycle control appears as a relevant mechanism involved in resveratrol-induced apoptosis.

Interestingly, in MCF-7 cells treated with concentrations of resveratrol around 150 μM the total rate of cell death (close to 40%) was above apoptosis levels (about 25%), suggesting that non-apoptotic processes could also be simultaneously taking place. In this regard, it has been shown that resveratrol, through the inhibition of reactive oxygen species production and the impairment of arachidonic acid release and prostaglandin (PGE_2) synthesis, could inhibit proliferation of 3T6 fibroblasts by cell cycle arrest in the absence of apoptosis [38]. Thus, resveratrol concentration could be an important parameter determining the mechanism of cell death in human cancer cells. The absence of apoptosis in MDA-MB-231 cells in presence of resveratrol, further suggests that apoptotic and/or non-apoptotic mechanisms could contribute to the antiproliferative activity of this molecule.

In summary, the antiproliferative activity of resveratrol in MCF-7 and MDA-MB-231 human breast cancer cells could result from the specific induction of apoptotic and/or non-apoptotic cell death. We suggest that the prevalence of either pathway could be regulated by differential control of the cell cycle, mainly at the G1/S transition. Among relevant factors that may influence one mechanism or another are the characteristics of the target cell and the concentration of resveratrol. Most of the information about resveratrol has been obtained from *in vitro* experiments after the application of a single, high dose of the molecule. Although these experiments are very important to understand the molecular mechanisms involved in the antiproliferative activity of resveratrol, several questions remain about the action of resveratrol *in vivo*. This aspect could be particularly relevant regarding the chemopreventive efficacy and use in tumor development of regular, low doses of this natural compound. Thus, the mechanisms by which resveratrol induces cell death in a particular tumor cell should be taken into consideration for the chemopreventive application of this molecule.

Acknowledgments

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