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Mechanisms Involved in Resveratrol-Induced Apoptosis and Cell Cycle Arrest in Prostate Cancer–Derived Cell Lines

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ABSTRACT: Resveratrol is a polyphenol found at high concentrations in grapes and red wine with reported anticarcinogenic effects. We studied the molecular mechanism of resveratrol-induced apoptosis and proliferation arrest in prostate derived cells PZ-HPV-7 (nontumorigenic line), LNCaP (androgen-sensitive cancer line), and PC-3 (androgen-insensitive cancer line). Apoptosis and cell cycle distribution were evaluated by flow cytometry and proliferation by MTT assay and direct cell counting. Caspases, bax, bcl-2, cyclins, Cdks, p53, p21, and p27 were measured by Western blot and kinase activities of cyclin/Cdk complexes by immunoprecipitation followed by kinase assays with appropriate substrates. Resveratrol induced a decrease in proliferation rates and an increase in apoptosis in cancer cell lines in a dose- and time-dependent manner. These effects were coincident with cell accumulation at the G0/G1 phase. In LNCaP and PC-3, the apoptosis induced by resveratrol was mediated by activation of caspases 9 and 3 and a change in the ratio of bax/bcl-2. Expressions of cyclin D1, E, and Cdk4 as well as cyclin D1/Cdk4 kinase activity were reduced by resveratrol only in LNCaP cells. In contrast, cyclin B and Cdk1 expression and cyclin B/Cdk1 kinase activity were decreased in both cell lines in the presence of resveratrol. However, modulator proteins p53, p21, and p27 were increased by resveratrol only in LNCaP cells. These effects probably result in the observed proliferation arrest and disruption of cell cycle control. In addition, the specific differences found between LNCaP and PC-3 suggest that resveratrol acts through different mechanisms upon the androgen or estrogen receptor cell status.

Key words: Cyclins/Cdk complexes, androgen sensitivity, caspases, p53, p21.

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Prostate carcinoma is one of the most common malignancies and represents the second most common cause of cancer-related deaths in men wide-world (Forti and Selli, 1996; Long et al, 1997). At the beginning, prostate cancer is asymptomatic, and late diagnosis is usual. Because many malignant prostate tumors are androgen-sensitive, testosterone ablation represents the main therapy of localized and early metastatic tumors (Cunha et al, 1987; Wilding, 1992; Aquilina et al, 1997). However, advanced stages usually become androgen-insensitive, with practically no therapeutic options.

Some epidemiologic studies have shown that nutritional factors may be related with carcinogenesis. Occidental fat-enriched diet correlates with high risk of prostate and other cancers, while oriental diet based on vegetables, fruit, and seeds is correlated with lower cancer risk (Adlercreutz and Mazur, 1997; Clinton and Giovannucci, 1998; Hsing et al, 2000; Romero Cagigal et al, 2003). Considering these data, a chemopreventive role for natural compounds present in natural food has been proposed. Among these substances, polyphenolic antioxidants have received increasing attention in recent years. One of such naturally occurring polyphenols is resveratrol, which is present in peanuts, grapes, and red wine (Sobolev and Cole, 1999; Fremont, 2000; Sanders et al, 2000). Also, epidemiologic studies have suggested that regular and moderate red wine consumption could have beneficial effects on human health (Maxwell et al, 1994; Goldberg et al, 1995).

Resveratrol has been shown to inhibit cancer initiation, promotion, and progression in several experimental models (Jang et al, 1997). Activation of apoptosis has been proposed as a probable mechanism for chemotherapeutic agents to eliminate cancerous cells (Naik et al, 1996; Deigner and Kinscherf, 1999). For that reason,

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recent studies have focused on apoptosis, as a potential process targeted by resveratrol in cancer cells. In the prostate cancer–derived cell line DU-145, a polyphenolic fraction isolated from grape seeds induces apoptosis and proliferation arrest (Agarwal et al, 2000). Also, resveratrol induces apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in leukemia cells (Dorrie et al, 2001). However, in the prostate cancer cell line LNCaP the effect of resveratrol on caspase activation has not been conclusively demonstrated.

Another suggested target for resveratrol action is the control of the cell cycle. Recent data have shown that resveratrol inhibits tumor development and cellular proliferation in cancer-derived cell lines (Mgbonyebi et al, 1998; Dorrie et al, 2001; Tinhofer et al, 2001). This antiproliferative activity of resveratrol has also been shown to involve apoptosis induction in cancer cells (Ahmad et al, 2001; Dorrie et al, 2001; Tinhofer et al, 2001). In the estrogen–dependent mammary MCF-7 cells, resveratrol diminishes cell viability and induces apoptosis by modulating the expression and activity of relevant cell cycle regulators (Pozo-Guisado et al, 2002). It has been shown that resveratrol induces cell cycle arrest in prostate cell lines PC-3 and DU-145 (androgen-insensitive) but not in LNCaP (androgen-sensitive), probably by disruption of the G1/S transition (Sgambato et al, 2001). Similar results have been reported in DU-145 cells using polyphenolic extracts from grape seed (Agarwal et al, 2000). Resveratrol has structural similarity with both androgens and estrogens, and there is evidence that some of its effects might be mediated by steroid hormone receptors (Gehm et al, 1997; Bowers et al, 2000; Kampa et al, 2000). Normal prostate cells express androgen and, to a lesser extent, estrogen receptors (Latil et al, 2001; Leav et al, 2001; Royuela et al, 2001). However, in prostate cancer cells, estrogen receptors are usually overexpressed and androgen receptors, although present in sensitive stages, are normally mutated or absent in advanced metastatic disease (Veldscholte et al, 1990; Culig et al, 1993). Moreover, some effects of resveratrol might be mediated by other mechanism related with its antioxidant capacity.

In the present work, we studied the mechanisms involved in resveratrol-induced proliferation arrest and apoptosis in prostate cancer, comparing nontumorigenic, androgen-sensitive, and androgen-insensitive cancer cell lines.

Material and Methods

Reagents

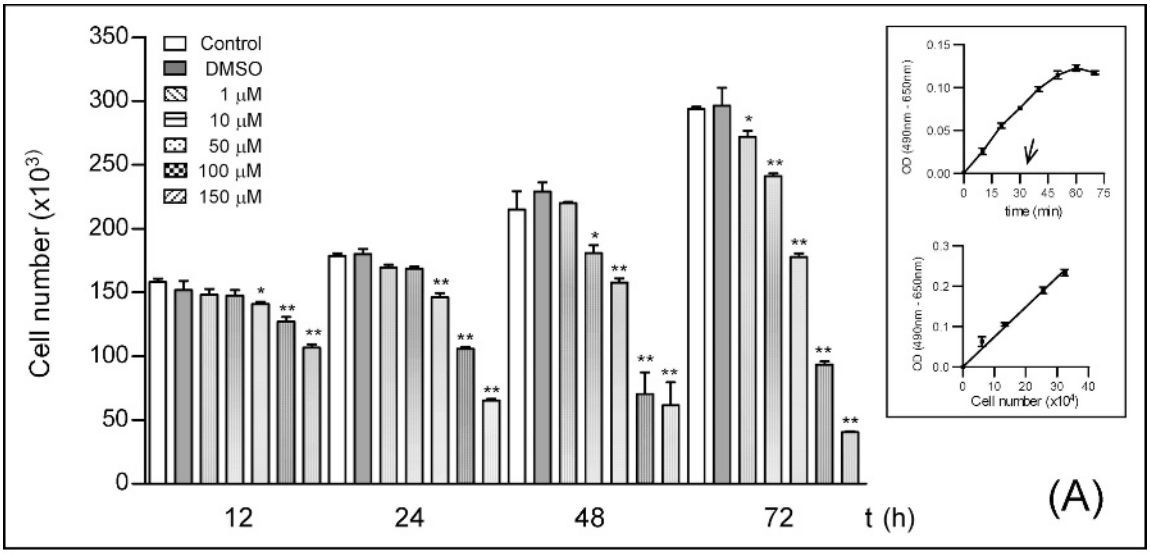
Trans-resveratrol, ATP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and human polyclonal β-actin antibody (A2066) were purchased from Sigma-Aldrich Chemical (St Louis, Mo). Dulbecco Modified Eagle Medium: Nutrient mixture F-12 (Ham) (1:1) (DMEM/F-12), keratinocyte serum-free medium, bovine pituitary extract (BPE), recombinant epidermal growth factor (rEGF), and fetal bovine serum (FBS) were obtained from Invitrogen Corporation (Carlsbad, Calif). Histone H1 protein was from Stress Gen (San Diego, Calif). Protein A/G plus agarose, gluthathione-S-transferase-retinoblastoma protein (GST-Rb), and 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), Cdk1 (sc-54), and bax (sc-526) were from Santa Cruz Biotechnology (Santa Cruz, Calif). Human monoclonal β-actin antibody (69100) was obtained from MP Biomedicals, Inc (Irvine, Calif). Antibodies against caspase-3 (551150) and caspase-8 (559932) were purchased from BD PharMingen (San Diego, Calif). Anti-caspase-9 (218794) and anti-bcl-2 (OP60) were from Calbiochem (San Diego, Calif), and peroxidase-conjugated goat anti-rabbit (111035003) and goat anti-mouse (115035003) were purchased from Jackson ImmunoResearch (West Grove, Pa).

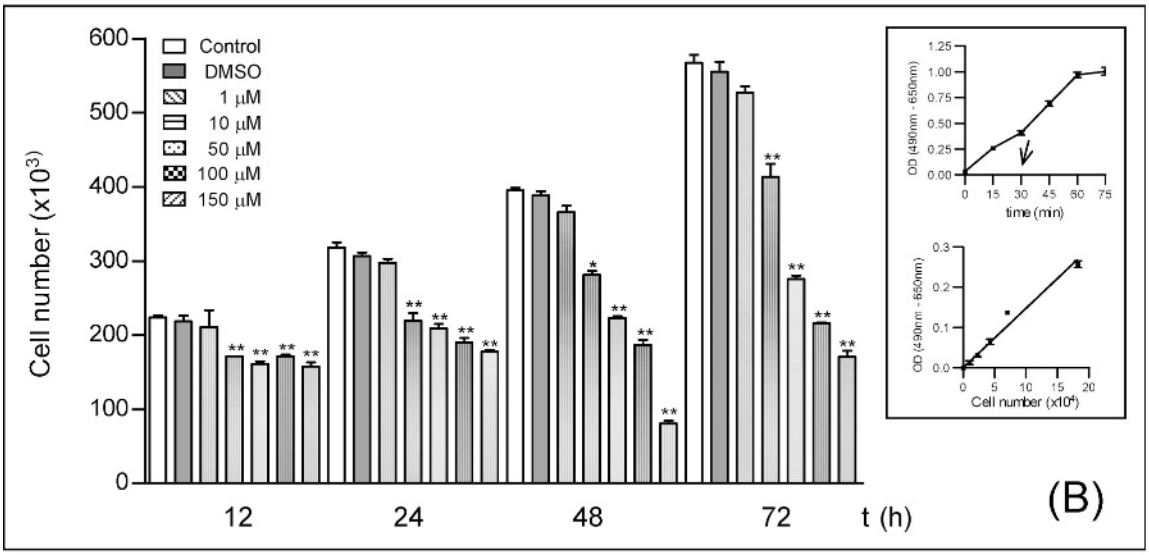
Cell Cultures

Androgen-sensitive LNCaP and androgen-insensitive PC-3 cell lines from human prostate cancer (American Type Culture Collection [ATCC], Manassas, Va) were cultured in DMEM/F-12 supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. PZ-HPV-7 human nontumorigenic prostate epithelial cells (ATCC) were cultured in keratinocyte-serum free medium supplemented with 0.2 ng/mL rEGF, 30 μg/mL BPE, and 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. Cells were counted and plated at the same initial density for treatments with resveratrol. At 60%–80% confluence, cells were treated with the indicated concentrations of resveratrol dissolved in sterile DMSO. Treatments were carried out for time periods ranging from 12 to 72 hours at *trans*-resveratrol concentrations of 1 to 150 μM. Culture media were changed every day containing fresh resveratrol. All experiments were carried out with cells at passage 3–9.

Cell Growth Inhibition Studies

Cell growth inhibition was analyzed by the spectrophotometric measurement of the mitochondrial dehydrogenase activity using MTT (Mosmann, 1983). PC-3, LNCaP, and PZ-HPV-7 cells were seeded at densities of 20, 25, and 30 $\times$ 10^3 cells/well, respectively, in 24-well culture plates. An incubation time with MTT of 30 minutes was optimized for these cell lines by determining the increase in OD (490–650 nm) with respect to time. Using these conditions, the increase in OD (490–650 nm) was also related to cell number by constructing linear curves with known amounts of cells (Figure 1A through C, inserts). After 24 hours of culture, the cells were treated with 0, 1, 10, 50, 100, or 150 μM of resveratrol for 12, 24, 48, or 72 hours. At the end of each treatments, culture media were aspirated and cells incubated at 37°C in Locke solution (2.3 mmol


Control
DMSO
1 μM
10 μM
50 μM
100 μM
150 μM
Cell number (x10³)
t (h)
12
24
48
72
OD (490nm - 650nm)
time (min)
Cell number (x10⁴)
(A)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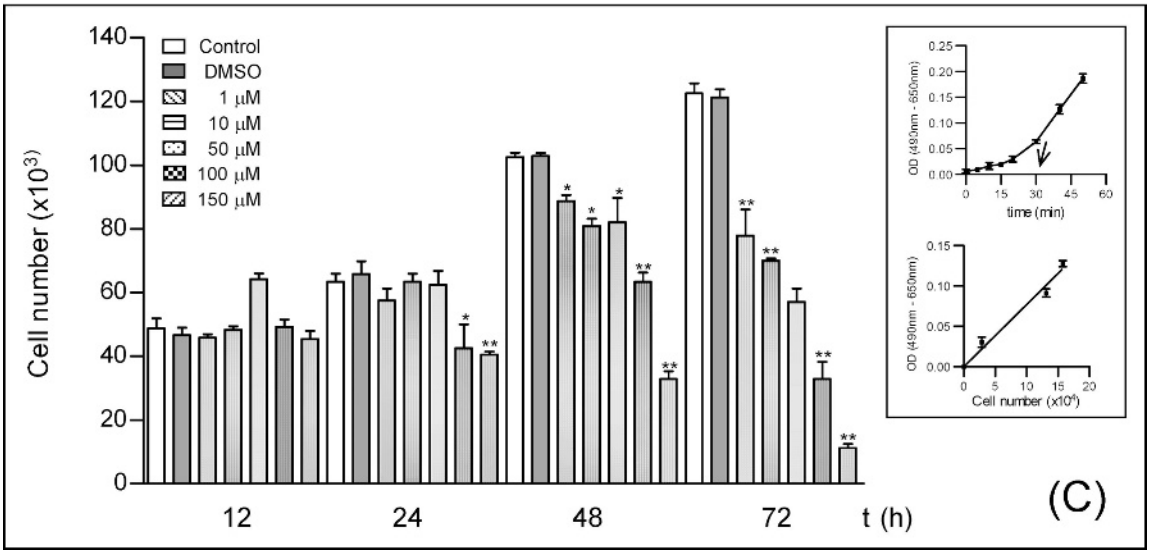
Control
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OD (490nm - 650nm)
time (min)
Cell number (x10⁴)
(C)

$CaCl_2$, 1 mmol $MgCl_2$, 5 mmol KCl, 134 mmol NaCl, 4 mmol $NaHCO_3$, 5 mmol glucose, 10 mmol Hepes, pH 7.4), containing 0.35 mmol of MTT. Then, the solution was aspirated and the formazan salts produced by active mitochondrial dehydrogenases were dissolved in DMSO and the absorbance at 490 and 650 nm determined. The cell number was estimated using standard curves for each cell line. Based on these experiments, treatments with resveratrol were performed for 36 hours in LNCaP and PC-3 cells. For direct determination of cell number, cells were plated at the density described above and 24 hours later treated during 36 hours with different resveratrol concentrations. Medium was aspirated and cells tripsynized and counted. Untreated and DMSO treated cells were used as controls. All the experiments were performed in triplicate in at least 3 cultures for each cell line.

Flow Cytometry

Cell cycle distribution and ploidy status of cells after treatment with resveratrol were determined by flow cytometry DNA analysis. At the end of treatments, cells were detached from the plates by the addition of 0.25% trypsin, washed in PBS, fixed in 70% ethanol at 4°C, and treated with 10 mg/mL RNAse for 30 minutes at 37°C. The DNA content was evaluated in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) after staining the cells with 50 μg/mL propidium iodide for 15 minutes in the dark at room temperature. For cell cycle analysis, only single cells were considered.

SDS-PAGE and Western Blot

At the end of treatments with resveratrol, cells were washed with cold PBS and lysed in ice-cold lysis buffer (50 mmol Tris-HCl pH 7.5, 2 mmol EDTA, 2 mmol EGTA, 10 mmol β-glycerophosphate, 150 mmol NaCl, 0.5% NP40, 1 mmol phenyl-methyl sulfonyl fluoride, 1 mmol NaF, 1 mmol DTT, 1% β-mercaptoethanol, and 4 μg/μL complete protease inhibitor cocktail [Roche, Penzberg, Germany]). Cell lysates were centrifuged at 15 000 × *g* for 15 minutes at 4°C, and protein concentration was determined in the supernatants using the Coomassie Plus protein assay reagent (Pierce, Rockford, Ill) using bovine serum albumin as standard. Fifteen micrograms of protein were mixed with SDS sample buffer, denatured, and electrophoresed in 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis, gels were transferred to nitrocellulose membranes by electroblotting and blocked for 2 hours at room temperature in TBS-T (50 mmol Tris-HCl pH 7.5, 150 mmol NaCl, 0.2% Tween-20) containing 7% nonfat milk. Blots were sequentially incubated with the primary and secondary antibodies, washed in TBS-T, and developed using the SuperSignal Substrate (Pierce) and a chemiluminescence imaging screen (Bio-Rad Labs, Hercules, Calif). The screen was scanned using a Molecular Imager FX system from Bio-Rad Laboratories. Some membranes were developed by enhanced chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to Kodak BioMax Light films for 1–10 minutes. In order to detect a second protein, some blots were stripped by incubation with 100 mmol Tris-HCl, pH 7.4, 100 mmol β-mercaptoethanol, and 2% SDS at 60°C for 30 minutes.

Immunoprecipitation and Kinase Assay

Cultures were grown in 100-mm dishes and treated with solvent (DMSO), 1, 10, 50, 100, or 150 μM resveratrol for 36 hours. Cells were then lysed on ice for 10 minutes with 500 μL IP buffer (50 mmol Tris–HCl pH 7.5, 150 mmol NaCl, 0.5% Nonidet P40) containing 1 mmol phenyl-methyl sulfonyl fluoride, 1 mmol sodium orthovanadate, 1 mmol NaF, 10 mmol β-glycerophosphate, 1 mmol DTT, and 4 μg/μL complete protease inhibitor cocktail. After brief mixing, lysates were centrifuged at 15 000 × *g* for 15 minutes at 4°C, pellets were discarded, and protein concentration determined in the supernatants as indicated above. An amount of 500 μg protein was used for each immunoprecipitation reaction. To eliminate nonspecific binding, lysates were preincubated for 1 hour at 4°C with 20 μL protein A/G Plus agarose beads. After removing the beads by centrifugation, cell lysates were rotated overnight at 4°C with 1 μg anti-cyclin D1 or anti-Cdk1 polyclonal antibodies. Extracts were then incubated at 4°C for an additional 60 minutes with 30 μL protein A/G Plus agarose beads. Beads were washed 3 times in IP buffer and twice in kinase buffer (20 mmol Tris–HCl pH 8.0, 10 mmol MgCl2, 1 mmol EGTA, 1 mmol DTT) and finally resuspended in a total volume of 10 μL of kinase buffer. In vitro kinase activity was assayed at 30°C for 30 minutes in 25 μL kinase buffer containing 10 μL protein A/G Plus agarose beads, 1 mmol NaF, 1 mmol 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 mmol nonlabeled ATP. After the reaction was completed, SDS-sample buffer was added and samples boiled for 5 minutes. The levels of phosphorylated GST-Rb or Histone H1 were determined by SDS–PAGE electrophoresis and autoradiography using phosphorscreens on a Molecular Imager FX System (BioRad Laboratories).

Statistical Analysis

Data were expressed as mean ± SEM. Statistical comparison between treatments was carried out using GraphPad Prism 4.0 (GraphPad Software, Inc, San Diego, Calif). One-way ANOVA followed by Dunn test was applied, with significances of $P < .05$, $P < .01$, or $P < .001$.

←

Figure 1. Effect of resveratrol on the growth of prostatic cell lines. LNCaP **(A)**, PC-3 **(B)** and PZ-HPV-7 **(C)**. Cell cultures were treated with different concentrations of resveratrol (1–150 μM) during different time intervals (12–72 hours). At the end of each experiment MTT assays were performed to estimate cell number, as described in Experimental Procedures. Calibration curves are represented in each insert, with arrows indicating the selected time for MTT incubation. Data were expressed as means ± SEM of 3 different experiments.

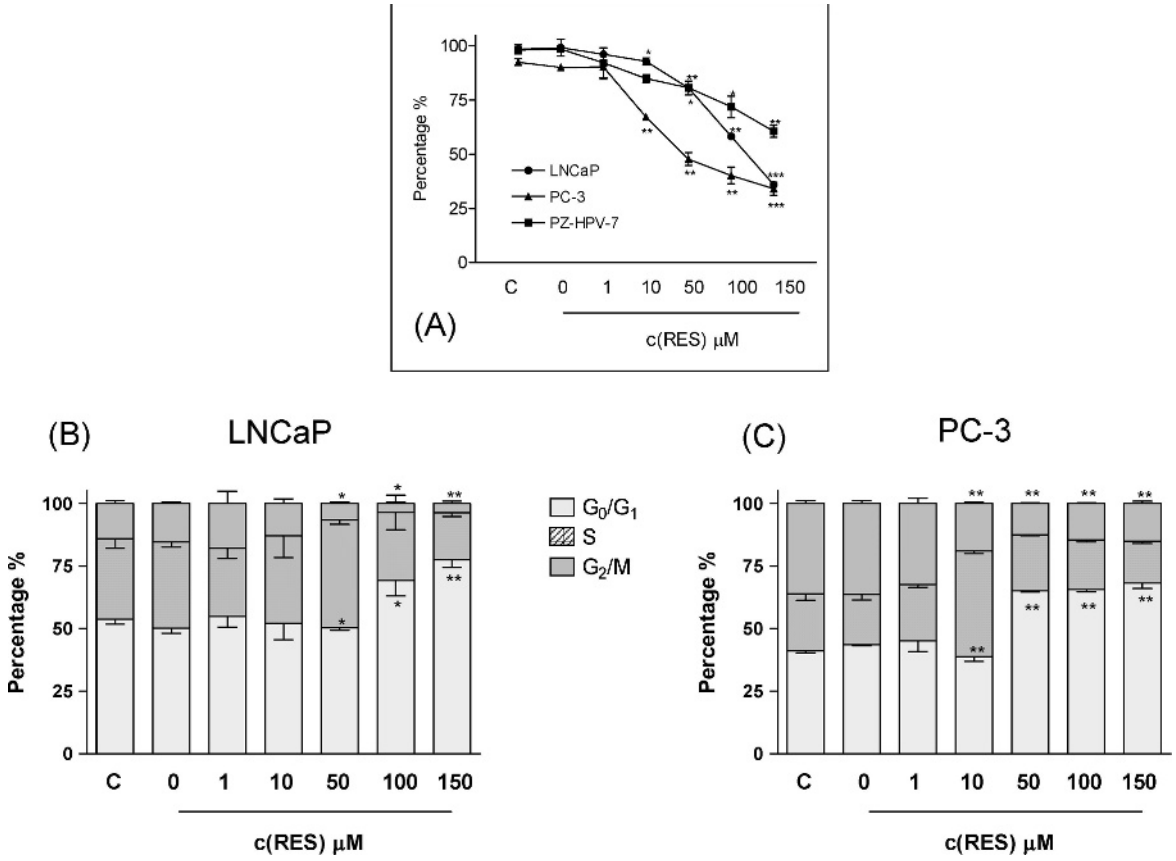

Figure 2. Effect of resveratrol on cell cycle distribution in prostate cancer cell lines. Cell cultures were treated with 0–150 μM of resveratrol during 36 hours. The effect of resveratrol on cell proliferation was estimated by direct cell counting in PZ-HPV-7, LNCaP, and PC-3 cells **(A)**. Cell distribution in the G0/G1, S, and G2/M phases of cell cycle was evaluated by flow cytometry in LNCaP **(B)**, and PC-3 **(C)** cell cultures in absence or presence of resveratrol. Data were expressed as means ± SEM of 3 different experiments. * indicates $P < .05$; **, $P < .01$; ***, $P < .001$.

Results

Resveratrol Inhibited Cell Growth in Normal and Prostate Cancer Cell Lines

Prostate cancer cells (LNCaP and PC-3) and PZ-HPV-7 nontumorigenic human prostate epithelial cells were treated with 0–150 μM of resveratrol during 12–72 hours and the cell number was estimated by MTT assay, as indicated in the methods. The treatment of LNCaP (Figure 1A) and PC-3 (Figure 1B) prostate cancer cells with resveratrol resulted in a dose-dependent and time-dependent inhibition of cell proliferation, as previously reported (Hsieh and Wu, 1999; Kampa et al, 2000). However, growth inhibition was more potent in cancer cells than nontumorigenic cells. Thus, whereas in LNCaP and PC-3 cells resveratrol significantly decreased their proliferation rate after a short time, in PZ-HPV-7 cells no effect was found until after 24 hours of treatment, with concentrations of resveratrol above 100 μM (Figure 1C). From these results, we selected resveratrol treatments for 36 hours in all cell lines. A similar effect of resveratrol on the cell number was also determined by cell counting using a hemocytometer (results not shown). When these cell lines were treated with resveratrol for 36 hours (Figure 2A), a reduction in the number of cells could be observed that was more pronounced in PC-3 than in LNCaP, and more pronounced in LNCaP than in PZ-HPV-7. These results are in agreement with those presented in Figure 1.

Resveratrol-Induced Changes in Cell Cycle Distribution in Prostatic Cancer Cells

To analyze whether resveratrol-induced inhibition of cell growth in prostate cancer cell lines was accompanied by alterations in cell cycle distribution, we analyzed the percentage of cells in the different stages of cell cycle by flow cytometry. As shown in Figure 2, resveratrol induced an accumulation of cells in G0/G1 both in LNCaP (from 100 μM) and PC-3 cells (from 50 μM). Similarly, the fraction of cells at G2/M also decreased in both cell lines treated with increasing concentrations of resveratrol (Figure 2B and C). However, a larger

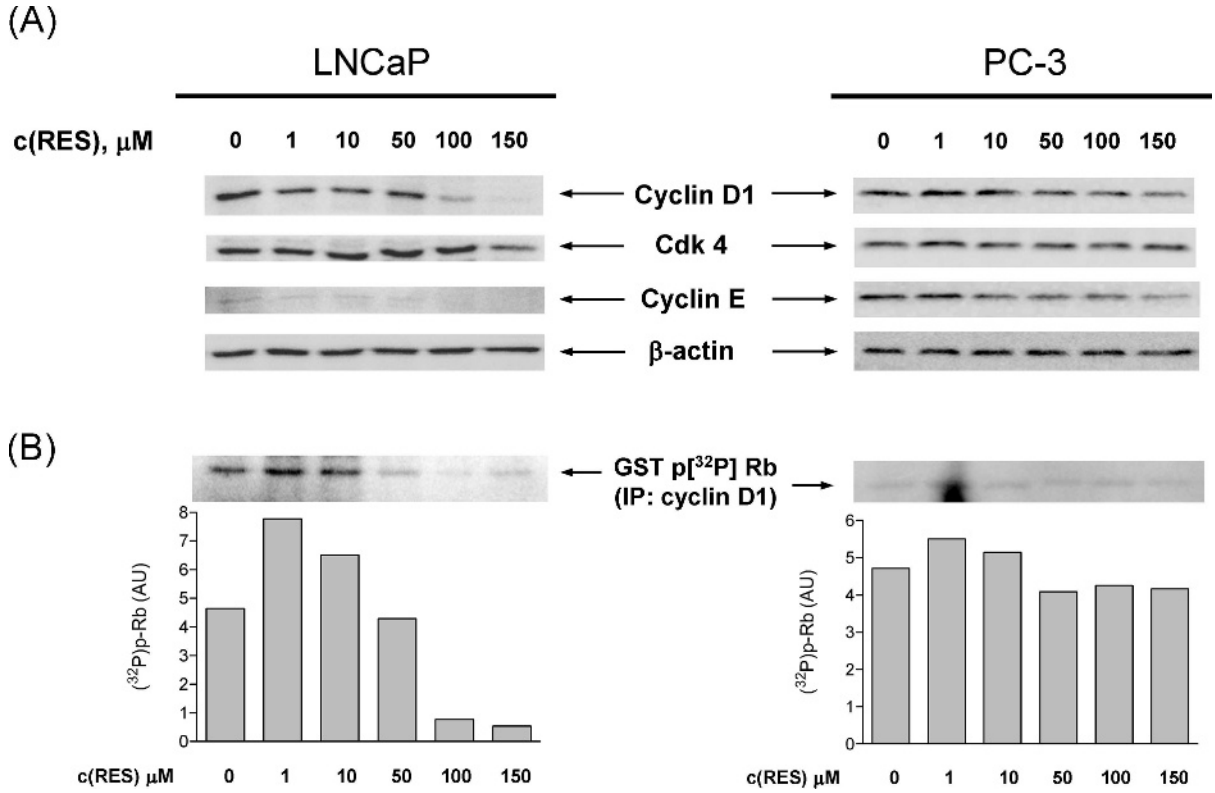

Figure 3. Effect of resveratrol on G1/S transition in prostate cancer cells. LNCaP and PC-3 cell cultures were treated with 0–150 μM of resveratrol during 36 hours. Expression of cyclin D1, E, and Cdk4 was determined by Western blot in absence or presence of resveratrol **(A)**. β-actin was used as control. Kinase activity of the G1/S transition complex, cyclin D1/Cdk4, was measured by immunoprecipitation with anti-cyclin D1 antibody followed by kinase assay using GST-RB and ^{32}P-γ- ATP as substrates **(B)**. Experiments were carried out at least 3 times with equivalent results. Photographs shown correspond to representative experiments.

number of PC-3 cells remained at G2/M phase after resveratrol treatments compared with LNCaP cells. Further, concentrations of resveratrol of 10 μM (PC-3) or 50 μM (LNCaP) induced a transient increase in cells at the S phase.

Effect of Resveratrol on Expression and Activity of Cyclin/Cd-Kinase Complexes in Prostate Cancer Cell Lines

In order to investigate the role of cyclin/Cdk complexes in the antiproliferative activity of resveratrol, the expression of G1/S regulators cyclin D1, cyclin E, and Cdk4 and of G2/M regulators cyclin B and Cdk1 and the kinase activity of cyclin D1/Cdk4 and cyclin B/Cdk1 complexes were analyzed in LNCaP and PC-3 cells. With regard to G1/S transition in androgen-sensitive LNCaP cells, resveratrol induced a significant decrease in protein expression of cyclins D1 and E without a significant change in Cdk4 expression (Figure 3A, left panel). Kinase activity of cyclin D1/Cdk4 complex in androgen-dependent LNCaP showed a biphasic pattern that increased at lower concentrations of resveratrol (1 μM) to gradually decrease below basal levels by 100–150 μM (Figure 3B, left panel). In contrast, in androgen-independent PC-3 cells only a slight decrease in expression and kinase activity of this complex was observed upon treatment with high concentrations of resveratrol (Figure 3A and B, right panels). With regard to G2/M transition, resveratrol decreased, in a concentration-dependent manner, protein expression of cyclin B1 and Cdk1 in either LNCaP or PC-3 (Figure 4A). The effect of resveratrol on cyclin B1/Cdk1 kinase activity also showed a similar biphasic pattern in both cell lines, with an slight increase in activity at low concentrations (1 μM) and a gradual decrease below basal levels at high concentrations of this molecule (Figure 4B).

Effect of Resveratrol on Modulators of the Cell Cycle p53, p21, and p27

Since inhibitory mechanism of resveratrol on cell proliferation might also affect the expression of negative regulators of the cell cycle, we evaluated protein expression of p53, p21, and p27. The p21 and p27 are inhibitors of cyclin/Cdk complexes mainly involved in

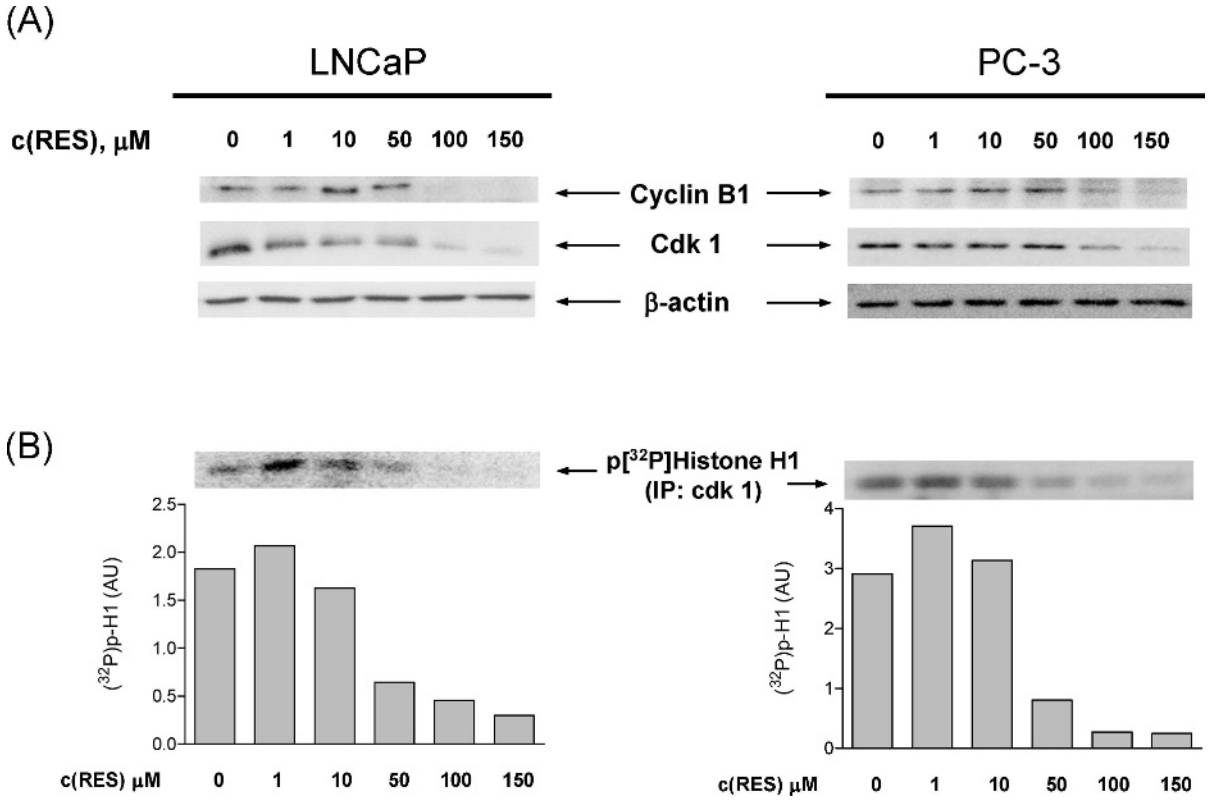

Figure 4. Effect of resveratrol on G2/M transition in prostate cancer cells. LNCaP and PC-3 cell cultures were treated with 0–150 μM of resveratrol during 36 hours. Expression of cyclin B1 and Cdk1 was determined by Western blot in absence or presence of resveratrol **(A)**. β-actin was used as control. Kinase activity of the G2/M transition complex, cyclin B1/Cdk1, was measured by immunoprecipitation with anti-Cdk-1 antibody followed by kinase assay using Histone H1 and ^{32}P-γ- ATP as substrates **(B)**. Experiments were carried out at least 3 times with equivalent results. Photographs shown correspond to representative experiments.

G1/S transition. All 3 proteins were significantly increased by resveratrol in a dose-dependent manner in LNCaP cells (Figure 5A), while in PC-3 cells there were no significant changes in protein expression of p21 and p27 at the concentrations of resveratrol used (Figure 5B).

Apoptosis Induction by Resveratrol

To analyze whether the antiproliferative activity of resveratrol in LNCaP and PC-3 cell lines was associated with increased apoptosis, the apoptotic index was determined by flow cytometry. By measuring the fraction of cells with sub G0/G1 DNA content, we observed that concentrations of resveratrol below 50 μM did not induce a significant level of apoptosis in any of the cell lines analyzed. Increasing the concentration of this molecule to 100 μM and 150 μM increased apoptosis rates to maximum values of 50% and 20% for LNCaP and PC-3, respectively (Figure 6A and B). The nontumorigenic prostate PZ-HPV-7 cell line had a higher level of basal apoptosis (about 10%) that was not affected by resveratrol at any of the concentrations used. Thus, the antiproliferative effect of resveratrol was associated with apoptosis mainly in androgen-dependent LNCaP cells.

Resveratrol-Induced Apoptosis Is Mediated by Caspase Activation

To further characterize this cell-specific apoptotic effect of resveratrol in prostatic cancer cells, we analyzed the activation of caspases and the levels of total bax and bcl-2 proteins. For this purpose, LNCaP and PC-3 cells were plated on 100-mm culture dishes and treated with resveratrol or DMSO for 36 hours, and total protein was analyzed by SDS-PAGE. We observed that caspase 9 was significantly activated by resveratrol in LNCaP and PC-3 cells. Active caspase 8 was slightly increased by high doses of resveratrol only in PC-3 cells. As expected, effector caspase 3 was activated in both cell lines by resveratrol in a dose-dependent manner (Figure 6C). In LNCaP cells, proapoptotic regulator bax increased with resveratrol treatments, whereas antiapoptotic bcl-2 protein markedly decreased even at low concentrations of resveratrol. In contrast, in PC-3

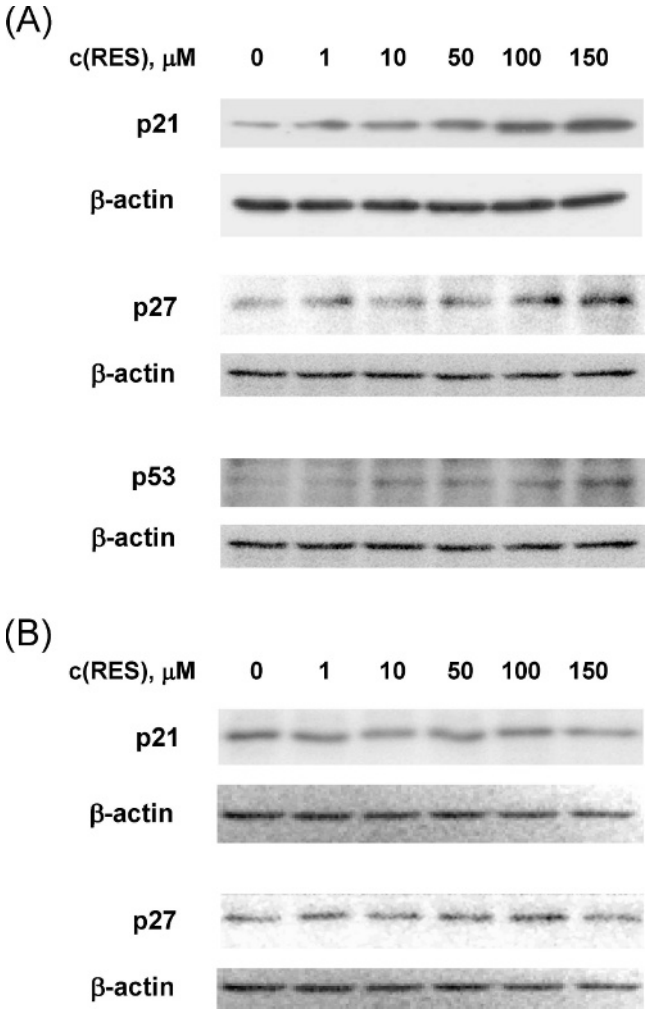

Figure 5. Effect of resveratrol on negative regulators of cell cycle in prostate cancer cells. The expression of tumor suppressor p53, p21, and p27 was analyzed by Western blot in LNCaP **(A)** and PC-3 **(B)** cells. Cell cultures were treated with 0–150 μM of resveratrol during 36 hours. β-actin was used as control. Experiments were carried out at least 3 times with equivalent results. Photographs shown correspond to representative experiments.

cells only bcl-2 significantly decreased by resveratrol treatment with no important changes in bax level (Figure 6D).

Discussion

Polyphenolic compounds naturally occurring in diets rich in fruits, vegetables, and seeds have been proposed as chemopreventive agents against a variety of diseases, including cancer (Clement et al, 1998; Damianaki et al, 2000; Schneider et al, 2000; Kang et al, 2003; Mazzon et al, 2005; Scalbert et al, 2005; Yi et al, 2005). Resveratrol, one of these polyphenols present at high levels in grapes and red wine, has been extensively studied regarding its effects on several malignancies, including prostate cancer (Hsieh and Wu, 1999; Schneider et al, 2000; Boissy et al, 2005; Signorelli and Ghidoni, 2005; Zunino and Storms, 2005; Garvin et al, 2006; Vyas et al, 2006). Resveratrol has a high structural homology with androgens and estrogens, and there is convincing evidence that at least some of its effects might be mediated by steroid receptors (Bowers et al, 2000; Gao et al, 2004). Apoptosis induction and proliferative arrest are the main effects proposed for resveratrol, as well as for other similar compounds (Pozo-Guisado et al, 2002; Jiang et al, 2005; Poussier et al, 2005; Pozo-Guisado et al, 2005; Sheng et al, 2005; Zunino and Storms, 2005). In prostate cancer, however, there are no conclusive data about the mechanisms involved in resveratrol action, probably because of the use of different experimental models. Most of our knowledge about molecular aspects of prostate cancer comes from metastatic prostate-derived cell lines displaying a variety of specific molecular characteristics and representing different malignancy grades. For these reasons, more comparative studies are needed. We have studied the effect of different concentrations of resveratrol on a panel of prostate-derived cell lines representing nontumorigenic (PZ-HPV-7) epithelium and androgen-responsive (LNCaP) and androgen-unresponsive (PC-3) stages of prostate cancer. It was found that resveratrol induced apoptosis, detected by flow cytometry, at concentrations over 100 μM in both cancer cell lines without a significant effect on the nontumorigenic cell line. Our data on capase-3 activation strongly suggest that the mechanism of resveratrol-induced apoptosis in these prostate cancer-derived cell lines might be mediated by caspase-dependent signaling leading to caspase-3 activation. Androgen-responsive LNCaP cells showed the highest sensitivity to resveratrol-induced apoptosis, reaching more than 50% of cell death at 150 μM of resveratrol as compared to 20% of apoptotic cells in androgen-insensitive PC-3 cells. In addition, resveratrol consistently induced an increase in the level of active form of caspase-9, indicative of an activated intrinsic apoptotic pathway, suggesting that this might be the main, but not the unique, mechanism of resveratrol-induced apoptosis in LNCaP and PC-3 cells. However, a slight increase in caspase-8 was also noted in PC-3 cells, suggesting that the extrinsic pathway might also be involved in this cell line. Interestingly, in breast cancer–derived cell lines, resveratrol induced apoptosis in estrogen receptor expressing MCF-7 cells, but not in estrogen-unresponsive MDA-MB-231 cells, suggesting that resveratrol has a higher potency to induce apoptosis in cancer cells expressing steroid hormone receptor (Pozo-Guisado et al, 2002). However, the molecular mechanism involved in resveratrol-induced apoptosis appears to be different,

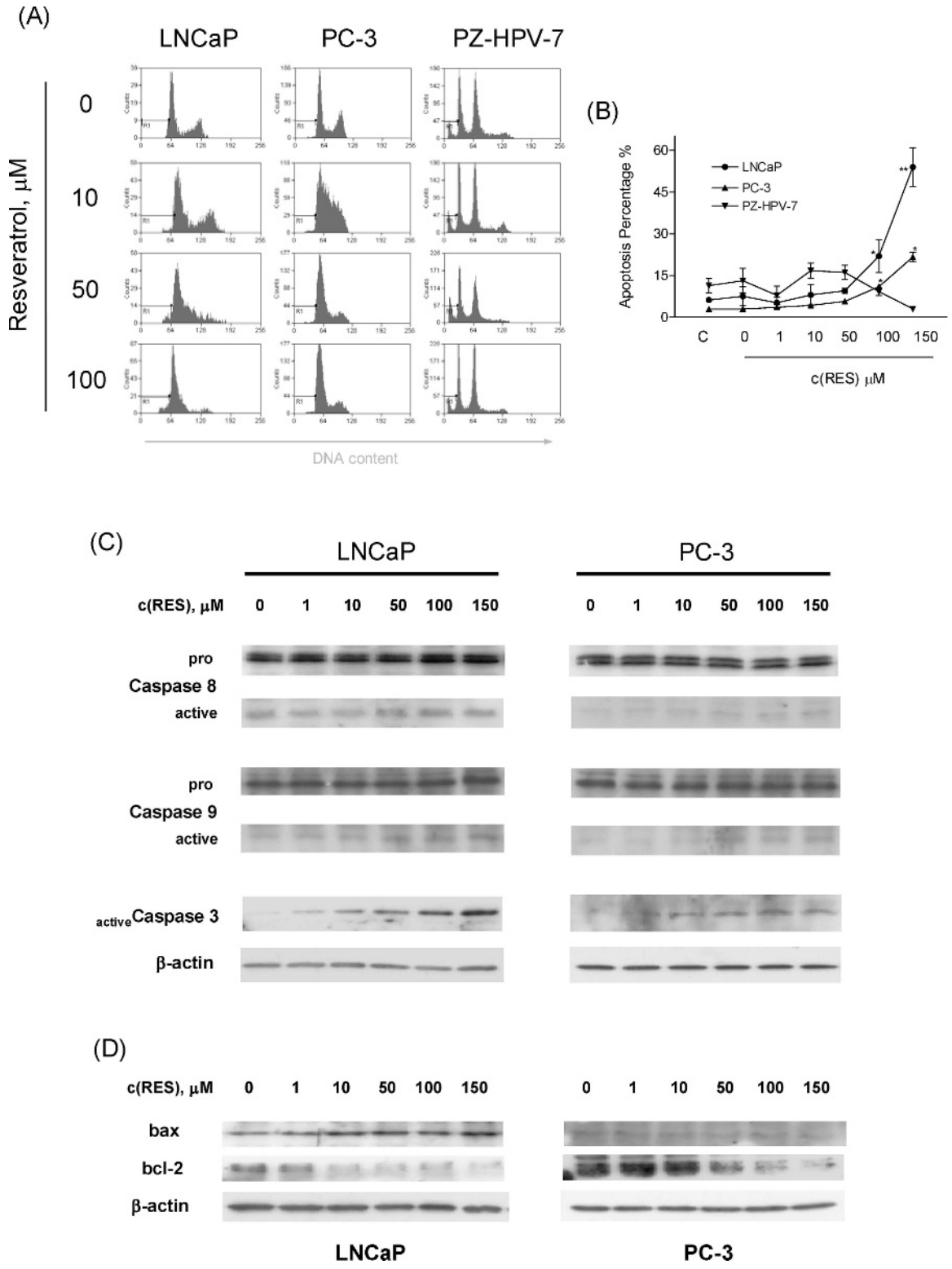

Figure 6. Effect of resveratrol on apoptosis index, caspase activation, and bax/bcl-2 expression in prostate cancer cell lines. LNCaP, PC-3, and PZ-HPV-7 cell cultures were treated with 0–150 μM of resveratrol for 36 hours. Representative histograms of flow cytometry experiments showing cell distribution upon DNA content in absence or presence of resveratrol **(A)**. The region corresponding to cells with sub-G0/G1 DNA content (considered as apoptotic cells) is indicated as R1. Percentage of apoptotic cells versus resveratrol concentrations in the 3 cell lines **(B)**. Pro- and active forms of caspase-9, -8, and -3 were determined by Western blot **(C)**. Protein expression of bax and bcl-2 were assayed by Western blot **(D)**. β-actin was used as control. Experiments were carried out at least 3 times with equivalent results. Data were expressed as means ± SEM of 3 different experiments. Photographs shown correspond to representative experiments.

since it was not mediated by caspase activation in MCF-7 breast tumor cells (Pozo-Guisado et al, 2005).

Data on caspase activation by resveratrol in prostate cell lines are not conclusive, but in leukemia cells, resveratrol-induced activation of the mitochondrial pathway involving caspase-9 activity has been reported (Dorrie et al, 2001). On the other hand, a marked increase in proapoptotic bax protein and a decrease in antiapoptotic bcl-2 were induced by resveratrol in LNCaP. This result is in accordance with a recent report showing that resveratrol induced an inhibition of PI3K pathway followed by a decrease in the level of bcl-2 and an increase in proapoptotic proteins bax, bak, bid, and bad in LNCaP cells (Aziz et al, 2006). In PC-3, only a decrease in bcl-2 was noted in the presence of resveratrol without important changes in bax. However, the activation of the intrinsic apoptotic pathway is correlated with changes in the ratio of both proteins rather than with individual variations (Korsmeyer et al, 1993; Pozo-Guisado et al, 2002; Reed, 1994). Therefore, our results suggest that resveratrol induced apoptosis in LNCaP and PC-3 prostate cancer cells through alteration of the bax/bcl-2 balance and through activation of caspase-9, both processes leading to increased caspase-3 activity. In agreement, LNCaP cells, which had higher levels of caspase-9, -8, and -3 activities and elevated bax/bcl-2 ratio, also had increased apoptosis rates with respect to PC-3 cells.

Proliferation arrest is another main effect of resveratrol on cancer cells from different origins (Schneider et al, 2000; Pozo-Guisado et al, 2002; Kaneuchi et al, 2003; Larrosa et al, 2003; Kim et al, 2004; Poussier et al, 2005). Using different prostate cancer cell lines, it has been suggested that resveratrol induces disruption of the G1/S transition of the cell cycle (Sgambato et al, 2001). We have confirmed the marked effect of resveratrol on cell proliferation in nontumorigenic prostate epithelial cell line and in both prostate cancer-derived cell lines used. This inhibitory effect was dose- and time-dependent. Our data on cell cycle distribution indicate that high concentrations of resveratrol induced a marked increase in cell number in G0/G1 phase, with a corresponding decrease in the other phases in both prostate cancer cell lines. Interestingly, concentrations of this molecule between 10 and 50 μM induced a moderate, although reproducible, increase in cells at the S phase. We have reported a similar biphasic effect of resveratrol in mammary cancer cell lines (Pozo-Guisado et al, 2004). Agonist action for androgen/estrogen receptors at low concentration and antagonist activity at high concentration may be proposed as a possible explanation. The highest concentration of resveratrol resulted in almost no LNCaP cells in G2/M, while in PC-3, about 20% of cells still remained at that cell cycle phase, suggesting that G2 checkpoints might be also involved in the resveratrol effect. Studying the cyclin/Cdk complexes that regulate the 2 major cell cycle transitions, we have found that the expression of cyclins D1/E and the kinase activity of cyclin D1/Cdk4 complex, which regulates G1/S transition, were markedly reduced by resveratrol in LNCaP cells, with only a slight effect on PC-3 cells. In contrast, the expression and kinase activity of the cyclin B1/Cdk1 complex, which regulates G2/M transition, showed a significant decrease in both cell lines at concentrations of resveratrol above 50 μM. These results suggest that the G1/S and the G2/M transitions of the cell cycle might be differentially affected by resveratrol in either prostate cancer cell line. Considering that resveratrol can interact with both androgen and estrogen receptors (Gehm et al, 1997) and that LNCaP cells express androgen receptor whereas PC-3 cells have estrogen receptors alpha/beta, the differential interaction of resveratrol with these 2 type of receptors may account for the differences in the molecular mechanism showed in both cell lines. In this regard, resveratrol appears to inhibit the G1/S transition more efficiently in LNCaP than in PC-3 cells, an effect that might also help to explain the higher sensitivity to apoptosis in androgen-responsive LNCaP cells. A cell-specific mechanism of resveratrol action has been also shown in MCF-7 and MDA-MB-231 human breast cancer cells, in which apoptosis was also induced in estrogen-responsive MCF-7 but not in estrogen-unresponsive MDA-MB-231 cells (Pozo-Guisado et al, 2002; Pozo-Guisado et al, 2004). However, the presence of a very low level of beta-estrogen receptor expression in LNCaP cells may not rule out completely the influence of this receptor, but taking into account the differences in the level of expression in both cells, we consider that our suggestion is highly probable. We are currently studying this issue. Analyzing upstream modulators of cyclin/Cdk complexes, we have found that p21 and p27 were increased by resveratrol only in LNCaP cells. Consistently, the key cell cycle regulator p53 was also increased by resveratrol in this cell line. P53-dependent gene expression, as androgen receptor coactivators and activation of NFkB induced by resveratrol in LNCaP cells have also been reported (Narayanan et al, 2003). In addition, resveratrol induced, via MAPK pathway, a phosphorylation of p53 followed by an increase in p21 in a mutant p53 prostate cancer cell line (Lin et al, 2002). These data confirm that p53 and p21 (like p27 and other cell cycle regulators) are very pleiotropic proteins and are related to several molecular pathways. Considering that p21 and p27 proteins inhibit mainly G1-S cyclin/Cdk complexes, we speculate that resveratrol may be affecting the cell cycle of PC-3 cells through other regulators. Taken together,

the data presented in this work indicate that resveratrol induces caspase-9-mediated apoptosis with changes in the bax/bcl-2 ratio and proliferation arrest through disruption of the G1/S and G2/M transitions. Nevertheless, the molecular mechanism of resveratrol may be different in cancer cells representative of different malignancy grades. We suggest that these differences might be explained by the distinct profile of steroid receptors present in these cell lines. Our results reinforce the use of resveratrol in the chemoprevention of prostate cancer. Although the chemopreventive action of grape juice or red wine on healthy cells is not only due to resveratrol but also to other numerous polyphenolic compounds like gallic acid, quercetin, and catechin, among others, resveratrol concentration in red wine may reach over 4 mg/L. However, data presented in this work strongly suggest that resveratrol may have therapeutic potential on malignant cells and that it could be used as a pharmacological preparation. From this perspective the potential toxicity of resveratrol in vivo is a major issue. There are very few studies on resveratrol toxicity in vivo. Recently, it has been reported that only at high doses (3 g/Kg/day), resveratrol causes moderate liver toxicity as measured by DNA array and induction of phase II metabolizing enzymes (Hebbar et al, 2005). This potentially toxic concentration is far beyond the doses used in this study. Finally, it is important to note that comparing these aspects of resveratrol effects, in the same experimental conditions, between LNCaP and PC-3 cells is very relevant considering that these cells are representative of the 2 main stages of prostate cancer, namely androgen-sensitive and androgen-insensitive tumors.

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