Resveratrol Inhibits the Expression and Function of the Androgen Receptor in LNCaP Prostate Cancer Cells

Susan H. Mitchell, Wen Zhu, and Charles Y. F. Young

Materials and Methods

**Growth Response.** LNCaP cells were seeded at $4 \times 10^4$ cells/well in 24-well plates. After 2 days, they were treated with varying amounts of resveratrol with or without 1 nM Mib. Mib is a nonmetabolizable, synthetic androgen. One study removes the spent media after 24 h and replenishes the wells with fresh, untreated media. Then after 24 h, an MTS [3-(4,5-dimethyl-2-yl)-5-(3carboxymethoxyphosphonyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay is performed (Promega Corp., Madison, WI) as per manufacturer’s instructions. In other studies, 6 days after treatment 400 μl of spent medium were collected and sent to the Mayo Immunochannel Core facility and assayed for total hK2 and PSA levels. Then an MTT assay was performed (Sigma Chemical Co., St. Louis, MO) for measuring cell viability. Four wells/treatment were used, and it was repeated twice.

**Western Blot Analysis.** LNCaP cells were plated in 10-cm dishes at $9 \times 10^5$ cells/dish in RPMI 1640 (Mediatech, Herndon, VA) and 5% FCS (Biofluids, Rockville, MD). After 48 h, the cells were treated with 1.0 nM Mib and varying concentrations of resveratrol. After 24 h, cells were collected according to Santa Cruz Biotechnology research applications. Protein levels were measured with a DC protein assay (Bio-Rad, Hercules, CA). Ten μg of protein were loaded into precast 4–12% NuPage gels (Novex, San Diego, CA), run with 4-morpholinepropanesulfonic acid buffer and transferred according to the manufacturer’s instructions onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). A Ponceau S stain was performed for total protein staining and visualized on a digital camera. The membranes were blocked overnight at 4°C in TBST [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.1% Tween 20] and 5% dry milk. The membranes were washed three times for 10 min each with TBST. Primary antibody for AR and p21 (PharMingen, San Diego, CA) at a 1:2000 dilution was incubated at room temperature for 1 h. The membranes were washed three times for 10 min each with TBST. Antimouse horseradish peroxidase secondary antibody (Amersham, Piscataway, NJ) used at 1:10,000 dilution was also incubated for 1 h at room temperature. The membranes were washed again, and Renaissance chemiluminescence (DuPont NEN, Boston, MA) was used according to the manufacturer’s instructions.

**Transient Transfections.** LNCaP cells were plated in 60-mm dishes until they reached a confluence of 50–70%. Cells were transiently transfected as described previously with the appropriate constructs (7). After 24 h, the cells were treated with 50, 100, or 150 μM resveratrol with or without Mib. Whole-cell extracts were prepared, and a luciferase assay was performed according to the manufacturer’s instructions (Promega) for the PSA promoter/luciferase or AR promoter/luciferase transfection. The hK2 ARE/minimal thymidine kinase promoter/CAT transfection used the CAT ELISA from Boehringer Mannheim as per the instructions. CMV-β-gal expression vector and parental vectors (pGL3 and pBLCAT2) as controls were also included in the above transfections. β-gal activity was assayed for normalization purposes (7). Each transfection was done three times, and SDs were calculated.

**Northern Blots.** LNCaP cells were treated with varying amounts of resveratrol, 1 nM Mib as indicated, and RNA was collected by the guanidine isothiocyanate method (8). An RNA gel was run and transferred onto a nylon membrane according to the GeneScreen protocol by New England Nuclear. Fifteen μg of total RNA were loaded in each lane. cDNAs for ARA70 and glyceraldehyde-3-phosphate dehydrogenase were used as probes labeled with $[^35]P$dCTP by random priming. The hybridization was performed according to Clontech protocols with ExpressHyb hybridization solution. The films were autoradiographed at –70°C.

Hoechst 33258 Staining for Apoptosis. LNCaP cells were seeded at $9 \times 10^5$ cells/10-cm dish for 48 h. Cells were then treated with 1 nM Mib and 0, 50, 100 or 200 μM of resveratrol for 24 and 32 h. Then the plates were
treated with Hoechst 33258 (15 μg/ml in PBS) for 5 min at 37°C. Cells were washed carefully in PBS twice and visualized by fluorescence microscopy using an Axioplan II microscope (Zeiss, Inc.). An appropriate excitation filter was used (365 nm excitation; 420 nm emission). The number of apoptotic cells per 500 total cells were counted and expressed as a percentage of the total. A cell was counted as being apoptotic if nuclear fragmentation and chromatin condensation was observed (9).

Results and Discussion

The LNCaP cell line is a well-established, androgen-responsive prostate cancer cell line obtained from a lymph node metastasis of a prostate cancer patient (10). LNCaP cells express an AR and a number of androgen-inducible genes (e.g., PSA and hK2), and their growth is stimulated by androgens. We first examined the effects of resveratrol on androgen-stimulated growth and gene expression in LNCaP cells. It was observed that resveratrol-treated cells showed a decrease in the cell number, indicating decreased cell growth with a statistically significant $P < 0.05$ (Fig. 1). Spent medium was collected for assays of total PSA and hK2 proteins. The normalized data in Fig. 1b shows the dramatic decrease in both PSA and hK2 in the presence of androgens and resveratrol.

Given the large decrease in two androgen-inducible proteins, we transfected a construct containing a 6-kb PSA promoter fragment in front of a luciferase reporter gene into LNCaP cells with or without Mib to test whether resveratrol can directly affect androgen-mediated transcriptional activity of the PSA gene. Mib is a synthetic androgen that is not metabolized in cell culture. The upper panel of Fig. 2 shows the fold of androgenic induction of empty control vector, pGL3 basic, versus the 6-kb PSA promoter. As is usually seen in Mib-treated cells, the PSA promoter gives a strong androgenic induction of the luciferase activity. However, resveratrol treatment abolishes the androgenic induction of the PSA promoter ($P > 0.05$). To further examine whether the AR binding site, referred to as an ARE, is the actual target in androgen-inducible genes, the lower panel shows a transfection with a construct that contains three copies of an ARE inserted in front of a minimal thymidine kinase promoter with a $CAT$ reporter gene. LNCaP cells were treated with or without Mib, and fold androgen induction is depicted compared with the empty control vector $CAT2$. Once again, treatment with resveratrol is sufficient to completely abolish androgen induction.

The above transfections suggest that the AR must be involved in resveratrol inhibition of LNCaP cells. Therefore, a Western blot was performed to see whether AR protein levels changed with resveratrol treatment (Fig. 3a). Fig. 3a shows that AR protein levels are decreased in a dose- and time-dependent manner with resveratrol treatment.

A recent study showed that resveratrol can affect gene expression at the transcriptional level (11). The above results demonstrate that is the case for the androgen-regulated genes. To determine whether resveratrol can also affect the AR at the transcriptional level, gene transfer assays were performed with an AR promoter. Transient transfections of an AR promoter luciferase construct (Fig. 3b) show a significant decrease in the transcription activity of the AR promoter by resveratrol treatments ($P < 0.05$).

We have demonstrated that resveratrol can inhibit the expression of the AR and its functions, including gene induction and cell proliferation. To further ascertain that resveratrol has a broad effect on androgen-regulated genes, Northern or Western analysis was performed to see whether different classes of the androgen-regulated genes are affected. Fig. 3c shows that the AR-specific coactivator
ARA70 mRNA level is decreased with resveratrol treatment. The graph shows the normalization data as a percentage of the Mib-treated control. It seems that the maximal reduction of ARA70 mRNA is achieved at 100 μM resveratrol. It has been suggested that the decrease in ARA70 could affect AR-mediated gene expression and cellular processes (12–14). Therefore, it is possible that the repression of the expression of ARA70 may further enhance the inhibitory effects of resveratrol on AR-mediated gene expression.

Recently, it has been reported that p21Waf1/Cip1, a cyclin-dependent kinase inhibitor, is also an androgen up-regulated gene (15). p21 has been reported as a powerful cell proliferation inhibitor or cell death facilitator (16). On the other hand, in many cases it has been shown that this protein can protect cells from death (17), and reducing its intact protein levels may enhance apoptosis (16). The authors of the above report (15) suggested that the increase in p21 by androgens may facilitate LNCaP cell proliferation. A Western blot of LNCaP cells treated with 0, 100, or 200 μM resveratrol for p21 protein was performed. Fig. 4a shows that resveratrol indeed decreases p21 in a dose-dependent manner. Our study seems to suggest that the reduction of p21 by resveratrol may be at least in part because of the repression of AR. Moreover, the higher the concentrations of resveratrol, the more profound effects it has on p21 levels. This may not solely be from the repression effect on the AR. Also, it will be interesting to determine whether any other factors can be affected by resveratrol that subsequently reduce the expression of p21.

We further examined the growth-inhibitory effect of resveratrol on LNCaP cells treated with varying amounts of resveratrol at 24- and 72-h treatments. The graph shows the normalized densitometric results as a percentage of control (no resveratrol treatment). The cells were treated with the indicated amounts of resveratrol for 24 h. Luciferase activities were normalized by β-gal activities and presented as relative light units/milliunits of β-gal. The transfection was repeated three times; bars, SD. Northern blot analysis of the AR coactivator ARA70 in LNCaP cells treated with resveratrol and Mib. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, as depicted in the graph.
LNCaP cells after 24 h treatment. We found that a 100 μM treatment of resveratrol caused inhibition of cell growth in the presence of androgens with little detection of apoptosis, whereas 200 μM resveratrol induced massive apoptotic cell death (Fig. 4b). Thus, from the study we intend to suggest that the reduction of p21 may be related to negative growth effects of resveratrol, and that the occurrence of either growth inhibition or apoptosis depends on the degree of the extent of p21 reduction. Further study of whether and how the reduction of p21 by resveratrol indeed can negatively affect the growth of prostate cancer cells will be needed to clarify our points.

To study whether the effects of resveratrol are reversible, LNCaP cells were treated with resveratrol for 24 h, and then the treated medium was removed and fresh medium was added. Fig. 4c shows that with up to the 100 μM concentration of resveratrol, the growth-inhibitory effects on the cells can be reversed (P = 0.06). However, at 150 μM, the cells are still inhibited and similar to effects without medium removal (P < 0.05). Because these studies were conducted without Mib, it shows that at high concentrations, resveratrol could inhibit cell growth.

Androgens are important regulators of the prostate gland. Androgens are so essential in the carcinogenesis of prostate cancer that it does not develop unless a man produces and uses androgens (18, 19). One important question not yet addressed is whether resveratrol is able to accumulate in tissues such as the prostate at concentrations as high as 100 and 200 μM. No conclusive human studies have been executed. Other chemically related compounds, such as green tea polyphenols, appear in the blood stream at relatively low concentrations after oral administration (20). Their in vitro concentrations to achieve biological effects are often much higher than those in plasma. However, their in vivo administration seems to be able to show certain biological effects, as predicted in the in vitro system. For example, in a very recent report (21), green tea polyphenols seem to show a repressive effect on androgen action in mice. Further study of in vivo effects of resveratrol on the expression of the AR and subsequent androgen action will clarify this question. Nevertheless, in this study we have shown a novel aspect of resveratrol that presents as a potent inhibitor for androgen action in prostate cancer cells. We showed that the inhibition of androgen action by resveratrol is mediated via the reduced expression of several important genes such as AR, ARA70, and p21. This study strongly suggests that resveratrol can modulate AR-mediated action in the prostate. It has the potential to become a chemopreventive and/or chemotherapeutic agent for prostate cancer.

References


Resveratrol Inhibits the Expression and Function of the Androgen Receptor in LNCaP Prostate Cancer Cells

Susan H. Mitchell, Wen Zhu and Charles Y. F. Young