Effects of resveratrol and other wine polyphenols on the proliferation, apoptosis and androgen receptor expression in LNCaP cells

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Received 10 February 2014; accepted 19 February 2014
Available online 22 May 2014

Keywords
Cell proliferation; Apoptosis; Androgen receptor; LNCaP; Resveratrol; Polyphenols

Abstract
Purpose: To address the effect of resveratrol and other red wine polyphenols on cell proliferation, apoptosis and androgen receptor (AR) expression in human prostate cancer LNCaP cells.

Materials and methods: LNCaP cells (5 x 10²) were cultured in microtiter plate modules and treated with gallic acid, tannic acid and quercetin (1, 5 and 10 µM), rutin and morin (25, 50 and 75 µM) and resveratrol (5, 10 and 25 µM). To address the extent of proliferation at 24, 48, 72 and 96 h, a colorimetric immunoassay method was used. An activity caspase 3/7 detection assay was used to disclose apoptosis at 24, 48 and 72 h. AR mRNA levels were determined by real time RT-PCR.

Results: All polyphenols studied significantly inhibited (P<.05) cell proliferation compared to control. However, there were moderate differences between them. Resveratrol was the strongest inhibitor at different times and doses. Also, caspase-3 and caspase-7 activity was significantly higher (P<.05) than control in the presence of all the compounds, but the earlier response was achieved by resveratrol. Resveratrol, quercetin and morin were the only nutrients that significantly inhibited AR mRNA expression. Again resveratrol produced the highest inhibition (90–250 times less than control), followed by morin (67–100 times) and quercetin (55–91 times).

Please cite this article as: Ferruelo A, Romero I, Cabrera PM, Arance I, Andrés G, Angulo JC. Los efectos de resveratrol y otros polifenoles del vino sobre la proliferación, apoptosis y expresión de receptor androgénico en células LNCaP. Actas Urol Esp. 2014;38:397–404.

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2173-5786/$ - see front matter © 2014 AEU. Published by Elsevier España, S.L. All rights reserved.
Conclusions: All polyphenols studied showed important antiproliferative effects and induced apoptosis when added to LNCaP cells culture. We confirm that resveratrol, morin and quercetin may achieve such effect through reduced expression of AR. The synergistic effects of these compounds and their potential to prevent progression of hormone-dependent prostate cancer merit further study.

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Introduction

Prostate cancer is the most common neoplasm diagnosed in men in industrialized countries. Since the introduction of prostate-specific antigen (PSA) screening test in the late 1980s and because of the increasing public awareness with respect to this disease in the early 1990s, the number of new cases has increased dramatically during the last decade. Besides PCA screening, other possible causes for its increasing incidence are aging of the population, the high consumption of meat and fat and the higher rates of obesity.

In Mediterranean countries, with a high intake of olive oil, fruits, vegetables and wine, the incidence of prostate cancer is much lower than that in other western countries. All those food products contain important amounts of phenolic compounds, commonly referred to as polyphenols. Flavonoids are the most common polyphenolic compounds present in vegetables. An important effect of flavonoids is their strong antioxidant activity: they are scavengers of oxygen-derived free radicals which are involved in tumor promotion. Flavonoids have also shown many biological properties that may account for cancer chemoprevention as apoptosis enhancement, cell growth arrest, inhibition of DNA synthesis, and modulation of signal transduction pathways. The authors conducted a previous study that showed that some red wine polyphenols inhibited the proliferation and induced apoptosis in human prostate cancer LNCaP cells cultured in medium containing androgens and also in murine bladder cancer cell line MB-49.

Polyphenols are usually absorbed from the upper gastrointestinal tract, showing an increased uptake at the liver, heart and kidney. A regular ingestion of polyphenols is necessary to obtain biologically important concentrations. Red wine is a rich source of polyphenolic substances and more than 200 individual phenolic compounds have been identified, catechin, quercetin, phenolic acid, and resveratrol being the most important.

On the other hand, evidence has shown that androgens are involved in the development and progression of prostate cancer, and their biological effects in the prostate are mediated by the AR, a ligand-activated transcription factor of the nuclear receptor superfamily. Most of the molecular
mechanisms responsible for the development of recurrent hormone-refractory tumors involve alterations in the function of the AR and its complex signaling pathways. Thus, the AR could still play a role in the development of prostatic malignancy and strategies to minimize the AR function are expected to play a beneficial role. We investigate the effect of some red wine polyphenols on cell proliferation, apoptosis, and androgen receptor expression in human prostate cancer LNCaP cells.

Materials and methods

Cell cultures and treatments

The androgen-responsive LNCaP human prostate cancer cell line (American Type Culture Collection, Rockville, MD, USA) was used. This cell line shows functional AR. It was maintained in RPMI 1640 (Gibco BRL, Barcelona, Spain) supplemented with 5% heat-inactivated fetal bovine serum (FCS, LINUS, Cultek, Madrid, Spain), 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 50 µg/ml gentamicin, and 2 mM L-glutamine (Gibco BRL, Barcelona, Spain). All cells were cultured in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. For the assays, the FCS in the medium was replaced by 5% charcoal-stripped FCS (cFCS, LINUS, Cultek, Madrid, Spain) and the cells were treated with several concentrations of the following polyphenols: tannic acid (1–10 µM), gallic acid (1–10 µM), quercetin (1–10 µM), rutin (25–75 µM), morin (25–75 µM) and resveratrol (5–25 µM) during 24, 48, 72 and 96 h. All compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in either ethanol (tannic acid, gallic acid, quercetin and resveratrol) or DMSO (rutin and morin) and stored at −20 °C. The final concentrations used for different experiments were prepared by diluting the stock with RPMI before use. Non-treated LNCaP cells in a carrier solvent (0.1% DMSO or ethanol) were used as control.

Assay for cell proliferation

Cells were subcultured into a 96-well plate with 1 × 10^3 cells per well in 100 µl medium. After overnight incubation, the medium in each well was discarded and replaced by fresh medium with the above-mentioned polyphenols at the indicated concentrations. The medium was changed and the same polyphenol concentration added every 24 h. The results were obtained after 24, 48, 72 and 96 h of incubation. The extent of proliferation was assessed using a colorimetric immunoassay (Cell Proliferation ELSA BrdU, Roche, Barcelona, Spain). This technique is based on the incorporation of the pyridine analog bromodeoxyuride (BrdU) instead of thymidine into the DNA of proliferating cells. This BrdU is detected by immunoassay, by measuring the absorbance at 450 nm in an ELISA microplate auto reader (Bio-Tek Instruments, Winooski, USA). Absorbance values directly correlate with the amount of DNA synthesis and therefore with the number of proliferating cells. Experiments were repeated a minimum of three times and the results expressed in absorbance units (AU).

Caspase-3/7 activity assays

The cells were grown in 6-well plates at a density of 1 × 10^5 cells per well and treated with the highest doses of each polyphenol used in the proliferation study. At 24, 48, 72 and 96 h of treatment, the media was aspirated and the cells were washed once with PBS. Then we added M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA) with fresh protease inhibitor cocktail (Boehringer Mannheim, Germany). The cells were gently stirred for 5 min and the lysate was collected in a microfuge tube. The lysate was cleared by centrifugation at 13,000 rpm for 10 min and the supernatant was either used or immediately stored at −80 °C. The protein concentration was determined by BCA Protein Assay using the manufacturer’s protocol (Pierce, Rockford, IL, USA).

For apoptosis quantification we used a luminescent assay that measures caspase-3 and -7 activities (Caspase-Glo 3/7 Assay. Promega, Madison, WI, USA). Briefly, the assay provides a proluminescent substrate for caspase 3/7 in a reagent optimized for caspase activity, luciferase activity, and cell lysis. The addition of reagent results in cell lysis, caspase cleavage of the substrate, and generation of a “glow-type” luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present. For this assay we used 1 µg protein for each determination.

Androgen receptor real-time RT-PCR

RNA was prepared from cell samples using Rneasy (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The RNA was then quantitated using a spectrophotometer (absorbance to 260/280 nm). To analyze the expression of AR by real-time PCR, 1 µg of total RNA from each sample was used for first-strand cDNA using random primers and Moloney murine leukemia virus (MMuLV) reverse transcriptase (Sigma, St. Louis, MO, USA). All PCR reactions were performed using a fluorescence temperature cycler (iCycler, Bio-Rad Laboratories, Hercules, CA, USA) with 25 µl reaction mixture containing 12.5 µl Universal PCR Master Mix (Applied Biosystems, Madrid, Spain), 1.25 µl of a mix of unlabelled PCR primers (AR or 18S) and Taqman MGB probe (Assays-on-Demand, Applied Biosystems, Madrid, Spain). PCR reactions were cycled according to the manufacturer’s instructions. To determine the relative expression of AR in each sample, the results were normalized to 18S (used as an internal standard) and the comparative Ct method was used (User Bulletin #2 (2001), Applied Biosystem, Madrid, Spain). For the comparative Ct calculation to be valid, the efficiency of the target amplification should approximately equal the reference amplification. Finally, template dilution was made for AR (range: 0.025–1 ng) and 18S (range: 0.001–0.025 ng) and the PCR efficiency of the target amplification was approximately equal to the reference amplification.

Cells were treated with control and the highest concentrations of different polyphenols for 24 and 48 h. At the end of each incubation period, total RNA was reverse-transcribed to cDNA and quantitative real-time PCR was
Figure 1  Effect of polyphenols on cell proliferation of human prostate cancer cells (LNCaP) over 96 h of incubation. Cells were treated with different concentrations of polyphenols (μM) and proliferation cell determined by the cell proliferation ELISA BrdU assay. (a) Tannic acid, (b) rutin, (c) gallic acid, (d) morin, (e) quercetin and (f) resveratrol. Values are means ± SE of a typical experiment in triplicate. *P < 0.05 and **p < 0.01 vs. control.

performed. Values were calculated as mean ± SE of a typical experiment performed in triplicate.

Statistical analysis

One-way ANOVA with Dunnett t-test post hoc analysis was used to compare and AR expression analysis between treated and control groups. Comparisons with regard to caspase 3 and 7 activity were carried out with Student’s unpaired t test. Significance level was reached when p < 0.05.

Results

Effect of polyphenols on cell proliferation

Fig. 1 shows the effect of different polyphenols on LNCaP cells proliferation. Although all the tested polyphenols
Resveratrol and LNCaP cell

**Figure 2** Polyphenol-induced apoptotic cell death in LNCaP cells. Cells were treated with the highest concentrations of polyphenols for 24, 48 and 72 h. At the end of the different incubation period, cells were lysated and analyzed for activity of caspase-3 and -7 with the aid of a luminometric assay. (a) Tannic acid, (b) rutin, (c) gallic acid, (d) morin, (e) quercetin and (f) resveratrol. Values are means ± SE of a typical experiment performed in triplicate. *P < 0.05 and **P < 0.01 vs. control.

Successfully inhibited cell proliferation, there were moderate differences between them. Resveratrol was the strongest inhibitor at different times and doses. Cell proliferation was significantly inhibited by tannic and gallic acid at 5 and 10 µM at any incubation time. The weakest effect was produced by quercetin; however, it was able to inhibit cell proliferation at 96 h (as the rest of nutrients).

**Effect on caspase-3 and caspase-7 activity**

We added flavonoids at highest concentrations that inhibited proliferation in this study, during 24, 48, and 72 h. The cell lysates were then analyzed for caspase-3/7 activity by means of a luminometric assay (Fig. 2). All the polyphenols tested significantly increased the caspase-3/7 activity. At 24 h, the activity of both enzymes increased and was highest at 72 h after the addition of resveratrol. With the rest of polyphenols, the activity of caspase-3/7 increased at 48 h and it also reached its maximum at 72 h.

**Effect on androgen receptor expression**

To determine changes in AR mRNA expression after polyphenol exposure, real time RT-PCR was used. LNCaP cells were exposed to a nutrient concentration known to induce caspase-3/7 activity as revealed in the previous phase of the present study. As shown in Table 1, quercetin, morin and resveratrol were the only nutrients that significantly inhibited AR mRNA expression. Again, resveratrol produced the highest inhibition (90–250 times less than control). Quercetin and morin also produced inhibition, but the lower magnitude (55–91 and 67–100 times, respectively).

**Discussion**

Epidemiological studies have demonstrated a considerable geographical variation in the age-adjusted incidence of prostate cancer and environmental agents play a very important role. Many studies have addressed the relationship between nutrients (some of them included in the present study) and anticancer protection. Nutritional agents as
soy isoflavones, lycopene, vitamin E, selenium, vitamin D, and other polyphenols have been found to inhibit proliferation or to induce apoptosis in some prostate cancer cell lines. Grapes and wine are known natural sources of dietary polyphenols, and tannins as tannic and gallic acid, both present in red wine, have also been found to have anti-initiating and antipromoting properties. In a previous study the authors demonstrated the inhibitory effect of some of the mentioned polyphenols on the proliferation of LNCaP cells.

In our study we focussed on six polyphenols (quercetin, rutin, morin, resveratrol, gallic acid and tannic acid) that are invariably present in red wine, an important constituent of Mediterranean diet. We have demonstrated a growth inhibition of LNCaP cells at different concentrations and time of exposure to those nutrients. Resveratrol, tannic and gallic acid were the most successful inhibitors of the growth of LNCaP cells, while quercetin, rutin and morin required higher concentrations and longer exposure times to inhibit cell proliferation. Our findings were consistent with reports available in the literature. Suppression of cell proliferation through alterations in division and apoptosis has been documented as well. Caspase-3/7 ultimately appears to function downstream in the apoptosis pathway and is associated with the cleavage of many critical cellular substrates. In our experiment we found an increase in the activity of both enzymes in LNCaP cells after exposure to the studied polyphenols. This finding supports apoptosis as a major mechanism of polyphenol-induced antiproliferative effects in our study.

On the other hand, we used a real-time RT-PCR method for relative quantification of AR gene expression. We observed a reduced expression of AR mRNA in LNCaP cells induced by the flavonoids rutin and morin and the phytalexin resveratrol at 24 and 48 h after exposure. Other studies have demonstrated the inhibition capacity of resveratrol and quercetin over AR mRNA expression, but not by the flavonoid morin. LNCaP cells are known to be androgen sensitive and proliferate in response to AR activation, and down-regulation of the AR protein using antisense knockdown produced an inhibition of growth and an increase of the apoptosis rate. This could indicate that the effects of resveratrol, quercetin and morin could be, at least in part, mediated by regulation of the expression of the AR gene. On the other hand, such hypothesis is not likely to be valid for the other studied nutrients. Androgens are involved in the development and progression of prostatic neoplasia and AR is the essential mediator for the androgen action. This nuclear receptor is activated by its ligands, testosterone and 5α-dihydrotestosterone, and consequent dissociation by the heat shock proteins. The activation process includes recruitment of coactivators. Finally, activation of AR upregulates the transcription of genes containing androgen-response elements in their promoters. So, the suppression of androgen signaling is a therapeutic target for prostate cancer. Resveratrol inhibits the function of the androgen receptor and also modulates AR transcriptional activity, but this is not the only nutrient to downregulate expression of androgen synthesizing genes as revealed by studies conducted with pomegranate and green tea polyphenols in LNCaP-AR, LNCaP 104-S, LNCaP R1Ad and LNCaP 104-R1 cells.

Apart from the reduction of AR gene expression, cell cycle arrest and promotion of apoptosis, many other mechanisms are likely to mediate the action of polyphenols on prostate cancer cell lines. Resveratrol, quercetin and other nutrients have also been reported to inhibit the hedgehog cascade and GLI-1 expression and suppress the in vitro growth of androgen-dependent LNCaP and androgen-independent PC3 cell lines. According to the National Cancer Institute, about 400 compounds are considered potentially chemopreventive agents. Data regarding the accumulated evidence of polyphenolic substances and flavonoids limiting presentation and or progression of prostate cancer in humans

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Mean C T RA</th>
<th>Mean C T 18S</th>
<th>ΔCT (AR-18S)</th>
<th>ΔΔCT</th>
<th>RA levels (2−ΔΔCT)</th>
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<tr>
<td>C</td>
<td>28.4 ± 0.1</td>
<td>25.63 ± 0.21</td>
<td>2.77 ± 0.23</td>
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<td>T10</td>
<td>26.83 ± 0.21</td>
<td>24.47 ± 0.21</td>
<td>2.34 ± 0.29</td>
<td>−0.4 ± 0.29</td>
<td>1.32</td>
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<td>M25</td>
<td>32.9 ± 0.1</td>
<td>24.07 ± 0.21</td>
<td>8.84 ± 0.23</td>
<td>6.06 ± 0.23</td>
<td>0.015</td>
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<tr>
<td>G10</td>
<td>27.53 ± 0.06</td>
<td>23.93 ± 0.15</td>
<td>3.57 ± 0.16</td>
<td>0.83 ± 0.16</td>
<td>0.58</td>
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<tr>
<td>R75</td>
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<td>25.7 ± 0.17</td>
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<td>−0.6 ± 0.23</td>
<td>1.52</td>
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<tr>
<td>Q10</td>
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<td>24.07 ± 0.15</td>
<td>8.57 ± 0.22</td>
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<tr>
<td>RV25</td>
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<td>24.17 ± 0.1</td>
<td>9.23 ± 0.12</td>
<td>6.46 ± 0.12</td>
<td>0.011</td>
</tr>
</tbody>
</table>

C T; cycle threshold; C: control; T: tannic acid; M: morin; G: gallic acid; R: rutin; Q: quercetin; RV: resveratrol.
are very scarce, although resveratrol, epigallocatechin-3-gallate, curcumin, genistein, and quercetin are under clinical evaluation. Preclinical investigations have established the anticarcinogenic effects induced by some dietary compounds using TRAMP and PTEN knockout transgenic mouse models of prostate cancer and also the potential to reverse treatment resistance.

In conclusion, the polyphenols studied have important antiproliferative effects and can induce apoptosis when added to LNCaP cells in culture. Resveratrol appears to be the most potent of these compounds and one of the mechanisms involved in the effect of this substance is mediated through a reduced expression of the androgen receptor. Very likely the effect of resveratrol is synergistic with that of other polyphenols, such as morin and quercetin. The results presented provide a rationale to more deeply study the in vivo effects of these nutrients. Possibly, with the aid of animal and epidemiological studies, more firm recommendations about the intake of these substances as chemopreventive agents could be made in the future.

Funding

This work was funded by the Becas Fundación para la Investigación en Urología (FIU) of the Spanish Urological Association.

Conflict of interest

The authors declare that they have no conflict of interest.

References

30. Mimeault M, Batra SK. Animal models relevant to human prostate carcinogenesis underlining the critical implication
