

# Enterolactone induces apoptosis in human prostate carcinoma LNCaP cells via a mitochondrial-mediated, caspase-dependent pathway

Li-Hua Chen,<sup>1</sup> Jing Fang,<sup>1</sup> Huaixing Li,<sup>1</sup>  
Wendy Demark-Wahnefried,<sup>2</sup> and Xu Lin<sup>1</sup>

<sup>1</sup>Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and Graduate School of the Chinese Academy of Sciences, Shanghai, China; and <sup>2</sup>School of Nursing and Department of Surgery, Duke University Medical Center, Durham, North Carolina

## Abstract

The mammalian lignan enterolactone is a major metabolite of plant-based lignans that has been shown to inhibit the growth and development of prostate cancer. However, little is known about the mechanistic basis for its anti-cancer activity. In this study, we report that enterolactone selectively suppresses the growth of LNCaP prostate cancer cells by triggering apoptosis. Mechanistic studies showed that enterolactone-induced apoptosis was characterized by a dose-dependent loss of mitochondrial membrane potential, release of cytochrome *c* and cleavage of procaspase-3 and poly(ADP-ribose)-polymerase (PARP). Caspase dependence was indicated by the ability of the pan-caspase inhibitor z-VAD-fmk to attenuate enterolactone-mediated apoptosis. Mechanistic studies suggested roles for Akt, GSK-3 $\beta$ , MDM2, and p53 in enterolactone-dependent apoptosis. Our findings encourage further studies of enterolactone as a promising chemopreventive agent against prostate cancer. [Mol Cancer Ther 2007;6(9):2581–90]

## Introduction

Prostatic carcinoma is the second most frequently diagnosed cancer worldwide and is one of the major life-threatening diseases among men (1). Although the incidence of latent prostate cancer is similar across all ethnic populations, there is an 80-fold difference in incidence and a 16-fold difference in mortality of prostate cancer between the

United States and China (1, 2). Diet is considered a primary factor contributing to the huge differential in the prevalence of prostatic carcinoma (3). Although there are several dietary factors that may be important for this disease, we propose a study that specifically focuses on dietary lignans because the traditional plant-based diet in Asia is rich in lignans as compared with the omnivorous diet of the United States and Northern Europe (4). Moreover, our previous studies suggest an inhibitory effect of this phytochemical on prostate cancer growth (5).

Dietary lignans have phytoestrogenic properties (6) and are broadly available in cereals, legumes, fruits, vegetables, and grains, with the highest concentration in flaxseed and sesame seeds (7, 8). Plant-based lignans, secoisolariciresinol and matairesinol, are converted by the intestinal microflora to mammalian lignans of enterodiol and enterolactone, the major forms in the biological fluids of humans and animals (ref. 9; Fig. 1A). Enterolactone was reported to inhibit mammary carcinogenesis in rats (10), and enterolactone, enterodiol, and other mammalian lignan derivatives were also found to suppress breast and colon cancer cell growth *in vitro* (11, 12).

The association between enterolactone and prostate cancer risk in epidemiologic studies is limited and remains controversial. Kilkkinen et al. (13) reported the results from a nested case-control study, in which serum enterolactone levels were not significantly different between prostate cancer patients and controls. In contrast, in a recent population-based study from Sweden, Hedelin et al. (14) found that serum levels of enterolactone were inversely associated with prostate cancer risk. However, due to the limited availability of purified lignans, only a few studies have investigated the causal relationship between lignans and prostate cancer. Demark-Wahnefried et al. (15) observed lower rates of proliferation and higher rates of apoptosis in the tumors of patients following a flaxseed-supplemented, fat-modified diet before prostatectomy; they also found suppressed levels of total and free testosterone from baseline to follow-up. Reduced free androgen index was also reported by Dalais et al. (16) in patients treated with flaxseed and soy grits. Our previous animal study also showed that 5% flaxseed supplementation significantly inhibited tumor burden and malignancy in male transgenic adenocarcinoma mouse prostate mice (17). Moreover, our *in vitro* study also showed that enterodiol and enterolactone significantly decreased cell viability in three human prostate cancer cell lines, and enterolactone was more potent than enterodiol (5). More recently, the plant lignan 7-hydroxymatairesinol, a precursor of enterolactone, was found to significantly reduce tumor burden in mice with LNCaP xenografts (18).

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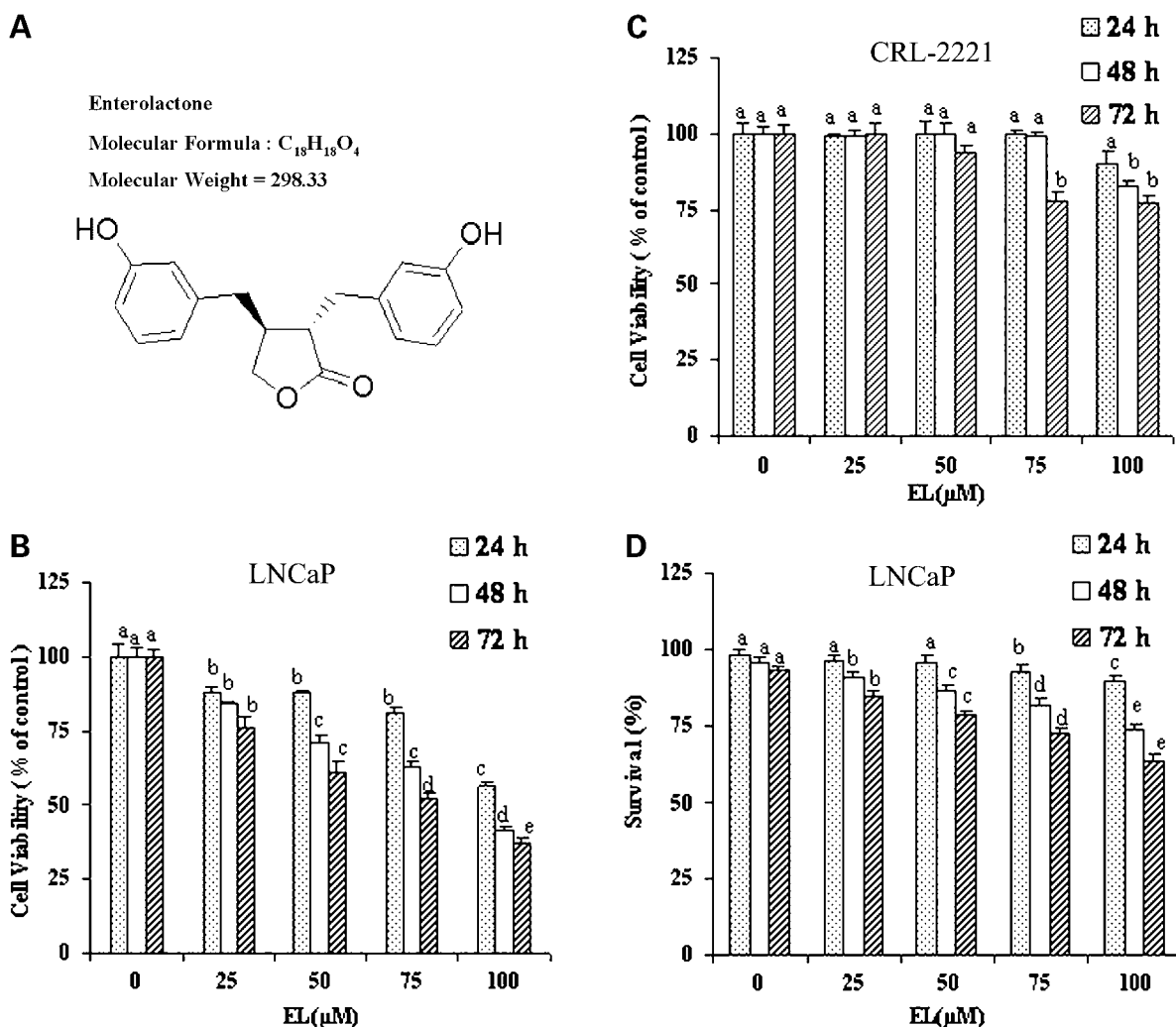
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**Requests for reprints:** Xu Lin, Institute for Nutritional Sciences, 294 Tai Yuan Rd., Shanghai 200031, China. Phone: 86-21-5492-0249; Fax: 86-21-5492-0291. E-mail: xlin@sibs.ac.cn

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**Figure 1.** Enterolactone decreases cell viability in human prostate carcinoma LNCaP cells but not in human nontumorigenic prostate epithelial cells. **A**, chemical structure of enterolactone. **B**, MTT assay on LNCaP cells. **C**, MTT assay on human nontumorigenic prostate epithelial CRL-2221 cells. **D**, trypan blue dye exclusion assay on LNCaP cells. Columns, mean of three independent experiments; bars, SE. Non-corresponding letters indicate significant differences at  $P < 0.05$ .

Together, the evidence from *in vivo* and *in vitro* research suggests anticancer activity of lignans in prostate cancer; however, the specific mechanisms are not yet well understood. In general, the modulation of cell proliferation and apoptosis have been highlighted as key players for the overall efficacy of anticancer agents (19). Lower proliferation rates and higher apoptotic indices have been observed in both humans and animals treated with lignan-rich diets (15, 17, 18, 20, 21). Accumulating evidence suggests that reduced cancer cell apoptosis is essential for the transition of prostatic carcinoma from latent to clinically overt and from hormone-sensitive to hormone-insensitive disease (22, 23). Therefore, the objective of the present study was to thoroughly investigate enterolactone-induced apoptosis and explore potential mechanism(s) by which enterolactone inhibits prostate cancer cell survival and stimulates apoptosis.

## Materials and Methods

### Reagents

This study used JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes) and the pan-caspase inhibitor, z-VAD-fmk (Promega). We also purchased antibodies from Cell Signaling [phospho-Akt (Ser<sup>473</sup>), Akt, phospho-GSK-3β and phospho-MDM2], Santa Cruz Biotechnology [p53, MDM2, caspase-3 (cleaved), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and BD PharMingen [cleavage site-specific poly(ADP-ribose)-polymerase (PARP) and cytochrome *c*]. Enterolactone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue dye, and other chemicals that were not specifically indicated were purchased from Sigma.

### Cell Culture and Treatment

The androgen-dependent human LNCaP prostate cancer cells and human nontumorigenic CRL-2221 prostate

epithelial cells were obtained from American Type Culture Collection. The normal human ovarian epithelial cell line IOSE80 and hepatocyte cell line HL-7702 (gifts from Dr. Dong Xie, Institute for Nutritional Sciences, Shanghai, China) and LNCaP cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mmol/L HEPES, and 1% penicillin and streptomycin. CRL-2221 cells were cultured in keratinocyte-SFM media supplemented with epidermal growth factor (0.2 ng/mL), bovine pituitary extract (30 µg/mL), and 1% penicillin and streptomycin (all components were purchased from Life Technologies BRL). The cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator. Enterolactone was dissolved in DMSO (Sigma Chemical) at a concentration of 100 mmol/L and stored at -20°C. Working solutions of enterolactone were then made by serial dilutions of the stock solutions with cell culture medium. DMSO was employed as negative control, and the final concentration of DMSO was 0.1% or lower (v/v) in cell culture experiments.

#### Cell Growth Assay

Cell growth was determined using the MTT assay and trypan blue dye exclusion assay. For the MTT assay,  $5 \times 10^3$  cells per well were cultured in 96-well plates and treated with 0 to 100 µmol/L of enterolactone for 24, 48 and 72 h. After incubation for specified times at 37°C in a humidified incubator, 10 µL of MTT reagent (5 mg/mL) was added to each well and further incubated for 2 h. The reaction was stopped by adding 100 µL of DMSO. Absorbance was measured at 570 nm on a microplate reader (SpectroMax 190, Molecular Devices). Data were presented from three separate experiments, and the percentage of enterolactone-induced cell growth inhibition was determined where DMSO-treated cells (control) were taken as 100%.

For the trypan blue dye exclusion assay,  $3 \times 10^5$  cells were plated in 60-mm plates and treated with specified doses of enterolactone or DMSO (control) for 24, 48, or 72 h. Both floating and adherent cells were collected and counted in triplicate using a hemocytometer. The viable and nonviable cells were discriminated by trypan blue dye exclusion.

#### Hoechst 33258 Staining Assay

To analyze apoptosis qualitatively, the Hoechst 33258 staining assay was done. LNCaP cells were seeded in 12-well plates at a density of  $5 \times 10^4$  cells per well. After treatment with 0, 50, and 100 µmol/L of enterolactone for 48 h, cell morphology was assessed using a Hoechst 33258 staining kit (Beyotime Institute of Biotechnology). Briefly, cells in 12-well plates were fixed with 4% formaldehyde in PBS for 10 min, washed twice with PBS, and then stained by Hoechst 33258 (10 ng/mL) for 5 min. The condensed or fragmented nuclei of apoptotic cells were observed under fluorescence microscopy (excitation, 365 nm; emission, 480 nm).

#### Propidium Iodide Staining Assay

To quantify enterolactone-induced apoptotic death of LNCaP cells, propidium iodide (PI) staining was done by flow cytometry. The cells were treated with 0, 25, 50, and

100 µmol/L of enterolactone for 72 h. The floating and trypsinized adherent cells were collected and washed with PBS. The cell pellets were resuspended in 300 µL of PBS and fixed by the addition of 700 µL of ice-cold ethanol at -20°C. After incubation for 30 min, the cells were re-pelleted by centrifugation and resuspended in 0.5 mL of PBS containing 100 µg/mL of RNase and then incubated at 37°C for 30 min. Finally, the cells were stained with 0.5 mL of PI solution (100 µg/mL in PBS) for 30 min. Cell cycle distribution was detected by a FACScalibur flow cytometer (Becton Dickinson), and data were analyzed using the ModFit LT for Mac V1.01 software. Cells with sub-G<sub>0</sub>-G<sub>1</sub> DNA were classified as apoptotic cells.

#### Terminal Nucleotidyl Transferase – Mediated Nick End Labeling Assay

To further discriminate apoptotic cells from necrotic cells, LNCaP cells were treated with 0, 25, 50, 75, and 100 µmol/L enterolactone for 72 h, and apoptotic cells were measured by terminal nucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) with the APO-BRDU staining kit (Phoenix Flow Systems) according to the manufacturer's instructions. In brief, the cells were fixed in 4% paraformaldehyde for 1 h at 4°C, and then enterolactone-treated cells were washed with PBS and permeabilized with 70% ethanol. After two washings with wash buffer, permeabilized cells were incubated with TdT enzyme and Br-dUTP for labeling DNA breaks at 37°C for 1 h. At the end of the incubation time, the cells were rinsed and resuspended with fluorescein ~ PRB-1 antibody solution. PI/RNase A solution was added to the tube for 30 min in the dark, and then cells were analyzed by flow cytometry.

#### Quantitation of Mitochondrial Membrane Potential ( $\Delta\Psi_m$ )

The effect of enterolactone on mitochondrial membrane potential was assessed by JC-1 dye, a radiometric, dual-emission fluorescent dye that generates fluorescence red (excitation, 550 nm; emission, 600 nm) within the mitochondria in proportion to  $\Delta\Psi_m$ . When  $\Delta\Psi_m$  dissipates, JC-1 dye leaks into the cytoplasm and emits fluorescence green (excitation, 485 nm; emission, 535 nm). LNCaP cells ( $5 \times 10^4$ ) were grown on glass chamber slides and treated with 0, 50, and 100 µmol/L of enterolactone for 24 h. After treatment, the cells were incubated in RPMI 1640 containing 10 µg/mL of JC-1 dye for 15 min at 37°C in the dark. The cells were washed and  $\Delta\Psi_m$  was determined by fluorescence microscopy.

To further quantify the effect of enterolactone on mitochondrial membrane potential, LNCaP cells were seeded in 24-well plates ( $5 \times 10^4$  cells per well) overnight. Following treatment with 0, 25, 50, 75, and 100 µmol/L of enterolactone for 24, 48, and 72 h, the cells were incubated with JC-1 as described above. The cells were then trypsinized, and 100 µL of the cell suspension was transferred to black 96-well plates. Red and green fluorescence was measured using a fluorescence plate reader. The ratios of red/green fluorescence (% of control) were calculated

according to following equation (a decreased ratio of red/green cells indicative of apoptosis):

Red/green ratio (% of control)

$$= \frac{\text{Ratio of red/green fluorescence in enterolactone - treated cells}}{\text{Ratio of red/green fluorescence in DMSO control cells}} \times 100\%.$$

### Protein Extraction and Immunoblotting

LNCaP cells were cultured in 60-mm dishes with standard culture medium for 24 h and then treated with stepped doses of enterolactone for varying lengths of time. Upon washing with PBS, the cells were scraped from the dish and centrifuged at 4,000 rpm for 5 min. The cell pellet was incubated for 30 min in radioimmune precipitation buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH, 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mmol/L EDTA and 10 mmol/L NaF] supplemented with 1 mmol/L sodium vanadate, 2 mmol/L leupeptin, 2 mmol/L aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, and 2 mmol/L pepstatin A. After centrifugation at 14,000 rpm for 15 min, the supernatant, which contained the total cellular protein extract, was collected and stored at  $-70^{\circ}\text{C}$ . For Western blotting of cytochrome *c*, the cytosolic and mitochondrial fractions were prepared as previously described (24). The protein concentration was determined using the Bradford assay (Sigma). About 60  $\mu\text{g}$  of protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with target antibodies. Protein bands were then detected by incubation with horseradish peroxidase-conjugated antibodies and visualized through an enhanced chemiluminescence reagent (Perkin-Elmer). GAPDH was used as a loading control.

### Statistical Analysis

One-way ANOVA was used to test statistical differences among treatment groups followed by Tukey's multiple comparisons. All experiments in the present study were done at least thrice, and the results were expressed as the mean  $\pm$  SE and considered significant when  $P < 0.05$ .

## Results

### Enterolactone Decreases Cell Viability

MTT assay results indicated that the viability of LNCaP cells was significantly inhibited by enterolactone with concentrations  $\geq 25$   $\mu\text{mol/L}$  (Fig. 1B). The treatment of LNCaP cells with 0 to 100  $\mu\text{mol/L}$  of enterolactone resulted in a dose- and time-dependent inhibition of cell growth, accounting for 12% to 44% (24 h,  $P < 0.05$ –0.001), 16% to 59% (48 h,  $P < 0.05$ –0.001), and 24% to 63% (72 h,  $P < 0.05$ –0.001), respectively.

Furthermore, the trypan blue exclusion assay also showed enterolactone-induced cell death in a dose- and time-dependent manner (Fig. 1D). In contrast to the significant inhibition observed in cell viability of cancer cells, no obvious cytotoxicity was detected in normal epithelium (CRL-2221 cells) treated with comparable doses.

When CRL-2221 and LNCaP cells were exposed to 100  $\mu\text{mol/L}$  of enterolactone for 48 and 72 h, the inhibition of cell viability was 17% and 23% in CRL-2221 cells and 59% and 63% in LNCaP (Fig. 1C). In addition, the trypan blue dye exclusion assay also showed little toxicity of enterolactone exposure in the normal human ovarian epithelial cell line IOSE80 and hepatocyte cell line HL-7702 (Supplementary Data).<sup>3</sup> Thus, the growth of human cancer cells was apparently more sensitive to enterolactone treatment than that of normal cells.

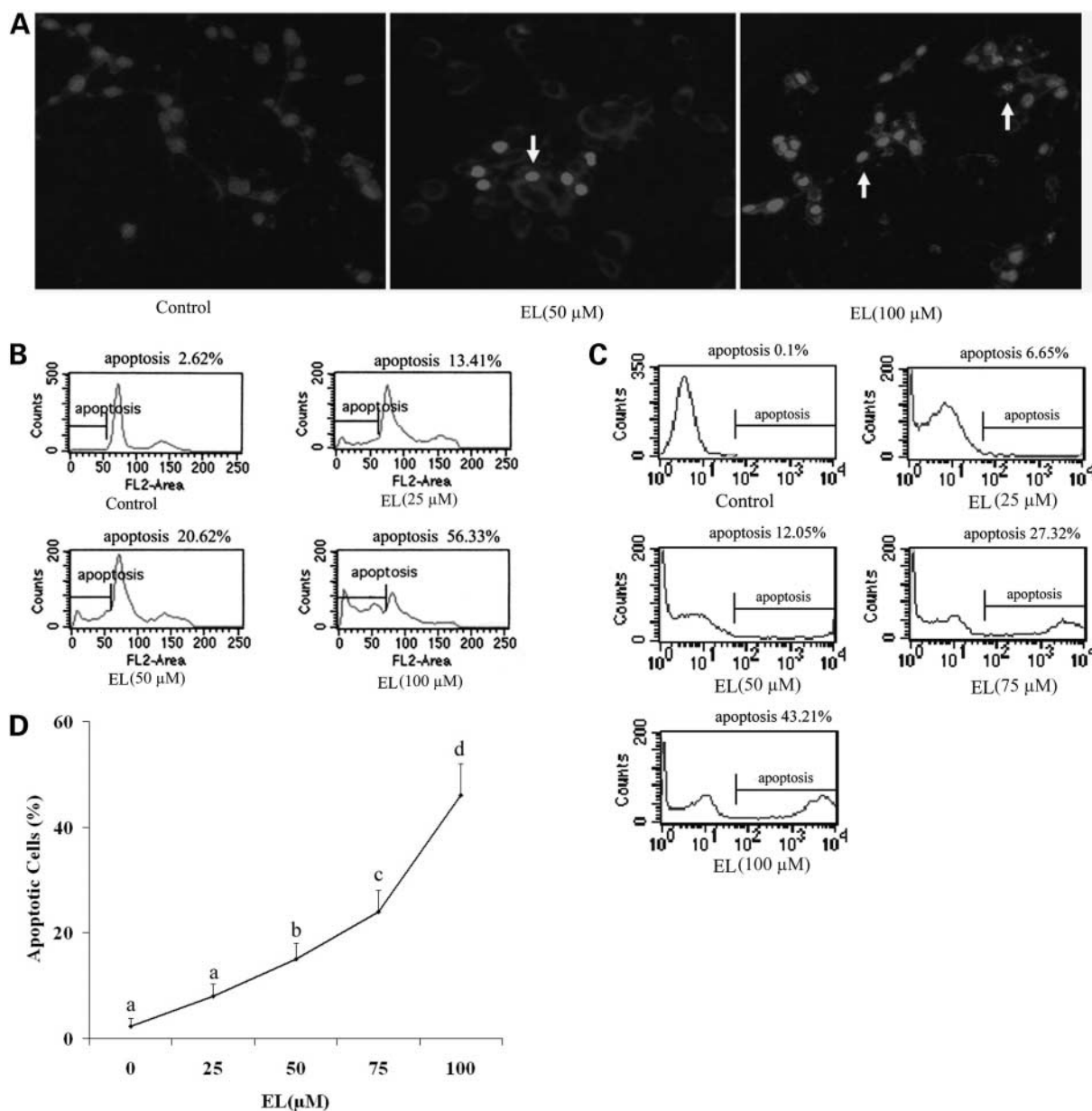
### Enterolactone Induces Cell Apoptosis

Figure 2A shows the results of the Hoechst 33258 staining assay, which was used to confirm that inhibited cell viability resulted from enterolactone-induced cell apoptosis. In this case, the apoptotic cells, characterized by condensed nuclei, were observed after the exposure of LNCaP cells to 50 and 100  $\mu\text{mol/L}$  of enterolactone. Data from the PI staining assay are featured in Fig. 2B. The sub- $G_0$ - $G_1$  DNA contents (apoptotic cells) were 2.6%, 13.4%, 20.6%, and 56.3%, corresponding to the doses of 0, 25, 50, and 100  $\mu\text{mol/L}$  enterolactone for 72 h in representative flow cytometer profiles. Owing that PI staining may yield false positive data, the TUNEL assay was employed as a more discriminating measure. Consistent with the results of PI staining, a dose-dependent increase of apoptosis also was observed (Fig. 2C and D). The data from three independent experiments showed that the percentages of total apoptotic cells increased from 8% to 46% when enterolactone concentrations increased from 25 to 100  $\mu\text{mol/L}$ , with significantly higher apoptotic cell death occurring with enterolactone concentrations  $\geq 50$   $\mu\text{mol/L}$  (Fig. 2D). Taken together, these consistent findings provide evidence that the inhibitory effect of enterolactone on LNCaP cell growth may be due to its apoptogenic properties.

### Enterolactone Disrupts Mitochondrial Membrane Potential and Leads to Cytochrome *c* Release

One critical event in the initiation of apoptosis is the disruption of mitochondrial membrane potential, which also is one of the mechanisms related to the activation of apoptotic cascades. The mitochondrial dysfunction is induced by various cellular stimuli, such as the translocation of Bax from the cytosol to the mitochondria, which subsequently triggers the release of cytochrome *c* from the mitochondria to the cytosol, an event which activates caspases and results in apoptosis (25). As shown in Fig. 3A, the treatment of LNCaP with enterolactone for 24 h resulted in dissipating mitochondrial potential. Figure 3B shows that LNCaP cells treated with 25 to 100  $\mu\text{mol/L}$  of enterolactone had dose-dependent decreases in mitochondrial membrane potential as indicated by reduced ratios of red/green fluorescence from 95% to 57% (24 h,  $P < 0.05$ –0.001), 99% to 53% (48 h,  $P < 0.05$ –0.001), and 90% to 49% (72 h,  $P < 0.05$ –0.001), respectively. Enterolactone also produced a dose-dependent release of cytochrome *c* (Fig. 3C).

<sup>3</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).



**Figure 2.** Enterolactone induces apoptosis in LNCaP cells. **A**, representative photomicrographs (Olympus, magnification, 200 $\times$ ) of LNCaP cells stained by Hoechst 33258 fluorescent dye after exposure of the cells to 0, 50, and 100  $\mu$ mol/L enterolactone for 48 h. *Arrows*, apoptotic cells were characterized as condensed or fragmented nuclei. **B**, representative flow-cytometric analysis of LNCaP cells stained by PI after exposure of the cells to 0 to 100  $\mu$ mol/L enterolactone for 72 h. Data are representative of at least three independent experiments with similar results. **C**, representative flow-cytometric analysis of LNCaP cells stained by TUNEL after exposure of the cells to 0 to 100  $\mu$ mol/L enterolactone for 72 h. **D**, summarized data from **C**. *Points*, mean of three independent experiments; *bars*, SE. Non-corresponding letters indicate significant differences at  $P < 0.05$ .

### Enterolactone Induces Cleavage of Caspase-3 and PARP

Enterolactone treatment of LNCaP cells resulted in a dose-dependent increase of the cleavage of caspase-3 and PARP (Fig. 4A), events that are considered biochemical hallmarks of apoptosis. Flow cytometry results are depicted in Fig. 4B and also lend support to a caspase-dependent pathway; the percentages of apoptosis were 3% and 21%

in the control and enterolactone-treated cells, compared with 7% in the cells coincubated with z-VAD-fmk and enterolactone. Data from three independent experiments also showed that pretreatment with z-VAD-fmk significantly antagonized enterolactone-induced apoptotic cell death (Fig. 4C). Collectively, these results suggest that enterolactone induces apoptosis via a caspase-dependent pathway.

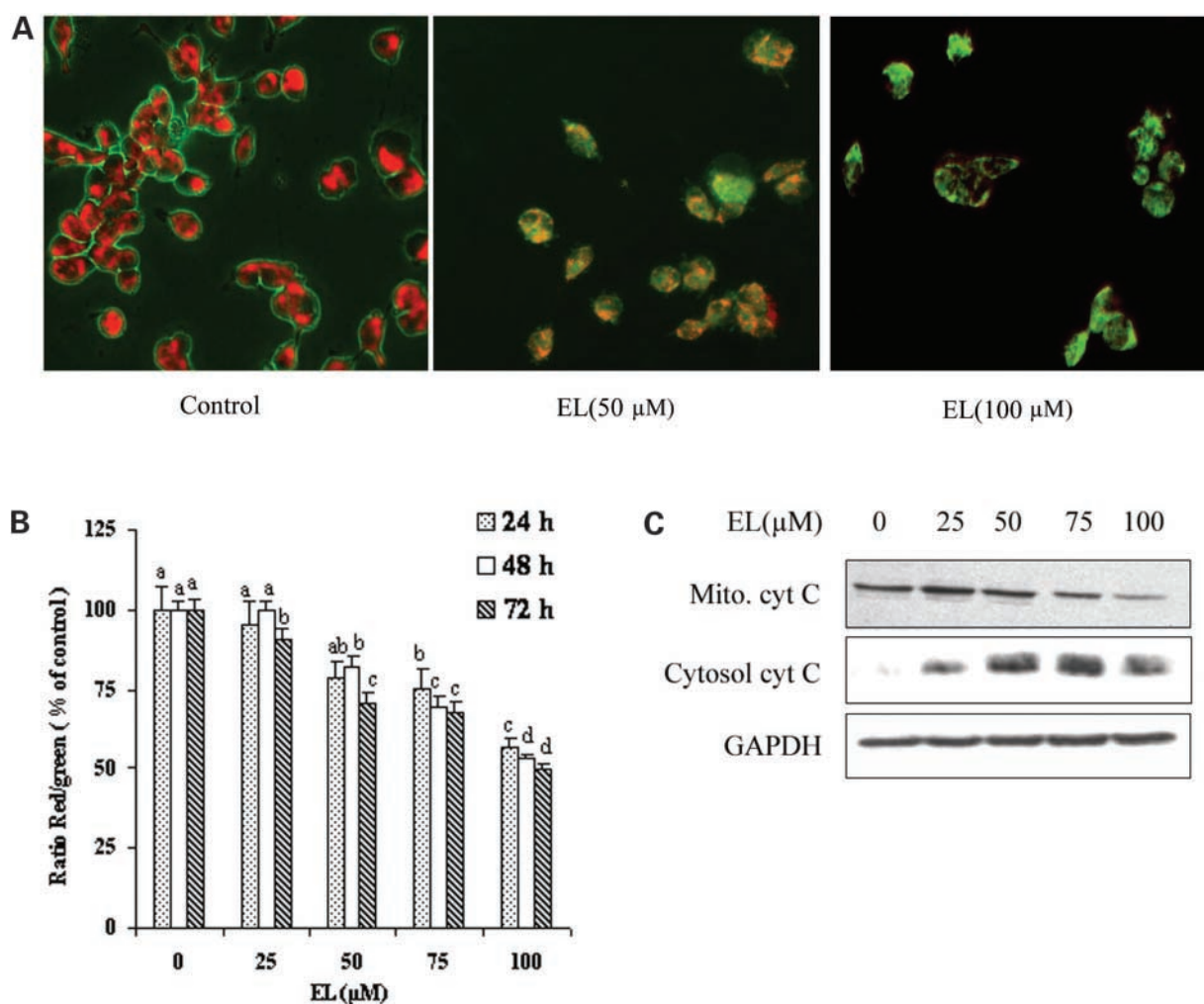
### Enterolactone Inhibits Akt Activation

Recent studies indicate that the Akt signaling pathway plays a critical role in controlling cell survival and apoptosis. Activated Akt may promote cell survival by inhibiting apoptosis through its ability to phosphorylate downstream targets (26). To determine the potential involvement of the Akt pathway in enterolactone-induced apoptosis, we determined the phosphorylation status of Akt in LNCaP cells via a dose- and time-response experiment. As shown in Fig. 5A and B, Akt phosphorylation levels were reduced by enterolactone in a dose- and time-dependent manner. Evidence of the inhibitory effect of enterolactone on Akt phosphorylation was further supported by the results of Western blotting in

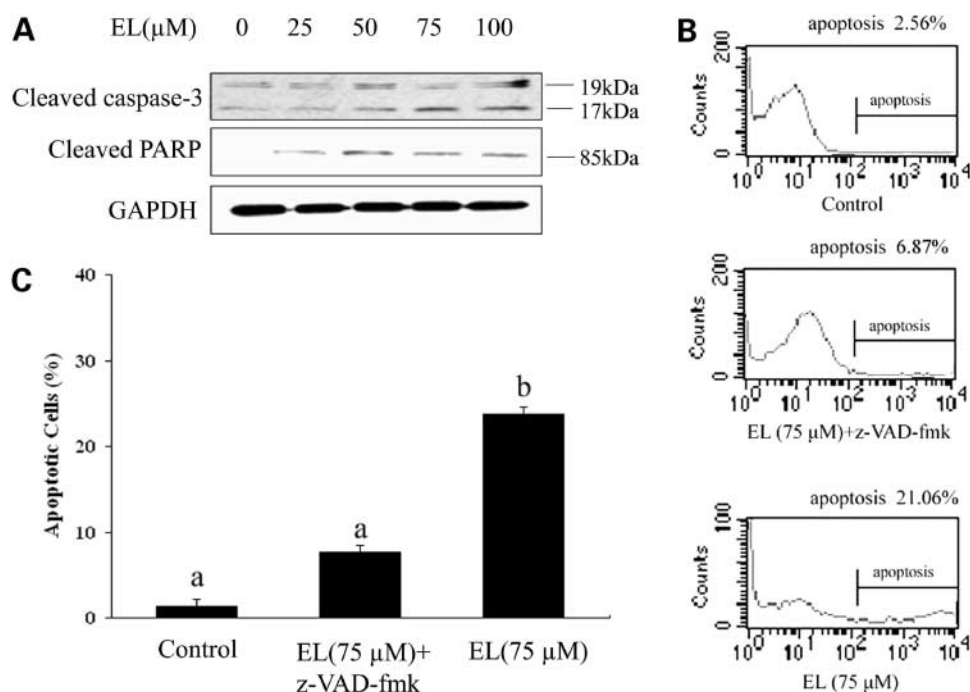
which the levels of phosphorylated GSK-3 $\beta$ , one of the key downstream elements of the Akt signaling pathway (27), were decreased with increased doses of enterolactone, concomitantly with reduced levels of phosphorylated Akt.

### Enterolactone Promotes p53 Expression while Inhibiting MDM2 Expression

The interrelated effects of enterolactone on tumor suppressor p53 and oncoprotein MDM2 also were evaluated due to their important role in apoptosis and their association with caspase activation and cytochrome *c* release (28–30). Thus, we investigated the effect of enterolactone on p53 and MDM2 in LNCaP cells. Figure 6 illustrates that enterolactone attenuates the expression of



**Figure 3.** Enterolactone induces disruption of mitochondrial membrane potential and cytochrome *c* release. **A**, representative photomicrographs of LNCaP cells stained by JC-1 fluorescent dye after exposure of the cells to 0, 50, and 100  $\mu$ mol/L enterolactone for 24 h (detailed information is described in Materials and Methods). The images illustrate the dissipation of mitochondria potential, i.e., increasing ratio of green fluorescent cells (damaged mitochondria) to orange-red fluorescent cells (intact mitochondria) under fluorescence microscopy (magnification, 200 $\times$ ). **B**, quantified effect of enterolactone on dissipation of mitochondrial membrane potential in LNCaP cells treated with indicated doses of enterolactone for 24, 48, and 72 h. Non-corresponding letters indicate significant differences at  $P < 0.05$ . **C**, Western blot analysis for the effect of enterolactone on releasing cytochrome *c* in LNCaP cells after treated with indicated doses of enterolactone for 24 h. *Mito. cyt C*, mitochondrial cytochrome *c*.



**Figure 4.** Enterolactone induces cleavage of caspase-3 and PARP. **A**, Western blot analysis for the effect of enterolactone on the cleavage of caspase-3 and PARP in LNCaP cells after 24 h exposure to indicated doses of enterolactone. **B**, effect of pretreatment of pan-caspase inhibitor (z-VAD-fmk) on enterolactone-induced apoptosis as determined by the TUNEL assay. The LNCaP cells were incubated for 72 h with DMSO control, 75 μmol/L enterolactone, or pretreated with 100 μmol/L z-VAD-fmk for 2 h before treatment of 75 μmol/L enterolactone. **C**, summarized data from **B** and the percentage of apoptotic LNCaP cells in different treatment groups presented as mean  $\pm$  SE of three independent experiments. Non-corresponding letters indicate significant differences at  $P < 0.05$ .

total and phospho-MDM2 and promotes p53 expression in a concentration-dependent manner. These results suggest that p53 and MDM2 influence enterolactone-induced apoptosis.

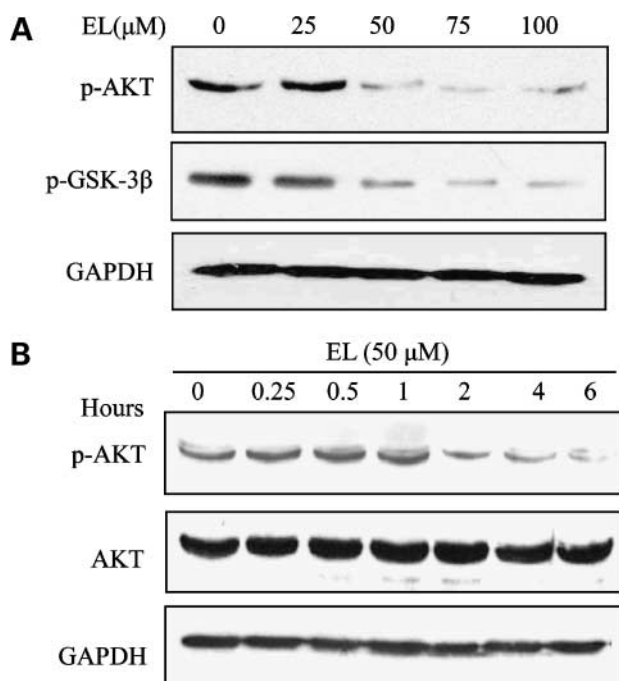
## Discussion

To the best of our knowledge, this is the first published report to document that enterolactone induces apoptosis in LNCaP human prostate cancer cells via disruption of mitochondrial membrane potential, elevation of cytochrome *c* release, and activation of caspase-3. Enterolactone-induced apoptosis also may be mediated through the inactivation of the Akt signaling pathway, as suggested by the reduced phosphorylation of Akt and its downstream targets (GSK-3 $\beta$  and MDM2), and enhanced p53 expression.

Prostate cancer is a major life-threatening malignant disease in men, particularly in western countries. Although men in China and Japan traditionally were considered at low risk, the prevalence of prostate cancer has increased substantially in recent years owing to the adoption of western lifestyles and a rapidly aging population (31). To more effectively prevent and control this disease, there is a need to identify chemopreventive approaches that induce apoptosis (32). Apoptosis is an important cellular defense mechanism that removes unnecessary and damaged cells, and the lack of ability to undergo apoptosis has been

viewed as a critical contributor to prostate carcinogenesis (33). As major metabolites of dietary lignans, enterolactone and enterodiols alone or in combination were reported to induce apoptosis in colon cancer cells (34, 35). However, little is known whether enterolactone could induce apoptosis in prostate cancer. In the present study, the data from the MTT and trypan blue exclusion assay showed that enterolactone blocked the growth of LNCaP cells in a dose- and time-dependent manner. These data are in agreement with our previous findings that enterolactone significantly hindered the growth of three prostate cancer cell lines, including LNCaP (5). In the current study, we confirmed that enterolactone-induced cell loss was mainly due to enhanced apoptosis as shown using three independent methods. The data of present study also parallel the results of previous animal experiments from our group and others in which lignans or lignan-rich diets promoted apoptotic activity in LNCaP human prostate cancer xenografts or transgenic adenocarcinoma mouse prostate mice (15, 17, 18, 20, 21). Thus, it seems that enterolactone-induced apoptosis plays a critical role in its anticancer activity.

Disruption of mitochondrial membrane potential and subsequent release of apoptotic promoting factors such as cytochrome *c* are considered key cellular events that trigger apoptosis. In a previous study, Hausott et al. (36) found that the plant-derived lignan, nordihydroguaiaretic acid,



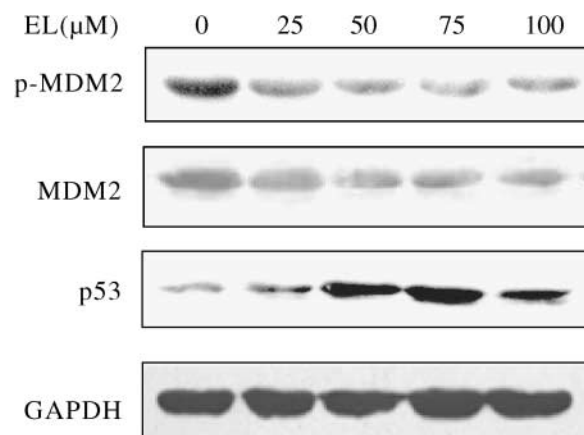
**Figure 5.** Enterolactone inhibits the phosphorylation status of Akt and GSK-3 $\beta$ . **A**, Western blot analysis for the effect of enterolactone on the levels of phosphorylated Akt and GSK-3 $\beta$  in LNCaP cells after 6 h exposure to indicated doses of enterolactone. **B**, the effect of enterolactone on the levels of phosphorylated Akt in LNCaP cells exposed to 50  $\mu$ mol/L of enterolactone at various time points.

reduced the mitochondrial membrane potential of colon cancer cells in a time- and dose-dependent manner. Similarly, we have documented not only a dose- and time-dependent dissipation in mitochondrial membrane potential, but also a dose-dependent increase of cytochrome *c* release when LNCaP cells were exposed to increasing concentrations of enterolactone. Existing studies have indicated that the release of cytochrome *c* could activate the caspase cascade by forming apoptosomes containing cytochrome *c*, Apaf-1, and procaspase-9 in the presence of dATP, and that the complex subsequently activates caspase-9 and caspase-3, resulting in the cleavage of PARP (37). We also observed that enterolactone activated caspase-3 and cleaved PARP in a dose-dependent manner. Moreover, the involvement of the caspase-dependent pathway was further confirmed by pretreatment with a pan-caspase inhibitor that significantly counteracted enterolactone-induced apoptosis. Thus, our findings suggest that the mitochondrial-mediated, caspase-dependent pathway is one possible mechanism underscoring enterolactone-induced apoptosis.

Prostate carcinogenesis is regulated by multiple signaling pathways, and the Akt signaling pathway is also a critical pathway in regulating cell survival and apoptosis. Phosphorylation of several cellular proteins, such as BAD, nuclear factor- $\kappa$ B, Bcl-2, MDM-2, and GSK-3 $\beta$ , can occur with the activation of Akt (38). Overexpression of Akt also

has been found in many human cancers, including prostate cancer (39). However, Akt activity can be reduced via an inhibitor or by genistein-derived chemopreventive agents (39, 40). Like its lignan counterpart, genistein, which is found in soy products, also exerts phytoestrogenic effects. As a key molecule in cell signal transduction pathways, Akt has been considered a potential target for novel anticancer therapies (41). In the present study, we found that enterolactone significantly inhibited Akt phosphorylation (activated form of Akt). We also found dose-dependent decreases in the levels of phospho-GSK-3 $\beta$ , a key downstream target protein that could be activated by reduced phosphorylation and induced apoptosis (42). Although no extant study has evaluated the role of enterolactone on Akt and GSK-3 $\beta$ , inhibition of cell growth and induction of apoptosis has been observed with genistein, and these effects were associated with suppressed levels of phosphorylated Akt protein and GSK-3 $\alpha/\beta$  (43). Thus, our data parallel the body of research related to genistein, and the inhibition of the Akt signaling pathway might be one of the essential mechanisms of enterolactone-induced apoptosis. However, it is currently not clear whether the dephosphorylation of Akt per se is sufficient or only one of multiple contributory signals involved in enterolactone-induced apoptosis.

Another noteworthy finding of present study was that elevated p53 expression in enterolactone-treated cells was concomitantly linked with reduced MDM2 levels. The tumor suppressor p53 has emerged as one of the critical molecules involved in induction of apoptosis by activating caspase-dependent mitochondrial cytochrome *c* release (28). Oncoprotein MDM2 is a key negative regulator of p53 and binds with p53 protein for ubiquitin proteolysis of p53 (29). In many cases, the expression of p53 is inhibited under conditions in which the Akt pathway is activated (44). It has been shown that inhibition of Akt activity could impair the phosphorylation of MDM2 and cause



**Figure 6.** Enterolactone increases expression p53 and decreases that of MDM2 Western blot analysis for the effect of enterolactone on expression of p53, phospho-MDM2, and MDM2 in LNCaP cells after 6 h exposure to indicated doses of enterolactone.



the destabilization of MDM2 or disruption of MDM2-p53 binding (30) and consequently up-regulate the levels of p53 (45). The human prostate LNCaP cell line is characterized by high Akt activity, phosphorylation of MDM2, and a wild type of p53 gene (39, 46). In the present study, when LNCaP cells were treated with apoptogenic doses of enterolactone, the phosphorylation levels of Akt were suppressed, along with reduced total and phospho-MDM2 and concomitantly increased levels of p53, suggesting that the inhibition of Akt-dependent phosphorylation may act as an essential mechanism-associated enterolactone-induced apoptosis that is mediated by the down-regulation of MDM2. Indeed, the critical role of MDM2 in chemoprevention has been underscored by a recent study of Li et al. (47) who found that genistein directly suppressed MDM2 and tumor growth in PC3 xenografts, whereas overexpression of MDM2 could eliminate genistein-induced apoptosis (47). Similar to our data, Li et al. (47) also found that genistein-induced down-regulation of MDM2 was accompanied by the up-regulated p53 expression in cancer cells with wild-type p53 such as MCF-7 cells. Nonetheless, further studies are warranted to gain insights into the mechanism of inhibition of prostate cancer by enterolactone and other lignans.

The discovery of chemopreventive agents from food or botanical sources that are effective and free of cytotoxic effects on normal cells has been declared a priority. In our study, the doses of enterolactone used to induce anticancer activity were 50  $\mu\text{mol/L}$  or higher, thus exceeding previously reported plasma enterolactone concentrations of 13.8 nmol/L in men who consumed a relatively lignan-rich diet (48) or  $358 \pm 67$  nmol/L in humans who used a secoisolariciresinol supplement of 500 mg/day (49). However, enterolactone in human prostatic fluid was found to be 200-fold higher than in plasma (50). Accordingly, it is likely that anticancer doses of enterolactone may be achievable through pharmacologic approach. Regarding the safety issue, nontumorigenic cells were insensitive to the enterolactone treatment in contrast to LNCaP cells. In addition, given the wider distribution, dietary lignans may be a more important source than isoflavone in prostate cancer prevention, particularly for many Westerners who do not habitually consume soy and soy products. Nonetheless, combining various phytoestrogens may enhance overall anticancer properties.

Taken together, the results of the present study provide the first solid evidence that enterolactone suppresses LNCaP cell growth by the induction of apoptosis via a mitochondrial-mediated, caspase-dependent pathway. Enterolactone-induced apoptosis may be mediated by the inhibition of Akt-dependent phosphorylation and promotion of p53 expression. This work, in tandem with previous studies, will enhance our knowledge regarding the mechanism(s) of dietary phytochemicals on prostate carcinogenesis and ultimately expand the scope of adopting alternative approaches in prostate cancer prevention.

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