Apoptotic markers in a prostate cancer cell line: Effect of ellagic acid

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Received June 25, 2013; Accepted August 12, 2013

DOI: 10.3892/or.2013.2757

Abstract. Ellagic acid (EA) inhibits cell growth and induces apoptosis in cultured cells; however, the precise molecular mechanism involved in EA-induced apoptosis in prostate cancer cells is unknown. The aim of the present study was to delineate possible apoptotic pathway(s) involved in the EA-mediated chemotherapeutic effects in the LNCaP human prostatic cancer cell line. EA produced anti-proliferative effects through inhibition of rapamycin (mTOR) activation and a reduction in intracellular levels of β-catenin. Moreover, we demonstrated that EA induced apoptosis via downregulation of the anti-apoptotic proteins, silent information regulator 1 (SIRT1), human antigen R (HuR) and heme oxygenase-1 (HO-1). EA modulated the expression of apoptosis-inducing factor (AIF) resulting in a significant increase in reactive oxygen species (ROS) levels and the activation of caspase-3. Finally, we demonstrated that EA reduced both transforming growth factor-β (TGF-β) and interleukin-6 (IL-6) levels. EA treatment resulted in the increased expression of the tumor suppressor protein p21 and increased the percentage of apoptotic cells. In conclusion, the results suggest that EA treatment represents a new and highly effective strategy in reducing prostate cancer carcinogenesis.

Introduction

Prostate cancer (PC) is the most common cancer in men over the age of 50 years. PC represents one of the leading causes of cancer-related mortality in Western countries (1-3) and is associated with the most rapidly increasing rate of diagnosis in Japanese men (4). Thus, it is essential to clearly delineate the risk factors, diagnosis, treatment options and emerging therapies to better understand and to detect the onset of PC. A diet rich in fruits and vegetables has been reported to reduce the risk of common types of cancer and may prove useful in cancer prevention. Moreover, since less-differentiated tumors become resistant to a wide variety of cytotoxic drugs, considerable attention has been focused on chemoprevention with natural compounds as a new and alternative approach to cancer control. Epidemiological studies have shown the ability of dietary compounds to act epigenetically against cancer cells and to influence an individual's risk of developing cancer (5). Several natural antioxidants, in particular polyphenols, have been reported to exhibit chemotherapeutic activity both *in vivo* and *in vitro* (6-10). EA (2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) is a natural polyphenol found, as both free and bound forms, in numerous fruits and vegetables, particularly in pomegranate (11-15). EA exhibits antioxidant and anticarcinogenic properties including inhibition of tumor formation and growth both *in vitro* and *in vivo* (16-25). Moreover, EA was found to inhibit human prostate cancer cell invasion (23).

Our previous research demonstrated a dose-dependent cytotoxic effect of EA, resulting in a reduction in the proliferation rate and a marked increase in DNA damage in prostatic cancer cell lines (26). Moreover, EA reduced chromogranin A (CgA) levels and increased p75 nerve growth factor receptor (p75NGFR) expression, resulting in the reversion of prostatic cancer cell lines from a proliferating to a differentiated state (26). Therefore, EA with both anti-proliferative and pro-differentiation properties is promising as a cancer therapeutic agent.

Tumorigenesis is a multistep process activated by various environmental carcinogens, inflammatory agents and tumor promoters. These carcinogens modulate transcription factors, anti-apoptotic proteins, pro-apoptotic proteins, protein kinases, cell cycle proteins, cell adhesion molecules and growth factor signaling pathways. EA was found to inhibit cell growth and induce apoptosis in a variety of cell cultures (16,18,23). A multitude of factors modulate apoptosis including growth factors, intracellular mediators of signal transduction, nuclear proteins regulating gene expression, DNA replication and cell cycle regulatory genes (27-31). Moreover, the implications for ROS regulation are highly significant for cancer therapy.

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Key words: ellagic acid, prostate cancer, apoptosis

Commonly used radio-therapeutic and chemotherapeutic drugs influence cancer outcome through ROS modulation.

The present study examined the involvement of apoptotic markers in the cytotoxic effects exerted by EA on the LNCaP human prostatic cancer cell line. In particular, we investigated the anti-carcinogenic properties of EA by evaluating its ability to induce cell cycle arrest and apoptosis. We evaluated mTOR, SIRT1, β-catenin, HUR, AIF, caspase-3, p21, IL-6 and TGF-β. In addition we examined the effects of EA on the cell cycle and showed that EA regulates apoptosis in the LNCaP prostatic cancer cell line.

Materials and methods

Cell culture and treatments. Frozen LNCaP cells were purchased from the American Type Culture Collection (Rockville, MD, USA). After thawing, LNCaP cells were re-suspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (both from Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were plated at a density of 1-5x10^6 cells/T75 flask. Cell cultures were maintained at 37˚C in a 5% CO_2 incubator, and the medium was changed after 3-4 days. Subconfluent cells were treated for 48 h with 2 different concentrations (25 and 50 μM) of freshly prepared EA dissolved in dimethyl sulfoxide (DMSO). Control groups received DMSO alone.

Immunoblot analysis. Cells were cultured in T75 flasks for 48 h. They were then washed with PBS and trypsinized (0.05% trypsin w/v with 0.02% EDTA). The pellets were lysed in buffer (50 mM Tris-HCl, 10 mM EDTA, 1% v/v Triton X-100, 1% PMSF, 0.05 mM pepstatin A and 0.2 mM leupeptin) and after mixing with sample loading buffer (50 mM Tris-HCl, 10% w/v SDS, 10% v/v glycerol, 10% v/v 2-mercaptoethanol and 0.04% bromophenol blue) at a ratio of 4:1, were boiled for 5 min. Samples (20 μg proteins) were loaded onto 8 or 12% SDS-polyacrylamide (SDS-PAGE) gels and subjected to electrophoresis (120 V, 90 min). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After transfer, the blots were incubated with Li-Cor blocking buffer for 1 h, followed by overnight incubation with a 1:1,000 dilution of the primary antibody. Primary polyclonal antibodies directed against AIF, β-catenin, p-mTOR, SIRT-1, caspase-3 and p21 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) while HuR and TGF-β were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). After washing with TBS, the blots were incubated for 1 h with the secondary antibody (1:1,000). Protein detection was carried out using a secondary infrared fluorescent dye-conjugated antibody absorbing at 800 and 700 nm as described below. The blots were visualized using an Odyssey Infrared imaging scanner (LI-COR Biosciences) and quantified by densitometric analysis performed after normalization with β-actin (Santa Cruz). Results are expressed as arbitrary units (A.U.).

Cell cycle analysis. Cells were cultured as previously described, fixed in 70% ethanol overnight at -20˚C and washed with phosphate-buffered saline (PBS). Aliquots of 1x10^6 cells were re-suspended in 1 ml of PBS containing 1 mg/ml of RNase A and 0.5 mg/ml propidium iodide (PI). After a 30-min incubation, the cells were analyzed by flow cytometry using a FACScan flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and evaluated by fluorescence-activated cell sorting (FACS) analysis to identify the cells at different stages of the cell cycle.

IL-6 measurements. IL-6 levels were determined in the culture supernatant using an ELISA kit (AssayGate Inc., Ijamsville, MD, USA). The assays were performed according to the manufacturer's guidelines. Results are expressed as pg/ml.

In-cell western blotting. Cells were seeded in a 96-well cell culture plate. After a 48-h treatment with 25 or 50 μM EA, cells were washed in PBS and directly fixed with 4% of paraformaldehyde (PFA) in PBS for 20 min. Cells were permeabilized with 0.2% Triton X-100, blocked with Li-Cor blocking buffer for 60 min at room temperature, followed by overnight incubation with rabbit HO-1 (1:500) and mouse β-actin primary antibody (1:1,000). β-actin was used as a housekeeping gene to normalize the HO-1 signal for the cell number. After 3 washes, protein detection was carried out using a secondary infrared fluorescent dye-conjugated antibody absorbing at 800 or 700 nm. The whole plate was visualized using an Odyssey Infrared imaging scanner with a 700-nm fluorophore (red dye) and 800-nm fluorophore (green dye). Relative fluorescence units from the scanning allowed a quantitative analysis of the proteins.

ROS measurement. Determination of ROS was performed using a fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described (32). The fluorescence [corresponding to the oxidized radical species 2',7'-dichlorofluorescein (DCF)] was monitored spectrofluorometrically (excitation, λ=488 nm; emission, λ=525 nm). Thus, the intensity of fluorescence was proportional to the levels of oxidant species. The results are reported as fluorescence intensity/mg protein. Protein content was measured according to the method described by Bradford (33).

Statistical analyses. Statistical significance between experimental groups was determined by the Fisher's method of analysis of multiple comparisons ($p<0.05$). For comparisons among treatment groups, the null hypothesis was tested by a 2-factor ANOVA for multiple groups or unpaired t-test for 2 groups. Data are presented as means ± SD.

Results

Effect of EA on β-catenin, p-mTOR, HuR and SIRT1 expression. We assessed the levels of β-catenin, p-mTOR, and SIRT1 after 48 h of culture in the presence of EA. As shown in Fig. 1, EA treatment (25 and 50 μM) resulted in a decrease in both β-catenin and p-mTOR protein expression ($p<0.05$). Similarly, EA exposure showed a significant ($p<0.05$) reduction in the RNA-binding protein HuR and consequently in SIRT1 ($p<0.05$). m-TOR protein expression remained unchanged upon EA treatment (data not shown).

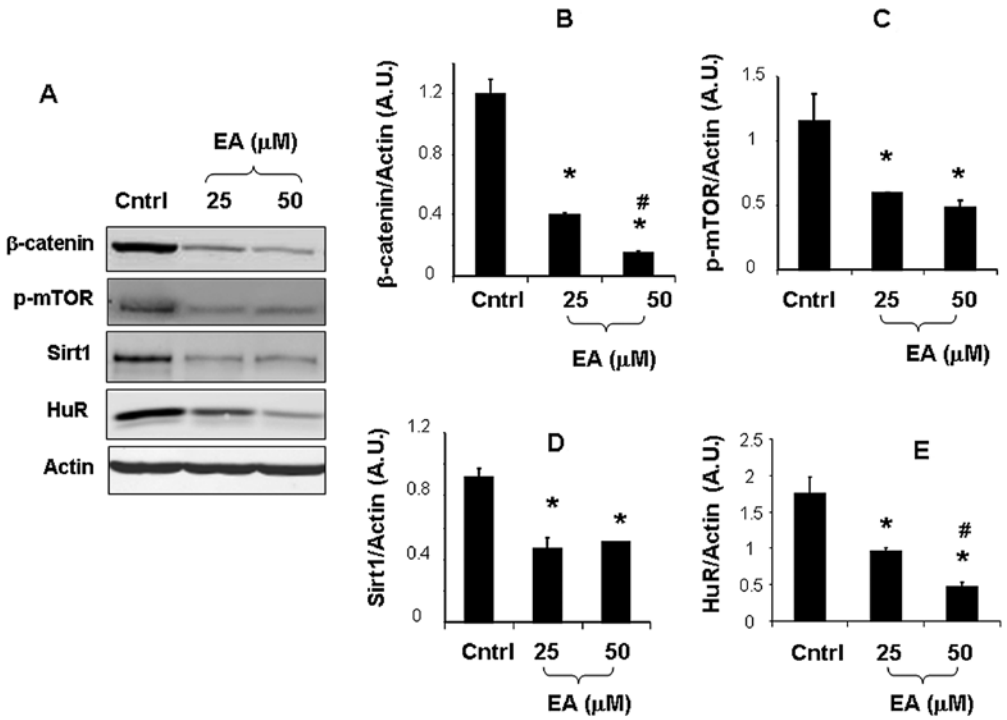

Figure 1. (A) Representative western blotting of β-catenin, p-mTOR, Sirt1 and HuR protein expression in cultured LNCaP cells. (B-E) Effect of EA (25 and 50 μM) on β-catenin, p-mTOR, Sirt1 and HuR expression in cultured LNCaP cells. Results are expressed as arbitrary units (A.U.), and represent the means ± SD of 4 experiments performed in triplicate. *p<0.05, significant result of 25 and 50 μM EA vs. control. #p<0.005, significant result of 50 μM EA vs. 25 μM EA. EA, ellagic acid; Sirt1, silent information regulator 1; HuR, human antigen R; Cntrl, control.

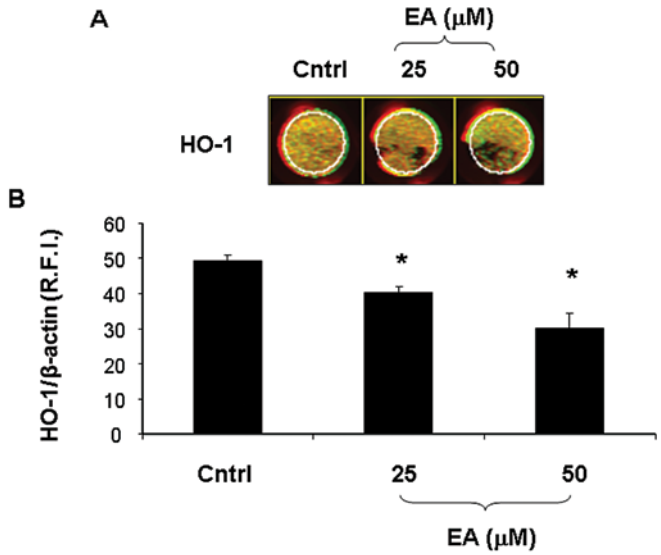

Figure 2. (A) Representative image of HO-1 protein expression in cultured LNCaP cells by in-cell western blotting. (B) Effect of 25 and 50 μM EA on HO-1 expression in cultured LNCaP cells. Results are expressed as relative fluorescence intensity (R.F.I.) and represent the means ± SD of 4 experiments performed in triplicate. *p<0.005, significant result of 25 and 50 μM EA vs. control. EA, ellagic acid; HO-1, heme oxygenase-1; Cntrl, control.

Effect of EA on HO-1 expression. HO-1 protein levels were examined by in-cell western blot analysis, to quantify total endogenous cellular protein (Fig. 2). LNCaP cells treated with EA (25 and 50 μM) showed a marked decrease (p<0.005) in HO-1 fluorescence. HO-1 values were background subtracted from wells treated only with secondary antibody, and then normalized to the cell number by dividing the total actin fluorescence signal.

Effect of EA on AIF, caspase-3, p21 and TGF-β expression. Fig. 3 shows the densitometric analysis of AIF, caspase-3, p21 and TGF-β expression in untreated and EA-treated LNCaP cells. EA treatment induced a significant (p<0.05) decrease in total AIF, caspase-3 and TGF-β expression. In contrast p21 expression was increased (p<0.05) in the EA-treated group when compared to the controls. Immunoblot analysis showed an increase (p<0.05) in cleaved-AIF and cleaved-caspase-3 in cells treated with EA.

ROS levels. As shown in Fig. 4, exposure of LNCaP cells to both 25 and 50 μM EA resulted in a significant increase (p<0.005) in ROS levels when compared to the untreated cells. The effect appeared to be dose dependent and did not reach a plateau at the doses examined.

Effect of EA on IL-6 levels. The effect of EA on IL-6 levels is shown in Fig. 5. EA treatment induced a significant (p<0.05) concentration-dependent decrease IL-6 in levels when compared to the control cells.

Cell cycle analysis. Flow cytometric analysis of the cell cycle distribution is shown in Fig. 6. Our results revealed that EA (50 μM) induced a slight but significant (p<0.005) increase in the percentage of cells in the G_0/G_1 or resting phase, a limited but significant (p<0.005) decrease in the percentage of cells in the S (or synthetic) and in the G_2/M (or mitotic) phases with concomitant increase (p<0.005) in the percentage of apoptotic

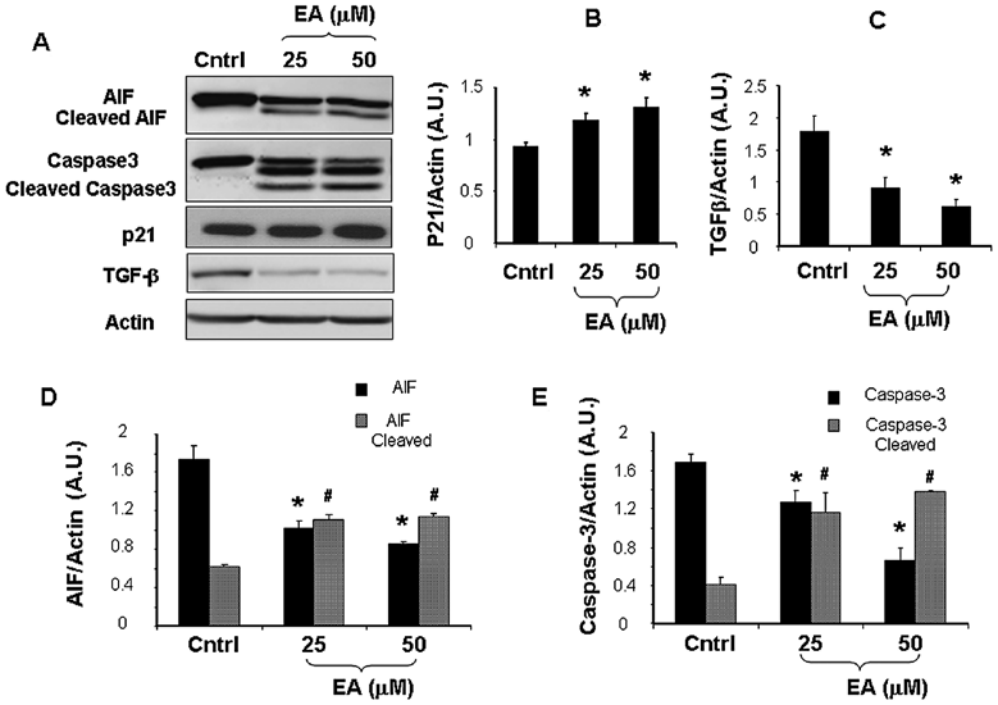

Figure 3. (A) Representative western blotting of AIF, caspase-3, p21, TGF-β protein expression in cultured LNCaP cells. (B-E) Effect of EA (25 and 50 μM) on AIF, caspase-3, p21, TGF-β expression in cultured LNCaP cells. Results are expressed as arbitrary units (A.U.), and represent the means ± SD of 4 experiments performed in triplicate. *p<0.05, significant result of 25 and 50 μM EA vs. (black) control; #p<0.05, significant result of 25 and 50 μM EA vs. (grey) control. EA, ellagic acid; AIF, apoptosis-inducing factor; TGF-β, transforming growth factor-β; Cntrl, control.

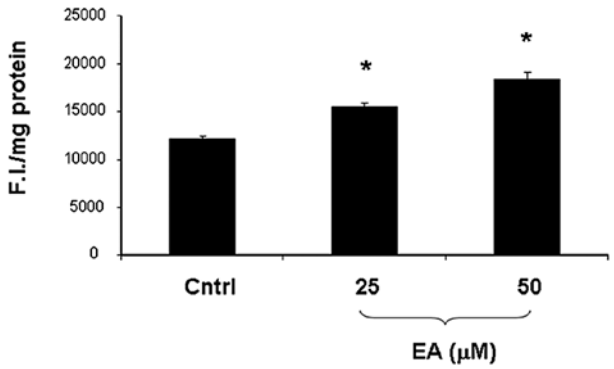

Figure 4. Intracellular oxidants in LNCaP cells untreated and treated for 48 h with EA at different concentrations (25 and 50 μM). Values represent the means ± SD of 4 experiments performed in triplicate. *p<0.005, significant result vs. untreated control cells. EA, ellagic acid; Cntrl, control.

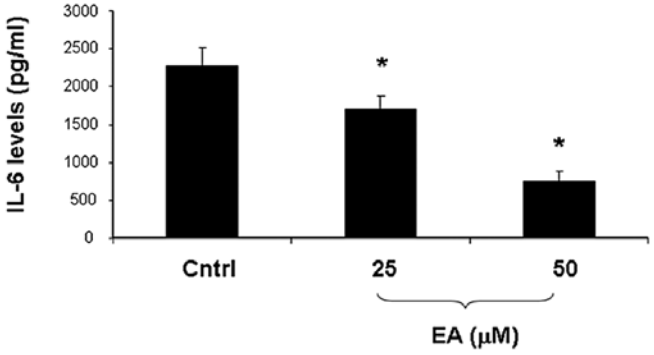

Figure 5. IL-6 levels in LNCaP cells untreated and treated for 48 h with EA at different concentrations (25 and 50 μM). Values represent the means ± SD of 4 experiments performed in triplicate. *p<0.05, significant result vs. untreated control cells. IL-6, interleukin-6; EA, ellagic acid; Cntrl, control.

cells. EA at 25 μM did not induce any modification in cell cycle distribution (data not shown).

Discussion

Prostrate cancer is a chronic disease that develops from a small lesion to clinical manifestation over an extended period of time. However, once the disease is metastatic, patient prognosis is poor. Thus, the development of new strategies to fight PC has become an important therapeutic mission. The administration of both synthetic and naturally occurring agents to suppress, reverse and delay carcinogenesis, is increasingly being touted as an effective approach for the management of prostatic neoplasia (34-37). In recent years, naturally occurring antioxidant compounds present in the human diet have gained considerable attention as cancer-chemopreventive and chemotherapeutic agents (34,36-38). As previously reported (26), naturally occurring polyphenol EA is regarded as a promising new class of cancer therapeutic agents, with both anti-proliferative and pro-differentiating properties.

β-catenin is a subunit of a protein complex acting as a signal transducer, and aberrant accumulation of intracellular β-catenin is a well-recognized characteristic of several types of cancers, including prostate, colon and liver (39-42). The reduction in β-catenin levels, found in the present study, strongly suggests that this represents a potential mechanism implicated in the anti-proliferative effects of EA.

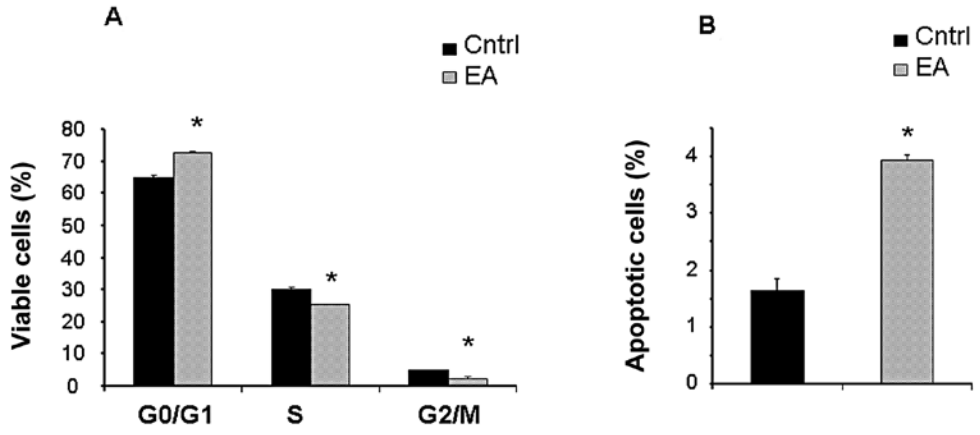

Figure 6. Effect of EA (50 μM) on cell cycle distribution as determined by FACS analysis. (A) Percentage of viable cells. (B) Percentage of apoptotic cells. Values are representative from triplicate experiments ± SD. *p<0.005, significant result vs. untreated control cells. EA, ellagic acid; FACS, fluorescence-activated cell sorting; Cntrl, control.

We showed that EA treatment exerts anti-proliferative effects by reducing intracellular levels of β-catenin. We previously demonstrated that EA reduced Akt activation/phosphorylation in prostate cancer cell lines. The capacity of p-Akt to phosphorylate/activate mTOR has been described in several cancer cell lines (43-45). Dysregulation of the mTOR pathway occurs in many types of cancers including prostate (43). The inhibition of mTOR activation is considered as a potential target for the development of anticancer therapeutics (43-49). In view of these observations, the significant reduction in mTOR activation, observed in the present study, indicates that EA exerts an anti-proliferative effect by reducing PI3K/Akt downstream signaling through inhibition of mTOR phosphorylation. In addition, acetylation of Akt blocks Akt binding to PIP_3, thereby preventing membrane localization and phosphorylation of Akt. Deacetylation by SIRT1 enhances Akt binding to PIP_3 and promotes activation/phoshorylation (50).

SIRT1 functions as an oncogenic protein and plays a role in tumorigenesis (51). SIRT1 is overexpressed in human PC cells (DU145, LNCaP, 22Rv1 and PC3) when compared to normal prostate epithelial cells (PrEC), and inhibition of SIRT1 results in anti-proliferative effects in human PC cells. SIRT1 was found to be overexpressed in human PC tissues when compared with adjacent normal prostate tissue (52). A link between SIRT1 and HuR levels and the anti-apoptotic influence of these 2 proteins (53) has been reported. The RNA-binding protein HuR regulates the stability of many target mRNAs, including SIRT1 mRNA. The reduction in SIRT1 and HuR, observed in the present study following EA treatment, suggests that EA may induce apoptosis via the reduction of the anti-apoptotic proteins SIRT1 and HuR. Moreover, HO-1 translation was found to be diminished in HuR-depleted cells (54). An increase in HO-1 protein levels is associated with a parallel increase in EC-SOD, eNOS (55), increased activation of Akt and an increase in mitochondrial function. HuR promotes HO-1 expression through mRNA stabilization and translational upregulation. Our results confirmed that decreased levels of HuR, after EA treatment, were associated with a reduction in HO-1 protein expression suggesting that HO-1 is a focal target in cancer treatment. Thus, the anti-proliferative effect of EA includes the inactivation of PI3K/Akt signaling cascade, i.e. the inhibition of Akt, mTOR and SIRT1, a decrease in HO-1 levels and, consequently, the activation of the mitochondrial-mediated apoptotic pathway.

AIF is a flavoprotein anchored to the mitochondrial inner membrane. Under physiological conditions, AIF exhibits NADH oxidase activity, important for mitochondrial respiration (56). A previous study with mice characterized by reduced AIF expression, the 'Harlequin' (Hq) mice, suggested that AIF may also protect against oxidative stress (57). Although mislocalization of the AIF protein leads to nuclear condensation and apoptosis, *in vivo* models of AIF loss result in oxidative stress and neuronal degeneration (57). Upon a specific death signal, AIF is cleaved at the N-terminus releasing the protein from its membrane anchor (58). AIF translocation from the cytosol to the nucleus results in chromatin condensation and large-scale DNA fragmentation, and apoptotic cell death (59) in a caspase-independent manner (60). We noted a decrease in AIF expression and an increase in cleaved AIF upon exposure of the LNCaP cells to EA. The observation that EA modulates AIF expression has at least 2 important conceptual implications. Firstly, EA has a pro-oxidant potential; secondly, a direct link exists between EA and the induction of cleaved AIF followed by activation of the apoptotic pathways. In addition, cancer chemopreventive agents induce apoptosis through ROS generation and disruption of redox homeostasis leading to the activation of the caspase cascade (61,62). In agreement with previous findings (63), our results showed that EA increased ROS levels and activation of caspases, as demonstrated by a decrease in caspase-3 with a concomitant increase of its cleaved form. Therefore, EA-induced ROS production and activation of caspase-3 may be due to AIF cleavage.

IL-6 is a multifunctional cytokine and a major activator of different signaling pathways. It regulates growth of prostate cancer (64) and inhibits apoptosis in several prostate cancer cell lines (65). Increased IL-6 levels, found in prostate cancer, may be due to enhanced expression of TGF-β (66), which regulates cell proliferation, phenotype and matrix synthesis. In cancer cells, TGF-β acts on the surrounding stromal, immune, endothelial and smooth-muscle cells, causing

immunosuppression and angiogenesis, resulting in a more invasive form of cancer (67). We demonstrated in the present study that EA reduces both TGF-β and IL-6 levels. Overall, EA induces both caspase-independent and caspase-dependent apoptotic cell death. EA caused a significant increase in p21 expression, a negative regulator of the cell cycle. In addition, FACS analysis revealed that EA treatment induced a different distribution of cells in the various phases of the cell cycle, with a concomitant increase in apoptotic cells. Moreover, in addition to pro-differentiating properties, the cytotoxic effects of EA may be due to its ability to modulate the expression of multiple apoptotic markers. In conclusion, the present study strongly supports the hypothesis that EA regulates apoptosis through activation/inactivation of several proteins involved in tumor growth and cell invasiveness. EA may represent a new approach and a highly effective strategy for reducing the occurrence of prostate cancer.

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