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ORIGINAL ARTICLE

γ-Tocopherol inhibits human prostate cancer cell proliferation by up-regulation of transglutaminase 2 and down-regulation of cyclins

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Abstract To establish a system to study differentiation therapy drugs, we used the androgen-independent human prostate PC-3 tumor cell line as a target and α- and γ-tocopherol as inducers. Effects of α- and γ-tocopherol on the cell cycle, proliferation and differentiation, were examined. A more significant growth inhibition activity for γ- than for α-tocopherol was observed. Flow cytometry analysis of α- and γ-tocopherol-treated prostate carcinoma PC3 cells showed decreased progression into the S-phase. This effect, particularly evident for γ-tocopherol, was associated with an up-regulation and increased activity of transglutaminase 2 (TG2), a reduced DNA synthesis and a remarkable decreased levels of cyclin D1 and cyclin E. Activation of TG2 suggests that γ-tocopherol has an evident differentiative capacity on PC3 cells, leading to an increased expression of TG2, and reduced cyclin D1 and cyclin E levels, affecting cell cycle progression. It is feasible that up-regulation and activation of TG2, associated with a reduced proliferation, are parts of a large-scale reprogramming that can attenuate the malignant phenotype of PC3 cells in vitro. These data suggest further investigation on the potential use of this γ-form of vitamin E as a differentiative agent, in combination with the common cytotoxic treatments for prostate cancer therapy.

Keywords Human prostate cancer · Transglutaminase · Cyclins · Vitamin E

This work is dedicated to Alberto Abbruzzese, who died in 2011.

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Introduction

Prostate cancer originates as an androgen-dependent hyperproliferation of the epithelial cells of the gland and it evolves in an androgen-independent, highly aggressive cancer for which no successful therapy is available to date. Neuroendocrine differentiation plays an important role in the progression of prostate cancer to an androgen-independent state with profound impact on prostate cancer therapies. A potential innovative cancer treatment that may eliminate some drawbacks of the current prostate cancer chemotherapy is differentiation therapy (Leszczyniecka et al. 2001). In this treatment approach, the desired drug causes the malignant cells to undergo terminal differentiation instead of killing the tumor cells. The concept behind this therapy is based on the observations that cells of most tumors, including prostate cancers, are blocked at an early stage of cellular differentiation and that certain agents can bypass or correct this block in vitro (Torricelli et al. 2011). Based on these studies, a number of drugs have been identified, tested, and found to show promise in the treatment of human myeloid leukemia (James et al. 1999; Sachs 1978). An obstacle in identifying drugs for prostate cancer differentiation therapy is the absence of an appropriate in vitro cell maturation system and useful markers that truly define the normal mature prostate cell. A number of attempts have been made to develop such a system (Ellerhorst et al. 1999; Maier et al. 2000). These studies,

however, used apoptosis indicators and/or a limited number of neuroendocrine markers rather than an array of indicators that define the particular function of normal prostate cells. Several lines of evidence show a key role of TGs in the antineoplastic activity of some chemical agents, through the induction of terminal cell differentiation followed by apoptosis (Lentini et al. 2004). The apoptotic functions of TG2 are linked to the ability of this enzyme to irreversibly cross-link proteins in the presence of Ca^{2+} (Folk and Chung 1985). It is likely that differentiating agents are able to induce a stressful condition, followed by a massive release of Ca^{2+} from intracellular stores or influx of Ca^{2+} from outside the cell, leading to activation of TG2 resulting in post-translational modification of key proteins and onset of apoptosis (Lentini et al. 2009). It has been reported that vitamin E reduces the growth of prostate carcinoma cells in vitro and cancer disease progression in vivo (Conte et al. 2004) and this protective action has been attributed to activation of TG2 (Torricelli et al. 2011).

There are different forms of vitamin E, four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ). The α-tocopherol is the only form that is maintained in human plasma and is the more abundant form found in nature and in human tissues (Brigelius-Flohé and Galli 2010). After passing to the liver, only α-tocopherol appears in the plasma due to its specific selection by the hepatic α-tocopherol transfer protein (α-TTP). Non-α-tocopherols are poorly recognized by α-TTP. The α-TTP and plasma phospholipid transfer protein have a well-characterized importance in cytosol, because they are responsible for the homeostasis of vitamin E levels in the body (Lee et al. 2005). Studies show that γ-tocopherol is absorbed from the intestine almost as efficiently as α-tocopherol. Tissues took up the two tocopherols at almost the same initial rate, but γ-tocopherol eventually disappeared faster. When rats were fed corn or soybean oils in the diet for several months, tissue γ-tocopherol equaled α-tocopherol in some tissues. In conclusion, valuation of the vitamin E activity of γ-tocopherol in animal fed vegetable oil showed it to be nearly 10 % as active as α-tocopherol (Bieri and Evarts 1974).

In the last two decades, many studies demonstrated the potential anticancer effects of vitamin E in prostate cancer, alone or combined with other nutrients. It was believed that vitamin E, alone or combined with selenium, has protective effects against prostate cancer due to both antioxidant and anticancer properties that inhibit specific cellular processes in the development of this cancer. A recent clinical trial concluded that selenium, vitamin E, or selenium plus vitamin E did not prevent prostate cancer in the generally healthy, heterogeneous population of men (Lippman et al. 2009).

Since little is known of the activity of tocopherols in the prevention of prostate cancer cell proliferation and metastasis, the present study was undertaken to investigate the effects of α-, and γ-tocopherol on prostate cancer cell proliferation, collecting evidence on the mechanism of action of these two forms of vitamin E.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer saline (PBS), glutamine, penicillin (10,000 UI/ml), and streptomycin (10,000 μg/ml) were from Eurobio Laboratoires (Le Ulis Cedex, France). Fetal calf serum (FCS) was from Gibco (Grand Island, NY, USA). [^{14}C]-Methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). All solvents came from Mallinckrodt Baker (Milan, Italy). Sodium citrate, phenylmethylsulfonylfluoride (PMSF), Tris, ethylenediaminotetraacetic acid (EDTA), trichloroacetic acid (TCA) and all reagents were from Sigma Chemicals (St. Louis, MO, USA). Human prostate carcinoma cells (PC3) were purchased from ATCC (Manassas, VA). Specific antibodies for cyclin D1 (A-12), cyclin E (C-19), and TG2 (G-8) were purchased from Santa Cruz Biotechnology, Inc (CA, USA). An anti-β-actin (C4) antibody was obtained from Abcam Ltd. (UK). All reagents, (+)-α-tocopherol, and (+)-γ-tocopherol were from Sigma (St. Louis, MO, USA), and horse radish peroxidase from Pierce (Rockford, IL, USA). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Italy). Dead Cell Apoptosis Kit with Annexin V PE and SYTOX® Green for flow cytometry were from Invitrogen S.R.L (Milano, Italy).

Cell culture

PC3 cells were cultured at 37 °C, in DMEM with 5 % FCS and 5 % CO_2. Before each treatment, the cell line was starved for 24 h to rule out possible interferences with cell growth due to serum components and promote its synchronization. Trypan blue was used for cell viability detection. Lactate dehydrogenase (LDH) release was determined for plasma membrane integrity. PC3 cells subcultured into nine flasks were grown for 12 h and divided into three batches of three flasks each: (1) 25–50 μM α-tocopherol; (2) 25–50 μM γ-tocopherol; and (3) 0.05 % ethanol-treated samples. Samples were incubated for 48 and 72 h with tocopherols in ethanol (0.05 %). The concentration of tocopherols in samples were tested every 24 h and maintained as the original. LDH assay was performed detecting the absorbance at 340 nm. Dead Cell Apoptosis Kit with Annexin V PE and SYTOX® Green was used because this product detects the externalization of

phosphatidylserine in apoptotic cells using recombinant annexin V conjugated to the orange fluorescent phycobiliprotein R-PE, and dead cells using SYTOX® Green nucleic acid stain. After treatment with both probes, apoptotic cells show orange fluorescence, dead cells show green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished in the 530/30 and 585/42-nm bandpass filters with a 488-nm laser flow cytometer.

Cell proliferation assay

PC3 cells were incubated and treated as reported above. Following the treatments, cells were washed with PBS, trypsinized, collected in PBS, and counted. Control cells were incubated with 0.05 % ethanol final concentration. Cells were harvested and counted in duplicate with a Neubauer modified chamber, after trypan blue staining for cytotoxicity evaluation.

DNA synthesis detection

A total of 3×10^6 PC3 cells were treated with tocopherols or ethanol, at the concentration noted above, for 72 h in medium with 5 % FCS. The cells were then pulsed with 20 μm BrdU (Sigma, St. Louis, MO) for 1 h at 37 °C in 10 % FCS. The cells were then fixed with cold 95 % ethanol and washed with PBS. Extinction was detected with a microplate reader at 405 nm with a reference at 490 nm.

Transglutaminase 2 activity assay

Transglutaminase 2 assay was performed according to a previously reported method (Gismondi et al. 2010), by treating PC3 cells with tocopherols (20–50 μM) or ethanol (0.05 %), in the presence of [^{14}C]-methylamine (46.6 Ci/mmol, 0.5 μl/ml DMEM). Then, cells were harvested, counted, and washed twice in PBS. Cell proteins were precipitated in 10 % TCA, washed extensively, solubilized in 0.1 N NaOH at 37 °C, and measured for radioactivity with a scintillation counter (TRICARB 2100, PACKARD, efficiency 70–90 %).

Cell cycle analysis

PC3 cells were grown and treated as described above. Tocopherols- or ethanol-treated cells were collected in PBS, centrifuged (10 min 500×*g* at 4 °C), and washed twice with PBS. Pellets were resuspended in 500 μl of PBS, then fixed with cold 95 % ethanol and stored at 4 °C. Pellets were centrifuged (30 min, 12,000×*g* at 4 °C) and washed with PBS; 500 μl staining solution (PBS 1 ml, propidium iodide 50 ng, RNase A 10 ng) was added to the resuspended cells. The mixture was kept in the dark for 20 min at room temperature and stored on ice. Flow cytometry was executed by a FACS canflow cytometer (Becton Dickinson, Oxford, Oxfordshire, UK).

Western-blot analysis of TG2 and cyclins D1, E

Dissociated PC-3 cells were washed three times with cold PBS and lysed in ice-cold radioimmunoprecipitation assay buffer [50 mmol/l Tris (pH 7.4), 1 % Nonidet P-40, 0.25 % sodium deoxycholate, and 150 mmol/l NaCl] containing a mixture of protease inhibitors (Invitrogen) at 4 °C for 10 min. Cell lysates were centrifuged at 13,000×*g* for 10 min at 4 °C. Protein concentrations were measured with a Bio-Rad Protein Assay Kit, based on the method of Bradford (Bradford 1976). For electrophoresis, solubilized proteins (100 μg per well) were separated on a 12 % SDS-polyacrylamide gel and transferred onto a Hybond-C extra membrane (Amersham, Piscataway, NJ). The membrane was blocked with freshly prepared 5 % dry milk in PBS 0.05 % Tween (milk/PBS/Tween) for 10 min at room temperature and then incubated overnight at 4 °C with a primary antibody against TG2, cyclin D1, and cyclin E. The membrane was washed three times with milk/PBS/Tween at room temperature, and incubated with goat antimouse secondary antibody labeled with horseradish peroxidase (1:5,000) for 60 min at room temperature. Chemiluminescence was obtained by enhanced chemiluminescence detection kit (Amersham Biosciences). Images were analyzed by a trial version of MCID analysis software (Imaging Research, St. Catharines, Ontario, Canada).

Statistical analysis

All data represented at least four independent experiments and were expressed as mean ± SD, unless otherwise indicated. Statistical comparisons were made using two-tail student's *t* test. *P* values of 0.05 or less were considered to represent a statistically significant difference.

Results

PC3 cancer cell proliferation inhibition by α- and γ-tocopherol

PC3 cancer cell proliferation was inhibited by α- and γ-tocopherol (Fig. 1). However, γ-tocopherol showed a stronger growth inhibition with respect α-tocopherol. PC3 cells proliferation was reduced by about 24.5 and 50 %, when cells were treated for 72 h, with 25 μM α-tocopherol and γ-tocopherol, respectively. Raising the concentration of the two tocopherols (50 μM), PC3 cell proliferation was

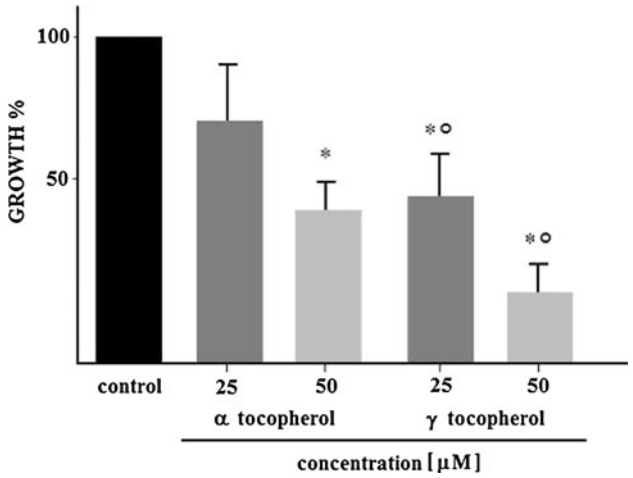

Fig. 1 Cell proliferation rate of α- or γ-tocopherol-treated PC3 cells. PC-3 cells were treated for 72 h with α- and γ-tocopherols (25 or 50 μM) or ethanol (0.05 %). Values are mean ± SD of four independent experiments. *$P < 0.05$, as compared to control. *Open circles* $P < 0.05$, as compared to α- and γ-tocopherol at the respective concentrations

further decreased (α-tocopherol 53.0 % and γ-tocopherol 78.7 %). Negligible LDH release or trypan blue uptake was observed among control and treated PC3 cells suggesting that the decrease in cell number induced by α- or γ-tocopherol was not the result of apoptosis or necrosis. These data were confirmed by analysis following staining with SYTOX Green (data not shown).

PC3 cell cycle progression and DNA synthesis inhibition by α- and γ-tocopherol

The effect of α- and γ-tocopherol on PC3 cell cycle and DNA synthesis was investigated. α- and γ-tocopherols (50 μM)-treated cells presented after 72 h of treatment an increased number of cells on G1-phase of the cell cycle with a lower S-phase population with respect to the untreated PC3 cells.

Table 1 shows the cell cycle distribution, with an evident S-phase population, lower in the α- and γ-tocopherols 72 h-treated PC3 cells (28.0 ± 5.0 and 15.0 ± 2.7 % respectively) with respect to the control cells (45 ± 3.0 %). The number of cells in the G1-phase was lower in control cells (40.0 ± 5.0 %) than in α- and γ-tocopherols-treated cells (60.0 ± 4.0 and 72.0 ± 5.0 % respectively). Apoptotic cells were present in a low number. BrdU incorporation assay on PC-3 cells treated 72 h with tocopherols revealed a decreased activity of DNA synthesis (Fig. 2). Indeed, in agreement with the G1–S transition delay, α-tocopherol (25 μM) inhibited DNA synthesis by 14.9 ± 7 %, and γ-tocopherol (25 μM) inhibited it by 48.9 ± 8 % ($P < 0.05$). The activity of DNA synthesis was inhibited by 42.5 ± 10 % following α-tocopherol treatment (50 μM) and 74.4 ± 9 % after γ-tocopherol (50 μM) treatment ($P < 0.05$). In conclusion, the inhibition effect of γ-tocopherol on DNA synthesis and cell proliferation was significantly higher than that of α-tocopherol.

TG2 up-regulation and activity in α- and γ-tocopherol-treated PC3 cells

Since TGs are commonly considered differentiation markers, a correlation between α- and γ-tocopherol antiproliferative activity and stimulation of differentiation in PC3 cell line was studied. Treatment with γ-tocopherol, 50 μM, significantly induced, compared to the control, an enhancement in TG2 activity of about threefold after 48 h and of fourfold after 72 h of treatment (Table 2). α-Tocopherol treatment (50 μM) showed an increase of TG2 activity of about twofold only after 72 h of incubation. The enhanced differentiation induced by α- and γ-tocopherol treatments was paralleled by an increased expression of the enzymatic protein as shown in Fig. 3a. The expression of TG2 protein was increased fourfold by 48 h and of sixfold by 72 h of treatment of PC3 cells with 50 μM γ-tocopherol (Fig. 3b).

Cyclin E and cyclin D1 levels of α- and γ-tocopherol-treated PC3 cells

Cyclin E and cyclin D1 of 50 μM γ-tocopherol-treated PC-3 cells were significantly down-regulated during 48 and 72 h of incubation with respect to control cells (Fig. 4a). On the contrary, a slight inhibition was observed for α-tocopherol-treated PC-3. In PC-3 cells incubated with 50 μM α-tocopherol, expression of cyclin E (Fig. 4b), was inhibited after 48 h of treatment by 14.3 %, and after 72 h by 30.2 %. Incubating PC-3 cell with 50 μM γ-tocopherol, the amount of cyclin E was decreased after 48 h of treatment by 56.1 % and after 72 h by 68.3 % ($P < 0.05$). Cyclin D1 protein expression, in PC-3 incubated with 50 μM α-tocopherol, was reduced by about 30.8 % only after 72 h of treatment. γ-Tocopherol (50 μM) decreased cyclin D1 protein expression by about 70 % in both the times of incubation (Fig. 3c).

Discussion

γ-Tocopherol possesses a higher inhibitory activity with respect to α-tocopherol during the neoplastic transformation of C3H/10T1/2 embryonic fibroblasts (Cooney et al. 1993). Accordingly, our results clearly indicate a more potent growth inhibition effect of γ- than α-tocopherol in PC3 prostate cell line. A possible explanation is that some components of the proliferation pathways of tumors may

Table 1 Cell cycle distribution of α- and γ-tocopherols-treated PC-3 cells

	G_0/G_1 (%)	S (%)	G_2/mitosis
Control	40.0 ± 5.0	45.0 ± 3.0	15.0 ± 4.0
α-Tocopherol	60.0 ± 4.0	28.0 ± 5.0	12.0 ± 3.5
γ-Tocopherol	72.0 ± 5.0*	15.0 ± 2.7*	13.0 ± 2.9

Cell cycle distribution of a representative experiment of ethanol (0.05 %) or α/γ-tocopherols (50 μM) in 72 h-treated PC-3 cells. Values are mean ± SD of four independent experiments

* $P < 0.05$, as compared to control

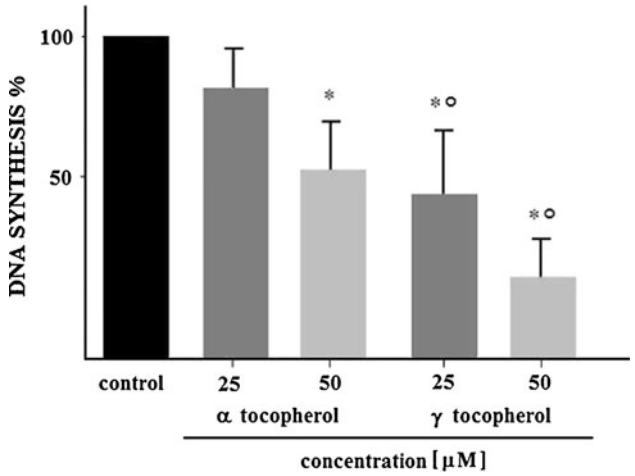

Fig. 2 DNA synthesis inhibition by α- and γ-tocopherol. PC-3 cells were treated for 72 h with α- and γ-tocopherols (25 or 50 μM) or ethanol (0.05 %). Values are mean ± SD of four independent experiments. *$P < 0.05$, as compared to control. *Open circles* $P < 0.05$, as compared to α- and γ-tocopherol at the respective concentrations

Table 2 TG2 activity in α- and γ-tocopherols-treated PC-3 cells

Incubation time (h)		48	72
Treatment	μM		
Control	–	5.7 ± 2	6.5 ± 1
α-Tocopherol	25	7.2 ± 3	8.2 ± 2
	50	9.5 ± 3	15.3 ± 4*
γ-Tocopherol	25	10.2 ± 2	12.5 ± 4
	50	18.3 ± 5*	27.0 ± 3*

TG activity is expressed as pmoles of [^{14}C]methylamine incorporated per mg of PC-3 total proteins. Values are mean ± SD of four independent experiments

* $P <$ 0.05, as compared to control

be more sensitive to γ-tocopherol than to other tocopherols. It is also interesting to consider that some tocopherol metabolites, like the γ-homologs (γ-CEHC), are more active than their vitamin precursors to inhibit PC-3 growth by specific down-regulation of cyclins' expression (Galli et al. 2004). Our findings that γ-tocopherol inhibition of proliferation of PC-3 prostate cancer cells is paralleled by a remarkable up-regulation of TG2 and by an increased activity of the enzyme provide a cellular mechanism supporting the concept brought forth from recent studies: that an overexpression of TG2 may be related to risk reduction for cancer, only when both TG2 protein and its activity are increased (Beninati et al. 2009). Cyclin D1 overexpression is associated with tumorigenesis, and cyclin D1 amplification and/or overexpression has been demonstrated in a variety of human tumors, including mantle cell lymphomas, breast carcinomas, head and neck squamous cell carcinomas and esophageal cancers (Reis-Filho et al. 2006). Since we observed delay in G1-phase of α- and γ-tocopherol-treated PC3 cells, we analyzed the effect of the two tocopherols on the regulation of the G1-phase cyclins E and D1. Western-blot analysis showed that γ-tocopherol treatments led to a higher decrease of cyclin D1 levels. As the cyclin E promoter contains multiple E2F sites, and disruption of pRB function was shown to up-regulate cyclin E levels (Dyson 1998), we investigated whether the down-regulation of cyclin D1 in PC3 was connected with a down-regulation of cyclin E levels. Like cyclin D1 protein levels, cyclin E decreased in γ-tocopherol-treated cells more than in α-tocopherol-treated cell. The two cyclins were decreased differently in time during 50 μM γ-tochopherol treatments. Cyclin E protein levels were decreased twofold at 48 h and threefold after 72 h of treatment. On the contrary, cyclin D1 showed a decrease of threefold already at 48 h and similarly after 72 h of γ-tochopherol treatment. In conclusion, our data suggest that γ-tocopherol is more potent than α-tocopherol in affecting proliferation of the androgen-independent human prostate PC-3 tumor cell line through the inhibition of cell cycle progression, via reduction of cyclin E and cyclin D1 levels. Since cyclins have a regulatory role for the G1–S transition, these data may explain the higher reduction of proliferation observed by γ-tochopherol treatments.

The reported evidence for γ-tocopherol-treated PC3 cells, like reduction of both cancer cell growth and levels of DNA synthesis, together with G1–S transition delay, with lower levels for proteins critical in G1–S transition, resulted in an impairment of cell proliferation without apparent apoptosis or necrosis. It was previously suggested that γ-tocopherol inhibits cell cycle progression

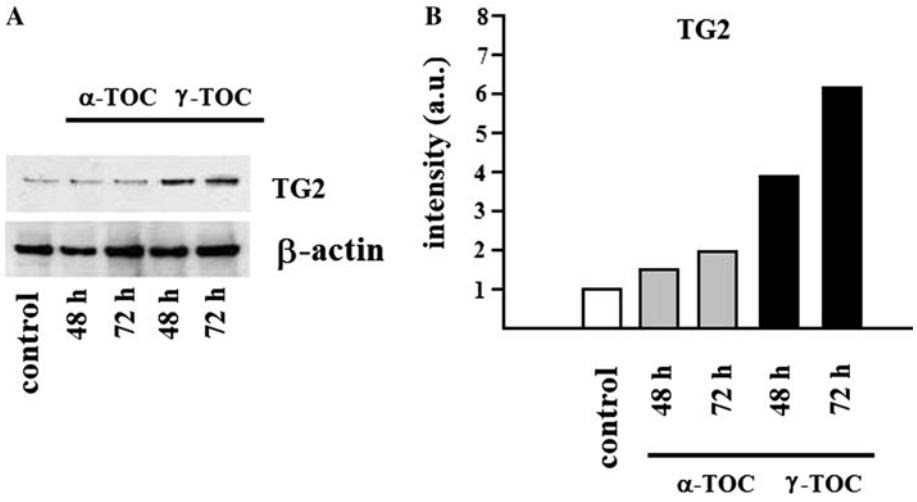

Fig. 3 TG2 up-regulation and activity in α- and γ-tocopherol-treated PC3 cells. **a** Induced expression of TG2 proteins in PC-3 cells treated for 48 and 72 h with 50 μM α- (α-TOC) or γ- (γ-TOC) tocopherols. **b** MCID software was used to analyze the intensities of the bands in the blots and the relative increase in expression was calculated in arbitrary units (*a.u.*). Values are *β*-actin corrected

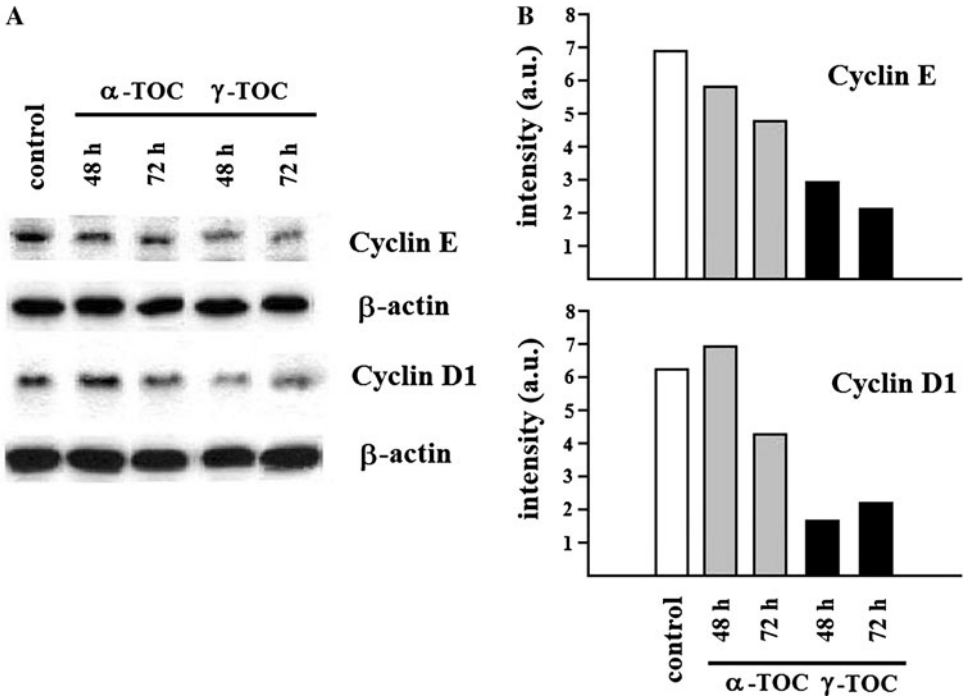

Fig. 4 Cyclin E and cyclin D1 levels in α- and γ-tocopherol-treated PC3 cells. **a** Decreased expression of cyclin E and cyclin D1 proteins in PC-3 cells treated for 48 and 72 h with 50 μM α- (α-TOC) or γ- (γ-TOC) tocopherols. **b** Cyclin E: intensities of the bands of blots from α- and γ-tocopherol-treated PC3 cells. **c** Cyclin D1: intensities of the bands of blots from α- and γ-tocopherol-treated PC3 cells. MCID software was used to analyze the intensities of the bands in the blots and the relative increase in expression was calculated in arbitrary units (*a.u.*). Values are *β*-actin corrected

via reduction of cyclin D1 and cyclin E levels, with a non-antioxidant mechanism (Gysin et al. 2002; Galli et al. 2004). Our data support previous observation that tocopherols merit a concern beyond their mere antioxidant properties.

We have shown that up-regulation and activation of TG2 induces down-regulation of cyclin E and cyclin D1 with G1–S transition delay in human PC3 prostate cell line. In conclusion, we suggest that the vitamin E γ-analog may be active in triggering the cell differentiation program, which may represent a potential approach to the treatment of prostate cancer.

Conflict of interest Authors declare that they have no conflict of interest.

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