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ScienceDirect
Journal of Nutritional Biochemistry 19 (2008) 263–268
Journal of Nutritional Biochemistry

The vitamin E analog tocopherol succinate strongly inhibits gap junctional intercellular communication in rat liver epithelial cells (IAR203)

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Received 6 December 2006; received in revised form 1 February 2007; accepted 27 March 2007

Abstract

Vitamin E is a scavenger molecule trapping free radicals in biological membranes. However, it has also been shown to elicit the formation of reactive oxygen species and apoptosis in cancer cells. In this study, we tested the ability of α-tocopherol, tocopherol acetate, tocopherol phosphate and tocopherol succinate (TS) to modulate gap junctional intercellular communication in the rat liver epithelial cell line IAR203, as measured by the transfer of Lucifer yellow. While α-tocopherol, tocopherol acetate and tocopherol phosphate moderately reduced the dye transfer, TS at 10 and 25 μM strongly inhibited it, probably via the induction of the hypophosphorylation of connexin 43. Our results show that, besides their interesting antioxidant properties, vitamin E analogs, especially TS, can exert adverse effects on gap junctional intercellular communication, which could explain their controversial effects in carcinogenesis.

Keywords: Vitamin E; Tocopherol succinate; Gap junction; Connexin 43; Liver cells

1. Introduction

Presently, there is major interest in the possibility that free radical-mediated events in biochemical processes may lead to a number of disorders, such as cardiovascular diseases and cancer. Therefore, the control of free radical generation is of importance in considering the prevention of these diseases. Nutritional factors play an important role in this control; among the nutrients to be considered is vitamin E [1]. Vitamin E is the generic term for a group of lipid-soluble derivatives, including four tocopherols (α-, β-, γ-, δ-) and four tocotrienols (α-, β-, γ-, δ-) [2]. The form α-tocopherol is the most abundant form in nature and possesses the most powerful antioxidant property (for a review, see Reference [3]). Vitamin E esters are used in pharmacological formulations and dietary supplements because they are much more resistant to oxidation as compared with the unesterified form. Vitamin E is a scavenger molecule trapping free radicals in biological membranes. The particular function of vitamin E is to protect biological membranes from oxidative damage [4]. However, an effect opposite of the antioxidative effect of vitamin E, especially of tocopherol succinate (TS), is to elicit the formation of reactive oxygen species and apoptosis in cancer cells [5]. Until now, clinical studies have shown contradictory results regarding the benefits of vitamin E in the prevention of cardiovascular disease and cancer [6–8]. Thus far, many studies focused on TS, which seems to be a promising molecule for cancer treatment [9,10].

Gap junctions are membrane channels contributing to intercellular communication in mammalian tissues [11,12]. They allow the transfer of ions and other cytoplasmic molecules (molecular weight <1000 Da, small metabolites and second messengers) between adjacent cells [13]. They are protein structures composed of six subunits called connexins (Cxs). The functionality of gap junctions can be regulated at different levels, such as transcription, translation and Cx posttranslational processing, as well as assembly, gating, internalization and degradation [14]. Gap junctional intercellular communication (GJIC) is believed to be important in controlling cellular homeostasis, normal embryonic development [15,16], differentiation and regulation of cell proliferation [17,18]. Inhibition of GJIC has been postulated to contribute to teratogenesis [19] and carcinogenesis [20]. Moreover, it has been shown that transformed

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0955-2863/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.jnutbio.2007.03.005

and tumoral cell lines display reduced communication capacity [21,22]. These observations suggest that GJIC disorders may play an important role in tumor development [23]. GJIC can be modulated by endogenous and exogenous agents. Most tumor promoters inhibit GJIC [24,25]; conversely, molecules having antipromoter activities can enhance GJIC [26,27].

To evaluate the impact of vitamin E analogs on GJIC, we chose to test the naturally occurring form α-tocopherol (αT) with or without ascorbic acid (AA) and three commercially available forms, TS, tocopherol acetate (TA) and tocopherol phosphate (TP), on well-communicating cells (IAR203). To follow-up GJIC, we used the fluorescent dye transfer assay. As TS displayed the strongest capacity to inhibit GJIC, we analyzed its impact on the phosphorylation state and the immunolocalization of the Cx 43 protein, the main Cx expressed in the IAR203 cell system.

2. Materials and methods

2.1. Chemicals

αT, TS, TA, TP, AA, Lucifer yellow CH (LY) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Quentin Fallavier, France). αT, TS, TA and TP were dissolved in DMSO and stored at −20°C. AA was incorporated directly in the medium at the same concentration as was αT (10 or 25 μM). Both test and control solutions were dissolved into the culture medium at the same DMSO concentration (0.1%). Lucifer yellow CH was prepared as a 5% (w/v) solution in 0.33 M of lithium chloride. Medium components were purchased from Invitrogen (Cergy Pontoise, France).

2.2. Cell culture

IAR203 cells were cloned from a rat liver epithelial cell line named IAR20 (obtained from IARC, Lyon, France). This cell line was maintained in F10 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 μg/ml) and gentamycin (10 μg/mL). The cells were cultured at 37°C in a humidified incubator in a 5% CO_2 atmosphere.

The basal F10 medium, used for the IAR203 cell culture, did not contain any form of vitamin E. For cell density measurement, dye transfer and western blotting, 3×10^5 cells were seeded into 35-mm petri dishes. Twenty-four hours after seeding, the cells were incubated with DMSO or vitamin E analogs for 24 h for cell density measurement and for 1 or 24 h for western blotting and dye transfer assay.

2.3. Cell density

Cells were counted after a 24-h incubation. Cell numbers were determined by means of a Coulter Counter Channelizer (Beckman Coulter, Villepinte, France) after trypsinization with 0.05% trypsin and 0.02% EDTA.

2.4. Neutral red uptake

For each treatment, incorporation of neutral red was measured into eight wells from two 96-well microplates. Twenty-four hours after seeding, the cells were treated for 24 h with a control medium supplemented or not with αT, TS, TA or TP. The cells were incubated with neutral red solution (50 μg/mL) for 3 h at 37°C, washed three times with warm phosphate-buffered saline (PBS; 37°C) and fixed with destain solution (1% glacial acetic acid, 50% ethanol and 49% distilled water). Signals were assessed with SpectraFluor Plus (absorption filter=540 nm; Tecan, Trappes, France).

2.5. Dye transfer assay

GJIC was tested by measurement of dye transfer after the microinjection of 5% LY as previously described [28]. The intercellular transfer of LY was determined quantitatively by counting fluorescent cells with an epifluorescent microscope (Olympus IMT2, Tokyo, Japan). The cells were maintained at room temperature during injection and fixed with 4% formaldehyde (diluted in PBS) to defer scoring. For each treatment, data are the mean±S.D. of at least 14–25 microinjection trials. Data were expressed as the percentage of the control cells to compare results from independent experiments.

2.6. Western blot analysis

Cx 43 expression was performed on IAR203 total protein extracts. Cells were washed twice with PBS and scrapped off into 75 μl of a 2× sample buffer: 50 mM of Tris–Cl and 2% sodium dodecyl sulfate (SDS). After sonication, lysates were assayed for protein quantification by the Lowry method [29]. β-Mercaptoethanol at a final concentration of 5% and 0.1% bromophenol blue were added to the samples. Proteins (25 μg/well) were submitted to electrophoresis in 10% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane (Schleicher and Schuell, Kenne, NH, USA) at 55 V for 2 h at 4°C using a mini electroblotting apparatus (Bio-Rad, Richmond, CA, USA). Equal protein loading was verified on gel by Coomassie Blue solution (R250/G250, 1:1), and membranes were stained with Ponceau S (Fluka, Buck, Switzerland) to control the quality of protein transfer. Membranes were blocked for 4 h with 5% skimmed milk powder in Tris-buffered saline-Tween (TBS-T) containing 50 mM of Tris–HCl, 200 mM of NaCl, pH 7.4, and 0.1% Tween (Sigma, St. Louis, MO, USA). They were then incubated overnight at 4°C with rabbit anti-Cx 43 serum (1:500), kindly provided by Dr. K. Willecke and Dr. O. Traub (University of Bonn, Bonn, Germany). Then, they were washed three times in TBS-T for 15 min at room temperature. Membranes were incubated with a secondary peroxidase antirabbit antibody (1:6000) and then washed three times for 15 min with TBS-T. Cx 43 was detected using an enhanced chemiluminescence kit (Amersham Biosciences, Saclay,

France). Western blot analysis was repeated on extracts from four independent cultures. Since Cx 43 is expressed in the heart and not in normal liver, in vivo, heart and liver proteins were used as positive and negative controls, respectively.

2.7. Immunolocalization

For immunochemistry, cells were seeded onto epoxy-treated slides to avoid cell diffusion. Twenty-four hours after seeding, cells were treated with 0.1% DMSO or 10 or 25 μM of TS for 24 h. They were then briefly washed three times with PBS, permeabilized with 0.25% Triton X-100–4% paraformaldehyde for 2 min and fixed with 4% paraformaldehyde in PBS for 30 min. The cells were prehybridized for 1 h in PBS/2% bovine serum albumin and then incubated with anti-Cx 43 (1:50), followed by fluorescein isothiocyanate antimouse immunoglobulin G (1:200). After mounting in a Vecta-Shield (Vector Laboratories, Biovalley, Marne la Vallée, France), samples were observed (×400) using an Olympus fluorescent microscope equipped with a camera (DP50; Olympus, Rungis, France).

3. Results

3.1. Effects of vitamin E analogs on cell density and neutral red uptake

Since the monolayer density can influence communication between cells, we evaluated the potential cytotoxic effects of vitamin E analogs on cell density at a concentration range of 0.1–100 μM during a 24-h treatment. Results concerning αT and TS are shown in Table 1. αT up to 100 μM had no toxic or cytostatic effect in cells treated for 24 h. The lack of cytotoxic effects was confirmed using the neutral red uptake assay, which measures the number of viable cells. In the case of TS, a weak decrease in neutral red uptake was seen for concentrations higher than 25 μM, with no consequence on cell density. Thus, the microinjection experiments could be performed with αT and TS at concentrations of up to 25 μM. For the other tocopherol analogs, no effect on cell density or neutral red uptake was observed (data not shown). Succinic acid was tested at the same concentration range as was TS since TS could be degraded into tocopherol and succinic acid: no effect was detectable up to 100 μM (data not shown). In addition, no effect on cell density was observed with AA at 10 or 25 μM for 24 h of incubation (data not shown).

Table 1
Effects of αT and TS on cell density and neutral red incorporation

	Concentration (μM)					
	0.1	1	10	25	50	100
αT						
Cell number[a]	99±4	114±5	119±8	124±19	132±14	134±15
Neutral red uptake[b]	90±5	102±1	103±9	99±9	99±7	107±8
TS						
Cell number[a]	137±17	133±13	118±3	112±5	114±2	100±4
Neutral red uptake[b]	127±20	121±21	95±5	83±1	77±4	81±3

Data are the mean±S.E.M. of three independent experiments. Results are expressed as percentage of the control.

[a] For cell density measurement, 300,000 cells were seeded in 35-mm petri dishes. Twenty-four hours after seeding, the cells were treated for 24 h with the different concentrations of αT and TS, trypsinized and counted.

[b] For the neutral red assay, 20,000 cells were seeded by well (8 mm) in a 96-well microplate. Cells were fixed and incubated with neutral red and then washed; signals were measured at 540 nm.

Table 2
Effects of αT, TA, TP, TS and αT+AA on GJIC

Concentration	αT	TA	TP	TS	αT+AA[a]
10 μM					
1 h	85±4	76±16	85±1	78±18	77±23
24 h	93±7	96±3	90±2	16±5[b]	88±13
25 μM					
1 h	98±5	69±15	62±13[b]	19±2[b]	68±7[b]
24 h	98±5	70±9	58±6[b]	2±1[b]	60±15[b]

Data are the mean±S.E.M. of three independent experiments. Values are expressed as percentage of the control. For all conditions, 300,000 cells were seeded in 35-mm petri dishes. Twenty-four hours after seeding, the cells were treated for 1 or 24 h with the different concentrations of αT, TA, TP and TS. After microinjection, IAR203 control cells communicated with 100–140 surrounding cells.

[a] AA was added at the same concentration as was αT (10 or 25 μM).

[b] The average number of dye-coupled cells was significantly (by Dunnett's method) lower than the corresponding control value ($P < .05$).

3.2. Effects of αT, αT+AA, TA, TP and TS on GJIC in IAR203 cells

Table 2 summarizes the effects of vitamin E analogs on GJIC. αT alone had no effect on GJIC at whatever the concentration for 1- and 24-h treatments. A significant slight effect was observed when cells were incubated with 25 μM of αT in combination with AA. No effect was observed with AA alone (10 or 25 μM; data not shown).

TA and TP slightly inhibited the GJIC (~40% of inhibition), and this inhibition was significant only for TP at 25 μM.

TS at 25 μM drastically inhibited dye transfer (81% of inhibition) from 1 h, and the cells were totally uncoupled after a 24-h treatment (98% of inhibition). At 10 μM, the inhibition was delayed, showing a dose-dependent effect.

We checked that the inhibition observed with TS was effectively due to TS and not to succinate alone after dissociation. Free succinate was added to the culture medium at 25 μM for 1 and 24 h; no inhibition of GJIC was detected (data not shown).

3.3. Effects of αT, TA, TP and TS on Cx 43 expression

Only TS was able to interfere drastically with GJIC. Thus, the link between this inhibition and the expression of the Cx present in IAR203 cells, Cx 43, was explored. When protein extracts were separated in 10% polyacrylamide gels, several

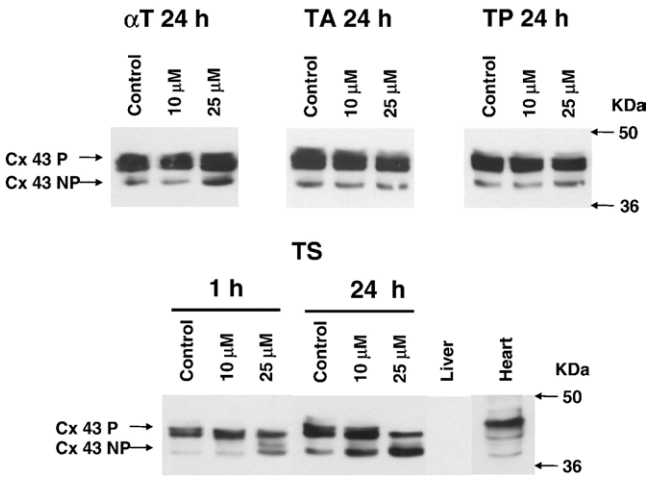

Fig. 1. Effects of vitamin E analogs on the phosphorylation of the Cx 43 protein. Western blot analysis was performed with total proteins extracted from untreated cells and cells treated with 10 or 25 μM of αT, TA or TP for 24 h, treated with 10 or 25 μM of TS for 1 or 24 h or extracted from the rat heart or liver.

bands were detected around 43 kD: one corresponded to the nonphosphorylated (NP) form (Cx 43-NP), whereas the other bands corresponded to the phosphorylated (P) forms (Cx 43-P; Fig. 1). Under these conditions, for αT, TA or TP, no change in the quantity or in the distribution of the P forms was observed. In contrast, at 1 h, 25 μM of TS induced a decrease in the Cx 43-P in favor of the NP form. At 24 h, the TS-induced decrease in the P form was observed for 10 and 25 μM.

3.4. Effect of TS on immunolocalization of Cx 43

As expected, Cx 43 was located in the cell–cell contact region: in control cells, discontinuous beaded strands of punctate fluorescence labeled outlined cells. In comparison, in TS-treated cells at 10 or 25 μM for 24 h, the fluorescence label was continuous (Fig. 2). Thus, TS did not induce any decrease in the Cx 43 protein amount or any change in its localization.

4. Discussion

Since in previous studies we had observed that various micronutrients or food-borne microconstituents, such as retinoic acid, apigenin and diallyl disulfide, enhance cell–cell communication in vitro [26,30], we studied the ability of αT and other vitamin E analogs to modulate GJIC. Unexpectedly, we observed that these micronutrients do not increase the dye transfer in IAR203 cells. Conversely, for the first time, we showed a strong inhibitory effect of TS on GJIC (98% of inhibition at 25 μM for a 24-h treatment) and a moderate but significant inhibitory effect of αT in combination with AA as well as of TP (~35% inhibition at 25 μM for 1 h). The slight effect of TA was not significant and αT alone

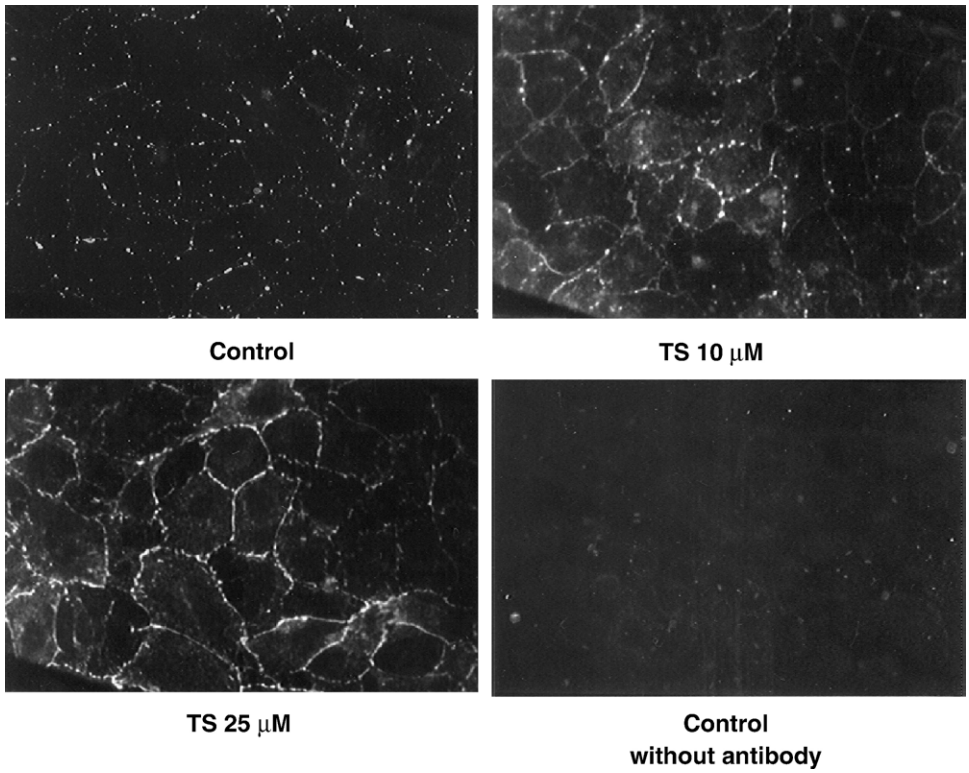

Fig. 2. Immunofluorescent staining of the Cx 43 protein in control cells and cells treated with TS for 24 h. Cells were plated on epoxy-treated slides and fixed with paraformaldehyde; the Cx 43 proteins were detected with Cx 43 antibodies followed by a fluorescein isothiocyanate-conjugated second antibody.

had no effect, which could be explained partly by the instability of this vitamin in the culture medium [31].

The drastic inhibitory effect of TS is dose and time dependent. In addition, TS inhibition can be maintained during 24 h, whereas the effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the well-known GJIC inhibitor, has been shown to be reversed after 4 h [32]. TPA-induced GJIC inhibition is attributed to its capacity to stimulate protein kinase C (PKC), which results in Cx hyperphosphorylation [33]. In contrast, we observed that TS inhibits the phosphorylation of Cx 43. This indicates that TS and TPA, both inhibitors of GJIC, act through different mechanistic pathways. Another class of tumor promoters, organic pollutants, such as polychlorinated biphenyls, elicits a similar hypophosphorylation of Cx 43 involving the Src kinases and phospholipase C [34]. To our knowledge, as no information is available about the effect of TS on these enzymes, it deserves to be investigated. Indeed, studies on the effects of TS on enzyme activities are scarce. On one hand, it has been shown that αT inactivates cellular PKC in smooth muscle cells [35] and that TS activates PKC in HL60 cells [36]. On the other hand, αT could also activate protein tyrosine phosphatases [37]. A limited number of studies have examined the contribution of protein phosphatases to GJIC regulation, but it is not clear whether their activation has a stimulating effect on GJIC [38]. Thus, we can hypothesize that the TS effect could be due to the activation of a phosphatase that could result in the hypophosphorylation of Cx 43 or that of other proteins interacting with the regulation of Cx 43-constituted gap junctions. In TS-treated cells, the amount of Cx 43 and its localization in the membrane were maintained when gap junction functionality was lost, suggesting that the decrease in Cx 43 phosphorylation is involved in the gating of the gap junctions. Indeed, Cx phosphorylation has been implicated in the regulation of GJIC at several stages of the Cx life cycle, such as trafficking, assembly/disassembly, degradation and gating of gap junction channels [39].

Interestingly, among the different vitamin E analogs tested on GJIC, TS is the more efficient. Prasad and Prasad [40] established in 1982 that TS was the form of vitamin E that was the most active on tumor cell proliferation. TS is not hydrolyzed by tumor cells in vitro [41]. It possesses unique biological properties: its antiproliferative mechanism of action results from the intact molecule and not from the release of αT [42]. These observations are in agreement with the results of studies showing selective inhibition of tumor cell growth by TS in vitro [43–46] and in vivo [47]. Contrary to the poor effect of αT on cancer cells, TS exerts a strong proapoptotic effect on malignant cells and is now proposed as a good adjuvant for cancer treatment, particularly for prostate cancer [5,9,10]. It will be interesting to complete our findings by investigating (1) whether the mechanisms involved in the induction of apoptosis by TS are linked to those leading to GJIC inhibition; (2) whether TS has the same effect on other cell lines competent for GJIC, either normal or tumoral cells, for which blocking cell–cell communication could prevent resistance to chemotherapeutic agents; and (3) whether γ-tocopherol, which is the vitamin E form mainly present in food, has a similar effect on GJIC.

From a general point of view, the impact of the strong inhibitory activity of TS on GJIC in vitro and its significance in the modulation of carcinogenesis in vivo deserve to be discussed.

First, it is important to recall that vitamin E analogs are currently added in culture media to prevent oxidation. We showed in this study that besides this expected effect, vitamin analogs, especially TS, can inhibit GJIC with possible repercussions on the control of intercellular homeostasis [17] and the control of cell growth and cell death [18]. We assume that such an adverse effect could introduce a bias in some mechanistic vitro experiments and therefore should be taken into account by investigators.

Second, the ability of TS to inhibit GJIC suggests that, at least in some conditions, it behaves like a tumor promoter [24,25]. Our results support epidemiological data suggesting the absence of a protective effect of vitamin E against cancer and even more its potential adverse effect: for example, a pooled analysis of prospective studies reported no protective effect of dietary vitamin E on lung cancer [48]. Supplementation with αT does not prevent lung cancer in cigarette smokers either [49]. Moreover, a recent meta-analysis of 19 randomized trials clearly emphasized that high-dose vitamin E supplementation may increase all-cause mortality [50].

Taken all together, mechanistic and epidemiological data point out the necessity of various additional in vitro and in vivo experiments for us to better understand whether vitamin E supplementation is beneficial or harmful toward cancer risk.

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