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Vitamin E deficiency induces liver nuclear factor-κB DNA-binding activity and changes in related genes

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Abstract

The biological functions of vitamin E have been classically attributed to its property as a potent inhibitor of lipid peroxidation in cellular membranes. However, in 1991, Azzi's group first described that α-tocopherol inhibits smooth muscle cell proliferation in a protein kinase C (PKC)-dependent way, demonstrating a non-antioxidant cell signalling function for vitamin E. More recently, the capacity of α-tocopherol to modulate gene expression with the implication of different transcription factors, beyond its antioxidant properties, has also been established. This study was to determine the effect of vitamin E-deficiency on liver nuclear factor-kappa B (NF-κB) DNA-binding activity and the response of target antioxidant-defense genes and cell cycle modulators. Rats were fed either control diet or vitamin-E free diet until 60 or 90 days after birth. Vitamin E-deficiency enhanced liver DNA-binding activity of NF-κB [electrophoretic mobility-shift assay, (EMSA)] and up-regulated transcription of γ-glutamylcysteine synthetase (*γ-GCSM; γ-GCSC)*, *cyclin D1* and *cyclin E*. We also showed down-regulation of *p21* $^{(\mathrm{Waf1/Cip1})}$ transcription. Western-blot analysis demonstrated that γ-glutamylcysteine synthetase catalytic subunit (γ-GCSC) and cyclin D1 showed a similar pattern to that found in the RT-PCR analysis. Moreover, chromatin immunoprecipitation (ChIP) assay demonstrated that NF-κB directly regulates transcription of *γ-GCS* (both subunits) and *cyclin D1* through the binding of NF-κB to the corresponding gene promoters, which was enhanced in vitamin E-deficiency. These findings show that vitamin E-deficiency induces significant molecular regulatory properties in liver cells with an altered expression of both antioxidant-defense genes and genes that control the cell cycle and demonstrate that liver NF-κB activation is involved in this response. Our results emphasize the importance of maintaining an adequate vitamin E consumption not only to prevent liver oxidative damage but also in modulating signal transduction.

Keywords: *Vitamin E-deficiency, liver, nuclear factor-kappa B (NF-κB), redox status, gamma-glutamylcysteine synthetase*

Abbreviations: *ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; γ-GCS, γ-glutamylcysteine synthetase; γ-GCSC, γ- glutamylcysteine synthetase catalytic subunit; γ-GCSM, γ-glutamylcysteine synthetase modifier subunit; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; NF-κB, nuclear factor-κB; PKC, protein kinase C; ROS, reactive oxygen species*

Introduction

Reactive oxygen species (ROS) participate in the development of cardiovascular disease, cancer and neurodegenerative disorders [1,2] and also as initiating factors and modulators of liver cell injury [3,4] although the precise role they play in disease mechanisms is not known. Cells have a variety of antioxidant defenses, including glutathione (GSH), antioxidant vitamins and protective enzymes which, when in balance with the

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generation of free radicals, protect the cell against oxidative damage. This protection is adequate when low levels of ROS are produced during normal aerobic respiration; however, when the oxidant levels exceeds the cell's capacity to detoxify, damage occurs and promotes acute or chronic responses if the insult is sustained.

Among the cellular antioxidant defenses, vitamin E has been classically considered the most important lipid-soluble antioxidant [5]. This generic term includes at least eight natural isoforms with biological activity, i.e. four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ). The α-tocopherol is quantitatively the major form of vitamin E in human and animal plasma and tissues where it plays an essential role in maintaining the integrity of biological membranes. The α-tocopherol has been clearly recognized as a potent inhibitor of lipid peroxidation in cellular membranes where it acts as a powerful chain-breaking agent through the scavenging of peroxyl radicals by undergoing a one-electron oxidation to form tocopheroxyl radicals [6]. However, besides its well-known chain-breaking antioxidant activity, in 1991, Azzi's group first described that α-tocopherol inhibits smooth muscle cell proliferation in a protein kinase C (PKC)-dependent way [7] and, since that, other diverse effects had been related to its non-antioxidant properties including specific effects of α-tocopherol in cell signalling and regulation of gene expression. PKC inhibition is achieved through protein phophatase 2A activation by α-tocopherol, which will catalyze PKC dephosphorylation; in all the observed vitamin E effects, the involvement of PKC and others enzymes has not always been studied and it remains to be investigated whether the regulation of transcription of some genes is the consequence of PKC inhibition or is mediated by several tocopherol regulatory proteins (i.e. α-tocopherol-associated proteins), that may play a role in cellular signalling by regulation of the intracellular tocopherol distribution. However, the inhibition of PKC is responsible for the α-tocopherol induced diminution of release of ROS, the inhibition of thrombocyte aggregation and most of the α-tocopherol effects on cell signalling [8–10].

Vitamin E deficiency in humans gives rise to a specific neurological syndrome clinically similar to Friedreich ataxia phenotype [11]. Epidemiological studies have also shown that vitamin E inadequacy is implicated in the pathogenesis of atherosclerosis, diabetes, cancer, inflammatory processes and immune responses [9,12]. A similar disorder to human vitamin E deficiency can be induced in rats and other animal species by a long-term vitamin E deprivation in the diet [13] or, more recently, by deleting the α-tocopherol transfer protein gene [14,15]. It has been suggested that the neuromuscular damage induced by vitamin E deficiency could be the consequence of an increase in oxygen-derived free radicals [13–16] and, in fact, the evidence for altered intracellular redox status induced by vitamin E deficiency had been demonstrated in different tissues [17–20]. In this context, it has been reported that neuronal and skeletal muscle tissues are greatly vulnerable in response to vitamin E depletion whereas liver morphology and liver mitochondrial function seems not to be affected in vitamin E-deficient rats [11,13,15,16]. However, the lack of changes in liver morphology and respiratory chain does not imply that other manifestations either related to oxidative stress or to other specific biological effects of hepatic vitamin E-depletion could not take place. In fact, during the last years, specific effects of vitamin E in cell signalling and regulation of gene expression have been demonstrated beyond its antioxidant function [7–10] and, at present, little is known about liver molecular events in response to hepatic vitamin E-depletion, a circumstance which have been associated to several chronic liver diseases[21].

Recent attention has been focused on the transcription factor nuclear factor-κB (NF-κB) as mediator in the process of inflammation, apoptosis, cell survival and proliferation, controlling the expression of numerous genes, and its altered modulation has been related with liver damage, among other diseases. Several lines of evidence, including either its inhibition caused by various antioxidants and its induction after *in vivo* treatment with oxidants and agents that deplete GSH, suggest that NF-κB is subject to redox regulation [22–27]. In this context, experimental data is accumulating to suggest that the anti-inflammatory properties of vitamin E are partly due to its ability to down-regulate NF-κB, either by directly affecting key steps in its activation pathway or by modulating the intracellular redox status, one of the major determinants of NF-κB activation [27].

This study was to investigate the effect of *in vivo* vitamin E deficiency on hepatic intracellular redox status, the liver response of the transcription factor NF-κB DNA-binding activity and the effect of hepatic vitamin E depletion on NF-κB target genes gamma-glutamylcysteine synthetase (γ-*GCS* modifier or light subunit, γ-*GCSM*; γ-*GCS* catalytic of heavy subunit, γ-GCSC) and cyclin D1, which have been implicated in antioxidant defense and cell cycle progression, respectively. We show that hepatic vitamin E deficiency transcriptionally activates γ-*GCS* (both subunits) and *cyclin D1* by mechanisms that implies the activation in the depleted liver of the transcription factor NF-κB (electrophoretic mobility-shift assay, (EMSA)) and the binding of NF-κB to their corresponding gene promoters (chromatin immunoprecipitation (ChIP) assay), which was enhanced in vitamin E-deficiency.

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Material and methods

Chemicals

Biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Salts and solvents were from Merck (Darmstadt, Germany) and Fluka (Germany). Plasma aminotransferases, cholesterol and triglycerides were analyzed by commercial kits from Spinreact (Girona, Spain). Control and vitamin-E free diets were from ICN Biomedicals (Cleveland, OH, USA).

Animals and diets

All animal use procedures were according to the NIH Guide for the Care and Use of Laboratory Animals. Male specific pathogen-free Wistar rats were made deficient in vitamin E by feeding a vitamin E-free diet, as follows. Pregnant rats (Charles River, Barcelona, Spain) were housed in individual cages in a room maintained at 22°C with a 12-h light–dark cycle. After pup birth, the dams with their litter were randomly divided in two groups. The first one was fed on a complete purified diet following the recommendations of the American Institute of Nutrition [28]. The composition of this diet was (in g/Kg): corn starch 397.5, casein (vitamin-free) 200, dextrinized corn starch 132, sucrose 100, soybean oil 70, alphacel non-nutritive bulk 50, AIN-93 vitamin mix 10, AIN-93G mineral mix 35, choline bitartrate 2.5, L-cystine 3.0 and T-butyl hydroquinone 0.014. The composition of AIN-93 vitamin mix (g %) was: niacin, 0.300; D calcium pantothenate, 0.160; pyridoxine HCl, 0.070; thiamine HCl, 0.060; riboflavin, 0.060; folic acid, 0.020; biotin, 0.002; vitamin B-12, 0.250; all-*trans*-retinyl palmitate (250,000 U/g), 0.160; all-rac-α-tocopheryl acetate powder (250 U/g), 3; vitamin D-3 (400,000 U/g) 0.025; menadione, 0.008; powder sugar, 95.885. The concentration of tocopherols analogues was analyzed in the diet and the average amount of major tocopherol analogues, α-tocopherol and γ-tocopherol, per gram of control diet was 67 and 15 μg/g, respectively. The amount of the other analogues, i.e. β and δ tocopherol was unappreciable in our experimental conditions. The α-tocopherol values are in agreement with the amount reported in AIN-93G diet. The γ-tocopherol could be a component present in the soybean oil. The second group was fed on the same diet but devoid of vitamin E. Milk production was controlled during lactation in both groups [29]. At 21 days of age, male pups from mothers fed the complete diet (control group) and male pups from mothers fed the vitamin E-free diet (deficient group) were weaned into their corresponding mother diet until they were 60 or 90 days old. These periods were appropriate to induce a severe deficiency in vitamin E without significantly alter the growth of the animals. The amount of food intake and the body and liver weights were evaluated in control and deficient rats.

Sampling procedure

The experiments were performed between 10:00 and 12:00 a.m. Rats were anaesthetised with Pentothal (50 mg/kg body weight, ip). Blood was collected from the aorta in heparinized syringes and then liver samples were taken and processed immediately.

Tocopherol determination

Samples of plasma were prepared for tocopherols determination following the extraction procedures described by Arnaud et al. [30]. The extraction of tocopherols from diets and for liver tissues was made as described by Podda et al. [31]. Briefly, tocopherols were quantified in aliquots of the hexane phase by the HPLC method described previously [30].

Mitochondria isolation

Liver mitochondria were isolated as has been described [32].

Glutathione determination

Reduced glutathione (GSH) was measured using the glutathione-S-transferase assay [33]. Oxidized glutathione (GSSG) was measured using an HPLC method with UV–Vis detection, which was developed to measure GSSG in the presence of a large excess of GSH [34].

Lipid peroxidation

Lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) using the HPLC method described by Wong et al. [35].

RT-PCR studies

Total RNA from rat liver was isolated by guanidinium thiocyanate method [36]. Aliquots of 2 μg were reverse transcribed using SuperScript™ RNase H^- (GibcoBRL®, USA) and subsequently amplified by polymerase chain reaction (PCR) using AmpliTaq® DNA polymerase (Perkin-Elmer, USA). The primers used for the different genes studied were: 5′-ATGCCATGGGATTTGGGA-3′ and 5′-CATTGTCTGCCAGTTTGTGGA-3′ for γ-*GCSM*; 5′-TGGATTCACACTGCCAGAGC-3′ and 5′-CACCTGAAGACAGCAGTTGC-3′ for γ-*GCSC*; 5′-CCTCCAAAGTTGCACCAGTTT-3′ and 5′-TTGCTTGGGCTTTGTCCA-3′ for *cyclin E*; 5′-TGTTCGTGGCCTCTAAGATGA-3′ and 5′-GCTTGACTCCAGAAGGGCTT-3′ for *cyclin D1*; 5′-ACAGCGATATCGAGACACTCA-3′ and 5′-GTGAGACACCAGAGTGCAAGA-3′ for *p21* $^{(Waf1/Cip1)}$. 18S rRNA was simultaneously amplified and used as an internal control.

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Reactions were resolved in a 2% agarose gel, stained with ethidium bromide and quantified using the GeneGenius System and the Gene Tools analysis software (Syngene).

Immunoblot analysis

Tissue was homogenized in 10 ml of ice cold RIPA buffer consisting in 1.85 mM NaH_2PO_4, 8.4 mM Na_2HPO_4 pH 7.9, 0.1 M NaCl, 12 mM deoxycholate, 0.1% SDS, 1% Triton X-100, 0.5% NaN_3 in the presence of protease inhibitors (Sigma) in a proportion of 10 ml/g of tissue. The resulting homogenate was centrifuged at 160,000*g*. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins were detected by using the corresponding antibody (Santa Cruz, γ-GCSC sc-22755 and cyclin D1 sc-246) and secondary horseradish peroxidase-conjugated antibody. Blots were developed by enhanced chemiluminescence (Dupont). The loading of the gels was checked with an antibody specific for β-actin.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay method was used to characterize the binding activities of NF-κB transcription factor in nuclear extracts [37] prepared according to Digman et al. [38] with modifications [39,40]. The oligonucleotide containing the consensus sequence for NF-κB (5′-GTACGGAGTATCCA-GCTCCGTAGCATGCAAATCCTCTGG-3′) was 3′- labeled with digoxigenin. Protein DNA binding assay were performed with 25 μg of nuclear protein. Unspecific binding was blocked by using 1 μg of poly [deoxy(inosine-cytosine)] and 1 μg of poly L-lysine. The preparations were incubated with the labelled probes at room temperature for 30 min. DNA–protein complexes were separated from unbound probe by electrophoresis on 8% polyacrylamide gels. Complexes formed were identified by autoradiography of the gels. Cold competitions were performed by adding 100-fold molar excess of specific unlabeled NF-κB oligonucleotide probe 15 min prior to the addition of the digoxigenine-labeled oligonucleotide probes.

Chromatin immunoprecipitation assay

ChIP assays were performed according to Borrás et al. [41], modified for tissue samples. Liver tissues were excised and immersed into 10 ml of 1 × PBS pH 7.4 and 1% formaldehyde, and gently stirred for 12 min at room temperature to crosslink the transcription factors to DNA. The reactions were stopped by addition of glycine to a final concentration of 0.125 mol/l. The samples were washed twice with 10 ml of cold 1 × PBS pH 7.4 and immersed into 8 ml of 1 × PBS pH 7.4 supplemented with 2μl/ml of a protease inhibitor cocktail (Sigma). The liver tissues were disaggregated with a Dounce homogenizer and centrifuged at 1500g for 5 min. The cell pellets were resuspended in 3 ml of cell lysis buffer (5 mmol/l HEPES pH 8.0, 85 mmol/l KCl, 0.5% NP_{40}) supplemented with protease inhibitor cocktail, incubated on ice for 15 min and centrifuged at 3500*g* for 5 min to pellet the nuclei. The pellets were resuspended in nuclear lysis buffer (50 mmol/l Tris–HCl pH 8.1, 10 mmol/l EDTA, 1% SDS), supplemented with the same protease inhibitors mentioned above, at a ratio 1:1 (v/wt) relative to the initial weight, incubated on ice for 20 min, and stored at −20° C.

One millilitre of each sample was sonicated on ice with 10 pulses of 10 s in a Vibra-Cell VCX-500 sonicator (Sonics and Materials), centrifuged at 14,000*g* for 10 min at 4°C, and its DNA concentration estimated. Equivalent amounts of the samples were diluted 10-folds and precleared by adding 30 μl/ml of 1:1 (v/v) protein A/G Sepharose (Amersham Bioscience) (blocked with 100 μg/ml λDNA, 500 μg/ml tRNA and 1 mg/ml BSA). The samples were incubated at 4°C for 3 h in a rotating plate, centrifuged at 12,000*g* for 1 min to discard protein A/G Sepharose, and fractioned in aliquots equivalent to 50 μg of DNA.

The immunofractionation of complexes was performed by adding to each aliquot 2 μg of the corresponding antibodies (Santa Cruz Biotechnology, NF-κB (p65) sc-109 and RNA polymerase II sc-899) and incubating at 4°C overnight in a rotating plate. The samples were next incubated with 50 μl of blocked protein A/G Sepharose for 3 h at 4°C under gentle rotation, and the immunocomplexes, containing chromatin fragments/antibody/protein A/G Sepharose were collected by centrifugation at 12,000*g*, 1 min. Antibody-bound fractions were washed twice with low-salt buffer (150 mmol/l NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mmol/l EDTA, 50 mmol/l Tris–HCl pH 8.0), twice with high-salt buffer (500 mmol/l NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mmol/l EDTA, 50 mmol/l Tris–HCl pH 8.0), twice with LiCl buffer (250 mmol/l LiCl, 1 mmol/l EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 50 mmol/l Tris–HCl pH 8.0) and twice with Tris EDTA buffer pH 8.0. The immunoselected chromatin was eluted from the protein A/G Sepharose by two consecutive extractions with 100 μl of elution buffer (1% SDS, 100 mmol/l $NaHCO_3$) each, with vigorous shaking in a vortex, and centrifugation at 12,000*g*, 2 min. An aliquot of the crosslinked chromatin was treated as above in the absence of antibody (No Ab fraction) and the first supernatant,

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after the incubation with protein A/G Sepharose, was saved as input fraction. The cross-links were reversed by heating the eluate at 65°C overnight. The DNA from all fractions was extracted, after proteinase K incubation, with a DNA purification kit (PCR Purification Kit, Quiagen) and used for PCR analysis of the target genes. For PCR analysis 1:5000 of the initial DNA was used in the case of input fraction, and 1:30 of the immunoprecipitated fractions.

Analysis of immunoprecipitated DNA

To check if the inmunoprecipitated chromatin fraction contains the γ-*GCSC*, γ-*GCSM* and *cyclin D1* promoter among the pool of DNA, the DNA samples (input, bound and no antibody) were analyzed by PCR using the following primers: γ-*GCSC* (promoter) 5′-TGTAAGCATGAGGCTCCCTCC-3′ and 5′-TTC-CTACTTGCGACCCAAGG-3′. γ-*GCSC* (intron 1–exon 2) 5′-TGTCTCTGAGCAGCACTTGC-3′ and 5′-AGAACATCGCCGCCATTCAG-3′. γ-*GCSM* (promoter) 5′-TCGACCAGTTTCAATTCTCT-ATCC-3′ and 5′-GAGTATTTGGGCTTCCTGA-CATT-3′. γ-*GCSM* (intron 3) 5′-GCAGTGGGGAT-GAGCCTCTG-3′ and 5′-TACTCCCAGGCAACC-CGAGC-3′. *Cyclin D1* (promoter) 5′-CCCCGCTC-TTTCCCAGC-3′ and 5′-GCTGCCTCGCGCTC-TACG-3′. *α-actin* (promoter) 5′-AGGGACTCTAG-TGCCCAACACC-3′ and 5′-CCCACCTCCACCC-TACCTGC-3′. *α-actin* (coding region) 5′-AGGATT-CCTACGTGGGCGAC-3′ and 5′-TAGAGAGACA-GCACCGCCTG-3′.

Statistics

Values in the tables are presented as means ± SEM for the number of animals indicated. Data were analyzed by one-way ANOVA. When the *F*-test were significant ($P < 0.05$), post-hoc comparisons of means were made using Tukey's multiple comparison test. Significant differences in vitamin E deficient group compared to its respective control group at each time point (60 or 90 days) are shown. Means without a common letter differ, $P < 0.05$. All statistical calculations were carried out using Graph-Pad Prism, version 3.0 (GraphPad Software, San Diego, CA).

Results

Physiological parameters in vitamin E-deficient rats

Milk production was evaluated at the peak of lactation in dams fed either control diet or vitamin-E deficient diet and no difference was found between groups. After weaning, rats ate the solid control diet or vitamin E-deficient diet and no statistically difference in food intake was found. Rats were weighted weekly over the period studied and those fed on vitamin E-free diet gained weight at a similar rate that controls. Average body weight and diet ingested every 15 days by control and vitamin E-deficient rats are showed in Table I. The liver weights were also similar in control and deficient rats at 60 or 90 days (results not shown). Standard liver function tests, including aspartate aminotransferase and alanine aminotransferase plasma activities were measured as an index of hepatic injury. Aspartate aminotransferase activity (UI/l) was significantly increased in vitamin E deficient rats for 90 days (control rats: 37 ± 2, $n = 5$; vitamin E-deficient rats: 64 ± 9, $n = 6$; $P < 0.05$). Cholesterol and triglycerides were also significantly elevated in plasma from vitamin E-deficient rats (result not shown).

***α**-Tocopherol concentration in plasma and liver*

The concentration of α-tocopherol in plasma from control and vitamin E-deficient rats is shown in Figure 1. Rats fed on vitamin E-free diet from their birth (through the dam's milk) to 90 days of age (vitamin E-deficient rats) showed a progressive decrease in plasma α-tocopherol concentration when compared, at each time, with values found in control rats. This decrease was significant from 60 days of age, when plasma α-tocopherol concentration from deficient rats was less than 10% of its corresponding control values. In parallel with plasma tocopherol, the dietary deficiency of vitamin E for 60 or 90 days decreased significantly liver α-tocopherol concentration to about 20 and 10% of its

Table I. Average body weight and diet ingested by control and vitamin E- deficient rats.

	Body weight (g)		Food intake (g/d)	
Days	Control	Vitamin E- deficient	Control	Vitamin E-deficient
21–34	80.1 ± 6.1 (20)	88.6 ± 5.9 (18)	16.7 ± 0.9 (15)	16.6 ± 0.6 (17)
35–48	161.7 ± 6.8 (15)	177.9 ± 4.5 (11)	17.5 ± 0.6 (6)	15.7 ± 0.6 (8)
49–62	233.2 ± 6.0 (6)	248.4 ± 5.2 (8)	17.3 ± 0.2 (11)	17.5 ± 0.5 (18)
63–76	297.6 ± 6.4 (7)	316.5 ± 10.1 (8)	18.7 ± 0.5 (6)	18.9 ± 0.3 (6)
77–90	330.8 ± 7.9 (6)	341.8 ± 14.7 (4)	18.6 ± 0.7 (7)	19.6 ± 0.7 (7)

The data are means ± SEM with the number of animals indicated in parentheses. There are no significant differences between groups.

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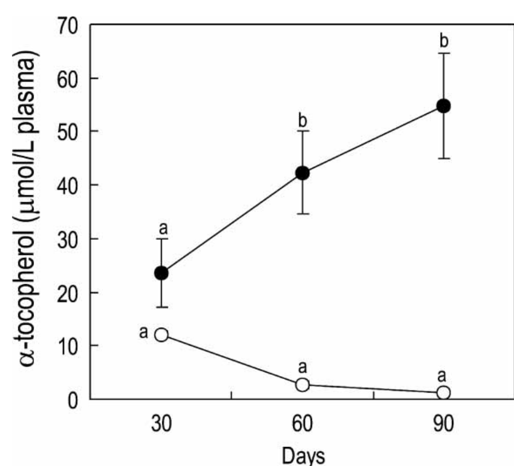

Figure 1. Plasma α-tocopherol levels in rats fed with control diet (●) and vitamin E-deficient diet (○) over 90 days. Values are means ± S.E.M. for $n = 4$ animals. Results in vitamin E-deficient rats significantly different from those of respective control group at each time point (30, 60 or 90 days) are shown. Means without a common letter differ, $P < 0.05$.

corresponding control values, respectively. Other tocopherol analogues were not detectable in liver from control and deficient rats (Table II).

GSH and MDA levels in liver mitochondria

Isolated liver mitochondria from vitamin E-deficient animals for 60 or 90 days showed a significant increase in MDA concentration and a significant decrease in GSH levels (Table II) when compared with its control values.

Glutathione status and MDA concentration in total liver

Vitamin E deficiency for 90 days induced a significant decrease in liver GSH concentration (Table II). Accordingly, liver GSH/GSSG ratio decreased significantly in vitamin E-deficient rats (control rats: 125 ± 15, $n = 3$; vitamin E-deficient rats: 45 ± 8, $n = 5$; $p < 0.05$). We also found an increase in MDA levels in liver from 90 days vitamin E-deficient rats (Table II).

NF-κB DNA-binding activity in liver of vitamin E-deficient rats

Experimental data is accumulating to suggest that the anti-inflammatory properties of vitamin E are partly due to its ability to down-regulate NF-κB, either by directly affecting key steps in its activation pathway or by modulating the intracellular redox status, one of the major determinants of NF-κB activation [27]. Moreover, NF-κB had been established as mediator in the process of inflammation, immune response, apoptosis, cell survival and proliferation [24,25]. We have found changes in the liver intracellular redox status with an increase in MDA and a decrease in GSH concentrations both in liver and in hepatic isolated mitochondria from vitamin E-deficient rats. Moreover, we also found a decreased GSH/GSSG ratio in total liver. These observations prompted us to examine the DNA-binding activity of NF-κB which is modulated by the intracellular redox status and plays a critical role in mediating the liver response to redox change [22,23,25]. For this purpose, nuclear extracts from liver control and either 60 or 90 days vitamin E-deficient rats were prepared and subjected to EMSA analysis by using the oligonucleotide containing the consensus NF-κB sequences. The effect of hepatic vitamin E-depletion for 60 or 90 days on DNA-binding activity of NF-κB is shown in Figure 2 *Panel A*. Standard EMSA analysis is shown in Figure 2 *Panel B*. Hepatic vitamin E-depletion for 60 or 90 days resulted in an induction, more important at 90 days, of a specific bandshift that competes with an excess of the same unlabeled oligonucleotide harbouring the consensus NF-κB binding site.

mRNA Levels of γ-GCS, cyclin D1, cyclin E and p21

The enzyme γ -GCS is a major determinant in the GSH synthesis, which concentration is decreased in the liver and in hepatic isolated mitochondria from vitamin E-deficient rats. Vitamin E-deficiency for 90 days increased γ-GCSM and γ-GCSC mRNA levels (Figure 3). The increased expression of *γ-GCSM*

Table II. Levels of α-tocopherol, glutathione and malondialdehyde in total liver and concentrations of malondialdehyde and glutathione in isolated liver mitochondria from control and vitamin E-deficient rats.

	60 days		90 days	
	Control	Vitamin E- deficient	Control	Vitamin E-deficient
Total liver				
α-Tocopherol (μg/g liver)	24.7 ± 1.9 (4)^b	4.7 ± 0.4 (5)^a	31.1 ± 5.5 (3)^b	3.2 ± 0.5 (3)^a
GSH (μmol/g liver)	6.6 ± 0.2 (4)^a	6.6 ± 0.2 (5)^a	6.9 ± 0.2 (7)^b	5.7 ± 0.3 (8)^a
MDA (nmol/g liver)	28 ± 11 (3)^a	115 ± 19 (3)^b	42 ± 12 (3)^a	620 ± 190 (3)^b
Liver mitochondria				
GSH (nmol/mg protein)	7.6 ± 0.4 (2)^b	5.3 ± 0.5 (3)^a	7.8 ± 0.4 (5)^b	4.5 ± 0.4 (7)^a
MDA (nmol/mg protein)	2.9 ± 0.3 (4)^a	6.7 ± 0.8 (5)^b	1.6 ± 0.5 (4)^a	8.7 ± 1.2 (3)^b

Results are means ± SEM with the number of animals indicated in parentheses. Values in vitamin E-deficient rats significantly different from those of respective control group at each time point (60 or 90 days) are shown. Means without a common letter differ, $P < 0.05$.

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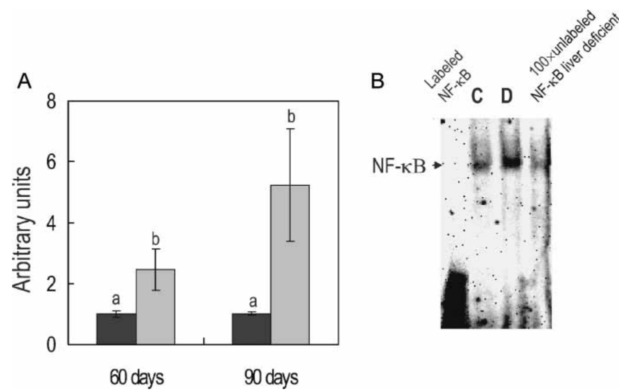

Figure 2. Effect of vitamin E-deficiency on liver NF-κB DNA-binding activity by EMSA analysis (*Panel A*). Representative EMSA analysis from 90 days vitamin E-deficient livers (*Panel B*). Liver nuclear extracts from control rats (C) and vitamin E-deficient rats (D) were incubated with digoxigenin-labeled NF-κB oligonucleotides containing the NF-κB consensus motifs and analysed by EMSA. The position of specific complexes is indicated. Hundred fold molar excess of specific unlabeled oligonucleotide NF-κB probe were added to the binding reactions to show the specificity of the complexes. The data shown in *panel A* are means ± S.E.M. of at least three independent experiments. The mean value for the control group was defined as 1.0 and results in vitamin E-deficient rats were expressed relative to their control group. Means without a common letter differ, $P < 0.05$.

occurred early because it was already evident at 60 days of deficiency. Vitamin E-deficiency for 90 days decreased mRNA levels of p21, critical in the G1 arrest and enhanced cyclin D1 and cyclin E mRNA levels, positive regulators of G1 to S phase transition (Figure 3).

Western blot analysis in liver of control and vitamin E-deficient rats

Liver samples were electrophoresed and immuno-blotted with specific antibodies. The amount of γ-GCSC protein was significantly higher in vitamin E-deficient rats than in controls. Vitamin E-deficiency also increased the amount of cyclin D1 protein when compared with controls (Figure 4). These changes induced by vitamin E deficiency in the pattern levels followed the pattern of gene expression showed in Figure 3.

Chromatin immunoprecipitation assay

These results showed that vitamin-E deficiency induced changes in the liver intracellular redox status, NF-κB activation and up-regulation of γ-*GCS*. Moreover, it is known that γ-GCSM and γ-GCSC genes have in their promoters the consensus binding sites for NF-κB [42,43]. To establish the potential role

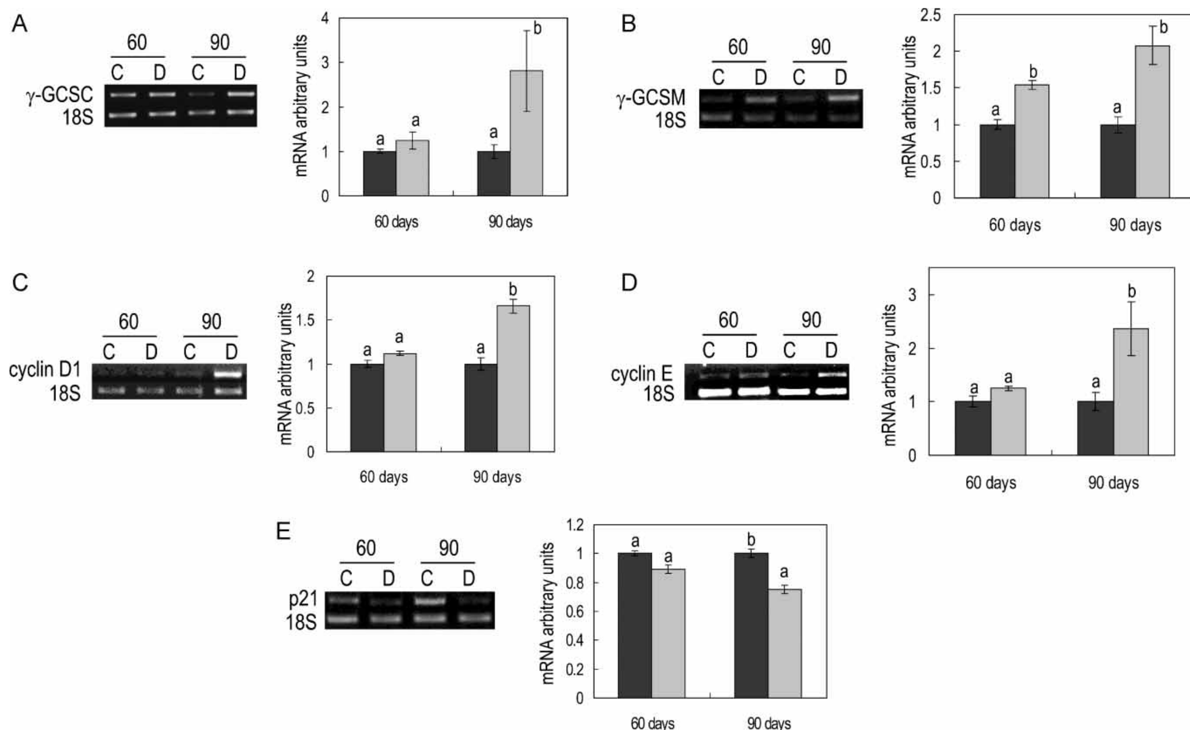

Figure 3. Gene expression of γ-*GCSC (Panel A)*, γ-*GCSM (Panel B)*, *Cyclin D1 (Panel C)*, *Cyclin E (Panel D)* and *p21* $^{(Waf1/Cip1)}$ (*Panel E*) in liver from control rats (C) and vitamin E-deficient rats (D). Values were normalized to 18S ribosomal RNA levels. The data shown are means ± S.E.M. of at least three independent experiments. The mean value for the control group was defined as 1.0 and results in vitamin E-deficient rats were expressed relative to their control group. Means without a common letter differ, $P < 0.05$.

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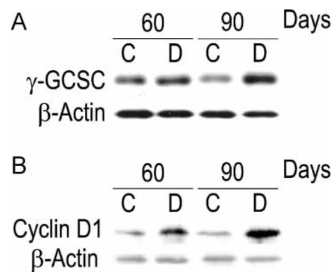

Figure 4. Western blot analysis of γ-GCSC and cyclin D1 in liver from control rats (C) and vitamin E-deficient rats (D). Total protein extracts were obtained as described in experimental procedures. The amount of both proteins, γ-GCSC and cyclin D1, were significantly increased in the liver from vitamin E-deficient rats when compared with their controls.

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of NF-κB in the regulation of γ-GCSM and γ-GCSC genes we carried out a ChIP assay whit an antibody against RelA/p65 a subunit component of NF-κB complex present in the most widely expressed form of NF-κB. Figure 5 *Panel A* shows that NF-κB is present in the promoter of both genes either at 60 and 90 days of vitamin E deficiency.

To determine if γ-GCSM and γ-GCSC genes were transcribed as result of the NF-κB binding to their promoters, we performed an alternative method, *RNAPol-ChIP*, to nuclear run-on assay developed in our laboratory based in formaldehyde ChIP assay, using an antibody against RNA polymerase II [44]. In the present work, we have screened for the presence of this enzyme in the first intron and second exon of the γ-GCSM gene and in the third intron of γ-GCSC gene. As is shown in the Figure 5 *Panel B* RNA polymerase II was present in the region to be transcribed of both genes concomitantly with the NF-κB binding and this is in good agreement with the RT-PCR experiments showed in the Figure 3.

Furthermore, several reports [45,46] have described an association between NF-κB activation and proliferation by promoting *cyclin D1* transcription. Therefore, we have studied NF-κB binding to *cyclin D1* promoter. As is shown in Figure 6, NF-κB binding site is also present clearly in the promoter of this gene either at 60 and 90 days of vitamin E-deficient livers.

Discussion

Epidemiological studies have shown that vitamin E inadequacy is implicated in the pathogenesis of atherosclerosis, diabetes, inflammatory processes, immune responses and different tumors [9,12]. Since the discovery of vitamin E, studies with different tocopherols and tocotrienols have focused principally on their antioxidant properties. The α-tocopherol is quantitatively the major form of vitamin E in human and animal tissues where it plays an essential role in

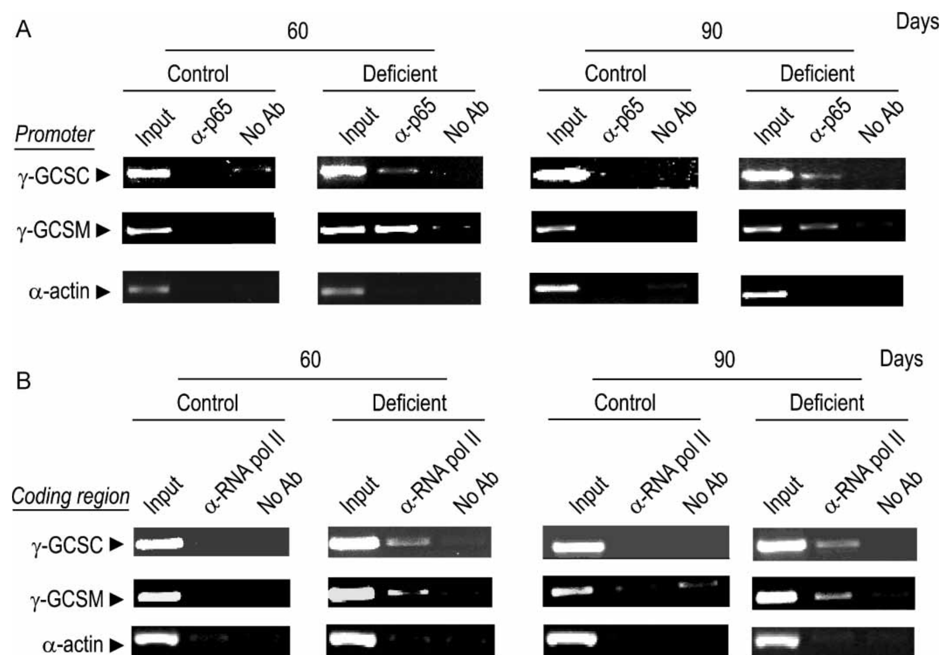

Figure 5. *In vivo* binding analysis of RelA/p65 to the *γ-GCSC* and *γ-GCSM* promoters *(Panel A)* and *in vivo* binding analysis of RNA polymerase II to the region to be transcribed of *γ-GCSC* and *γ-GCSM (Panel B)* in control and vitamin E-deficient livers. Formaldehyde-crosslinked chromatin immunoprecipitation was performed from control and vitamin E deficient livers. Immunoprecipitates obtained with each antibody were aliquoted and subsequently analyzed by PCR with primers specifics for *γ-GCSC* and *γ-GCSM* promoters and for the region to be transcribed of both genes (see "Material and methods" section). For each experiment, a sample on total chromatin (input) was included in the PCR reactions. No Ab: no antibody. PCR products obtained with oligonucleotides specific for α-actin promoters and coding region were included as a negative control.

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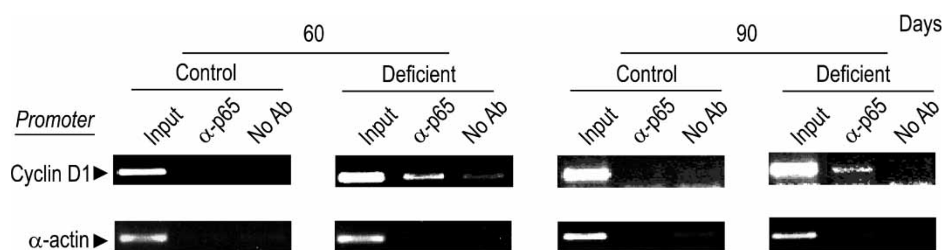

Figure 6. *In vivo* binding analysis of RelA/p65 to the *cyclin D1* promoter in control and vitamin E-deficient livers. The experiment was conducted and the results depicted as in Figure 5 *Panel A*.

maintaining the integrity of biological membranes by acting as a potent inhibitor of lipid peroxidation[6]. However, during the last years specific effects of vitamin E on signal transduction and gene expression have been demonstrated beyond its antioxidant function. Identifying the novel cellular reactions as well as the mechanism of modulation of gene expression by vitamin E will probably help to better link molecular and clinical process [7–10] and, at present, little is known about liver molecular events in response to hepatic vitamin E-depletion.

Novel experimental data is now accumulating to suggest that the anti-inflammatory properties of vitamin E are partly due to its ability to down-regulate NF-κB, either by directly affecting key steps in its activation pathway or by modulating the intracellular redox status [27]. NF-κB plays a critical role in cell growth and differentiation, apoptosis and adaptive response to changes in cellular redox balance, and its altered regulation has been associated with the pathogenesis of several diseases, including liver damage [22–27,47]. It has been reported recently that the liver morphology and respiratory function was not affected by hepatic vitamin E-depletion, perhaps reflecting the high levels of antioxidant enzymes in the liver, more than levels found in other tissues [16]. Nevertheless, other manifestations either related to oxidative stress or to other biological effects of vitamin E-depletion could take place. Similarly, in previous papers, we had reported that hepatic vitamin A-depletion induced liver oxidative injury without appreciable alterations in liver morphology and respiratory chain but with an important decrease in mitochondrial membrane potential, damage of mitochondrial DNA and changes in proliferative control genes [41,48,49].

This study showed that diet-induced liver vitamin E-depletion for 60 or 90 days correlated with a decrease in the concentration of GSH, which plays a crucial role in protecting cells against oxidative stress, and with an increase in the levels of MDA, an end product of lipid peroxidation, in isolated mitochondria, the major physiological source and target for ROS. Moreover, we also found a decreased GSH/GSSG ratio and an increase in MDA concentration in total liver.

Oxidative stress potentially up-regulates antioxidant defense capacity. Since gam-maglutamylcysteine synthetase (γ-GCS, also known as glutamate-cysteine ligase, γ-GCL) catalyses the first step in the biosynthesis of GSH and is considered the rate-limiting enzyme in this pathway, the regulation of γ-GCS subunits (catalytic subunit, γ-GSCC; modifier subunit, γ-GCSM) has been a topic of extensive research [50]; in our study, vitamin E-deficient rats showed a significant increase in liver mRNA expression of both γ-GCS subunits. This effect was confirmed by the RNApol-ChIP. Using Western blot analysis it was also found that γ-GCSC protein was increased in liver of vitamin E-deficient rats when compared to controls. The fact that liver from vitamin E-deficient rats had increased levels of mitochondrial MDA and decreased concentrations of GSH clearly shows that the compensatory activation in γ-*GCS* expression is insufficient to counterbalance the elevated oxidative stress generation.

Recently, Barella et al. [51] using rats fed vitamin E sufficient diet or control deficient diet had reported that vitamin E supplementation up-regulated γ-*GCSM*; in fact, the diversity of chemical and physical agents that are capable of inducing γ-GCS is quite astounding [52] and includes stresses that produce ROS, glutathione depleting agents and also some antioxidants; nevertheless, at present, it is well established that oxidative stress increased the transcription of γ-*GCS* subunits [50,52], as occurs in our model of vitamin E deficiency. Furthermore, in the signalling process of γ-GCS gene expression, NF-κB plays an important role, since it binds to elements in the promoters of the rat γ-GCSM and γ-GCSC genes [42,43]. Interestingly, we also found a significant increase in the binding of NF-κB in vitamin E-deficient rats for 60 or 90 days, especially at 90 days.

In this work, we also demonstrated an altered expression pattern of genes that participate in the cell cycle control. We found a decrease in the expression of $p21^{(Waf21/Cip1)}$ that encodes a cyclin dependent kinase inhibitor, which is critical in the G1 arrest, and a significant increase in the expression of either cyclin D1 and cyclin E, positive regulators of G1 to S phase transition, which are under control of oxidative

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stress responsive transcription factors, such as NF-κB [53,54]. Western stern blot analysis revealed that the amount of cyclin D1 protein, similar to the results found in the amount of γ-GCSC protein, was also increased in liver of vitamin E-deficient rats when compared to controls. Recently, Fischer et al. [55] reported that the expression of γ-GCSC and G1/S-specific cyclin D1 did not changed in their vitamin E-deficient model, although in combined Se and vitamin E deficiency these genes were differentially expressed. However, in our work we used different experimental techniques and more prolonged period of vitamin E-deficiency (rats are fed on vitamin E-free diet from their birth, through the dam's milk, to 90 days of age, which is longer that the period of deficiency induced in the previous work where rats were fed on vitamin E-free diet from a live weight of 35 g for 49 days).

Our results are in agreement with the mechanism demonstrated for the *in vivo* anticancer protection induced by tocopherols, (α- and γ-tocopherol, mainly the γ isoform), which implies a decreased progression into the S-phase associated with a diminished DNA synthesis via reduction of cyclin D1 and E levels [56,57]. In this context, results from experimental and clinical studies on the anticancer activity of tocopherol analogues have led to their use in order to protect particular populations from cancer hazard [58]. Even so, recent controlled and randomized trials in patients at risk of certain types of cancer—essentially lung and digestive tract cancer—have produced contradictory results about the protective role of liposoluble vitamin supplementation in the incidence of cancer and mortality [59,60]. Nevertheless, in primary malignancies of the liver, as hepatocellular carcinoma, the hepatic tocopherol concentration was significantly reduced [61] and it seems quite recognized that the administration of vitamin E has a protective role either *in vivo*—in chemically induced hepatocarcinogenesis in experimental animals [62] and in transgenic rodents with high prevalence of liver tumors [63]—and *in vitro*—in normal hepatocyte and hepatoma cells [64]. Moreover, other studies had also revealed that the addition *in vivo* or *in vitro* of vitamin E inhibited the NF-κB activation and the altered cell-cycle related gene expression induced by prooxidants as β-carotene [65] or phenobarbital, an efficacious hepatic tumor promoter [66]. However, the potentially chemopreventive effect of tocopherol on human hepatocarcinogenesis is still developing and deserves further investigations.

Moreover, using the ChIP assay analysis we have established that in vitamin E deficient livers NF-κB is bound to the γ-*GCSC* and γ-*GCSM* promoters. The presence of NF-κB in both promoters allows to RNA polymerase II to move to coding region and carry out gene transcription. Accordingly with these results we propose that NF-κB activation directly drives transcription of the genes in response to hepatic vitamin E depletion. NF-κB has been also implicated in the control of cell cycle through transcriptional regulation of *cyclin D1* [45,46,54]. The fact that in our model, NF-κB appears attached to *cyclin D1* promoter simultaneously with gene overexpression may explain the *cyclin D1* expression pattern observed in our vitamin E deficiency model and point out the importance of tocopherols in the control of cell cycle through NF-κB activation.

In conclusion, this study demonstrates that *in vivo* depletion of liver α-tocopherol enhances lipoperoxidation in this tissue, induces liver NF-κB activation and up-regulates *gamma-glutamylcysteine synthetase* (γ-*GCSM*; γ-*GCSC*), *cyclin D1* and *cyclin E*. We also show a down-regulation of *p21 (Waf1/Cip1)* transcription. This altered pattern of gene expression affects both antioxidant-defense genes and genes that control the cell cycle. Besides its well-known chain-breaking antioxidant activity, alternative functions of vitamin E unrelated to its radical chain-breaking potential have been proposed [7–10,67], especially the capacity of α-tocopherol to modulate gene expression. Thus, vitamin E could have, similarly to vitamin A and vitamin D derivatives, cell regulatory properties acting as a "gene regulator" [67]. In fact, we had reported in previous papers that hepatic depletion of vitamin A also alters intracellular redox status and induces liver regulatory properties with changes in proliferative control genes, unrelated specifically with its antioxidant function [41,49].

Furthermore, regulation of gene expression requires transcription factors. Our findings show that chronic vitamin E-deficiency induces significant molecular regulatory properties in liver cells and demonstrate a role for NF-κB activation in this response. Several mechanisms may be responsible for this function. In some studies it has been demonstrated that PKC has been implicated in the signal transduction mediated by vitamin E due to its deactivation by α-tocopherol and its participation in the regulation of different transcription factors [68]. In our study using ChIP assay, we demonstrated that NF-κB directly regulates the transcription of γ-*GCS* (both subunits) and *cyclin D1* through the binding of NF-κB to their corresponding gene promoters, which was enhanced in vitamin E-deficiency. Further investigation would be needed to provide information about the initial events underlying the hepatic response to vitamin E-depletion.

Our results are in accordance with experimental and epidemiological studies that support the protective role of tocopherols for cellular integrity [56,57,69] and emphasize the importance, in a physiological context, of maintaining an adequate vitamin E consumption not only to prevent liver oxidative damage but also to modulate gene expression and signal transduction.

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