Novel Transcriptional Activities of Vitamin E: Inhibition of Cholesterol Biosynthesis†

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ABSTRACT: Vitamin E is a dietary lipid that is essential for vertebrate health and fertility. The biological activity of vitamin E is thought to reflect its ability to quench oxygen- and carbon-based free radicals and thus to protect the organism from oxidative damage. However, recent reports suggest that vitamin E may also display other biological activities. Here, to examine possible mechanisms that may underlie such nonclassical activities of vitamin E, we investigated the possibility that it functions as a specific modulator of gene expression. We show that treatment of cultured hepatocytes with (*RRR*)-α-tocopherol alters the expression of multiple genes and that these effects are distinct from those elicited by another antioxidant. Genes modulated by vitamin E include those that encode key enzymes in the cholesterol biosynthetic pathway. Correspondingly, vitamin E caused a pronounced inhibition of de novo cholesterol biosynthesis. The transcriptional activities of vitamin E were mediated by attenuating the post-translational processing of the transcription factor SREBP-2 that, in turn, led to a decreased transcriptional activity of sterol-responsive elements in the promoters of target genes. These observations indicate that vitamin E possesses novel transcriptional activities that affect fundamental biological processes. Cross talk between tocopherol levels and cholesterol status may be an important facet of the biological activities of vitamin E.

The term vitamin E refers to a family of structurally related neutral plant lipids that are critical for vertebrate health and fertility. Numerous studies established that members of the vitamin E family are efficient chain-breaking radical scavengers both in vitro and in vivo and led to the definition of vitamin E as the most important lipid-soluble antioxidant (*1*). While all isoforms of vitamin E possess comparable radical trapping activity in vitro (*2*), (*RRR*)-α-tocopherol (denoted herein as αTOH[1]) exhibits the highest potency in biological assays (*3*). This vitamer preference stems from the combined activities of the hepatic α-tocopherol transfer protein (TTP) that selectively retains αTOH (*3*−*5*) and the catabolic actions of hepatic enzymes that degrade other forms of vitamin E to water-soluble products (e.g., ref *6*).

The selectivity for αTOH raises the possibility that this vitamer has unique biological functions. Indeed, novel regulatory properties have been ascribed in recent years to αTOH, including the modulation of apoptosis, cell adhesion, and specific enzymatic activities (cf. refs *7* and *8*). Moreover, αTOH was shown to regulate the expression levels of several mRNAs and proteins, such as collagen α1 (*9*), the scavenger receptors SR-BI (*10*) and CD36 (*11*, *12*), α-tropomyosin (*13*), the nuclear receptor PPARγ (*14*), and the adhesion molecule VCAM-I (*15*). These anecdotal observations were further supported by several studies that documented genomic responses to vitamin E using expression-profiling approaches (*16*−*23*). However, interpretation of such analyses is complicated by the known genomic responses to changes in cellular redox status (*24*). Indeed, treatment with vitamin E elicits pronounced changes in the expression of known antioxidant-responsive genes, such as glutathione S-transferase, cytochrome P450-1A2, catalase, superoxide dismutase, and metallothioprotein (*18*, *19*, *22*). Thus, at present, only very limited information is available regarding the molecular mechanisms that mediate genomic responses to vitamin E. Here, we report the identification of specific genomic activities of αTOH that are distinct from those induced by another antioxidant and provide insights into the molecular mechanisms that mediate these activities.

EXPERIMENTAL PROCEDURES

Cell Culture. HepG2 C3A cells were cultured at 37 °C and 5% CO_2 with Dulbecco's modified Eagle's medium (CellGro) supplemented with 10% FBS (Atlanta Biologicals) or LPDS (Sigma Chemical Co.). Antioxidants were added either as preformed serum complexes (*25*−*27*) or from ethanol stocks directly to the media. Both methods resulted in comparable uptake efficiencies as determined by accumulation of radioactively labeled tocopherol. Final ethanol concentrations did not exceed 0.2% (v/v). Unless otherwise indicated, treatments lasted 48 h, with fresh medium change

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; αTOH, (*RRR*)-α-tocopherol; FBS, fetal bovine serum; LPDS, lipoproein-deficient serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; DCFDA, 2′,7′-dichlorodihydrofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; ROS, reactive oxygen species; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A, SREBP-2, sterol-responsive element-binding protein 2; BHT, butylated hydroxytoluene.

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after 24 h. Cell viability was evaluated using an MTT reduction conversion assay (*28*; Sigma Chemical Co.).

Microarray Analyses. Total RNA was isolated using the TRIzol reagent (Invitrogen), and RNA quality was evaluated using the 2100 bioanalyzer (Agilent Technologies). Double-stranded cDNA was synthesized using a SuperScript II reverse transcriptase kit (Invitrogen). Phase Lock Gel, phenol/chloroform extraction, and ethanol precipitation were employed to purify the resultant cDNA. cRNA was synthesized using the GeneChip IVT labeling kit (Affymetrix). In vitro transcription was carried out for 4 h at 37 °C, using biotinylated ribonucleotides (Enzo Diagnostics) for labeling. Labeled cRNA was purified with an RNeasy kit (Qiagen) and fragmented in fragmentation buffer for 30 min at 94 °C. Fragmented cRNA was hybridized to human U133A GeneChip microarrays (Affymetrix) in a rotating hybridization oven for 16 h at 45 °C. Staining was performed on an Affymetrix fluidics station utilizing streptavidin/phycoerythrin conjugate (Molecular Probes), followed by biotinylated antibody to streptavidin (Vector Laboratories), and finally via a second streptavidin/phycoerythrin conjugate. Stained microarrays were scanned on a Hewlett-Packard GeneArray scanner, and data were compiled with Affymetrix Microarray Suite 5.0 software. Each treatment condition was repeated in three independent cultures, each of which was subjected to three independent hybridization reactions, for a total of nine microarrays per experimental condition.

Data Analyses. Intensity data from each of the nine microarrays were uploaded to GeneTraffic version 2.8 (Iobion). Raw data were normalized using robust multichip analysis, and *p* values were calculated using an F-class ratio with variance stabilization, which served as a measure of the difference between the normalized intensity value means of various treatment groups relative to the variability of intensity values of each replicate within a given treatment group. Significantly regulated gene lists ($p < 0.05$, absolute fold change greater than 1.2) were then manually clustered on the basis of known cellular function.

Measurements of Intracellular ROS. Triplicate wells of HepG2 C3A cells in 96-well plates were serum-starved for 72 h, washed twice with minimum essential medium lacking phenol red (CellGro), and supplemented with various concentrations of *N*-acetyl-L-cysteine (NAC) or αTOH (delivered from ethanolic stocks) for 24 h. The cell-permeable dye DCFDA (Molecular Probes) was then added to 5 μg/mL in Hank's buffered saline solution lacking phenol red (Sigma Chemical Co.) for 2 h at 37 °C. DCFDA rapidly diffuses across cell membranes, where the diacetate moiety is cleaved by intracellular esterases to yield 2′,7′-dichlorodihydrofluorescein that oxidizes upon interaction with ROS to the highly fluorescent DCF that serves as a sensitive quantitative readout for ROS levels in live cells (*29*, *30*). DCF fluorescence was measured with a Tecan SpectraFluor Plus microplate reader (excitation, 485 nm; emission, 535 nm). The assay range and linearity were ensured using known concentrations of hydrogen peroxide.

Validation of Microarray Results. Total RNA was isolated from treated cells, and cDNA was prepared as described above. The expression profiles of selected genes were quantified, in triplicate, on a 7500 Real Time RT-PCR system instrument (Applied Biosystems). FAM dye-labeled Taqman MGB probes (Applied Biosystems) were utilized to assess mRNA levels of the genes encoding HMG-CoA reductase, the low-density lipoprotein (LDL) receptor, and A-kinase anchor protein 12 (AKAP12). Threshold cycle (Ct) values were determined for each replicate and normalized to β-actin or 18S mRNA levels detected by the same procedure. These measurements were then converted to fold change values. Where indicated, actinomycin D (Sigma) was added from DMSO stock to 1 μM.

Reporter Gene Assays. HepG2 C3A cells grown in MEM/10% LPDS were transiently transfected with a pGL3-basic vector encoding firefly luciferase under the control of the SREBP2 promoter (generous gift of Dr. Timothy Osborne, University of California Irvine; see ref *31* for details) using the Fugene6 reagent (Roche). Two hours after transfection, triplicate wells were treated as indicated for 36 h and lysed, and luciferase expression was assayed using a commercial kit (Promega) on a Berthold luminometer. Where indicated, the single sterol response element (SRE) sequence in this promoter was mutated to change the wild-type SRE (5′-ATCACCCCAT-3′) to a nonfunctional response element (5′-ATAAAAAAAT-3′) using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing.

Measurements of SREBP-2 Processing. Chinese hamster ovary (CHO) cells were propagated in Ham's F-12 medium supplemented with 10% FBS and then adapted to LPDS for 2 days. Mevalonic acid (50 μM, Sigma Chemical Co.) and other treatments were added as indicated, after which the cells were treated with 10 μM MG132 for an additional 5 h. The cells were then harvested and nuclear extracts prepared as described previously (*32*). Briefly, scraped cells were washed with cold PBS and resuspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 M EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After the cells were swelled for 15 min on ice, NP-40 was added to 0.58% and the samples were mixed vigorously and microfuged at 3000*g* for 30 s. The pellet was resuspended in 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF and rocked at 4 °C for 15 min, and the nuclear extracts were obtained after microcentrifugation at 14 000 rpm for 5 min at 4 °C. The presence of processed SREBP-2 in the nuclear fraction was measured by anti-SREBP-2 immunoblotting (Caymen Chemicals, antibody 10007663). Equal loading was ensured by immunoblotting with antibodies for proliferating cell nuclear antigen (PCNA; cell signaling clone PC10). Fractionation of HepG2 cells into nuclear and membranous fractions (Figure 4A) was done according to the method of Goldstein and Brown (*33*). Immunoblots were quantified by densitometry using Scion Image software (Scion Corp.).

Measurements of de Novo Cholesterol Biosynthesis. De novo biosynthesis of cholesterol was measured by following the incorporation of radiolabeled acetate into cholesterol following the protocol of Goldstein and Brown (*34*). HepG2 C3A cells were cultured in six-well plates for 18 h and washed with PBS, and the medium was changed to DMEM + 10% lipoprotein-deficient serum (LPDS; Sigma Chemical Co.). Twenty-four hours later, triplicate wells were treated with one of the following: 1−100 μM αTOH, 10 μg/μL cholesterol + 0.1 μg/μL 25-hydroxycholesterol, 1 mM NAC, or vehicle control (ethanol). After 24 h, the cells were pulsed with 2 μCi of [1-^{14}C]acetic acid (2.11 GBq/mmol, 57.0 mCi/

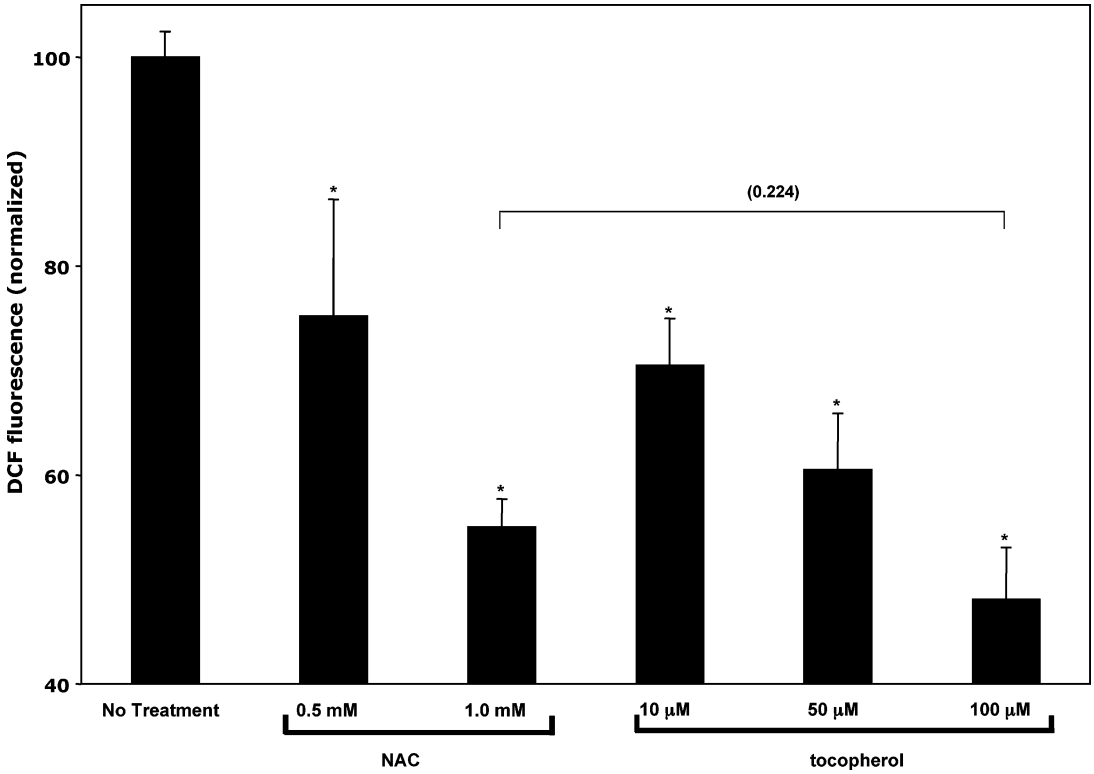

FIGURE 1: Quenching of intracellular reactive oxygen species by NAC and αTOH. Confluent HepG2 cells were serum-starved for 72 h, washed twice with minimum essential medium lacking phenol red, and treated with the indicated concentrations of NAC or αTOH for 24 h. Reactive oxygen species levels were measured using DCFDA fluorescence as described in the Experimental Procedures. Shown are means and standard deviations from three independent experiments. Fluorescence values were normalized to those obtained from untreated cells (36 011 fluorescence units). Asterisks denote statistically significant changes from control samples, as determined by a Student's *t*-test. *P* values obtained from statistical analyses are shown in parentheses.

mmol, GE Amersham) for 6 h. The cells were washed with PBS, and lipids were extracted by incubation with 2 mL of 3:2 hexane/2-propanol at 25 °C for 30 min. Solvent was then evaporated under a N_2 stream, and the lipids were resuspended in 60 μL of hexane and resolved by thin layer chromatography (TLC) developed with 80:20:1 petroleum ether/diethyl ether/acetic acid. Resolved lipids were visualized with iodine vapor, and radioactive incorporation into cholesterol was quantified by scintillation counting.

RESULTS

The main goal of the studies presented here was to evaluate the impact of vitamin E (and, specifically, αTOH) on gene expression in human hepatocytes. A possible confounding factor in such experiments stems from the nonspecific effects that antioxidants have on multiple redox-responsive cellular targets. To examine the unique activity of αTOH, we compared its transcriptional activity effects to those of another established antioxidant, NAC (*35*). We hypothesized that specific targets of vitamin E actions would respond to vitamin E treatment, but not to NAC treatment. To compare the efficacy of αTOH and NAC in quenching reactive oxygen species in our cell system, we employed DCF, a cell-permeable fluorescent probe that quantitatively reports on intracellular ROS levels (*29*, *36*−*38*). Data in Figure 1 show that treatment of HepG2 cells with either NAC or αTOH caused a dose-dependent decrease in cellular ROS levels. Importantly, 100 μM αTOH elicited a ca. 50% reduction in cellular ROS levels, similar to the change induced by NAC. These results show that treatment of HepG2 cells with 1 mM NAC is an appropriate control for the antioxidant effects of 100 μM αTOH.

To evaluate the specific effects of vitamin E on hepatic gene expression, we determined the global expression profile of HepG2 cells that were treated with 100 μM αTOH and compared it with the expression profile of untreated cells or those treated with 1 mM NAC. RNA extracted from the different experimental groups was processed as described in the Experimental Procedures and hybridized to Affymetrix U133A expression microarrays (Affymetrix) on which ca. 22 000 transcripts and ESTs are displayed, representing approximately 14 500 unique human genes.

In three independent array experiments, 57 genes displayed statistically significant differences in expression levels between the αTOH treatment group and untreated controls ($p < 0.05$). Of these, 29 were up-regulated by αTOH, while the expression of 28 genes was repressed by this treatment. Changes in the expression levels of these genes were in the 1.2−2.0-fold range. The affected genes can be grouped into four major categories when clustered on the basis of known cellular functions: cell proliferation, lipid status, protein stability and metabolism, and transcriptional regulation (Table 1). Treatment with αTOH caused specific and significant reduction in the expression levels of 17 genes that play central roles in regulating cellular lipid status (Table 2). Interestingly, 10 of these αTOH-responsive gene products catalyze key steps in the de novo biosynthesis of cholesterol: dehydrocholesterol reductase, farnesyl diphosphate synthase, HMG-CoA synthase, isopentenyl-diphosphate δ isomerase, lanosterol synthase, lathosterol oxidase, squalene

Table 1: Functional Clustering of TOH-Responsive Genes

cellular function	total no. of TOH-responsive genes[a]	no. of genes up-regulated by TOH treatment	no. of genes down-regulated by TOH treatment
cell proliferation	23	19	4
lipid status	17	0	17
protein stability and metabolism	9	6	3
transcriptional regulation	5	3	2
other	3	1	2
total	57	29	28

[a] $p < 0.05$, absolute fold change >1.2.

monooxygenase, squalene synthase, and sterol-*C*4-methyl oxidase (Table 2). Notably, mRNA levels of the HMG-CoA reductase gene, encoding the rate-limiting enzyme in cholesterol biosynthesis (*39*), and of the low-density lipoprotein (LDL) receptor, a key regulator of cholesterol transport (*40*), were also significantly reduced by TOH treatment (Table 2). Importantly, we observed that none of these transcripts were affected by NAC treatment.

To verify the results obtained from microarray analyses, we employed quantitative, real-time RT-PCR. The data show that αTOH treatment caused a significant decrease in the levels of mRNAs encoding the HMG-CoA reductase and the LDL receptor and that the magnitude of the effect was similar to that observed in expression arrays (Figure 2). Real-time RT-PCR of the signal transducer AKAP12 transcript revealed that αTOH treatment caused an effect similar to that observed in the expression arrays (i.e., 2−3-fold increase in mRNA levels, Figure 2A). These results indicate that the effects of αTOH on gene expression observed in the microarray experiment were both significant and reproducible. Additional experiments revealed that the vitamin E-induced changes in the HMG-CoA reductase transcript were specific and did not occur following treatments with the soluble antioxidant NAC or the lipid-soluble antioxidant BHT (Figure 2B). These observations raise the possibility that the regulation of HMG-CoA reductase transcript stems from specific actions of vitamin E.

The vitamin E-induced decreases in transcript levels observed in cholesterol homeostatic genes could in principle result from inhibition of transcription or, alternatively, from facilitation of mRNA degradation. To discriminate between these possibilities, we measured the effect of vitamin E on gene expression in the presence of actinomycin D, a selective inhibitor of DNA-directed RNA synthesis (*41*). Cells were treated with 1 μM actinomycin D together with 100 μM αTOH (or ethanol), and the levels of mRNAs encoding HMG-CoA reductase and the LDL receptor were measured using real-time RT-PCR. We observed that, in the presence of the transcriptional inhibitor actinomycin D, vitamin E did not affect the expression of these transcripts (data not shown), demonstrating that αTOH attenuates the mRNA levels of cholesterol homeostatic genes by inhibiting transcription and not by affecting mRNA stability.

Molecular Mechanisms of α*TOH Action.* The 10 cholesterol homeostatic genes that are affected by vitamin E treatment share common regulatory features in their promoter regions. Specifically, the sequence 5′-ATCACCCCAT-3′ is found upstream of the transcription start sites of all of these genes. This conserved regulatory sequence, known as the SRE, is the binding site for the transcription factor SREBP-2, which mediates many established responses to sterols (*42*). To examine whether SREBP-2 mediates the transcriptional activities of vitamin E, we employed a reporter construct in which the luciferase gene is under the regulation of an SRE-containing promoter (the SREBP-2 promoter, possessing a single SRE at position −293 (*31*)). We transfected the reporter construct into HepG2 cells and monitored the effect of αTOH treatment on luciferase expression. Inhibiting cholesterol biosynthesis by treatment of the cells with the HMG-CoA reductase inhibitor lovastatin caused a ca. 2-fold increase in luciferase expression (Figure 3A), as anticipated from the established response of the SREBP-2 promoter to depletion of cellular sterol pools (*43*). Conversely, increasing cholesterol levels by the addition of exogenous (cholesterol-rich) serum lipoproteins caused a ca. 20% repression of promoter activity (Figure 3A). This feedback inhibition of SRE-containing promoters by sterols is an established feature of cholesterol homeostatic regulation (*31*, *44*−*46*). Importantly, we observed that treatment of the cells with vitamin E led to a dose-dependent attenuation of reporter gene expression, reaching approximately 50% inhibition at 100 μM αTOH. The inhibitory effect of αTOH on promoter

Table 2: Effect of αTOH on the Expression of Lipid Homeostatic Genes

fold change[a]	gene identity	accession no.
−1.37	7-dehydrocholesterol reductase[b]	NM_001360
−1.33	acetoacetyl coenzyme A thiolase	NM_005891
−1.28	cytochrome P450, family 51, A1	NM_000786
−1.34	farnesyl diphosphate synthase[b]	NM_002004
−1.41	HMG-CoA reductase[b]	NM_000859
−1.55	HMG-CoA synthase[b]	NM_002130
−1.42	hydroxysteroid (17-β) dehydrogenase	NM_016371
−1.47	isopentenyl-diphosphate δ isomerase[b]	NM_004508
−1.27	lanosterol synthase[b]	NM_002340
−1.29	lathosterol oxidase[b]	NM_006918
−1.21	low-density lipoprotein receptor	NM_000527
−1.22	phosphate cytidylyltransferase 2, ethanolamine	NM_002861
−1.32	squalene monooxygenase[b]	NM_003129
−1.27	squalene synthase[b]	NM_004462
−1.28	stearoyl-CoA desaturase (δ-9-desaturase)	NM_005063
−1.58	sterol-*C*4-methyl oxidase[b]	NM_006745

[a] $p < 0.05$. [b] Gene products that catalyze reactions in the de novo cholesterol biosynthetic pathway.

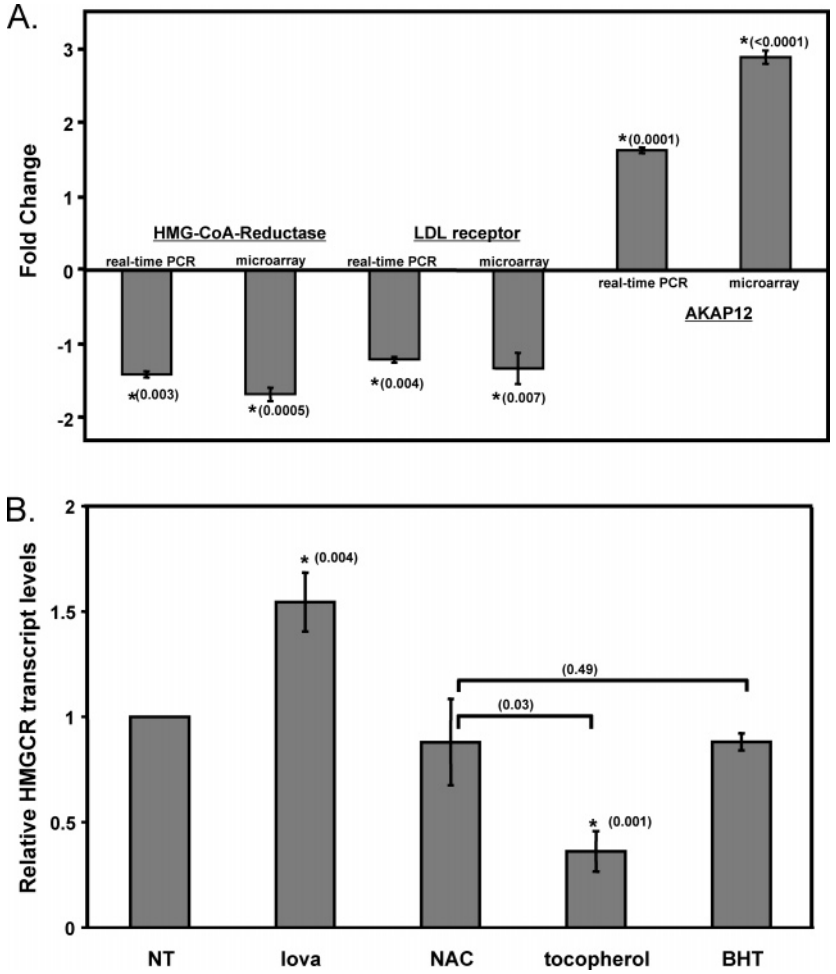

FIGURE 2: Validation of microarray data: (A) comparison of real-time RT-PCR and microarray data, (B) effect of different antioxidants on HMG-CoA reductase gene expression. HepG2 cells were treated for 24 h with 100 μM αTOH (or ethanol as the vehicle control), 50 μM lovastatin, 1 mM NAC, or 200 μM BHT, and total RNA was isolated and utilized for cDNA synthesis. Expression levels of the indicated genes were quantified by real-time RT-PCR, as described in the Experimental Procedures. Shown are averages and standard deviations of three independent experiments. Asterisks denote statistically significant changes from control samples, as determined by a Student *t* test. *p* values obtained from statistical analyses are shown in parentheses. NT = nontreated.

activity does not reflect a general cell response to antioxidants or the redox state, as NAC did not affect promoter activity (Figure 3A). To implicate specific promoter regions in the transcriptional responses to vitamin E, we ablated the single SRE sequence in the luciferase reporter construct using site-directed mutagenesis (see the Experimental Procedures). As expected, the SRE-defective promoter no longer responded to alterations in cholesterol status induced by lovastatin and serum (Figure 3B). Importantly, mutating the SRE sequence also completely abolished transcriptional responses to αTOH (Figure 3B). As the experimental treatments used in these experiments did not affect cell viability (data not shown), we conclude that αTOH inhibits the expression of these cholesterol homeostatic genes by attenuating SRE-mediated transcriptional responses.

Physiological regulation of SREBP-2 by sterols occurs through post-translational proteolytic activation of the SREBP-2 transcription factor that controls its translocation from the endoplasmic reticulum to its site of action in the nucleus (*47*). We examined whether the transcriptional effects of vitamin E, like those of sterols, are mediated by post-translational processing of SREBP-2. To this end, we fractionated treated HepG2 and CHO cells and assayed the levels of proteolytically activated SREBP-2 by immunoblotting. As expected, processing of SREBP-2 was enhanced when cholesterol levels were depleted (i.e., in the presence of lovastatin) and reduced upon the addition of exogenous sterols, as shown in Figure 4. Treatment with vitamin E elicited a dose-responsive decrease in the amount of activated (nuclear) SREBP-2, reaching ca. 50% inhibition at 100 μM αTOH, similar to the magnitude of inhibition elicited by sterols (Figure 4).

These results indicate that vitamin E attenuates the transcriptional responses of sterol response elements in the promoter regions of cholesterol homeostatic genes and that these events are mediated by the actions of SREBP-2.

Attenuation of de Novo Cholesterol Biosynthesis by αTOH in Vivo. The observations that tocopherol specifically attenuates the expression of key regulators of cholesterol homeostasis raise the possibility that vitamin E levels impact the cellular cholesterol status. To evaluate the physiological significance of these findings, we examined whether treatment with vitamin E influences the rate of de novo cholesterol biosynthesis in cultured cells. HepG2 cells were incubated with the radiolabeled precursor [^{14}C]acetate for 6 h, and incorporation of the label into cholesterol was measured after lipid extraction and thin layer chromatography. Treatment with vitamin E resulted in a dose-dependent

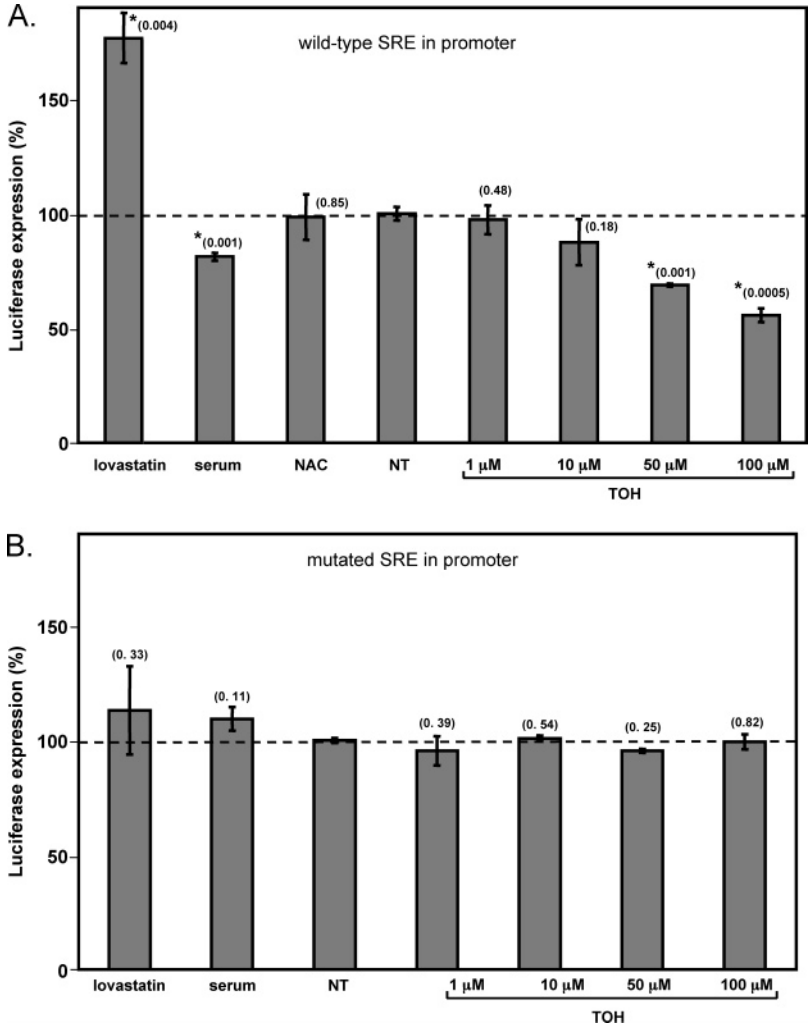

FIGURE 3: Vitamin E inhibits transcription of an SRE-driven reporter gene. HepG2 cells were transiently transfected with 0.5 μg of a pGL3-basic plasmid encoding the structural gene for firefly luciferase driven by the SREBP-2 promoter containing either the wild-type (A) or a mutated (B) SRE sequence. Two hours after transfection, the cells were treated with 10% FBS, 1 mM NAC, 1 μM lovastatin, or the indicated tocopherol concentration for 36 h. Luciferase expression was measured in clarified lysates as described in the Experimental Procedures. Shown are means and standard deviations of three independent experiments. Luminescence readings were normalized to those measured in untreated lysates (1.2×10^6 luminescence units). Asterisks denote statistically significant changes from control samples, as determined by a Student *t* test. *p* values obtained from statistical analyses are shown in parentheses. NT = nontreated.

inhibition of cholesterol biosynthesis, reaching ~30% inhibition at 100 μM αTOH (Figure 5). The magnitude of inhibition by αTOH was comparable to that induced by established physiological attenuators of the cholesterol biosynthetic pathway, namely, high sterols (10 μg/μL cholesterol + 0.1 μg/μL 25-hydroxycholesterol). Importantly, suppression of cholesterol biosynthesis was a specific attribute of vitamin E, as treatment with the vehicle control (ethanol) or the general antioxidant NAC did not affect incorporation of acetate into cholesterol (Figure 5). These findings indicate that the specific effects of αTOH on the cholesterol biosynthetic pathway are significant at the physiological end point of the pathway, namely, the production of cholesterol.

DISCUSSION

It is commonly believed that the biological requirement for vitamin E stems from the ability of the vitamin to scavenge intracellular free radicals. By counteracting the harmful actions of reactive oxygen species and lipid radicals, the vitamin is thought to prevent cellular damage and the onset of related pathologies. In support of this notion, vitamin E-deficient humans and animals display pathologies that are commonly attributed to elevated oxidative stress, such as atherosclerosis and neuronal degeneration. However, recent observations suggest that members of the vitamin E family possess biological activities in addition to those elicited by other antioxidants. Thus, tocopherol was shown to influence apoptosis (*48*−*50*) and proliferation (*51*) and to modulate the activity of some enzymes (*52*−*55*). An additional striking "nonclassical" effect of vitamin E is its documented ability to modulate the expression of certain mRNAs and proteins. Recently, a number of groups utilized cDNA microarray approaches to determine the effect of vitamin E on mRNA expression profiles. Gohil et al. (*16*, *21*−*23*) compared the gene expression profiles in the brains and livers from $TTP^{-/-}$ (vitamin E-deficient) mice with those from wild-type mice and reported pronounced differences (2−20-fold) in the levels of multiple transcripts that play important roles in myriad biological functions. Similarly, Barella and colleagues compared the expression profile of vitamin E-depleted rats with those obtained from normal animals (*17*−*19*, *56*). A

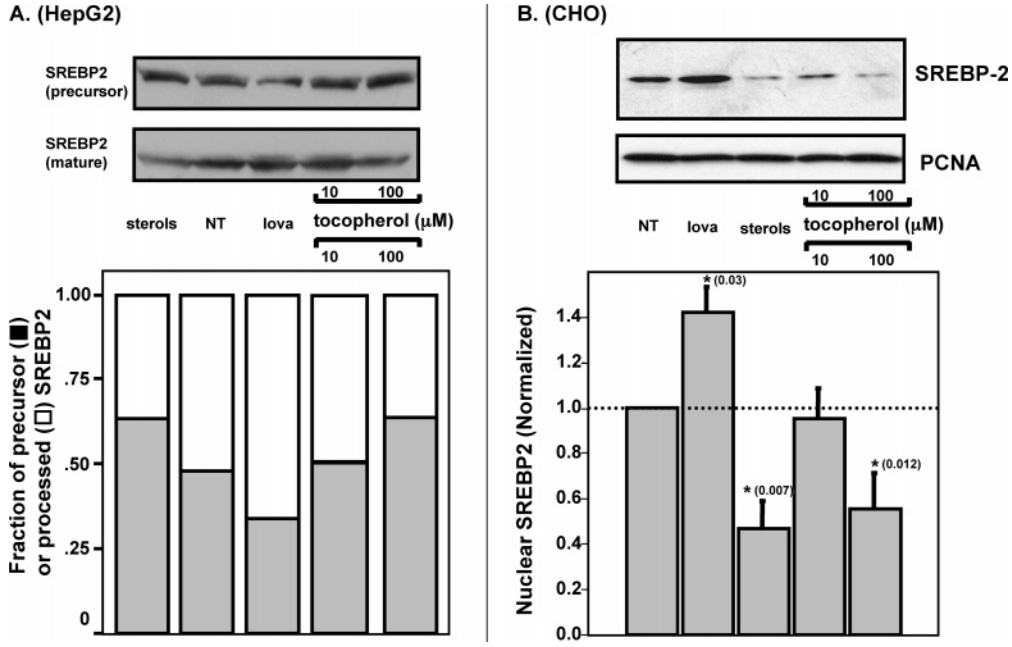

FIGURE 4: Vitamin E inhibits proteolytic activation of SREBP-2. HepG2 cells (A) or CHO cells (B) were cultured in media containing 10% LPDS and treated with 50 μM lovastatin, 10 μg/μL cholesterol + 0.1 μg/μL 25-hydroxycholesterol, or the indicated concentration of αTOH for 24 h. The cells were then treated with 10 μM MG132 for 5 h and washed and SREBP-2 levels assessed by anti-SREBP-2 immunoblotting. (A) Representative analysis of nuclear and membrane fractions from treated HepG2 cells. (B) Representative analyses of nuclear fractions from treated CHO cells. Shown are averages and standard deviations. Asterisks denote statistically significant changes from control samples, as determined by a Student *t* test. *p* values obtained from statistical analyses are shown in parentheses. NT = nontreated.

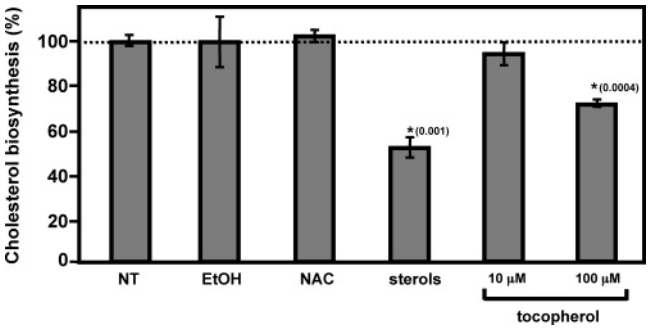

FIGURE 5: Vitamin E attenuates de novo cholesterol biosynthesis. HepG2 cells were cultured in 10% LPDS and treated for 24 h as indicated in the text. [1-^{14}C]Acetic acid (2 μCi) was added to the culture medium for 6 h, prior to lipid extraction and chromatographic analysis of labeled cholesterol. Shown are averages and standard deviations of three independent experiments, after normalization to nontreated samples (12 104 cpm). Asterisks denote statistically significant changes from control samples, as determined by a Student's *t*-test. *P* values obtained from statistical analyses are shown in parentheses. NT = nontreated.

major confounding factor in interpreting the data from these studies stems from the established genomic responses to changes in the redox status. Vertebrate cells have evolved sensitive mechanisms to detect changes in the levels of oxygen, oxygen radicals, and reducing agents and to relay this information to specific transcription factors, such as the hypoxia-responsive HIF-1 (*24*), the redox-responsive NF-kB (*57*), and the antioxidant-responsive Nrf2 (*58*, *59*). Thus, treatment of cells with vitamin E can (and is likely to) lead to transcriptional responses that originate from changes in the cellular redox status and are *not* vitamin E-specific. Indeed, the aforementioned expression profiling experiments documented pronounced changes in the expression of multiple known redox-sensitive genes.

To isolate genomic responses that are specific to vitamin E, we focused our attention on αTOH-responsive mRNAs that were not affected by treatment with another antioxidant, NAC. The data revealed a limited number of transcripts that are attenuated specifically by vitamin E. Of special interest are the observations that genes involved in maintaining lipid homeostasis are down-regulated by vitamin E. Of these, 10 encode enzymes that catalyze important steps in the de novo biosynthesis of cholesterol, including the rate-limiting enzyme of this pathway, HMG-CoA reductase. The data further demonstrate that these specific genomic responses to vitamin E are mediated by the sterol-responsive transcription factor SREBP-2. Moreover, αTOH appears to elicit its regulatory action on SREBP-2 through a mechanism similar to that employed by sterols, namely, by attenuating the proteolytic activation of SREBP-2 and its translocation into the nucleus. The transcriptional activities of vitamin E have a marked physiological impact in that treatment with αTOH caused a pronounced inhibition of de novo cholesterol biosynthesis.

The precise molecular mechanisms by which vitamin E influences SREBP-2 activity are not completely understood. It is possible that αTOH shares the sensing mechanism utilized for sterols, i.e., binding to sterol-sensing domains such as that found in the sterol sensor SCAP (*60*, *61*). Alternatively, novel and specific mechanisms may exist that convey information about tocopherol levels to the transcriptional machinery.

Our observations suggest that a "cross talk" exists between the physiological levels of vitamin E and the mechanisms that regulate cholesterol homeostasis. Thus, one would expect that, in whole animals, vitamin E levels will impact the overall cholesterol status. Indeed, it was reported that increased dietary intake of vitamin E is accompanied by a dose-dependent reduction in total serum cholesterol in the rat, quail, and rabbit (*62*−*67*). The impact of vitamin E on

the health risk in human populations still awaits complete resolution (*68*−*73*). The observations described in this study reinforce the notion of physiologically relevant nonclassical activities of vitamin E and reveal that key roles of the vitamin in supporting human health may be mediated by its ability to regulate the rates of transcription of specific target genes.

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