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Expression of the Alpha Tocopherol Transfer Protein gene is regulated by Oxidative Stress and Common Single Nucleotide Polymorphisms

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Abstract

Vitamin E (α -tocopherol) is the major lipid soluble antioxidant in most animal species. By controlling the secretion of vitamin E from the liver, the α -tocopherol transfer protein (α TTP) regulates whole-body distribution and levels of this vital nutrient. However, the mechanism(s) that regulate the expression of this protein are poorly understood. Here we report that transcription of the *TTPA* gene in immortalized human hepatocytes (IHH) is induced by oxidative stress and by hypoxia, by agonists of the nuclear receptors PPAR α and RXR, and by increased cAMP levels. The data show further that induction of *TTPA* transcription by oxidative stress is mediated by an already-present transcription factor, and does not require *de novo* protein synthesis. Silencing of the cAMP response element binding (CREB) transcription factor attenuated transcriptional responses of the *TTPA* gene to added peroxide, suggesting that CREB mediates responses of this gene to oxidative stress. Using a 1.9 Kb proximal segment of the human *TTPA* promoter together with site-directed mutagenesis approach, we found that single nucleotide polymorphisms (SNPs) that are commonly found in healthy humans dramatically affect promoter activity. These observations suggest that oxidative stress and individual genetic makeup contribute to vitamin E homeostasis in humans. These findings may explain the variable responses to vitamin E supplementation observed in human clinical trials.

Keywords

tocopherol; oxidative stress; single nucleotide polymorphism

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INTRODUCTION

Vitamin E is a collective name for a family of neutral plant lipids (tocols), of which α -tocopherol is selectively retained in the vertebrates and hence is considered the most biologically active form. α -tocopherol's characteristic hydrophobicity, localization in cell membranes and efficacy in scavenging lipid radicals contributed to its common definition as an important lipid-soluble antioxidant in humans (1–4). Accordingly, vitamin E status is thought to influence risk for diseases with an oxidative stress component, including Alzheimer's Disease (AD), Downs syndrome (DS), cardiovascular disease, diabetes, and ataxia (5-13). Possible antioxidant-independent actions of vitamin E have also been raised (see (14,15) for discussions).

The extreme hydrophobicity of α -tocopherol poses a major thermodynamic barrier to its distribution and transport through the aqueous milieu of the cytosol and circulation. In the liver, α -tocopherol is bound to alpha tocopherol transfer protein (α TTP) that catalyzes its transport between intracellular membranes and facilitates incorporation of the vitamin into lipoproteins for delivery of the vitamin to other tissues (16,17). Consequently, α TTP regulates whole-body levels and distribution of α -tocopherol. The critical function of α TTP in regulating vitamin E homeostasis is underscored by observations that heritable mutations in the *TTPA* gene result in ataxia with vitamin E deficiency (AVED; OMIM #277460), characterized by progressive spinocerebellar ataxia and low serum α -tocopherol levels (18,19). Similarly, α TTP-null mice present low vitamin E levels, ataxic phenotype and increased levels of oxidative stress markers in the plasma, brain, heart, lung and placenta (20–23). Importantly, the linear correlation between plasma concentrations of α -tocopherol and α TTP dosage in the *Ttpa*^{+/+}, *Ttpa*^{+/-} and *Ttpa*^{-/-} mice (24) indicate that α TTP determines systemic vitamin E levels. In support of such relationship we recently demonstrated that the anti-proliferative effect of α -tocopherol in prostate cancer cells is linearly correlated to cellular α TTP expression levels (25).

α TTP displays a distinctly narrow tissue expression profile. It is highly expressed in the liver and to a lesser extent in the cerebellum and prefrontal cortex of the brain, kidney, and lung (21,26). α TTP is also expressed in human placental trophoblasts and in murine uterus (24,27–29), where it may regulate delivery of α -tocopherol to the developing embryo. Despite the well-documented role of α TTP as an indispensable protein in maintaining normal α -tocopherol levels, the mechanism(s) that control the tissue-specific expression of the protein remain incompletely understood.

The majority of studies that address the regulation of α TTP levels focused on the relationship between the protein and its ligand, α -tocopherol. It has been reported that dietary vitamin E deficiency lowered α TTP protein levels in the rat, suggesting that α -tocopherol stabilizes the protein (30). Indeed, we recently found that α -tocopherol protects α TTP from ubiquitination and proteosomal degradation (31). While in some *in vivo* studies vitamin E status did not affect α TTP protein levels (32–34), other reports indicated that dietary vitamin E depletion (35) or repletion (33) markedly influenced mRNA levels. Dietary conjugated linoleic acid was recently shown to increase expression of hepatic α TTP (34,36). Other studies examined whether oxidative stress modulates α TTP expression. Thus, it was reported that hyperoxia decreased α TTP expression in rat liver (37), that smoke-induced oxidative stress did not affect hepatic α TTP protein levels in the mouse (32), and that in the zebrafish and in cultured human trophoblast, pro-oxidants increased α TTP expression (38,39). In summary, available information does not provide us with a thorough and consistent understanding regarding the mechanisms that regulate α TTP levels.

In the studies described here, we began to uncover the mechanisms that regulate transcription of the *TTPA* gene. Specifically, we report our findings regarding the transcriptional responses of the *TTPA* gene to oxidative stress, vitamin E, cAMP, and modulators of two nuclear receptors. Furthermore, we report on the functional outcomes of single nucleotide polymorphism in the *TTPA* promoter that are commonly found in healthy humans.

METHODS

Cell lines

Since expression of aTTP in primary hepatocytes dramatically decreases following isolation (REF), we employed immortalized human hepatocytes (IHH) that endogenously express the protein (31) as a model system. IHH (generous gift from R. Ray, St. Louis University, St. Louis, MO) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (Hyclone Laboratories, Logan, UT) as described in (40).

CREB knock-down

Lentiviral shRNA constructs targeted against human CREB (TRCN000007308, or a control shRNA) in the pLKO vector were transfected into HEK293T cells using Lipofectamine-Plus (Invitrogen, Carlsbad, CA). Culture media from 100-mm dishes were harvested 24 and 48 hours post-transfection, pooled, and virus particles pelleted by centrifugation at $100,000 \times g$ for 1.5 hours. The resuspended lentivirus was transduced with polybrene (4 $\mu\text{g}/\text{ml}$) into IHH cells. Twenty-four hours after infection, the cells were infected with another lentivirus dose and treated with 200 μM of hydrogen peroxide for 3 hours, and lysed 24 hours later. Knock-down efficiency was evaluated by immunoblotting using a rabbit anti-human anti-CREB antibody (generous gift of Dr. Ed Greenfield; CWRU, Cleveland, OH).

Cell treatments and RNA harvest

To identify chemical modulators of *TTPA* transcription, IHH cells were treated for 3 hours with 1 μM of GW0742 (PPAR δ agonist), WY14643 (PPAR α agonist), TNF α , Troglitazone (PPAR γ agonist), 9-*cis* retinoic acid, all-*trans* retinoic acid or 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor), 24 hours with 200 μM deferoxamine (DFX; a hypoxic mimetic; (41)); 3 hours with 200 μM hydrogen peroxide; 16 hours with 1 μM dexamethasone or 100 μM d- α -tocopherol (Acros Organics, NJ), or 30 minutes with 2.5 μM or 10 μM Bay 11-7085 (NF- κB inhibitor). Appropriate solvent was used as vehicle control. RNA was harvested with Trizol reagent (Invitrogen, Grand Island, NY) and reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Taqman expression assays for Fam-labeled *TTPA* (Hs00609398_m1), *MT1A* (Hs00831826_s1) and 18s (Hs99999901_s1) were used in combination with Fast Universal PCR Master Mix (Applied Biosystems) in a 96 well format on a StepOnePlus real time PCR machine (Applied Biosystems). The data were analyzed according to the Livak method for comparative real-time PCR (42).

Generation and measurements of reactive oxygen species (ROS)

IHH cells were pre-treated with 100 μM d- α -tocopherol (in ethanol), or 1 mM N-acetyl cysteine (NAC) for 16 hours. Oxidative stress was induced by challenging the cells with the indicated concentrations of H_2O_2 for 3 hours. 10 $\mu\text{g}/\text{ml}$ dichloro-fluorescein diacetate (DCF-DA, Invitrogen) was added in HBSS without phenol red during the final hour of H_2O_2 treatment and DCF fluorescence was read on a plate reader (excitation = 485 nm; emission = 535 nm). Raw fluorescence was normalized to DNA content as determined by bisbenzamide

assay (43). Bisbenzamide fluorescence was measured with a plate reader (excitation = 365 nm; emission = 460 nm).

Western blotting

Endogenous α TTP expression was determined in lysates prepared from liver, heart, cerebellum, prefrontal cortex, kidney, and intestine of 8 days old C57Bl/6 mice using a rat polyclonal 12D7 antibody (generous gift of H. Arai, University of Tokyo). Expression of α TTP in IHH cells was determined using the CW201P antibody generated in rabbits against the purified recombinant human α TTP.

Molecular biology

A 1904 bp fragment of human genomic DNA immediately 5' of the ATG translational start site of *TTPA* (NC_000008.10 chromosome 8, 8q12.3; range 63998612-64000612) was amplified from genomic human DNA, shuttled through the pDRIVE vector (Qiagen, Valencia, CA) and ligated into the pGL3-Basic vector (Promega) where it drives expression of the luciferase reporter gene. Potential regulatory cis-acting elements in this promoter sequence were annotated by identifying putative liver-specific transcription factor binding sites that are conserved in human, mouse, zebrafish, chicken, and chimpanzee using the web-based algorithms MatInspector (44) and MultAlin (45). Single nucleotide polymorphisms (SNPs) that commonly occur in the *TTPA* promoter of healthy humans were identified in public databases (dbSNP), and introduced into the 1.9 Kb promoter construct by site directed mutagenesis. All final constructs were verified by sequencing.

Reporter assays

IHH cells were plated in triplicate 24-well plates and co-transfected with the luciferase reporter construct (pGL3B-*TTPA*), together with the β -galactosidase-expressing pCH110 (Pharmacia) using Lipofectamine Plus (Invitrogen). Promoter-less pGL3B was used as a measure of background luciferase activity. Twenty-four hours after transfection, cells were treated with indicated concentrations of chemicals for twenty-four hours. Following lysis, luciferase activity was measured according to manufacturer instructions (Promega), and normalized to β -galactosidase activity to account for variations in transfection efficiency.

Results

Transcriptional regulation of the tocopherol transfer protein

Examination of murine *Ttpa* gene expression in 8 days old C57Bl/6J mice revealed, in agreement with previous reports (21,46), that α TTP mRNA and protein levels are highest in the liver, significant in the cerebellum, cortex, and kidney, and absent from the intestine and heart (Figure 1). In all tissues, we observed close correspondence between the levels of the mRNA detected by RT-PCR, and those of the protein, revealed by immunoblotting. These data indicate that the *Ttpa* gene is differentially expressed at the transcriptional level in various tissues.

To begin to examine the mechanisms by which the expression of *TTPA* is regulated, a computational sequence algorithm (MatInspector; (44)) was employed to identify putative transcription factor binding sites in the proximal promoter region of the human *TTPA* gene. Figure 2A depicts the location of the putative response elements in the *TTPA* promoter, transcription factors known to associate with these elements, and ligands and compounds that are known to activate these factors (47–59). Immortalized human hepatocytes (IHH) cells were used to investigate the transcriptional responses of *TTPA* to these compounds using real-time RT-PCR to measure the expression level of the *TTPA* mRNA. As shown in

Figure 2B, treatment of IHH cells with the RXR agonist 9-cis RA or with the synthetic PPAR α -selective ligand WY14643 increased the level of *TTPA* mRNA by approximately 2- and 3-folds, respectively. These findings indicate that PPAR α and RXR control *TTPA* expression, perhaps through their mutual heterodimerization (60). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), which raises intracellular cAMP levels, also upregulated the expression levels of *TTPA*, suggesting that the cAMP response element-binding transcription factor CREB (61) regulates *TTPA* transcription.

In line with the reports that the only known high-affinity ligand of α TTP, α -tocopherol, affects *TTPA* levels [16, 18], treatment with vitamin E caused a marked increase in the level of *TTPA* mRNA (3-fold; Figure 2B). Additionally, the expression of *TTPA* mRNA increased in response to two potent inducers of oxidative stress, H₂O₂ (ca. 3-fold) and the hypoxia mimetic deferoxamine (DFX; 4-fold; Figure 2B). Expression levels of the α TTP protein also increased in response to these treatments, evidenced from immunoblotting the various lysates using anti- α TTP antibodies (Figure 2C).

Transcriptional regulators of the proximal *TTPA* promoter

To address the molecular mechanisms by which the *TTPA* gene responds to the various inducers, we cloned the proximal human *TTPA* promoter (1.9 Kb upstream of the translation start site) into the pGL3-Basic vector, where it drives expression of the luciferase reporter gene (Figure 3A). IHH cells transfected with this construct were treated as in Figure 2, and promoter activity was determined as described in Methods (Figure 3B). Similar to our observations with the endogenous promoter (Figure 2B), H₂O₂, DFX and IBMX significantly stimulated the transcriptional activity of the isolated promoter. Interestingly, we found that inflammatory mediator TNF α also enhanced promoter activity. However, treatment with α -tocopherol, which activated transcription of endogenous *TTPA* mRNA, had no significant effect on transactivation of the cloned *TTPA* promoter. This observation suggests that a transcriptional element(s) mediating responsiveness to vitamin E reside outside the 1.9 Kb promoter region we cloned. Taken together, these results implicate the transcription factors CREB, NF- κ B, Sp1, GR and STAT, as candidate mediators of the transcriptional response of the *TTPA* gene.

Oxidative stress directly stimulates transcription of the *TTPA* gene

Upregulation of *TTPA* expression by H₂O₂ (Figure 2B) may represent an important physiological feedback mechanism in which α TTP levels increase in response to oxidative stress, resulting in distribution of vitamin E to prevent further oxidative damage. We examined the effect of vitamin E on H₂O₂-induced oxidative stress using DCF-DA, a cell-permeable oxidation-sensitive fluorescent dye widely used as an indicator of intracellular reactive oxygen species levels (62). As anticipated, treatment of IHH cells with hydrogen peroxide yielded a dose-responsive increase in dye fluorescence (Figure 4A). Pretreatment of the cells with vitamin E (100 μ M α -tocopherol) or with the water-soluble antioxidant N-acetyl cysteine (1 mM, (63)) markedly inhibited the H₂O₂-induced increase in DCF fluorescence (Figure 4A), confirming the established antioxidant action of these molecules. Finally, H₂O₂ treatment markedly increased expression of the oxidative stress-responsive gene metallothionein-1A (*MT1A*; (64)), concomitant with increased *TTPA* mRNA (Figure 4B). These data verify that treatment with H₂O₂ caused marked elevation of intracellular oxidative stress in IHH cells, and that the endogenous *TTPA* promoter is activated by this stimulus.

In principle, the H₂O₂-induced increase in *TTPA* mRNA could result from enhanced transcription of the gene, or from stabilization of the *TTPA* transcript. To distinguish between these possibilities, cells were treated with the transcriptional inhibitor actinomycin

D and the rate of degradation of *TTPA* mRNA was examined in the presence or absence of H₂O₂ (Figure 5A). The data showed that the half-life of the *TTPA* mRNA (approximately 3 hours) is not affected by treatment with hydrogen peroxide. We therefore conclude that oxidative stress-induced increase in *TTPA* transcript results from upregulation of gene transcription.

To examine whether the increase in *TTPA* mRNA reflects a direct nutrient-gene interaction or whether it is a secondary response that requires *de novo* protein synthesis, we examined the effect of the translational inhibitor cyclohexamide (65) on H₂O₂-stimulated transcription of *TTPA*. As shown in Figure 5B, pretreatment of IHH cells with cyclohexamide did not alter the transcriptional response of *TTPA* to hydrogen peroxide, indicating that *TTPA* expression is regulated by already-present transcription factor(s) which respond to oxidative stress. In light of two putative NF- κ B binding sites in the *TTPA* promoter (Figure 3A), and the established oxidative stress-responsiveness of this transcription factor (66), we examined the possible involvement of NF- κ B in the transcriptional response of *TTPA* to H₂O₂. Treatment of IHH cells with the NF- κ B inhibitor Bay 11-7085 (67,68) did not abrogate the transcriptional response of *TTPA* expression to oxidative stress (data not shown), indicating that a transcription factor other than NF- κ B mediates this response. To further interrogate the elements in the promoter region that may be responsible for the oxidative stress-induced *TTPA* transcriptional response we investigated involvement of the cAMP response element-binding (CREB) transcription factor, which is known to mediate oxidative stress responses in α TTP-expressing tissues (liver and the central nervous system (69,70)). Specifically, we examined CREB's role using lentivirus-mediated delivery of CREB shRNA in IHH cells prior to measuring the effect of H₂O₂ on *TTPA* mRNA. The targeted knockdown approach attenuated the expression of CREB significantly as demonstrated by anti-CREB immunoblotting (>40% decrease; Figure 6A). Strikingly, shRNA-mediated knockdown of CREB significantly attenuated the transcriptional response of *TTPA* to oxidative stress (approximately 2-fold reduction; Figure 6B). These results show that CREB is a key mediator of the transcriptional response of the *TTPA* promoter to oxidative insults.

Effects of common promoter single nucleotide polymorphisms (SNPs) on transcription of *TTPA* gene

Examination of the NCBI database (<http://www.ncbi.nlm.nih.gov/snp>) revealed the presence of 37 SNPs in the 2 Kb region upstream of the *TTPA*'s initiating ATG codon. We focused on the 12 variants that have been validated by multiple independent studies and were found to occur in a significant fraction of healthy human populations (penetrance between 1 and 50%; Table 1). Using site-directed mutagenesis, we introduced these SNPs into the luciferase reporter construct that harbors the *TTPA* promoter, and measured their effects on promoter activity. We found that the various SNPs had profound impact on the transcriptional activity of the *TTPA* promoter (Figure 7B). Thus, the -1752C/T, -1408G/T, and -345C/T substitutions resulted in a ca. 3-fold increase in promoter activity compared to the 'parental' construct. In contrast, the -1408A/T, -980A/T, -943A/G, -674C/T, -439A/G and -344 C/T SNPs significantly repressed promoter activity. These alterations in transcriptional activities may reflect changes in the specific transcription factors that bind to the different *TTPA* promoter variants (Figure 7C). These results indicate that common SNPs in the *TTPA* promoter greatly affect the expression levels of α TTP, and thereby may critically alter circulating levels of vitamin E. In support of this notion, it has been previously reported that the -980A/T *TTPA* variant, which in our hands greatly suppressed promoter activity, is associated with reduced plasma vitamin E levels (71).

DISCUSSION

α TTP is the only known direct regulator of whole-body vitamin E status, yet the mechanisms that regulate its expression are by and large unknown. Our results reveal that expression of the *TTPA* gene is induced by WY14643 and 9-cis-retinoic acid, ligands that activate the nuclear receptors PPAR α and RXR, respectively. Together with the identification of a putative PPAR response element in the *TTPA* promoter, these data suggest that *TTPA* transcription is regulated by PPAR α -RXR heterodimers (72). The involvement of these heterodimers in specifically regulating *TTPA* expression and its physiological consequences remain to be clarified.

Of critical importance are our findings showing that oxidative stress enhances expression levels of the α TTP mRNA and protein in cells (Figures 2B and 5B), and increases the transcriptional activity of the isolated *TTPA* promoter (Figure 3B). Since oxidative stress did not influence the stability of the *TTPA* mRNA (Figure 5A) and since stimulation of *TTPA* transcription by peroxide did not require *de novo* protein synthesis (Figure 5B), we conclude that oxidative stress-induced enhancement of *TTPA* expression is mediated by an already-present oxidative stress-responsive transcription factor. The lack of inhibition by the NF- κ B antagonist Bay 11-7085 suggests that the transcription factor responsible for this effect is not NF- κ B. Possibly, other oxidative stress-responsive transcription factors such as SP1, STAT and GR, in addition to CREB (see below) mediate the transcriptional responses of *TTPA* to oxidative stress.

The data show that *TTPA* expression was induced upon treatment with the phosphodiesterase inhibitor IBMX, which elevates intra-cellular levels of cAMP (Figure 2B). Moreover, silencing CREB expression with a specific shRNA abrogated the transcriptional response of *TTPA* to oxidative stress (Figure 6B). These data demonstrate that CREB plays an important role in mediating the effects of oxidative stress onto the *TTPA* promoter. Further support to this conclusion is found by the presence of multiple CREB binding sites in the *TTPA* promoter (Figure 3A), and by published reports of the effect of vitamin E on the expression levels (73) or phosphorylation status (74) of CREB. Future studies will delineate the specific CREB binding site in the *TTPA* promoter that mediates this transcriptional response.

Do the transcriptional responses of the *TTPA* gene to oxidative stress represent a feedback regulatory mechanism, in which increased α TTP levels increase antioxidant bioavailability? Bella et al reported that hepatic α TTP levels in mice do not change upon exposure to oxidative stress in the form of environmental tobacco smoke (32). However, we note that α TTP is also expressed in the brain (26) and placenta (27), where it is thought to regulate localized vitamin E status. It is possible that oxidative-stress responses of the *TTPA* gene are more relevant in these tissues, where α TTP levels are significantly lower than in the liver.

A puzzling enigma in present-day vitamin E research is an apparent contradiction between the beneficial effects of α -tocopherol supplementation observed in laboratory animals, and the lack of consistent efficacy in controlled clinical trials. For example, vitamin E exhibits remarkable anti-proliferative effects in cell culture models of prostate cancer (25,75–78). Moreover, supplementation with α -tocopherol is highly effective in reducing prostate tumor load in rodent models of the disease (79–81). Nevertheless, clinical trials in human population proved inconsistent at best: both the Alpha Tocopherol Beta Carotene cancer prevention study (ATBC; (82)), and Carotene and Retinol Efficacy Trial (CARET; (83)) demonstrated protective effects for vitamin E against prostate cancer. On the other hand, the randomized, placebo-controlled clinical trial SELECT showed that vitamin E supplementation *increased* prostate cancer risk (84). Our data show that commonly

occurring SNPs have profound impact on the transcriptional activity of the *TTPA* promoter (Figure 7B). On the molecular level, these functional outcomes of the SNPs likely reflect alterations in transcription factor binding, such as the new putative binding site for the transcriptional inhibitor BCL6 that is generated by the -980 A/T polymorphism (Figure 7C). It is tempting to speculate that such polymorphic variations manifest in variable baseline vitamin E levels among individuals, and in heterogeneous response to supplementation. Indeed, Kelly and colleagues demonstrated that circulating vitamin E levels in healthy humans is a genetically determined trait that is subject to great inter-individual variations (85,86). Furthermore, one common promoter variant (rs6994076) was reported to associate with reduced plasma vitamin E levels in healthy humans (71). Determinations of *TTPA* genotype in samples from clinical intervention trials stand to provide conclusive testing of this hypothesis.

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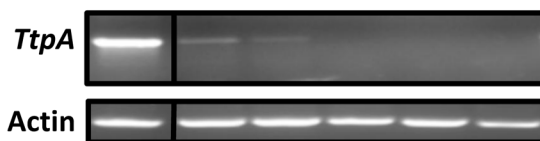
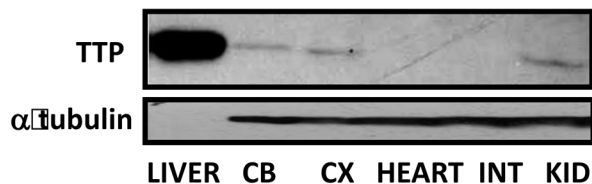
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Highlights

- Vitamin E is the major lipid-soluble antioxidant
- α TTP is the only known regulator of vitamin E status
- Oxidative stress and promoter SNPs regulate expression of the *TTPA* gene
- Our findings may explain perplexing results regarding responses to vitamin E supplementation observed in clinical trials.

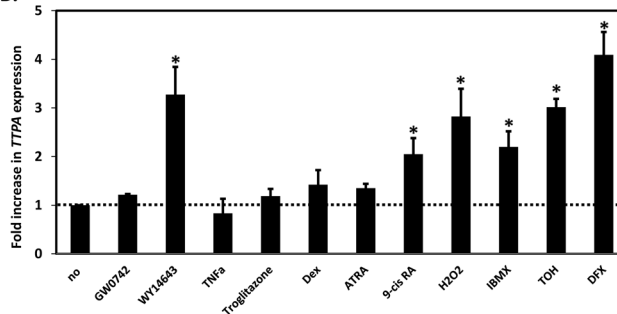
A. mRNA:**B. Protein:****Figure 1. Tissue-specific expression of the tocopherol transfer protein**

RNA was harvested from indicated tissues of an 8 days old mouse, and used to determine mRNA levels of α TTP and actin using RT-PCR (top two panels) or for measuring α TTP and α -tubulin protein levels using immunoblotting (bottom two panels). Ten μ g of liver protein and 200 μ g of cerebellum (CB), cortex (CX), heart, intestine (INT) and kidney (KID) were loaded on the indicated lanes.

A.

Position in <i>TTPA</i> promoter	Target Transcription factor	Compound	Cellular action	References
-791	PPAR α	WY14643	lipid metabolism	47
-789	RXR	9-cis-RA	heterodimerization with PPAR	48
-512, -733, -1481, -1507	CREB	IBMX	Responses to cAMP and Ca ²⁺	52, 54
-944 -156, -1744 -187, -1117, -1568 -836, -1786	P53 ARNT STAT NF- κ B	DFX	Responses to hypoxia	53 49 55 50
-512, -733, -1481, -1507 -1560, -1751 -85 -187, -1117, -1568 -836, -1786	CREB GR Sp1 STAT NF- κ B	H ₂ O ₂	Responses to oxidative stress	54 56 57 58 59

B.



C.

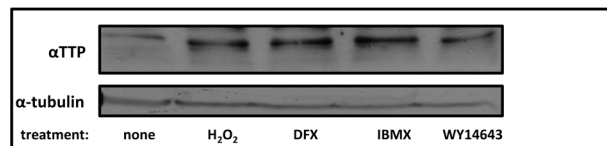


Figure 2. Modulators of *TTPA* transcription

A. Locations of putative transcription factors in the proximal human *TTPA* promoter were identified using sequence homology algorithms and are listed with their activator and physiological context. **B.** Real-time PCR results of *TTPA* expression. IHH cells were treated with 1 μ M GW0742, 1 μ M Wy14643, 1 μ M TNF α , 1 μ M Troglitazone, 1 μ M ATRA (all trans retinoic acid) or 1 μ M 9-cis RA (9-cis retinoic acid) for 4 hours; 200 μ M H₂O₂ (hydrogen peroxide) for 3 hours; 0.5 mM IBMX (3-isobutyl-1-methylxanthine) for 24 hours; 100 μ M TOH (d- α -tocopherol) or 1 μ M Dex (Dexamethasone) for 16 hours; or 200 μ M DFX (desferrioxaminemesylate) for 24 hours prior to RNA isolation. Shown are averages and standard deviations of three independent experiments. Asterisks indicate statistical significance at $p < 0.05$ compared to the untreated cells, determined by a Student's T-test. **C.** α TTP protein expression. IHH cells were treated with 200 μ M H₂O₂ for 3 hours or 0.5 mM IBMX, 1 μ M Wy14643, 200 μ M DFX for 24 hours prior to protein extraction and SDS-PAGE. Three hundred μ g of lysate protein were immunoblotted against α TTP and tubulin.

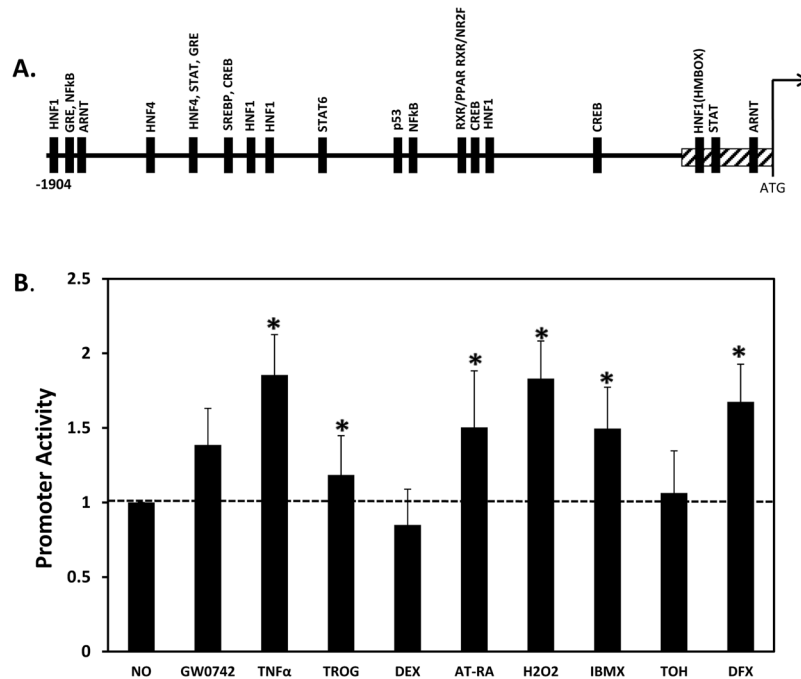


Figure 3. Transcriptional responses of the cloned *TTPA* promoter

A. Locations of putative transcription factor binding sites in the proximal human *TTPA* promoter were identified using sequence homology algorithm (MatInspector; TransFac). Transcription factor binding sites are shown as solid boxes. A proximal GC-rich region is shown as a gray-hatched box. **B.** A luciferase reporter construct driven by the cloned human *TTPA* promoter (1904 bp) was co-transfected with β -galactosidase into IHH cells. Twenty-four hours after transfection, cells were treated with 200 μ M H₂O₂ for 3 hours or 1 μ M GW0742, 1 μ M Wy14643, 1 μ M TNF α , 1 μ M Troglitazone, 1 μ M ATRA (all trans retinoic acid) or 1 μ M 9-cis RA (9-cis retinoic acid), 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 100 μ M TOH (d- α -tocopherol), 1 μ M Dex (Dexamethasone) or 200 μ M DFX (desferrioxaminemesylate) for 24 hours prior to harvest. Shown are average values from three independent transfections, after normalization to the untreated condition. Asterisks indicate statistical significance of p < 0.05 compared to the untreated -1904 construct, as determined from a Student's T-test.

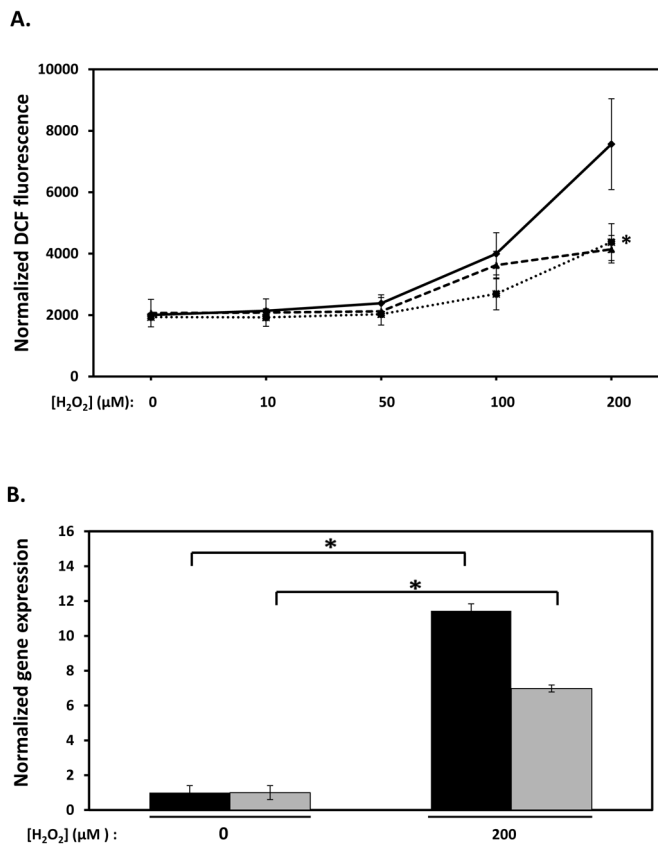


Figure 4. Transcriptional response of the α TTP gene to oxidative stress

A. Measurement of hydrogen peroxide-induced oxidative stress. The DCF-DA assay was used to measure intracellular reactive oxygen species as described in Methods. IHH cells were treated with 100 μ M tocopherol (dotted line) or 1 mM N-acetyl cysteine (dashed line), or vehicle control (solid line) for 16 hours prior to challenge with 200 μ M H₂O₂ for a 3 hours. Asterisks indicate statistical significance of $p < 0.05$ versus vehicle-treated samples, determined by a Student's T-test. **B.** mRNA levels of the *MT1A* (black bars) and *TTPA* (gray bars) genes was measured by real-time RT-PCR in cells treated as with 200 μ M hydrogen peroxide (or with control vehicle) for 3 hours. Asterisks indicate statistical significance at $p < 0.05$, determined from a Student's T-test.

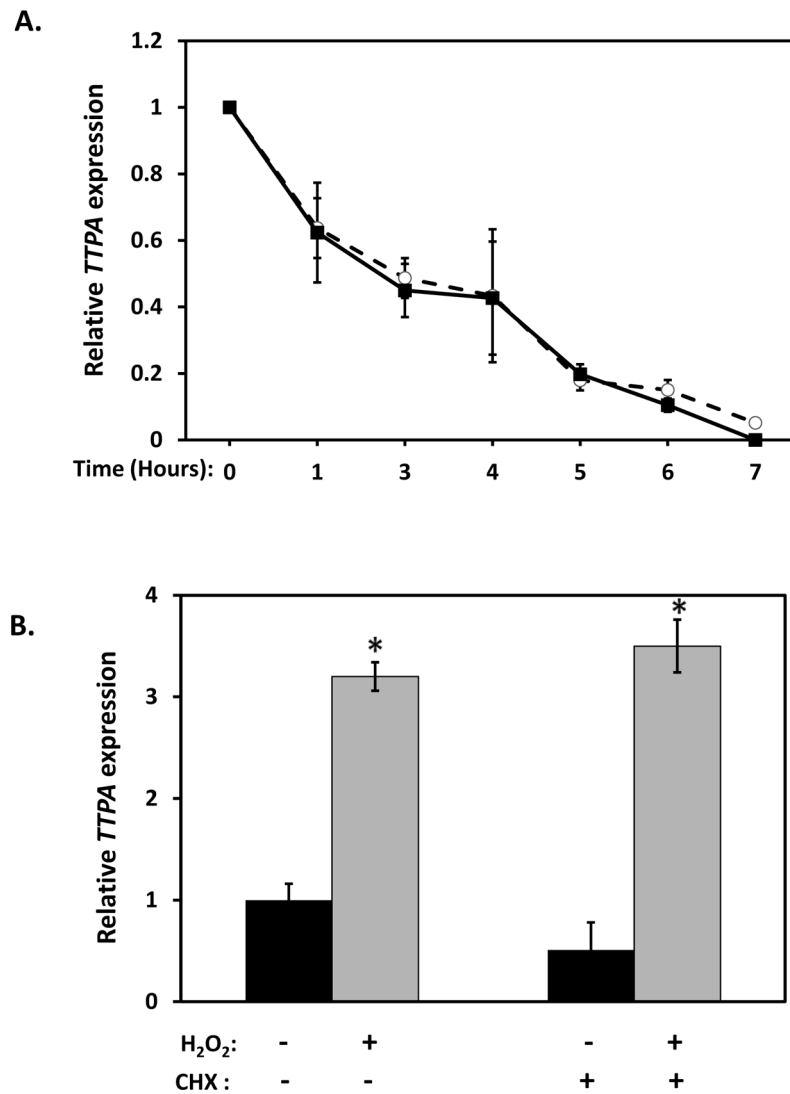
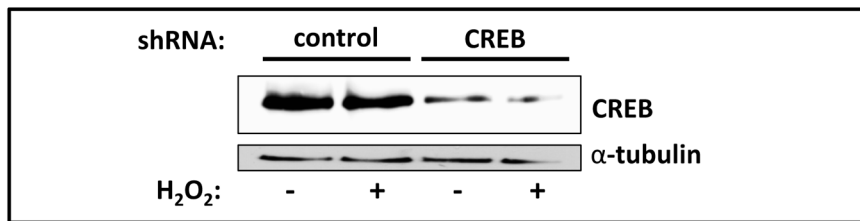


Figure 5. Effect of oxidative stress on *TTPA* mRNA expression

A. mRNA stability. Real-time RT-PCR was used to quantitate *TTPA* mRNA after treatment with 10 μ g/ml actinomycin D for the indicated duration in the presence (open circles) or absence (solid squares) of 200 μ M H₂O₂. **B. Requirement for de novo protein synthesis.** Real-time RT-PCR was used to measure *TTPA* mRNA levels following treatment with 200 μ M H₂O₂ (or vehicle) for three hours. Where indicated, cells were pre-treated with the protein synthesis inhibitor cyclohexamide (100 μ g/ml) for 15 minutes prior to H₂O₂ challenge. Shown are averages and standard deviations of three independent experiments. Asterisks indicate statistical significance of $p < 0.05$ versus untreated samples, determined from a Student's T-test.

A.



B.

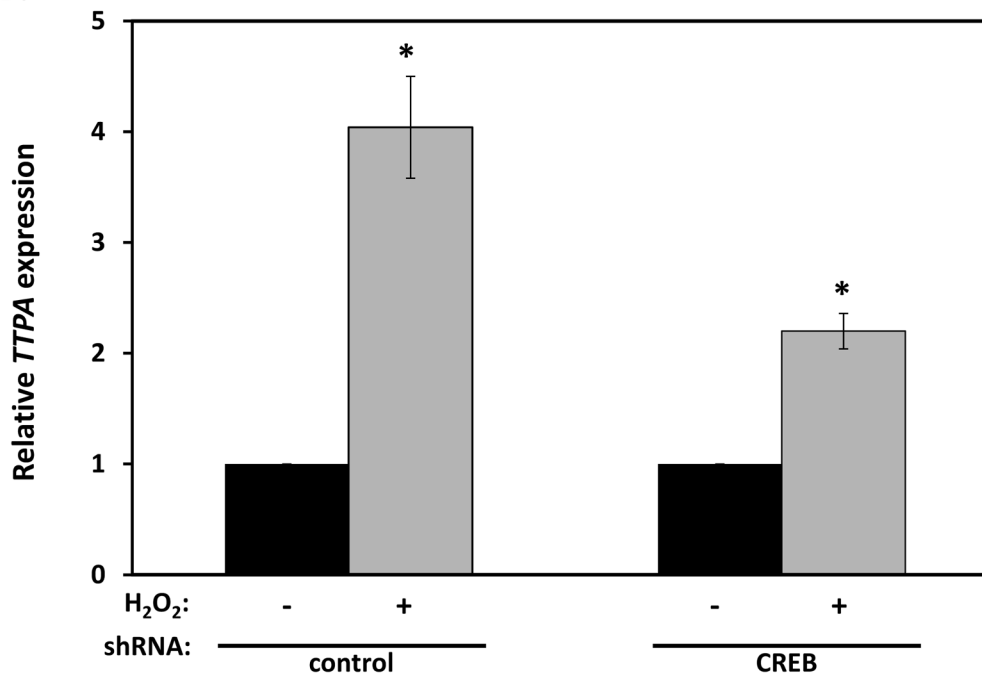


Figure 6. CREB mediates transcriptional response of the *TTPA* gene to oxidative stress
A. IHH cells were transduced with lentiviruses harboring shRNA directed at CREB (or control shRNA) for 48 hours. Where indicated, transduced cells were treated with 200 μ M H₂O₂ for 3 hours prior to lysis and immunoblotting. Densitometric analysis revealed shRNA-mediated knockdown efficiency >40%. **B.** IHH cells were transduced with lentiviruses as in panel (A) and Real-time RT-PCR used to measure *TTPA* mRNA levels. Asterisks indicate statistical significance of p<0.05 compared to untreated samples, determined from a Student's T-test.

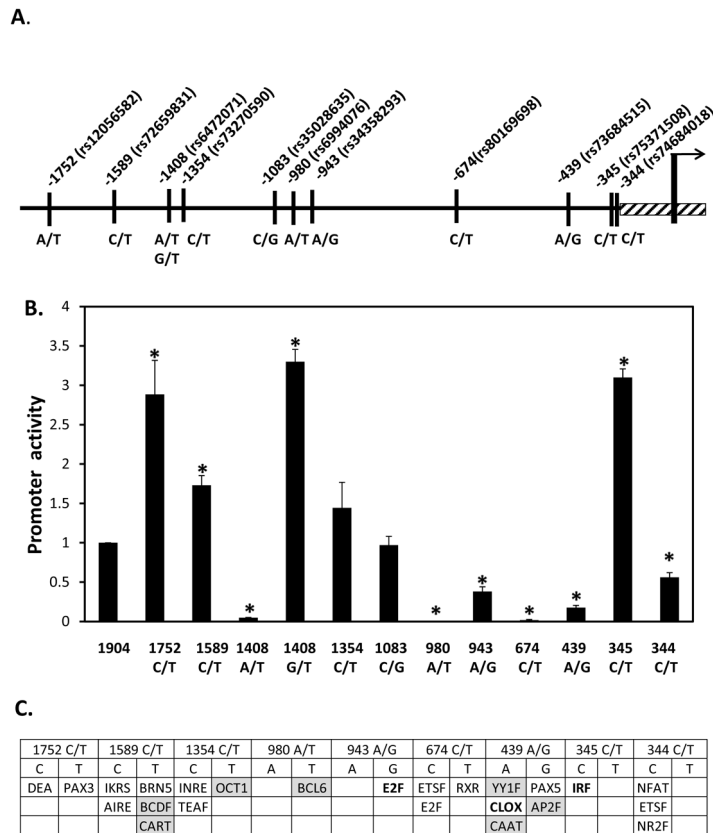


Figure 7. Effect of common SNPs on the transcriptional activity of the *TTPA* promoter
A. Schematic depiction of the 1.9 Kb proximal *TTPA* promoter, showing position and reference ID (rs#) of validated single nucleotide polymorphisms. **B.** Transcriptional activities of SNP-mutated *TTPA* promoter. SNPs were introduced into the wild-type (1904 bp) *TTPA* promoter in the luciferase reporter construct, and luciferase activities measured as described in Methods. A promoter-less pGL3B vector served as a background control. Shown are the averages and standard deviations of three independent experiments. Asterisks indicate statistical significance of $p < 0.05$, compared to the wild-type promoter construct, as determined by Student's T-test. **C.** Transcription factor binding sites that may be altered by SNPs in the proximal *TTPA* promoter. Repressors are shown in shaded boxes and activators in bold typeface.

Table 1
 Characteristics of validated SNPs located in the *TTPA* promoter (proximal 1.9 KB).

rs#	SNP location	Population	Study	Sample Count	Penetrance	
					% major allele	% minor allele
rs73684515	-439 A/G	Yoruba in Ibadan, Nigeria	1000Genomes pilot low-coverage panel	59	A=10.2	G=89.8
rs80169698	-674 C/T	Yoruba in Ibadan, Nigeria	1000Genomes pilot low-coverage panel	59	C=94.9	T=5.1
rs34358293	-944 A/G	Caucasian	SNP500Cancer	30		
		African/African American	SNP500Cancer	24		
		Hispanic	SNP500Cancer	23		
		Pacific Rim	SNP500Cancer	24	A=2.1	G=97.9
rs6994076	-980 A/T	Utah residents with Northern and Western European ancestry	HapMap	113	A=52.2	T=47.8
		Han Chinese in Beijing, China	HapMap	45	A=24.4	T=75.6
		Japanese in Tokyo, Japan	HapMap	86	A=29.7	T=70.3
		Yoruba in Ibadan, Nigeria, Sub-Saharan	HapMap	113	A=27.0	T=73.0
		African ancestry in Southwest USA	HapMap	49	A=36.7	T=63.3
		Caucasian	SNP500Cancer	30	A=50.0	T=50.0
		African/African American	SNP500Cancer	24	A=29.2	T=70.8
		Hispanic	SNP500Cancer	23	A=34.8	T=65.2
rs73270590	-1356 C/T	Pacific Rim	SNP500Cancer	24	A=26.1	T=73.9
		Yoruba in Ibadan, Nigeria	1000Genomes pilot low-coverage panel	59	A=12.7	T=87.3
rs6472071	-1409 A/G/T	Utah residents with Northern and Western European ancestry	1000Genomes pilot low-coverage panel	60	A=44.2	T=55.8
		Han Chinese in Beijing, China + Japanese in Tokyo, Japan	1000Genomes pilot low-coverage panel	60	A=40.0	T=60.0
		Yoruba in Ibadan, Nigeria	HapMap	60	A=47.5	T=52.5
		Yoruba in Ibadan, Nigeria	1000Genomes pilot low-coverage panel	59	C=11.9	G=88.1
rs35028635	-1083 C/G	Caucasian	SNP500Cancer	30	C=0	G=100
		African/African American	SNP500Cancer	24	C=2.1	G=97.9
		Hispanic	SNP500Cancer	23	C=0	G=100
rs12056582	-1752 C/T	Pacific Rim	SNP500Cancer	24	C=0	G=100
		Utah residents with Northern and Western European ancestry	1000Genomes pilot low-coverage panel	60	C=95.8	T=4.2
		Han Chinese in Beijing, China + Japanese in Tokyo, Japan	1000Genomes pilot low-coverage panel	60	C=64.2	T=35.8