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Lifelong α-Tocopherol Supplementation Increases the Median Life Span of C57BL/6 Mice in the Cold but Has Only Minor Effects on Oxidative Damage

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ABSTRACT

The effects of dietary antioxidant supplementation on oxidative stress and life span are confused. We maintained C57BL/6 mice at 7 ± 2°C and supplemented their diet with α-tocopherol from 4 months of age. Supplementation significantly increased ($p = 0.042$) median life span by 15% (785 days, $n = 44$) relative to unsupplemented controls (682 days, $n = 43$) and also increased maximum life span (oldest 10%, $p = 0.028$). No sex or sex by treatment interaction effects were observed on life span, with treatment having no effect on resting or daily metabolic rate. Lymphocyte and hepatocyte oxidative DNA damage and hepatic lipid peroxidation were unaffected by supplementation, but hepatic oxidative DNA damage increased with age. Using a cDNA macroarray, genes associated with xenobiotic metabolism were significantly upregulated in the livers of female mice at 6 months of age (2 months supplementation). At 22 months of age (18 months supplementation) this response had largely abated, but various genes linked to the p21 signaling pathway were upregulated at this time. We suggest that α-tocopherol may initially be metabolized as a xenobiotic, potentially explaining why previous studies observe a life span extension generally when lifelong supplementation is initiated early in life. The absence of any significant effect on oxidative damage suggests that the life span extension observed was not mediated via any antioxidant properties of α-tocopherol. We propose that the life span extension observed following α-tocopherol supplementation may be mediated via upregulation of cytochrome p450 genes after 2 months of supplementation and/or upregulation of p21 signaling genes after 18 months of supplementation. However, these signaling pathways now require further investigation to establish their exact role in life span extension following α-tocopherol supplementation.

INTRODUCTION

IT HAS BEEN LONG SUGGESTED that reactive oxygen species (ROS)-induced damage to proteins, lipids, and DNA is a major contributor to the biochemical, physiological, and functional declines associated with aging.[1,2] Nevertheless, the exact role oxidative stress plays in mammalian aging remains controversial, despite comparative data suggesting extended life

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span is associated with reduced ROS production and/or a greater resistance to oxidative stress,[3–6] (but also[7]). Organisms possess a suite of endogenous antioxidants, including superoxide dismutase, catalase, glutathione, and glutathione peroxidase, to protect against oxidative-induced damage. Moreover, exogenous antioxidants such as α-tocopherol and ascorbic acid may confer protection against ROS damage.[1] Theoretically, dietary supplementation with exogenous antioxidants may help support the endogenous antioxidant defenses by scavenging ROS, thereby reducing oxidative damage. This has resulted in dietary supplementation, with various antioxidants being advocated as a potential therapy to extend both life span and health span.[8,9]

Vitamin E is a lipid-soluble antioxidant consisting of at least eight tocopherol and tocotrienol isomers (including α- and γ-tocopherol), which acts as a chain-breaking antioxidant and a peroxyl radical scavenger *in vivo*.[10] Vitamin E's effects are however not restricted to its role as an antioxidant as, for example, it is known to be a potent stimulator of xenobiotic metabolism both *in vivo* and *in vitro*.[11,12] However, the long-term benefits of vitamin E supplementation appear confused. Some studies suggest supplementation can ameliorate various cardiovascular, neurological, and oxidative stress parameters in mammals,[13–15] and others report little or no effect.[16] Indeed, dietary vitamin E deficiency is associated with various pathologies, including diabetes, atherosclerosis, and various cancers.[11] The effects on life span following vitamin E supplementation in animal models are similarly confused. Using a comparative approach, Zou et al.[17] reported that γ-tocopherol, but not α-tocopherol, extended life span in *Caenorhabditis elegans*, but that neither isoform had any life span effect in *Drosophila melanogastor* or *Anastrepha ludens*. An increase in median/mean life span has been reported in various strains of mice following vitamin E supplementation[18–21] or following supplementation with a mixed antioxidant diet containing vitamins E and C.[22] However, other studies report no effect on life span or on oxidative stress following vitamin E supplementation or when using a mixed antioxidant diet.[14,23,24]

There are several potential reasons why dietary antioxidants, such as vitamin E, have not become robust and repeatable pro-longevity treatments. Firstly, vitamin E supplementation may indeed quench ROS and reduce oxidative stress, but this may have no or little impact on aging and life span if the importance of these processes has been overestimated *in vivo*.[17] Secondly, Vitamin E may scavenge ROS, and ROS may be important in the aging process, but this effect might be offset by a parallel compensating impacts on the endogenous antioxidant system, or indeed on any system involved in redox status or oxidative damage, similar to those reported following vitamin C supplementation.[25,26] Thirdly, because body mass and energy metabolism affect both free-radical production and life span,[7,27,28] if vitamin E supplementation influences one or both of these factors, this could offset any potential benefits. Finally, age of initiation of supplementation may be important. A significant increase in mean life span was only observed in mice supplemented from 2 and 9 months, but not at 16 or 22 months of age.[19]

It is well established that cold exposure elevates metabolic rate, upregulates various antioxidants, and increases both ROS production and oxidative stress.[29–32] Therefore, to examine whether lifelong α-tocopherol supplementation (initiated at 4 months of age) had a beneficial effect on oxidative stress and life span, we maintained C57BL/6 mice at 7 $\pm$ 2°C over their lifetime to potentially increase metabolic rate, ROS production, and oxidative damage. We have previously hypothesized[26] that under conditions of cold exposure, we would be more likely to detect an effect of antioxidant supplementation on oxidative stress and life span if such an effect existed. We measured life span, energy balance (resting metabolic rate, daily energy expenditure by doubly-labeled water[33]) hepatic lipid peroxidation (thiobarbituric acid-reactive substances [TBAR]) using an HPLC method,[26,34] and lymphocyte and hepatocyte DNA oxidative damage (modified Comet assay) in male and female C57BL/6 mice maintained in the cold (7 $\pm$ 2°C) with *ad libitum* access to either a control or an α-tocopherol (550 mg/kg) supplemented diet. In addition, Clontech cDNA- macroarrays were used to exam-

ine hepatic gene expression changes with age and α-tocopherol supplementation in female mice.

MATERIALS AND METHODS

Experimental design and mouse husbandry

C57BL/6 mice were purchased at 8 weeks of age from a commercial breeder (Harlan, Bicester, United Kingdom), individually housed in cages (48 × 15 × 13 cm) at 22 ± 2°C and under a 16L/8D light/dark cycle (lights on 0600). From 12 weeks of age onward, and as previously described,[26] mice were maintained at 7 ± 2°C within a controlled temperature room (Atlantic Cooling Services, Scassow, UK), with access to bedding material and water and ad libitum access to standard mouse diet (Rat and Mouse Maintenance (RMI), Special Diets Services, BP Nutrition, Witham, UK). Over a 4 week period of acclimation to 7 ± 2°C, bedding material was gradually removed and then at 16 weeks of age the mice were randomly assigned to a control RM1 diet group (22 mg/kg *ad libitum* α-tocopherol) or a α-tocopherol supplemented group (RM1 + 550 mg/kg of α-tocopherol) equivalent to 25 × the control diet. All experiments followed institutional guidelines for laboratory animal care and welfare under license from the UK Home Office.

Longevity analysis

A total of 40 female (19 α-tocopherol supplemented, 21 control) and 47 male (25 α-tocopherol supplemented, 22 control) mice were used in the life span analysis. All individuals were monitored daily following the guidelines described previously,[26] with Kaplan-Meier survival curves constructed using birth (mice purchased at 8 weeks of age, see above) and known death dates of each individual. The oldest 10% survival was determined as the age of death of the oldest 10% of mice in each experimental cohort.

An additional group of male and female mice that followed the same husbandry protocol described above were used to examine the effects of lifelong α-tocopherol supplementation on gene expression profiles (females only), metabolism (males and females), and oxidative stress (males and females).

Metabolic studies

Resting metabolic rate (RMR) was determined at 6 and 22 months of age (2 and 18 months following initiation of α-tocopherol supplementation), using open-flow respirometry.[35] All individuals had *ad libitum* access to food and water prior to, but not during, measurements. Mice were weighed (0.01 g) and then placed in an airtight Plexiglas chamber within a temperature-controlled incubator (INL-401N-010, Gallenkamp, Loughborough, UK) at 7 ± 0.1°C. Silica gel-dried air was pumped (Charles Austin Leighton Buzzard, UK) at 600–800 mL min^{-1} (DM3A, Alexander Wright Flow Meter, UK), redried, and then a sub-sample (~150 mL min^{-1}) was passed through a paramagnetic oxygen and an infrared carbon dioxide analyzer (Series 1400, Servomex Group, Crowburgh, UK). Energy expenditure, corrected for standard temperature and pressure, was calculated[36] using the lowest 10 consecutive readings in oxygen concentration (equivalent to 5 min within the chamber) and used to calculate resting metabolic rate (mL O_2 min^{-1}).

Daily energy expenditure (DEE) was estimated in the same mice as RMR using the doubly labeled water technique, as previously described.[33,37,38] On day one, the animals were weighed (±0.1 g Sartorius balance) and ~0.15 mL of DLW (30 APE ^{18}O, 15 APE ^{2}H) was administered (IP). Syringes were weighed before and after administration (±0.0001 g, Sartorius balance) to calculate the mass of DLW injected. Initial blood samples were taken after 1 h of isotope equilibration to estimate initial isotope enrichments. Final blood samples (80 μL) were taken as close as feasible to either 24 or 48 h after the initial blood sample to estimate isotope elimination rates. Background enrichments were evaluated from blood samples taken from unlabeled animals in the same protocols.[39] The isotope ratios ^{18}O:^{16}O and ^{2}H:^{1}H were analyzed using gas source isotope ratio mass spectrometry (Optima, Micromass IRMS and Isochrom μG, Manchester, UK). Three high enrichment standards were run each day alongside the samples, with all raw data corrected to these

standards to minimize any problems of inlet cross contamination. Isotope enrichments were converted to values of daily energy expenditure using a single pool model.[33]

Comet assay

A modified Comet assay was employed to determine hepatic and lymphocyte DNA oxidative damage in mice at 6 and 22 months of age, as previously described.[26] We used site-specific bacterial repair enzymes endonuclease III (ENDO III) and formamidopyrimidine-DNA glycosylase (FPG) to increase the sensitivity and specificity of the Comet assay.[40] Damage was scored visually, with 100 comets on each slide assigned a score from 0 to 4, depending on the fraction of DNA pulled out into the tail. The overall score for each slide was therefore between 0 (undamaged) and 400 (completely damaged).

Thiobarbituric acid–reactive substances

Hepatic lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances (TBAR) using HPLC in 6- and 22-month-old α-tocopherol supplemented and control mice as previously described.[41] In brief, liver homogenates (0.1 mL) in 0.05 M potassium phosphate (pH 7.4) were incubated at 37°C in 2.5 mL buffer (final volume) and expressed as (nmol malonaldehyde/mg protein.

Hepatic α-tocopherol

The levels of α-tocopherol were measured in liver homogenates using reverse-phase HPLC and a fluorescent and visible detection method adapted from Hess et al.[42]

Macroarray analysis

A sample of female mice ($n = 6$ in each case) from the α-tocopherol supplemented and control diet groups were used for the macroarray analysis at 6 and 22 months of age (i.e., 2 and 18 months following start of α-tocopherol supplementation). Samples were collected at the same time each day to avoid any circadian changes in liver metabolism potentially confounding treatment effects. Samples were dissected, immediately frozen in liquid N_2, and stored at −80°C until use, with no pathology being evident in any animal at this time. Total RNA was extracted using standard protocols,[43] suspended in 300 μL of ultra-pure water and frozen at −80°C. Each sample (250 μL of total RNA) was treated following the Clontech Atlas Pure System (Clontech Laboratories, Mountain View, CA, <http://www.clontech.com/clontech>) protocol, including digestion in 25 μL of RNase-free Dnase 1. Isolated RNA was re-suspended in 30 μL ultra-pure water, yield and purity determined (Agilent Technologies, Wokingham, UK), and stored at −80°C until use. Clontech Atlas mouse stress cDNA expression arrays (Cat #7749-1) were used containing a grid of 140 cDNAs spotted in duplicate on a nylon membrane. Probe preparation, hybridization, and analysis are described elsewhere.[26]

For the macroarray analysis, spot intensities were log-transformed with respect to the base 2, resulting in differences of 1, 2, and 3 on the log scale corresponding to fold changes of 2, 4, and 8 on the original scale.[26] An analysis of correlations between the 24 slides revealed that 1 array from the 6-month α-tocopherol supplemented mice did not correlate well with the other 23 arrays, therefore this slide was eliminated from further analysis. Normalization employed a two-stage strategy. The first normalization stage involved calculating the average intensity across the six slides or, in the case of the 6-month α-tocopherol mice, across five slides (see above). Although numerous genes might change in their average intensity, we assumed that the overall distribution across genes should more or less stay the same. For this reason the group average was standardized such that they had the same median and the same inter-quartile range (used as a robust measurement of scale). In the second normalization stage, within an experimental group, there was no reason to assume that genes were systematically differentially expressed between individual animals. For this reason, we used the standardized group average (see first stage) and examined the six scatter-plots of this average against each of the individual arrays and assumed the data to scatter around the 45° line of this plot. A nonlinear robust regression (loess regression/normalization) was employed to remove any systematic departure

from this expected behavior (see Yang et al.[44] for comparison). The 23 values were analyzed in two-way analysis of variance (ANOVA) with age (6 months, 22 months) and treatment (control, α-tocopherol supplemented) as factors, also allowing for interaction between age*treatment (see Kerr et al.[45] for a discussion of ANOVA methods in a gene expression context), resulting in three p values per gene. A total of 54 genes were identified with significant differences in expression ($p < 0.05$). By chance alone, we predicted that only seven genes (140*5%) would be expected to be significant, indicating that most of the genes identified are likely to be true positives.

Northern analysis

Total RNA was extracted from the same liver samples used for the macroarrays using a guanidium isothiocyanate/phenol method, fractionated on a 1.4% denaturing agarose gel, and transferred overnight to a positively charged nylon membrane (Amersham Biosciences, Little Chalfont, UK). Following cross-linking and hybridization at 42°C, we probed sequentially for Cu-Zn-superoxide dismutase (5′ CTGCACGCCGCCCGACACAACATTATTGAGGT), catalase (5′ GAGAATCCATCCAGCGTTGATTACAGGTGA), and glutathione peroxidase (5′ AGACCAAATGATGTACTTGGGGTCGGTCAT) mRNA using 5′ digoxigenin end-labeled oligonucleotides (Eurogentec, Southhampton, UK). Membranes were subsequently stripped and hybridized for 18S rRNA (5′ CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG). Signals were detected by chemiluminescence (Applied Biosystems, Framingham, UK), and quantified using ImageJ (Microsoft Java Image, Redmond, WA).

Western analysis

Liver samples were homogenized in lysis buffer, subsequently resolved on 12% Tris glycine gels (Invitrogen, Paisley, UK), and transferred to nitrocellulose. Blots were probed using the primary antibodies p53 (#2524, Cell Signaling Technology, Danvers, MA), phospho-p53 (serine 15, #9284, Cell Signaling Technology), and α-Actin (#8277, Abcam, Cambridge, UK); and secondary antibodies anti-rabbit HRP (#7074, Cell Signaling Technology) or anti-mouse HRP (#7076, Cell Signaling Technology). Enhanced chemoluminescence was performed (Pierce Perbio, Cramlington, UK) and blots were subsequently stripped (Pierce Perbio).

Statistical analysis

All values reported are mean ± standard error of the mean (SEM), except where indicated. All data were analyzed using Minitab statistical software, version 13 (Minitab, State College, PA). Significance is indicated by p values < 0.05.

RESULTS

We observed no significant sex effect on life span ($p = 0.488$) and no sex by treatment interaction ($p = 0.495$), therefore life span data for male and female mice were pooled. Kaplan-Meier analysis of survival (44 α-tocopherol supplemented and 43 control mice) indicated that α-tocopherol supplemented mice lived significantly longer (Fig. 1; $X^2 = 4.157$; $p = 0.042$) than control mice. Median life span of α-tocopherol supplemented mice (785 days, range 367–952 days) was 103 days (15%) greater than control mice (682 days, range 373–917 days). The oldest 10% survival (mean ± SEM) was

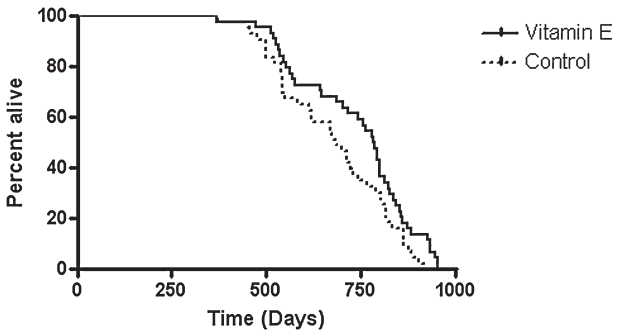

FIG. 1. Life span of life-long α-tocopherol supplemented (19 female and 25 male) and control mice (21 female and 22 male) maintained at 7 ± 2°C. α-tocopherol supplemented mice lived significantly longer (log rank test, $X^2 = 4.157$; $p < 0.05$) than control mice when maintained in the cold (7 ± 2°C). Median life span of α-tocopherol supplemented mice was 785 days (range 367–952 days) and control mice was 682 days (range 373–917 days). There was no significant sex or sex by treatment interaction effects on life span ($p > 0.05$). α-tocopherol supplemented mice indicated by solid line and control mice by dotted line.

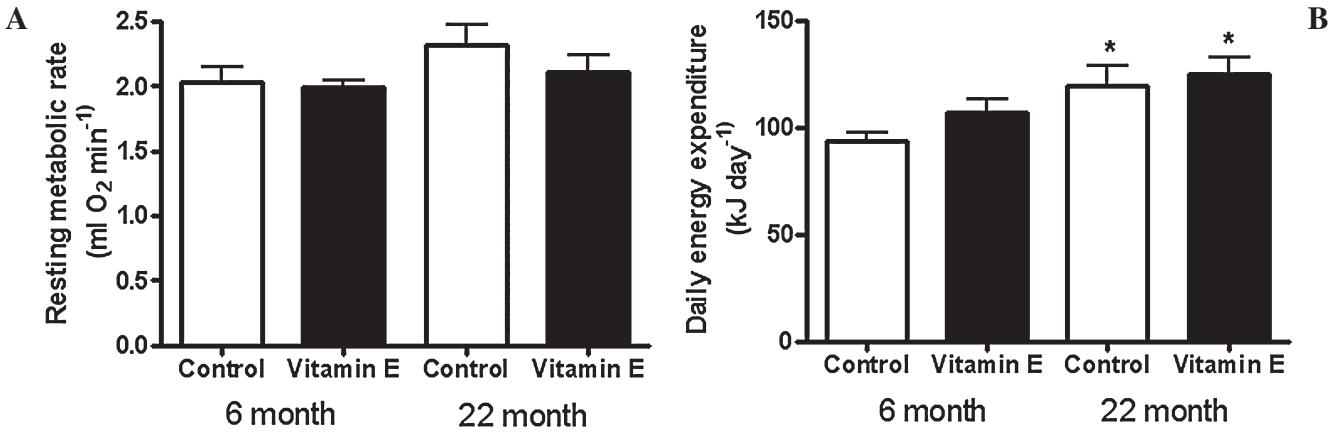

FIG. 2. (**A**) There were no significant age or treatment effects on resting metabolic rate and no age by treatment ($p > 0.05$). (**B**) Daily energy expenditure significantly increased with age ($F = 4.89$, $p < 0.05$) but no significant treatment or age-by-treatment interaction effect on DEE was observed ($n = 6$–13).

significantly greater ($p = 0.028$) at 945 ± 5 days in α-tocopherol supplemented mice compared to control mice (894 ± 9 days).

To determine the effects of α-tocopherol supplementation on metabolism (RMR and DEE), we employed a general linear model with age and treatment as factors and body mass as a covariate in mice at 6 or 22 months of age (2 or 18 months following supplementation). There were no significant age or treatment effects on RMR, and no treatment by age interaction (Fig. 2A; $p > 0.05$ in all cases). There was a significant age-associated increase in DEE (Fig. 2B; $F = 4.89$, $p < 0.05$) but no significant treatment or age by treatment interaction effect.

Hepatocyte oxidative DNA damage increased significantly with age (6 to 22 months of age) when using the site-specific enzymes endonuclease III (ENDO III, Fig. 3A; $F = 8.45$, $p = 0.008$) and formamidopyrimidine-DNA glycosylase (FPG; Fig. 3B; $F = 8.99$, $p = 0.007$), but no treatment effect was observed ($p > 0.05$). In lymphocytes, α-tocopherol did not alter oxidative DNA damage compared to control mice, either when using the site-specific enzyme ENDO III (Fig. 3C; $F = 4.20$, $p = 0.054$) or FPG (Fig. 3D; $p > 0.05$). A significant age-related decrease in lipid peroxidation was seen (Fig. 3E; $F = 22.14$, $p < 0.001$), but again no treatment effect was observed. Hepatic α-tocopherol was significantly elevated in the supplemented mice (Fig. 3F; $F = 8.37$, $p = 0.007$) relative to controls, with both a significant age ($F = 7.03$, $p = 0.013$) and an age by treatment interaction effect ($F = 5.95$, $p = 0.021$).

Table 1 summarizes those genes, their p values, and fold changes altered significantly in expression by age, but not treatment (α-tocopherol supplementation) or by an age by treatment interaction. Table 2 shows the p values and fold changes of genes where there was a significant treatment effect in combination with an age effect, an age-by-treatment interaction, or a direct treatment effect in the absence of any age-related effect. We present the fold changes for the four-way comparisons of 2 months against 18 months following initiation of experiment (equivalent to 6 and 22 months of age) for both control and α-

FIG. 3. Employing a general linear model with age and treatment as factors and body mass as a covariate indicated that hepatic oxidative DNA damage increased significantly with age when using the site-specific enzymes endonuclease III (Endo III, 8.45, $p < 0.01$) (**A**) and formamidopyrimidine-DNA glycosylase (FPG, $F = 8.99$, $p < 0.01$) (**B**), but treatment had no effect. Lymphocyte oxidative DNA damage was unaffected by treatment and by age when using ENDO III ($p > 0.05$) (**C**) and FPG ($p > 0.05$) (**D**). Mice supplemented with α-tocopherol did not have significantly different levels of hepatic lipid peroxidation (thiobarbituric acid(reactive substances) compared to control mice ($p > 0.05$), although there was a significant age effect ($F = 22.14$, $p < 0.001$) (**E**). Hepatic α-tocopherol was significantly elevated in supplemented mice ($F = 8.37$, $p = 0.007$) (**F**), with a significant age ($F = 7.03$, $p = 0.013$) and age-by-treatment interaction ($F = 5.95$, $p = 0.021$) observed. All mice are denoted by age, that is, 6 or 22 months of age, corresponding to 2 or 18 months of α-tocopherol supplementation. Mean ± SEM; $n = 5$–12 in all cases.

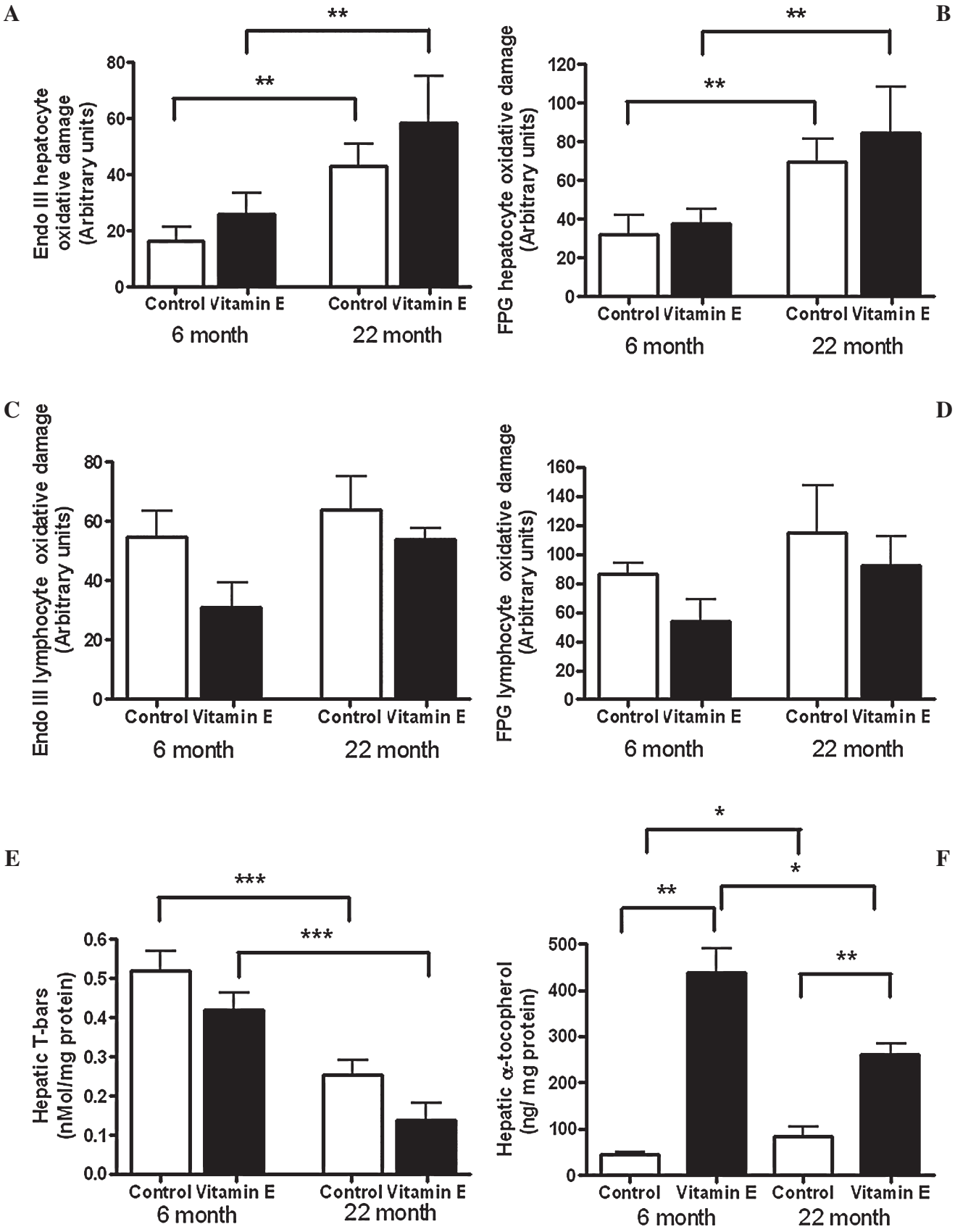
A
Endo III hepatocyte oxidative damage (Arbitrary units)
80
60
40
20
0
**
**
Control Vitamin E
Control Vitamin E
6 month
22 month
B
FPG hepatocyte oxidative damage (Arbitrary units)
120
100
80
60
40
20
0
**
**
Control Vitamin E
Control Vitamin E
6 month
22 month
C
Endo III lymphocyte oxidative damage (Arbitrary units)
80
60
40
20
0
Control Vitamin E
Control Vitamin E
6 month
22 month
D
FPG lymphocyte oxidative damage (Arbitrary units)
160
140
120
100
80
60
40
20
0
Control Vitamin E
Control Vitamin E
6 month
22 month
E
Hepatic T-bars (nMol/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
Control Vitamin E
Control Vitamin E
6 month
22 month
F
Hepatic α-tocopherol (ng/ mg protein)
500
400
300
200
100
0
*
*
**
**
Control
Vitamin E
Control
Vitamin E
6 month
22 month

Table 1. Significant Hepatic Gene Expression Changes with Age

Gene	*Gene symbol*	p	*Age fold-change control*	*Age fold-change α-tocopherol*
(A) Up-regulated				
G/T-mismatch binding protein	Gtbp	0.0011	1.93	1.88
Microsomal UDP-glucuronosyltransferase 1-7 precursor	Udpgt	0.0077	1.60	1.58
Uracil-DNA glycosylase	Ung1	0.0376	1.33	1.11
Yeast DNA repair protein Rad52 homolog	MmRad52	0.0356	1.45	1.30
(B) Down-regulated				
Islet of langerhans regenerating protein 3 beta	Reg III-beta	0.0013	−2.42	−1.47
DNA-repair protein	Xrcc1	0.0019	−1.68	−1.43
T-complex protein 1 zeta subunit 2	Tcp1-zeta-2	0.0076	−1.38	−1.38
Islet of langerhans regenerating protein 3 alpha	Reg 111-alpha	0.0081	−1.89	−1.54
Beta crystallin B2	Crybb2	0.0093	−2.03	−1.23
Gamma crystallin A-F	Cryga-f	0.0104	−1.50	−1.84
7,8-dihydro-8-oxoguanine triphosphatase homolog	8-oxo-dGTPase	0.0126	−1.37	−1.32
Stress-activated c-jun N-terminal kinase 3	p49	0.0138	−1.73	−1.37
Extracellular superoxide dismutase precursor (Cu-Zn)	Cu-Zn SOD	0.0144	−1.52	−2.09
Probable protein disulfide isomerase	Erp60/Calr	0.0153	−1.17	−1.57
Extracellular signal-regulated kinase 6	p38 gamma	0.0175	−1.42	−1.36
Cytochrome P450 IA2	Cyp1a2	0.0211	−1.52	−1.53
5,6-dihydroxyindole-2-carboxylic acid oxidase precursor	Tyrp1/Cat B	0.0215	−1.37	−1.43
DNAJ-like heat-shock protein from mouse tumor	Mtj1	0.0236	−1.11	−1.66
DNA mismatch repair protein	Msh2	0.0257	−1.14	−1.26
T-complex protein 1 eta subunit	Tcp1-eta	0.0281	−1.22	−1.80
Catalase 1	Cat	0.0363	−1.25	−1.70
Osmotic stress protein;	Osp94	0.0418	−1.31	−1.27
General transcription factor IIH polypeptide 1	Tfllh	0.0452	−1.10	−1.43

tocopherol supplemented mice and the treatment against control comparisons at 2 and 18 months. Of the 23 genes that significantly changed expression with age (Table 1) independent of a treatment effect, only four genes, Gtbp, Udpgt, Ung1, and Rad54, significantly increased in expression. The remaining 19 genes all showed a significant decrease in expression with age (Table 2), several of which were involved in cellular/oxidative stress and DNA repair, including Cu-Zn SOD, Cat, Osp94, Mtj1, Xrcc1, 8-oxo-dGTPase, and Msh2. Northern analysis showed good agreement with our microarray gene expression data (Supplemental Fig. 1), with Cu-Zn SOD, Cat, and indeed glutathione peroxidase (Gpx was not present on the macroarray), all elevated in the livers of 6-month-old mice relative to 22-month-old mice.

A total of 29 genes were significantly altered in expression following α-tocopherol supplementation (Table 2), either as a direct treatment effect (26 genes) or as a treatment-by-age interaction (14 genes). In agreement with Table 1, the majority of the treatment-by-age effects on gene expression were decreased (Table 2). We grouped the 29 differentially regulated genes into five broad functional sub-groups—cytochrome p450s, detoxification, p21 signaling cascade, repair/protection, and those genes unrelated to the above four functions. The cytochrome p450 group included several associated with the p450 II subgroup, and along with the genes in the phase 2 detoxification group, the majority were significantly upregulated in the α-tocopherol treated group by around 2- to 3-fold after 2 months of supplementation (6 months of age). Only one cytochrome p450 (VIIb1) was downregulated following 2 months of α-tocopherol supplementation (Table 2), but even including this down-regulated gene the average expression difference of these P450 and detoxification genes between α-tocopherol and control mice following 2 months of treatment was 235% (2.35 fold). However, by 18 months of treatment (22 months of age), the transcriptional changes between treatment and control groups for these P450 and detoxification genes were much reduced (Table 2).

Table 2. Significant Hepatic Gene Expression Changes with Treatment (α-Tocopherol Supplementation), or with an Age by Treatment Interaction

Gene	*Gene symbol*	p *treatment*	*Treatment fold-change 6 months*	*Treatment fold-change 22 months*	p *age*treatment*	*Age fold-change control*	*Age fold-change α-tocopherol*
Cytochrome P450							
Cytochrome P450 IIIC29	Cyp2c29	<0.0001	3.04	1.09	<0.0001	1.31	−2.12
Cytochrome P450 IIF2	Cyp2f2	<0.0001	3.07	1.10	<0.0001	1.29	−2.16
Cytochrome P450 IIB9	Cyp2b9	0.0006	2.15	1.24	0.0207	−1.60	−2.77
Cytochrome P450 IID9	Cyp2d9	0.0090	1.78	1.20	0.1368	1.10	−1.35
Cytochrome P450 IIIA11	Cyp3a11	0.0141	2.57	−1.11	0.0007	−1.46	−4.17
Cytochrome P450 IIA4	Cyp2a4	<0.0001	3.11	1.32	<0.0001	1.10	−2.13
Cytochrome P450 IIE1	Cyp2e1	<0.0001	3.05	1.17	<0.0001	1.12	−2.33
Cytochrome P450 VIIB1	Cyp7b1	0.0496	−1.81	−1.62	0.8404	−1.44	−1.30
Detoxification							
Microsomal UDP-glucuronosyltransferase 1-1 precursor	Udpgt/Ugt1a1	<0.0001	2.88	1.22	<0.0001	1.07	2.21
Microsomal UDP-glucuronosyltransferase 2B5 precursor	Udpgt/Ugt2b5	0.0253	1.61	1.08	0.0746	−1.02	−1.52
Liver carboxylesterase precursor; esterase 31	Es31	<0.0001	3.13	1.05	<0.0001	−1.06	−3.18
Liver carboxylesterase precursor; esterase 1	Es1	<0.0001	3.03	1.10	<0.0001	1.30	−2.11
Alcohol sulfotransferase 1	Sta1	0.0344	2.98	1.81	0.5287	3.67	2.24
Thiosulfate sulfurtransferase	Tst	0.0193	1.30	1.34	0.8901	−1.24	−1.20
Quinone oxidoreductase	Cryz	0.1321	−2.10	1.12	0.0216	1.10	2.62
p21 signaling cascade							
Cyclin-dependent kinase inhibitor 1	Cdkn1a/p21	<0.0001	1.29	5.54	0.0007	−4.78	−1.11
MAP kinase-activated protein kinase 2	Mapkapk-2	0.0028	1.76	1.36	0.3045	−1.29	−1.67
Mitogen-activated protein kinase p38	p38	0.0409	1.20	1.92	0.2289	−1.92	−1.20
Mouse double minute 2	Mdm2	0.5880	−1.51	1.89	0.0395	−2.07	1.38
Repair/protection							
DNA repair protein homolog 1	Rad51	0.0038	−1.04	−1.53	0.0163	1.28	−1.15
Xeroderma pigmentosum group C complementing protein	Ercc5	0.0311	−1.40	−1.44	0.9268	−1.23	−1.26
DNA polymerase alpha catalytic subunit	Pola-1	0.0157	1.38	1.09	0.1296	−1.07	−1.34
V(D)J recombination activating protein	Rag-1	0.0176	1.20	1.58	0.2957	−1.13	1.16
Mitochondial matrix protein P1 precursor	Hsp-60/Hspd1	0.0182	2.24	1.24	0.1347	1.11	−1.63
T-complex protein 1 gamma subunit	Tcp1-gamma	0.0424	1.46	1.20	0.4358	−1.15	−1.39
Endoplasmin precursor; endoplasmic reticulum protein 99	Erp99/Hsp90b1	0.0152	1.87	1.13	0.0724	−1.05	−1.74
Calnexin precursor	Canx	0.0029	1.93	1.23	0.0701	−1.15	−1.81
Replication factor C1	Recc1/lbf-1	0.9170	1.22	−1.17	0.0046	1.02	−1.14
Other							
Vimentin	Vim	0.0304	1.38	1.11	0.2369	1.08	−1.15

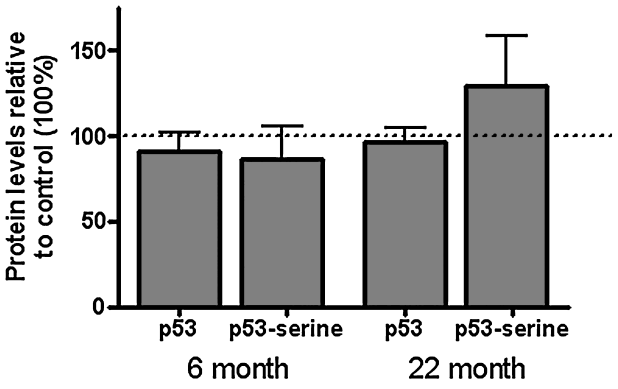

FIG. 4. Western blot scanning densitometry quantification of the levels of total p53 and phosphorylated p53 at serine[15] in liver of α-tocopherol supplemented mice at 6 months and 22 months of age relative to age-matched controls. Protein levels were not significantly affected by age or by treatment ($p > 0.05$). Mean ± SEM, with protein levels expressed as a percentage of control values (100%); $n = 5–6$.

In contrast, supplementation on genes associated with the p21 signaling pathway were only slightly upregulated by α-tocopherol supplementation at 6 months of age (20–76% above control), with Mdm2 downregulated relative to control levels (Table 2). However, by 18 months of supplementation (22 months of age) the four genes associated with this pathway were all upregulated by on average 268% (2.68 fold), with p21 upregulated 5.5 fold (554%), although p53 transcript levels were not altered by treatment. Using Western analysis, we show there were no significant age, treatment, or age-by-treatment interaction effects (Fig. 4; $p > 0.05$ in all cases) on either p53 or p53-serine protein levels in the liver relative to control mice. The effects of α-tocopherol supplementation on DNA repair and cellular protection against oxidative stress were less clear. After two months of supplementation, several chaperones and heat shock protein increased significantly in expression, but following 18 months of treatment the fold-change of all these chaperones were reduced relative to the control group (Table 2).

DISCUSSION

The consumption of diets rich in antioxidants have been advocated as a method to improve and enhance health span and life span.[9] Dietary supplementation with α-tocopherol increased significantly the median and maximum (oldest 10%) life span of mice in this study housed at 7 ± 2°C, in accord with previous studies that maintained mice at more benign temperatures.[18,19,21,46] We initiated supplementation at 4 months of age, and the age of initiation and duration of supplementation may be critical in achieving positive life span effects,[19] with studies observing a life span extension generally starting vitamin E/α-tocopherol supplementation earlier[18,19,46] rather than later in life.[14,24,47]

The oxidative stress theory of aging suggests that damage to proteins, lipids, and DNA are a causal factor in the aging process.[1] Increases in lifetime metabolic rate are often assumed to be positively associated with elevated ROS production and hence oxidative stress, although considerable debate exists on how metabolic rate is related to both ROS production and to life span.[27,48–51] Whatever direction these effects take, we observed no consistent difference in metabolic rate parameters following α-tocopherol supplementation, suggesting the life span effects were not mediated via this route. Perhaps surprisingly, given the positive effect on life span, there was no reduction in the oxidative stress parameters measured following supplementation, with hepatic and lymphocyte DNA oxidative damage and hepatic lipid peroxidation being unaffected relative to controls, although hepatic DNA oxidative damage increased with age. Our data confirms previous studies showing variable effects of vitamin E/α-tocopherol supplementation on oxidative stress.[14,23,24,52,53]

Consistent with the absence of a dramatic effect of α-tocopherol supplementation on various oxidative stress parameters, alterations in expression of genes associated with protection and repair against oxidative stress were similarly small and inconsistent. Several genes associated with protection against oxidative stress and DNA damage, including Cu-Zn SOD, catalase, 8-oxo-dGTPase, and Xrcc1 decreased with age but were not affected by treatment. The age-associated declines in Cu-Zn SOD and catalase expression were correlated with Northern blot analysis of these same genes in the same animals,[26] with glutathione peroxidase (Gpx was absent from the macroar-

ray) also downregulated with age. The DNA repair genes Rad51 and Ercc1 were downregulated by treatment, while several molecular chaperones were upregulated by α-tocopherol supplementation. These changes were greatest at 2 months of supplementation (6 months old), but by 18 months the differences in expression of most of these genes between supplemented and control animals were reduced.

Our macroarray analysis indicated that eight cytochrome P450 genes and five phase 2 detoxification genes were upregulated by 2- to 3-fold after 2 months of supplementation. The initial step of α-tocopherol degradation is a w-hydroxylation of the side chain element by cytochrome P450 hydroxylases.[54,55] Supplementation with α-tocopherol increases cytochrome P450s levels in mice[12,56] and activates the nuclear pregnane X receptor,[57] which itself regulates many genes linked to xenobiotic detoxification, including several P450 enzymes.[58] It is feasible that α-tocopherol is initially metabolized as a xenobiotic, perhaps explaining why previous studies generally only observe a life span extension when supplementation is initiated early in life. However, it has also been suggested that induction of genes linked to xenobiotic metabolism plays a significant role in longevity assurance in animals.[59–61] Interestingly, the induction of hepatic P450 genes after 18 months of supplementation was largely abrogated, although as we examined only two time-points, we do not know exactly when this actually occurred. However, by 18 months of supplementation, a significant upregulation of several genes associated with the cyclin dependent kinase inhibitor 1A (Cdki1a/p21) signaling pathway was observed, with p38 Mapk up 92%, Mdm2 up 89%, and p21 upregulated 554% (5.5-fold). The main regulator of p21 is p53, with the antitumorigenic action of p53 mediated via p21. However, there was no evidence that p53 protein levels (total and phosphorylated) were altered by α-tocopherol treatment. This suggests that α-tocopherol may modulate p21 expression independently of p53, possibly via the promoter region of the p21 gene, which contains a C/EBPb response element sensitive to α-tocopherol.[62]

In summary, we demonstrate that lifelong supplementation with α-tocopherol (initiated at 4 months of age) significantly extends median life span by 15% in cold exposed mice. No treatment effects were observed on oxidative stress or on oxidative stress-related gene expression. The induction of cytochrome P450 and detoxification genes early in the treatment phase (after 2 months) suggests exogenous α-tocopherol may initially be identified as a xenobiotic and detoxified, and this upregulation may be important in the life span extension observed. By 18 months of supplementation (22 months of age) the expression of these genes was almost normalized, at a time when the treatment group showed upregulation of genes involved in the p21 signaling pathway. Our experimental approach has therefore identified two distinct signaling pathways, which are induced in a temporal fashion, that may potentially underlie the life span extension following α-tocopherol supplementation in cold-exposed mice. These findings now require further investigation to establish their specific role in life span extension following lifelong α-tocopherol supplementation in mice.

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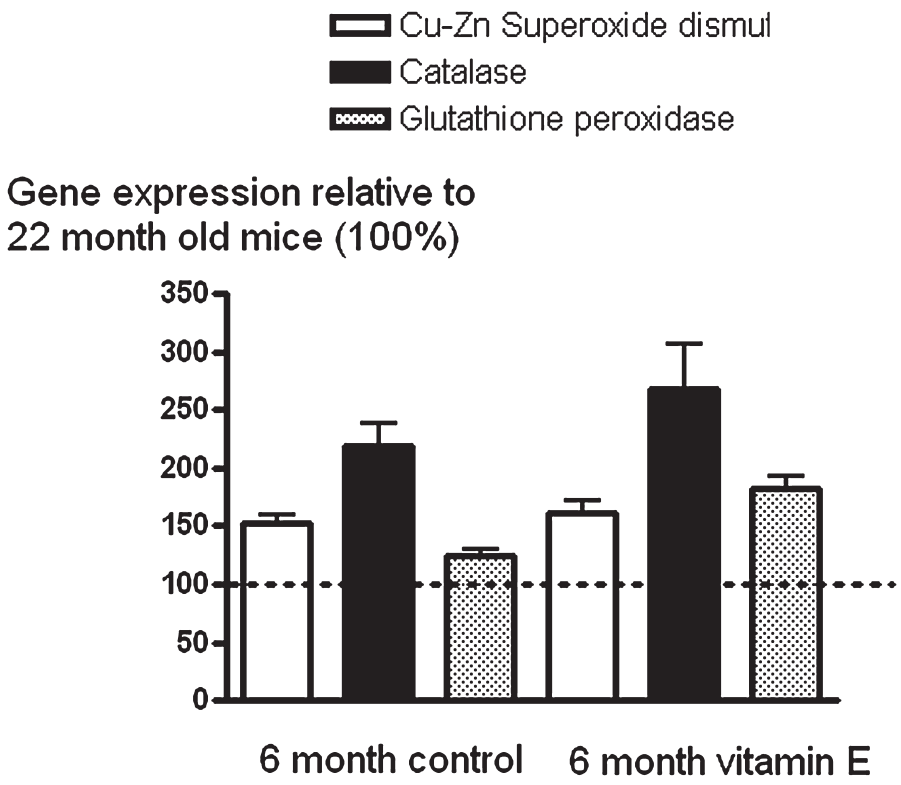

Supplementary FIG. 1. Age-related gene expression as determined by Northern analysis. mRNA levels of Cu-Zn superoxide dismutase, catalase, and glutathione peroxidase (not present on macroarray) at 6 months of age in control and α-tocopherol supplemented mice relative to control or α-tocopherol supplemented mice at 22 months of age. Values expressed as percentage mean ± SEM relative to expression levels at 22 months of age (100%, indicated by line). All samples relative to 18S; $n = 5–6$.