

Mechanisms underlying the radioprotective properties of γ -tocotrienol: comparative gene expression profiling in tocol-treated endothelial cells

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Received: 28 March 2011 / Accepted: 8 April 2011 / Published online: 24 April 2011
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Abstract Among the eight naturally occurring vitamin E analogs, γ -tocotrienol (GT3) is a particularly potent radioprophylactic agent *in vivo*. Moreover, GT3 protects endothelial cells from radiation injury not only by virtue of its antioxidant properties but also by inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and by improving the availability of the nitric oxide synthase cofactor tetrahydrobiopterin. Nevertheless, the precise

mechanisms underlying the superior radioprotective properties of GT3 compared with other tocols are not known. This study, therefore, examined the differences in gene expression profiles between GT3 and its tocopherol counterpart, γ -tocopherol, as well as between GT3 and α -tocopherol in human endothelial cells. Cells were treated with vehicle or the appropriate tocol for 24 h, after which total RNA was isolated and genome-wide gene expression profiles were obtained using the Illumina platform. GT3 was far more potent in inducing gene-expression changes than α -tocopherol or γ -tocopherol. In particular, GT3 induced multiple changes in pathways known to be of importance in the cellular response to radiation exposure. Affected GO functional clusters included response to oxidative stress, response to DNA damage stimuli, cell cycle phase, regulation of cell death, regulation of cell proliferation, hematopoiesis, and blood vessel development. These results form the basis for further studies to determine the exact importance of differentially affected GO functional clusters in endothelial radioprotection by GT3.

This paper is part of a Special Issue entitled “Tocotrienols in health and disease” (Guest editors: Bharat B. Aggarwal and Kalaniti Nesaretnam).

Electronic supplementary material The online version of this article (doi:10.1007/s12263-011-0228-8) contains supplementary material, which is available to authorized users.

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Keywords Radiation injuries · Tocotrienol · Tocopherol · Vitamin E · Genome expression profiling

Introduction

Exposure to high doses of ionizing radiation can cause severe, sometimes even life threatening injury to normal tissues. Radiation-induced tissue injury may occur both in clinical and nonclinical settings. In patients treated with radiotherapy, normal tissue radiation toxicity is the most important cause of treatment-related side effects, and thus limits the uncomplicated cancer cure rate. Nonclinical exposure with high radiation doses may occur in

emergency situations, such as radiological/nuclear accidents or terrorist attacks. Under such circumstances, radiation exposure may cause substantial mortality.

Because of the potential impact of radiation injury and the limited availability of effective radioprotectants, there is an ongoing quest to develop novel pharmacological agents that can prevent or mitigate normal tissue radiation injury. Because of relatively potent antioxidant properties and lack of performance-degrading side effects, vitamin E analogs, collectively referred to as tocopherols, are considered to be attractive candidate agents for radioprotection. In recent years, various tocopherols have been tested for their radioprotective potential (Berbee et al. 2009; Kumar et al. 2002; Singh et al. 2006). Important structural and functional differences exist between the different tocopherols. Tocopherols and tocotrienols differ in the side chain that is attached to the chroman ring. Whereas tocopherols have a saturated phytyl side chain, tocotrienols have an unsaturated isoprenoid chain. The four isoforms, α -, β -, δ - and γ -, differ in the number and position of methyl groups on the chroman ring.

Most studies on the effects of vitamin E on radiation injury were conducted with α -tocopherol, the most commonly used vitamin E supplement and the vitamin E isoform that is most abundant in human and animal tissues (Bichay and Roy 1986; Felemovicius et al. 1995; Odagiri et al. 1992; Ramos et al. 2006; Singh et al. 2006; Srinivasan and Weiss 1992). However, recent studies have shown that other members of the tocopherol family, γ -tocotrienol (GT3) in particular, are far more effective in reducing acute radiation toxicity and protecting from lethality. In a direct side-by-side comparison, Kumar et al. demonstrated that GT3 is superior to α -tocopherol in reducing lethality after total body irradiation (TBI) in mice (Kumar et al. 2008). Subsequent studies have shown that the radioprotective properties of GT3 not only depend on its antioxidant action but also on the modulation of endothelial cell function (Berbee et al. 2009). Endothelial dysfunction is a prominent feature of early as well as late radiation injury and has been shown to play an important role in the pathogenesis of radiation toxicity in various organ systems (Baker and Krochak 1989; Hopewell et al. 1993; Jaenke et al. 1993; Lyubimova and Hopewell 2004; Maj et al. 2003; Paris et al. 2001; Rezvani et al. 1995; Wang et al. 2002, 2007).

The exact mechanisms underlying the superior radioprotective effects of GT3 and, in particular, how GT3 reduces radiation-induced endothelial dysfunction has not been elucidated. GT3, in contrast to α -tocopherol, is a potent inhibitor of endothelial 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (Parker et al. 1993; Song and DeBose-Boyd 2006). Moreover, after radiation exposure of mice in vivo, GT3 reduces radiation-induced vascular oxidative stress in an HMG-CoA reductase dependent

manner (Berbee et al. 2009). GT3 may also exert its beneficial inhibitory effects on postirradiation free-radical production by improving the availability of the endothelial nitric oxide synthase (eNOS) cofactor BH4 through downregulation of GTP-cyclohydrolase 1 regulatory protein (GFRP), thereby enhancing the production of NO and reducing the production of ONOO⁻ by eNOS (Berbee et al. 2011). Finally, GT3 has been shown to improve the post-irradiation recovery of plasma markers of endothelial function, an effect that is not dependent of HMG-CoA reductase inhibition (Berbee et al. 2009).

In order to improve our understanding of the mechanisms responsible for the differences in radioprotective potential of the various tocopherols, we performed a series of gene-expression experiments to compare GT3, γ -tocopherol, and α -tocopherol. By identifying the genomic functional pathways that are differentially regulated by GT3 compared to the other two tocopherols, several candidate pathways and genes were identified. These findings offer opportunities for future mechanistic studies to determine the exact mechanism by which GT3 confers superior radioprotection in vivo and for development of novel analogs to further enhance these properties.

Methods

Cell culture and reagents

Unless otherwise specified, chemicals were obtained from Sigma–Aldrich (St. Louis, MO). α -tocopherol, γ -tocopherol, and GT3 were acquired from Yasoo Health Inc. (Johnson City, TN) and were dissolved in ethanol to obtain stock solutions of 50 mM. Tocol stock solutions were stored in liquid nitrogen.

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD), and cell cultures were performed as described previously (Boerma et al. 2006). HUVECs were cultured in EGM-2 medium with growth supplements (Lonza). Passage 4–6 cells were used in the experiments. After 2 days (confluence 90%), the cells were incubated for 24 h in 3 ml culture media containing vehicle (ethanol), 5 μ M α -tocopherol, 5 μ M γ -tocopherol, or 5 μ M GT3. The cells were harvested after 24 h of incubation. Total RNA was isolated using Ultra-spec. The experiments were performed in triplicate.

Arrays

Microarrays were used to determine tocol-induced changes in gene expression profiles in the HUVEC cells. Both the generation of aRNA and microarray hybridization were performed by the Microarray Core Laboratory of the

University of Texas Health Science Center (Houston, TX). All RNA samples were tested for integrity and minimal degradation using an Agilent Bioanalyzer 2100. Two hundred nanogram of total RNA was reverse transcribed and amplified overnight with T7 RNA polymerase and labeled with biotin following the manufacturer's protocol, in which 1.5 mg of biotin labeled aRNA was hybridized to microarrays at 58°C overnight. Illumina Genome-Wide Expression BeadChips (Human Ref- 6 v 2.0, Illumina, San Diego, CA), representing ~43,000 human transcripts (Kuhn et al. 2004), were used. Arrays were incubated with Cy3 streptavidin and washed according to the manufacturer's protocol. Initial analysis of the microarray data was done using Illumina's Beadstudio V1. After background subtraction, arrays were normalized to each other by rank-invariant normalization. Changes in gene expression were tested using a modified *t* test that employs estimates of variation that include sequence-specific biological variation (σ_{bio}), nonspecific biological variation (σ_{neg}), and technical error (σ_{tech}), according to the Illumina User Guide (2005), rev B. Genes were considered differentially regulated at $P < 0.001$.

Genes that were up- or downregulated by α -tocopherol, γ -tocopherol, or GT3 were classified according to Gene Ontology (GO) biological processes and KEGG pathways using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Ashburner et al. 2000; Huang et al. 2009). GO biological processes with a $P < 0.05$ were considered to be differentially regulated.

Results

No signs of cell toxicity were observed after incubation with 5 μ M of α -tocopherol, γ -tocopherol, or GT3 for 24 h. There were major differences in the quantity of genes up- or downregulated by the different tocopherols. GT3 was by far the most potent, with a differential expression of 898 genes (Fig. 1). In comparison, at the 5 μ M concentration level, α -tocopherol and γ -tocopherol only changed the expression of 39 and 172 genes, respectively. There was limited overlap between the genes differentially expressed after treatment with the different tocopherols. There were only 2 genes upregulated by all 3 tocopherols: PMPCA and LOC642755.

In order to obtain information about the functional effects of up- and downregulated genes, the differentially regulated genes were classified according to GO biological processes. Both γ -tocopherol and GT3 affected genes involved in the regulation of cell death (Table 1). GT3 modified a total of 233 GO biological processes (Fig. 2). The affected GO functional clusters included several processes known to be of importance in the cellular response

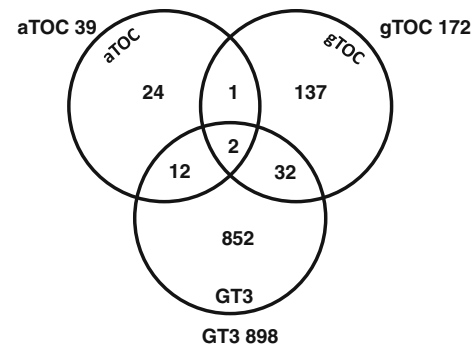


Fig. 1 Venn diagram of up- and downregulated genes in endothelial cells 24 h after treatment with α -tocopherol (aTOC) (5 μ M), γ -tocopherol (gTOC) (5 μ M), or γ -tocotrienol (GT3) (5 μ M). GT3 affected a total number of 898 genes, whereas aTOC and gTOC only affected 39 and 172 genes, respectively. There was limited overlap in up- and downregulated genes between the different tocopherols. Only 2 genes were affected by all 3 tocopherols. Genes were considered differentially regulated at $P < 0.001$

to ionizing radiation: response to oxidative stress (GO: 0006979) (see supplemental data for gene list), response to DNA damage stimuli (GO:0006974) (see supplemental data for gene list), cell cycle phase (GO:0022403), regulation of cell death (GO: 0010941), regulation of cell proliferation (GO:0042127), regulation of inflammatory response (GO:0050727), hematopoiesis (GO:0030097), and blood vessel development (GO:0001560) (Table 2; Fig. 3).

Gene expression in GT3-treated cells was also directly compared with gene expression in cells treated with α -tocopherol and γ -tocopherol. Relative to α -tocopherol and γ -tocopherol, GT3 up- or downregulated 102 and 84 genes, respectively. These genes corresponded to 90 and 42 GO biological pathways, respectively (Fig. 4). Compared to α -tocopherol, GT3 regulated various processes involved in the regulation of cell death. Compared to both α -tocopherol and γ -tocopherol, GT3 regulated several processes that are of importance in the response to and the recovery from radiation exposure, like regulation of cell proliferation (GO:0042127), regulation of inflammatory response (GO:0050727), and leukocyte migration (GO:0050900) (see supplemental data for a complete list of the differentially regulated pathways).

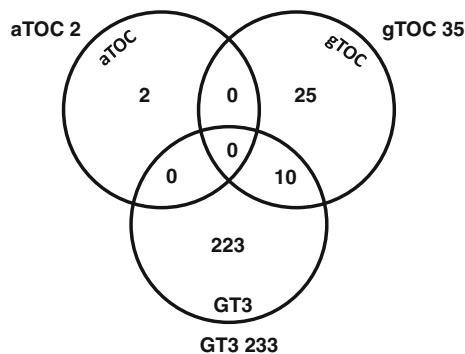
Discussion

The potent radioprotective properties of the vitamin E analog GT3 depend, at least partially, on protection of endothelial cell function. Recent gene expression studies using tumor cell lines and immune cells have shown that different tocopherols may induce distinct differential changes in these cell lines (Campbell et al. 2009; Han et al. 2010;

Table 1 Gene Ontology (GO) biological functional gene clusters regulated by both γ -tocopherol (gTOC) (5 μ M) and γ -tocotrienol (GT3) (5 μ M)

Term	GT3 count	<i>P</i>	gTOC count	<i>P</i>
GO:0006916 ~ antiapoptosis	16	0.03	8	0.004
GO:0042981 ~ regulation of apoptosis	56	0.0005	16	0.01
GO:0043067 ~ regulation of programmed cell death	57	0.0004	16	0.01
GO:0010941 ~ regulation of cell death	58	0.0002	16	0.01
GO:0043066 ~ negative regulation of apoptosis	27	0.006	9	0.0
GO:0006357 ~ regulation of transcription from RNA polymerase II promoter	51	0.0008	14	0.02
GO:0043069 ~ negative regulation of programmed cell death	28	0.004	9	0.02
GO:0060548 ~ negative regulation of cell death	29	0.002	9	0.02
GO:0012501 ~ programmed cell death	45	0.0006	12	0.03
GO:0051129 ~ negative regulation of cellular component organization	12	0.04	5	0.05

GO functional cluster were considered differentially regulated at $P < 0.05$

**Fig. 2** Venn diagram of differentially regulated Gene Ontology (GO) biological functional gene clusters in endothelial cells 24 h after treatment with α -tocopherol (aTOC) (5 μ M), γ -tocopherol (gTOC) (5 μ M), or γ -tocotrienol (GT3) (5 μ M). GT3 affected a total number of 233 GO functional clusters, whereas aTOC and gTOC only affected 2 and 25 clusters, respectively. Ten GO functional clusters were identified that were both affected by GT3 and gTOC. GO functional cluster was considered differentially regulated at $P < 0.05$

Wu and Ng 2010), suggesting that the same may apply to endothelial cells. With this study, we aimed to explore whether there are marked differential changes in endothelial gene expression after treatment with GT3, γ -tocopherol, and α -tocopherol and whether these tocopherols differentially regulate functional gene clusters known to be important in the cellular response to radiation. The present study thus sheds light on the mechanism underlying the superior radioprotective properties of GT3 compared to other tocopherols.

The specific comparison of GT3 with α -tocopherol and γ -tocopherol was performed because α -tocopherol is the most abundant tocopherol and because γ -tocopherol and GT3 share structural similarities with regard to the chromanol ring. However, γ -tocopherol and GT3 have different side chains: tocotrienols contain an isoprenoid side chain with three double bonds, whereas tocopherols have a (saturated) phytyl side chain. These structural variations are believed

Table 2 Selection of Gene Ontology (GO) biological functional gene clusters regulated by γ -tocotrienol (GT3) (5 μ M) and expected to be of importance in the response to and recovery from radiation exposure

Term	GT3 count	<i>P</i>
GO:0001568 ~ blood vessel development	25	2E-04
GO:0006974 ~ response to DNA damage stimulus	27	0.01
GO:0006979 ~ response to oxidative stress	14	0.03
GO:0010941 ~ regulation of cell death	58	0.0002
GO:0022403 ~ cell cycle phase	28	0.02
GO:0030097 ~ hematopoiesis	20	0.007
GO:0042127 ~ regulation of cell proliferation	64	1E-06
GO:0042981 ~ regulation of apoptosis	56	0.0005

GT3 differentially regulated a total 233 GO biological functional clusters

GO functional cluster were considered differentially regulated at $P < 0.05$

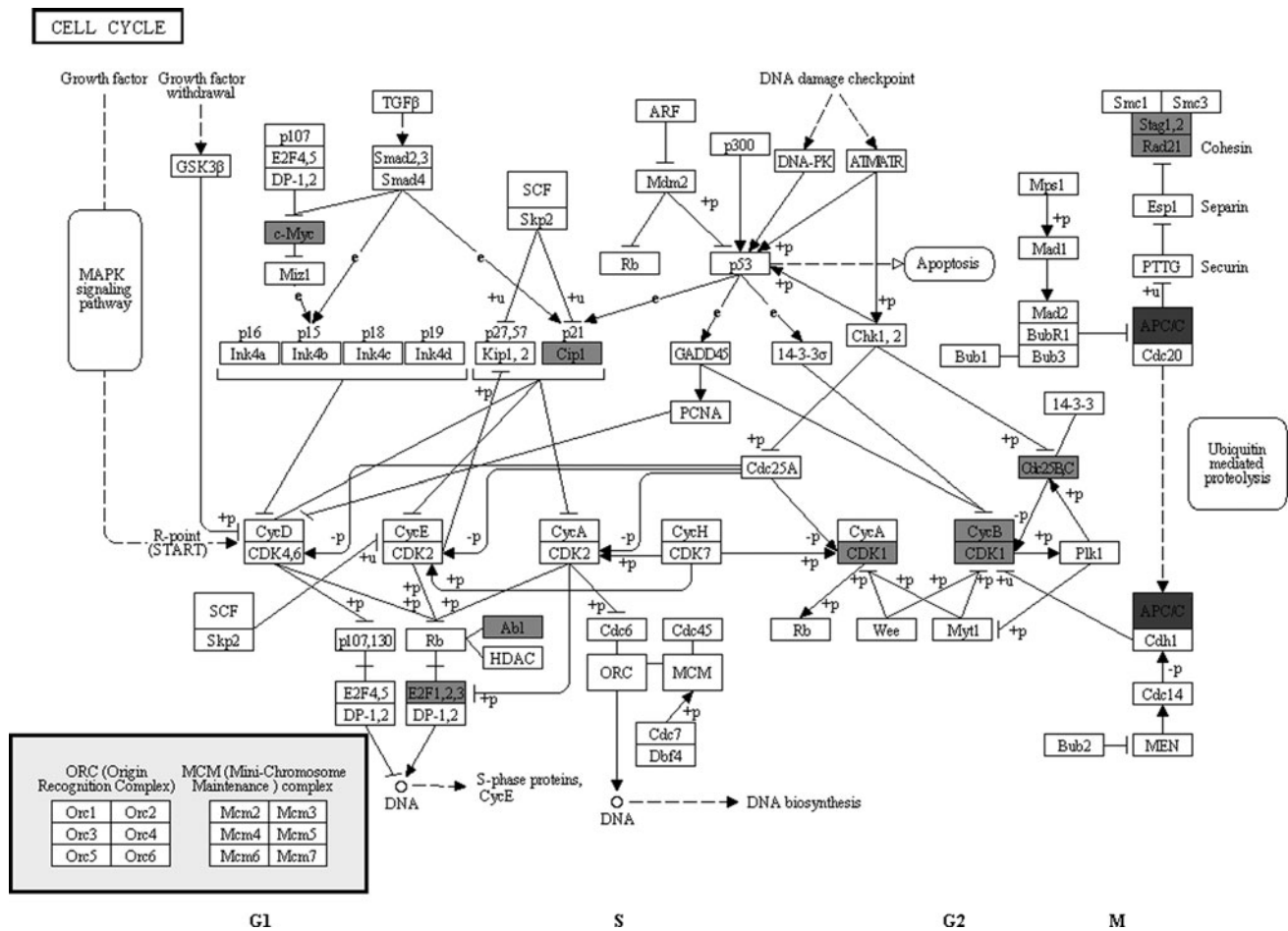


Fig. 3 Visualization of the quantity of genes differentially regulated by γ -tocotrienol (GT3) and α -tocopherol (aTOC) using the KEGG cell cycle pathway. α -tocopherol (aTOC) (5 μ M) only affects the gene expression of 1 gene, APC/C. γ -tocotrienol (GT3) (5 μ M) induces

changes in gene expression of APC/C and various other genes. *Dark gray*: genes regulated by both aTOC and GT3. *Light gray*: genes only regulated by GT3. Genes were considered differentially regulated at $P < 0.001$

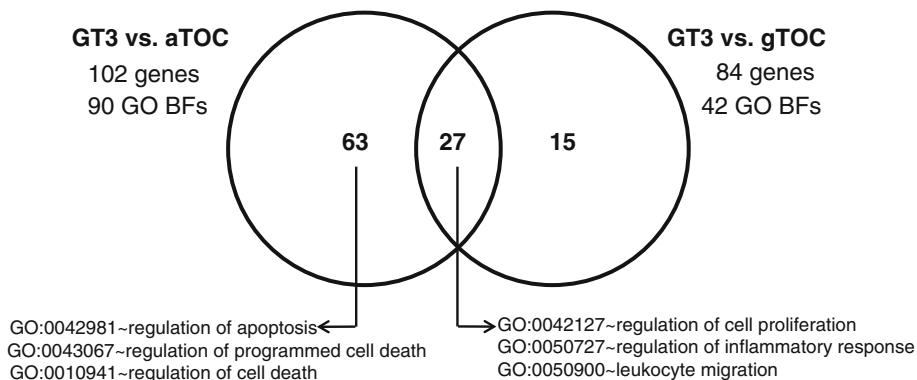


Fig. 4 Venn diagram of differentially regulated Gene Ontology (GO) biological functional gene clusters of genes up- or downregulated by γ -tocotrienol (GT3) (5 μ M) compared with α -tocopherol (aTOC) (5 μ M) and γ -tocopherol (gTOC) (5 μ M). GT3 regulated several

Gene Ontology (GO) biological functional gene clusters that are expected to be of importance in the response to and recovery from radiation exposure. GO functional cluster was considered differentially regulated at $P < 0.05$

to result in functional differences between the different tocotols that are thought to be due to differences in distribution in lipid bilayers, more efficient interaction with the

chromatin ring in tocotrienols with the lipid bilayer, and differences in cellular uptake (Aggarwal et al. 2010). Remarkably, as a result of the latter effect, GT3

accumulates in endothelial cells to levels that are 30–50 fold greater compared with α -tocopherol (Naito et al. 2005; Noguchi et al. 2003).

Comparative gene expression profiling in endothelial cells treated with different tocopherols revealed major differences in gene expression between the different treatment groups. Notably, GT3 was far more potent in inducing gene-expression changes than α -tocopherol or γ -tocopherol. Relative to vehicle-treated control cells, both GT3 and γ -tocopherol-induced gene clusters associated with the regulation of cell death and apoptosis, processes that clearly play an important role in the development of acute radiation toxicity. Moreover, compared to vehicle, GT3 induced multiple changes in several other pathways known to be important in the cellular response to radiation. Affected GO functional clusters included response to oxidative stress, response to DNA damage stimuli, cell cycle phase, regulation of cell proliferation, regulation of inflammatory response, blood vessel development, and hematopoiesis. Modification of these processes, as seen after treatment with GT3, can be expected to alter cellular radiation sensitivity and/or the ability to recover from the effects of radiation exposure. Further research is needed to determine the exact importance of the different processes in the mechanism underlying the radioprotective properties of GT3 and to identify the key regulatory genes.

GT3 is significantly more effective as a radioprotectant than most other tocopherols. By comparing gene expression in GT3-treated cells directly with gene expression in cells treated with α -tocopherol and γ -tocotrienol, as well as by analyzing differentially regulated functional gene clusters, we aimed to identify pathways that may be responsible for the difference in radioprotective effects of these tocopherols. With respect to processes that are known to regulate important cellular responses to radiation, GT3 distinguished itself from both α -tocopherol and γ -tocopherol by regulating gene clusters involved in cell proliferation and the inflammatory response. Moreover, compared to α -tocopherol, GT3 regulated several gene groups involved in the regulation of cell death.

Tocotrienols exert their effect on several different molecular levels. Hence, they modulate their targets not only at the transcriptional level but also at the translational or post-translational levels or by direct interaction with the substrate (Aggarwal et al. 2010). Therefore, the radioprotective effects of GT3 can also be a result of modifications at various levels. For example, this may explain why, in the current study, we did not observe changes in the gene expression levels of HMG-CoA reductase, even though the inhibition of HMG-CoA reductase is known to play an important role in the mechanism underlying GT3-induced regulation of endothelial cell function and radioprotection. In fact, GT3 regulates the activity of HMG-CoA reductase

by enhancing proteasomal degradation of the enzyme (Parker et al. 1993; Song and DeBose-Boyd 2006). GT3, like δ -tocotrienol, stimulates ubiquitination of HMG-CoA reductase by Insig proteins, thus targeting the enzyme for degradation by the 26S proteasome. Therefore, a direct change in gene expression levels of the enzyme would not be expected.

In a previous study, we provided evidence to support that GT3 exerts some of its beneficial effects on postirradiation free-radical production by improving the availability of the endothelial nitric oxide synthase (eNOS) cofactor BH4 through downregulation of GTP-cyclohydrolase 1 regulatory protein (GFRP) (Berbee et al. 2011). We showed that GT3 induced a small, but highly significant, decrease in GFRP transcription, gene expression, and protein level *in vitro* and abrogated the early decrease in BH4 levels observed *in vivo*. The observation from the current experiment that GFRP was not among the genes downregulated by GT3 seemingly contradicts these data. However, this might be caused by a difference in sensitivity of the methods used in our previous experiments and the microarray analysis used in the current study or because of a difference in GT3 concentration between the two studies.

A recent study by Li et al. showed that δ -tocotrienol protects mouse and human hematopoietic progenitors from γ -irradiation through Erk activation-associated mTOR survival pathways (Li et al. 2010). In contrast, in our current experiment, no evidence was found suggesting that GT3 conveys endothelial radioprotection through Erk/mTOR signaling. This difference may be either a result of differences between γ -tocotrienol and δ -tocotrienol or, alternatively, depend on the studied cell type.

Taken together, our data demonstrate that GT3 is substantially more effective in inducing gene-expression changes in endothelial cells than α -tocopherol, the standard vitamin E compound, or γ -tocopherol, its saturated structural analog. Moreover, GT3 induced multiple changes in functional genetic pathways known to be of critical importance in the regulation of cellular responses to radiation exposure, such as response to oxidative stress, response to DNA damage stimuli, cell cycle phase, regulation of cell death, regulation of cell proliferation, hematopoiesis, and blood vessel development. These results form the basis for further studies to assess the importance of the different affected GO functional clusters in *in vivo* GT3-induced radioprotection.

Acknowledgments Dr. Berbée was enrolled in the PhD program in the Department of Radiation Oncology, GROW Research Institute, Maastricht University Medical Center, The Netherlands. This work was supported by the National Institutes of Health (grants AI67798, CA83719, and CA71382 to MH-J), Defense Threat Reduction Agency (grants H.10027_07_AR_R and H.10045_07_AR_R to KSK) and the Veterans Administration. Presented in part at the 1st

International Conference on Tocotrienols and Chronic Disease, Las Vegas, NV, July 29–30, 2010.

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