IJABBR- 2014- eISSN: 2322-4827

International Journal of Advanced Biological and Biomedical Research

Journal homepage: www.ijabbr.com

Original Article

Tocotrienol Modulates the Expression of Proteins in Oxidative Stress-Induced Caenorhabditis Elegans

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A R T I C L E I N F O

Article history:
Received: 09 Apr, 2015
Revised: 30 May, 2015
Accepted: 28 Jun, 2015
ePublished: 30 Jul, 2015
Key words:
Development of cities
Green space
Promotion of environmental

A B S T R A C T

Objective: Oxidative stress that damages proteins result in aging and age related diseases. The aim of this study is to determine the effect of tocotrienol rich fraction (TRF) on the expression of proteins in oxidative stress-induced caenohabditis elegans (C.elegans) which has homologous genes to humans. **Methods:** The worms were treated with TRF prior to, after and continuously in separate groups upon induction of oxidative stress with hydrogen peroxide. The expression of proteins were analyzed with 2D-gel electrophoresis and identified with mass spectrophotometry. Results showed that induction of oxidative stress and TRF treatment separately modulated the expression of 11 proteins. Pre-treatment of TRF altered the expression of 5 proteins while post-treatment and a continuous treatment of TRF in oxidative stress-induced worms affected the expression of 9 and 10 proteins respectively. **Results:** From these differentially expressed proteins, a total of 12 were successfully identified. TRF was found to increase the expression of glutathione-S transferase but decreased the expression of mRNA cap guanine-N7 methyltransferase, inorganic pyro-phosphatase, enoyle-CoA hydratase, vitellogenin 6 precursor, cathepsin B-like cysteine proteinase 4 precursor, triosephosphatase isomerase, tubulin-specific chaperon B and putative D-amino acid oxidase. In conclusion, TRF modulated the expression of proteins involved in energy metabolism, oxidative stress, proteolysis and biosynthesis of mRNA in C.elegans.

1.INTRODUCTION

Oxidative stress is a pathological condition associated with increased biomolecule damages caused by a group of oxidants known as reactive oxygen species (ROS). ROS encompasses free radicals and molecular species that generate free radicals such as hydrogen peroxide (H_2O_2). Oxidative stress has been reported to be involved in a number of human diseases such as cancer, atherosclerosis, diabetics, and neurological diseases as well as in the aging process (Kunwar et al., 2011). Despite having a remarkable repertoire of antioxidants, biomolecules in the cells are constantly oxidized by overwhelming production of free radicals (Fusco et al., 2007).

Vitamin E is a generic term for lipid-soluble, chain breaking antioxidants which consists of four tocopherol isomers (α, β, γ, δ) and four tocotrienol isomers (α, β, γ,

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δ). Although tocopherols and tocotrienols are closely related chemically, the later has been found to be more potent as an antioxidant (Muid et al., 2013). Researches over the past decade have showed the significance of tocotrienol in modulating oxidative stress response in aging and age related diseases (Budin et al., 2009; Chin et al., 2011; Makpol et al., 2010; Nazrun et al., 2008). The role of tocotrienol in disease prevention has since been further explored where it has been found to affect signalling pathways such as nuclear factor-κB, signal transducer and activator of transcription (STAT) 3, death receptors, apoptosis, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), hypoxia-inducible factor (HIF) 1, growth factor receptor kinases, and angiogenic pathways which leads to cancer prevention (Kannappan et al., 2012). In establishing the molecular mechanism of tocotrienol in oxidative stressed conditions, the changes in gene expression with tocotrienol treatment has been the core objective of recent studies (Berbée et al. 2012; Makpol et al., 2012; Then et al., 2012). Selected proteins such as cell cycle regulating proteins were also measure on a hypothesis-driven basis in some of these studies but none have addressed the whole proteome response to oxidative stress induction which elucidates the cellular network response to oxidative stress.

Oxidative stress damages proteins by modification of its side chains depending on the nature of the amino acid present. Aliphatic side chains which consist of valine and leucine can only be oxidized by very reactive hydroxyl radicals while more reactive side chains mainly made up of tryptophan can be oxidized by less active oxidants (Requena et al., 2001; Requena & Stadtman, 1999). Our earlier findings also suggest that post-translational modifications may alter the expression of proteins as compared to their coding genes in response to oxidative stress (Goon et al., 2013). This study was therefore aimed to elucidate a wider proteome response to oxidative stress induction *in vivo* and to identify the probable proteins targeted by tocotrienol which has been found to protect Caenorhabditis elegans (*C.elegans*), a nematode with highly conserved genome with humans, from oxidative stress (Cutter et al., 2009; Goon et al., 2013).

2. MATERIAL AND METHODS

2.1. Nematode strain and culture conditions

The wild type *C. elegans* strain (N2) used in this experiment was obtained from the UKM Molecular Biology Institute (UMBI). All of the maintenance and handling procedures for the nematode were conducted as described previously (Brenner, 1974). The nematodes were maintained at 20°C on nematode growth media with *E. coli OP50* as a food source (Stiernagle 2006). To prevent the production of progeny, the nematodes were transferred onto plates containing 40 mM 5-fluoro-29-deoxyuridine (Sigma-Aldrich, St. Louis, MO, USA) after treatment with H_2O_2. The nematodes were divided into six groups and treated accordingly: control, H_2O_2 induction, TRF treatment, TRF treatment pre-H_2O_2 induction (TRF+ H_2O_2), TRF treatment post-H_2O_2 induction (H_2O_2+TRF), and TRF treatment pre and post-H_2O_2 induction (TRF+ H_2O_2+TRF). The TRF treatments were given from hatching to day 3 of adulthood to determine the ability of *C. elegans* to recover from H_2O_2-induced oxidative stress during the developmental phase.

2.2. Nematode growth medium (NGM) with TRF

The TRF was supplied by Sime Darby Bioganic, previously known as Golden Hope Bioganic (Selangor, Malaysia), in its commercial form, TriE, which consists of 15% a-tocopherol, 23% a-tocotrienol, 2% b-tocotrienol, 20% c-tocotrienol and 11% d-tocotrienol with 70% vitamin E purity. The optimal dose of TRF for the treatment of C. elegans, 50 μg/ml, was ascertained based on the highest rate of nematode survival after exposure to various concentrations of TRF. NGM containing 50 μg/ml TRF was then prepared according to a previous study (Goon et al., 2013). Briefly, the TRF was dissolved in absolute ethanol containing Tween 80 and then mixed with MilliQ water at a ratio of 1:1, followed by sonication. The TRF solution was then added aseptically to autoclaved nematode growth media before solidification in petri dishes.

2.3. Oxidative stress induction with H_2O_2

C.elegans nematodes were treated with H_2O_2 for two hours at the L4 stage (Larsen, 1993). The dose of H_2O_2 used to induce oxidative stress in *C.elegans* was predetermined to be 0.3 mM because this dose resulted in greater than 90% nematode survival compared with the control as shown in our previous study (Goon et al., 2013).

2.4. Protein extraction and quantification

Protein samples were extracted from *C.elegans* by sonication. The nematodes were washed from respective plates using M9 buffer and transferred into microcentrifuge tube. The washing process was repeated using deionized water to remove the bacteria. The nematodes were resuspended in lysis buffer containing 8 M urea, 4% 3-[(cholamidopropyl) dimethyl ammonio]-1-propanesulfonate, 40 mM dithiothreitol (DTT), 0.01% protease inhibitor cocktail and 2% immobilized pH gradient (IPG) buffer (pH 3–10) and later sonicated on ice for 2 minutes. Supernatant was collected after centrifugation and protein content was estimated by Bradford assay. Aliquots of protein samples were stored at -80°C.

2.5. Two-dimensional gel electrophoresis

2.5.1. First-dimension isoelectric focusing

First-dimension isoelectric focusing was performed using 24-cm Immobiline DryStrips Linear (pH3-10) on an Ettan IPGPhor 3 isoelectric focusing System (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instruction manual. Briefly, dry strips were first rehydrated for 16h with 450μl of rehydration buffer containing 8 M urea, 2% 3-[(cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (CHAPS), 0.5% IPG buffer (pH 3–10) and trace amounts of bromophenol blue. Protein extracts were applied on the acidic end of the dry strip using sample cup. Isoelectric focusing (IEF) was performed at 20°C under a current limit of 50 μA/strip for 38.1 kVh.

2.6.Second-dimension sodium dodecyl polyacrylamide gel electrophoresis

Focused IPG strips were equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29% glycerol, 2% SDS and trace amounts of bromophenol blue) containing 1% DTT. Next, the strips were alkylated in the same buffer containing 2.5% iodoacetamide for another 15 min. Second dimensional gel electrophoresis was then performed on an ETTAN DALT II electrophoresis system (GE Healthcare Bio-Sciences, Uppsala, Sweden) on 12.5% SDS slab gels with the IPG strips sealed on the top of the gels using 0.5% agarose. The gels were run at 40 mA per gel until the bromophenol blue dye reached the bottom of the gel.

2.7. Silver staining

Analytical gels were stained with silver nitrate as described in the Plus One Silver Staining Kit (GE Healthcare) with modifications. The complete protocol was followed for analytical gels. The protocol was modified to omit glutaraldehyde from the sensitization step and formaldehyde from the silver reaction step (Yan et al., 2000). Silver stained gels were scanned using a UMAX scanner (model UTA-2100 XL). Gel images were then analyzed using Image Master 2D Platinum software Version 6.0 (GE Healthcare Bio-Sciences). Following background subtraction, protein spots were automatically defined and the volume of each spot was normalized as a percentage of the total volume of all spots detected on the gel.

2.8. Image analysis

All experiments were performed in three biological and three experimental replicates. For every sample, a total of nine gels were run (three separate cultures, three gels run per culture) to account for experimental and biological variations. The expression of proteins were compared between the control, hydrogen peroxide and TRF treated groups while TRF+H_2O_2, H_2O_2+TRF and TRF+ H_2O_2+TRF groups were compared with the H_2O_2-induced group. Statistical analysis was performed by two-tailed unpaired t-test. A value of $p<0.05$ was considered as statistically significant.

2.9. In-Gel tryptic digestion

Protein spots were excised, and in-gel was digested with trypsin (Promega) for mass spectrometric analysis according to published protocols (Shevchenko et al. 1996, Wilm et al. 1996). Excised spots were washed in destaining solution [15 mM potassium ferricyanide/50 mM sodium thiosulphate, 1:1 (vol/vol)]. Next, the spots were reduced in a solution containing 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60°C and alkylated in 55 mM iodoacetamide/100 mM ammonium bicarbonate for 20 min in the dark. The gel pieces were then washed (3×20 min) in 50% acetonitrile/100 mM ammonium bicarbonate. The gel pieces were rehydrated with 100% acetonitrile and dried in a vacuum centrifuge (SpeedVac, Thermo Scientific, Savant DNA 120). The dried gel pieces were rehydrated again with 25 μl of 7 ng/μl trypsin (Promega trypsin gold) in 50 mM ammonium bicarbonate buffer and digested at 37°C for 18–20 h. Tryptic peptides were then extracted using 50% acetonitrile for 15 min, followed by 100% acetonitrile for 15 min. The extracted solutions were pooled into a single tube and dried in a SpeedVac concentrator and solubilized with 10 μl of 10% acetonitrile/40 mM ammonium bicarbonate.

2.10. MALDI-TOF/TOF mass spectrometry analysis and database searching

Extracted peptides were first desalted using ZipTip C18 (Millipore, Billerica, MA, USA) according to protocols described by the manufacturer. The final elution volume was 1.5 μl after ZipTip cleanup. The peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid; Sigma) prepared in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Aliquots of samples (0.7 μl) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained on a MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight/time-of-flight) mass spectrometer (ABI 4800 plus; Applied Biosystems) in the positive ion reflector mode. All fractions were measured in single mass spectrometry (MS) for precursor ion selection before MS/MS was performed. The 20 most abundant precursor ions for each sample were selected for subsequent fragmentation by high-energy collision-induced dissociation (CID). The collision energy was set to 1 keV, and the air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. Known contamination peaks were removed before database searches. Spectra were processed and analyzed

by the Global Protein Server Explorer 3.6 software (Applied Biosystems) which uses an internal MASCOT (Matrix Science, London, UK) program for matching MS and MS/MS data against database information.

3. RESULTS

3.1. Resolving the lymphocyte proteome by 2DE

Image analysis by Image Master Software revealed a total of ~2000 spots in silvered stain gels at pH 3-10. A total of 35 protein spots were found to be differentially expressed (Figure 1 and Figure 2).

3.2. Identification of protein

MALDI-TOF/TOF was able to identify only 12 protein spots (Table I). Other protein spots were unidentifiable due to low abundance. The indexed numbers in Table I correspond to the numbers in Figure 1 and Figure 2, and they indicate the location of the proteins on 2DE.

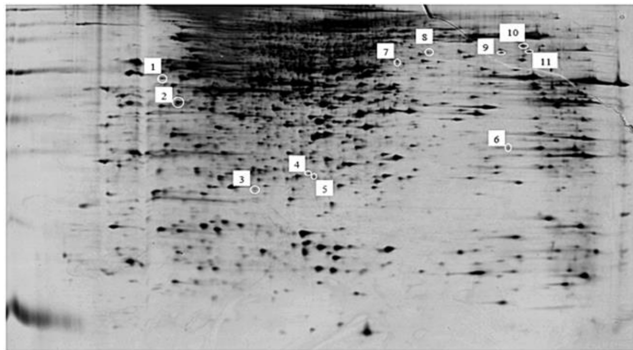

Figure 1:

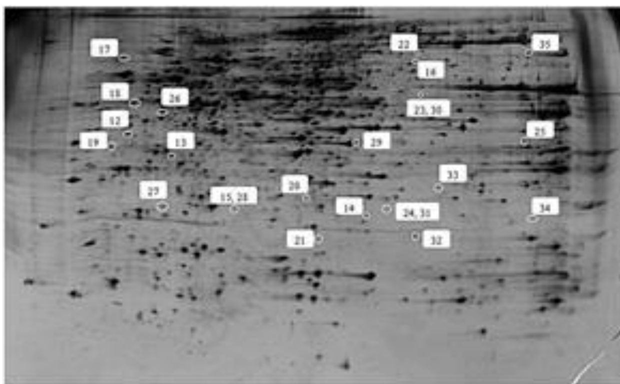

Figure 2:

4. DISCUSSION

C.elegans is a useful animal model for in vivo studies of biological problems associated with human diseases. It is a multicellular organism that undergoes a mulmultifaceted developmental process including embryogenesis, morphogenesis, and growth until it reaches the adulthood. Biological information that is obtained from *C. elegans* has been suggested to be directly applicable to humans (Cox-Paulson et al., 2012; Markaki&Tavernarakis, 2010). In the present study, *C.elegans* exposed to H_2O_2 was found to exhibit differential expression of only one protein identified as vitellogenin 6. This protein is a phospholipoglycoprotein which functions as a lipid transfer protein (Avarre et al., 2007). Its production is regulated transcriptionally and post-transcriptionally by *daf-2*/IIS pathway in C.elegans (Depina et al., 2011). Vitellogenin has been suggested to act as an antioxidant which serve as a preferential target for carbonylation due to their metal-binding properties (Nakamura et al., 1999). The antioxidative capacity of this protein together with its cholesterol transporting properties have also been acclaimed for increased stress resistance against pathogen infection in *C. elegans* (Fischer et al., 2013).

Interestingly, TRF treatment resulted in unfavourable changes of protein expressions in worms that were not induced with oxidative stress. *C.elegans* that were treated with TRF had decreased expression of mRNA cap guanine-N7 methyltransferase which catalyzes the transfer of a methyl group from S-adenosylmethionine (AdoMet) to GpppRNA to form m^7GpppRNA and S-adenosylhomocysteine (AdoHcy) (Zheng & Shuman, 2008). The 5' cap is an essential feature of eukaryotic messenger RNA because it increases the stability of mRNA ((Fabrega et al., 2004)). Thus, reduction in the expression of this enzyme may disrupt the translation of mRNA in *C.elegans*. However, results of our preliminary study which was published earlier found that the lifespan of *C.elegans* was increased significantly following TRF treatment (Goon et al., 2013). The reason for this unforeseen result is unclear since no studies have related the significance of mRNA capping to the lifespan of any living organisms.

TRF was also found to decrease the expression of inorganic pyrophosphatase in *C.elegans*. The hydrolysis of inorganic pyrophosphate (PPi) catalyzed by this enzyme provides thermodynamic forces that is required for the synthesis of DNA, RNA, protein and polysaccharide (Yi et al., 2012). Apart from that, it is also involved in larval development and intestinal function of *C.elegans*. The *C.elegans* inorganic pyrophosphatase has previously been reported to be similar to the human pyrophosphatase at the amino acid levels. Decreased pyrophosphatase activity in *C.elegans* causes developmental arrest at early larval stages and defects in intestinal morphology and function (Ko et al., 2007). Treatment with TRF also affected the fatty acid metabolism of *C.elegans* where it decreased the expression of enoyl-CoA hydratase which is essential for beta-oxidation. This enzyme catalyzes the formation of a β-hydroxyacyl-CoA thioester which is further metabolized into acetyl CoA (Agnihotri&Liu 2003). Interestingly, TRF treatment increased the expression of glutathione S-transferase 7, which is a member of glutathione S-transferase family, that plays a crucial role

in the reduction of reactive oxygen species (Gao et al., 2001; Hayes et al., 2005; Lindblom&Dodd, 2006; Nguyen et al., 2003). Glutathione S-transferase is also involved in steroid and eicosanoid biosynthesis as well as amino acid metabolism (Hayes et al., 2005; Johansson&Mannervik, 2001; Jowsey et al., 2001). Though the functions of glutathione S-transferase 7 have not been fully characterized, the gene encoding this enzyme has been regarded as a stress inducible gene (Shukla et al., 2012). Treatment of TRF after oxidative stress induction decreased the expression of triosephosphate isomerase and cysteine protease which are involved in energy metabolism and proteolysis respectively (Grant, 2008; Mohamed&Sloane, 2006). Triosephosphate isomerase catalyses the isomerization of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate in glycolysis. Deficiency of this enzyme is associated with progressive neurological dysfunction leading to premature death in humans (Orosz et al., 2006). The function of this enzyme is conserved across eukaryotes to bacteria (Symersky et al. 2003). Decreased expression of this enzyme leads to the accumulation of ketones in the cells which triggers the synthesis of glycerol from NADH-dependent glucose 3-phosphate dehydrogenase. This results in increased gluconeogenesis and HMP pathway (Compagno et al., 2001). NADPH that is produced from HMP pathway, is important for the maintenance of redox balance in the body (Lee et al., 2009). Diversion of the glucose metabolism with decreased triosephosphate isomerase expression shows that TRF treatment helped to enhance the anti-oxidative mechanism in oxidative stress induced worms. Cysteine proteases has widely been used as targets for drug and vaccine development due to their essential roles in feeding (Delcroix et al., 2006; Williamson et al., 2003; Williamson et al., 2004), molting (Guiliano et al., 2004), embryogenesis (Hashmi et al., 2004) and immune functions (Dalton et al., 2003). Two types of cysteine proteases which have been extensively studied in *C.elegans* are cathepsin B-like protease and cathepsin L protease which are involved in regulating catabolic functions (Miranda-Miranda et al., 2009) and yolk processing during embryogenesis (Britton & Murray, 2004). Knock-out of cathepsin L protease gene in *C.elegans* was reported to produce early embryonic lethal phenotype (Louise Ford, 2009). These results raised the intriguing possibility that treatment of TRF prior to oxidative stress induction disrupts the embryogenesis of *C.elegans*.

Treatment of TRF after oxidative stress induction decreased the expression of tubulin-specific chaperon B in *C.elegans*. Tubulin-specific chaperon B plays a role in microtubule dynamics and plasticity during neurogenesis. Nevertheless, it has been found to be non-essential in the development of many organisms (Strayko et al., 2005) Over expression of this protein however, can lead to neuronal degeneration (Lopez-Fanarraga et al., 2007). Meanwhile, D-amino acid oxidase which is also found to decrease in expression with TRF treatment, is involve in oxidative deamination of D-amino acids (Tedeschi et al., 2012). A recent investigation on the role of this D-amino acid oxidase in *C. elegans* revealed that it is important for the development and maturation of germ cells (Saitoh et al., 2012). Research with mammals also claimed that this enzyme has antibacterial effect because it generated hydrogen peroxide, which is an important molecule in the defense systems (Nakamura et al., 2012). The reason for decreased expression of D-amino acid oxidase as well as tubulin-specific chaperon with oxidative stress induction and TRF treatment in this study is still unclear.

Table 1:
List of proteins identified as being differentially expressed following H_2O_2 induction and TRF treatment

Index No.	Protein name	Abbreviation	Swiss-Prot accession no.	MW/pI Experimental	MW/pI Theoritical	Protein Score	Peptide (% cov)
1	mRNA cap guanine-N7 methyltransferase	MCES_CAEEL	**Q9XVS1**	65.1/4.45	43.384/4.82	180	22
2	Inorganic pyro-phosphatase	IPYR_CAEEL	**Q18680**	55/4.6	44.125/5.47	154	18
4	Enoyle-CoA hydratase	ECHM_CAEEL	P34559	21/6.25	31.152/8.58	121	11
5	Glutathione-S transferase P1	GSTP1_CAEEL	P09211	20.2/6.3	23.886/5.91	156	19
6,8,9	Vitellogenin 6 precursor	VIT6_CAEEL	P18948	25/8.55	193.2/6.85	115	8
7	Hypothetical protein F02A9	YLPD_CAEEL	P34384	79.5/7.2	66.482/8.56	178	12
12	Cathepsin B-like cysteine proteinase 4 precursor	CPR4_CAEEL	P43508	30/4.85	36.47/4.41	234	17
14	Triosephosphatase isomerase	TPIS_CAEEL	**Q10657**	19.5/7.15	26.558/6.23	211	15
19	Tubulin-specific Chaperon B	TBCB_CAEEL	**Q20728**	34.5/4.1	25.42/4.7	122	12
23	Putative D-amino acid oxidase	OXDA1_CAEEL	O01739	35.5/9.1	37.584/8.13	222	15

CONCLUSION

TRF treatment modulated the expression of proteins involved in energy metabolism, oxidative stress, proteolysis and biosynthesis of mRNA in *C.elegans*. These proteins may be responsible for the restoration of lifespan in *C.elegans* subjected to oxidative stress as found in our earlier report (Goon et al., 2013). Future studies are implicated to determine the functional changes in *C.elegans* with altered expression of these proteins to establish the role of TRF *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Science, Technology and Innovation, Malaysia (Science Fund, 02-01-02-SF 0531) and facilitated by the Department of Biochemistry, UKM Medical Center, UKM Molecular Biology Institute and Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

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