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Original Communication

Liver heme oxygenase-1 expression is positively induced by palm oil-derived tocotrienol-rich fraction (TRF) supplementation in mice

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ABSTRACT

The antioxidant activities of tocotrienols are more robust than that of tocopherols. Palm oil is a rich natural source of tocotrienols. Heme oxygenase-1 (HO-1) is an enzyme that has antioxidant, anti-inflammatory and cytoprotective functions. The objective of this study is to determine the effects of different doses of TRF oral supplementation on HO-1 gene and protein expression in mice livers. Thirty male ICR white mice (25-30 g) were divided into five groups; three groups were administered TRF orally for 14 days at doses of 200, 500 and 1000 mg/kg respectively (n = 6 per group), a positive control group was administered 100 mg/kg butylated hydroxyanisole (BHA) orally for 14 days (n = 6), and a control group (n = 6) was only administered the vehicle (corn oil). At day 15, the mice were sacrificed and their livers isolated. Total RNA was extracted from the livers and quantitative real-time polymerase chain reaction (qPCR) assays were performed to analyse HO-1 gene expression. The livers were then homogenized and HO-1 protein expression was analysed by Western blotting. It was observed that TRF oral supplementation at concentrations of 200, 500 and 1000 mg/kg for 14 days resulted in significant concentration-dependent increase in HO-1 gene and protein expression in mice livers, compared to controls. In conclusion, TRF supplementation induced HO-1 gene and protein expression in mice livers dose-dependently, with the highest expression seen in mice receiving 1000 mg/kg TRF.

KEYWORDS: heme oxygenase-1, liver, mice, gene expression, protein expression, TRF.

INTRODUCTION

Vitamin E is an interesting group of compounds with diverse biological effects. It is widely accepted to be the first line of antioxidant defence against lipid peroxidation, protecting polyunsaturated fats in cellular membranes through its free-radical-scavenging activity at the early stages of free-radical attack [1]. A total of eight different isoforms of vitamin E that belong to two groups are known to occur in nature: α, β, γ, δ tocopherols and α, β, γ, δ tocotrienols. These compounds are characterized by a 6-chromanol ring structure and an isoprenoid side chain [2]. A standardized tocotrienol-rich fraction (TRF) consists of 68% tocotrienols and 32% α-tocopherol. The tocotrienols found in TRF are a mixture of α, β, γ, and δ-tocotrienols. TRF is obtained from palm oil after it has undergone esterification, distillation, crystallization and chromatography [3]. Most of the tocopherols have saturated side chains, whereas tocotrienols side chains possess double bonds at the 3', 7', and 11' positions [4]. Tocopherols

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are abundantly found in oils extracted from the leaves and seeds of most plants, e.g. corn, olive, soybean, sesame, peanut and sunflower. Tocotrienols are less abundant and found only in the oil fractions of some cereal grains such as barley, rice, annatto, wheat, and most abundantly, in palm fruit [5].

Vitamin E has been given a thorough consideration as an important component of the antioxidant network. As such, it is assumed to protect cells from potentially dangerous by-products of metabolism such as reactive oxygen species (ROS) [6]. All forms of vitamin E have antioxidant activities; however, tocotrienols exert enhanced antioxidant activities compared to tocopherols [4]. In addition, tocotrienols have other significant functions, particularly in maintaining a healthy cardiovascular system, and a potential role in protection against cancer and other diseases [7].

Some chemicals found in food and phenolic antioxidants are chemoprotective, i.e. they induce the expression of genes and proteins involved in cellular defence. The increased expression of cellular defence proteins provided added protection against oxidative/chemical stress. Some of these proteins are phase II drug-metabolizing enzymes, although other enzymes and antioxidant proteins are also involved. These proteins are known as phase II proteins [8]. Phase II proteins are mainly regulated by the Nrf2/ARE system [9, 10]. Heme oxygenase-1 (HO-1) is an example of phase II proteins regulated by Nrf2 [11]. HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme (a pro-oxidant) to carbon monoxide, biliverdin, and free iron [12]. HO-1 induction is important in terms of cellular defense mechanism due to the fact that HO-1 expression is inducible in response to various forms of cellular insult. Moreover, the end products of HO-1 catabolism exhibit anti-oxidative, anti-inflammatory and anti-apoptotic properties [12].

Two earlier studies had attempted to link the antioxidant activities of vitamin E with the induction of several phase II antioxidant enzymes [13, 14]. These two studies [13, 14] have given hints of the connection between the antioxidant activity of tocopherols and tocotrienols with Nrf2 activation of chemoprotective enzymes. The first study, which is an *in vitro* study using MDA-MB-231 breast cancer cells suggested that tocotrienols were responsible for regulating the expression of Nrf2-regulated cytoprotective enzymes such as catalase and glutathione peroxidase [13]. The second study, which is also an *in vitro* study, was performed using human retinal pigment epithelial cells. The results of the second study showed that tocopherols were able to regulate the expression of several Nrf2-related cytoprotective enzymes such as glutamate cysteine ligase, NAD(P)H:quinone oxidoreductase 1, glutathione S-transferase and superoxide dismutase [14]. However, not many *in vivo* studies have been done to explore the effect of TRF on Nrf2-regulated chemoprotective enzymes in the liver. In the present work, HO-1 gene and protein levels were measured in the livers of mice supplemented with different doses of palm TRF.

MATERIALS AND METHODS

Materials

Palm oil tocotrienol-rich fraction (TRF) (Gold Tri.E 70) was purchased from Sime Darby Bioorganic (Kuala Lumpur, Malaysia) and consists of α-tocopherol at 159.5 mg/g, α-tocotrienol at 205.1 mg/g, β-tocotrienol at 32.9 mg/g, γ-tocotrienol at 249.8 mg/g and δ-tocotrienol at 119 mg/g. TRIzol reagent was purchased from Life Technologies (Carlsbad, California, USA). iScript™ cDNA synthesis kit and iQ™ SYBR® Green supermix (2X) kit were purchased from Bio-Rad (Hercules, California, USA). Gel Red Nucleic Acid Gel Stain (10,000X in water) was purchased from Biotium (Hayward, California, USA). HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Vivantis Technologies (Oceanside, CA, USA). HO-1 mouse polyclonal primary antibody and β-actin rabbit polyclonal antibody were purchased from Abcam Biotechnology (Cambridge, UK). Radioimmunoprecipitation assay (RIPA) lysis buffer and goat anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology (USA). Chemiluminescence Western blotting detection reagents were purchased from Amersham (Uppsala, Sweden). Nitrocellulose membrane and Ponceau S solution were purchased from Sigma-Aldrich (Seelze, Germany).

Animals and treatment

Male ICR white mice (25-30 g) were used in this study. The mice were kept in clean polypropylene cages in a ventilated room with a 12-hour light-

dark cycle, with food and water available *ad libitum.* Animals were treated with three different doses of TRF (dissolved in corn oil). The mice were divided into 5 groups. Mice in the first group (n = 6) was designated as the control group and were given only the vehicle, i.e. corn oil. Mice in the second group (n = 6) were treated with 200 mg/kg TRF. Mice in the third group (n = 6) were treated with 500 mg/kg TRF. Mice in the fourth group (n = 6) were treated with 1000 mg/kg TRF. Mice in the fifth group (n = 6), i.e. the positive control group, were treated with 100 mg/kg butylated hydroxyanisole (BHA). All treatments were administered *via* oral gavage for 14 consecutive days. At day 15, mice were sacrificed *via* cervical dislocation. Their livers were subsequently isolated, snapped frozen in liquid nitrogen and stored at -80 °C until further use. All experimental procedures were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

RNA extraction

Total RNA from frozen liver tissues was isolated using TRIzol reagent, according to the manufacturer's instructions. Isopropyl alcohol (Sigma, USA) was added during each extraction step to precipitate the total RNA. The extracted total RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase-free water. Total RNA was stored at -80 °C immediately after extraction. Concentration and purity of the extracted RNA were determined by NanoDrop spectrophotometer 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A260 to A280 ranging from 1.5 to 2.0 was used for cDNA synthesis.

Reverse transcription

Generation of cDNA from RNA was done using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, a volume (containing 1 µg) of total RNA from each sample was added to a mixture of 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, and a suitable volume of nuclease-free water (the final reaction mix volume is 20 µl). The final reaction mix was kept at 25 °C for 5 min, 42 °C for 30 min, and heated to 85 °C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by polymerase chain reaction (PCR).

Quantification of HO-1 gene expression by quantitative real-time PCR

Quantitative real-time PCR was performed on the MiniOpticon cycler (Bio-Rad, USA). The total reaction volume used was 20 µl, consisting of 1 µl of 10 µM forward primer and 1 µl of 10 µM reverse primer (500 nM final concentration of each primer), 10.0 µl of iQ™ SYBR® Green Supermix (2X) (Bio-Rad, USA), 6.0 µl of nuclease-free water and 2.0 µl of cDNA. Both forward and reverse primers for the genes of interest in this study were designed according to previous studies and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for our genes of interest are shown in Table 1.

The thermocycling conditions were initiated at 95 °C for 30 sec, followed by 40 PCR cycles of denaturation at 95 °C for 15 sec and annealing/ extension at 60 °C for 30 sec. At the end of each cycle, a melting curve (dissociation stage) analysis was performed in order to determine the specificity of the primers and the purity of the final PCR product. All measurements were performed in triplicate and no-template controls (NTC) were incorporated onto the same set of PCR tubes to test for contamination by any assay reagents. Threshold cycles were determined for each gene and quantification of templates was performed

Table 1. Primer sequence for GAPDH and HO-1.

Gene description	Primer sequence
GADPH	F: 5'-GTGGAGTCTACTGGTGTCTTCA-3' R: 5'-TTGCTGACAATCTTGAGTGAGT-3'
HO-1	F: 5´-CCTCACTGGCAGGAAATCATC -3´ R: 5´-TATGTAAAGCGTCTCCACGAGG -3´

according to the relative standard curve method. The relative gene expression (ΔΔCt) technique, as defined in the Applied Biosystems User Bulletin No. 2 [15], was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as relative amount normalized against GAPDH standard controls. Subsequently, agarose gel electrophoresis was performed to determine the reliability of the melting curve analysis and to confirm the size of the PCR product. Briefly, electrophoresis was performed using 1% agarose gel in order to separate the real-time PCR products. Gel Red™ nucleic acid gel stain (Biotium, USA) was used to stain the gels for 30 min and the gels were subsequently de-stained in distilled water for 30 min. Bands were then visualized under ultraviolet light using a gel documentation system (FluorChem FC2, Alpha Innotech, USA).

Sample preparation for Western blotting

Liver tissue samples were homogenized in RIPA lysis buffer (which contained 10 µl phenylmethylsulfonyl fluoride (PMSF), 10 µl sodium orthovanadate and 10 µl protease inhibitor cocktail solution per 1 ml of 1X RIPA lysis buffer). After centrifugation, the supernatants were collected and their protein concentrations were determined.

Western blotting

Standard Western blotting procedure was used for the immunodetection of proteins. Briefly, 100 µg of liver protein was separated using 15% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) electrophoresis. The proteins in the gel were then transferred to a nitrocellulose membrane. The membrane was then incubated for 20 minutes at room temperature in a blocking solution (150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% (v/v) tween-20 and 10% non-fat milk powder (pH 7.4)). After blocking, the membrane was incubated with the following antibodies: primary polyclonal rabbit anti-mouse HO-1 and primary polyclonal rabbit anti-mouse actin for 1 hour at room temperature. Subsequently, incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody was carried out for another hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Significant differences between mean values of multiple groups were determined using one-way ANOVA and Student's t-test. Statistical analysis was conducted using the SPSS software version 22. The result was considered statistically significant when $p < 0.05$.

RESULTS

Body weight gain and food intake after palm TRF treatment

To investigate whether different doses of palm TRF affect body weight gain and food intake in the control and experimental mice, body weight of mice in each group were recorded and food intake of each mouse was individually measured every two days till the end of the treatment period. Table 2 shows that in palm TRF-treated groups, body weight gain and food consumption were only slightly inhibited by the administration of 1000 mg/kg TRF but they were not inhibited by the administration of 500 and 200 mg/kg TRF. However, the difference in body weight gain and food consumption after 14 days was not statistically significant for all groups. Administration of BHA alone did not induce any significant alterations in those parameters and the results did not differ significantly from those of the control and TRF-treated groups.

Liver HO-1 gene expression

To examine the effect of palm TRF on HO-1 mRNA, mice were fed with different doses of palm TRF (200, 500, 1000 mg/kg) in the presence of vehicle-treated control mice (fed corn oil) and a positive control group (administered BHA (100 mg/kg)) for 14 days. After 14 days of treatment, the mice were sacrificed and HO-1 gene expression in the liver was measured using quantitative real-time PCR. As shown in Figure 1, palm TRF at concentrations of 200, 500, and 1000 mg/kg caused a significant concentration-dependent increase in the fold change of HO-1 gene expression levels [1.4-, 2.6-, and 3.9-fold respectively, as compared to controls;

Table 2. Body weight and food intake of control and treated mice.

Groups	Body weight		Food intake
	Day 1 (g)	Day 14 (g)	(g/bw/day)
Control	28.67 ± 2.13	33.33 ± 2.27	4.50 ± 1.11
T200	27.67 ± 1.96	32.33 ± 2.11	4.37 ± 1.85
T500	28.33 ± 1.25	30.33 ± 1.84	4.17 ± 1.92
T1000	31.67 ± 1.17	30.33 ± 2.18	2.09 ± 1.16
BHA	30.00 ± 1.32	30.67 ± 1.43	4.93 ± 1.23

T200, T500, T1000: Groups of mice treated with oral TRF at a daily dose of 200, 500 and 1000 mg/kg body weight, respectively. Control: control mice, BHA: positive control group that was given butylated hydroxyanisole (100 mg/kg). Values are given as mean ± SEM (n = 6 for each group). No statistical significance was found between control and treated groups (ANOVA).

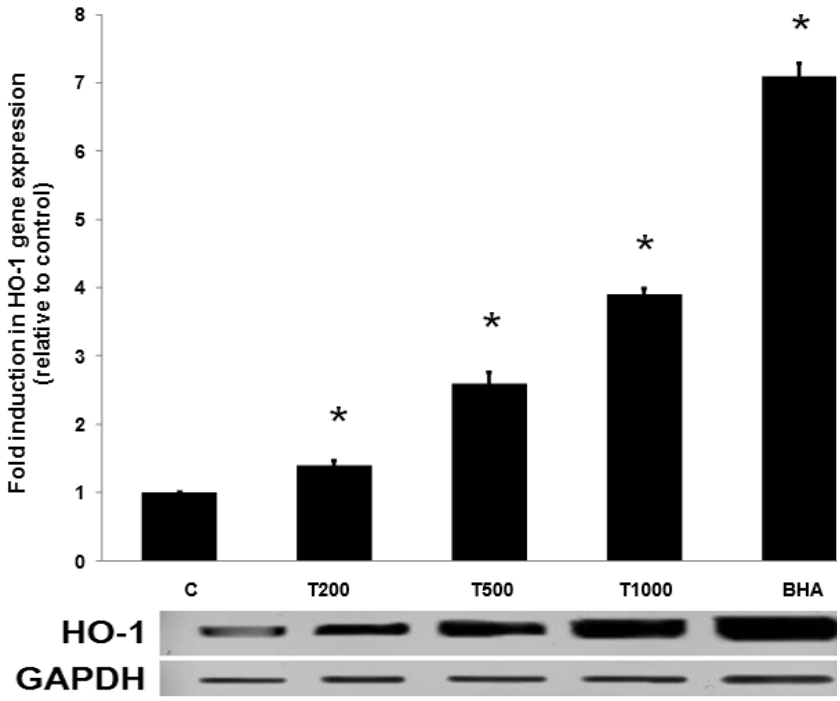

Figure 1. Effect of different doses of TRF on HO-1 gene expression in the livers of mice, evaluated using real-time PCR (qPCR). Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Data is presented as mean ± SEM. Amplified products were also visualized by agarose gel electrophoresis and gene expression was confirmed by identification of the appropriate bands. GAPDH served as the reference gene. T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg). Asterisk (*) indicates statistically significant difference from the control group ($p < 0.05$).

$p < 0.05$]. Mice treated with BHA (100 mg/kg) also showed significant increase in HO-1 gene expression levels (7.1-fold), compared to controls ($p < 0.05$).

Liver HO-1 protein expression

In agreement with the HO-1 gene expression results, administration of palm TRF at concentrations of 200, 500, and 1000 mg/kg to mice for 14 days through oral gavage significantly increased liver HO-1 protein expression levels by 1.6-, 2.5-, and 3.6-fold, respectively, compared to controls ($p < 0.05$). After 14 days, mice treated orally with BHA (100 mg/kg) for 14 days showed a significant increase in HO-1 protein expression levels (6.6-fold; $p < 0.05$), compared to controls (Figure 2).

DISCUSSION

HO-1 inhibits oxidative stress by removing excess heme. HO-1 and the catabolic products of heme work in tandem to decrease inflammation and cytotoxic reactions [16]. HO-1 knockout mice are characterized by enlarged spleens and hepatic lesions due to chronic inflammatory processes [17]. Humans with HO-1 deficiency have been shown to be susceptible to oxidative stress and inflammation which will eventually lead to severe endothelial damage [18]. Increased HO-1 expression has been shown to prevent hepatocyte damage due to oxidative stress [19]. TRF increased liver HO-1 gene and protein levels in a dose-dependent manner, with the highest levels observed in mice that were administered 1000 mg/kg TRF, followed by those that were administered 500 mg/kg and 200 mg/kg, respectively. However, the highest observed increase in HO-1 gene and protein levels due to TRF administration was still below the expression levels induced by BHA (positive control) treatment. Since BHA is a classical inducer of phase II detoxifying enzymes (e.g., HO-1) through the activation of the Nrf2 pathway, it is suggested that

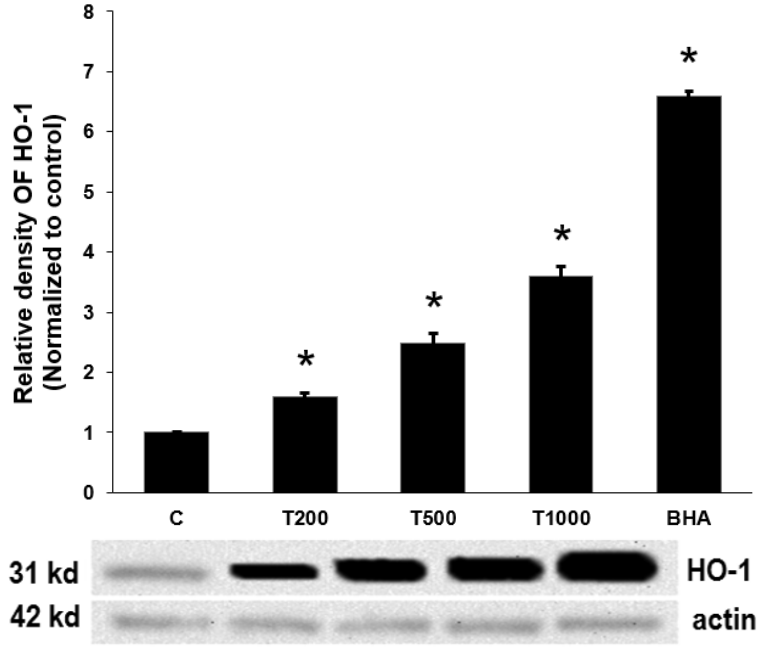

Figure 2. Effect of different doses of TRF on HO-1 liver protein expression. Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Their livers were then harvested and HO-1 protein expression levels were determined by Western blotting. The intensity of protein bands were quantified relative to the signals obtained for actin using ImageJ software, and was normalized to control. The graph represents the average optical density (± S.E.M.) of bands from three different experiments. Asterisk (*) indicates statistically significant difference from the control group ($p < 0.05$). T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg).

the antioxidant activity of tocotrienols might also partly be mediated through the induction of phase II enzymes (such as the HO-1) by means of Nrf2 induction. Classical inducers of HO-1 triggers increased HO-1 expression through the binding of Nrf2 to the antioxidant response element (ARE) at the HO-1 promoter region [20]. HO-1 was found to be less inducible in Nrf2-deficient mice, which implied that Nrf2 is important for HO-1 induction [21]. The antioxidant activities of tocotrienols (the major component of TRF) have been linked to Nrf2 activation in an *in vitro* study [13]. However, the exact mechanism by which tocotrienol (or TRF) induces HO-1 expression in liver has not been conclusively studied. Since HO-1 is a phase II enzyme and phase II enzymes are regulated by Nrf2, it is suggested that tocotrienols (which are the major components of palm oil TRF) are able to dissociate the Nrf2/Keap1 complex, allowing Nrf2 to translocate to the nucleus and increase the expression of phase II enzymes (including HO-1) in the liver. We therefore postulate that consumption of palm oil TRF might be beneficial in terms of general health and cancer chemopreventive strategy. However, further studies are needed to confirm this mechanism. At present, not much can be done to prevent liver cell damage and degeneration. Induction of HO-1 expression through supplementation with natural products may be effective (and safer compared to synthetic drugs) to prevent liver carcinogenesis and other liver diseases [22]. In this study, we found that palm oil TRF was able to induce HO-1 expression in mice liver, which indicates that palm oil TRF might have potential chemoprevention/chemoprotective properties.

CONCLUSION

HO-1 is transcriptionally upregulated by a large variety of stimuli, e.g. heme, oxidative stress, signaling proteins and organic chemicals. Therefore, the induction of HO-1 by pharmacological means, preferentially through supplementation by various forms of natural products, offers a potential new therapeutic target for liver degenerative diseases. In this respect, the recommended therapeutic dose for palm oil TRF which is effective in the prevention or treatment of liver diseases should be aggressively investigated. Consuming palm oil TRF in the form of supplements could be an affordable chemoprotective measure in the long run. Further studies are needed to conclusively support this strategy.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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