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ORIGINAL CONTRIBUTION

Supplementation with tocotrienol-rich fraction alters the plasma levels of Apolipoprotein A-I precursor, Apolipoprotein E precursor, and C-reactive protein precursor from young and old individuals

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Abstract
Purpose Tocotrienol possess beneficial effects not exhibited by tocopherol. In vitro studies using animal models have suggested that these effects are caused via modulation of gene and protein expression. However, human supplementation studies using tocotrienol-rich isomers are limited. This study aims to identify plasma proteins that changed in expression following tocotrienol-rich fraction (TRF) supplementation within two different age groups.
Methods Subjects were divided into two age groups—32 ± 2 (young) and 52 ± 2 (old) years old. Four subjects from each group were assigned with TRF (78 % tocotrienol and 22 % tocopherol, 150 mg/day) or placebo capsules for 6 months. Fasting plasma were obtained at 0, 3, and 6 months. Plasma tocopherol and tocotrienol levels were determined. Plasma proteome was resolved by 2DE, and differentially expressed proteins identified by MS. The expressions of three proteins were validated by Western blotting.
Results Six months of TRF supplementation significantly increased plasma levels of tocopherols and tocotrienols. Proteins identified as being differentially expressed were related to cholesterol homeostasis, acute-phase response, protease inhibitor, and immune response. The expressions of Apolipoprotein A-I precursor, Apolipoprotein E precursor, and C-reactive protein precursor were validated. The old groups showed more proteins changing in expression.
Conclusions TRF appears to not only affect plasma levels of tocopherols and tocotrienols, but also the levels of plasma proteins. The identity of these proteins may provide insights into how TRF exerts its beneficial effects. They may also be potentially developed into biomarkers for the study of the effects and effectiveness of TRF supplementation.

Keywords Tocotrienol-rich fraction · Supplementation · Plasma proteins · Two-dimensional electrophoresis · Matrix-assisted laser desorption/ionization

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Introduction

Tocotrienol (α, β, γ, δ) along with tocopherol (α, β, γ, δ) stereoisomers represent the two naturally occurring subclasses of vitamin E. Supplementation with vitamin E has beneficial effects such as boosting the immune response [1], lowering blood cholesterol [2], and inhibiting cancerous cell proliferation [3]. However, the beneficial effects of dietary supplementation of vitamin E (mainly tocopherol) in human studies have remained controversial with some

showing beneficial effects [4, 5], no effect [6, 7], or even detrimental effects [8, 9]. Natural forms of tocotrienol have been reported to be more potent than tocopherol in terms of antioxidative potential [10, 11], cholesterol-lowering effect [12, 13], and cancer inhibition [14, 15]. Tocotrienol has also been reported to affect gene and protein expression [16]. Tocotrienols extracted from crude palm oil consist mainly of a mixture of α-, γ-, and δ-tocotrienols and a small proportion of α-tocopherols. This is our extract of interest and is referred to as tocotrienol-rich fraction (TRF).

Although tocopherols and the less-studied tocotrienols are members of the vitamin E family, they have been reported to have distinct properties. The unsaturated phytyl tail of the tocotrienols allows greater intramembrane mobility resulting in more efficient scavenging of free radicals [19]. Supplementation studies using α-tocopherol alone have been suggested to compromise the absorption and action of other tocopherol isomers such as gamma-tocopherol [20]. Plasma α-tocopherol has been shown to be the main isomer to increase after supplementation, but smaller increases in tocotrienols have been associated with a reduction in DNA damage, improvements in lipid profile, and oxidative status in older adults [21, 22]. Since these isomers are present as a mixture in nature, it is possible that both isomers have functions that complement each other suggesting the need to further investigate their effects in the human body.

The response of individuals from different age groups toward vitamin E supplementation has been reported to be different [1, 17, 18]. Epidemiological studies may to certain degree provide ideas as to the beneficial effects of nutrients. However, studies performed at the molecular level will provide a more definitive conclusion as to how these effects are brought about. It is likely that the effects of vitamin E involve a variety of sophisticated pathways associated with a number of different biological processes. This in turn would involve the regulation of a number of different proteins. Thus, one way to study the molecular mechanisms involved would be to study changes in protein expression following vitamin E supplementation within the different age groups. Proteomics is a technique that allows for both the identification and quantification of changes in global protein expression.

Plasma proteins originate from a variety of sources which include proteins secreted by solid tissues and that act in plasma, immunoglobulins, receptor ligands, tissue leakage products, aberrant secretions, and proteins. Therefore, changes in the level of plasma proteins may reflect changes occurring within the body. The ease of sampling and ready availability makes plasma an ideal sample for proteomics analysis. Thus, this study was performed with the aim of identifying human plasma proteins that changed in expression following supplementation with TRF from two different age groups. Identifying and understanding these changes may provide insights into how TRF exerts its effects.

Materials and methods

Study subjects

This study was approved by the Research and Ethics Committee of the Medical Faculty, Universiti Kebangsaan Malaysia. To minimize biological variation due to the effects of gender, race, and age, volunteers were recruited only from among females and ethnic Malays aged between 33 and 52 years old. A total of 48 volunteers were subjected to a full medical examination and biochemical tests prior to recruitment. Only 16 subjects fulfilled all criteria and were enrolled for the study. Exclusion criteria included smoking, drinking, suffering from any disease, pregnant, and taking any other medication or supplementation. Written informed consent was obtained from all subjects.

Experimental design

Subjects were divided into two age groups—32 ± 2 years old (young) and 52 ± 2 years old (old). Each group was further divided into placebo and supplemented groups. Four subjects from each group were randomly assigned with either TRF—(TRF extract is a commercial product, commercial name Tri® E, obtained from Sime Darby Bioganics. Composition: 74 % tocotrienol, 26 % tocopherol)—or placebo capsules, for a duration of up to 6 months. Subjects consumed TRF at a dosage of 150 mg/day after dinner to ensure proper absorption. Two young subjects dropped out from the study for personal reasons. Overall compliance of the subjects with TRF supplementation was determined by counting the returned capsules at the end of each month and by assessing plasma total vitamin E.

Collection of blood plasma

Fasting blood (15 ml) was collected at 0, 3, and 6 months of supplementation. Blood was collected in heparin tubes between 08:30 and 09:30 and processed within 1 h. Plasma was separated by centrifugation (1,200 rpm for 30 min at 4 °C), and aliquots were stored at −80 °C until use. Total protein concentration of the plasma was determined according to the Bradford method.

Two-dimensional gel electrophoresis (2DE)

First-dimension IEF was performed on an Ettan IPGPhor 3 IEF System (GE Healthcare) as previously described with

slight modifications [19]. Briefly, Immobiline DryStrip pH 4–7, 24 cm were rehydrated (10 h) using buffer containing 7 M urea, 2 M thiourea, 2 % (v/v) IPG buffer pH 4–7, 2 % (w/v) CHAPS, and bromophenol blue (trace). Plasma (120 μg of protein) was loaded by in-gel rehydration. IEF was performed as follows: 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 3 h, and finally 10,000 V for 4 h. Focused IPG strips were equilibrated for 15 min in equilibration solution (6 M urea, 75 mM Tris-HCl buffer, 2 % (w/v) SDS, 29.3 % (v/v), 30 % glycerol, 0.002 % (w/v), bromophenol blue) containing 1 % DTT. DTT was replaced with 2.5 % iodoacetamide in equilibration solution for another 15 min. Electrophoresis of reduced and alkylated samples was carried out using 24 cm 12.5 % SDS-PAGE gels (Ettan DALT II, GE Healthcare) and subjected to 80 V, 1 W/gel for 1 h and 500 V, 13 W/gel for 5 h until the bromophenol blue dye reached the bottom edge of the gel.

Silver staining and image analysis

Protein spots were visualized using protocols described in the PlusOne^{TM} Silver staining kit (GE Healthcare). The complete protocol was followed for analytical gels. For preparative gels, a modified protocol was used. Glutaraldehyde was omitted from the sensitization step, and formaldehyde omitted from the silver reaction step [20]. Silver-stained gels were scanned (UMAX PowerLook 1000), and protein profiles compared (Image Master Platinum v 6.0, GE Healthcare). The volume of each spot was normalized as a percentage of the total volume of all spots detected on the gel. A saliency of three was used to filter out noise on the background and detect variations in spot protein profile.

Optimization of 2DE gel electrophoresis

Gels with different sample loads (40, 90, 120, and 130 μg) were run to determine optimal loading.

In-Gel tryptic digestion

Protein spots were excised and in-gel digested with trypsin (Promega) for mass spectrometric identification according to the published protocols [21–23]. Briefly, excised spots were first destained in destaining solution (15 mM potassium ferricyanide/50 mM sodium thiosulphate, 1:1 [v/v]). The spots were then 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. This was followed by dehydration pieces in 100 % acetonitrile and drying in a vacuum centrifuge. Subsequently, the dried gel pieces were rehydrated 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 then pooled into a single tube and dried in a Speed-Vac concentrator and solubilized with 10 μl of 10 % acetonitrile/40 mM ammonium bicarbonate.

MALDI-TOF/TOF mass spectrometry analysis and database searching

Protein identification was performed as previously described [18]. Briefly, extracted peptides were first desalted using ZipTip C18 (Millipore). The final elution volume following ZipTip cleanup was 1.5 μl. Peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) prepared in 50 % ACN/0.1 % TFA. Samples (0.7 μL) were spotted onto stainless-steel sample target plates. Peptide mass spectra were acquired on a MALDI-TOF/TOF mass spectrometer (ABI 4800 plus, Applied Biosystems) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions *per* sample were selected for subsequent fragmentation by high-energy CID. The collision energy was set to 1 keV, and air 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. The other parameters for searching were of trypsin, one missed cleavage, variable modification of carbamidomethyl, oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks for trypsin were removed before searching. Spectra were processed and analyzed using the Global Protein Server Explorer 3.6 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against human databases downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot).

Western blotting

Validation of protein expression was performed by Western blotting on three proteins—APO E, APO A1, and CRP. These proteins were selected due to the marked difference in their expression dynamics following TRF

supplementation. Plasma protein (30 μg) was separated by 12.5 % SDS-PAGE. Resolved proteins were then electrotransferred to a polyvinylidene fluoride (PVDF) microporous membrane (GE Healthcare) and blocked with 5 % skim milk. The membrane was probed with mouse monoclonal anti-APO E (Santa Cruz, CA, USA), mouse monoclonal anti-APO A1 (Santa Cruz, CA, USA), and mouse monoclonal anti-CRP (Santa Cruz, CA, USA) in 5 % skim milk. The blots were then washed 3 × 10 min each in TBST incubated for 1 h with goat anti-mouse IgG-HRP (Santa Cruz, CA, USA) in 5 % skim milk at room temperature. The blots were developed and visualized with the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA). Quantitation of protein expression was performed using Image Master Total Lab V1.11 (Amersham, USA) as integrated intensity units relative to the protein signal detected in baseline plasma.

Statistical analysis

Proteins that differ in their expression by at least twofold were considered as being differentially expressed. Results were expressed as mean volume ratio. Statistical analysis was performed by analysis of variance (ANOVA) with significance level set at $p < 0.05$.

Results

Effects of TRF supplementation on plasma tocopherol and tocotrienol levels

Demographic information for young and old subjects at day 0 (before supplementation) are provided in Table 1. No significant differences were observed during the baseline period for both the young and old groups. There was also no significant change in the placebo group. Following TRF supplementation, a significant increase in total tocopherols and tocotrienols (Table 2) was observed in both the old and young groups. The level of tocotrienol was much higher in the old group after 6 months of supplementation.

Resolving the plasma proteome by 2DE

A sample loading of 120 μg resulted in the best resolution. More than 1000 well-resolved spots were detected on silver-stained 2DE gels. All gels were highly reproducible within and between samples (experimental and biological replicates). Comparison of the plasma proteome profiles of subjects before and following supplementation with TRF at three and 6 months revealed significant changes in 2DE proteome profiles. A representative 2DE gel showing differentially expressed protein spots is shown in Fig. 1.

The effect of TRF supplementation on plasma proteins from different age groups

Twelve protein spots showed significant change in expression ($P < 0.05$). These proteins were unambiguously identified by MALDI-TOF/TOF. The 12 spots represented eight proteins (more than one protein spot were identified as FHR1, APOE, and AMBP). The identity (and abbreviation, in brackets), accession number, protein score, sequence coverage, theoretical and experimental molecular mass and pI, and change in expression are shown in Table 3a, b. The "Spot" column in Table 3a, b refers to the spots labeled in Fig. 1. From this point forward, these proteins will be referred to by their abbreviations as shown in Table 3a, b. Based on their known functions, the differentially expressed proteins were classified into four functional categories—cholesterol homeostasis (APOA1, APOE), acute-phase response (CRP, HTPR), protease inhibitor (CBPN, AMBP), and immune response (FHR1, FHR2).

In both groups, changes in protein expression were first observed after 3 months. In the young group, three proteins (APOE, FHR2, and AMBP) were upregulated after 3 months. For the old group, six proteins (CBPN, FHR1, HPTR, APOE, FHR2, and AMBP) were upregulated after 3-month supplementation. The remaining regulated proteins only changed in expression after 6 months of TRF supplementation. All proteins were upregulated in expression with the exception of CRP which was downregulated. The expression of three proteins—APOA1, APOE, and CRP—was validated by Western blotting across all 42 samples (Fig. 2a–c). All three proteins showed similar expression profiles as observed in 2DE ($p < 0.05$).

Discussions

The presence/absence of a particular protein and/or its isoforms represents the likelihood of other biologically active molecules. This in turn corresponds to cellular functions. Thus, identifying proteins whose expressions were affected by TRF supplementation within the two different age groups may give us insights into the events that lead to the protective effects of TRF. After 6 months of TRF supplementation, the levels of tocopherol in the plasma of young and old individuals were comparable. However, tocotrienol levels were much higher in young individuals (Table 2). On the other hand, it was found that a greater number of proteins changed in expression within the old group. This suggested a higher sensitivity toward TRF supplementation among old individuals. A lower plasma level of tocotrienol was apparently needed to exert change in protein expression in old individuals compared

to young individuals. However, as to why the levels of tocotrienols were lower in old individuals was unclear. Here, we discuss the changes in plasma protein profile with the proteins classified under different functional categories.

Proteins involved in cholesterol homeostasis

APOA1 was found to be significantly increased in both age groups following TRF supplementation. Its expression was validated by Western blotting (Fig. 2a). APOA1 is a

Table 1 Demographic information for young and old subjects at day 0 (before supplementation)

	Young	Old
Age (years)	34.6 ± 0.8	49.5 ± 0.9
BMI (kg/m^2)	25.9 ± 3.1	26.0 ± 1.6
Blood pressure (mmHg)		
Systolic	122.2 ± 2.5	126.1 ± 1.7
Diastolic	81.2 ± 2.6	86.0 ± 2.7
Heart rate	73.1 ± 2.0	72.5 ± 2.3
Fasting blood glucose (mmol/L)	4.6 ± 0.2	4.9 ± 0.2
LDL (mmol/L) (<2.58)	2.4 ± 0.1	2.4 ± 0.1
HDL (mmol/L) (>1.03)	1.4 ± 0.2	1.6 ± 0.2
Triglycerides (mmol/L) (<1.68)	1.1 ± 0.4	1.0 ± 0.3

All values are shown as mean ± standard deviation. There was no significant difference in values between individuals from the young and old groups

Table 2 Plasma total tocopherols and total tocotrienols for young and old groups following TRF supplementation

Plasma total tocopherols for both young and old groups

Group	Month		
	0	3	6
Younger			
Placebo	10.11 ± 1.27	9.07 ± 0.92	7.88 ± 0.26
TRF	9.89 ± 1.12	12.40 ± 0.34*	11.99 ± 0.74*
Older			
Placebo	9.05 ± 6.2	9.85 ± 0.94	11.11 ± 0.96
TRF	9.45 ± 0.98	14.67 ± 1.26*	15.84 ± 1.64*

Plasma total tocotrienols for both young and old groups

Group	Month		
	0	3	6
Younger			
Placebo	0.00 ± 0.00	0.09 ± 0.05	0.00 ± 0.00
TRF	0.00 ± 0.00	0.08 ± 0.05	8.14 ± 4.10*
Older			
Placebo	0.10 ± 0.00	0.43 ± 0.26	0.50 ± 0.41
TRF	0.09 ± 0.02	0.63 ± 0.38	2.20 ± 1.16*

All values are mean ± SEM

$n = 3$ subjects in the placebo group and 3 in the TRF young group

$n = 4$ subjects in the placebo group and 4 in the TRF old group

All values are in μmoles/L

* The mean difference is significant at $p < 0.05$

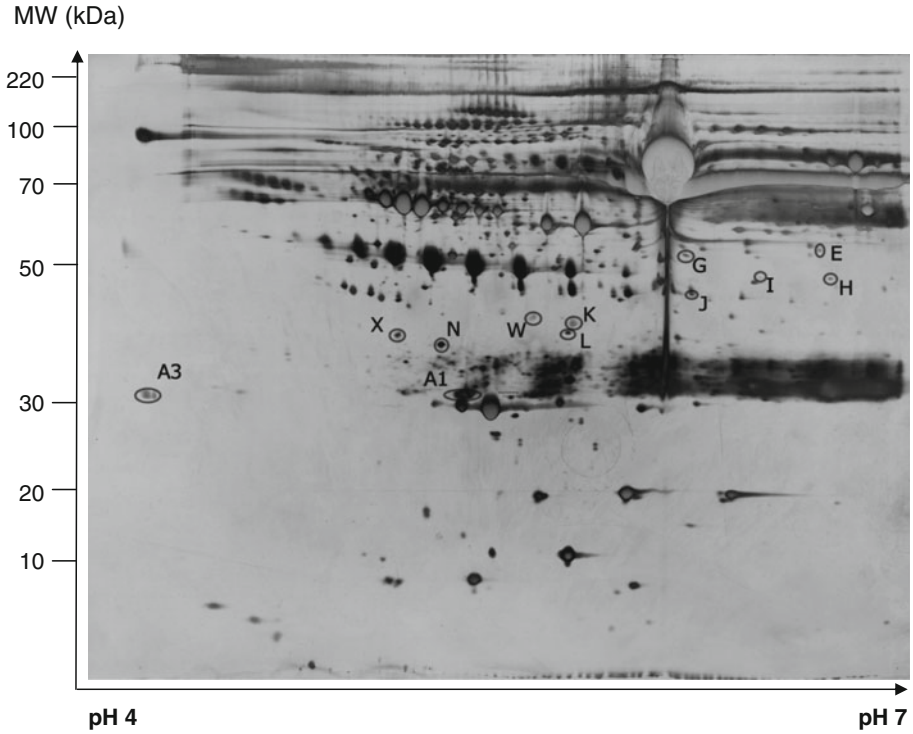

Fig. 1 Representative 2DE of human plasma. Protein from the human plasma was resolved by 2DE. The resulting gels were visualized by silver staining. *Circles* indicate protein spots that changed in *volume* after TRF supplementation. The labeling corresponds to proteins in Table 3a, b

Table 3 List of plasma proteins identified as being differentially expressed following TRF supplementation

Spot	Protein	Accession No. (Uniprot)	Score/% coverage	Mass (kDa)/pI	Expression
A: Differently expressed plasma proteins following TRF supplementation at 3 versus 0 month and 6 versus 0 month for young subjects identified by MALDI-TOF MS/MS					
G	Complement factor H-related protein 1 precursor (FHR1)	Q03591	230/17 %	37,637/7.75	6 versus 0 (↑)
K	Apolipoprotein E precursor (APOE)	P02649	1,260/60 %	36,132/5.65	3 versus 0 (↑)
					6 versus 0 (↑)
L	Complement factor H-related protein 2 precursor (FHR2)	P36980	109/22 %	30,631/6.00	3 versus 0 (↑)
					6 versus 0 (↑)
X	AMBP protein precursor [contain alpha-1 microglobulin] (AMBP)	P02760	295/40 %	38,974/5.95	3 versus 0 (↑)
					6 versus 0 (↑)
W	Apolipoprotein E precursor (APOE)	P02649	901/56 %	36,132/5.65	6 versus 0 (↑)
A3	Apolipoprotein A-I precursor (APOA1)	P02647	592/43 %	30,759/5.56	6 versus 0 (↑)
A1	C-reactive protein precursor (CRP)	P02741	410/31 %	25,023/5.45	6 versus 0 (↓)
B: Differently expressed plasma proteins following TRF supplementation at 3 versus 0 month and 6 versus 0 month for old subjects identified by MALDI-TOF MS/MS					
E	Carboxypeptidase N catalytic chain precursor (CBPN)	P15169	190/20 %	52,253/6.86	3 versus 0 (↑)
					6 versus 0 (↑)
G	Complement factor H-related protein 1 precursor (FHR1)	Q03591	230/17 %	37,637/7.75	3 versus 0 (↑)
					6 versus 0 (↑)
H	Complement factor H-related protein 1 precursor (FHR1)	Q03591	399/21 %	37,637/7.75	3 versus 0 (↑)
I	Complement factor H-related protein 1 precursor (FHR1)	Q03591	212/19 %	37,637/7.75	3 versus 0 (↑)
J	Haptoglobin-related protein precursor (HPTR)	P00738	109/26 %	38,983/6.42	3 versus 0 (↑)
					6 versus 0 (↑)
K	Apolipoprotein E precursor (APOE)	P02649	1,260/60 %	36,132/5.65	3 versus 0 (↑)
					6 versus 0 (↑)
L	Complement factor H-related protein 2 precursor (FHR2)	P36980	109/22 %	30,631/6.00	3 versus 0 (↑)
					6 versus 0 (↑)
N	AMBP protein precursor [contain alpha-1 microglobulin] (AMBP)	P02760	352/28 %	38,974/5.95	3 versus 0 (↑)
					6 versus 0 (↑)
A3	Apolipoprotein A-I precursor (APOA1)	P02647	592/43 %	30,759/5.56	6 versus 0 (↑)
A1	C-reactive protein precursor (CRP)	P02741	410/31 %	25,023/5.45	6 versus 0 (↓)

The accession number (Swiss-Prot), score/% coverage, Mass (kDa)/pI, and expression are shown in the table

28 kDa apolipoprotein with a major role in the functions of high-density lipoprotein (HDL) during the transport of excess cholesterol from peripheral tissues toward the liver for excretion [24]. In addition, APOA1 also interacts with paraoxonase, an enzyme located on HDL which reduces oxidative modification of LDL [25, 26], thereby decreasing the occurrence of atherosclerosis. Aldred et al. [27] reported increased levels of APOA1 in blood plasma following supplementation with α-tocopherol.

APOE was increased in the plasma of both younger and old individuals following TRF supplementation. The expression of APOE was validated by Western blotting (Fig. 2b). APOE is a 34 kDa apolipoprotein with roles in reverse cholesterol transport (RCT) and plasma lipoprotein metabolism [28]. It can serve as a ligand for the LDL receptor and for specific APOE receptor in liver tissues. APOE functions in the clearance of VLDL and chylomicron remnants, thus affecting circulating concentrations of lipoproteins and plasma levels of cholesterol [29]. Low concentration of APOE represented a risk in developing cardiovascular disease [30].

Acute-phase proteins

TRF supplementation lowered CRP levels in both old and young subjects. The expression of CRP was validated by Western blotting (Fig. 2c). CRP is a pentameric acute-phase protein. Its expression has been shown to be regulated in response to tissue injury, infection, or other inflammatory stimuli. It has been proposed to be an important predictive

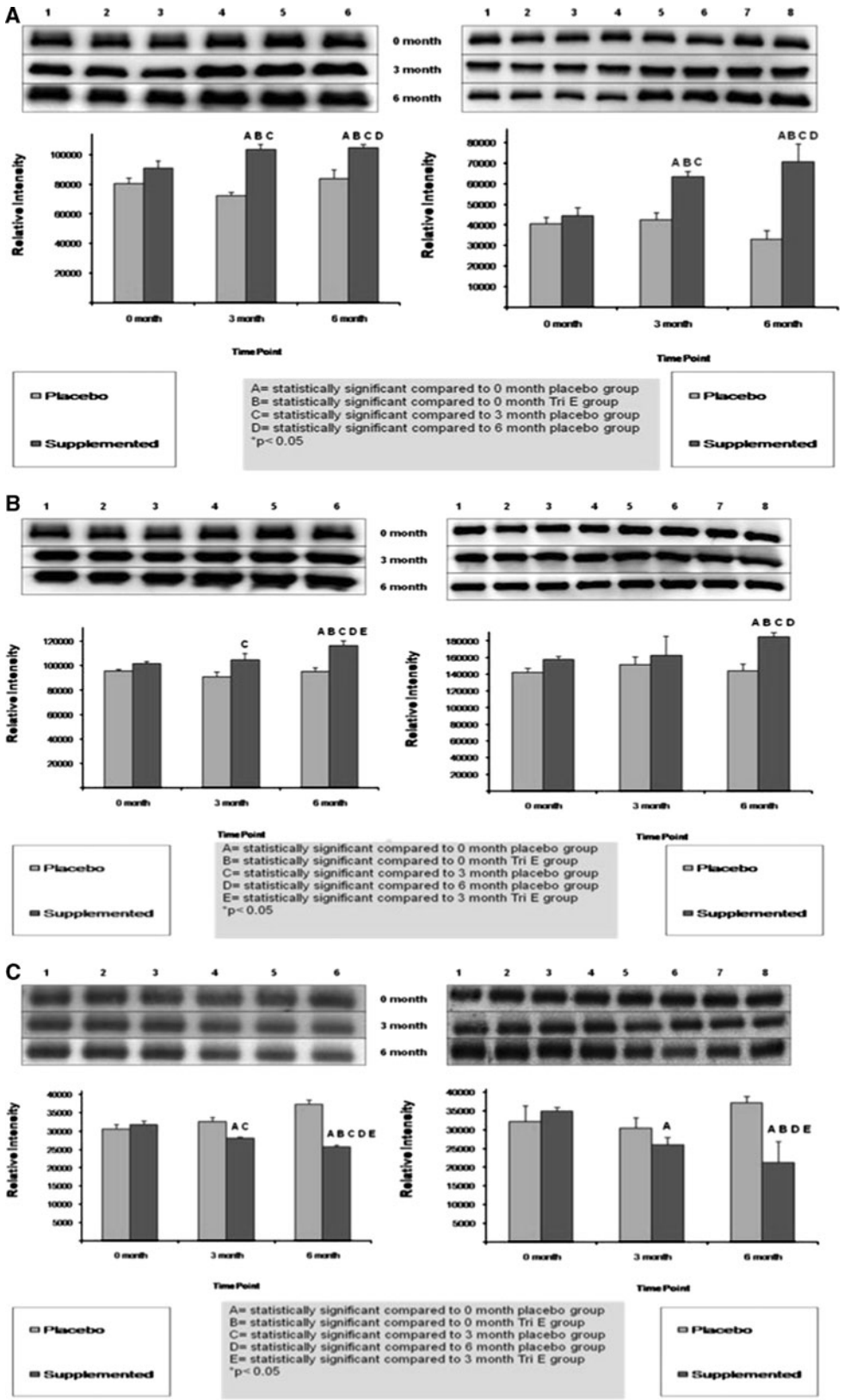

Fig. 2 Results of Western blotting showing relative expressions of APO A1, APO E, and CRP for both young and old subjects. **a** Effect of TRF supplementation on the relative expression of APO A1, **b** Effect of TRF supplementation on the relative expression of APO E, **c** Effect of TRF supplementation on the relative expression of CRP. Results represent the mean ± SEM. $*p < 0.05$

and prognostic marker for cardiovascular events including atherosclerosis, stroke, and thrombosis [31–33]. CRP directly exhibits pro-inflammatory effects on peripheral vascular cells such as activation of the classical complement pathway, increase in cellular adhesion, and reduction of endothelial nitric oxide [34, 35]. A strong correlation between increased plasma CRP level and impaired endothelial function has been reported in patients with coronary artery disease [36]. CRP and total cholesterol levels decreased significantly after simvastatin (a hypolipidemic drug) treatment, but increased when simvastatin therapy was terminated [37]. Thus, CRP level may potentially be used as a novel biomarker of vascular risk factor and monitoring the effect of pharmacologic intervention on cardiovascular disease.

Protease inhibitor

CBPN was found to be up-regulated in both young and old subjects 6 months after TRF supplementation. CBPN is an important inactivator of several potent peptides including bradykinin [38], anaphylatoxin [39], creatine kinase MM [40], and fibrinopeptides [41]. Bradykinin is a nine-amino peptide involved in the inflammatory response and tissue injury [38, 42].

AMBP was up-regulated in young and old subjects after 6 months of TRF supplementation. This protein is synthesized in the liver and is rapidly spread to extracellular compartments [43] and interstitial fluids [44]. Recent findings have suggested AMBP to be involved in the reduction and scavenging of radicals, thereby decreasing oxidative damage potential [45].

Immune response

Deterioration of the immune system has been attributed to high incidence of infection especially in old individuals [46]. Optimal concentrations of antioxidants are needed for proper function of the immune system. Vitamins with antioxidant properties have been shown to enhance immune response [47]. TRF supplementation was shown to increase the protein plasma levels of complement factors such as FHR1 and FHR2. These proteins are involved in the elimination of foreign pathogens.

In conclusion, our results have demonstrated that TRF supplementation increased plasma concentrations of tocopherols and tocotrienols after 6 months. More proteins were found to have changed in expression following TRF treatment in old individuals suggesting that old individuals were more sensitive to the effects of TRF supplementation. Among proteins that changed in expression, three were of particular interest—APOA1 (up-regulated), APOE (up-regulated), and CRP (down-regulated). The up-regulation (APOA1 and APOE) and down-regulation (CRP) of these proteins are known to have positive effects against atherosclerosis. Thus, it can be concluded that the consumption of TRF appears to not only affect plasma levels of tocopherols and tocotrienols, but also affect the expression of proteins that are beneficial against atherosclerosis. These proteins may also potentially be developed into biomarkers for the study of the effects and effectiveness of TRF supplementation.

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