

Tocotrienols reduce monocytes adhesion to stimulated human endothelial cells

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Abstract

The anti-atherosclerotics activity of tocotrienols (TCT) compared to alpha-Tocopherol (α -TOC) in in vitro study is not much being reported especially in human endothelial cells. The aim of the present study was to study the effects of TTMF, TCT and α -TOC on monocytes adherence to stimulated endothelial cells and to investigate the correlation between monocytes adherence and adhesion molecules in endothelial cells treated with TTMF, pure TCT isomers and α -TOC. Human umbilical vein endothelial cells (HUVECs) were incubated with TTMF, TCT isomers and α -TOC (0.3-10 μ M) together with lipopolysaccharide, LPS (1 μ g/ml). Monocytes adherence was measured by Rose Bengal staining. Soluble ICAM-1, VCAM-1 and e-selectin and NF κ B binding were measured by ELISA. TTMF and TCT isomers inhibit monocytes adhesion to LPS-stimulated HUVECs but not α -TOC. δ -TCT exhibit the highest % inhibition of monocytes adhesion compared to the other TCT isomers. Only TCT isomers show positive correlation of monocytes adhesion with certain adhesion molecules and NF κ B but not TTMF and α -TOC. In conclusion, TTMF and TCT isomers exhibit reduction of adhesion of monocytes to LPS stimulated endothelial cells. The reduction of monocytes adhesion by TCT isomers especially δ -TCT are positively correlated with reduction of adhesion molecules and NF κ B deactivation. It can be suggested that TCT especially the δ -TCT isomers is beneficial in the prevention of early atherogenesis in human.

Keywords

Tocotrienol

Monocyte adherence

Endothelial cells

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Introduction

The adherence of monocytes to the vascular endothelium and subsequent migration of cells into the vessel wall are an important early event in atherogenesis. Monocytes adherence to ECs is mediated by cell adhesion molecules such as ICAM-1, VCAM-1 and e-selectin (Vasanthi *et al.*, 2012). Pro-inflammatory cytokines such as IL-6 induces ECs to release chemotactic factors and cell adhesion molecules and subsequently contributes to the inflammatory process (Ludwig *et al.*, 2004).

Modulation of monocyte-endothelial adhesion could be an important target in atherosclerosis. It has also been shown that agonist-induced adhesion of monocytes to endothelium is mediated by translocation of the transcription factor, nuclear factor kappa B, NF κ B (Lee *et al.*, 2014). NF κ B is a mammalian transcription factor that is directly involved in the activation of genes responsible for immune, inflammatory or acute phase responses (Oeckinghaus and Ghosh, 2009).

It has been suggested that suitable therapeutics that can block leukocyte-endothelial interactions are the point of interest in the prevention of atherosclerosis (Lawson and Wolf, 2009). Previously it has been reported that alpha-tocopherol inhibits monocyte-endothelial cell adhesion by deactivation of NF κ B (Islam *et al.*, 1998). However, epidemiological studies have indicated the beneficial effects of Vitamin E in reduction of cardiovascular events but in various clinical trials, the results were contradictory (Rimm *et al.*, 1993). In a meta-analysis report, intervention with high dose of Vitamin E in humans (> 150 IU/day or 100 mg/day) have increased all cause of mortality and should be avoided (Miller *et al.*, 2005). However, such conclusions may not be appropriate when only α -TOC was tested in that meta-analysis (Gee, 2011). In addition, α -TOC is often incorrectly referred to as Vitamin E when Vitamin E actually consists of both TOCs and TCTs. The existence of TCTs is almost always disregarded in Vitamin E research (Khanna *et al.*, 2005).

TCT is one of the vitamin E compounds which

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consist of four isomers, α -, β -, γ - and δ -TCT are present in the seed endosperm of most monocots, including agronomically important cereal grains such as wheat, rice and barley. Crude palm oil, extracted from the fruits of *Elaeis guineensis* particularly contains a high amount of TCT (up to 800 mg/kg), mainly consists of γ - and α -TCT (Sen *et al.*, 2007). TCT has been reported to have beneficial effects in terms of hypercholesterolemic activity, potent antioxidant activity, anti cancer, anti aging, anti thrombotic and anti angiogenic and were reported to be more potent than TOC (Ahsan *et al.*, 2014). TCT are the most effective vitamin E for reducing cytokine and endothelial expression of adhesion molecules (ie. sVCAM-1, sICAM-1 and e-selectin) and adhesion to monocytes (Naito *et al.*, 2005; Qureshi *et al.*, 2011).

Although TCT activity is superior to TOC, the potential role of TCT in the prevention of atherosclerosis has received minimal public attention. It has been suggested that TCT is expected to accomplish as an important therapeutic option in atherosclerotic disease complications such as coronary artery disease (Vasanthi *et al.*, 2012). The information on TTMF and TCT isomers on inhibition of monocytes binding to endothelial cells and its correlation with adhesion molecules are still lacking. Therefore, the objectives of this study were to study the effects of TTMF, TCT isomers and α -TOC on i) monocytes binding activity ii) adhesion molecules production and iii) NF κ B transcription in stimulated endothelial cells. Then correlation of monocytes binding and adhesion molecules in endothelial cells treated with TTMF, TCT isomers and α -TOC were also being investigated in this study.

Materials and Methods

Materials

TTMF was provided by Golden Hope Jomalina Sdn. Bhd. Malaysia. TCT isomers and α -TOC were provided by Davos Life Science, Singapore. Medium 200 and low serum growth supplements (LSGS) were obtained from Cascade Biologics, USA. RPMI-1640 medium (with glutamax-I and HEPES), L-glutamine and fetal bovine serum (FBS) were purchased from Gibco-Life Technologies, USA. Penicillin/streptomycin was purchased from PAA laboratories, Austria. MTT was purchased from Fluka, Germany. PBS and Accutase were purchased from ICN Biomedical, USA. Phosphate buffer saline (PBS) was obtained from MP Biomedicals, France. Rose Bengal staining was obtained from Sigma Aldrich, USA. ELISA test kits for sICAM-1, sVCAM-1 and E-selectin were purchased from Bender Medsystems,

Austria. NF κ B binding assay kit was obtained from Cayman Chemicals, USA.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics, USA. HUVEC were cultured in medium 200 supplemented with LSGS in a humidified incubator set at 37°C and 5% carbon dioxide (CO₂) until confluent. Cells were grown in 25 cm² flasks (BD Falcon, UK). Cells were harvested by cell detachment solution (Accutase) and sub-cultivation ratio was 1:3 (culture:medium).

Cell cytotoxicity assay

TTMF was first tested for its toxicity concentrations against HUVEC cell by using Microculture Tetrazolium Salt (MTT) assay (Mosmann, 1983). Briefly, 100 μ l of 1 x 10⁵ cells/ml were seeded into 96 well micro plates in the presence of varying concentration of TTMF (0.3 – 200 μ M) prior to incubation for 24 hours in a humidified incubator set at 37°C and 5% CO₂. Control wells of untreated cell population were also included. Twenty μ l of MTT solution (5 mg in 1 ml PBS) was added to each well followed by 4 hours incubation at 37°C. Then, 170 μ l of the medium was removed from each well before the addition of 100 μ l of DMSO to solubilize the formazan crystal formed after the incubation with MTT. The plate was then incubated at room temperature for 30-50 minute. The absorbance of each well was measured at 550 nm wavelength using a microplate reader (Micro Quant, Biotek Instruments).

Preparation and Supplementation of HUVEC with TTMF, TCT isomers and α -TOC

A stock solution of TTMF, TCT isomers and α -TOC stock solution of were prepared in absolute ethanol and stored at -80°C. The stock solutions were then mixed with FCS at a ratio of 1:20 and incubated at 37°C for 15 minutes during which time a brief vortex was conducted every 5 minutes. By using this method, it has been shown that there was an increased of cellular vitamin E uptake in a dose-dependent manner (Martin *et al.*, 1997). The FCS-TTMF, TCT isomers and α -TOC were then diluted into various working concentrations (0.3 – 10 μ M) with culture medium. HUVEC in 25 cm² culture flask were treated with different concentrations of TTMF or TCT isomers or α -TOC (0.3 – 10 μ M) together with LPS (1 μ g/ml) for 16 hours in CO₂ humidified incubator set at 37°C. At the end of the incubation period, the supernatant from each sample were collected, centrifuged and stored at -70°C until

used. The HUVECs were collected to obtain nuclear lysates.

Monocyte adherence assay

Firstly, HUVEC were seeded in 96-well micro titer plates at a seeding density of 1×10^5 cells/ml and incubated overnight in a humidified incubator set at 37°C and 5% CO₂. HUVEC were then treated with varying concentrations of TTMF or TCT isomers or α -TOC (0.3 – 10 μ M) together with LPS (1 μ g/ml) prior to incubation for 16 hours in a humidified incubator set at 37°C and 5% CO₂. After that, an amount of 0.5×10^6 /ml U937 cells was added into each well and kept for an hour in a humidified incubator set at 37°C and 5% CO₂. At the end of incubation, the unbound U937 cells were washed away with RPMI-1640 medium. Then, 100 μ l of 0.25% Rose Bengal in PBS was added into each well and incubated for 10 minutes in room temperature. Excess stain were washed away for 3 times with PBS containing 10% FBS. After washing, 200 μ l of ethanol: PBS (1:1 v/v) solution was added into each well and incubated for an hour at room temperature. After incubation, the absorbance in each well was read at 570 nm wavelength with a microplate reader (Tecan Safire, Männedorf, Switzerland)

Enzyme-Link immunosorbent assay (ELISA)

Concentrations of surface levels of adhesion molecules in supernatant of HUVEC cells were measured by ELISA standard kit (Bender Med System, Vienna, Austria). Tests were performed according to the instructions provided by the manufacturer. At the end of ELISA testing, absorbance was obtained by spectrophotometer (Micro Quant, Biotek Instruments) at 405 nm wavelength.

Quantitation of NF κ B (p50) protein level in cell lysates

HUVEC in 25 cm² culture flasks were treated with different concentrations of TTMF or TCT isomers or α -TOC (0.3 – 10 μ M) together with LPS (1 μ g/ml) for 16 hours in CO₂ incubator set at 37°C. After incubation, HUVEC were harvested with cell detachment solution (Accutase) and transferred into pre-chilled 15 ml centrifuge tubes to perform nuclear extraction process according to the manufacturer protocols. Quantitation of NF κ B (p50) protein binding in nuclear extract was performed by ELISA method according to the manufacturer instruction manual (Cayman Chemicals, USA). The absorbance in each well was measured at 450 nm with a microplate reader (Tecan Safire, Männedorf, Switzerland)

Statistical analysis

Results are expressed as mean + SD. The analysis of variance (ANOVA) with post-Hoc test was performed. The differences between each concentration of TCT isomers and LPS controls were analysed with Bonferroni post Hoc analysis. Correlation was performed using Pearson correlation test. All data was analyzed by a statistical package program, SPSS version 16.0. Level of significance was set at $p < 0.05$. To compare the effectiveness and identify the most potent vitamin E vitamers, area under the curve (AUC) analysis for each vitamin E vitamers across all concentrations combined (0.3 μ M – 10 μ M) was performed for each biomarker using the Graph Version 4.3 software. The AUC analysis was used because the effects of vitamin E vitamers on monocytes adhesion to endothelial cells were not in the dose-dependent manner. After obtaining the AUC for each vitamin E vitamers, the percentage (%) inhibition against their respective controls was calculated for each biomarker.

Results

Effects of TTMF on cell viability

Effects of varying concentrations of TTMF on cell viability were observed by MTT assay. TTMF greater than 10 μ M have shown reduced cell viability as when compared to control untreated cell population (Figure 1). As a result, TTMF, TCT isomers and α -TOC no greater than 10 μ M were used to treat the cells for this experiment.

Effects of TTMF, TCT isomers and α -TOC on adhesion of monocytes to LPS-stimulated HUVEC

The effects of TTMF, TCT isomers and α -TOC on adhesion of monocytes to LPS-stimulated HUVEC are illustrated in Figure 2. It was found that TTMF, α -TCT (one concentration), β -, γ -, δ -TCT ($p < 0.0001$) but not α -TOC lead to reduction of monocytes adhesion to LPS-stimulated HUVEC compared to LPS controls. TTMF at 0.3 μ M showed the lowest monocytes adhesion. For α -TCT, significant reduction of monocytes adhesion was only observed at 1.3 μ M concentration compared to LPS controls. At the concentrations of 0.3 μ M – 10 μ M, the monocytes adhesion was significantly reduced by β -, γ - and δ -TCT. In contrast, α -TOC at 0.3 μ M and 5 μ M showed a significant increment on monocytes adhesion to LPS-stimulated HUVEC. Overall % inhibition of TTMF, α -, β -, γ -, δ -TCT and α -TOC towards LPS controls was assessed by AUC analysis. δ -TCT exhibited the highest % inhibition of monocytes adhesion followed by γ -TCT, β -TCT,

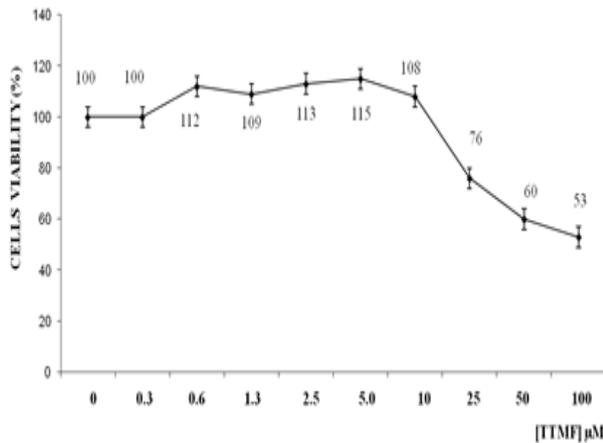


Figure 1. Effects of TTMF on cells viability. Data are expressed as percentage of cells viability in Mean + SD (n=3).

TTMF and α -TCT. However, α -TOC did not show any inhibitory effects on monocytes adhesion to LPS-stimulated HUVEC.

Correlation of monocyte adhesion to LPS-stimulated HUVECs with adhesion molecules protein expression by TTMF, TCT isomers and α -TOC.

Correlation of monocytes adhesion to LPS-stimulated HUVECs with adhesion molecules protein expression by TTMF, TCT isomers and α -TOC is illustrated in Table 1. For TTMF, there is no significant correlation of monocytes adhesion with adhesion molecules protein expression. There is a significant positive correlation of monocytes adhesion with soluble ICAM-1 and VCAM-1 as shown by α -TCT. β -TCT shows a significant positive correlation between monocytes adhesion and NF κ B transcription. For γ -TCT, significant positive correlation was observed between monocytes adhesion with VCAM-1 and NF κ B. For δ -TCT, significant positive correlation was observed between monocytes adhesion and VCAM-1, e-selectin and NF κ B. Finally, for α -TOC, no significant correlation was observed between monocytes adhesion with all markers.

Discussion

This present study indicates that, TTMF and TCT isomers reduce monocytes adhesion to LPS-stimulated HUVECs but this is not so for α -TOC. This study also shows that δ -TCT exhibit the highest % inhibition of monocytes adhesion compared to the other TCT isomers. Only TCT isomers have shown positive correlation of monocytes adhesion with certain adhesion molecules and NF κ B but this is not for TTMF and α -TOC.

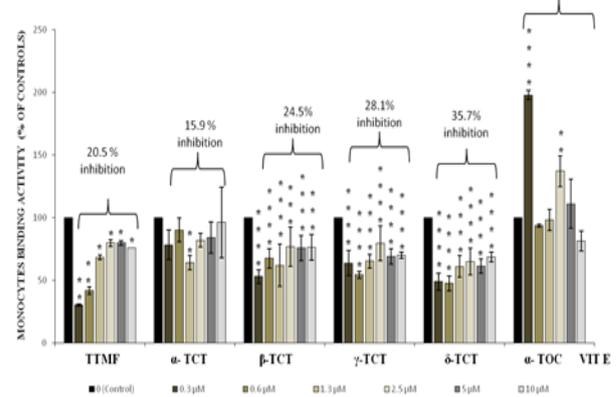


Figure 2. Effects of TTMF, TCT Isomers and α -TOC (0.3 μ M – 10 μ M) on the monocytes binding activity in LPS stimulated cultured HUVECs. Data are expressed as percentage (%) of LPS Controls. Data are expressed as Mean + SD (n=3). * p<0.05, ** p<0.01, *** p<0.001 and **** p< 0.0001 compared to HUVECs incubated with LPS alone.

Previously, it has been reported that in TNF- α stimulated endothelial cells, δ -TCT exerted the most profound inhibitory effects on monocytes adhesion compared to the other TCT isomers (Cho *et al.*, 2002). Consistently, Naito *et al.* (2005) also reported similar finding where δ -TCT acts as the most potent TCT isomers for the reduction of monocytes adhesion to 25-hydroxycholesterol stimulated human endothelial cells. It has been reported that, α – TOC compared to TCTs has less beneficial effects in inhibiting adhesion molecules in stimulated endothelial cells (Mutalib *et al.*, 2014). Therefore it can be postulated that the reduced potency of α -TOC in inhibiting monocytes adherence is due to less beneficial effects in the reduction of adhesion molecule as stated above. In addition, α -tocopherol supplementation in healthy subjects does not affect its monocyte adhesion to endothelial cells (Wollard *et al.*, 2006).

Adhesion of leucocytes with vascular endothelial cells is an important process during an inflammatory response. The first event involves rolling of leukocytes along the endothelial membrane, a process which is mediated by e-selectin. E-selectin, which is expressed by endothelial cells, mediates the interaction between endothelial cells and monocytes (Lee *et al.*, 2011). After rolling, firm adhesion is accomplished by endothelial adhesion molecules (VCAM-1 and ICAM-1). Previously we have reported that TTMF and TCT isomers significantly inhibited LPS-induced endothelial cells production of VCAM-1, ICAM-1, and e-selectin via deactivation of NF κ B (Muid *et al.*, 2011).

Therefore we wanted to further investigate

Table 1. Correlation of monocyte binding activity with TTMF, TCT isomers and α -TOC

	ICAM-1	VCAM-1	e-selectin	NF κ B
TTMF	r=0.039 NS	r=-0.344 NS	r=0.420 NS	r=0.488 NS
α -TCT	r=0.519 p<0.05	r=0.458 p<0.05	r=0.060 NS	r=0.301 NS
β -TCT	r=0.314 NS	r=0.014 NS	r=0.135 NS	r=0.691 p<0.01
γ -TCT	r=0.096 NS	r=0.522 p<0.01	r=0.030 NS	r=0.671 p<0.0001
δ -TCT	r=0.420 NS	r=0.691 p<0.01	r=0.592 p<0.01	r=0.705 p<0.0001
α -TOC	r=0.402 NS	r=-0.324 NS	r=-0.090 NS	r=-0.251 NS

whether the reduction of monocytes adhesion by TTMF and TCT isomers is correlated with reduction of these endothelial adhesion molecules. Scarce data have been reported on the correlation of monocyte adhesion with soluble ICAM, VCAM-1 and e-selectin and NF κ B transcription. In this study, we found that monocytes adhesion to LPS stimulated HUVECs by TCT isomers shows positive correlation with either one or two adhesion molecules (ICAM-1, VCAM-1 and e-selectin) and NF κ B. These suggest that, the reduction of monocytes adhesion by TCT isomers in this study is correlated with reduction of adhesion markers as well.

Conclusion

TTMF and TCT isomers inhibit the adhesion of monocytes to LPS stimulated endothelial cells but not so α -TOC. The reduction of monocytes adhesion by TCT isomers especially δ -TCT are positively correlated with reduction in adhesion molecules and NF κ B deactivation. Findings from this present study is highly promising for TTMF and TCT isomers as potential anti-atherogenic agents since adherence of circulating monocytes to the endothelium is the first step in the formation of fatty streaks in atherogenesis. Therefore, it can be suggested that TCT especially the δ -TCT isomer is beneficial in the prevention of early atherogenesis. However, further *in vivo* studies using TCT isomers in the absence of α -TOC need to be conducted to further determine to beneficial effects of TCT as a potential anti-atherogenic agent.

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