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The two faces of α- and γ-tocopherols: an *in vitro* and *ex vivo* investigation into VLDL, LDL and HDL oxidation

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Abstract

Background: Vitamin E and its derivatives, namely, the tocopherols, are known antioxidants, and numerous clinical trials have investigated their role in preventing cardiovascular disease; however, evidence to date remains inconclusive. Much of the *in vitro* research has focused on tocopherol's effects during low-density lipoprotein (LDL) oxidation, with little attention being paid to very LDL (VLDL) and high-density lipoprotein (HDL). Also, it is now becoming apparent that γ-tocopherol may potentially be more beneficial in relation to cardiovascular health.
Objectives: Do α- and γ-tocopherols become incorporated into VLDL, LDL and HDL and influence their oxidation potential in an *in vitro* and *ex vivo* situation?
Design: Following (i) an *in vitro* investigation, where plasma was preincubated with increasing concentrations of either α- or γ-tocopherol and (ii) an *in vivo* 4-week placebo-controlled intervention with α- or γ-tocopherol. Tocopherol incorporation into VLDL, LDL and HDL was measured via high-pressure liquid chromatography, followed by an assessment of their oxidation potential by monitoring conjugated diene formation.
Results: In vitro: Both tocopherols became incorporated into VLDL, LDL and HDL, which protected VLDL and LDL against oxidation. However and surprisingly, the incorporation into HDL demonstrated pro-oxidant properties. *Ex vivo*: Both tocopherols were incorporated into all three lipoproteins, protecting VLDL and LDL against oxidation; however, they enhanced the oxidation of HDL.
Conclusions: These results suggest that α- and γ-tocopherols display conflicting oxidant activities dependent on the lipoprotein being oxidized. Their pro-oxidant activity toward HDL may go some way to explain why supplementation studies with vitamin E have not been able to display cardioprotective effects.

Keywords: Tocopherol; Lipoprotein oxidation; Pro-oxidant activity; Antioxidants

1. Introduction

The oxidation of plasma lipoproteins is a pivotal event in the development of atherosclerosis [1,2], and numerous experimental studies have persuasively suggested a major role for oxygen-derived free radicals in the pathogenesis of this disease [3]. It has been demonstrated that antioxidant vitamins can protect against oxidative injury and are therefore believed to provide protection against a myriad of diseases. *In vitro* findings have demonstrated that vitamin E (i) reduces the oxidative modification of low-density lipoprotein (LDL) [4], (ii) decreases LDL deposition in arterial walls, (iii) reduces monocyte adhesion onto the endothelium and (iv) inhibits platelet activation [2]. These findings, together with the oxidative hypothesis of atherosclerosis, have fueled numerous epidemiological studies and clinical trials investigating the role of vitamin E in the prevention and treatment of cardiovascular disease. However, randomized controlled trials have provided conflicting evidence, with some reporting beneficial effects [5–7], while others demonstrating a neutral or deleterious effect [8–10]. Thus, the oxidation hypothesis remains at an impasse, and the public remains confused about vitamin E supplementation.

Analysis of naturally occurring vitamin E reveals that it comprises eight structurally related forms, viz. four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ). As the predominant form of vitamin E in humans and animals, α-tocopherol has been extensively studied; however, to date, little work has been carried out to identify the properties of γ-tocopherol, the major form of vitamin E ingested in the US diet [11,12]. The studies of Ohrvall et al. [13] and Kontush et al. [14], reported that serum concentrations of γ-tocopherol, but not α-tocopherol, were found to be lower in patients with cardiovascular disease when compared with control subjects. Saldeen et al. [15] reported γ-tocopherol to be more potent than α-tocopherol in delaying arterial thrombus formation and reducing LDL oxidation, superoxide generation and lipid peroxidation. It has also been indicated that regular consumption of nuts, a major source of γ-tocopherol, lowers the risk of myocardial infarction and death from ischemic heart disease [16].

As described, investigations into lipoprotein oxidation and the antioxidant properties of α- and γ-tocopherols have mainly focused on LDL oxidation, with little attention being given to very LDL (VLDL), the precursor molecule of LDL, or high-density lipoprotein (HDL). Studies have demonstrated that oxidized VLDL, like oxidized LDL, plays a role in the development of atherosclerotic lesions. For example,

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modified VLDL is chemotactic for circulating monocytes [17], toxic to endothelial and smooth muscle cells [18], and can be taken up by macrophage scavenger receptors [17]. Furthermore, the focus of research is shifting from LDL to HDL, with this latter lipoprotein showing considerable potential for improving cardiovascular health. Low levels of HDL have been identified in over 40% of patients with acute myocardial infarctions [19]. Moreover, HDL has been identified as the most important risk factor for coronary events in patients with confirmed coronary artery disease [20]. The most clearly defined atheroprotective role of HDL is the process of reverse cholesterol transport [21], although HDL can also directly prevent LDL oxidation [22,23]. Oxidation of HDL results in it having a reduced capacity to remove excess free cellular cholesterol from peripheral tissues, hence highlighting the possible involvement of oxidized HDL in the pathogeneses of atherosclerosis [24,25]. HDL is also known for its ability to inhibit monocyte chemotaxis and leukocyte adhesion to endothelial cells [23]. For these reasons, it is vital to understand and investigate the effects such antioxidants have on all three lipoproteins, particularly HDL due to its established cardioprotective attributes.

Therefore, the aims of this study are (i) to investigate the oxidation potential of lipoproteins isolated from plasma preincubated with either α- or γ-tocopherol and (ii) to examine the effect of a 4-week placebo-controlled intervention with either α- or γ-tocopherol supplementation in healthy volunteers on (a) the incorporation of either α- or γ-tocopherol into VLDL, LDL and HDL, (b) VLDL, LDL and HDL oxidation and (c) serum hydroperoxide (HPO) levels.

2. Subjects and methods

2.1. In vitro Investigation

2.1.1. Plasma separation

Fasting peripheral venous blood was obtained from healthy volunteers with no known history of cardiovascular disease and not taking dietary supplements known to affect oxidation. Blood was collected into lithium heparin containing tubes (50 kU/l) and immediately stored on ice and was immediately subjected to centrifugation at 1100×*g* maximum for 20 minutes at 4°C in a Beckman J-6B centrifuge. Plasma was harvested, pooled and separated into two aliquots for (i) preincubation with increasing concentrations of α- or γ-tocopherol or (ii) not preincubated with α- or γ-tocopherol. Plasma was then stored at −70°C.

2.1.2. Lipoprotein isolation

Lipoproteins were isolated from both preincubated (with increasing concentrations of either α- or γ-tocopherol, as described below) and non preincubated plasma, VLDL by floatation ultracentrifugation [26] and LDL and HDL by nonequilibrium density gradient ultracentrifugation [27,28].

Lipoprotein purity was assessed by agarose electrophoresis using a Beckman Paragon Electrophoresis Kit (Beckman, UK) and following the manufacturer's instructions.

2.1.3. Lipoprotein purification and protein determination

Small contaminating molecules were removed from isolated lipoproteins by size exclusion chromatography using a prepacked column containing Sephadex G25 (PD10; Pharmacia Ltd, Milton Keynes, UK). The total protein concentration of each lipoprotein was determined using a commercial kit based on the Coomassie Blue reaction with proteins (Bio-Rad, Munich Germany; 500-006), which permitted the standardization of lipoproteins (VLDL to 25 μg/ml, LDL and HDL to 50 μg/ml) prior to oxidation.

2.1.4. Lipoprotein oxidation

Oxidation was mediated by the addition of copper (II) chloride solution ($CuCl_2$), final concentration 17.5 μM for VLDL, 2 μM for LDL and 5 μM for HDL [24–26] and was monitored in a thermostatically controlled spectrophotometer (Molecular Devices Spectra Max 190) at 37°C by measuring absorbance change at 234 nm; oxidation was followed for 24 h. Results are expressed as time at half maximum (an equivalent of lag time).

2.1.5. In vitro assessment of α- and γ-tocopherols' antioxidant properties

2.1.5.1. Preincubated plasma. Prior to isolation by ultracentrifugation (as described above), plasma was preincubated for 2 h, at 4°C or 37°C, with increasing concentrations of α- or γ-tocopherol. Tocopherol was added in a volume of 20 μl/ml plasma (final concentration range, 0–5 μM; stock solutions were dissolved in ethanol with 0 μM containing ethanol alone).

2.1.5.2. Postincubated lipoproteins. VLDL, LDL and HDL were isolated from non-incubated plasma and oxidation mediated in the presence of increasing concentrations of α- and γ-tocopherols (0–5 μM) in a volume of 20 μl/ml lipoprotein.

2.1.6. α- and γ-tocopherol concentration

Percent incorporation of α- and γ-tocopherols within lipoproteins preincubated with the tocopherols was measured via high-pressure liquid chromatography (HPLC) with electrochemical diode array detection according to Craft [29]. Levels of α- and γ-tocopherols are expressed as nmol tocopherol/mg protein.

2.2. Ex vivo investigation

This was a double-blind, randomized, placebo-controlled trial carried out on 30 healthy subjects with no known history of atherosclerosis and not taking dietary supplements. Volunteers were randomized (using computer-generated random numbers and a block design with block sizes of 9) to receive either α- or γ-tocopherol (420 mg/day) or placebo for 4 weeks. There were 10 subjects in each of the three groups. Fasting blood was taken before and after the supplementation period; plasma (heparinized) and serum were isolated and stored at −70°C until required. This study was approved by the Research Ethics Committee, Queen's University, Belfast, and informed written consent was given by all participants.

2.2.1. Ex vivo lipid analysis

Serum triglyceride and total, LDL and HDL cholesterol were measured using enzymatic assays (Boehringer Mannheim) on a Cobas Fara Auto Analyser.

2.2.2. Ex vivo serum and lipoprotein levels of α- and γ-tocopherols

The concentrations of α- and γ-tocopherols in serum, VLDL, LDL and HDL from the pre- and postsupplemented samples were measured by HPLC, as described by Hasselwander et al. [28]. Levels of α- and γ-tocopherols are expressed as nmol tocopherol/mg protein.

2.2.3. Ex vivo markers of oxidative stress

(a) VLDL, LDL and HDL were isolated and subjected to copper-mediated oxidation as described above.
(b) Preformed HPOs: Serum HPOs were measured using the *F*errous *O*xidation of *X*ylenol orange method I according to Wolff [30] and as described by McEneny et al. [31].

2.2.4. Statistical analysis

Analyses were performed using the statistics software package for Social Sciences (SPSS), version 17.0, for Windows. For the *in vitro* investigation, comparison between tocopherol concentrations was assessed using an independent-samples *t* test or paired-samples *t* test, where appropriate. For the *ex vivo* study, pre- and post-values were compared within each intervention group by paired-samples *t* tests. Significance for all tests was established at $P<.05$, and results are expressed as mean±standard deviation. Associations between continuous variables were measured using Pearson's correlation coefficient.

3. Results

3.1. VLDL, LDL and HDL purity

Following ultracentrifugation, electrophoresis demonstrated that each lipoprotein migrated as a single band with characteristic mobility of pre-β for VLDL, β- for LDL and α- for HDL, hence indicating that all lipoproteins were void of contamination (results not shown).

3.2. In vitro assessment of α- and γ-tocopherols' incorporation into VLDL, LDL and HDL and their effect on their antioxidant potential

3.2.1. VLDL and tocopherol

3.2.1.1. VLDL isolated from preincubated plasma. All concentrations of α- and γ-tocopherols lead to an increase in their incorporation into VLDL, over baseline ($P<.05$; Table 1). When VLDL was isolated from plasma incubated with α- and γ-tocopherols at 4°C, the VLDL was totally resistant toward oxidation; however, when isolated from plasma incubated at 37°C, α- and γ-tocopherols could only protect VLDL against oxidation at their highest concentrations (Fig. 1). Comparison between the two tocopherols demonstrated that although more γ-tocopherol became incorporated into VLDL ($P<.05$; Table 1), it was α-tocopherol that was more able to protect VLDL against oxidation at their lower concentrations (Fig. 1).

3.2.1.2. VLDL isolated from nonincubated plasma. We found that α- and γ-tocopherols protected VLDL against oxidation when added to the reaction mixture following its isolation from plasma. In addition, we observed a dose-dependant increase in time at half maximum and hence a decrease in the lipoproteins susceptibility to oxidation as the antioxidants increased (results not shown).

3.2.2. LDL and tocopherol

3.2.2.1. LDL isolated from preincubated plasma. We found that α-tocopherol only became significantly incorporated into LDL at 100 nmol α-tocopherol/mg protein, while γ-tocopherol became significantly incorporated at all concentrations ($P<.05$; Table 1). With regard to oxidation, α- and γ-tocopherols could protect LDL against oxidation when isolated from plasma incubated at 4°C ($P<.05$; Fig. 2A), however, at 37°C, only the highest concentrations of α- and γ-tocopherols could protect LDL against oxidation (Fig. 2B). Comparison between the two tocopherols demonstrated that although γ-tocopherol's incorporation into LDL was significantly greater than that of α-tocopherol ($P<.05$; table 1), again it was α-tocopherol that was more efficient in protecting LDL against oxidation ($P<.05$), at both temperatures, but more notably at 4°C.

3.2.2.2. LDL isolated from nonincubated plasma. Similarly to results previously published [32], we found that α- and γ-tocopherols protected LDL against oxidation when added to the reaction mixture following their isolation from plasma. There was a dose-dependant increase in time at half maximum and hence a decrease in the lipoproteins susceptibility to oxidation as the antioxidants increased (results not shown).

3.2.3. HDL and tocopherol

3.2.3.1. HDL isolated from preincubated plasma. α-Tocopherol became significantly incorporated into HDL at and above 50 nmol α-tocopherol/mg protein (Table 1), while γ-tocopherol was significantly incorporated at all concentrations. However, and surprisingly when HDL was isolated from plasma incubated with either tocopherol at 4°C or 37°C, its incorporation promoted HDLs oxidation, especially at the higher concentrations ($P<.05$; Fig. 3A, B). Comparing the incorporation of the two tocopherols into HDL, we found that the incorporation of γ-tocopherol was greater than α-tocopherol ($P<.05$ at all concentrations). However, γ-tocopherol displayed greater pro-oxidant properties, especially when HDL was isolated from plasma incubated at 37°C (Fig. 3B).

3.2.3.2. HDL isolated from nonincubated plasma. Similarly to results previously published [33], we again found that α- and γ-tocopherols protected HDL against oxidation when added to the reaction mixture following their isolation from plasma. We found a dose-dependant increase in time at half maximum and hence a decrease in the lipoproteins susceptibility to oxidation as the antioxidants increased (results not shown).

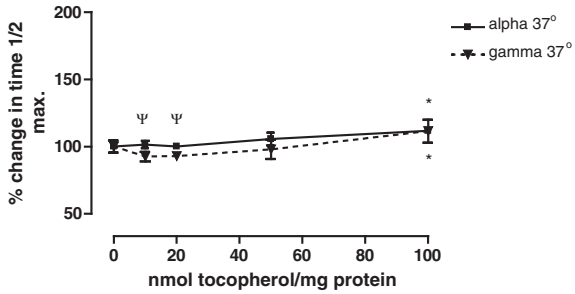

Fig. 1. Percent change in the oxidation potential of VLDL isolated from plasma incubated with either α- or γ-tocopherol at 37°C. Results: mean±S.D ($n=9$). *Tocopherol versus baseline, $^{\Psi}$α- versus γ-tocopherol.

3.3. Ex vivo investigation

3.3.1. Ex vivo lipid analysis

Following the 4-week supplementation period, no significant differences in lipid levels were detected in all three groups studied (results not shown).

3.3.2. Ex vivo serum levels of α- and γ-tocopherols

The results in Fig. 4A illustrate that, following the 4-week α-tocopherol supplementation, there was a significant increase in serum levels of α-tocopherol ($P=.004$) and a concomitant decrease in the level of γ-tocopherol ($P=.001$). We found that in the γ-tocopherol-supplemented group, while there was no significant change in the level of serum α-tocopherol, there was, as expected, a significant increase in the level of serum γ-tocopherol ($P=.001$; Fig. 4B). No significant differences were found in the levels of either α- or γ-tocopherol in the placebo group (results not shown).

3.4. Ex vivo lipoprotein incorporation of α- and γ-tocopherols

3.4.1. VLDL

Following the 4-week supplementation with α-tocopherol, there was a substantial increase in the level of α-tocopherol within VLDL (pre 130.67±52 vs. post 224.91±80.5 nmol α-tocopherol/mg protein; $P=.001$) and no significant difference in the level of γ-tocopherol (pre 8.59±4.3 vs. post 11.52±7.2 nmol γ-tocopherol/mg protein; $P=$NS). In the γ-tocopherol-supplemented group, there was a significant increase in the concentration of γ-tocopherol within VLDL (pre 6.16±1.5 vs. post 21.23±10.6 nmol γ-tocopherol/mg

Table 1
Percent incorporation above baseline of α- and γ-tocopherols into VLDL, LDL and HDL isolated from plasma incubated at 4°C with increasing concentrations of α- and γ-tocopherols, measured via HPLC

Tocopherol concentration (nmol/mg protein)	VLDL		LDL		HDL	
	α-Tocopherol % incorporation	γ-Tocopherol % incorporation	α-Tocopherol % incorporation	γ-Tocopherol % incorporation	α-Tocopherol % incorporation	γ-Tocopherol % incorporation
0	0	0	0	0	0	0
10	13.8±4.5 *	33.9±22.7*,†	0.6±1.2	27.7±8.2*,†	1.0±0.5	24.0±7.9*,†
20	20.2±8.7 *	48.3±18.3*,†	0.1±3.2	44.2±0.6*,†	0.4±1.0	37.3±3.8*,†
50	22.5±6.2*,†	76.4±7.7*,†	2.2±0.4	71.8±4.1*,†	2.4±1.4 *	72.2±3.0*,†
100	34.2±3.4 *	87.0±4.7*,†	4.8±0.8 *	71.9±2.2*,†	3.3±0.5 *	83.2±1.7*,†

Results: mean±S.D ($n=3$).
* $P<.05$ versus baseline.
† $P<.05$ α- versus γ-tocopherol.

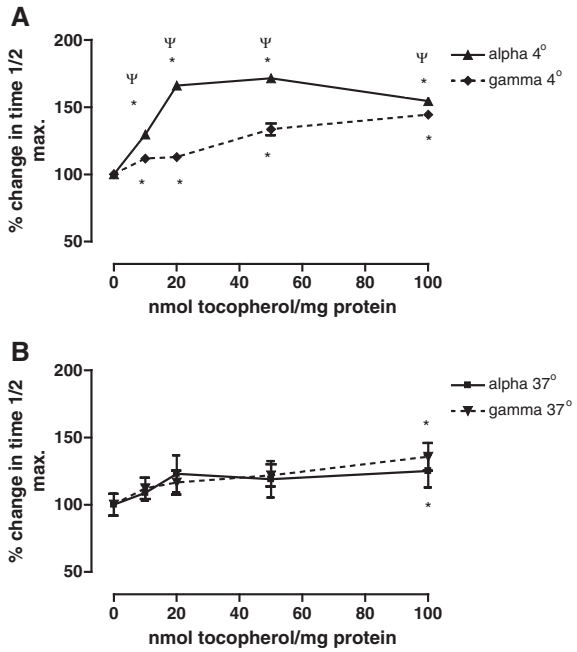

Fig. 2. (A) Percent change in the oxidation potential of LDL isolated from plasma incubated with either α- or γ-tocopherol at 4°C. Results: mean±S.D (n=9). *Tocopherol versus baseline, $^{\Psi}$α- versus γ-tocopherol. (B) Percent change in the oxidation potential of LDL isolated from plasma incubated with either α- or γ-tocopherol at 37°C. Results: mean±S.D (n=9). *Tocopherol versus baseline, $^{\Psi}$α- versus γ-tocopherol.

protein; P=.008) and no significant difference in the level of α-tocopherol (pre 132±41.3 vs. post 152.9±79.4 nmol α-tocopherol/mg protein; P=NS). No differences were found within VLDL from the placebo group (results not shown).

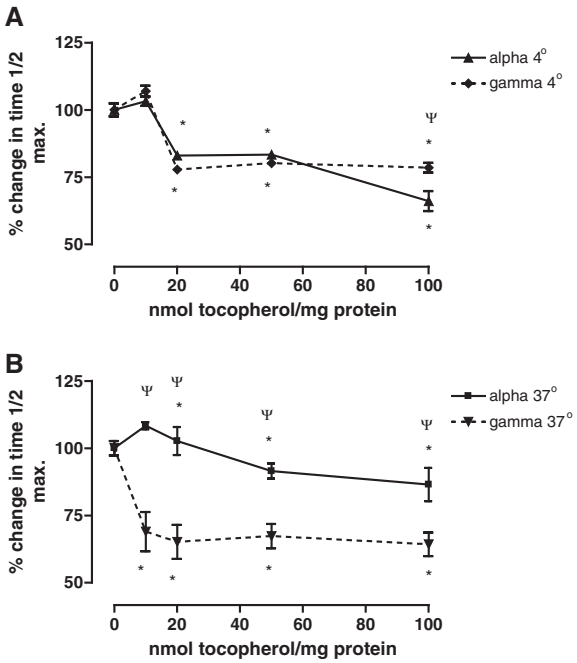

Fig. 3. (A) Percent change in the oxidation potential of HDL isolated from plasma incubated with either α- or γ-tocopherol at 4°C. Results: mean±S.D (n=9). *Tocopherol versus baseline, $^{\Psi}$α- versus γ-tocopherol. (B) Percent change in the oxidation potential of HDL isolated from plasma incubated with either α- or γ-tocopherol at 37°C. Results: mean±S.D (n=9). *Tocopherol versus baseline, $^{\Psi}$α- versus γ-tocopherol.

3.4.2. LDL

Following the 4-week supplementation with α-tocopherol, there was a significant increase in the level of α-tocopherol within LDL (pre 53.46±10.5 vs. post 102.3±21 nmol α-tocopherol/mg protein; P<.001) and no difference in the level of γ-tocopherol (pre 2.78 ±0.5 vs. post 2.94±0.6 nmol α-tocopherol/mg protein; P=NS). In the γ-tocopherol-supplemented group, there was an increase in the level of γ-tocopherol (pre 2.98±1.2 vs. post 10.04±3 nmol γ-tocopherol/mg protein; P=.002), and surprisingly, the level of α-tocopherol also increased in this group (pre 56.65±12.2 vs. post 69.44±15.4 nmol α-tocopherol/mg protein; P=.009). No differences were found within LDL from the placebo group (results not shown).

3.4.3. HDL

Following the 4-week supplementation with α-tocopherol, there was a significant increase in the level of α-tocopherol within HDL (pre 4.98±1.7 vs. post 11.92±2.5 nmol α-tocopherol/mg protein; P<.001) and no difference in the level of γ-tocopherol (pre 0.31±0.1 vs. post 0.42±0.1 nmol γ-tocopherol/mg protein; P=NS). In the γ-tocopherol-supplemented group, there was, as expected, a significant increase in the level of γ-tocopherol within HDL (pre 0.31±0.07 vs. post 1.02±0.34 nmol γ-tocopherol/mg protein; P=.003), with no significant difference in the level of α-tocopherol within HDL (pre 5.09±0.98 vs. post 6.03±1.8 nmol α-tocopherol/mg protein; P=NS). No differences were found within HDL from the placebo group (results not shown).

3.5. Ex vivo markers of oxidative stress

3.5.1. Lipoprotein oxidation

3.5.1.1. α-Tocopherol-supplemented group. In the α-tocopherol-supplemented group, both VLDL and LDL oxidations were reduced, as represented by an increase in time at half maximum (pre vs. post,

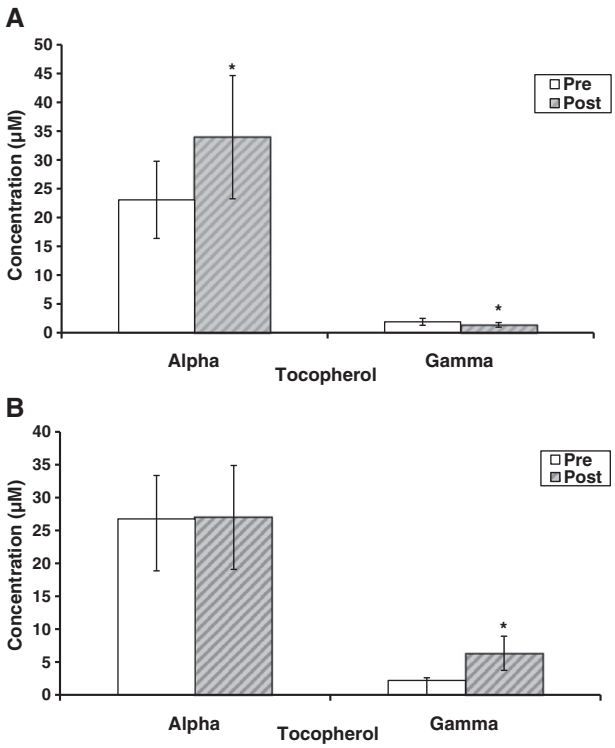

Fig. 4. (A) Serum levels of α- and γ-tocopherols in the α-tocopherol-supplemented group. (B) Serum levels of α- and γ-tocopherols in the γ-tocopherol-supplemented group.

VLDL 156±22 vs. 231±79 min; P=.028; LDL 74.5±4.5. vs. 85.5±9.0 min; P=.009). During HDL oxidation, there was a tendency for time at half maximum to decrease, indicating a trend toward a negative or pro-oxidant effect of the supplementation; however, this did not reach significance (pre vs. post, 49.6±9.3 vs. 45.9±5.2 min; P=.139).

3.5.1.2. γ-Tocopherol-supplemented group. In this supplemented group, only the oxidation potential of LDL was enhanced, with no significant effect being displayed for VLDL (pre vs. post, VLDL 181.6±64 vs. 218.7±75 min; P=.169; LDL 76.4±9.3 vs. 85.8±11.9 min; P=.032). Interestingly, HDL displayed significant pro-oxidant potential, (pre vs. post, 51.4±3.4 vs. 46.1±1.9 min; P=.012).

3.5.1.3. Placebo-supplemented group. No change was observed in the oxidation potential for any lipoprotein in this group (results not shown).

3.5.1.4. Correlations between change in time at half maximum and incorporation of tocopherol into VLDL, LDL and HDL.

(i) α-Tocopherol-supplemented group: results demonstrated that the increase in VLDL time at half maximum and α-tocopherol incorporation into VLDL were positively associated, (r^2=0.817, P=.007), as was the incorporation into LDL (r^2=0.714, P=.111), while the decrease in HDL time at half maximum and increase in α-tocopherol incorporation were negatively associated; (r^2=−0.886, P=.019).

(ii) γ-Tocopherol-supplemented group: results demonstrated that the increase in VLDL time at half maximum and γ-tocopherol incorporation into VLDL were not positively correlated (r^2=0.321, P=.482); however, they were for LDL (r^2=0.943, P=.005). With HDL, although, there was a tendency toward a negative correlation between decrease in time at half maximum and incorporation of γ-tocopherol into HDL, the results were not significant, (r^2=−0.657, P=0.156).

(iii) Placebo group: as expected, no significant correlations were found between VLDL, LDL or HDL oxidation and α- and γ-tocopherol levels.

3.5.2. Preformed HPOs

No change was observed in the level of HPOs in all three groups studied (results not shown).

4. Discussion

4.1. In vitro study

These results have shown for the first time the oxidation potential of the three major lipoproteins (VLDL, LDL and HDL) when isolated from plasma preincubated with increasing concentrations of either α- or γ-tocopherol at 4°C and 37°C. Normally, investigators study the antioxidant properties of tocopherol following their isolation from plasma [31,32]; however, this may essentially be erroneous, since in this situation, the tocopherols are located in the aqueous environment surrounding the lipoproteins and not incorporated within the lipoproteins. Therefore, and for this reason, as well as repeating postincubated experiments, this study examined the oxidation potential of VLDL, LDL and HDL when isolated from plasma following its incubation with tocopherol, a procedure that may better reflect the *ex vivo* situation following tocopherol supplementation, therefore, being more physiologically relevant. In accordance with other researchers [32,33], this study found that VLDL, LDL and HDL were protected against oxidation when the tocopherols were added after isolation.

During the preincubated experiments, VLDL, the largest of the endogenous lipoproteins, had the greatest capacity to incorporate α- and γ-tocopherols, and this incorporation completely protected VLDL against oxidation when isolated from plasma preincubated at 4°C, which was in keeping with Arai et al. [34], who also used this incubation temperature; however, and possibly due to their prolonged 24-h incubation step, this group found that the incorporation did not completely prevent the peroxidation process. Regarding VLDL isolated from plasma preincubated with tocopherol, at 37°C, the results described here demonstrate that VLDL was only protected against oxidation when the tocopherols were at their highest concentration (100 nmol tocopherol/mg protein). This suggests that either the lipoprotein had become auto-oxidized within the plasma prior to its isolation, which was not the case, as conjugated dienes and HPOs were unaffected during the incubation period (results not shown), or the tocopherols themselves had become less efficient at this temperature; this latter factor may relate, in part, to why intervention trials have shown tocopherol to be inefficient in reducing cardiovascular events. No study, to the author's knowledge, has investigated the role of γ-tocopherol during VLDL oxidation; therefore, this study has shown for the first time that γ-tocopherol functions similarly to α-tocopherol. However, although VLDL had the capacity to incorporate more γ-tocopherol than α-tocopherol, this incorporation did not correlate with its degree of protection toward VLDL oxidation, showing that α-tocopherol was more efficient, which, in turn, may be related to their chemical structures, as α-tocopherol contains an additional methyl group on its chromanol ring and thus has the ability to donate additional phenolic hydrogens to free radicals and is therefore a more potent electron-donating molecule [35,36].

Similarly to VLDL, incorporation of tocopherol into LDL translated as oxidative protection, and again, this protection was less marked when plasma was preincubated at 37°C. Although to the author's knowledge, no studies have investigated the role of tocopherol in protecting LDL against oxidation when isolated from plasma preincubated at 4°C, the protection afforded to LDL from preincubated plasma at 37°C was in agreement with other researchers [37,38] who, similarly to the results presented here, found that tocopherol protected this lipoprotein against oxidation. Another novel aspect of this work was the inclusion of γ-tocopherol during LDL oxidation; however, although more γ-tocopherol became incorporated into LDL, both tocopherols afforded a similar level of protection toward oxidation, which again suggests that α-tocopherol was the more efficient antioxidant. Overall, the results for LDL suggest that preincubating plasma at 37°C leads to a reduction in the antioxidant activity of the tocopherols, which again may assist in our understanding of the lack of cardioprotective effect following tocopherol supplementation.

Interestingly, at both temperatures, the *in vitro* oxidation of HDL isolated from plasma preincubated with either α- or γ-tocopherol demonstrated a significant decrease in its time at half maximum, indicating that the tocopherols exhibited an overt pro-oxidant effect. In the last decade, the study of the antiatherogenic potential of HDL has become a hugely important field with the cardioprotective attributes of HDL documented in nearly every step of the atherosclerotic process [39], most importantly HDLs involvement in the 'reverse cholesterol transport' pathway [23]. Consequently, even mildly oxidized HDL has a diminished capacity to accept cellular cholesterol for removal, and furthermore, oxidized HDL loses its capacity to perform other protective antiatherosclerotic actions [24,40,41]; therefore, these results have important clinical implications in that they may help explain the contradictory and inconclusive outcomes of the many clinical trials with vitamin E [5–10].

These *in vitro* results show that the two tocopherols may function differently, depending on the environment in which they are found and in which lipoprotein they are associated with, and although tocopherol supplementation improves VLDL and LDL oxidation, any

beneficial effect that this may have is negated by the detrimental effects shown during HDL oxidation.

4.2. Ex vivo study

The *ex vivo* study was designed to assess the effect of a 4-week dietary supplement of either α or γ-tocopherol or placebo on the susceptibility of VLDL, LDL and HDL to oxidation and to examine if there was any alteration in HPO levels.

4.3. Ex vivo serum levels of α- and γ-tocopherols

Serum analysis of α- or γ-tocopherol confirmed subject compliance; results demonstrated a significant increase in the tocopherol corresponding to the supplement taken. Those subjects taking placebo showed no change in serum levels of either tocopherol.

4.4. Ex vivo α- and γ-tocopherol levels within lipoproteins

Following the 4-week supplementation with α-tocopherol, there was a substantial increase in α-tocopherol levels within VLDL, LDL and HDL. Following the γ-tocopherol supplementation, there was a significant increase in γ-tocopherol levels within all lipoproteins; however, surprisingly in LDL, there was also a substantial increase of α-tocopherol. These results suggest that supplementation with γ-tocopherol produced an increase in the uptake of α-tocopherol into the lipoprotein particle from other dietary sources. The studies of Traber and Kayden [42] demonstrated a similar finding; their study investigated the effects of ingestion of a single dose of both α- and γ-tocopherols and found that 12 h following ingestion, plasma and lipoproteins contained equal quantities of each tocopherol, but by 24 h, there was a sharp decline in the concentration of γ-tocopherol. They concluded that in the presence of γ-tocopherol, α-tocopherol is preferentially secreted by the liver in nascent lipoprotein particles and that α-tocopherol transfer protein is responsible for this preferential incorporation. Therefore, these results indicate that increased γ-tocopherol levels caused enhanced uptake of α-tocopherol into LDL. This finding may help explain why *in vivo* studies have demonstrated that γ-tocopherol intake is more closely associated with a reduction in cardiovascular disease [13, 14]. Supplementing subjects with γ-tocopherol may in fact result in a change in the distribution of the tocopherols within endogenous lipoproteins and may lead to enhanced uptake of α-tocopherol into LDL, which in turn may assist in reducing cardiovascular disease risk, although this remains to be confirmed in randomized controlled clinical trials.

4.5. Ex vivo oxidation potential

With regard to VLDL, only in the α-tocopherol-supplemented group was the oxidation potential of VLDL significantly decreased. No previous research has examined the resistance of VLDL to oxidation when supplemented with either α- or γ-tocopherol, although Porkkala-Sarathaho et al. [43] were able to show that the oxidative resistance of a mixture of VLDL+LDL was increased when supplemented with 200 mg α-tocopherol/day; however, it is feasible that this increase was enhanced due to the presence of LDL within the mixture. During LDL oxidation, both tocopherols afforded oxidative protection. However, the effect shown in the γ-tocopherol-supplemented group may not be directly due to γ-tocopherol alone but may be due to an additive effect of both α- and γ-tocopherols within the LDL; as described above, both tocopherols increased in LDL following γ-tocopherol supplementation. These LDL results are consistent with many of the vitamin E supplementation trials, showing that vitamin E (in particular, α-tocopherol) leads to an increase in the resistance of LDL to oxidation [44,45]; however, this is not enough to translate into clinical improvements in atherosclerosis, as the beneficial effects afforded to VLDL and LDL may be counteracted by the detrimental effects on the important antiatherogenic lipoprotein, HDL as discussed below. Regarding the oxidation potential of HDL, in agreement with the *in vitro* results, HDL from both α- and γ-tocopherol-supplemented groups displayed a decrease in the time at half maximum (although this was only significant in the γ-tocopherol group), indicating a negative effect of tocopherol supplementation (i.e., pro-oxidant activity). Few studies have examined the oxidation potential of tocopherol supplementation on HDL. However, one trial coadministered vitamins C and E (DL-α-tocopherol acetate) to the subjects for 10 days and showed that this mixture of antioxidants inhibited the *ex vivo* oxidation of HDL [25]. It is difficult to attribute this antioxidant property to the tocopherol alone, as it may be due to vitamin C's synergistic relationship with vitamin E. Another trial supplemented healthy volunteers with either 200 or 400 mg of vitamin E/day (DL-α-tocopherol) and showed that both vitamin E concentrations protected HDL against oxidation [45]. However, this investigation used vastly different oxidation conditions to those presented here, including a very low Cu^{2+} concentration (1.66 pM), which would stimulate the oxidation process by a totally different kinetic mechanism [46] to that of the higher Cu^{2+} concentration used in the study described here. In agreement with the current findings, Raveh et al. [46] also demonstrated that supplementation with α-tocopherol was detrimental to HDL oxidation. Therefore, although the *ex vivo* results were in disagreement with the first two studies, they do confirm the *in vitro* findings and those of Raveh et al. [46]. The results described here indicate that supplementation with vitamin E may be producing a proinflammatory or dysfunctional HDL, which several lines of evidence suggest will contribute to the pathophysiology of cardiovascular disease. Further research would be required to investigate if and which region of the HDL particle had become dysfunctional; however, from these results, it is noticeable that the HDL, after supplementation with vitamin E, is not functioning as an efficient antioxidant. Thus, these results again reiterate a possible explanation as to why large-scale supplementation studies with vitamin E have, to date, been inconclusive in demonstrating a beneficial or cardioprotective effect [8–10].

5. Conclusion

These results demonstrated that α- and γ-tocopherols displayed conflicting antioxidant activities, which was dependent on the lipoprotein being oxidized. Equally, in both the *in vitro* and *ex vivo* situations, both tocopherols protected VLDL and LDL against oxidation; however, both tocopherols displayed pro-oxidant properties during HDL oxidation. These results may have physiological implications in that HDL is suggested to be responsible for the majority of reverse cholesterol transport in humans and is considered to be an atheroprotective molecule; however, when it becomes dysfunctional, for example, by oxidation, it loses these protective properties and may instead yield detrimental effects. These results have helped tease out possible mechanisms whereby tocopherol is ineffective during large-scale supplementation studies in bringing about cardioprotective benefits.

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