RESEARCH COMMUNICATION

Evidence for the nitration of γ -tocopherol *in vivo*: 5-nitro- γ -tocopherol is elevated in the plasma of subjects with coronary heart disease

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This study investigated the hypothesis that nitration of γ -tocopherol may be an important mechanism for the detoxification of reactive nitrogen oxide species *in vivo*. Using liquid chromatography–tandem MS we have shown that γ -tocopherol can be nitrated *in vivo* to form 5-nitro- γ -tocopherol and that concentrations of this compound are elevated in the plasma of subjects with coronary heart disease. In addition, we demonstrate in

carotid-artery atherosclerotic plaque that nitration of γ -tocopherol is also evident at levels similar to that seen in the plasma of subjects with coronary heart disease.

Key words: antioxidant, atherosclerotic plaque, carotid artery, reactive nitrogen oxide species, vitamin E.

INTRODUCTION

The role of reactive nitrogen oxide species (RNOS) in human disease states characterized by inflammation, such as coronary atherosclerosis, has largely been obtained from the quantification of 3-nitrotyrosine in diseased tissues [1]. Recent evidence has suggested that one form of the lipid-soluble antioxidant vitamin E (γ -tocopherol) can scavenge membrane soluble RNOS, an activity that is not shared by the other major form of vitamin E, α -tocopherol [2]. This has led to speculation that nitration of γ -tocopherol may be an important mechanism for detoxification of RNOS *in vivo* [2]. An *in vivo* role for such a mechanism in coronary atherosclerosis is supported by the observation that patients with coronary heart disease have a higher α -tocopherol-to- γ -tocopherol ratio than healthy controls [3–5], but no difference in α -tocopherol, suggesting selective depletion of γ -tocopherol. The chemical structure of γ -tocopherol may afford a unique biochemical role in preventing the initiation or progression of diseases involving inflammation by acting as a renewable substrate for nitration by RNOS in endogenous lipid structures.

Vitamin E is the major lipid-soluble dietary antioxidant and occurs in two structurally related groups, namely the tocopherols and tocotrienols. The tocopherol forms differ from each other in the substitution on the chromanol ring. For example, α -tocopherol is fully substituted on the chromanol ring, while the other major dietary form, γ -tocopherol, is unsubstituted at the 5 position. This difference leads to different chemical reactivity, such that α -tocopherol is a stronger antioxidant than γ -tocopherol. However, unlike γ -tocopherol, α -tocopherol cannot trap damaging species such as RNOS produced by various cells [2]. Although γ -tocopherol is not stored in the body at the same concentration as α -tocopherol, this unique property of being able to trap RNOS may make it important for human health [6].

There is evidence that nitration could occur in the lipid phase. NO partitions substantially in favour of the lipid phase, as do other gaseous molecules [7,8]. Thus oxidation of NO to higher oxides has been demonstrated by Liu et al. [7] to require a

discrete lipid phase in which oxygen and NO may co-localize and interact to produce more reactive, nitrogen-based compounds at a rate which is greatly accelerated compared with the aqueous phase. Cooney et al. [9] demonstrated that NO₂, also a lipid-soluble species, was sequestered through nitration of γ -tocopherol, which was superior to α -tocopherol in the detoxification of NO₂. The co-existence of γ -tocopherol and NO in the lipid phase of cellular membranes implicates oxidation of NO as a source of RNOS and γ -tocopherol nitration as a means of detoxification of such species.

The nitration of γ -tocopherol has been observed *in vitro* by treatment of lipoproteins with peroxynitrite and in lipopolysaccharide-stimulated rat astrocytes [2,10]. We decided to investigate the possible existence of 5-nitro- γ -tocopherol in human plasma and atherosclerotic tissue. We now show that γ -tocopherol can be nitrated *in vivo* to form 5-nitro- γ -tocopherol and that this nitration product of γ -tocopherol is elevated in the plasma of subjects with coronary disease. In addition, we demonstrate, in carotid-artery atherosclerotic plaque, that nitration of γ -tocopherol is also evident at levels similar to that seen in plasma of subjects with coronary heart disease.

EXPERIMENTAL

Materials

α -, β - and γ -tocopherol and α -tocopherol acetate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), whereas NaNO₃ was obtained from Ajax Chemicals (Sydney, Australia). All solvents were HPLC-grade and were distilled before use.

Synthesis of 5-nitro- γ -tocopherol and 7-nitro- β -tocopherol

The method of Christen et al. [2] was used with some alterations. Briefly, to an ethanolic solution of the tocopherol (20 ml, 1 mg/ml) was added two mol-equiv. of NaNO₃ in water (2%, w/v) and glacial acetic acid (4%, v/v, final vol.). The solution was manually mixed and left for 10 min. Water (a quarter of the

Abbreviations used: RNOS, reactive nitrogen oxide species; LC-UV, liquid chromatography with UV detection; LC-MS/MS, liquid chromatography–tandem MS.

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final volume) was added and the mixture was extracted with hexane (5×30 ml). The organic extract was washed with water (2×30 ml), dried (using anhydrous MgSO_4) and the solvent was evaporated to apparent dryness. The bright orange oil which remained was purified by reverse-phase HPLC using methanol as the mobile phase. The solution was dried under vacuum with heating. The residue was taken up into diethyl ether, dried again, and left under vacuum at room temperature for 36 h. The yield was approx. 75%.

Standard curves

Standard curves were prepared for α - and γ -tocopherol using α -tocopherol acetate as the internal standard and liquid chromatography with UV detection (LC-UV) as the method for quantification. α -Tocopherol acetate (75 nmol) was added to each tocopherol in the range 0–200 nmol. A plot of the response of the tocopherol relative to the response of the internal standard was obtained and used to quantify sample concentrations. For 5-nitro- γ -tocopherol, the internal standard used was 7-nitro- β -tocopherol (25 pmol), since it is isomeric and should exhibit similar properties to 5-nitro- γ -tocopherol. A standard curve in the range 0–500 pmol was established. A detection limit of 5 pmol injected on to the column was observed for 5-nitro- γ -tocopherol.

Sample preparation

Blood from fasting subjects was collected into EDTA-treated tubes, centrifuged (3000 g, 10 min), transferred to a fresh tube and stored (-80°C) until analysis. Upon thawing, internal standards (75 nmol of α -tocopherol acetate, 25 pmol of 7-nitro- β -tocopherol) were added to plasma (1 ml) and this was added to methanol (5 ml). The samples were mixed vigorously for 30 s, water was added (2 ml) and the mixing was repeated. Heptane (2 ml) was added, mixed for 1 min using a vortex mixer, and the heptane layer was obtained after brief centrifugation. Samples were dried under vacuum and reconstituted in ethyl acetate (40 μl). Plaque samples were extracted as previously described [11]. The internal standard was added to a portion of the extract, which was then analysed as for plasma samples.

LC-UV tandem MS (LC-UV-MS/MS) analysis

Separation of 8 μl sample volumes was performed with an Agilent LiChrosphere C_{18} column (125 mm length $\times$ 4 mm column diameter; 5 μm pore size) using a mobile phase which commenced at 100% methanol for 12 min and was modified to 100% ethyl acetate over 7 min. This was maintained for 10 min and then returned to 100% methanol over 5 min. Equilibration required 4 min. Solvent flow was constant at 400 $\mu\text{l}/\text{min}$. UV detection of tocopherols was achieved using absorbance at 296 nm. The (negative mode) electrospray nebulizer of the Agilent MSD Trap mass spectrometer was maintained at 276 kPa (40 lbf/in²) and the drying gas at 350°C and 12 litres/min. Detection of 7-nitro- β -tocopherol and 5-nitro- γ -tocopherol was achieved by selecting and fragmenting (amplitude = 0.8) the $M-1$ ion (m/z 460) to produce the product ion (m/z 194). All quantification was performed using the peak due to the product ion.

Subjects

Subjects were recruited from the general population and the study had the approval of the Ethics Committee of Royal Perth Hospital. All subjects were non-smokers and were free of any

antioxidant supplements for at least 1 month prior to the study. The coronary-disease group were all treated hypertensives; six had hypercholesterolaemia, two had non-insulin-dependent diabetes. Four subjects had previous by-pass surgery or angioplasty for blocked coronary arteries, two subjects had previous myocardial infarction and one subject transient ischaemic attacks. No subjects were taking nitrates. Control subjects had no risk factors for, or clinical evidence of, cardiovascular disease. The mean age of the subjects with coronary disease was 67.7 ± 4.0 years and for the control subjects 50.8 ± 3.2 years. Subjects were matched for body/mass index (coronary disease, 27.6 ± 1.6 ; controls, 26.7 ± 0.8 kg/m²).

RESULTS AND DISCUSSION

We first detected 5-nitro- γ -tocopherol in isolated human low-density lipoproteins using HPLC with electrochemical detection (L. W. Morton, unpublished work). The primary problem with this technique is that it is not possible to be entirely certain of the identity of the peak on the chromatogram. Using LC-MS/MS, a technique combining the selectivity and sensitivity of MS with the resolving power of LC, we have now confirmed the existence of endogenous 5-nitro- γ -tocopherol for the first time in human plasma.

Figure 1 Mass spectrum (A) and fragmentation pattern (B) of 5-nitro- γ -tocopherol

From a molecular mass of 461, a proton is lost during electrospray ionization to generate the $[M-1]^-$ ion, which is selected and fragmented to generate the product ion (m/z 194) used for quantification.

Figure 2 LC/MS/MS chromatogram of (A) standards of 7-nitro- β -tocopherol and 5-nitro- γ -tocopherol, (B) human plasma extract and (C) plaque lipid extract, showing the measurement of the internal standard 7-nitro- β -tocopherol and 5-nitro- γ -tocopherol

Standards and extracts were separated by reverse-phase HPLC before analysis using tandem MS in which the $[M-1]^-$ ion at m/z 460 is isolated and fragmented to the major product ion m/z 194.

An authentic standard of 5-nitro- γ -tocopherol was prepared by chemical nitration of γ -tocopherol. This enabled us to establish the mass spectra and LC retention time of this com-

pound. From a molecular mass of m/z 461 this compound loses a proton in electrospray ionization to generate a $[M-1]^-$ ion, which can be selected and further fragmented to generate the product ion (m/z 194) due to fission of the heterocyclic ring (Figure 1). This characteristic fragmentation of 5-nitro- γ -tocopherol provides the basis for its unequivocal detection in lipid extracts from plasma and plaque tissue. Although we also detected 5-nitro- γ -tocopherol in the low-density-lipoprotein fraction of human plasma, we have not yet attempted to determine its natural distribution throughout other lipoprotein fractions. This could provide information as to whether certain lipoprotein classes might be specifically targeted by RNOS.

In order to quantify the concentration of 5-nitro- γ -tocopherol in plasma, and due to the lack of commercially available stable-isotope-labelled standards, we used 7-nitro- β -tocopherol as an internal standard. Since plasma does not contain detectable levels of 7-nitro- β -tocopherol, this compound was added as an internal standard to each sample. Although it shares common fragmentation pathways with 5-nitro- γ -tocopherol in LC-MS/MS, the two are separated by reverse-phase HPLC. Figure 2 shows clear separation of the internal standard and 5-nitro- γ -tocopherol on chromatograms obtained from standards, a lipid extract of human plasma and plaque tissue for the specific mass transition m/z 460 to 194. Using this method we report data obtained from seven male subjects with coronary artery disease compared with nine normal healthy male controls. As well as quantification of 5-nitro- γ -tocopherol, the plasma concentration of α - and γ -tocopherols was simultaneously determined by inline UV detection. Table 1 shows the characteristics of the subjects and controls together with the concentration of α - and γ -tocopherol, tocopherol ratio and the level of 5-nitro- γ -tocopherol expressed per mol of γ -tocopherol. The levels of 5-nitro- γ -tocopherol in subjects with coronary disease were more than double that of controls ($P < 0.001$). The α -tocopherol-to- γ -tocopherol ratio was also slightly elevated in subjects with coronary disease, but this did not reach statistical significance ($P = 0.09$).

To determine whether this apparent elevation in γ -tocopherol nitration was reflected in atherosclerotic tissue, carotid plaque samples were analysed for 5-nitro- γ -tocopherol content and the tocopherol ratio was calculated. It was found that γ -tocopherol nitration was present and in some individuals, there was more 5-nitro- γ -tocopherol than γ -tocopherol. The molar ratio of 5-nitro- γ -tocopherol to γ -tocopherol is almost 1:2 (Table 1). There was a similar tocopherol ratio in plaque and plasma consistent with no particular depletion of γ -tocopherol in plaque compared with plasma. Suarna et al. [12] determined α - and γ -tocopherol levels in 11 carotid plaques and noted that α -tocopherol was not depleted compared with normal human plasma. From their data a mean α -tocopherol-to- γ -tocopherol ratio of approx. 26 can be calculated, which is similar to the ratio observed in plaque samples from our study. The level of

Table 1 Measures of γ -tocopherol nitration and tocopherol ratio in plasma and carotid plaque

The 5-nitro- γ -tocopherol and α - and γ -tocopherol levels were assessed using LC-UV-MS/MS. The tocopherol ratio was calculated from individual data as the molar ratio α -tocopherol/ γ -tocopherol and represents a measure of depletion of γ -tocopherol. Similarly, the degree of nitration of γ -tocopherol was calculated relative to the parent molecule. Subject characteristics are outlined in the Experimental section. Results are means $\pm$ S.E.M. * $P < 0.001$ Coronary disease versus control.

Subject group	$[\alpha\text{-Tocopherol}]$ ($\mu\text{mol/litre}$)	$[\gamma\text{-Tocopherol}]$ ($\mu\text{mol/litre}$)	$\alpha\text{-Tocopherol-to-}$ $\gamma\text{-tocopherol ratio}$	5-Nitro- γ -tocopherol/ γ -tocopherol (mmol/mol)
Coronary disease ($n = 7$)	21.0 (2.2)	0.88 (0.13)	26.3 (3.0)	487 (54)*
Controls ($n = 9$)	21.3 (2.4)	1.03 (0.12)	21.4 (1.6)	163 (20)
Carotid plaque ($n = 8$)	—	—	19.1 (5.0)	460 (134)

γ -tocopherol nitration in the plasma of subjects with coronary disease appears to reflect that seen in atherosclerotic plaque and may reflect the degree of disease in the arterial wall.

The strength of this relationship clearly needs to be tested in a larger cohort, but certainly indicates the potential of 5-nitro- γ -tocopherol as a marker of RNOS in subjects with heart disease. These data have substantial implications in the field of human health. There is a growing body of evidence that γ -tocopherol may be substantially more important than previously thought in homeostasis, yet recommended dietary intakes have not yet been established [6]. The epidemiological relationship of coronary heart disease and cancer with fruit and vegetable intake [13] supports the notion that components of these foodstuffs are biologically active in more complex mechanisms than are currently understood. We propose that γ -tocopherol could be a central component of these diets, which connects the diet to the epidemiological evidence. We have demonstrated that γ -tocopherol becomes nitrated and that elevated plasma concentrations of 5-nitro- γ -tocopherol in subjects with coronary heart disease appear to reflect the levels seen in atherosclerotic plaque. It is possible that nitration prevents γ -tocopherol from carrying out other physiological function(s) such as inhibition of cyclooxygenase [14], and this could be crucial in many disease states where inflammation is a characteristic. The nitration of γ -tocopherol in carotid plaque, a focal point of inflammation, is an important observation, and the consequences of this, in terms of onset and/or progression of a number of inflammation-based disease states, are yet to be established.

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