methods

Analysis of plasma tocopherols α, γ, and 5-nitro-γ in rats with inflammation by HPLC coulometric detection

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Abstract Reactive nitrogen oxide species (RNOS) have been implicated as effector molecules in inflammatory diseases. There is emerging evidence that γ-tocopherol (γT), the major form of vitamin E in the North American diet, may play an important role in these diseases. γT scavenges RNOS such as peroxynitrite by forming a stable adduct, 5-nitro-γT (NGT). Here we describe a convenient HPLC method for the simultaneous determination of NGT, αT, and γT in blood plasma and other tissues. Coulometric detection of NGT separated on a deactivated reversed-phase column was linear over a wide range of concentrations and highly sensitive (~10 fmol detection limit). NGT extracted from blood plasma of 15-week-old Fischer 344 rats was in the low nM range, representing ~4% of γT. Twenty-four h after intraperitoneal injection of zymosan, plasma NGT levels were 2-fold higher compared to fasted control animals when adjusted to γT or corrected for total neutral lipids, while α- and γT levels remained unchanged. These results demonstrate that nitration of γT is increased under inflammatory conditions and highlight the importance of RNOS reactions in the lipid phase. The present HPLC method should be helpful in clarifying the precise physiological role of γT.—Christen, S., Q. Jiang, M. K. Shigenaga, and B. N. Ames. **Analysis of plasma tocopherols α, γ, and 5-nitro-γ in rats with inflammation by HPLC coulometric detection. *J. Lipid Res.* 2002.** 43: **1978–1985.**

Supplementary key words reactive nitrogen oxide species • nitration • vitamin E

Reactive nitrogen oxide species (RNOS) have been implicated as important effector molecules in inflammatory diseases (1, 2). On the other hand, antioxidants such as vitamin E have been proposed to modulate the effects mediated by RNOS and thus provide protection against inflammation-induced pathology.

Vitamin E is a family of lipid-soluble chain-braking antioxidants that share a similar chromanol ring structure (**Fig. 1**). Members of this family differ only in the number and position of methyl substitutents on the phenolic ring, and the degree of saturation of the phytyl side chain. The two principal forms of vitamin E in the diet and tissues are α-tocopherol (αT, 2) and γ-tocopherol (γT, 1). αT is the most effective antioxidant in vitro (3) and has the highest bioactivity in vivo among the different vitamers (4). Several human intervention studies have been conducted to demonstrate, despite generally inconclusive results, a protective effect of αT in inflammatory diseases, such as atherosclerosis (5). However, a growing body of evidence suggests that γT may also play an important role in these diseases (6).

In a seminal study, Cooney et al. found that γT is superior to αT in the detoxification of nitrogen dioxide (7). We later demonstrated that the reaction of γT with RNOS such as peroxynitrite is fundamentally different to that of αT (8). Thus, while RNOS oxidize αT near-quantitatively to the para-quinone, α-tocopheryl quinone, the major reaction product of γT with RNOS is the nitro-phenol, 5-nitro-γ-tocopherol (NGT, 3) (Fig. 1). Most importantly, nitration of γT is not affected by the simultaneous presence of αT, suggesting that the two forms of vitamin E may complement each other (8). Based on this observation, we speculated that γT is a target for nitration in vivo and may be involved in the detoxification of RNOS. To be able to test this hypothesis, we developed a highly specific and sensitive HPLC method for the detection of NGT in biological samples. The method is based on isocratic reversed-phase separation of extracted tocopherols and electrochemical detection using a dual-electrode coulometric cell. Using a zymosan-induced peritonitis model in rats, we found that NGT is present in normal blood plasma at a level of a few percent of its parent, γT, and is

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Abbreviations: αT, α-tocopherol; BHT, butylated hydroxytoluene; Ch18:1, cholesteryl oleate; Ch18:2, cholesteryl linoleate; Ch20:4, cholesteryl arachidonate; FC, free cholesterol; γT, γ-tocopherol; NGT, 5-nitro-γ-tocopherol; TC, total cholesterol; TG, triglycerides; TNL, total neutral lipids.

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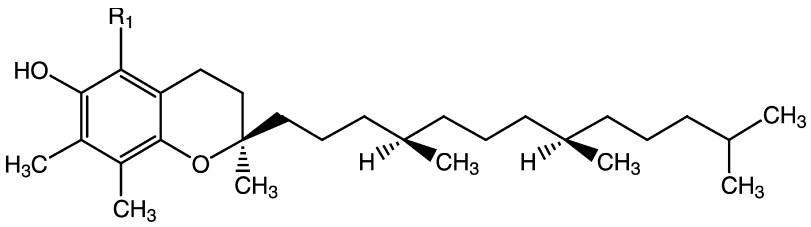

1: $R_1 = H$ (γ-tocopherol, γT)
2: $R_1 = CH_3$ (α-tocopherol, αT)
3: $R_1 = NO_2$ (5-nitro-γ-tocopherol, NGT)

Fig. 1. Structures of tocopherols analyzed in this study.

increased by 2-fold during acute inflammation. This relatively simple and highly specific HPLC method should be useful in clarifying the precise physiological role of γT.

MATERIALS AND METHODS

Materials

αT and γT (purity >99.7%) were from Fluka. Their concentration was determined using $\varepsilon 294\ nm^{EtOH} = 3{,}265\ M^{-1}\ cm^{-1}$ and $\varepsilon 298\ nm^{EtOH} = 3{,}810\ M^{-1}\ cm^{-1}$ (9), respectively. Butylated hydroxytoluene (BHT) and lithium acetate were from Aldrich, zymosan A from Sigma. Sodium dodecyl sulfate (SDS) was from US Biological and purified with hexane (10). All aqueous solutions were made with analytical-grade water prepared using a Milli-Q water purification system (Millipore). All solvents used were HPLC grade (Fisher Scientific), except for hexane (GC quality, from J. T. Baker) and ethanol [reagent grade, containing 5% (v/v) isopropanol, Fisher Scientific]. Mobile phases were filtered through 0.22 μm Durapore membranes (Millipore), degassed by sonication, and kept oxygen-free by continuous sparging with helium gas when in use.

Zymosan peritonitis model

Animal experiments were approved by the UC Berkeley Animal Care and Use Committee. In addition, experiments were carried out according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male Fischer 344 rats obtained from Simonsen Laboratories (Gilroy, CA) were used at 15-weeks-of-age (~320 g body weight) after acclimatization for 7 weeks on a standard Purina 5011 chow diet (Dyets, Bethlehem, PA). Peritonitis was induced as previously described (11). Briefly, zymosan A prepared in PBS and treated for 30 min at 95°C was injected intraperitoneally at 250 mg/kg body weight. Control animals received nonpyrogenic PBS. Since this treatment causes a marked decline in food consumption (12), one control group consisted of animals fasted for the entire duration of the experiment. Twenty-four h after injection of zymosan, animals were anesthetized under ether and sacrificed by cervical dislocation after blood removal by subclavicular artery section. Heparinized plasma and excised tissues were stored immediately at −80°C until analysis.

Synthesis of 5-nitro-γT

NGT (2,7,8-trimethyl-2-[4′,8′,12′-trimethyltridecyl]-5-nitro-6-chromanol) was synthetized by the nitrous acid method as described previously (8). Briefly, an ethanolic solution of γT (1 mg/ml) was acidified with 0.04 vol glacial acetic acid and nitration induced by the addition of 0.6 vol of a 2% sodium nitrite solution. After 2 min, the reaction was stopped with 0.4 vol of 20% potassium hydroxide. Two volumes of water were added, the crude product extracted into hexane and further purified by semi-preparative reversed-phase HPLC using 100% methanol as the eluent, and peak-collected using diode-array detection (HP 1090). The concentration of the pooled fractions was determined using $\varepsilon 410\ nm^{EtOH} = 1{,}976\ M^{-1}\ cm^{-1}$ (13). The purity of the product was >99%.

Extraction procedure

NGT and all other tocopherols were extracted from plasma into hexane based on previously published procedures (14, 15). It is important that the extraction mixture has not been acidified, since even weak acidic conditions promote the nitration of γT when nitrite is present (cf. NGT synthesis). Generally, 0.2 ml of blood plasma was extracted with 7 ml of a 2:5 mixture (v/v) of methanol-hexane to which 20 μl of 0.1 M BHT was added. BHT is necessary to inhibit the formation of substances that interfere with NGT analysis. The hexane phase was evaporated under a stream of high-purity nitrogen gas. Extracts were redissolved in 160 μl reagent grade ethanol and immediately subjected to HPLC analysis. For low NGT levels, 0.4 ml of blood plasma can be extracted with double the volume of methanol-hexane, but redissolving in the same volume of ethanol. Although we recommend that extracts are analyzed immediately, they can be stored at −20°C for a few hours, without noticeable changes in analyte concentrations.

HPLC analysis

The HPLC system consisted of a Hitachi (Tokyo, Japan) L-6200 gradient pump and a Hitachi L-7200 autosampler with built-in Peltier cooling unit set to 2°C. The electrochemical detection system consisted of a Model 5200A Coulochem II controller (ESA Inc., Chelmsford, MA) and a Model 5011 high sensitivity analytical cell. A membrane pulse damper (SSI) and Model 5020 guard cell were installed between the pump and the autosampler. The whole system was assembled with PEEK tubing and passivated with 20% nitric acid prior to use. UV detection was performed on a Spectroflow 757 detector (Applied Biosystems) connected in series after the electrochemical cell.

NGT was separated from the other tocopherols on a 15 × 0.46 cm Supelcosil™ (Supelco, Bellefonte, PA) LC-18-DB column (deactivated octadecyl silane, 3 μm particle size) equipped with an LC-8 precolumn cartridge assembly and eluted isocratically with 95:5 (v/v) methanol-0.5 M lithium acetate (pH 4.75) at a flow rate of 1.3 ml/min. Generally, 50 μl of extract was injected. β-tocopherol is not separated from γT under reversed-phase conditions, but constitutes only a minor fraction (<10%) in blood plasma and other tissues of rats (16) and humans (17). Also, 7-nitro-β-tocopherol elutes before NGT under reversed-

phase conditions (18). After the first 20 min, non-polar lipids and other interfering substances were washed off the column by running a linear gradient from the initial mobile phase to *tert*-butanol-methanol (50:50, v/v) within 20 min. After this step, initial conditions were restored within another 20 min. Coulometric detection was performed at +300 (E1; upstream) and +500 mV (E2; downstream electrode), unless indicated otherwise. Free cholesterol (FC) was monitored at 210 nm and used as an internal standard in the recovery studies.

Lipid analysis

The quantitatively major cholesterol esters, cholesteryl oleate (Ch18:1), cholesteryl linoleate (Ch18:2), and cholesteryl arachidonate (Ch20:4) were determined together with FC in hexane extracts by HPLC with UV detection (15). Total triglycerides (TG) were determined enzymatically (19) using a commercial kit (Stanbio Laboratories, San Antonio, TX).

Statistical analysis

Statistical tests were performed using GraphPad Prism (San Diego, CA). Comparisons between the different groups were done by using the unpaired Student's t-test. A two-tailed P value <0.05 was considered statistically significant. All results are expressed as mean ± SD.

RESULTS

HPLC separation and electrochemical detection

Separation of NGT was achieved by slight modification of methods described previously for analyzing unmodified tocopherols (10, 15). NGT is conveniently separated on reversed-phase columns using short-chain alcohols as solvent, as are all other tocopherols (20). **Figure 2** shows a typical chromatogram of authentic tocopherols resolved on a deactivated octadecylsilane column using a mobile phase containing methanol/water. NGT is retained much longer under reversed-phase conditions than its parent compound, similar to the situation with 3-nitrotyrosine. A deactivated column was required for adequate separation of biological samples. The cleaning cycle with *tert*-butanol/methanol after each injection was necessary for maintaining the high sensitivity of the assay.

Electrochemical detection was chosen for analysis, because NGT is, in contrast to αT and γT, not fluorescent. Initially, we tried to chemically (using dithionite, borohydride, or Sn-borohydride under phase transfer conditions) or electrochemically reduce NGT to 5-amino-γT, a strategy previously employed for the detection of 3-nitrotyrosine (11, 21, 22), since nitration is known to increase the oxidation potential of phenolic compounds, and aminophenols are much more electrochemically active. However, none of these approaches nor UV photolysis (21) was successful. We therefore chose to analyze NGT directly in the oxidative mode. To achieve the highest possible sensitivity, we used coulometric detection using a high-sensitivity dual-electrode. One of the main advantages of using electrodes in series is that the near-quantitative conversion efficiency of coulometric detection can be used to selectively filter out interfering substances. Although amperometric detection can be readily used for measuring

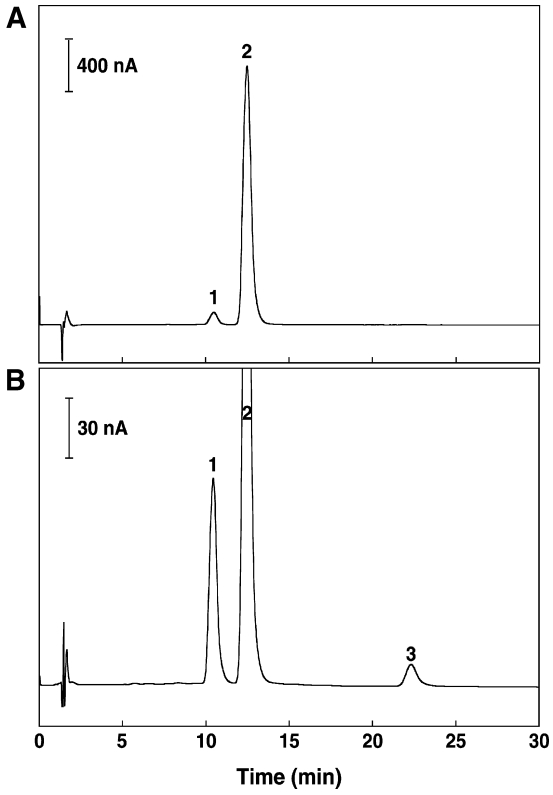

Fig. 2. Chromatogram of a standard mixture of tocopherols using coulometric detection. A methanolic mixture of 16.6 pmol γ-tocopherol (γT) [1], 225 pmol α-tocopherol (αT) [2], and 3.4 pmol 5-nitro-γ-tocopherol (NGT) [3] was separated on a deactivated C18 column using 95:5 (v/v) methanol-0.5 M lithium acetate (pH 4.75) at a flow rate of 1.3 ml/min as described under Materials and Methods. Eluent was monitored by coulometric detection using a potential of +300 and +500 mV on the upstream (A) and downstream (B) electrode, respectively. After 20 min, the solvent was gradually changed to *tert*-butanol-methanol (50:50, v/v) to remove non-polar lipids and other interfering substances from the column.

NGT in biochemical experiments (8), this type of detection was not sensitive enough to detect basal levels of NGT in biological samples.

Sensitivity of coulometric detection (i.e., high signal to noise ratio) was greatly affected by the choice of electrolyte. Lithium acetate was found to be the best choice. Other electrolytes such as sodium perchlorate, lithium perchlorate, or ammonium acetate commonly used for electrochemical detection-based tocopherol HPLC assays (20) gave inferior results. To determine the ideal working potentials, hydrodynamic voltammograms of NGT, αT, and γT were run. The voltammograms in **Fig. 3** clearly show that nitration increases the oxidation potential of γT. However, in contrast to 3-nitrotyrosine, this potential is still low enough to detect NGT directly in the oxidative mode without increasing background noise to unacceptably high levels. A potential of +300 mV was chosen on the first electrode to oxidize low-potential compounds such as αT, and to allow NGT to be selectively oxidized on the second electrode. A potential of +500 mV on the second electrode was chosen to give a good signal while maintaining high specificity for the analyte. Using these

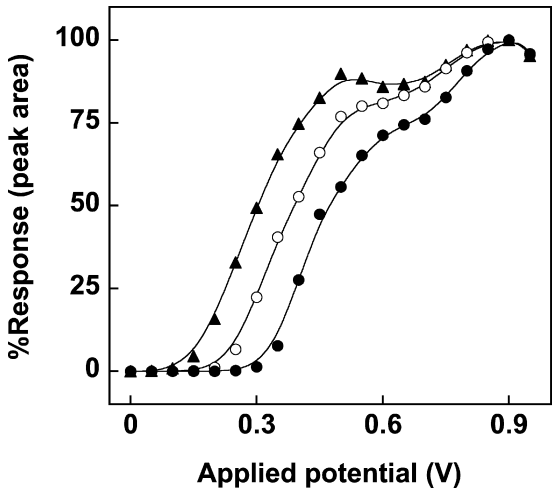

Fig. 3. Hydrodynamic voltammogram of tocopherols. A standard mixture containing 1 nmol each of αT (closed triangles), γT (open circles), and NGT (closed circles) was separated by HPLC as described in the legend to Fig. 2, and the eluant monitored with coulometric detection at increasing potentials up to +950 mV. Hydrodynamic voltammograms were obtained by calculating the percentage of the maximum peak area for each tocopherol and plotting these values versus the applied potential.

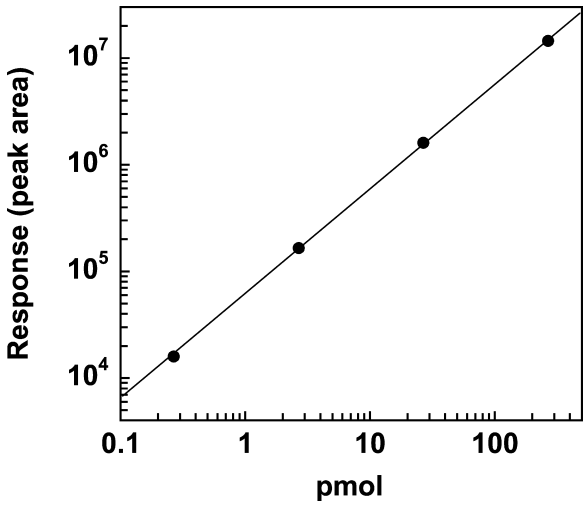

Fig. 4. Standard curve of NGT. NGT dissolved in methanol was analyzed under the same HPLC conditions as described in the legend to Fig. 2. Please note that detector response (peak area) was normalized to a full scale of 100 nA.

settings, detection of NGT was linear over more than four orders of magnitudes (**Fig. 4**, $r^2 = 0.9999$) and highly sensitive (~10 fmol detection limit).

Recovery, reproducibility, and stability

Using the extraction procedure described under Materials and Methods, NGT, γT, and αT were recovered from methanol with an efficiency of 94.6 ± 0.01, 95.3 ± 0.02, and 96.5 ± 0.01% (n = 4). NGT added to rat plasma and extracted several times on the same day was recovered with 84.7 ± 2.1% (n = 6) efficiency (2.5% CV). When the sample was extracted on different days, recovery was 84.6 ± 2.1% (n = 3) (2.5% CV). Repetitive analysis (n = 6) of the same plasma sample for endogenous NGT (~0.01 μM) also had a low CV (5%). NGT added to rat plasma and incubated at 37°C was stable for at least 30 min (data not shown). Addition of physiological concentrations of nitrite (0.1 mM) to rat or human plasma supplemented with γT in vivo and incubation at 37°C did not result in the formation of artefactual NGT (data not shown). Evaporation of extracts by a stream of nitrogen did also not result in unwanted nitration of γT using the extraction procedure described under Materials and Methods. These results demonstrate that the extraction and analysis method described here are highly specific for NGT formed in vivo.

Concentrations of tocopherols in rat blood plasma

We next determined the levels of NGT, γT, and αT in rats injected intraperitoneally with zymosan. This treatment provokes a systemic inflammatory response characterized by bacterial translocation (23) and marked increases in circulating cytokines (24), nitrite/nitrate (11, 25), and 3-nitrotyrosine (11), and a profound decline in antioxidants such as ascorbate (26). Consistent with previous reports (12), zymosan treatment led to a significant decrease in plasma αT (**Table 1**). The same was true for γT. However, this decrease could entirely be attributed to the complete inflammation-induced decline in food consumption, because plasma levels of αT and γT in zymosan-treated animals were identical to those in control animals fasted for 24 h. In contrast, plasma NGT levels were significantly higher in zymosan-treated animals compared to fasted controls. A representative HPLC chromatogram of plasma from a zymosan-treated animal is shown in **Fig. 5**. NGT (Fig. 5B, peak 3) was identified by coelution with an authentic standard and identical electrochemical behavior (i.e., voltammogram). The present method can also be used for the detection of NGT in tissue homogenates. The only difference is that SDS has to be added to the extraction mixture (14). **Figure 6** shows a typical chromatogram obtained from a liver homogenate.

Since tocopherols are distributed within lipoproteins in the circulation, vitamin E levels generally vary with the concentration of lipids (27). We therefore measured the concentrations of FC, major cholesterol esters, and TG in plasma to correct the measured tocopherol values for changes in above lipids.

While free FC and Ch18:2 were significantly decreased by fasting (Table 1), the decrease in TG and total neutral lipids (TNL, TC + TG) was not significant ($P = 0.22$ and 0.23, respectively). In addition to the effects of fasting, zymosan treatment led to a significant shift from polyunsaturated cholesterol esters to FC, possibly as a result of increased energy demand and formation of inflammatory lipid mediators (28). Adjustment of tocopherol levels to FC or total cholesterol (TC) alone was therefore not appropriate. Finally, correction of tocopherol levels to the observed changes in lipid profile by using TNL revealed that αT and γT levels did not actually change in zymosan-

TABLE 1. Plasma tocopherol and lipid concentrations 24 h after zymosan treatment

	Control (5)	Control, Fasted (4)	Zymosan (4)
		μmol/l	
Tocopherols			
αT	11.1 ± 2.2	6.9 ± 0.6[a]	6.9 ± 1.3
γT	0.42 ± 0.15	0.11 ± 0.02[a]	0.11 ± 0.03
NGT	0.012 ± 0.004	0.005 ± 0.001[a]	0.009 ± 0.002[b]
		mmol/l	
Lipids			
FC	0.28 ± 0.04	0.20 ± 0.02[a]	0.36 ± 0.06[b]
Ch18:1	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.03
Ch18:2	0.26 ± 0.04	0.19 ± 0.03[c]	0.09 ± 0.04[b]
Ch20:4	0.43 ± 0.08	0.53 ± 0.04	0.23 ± 0.10[b]
TC	1.01 ± 0.14	0.98 ± 0.10	0.73 ± 0.19
TG	2.67 ± 1.60	1.61 ± 0.57	1.61 ± 0.18
TNL	3.62 ± 1.46	2.59 ± 0.65	2.34 ± 0.22
Tocopherols, corrected			
αT (per 10^3 TNL)	3.3 ± 0.7	2.8 ± 0.6	2.9 ± 0.3
γT (per 10^3 TNL)	0.12 ± 0.03	0.05 ± 0.00[d]	0.05 ± 0.01
NGT (per 10^3 TNL)	0.004 ± 0.001	0.002 ± 0.000[a]	0.004 ± 0.001[b]
NGT (%γT)	3.7 ± 0.4	4.0 ± 0.6	7.7 ± 0.5[e]

αT, α-tocopherol; Ch18:1, cholesteryl oleate; Ch18:2, cholesteryl linoleate; Ch20:4, cholesteryl arachidonate; FC, free cholesterol; γT, γ-tocopherol; NGT, 5-nitro-γ-tocopherol; TC, total cholesterol; TG, triglycerides; TNL, total neutral lipids. Total cholesterol (TC) = [FC] + [Ch18:1] + [Ch18:2] + [Ch20:4]. Total neutral lipids (TNL) = [TC] + [TG]. Values represent mean ± SD. Number of animals used in each group are indicated in parentheses.

[a] Significantly different from control, $P < 0.01$.
[b] Significantly different from control, fasted, $P < 0.01$.
[c] Significantly different from control, $P < 0.05$.
[d] Significantly different from control, $P < 0.001$.
[e] Significantly different from control, fasted, $P < 0.001$.

treated animals compared to fasted control animals (Table 1). Interestingly, fasting by itself had a significant effect on TNL-adjusted γT but not αT.

In contrast to the lack of change in TNL-adjusted αT and γT, NGT levels adjusted for TNL were significantly increased in zymosan-treated animals compared to fasted controls. An ~2-fold increase was also apparent when NGT levels were expressed as a percentage of γT, an approach analogous to the adjustment of 3-nitrotyrosine to tyrosine (11). Since there was no difference in NGT between fasted and unfasted control animals when adjusted for γT, this adjustment is probably the best way of expressing NGT data (i.e., reflecting the degree of nitration of available substrate rather than presence of absolute amount). Despite the fact that γT levels were almost 30-fold lower than those of αT, nitration of γT was quite extensive (compared to 3-nitrotyrosine, which is in the ppm range) (11). These results indicate that γT constitutes an important target for nitration in vivo. Our data further support the recent findings of elevated NGT in Alzheimer disease brains (29) and blood plasma from subjects with coronary heart disease (18), both conditions associated with inflammation.

DISCUSSION

In the present study, a highly sensitive, specific, and reliable HPLC method for the analysis of NGT in blood plasma and other tissues is described. Increased levels of plasma NGT were demonstrated in rats with zymosan-induced peritonitis. Possible artefactual nitration of γT during analysis was excluded by appropriate in vitro experiments. Compared to the analysis of NGT using LC-MS (18) or CoulArray detection (29), the present method requires somewhat less expensive equipment and, at the same time, offers an at least 50-fold higher sensitivity.

Because NGT was, unlike 3-nitrotyrosine (11, 21), not readily amenable to chemical or electrochemical reduction, the nitro-phenol was detected directly in the oxidative mode. Compared to 3-nitrotyrosine, NGT is still relatively easy to oxidize (cf. Fig. 3). Using a Model 5011 coulometric cell, high sensitivity was achieved (~10 fmol detection limit). Our method proved sensitive enough to detect basal levels of NGT in as little as 0.2 ml of blood plasma from rats. Direct detection of NGT in the oxidative mode has previously been described (29, 30) using CoulArray detection, in which the analyte passes through four to 16 consecutive electrodes. Although this method has the advantage that more electrochemically different analytes can be detected in a single run, its sensitivity appears to be lower (~0.5 pmol for NGT) than that using the dual-electrode setup used here. Also, a CoulArray system is considerably more expensive. More recently, an LC-MS/MS method was described and used for the measurement of NGT in plasma of normal human subjects and subjects with coronary heart disease (18). The advantage of this method is that NGT is unambiguously analyzed by specific detection of the nitrated chomanol ring fragment. However, the method is considerably less sensitive (~5 pmol detection limit) than using electrochemical detection, and requires equipment not readily available to

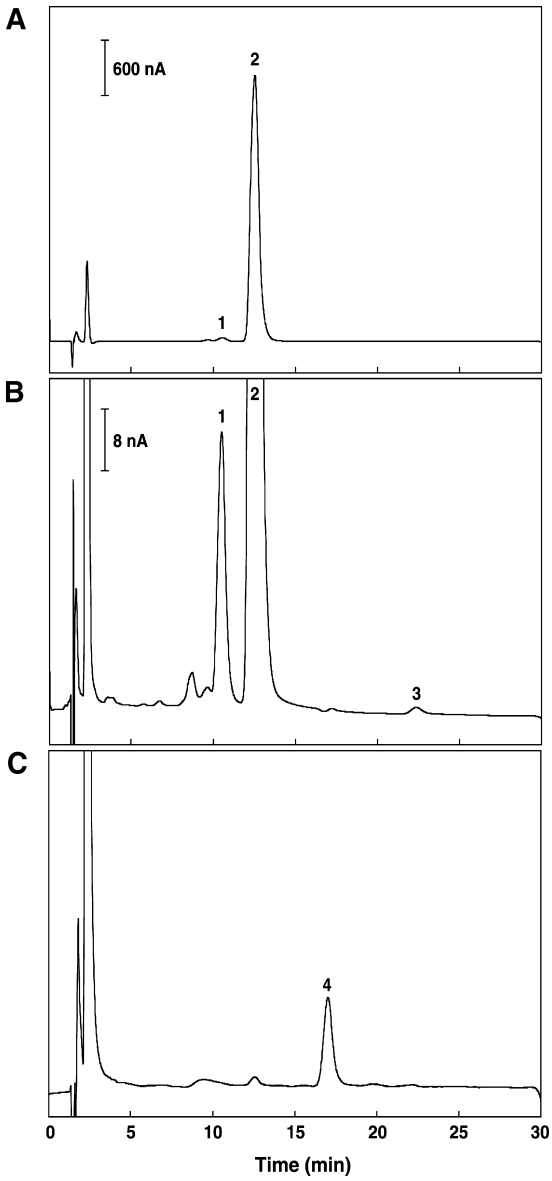

Fig. 5. Typical chromatogram of tocopherols in blood plasma of a zymosan-treated rat. Hexane phase of plasma extract was redissolved in ethanol and separated by HPLC as described in the legend to Fig. 2. Eluant was monitored simultaneously by coulometric (A and B) and UV detection at 210 nm (C). Peak identification: γT [1], αT [2], NGT [3], and free cholesterol (FC) [4]. The NGT peak in this chromatogram represents ~0.5 pmol of analyte on column.

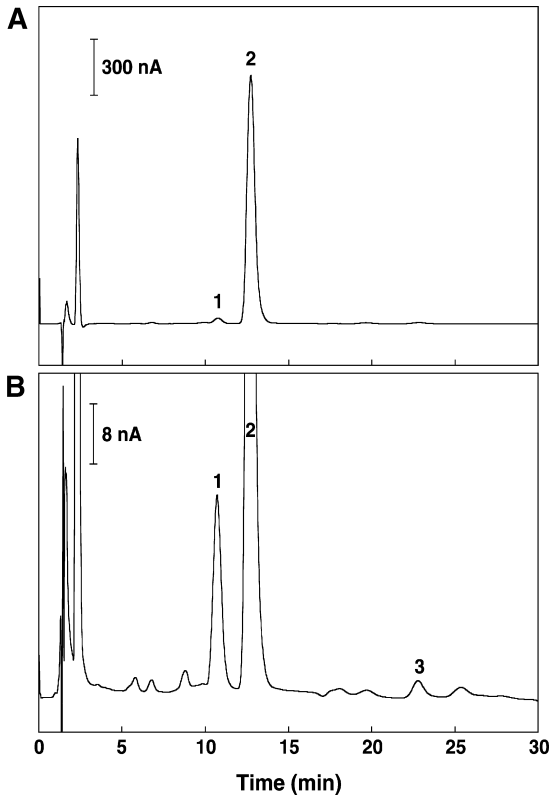

Fig. 6. Typical chromatogram of tocopherols in liver homogenate of a zymosan-treated rat. Tissue was homogenized in 10 vol (v/w) sodium acetate buffer (0.1 M, pH 7.2). One tenth of a milliliter SDS (1%, w/v) and 20 μl butylated hydroxytoluene (0.1 M) were added to 0.4 ml homogenate and mixed vigorously. Then, 2 ml of ice-cold methanol was added and mixed for 30 s. Finally, lipophilic antioxidants were extracted into 5 ml of ice-cold hexane by vigorous vortexing for 60 s. After evaporation of the hexane phase, the extract was redissolved in ethanol and subjected to HPLC as described in the legend to Fig. 5. Under these conditions, NGT is well separated from other eluting compounds, while interfering substances were removed on the first electrode. UV trace not shown.

most investigators. The availability of appropriate stable isotope-labeled internal standards (NGT and γT) may further enhance the specificity of this assay.

We have limited experience with using GC-MS for measuring NGT. Although tocopherols can be conviently assayed by GC (20), derivatization of NGT to a product that can be detected in negative chemical ionization mode (which provides sensitivity similar to that of HPLC with electrochemical detection) proved to be difficult. In particular, such derivatives were extremely heat-labile and reasonable reproducibility was achieved only by using tedious on-column injection.

Similar to the data obtained with brain samples and blood plasma from normal human subjects (18, 29), NGT represented a few percent of γT in normal rat plasma, which is several orders of magnitudes higher than the extent of protein tyrosine nitration, even during inflammation (11). These data indicate that γT constitutes an important target for nitration in vivo. One reason for the "preferential" nitration of γT is most likely nitric oxide's increased solubility and accelerated reaction with oxygen in lipid membranes (31). Another reason is that γT is more easily nitrated by peroxynitrite compared to tyrosine (32). We have previously shown that nitration of γT in liposomes prepared with soybean phosphatidylcholine is not affected by the presence of αT (8). Based on similar experiments in liposomes prepared with synthetic, saturated phospholipid, it was reasoned that nitration of γT by peroxynitrite or nitrating species derived from it (e.g., nitrogen dioxide) may not be relevant, unless αT levels have been compromised (32). However, our results clearly demonstrate that increased nitration of γT occurs in vivo despite unchanged levels of αT (Table 1).

Whether the measurement of NGT in blood plasma

may offer a better alternative to 3-nitrotyrosine as a pathophysiological marker of RNOS remains to be determined. In separate experiments we found that although NGT levels are elevated 24 h after zymosan treatment, they rapidly return to baseline levels and even below 2 weeks after treatment (unpublished data). A possible explanation for this observation is that NGT is metabolized in a similar fashion to the P_{450}-dependent hepatic degradation of γT (33), and excreted into urine. The main question that remains is whether γT has any potential health effects. There is some epidemiological evidence that γT may protect from heart disease and certain types of cancer (6). However, no controlled human supplementation studies have been conducted so far to document a protective effect of γT. We recently found that supplementation with moderate levels of γT inhibits protein nitration and oxidation of ascorbate in zymosan-treated rats while αT levels remained unaffected (34). In another study, supplementation of rats with γT has been shown to have a greater effect on inhibition of platelet aggregation and delay of arterial thrombogenesis than supplementation with αT (35). These results could be explained by the recently reported anti-inflammatory properties of γT (36). Further studies into the specific biological effects of γT (compared to αT) are therefore clearly warranted. The HPLC method described here should be useful in assisting this process.

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REFERENCES

1. Christen, S., T. M. Hagen, M. K. Shigenaga, and B. N. Ames. 1999. Chronic inflammation, mutation, and cancer. *In* Microbes and Malignancy: Infection as a Cause of Human Cancers. J. Parsonnet, editor. Oxford University Press, New York, Oxford. 35–88.
2. Laroux, F. S., K. P. Pavlick, I. N. Hines, S. Kawachi, H. Harada, S. Bharwani, J. M. Hoffman, and M. B. Grisham. 2001. Role of nitric oxide in inflammation. *Acta Physiol. Scand.* **173:** 113–118.
3. Burton, G. W., and K. U. Ingold. 1981. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J. Am. Chem. Soc.* **103:** 6472–6477.
4. Brigelius-Flohe, R., and M. G. Traber. 1999. Vitamin E: function and metabolism. *FASEB J.* **13:** 1145–1155.
5. Ricciarelli, R., J. M. Zingg, and A. Azzi. 2001. Vitamin E: protective role of a Janus molecule. *FASEB J.* **15:** 2314–2325.
6. Jiang, Q., S. Christen, M. K. Shigenaga, and B. N. Ames. 2001. gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. *Am. J. Clin. Nutr.* **74:** 714–722.
7. Cooney, R. V., A. A. Franke, P. J. Harwood, V. Hatch-Pigott, L. J. Custer, and L. J. Mordan. 1993. γ-Tocopherol detoxification of nitrogen dioxide: Superiority to α-tocopherol. *Proc. Natl. Acad. Sci. USA.* **90:** 1771–1775.
8. Christen, S., A. A. Woodall, M. K. Shigenaga, P. T. Southwell-Keely, M. W. Duncan, and B. N. Ames. 1997. gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. *Proc. Natl. Acad. Sci. USA.* **94:** 3217–3222.
9. Bourgeois, C. 1992. Determination of Vitamin E: Tocopherols and Tocotrienols. Elsevier Applied Science, London and New York.
10. Lang, J. K., K. Gohil, and L. Packer. 1986. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Anal. Biochem.* **157:** 106–116.
11. Shigenaga, M. K., H. H. Lee, B. C. Blount, S. Christen, E. T. Shigeno, H. Yip, and B. N. Ames. 1997. Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc. Natl. Acad. Sci. USA.* **94:** 3211–3216.
12. Demling, R., C. LaLonde, K. Ikegami, L. Picard, and U. Nayak. 1995. Alpha-tocopherol attenuates lung edema and lipid peroxidation caused by acute zymosan-induced peritonitis. *Surgery.* **117:** 226–231.
13. Marcinkiewicz, S. 1972. Complete analysis of tocopherol mixtures. III. New method of determination of tocopherols based on chromatography of their hydrogenation products and on spectrometry of their nitro derivatives. *Chem. Anal.* **17:** 13–20.
14. Burton, G. W., A. Webb, and K. U. Ingold. 1985. A mild, rapid, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids.* **20:** 29–39.
15. Sattler, W., D. Mohr, and R. Stocker. 1993. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography postcolumn chemiluminescence. *Methods Enzymol.* **233:** 469–489.
16. Schuep, W., and R. Rettenmaier. 1994. Analysis of vitamin E homologs in plasma and tissue: high-performance liquid chromatography. *Methods Enzymol.* **234:** 294–302.
17. Handelman, G. J., L. J. Machlin, K. Fitch, J. J. Weiter, and E. A. Dratz. 1985. Oral alpha-tocopherol supplements decrease plasma gamma-tocopherol levels in humans. *J. Nutr.* **115:** 807–813.
18. Morton, L. W., N. C. Ward, K. D. Croft, and I. B. Puddey. 2002. Evidence for the nitration of gamma-tocopherol in vivo: 5-nitro-gamma-tocopherol is elevated in the plasma of subjects with coronary heart disease. *Biochem. J.* **364:** 625–628.
19. Warnick, G. R. 1986. Enzymatic methods for quantification of lipoprotein lipids. *Methods Enzymol.* **129:** 101–123.
20. Lang, J. K., M. Schillaci, and B. Irvin. 1992. Vitamin E. *In* Modern Chromatographic Analysis of Vitamins. A. P. De Leenheer, W. E. Lambert, and H. J. Nelis, editors. Marcel Dekker, Inc., New York. 153–195.
21. Liu, H., T. Huang, C. B. Kissinger, and P. T. Kissinger. 1998. Comparison of detection methods for liquid chromatographic determination of 3-nitro-L-tyrosine. *J. Chromatogr. B Biomed. Sci. Appl.* **713:** 289–295.
22. Sodum, R. S., S. A. Akerkar, and E. S. Fiala. 2000. Determination of 3-nitrotyrosine by high-pressure liquid chromatography with a dual-mode electrochemical detector. *Anal. Biochem.* **280:** 278–285.
23. Deitch, E. A., R. D. Specian, M. B. Grisham, and R. D. Berg. 1992. Zymosan-induced bacterial translocation: a study of mechanisms. *Crit. Care Med.* **20:** 782–788.
24. Shayevitz, J. R., C. Miller, K. J. Johnson, and J. L. Rodriguez. 1995. Multiple organ dysfunction syndrome: end organ and systemic inflammatory response in a mouse model of nonseptic origin. *Shock.* **4:** 389–396.
25. Cuzzocrea, S., A. Filippelli, B. Zingarelli, M. Falciani, A. P. Caputi, and F. Rossi. 1997. Role of nitric oxide in a nonseptic shock model induced by zymosan in the rat. *Shock.* **7:** 351–357.
26. Demling, R., K. Ikegami, L. Picard, and C. Lalonde. 1994. Administration of large doses of vitamin C does not decrease oxidant-induced lung lipid peroxidation caused by bacterial-independent acute peritonitis. *Inflammation.* **18:** 499–510.
27. Gey, K. F., P. Puska, P. Jordan, and U. K. Moser. 1991. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *Am. J. Clin. Nutr.* **53:** 326S–334S.
28. Larsson-Backstrom, C., E. Arrhenius, K. Sagge, L. Lindmark, J. Paprocki, and L. Svensson. 1990. Sequential changes in lipid metabolism and the fatty acid profile in liver lipids during fasting and sepsis. *Circ. Shock.* **30:** 331–347.
29. Williamson, K. S., S. P. Gabbita, S. Mou, M. West, Q. N. Pye, W. R. Markesbery, R. V. Cooney, P. Grammas, U. Reimann-Philipp, R. A. Floyd, and K. Hensley. 2002. The nitration product 5-nitro-gamma-tocopherol is increased in the Alzheimer brain. *Nitric Oxide.* **6:** 221–227.
30. Hensley, K., K. S. Williamson, and R. A. Floyd. 2000. Measurement

of 3-nitrotyrosine and 5-nitro-gamma-tocopherol by high-performance liquid chromatography with electrochemical detection. *Free Radic. Biol. Med.* **28:** 520–528.
31. Liu, X., M. J. Miller, M. S. Joshi, D. D. Thomas, and J. R. Lancaster, Jr. 1998. Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. USA.* **95:** 2175–2179.
32. Goss, S. P., N. Hogg, and B. Kalyanaraman. 1999. The effect of alpha-tocopherol on the nitration of gamma-tocopherol by peroxynitrite. *Arch. Biochem. Biophys.* **363:** 333–340.
33. Sontag, T. J., and R. S. Parker. 2002. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin e status. *J. Biol. Chem.* **277:** 25290–25296.
34. Jiang, Q., J. Lykkesfeldt, M. K. Shigenaga, E. T. Shigeno, S. Christen, and B. N. Ames. 2002. γ-Tocopherol supplementation inhibits protein nitration and ascorbate oxidation in rats with inflammation. *Free Radic. Biol. Med.* In press.
35. Saldeen, T., D. Li, and J. L. Mehta. 1999. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. *J. Am. Coll. Cardiol.* **34:** 1208–1215.
36. Jiang, Q., I. Elson-Schwab, C. Courtemanche, and B. N. Ames. 2000. gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc. Natl. Acad. Sci. USA.* **97:** 11494–11499.