γ -Tocopherol detoxification of nitrogen dioxide: Superiority to α -tocopherol

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ABSTRACT In the vitamin E group, α -tocopherol is generally considered to be the most potent antioxidant with the highest vitamin bioactivity, yet γ -tocopherol is produced in greater amounts by many plants and is the principal tocopherol in the United States diet. This report describes a fundamental difference in the chemical reactivities of α -tocopherol and γ -tocopherol with nitrogen dioxide (NO_2), which leads to the formation of a nitrosating agent from α -tocopherol, but not from γ -tocopherol. Nitric oxide (NO) is a major product of the reaction of γ -tocopherol with NO_2 , while α -tocopherol reacts with NO_2 to form an intermediate tocopheroxide analogue. The biological significance of γ -tocopherol is suggested by limited epidemiological data as well as the observation that it is a more potent inhibitor than α -tocopherol of neoplastic transformation during the postinitiation phase in 3-methylcholanthrene-treated C3H/10T $\frac{1}{2}$ murine fibroblasts. This latter property suggests the superiority of γ -tocopherol in a mammalian biological assay and a role for endogenous NO production in promotion of neoplastic transformation.

γ -Tocopherol (γ -T) is the primary tocopherol (Structure 1) found in the lipid fraction of many seeds and nuts, such as soybeans, walnuts, and corn (1, 2). As a consequence of the dietary use of oils derived from these sources, γ -T accounts for as much as 70% of the total intake of tocopherols in the United States diet (3), yet little is known concerning its biological function or metabolic fate. γ -T possesses only 10–20% of the vitamin E activity of α -tocopherol (α -T) in traditional bioassays (4), is less efficient as an antioxidant (5, 6), and serum levels average 5-fold lower than α -T (7), despite the higher dietary intake of γ -T (3) and similar rates of absorption from the gastrointestinal tract (8). γ -T is preferentially taken up by human endothelial cells in culture, yet it

disappears from cells at a faster rate (9). While some animal and epidemiologic studies show α -T consumption to be inversely associated with cancer incidence, other studies are equivocal (10, 11). α -T inhibits neoplastic transformation in cell culture during both initiation (12) and promotion (13) phases, although the effects of α -T in the latter case are limited compared to other antioxidants such as the carotenoids (13) and vitamin C (14), despite the potent antioxidant activity of α -T (6, 15, 16). At high doses, α -T alone (17) and in combination with other tocopherols (18) is associated with enhanced tumor formation.

In contrast to α -T, there have been few epidemiologic studies evaluating the relationship between γ -T consumption and disease incidence. Serum γ -T levels are $\approx$ 2-fold higher in the population of Fiji compared to those from populations of the Cook Islands, while α -T levels are similar in the two populations (ref. 19; L. Le Marchand, J. Hankin, F. Bach, B. H. Henderson, and L. N. Kolonel, personal communication). Interestingly, lung cancer incidence rates in Fiji are 10–20 times lower than in the Cook Islands, despite similar patterns of smoking (19, 20). Potischman *et al.* (21) reported that serum γ -T levels were lower in patients with early-stage cervical cancer but higher in patients with late-stage disease compared to controls.

At present there is no mechanistic explanation for any unique biological or chemical properties of γ -T, compared to α -T, that might account for either its presence in seed oils or its association with cancer. The tocopherols function as membrane antioxidants (22) and are concentrated in the germ plasma of seeds (2), where they may also protect the genetic material of the plant, as suggested by their binding to the nucleoprotein complex (23). Two major sources of γ -T, soybeans and corn, are known to produce nitrogen oxides (24, 25). Stored corn can generate lethal concentrations of nitrogen dioxide (NO_2), a molecule that reacts with unsaturated membrane lipids (26) and as a nitrosating agent (27). Animals are exposed to nitrogen oxides from a variety of sources. Nitric oxide (NO) is produced endogenously by macrophages (28) in response to infection, by endothelial cells to control vascular tone (29), and by postsynaptic neurons (30), while a major exogenous source of exposure is cigarette smoke (31). Nitrogen oxides cause mutations in bacteria (32–34) and mammalian cells (35), possibly through deamination of DNA bases as a consequence of nitrosation of primary amines on those bases (33, 35). GC $\rightarrow$ AT transition mutations observed in the p53 gene of lung tumors from smokers (36) may be the result of cytosine deamination by this mechanism. Both nitrosation (27, 37) and mutation (33, 34) by NO require oxidation, suggesting a possible role for NO_2 in these processes.

Selander and Nilsson reported in 1972 (38) that NO_2 reacts with α -T model compounds to produce quantitative yields of

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Abbreviations: α -T, α -tocopherol; γ -T, γ -tocopherol; NMOR, *N*-nitrosomorpholine.

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the nitrite ester of α -tocopherylquinone. Organic nitrite esters are potent nitrosating agents (39–41) and are not inhibited by α -T (42), although α -T is reported to block nitrosation by nitrous acid and nitrogen oxides (43, 44). In contrast, it has been suggested that γ -T may catalyze nitrosation reactions through formation of a C-nitroso intermediate (43, 44), although this has not been verified experimentally. In light of the occurrence of γ -T in plants and seeds, known to produce nitrogen oxides, and the demonstrated ability of α -T to form a nitrite ester, we sought to identify the relative reactivities of these tocopherol homologues with NO_2 and the products formed in order to assess their role in nitrosation. In addition, the epidemiologic data from South Sea Island populations prompted us to evaluate the chemopreventive potential of γ -T in the widely used C3H/10T $\frac{1}{2}$ transformation assay.

MATERIALS AND METHODS

Chemicals. Morpholine, α -T, and γ -T standards were from Sigma. Tocopheroxide and α -tocopherylquinone were synthesized from α -T according to the methods of Boyer (45) and Issidorides (46), respectively. Stock solutions of α -T and γ -T in hexane were quantitated at 292 and 298 nm using $E^{1\%}$ values of 75.8 and 91.4 (100 ml/g-cm), respectively (47). Chromatography solvents were HPLC grade from Fisher Scientific. *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine standards were from Chem Service (West Chester, PA). EPA grade NO_2 (55 ppm in N_2 containing 3.5 ppm NO) and EPA grade NO (22.5 or 91 ppm in N_2 containing <1 ppm NO_2) certified standards were from Scott Specialty Gases (Plumsteadville, PA).

Reaction Conditions. Five-milliliter solutions of tocopherol (1 mM) in hexane were exposed to 27.5 ppm NO_2 in N_2 by bubbling (60 ml/min) for 15 min. NO_2 exposure was stopped and the volume was adjusted to 5 ml with hexane, followed 5 min later by the addition of 50 μ l of 100 mM morpholine and *N*-nitrosopyrrolidine (200 μ g/ml as internal standard) in hexane. Aliquots were placed into amber vials, capped, and incubated at either 0°C or 37°C for the indicated times. One-microliter samples were removed at intervals and analyzed by gas chromatography/thermal energy analysis (Thermedics, Waltham, MA) for NMOR as described (27). In some experiments, morpholine was omitted to allow HPLC analysis of the tocopherol reaction products in its absence.

HPLC. Reaction products of the tocopherols were determined by using a Spherex 5 C-18 (250 $\times$ 4.6mm) reversed-phase column (Phenomenex, Belmont, CA) with a 1.5-ml/min flow of $\text{CH}_3\text{CN}/\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{hexane}$ (60:20:10:5). Compounds were monitored at 240 nm (tocopheroxides), 266 nm (tocopherylquinone), and 295 nm (tocopherols) with a Beckman 168 multiple wavelength diode array detector.

Nitrogen Oxide Analysis. Nitrite was analyzed according to the method of Saltzman (48). NO_2 was measured in the gas phase by bubbling a known volume of gas through 10 mM vanillin (pH 9.0) and measuring the nitrite formed (48). NO was measured (27) by direct injection of gas samples (10 μ l) into the thermal energy analyzer ($t = 25^\circ\text{C}$).

Transformation Assay. C3H/10T $\frac{1}{2}$ murine fibroblasts were seeded at 2000 cells per 60-mm dish in 5 ml of Eagle's basal medium containing 2.5 μ g of gentamicin per ml and 5% calf serum. The next day, cells were treated with 3-methylcholanthrene (2.5 μ g/ml in acetone) or vehicle alone (0.5% acetone) for 24 hr. The carcinogen-containing medium was then replaced with fresh medium and the cells were grown to confluence (8–10 days). Culture medium was then replenished and the tocopherols in acetone (final acetone concentration, 0.5%) were added weekly at the time of medium change for 4 weeks after confluence. Types II and III transformed foci, which have previously been shown to be tumorigenic, were scored from fixed and stained cultures

according to the guidelines described by Reznikoff *et al.* (49) and are reported as transformed foci per dish $\pm$ SEM.

RESULTS AND DISCUSSION

Fig. 1 illustrates the time- and temperature-dependent formation of NMOR from the reaction of morpholine with a nitrosating agent formed by exposure of 1 mM α -T to NO_2 . Although no nitrosation was observed at 0°C, the nitrosating agent was stable at this temperature for at least 5 days. After this time, raising the temperature to 37°C resulted in rapid nitrosation of morpholine (Fig. 1). In contrast, no significant amount of NMOR formed when morpholine was added to NO_2 -exposed γ -T in hexane. A hexane control exposed to NO_2 yielded only small amounts of NMOR with no time-dependent increase.

α -T and γ -T removed 43% and 39%, respectively, of the NO_2 from the gas stream; however, a significantly greater amount of nitrous acid and/or nitrite ester formed in the α -T solution (51 μM vs. 10 μM for γ -T) as detected by reaction with Griess–Saltzman reagent (48). In addition, γ -T, but not α -T, solutions exposed to NO_2 turned yellow and an additional peak in the absorption spectrum was observed at 432 nm (in hexane), consistent with formation of a C-nitroso aromatic compound, as reported for reaction of γ -T with nitrous acid (50), or formation of C-nitro tocopherol.

Measurement of NO in the gas stream exiting tocopherol solutions during NO_2 exposure revealed a striking difference in NO evolution (Fig. 2), with γ -T reducing NO_2 to NO at a significantly greater rate than did α -T. It has been postulated that the ability of α -T to react with nitrosating agents to produce NO accounts for its ability to prevent nitrosation reactions (43, 44). While limited NO formation by α -T under our reaction conditions was observed, γ -T was superior in this regard. Cessation of NO_2 exposure, but with continued bubbling of N_2 , resulted in decreasing NO evolution over several minutes.

Chromatographic analysis of the reaction products of α -T with NO_2 showed that α -T levels decreased during both the

FIG. 1. Nitrosating potential of α -T and γ -T exposed to NO_2 . Reaction conditions are described in *Materials and Methods*. Tocopherol (1 mM in hexane) was exposed to NO_2 (27.5 ppm in N_2) for 15 min. After cessation of NO_2 exposure, morpholine was added (final concentration, 1 mM), the sample was placed in a 37°C heating block (time 0), and NMOR was measured over time. ●, 1 mM α -T incubated at 37°C; ○, 1 mM α -T incubated at 0°C for 137 hr and then switched to 37°C; ▲, 1 mM γ -T at 37°C; ▽, hexane control (37°C) exposed to NO_2 . Yield of NMOR was 19% of the theoretical maximum based on absorption into 5 ml of hexane of 0.43 μmol of NO_2 as the limiting reagent.

FIG. 2. NO formation from the reaction of tocopherols with NO_2 . Hexane solutions containing 1 mM α -T (●), 1 mM γ -T (▲), or 1 mM each α -T and γ -T (○) were exposed to 27.5 ppm NO_2 (flow rate, 60 ml/min) from time 0 and the exit gas was monitored for NO. Each point represents mean $\pm$ SEM ($n \geq 5$ for individual tocopherols; $n \geq 3$ for combined tocopherols).

15-min exposure to NO_2 and for ≈ 5 hr afterward when incubated at 37°C (Fig. 3). The major initial product in α -T solutions exposed to NO_2 was similar in retention time and absorption spectrum (Fig. 4) to tocopheroxide (Structure 2). This is consistent with published reports of unstable tocopheroxide intermediates formed during oxidation of α -T by various oxygen-centered radicals (51). Although stable at 0°C , the tocopheroxide analogue decomposed at 37°C , resulting in simultaneous formation of α -tocopherylquinone

FIG. 3. Changes in reactants and products as a function of time for NO_2 -exposed α -T in hexane. Solutions were exposed as described in Fig. 1. NO_2 was stopped and samples were incubated at 37°C (time 0) and changes in reactant (α -T, ●) and product (α -tocopherylquinone, ▼; tocopheroxide analogue, ▽; MMOR, □ concentrations in the absence (A) or presence (B) of 1 mM morpholine were measured.

FIG. 4. Chromatography of α -T after exposure to NO_2 . Reaction products of α -T with NO_2 were chromatographically separated, as described in *Materials and Methods*, and monitored at 266 nm (0–3.6 min), 295 nm (3.6–4.6 min), and 240 nm (4.6–5.4 min). Peaks appearing at 3.2 and 4.8 min were identified as α -tocopherylquinone and a tocopheroxide analogue, respectively, by comparing retention times and absorption spectra (Upper Left Inset, α -tocopherylquinone and peak at 3.2 min; Upper Right Inset, tocopheroxide and peak at 4.8 min).

and further loss of α -T (Fig. 3A), consistent with the reaction mechanism outlined in Scheme 1 for reaction of the tocopheroxide nitrite (Compound II) with α -T (Compound I) to yield tocopherylquinone (Compound V) and the nitrite ester of α -T (Compound III). Morpholine accelerated destruction of the tocopheroxide analogue while diminishing both tocopherylquinone formation and α -T destruction (Fig. 3B). The nitrosating agent(s) could not be recovered after chromatography, probably because of reaction with methanol in the mobile phase (52). The nitrite esters of α -T (Compound III), tocopheroxide (Compound II), and tocopherylquinone (Compound IV) shown in Scheme 1 could each be a potential nitrosating agent, analogous to other nitrous acid esters previously described (39–41, 52). γ -T, lacking a C-5 methyl group, is more reactive than α -T at this position and forms 5-nitroso γ -T in the presence of HONO (50). The lack of significant nitrous acid formation along with the rapid evolution of NO by γ -T solutions suggests a reaction pathway distinct from that observed for α -T.

Although α -T is a more effective inhibitor of lipid oxidation in cells than carotenoids (15), it is significantly less effective than other antioxidants at inhibiting neoplastic transformation (13, 14). This suggests that general antioxidant properties, as assessed by prevention of lipid oxidation, cannot completely explain the inhibition of transformation by antioxidants (15). An alternative hypothesis is that nitrogen oxides, produced by cells during the promotional phase of neoplastic transformation, form mutagenic nitrosamines or induce deamination of nucleic acids, and α -T may be limited in preventing these mutagenic events. The demonstrated endogenous production of NO by cells of mesenchymal origin, including vascular smooth muscle cells (53), suggests that genotoxic damage due to endogenous nitrosation is plausible. NO requires oxidation to act as a nitrosating agent

(27, 35, 54); therefore, reduction of NO_2 to NO by lipid-phase antioxidants could be protective. Consequently, γ -T may be a more effective inhibitor than α -T of neoplastic transformation because of its superior ability to scavenge and chemically reduce NO_2 without forming a nitrosating intermediate. As shown in Fig. 5, γ -T added during the promotion phase of the C3H/10T $\frac{1}{2}$ transformation assay was significantly more effective than α -T at inhibiting transformation. Because tocopherols were added during the postconfluence promotional phase of the transformation assay, and not during carcinogen

exposure, these data suggest that generation of NO may play a significant role during the promotional phase of neoplastic transformation. Preliminary evidence indicates that C3H/10T $\frac{1}{2}$ fibroblasts do in fact generate nitrogen oxides as predicted (data not shown).

While the ability of nitrous acid esters to act as nitrosating agents is well established, our observation that α -T can form such a nitrosating agent is unusual in light of its perception as a potent antioxidant and cancer chemopreventive agent. α -T functions effectively as an inhibitor of lipid peroxidation and inhibits nitrosation when assessed in short-term assays (43, 44, 55); however, simultaneous formation of a nitrosating agent from reaction with NO_2 suggests that long-term protection from genotoxic deamination reactions may be incomplete. This may explain enhanced tumor formation when α -T is administered to animals at high doses (17, 18). Nitta *et al.* (18) observed an inflammatory response at the site of tocopherol injection, suggesting that generation of free radicals by invading macrophages may have contributed to tumor formation.

The unique chemical and biological properties among the tocopherols described in this report offer a plausible explanation to intriguing questions concerning the natural occurrence of γ -T. The results of our investigations suggest that accumulation of γ -T in grains and seeds may protect against both general oxidation reactions and NO_2 -mediated damage. The efficacy of γ -T, relative to α -T as an inhibitor of neoplastic transformation, implies that NO_2 may act as an endogenous mutagen in the C3H/10T $\frac{1}{2}$ carcinogenesis assay, and further study in this area is warranted. While the chemistry of NO_2 reaction with tocopherols in hexane may provide a useful lipid-phase model, it remains to be determined whether the reactions described here are relevant to cellular mutation and human cancer. In particular, other oxidative processes may be more important in the etiology of cancers at various sites. In those instances, α -T, the caro-

FIG. 5. Effects of tocopherols on neoplastic transformation. Effects of α -T (●) and γ -T (▲) on morphological transformation of C3H/10T $\frac{1}{2}$ fibroblasts are shown. Each point represents mean $\pm$ SEM ($n = 24$ for each γ -T concentration; $n = 12$ for each α -T concentration). Negative controls (no 3-methylcholanthrene treatment) yielded an average of 0.1 focus per dish. Previously published data for α -T (13) showed inhibition of 44% and 56% at 3 and 30 μM , respectively, vs. 40% and 59% for α -T in the current study; $P = 0.001$ for difference in means between α -T and γ -T (30 μM) using a two-tailed unpaired t test.

tenoids, or other antioxidants may be superior in preventing genetic damage, while in some cases the antioxidant properties of a molecule may be unrelated to its chemopreventive properties, as appears to be the case for carotenoids (15). The present research emphasizes the importance of defining relevant reactants and mechanisms involved with oxidative damage and its prevention. The observations reported here provide a chemical and biological rationale supportive of the epidemiologic data from Fiji, and it is hoped that these studies will stimulate further prospective studies into the relationship of lung cancer with dietary intake and serum levels of both α -T and γ -T, as well as the general physiologic relevance of NO exposure to carcinogenesis.

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