PALM OIL TOCOTRIENOLS AS ANTIOXIDANTS AND CHEMOPREVENTIVE AGENTS

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ABSTRACT

Breast cancer leads all cancer incidence among American women, accounting for 32% of the 2005 estimated new cases in the United States. It is the second leading cause of cancer deaths, estimated at 40 000 yr^{-1}. Estrogens, natural or synthetic, used widely in a variety of clinical conditions, from estrogen replacement therapy to cancer treatment, are themselves carcinogenic, causing uterine and breast cancers. However, the mechanism of their carcinogenic action is still not well understood. We found that both 17β-estradiol (E_2) and estrone (E_1) could be activated by the versatile epoxide-forming oxidant dimethyldioxirane (DMDO) to inhibit nuclear RNA synthesis and to bind DNA forming estrogen-DNA adducts both in vitro *and* in vivo*. Since DNA adducts can cause mutation, and mutation is the molecular basis for the initiation of carcinogenesis, our findings strongly suggest the possibility that both E_2 and E_1 are the initiators for uterine and breast carcinogenesis. Based on this new insight, a method to screen chemopreventive agents against breast cancer, at the initiation, was developed. This screening test determines whether a chemical is able to prevent the formation of E_2 or E_1 epoxide as measured by both the loss of the ability of E_2 or E_1 to inhibit nuclear DNA-dependent RNA synthesis and the ability of [^{3}H] E_2 or E_1 to bind DNA. This article summarizes the results of our recent studies on the preventive effects of Red Palm Oil, tocopherols and tocotrienols on the epoxidation of several carcinogens including E_2, E_1 and aflatoxin B_1 (AFB_1). Our data suggest that tocotrienols are more potent chemopreventive agents than tocopherols against the epoxide formation of E_2, E_1 and AFB_1. As a dietary supplement, tocopherols and especially tocotrienols may have the potential to prevent breast and liver cancers.*

Keywords: tocotrienol, breast cancer, 17β-estradiol, estrone, aflatoxin.

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INTRODUCTION

Breast cancer leads all cancer incidences among American women, accounting for 32% of the 2005 estimated new cases in the United States (Jemal *et al.*, 2005). It is the second leading cause of cancer deaths, estimated at 40 000 yr^{-1} (Jemal *et al.*, 2005). Epidemiological studies have shown that 70%-80% of all cancer incidence is due to environmental factors and personal lifestyle (Doll *et al.*, 1981), and is therefore, in theory, preventable. However, in order to prevent a disease, it is necessary to understand the cause of the disease. Estrogens, natural or synthetic, used widely in a variety of clinical conditions, from estrogen replacement therapy to cancer treatment, are themselves carcinogenic, causing uterine and breast cancers (Mcgonigle *et al.*, 1994; Grady *et al.*, 1995; Toniolo *et al.*, 1995; Colditz *et al.*, 1998; Schairer *et al.*, 1998; Rossouw *et al.*, 2002). However, the mechanism of their carcinogenic action is still not well understood. Because estrogens are required for the growth and development of target cells, it has long been believed that estrogens are promoters for carcinogenesis (Feigelson *et al.*, 1996). However, this promotional hypothesis is not able to explain how the cancer cells are developed initially. Chemical carcinogenesis is a multistage process

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including initiation, promotion and progression (Miller *et al.*, 1981; Weisburger, 1980; Farber, 1984; Hemminki, 1993; Dipple, 1995). Initiation is the first critical and irreversible step in carcinogenesis, it requires the covalent binding of a carcinogen to DNA forming DNA adducts (Miller *et al.*, 1981;Weisburger, 1980; Farber, 1984; Hemminki, 1993; Dipple, 1995). DNA adducts can cause mutation and mutation is the molecular basis of carcinogenesis (Miller *et al.*, 1981; Weisburger, 1980; Farber, 1984; Hemminki, 1993; Dipple, 1995).

Several years ago, we found that estrone (E_1) and 17β-estradiol (E_2) similar to the well established initiating chemical carcinogens, *e.g.* benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, aflatoxins, *etc.* (Miller *et al.*, 1981; Weisburger, 1980; Farber, 1984; Hemrninki, 1993; Dipple, 1995; Yu *et al*., 1994; 1996; Raney *et al*., 1992; Iyer *et al*., 1992), could be activated by epoxidation and to bind DNA forming estrogen-DNA adducts both *in vitro* (Yu *et al.*, 1996;1998a,b; 1999a) and *in vivo* (Yu *et al.*, 1998c; 1999b; 2001), and to inhibit nuclear DNA-dependent RNA synthesis (Yu *et al*., 1996; 1998a, b). These findings strongly suggest that both E_1 and E_2 are potential initiators for uterine and breast cancers. Based on this new insight, a method to screen chemo-preventive agents against breast cancer, at the initiation, was developed (Yu, 2002). This screening test determines whether a chemical is able to prevent the formation of E_2 or E_1 epoxide as measured by both the loss of the ability of E_2 or E_1 to inhibit nuclear DNA-dependent RNA synthesis and the ability of [^{3}H]- labelled E_2 or E_1 to bind to DNA (Yu, 2002). Using this screening protocol, we have found that the breast cancer preventive effect of tamoxifen (TAM) is through a competitive epoxidation mechanism that prevents the formation of E_1 and E_2 epoxides and consequently , the initiation of breast cancer (Yu *et al.*, 2003). We provided evidence suggesting that the prevention of chemical carcinogen DNA binding and inhibition of nuclear RNA polymerase activity by organosulfur compounds from garlic, *e.g.* diallyl disulfide (DADS) and dialyl trisulfide (DATS), as the possible mechanisms for their anti-cancer initiation and proliferation effects (Yu *et al.*, 2003). We studied in detail the chemopreventive potentials of vegetable oils and unsaturated fatty acids against breast cancer carcinogenesis at the initiation (Yu *et al*., 2004). And we provided a molecular explanation why the tocotrienols are more potent cancer preventive agents than tocopherols (Yu *et al.*, 2005).

This article summarizes the results of our recent studies on the preventive effects of Red Palm Oil, tocopherols and tocotrienols on the epoxidation of several carcinogens including E_1 and E_2, and aflatoxin B_1 (AFB_1). Our data confirm that tocotrienols are more potent chemopreventive agents than tocopherols against the epoxide formation of not only E_2 but also of E_1 and AFB_1.

MATERIALS AND METHODS

Chemicals

The 17 β-estradiol (E_2) and estrone (E_1) were purchased from Sigma Chemical Company (St Louis, MO) while [2,4,6,7- ^{3}H]E_2 (94 Ci mmol^{-1}), [2,4,6,7-^{3}H]E_1 (98 Ci mmol^{-1}) and 5'-[α-^{32}P]GTP (3000 Ci mmol^{-1}) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Aflatoxin B_1 (AFB_1) and [^{3}H (G)]AFB_1 [29.2 Ci mmol^{-1}) were from Moravek Biochemicals (Brea, CA). Red Palm Oil (RPO), α-, γ- and δ-tocopherols, α-, γ- and δ-tocotrienols were generous gifts from Malaysian Palm Oil Board.

Isolation of Rat Liver Nuclei and Phenobarbital Induced Liver Microsomes

Rat liver nuclei were isolated by the hypertonic sucrose method as described previously (Yu, 1974; 1977). The phenobarbital induced liver microsomes were isolated the same way as described before (Yu, 1983; Yu *et al*., 1986; 1990).

Assays for the Preventive Effect of α-Tocopherol, α-Tocotrienol and Red Palm Oil (RPO) on the formation of E_2 Epoxide as Reflected by the Loss of E_2 Inhibition on Nuclear RNA Synthesis After Dimethyldioxirane (DMDO) Activation *in vitro*

The E_2, 1 mg, was mixed with α-tocopherol, α-tocotrienol, or RPO in the amount as indicated (*Table 1*), and reacted with 1 ml DMDO solution prepared according to Adam *et al.* (1987) at room temperature for 1 hr. The reaction mixture was then vacuum dried and dissolved in 200 µl dimethylsulfoxide (DMSO). For DNA binding, 50 µ1 of this reaction mixture in DMSO was added to 0.1 ml rat liver nuclei (150 µg DNA), and incubated on ice for 30 min. Then, 0.5 ml of the RNA assay medium [l00 mM Tris-HCl (pH 7.9 at 23ºC), 2 mM $MnCl_2$,28 mM 2-mercaptoethanol, 70 mM $(NH_4)_2SO4$ and 0.2 mM each ATP, GTP, UTP and CTP] containing 0.1 pCi [α-^{32}P]GTP was added to start RNA synthesis at 37ºC for 15 min with shaking. At the end of 15 min incubation, RNA synthesis was terminated by the addition of 3 ml of 10% TCA containing 1% pyrophosphate. The radioactive RNA, after TCA precipitation, was collected onto Whatman GF/C filters, which were washed and counted in 5 ml Bio-Safe II (Research Products International Corp., Mount Prospect, IL) as previously described (Yu *et al*., 1996; 1998a, b). The specific activity of RNA polymerase was expressed as picomoles [α-^{32}P]GMP incorporated/mg DNA.

Activation of [^{3}H]E_2 by DMDO for Rat Liver Nuclear DNA Binding

The procedure for [^{3}H]E_2 binding to nuclear DNA after DMDO activation was essentially the same as described previously (Yu *et al*., 2002; 2004; 2005). Briefly, 1 mg E_2 containing 10 μCi [^{3}H]E_2 was either activated alone, or in the presence of RPO in the amount as indicated (*Figure 1*) with 1 ml DMDO solution at room temperature for 1 hr. After activation, the samples were vacuum dried. The samples, dissolved in 200 μl DMSO, were mixed with 400 μ1 of the isolated rat liver nuclei (600 μg DNA), and incubated on ice for 2 hr for DNA binding. The samples were then digested sequentially with RNase (50 μg, l hr), pronase (50 μg, 2 hr), and followed by phenol-chloroform extraction of the DNA (Yu, *et al*., 1995; 2002; 2004; 2005). The DNA in the final aqueous phase was precipitated with 1/10 volume 30% sodium acetate (pH 5.0) and four volumes of 95% ethanol at -20°C overnight. The DNA pellet was repeatedly washed with 95% alcohol containing 3% sodium acetate to get rid of the free [^{3}H] labelled E_2. Finally the DNA from each group was re-dissolved in 0.5 ml H_20 for optical density measurement and radioactivity counting.

Activation of [^{3}H]E_2 by Liver Microsomes for Calf Thymus DNA Binding

The procedure for the binding of [^{3}H]AFB_1 to DNA after activation by liver microsomes as described previously (Yu, 1983; Yu, *et al*., 1986; 1990; 1994; 1996) was adopted for the present study (*Figure 2*). The 1 mg E_2 containing 10 μCi [^{3}H]E_2 was mixed either with 100 μl DMSO alone (the control group), or with 100 μl DMSO containing the amount of RPO as indicated (*Figure 2*). The samples were incubated together with 20 μl microsomes (0.5 mg protein) in buffer A (0.05 M Tri-HC1, pH 7.5 containing 0.25 M sucrose), 20 μl binding buffer (0.9 M Tris-HC1, pH 7.5,0.06 M $MgCl_2$, 16 mM NADP, 100 mM glucose-6-phosphate) containing 0.2 units glucose-6-phosphate dehydrogenase, and 600 μg calf thymus DNA in 100 μl H_2O for 1 hr at 37°C. The samples were then digested sequentially with RNase A (50 μg, 1 hr), pronase (50 μg, 2 hr), and the DNA was purified and counted as described in *Figure 1*.

Activation of [^{3}H]AFB_1 by DMDO for Calf Thymus DNA Binding

The procedure for [^{3}H]AFB_1 binding to calf thymus DNA after DMDO activation was essentially the same as described previously for [^{3}H]E_2 binding (Yu *et al*., 1998a, b; 2003). Briefly, AFB_1 ,40 μg, containing 5 μCi [^{3}H] labelled AFB_1 was incubated either alone or together with 40, 200, or 400 μg α-tocopherol or one of the tocotrienols as indicated (*Figure 3*) in 0.5 ml DMDO at room temperature for 1 hr. After incubation, the reaction mixtures were vacuum dried. The samples were then dissolved in 160 μl DMSO, together with 160 μ1 calf thymus DNA (400 μg), 160 μ1 H_2O and incubated on ice for 2 hr

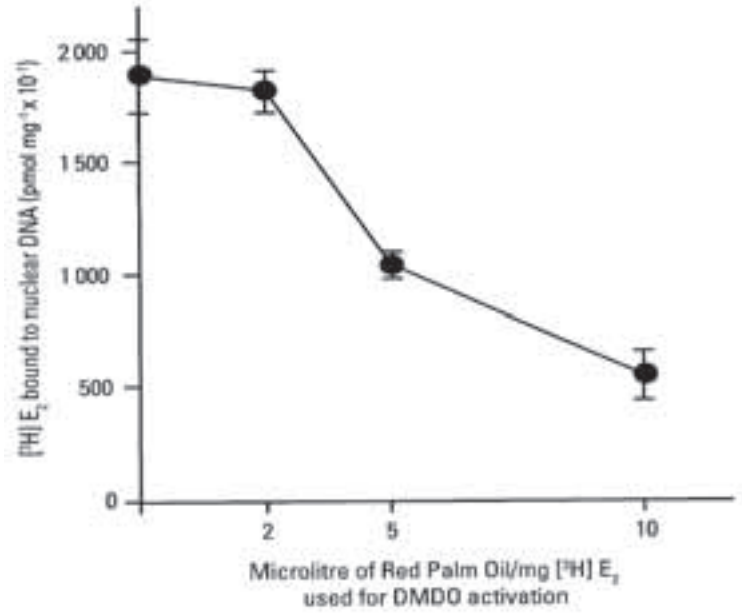

Figure 1. The preventive effect of Red Palm Oil on the formation of E_2 epoxide as reflected by the reduced binding of [^{3}H] labelled E_2 to rat liver nuclear DNA after dimethyldioxirane (DMDO) activation in vitro.

Notes: The 1 mg of E_2 containing 10 μCi [^{3}H] labelled E2 was mixed with 0, 2, 5 or 10 μl Red Palm Oil as indicated and incubated in 1 ml DMDO at room temperature for 1 hr, and vacuum dried. The samples were dissolved in 200 μl DMDO, mixed with 400 μl of the isolated rat liver nuclei (600 μg DNA), and incubated on ice for 2 hr for DNA binding. The nuclear DNA was then purified after RNase and pronase digestion followed by phenol extraction. The purified DNA from each group was re-dissolved in 0.5 rnl H_2O for A_{260} measurement and radioactivity counting. Values given are mean ± SE of 2-3 independent experiments.

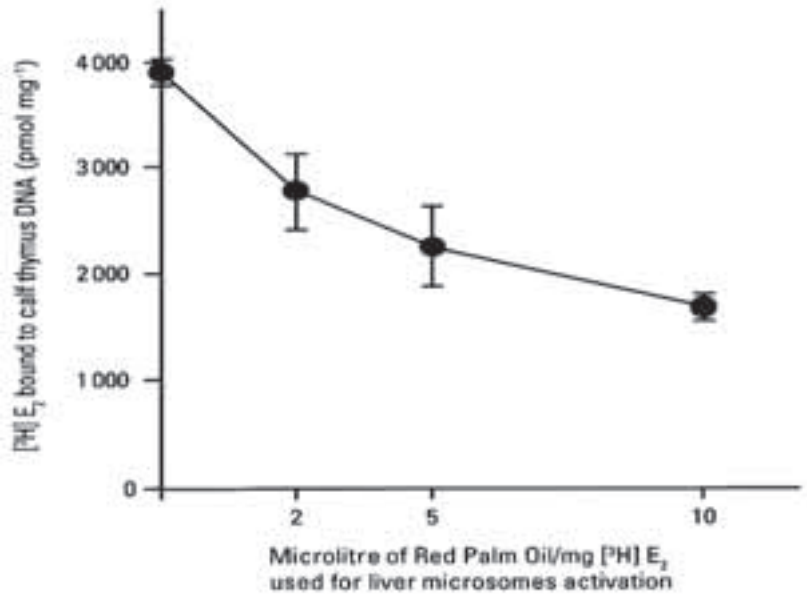

Figure 2. The preventive effect of Red Palm Oil on the formation of E_2 epoxide as reflected by the reduced binding of [^{3}H]labelled E_2 to calf thymus DNA after liver microsomes activation in vitro.

Notes: The 1 mg of E_2 containing 10 μCi [^{3}H]labelled E_2 was mixed with 100 μl dimethylsulfoxide (DMSO) alone (the control group), or with 100 μl DMSO containing 0, 2, 5 or 10 μl Red Palm Oil as indicated. The samples were incubated together with 20 μl microsomes (0.5 mg protein) in buffer A (0.05 M Tri-HC1, pH 7.5 containing 0.25 M sucrose), 20 μl binding buffer (0.9 M Tris- HCl, pH 7.5, 0.06 M $MgCl_2$, 16 mM NADP, l00 mM glucose-6-phosphate) containing 0.2 units glucose-6-phosphate dehydrogenase, and 600 μg calf thymus DNA in 100 μl H_2O for 1 hr at 37°C. The samples were then digested sequentially with RNase A (50 μg, 1 hr), pronase (50 μg, 2 hr) and followed by phenol-chloroform extraction of the DNA. The DNA was precipitated, washed, and counted. Values given are mean ± SE of 2-3 independent experiments.

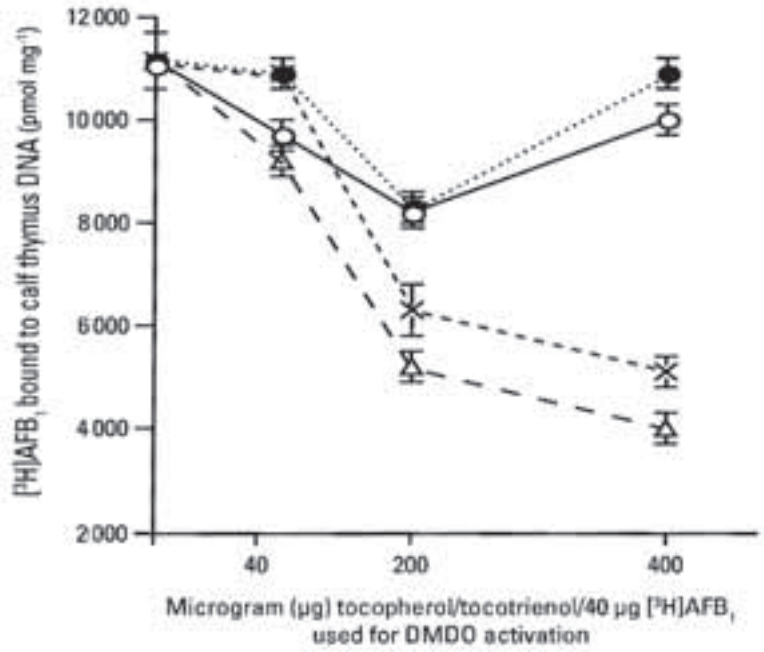

Figure 3. Compare the preventive effect of α-tocopherol, α-, γ- and δ-tocotrienols on the formation of aflatoxin B_1 (AFB_1) epoxide as reflected by the reduced binding of [^{3}H]AFB_1 to calf thymus DNA after dirnethyldioxirane (DMDO) activation in vitro.

Notes: The AFB_1 ,40 μg, containing 5 μCi [^{3}H]AFB_1 was incubated either alone or together with 40, 200 or 400 μg α-tocopherol or one of the tocotrienols as indicated in 0.5 ml DMDO at room temperature for 1 hr. After incubation, the reaction mixtures were vacuum dried. The samples were then dissolved in 160 μl dimethylsulfoxide (DMSO), together with 160 μl calf thymus DNA (400 μg), 160 μl H_2O and incubated on ice for 2 hr for DNA binding. After binding, 1/10 volume of 30% sodium acetate (pH 5.5) was added to precipitate the DNA. The DNA pellet was washed with 70% alcohol to get rid of free [^{3}H]AFB_1. The final DNA precipitate was dissolved in 0.5 ml H_2O for A_{260} measurement and radioactivity counting. The [^{3} H]AFB_1 +α-tocopherol (●- - -●); [^{3}H]AFB_1 + α- tocotrienol (O- - -O); [^{3}H]AFB_1 + γ-tocotrienol (×- - -×); [^{3}H]AFB_1 +, δ-tocotrienol (△- - -△). Values given are means ± SE of 2- 3 independent experiments.

for DNA binding. After binding, 1/10 volume of 30% sodium acetate (pH 5.5) was added, and the DNA was precipitated with four volumes of 100% alcohol for 30 min at -20ºC. The DNA pellet was washed three times with 2 ml 70% alcohol after each centrifugation. The final DNA precipitate was dissolved in 0.5 ml H_2O for A_{260} measurement and radioactivity counting.

Activation of [^{3}H]AFB_1 by Liver Microsomes for Calf Thymus DNA Binding

The procedure for the binding of [^{3}H]AFB_1 to DNA after activation by liver microsomes is essentially the same as described previously (Yu, 1983, Yu, *et al*., 1986; 1990; 1994; 1996). AFB_1 40 μg, containing 5 μCi [^{3}H] labelled AFB_1 was mixed with 60 μl DMSO alone, or with 60 μl DMSO containing 40 200 or 400 μg α-tocopherol or one of the tocotrienols as indicated (*Figure 4*). Then, 400 μg calf thymus DNA in 170 μ1 H_2O was added, the samples were incubated together with 10 μl microsomes (0.5 mg protein) in buffer A (0.05 M TriHCl, pH 7.5 containing 0.25 M sucrose), and 20 μl binding buffer (0.9 M Tris-HC1, pH 7.5, 0.06 M MgCl, 16 mM NADP, 100 mM glucose-6-phosphate) containing 1.0 units glucose-6-phosphate dehydrogenase for 1 hr at 37ºC. They were then digested sequentially with a mixture of RNase A (50 μg) and T_1 (5 units) for 1 hr at 37ºC, and followed with pronase K (50 μg) for 2 hr at 37ºC. The DNA, after phenol-chloroform extraction, was precipitated, washed, and finally dissolved in 0.5 ml H_2O for A_{260} measurement and radioactivity counting.

Activation of [^{3}H]E_1 by DMDO for Rat Liver Nuclear DNA Binding

The procedure for [^{3}H]E_1 binding to nuclear DNA after DMDO activation was essentially the same as described previously for [^{3}H]E_2 activation and binding (Yu *et al.,* 2002; 2004; 2005). Briefly, 1 mg E_1 containing 10 μCi [^{3}H]E_1 was activated either alone, or in the presence of 5 mg of one of the tocopherols

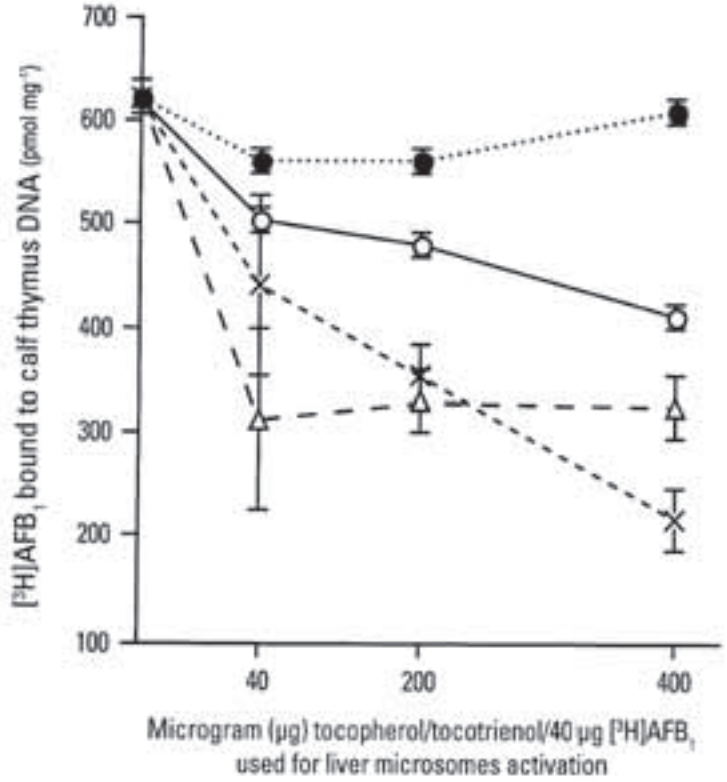

Figure 4. Compare the preventive effect of α-tocopherol, α-, γ- and δ-tocotrienols on the formation of aflatoxin B_1(AFB_1) epoxide as reflected by the reduced binding of [^{3}H]AFB_1 calf thymus DNA after rat liver microsomes activation in vitro.

Notes: The AFB_1, 40 μg, containing 5 μCi [^{3}H] AFB_1 was mixed with 60 μl dimethylsulfoxide (DMSO) alone, or with 60 μl DMSO containing 40, 200 or 400 μg α-tocopherol or one of the tocotienols as indicated. Then, 400 μg calf thymus DNA in 170 μl H_2O was added and the samples were incubated together with 10 μl microsomes (0.5 mg protein) in buffer A (0.05 M Tri-HCl, pH 7.5 containing 0.25 M sucrose), and 20 μl binding buffer (0.9 M Tris-HCl, pH 7.5, 0.06 M MgCl, 16 mM NADP, 100 mM glucose-6-phosphate) containing 1.0 units glucose-6-phosphate dehydrogenase for 1 hr at 37ºC. They were then digested sequentially with a mixture of RNase A (50 μg) and T, (5 units) for 1 hr at 37ºC, and followed with pronase K (50 μg) for 2 hr at 37ºC. The DNA, after phenol-chloroform extraction, was precipitated, washed, and finally dissolved in 0.5 ml H_2O for measurement and radioactivity counting. The [^{3}H]AFB_1 + α- tocopherol (●- - -●) ; [^{3} H] AFB_1 + α-tocotrienol (O- - -O); [^{3}H]AFB_1 + γ-tocotrienol (×- - -×); [^{3}H] AFB_1+ δ-tocotrienol (△- - -△). Values given are means ± SE of 2-3 independent experiments.

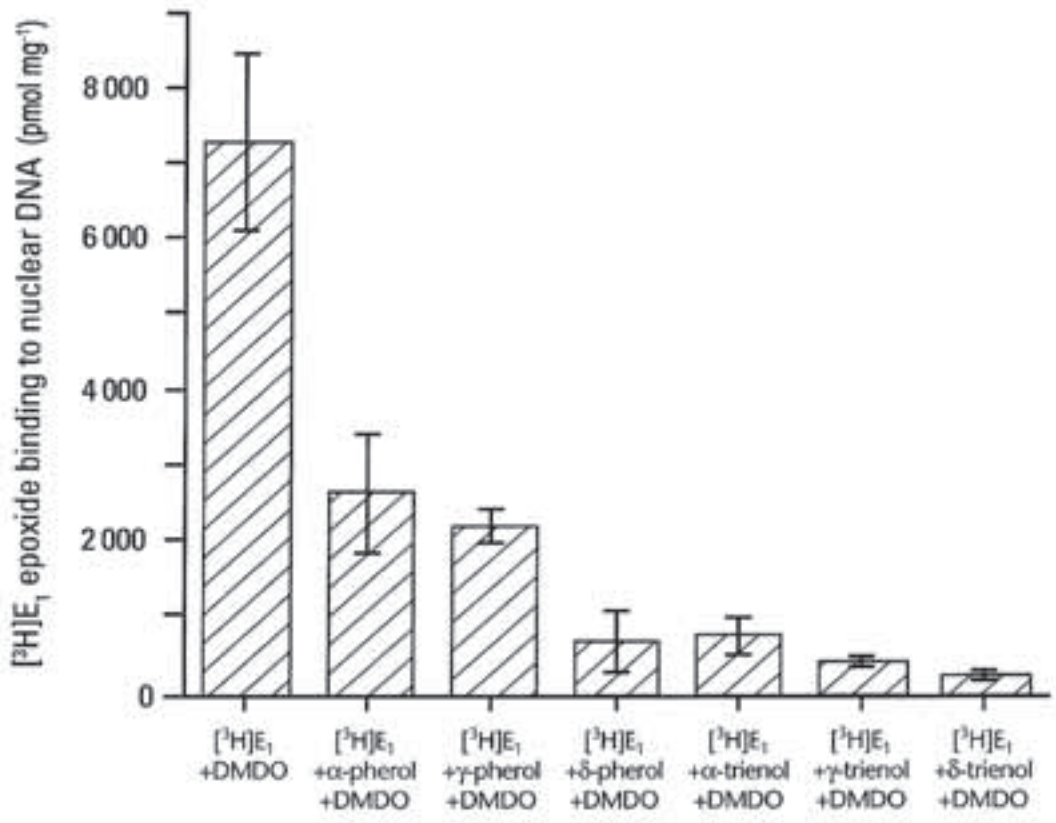

Figure 5. Compare the preventive effect of α-, γ- and δ-tocopherols, and α-, γ- and δ-tocotrienols on the formation of E_1 epoxide as reflected by the reduced binding of [³H]E_1 to rat liver nuclear DNA after DMDO activation in vitro.

Notes: The 1 mg E_1 containing 10 μCi [³H]E_1 was activated either alone, or in the presence of 5 mg of one of the tocopherols or tocotrienols as indicated with 1 ml DMDO at room temperature for 1 hr, and vacuum dried. The samples, dissolved in 200 μl DMSO, were mixed with 400 μl of the isolated rat liver nuclei (600 μg DNA), and incubated on ice for 2 hr for DNA binding. The nuclear DNA was then purified after RNase, T_1 and pronase digestion followed by phenol extraction. The purified DNA from each group was redissolved in 0.5 ml H_2O for A_{260} measurement and radioactivity counting. Values given are mean ± SE of 2-3 independent experiments.

or tocotrienols as indicated (*Figure 5*) with 1 ml DMDO at room temperature for 1 hr, and vacuum dried. The samples, dissolved in 200 μl DMSO, were mixed with 400 μl of the isolated rat liver nuclei (600 μg DNA), and incubated on ice for 2 hr for DNA binding. After binding, the samples were then digested sequentially with a mixture of RNase (100 μg), and T_1 (5 units) for 1 hr, and pronase (50 μg) for 2 hr followed by phenol-chloroform extraction of the DNA (Yu *et al*., 1995; 2002; 2004; 2005). The DNA extract in the final aqueous phase was precipitated with 1/10 volume 30% sodium acetate (pH 5.0) and four volumes of 95% ethanol at -20°C overnight. The DNA pellet was repeatedly washed with 95% alcohol containing 3% sodium acetate to get rid of the free [³H] labelled E_1. Finally, the DNA from each group was re-dissolved in 0.5 ml H_2O for optical density measurement and radioactivity counting.

RESULTS

Table 1 shows that the rate of RNA synthesis for 0.1 ml rat liver nuclei (150 μg DNA) in the presence of 1 mg E_2 under the assay conditions used was 634 pmol [^{32} P]GMP incorporated/mg DNA (100%) after 15 min incubation *in vitro.* However, the rate of RNA synthesis was reduced to only 209 pmol [^{32} P]GMP incorporated/mg DNA (33%) after the nuclei was treated with 1 mg E_2 activated by DMDO under identical assay conditions. This result is in good agreement with our earlier reports (Yu *et al*., 1996, 1998a, b; 2002; 2003; 2004; 2005) suggesting that E_2 after converting to E_2 epoxide by DMDO is able to bind DNA forming DNA adducts impairing the DNA template resulting in the inhibition of DNA-dependent nuclear RNA synthesis. Furthermore, as shown also in *Table 1*, when nuclei were treated with 1 mg E_2 activated by DMDO in the presence of either 5 mg α-tocopherol, 5 mg α-tocotrienol, or 10 μl RPO, the rate of nuclear RNA synthesis was restored to 330 pmol (52%) 646 pmol (102%) and 468 pmol (74%), respectively. These data suggest that α-tocopherol, α-tocotrienol and RPO are all effective in the prevention of E_2 epoxide formation as reflected by the loss of the ability of E_2 to inhibit nuclear RNA synthesis. But there are great differences in this prevention. At 5:1 ratio of tocopherol or tocotrienols to E_2 , α-tocotrienol is able to completely prevent the formation of E_2 epoxide, and α-tocopherol is only

TABLE 1. THE PREVENTIVE EFFECT OF α -TOCOPHEROL, α -TOCOTRIENOL AND RED PALM OIL (RPO) ON THE FORMATION OF E_2- EPOXIDE AS REFLECTED BY THE LOSS OF E_2 INHIBITION ON NUCLEAR RNA SYNTHESIS AFTER DIMETHYLDIOXIRANE (DMDO) ACTIVATION *in vitro*

Group	Nuclear RNA synthesis* (pmol [^{32}P] GMP incorporated/mgDNA)	%
E_2	634 ± 6	100
E_2 + DMDO	209 ± 6	33
E_2 + α-tocopherol (5 mg) + DMDO	330 ± 25	52
E_2 + α-tocotrienol (5 mg) + DMDO	646 ± 13	102
E_2 + Red Palm Oil (10 µl) + DMDO	468 ± 2	74

Notes: *E_2 1 mg, was mixed with α-tocopherol, α-tocotrienol, or Red Palm Oil in the amount as indicated, and reacted with 1 ml DMDO at room temperature for 1 hr. The reaction mixture was then vacuum dried and dissolved in 200 µl dimethylsulfoxide (DMSO). Then, 50 µl of this reaction mixture in DMSO was added to 0.1 ml rat liver nuclei (150 µg DNA), and incubated on ice for 30 min. RNA synthesis was assayed in 0.5 ml assay medium containing 0.1 µCi [α- ^{32}P]GTP at 37°C for 15 min. The radioactive RNA, after TCA precipitation, was collected onto Whatman GF/C filters, which was washed and counted. Values given are mean ± SE of 3-4 independent experiments.

able to prevent the formation around 50%. RPO is also clearly capable to prevent the activation of E_2 to epoxide by DMDO, but the underlying mechanism is more complicated because it contains several powerful antioxidants such as vitamin A precursor carotenoids, tocopherols, tocotrienols as well as the monounsaturated oleic and polyunsaturated linoleic acids, *etc*. (Yu *et al*., 2004).

The evidence that RPO is able to effectively prevent the formation of E_2 epoxide is further demonstrated by direct [^{3}H]E_2 binding to DNA experiments. As shown in *Figure 1*, when 1 mg E_2 containing 10 µCi [^{3}H]E_2 was activated by DMDO, it was able to bind rat nuclear DNA at a level of 18 930 (100%) pmol mg^{-1} DNA. The binding values were reduced to 18 173 (96%), 10 412 (55%), and 5490 (34%) pmol mg^{-1} DNA when 2, 5 and 10 µl RPO respectively, was mixed with the 1 mg of [^{3}H]E_2 for epoxidation by DMDO.

The preventive effect of RPO on the formation of E_2 epoxide was further verified using rat liver microsomes as the activation system (Yu *et al*., 1994; 1996; 1983; 1986; 1990). The results in *Figure 2* show that when 1 mg of E_2 containing 10 µCi [^{3}H] E_2 was activated by rat liver microsomes in the presence of 600 µg calf thymus DNA for 1 hr at 37ºC, the binding of the [^{3}H]E_2 to DNA was 3912 (100%) pmol/mg DNA. However, the values of the binding were reduced to 2775 (71%) , 2228 (57%) and 1682 (43%) pmol mg^{-1} DNA respectively, when 2, 5 and 10 µl RPO were mixed with E_2 at the very beginning for the activation.

Aflatoxin B_1 (AFB_1), a naturally occurring mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasitcus*, is a potent rat liver carcinogen (Busby Jr., *et al*., 1984). Similar to E_2, it requires the activation either by microsomal P450 enzymes (Lin *et al*., 1977; Essigmann *et al*., 1977; Yu, 1983; Yu *et al*., 1986) or by the versatile epoxide-forming oxidant DMDO (Baertschi *et al*., 1988; Yu *et al*., 1994; 1996) to the ultimate carcinogen AFB_1 exo-8,9-epoxide (AFB_1 epoxide) before it is able to bind DNA forming DNA adducts (Lin, *et al*., 1977; Essigmann *et al*., 1977; Baertschi *et al*., 1988;Yu *et al*., 1990). This is believed to be the molecular mechanism for AFB_1 epoxide to initiation of liver cancer carcinogenesis. Based on this fact, the method developed for E_2 was adopted for AFB_1 to screen chemopreventive agents against liver cancer carcinogenesis at the initiation. This screening test determines whether a chemical is able to prevent the formation of AFB_1 epoxide as measured by the loss of the ability of [^{3}H]AFB_1 to bind DNA.

Figure 3 summarizes the preventive effect of α-tocopherol, α-, γ- and δ-tocotrienols on the formation of AFB_1 epoxide activated by DMDO. As the data indicate that when AFB_1 40 µg, containing 5 µCi [^{3}H] AFB_1 was activated alone by DMDO, the binding of [^{3}H]AFB_1 to calf thymus DNA was 11 123 pmol mg^{-1} DNA (100%). However, when the activation was carried out in the presence of 40, 200 and 400 µg α-tocopherol, the values of the binding of [^{3}H]AFB_1 to calf thymus DNA were 9455 (85%), 823l (74%), and 10 901 (98%), respectively. When the activation was carried out in the presence of 40, 200 and 400 µg α-tocotrienol, the values of the binding of [^{3}H]AFB_1 to calf thymus DNA were 10 901 (98%), 8324 (75%), and 8453 (76%), respectively. When in the presence of 40, 200 and 400 µg γ-tocotrienol, the binding values were 10 122 (91%), 6340 (57%), and 5117 (46%), respectively. And in the presence of 40, 200 and 400 µg δ-tocotrienols, the binding values were 9232 (83%), 5228 (47%), and 4004 (36%), respectively. The results from these studies indicate: 1) α-tocopherol is not effective in the prevention of AFB_1 epoxide formation activated by DMDO even with a 10:1 weight ratio of α-tocopherol (mol. wt., 430) over AFB_1 (mol. wt., 312); 2) by comparison, tocotrienols were more effective in the prevention of AFB_1 epoxide formation especially when γ- or δ-tocotrienols were used.

The above conclusion is reconfirmed by experiments using liver microsomes as the activation system. As shown in *Figure 4*, when AFB_1 40 µg, containing 5 µCi [^{3}H]AFB_1 was activated by liver microsomes alone, the binding of [^{3}H]AFB_1 to calf thymus DNA was 622 pmol mg^{-1} DNA (100%). However, when the activation was carried out in the presence of 40, 200 or 400 µg α-tocopherol, the values of the binding of [^{3}H]AFB_1 to calf thymus DNA were

560 (90%), 560 (90%), and 610 (98%), respectively. When the activation was carried out in the presence of 40, 200, or 400 μg α-tocotrienol, the values of the binding of [^{3}H]AFB$_1$ to calf thymus DNA were 504 (81%), 479 (77%), and 411 (66%), respectively. When in the presence of 40, 200, or 400 μg γ-tocotrienol, the binding values were 442 (71%), 330 (53%), and 249 (40%), respectively. And in the presence of 40, 200, or 400 μg δ-tocotrienol, the binding values were 311 (50%), 330 (53%), and 323 (52%), respectively. These results again indicate that α-tocopherol is relatively ineffective in the prevention on the formation of AFB$_1$ epoxide and hence the binding of AFB$_1$ to DNA, and tocotrienols are much more effective in the prevention of AFB$_1$ epoxide formation, especially γ- and δ-tocotrienols.

Breast cancer incidence increases dramatically in women after menopause (Jemal *et al.,* 2005). And since the conversion of androstenedione to E$_1$ is major source of estrogens after the synthesis of E$_2$ in the ovaries ceases in post-menopausal women, the possibility that E$_1$ plays a contributing role in the observed increase in the incidence of breast cancer in women after menopause can not be ruled out. We have reported previously that E$_1$, like E$_2$, could also be activated to epoxide to bind DNA forming DNA adducts, and to inhibit nuclear DNA-dependent RNA synthesis (Yu *et al*., 1998a, b), it is important for these reasons to study and compare the preventive effects of tocopherols and tocotrienols on the formation of E$_1$ epoxide and therefore the potential against E$_1$ induced breast cancer carcinogenesis at the initiation.

Figure 5 summarizes the results from this study. The data indicate that when 1 mg E$_1$ containing 10 μCi [^{3}H]E$_1$ was activated alone by DMDO, the binding of the radioactive E$_1$ to rat liver nuclear DNA was 7271 pmol mg^{-1} DNA (100%). When activated in the presence of 5 mg α-, γ- or δ- tocopherols, the binding values reduced to 2618 (36%), 2181(30%) and 654 (9%) pmol mg^{-1} DNA, respectively. On the other hand, when activated in the presence of 5 mg α-, γ- or δ-tocotrienols, the binding values were further reduced to 727 (10%), 364 (5%) and 218 (3%) pmol mg^{-1} DNA, respectively. These results suggest: 1) both tocopherols and tocotrienols are effective in the prevention of E$_1$ epoxide formation; 2) tocotrienols are more potent than tocopherols in the prevention of [^{3}H]E$_1$ binding to liver nuclear DNA; 3) within the tocopherol and tocotrienol sub-family groups, the order of potency may be arranged as the following: δ-tocopherol > γ-tocopherol > α-tocopherol; and δ-tocotrienol > γ-tocotrienol > α- tocotrienol.

DISCUSSION

RPO is one of the richest natural sources for carotenoids and vitamin E (Cottrell, 1991; Sambanthanmurthi *et al.,* 2000; Edem, 2002; Sundram *et al*., 2003). Since both vitamins A and E are known to play an important role in the prevention of many health-related diseases (Sambanthanmurthi *et al*., 2000; Edem, 2002; Sundram *et al*., 2003; Radhika *et al*.; Benade, 2003; Kritchevsky *et al*., 2002; Stampfer, 1993; Rimm *et al*., 1993; Marchioli *et al*., 1999; Pruthi *et al*., 2001; Yusoff, 2002; Yusuf *et al*., 2000), including cancer (Gaziano *et al*., 2004; Gysin *et al*., 2002; Goh *et al*., 2002; Rahmat *et al*., 1993; Ngah *et al*., 1991), it should be obvious for this basic reason that the use of RPO has significant advantage over other vegetable oils. Our results presented in *Table 1* and *Figures 1* and *2* have clearly shown that RPO has a strong preventive effect on the formation of E$_2$ epoxide as reflected by the loss of the ability of E$_2$ in the inhibition of RNA synthesis and of the ability of E$_2$ to bind DNA. Since RPO contains a mixture of the powerful antioxidants including carotenoids, tocopherols and tocotrienols, these may be the major contributors for the observed preventive effect. Another potential contributing factor should be considered is the fact that RPO contains both mono-unsaturated oleic and the polyunsaturated linoleic acids. From our earlier studies we have demonstrated that these mono- and polyunsaturated fatty acids are effective competitive epoxidation substrates and are able to prevent E$_2$ epoxidation *in vitro* (Yu *et al*., 2004).

AFB$_1$ is a potent liver carcinogen (Busby Jr *et al*., 1984). Similar to the female hormones E$_2$ and E$_1$, it requires the activation to AFB$_1$ epoxide before it is able to bind DNA (Lin *et al*., 1977; Essigmann *et al*., 1977; Yu, 1983; Yu *et al*., 1986; Baertschi *et al*., 1988; 1994; 1996). It is important, for this reason, to find out whether α-tocopherol and tocotrienols are also able to prevent the AFB$_1$ epoxidation and therefore the potential to prevent AFB$_1$ induced liver cancer at the initiation. Results presented in *Figures 3* and *4* indicate that α-tocopherol is not effective in the prevention of AFB$_1$ epoxide formation under the experimental conditions used. Tocotrienols, on the other hand, are more effective in the prevention using either DMDO (*Figure 3*), or rat liver microsomes activation system (*Figure 4*).

We have reported previously that E$_1$, like E$_2$ could be activated by DMDO to epoxide and to bind DNA (Yu *et al*., 1988a, b; 2002). It is known that E$_1$ is the major female hormone for post-menopausal women, and since breast cancer incidence increases sharply after menopause (Jemal *et al*., 2005), it is an interesting but unsettled question whether E$_1$ is causally related to this phenomenon. That aside, it is basically important to find out whether tocopherols and tocotrienols are able to prevent E$_1$ epoxide formatiom. The results as shown in *Figure 5* clearly indicate that both tocopherols and tocotrienols are very effective in the prevention of E$_1$

epoxide formation, although by comparison tocotrienols are still more effective chemopreventive agents than tocopherols.

The underlying mechanism in the prevention of epoxidation of a chemical carcinogen by chemopreventive agents is believed through a competitive epoxidation inhibition mechanism (Yu, 2002; Yu *et al*., 2002). We further believe that there are differences in epoxidation potentials among various chemical carcinogens. It is for this reason that tocopherols and tocotrienols are able to effectively prevent the formation of E_2 and E_1 epoxides , and are less effective against the formation of AFB_1 epoxide.

Prevention is the best way to win the war on cancer. In this article, data are presented to show the preventive effect of tocopherols and tocotrienols on the formation of E_2, E_1 and AFB_1 epoxides. As a dietary supplement, tocopherols and especially tocotrienols may have the potential to prevent breast and liver cancers.

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