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Dose-Dependent Cholesterolemic Activity of Tocotrienols and α-Tocopherol

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ABSTRACT

Tocotrienols and tocopherols are isoforms of vitamin E. Vitamin E may exhibit antioxidant, prooxidant and non-antioxidant activities depending upon circumstances. In this study, the effect of tocotrienols and α-tocopherol on the activities of HMG CoA reductase and cholesterol 7 α-hydroxylase was investigated. Pure tocotrienols were isolated from palm fatty acid distillate and pure α-tocopherol was obtained commercially. Guinea pigs were treated with different dosages of tocotrienols and α-tocopherol. After the treatment period, animals were sacrificed and liver microsomes were prepared. HMG CoA reductase and cholesterol 7α-hydroxylase were assayed using tracer techniques. Our results showed that the effects of tocotrienols and α-tocopherol on the activities of both the enzymes were dose-dependent. At low dosages, both tocotrienols and α-tocopherol exhibited an inhibitory effect on both the enzymes. Moreover, tocotrienols were a much stronger inhibitors than α-tocopherol. At high dosages, on the other hand, tocotrienols and α-tocopherol showed opposite effects on the enzymes. While tocotrienols continued to exhibit an inhibitory effect, α-tocopherol actually exhibited a stimulatory effect on both the enzymes. A possible explanation for this observation is suggested.

INTRODUCTION

Tocotrienols and tocopherols are isoforms of vitamin E, the well-known fat-soluble natural antioxidant. The distribution of tocotrienols is restricted to a few plant sources, palm oil being the most abundant source of tocotrienols. Tocopherols, on the other hand, are widely distributed in nature being found in all plant and animal products (Kammal-Eldin & Appelqvist, 1996).

The antioxidant function of α-tocopherol is well documented. But it is now evident that depending on the dose levels, α-tocopherol could exhibit pro-oxidant activity. More recently evidence has accumulated to show that vitamin E has biological functions other than its classical antioxidant activity (Traber & Packer, 1995; Azzi & Stocker, 2000).

α-Tocopherol or vitamin E has been implicated in atherogenesis and cardiovascular disease in humans, though the exact nature of involvement of vitamin E in atherogenesis and cardiovascular disease is still unclear (Chan, 1998; Pryor, 2000). High blood cholesterol level is a recognized risk factor in atherosclerosis and cardiovascular disease (Grundy, 1986). Blood cholesterol level is modulated by the activities of cholesterol metabolizing enzymes in the body. This paper reports results of our studies on the effect of tocotrienols and α-tocopherol

administration on two key regulatory enzymes of cholesterol metabolism namely HMG CoA reductase and cholesterol 7 α-hydroxylase activities in the guinea pig.

MATERIALS AND METHODS

Treatment of animals

Male and female albino Harley guinea pigs were obtained from the Animal Breeding Unit, Faculty of Medicine, University of Malaya. On arrival, their body weights ranged from 700 to 900g and they were kept individually in cages in the experimental room with controlled temperature, 25±2°C, and lighting, 12-hour light and dark cycles. The animals were acclimatized for two weeks in the experimental room. Food and water were given *ad libitum.*

The guinea pigs were divided into groups of approximately equal mean body weights. The control group was injected with 200 μl of vitamin E-free palm olein triglycerides (POTG) (Khor & Tan, 1992) and the treated groups were injected intra-peritoneally with different doses of tocotrienols (T3) or α-tocopherol (α-T) dissolved in 200 μl of POTG for six consecutive days. The animals were then sacrificed at midnight. Blood was taken by cardiac puncture after ether anesthesia. Liver was excised, wiped clean, weighed and stored at -80°C. Sera were prepared by centrifugation at 2,000 g for 20 min after standing the blood for 45 min at room temperature (Tan *et al.*, 1991).

Sources of α-T and T3

α-T was purchased from Sigma Chemical Co., U. S. A. and T3 was isolated from palm oil fatty acid distillate (PFAD) as described by Khor, Chieng & Ong (1995a; 1995b).

Assay of microsomal HMG CoA reductase (HMGCR) activity

Liver microsomes were prepared and HMGCR activity was assayed using a tracer technique described by Shapiro & Rodwell (1969) and modified by Khor, Chieng & Ong (1995a; 1995b) for the guinea pig system.

Assay of microsomal cholesterol 7 α-hydroxylase (C7aH) activity

Liver microsomes were prepared according to Einarsson *et al.* (1987) and C7αH activity was assayed in the microsomes as described by Van Cantfort & Gielen (1975) with some modifications in order to optimize the assay conditions for the present system (Raajeswari, 2000).

Protein determination

The protein content in the liver microsomes was determined according to the method of Bradford (1976).

Serum vitamin C determination

Serum vitamin C level was determined in the deproteinised serum by a titrimetric method based on the reduction of 2,6-dichlorophenol-indolphenol (DCPIP) by ascorbic acid (Boyer, 1986). Vitamin C concentration was estimated by comparing with ascorbic acid standards.

Statistical analysis

The significance of differences of means of different groups was assessed by ANOVA and student *t* test. $P < 0.05$ was considered significant.

RESULTS

Several series of experiments were carried out. All animals appeared healthy during the course of the experiments and there was no significant difference in body weight gains at the end of the treatment periods. The vitamin C status of treated animals was no different from that of the control.

Results of administration of 1, 3 and 5 mg of T3 and α-T on liver HMGCR activity are shown in Figure 1. At a dosage of 1 mg/day palm T3, the results showed very strong inhibitory effect (67.4% inhibition) on the hepatic HMGCR activity compared to the control group ($P<0.001$). As the dosages of T3 were increased from 1 mg/day to 3 or 5 mg/day, the inhibitory effect of palm T3 on hepatic HMGCR activity decreased gradually from 67.4% to 48.6% and 23.7% respectively; but the inhibition on HMGCR was still significant ($P<0.01$). However, when α-T was administered at 1 mg/day, a small and marginally significant ($P<0.05$) inhibitory effect (15.6%) on hepatic HMGCR activity compared to the control group was observed. However, when the dosages of α-T were increased from 1 mg/day to 3 or 5 mg/day, the inhibitory effect of α-T on HMGCR activity was completely lost. In fact, at 5 mg/day α-T exhibited 2.4% stimulation on HMGCR activity (Figure 2).

Another experiment was carried out to investigate the effect of 5, 20 and 50 mg of α-T on HMGCR activity and the results are shown in Figure 3. These results show that at a dosage of 5 mg/day, α-T produced a significant inhibition (44%) on HMGCR compared to the control. At a dosage of 20 mg/day α-T did not produce any significant inhibition on HMGCR compared to the control. At a dosage of 50 mg/day, α-T caused significant activation (90%) of HMGCR activity compared to the control.

Results from the above experiments clearly demonstrate that tocotrienols inhibit HMGCR activity at all dosages but as to whether α-T inhibits or stimulates HMGCR activity would depend on the dosages.

Another experiment was carried out to investigate if α-T has the ability to attenuate the inhibitory effect of T3 on HMGCR when T3 and α-T are given together in a mixture. The results (Figure 4) show that T3 at 10 mg level significantly inhibited HMGCR activity compared to the

control. When T3 at 10 mg was given together with 5 mg of α-T, the inhibition on HMGCR activity was significantly reduced compared to that of T3 alone.

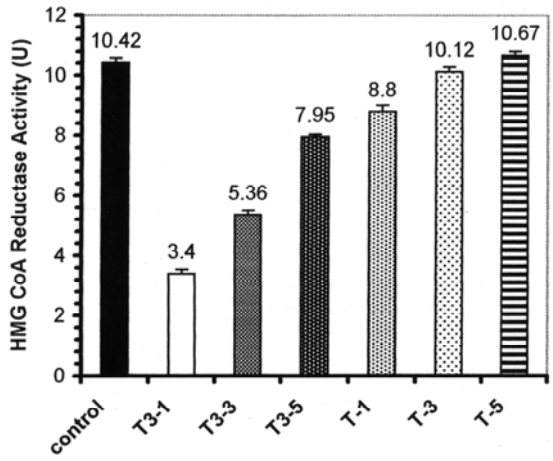

Figure 1. Effect of tocotrienols (T3) and α-tocopherol (T) on HMG CoA reductase activity

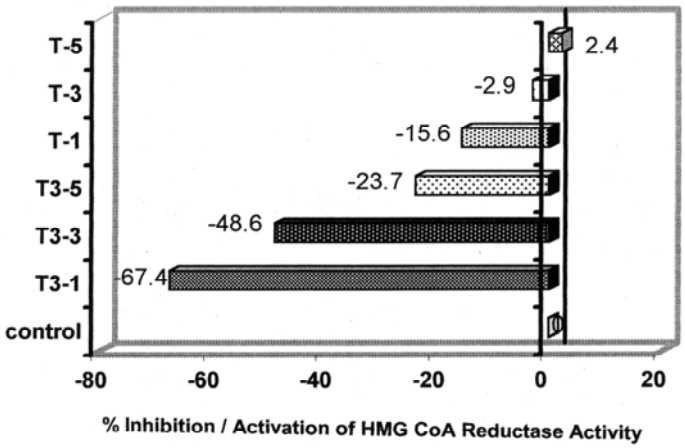

Figure 2. Percentage inhibition / activation of HMG CoA reductase by tocotrienols (T3) and α-tocopherol (T)

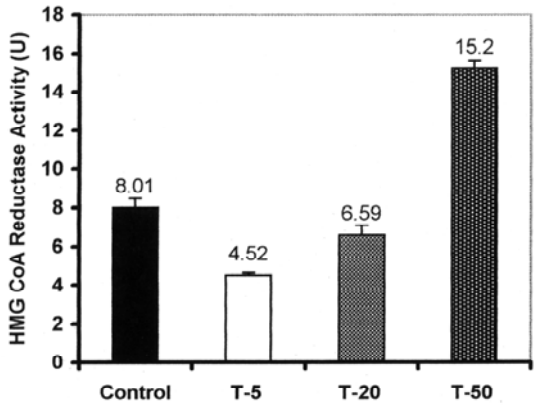

Figure 3. Effect of α-tocopherol (T) on HMG CoA reductase activity

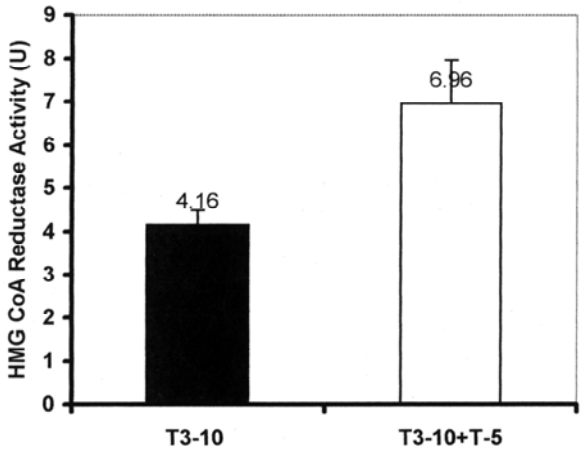

Figure 4. α-Tocopherol (T) attenuates inhibitory activity of tocotrienols (T3) on HMG CoA reductase

Figure 5 shows that T3 at 10 mg level produced about 48% inhibition of HMGCR activity compared to the control. When T3 was given together with 5 mg of α-T only 13% of inhibition on HMGCR activity was achieved. These results clearly demonstrate that α-T could attenuate the hypocholesterolemic activity of T3 if the ratio of α-T to T3 is high.

The effects of administration of palm T3 and α-T on hepatic microsomal C7αH activity are shown in Figure 6. Palm T3 given at a dose of 1 mg/day significantly inhibited hepatic C7αH activity (78 % inhibition) compared to the control values ($P<0.001$). When the dosages of palm T3 were increased from 1 mg to 3 and 5 mg/day, the inhibition on C7αH activity decreased progressively from 78 % to 59.8 % and to 33.3 % respectively. However, the inhibition on C7αH by palm T3 was still significant ($P<0.05$) compared to the control. On the other hand, when α-T was given at a dose of 1 mg/day, it resulted in significant inhibition (42%) on the hepatic C7αH activity compared to the control ($P<0.01$). When the dose of α-T was increased from 1 mg to 3 mg/day, the inhibition on C7αH (14.6%) was much reduced compared to the control group. When the dosage of α-T was increased to 5 mg/day, a significant activation (14.4 %) of the hepatic C7αH activity in comparison to the control ($P<0.05$) was observed (Figure 7).

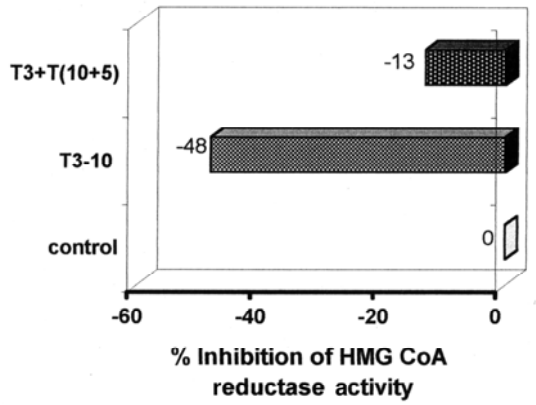

Figure 5. Percentage inhibition / activation of HMG CoA reductase activity by tocotrienols (T3) and a mixture of tocotrienols (T3) and α-tocopherol (T)

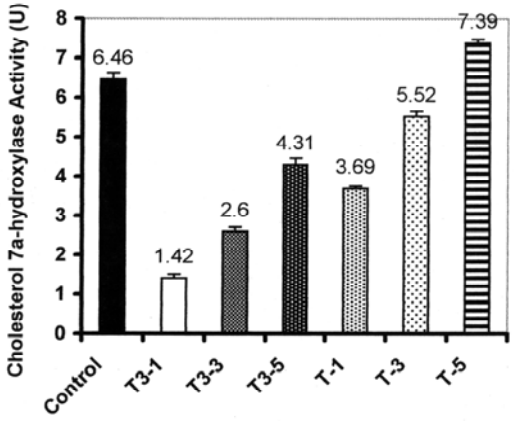

Figure 6. Effect of tocotrienols (T3) and a-tocopherol (T) on cholesterol 7 α-hydroxylase activity

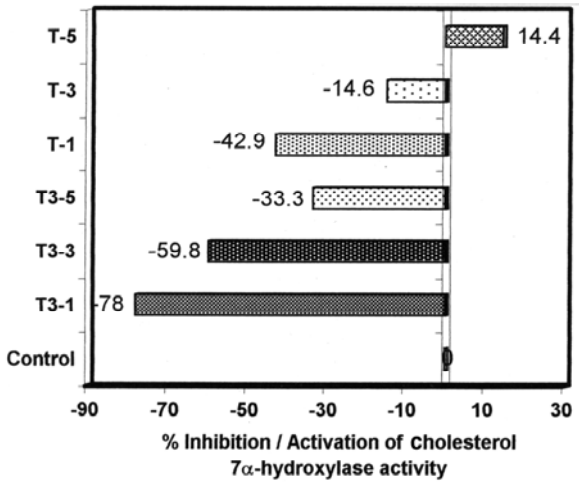

Figure 7. Percentage inhibition / activation of cholesterol 7 α-hydroxylase activity by tocotrienols (T3) and a-tocopherol (T)

The results presented here clearly demonstrate that T3 and α-T have an effect on the activities of hepatic HMGCR and C7αH in the guinea pigs. While palm T3 consistently inhibited both HMGCR and C7αH activities at all three dosages, α-T showed inhibitory effect only at a low dose (1 mg/day) and stimulated both the enzymes at a high dose of 5 mg/day.

DISCUSSION

The guinea pig was chosen as the animal model because cholesterol metabolism in the guinea pig is in many ways similar to that of humans (Fernandez, 2001). However, cholesterol metabolism in the guinea pig is modulated by vitamin C status. Hence in our studies, the vitamin C status of the guinea pigs was closely monitored and our results show no significant difference between the treated and the control groups. Therefore the vitamin C factor did not contribute to the alterations in cholesterol metabolism in our studies.

Recent large-scale human trials such as the CHOAS (Stephens *et al.,* 1996) and Health Professional Follow-Up Study (Rim *et al.,* 1993) clearly demonstrate the protective role of vitamin E or more specifically α-T intake and reduction in mortality from coronary heart disease (CHD). But the protective action of vitamin E or α-T against CHD is still unresolved. Previous attempts to investigate the hypocholesterolemic activity of vitamin E or α-T have produced inconsistent results in several human trials. Several groups reported that α-T supplementation produced no effect on serum cholesterol levels (Tsai *et al.,* 1978; Schwartz & Rutherford, 1981; Ehnholm *et al.,* 1982; Kesaniemi & Grundy, 1982; Stephens *et al.,* 1996) while other groups had observed slight increased serum cholesterol level after α-T supplementation (Chase, Dupont & Mathias, 1981; Howard, Rundell & Batsakis, 1982). Therefore whether α-T has any hypocholesterolemic activity in humans is still unresolved.

Previous studies (Pearce *et al.,* 1992) showed that α-T had no inhibitory activity on HMGCR in chickens and rat hepatocytes in culture. Qureshi *et al.* (1996) reported that adding 120 mmol/g of α-T to the control diet stimulated hepatic HMGCR activity by 7.5% in young chicken. In the guinea pig model, α-T showed some weak inhibitory activity on HMGCR at low dose level (1 mg/day) and gave a very strong stimulatory effect at high dose level (5 mg/day) (Figures 1 & 2). This observation is in agreement with our earlier reports (Khor *et al.,* 1995b; Khor & Ng, 2000) and with that of Qureshi *et al.* (1989) who observed stimulation of cholesterol metabolism by α-T in the avian model. Thus, it appears that the cholesterolemic activity of α-T was dose-dependent; at low dose level α-T exhibited weak inhibitory activity on HMGCR whereas at high dose level α-T showed strong stimulatory activity on HMGCR in the guinea pig. (Khor *et al*, 1995b; Khor & Chieng, 1996; Khor & Ng, 200) The dose-dependent nature of action of α-T on endothelial functions was observed by Keaney *et al.* (1994) who reported that at low dose level, α-T improved and at high dose α-T worsened endothelial vasodilator function. The mechanism of this dose-dependent, dual antagonistic action of α-T is still unknown.

Tocotrienols, the less common isoform of vitamin E, were first isolated from barley flour and demonstrated to inhibit HMG CoA reductase and lower plasma cholesterol levels in young broilers (Qureshi *et al.,* 1986). Since then, the interest in tocotrienols as a potential cholesterol-lowering agent has increased with time. Pearce *et al.* (1992) isolated tocotrienols from tocotrienol-rich fractions from palm oil and demonstrated that tocotrienols inhibited HMGCR activity in rat hepatocytes and HepG2 cells in culture. Khor *et al.* (1995a; 1995b) and Khor & Ng (2000) reported that pure tocotrienols isolated from palm oil fatty acid distillate inhibited strongly liver HMGCR activity in the guinea pig. The inhibitory activity of palm tocotrienols was further confirmed in the present series of study (Figures 1 & 2). The observation that tocotrienols is a more potent inhibitor of HMGCR at low doses than at higher doses (Figures 1 & 2) confirms earlier observations by Pearce *et al*. (1992) and Khor *et al.* (1995a; 1995b). It was thereby postulated that if there was a mechanism for *in vivo* conversion of tocotrienols to α-T, the reduction in inhibitory activity of tocotrienols at higher dose could be explained because α-T would accumulate under treatment condition of high doses of tocotrienols and would stimulate HMGCR activity. In fact it was reported that α-T accumulated several folds higher compared to the control in the serum and liver of guinea pigs treated with pure tocotrienols only (Khor *et al*. 1995a; 1995b) suggesting that some tocotrienols might have been converted to α-T. This

postulation received confirmative support recently from Qureshi *et al.* (2001) who reported in vivo conversion of α-[^{3}H]- and [^{14}C]-desmethyl tocotrienols to α-tocopherol.

From the above discussion, it is now clear that tocotrienols can be converted to α-T *in vivo*, but the rate of this conversion is still not resolved. Since α-T can exhibit stimulatory activity at high doses, it would be expected to reduce the positive hypocholesterolemic activity of tocotrienols. The evidence that α-T could attenuate the hypocholesterolemic effect of T3 was provided by Qureshi *et al.* (1996) and Khor & Ng (2000) who separately reported that α-T given in sufficient quantities together with tocotrienols attenuated the hypocholesterolemic activity of tocotrienols in chicken and in guinea pigs respectively.

The results of the present series of studies and those of Qureshi *et al.* (1996) would explain the dilemma encountered in the human trials of Palm-Vitee, which yielded conflicting results. Palm-Vitee, a product of MPOB, is a tocotrienol-rich preparation containing substantial amounts of α-T in addition to tocotrienols (Tan *et al*., 1991). In the human trials, different research groups gave one to four capsules of Palm-Vitee to their human subjects. Those groups (Tan *et al.*, 1991; Qureshi *et al.*, 1991) that used one and two capsules of Palm-Vitee for their experiments obtained positive hypocholesterolemic results while those groups (Wahlqvist *et al.*, 1992; Mensink *et al.*, 1999) that used four capsules of Palm-Vitee for their experiments obtained no positive results. The discrepancy of the observations could be explained on the basis of our present findings and that of Qureshi *et al.* (1996) that α-T could attenuate the positive effect of tocotrienols if present in sufficiently high quantities in the system.

Although the effect of tocotrienols on HMG CoA reductase is now established at least in experimental animal models, the effect of tocotrienols on cholesterol 7α-hydroxylase (C7αH) activity has not been reported before. Our present results (Figures 6 & 7) showed that tocotrienols inhibited significantly liver C7αH activity at 1, 3 and 5 mg levels and that the inhibition was stronger at low doses than at high doses. On the other hand, α-T inhibited significantly liver C7αH activity only at 1 mg level, showed no effect at 3 mg level and showed strong activation of C7αH activity at 5 mg level. (Figure 7)

HMGCR and C7αH are two key regulatory enzymes in the cholesterol metabolic pathway. The activity of these two enzymes is affected in parallel manner and in separate ways by different treatment methods or agents (Bjorkhem, Lund & Rudling, 1997). The present results show that HMGCR and C7αH activities are affected by tocotrienols and α-T either in a parallel manner or in separate ways depending on the doses of tocotrienols and α-T administered.

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