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# Effect of Dietary Vitamin E on Platelet Tocopherol Values and 12-Lipoxygenase Activity (42654)

ALVIN C. CHAN, SUSAN T. ST. MAURICE, AND CHERYL E. DOUGLAS

*Department of Biochemistry, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5*

*Abstract.* This study was designed to evaluate the effects of different amounts of dietary vitamin E on platelet tocopherol levels and 12-lipoxygenase activity when exogenous arachidonic acid was used as substrate. Weanling male Sprague–Dawley rats were fed diets containing 0, 50, and 5000 ppm of D-$\alpha$-tocopherol acetate for 4 months. Platelet tocopherol was increased with increasing concentrations of dietary vitamin E; however, the conversion of exogenously added arachidonate by platelet to 12-HETE (12-hydroxyeicosatetraenoic acid) and thromboxane B2 from these three dietary groups was essentially the same. This study provides direct evidence that platelet 12-lipoxygenase activity is independent of its vitamin E content when exogenously added arachidonate was used as substrate. 

Platelets are capable of metabolizing arachidonic acid via two major pathways leading to the formation of biologically active eicosanoids. One pathway is catalyzed by cyclooxygenase, a membrane-bound enzyme responsible for the biosynthesis of thromboxanes and prostaglandins (1, 2), and the other by 12-lipoxygenase, a soluble enzyme which catalyzes the oxidative conversion of arachidonate to 12-HPETE (12-hydroperoxy-eicosatetraenoic acid) which is rapidly reduced by peroxidase to 12-HETE (12-hydroxy-eicosatetraenoic acid) (3, 4). Although it is well established that the products of the cyclooxygenase reaction are mediators of platelet aggregation (2, 5), the precise biological role of the lipoxygenase products is less clear. Traditionally 12-HETE has been thought to act solely as a stimulator of neutrophil chemotaxis (6), but recently 12-HETE has been reported to be a potent inhibitor of platelet phospholipase $A_2$ (7) and may, therefore, regulate arachidonate metabolism at the level of its release from phospholipids.

The enzymic oxygenation of arachidonic acid is a free radical-initiated reaction requiring a small but continuous presence of cellular peroxides for activation (8). Endogenous compounds that have pro- or antioxidant properties can alter the rate of peroxide formation and, hence, the rate of the eicosanoid pathways. We have previously demonstrated that in rat platelets, thrombin-stimulated thromboxane synthesis from endogenous arachidonate can be suppressed by increasing amount of vitamin E in the diet (9). However, there is conflicting evidence on the effect of vitamin E on lipoxygenase. For example, platelets from vitamin E-deficient rabbits have been shown to form greater amounts of 12-HETE from exogenous arachidonate than controls (10); other studies indicate that vitamin E has no effect on 12-HETE formation in rat or human platelets, respectively (11, 12). The present study was undertaken to determine (a) the extent by which tocopherol levels in platelets can be regulated by the intake of this vitamin and (b) the effect of such alteration on platelet metabolism of exogenously added arachidonic acid.

**Materials and Methods.** *Animals, diets, and chemicals.* Male weanling Sprague–Dawley rats (average body weight 50 g) were fed a semi-purified diet which contained either 0, 50, or 5000 ppm of D-$\alpha$-tocopherol acetate for 4 months. The composition of the basal diet was (% weight): vitamin-free casein (20%), DL-methionine (0.3%), corn starch (10.2%), dextrose (50%), alphacel fiber (5%), AIN salt mix (3.5%), AIN vitamin mix without tocopherol (1.0%), stripped corn oil (10%). Food and tap water were provided at all times. 1-[$^{14}$C]Arachidonic acid, specific activity 56.5 mCi/mmole, was obtained from



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New England Nuclear Corp. (Boston, MA). Unlabeled arachidonic acid (AA) was from Sigma Chemical Co. (St. Louis, MO).

*Isolation of platelets.* Blood was obtained via aortic puncture from rats under light diethyl ether anesthesia and collected into citrate-containing tubes to a final concentration of 0.38% citrate. Platelets were isolated by differential centrifugation as described previously (13). The isolated platelets were washed and resuspended in a calcium-free Ringer–Tyrode buffer containing 2 m*M* EDTA.

*Determination of 12-lipoxygenase activity.* Both labeled and unlabeled arachidonate were mixed to give a specific activity of 3.42 $\mu$Ci/$\mu$mole and was purified by silicic acid column before use. In a routine assay, platelet protein was predetermined by the method of Lowry *et al.* (16) and 150 $\mu$g of washed intact platelet was incubated with 30 $\mu M$ of AA (120,000 dpm) for 3 min at 37°C with gentle stirring. AA was added in 5 $\mu$l ethanol to a volume of 500 $\mu$l platelet suspension. Incubation was stopped with the addition of 1 *N* formic acid to pH 3.5. AA and its products were recovered using two diethyl ether extractions of six times the assay volume, separated by thin-layer chromatography (250 $\mu$m silica gel G-coated, J. T. Baker Chemical Co., Phillipsburg, NJ), and developed in chloroform:methanol:acetic acid: water (90:8:1:0.8, v/v/v/v). Products were visualized and quantitated by a Berthold thin-layer chromatography linear analyzer equipped with a radiodetector. All extractions and separation by thin-layer chromatography were done immediately after incubation of platelets. However, we found that 12-HETE and $TxB_2$ were stable in ether extract when stored at 4°C overnight.

*Determination of platelet tocopherol.* Total lipid from an appropriate amount of platelets was extracted by the method of Bligh and Dyer (14) and a known amount of tocopherol acetate was added as internal standard. These compounds were separated by HPLC with a reverse-phase column (C-18 Bondapac) using a solvent system containing methanol:water 95:5, v/v. Tocopherol was quantitated by using the peak height ratio between tocopherol and tocopherol acetate as previously described (15). Protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as standard. Statistical analysis was performed by the analysis of variance.

**Results.** *Metabolism of arachidonate by rat platelet and effects of inhibitors.* In order to optimize the incubation conditions, the time course and amount of platelet protein in the conversion of arachidonic acid (AA) by washed intact rat platelets from chow-fed rats were studied (Figs. 1 and 2). Conversion of both lipoxygenase product 12-HETE and cyclooxygenase product $TxB_2$ were linear up to 4 min of incubation time. This was accompanied by a linear loss of added AA (Fig. 1). However, these conversions were extremely sensitive to the amount of platelets present in the incubation. Figure 2 shows that 12-HETE production was linear below 300 $\mu$g of platelet protein whereas $TxB_2$ generation was linear below 100 ug of platelet protein.

In order to verify that the products detected were indeed from the lipoxygenase and cyclooxygenase pathways, the known lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) and the cyclooxygenase inhibitor indomethacin were tested in the incubation system. Table I shows that both compounds were effective but nonspecific inhibitors of the lipoxygenase and cyclooxygenase

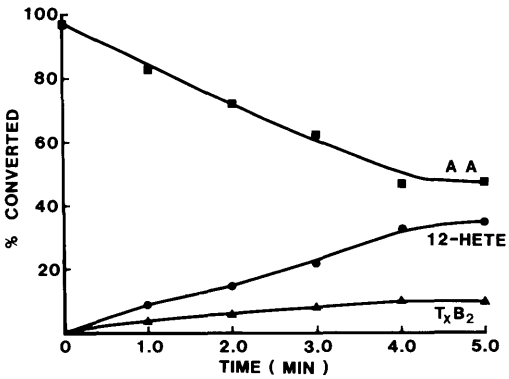


FIG. 1. Time course of the conversion of arachidonate (AA) by washed intact platelets from chow-fed rats (150 $\mu$g protein) via the lipoxygenase and cyclooxygenase pathways. 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; $TxB_2$, thromboxane $B_2$. Details of incubation conditions and quantitation of products are described under Materials and Methods.

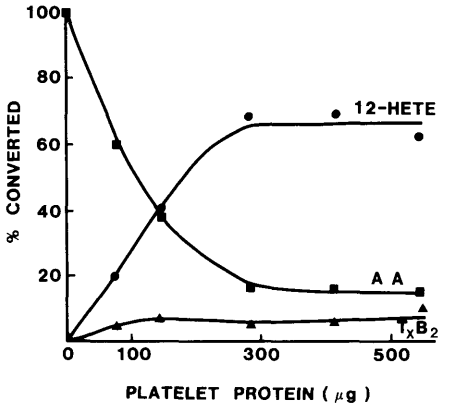


FIG. 2. Conversion of arachidonate (AA) by washed intact platelets from chow-fed rats via the lipoxygenase and cyclooxygenase pathways; dependency of amount of platelet protein. Incubation time was 5 min. Details of incubation conditions and quantitation of products are described under Materials and Methods.

pathways. At 5 $\mu M$, indomethacin exerted a 45% inhibition in both 12-HETE and $TxB_2$ conversion while NDGA inhibited 67 and 69% of lipoxygenase and cyclooxygenase products, respectively. It is worth noting that at the concentrations tested, none of the inhibitors was able to completely abolish the enzymic conversion of AA.

*Effect of dietary vitamin E on platelet vitamin E and AA metabolism.* After 4 months of feeding similar diets containing different levels of vitamin E, rat platelet tocopherol showed a trend which reflects the amount of vitamin E present in the diets (Table II). However, raising the dietary vitamin E content 100-fold (represented by the 5000 ppm supplemented diet) only resulted in a 2-fold increase in platelet tocopherol concentration. There is significant difference ($P < 0.05$) among platelet tocopherol values for the three dietary groups. In this study, the platelet tocopherol level from animals fed 50 ppm $\alpha$-tocopherol were in complete agreement with that reported by Lehmann (18).

The conversion of AA by washed intact platelets from the three dietary groups was investigated. In spite of an increase in tocopherol levels which is reflective of dietary intake, the conversion of AA into 12-HETE via the 12-lipoxygenase pathway was essentially unaltered. The conversion of AA into $TxB_2$ via the cyclooxygenase pathway, however, showed a decreasing trend against increasing dietary intake, but the difference is not statistically significant (Table II). Hence, the data indicate that the activities of 12-lipoxygenase and cyclooxygenase in rat platelet are not dependent on the tocopherol level in these cells when exogenous arachidonate is used as substrate.

**Discussion.** Results from this study show that intact unstimulated rat platelets rapidly metabolize exogenously added arachidonate via the lipoxygenase and cyclooxygenase pathways and the rate of these reactions is highly dependent on the reaction time as well

TABLE I. INHIBITORY EFFECT OF INDOMETHACIN AND NDGA (NORDIHYDROGUAIARETIC ACID) ON RAT PLATELET LIPOXYGENASE AND CYCLOOXYGENASE PATHWAYS[a]

| | 12-HETE[b] | % Inhibition | $TxB_2$[b] | % Inhibition |
|---|---|---|---|---|
| Indomethacin ($\mu M$) | | | | |
| 0 | 18.08 | 0 | 6.84 | 0 |
| 2.5 | 13.11 | 28 | 5.03 | 26 |
| 5.0 | 9.97 | 45 | 3.79 | 45 |
| NDGA ($\mu M$) | | | | |
| 0 | 21.49 | 0 | 7.86 | 0 |
| 2.5 | 9.95 | 54 | 3.70 | 53 |
| 5.0 | 7.16 | 67 | 2.41 | 69 |
| 10.0 | 3.06 | 86 | 2.02 | 74 |

[a] Washed intact rat platelets (150 $\mu$g protein) were preincubated for 5 min with various amounts of drug in 5 $\mu$l of dimethylsulfoxide (DMSO). Reaction was initiated with the addition of labeled arachidonate for 3 min. Extraction and quantitation of products were as described under Materials and Methods.

[b] Represents the percentage conversion of added labeled arachidonate. 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; $TxB_2$, thromboxane, $B_2$.

TABLE II. EFFECTS OF VARYING DIETARY VITAMIN E CONCENTRATIONS ON PLATELET TOCOPHEROL LEVELS AND 12-LIPOXYGENASE ACTIVITY[a]

| Dietary vitamin E (ppm) | Platelet tocopherol[b] (μg/mg protein) | 12-HETE[c] (% conversion) | $TxB_2$[c] (% conversion) |
|---|---|---|---|
| 0 | 0.06 ± 0.02 (5)[d] | 19.7 ± 2.7 (5) | 13.6 ± 0.9 (6) |
| 50 | 1.36 ± 0.20 (6) | 21.2 ± 4.0 (5) | 12.0 ± 0.5 (6) |
| 5000 | 2.62 ± 0.30 (6) | 21.6 ± 3.0 (4) | 10.9 ± 1.3 (6) |

[a] All values are means ± SEM; the methods of determing platelet tocopherol and 12-lipoxygenase activity are described under Material and Methods.
[b] Platelet tocopherol values are significantly different ($P < 0.05$) among all three dietary groups.
[c] Reaction was initiated with the addition of labeled arachidonate to 150 μg platelet protein for 3 min.
[d] Number in parentheses is number of rats.

as the amount of platelets present. It is worth noting that under the present experimental conditions, the "known" inhibitors for cyclooxygenase and lipoxygenase (indomethacin and NDGA) were found to be effective but nonspecific inhibitors for these two pathways. That these findings are not in complete agreement with the literature may in part be due to the relatively low concentrations of the inhibitors used in the incubations.

When fed an increasing level of vitamin E, the retention of tocopherol by rat platelets appears to be logarithmatically related to the vitamin concentration in the diets. Thus the level exceeding the requirement by 100-fold resulted in only a 2-fold increase in platelet tocopherol concentration. In spite of such an increase in tocopherol level, the conversion of exogenously added arachidonate to 12-HETE and thromboxane was essentially the same among the three dietary groups. This lack of difference strongly supports observations from other laboratories (11, 12) and furthermore, this study provides direct evidence that the activity of the soluble 12-lipoxygenase is independent of the amount of tocopherol present in rat platelets. However, we have previously reported that in the humans, tocopherol supplementation (400 mg/day) exerted a transitory stimulation of platelet 12-lipoxygenase activity during the first 3 weeks of supplement (17). While the duration of vitamin E feeding in the present study would have been too long to allow observation of any transient alteration in lipoxygenase activity, the results from these two studies suggest that factors affecting lipoxygenase activity may be different for rat and human platelets. It is also plausible that in the human platelets, tocopherol may act as a short-lived activator(s) on 12-lipoxygenase activity.

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