Relationship of Blood Thromboxane-B_2 (TxB_2) With Lipid Peroxides and Effect of Vitamin E and Placebo Supplementation on TxB_2 and Lipid Peroxide Levels in Type 1 Diabetic Patients

SUSHIL K. JAIN, PHD
K. STEPHAN KRUEGER, PHD
ROBERT MCVIE, MD
JOHN J. JARAMILLO, MD
MELISSA PALMER, MS
TINEY SMITH, RN

OBJECTIVE — To study the effect of vitamin E supplementation on platelet hyperaggregability in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS — Written informed consent according to the Institutional Review Board on Human Experimentation guidelines was obtained from diabetic patients ($n = 29$) and their age-matched normal siblings ($n = 21$) to participate in this study. Diabetic patients were supplemented with DL-α-tocopherol (vitamin E) capsule (orally, 100 IU/day) or placebo for 3 months in a double-blind clinical trial. Alternate diabetic patients were assigned to vitamin E or placebo during regular visits to the clinic. Fasting blood was collected from each diabetic patient before the start and after the vitamin E or placebo supplementation. Platelet aggregability was assessed by competitive enzyme-linked immunosorbent assay of the blood TxB_2 (a stable thromboxane metabolite). Plasma vitamin E and MDA (malondialdehyde, a product of lipid peroxidation) was assessed by high-performance liquid chromatography. Data were analyzed statistically on 12 diabetic patients on vitamin E and 12 on placebo supplementation.

RESULTS — Diabetic patients ($n = 29$) had 62% higher ($P < 0.05$) levels of TxB_2 and 15% higher levels ($P < 0.05$) of MDA in comparison to normal subjects ($n = 21$). Plasma TxB_2 levels had a significant correlation with MDA levels ($r = 0.45$, $P < 0.02$) but not with the HbA_1 ($r = -0.08$), glucose ($r = -0.13$), duration of diabetes ($r = -0.04$), or age ($r = 0.12$) of diabetic patients. Vitamin E supplementation lowered MDA levels by 30% ($P < 0.04$), TxB_2 levels by 51% ($P < 0.03$), and triglyceride levels by 22% ($P < 0.04$) in diabetic patients. There were no differences in these parameters before versus after placebo supplementation.

CONCLUSIONS — The elevated blood level of TxB_2 (hyperaggregability of platelets) is significantly related to the level of lipid peroxidation products (oxidative stress) in type 1 diabetic patients. Supplementation of modest doses of vitamin E (100 IU/day) significantly lowers blood TxB_2 and lipid peroxidation products levels in type 1 diabetic patients.

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Hyperaggregability of platelets is known to contribute to the development of thrombotic disease (1–7). Platelets of diabetic patients have increased aggregability when exposed in vitro to antagonists, such as collagen, ADP, and epinephrine (4–7). The mechanisms accounting for platelet activation in diabetes are not yet clear.

Recent studies have documented that hyperglycemia can cause generation of oxygen radicals, increased oxidative stress, and accumulation of lipid peroxidation products, such as malondialdehyde (MDA) in the blood of diabetic animals and patients (8–19). Oxidative stress is known to increase phospholipase-A_2 activity and stimulate the release of arachidonic acid, a powerful agonist that induces platelet activation and generation of thromboxane-A_2 (TxA_2) (20–22). Arachidonic acid can also be converted by cycloxygenase to prostaglandins. TxA_2 is primarily produced by platelets and formed from hydroperoxide prostaglandin (PGH_2) by prostaglandin synthetase. TxA_2 can induce aggregation of platelets, formation of clots, and smooth muscle contraction. TxB_2 is a stable metabolite of TxA_2.

Different studies have shown that both blood levels of TxB_2 and TxB_2 generation in platelets ex vivo in response to antagonists are lower after vitamin E supplementation in animals (23–25). Vitamin E addition to normal plasma in vitro (26,27) and vitamin E supplementation in normal volunteers inhibit Tx generation in parallel with suppression of platelet aggregability (28–33). The degree of in vitro aggregability and TxB_2 production is inversely related to vitamin E levels of platelets from diabetic patients (34,35). In diabetic rats, vitamin E supplementation lowers TxA_2 generation by platelets back to normal levels (36–38). In diabetic patients, vitamin E supplementation (600 mg/day for 2–4 weeks) lowered ADP-induced platelet aggregation and TxB_2 production in 14 type 2 diabetic patients with proliferative retinopathy (39). Similarly, diminution in ADP-induced Tx production was reported in platelets of 22 type 1 diabetic patients receiving 400 mg DL-α-tocopherol acetate daily for 4 weeks (40) and 9 insulin-requiring patients receiving 1,000 mg vitamin E daily for 5 weeks (41). There is no study on the role of increased oxidative stress in platelet aggregability in diabetic patients.

From the Department of Pediatrics, Louisiana State University Medical Center, Shreveport, Louisiana.
Address correspondence and reprint requests to Dr. Sushil K. Jain, Department of Pediatrics, LSU Medical Center, 1501 Kings Highway, Shreveport, LA 71130.
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Abbreviations: MDA, malondialdehyde; Tx, thromboxane.
A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Age, HbA_1, duration of diabetes, glucose, TxB_2, MDA, and vitamin E levels of normal subjects and type 1 diabetic children*

	Diabetic children	Normal subjects	*P* value
n	29	21	
Age (years)	12.7 ± 0.8	10.9 ± 0.9	NS
M/W	16/13	14/8	NS
Duration of diabetes (years)	5.2 ± 0.7	—	
HbA_1 (%)	12.3 ± 0.6	5.9 ± 0.1	0.001
Fasting glucose (mmol/l)	10.5 ± 1.1	4.9 ± 0.1	0.001
Platelet counts	269 ± 7	278 ± 9	NS
TxB_2 (pg/ml)	1,501 ± 237	924 ± 151	<0.05
MDA (nmol/ml)	0.43 ± 0.02	0.38 ± 0.04	<0.05
MDA (nmol/μmol total lipids)	0.05 ± 0.003	0.04 ± 0.003	<0.03
α-Tocopherol (nmol/ml)	17.3 ± 0.8	15.2 ± 1.0	NS
α-Tocopherol (nmol/μmol total lipids)	2.07 ± 0.07	1.94 ± 0.12	NS

Data are means ± SEM. Note significant increases in HbA_1, fasting glucose, MDA, and TxB_2 levels in diabetic patients in comparison to normal subjects.

The present study was undertaken to test the hypothesis that elevated oxidative stress increases the blood Tx level and platelet aggregability in type 1 diabetic children. Specific aims of this study were to examine any correlation between the MDA and TxB_2 levels in the blood and to ascertain whether modest vitamin E supplementation can reduce MDA and TxB_2 levels in type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

— Informed written consent of all patients was obtained in accordance with the protocol approved by the Institutional Review Board. Diabetic patients who agreed to participate in this study were asked to come to the clinic after overnight fasting and before taking any insulin. They were told to bring their insulin and syringes for use after drawing the blood. All patients were provided with free breakfast at the medical center cafeteria after the blood drawing. The routine check-up of the patients was done after they ate their breakfast. Alternate diabetic patients visiting the clinic were assigned to oral vitamin E (100 IU) or placebo capsule daily till the next visit (after 3 months). There was no control on the diet of these patients, and there is no apparent reason to believe that dietary intake was different between the two groups. Except for the nurse in charge, nobody knew about the assignment of patients to vitamin E or placebo. Both placebo and vitamin E capsules used in this study were similar in appearance, taste, texture, and smell. Enrolled in this study were 29 diabetic patients. Twenty-one normal subjects (healthy siblings) were also enrolled to serve as controls. Fasting blood from each patient was collected into tubes with and without EDTA before the start of and after the vitamin E or placebo supplementation. All analyses were done immediately after blood collection.

Tx was determined by the competitive enzyme-linked immunosorbent assay of the stable analog TxB_2 (42). Platelet counts were obtained by Coulter Counter. The glycosylated hemoglobin (HbA_1) value was measured by Glyc-affinity columns (Iso-Lab, Akron, OH); MDA and α- and γ-tocopherol were measured by high-performance liquid chromatography (43,44); serum glucose, triglycerides, and total cholesterol were measured by auto-analyzer; and total phospholipid was measured as described previously (45). Total lipid concentration was determined by adding total phospholipid, cholesterol, and triglyceride concentrations. Before the codes were opened, five subjects were deleted for noncompliance for the following reasons: a lost bottle, lost contact with the patient, taking of another medicine, and the finding, in one patient, of a thyroid disorder. After the biochemical analyses and the breaking of the code, 12 diabetic patients (D_1) were on vitamin E and 12 (D_2) were on placebo supplementation. These diabetic patients did not have signs of any clinical complications. No electrocardiograms were obtained. Fundoscopic exams were normal in all patients, with no exudates or hemorrhage. No patients had proteinuria. Sensation to light touch was normal in all patients. Vitamin E (DL-α-tocopherol) and placebo capsules were supplied by the Hoffmann-La Roche (Paramus, NJ). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Data were analyzed using a Mann-Whitney *U* test, paired Student's *t* test, and Pearson correlation coefficient with the Sigma Stat 1 statistical software.

RESULTS — Table 1 shows data on age, HbA_1, fasting blood glucose, duration of diabetes, sex ratio, and the levels of TxB_2, MDA, and vitamin E in the diabetic patients and normal siblings. This shows that the ages and sex distribution of the diabetic population are similar to those of the control subjects. Diabetic patients had a 62% higher level ($P < 0.05$) of TxB_2 and a 25% higher level ($P < 0.03$) of MDA than did normal subjects. Figure 1 illustrates that plasma TxB_2 levels had a significant correlation with the MDA levels. This relationship was significant irrespective of whether MDA was expressed as per milliliter plasma ($r = 0.38$, $P < 0.04$) or after the normalization with lipid concentration ($r = 0.45$, $P < 0.02$). The TxB_2 level did not show any relationship with the HbA_1 ($r = -0.08$), glucose ($r = -0.13$), duration of diabetes ($r = -0.04$), or age ($r = 0.12$) of diabetic patients. Nor was there any relationship between HbA_1 and plasma MDA levels ($r = -0.04$ with MDA expressed as per ml, $r = -0.17$ after normalization of MDA with lipid). There was no difference in the vitamin E levels between diabetic and normal subjects, nor was there any relationship ($r = 0.02$) of TxB_2 with vitamin E levels in diabetic patients.

Figure 2 illustrates TxB_2 levels in diabetic patients before and after vitamin E or placebo supplementation. There was no difference in the baseline level of TxB_2 between the two diabetic groups. However, TxB_2 levels were significantly lower ($P < 0.03$) in patients supplemented with vitamin E (771 ± 90 pg/ml) compared with respective baseline values (1,569 ± 319 pg/ml). There was no effect on TxB_2 levels in placebo-supplemented diabetic patients compared with baseline values. Table 2 shows that α-tocopherol levels were 79% higher in the vitamin E–supplemented group compared with baseline values. Placebo-supplemented patients did not have any change in α-tocopherol levels compared with their respective baseline level.

Table 3 shows data on MDA, lipids, insulin use, body weight, and blood pres-

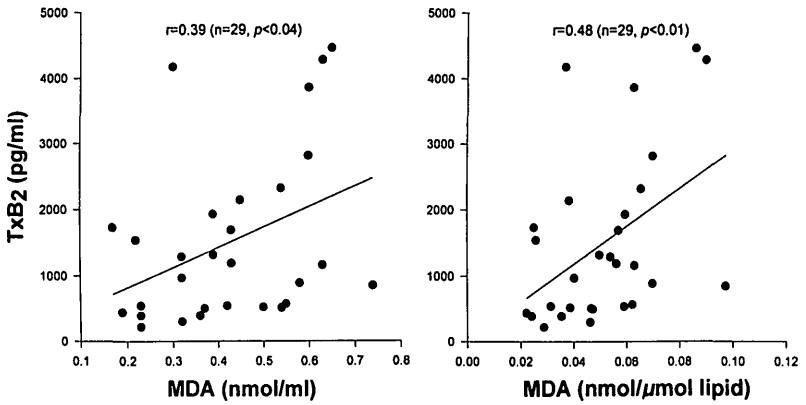

Figure 1—*Relationship between plasma TxB_2 and MDA levels in type 1 diabetic patients. Note a significant relationship of TxB_2 with the MDA levels even after normalization to the lipid concentration.*

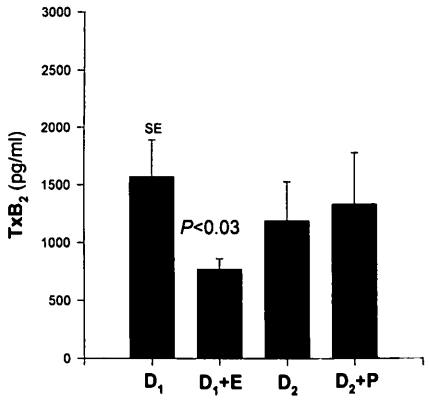

Figure 2—*Plasma TxB_2 level of diabetic patients before and after the vitamin E or placebo treatments. D_1, baseline level of diabetic patients on vitamin E supplementation; D_2, baseline level of diabetic patients on placebo supplementation. Note a significant reduction of TxB_2 levels after the vitamin E supplementation but not after placebo supplementation in diabetic patients.*

sure levels in diabetic patients before and after vitamin E or placebo supplementation. There was no difference in the baseline levels of these parameters between the two diabetic groups. However, MDA and triglycerides were significantly lower after vitamin E supplementation compared with respective baseline values. There was no effect on MDA and triglycerides levels in placebo-supplemented diabetic subjects compared with baseline values. Total cholesterol, insulin use, or gain in body weight did not differ in the vitamin E– and placebo-supplemented groups compared with respective baseline values.

There was no difference between vitamin E– and placebo-supplemented diabetic patients in the distribution of age (12.1 ± 1.0 vs. 12.7 ± 1.0 years), duration of diabetes (4.7 ± 1.0 vs. 5.7 ± 1.1 years), duration of supplementation (13.3 ± 0.4 vs. 13.6 ± 0.4 weeks), platelet counts (273 ± 10 vs. 256 ± 10), mean insulin dosage intake (0.88 ± 0.11 vs. 1.01 ± 0.10), or weight gain (2.39 ± 0.57 vs. 2.04 ± 0.49 kg). The range of HbA_1 was 7.5–18.4% in the vitamin E group and 8.8–17.4% in the placebo group.

CONCLUSIONS — Evidence for increased in vivo platelet activation in diabetic patients was demonstrated by Davi et al. (46) by measuring high levels of 11-dehydro-TxB_2, the stable metabolite of TxA_2. TxB_2 levels in plasma correlate significantly with TxB_2 production in platelet-rich plasma, indicating that plasma TxB_2 levels assess platelet synthesis of TxB_2, and its blood levels are widely used to determine the degree of platelet aggregability (29,43).

In agreement with previous studies, the present study also found elevated blood TxB_2 levels in diabetic patients in comparison with age-matched healthy control subjects. These diabetic patients did not have signs of any clinical complications, which suggests that hyperaggregability of platelets in diabetes is not related to complications. The mechanisms for the increased platelet aggregability in diabetes are not clear. The present study found a significant relationship of TxB_2 with the MDA levels in diabetic patients. The relationship of TxB_2 with endogenous vitamin E levels before any supplementation in diabetic patients was not significant, which suggests that the balance between the effects of oxidants and all of the antioxidants—not just vitamin E—determines level of oxidative stress. The lack of any relationship of TxB_2 with HbA_1 or duration of diabetes does not support a role of glycosylation and duration of diabetes in the hyperaggregability of platelets. Supplementation with modest vitamin E (100 IU/day, 3 months) significantly lowered both plasma MDA and TxB_2 levels in diabetic patients. The present study did not determine vitamin E or MDA levels or TxB_2 generation in response to agonists in the platelets; however, the blood levels of TxB_2 are widely used to determine the degree of platelet aggregability in vivo (29,43). Vitamin E supplementation can decrease effects of oxidative stress and thereby platelet aggregability and TxB_2 generation. Theoretically, MDA and TxB_2 are formed in equimolar amounts during prostaglandin metabolism (47,48). Thus, increased platelet activity can, in part, contribute to elevated MDA levels in diabetic patients. However, the absolute reduction in molar levels of MDA by vitamin E supplementation was 50-fold more than TxB_2. This argument—along with data from previous

Table 2—*Effect of oral vitamin E or placebo supplementation on plasma vitamin E levels in type 1 diabetic patients*

	Baseline (D_1)	Vitamin E (D_1+E)	Baseline (D_2)	Placebo (D_2+P)
n	12	12	12	12
α-Tocopherol (nmol)				
Per milliliter	16.3 ± 1.1*	29.1 ± 1.7†	18.5 ± 1.2	17.6 ± 1.8
Per micromole total lipids	1.96 ± 0.10*	3.77 ± 0.18†	2.26 ± 0.11	1.99 ± 0.10
γ-Tocopherol (nmol)				
Per milliliter	5.5 ± 0.7*	2.0 ± 0.3†	6.8 ± 0.8	5.5 ± 1.1
Per micromole total lipids	0.62 ± 0.06*	0.26 ± 0.04†	0.77 ± 0.06	0.55 ± 0.07

Data are means ± SEM. *n* is the number of patients in each diabetic group. Differences in values between * and † are significant ($P < 0.0001$). Note a significant increase in α-tocopherol and decrease in γ-tocopherol in vitamin E–supplemented but not in placebo-supplemented diabetic subjects.

Table 3—*Effect of oral vitamin E or placebo supplementation on plasma MDA, lipids, insulin use, body weight, and blood pressure levels in type 1 diabetic patients*

Group	Baseline (D_1)	Vitamin E (D_1+E)	Baseline (D_2)	Placebo (D_2+P)
n	12	12	12	12
MDA (TBA reactivity)				
Nanomoles per milliliter	0.44 ± 0.05*	0.34 ± 0.02†	0.38 ± 0.03	0.36 ± 0.02
Nanomoles per micromole total lipids	0.05 ± 0.006*	0.04 ± 0.004†	0.05 ± 0.005	0.05 ± 0.003
Total cholesterol (mmol/l)	3.97 ± 0.22	3.95 ± 0.17	4.07 ± 0.21	4.18 ± 0.18
Triglycerides (mmol/l)	2.98 ± 0.29*	2.32 ± 0.23†	2.42 ± 0.24	2.64 ± 0.29
Body weight (kg)	44.4 ± 5.6*	46.6 ± 5.7†	52.0 ± 5.2*	57.7 ± 5.3†
Insulin use (IU · kg^{-1} · day^{-1})	0.91 ± 0.12	0.89 ± 0.10	0.99 ± 0.08	0.91 ± 0.09
Blood pressure (mmHg)				
Systolic	102 ± 4	106 ± 4	102 ± 2	112 ± 4
Diastolic	62 ± 3	65 ± 3	65 ± 2	61 ± 3

Data are means ± SEM. *n* is the number of patients in each diabetic group. Differences in values between * and † are statistically significant ($P < 0.04$). Note a significant reduction in blood levels of lipid peroxidation and triglycerides after vitamin E supplementation but not after placebo supplementation.

in vitro studies (20–22) that hydrogen peroxide and other agents known to generate active oxygen species and induce lipid peroxidation can enhance platelet aggregation—suggests that increased oxidative stress can contribute to increased platelet aggregability of diabetic patients and that vitamin E supplementation may be beneficial in reducing platelet aggregation.

The present study also found an inhibitory effect on triglyceride accumulation after vitamin E supplementation in diabetic patients. Previous studies have shown an increase in the activity of lipoprotein lipase and inhibition of triglyceride accumulation after vitamin E supplementation in diabetic rats. Whether vitamin E supplementation increases lipoprotein lipase activity and thereby causes inhibition of triglyceride accumulation in the vitamin E–supplemented diabetic patients of the present study is not known (49,50).

Free radical–generating agents can promote coagulability of blood (51,52). Ceriello et al. (53) have shown a relationship between the in vitro oxidative susceptibility to 2,2′-azobis-(2-amidinopropane) dihydrochloride and elevated fibrinogen, prothrombin fragments 1+2, and D-dimer levels of plasma in type 2 diabetic patients. Whether there is any relationship between changes in TxB_2 and the above hemostatic variables in diabetes is not yet known. Whether increasing the dose of vitamin E supplementation will further reduce level of platelet aggregability in diabetic patients is not known. Figure 3 summarizes the proposed scheme by which hyperglycemia can cause lipid peroxidation and thereby generation of TxB_2. It also outlines how vitamin E supplementation can scavenge lipid peroxidation and lower the levels of MDA and TxB_2, thereby possibly lowering the risk of platelet aggregation and thromboses in diabetic patients.

The present study demonstrating a significant relationship between the level of lipid peroxidation products and TxB_2 and the effect of vitamin E supplementation on ameliorating the levels of both MDA and TxB_2 suggests that platelet aggregation is favored by an oxidant-antioxidant imbalance. Daily supplementation with a modest dose of vitamin E (100 IU/day) may reduce the risk for platelet aggregability and thrombotic disease in type 1 diabetic patients.

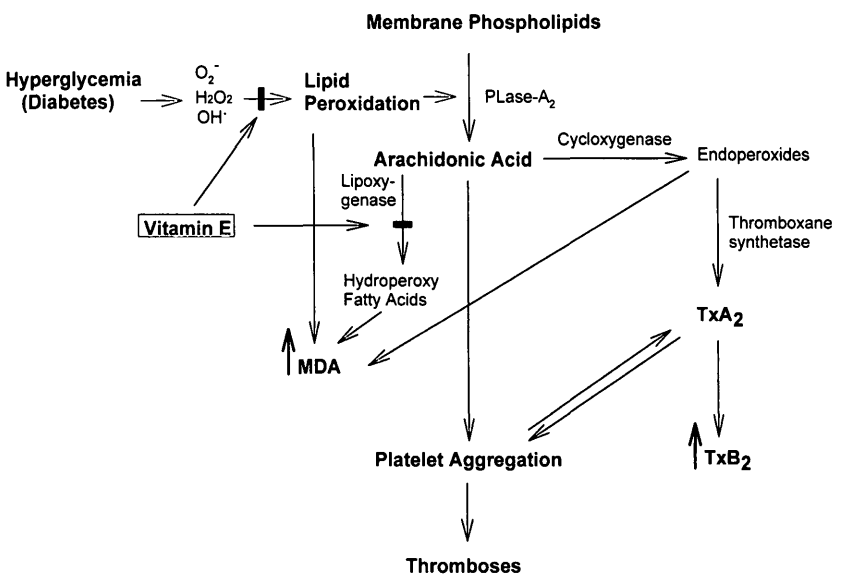

Figure 3—*Proposed scheme for a role of hyperglycemia in the increased levels of MDA and TxB_2 and for the effect of vitamin E in lowering these levels and the risk of thromboses in diabetic patients. PLase-A_2, phospholipase-A_2. Solid boxes indicate sites where vitamin E can have inhibitory/beneficial effects.*

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