

# Interrelation of Platelet Vitamin E and Thromboxane Synthesis in Type I Diabetes Mellitus

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## SUMMARY

**Platelet vitamin E content and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) synthesis have been investigated in type I diabetic subjects and age- and sex-matched controls. Platelets, but not plasma, from diabetic subjects contained significantly lower vitamin E levels and synthesized significantly greater amounts of TxA<sub>2</sub> when challenged with collagen or thrombin than platelets from control subjects. Conversion of exogenously added arachidonic acid to TxA<sub>2</sub> was unaltered between platelets from control and diabetic groups. Platelet vitamin E content from control and diabetic groups combined exhibited a significant negative linear correlation with collagen- and thrombin-induced TxA<sub>2</sub> production. These data suggest that low platelet vitamin E levels could be a contributing factor to the increased thromboxane synthesis demonstrated by platelets from the above type I diabetic subjects. DIABETES 33:239-243, March 1984.**

**B**oth macrovascular and microvascular complications are major causes of morbidity and mortality resulting from diabetes mellitus.<sup>1,2</sup> Hyperactive platelets are believed to be involved in the etiology of diabetic atherosclerotic complications<sup>2</sup> as well as microangiopathy and microthromboses.<sup>3</sup> Increased platelet aggregation has been reported in human diabetic subjects<sup>4,5</sup> and experimental animal diabetes.<sup>6</sup> Increased phospholipase activity<sup>7</sup> and increased synthesis of prostaglandin E (PGE)<sup>8</sup> and the highly potent platelet aggregator thromboxane A<sub>2</sub><sup>4,9</sup> have been reported in platelets from human diabetic subjects. We have reported increased thrombin-induced TxA<sub>2</sub> and PGE<sub>2</sub> biosynthesis in platelets from streptozotocin-diabetic rats.<sup>10</sup>

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Vitamin E therapy has been used, with varying degrees of success, in the treatment of such disorders as thrombophlebitis and thromboembolism<sup>11</sup> and peripheral vascular disease.<sup>12</sup> Favorable results with this therapy have been attributed to effects of vitamin E on platelet function.<sup>11</sup> Platelet hyperaggregability and increased malonyldialdehyde production have been demonstrated in vitamin E-deficient children, with complete reversal by supplemental dietary vitamin E.<sup>13</sup> Supplementation with dietary vitamin E has been shown to inhibit platelet aggregation,<sup>14,15</sup> PGE<sub>2</sub> and PGF<sub>2α</sub> synthesis,<sup>16</sup> and TxA<sub>2</sub> synthesis.<sup>17,18</sup> We have recently reported that platelets from streptozotocin-diabetic rats, when compared with platelets from nondiabetic controls, contain decreased vitamin E content. When these diabetic rats received supplemental vitamin E in their diet, platelet vitamin E levels increased and thrombin-induced platelet TxA<sub>2</sub> synthesis, otherwise increased, returned to levels found in the controls.<sup>19</sup>

In view of the above findings, the present study was designed to investigate vitamin E status and its possible relationship to thromboxane synthesis in platelets from human subjects with type I diabetes mellitus.

## MATERIALS

Bovine thrombin and bovine albumin were purchased from Sigma Chemical Co. (St. Louis, Missouri). Tritiated thromboxane B<sub>2</sub> (100 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Massachusetts). Unlabeled thromboxane B<sub>2</sub> (TxB<sub>2</sub>) and prostaglandins were generously supplied by Dr. John Pike of Upjohn (Kalamazoo, Michigan). Arachidonic acid was purchased from Nu Chek Prep, Inc. (Elysian, Minnesota). Vitamin E (d-alpha tocopherol) was purchased from Eastman Kodak Co. (Rochester, New York). Soluble calfskin collagen was purchased from Chemalog Chemical Dynamics Corp. (South Plainfield, New Jersey) and standardized using bovine serum albumin and the protein determination of Lowry et al.<sup>20</sup>

## METHODS

**Subjects.** This project was approved by the Ohio State University Human Subjects Committee. Type I diabetic subjects

TABLE 1  
Characterization of control and diabetic groups

	Control	Diabetic	P
Glucose (mg/dl)	71 ± 6 (56–93)	206 ± 22 (71–355)	<0.001
Triglyceride (mg/dl)	61 ± 4 (36–90)	73 ± 10 (25–120)	NS
Cholesterol (mg/dl)	142 ± 5 (110–180)	176 ± 7 (143–205)	<0.005
Platelets/nl blood	243 ± 8 (172–301)	303 ± 11 (217–381)	<0.001
Weight (kg)	64 ± 2 (50–80)	68 ± 4 (49–119)	NS

were selected from the Ohio State University Hospital's outpatient diabetes clinic and inpatient Clinical Research Center. Sixteen type I diabetic subjects (10 women, 6 men) and 16 age- and sex-matched healthy controls were studied. The mean ages for the diabetic and control groups were 32 yr (19–58 yr) and 31 yr (23–60 yr), respectively. None of the subjects was receiving supplemental vitamin E or any other medication known to interfere with vitamin E determinations. No subject had ingested aspirin for at least 2 wk or was receiving any other medication known to interfere with platelet thromboxane measurements. All diabetic subjects were receiving insulin. Duration of diabetes ranged from 1 mo to 36 yr (mean = 15 yr). In the diabetic study group, all but one had peripheral neuropathy, seven had mild proteinuria (< 0.5 g/24 h), and eight had retinopathy (two proliferative and six background).

**Preparation of washed platelets.** Following an overnight fast and after informed consent was obtained, blood (9.2 parts) was drawn into 77 mM EDTA (0.8 parts) and centrifuged at 250 × *g* for 15 min to prepare platelet-rich plasma (PRP). PRP was centrifuged (1950 × *g*) and platelets washed twice with TRIS (50 mM)-NaCl (150 mM)-EDTA (1.5 mM) buffer, pH 7.4. The final platelet pellet was suspended in calcium-free Krebs-Henseleit buffer, and the platelets counted using phase-contrast microscopy.

**Platelet thromboxane biosynthesis.** Platelet synthesis of TxA<sub>2</sub> was measured using radioimmunoassay (RIA) of TxB<sub>2</sub>, the stable hydrolysis product of TxA<sub>2</sub>, as previously described.<sup>17</sup> Platelets (10<sup>8</sup>/0.5 ml incubation volume) were stirred at 37°C with thrombin, collagen, or arachidonic acid. After termination with 1 N HCl, reaction products were extracted into diethyl ether, the ether evaporated under N<sub>2</sub>, and the residue suspended in TRIS (50 mM)-albumin (0.1%) RIA buffer (pH 7.6). RIA was performed using an antiserum against TxB<sub>2</sub>, which demonstrated the following cross-reactivities: PGD<sub>2</sub>, 1.6%; PGE<sub>2</sub>, 0.021%; PGF<sub>2α</sub>, 0.26%; arachidonic acid, < 0.001%; vitamin E, < 0.0015%; and 12-hydroxyeicosatetraenoic acid (HETE), < 0.08%.

**Plasma and platelet vitamin E.** Vitamin E was measured in plasma and platelets using HPLC as described by Hatam and Kayden.<sup>21</sup> Plasma (0.5 ml) or platelets (0.4–1.0 × 10<sup>9</sup> in 0.5 ml Krebs buffer) was subjected to ethanolic KOH saponification in the presence of ascorbic acid, the nonsaponifiable fraction extracted into hexane, the hexane evaporated under N<sub>2</sub>, and the residue dissolved in methanol. HPLC was performed using isocratic elution (methanol) and a reverse-phase Beckman Ultrasphere ODS column (Beckman Instruments, Inc., Novi, Michigan). Vitamin E standards and a pooled human plasma control were included with each run. The retention time for vitamin E is 4 min and the assay

is linear with vitamin E concentrations ranging from 0.3 to 25 μg/ml.

**Data analysis.** Differences between means were evaluated using Student's two-tailed *t* test. All data are presented as mean ± SEM. Numbers in parentheses indicate numbers of subjects in each group. NS denotes that differences are not significant.

## RESULTS

Table 1 presents information concerning the control and diabetic groups. Mean plasma glucose and cholesterol levels and whole blood platelet counts were significantly elevated in the diabetic group, while mean weight and plasma triglyceride levels were not different between the two groups.

**Platelet thromboxane synthesis.** Synthesis of platelet TxB<sub>2</sub> was measured after addition of thrombin, collagen, or arachidonic acid to washed platelets. Figure 1 illustrates thrombin-induced TxB<sub>2</sub> production in platelets from control

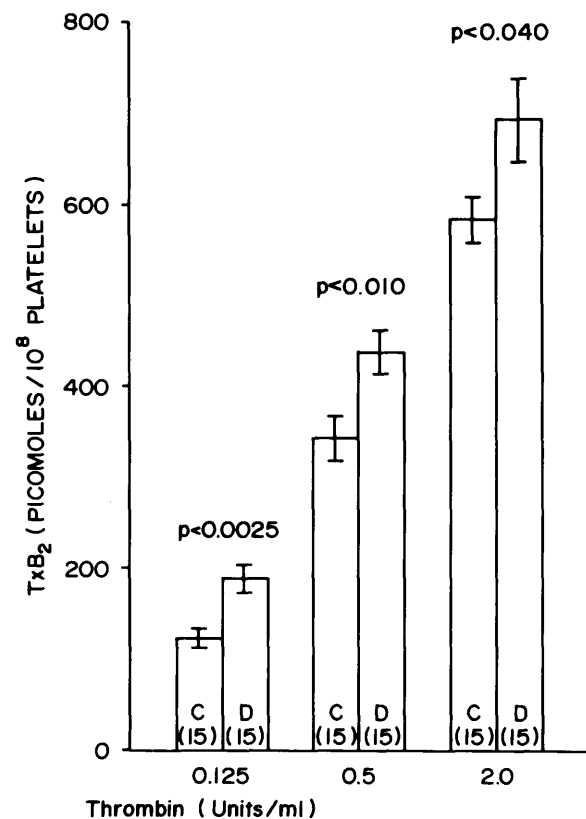


FIGURE 1. Thrombin-induced platelet TxB<sub>2</sub> synthesis in control (C) and diabetic (D) group platelets. Incubation time = 5 min.

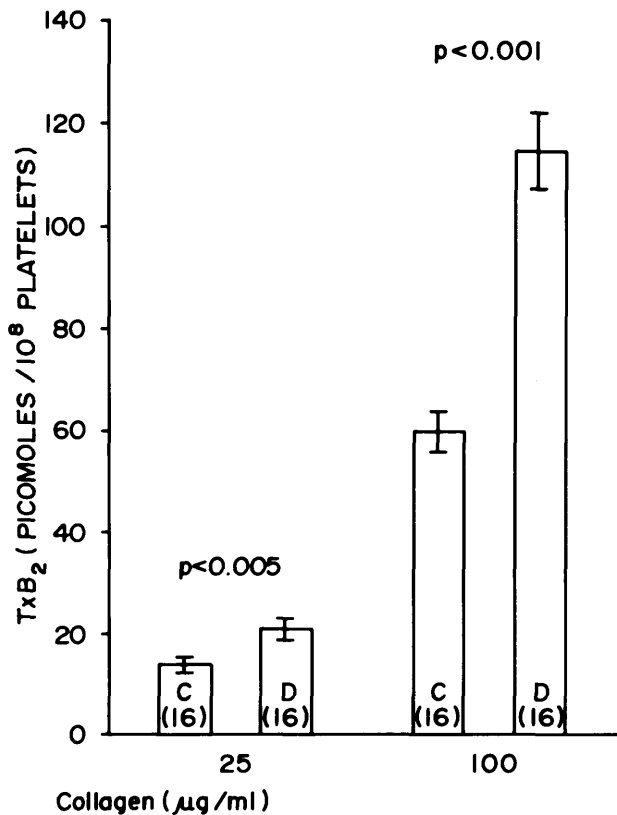


FIGURE 2. Collagen-induced platelet TxB<sub>2</sub> synthesis in control (C) and diabetic (D) group platelets. Incubation time = 2.5 min.

and diabetic groups. TxB<sub>2</sub> production in the platelets from the diabetic group was significantly increased with each thrombin concentration tested. Figures 2 and 3 denote collagen-induced TxB<sub>2</sub> synthesis at 2.5 and 5 min incubation time, respectively. At each collagen concentration tested, and at both incubation times, platelets from the diabetic group synthesized significantly greater amounts of TxB<sub>2</sub> than did platelets from the control group. There was no significant difference between platelets from the two groups when conversion of exogenously added arachidonic acid (AA) to TxB<sub>2</sub> was measured. At 0.5 min incubation time and in the presence of 1.25 µM AA, 181 ± 7 and 181 ± 12 pmol TxB<sub>2</sub>/10<sup>8</sup> platelets were produced by platelets from the control (N = 16) and diabetic (N = 16) groups, respectively. At 5 min incubation time and in the presence of 10 µM AA, 821 ± 46 and 856 ± 42 pmol TxB<sub>2</sub>/10<sup>8</sup> platelets were produced by platelets from the control (N = 16) and diabetic (N = 16) groups, respectively.

**Plasma and platelet vitamin E.** Figure 4 denotes vitamin E levels in platelets from control and diabetic groups. Vitamin E content was significantly decreased in platelets from diabetic subjects when compared with platelets from the non-diabetic controls. This decrease in platelet vitamin E, however, was not reflected by plasma vitamin E levels, which were not significantly different between control and diabetic groups. Plasma from the control group (N = 16) contained 5.29 ± 0.37 and plasma from the diabetic group (N = 16) contained 4.95 ± 0.36 µg vitamin E/ml.

**Correlation of platelet vitamin E and thromboxane synthesis.** When data from both control and diabetic groups

were combined, platelet vitamin E levels demonstrated a significant negative linear correlation with thrombin-induced platelet TxB<sub>2</sub> synthesis ( $r = -0.61$ ,  $P < 0.001$ , with 0.125 U/ml thrombin at 5 min incubation;  $r = -0.63$ ,  $P < 0.001$ , with 0.5 U/ml thrombin at 5 min incubation;  $r = -0.40$ ,  $P < 0.050$ , with 2.0 U/ml thrombin at 5 min incubation). A significant negative linear correlation also existed between platelet vitamin E levels and collagen-induced platelet TxB<sub>2</sub> synthesis ( $r = -0.51$ ,  $P < 0.005$ , with 25 µg/ml collagen at 2.5 min incubation;  $r = -0.65$ ,  $P < 0.001$ , with 100 µg/ml collagen at 2.5 min incubation;  $r = -0.53$ ,  $P < 0.005$ , with 12.5 µg/ml collagen at 5 min incubation;  $r = -0.57$ ,  $P < 0.005$ , with 25 µg/ml collagen at 5 min incubation;  $r = -0.42$ ,  $P < 0.030$ , with 100 µg/ml collagen at 5 min incubation). Figures 5 and 6 are representative diagrams of the relationship of platelet vitamin E with thrombin- and collagen-induced platelet TxB<sub>2</sub> synthesis, respectively.

## DISCUSSION

This study is the first to report, to our knowledge, in platelets from diabetic subjects, an increased synthesis of TxA<sub>2</sub> derived from arachidonic acid that was present in the platelet membrane phospholipids *in vivo*. These results are in agreement with those of Halushka et al., who reported, in platelets from human diabetic subjects, increased synthesis of PGE-like material derived from arachidonic acid that was present in platelet membrane phospholipids *in vivo*.<sup>8</sup> The increased collagen-induced TxA<sub>2</sub> synthesis in washed platelets from the diabetic group in this report is in support of

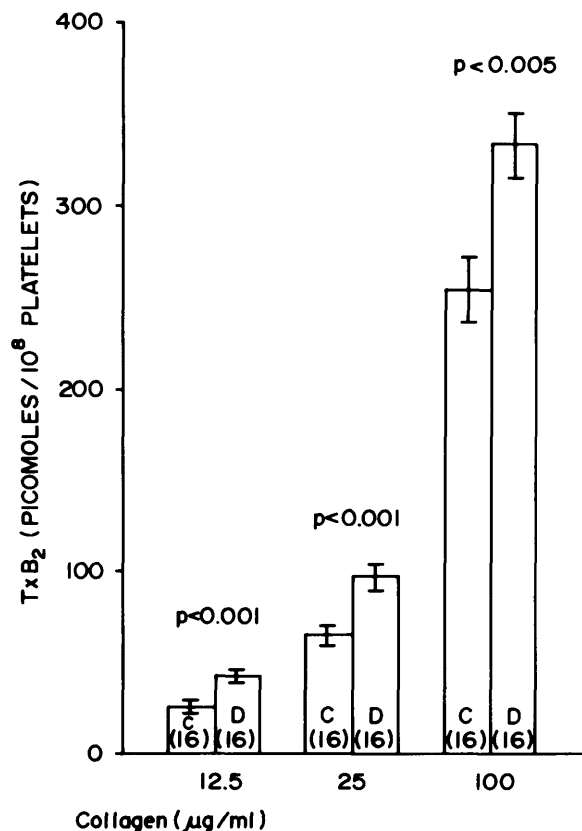


FIGURE 3. Collagen-induced platelet TxB<sub>2</sub> synthesis in control (C) and diabetic (D) group platelets. Incubation time = 5 min.

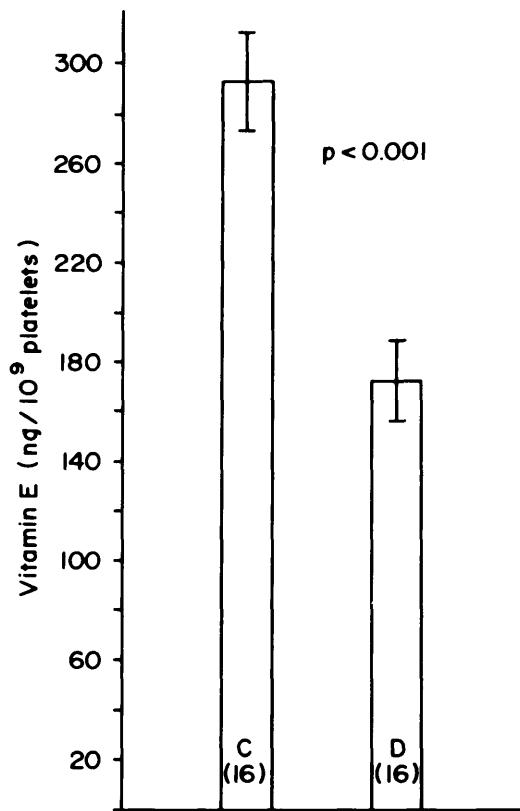


FIGURE 4. Vitamin E levels in platelets from control (C) and diabetic (D) groups.

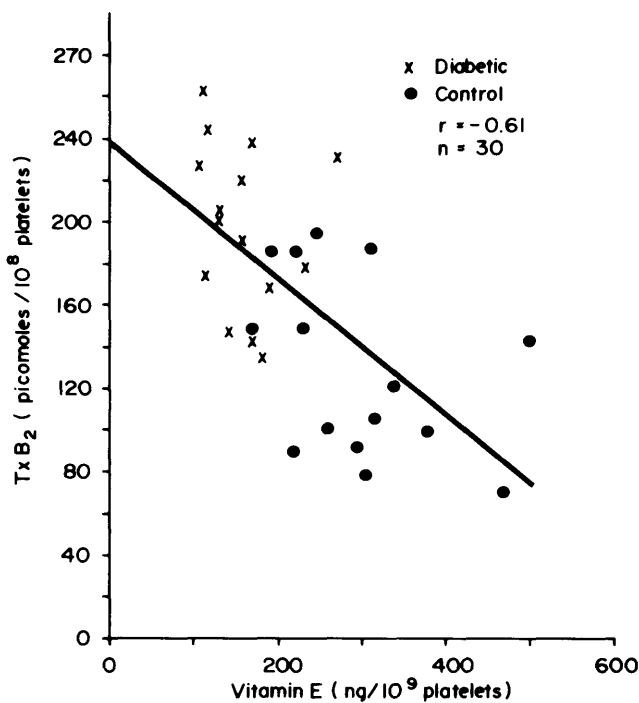


FIGURE 5. Relationship of vitamin E levels and thrombin-induced platelet thromboxane B<sub>2</sub> synthesis in platelets from control and diabetic groups combined. Thrombin concentration = 0.125 U/ml at 5 min incubation.

findings demonstrating increases in collagen-induced aggregation by platelets from diabetic subjects when these platelets were either suspended in plasma<sup>5,22</sup> or isolated from plasma.<sup>5</sup> The increased thrombin-induced TxA<sub>2</sub> synthesis reported here in platelets from the diabetic group is in support of Takeda et al.,<sup>7</sup> who, using platelets prelabeled with <sup>14</sup>C-arachidonic acid, found increased thrombin-induced phospholipase activity in platelets from diabetic subjects, and LaGarde et al.,<sup>5</sup> who, also using platelets prelabeled with <sup>14</sup>C-arachidonic acid, reported increased thrombin-induced synthesis of TxA<sub>2</sub> in platelets from diabetic subjects.

The failure to detect differences in conversion of exogenously added arachidonic acid to TxA<sub>2</sub> between platelets from control and diabetic groups is consistent with our previous reports with diabetic rats.<sup>10,19</sup> Halushka et al.<sup>9</sup> have reported increased arachidonic acid conversion to TxA<sub>2</sub> by platelets from human diabetic subjects when platelet-rich plasma was used. LaGarde et al.,<sup>5</sup> analyzing platelets from human diabetic subjects, have reported increased arachidonic acid-induced platelet aggregation when platelets were suspended in PRP but not when washed platelets were used. LaGarde also failed to detect differences in conversion of exogenously added arachidonic acid to TxB<sub>2</sub> between washed platelets from control and diabetic groups.<sup>5</sup> The above data indicate that the platelet cyclooxygenase/thromboxane synthetase enzyme system in the human diabetic subject is unaltered, although a factor(s) extrinsic to the platelet in diabetic plasma may affect this enzyme system. The derangement of arachidonic acid metabolism detected in this report is apparently at a step before the cyclooxygenase, which, as reported by Takeda et al.,<sup>7</sup> is most likely at the release of arachidonic acid from platelet membrane phospholipids.

The decreased vitamin E content in platelets of human diabetic subjects reported in this study is in support of our earlier findings with diabetic rats.<sup>19</sup> The reason for a decreased vitamin E in platelets but not plasma of these dia-

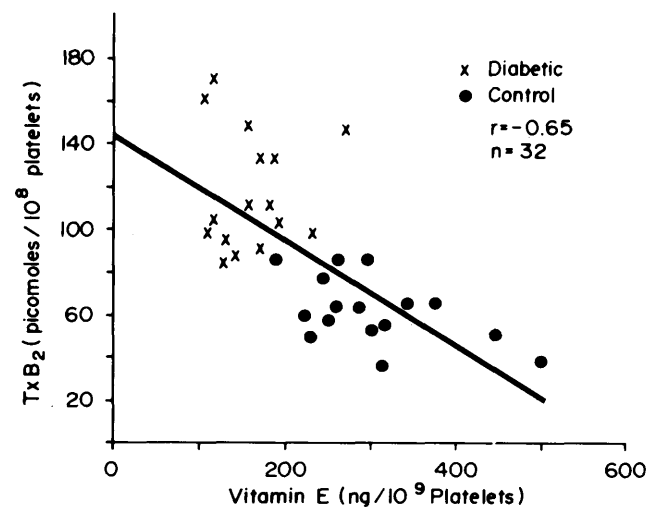


FIGURE 6. Relationship of vitamin E levels and collagen-induced platelet thromboxane B<sub>2</sub> synthesis in platelets from control and diabetic groups combined. Collagen concentration = 100 μg/ml at 2.5 min incubation.

betic subjects is unclear. Lehmann claims that platelet vitamin E content is a more sensitive and reproducible indicator of vitamin E status than the vitamin E content of plasma,<sup>23</sup> since plasma vitamin E levels are known to fluctuate in direct relation with plasma lipids.<sup>24</sup> The explanation for the decreased vitamin E content in platelets from the diabetic group is unknown at the present time. It is possible that some factor(s) present in the diabetic state interferes with the uptake or metabolism of vitamin E by the platelet. Vitamin E quinone, the biologic oxidation product of vitamin E, was not detected, when analyzed for by HPLC, in platelets from either the control or diabetic groups. Further studies need to be performed to settle the question as to why platelet vitamin E is decreased in these diabetic subjects and why this decrease is not reflected by decreases in plasma vitamin E.

The negative correlation between platelet vitamin E and  $\text{TxA}_2$  synthesis adds support to the hypothesis that vitamin E is a modulator of platelet  $\text{TxA}_2$  synthesis.<sup>17</sup> The results obtained with thrombin-, collagen-, and arachidonic acid-induced  $\text{TxB}_2$  synthesis suggest that vitamin E exerts its effect on platelet  $\text{TxA}_2$  synthesis at a step before cyclooxygenase/thromboxane synthetase, presumably at the release of arachidonic acid from the platelet membrane. We have previously reported that vitamin E modulates platelet thromboxane synthesis, with data consistent with an effect of vitamin E on the release of arachidonic acid from the platelet membrane, in rat<sup>17</sup> and rabbit<sup>18</sup> models. Increased deacylation of phospholipids has been reported in platelets from vitamin E-deficient rabbits.<sup>25</sup> The *in vitro* addition of vitamin E to platelet membrane fractions has been shown to decrease calcium release from the membrane,<sup>26</sup> which would presumably decrease phospholipase activity.

The importance of the above findings to the etiology and treatment of diabetic vascular complications awaits further investigation. Further studies involving the effects of dietary vitamin E supplementation may lend stronger support to a relationship between vitamin E content and thromboxane synthesis in platelets from type I diabetic subjects.

#### ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Lawrence Levine (Brandeis University) for supplying  $\text{TxB}_2$  antiserum and Dr. Robert Bryant (George Washington University) for providing HETE. We thank John Arnold, Kirkwood Pritchard, Jr., Nicholas Greco, Lois Jeffers, and the staff of OSU Clinical Research Center and outpatient diabetic clinic for technical assistance. We express sincere thanks to the volunteers who made this study possible.

This project was supported by grants HL 23439 and GCRC RR-34 from the National Institutes of Health. C.W.K. was supported in part by the Samuel J. Roessler Memorial Scholarship Fund.

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