Production of 12-Hydroxyeicosatetraenoic Acid and Vitamin E Status in Platelets from Type I Human Diabetic Subjects

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SUMMARY

Vitamin E content and biosynthesis of 12-hydroxyeicosatetraenoic acid (12-HETE) have been measured in platelets from type I diabetic subjects and age- and sex-matched, nondiabetic control subjects. Platelets from diabetic subjects synthesized significantly greater quantities of 12-HETE than did platelets from control subjects when 12-HETE synthesis was induced by thrombin or collagen, either in the presence or absence of indomethacin. Platelet conversion of exogenously added arachidonic acid (AA) to 12-HETE was not significantly different between the diabetic and control groups in the absence of indomethacin, although a small but significant increase in the conversion of AA to 12-HETE was present in the diabetic group platelets when indomethacin was added to the reaction. Vitamin E content was significantly reduced in platelets from the diabetic subjects, when compared with platelets from the control subjects, although plasma vitamin E levels were not significantly different between the two groups. Thrombin- and collagen-induced platelet 12-HETE synthesis demonstrated a significant negative linear correlation with platelet vitamin E content when measurements from both diabetic and control groups were combined. The above data suggest a relationship between low vitamin E content and increased 12-HETE synthesis in platelets from type I diabetic subjects.

DIABETES 1985; 34:526–31.

Numerous studies have presented evidence showing that platelets from subjects with diabetes mellitus display increased sensitivity to platelet-aggregating agents and produce increased amounts of prostaglandin E-like compounds and thromboxane A_2.[1–5] We have previously demonstrated that washed platelets from diabetic rats[6,7] and human subjects with type I diabetes mellitus[8] generate larger amounts of TxA_2 when induced by different agents. The increased TxA_2 production was correlated with decreased platelet vitamin E levels.[7,8] There are no studies reporting effects of the relationship of platelet vitamin to HETE production in the diabetic state. The present investigation regards the synthesis of 12-HETE and its relation to vitamin E status in platelets from human subjects with type I diabetes mellitus.

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Received for publication 6 April 1984 and in revised form 25 August 1984.

MATERIALS AND METHODS

Materials. Tritiated 12-HETE (48 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, Massachusetts). Unlabeled 12-HETE was generously supplied by Dr. Robert Bryant (George Washington University). Prostaglandins were a kind gift from Dr. John Pike of Upjohn Company (Kalamazoo, Michigan). Arachidonic acid was purchased from Nu Chek Prep, Inc. (Elysian, Minnesota). Indomethacin was purchased from Merck, Sharp, and Dohme Research Lab (Rahway, New Jersey). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a kind gift from Hoffman-LaRoche (New Jersey). Bovine thrombin, type IV gelatin from calf skin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, Missouri), and soluble calf skin collagen purchased from Chemalog Chemical Dynamics Corp. (S. Plainfield, New Jersey). The soluble calf skin collagen suspension was standardized against bovine serum albumin using the protein assay of Lowry et al.[9] Vitamin E (d-alpha-tocopherol) was purchased from Eastman Kodak Co. (Rochester, New Jersey).

Subjects. All subjects participated in this project after written, informed consent was obtained according to protocol approved by the Ohio State University Human Subjects Committee. Thirteen type I diabetic subjects (9 female, 4 male) were selected from patients of the Ohio State University Clinical Research Center, along with 13 age- and sex-matched, healthy control subjects. The mean age of the diabetic group was 31 yr (18–58 yr) and of the control group 32 yr (24–60 yr). All diabetic subjects were receiving insulin. The average

duration of their diabetes was 16 yr, ranging from 1 mo to 28 yr. Seven diabetic subjects had mild proteinuria (<0.5 g/24 h), 8 had retinopathy, and 9 had peripheral neuropathy. None of the subjects had ingested aspirin, or any other drug known to interfere with prostaglandin or HETE determinations, for at least 2 wk before the study. None of the subjects were receiving vitamin E supplementation.

Preparation of washed platelets. After an overnight fast, 9.2 parts blood was drawn into 0.8 parts 0.77 mM EDTA. Platelet-rich plasma (PRP) was prepared by centrifugation at 250 × *g* for 15 min. Platelets were pelleted at 1950 × *g* for 20 min and washed twice with Tris (50 mM)-NaCl (150 mM)-EDTA (1.5 mM) buffer (pH 7.4), the final platelet pellet being suspended in calcium-free Krebs-Henseleit buffer. Final platelet suspensions were adjusted to counts of 200/nl using phase-contrast microscopy.

HETE radioimmunoassay. Radioimmunoassay (RIA) for 12-HETE was performed by incubating platelets (10^8 in 0.5 ml Krebs buffer) at 37°C after the addition of thrombin, collagen, or arachidonic acid. In incubations performed in the presence of indomethacin, a cyclooxygenase inhibitor, the platelet suspension was preincubated with indomethacin for 3 min in an ice bath. Incubations were terminated with the addition of 100 μl of 1 N HCl, reaction products extracted once into 3 ml diethyl ether, the ether evaporated under N_2, and the residue dissolved in Tris (150 mM)-gelatin (0.1%) RIA buffer, pH 7.8. Recovery of tritiated HETE by this method is 88.4 ± 0.66% (mean ± SD). RIA of 12-HETE was performed using a rabbit 12-HETE antiserum kindly provided by Dr. Lawrence Levine (Brandeis University). Cross-reactivity of this antiserum with related compounds is as follows: 12-hydroxyheptadecatrienoic acid (HHT), 8.6%;[10] AA, 0.085%; PGD_2, <0.0017%; PGE_2, <0.0046%; $PGF_{2\alpha}$, <0.0019%; thromboxane B_2, <0.014%; vitamin E, <0.0004%; and 12-hydroperoxyeicosatetraenoic acid (HPETE), indeterminate. The generation of 12-HETE immunoreactivity was inhibited by ETYA, a competitive inhibitor of both platelet lipoxygenase and cyclooxygenase enzymes, in a dose-dependent manner. Unlabeled 12-HETE, provided by Dr. Robert Bryant, was standardized spectrophotometrically using an extinction coefficient of 28,000 M^{-1}/cm for 12-HETE in ethanol at a wavelength of 235 nm. This standard solution was diluted in Tris-gel buffer, aliquoted, and stored at −20°C. Tritiated 12-HETE was stored in ethanol at −20°C and diluted in Tris-gel buffer before use. HETE antiserum was stored at 4°C and diluted in Tris-gel buffer before use. After combination of standards, or appropriate dilutions of unknowns, with labeled 12-HETE and antiserum (total volume = 600 μl), tubes were incubated at 4°C for 6–10 h and then separation of bound from unbound 12-HETE accomplished with the addition of 500 μl of a charcoal (250 mg)-dextran (25 mg)-Tris-gel buffer (50 ml) slurry. After centrifugation, the eluant was decanted and counted using liquid scintillation. The detection limit for 12-HETE in this assay is 1.0 pmol. Interassay variability, evaluated with the use of two pooled extracted human platelet controls, was 8.2% and 9.1% (CV) for low- and high-range controls, respectively.

TABLE 1
Information concerning control and diabetic groups

	Control	Diabetic	P-value
Glucose (mg/dl)	79 ± 2	201 ± 25	$P < 0.001$
Triglyceride (mg/dl)	59 ± 4	84 ± 9	$P < 0.050$
Cholesterol (mg/dl)	141 ± 6	180 ± 6	$P < 0.001$
Platelets/nl blood	237 ± 10	315 ± 18	$P < 0.005$
Weight (kg)	63 ± 2	68 ± 5	NS

Glucose, triglyceride, and cholesterol are fasting values.

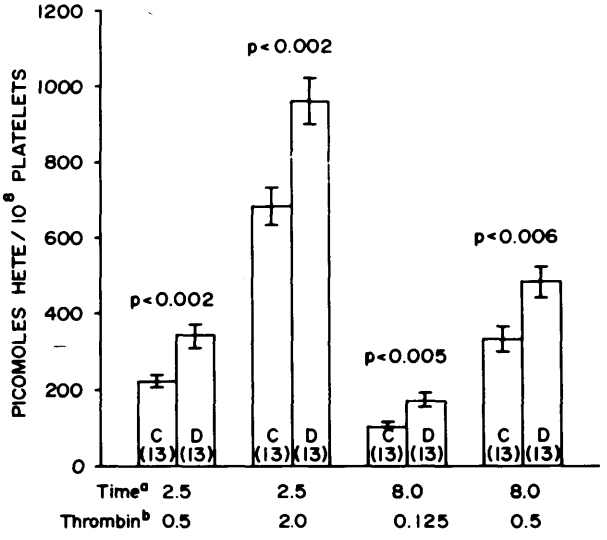

FIGURE 1. Thrombin-induced HETE production from platelets of control and diabetic groups. (a) Incubation time in minutes, and (b) thrombin concentration in U/ml.

Vitamin E. Vitamin E was measured in platelets and plasma using the extraction and HPLC procedure of Hatam and Kayden.[11] Platelets ($0.4–1.0 \times 10^9$ in 0.5 ml Krebs buffer) or plasma (0.5 ml) were saponified with ethanolic KOH at 55°C in the presence of ascorbic acid. The nonsaponifiable fraction was extracted into hexane, the hexane evaporated under N_2, and the residue dissolved in HPLC-grade methanol. Vitamin E was measured using isocratic elution (methanol) with a reverse-phase Beckman Ultrasphere ODS column (Beckman Instruments, Inc., Novi, Michigan). Absorbance was measured at 294 nm and peak area determined with an electronic integrator. Vitamin E standards and a pooled human control were included with each batch. Interassay variation, as evaluated with the control, was 2% (CV). Recoveries of alpha-tocopherol using this method are 99% with plasma or platelets.[11] Vitamin E retention time is 4 min and the assay demonstrates linearity in the range of 0.3–25 μg vitamin E/ml. This procedure separates alpha- from gamma- and beta-tocopherols but does not distinguish between the latter two. The procedure also separates vitamin E quinone, and some samples were run at 264 nm (absorbance maximum for E quinone) to check for the presence of this compound.

Data analysis. Student's two-tailed *t*-test was used to evaluate differences between means. All data are presented as mean ± SEM except where specifically indicated. NS indicates that differences are not significant. In all figures, (C) = control group, (D) = diabetic group, and numbers of subjects in each group are in parentheses.

RESULTS

Information pertaining to the diabetic and control groups is presented in Table 1. Mean values for plasma glucose, tri-

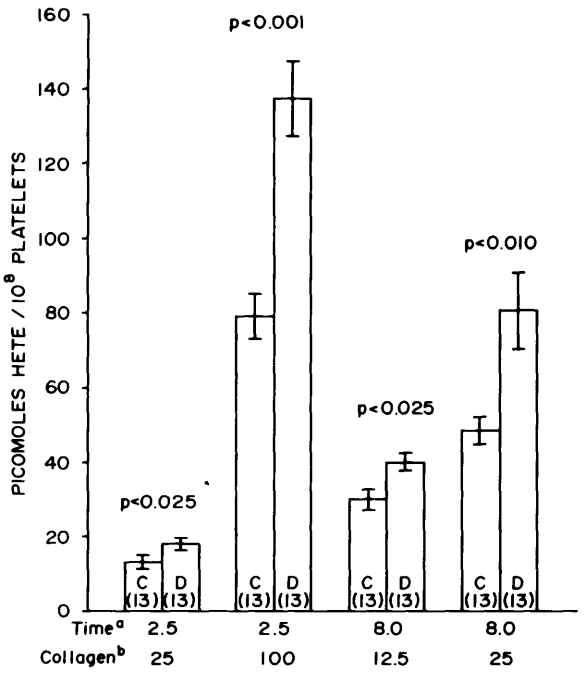

FIGURE 2. Collagen-induced HETE production from platelets of control and diabetic groups. (a) Incubation time in minutes, and (b) collagen concentration in μg/ml.

glyceride, cholesterol, and whole blood platelet counts were significantly increased in the diabetic group. Mean body weight was not different between the two groups.

Biosynthesis of platelet HETE. Thrombin-, collagen-, and AA-induced platelet HETE synthesis was evaluated. Incubation times for thrombin- and collagen-induced HETE production were selected to monitor both the initial velocity of the reaction (2.5-min incubation) and the maximal amount of products formed at the completion of the reaction (8-min incubation). At the 8-min reaction time for arachidonic acid-induced HETE production, there are still significant amounts of HETE being formed but the velocity of the reaction is decreasing and the amount of HETE formed by 8 min is representative of the total amount of HETE formed at completion of the reaction. Figures 1 and 2 illustrate thrombin- and collagen-induced platelet HETE synthesis, respectively, from control and diabetic groups. Platelets from the diabetic group synthesized significantly greater amounts of HETE at all thrombin and collagen concentrations tested and at both incubation times. When conversion of exogenous AA to HETE was evaluated, there was no significant difference between platelets from control and diabetic groups. In the presence of 10 μM AA at 8 min incubation time, platelets from the control group synthesized 4562 ± 296 pmol HETE/10^8 platelets, while platelets from the diabetic group synthesized 5065 ± 383 pmol HETE/10^8 platelets. To isolate lipoxygenase activity from cyclooxygenase activity, HETE biosynthesis was also evaluated in the presence of 140 μM indomethacin. Thrombin-, collagen-, and AA-induced thromboxane production in the presence of 140 μM indomethacin was <1 pmol/10^8 platelets, and indomethacin at this concentration caused no significant inhibition of lipoxygenase activity in our system. As indicated in Figure 3, thrombin-, collagen-, and AA-induced HETE production in the presence of indomethacin were significantly increased in platelets from the diabetic group. Differences, however, were greater between the two groups when thrombin and collagen were used to induce HETE production than when conversion of AA to HETE was measured.

Platelet and plasma vitamin E. Vitamin E levels in the platelets of control and diabetic groups are diagrammed in Figure 4. Vitamin E content of platelets from the diabetic group was significantly reduced when compared with platelets from the control group. There was no difference, however, when plasma vitamin E levels were compared. Plasma from the control group contained 5.2 ± 0.4 μg vitamin E/ml and from the diabetic group 5.0 ± 0.5 μg vitamin E/ml. Neither gamma-tocopherol, beta-tocopherol, nor vitamin E quinone were detected in plasma or platelets of either the control or diabetic groups.

Relationship between platelet vitamin E and HETE synthesis. A significant negative linear correlation existed between platelet thrombin-induced HETE synthesis and platelet vitamin E content ($r = -0.75$, $P < 0.001$ with 0.5 U/ml thrombin at 8-min incubation in the presence of 140 μM indomethacin). Correlation coefficients between platelet vitamin E and thrombin-induced HETE production in the absence of indomethacin with the various thrombin concentrations and incubation times used ranged from −0.50 to −0.64 and were all statistically significant. Collagen-induced platelet HETE synthesis also exhibited a significant negative linear correlation with platelet vitamin E content ($r = -0.70$, $P < 0.001$ with 100 μg/ml collagen at 8-min incubation in the presence of 140 μM indomethacin). Correlation coefficients between platelet vitamin E and collagen-induced HETE production in the absence of indomethacin with the various collagen concentrations and incubation times used ranged from −0.58 to −0.64 and were all statistically significant. Figures 5 and 6 are diagrams of the relationship between platelet vitamin E and thrombin- and collagen-induced HETE synthesis, respectively, in the presence of indomethacin.

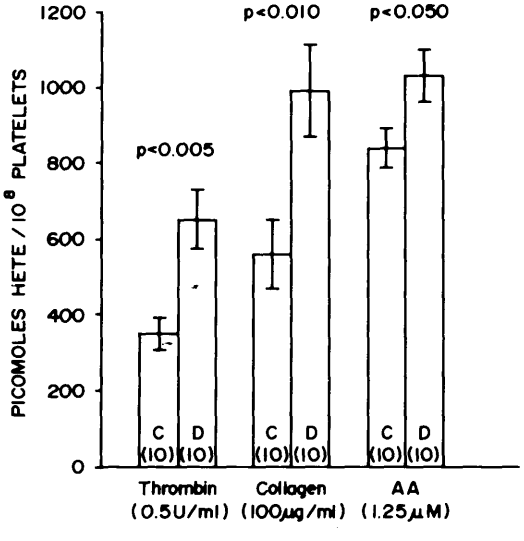

FIGURE 3. Thrombin-, collagen-, and arachidonic acid-induced HETE production, in the presence of 140 μM indomethacin, from platelets of control and diabetic groups. Incubation time with all three inducing agents = 8 min, after a 3-min preincubation with indomethacin in an ice bath.

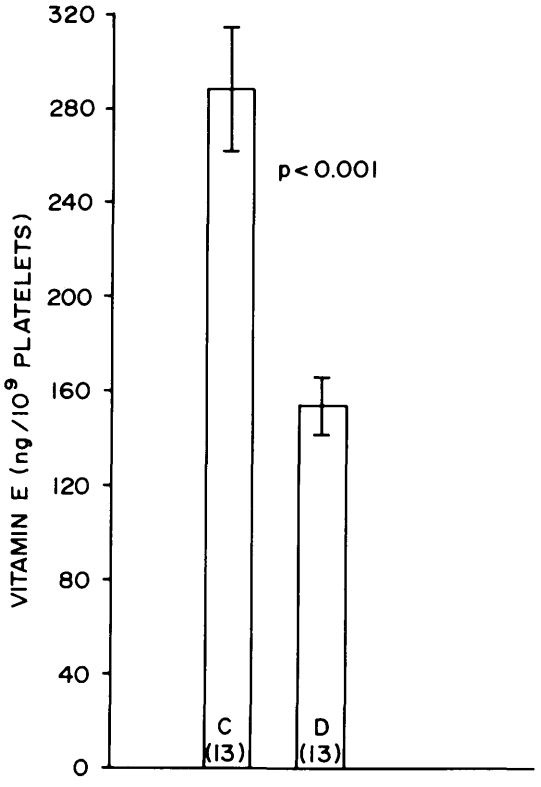

FIGURE 4. Vitamin E content in platelets from control and diabetic groups.

Relationship of other parameters with platelet vitamin E and HETE. Of the parameters including fasting plasma triglyceride, fasting plasma cholesterol, fasting plasma glucose, vascular complications, and insulin dosage, significant correlations were found only between cholesterol and thrombin- and collagen-induced HETE production ($r = 0.54$, $P < 0.020$) and between plasma glucose and thrombin-induced HETE production ($r = 0.59$, $P < 0.010$).

DISCUSSION

There is increasing evidence that platelets from diabetic subjects are more sensitive to aggregating agents and generate greater quantities of TxA_2 when compared with platelets of nondiabetic subjects.[1,3-5] Unlike many earlier studies, we measured platelet thromboxane and 12-HETE synthesis in washed platelets, using a radioimmunoassay system that allowed for the quantification of thromboxane and 12-HETE derived from arachidonic acid that was incorporated into platelet membrane phospholipids in vivo. We recently demonstrated that washed platelets from type I diabetic subjects, when challenged with collagen or thrombin but not arachidonic acid, synthesized significantly greater amounts of TxA_2 than did platelets from nondiabetic subjects.[8] We presented data that demonstrated a negative linear correlation between TxA_2 synthesis and vitamin E content in platelets from both control and diabetic groups combined.[8] A similar finding was also reported in rats with streptozocin-induced diabetes.[7]

The present study complements and extends the above findings by demonstrating a similar relationship between the synthesis of 12-HETE, a product of 12-lipoxygenase, and vitamin E content of platelets from type I human diabetic subjects and nondiabetic control subjects. Thrombin- and collagen-induced HETE synthesis was significantly elevated in platelets from diabetic subjects when compared with platelets from nondiabetic subjects both in the absence and presence of indomethacin. Previously, we attributed an analogous increase in TxA_2 synthesis in platelets from type I diabetic subjects to enhanced phospholipase-dependent deacylation of arachidonic acid from platelet membrane phospholipid.[8] The present data support the evidence for an increased thrombin- and collagen-induced platelet phospholipase activity, and rule out a channeling of substrate arachidonic acid between cyclooxygenase and lipoxygenase pathways, since the pattern for channeling would be expected to be an increase in one pathway and decrease in the other, rather than an increase in both pathways. Such an increase in phospholipase activity has been demonstrated in platelets from human diabetic subjects[12] and in platelets from rats with chemically induced diabetes mellitus[13] using the technique of prelabeling membrane phospholipids with ^{14}C-arachidonic acid.

Alternately, or in addition to increased release of platelet membrane arachidonic acid, our results could be explained by an increased platelet membrane arachidonic acid content. Reports addressing platelet membrane arachidonic acid content in diabetes are conflicting. Kalofoutis and Lekakis[14] reported small increases in platelet phospholipid arachidonic acid in poorly and well-controlled human diabetic subjects, and Morita et al.[15] reported a 20% increase in platelet phospholipid arachidonic acid content in well-controlled type II diabetic patients. Furthermore, Takahashi et al.[16] demonstrated increased in vitro uptake of ^{14}C-arachidonic acid into platelet phospholipids in type II diabetic subjects. Jones et al.,[17] however, reported decreased platelet phospholipid arachidonic acid content in platelets from diabetic subjects, and demonstrated a negative correlation between glycosylated hemoglobin levels and platelet phospholipid arachidonic acid content. Metz[18] attributes the conflicts in these reports to differences in insulin therapy and

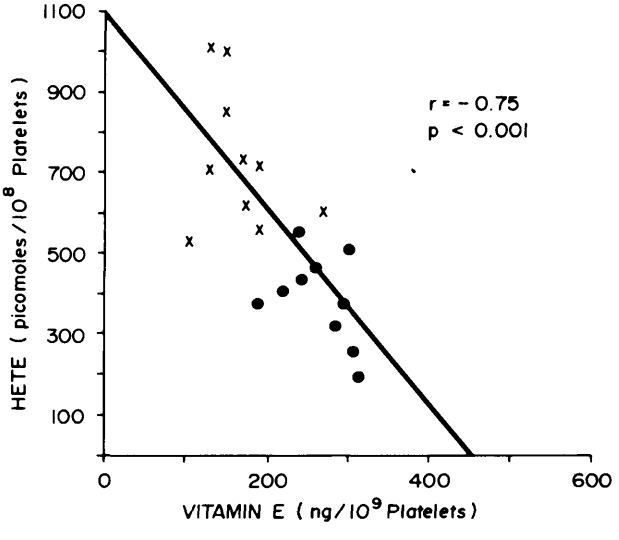

FIGURE 5. Thrombin-induced HETE production as a function of vitamin E content in platelets from control (●) and diabetic (x) groups. Thrombin concentration = 0.5 U/ml, incubation time = 8 min. HETE production was measured in the presence of 140 μM indomethacin.

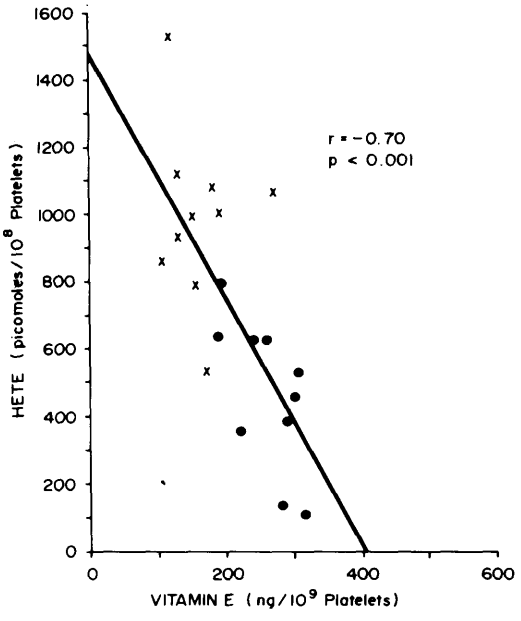

FIGURE 6. Collagen-induced HETE production as a function of platelet vitamin E content in platelets from control (●) and diabetic (x) groups. Collagen concentration = 100 μg/ml, incubation time = 8 min. HETE production was measured in the presence of 140 μM indomethacin.

adequacy of control in the diabetic subjects studied. He hypothesizes that, in type II diabetic subjects with normal or increased basal insulin levels, or in type I diabetic subjects under good control and receiving pharmacologic doses of insulin with increased basal insulin levels, platelet arachidonic acid levels are increased due to increased lipogenesis and desaturase activity. Conversely, Metz hypothesizes that in poorly controlled, insulin-deficient diabetic subjects, platelet arachidonic acid levels are decreased. Increased platelet arachidonic acid content would be consistent with the good degree of control that existed in most of the diabetic patients in our study. Because our patients were for the most part well-controlled, our results may not apply to poorly controlled or untreated diabetic subjects.

In the current study, HETE synthesis from exogenously added arachidonic acid is significantly greater, in washed platelets of diabetic subjects when compared with nondiabetic subjects, in the presence of but not in the absence of indomethacin. Apparently, the marginally increased activity of platelet lipoxygenase activity in the diabetic group is only detectable after indomethacin is used to isolate lipoxygenase activity from cyclooxygenase activity. Arachidonic acid-induced HETE production tended to be higher in the diabetic group even in the absence of indomethacin, but the differences did not reach statistical significance. The contribution of the increase in lipoxygenase activity, in the platelets from the diabetic group, to the increased HETE production appears to be minor when compared with the contribution of the increased phospholipase activity.

We have previously demonstrated an increased availability of platelet membrane arachidonic acid in rat and rabbit platelets deficient in vitamin E by measuring the synthesis of TxA_2 from arachidonic acid incorporated into the platelet membrane in vivo.[19,20] We have also demonstrated that rabbit platelets deficient in vitamin E have higher lipoxygenase activity.[21] Our studies are supported by those of Tagney and Driskell,[22] who prelabeled vitamin E-deficient platelets with ^{14}C-arachidonic acid and demonstrated an increase in its thrombin-induced release. In the present study, we report low vitamin E content in platelets from diabetic subjects, and demonstrate a significant negative linear correlation between platelet vitamin E content and thrombin- and collagen-induced HETE production. The mechanism for the decreased vitamin E content in platelets from the diabetic group is unknown. We postulate that low vitamin E content in platelets from diabetic subjects is responsible for increased HETE synthesis as well as increased TxA_2 synthesis,[8] and that the mechanism by which low platelet vitamin E promotes increases in HETE and TxA_2 synthesis is related to an increased thrombin- and collagen-induced release of arachidonic acid from membrane phospholipids and/or an increased platelet membrane arachidonic acid content. The existence of a significant correlation between platelet vitamin E and HETE production does not guarantee a cause-and-effect relationship, however, and could be coincidental. Also, a correlation coefficient of −0.050 to −0.075 does not exclude the possibility that other factors are important in the regulation of platelet HETE production in addition to platelet vitamin E. The in vivo effects of dietary vitamin E on cyclooxygenase and lipoxygenase activity in platelets from diabetic subjects remains to be explored.

Although we report significant correlations between fasting plasma cholesterol and platelet HETE production and also between fasting plasma glucose and thrombin-induced HETE production, this study was not designed to evaluate these parameters and the correlation coefficients may be misleading due to the good degree of diabetes control in our diabetic subjects and the narrow ranges for plasma glucose and cholesterol in both the control and especially the diabetic group. Only three patients had plasma glucoses in the 300s, and the mean ± SEM for the control group plasma cholesterol was 140 ± 6 and for the diabetic group 180 ± 6, both of the latter in the normal range. A study designed to evaluate these relationships requires a wider and more dispersed range of plasma glucose and cholesterol.

The significance of increased platelet lipoxygenase activity may stem from the effects of 12-hydroperoxyeicosatetraenoic acid (HPETE), the hydroperoxy precursor of HETE. Increased platelet HPETE production and release near the vascular endothelium may be significant in light of reports showing that lipid hydroperoxides inhibit endothelial prostacyclin synthetase.[23,24] We have previously reported that lipid peroxides are increased in plasma of rats with streptozocin diabetes, with concomitant decreases in aortic prostacyclin production.[7] It is a well-accepted hypothesis that decreased vascular prostacyclin production may lead to platelet hyperactivity, which may be a significant risk factor in the development of atherosclerosis.[25]

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Lawrence Levine of Brandeis University for kindly supplying the HETE antiserum and Dr. Robert Bryant of George Washington University for supplying the 12-HETE. We express sincere thanks to John Arnold, Dr. Kirkwood Pritchard, Jr., Nicholas Greco, and the staff of OSUH Clinical Research Center and outpatient diabetes clinic

for technical assistance. We sincerely thank the volunteers who made this study possible.

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

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