Decreased Activity of Arachidonate 12-Lipoxygenase in Platelets of Japanese Patients With Non–Insulin-Dependent Diabetes Mellitus

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To study the metabolism of the platelet 12-lipoxygenase pathway in diabetes, we evaluated the correlation between the activity and amount of arachidonate 12-lipoxygenase in the platelets of patients with non–insulin-dependent-diabetes mellitus (NIDDM). There were four parts in this investigation: (1) examination of abnormalities in platelet 12-lipoxygenase in patients with NIDDM recruited from the Hospital of Juntendo University School of Medicine; (2) comparison of 12-lipoxygenase in the platelets of non-obese NIDDM patients without angiopathy versus normal subjects matched for age, sex, and body mass index (BMI); (3) evaluation of gender differences; and (4) assessment of the potential influence of glycemic control. The activity of 12-lipoxygenase was assayed by incubation of [1-^{14}C]arachidonic acid with the platelet cytosol. The reaction mixture was extracted and separated by thin-layer chromatography, and the radioactive end products were detected. The activity of 12-lipoxygenase in the platelets of patients with NIDDM was significantly less than in normal subjects ($P < .003$), whereas the amount of 12-lipoxygenase protein did not differ between the two groups. Thus, the specific activity of 12-lipoxygenase in diabetic patients was significantly less than that of normal subjects ($P < .001$). The enzyme activity and the specific enzyme activity of 12-lipoxygenase in non-obese NIDDM patients without angiopathy were significantly lower than the values in normal subjects matched for gender, age, and BMI ($P < .006$ and $P < .0007$, respectively). No significant difference in the activity or amount of platelet 12-lipoxygenase was observed between males and females matched for age, BMI, and disease. In addition, no relationship was observed between 12-lipoxygenase activity and blood glucose levels measured at the time of specimen collection. However, slight negative correlations were noted between 12-lipoxygenase activity and 1,5-anhydroglucitol, hemoglobin A_{1c} (HbA_{1c}), and fructosamine ($r = .369$, $-.354$, and $-.279$, respectively). When recombinant 12-lipoxygenase was incubated with varying concentrations of glucose or fructose, enzyme inactivation was related to the length of incubation, and was unaffected by glucose or fructose. These observations suggest that the activity of 12-lipoxygenase in the platelets of patients with NIDDM is decreased by prolonged hyperglycemia. The mechanism involved requires further investigation.

THE ENHANCED RELEASE of platelet agonists in patients with diabetes mellitus may be important in the initiation of thrombosis, and may also accelerate the development of vascular complications in this disease.[1] The metabolism of arachidonic acid in human platelets is initiated by the reaction of cyclooxygenase or 12-lipoxygenase. The production of thromboxane A_2, which precedes aggregation, occurs via the cyclooxygenase pathway. Arachidonate 12-lipoxygenase was first identified in human platelets[2] and then in leukocytes[3]; it was then found in a variety of organs, including the pituitary gland, pineal gland, and trachea of different species.[4] The enzyme catalyzes the oxygenation of arachidonic acid to 12(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE). The majority of the hydroperoxy acid is reduced to the corresponding hydroxy acid (12-HETE), as observed in whole-cell preparations.[4]

The agents 12-HETE and 12-HPETE exhibit a variety of biological activities.[5] For instance, the migration of rat aortic smooth muscle cells is stimulated specifically by 12-HETE at low concentration of 3 fg/mL.[6] At higher concentrations (10 nmol/L) of 12-HETE, the release of luteinizing hormone–releasing hormone is maximally stimulated from the rat median eminence.[7] The synthesis of melatonin is stimulated by 12-HPETE at a concentration of 1 μmol/L.[8] This agent also enhances the adhesion of Lewis lung carcinoma cells to endothelial cells, subendothelial matrix, and fibronectin, presumably by stimulating the expression and/or activation of the glycoprotein IIb/IIIa receptor.[9] The agent 12-HETE and its derivatives activate the lysosomal and cytoplasmic hydrolytic activities of cholesteryl ester.[10] 12-HETE increases the invasiveness of rat prostate carcinoma cells via the selective activation of protein kinase Cα.[11] Siegel et al[12] reported that 12-HPETE blocks the aggregation of platelets induced by collagen and arachidonate. Sekiya et al[13] recently reported that 12-HETE exerts negative feedback to prevent excess aggregation by interfering with the liberation of arachidonic acid from membrane phospholipids.

Some patients with myeloproliferative disorders such as essential thrombocythemia, polycythemia vera, and chronic myelogenous leukemia show a reduction in 12-lipoxygenase activity in platelets.[14-16] The increased production of proaggregatory thromboxane A_2 by the cyclooxygenase pathway is well known in diabetes[17,18]; however, the 12-lipoxygenase pathway has received less study. The present study therefore compared the activity and amount of arachidonate 12-lipoxygenase in platelets from Japanese patients with non–insulin-dependent diabetes mellitus (NIDDM) versus healthy Japanese subjects.

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SUBJECTS AND METHODS

Trial Design and Subjects

This investigation consisted of four parts as follows.

Part 1. Part 1 was an examination of any abnormalities in platelet 12-lipoxygenase in Japanese patients with NIDDM recruited from the Hospital of Juntendo University School of Medicine in Japan. In a cross-sectional comparison, we evaluated the activity and content of 12-lipoxygenase in platelets between 54 patients with NIDDM and 26 normal subjects. The patients with diabetes were classified as NIDDM according to the criteria of the American Diabetes Association.[19] Their characteristics are summarized in Table 1. There were 30 males and 24 females aged 23 to 86 years (mean, 57.2). The mean body mass index (BMI) was 23.2 kg/m^2. Complications included diabetic retinopathy (n = 22), persistent proteinuria (n = 15), and hypertension (n = 19). The following agents were administered to treat the NIDDM (in rank order): oral hypoglycemic drugs (sulfonylureas) (n = 34), dietary restriction (n = 12), insulin alone (n = 6), and both insulin and an oral hypoglycemic agent (n = 2). None of the patients had a history of macrovascular complications and cardiac failure. The normal subjects consisted of Japanese volunteers (16 males and 10 females) with a mean age of 35.1 ± 9.0 years. They had no known history of diabetes and no evidence of abnormalities upon clinical examination. The mean BMI was 22.2 ± 2.3 kg/m^2.

Part 2. Part 2 was a cross-sectional comparison of the activity and amount of 12-lipoxygenase in platelets obtained from non-obese subjects NIDDM versus 12 normal controls. Subjects were matched for age, sex, and BMI. Such subjects were selected from the groups described in part 1 (Table 2). NIDDM patients who participated in part 2 had no diabetic retinopathy, persistent proteinuria, hypertension, or history of ischemic vascular disease. None of them were receiving insulin or antihypertensive agents. Nine patients were receiving an oral hypoglycemic agent (sulfonylurea), and three were controlled on diet alone.

Part 3. Part 3 was an evaluation of the relationship between gender and 12-lipoxygenase in subjects selected from the population studied in part 1. A cross-sectional comparison of the activity and amount of platelet 12-lipoxygenase was conducted in 26 males and 24 females matched for age, BMI, and disease.

Part 4. This investigation assessed the potential influence of glycemic control, BMI, and duration of diabetes in the 54 patients with NIDDM studied in part 1. Patients fasted overnight before blood was drawn. For 2 weeks before sampling, none of the subjects took nonsteroidal antiinflammatory drugs or other medications that may affect platelet function.

Methods

Preparation of platelets. Blood samples were drawn by sterile venipuncture into polypropylene syringes fitted with 19-gauge needles. Sampling was conducted in the early morning after an overnight fast. To 27 mL whole blood was added 3 mL of a mixture of 0.14-mol/L citric acid and 0.2-mol/L trisodium citrate. The following steps were performed at room temperature. The mixture was centrifuged at 320 × *g* for 10 minutes for preparation of the platelets. The upper layer (platelet-rich plasma [PRP]) was carefully collected by aspiration. The lower layer (red blood cell–rich plasma) was again centrifuged at 320 × *g* for 10 minutes after addition of phosphate-buffered saline containing 10 mmol/L EDTA at pH 6.5 (EDTA-PBS). The resulting upper layer was also carefully collected by aspiration and pooled with PRP. The PRP was then centrifuged at 1,300 × *g* for 10 minutes, and the pellets were resuspended and washed in EDTA-PBS. The pellets were then subjected to hypotonic treatment for 30 seconds to lyse any contaminating erythrocytes and then centrifuged at 1,300 × *g* for 10 minutes. The isolated platelets were suspended in 10 vol (vol/wt) ice-cold 20-mmol/L Tris-HCl buffer (pH 7.4) and sonicated twice at 20 kHz for 15 seconds using a Branson (Danbury, CT) model 185D sonicator. Before sonication, platelet numbers were determined with a Coulter (Miami, FL) STKS platelet analyzer. The cell homogenates were centrifuged at 109,000 × *g* for 50 minutes at 4°C. The resulting high-speed supernatant solutions were stored at −60°C and labeled as the cytosolic fraction of platelets.

Table 1. Clinical Data on the Patients With Diabetes

Parameter	
No. of subjects	54
Age (yr)	57.2 ± 14.4
BMI	23.2 ± 3.2
Diabetes duration (yr)	11.6 ± 8.1
Blood glucose (mg/dL)*	163.0 ± 61.6
HbA_{1c} (%)	9.1 ± 2.0
Fructosamine (μmol/L)	379.9 ± 88.4
1,5-Anhydroglucitol (μg/mL)	6.1 ± 5.7

NOTE. Values are the mean ± SD.
*At the time of blood collection.

Assay for 12-lipoxygenase activity. Since 12-lipoxygenase was localized mainly in the platelet cytosol,[20] we examined the enzyme activity in the cytosol fraction. For the assay of 12-lipoxygenase, [1-^{14}C]arachidonic acid (5 nmol, 80,000 cpm in 5 μL ethanol) was incubated with 100 μL of the platelet cytosol fraction, 75 μL water, and 20 μL 1-mol/L Tris-HCl (pH 7.4) at 30°C for 10 minutes. The reaction was stopped by addition of 300 μL diethyl ether:methanol:0.2-mol/L citric acid (30:40:1). The mixture was then centrifuged at 1,500 × *g* at 4°C for 3 minutes, and the upper phase was spotted onto 20 × 20-cm silica gel-60 F_{254} plates. The plates were developed in the organic phase of ethyl acetate:isooctane:acetic acid:water (110:50:20:100) for 70 minutes at room temperature and then scanned for the presence of radioactivity products.

Determination of protein in platelet cytosol. The protein concentration of the platelet cytosol was determined using the Bio-Rad (Hercules, CA) Protein Assay based on the method of Bradford.[21]

Measurement of 12-lipoxygenase using an enzyme-linked immunosorbent assay. A peroxidase-linked, sandwich-type immunoassay for human 12-lipoxygenase was performed using a monoclonal antibody directed against human platelet 12-lipoxygenase (HPLO-3).[22] The surface of an immunoplate was coated with HPLO-3 to trap 12-lipoxygenase present in the platelet cytosol. The amount of 12-lipoxygenase was quantified using the biotin-avidin system as described previously.[22]

Clinical laboratory examinations. Plasma glucose was assayed by the glucose oxidase method, with values given in milligrams per deciliter. Hemoglobin A_{1c} (HbA_{1c}) was evaluated by the column chromatographic method. The normal range was 5.2% to 6.4% as described by Peacock.[23] The plasma fructosamine level was evaluated by the method of Jonson et al.[24] The normal range was 205 to 285 μmol/L. The 1,5-anhydroglucitol level was determined by enzyme immunoassay. The normal range was 15 to 40 μg/mL as described by Fukuyama et al.[25]

Effect of glucose and fructose on activity of recombinant 12-lipoxygenase. Purified recombinant 12-lipoxygenase (16 μg protein in 200 μL) with a specific activity of 75 μmol/min/mg protein was incubated at 4°C for 10 or 23 hours with varying concentrations of glucose or fructose (100 to 900 mg/mL). After incubation, a 20-μL aliquot was removed and used for the standard assay of 12-lipoxygenase activity as already described.

Materials. [1-^{14}C]Arachidonic acid (1.85 GBq/mmol) was purchased from Amersham International (Little Chalfont, UK), immunoplates (Micro Well Plates) were from Nunc (Roskilde, Denmark), avidin horseradish peroxidase-conjugate and *N*-hydroxysuccinimidebiotin were

Table 2. Comparison of Activity and Amount of Platelet 12-Lipoxygenase in Non-Obese NIDDM Patients Without Angiopathy and Non-Obese Normal Subjects Matched for Sex and Age

Group	No.	Sex		Age (yr)	BMI (kg/m²)	Enzyme Activity (nmol/10 min/ mg protein)	Amount of Enzyme (μg enzyme/ mg protein)	Specific Enzyme Activity (nmol/10 min/ μg enzyme)
		Male	Female					
NIDDM	12	7	5	42.3 ± 8.4	22.8 ± 2.6	19.6 ± 9.7	4.2 ± 0.9	4.6 ± 1.7
Normal	12	7	5	41.8 ± 7.4	22.0 ± 2.1	37.4 ± 17.8	4.3 ± 1.1	8.5 ± 2.8
Statistical significance				NS	NS	$P < .006$	NS	$P < .0007$

NOTE. Values are the mean ± SD. Angiopathy includes microangiopathy (retinopathy and nephropathy) and macroangiopathy (hypertension and vascular ischemia).

Abbreviation: NS, no significance.

from Pierce (Rockford, IL), and *o*-phenylenediamine was from Nacalai Tesque (Kyoto, Japan).

Monoclonal antibody against human platelet 12-lipoxygenase (HPLO-3) was prepared as described previously.[22] Protein assay reagent[21] was obtained from Bio-Rad Laboratories. Silica gel-60 F_{254} precoated thin-layer chromatography plates (200 × 200 × 0.25 mm) were purchased from Merck (Darmstadt, Germany). Recombinant 12-lipoxygenase (human) was purified as described previously.[26]

Statistical Analysis

Statistical analyses were performed using Student's *t* test for unpaired data. For testing patients in different groups, the chi-square test was used. The correlations between platelet 12-lipoxygenase and other parameters were tested with linear regression analysis. Results are expressed as the mean ± SD. A level of P less than .05 was accepted as statistically significant.

RESULTS

Platelet 12-Lipoxygenase in NIDDM Patients and Normal Subjects

The platelet activity of 12-lipoxygenase in patients with NIDDM was significantly lower than in normal subjects (Student's *t* test, $P < .003$; Fig 1A). However, as determined by ELISA, the amount of 12-lipoxygenase in the platelets of patients with NIDDM did not differ significantly from that of the normal subjects (Fig 1B). Thus, the specific activity of 12-lipoxygenase in the patients with diabetes was significantly less than that in the normal subjects (Student's *t* test, $P < .001$; Fig 1C).

Although 12-lipoxygenase activity in the platelets of eight NIDDM patients who had both retinopathy and nephropathy was less than that observed in 25 NIDDM patients without retinopathy (enzyme activity, 18.8 ± 5.5 and 25.4 ± 17.9 nmol/10 min/mg protein, respectively), the difference was not statistically significant.

Comparison of Non-Obese NIDDM Patients With Normal Subjects Matched by Gender, Age, and BMI

The 12-lipoxygenase activity in the platelets of 12 non-obese NIDDM patients was significantly lower than that of normal subjects matched by gender, age, and BMI (Student's *t* test, $P < .006$; Table 2). The amount of 12-lipoxygenase in patients with NIDDM did not differ from that of the normal subjects. Thus, the specific activity of 12-lipoxygenase was significantly less in the platelets of patients with diabetes than in normal subjects (Student's *t* test, $P < .0007$).

Gender and 12-Lipoxygenase

No significant difference in the activity or amount of platelet 12-lipoxygenase was observed between the 26 males and 26 females matched for age, BMI, and disease (Table 3).

Comparison of Platelet 12-Lipoxygenase Activity With Other Parameters of Diabetes

Weak correlations of platelet 12-lipoxygenase activity were observed with 1,5-anhydroglucitol (Fig 2A; $r = .369$, $P < .01$), HbA_{1c} (Fig 2B; $r = -.354$, $P < .01$), and fructosamine (Fig 2C; $r = -.279$, $P < .04$). There was no significant correlation between the activity of 12-lipoxygenase and other parameters such as age, BMI, duration of diabetes, or blood sugar level measured at the time of collection (Fig 2D).

Effect of Glucose and Fructose on 12-Lipoxygenase Activity

When 12-lipoxygenase was incubated with varying concentrations of glucose or fructose before the activity determination, the inactivation of the enzyme showed a dependence on the length of incubation but was unaffected by the presence of glucose or fructose (Fig 3).

DISCUSSION

There are only a few reports on the 12-lipoxygenase pathway in the platelets of diabetic patients. Abbate et al[27] reported an increase in the production of thromboxane A_2 in response to thrombin stimulation of platelets obtained from patients with IDDM and NIDDM, and found a positive correlation between the synthesis of thromboxane A_2 and HbA_{1c}. In addition, the production of 12-HETE is reduced in NIDDM after incubation of arachidonic acid with thrombin-stimulated platelets. Yue et al[28] showed that the prevention of collagen abnormalities in diabetic rats may be regulated by a balance of cyclooxygenase and lipoxygenase pathways. The present study is the first to measure the activity and amount of arachidonate 12-lipoxygenase in the platelets of diabetic patients. Using a monoclonal antibody against human platelet 12-lipoxygenase, we found that platelet 12-lipoxygenase activity was decreased in diabetic patients compared with normal subjects, but the enzyme level remained unchanged. Some patients with myeloproliferative disorders exhibit a decrease in platelet 12-lipoxygenase activity.[14] The decrease is attributed to a decrease in 12-lipoxygenase mRNA and protein.[22] In contrast, as demonstrated in the present study, platelet levels of 12-lipoxygenase were unchanged in diabetic patients. Therefore, the mechanism responsible for the

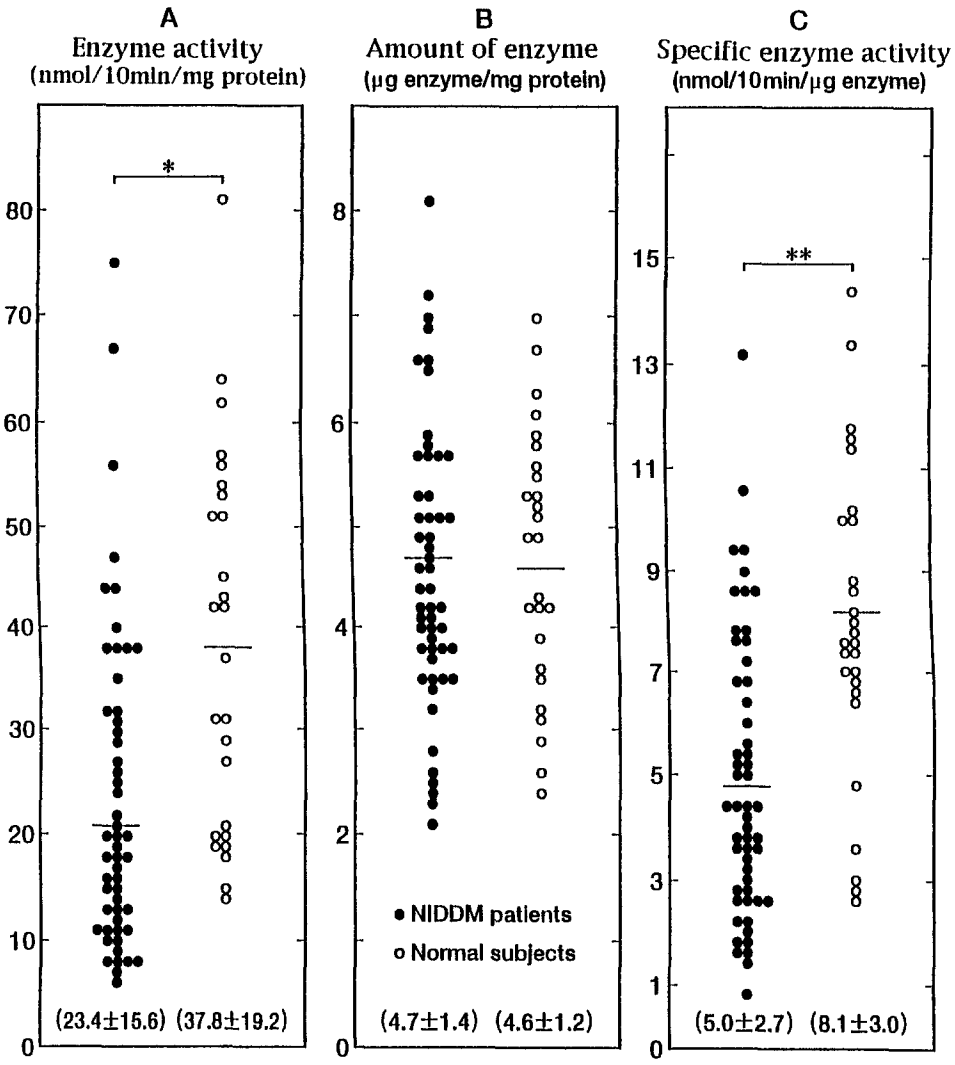

Fig 1. Activity and amount of platelet 12-lipoxygenase in Japanese NIDDM patients (●) and normal subjects (○). Line represents the mean for each group. Numerical value is the mean ± SD; n = 56 for NIDDM patients and 26 for normal subjects. *$P < .003$, **$P < .001$.

decrease in 12-lipoxygenase activity in the platelets of diabetic patients appears to differ from that in patients with myeloproliferative disorders.

Previous reports have demonstrated that platelet function is variously influenced by glycemic control,[29] age,[30] insulin,[31] antihypertensive drugs,[32] obesity,[33] and the presence of microangiopathy and macroangiopathy.[34] We therefore compared platelet 12-lipoxygenase activity between normal subjects and non-obese NIDDM patients who were not receiving insulin and who had no angiopathy. A significant decrease was found in the activity of 12-lipoxygenase in the platelets of patients with diabetes. These observations suggest that the observed decrease in 12-lipoxygenase activity is not associated with insulin administration. We also investigated the relationship between the decrease in 12-lipoxygenase activity and the severity of diabetes, particularly with respect to hyperglycemia. We found

Table 3. Comparison of Activity and Amount of Platelet 12-Lipoxygenase in Males and Females Matched for Age

Sex	No.	Age (yr)	Enzyme Activity (nmol/10 min/ mg protein)	Amount of Enzyme (μg enzyme/ mg protein)	Specific Enzyme Activity (nmol/10 min/ μg enzyme)
Males					
Total	26	54.0 ± 15.9	24.1 ± 17.4	4.7 ± 1.8	5.3 ± 3.4
NIDDM patients	20	59.9 ± 12.5	20.0 ± 14.1	4.7 ± 2.0	4.7 ± 3.2
Normal subjects	6	34.7 ± 9.2	37.9 ± 20.0	4.8 ± 0.9	7.6 ± 3.3
Females					
Total	26	53.9 ± 15.8	23.5 ± 14.0	4.5 ± 1.2	5.2 ± 3.1
NIDDM patients	20	59.7 ± 12.3	19.9 ± 12.3	4.6 ± 1.2	4.6 ± 3.2
Normal subjects	6	34.7 ± 9.8	35.3 ± 12.5	4.4 ± 1.2	7.3 ± 0.6
Significance		NS	NS	NS	NS

NOTE. Values are the mean ± SD. NS (no significance) for total males *v* total females, male NIDDM patients *v* female NIDDM patients, and normal males *v* normal females.

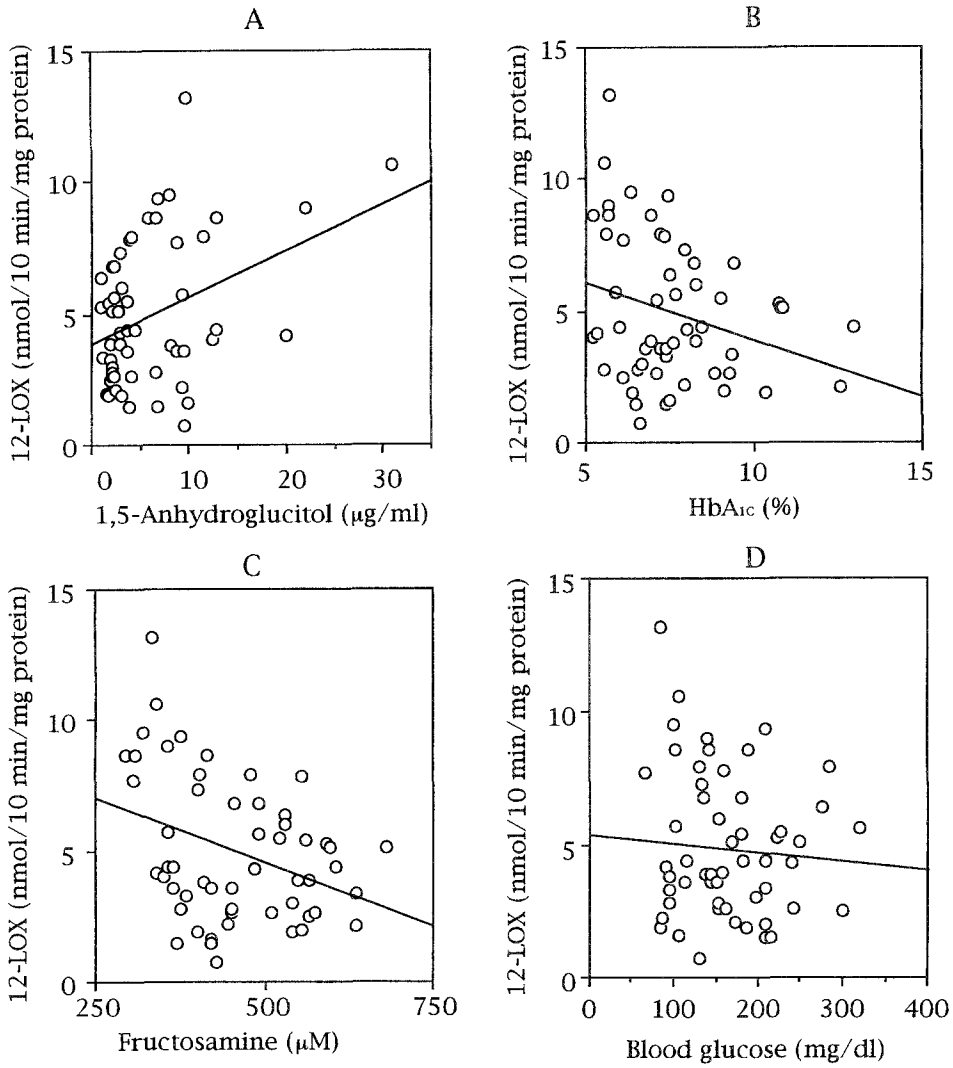

Fig 2. Relationship between platelet 12-lipoxygenase activity (12-LOX) and other parameters of glycemic control: (A) plasma 1,5-anhydroglucitol level, (B) HbA_{1c} value, (C) plasma fructosamine value, (D) blood glucose level at the time of specimen collection. Correlation coefficients for the scattergram were calculated (A, $r = .369$, $P < .01$; B, $r = -.354$, $P < .01$; C, $r = -.29$, $P < .05$; D, $r = .000$, $P > 1.0$).

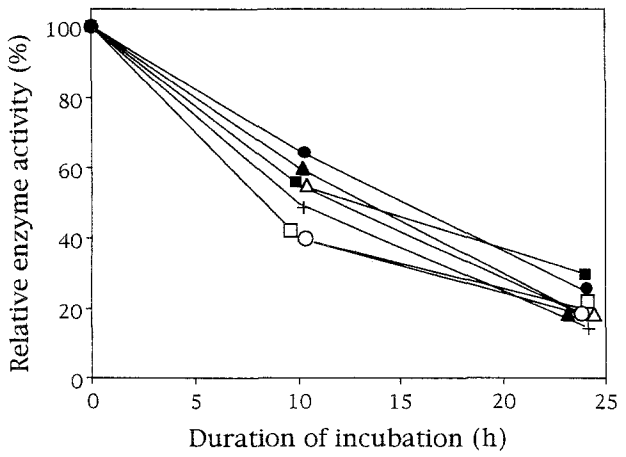

Fig 3. Effect of glucose and fructose on 12-lipoxygenase activity. Purified recombinant 12-lipoxygenase (16 μg protein in 200 μL) was incubated with varying concentrations of glucose or fructose at 4°C for 10 or 23 hours before addition of [1-^{14}C]arachidonic acid for assay of 12-lipoxygenase. Glucose: 100 mg/dL (○), 300 mg/dL (■), 500 mg/dL (△); fructose: 100 mg/dL (●), 300 mg/dL (■), 900 mg/dL (▲), none (+).

no relationship between 12-lipoxygenase activity and blood glucose levels measured at the time of specimen collection. However, we observed weak inverse correlations between 12-lipoxygenase activity and serum levels of fructosamine and HbA_{1c}. Additionally, a weak positive correlation was observed between 12-lipoxygenase and 1,5-anhydroglucitol. Plasma concentrations of 1,5-anhydroglucitol are considered a marker for hyperglycemia.[35] These findings suggest that the activity of 12-lipoxygenase may be reduced by poor glycemic control. Although the activity of this enzyme appeared to be lower in diabetic patients with retinopathy and nephropathy compared with patients without microangiopathy, the difference was not statistically significant. In view of the possible inactivation of 12-lipoxygenase in the presence of hyperglycemia, we examined the effect of acute hyperglycemia on 12-lipoxygenase activity in vitro. We found that the rate of 12-lipoxygenase inactivation was unaffected by the presence of glucose or fructose at varying concentrations (100 to 900 mg/dL).

The present in vivo and in vitro data suggest that platelet 12-lipoxygenase activity is unchanged during acute hyperglycemia. The lipoxygenase activity may be decreased during prolonged hyperglycemia, although this has not been experimen

tally substantiated. However, the correlations between 12-lipoxygenase activity and the markers for glycemic control were weak; this issue therefore requires further investigation.

It has been reported that in diabetes, the life span of platelets is reduced[36,37] and the platelets are subjected to increased oxidative stress.[38] If platelet turnover is increased in diabetes, one might expect higher enzyme protein or activity levels due to the increased metabolic capacity usually associated with younger platelets. Therefore, the inactivation of 12-lipoxygenase may not be explained by a change in the life span of the platelets in patients with diabetes.

However, glucose itself may undergo autoxidation, leading to the production of hydrogen peroxide and hydroxyl radicals.[39] Glycation may also generate free radicals.[40] Intracellular oxidant stress induced by the interaction of advanced glycation end products with specific receptors has been reported.[41] Recent study has demonstrated that the plasma level of 8-epi-prostaglandin $F_{2\alpha}$, a specific nonenzymatic peroxidation product of arachidonic acid and an indicator of oxidative stress, is elevated in diabetes.[42] However, the effect of oxidative stress on 12-lipoxygenase has not been clarified.

Lipoxygenase products inhibit the conversion of free arachidonic acid to prostanoids via the cyclooxygenase pathway in several in vitro cell systems such as endothelial cells,[43] macrophages,[44] and platelets.[12] In fact, Setty and Stuart[45] observed an inverse correlation between lipoxygenase and cyclooxygenase products. Antonipillai et al[46] demonstrated that the urinary excretion of 12-HETE increases and that of 6-keto-prostaglandin $F_{1\alpha}$ decreases in diabetic patients in vivo, suggesting that arachidonate metabolism in the lipoxygenase and cyclooxygenase pathways may be inversely related.

However, other studies have found increased urinary 6-keto-$PGF_{1\alpha}$ in newly diagnosed type I diabetic patients compared with age-matched controls,[47] and increased urinary TXA_2 in albuminuric diabetic patients.[48] Since we did not determine the urinary excretion of 12-HETE and Antonipillai et al[46] did not assay 12-lipoxygenase activity in platelets, we cannot presently reconcile the two observations.

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