The Role of Adenosine Triphosphate–Citrate Lyase in the Metabolism of Acetyl–Coenzyme A and Function of Blood Platelets in Diabetes Mellitus

Anna Michno, Anna Skibowska, Anna Raszeja-Specht, Justyna Ćwikowska, and Andrzej Szutowicz

Diabetes is known to increase blood platelet activity. Activities of pyruvate dehydrogenase (PDH), adenosine triphosphate (ATP)–citrate lyase (ATPCL), acetyl–coenzyme A (acetyl-CoA) content, malonyl dialdehyde (MDA), synthesis, and platelet aggregation in resting conditions and after activation with thrombin were measured in diabetic subjects and in age- and sex-matched healthy subjects. Activities of ATPCL and PDH, acetyl-CoA content, and thrombin-evoked MDA synthesis as well as platelet aggregation in diabetes were 31%, 51%, 62%, 35%, and 21%, respectively, higher than in healthy subjects. In addition, activation of diabetic platelets caused 2 times greater release of acetyl-CoA from their mitochondria than in controls. Both 1.0 mmol/L (−)hydroxycitrate and 0.1 mmol/L SB-204490 decreased acetyl-CoA content in platelet cytoplasm along with suppression of MDA synthesis and platelet aggregation. These inhibitory effects were about 2 times greater in diabetic than in control platelets. The data presented indicate that the ATPCL pathway is operative in human platelets and may be responsible for provision of about 50% of acetyl units from their mitochondrial to cytoplasmic compartment. Increased acetyl-CoA synthesis in diabetic platelets may be the cause of their excessive activity in the course of the disease. ATPCL may be a target for its specific inhibitors as factors decreasing platelet activity.

CHRONIC HYPERGLYCEMIA is recognized as a primary cause of cardiovascular complications developing in patients with diabetes mellitus. Several mechanisms may be involved in appearance of diabetic microangiopathy and macroagiopathy. They include excessive glycation of plasma and cell structural proteins, free radical generation, disturbances of lipid metabolism, and excessive platelet activity.[1-3] Diabetes was reported to increase platelet aggregation, production of platelet-activating factor, thromboxane A_2, and expression of some surface receptors, as well as to decrease platelet NO synthesis.[4-6] These changes in platelet activity could be secondary to platelet contacts with pathologically modified vascular epithelium.[7] However, primary changes in platelets themselves should also be taken into consideration. Such a possibility is supported by the fact that platelet hyperactivity was found in diabetic patients who did not display vascular changes.[2] On the other hand, insulin may normalize platelet function, despite persistent vascular changes.[8] Thus, normalization of glucose concentration may be crucial for return of platelet parameters to normal values.

Such a claim is supported by the fact that glucose is a principal energy substrate for platelets, providing about 50% of adenosine triphosphate (ATP) through pyruvate-derived acetyl–coenzyme A (acetyl-CoA) metabolism in the tricarboxylic acid cycle.[9] Glucose is transported into the platelets through the insulin-independent Glut-3 transporter, the Km of which is about 10 mmol/L. Therefore, hyperglycemia may increase glucose influx and its metabolism inside blood platelets.[10] If this is the case, then increased production of acetyl-CoA in platelet mitochondria and subsequent activation of acetyl-CoA–utilizing pathways in cytoplasm would be expected. Our preliminary studies revealed that diabetes increased activities of hexokinase and pyruvate dehydrogenase (EC 1.2.4.1., PDH) in platelets.[11] The disease also led to elevated activity of ATP–citrate lyase (EC 4.1.3.8., ATPCL), a cytosolic enzyme involved in indirect transport of acetyl units from mitochondria to cytoplasm.[11] However, it is not known whether ATPCL plays any role in the regulation of synthesis of fatty acids and derived thromboxanes in human blood platelets. Other pathways of acetyl-CoA transport include L-carnitine acetyltransferase and acetyl-CoA synthase, as well as direct transport of acetyl-CoA through calcium-dependent permeability transition mechanism.[12,13]

On the other hand, studies on ATPCL inhibitors such as (−)hydroxycitrate and SB-201076 demonstrated that citrate is a precursor of cytoplasmic acetyl-CoA for cholesterol and fatty acid synthesis in liver and adipose tissue, as well as for acetylcholine synthesis in the brain.[12,14,15] ATPCL inhibitors decreased cholesterol and triglyceride plasma levels, indicating their potential utility as hypolipidemic drugs.[14] However, there is no information whether these compounds could affect blood platelet activity that might depend on provision of cytoplasmic acetyl-CoA for synthesis of metabolites such as platelet-activating factor or arachidonic acid/thromboxane A_2. If this is the case, then platelet ATPCL could be a potential target for interventions on suppressing platelet activity. Therefore, the aim of this study was to investigate whether ATPCL may play a role in the metabolism of cytoplasmic acetyl-CoA in blood platelets and thereby affect their function in healthy and diabetic subjects.

MATERIALS AND METHODS

Patients

Our study group consisted of patients with type I and II diabetes from the Diabetology Center of Public Clinical Hospital No.1, Medical University of Gdańsk attending laboratory for scheduled check-ups. The control group consisted of healthy people coming for control blood examination from Outpatient Occupational Health Unit of the same

From the Chair of Clinical Biochemistry, Department of Laboratory Medicine, Medical University of Gdańsk, Gdańsk, Poland.

Submitted March 24, 2003; accepted July 2, 2003.

Supported by the Committee for Scientific Research Projects No. 4P05A 086 15, 3P05B 082 23, and W-78 from the Medical University of Gdańsk.

Address reprint requests to Andrzej Szutowicz, Prof MD, Chair of Clinical Biochemistry, Department of Laboratory Medicine, Medical University of Gdańsk, Dębinki 7 str., 80-211 Gdańsk, Poland.

0026-0495/04/5301-0035$30.00/0

doi:10.1016/j.metabol.2003.07.012

hospital. Patients with diabetes complicated by albuminuria above 0.03 g/d, or with evident macroangiopathy, as well as those with type II diabetes receiving insulin, were excluded from the study.

An additional sample of 10 mL of blood was collected from each subject in vacuum tubes containing 1 mg of K_2-EDTA per 1 mL of blood and used for isolation of platelets for enzyme, acetyl-CoA, and aggregation assays. Remaining tests were performed on the samples of blood collected by physician request. The study protocol was approved by the Regional Commission on Ethics of Human Studies at the Medical University of Gdańsk.

Reagents and Materials

Chemicals for enzyme and acetyl-CoA assays were purchased from Sigma Chemicals Co (Poznań, Poland); thrombin was from Bio-Med, Lublin, Poland; Commassie Brillant Blue G-250 was from Bio-Rad (Munchen, Germany); glycine-proline-arginine-proline-NH_2 tetrapeptide (GPRP) from Bachem AG (Bubendorf, Switzerland); SB-204990 was kindly provided by Smith Kline Beecham Pharmaceuticals (Harlow, UK). (−)Hydroxycitrate lactone was gift from Dr L.Y. Lewis (Mysore, India); it was converted into trisodium salt by hydrolysis with an equivalent amount of sodium hydroxide. All other chemicals were of analytical grade. Venoject tubes used for blood collection were from Becton-Dickinson (Oxford, UK).

Diagnostic kits for hemoglobin A_{1c} (HbA_{1c}) (1488414) and fructosamine (67246901) assays were from Roche (Zurich, Switzerland) Determinations were performed on a Hitachi 917 biochemical analyzer (Roche, Zurich, Switzerland). Plasma glucose was determined using commercial kit on a Dimension RXL biochemical analyzer (Dade Behring, Warsaw, Poland).

Platelet Isolation

Whole blood was centrifuged at 4°C at 100 × *g* for 15 minutes in a Jouan CR 3.12 centrifuge (Jouan S.A., Saint-Herbloin, France). The platelet-rich plasma obtained was collected into plastic tubes and centrifuged at 500 × *g* for 15 minutes to obtain platelet-poor plasma and platelet pellet. The pellet was washed 3 times with solution containing 140 mmol/L NaCl, 5 mmol/L NaHEPES buffer (pH 7.4), and 5 mmol/L glucose, and finally suspended in a small volume of the same solution to obtain a protein concentration of approximately 10 mg/mL. The amount of platelets and yield of separation procedure as well as contamination by other blood cells was assessed using a CELL-DYN 3200 automatic haematology analyzer (Abbott, Abbott Park, IL).

Enzyme Assays

ATPCL was assayed within 3 hours after platelet isolation, as described elsewhere.[16] PDH activity was determined by assessment of formed acetyl-CoA.[17] Immediately before the assays, platelet membranes were solubilized by the addition of Triton X-100 (final concentration, 0.2% vol/vol). Assays were performed at 37°C in Shimadzu UV 1202 spectrophotometer (Shimadzu Europa, Duisburg, Germany).

Acetyl-CoA Assay

Acetyl-CoA content was assessed in freshly isolated platelets that were incubated in medium containing glucose in order to obtain its steady-state level under controlled conditions.[18] Incubation medium in a final volume of 1.0 mL contained 20 mmol/L NaHEPES buffer, 1.7 mmol/L Na-phosphate buffer (final pH of the medium, 7.4), 140 mmol/L NaCl, 5.5 mmol/L KCl, and 2 mmol/L glucose. For studies of acetyl-CoA metabolism in activated platelets 0.1 IU of thrombin was added along with 2.5 mmol/L GPRP peptide to prevent aggregation, as indicated.[19] Incubation was started by the addition of platelet suspension (1 mg of protein) and caried on for 20 minutes at 37°C in polystyrene flat-bottom vessels in the water bath with continuous shaking for 100 cycles per minute. Incubation was terminated by transfer of 0.5-mL samples of the medium to Eppendorf tubes placed in an ice bath followed by centrifugation for 1 minute at 12,000 × *g*. Whole platelet pellet was deproteinized by the addition of 0.08 mL of 5 mmol/L HCl and placement for 1 minute in a boiling water bath. After centrifugation, the clear supernatants were taken for acetyl-CoA determinations by the cycling method using phosphotransacetylase and citrate synthase.[18]

For studies of intracellular distribution of acetyl-CoA, the remaining 0.5 mL of platelet suspension was mixed with an equal volume of ice-cold lysing medium containing 20 mmol/L Tris-HCl buffer, pH 7.4, 125 mmol/L KCl, 3 mmol/L EDTA, and 1.4 mg/mL digitonin, layered over 0.5 mL mixture of silicon oils (AR-20 and AR-200, 1:2) in 1.5-mL Eppendorf tubes. After 30 seconds, the particulate fraction was separated by centrifugation for 1 minute at 12,000 × *g*. The upper layer was collected for protein and lactate dehydrogenase assays. Silicon oil layer was discarded and the pellet was deproteinized by the addition of 5 mmol/L HCl and incubation in a boiling bath for 1 minute and used for determination of particulate (mainly mitochondrial) acetyl-CoA. Cytoplasmic acetyl-CoA content was calculated by subtraction of the mitochondrial acetyl-CoA content from that found in whole platelets. Occasionally, to check reliability of the separation procedure, glutamate dehydrogenase and lactate dehydrogenase activities were determined in particulate and soluble fractions.[20]

Platelet Aggregation and Malonyl Dialdehyde Assays

Platelets were suspended in 0.5 ml of medium containing 140 mmol/L NaCl, 20 mmol/L NaHEPES buffer (pH 7.4), and 2 mmol/L glucose to obtain a platelet concentration of 200 to 300 × $10^3/\mu$Ll and were preincubated for 5 minutes at 37°C in an APACT aggregometer (Labor, Ahrensburg, Germany), with simultaneous recording of spontaneous aggregation. Platelets were activated by the addition of 0.05 mL thrombin (final concentration, 0.1 IU/ml) and aggregation was recorded for 10 minutes at 37°C against parallel blank without thrombin. Both samples were deproteinised by the addition of 0.55 mL of 20% (wt/vol) trichloroacetic acid; the mixture was shaken for 30 minutes at 4°C and centrifuged. Clear supernatants were taken for malonyl dialdehyde (MDA) assay.[21] Accumulation of MDA in thrombin-activated platelets was calculated by subtraction of its amount in the activated sample from that present in sample deproteinized at zero time.

Protein Assay

Protein was determined by the method of Bradford with bovine immunoglobulin as a standard.[22]

Statistical Analysis

The differences between groups were tested by 1-way analysis of variance (ANOVA) followed by Bonferroni *t* multiple comparisons test or nonpaired Student's *t* test, using the Graph Pad Prism 2.01 statistical package (San Diego, CA).

RESULTS

Experimental Groups

The average age of diabetic patients was similar with that of the healthy controls (Table 1). Fasting glycaemia levels, HbA_{1C}, and fructosamine concentrations in the diabetic group were found to be 115%, 57%, and 59% higher than those in controls (Table 1). On the other hand, serum cholesterol was the same and triglyceride levels in the diabetic group were only slightly higher than those in the healthy group (Table 1).

Table 1. Demographic and Metabolic Parameters of the Experimental Groups

Parameter	Healthy Subjects	Diabetes
No. of subjects	50	48
Diabetes duration (yr)	—	10.9 ± 1.1
Age (yr)	43.6 ± 2.8	46.6 ± 1.5
Platelet count ($10^3/\mu L$)	270 ± 12	258 ± 9
Plasma glucose (mg/dL)	89.3 ± 1.3	211 ± 9*
Blood HbA_{Ic} (%)	5.1 ± 0.1	8.9 ± 0.2*
Fructosamine (μmol/L)	234 ± 3	372 ± 9*
Serum cholesterol (mg/dL)	216 ± 4	222 ± 7
Serum triglycerides (mg/dL)	104 ± 6	147 ± 24†

NOTE. Data are means ± SEM from number of observations given in the table; each group consisted of equal numbers of women and men.

Significantly different from healthy subjects: *$P < .001$, †$P < .05$.

Platelet counts in whole blood were also similar in both groups. Contamination of platelet preparations by red and white blood cells was below 1% (not shown).

Effect of Diabetes on Enzyme Activities

Activities of glutamate and lactate dehydrogenases, marker enzymes for mitochondrial and cytosolic fractions, respectively,were found to be similar in both groups. On the contrary, in diabetic platelets, PDH and ATPCL activities were 35% and 51%, respectively higher than in the control group (Table 2).

Effect of Inhibitors on ATPCL Activity

It is known that SB-204990 is an inactive cell-penetrant lactone precursor of potent ATPCL inhibitor SB-201076. The latter is formed inside the cells after lactone hydrolysis.[14] It has been shown that 30 minutes preincubation of platelets suspension at 37°C does not affect ATPCL activity in either diabetic or control platelets. However, longer, 60 minutes, preincubation caused a 50% decrease of enzyme activity (not shown). Therefore, platelet preincubation with SB-204990 to allow its influx and hydrolysis to SB-201076 inside the cells had to be limited to 30 minutes. Such treatment caused 61% and 37% inhibition of ATPCL activity in diabetic and control platelets, respectively (Table 3). On the other hand, 1 mmol/L (−)hydroxycitrate caused immediate 100% inhibition of ATPCL activity in Triton X-100–treated platelets from both groups (Table 3).

Table 2. Effect of Diabetes on Enzyme Activities in Diabetic Platelets

Enzyme	Specific Activity (nmol/min/mg protein)	
	Control	Diabetes
PDH	3.82 ± 0.17 (21)	5.01 ± 0.28* (34)
ATPCL	3.57 ± 0.28 (27)	5.39 ± 0.26* (33)
Lactate dehydrogenase	2,375 ± 115 (12)	2,551 ± 92 (14)
Glutamate dehydrogenase	14.1 ± 1.1 (12)	16.1 ± 1.0 (14)

NOTE. Data are means ± SEM from number of experiments given in parentheses.

*Significantly different from controls, $P < .01$.

Table 3. Effect of Inhibitors on ATPCL Activity in Blood Platelets

Additions (mmol/L)	ATPCL Activity (nmol/min/mg protein)	
	Control	Diabetes
No additions	2.98 ± 0.27	4.51 ± 0.30†
SB-204990 0.1	1.90 ± 0.21*	1.84 ± 0.21*
(−)Hydroxycitrate 1.0	0*	0*

NOTE. Data are means ± SEM from 12 experiments. Assay medium contained low 1 mmol/L instead saturating 20 mmol/L citrate concentration.

Significantly different from respective values: *without inhibitor $P < .001$; †control, $P < .001$ by Student's t test.

Effect of Diabetes on Platelet Acetyl-CoA

Treatment of blood platelets with digitonin in 0.7 mg/mL concentration solubilized plasma membranes, as indicated by 89% recovery of lactate dehydrogenase in the soluble fraction (Table 4). On the other hand, mitochondrial membrane integrity was well preserved as 92% of glutamate dehydrogenase remained inside of mitochondria (Table 4).

It is known that only acetyl-CoA present in the cytoplasmic compartment may be used for thromboxane A_2 and MDA or platelet-activating factor synthesis. Therefore, the acetyl-CoA distribution in mitochondrial and cytoplasmic platelet compartments was tested in this work.

Diabetes caused 62% increase of overall platelet acetyl-CoA (Table 5). However, this effect was confined to platelet mitochondria, where diabetes brought about a 113% increase of acetyl-CoA content. In the cytoplasmic compartment, no changes in acetyl-CoA content were found (Table 5). The addition of (−)hydroxycitrate resulted in an approximately 50% decrease of acetyl-CoA content in platelet cytoplasm of both groups and no significant changes of its level in mitochondria (Table 5). On the other hand, thrombin activation decreased acetyl-CoA content in platelet mitochondria of both healthy and diabetic groups by 3.5 and 8.0 pmol/mg of protein, respectively. Such treatment did not cause any changes in acetyl-CoA level in the platelet cytoplasmic compartment in either group (Table 5).

Effect of ATPCL Inhibitors on MDA Synthesis and Platelet Aggregation

MDA is an indicator of thromboxane A_2 synthesis in platelets, as it is produced by platelets in equivalent amounts with this platelet activator.[23] In resting conditions MDA synthesis in diabetic platelets was 63% higher that in control conditions

Table 4. Effect of Digitonin on Marker Enzyme Content in Blood Platelet Subcellular Fractions

Parameter	Whole Platelets	Mitochondria	Cytosol
Protein (mg/mL)	0.61 ± 0.02	0.15 ± 0.04	0.46 ± 0.01
Lactate dehydrogenase (nmol/min/mg protein)	1845 ± 37	204 ± 8	1641 ± 31
Glutamate dehydrogenase	13.3 ± 0.3	12.3 ± 0.1	1.0 ± 0.2

NOTE. Data are means ± SEM from 5 experiments.

Table 5. Effect of Diabetes on Acetyl-CoA Distribution in Blood Platelets: Effect of (−)Hydroxycitrate and Thrombin Activation

Additions (mmol/L)	Acetyl-CoA Content (pmol/mg platelet protein)		
	Whole Platelets	Mitochondria	Cytosol
No additions			
Control	13.2 ± 0.7	7.3 ± 0.4	5.9 ± 0.4
Diabetes	21.6 ± 0.8§	15.5 ± 0.5§	5.9 ± 0.4
(−)Hydroxycitrate 1.0			
Control	10.7 ± 1.1	7.6 ± 0.9	3.1 ± 0.8*
Diabetes	16.2 ± 0.7†	13.5 ± 0.8§	2.7 ± 0.6*
Thrombin 0.1 U/mL, GPRP 2.5			
Control	9.8 ± 0.9*	3.8 ± 1.0*	6.0 ± 0.9
Diabetes	13.1 ± 1.0†‡	7.5 ± 0.9†‡	5.6 ± 1.1

NOTE. Data are means ± SEM from 11 (no additions) and 6 experiments (additions).

Significantly different from: respective data with no additions, *$P < .01$, †$P < .001$; respective control, ‡$P < .05$, §$P < .005$.

(Table 6). Neither (−)hydroxycitrate nor SB-204990 had a significant influence on resting MDA synthesis in platelets of healthy and diabetic subjects. Thrombin addition brought about 5- and 3.8-fold increases in MDA synthesis in control and diabetic platelets, respectively. Absolute MDA synthesis in thrombin-activated platelets was 35% higher in diabetic than in control subjects (Table 6). Addition of (−)hydroxycitrate or SB-204990 resulted in 27% and 59% inhibition of thrombin-evoked MDA synthesis in control and diabetic platelets, respectively (Table 6). In the healthy group, (−)hydroxycitrate caused no change, whereas SB-204990 inhibited MDA synthesis by 54%. It should be noted that both inhibitors decreased MDA synthesis to similar absolute values (Table 6). Significant correlation was found between acetyl-CoA levels and spontaneous and thrombin-activated MDA synthesis in both platelet groups in different experimental conditions (Fig 1).

Diabetes caused a 46% increase in spontaneous and 21% increase in thrombin-evoked platelet aggregation. Neither (−)hydroxycitrate nor SB-204990 caused significant changes in platelet spontaneous aggregation in the diabetic or healthy groups (Table 7). On the other hand, in thrombin-activated platelets, (−)hydroxycitrate decreased aggregation of both diabetic and control platelets by 46% and 29%, respectively. The respective inhibitory effects of SB-204990 were 48% and 32% (Table 7).

DISCUSSION

The high values of HbA_{1c}, fructosamine, and fasting glucose in the group of diabetic patients selected for this study indicate that their hyperglycemia was not well controlled for either the short or long term (Table 1). Hence, increased activities of PDH and ATPCL may reflect adaptative changes in the expression of these enzymes caused by increased inflow of glucose into megakaryocytes of diabetic patients (Table 2). Such an explanation results from the fact that platelets express insulin-independent, type 3 glucose transporter of relatively high, 10 mmol/L Km, value for this metabolite.[10] Therefore, hyperglycemic conditions may activate glucose influx into the platelets, where its utilization is not limited due to relatively high hexokinase activity.[11] This claim is in accord with experiments performed on brain nerve terminals, which like platelets have insulin-independent Glut3 transporter.[24] They revealed that streptozotocin-evoked diabetes caused an increase in energy substrates utilization, acetyl-CoA content, and acetylcholine synthesis in brain nerve terminals.[25] Thus, the increased level of platelet acetyl-CoA (Table 5) shown here could be due to activation of its synthesis from pyruvate inside of platelet mitochondria. Such a possibility is supported by the fact that the diabetes-evoked increase in acetyl-CoA was confined exclusively to platelet mitochondria (Table 5). This indicates that the increased activity of PDH in diabetic platelets may be a main cause of its elevated level in the mitochondrial compartment (Tables 2 and 5).

For studies of intraplatelet distribution of acetyl-CoA, the digitonin solubilization method has been applied, which was previously used for fractionation of hepatocytes and nerve terminals.[20] Digitonin, used here for platelet subfractionation, solubilized cholesterol/sphingomyelin rich plasma membranes leaving cholesterol-poor mitochondrial membranes untouched. Data presented here prove that digitonin yielded reliable fractionation of the platelets, since 90% of lactate dehydrogenase was released to cytosol and more than 90% of glutamate dehydrogenase was retained inside mitochondria (Table 3).[20]

An increased level of mitochondrial acetyl-CoA may facilitate its indirect and direct transport to platelet cytoplasm. However, levels of cytoplasmic acetyl-CoA are not changed in diabetes. This might be due to the fact that either its transport and utilization in cytoplasm were not increased, or both of them were activated in the course of the disease. This second possibility is supported here by the fact that thrombin activation caused a 2 times greater decrease of mitochondrial acetyl-CoA in diabetic platelets than in control platelets (Table 5). This phenomenon is similar to that observed in nerve terminals, where depolarization resulted in a decrease of acetyl-CoA in mitochondria due to its release by a direct, calcium-dependent transport triggered by activation of permeability transition state in mitochondrial membranes.[26,27] It is possible that thrombin activation generated similar conditions in blood platelets. It was demonstrated here by increased spontaneous and thrombin-evoked platelet aggregation in diabetic individuals (Table 7).

Table 6. Effect of ATPCL Inhibitors on Resting and Thrombin-Evoked MDA Synthesis in Platelets

Additions (mmol/L)	MDA Synthesis (nmol/10 min/mg protein)	
	Control	Diabetes
Resting conditions		
No additions	0.62 ± 0.07	1.01 ± 011‡
SB-204990 0.1	0.55 ± 0.05	0.82 ± 0.06‡
(−)Hydroxycitrate 1.0	0.58 ± 0.03	0.74 ± 0.03‡
Thrombin 0.1 U/mL		
No additions	2.80 ± 0.22	3.80 ± 0.19‡
SB-204990 0.1	1.30 ± 0.18†	1.57 ± 0.19†
(−)Hydroxycitrate 1.0	2.50 ± 0.15	2.81 ± 0.25*

NOTE. Data are means ± SEM from 11-18 experiments.

Significantly different from: respective data without additions, *$P < .01$, †$P < .001$; respective control, ‡$P < .01$.

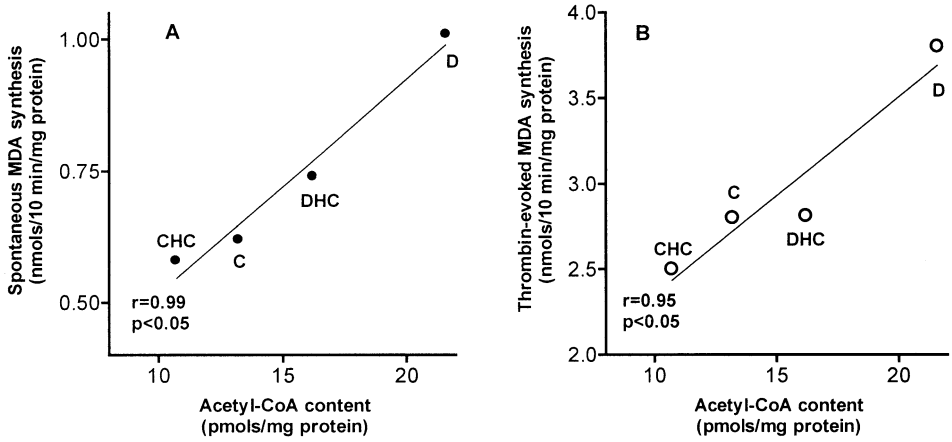

Fig 1. Correlations between acetyl-CoA content and spontaneous (A) and thrombin-evoked (B) MDA synthesis in platelets isolated from blood of healthy and diabetic people. Calculated from data presented in Tables 5 and 6. Abbreviations: D, diabetic; DHC, diabetic with 1 mmol/L (−)hydroxycitrate; C, control; CHC, control with 1 mmol/L (−)hydroxycitrate.

Our data indicate that much higher quantities of acetyl-CoA were transported into platelet cytoplasm where they could activate cytoplasmic pathways of fatty acids/thromboxanes and platelet-activating factor synthesis (Table 5).[4,28] Therefore, no changes in cytoplasmic level of acetyl-CoA were found (Table 5).

On the other hand, suppression of cytoplasmic acetyl-CoA content by (−)hydroxycitrate indicates that ATPCL plays a significant role in the maintenance of a stable level of this metabolite in the cytoplasmic compartment. About 50% of acetyl units were provided to cytoplasm by the ATPCL pathway, since this inhibitor caused a respective decrease of the acetyl-CoA level in platelet cytoplasm (Table 5).

The increase of both resting and thrombin-evoked MDA accumulation and aggregation of diabetic platelets reported here is in accord with other data revealing increased platelet activity in the course of the disease (Tables 6 and 7).[4,5,6,29,30] Values of spontaneous and thrombin-evoked aggregation of our platelet preparations were similar to those reported previosly for platelets isolated from citrate blood[29,30] (A. Szutowicz, unpublished data). It indicates that the procedure of platelet isolation from EDTA-blood used here did not artificially affect platelet activity (Table 7). Citrated blood could not be used in this experiment, as citrate interfered with our methods for acetyl-CoA and PDH assays.[17,18]

Table 7. Effect of ATPCL Inhibitors on Spontaneous and Thrombin-Evoked Platelet Aggregation

Additions (mmol/L)	Aggregation (%)	
	Control	Diabetes
Resting conditions		
No additions	10.3 ± 0.3	15.0 ± 0.7†
SB-204990 0.1	8.8 ± 0.4	13.6 ± 0.6†
(−)Hydroxycitrate 1.0	8.8 ± 0.4	12.3 ± 0.6†
Thrombin 0.1 U/mL		
No additions	78.5 ± 2.0	95.2 ± 2.0†
SB-204990 0.1	53.5 ± 2.8*	49.6 ± 4.0*
(−)Hydroxycitrate 1.0	56.1 ± 3.0*	51.3 ± 2.5*

NOTE. Data are means ± SEM from 12 to 24 experiments.

Significantly different from: respective data without additions, *$P < .001$; respective control, †$P < .001$.

Lack of significant inhibition of spontaneous MDA synthesis and platelet aggregation by (−)hydroxycitrate and SB-204990 indicates that the ATPCL pathway may not play an important role in the maintenance of these platelet functions under resting conditions either in healthy or in diabetic subjects. Presumably, provision of acetyl-CoA by other transport pathways was sufficient to maintain basal rates of thromboxane A_2 synthesis and spontaneous aggregation (Tables 6 and 7).

The fact that the thrombin activation–evoked decrease in mitochondrial acetyl-CoA was accompanied by an increase in MDA synthesis and platelet aggregation, and that these shifts were greater in diabetic than in control platelets, may indicate that these processes are interdependent. Namely, increased transport of acetyl units to cytoplasm in diabetic platelets could activate thromboxane A_2/MDA synthesis followed by increased platelet aggregation (Tables 5, 6, and 7).[4,5,6,30] Thus, the increased level of acetyl-CoA in diabetic platelets reported here would explain, at least in part, the pathomechanism of increased platelet activity in this pathology.[5,6,30] Highly significant correlation between acetyl-CoA levels and MDA synthesis in normal and diabetic platelets strongly supports the hypothesis that this metabolite plays an important role in the regulation of their activity by changes in platelet-activating lipids synthesis (Fig 1).[4,8,28,30]

On the other hand, increased inhibition of MDA synthesis in thrombin-activated diabetic platelets by SB-204490 indicates that in these conditions respective additional pool of acetyl groups was provided to cytoplasm via the ATPCL pathway (Tables 6 and 7). This means that metabolic flow through the ATPCL pathway was activated by the disease (Table 6). The fact that SB-204990 decreased absolute values of MDA synthesis and aggregation of normal and diabetic platelets to the same levels indicates that this compound may bring these functions of diabetic platelets back to normal range (Tables 6 and 7). However, in this in vitro study, relatively high (0.1 to 1.0 mmol/L) concentrations of inhibitors were used to assure significant inhibition of ATPCL activity (Table 3).[14] This raises the question whether similar inhibitor concentrations can be attained under in vivo conditions. Past studies demonstrated that 0.05- to 0.150-mmol/L concentrations of SB-201076 and nearly milimolar levels of (−)hydroxycitrate were present in

blood and tissues of experimental animals in which hypolipidemic effects of these compounds were evident.[14,31] Moreover, these conditions caused no apparent toxic effects in experimental animals. Therefore, ACL inhibitors may be considered as potential drugs that downregulate not only lipid synthesis in the liver of hyperlipidemic individuals,[14,31] but also platelet activity in diabetic subjects (Tables 6 and 7).

However, it should be emphasized that in our study disturbances in lipid metabolism did not contribute to changes in platelet acetyl-CoA metabolism, as serum cholesterol was the same and triglyceride level was slightly higher in diabetic compared to healthy subjects (Table 1). The absence of hypercholestrolemia in our diabetic group was apparently caused by selection of individuals free of vascular complications. On the other hand, the increase in serum triglycerides in the diabetic group resulted from the fact that half of the patients had type II diabetes. However, PDH and ATPCL activities in platelets of patients with diabetes I II were the same (data not shown). Thus, lasting hyperglycemia remains a major factor involved in the activation of enzymes of acetyl-CoA metabolism and accumulation of increased amounts of acetyl-CoA in diabetic platelets (Table 5, Fig1).

It is important to note that strong, 100%, inhibition of ATPCL by (−)hydroxycitrate remained in conflict with its relatively poor ability to inhibit MDA synthesis. The reverse was true for SB-204990 (Tables 2 and 6).[14] This discrepancy may result from the relatively poor permeability of platelet plasma membrane to (−)hydroxycitrate and the good permeability for SB-204990. It could cause intracellular concentration of SB-201076 to be higher than that of (−)hydroxycitrate.[14] On the other hand, the effect of inhibitors on ATPCL activity was tested in medium containing cells solubilized with Triton X-100, in which the effective concentration of (−)hydroxycitrate (Ki 0.15 μmol/L) was apparently higher than that of active derivative SB-201076 (Ki 1.0 μmol/L)[14] (Table 3). The ATPCL inhibitors used here are quite specific for this enzyme and not for other enzymes utilizing citrate.[14,32] Therefore, the suppressory effects of these inhibitors on MDA synthesis and platelet aggregation are likely to be due to their influence exclusively on ATPCL activity (Tables 6 and 7). Generally, the data presented here are consistent with the hypothesis that the ATPCL pathway is operative in human blood platelets. It may play an important role in the provision of acetyl-CoA to platelet cytoplasm and in the pathomechanism of their excessive activity in diabetes. Our data also indicate that changes in transport of acetyl-CoA from platelet mitochondria to cytoplasm caused by diabetic hyperglycemia may be responsible, at least in part, for platelet hyperactivity due to stimulation of production of thromboxane A_2 and presumably other acetyl-CoA–containing platelet activators.

ACKNOWLEDGMENT

We are indebted to Smith Kline and Beecham, Harlow, UK for free sample of SB-204990.

REFERENCES

1. Brownlee M: Glycation and diabetic complications. Diabetes 43:836-841, 1994

2. Cohen I, Burk D, Fullerton RJ, et al: Nonenzymatic glycation of human blood platelet proteins. Thromb Res 55:341-349, 1989

3. Jornesskog G, Egberg N, Fagrell B, et al: Altered properties of fibrinogen gel structure in patients with IDDM. Diabetologia 39:1519-1523, 1996

4. Davi G, Catalano I, Averna M, et al: Thromboxane biosynthesis and platelet function in type II diabetes mellitus. N Engl J Med 322:1769-1774, 1990

5. Halushka PV, Mayfield R, Wohltmann HJ, et al: Increased platelet arachidonic acid metabolism in diabetes mellitus. Diabetes 30:44-48, 1981

6. Rabini RA, Staffolani R, Fumelli P, et al: Decreased nitric oxide synthase activity in platelets from IDDM and NIDDM patients. Diabetologia 41:101-104, 1998

7. Colwell JA: Vascular thrombosis in type II diabetes mellitus. Diabetes 42:8-15, 1993

8. Halushka PV, Pawate S, Martin ML: Thromboxane A_2 and other eicosanoids, in Bruchhausen F, Walter U (eds): Handbook of Experimental Pharmacology, Platelets and Their Factors, vol 21. Berlin, Germany, Springer, 1997, pp 459-482

9. Holmsen H: Metabolism of platelets, in Williams WJ (ed): Hematology. New York, NY, McGraw-Hill, 1990, pp 1200-1233

10. Craik JD, Stewart M, Cheeman CI: GLUT-3 (brain-type) glucose transporter polypeptides in human blood platelets. Thromb Res 79:461-469, 1995

11. Nowacka M, Skibowska A, Raszeja-Specht A, et al: Relationship between acetyl-CoA metabolism and function of blood platelets from diabetic subjects. Clin Chem Lab Med 45:S105, 2002 (suppl, abstr)

12. Szutowicz A, Bielarczyk H, Skulimowska H: Effect of dichloroacetate on acetyl-CoA content and acetylcholine synthesis in rat brain synaptosomes. Neurochem Res 9:1107-1112, 1994

13. Srere PA: The molecular physiology of citrate. Nature 205:766-770, 1965

14. Pearce J, Yates W, Berkhout A, et al: The role of ATP citrate-lyase in the metabolic regulation of plasma lipids. Biochem J 334:113-119, 1998

15. Sullivan AC, Hamilton JG, Miller ON, et al: Inhibition of lipogenesis in rat liver by (−)hydroxycitrate. Arch Biochem Biophys 150:183-190, 1972

16. Szutowicz A, Stepień M, Bielarczyk H, et al: ATP–citrate lyase in cholinergic nerve endings. Neurochem Res 7:798-910, 1972

17. Szutowicz A, Stepień M, Piec G: Determination of pyruvate dehydrogenase and acetyl-CoA synthetase activities using citrate synthase. Anal Biochem 115:81-87, 1981

18. Szutowicz A, Bielarczyk H: Elimination of CoASH interference from acetyl-CoA cycling assay by maleic anhydride. Anal Biochem 164:292-296, 1987

19. Kawasaki K, Miyano M, Hirase K, et al: Amino acids and peptides. XVIII. Synthetic peptides related to N-terminal portion of fibrin alpha-chain and inhibitory effects on fibrinogen/thrombin clotting. Chem Pharm Bull 41:975-977, 1993

20. Bielarczyk H, Szutowicz A: Evidence for the regulatory function of synaptoplasmic acetyl-CoA in acetylcholine synthesis in nerve endings. Biochem J 262:377-380, 1989

21. Pense M, Black H, Furster W, et al: An improved malonyldialdehyde assay for estimation of thromboxane synthetase activity in washed human blood platelets. Prostaglandins 30:1031-1038, 1985

22. Bradford M: A rapid sensitive method for the quantitation of microgram quantities of protein utilizing principle of protein dye binding. Anal Biochem 72:248-254, 1976

23. Makris P, Tsakiris D, Papadoulos A, et al: The ratio MDA/

MDAa as a new index of platelet hyperactivity. Haemostasis 15:331-336, 1985

24. Reagan LP, Magarinos AM, McEwen BS: Neurological changes induced by stress in streptozotocin diabetic rats. Ann NY Acad Sci 893:126-137, 1999

25. Szutowicz A, Tomaszewicz M, Jankowska A, et al: Acetylcholine synthesis in nerve terminals of diabetic rats. Neuroreport 5:2421-2424, 1994

26. Green DR, Reed JC: Mitochondria and apoptosis. Science 281: 1309-1315, 1998

27. Bielarczyk H, Tomaszewicz M, Szutowicz A: Effect of aluminium on acetyl-CoA and acetylcholine metabolism in nerve terminals. J Neurochem 70:1175-1181, 1998

28. Shukla SD, Paul A, Klaschko DM: Hypersensitivity of diabetic human platelets to platelet activating factor. Thromb Res 66:239-246, 1992

29. Udvardy M, Kaplar M, Rejto L, et al: Increased in vivo platelet activation and reduced intravascular endothelium derived relaxing factor and nitrate/nitrite production in patients with diabetes mellitus. Platelets 9:257-260, 1998

30. Iwase E, Tawata M, Aida K, et al: A cross-sectional evaluation of spontaneous platelet aggregation in relation to complications in patients with type II diabetes mellitus. Metabolism 47:699-705, 1998

31. Sullivan AC, Triscari J, Spiegel HE: Metabolic regulation as a control for lipid disorders. II: Influence of (−)hydroxycitrate on genetically and experimentally induced hypertriglyceridemia in the rat. Am J Clin Nutr 30:777-784, 1977

32. Sullivan AC, Singh M, Srere PA, et al: Reactivity and inhibitor potential of hydroxycitrate isomers with citrate synthase, citrate lyase and ATP citrate lyase. J Biol Chem 252:7583-7599, 1977