Original Article

Alterations of Adenine Nucleotide Metabolism and Function of Blood Platelets in Patients With Diabetes

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Increased activity of blood platelets contributes to vascular complications in patients with diabetes. The aim of this work was to investigate whether persisting hyperglycemia in diabetic patients generates excessive accumulation of ATP/ADP, which may underlie platelet hyperactivity. Platelet ATP and ADP levels, thiobarbituric acid–reactive species synthesis, and aggregation of platelets from patients with diabetes were 18–82% higher than in platelets from healthy participants. In patients with diabetes, platelet stimulation with thrombin caused about two times greater release of ATP and ADP than in the healthy group while decreasing intraplatelet nucleotide content to similar levels in both groups. This indicates that the increased content of adenylate nucleotides in the releasable pool in the platelets of diabetic patients does not affect their level in metabolic cytoplasmic/mitochondrial compartments. Significant correlations between platelet ATP levels and plasma fructosamine, as well as between platelet ATP/ADP and platelet activities, have been found in diabetic patients. In conclusion, chronic hyperglycemia-evoked elevations of ATP/ADP levels and release from blood platelets of patients with diabetes may be important factors underlying platelet hyperactivity in the course of the disease. ***Diabetes*** **56:462–467, 2007**

Atherosclerosis and microangiopathy are common complications of long-lasting, insulin-dependent and -independent diabetes. Several factors contribute to the self-propelling mechanism of atheromatic lesions in the course of the disease, including dyslipoproteinemia; excessive synthesis of inflammatory cytokines, free radicals, and lipid peroxides; as well as blood platelet hyperactivity (1–3). Increased spontaneous and thrombin-, collagen-, or ADP-evoked aggregation has been demonstrated in diabetic patients (4). The elevation in the synthesis of thromboxane A_2, lipid peroxides, and platelet-activating factors in platelets correlates with the gravity of angiopathy in diabetic individuals (5,6).

Increased synthesis of unsaturated long-chain fatty acids in platelets from diabetic patients may also contribute to the excessive platelet activity in the course of the disease (7). Synthesis of endogenous fatty acids requires acetyl-CoA, which derives mainly from glucose transported into the platelets through the insulin-insensitive GLUT3 transporter (8). Increased glucose influx into megacariocytes in the course of sustained diabetic hyperglycemia is a likely cause of the increased activity of several enzymes involved in glycolytic, energy-producing, and fatty acid–synthesizing pathways, and it activates acetyl-CoA synthesis (9,10). Elevated levels of acetyl-CoA in the platelets of diabetic subjects correlate with increased synthesis of thromboxane A_2/malonyl dialdehyde and lipid peroxidation as well as spontaneous and thrombin-, ADP-, or collagen-evoked aggregation (9,10).

Oxidative metabolism of pyruvate-derived acetyl-CoA in platelet mitochondria is the main source of ATP when coupled with oxidative phosphorylation. Large pools of ATP/ADP are sequestered in secretory granules during platelet formation, and the release of ADP from activated platelets is one of the important signals stimulating their aggregation. Furthermore, ATP coreleased with ADP from dense granules may increase platelet aggregation in whole blood because of its cleavage to ADP by ecto-ATPases present on the surface of leukocytes (11,12). In addition, platelet exocytosis and aggregation require metabolic energy in the form of ATP (13). Therefore, in diabetes the increase of acetyl-CoA–dependent oxidative metabolism in platelets or in their precursors might cause an excessive accumulation of ATP/ADP (7,9,10,14). However, the relationships between ATP/ADP accumulation in platelets and their activity in patients with diabetes have not yet been examined.

The aim of this work was to investigate whether persisting hyperglycemia in diabetic patients may generate excessive accumulation of ATP/ADP, which may underlie platelet hyperactivity. Data presented indicate that this might be the case.

RESEARCH DESIGN AND METHODS

At the Blood Collection Unit of the Central Clinical Laboratory, we enrolled study patients with type 1 and 2 diabetes coming from the Diabetology Center of the Academic Clinical Center, Medical University of Gdańsk, for scheduled check ups. Type 1 diabetic patients received different insulin preparations, whereas type 2 diabetic patients obtained oral antidiabetic drugs or a combination of oral antidiabetics and insulin. Also, ~60% of patients obtained statins or/and other hypolipidemic drugs. The reference group consisted of healthy people from the Outpatient Occupational Health Unit of the same hospital attending the laboratory for routine blood examination. Their fasting

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GPRP, glycine-proline-arginine-proline-NH_2; TBARS, thiobarbituric acid–reactive species.

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plasma glucose, serum fructosamine, and blood A1C levels were <103 mg/dl, 285 μmol/l, and 6%, respectively. Diabetic patients with albuminuria >0.03 g/day, or with evident micro- and macroangiopathy, were not admitted to the study. None of the investigated participants took aspirin, phosphodiesterase inhibitors, calcium channel blockers, or nonsteroidal anti-inflammatory drugs for at least 2 weeks before blood drawing. Blood was collected with a vacuum tube system between 7 and 9 A.M. after overnight fasting and after obtaining written consent from each donor. The study protocol was approved by the regional bioethical commission at the Medical University of Gdańsk (permission NKEBN/350-683/2001).

Reagents and materials. Reagents for ATP/ADP, acetyl-CoA assays, and suramin, a nonselective purinergic receptors antagonist (15,16), were supplied by Sigma Chemicals (Poznań, Poland); thrombin was from Bio-Med (Lublin, Poland); Commassie Brillant Blue G-250 was from Bio-Rad (Munchen, Germany); and glycine-proline-arginine-proline-NH_2 (GPRP) tetrapeptide was from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade. Venoject tubes used for blood collection were from Becton Dickinson (Oxford, U.K.).

Biochemical tests were performed using samples of blood collected on physician's request. Serum fructosamine was assayed with a kit from Roche (Zurich, Switzerland) (no. 67246901) using an Ultrospec 3100 spectrophotometer (Amersham-Pharmacia-Biotech, Cambridge, U.K.) at a wavelength of 530 nm. Serum total cholesterol, HDL cholesterol, triglycerides, and plasma glucose were determined using commercial kits on an Architect 8000 analyzer (Abbott, Warsaw, Poland).

Washed platelet isolation. We collected 10-ml samples of blood from each person in vacuum tubes containing 1 mg of tripotassium-EDTA per 1 ml of blood, and we used them for platelet isolation. Whole blood was centrifuged in 4°C at 150*g* for 15 min in a centrifuge (223e; MPW, Warsaw, Poland). Blood cells were washed once with an amount of 140 mmol/l NaCl solution equivalent to plasma volume to obtain platelet recovery of 66 ± 8%. Platelet-rich plasma and subsequent washings were collected into the plastic tube and concentrated by centrifugation at 500*g* for 15 min. The pellet was washed once with solution containing 140 mmol/l NaCl, 15 mmol/l NaHEPES buffer (pH 7.4), 0.1 mmol/l EDTA, and 5 mmol/l glucose and finally suspended in a small volume of the same solution to obtain protein concentrations of ~10 mg/ml. The morphologic parameters of collected blood, yield of separation procedure, and purity of platelets were assessed with an HMX automatic hematologic analyzer (Beckman-Coulter, Miami, FL). The washed platelet preparation contained <0.1% leukocyte and <0.5% erythrocyte contamination, respectively.

ATP/ADP assay. ATP/ADP content was assessed in freshly isolated platelets that were incubated for 30 min in medium containing glucose to obtain steady-state levels under controlled conditions (9). Incubation medium in a final volume of 1.5 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, 5.0 mmol/l KCl, and 2.5 mmol/l glucose. For studies of ATP/ADP metabolism in activated platelets, 0.15 IU of thrombin was added along with 2.5 mmol/l GPRP peptide (17). Such conditions prevented platelet aggregation and decomposition but did not affect platelet activation by the thrombin (10,18). Incubation was started by the addition of platelet suspension (0.7–0.8 mg of protein) and carried for 30 min at 37°C in polystyrene flat-bottom vessels in a water bath with continuous shaking at 100 cycles/min. Incubation was terminated by transfer of 0.5-ml samples of the medium to Eppendorf tubes placed in the ice bath, followed by centrifugation for 1 min at 12,000*g*. The whole-platelet pellet was suspended in 0.1 ml of double-distilled water and deproteinized by boiling for 3 min. After centrifugation, clear supernatants were taken for ATP determinations by luciferin/luciferase luminometric method in a Berthold Junior LB 9509 luminometer (Berthold Technology, Bad-Wildbad, Germany) (19). ADP was detected after its conversion to ATP in the presence of phosphoenolpyruvate and pyruvate kinase (19).

Acetyl-CoA assay. Platelet acetyl-CoA was assayed by phosphotransacetylase-citrate synthase cycling assay as described previously (10,20).

Platelet aggregation and thiobarbituric acid–reactive species assays. Platelet aggregation was measured at 37°C on an APACT 2 aggregometer (Labor, Ahrensburg, Germany). Platelets were suspended in 0.3 ml of the medium containing 140 mmol/l NaCl, 20 mmol/l NaHEPES buffer, pH 7.4, and 2.5 mmol/l glucose density of 200–300 × 10^3 per μl and preincubated for 5 min at 37°C in an aggregometer. Platelets were activated by the addition of 0.03 ml thrombin (final concentration 0.1 IU/ml), and aggregation was recorded for 10 min at 37°C against a parallel blank without thrombin. Spontaneous aggregation was measured by recording turbidity changes in platelet suspension in medium without thrombin. Both samples were deproteinized by the addition of 0.05 ml of 20% (wt/vol) trichloroacetic acid; the mixture was shaken for 30 min in 4°C and centrifuged. Clear supernatants were taken for TBARS assay (21). Accumulation of thiobarbituric acid–reactive species (TBARS), including malonyl dialdehyde and other lipid peroxides, in resting and thrombin-activated platelets, was calculated by subtracting the amount of TBARS accumulated after 10 min of incubation from the amount present in a sample deproteinized at time 0 min.

TABLE 1
Basic laboratory parameters of healthy donors and patients with diabetes

Parameter	Healthy donors	Patients with diabetes
n	29	32
M/F (*n*)	15/14	16/16
Duration of the disease (years)	—	11.5 ± 1.4
Age (years)	48.0 ± 2.9	48.6 ± 2.4
Plasma glucose (mg/dl)	90 ± 2	162 ± 6.8*
Plasma fructosamine (μmol/l)	224 ± 7	334 ± 11*
A1C	5.0 ± 0.1	8.9 ± 0.4*
Total cholesterol (mg/dl)	213 ± 15	188 ± 5
Triglycerides (mg/dl)	114 ± 13	133 ± 6
HDL cholesterol (mg/dl)	59 ± 4	56 ± 2
LDL cholesterol (mg/dl)	131 ± 15	106 ± 5
Erythrocytes (tera/l)	4.80 ± 0.11	4.65 ± 0.08
Hemoglobin (g/l)	14.6 ± 0.3	14.5 ± 0.3
Platelets (giga/l)	264 ± 15	232 ± 11
Mean platelet volume (whole blood) (femtoliter)	8.7 ± 0.2	8.8 ± 0.2

Data are means ± SE except for the number of participants and sex. LDL cholesterol was calculated by Friedewald's formula. *$P < 0.001$ for difference from healthy group (unpaired Student's *t* test).

Protein assay. Protein was determined by the method of Bradford (22) with bovine immunoglobulin as a standard.

Statistical analysis. The data distribution was tested by Kolmogorov-Smirnov test. $P > 0.1$ was considered to be indicative for normal distribution. Differences between groups were tested by nonpaired Student's *t* test and within group by paired Student's *t* test. Correlations assessment was performed with Pearson's test. Calculations were performed using the Graph Pad Prism 4.0 statistical package (Graph Pad Software, San Diego, CA).

RESULTS

Experimental groups. The group of diabetic patients consisted of 13 subjects with type 1 diabetes and 19 subjects with type 2 diabetes. The average age of both the healthy and diabetic subjects was ~48 years (Table 1). Fasting plasma glucose, fructosamine, and A1C levels in the group of diabetic patients were 80, 49, and 76% higher, respectively, than in the healthy group. Total, HDL, and LDL cholesterol and triglyceride levels in sera of patients with diabetes did not differ significantly from values in healthy subjects (Table 1). No significant differences in platelet, erythrocyte count, hemoglobin concentration, or platelet volume were found between the healthy group and patients with diabetes (Table 1). Acetyl-CoA levels in whole resting platelets, as well as spontaneous and thrombin-evoked aggregation and TBARS accumulation in platelets of subjects with diabetes were 60, 64, 11, 17, and 18% higher, respectively, than in healthy control subjects (Table 2). A subgroup of patients treated with statins presented no differences in their lipids, glycemia control, platelet function, or metabolic parameters in comparison with a subgroup of statin-untreated diabetic subjects (not shown).

ATP/ADP levels in platelets. GPRP in 0.5- to 5.0-mmol/l concentrations caused dose-dependent inhibition of thrombin-evoked aggregation but had no effect on the increased TBARS accumulation or the decrease in ATP/ADP levels in thrombin-stimulated platelets. Maximal 80% inhibition of thrombin-evoked aggregation was attained at

TABLE 2
Selected parameters of platelet metabolism and function in healthy donors and patients with diabetes

Parameter	Healthy donors	Patients with diabetes
Acetyl-CoA (whole platelets) pmol/mg protein	14.9 ± 0.8	23.8 ± 1.0*
No. of observations	29	29
Resting TBARS synthesis (nmol · 10 min^{-1} · mg protein^{-1})	0.90 ± 0.03	1.05 ± 0.03*
No. of observations	13	17
Thrombin-stimulated TBARS synthesis (nmol · 10 min^{-1} · mg protein^{-1})	2.11 ± 0.07	2.47 ± 0.08†
No. of observations	17	21
Resting aggregation (%)	10.7 ± 0.8	17.5 ± 1.1‡
No. of observations	21	23
Thrombin-stimulated aggregation (%)	73.7 ± 1.7	82.0 ± 1.2*
No. of observations	17	23

Data are means ± SE. *$P < 0.005$, †$P < 0.05$, ‡$P < 0.0001$ for difference from healthy group (unpaired Student's t test).

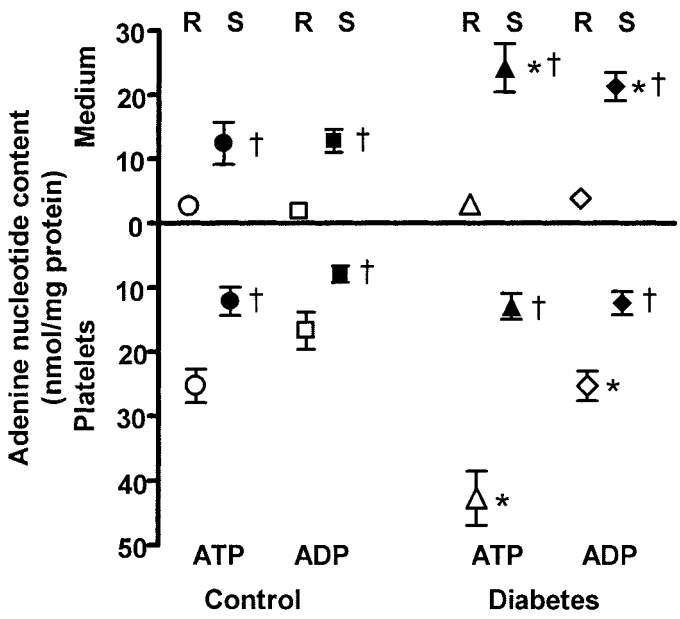

FIG. 1. Effect of thrombin stimulation (0.1 unit/ml) on ATP and ADP content in platelets from healthy control subjects and patients with diabetes and their release into the incubation medium. Data are the means ± SE (error bars) from eight experiments performed in duplicate. *$P < 0.01$ for difference from respective healthy group (unpaired Student's t test); †$P < 0.001$ from respective resting levels (paired Student's t test). R, resting conditions (white symbols); S, thrombin stimulation (black symbols).

2.5 mmol/l GPRP concentration (not shown). This concentration of GPRP was used in ATP/ADP studies described below.

Levels of ATP and ADP in resting platelets of diabetic patients were >80% higher than in the platelets from healthy subjects (Table 3). Activation of platelets from diabetic patients with thrombin (0.1 IU/ml) caused an approximately three times greater decrease of platelet ATP and ADP compared with healthy subjects (Table 3).

In selected cases secretion of adenine nucleotides from platelets to the medium was quantified (Fig. 1). Under resting conditions platelets of healthy and diabetic subjects released similar, small amounts of ATP and ADP to the medium, and in both groups thrombin evoked a severalfold increase of nucleotide release to the medium (Fig. 1). However, under these conditions absolute amounts of ATP and ADP released to the medium from platelets of diabetic subjects were 95 and 66% higher, respectively, than those released from platelets of healthy subjects (Fig. 1). Thus, after stimulation with thrombin, 65% of ATP and ~63% of ADP were released from patients' platelets (Fig. 1). In the case of platelets from healthy people, ATP and ADP releases to the medium were 52 and 62%, respectively (Fig. 1). We found ~70, 140, and 100% recoveries for ATP, ADP, and sum of ATP and ADP released to the medium, respectively (Fig. 1).

Suramin caused a dose-dependent inhibition of thrombin-evoked aggregation of platelets from diabetic patients down to the values seen in resting platelets (Fig. 2) (21). On the other hand, thrombin-activated TBARS synthesis was suppressed by 0.1–0.5 mmol/l suramin to values ~60% higher from its resting values (Fig. 2). Suramin also partially (80%) reversed the thrombin-evoked decrease of platelet ATP and completely prevented the reduction in ADP levels (Fig. 2). Suramin did not alter the above parameters in resting platelets (not shown). Similar, but quantitatively smaller, effects of suramin were observed in platelets from healthy subjects (not shown).

Relationship between diabetes control and platelet ATP/ADP levels. In accord with past data (10), acetyl-CoA levels in platelets of diabetic subjects correlated significantly with serum fructosamine concentration, a retrospective indicator of average glycemia, within 2 weeks preceding blood collection ($r = 0.44$, $P = 0.031$). In addition, ATP but not ADP levels in resting platelets from diabetic subjects correlated significantly ($r = 0.59$, $P =$

TABLE 3
Adenine nucleotide content in platelets of healthy donors and patients with diabetes

Experimental group/adenine nucleotide	Adenine nucleotide content		Thrombin-evoked decrease in nucleotide content (nmol/mg platelet protein)
	Resting conditions (nmol/mg platelet protein)	Thrombin stimulation (nmol/mg platelet protein)	
Healthy donors			
ATP	20.0 ± 1.8	13.3 ± 1.4*	6.7 ± 1.3
ADP	13.8 ± 1.4	10.0 ± 1.1*	3.8 ± 0.9
Patients with diabetes			
ATP	36.4 ± 2.5†	13.4 ± 1.4*	23.0 ± 2.3†
ADP	25.1 ± 1.9†	11.4 ± 1.3*	13.7 ± 1.5†

Data are means ± SE from 29 experiments performed in duplicate. *$P < 0.001$ from respective resting level; †$P < 0.001$ from respective healthy group.

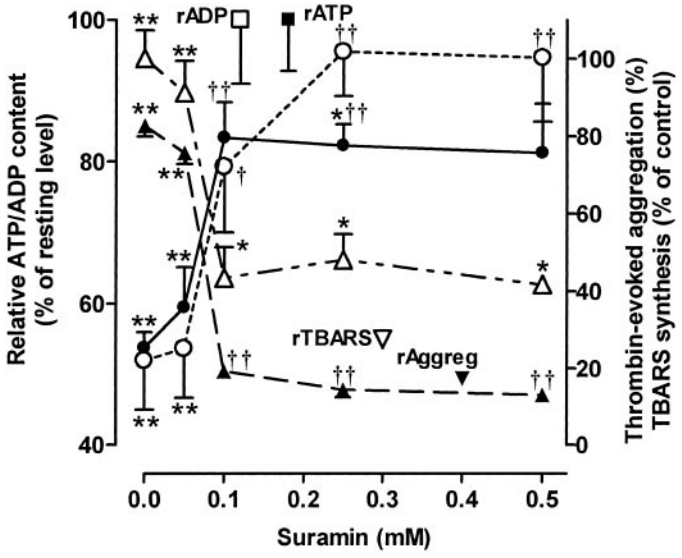

FIG. 2. Concentration-dependent effects of suramin on metabolic and functional parameters in thrombin-activated platelets from diabetic patients. Data are means ± SE from five experiments performed in duplicate. *P < 0.05, **P < 0.001 from resting conditions; †P < 0.05, ††P < 0.001 from no suramin conditions (paired Student's t test). Resting platelet levels are marked for resting ATP (rATP); resting ADP (rADP); resting aggregation (rAggreg), and resting TBARS (rTBARS). ○, ADP; ●, ATP; △, TBARS; ▲, aggregation.

0.001; and $r = 0.09$, $P = 0.665$) with fructosamine concentrations in their sera (Fig. 3).

Neither ATP nor ADP levels in platelets of diabetic subjects correlated with fasting plasma glucose ($r = 0.001$, $P = 0.85$; and $r = 0.002$, $P = 0.83$), blood hemoglobin A1C ($r = 0.114$, $P = 0.15$; and $r = 0.020$, $P = 0.55$), serum total cholesterol ($r = 0.060$, $P = 0.38$; and $r = 0.090$, $P = 0.29$), serum LDL cholesterol ($r = 0.016$, $P = 0.70$; and $r = 0.007$, $P = 0.80$), and serum triglyceride levels ($r = 0.0004$, $P = 0.94$; and $r = 0.0003$, $P = 0.95$). No significant correlation between these parameters was found in the group of healthy people (not shown).

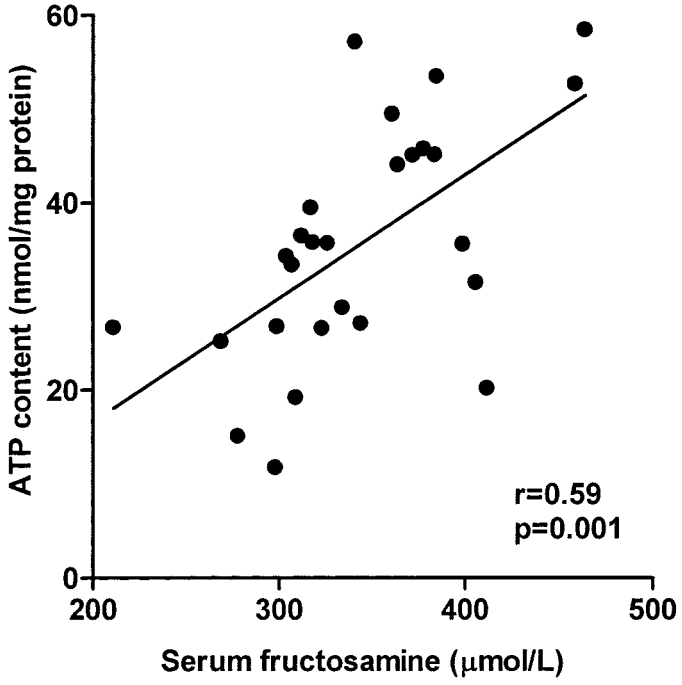

FIG. 3. Correlation between serum fructosamine concentration and whole-platelet resting ATP content in patients with diabetes.

ATP/ADP levels and platelet function. Diabetes increased the resting as well as thrombin-stimulated aggregation and TBARS synthesis in the platelets (Table 2) (9,10). In the group of healthy subjects, we found no significant correlations between platelets' functional parameters and their ATP/ADP resting levels (Table 4). In diabetic patients significant correlations existed between ATP or ADP plus ATP levels in resting platelets and spontaneous aggregation, thrombin-evoked aggregation, or resting and thrombin-evoked TBARS synthesis (Table 4). Statistically significant relationships have been also found between ADP concentrations and spontaneous aggregation or TBARS synthesis in platelets of diabetic patients (Table 4). In patients, significant correlations existed between amounts of ATP released from thrombin-stimulated platelets and their aggregation ($r = 0.66$, $P = 0.005$) or TBARS synthesis ($r = 0.71$, $P = 0.003$) (Table 5). No such correlations were found for released ADP (Table 5). In healthy subjects, thrombin-evoked depletion of nucleotides from platelets also showed no correlation with their aggregation or TBARS synthesizing capacity (Table 5).

DISCUSSION

In this report, type 1 and 2 diabetic patients were considered as one experimental group. Such an experimental design was justified by our previous data (10) showing that diabetes-evoked changes both in the activities of enzymes of acetyl-CoA metabolism and in acetyl-CoA level depend on the degree of chronic hyperglycemia and not on other factors specific for a particular type of diabetes (9,10,23). Also, this study revealed that all laboratory markers of the disease, as well as levels of ATP/ADP in platelets, were increased to a similar extent in both type 1 and 2 diabetic patients (Tables 1 and 2 and data not shown). As in our past report, both patient subgroups enrolled in the current study demonstrated similar fasting hyperglycemia as well as a similar elevation of serum fructosamine, indicating unsatisfactory control of the disease within the average time of platelet survival (Table 1) (7,10).

On the other hand, total and LDL cholesterol levels in sera of diabetic patients tended to be lower than those found in the healthy group (Table 2). Such results were apparently attributable to intensive antilipemic treatment with statins applied to some of the patients in an effort to reach goals set by Adult Treatment Panel III guidelines. It excludes disturbances in lipid metabolism, and statins pleiotropic effects, as a cause of alterations of platelet ATP/ADP metabolism in this group of diabetic subjects (Tables 2 and 3 and Fig. 1).

The increases of acetyl-CoA and ATP/ADP levels in platelets from diabetic subjects should therefore be considered a consequence of their chronic exposure to hyperglycemia (Tables 1, 2, and 3). Average life span of albumin and platelets in the blood are similar. It would explain the existence of significant correlations between platelet acetyl-CoA and ATP levels and serum glycated albumin (fructosamine) and the lack of such relationships with fasting plasma glucose and blood A1C in diabetic patients (Fig. 3) (23).

Our results are in disagreement with the results of Collier et al. (24), who showed no change in platelet ATP/ADP content in young insulin-dependent diabetic

TABLE 4
Correlations between ATP/ADP content and function of blood platelets in healthy donors and patients with diabetes

Parameter	ATP correlation (r)	ADP correlation (r)	ATP + ADP correlation (r)
Healthy donors			
Spontaneous TBARS synthesis	0.04	0.01	0.03
Thrombin-stimulated TBARS synthesis	−0.22	−0.17	−0.35
Spontaneous aggregation	0.19	0.48*	0.32
Thrombin-stimulated aggregation	−0.32	−0.25	−0.34
Patients with diabetes			
Spontaneous TBARS synthesis	0.53†	0.58*	0.67*
Thrombin-stimulated TBARS synthesis	0.48†	0.36	0.56‡
Spontaneous aggregation	0.53‡	0.55*	0.62†
Thrombin-stimulated aggregation	0.42†	0.38	0.51†

Data were calculated from 13–23 pairs of observations. Nucleotide contents were expressed as nmol/mg protein; TBARS synthesis as nmol · 10 min^{-1} · mg $protein^{-1}$; and aggregation as %. *$P < 0.02$; †$P < 0.005$; ‡$P < 0.05$.

patients. However, that study used a percoll gradient containing 10 mmol/l EDTA for platelet isolation, whereas in our study simple centrifugation in medium containing 0.1 mmol/l EDTA was used (see RESEARCH DESIGN AND METHODS) (Fig. 1 and Table 3) (24). On the other hand, our data are in accord with findings demonstrating that inherited or acquired deficiency of energy metabolism in platelets results in reduced ATP/ADP secretory pools (25,26).

In this study, amounts of adenine nucleotides secreted by activated platelets presumably reflect the size of the granular/releasable pool (Fig. 1 and Table 3), whereas their amounts remaining in the cells presumably correspond to the sum of mitochondrial and cytoplasmic metabolic pools (Fig. 1 and Table 3) (25,27). Indeed, consistent with this interpretation, the intragranular releasable pools of ATP/ADP were increased in platelets of diabetic patients (Fig. 1) (24,28). Accordingly, cytoplasmic pools of ATP/ADP did not increase in platelets of diabetic subjects because the intraplatelet content of these nucleotides after thrombin activation fell to the same levels in both groups (Fig. 1 and Table 3).

Significant correlations between ATP/ADP resting concentrations or their thrombin-evoked release and thrombin-evoked aggregation or TBARS synthesis in platelets from diabetic patients indicate that these nucleotides may contribute to excessive platelet activity in this disease (Tables 4 and 5). However, similar correlations have been found between diabetic patients' platelet function and their acetyl-CoA content (see RESULTS) (9). This indicates that the latter may be a primary signal increasing platelet activity in diabetes through the elevation of both ATP/ADP releasable pools and synthesis of thromboxane A_2 and other lipid platelets agonists (Fig. 1 and Tables 3 and 5) (2,5,9).

That the sum of ATP and ADP that was lost from thrombin-stimulated platelets was fully recovered in the postincubation medium indicates that in these conditions nucleotides were not converted to AMP, adenosine, and other products of their degradation (Fig. 1). This may be attributable to the fact that purified platelets do not possess all significant adenine nucleotide–degrading activities (11). Therefore, we postulate that in addition to acetyl-CoA–dependent overproduction of thromboxane A_2/malonyl dialdehyde and other thiobarbituric acid–reacting lipid peroxides, increased release of ADP/ATP from platelets may be responsible for their excessive thrombin-stimulated aggregation in diabetic patients (Fig. 1 and Table 5) (5,9,10). In addition, increased release of ATP from patients' platelets may also support their aggregation because of its breakdown to ADP by ecto-ATPases present on the surface of platelets, as evidenced by respective negative and positive recoveries of ATP and ADP released into the medium (Fig. 1). Moreover, ATP itself could directly activate platelets through $P2X_1$ receptors and augmentation of secretory vesicle docking and serotonin exocytosis (13,29). This could explain why in diabetic patients strongest correlations were revealed between ATP, but not ADP, platelet levels and different platelet activities (Tables 4 and 5). That suramin, a nonspecific purinergic receptor antagonist (15,16), inhibited thrombin-evoked platelet activation and aggregation indicates that ATP/ADP released from activated platelets exert their effects through purinergic receptors (Fig. 2) (29). Thus, the excessive releases of ATP/ADP would mediate thrombin-

TABLE 5
Correlations between thrombin-stimulated ATP/ADP release and platelet function in healthy donors and patients with diabetes

Parameter	ΔATP correlation (r)	ΔADP correlation (r)	ΔATP + ADP correlation (r)
Healthy donors			
Thrombin-stimulated TBARS synthesis	−0.17	−0.23	−0.29
Thrombin-stimulated aggregation	−0.08	−0.05	−0.04
Patients with diabetes			
Thrombin-stimulated TBARS synthesis	0.71*	0.07	0.42
Thrombin-stimulated aggregation	0.66†	0.10	0.53‡

Data were calculated from 12–16 pairs of observations. Δ = nucleotide release from platelets calculated as a difference between their intraplatelet level in resting and thrombin-stimulated conditions. Nucleotide content was expressed as nmol/mg protein; TBARS synthesis as nmol · 10 min^{-1} · mg $protein^{-1}$; and aggregation as %. *$P < 0.005$; †$P < 0.01$; ‡$P < 0.05$.

evoked platelet hyperactivation in diabetic subjects (Figs. 1 and 2). Saturating levels of suramin did not completely suppress the activating effects of thrombin on TBARS synthesis (Fig. 2), suggesting that a significant part of thrombin-evoked platelet activation is independent of ADP release (Fig. 2).

Presented experiments were performed with plasma-free platelets isolated from blood using EDTA under cold conditions to avoid interference of citrate with acetyl-CoA assay (9,10). Platelets were aggregated with a suboptimal concentration of thrombin (0.1 IU/ml; see RESEARCH DESIGN AND METHODS) to enable observation of eventual activating effects (Table 2) (30). ATP/ADP levels and spontaneous and thrombin-evoked aggregation for our EDTA-isolated platelets appeared to be the same as for platelets isolated from blood using either citrate or EDTA (Tables 2 and 3) (24,28,30–32). These data evidence reliability of the employed isolation procedure, competence of obtained washed plasma-free platelet preparation and thereby credibility of results (Figs. 1–3 and Tables 2 and 3).

In conclusion, our current and previous data suggest that excessive activity of blood platelets in diabetic patients may be caused by two different mechanisms: *1*) an increased intravesicular accumulation and release of ATP/ADP (Table 3 and Fig. 1) and *2*) increased acetyl-CoA–dependent synthesis of thromboxane A_2 and other lipid activators in the cytoplasmic compartment (9,10). The putative mechanism that might link excessive acetyl-CoA synthesis to increased accumulation of granular pools of ATP/ADP in platelets of diabetic patients remains to be established.

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