Effect of L-Carnitine on Acetyl-CoA Content and Activity of Blood Platelets in Healthy and Diabetic Persons

ANNA Michno,1 ANNA Raszeja-Specht,1 AGNIESZKA Jankowska-Kulawy,1 TADEUSZ Pawelczyk,2 and ANDRZEJ Szutowicz1*

Background: Excessive blood platelet activity contributes to vascular complications in diabetic persons. Increased acetyl-CoA in platelets from diabetic persons has been suggested to be a cause of this hyperactivity. We therefore investigated whether L-carnitine, which up-regulates metabolism of acetyl-CoA in muscles and brain, may affect platelet function in healthy and diabetic individuals.

Methods: We obtained platelets from healthy and diabetic persons and measured acetyl-CoA concentrations, malonyl dialdehyde (MDA) synthesis, and platelet aggregation in the absence and presence of L-carnitine. Activities of selected enzymes involved in glucose and acetyl-CoA metabolism were also assessed.

Results: Fasting glucose, fructosamine, and hemoglobin A1c were present in significantly higher amounts in the blood of diabetic patients than in healthy individuals. Activities of carnitine acetyltransferase, glucose-6-phosphate dehydrogenase, oxoglutarate dehydrogenase, and fatty acid synthase were 17%–62% higher in platelets from diabetic patients. Mitochondrial acetyl-CoA was increased by 98% in platelets from diabetic patients, MDA synthesis was increased by 73%, and platelet aggregation by 60%. L-Carnitine had no or only a slight effect on these indices in platelets from healthy individuals, but in platelets from diabetic persons, L-carnitine caused a 99% increase in acetyl-CoA in the cytoplasmic compartment along with increases in MDA synthesis and platelet aggregation.

Conclusions: Excessive platelet activity in persons with diabetes may result from increased acetyl-CoA, which apparently increases synthesis of lipid activators of platelet function. L-Carnitine may aggravate platelet hyperactivity in diabetic persons by increasing the provision of surplus acetyl-CoA to the cytoplasmic compartment.

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Vascular complications in diabetes mellitus develop as a result of several disturbances in lipoprotein metabolism, increased protein glycation, free radical production, and chronic inflammation (1, 2). Endothelial cell damage leads to decreased production of antiaggregating and vasodilating substances. Hyperglycemia itself may also trigger primary disturbances in platelet activity by the activation and excessive production of lipid proaggregatory compounds (2). Therefore, pharmacologic or biochemical interventions that reduce synthesis of lipid mediators of aggregation could prevent the development of diabetic angiopathy (1).

L-Carnitine is a metabolite involved in indirect transport of cytoplasmic long-chain acyl-CoA through the mitochondrial membrane. In the outer mitochondrial membrane, carnitine acyl-CoA transferase I (EC 2.3.1.20) catalyzes reactions in which L-carnitine forms acylcarnitine intermediates. These intermediates are converted back to acyl-CoA on the inner mitochondrial membrane in reactions driven by acyl carnitine translocase and carnitine acyl-transferase II (EC 2.3.1.21). Fatty acids supplied to the mitochondrial matrix are subsequently used in the beta-oxidation cycle (3). Some of the acetyl-CoA groups formed in mitochondria by pyruvate dehydrogenase (PDH; EC 1.2.4.1) reactions and/or in the beta-

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Nonstandard abbreviations: PDH, pyruvate dehydrogenase, ACL, ATP-citrate lyase; Hb A1c, hemoglobin A1c; and MDA, malonyl dialdehyde.
oxidation cycle are transported to the cytoplasm, where they serve in synthetic pathways as precursors for several acetyl groups (3). In insulin-dependent tissues of omnivore animals, the indirect ATP-citrate lyase (ACL; EC 2.3.3.8) pathway was found to provide >70% of acetyl-CoA units for fatty acid synthesis (4, 5). In the brain, which in bulk is an insulin-independent organ, the indirect ACL pathway and the direct, permeability transition-dependent transport of acetyl-CoA have been reported to provide acetyl-CoA for structural lipid synthesis in the cytoplasmic compartment of glial and noncholinergic neuronal cells (6, 7). Neurons contain insulin-independent GLUT3 and GLUT1 glucose transporters (8); therefore, streptozotocin-evoked diabetic hyperglycemia increases conversion of glucose to pyruvate and acetyl-CoA and stimulates acetylcholine synthesis in brain nerve terminals (7).

Like neurons, uptake of glucose into blood platelets is independent of insulin and is mediated through GLUT3 (9). It has been shown that diabetic hyperglycemia significantly increases the activity of key glucose and acetyl-CoA metabolism enzymes, including hexokinase, ACL, and PDH in platelets (10). On the other hand, there are no data on possible changes in the activity of acetyl-metabolizing enzymes such as carnitine acetyltransferase (2.3.1.7), fatty acid synthetase, and citrate synthase (EC 2.3.3.1) in platelets from persons with diabetes.

Our earlier data revealed that diabetes produces an almost 2-fold increase in acetyl-CoA content in platelets (10). This increase correlated with the degree of medium-term hyperglycemia assessed by the fructosamine concentration in diabetic plasma (11). We therefore postulated that increased provision of acetyl-CoA to the platelet cytoplasmic compartment through the ACL pathway activates synthesis of platelet proaggregatory factors such as polyunsaturated fatty acids in the course of this disease (10, 11).

L-carnitine derivatives were reported to suppress platelet activity in healthy people (12, 13). Oral application of propionyl-l-carnitine inhibited arachidonic acid turnover and reactive oxygen species production by the platelets (12). Another study demonstrated that inhibition of the synthesis of platelet-activating factor led to both in vivo and in vitro suppression of platelet activity by propionyl-l-carnitine. The authors suggested that the suppressed platelet activity was caused by intracellular hydrolysis of propionyl-l-carnitine to l-carnitine with subsequent depletion of acetyl-CoA as a result of acetyl-l-carnitine formation (13). This explanation, however, has not been confirmed by experimental findings. Other studies have demonstrated that inhibition of long-chain carnitine acyltransferase-1 by perhexiline, amiodarone, or 2-tetradecylglycic acid inhibits platelet aggregation (14, 15), suggesting that stimulation of acylcarnitine metabolism activates rather than inhibits platelet activity (15). However, there are no data on the effect of l-carnitine on the concentration and distribution of acetyl-CoA in platelets of healthy or diabetic individuals.

Our recent data demonstrated that excessive platelet activity in diabetes may also be linked to increased protein glycation and acetyl-CoA content (10, 11). In addition, l-carnitine was found to increase the acetyl-CoA concentration and cholinergic activity in the brain (16). Thus, data demonstrating the suppression of platelet function by l-carnitine derivatives conflict with those demonstrating increased platelet and cholinergic neuron activity in hyperglycemic states (6, 11–13, 16). The aim of this study, therefore, was to investigate whether l-carnitine changes the amounts of acetyl-CoA in blood platelets and, if so, how it would affect basic indicators of platelet activity.

Materials and Methods

Patients

At the Academic Clinical Center, Medical University of Gdańsk, we enrolled study patients with diabetes type 1 and 2 who visited the Diabetology Center laboratory for scheduled check-ups; we also enrolled a reference group of healthy individuals who visited the Occupational Health Unit laboratory for routine blood examination. Diabetic patients with albuminuria (albumin excretion in urine >0.03 g/day) or with evident macroangiopathy were not admitted to the study. None of the study participants had taken aspirin, phosphodiesterase inhibitors, calcium channel blockers, or nonsteroidal antiinflammatory drugs for at least 2 weeks before blood drawing. Patients with type 2 diabetes were treated with a combination of diet and oral antidiabetic drugs. Additional 10-mL samples of blood were collected from each participant into morphology evacuated tubes containing 1 mg of tripotassium EDTA per 1 mL of blood. These samples were used for isolation of platelets for enzyme, acetyl-CoA, and aggregation assays. Remaining tests were performed with blood samples collected on physician request. The study protocol was approved by the Regional Bioethical Commission at the Medical University of Gdańsk (permission: TKE BN/350/97).

Reagents and Materials

Reagents for enzyme and acetyl-CoA assays were supplied by Sigma Chemical Co., Thrombin was from BioMed, Coomassie Brilliant Blue G-250 was from Bio-Rad, and GPRP tetrapeptide was from Bachem AG. All other chemicals were of analytical grade. Venoject tubes used for blood collection were from Becton Dickinson.

Diagnostic assays for hemoglobin A1c (Hb A1c; product no. 1488414) and fructosamine (product no. 67246901) were from Roche. Determinations were performed on a Hitachi 917 biochemical analyzer (Roche). Plasma glucose was measured by a commercial assay on a Dimension RxL biochemical analyzer (Dade Behring).
PLATELET ISOLATION

Blood cells and plasma were separated by centrifugation of whole blood at 150g at 4 °C for 15 min in a Jouan CR 3.12 centrifuge. The cells were washed twice with a volume of saline solution (9 g/L NaCl) equivalent to the plasma volume to increase platelet recovery (70%). The resulting platelet-rich plasma and subsequent washings were collected into a plastic tube and centrifuged at 500g for 15 min to obtain platelet-poor plasma and a platelet pellet. The pellet was washed 3 times with a solution containing 140 mmol/L NaCl, 5 mmol/L sodium HEPES buffer (pH 7.4), 0.1 mmol/L EDTA, and 5 mmol/L glucose and then suspended in a small volume of the same solution to obtain a protein concentration of ~10 g/L. The platelet amount and yield of the separation procedure as well as contamination by other blood cells was assessed with the HMX automatic hematologic analyzer (Beckman-Coulter).

ENZYME ASSAYS

The activities of fatty acid synthetase, ketoglutarate dehydrogenase (EC 1.2.4.2), carnitine acetyltransferase, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and citrate synthase were assayed by methods described elsewhere (17–21). Immediately before the assays, platelet membranes were solubilized by the addition of Triton X-100 (final concentration, 0.2% by volume). Assays were performed at 37 °C in an Ultraspec 3 spectrophotometer (Amersham-LKB).

ACETYL-COA ASSAY

The acetyl-CoA content was measured in freshly isolated platelets that were incubated in medium containing glucose to obtain a steady-state concentration under controlled conditions (10). Incubation medium (final volume, 1.0 mL) contained 20 mmol/L sodium HEPES buffer, 1.7 mmol/L sodium 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 acetyl-CoA metabolism in activated platelets, 0.1 U/mL of thrombin was added along with 2.5 mmol/L GPRP peptide to prevent aggregation, as indicated (22). Changes in the composition of the basic medium are indicated in the text. Incubation was started by the addition of platelet suspension (1 mg of protein) and continued for 30 min at 37 °C in polystyrene flat-bottomed vessels in a water bath with continuous shaking at 100 cycles/min. Incubation was terminated by transfer of 0.5-mL samples of the cell suspension to Eppendorf tubes placed in an ice bath followed by centrifugation for 1 min at 12,000g. The whole platelet pellet was deproteinized by addition of 0.08 mL of 5 mmol/L HCl and 30 min at 4 °C and centrifuged. Clear supernatants were taken for a malonyl dialdehyde (MDA) assay (25). Accumulation of MDA in thrombin-activated platelets was calculated by subtraction of the amount accumulated after 10 min in the activated sample from that present in the sample deproteinized at time zero.

PROTEIN ASSAY

Protein was quantified according to the method of Bradford (26) with bovine immunoglobulin as the calibrator.

STATISTICAL ANALYSIS

The data distribution was tested by Kolmogorov–Smirnov test. A P value >0.1 was considered to be indicative of gaussian distribution. Differences between 2 experimental groups were tested by unpaired Student t-test. Results obtained on platelets isolated from the same person and exposed to different treatments were compared by use of the paired Student t-test. Correlations were assessed with the Pearson test. Calculations were performed with the CoA calibrator did not interfere with its determination [Ref. (23) and our unpublished data].

To assess the intracellular distribution of acetyl-CoA, we mixed the remaining 0.5 mL of platelet suspension with an equal volume of ice-cold lysis solution containing 20 mmol/L Tris-HCl buffer (pH 7.4), 125 mmol/L KCl, 3 mmol/L EDTA, and 1.4 g/L digitonin, layered over a 0.5-mL mixture of silicon oils (AR-20 and AR-200, 1:2 by volume) in a 1.5-mL Eppendorf tube. After 30 s, the particulate fraction was separated by centrifugation for 1 min at 12,000g. The upper layer was collected for protein and lactate dehydrogenase assays. The 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 min. It was then used for determination of particulate (mainly mitochondrial) acetyl-CoA. Cytoplasmic acetyl-CoA content was calculated by subtraction of mitochondrial acetyl-CoA content from that found in whole platelets. To check the reliability of the separation procedure, we occasionally determined the glutamate dehydrogenase and lactate dehydrogenase activities in the particulate and soluble fractions (10, 24).
Results

CHARACTERIZATION OF EXPERIMENTAL GROUPS

Persons with type 1 or type 2 diabetes were enrolled in the studies. Basic morphometric blood values, including platelet counts, volume, and protein content (Table 1) as well as erythrocyte and leukocyte counts and hemoglobin concentration (not shown) in the diabetic patients were similar to those for healthy individuals. In both diabetic groups, fasting plasma glucose, serum fructosamine, and blood Hb A1c concentrations were 110%, 52%, and 77% higher, respectively, than in healthy participants (Table 1). Serum cholesterol and triglyceride concentrations were significantly higher in the patients with type 2 diabetes than in the healthy participants and patients with type 1 diabetes. On average, the group with type 1 diabetes was younger and the group with type 2 diabetes was older than the healthy group (Table 1). Mean disease duration varied from 11.4 years for the group with type 1 diabetes to 14.5 years for the group with type 2 diabetes.

Our previous studies revealed that disturbances in acetyl-CoA metabolism do not depend on the degree of dyslipidemia and age differences, but only on the degree of lasting hyperglycemia (10, 11). Accordingly, the distribution of individual values for fructosamine (Table 1) and other markers of hyperglycemia (not shown) in blood were similar in persons with type 1 or type 2 diabetes. Therefore, in further studies, patients with either type 1 or type 2 diabetes were considered as a chronically hyperglycemic group.

ENZYMES OF ACETYL-COA AND ENERGY METABOLISM

Our previous studies demonstrated that diabetic hyperglycemia caused increased activity of several enzymes involved in glucose-derived acetyl group metabolism in platelets, such as hexokinase, PDH, and ACL (11). As shown in Table 2, diabetes caused increases in the activities of glucose-6-phosphate dehydrogenase (17%) carnitine acetyltransferase (24%), 2-oxoglutarate dehydrogenase (36%), fatty acid synthetase (55%), and citrate synthase (98%) relative to healthy controls.

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Table 1. Demographic and basic laboratory data of healthy and diabetic participants.a

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Type 1 diabetes</th>
<th>Type 2 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of individuals</td>
<td>29</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Men/Women</td>
<td>13/16</td>
<td>6/6</td>
<td>10/8</td>
</tr>
<tr>
<td>Duration of the disease, years</td>
<td>47.4 (2.1)</td>
<td>39.7 (2.6)b</td>
<td>57.6 (2.4)b</td>
</tr>
<tr>
<td>Age, years</td>
<td>950 (30)</td>
<td>2130 (200)c</td>
<td>1910 (90)c</td>
</tr>
<tr>
<td>Fructosamine, μmol/L</td>
<td>245 (5)</td>
<td>369 (15)c</td>
<td>379 (15)c</td>
</tr>
<tr>
<td>Hb A1c, %</td>
<td>5.1 (0.1)</td>
<td>9.0 (0.4)c</td>
<td>9.1 (0.3)c</td>
</tr>
<tr>
<td>PLTs, 10^11/μL</td>
<td>256 (8)</td>
<td>274 (26)</td>
<td>245 (12)</td>
</tr>
<tr>
<td>MPV, fL</td>
<td>8.8 (0.2)</td>
<td>9.1 (0.2)</td>
<td>9.1 (0.2)</td>
</tr>
<tr>
<td>Cholesterol, mg/L</td>
<td>1960 (100)</td>
<td>1970 (90)</td>
<td>2300 (100)c</td>
</tr>
<tr>
<td>Triglycerides, mg/L</td>
<td>1060 (60)</td>
<td>1110 (80)</td>
<td>1800 (100)c</td>
</tr>
</tbody>
</table>

*a All data are the means (SE) except for the number of participants and sex.

*b Significantly different from healthy persons (unpaired Student t-test); P < 0.05.

*c Significantly different from healthy persons (unpaired Student t-test); P < 0.001.

Table 2. Activities of selected enzymes involved in acetyl-CoA metabolism in blood platelets from healthy and diabetic persons.a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Healthy persons</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>70.7 (3.6)</td>
<td>82.9 (3.7)b</td>
</tr>
<tr>
<td>No. of observation</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Carnitine acetyltransferase</td>
<td>14.7 (0.5)</td>
<td>17.7 (0.7)c</td>
</tr>
<tr>
<td>No. of observation</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>2-Oxoglutarate dehydrogenase</td>
<td>2.76 (0.10)</td>
<td>3.77 (0.19)c</td>
</tr>
<tr>
<td>No. of observation</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Fatty acid synthetase</td>
<td>1.42 (0.10)</td>
<td>2.29 (0.20)c</td>
</tr>
<tr>
<td>No. of observation</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>39.3 (4.8)</td>
<td>44.8 (3.3)</td>
</tr>
<tr>
<td>No. of observation</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

*a Data are the means (SE) from number of observations given.

b,c Significantly different from healthy group (unpaired Student t-test); b P < 0.05; c P < 0.001.
tine acetyltransferase (21%), 2-oxoglutarate dehydrogenase (37%), and fatty acid synthetase (62%), but we found no significant changes in citrate synthase activity (Table 2).

EFFECT OF GLUCOSE CONCENTRATION ON PLATELET ACETYL-CoA CONTENT

Increasing glucose concentrations in the incubation medium caused a gradual increase in acetyl-CoA content in platelets from both groups. Maximum concentrations of acetyl-CoA were achieved at a glucose concentration of 2.5 mmol/L (Fig. 1). At this experimental point, the acetyl-CoA content in platelets from diabetic individuals was 60% higher than in the platelets from the healthy individuals (Fig. 1). In both diabetic and healthy persons, no significant changes in platelet acetyl-CoA concentrations occurred with further increases of the glucose concentration to 10 mmol/L.

EFFECT OF GLUCOSE ON ACETYL-CoA DISTRIBUTION IN PLATELETS

To examine the role of glucose in provision of acetyl-CoA to the cytoplasmic compartment, intraplatelet distribution of this metabolite was assessed in platelets incubated in medium without and with 2.5 mmol/L glucose. In platelets obtained from both diabetic and healthy individuals, glucose caused no significant changes in mitochondrial acetyl-CoA concentration but caused a 2- to 3-fold increase in cytoplasmic acetyl-CoA (Fig. 2). On the other hand, for the group with diabetes, there was a 98% increase in mitochondrial acetyl-CoA but no significant changes in the cytoplasmic pool of this metabolite in platelets incubated in glucose-containing medium (Fig. 2).

CONCENTRATION-DEPENDENT EFFECTS OF L-CARNITINE ON ACETYL-CoA CONTENT

In platelets from the diabetic group, incubated in medium containing 2.5 mmol/L glucose, L-carnitine (0.05–10.0 mmol/L) caused concentration-dependent increases in acetyl-CoA content. A half-maximal effect was observed at 1 mmol/L, whereas a maximal 53% increase was achieved at 2.5 mmol/L L-carnitine (Fig. 3). In platelets from the healthy group, the maximal increase in acetyl-CoA was lower (25%) at the same L-carnitine concentration (Fig. 3). An increase of L-carnitine to 10 mmol/L produced no change in acetyl-CoA content (Fig. 3). Therefore, in further experiments, a saturating concentration of L-carnitine (2.5 mmol/L) was used.

EFFECTS OF L-CARNITINE ON ACETYL-CoA COMPARTMENTALIZATION

There was no difference between mean platelet acetyl-CoA content assessed in the absence or presence of L-carnitine in patients with type 1 or 2 diabetes (not shown). The distributions of individual acetyl-CoA measurements were also similar in both groups (not shown). Combine with the uniform distribution of plasma fructosamine concentrations (Table 1), these similarities justified consideration of both types of diabetes as a single experimental group.

For both the diabetic and healthy experimental groups, in the absence of glucose, 2.5 mmol/L L-carnitine caused no changes in acetyl-CoA content in the platelets (Table...
3). For the healthy group, in platelets incubated with glucose, l-carnitine caused a 32% increase in acetyl-CoA content (Table 3). In platelets from the diabetic group, l-carnitine caused a much higher increase (69%) in total acetyl-CoA. Under these conditions, platelets from patients with diabetes contained 2-fold more acetyl-CoA than did platelets from the healthy group (Table 3).

l-Carnitine produced no significant changes in intraplatelet distribution of acetyl-CoA in the healthy group (Fig. 4). On the other hand, in the diabetic group, l-carnitine increased cytoplasmic and mitochondrial acetyl-CoA by 100% and 26%, respectively (Fig. 4).

In platelets from the healthy group, incubated with or without l-carnitine, activation with thrombin did not change the acetyl-CoA content (Fig. 5). In contrast, in platelets from the diabetic group, incubated without or with l-carnitine, the addition of thrombin caused 24% and 51% decreases, respectively, in total acetyl-CoA content compared with the values for platelets from the healthy group (Fig. 5).

**EFFECT OF L-CARNITINE ON PLATELET AGGREGATION**

In the medium containing glucose, the spontaneous aggregation of platelets from the diabetic group was 57% higher than that of platelets from the healthy group (Table 4). l-Carnitine increased spontaneous aggregation of platelets from the healthy and diabetic groups by 33% and 61%, respectively (Table 4). Under these conditions, platelet aggregation was 90% higher in samples from the diabetic group than in samples from the healthy participants. Compared with the platelets from the healthy group, thrombin-evoked aggregation of platelets from the diabetic group was 14% and 20% higher in l-carnitine-free and l-carnitine-supplemented medium, respectively (Table 4). l-Carnitine alone caused a slight but significant 10% increase in thrombin-evoked aggregation of platelets from the diabetic group but not the healthy group.

ADP- and collagen-induced platelet aggregation rates were 16% and 12% higher, respectively, in the diabetic group than in the healthy group (Table 4). l-Carnitine had no effect on these rates in platelets from the healthy group. On the other hand, l-carnitine increased ADP- and collagen-stimulated platelet aggregation in samples from the diabetic group by 10% and 14%, respectively (Table 4). In the presence of l-carnitine, both ADP- and collagen-

![Fig. 3. Concentration-dependent effect of L-carnitine on acetyl-CoA content in platelets isolated from the blood of healthy (□) and diabetic (○) persons and incubated with 2.5 mmol/L glucose.](image)

Data are the means (SE; error bars) from 3 duplicate estimations. Significant differences: *, P <0.05; **, P <0.005 for difference from healthy group at the same l-carnitine concentration (unpaired Student t-test); +, P <0.05 for difference from no l-carnitine within the same group (healthy or diabetic; repeated-measures ANOVA test for multiple comparisons).

![Fig. 4. Effect of 2.5 mmol/L L-carnitine on acetyl-CoA content and distribution in platelets isolated from blood of healthy or diabetic persons and incubated with 2.5 mmol/L glucose.](image)

Data are the means (SE; error bars) from 11–12 experiments. □, control (no l-carnitine); □, 2.5 mmol/L l-carnitine. Significant differences: *, P <0.0001 compared with respective values in healthy persons (unpaired Student t-test); +, P <0.0005 compared with the respective control (paired Student t-test).

| Table 3. Effect of l-carnitine on acetyl-CoA concentrations in resting platelets incubated in medium without glucose or containing 2.5 mmol/L glucose. |
|-----------------|-----------------|
|                  | Healthy group   | Diabetic group |
| Medium with no glucose |                  |                |
| Control          | 11.0 (2.1)      | 17.6 (2.3)      |
| 2.5 mmol/L l-carnitine | 13.4 (1.1)      | 19.1 (1.7)      |
| Medium containing 2.5 mmol/L glucose |                  |                |
| Control          | 16.1 (0.9)      | 25.5 (1.1)      |
| 2.5 mmol/L l-carnitine | 21.4 (1.4)      | 43.1 (2.8)      |

* Data are the means (SE) from 17 (no glucose) and 32 (2.5 mmol/L glucose) duplicate observations.

** Significantly different from healthy group (unpaired Student t-test): * P <0.01; ** P <0.001.

Significantly different from respective control (paired Student t-test): * P <0.0001.
EFFECT OF L-CARNITINE ON MDA SYNTHESIS

Under resting conditions, l-carnitine induced 25% and 61% increases in MDA production in platelets from the healthy and diabetic groups, respectively (Table 5). On the other hand, resting MDA production in platelets from the group with diabetes incubated without and with l-carnitine was 60% and 106% higher, respectively, than in platelets from the healthy group.

Stimulation with thrombin led to severalfold increases in MDA synthesis in both groups (Table 5). In the absence and presence of l-carnitine, thrombin-stimulated MDA synthesis was 73% and 104% higher, respectively, in platelets from the diabetic group than in platelets from the healthy group (Table 5). l-Carnitine caused an 11% increase in MDA accumulation in platelets from the healthy group and a 31% increase in platelets from the diabetic group (Table 5).

CORRELATIONS BETWEEN PLATELET ACTIVITIES AND ACETYL-COA CONTENT

To investigate the correlation between acetyl-CoA and platelet activity, indicators of platelet activity in individual participants were compared with the respective acetyl-CoA values (Table 6, Fig. 6). In healthy persons, only 1 of 4 tested indicators of platelet activity displayed a moderately significant correlation (P < 0.05) with platelet acetyl-CoA in either the absence or presence of l-carnitine (Table 6 and Fig. 6A). In platelets from diabetic participants incubated with l-carnitine, both spontaneous and thrombin-evoked MDA synthesis and aggregation displayed highly significant correlations (P < 0.001) with platelet acetyl-CoA (Table 6 and Fig. 6B). In the absence of l-carnitine, 3 of 4 indicators of excessive platelet activity in samples from diabetic patients correlated significantly (P < 0.01 and P < 0.0001) with platelet acetyl-CoA content (Table 6).

**Table 4. Effect of l-carnitine on resting antagonist-evoked aggregation of blood platelets incubated in medium containing 2.5 mmol/L glucose.**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Healthy group</th>
<th>Diabetic group</th>
</tr>
</thead>
</table>
| Resting aggregation | 5.7 (0.2) | 9.1 (0.4)^
| 2.5 mmol/L L-carnitine | 7.7 (0.3) | 14.6 (0.8)^b,d |
| Thrombin (0.1 U/mL)-evoked aggregation | 70.9 (0.8) | 80.8 (0.7)^p |
| 2.5 mmol/L L-carnitine | 74.4 (1.0)^d | 89.4 (0.8)^p,b,d |
| ADP (0.01 mmol/L)-evoked aggregation | 56.8 (2.4) | 65.7 (2.4)^e |
| 2.5 mmol/L L-carnitine | 57.5 (2.3) | 72.3 (2.1)^p,b,d |
| Collagen (0.05 mmol/L)-evoked aggregation | 64.3 (1.6) | 72.3 (1.7)^c |
| 2.5 mmol/L L-carnitine | 65.7 (2.9) | 82.5 (1.3)^p,b,d |

^ Data are the means (SE) from 30 (thrombin) and 6 (ADP and collagen) duplicate observations.

^b Significantly different from healthy persons (unpaired Student t-test); ^p < 0.001; ^c P < 0.001.

^d Significantly different from respective control (paired Student t-test); ^d P < 0.001; ^c P < 0.001.

**Table 5. Effect of l-carnitine on resting and thrombin-evoked MDA synthesis in blood platelets incubated in medium containing 2.5 mmol/L glucose.**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Healthy group</th>
<th>Diabetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting MDA synthesis</td>
<td>0.40 (0.02)</td>
<td>0.64 (0.03)^p</td>
</tr>
<tr>
<td>2.5 mmol/L L-carnitine</td>
<td>0.50 (0.02)^c</td>
<td>1.03 (0.05)^p,c</td>
</tr>
<tr>
<td>Thrombin (0.1 U/mL)-evoked MDA synthesis</td>
<td>1.67 (0.05)</td>
<td>2.89 (0.08)^p</td>
</tr>
<tr>
<td>2.5 mmol/L L-carnitine</td>
<td>1.85 (0.08)^c</td>
<td>3.78 (0.09)^p,c</td>
</tr>
</tbody>
</table>

^ Data are the means (SE) from 30 duplicate observations.

^b Significantly different from healthy group (unpaired Student t-test), P < 0.001.

^c Significantly different from respective control (paired Student t-test), P < 0.0001.
The increased Hb A1c and fructosamine concentrations in the blood of diabetic patients in this study indicated that they remained chronically hyperglycemic within the 2- to 3-month and 2-week periods preceding the investigation, respectively (Table 1). The similar mean values and distributions of individual plasma values for fructosamine, blood glucose, Hb A1c, and platelet acetyl-CoA in patients with type 1 and 2 diabetes allowed for their assessment as a single hyperglycemic group (Table 1).

In platelets collected from diabetic patients for in vitro studies, increased fructosamine indicates that the platelets have been exposed to hyperglycemia at some time during their lifetime in circulation (Table 1). One may therefore postulate that increased activities of fatty acid synthetase, ketoglutarate, glucose-6-phosphate dehydrogenase, and carnitine acetyltransferase may be part of an adaptive response of megakaryocytes to the increased influx of glucose through the GLUT 3 glucose transporter (Table 2). These data are also consistent with our previous findings demonstrating increases in hexokinase, PDH, and ATP-citrate lyase activities in platelets from patients with diabetes (11), allowing the conclusion that chronic hyperglycemia in megakaryocytes causes adaptive up-regulation of multiple key enzymes involved in the incorporation of acetyl units derived from glucose into fatty acids and, subsequently, to their biologically active signaling derivatives (25, 27). Increased glucose-6-phosphate dehydrogenase activity may suggest an increase in provision of NADPH for fatty acid synthesis in platelets of diabetic patients (Table 2). The increase in fatty acid synthetase activity in platelets from diabetic patients is consistent with this finding (Table 2), which could indicate the activation of fatty acid synthesis and use for energy production and peroxidation-dependent MDA synthesis in these platelets (Table 5) (28). In addition, the increases in ketoglutarate dehydrogenase (Table 2) and PDH (10) activity in platelets from diabetic patients indicates that energy production was also activated under these conditions.

Glucose-derived pyruvate from the mitochondrial PDH reaction is a main source of acetyl-CoA in platelets (29). Therefore, the increase in total acetyl-CoA content in platelets from diabetic patients might be attributable to increased metabolic flow of glucose through the glycolytic cycle to pyruvate and activation of its decarboxylation by PDH in mitochondria (Fig. 2 and Table 3) (10, 11). This assumption is supported by the finding that a diabetes-evoked increase of acetyl-CoA in platelets was confined to their mitochondrial compartment (Fig. 2). Thus, mitochondria would be a primary site of excessive acetyl-CoA accumulation in disease-affected platelets (Fig. 2 and Table 3). On the other hand, the glucose-induced increase of the acetyl-CoA concentration in platelet cytoplasm indicates that this metabolite was efficiently transported from mitochondria to cytoplasm in both healthy and diabetic participants (Fig. 2).

L-Carnitine has been reported to increase acetyl-CoA provision for fatty acid synthesis in the liver and for fatty acid and acetylcholine production in the brain (3, 16), apparently through activation of the carnitine acetyltransferase pathway (3, 16). Lack of an l-carnitine effect on platelet acetyl-CoA in the absence of glucose indicates that only acetyl groups synthesized in the PDH reaction are available for carnitine acetyltransferase-dependent transport through the mitochondrial membrane (Table 3). Moreover, the absence of or weak stimulatory effects of l-carnitine on acetyl-CoA content and distribution as well as on the functional indicators of platelets from the healthy group indicate that carnitine acetyltransferase-mediated transport is relatively slow under physiologic conditions (Figs. 3 and 4; Tables 4 and 5). In contrast, the marked increases in total and cytoplasmic acetyl-CoA content induced by l-carnitine in platelets from the diabetic group indicate that substrate flow through the carnitine acetyltransferase pathway was markedly increased by chronic hyperglycemia (Table 3; Figs. 3 and 4).
The mechanism of this activation remains obscure because the activity of carnitine acetyltransferase in platelets from the diabetic group was only 20% higher, whereas the carnitine-evoked increase in acetyl-CoA was more than 4-fold higher than in platelets from the healthy group (Tables 2 and 3).

Nevertheless, the activation of resting platelets by l-carnitine and thrombin, ADP- or collagen-evoked platelet aggregation, and MDA synthesis in platelets from the diabetic patients lead us to conclude that these phenomena are caused by an increased supply of acetyl-CoA to the cytoplasm (Fig. 4; Tables 4 and 5). Moreover, these data indicate that the provision of acetyl-CoA to cytoplasm is necessary for excessive platelet aggregation irrespective of the triggering mechanism (Table 4).

Activation by thrombin of platelets from diabetic patients led to the total elimination of l-carnitine-evoked increases in cytoplasmic acetyl-CoA (Fig. 5), possibly because of excessive use of this metabolite to support the undue platelet activity taking place under these pathologic conditions (Tables 4 and 5). In addition, the importance of acetyl-CoA for excessive activities of platelets in patients with diabetes is evidenced here by the existence of significant correlations between acetyl-CoA concentrations and MDA synthesis or platelet aggregation (Fig. 6B and Table 6).

In platelets, MDA is synthesized from arachidonic acid in equivalent amounts with thromboxane A2 and derived thromboxane B2 (25, 30). However, much greater amounts of MDA are formed in peroxidation processes of other platelet lipids (31). Nevertheless, a strong correlation between MDA and thromboxane B2 was found in diabetic patients (31). Thus, the diabetes/l-carnitine-evoked increases in MDA synthesis reported here apparently reflect changes in thromboxane A2 synthesis taking place under these conditions (Table 5). Therefore, the marked increases in both acetyl-CoA concentrations and MDA synthesis in platelets from the diabetic patients (Tables 3 and 5) as well as the significant correlation between these 2 indicators (Table 6) suggest that surplus acetyl-CoA may trigger excessive platelet activity through stimulation of thromboxane A2 synthesis and/or lipid peroxidation (Figs. 4 and 5; Tables 4 and 5) (31).

The lack of evident effects of l-carnitine on platelets from healthy persons and the presence of significant stimulatory effects on cytoplasmic acetyl-CoA amounts and platelet function in platelets from diabetic patients indicate that activation of acetyl-CoA transport to the cytoplasm through the carnitine acetyltransferase pathway plays a principal role in excessive platelet activity in diabetes (Fig. 4; Tables 3, 4, and 5). These data are in accordance with reports on the absence of stimulatory effects of propionyl-l-carnitine on platelet-activating factor synthesis and arachidonic acid consumption in healthy individuals (12, 13). However, we found no reference data on platelets from patients with diabetes.

The data presented here show a similar increase in MDA synthesis and other platelet markers in resting and thrombin-activated platelets from patients with type 1 and 2 diabetes (Tables 1 and 5), whereas Vericel et al. (32) found activation of resting MDA synthesis only in patients with type 1 diabetes. The reasons for this discrepancy remain unknown. One can also assume that l-carnitine–induced increases in cytoplasmic acetyl-CoA in platelets of diabetic persons (Fig. 5) promote synthesis of platelet-activating factor. However, the available data instead indicate that in resting platelets from healthy individuals, l-carnitine causes slight inhibition of platelet-activating factor synthesis (13), which is consistent with our finding of no significant effects of l-carnitine on platelet function in the resting state (Fig. 4; Tables 4 and 5).

Our results indicate a causative relationship between increased acetyl-CoA and excessive activity of platelets in persons with diabetes (Figs. 2, 4, 5, and 6; Tables 4, 5, and 6). This conclusion is supported by the existence of significant positive correlations between acetyl-CoA content in platelets from diabetic patients and the indicators of their function either under resting conditions or after activation with thrombin (Table 6; Fig. 6). The absence of such relationships from platelets from healthy individuals supports the notion that the supply of acetyl-CoA is not a rate-limiting factor for physiologic platelet functions (Table 6; Fig. 6). In platelets from diabetic patients, l-carnitine stimulates formation of an additional pool of acetyl-CoA that could easily reach the cytoplasmic compartment and trigger further, proportional increases in platelet activity (Tables 3 and 6; Figs. 4 and 6).

In conclusion, our data suggest that l-carnitine may exert an undesirable effect in persons with diabetes, aggravating excessive platelet activity by increasing the acetyl-CoA supply to their cytoplasmic compartment. On the other hand, the generally beneficial effects of l-carnitine supplementation in diabetes might be attributable to increased turnover and use of free fatty acids, improving the energy balance in insulin-dependent and -independent tissues.

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