Rapid Change of Platelet Aggregability in Acute Hyperglycemia

Detection by a Novel Laser-light Scattering Method

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Key words
Platelet aggregability, hyperglycemia, glucose tolerance test

Summary
We examined the alteration of platelet aggregability in acute hyperglycemia during 75-gram oral glucose tolerance tests (OGTT). Twenty subjects underwent 75-gram OGTT and venous blood samples were obtained before (0 min), 60, 120 and 180 min postload. Platelet aggregability shown as the number of small platelet aggregates was measured with a novel laser-light scattering (LS) method. Platelet aggregability increased in parallel with both glucose and immuno-reactive insulin (IRI) levels. The number of mean small aggregates at 60 min (12.30 ± 1.10 × 10⁴) was significantly higher than the one at 0 min (8.32 ± 0.88 × 10⁴, p < 0.001), 120 min (10.63 ± 0.98 × 10⁴, p < 0.005) and 180 min (8.28 ± 0.84 × 10⁴, p < 0.001) (mean ± SEM). Small aggregates correlated positively with plasma glucose levels at 60 min postload (r = 0.67, p = 0.001) while not with IRI. It might be important to suppress transient hyperglycemia for preventing the onset of acute coronary syndromes that could be closely related to platelet hyperaggregability.

Introduction
Diabetes mellitus is associated with accelerated atherosclerosis and an increased prevalence of cardiovascular disease (1). The major cause of morbidity and death in diabetic patients is cardiovascular disease, which includes coronary heart disease, cerebrovascular disease and peripheral vascular disease (2). There is a hypothesis that high glucose levels per se directly accelerates atherosclerosis in diabetic patients. The de novo synthesis of diacylglycerol with subsequent stimulation of protein kinase C (3), increased advanced glycosylation end-products (4), and activation of the polyol pathway (5) are thought to support the hypothesis.

Increased platelet aggregability and adhesiveness, which possibly increase thrombosis, are known to be novel risk factors in coronary heart disease in type 2 diabetes mellitus (6-8). Alteration of platelet function in diabetic patients may influence acceleration of vasculopathy found in those patients. However, precise mechanism(s) for the alteration of platelet function in diabetes patients has not been well understood.

It is well known that intracoronary thrombus formation plays a significant role in the onset of acute coronary syndromes such as unstable angina or acute myocardial infarction. Impaired fibrinolytic capacity (9, 10), increased coagulation (11, 12), and increased platelet aggregation and adhesiveness (13, 14) were reported in acute coronary syndromes.

Currently platelet aggregation is measured using an optical density (OD) method or an impedance method. A novel platelet aggregability measurement system with a laser-light scattering method was developed recently by Ozaki et al. (15) and the system is capable of monitoring the increase in size of small aggregates which cannot be detected with the conventional methods.

The aim of the present study was to elucidate whether the dynamic alteration of plasma glucose during oral glucose tolerance tests (OGTT) directly affects platelet aggregability using the highly sensitive platelet aggregometer.

Materials and Methods

Population
The present study included 20 subjects (11 men, 9 women; mean age 61) who were admitted to our institution for diagnostic evaluation of their ischemic heart disease. They had not been diagnosed as having diabetes mellitus before and they had not been treated with anti-platelet agents for at least a month before the study. Patient profiles are listed in Table 1. Protocols were approved by the Kumamoto University School of Medicine Ethical Committee and written informed consent was obtained from all the study subjects.

Protocol
The study subjects underwent 75-gram oral glucose tolerance tests (OGTT) starting at 6 a.m. after an overnight fast. Venous blood sampling was obtained before (0 min), 60, 120 and 180 min postload to examine the serial changes of plasma glucose and immuno-reactive insulin levels as well as platelet aggregability. According to the criteria published by the American Diabetic Association (16), the study subjects were divided into 2 subgroups: the normal and abnormally glucose tolerant (diabetes or impaired glucose tolerance) groups. Four of the study subjects were given a same quantity of water without glucose orally as placebo controls and blood sampling was done in the same way.
Coronary Angiography

Coronary angiography (CAG) was performed in all the study subjects to examine whether they had significant coronary artery lesions. Intracoronary injection of acetylcholine was done to provoke coronary spasm (17). After the provocation, isosorbide dinitrate was administered to detect organic coronary artery lesions. The study subjects were divided into 2 subgroups according to CAG findings: the coronary artery disease (CAD) (coronary spasm and/or organic lesions) and non-CAD (neither coronary spasm nor organic lesions) groups.

Platelet Aggregometry

Venous blood was collected into tubes containing sodium citrate, and then centrifuged at 150 g for 10 min to obtain platelet-rich plasma (PRP). PRP aggregation was simultaneously determined by evaluating maximum percent decrease in optical density (OD), and by assessing laser-light scattering (LS) intensity using an aggregometer, PA-200 (Kowa, Tokyo, Japan). ADP 1.0 μM was used as an agonist for platelet aggregation and added to PRP 60 s after the start of measurement.

The principles of the LS method have been described previously (15). This method is based on the fact that the intensity of scattered light emitted from a particle increases in proportion to the square of its diameter. Briefly, a diode laser-light beam (width, 40 μm; wave length, 675 nm) was passed through PRP (300 μL) stirred in a cylindrical glass cuvette with a 5 mm internal diameter. The light scattered from the observation volume (48 × 140 × 20 μm) was detected by a photocell array. Light intensity corresponds to particle size. Signal frequency for every 10 s represents the number of aggregates (counts/10 s). The LS signals obtained were digitized with an A/D (analogue/digital) converter and processed by a computer (PC-9821 Xa16, NEC, Tokyo, Japan). Data were recorded as a two-dimensional graph showing the change over time(s) of number of aggregates (counts/10 s) for 5 min.

Particles with an intensity of 25 to 400 mV represented small aggregates (9-25 μm), those with an intensity of 400 to 1,000 mV represented medium aggregates (25-50 μm), and those with an intensity of 1,000 to 2048 mV represented large aggregates (50-70 μm). Small aggregates contain approximately 70-1,400 platelets, and medium aggregates contain approximately 1,000-11,000 platelets, and large aggregates contain approximately 11,000 to 31,000 platelets. Generally, aggregates smaller than 10 μm are formed in the early phase of aggregation, larger aggregates are formed in the following phase (15). Quantitative estimation of platelet aggregation was performed by determining the peak intensity of LS produced by small aggregates.

Glucose, Immuno-reactive Insulin and Lipids Measurements

Plasma glucose was measured by an autoanalyzer using the enzyme-electrode method. Immuno-reactive insulin was measured by a radio immunoassay kit (Eiken Chemical, Tokyo, Japan). Lipids were measured by autoanalyzers, AU5200 (Olympus, Tokyo, Japan) and 7070 (Hitachi, Tokyo, Japan).

Statistical Analysis

Data are expressed as mean ± SEM. Serial changes of platelet aggregation were analyzed by 1-way ANOVA for repeated measures, followed by the Tukey-Kramer multiple comparison test. Serial changes of platelet aggregation between subgroups were analyzed by 2-way ANOVA for repeated measures. Mean platelet aggregability between the subgroups at each sampling point was analyzed by the Student t-test. A linear regression analysis was performed to analyze the relationship between plasma glucose and IRI levels and platelet aggregation. P values less than 0.05 were considered statistically significant.

Results

Clinical characteristics of the study subjects are listed in Table 1. OGTT revealed that 10 subjects had normal glucose tolerance, 6 had DM, and the remaining 4 had impaired glucose tolerance. CAG showed

<table>
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<th>Table 1 Clinical characteristics of the study subjects. Values are shown as number (%) or mean ± SEM. Diseased artery is defined as having lumen diameter stenosis more than 75%. Coronary spasm was provoked by intracoronary injection of acetylcholine (17)</th>
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that there were 2 subjects with 2-vessel disease and 4 with single-vessel disease. Coronary spasm was induced in 4 of 20 subjects by intracoronary injection of acetylcholine (17). Two of those had no stenosed coronary lesions and 2 with single-vessel disease (organic lesion with spasm). In all the 20 subjects, the mean plasma glucose level (mg/dl) at baseline (92 ± 4) was increased and peaked at 60 min (187 ± 10) during OGTT. The mean IRI level (μU/ml) was also increased from 9.2 ± 1.3 at baseline to 64.5 ± 9.1 at 60 min.

ADP 1.0 μM-induced platelet aggregability as shown by the numbers of small platelet aggregates changed dynamically during OGTT in all subjects (Fig. 1). The numbers of small aggregates reached the peak level at 60 min in 16 subjects and at 120 min in the remaining 4. Small platelet aggregates were increased in parallel with the glucose and IRI levels which also peaked at 60 min (Fig. 2). The number of mean small aggregate at 60 min (12.13 ± 1.05 × 10^4) was significantly higher than that at 0 min (8.32 ± 0.88 × 10^4, p < 0.001), 120 min (10.63 ± 0.98 × 10^4, p < 0.05), and 180 min (8.28 ± 0.84 × 10^4, p < 0.001).

A representative aggregation pattern during OGTT is shown in Fig. 3. On the other hand, platelet aggregability did not change when water was administered as a placebo control (n = 4) (7.4 ± 2.7 × 10^4 at 0 min, 7.0 ± 2.5 × 10^4 at 60 min, 7.4 ± 2.7 × 10^4 at 120 min and 6.9 ± 2.2 × 10^4 at 180 min).

The alteration in the pattern of platelet aggregability during OGTT could be linked to the results of OGTT. Abnormal glucose tolerant subjects had higher platelet aggregability as compared with normal subjects all through OGTT (p < 0.001) (Fig. 4, left panel) while the results of CAG did not show a relation with the alteration in platelet aggregability (Fig. 4, right panel). Platelet aggregability at 60 min and 120 min was significantly higher than that at 0 and 180 min in
each subgroup (p < 0.001, 60 min vs 0 and 180 min in all subgroups; p < 0.01, 120 min vs 0 and 180 min in the abnormal glucose tolerant subjects; p < 0.05, 120 min vs 0 and 180 min in the normal glucose tolerant subjects; p < 0.01, 120 min vs 0 min and p < 0.05, 120 min vs 180 min in the CAD group; p < 0.05, 120 min vs 0 min and p < 0.01, 120 min vs 180 min in the non CAD group) (Fig. 4). Platelet aggregability in abnormal glucose tolerant subjects was significantly higher than in the normal glucose tolerant subjects at all the sampling points (p < 0.01 at 0 and 180 min, p < 0.001 at 60 and 120 min) (Fig. 4, left panel). Platelet aggregability was also higher in the CAD group than in the non-CAD group at 60 min (p < 0.05). (Fig. 4, right panel).

The number of small aggregates at 60 min correlated positively with plasma glucose levels at 60 min (r = 0.67, p = 0.001) (Fig. 5, left panel) while not with IRI levels (Fig. 5, right panel). There were no significant correlations between platelet aggregability and glucose or insulin levels at 0, 120 and 180 min. Platelet aggregability measured by a conventional optical density method did not alter during OGTT.

Discussion

In this study we demonstrated that ADP-induced platelet aggregation changed dynamically during OGTT in parallel with glucose and insulin levels in blood. The peak levels of platelet aggregability were significantly correlated with those of glucose. It is likely that platelet function is affected by plasma glucose levels in a rapid fashion.

In a previous study, Virgolini et al studied platelet aggregation and platelet sensitivity during an intravenous glucose tolerance test (IVGTT) (18). They reported that the maximal amplitude (Tmax) of the response curve for ADP-induced platelet aggregation did not alter during IVGTT. Their data is consistent with ours using an optical density method to examine platelet aggregation. Giugliano et al reported that acute hyperglycemia induced by hyperglycemic glucose clamp increased platelet aggregation determined by a conventional aggregometer (19). The glucose levels in their study reached almost 270 mg/dl, which is thought to be above the physiologic range. In the present study, platelet aggregation was increased even by hyperglycemia within the physiologic range, which may occur in a postprandial period.

A highly sophisticated method to determine platelet aggregability was developed recently using a laser-light scattering technique (15). The method enables to detect a minimum change of platelet aggregability because the method is capable of detecting small platelet aggregates containing approximately 70-1,400 platelets, which are formed at the early stage of platelet aggregation. This method can distinguish even between the different antiplatelet mechanisms of ticlopidine and aspirin (20). Spontaneous platelet aggregation without agonists such as ADP or epinephrine was observed in patients with diabetes by the laser-light scattering method while not by a conventional optical density method (8). This increased sensitivity for detecting platelet aggregation over the conventional method may produce the significant results of the present study, where platelet aggregability could not be determined by the previous study (18).

In the present study, platelet aggregation during OGTT was altered not only in abnormal glucose tolerant subjects but also in normal glucose tolerant subjects according to the changes of their plasma glucose levels. Moreover, the increase was more obvious in the abnormal glucose tolerant group as compared with the normal glucose tolerant group. Platelet aggregability tended to be higher in the subjects with coronary artery diseases as compared with those with normal coronary arteries.

Increased platelet aggregation is one of the important initial processes in the development of cardiovascular diseases including acute myocardial infarction (21) and cerebral infarction (22). Overeating is reported as one of the possible triggers for acute myocardial infarction (23). From the results of our present study, the reason why overeating could be a trigger may be in part due to the transitoriely increased platelet aggregability induced by postprandial hyperglycemia.

The precise mechanism(s) of increased platelet aggregability induced by hyperglycemia is not elucidated clearly in this study. However, from the fact that platelet aggregability correlates to plasma glucose levels only at 60 min when insulin levels increased maximally, we can speculate that platelet aggregability is affected directly by plasma glucose levels under a hyperinsulinemic state. To investigate this speculation, PRP was incubated with insulin (100 μU/ml) and/or glucose (300 mg/dl) for 60 min and then ADP-induced platelet aggregability was measured (n = 5). Insulin alone had no significant effect on platelet aggregability as compared with control while glucose did increase an increase in platelet aggregability (p < 0.05, 100 ± 10% vs 202 ± 17%). Concomitant use of insulin with glucose significantly increased platelet aggregability (275 ± 20%, p < 0.01 vs control and insulin alone, p < 0.05 vs glucose alone). It is suggested that glucose has a direct effect on platelet aggregability and that the additional presence of insulin enhances hyperglycemia-induced platelet aggregation.

Insulin receptors on platelets (24) might be involved in the increase of platelet aggregability during OGTT.

Nitric oxide is known to inhibit platelet aggregation (25) and L-arginine normalizes increased platelet aggregation induced by hyperglycemia (19). These findings suggest that reduced nitric oxide availability induced by hyperglycemia may be related to the increased platelet aggregation. It is also reported that free radical production is increased in type 2 diabetes with poor glycemic control (26). Therefore, nitric oxide might be depleted by reactive oxygen species that could be...
increased when hyperglycemia exists. That may lead to increased platelet aggregability.

In conclusion, we report for the first time that platelet aggregability altered dynamically and concomitantly with plasma glucose level during OGTT both in normal and abnormal glucose tolerant subjects. This finding suggests that transitory increased plasma glucose levels observed in a postprandial period may be a trigger of cardiovascular events through increasing platelet aggregability. It might be important to suppress postprandial hyperglycemia causing hyperaggregability for preventing the onset of acute coronary syndromes, especially in diabetic subjects.

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