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Platelet Aggregation is not Enhanced in Patients with Prediabetes

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Abstract

Prediabetes [impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG)] is a major risk factor for T2DM as well as for cardiovascular disease and mortality. In the present study, the platelet aggregation and fibrinogen levels were investigated in prediabetic subjects who had no confounding factors such as hypertension, obesity or dyslipidemia. Thirty-nine subjects with prediabetes (24 IFG and 15 IGT) and age, sex and BMI matched 36 healthy controls were enrolled. Platelet aggregation, fibrinogen and hsCRP levels, HOMA-IR and HOMA-β indexes were determined. Platelet aggregation induced by collagen, epinephrine or ADP was not different (p=0.93, p=0.90 and p=0.29, respectively) between two groups, whereas fibrinogen levels were significantly higher (p=0.006) in the prediabetics when compared to controls. hsCRP levels, HOMA-IR and HOMA-β indexes in the two groups were not different. The power of the study was calculated according to the results and established as 0.97 for collagen, 0.95 for epinephrine and 0.83 for ADP. Despite the high plasma fibrinogen levels, the platelet aggregation in prediabetics was not different when compared to healthy controls. These data suggest that platelet aggregation may not be involved in the mechanism of prothrombotic state in prediabetic state.

Introduction

Diabetes mellitus is accompanied by microvascular and macrovascular complications, which are associated with accelerated atherotrombosis resulting in premature coronary artery disease (CAD) and increased risk of cerebrovascular and peripheral vascular disease [1-3]. The causal relationship between diabetes mellitus and cardiovascular diseases starts before the establishment of overt disease in the course of progression from normal glucose tolerance that is associated with resistance to the biologic activity of insulin [4]. Subjects exhibit excessive increases in postprandial plasma glucose levels and compensatory increases in fasting plasma insulin levels, many years before the onset of type 2 diabetes (T2DM).

Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), which represent the "prediabetes", are early stages of glucose intolerance and major risk factors for the development of T2DM. Moreover, individuals with prediabetes have

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Abbreviations IGT: Impaired glucose tolerance; IFG: Imparied fasting glucose; T2DM: Type 2 diabetes mellitus; BMI: Body mass index; hsCRP: High sensitivity C – reactive protein; HOMA: Homeostasis model assessment model; ADP: Adenosine diphosphate; CAD: Coronary artery disease; OGGT: Oral glucose tolerance test; PRP: Platelet rich plasma; TG: Triglyceride; LDL: Low-density lipoprotein; HDL: High-density lipoprotein

been reported to be at increased risk of cardiovascular diseases and mortality due mainly to hyperglycemia, insulin resistance and other cardiovascular risk factors [5-7]. Abnormalities in platelet functions (increased platelet adhesion, aggregation, etc.) and high fibrinogen concentrations have been reported in diabetic patients [8,9]. However, the role of platelet aggregation in the pathogenesis of increased cardiovascular risk in prediabetic period is unclear. To our knowledge, there is only one report about platelet aggregation in early diabetes, which was performed in a relatively small group of patients [10].

We hypothesized that alterations in platelet aggregation and fibrinogen levels may play role in prediabetic subjects regarding the prothrombotic state. To address this question, we investigated platelet aggregation and fibrinogen levels in prediabetic subjects without hypertension, obesity, and/or dyslipidemia and family history of T2DM.

Materials and Methods

Patients and controls

A total of 75 subjects (41 men and 34 women) were selected from 215 subjects, which were screened for impaired glucose tolerance by a 75 g oral glucose tolerance test (OGTT) according to the American Diabetes Association criteria [11]. Exclusion criteria included presence of diabetes mellitus, medications that affect glucose tolerance, platelet aggregation or fibrinogen levels, and liver, kidney or thyroid disorders.

All individuals undergoing OGTT were subjected to high carbohydrate diet three days before the test. Venous blood samples were taken at 0 and 120 min for measurements of plasma glucose concentrations. IFG was defined as fasting plasma glucose of 100–125 mg/dl and below 140 mg/dl by OGTT/120 min. IGT was defined as plasma glucose 140–199 mg/dl by OGTT/120 min. Normal glucose tolerance was defined as fasting and OGTT/120 min plasma glucose below 100 mg/dl and 140 mg/dl, respectively. Only the subjects with isolated IFG or IGT were enrolled.

Systolic and diastolic blood pressure was measured according to the standard technique with the subject in the supine position. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or current use of antihypertensive medication. Standing height and body weight were measured in light indoor clothing and without shoes. Body mass index (BMI) was calculated as weight divided by squared height (kg/m2). Smoking status was recorded as “none” or “current” using a standard questionnaire.

The Ethical Committee of Gulhane Medical School approved the study, and all patients and controls gave written informed consent. Characteristics of the patients and controls are shown in Table 1.

Platelet Aggregation

To evaluate platelet aggregation, 12-hour overnight fasting venous blood samples

were drawn in the morning (between 8:00 AM and 9:00 AM). The test was performed within 30 minutes from the time blood was withdrawn using sodium citrate (3.8%) as an anticoagulant (1:9 citrate to blood).

The samples were centrifuged at 60 g (r=85 mm, 800 rpm) for 10 minutes at room temperature and yielded platelet rich plasma (PRP). The remnant of PRP was recentrifuged at 856 g (r=85 mm, 3000 rpm) for 10 minutes, and the PRP was adjusted with its own platelet poor plasma to obtain platelet count of 250x109 L-1. A teflon coated stir bar was inserted into the cuvette with PRP then into the heater block to a temperature of 37 oC, and the aggregation procedure was done in a computerized aggregometer (model 560-Ca, Chrono-log Corporation, Havertown, PA, USA). The stimulating agents including collagen (2 μg/mL), epinephrine (10 μM) and adenosine diphosphate (ADP) (20 μM) (Sigma, St. Louis, MO, USA), were added to the cuvette with PRP, and changes in light transmission were observed for 6 minutes. The maximal amplitudes of the aggregation curves, expressed as percentage, were used for quantitative analysis.

Biochemical tests

The serum samples collected at the same time were promptly centrifuged, and the plasma was separated and stored at -70°C for measurement of glucose, total cholesterol, triglyceride (TG), high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol. Glucose, total cholesterol, TG and HDL-cholesterol levels were measured by the enzymatic colorimetric method with Olympus AU 600 autoanalyzer using reagents from Olympus Diagnostics, (GmbH, Hamburg, Germany). All plasma samples were run in the same assay. LDL-cholesterol was calculated by Fridewald's formula when TG levels were below 400 mg/dL [12]. Serum basal insulin value was determined in duplicate by the coated tube method (DPC-USA).

High sensitivity C-reactive protein

Serum hsCRP was measured by turbidimetric fixed rate method [13] by an automated analyzer (Olympus AU- 2700, Mishima, Japan). All assays were performed in duplicate.

Fibrinogen

Fibrinogen levels were determined in citrated plasma with HemosIL Fibrinogen-C Reagent on ACL Advance Analyzer (Beckman Coulter) according to the manufacturer's instructions (normal range 200-400 mg/dl).

Assessment of insulin sensitivity and β-cell function

Homeostasis Model Assessment Model (HOMA) was used to determine the insulin sensitivity index with formula: HOMA-IR= fasting insulin (μU/ml) x fasting glucose (mg/dl) /405 [14]. Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate low insulin sensitivity (insulin resistance). β-cell function was determined by homeostasis model assessment of β-cell func-

tion (HOMA-β). HOMA-β was calculated with formula: HOMA-β= [20 × fasting insulin (μU/ml)] / [fasting glucose (mmol/l) − 3.5] [15].

Statistical analysis

Results are reported as the mean±SD and median (for the skewed data). Differences between prediabetic and control groups were tested for significance by independent samples T-test and Mann-Whitney U test. Differences were considered significant at $p<0.05$. Because there has been no former data regarding platelet aggregation in such a study population, the sample size was not calculated by power analyzing. The power of the study was calculated according to the results.

Results

According the glucose tolerance status the subjects were grouped as prediabetes (n=39; n=24 for IFG and n=15 for IGT) and normal controls (n=36). The two groups had similar age, sex and BMI. The characteristics of the prediabetic group and the controls are described in Table 1.

Platelet aggregation induced by collagen, epinephrine or ADP was not significantly different in the prediabetic group compared to healthy controls (p=0.93, p=0.90 and p=0.29, respectively) (Figure 1). HOMA-IR and HOMA-β indexes,

Table 1: Characteristics of the prediabetic subjects and normal controls.

	Prediabetes (n=39)	Control (n=36)	P
Age, year	47.1 ± 10.2	45.5 ± 9.1	0.48*
Sex, M/F	20/19	21/15	0.54***
BMI (kg/m2)	27.6 ± 2.5	26.7 ± 3.5	0.19*
Total cholesterol (mg/dl)	212 (97-302)	183 (105-281)	0.09**
HDL cholesterol (mg/dl)	40 (32-69)	45 (28-68)	0.14**
LDL cholesterol (mg/dl)	129.5 (50-223)	110 (49-177)	0.10**
Triglycerides (mg/dl)	138 (61-496)	134 (30-495)	0.52**
Fasting glucose (mg/dl)	112.4 ± 8.1	87.2 ± 8.6	< 0.001*
hsCRP (mg/L)	1.47 (0.19-2.96)	0.90 (0.06-2.93)	0.06**
HOMA-IR	2.30 (0.23-8.38)	1.75 (0.08-5.79)	0.07**
HOMA-β	1.6 (0.22-4.62)	2.3 (0.1-18.1)	0.22**
Fibrinogen (mg/dl)	312.6 ± 55.2	278.6 ± 44.9	0.006*
PA by collagen (μg/mL)	73 (55-99)	75 (31-110)	0.93**
PA by epinephrine (μM)	70 (3-92)	71 (9-91)	0.90**
PA by ADP (μM)	88 (36-125)	77 (5-115)	0.29**
Smokers (%)	20.5	27.8	0.46***

Results are expressed as mean ± SD, median (min-max).
Independent sample-t test*, Mann-Whitney U test**, Chi-square test***
hsCRP: High sensitive C-reactive protein, HOMA-IR: Homeostasis model assessment insulin resistance, PA: Platelet aggregation, ADP: Adenosine diphosphate

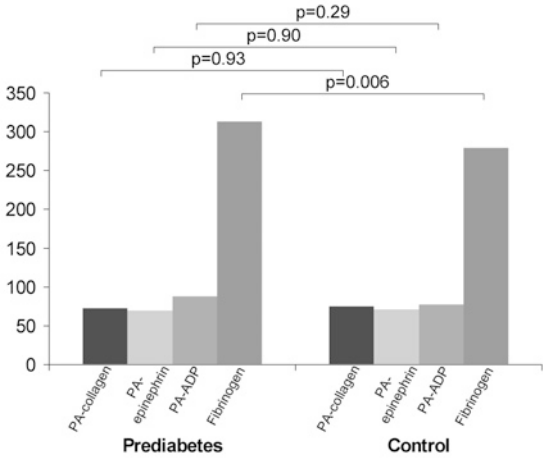

Figure 1. Platelet aggregation induced by collagen, epinephrine and adenosine diphosphate was similar in the prediabetics and healthy controls. Fibrinogen concentrations were significantly high in the prediabetic group.

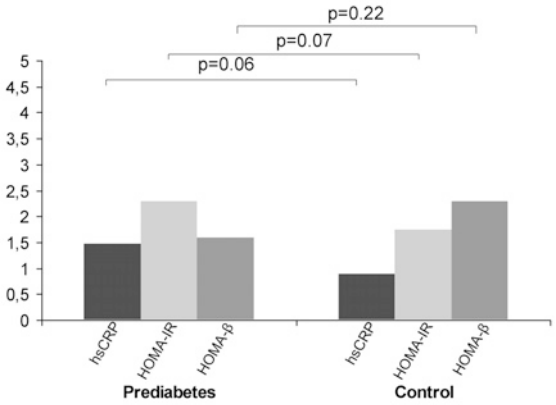

Figure 2. hsCRP levels and HOMA indexes were not different between prediabetics and controls.

and hsCRP levels were not different between the two groups as well (p=0.07, p=0.22 and p=0.06 respectively) (Figure 2). Fasting glucose and fibrinogen levels were significantly higher in the prediabetic group than the controls (p<0.001 and p=0.006, respectively).

In subgroup analysis, HOMA-IR and HOMA-β indexes, and hsCRP levels were also similar in subjects with IGT and IFG (p=0.15, p=0.3 and p=0.15 respectively). The power of the study calculated according to the results established as 0.97 for collagen, 0.95 for epinephrine and 0.83 for ADP.

Discussion

In this preliminary study, no significant difference was found between the prediabetic subjects and controls regarding platelet aggregation as well as plasma hsCRP levels and HOMA indexes. On the other hand, the fibrinogen levels were significantly higher in prediabetic subjects than the controls.

The onset of macrovascular complications in diabetes mellitus is likely to begin years or even decades earlier. Increased fasting and 2-hour post-challenge plasma glucose levels are associated with increased cardiovascular mortality [16–17]. A recent meta-analysis concluded that in nondiabetic individuals the relative risk of cardiovascular events was 1.26 for subjects in the highest category of blood glucose level compared with subjects in the lowest blood glucose category [18]. Prediabetes not only identifies individuals at risk for diabetes, but also predicts cardiovascular disease and mortality. However, the pathogenesis of the increased cardiovascular risk in prediabetic subjects is still poorly understood. The possible inciting mechanisms involved are insulin resistance, sub-clinical inflammation and other CAD risk factors in the prediabetic state [19, 20]. Insulin resistance is a pronounced defect in the prediabetic state and is associated with the increased risk of CAD [6, 18]. Chronic sub-clinical inflammation is also associated with increased CAD risk and appears to be associated with insulin resistance and other features of the insulin resistance syndrome in the prediabetic state [21–23]. In the present study, we did not find any difference in hsCRP levels and HOMA indexes between the prediabetic subjects and controls. The absence of the confounding factors such as hypertension, obesity, hyperlipidemia as well as diabetic complications (retinopathy, nephropathy and neuropathy) in our study groups is an important and distinct feature of the present work from the previous studies addressing the same issue [23, 24]. Moreover, we have recently reported in a group of selected people with IFG that circulating levels of soluble CD40 ligand which has been shown to be involved in the atherosclerotic conditions are not different from the healthy controls [25]. Therefore, the discrepancies between our results and the other studies might be due to the selection criteria of the subjects.

Platelets are heterogeneous in size, density, and reactivity and these changes arise at or before thrombopoiesis. Alterations in these variables may be involved in the natural history of vascular disease. Larger and functionally more reactive platelets have been found in diabetics and also prediabetics [26, 27]. Although platelet counts are normal in diabetics, multiple studies offer evidence of enhanced activation or increased platelet activity in patients with T2DM [9]. Garcia et al. found that platelets from patients with T2DM maximally aggregated with low concentrations of ADP and arachidonic acid [8]. Rosove et al. demonstrated an increased response to ADP in several insulin-dependent diabetics [28]. Hughes et al. found evidence of enhanced platelet hyperaggregability in vitro or increased circulating platelet aggregates in more than one third of patients with T2DM [29]. However, the role of platelets in the pathogenesis of increased cardiovascular risk in prediabetes is not clear. In an early study, Sagel et al. reported an increase in platelet aggregation in response to ADP in a group of latent diabetics with 100 g OGTT [10]. However, the number of the subjects was relatively small, and collagen and epinephrine stimulations were not applied in that study. In our study, we did not find any difference in response of platelets to collagen, ADP or epinephrine between the prediabetic subjects and controls. The absence of insulin resistance, sub-clinical inflammation and/or other risk factors for CAD may explain the difference of our results from the

previous study. These data imply that platelet aggregation may not have a significant role in prothrombotic state in patients with prediabetes.

Fibrinogen is a powerful, independent risk factor for cardiovascular diseases. It has an influence on thrombogenesis, affecting the blood viscosity, and platelet aggregation [30]. Prospective studies have consistently shown elevated fibrinogen levels to be a powerful predictor of future cardiovascular events, independent to that of other cardiovascular risk factors [31, 32]. A number of studies have reported elevated plasma fibrinogen levels in patients with type 1 [33], T2DM [34] and prediabetic subjects in association with insulin resistance and sub-clinical inflammation [19, 21]. In our study, despite the lack of insulin resistance, sub-clinical inflammation and/or other risk factors for CAD, plasma fibrinogen levels were significantly higher in prediabetic subjects than the controls. This observation suggests that hyperfibrinogenemia per se may take place in impaired haemostasis in these patients.

The present study, however, has several limitations. Our data may not be representative for all subjects with prediabetes due to the narrow selection criteria and relatively small sample size. In addition, HOMA formula used for the measurement of insulin sensitivity and beta cell function is only an estimate and may not be as accurate as glucose clamp test.

In conclusion, platelet aggregation may not be involved in the mechanism of the prothrombotic state in prediabetes. Hyperfibrinogenemia seems to be a constant feature of this hyperglycemic period regardless of the absence of confounders. Further studies with larger populations are needed to evaluate platelet aggregation and other risk factors in the prediabetic state.

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