STREPTOZOTOCIN-INDUCED DIABETES MELLITUS – A PARADOX OF HIGH INTRINSIC PLATELET REACTIVITY AND LOW *IN VITRO* PLATELET AGGREGATION

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Abstract

Context. Studies of platelet function in diabetics are inconsistent, some studies reporting higher platelet reactivity, while others showed no change.

Objective. We aimed to evaluate platelet indices and *in vitro* platelet aggregation in rats with long-lasting (28 weeks) diabetes mellitus.

Design. Twelve controls and 14 diabetic rats were investigated. Diabetes was induced in 11-week-old rats using streptozotocin (60 mg/kg,i.p.). Platelet indices and *in vitro* adenosine diphosphate (ADP)-, protease-activated receptor 4 (PAR4) agonist-, and arachidonic acid (AA)-induced platelet aggregation were assessed at the age of 38 weeks.

Results. Compared to controls, diabetic rats presented lower platelet count and plateletcrit (both $p \leq 0.001$), and higher mean platelet volume ($p < 0.01$). ADP- ($p = 0.04$) and AA-induced ($p < 0.01$) platelet aggregation were lower in diabetic compared with control rats, whereas PAR4 agonist-induced platelet aggregation was similar between the two groups ($p = 1.00$).

Conclusions. This study demonstrates a paradox of high intrinsic platelet reactivity and low *in vitro* ADP- and AA-induced platelet aggregation in diabetic rats compared with non-diabetic controls. The relevance of *in vitro* platelet aggregation to the contribution of platelets to *in vivo* thromboembolic events in diabetic rats remains questionable.

Key words: animal model, diabetes mellitus, platelet aggregation, platelet indices, streptozotocin.

from *in vitro* studies, that platelet activity is enhanced in the presence of diabetes (3). Platelet indices commonly used in clinical practice have also been shown to reflect the degree of platelet activation. Studies have demonstrated that larger platelets, commonly seen in diabetic patients, reflect higher platelet turnover rate, are more rapidly recruited to thrombus formation, and have greater agonist-induced aggregation (4).

Although higher platelet reactivity appears to be accepted as an ubiquitous feature in diabetic patients, studies of platelet function are rather inconsistent, particularly in patients with type 1 diabetes (5,6). In addition, numerous *in vitro* studies of platelet aggregation in murine models of streptozotocin (STZ)-induced diabetes mellitus have failed to show increased platelet aggregation in the diabetic animals compared to their non-diabetic controls (7-9). However, in all those experimental studies, platelet function was assessed after rather short duration of diabetes (up to 4 weeks), and the relationship between platelet indices and *in vitro* platelet aggregation has not been assessed in this setting.

Accordingly, we aimed to evaluate platelet indices and *in vitro* platelet aggregation in rats with long-lasting (28 weeks) STZ-induced diabetes mellitus.

MATERIALS AND METHODS

Animals and housing

Eight-week-old male Wistar rats (bodyweight 234.7 ± 5.3 g) were randomly assigned to two groups: control ($n = 12$) and diabetics ($n = 14$). All animals were housed individually in a climate-controlled room (21°C - 24°C), with a 12-hour light/12-hour dark photoperiod, in an accredited animal facility, and had free access to food and water.

All experiments complied with the International Council for Laboratory Animal Science guidelines

INTRODUCTION

Patients with diabetes mellitus have a 2-to-4-fold higher risk of ischemic disease than their non-diabetic counterparts (1). In individuals with type 1 diabetes, the risk appears to be even higher (2). Platelet hyperreactivity has been postulated as a potential contributor to the increased thrombogenicity observed in the diabetic population.

There is increasing evidence, largely derived

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(Directive 2010/63/EU) and were approved by the local Ethics Committee.

Induction of diabetes mellitus

Diabetes was induced at the age of 11 weeks by a single intraperitoneal injection of STZ (60 mg/kg of bodyweight; Sigma-Aldrich, St Louis, MO) in citrate-buffered saline (0.1 mol/L, pH 4.5; Sigma-Aldrich), as described previously (10-12). Insulin was not administered. Glucose levels were measured one week post-STZ administration, as described previously (10). Rats were considered diabetics if fasting plasma glucose exceeded 250 mg/dL. Age-matched control rats received an equal volume of citrate buffer, without STZ.

Blood sampling and analysis

At 38 weeks of age, all animals were anesthetized, and non-fasting plasma glucose was measured using a clinical glucometer (SensoCard; Elektronika Kft., Budapest, Hungary) and commercially available test strips (SensoCard), as described previously (10,12). The abdominal cavity was opened, the aorta was cannulated, and blood samples were collected into two vacutainers containing ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lakes, New Jersey) and hirudin (Roche Diagnostics International Ltd, Rotkreuz, Switzerland), respectively.

Platelet count was performed in all rats using the direct current detection method and platelet indices were derived from the automated cell counter (Sysmex XP-300 Automated Hematology Analyzer; Sysmex Corporation, Japan).

Platelet aggregation was assessed *in vitro* in six control and five diabetic rats using whole-blood

impedance aggregometry (Multiplate analyzer, Roche Diagnostics, Mannheim, Germany). After sampling, the blood was kept at room temperature for 30 min to 2 h prior to platelet aggregation testing. Whole-blood samples were diluted in the test cells with NaCl (0.9%) and stirred at 37°C for 3 min. Aggregation was then stimulated with adenosine diphosphate (ADP; 9.7 µM), protease-activated receptor 4 (PAR4) agonist (442 µM), and arachidonic acid (AA; 60 µM), respectively. Agonists' concentrations were chosen to ensure the highest mean area under the aggregation curve (AUC) values (13). Changes in electrical impedance were recorded for 6 min and the AUC, reflecting overall platelet activity, was determined.

Statistics

Data are expressed as means ± standard error of the mean, and were compared using the Mann-Whitney U test. Correlations were ascertained with Spearman's correlation method. All tests were two-sided and a p-value < 0.05 was considered statistically significant. All data were computed using the GraphPad Prism software (GraphPad Software; San Diego, CA).

RESULTS

Platelet indices

At the end of the study, non-fasting plasma glucose was significantly higher in diabetic compared to control rats (459.4 ± 18.5 mg/dL *versus* 128.1 ± 4.8 mg/dL, $p < 0.0001$). Compared to their non-diabetic controls (Table 1), diabetic rats presented significantly lower platelet count and plateletcrit (both $p \leq 0.001$). The analysis of platelet indices revealed significantly higher mean platelet volume (MPV) in diabetic compared to

Table 1. Platelet indices in diabetic and control rats

Parameter	Control (n = 12)	Diabetes (n = 14)	p-value
Platelet count ($\times 10^4/\text{mm}^3$)	86.1 ± 2.2	70.9 ± 3.1	0.001
Plateletcrit (%)	57.3 ± 2.0	45.8 ± 1.9	< 0.001
Mean platelet volume (fL)	6.7 ± 0.1	7.1 ± 0.1	< 0.01
Platelet distribution width (fL)	7.4 ± 0.1	7.8 ± 0.2	0.09
Platelet-large cell ratio (%)	4.7 ± 0.4	5.7 ± 0.5	0.19

Data are expressed as means ± standard error of the mean. p-values refer to between-group comparisons using the Mann-Whitney U test.

Table 2. Adenosine diphosphate, protease-activated receptor 4 agonist, and arachidonic acid-induced platelet aggregation in diabetic and control rats

Parameter	Control (n = 6)	Diabetes (n = 5)	p-value
ADP-induced aggregation (AUC; AU)	67.7 ± 2.5	58.2 ± 2.0	0.04
PAR4 agonist-induced aggregation (AUC; AU)	63.5 ± 2.0	62.6 ± 1.4	1.00
AA-induced aggregation (AUC; AU)	59.3 ± 2.3	45.0 ± 0.9	< 0.01

AA – arachidonic acid; ADP – adenosine diphosphate; AU – arbitrary units; AUC – area under the curve; PAR4 – protease-activated receptor 4. Data are expressed as means ± standard error of the mean. p-values refer to between-group comparisons using the Mann-Whitney U test.

non-diabetic rats ($p < 0.01$). Platelet distribution width (PDW) also tended to be higher in the diabetic rats ($p = 0.09$). There was no significant correlation between plasma glucose and any of the platelet indices (all $p \geq 0.16$).

In vitro whole-blood platelet aggregation

Figure 1 depicts typical aggregometry tracings obtained in control and diabetic rats. Compared to non-diabetic controls, diabetic rats presented significantly lower mean AUC for both ADP- ($p = 0.04$) and AA-induced ($p < 0.01$) platelet aggregation (Table 2). Meanwhile, the AUC for PAR4 agonist-induced platelet aggregation was similar in diabetic and non-diabetic rats ($p = 1.00$). There was no significant correlation between plasma glucose and platelet aggregation among the diabetic rats (all $p \geq 0.13$). No thromboembolic event was recorded in any of the studied animals.

DISCUSSION

Platelet indices have emerged as biomarkers

Figure 1. Representative aggregometry tracings obtained in a control and a diabetic rat (measurements in duplicate) using (a) adenosine diphosphate, (b) protease-activated receptor 4 agonist, and (c) arachidonic acid as agonists. AU – arbitrary units.

of platelet activation, providing a valid instrument for easy, widely available evaluation of thromboembolic risk in different clinical settings, including myocardial infarction, arterial hypertension, chronic kidney disease, atrial fibrillation, or diabetes (14–16). Larger platelets, reflected by increased MPV and PDW values, have been shown to contain more dense granules and higher thromboxane A₂ levels, to release larger amounts of agonists, to be metabolically and enzymatically more active, to have increased expression of glycoprotein Ib and IIb/IIIa receptors and thus to adhere and aggregate more easily, and to have higher thrombotic potential than smaller platelets (17,18). When associated with reduced platelet count, elevated platelet size has been related to increased platelet consumption (19). In line with these concepts, in the present study, rats with long-lasting diabetes mellitus presented significantly lower platelet count and significantly higher MPV, and also tended to have higher PDW compared to their non-diabetic controls, indicating higher platelet reactivity and platelet turnover.

Paradoxically, however, this increased platelet reactivity observed in the diabetic rats was not reflected in higher *in vitro* platelet aggregation. On the contrary, ADP- and AA-induced platelet aggregation were significantly lower in the diabetes group, whereas PAR4 agonist-induced aggregation was similar between diabetic and non-diabetic rats. Together, these results suggest that platelet inhibitors present in the diabetic plasma may counterbalance, or even exceed the intrinsic platelet hyperreactivity. The same hypothesis emerges from several previous experimental studies. In the study by Judge *et al.*, despite larger platelet size and higher ATP release in response to ADP in diabetic compared to non-diabetic rats, reflecting increased platelet reactivity, ADP-, collagen-, and AA-induced platelet aggregation was not different in diabetic compared with control rats (8). In numerous other *in vitro* studies, diabetic rats platelets suspended in diabetic plasma displayed similar aggregability to that observed in non-diabetic rats platelets. However, when diabetic platelets were suspended in plasma from control rats, enhanced aggregation was noted (7,20,21), suggesting the presence of yet unidentified antiplatelet factors in the diabetic plasma. Nevertheless, none of these studies, in which platelet function was assessed after rather short duration of diabetes (up to 4 weeks), reported lower *in vitro* agonist-induced platelet aggregation in diabetic rats, as seen in our rats with long-term diabetes. Therefore, the present study brings additional insights regarding the platelet reactivity - *in*

vitro platelet aggregation controversy, suggesting that, if present, the amounts and/or the activities of diabetes-related platelet inhibiting factors are likely to increase with longer duration of diabetes.

Compared to other platelet function tests, whole-blood aggregometry has the advantage of assessing platelet function under more physiological conditions, in the presence of other blood elements that have the potential to affect platelet function. However, given the indisputably higher thromboembolic risk of diabetic compared with non-diabetic patients, it is questionable whether the lower *in vitro* platelet aggregation observed in the diabetic rats is an accurate reflection of *in vivo* platelet function. Indeed, experimental data indicate that platelet function assessed under *in vitro* conditions can be significantly lower than that observed under *in vivo* or *ex vivo* conditions. Using an *ex vivo* model of extracorporeal circulation, Yeom *et al.* demonstrated significantly higher platelet aggregation in rats with STZ-induced diabetes mellitus (22), whereas in the study by Paul *et al.*, despite similar *in vitro* platelet aggregation, *in vivo* ADP-induced platelet aggregation was significantly higher in the Zucker Diabetic Fatty rats compared with the non-diabetic rats (9). Significantly higher *in vivo* ADP-induced platelet aggregation was also reported in rabbits with STZ- and alloxan-induced diabetes (23), whereas in a model of FeCl₃-induced arterial injury, arterial thrombi were present in 64% and complete stable thrombotic occlusion developed in 58% of the diabetic mice, compared with only 28%, and 5% of the non-diabetic mice, respectively (24).

Platelet hyperreactivity in the setting of diabetes mellitus has been linked to a multitude of biochemical factors, including hyperglycaemia and hyperlipidaemia, insulin resistance, inflammation, or oxidative stress, and the impact of such factors on *in vitro* platelet aggregation has been extensively studied. However, the range of factors affecting platelet function is much larger, including blood rheology and hemodynamics, platelet-vessel wall interactions, or sympathetic tone. The impact of such factors, which cannot be assessed using currently available *in vitro* techniques, on platelet function, may explain, at least partially, this diabetes-related *in vivo* - *in vitro* platelet aggregation controversy. Shear stress has been shown to affect platelet adhesiveness and activation (25), and significantly higher shear-induced platelet adhesion and aggregation to the vascular endothelium have been reported in the setting of hyperglycaemia (26). Meanwhile, shear-independent parameters of

platelet function, such as *in vitro* aggregometry, or the expression of activation antigens on platelets circulating in the venous blood, were not affected by hyperglycaemia (26). Shear stress-induced thrombus formation on the tunica media of an injured vessel was also significantly higher when blood was obtained from diabetic patients (27). Moreover, sudden shear acceleration coupled with deceleration, as seen at sites of arterial atherosclerotic narrowing, has been shown to directly initiate platelet aggregation, independent of biochemical triggers, and to represent the main mechanism responsible for initial thrombus formation, whereas agonist release appears to intervene later and to be mainly responsible for stabilizing the previously formed platelet aggregates (28). This emerging concept may explain the limited efficacy of agonist inhibitors in patients with diabetes (29), in which extensive atherosclerotic disease creates shear microgradients. Sympathetic overactivity has also been demonstrated in patients with diabetes mellitus, particularly when associated with arterial hypertension (30), and adrenoceptor activation has been linked to increased platelet reactivity (31). In the study by Rauch *et al.*, diabetic cardiovascular autonomic neuropathy was associated with increased platelet activation (32). Meanwhile, adrenergic inhibition has been shown to attenuate diabetes-related platelet hyperreactivity (20).

Species differences obviously mandate caution in extrapolating results from experimental studies to human disease, and the possibility that decreased *in vitro* platelet aggregation is a feature of the diabetes mellitus models used in experimental studies and not of diabetes per se cannot be excluded. However, several *in vitro* studies on human diabetes have also failed to show increased platelet aggregation (33, 34). Given the obvious limitations of *in vitro* techniques (35), the pathophysiological relevance of *in vitro* platelet aggregation to the contribution of platelets to *in vivo* diabetes-related thromboembolic complications remains questionable. Mechanisms present exclusively *in vivo*, such as shear stress or sympathetic activity, could account for the paradox of high intrinsic platelet reactivity – low *in vitro* platelet aggregation – high *in vivo* thromboembolic risk seen in this setting. Such mechanisms may also explain the inconsistent ability of “laboratory resistance” to antiplatelets, defined as incomplete *in vitro* inhibition of platelet aggregation despite the use of oral antiplatelet drugs, to predict “clinical resistance” (i.e. ongoing thrombotic events despite antiplatelet therapy).

Potential limitations

There are several limitations to our study. Although major thromboembolic events were not observed in the present study, occurrence of microthrombosis was not assessed. In the present study, platelet aggregation was not assessed in the absence of plasma. However, previous studies have already demonstrated that larger platelets have greater agonist-induced aggregation. Also, since *in vivo* platelet function was not assessed in this study, the relevance of our *in vitro* findings to *in vivo* platelet activity could not be evaluated. However, previous studies have already demonstrated higher *in vivo* and *ex vivo* platelet aggregation in animals with STZ-induced diabetes mellitus. Assessing platelet function in diabetic rats treated with insulin and/or with antiplatelet agents would have also been of interest.

In conclusion, the present study demonstrates a paradox of high intrinsic platelet reactivity and low *in vitro* ADP- and AA-induced platelet aggregation in rats with STZ-induced diabetes mellitus compared with non-diabetic controls. These results suggest that platelet inhibitors present in the diabetic plasma may counterbalance, or even exceed the intrinsic platelet hyperreactivity, and that the amounts and/or the activities of such inhibiting factors could increase with longer duration of diabetes. It remains questionable whether *in vitro* platelet aggregation is an accurate reflection of *in vivo* platelet function in diabetics.

Conflict of interest

The authors declare that they have no conflict of interest.

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