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Reduced platelet response to aspirin in patients with coronary artery disease and type 2 diabetes mellitus

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ABSTRACT

Introduction: Diabetes mellitus is complicated by accelerated atherosclerosis, resulting in an increased risk of coronary artery disease (CAD) and thrombosis. Despite the proven benefits of aspirin, previous studies indicate a reduced cardiovascular protection from aspirin in diabetic patients. We aimed to investigate whether diabetes mellitus influenced the platelet response to aspirin in patients with CAD.

Materials and Methods: Platelet aggregation and activation were evaluated during aspirin treatment in 85 diabetic and 92 non-diabetic patients with CAD. Adherence to aspirin was carefully controlled. All patients had CAD verified by coronary angiography and were taking 75 mg non-enteric coated aspirin daily.

Results: Diabetic patients showed significantly higher levels of platelet aggregation compared to non-diabetic patients evaluated by VerifyNow® Aspirin ($p = 0.03$) and Multiplate® aggregometry using arachidonic acid (AA) 0.5 mM ($p = 0.005$) and 1.0 mM ($p = 0.009$). In addition, platelet activation determined by soluble P-selectin was significantly higher in diabetics compared to non-diabetics ($p = 0.005$). The higher AA-induced aggregation was associated with higher levels of HbA_{1c}. Compliance was confirmed by low levels of serum thromboxane B_2 (below 7.2 ng/mL). Diabetics had significantly higher levels of serum thromboxane B_2 ($p < 0.0001$).

Conclusions: Diabetic patients with CAD had significantly higher levels of both platelet aggregation and activation compared to non-diabetic patients with CAD despite treatment with the same dosage of aspirin. These findings may partly explain the reduced cardiovascular protection from aspirin in diabetic patients.

Introduction

Diabetes mellitus (DM) is complicated by an increased risk of coronary artery disease (CAD) and cardiovascular events [1–3]. Furthermore, diabetic patients without previous myocardial infarction (MI) carry a similarly high risk of developing acute MI as non-diabetic patients with previous MI [4].

Low-dose aspirin is recommended for patients with CAD and diabetes to prevent cardiovascular events [5,6]. Despite the proven benefits of aspirin in patients at risk of suffering vascular events [6], randomized controlled trials have failed to show a clear benefit of aspirin in diabetic patients with a non-significant reduction in cardiovascular events [7,8]. These findings have increased the focus on the effects of aspirin on platelet function and cardiovascular events in diabetic patients.

A high inter-individual variation in platelet response to aspirin has been reported and is referred to as aspirin resistance or aspirin low-responsiveness. Low-responsiveness is a controversial phenomenon, and the prevalence has been reported to vary from 0 to 60% depending on definition, study population, method and compliance [9]. It has been hypothesized that patients with an inadequate inhibition of platelet aggregation might have an increased risk of cardiovascular events [10–13]. This problem may be particularly prominent in diabetic patients.

We aimed to compare platelet aggregation and activation in response to aspirin among type 2 diabetic patients with stable CAD versus non-diabetic CAD patients.

Materials and Methods

Study population

177 patients with stable CAD (85 diabetics and 92 non-diabetics) were recruited from the Western Denmark Heart Registry from November 2007 to April 2008.

Abbrevations: AA, Arachidonic acid; ARU, Aspirin reaction units; AUC, Area under the curve; CAD, Coronary artery disease; COX-1, Cyclooxygenase-1; DM, Diabetes mellitus; HbA_{1c}, Haemoglobin A_{1c}; MI, Myocardial infarction; TxA_2, Thromboxane A_2; TxB_2, Thromboxane B_2; sP-selectin, Soluble P-selectin.

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The hypothesis was that diabetic patients did not differ from non-diabetic patients as regards aspirin response determined by platelet aggregation using the VerifyNow® Aspirin Assay. From previous studies we estimated the mean ARU to be around 440 ARU and the standard deviation to be 30 ARU [11]. The MIREDIF was chosen to be 15 ARU, and the level of significance to be 5% (2alfa) and the power to be 90% (1-beta). This required 85 participants in each group (diabetics and non-diabetics, respectively).

Patients were eligible for the study if they were 18 years or older and had significant CAD verified by coronary angiography. All diabetic patients had been diagnosed with type 2 diabetes and were treated with oral hypoglycaemic agents and/or insulin. All non-diabetic patients had fasting plasma glucose <7 mmol/L at the time of inclusion. Patients were excluded if they met any of the following criteria: treatment with any drug with known effect on platelet function (e.g. clopidogrel, NSAID, ticlopidin, dipyridamol) or warfarin, previous ischaemic vascular events, percutaneous coronary intervention or coronary artery bypass grafting within the previous 12 months or platelet count $<120 \times 10^9$/L.

All patients were taking 75 mg aspirin tablets (Hjerdyl®, Sandoz, Denmark) daily for at least 7 days prior to blood sampling. To optimize compliance and uniform pharmacokinetics each patient received a package with seven tablets of 75 mg non-enteric coated aspirin. All patients ingested 75 mg of aspirin 1 hour before blood sampling. Compliance was optimized by face-to-face interviews, pill-counting and confirmed by measurements of serum TxB_2.

The study was conducted in agreement with the Helsinki-II-declaration and approved by The Central Denmark Region Committees on Biomedical Research Ethics (M-20070180). All participants gave written informed consent.

Laboratory investigations

Blood samples were obtained from the antecubital vein with patients in supine position after 30 minutes of rest using vacuum tubes, a large bore needle (19 G) and a minimum of stasis.

VerifyNow® Aspirin and Multiplate® aggregometry

Tubes containing 3.2 % sodium citrate (Terumo, Leuven, Belgium) were used for platelet aggregation analyses by the VerifyNow® Aspirin assay and the Multiplate® aggregometer. The tubes were filled to capacity and gently inverted 5 times to ensure mixing with the anticoagulant. Blood samples rested for at least 30 minutes but no longer than 2 hours before analysis.

TheVerifyNow® Aspirin system (Accumetrics, San Diego, CA, USA) is based on turbidimetric optical detection to measure platelet aggregation. Analysis was performed by inserting the tubes with whole blood into the system containing fibrinogen-coated beads and 1.0 mM arachidonic acid (AA) [14,15]. Platelet aggregation was expressed as Aspirin Reaction Units (ARU) [15,16]. The manufacturer recommends a cut-off at ≥550 ARU to classify patients as aspirin low-responders [14].

The Multiplate® aggregometer (Dynabyte, Munich, Germany) is based on impedance aggregometry. We used AA in final concentrations of 0.5 and 1.0 mM, and collagen (Horm collagen, Nycomed) in final concentration 1.0 μg/mL as agonists. Analysis was performed by mixing anticoagulated whole blood (300 μL) and preheated saline (270 μL, 37 °C). After 3 minutes of incubation, the measurements were initiated by adding an agonist (30 μL). The test time was 6 minutes. The area under the aggregation curve (AUC) expressed the aggregation response during the test time (AU*min) [17]. Weisser et al [18] have suggested a cut-off at ≥300 AUC using the Multiplate® aggregometer with 0.5 mM AA to classify patients as aspirin low-responders.

Soluble P-selectin

Non-siliconized 5 mL glass tubes without anticoagulants (Terumo, Europe) were used for measurements of soluble P-selectin in serum (sP-selectin). The tubes were allowed to clot for 30 minutes at room temperature before serum was separated by centrifugation at 1500 *g* for 15 minutes. Serum was stored at -80 °C before analysis. Soluble P-selectin concentrations were determined by an ELISA according to manufacturer's instructions (R&D systems, MN, USA [19]).

Serum thromboxane B_2

Non-siliconized 5 mL glass tubes without anticoagulants (Terumo, Europe) were used for measurements of serum TxB_2. The tubes were allowed to clot for one hour at 37 °C before serum was separated by centrifugation at 2600 *g* for 10 minutes. Serum was stored at -80 °C for 2-4 months before analysis. Serum TxB_2 concentrations were measured according to Patrono et al. [20] with the modification that serum was collected after 1 hour of clotting, and serum TxB_2 was measured by ELISA (Cayman Chemical, MI, USA [21]).

Statistical analysis

All data were checked for normality and equality of variances. Continuous data are represented as mean and standard deviation (SD) if data were normally distributed. If not, data are presented as median and percentiles or log-transformed to obtain normal distribution. For data approximating a normal distribution, a two-sided t-test was used to test the difference between two unpaired groups. For data that were not normally distributed, the Mann-Whitney test was used for comparison of two unpaired groups. To test differences in proportions between two or more groups we used Fisher's exact test or the Chi-square test. Pearson´s correlation was used to test for correlation between normally distributed data. A two-sided p-value<0.05 was considered statistically significant. Graphs are presented with mean bar if normally distributed and median bar if not.

Data were registered in Epidata version 3.1 (EpiData Association, Denmark). Statistical analyses were performed using GraphPad Prism® version 5.0 (GraphPad Software, CA, USA) and R version 2.6.1 [22].

Results

Clinical characteristics of the study population are shown in Tables 1 and 2. Patients with diabetes were significantly older than non-diabetics. However, the age difference was only 3 years. The prevalence of previous MI was high among both non-diabetic (66%) and diabetic patients (65%), demonstrating that our study population was a group of high-risk CAD patients. More diabetic patients had undergone by-pass surgery compared with non-diabetic patients (31 vs. 16 %, p = 0.02).

Pill counting and face-to-face interviews did not reveal any non-compliant patients, and optimal compliance was confirmed by serum TxB_2 levels below 7.2 ng/mL in all patients [23].

Diabetic patients with CAD had significantly higher levels of platelet aggregation and activation than non-diabetic patients with

Table 1
Clinical and biochemical characteristics of the study population. Mean ± standard deviations are indicated.

Variables	Non-diabetics $n = 92$	Diabetics $n = 85$	*p*-value
Age, years	64 ± 8	67 ± 8	0.0053
Female, *n* (%)	16 (17)	15 (18)	0.96
Body Mass Index, kg/m^2	27 ± 4	30 ± 4	0.001
Systolic blood pressure, mmHg	143 ± 21	146 ± 23	0.46
Diastolic blood pressure, mmHg	87 ± 12	83 ± 12	0.04
Current smokers, *n* (%)	23 (25)	18 (21)	0.60
Time since last aspirin intake, min	77 ± 35	81 ± 49	0.51
B-Leukocytes, 10^9/L	6.7 ± 1.6	7.9 ± 2	< 0.0001
B-Haemoglobin, mmol/L	8.9 ± 0.7	8.6 ± 0.8	0.007
B-Platelet count, 10^9/L	233 ± 49	238 ± 64	0.55
P-Creatinine, μmol/L	84 ± 41	92 ± 31	0.12
B-Haemoglobin A_{1c}, %	5.8 ± 0.4	7.4 ± 1.1	<0.0001

Table 2
Morbidity and medication of the study population.

	Non-diabetics $n=92$	Diabetics $n=85$	*p*- value
Morbidity	*n*(%)	*n* (%)	
Myocardial infarction	61 (66)	55 (65)	0.82
By-pass surgery	15 (16)	26 (31)	0.02
Percutanous coronary intervention	87 (95)	76 (89)	0.20
Stroke	9 (10)	8 (9)	0.93
Medication			
Statins	80 (87)	75 (88)	0.80
Beta-blockers	65 (71)	68 (80)	0.15
ACE inhibitors	34 (37)	41(48)	0.13
AT2 inhibitors	6 (7)	20 (24)	0.001
Calcium antagonists	19 (21)	24 (28)	0.24
Diuretics	15 (16)	36 (42)	0.0001
Proton pump inhibitors	5 (5)	12 (14)	0.05
Insulin	0 (0)	31 (36)	< 0.0001
Oral antidiabetica	0 (0)	68 (80)	< 0.0001

ACE: angiotensin-converting enzyme.
AT2: angiotensin 2.

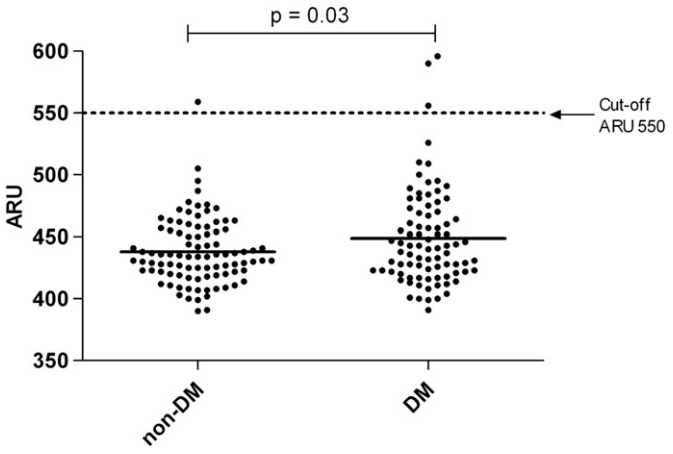

Fig. 1. Platelet aggregation in non-diabetic and diabetic patients assessed by VerifyNow® Aspirin. The dotted line indicates the cut-off level. Patients above this line were aspirin low-responders.

CAD despite treatment with the same dosage of aspirin. As illustrated in Table 3, these differences were significant and were seen both for evaluation of platelet aggregation by VerifyNow® Aspirin (Fig. 1), by Multiplate® aggregometry using AA as agonist (Fig. 2), as well as by platelet activation determined by sP-selectin and serum TxB_2. An association, though non-significant, was seen for platelet aggregation using collagen as agonist.

When comparing sP-selectin and AA-induced aggregation we found no significant correlation using VerifyNow® ($r=0.08$, $p=0.32$) and Multiplate® (0.5 mM AA) ($r=0.12$, $p=0.13$), respectively. A weak but significant correlation was found between serum TxB_2 and Multiplate® (0.5 mM AA) ($r=0.38$, $p=0.03$), but no significant correlation was observed between serum TxB_2 and VerifyNow® ($r=0.12$, $p=0.10$).

There was a significant, though weak, correlation between the level of haemoglobin A_{1c} (HbA_{1c}) and AA-induced aggregation using Multiplate® (0.5 mM AA) ($r=0.23$, $p=0.002$) as well as serum TxB_2 ($r=0.21$, $p=0.005$), and a weak non-significant correlation using VerifyNow® ($r=0.10$, $p=0.17$).

Fig. 1 shows that 4 patients were classified as aspirin low-responders using the recommended cut-off by VerifyNow®. Using a cut-off at ≥300 AUC, 2 patients were classified as aspirin low-responders by Multiplate® (Fig. 2). One patient was classified as aspirin low-responder by both VerifyNow® and Multiplate® (Table 4).

As illustrated in Table 4, a total of 5 (2.8%) patients were classified as aspirin low-responders by either VerifyNow® or Multiplate® using 0.5 mM AA. Among these, 3 patients were diabetics and 2 were non-diabetics. The 5 aspirin low-responders did not differ from the aspirin sensitive patients concerning HbA_{1c} (6.4 ± 0.5 vs. 6.6 ± 1.1, $p=0.76$), sP-selectin (ng/mL: 67 ± 30 vs. 72 ± 27, $p=0.36$) or serum TxB_2 (ng/mL: 0.5 (0.2; 2.4) vs. 0.7 (0.4; 1.5), $p=0.69$).

Table 3
Platelet aggregation and related serum metabolites in diabetic and non-diabetic patients.

Assay	Non-diabetics $n=92$	Diabetics $n=85$	*p*-value
VerifyNow® Aspirin, ARU: mean ± SD	438 ± 27	449 ± 39	0.03
Multiplate, AUC, median (25;75 percentiles)[a]			
AA, 0.5 mM	69 (36;104)	91 (52;144)	0.005
AA, 1.0 mM	120 (63;180)	159 (91;232)	0.009
Collagen, 1.0 μg/mL	264 (182;386)	281 (194;438)	0.26
Serum sP-selectin, ng/mL, mean ± SD	66 ± 28	78 ± 25	0.005
Serum TxB2, ng/mL, n=177, median (25;75 percentiles)	0.6 (0.4;0.9)	1.0 (0.5;2.2)	<0.0001

[a] Values missing for three patients.

Discussion

The main finding in this study was a significantly lower platelet response to aspirin in diabetic patients with CAD compared with non-diabetic CAD patients. This was investigated by AA- and collagen induced-platelet aggregation using the Multiplate® aggregometer and the VerifyNow® Aspirin and by platelet activation determined by sP-selectin as well as serum TxB_2. These findings may indicate the presence of more reactive and prothrombotic platelets in diabetic patients with CAD, and might partly explain the reduced cardiovascular protection from aspirin in diabetic patients [6–8].

Platelet aggregation was significantly higher in diabetic patients using the COX-1 dependent agonist AA and showed a trend when collagen was used as agonist. Our study population were on aspirin mono-therapy, therefore AA was the most relevant agonist [24].

Increased levels of sP-selectin has been suggested as a predictor of future cardiovascular events [25]. In accordance with previous studies [26–28] we found that diabetic patients with CAD had higher levels of sP-selectin during aspirin treatment compared with non-diabetic patients. Thus, diabetic patients may have more reactive platelets

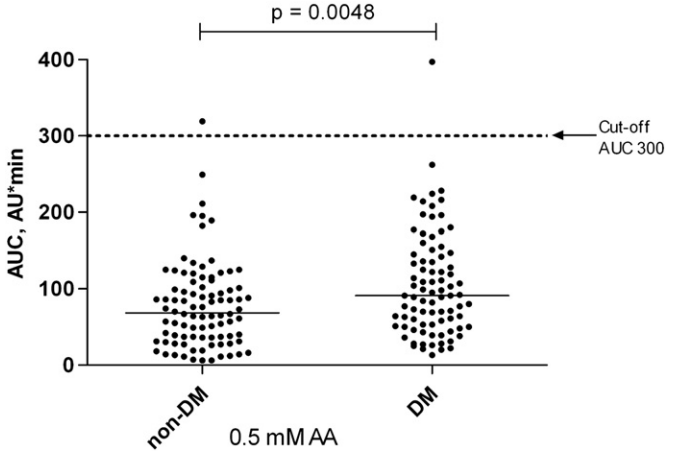

Fig. 2. Platelet aggregation in diabetic and non-diabetic patients assessed by the Multiplate® aggregometer using 0.5 mM AA as agonist. The dotted line indicates the cut-off level. Patients above this line were aspirin low-responders.

Table 4
Low-responders and responders to aspirin assessed by 0.5 mM AA Multiplate® and VerifyNow® Aspirin.

	VerifyNow® Aspirin	
Multiplate®	Responder	Low-responder
Low-responder	1	1
Responder	172	3

despite aspirin therapy and thereby an increased risk of future cardiovascular events.

Thromboxane A_2 (TxA_2) is a potent platelet activator and vasoconstrictor, and serum TxB_2 is a measure of maximum platelet capacity to produce TxA_2 [29]. Despite low levels of serum TxB_2 in all patients, we found significantly higher serum TxB_2 levels in diabetic patients compared with non-diabetic patients during aspirin therapy. The higher level of TxB_2 may indicate a reduced COX-1 inhibition by aspirin in diabetic patients. Alternatively, it may reflect a COX-2 source of TxA_2 production in response to inflammatory stimuli in diabetic patients [29,30]. We observed a significant, though weak, correlation between serum TxB_2 and AA-induced aggregation using Multiplate® aggregometry. Accordingly, it has previously been shown that even small amounts of uninhibited COX-1 may be important for induction of platelet aggregation [31].

Overall, our findings are in agreement with previous studies reporting a reduced response to aspirin [26,28,32–34] and increased prevalence of aspirin low-responders [32,35] in diabetic patients compared with non-diabetic patients.

Several mechanisms may explain the reduced response of diabetic platelets to aspirin. Hyperglycemia together with dyslipidaemia and insulin resistance in diabetic patients may all affect the response to antiplatelet drugs [3,36,37]. In accordance with previous studies we found that higher levels of glycated haemoglobin were related to higher AA-induced platelet aggregation [36] and higher levels of serum TxB_2 [34]. Hyperglycemia may cause a competition between acetylation and glycation of platelet proteins, thus making the acetylation of COX-1 less efficient and causing a reduced effect of aspirin in diabetic patients [3,36]. Diabetics might also have an increased platelet turnover and be hypersensitive to pro-aggregatory agonists causing an increased platelet activation and aggregation [38–40]. DiChiara et al. have suggested that higher doses of aspirin may overcome the reduced platelet response to aspirin observed in diabetic patients [32]. However, it remains uncertain whether the use of higher doses of aspirin in diabetic patients lead to a reduction in cardiovascular mortality [41].

A low response to aspirin may be clinically important. Two recent meta-analyses have reported an association between a reduced response to aspirin and an increased risk of cardiovascular events [42,43]. We identified 5 (2.3%) patients as aspirin low-responders using the recommended cut-off at ARU≥550 for the VerifyNow® Aspirin assay [14] and a cut-off at≥300 AUC for the Multiplate® aggregometer with 0.5 mM AA [18]. Previous studies reporting a higher prevalence (up to 60%) of aspirin low-responsiveness among patients with CAD have not used COX-1-dependent agonist like AA to assess platelet function [10] or did not report systematic control of compliance [10,42,44].

Optimizing compliance is crucially important when evaluating aspirin response and lack of confirmation of compliance may partly explain the differing estimates of low-responsiveness. We used optimal methods for ensuring compliance: face-to-face interviews, pill-counting and measurements of serum TxB_2, which is the most sensitive method for confirmation of compliance [24]. According to serum TxB_2 measurements, all patients in the present study were fully compliant. In studies with thorough compliance control, the prevalence of aspirin low-responsiveness among patients with CAD is only 0% -14% using VerifyNow® [11,32,35], whereas studies with poor or no evaluation [44,45] of compliance report a prevalence of aspirin low-responsiveness up to 27% using VerifyNow®. Obviously, there is a need for standardized methods to assess both platelet function and compliance.

Elevated levels of HbA_{1c} and serum TxB_2 in aspirin resistant patients have previously been reported [46,47]. However, in the present study, aspirin low-responsive patients did not differ from the aspirin sensitive patients with regard to HbA_{1c}, sP-selectin and serum TxB_2. Due to the low number of aspirin low-responsive patients, our results were not conclusive on this point.

The strengths of this study are inclusion of a study population of high-risk CAD patients with a large proportion of diabetic patients and thorough assessment of compliance. Yet, some limitations should be acknowledged including differences in baseline characteristics between groups. Assessment of platelet function at baseline before aspirin treatment was not performed, since we chose not to withdraw aspirin in high-risk CAD patients for ethical reasons. The low number of aspirin low-responsive patients caused a relatively low statistical power, when comparing aspirin low-responsive patients with aspirin sensitive patients.

The present study demonstrates a lower response to aspirin in diabetic patients with CAD compared to non-diabetic CAD patients, indicating that platelets are more reactive and less responsive to aspirin in diabetic patients. This may partly explain the reduced cardiovascular protection by aspirin in this patient group. Further studies are needed to investigate the relationship between low platelet inhibition and cardiovascular events in diabetic patients with CAD. Randomized clinical trials with diabetic patients should be undertaken to investigate whether anti-platelet therapy can be optimized in order to reduce the risk of cardiovascular events.

Conflict of interest statement

We confirm that there are no financial or other conflicts of interest for any of the authors related to the material contained in this manuscript.

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