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Inhibition of platelet aggregation by prostaglandin E1 (PGE1) in diabetic patients during therapy with clopidogrel and aspirin

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Abstract

Diabetes mellitus (DM) is associated with increased platelet activation and reduced platelet inhibition by clopidogrel. Prostaglandin E1 (PGE1) stimulates adenylyl cyclase activity in platelets and increases cyclic AMP concentrations, which inhibit Ca²⁺ release and platelet aggregation induced by P2Y1 receptor activation. PGE1 is included in the VerifyNow P2Y12 assay to suppress P2Y1 induced platelet aggregation. We hypothesized that diabetes mellitus may be associated with altered response to PGE1 in subjects treated with clopidogrel. Subjects with established coronary artery disease who were taking clopidogrel 75 mg daily and aspirin for >14 days were enrolled ($n = 96$). Diabetic ($n = 34$) were compared with non-diabetic subjects ($n = 62$). VerifyNow P2Y12 assay and light transmittance aggregometry (LTA) were performed using ADP as agonist with and without addition of PGE1. Genomic DNA was genotyped for common cytochrome P450 (CYP) 2C19 variants using Taqman assays. Residual on-treatment platelet aggregation induced by 20 μ M ADP was not significantly different between subjects with and without DM. Addition of 22 nM and 88 nM PGE1 to 20 μ M ADP resulted in a significant reduction of maximal platelet aggregation (MPA). Residual LTA platelet aggregation with PGE1 and VerifyNow P2Y12 platelet reactivity were significantly higher in subjects with DM than those without DM and in carriers of CYP 2C19*2 polymorphism. We conclude that an impaired inhibitory response to PGE1 may contribute to the high platelet reactivity phenotype in subjects with DM treated with clopidogrel. Addition of PGE1 to ADP agonist platelet assays may identify subjects with blunted inhibitory response to prostaglandins and result in a higher proportion of subjects with DM being classified as non-responders.

Keywords

Platelet; clopidogrel; aggregation; prostaglandin; diabetes

Introduction

High on treatment platelet reactivity during therapy with clopidogrel and insufficient platelet inhibition are associated with increased ischemic events after coronary stenting, and in particular stent thrombosis [1–5]. Platelet inhibition by clopidogrel is attenuated in carriers of cytochrome CYP 2C19 non-functional polymorphisms that are associated with reduced active clopidogrel thiol metabolite generation. Carriers of *2 non-functional alleles are at increased risk of adverse ischemic events and stent thrombosis after coronary stenting [5–9]. In addition to variable exposure to the active clopidogrel metabolite, other clinical variables have been demonstrated to influence on-treatment platelet aggregation. In particular, a blunted inhibitory response to clopidogrel has been demonstrated in subjects with diabetes mellitus [10–12], which is associated with spontaneous platelet activation, and increased sensitivity of platelets to agonists. Diabetes has also been demonstrated to be associated with increased production of platelet activating factor, thromboxane A₂, and reduced platelet NO synthesis [13, 14].

Prostaglandins are important mediators of inflammation and thrombosis. Prostaglandin E₁ (PGE₁) stimulates adenylyl cyclase activity and is a potent inhibitor of platelet aggregation *in vitro* [15, 16]. In specific, PGE₁ binds to prostacyclin receptor (IP) on platelets, raises intracellular cAMP which inhibits phospholipase C activation, and reduces Ca²⁺ mobilization from intracellular stores. PGE₁ thus antagonizes the effects of P2Y₁ receptor activation. Activation of P2Y₁, which is a Gq-coupled receptor linked to phospholipase C generates inositol 1,4,5-trisphosphate and is followed by release of Ca²⁺, resulting in platelet activation and aggregation [16, 17]. Due to these qualities, it is used in the VerifyNow point-of-care P2Y₁₂ platelet assay at a concentration of 22 nM along with ADP to suppress aggregation induced by P2Y₁ and measure P2Y₁₂ inhibition as previously described [18]. Diabetes mellitus is associated with altered response of platelets to prostacyclin *in vitro*, and also a blunted response to vasodilating properties of PGE₁ *in vivo* [14, 15, 19–21], which suggests that DM patients may be less sensitive to the inhibitory effects of PGE₁ *in vitro*.

In this study, we tested the hypothesis that diabetes mellitus may be associated with reduced response to PGE₁ in subjects treated with clopidogrel. Thus, addition of PGE₁ to assays measuring platelet inhibition by clopidogrel may differentially impact results in diabetics vs. non-diabetic patients.

Methods

Patients

The study protocol was approved by the Indiana University School of Medicine Institutional Review Board (IRB) for research. Informed consent was obtained from all subjects. Subjects were recruited if they had a history of coronary artery disease and were taking clopidogrel 75 mg and aspirin 81–325 mg daily. Subjects had to be at least 21 years of age. Subjects were excluded if they had a history of medication noncompliance as defined below, used greater than clopidogrel 75 mg daily or did not use the drug for 14 consecutive days before platelet testing. Other exclusions included drug or alcohol abuse, pregnancy, bleeding disorder, platelet count less than 150 000/mm³, myelodysplastic or myeloproliferative

disorders, concomitant dipyridamole or warfarin use, chronic liver disease (hepatic transaminases greater than or equal to 3xULN), or renal disease (serum creatinine greater than 2.0 mg/dl). Compliance with clopidogrel and aspirin during the 14 days preceding the blood draw was ascertained by review of a medication logbook provided by the study subjects. Because the recruited subjects were known to have cardiovascular disease requiring dual anti-platelet pharmacotherapy, baseline predrug platelet aggregation studies were not performed.

Blood samples

Phlebotomy was performed on day 15, 24 hours after the last dose of clopidogrel and aspirin. Blood was obtained on all 96 subjects from an antecubital or forearm vein using a 21 gauge needle with vacutainer collection tubing. The first 4 ml of blood drawn was discarded to avoid spontaneous platelet activation. Whole blood was collected in 3.2% sodium citrate tubes. VerifyNow P2Y12 assay was completed within 30 minutes after blood draw but no sooner than 10 minutes according to testing instructions, and LTA was completed within 2 hours of the blood draw.

Platelet aggregation studies

Ex vivo platelet function was assessed by light transmittance aggregometry (LTA) at 37°C with an Optical Lumi-Aggregometer (Model 700 with AggroLink 8 software, Chrono-Log Corporation, Havertown, PA, USA). Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by differential centrifugation as previously described [22, 23]. Platelet aggregation in PRP was induced with 20 µM adenosine diphosphate (ADP). For the PGE1 inhibitory LTA studies, PRP was incubated for 1 minute with PGE1 [(11α,13 E,15 S)-11,15-Dihydroxy-9-oxoprost-13-enoic acid, Alprostadil, Sigma-Aldrich, USA] at a final concentration of 22 nM and 88 nM, prior to performance of LTA with ADP 20 µM.

VerifyNow™(VN) P2Y12 (Accumetrics Inc., San Diego, CA, USA) point-of-care assay was used to assess platelet inhibition in whole blood in subjects during maintenance clopidogrel dosing as previously described [24].

Genotyping of CYP450 variants

Genomic DNA was isolated from whole blood using Qiagen's QIAamp® DNA Blood Midi Kit (Germantown, MD, USA). Subjects were genotyped for *CYP2C19**2 (681 G>A; rs4244285), and *CYP2C19**3 (636 G>A; rs4986893) SNPs using a Bio-Rad Laboratories real-time PCR system. Sequence specific primers were used to amplify the alleles of interest, along with two allele-specific TaqMan probes (Applied Biosystems, Foster City, CA, USA). Allelic discrimination was used to determine individual genotypes (Optical System 3.1 software, Bio-Rad Laboratories).

Statistical Analysis

Categorical variables were compared using the χ^2 test. Normal distribution of continuous data was assessed by the Kolmogorov–Smirnov test. Unpaired two-sided Student's *t*-test was used to compare normally distributed continuous data between two groups. Non-parametric continuous data were compared across two groups with the two-sided unpaired Wilcoxon

rank sum test. Correlation between VN P2Y₁₂ platelet reactivity and ADP LTA was tested using Pearson's correlation coefficient. Spearman's correlation coefficient was performed to test for correlations between PGE₁ and ADP LTA with other measures of platelet aggregation. All tests were conducted two-sided and values are represented as mean±SD unless otherwise specified. Statistical significance was defined as $p < 0.05$.

Results

Baseline characteristics of diabetic and non-diabetic study subjects are described in Table I. Overall clinical variables were well balanced between subjects with and without DM.

Among the 96 patients included in this study, 68 (71%) were wild-type homozygous for the *2 allelic variant (wt/wt), 24 (25%) were heterozygous *2 allele carriers (wt/*2), and four patients (4%) were homozygous *2 allele carriers (*2/*2). For genotype distributions, no significant deviations from Hardy–Weinberg equilibrium were observed ($p = 0.32$ for CYP2C19*2 genotypes). There were no CYP2C19*3 alleles detected in the study population which is consistent with the very rare frequency (<1%) of this loss-of-function allele in Caucasians and African-Americans.

Platelet aggregation induced by ADP demonstrated wide interindividual variability during therapy with clopidogrel as previously documented (Tables II and III; and Figures 1 and 2).

There was no statistically significant difference in platelet aggregation induced by 20μM ADP between subjects with and without diabetes mellitus ($46.9 \pm 12\%$ vs. $45.2 \pm 12\%$; $p = 0.51$) (Table II, Figure 1). However, the VNP2Y₁₂ platelet reactivity was significantly higher in patients with DM (231.1 ± 69 vs. 192.4 ± 78 PRU, $p = 0.018$) (Table II). Addition of PGE₁ 22nM and 88nM to 20μM ADP during light transmission aggregometry resulted in significant inhibition of platelet aggregation in both diabetic and non-diabetic patients in a dose dependent fashion. Patients with diabetes demonstrated significantly higher residual aggregation as compared to non-diabetics (PGE₁ 22nM: $24.5 \pm 15\%$ vs. $18.4 \pm 13\%$; $p = 0.038$; PGE₁ 88nM: $12.1 \pm 11\%$ vs. $6.8 \pm 8\%$; $p = 0.01$) (Table II, Figure 1)).

CYP 2C19 *2 carrier status was associated with significantly higher on-treatment platelet aggregation induced by 20μM ADP, as well as higher VNP2Y₁₂ platelet reactivity (Table III).

VerifyNow platelet reactivity (PRU) correlated well with 20μM ADP induced platelet aggregation by LTA ($r = 0.72$; $p < 0.00001$). VerifyNow platelet reactivity (PRU) also correlated well with 20μM ADP induced LTA with 22nM PGE₁ ($r = 0.62$; $p < 0.00001$) and 88nM PGE₁ ($r = 0.59$; $p < 0.00001$) (Figure 2).

Discussion

Diabetes mellitus is an important risk factor for coronary artery disease and associated with disease progression and higher rates of recurrent ischemic events after coronary stenting. It has long been recognized that diabetes mellitus is associated with platelet activation and higher platelet reactivity during antiplatelet therapy, and this association has been in support

of higher thrombotic events observed in patients with diabetes mellitus [1, 4, 10–12]. Multiple studies examining platelet inhibition during therapy with clopidogrel have demonstrated higher on-treatment platelet reactivity in diabetics as compared to non-diabetics [4, 10–12]. This phenomenon has been documented using several modalities of platelet reactivity testing during therapy with clopidogrel including light transmission aggregometry, the vasodilator-stimulated phosphoprotein assay (VASP) and the point of care device VerifyNow [4, 10–12, 25]. The VNP2Y12 assay includes ADP as agonist along with PGE1 with the intention to suppress platelet aggregation induced by activation of P2Y1 and measure residual activity related to activation of P2Y12. In the VASP assay, PGE1 is used to increase cAMP and calculate the P2Y12 reactivity ratio [25]. In our study platelet aggregation induced by ADP alone was not statistically different between diabetics and non-diabetics during clopidogrel maintenance therapy with slight trend towards higher residual activity in diabetics. After addition of PGE1 at concentrations at or above the one used in the VN device, significantly higher residual platelet reactivity was observed in diabetics compared to non-diabetic subjects. Similarly, VN platelet reactivity was higher in subjects with diabetes mellitus as compared to non-diabetic subjects. These findings may suggest a blunted inhibitory response of platelets from diabetics to PGE1, a finding that has previously been documented in platelets from diabetics subjects without antiplatelet therapy [15, 19, 21]. These previously published findings support an altered response of diabetic platelets to prostacyclin and reduced vasodilator response to prostaglandins. Thus, inclusion of PGE1 in assays that examine residual platelet reactivity during dual antiplatelet therapy with P2Y12 inhibitors may potentially lead to a larger proportion of individuals with blunted inhibitory response to PGE1 to be classified as non-responders. This may also lead to a larger proportion of diabetic subjects being classified as non-responders if indeed a blunted inhibitory response to PGE1 is generally found in subjects with diabetes mellitus during dual antiplatelet therapy.

Because P2Y12 antagonists reduce the ability of ADP to lower cAMP, it has been suggested that P2Y12 antagonists amplify the platelet inhibitory response of naturally occurring platelet inhibitors such as PGE1 and PGI2 that act by raising cAMP [16, 26]. Both Iyu and Cattaneo et al. demonstrated that cangrelor, a selective P2Y12 inhibitor, potentiates the antiaggregatory effects of PGI2 in response to thrombin or TRAP [26, 27]. Addition of PGE1 to P2Y12 receptor inhibition results in a synergistic effect on inhibition of Ca^{2+} release and subsequent platelet aggregation, a synergism that may be especially important at low levels of PGE1 [16].

Recently, Iyu et al published data suggesting that PGE1 inhibits platelet aggregation via its interaction with IP receptors and promotes platelet aggregation through activation of EP3 receptor [28]. Antagonists to EP3 or agonists to IP receptors may thus be potential targets for antithrombotic drug development in the future.

Consistent with findings from other investigators, CYP 2C19 *2 carrier status was associated with increased on-treatment platelet reactivity during therapy with clopidogrel by both LTA and VNP2Y12 assay [5–9, 29, 30]. Since the distribution of CYP2C19*2 genotypes was not significantly different among subjects with and without diabetes, it is unlikely to be responsible for the difference in platelet response observed with PGE1.

Limitations of our study include a relatively small sample size and lack of pharmacokinetic analysis to detect differences in active metabolite concentrations. In addition, adherence to daily clopidogrel administration through use of a drug diary cannot be completely ascertained.

In conclusion, our study confirms previous observation about the impact of reduced function *CYP2C19**2 polymorphism on platelet inhibition by clopidogrel. The findings of our study suggest a blunted inhibitory response of platelets to PGE1 during dual antiplatelet therapy with clopidogrel and aspirin in subjects with diabetes mellitus.

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Figure 1. Light transmittance aggregometry induced by ADP 20μM with and without addition of PGE1 at 22nM and 88 nM in subjects with and without diabetes mellitus. Values represent mean±SEM.

Figure 2.

Scatterplots of VerifyNow (VN) P2Y12 platelet reactivity (PRU) vs. maximal platelet aggregation (MPA) induced by ADP 20 μ M (Panel a), VN P2Y12 PRU vs. MPA induced by ADP 20 μ M and PGE1 88nM (Panel b), VN P2Y12 PRU vs. MPA induced by ADP 20 μ M and PGE1 22nM (Panel c), and MPA induced by ADP 20 μ M vs. MPA induced by ADP 20 μ M and PGE1 88 nM (Panel d) in subjects with () and without (◇) diabetes mellitus.

Table I

Diabetes mellitus and platelet aggregation.

Clinical variables	Subjects with DM (<i>n</i> = 34)	Subjects without DM (<i>n</i> = 62)	<i>p</i> -value
Age (years)	58 ± 8.4	55.3 ± 10	0.19
Body mass index (kg/m ²)	34 ± 6.8	31.5 ± 6.7	0.09
Male gender (%)	19/34 (55.9)	32/62 (51.6)	0.69
Prior myocardial infarction (%)	29/34 (85.3)	50/62 (80.6)	0.57
Coronary artery bypass grafting (%)	4/34 (11.8)	11/62 (17.7)	0.44
Peripheral vascular disease (%)	9/34 (26.5)	10/62 (16.1)	0.22
Hypertension (%)	34/34 (100)	58/62 (93.5)	0.13
Hyperlipidemia (%)	34/34 (100)	62/62 (100)	0.99
Current smoking (%)	14/34 (41.1)	35/62 (56.5)	0.15
Family history of CAD	8/26 (30.8)	16/62 (25.8)	0.81
Proton pump inhibitor use (%)	9/34 (26.5)	20/62 (41.9)	0.55
CYP 2C19 genotype (%)			
*1/*1	23/32 (71.9)	45/62 (72.6)	
*1/*2	10/32 (31.3)	14/62 (22.6)	
*2/*2	1/32 (3.1)	3/62 (4.8)	0.71

Table II

CYP 2C19 *2 genotype status and platelet aggregation.

	ADP 20 μ M (MPA)	ADP 20 μ M & PGE1 22 nM (MPA)	ADP 20 μ M & PGE1 88 nM (MPA)	VN P2Y12 reactivity (PRU)
DM	46.9 $\pm$ 12 %	24.5 $\pm$ 15 %	12.1 $\pm$ 11 %	231.1 $\pm$ 69
No DM	45.2 $\pm$ 12 %	18.4 $\pm$ 13 %	6.8 $\pm$ 8 %	192.4 $\pm$ 78
<i>p</i> -value	0.51	0.038	0.01	0.018

Table III

CYP 2C19 *2 genotype status and platelet function.

	ADP 20 μ M (MPA)	ADP 20 μ M & PGE1 22 nM (MPA)	ADP 20 μ M & PGE1 88 nM (MPA)	VN P2Y12 reactivity (PRU)
CYP 2C19 wt/wt	43.6 $\pm$ 12%	17.7 $\pm$ 13%	6.7 $\pm$ 13%	195 $\pm$ 78
CYP 2C19 *2 carriers	51 $\pm$ 11%	27.9 $\pm$ 14%	13.9 $\pm$ 14%	234.6 $\pm$ 67
<i>p</i> -value	0.005	0.002	0.01	0.017