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Determinants of subacute response to clopidogrel: relative impact of CYP2C19 genotype and PGE_1/adenylate cyclase signalling

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ABSTRACT

Background: and Hypotheses: The signal transduction pathway modulated by activation or blockade of platelet $P2Y_{12}$ receptors is linked to PGE_1-stimulated adenylate cyclase effects, but this link's impact on $P2Y_{12}$ receptor antagonist response is uncertain. We therefore tested the hypothesis that pre-treatment platelet responsiveness to PGE_1 predicts subsequent responsiveness to clopidogrel.
Methods: In order to maximise heterogeneity of platelet responsiveness to PGE_1 we investigated both healthy subjects (n = 30) and patients with CHD undergoing elective coronary stenting (n = 22), all genotyped for common CYP2C19 variants associated with clopidogrel sensitivity (CS). We determined baseline pre-clopidogrel platelet sensitivity to the inhibitory effects of PGE_1 by ADP-induced whole blood aggregation. Clopidogrel was administered for 7 days utilising a weight-based regimen. CS was expressed as change (Δ) in ADP-induced aggregation and in VASP-phosphorylation (VASP-P). We used univariate and multivariate analysis to correlate such parameters with PGE_1 sensitivity, BMI and presence/absence of CHD.
Results: In the study cohort, pre-treatment responsiveness to PGE_1 varied widely (70 ± 28 [standard deviation (SD)]% inhibition of aggregation: range 10 to 100%). In the entire study cohort, pre-treatment PGE_1 sensitivity correlated with CS irrespective of genotype. On univariate analysis, CS was not significantly greater for patients without than those with loss-of-function mutations. Moreover, at multivariate analysis, PGE_1 sensitivity, but not genotype, was a strong correlate of ΔADP and ΔVASP-P ($P < 0.0001$ for both).
Conclusions: The integrity of the cAMP pathway is a major determinant of subacute CS.

Introduction

The $P2Y_{12}$ receptor antagonist clopidogrel has a proven role in the prevention of adverse events in acute coronary syndromes and stent thrombosis [1,2]. However, some patients still develop thrombosis despite compliance with clopidogrel therapy [3–5]. Part of these treatment failures have been ascribed to impaired clopidogrel sensitivity (CS) [6], also described as "high-on-treatment platelet reactivity" or, in clinical jargon, "clopidogrel resistance". Response to clopidogrel may be estimated via changes in ADP-induced aggregation (ΔADP) or changes in phosphorylation of vasodilator-stimulated phosphoprotein (ΔVASP-P). However it is known that VASP-P at Ser-239 occurs primarily in response to nitric oxide (NO) rather than PGE_1 activation [7]. Many investigations have suggested that impaired CS is engendered primarily by genetically determined impairment of clopidogrel bioactivation [8,9]. However, determinants of CS are multiple and still incompletely defined. The previously described risk factors for impaired CS, including cytochrome P450 (CYP) 2C19 loss-of-function genotype [9–12], have recently been shown to explain only a fraction of these findings [13–15]. The incidence of impaired CS is higher in individuals with coronary heart disease (CHD) [16,17], diabetes [18–21] and obesity [18,22] compared with healthy subjects, and these differences evidently cannot be the result of genotype alone [6].

The $P2Y_{12}$ receptor is linked, via G_i protein, to adenylate cyclase, inducing effective inhibition of cAMP mediated negative control over platelet aggregation [23]. Blockade of $P2Y_{12}$ receptors by clopidogrel therefore in principle restores this negative control (reviewed [24]). A number of prostanoids, including prostacyclin and prostaglandin E_1 (PGE_1), function as physiological activators of adenylate cyclase. Furthermore, PGE_1 has previously been shown to potentiate the effects of $P2Y_{12}$ inhibitors [25,26]. The integrity of adenylate cyclase signalling is impaired in patients with stable and unstable angina [27]. We therefore tested the hypothesis that the integrity of the PGE_1/cAMP pathway is a genotype-independent correlate of CS.

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Methods

Subject/Patient Selection

The study was performed within the Cardiology Unit, Queen Elizabeth Hospital, Adelaide with subjects recruited between September 2011 and January 2013. Patient selection criteria were: (1) known CHD; and (2) intention to perform PCI with associated stent insertion and initiation of clopidogrel therapy. A cohort of adults without any history of symptomatic CHD were also evaluated. For all subjects exclusion criteria were: (1) currently taking, or prior adverse reaction to clopidogrel or other $P2Y_{12}$ antagonists; (2) concomitant therapy with oral anticoagulants; (3) current regular treatment with non-steroidal anti-inflammatory drugs excluding aspirin; (4) known bleeding diathesis.

Normal subjects were recruited by local advertisement. Selection criteria were: age >35 years (to correspond to the anticipated age of patients); and the absence of known CHD.

In order to minimise the impact of heterogeneous patient weight on response to clopidogrel, a weight-adjusted dosing regimen was utilised with daily doses of either 75 or 150 mg. Patients/subjects with extreme weights, (<45 kg or >160 kg) were excluded.

The protocol was approved by the institutional Human Research Ethics Committee and informed consent was obtained prior to study entry.

Protocol

CYP2C19 Genotyping

In order to identify individuals with loss-of-function mutations, buccal smears were obtained at entry. The CYP2C19 genotype protocol used specific polymerase chain reactions (PCR) followed by single base extension assays that was read on a MALDI-TOF mass spectrometer (Sequenom MassARRAY San Diego, CA). The following alleles were identified: CYP2C19*1, *2, *3 and *17. Analysis of genotype was performed with extensive and ultrarapid genotypes considered "good metabolisers" of clopidogrel (*1/*1; *1/*17; *17/*17); with all other combinations containing loss-of-function genes excluded from the "good metaboliser" group (*1/*2 and *2/*17). DNA extraction and genotype identification via PCR was performed by Healthscope Laboratories, Melbourne, Australia.

Pre-clopidogrel Assessment of Platelet Physiological Status

Prior to initiation of clopidogrel therapy and potential stent insertion, blood was obtained from fasting patients/subjects via a femoral venous line or an antecubital vein, and utilised for the following investigations:

(i) determination of ADP-induced aggregation;
(ii) inhibition of ADP-induced aggregation by PGE_1 and sodium nitroprusside (SNP);
(iii) quantitation of VASP phosphorylation (VASP-P).

Furthermore, complete blood examination, blood glucose level and plasma insulin level were routinely obtained from these fasting patients/subjects at baseline.

Clopidogrel Dosing Regimen

After completing baseline investigations, clopidogrel therapy was initiated according to a weight-based regimen. Patients/subjects weighing 45–80 kg received a loading dose of 600 mg clopidogrel followed by 75 mg daily, while patients weighing >80 kg received the same loading dose, but a maintenance dose of 150 mg. This regimen was continued for 7 days.

Assessment of Clopidogrel Effect

Follow-up evaluation was performed 7 days post-initiation of clopidogrel therapy. Platelet aggregometry and evaluation of VASP-P were repeated. Clopidogrel effect was expressed as:

a) ΔADP, i.e. percent inhibition of ADP-induced aggregation when taking clopidogrel compared with baseline ADP-induced aggregation.
b) ΔVASP-P, i.e. difference between pre-clopidogrel VASP-PRI and VASP-PRI on clopidogrel.

Whole Blood Platelet Aggregometry

Whole blood aggregometry was performed utilising a Chrono-log model 700 CA (Chronolog, Havertown, PA, USA) as previously described [28]. Baseline aggregation responses to both 2.5 μM and 5 μM ADP were determined. Inhibition of platelet aggregation by PGE_1 and SNP were assessed by adding 30 nM PGE_1 and 10 μM SNP 1 minute prior to ADP. Inhibition of aggregation was calculated as percent reduction relative to the effects of ADP alone. Inhibition of aggregation by clopidogrel was expressed as percent reduction in ADP response.

Quantitation of VASP-P

VASP-P analysis was performed using a commercial kit (Biocytex, France), with flow cytometry performed using a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Platelet reactivity index (PRI) was calculated as previously described [29]. Testing was performed within 24 hours of blood collection, with the majority of samples processed within 4 hours. VASP phosphorylation was detected at Ser-239 using the 16C2 antibody.

Statistical Methods

1. Clopidogrel effect (measured as ΔADP or ΔVASP-P) was correlated with the dose-per-unit weight via linear regression, while extent of response for subjects with and without loss-of-function mutations was compared utilising non-paired Wilcoxon rank sum test for ΔADP and non-paired T-test for ΔVASP-P.
2. Evaluations of responses to PGE_1 and SNP at baseline were utilised to perform linear regression analyses comparing these responses and subsequent responses to clopidogrel therapy.

Table 1
Participant Demographics.

	Healthy subjects		CHD	
Genotypes*	All (n = 30)	Good metaboliser (n = 18)	All (n = 22)	Good metaboliser (n = 15)
Median age (years)	49	44	64	64
Age range (years)	37-74	40-63	48-85	48-83
Gender (M:F)	17:14	12:6	13:9	8:7
Received double dose clopidogrel (n)	15	8	7	4
BMI median (kg/m²)	27.5	24.1	26.6	25.7
BMI range (kg/m²)	17.4-43.3	17.4-33.3	18.7-49.4	18.7-49.4
PPIs	3%	0%	23%	26%
Fat soluble statins	3%	0%	27%	13%
CCBs	3%	0%	55%	53%
Current smoker	3%	0%	18%	13%
T2DM	3%	0%	9%	20%
BMI ≥ 30 (kg/m²)	26%	11%	32%	33%

PPI = Proton pump inhibitor.
BMI = Body Mass Index.
T2DM = Type 2 diabetes mellitus.
CCB = calcium channel blocker.
* Loss-of-function mutations CYP2C19*1/*2 (n = 15) and CYP2C19*2/*17 (n = 4).