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Post receptor determinants of acute platelet response to clopidogrel in patients with symptomatic myocardial ischemia

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ABSTRACT

Background: Clopidogrel resistance is more common in patients with loss-of-function CYP2C19 genotypes. Since adenylate cyclase (AC) and soluble guanylate cyclase (sGC) pathways are variably impaired in patients with ischaemic heart disease, we tested the relevance of these determinants in patients undergoing acute loading with clopidogrel (600 mg) prior to non-emergent coronary stenting.
Methods: Inhibitory effects of prostaglandin E_1 (PGE_1, an AC activator) and sodium nitroprusside (NP, a sGC activator) on platelet aggregation were determined at baseline and compared with platelet responses to clopidogrel (4 h after administration) assessed as ΔADP, and Platelet Reactivity Index (ΔPRI). Data were analysed according to CYP2C19 genotype.
Results: In patients without loss of function mutations ($n = 18$), ΔADP but not ΔPRI, was directly correlated with baseline PGE_1 responsiveness ($r_s = 0.62$, $p = 0.005$)). NP responsiveness did not predict ΔADP. However there was no relationship between clopidogrel responses and either PGE_1 or NP responsiveness in patients with loss of function mutations. Multivariate correlates of clopidogrel response were both the genotype status ($\beta = -0.609$, $p < 0.001$) and the baseline response to PGE_1 ($\beta = 0.303$, $p = 0.03$).
Conclusions: While genetically impaired bio-activation markedly limits acute (4 h) clopidogrel response, impaired AC signalling provides an additional cause for clopidogrel resistance.

1. Introduction

Effective antiplatelet therapy plays a critical role in the prevention of coronary stent thrombosis [1], and in the management of acute coronary syndromes [1]. Agents such as clopidogrel, which inhibit binding of ADP to platelet $P2Y_{12}$ receptors, have proved particularly valuable in this context [2]. However several studies have shown that there is marked inter-individual variability in the response to clopidogrel [3–5], and that impaired clopidogrel response is associated with poor clinical outcomes [6,7]. Enzymatic bio-activation of clopidogrel is mediated by the cytochrome P450 system, especially cytochrome P450 (CYP) 2C19. Genotypes associated with reduced bio-activation are the CYP2C19*2*2 homozygosity and the CYP2C19*1*2 heterozygosity [8,9]. In such cases, reduced function of CYP2C19 leads to impaired bio-activation, therefore predisposing to reduced platelet responsiveness to clopidogrel ("clopidogrel resistance").

While there is no doubt that loss-of-function mutations of CYP2C19 contribute to clopidogrel "resistance" [8], genotype differences may account for only part of the variability in platelet responses to clopidogrel [10]. Furthermore epidemiological data suggest that other non-genetic factors may be involved in determining variability in the platelet response to clopidogrel, including ischemia [11,12], obesity [13–15], diabetes mellitus [16,17], chronic renal insufficiency [18,19] and smoking status [20,21]. While the mechanisms underlying the association of these factors with clopidogrel "resistance" remain uncertain, their very existence should stimulate interest in other sources of variability in clopidogrel antiplatelet effects.

Through the generation of an active metabolite, clopidogrel antagonizes binding of ADP to one of the two purinergic receptor types on the platelets. While pro-aggregatory effects of the $P2Y_1$ receptor are unaffected, those of the $P2Y_{12}$ receptor are inhibited. Intracellular signal transduction after $P2Y_{12}$ receptor stimulation is mediated via G_i-protein linked suppression of adenylate cyclase [22]. In turn, decreased adenylate cyclase activity results in diminished cyclic AMP (cAMP) formation and diminished phosphorylation of VASP, particularly at Ser 157, but also at Ser

Abbreviations: ADP, adenosine-5′-diphosphate; c AMP, cyclic adenosine 3′,5′-monophosphate; c GMP, cyclic guanosine 3′,5′-monophosphate; INR, international normalized ratio; NO, nitric oxide; PRI, Platelet Reactivity Index; PGE_1, prostaglandin E_1; PGI_2, prostacyclin; sGC, soluble guanylate cyclase; NP, sodium nitroprusside; STEMI, ST elevated myocardial infarction; VASP, vasodilator stimulated phosphoprotein; VASP-P, vasodilator stimulated phosphoprotein-phosphorylated.

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239 [23]. VASP phosphorylation particularly at Ser 239 [23] is also modulated by cGMP [24,25], a product of soluble guanylate cyclase upon activation by nitric oxide (NO). Therefore in theory, the effects of ADP binding to the $P2Y_{12}$ receptor also reflect an attenuation of the physiological anti-aggregatory actions of adenylate cyclase (and perhaps soluble guanylate cyclase) activators.

If this is the case, the effectiveness of $P2Y_{12}$ antagonists such as clopidogrel should be related to the integrity of adenylate cyclase signaling [26]. Indeed, a number of investigators have previously provided evidence that prostacyclin (PGI_2) and prostaglandin E_1 (PGE_1), powerful activators of platelet adenylate cyclase, potentiate clopidogrel effect [27–29].

It is also likely that the integrity of adenylate cyclase signaling varies between individuals, as does that of soluble guanylate cyclase signaling. We have indeed previously shown [30–32] that both these pathways are frequently impaired in patients with angina pectoris.

In the current study we therefore examined the relationship between the integrity of the adenylate cyclase and guanylate cyclase signaling pathways in platelets and the effects of clopidogrel, in the presence or absence of loss of function genotypes. We studied physiological determinants of the effects of clopidogrel acutely (4 h) after a loading dose in order to identify the molecular basis of the non-genetic components of clopidogrel resistance.

2. Methods

2.1. Patient selection

Patients with stable or unstable angina pectoris included for the study were admitted to The Queen Elizabeth Hospital, Adelaide to have at least one stent inserted in the coronary arteries. The following exclusion criteria were applied: 1. concomitant therapy with $P2Y_{12}$ receptor antagonists (clopidogrel, ticagrelor, prasugrel) or GPIIb/IIIa receptor antagonists that would interfere with baseline ADP induced aggregation and 2. risk of bleeding (including patients who were on warfarin prior to the procedure and having their INR, above 1.6) or severe renal insufficiency (creatinine >0.3 mmol/L). All patients suitable for inclusion in the study received a loading dose of 600 mg of clopidogrel just before PCI. The study was approved by the Institutional Human Research and Ethics Committee. Informed consent was obtained from all patients prior to the coronary angiogram.

2.2. Study protocol

Thirty-four consecutive patients were enrolled in the study. Blood was collected at baseline and 4 h after receiving a 600 mg oral loading dose of clopidogrel. The baseline blood sample was collected from the femoral arterial sheath during the angiographic procedure and the 4-h sample was taken from an antecubital vein. The intravenous cannula was flushed with 0.9% sodium chloride and the first 5 mL of blood was discarded. Blood was collected in plastic tubes containing 1:10 volume of citrate anticoagulant (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate, pH 5) for aggregation studies and in Vacutainer tubes (with trisodium citrate) for flow cytometry-based VASP phosphorylation (VASP-P) assessment.

2.3. Chemicals

PGE_1, NP and ADP were purchased from Sigma Aldrich (St. Louis, Missouri, USA). VASP kits were purchased from Biocytex (Marseille, France).

2.4. Platelet aggregation studies

Aggregation in whole blood was examined utilizing a two-channel Model 560 impedance aggregometer (Chrono-Log, Havertown, Pennsylvania, USA) as described previously [31]. In brief, tests were performed at 37 °C and a stirring speed of 900 rpm. Blood samples were diluted two-fold with normal saline (final volume 1 mL) and pre-warmed for 5 min at 37 °C. Aggregation was induced with ADP (final concentration of 2.5 μmol/L and 5 μmol/L). Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance in ohms. Both NP and PGE_1 (final concentration of 10 μmol/L and 33 nmol/L, respectively) were added to samples 1 min before ADP 2.5 μmol/L. Inhibition of aggregation was evaluated as a percentage of the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied. Construction of complete concentration response curves for NP and PGE_1 was precluded because of limited duration of whole blood stability. ADP responses were evaluated before and after clopidogrel, using 2.5 μmol/L and 5 μmol/L ADP, respectively, and results were stratified according to genotype status.

For the purpose of correlating high on-treatment platelet reactivity (HTPR) with clopidogrel response, patient's ADP response on treatment was divided into ≥ or ≤ mean (2.7 Ω). Mean on-treatment response for those defined as HTPR was 5.4 ± 1.9 Ω, while the absence of such a response was associated with mean ADP response of 0.6 ± 0.8 Ω.

2.5. Platelet Reactivity Index (PRI)

VASP-P analysis was performed within 24 h of blood collection using the VASP kits (BioCytex, Marseille, France). Blood samples were incubated with PGE_1 alone or PGE_1 and ADP. After cell permeabilization, cells were initially labeled with the primary monoclonal antibody (MAb) for serine 239 phosphorylated VASP (16C2). This was followed by staining with goat anti-mouse IgG-fluorescence isothiocyanate polyclonal reagent. The last step was to label the platelets with a counter staining reagent against CD61-PE. Flow cytometric analysis was performed using a Becton Dickinson FACS CANTO II flow cytometer (BD Biosciences, San Jose, California, USA). Platelet population was assessed on forward and side scatter, and 10,000 gated events were analyzed for corrected mean fluorescence intensity (MFI_c) using the BD FACS Diva software. MFI_C was obtained by subtracting MFI value obtained for the negative isotypic control to the MFI obtained for anti VASP-P MAb in the presence of PGE_1 alone or in the presence of ADP. The Platelet Reactivity Index was calculated according to the following equation [33]:

$$PRI = \left[\left(MFI_{C\ PGE1} - MFI_{C\ PGE1+ADP}\right)/MFI_{C\ PGE1}\right] \times 100.$$

2.6. Genetic testing

Buccal swabs were taken from patients to collect DNA and test for polymorphisms identified via polymerase chain reaction (PCR). All such tests were conducted at Healthscope Pathology, Melbourne, Australia. The polymorphic sites were identified by single nucleotide polymorphisms using the Sequenom Mass ARRAY iPLEX platform.

The CYP2C19 alleles tested were: *2 (681G>A), *3 (636G>A) and *17 (−808C>T). Based on the test results, genotypes were classified as extensive (*1/*1), intermediate (*1/*2,*2/*17), poor (*2/*2) and ultra metabolizers (*1/*17, *17/*17). However in order to avoid multiple comparisons, patient's genotypes were classified either as “genetically impaired bio-activation” or “no genetically impaired bio-activation”.

3. Statistical analysis

Data were checked for Gaussian or non-Gaussian distribution using the D'Agostino test. Based on the nature of distribution, either Pearson's (Gaussian) or Spearman's (non-Gaussian) correlation analyses were