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Full Length Article

Impaired adenylate cyclase signaling in acute myocardial ischemia: Impact on effectiveness of $P2Y_{12}$ receptor antagonists

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ABSTRACT

Introduction: $P2Y_{12}$ receptor antagonists reduce risk of thrombotic complications after stent implantation but increase bleeding risk. Activation of $P2Y_{12}$ receptors by ADP causes Gi-protein-mediated inhibition of adenylate cyclase (AC), thus limiting platelet response to anti-aggregatory effect of prostacyclin (PGI_2). However, $P2Y_{12}$ blockade reverses this ADP-induced suppression of the platelet PGI_2/AC signaling pathway. We previously demonstrated that impairment of this pathway predicts poor response to clopidogrel.

Objectives: To identify clinical correlates of variability in PGI_2/AC signaling, and to assess the impact of such variability on individual responses to the direct $P2Y_{12}$ receptor antagonists ticagrelor (*in vivo*) and 2-methylthioadenosine-monophosphate (2MeSAMP) (*in vitro*).

Patients/Methods: We compared the inhibitory effects of prostaglandin E_1 (PGE_1) and the PGI_2 analog Iloprost (Ilt) on platelet aggregation in whole blood samples from healthy control subjects ($n = 17$), and patients with stable angina pectoris (SAP; $n = 35$) or acute coronary syndromes (ACS; $n = 23$), with or without associated diabetes/hyperglycemia.

Results: Compared to control subjects, patients with ACS and – to a lesser extent – those with SAP, exhibited impaired responses to PGE_1, accentuated in the presence of hyperglycemia. Efficacy of ticagrelor treatment, measured as change in platelet reactivity index, was directly related to pre-treatment PGE_1 response, both at univariate and multivariate analysis. There was a strong correlation between extent of inhibition of platelet aggregation, whether by PGE_1 or Ilt, and the anti-aggregatory effect of 2MeSAMP *in vitro*.

Conclusions: The integrity of PGI_2/AC signaling, which is impaired in the presence of ACS and hyperglycemia, predetermines the anti-aggregatory efficiency of $P2Y_{12}$ receptor antagonists.

1. Introduction

The establishment of coronary artery angioplasty plus stent insertion has improved short and long-term outcomes for patients with evolving acute myocardial infarction and unstable angina pectoris [1,2], although its benefits in patients with stable angina pectoris (SAP) remain more controversial [3–5]. Part of the usual therapeutic strategy associated with coronary stenting has been the introduction of "dual anti-platelet therapy", comprising the combination of low-dose aspirin plus an ADP $P2Y_{12}$ receptor antagonist [6], in order to reduce the short and medium-term risk of stent thrombosis.

Although clopidogrel was the first $P2Y_{12}$ receptor antagonist to be used extensively in this regard, anti-aggregatory responses to the usual (75 mg/day) treatment regimen vary widely, and this variability has been implicated as a potential risk factor for the occurrence of thrombotic events [7]. Clopidogrel is a prodrug, and there is evidence to suggest that genetically determined impairment of its enzymatic bioactivation may contribute to this variability in response ("clopidogrel resistance") in some patients [7,8]. Variable impairment in the formation of clopidogrel active metabolite largely reflects "loss-of-function" mutations in the bio-activating enzyme CYP2C19 [7–10]. However, we have recently shown that this is not the major source of inter-individual variability of response to clopidogrel [11], consistent with the results of previous investigations [12,13].

Activation of $P2Y_{12}$ receptor-associated signaling by ADP results in extensive intracellular biochemical changes, including Gi protein-

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mediated inhibition of platelet adenylate cyclase (AC) [14,15], which generates cAMP in response to the anti-aggregatory autacoids prostacyclin (prostaglandin I_2, PGI_2) and prostaglandin E_1 (PGE_1) *via* activation of IP receptors [16]. Since blockade of the $P2Y_{12}$ receptor by clopidogrel active metabolite would reverse ADP-induced inhibition of AC, we have previously postulated [14] that the efficacy of clopidogrel in individual patients could be predicted by pre-treatment responses to PG-induced indirect activation of AC, for example by PGE_1 [11,14,15,17]. Indeed, for both acute [15] and subacute [11] responses, a close association emerged, which in the case of weight-adjusted subacute therapy, exceeded the importance of clopidogrel activator genotype [11].

In the past 10 years, major changes in the patterns of clinical utilization of $P2Y_{12}$ receptor antagonists have occurred. Evidence has emerged that both prasugrel [18] and ticagrelor [19] may be more effective than clopidogrel (in the then usual dosing regimen of 300 mg loading dose, followed by 75 mg/day) in the management of patients with ACS in terms of protection against thrombotic complications post stent insertion. On the other hand, it has become apparent that the use of such agents, especially in the long-term [20,21], may substantially increase bleeding risk.

As therapy with ticagrelor [19] and prasugrel [18] is usually initiated in the context of ACS, and many such patients also suffer from poorly controlled diabetes [22], it is particularly important to evaluate potential impairment of PGI_2/AC signaling in such individuals. We have previously demonstrated that platelet responsiveness to PGE_1 is impaired in the presence of myocardial ischemia [23]. Furthermore, diminished platelet responsiveness to clopidogrel and to its active metabolite has been documented in patients with coronary artery disease and concomitant diabetes [24], suggesting that diabetes may represent an incremental cause of impaired efficacy of $P2Y_{12}$ receptor inhibition.

To date, possible heterogeneity in individual patient responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel has not been extensively studied, largely because normal clinical doses of both ticagrelor and prasugrel consistently produce greater inhibition of platelet aggregation than that seen with clopidogrel. However, the emergence of bleeding risk provides a strong additional rationale for establishment of general principles in the area. Therefore, the current study tested two major hypotheses:

(1) that integrity of the anti-aggregatory prostaglandin/adenylate cyclase (PGI_2/AC) pathway is variably impaired in the presence of SAP and/or ACS, and that this impairment is accentuated with concomitant diabetes/hyperglycemia;
(2) that responses to $P2Y_{12}$ receptor antagonists other than clopidogrel, namely to the directly acting antagonist ticagrelor (*in vivo*) and the short-acting direct antagonist 2-methyl thioadenosine monophosphate (2MeSAMP) (*in vitro*), are also dependent on pre-treatment integrity of PGI_2/AC signaling.

2. Methods

2.1. Study protocol

Inhibition of platelet aggregation by activators of PGI_2/AC signaling (PGE_1 and PGI_2) and the direct AC activator forskolin (Fsk) was evaluated in blood samples obtained from 17 healthy (control) subjects, 35 patients with SAP and 23 patients with ACS attending The Queen Elizabeth Hospital (Adelaide, Australia), and currently receiving no concomitant therapy with $P2Y_{12}$ receptor antagonists or GPIIb/IIIa receptor blockers. Non-ischemic control subjects (or simply, Controls, $n = 17$) were recruited consecutively by local advertisement; selection criteria were: age $>$ 30 years (to correspond to the anticipated age of patients); and the absence of anginal symptoms. In ACS patients, treatment with ticagrelor (180 mg loading dose and 90 mg bd maintenance dose) was initiated post initial blood sampling. No other patients, nor control subjects, received ticagrelor or any other $P2Y_{12}$ receptor antagonists. We sought to maximize recruitment of SAP patients with concomitant diabetes mellitus in order to better evaluate possibly incremental deleterious impact of this condition on PGI_2/AC signaling. Concomitant blood glucose levels were measured in all diabetic patients. All human studies were performed in accordance with the Declaration of Helsinki. The study was approved by the Institutional Human Research and Ethics Committee, and informed consent was obtained from all subjects.

Blood samples were collected either from the arterial sheath prior to contrast injection during the angiographic procedure (in patients) or from an antecubital vein (in control subjects) into plastic tubes containing 1:10 volume of citrate anticoagulant (2 parts of 0.1 M citric acid to 3 parts of 0.1 M trisodium citrate, pH 5) for platelet aggregation studies, and into Vacutainer tubes with trisodium citrate for flow cytometry-based VASP phosphorylation (VASP-P) assays.

2.2. Platelet aggregation studies

Aggregation in whole blood was examined utilizing a 4-channel Model 700 impedance aggregometer (Chrono-Log, Havertown, Pennsylvania, USA) as we described previously [11]. 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 prewarmed for 5 min at 37 °C. Aggregation was induced with ADP or the thrombin receptor activating peptide (TRAP). PGE_1 (30 nM) or the PGI_2 mimetic Iloprost (Ilt, 0.3 nM) were added to samples 1 min before ADP (final concentration of 2.5 μM), and Fsk (5 μM) 5 min before ADP. Similarly, anti-aggregatory responses to the NO donor sodium nitroprusside (SNP, 10 μM), was utilized to assess nitric oxide/soluble guanylate cyclase signaling. Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance in ohms. Results obtained from aggregometry assays with respect to responses to PGE_1, Ilt and SNP were evaluated as a percentage of the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied, and reported as percent inhibition of aggregation (%). *Chemicals*: PGE_1, Ilt, Fsk, 2MeSAMP and ADP were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

2.3. Platelet Reactivity Index (PRI)

Vasodilator-stimulated phosphoprotein (VASP-P) analysis was performed within 24 h of blood collection using VASP kits (BioCytex, Marseille, France) according to the manufacturer's instructions. Blood samples were incubated with PGE_1 alone or PGE_1 + ADP, and then with a specific monoclonal antibody (clone 16C2) to label VASP in its phosphorylated state. This was followed by staining with the goat anti-mouse IgG-fluorescence isothiocyanate polyclonal reagent, labeling 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), as described by us previously [15]. Platelet population was assessed on forward and side scatter, and 10,000 gated events were analyzed for corrected mean fluorescence intensity (MFIc) using the BD FACS Diva software. Dual color flow cytometry analysis permits evaluation of the capacity of ADP to inhibit VASP phosphorylation. Platelet reactivity index (PRI) was calculated using corrected MFIc in the presence of PGE_1 alone or PGE_1 + ADP. Changes in PRI (δPRI) were utilized as an index of individual responsiveness to $P2Y_{12}$ receptor antagonist therapy [11,15].

2.4. Assessment of $P2Y_{12}$ receptor blockade

a) With ticagrelor *in vivo*: blood samples were collected before initiation and 7 days after therapy with ticagrelor, and both VASP-P flow cytometry and aggregometry were performed. Responses to

ticagrelor treatment were expressed both as δPRI and δADP (decrease in ADP (5 μM) –induced platelet aggregation) respectively, as for previous investigations [11,15]. Because of better specificity and reproducibility, changes in PRI were utilized as the primary measure of response to ticagrelor *in vivo*.

b) With 2MeSAMP *in vitro*: blood samples, taken from individuals not currently receiving therapy with $P2Y_{12}$ receptor antagonists, were preincubated for 5 min with 10 μM 2MeSAMP before stimulation with 5 μM ADP for aggregometry assay; as for *ex vivo* studies, the extent of aggregation was compared to that with ADP alone.

2.5. Statistical analysis

Data analysis utilized one-way ANOVA or Chi squared test for group comparisons as appropriate, with the Bonferroni test for post-hoc comparisons of data subjected to ANOVA, and with Pearson's coefficients for bivariate correlation tests. Comparisons of aggregation parameters before and after initiation of ticagrelor therapy were made using paired *t*-tests. Backwards stepwise multiple logistic regression was performed to identify determinants of variability in individual responsiveness to PGE_1 across the entire range of patients and control subjects, utilizing patient *vs* normal status, acuity of ischemia, blood glucose level, age, gender, therapy with ACE inhibitors and past history of hypertension as covariates. Determinants of sensitivity to ticagrelor were identified by utilizing response to PGE_1 and SNP, age and gender as covariates in multivariate backwards multiple linear regression analysis. All tests were 2-tailed, and data were expressed as mean ± SEM, unless otherwise stated. *P* values < 0.05 were considered statistically significant. Data analysis was performed using SPSS-23 software.

3. Results

3.1. Subject/patient characteristics: implications of ischemia and diabetes/hyperglycemia on PGI_2/AC signaling

3.1.1. Responsiveness to PGE_1

A total of 35 patients with SAP and 23 with ACS were compared with 17 age-matched non-ischemic control subjects. Demographics are summarized in Table 1. In general, this was a middle-aged group of individuals: the patients with symptomatic ischemic heart disease were extensively, but not universally, treated with anti-anginal and cardioprotective agents, but none with $P2Y_{12}$ receptor antagonists at initial assessment.

ACS = acute coronary syndrome; ARB = angiotensin receptor blockers; CAD = coronary artery disease; DM = diabetes mellitus; SAP = stable angina pectoris; SD = standard deviation; *p* values refer to results of ANOVA.

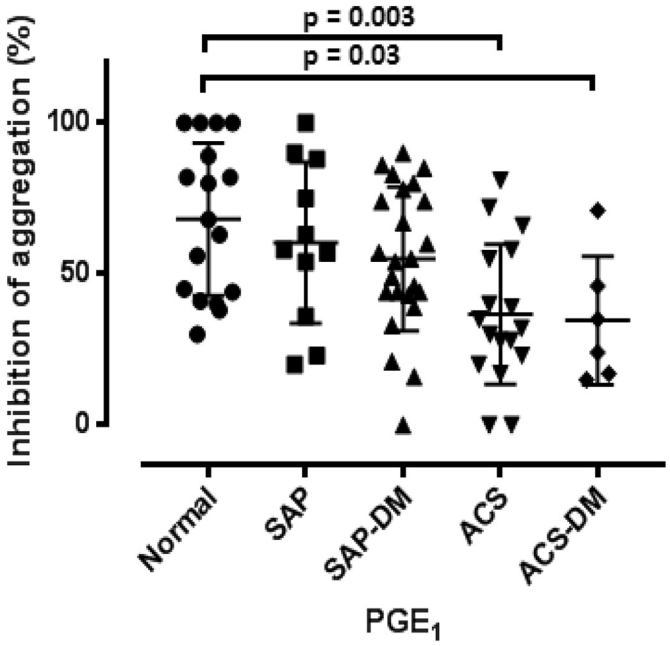

Fig. 1. Inhibition of ADP (2.5 μM)-induced platelet aggregation by PGE_1 (30 nM) and in whole blood samples from control subjects and patients with stable angina pectoris (SAP) (without and with diabetes mellitus (DM)) or acute coronary syndrome (ACS) (without and with DM): $p = 0.002$, one-way ANOVA for differences among groups with regard to PGE_1 responses: $p = 0.003$ and $p = 0.03$ for ACS and ACS/DM respectively *vs.* normal subjects (Bonferroni post-hoc test).

Among patients with SAP in particular, there was, by intention, a high proportion of diabetics. Many diabetic patients, particularly those with ACS, were hyperglycemic at the time of study. In whole blood samples, ADP induced platelet aggregation of 6.5 ± 1.8, 7.27 ± 2.3 and 7.29 ± 2.0 Ω for the control, SAP and ACS groups respectively (p = NS). Results re inhibition of platelet aggregation by PGE_1 are depicted in Fig. 1, with patients also stratified according to diabetic status. There was substantial heterogeneity of PGE_1 responses, with diminution of platelet responses to PGE_1 for SAP and ACS patients, relative to normal controls ($p = 0.002$, 1-way ANOVA). However, on *post hoc* testing this reached statistical significance only for ACS patients (with/without concomitant diabetes mellitus) compared to controls.

3.1.2. Responsiveness to the direct AC activator Fsk

With the concentration of Fsk utilized (5 μM), across the spectrum of

Table 1
Demographics of patients and control subjects recruited.

Clinical characteristics	Controls	SAP without DM	SAP with DM	ACS without DM	ACS with DM	p
	(*n* = 17)	(*n* = 11)	(*n* = 24)	(n = 17)	(n = 6)	
Age (years: mean ± SD)	59 ± 8	63 ± 10	70 ± 14	66 ± 16	63 ± 17	NS
Gender (male; %)	41.2	90.9	54.2	70.6	83.3	0.05
CAD risk factors						
Ex/current smoker (%)	0	9.1	4.2	52.9	33.3	0.0001
Hypertension (%)	17.6	54.5	62.5	23.5	66.7	0.01
Hyperlipidaemia (%)	35.3	81.8	29.2	41.2	83.3	0.01
Medications (%)						
Aspirin	11.8	81.8	37.5	100	100	0.001
β-Blockers	0	36.4	33.3	23.5	0	0.04
Ca^{++} antagonists	5.9	36.4	29.2	5.9	50	0.04
ACE-inhibitors/ARB	11.8	54.5	79.2	58.8	33.3	0.001
Statins	35.3	81.8	66.7	47.1	66.7	0.1

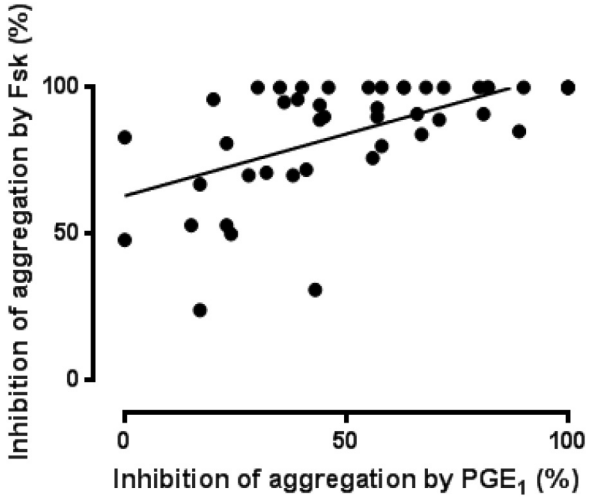

Fig. 2. Correlation between anti-aggregatory effects of PGE_1 (30 nM) and forskolin (Fsk, 5 μM); $r = 0.58$, $p < 0.001$; $n = 46$ (patients/normal subjects combined).

subject/patient groups studied, there was a direct and significant ($p < 0.001$) correlation between inhibitory effects of PGE_1 and those of Fsk on platelet aggregation (Fig. 2).

Although PGE_1-induced activation of AC is initiated by its binding to IP receptors, these data thus imply that heterogeneity in responsiveness to PGE_1 at least partially involves variable functionality of AC.

3.1.3. Determinants of PGI_2/AC signaling

The presence of diabetes tended to be associated with further impairment of PGI_2/AC signaling, but this primarily reflected a significant ($r = -0.42$; $p = 0.0002$) inverse relationship between blood glucose level and responsiveness to PGE_1 (Fig. 3).

Multivariate analysis was performed to identify determinants of variability in individual platelet responsiveness to PGE_1 (Table 2). We found on initial evaluation that, surprisingly, diabetes mellitus, was associated with significant increases in anti-aggregatory responses to PGE_1, while these were diminished in the presence of SAP, hyperglycemia, and in women.

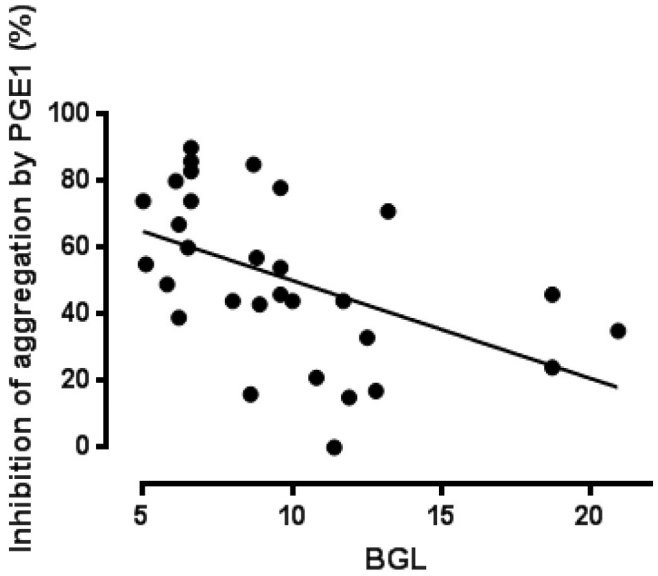

Fig. 3. Correlation between anti-aggregatory effects of PGE_1 and blood glucose levels (BGL, mmol/L) in the 30 diabetic patients in the study; $r = -0.49$, $p = 0.006$.

Table 2
Determinants of variability in platelet response to the anti-aggregatory effects of PGE_1 (multivariate analysis).

Covariates	β	p
SAP	−0.35	0.01
ACS	−0.15	0.33
Diabetes	0.29	0.02
Hypertension	0.15	0.89
Age	−0.03	0.78
Female gender	−0.37	0.001
Blood glucose	−0.37	0.005
ACEi/ARB	0.09	0.45

ACEi = ACE inhibitors; ACS = acute coronary syndrome; ARB = angiotensin receptor blockers; SAP = stable angina pectoris.

Because the majority of cases of severe hyperglycemia occurred in patients with ACS, it appeared possible that the inclusion of both hyperglycemia and ACS as components of the multivariate analysis might induce a confounding influence on results. Removal of blood glucose level from the multivariate analysis resulted in emergence of ACS ($\beta = -0.40$, $p = 0.0001$) as a strong predictor of poor responsiveness to PGE_1, and the disappearance of diabetic status *per se* as a significant determinant of responsiveness.

3.2. Correlations between pre-treatment platelet responses to PGE_1 and subsequent responses to $P2Y_{12}$ blockade

3.2.1. Responses to ticagrelor in vivo

Treatment with ticagrelor for 7 days in patients with ACS ($n = 18$) induced a marked reduction in PRI, thus indicating a very adequate suppression of ADP-induced platelet aggregability *in vivo* (Table 3).

Importantly, there was a direct and significant correlation between pre-treatment platelet responses to the inhibitory effect of PGE_1 on ADP-induced aggregation *in vitro*, and the efficacy of 7-day therapy with ticagrelor, assessed *ex vivo* as fall in PRI (Fig. 4).

In all ticagrelor-treated patients, there was virtually complete suppression of ADP-induced platelet aggregation in blood samples at follow-up, thus precluding utilization of δADP response, as a parameter of heterogeneity of ticagrelor effect (Table 3). There was also a minor decrease in TRAP-induced platelet aggregation in blood samples at follow-up in comparison with baseline.

Data re heterogeneity of impact of ticagrelor on PRI were also analyzed with multiple logistic regression (Table 4) in the model utilizing PGE_1 response, SNP response, patient age and gender confirmed that the extent of pre-treatment platelet response to PGE_1 was a major and direct determinant ($\beta = 0.82$; $p = 0.002$) of individual δPRI in patients on ticagrelor.

There was also a trend towards an inverse relationship with platelet response to the nitric oxide donor SNP, as previously reported by us [11]. If age was replaced in this model by diabetic status, response to PGE_1 ($\beta = 1.32$; $p = 0.004$) and that to SNP ($\beta = -0.89$; $p = 0.034$) were now both significant, but opposing, determinants of response.

Table 3
Changes in platelet reactivity index (PRI) and aggregability (in response to 5 μM ADP and 1 μM TRAP) following ticagrelor therapy for 7 days in patients with acute coronary syndrome ($n = 18$).

Parameter	Baseline	Follow-up	p*
PRI (%)	77.3 ± 2.2	13.8 ± 1.8	< 0.001
ADP (ohms)	7.8 ± 0.5	0.5 ± 0.3	< 0.001
TRAP (ohms)	9.3 ± 0.6	7.5 ± 0.8	0.047

* P values refer to results of paired *t*-tests.

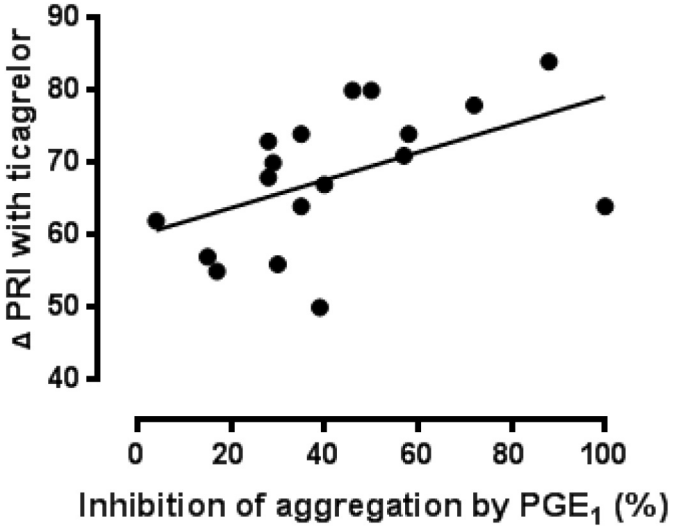

Fig. 4. Correlation between the inhibitory effect of PGE_1 (30 nM) on ADP-induced aggregation, assessed before the initiation of ticagrelor therapy, and the efficacy of 7-days' therapy with ticagrelor, measured as fall in PRI (δPRI), in patients with acute coronary syndrome ($r = 0.52$, $p = 0.02$; $n = 18$).

Table 4
Association of patient characteristics with response to ticagrelor therapy for 7 days (measured as a change in platelet reactivity index, δPRI) in patients with acute coronary syndrome ($n = 18$, multivariate analysis).

Covariates	β	p
PGE_1 response	0.82	0.002
SNP response	−0.53	0.16
Age	−0.24	0.22
Female gender	−0.42	0.21

3.2.2. Responses to 2MeSAMP in vitro

As regards the short acting direct $P2Y_{12}$ antagonist 2MeSAMP, additional investigations were carried out *in vitro* utilizing blood samples obtained both from patients with SAP ($n = 6$) and ACS ($n = 17$), not receiving any ADP-receptor antagonist *in vivo*. Responsiveness to the inhibitory effects of both PGE_1 and Ilt on ADP-induced platelet aggregation predicted ($r = 0.45$, $p = 0.03$ and $r = 0.80$, $p = 0.02$ respectively) the extent of the decrease in platelet aggregation following incubation with 2MeSAMP (Fig. 5).

4. Discussion

The current study had two major underlying rationales:

(i) our previous finding that platelets from patients with SAP are hyporesponsive to the anti-aggregatory effects of PGE_1 [23]; and
(ii) our recent studies focusing on the importance of post-receptor, AC-dependent signaling as a major source of variability in individual patients' responsiveness to the $P2Y_{12}$ receptor antagonist clopidogrel [11,15].

These data suggested that inter-individual variability in responsiveness of the PGI_2/AC signaling pathway might be of considerable importance with regard to both safety and efficacy of other $P2Y_{12}$ receptor antagonists. However, no data were available concerning the variability in PGI_2/AC signaling in patients with symptomatic myocardial ischemia. Diminished anti-aggregatory response to clopidogrel in diabetics with concomitant ischemia has been observed previously [24,25], with the additional finding that VASP phosphorylation in response to its active metabolite is diminished [24], although the role of

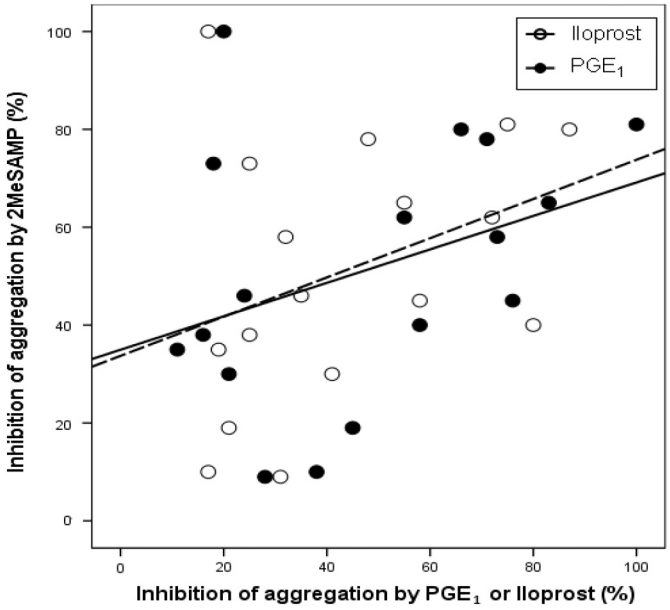

Fig. 5. Correlations between anti-aggregatory effect of 2MeSAMP (10 μM) *in vitro* and inhibition of platelet aggregation by 30 nM PGE_1 ($r = 0.45$, $p = 0.03$; $n = 23$) or 0.3 nM Iloprost ($r = 0.8$, $p < 0.001$; $n = 17$) before the application of 2MeSAMP.

hyperglycemia in this process has not previously been investigated. These data therefore suggested that diabetes might be associated with impairment of post-receptor signaling in response to clopidogrel active metabolite, and, analogously, to other directly active $P2Y_{12}$ receptor antagonists such as ticagrelor. Furthermore, in view of our previous data with NO signaling [22,26], it was appropriate to consider the impact of hyperglycemia *per se* as a potential negative modulator of post-receptor signaling within the PGI_2/AC pathway.

The first part of the current study was therefore an evaluation of the relative impact of symptomatic stable or unstable myocardial ischemia on platelet responsiveness to PGE_1, and the potential impacts of both diabetes mellitus *per se* and of hyperglycemia on these responses. As shown in Fig. 1, the presence of ACS in particular was associated with marked impairment of responses to PGE_1. Multivariate analyses confirmed that the presence of angina pectoris and of hyperglycemia were associated with impairment of PGI_2/AC signaling. Furthermore, when the "confounder" of the close association between hyperglycemia and the presence of ACS was taken into account, it emerged that ACS was strongly associated with impaired PGE_1 responses.

The second objective of the study was an investigation of the relationship between integrity of the PGI_2/AC pathway and individual responsiveness to $P2Y_{12}$ receptor antagonists other than clopidogrel. We undertook this investigation through both an *ex vivo* study of ticagrelor therapy in ACS patients, and an *in vitro* study with the short-acting direct $P2Y_{12}$ receptor antagonist 2MeSAMP.

With ticagrelor, we used the dosing schedule for treatment of ACS patients utilized in the PLATO study [19]: 180 mg as loading dose and 90 mg twice daily as maintenance dose for 7 days prior to assessment of patient response. With this regimen, the resultant inhibition of ADP-induced aggregation approached 100% in most patients, thus impeding data analysis. However, heterogeneity of response could be detected and assessed *via* PRI determination, as a measure of VASP phosphorylation, the corresponding biochemical process. Pre-treatment platelet response to PGE_1 was a strong and direct correlate of a reduction in PRI (δPRI) following initiation of ticagrelor treatment, both on univariate (Fig. 4) and multivariate (Table 4) analyses.

An accurate *in vitro* analysis of the putative relationship between integrity of the PGI_2/AC axis and response to $P2Y_{12}$ receptor blockade is

difficult for prodrugs such as clopidogrel or prasugrel, but feasible for directly acting agents. Therefore, in the current study we also utilized the direct short-acting antagonist 2MeSAMP. As shown in Fig. 3, pretreatment platelet responses to both PGE_1 and the PGI_2 analog Ilt (a "pure" IP receptor agonist) were strong predictors of extent of suppression of ADP-induced aggregation by 2MeSAMP. These results therefore imply that an individual patient's responsiveness to AC stimulation by PGE_1 or PGI_2 is a strong predictor of subsequent responsiveness to all $P2Y_{12}$ receptor antagonists, not just clopidogrel. Furthermore, impairment of PGI_2/AC signaling is a particularly common and severe problem in patients with ACS. On multivariate analyses, SAP, ACS, hyperglycemia and gender were found to independently predict PGE_1 response. As regards gender differences, responses to PGE_1 were both significantly and substantially impaired in women. No previous studies appear to have evaluated this issue, and indeed clinical evaluations [27,28] have shown less than definitive increases in thrombotic risk for $P2Y_{12}$ receptor antagonists in women. Given that *in vitro* estimation of anti-aggregatory responses to PGE_1 are independent of patient body weight, while most women included in studies with ticagrelor were of lower body weight than men, it is possible that impaired efficacy of PGI_2/AC signaling in women might be obscured clinically by utilization of greater doses per unit weight.

There are several limitations to this study. First, from a mechanistic point of view, we were able to determine, *via* correlation between responses to PGE_1 and those to the direct AC stimulator Fsk (Fig. 2), that at least part of the basis for platelet resistance to PGI_2/AC pathway activation is dysfunction of AC. However, we did not explore whether problems with receptor activation and Gi/Gs protein function might also contribute, and/or whether AC dysfunction was due to oxidative stress. The multivariate analysis of determinants of response to ticagrelor was limited by small sample size. There must also be some residual uncertainty as regards the relative impacts of ACS and hyperglycemia on integrity of PGI_2/AC signaling, given their apparently reciprocal confounding effects in multivariate analysis. However, the strong negative correlation between blood glucose level and PGE_1 response (Fig. 3), as well as the lack of impact of diabetic status on response to ticagrelor in modified multivariate analysis suggest that hyperglycemia, perhaps *via* impact on tissue redox state, represents the major modulator of this signaling pathway [22,26]. Finally, it remains to be determined from more extensive clinical data to what extent variability in platelet PGI_2/AC signaling translates into risks of thrombotic and hemorrhagic complications during treatment with $P2Y_{12}$ receptor antagonists.

The main implication of the current findings is that some diminution of ticagrelor effect is likely to occur particularly in patients with ACS, and to a lesser extent in those with SAP. The presence of diabetes with associated hyperglycemia accentuates the extent of this problem. While this is likely to reduce the level of protection by ticagrelor against thrombosis, especially in hyperglycemic patients with ACS, the occurrence of bleeding complications during acute ischemia predictably is a relatively minor concern, as was observed in PLATO [19]. On the other hand, there was a somewhat greater response to PGE_1 in patients with SAP, suggesting that the relative risk of bleeding, rather than thrombotic, complications would be a greater concern here, as proved to be the case in PEGASUS [20].

Furthermore, it is now customary, in order to reduce risk of late thrombotic complications, to continue dual antiplatelet therapy in some patients for as long as 2 years or even more after an ACS or in patients with stenting performed in complex anatomy, such as at bifurcations in case of extensive dependent myocardial territory [29]. It is likely that responsiveness to any $P2Y_{12}$ antagonist utilized here will increase with the transition to chronic ischemic heart disease, with associated decreases in thrombotic, but increases in bleeding, risks. The current data therefore also provide an argument for decreased dosing with $P2Y_{12}$ antagonists beyond the first year of therapy.

The results of the current study also have implications for a better understanding of mechanisms maintaining cardiovascular homeostasis in general. It has previously been reported that the presence of endothelial dysfunction, as reflected in diminution of flow-mediated dilatation, is associated with platelet resistance to clopidogrel [30]. It is now well-established that this condition of "endothelial dysfunction" reflects, *inter alia*, a variable combination of disordered nitric oxide generation and soluble guanylate cyclase signaling, and that this latter component, reflected in platelets as "nitric oxide resistance" [31], is amenable to therapeutic amelioration [31,32]. The overall benefits, at least in theory, of such manipulation would include decreased risk of thrombotic events in high risk patients [31–33]. Analogously, disordered PGI_2/AC signaling is likely to represent a thrombotic risk factor, and its potential amelioration may represent an attractive future therapeutic target. Incorporation of consideration of the biochemical bases underlying variability of patient responsiveness to $P2Y_{12}$ antagonist therapy therefore offers the potential to address some of the ongoing dilemmae limiting therapeutic efficacy of coronary stenting in ischemic heart disease [21,34].

Addendum

H. Imam performed the majority of the experiments and participated in data analysis and manuscript preparation.

T.H. Nguyen participated in data analysis and manuscript preparation.

R. De Caterina helped with the study design and manuscript preparation.

V. Nooney participated in study design and co-supervised some of the experiments.

C-R. Chong supervised patients with stable angina and diabetes, and performed experiments with sodium nitroprusside.

J.D. Horowitz participated in study design, patient supervision and helped with manuscript preparation.

Y.Y. Chirkov supervised the majority of the experiments, data acquisition and manuscript preparation.

Declaration of Competing Interest

R. De Caterina received personal fees for participation into Advisory Boards and non-financial support from AstraZeneca; grants, consultancy fees and support for participation into meetings from Bayer, BMS/Pfizer and Daiichi Sankyo; consultancy fees from Novartis. J. D. Horowitz received speaker's honoraria from AstraZeneca and BMS/Pfizer. None of the other authors have any conflict of interest with regard to this study.

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