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Effects of clopidogrel on ''aspirin specific'' pathways of platelet inhibition

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Abstract

The most widely accepted methods of assessing response to clopidogrel involve isolated ADP-induced platelet aggregation. Whilst poor response determined by these assays correlates with adverse clinical events, the number of ''poor responders'' is far higher than the number of events attributed to treatment failure. Clopidogrel may have effects that cannot be assessed using isolated ADP-induced aggregation. We have investigated the effect of clopidogrel on Arachidonic Acid (AA) induced platelet activation – an ''aspirin specific'' pathway using a novel near patient assay. Thirty four volunteers on no medication and 36 patients, on maintenance therapy with aspirin 75 mg daily, were recruited. Blood tests for Thrombelastogram PlateletMapping were taken immediately prior to and 6 hours after administration of a 600 mg clopidogrel loading dose. Changes in the area under the response curve at 15 minutes (AUC15) with both ADP- and AA-stimulation were calculated as were the corresponding percentage platelet and percentage clotting inhibition (%PIn and %CIn). There were predictable and significant changes in the AUC15 of the ADP channel in response to clopidogrel and the corresponding %PIn and %CIn in both volunteers and patients. There were also significant reductions in the AUC15 of the AA channel (presented as Mean +/− 95%CI), by 27.2 +/− 11.8%, $p = 0.005$ in volunteers and 35.0 +/− 8.2%, $p < 0.001$ in patients) and increases in the %PIn and %CIn calculated using the AA channel in volunteers (by 20.0 +/− 11.4%, $p + 0.02$ and 32.3 +/− 12.8%, $p < 0.001$ respectively) and patients (by 24.2 +/− 8.6%, $p < 0.001$ and by 18.0 +/− 8.6, $p < 0.001$ respectively). Clopidogrel has both independent and aspirin-synergistic effects on AA-induced platelet activation suggesting potentiation of the antiplatelet activity of aspirin. This effect may be clinically important and is not detected by current ''gold standard'' methods of assessing response to clopidogrel.

Keywords: *Platelets, aspirin, angioplasty, stents, clopidogrel*

Introduction

Clopidogrel, a thienopyridine antiplatelet agent, is widely used in patients with cardiovascular disease. There is a sound evidence base to support its use (a) instead of aspirin in the prevention of vascular events in high-risk individuals [1]; (b) in addition to aspirin in patients with acute coronary syndrome [2]; (c) in ST elevation myocardial infarction [3, 4]; and (d) in patients treated with coronary stents [5]. Despite these data there remains concern about responses of individual patients to standard dose clopidogrel therapy. These uncertainties stem from (a) the diversity of methods available for monitoring its effects; (b) the marked heterogeneity in observed responses to clopidogrel using these methods; and (c) an incomplete understanding of how clopidogrel exerts its full clinical effect.

Clopidogrel is a pro-drug that is metabolized by hepatic CYP3A4 to its active metabolite. The latter is thought to predominantly exert its effect by irreversibly binding platelet P2Y12 receptors, thereby inhibiting (a) ADP-induced platelet aggregation and (b) the conformational change that allows fibrinogen to bind to the platelet glycoprotein (GP) IIb/IIIa receptor. The most widely accepted method of assessing responses to clopidogrel is light transmission aggregometry using 10 mmol ADP stimulation, with residual aggregation of >50% taken to demonstrate lack of therapeutic efficacy. Aggregation, however, is just one aspect of platelet function. For example, changes in platelet adhesion and the release of platelet granules are not directly assessed using this technique. Importantly, there are large disparities between apparent rates of ''resistance'' as assessed by isolated platelet

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function tests such as aggregometry and the subsequent occurrence of adverse clinical outcomes. Thus, some aggregometry studies show far higher numbers of poor responders than clinical treatment failure would suggest and others, in which the vast majority of participants would be ''resistant'' using current criteria, show important benefits of clopidogrel on outcome [3]. It is therefore likely that there are clinically important effects of clopidogrel that cannot be assessed using ADP-induced platelet aggregation. Indeed there is now substantial evidence that clopidogrel has other properties including an anti-inflammatory effect [6], and effects on enzymatic components of coagulation [7].

It is likely that clopidogrel plays an important role in preventing the amplification of platelet activation by other platelet activators by inhibiting the effect of ADP released in dense platelet granules. There is some indirect evidence for a clinically important action of clopidogrel on Arachidonic Acid (AA)-induced platelet activation. In a small study Dropinski et al. showed, using aggregometry, that in four of five patients initially labelled as 'non-responders' to aspirin, clopidogrel therapy increases inhibition of AA-induced platelet activation enough to convert them to responders [8]. However, another study using aggregometry showed that in a group of 36 patients on aspirin therapy, clopidogrel therapy resulted in no significant change in AA-induced platelet aggregation [9]. The observed interaction may be dependent on the dose of aspirin. Serebruany et al. showed using aggregometry that whilst raising the dose of aspirin from 81 mg to 325 mg increases the antiplatelet effect of aspirin monotherapy, the same dose adjustment in patients concurrently treated with clopidogrel did not increase its antiplatelet effect further [10].

We have used a novel modification of Thrombelastograph PlateletMapping (TEG), previously described by this group, to investigate the hypothesis that clopidogrel has important, independent and aspirin synergistic effects, through inhibition of AA-induced platelet activation. TEG has previously been shown to correlate with optical aggregation in assessment of responses to antiplatelet therapy [11, 12] and to correlate with clinical outcomes [13, 14]. We have investigated the activity of clopidogrel on both ADP-induced platelet activation and AA-induced platelet activation (previously considered as specific for detecting the effects of aspirin), in the context of both clopidogrel monotherapy and in patients already established on aspirin therapy.

Elucidating the precise mechanisms by which clopidogrel exerts its full antiplatelet effect could have important implications in determining the optimal methods of assessing response to clopidogrel and aspirin, and potentially in guiding therapeutic manipulation and identifying individuals at risk from clopidogrel withdrawal.

Methods

Approval was obtained from the Southampton and South West Hampshire Research Ethics Committee B and the Isle of Wight, Portsmouth and South East Hampshire Research Ethics Committee prior to commencing the study. All subjects provided written informed consent. Procedures followed were in accordance with the Declaration of Helsinki 2008.

Thirty four volunteers on no regular medication and 36 patients on maintenance therapy with aspirin were recruited. Volunteers were excluded if they had taken antiplatelet medication or non-steroidal anti-inflammatory medication within 14 days or if they had a history of peptic ulceration, bronchial asthma, or bleeding. Patients were all non-smokers with angiographically proven coronary artery disease and were on maintenance therapy with aspirin 75 mg daily.

In all subjects, venesection was performed from the antecubital fossa immediately prior to and 6 hours after witnessed administration of a clopidogrel 600 mg loading dose. After the first 2 mls were discarded blood was drawn into a 6 ml Lithium Heparin Vacutainer.

The samples were analysed using TEG PlateletMapping (Haemoscope, Il, USA) according to manufacturer's instructions. TEG is a bedside test that provides an overall assessment of haemostatic function, providing a graphic representation of the speed of clot formation and clot strength. TEG PlateletMapping utilises four channels to detect the effects of antiplatelet therapy [15]. The percentage platelet inhibition (%Pin) in response to AA- or ADP-stimulation is calculated by comparing the Maximum Amplitudes (MA) of a fibrin channel (generated without thrombin generation by the addition of reptilase and factor XIIIa to a heparinised sample) with maximal platelet activation due to thrombin stimulation (the thrombin channel) and platelet activation due to AA (for aspirin, the AA channel) or ADP (for clopidogrel, the ADP channel) in the presence of fibrin stimulation [12].

The short TEG percentage clotting inhibition (%CIn), is calculated by comparing the Area under the response curve at 15 minutes (AUC15) of AA- or ADP-induced clotting responses (the AA or ADP channels) with the response to maximal thrombin stimulation [16]. This has potential advantages as it incorporates the effects of fibrin, is measured in only 15 minutes and incorporates information on the speed of clot formation as well as maximal clot strength. The methods have previously been described in detail [12, 16].

All samples were run until the MA (representing maximal clot strength) was obtained or at least

60 minutes had elapsed. The AUC15 was also calculated as previously described by this group [16]. For each sample the MA and AUC15 were recorded for all channels. The percentage change from baseline in the AUC15 of the AA and ADP channels were calculated as were the percentage platelet inhibition (%PIn) and percentage clotting inhibition (%CIn) (i) calculated from the AA channel and considered to represent response to aspirin and (ii) calculated from the ADP channel and considered to represent response to clopidogrel [12, 16].

"Non-response" to aspirin (defined as a %PIn or a %CIn of <50%) was calculated in the patient group for both the pre-clopidogrel and post-clopidogrel samples.

Data are presented as the Mean +/− 95% Confidence Interval. Significance was determined using paired two-tailed, *t*-tests with a p value of <0.05 considered to represent significance.

For comparison of the number of non-responders significance was determined using Fisher's Exact test with a p value of <0.5 considered to represent significance.

We have established that both TEG MA and short TEG AUC15 provide reliable and reproducible results. Intra-individual baseline reproducibility ($n=20$) is <4% for MA and 7% for AUC15. Intra-individual variability in response to treatment ($n=10$) is <5% for both MA and AUC15. Inter-individual baseline variability ($n=56$) is 5% for MA and 8% for AUC15.

Results

Effects of clopidogrel loading dose in healthy volunteers

There were no significant differences in the AUC15 of the thrombin or fibrin Channels after clopidogrel compared to baseline.

Effects of clopidogrel on ADP-induced platelet activation. There was a significant reduction in the AUC15 of the ADP channel following clopidogrel (by $47.2 \pm 9.7\%$, from 970 ± 87 to 553 ± 126, $p<0.001$). There were significant increases in both the %PIn (from -11.7 ± 13.7 to 39.6 ± 14.7, $p<0.001$), and the %CIn (from 0.11 ± 8.1 to 49.1 ± 11.3, $p<0.001$, Figure 1) due to clopidogrel.

Effects of clopidogrel on AA-induced platelet activation. There was a significant reduction in the AUC15 of the AA channel following clopidogrel (by $27.2 \pm 11.8\%$, from 854 ± 103 to 619 ± 117, $p=0.005$). There were significant increases in the %PIn (from 3.5 ± 11.6 to 16.5 ± 11.3, $p=0.02$) and %CIn (from 2.2 ± 13.8 to 34.5 ± 11.7, $p<0.001$, Figure 1) calculated from the AA channel.

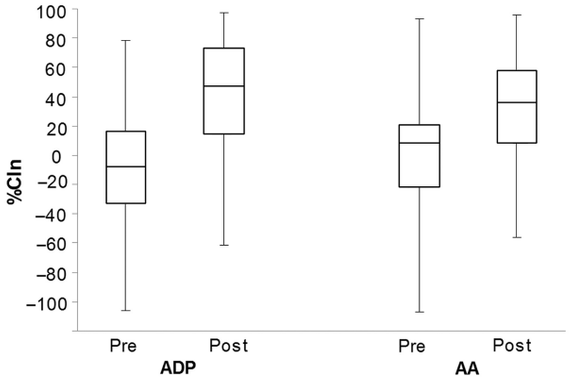

Figure 1.

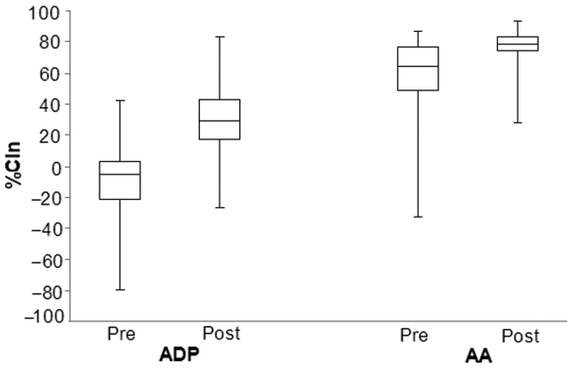

Figure 2.

Effects of clopidogrel loading dose in patients on maintenance therapy with aspirin

There were no significant differences in the AUC15 of the thrombin or fibrin channels following clopidogrel compared to baseline.

Effects of clopidogrel on ADP-induced platelet activation. There was a $34.2 \pm 9.2\%$ reduction in the AUC15 of the ADP channel (from 1074 ± 58 to 700 ± 99, $p<0.001$). This corresponded to significant increases in %PIn (from 0.58 ± 5.0 to 39.5 ± 9.1, $p<0.001$) and %CIn (from -10.1 ± 27.4 to 31.9 ± 8.3, $p<0.001$, Figure 2) due to clopidogrel.

Effects of clopidogrel on AA-induced platelet activation. There was a $35.0 \pm 8.2\%$ decrease in the AUC15 of the AA channel (from 401 ± 71 to 234 ± 33 $p<0.001$). The %PIn and %CIn calculated from the AA channel increased significantly (from 66.0 ± 10.1 to 90.2 ± 5.5, $p<0.001$, and from 58.4 ± 8.2 to 76.4 ± 4.1, $p<0.001$, Figure 2).

Effect of clopidogrel on non-responders to aspirin. At baseline, 10 of 36 patients (by %CIn) were "non-responders" to aspirin, compared to two of 36 after clopidogrel administration ($p=0.02$). By %PIn four of six non-responders to aspirin were converted to

responders by the addition of clopidogrel therapy. All initial non-responders by %PIn were also non-responders by %CIn.

Discussion

It is well established that there is significant variability in patient responses to clopidogrel and furthermore that ''poor response'' is associated with adverse clinical outcomes [17–23]. However, there is as yet no generally accepted clinically relevant technique for assessing patient responses to clopidogrel, particularly in the context that estimates of poor response or ''resistance'' using isolated platelet function tests are much higher than clinical treatment failure would suggest. Furthermore, there are concerns about an increase in clinical events such as stent thrombosis upon clopidogrel withdrawal, even in the ongoing presence of aspirin.

In this study we have used short TEG to determine the effect of clopidogrel on ADP- and AA-induced platelet activation, in volunteers on no other medication, and in patients on aspirin maintenance therapy. We have demonstrated a significant effect of clopidogrel on AA-induced platelet aggregation as well as the predictable inhibition of ADP-induced clot formation. Furthermore, the effect on AA-induced platelet aggregation significantly potentiates the effect of aspirin, to the extent that eight out of 10 aspirin non-responders by %CIn (and four out of six aspirin non-responders by %PIn) are converted to responding normally to aspirin as a result of additional clopidogrel therapy. This result is in keeping with a previous study using optical aggregation [8].

The ability of clopidogrel to inhibit AA-induced platelet activation is as yet unexplained but it may relate at least in part to platelet granule release (containing ADP) during platelet activation. Platelet activation by diverse stimuli, including AA, results in platelet granule release and the subsequent action of released ADP on P2Y12 receptors is then blocked by clopidogrel, hence perhaps potentiating the antiplatelet activity of aspirin.

However, the mechanism of action of clopidogrel is undoubtedly complex. Clopidogrel is likely to have an anti-inflammatory action [6], inhibit multiple platelet agonists in addition to ADP, and have an effect on enzymatic components of coagulation [7]. Recent studies have suggested that clopidogrel may even have important clinical effects months after its cessation [21].

The effects of clopidogrel that we have demonstrated on AA-induced platelet activation may have important clinical implications. Clopidogrel withdrawal can precipitate adverse events despite ongoing maintenance therapy with aspirin [22]. For example: a temporal relationship has been reported between cessation of clopidogrel therapy and the onset of stent thrombosis [23]. These findings suggest that one possible mechanism would be that removal of clopidogrel leads to a rebound attenuation of the antiplatelet effect of aspirin. This study raises questions about how to best assess patient responses to aspirin and clopidogrel therapy. Isolated tests of platelet function, even the current gold standard of ADP-induced light transmission aggregation, potentially ignore other such clinically relevant effects of clopidogrel. Short TEG has potential advantages in that it is (a) rapid, (b) provides information on the effects of both aspirin and clopidogrel from a single sample, (c) is a whole blood assay incorporating the effects of platelets, other cellular components, thrombin and clotting factors, and (d) provides information on maximal clot formation due to thrombin stimulation, itself a marker of risk for adverse thrombotic events [17].

This study has some limitations. Whilst the administration of clopidogrel was witnessed we cannot exclude the use of other medications not reported by study participants that could have influenced baseline results, or clopidogrel efficacy. In the patient group, although only those that reported compliance with aspirin therapy were included, the presence of aspirin was not assessed objectively.

Conclusions

Short TEG demonstrates that clopidogrel has both independent and aspirin-synergistic effects, on AA-induced platelet activation. These data may have clinical relevance.

The current gold standard method of assessing response to clopidogrel, ADP-induced platelet aggregation, is unlikely to accurately determine the true clinical effect of clopidogrel therapy, particularly in individuals administered additional antiplatelet therapies.

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