Specific Impairment of Human Platelet P2\textsubscript{Y\textsubscript{AC}} ADP Receptor–Mediated Signaling by the Antiplatelet Drug Clopidogrel

J. Geiger, J. Brich, P. Höning-Liedl, M. Eigenthaler, P. Schanzenbächer, J.M. Herbert, U. Walter

Abstract—Clopidogrel is an effective new antiplatelet agent useful for the treatment of ischemic cerebrovascular, cardiac, infarction, and vascular death. Clopidogrel and the chemically related ticlopidine are thienopyridines that selectively and specifically interfere with ADP-mediated platelet activation. In contrast to ticlopidine, which may cause neutropenia, clopidogrel appears to be a safe and well-tolerated drug. Thienopyridines are inactive in vitro, require in vivo metabolism, and cause an irreversible inhibition of platelet function. However, the mechanism of thienopyridine action is not well established owing to the limited understanding of platelet ADP receptors and their intracellular signaling.

Key Words: platelet inhibition, purinergic receptors, vasodilator-stimulated phosphorylant.

Increased platelet activation and aggregation are central to the pathophysiology of acute and chronic arterial vascular diseases. This concept has gained broad acceptance since platelet inhibitors have been proven as effective agents for the treatment of both chronic and acute diseases of the arterial vessel wall. Platelets are activated by numerous agents and conditions, but ADP is thought to play a key role in the development of arterial thrombosis. Very recently, several laboratories, including those of our own groups, provided evidence for the existence of 3 distinct human platelet ADP receptors, which mediate ADP-caused cation influx (P2X1 receptor), calcium mobilization (P2Y1 receptor), and adenylyl cyclase inhibition (P2Y\textsubscript{AC} receptor). Earlier studies with ticlopidine and clopidogrel had suggested that thienopyridines do not inhibit the ADP receptor pathways coupled to calcium influx and mobilization but instead, prevent the inhibitory ADP effects on adenylyl cyclase stimulation in platelets of different species. In the current study, we addressed the question as to which of the 3 now pharmacologically defined human platelet ADP receptors is affected by a clinically used clopidogrel dosage. Moreover, we analyzed the effect of clopidogrel on ADP-regulated intracellular signaling and protein phosphorylation. These studies were made possible because of the recent development of phosphorylation-specific monoclonal antibodies that allow quantitative analysis of vasodilator-stimulated phosphorylation in intact human platelets. Previously, we showed that NO- and prostaglandin-stimulated phosphorylation of VASP, a

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cytoskeleton/integrin-associated protein present at high concentrations in human platelets,27–30 is closely correlated with the inhibition of platelet and fibrinogen receptor (glycoprotein [GP] IIb/IIIa) activation.30 This study now demonstrates that clopidogrel selectively impairs the human platelet P2Y<sub>AC</sub> ADP receptor and its inhibitory effect on prostaglandin E<sub>2</sub>–stimulated, cAMP-mediated VASP phosphorylation.

**Methods**

**Volunteers**

Six healthy volunteers (2 female and 4 male, with an age range of 23 to 48 years) who were not take any platelet-acting drugs entered this study after their written, informed consent was obtained and after approval of this study by the ethics committee of our university. The volunteers took 75-mg clopidogrel tablets (provided by Sanofi and Bristol-Myers Squibb) daily for 7 days. Blood samples were taken before treatment, after 7 days of treatment, and 4 weeks after treatment had been discontinued. Blood counts of the volunteers were monitored throughout the study and were essentially unaltered.

**Platelet Preparation, Platelet Aggregation, Platelet Calcium, and cAMP Regulation**

Platelet-rich plasma and washed platelets were prepared from whole human blood, and platelet aggregation was determined with the aggregometer PAP-4 (Biodata) as previously described.15 Aggregation responses were determined in 0.3-mL samples of platelet-rich plasma. Aggregation was stimulated with either 20 μmol/L ADP, 5 μmol/L U-46619, or a combination (2.5 μmol/L ADP + 5 μmol/L epinephrine). Aggregation responses are relative to the aggregation before treatment. cAMP levels were stimulated with either 30 nmol/L PGE<sub>1</sub>, or combinations (30 nmol/L PGE<sub>1</sub> + 5 μmol/L ADP; 30 nmol/L PGE<sub>1</sub> + 55 μmol/L epinephrine) as described in “Methods.” cAMP levels are relative to those detected in platelets incubated with ADP alone. Data represent the means of experiments (number of volunteers) as indicated.

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<th>Before Treatment</th>
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<td>simulated with</td>
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</table>

Values are mean±SEM. Platelet aggregation and cAMP levels were analyzed using platelets from volunteers before, during (after 7 days), and after (after 4 weeks) of clopidogrel treatment. Aggregation was stimulated with either 20 μmol/L ADP, 5 μmol/L U-46619, or a combination (2.5 μmol/L ADP + 5 μmol/L epinephrine). Aggregation responses are relative to the aggregation before treatment. cAMP levels were stimulated with either 30 nmol/L PGE<sub>1</sub>, or combinations (30 nmol/L PGE<sub>1</sub> + 5 μmol/L ADP; 30 nmol/L PGE<sub>1</sub> + 55 μmol/L epinephrine) as described in “Methods.” cAMP levels are relative to those detected in platelets incubated with ADP alone. Data represent the means of experiments (number of volunteers) as indicated.

**Western Blot Analysis of VASP Phosphorylation**

The extent of VASP phosphorylation was determined in platelets obtained from platelet-rich plasma, which either was left untreated or incubated for 3 minutes with 5 μmol/L ADP, 55 μmol/L epinephrine, 30 nmol/L PGE<sub>1</sub>, combinations thereof (30 nmol/L PGE<sub>1</sub> + 5 μmol/L ADP; 30 nmol/L PGE<sub>1</sub> + 55 μmol/L epinephrine), or vehicle alone. Platelets were then sedimented by centrifugation, the supernatant plasma was rapidly removed, and the platelet pellet was solubilized in a hot, SDS-containing stop solution. Platelet proteins were separated by SDS–polyacrylamide gel electrophoresis, blotted on nitrocellulose, and analyzed for the extent of VASP serine 239 phosphorylation by the monoclonal antibody 16C2 as described.26

**Results**

**Platelet Aggregation**

Clopidogrel treatment caused the nearly complete inhibition of ex vivo ADP-stimulated platelet aggregation (the Table). This treatment also abolished the ex vivo platelet aggregation in response to the combination of ADP and epinephrine at concentrations that, when used alone, did not activate platelets (the Table). The ADP-induced shape change, U-46619 (a stable thromboxane A<sub>2</sub> analogue)-stimulated aggregation, and thrombin-stimulated aggregation were not significantly inhibited (the Table; shape change and thrombin data not shown). A small inhibitory effect on thromboxane-induced aggregation may have been due to the secondary inhibition of ADP released by thromboxane-activated platelets. Platelet aggregation in response to ADP was essentially normal 4 weeks after clopidogrel treatment was discontinued (the Table). In 1 of our volunteers (a 23-year-old healthy male of normal weight), clopidogrel had little effect on ADP-stimulated platelet aggregation and the other tests analyzed in this study were performed using a Perkin-Elmer LS-50 fluorometer. Platelets were stimulated with 1 μmol/L ADP or 1 μmol/L U-46619 in the presence of 1 nmol/L Ca<sup>2+</sup> or 4 nmol/mL EGTA, respectively.
Interestingly, a regular treatment with ticlopidine was also essentially ineffective in this individual (data not shown). This volunteer had no obvious blood and platelet abnormalities and was not included in our further data analyses. The frequency and mechanisms of impaired clopidogrel (and ticlopidine) responses in some individuals need further investigation.

Stimulated-Platelet Calcium Responses
In agreement with our previous study with ticlopidine, 15 platelet calcium responses evoked by ADP or U-46619 were not affected by clopidogrel treatment. Neither calcium influx nor mobilization of intracellularly stored calcium ions was affected by thienopyridines 15 (Figure 1).

Platelet cAMP Content
Clopidogrel treatment did not significantly alter the basal and PGE1-stimulated cAMP content in platelets. In contrast, the inhibitory effects of ADP (but not those of epinephrine) on PGE1-stimulated cAMP levels were abolished by clopidogrel treatment (the Table).

VASP Phosphorylation
Both ADP and epinephrine inhibited PGE1-stimulated VASP phosphorylation at serine 157 (data not shown; detected by the phosphorylation-induced shift of VASP from the 46- to the 50-kDa form50) and at serine 239 (detected by the phosphorylation-specific monoclonal antibody 16C226) as demonstrated in Figures 2 and 3. Clopidogrel treatment did not alter basal and PGE1-stimulated VASP phosphorylation but strongly attenuated the inhibitory effect of ADP on PGE1-stimulated VASP phosphorylation (Figures 2 and 3). All ADP responses were essentially restored 4 weeks after treatment was discontinued. The inhibitory effects of epinephrine on PGE1-induced VASP phosphorylation were not affected by clopidogrel treatment (Figure 3).

Discussion
The data presented show that a clinically effective dose of clopidogrel9 selectively inhibits ADP-stimulated platelet aggregation and impairs the inhibitory ADP effects on platelet cAMP levels and cAMP-mediated VASP-phosphorylation. In agreement with earlier studies,21–25 clopidogrel inhibits the effects of ADP on adenylyl cyclase stimulation without affecting ADP-induced calcium influx or calcium mobilization in platelets tested ex vivo. As summarized in Figure 4, clopidogrel thus inhibits the G,, protein–coupled P2Y1 ADP receptor but neither the cation channel–coupled P2X1 ADP receptor nor the G,, protein–coupled P2Y1 ADP receptor, which were only very recently identified and characterized.12–20 Interestingly, clopidogrel did not affect the inhibitory, also G,, protein–mediated, effect of epinephrine on platelet cAMP levels and VASP phosphorylation. These findings strongly suggest that clopidogrel impairs the platelet P2Y1 ADP receptor at the receptor level directly or at a level preceding G,, protein(s). This conclusion is also supported by earlier observations that clopidogrel reduces the number of binding sites for 2-methylthio-ADP in human and rat platelets.23,31 The time course of clopidogrel effects (although not extensively studied here) appears to be similar to that of ticlopidine (the Table and other data not shown). The practically irreversible anti-ADP effects of ticlopidine and clopidogrel (which are inactive in vitro) reach their maximum within 3 to 4 days of treatment and are completely gone.

Figure 1. ADP-stimulated calcium responses of clopidogrel-treated platelets. Fura 2–loaded, washed, human platelets were stimulated with 1 μmol/L ADP in the presence of 1 mmol/L CaCl2 or 4 mmol/L EGTA in the buffer medium as indicated. The traces shown are the responses obtained from the same individual before, during (7 days), and after (4 weeks) treatment with clopidogrel. Calcium experiments were performed with the platelets of 2 volunteers, the results being virtually identical for both. arb. indicates arbitrary.

Figure 2. Western blots showing the effect of clopidogrel on PGE1−, ADP−, and epinephrine-regulated VASP serine 239 phosphorylation in platelets. PGE1−, ADP−, and epinephrine-regulated VASP serine 239 phosphorylation was analyzed as described in Methods by using platelets obtained from volunteers before, during (7 days), and after (4 weeks) clopidogrel treatment. A, Lane 1, control; lane 2, PGE1; lane 3, ADP treated; and lane 4, ADP- and PGE1-treated platelets. B, Lane 1, control; lane 2, PGE1; treated; lane 3, epinephrine treated; and lane 4, epinephrine- and PGE1-treated platelets. Western blot data of 1 experiment representative of the results obtained with platelets from 5 volunteers are shown.
The specificity of clopidogrel, which (under the conditions used) impairs the P2Y_{AC} ADP receptor only without detectable effect on many other platelet receptors involved in the activation or aggregation of platelets, is remarkable.

The mechanisms of G protein–coupled receptor regulation of platelet GP IIb/IIIa activation and ultimately aggregation have not been fully elucidated but appear to involve more than 1 intracellular pathway and distinct regulatory molecules, as summarized in Figure 4. Activation of the platelet P2Y_{AC} ADP receptor by ADP liberates the G_{i} protein subunits α_{i} and βγ, which couple to independent signaling events (Figure 4). Subunit α_{i} decreases platelet cAMP levels and (among various cAMP-regulated events, including inhibition of ADP receptor activation of phospholipase C) reduces the level of phospho-VASP. Phosphorylation of VASP in response to cAMP-elevating agents is closely correlated with the inhibition of GP IIb/IIIa. In vivo intact endothelium serves as a source of cAMP-elevating factors such as prostacyclin, which stimulate VASP phosphorylation and inhibit platelet aggregation. Indeed, endothelium-dependent platelet VASP phosphorylation has been demonstrated in platelet–endothelial cell coinclusions and in the intact coronary system. The important regulatory role of VASP in platelet activation/aggregation is supported by very recent data obtained with platelets from VASP-deficient mice. VASP-deficient murine platelets, when compared with wild-type murine platelets, displayed an impaired cyclic nucleotide–mediated inhibition of aggregation and an enhanced thrombin-and-collagen-induced integrin α_{IIb}β_{3} activation. Other recent in vitro data suggest that the platelet P2Y_{AC} and P2Y1 ADP receptors and their G_{i}- or G_{q}-coupled signaling are both required but alone are insufficient to mediate ADP-evoked platelet aggregation. For example, platelet aggregation was not induced by 10 μmol/L ADP in the presence of the P2Y_{AC} blocker ARL 66096 but occurred when an additional 1 μmol/L epinephrine was used. Similarly, 2-methylthio-ADP–induced platelet aggregation was inhibited by the P2Y1 blocker A3P5PS but was restored by the addition of 2.5 μmol/L epinephrine. In our present experiments (the Table), the P2Y_{AC} blocker clopidogrel not only inhibited ADP (20 μmol/L)–evoked aggregation but also the aggregation response by the combination of low-dose (2.5 μmol/L) ADP and 3 μmol/L epinephrine. Whereas 10 to 20 μmol/L ADP alone is sufficient to induce aggregation (see also the Table), low-dose (2.5 μmol/L) ADP concentrations (which alone did not cause aggregation) were chosen to demonstrate the synergism between ADP and epinephrine and its possible sensitivity to clopidogrel treatment. Our data (the Table) demonstrate that the P2Y_{AC} receptor is required for the synergism between ADP and epinephrine with respect to aggregation when low concentrations of ADP are used. Our present data with clopidogrel are in full agreement with the suggestion that both the P2Y_{AC} ADP receptor and the P2Y1 ADP receptor are required and essential for ADP-induced platelet aggregation. The relative contribution of each of these 2 ADP receptors in mediating the in vivo effects of ADP may depend on

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**Figure 3.** Clopidogrel effects on PGE1-, ADP-, and epinephrine-regulated VASP serine 239 phosphorylation in platelets. PGE1, ADP, and epinephrine-regulated VASP serine 239 phosphorylation was analyzed as described (see Methods and Figure 2) by using platelets obtained from volunteers before, during (7 days), and after (4 weeks) clopidogrel treatment. VASP serine 239 phosphorylation data (reported relative to the extent seen in platelets treated with ADP or epinephrine alone) are the mean ± SEM of the results obtained with platelets from 5 volunteers.

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**Figure 4.** Sites of platelet inhibition by clopidogrel, aspirin, and GP IIb/IIIa antagonists. Clopidogrel selectively impairs the molecularly not yet cloned P2Y_{AC} ADP receptor without affecting ADP-gated cation channels (P2X1 ADP receptor), the phospholipase C (PLC)/G_{q}-protein–coupled P2Y1 ADP receptor, or the G_{i}-protein–coupled epinephrine (α_{2A}) adrenergic receptor (α_{2A}-R). G_{i} protein–coupled receptors inhibit and G_{q} protein–coupled receptors promote cAMP-mediated phosphorylation of VASP (P-VASP), which is known to be closely correlated with the inhibition of aggregation and fibrinogen receptor (GP IIb/IIIa) activation. The detailed mechanisms of GP IIb/IIIa activation and inhibition have not been fully elucidated at the molecular level but involve multiple receptors and signaling events as discussed in the text. Aspirin inhibits the generation of platelet thromboxane A_{2} (TXA_{2}) and thereby its receptor (TXA_{2}-R)–mediated events. PI3K indicates phosphoinositide 3-kinase; PTK, phosphotyrosine kinase; and PKC, protein kinase C.
the local concentrations of ADP and the regulation of these ADP receptors by other signaling pathways. In this respect, it is of interest to note that the P2Y1 ADP receptor linked to calcium mobilization is strongly inhibited by both cAMP- and cGMP-regulated pathways.12 P2Y1c receptor–mediated inhibition of VASP phosphorylation may be an important component of ADP-stimulated platelet aggregation in vivo. However, activation of Gi proteins by ADP may also liberate the βγ subunit complex, which is known to activate the C protein kinases, phosphoinositol 3-kinase, and the phosphotyrosine kinases,37 all of which are thought to be linked to GP IIb/IIIa activation.29 Clearly, elucidation of the molecular mechanisms of platelet activation and aggregation mediated by G protein–coupled receptors needs further investigation. In conclusion, the clinical efficacy of clopidogrel and our present data indicate an important role of the platelet P2Y1c ADP receptor in mediating the in vivo effects of ADP that are associated with arterial thrombosis in patients at high risk for arterial cardiovascular complications.

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