Clopidogrel Inhibits the Binding of ADP Analogues to the Receptor Mediating Inhibition of Platelet Adenylate Cyclase


Clopidogrel, like the homologous thienopyridine derivative ticlopidine, selectively inhibits platelet aggregation induced by ADP. We have previously described two nucleotide-binding sites on platelets related to ADP-mediated platelet responses. The first is a high-affinity binding site for 2-methylthio-ADP (2-MeSADP) that is linked to the inhibition of stimulated adenylate cyclase. The second is the 100-kd exofacial membrane protein aggregin, which is labeled by the reactive ADP analogue 5'-p-fluorosulfonylbenzoyladenosine (FSBA) that is related to shape change and aggregation. We set out to determine if either of these sites is blocked in vivo by clopidogrel or its active metabolite. Six subjects were given clopidogrel (75 mg/day for 10 days) in a double-blind crossover experiment. All of the subjects developed prolonged bleeding times while taking the drug. The rate of onset of the effect on bleeding time varied among subjects. Platelet aggregation induced by ADP or thrombin was significantly impaired by the drug treatment, but no effect was detected on shape change. Total incorporation of [32P]2-MeSADP into aggregin was also unaffected. Inhibition of adenylate cyclase by ADP or by 2-MeSADP was greatly reduced in all subjects, and in the case of 2-MeSADP, there was evidence for a noncompetitive effect. Inhibition of adenylate cyclase by epinephrine was unaffected. In the three subjects for whom binding measurements were made, the number of binding sites for [32P]2-MeSADP was reduced from 534±44 molecules per platelet during control and placebo periods (11 determinations) to 199±78 molecules per platelet during drug treatment (three determinations). There was no consistent change in the affinity of platelet function by clopidogrel is associated with a selective reduction in the number of functional receptors mediating the inhibition of stimulated adenylate cyclase activity by ADP.

KEY WORDS • ADP receptor • adenylate cyclase • platelets • thienopyridine • aggregation • aggregin • clopidogrel

Platelets from humans and other mammalian species aggregate in response to exposure to ADP at micromolar concentrations. Aggregation is contingent on the presence of fibrinogen and calcium ions in the medium, the metabolic activity of the platelets, and exposure of the functional integrin GPIIb/IIIa complex that acts as a fibrinogen-binding site on activated platelets. In contrast, ADP-induced shape change, which is associated with a transient increase in cytoplasmic Ca²⁺ and activation of calmodulin-dependent myosin light-chain kinase, does not require fibrinogen, integrins, or extracellular Ca²⁺. ADP, which does not normally cross the cell membrane, is assumed to raise cytoplasmic Ca²⁺ by activating a specific transmembrane receptor coupled to Ca²⁺ gating. ADP may also activate phospholipase C, leading to formation of diacylglycerol and inositol-1,4,5-trisphosphate and mobilization of Ca²⁺ from its intracellular depots (for recent reviews, see References 2 and 4).

Like several other aggregating agents, ADP inhibits platelet adenylate cyclase, also presumably by a receptor-mediated action. In intact cells this is demonstrated as a reduction of the increase in platelet adenosine 3', 5'-cyclic monophosphate (cAMP) concentration that follows exposure to prostacyclin (prostaglandin I₂ [PGI₂]) or other stimulators of adenylate cyclase. Inhibition by ADP of a particulate preparation of adenylate cyclase from platelet membranes has been observed, with evidence for a requirement for guanine nucleotides. A receptor for ADP that mediates this effect has been identified by the binding and displacement of 2-azido-ADP (2-N₃ADP) and 2-methylthio-ADP (2-MeSADP). Normal human platelets have between 500 and 1,000 of these receptors per cell, with a binding affinity for 2-MeSADP on the order of 10 nM, and a half-maximal displacement of 2-MeSADP binding by 10 μM ADP.

Aggregation of platelets by ADP is inhibited by the reactive analogue 5'-p-fluorosulfonylbenzoyladenosine (FSBA). Under conditions that lead to inhibition of shape change (incubation of washed platelets for 30 minutes with 40 mM FSBA), initiated FSBA ([3H]FSBA)
is incorporated into a platelet membrane protein of about 100 kd, a reaction inhibited by ADP and ATP. This protein, a putative ADP receptor, has been given the name aggregin. As FSBA neither blocks the effect of ADP on adenylyl cyclase nor inhibits the binding of 2-MeSADP, it is clear that aggregin and the high-affinity 2-MeSADP receptor previously identified are functionally distinct.

Thienopyridine compounds, including the antithrombotic drugs ticlopidine and clopidogrel (SR 25990C), have been found to selectively inhibit platelet aggregation induced by ADP. To elucidate the relations between aggregin, the 2-MeSADP receptor, and the various responses of platelets to ADP, we have studied platelet responses before and after administration of clopidogrel or a placebo to human volunteers.

Methods

Materials

Human thrombin (3,203 National Institutes of Health units/mg protein) was kindly supplied by Dr. John W. Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. Human fibrinogen was from Kabi, Franklin, Ohio. [2,8-3H]adenine (1.06 TBq/mmol) and [32P]phosphate (carrier free) were from ICN Biomedicals, Costa Mesa, Calif., and [U-14C]adenine (10 GBq/mmol) was from Amersham Corp., Arlington Heights, Ill. Dowex AG50, urea, and acrylamide were from Bio-Rad, Richmond, Calif. [H]FSBA (500 GBq/mol) was prepared as reported.

Design of the Experiment

Six healthy male volunteers, aged 22–35, gave informed written consent to a protocol approved by the Temple University School of Medicine Institutional Review Board. They were studied for a period of 73 days divided into three segments. In the first period each subject was given either placebo or clopidogrel, 75 mg daily by mouth for 10 days. After a washout period of 50 days without treatment, in the third period each subject was given either placebo or clopidogrel, 75 mg daily by mouth for 10 days. After a further 10-minute incubation at 37°C, the centrifugation was repeated and the pellet finally resuspended in buffer A containing 18.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.35.

Platelet Aggregation and Shape Change

These measurements were performed at 37°C in a Chronolog Lumiaggregometer. Shape change was measured after dilution to 1 x 10^8 platelets/ml and addition of 4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid to prevent aggregation. Aggregation was measured at a standard platelet count of 5 x 10^10 platelets/ml after addition of 1 mg/ml fibrinogen. Aggregation and shape change are expressed as the rate of increase or decrease. respectively, of light transmission in millivolts per minute during 1 minute after the addition of an agonist.

Labeling of Aggregin

Washed platelets (6 ml, 5 x 10^10 platelets/ml) were incubated with 100μM [H]FSBA for 40 minutes at 23°C. The labeled platelets were sedimented and washed. Membranes were prepared by the glycerol osmotic lysis method; solubilized in a solution of sodium dodecyl sulfate (SDS), urea, and diithiothreitol; and dialyzed; and the retentate was assayed for radioactivity. With each volunteer, a portion of the retentate obtained from at least one experiment was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 7.5% gels to confirm the labeling pattern. The gels were sliced and assayed for radioactivity. The radioactivity peak at 100 kd corresponds to [H]FSBA–labeled aggregin.

Adenylate Cyclase Inhibition

PRP was incubated for 1 hour with 1 μM [U-14C]adenine to label intracellular ATP. Duplicate samples of this labeled PRP (0.45 ml) were incubated for 2 minutes with 50 μl saline containing 50 mM EDTA and five concentrations of ADP (final concentration, 0.1–10 μM), five concentrations of 2-MeSADP (1–100 nM), and three concentrations of epinephrine (0.01–1 μM). PGI2 (0.1 μM) was then added, and after a further 2-minute incubation the reaction was stopped with trichloroacetic acid; the radioactivity of cAMP was then measured after purification by chromatography on Dowex AG50 and alumina. An internal standard of [H]cAMP was used to measure recovery. Hill plots of the data were used to interpolate, where possible, the
concentration of each inhibitor required for 50% inhibition (ED$_{50}$). In one experiment in which adenylate cyclase was measured in isolated platelets, the platelets were labeled by incubation for 1 hour with 0.2 nM [2,8-$^3$H]adenine, acidified to pH 6.4, centrifuged at 1,030g for 15 minutes at room temperature, and resuspended in isotonic saline buffered to pH 7.4 with 10 mM HEPES. [14C]cAMP was used as the recovery standard.

Equilibrium Binding of $\beta$-$[^32P]2$-MeSADP

2-MeSAMP was converted to the imidazolate and phosphorylated with tri-n-butylammonium phosphate (37 GBq/mmol). The diphosphate was isolated by paper electrophoresis followed by anion-exchange high-performance liquid chromatography. Samples of PRP (0.45 ml) were incubated for 10 minutes at 37°C with 50 μl saline containing 50 mM EDTA and the radioligand at 10 concentrations in the range 2–120 nM (final). Duplicate aliquots (0.2 ml) were layered over a cushion of 20% sucrose in tris(hydroxymethyl)aminomethane-buffered saline containing 10 mM EDTA in Sarstedt microsediment collection tubes and centrifuged for 2 minutes at full speed in a modified Fisher Model 59 microcentrifuge. The tubes were frozen in solid CO$_2$ and cut 1 mm above the pellet. Pellet and supernatant were assayed for radioactivity by Cerenkov counting. Nonspecific binding was determined at two concentrations of the radioligand in the presence of 1 mM ADP.

Statistical Analysis

In each case the mean of observations on all subjects made on a given day, placebo, pre 2, or drug, was compared with the mean for day 1 (pre 1) by the unpaired $t$ test. Alternatively, the results obtained for each subject for the drug period were compared, using the paired $t$ test, with the average results for all other periods for the same donor.

Results

Treatment with 75 mg clopidogrel/day for 10 days caused a sharp increase in the Ivy bleeding time in all of the six subjects, although the pattern of increase was diverse (Figure 1). In two subjects (C and F) the increase in bleeding time was rapid but small. In two others (A and B) it was slower but more pronounced. In the remaining two subjects (D and E) the increase was rapid and large. In five of the six subjects the bleeding time had returned to near normal within 4 days of withdrawing the drug. In the sixth (D) it was still abnormal 4 days after withdrawing the drug. The mean bleeding time (±SEM) for all observations in the pre 1, pre 2, and placebo periods was 5.4±0.2 minutes ($n=42$). The mean bleeding time was increased in the drug period to 18.1±2.3 minutes ($n=18$), recording “30 minutes” for bleeding times greater than or equal to 30 minutes. There was no significant difference between the bleeding times measured in pre 1 and those in pre 2 or placebo. No other hematological abnormalities were detected, and there was no evidence of blood loss or other consequences of this marked effect on cutaneous hemostasis. All of the other routine blood chemistry tests, including serum electrolytes, liver and kidney function, blood glucose, hematocrit and hemoglobin, and whole-blood platelet and leukocyte counts, remained within normal limits throughout the study.

Platelet aggregation and shape change in response to ADP and thrombin (Figure 2) were studied in washed platelet suspensions so that the results would be directly comparable to those of the aggregin labeling experiments. No significant difference in the shape change response to either 1 μM ADP or 1 nM thrombin was seen when the drug period was compared with the
Incorporation of the reactive ADP analogue \(^{[3]H}\)FSBA into platelet membranes was unaffected by drug treatment. The mean extent of incorporation during the drug period (53.0±3.2 cpm/10^8 platelets) was essentially the same as the mean in the other three periods (51.1±1.8). This value corresponds to the incorporation of about 31.8×10^3 molecules of FSBA per platelet, without allowance for possible losses during membrane isolation. Samples of labeled membranes analyzed on nonreduced SDS-PAGE confirmed that the radioactivity was predominantly in the 100-kd protein.

When platelets are incubated in PRP with radioactive adenine, the label is rapidly incorporated into the cytoplasmic pool of rapidly equilibrating adenine nucleotides, predominantly as ATP; stored nucleotides in the dense granules and actin-bound ADP are labeled much more slowly. When prelabeled platelets are incubated with 0.1 \(\mu\)M PGI\(_2\), within 2 minutes the radioactivity of cAMP in the cells increases from the basal level (<0.2%) to 1.5–3.5% of the total intracellular radioactivity. Preincubation of the platelets for 2 minutes with epinephrine, ADP, or 2-MeSADP causes a dose-dependent reduction in the extent of cAMP accumulation. The \(ED_{50}\) for ADP for the inhibition of adenylate cyclase in PRP from control subjects was 2.7±0.3 \(\mu\)M (\(n=18\)). After drug ingestion the value obtained was 7.8 \(\mu\)M in one case (subject E) and was greater than the highest concentration tested (10 \(\mu\)M) in the other five (Figure 3). The \(ED_{50}\) for 2-MeSADP for the inhibition of adenylate cyclase in PRP from control subjects was 4.4±1.2 nM (\(n=18\)). This value was increased during drug treatment to 14.8 nM in subject E. In the other five subjects less than 50% inhibition was seen at 100 nM, the highest concentration used. In PRP from normal donors the Hill coefficient for inhibition by ADP or 2-MeSADP, as obtained from the slope of the plot of logits of these effects against the log of the dose, is generally between 0.8 and 1.0. In most cases Hill plots of the drug period data were notably curved, indicating a loss of efficacy of the agonist and a noncompetitive effect of clopidogrel (data not shown). In contrast, the Hill plots for epinephrine inhibition of adenylate cyclase were linear and parallel. There was a small increase in the mean \(ED_{50}\) for epinephrine from 88±11 to 151±44 nM, but the effect was not statistically significant.
In one experiment with blood from subjects E and F and a third normal donor who was not included in the trial, we compared the inhibition of adenylate cyclase in PRP (Figure 4A) and in the same platelets isolated by centrifugation and resuspended in HEPES-buffered saline (Figure 4B). With all three donors, the isolated platelets showed the same or greater sensitivity to the inhibitory effect of epinephrine as platelets in PRP; their sensitivity to ADP and 2-MeSADP was somewhat reduced. The two experimental subjects, E and F, were both receiving clopidogrel on the day this experiment was done. Subject F showed a very strong response to the drug so that no estimate of ED50 was possible for either ADP or 2-MeSADP; subject E showed only a modest decrease in response to ADP or 2-MeSADP. These responses were essentially the same in PRP and washed platelets.

We decided to study the binding of 2-MeSADP to platelets in PRP, in part because a competitive effect of clopidogrel or some metabolic derivative on binding could be missed by isolating the platelets from their plasma. Measurements of binding affinity (20.8±4.4 nM) and of the number of binding sites (534±44 per platelet) from 11 separate determinations in four subjects during control or placebo phases of the trial were in agreement with published figures for normal donors.8 Only three measurements were possible (on subjects A, E, and F) during the drug treatment phase. In all three of these subjects the number of sites was considerably lower than estimates made on the same subject during the control phase, and the mean of the treatment measurements (199±78 sites per cell) was significantly lower than the control mean (p<0.01). In subject F, binding was so low that it was close to the limits of detection, and no estimate for the binding affinity was made (Figure 5). For subjects A and E the binding affinity during drug treatment was at the higher end of the normal range.

**Discussion**

The thienopyridine drugs ticlopidine and clopidogrel have been proposed as potential antithrombotic agents.
Clopidogrel Blocks ADP Receptors on Platelets

by Mills et al

We have demonstrated two distinct nucleotide-binding sites on platelet membranes. The first of these is a high-affinity binding site for ADP and certain 2-substituted ADP analogues. This site was identified by equilibrium binding to platelets of 2-N\textsubscript{3}ADP labeled with \textsuperscript{32}P in the β-phosphate\textsuperscript{7} and was confirmed with the tighter binding analogue, 2-MeSADP, that was similarly labeled.\textsuperscript{8} The binding affinity of 2-MeSADP in control platelets estimated from binding isotherms performed in PRP was 20.8 nM compared with an ED\textsubscript{50} for inhibition of adenylate cyclase, also in PRP, of 4.4 nM, values that are close to published figures.\textsuperscript{5,8} The ED\textsubscript{50} for ADP for inhibition of adenylate cyclase was 2.7 ±0.3 μM (n = 18) in control platelets, whereas the K\textsubscript{D} for displacement of bound 2-MeSADP by ADP was 3.5 μM.\textsuperscript{8} 2-NADP, with intermediate affinity, binds to platelets with an apparent K\textsubscript{D} of 140 nM, and inhibits adenylate cyclase with an ED\textsubscript{50} of 88 nM.\textsuperscript{7} These figures suggest that the site identified by 2-MeSADP binding is the receptor through which ADP inhibits adenylate cyclase. This conclusion is supported by the observation that the non-cell-penetrating thiol reagent p-chloromercuribenzenesulfonate blocks the binding of both 2-N\textsubscript{3}ADP\textsuperscript{7} and 2-MeSADP\textsuperscript{9} to platelets. This reagent blocks the inhibitory effect of ADP and its analogues on adenylate cyclase without inhibiting ADP-induced shape change.

The reactive nucleotide analogue FSBA has been used to identify the binding sites for adenine nucleotides on a large number of purified proteins.\textsuperscript{16} FSBA inhibits platelet aggregation and shape change induced by ADP, although it has no effect on adenylate cyclase or on the inhibition of adenylate cyclase by ADP.\textsuperscript{12} Inhibition of shape change by FSBA is progressive, corresponding to the covalent incorporation of label from \textsuperscript{3}H\textsubscript{FSBA} into a platelet membrane protein, aggregin, of M\textsubscript{r} 100 kd.\textsuperscript{17} No other proteins become labeled under these conditions. ADP prevents the incorporation of FSBA, and at high concentrations FSBA causes a rapid shape change,\textsuperscript{18} supporting the conclusion that FSBA acts at the same site as ADP.

Our results confirm that clopidogrel has a dramatic effect on the bleeding time when given orally to human volunteers and that platelet aggregation induced by ADP and thrombin is markedly inhibited. We saw no effect of the drug on shape change induced by either agent, although wide variations in the responsiveness of control samples of washed platelets made accurate comparisons difficult. The incorporation of label from \textsuperscript{3}H\textsubscript{FSBA} into platelet membrane proteins was entirely unaffected by treatment with clopidogrel, indicating that the drug has no effect on the availability or the nucleotide-binding properties of aggregin.

Adenylate cyclase inhibition by ADP and by 2-MeSADP was severely curtailed by clopidogrel in all of the six volunteers studied, in agreement with recent findings in experimental animals that clopidogrel given orally reduces the inhibitory effect of ADP on platelet adenylate cyclase stimulated by PGs.\textsuperscript{14} Similar results have been seen in human volunteers given ticlopidine.\textsuperscript{15}

The similarity between the results obtained in PRP and after isolation of the platelets indicates that the effects of clopidogrel are not easily reversed during short-term in vitro experiments and suggest either a permanent modification of the receptor or a defect in its biosynthesis. The effect is apparently indirect, as neither

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**FIGURE 5.** Panel A: Binding isotherms for 2-methylthio-ADP (2-MeSADP) with the platelets of one subject (F) in platelet-rich plasma, showing three measurements made during control periods (pre 1, placebo, pre 2) and one measurement at the end of the drug treatment period (drug). Results are corrected for nondisplaceable binding measured in the presence of 1 mM ADP, equivalent to 0.1–0.3% of the added isotope, or less than 5% of the specific binding at half saturation in controls. Panel B: Scatchard plots of the data from panel A. The lines were drawn by eye.
the drugs themselves nor their principal metabolites have demonstrable effects on platelets in vitro.13

It has previously been shown that ticlopidine, which is similar to clopidogrel in structure and pharmacology, blocks the binding of [3H]ADP to a low-affinity site (Kd > 100 μM) on human platelets.19 Experiments with ring-labeled nucleotides are complicated by the possibility that dephosphorylation by enzymes in plasma or on the platelet surface would lead to the generation of labeled adenosine, which is rapidly accumulated into the cell and phosphorylated to ATP. It is possible that inhibition of this nonsaturable binding may in fact represent the blockade of nucleoside uptake or of ADP metabolism.

We have found no correlation between the severity of the bleeding time response and the effect on adenylate cyclase activity or, indeed, between the cyclase effect and the impairment of 2-MeSADP binding; more precise measurements on larger numbers of subjects will be required to examine this question. The variability in the speed of onset and the extent of the increase in the bleeding time suggest that clopidogrel is metabolized at a variable rate in different subjects; the appearance in four subjects of prolonged bleeding times within 2 days of drug treatment suggests that this effect, if mediated by platelets, is due to an action on the mature cell rather than to a failure of normal development of the megakaryocyte.

These results do not offer a convincing mechanism of action for the antithrombotic thienopyridine compounds, but they do suggest some novel possibilities. Aggrelin appears to be involved in ADP-induced shape change and exposure of fibrinogen-binding sites, but it plays no role in the action of clopidogrel. On the other hand, the fact that clopidogrel blocks the 2-MeSADP binding site, a receptor through which ADP inhibits adenylate cyclase, is clear; however, it is not known how the blocking of this receptor modulates ADP-induced aggregation. Although many aggregating agents, including platelet-activating factor, epinephrine, thrombin, and vasopressin, share the ability to inhibit adenylate cyclase, inhibition of this enzyme by the intracellular "P"-site agonist 2'5'-dideoxycadenosine does not cause or potentiate aggregation.20 2-MeSADP inhibits adenylate cyclase at concentrations 100–200-fold lower than are required to cause aggregation. Our data do, however, suggest that a defect in the receptor mechanism for adenylate cyclase inhibition may be associated with impaired aggregation.

References
Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase.

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