



Specific inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro*

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1 The thienopyridine clopidogrel is a specific inhibitor of ADP-induced platelet aggregation *ex vivo*. No direct effects of clopidogrel ($\leq 100 \mu\text{M}$) on platelet aggregation *in vitro* have been described so far.

2 Possible *in vitro* antiaggregatory effects (turbidimetry) of clopidogrel were studied in human platelet-rich plasma and in washed platelets.

3 Incubation of platelet-rich plasma with clopidogrel ($\leq 100 \mu\text{M}$) for up to 8 h did not result in any inhibition of ADP ($6 \mu\text{M}$)-induced platelet aggregation.

4 Incubation of washed platelets with clopidogrel resulted in a time- (maximum effects after 30 min) and concentration-dependent ($\text{IC}_{50} 1.9 \pm 0.3 \mu\text{M}$) inhibition of ADP ($6 \mu\text{M}$)-induced platelet aggregation. Clopidogrel ($30 \mu\text{M}$) did not inhibit collagen ($2.5 \mu\text{g ml}^{-1}$), U46619 ($1 \mu\text{M}$)- or thrombin (0.1 u ml^{-1})-induced platelet aggregation. The inhibition of ADP-induced aggregation by clopidogrel ($30 \mu\text{M}$) was insurmountable indicating a non-equilibrium antagonism of ADP actions. The R enantiomer SR 25989 C ($30 \mu\text{M}$) was significantly less active than clopidogrel ($30 \mu\text{M}$) in inhibiting platelet aggregation ($32 \pm 5\%$ vs $70 \pm 1\%$ inhibition, $P < 0.05$, $n = 5$).

5 In washed platelets, clopidogrel ($\leq 30 \mu\text{M}$) did not significantly reverse the inhibition of prostaglandin E_1 ($1 \mu\text{M}$)-induced platelet cyclic AMP formation by ADP ($6 \mu\text{M}$).

6 The antiaggregatory effects of clopidogrel were unchanged when the compound was removed from the platelet suspension. However, platelet inhibition by clopidogrel was completely abolished when albumin (350 mg ml^{-1}) was present in the test buffer.

7 It is concluded that clopidogrel specifically inhibits ADP-induced aggregation of washed platelets *in vitro* without hepatic bioactivation. Inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro* occurs in the absence of measurable effects on the reversal of PGE_1 -stimulated cyclic AMP by ADP.

Keywords: Clopidogrel; SR 25989 C; platelets; aggregation; shape change; ADP

Abbreviations: ADP, adenosine 5'-diphosphate; cyclic AMP, adenosine 3':5'-cyclic monophosphate; PGE_1 , prostaglandin E_1

Introduction

Clopidogrel is an antiplatelet compound that has recently been shown to be effective in the secondary prevention of cardiovascular complications of atherosclerosis (CAPRIE Steering Committee, 1996). The thienopyridines clopidogrel and ticlopidine are specific inhibitors of ADP-induced platelet aggregation but not of ADP-induced shape change or Ca^{2+} transients (for reviews see Coukell & Markham, 1997; Schrör, 1998). Although the exact mechanisms are not known, treatment with clopidogrel reduces the binding of ADP or stable ADP analogues to high-affinity binding sites on platelets (Mills *et al.*, 1992; Savi *et al.*, 1994b; 1997; Gachet *et al.*, 1995). The ADP receptors that are modified by clopidogrel are also believed to mediate the inhibition of prostaglandin I_2 - or E_1 -induced cyclic AMP formation by ADP (Defreyn *et al.*, 1991; Mills *et al.*, 1992).

In platelet-rich plasma, clopidogrel ($\leq 100 \mu\text{M}$) does not inhibit platelet aggregation *in vitro* (Herbert, 1994). In rats, the *in vivo* activity of clopidogrel has been proposed to be dependent on hepatic biotransformation to an active metabolite (Savi *et al.*, 1992). In these studies, clopidogrel (40 mg kg^{-1}) was less effective in hepatectomized rats as compared to normal control rats. In addition, clopidogrel did

inhibit platelet aggregation in isolated, blood-perfused rat livers. The bioactivation of clopidogrel has been suggested to be mediated by the hepatic cytochrome P450 system (Savi *et al.*, 1994a).

However, an active metabolite of clopidogrel has not been published so far and the need for hepatic activation of clopidogrel has not been demonstrated in humans. In this context, it is noteworthy that in animal studies much higher doses of clopidogrel were used as compared to the standard dosage for humans (75 mg d^{-1}). Thus, different mechanisms may account for platelet inhibition in humans as compared to animal studies.

This study demonstrates that clopidogrel specifically inhibits ADP-induced aggregation of washed human platelets *in vitro* without the need of hepatic bioactivation. Inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro* occurs in the absence of measurable effects on the reversal of PGE_1 -stimulated cyclic AMP by ADP.

Methods

Preparation of washed human platelets

Washed human platelets were prepared as previously described (Weber *et al.*, 1993). Fresh citrated blood was obtained from a local blood-bank. Platelet-rich plasma was prepared by

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centrifugation at $250 \times g$ for 10 min at room temperature. The pH was adjusted to 6.5 with acidic citrate dextrose (Biotest, Frankfurt, Germany). The platelets were washed twice in a buffer (pH 6.5) containing [mM]: NaCl 113, Na_2HPO_4 4, NaH_2PO_4 24, KH_2PO_4 4, supplemented with 0.05 u ml^{-1} apyrase (Grade V, Sigma, Deisenhofen, Germany), 5 mM glucose and 50 nM prostaglandin E_1 . Washed platelets were resuspended in HEPES-buffered Tyrode solution (pH 7.4) of the following composition [mM]: NaCl 134, NaHCO_3 12, KCl 2.9, NaH_2PO_4 0.36, MgCl_2 1, CaCl_2 2, HEPES 5, supplemented with 5 mM glucose. When indicated, clopidogrel (0.3–30 μM) or vehicle was incubated with the platelets for 2.5–120 min at 37°C prior to stimulation. In some experiments, platelet-rich plasma was pre-incubated with acetylsalicylic acid (10 μM) for 15 min.

Platelet aggregation and shape change

Platelet function was measured as previously described (Weber *et al.*, 1993). Briefly, 400 μl of washed platelet suspension or 400 μl of platelet-rich plasma were incubated with 90 μl of test buffer (HEPES-buffered Tyrode, see above) supplemented with 1.5 mg ml^{-1} fibrinogen (final concentration 0.3 mg ml^{-1}) in a 2-channel aggregometer (Labor, Hamburg, Germany) for 2 min at 37°C . Platelets were stimulated by adding 10 μl of the respective stimulus (0.2–600 μM ADP, $2.5 \mu\text{g ml}^{-1}$ collagen, 1 μM U46619, or 0.1 u ml^{-1} thrombin, respectively). Changes in light transmission were recorded during constant stirring of the samples (1,200 r.p.m., 37°C). Aggregation responses were assessed by measuring the maximum change of light transmission (% of control) and the change of light transmission (% of control) 4 min after addition of the stimulus. For shape change measurements, washed platelets were resuspended in Ca^{2+} -free test buffer. 400 μl of washed platelet suspension were incubated with 90 μl of test buffer supplemented with 10 mM EGTA (final concentration 2 mM) in a 2-channel aggregometer (Labor, Hamburg, Germany) for 2 min at 37°C . Platelets were stimulated by adding 10 μl of the stimulus (0.2–20 μM ADP). Shape change responses were assessed by measuring the decrease in light transmission.

Determination of cyclic AMP in washed platelets

Washed platelets (480 μl) were incubated in an aggregometer for 1 min at constant stirring (1,200 r.p.m., 37°C). Then, 1 μM prostaglandin E_1 (10 μl) or buffer (10 μl) were added for 1 min. Subsequently, the platelets were stimulated with 6 μM ADP (10 μl). After 1 min, the reaction was terminated by adding 100 μl 25% HClO_4 . The samples were neutralized with 125 μl 2 M K_2CO_3 and centrifuged at $10,000 \times g$ for 5 min. Cyclic AMP was determined in the supernatants by radioimmunoassay as previously described (Schröder & Schrör, 1993).

Materials

Acidic citrate-dextrose (Biostabil[®], Biotest, Frankfurt, Germany); ADP (Boehringer, Mannheim, Germany); clopidogrel, SR 25989 C (Sanofi Recherche, Toulouse, France); collagen (Collagenreagent Horm[®], Nycomed, München, Germany); [^3H]-cyclic AMP (New England Nuclear, Dreieich, Germany); U46619 (Upjohn Diagnostics, Heppenheim, Germany). α -Thrombin was a gift from Dr J. Stürzebecher, Erfurt, Germany. All other reagents were obtained from Sigma (Deisenhofen, Germany).

Statistics

Data are means \pm s.e. mean from n experiments. Statistical analysis was performed using two-tailed Student's t -test for paired or unpaired data, as applicable. $P < 0.05$ was considered significant.

Results

Clopidogrel does not inhibit platelet aggregation in platelet-rich plasma

When clopidogrel ($\leq 100 \mu\text{M}$) was incubated with platelet-rich plasma, no inhibition of ADP (6 μM)-induced platelet aggregation was observed. Even an incubation of clopidogrel with platelet-rich plasma for up to 8 h did not result in any antiaggregatory activity of the compound (not shown).

Clopidogrel selectively inhibits ADP-induced aggregation in washed platelets

In contrast, when clopidogrel (0.3–30 μM) was incubated with washed platelets for 1 h, a concentration-dependent inhibition of ADP (6 μM)-induced platelet aggregation was seen (Figure 1). Incubation of washed platelets with clopidogrel resulted in a reduction of the maximum aggregation amplitude. In addition, there was a marked effect on the reversibility of ADP-induced aggregation in clopidogrel-treated platelets. Thus, both, maximum aggregation amplitudes as well as aggregation amplitudes 4 min after addition of the stimulus, were used for quantification of clopidogrel effects (Figure 2). Data analysis revealed a significant ($P < 0.05$) inhibition of ADP-induced platelet aggregation by $\geq 0.3 \mu\text{M}$ clopidogrel. From the data presented in Figure 2B, an IC_{50} value of $1.9 \pm 0.3 \mu\text{M}$ was calculated. Importantly, the inhibitory effects of clopidogrel on aggregation of washed platelets were selective for ADP. Platelet aggregation, induced by collagen ($2.5 \mu\text{g ml}^{-1}$), the thromboxane A_2 mimetic U46619 (1 μM) or thrombin (0.1 u ml^{-1}), was not significantly inhibited by clopidogrel (30 μM) (Figure 2). Interestingly, in the aggregation experiments, ADP (6 μM)-induced platelet shape change did not seem to be affected by clopidogrel. However, because platelet aggregation interferes with the measurement of platelet

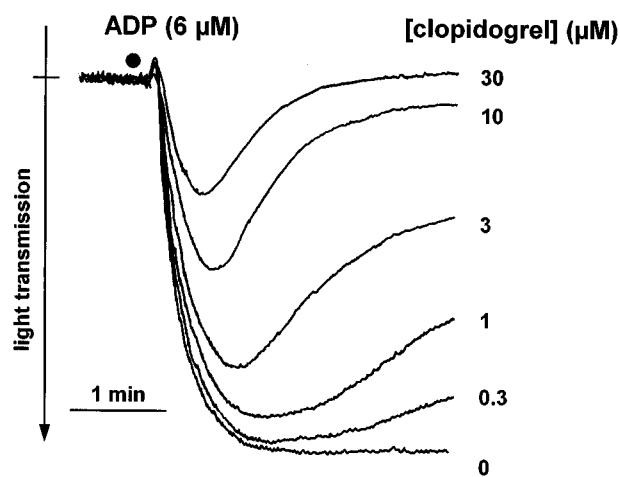


Figure 1 Representative original tracings demonstrating the effects of clopidogrel (0.3–30 μM) on ADP (6 μM)-induced aggregation and shape change of washed human platelets.

shape change, possible effects of clopidogrel on platelet shape change were studied in a separate series of experiments, performed in the presence of EGTA. In these studies, a complete inhibition of platelet shape change by clopidogrel was observed at low concentrations of ADP (0.2 μM) (Figure 3). At higher ADP concentrations (2 μM , 20 μM), the inhibitory effects of clopidogrel on ADP-induced platelet shape change were less obvious (Figure 3).

The inhibitory effects on platelet aggregation are restricted to apyrase-sensitive mechanisms

In order to further characterize if the *in vitro* actions of clopidogrel are specific for ADP-mediated processes, the antiaggregatory effects of clopidogrel were compared with apyrase, an ADP/ATP-degrading enzyme. In control experiments, apyrase (1 u ml⁻¹) has been shown to completely inhibit ADP-induced platelet aggregation. The effects of apyrase on collagen (2.5 $\mu\text{g ml}^{-1}$)- and U46619 (1 μM)-induced platelet aggregation were studied. Similar to clopidogrel, (see Figure 2), apyrase (1 u ml⁻¹) did not inhibit collagen (2.5 $\mu\text{g ml}^{-1}$)- or U46619 (1 μM)-induced aggregation of washed platelets (not shown). Therefore, we have repeated the experiments with acetylsalicylic acid

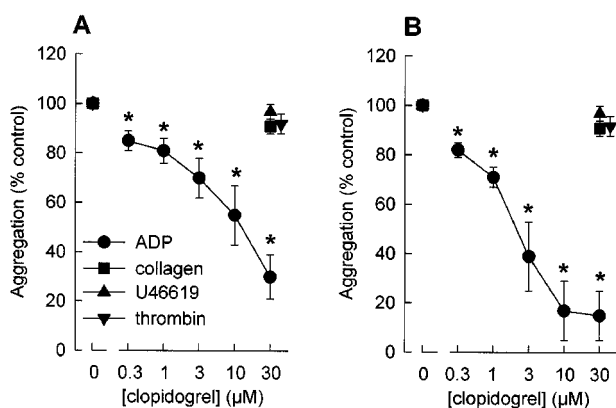


Figure 2 Effects of clopidogrel (0.3–30 μM) on ADP (6 μM)-, collagen (2.5 $\mu\text{g ml}^{-1}$), U46619 (1 μM) or thrombin (0.1 u ml⁻¹)-induced aggregation of washed human platelets. The aggregation was assessed by measuring the maximum change of light transmission (A) and the change of light transmission 4 min after addition of the stimulus (B). Data are given as % of aggregation response induced by the respective agonists without clopidogrel (control=100%). (means \pm s.e. mean of $n=5-6$ independent experiments, * $P<0.05$ vs control, Student's *t*-test for paired data).

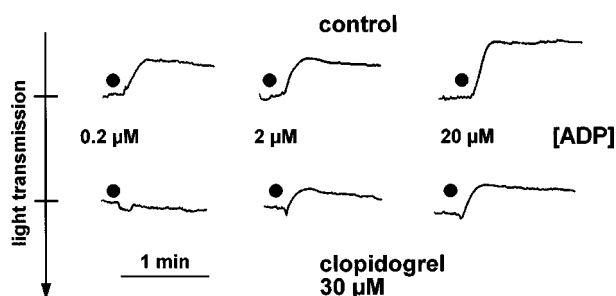


Figure 3 Original tracings demonstrating the effects clopidogrel (30 μM) on ADP (0.2–20 μM)-induced platelet shape change. The experiments were carried out in a Ca^{2+} - and fibrinogen-free medium supplemented with 2 mM EGTA. Shown is one representative experiment of $n=3$ with similar results.

(10 μM) pre-treated platelets. In this system, apyrase significantly ($P<0.05$) inhibited collagen-induced platelet aggregation, indicating that ADP is an essential amplification mechanism for collagen-induced aggregation of acetylsalicylic acid-treated platelets (Figure 4). Interestingly, clopidogrel (30 μM) also significantly reduced the aggregation of acetylsalicylic acid-treated platelets to an extent similar to the effects of apyrase (Figure 4). In contrast, when the effects of apyrase and clopidogrel, respectively, on U46619-induced aggregation of acetylsalicylic acid-treated platelets were studied, apyrase did not inhibit U46619-induced platelet aggregation, indicating that the thromboxane A₂ mimetic is capable of stimulating platelet aggregation independently of ADP-mediated amplification mechanisms even after cyclooxygenase inhibition. Consistently, there was no inhibition of U46619-induced aggregation of acetylsalicylic acid-treated platelets by clopidogrel (30 μM). Thus, antiaggregatory actions of clopidogrel can only be observed in apyrase-sensitive systems. These data provide further evidence for the ADP selectivity of clopidogrel actions *in vitro*.

The inhibition of ADP-induced aggregation by clopidogrel is insurmountable

In order to further characterize the effects of clopidogrel (30 μM , 60 min) on ADP-induced platelet aggregation *in vitro*, ADP concentration-effect curves had been constructed (Figure 5). In these experiments, the maximum response to ADP was reduced by clopidogrel indicating a non-equilibrium antagonism of ADP actions.

The antiaggregatory effects of clopidogrel are dependent on the incubation time but not on the presence of the compound in the test medium

The inhibition of ADP-induced platelet aggregation was time-dependent (Figure 6). Measurable effects could be detected after 2.5 min. However, there was an increase in the inhibitory effects of clopidogrel upon further incubation. Maximum and stable inhibition was observed after 30 min (Figure 6). In order to study if the presence of clopidogrel is required for the antiaggregatory effects of the compound, clopidogrel (30 μM , 60 min) pre-incubated washed platelets

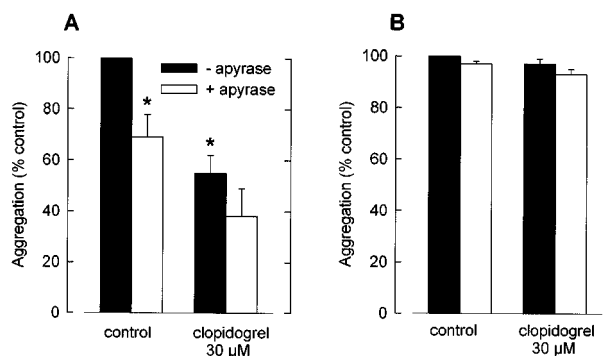


Figure 4 Effects of clopidogrel (30 μM) on collagen (2.5 $\mu\text{g ml}^{-1}$) (A) and U46619 (1 μM) (B)-induced aggregation of acetylsalicylic acid (10 μM)-treated washed human platelets. Experiments were carried out in the absence and presence of apyrase (1 u ml⁻¹). The aggregation was assessed by measuring the maximum change of light transmission. Data are given as % of aggregation response induced by the respective agonists without clopidogrel (control=100%). (means \pm s.e. mean of $n=4$ independent experiments, * $P<0.05$ vs control, Student's *t*-test for paired data).

were washed again and resuspended in clopidogrel-free buffer. Untreated control platelets were processed in the same way and served as controls. In these experiments, the inhibitory effects of clopidogrel were not changed (not shown).

The antiaggregatory effects of clopidogrel are stereoselective

The *in vitro* antiaggregatory effects of clopidogrel were also compared with the R enantiomer SR 25989 C. In these experiments, SR 25989 C (30 μM) was significantly less active as compared to clopidogrel (30 μM) with respect to the inhibition of ADP (6 μM)-induced platelet aggregation (32 \pm 5% vs 70 \pm 1% inhibition, $P < 0.05$, Student's *t*-test for paired data, $n = 5$), indicating a relative stereoselectivity of clopidogrel effects.

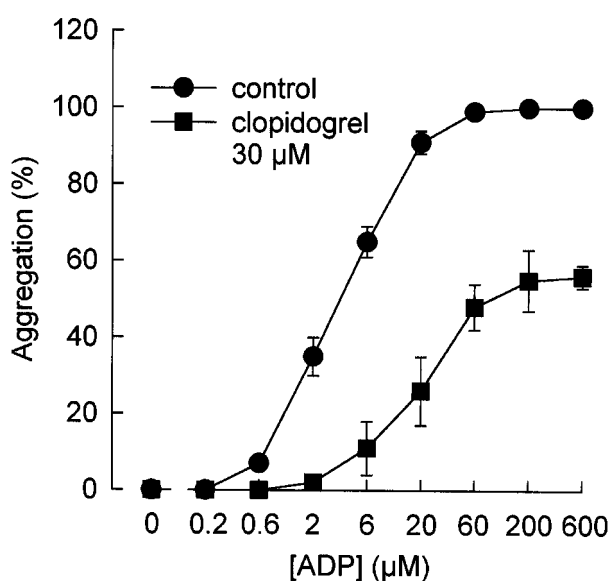


Figure 5 ADP (0.2–600 μM)-induced platelet aggregation in control and in clopidogrel (30 μM)-treated washed platelets. Data are given as % of maximum aggregation response (control=100%). (means \pm s.e.mean of $n = 3$ independent experiments).

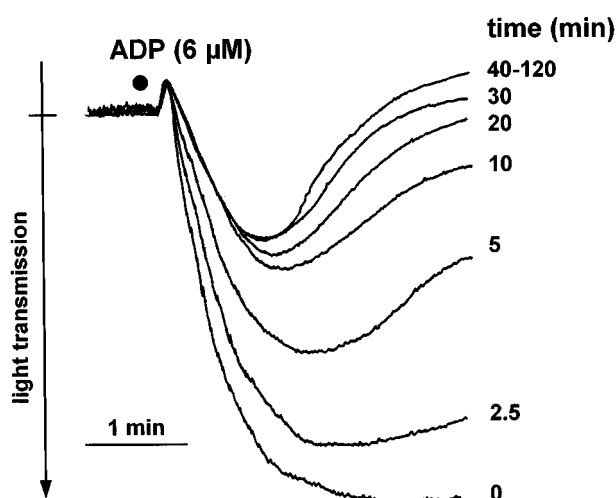


Figure 6 Original tracings demonstrating the time course of the inhibitory actions of clopidogrel (30 μM) on ADP (6 μM)-induced platelet aggregation. Shown is one representative experiment of $n = 4$ with similar results.

Clopidogrel does not reverse the inhibitory effects of ADP on prostaglandin E_1 -induced cyclic AMP formation

Stimulation of washed platelets with prostaglandin E_1 (1 μM) resulted in a significant ($P < 0.05$) increase in cyclic AMP levels (Figure 5). When ADP (6 μM) was added to prostaglandin E_1 -stimulated platelets, the stimulatory effects on cyclic AMP formation were completely abolished. Pretreatment of the platelets with clopidogrel (3–30 μM) for 1 h did not significantly reverse the ADP-mediated inhibition of platelet adenylylase. The data are summarized in Figure 7.

Protein prevents *in vitro* antiaggregatory actions of clopidogrel in platelet-rich plasma

Finally, we have tested the hypothesis that proteins, present in platelet-rich plasma, might prevent the *in vitro* antiaggregatory actions of clopidogrel. Therefore, experiments were carried out, using washed human platelets in test buffer supplemented with albumin (350 mg ml^{-1}). When protein was present in the buffer, the antiaggregatory effects of clopidogrel (30 μM) were completely abolished (not shown). Interestingly, when washed platelets were preincubated with clopidogrel for 1 h, subsequent addition of albumin was not able to reverse the inhibition of platelet aggregation by clopidogrel (not shown).

Discussion

The thienopyridine clopidogrel is believed to be inactive *in vitro* and it has been suggested that its activity is dependent on hepatic biotransformation (for review see Coukell & Markham, 1997). The present study is the first to demonstrate direct inhibition of platelet aggregation by clopidogrel *in vitro*. The *in vitro* inhibition of platelet aggregation by clopidogrel is selective for ADP and does not require hepatic bioactivation.

In line with previous reports (for review see Herbert, 1994), we were not able to detect any direct antiaggregatory effects of clopidogrel ($\leq 100 \mu\text{M}$) in platelet-rich plasma. However, when washed human platelets were studied, a concentration-dependent inhibition of ADP-induced platelet aggregation by clopidogrel was observed. Several lines of evidence indicate that the observed *in vitro* antiaggregatory effects of clopidogrel

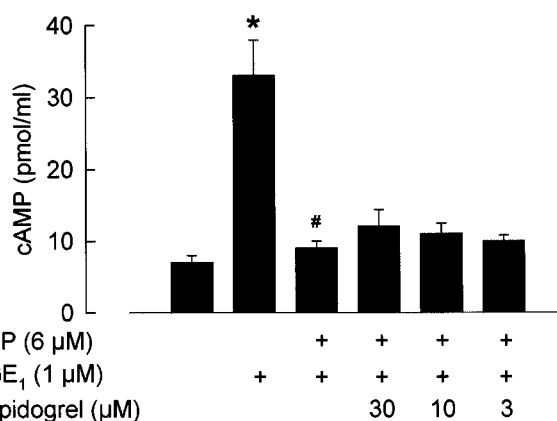


Figure 7 Effects of clopidogrel (3–30 μM) on the inhibitory effects of ADP (6 μM) on prostaglandin E_1 (PGE₁, 1 μM)-stimulated platelet adenylylase. (means \pm s.e.mean of $n = 4$ independent experiments, * $P < 0.05$ vs control, # $P < 0.05$ vs PGE₁, Student's *t*-test for unpaired data).

are due to a specific mechanism that is similar to the *ex vivo* inhibition of platelet aggregation by the compound.

The inhibitory effects of clopidogrel were selective for ADP, because no inhibition of platelet aggregation was seen when collagen, thrombin, or the thromboxane A₂ agonist U46619 were used as proaggregatory stimuli. In addition, the selectivity of clopidogrel for ADP-induced platelet activation was confirmed using apyrase, an ADP-degrading enzyme (Cattaneo *et al.*, 1991). In these experiments, acetylsalicylic acid-treated platelets were sensitive to apyrase, when aggregation was stimulated with collagen. In contrast, U46619-induced aggregation of acetylsalicylic acid-treated platelets was not affected by apyrase. These data indicate that, when acetylsalicylic acid-treated platelets are used, collagen- but not U46619-induced platelet aggregation partially depends on endogenous ADP. Interestingly, in the presence of acetylsalicylic acid, clopidogrel inhibited collagen-induced platelet aggregation to an extent similar to apyrase. In contrast, there was no inhibition of U46619-induced aggregation of acetylsalicylic acid-treated platelets by clopidogrel. Thus, *in vitro* antiaggregatory actions of clopidogrel are restricted to apyrase-sensitive systems and involve the suppression of the effects of released ADP. This is in line with previous reports on the *ex vivo* inhibition of platelet aggregation by the compound (Feliste *et al.*, 1987). The demonstration of synergistic effects of clopidogrel and acetylsalicylic acid is interesting and corresponds to experimental and clinical data with ticlopidine (De Caterina *et al.*, 1991; Neumann *et al.*, 1997). In addition, in accordance to *ex vivo* data that demonstrate antiaggregatory effects of clopidogrel in washed platelets (Gachet *et al.*, 1990), *in vitro* platelet inhibition persisted even when clopidogrel-treated platelets were washed again in order to remove any free compound from the platelet suspension. Thus, the antiaggregatory activity of clopidogrel was associated with the platelets and was not dependent on the presence of the compound in the test buffer. Since the time periods to study inhibition of washed human platelets are limited (washed platelet preparations are stable only for several hours), no statement about possible irreversibility of platelet inhibition by clopidogrel *in vitro* can be made. In line with previous *ex vivo* studies (Defreyn *et al.*, 1991; Savi *et al.*, 1992), clopidogrel (SR 25990 C) was significantly less effective in inhibiting ADP-induced platelet aggregation as compared to its levorotatory enantiomer (SR 25989 C). Finally, the inhibitory effects of clopidogrel on ADP-induced platelet aggregation were insurmountable. Thus, the *in vitro* actions of clopidogrel on human platelets involve a non-equilibrium antagonism, similar to the effects observed *ex vivo* (Mills *et al.*, 1992). In line with the *ex vivo* data (Gachet *et al.*, 1990; 1992; Mills *et al.*, 1992), incubation of clopidogrel with washed platelets did not appear to inhibit ADP (6 μ M)-induced platelet shape change. However, when shape change was measured separately, i.e. under conditions precluding aggregation, a marked inhibition of ADP (0.2 μ M)-induced platelet shape change was seen. With higher ADP concentrations (2 μ M, 20 μ M), the inhibition of platelet shape change by clopidogrel was less obvious.

Taken together, several lines of evidence indicate that clopidogrel specifically and selectively inhibits ADP-induced platelet aggregation *in vitro*. These data are in contrast to the general assumption that hepatic biotransformation is required for antiaggregatory activity of clopidogrel (Savi *et al.*, 1992; 1994a). However, this assumption is based on animal studies only, and the requirement of hepatic biotransformation in humans has not yet been demonstrated. Although several metabolites are known, a biologically active metabolite has not been published so far. Our data indicate that hepatic

biotransformation is not required for platelet inhibition by clopidogrel. It is well possible that clopidogrel itself and not an unknown metabolite accounts for the antiaggregatory activity of the compound. This view is supported by early pharmacokinetic studies with ticlopidine, a sister-compound of clopidogrel. In these studies (Di Perri *et al.*, 1991), a single dose (500 mg) was given to healthy volunteers and the time course of platelet inhibition was correlated with the plasma concentrations of ticlopidine as well as with the amount of cell-associated (unchanged) compound. The maximum concentration of erythrocyte- and neutrophil-associated ticlopidine clearly paralleled the peak plasma concentration of the compound. In contrast, maximum inhibition of platelet aggregation occurred several hours later and paralleled the concentration of platelet-associated (unchanged) compound. Thus, it has been speculated that a redistribution into a platelet-compartment, possibly *via* a specific uptake mechanism, can explain the delayed platelet inhibition by ticlopidine (Di Perri *et al.*, 1991). Alternatively, it is also possible that platelets are capable to generate the putative active metabolite of clopidogrel. This possibility is supported by the time-dependence of platelet inhibitory actions of clopidogrel, as demonstrated in the present study. Clearly, further studies are needed to address this issue.

The second interesting point of our study is the finding that the *in vitro* inhibition of ADP-induced platelet aggregation by clopidogrel can be observed without a significant reversal of the inhibitory effects of ADP on platelet adenylate cyclase. It is well known that clopidogrel treatment selectively reduces the number of functional ADP receptors mediating the inhibition of stimulated adenylate cyclase (Defreyn *et al.*, 1991; Mills *et al.*, 1992; for review see Coukell & Markham, 1997). However, in a recent study, Savi *et al.* (1996) have proposed that cyclic AMP is not an essential messenger for ADP-induced platelet aggregation. Although clopidogrel reversed the inhibitory effects of ADP on platelet adenylate cyclase, this mechanism was not responsible for the *ex vivo* antiaggregatory actions of the compound (Savi *et al.*, 1996). Our data support this conclusion and demonstrate that inhibition of ADP-induced platelet aggregation by clopidogrel can occur without a measurable alteration of cyclic AMP metabolism. However, it is well possible that the measurement of cyclic AMP by radioimmunoassay is not sensitive enough to detect small, though functionally relevant changes in platelet cyclic AMP concentrations. Furthermore, additional studies examining the effects of clopidogrel using a wider range of ADP concentrations are also required.

Finally, the discrepancy between the antiaggregatory effects of clopidogrel in washed platelets as compared to platelet-rich plasma needs to be addressed. Using platelet-rich plasma, we were not able to detect any *in vitro* inhibition of platelet aggregation by clopidogrel. This is in line with previous studies (for review see Coukell & Markham, 1997). In contrast, when washed human platelets were used, clopidogrel concentration-dependently inhibited platelet aggregation. There is an early study with ticlopidine, demonstrating no *in vitro* antiaggregatory effects in washed platelets (Di Minno *et al.*, 1985). However, in this study, albumin was present in the test medium. We have, therefore, hypothesized that the presence of protein might explain the lack of platelet inhibition of clopidogrel in platelet-rich plasma. In fact, the addition of albumin to the test medium completely prevented the antiaggregatory actions of clopidogrel. Although these data can explain why *in vitro* antiaggregatory effects of clopidogrel have not yet been described, the question why clopidogrel is active *in vivo*, i.e. in the presence of high protein concentrations, remains open. However, it is known that several days of

treatment with clopidogrel are needed to achieve maximum platelet inhibitory effects (Coulkell & Markham, 1997). Thus, one might speculate that saturation of protein binding sites for clopidogrel might occur during this time. Alternatively, specific uptake or intercellular redistribution mechanisms (Di Perri *et al.*, 1991) might explain the delayed onset of platelet inhibition *in vivo*.

In summary, we demonstrate that clopidogrel specifically and selectively inhibits ADP-induced aggregation of washed platelets *in vitro* without hepatic bioactivation. It is difficult to speculate about the ADP receptor(s) involved in the antiaggregatory actions of clopidogrel *in vitro*. At least three different ADP receptors are expressed in platelets (Hechler *et al.*, 1998; Geiger *et al.*, 1998): (i) the Gq-coupled P2Y₁ receptor which is thought to be involved in ADP-induced platelet shape change and aggregation; (ii) the Gi-coupled P2Y receptor, which has been termed 'P2Y_{cyc}' and which is thought to complete and to potentiate the initial P2Y₁-mediated platelet activation; and (iii) P2X₁, a receptor-operated Ca²⁺ channel the role of which is unknown. Hechler *et al.* (1998) speculate

that the Gi-coupled P2Y receptor is involved in the antithrombotic actions of clopidogrel. A significant reversal of the inhibitory effects of ADP on platelet adenylate cyclase was not involved in the inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro*. Thus, our data do not support the concept of a Gi-coupled P2Y receptor-mediated inhibition of platelet function by clopidogrel. On the other hand, the marked inhibition of ADP (0.2 μM)-induced platelet shape change by clopidogrel is also compatible with the concept that the P2Y₁ receptor is the target of the compound. However, the present data do not allow definitive statements about the receptor(s) clopidogrel is acting on and further studies are needed to resolve this issue. The demonstration of an inhibition of platelet aggregation by clopidogrel *in vitro* should facilitate the investigation of many open questions regarding the mode of action of this exciting antiplatelet compound.

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