Combined ADP and Thromboxane A<sub>2</sub> Antagonism Prevents Cyclic Flow Variations in Stenosed and Endothelium-Injured Arteries in Nonhuman Primates

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Background. This study was designed to test the hypothesis that clopidogrel, a potent inhibitor of platelet aggregation, can eliminate cyclic flow variations in stenosed and endothelium-injured coronary and femoral arteries in nonhuman primates.

Methods and Results. We studied five anesthetized, open-chest baboons. Blood flow velocity in the coronary and femoral arteries was monitored by pulsed Doppler flow probes placed around the arteries. Cyclic flow variations were established by mechanically injuring the endothelium of the arteries and by narrowing the arteries with external constrictors. Clopidogrel (10 to 20 mg/kg IV bolus plus 2.5 mg·kg<sup>-1</sup>·h<sup>-1</sup> continuous infusion) was administered 60 minutes after cyclic flow variations were established. Clopidogrel abolished cyclic flow variations in the coronary and femoral arteries of all five baboons (frequency of cyclic flow variations, 0/h versus 14/h at baseline, P<.001). Then epinephrine was infused (maximum average dose, 2.2 µg·kg<sup>-1</sup>·min<sup>-1</sup>·IV). Epinephrine did not restore cyclic flow variations in the coronary or femoral arteries of any baboon. Before treatment with clopidogrel, ADP, collagen, and U46619, a thromboxane A<sub>2</sub> mimetic, induced dose-dependent platelet aggregation in vitro. Serotonin, however, did not induce platelet aggregation in vitro. Clopidogrel given in vivo completely inhibited ADP-induced platelet aggregation and significantly diminished collagen- and U46619-induced platelet aggregation in vitro.

Conclusions. Clopidogrel eliminates cyclic flow variations in stenosed and endothelium-injured coronary and femoral arteries of nonhuman primates at least in part by antagonizing the platelet proaggregatory effects of ADP and thromboxane A<sub>2</sub>. (Circulation. 1993;88:2888-2893.)

Key Words • ADP • clopidogrel • epinephrine • platelet aggregation inhibitors • serotonin • thromboxane

Platelet aggregation and the release of platelet-derived mediators in atherosclerotic coronary arteries appear to be important in the conversion from chronic to acute coronary artery disease syndromes. Recent studies also suggest that platelet aggregation may contribute to the development of acute coronary artery occlusion and chronic arterial restenosis after percutaneous transluminal coronary angioplasty.2-8 Experimental studies in animals have shown that several platelet-derived substances are important mediators of platelet aggregation, including ADP, serotonin, and thromboxane A<sub>2</sub>. Studies in dogs have demonstrated that these substances induce coronary cyclic flow variations, which are caused by recurrent platelet aggregation and dislodgment and dynamic vasoconstriction in severely stenosed and endothelium-injured arteries.9-14 Platelet aggregation–associated cyclic flow variations may be eliminated by antagonizing the effects of these platelet-derived factors.9-14 Clopidogrel, a novel antiplatelet agent and an analogue of ticlopidine,9,15-17 is a potent inhibitor of ADP-induced platelet aggregation in humans.16 In dogs, clopidogrel diminishes platelet aggregation induced by serotonin and thromboxane A<sub>2</sub> mimetic (U46619); it also inhibits ADP-induced platelet aggregation.9

However, platelets from different species may respond differently to the same stimulus. The effect of a given drug on platelet aggregation may also be different from species to species. Therefore, in the present study, we have evaluated the effect of clopidogrel on platelet aggregation and cyclic flow variations in the femoral and coronary arteries of nonhuman primates.

Methods

All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston, Tex.
Surgical Preparation

Five female baboons weighing 15 to 25 kg were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and placed on mechanical ventilators (Harvard model 60, Natick, Mass). Plastic catheters were inserted into the carotid artery for monitoring blood pressure and into the jugular vein for administering fluids and drugs. A thoracotomy was performed in the fifth left intercostal space, and the heart was suspended in a pericardial cradle. A 1- to 2-cm segment of the left anterior descending coronary artery was carefully exposed, and nearby branches were ligated. A 1- to 2-cm segment of the femoral artery was also exposed. An ultrasonic Doppler flow probe (Harley Instruments, Houston, Tex) was placed around the exposed coronary and femoral arteries to monitor blood flow velocity.

Experimental Procedures

Baseline hemodynamic values were recorded on an eight-channel recorder (model 3000, Gould, Inc, Cleveland, Ohio). Hemodynamic variables included heart rate, systolic and diastolic aortic pressures, and phasic and mean blood flow velocities in the coronary and femoral arteries. Then the endothelium of the coronary and femoral arteries was injured by gently squeezing the artery with cushioned forceps ("Folts model"). A plastic constrictor was placed around injured arteries to reduce phasic arterial flow velocities to between 50% and 60% of baseline levels.

Clopidogrel (Sanofi Recherche, Toulouse, France)\textsuperscript{15,16} was administered 60 minutes after cyclic flow variations were established. Initially, a 10-mg/kg intravenous bolus of clopidogrel was administered, followed by a continuous infusion of 2.5 mg \cdot kg\textsuperscript{-1} \cdot h\textsuperscript{-1} IV. The total dose of clopidogrel was dissolved in 0.5 mL of methanol and diluted with saline to 50 mL. If the initial dose of clopidogrel did not completely abolish cyclic flow variations, an additional bolus of 10 mg/kg IV was given. After cyclic flow variations were eliminated, baboons were monitored for 30 minutes. Then an infusion of epinephrine was begun at 0.76 \mu g/min IV. At 20-minute intervals, the concentration of epinephrine was incrementally increased (1.53, 3.82, 7.64, 15.28, and 38.2 \mu g/min) until cyclic flow variations returned. Baboons were monitored for 30 minutes after the restoration of cyclic flow variations or after the highest dose of epinephrine was given.

Platelet Aggregation Study

Ex vivo platelet aggregation was examined before and after the administration of clopidogrel. Blood samples were collected in plastic tubes containing 3.8% sodium citrate (9 vol blood:1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging whole blood at 200g for 20 minutes at 37°C. An impedance method was used to determine platelet aggregation on a dual-channel aggregometer (Chrono Log Instruments, Havertown, Pa). Agonists and their final concentrations were collagen at 5, 10, and 20 \mu g/mL; ADP at 5, 10, and 20 \mu mol/L; U46619 (thromboxane mimetic) at 100, 200, and 400 ng/mL; and serotonin at 0.5, 1, and 2 \mu mol/L. Before U46619 and serotonin were added, epinephrine was added at 10 \mu mol/L. The degree of platelet aggregation was reported as the increase in electric resistance between two electric leads placed in platelet-rich plasma.

Additional studies were performed to compare platelet aggregation in platelet-rich plasma of humans, baboons, and dogs. Blood samples were collected from four healthy human subjects, three baboons, and four dogs. We evaluated platelet responses to epinephrine, collagen, ADP, and U46619 and to the combination of epinephrine with collagen, with ADP, and with U46619. In these additional studies, the degree of platelet aggregation was reported as the maximal percentage that light transmission increased in platelet-rich plasma after the addition of an agonist.

Statistical Analyses

All values were expressed as mean±SD. A one-way ANOVA with repeated measurements was used for the comparison of hemodynamic values obtained at different time periods. The changes in frequency of cyclic flow variations and platelet aggregation before and after clopidogrel treatment were compared by paired Student’s \textit{t} tests. A value of \textit{P}<.05 was considered significant.

Results

Cyclic flow variations developed in both the coronary and femoral arteries of all five baboons after endothelial injury and external constriction. The frequency of cyclic flow variations in the coronary and femoral arteries was the same in each animal (Fig 1). Heart rates and aortic blood pressures were not significantly affected by the development of cyclic flow variations (Fig 2A). The severity of cyclic flow variations as indicated by the nadir flow velocity was not significantly different in the coronary and femoral arteries (Fig 2B).

The intravenous administration of clopidogrel (10 mg/kg plus 2.5 mg \cdot kg\textsuperscript{-1} \cdot h\textsuperscript{-1} continuous infusion) eliminated cyclic flow variations in 5 to 10 minutes in the
femoral arteries of all five baboons and in the coronary arteries of four of the five. The additional 10 mg/kg bolus of clopidogrel administered to the fifth baboon abolished cyclic flow variations in its coronary artery as well. The frequencies of cyclic flow variations before and after clopidogrel treatment were significantly different (Fig 1).

Epinephrine did not restore cyclic flow variations in the coronary or femoral arteries of any animal at an average dose of 2.2 μg·kg⁻¹·min⁻¹. Epinephrine did, however, increase heart rates and systolic and diastolic aortic pressures; these increases were statistically significant (Fig 2A).

Before clopidogrel was administered to the baboons, collagen, ADP, and U46619 induced platelet aggregation in vitro in platelet-rich plasma in a dose-dependent manner (Fig 3). Serotonin did not induce platelet aggregation in vitro in any dose tested in baboon platelets. In vivo treatment with clopidogrel completely inhibited ADP-induced platelet aggregation in vitro and partially inhibited collagen- and U46619-induced platelet aggregation (Fig 3).

In vitro platelet aggregation in platelet-rich plasma of different species showed significant differences (Table). Human platelets aggregated in response to epinephrine, collagen, ADP, and U46619 in a dose-dependent manner. Baboon platelets aggregated in response to collagen, ADP, and U46619. Canine platelets aggregated only in response to collagen and ADP. Despite these differences, however, the addition of epinephrine to collagen, ADP, and U46619 significantly enhanced platelet aggregation in all species (Table).

**Discussion**

This study demonstrates that in vivo treatment with clopidogrel, a potent inhibitor of platelet aggregation, eliminates cyclic flow variations in stenosed and endothelium-injured coronary and femoral arteries in baboons.

Cyclic flow variations have been observed in severely stenosed and endothelium-injured coronary arteries and femoral arteries in dogs.9–14,18 Clinically, cyclic flow variations have been observed before and after percutaneous transluminal coronary angioplasty in coronary arteries in some patients with limiting angina19 and in the femoral arteries of some patients with intermittent claudication.18 Histological and radioisotope-labeled platelet studies have revealed that cyclic flow reductions are caused by recurrent platelet aggregation and dislodgment in endothelium-injured arteries.11,13,15 Several platelet-derived factors, including ADP, serotonin, and thromboxane A₂, mediate cyclic flow reductions in the canine model.9–14 Inhibiting the production of these platelet-derived factors or blocking their receptors eliminates cyclic flow variations in dogs.9–14 Schumacher et al.20 have also reported cyclic flow variations in the renal arteries of cynomolgus monkeys and, further, that thromboxane A₂ receptor antagonists inhibit these cyclic flow variations.

Clopidogrel is a potent inhibitor of ADP-induced platelet aggregation in humans.16 In previous studies in dogs, we have shown that clopidogrel reduces platelet aggregation induced by serotonin and thromboxane A₂ and that it inhibits platelet aggregation induced by ADP.9 Clopidogrel eliminates cyclic flow variations in coronary arteries in dogs and also prevents epinephrine-restored cyclic flow variations in the same experimental model.9 In the present study, clopidogrel abolished cyclic flow variations in both coronary and femoral arteries in all five baboons. Epinephrine, even at high doses, failed to restore cyclic flow variations in any animal. In previous studies, epinephrine easily restored cyclic flow variations in dogs after the flow variations

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**Fig 2.** Graphs showing changes in heart rate (HR) and systolic (AOS) and diastolic (AOD) aortic pressures (A) and mean blood flow velocities in the left anterior descending coronary artery (LAD) and femoral (FEM) arteries (B) before and after clopidogrel treatment. Peak indicates the highest, and nadir the lowest average flow during the 60 minutes of cyclic flow variations (CFVs). *P<.05, **P<.01, ***P<.001 compared with baseline.
were abolished by aspirin, a thromboxane antagonist, or a serotonin receptor antagonist.21-23 Cyclic flow variations were less easily restored, however, when a thromboxane A2 antagonist and a serotonin antagonist were administered concomitantly.22 These data demonstrate that in baboons, clopidogrel prevents cyclic flow variations and also protects against epinephrine-restored cyclic flow variations.

In vitro platelet aggregation studies demonstrated that baboon platelets respond well to stimulation with ADP, collagen, and the thromboxane A2 mimetic U46619 but not to serotonin. In humans and dogs, however, serotonin, ADP, and thromboxane A2 are important mediators of platelet aggregation.10,24,25 In this study, treatment with clopidogrel completely inhibited platelet aggregation induced by ADP and significantly reduced platelet aggregation induced by collagen and the thromboxane A2 mimetic.

Also in this study, the addition of epinephrine in vitro to the other agonists enhanced platelet aggregation in all three species tested. This finding may help explain how epinephrine restores cyclic flow variations after they have been abolished. If only one major factor of platelet aggregation is inhibited, cyclic flow variations often disappear. Epinephrine enhances other platelet aggregation factors and can restore the abolished cyclic flow variations even though epinephrine alone does not cause platelet aggregation in vitro in the baboon. The simultaneous inhibition of more than one mediator of platelet aggregation protects against epinephrine-restored cyclic flow variations in canine coronary arteries.9,12 Baboon platelets, like canine platelets, do not aggregate in response to epinephrine alone. However, epinephrine enhances platelet aggregation induced by other agonists. Epinephrine may not be able to restore cyclic flow variations after clopidogrel treatment, because clopidogrel simultaneously inhibits both ADP- and thromboxane A2-induced platelet aggregation.

Clopidogrel, like the homologous thienopyridine derivative ticlopidine, selectively inhibits platelet aggregation induced by ADP, and others have shown that clopidogrel inhibits ADP binding to a high-affinity platelet binding site that is linked to the inhibition of stimulated adenylate cyclase.17 The inhibition of platelet function by clopidogrel in previous studies was associated with a selective reduction in the number of functional receptors mediating the inhibition of stimulated adenylate cyclase by ADP.17 Others have also demonstrated a broad-spectrum antiplatelet activity of ticlopidine and its analogues that appears related to the suppression of the effects of released ADP.16 Finally, other investigators have shown that the thienopyridines

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**Fig 3.** Graphs showing ex vivo platelet aggregation induced by ADP (A), collagen (B), and U46619, a thromboxane A2 mimetic (C) before and after clopidogrel treatment. *P<.05, **P<.01, ***P<.001 compared with control.
In Vitro Platelet Aggregation in Platelet-Rich Plasma of Healthy Humans, Baboons, and Dogs in Response to Various Agonists and to the Addition of Epinephrine to Each Agonist

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Platelet Aggregation</th>
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<tr>
<td></td>
<td>Humans, % (n=4)</td>
<td>Baboons, % (n=3)</td>
<td>Dogs, % (n=4)</td>
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<tr>
<td>Epinephrine</td>
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<td>25 µmol/L</td>
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<td>Collagen</td>
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<td>48±10</td>
<td>22±19</td>
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<td>71±7</td>
<td>67±3</td>
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<td>26±8</td>
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<td>55±5</td>
<td>21±2†</td>
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<td>8 µmol/L</td>
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<td>U46619</td>
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<td>43±12*</td>
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<td>Collagen+epinephrine (1 µmol/L)</td>
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<tr>
<td>2 µg/mL</td>
<td>73±2‡</td>
<td>70±7†</td>
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<td>70±8</td>
<td>51±5‡</td>
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Platelet aggregation is reported as the maximal percentage that light transmission increased in platelet-rich plasma after the addition of an agonist or combination of agonists.

*P<.05, †P<.01, ‡P<.001 compared with human.
§P<.05, ¶P<.01, †P<.001 compared with the single agonist without epinephrine.

In conclusion, clopidogrel effectively eliminated cyclic flow variations associated with platelet aggregation in stenosed and endothelium-injured coronary and femoral arteries in baboons. This effect may arise from the ability of clopidogrel to antagonize the platelet proaggregatory function of ADP and thromboxane A₂.

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