PlA2 Polymorphism of β3 Integrins Is Associated With Enhanced Thrombin Generation and Impaired Antithrombotic Action of Aspirin at the Site of Microvascular Injury

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Background—Mechanisms by which the PlA2 (Leu33Pro) polymorphism of β3 integrins could lead to an increased risk for coronary events are unclear. This study was designed to examine the effect of this polymorphism on blood coagulation.

Methods and Results—In normal subjects (12 with PlA1A1, 9 with PlA1A2, and 3 with PlA2A2), we evaluated the activation of prothrombin, factor V, and factor XIII and fibrinogen removal by quantitative immunoblotting; thrombin-antithrombin III complex generation using ELISA; and levels of fibrinopeptide A and B by high-performance liquid chromatography in blood collected every 30 seconds at sites of standardized microvascular injury before and after 7 days of aspirin ingestion (75 mg/d). Compared with the PlA1A1 subjects, the Pl A2 carriers exhibited higher maximum rates of thrombin B-chain generation (by 31.6%; \(P=0.005\)), thrombin-antithrombin III complex generation (by 30.7%; \(P=0.003\)), fibrinogen consumption (by 31.3%; \(P=0.002\)), prothrombin consumption (by 26.1%; \(P=0.011\)), and activation of factor V (by 14.1%; \(P=0.033\)) and factor XIII (by 27.0%; \(P=0.012\)). In the PlA1A1 homozygotes, aspirin ingestion resulted in reductions in the velocity of thrombin B-chain formation (by 32.1%; \(P=0.007\)), prothrombin consumption (by 30.4%; \(P=0.018\)), factor Va generation (by 28.9%; \(P=0.014\)), fibrinogen removal (by 41.2%; \(P=0.001\)), and factor XIII activation (by 22.6%; \(P=0.026\)). In the PlA2 carriers, aspirin did not alter the velocity of all these processes. After aspirin ingestion, fibrinopeptide A and B concentrations in the last 30-second interval were significantly reduced, but only in the PlA1A1 subjects.

Conclusions—The presence of the PlA2 allele is associated with enhanced thrombin formation and an impaired antithrombotic action of aspirin, which might favor coronary thrombosis in the PlA2 carriers. (Circulation. 2001;104:2666-2672.)

Key Words: glycoproteins ■ genetics ■ coagulation ■ thrombin ■ aspirin

Glycoprotein (GP) IIb/IIIa molecules play an important role in platelet aggregation and adhesion and determine efficient thrombus formation.\(^1\) Clinical studies have shown the efficacy of GP IIb/IIIa antagonists.\(^2\) Evaluation of blood clotting in blood emerging from skin bleeding-time wounds seems to be a useful approach when alterations in \(\beta_3\) integrin function or thrombin generation are suspected.\(^3,4\)

A common polymorphism of GP IIIa, HPA-1, is characterized by a thymidine to cytosine transition at nucleotide 1565, which results in Leu33 to Pro substitution, which defines the PlA1 (HPA-1a) and PlA2 (HPA-1b) alleles, respectively.\(^5\) The PlA2 allele is present in 20% to 30% of the European population.\(^6\)

In 1996, Weiss et al\(^7\) found that the PlA2 allele is associated with a 2.8-fold increase in the risk of first myocardial infarction. Sudden cardiac death was reported to occur more frequently in PlA2 carriers.\(^8\) Epidemiological studies, however, gave inconsistent results, questioning the role of the PlA2 polymorphism as a genetic coronary risk factor.\(^9\)

All the observations suggesting that the PlA2 allele is a risk factor for arterial thrombosis rather than for atherosclerosis led to the concept that the Pro33 substitution in β3 integrins may induce a hypercoagulable state, most likely through platelet hyperaggregability. Platelet reactivity, however, was reported to be increased,\(^10\) decreased,\(^11\) or unaltered\(^12\) in the PlA2 carriers. Binding of exogenous fibrinogen to PlA2-positive platelets was found to be increased\(^10,13\) or unaltered\(^14\) in the PlA2 carriers. Binding of exogenous fibrinogen to PlA2-positive platelets was found to be increased\(^10,13\) or unaltered\(^14\) in the PlA2 carriers. Binding of exogenous fibrinogen to PlA2-positive platelets was found to be increased\(^10,13\) or unaltered\(^14\) in the PlA2 carriers. Binding of exogenous fibrinogen to PlA2-positive platelets was found to be increased\(^10,13\) or unaltered\(^14\) in the PlA2 carriers. Binding of exogenous fibrinogen to PlA2-positive platelets was found to be increased\(^10,13\) or unaltered\(^14\) in the PlA2 carriers.
It has been hypothesized that the clinical efficacy of antiplatelet drugs (eg, aspirin) might be related to the PI\textsuperscript{A2} polymorphism.\textsuperscript{15} In PI\textsuperscript{A2} carriers, we reported impaired inhibition of thrombin generation by low-dose aspirin\textsuperscript{16} and shorter bleeding times both before and after 300 mg of aspirin.\textsuperscript{17} In contrast, Cooke et al\textsuperscript{18} found a weaker aggregation of PI\textsuperscript{A2}-positive platelets in response to aspirin in vitro.

The present study was undertaken to determine whether the PI\textsuperscript{A2} polymorphism affects the activation of prothrombin, factor V, factor XIII, and fibrinogen cleavage at sites of microvascular injury. We also sought to evaluate the effect of aspirin on the coagulant reactions in relation to the PI\textsuperscript{A2} allele.

We found that the presence of the PI\textsuperscript{A2} allele is associated with enhanced thrombin formation and, consequently, faster activation of factor V, factor XIII, and cleavage of fibrinogen. In addition, the PI\textsuperscript{A2} carriers exhibited an impaired anticoagulant action of aspirin.

Methods

Genotyping for PI\textsuperscript{A1A2} Polymorphism

The PI\textsuperscript{A1} and PI\textsuperscript{A2} alleles of the integrin β\textsubscript{3} gene were detected using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique. A pair of primers (5'-GCT CCA ATG TAC GGG GTA AAC and 5'-GGG GAC TGA CTT GAG TGA CCT) was designed to amplify a 282-bp fragment of intron 2 and exon 2 of the gene. Digestion of the fragment with MspI restriction endonuclease produced 125- and 157-bp fragments only if the PI\textsuperscript{A2} allele was present; this allele has the enzyme recognition site due to a T→C transition. Fragments were separated on a 2% agarose gel and visualized using ethidium bromide.

Subjects

Participants of the study were recruited from symptom-free, non-smoking, male medical students aged 21 to 24 years who did not take any drug for at least 4 weeks. Among 50 randomly selected volunteers, we identified 12 PI\textsuperscript{A2} carriers, including 3 PI\textsuperscript{A2A2} homozygotes. Twelve age-matched PI\textsuperscript{A1}-negative men were investigated as controls. Groups were similar with respect to demographic characteristics. Results of routine laboratory blood tests were normal. All volunteers gave informed consent, and the protocol was approved by the University Ethics Committee.

All analyses were performed before and after a 7-day administration of aspirin (75 mg/d). We determined platelet count, as well as plasma fibrinogen and antithrombin-III concentrations, using nephelometry (Dade Behring). The PI\textsuperscript{A2}-positive and -negative subjects than in the PI\textsuperscript{A2} carriers (\textit{P}<0.003). Neither genotype- nor aspirin-related differences in concentrations of products were analyzed as a function of time. Changes in maximum rates were analyzed using IGOR Pro Version 3.1 software (WaveMetrics Inc). To make our analyses independent of sample volumes, values calculated for total yields were used in the Results section.

High Performance Liquid Chromatography Analysis for Fibrinopeptides

The separation of peptides in supernatants from the last 30-second interval was performed by reverse-phase chromatography at 214 nm. Quantitation of fibrinopeptides A and B, which were identified using matrix-assisted laser desorption ionization-time flight (MALDI-TOF) mass spectrometry, was performed as described previously.\textsuperscript{22}

Statistical Analysis

Data are expressed as mean±SEM. Comparisons between different genotypes were performed using the Mann-Whitney U test. Differences between parameters measured before and after aspirin treatment were calculated with the Wilcoxon signed rank test for paired data. \textit{P}<0.05 was considered significant.

Results

The initial bleeding time was shorter in the PI\textsuperscript{A2} carriers than in the PI\textsuperscript{A1A1} homozygotes (307±18 versus 345±19 seconds; \textit{P}=0.046). Aspirin (75 mg/d) prolonged the bleeding time by 52±6 seconds (\textit{P}=0.032) and 122±8 seconds (\textit{P}=0.002), respectively; the posttreatment difference was significant (\textit{P}=0.003). Neither genotype- nor aspirin-related differences in concentrations of the products determined in the first 30-second interval were observed (data not shown).

Prothrombin and Its Activation Products

Before aspirin ingestion in the PI\textsuperscript{A1A1} subjects, consumption of prothrombin, which was detected on nonreduced gels as a molecular weight (M\textsubscript{r})=72 000 band (Figure 1A, A lines), was complete 60 seconds later than it was in the PI\textsuperscript{A2} carriers (\textit{P}=0.02). This reaction was retarded by 60 seconds after aspirin ingestion in the former group (\textit{P}=0.002), but unaltered in the latter group (Figure 1A, B lines). Densitometry of immunoblots revealed that a pretreatment maximum rate of prothrombin consumption was lower by 26.1% in the PI\textsuperscript{A1A1} subjects than in the PI\textsuperscript{A2} carriers (\textit{P}=0.011; Figure 1B and the Table). Aspirin ingestion decreased the velocity of this reaction by 30.4% only in the former group (Figure 1B and the Table), which resulted in a higher posttreatment rate of prothrombin consumption in the PI\textsuperscript{A2} carriers (\textit{P}=0.008).
Before aspirin ingestion, total amounts of thrombin-antithrombin III complexes rose with time at a slower rate (by 30.7%) in the Pl A1A1 homozygotes than in the Pl A2 carriers ($P < 0.003$; Figure 1C and the Table). In the Pl A1A1 subjects, aspirin lowered the velocity of thrombin-antithrombin III complex generation (by 33%; $P < 0.004$); generation was unaltered by aspirin in the Pl A2 carriers (Table).

Figure 2A shows immunoblots representing thrombin B-chain, prethrombin 2, and F1.2, which were identified by comparison with purified standards. In the Pl A2-negative subjects, all the products of prothrombin activation appeared slower compared with the Pl A2 carriers ($P=0.007$; Figure 2A, A lines). After aspirin ingestion, the release of these products was retarded by 60 seconds in the Pl A1A1 homozygotes ($P=0.004$) and was unaltered in the Pl A2 carriers (Figure 2A, B lines). An initial maximum rate of thrombin B-chain generation was lower by 31.6% in the Pl A1A1 subjects compared with the Pl A2 carriers ($P=0.005$; Figure 2B). Aspirin ingestion resulted in a significant reduction in this variable (by 32.1%) in the former, but not in the latter, group (Table). The kinetics of prethrombin 2 generation were similar to those of thrombin B-chain before and after aspirin ingestion (Table).

**Fibrinogen**
Fibrinogen, which is seen to migrate at $M_r = 340,000$ on nonreduced gels, disappeared earlier in the Pl A2 carriers (Figure 3A, A lines). Aspirin ingestion retarded fibrinogen consumption ($P=0.013$) in the Pl A2-negative subjects, but this delay was negligible in the Pl A2 carriers (Figure 3A, B lines).

Before aspirin ingestion, the rate of fibrinogen consumption was higher by 31.3% in the Pl A2 carriers than in the Pl A1A1 subjects ($P=0.002$; Figure 3B and the Table). Aspirin slowed down fibrinogen cleavage in both groups; however, only in the Pl A1A1 homozygotes was the reduction in the maximum velocity (by 41.2%) significant (Table).

**Factor V/Va**
Factor Va heavy and light chains appeared 30 seconds later in the Pl A2-negative group compared with the Pl A2 carriers ($P=0.027$ and $P=0.034$, respectively; data not shown). A maximum rate of factor Va heavy chain generation was
slightly lower (by 11.4%) in the PlA1A1 subjects than in the PlA2 carriers (P = 0.044). After aspirin administration, this parameter did not change in the PlA2 carriers, but a significant reduction (by 41.6%) in the velocity of factor Va heavy chain formation was observed in the PlA1A1 homozygotes (Table). Formation of factor Va light chain, the limiting component in factor Va generation, was enhanced in the PlA2-positive group by 14.1% (P = 0.033) before drug administration and by 31.8% (P = 0.005) after aspirin ingestion. Posttreatment maximum rates of factor Va light chain generation fell significantly (by 28.9%) in the PlA1A1 subjects but not in the PlA2 carriers (Table).

Factor XIII/XIIIa
An Mr = 74 000 band on nonreduced gels, corresponding to the product of thrombin-mediated activation of factor XIIIa, was first seen 30 to 60 seconds later in the PlA1A1 homozygotes than in the PlA2 carriers (P = 0.012; data not shown). Quantitative analyses of immunoblots showed a more rapid cleavage of factor Va light chain, the limiting component in factor Va generation, was enhanced in the PlA2-positive group by 14.1% (P = 0.033) before aspirin ingestion and by 31.8% (P = 0.005) after drug administration. Posttreatment maximum rates of factor Va light chain generation fell significantly (by 28.9%) in the PlA1A1 subjects but not in the PlA2 carriers (Table).

Fibrinopeptides A and B
Chromatograms (Figure 4) showed that before aspirin ingestion, levels of fibrinopeptide A, released by thrombin from the Aβ chain of fibrinogen, did not differ between the PlA2-positive and -negative subjects (9.4 ± 1.9 versus 9.2 ± 2.2 μmol/L; P = 0.5). Aspirin ingestion resulted in a marked decrease in fibrinopeptide A concentrations to 2.4 ± 0.4 μmol/L only in the latter group (P = 0.029); they remained unaltered in the former group (7.8 ± 0.9 μmol/L; P = 0.5). Final posttreatment fibrinopeptide A concentrations were higher in the PlA2 carriers (P = 0.016). Levels of fibrinopeptide B, which is released at a much slower rate than fibrinopeptide A from the Bβ chain of fibrinogen, could be estimated in 5 of 12 PlA2-positive subjects. Unexpectedly, none of the PlA1A1 homozygotes had fibrinopeptide B levels above the detection limit (0.05 μmol/L).

Comparison Between PlA2A2 Homozygotes and PlA1A2 Heterozygotes
There was a slight trend toward higher maximum rates of the coagulant reactions in the PlA2A2 homozygotes compared with...
the PlA1A2 heterozygotes, both before and after aspirin administration ($P=0.1$ to 0.3).

**Discussion**

Our results demonstrate that the PlA2 polymorphism affects blood clotting at the site of microvascular injury in healthy subjects. We provide evidence for a more rapid thrombin generation in the PlA2 carriers by an evaluation of prothrombin activation products. The Pro33 substitution in $\beta_3$ integrins was also associated with faster thrombin-mediated fibrinogen proteolysis and factor XIII activation, which can give rise to enhanced stabilization of the fibrin formed by factor XIII-mediated cross-linking. Likewise, factor V activation, which is largely mediated by thrombin, was enhanced in the PlA2 carriers.

Replacement of leucine at position 33 with proline confers the altered conformation of GP IIIa and spatial orientation of the fibrinogen-binding region. The PlA2-expressing cells exhibited increased adhesion, enhanced spreading, more extensive actin polymerization, and enhanced fibrin clot retraction, reflecting PlA2-related differences in outside-in signaling. Available data on platelet function are conflicting. The PlA2-related changes in thrombin generation reported here may reflect alterations in platelet reactivity, which is different from aggregability. It is known that platelets provide a catalytic surface for the formation of the prothrombinase complex, which is responsible for explosive thrombin generation. It might be speculated that the PlA2 allele is related to the enhancement of this specific procoagulant function of platelets, because an inherited deficiency in GP IIb/IIIa (Glanzmann thrombasthenia) is associated with reduced thrombin formation in bleeding-time blood. Another possibility is that the PlA2 polymorphism is linked to another pathogenic allele that accounts for the alterations in blood coagulation reported here.

It remains to be determined whether the procoagulant effects of the PlA2 allele could be detected in a hemostatic microenvironment different from microvessels or in older CAD patients, in whom metabolic risk factors (eg, hypercholesterolemia) may influence coagulant reactions.

Prothrombin may bind, via the Arg-Gly-Asp (RGD) sequence within its catalytic domain, to $\alpha_{IIb}\beta_3$ and possibly $\alpha_v\beta_3$ integrins, which are also expressed on the endothelium, leukocytes, and vascular smooth muscle cells. It might be speculated that the PlA2 allele affects prothrombin activation and leads to procoagulant alterations in the function of $\beta_3$ integrins.

The principal mode of action of aspirin involves the inhibition of thromboxane $A_2$ production by acetylation of platelet cyclooxygenase. Aspirin was found to decrease...
thrombin generation in vitro and in vivo. Our current and previous studies show that, unlike the Pl A1A1 homozygotes, thrombin formation at the site of injury is not impaired by low-dose aspirin in healthy Pl A2 carriers. Interestingly, aspirin enhanced the differences in blood clotting observed between the Pl A2-positive and -negative subjects. Platelets of the Pl A2 heterozygotes, in contrast to the Pl A2 homozygotes, were reported to be more sensitive to 2.5 and 5 μmol/L aspirin. These in vitro observations seem to differ from our in vivo findings, and the issue has been discussed in detail recently.

How could the Pro33 substitution in β3 integrins influence the anticoagulant effects of aspirin? Aspirin has been reported to inhibit GP IIb/IIIa activation by interfering with intracellular signaling events and by acetylating GP IIb and GP IIIa molecules. Moreover, GP IIb/IIIa inhibitors seem to reduce factor Va binding to platelets, resulting in a significant decrease in thrombin generation. It is unclear whether the alterations in GP IIb/IIIa function induced by aspirin and GP IIb/IIIa blockers bear any similarities. Molecular mechanisms underlying the effects of aspirin described here await elucidation.

In summary, our study provides evidence that, compared with the Pl A1A1 homozygotes, thrombin is generated more rapidly at sites of microvascular injury in Pl A2 carriers and that the difference between genotypes becomes more pronounced by aspirin. Hence, the Pl A2 polymorphism may lead to life-long procoagulant effects. Because thrombin stimulates smooth muscle cell migration and other atherogenic processes, the Pl A2 polymorphism may also promote atherothrombosis via indirect, nonthrombotic actions. Our observations could also have important therapeutic implications, because the antithrombotic effects of aspirin may be blunted in patients with coronary artery disease.

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Figure 4. High performance liquid chromatography chromatograms showing fibrinopeptide A (FPA) in the last 30-second bleeding-time blood sample taken from a PlA2A2 (A) and PlA1A1 (B) subject. In both panels, upper chromatograms depict fibrinopeptide A before aspirin administration, and lower chromatograms show fibrinopeptide A after 7 days of aspirin therapy (ASA; 75 mg/d). Fibrinopeptide B is invisible in both chromatograms.

References


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