PlA2 Polymorphism of \( \beta_3 \) Integrins Is Associated With Enhanced Thrombin Generation and Impaired Antithrombotic Action of Aspirin at the Site of Microvascular Injury

Anetta Undas, MD, PhD; Kathleen Brummel, PhD; Jacek Musial, MD, PhD; Kenneth G. Mann, PhD; Andrew Szczeklik, MD, PhD

**Background**—Mechanisms by which the PlA2 (Leu33Pro) polymorphism of \( \beta_3 \) 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
It has been hypothesized that the clinical efficacy of antiplatelet drugs (eg, aspirin) might be related to the PlA2 polymorphism.15 In PlA2 carriers, we reported impaired inhibition of thrombin generation by low-dose aspirin16 and shorter bleeding times both before and after 300 mg of aspirin.17 In contrast, Cooke et al.18 found a weaker aggregation of PlA2-positive platelets in response to aspirin in vitro.

The present study was undertaken to determine whether the PlA2 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 PlA2 allele.

We found that the presence of the PlA2 allele is associated with enhanced thrombin formation and, consequently, faster activation of factor V, factor XIII, and cleavage of fibrinogen. In addition, the PlA2 carriers exhibited an impaired anticoagulant action of aspirin.

Methods

Genotyping for PlA1A2 Polymorphism

The PlA1 and PlA2 alleles of the integrin β3 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 TCA C) 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 PlA2 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 PlA2 carriers, including 3 PlA1A2 homozygotes. Twelve age-matched PlA2-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 PlA2-positive and -negative subjects were similar with respect to all 3 variables, both before and after aspirin ingestion.

Model of Microvascular Injury

Evaluation of tissue factor–initiated blood coagulation at the site of microvascular injury was adopted from the method described by Szczeklik et al.19 Briefly, a sphygmomanometer cuff placed on the upper arm was inflated to 40 mm Hg and 2 standardized incisions were made on the forearm using a Simplate II device (Organon Teknika). The procedure was performed by the same investigator, who was blinded to the results of genotyping. Blood oozing from bleeding-time wounds was collected into heparinized capillary tubes (Kabe Labortechnik) every 30 seconds until bleeding stopped. Blood was passed into an anticoagulant cocktail (1:9 v/v) containing sodium citrate, aprotinin, chloromethyl ketone, and heparin (Diagnostica Stago). After centrifugation at 2000g for 20 minutes, the supernatants were stored at −80°C. We determined (1) thrombin-antithrombin III complex concentrations by using commercially available assay kits (Enzymost, Dade Behring) and (2) prothrombin activation, factor Va light and heavy chain generation, fibrinogen cleavage, and factor XIII activation by quantitative immunoblotting.

Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel analysis (SDS-PAGE) was performed as described previously.20,21 Samples were separated on 5% to 15% linear gradient gels and transferred to nitrocellulose membranes (Bio-Rad). We used the following primary antibodies: (1) burro prethrombin 1 polyclonal antibody, which recognizes prothrombin, prethrombin 1, prethrombin 2, prethrombin fragment 2, and α-thrombin B-chain; (2) murine monoclonal α-fibrinogen 3A, which is raised against the αα chain of fibrinogen; (3) murine monoclonal α-factor Vα C No. 17 and α-factor Vα C No. 9, which recognize the human factor Va heavy chain and factor Va light chain, respectively; (4) rabbit polyclonal α-factor XIII (D46/79), which is raised against the subunit A.21 Horseradish peroxidase–labeled IgG secondary antibodies were used (Southern Biotechnology). Densitometry of immunoblots was performed as described previously.20

Concentrations in each 30-second interval were estimated from serial dilutions of purified standard proteins by horizontal comparison of sample band density. Both concentrations and total amounts 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-of-flight (MALDI-TOF) mass spectrometry, was performed as described previously.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. P<0.05 was considered significant.

Results

The initial bleeding time was shorter in the PlA2 carriers than in the PlA1A1 homozygotes (307±18 versus 345±19 seconds; P=0.046). Aspirin (75 mg/d) prolonged the bleeding time by 52±6 (P=0.032) and 122±8 seconds (P=0.002), respectively; the posttreatment difference was significant (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 PlA1A1 subjects, consumption of prothrombin, which was detected on nonreduced gels as a molecular weight (Mr )=72 000 band (Figure 1A, A lines), was complete 60 seconds later than it was in the PlA2 carriers (P=0.02). This reaction was retarded by 60 seconds after aspirin ingestion in the former group (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 PlA1A1 subjects than in the PlA2 carriers (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 PlA2 carriers (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 PI A1A1 subjects than in the PI A2 carriers (P = 0.044). After aspirin administration, this parameter did not change in the PI A2 carriers, but a significant reduction (by 41.6%) in the velocity of factor Va heavy chain formation was observed in the PI A1A1 homozygotes (Table). Formation of factor Va light chain, the limiting component in factor Va generation, was enhanced in the PI A2-positive group by 14.1% (P = 0.033) before drug administration 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 PI A1A1 subjects but not in the PI A2 carriers (Table).

Factor XIII/XIIIa

An M r = 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 PI A1A1 homozygotes than in the PI A2 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 PI A2-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 PI A1A1 subjects but not in the PI A2 carriers (Table).

Fibrinopeptides A and B

Chromatograms (Figure 4) showed that before aspirin ingestion, levels of fibrinopeptide A, released by thrombin from the Aα chain of fibrinogen, did not differ between the PI A2-positive and -negative subjects (9.4 ± 1.9 versus 9.2 ± 2.2 μmol/L; P = 0.6). 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 PI A2 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 PI A2-positive subjects. Unexpectedly, none of the PI A1A1 homozygotes had fibrinopeptide B levels above the detection limit (0.05 μmol/L).

Comparison Between PI A2A2 Homozygotes and PI A1A2 Heterozygotes

There was a slight trend toward higher maximum rates of the coagulant reactions in the PI A2A2 homozygotes compared with
the PI-A2 heterozygotes, both before and after aspirin administration ($P$=0.1 to 0.3).

**Discussion**

Our results demonstrate that the PI-A2 polymorphism affects blood clotting at the site of microvascular injury in healthy subjects. We provide evidence for a more rapid thrombin generation in the PI-A2 carriers by an evaluation of prothrombin activation products. The Pro33 substitution in IIb/IIIa 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 PI-A2 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 PI-A2-expressing cells exhibited increased adhesion, enhanced spreading, more extensive actin polymerization, and enhanced fibrin clot retraction, reflecting PI-A2-related differences in outside-in signaling. Available data on platelet function are conflicting. The PI-A2-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 PI-A2 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 PI-A2 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 PI-A2 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 IIb/IIIa integrins, which are also expressed on the endothelium, leukocytes, and vascular smooth muscle cells. It might be speculated that the PI-A2 allele affects prothrombin activation and leads to procoagulant alterations in the function of IIb/IIIa integrins.

The principal mode of action of aspirin involves the inhibition of thromboxane A2 production by acetylation of platelet cyclooxygenase. Aspirin was found to decrease...
Effects of the PlA2 Polymorphism on the Kinetics of the Coagulant Reactions in Bleeding-Time Blood, Expressed as a Function of Total Yields and Concentrations Versus Time

<table>
<thead>
<tr>
<th>Maximum Rate of</th>
<th>PlA1A1 (n=12)</th>
<th>PlA2A2 + PlA1A2 (n=12)</th>
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<tbody>
<tr>
<td></td>
<td>Before ASA</td>
<td>After ASA</td>
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<tr>
<td>Prothrombin consumption†</td>
<td>0.231±0.032</td>
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<tr>
<td></td>
<td>nmol/s</td>
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<tr>
<td>TAT generation*</td>
<td>12.8±1.1</td>
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<td>Thrombin B-chain generation†</td>
<td>1.53±0.12</td>
<td>0.97±0.08</td>
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<tr>
<td></td>
<td>pmol/L per s</td>
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<td>Prothrombin 2 generation†</td>
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<td>31.3±4.2</td>
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<td>Prothrombin 2 generation†</td>
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<td>pmol/L per s</td>
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<td>Factor Va heavy chain formation†</td>
<td>1.92±0.21</td>
<td>1.36±0.15</td>
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<tr>
<td></td>
<td>fmol/s</td>
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<tr>
<td>Factor Va light chain formation†</td>
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<td>31.4±4.2</td>
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<td>nmol/L per s</td>
<td>0.204±0.02</td>
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<tr>
<td>Factor XIII removal†</td>
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<td>0.531±0.07</td>
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<tr>
<td></td>
<td>fmol/s</td>
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<tr>
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<td>0.022±0.002</td>
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<td>0.018±0.004</td>
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<td>18.88±2.1</td>
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<tr>
<td></td>
<td>pmol/L per s</td>
<td>0.731±0.06</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. ASA indicates aspirin (75 mg/d for 7 days); TAT, thrombin-antithrombin III complexes.
* Determined by immunoenzymatic assay (ELISA).
† Determined by quantitative Western blotting.

thrombin generation in vitro and in vivo. Our current and previous studies show that, unlike the PlA1A1 homozygotes, thrombin formation at the site of injury is not impaired by low-dose aspirin in healthy PlA2 carriers. Interestingly, aspirin enhanced the differences in blood clotting observed between the PlA2-positive and -negative subjects. Platelets of the PlA1A2 heterozygotes, in contrast to the PlA2A2 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 PlA1A1 homozygotes, thrombin is generated more rapidly at sites of microvascular injury in PlA2 carriers and that the difference between genotypes becomes more pronounced by aspirin. Hence, the PlA2 polymorphism may lead to life-long procoagulant effects. Because thrombin stimulates smooth muscle cell migration and other atherogenic processes, the PlA2 polymorphism may also promote atherosclerosis via indirect, nonthrombotic actions. Our observations could also have important therapeutic implications, because the antithrombotic effects of aspirin may be blunted in the PlA2 carriers. Whether this holds true for patients with coronary artery disease is currently being investigated.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL-46703) and the National Institutes of Health Grant Training (T32 HL-07594). We thank Marek Sanak and Michal Zolnowski for genotyping the PlA2 polymorphism. We are indebted to Wojciech Sydor and Jed Pauls for their excellent technical assistance.
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 subject (B). 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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