Modulation of Thrombotic Responses in Moderately Stenosed Arteries by Cigarette Smoking and Aspirin Ingestion

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Abstract  Cigarette smoking is a known risk factor for cardiovascular disease in men and women, and it has been suggested that this risk is linked to enhanced formation of platelet thromboxane A₂ (TXA₂). This led us to investigate the effect of cigarette smoking and TXA₂ formation on collagen-induced thrombogenesis in flowing nonanticoagulated human blood. Thrombus formation in blood from smokers and nonsmokers was compared before and 2 hours after ingestion of a single oral dose of 990 mg aspirin, which is sufficient to block platelet TXA₂ formation. Nonanticoagulated blood was drawn directly from an antecubital vein over collagen fibrils in a parallel-plate perfusion chamber by a peristaltic roller pump placed distally to the chamber. Wall shear rates at the collagen surface were characteristic for medium-sized (650 s⁻¹) and moderately stenosed (2600 s⁻¹) arteries. Blood-collagen interactions were morphologically quantified, and markers of platelet release, β-thromboglobulin (β-TG), and activation of coagulation, fibrinopeptide A (FPA), were measured immediately distal to the perfusion chamber. The thrombus volume in blood from cigarette-smoking individuals was nearly twofold larger than in blood from nonsmokers at 2600 s⁻¹ (37.4 and 19.4 μm³/μm²; P<.03). However, ingestion of aspirin reduced the thrombus volume in blood from smokers by 61.8% (P<.01), which was substantially more than the 37.6% reduction in blood from nonsmokers (P<.03). Neither cigarette smoking nor aspirin ingestion affected thrombus formation at 650 s⁻¹. The plasma levels of FPA and β-TG were slightly lower in nonsmokers and after aspirin ingestion. Thus, it appears that cigarette smoking increases the thrombotic response at high arterial shear conditions. However, blocking the TXA₂ formation by aspirin reduces this hyperresponsiveness to the range observed in healthy nonsmoking individuals. The antithrombotic effect of aspirin in smokers and nonsmokers is observed only at a high, nonphysiological arterial wall shear rate (2600 s⁻¹). (Arterioscler Thromb. 1994;14:617-621.)

Key Words: • cigarette smoking • aspirin • thrombus formation • arterial blood flow • thromboxane A₂ • wall shear rate

Several prospective studies have demonstrated that cigarette smoking is a major risk factor for cardiovascular disease. Enhanced thromboxane A₂ (TXA₂) synthesis in platelets has been implicated as a possible source of risk, since biochemical studies have shown increased TXA₂ production in cigarette smokers. The TXA₂ molecule is a potent vasoconstrictor and platelet aggregation agonist. Both events are closely associated with cardiovascular disorders.

A single oral dose of 1 g aspirin blocks the platelet production of TXA₂ by irreversible acetylation of platelet cyclooxygenase. The effect of blocking TXA₂ is partial inhibition of the aggregation response to low concentrations of ADP and collagen. Ingestion of aspirin abolishes acute and chronic effects of enhanced platelet aggregation observed in healthy habitual smokers.

The goal of the present study was to investigate the effect of cigarette smoking on collagen-induced thrombogenesis in flowing nonanticoagulated human blood and to evaluate the effect of a 990-mg single oral dose of aspirin in both smokers and nonsmokers at arterial blood flow conditions. A well-characterized human ex vivo model of arterial thrombogenesis, previously validated with in vivo induced coronary thrombosis in dogs, was used. Data obtained with this model with blood from patients with various subtypes of von Willebrand disease and hemophilia A correlate well with the patients’ clinical findings. Experimental antiplatelet agents have also been evaluated with this model.

Methods

Design of the Study

Twenty-four healthy men 24 to 47 years old were included in this open study. Twelve of the volunteers were smokers, and 12 were nonsmokers. None of the donors had ingested aspirin for at least 10 days before the perfusion experiment. The volunteers had been fully informed about the study and had given their free, informed consent to participate.

Platelet adherence and thrombus formation on an immobilized fibrillar collagen type III surface were measured before and 2 hours after ingestion of aspirin according to the ex vivo model of thrombogenesis with the parallel-plate perfusion chambers developed by Sakariassen et al. The smokers were those who smoked at least 10 cigarettes a day, and all had been habitual smokers for years. Within 5 minutes before each perfusion they smoked a cigarette.

The volunteers took three aspirin tablets orally once (3×330 mg; Novid, Nycomed), which were dissolved in water immediately before administration. The local ethics committee approved the protocol, which was performed according to the Declaration of Helsinki.

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Blood Samples

Venipuncture of an antecubital vein was performed with a No. 19 butterfly infusion set (Abbott Laboratory). The first 5 mL of blood was collected into EDTA for determination of platelet count and hematocrit (Auto Counter AC 920, Swelab Instruments).

Plasma fibrinogen was analyzed in citrated plasma according to the method of Claus.\textsuperscript{19} Clotting time was recorded with a coagulometer KC10A (Amelung GmbH). A standard curve was prepared with human fibrinogen (Baxter Dade AG).

Preparation of Collagen Surfaces

Type III collagen was purified by selective salt precipitation from a human placental extract of a pepsin digest.\textsuperscript{20} The collagen concentration was estimated from the amino acid composition analyzed by a Biotronik LC 5000 amino acid analyzer (Biotronik) after 24 hours of hydrolysis of a purified sample in 6 mol/L HCl under vacuum. Collagen fibrils were made by dialysis at 4°C against 20 mmol/L Na₂HPO₄, pH 7.5, for 48 hours.\textsuperscript{21}

The fibrillar collagen suspension was sprayed onto washed Thermanox plastic coverslips (Miles Laboratories) to a final density of about 20 μg/cm². The coating was done with an air brush at a nitrogen operating pressure of 1 atm, and the collagen-coated coverslips were stored at 22°C for 16 to 20 hours before they were used in the perfusion experiments.\textsuperscript{22} These fibrils give a maximal thrombogenic stimulus at 10 μg/cm² (final density on spray coated coverslips), but they do not trigger coagulation. The thrombogenicity of these fibrils is much like that of intact artery subendothelium, although the latter surface triggers much more fibrin deposition.\textsuperscript{23}

Ex Vivo Perfusions, Fixation, and Embedding

Ex vivo perfusions\textsuperscript{24} were performed at 37°C with a collagen-coated coverslip positioned in a parallel-plate perfusion chamber.\textsuperscript{12,13} After venipuncture of an antecubital vein, the first 5 mL was discarded; subsequently, 50 mL of blood was drawn directly over the collagen surface by an occlusive roller pump (model M312, Gilson) at a constant flow rate of 10 mL/min. The pump was placed distal to the chamber. Wall shear rates characteristic of healthy medium-sized (650 s⁻¹) and moderate (500 s⁻¹) arteries were maintained at the collagen surface for 5 minutes in two parallel-plate perfusion chambers having different cross-sectional dimensions of the rectangular blood flow channel.

After 5 minutes, blood perfusions were terminated by a 20-second perfusion at 10 mL/min with a buffer containing (mmol/L) NaCl 130, KCl 2, NaHCO₃ 12, CaCl₂ 2.5, and MgCl₂ 0.9 (pH 7.4), followed by a 40-second perfusion (10 mL/min) with a fixation solution consisting of 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). Postfixation was performed with freshly prepared fixation solution at 4°C for 1 hour. The coverslips with the fixed thrombotic deposits were finally embedded in epoxy resin.\textsuperscript{22}

Morphometry

Thrombus formation was light-microscopically quantified on sections 1 μm thick. The sections were cut periodical to the direction of the blood flow 1 mm downstream from the upstream edge of the coverslip. The sections were stained with basic fuchsin and toluidine blue.\textsuperscript{25}

Standard morphometry was used to assess the percentage of the surface covered by platelets (percent platelet adhesion) and by fibrin (percent fibrin deposition).\textsuperscript{22} The evaluations were performed on a Kontron Vidas image analyzing unit (Zeiss) at ×500 or ×2000 magnification, depending on the size of the thrombi.

Fibrinopeptide A and β-Thromboglobulin

Plasma levels of fibrinopeptide A (FPA) and β-thromboglobulin (β-TG) were measured distal to the perfusion chamber. At 4 minutes of perfusion, blood samples (2×0.9 mL) were collected immediately distal to the chamber. The blood samples were aspirated successively during 15 seconds into 1-mL syringes prefilled with 0.1 mL of 1000 U heparin plus 1000 U aprotinin per milliliter of saline for FPA and anticoagulant mixture according to Ludlam and Cash\textsuperscript{27} for β-TG. The samples were immediately chilled on ice. The blood sampling was performed in a rubber-coated area of the tubing to prevent leakage of blood after the puncture. The sampling occurred without the pump’s being switched off. Further processing of the blood samples for quantification of the plasma levels of FPA and β-TG were according to the manufacturers of the respective kits, FPA from IMCO, and β-TG from Amersham.

Statistical Analysis

The significance of difference was calculated with paired and unpaired Student’s t test or Wilcoxon’s signed-rank test (nonparametric data). Values of P<.05 were considered significant. Two-tailed probabilities were used throughout the analysis.

Results

Clinical Data

All 24 volunteers recruited for the study completed it according to the protocol. There were no adverse events related to the ingestion of aspirin.

Hematocrit and Platelet Count

The mean platelet count was significantly higher in smokers than in nonsmokers (Table 1). A drop in average hematocrit of 2% was observed at the second blood donation (P<.05), but no statistically significant change in mean platelet count was observed. All individual platelet counts were within the normal range (1.5 to 4.5×10ⁱ²/L). The hematocrit of one of the smokers was 50.5%, which was above the normal range (38% to 48%).
Fibrinogen

Plasma fibrinogen was analyzed in samples collected before aspirin ingestion only. No significant difference was observed between smokers and nonsmokers (Table 1).

Platelet-Collagen Adhesion

The platelet-collagen adhesion in blood from smokers and nonsmokers was similar before ingestion of aspirin. However, aspirin significantly increased platelet-collagen adhesion at each wall shear rate in both smokers and nonsmokers (Fig 1). The increases at 650 s⁻¹ were 42% (P<.01) in smokers and 14% (P<.05) in nonsmokers. At 2600 s⁻¹, the corresponding increases were 30% (P<.0005) and 19% (P<.001). After ingestion of aspirin there was a significantly higher platelet adhesion at 650 s⁻¹ in smokers compared with nonsmokers ('].005), whereas no difference was observed at 2600 s⁻¹.

Thrombus Volume

The average thrombus volume at the wall shear rate of 2600 s⁻¹ in blood from smokers before aspirin ingestion was nearly twofold that observed in blood from nonsmokers (P<.03). However, no significant difference was found at 650 s⁻¹ (Fig 2).

Aspirin ingestion decreased the thrombus volume at 2600 s⁻¹ by 62% (P<.01) in blood from cigarette smokers and by 38% (P<.003) in blood from nonsmokers. The average thrombus volume at 650 s⁻¹ was not affected by aspirin.

β-TG Plasma Levels

The median postchamber β-TG levels were not significantly different between smokers and nonsmokers. No significant changes were recorded in the median

| Table 2. Effect of Aspirin on Activation of Platelets (β-Thromboglobulin) and Coagulation (Fibrinopeptide A) in Smokers and Nonsmokers |
|---------------------------------|----------------|----------------|----------------|----------------|
| **Shear Rate, s⁻¹**  | **Marker** | **Smokers, ng/mL** | **Smokers, ng/mL** | **Nonsmokers, ng/mL** |
| 650 (n=6)  | β-TG | 100 (68-301) | 70 (43-141) | 61 (39-89) |
| 2600 (n=12)  | β-TG | 99 (74-155) | 65 (46-73) | 73 (45-109) |
| 650 (n=6)  | FPA | 19 (6-142) | 5 (2-9) | 8 (6-55) |
| 2600 (n=12)  | FPA | 19 (8-29) | 7* (5-12) | 9 (6-21) |

β-TG indicates β-thromboglobulin; FPA, fibrinopeptide A. Data are medians (quartiles). Five-minute perfusion at 10 mL/min with nonanticoagulated blood. Blood samples were collected immediately distal to perfusion chamber at 4 minutes of perfusion time. Note that the area of the collagen surface exposed to blood at 2600 s⁻¹ is 62.5% of the area at 650 s⁻¹.

*P<.05; comparison with corresponding control values before aspirin treatment (Wilcoxon's signed-rank test).
postchamber β-TG plasma levels after ingestion of aspirin (Table 2).

**Fibrin Deposition and FPA Plasma Levels**

Fibrin deposition was <5% at each wall shear rate in blood from both smokers and nonsmokers before and after aspirin ingestion, and no significant change was detected after aspirin in any of the groups (data not shown).

The median postchamber FPA plasma levels were not significantly different between smokers and nonsmokers before aspirin ingestion. Aspirin reduced the median FPA level significantly, by 63% (P<.05), in smokers at 2600 s⁻¹ but not in nonsmokers. The median FPA levels at 650 s⁻¹ were not affected by aspirin in either smokers or nonsmokers (Table 2).

**Discussion**

Cigarette smoking is a known risk factor for cardiovascular disease in men and women. Increased urinary excretion of the stable thromboxane metabolite 2,3-dinor-thromboxane B₂ has been observed in smokers of both sexes, suggesting that cigarette smoking may increase platelet reactivity and vasoconstriction through augmented TxA₂ formation.

The present study was carried out to investigate whether cigarette smoking affected collagen-induced thrombogenesis in flowing nonanticoagulated human blood and to quantify the effect of aspirin-blocked TxA₂ synthesis on thrombus formation. The study was performed with a human ex vivo model of thrombogenesis at blood flow conditions comparable to those encountered in medium-sized and moderately stenosed arteries.

The effect of cigarette smoking on thrombus formation was shear rate dependent and apparent only at high-shear conditions, as found in atherosclerotic arteries. The thrombus volume was increased nearly twofold in blood from cigarette smokers at the highest shear condition of 2600 s⁻¹, whereas no difference was noted at 650 s⁻¹. A protective effect of aspirin against thrombus formation in blood from cigarette smokers and nonsmokers was observed only at the highest shear rate and was much more pronounced in smokers. Thus, the apparent significance of TxA₂ in thrombogenesis appeared to be shear-rate dependent.

The pronounced reduction in thrombus formation by aspirin in blood from cigarette smokers indicates that smoking enhances the platelet reactivity through TxA₂. However, it should be realized that the increased TxA₂ response could be a consequence and not a primary cause of platelet activation. Nevertheless, aspirin ingestion reduced thrombus formation in blood from cigarette smokers to the level observed in blood from nonsmokers.

Although aspirin ingestion lowers platelet activation, it apparently increases platelet-collagen adhesion in our model (Fig 1). This seems contradictory, but it should be realized that rapid thrombus growth depletes the blood layers streaming adjacent to the surface of platelets. Thus, ingestion of a platelet inhibitor affecting the platelet-collagen interaction gives a secondary, enhancing effect on platelet adhesion. Lower consumption of platelets by growing thrombi after ingestion of a platelet inhibitor increases the platelet concentration in the blood layers streaming adjacent to the collagen surface, thus leaving more platelets available to adhere to the collagen. This physical effect was apparently enhancing the platelet-collagen adhesion at 2600 s⁻¹ and probably also at 650 s⁻¹, even though aspirin had only marginal effect on the thrombus volume. Similar findings were previously observed in studies with the platelet inhibitors clopidogrel and the reversible TxA₂ receptor antagonist HN-11500 in the same experimental model and at similar experimental conditions.

Cigarette smoking had only minor, nonsignificant effects on the coagulation events at the collagen surface. Thus, it appears that cigarette smokers have increased thrombotic response at high arterial shear conditions, which can be reduced by aspirin. However, the enhanced thrombotic response and the antithrombotic effect of aspirin were observed only at high wall shear rate, as encountered in moderately stenosed arteries (2600 s⁻¹). These experimental findings support the notion of cigarette smoking as a risk factor for cardiovascular disease. Our findings indicate also that exposure of collagen fibrils of ruptured arterial stenotic plaques to flowing blood from smokers is more likely to produce critical thrombotic occlusion than blood from nonsmoking individuals. However, it should be emphasized that no distinction was made between acute and chronic effects of cigarette smoking. The antithrombotic effect of aspirin is apparently mediated exclusively through platelet inhibition by blockage of TxA₂ formation. However, the collagen surface used is nonprocoagulant, whereas situations with plaque rupture also may include exposure of tissue factor to the blood.

The effect of cigarette smoking and aspirin on platelet function and coagulation when a procoagulant surface is exposed to the bloodstream at high arterial wall shear rates remains to be established.

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