Differences Between Mainstream and Sidestream Cigarette Smoke Extracts and Nicotine in the Activation of Platelets Under Static and Flow Conditions

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Background—Cigarette smoke is a primary risk factor for cardiovascular diseases. Enhanced function of the hemostatic system, in which platelets play a major role, is a significant underlying mechanism in cardiovascular disease and its progression. Epidemiological studies, complemented by physiological and biochemical data, show that cigarette smoke adversely affects platelet function, both in smokers and in nonsmokers exposed to sidestream smoke.

Methods and Results—The thrombogenic potential of platelets subjected to mainstream smoke extracts, sidestream extracts, and nicotine was measured in vitro under static and dynamic flow conditions. Platelet activation state was measured with a modified prothrombinase-based method. Mainstream and sidestream smoke extracts caused increased platelet activation. Although low-tar mainstream extracts activated platelets less than high-tar extracts, the sidestream extracts were almost equally potent. Modification of the filters of low-tar cigarettes, by blocking the air-bypass holes, raised activation rates by mainstream extracts to the level of high-tar extracts. Nicotine (50 nmol/L and 5 μmol/L) inhibited platelet activation under both flow and static conditions.

Conclusions—Cigarette smoke extracts directly cause platelet activation but also markedly increase the susceptibility of platelets to activation by shear stress. In contrast, nicotine, although also a constituent of cigarette smoke, significantly reduces platelet susceptibility to shear stress. **(*Circulation*. 2004;109:78-83.)**

Key Words: platelets ■ smoking ■ thrombosis ■ cardiovascular diseases

Cigarette smoke is one of the primary risk factors in cardiovascular disease, including the progression of atherosclerosis.[1] Apart from the risk to smokers, it is a major risk for nonsmokers subjected to high levels of secondhand smoke, produced largely by smoldering cigarettes. A key focus of the present study was the differences between "light" (low-tar) and normal (high-tar) cigarettes. The former dilute the inhaled (mainstream) smoke with air that enters the filter through side holes and bypasses the burning tobacco.[2] As long as these are not blocked, as is the case with standard smoking-machine usage and with smokers who do not block the holes with lips or fingers, and as long as the puff volume remains constant, these cigarettes deliver less smoke per puff.

Although the risk from smoking is multifactorial, it has been shown that cigarette smoke acutely increases platelet thrombus formation in patients with coronary artery disease.[3] Platelet-dependent thrombin generation is elevated in smokers even after they abstain from smoking for several hours.[4] It has been suggested that a higher number of active platelets in chronic smokers leads to increased thrombin generation and increased thrombotic risk.[5] Many studies, mainly of platelet aggregation in response to exogenous agonists like ADP and serotonin, show increased response after exposure to cigarette smoke.[6–10] This is despite the fact that nicotine, which is the major alkaloid of cigarette smoke, actually inhibits platelet activity.[11,12] In addition to their role in aggregation and formation of the hemostatic plug, activated platelets are centrally involved in the clotting process: factor X activation by factors IXa+VIIIa requires negative phospholipid, and prothrombin activation requires both negative phospholipid and the activated cofactor, factor Va.

In this study, we examine the prothrombotic properties of platelets exposed to cigarette-smoke extracts of both the mainstream and sidestream type and describe the importance of platelet activation by these agents when exposed to flow conditions that mimic the stresses that may be encountered under normal arterial flow.

Methods

Smoke Extracts

Two cigarette brands, 1 standard and 1 "ultralight," were used for preparing the smoke extracts: high-tar Marlboro 100s, with 16 mg of

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Drs Jesty and Bluestein have an interest in a pending US patent application concerning the modified prothrombinase method used in this study to measure platelet activation.

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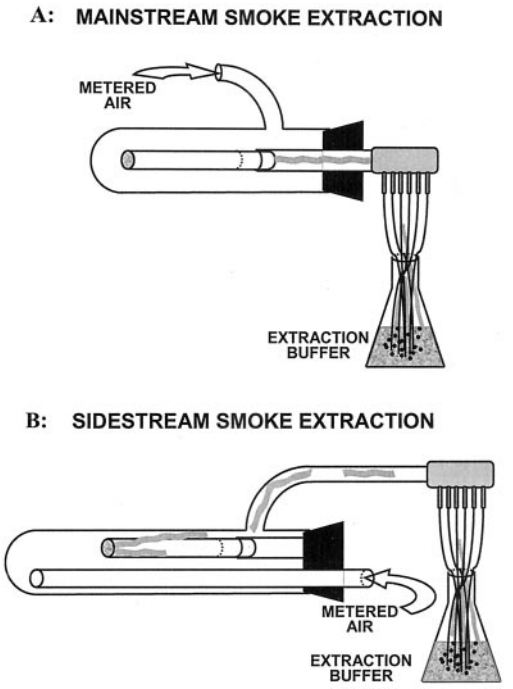

Figure 1. Schematic of smoke extraction methods. A, In mainstream extraction, all smoke passes through cigarette filter. B, In sidestream extraction, no smoke passes through filter. Other details are given in Methods.

tar and 1.2 mg nicotine (Philip Morris) and low-tar Kent III Ultralight 100s, with 5 mg of tar and 0.5 mg of nicotine (Lorillard). Extracts were prepared by modifications of previous methods[13] and do not correspond with those made by smoking machines (Figure 1). In the mainstream extraction, all the smoke passes through the cigarette and the filter, whereas in the sidestream extraction, all smoke is collected from the smoldering end of the cigarette, and none passes through the cigarette or filter. The bypass ventilation holes in the filters of low-tar cigarettes remain unobstructed, except in the case of 1 modified mainstream extraction process (low-tar, modified filter), in which the holes were blocked with transparent tape.

In all preparations, puffing was mimicked by alternating the airflow rate every 30 seconds, from 200 mL/min for 25 seconds to 600 mL/min for 5 seconds, measured by an in-series flowmeter (Cole-Parmer). This procedure was followed until the cigarette burned to 2 to 3 mm short of the filter, which took ≈5 minutes. Smoke was distributed via a manifold (Small Parts Inc) to 6 0.9-mm capillaries submerged in HEPES-buffered saline (130 mmol/L NaCl, 20 mmol/L HEPES-NaOH, pH 7.4). A "standard" extract of each type was prepared by extracting the smoke from 2 cigarettes into 100 mL of buffer (depth ≈4.5 cm). Unlit control extracts were similarly prepared with unlit cigarettes. Extracts were separated into aliquots and stored at −20°C.

Platelets

Informed consent, approved by the Stony Brook University Institutional Review Board, was obtained from healthy nonsmoking adults of both sexes who had taken no aspirin or ibuprofen for 2 weeks. Blood was drawn by venipuncture and collected into 1/100 volume 40% trisodium citrate. Platelet-rich plasma was prepared by centrifugation at 700*g* for 2.5 minutes. Platelet-rich plasma (10 mL) was gel filtered at 3 mL/min through a 220-mL column of Sepharose 4B (Sigma-Aldrich) that had been equilibrated with a HEPES-modified Ca^{2+}-free Tyrode's buffer as described previously.[14,15] The gel-filtered platelets were counted (Z1 particle counter; Coulter) and maintained with gentle agitation at room temperature. Platelets were used within 4 hours of gel filtration. Addition of 3 mmol/L Ca^{2+} and treatment with smoke extracts or nicotine was started 15 minutes before each experiment.

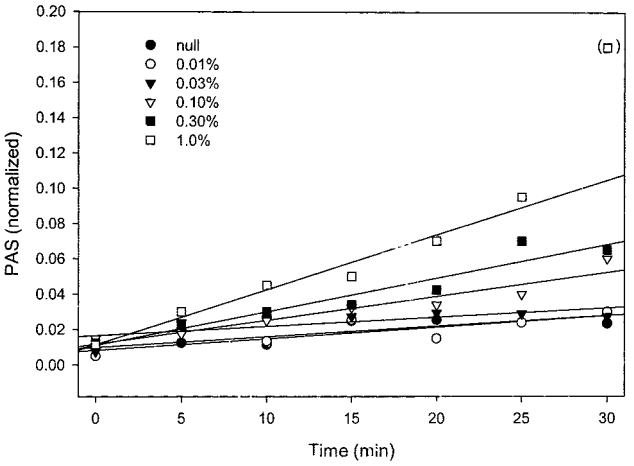

Figure 2. Effect of varying concentrations of high-tar mainstream smoke extracts on platelets under flow conditions. Platelets were circulated at 37°C in capillary flow loop at intermittent shear stress of 4 dyne/cm^2 at varying dilutions of "standard" smoke extract (see Methods). Samples were removed every 5 minutes for PAS assay. Lines were fitted by unweighted linear regression.

Platelet Activation State Assay

Platelet activation state (PAS) was measured by a modified prothrombinase method that measures the generation of acetylated thrombin from acetylated prothrombin by factor Xa.[15,16] Activated, but not unactivated, platelets contribute 2 essential cofactors in this reaction, negative phospholipid and factor Va. Thus, the rate of thrombin generation reflects the activation state of the platelets. We have shown that prothrombinase-based PAS measurements correlate with negative phospholipid exposure, measured by annexin V binding.[16] To circumvent some of the PAS variability in individual platelet preparations, which vary significantly in base activation state and maximal activity, PAS data values from individual experiments ($PAS_{observed}$) were divided by the activity of those same platelets subjected to maximal activation with 5 μmol/L calcium ionophore A23187 (PAS_{max}), to generate a normalized PAS value.[15,16] PAS data are dimensionless values with a maximum value of 1.

Circulation Loop

Platelets (10^5 per μL) were circulated in a flow loop that contained a 1-m section of 0.86-mm PTFE capillary tubing, as described previously.[16,17] The platelets were exposed intermittently to shear in the capillary section for ≈25% of the circulation period. Thus, for 30-minute experiments, the integrated time of shear exposure was ≈7.5 minutes. Except in initial dose-response experiments (Figure 2), which were conducted at a shear stress of 4 dyne/cm^2, the flow rate was adjusted to produce a shear stress of 12 dyne/cm^2 in the capillary section. All circulation experiments were done at 37±2°C, with samples being removed for PAS assay every 5 minutes.

Statistics

The final result of a given experiment is a platelet activation rate (PAR), which is the rate of increase in the normalized PAS value per unit of time. Because the normalized PAS is dimensionless, the units of PAR are reciprocal time (min^{-1}). For each experimental run, PAR values were determined by linear regression. These were then collected for each experimental condition to generate a mean PAR value and SD. Pairwise comparisons on these data were done by the Student paired *t* test (Tables 1 and 2).[18]

TABLE 1. PARs in the Presence of Smoke Extracts Under Static Conditions

Extract	Unlit Mainstream	High-Tar Mainstream	Low-Tar Mainstream	Unlit Sidestream	High-Tar Sidestream	Low-Tar Sidestream
PAR, min^{-1}	0.000335	0.00076	0.00044	0.00046	0.00082	0.00072
Low-tar sidestream	...	...	$P<0.001$	$P<0.001$	$P=0.2$	
High-tar sidestream	...	$P=0.6$	...	$P<0.001$		
Low-tar mainstream	$P=0.002$	$P=0.003$				
High-tar mainstream	$P<0.001$					

PARs are means of individual regression lines in Figure 3 for each condition. Probabilities (*P*) for all relevant pair comparisons were calculated as described in Methods against the null hypothesis that PAR values were identical.

Given the number of independent variables in the study, confirmatory ANCOVA was also performed on the PAR values of each experimental set, under 1 set of conditions, compared with all other PAR values obtained with extracts of the same type. For example, the 4 PAR values obtained with the high-tar mainstream extract under flow conditions were compared with all other PAR values obtained with mainstream extracts under flow conditions.[19]

Results

Dose-Response

Initial dose-response experiments with high-tar mainstream extracts were done to establish the relation between extract concentration and PAR and to select conditions for subsequent experiments. Platelets were circulated at a shear stress of 4 dyne/cm^2, which characterizes the lower end of normal arterial flow conditions. An unlit cigarette extract was used as a control. The activation rates of platelets at 6 concentrations of extract are shown in Figure 2. Recalling that the "standard" smoke extract is defined here as having a "concentration" of 1 cigarette per 50 mL of extract buffer, we see that even a concentration of 0.1% of this (1 cigarette per 50 L) causes a detectable rise in PAR. A concentration of 1% (1 cigarette per 5 L) was chosen for subsequent experiments. In the same experiments, platelets were counted before and after 30 minutes of circulation. The average loss under these conditions was 11.1±2.3% (SEM).

Static Conditions

The effects of the 4 types of smoke extract (high- and low-tar extracts prepared by mainstream and sidestream methods) were first tested under static conditions. These results measured the direct activation of platelets and served as controls for the flow experiments. The slow activation of platelets in the absence of other exogenous stimulus is well known, particularly in the presence (as here) of Ca^{2+} ions. The pooled results for 3 comparisons of mainstream and sidestream smoke extracts are shown in Figure 3, with mean PARs and probabilities for pairwise comparisons shown in Table 1. The following points are noteworthy: (1) for the high-tar cigarettes, mainstream and sidestream extracts were nearly equipotent in activating platelets; (2) in contrast, mainstream and sidestream extracts of low-tar cigarettes showed significant differences; whereas the low-tar mainstream extract activated platelets less than the high-tar extract, the more potent sidestream extracts were very similar. With the major proviso that we limit ourselves here to platelet activation and to the particular cigarettes used, we concluded that these cigarettes differed significantly only in their mainstream smoke. They did not differ in the sidestream smoke, which has maximum platelet-activating potency. ANCOVA analysis yielded a significant *F* value ($\alpha=0.05$) that was greater than the critical value for both extraction methods (mainstream $F=9.73$, sidestream $F=11.21$; critical value=5.79).

Flow Conditions

Although the static studies showed that smoke extracts directly caused slow platelet activation, a major purpose of the present study was to determine whether they rendered the platelets more sensitive to flow. Activation was studied in the presence of smoke extracts for 30 minutes at an intermittent shear stress of 12 dyne/cm^2, the upper range of normal coronary artery flow conditions. Figure 4 shows the results for mainstream and sidestream extracts, with the results of pairwise comparisons shown in Table 2. Shear stress caused a major increase in PAR, as expected, but the key qualitative results of the static studies remained: (1) Although low-tar and high-tar mainstream smoke extracts differed signifi-

TABLE 2. PARs in the Presence of Smoke Extracts Under Flow Conditions at a Shear Stress of 12 dyne/cm^2

Extract	Unlit Mainstream	High-Tar Mainstream	Low-Tar Mainstream	Modified Mainstream	Unlit Sidestream	High-Tar Sidestream	Low-Tar Sidestream
PAR, min^{-1}	0.00365	0.00745	0.00565	0.00785	0.0027	0.00895	0.00795
Low-tar sidestream	...	...	$P=0.001$	...	$P<0.001$	$P=0.5$	
High-tar sidestream	...	$P=0.4$	...	...	$P<0.001$		
Modified mainstream	$P=0.002$	$P=0.64$	$P<0.001$				
Low-tar mainstream	$P=0.001$	$P=0.03$					
High-tar mainstream	$P<0.001$						

PARs are the regression slopes of the lines in Figure 4. Other details are as for Table 1.

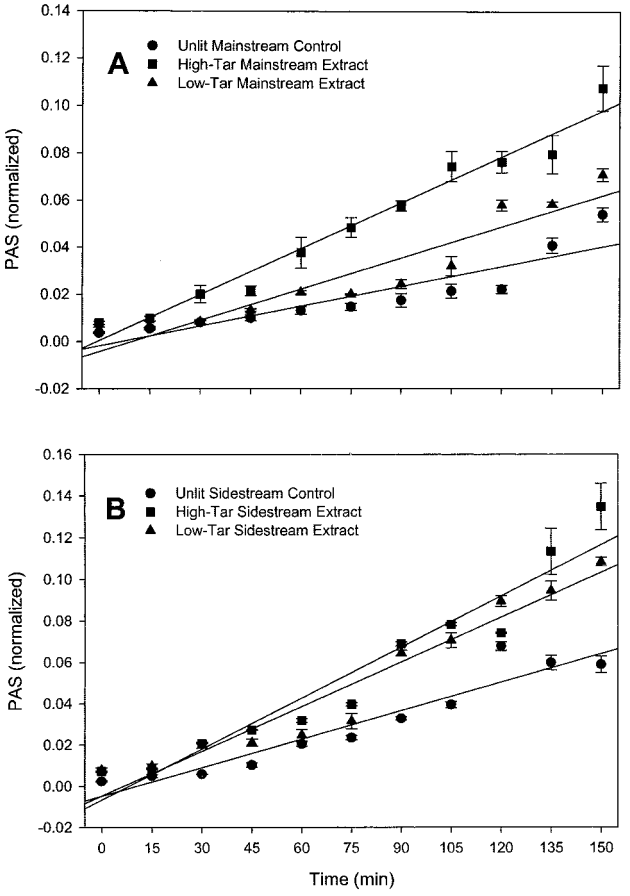

Figure 3. Platelet activation under static conditions in presence of (A) mainstream and (B) sidestream smoke extracts from unlit, high-tar, and low-tar cigarettes. Platelets were incubated in presence of smoke extract (1% of "standard" extract) at 37°C for 150 minutes, and samples were removed every 15 minutes for PAS assay. Data points and lines represent collected results of triplicate experiments performed on 3 different platelet preparations. Data points are means (±SEM) of normalized PAS values for platelets subjected to unlit extract (●), high-tar extract (○), and low-tar extract (■).

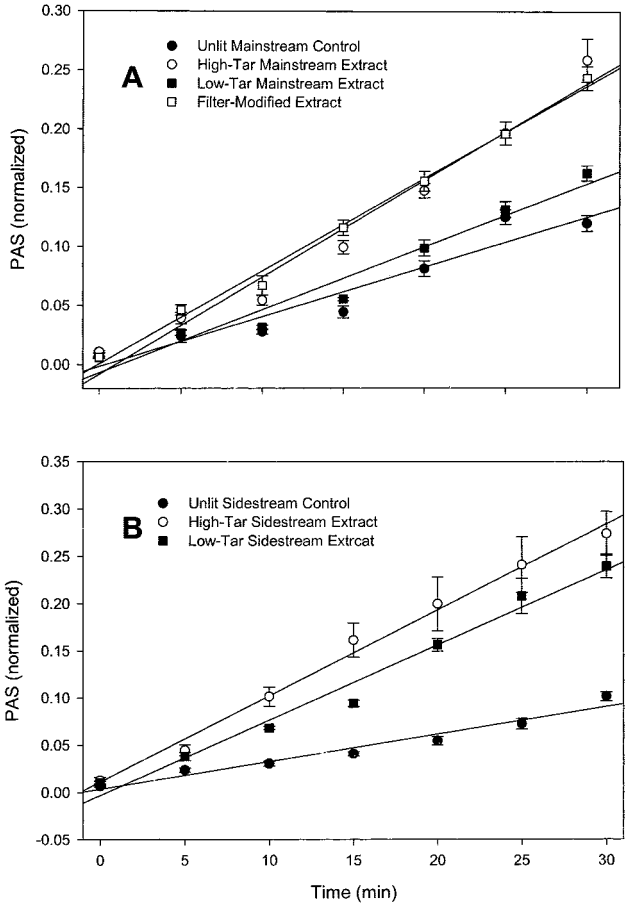

Figure 4. Platelet activation under intermittent shear stress of 12 dyne/cm^2 in presence of mainstream smoke extracts from (A) unlit, high-tar, low-tar, and low-tar modified-filter cigarettes and (B) sidestream smoke extracts from unlit, high-tar and low-tar extracts. Platelets were circulated at 37°C for 30 minutes through capillary loop, with samples being removed every 5 minutes. Each data point and fitted line represents collected results of experiments performed on 4 different platelet preparations. Data are means (±SEM) of normalized PAS values for platelets subjected to unlit extract (●), high-tar extract (○), low-tar extract (■), and low-tar modified-filter extract (□).

cantly, the sidestream extracts were maximally potent and nearly identical. (2) Although the relative effects of the extracts under flow conditions were of the same order as seen under static conditions, the absolute rates of shear-induced platelet activation were greatly increased in the presence of smoke extracts, even under shear conditions equivalent to normal coronary flow. ANCOVA again yielded significant F values (α=0.05; mainstream F=9.11, critical value=3.59; sidestream F=5.68, critical value=4.46).

Modified Filter

Other parameters being equal (eg, puff volume, smoke extraction by the filter material, etc), low-tar cigarettes reduce the delivery of mainstream smoke per puff by diluting it with air that enters through holes in the filter. Although the airflow in our smoke extraction remains the same, and the method ensures that all smoke is extracted (Figure 1A), the volume of air that actually passes through the cigarette is reduced by filter bypass. To determine the effect of bypass, we blocked the holes of the filter with tape and measured PAR under flow conditions (Figure 4A). Table 2 shows that under these conditions, platelet activation increased to the rates seen with unmodified high-tar cigarettes.

Nicotine

The effects of nicotine on platelet activation were measured under static and flow conditions at 2 concentrations, 1 very high (5 μmol/L) and 1 within the range expected in the plasma immediately after smoking 1 cigarette (50 nmol/L). The results of triplicate (static) and quadruplicate (flow) experiments are summarized in Figure 5. Platelet activation rate was reduced ≈50% at both nicotine concentrations (P<0.001 in both cases). ANCOVA yielded significance (α=0.05) at both nicotine concentrations (static F=9.06, critical value=5.79; flow F=113.57, critical value=4.46).

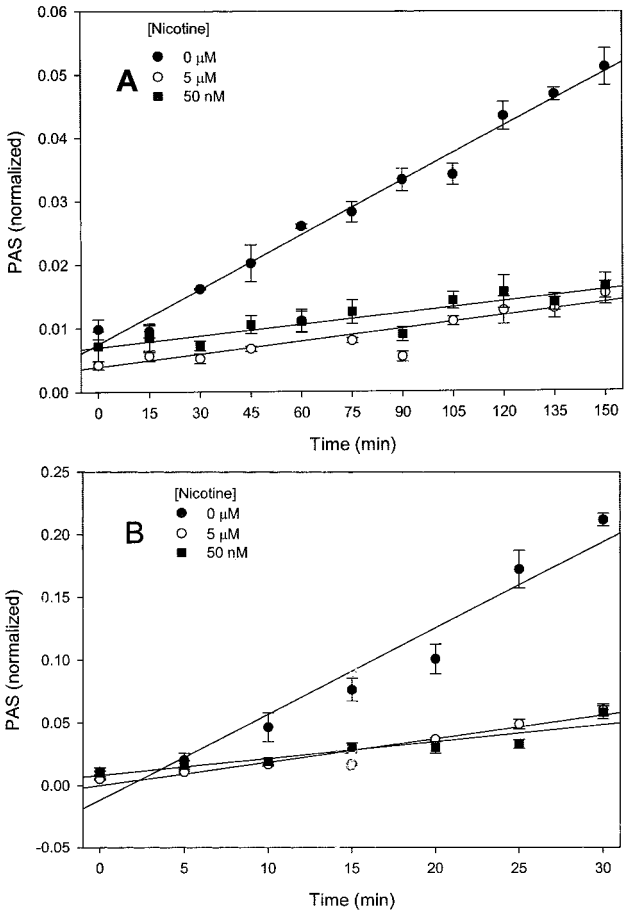

Figure 5. Effect of nicotine on platelet activation under static and flow conditions. Nicotine was added to platelet preparations to concentrations shown, and platelet activation was followed under (A) static or (B) flow conditions as described for Figures 3 and 4. Data points and lines represent collected results of (A) triplicate or (B) quadruplicate experiments performed on different platelet preparations. Data points are means (±SEM) of normalized PAS values for platelets in presence of 0 μmol/L nicotine (●), 50 nmol/L nicotine (■) and 5 μmol/L nicotine (○). Note different ordinate and abscissa scaling for 2 conditions.

Discussion

Platelet Activation

The prothrombinase assay measures the exposure on activated platelets of 2 required cofactors of prothrombin activation: anionic phospholipid and factor Va. Importantly, we note that this activity is not simply an indicator; support of thrombin generation constitutes a major part of any summation of the thrombogenic potential of platelets. The PAS assay does not discriminate between the activity of whole platelets and that of microparticles that may be released on activation and/or mechanical damage; both contributions are measured, and it thus gives a global activation measure. Measurements of platelet recovery after flow experiments show that platelet loss is quite small (≈11%), and we conclude that microparticle formation is probably not a major confounding variable.

Smoke Extraction

Although the use of smoke extracts is standard in studies of the effects of tobacco smoke on physiological systems, differences between extract preparation and the physiological processes in a smoker's lungs and vascular system are obvious, and we cannot extrapolate the results obtained here to the physiological response to cigarette smoke. Yet, recalling that the "standard" smoke extract is defined here as having a "concentration" of 1 cigarette per 50 mL of extract buffer, we see that even a concentration of 0.1% of this (ie, 1 cigarette per 50 L) causes a detectable rise in PAR (Figure 2). In contrast, the comparative results of the present study should be valid because of the identical extraction methods used for the 2 types of cigarettes. Moreover, by comparing platelet activation under static and flowing conditions, we demonstrate unequivocally that cigarette smoke sensitizes the platelets to flow-induced activation.

Static Conditions

The conditions in this part of the study approximate those of other investigators who have studied other effects of smoke on platelets. One critical result is clear: whereas the high-tar and low-tar mainstream extracts differ, the sidestream extracts are equally and maximally potent in platelet activation.

Flow Conditions

As we[16,17] and others have shown, using a variety of shear-inducing techniques and measures of activation state, shear stress activates platelets. The shear stress selected for the main part of this study, 12 dyne/cm^2, characterizes blood flow in healthy coronary arteries, and we asked whether smoke extracts render platelets more sensitive to such a stimulus. The data shown in Figure 4 and Table 2 show that they do, with a 30-minute exposure to intermittent shear in the presence of smoke extracts activating platelets to 50% of maximum. In contrast, the smoke-free controls (Figure 4) showed activation over the same time period of just 20%.

We also used the flow system to examine the effect of filter modification of low-tar cigarettes, and the results (Figure 4A) show that in the absence of bypass filter ventilation, the extracts are as potent as those of high-tar cigarettes in causing platelet activation. (Sidestream extracts from modified cigarettes were not prepared or studied, because the sidestream extraction method allows no smoke through the filter.) Several studies have shown that when smoking low-tar cigarettes, smokers often compensate for the reduction in smoke concentration by increasing puff frequency and puff volume.[2] Some smokers may also partially block the filter bypass holes of these cigarettes with fingers or lips. Although the prevalence of this latter behavior is unclear, we addressed its possible effect by completely blocking the filter holes, and indeed, under these conditions, platelet activation rose to levels equal to those with high-tar cigarettes.

Despite the much higher rates of platelet activation than under static conditions, the qualitative differences between the different extracts remain. Only the low-tar mainstream extract showed any significant reduction in platelet-activating potential, and this requires the filter bypass holes to remain unobstructed during extraction (Table 2). The remainder (including, especially, the sidestream low-tar extract and modified-filter low-tar extract) were equally and maximally potent.

Nicotine

The observation that nicotine desensitizes platelets is not new,[11,12] and we demonstrate that desensitization remained under flow conditions (Figure 5). The effect was a reduction of 50% or more in PAR under both static and dynamic conditions at a nicotine concentration of only 50 nmol/L (8 ng/mL), a level that corresponds approximately with the blood level immediately after smoking a low-tar cigarette.[20] These results also imply that if nicotine were absent, as it is in some recently marketed cigarettes, platelet activation by smoke extracts might be substantially higher than observed here.

Summary

The results demonstrate that cigarette-smoke extracts substantially increase platelet activation caused by exposure to shear stress and do so even under normal flow conditions, approximately equivalent to the conditions in healthy coronary arteries. Nicotine is not the cause, because nicotine in the absence of smoke significantly protects platelets against activation. The results address differences between mainstream smoke, which is the small proportion of smoke that smokers inhale, and sidestream smoke, the majority of which is formed by smoldering cigarettes and which is the major component of secondhand smoke. We show that at equivalent concentrations, sidestream smoke can be significantly more potent than mainstream smoke in the activation of platelets under both static and flow conditions. Although the differences are small for high-tar cigarettes, in which mainstream smoke contains high levels of tar and nicotine anyway, they are highly significant for low-tar cigarettes. The lower potency of low-tar mainstream smoke depends on the filters of these cigarettes, which allow bypass air to dilute the smoke. When bypass is prevented, as it may be if smokers block these holes during inhalation, the mainstream smoke of low-tar cigarettes is as potent in activating platelets as that of high-tar ones. Thus, in this study of a major thrombogenic effect, we find that the “light” designation for such cigarettes refers only to the mainstream smoke, which is not a significant constituent of secondhand smoke.

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