Acute effects of cigarette smoking on platelet-dependent thrombin generation


Second Department of Internal Medicine, Kyorin University School of Medicine, Tokyo, Japan

Aims Thrombin is an important factor in the pathogenesis of thrombotic diseases. To clarify whether smoking has an effect in platelet-dependent thrombogenesis, we studied the acute effects of smoking on platelet-dependent thrombin level in smokers.

Methods and Results Subjects consisted of ten smokers and nine non-smokers. Platelet-dependent thrombin level measured after overnight fasting was greater in smokers than in non-smokers (smokers vs non-smokers, 121 ± 47 vs 56 ± 5 mIU ml⁻¹, P<0·01). When subjects in the smokers group smoked two cigarettes containing 0·9 mg of nicotine per cigarette, platelet-dependent thrombin levels showed a transient three-fold increase in blood samples obtained immediately after smoking (365 ± 76 mIU ml⁻¹, P<0·001). Thrombin levels in the blood samples obtained 10 min and 30 min after smoking were less than that in the samples obtained immediately after smoking ceased, but were not significantly different from those in the samples obtained before smoking. Blood nicotine level increased significantly immediately after smoking (P<0·001), and plasma protein C activity decreased significantly 30 min after smoking (P<0·05). When nicotine or cotinine was added to the platelet-rich plasma of non-smokers ex vivo, the platelet-dependent thrombin level increased significantly (P<0·002).

Conclusion Platelet-dependent thrombin level is enhanced in smokers, even when not smoking, when compared with non-smokers and increases immediately after smoking. Increases in nicotine and cotinine levels caused by smoking induced a prothrombotic state in smokers via increased platelet-dependent thrombogenesis.


Key Words: Thrombin, smoking, platelet, thrombogenesis, cigarette.

See page 16 for the Editorial comment on this article

Introduction

Epidemiological studies have shown that smoking correlates significantly with both incidence and increased mortality in acute myocardial infarction, and has subsequently been established as a coronary risk factor[1,2]. The predisposition of smokers to develop ischaemic heart disease is related to endothelial cell damage[3–5] and increased platelet aggregation, via elevated fibrinogen[6,7]. However, the direct effects of smoking on platelets have not been fully evaluated. Activated platelets form platelet thrombi through adhesion to vascular endothelial cells and platelet aggregation, and promote thrombin generation and fibrin strand formation by activating the coagulation cascade via platelet factor 3 (phospholipid)[8]. We have reported previously that platelet-dependent thrombin levels are increased in patients with hyperlipidaemia[9] and diabetes mellitus[10], using the method previously reported by Aronson[11]. The prothrombotic state caused by smoking has not been studied with respect to platelet-dependent thrombin generation. The objectives of the present study were to determine whether platelet-dependent thrombin levels are increased in smokers, and to explore whether changes in platelet-dependent thrombin levels occur immediately after smoking.

Methods

Subjects

Ten healthy male smokers (mean age 31·4 ± 1·0 years, range 27 to 35 years) and nine healthy male non-smokers
(mean age 31·8 ± 1·7 years, range 26 to 39 years) from the medical staff of the Kyorin University Hospital participated in this study. All subjects had normal thyroid, hepatic and renal function, and no history of ischaemic heart disease, heart failure, inflammatory disease, malignancy or diabetes mellitus. Subjects were not taking drugs known to affect platelet function, coagulation, the fibrinolytic system or lipid metabolism, during the 2 weeks prior to the study. Participants in the smokers group smoked 20 to 40 cigarettes per day (median 25) and had a smoking history of 4 to 16 years (median 10 years). Written informed consent was obtained from all of subjects in accordance with the requirements of the Kyorin University Hospital Ethics Committee.

**Experimental protocol**

The study commenced at 8 a.m. after the subjects had fasted for at least 10 h and abstained from smoking (smokers group) for at least 12 h. After resting in the sitting position for 30 min, subjects in the smokers group smoked two cigarettes containing 0·9 mg of nicotine per cigarette successively within 15 min. The smokers were instructed to smoke two-thirds of the cigarette. Venous blood samples were collected, just before and immediately after smoking, as well as 10 and 30 min after smoking. Platelet-dependent thrombin level, plasma nicotine concentration and protein C activity were determined.

Blood samples from non-smokers were obtained after 10 h of fasting. Platelet-dependent thrombin levels before and after the addition of nicotine or cotinine were determined 

**Blood samples**

Platelet-dependent thrombin level was measured according to the method of Aronson[11], with slight modifications. Venous blood (3·13% sodium citrate: blood 1:9) was centrifuged at 800 g for 10 min at 22°C. Platelet-rich plasma was separated from the upper two-thirds of the supernatant to avoid contamination by other cells, such as monocytes. The remaining blood was centrifuged again at 1500 g for 15 min to obtain platelet-poor plasma. The platelet count in the platelet-rich plasma was determined using a Coulter counter (S-Plas IV, Coulter Electronics, Hialeah, FL, U.S.A.), and the platelet concentration in the platelet-rich plasma was adjusted to 15 x 10^6 μl^-1 with platelet-poor plasma. The platelet-rich plasma aliquots (0·5 ml) were placed into round-bottomed polypropylene tubes (12 x 75 mm), and 20 μl of 1 mol . l^-1 calcium chloride was added to initiate clotting. Glass tubes were not used to prevent activation of the intrinsic pathway of the coagulation cascade. Samples (10 μl) were then added at 10-min intervals for 30 min to the wells of a microtitre plate containing 90 μl of 3·8% sodium citrate solution. Colour developed 2 min after the addition of 50 μl of 0·5 mmol . l^-1 S-2238 H-D-Phe-Arg-NH₂-NO₂-HCl, a thrombin-specific substrate (testozyme, Chromogenix AB, Umeå, Sweden) at 1 mol . l^-1 Tris (pH=8·1). The absorbance of the released colour product was measured with a spectrophotometer at a wavelength of 405 nm using a Vmax microtitre plate reader (Easy Reader, EAR 340AT, SLT Lab Instruments GmbH, Vienna, Austria). Measurements were obtained in triplicate at each time-point. The amount of thrombin generation was calculated from a standard curve.

The plasma nicotine concentration was measured using a gas chromatographic method[12]. Protein C activity was measured according to the method described by Mattioli[13]. Platelet-rich plasma from non-smokers was prepared by the method described above and 500 μl aliquots of platelet-rich plasma were placed into round-bottomed polypropylene tubes. Nicotine (Sigma Chemical Co, St. Louis, MO, U.S.A.) or cotinine (Sigma Chemical Co, St. Louis, MO, U.S.A.) was added at a final concentration of 2 to 200 ng . ml^-1 or 20 ng to 2 μg . ml^-1 respectively, and thrombin generation was measured after incubation for 60 min at room temperature.

**Statistics**

Age is expressed as the mean ± standard deviation. Other data are expressed as mean ± standard error. Thrombin generation was compared using the repeated-measures analysis of variance, and the Bonferroni/Dunn method was used for post hoc analysis. Kruskal-Wallis non-parametric analysis of variance was used for comparisons of other variables. A P value <0·05 was considered statistically significant.

**Results**

**Baseline levels of platelet-dependent thrombin in smokers and non-smokers**

Platelet-dependent thrombin levels at 0, 10, 20 and 30 min after the addition of calcium chloride were 34 ± 3 mIU . ml^-1, 46 ± 6 mIU . ml^-1, 49 ± 4 mIU . ml^-1, and 56 ± 5 mIU . ml^-1, respectively, in non-smokers, and 64 ± 5 mIU . ml^-1, 72 ± 10 mIU . ml^-1, 72 ± 8 mIU . ml^-1 and 121 ± 47 mIU . ml^-1 in smokers. Thus, platelet-dependent thrombin level increased significantly in the smokers (P<0·01 at 30 min after addition of calcium chloride).

**Acute effects of smoking on platelet-dependent thrombin level**

Platelet-dependent thrombin levels immediately after smoking were increased significantly compared with the
samples obtained before smoking (365 ± 76 mIU . ml⁻¹, 121 ± 4 mIU . ml⁻¹, *P*<0·001). The concentrations in samples obtained 10 and 30 min after smoking were 233 ± 71 mIU . ml⁻¹ and 215 ± 69 mIU . ml⁻¹, respectively, which were lower than those obtained immediately after smoking and not significantly different from that in the samples obtained before smoking (Fig. 1).

**Acute effects of smoking on plasma nicotine concentration and protein C activity**

The plasma nicotine concentration increased from 4·4 ± 1·6 ng . ml⁻¹ prior to smoking to 25·5 ± 5·4 ng . ml⁻¹ immediately after smoking (*P*<0·01). At 10 min after smoking, the value was still significantly higher than the baseline value, (26·7 ± 4·9 ng . ml⁻¹), and 30 min after smoking the concentration (6·6 ± 1·5 ng . ml⁻¹) decreased to the baseline level (Table 1).

Plasma protein C level did not differ significantly among samples obtained before smoking, immediately after smoking, or 10 min after smoking (106·6 ± 4·0%, 106·6 ± 4·4% and 107·6 ± 4·4%, respectively), but decreased to 92·5 ± 1·6% 30 min after smoking (*P*<0·05).

**Figure 1** Platelet-dependent thrombin level before and after smoking: ■ before smoking, ○ =0 min after smoking, △ =10 min after smoking, ■ =30 min after smoking. Platelet-dependent thrombin level increased immediately after smoking. *P*<0·001 vs before smoking. Data are expressed as the mean ± SEM.

**Figure 2** Effect of nicotine on platelet-dependent thrombin level in vitro: ■ =baseline, ○ =2 ng . ml⁻¹ of nicotine, △ =20 ng . ml⁻¹ of nicotine, ■ =200 ng . ml⁻¹ of nicotine. Non-smokers platelet-rich plasma with pretreatment of nicotine (2–200 ng . ml⁻¹) significantly increased platelet-dependent thrombin level. *P*<0·001 vs baseline. Data are expressed as the mean ± SEM.

**Table 1** Acute effects of smoking on plasma concentrations of nicotine and protein C

<table>
<thead>
<tr>
<th></th>
<th>Before smoking</th>
<th>After smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nicotine (ng . ml⁻¹)</strong></td>
<td>4·4 ± 1·6</td>
<td>25·5 ± 5·4**</td>
</tr>
<tr>
<td><strong>Protein C (%)</strong></td>
<td>106·6 ± 4·0</td>
<td>106·6 ± 4·4</td>
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</table>

Values are expressed as the mean ± SEM. *P*<0·05 vs before smoking, **P*<0·001 vs before smoking.

Platelet-dependent thrombin levels 30 min after the addition of calcium chloride, when 2 ng . ml⁻¹ of nicotine was added to the platelet-rich plasma of healthy non-smokers, were 301 ± 80 mIU . ml⁻¹, which was significantly higher than the baseline value (Fig. 2, *P*<0·001). A similar increase was noted with the addition of either 20 ng . ml⁻¹ or 200 ng . ml⁻¹ of nicotine (*P*<0·001). Similarly, the platelet-dependent thrombin level after the addition of 20 ng . ml⁻¹ of cotinine to the platelet-rich plasma of healthy non-smokers was 234 ± 76 mIU . ml⁻¹ which was increased compared with the baseline value (Fig. 3, *P*<0·001). Thrombogenesis increased similarly with the addition of either 200 ng . ml⁻¹ or 2 µg . ml⁻¹ of cotinine (*P*<0·001).

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Discussion

In the present study, the platelet-dependent thrombin level was increased significantly in healthy male smokers compared with non-smokers, even before smoking. Platelet-dependent thrombogenesis also showed a transient, but sharp, increase immediately after smoking.

Smoking and thrombin generation

Smoking is widely recognized as a coronary risk factor. According to the Framingham study, the relative risk of sudden cardiac death in male smokers is about 10 times greater than that in non-smokers, while in female smokers, this risk was 4.5 times greater\textsuperscript{14,15}. A similar trend was also noted in the Gotenborg study in Sweden\textsuperscript{2}.

The mechanisms by which smoking induces ischaemic heart disease have not been established. Factors involved in the predisposition of smokers to ischaemic heart disease include endothelial cell damage caused by nicotine\textsuperscript{16}, carbon monoxide\textsuperscript{17}, free radicals\textsuperscript{18}, promotion of atherosclerosis by increasing serum total cholesterol and triglyceride and decreasing high-density lipoprotein cholesterol\textsuperscript{19}, increases in platelet aggregation\textsuperscript{7} and coagulation activity\textsuperscript{20} caused by plasma fibrinogen elevation\textsuperscript{6} and platelet activation. Previous reports have observed an increase\textsuperscript{21,22}, decrease\textsuperscript{23}, or no change\textsuperscript{24} in platelet aggregability following smoking. Platelet aggregability is apparently increased following smoking only in patients with coronary artery disease\textsuperscript{25}.

The formation of platelet thrombi associated with disruption of atherosclerotic plaques has been reported during the onset of acute coronary syndromes\textsuperscript{26,27}. The role of platelets in the initiation of ischaemic heart disease has received much attention. It appears that the formation of platelet and fibrin thrombi in the coronary arteries during an acute coronary event is promoted by the adhesion of platelets to endothelial cells, platelet aggregation and increased procoagulant activity of platelets.

We have reported previously that platelet-dependent thrombogenesis is increased in patients with hyperlipidaemia\textsuperscript{29} or diabetes mellitus\textsuperscript{30}, suggesting that the increased procoagulant activity of platelets is involved in the predisposition of patients with these conditions to coronary thrombosis. In the present study, we observed a sharp increase in the platelet-dependent thrombin level following smoking. Smoking apparently promotes the procoagulant activity of platelets and enhances the prothrombotic state. The sharp exacerbation of the prothrombotic state following smoking is also assumed to be related closely pathophysiologically to the onset of the acute coronary syndrome.

Role of nicotine and cotinine in smoking-induced platelet-dependent thrombogenesis

In the present study, platelet-dependent thrombin levels increased immediately after smoking and then decreased soon after. Plasma nicotine concentrations also increased immediately after smoking and decreased in a similar manner to the pre-smoking levels 30 min after smoking, in parallel with platelet-dependent thrombin levels. When nicotine or cotinine was added ex vivo to the platelet-rich plasma of non-smokers at concentrations similar to those observed in the plasma of smokers, the platelet-dependent thrombin level increased significantly. Therefore, nicotine and its metabolite cotinine play an important role in increasing platelet-dependent thrombin levels in smokers. The basal plasma concentration of nicotine in smokers (after 12 h of abstinence from smoking) was comparable to the concentration which exhibited the maximum increase in the thrombin level ex vivo. The reason why this concentration did not increase the thrombin level in vivo was not clear. There might be inhibitory factors to thrombin generation in the plasma of smokers.

Increases in blood nicotine cause platelet activation by increasing levels of catecholamines, especially epinephrine\textsuperscript{28,29}. Kimura \textit{et al.}\textsuperscript{20} have reported that smoking increased levels of epinephrine, fibrinopeptide A and the thrombin-antithrombin III complex, but excluded the involvement of platelets, since \(\beta\)-thromboglobulin and platelet factor IV levels were not increased. However, no correlation exists between platelet-dependent thrombin level and the release of \(\beta\)-thromboglobulin or platelet factor IV from the platelets\textsuperscript{29}. Thus, it is possible that increases in levels of fibrinopeptide A and the thrombin-antithrombin III complex in smokers are induced by an increase in platelet-dependent thrombin level. In the present study,
plasma protein C activity decreased significantly within 30 min after smoking. This is probably attributable to increases in the platelet-dependent thrombin level we observed ex vivo, now occurring in vivo, and protein C, a physiological anticoagulant factor, being consumed reactively.

**Study limitations**

Various humoral factors may be involved in the increase of platelet-dependent thrombin level during smoking. It is possible that increased plasma catecholamine levels induced by enhanced sympathomimetic activity during smoking are important\(^\text{[30]}\). However, plasma catecholamine levels were not measured in this study. In the present study, the plasma concentration of nicotine in smokers after 12 h of abstinence from smoking (i.e., 4.4 ng . ml\(^{-1}\)) was slightly higher than the concentration which already exhibited the maximum increase in thrombin level ex vivo. In other words, it is possible that factors which inhibit the increase in thrombin level due to nicotine also are present in the plasma of smokers. Future studies of factors which promote or inhibit platelet-dependent thrombin generation are necessary.

**Conclusion**

Smokers exhibit increased platelet-dependent thrombin levels compared with non-smokers, even before smoking. Platelet-dependent thrombin level increases sharply, immediately after smoking. Increases in plasma nicotine and cotinine levels due to smoking may be important factors in the pathophysiology of increased platelet-dependent thrombin generation.

**References**


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