Smoking Is Associated With Altered Endothelial-Derived Fibrinolytic and Antithrombotic Factors
An In Vitro Demonstration

Rajat S. Barua, MD; John A. Ambrose, MD; Dhanonjoy C. Saha, PhD; Lesley-Jane Eales-Reynolds, PhD

Background—Data about the effects of smoking on thrombo-hemostatic factors (tissue factor [TF] and tissue factor pathway inhibitor [TFPI-1]) are limited and on fibrinolytic factors (tissue plasminogen activator [t-PA] and plasminogen activator inhibitor-1 [PAI-1]) are debatable. The present study investigated the smoking-related, endothelial cell (EC)–specific responses for these factors and their relation to nitric oxide (NO) production in vitro.

Methods and Results—Serum from 8 nonsmokers and 15 smokers were incubated with confluent (~85%) human umbilical vein endothelial cells (HUVECs) in 24-well tissue-culture plates for 12 hours. After the incubation, basal NO, t-PA, PAI-1, TF, TFPI-1 production, and substance P (SP)–stimulated NO, t-PA, and PAI-1 production were determined. HUVECs treated with smokers’ serum showed lower basal (P<0.02) and SP-stimulated (P=0.059) t-PA production but similar basal and stimulated PAI-1 production (P=0.9 and P=0.6) compared with nonsmokers. Basal t-PA/PAI-1 molar ratio was significantly reduced in smokers (P<0.005). TFPI-1 level in the cell culture supernatant was also significantly lower in smokers compared with the nonsmoker group (P<0.05) with no difference in TF level between both groups (P=0.5). As previously reported, both basal (P<0.001) and SP-stimulated (P<0.05) NO production were significantly reduced in smokers. Basal TFPI-1 in culture correlated positively with basal NO production (r=0.42, P=0.04) and negatively with serum cotinine level (r=−0.6, P=0.01).

Conclusions—These results indicate that cigarette smoking is associated with alterations in EC-derived fibrinolytic (t-PA) and antithrombotic (TFPI-1) factors. To our knowledge, this is the first demonstration that EC-derived TFPI is affected by smoking and endogenous NO or that the degree of smoke exposure may influence TFPI levels in an EC milieu.

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Key Words: smoking • thrombosis • fibrinolysis • nitric oxide

Cigarette smoking is associated with an increased risk of acute myocardial infarction and sudden cardiac death.1 It has been hypothesized that alterations of antithrombotic and fibrinolytic functions in the blood or at the vessel-wall interface are responsible for most of these acute events in smokers.1,2 The exact mechanisms underlying this dysfunction are yet to be elucidated. Cigarette smoke–induced alterations of endothelial cell (EC) function seem to be one of the potential mechanisms for this dysfunction.

ECs play a central role in vascular homeostasis by maintaining a delicate balance between vasodilating (NO) and vasoconstricting (endothelin-1) factors, thrombotic (tissue factor [TF]) and antithrombotic (tissue factor pathway inhibitor-1 [TFPI-1]) factors, as well as fibrinolytic (tissue plasminogen activator [t-PA]) and antifibrinolytic factors (plasminogen activator inhibitor-1 [PAI-1]).3 Data about the effects of smoking on thrombo-hemostatic factors (TF and TFPI-1) are limited and on fibrinolytic factors (t-PA and PAI-1) are debatable, with increase, decrease, or change of these molecules having been reported.2,4–6

Nitric oxide (NO), in addition to its vasoregulatory function, is involved in maintaining antithrombotic properties of various cells, including ECs and platelets.3 We and others have shown that the NO biosynthesis or bioavailability is reduced in smokers.7 However, the possible interrelations between endothelial NO, TF, TFPI-1, t-PA, and PAI-1 production in smokers are not known. In this study, using a previously validated in vitro model,7 the effect of smokers’ serum on endothelial production of these hemostatic molecules was examined.

Methods

Subjects
Healthy male nonsmokers (n=8) and active smokers (n=15) (mean age, 33.0±1.4 and 31.8±2.2, respectively) were requested to abstain from smoking and foods or caffeinated drinks overnight. The
following morning, after a 10-minute rest period, their serum was collected and stored at −70°C until use. The mean cigarette exposure in smokers was 8±3 pack-years and 12±3 cigarettes per day. All subjects were free of other traditional cardiovascular risk factors (ie, hypertension, diabetes, low HDL, hyperlipidemia, or a family history of premature vascular disease) and were not taking any medication. The serum cotinine concentrations were determined by ELISA (STC Technologies). Saint Vincent Catholic Medical Centers of New York Ethics Committee approved the study protocol, and each subject gave written informed consent.

**Endothelial Cell Culture and Determination of Homeostatic Parameters In Vitro**

Primary human umbilical endothelial cells (HUVECs) were purchased (Clonetics). The vitro experiments were performed as previously described. In brief, serum from nonsmokers and smokers was incubated with confluent (~85%) HUVECs in 24-well tissue-culture plates for 12 hours. After the incubation, basal NO, t-PA, PAI-1, TF, TFPI-1 production, and substance P (SP)–stimulated NO, t-PA, and PAI-1 production were determined. All parameters were measured from the same cell culture supernatant. NO concentration in each sample was determined using an NO analyzer (Sievers). Hemostatic variables were determined using commercially available ELISA kits (American Diagnostica [TF and TFPI-1] and Biopool AB [t-PA and PAI-1]). The parameters were expressed in their respective units after adjusting for the background. t-PA/PAI-1 molar ratio was calculated by using a formula published previously.

**Statistical Analyses**

Results are presented as the mean±SEM. Unpaired Student’s t test was used to compare the group differences. Linear regression analysis was used to examine the association between in vitro EC-derived thrombo-hemostatic parameters, NO, and smokers’ serum cotinine level. P<0.05 was considered statistically significant.

**Results**

**In Vitro Effect of Smokers’ Serum on EC-Derived NO and Hemostatic Parameters**

HUVECs exposed to smoker’s serum in vitro showed a significantly lower basal and SP-stimulated NO production compared with nonsmokers (1.3±0.2 versus 4.0±0.5 μmol/L, P<0.001; and Δ baseline, 0.1±0.1 versus 1.1±0.5 μmol/L, P<0.05, respectively), as previously reported.

In the cell culture supernatant, TF antigen level from HUVECs treated with smokers’ serum was not different from that of nonsmokers (19.0±3.5 versus 15.0±5.8 pg/mL, respectively, P=0.6). However, the smokers showed a significantly lower TFPI-1 level compared with the nonsmokers (5.7±0.4 versus 8.2±1.3 ng/mL, respectively, P=0.04).

HUVECs treated with smokers’ serum showed significantly lower basal t-PA production compared with the nonsmokers (2.3±0.3 versus 4.9±1.1 ng/mL, P=0.02). Similarly, SP-stimulated t-PA release was lower in smokers compared with nonsmokers (Δ% baseline, 15.0±4.4% versus 36.0±12.0%), although this difference did not quite reach significance (P=0.059). Additionally, both basal and SP-stimulated PAI-1 concentrations were similar between smokers and nonsmokers (81.6±6.1 versus 82.5±16.6 ng/mL, P=0.95; and 7.3±1.4 versus 6.0±1.7 ng/mL, P=0.6, respectively). Basal t-PA/PAI-1 molar ratio was significantly reduced in smokers compared with nonsmokers (0.02±0.01 versus 0.04±0.01, respectively, P<0.005 (Figure 1).
Relationship Between Endothelial-Derived Hemostatic Parameters, Nitric Oxide, and Serum Cotinine

On linear regression analysis, no significant correlation was found between basal NO and t-PA ($r = 0.33$, $P = 0.12$), PAI-1 ($r = 0.05$, $P = 0.84$), or TF ($r = 0.17$, $P = 0.43$). Only basal TFPI-1 positively correlated with basal NO ($r = 0.42$, $P = 0.04$). In the smokers’ group, serum cotinine correlated negatively with basal TFPI-1 ($r = -0.6$, $P = 0.01$), and no apparent association with other parameters was found (Figure 2).

Discussion

Thrombosis seems to be the main cause of mortality and morbidity in cigarette smokers. Burke et al have demonstrated that sudden cardiac death in smokers was mainly associated with thrombosis regardless of the underlying plaque pathology (erosion or rupture) and was not associated with the number of lipid-rich vulnerable plaques. ECs are an important source of opposing vascular homeostatic factors that regulate important physiological functions. Using serum from male smokers, this study provides additional evidence of the potential alteration in endothelial antithrombotic and fibrinolytic properties that may contribute to an acute thrombotic event in smokers.

The classical view that thrombus formation occurs at a site of injury because of a nidus provided by the cell surface–bound TF has been broadened by the observation that TF antigen is also found circulating in the blood and has the potential to initiate thrombosis. Several studies have suggested that significantly higher levels of circulating soluble TF are found in patients with acute myocardial infarction and unstable angina. Potential sources of these soluble circulating TF include EC, vascular smooth muscle cell, monocyte, and leukocytes. It has been suggested that increased expression of TF on the cellular surface is associated with increased soluble TF. Data concerning the effect of smoking on TF expression or level are limited, and the contribution of ECs in this regard is not known. Matetzky et al have shown that smoking is associated with increased TF expression in atherosclerotic plaques from human carotid and aortic root plaques from apoE−/− mice. On the other hand, Blann et al reported no significant difference in soluble TF between smokers and nonsmokers either in controls or in subjects with peripheral arterial disease. In our in vitro study (12-hour incubation), there was no significant difference in EC-derived soluble TF antigen between smokers’ and nonsmokers’ group. It has been proposed that apoptotic ECs are one of the potential sources of TF. We have previously reported that in the present model, smokers’ serum did not alter EC viability or promote EC apoptosis, and this may explain why there was no difference in EC-derived TF between smokers and nonsmokers in the present study.

Nevertheless, in the same cell culture supernatant there was a significant decrease in EC-derived TFPI-1 secretion in smokers. TFPI-1 is not only EC-surface bound, but a substantial amount is also secreted in the blood. The soluble form of TFPI-1 directly inhibits factor Xa, whereas surface-bound TFPI-1 on ECs inhibits TF-factor VIIa complex in conjunction with factor Xa by translocation into caveolae. Thus, the lower TFPI-1 antigen level in smokers as observed in this study may indicate either decreased production or an increased translocation of TFPI from the cell surface to the cytoplasm. Regardless of the mechanism, smokers had a relative increase of the TF/TFPI-1 ratio and conceivably an increase in thrombotic potential. Additionally, TFPI-1 showed a significant negative correlation with the serum cotinine level, suggesting a possible dose-related effect of smoking on TFPI.

The structural support of a thrombus is provided by a matrix of cross-linked fibrin. A timely dissolution of the fibrin matrix is essential to inhibit pathological propagation of a thrombus. The lysis of fibrin is mediated by plasmin that is activated by t-PA, which in turn can be inhibited by PAI-1. It has been suggested that t-PA/PAI-1 ratio, or more appropriately, their molar ratio, is a useful indicator of fibrinolytic balance. The effects of smoking on t-PA and PAI-1 are controversial, and conflicting results have been reported. More recently, Newby et al have shown that in smokers, SP-stimulated t-PA antigen and activity are decreased with no change in PAI-1 antigen or activity in both the peripheral and coronary circulation. Our in vitro data are consistent with their finding and indicate that SP-stimulated t-PA release was decreased in smokers with no change in basal and stimulated PAI-1 antigen. However, in our in vitro model, basal t-PA production and t-PA/PAI-1 molar ratio were also significantly reduced in smokers. This is inconsistent with the data of Newby et al who found increased basal plasma level of t-PA in smokers. The cause of this discrepancy is not known, but this could be attributable to differences between the models (in vivo versus in vitro).

Yang and Loscalzo, using L-arginine, proposed that NO may be involved in regulation of TF expression in ECs, but they also found that basal levels of NO have little effect on TF induction. In the present in vitro model, there was no correlation between basal NO and TF levels. On the other hand, a relatively weak but significant positive correlation was found between TFPI-1 antigen and NO. Because a significant portion of TFPI-1 resides in the same microdomain (caveolae) as eNOS, cross-talk or an overlapping of the signal transduction pathways of these two molecules is conceivable. Whether this correlation represents such an effect or simply a general manifestation of endothelial dysfunction requires additional investigation.
Study Limitations
First, the finding in this study should be interpreted as only EC-specific responses, because other types of cells can also produce these hemostatic factors under inquiry. Additionally, whether these observed changes in HUVECs extend to arterial ECs requires additional confirmation.

Second, a limitation of sample volume did not allow us to examine the activities of the thrombo-hemostatic parameters. However, because one molecule was the specific counter-regulator of the other and was examined in a static environment, it is likely that the relative concentration of each molecule would reflect the specific thrombo-hemostatic function.

Conclusion
The present study investigated the potential effect of smoking on EC-derived factors that could influence local thrombo-hemostasis. The data suggest that the endothelial contribution to local thrombo-hemostasis is dysfunctional in smokers both in terms of limiting the initiation of thrombus formation (TFPI-1) and also in promoting its effective dissolution (t-PA). Future studies will be needed to confirm whether such alterations of hemostatic molecules exist in vivo and contribute to the increased acute cardiac events in smokers.

References
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