Original article

Effects of chronic cigarette smoking on endothelial function in young men

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Summary The aim of this study was to elucidate endothelial dysfunction due to chronic cigarette smoking in young smokers and to determine practical markers of the functional derangement. The subjects were young, healthy, male non-smokers (\(n=11\)) and smokers (\(n=9\)). Endothelium-dependent and -independent vasodilation was assessed by flow-mediated vasodilation (FMD) and nitroglycerine-induced vasodilation (NID), respectively, and possible markers of endothelial function were measured. FMD in smokers was significantly lower than in control subjects (5.0 ± 2.6% and 9.5 ± 5.2%, \(p<0.05\)). Plasminogen activator inhibitor type 1 (PAI-1) and tissue plasminogen activator levels were significantly (\(p<0.05\)) higher in smokers (6.7 ± 4.5 ng/ml and 4.3 ± 2.0 ng/ml) compared with control subjects (2.9 ± 1.9 ng/ml and 3.0 ± 0.6 ng/ml). Furthermore, PAI-1 levels correlated inversely with FMD (\(r=-0.451, p<0.05\)). No significant differences were observed for NID, or plasma NO\(_2^−\), NO\(_X\), thrombomodulin, von Willebrand factor, and tissue factor pathway inhibitor levels. Chronic cigarette smoking-induced endothelial dysfunction and the PAI-1 level could be a good marker of endothelial dysfunction in young smokers.

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Introduction

The vascular endothelium plays a fundamental role in the regulation of vascular tone, thrombotic balance, and inflammatory responses [1], and its dysfunction is considered to be one of the causes of pathological conditions, such as atherosclerosis, as is shown at an early stage in the development of atherosclerosis [2,3]. Moreover, recent studies demonstrated that the severity of
endothelial dysfunction relates to cardiovascular events [4]. Among the risk factors for atherosclerosis, cigarette smoking is a major factor that also exhibits a close association with coronary artery disease [5]. Indeed, impairment of endothelial function, such as reduced reactive hyperemia and attenuated vasodilatory response to acetylcholine, has been shown by cigarette smoking, hypercholesterolemia, hypertension, and diabetes mellitus [6–11]. Although the precise mechanisms by which smoking could affect endothelial function are not clear, cigarette smoking is known to impair endothelial prostacyclin production, increase monocyte–endothelial cell adhesion and endothelial adhesion molecule expression, and increase endothelial production of angiotensin II [12,13]. The oxidants and free radicals [14–16], as well as nicotine [17] in the smoke, likely contribute to the endothelial dysfunction. Moreover, impaired fibrinolysis in plasma with chronic smoking may affect endothelial function [18]. In principle, changes in intrinsic substances above pathophysiological changes could be detected as indices of endothelial impairment by cigarette smoking. Several studies have identified the specific and measurable markers of endothelial dysfunction, including soluble adhesion molecules, von Willebrand factor (vWF) and thrombomodulin (TM) [19]. However, such clinical and practical markers have not yet been established in smokers. Therefore, the purpose of this study was to examine the effect of cigarette smoking on endothelial function in healthy young adults and search for practical markers for endothelial dysfunction in young smokers.

Methods

Subjects

The subjects of this study were young, healthy, male non-smokers (control subjects: n = 11, mean age 27 ± 3 years) and smokers (n = 9, mean age 30 ± 4 years) (Table 1). Smokers were defined as any who had smoked at least 20 cigarettes per day for the past 5 years. A complete physical examination was performed prior to the study. The participants were free from other risk factors for coronary artery disease and none was treated with any medications during the study. All participants provided informed consent.

Study design

Cigarette smokers refrained from smoking for at least 2 h before arrival in the vascular laboratory. The participants rested on beds in supine position for at least 20 min before the study. Endothelium-dependent vasodilation and endothelium-independent vasodilation were measured according to methods that have been described previously [20,21]. SONOS-2000 (Hewlett Packard, Andover, MA, USA) equipped with a 7.5 MHz ultrasound probe was used to measure the internal diameter and flow velocity of the right brachial artery at a position several centimeters below the elbow, where the artery could easily be detected. Next, a Manchette tourniquet was used to apply 300 mm Hg of pressure for 5 min, and the internal diameter of the brachial artery was measured at the same location 45–60 s after release of the tourniquet in order to compare the rate of dilation of the artery before and after reactive hyperemia (endothelium-dependent vasodilation). Arterial diameter measurements were made at end-diastole (peak of R wave on electrocardiogram) using electronic calipers. Arterial flow velocity was also measured at baseline and during reactive hyperemia using a pulsed Doppler signal at 70° in the center of the artery. Blood flow was calculated based on the flow velocity and the cross-sectional area of the vessel. After a 15-min recovery period, the internal diameter of the brachial artery was measured again at the same location. Next, 0.3 mg of nitroglycerine (NTG) was sprayed into the mouth, and the internal diameter of the brachial artery was measured at the same location 5 min later, to compare the degree of dilation of the artery before and after endothelium-dependent vasodilation.

Before endothelium-dependent vasodilation was measured, blood samples were taken to measure the plasma levels of NOX, NOX (nitrate + nitrite), plasminogen activator inhibitor type 1 (PAI-1), tissue plasminogen activator (t-PA), TM, tissue factor pathway inhibitor (TFPI), and vWF as indices of endothelial function. NOX and NOX concentrations were evaluated by the HPLC-Griess system (ENO-10; EICOM, Kyoto, Japan) as described previously [22,23]. NOX and NOX contamination in the laboratory ware or during measurement procedure was actively excluded [23,24]. PAI-1, t-PA, TM, and TFPI were measured using enzyme-linked immunosorbent assay. vWF was measured by the ristocetin cofactor assay.

Statistical analysis

Values are expressed as the mean ± S.D. Unpaired Students’ t-test was used to compare between the two groups. Linear regression analysis was used to examine the association between change of FMD and each marker of endothelial

<table>
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<th>Table 1 Baseline characteristics of study subjects.</th>
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<tr>
<td>Control (n=11)</td>
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<td>Age (years)</td>
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<td>BP (mm Hg)</td>
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<td>HR (bpm)</td>
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<td>TC (mg/dl)</td>
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<td>TG (mg/dl)</td>
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<td>HDLC (mg/dl)</td>
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<tr>
<td>BP: blood pressure (systolic/diastolic); HR: heart rate; TC: total cholesterol; TG: triglyceride; HDLC: high-density lipoprotein cholesterol.</td>
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Figure 1  Changes in arterial diameter in flow-mediated (FMD) and nitroglycerine (NTG)-induced vasodilation. Changes in brachial arterial diameters (%) as a result of FMD and NTG-induced vasodilation are expressed as the mean ± S.D. Controls, control subjects (n = 11); smokers, subjects who currently smoke (n = 9). *Significantly (p < 0.05) different from the control group.

function. p-Values < 0.05 were considered to indicate statistical significance.

Results

There were no differences in age, systolic and diastolic blood pressure, total cholesterol, triglyceride, or high-density lipoprotein cholesterol between smokers and control subjects (Table 1). Heart rate (HR) was significantly higher in smokers compared with control subjects. The baseline diameter of the brachial artery was not different between smokers and control subjects (4.11 ± 0.31 mm and 3.92 ± 0.24 mm, respectively). FMD in the smokers was significantly smaller than those in the control subjects (5.0 ± 2.6% and 9.5 ± 5.2%, respectively, p < 0.05) (Fig. 1). Baseline blood flow velocity and the increase in the blood flow during reactive hyperemia were not different between the two groups (smokers: 181 ± 87 ml/min, 639 ± 245% and control subjects: 171 ± 97 ml/min, 718 ± 363%, respectively). Prior to NTG administration, the brachial artery diameter of the two groups was not different (4.10 ± 0.31 mm and 3.94 ± 0.20 mm, respectively). NTG-induced vasodilation (NID) was not significantly different between the two groups (smokers: 15.5 ± 4.4% and control subjects: 14.8 ± 3.8%, respectively) (Fig. 1). Significantly higher levels (p < 0.05) of plasma PAI-1 and t-PA were observed in the smokers (6.7 ± 4.5 ng/ml and 4.3 ± 2.0 ng/ml, respectively) compared with the control subjects (2.9 ± 1.9 ng/ml and 3.0 ± 0.6 ng/ml, respectively) (Fig. 2). Plasma NO$_2^-$ (0.169 ± 0.047 μmol/l and 0.152 ± 0.095 μmol/l, respectively) and NO$_X$ (26.1 ± 8.6 μmol/l and 32.3 ± 9.6 μmol/l, respectively) levels were not different between smokers and control subjects (Fig. 3). TM (smokers: 1.9 ± 0.3 FU/ml and control: 2.1 ± 0.3 FU/ml, respectively), TFPI (smokers: 15.8 ± 4.4 ng/ml and control: 18.4 ± 5.2 ng/ml, respectively), and vWF (smokers: 62.8 ± 21.9% and control:

Figure 2  Plasma levels of plasminogen activator inhibitor type 1 (PAI-1) and tissue plasminogen activator (t-PA) in smokers and control subjects. Plasma levels of PAI-1 and t-PA in the control state are expressed as the mean ± S.D. Controls, control subjects (n = 11); smokers, subjects who currently smoke (n = 9). *Significantly (p < 0.05) different from the control group.
Figure 3  Plasma $\text{NO}_2^-$ and $\text{NO}_X$ levels in smokers and control subjects. Plasma $\text{NO}_2^-$ and $\text{NO}_X$ levels in the control state are expressed as the mean ± S.D. Controls, control subjects ($n=11$); smokers, subjects who currently smoke ($n=9$).

Figure 4  Relationship between plasma levels of plasminogen activator inhibitor type 1 (PAI-1) and flow-mediated vasodilation (FMD). FMD in smokers ($n=11$) and non-smokers ($n=9$) were plotted against the PAI-1 levels. There was a significant inverse correlation between these two parameters. Open circle, control subjects (non-smokers); closed circle, smokers.

Figure 5  Relationship between plasma thrombomodulin (TM) levels and flow-mediated vasodilation (FMD). FMD in smokers ($n=11$) and non-smokers ($n=9$) were plotted against TM levels. There was a significant correlation between these two parameters. Open circle, control subjects (non-smokers); closed circle, smokers.

66.3 ± 36.6%, respectively) levels in plasma were not significantly different between the two groups. PAI-1 levels correlated inversely with FMD ($r=−0.451$, $p<0.05$) (Fig. 4) and TM levels correlated directly with FMD ($r=0.468$, $p<0.05$) (Fig. 5), respectively. Meanwhile, $\text{NO}_2^-$ ($r=−0.228$), $\text{NO}_X$ ($r=−0.213$), t-PA ($r=−0.153$), TFPI ($r=0.265$), and vWF ($r=0.170$) did not correlate with the FMD. The amount and duration of smoking did not correlate significantly with either the FMD or the various biomarkers. We did not find any significant correlation among the biomarkers and lipid profiles in this study.

Discussion

In this study, endothelium-dependent (flow-mediated) dilation was impaired significantly in the smokers compared with the control subjects, which was consistent with an early report suggesting an association between smoking and endothelial dysfunction [20,25]. The mechanism of smoking-induced endothelial dysfunction could be complex and remains unclear. A large number of oxidants and oxygen-derived free radicals in the smoke likely contribute towards endothelial dysfunction [12—16]. Moreover, smoke of cigarette is a source of nicotine and chronic exposure results in nicotine-induced impairment of endothelium-dependent arteriolar dilation [17]. From these points of view, it would be reasonable to postulate that smoking-induced endothelial dysfunction most likely depends on increased oxidative stress and its toxic products.

$\text{NO}_X$ was measured as a possible marker of endothelial function in all subjects, because plasma $\text{NO}_X$ levels in smokers have been shown previously to be lower compared with non-smokers [26]. A tendency towards lower $\text{NO}_X$ lev-
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els in smokers compared with non-smokers was found in our study. However, this difference was not statistically significant. Moreover, NOx levels did not correlate with FMD. Thus, it would not be appropriate to use NOx levels to indicate endothelial function, because NOx concentrations in plasma are known to be influenced by various factors, including food and drink [27] and because a recent study showed that steady state NOx concentrations in whole blood and plasma preferentially imply NOx elimination rather than NOx formation [28]. Seventy to ninety percent of plasma NOx−, but not NO3−, has been reported to originate from endothelial nitric oxide synthase activity [29] and is now considered to reflect nitric oxide synthase activity, based on several investigations [30—32]. Therefore, we evaluated this substance separately. NOx− levels were not different between the two groups and NOx− levels did not correlate with FMD. NOx levels were about 100-fold of NOx− levels in this study. Thus, more strict conditions would be necessary to evaluate plasma NOx− levels in future.

Imbalances in the fibrinolytic system may cause microthrombi that will cause occlusion of the microcirculation, and higher PAI-1 levels were observed in smokers vs. healthy subjects [18] and in patients with angina pectoris [33], which is consistent with our findings. This higher level of PAI-1 would be partly explained by the pharmacological effect of nicotine to increase PAI-1 mRNA expression and protein production in endothelial cells [34]. Moreover, oxidative stress in endothelial cells has been reported to cause significant increases in PAI-1 mRNA expression and protein production [35]. Although we did not investigate the mechanism of the increased PAI-1 in young smokers any further, possible sources to increase PAI-1 would be soluble substances in the serum, as Barua et al. have reported that endothelial cells treated with serum isolated from cigarette smokers exhibited enhanced PAI-1 production compared with non-smokers [36]. Interestingly, recent studies have revealed inhibitory effects of H2O2 and nicotine on the expression and secretion of adiponectin in 3T3-L1 adipocytes [37] and impaired endothelium-dependent vasorelaxation in adiponectin-knockout mice [38]. In smokers, we observed higher levels of both PAI-1 and t-PA. Increased plasma t-PA levels have been reported in smokers with coronary risk factors [39], which would appear to be somewhat contradictory. However, in the present study, the increase in t-PA was about 30% of the value in non-smokers, while the increase in PAI-1 was about 130% (Fig. 2). In other words, the degree of the increase in PAI-1 largely exceeded that of t-PA and the imbalance could shift blood away from fibrinolysis. Extracellular generation of oxygen radicals by smoking may be responsible for the increase in t-PA release and in t-PA mRNA synthesis [40]. However, the higher level of plasma t-PA does not necessarily imply a higher output (release) of t-PA from endothelial cells. Indeed, such patients exhibit impaired release of active t-PA after stimulation and an inverse correlation exists between basal t-PA concentration and active t-PA release after endothelial stimulation by substance P [39]. Therefore, the reduction in fibrinolytic capacity appears to reflect both impairment of acute t-PA release and elevation of plasma PAI-1 concentrations. The subsequent depletion of endothelial cell t-PA stores and concurrent increases in PAI-1 would impair the capacity of the vasculature to lyse intraluminal thrombi, leading to future coronary events [41,42]. Further evaluation of any correlation between functional changes (FMD) and its biochemical markers revealed that plasma PAI-1 levels correlated significantly with the endothelial function (FMD) among all subjects (smokers and non-smokers). Few prior studies have related plasma PAI-1 levels to measures of vascular function and an inverse correlation between plasma PAI-1 and FMD has been observed [43]. Therefore, increases in PAI-1 would reflect the smoking-induced endothelial dysfunction in the young male adults. Barua et al. demonstrated that the basal t-PA/PAI-1 ratio was significantly reduced in smokers [36]. We assessed the t-PA/PAI-1 ratio between smokers and control subjects. T-PA/PAI-1 tended to be higher in control subjects; however, the relationship was not statistically significant.

Although TM levels in plasma were not significantly different between smokers and non-smokers, TM levels correlated with FMD among all subjects (smokers and non-smokers) in this study. As correlations between TM and several biochemical markers of endothelial dysfunction have been indicated [44], TM, as well as its oxidative modification, would be regarded as useful biomarkers for cigarette smoking [45].

There are several limitations to this study. First, the sample size was relatively small. Therefore, the data would not bear thorough analysis, i.e., stepwise multivariate analysis. Second, we did not measure any markers of oxidative stress. Third, we did not measure C-reactive protein (CRP), as a correlation between FMD in subcutaneous resistance arteries and CRP in male patients with coronary heart disease has been reported previously [46]. Fourth, a possible contribution of higher HR on FMD in smokers remains unknown, as some studies indicated an interaction [47,48].

In conclusion, we demonstrated that FMD was attenuated and plasma levels of PAI-1 and t-PA were elevated in smokers. These results suggest that chronic cigarette smoking could induce endothelial dysfunction. Thus, the PAI-1 level could be a good marker of endothelial dysfunction in these young smokers.

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