Title Page

Title:

VISUALLY EVOKED CEREBRAL VASOMOTOR RESPONSE IN SMOKING AND NON-SMOKING YOUNG ADULTS, INVESTIGATED BY FUNCTIONAL TRANSCRANIAL DOPPLER

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ABSTRACT

Smoking has been known to cause endothelial dysfunction and is an important risk factor for ischemic stroke. In our study we investigated whether chronic cigarette smoking affects the cerebral blood flow velocity response to a physiological, visual stimulus. By using a visual cortex stimulation paradigm, the flow velocity response in the posterior cerebral arteries (PCA) was measured bilaterally, in 32 young healthy adults (16 smokers, 16 non-smokers). The stimulation protocol consisted of 10 cycles with a resting phase of 20 seconds and a stimulating phase of 40 seconds for each cycle. Besides functional transcranial Doppler (TCD), laboratory tests and measurement of intima-media-thickness (IMT) were also performed. Repeated measure ANOVA was used to detect differences in visually evoked relative flow velocity time courses between smokers and non-smokers. Repeated measure ANOVA revealed marked difference in the peak systolic flow velocity time courses between smokers and non-smokers ($p<0.001$). Maximum percent change of visually evoked flow velocity after visual stimulation was 19±4% and 30±3% in smokers and non-smokers, respectively ($p<0.0001$). IMT values did not indicate atherosclerosis in young smokers. Infectious disease and hyperlipidaemia were also ruled out by measurement of sensitive C-reactive protein and serum lipids. This is the first functional TCD study demonstrating impaired visually evoked flow velocity response caused by chronic cigarette smoking in otherwise healthy, young subjects. The impaired cerebral vasodilatory mechanism together with atherosclerosis may influence stroke occurrence and outcome in chronic smokers.

INTRODUCTION

Cigarette smoking remains highly prevalent in most parts of the world, and passive exposure to environmental tobacco smoke is common and often unavoidable. Smoking has been known to be a major risk factor for coronary heart disease, stroke, and peripheral vascular disease. The cardiovascular risks increase with the amount of cigarettes and the duration of smoking (Burns, 2003).

The mechanisms by which cigarette smoking contributes to damage of the vascular system are complex and multifactorial. Smoking may damage the endothelium, leading to endothelial dysfunction, increased platelet function, inflammatory processes, hypercoagulability, and eventually atherosclerosis (Vapaatalo & Mervaala, 2001). Cigarette smoking causes an increase in vascular superoxide production which results in decreased nitric oxide (NO) bioactivity (Raij, DeMaster & Jaimes, 2001). Both aqueous extracts of tobacco and cigarette smoke contain highly reactive glycation products, the so called glycotoxins, that can rapidly induce advanced-glycation-end-products formation in proteins, and cause DNA mutations within hours (Cerami et al., 1997). Glycotoxins were shown to cross-link connective tissue collagen, inhibit cell-derived nitric oxide activity, and modify ApoB, thereby preventing the normal uptake of LDL-cholesterol by tissue LDL receptors (Kohn, Cerami & Monnier, 1984; Bucala, Tracey & Cerami, 1991; Bucala et al., 1995). Tobacco smokers show signs of activated platelet function and increased sensitivity to vasoconstriction (Lassila et al., 1988). Smoking is also a major risk factor for periodontitis and chronic bronchitis. Bacterial endotoxins that stimulate the release of pro-inflammatory cytokines can have systemic effects and may lead to cardiovascular diseases such as atherosclerosis (Greenwell & Bissada, 2002).

Smoking is believed to cause not only structural alteration of the vessels but deterioration of the vasomotor response to different stimuli as well. Basal cerebral blood flow

(CBF) in chronic smokers (Kubota et al., 1983; Rogers et al., 1983; Rogers, Meyer, Judd & Mortel, 1985; Yamashita, Kobayashi & Yamaguchi, 2000), as well as acute effect of cigarette smoking on CBF (Wennmalm, 1982; Boyajian & Otis, 2000; Terborg, Bramer, Weiller & Rother, 2002) were examined in humans. However, only few studies have investigated the chronic effects of cigarette smoking on cerebral vasomotor reactivity and reported contradictory results (Rogers, Meyer, Shaw, Mortel & Thornby, 1984; Silvestrini, Troisi, Matteis, Cupini & Bernardi, 1996; Terborg et al., 2002). Whether smokers have impaired cerebral vasodilatory mechanism is an important question since it might contribute to increased stroke risk and worse outcome in cerebral ischemia. Therefore, our aim was to test whether chronic cigarette smoking affects the cerebral blood flow (CBF) response to a visual stimulus in healthy, young subjects. To our best knowledge, physiological stimulus evoked flow changes have never been tested in human smokers.

METHODS

Subjects

Thirty-two young healthy adults (16 smokers, 16 non-smokers; 8 males and females in both groups) between 18 and 38 years of age were included in the study. The study was approved by the local ethics committee, and each volunteer gave written, informed consent. Cerebrovascular risk factors, history of migraine, coronary and peripheral artery diseases were screened and patients with vascular risk factors other than smoking were excluded. The included subjects did not take any medicine regularly. Persons were considered as smokers, if they smoked at least 15 cigarettes a day for at least 3 years. Non-smokers did not smoke any cigarette. The study protocol included routine clinical laboratory tests, haemostasis screening test, serum lipids, and inflammatory markers. Blood was drawn after an overnight fast between 8 and 9 a.m., after the TCD examination. All volunteers underwent a complete neurological examination, and extra- and transcranial duplex scans were also performed to

exclude neurological and vascular abnormalities. Intima-media thickness (IMT) was measured in both common carotid arteries. These ultrasound examinations were performed using SONOS 4500 (Agilent Technologies) equipment with a 7.5 MHz linear transducer. On-line measurements of IMT were performed in the far artery wall of the common carotid arteries, 10 mm proximal to the carotid bulb, with the transducer in the mediolateral direction. All measurements were performed on frozen, enlarged images at end-diastole. IMT was measured on a 1-cm segment. In each of these 1-cm segments, 11 measurements of IMT were performed at 1-mm increments on both sides. The mean IMT of the 22 values in each subject was calculated.

The functional TCD tests were performed between 7 and 8 a.m. in a quiet room at about 25°C while the subjects were sitting comfortably. All volunteers had abstained from caffeine overnight before the study. Volunteers were asked not to smoke from 10 p.m. before the day of the TCD test till the end of the the examination. Blood pressure was measured noninvasively before the TCD examination and at the end of the experiment. Ultrasound examinations were always performed by the same examiner (L.O.) who was unaware of the volunteers' smoking habits.

Functional TCD Study

Two 2-MHz probes were mounted by an individually fitted headband. In all cases, the P2 segment of the PCA was insonated on both sides at a depth of 58 mm. Peak systolic and end-diastolic blood flow velocities were recorded with a Multidop T2 Doppler device (DWL). The reason for the separated evaluation of systolic and end-diastolic blood flow velocities was that the indices show different time courses in dynamic blood flow regulation. Being less influenced by Doppler artefacts, the peak systolic velocity index was used (Rosengarten, Aldinger, Kaufmann & Kaps, 2001).

As a stimulation paradigm, we used a news magazine with emotionally neutral text that the volunteers could read freely. This "reading" test has been previously validated against a checkerboard stimulation paradigm (Rosengarten, Aldinger, Spiller & Kaps, 2003). The stimulation protocol consisted of 10 cycles with a resting phase of 20 seconds and a stimulation phase of 40 seconds for each cycle. During the resting periods, volunteers were instructed to close their eyes; during the stimulation phases, they read silently. Changes between phases were signaled acoustically with a tone.

Beat-to-beat intervals of cerebral blood flow velocity data were interpolated linearly with a "virtual" time resolution of 50 ms for averaging procedures. Within one person, flow velocity data of ten cycles and of the right and left sides were averaged. To ensure independence from the insonation angle and to allow comparisons between volunteers, absolute data were transformed into relative changes of cerebral blood flow velocity in relation to baseline. Baseline was calculated from the blood flow velocity averaged for a time span /-5/ to 0 seconds before the beginning of the stimulation phase. Four main phases could be differentiated during the cycles (Figure 1).

<Insert Figure 1 about here>

After closure of the eyes, the flow velocity decreases (phase1: /-20s/–/-5s/ period), and becomes stable in some seconds during the resting period (phase2: /-5s/–/0s/ period, baseline). After opening the eyes, the velocity increases and reaches its maximum (phase3: overshooting). Later, the velocity slightly decreases and becomes stable in the second half of the stimulation phase (phase4: /+30s/–/+40s/ period, plateau).

Maximum relative change of visually evoked flow velocity was determined during the visual stimulation in the overshooting phase (v_{max}). Relative change of visually evoked flow velocity was also calculated after stabilization of the velocity during the stimulation phase

(plateau phase) by averaging the relative flow velocities between /+30s/–/+40s/ ($v_{plateau}$). Relative velocities were expressed in % of baseline (Figure 1).

Statistical Analysis

Data were expressed as means±standard deviation (SD). Results of bilateral measurements were averaged within one patient. Normality of continuous variables (blood pressure, age, maximum increase of relative changes of flow velocity /v_{max}/, increase in relative flow velocity in the plateau phase /$v_{plateau}$/, IMT, and laboratory values) was checked by the *Saphiro-Wilk test*. In case of normal distribution, *analysis of variance (ANOVA)*, in case of non-normal distribution, the *Mann-Whitney U test* was used to detect differences between smokers and non-smokers.

Repeated measure ANOVA was used to detect differences in visually evoked relative flow velocity time courses between the smokers and non-smokers, and males and females. Statistical significance was assumed at $p<0.05$.

RESULTS

Data from all volunteers were used for evaluation. The mean age was 31±5 years in the smokers and 31±6 years in the non-smokers (Table 1). Comparison of blood pressure and laboratory values (including erythrocyte sedimentation rate and C-reactive protein) revealed no significant differences between the two groups (Table 1).

<Insert Table 1 about here>

The C-reactive protein levels were 1.47±1.25 and 1.51±2.20 mg/L in smokers and non-smokers, respectively (*p=0.957*). No significant difference in IMT values (*p=0.1794*) was found between smokers (0.420±0.045 mm) and non-smokers (0.392±0.049 mm).

The relative flow velocity time courses in smokers and non-smokers for visually evoked functional TCD test are shown in Figure 2 for the peak systolic data.

<Insert Figure 2 about here>

Repeated measure ANOVA revealed marked difference in the flow velocity time courses between smokers and non-smokers (*p value for the interaction<0.001*). Both the maximum reactivity during the overshooting phase (v_{max}) and the reactivity during the plateau phase ($v_{plateau}$) were significantly higher in non-smokers than in smokers (Table 1). No significant difference was found between males and females neither in smokers nor in non-smokers.

DISCUSSION

Our results proved that smoking, even hours after smoking the last cigarette, inhibits the visually evoked cerebral vasomotor reactivity. To our best knowledge this is the first functional TCD study demonstrating impaired visually evoked flow velocity response in young smokers without any obvious underlying cause except for chronic cigarette smoking. The acute effects of smoking can be excluded, in as much as the volunteers were asked not to smoke for at least 9 hours before the examination. In order to minimise the effect of overnight abstinence and nicotine withdrawal, the experiments were performed early in the morning. Although chronic smoking has an important role in development of atherosclerosis, the IMT in the young subjects enrolled in our study did not differ between smokers and non-smokers. It indicates that atherosclerosis in our study population probably did not play a significant role in the worse reactivity. Infectious disease and hyperlipidaemia were also ruled out by measurement of sensitive C-reactive protein and serum lipids. In order to avoid administration of external agents (CO_2 or acetazolamide) and alteration of the physiological parameters (pCO_2, pO_2,), we applied visual stimulus in our study to induce vasodilation and to evoke flow velocity changes.

Effect of cigarette smoking on blood flow in previous studies

In humans, resting CBF in chronic smokers as well as CBF changes induced by acute cigarette smoking were examined. Acute inhalation of cigarette smoke has been reported to increase CBF levels in most studies (Wennmalm, 1982; Boyajian & Otis, 2000; Terborg et al., 2002). In contrast, chronic exposure to tobacco smoke decreases resting CBF, which has been shown to be reversible several years after cessation of smoking (Kubota et al., 1983; Rogers et al., 1983; Rogers et al., 1985; Yamashita et al., 2000). Contrary to these reports, only few studies investigated the chronic effect of cigarette smoking on cerebrovascular reactivity. Using a Xe inhalation method, Rogers et al. (1984) reported worse vasodilator and vasoconstrictor reactivity in chronic smokers to 5% CO_2 and 100% O_2, respectively. Quite to the contrary, others did not find significant difference in the vasomotor reactivity, when the chronic effect of cigarette smoking was assessed by breath holding test, between healthy smokers and age-matched controls (Silvestrini et al., 1996; Terborg et al., 2002).

Outside the central nervous system, the results are more consistent: impaired flow response to different vasodilatory stimuli was described in a number of arteries as a long term effect of cigarette smoking, including impaired microcirculatory regulation in the skin (Dalla Vecchia et al., 2004), worse flow-mediated vasodilation of the brachial artery (Celermajer et al., 1993), and impaired endothelium-dependent coronary vasoreactivity (Zeiher, Schachinger & Minners, 1995). Our results, based on visually evoked flow velocity response, support the theory that chronic smoking deteriorates the vasodilatory mechanism also in the cerebral vessels.

Possible causes of impaired vasodilation in chronic smokers

Smoking was shown to increase the number of damaged endothelial cells in circulation and causes morphological changes within the endothelium (Vapaatalo & Mervaala, 2001). Besides morphological changes, functional alterations of the endothelium are also induced by cigarette smoke (Celermajer et al., 1993). Oxidants from cigarette smoke

inactivate NO and promote the oxidation of LDL-cholesterol (Bucala et al., 1991; Bucala et al., 1995; Raij et al., 2001). Smoking was reported to inhibit endothelial and neuronal NO synthases, impair NO signaling due to increased release of superoxide anion and subsequent inactivation of nitric oxide, and reduce exhaled NO in man, which is an indicator of decreased endogenous NO production (Raij et al., 2001; Demady et al., 2003; Landmesser, Harrison & Drexler, 2005). Inhibition of neuronal or endothelial nitric oxide synthase was shown to attenuate the stimulus evoked flow response in different animal studies, indicating the substantial role of NO in the coupling of neuronal activation to regional cerebral blood flow (Kharitonov, Robbins, Yates, Keatings & Barnes, 1995; Cholet, Seylaz, Lacombe & Bonvento, 1997; Yang & Iadecola, 1998; Demady et al., 2003). Dysregulation of Ca^{2+}-channels was also demonstrated in nicotine treated animals, mimicking the effect of an apparent decrease in bioavailability of endogenous NO (Gerzanich, Zhang, West & Simard, 2001).

The decreased NO release in smokers inversely parallels the increased production of a vasoconstrictive/proliferative peptide, endothelin (Haak, Jungmann, Raab & Usadel, 1994). Platelet activation, increased thromboxane levels have also been shown as an effect of long term cigarette smoking (Lassila et al., 1988; Patel & Kent, 1988). The imbalance between these antagonistic factors (NO versus endothelin and thromboxane), as well as the increased sympathetic tone in smokers may contribute to the impaired vasodilation and enhanced vasoconstriction in smokers.

Possible consequences of impaired vasodilatory mechanism in smokers

Our study indicates that smoking might contribute to ischemic stroke not only by atherothrombosis but also through an impaired cerebral vasodilatory mechanism. Impaired vasodilation may lead to compromised cerebral hemodynamics even at an earlier stage of carotid stenosis or at lower cerebral perfusion pressures, causing cerebral ischemia (Vernieri,

Pasqualetti, Passarelli, Rossini & Silvestrini, 1999). Moreover, the decreased vasodilatory capacity may result in progression of ischemic damage in the penumbra, leading to worse outcomes. Thus, impairment of cerebral vasomotor reactivity, demonstrated by this and previous studies (Rogers et al., 1984), could act together with atherosclerosis and may influence stroke occurrence and outcome in chronic smokers.

Further studies are needed to investigate whether impaired cerebral vasodilation is reversible after cessation of smoking. Since smoking habits may influence cerebral flow response to a vasodilatory physiological stimulus, this should be taken into consideration when a human study is planned.

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Table 1. Examined parameters of smokers and non-smokers (*:p<0.001; ANOVA*)

Examined parameters	Smokers (n=16)	Non-smokers (n=16)
Age (years)	31.1±4.7	30.6±5.6
Systolic blood pressure before the experiment (mmHg)	122±11	121±12
Diastolic blood pressure before the experiment (mmHg)	77±10	76±10
Systolic blood pressure after the experiment (mmHg)	123±10	122±11
Diastolic blood pressure after the experiment (mmHg)	78±10	76±9
Erythrocyte sedimentation rate (mm/h)	9±6	7±5
Hematocrit	0.435±0.039	0.428±0.042
Hemoglobin (g/L)	143.4±13.1	141.2±15.2
White blood cell (G/L)	6.6±1.5	6.8±2.2
Thrombocyte (G/L)	243±50	261±53
Prothrombin time (s)	9.9±0.8	9.8±0.4
APTT (s)	36.4±4.0	36.2±2.9
Thrombin time (s)	16.5±1.8	16.5±1.2
Fibrinogen (g/L)	3.1±0.6	2.9±0.9
Sodium (mmol/L)	142.3±2.8	142.8±3.6
Potassium (mmol/L)	4.3±0.2	4.2±0.4
Glucose (mmol/L)	4.6±0.8	4.5±0.3
Blood urea nitrogen (mmol/L)	4.9±1.1	4.8±1.2
Creatinine (μmol/L)	70.9±16.3	67.4±16.2
Triglyceride (mmol/L)	1.19±0.73	0.90±0.53
Cholesterol (mmol/L)	4.43±0.94	4.26±0.80
LDL-cholesterol (mmol/L)	2.49±0.91	2.36±0.75
HDL-cholesterol (mmol/L)	1.51±0.52	1.55±0.33
v_{max} (%)	**19±4**	**30±3 ***
$v_{plateau}$ (%)	**14±5**	**21±3 ***

FIGURE LEGENDS:

Figure 1. Schematic picture of the visually evoked relative flow velocity time course in relation to baseline.

Figure 2. Visually evoked relative flow velocity time courses in smokers and non-smokers for the peak systolic data. Error bars indicate standard deviation. (*Repeated mesare of ANOVA*)

Figure 1.

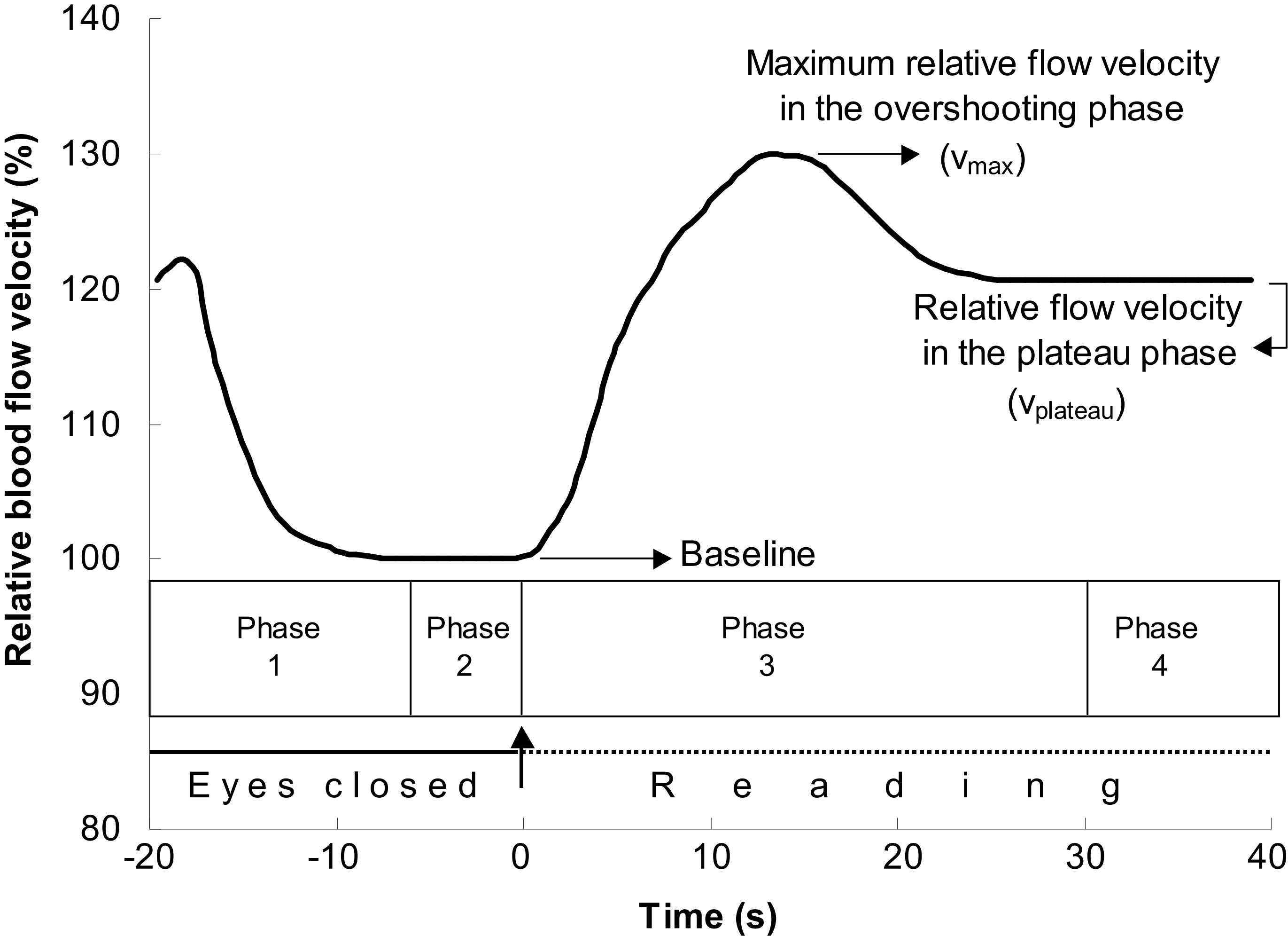

Figure 2.