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Effects of nicotine, its metabolites and tobacco extracts on human platelet function *in vitro*

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ABSTRACT

Cigarette smoking is a leading cause of cardiovascular disease. The cardiovascular effects of smoking are probably multifactorial, including effects on platelets. Previous reports investigating the effects of nicotine and tobacco on platelet function are inconsistent.

The present study investigated *in vitro* effects of nicotine, its major metabolites, tobacco extracts and extract of tobacco-free snuff on human platelets.

None of the metabolites cotinine, cotinine-N-oxide, nicotine-1′-N-oxide or trans-3′-hydroxycotinine (0.1–10 μM) affected platelet aggregation or P-selectin expression. Nicotine (10 μM) weakly increased platelet aggregation, whereas trans-3′-hydroxycotinine (0.1 μM) and nicotine-1′-N-oxide (1–10 μM) weakly inhibited adhesion to fibrinogen. To elucidate the influence of other tobacco compounds, we investigated the impact of moist tobacco and smoke extracts on platelet function. Filtered extracts of oral snuff, cigarette smoke and tobacco free snuff inhibited platelet adhesion concentration-dependently. The inhibitory effects of tobacco extracts on platelet adhesion were independent of nicotine content and the nitric-oxide-pathway and not mediated through a platelet-nicotine-receptor.

Taken together, tobacco extracts inhibit platelet activation during short-term *in vitro* challenge. As only limited effects of nicotine and nicotine metabolites were seen, the tobacco-induced platelet inhibition are likely induced by other compounds present in tobacco and tobacco free snuff.

1. Introduction

There is no doubt about the harmful effects of smoking on the cardiovascular system. The exact mechanisms by which smoking induces cardiovascular disease are not entirely known, but are most likely multifactorial. The effects of cigarette smoke on platelets have been investigated in numerous studies, but the results are somewhat contradictory. Cigarette smoke has been shown to activate platelets, which results in increased clot strength *ex vivo* (Barua et al., 2010) and increased P-selectin expression (Lupia et al., 2010). In addition, smokers show increased spontaneous aggregation *ex vivo* (Fusegawa et al., 1999) and smoking has been found to increase agonist induced platelet aggregation *in vitro* (Hung et al., 1995). However, there are also studies showing that platelet aggregation after *in vitro* activation, as well as *in vitro* bleeding time is either unchanged or decreased in smokers compared to non-smokers (Brockmann et al., 2001; Nair et al., 2001). Smokeless oral tobacco is probably less harmful than cigarettes, although long-term use of oral snuff, has been shown to increase the risk of fatal myocardial infarction (Hergens et al., 2007). Studies on the influence of oral snuff on platelet function are scarce, but one study showed unchanged urinary levels of a metabolite of the platelet activation marker thromboxane A_2, after use of oral snuff (Wennmalm et al., 1991).

Tobacco products are complex mixtures of compounds, containing not only nicotine, but also numerous other pharmacologically active substances (Benowitz and Gourlay, 1997). Nicotine affects the cardiovascular system in many ways, some mechanisms being well characterized. By activating the sympathetic nervous system, nicotine induces increased heart rate and myocardial contraction, vasoconstriction in the skin and adrenal and release of catecholamines (Benowitz, 1996). Nicotine can also affect lipid metabolism (Cluette-Brown et al., 1986), accelerate the development of atherosclerosis (Strohschneider et al., 1994) and induce endothelial

Abbreviations: ADP, adenosine 5-diphosphate; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; ODQ, 1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline.

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dysfunction (Chalon et al., 2000). After entering the circulation, nicotine is subjected to extensive metabolism, resulting in a number of major and minor metabolites. On average, 70–80% of the nicotine is metabolized to cotinine, about 4% is converted to nicotine-1′-N-oxide and 0.4% to nornicotine (Benowitz and Jacob, 1994). Cotinine is further metabolized to cotinine-N-oxide and trans-3′-hydroxycotinine, among others (Benowitz et al., 1994). Trans-3′-hydroxycotinine is the most abundant metabolite in urine, accounting for on average 38% of the metabolites (Benowitz et al., 1994). As most of the metabolites have a considerably longer physiological half-life compared to nicotine, plasma concentrations of nicotine metabolites in tobacco users tend to accumulate throughout the day (Benowitz and Jacob, 1994, 2001). Although the concentration of some of the nicotine metabolites in the blood is far higher than nicotine in tobacco users, few earlier studies have examined their effect on the cardiovascular system. Also, the direct effects of tobacco on platelets are relatively unknown. This study aimed to investigate the effects of nicotine, nicotine metabolites, tobacco extracts and tobacco free snuff on platelet function *in vitro*.

2. Methods

2.1. Experimental design

This study investigates the effect of tobacco on platelet function *in vitro*. Platelet adhesion, aggregation and P-selectin expression were evaluated after exposure to nicotine and its four most abundant metabolites in plasma. As tobacco products contains not only nicotine, but also a huge number of other substances, the effect of tobacco extracts (cigarette smoke and moist tobacco) on platelet adhesion were investigated. Moreover, a tobacco free substitute (Choice apple) was also studied to investigate possible platelet effects of a plant extract that is used as an aid to stop smoking or using snuff. The impact of tobacco extracts on platelets was evaluated alone or in combination with the known platelet activators adenosine 5-diphosphate (ADP) or adrenaline (Sigma–Aldrich, St. Louis, MO, USA). In addition, in order to study if the effect of the tobacco extracts were mediated via the nitric oxide (NO) system, which is known to inhibit platelets *in vitro* and *in vivo*, platelets were pre-treated with (1) the phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine, (IBMX; Sigma–Aldrich) which inhibits degradation of cGMP resulting in augmented effect of NO, or (2) the guanylyl cyclase inhibitor 1,2,4-Oxadiazolo[4,3-a]quinoxalin-1-one, (ODQ; Sigma–Aldrich) which inhibits the synthesis of cGMP and thus inhibits the effect of NO. Finally, platelets were pre-treated with the nicotine-receptor antagonist mecamylamine, to study if the effect of the extracts were mediated through the platelet nicotine receptor.

2.2. Nicotine and nicotine metabolites

Nicotine and four of its most abundant liver metabolites, cotinine, nicotine-1′-N-oxide, cotinine-N-oxide and trans-3′-hydroxycotinine, found in plasma were studied. Nicotine and cotinine were bought from Sigma–Aldrich, while nicotine-1′-N-oxide, cotinine-N-oxide and trans-3′-hydroxycotinine were kind gifts from Dr. Georg B. Neurath (Hamburg, Germany). All drugs were dissolved in distilled water, except for cotinine which was dissolved in ethanol and dilutions were made in 0.9% NaCl. Nicotine and nicotine metabolites were used in concentrations similar to those found in plasma from tobacco users (Benowitz et al., 1994).

2.3. Preparation of tobacco extract

Extract of oral snuff and tobacco free snuff was prepared using a protocol previously described (Petro et al., 2002), with a few modifications. Ten grams of oral tobacco (Ettan moist, Swedish Match, Stockholm, Sweden, and Copenhagen snuff fine cut, US Smokeless Tobacco Company, Richmond, VA, USA) or tobacco free snuff (Choice apple, Nicofree, Trångsviken, Sweden) were mixed with 100 ml phosphate-buffered saline (PBS) and incubated for 2 h at 37 °C. The mixture was centrifuged for 10 min at 450*g* followed by collection of the supernatant and re-centrifugation for 1 h at 13,000*g*. The suspension was filtered through a 0.2-μm filter and pH was adjusted to 7.4 before being aliquoted and stored at −70 °C. The concentration of the filtered solution was considered as 100%.

Cigarette smoke extract was prepared as described (Su et al., 1998), with few modifications. Smoke from two Camel filter cigarettes (R.J. Reynolds Tobacco Company, Winston-Salem, NC, USA) was drawn through 10 ml PBS, pre-warmed to 37 °C, using water suction at a constant flow. All cigarettes were smoked to the same level (approximately 80% of the cigarette) and each cigarette was smoked for 5 min ± 30 s. The solution was sterilized using a 0.2 μm filter and the obtained solution was considered as 100%. Cigarette smoke extract was prepared 30 min prior to use. A broad concentration range was used for the tobacco extracts (0.001–10%) to cover the plasma concentrations of the different constituents in tobacco.

2.4. Analysis of nicotine content in tobacco extract

High-performance liquid chromatography (HPLC) was used to separate nicotine from other constituents and nicotine content was quantified using a UV detector. The system consisted of a P680 HPLC pump from Dionex (Sunnyvale, CA, USA), a Gina 50 autosampler and a photo-diode array UV-detector UVD340U from Gynkotek (Germinger, Germany). The column was an X-bridge C18 3 μm, 3 × 100 mm from Waters (Milford, MA, USA). Samples were separated using a mobile phase consisting of 5:95 (v/v) acetonitrile:ammonium formiate 10 mM, pH 4.2, at a flow rate of 500 μl/min. Each sample was injected into the HPLC system in a volume of 20 μl and nicotine was detected at a wavelength of 260 nm. The run time for each sample was 4 min and the retention time was 2.3 min. A standard curve was constructed using 25, 50, 100 and 250 μM of nicotine. Cigarette smoke extract was diluted 1:1–1:5 in mobile phase, while oral snuff extract was diluted 1:25.

2.5. Subjects

The study conforms with the principles outlined in the Declaration of Helsinki, Finland 1964 and later revisions, and was approved by the Regional Ethical Review board in Linköping, Sweden. Blood was consecutively collected from healthy blood donors at the Blood Transfusion Centre, University Hospital, Linköping, Sweden. Blood donors were included only if they declared that they were non-tobacco users; had not used any antiplatelet drug such as aspirin for 2 weeks prior to the study; during the 3 previous months not suffered fever after visiting malaria region; suffered medical treatment-required conditions; been pregnant; used acupuncture, tattoo or piercing; been treated by dentist the previous 14 days; or had been vaccinated or suffered infection the previous month. In total, blood from 60 different blood donors was used.

2.6. Platelet aggregation

Venous blood was collected in silicone-coated vacutainer tubes with 3.8% trisodium citrate (blood/anticoagulant 9:1; BD Vacutainer®, Plymouth, UK). Platelet-rich plasma was obtained by centrifugation at 220*g* for 20 min; platelet poor plasma was prepared by further centrifugation at 1500*g* for 10 min. *In vitro* platelet aggregation induced by 10 μM ADP was measured as previously described (Persson et al., 2000) in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA).