Original Article

Nicotine Modulation of Factor VII Activating Protease (FSAP) Expression in Human Monocytes

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Aim: Factor VII activating protease (FSAP) is a plasma serine protease involved in hemostasis and remodeling processes. Increased levels of circulating FSAP during pregnancy and in women using oral contraceptives (OCs) indicate that the hormonal status critically influences FSAP expression. In this respect, the aim of this study was to quantify nicotine modulation of FSAP expression in human monocytes/macrophages isolated from healthy female smokers and non-smokers, and from women who use OCs and smoke.

Methods: FSAP concentration and activity were measured in plasma samples obtained from healthy non-pregnant, pre-menopausal, non-smoking women who did not use OCs ($n=69$), non-pregnant, pre-menopausal women who currently smoke and use OCs ($n=43$), and women who are only smokers ($n=40$) or currently use OCs ($n=48$). Expressions of FSAP mRNA and protein in monocytes isolated from healthy non-pregnant female or healthy male donors were analyzed.

Results: Strongest circulating FSAP concentration and activity occurred in women with combined smoking and use of OCs compared to the control group. Enhanced FSAP levels were also observed in smoking women when compared to non-smokers. *Ex vivo* experiments demonstrated enhanced FSAP expression in monocytes isolated from women using OCs and currently smoking. Nicotine enhanced FSAP mRNA and protein levels in monocytes.

Conclusions: Monocytes from healthy female smokers show a constitutively enhanced FSAP expression and this effect could be replicated *in vitro* by stimulating monocytes with nicotine. The upregulation of FSAP due to nicotine and OC usage may be linked to a higher incidence of arteriothrombotic diseases related to their usage.

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Key words; FSAP, Monocytes, Smoking, Oral contraceptives

Introduction

Experimental and clinical evidence suggests that women using oral contraceptives (OCs) have an increased risk for both venous and arterial thrombotic

disease^{1, 2}). Smoking is known to increase the risk of arteriothromboembolic events in women using OCs³⁻⁵). Existing data are mixed with regard to possible protection provided by OCs against atherosclerosis and cardiovascular events^{6, 7}). Although there is a general consensus that hormonally-mediated alterations of vascular homeostasis account for hypercoagulability under OC treatment, a precise correlation between changes in coagulation related to female sex hormonal levels and thrombosis have not been fully established⁸). Furthermore, specific effects of nicotine that are primarily responsible for initiating coagulation activation remain

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Table 1. Baseline characteristics of study participants

Parameters	Controls (<i>n</i> =69)	Smoking OC non-users (<i>n</i> =40)	OC users (<i>n</i> =48)	Smoking OC users (<i>n</i> =43)	<i>p</i> -value
Age (years)	31 ± 10	29 ± 11	27 ± 9	32 ± 10	ns
BMI, kg/m ²	23.9 ± 4.5	24.3 ± 4.3	24.9 ± 5.3	25.2 ± 3.4	ns
Systolic BP, mmHg	114 ± 4	110 ± 10	115 ± 9	108 ± 12	ns
Diastolic BP, mmHg	70 ± 3	75 ± 6	73 ± 7	70 ± 8	ns
Cholesterol, mmol/L	5.11 ± 0.8	4.80 ± 0.7	5.81 ± 0.9	5.30 ± 1.0	ns
Triglycerides, mmol/L	1.20 ± 0.4	1.10 ± 0.5	1.30 ± 0.7	1.50 ± 0.6	ns
LDL cholesterol, mmol/L	3.1 ± 1.0	3.2 ± 0.8	3.5 ± 0.6	3.2 ± 0.2	ns
HDL cholesterol, mmol/L	1.4 ± 0.4	1.3 ± 0.2	1.2 ± 0.3	1.4 ± 0.5	ns
Cigarettes/day	0	15 ± 8	0	17 ± 7	***
Smoking period (years)	0	7 ± 4	0	8 ± 5	***

Values are mean ± SD. Data were compared and *p* values are calculated by Kruskal-Wallis test and indicate the difference between a control group and all other groups.

****p* < 0.001 versus control group, ns not significant.

unknown. Although nicotine is a major constituent of cigarette smoke it is also part of the treatment to wean people off smoking. Previous studies have shown the effects of nicotine on the expression of plasminogen activator inhibitor-1 (PAI-1) and urokinase-plasminogen activator (u-PA)^{9, 10}, but little is known about how it influences coagulation.

Factor VII activating protease (FSAP) was identified as a plasma serine protease that activates coagulation factor VII as well as pro-urokinase (pro-uPA)^{11, 12}. In addition to hepatocytes, the major cellular source of intravascular FSAP is monocytes, the only blood cells capable of synthesizing FSAP in response to various inflammatory mediators, such as cytokines and bacterial endotoxins¹³. The protease expressed by the FSAP gene carrying the single nucleotide polymorphism (SNP) Marburg I (MI; G534E, 1601 G/A) has reduced ability to activate pro-uPA and FVII^{14, 15}. FSAP also inactivates tissue factor pathway inhibitor (TFPI), which would also have a pro-coagulant effect on blood clotting¹⁵. Furthermore, high levels of FSAP are found in monocytes/macrophages of atherosclerotic plaques¹³.

MI-SNP was found to be an independent risk factor for the development of late complications of carotid stenosis¹⁶, may also be related to the occurrence of venous thromboembolism^{17, 18} and represents a general cardiovascular risk factor¹⁹. Recently, measurements of circulating FSAP antigen and activity demonstrated a modest association between elevated FSAP and venous thromboembolism¹⁸. We and others have shown that the levels of circulating FSAP during pregnancy and in women using OC were increased, indicating that the hormonal status criti-

cally influences FSAP expression^{20, 21}. Furthermore, SNPs in the FSAP gene were also correlated to a higher incidence of venous thrombosis in the elderly²². It was shown that FSAP inhibits the activity of platelet-derived growth factor-BB (PDGF-BB), which in turn influences remodelling processes in the vessel wall^{23, 24}.

The main focus of the present study was to determine the alterations in circulating FSAP levels and FSAP expression in monocytes exposed to OCs and/or cigarette smoking in healthy, non-pregnant, pre-menopausal women. Finally, FSAP mRNA expression in cultured monocytes/macrophages was evaluated to test the hypothesis that cigarette consumptions has a direct influence on FSAP expression and that cigarette smoking and OC use act synergistically to increase the risk of enhanced FSAP expression.

Materials and Methods

Study Population

Four groups of healthy, non-pregnant, pre-menopausal women, aged 18-40 years (median age of 25) were included in this study (**Table 1**). OC users who smoked (*n*=43) or not (*n*=48) had been on low-oestrogen OCs (<35 µg ethinylestradiol) containing either second- (levonorgestrel; *n*=22) or third-generation (desogestrel, *n*=21) progestagens for 21 days per cycle for 18 months before the examination. The smoking OC non-users were defined as women who smoked 10 or more cigarettes per day and were not using OC (**Table 1**). None of the women had a history of deep vein thrombosis, pulmonary embolism, stroke, acute myocardial infarction, pregnancy, sur-

gery, major illness or natural or surgical menopause. All women were free of infection and were not using any other medication at the time of blood collection. A reference group comprised age-matched healthy women ($n=69$) who had no history of pregnancy or recent disease and had equivalent blood pressure and other risk factors of atherosclerosis (**Table 1**). This study was approved by the Ethics Committee of the University of Giessen, and informed consent was obtained from all subjects.

Blood Sampling and FSAP Assay Systems

Citrated blood samples were collected and plasma was prepared by centrifugation (1,500 g, 20 min, 20°C) and stored at -80°C. Plasma FSAP antigen and activity levels were determined by ELISA and heterogeneous immuno-activity assay, respectively, as described previously^{25, 26}. The plasma antigen and activity contents were expressed as plasma-equivalent units (PEU).

Cell Isolation and Cell Culture

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from 5 healthy non-pregnant female or healthy male donors using Ficoll-Hypaque density gradient centrifugation. The PBMC phase was collected and washed with PBS for 10 min at 1100 rpm. The mononuclear cells were suspended in eluting buffer and processed to counter flow-centrifugation elution using a Beckman centrifuge as described previously^{13, 20}. The monocytes were then suspended at a final concentration of 1×10^6 cells/mL, washed 3 times and then cultured in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA) containing L-glutamine (2 mM), penicillin (100 µg/mL) and streptomycin (0.1 µg/mL) at 37°C in 5% CO₂ atmosphere. The monocytes were incubated with nicotine at concentrations ranging from 0 to 100 µM in the presence or absence of a selective nicotine acetylcholine receptor $\alpha 7$ subunit antagonist ($\alpha 7$ -nAChR), α -bungarotoxin at a concentration of 10 nM for 24 hours.

Monocytes from the different study groups (control group, smoking OC users and non-users, and only OCs users) were isolated with a RosetteSep kit according to the manufacturer's instructions. Two different methods were used due to different requirements for the two sets of experiments. Isolation of monocytes by centrifugal elutriation is suitable for processing one sample at a time but a large blood volume, whereas the column method is suitable for processing many samples simultaneously, although only a small blood volume can be processed.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including DNase treatment. cDNA synthesis was performed using a kit from Perkin Elmer as described previously¹³ and the cDNA was stored at -20°C. Relative mRNA quantification was performed by real-time RT-PCR using CFX 96 real-time system Bio-Rad (Bio Rad, Munich, Germany). For FSAP, the following primer sequences were used: sense: CCCACTGAGCCATCAACCAA; antisense: TGGGGCATGGAGATGGTC with a product size of 169 bp. The expression was normalized to the internal control (GAPDH) sense: CCT CAA GAT CAT CAG CAA TGC CTC CT; antisense: GGT CAT GAG TCC TTC CAC GAT ACC AA) and its relation to the expression in the control group was calculated.

Western Blot Analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli method, as described previously⁴. The cell pellets were lysed on ice in lysis buffer containing 1% (v/v) NP40, 1M Hepes (pH 7.8), 5M NaCl, 0.5M EDTA, 1M Na₂MoO₄, 2.5 mL glycerin and complete protease inhibitor cocktails (1M DTT, PMSF, leupeptin, aprotinin). The protein concentration was determined using the colorimetric protein assay based on the Lowry protein assay according to the protocol of the Bio-Rad protein assessment kit (Bio Rad). The lysates (50 µg) were separated on a 13% (w/v) SDS-PAGE gel and electroblotted onto 0.2-µm-pore Immobilon-PVDF membranes (Bio Rad). Blots were blocked with 5% non-fat milk in PBS buffer (1 x PBS, 0.1% (v/v) Tween-20, distilled water) for 1 hour and were then probed with primary antibodies (1:6000, anti-FSAP clone 1102/1189-2) overnight at 4°C. The blots were washed and then probed with secondary antibodies (anti-mouse Ig G horseradish peroxidase-linked whole antibody from sheep; Amersham Biosciences) diluted 1:3000 in wash buffer with 5% non-fat dry milk and incubated for 1 hour at room temperature. To confirm equal protein loading, blots were stripped and Western blot analysis was performed with anti-vinculin antibody produced in mice (clone V9131; Sigma-Aldrich, Hamburg, Germany). The proteins were detected using a chemiluminescence ECL detection system (Amersham Pharmacia Biotech) and blot was immediately exposed to a film for 60 seconds and developed in Curix HT-530 U (Agfa).

Fig. 1. Increased plasma FSAP levels and gene expression in monocytes from women using OCs and/or smoking.

Box/whisker plots of FSAP concentration (A) and activity (B) levels in plasma of non-smoking OC non-users (control group, $n=69$), smoking OC non-users ($n=40$), OC users ($n=48$) or smoking OC users ($n=43$). Boxes mark the 25th and 75th percentile values, the line inside each box represents the median, and whiskers indicate the spread of 5th and 95th percentiles. FSAP mRNA expression (C) was analyzed by real-time RT-PCR in monocytes obtained from non-smoking OC non-users, smoking OC non-users, OC users or smoking OC users. Relative mRNA quantification was performed by real-time RT-PCR and the fold change of the target gene (FSAP) was normalized to the internal control (GAPDH) and related to the expression in non-smoking OC non-users (control group), and finally calculated and presented as box/whisker plots. * $p < 0.05$, *** $p < 0.001$, compared to the control group.

Fig. 2. Nicotine modulation of FSAP gene and protein expression in human monocyte-derived macrophages.

The effect of nicotine at increasing concentrations from 1 to 100 μM on the expression of FSAP in monocyte-derived macrophages cultured *in vitro* for 24h was assessed using real-time RT-PCR (A). Relative mRNA quantification was performed and the fold change of the target gene (FSAP) was normalized to the internal control (GAPDH) and related to the expression in the control group. Data are the mean $\pm$ SD of four independent experiments involving 5 donors. * $p < 0.05$, compared to unstimulated cells.

Statistical Analysis

All data sets were subjected to normality testing using the Kolmogorov-Smirnov test. As the results were not normally distributed, the non-parametric Kruskal-Wallis test was used to test differences between groups with repeated measurements for the different time points of FSAP antigen and activity. FSAP levels were plotted as box/whisker plots, and differences in the median values across the indicated periods of pregnancy were examined by the Kruskal-Wallis test. The Mann-Whitney *U*-test was used to evaluate differences in relative FSAP mRNA expression among the groups. $P \leq 0.05$ was considered significant. Statistical analyses were carried out using SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

Conflict of Interest

No.

Results

The combined usage of OCs as well as smoking was associated with the elevation of plasma FSAP antigen and activity (**Fig. 1A and B**) compared to the control group. Smoking alone did not influence FSAP levels but OC usage alone increased FSAP levels significantly. The level of plasma FSAP did not differ between users of second- and third-generation OCs (data not shown). Significant elevation of FSAP mRNA expression was observed in monocytes obtained from women who smoked and used OCs ($p < 0.001$ vs non-

smoking/non-OCs users) (**Fig. 1C**). Increased FSAP mRNA expression (**Fig. 1C**) was also found in monocytes obtained from smoking non-OC users ($p < 0.05$ vs non-smoking/non-OCs users).

Untreated freshly isolated human monocytes produced very low levels of FSAP mRNA and protein but it was inducible by LPS, as described previously¹³. Cells were incubated for 24h in the presence of nicotine at the indicated doses, ranging from 1 to 100 μM , and there was a dose-dependent increase in FSAP mRNA levels (**Fig. 2**).

In order to evaluate whether the increase in plasma FSAP concentration observed in women with combined smoking and use of OCs was related to enhanced monocyte FSAP expression, we explored the effect of nicotine in combination with 17β -estradiol and progesterone on FSAP expression in primary cultures of human monocyte-derived macrophages (**Fig. 3A and B**). Furthermore, treatment with estradiol for 24h resulted in an increase of FSAP mRNA and protein levels (**Fig. 3A and B**), whereas progesterone administration at physiological concentration was less effective (**Fig. 3A and B**). Nicotine enhanced the effect of estrogen and progesterone on FSAP in human monocytes/macrophages. A selective $\alpha 7$ -nAChR antagonist, α -bungarotoxin, reversed the stimulating effects of nicotine (**Fig. 2**), but not of estrogen and progesterone, suggesting the specific involvement of $\alpha 7$ -nAChR receptors (**Fig. 3A and B**).

Fig. 3. Effect of nicotine, 17β-estradiol and progesterone on FSAP mRNA and protein level in cultured monocyte-derived macrophages.

The effect of a selective α7-nAChR antagonist, α-bungarotoxin, at 10 nM concentration (solid columns) in the presence of 100 µM nicotine and 1000 ng/mL 17β-estradiol and 100 ng/mL progesterone on the mRNA level (A) and on the protein level (B) was determined by real-time RT-PCR and Western blotting. Data are the mean ± SD of four independent experiments, involving 5 donors.

Discussion

Numerous studies have attempted to find a link between the pathogenesis of thromboembolic disease and FSAP^{17, 18}. We hypothesize that the expression of FSAP in monocytes and their activation status in atherosclerosis and cardiovascular events are closely related to each other^{13, 16}. FSAP antigen and activity levels were significantly enhanced during pregnancy and in women with oral hormone contraceptive therapy²⁰. Furthermore, biochemical evidence indicates that FSAP is potentially involved in regulating coagulation and fibrinolysis^{11, 12, 15}. The aim of this study

was to determine the level of FSAP expression in young pre-menopausal female smokers and non-smokers without any additional risk factors for atherosclerosis, but using OC therapy.

Current use of OCs as well as smoking were found to be associated with increased plasma FSAP levels (antigen and activity). No differences were observed in plasma FSAP levels between users of second- and third-generation OCs (data not shown). We also found that monocytes from women with combined smoking and use of OCs showed higher FSAP mRNA expression even when compared to the control non-user group. Moreover, we report here for the first

time that monocytes isolated from healthy female smokers show a significantly higher constitutive expression of FSAP than those from non-smokers (1.5-fold, $p < 0.05$).

FSAP mRNA and protein are significantly increased in the presence of estradiol and progesterone in cultured human monocytes/macrophages²⁰. This is in line with the epidemiological observation of differences in FSAP plasma concentration between healthy men and women²⁵. To determine whether the impact of smoking on monocyte FSAP generation might result from a direct nicotine effect, cultured monocytes/macrophages derived from healthy human blood donors were treated with nicotine *in vitro* and analyzed for FSAP mRNA and protein expression. Nicotine is reported to inhibit the activation of monocytes via nicotinic acetylcholine receptor $\alpha 7$ subunit ($\alpha 7$ -nAChR)²⁷, and this can be blocked by a selective antagonist, α -bungarotoxin. The $\alpha 7$ -nAChR antagonist prevented nicotine-induced enhancement of FSAP expression in monocytes in the presence and absence of estrogen and progesterone stimulation. These results suggest that the effect of nicotine on FSAP production in human monocytes depends on $\alpha 7$ -nAChR. It is important to emphasize that the nicotine concentrations used in our *in vitro* experiments were in the same range as those measured in the blood (around 70 ng/mL)²⁸ and tissues (0.5-2.6 times serum levels)²⁹ of smokers. Therefore, in spite of continuous exposure to a number of bioactive compounds and a subsequent inflammatory state which, in our opinion, largely underlies the enhanced FSAP expression in healthy female smokers, a relevant role for nicotine is possible.

In conclusion, FSAP levels were elevated in young pre-menopausal female smokers and non-smokers without any additional risk factors for atherosclerosis, but using oral contraceptive therapy. Central mechanisms by which FSAP can modulate the structural and functional integrity of the vessel wall have been characterized and increase our understanding of monocyte-derived changes in vascular homeostasis in response to smoking and nicotine, respectively.

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