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CLINICAL PHARMACOLOGY OF EICOSANOIDS, NICOTINE INDUCED CHANGES IN MAN*

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Smoking is an important risk factor for respiratory and cardiovascular diseases. The role of numerous chemical, partly uncharacterised compounds existing in tobacco smoke is not known. (−)-Nicotine, its stereoisomer (+)-nicotine and main metabolite cotinine are biologically active compounds influencing *e.g.* catecholamine and eicosanoid systems. The precise mechanisms are not well known. The purpose of the present study consisting of a PhD thesis (11) and five original papers was to investigate the *in vitro* effects of nicotine isomers and cotinine on eicosanoid production in polymorphonuclear leukocytes, platelets and whole blood *in vitro*, and to clarify the effects of smoking without and with nicotine substitution on eicosanoid production *in vivo* and *ex vivo*. It was found that all the tested compounds modulated blood cell eicosanoid synthesis. Nicotine isomers and cotinine increased PGE_2 but decreased TXB_2, LTB_4 and LTE_4 synthesis *in vitro*. Eicosanoid synthesis *in vivo* and *ex vivo* was higher in smokers (n = 60) than in non-smoking controls (n = 20). This may contribute to the harmful cardiovascular effects of smoking. Cessation of smoking without, but not with, nicotine substitution reduced eicosanoid synthesis measured *ex vivo* as whole blood production or *in vivo* as urinary excretion of eicosanoid metabolites after 3, 7 and 14 days. Thus long-term nicotine substitution diminishes the beneficial effects of smoking cessation.

Key words: *eicosanoids, prostaglandins, thromboxane, leukotrienes, smoking, nicotine, cotinine, smoking cessation, nicotine substitution.*

INTRODUCTION

Smoking is a major risk factor for pulmonary and many cardiovascular diseases, such as myocardial infarction, stroke and peripheral arteriopathy, but the precise mechanisms involved are not known. Different components in

*This autoreview is based on the PhD thesis of Saareks (11) and the five original articles referred in the list of references.

tobacco smoke, including nicotine, may alter arachidonic acid metabolism in addition to increasing catecholamine levels (1) and activating platelets (2, 3) and leukocytes (4, 5).

Smoking cessation reduces the risk of cardiovascular diseases (6) and reverts inflammatory changes in the respiratory tract (7). These changes can also be related to alterations in eicosanoid synthesis. Nicotine replacement therapy reduces nicotine withdrawal symptoms and improves the quit rate in persons abstaining from tobacco (8), but cardiovascular complications can occur even during nicotine replacement therapy (9, 10, for further references, see 11). The mechanisms of drawbacks of chronic nicotine substitution related to eicosanoid synthesis are not well established.

In tobacco smoke, in addition to the "normal" (−)-nicotine the (+)-isomer of nicotine is present in a proportion of 3—12 % (12) of the total amount of nicotine. The major metabolite of nicotine, cotinine, in the blood of smokers exceeds ten times that of nicotine (13).

The present study was designed to compare the effects of smoking cessation, nicotine chewing gum and nicotine patch as well as to evaluate the effects of nicotine isomers and cotinine on cyclooxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism in man.

STUDY DESIGN AND METHODS

Smoking cessation and nicotine substitution

Sixty healthy smoking volunteers and 20 age-matched healthy non-smoking controls (8 males and 12 females) participated in the study. Blood samples were taken after 12 h fasting in the morning and 12 h overnight urines collected before the trial (day 0) and on days 3, 7 and 14 after smoking cessation.

After smoking cessation, 60 subjects chose one of the following procedures: 1) to continue without nicotine substitution (smoking cessation group, n=15; 7 males and 8 females); 2) use nicotine chewing gum (nicotine chewing gum group, n = 15; 12 males and 3 females); 3) use nicotine patches (nicotine patch group, n = 30; 19 males and 11 females). The subjects were allowed to use nicotine chewing gum (Nicorette® 4 mg) or nicotine patches (Nicotinell®, alternatively 14 mg/24h or 21 mg/24 h) *ad libitum*.

The study protocols were approved by the Ethical Committee of Tampere University Hospital and informed consent was obtained from the subjects.

In vitro and ex vivo experiments

These experiments were performed to clarify the effects of the test compounds [(−)-nicotine and cotinine 0.5 nM—0.5 mM; (+)-nicotine 0.5 nM—0.05 mM] and smoking on the capacity of blood cells to synthesise different eicosanoids.

Polymorphonuclear leukocyte (PMN) experiments

PMN isolation from venous blood was done after 12 h fasting in the morning (8—9 a.m.) from freshly drawn whole blood by means of Ficoll-Paque density gradient centrifugation. The purity of PMNs was > 98% and viability as determined by Trypan blue > 98%.

PMNs (5×10^6) were preincubated in Dulbecco's phosphate-buffered saline for 15 min at 37°C without, and for a further 15 min with the test compound. Eicosanoid synthesis was triggered by a calcium ionophore A23187 (0.5 μM, 5 min, 37°C) and the reaction was stopped by centrifugation (10 000 × g, 2 min, 4°C).

Platelet rich plasma (PRP) experiments

PRP fraction was separated from freshly drawn venous blood after 12 h fasting in the morning (8—9 a.m.) by centrifugation at 200 × g for 20 min. PRP was adjusted to a final concentration of 15×10^6 platelets/100 μl with autologous platelet poor plasma.

Platelets (15×10^6) were preincubated in Dulbecco's phosphate buffered saline for 15 min at 37°C without, and for a further 15 min with the test compound. Eicosanoid synthesis was triggered by A23187 (10 μM, 60 min, 37°C) and the reaction was stopped by centrifugation (10 000 × g, 2 min, 4°C).

Whole blood experiments

Freshly drawn venous blood was taken after 12 h fasting in the morning (8—9 a.m.) into 10 ml heparinized glass vacutainer tubes. Eicosanoid synthesis was triggered by A23187 (10 μM), and the incubation was carried out for 60 min at 37°C either in the presence (*in vitro* samples) or absence (*ex vivo* samples) of the test compound. Plasma was separated by centrifugation (1600 × g, 10 min, +4°C).

Eicosanoid assays from PMN, PRP and whole blood samples

PGE_2 concentration was determined from the PMN incubation medium and from PRP by direct RIA using [^{125}I]-PGE_2 and antisera from the Institute of Isotopes Co, Budapest, Hungary (14).

TXB_2 concentrations from PRP incubation medium or from plasma (A23187-stimulation) were measured using direct [^{3}H]-TXB_2-RIA, in which the antiserum was from Prof. C. Taube (Martin Luther University, Halle, Germany) (14).

Cysteinyl leukotriene formation was determined as LTE_4-like immunoreactivity from plasma by direct RIA in a cross-reactivity way, using [^{3}H]-LTC_4 as the radiolabeled ligand and LTE_4 as the non-labeled ligand. An in-house rabbit antiserum, raised against the bovine serum albumin conjugate of LTC_4/LTD_4/LTE_4, was used for the assay (15).

Urinary 11-dehydro-TXB_2 was measured after selective one-step solid phase extraction on C_8-silica cartridges (Varian, Harbor City, CA, USA) by RIA using a specific antiserum and [^{125}I]-11-dehydro-TXB_2 tyrosine methyl ester tracer (Institute of Isotopes Co.) and nonlabeled 11-dehydro-TXB_2 (Cayman Chemical Co., Ann Arbor, MI, USA) (16).

2,3-dinor-6-keto-$PGF_{1\alpha}$ RIA was carried out after selective two-step solid-phase extraction on C_1-silica cartridges (Applied Separations, Allentown, PA, USA) using antiserum raised against 6-keto-$PGF_{1\alpha}$ with 100% cross-reaction with 2,3-dinor-6-keto-$PGF_{1\alpha}$ and [^{125}I]keto-$PGF_{1\alpha}$-tyrosine methyl ester as labeled radioligand (Institute of Isotopes Co.) and non-labeled 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Cayman Chemical Co.) (17).

Urinary leukotriene E_4 was measured by direct enzyme immunoassay using a commercial kit (Cayman Chemical Co.).

LTB_4 was measured after solid phase extraction on Bond Elut C_8 silica cartridges (Varian) by HPLC using PGB_2 (Cayman Chemical Co.) as internal standard and nonlabeled LTB_4 (Cayman Chemical Co.) for the calibration of the standard curve. PGB_4 and LTB_4 were monitored at 271 nm (18).

Other assays

Urinary creatinine was determined spectrophotometrically by the picric acid method using a commercial assay kit (Orion Diagnostics, Espoo, Finland).

Smoking cessation and the use of nicotine chewing gum were monitored by determining thiocyanate spectrophotometrically (19) and the main metabolites of nicotine, cotinine and trans-3'-hydroxycotinine by RP-HPLC (20) in urine. The subjects were ranked as smokers and excluded from the study, if no decrease was observed during the sampling time in urinary thiocyanate concentration.

RESULTS

Effects of nicotine stereoisomers and cotinine on eicosanoid synthesis in vitro (Table 1)

Table 1. Summary of the effects of nicotine stereoisomers and cotinine (50 μM) on the A23187-stimulated formation of cyclooxygenase and lipoxygenase pathway products in human whole blood *in vitro* (↑ increase over 2-fold; ↓ decrease over 25%). For details, see 11

	(−)-nicotine	(+)-nicotine	(−)-cotinine
PGE_2	↑	↑	↑
TXB_2	↓	↓	↓
LTE_4	↓	↓	↓

(−)-Nicotine stimulated PGE_2 production in PMNs and in whole blood. At the highest concentration applied (500 μM), PGE_2 production was increased about four-fold in PMNs and about seven-fold in whole blood. (−)-Nicotine inhibited TXB_2 formation in PRP and in whole blood, and LTB_4 production in PMNs and in whole blood. In whole blood LTE_4 formation was reduced only with the highest concentrations of (−)-nicotine applied.

(+)-Nicotine increased PGE_2 but inhibited TXB_2 and LTE_4 synthesis in whole blood. At the highest concentration applied (50 μM), (+)-nicotine increased PGE_2 synthesis more than two times over.

(−)-Cotinine stimulated PGE_2 production in PMNs and in whole blood. At the highest concentration applied (500 μM), PGE_2 production was increased about four-fold in PMNs and about seven-fold in whole blood. (−)-Cotinine inhibited TXB_2 formation in PRP and in whole blood. (−)-Cotinine inhibited LTB_4 production in PMNs and in whole blood. In whole blood LTE_4 synthesis was reduced only with the highest concentration.

Compliance control of smoking cessation

Urinary thiocyanate concentrations in non-smoking controls were about one-fourth of those observed in smokers. Smoking cessation brought a gradual decrease in these levels during the 14-day follow-up in all groups independently of nicotine substitution.

Measurable cotinine or trans-3'-hydroxycotinine concentrations were not found in any of the non-smokers. Smoking cessation without nicotine substitution reduced the levels below the detection limit within three days. In both groups using nicotine substitution, the concentrations remained more or less at the initial levels during the two-week observation period.

Effects of smoking cessation and nicotine substitution on eicosanoid synthesis (Table 2 and 3)

Table 2. Effects of smoking, smoking cessation and nicotine substitution (chewing gum, or patch) on the A23187-stimulated formation of cyclooxygenase and lipoxygenase pathway products *ex vivo* and *in vivo* (↑increase, ↓decrease) For details, see 11.

		Smoking	Smoking cessation	Nicotine substitution
Ex vivo (A23187-stimulation)	PGE_2	↑	↓	↑
	TXB_2	↑	↓	↑
	LTB_4	↑	↓	↑
	LTE_4	↑	↓	↑
In vivo (Urinary excretion)	11-dehydro-TXB_2	↑	↓	↑
	2,3-dinor-6-keto-$PGF_{1\alpha}$	↑	↓	↑
	LTE_4	↑	↓	↑

Table 3. Urinary eicosanoid excretion (pmol/μmol creatinine) in smokers before smoking cessation and in non-smoking controls (mean ± SEM). For details, see 11.

Intervention group (n)	2,3-dinor-6-keto-$PGF_{1\alpha}$	11-dehydro-TXB_2	LTE_4
Non-smokers (15)	14.7 ± 1.1	54.1 ± 6.6	13.5 ± 1.4
Smoking cessation (15)	45.3 ± 5.3	115.8 ± 13.2	53.6 ± 11.4
Nicotine gum (15)	27.0 ± 5.0	107.5 ± 14.4	57.5 ± 14.9
Nicotine patch (30)	54.2 ± 6.4	120.0 ± 16.0	78.6 ± 7.4

In whole blood *ex vivo*, A23187-stimulated PGE_2 and TXB_2 productions were about three and LTB_4 and LTE_4 synthesis about four times higher in smokers than in non-smoking controls. Three days after smoking cessation without nicotine substitution, PGE_2, TXB_2, LTB_4 and LTE_4 levels were lowered to about 70%, 80%, 45% and 60% of the initial values, and after

14 days 55%, 80%, 45% and 50%, respectively. In the group that gave up smoking but used nicotine chewing gum, no significant changes were seen during the two-week follow-up.

Urinary excretion of all eicosanoid metabolites measured was higher in smokers than in non-smoking controls. Smoking cessation without nicotine substitution reduced urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$, LTE_4 and 11-dehydro-TXB_2 near to non-smoking levels, whereas in the groups that gave up smoking but used nicotine substitution in the form of chewing gum or patches, no significant changes were observed in these analyses during the two-week follow-up.

DISCUSSION

Relevance of the variables measured

The eicosanoid concentrations present in unstimulated plasma or whole boold are so low that direct determination is not possible, even with the highly sensitive (radio)immunoassay methods currently available. Therefore, in the present study, eicosanoid synthesis in PMNs, PRP and whole blood was stimulated with calcium ionophore A23187, commonly used to determine the eicosanoid synthesis capacity.

In *in vitro* and *ex vivo* experiments, PGE_2 and TXB_2 productions reflect the cyclooxygenase 2 and 1 activity in leukocytes and platelets, respectively. LTB_4 and E_4 productions indicate the activity of 5-lipoxygenase, and in addition also the activity of LTA_4-hydrolase (LTB_4) and LTC_4-synthase (LTE_4).

In the whole blood model, the different cell-cell interactions affecting the eicosanoid spectrum are present. In this model, PGE_2 is synthesized by monocytes and neutrophils. TXB_2 is mainly derived from platelets, with a smaller proportion originating from monocytes. LTB_4 is formed in monocytes, eosinophils and neutrophils. Monocytes, basophils and eosinophils produce LTC_4, which is then degraded to LTD_4 and LTE_4 by cellular or plasma enzymes (for review, see 11, 21).

Certain urinary metabolites of eicosanoids are regarded as *in vivo* markers of eicosanoid production. In urine, 6-keto-$PGF_{1\alpha}$ and TXB_2 originate predominantly from the kidney under physiological conditions and do not represent the systemic formation of PGI_2 and TXA_2. It has been generally acknowledged that urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 11-dehydro-TXB_2 (together with 2,3-dinor-TXB_2) are the major compounds which reflect the total body production of PGI_2 and TXA_2 in man. Urinary excretion of 11-dehydro-TXB_2 is an index of platelet activation (22) and that of 2,3-dinor-6-keto-$PGF_{1\alpha}$ an index of PGI_2 formation by endothelium (23).

A significant proportion, approximately 4—13%, of leukotrienes is excreted in urine as LTE_4 (24). The measurement of urinary LTE_4 has therefore been used as a marker of *in vivo* cysteinyl leukotriene production (22, for review, see 25).

Nicotine stereoisomers and cotinine in vitro

Nicotine stereoisomers and cotinine stimulated PGE_2 production *in vitro*. (+)-Nicotine was included in the present study because there was no earlier evidence available on its effects on arachidonic acid metabolism, although it has been shown to be biologically active (26, 27). The effects of cotinine on eicosanoid production were also unknown, although cotinine has about a ten times higher blood and plasma concentration (1, 13) and a considerably longer half-life than the parent compound (1).

Regarding the effects of nicotine, our results are consistent with previous observations on the stimulation of PGE_2 formation induced by nicotine (28), by smokeless tobacco extract (29) in man, and by nicotine shown in animal models, *e.g.* in isolated rabbit heart (30). It may function as a cosubstrate for the peroxidase component of prostaglandin endoperoxide synthase (31).

Nicotine stereoisomers and cotinine inhibited TXB_2 formation *in vitro*. The effect of (+)-nicotine on TX synthesis has not been described previously. (−)-Nicotine and cotinine at submicromolar concentrations suppress TXB_2 synthesis in arachidonic acid-stimulated intact macrophage-like cells and in cell free microsomal preparations, possibly by direct inhibition of thromboxane synthase (32), as well as in human platelet-rich plasma (33).

Nicotine stereoisomers and cotinine inhibited LTB_4 and E_4 production *in vitro*, which conflicts with the reported nicotine-induced increase in LTC_4 production in A23187-stimulated human neutrophils (28). PGE_2 has been reported to inhibit 5-lipoxygenase pathway (34, 35). However, it is unlikely that this explains the reduction in LT production because in A23187-stimulated human polymorphonuclear leukocytes as well as in whole blood, acetylsalicylic acid almost completely blocked PGE_2 production, whereas the inhibition of LT production by nicotine or cotinine remained unaffected.

Since 5-lipoxygenase is the first enzyme in the biosynthesis pathway of LTB_4 as well as of LTC_4 and LTD_4, its impairment curtails the synthesis of all LTs. The mechanism of action of nicotine stereoisomers and cotinine *in vitro* is therefore probably to inhibit 5-LO as these electron donating compounds reduce the catalytically active ferric enzyme to the catalytically inactive ferrous form, as previously suggested for catecholamines and other phenolic compounds (18). However, the inhibition of LTA_4-hydrolase, glutathione-S-transferase, γ-glutamyl peptidase, cysteinyl glycinase cannot be excluded.

In general, nanomolar concentrations of (−)-nicotine and cotinine were more effective in stimulating PGE_2 production in PMNs and in inhibiting TXB_2 synthesis in PRP than in whole blood, whereas at high concentrations the drugs were more effective in whole blood.

Smoking cessation and nicotine substitution

PGE_2, TXB_2, LTE_4 and LTB_4, synthesis in A23187-stimulated whole blood *ex vivo* were all higher in smokers than in non-smoking subjects consistently with previous findings on increased cyclooxygenase activity (36), LTB_4 serum concentrations (37) and blood levels of LTC_4, LTD_4 and LTE_4 (38) in smokers. The increase in PGE_2 synthesis also seen *in vitro* is probably due to the stimulation of cyclo-oxygenase by nicotine and cotinine.

Urinary excretions of 11-dehydro-TXB_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ and LTE_4 were higher in smokers than in non-smoking subjects as reported earlier (39, 40, 41). However, smoking has also been associated with reduced (42, 43) or unchanged (44, 45) PGI_2 synthesis. The diverging findings may be related to methods (6-keto-$PGF_{1\alpha}$ antibodies, purification processes) age and vascular injuries of the subjects.

The increased PGI_2 synthesis observed in smokers has been suggested to be a compensatory mechanism of endothelium for the general vasoconstrictive properties of cigarette smoking (40). The increased systemic TX synthesis found in smokers in the present study and earlier (39, 44, 46, 47) reflects platelet activation (39). In the present study, smokers showed increased urinary excretion of LTE_4 in line with previous report of Fauler and Frölich in 1997 (48).

In our nicotine substitution groups urinary cotinine and trans-3'-hydroxycotinine concentrations remained more or less at the initial levels after 3 and 7 days, and no decrease was observed in eicosanoid synthesis either. From 7 to 14 days, some, but not all, of the eicosanoids measured were lowered parallel to urinary cotinine and trans-3'-hydroxycotinine concentrations, indicating a decreased use of substitution. Our findings suggest then that nicotine in the form of chewing gum and patches counteracts the decrease in systemic eicosanoid production upon cessation of smoking.

There are also studies whose results (for references, see 11) do not agree with the present results. It is easy to find differences in study design or in subjects participating or in route of administration and doses of nicotine which are good explanations for the disagreements. These have been recently discussed in detail by us (11).

Concerning the role of increased systemic eicosanoid synthesis in the development of atherosclerosis and cardiovascular diseases in general, cigarette smoking affects the important factors in the progression of atherosclerosis and

in the acute development of cardiovascular complications. One of these factors is decreased endothelial nitric oxide bioactivity in smokers (49, 50).

It was an interesting and important finding in the present study that no decrease was observed in eicosanoid synthesis during substitution therapy with nicotine chewing gum or nicotine patches. This suggests that nicotine/or cotinine could be major factors in explaining the differences found in eicosanoid synthesis between smokers and non-smokers. The increased level of systemic eicosanoid synthesis observed in volunteers using nicotine chewing gum or nicotine patches might partly explain the cardiovascular complications that have been reported to occur during nicotine replacement therapy (for references, see 11).

The increased eicosanoid synthesis observed in smokers and in persons quitting smoking but using nicotine substitution raises the question as to whether the expressions of cyclooxygenase-2 and 5-lipoxygenase are increased in smokers/in persons predisposed to nicotine. Both issues will be addressed in our further studies.

SUMMARY AND CONCLUSIONS

1. Eicosanoid synthesis both *ex vivo* and systemically was higher in smokers than in non-smoking controls, which may contribute to the harmful cardiovascular effects of smoking.

2. Cessation of smoking without nicotine substitution decreased eicosanoid synthesis with possibly decreased risk of cardiovascular diseases and improvement of lower respiratory tract inflammatory changes.

3. Quitting smoking but using nicotine substitution showed no significant changes in eicosanoid synthesis suggesting the crucial role of nicotine and/or cotinine.

4. Long-term nicotine substitution may diminish the beneficial effects of smoking cessation due to the stimulatory effects of nicotine and cotinine on eicosanoid synthesis.

5. All the test compounds modulated eicosanoid synthesis *in vitro*. Nicotine stereoisomers and cotinine stimulate cyclooxygenase, but inhibit 5-lipoxygenase and TX-synthase.

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