VIRPI SAAREKS

Nicotine-Induced Changes in Eicosanoid Synthesis in Man

Effects of Smoking Cessation, Nicotine Substitution, Pyridoxine and Nicotinic Acid

University of Tampere
Tampere 2000

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■

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of
Building B, Medical School of the University of Tampere,
Medisiinarinkatu 3, on March 31st 2000, at 12 o'clock.

University of Tampere
Tampere 2000

“Elo ihmisen huolineen ja murheineen,
se on vain väliaikainen...”
Tatu Pekkarinen, Väliaikainen

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals. Some previously unpublished data are also presented.

I **Saareks V, Riutta A, Mucha I, Alanko J and Vapaatalo H** (1993): Nicotine and cotinine modulate eicosanoid production in human leukocytes and platelet rich plasma. Eur J Pharmacol 248:345-349.

II **Saareks V, Mucha I, Sievi E, Vapaatalo H and Riutta A** (1998): Nicotine stereoisomers and cotinine stimulate prostaglandin E_2 but inhibit thromboxane B_2 and leukotriene E_4 synthesis in whole blood. Eur J Pharmacol 353:87-92.

III **Riutta A, Saareks V, Mucha I, Alanko J, Parviainen M and Vapaatalo H** (1995): Smoking cessation and nicotine substitution modulate eicosanoid synthesis ex vivo in man. Naunyn-Schmiedeberg's Arch Pharmacol 352:102-107.

IV **Saareks V, Mucha I, Sievi E and Riutta A** (1999): Nicotinic acid and pyridoxine modulate arachidonic acid metabolism in vitro and ex vivo in man. Pharmacol Toxicol 84:274-280.

V **Saareks V, Ylitalo P, Mucha I and Riutta A**: Effects of smoking cessation and nicotine substitution on systemic eicosanoid production in man. Naunyn-Schmiedeberg's Arch Pharmacol (submitted).

ABBREVIATIONS

A23187	calcium ionophore A23187 (Calcimycin)
AA	arachidonic acid
ASA	acetylsalicylic acid
COX	cyclooxygenase
cyp 450	cytochrome 450
EET	epoxyeicosatetraenoic acid
HETE	hydroxyeicosatetraenoic acid
(RP)-HPLC	(reversed-phase) high-performance liquid chromatography
LO	lipoxygenase
LT	leukotriene
NO	nitric oxide
PG	prostaglandin
PGG_2	prostaglandin endoperoxide G_2
PGH_2	prostaglandin endoperoxide H_2
PGHS	prostaglandin endoperoxide synthase
PGI_2	prostacyclin
PL	phospholipase
PMN(s)	polymorphonuclear leukocyte(s)
PRP	platelet rich plasma
RIA	radioimmunoassay
TX	thromboxane

INTRODUCTION

Smoking is a major risk factor for pulmonary and many cardiovascular diseases, such as myocardial infarction, stroke and peripheral arterial atherosclerosis, but the precise mechanisms involved are not clearly understood. As well as increasing catecholamine levels (Benowitz 1988) and activating platelets (Rival et al. 1987, Blache et al. 1992) and leukocytes (Bridges et al. 1993, Nowak et al. 1990), smoking and the different components in tobacco smoke, including nicotine, may contribute to the pathogenesis of these diseases by altering arachidonic acid (AA) metabolism. Pyridine derivatives nicotinic acid and pyridoxine have a pyridine moiety in common with nicotine and cotinine.

Eicosanoids are formed from polyunsaturated fatty acids (Crawford 1983, VanRollins et al. 1985, Ferretti and Flanagan 1986), mainly from AA, which is liberated from cell membrane phospholipids by phospholipases (PL) A_2 and C (Van den Bosch 1980, Irvine 1982). AA is metabolized to eicosanoids via prostaglandin H synthase (PGHS) (Bergström et al. 1964), different lipoxygenases (LOs) (Borgeat et al. 1976), cytochrome P450 (cyp 450) pathway (McGiff 1991) and free-radical catalyzed pathway (Morrow et al. 1990, Morrow et al. 1994, Harrison and Murphy 1995).

Eicosanoids are potent biological mediators, and it has been suggested that they are involved in the pathophysiology of many diseases including asthma, gastric ulcer, psoriasis, ischemic heart disease, migraine and thrombosis (Uotila and Vapaatalo 1984, Feuerstein and Hallenbeck 1987, Oates et al. 1988a, Oates et al. 1988b, Barnes and Smith 1999). Among eicosanoids, prostaglandin (PG) E_2 and leukotriene (LT) B_4 have a role in inflammatory diseases. Thromboxane (TX) A_2 has a potent proaggregatory activity on platelets and contractile activity on vascular smooth muscle (Hamberg et al. 1975, Ellis et al. 1976). The balance between antiaggregatory and vasodilatory prostacyclin (PGI_2) (Moncada et al. 1976) and proaggregatory TXA_2, the main cyclooxygenase products of AA metabolism in vascular endothelial cells and platelets, respectively, is of functional importance in platelet/vessel wall interactions (Fitzgerald et al. 1986, Vesterqvist et al. 1987). Cysteinyl LTs C_4, D_4 and E_4, which contract smooth muscle, are assumed to be of importance in asthma (Devillier et al. 1999a, Devillier et al. 1999b) and in reperfusion injury (Shappel et al. 1990).

Smoking cessation reduces the risk of cardiovascular diseases (Dobson et al. 1991) and reverts inflammatory changes in the lower respiratory tract (Rennard et al. 1990). The decrease in the occurrence of these diseases might be related to possible alterations in eicosanoid synthesis.

Nicotine replacement therapy reduces nicotine withdrawal symptoms and improves the quit rate in persons abstaining from tobacco (Thompson and Hunter 1998), but there are reports of cardiovascular complications during nicotine replacement therapy (Dacosta et al. 1993, Jackson 1993, Pierce 1994, Warner and Little 1994, Arnaot 1995, Ottervanger et al. 1995, Riche et al. 1995). The drawbacks of chronic nicotine substitution are not well established, nor are the effects of nicotine replacement on eicosanoid synthesis properly understood.

In tobacco smoke, the (+)-isomer of nicotine is present in a proportion of 3-12% (Klus and Kuhn 1977, Nwosu et al. 1988) of the amount of nicotine. This isomer has been reported to be physiologically effective in man. The concentrations of cotinine, the major mammalian metabolite of nicotine, in the blood and plasma of smokers are about ten times higher than those of nicotine (Benowitz et al. 1987, Höfer et al. 1992). Nicotinic acid is a potent hypolipidemic drug (Altschul et al. 1955) when administered in pharmacological doses in the range of one to eight grams per day. Pyridoxine is an antithrombotic compound (Subbarao et al. 1977, Kornecki and Feinberg 1978, van Wyk et al. 1992) which may have a possible therapeutic role in some diseases including asthma (Driskell 1994).

The evidence concerning the effects of smoking cessation, nicotine substitution, nicotinic acid and pyridoxine on eicosanoid synthesis is inconclusive. However, the possible alterations in eicosanoid production might contribute to the cardiovascular complications reported during nicotine replacement therapy. The present study was designed to compare the effects of smoking cessation, nicotine chewing gum and nicotine patch on cyclooxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism in man.

REVIEW OF THE LITERATURE

1. EICOSANOIDS

1.1. Arachidonic acid and phospholipases

Eicosanoids are synthesized from 20-carbon (eicosa-) polyunsaturated fatty acids (Crawford 1983, VanRollins et al. 1985, Ferretti and Flanagan 1986), mainly from AA, which is released from cell membrane phospholipids through the action of PLA_2 and PLC (Van den Bosch 1980, Irvine 1982). The other precursors of eicosanoids are dihomo-γ-linolenic, eicosapentaenoic and docosatetraenoic acids (Mitchell 1992). The amount of free arachidonic acid is a rate-limiting factor in eicosanoid synthesis. PLA_2 and C are activated by various physiological agents (e.g., angiotensin II, biogenic amines, bradykinin, cytokines, histamine, thrombin, endothelin) (Berridge 1993) and non-specific pharmacological stimuli (calcium ionophores, phorbol esters) via the increase of intracellular Ca^{2+} (Pickett et al. 1977, Exton 1994).

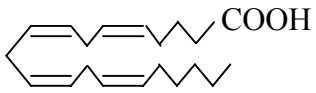


Figure 1. The structure of arachidonic acid.

1.2. Prostaglandin endoperoxide synthase pathways

Two forms of prostaglandin endoperoxide synthase (PGHS) are known. Constitutive PGHS I (cyclooxygenase I = COX-1) is involved in cell-cell signalling and in maintaining tissue homeostasis. Inducible PGHS II (cyclooxygenase II = COX-2), which is stimulated by different mediators of inflammation, growth factors and tumor promoters, is involved in inflammation and in mitogenesis. The PGHS I-pathway was discovered in 1964 by Bergström and coworkers, and human PGHS I cDNA was

cloned, sequenced and expressed by Funk and coworkers in 1991. Interleukin-1- and phorbol ester-induced increases in human fibroblast and endothelial cell PGHS protein were reported in 1988 by Raz and coworkers and by Wu and collaborators, and the hypothesis of the existence of PGHS II was presented in 1990 by Fu and coworkers. Human PGHS II cDNA was cloned, sequenced and expressed in 1992 (Hla and Neilson 1992). Both isozymes are expressed in most tissues (O'Neill and Ford-Hutchinson 1993). They are located subcellularly in endoplasmic reticulum, nuclear envelope and plasma membranes (Regier et al. 1993). PGHS II has been proposed to be constitutive in kidneys (Guan et al. 1997), lungs (Asano et al. 1996, Walenga et al. 1996, Petkova et al. 1999) and platelets (Weber et al. 1999).

PGHS includes a COX and a peroxidase activity (O'Brien and Rahimtula 1978, Miyamoto et al. 1976, Marshall and Kulmacz 1988). The COX activity catalyzes the bisoxygenation of AA to form endoperoxide G_2 (PGG_2) (Samuelsson 1972, Nugteren and Hazelhof 1973), and the peroxidase activity catalyzes the reduction of the 15-hydroperoxyl group of PGG_2 into PGH_2 (see Figure 2). Both PGG_2 and PGH_2 are chemically unstable. Heme is required as a cofactor for the formation of PGG (Hemler and Lands 1976, Van der Ouderaa et al. 1977, Ogino et al. 1978), and the presence of an electron donor is required for the synthesis of PGH (Van der Ouderaa et al. 1977). It has been reported that adrenaline and other catecholamines stimulate PG formation by acting as cosubstrates for the peroxidase component of PGHS and by defending PGHS against self-inactivation (Alanko et al. 1992).

Corticosteroids (Bailey et al. 1988) are known to suppress PGHS activity by inhibiting PGHS gene transcription and expression (Masferrer et al. 1992, DeWitt and Meade 1993). Glucocorticoids, however, only affect PGHS II but not significantly PGHS I (Fu et al. 1990, Coyne et al. 1992). Endogenous factors including cytokines (Raz et al. 1988, Toratani et al. 1999) and growth factors (Habenicht et al. 1985, Rich et al. 1998) can stimulate PGHS activity by inducing PGHS II gene expression and transcription.

The effect of acetylsalicylic acid (ASA) on PG biosynthesis, i.e. the inhibition of the COX activity of the PGHS enzyme, was shown in 1971 (Collier, Smith and Willis, Vane). ASA causes an irreversible inhibition of cyclooxygenase activity (Roth et al. 1975, Roth and Majerus 1975) by acetylating a $serine^{530}$ residue of the enzyme. Selective PGHS II-inhibitors, coxibs, e.g. rofecoxib and celecoxib, have been developed (Futaki et al. 1994, Masferrer et al. 1994, Mitchell et al. 1994) to avoid the side effects, especially gastric ulcers, caused by non-selective non-steroidal anti-inflammatory drugs.

PGH_2 ($t_{1/2}$ = 3 minutes) is metabolized into PGs (PGD_2, PGE_2, $PGF_{2\alpha}$), TXA_2 and PGI_2. PGs were "found" in the early 1930s when the biological actions of human semen on smooth muscle and on blood pressure were detected, and the biologically active lipid

constituents were named as "prostaglandins" (Kurzrok and Lieb 1930, von Euler 1935). The first PGs were isolated in the 1960s (Bergström and Sjövall 1960) and their structures were described by Bergström and coworkers (1963).

PGD_2 is formed by a PGD synthase or prostaglandin endoperoxide-PGD-isomerase (Hamberg and Fredholm 1976). PGD_2 synthesis in man has been demonstrated in the central nervous system, gastrointestinal tract, mast cells, alveolar macrophages, uterus, skin, platelets and kidney medulla (Urade et al. 1995). The half-life of PGD_2 in human plasma is ≤ 5 minutes (Hensby 1974).

PGE_2 is formed by prostaglandin endoperoxide PGE-isomerase (Nugteren and Hazelhof 1973, Miyamoto et al. 1974). Large amounts of PGE_2 are formed in the seminal vesicle. Other organs containing PGE-isomerase include the gastrointestinal system, uterus, kidney, vascular smooth muscle cells and endothelium of the microvasculature (Urade et al. 1995). Human airway epithelium (Churchill et al. 1989) and airway smooth muscle (Delamere et al. 1994) can produce large amounts of PGE_2. Small amounts of PGE_2 are produced in platelets, monocytes and granulocytes. The half-life of PGE_2 in plasma is less than one minute (Hamberg and Samuelsson 1971).

$PGF_{2\alpha}$ is formed either by direct reduction of PGH_2 and isomerization of PGE_2 (or PGD_2), with subsequent reduction of the 9-keto-group by the prostaglandin 9-keto-reductase, or nonenzymatically by spontaneous hydrolysis of prostaglandin endoperoxides (Urade et al. 1995). The half-life of $PGF_{2\alpha}$ is less than one minute (Granström and Samuelsson 1971a, Granström and Samuelsson 1971b).

TXA_2 is formed by TXA_2 synthase, a cyp 450 enzyme (Haurand and Ullrich 1985), which is present for example in platelets, endothelium (Ingerman-Wojenski et al. 1981), adrenal glands, brain, colon, duodenum, heart, kidney, liver, lungs, lymph node, and spleen (Nüsing and Ullrich 1990). TXA_2 was earlier known as rabbit aorta contracting substance (Piper and Vane 1969). The chemical structure for TXA_2 was described by Hamberg and coworkers (Hamberg and Samuelsson 1974, Hamberg et al. 1975). The half-life of TXA_2 at physiological pH is about 30 seconds (Hamberg et al. 1975), but it is increased via a non-covalent binding to plasma proteins, mainly albumin (Folco et al. 1977).

PGI_2 is formed through PGI_2 synthase, which is present at least in aorta, brain, endothelium, heart, kidney, liver, lungs, ovary, prostate, skeletal muscle, small intestine, stomach, thymus, and tongue (Smith et al. 1983, Miyata et al. 1994, Siegle et al. 1994). PGI_2 was named as prostaglandin X (Bunting et al. 1976, Moncada et al. 1976). It undergoes rapid chemical transformation in biological systems to 6-keto-$PGF_{1\alpha}$, the half-life of PGI_2 at physiological pH being about 3 minutes in aqueous solutions (Cho and Allen 1978).

1.3. Lipoxygenase pathways

Via the lipoxygenase pathways (Figure 2), AA is first metabolized by 5-LO into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Borgeat et al. 1976), which is converted into a 5,6-epoxide, LTA_4, by the same enzyme (Shimizu et al. 1984, Shimizu et al. 1986, Rouzer et al. 1986). The human 5-LO gene was isolated and characterized in 1989 by Funk and coworkers. In the chain reaction leading to leukotriene synthesis, 5-LO is first stimulated and translocated to the cell membrane (Rouzer and Kargman 1988). At the cell membrane the soluble enzyme interacts with 5-LO activating protein (Miller et al. 1990). Expression of both 5-LO and 5-LO activating protein are necessary for leukotriene synthesis (Dixon et al. 1990).

LTA_4 is transformed either into 5,12-dihydroxyeicosatetraenoic acid, LTB_4 by LTA_4-hydrolase (Borgeat and Samuelsson 1979a, Rådmark et al. 1980a), or into LTC_4, (Murphy et al. 1979, Rådmark et al. 1980b) by LTC_4 synthase (Mannervik et al. 1984, Bach and Brashler 1985, Söderström et al. 1985). The latter is a glutathionyl derivative, formed by the action of glutathione-S-transferase. LTD_4 is synthesized by the removal of glutamic acid from LTC_4 catalyzed by γ-glutamyl-transferase (Örning et al. 1980, Parker et al. 1980). LTE_4 results from the cleavage of the peptide chain of LTD_4 by cysteine-glycine dipeptidase (Lewis et al. 1980, Parker et al. 1980, Bernström and Hammarström 1981, Örning et al. 1981). Cysteinyl leukotrienes have bronchoconstricting properties, and they were previously known as a slow reacting substance of anaphylaxis (SRS-A), first described by Feldberg and Kellaway (1938).

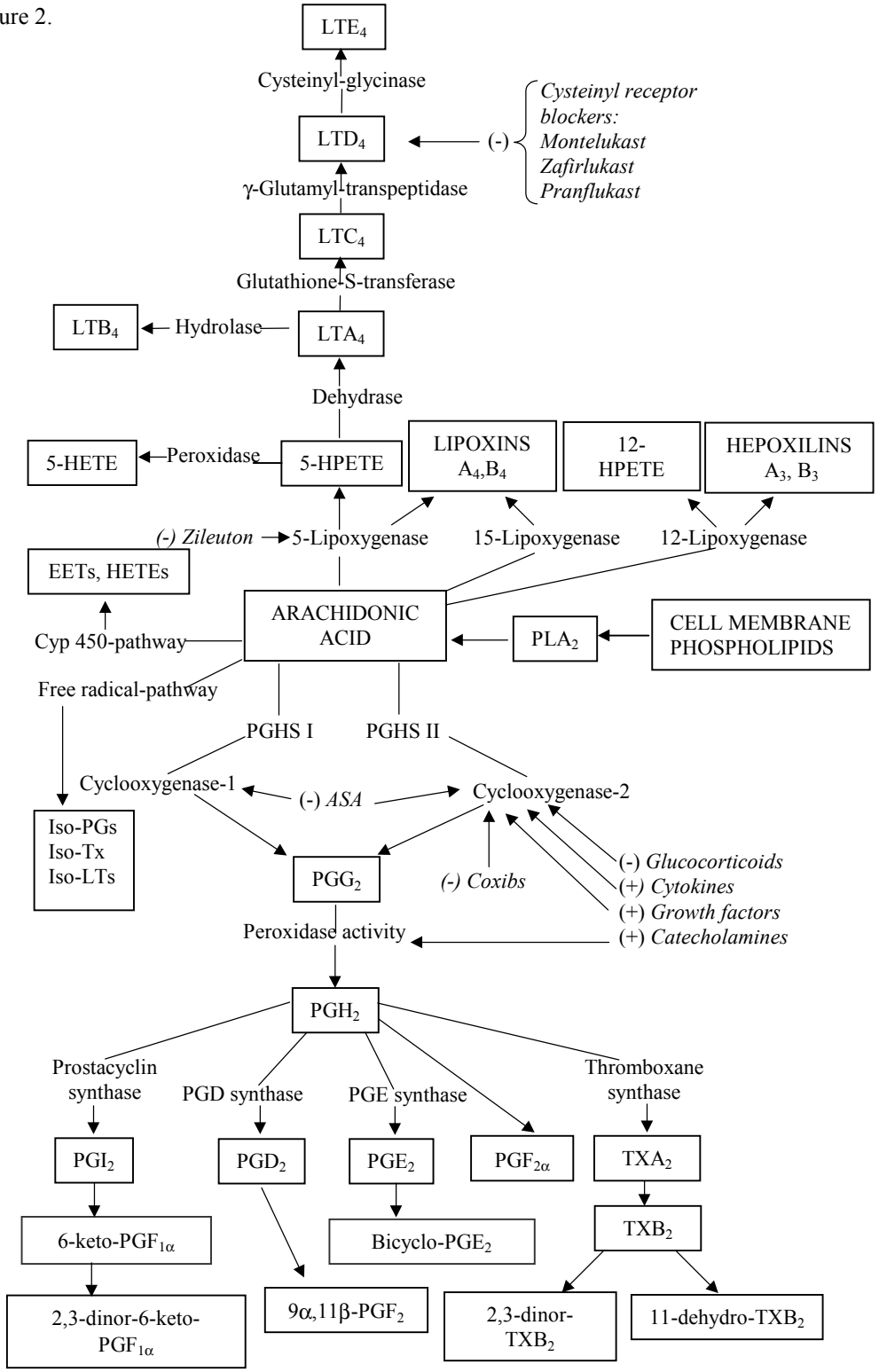
AA is metabolized into HPETEs, lipoxins and hepoxilins via the actions of other LOs. Among these metabolites, 12- (Hamberg et al. 1974) and 15-HPETE (Borgeat and Samuelsson 1979b) are formed by the actions of 12- and 15-LOs, lipoxins A_4 and B_4 by the combined action of 5- and 15-LO (Samuelsson 1983, Serhan et al. 1984a, Serhan et al. 1984b), and hepoxilins A_3 and B_3 via 12-LO-pathway (Pace-Asciak et al. 1983).

1.4. Other pathways

In cyp 450 catalyzed pathway (see McGiff 1991, Capdevila et al. 1992), AA is metabolized in three ways: allylic oxidation, resulting in the formation of racemic 5-, 8-, 9-, 11-, and 15-HETEs together with 12(R)-HETE; hydroxylation, resulting in the formation of 16-, 17-, 18-, 19-, 20-HETEs; and olefin epoxidation, resulting in the formation of 5,6-, 8,9-, 11,12-, 14,15-epoxyeicosatetraenoic acids (EETs). Elements of this pathway have been localized in the kidney and several extrarenal sites. In the free radical-catalyzed pathway isoprostanes D, E and F (Morrow et al. 1990, Morrow et al. 1994) and isoleukotrienes (Harrison and Murphy 1995) are generated from AA.

The major compounds of AA metabolism produced in different pathways are shown in Figure 2 on page 17.

Figure 2.


LTE4
Cysteinyl-glycinase
LTD4
(-)
Cysteinyl receptor blockers:
Montelukast
Zafirlukast
Pranflukast
γ-Glutamyl-transpeptidase
LTC4
Glutathione-S-transferase
LTB4
Hydrolase
LTA4
Dehydrase
5-HETE
Peroxidase
5-HPETE
LIPOXINS A4,B4
12-HPETE
HEPOXILINS A3, B3
(-) Zileuton
5-Lipoxygenase
15-Lipoxygenase
12-Lipoxygenase
EETs, HETEs
ARACHIDONIC ACID
Cyp 450-pathway
PLA2
CELL MEMBRANE PHOSPHOLIPIDS
Free radical-pathway
PGHS I
PGHS II
Cyclooxygenase-1
(-) ASA
Cyclooxygenase-2
Iso-PGs
Iso-Tx
Iso-LTs
PGG2
(-) Coxibs
(-) Glucocorticoids
(+) Cytokines
(+) Growth factors
(+) Catecholamines
Peroxidase activity
PGH2
Prostacyclin synthase
PGD synthase
PGE synthase
Thromboxane synthase
PGI2
PGD2
PGE2
PGF2α
TXA2
6-keto-PGF1α
Bicyclo-PGE2
TXB2
2,3-dinor-6-keto-PGF1α
9α,11β-PGF2
2,3-dinor-TXB2
11-dehydro-TXB2

1.5. Urinary markers of systemic eicosanoid production

The catabolism of eicosanoids to lower molecular weight, more hydrophilic compounds, occurs through several pathways. Most tissues, mainly the lungs, liver and kidney, are endowed with enzymes that rapidly and extensively inactivate eicosanoids. These include 9- and 15-hydroxyprostaglandin dehydrogenase and delta-13-reductase (for a review, see Granström and Kumlin 1987).

PGF-type compounds represent about two-thirds of all PGD_2-metabolites in primates. The single most abundant metabolite in man is 9α,11β-dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid (Liston and Roberts 1985). 9α,11β-PGF_2 is a biologically active metabolite of PGD_2, inhibiting platelet aggregation and causing both coronary and bronchial smooth muscle contraction in man (Pugliese et al. 1985, Seibert et al. 1987). This metabolite can be used as an indicator of systemic PGD_2 synthesis (Obata et al. 1994). A major urinary metabolite of PGE_2 is 11α-hydroxy-9,15-diketo-2,3,4,5-tetranorprostane-1,20-dioic acid (Samuelsson et al. 1975). An elegant procedure for monitoring PGE_2 production in vivo is the measurement of a bicyclo-metabolite which is formed from 13,14-dihydro-15-keto-PGE_2, the most prominent metabolite of circulating PGE_2, respectively, spontaneously at alkaline conditions (Granström et al. 1980, Starczewski et al. 1984). A major metabolite of $PGF_{2\alpha}$ in urine is 9α,11α-dihydroxy-15-keto-2,3,4,5-tetranorprostane-1,20-dioic acid (Hamberg 1973).

Out of the 20 extrarenal metabolites of TXA_2 found in urine, 11-dehydro-TXB_2 has been established as the most prominent breakdown product of TXB_2 (Schweer et al. 1987, Catella and FitzGerald 1987, Chiabrando et al. 1993). Other abundant degradation products of TXA_2 in urine are 2,3-dinor-TXB_2, 11-dehydro-2,3-dinor-TXB_2, 2,3-dinor-TXB_2, 2,3,4,5-tetranor-TXB_2, 11-dehydro-2,3-4,5-tetranor-TXB_2, TXB_2, and 11-dehydro-15-keto-13,14-dihydro-20-carboxy-TXB_2. Urinary excretion of 11-dehydro-TXB_2 is considered to be a reliable index of platelet activation (Granström 1988, Fischer 1989) and can be used to evaluate systemic TX production.

2,3-Dinor-6-keto-$PGF_{1\alpha}$ is the most prominent of the 16 extrarenal metabolites of PGI_2 found in urine (Rosenkranz et al. 1980, Brash et al. 1983). Other abundant degradation products of PGI_2 in urine include 2,3-dinor-6,15-diketo-13,14-dihydro-20-carboxy-$PGF_{1\alpha}$, 2,3-dinor-6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$, 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$, and 2,3,4,5-tetranor-$PGF_{1\alpha}$. Urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ is generally accepted as an index of systemic PGI_2 formation by endothelium in man (FitzGerald et al. 1983).

The degradation of LTC_4 occurs in the lungs, kidney, and liver (Denzlinger et al. 1986). The main urinary metabolites of LTs C_4, D_4 and E_4 consist of beta- and omega-

oxidized compounds. The end product of cysteinyl leukotriene pathway, LTE_4, is metabolized through ω-oxidation into 20-OH-LTE_4 and 20-COOH-LTE_4 or acetylated into N-acetyl-LTE_4 (Sala et al. 1990). A significant fraction of cysteinyl leukotrienes is excreted in urine as LTE_4 (Örning et al. 1985), and the measurement of urinary LTE_4 has been used as a marker of in vivo cysteinyl leukotriene production (Granström 1988, Nicolli-Griffith et al. 1990). LTB_4 is not excreted in urine because it is oxygenated to carbon dioxide and water in the liver.

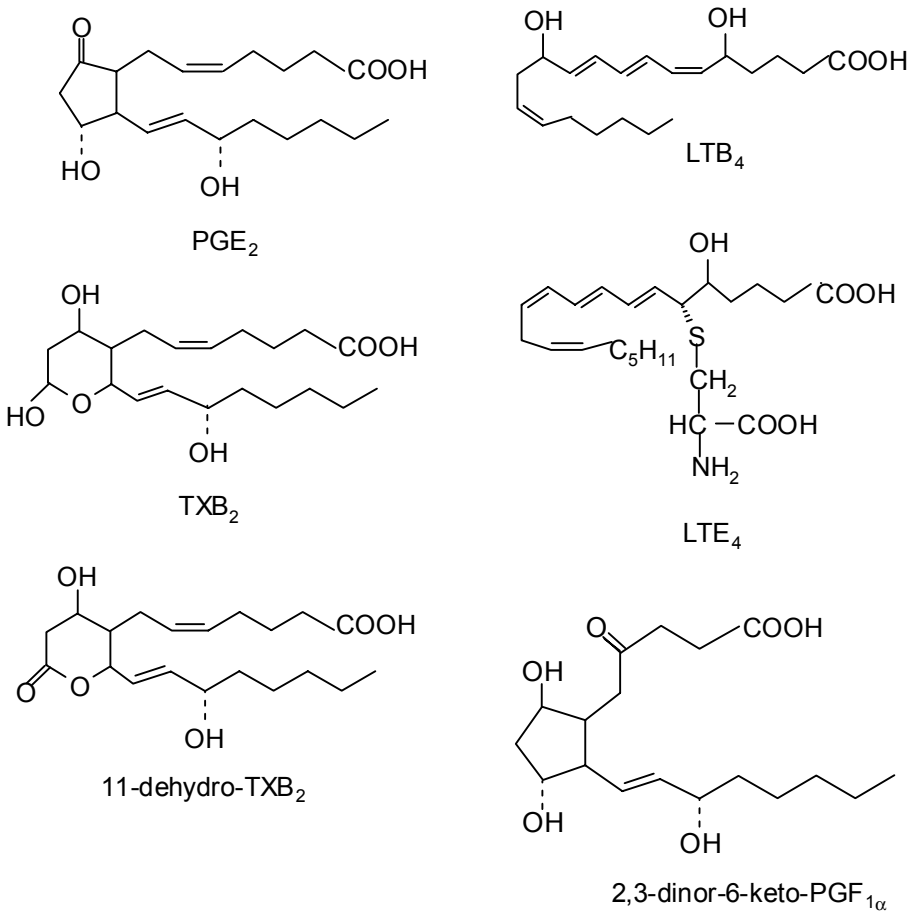


Figure 3. Structures of the eicosanoids measured in the present study.

1.6. Physiological and pathophysiological role of eicosanoids

The physiological effects of eicosanoids are well known, and it has been suggested that they are involved in the pathophysiology of many diseases. The main physiological effects of prostanoids and leukotrienes (for reviews, see Uotila and Vapaatalo 1984, Feuerstein and Hallenbeck 1987, Oates et al. 1988a, Oates et al. 1988b, Barnes and Smith 1999) are shown in Tables 1 and 2, respectively.

Table 1. Physiological effects of prostanoids.

PGD_2	Bronchoconstriction Platelet antiaggregation Vasodilation in systemic resistance vessels Pulmonary artery and vein constriction Uterus and gastrointestinal tract smooth muscle relaxation
PGE_2	Bronchodilation Gastric cytoprotection Enhancement of vascular permeability Inhibition of the release of proinflammatory mediators Vasodilation Platelet antiaggregation Uterus smooth muscle relaxation
$PGF_{2\alpha}$	Bronchoconstriction Increase of airway secretion Uterus smooth muscle contraction
PGI_2	Vasodilation Enhancement of fibrinolysis Platelet antiaggregation
TXA_2	Vasoconstriction Platelet aggregation Airway smooth muscle constriction

Table 2. Physiological effects of leukotrienes.

LTB_4	Chemotaxis Chemokinesis Leukocyte activation Plasma exudation Adhesion of neutrophils to vascular endothelium Secretion of lysosomal enzymes and superoxide anions
LTC_4, LTD_4, LTE_4	Smooth muscle contraction Bronchoconstriction Increase of microvascular permeability Increase of airway secretion

The effects of eicosanoids are mediated via different guanine nucleotide binding protein-linked receptors.

The receptors mediating the actions of prostanoids have been named according to the natural prostanoid for which they have the greatest apparent affinity. These receptors have recently been cloned. They include TXA_2, PGI_2, PGF, and PGD receptors, and four subtypes of PGE receptors, named EP_1, EP_2, EP_3 and EP_4, which all activate second messengers, such as cyclic adenosine monophosphate (cAMP). As yet there are no prostanoid receptor antagonists in routine clinical use (for reviews, see Negishi et al. 1995, Narumiya et al. 1999).

The effects of leukotrienes are mediated via leukotriene receptors. Cysteinyl leukotriene receptors $CysLT_1$ and $CysLT_2$ have been detected in the lungs (Gorenne et al. 1996); these may have subclasses (Rovati et al. 1997). $CysLT_1$ receptors have recently been cloned and characterized (Lynch et al. 1999). Since cysteinyl leukotrienes are involved in the pathogenesis of asthma, $CysLT_1$-selective receptor antagonists such as montelukast, pranflukast and zafirlukast and 5-LO inhibitors such as zileuton have been developed. Leukotriene receptor antagonists and leukotriene synthesis inhibitors reduce asthma symptoms induced by exercise, ASA, and allergen challenges, and reduce the need for adrenergic beta-agonists (for reviews, see Devillier et al. 1999a, Devillier et al. 1999b).

Synthetic derivatives of PGE_1 (e.g. misoprostol) and PGE_2 (e.g. enprostil) prevent and cure non-steroidal anti-inflammatory drug-induced injuries in stomach mucosa (Sontag et al. 1994, Koch 1999). Synthetic derivatives of PGE_2 with high affinity to uterus (sulproston, dinoproston, gemeprost) and synthetic analogues of $PGF_{2\alpha}$ (dinoprost) are used clinically to induce labor and for therapeutic abortion (Kanhai and Keirse 1989, Bygdeman et al. 1994, MacLennan et al. 1994).

The ratio between TXA_2 and PGI_2 is important in regulating platelet-vessel wall interaction and in thrombi formation (Moncada and Vane 1979). TXA_2 synthase inhibitors (Lefer et al. 1981), TXA_2/PGH_2 receptor antagonists (Schrör and Thiemermann 1986, Kotze et al. 1993, White et al. 1994), and combined type agents (De Clerk et al. 1989, Golino et al. 1990, Golino et al. 1993, Squadrito et al. 1993) have prevented thrombosis and reduced damages in myocardial ischemia in experimental investigations. In clinical investigations, substantial prevention of occlusive vascular diseases has been achieved with daily oral administration of low doses (50-300 mg) of ASA, blocking the TXA_2 formation in platelets (for reviews, see Viinikka 1990, Patrono 1994).

The first clinical application of PGI_2 administration in 1979 helped to improve circulation in patients with severe vascular disease (Szczeklik et al. 1979). PGI_2 and its stable analogs (e.g. cicaprost, epoprostenol, iloprost) have beneficial effects on atherosclerosis, myocardial ischemia, peripheral vascular disease, and thrombosis (Kaukinen et al. 1985, Sim et al. 1985, Ylitalo et al. 1990, Tanonaka et al. 1991, Grant and Goa 1992, Xin-Liang et al. 1992, Braun et al. 1993, Ranaut et al. 1993, Seiler et al. 1994).

Both TXA_2 and PGI_2 biosynthesis is increased in many pathophysiological conditions including myocardial infarction (Fitzgerald et al. 1986, Rebuzzi et al. 1992), peripheral vascular disease (Reilly and FitzGerald 1986, Knapp et al. 1988), pregnancy-induced hypertension (Ylikorkala et al. 1986, Minuz et al. 1988), sickle cell disease (Foulon et al. 1993), β-thalassemia major (Eldor et al. 1991), thrombocytopenic purpura (Rousson et al. 1989), and unstable angina (Fitzgerald et al. 1986). In addition, increased TXA_2 biosynthesis is associated with cerebral ischemia (Koudstaal et al. 1993), type II diabetes mellitus (Davi et al. 1993), homocysteinuria (Di Minno et al. 1993), type IIa hypercholesterolemia (Davi et al. 1992), polycytemia vera (Landolfi et al. 1992), and pulmonary hypertension (Christman et al. 1992).

Increased concentrations of LTB_4 are found in psoriasis (Brain et al.1984), in rheumatoid arthritis (Davidson et al. 1983), and in inflammatory bowel disease (Sharon and Stenson 1984). Increased synthesis of LTC_4, LTD_4, and LTE_4 has been reported in asthma (for a review, see Drazen 1998), juvenile rheumatoid arthritis (Fauler et al. 1994), in atopic dermatitis (Fauler et al. 1993), in hepatorenal syndrome (Moore et al. 1990), and in extrahepatic cholestasis (Mayatepek and Pecher 1993). Enhanced urinary excretion of 8-iso-$PGF_{2\alpha}$ has been described in association with cardiac reperfusion injury and cardiovascular risk factors, including cigarette smoking, diabetes mellitus, and hypercholesterolemia (Patrono and FitzGerald 1997). AA metabolites produced by the cyp 450-pathway have diverse physiological actions including vasodilation, vasoconstriction, inhibition of Na^+,K^+-ATPase and ion transport, and modulation of cell

growth. They may have a role in the pathogenesis of hypertension in spontaneously hypertensive rats; data for humans are limited. (Rahman et al. 1997)

2. NICOTINE AND SMOKING

Cigarette smoke contains several thousands of compounds in particulate and gas phases. These compounds include nicotine, nitric oxide (NO), carbon monoxide, phenols, hydrogen cyanide and polycyclic aromatic hydrocarbons (Hoffman and Wynder 1986), of which nicotine and carbon monoxide can be considered the major determinants in cardiovascular pathogenesis. Carbon monoxide is present in cigarette smoke in a proportion ranging from 2.7 to 6.0% of smoke (Cowie et al. 1973).

Nicotine is a natural liquid alcaloid, which was isolated from the leaves of tobacco plant, Nicotiana tabacum, in 1828 by Posselt and Reiman. Nicotine can also be obtained from the leaves of related species of tobacco plant (Solanaceae). Nicotine is a tertiary amine composed of a pyridine and pyrrolidine ring. It has two dissociation constants, one for the pyrrolidine (pKa 7.80) and the other for the pyridine nitrogen (pKa 2.97). Nicotine is a colorless, volatile base that turns brown (autooxidation) and acquires the odor of tobacco on exposure to air or light. It is water- and lipid-soluble and forms water-soluble salts.

Nicotine is the dependence-producing constituent of tobacco (Lee and D'Alonzo 1993). Nicotine dependence is associated with cigarette smoking, but it may also be associated with the use of nicotine substitution (Hatsukami et al. 1993, Benowitz and Henningfield 1994, Keenan et al. 1994, Kessler 1995, Slade et al. 1995).

Smoking is associated with cancer of the lungs, the cervix of uterus, oesophagus, larynx, bladder, pancreas and stomach, as well as with leukaemia (Wald and Hackshaw 1996). Smoking is a major risk factor of coronary and peripheral vascular disease, stroke and chronic obstructive lung disease (Ashton 1991, Wald and Hackshaw 1996). Considerable health hazards are also associated with maternal smoking during pregnancy and passive smoking (Lam 1989, Steenland 1992, DiFranza and Lew 1996, Law and Hackshaw 1996).

2.1. Pharmacokinetics of nicotine

Nicotine is readily absorbed to the arterial circulation from the respiratory tract, buccal and nasal mucosae (Temple 1976, Russell et al. 1983, West et al. 1984), the gastrointestinal tract (Malizia et al. 1983), skin (Gehlbach et al. 1974, Rose et al. 1984), and also the renal tubulae (Russell and Fayerabend 1978, Pilotti 1980), depending on the pH of the tissue and the nicotine delivery system (Benowitz 1988, Fiore et al. 1992). About 30% of nicotine is non-ionized at physiologic pH, which allows it to readily cross cell membranes (Benowitz 1992).

Pulmonary absorption of nicotine is extremely rapid, occurring at a rate similar to that after intravenous administration (Russell and Feyerabend 1978). Nicotine is rapidly absorbed when cigarette smoke, in which the (+)-isomer of nicotine is present in a proportion ranging from 3-12% (Klus and Kuhn 1977, Nwosu et al. 1988), is inhaled but negliaibly absorbed when the smoke is not inhaled (Haines et al. 1974). Nicotine crosses the blood-brain barrier, reaches the brain within 7-19 seconds and reaches peak blood concentrations within 5 minutes of smoking the cigarette (Schneider 1992, Rose 1996). Absorption from the stomach is limited at normal intragastric pH, whereas intestinal absorption is far more efficient. Depending on the brand, a cigarette contains 0.5-1 mg nicotine, which is well absorbed systemically. Absorption varies according to the intensity of inhalation and the smoker's technique (Benowitz and Henningfield 1994).

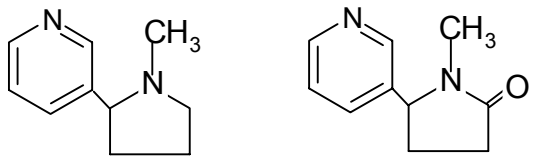
Cigarette smoking produces a background level of nicotine which builds up over the day. Superimposed on this are peaks corresponding to each cigarette (Schneider 1992), but these may not last for more than 30 minutes (Srivastava et al. 1990). Daytime blood and plasma levels of nicotine in habitual smokers are routinely maintained at a constant level of approximately 0.1 μM (Benowitz and Jacob 1985, Benowitz et al. 1987, Höfer et al. 1992).

The initial distribution half-life of nicotine is 7-10 minutes, and its elimination half-life ranges from 1 to 4 hours (Benowitz et al. 1982). Approximately 80 to 90% of nicotine is metabolized in the body, mainly in the liver. Part of the inhaled nicotine is metabolized by the lungs (Turner et al. 1975). Over 20 nicotine metabolites have been identified (for a review, see Kyerematen and Vesell 1991), the major mammalian metabolites being cotinine, nicotine-1-N-oxide and trans-3'-hydroxycotinine (Neurath et al. 1988, Benowitz et al. 1994, Benowitz and Jacob 1994). Cotinine has a more constant blood and plasma concentration of approximately 1.0 μM (Benowitz et al. 1987, Höfer et al. 1992), and a considerably longer half-life (about 20 hours) than that of the parent compound; no nicotine-like peak after smoking a cigarette can be seen (Benowitz et al. 1987). The profile of metabolites and the rate of metabolism (Benowitz and Jacob 1993)

do not differ between smokers and non-smokers. The concentrations of nicotine and cotinine are of course largely determined by cigarette consumption, the nicotine yield of the brand used and, to a lesser extent, by inhalation frequency.

Nicotine and its metabolites are eliminated by the kidney (Turner 1969). Renal excretion of unmetabolized nicotine accounts for 2-20% of total elimination, depending on urinary pH and urine flow (Beckett et al. 1972, Matsukura et al. 1979, Rosenberg et al. 1980, Benowitz and Jacob 1985).

Nicotine and cotinine can be measured in blood, saliva and urine (Benowitz 1983). The blood concentration of nicotine is an indicator of smoking over the last few hours, the cotinine concentration reflects the amount of smoking during the last week (Benowitz 1983).



Nicotine Cotinine

Figure 4. The structures of nicotine and cotinine.

2.2. Cardiovascular effects

Nicotine affects many organ systems in the body. Its effects are mediated via different guanine nucleotide binding protein-linked receptors. These nicotinic receptors exist in five subtypes, the M_1, M_2, M_3, M_4 and M_5 receptors (for a review, see Lindström et al. 1998).

Nicotine induces catecholamine release from adrenal medulla and primarily through this mechanism alters the function of the cardiovascular system by increasing heart rate, vascular resistance, stroke volume, cardiac output, myocardial contractility, blood pressure, and cardiac arrhythmia formation (Benowitz 1988, Benowitz 1991). These effects depend on the nicotine content of the cigarette and they can also be produced by intravenous nicotine administration (von Ahn 1960, Koch et al. 1980). It has been found that cotinine infusion in concentrations seen in moderately heavy smokers has no effect

on heart rate, blood pressure, or skin temperature (Benowitz et al. 1983). By contrast, cotinine infusion to abstinent smokers in concentrations similar to those commonly achieved during daily cigarette smoking has been reported to have psychological effects and also minimal effects on cardiovascular function (Keenan et al. 1994).

There are several mechanisms through which smoking may increase the risk of cardiovascular diseases. Smoking increases catecholamine levels and leads to vasoconstriction, especially in the presence of atherosclerosis (Hill and Wynder 1974, Winniford et al. 1986). Habitual smoking alters the lipid profile and increases white cell counts; increased hematocrit together with fibrinogen and platelet numbers imply an increased viscosity of blood (Kotamäki and Laustiola 1993, Netscher et al. 1995). The renin-angiotensin-aldosterone system is also activated in smokers (Laustiola et al. 1988a). Abstention from chronic smoking normalizes catecholamine levels, blood and plasma viscosities, hematocrit, plasma fibrinogen levels and total white cell count (Ernst and Martai 1987, Laustiola et al. 1991).

Chronic smoking leads to a lowered density of β_2-adrenoceptors in circulating lymphocytes (Laustiola et al. 1988b), and the blood pressure-lowering effect of β-adrenoceptor blockers is attenuated in cigarette smokers (Houben et al. 1981, Kotamäki et al. 1993). Monitored smoking cessation progressively increases lymphocytic β_2-adrenoceptor density; eight weeks after cessation the adrenoceptor density reaches that of nonsmokers (Laustiola et al. 1991). Both habitual (Mustard and Murphy 1963, Glynn et al. 1966, Hawkins 1972, Levine 1973) and acute smoking (Levine 1973, Grignani et al. 1977, Bierenbaum et al. 1978, Davis and Davis 1979) increase platelet aggregability. In habitual smokers platelet survival is shortened (Mustard and Murphy 1963, Glynn et al. 1966, Hawkins 1972, Levine 1973). Smoking induces platelet (Rival et al. 1987, Blache et al. 1992) and leukocyte (Bridges et al. 1993) activation, and activated leukocytes stimulate platelet function (de Gaetano et al. 1990). After exercise smokers' platelets are desensitized to adrenaline in vitro, which may be a consequence of continuous platelet activation during physical stress (Lassila and Laustiola 1988). Smoking-induced platelet and leukocyte activation is probably not affected by the NO of tobacco smoke, because there are observations indicating that NO is not absorbed from the inhaled smoke (Freeman et al. 1978, Rångemark and Wennmaln 1996).

The use of nicotine patches results in elevated noradrenaline levels (Netscher et al. 1995), but patches appear to produce less catecholamine release and platelet activation than smoking (Benowitz et al. 1993). It has been suggested that nicotine patches have no effect on vasopressin or fibrinogen concentrations, hematocrit, or white cell or platelet counts (Netscher et al. 1995). However, the use of nicotine chewing gum has been shown to counteract the decrease in lymphocyte counts after smoking cessation (Jensen et al. 1998). Some studies have reported that nicotine has no effect (Alster and

Wennmalm 1981), others suggest it enhances platelet aggregation in vitro (Cahao et al. 1983, Renaud et al. 1984). Platelets (Cahao et al. 1983) and leukocytes (Davies et al. 1982, Hoss et al. 1986) have been shown to possess high-affinity, non-cholinergic binding sites for nicotine.

Vascular endothelium is a principal target for the effects of risk factors in the early pathogenesis of atherosclerosis and cardiovascular diseases. It has been shown that smoking impairs endothelium-dependent dilation both in human arteries and veins (Celermajer et al. 1993, Lekakis et al. 1997, Moreno et al. 1998), even in the absence of atherosclerotic lesions (Zeiher et al. 1995). Smoking decreases NO bioactivity in vascular endothelium (Kiowski et al. 1994, Kugiyama et al. 1996) as well as platelet-derived NO release (Ichiki et al. 1996), and increases the sensitivity of the vascular smooth muscle to vasodilator stimuli (Rångemark and Wennmalm 1992). Cigarette smoking is also associated with reduced NO in the exhaled air of smokers (Persson et al. 1994, Kanazawa et al. 1996), whereas smoking cessation has been reported to increase the amount of exhaled NO (Robbins et al. 1997). As regards the mechanisms leading to endothelial dysfunction, cigarette smoke contains free radicals that may be responsible for endothelial cell injury (Powell 1998). Increased levels of F_2 isoprostanes in smokers indicate enhanced in vivo oxidative stress. Levels of F_2 isoprostanes have been shown to fall after two weeks' abstinence from smoking (Morrow et al. 1995). The increased plasma levels of oxidized low density lipoprotein observed in chronic smokers are related to the extent of endothelial dysfunction, raising the possibility that chronic smoking potentiates endothelial dysfunction by increasing circulating and tissue levels of oxidized low density lipoprotein (Powell 1998). Venous endothelial dysfunction seems to be rapidly reversible after smoking cessation (Moreno et al. 1998).

2.3. Nicotine replacement therapy

The pharmacologic actions of nicotine include pleasure, arousal, and relaxation. Smoking reduces anxiety and stress, relieves hunger, and prevents weight gain, and may also improve attention, reaction time, and the performance of individuals. Cessation of tobacco use is usually followed by a withdrawal syndrome, which includes a "craving" for tobacco, irritability, anxiety, restlessness, and concentration problems (Hughes et al. 1984, Benowitz 1988). Other symptoms include drowsiness, headaches, increased appetite and weight gain, sleep disturbances (insomnia), and gastrointestinal complaints.

Nicotine replacement therapy has been shown to reduce the severity of withdrawal symptoms (Thompson and Hunter 1998). Currently available forms of nicotine

substitution include chewing gum, patch, lozenge, nasal spray, oral inhaler, and sublingual tablet (Kunze et al. 1998, Fant et al. 1999). The long-term efficacy of nicotine replacement, however, is relatively low. Comparisons with placebo treatment show considerable benefits of nicotine substitution at 6 weeks, but verified abstinence rates at 12 months remain in the range of 5-20%. All therapies currently available appear to be equally effective, and concomitant behavioral or supportive therapy increases the quit rate. (for a review, see Silagy et al. 1999)

Nicotine chewing gum is available in 2 and 4 mg preparations, designed for users with different intensities of nicotine addiction. Nicotine gum does not produce the peak nicotine concentrations seen after smoking a cigarette (Benowitz et al. 1988, Srivastava et al. 1991). Average blood nicotine and cotinine concentrations in persons chewing nicotine gum giving 48 mg nicotine per day (i.e. 12-24 pieces of chewing gum) are 0.09 μM and 1.14 μM, respectively (Benowitz et al. 1987).

Nicotine patches are designed to be worn for 16 or 24 hours and to deliver a fixed dose of nicotine (total nicotine content from 5 to 22 mg) through the skin at a predetermined rate during the recommended wearing time. The steady state nicotine plasma concentration is achieved within 8-10 hours of applying the patch. Like nicotine gum, patches do not produce the peak nicotine concentrations seen after smoking a cigarette (Benowitz et al. 1988, Srivastava et al. 1991), but it may be possible to see fairly constant plasma nicotine concentrations (Palmer et al. 1992) comparable to moderate smoking or other smoking-cessation devices (Mulligan et al. 1990). In volunteers using nicotine patches giving 22 mg/day, serum nicotine and cotinine levels of 0.06 μM and 0.85 μM, respectively, have been reported (Hurt et al. 1993).

The nicotine nasal spray was developed to more closely emulate cigarette delivery and to improve the efficacy of replacement therapy, especially with strong nicotine addiction. Nicotine is rapidly absorbed from the nasal spray, the peak plasma concentration being achieved in about 11 to 13 minutes for 1 mg doses, which is slower than for smoking but faster than for other nicotine treatments. Venous plasma nicotine concentrations are generally lower than those in smokers. Venous plasma concentrations after a single dose range from 0.03 to 0.07 μM, but even concentrations ranging from 0.1 to 0.18 μM have been reported to occur during ad libitum self-administration. (Schneider 1996)

Since the method of delivering nicotine via an oral inhaler includes handling and active inhalation, it resembles smoking more closely than the other forms of nicotine substitution. However, the plasma concentration of nicotine does not rise as rapidly or to the same level as during smoking. When an oral inhaler is used ad libitum, the plasma nicotine concentration is about 0.04 - 0.07 μM, which is only one-third of the concentration observed during smoking (Lunell et al. 1997).

Nicotine substitution should be used with caution in patients with cardiovascular disease, and should not be used at all for patients with recent cerebrovascular disease, recent myocardial infarction, serious cases of arrhythmia or unstable angina pectoris. There are reports of cardiovascular events like myocardial infarction and stroke associated with the use of nicotine substitution (Dacosta et al. 1993, Jackson 1993, Pierce 1994, Warner and Little 1994, Arnaot 1995, Ottervanger et al. 1995, Riche et al. 1995), but in some studies nicotine replacement therapy was safely used by smokers with less severe cardiovascular disease (Working Group for the Study of Transdermal Nicotine in Patients with Coronary Artery Disease 1994, Joseph et al. 1996).

2.4. Potential beneficial effects of nicotine

The possible therapeutic uses of nicotine as a cholinergic agonist have been studied for many diseases, including Alzheimer's disease (van Duijn and Hofman 1992, Baldinger and Schroeder 1995, Snaedal et al. 1995, Wilson et al. 1995), Tourette's syndrome (McConville et al. 1992, Silver and Sanberg 1993, Dursun et al. 1994), extrapyramidal disorders of schizophrenic patients (Anfang and Pope 1997), spastic dystonia (Vaughan et al. 1997), Parkinson's disease (for a review, see Newhouse et al. 1997), and ulcerative colitis (Pullan et al. 1994, Thomas et al. 1996, Sandborn et al. 1997), but the results are inconclusive. The possible beneficial effects of nicotine may be important in clarifying the mechanisms of different diseases and in this way help to develop therapeutic interventions; they cannot be used as a rationale for cigarette smoking.

2.5. Effects of nicotine and smoking cessation on eicosanoid synthesis

The effects of nicotine and smoking on AA metabolism have been intensively studied both in vitro and in vivo in various animal species including rabbit, rat and hamster. However, there is only limited research evidence on the effects of smoking and nicotine on AA metabolism, especially on its 5-LO pathway, in man. Knowledge about the effects of cotinine is even more limited, and the effects of (+)-nicotine are unknown.

2.5.1. Nicotine in vitro

Nicotine has been found to inhibit PG synthesis in human kidney microsomes (Alster et al. 1983), but to have no effect on PGE_2 formation in macrophage-like cells (Goerig et al. 1992). In these studies exogenous arachidonic acid was included in incubations. In human umbilical vein endothelial cells stimulated with a calcium ionophore A23187, concentrations of nicotine comparable to the plasma levels of smokers did not affect the release of endothelial PGE_2 (Bull et al. 1988), whereas in lipopolysacharide-stimulated peripheral blood monocytes – i.e. in a model reflecting PGHS II activity - nicotine potentiated PGE_2 release (Payne et al. 1996). Nicotine at concentrations achievable in the lungs and oral tissues of smokers, i.e. several times higher than those in plasma, enhanced PGE_2 generation in concanavalin A–stimulated neutrophils (Seow et al. 1994). The differences found in the effects of nicotine on PGE_2 production may be due to the different stimuli used or the inclusion of exogenous arachidonic acid in incubations. The differences may also be due to tissue differences of cyclooxygenase (Alster et al. 1983).

There are some discrepancies in the evidence concerning the effect of nicotine on endothelial PGI_2 synthesis. It has been reported that nicotine inhibits PGI_2 production in human peripheral vein (Sonnenfeld and Wennmalm 1980), in umbilical arteries from newborn infants (Stoel et al. 1982) and in human endocardium (Alster et al. 1986). In human umbilical vein endothelial cells, nicotine in concentrations comparable to the plasma levels of smokers has been found to have no effect on PGI_2 formation (Bull et al. 1988). The same observation has been made in human umbilical arteries (Jeremy et al. 1985, Ylikorkala et al. 1985), whereas in primary cultures of human umbilical vein endothelial cells, nicotine increased PGI_2 production (Boutherin-Falson and Blaes 1990). The disparities in the literature may be due to differences in experimental conditions and in the nicotine concentrations applied (Bull et al. 1988, Boutherin-Falson and Blaes 1990).

In human platelets nicotine neither induced synthesis of TX in human platelets, nor affected the platelet synthesis of TX induced by AA (Wennmalm 1978). In platelet microsomes neither nicotine nor cotinine affected TXA_2 synthesis (Alster and Wennmalm 1981), and in platelets nicotine did not affect TX formation (Wennmalm and Alster 1983). However, in fetal platelets nicotine concentration-dependently inhibited platelet TXA_2 synthesis both after platelet-aggregation and stimulation with exogenous AA (Ylikorkala et al. 1985). Nicotine inhibited TXB_2 production during aggregation in platelet-rich plasma (PRP) and during whole blood clotting (Toivanen et al. 1986). Nicotine inhibited the formation of TX in promyelocytic leukaemia cells (HL-60) induced to differentiate into macrophages (Goerig and Habenicht 1988) and in macrophage-like cells (Goerig et al. 1992). Nicotine has been shown to selectively inhibit TX synthase at concentrations that are readily observed in the circulation of smokers (Goerig et al. 1992).

There is only limited knowledge about the effects of nicotine on the synthesis of LO-pathway products. In macrophage-like cells, with exogenous AA included in incubations, nicotine did not affect LTB_4 formation (Goerig et al. 1992). However, in calcium ionophore A23187 (A23187)-stimulated human neutrophils nicotine at concentrations achievable in lung and oral tissues of smokers increased LTC_4 production (Seow et al. 1994).

2.5.2. Cigarette smoke and smoking

The effects of smoking on PGI_2 and TXA_2 synthesis have been quite intensively studied, but as yet there are only limited data on the effects of smoking on other prostanoids and especially on leukotrienes in man. The role of the different components of tobacco and cigarette smoke in AA metabolism has been studied by using nicotine-free cigarettes, ethanolic and aqueous cigarette smoke extracts and cigarette smoke condensates.

In human umbilical artery ethanolic and aqueous cigarette smoke extracts have been found dose-dependently to inhibit the release of PGI_2 and the in vitro conversion of AA into PGI_2 (Jeremy et al. 1985). In cultured human endothelial cells cigarette smoke condensate inhibits basal, phorbol myristate acetate- and thrombin-induced PGI_2 production, but does not affect the production of PGI_2 from exogenous AA (Reinders et al. 1986). In vitro umbilical arteries of smokers' infants were found to produce significantly less PGI_2-like activity than those of controls (Ahlsten et al. 1986, Ahlsten et al. 1990). It has been reported that the production of various PGs is enhanced in the

bronchoalveolar lavage fluid of smokers, indicating increased COX activity (Zijlstra et al. 1992).

When nicotine-containing tobacco smoke is inhaled, urinary excretion of 6-keto-$PGF_{1\alpha}$ is reduced in smokers but not in non-smokers (Nadler et al. 1983). Smoking women show lower basal 6-keto-$PGF_{1\alpha}$ excretion than non-smoking women, and the excretion is further reduced after smoking four high-nicotine cigarettes in smokers using oral contraceptives (Mileikowsky et al. 1988). However, neither smoking (Wennmalm et al. 1990, Wennmalm et al. 1991, Benowitz et al. 1993) nor the regular use of oral snuff (Wennmalm et al. 1991) has an effect on 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion, although in some studies chronic smoking has been associated with increased biosynthesis of PGI_2 measured as urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Nowak et al. 1987, Lassila et al. 1988, Murray et al. 1990). It has been suggested that the increased PGI_2 production is a compensatory mechanism of healthy endothelium for the general vasoconstrictive properties of cigarette smoking (Lassila et al. 1988). The age of the subjects and possible underlying vascular injuries may alter PGI_2 synthesis, which may partly explain the different observations in the literature.

Ex vivo, serum concentrations of TXB_2 are increased immediately after smoking (Toivanen et al. 1986). Apparently healthy habitual smokers show increased urinary excretion of TXB_2 (Uedelhoven et al. 1991), 11-dehydro-TXB_2 (Uedelhoven et al. 1991, Rångemark et al. 1993) and 2,3-dinor-TXB_2 (Nowak et al. 1987, Lassila et al. 1988, Barrow et al. 1989, Wennmalm et al. 1990, Uedelhoven et al. 1991, Wennmalm et al. 1991). Lassila and co-workers (1988) studied monozygotic pairs of male twins whose only apparent discordant factor was smoking. As the genetic effects were controlled for, these subjects offered a unique opportunity to evaluate the effects of smoking on systemic eicosanoid synthesis. The increased urinary excretion of TXA_2 metabolites reflects augmented TX biosynthesis by activated platelets (Nowak et al. 1987). Thus, a 20 mg dose of ASA, known to selectively inhibit platelet COX, has been reported to abolish the difference in the excretion of 2,3-dinor-TXB_2 between smokers and non-smokers (Nowak et al. 1987). However, the exact mechanism of platelet activation during cigarette smoking is not known. The increased platelet activation in chronic smokers may be linked to smoking-induced vascular injury (Nowak et al. 1987), but cigarette smoking also influences platelet function directly (Wennmalm et al. 1991) through some constituent(s) of cigarette smoke. One potential candidate is nicotine.

There has been only very little research into the effects of smoking on LO-pathways in man. LTB_4 serum concentrations are higher in smokers than in non-smokers (Thornton et al. 1989), and it has been shown that acute smoking increases blood levels of LTC_4, LTD_4 and LTE_4 (Kobayashi et al. 1988). Urinary excretion of LTE_4 has been reported to be higher in smokers than in non-smokers and to correlate with the number

of cigarettes smoked daily (Fauler and Frölich 1997). In non-smokers, smoking six cigarettes within 12 hours results in a twofold increase in LTE_4 excretion (Fauler and Frölich 1997).

2.5.3. Smoking cessation, nicotine administration and nicotine substitution

The effects of smoking cessation on eicosanoid synthesis in man are not well established, and those of nicotine substitution on AA metabolism are largely unknown.

It has been reported that smoking cessation decreases the excretion of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2, but has no effect on the excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Rångemark et al. 1993).

As regards the effect of nicotine, snuff users, in contrast to cigarette smokers, display no increase in urinary excretion of 2,3-dinor-TXB_2, despite a comparable nicotine exposure as assessed by urinary cotinine level (Wennmalm et al. 1991). In a crossover study comparing the effects of cigarette smoking, transdermal nicotine and placebo transdermal nicotine (Benowitz et al. 1993), transdermal nicotine had no effect on the urinary excretion of 11-dehydro-TXB_2, even though the plasma levels of nicotine were nearly within the same range as those observed during smoking. Excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was not significantly influenced by either of the treatments. In non-smokers transdermal nicotine has been reported to have no effect on urinary LTE_4 excretion (Fauler and Frölich 1997).

Nicotine infusion to healthy non-smokers has been found to produce a weak platelet stimulation that is not accompanied by a significant change in urinary 2,3-dinor-TXB_2 excretion (Nowak et al. 1996). In this study the plasma nicotine concentration achieved during infusion was about 60% of the level that smoking one cigarette or chewing a 4 mg nicotine gum would yield (Benowitz 1988).

3. NICOTINIC ACID

Nicotinic acid (pyridine-3-carboxylic acid, pKa = 4.85), also known as niacin, is a water soluble B group vitamin. The daily human requirement of nicotinic acid is about 15 to 20 mg. Nicotinic acid deficiency causes a syndrome called pellagra (Italian for Pelle agra, or "rough skin") (Goldsmith 1958), which is characterized by skin lesions,

gastrointestinal symptoms and neurological disturbances. Nowadays nicotinic acid deficiency is rare but it may occur in chronic alcoholics, for instance. Nicotinic acid deficiency usually occurs simultaneously with other vitamin B-complex deficiencies.

3.1. Pharmacokinetics

Nicotinic acid is absorbed rapidly from the gastrointestinal tract and widely distributed in the body tissues. Plasma levels of free nicotinic acid reach a peak in 30-60 minutes, and its half-life is also short (Weiner 1979, Cayen 1985). In the body, nicotinic acid functions after conversion into either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). NAD and NADP are involved in electron transfer reactions in the respiratory chain. Nicotinic acid is metabolized to N-methylnicotinamide and 2-pyridone and 4-pyridone derivatives. These metabolites together with nicotinuric acid, unchanged nicotinic acid and nicotinamide are excreted in urine. With larger doses the amount excreted unchanged is increased. (Weiner 1979, Cayen 1985)

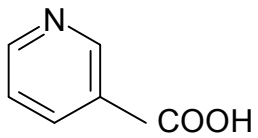


Figure 5. The structure of nicotinic acid.

3.2. Nicotinic acid in therapy

The hypolipidemic properties of nicotinic acid were discovered in 1955 by Altschul and coworkers. These properties are not related to its role as a vitamin. In pharmacological doses of 1-8 mg per day, nicotinic acid favourably affects all plasma lipid subtypes: it lowers plasma total and low density lipoprotein-cholesterol concentrations, lipoprotein$_a$ and triglycerides, and increases plasma high density lipoprotein-cholesterol (Grundy et al. 1981). Nicotinic acid has been shown to reduce mortality and non-fatal myocardial infarction, and, in combination with bile-acid binding resins, to prevent or cause

regression of atherosclerotic plaques (Canner et al. 1986, Blankenhorn et al. 1987, Brown et al. 1990).

The major drawbacks of nicotinic acid are its associated adverse effects, of which dermal (flushing) and gastrointestinal reactions are most common. Nicotinic acid derivatives (e.g. acipimox) were developed to overcome the problem of hindered compliance due to the side effects of nicotinic acid. They also have more prolonged effects on lipoprotein metabolism (Hotz 1983).

As a vitamin, nicotinic acid and its derivatives are used in the prophylaxis and in the treatment of pellagra.

3.3. Nicotinic acid and eicosanoid synthesis

Studies concerning the effect of nicotinic acid on eicosanoid synthesis originated in the observation that niacin-induced flushing was substantially attenuated by pre-treatment with ASA, suggesting that the vasodilation is mediated by a PG. Nicotinic acid infusion has been shown to increase the release of PGE (Eklund et al. 1979), whereas ingestion of 500 mg nicotinic acid produced no change in the excretion of the major urinary metabolite of PGE_2 (Morrow et al. 1989). It has been demonstrated that flushing is primarily due to the release of PGD_2 (Morrow et al. 1989, Morrow et al. 1992, Awad et al. 1994) from a nicotinic acid responsive cell that resides in the skin (Morrow et al. 1992).

Nicotinic acid increases the production of 6-keto-$PGF_{1\alpha}$ in human whole blood stimulated with exogenous and endogenous AA substrate (Pattison et al. 1987). Urinary excretion of 6-keto-$PGF_{1\alpha}$ (Olsson et al. 1983) and that of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Morrow et al. 1989, Edlund et al. 1990) are both modestly elevated after niacin ingestion.

To the best of our knowledge there has been no earlier research into the effects of nicotinic acid on TX synthesis and on 5-LO-catalyzed AA metabolism.

4. PYRIDOXINE

In 1926 Goldberger and Lillie showed that a diet deficient in vitamin B_2 caused dermatitis in rats. In 1934, however, György distinguished the water-soluble factor whose deficiency was responsible for the dermatitis from the diet deficient in vitamin B_2; he called it vitamin B_6. Vitamin B_6 was isolated in 1938 (György, Ichiba and Michi, Keresztesy and Stevens, Kuhn and Wendt, Lepkovsky) and its structure was demonstrated in 1939 (Harris and Folkers, Kuhn et al.). In 1939 György and Eckhartd called vitamin B_6 pyridoxine. Pyridoxal and pyridoxamine, both of which possess the same biological properties, were found in the 1940s (Snell et al. 1942, Snell 1944, Snell 1945). Vitamin B_6 is a water-soluble B-complex vitamin and the generic descriptor for all 3-hydroxy-2-methylpyridine derivatives possessing the biological activity of pyridoxine. The three forms of vitamin B_6 are pyridoxine (the alcohol), pyridoxamine (the amine) and pyridoxal (the aldehyde). These forms differ in the nature of the substituent on the carbon atom in position 4 of the pyridine nucleus. These compounds may also be phosphorylated.

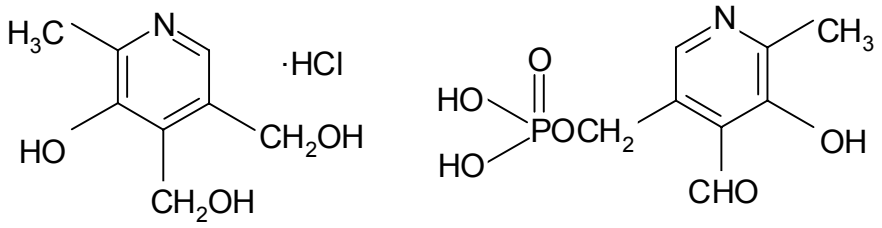
The human daily requirement of vitamin B_6 is about 1.5 to 2 mg which is easily covered by the normal western diet. Pyridoxine deficiency is rare, but it may be induced by drug use during isoniazid therapy, for instance. Pyridoxine at a dose of 200 mg/day may cause neurotoxicity (Schaumburg et al. 1983, Parry and Bredesen 1985). Sensory peripheral neuropathies occur when very high doses of pyridoxine are administered, but they may also develop with lower doses over a longer period of time (Berger and Schaumburg 1984).

4.1. Pharmacokinetics

In mammalian organisms pyridoxine, pyridoxamine and pyridoxal are absorbed from the gastrointestinal tract and converted into pyridoxal phosphate and pyridoxamine phosphate. They are stored mainly in the liver.

Pyridoxal-5'-phosphate is the biologically active coenzyme form of vitamin B_6, and represents at least 60% of vitamin B_6 in human plasma (Lumeng et al. 1980, Chrisley et al. 1988, Driskell and Chrisley 1991), where it is nearly completely bound to albumin (Anderson et al. 1974, Whyte et al. 1985). Pyridoxal-5'-phosphate functions as a coenzyme in over 100 different reactions (Sauberlich 1985). It is involved principally in amino acid metabolism but also in carbohydrate and fat metabolism.

The oxidation of pyridoxal phosphate and pyridoxamine phosphate into 4-pyridoxic acid and into other inactive metabolites occurs in the liver. Renal elimination is the main pathway for the excretion of vitamin B_6. 4-Pyridoxic acid is the principal excretory product of all three forms of the vitamin (Zempleni and Kübler 1995).



Pyridoxine hydrochloride Pyridoxal-5'-phosphate

Figure 6. The structures of pyridoxine hydrochloride and pyridoxal-5'-phosphate.

4.2. Pyridoxine in therapy

Pyridoxine is used for the treatment of pyridoxine deficiency, sideroblastic anaemia, and certain rare enzyme defects such as homocysteinuria (Boers et al. 1985) and primary hyperoxaluria (particularly type I) (Alinei et al. 1984, de Zegher et al. 1985, Bässler 1988, Milliner et al. 1994) and homocysteinemia (Brattström et al. 1990). Pyridoxine is commonly used to prevent isoniazid-induced peripheral neuropathy with high isoniazid doses, and it may also prevent seizures caused by isoniazid toxicity.

Pyridoxine has also been tried in the treatment of many other disorders, including alcoholism (Lumeng and Li 1974, Veitch et al. 1975, Labadarios et al. 1977, Mardel et al. 1994), chronic hemodialysis (Teehan et al. 1978, Kleiner et al. 1980, Kriley and Warady 1991), nausea and morning sickness in pregnancy (for a review, see Aikins Murphy 1998), premenstrual syndrome (for a review, see Wyatt et al. 1999) and essential hypertension (Aybak et al. 1995), but the results are inconclusive.

Pyridoxal-5'-phosphate has been shown to inhibit platelet aggregation both in vitro and following administration to man (Subbarao et al. 1977, Kornecki and Feinberg 1978, van Wyk et al. 1992), and polymorphonuclear leukocyte (PMN) aggregation in vitro (Schinella et al. 1995).

4.3. Pyridoxine and eicosanoid synthesis

Only limited data are available on the effects of pyridoxine and pyridoxal-5'-phosphate on the PGHS pathway of AA metabolism in man. In human platelets pyridoxal 5'-phosphate has been shown to inhibit TXB_2 generation induced by adenosine diphosphate (ADP), thrombin, adrenaline and platelet activating factor (PAF), but not that induced by AA (Krishnamurthi et al. 1982). On this basis it was suggested that pyridoxal-5'-phosphate may inhibit AA release via PLA_2 inhibition rather than AA metabolism.

Pyridoxine in prolonged administration (200 mg/day for 2 weeks) does not decrease TXB_2 production ex vivo (van Wyk et al. 1992). The effects of pyridoxine and pyridoxal-5'-phosphate on the 5-LO pathway remain unexplored.

AIMS OF THE STUDY

The main purpose of this study was to investigate the effects of smoking and nicotine on eicosanoid synthesis in man. Since nicotinic acid and pyridoxine have a pyridine moiety in common with nicotine, these compounds were included in this study to compare their effects on arachidonic acid metabolism with those of nicotine.

More specifically, the aims were:

1) To clarify the actions of nicotine stereoisomers, cotinine, nicotinic acid, pyridoxine and pyridoxal-5'-phosphate on prostanoid and leukotriene synthesis in vitro (I, II, IV);

2) To investigate the effects of nicotinic acid and pyridoxine on prostanoid and leukotriene synthesis ex vivo (IV) and in vivo (unpublished results) and to compare these effects to those of nicotine; and

3) To evaluate the effects of smoking, smoking cessation and nicotine substitution on prostanoid and leukotriene synthesis ex vivo (III) and in vivo (V).

MATERIALS AND METHODS

1. CHEMICALS AND DRUGS

Calcium ionophore A23187 (calcimycin), (-)-cotinine, (+)-nicotine, pyridoxine hydrochloride and pyridoxal-5'-phosphate were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). (-)-Nicotine was obtained from BDH Chemicals (Poole, England). Nicotinic acid was obtained from E. Merck (Darmstadt, Germany). Heparinized glass vacutainer tubes (143 U.S.P. heparin) were from Becton Dickinson (Meylan Cedex, France). Sustained-release nicotinic acid (Lipolyt retard®) and pyridoxine hydrochloride (Heksavit®) for the ex vivo study were from Leiras Pharmaceuticals (Turku, Finland). Nicotine chewing gum (Nicorette®) was from Kabi Pharmacia (Sweden) and nicotine patches (Nicotinell®) from Ciba-Geigy AG (Switzerland).

2. VOLUNTEERS

The whole blood used for PMNs and PRP isolation (I) and for in vitro incubations (II) was from the staff of our laboratory. The healthy volunteers in the nicotinic acid and pyridoxine study were medical students or medical doctors (IV), and those in the smoking cessation and nicotine substitution study staff of the Medical School and members of voluntary smoking cessation groups (III, V).

In (III), (IV) and (V) the study protocols were approved by the Ethical Committee of Tampere University Hospital and informed consent was obtained from the subjects. In all the studies the volunteers had abstained from all drugs for at least 14 days before sampling, and avoided any major changes in diet or other living habits during the study.

3. STUDY DESIGNS

3.1. Smoking cessation and nicotine substitution

Sixty healthy smoking volunteers, all of whom had smoked for several years (38 males and 22 females, age 20-45 years) 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 (8-9 a.m.) and 12 h overnight urines collected from all the subjects before the trial (day 0) and on days 3, 7 and 14 after smoking cessation.

After smoking cessation, the 60 subjects were allowed to choose whether they wanted to continue without nicotine substitution (smoking cessation group, n=15; 7 males and 8 females), use nicotine chewing gum (nicotine chewing gum group, n=15; 12 males and 3 females) or 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/24 h or 21 mg/24 h) ad libitum (II, V).

3.2. Nicotinic acid and pyridoxine administration

Eight healthy non-smoking male volunteers (age 25-40 years) participated in the nicotinic acid study, and 8 healthy non-smoking male volunteers of the same age took part in the pyridoxine study. Control blood samples were taken from all volunteers after 12 h fasting in the morning (8-9 a.m.) of the first day of the study and 12 h overnight urines collected from all subjects before drug administration. In the nicotinic acid study, 1000 mg nicotinic acid orally was administered at 7 p.m., 500 mg at 10 p.m., and 1000 mg at 7 a.m. the next morning. Blood samples were taken in the morning (8-9 a.m.) after the last dose of nicotinic acid. Twelve hour overnight urines were collected during drug administration between 7 p.m. and 7 a.m. In the pyridoxine study the drug was administered in a 300 mg dose orally to the volunteers twice daily for 7 days. Blood samples were taken in the morning (8-9 a.m.) after the last dose (IV). Twelve hour overnight urines were collected during the night (from 7 p.m. to 7 a.m.) before the last pyridoxine dose.

4. IN VITRO AND EX VIVO EXPERIMENTS

In vitro and ex vivo experiments were performed to clarify the effects of the test compounds and smoking on the synthesis capacity of different eicosanoids. The experimental conditions used in vitro and ex vivo were found to be optimal on the basis of preliminary studies.

4.1. Concentration ranges of the drugs used in vitro

The concentration ranges of the drugs used in whole blood incubations (II, IV) are given in Table 3.

Table 3. Concentration ranges of the drugs used in whole blood in vitro.

Compound	Whole blood
(-)-Nicotine	0.0005-500 μM
(+)-Nicotine	0.0005-50 μM
Cotinine	0.0005-500 μM
Nicotinic acid	10-1000 μM
Pyridoxine	0.1-500 μM
Pyridoxal-5'-phosphate	0.1-500 μM

The concentrations of (-)-nicotine and cotinine in PMN and PRP incubations (I) were the same as those tested in whole blood.

4.2. Experimental procedure for polymorphonuclear leukocytes

PMN isolation. Venous blood was collected after 12 h fasting in the morning (8-9 a.m.). PMNs were isolated from freshly drawn whole blood by means of Ficoll-Paque density gradient centrifugation. Red cells were removed by dextran sedimentation followed by lysis of the few remaining red cells. After the isolation procedure, the purity of PMNs

was > 98 %; the PMNs were contaminated with less than 2 % mononuclear cells. Viability as determined by Trypan blue was > 98 % (I).

PMN incubation. 5 x 10^6 PMNs were preincubated in 465 µl of Dulbecco's phosphate-buffered saline for 15 min at 37°C, and for a further 15 min with the test compound. Eicosanoid synthesis was triggered by A23187 (0.5 µM, 5 min, 37°C) and the reaction was stopped by centrifugation (10 000 x g, 2 min, 4°C) (I).

4.3. Experimental procedure for platelet rich plasma

PRP preparation. Venous blood was collected after 12 h fasting in the morning (8-9 a.m.). PRP was obtained from freshly drawn venous blood by centrifugation at 200 x g for 20 min to obtain platelet rich plasma, which was adjusted to a final concentration of 15 x 10^6 platelets / 100 µl with autologous platelet poor plasma (I).

PRP incubation. 15 x 10^6 platelets were preincubated with 365 µl of Dulbecco's phosphate buffered saline for 15 min at 37ºC and for a further 15 min with the test compound as for polymorphonuclear leukocytes. Eicosanoid synthesis was triggered by A23187 (10 µM, 60 min, 37ºC) and the reaction was stopped by centrifugation (10 000 x g, 2 min, 4°C) (I).

4.4. Experimental procedure for whole blood

Whole blood collection. Freshly drawn venous blood was collected after 12 h fasting in the morning (8-9 a.m.) into 10 ml heparinized glass vacutainer tubes (II, III, IV).

Whole blood incubation. 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 x g, 10 min, +4°C) (II, III, IV).

5. EICOSANOID ASSAYS

5.1. Radioimmunoassays

5.1.1. Platelet rich plasma, polymorphonuclear leukocytes and whole blood samples

PGE_2 determination. PGE_2 concentration was determined from the PMN incubation medium and from plasma by direct RIA using ^{125}I-PGE_2 and antisera from the Institute of Isotopes Co. (Budapest, Hungary) (I, II, III, IV). The drugs included in the study were checked for cross-reactivity either at the highest concentration (I, III) or at all concentrations (II, IV) employed. No interference was found. For the cross-reactivity values of the antibody used in PGE_2-radioimmunoassay, the detection limits, and the intra- and interassay coefficients of variation, see study III.

TXB_2 determination. TXB_2 concentrations from platelet-rich plasma 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) (I, II, III IV). The compounds included in the study were checked for cross-reaction at the highest concentration (I, III) or at all concentrations (II, IV) employed, and no interference was found. For the cross-reactivity values of the antibody used in TXB_2 radioimmunoassay, the detection limits, and the intra- and interassay coefficients of variation, see the study III.

LTE_4 determination. Cysteinyl leukotriene formation was determined as LTE_4-like immunoreactivity from plasma by direct RIA in a cross-reactive 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. The compounds included in the study were checked for cross-reaction at the highest concentration (III) or at all concentrations employed (II, IV), and no interference was found. For the cross-reactivity values of the antibody used in LTE_4 radioimmunoassay, the detection limits, and the intra- and interassay coefficients of variation, see the study III.

5.1.2. Urine samples

Urinary 11-dehydro-TXB$_2$. 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 (both from the Institute of Isotopes Co.) and nonlabeled 11-dehydro-TXB$_2$ (Cayman Chemical Co., Ann Arbor, MI) (V). The cross-reactivity values of the antibody used in the 11-dehydro-TXB$_2$ radioimmunoassay were as follows: 11-dehydro-TXB$_2$ 100%, and for other compounds tested less than 0.5% (see Riutta et al. 1992) (V).

Urinary 2,3-dinor-6-keto-prostaglandin F$_{1\alpha}$. After selective two-step solid-phase extraction on C$_1$-silica cartridges (Applied Separations, Allentown, PA, USA) 2,3-dinor-6-keto-PGF$_{1\alpha}$ RIA was carried out using antiserum raised against 6-keto-PGF$_{1\alpha}$ with 100% cross-reaction with 2,3-dinor-6-keto-PGF$_{1\alpha}$ and 6-[^{125}I]keto-PGF$_{1\alpha}$-tyrosine methyl ester as labeled radioligand (both from the Institute of Isotopes Co.) and non-labeled 2,3-dinor-6-keto-PGF$_{1\alpha}$ (Cayman Chemical Co.). The cross-reactivity values of the antibody used in the 6-keto-PGF$_{1\alpha}$-radioimmunoassay were as follows: 2,3-dinor-6-keto-PGF$_{1\alpha}$ 100%, 6-keto-PGF$_{1\alpha}$ 100%, and for other compounds tested less than 3% (see Riutta et al. 1994) (V).

Urinary LTE$_4$. Urinary leukotriene E$_4$ was measured by direct enzyme immunoassay using a commercial kit (Cayman Chemical Co.). Urine samples were added to microtiter plates at least in three serial dilutions in duplicate. The specificity of the assay was as follows: for LTE$_4$ 100%, LTE$_5$ 100%, N-acetyl-LTE$_4$ 20%, LTC$_4$ 10%, LTD$_4$ 9%, LTC$_5$ 5%, LTD$_5$ 4%, and for LTs B$_4$ and B$_5$ <0.01%. The intra- and interassay coefficients of variation were both below 10%. (V)

5.2. High performance liquid chromatography

LTB$_4$ was measured after solid phase extraction on Bond Elut C$_8$ silica cartridges (Varian, Harbor City, CA, USA) 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$_2$ and LTB$_4$ were monitored at 271 nm (Alanko et al. 1991). (I, III)

6. OTHER ASSAYS

Urinary creatinine. In order to avoid differences due to variations in diuresis, urinary creatinine was determined spectrophotometrically by the picric acid method using a commercial assay kit (Orion, Espoo, Finland). The excretion of urinary eicosanoids, thiocyanate, cotinine and trans-3'-hydroxycotinine was correlated to creatinine excretion (III, V).

Smoking cessation and the use of nicotine chewing gum were monitored by determining thiocyanate and the main metabolites of nicotine, cotinine and trans-3'-hydroxycotinine concentrations 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 (III, V).

Urinary thiocyanate. Urinary thiocyanate concentrations were measured spectrophotometrically (Giraudi and Grillo 1981). (V).

Urinary cotinine and trans-3'-hydroxycotinine. Urinary cotinine and trans-3'-hydroxycotinine were determined by RP-HPLC (Parviainen and Barlow 1988). (III, V).

7. STATISTICS

The results are given as means ± SEM. In communications I, II, III and IV, paired t-test was used to analyze statistical significances of the changes within the study groups. In study V comparisons between basal eicosanoid synthesis in smokers and non-smokers were based on the t-test. In pairwise comparisons within the treatment groups (V, unpublished), statistical analysis was based on ANOVA for repeated measures, supported by Bonferroni confidence intervals. One-way ANOVA was used for comparisons between the treatment groups. Because there is substantial interindividual variation in eicosanoid production, each volunteer served as his/her own control. The data were analyzed with BMDP Statistical Software (Los Angeles, CA, USA).

RESULTS

1. THE EFFECTS OF THE TEST COMPOUNDS IN VITRO

Under basal, unstimulated conditions, PMNs, PRP and whole blood did not release detectable amounts of PGE_2, TXB_2, LTB_4 and LTE_4 (I, II, IV).

Table 4. A23187-stimulated eicosanoid production (mean ± SEM) in samples from healthy non-smoking volunteers.

	10^6 PMNs 10^6 platelets (I, n = 6)	1 ml whole blood (II, n = 6)	1 ml whole blood (III, n = 15)	1 ml whole blood (IV, n = 8 in both groups*)
PGE_2	146 ± 10 pg	2.8 ± 0.4 ng	3.6 ± 0.4 ng	2.4 ± 0.5 ng 2.6 ± 0.4 ng
LTB_4	9.6 ± 0.6 ng	15.2 ± 3.1 ng	18.8 ± 3.6 ng	23.6 ± 4.2 ng 18.2 ± 3.8 ng
LTE_4	not measured	58.9 ± 11.8 ng	16.1 ± 3.1 ng	38.7 ± 7.0 ng 61.7 ± 10.7 ng
TXB_2	422 ± 35 pg	97.2 ±11.6 ng	61.4 ± 8.2 ng	114.0 ± 24.1 ng 110.7 ± 22.0 ng

* Groups receiving nicotinic acid and pyridoxine, respectively.

1.1. Effects of nicotine stereoisomers and cotinine on eicosanoid synthesis

Nicotine stereoisomers and cotinine were able to modulate eicosanoid synthesis in vitro (I, II).

(-)-Nicotine stimulated PGE_2 production in PMNs (I) and in whole blood (II). 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 (I) 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 (II). At the highest concentration applied (50 μM), (+)-nicotine increased PGE_2 synthesis more than two times over.

(-)-Cotinine stimulated PGE_2 production in PMNs (I) and in whole blood (II). 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 (I) 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.

1.2. Effects of nicotinic acid and pyridoxine on eicosanoid synthesis

In whole blood, nicotinic acid stimulated PGE_2 synthesis dose-dependently (IV). It also increased TXB_2 formation and enhanced LTE_4 synthesis. Pyridoxine (IV) stimulated PGE_2 production and increased TXB_2 formation, but had no effect on LTE_4 formation. Only the highest tested concentration of pyridoxal-5'-phosphate (500 μM) stimulated PGE_2 and TXB_2 production, and it had no effect on LTE_4 formation.

Table 5. Effects of nicotine stereoisomers, (-)-cotinine, nicotinic acid, pyridoxine and pyridoxal-5'-phosphate on arachidonic acid cyclooxygenase and 5-lipoxygenase pathways in human whole blood in vitro (II). 0 = no effect, ↑ = increased, ↓ = decreased.

	(-)-nicotine	(+)-nicotine	(-)-cotinine	nicotinic acid	pyridoxine	pyridoxal-5`-phosphate
PGE_2	↑	↑	↑	↑	↑	↑
LTE_4	↓	↓	↓	↑	0	0
TXB_2	↓	↓	↓	↑	↑	↑

The effects of (-)-nicotine and (-)-cotinine on PGE_2 synthesis in PMNs and on TXB_2 synthesis in PRP (I) were similar to those observed in whole blood.

2. EX VIVO AND IN VIVO RESULTS

2.1. Effects of smoking cessation and nicotine substitution on eicosanoid synthesis

Urinary thiocyanate concentration levels 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 (III, V).

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.

Table 6. Urinary cotinine, trans-3'-hydroxycotinine and thiocyanate concentrations (ng/μmol creatinine, mean ± SEM) in the persons quitting smoking (III, V).

		Days after smoking cessation			
	Substitution	0	3	7	14
Cotinine	No substitution	120 ± 20	<10	<10	<10
	Chewing gum	90 ± 25	120 ± 30	90 ± 20	70 ± 15
	Patch	105 ± 15	100 ± 25	90 ± 30	80 ± 35
Trans-3-hydroxy-cotinine	No substitution	580 ± 65	<10	<10	<10
	Chewing gum	460 ± 40	620 ± 125	420 ± 35	340 ± 35
	Patch	520 ± 45	500 ± 20	460 ± 35	420 ± 40
Thiocyanate	No substitution	1010 ± 260	620 ± 160	420 ± 60	230 ± 25
	Chewing gum	830 ± 85	540 ± 50	360 ± 15	250 ± 40
	Patch	1090 ± 100	670 ± 60	380 ± 35	320 ± 25

In whole blood, ex vivo PGE_2 and TXB_2 production was 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 to 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 (III).

Urinary excretion of all eicosanoid metabolites measured in the present study was higher in smokers than in non-smoking controls (V). (Table 7)

Table 7. Urinary eicosanoid excretion in smokers before smoking cessation and in non-smoking controls (mean ± SEM) (V).

Group (n)	2,3-dinor-6-keto-$PGF_{1\alpha}$ pg/µmol creatinine	11-dehydro-TXB_2 pg/µmol creatinine	LTE_4 pg/µmol creatinine
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

Smoking cessation without nicotine substitution reduced urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$, LTE_4 and 11-dehydro-TXB_2, 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 analytes during the two-week follow-up (V).

2.2. Effects of nicotinic acid and pyridoxine administration on eicosanoid synthesis

Nicotinic acid treatment increased ex vivo PGE_2, TXB_2 and LTE_4 synthesis to 185, 165 and 175% of the initial value, respectively. One-week pyridoxine treatment decreased PGE_2, TXB_2 and LTE_4 synthesis to 75, 65 and 45% of the initial value, respectively (IV).

Before drug administration, urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was 21.8 ± 5.5 pg/µmol creatinine in the nicotinic acid group and 18.9 ± 1.7 pg/µmol creatinine in the pyridoxine group. After acute nicotinic acid treatment, an increasing tendency (to about 110% of the basal value) was seen in 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion (P=0.07).

In the pyridoxine-treated subjects urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was increased after seven days to 165% of the initial value ($P<0.01$). (unpublished data)

Basal urinary excretion of 11-dehydro-TXB_2 was 72.8 ± 9.7 pg/µmol creatinine in the nicotinic acid group and 48.5 ± 25.3 pg/µmol creatinine in the pyridoxine group. After nicotinic acid treatment 11-dehydro-TXB_2 excretion was increased to 260% of the initial value ($P<0.05$). Pyridoxine-treatment decreased 11-dehydro-TXB_2 excretion to 70% of the initial value ($P<0.05$). (unpublished data)

In the nicotinic acid group basal urinary excretion of LTE_4 was 14.0 ± 2.1 pg/µmol creatinine in the nicotinic acid group and 18.1 ± 10.7 pg/µmol creatinine in the pyridoxine group. In the nicotinic acid-treated subjects urinary excretion of LTE_4 increased to 200% of the initial value ($P<0.05$). In the pyridoxine-treated group LTE_4 excretion was decreased to 65% of the initial value ($P<0.01$). (unpublished data)

Table 8. Effects of smoking, smoking cessation, nicotine substitution (nicotine chewing gum or nicotine patch), nicotinic acid and pyridoxine on arachidonic acid cyclooxygenase and 5-lipoxygenase pathways ex vivo and in vivo in man. ↑ = increased, ↓ = decreased, ( ) = tendency.

	Smoking	**Smoking Cessation**	**Nicotine Substitution**	**Nicotinic Acid**	**Pyridoxine**
Ex vivo					
PGE_2	↑	↓	↑	↑	↓
LTB_4	↑	↓	↑	↑	↓
LTE_4	↑	↓	↑	↑	↓
TXB_2	↑	↓	↑	↑	↓
In vivo					
11-dehydro-TXB_2	↑	↓	↑	↑	↓
2,3-dinor-6-keto-$PGF_{1\alpha}$	↑	↓	↑	(↑)	↑
LTE_4	↑	↓	↑	↑	↓

DISCUSSION

1. DISCUSSION ON METHODS

1.1. Calcium ionophore A23187

The eicosanoid concentrations present in unstimulated plasma or whole blood are so low that direct determination is not possible, even with the highly sensitive (radio)immunoassay methods currently available. Eicosanoid measurements from unstimulated plasma can only be done after extraction of relatively large volumes of blood. This is why in vitro studies apply various stimuli, such as opsonized zymosan, phorbol myristate acetate, lipopolysaccharide, N-formyl-Met-Leu-Phe, des-Arg-C5a, calcium ionophores, exogenous AA and PLA_2.

In the present study, eicosanoid synthesis in PMNs, PRP and whole blood was stimulated with calcium ionophore A23187. Studies concerned with eicosanoid synthesis capacity frequently use this compound to activate eicosanoid-producing cells. Some of the other triggers may be considered to be more physiological, but A23187 has the important advantage of simultaneously activating both COX and LO pathways in AA metabolism. Although both COX- and LO-products may also be synthesized after stimulation with other agonists like opsonized zymosan, efficient synthesis of LO-products, especially LTB_4, requires the presence of a calcium signal (A23187) (Tripp et al. 1985, Laegreid et al. 1989, Kaever et al. 1990, Yoss et al. 1990).

1.2. In vitro and ex vivo models

In in vitro and ex vivo experiments, PGE_2 and TXB_2 production indirectly reflects the activity of COX in leukocytes and platelets, respectively. LTB_4 and E_4 production indirectly reflects the activity of 5-LO, but also the activity of LTA_4-hydrolase (LTB_4) and LTC_4-synthase (LTE_4). The measurement of 5-HETE (which was not used in the present study) directly reflects the activity of 5-LO. PGE_2, LTE_4 and TXB_2 can be reliably measured by direct radioimmunoassay after stimulation with A23187. LTB_4 synthesis was determined with RP-HPLC due to the lack of specific (commercial) RIA.

As regards the sources of eicosanoids in the various models used in the present study, in PMNs PGE_2 and LTB_4 are synthesized by neutrophils. In PRP the source of TXB_2 is platelets.

In the whole blood model the various cell-cell interactions affecting the eicosanoid spectrum synthesized are present. In this model, PGE_2 is synthesized by monocytes and neutrophils. TXB_2 is mainly derived from platelets, with a smaller proportion being synthesized by monocytes (Djurup et al. 1993, Orlandi et al. 1994). LTB_4 is derived from 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. Via intercellular interactions, erythrocytes may metabolize LTA_4 to LTB_4 (Fitzpatrick et al. 1984) and platelets to LTC_4 (Maclouf and Murphy 1988). In A23187-stimulated whole blood LTE_4 is very stable and represents the end product of total cysteinyl leukotriene metabolism (Zakrzewski et al. 1989).

1.3. Urinary eicosanoids

Because of the chemical instability of the parent compounds, systemic eicosanoid synthesis is evaluated by the measurement of urinary metabolites. Certain metabolites of eicosanoids in urine 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. Since the description of the in vivo catabolism of PGI_2 (Rosenkranz et al. 1980, Brash et al. 1983) and TXA_2 (Roberts et al. 1981, Patrignani et al. 1989), 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 (Fischer 1989, Granström 1988) and that of 2,3-dinor-6-keto-$PGF_{1\alpha}$ an index of PGI_2 formation by endothelium (FitzGerald et al. 1983).

A significant proportion, approximately 4-13%, of cysteinyl leukotrienes is excreted in urine as LTE_4 (Örning et al. 1985, Maltby et al. 1990). The measurement of urinary LTE_4 has therefore been used as a marker of in vivo cysteinyl leukotriene production (Granström 1988, Nicolli-Griffith et al. 1990). This measurement appears to provide a reliable assessment of whole body cysteinyl leukotriene synthesis; it can be used to monitor the effects of diseases and drugs on cysteinyl leukotriene synthesis in man (for a review, see Frölich et al. 1994).

In the present study, urinary 11-dehydro-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ were measured by direct RIA after selective one-step (Riutta et al. 1992) and two-step (Riutta et al. 1994) solid phase extractions, respectively. Both 11-dehydro-TXB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ assays have been described in detail and compared to assays described previously in the literature (Riutta 1995).

Urinary LTE_4 was measured by direct enzyme immunoassay using a method previously validated and described in detail (Kumlin et al. 1995). The results of our study are at the same level as those reported by Kumlin and co-workers. Compared to the values of urinary LTE_4 excretion indicated in the literature (range about 4.5-8.5 pg/μmol creatinine), which are obtained from RIA after purification on solid-phase extraction followed by separation on RP-HPLC (Fauler et al. 1991, Asano et al. 1995, Sala et al. 1994, Kerttula et al. 1997), the results obtained with direct enzyme immunoassay of serially diluted urine samples are marginally higher.

In the present study, urinary LTE_4 was measured by RIA after purification on solid-phase extraction followed by separation on RP-HPLC from five persons quitting smoking without nicotine substitution and from five persons in both substituted groups, and the results obtained were slightly lower than those obtained with enzyme immunoassay. A possible explanation for this finding is the cross-reactivity of the antibody with N-acetyl LTE_4 and possibly also with other beta- and omega-oxidized cysteinyl leukotriene metabolites. However, this cross-reactivity can be considered advantageous because it means that the results more accurately reflect the total systemic cysteinyl leukotriene synthesis.

2. NICOTINE AND SMOKING

2.1. 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 AA metabolism, although the (+)-isomer has been shown to be biologically active (Aceto et al. 1979, Ikushima et al. 1982), and it is generally acknowledged that the effects of the optical isomers of a drug on eicosanoid production may differ. The effects of cotinine on eicosanoid production were also unknown, although cotinine has about a ten times higher blood and plasma concentration (Benowitz et al. 1987, Höfer et

al. 1992) and a considerably longer half-life than the parent compound (Benowitz et al. 1987).

As regards the effects of nicotine, our results are consistent with previous observations on the stimulation of PGE_2 formation induced by nicotine (Seow et al. 1994), by smokeless tobacco extract (Johnson et al. 1996) in man, and by nicotine shown in animal models, for instance in isolated rabbit heart (Wennmalm 1977). A possible explanation for the increased PGE_2 formation induced by nicotine is that it may function as a cosubstrate for the peroxidase component of PGHS (Mattammal et al. 1987).

Nicotine stereoisomers and cotinine inhibited TXB_2 formation in vitro. The effect of (+)-nicotine on TX synthesis has not been described previously, but (-)-nicotine and cotinine at submicromolar concentrations have been found to suppress TXB_2 synthesis in arachidonic acid-stimulated intact macrophage-like cells and in cell free microsomal preparations (Goerig et al. 1992), as well as in human platelet-rich plasma (Toivanen et al. 1986). One possible explanation for these findings is the direct inhibition of TX-synthase by nicotine and cotinine (Goerig et al. 1992).

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 (Seow et al. 1994). PGE_2 has been reported to inhibit 5-lipoxygenase pathway (Ney and Schrör 1989, Christman et al. 1993) but it is unlikely that this explains the reduction in LT production. This is due to the fact that in A23187-stimulated human polymorphonuclear leukocytes as well as in whole blood, ASA was found almost completely to block PGE_2 production, whereas the inhibition of LT production by nicotine or cotinine remained unaffected.

Since 5-LO is the first enzyme in the biosynthesis pathway of LTB_4 as well as LTC_4 and D_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. One possible explanation for the inhibition of LT synthesis by nicotine stereoisomers and cotinine is that these electron donating compounds inhibit 5-LO by reducing the catalytically active ferric enzyme to the catalytically inactive ferrous form, as previously suggested for catecholamines and other phenolic compounds (Alanko et al. 1992). However, the inhibition of LTA_4-hydrolase and the enzymes catalyzing the formation of LTB_4 and LTE_4 (glutathione-S-transferase, γ-glutamyl peptidase, cysteinyl glycinase) by nicotine stereoisomers and cotinine 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. However, at the concentrations measured in the plasma of smokers, the effects of

nicotine and cotinine on PGE_2 and TXB_2 synthesis were more or less the same in both experimental models.

2.2. Smoking cessation and nicotine substitution

PGE_2, LTB_4, LTE_4 and TXB_2 synthesis in A23187-stimulated whole blood ex vivo were all higher in smokers than in non-smoking subjects. This observation is consistent with previous findings which indicate that cyclooxygenase activity (Zijlstra et al. 1992), LTB_4 serum concentrations (Thornton et al. 1989) and blood levels of LTC_4, LTD_4 and LTE_4 (Kobayashi et al. 1988) are increased in smokers. The increase in PGE_2 synthesis is probably due to the stimulation of COX induced by nicotine and cotinine. This effect was also seen in vitro.

Urinary excretion of 11-dehydro-TXB_2, 2,3-dinor-6-keto-$PGF_{1\alpha}$ and LTE_4 was higher in smokers than in non-smoking subjects. There are also earlier reports of increased systemic PGI_2 synthesis in smokers (Nowak et al. 1987, Lassila et al. 1988, Barrow et al. 1989). However, smoking has also been associated with reduced (Nadler et al. 1983, Mileikowsky et al. 1988) or unchanged (Wennmalm et al. 1991, Benowitz et al. 1993) PGI_2 synthesis. The studies that reported reduced PGI_2 synthesis (Nadler et al. 1983, Mileikowsky et al. 1988) measured 6-keto-$PGF_{1\alpha}$, which means that these results reflect PGI_2 production in the kidneys more than systemic PGI_2 synthesis. The age of the subjects and possible underlying vascular injuries may also be of importance. Furthermore, it is important to remember that the use of several different purification methods prior to assays, different antibodies in RIA, and the variable specifity of the immunoassays and gas-chromatography-mass spectrometry methods affect the reliability of the results obtained.

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 (Lassila et al. 1988) possibly by a mechanism mediated by some constituent(s) of tobacco smoke. The increased systemic TX synthesis found in smokers found in the present study and earlier (Nowak et al. 1987, Lassila et al. 1988, Wennmalm et al. 1991, Rångemark et al. 1993) reflects platelet activation (Nowak et al. 1987) possibly by a mechanism mediated by some constituent(s) of cigarette smoke. In the present study smokers showed increased urinary excretion of LTE_4. This observation is line with previous report of increased systemic cysteinyl leukotriene synthesis in smokers (Fauler and Frölich 1997), where close correlation was found between urinary excretion of LTE_4 and the number of cigarettes smoked daily.

Three days after smoking cessation without nicotine substitution, a decrease was seen both in ex vivo PGE_2, LTB_4, LTE_4 and TXB_2 formation and in systemic PGI_2, TX and cysteinyl leukotriene synthesis. Neither cotinine nor trans-3'-hydroxycotinine were found in urine, which indicates compliance on the part of the subjects. In the present study, the decrease in thromboxane synthesis on day 3 was slightly weaker than in a previous study with healthy women, in which smoking cessation in three days decreased the excretion of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 to 55 and 60% of the initial values (Rångemark et al. 1993). However, in these women smoking cessation had no effect on the excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Rångemark et al. 1993).

In our nicotine chewing gum and nicotine patch 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, ex vivo PGE_2 and LTE_4 formation but not LTB_4 and TXB_2 formation, as well as 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 11-dehydro-TXB_2 excretion in the nicotine chewing gum group and 2,3-dinor-6-keto-$PGF_{1\alpha}$ and LTE_4 excretion in the nicotine patch group was slightly decreased compared to days 3 and 7. At the same time, urinary cotinine and trans-3'-hydroxycotinine concentrations were slightly diminished, 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.

In a previous crossover study with male smokers aged 31 to 65 years (Benowitz et al. 1993) urinary excretion of 11-dehydro-TXB_2 during transdermal nicotine treatment (21 mg/24 h) was at a similar level to that during placebo treatment but about 20% lower than during smoking. The treatments showed no differences in 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion. In this study each treatment block lasted no more than five days and it cannot be ruled out that during a longer treatment period it might be possible to see differences between the effects of placebo and transdermal nicotine treatments. In male non-smokers aged 25 to 35 years, nicotine infusion had no effect on urinary excretion of 2,3-dinor-TXB_2 (Nowak et al. 1996). The plasma nicotine concentration achieved during infusion, about 60% of what one cigarette or a 4 mg nicotine gum would give (Benowitz 1988), may not be high enough to alter systemic eicosanoid synthesis. During nicotine infusion the acute effect of nicotine on platelets is studied, but the in vivo effects of nicotine may be different during habitual smoking.

The present results also seem to conflict with the findings of Fauler and Frölich (1997), according to whom acute smoking but not transdermal nicotine increased urinary excretion of LTE_4 in non-smokers (Fauler and Frölich 1997). Fauler and Frölich studied the acute effects of cigarette smoking and nicotine on cysteinyl LT synthesis in non-

smokers. However, the effect of nicotine on cysteinyl LT synthesis may be different in habitual smokers abstaining from tobacco but using nicotine substitution.

The present ex vivo and in vivo effects of smoking and nicotine substitution seem to be in conflict with the decreases observed in LTE_4 and TXB_2 synthesis induced by nicotine stereoisomers in vitro. It must be borne in mind, however, that the lack of contact of blood cells with vascular endothelium is a major factor that differentiates in vitro and in vivo data. In vivo, the contact of blood cells with endothelial cells may lead to major changes in the resulting eicosanoid spectrum synthesized (Marcus 1990) when compared with the products of individual cell types or whole blood. Cross-over exchange of soluble mediators and direct blood cell - endothelial cell contacts determine the conditions of specific activation and expression of biosynthetic enzymes. In cocultures of endothelial and monocytic cells an increase in PGE_2 synthesis has been observed (Koll et al. 1997), whereas TX synthesis was found to be enhanced in coculture systems of platelets and endothelial cells (Karim et al. 1996), and LTB_4 and C_4 synthesis enhanced in cocultures of polymorphonuclear leukocytes and endothelial cells (Brady et al. 1995).

There are also other possible explanations for the differences in the effects of nicotine in vitro compared to those of nicotine substitution ex vivo/in vivo. Smoking may induce hypoxia which stimulates prostaglandin synthesis (Michiels et al. 1993) and induces COX-2 (Schmedtje et al. 1997, Ji et al. 1998). The responsiveness of leukocytes and platelets to a variety of agonists in vivo might be increased by smoking or by nicotine/cotinine. Thus, smoking- or nicotine-activated leukocytes (Bridges et al. 1993, Nowak et al. 1990) and platelets (Rival et al. 1987, Blache et al. 1992, Nowak et al. 1996) may be the origin of increased eicosanoid synthesis. Nicotine stimulates the liberation of catecholamines from the adrenal gland (Benowitz 1991) and the release of serotonin from gastrointestinal enterochromaffin cells (Racké and Schwörer 1992), which are thought to be the main source of platelet serotonin (Verbeuren 1989). Both catecholamines (Alanko et al. 1992) and serotonin (Sih et al. 1970) can increase eicosanoid synthesis. Nicotine increases the production of proinflammatory cytokines (Fischer and König 1994), which have been reported to augment the expression of 5-lipoxygenase and cyclooxygenases (for a review, see Serhan et al. 1996), leading to increased eicosanoid synthesis.

3. NICOTINIC ACID AND PYRIDOXINE

Nicotinic acid, pyridoxine and pyridoxal-5'-phosphate (the latter only at the highest concentration applied) stimulated PGE_2 production in vitro. The stimulation of PGE_2 production induced by nicotinic acid in vitro was dose-dependent, but considerably weaker than that induced by nicotine and cotinine. The stimulation of PGE_2 production induced by pyridoxine was dose-independent and weaker than that induced by nicotine, cotinine, and nicotinic acid. The enhancing effect of pyridoxal-5'-phosphate was very weak. Nicotinic acid has previously been reported to increase the release of PG-like substances from isolated perfused rabbit heart (Kaijser and Wennmalm 1978, Sahin et al. 1984). Pyridoxine has previously been reported to increase the generation of PGE_2 in isolated perfused rabbit heart (Sahin et al. 1984) and in rabbit kidney medulla slices (Fujimoto et al. 1987).

Nicotinic acid, pyridoxine, and pyridoxal-5'-phosphate (the latter only at the highest concentration applied) stimulated TXB_2 formation in vitro. The stimulation of TXB_2 synthesis induced by nicotinic acid was relatively weak and dose-independent, and that induced by pyridoxine even weaker. It is possible that the increased TXB_2 synthesis is due not to the stimulation of TXA_2 synthase, but the stimulation of cyclooxygenase. Nicotinic acid as a pyridine derivative may even inhibit TX synthase like nicotine and cotinine (Goerig et al. 1992), as shown in platelets by Vincent and Zijlstra (1978). Pyridine and its derivatives have also been reported to inhibit TXA_2 synthesis in human platelet microsomes (Tai et al. 1980).

Nicotinic acid stimulated LTE_4 synthesis in vitro but pyridoxine and pyridoxal-5'-phosphate had no effect on LTE_4 production. The mechanism of action of nicotinic acid in vitro might be to activate 5-LO, but the activation of LTA_4 hydrolase and the enzymes catalyzing the formation of LTE_4 (LTC_4-synthase, γ-glutamyl peptidase, cysteinyl glycinase) cannot be excluded.

Following nicotinic acid administration to the subjects, PGE_2, TXB_2, and LTE_4 syntheses ex vivo were enhanced. In addition, LTB_4 production (measured from 3 volunteers) was increased (data not shown). Urinary excretion of 11-dehydro-TXB_2 and LTE_4 was also increased, and a tendency to increase was seen in 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion. Stimulation of the cyclooxygenase pathway is consistent with earlier findings of increased $9_{\alpha},11_{\beta}$-PGF_2 production ex vivo (Morrow et al. 1989) as well as increased 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Morrow et al. 1989) and 6-keto- $PGF_{1\alpha}$ (Olsson et al. 1983) production in vivo. Plasma concentrations of nicotinic acid, administered according to our study protocol, can be estimated to be between 30 and 70 μM on the basis of a previous study (Carlson 1971).

The volunteers in the present study received a relatively high dose of pyridoxine. The treatment was found to decrease PGE_2 synthesis ex vivo. This can be considered to be consistent with previous results of higher kidney PGE_2 levels in vitamin B_6 deficient rats (Maranesi et al. 1993). The treatment markedly decreased LTE_4 synthesis as well as LTB_4 production (measured from three volunteers) (data not shown). Ex vivo TXB_2 synthesis was also decreased. Pyridoxine treatment decreased systemic TX and LTE_4 synthesis. Interestingly, urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was increased, reflecting increased systemic PGI_2 synthesis.

It has been shown in healthy volunteers that one week's oral treatment with 800 mg pyridoxine daily results in pyridoxine and pyridoxal-5'-phosphate plasma concentrations of 4 and 0.8 μM, respectively (Edwards et al. 1990). Consequently, the plasma concentrations of pyridoxine and pyridoxal-5'-phosphate reached after daily doses of 600 mg in our study do not exceed those used in vitro. This indicates that the ex vivo effects of vitamin B_6 compounds, in contrast to the in vitro effects of pyridoxine and pyridoxal-5'-phosphate on eicosanoid production, are due to mechanisms other than actions at the level of enzyme catalysis.

4. CLINICAL IMPORTANCE

As concerns the role of increased systemic eicosanoid synthesis in the development of cardiovascular diseases, cigarette smoking has many and varied effects on the factors that are considered to be important in the progression of atherosclerosis and in the acute development of cardiovascular complications. Among these effects are alterations in the lipid profile (Kotamäki and Laustiola 1993, Netscher et al. 1995), impaired endothelial function (Celermajer et al. 1993, Lekakis et al. 1997, Moreno et al. 1998), desensitization of β_2-adrenergic mechanisms (Lassila and Laustiola 1988, Laustiola et al. 1988b), and activation of the renin-angiotensin-aldosterone system (Laustiola et al. 1988a), as well as increases in catecholamine levels (Hill and Wynder 1974, Winniford et al. 1986), leukocyte and platelet counts and activation, and blood viscosity (Laustiola et al. 1991, Kotamäki and Laustiola 1993, Netscher et al. 1995).

It is assumed that platelets play an important role in atherosclerosis and its complications (Harker and Ritchie 1980, Trip et al. 1990). Among the stimuli initiating the process of platelet activation and leading to various platelet responses, including aggregation, thrombin and collagen are thought to be decisive in the pathology of plaque rupture and acute occlusive thrombus formation (Fuster et al. 1987). However, the prevention of occlusive vascular diseases achieved in clinical studies with daily oral

administration of low doses of ASA (for a review, see Viinikka 1990, Patrono 1994) clearly highlights the importance of TXA_2 in platelet interactions in vivo.

Endothelial NO bioactivity is decreased in smokers (Kiowski et al. 1994, Kugiyama et al. 1996). In the present and also in many previous (Nowak et al. 1987, Lassila et al. 1988, Murray et al. 1990) studies, smoking has been associated with increased systemic PGI_2 synthesis. It has been shown that the sensitivity of smokers' platelets to PGI_2 is preserved, which indicates that PGI_2 is able to counteract vasoconstriction and thrombus formation in smokers (Lassila 1989). The increased PGI_2 synthesis can be seen as a compensatory mechanism of endothelium for the general vasoconstrictive properties of cigarette smoking (Lassila et al. 1988). NO, by increasing the concentrations of intracellular cGMP, relaxes smooth muscle and inhibits platelet aggregation (for a review, see Moncada et al. 1991). NO probably has greater importance to endothelial function than PGI_2, but endothelial function might be even worse if there is no increase in PGI_2 synthesis. Cardiovascular diseases are not, however, manifested in all smokers, underscoring the importance of genetic, defensive and compensatory mechanisms in withstanding atherothrombotic processes.

The decreased risk of cardiovascular diseases (Dobson et al. 1991) and the improvement of lower respiratory tract inflammatory changes (Rennard et al. 1990) after smoking cessation may be related to the decrease in systemic 11-dehydro-TXB_2 and LTE_4 synthesis observed in the present study. However, smoking cessation also normalizes catecholamine levels, blood and plasma viscosities, plasma fibrinogen levels, and total leukocyte count (Ernst and Martai 1987, Laustiola et al. 1991); all these factors contribute to the decreased risk of cardiovascular diseases. Smoking cessation puts an end to exposure to nicotine, NO, carbon monoxide, phenols, hydrogen cyanide, and polycyclic aromatic hydrocarbons, which certainly has a beneficial effect on respiratory function.

It was an interesting and important finding in this study that no decrease was observed in eicosanoid synthesis during substitution therapy with nicotine chewing gum or nicotine patches. This suggests that nicotine and/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 (Dacosta et al. 1993, Jackson 1993, Pierce 1994, Warner and Little 1994, Arnaot 1995, Ottervanger et al. 1995, Riche et al. 1995). However, even in this respect the stimulating effects of nicotine on the cardiovascular system probably play a considerable role.

The increased eicosanoid synthesis observed in smokers and in persons quitting smoking but using nicotine substitution raises the question as to whether the expression

of COX-2 and 5-LO is increased in smokers/in persons predisposed to nicotine. Both issues will be addressed in our further studies.

The effect of nicotinic acid on AA metabolism was tested in the present study by administering a commonly used therapeutic dose. With this dose, a significant increase was observed in PG, TX and LT production ex vivo as well as in systemic TX and LTE_4 synthesis. The increase in TX synthesis is potentially unfavorable. The increase in LTE_4 formation may be unfavorable for asthmatic subjects, who already have increased leukotriene formation (Devillier et al. 1999a). Furthermore, nicotinic acid is known to increase the release of PGD_2 measured as $9_{\alpha},11_{\beta}$-PGF_2 (Morrow et al. 1989 and 1992). PGD_2 and $9_{\alpha},11_{\beta}$-PGF_2 are both potent bronchoconstrictors (Beasley et al. 1987) and involved in acute asthmatic attacks (Obata et al. 1994).

The decrease in TX and the increase in PGI_2 synthesis after pyridoxine administration might be beneficial for patients with enhanced TX formation. As regards the possible clinical relevance of reduced leukotriene production with pyridoxine, studies with asthmatic subjects have found significantly lower plasma and erythrocyte pyridoxal-5'-phosphate levels than in controls. The reported decrease in the frequency and severity of asthmatic attacks with pyridoxine treatment (Reynolds and Natta 1985) might be related to the inhibition of the 5-LO pathway observed in the present study. However, as far as the clinical relevance of the effects of pyridoxine is concerned it must be borne in mind that the dose used in the present study was relatively high and may induce neuropathy when administered on a long-term basis (Schaumburg et al. 1983).

SUMMARY AND CONCLUSIONS

The present study was designed to investigate the effects of nicotine, smoking, nicotine substitution, pyridoxine, and nicotinic acid on eicosanoid synthesis in man. The effects of nicotine stereoisomers, cotinine, nicotinic acid, pyridoxine, and pyridoxal-5'-phosphate were studied in vitro. The effects of smoking, smoking cessation, nicotine substitution, nicotinic acid, and pyridoxine were studied both ex vivo and in vivo.

The main results and conclusions are as follows:

1. All the test compounds modulated eicosanoid synthesis in vitro. Nicotine stereoisomers and cotinine stimulated PGE_2, but inhibited LTB_4, LTE_4 and TXB_2 synthesis in vitro. The results imply that nicotine stereoisomers and cotinine stimulate COX, but inhibit 5-LO and TX-synthase. Nicotinic acid stimulated PGE_2, TXB_2 and LTE_4 synthesis in vitro. In vitro pyridoxine and at high concentrations pyridoxal-5'-phosphate stimulated PGE_2 and TXB_2 production, but had no effect on LTE_4 synthesis.

2. Nicotinic acid stimulated but pyridoxine inhibited PGE_2, TXB_2, and LTE_4 synthesis ex vivo. Nicotinic acid increased systemic TX and LTE_4 synthesis, and tended to enhance systemic PGI_2 production. Pyridoxine treatment decreased TX and LTE_4 production in vivo, but increased production of PGI_2. The increased TX and LTE_4 synthesis caused by nicotinic acid is potentially unfavorable, while the inhibition of LT synthesis and the increase in PGI_2 production caused by pyridoxine might be beneficial.

3. Both ex vivo and systemic (in vivo) eicosanoid synthesis was higher in smokers than in non-smoking controls, which may contribute to the harmful cardiovascular effects of smoking. Cessation of smoking without nicotine substitution decreased eicosanoid synthesis; the decreases in TX and leukotriene synthesis may contribute to the decreased risk of cardiovascular diseases and to the improvement of lower respiratory tract inflammatory changes after smoking cessation.

   In persons quitting smoking but using nicotine substitution, no significant changes were observed in eicosanoid synthesis during the follow-up, which suggests that nicotine and/or cotinine could be major factors in explaining the differences found in eicosanoid synthesis between smokers and non-smokers. Long-term nicotine substitution may diminish the beneficial effects of smoking cessation due to

the stimulatory effects of nicotine and cotinine on eicosanoid synthesis. The increased level of systemic eicosanoid synthesis observed in volunteers using nicotine chewing gum or nicotine patches might partly explain the increased risk of cardiovascular complications reported to occur during nicotine replacement therapy.

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