Perturbations in Blood Phosphatidylcholine and Sphingomyelin Fatty Acid Composition in a Sample Population of Cigarette Smokers

Nicoleta Ionescu · Caterina de Freitas ·
Allain A. Bueno

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Abstract Docosahexaenoic (DHA) and arachidonic acids (AA) are polyunsaturated fatty acids (PUFAs), major components of brain tissue and neural systems, and the precursors of a number of biologically active metabolites with functions in inflammation resolution, neuroprotection and other actions. As PUFAs are highly susceptible to peroxidation, we hypothesised whether cigarette smokers would present altered PUFAs levels in plasma and erythrocyte phospholipids. Adult males from Indian, Sri-Lankan or Bangladeshi genetic backgrounds who reported smoking between 20 and 60 cigarettes per week were recruited. The control group consisted of matched non-smokers. A blood sample was taken, plasma and erythrocyte total lipids were extracted, phospholipids were separated by thin layer chromatography, and the fatty acid content analysed by gas chromatography. In smokers, dihomo-gamma-linolenic acid, the AA precursor, was significantly reduced in plasma and erythrocyte phosphatidylcholine. AA and DHA were significantly reduced in erythrocyte sphingomyelin. Relatively short term smoking has affected the fatty acid composition of plasma and erythrocyte phospholipids with functions in neural tissue composition, cell signalling, cell growth, intracellular trafficking, neuroprotection and inflammation, in a relatively young population. As lipid peroxidation is pivotal in the pathogenesis of atherosclerosis and neurodegenerative diseases such as Alzheimer disease, early effects of smoking may be relevant for the development of such conditions.

Keywords Docosahexaenoic acid · Arachidonic acid · Phosphatidylcholine · Sphingomyelin · Cigarette · Neurodegeneration

Introduction

Although smoking is to a certain extent a socially tolerable habit, consequences to health have been clearly identified, such as direct vascular actions [1], oxidative stress [2], thrombogenic effects [3], secondary dyslipidemia [4], and cancer [5].

Polyunsaturated fatty acids (PUFAs) are involved in a diversity of metabolic processes and bodily functions. Approximately 30 % of the fatty acids in the human brain are N-3 PUFAs and some other 30 % are N-6 PUFAs [6].

Docosahexaenoic acid (DHA) is an essential N-3 PUFA and one of the major components of the brain tissue and neural systems [7]. It is needed for adequate vision and normal brain development [8–10]. Neurite growth and synaptogenesis [11] as well as brain gene expression [12] are directly stimulated by DHA. DHA confers protection to the neural tissue, highly susceptible to peroxidation, serving as antioxidant to quench reactive oxygen species in the brain [13]. Neuroprotectin D1, a DHA metabolite, conveys neuroprotection during stress [14]. In a rat model of perinatal hypoxia–ischemia potentiated by lipopolysaccharide-induced inflammation, pre-treatment with DHA conferred protection [15].

Arachidonic acid (AA) is the most abundant N-6 PUFA in the brain [6], and has also been demonstrated as essential for brain development and for protection during inflammatory events. Enzymes of the cytochrome P450 system were found to metabolise AA to epoxyeicosatrienoic acid and hydroxy fatty acids, biologically active compounds

N. Ionescu · C. de Freitas
Faculty of Life Sciences, London Metropolitan University,
166-220 Holloway Road, London N7 8DB, UK

A. A. Bueno (✉)
Institute of Science and the Environment, University of
Worcester, Henwick Grove, Worcester WR2 6AJ, England, UK
e-mail: a.bueno@worc.ac.uk

with a variety of functions, including vasodilatation, angiogenesis and inflammation resolution, protecting the ischemic myocardium and brain [16–18].

Lipid peroxidation is a major factor in the pathogenesis of various disorders such as coronary heart disease (CHD), atherosclerosis and inflammation [19]. Low levels of certain PUFAs, particularly DHA and AA, have been associated with higher risk of CHD [20], type 2 diabetes [21] and chronic obstructive pulmonary disease [22], as well as neurological disorders such as schizophrenia [23] and Alzheimer disease [24].

A study found that male individuals who smoke >20 cigarettes per day presented decreased PUFA levels, followed by increased saturated (SFA) and monounsaturated fatty acids (MUFA) [25]. Reduced levels of eicosapentaenoic acid (EPA) and DHA in erythrocytes of smokers with schizophrenia, as compared to non-smokers, have also been observed [26]. Short-term cigarette smokers, otherwise healthy, presented dramatic perturbations in the bronchoalveolar lavages of membrane phospholipids released after cellular damage [27].

As positive correlations between cigarette smoking and Alzheimer's Disease have been identified [28, 29], the aim of the present study was to investigate the plasma and erythrocyte membrane phospholipid fatty acid profiles of young adult males of Indian, Sri-Lankan or Bangladeshi genetic backgrounds with a smoking habit. The population recruited is relatively young and smokes between 20 and 60 cigarettes per week. This particular male population is at higher risk to develop CHD as compared to men from other genetic backgrounds [30, 31]. Our aim is to reveal early signs of phospholipid perturbations induced by cigarette smoking.

Subjects and Methods

Subjects

Ethical approval to conduct this research was granted by the Camden and Islington Community Research Ethics Committee.

In February–March 2011, 22 healthy adult male students and members of staff from the London Metropolitan University (10 non-smokers, 12 smokers), between 20 and 35 years of age, voluntarily took part in this study. In this study, smokers recruited consumed between 20 and 60 cigarettes per week; whilst a non-smoker was defined as not smoking at all. The volunteers recruited were of Indian, Sri-Lankan or Bangladeshi genetic backgrounds only.

All volunteers were explained the aims of the project and the purpose of the blood collection; they verbally agreed to it and also signed a consent form before engaging

into the study. All volunteers were firstly asked to complete a short health and lifestyle questionnaire to check for eligibility, and were immediately excluded if the inclusion criteria were not fully met. Exclusion criteria include: use of self-prescribed medicines within the previous 2 weeks, alcohol intake, obesity, and any self-reported health condition; the use of other drugs (including prescribed medicines), the consumption of vitamin tables, protein supplements, fish oil supplements or other fatty acid supplements, vegetarian or not, within the previous 2 months.

Venous Blood

Non-fasting venous blood samples (5 mL) were collected from the median cubital or cephalic vein into EDTA Vacutainer® tubes (Becton & Dickinson, Oxford, UK), by a qualified phlebotomist. Plasma and red blood cells were immediately obtained by centrifugation at $1,000\times g$ for 30 min at 4 °C. Plasma was transferred into 2 mL sterile vials and stored at –80 °C. Erythrocytes were washed with phosphate buffered saline ($1\times$ PBS) and centrifuged; this washing step repeated twice. The remaining buffy coat was removed after every wash and erythrocyte aliquots were transferred into 2 mL sterile vials and stored at –80 °C until analyses.

Lipid Extraction

Lipids were extracted from plasma and erythrocytes using the modified method of Folch [32]. Briefly, 1 mL of freshly defrosted sample was homogenised in 45 mL of chloroform methanol (2:1 v/v) containing 0.01 % butylated hydroxytoluene (BHT), flushed with oxygen-free nitrogen (OFN), and kept at 4 °C overnight. The samples were filtered using qualitative filter paper (grade 1, 11 µm, Whatman, UK) into 100 mL separating funnels. Filters were rinsed with 15 mL of the same chloroform methanol solution and the rinse added into the funnel. Fifteen mL (25 % v/v) of 0.85 % NaCl solution were added, OFN was flushed through the samples with stoppers on, and the funnels were then stored at 4 °C overnight. The organic layer was drained into 100 mL round bottomed flasks. The solvent was removed under reduced pressure in a water bath at 37 °C using a rotary evaporator. Samples were handled under subdued light at all times. All solvents used were HPLC grade, obtained from Fisher Scientific UK (Loughborough, UK). All chemicals used were obtained from Sigma-Aldrich (Dorset, UK).

Phospholipids Separation and Gas Chromatography

Briefly, total lipids were applied onto a previously dried silica gel plate (250 µm layer thickness, 10–12 µm particle

size, 200 mm × 200 mm, obtained from VWR, UK), and thin layer chromatographically (TLC) separated with chloroform & methanol & methylamine solution (65:35:15 v/v/v) containing 0.01 % BHT. The phospholipid bands were visualised by spraying the developed plates with 2,7-dichlorofluorescein (0.01 % w/v in methanol) and identified by comparison with authentic standards (Sigma) under UV light. The phospholipid fractions analysed in erythrocytes were phosphatidylethanolamine (ePE), phosphatidylcholine (ePC), sphingomyelin (eSM) and phosphatidylserine (ePS); while those analysed in plasma were phosphatidylcholine (pPC) and sphingomyelin (pSM). Fatty acid methyl esters (FAMES) were obtained from the scrapped TLC bands by transmethylation with 15 % acetyl chloride in dried methanol for 3 h at 70 °C under OFN in an airtight glass vial. FAMES were extracted with petroleum spirit, dried, dissolved in 1 mL of heptane containing 0.01 % BHT and kept at 4 °C until gas chromatography. Phospholipid fractions were injected in a HRGC Mega 2 Series Gas Chromatograph (Fisons Instruments, Italy) and separated in a capillary column (60 m × 0.32 mm 0.25 µm film thickness, BPX70, SGE, UK) using hydrogen as carrier gas. FAMES were identified by comparison with the retention times of previously injected authentic standards (Sigma). The calculation of area percent is assumed to be equivalent to weight percent, as equal weight standards were used to regulate the instrument response to be equivalent across all FAMES, as standardized by Bueno et al. [33]. EZChrom Elite Chromatography Data System (Agilent Technologies, San Ramon, USA) was used to quantify the peak areas.

Statistical Analysis

Values are expressed as mean ± standard deviation. Normality tests were carried out using IBM® SPSS® Statistics Version 19. Statistical evaluation was carried out using Microsoft Office® Excel® 2010 with Analysis ToolPak add-in. The differences in phospholipid fatty acid profiles were assessed using independent samples Student's *t* test, equal variances assumed. Significant difference were assumed if *p* < 0.05.

Results

Self-assessed dietary intake revealed no differences in the consumption of fish per week among all the participants (data not shown). None of the volunteers included in the study reported consumption of dietary supplements (vitamins, fish oil, other essential fatty acid supplements of vegetarian origin or not) in the 2 months prior to blood collection. The age and BMI of all participants are statistically similar (Table 1).

The aminophosphoglycerides ePE and ePS, which are rich in PUFAs, did not present relevant alterations within the 51 fatty acids identifiable by our technique (data not shown).

The PC fractions of smokers presented no alterations in the amount of SFA, but did present a significant increase in the amount of c18:1n-7 (cis-vaccenic acid) in the plasma (pPC), and a significant increase in the total amount of MUFAs in erythrocytes (ePC) of smokers (Table 2). A significant reduction in c20:3n-6 (dihomo-gamma-linolenic acid, DGLA), the AA precursor, was found in both pPC and ePC of smokers, but no other relevant alterations in N-6 or N-3 PUFAs were found (Table 2).

The pSM of smokers presented significant decrease in c18:0 (stearic acid), c22:0 (behenic acid) and c24:0 (lignoceric acid), but these reductions did not reflect in a significant decrease in the total amount of SFA. In eSM, a significant increase in c16:0 (palmitic acid) was found, but this was followed by a decrease in c18:0 and no differences were found in the total amount of SFA (Table 3). Significant reduction in c18:2n-6 (linoleic acid) was found in eSM, and reduction in c20:3n-6 was found in both pSM and eSM in smokers. Reductions in c20:4n-6 (AA), in the total amount of N-6 PUFAs, in c22:6n-3 (DHA) and in the total amount of N-3 PUFAs were observed in the eSM of smokers (Table 3).

Discussion

In the UK, smoking is a major burden for the public health system and a leading cause of death. According to the National Health Service (NHS) Information Centre, in England only, in 2007–2008, there were 1.4 million hospital admissions with a primary diagnosis of a disease caused by the effects of smoking [34]. According to the UK Office for National Statistics [35], in 2010 in England and Wales, 16,807 deaths were registered for males with underlying cause of death as malignant neoplasm of trachea, bronchus and lung. Every year, the NHS spends ~£ 5.2 billion for treating illnesses associated with smoking [34]. Estimations of the UK Alzheimer's Society reveal that by 2,025 one million people in the UK will have

Table 1 Age (years) and body mass index (BMI) of healthy volunteers (control; *N* = 10) and smokers (*N* = 12)

	Control	Smokers
Age	27 ± 3.0	25 ± 1.8
BMI	22 ± 2.3	24 ± 3.9

Data are presented as mean ± SD

Table 2 Fatty acid composition of plasma and erythrocyte phosphatidylcholine of male healthy volunteers (control) as compared to smokers

Fatty acid	Plasma		Erythrocyte	
	Control	Smokers	Control	Smokers
c16:0	27.71 ± 1.37	27.74 ± 2.08	32.98 ± 1.17	33.20 ± 1.45
c18:0	14.65 ± 1.63	14.02 ± 1.38	11.86 ± 0.83	11.72 ± 0.88
Σ SFA	42.88 ± 1.70	42.34 ± 1.35	45.36 ± 1.25	45.48 ± 0.88
c18:1n-9	11.11 ± 1.84	12.06 ± 1.93	14.82 ± 1.13	15.70 ± 1.19
c18:1n-7	1.22 ± 0.31	1.46* ± 0.22	1.29 ± 0.27	1.48 ± 0.17
Σ MUFA	12.97 ± 2.25	14.26 ± 2.00	16.60 ± 1.28	17.74* ± 1.29
c18:2n-6	25.15 ± 3.31	24.05 ± 2.64	23.63 ± 2.32	22.32 ± 2.52
c20:3n-6	3.19 ± 0.73	2.65* ± 0.43	2.05 ± 0.39	1.73* ± 0.30
c20:4n-6	9.89 ± 1.58	10.55 ± 1.67	7.30 ± 1.06	7.70 ± 1.05
Σ N-6 PUFA	39.22 ± 3.18	38.21 ± 3.04	34.00 ± 2.20	32.64 ± 2.33
c18:3n-3	0.23 ± 0.22	0.33 ± 0.10	0.16 ± 0.06	0.21 ± 0.07
c20:5n-3	0.59 ± 0.42	0.68 ± 0.34	0.49 ± 0.30	0.48 ± 0.20
c22:6n-3	2.88 ± 1.04	2.69 ± 0.80	1.85 ± 0.76	1.68 ± 0.50
Σ N-3 PUFA	4.43 ± 1.62	4.58 ± 1.07	3.00 ± 1.18	2.95 ± 0.75

Healthy volunteers (control) $N = 10$; smokers $N = 12$. Data are presented as mean ± SD of total FAs (%)

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids

* $p < 0.05$

dementia; it costs £ 17 billion a year for the public health system and is expected to increase [36].

The population we have investigated in this study are healthy young males from Indian, Sri-Lankan or Bangladeshi genetic backgrounds. It has been described in south Asian men a higher risk to develop CHD as compared to men from other genetic backgrounds [30, 31]. Furthermore, cigarette smoking is positively correlated to CHD [1] and to Alzheimer's Disease [28, 29].

PUFAs play a crucial role in inflammation resolution [37], and are essential for cell differentiation, proliferation and regeneration. Phospholipids are major components of cell membranes, and are classified in species according to their distinctive structure and functions. Phosphatidylethanolamine and phosphatidylserine are richer in PUFAs and constitute mainly the inner cell membrane, presented also in DHA-rich receptor systems and as substrate for nuclear receptors. Distinctively, phosphatidylcholine and sphingomyelin are richer in SFA and constitute mainly the outer cell membrane [38]. Phosphatidylcholine plays a major role in the transport of essential fatty acids, and is a substrate for cell signalling [39]. Sphingomyelin plays a structural role as bilayer stabilizing lipid; and it is a major compound of the myelin sheath, which surrounds the axons of some neurons [40]. Furthermore, sphingomyelin is a substrate for ceramide synthesis, a lipid second messenger involved in cell growth, intracellular trafficking, inflammation and apoptosis [41].

Lipid peroxidation is induced by oxygen in the presence of initiators such as free radicals, heat, light, photosensitizing pigments and metal ions [42]. The primary promoters of

peroxidation are free radicals, for example reactive oxygen species such as alkyl, hydroxyl, alkoxyl and peroxy radicals [43], which have been identified in the cigarette smoke and condensed tar. Additionally, the cigarette gas phase presents high concentration of nitric oxide, which reacts with oxygen to generate nitrogen dioxide, or with superoxide to generate peroxynitrite [44–46]. These compounds are also involved in alterations and damage to proteins and nucleic acids [44, 47, 48].

PUFAs are highly susceptible to peroxidation [49], and links between their peroxidation and a number of neurodegenerative diseases, including Alzheimer Disease [24], have been documented [50]. Furthermore, the role of oxidative stress in demyelination has also been demonstrated [51].

In the present study, the population recruited is relatively young (25 ± 1.8 years) as compared to the general UK population (39.7 years in 2010; UK Office for National Statistics), presents normal BMI, no other underlying health conditions nor special dietary manipulations. However, some of the fatty acid perturbations found in the phospholipids studied are dramatic. We observed a significant reduction of DGLA in pPC, ePC, pSM and eSM. Experimental studies have demonstrated the anti-atherosclerotic [52], anti-inflammatory [53] and antidyskinetic [54] effects of DGLA.

Furthermore, we observed a significant reduction of AA and DHA in eSM. Not only both AA and DHA are major constituents of neural tissue, they are also the precursors of hydroxy fatty acids with roles in resolution inflammation

Table 3 Fatty acid composition of plasma and erythrocyte sphingomyelin of male healthy volunteers (control) as compared to smokers

Fatty acid	Plasma		Erythrocyte	
	Control	Smokers	Control	Smokers
c16:0	26.18 ± 3.42	27.62 ± 4.11	25.66 ± 5.26	30.37* ± 3.65
c18:0	10.84 ± 1.64	9.69* ± 0.84	13.96 ± 4.90	10.72* ± 2.28
c22:0	9.42 ± 2.12	7.57* ± 1.41	5.45 ± 1.15	5.76 ± 0.86
c24:0	7.53 ± 1.90	5.89** ± 0.96	16.80 ± 4.70	17.43 ± 2.22
Σ SFA	56.99 ± 4.11	53.74 ± 4.57	63.56 ± 5.19	66.21 ± 4.15
c18:1n-9	5.78 ± 2.09	7.33 ± 2.68	2.09 ± 0.29	2.82 ± 3.09
c18:1n-7	0.65 ± 0.11	0.70 ± 0.11	0.25 ± 0.06	0.30 ± 0.20
c24:1	11.13 ± 4.01	11.35 ± 1.97	16.98 ± 2.80	19.26 ± 3.09
Σ MUFA	18.05 ± 4.27	20.13 ± 3.00	19.54 ± 2.60	22.59 ± 4.49
c18:2n-6	12.34 ± 3.29	13.08 ± 3.28	2.46 ± 0.29	2.11* ± 0.37
c20:3n-6	1.20 ± 0.32	0.93* ± 0.14	0.43 ± 0.21	0.28* ± 0.08
c20:4n-6	3.72 ± 0.74	3.57 ± 0.64	5.49 ± 2.44	3.49* ± 1.23
Σ N-6 PUFA	18.03 ± 4.02	18.47 ± 3.77	10.22 ± 3.51	7.02** ± 1.99
c18:3n-3	0.24 ± 0.12	0.34 ± 0.16	0.09 ± 0.03	0.09 ± 0.03
c20:5n-3	0.47 ± 0.16	0.53 ± 0.21	0.25 ± 0.08	0.28 ± 0.26
c22:6n-3	1.03 ± 0.43	1.01 ± 0.34	4.60 ± 2.50	2.56* ± 1.38
Σ N-3 PUFA	2.01 ± 0.65	2.19 ± 0.65	6.29 ± 3.27	3.86* ± 1.78

Healthy volunteers (control) $N = 10$; smokers $N = 12$. Data are presented as mean ± SD of total FAs (%)

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids

* $p < 0.05$; ** $p \leq 0.01$

and tissue regeneration [55]. Abnormalities in the erythrocyte membrane are likely to reflect abnormality in other tissues [56].

We have not found significant alterations in the fatty acid composition of phosphatidylethanolamine and phosphatidylserine, which are naturally much richer in PUFAs, and located more abundantly on the cytosolic side of cell membranes.

We speculate the reasons for the lack of differences in PUFA content within these two species could be possibly due to a physiological protective mechanism which will to some extent preserve these phospholipids from harmful peroxidation in a first instance, sacrificing phosphatidylcholine and sphingomyelin instead, which are naturally much richer in SFA. If our hypothesis is correct, it would be reasonable to suggest that chronic cigarette smoking will most probably affect PUFA levels in other phospholipids at a later stage. Remarkably, the level of phospholipid perturbation found in our study is based on young volunteers who smoke between 20 and 60 cigarettes per week; this is a fraction in relation to what another study [25] has found in terms of plasma lipid perturbations in middle-aged men who smoked >20 cigarettes per day.

Another reasonable explanation for the reduced levels of AA and DHA observed in erythrocyte sphingomyelin could be related to the fact these two fatty acids are, respectively,

the precursors of lipoxins [57] and neuroprotectins [58], endogenous regulators of inflammatory responses. Due to the increased oxidative stress induced by cigarette consumption, their substrates might have been diverted from the normal flux to attend increased metabolic demand for neuroprotection.

The present study has shown significant decreased DGLA and increased MUFA levels in plasma and erythrocyte phosphatidylcholine of young smokers. Furthermore, significant reductions were observed in major N-6 and N-3 PUFAs in plasma and erythrocyte sphingomyelin. As sphingomyelin is a major constituent of the myelin sheath, it is reasonable to suggest that the phospholipid composition of other tissues, including the nervous tissue, may be affected or will be affected at some point after chronic exposure to the use of cigarettes. As PUFA peroxidation has been documented in demyelination and neurodegenerative diseases, further studies are needed to investigate the chronic effects of cigarette smoking in the fatty acid composition of membrane phospholipids.

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Conflict of Interests The authors have no conflict of interest to declare.

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