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Age-Related Accumulation of Free Polyunsaturated Fatty Acids in Human Retina

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Key Words

Fatty acids · Gas chromatography · Lipids · Retina

Abstract

The present study reports composition of free (nonesterified) as well as total (sum of free and esterified) fatty acids (FAs) in human retina (n = 13). For free fatty acid (FFA) analysis, retina tissue was homogenized, total lipids were partitioned with ethyl acetate and subsequently applied onto a aminopropyl (NH_2) cartridge to isolate FFAs from the bulk of other lipids. FFAs were converted to methyl ester derivatives and analysed by gas chromatography using flame ionization detector. Analysis of FFAs revealed that the mean percentage composition of the major components including palmitic acid (PA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA) and docosahexaenoic acid (DHA) were 17.2, 36.7, 15.6, 8.8 and 14.2%, respectively. There were significant correlations between age of the donors' and the content of both free AA and DHA ($r_{Pearson}$ = 0.69, p = 0.005, and $r_{Pearson}$ = 0.64, p = 0.009). The mean percentage of total PA, SA, OA, AA and DHA were 22.6, 23.2, 17.7, 11.4 and 21.9%, respectively. There was no association between age and any of the major FAs. The present study provides the first evidence for the presence of FFAs in the human retina as well as an age-related accumulation of polyunsaturated fatty acids (PUFAs). The latter finding suggests an alteration in the metabolism of retinal PUFAs which can be due to an increase of oxidative stress and/or decrease of antioxidant defences during ageing.

Introduction

The fatty acid (FA) profile of human retina has been the subject of several studies. The work was pioneered by Futterman and Andrews [1] in 1964. They reported that palmitic

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acid (PA), stearic acid (SA), oleic acid (OA), arachidonic acid (AA) and docosahexaenoic acid (DHA) were the major FAs in human retinal tissue. Of these, DHA was reported to be the most abundant. These findings have been confirmed by Martinez [2] examining composition of total FAs in retina during early human development. Van Kuijk and Buck [3] investigated the composition of total FAs in human macula and peripheral retina and reported similar findings to those in a previous study [1]. They also reported that AA and DHA content in the peripheral tissue were substantially higher when compared with the macula region. Unsaturated fatty acids account for over 50% of composition of total lipids [1–3]. Of these, AA and DHA account for about 60%. AA and DHA in nervous tissues are present and esterified to neutral lipids or phospholipids [4]. The ratio neutral lipid/phospholipid FA is estimated to be about 0.15 [4]. To the best of our knowledge, no information is available in the literature about the content of free (nonesterified) FAs in human retina. Free fatty acids (FFAs) in animal retina have been reported to account for approximately 5–7% of the composition of total FAs [5, 6].

The current study was undertaken to examine composition of free and total FAs in human retina. In addition, we provide the first evidence for an age-related increase in the content of free AA as well as free DHA.

Materials and Methods

Reagents

Butylated hydroxytoluene (BHT) and boron trifluoride were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Aminopropyl (NH_2) cartridges (500 mg) were from Waters Corp. (Milford, Mass., USA). Solvents were purchased from BDH Chemicals Ltd (Poole, UK). All chemicals and reagents were of the highest purity available.

Tissue Samples

Human retinas were obtained from the Coimbra University Hospital Eye Bank. The eyes were enucleated within 6 h after donors' death. Thereafter, the corneas were excised for penetrating keratoplasty. The lenses and vitreouses were subsequently removed. The retinas were carefully peeled from the eye and sealed in a vial under nitrogen. The samples were stored at −80°C and analyzed within 1–2 months after sample collection.

Preparation of Retina Homogenate

Retina samples were homogenized for 20 s at 4°C in a Potter-Elvehjem homogenizer in 2.5 ml of ice-cold phosphate-buffered saline (10 m*M* potassium phosphate, 10 m*M* sodium chloride, pH 7.4; PBS) containing BHT (100 μ*M*).

Analysis of FA Composition

Total (Sum of Free and Esterified)

Hydrolysis and Total Lipid Extraction. Aliquots (1 ml) of retina homogenate were placed into glass vials and aqueous KOH (1 ml; 1 *M*) was added. The sample was incubated for 40 min at 60°C. pH was adjusted to 2 using HCl (1 ml; 1 *M*) and heptadecaenoic acid (2 μg in 100 μl ethanol) was added as the internal standard. Ethyl acetate (10 ml) was added and the sample was vortex mixed for 30 s. After centrifugation at 2,500 *g* for 5 min at room temperature, the upper (organic) layer was transferred into a new glass tube.

Formation of FA Methyl Ester Derivatives. Total lipid extract was dried under a stream of nitrogen. Boron trifluoride-methanol (14%) solution (500 μl) was added and the sample was heated at 60°C for 30 min. Water (500 μl) and hexane (1 ml) were subsequently added, and the sample was vortex mixed. After centrifugation at 2,500 *g* for 5 min, the hexane layer was transferred into a glass vial and the solvent was removed under a stream of nitrogen. The sample was reconstituted in hexane (50 μl).

Free Fatty Acids

Total Lipid Extraction. An aliquot (1 ml) of retina homogenate was placed into a glass vial and heptadecaenoic acid (2 μg in 100 μl ethanol) was added. Total lipids were extracted as described above.

Solid-Phase Extraction Procedure (SPE). Total lipid extract from the previous step was applied onto an NH_2 cartridge preconditioned with hexane (10 ml). The cartridge was washed with 10 ml of hexane/ethyl acetate (30/70, v/v), acetonitrile/water (90/10, v/v) and acetonitrile, respectively. FFAs were eluted by washing the column with 5 ml of ethyl acetate/methanol/acetic

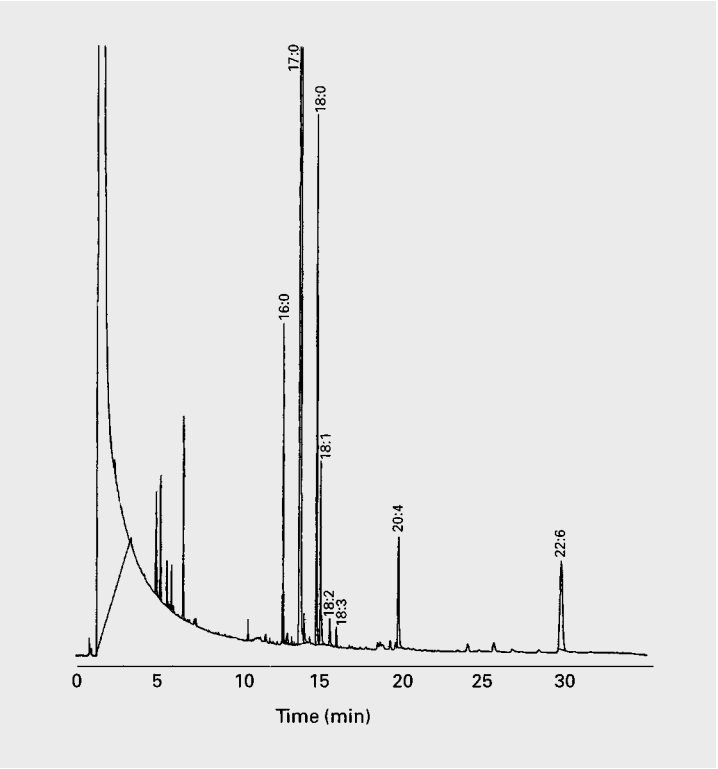

Fig. 1. Typical gas chromatographic separation of the FFA methyl esters (FAME) present in human retina. FAs were derivatized with boron trifluoride-methanol; methyl ester derivatives were separated by capillary gas chromatography on an Omegawax column (30 m × 0.53 mm) and detected by flame ionization detector. Quantification of each FAME was done automatically using heptadecaenoic acid (C17:0) as internal standard.

acid (10/85/5, by volume) [7]. The solvent was dried under nitrogen and methyl ester derivatives were prepared as described above.

Analysis of FA Composition. FA methyl esters were separated by capillary gas chromatography on Fisons GC 8000 series. Briefly, a sample (1 µl) was injected onto an Omegawax column (30 m × 0.53 mm, film thickness 1.0 µm; Supleco, Dorset, UK) and the signal was detected using a flame ionization detector (FID) at 250°C. Separation was carried out, using the following temperature program: initial temperature 170°C; initial time 2 min; rate 10°C/min; final temperature 200°C; final time 20 min.

Identification of the components in the total lipid extract was based on comparison of relative retention times with those of a standard mixture of FA methyl esters. Quantification of each component was done by integrating the area under each peak relative to that of heptadecaenoic acid (17:0) used as internal standard.

Results

Analysis of the retina samples for the composition of total FAs revealed that PA (16:0), SA (18:0), OA (18:1), AA (20:4) and DHA (22:6) were the major components. Figure 1 shows a typical gas chromatographic separation of the FFAs present in human retinas, following extraction on a NH_2 column. The peaks corresponding to the major FA methyl esters are indicated in the chromatogram (fig. 1). The mean percentage composition of PA, SA, OA, AA and DHA were 22.6, 23.2, 17.7, 11.4 and 21.9%, respectively. The samples also contained small amounts of linoleic acid (18:2) and linolenic acid (18:3), 1.5% each. Percentage composition of total FAs was not influenced by age of the donors.

Table 1. Composition of total FAs in human retina

Age, years	n	FAs, %				
		16:0	18:0	18:1	20:4	22:6
10–20	2	21.0	24.1	18.1	13.3	21.5
21–30	7	22.5 ± 3.2	23.9 ± 0.7	17.5 ± 1.6	10.3 ± 4.5	21.7 ± 4.2
31–40	3	24.2 ± 0.8	21.5 ± 1.0	18.6 ± 1.4	12.7 ± 0.7	21.1 ± 1.18
51–60	1	22.0	21.9	16.4	11.9	26.0
Average		22.6 ± 2.7	23.2 ± 1.3	17.7 ± 1.3	11.4 ± 3.4	21.9 ± 3.4

Table 2. Composition of FFAs in human retina

Age, years	n	FAs, %				
		16:0	18:0	18:1	20:4	22:6
10–20	2	13.3	35.1	11.9	5.7	7.8
21–30	7	18.6 ± 5.6	38.4 ± 4.5	16.9 ± 2.8	8.4 ± 1.6	13.3 ± 6.2
31–40	3	17.3 ± 1.5	34.1 ± 2.1	15.3 ± 1.3	11.5 ± 1.1	17.9 ± 2.6
51–60	1	14.9	34.7	14.2	9.9	22.5
Average		17.2 ± 4.8	36.7 ± 7.5	15.6 ± 3.0	8.8 ± 2.3	14.2 ± 6.1

Table 1 summarizes percentage composition of total FAs in the different age groups.

Analysis of composition of FFAs revealed that PA, SA, OA, AA and DHA were also the major components in the human retina samples. The mean percentage compositions of PA, SA, OA, AA and DHA were respectively 17.2, 36.7, 15.6, 8.8 and 14.2. The samples also contained small amounts of linoleic acid (18:2) and linolenic acid (18:3), 2.3 and 1.3% respectively. Table 2 summarizes percentage composition of FFAs in the different age groups.

There was a significant association between age of the donors and the relative abundance of free AA as well as the free DHA in the retina samples ($r_{Pearson}$ = 0.6879, p = 0.005, and $r_{Pearson}$ = 0.6398, p = 0.009). Age-related accumulation of polyunsaturated fatty acids in human retina samples is shown in figure 2.

Discussion

This study is first to report the presence of free (nonesterified) FAs in human retina as well as an age-related change in the relative abundance of free AA and as well as free DHA. The technique for the isolation of FFAs involved total lipid extraction with ethyl acetate followed by chromatography using an NH_2 cartridge. The NH_2 cartridge functions as an ion exchanger selectively binding organic molecules with a carboxylic moiety. The NH_2-chromatography step was initially used

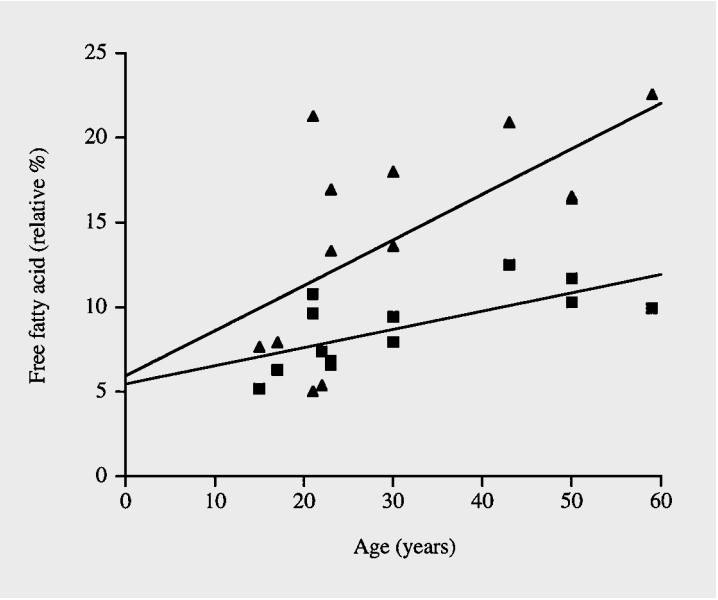

Fig. 2. Relationship between donors' age (years) and relative abundance of free AA (■) and DHA (▲) in human retina. Correlation for AA: $r_{Pearson} = 0.689$, $p = 0.005$; correlation for DHA: $r_{Pearson} = 0.640$, $p = 0.009$.

to isolate FFAs from neutral lipids such as cholesterol esters and/or triacylglycerides [8]. The NH_2-chromatography step has also been shown to be a valuable tool in isolating prostaglandin-like compounds from biological fluids such as plasma and isolated lipoproteins [7, 9]. Advantages of the NH_2-chromatography step over the conventional thin-layer chromatography (TLC) for isolation of FFAs and related compounds are higher recovery and speed. The latter is important in minimizing lipid peroxidation which could occur during TLC.

PA, SA, OA, AA and DHA were the major FFAs in the human retina with SA being the prominent component (37%). Other major FFAs were present in relatively similar amounts (12–17%). Unsaturated FAs accounted for approximately 37% of total FFA composition. Of these, polyunsaturated fatty acids (PUFAs) accounted for 62%. Significant correlations between age of the donors and the content of free AA and free DHA were found. The mechanism(s) for the age-related enhancement of free PUFAs during ageing in the human retina are not yet understood.

PA, SA, OA, AA and DHA were also the major FAs when the retina samples were analysed for composition of total FAs. PA, SA, OA and DHA present in relatively similar amounts (approximately 20–24% each). AA accounted for 12% of the total FA composition. Unsaturated FAs accounted for about 54% of the total FA composition. Among unsaturated FAs, PUFAs accounted for 61%. Our figures for the percentage composition of total FAs are in good agreement with those reported by other investigators [1–3].

Information on the presence of FFAs in human retina is rather limited. Bazan [10] has reported that, in nervous tissues, FFAs (e.g. AA and DHA) are not detected under non-stress conditions, due to the tight metabolic control of the various enzymatic steps involved in the release of esterified fatty acids. It should, however, be remarked that this

assumption is based on the analysis of brain tissues. In an attempt to settle the discrepancy between our findings on the presence of FFAs in human retina and those reported by Bazan [10], human brain homogenates (n = 3) were analysed for the content of FFAs following extraction of total lipid and the NH_2-chromatography step. The analysis revealed no quantifiable levels of FFA, confirming previous findings by Bazan [10]. The other difference between the two tissues is that PUFAs in the retina are predominantly esterified to the sn-1 position while those in the brain are mainly esterified to the sn-2 position [11].

It seems unlikely that FFAs are released during the postmortem time. In fact, retina samples incubated for 6 h at room temperature show only a slight increase on the amount of FFAs, which is whithin the coefficient of variation for the levels detected in human samples and without any statistical significance (data not shown). Moreover, analysis of all retina samples revealed that there is no correlation ($p > 0.1$) between postmortem time for collection of retinas (2–6 h) and the amount of FFAs in the tissue.

Other lines of evidence which argue against a role of activation of hydrolytic enzymes during isolation of tissue as a possible source for FFAs in the human retina are: (1) no changes in the content of SA as the predominant FFA in the different age groups; (2) no significant changes in the ratio free AA to free DHA in the retina from the three different age groups and (3) correlation between age and the content of free AA as well as free DHA (fig. 2).

There is no obvious age-related decrease on the relative abundance of the saturated FAs PA and SA or on the unsaturated oleic acid (table 2). It therefore appears that the age-related increase on the free unsaturated FAs is selective to AA and DHA and may either result from an increase in the total amount of these unsaturated FAs with age or to an age-related activation of enzymatic systems that selectively hydrolyse AA and DHA from phospholipids.

The exact mechanism(s) for age-related increase in the relative abundance of free PUFAs is not yet understood. One possibility could be progressive degradation of retinal structures during ageing, particularly light-stimulated degradation of photoreceptors. Photoreceptors are particularly enriched in AA and DHA. Photoreceptor cells have the highest AA and DHA content in the human body [12]. It has been consistently shown that prolonged exposure of animals to light appears to result in a progressive degeneration of photoreceptor cells, which is accompanied by a concomitant loss of esterified PUFAs [13]. Different levels of environmental exposure to light and/or reduced antioxidant defence mechanisms could further explain the requirement for higher free AA and DHA during ageing. In further support of an association between ageing and increased oxidative damage in retina, is the observation by Trevithick and Dzialoszynski [14] indicating an age-related increase of superoxide-like reactive oxygen species in retinal homogenates of rat. Oxidative stress also appears to influence the intracellular signalling pathways by increasing the production of inositol phosphates [15] increasing the calcium concentration and inducing the release of endogenous glutamate by a calcium-dependent mechanism [16]. In such context, it is also possible that accumulation of AA and DHA may reflect these disturbances on calcium homeostasis or other stress conditions resulting in the activation of membrane phospholipases and release of AA and DHA.

Release of FFAs during ageing and oxidative stress may modulate a variety of physiological processes by mechanisms that are still largely unknown. In retina, for example, it has

been shown that, under oxidative stress, there is an increased release of AA, which seems to inhibit Na^+-dependent transporters for glutamate and GABA [16, 17]. Accumulation of glutamate in the synaptic cleft leads to an overactivation of the glutamate receptors and possibly cell death by an excitotoxic mechanism. On the other hand, free AA has been reported to be a retrograde messenger, influencing the release of the neurotransmitters from the presynaptic ending [18]. Therefore, increased levels of free AA may contribute to retinal degeneration during ageing and also to a number of age-related pathologies of the retina.

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