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Low-temperature improved-throughput method for analysis of brain fatty acids and assessment of their post-mortem stability

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Abstract

Deficiency of docosahexaenoic acid (DHA) and other omega-3 (ω3) fatty acids may constitute an alterable risk factor for Alzheimer's disease (AD). Mechanisms of potential involvement of DHA in the disease process have been postulated primarily from studies in vitro and in mouse models of AD. Information on the fatty acid profile of the brain in AD itself is limited and in some respects contradictory. Interpretation of the findings is complicated by the diversity of methods used in previous studies and a lack of information as to the effect of post-mortem delay on the results. Here we report the development of a simple and highly reproducible method that enables relatively high-throughput measurement of the fatty acid composition in samples of brain tissue and using this method we have demonstrated that there is no significant change in fatty acid composition under conditions designed to model post-mortem delay of up to 3 days at 4 °C (or even at room temperature). The development of this method and the observation that delay of up to 3 days has no effect on fatty acid content will facilitate further studies of fatty acid composition on large cohorts of post-mortem brains.

Keywords: Docosahexaenoic acid; Methylation; Saponification; Post-mortem; Lipid

1. Introduction

Evidence that brain lipid metabolism may influence the development of several neurological diseases, including AD, includes the observations that (i) the gene encoding the cholesterol transporter apolipoprotein E type 4 is a major risk factor for both sporadic and familial AD, (ii) epidemiological (Barberger-Gateau et al., 2002; Conquer et al., 2000; Larrieu et al., 2004; Morris et al., 2003; Tully et al., 2003; Young and Conquer, 2005) and experimental studies have indicated that increased ω3 fatty acid consumption decreases the risk of developing a number of diseases including AD (reviewed in Assisi et al., 2006) and (iii) the modern human diet is deficient in ω3 fatty acids compared to the paleolithic diet on which humans evolved (Broadhurst et al., 2002; Muskiet et al., 2004).

Dietary ingestion of the brain-essential 22 carbon ω3 polyunsaturated fatty acid, docosahexaenoic acid (DHA; 22:6n-3), found predominantly in marine, oily fish and algae, has been repeatedly implicated as a potential modifier of the risk of development of AD. This fatty acid, which is concentrated in synaptic membranes (Breckenridge et al., 1972), is considered critical for normal learning and memory (Hashimoto et al., 2002; Moriguchi et al., 2000; Salem et al., 2001) and neurodevelopment (Lauritzen et al., 2001). DHA is necessary for normal membrane fluidity and function (Farkas et al., 2000; Valentine and Valentine, 2004). Dietary manipulation of DHA levels in a transgenic mouse model of AD has shown that DHA protects against dendritic degeneration (Calon et al., 2004). DHA deficiency is associated with oxidative stress and caspase-mediated cleavage of actin, which may be responsible for damage to dendritic scaffold proteins (Calon et al., 2004). Low brain DHA levels cause a decline in NMDA receptor density (Calon et al., 2005). DHA has anti-oxidant activity (Bazan, 2005; Calon et al., 2004; Cole et al., 2004) and is a potential stimulator of cerebral activities of catalase and glutathione (Hossain et al., 1999). DHA lowers cerebral Aβ load and potential downstream toxicity in an aged mouse model of AD (Lim et al., 2005) and, accompanied by the biosynthesis of, neuroprotectin D1, lowers neuronal secretion of Aβ (Lukiw et al., 2005).

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DHA released from membrane lipid stores by phospholipase A_2 provides substrate for the biosynthesis of D class resolvins, and neuroprotectins including neuroprotectin D1. These bioactive docosanoids inhibit the synthesis of pro-inflammatory arachidonic acid (20:4 n-6) derived prostaglandins leukotrienes and thromboxanes and are implicated in the prevention of neuroinflammation associated with acute neural trauma and neurodegenerative diseases (Farooqui et al., 2007). DHA metabolite neuroprotectin D1 also inhibits Aβ42-induced apoptosis of cultured human neural cells (Lukiw et al., 2005), is a potent stimulant for resolution of inflammation (Schwab et al., 2007) and a key component of survival signalling in AD (Lukiw and Bazan, 2006). Neuroprostanes, free radical-mediated derivatives of DHA, are increased in the brain and cerebrospinal fluid in AD (Montine et al., 2004; Reich et al., 2001).

Although much information is therefore available about the potential involvement of DHA in AD, it is presently unclear how DHA levels are affected in this disease, as the fatty acid composition of brain tissue has been analysed in only a few studies, using different methods and based on small numbers of samples with a range of post-mortem delays. Here we report the development of a method that enables relatively rapid and accurate measurement of cerebral fatty acid composition. We have, in addition, modelled post-mortem delay of various durations at 4 °C and at room temperature and shown that storage of brain tissue for up to 72 h, even at room temperature, has no effect on the fatty acid profile of frontal cortex as determined by this method.

2. Materials and methods

2.1. Materials

Brain tissue was obtained from the South West Dementia Brain Bank, University of Bristol, with local Research Ethics Committee approval. Two ml screw-top sealable glass vials purchased from Fisher Scientific (Loughborough, UK) were used during experimental procedures unless indicated. Solvents and butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-4-methylphenol) which was added to chloroform–methanol solutions at a concentration of 100 mg/l were also from this supplier.

2.2. Lipid extraction

Samples of anterior frontal cortex (Brodmann area 10) from four normal human brains with short post-mortem delays (4–10 h) were used for lipid extraction by a procedure based on the Folch method. Centrifugation steps were conducted for 5 min at 2000 × *g*. Four replicates – one sample from each normal brain – were used for analysis of every experimental condition.

2.2.1. Mechanical homogenisation

Brain tissue (50 mg of frontal cortex) in 350 μl of methanol at 0 °C was homogenised in a Bertin Technologies Precellys 24 mechanical homogeniser (Stretton Scientific, Stretton, UK) at 6000 rpm for 2 s × 30 s with 15–25 2.3 mm Biospec Products zirconia–silica beads (Stratech Scientific, Newmarket, UK) in a screw cap 2 ml microcentrifuge tube that had been washed by vortexing with 1 ml of chloroform three times. The sample was cooled on ice after homogenisation. Chloroform (400 μl) was added and the solution briefly vortexed. The homogenate was removed and the beads/microcentrifuge tube washed with a further 150 μl of chloroform:methanol 2:1 (v:v). The two solutions were combined and spun. The supernatant was removed and the pellet vortexed in 200 μl of chloroform:methanol 1:1 (v:v). The resuspended pellet was re-spun. The two supernatants were combined and after addition of a further 400 μl of chloroform and 325 μl of 0.9% (w/v) potassium chloride the solution was vortexed and spun. The lipid fraction obtained from the lower chloroform phase was used to determine the fatty acid composition of neural tissues.

2.2.2. Manual homogenisation

Brain tissue was homogenized in chloroform:methanol 2:1 (v:v) using a Potter-Elvehjem homogeniser and the lipids were extracted by the Folch method (Folch et al., 1957).

2.3. Saponification

The chloroform phase was removed under N_2 and 200 μl of ethanolic KOH (1 M) was added to the lipid residue. The solution was capped and left overnight at room temperature or capped under nitrogen and kept at 80 °C for 1 h. Distilled water (1 ml) was added to the saponification mixture along with 0.7 ml of hexane:diethylether 1:1 (v:v) and the biphasic solution was vortexed and spun for 5 min at 2000 × *g*. The cholesterol containing upper phase was discarded. The addition of hexane:diethylether, vortexing, centrifugation and removal of upper phase steps were repeated three times. The pH of the lower phase was adjusted to pH 3 with 5 M hydrochloric acid. 600 μl of diethyl ether was added followed by vortexing, centrifugation (as above) and removal of the upper phase repeated twice and the lower phase was discarded. The two upper phases (containing the free fatty acids) were combined and dried under N_2.

2.4. Methylation

Methyl esters of free fatty acids were prepared as described previously with a solution of 2.5% concentrated H_2SO_4 in anhydrous methanol either capped and left overnight at room temperature or capped under nitrogen and kept at 80 °C for 1 h (Fraser et al., 2004).

2.5. Analysis of fatty acid methyl esters

Fatty acid methyl esters were separated and quantified using a 30 m × 0.25 mm fused silica DB-23 column J and W Scientific capillary column supplied by Fisher Scientific (Loughborough, UK) and a Shimadzu GC-14B series gas chromatograph with flame ionization detection as described previously (Qi et al., 2004). Fatty acids methyl esters were identified by comparison of retention time with those of authenticated standards (Sigma–Aldrich, UK).