Detection of a homologous series of C_{26}–C_{38} polyenoic fatty acids in the brain of patients without peroxisomes (Zellweger's syndrome)

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The brains of patients with inherited abnormalities in peroxisomal structure and function contain greatly increased proportions of a homologous series of unique polyenoic fatty acids with carbon chain lengths ranging from 26 to 38. Based on evidence by chemical ionization and electron impact mass spectrometry before and after catalytic hydrogenation, and argentation t.l.c., these lipids have been tentatively identified as 26:5, 28:5, 30:5, 30:6, 30:7, 32:5, 32:6, 32:7, 34:5 and 34:6 fatty acids. A further two fatty acids eluting at very high temperatures from gas chromatography columns have been tentatively identified on the basis of their chemical ionization mass spectra as 36:6 and 38:6 fatty acids.

INTRODUCTION

It is well known that human brain is a rich source of polyenoic fatty acids. However, most investigations thus far have been confined to those fatty acids with carbon chain lengths > 22. Recent reports however, have confirmed the existence of polyenoic long chain fatty acids, i.e. those fatty acids with carbon chain lengths > 22 (VLCFA) in another rich source of polyenoic fatty acids, the rat testis (Grogan & Heath, 1983). In addition, it has been reported that human endothelial cells in culture synthesize and then excrete 24:4, 24:5, 26:4 and 26:5 fatty acids into the cell culture medium (Rosenthal & Hill, 1984). To our knowledge there have been no reports of these lipids in human brain.

We have recently been investigating the metabolism of saturated VLCFA in patients with the cerebro-hepato-renal (Zellweger) syndrome, a rare inherited disease which is characterized ultrastructurally by the virtual absence of peroxisomes in all tissues thus far examined (liver, kidney and fibroblasts), (Kelley, 1983; Arias *et al.* 1985) and biochemically by the accumulation of VLCFA in plasma and cultured skin fibroblasts (Moser *et al.* 1984). Preliminary analysis of brain fatty acids from these patients revealed the unexpected presence of a unique class of polyenoic fatty acids with carbon chain lengths ranging from 26 to 38 and indicates a possible role for these compounds in normal human brain. Their presence in elevated amounts in patients with ultrastructural evidence of peroxisomal abnormalities suggests that this organelle may be involved in their metabolism.

EXPERIMENTAL

Materials

All reagents were of analytical grade, and solvents were distilled prior to use. Silica gel 60 (20 cm × 20 cm) thin layer plates were obtained from Merck, Darmstadt, West Germany. Fused silica columns were obtained from SGE, Melbourne, Australia.

Methods

Lipids were extracted from 0.2–0.5 g of post-mortem whole brain from patients with Zellweger's syndrome, and from age-matched controls, (1–5 months) according to the procedure of Folch *et al.* (1957). The total lipids were transesterified with 0.27 M-H_2SO_4 in methanol and the fatty acid methyl esters were isolated by preparative t.l.c. as described by Poulos *et al.* (1985). The total fatty acid esters were applied under N_2 as 4 cm zones to $AgNO_3$-impregnated silica gel 60 t.l.c. plates prepared as described by Inomata *et al.* (1982) and chromatograms were developed in hexane/diethyl ether/acetic acid (90:10:2, by vol.). A mixture of 20.0, 20:1, 20:2, 20:3, 20:4 and 22:6 fatty acid methyl esters (purchased from NuChek Prep, Elysian, MN, U.S.A.) was used as a chromatographic marker. After development the plates were allowed to air dry briefly and were then sprayed with 0.2% (w/v) dichlorofluorescein in ethanol in order to locate the various methyl ester zones. Under these chromatographic conditions the brain fatty acids split into seven zones (i.e. saturated, and with one to six double bonds). Each zone was quickly scraped from the plate and eluted with 4 ml of chloroform/methanol (2:1, v/v). Dichlorofluorescein was removed by partitioning (Folch *et al.*, 1957) after the addition of two drops of 2 M-NH_4OH. The purified fatty acid methyl esters were stored at −10 °C under N_2 until ready for g.l.c. or hydrogenation. In later experiments the antioxidant butylated hydroxy-toluene (BHT) was included in all solvents used, at a concentration of 50–100 mg/l.

Hydrogenation was performed in the following manner. The combined tetra-, penta-, and hexa-enoic

Abbreviation used: VLCFA, very long chain fatty acid.

acid fractions isolated by argentation t.l.c. were dissolved in 15 ml of hexane and reacted with H_2 gas (1 atm; 101325 Pa) for 16 h at room temperature in the presence of 15 mg of 10% (w/w) palladium on charcoal. To ensure suspension of the catalyst the mixture was stirred continuously. At the end of the reaction period the mixture was filtered through Whatman No. 1 filter paper [previously washed with chloroform/methanol (1:1, v/v)] and the filtrate was collected and evaporated to dryness under N_2 at 40 °C. The residue was dissolved in a small volume of chloroform and stored at −10 °C.

G.l.c. was performed on a Perkin–Elmer Sigma 2B gas chromatograph equipped with a 12 m × 0.3 mm (i.d.) fused silica BP-1 column (equivalent to SE-30) (1 μm phase thickness) and a splitless injector. The average linear velocity of the H_2 carrier gas was 45 cm/s. The temperature program employed was 165 °C for 0.5 min and 4 °/min to 320 °C.

The peak heights of the various fatty acid methyl ester peaks were determined with a Hewlett–Packard 3390 integrator. Data were expressed as the ratio of the peak height of a particular component relative to the peak height of docosanoic acid. This measurement has been found to be a useful biochemical index for abnormal VLCFA metabolism (Moser *et al.* 1984). Data were also expressed as a percentage of the peak height of an individual fatty acid relative to the combined peak heights of all fatty acids with carbon chain lengths > 14.

Combined gas chromatography–chemical ionization mass spectrometry was performed using a Finnegan MAT quadruple model 3200 instrument equipped with a 12 m × 0.3 mm (i.d.) fused silica BP-1 column (1 μm phase thickness). Methane was used as the chemical ionization reagent gas. Combined gas chromatography – electron impact mass spectrometry was performed using a Hewlett–Packard quadrupole model 5992B instrument equipped with a gas chromatograph which had been modified to include an SGE OC3-1 on-column injection system connected to a fused silica 12 m × 0.3 mm (i.d.) BP-1 column.

Patients

The diagnosis of Zellweger's syndrome was based on clinical history, examination, post-mortem findings and laboratory examination (Kelley, 1983). Post-mortem samples were stored at either −20 °C or −70 °C and had been stored for periods ranging from 6 months to 10 years prior to extraction.

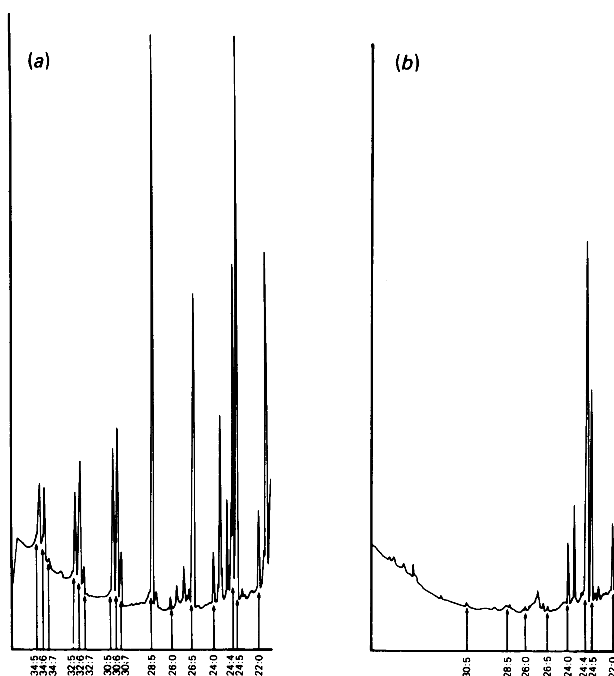

Fig. 1. G.l.c. of brain VLCFA

Fatty acid methyl esters were prepared from the total lipids extracted from whole brain and were subjected to g.l.c. as described in the text. (*a*) Zellweger brain, (*b*) control brain.

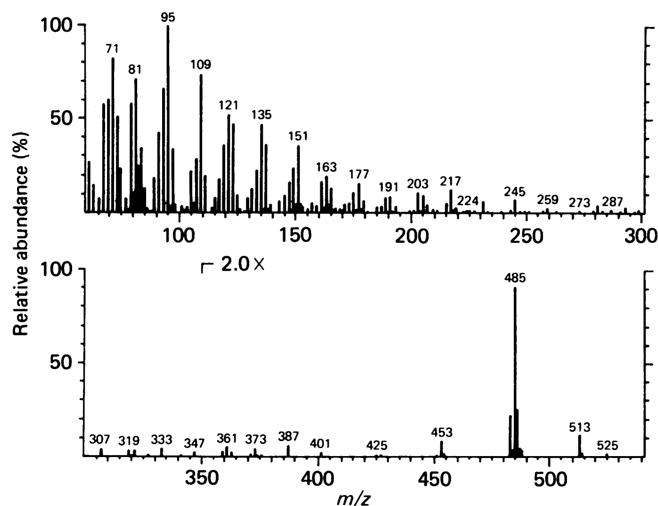

Fig. 2. Chemical ionization mass spectrometry of brain polyenoic VLCFA

Combined g.l.c.–chemical ionization mass spectrometry was carried out as described in the text. The spectrum shown above was derived from the fatty acid tentatively identified as 32:5.

RESULTS AND DISCUSSION

As expected, analysis by g.l.c. of the fatty acid methyl ester fractions (previously isolated by preparative t.l.c.) revealed that saturated VLCFAs are only trace components of normal neonatal human brain and further that the brain of patients with Zellweger's syndrome contain increased proportions of these lipids, mainly 26:0. However, a number of other compounds which collectively comprised < 2% of fatty acids of carbon chain lengths > 14 (based on peak height measurements) and which because of their elution times were originally though to be VLCFA with an odd number of carbon atoms, were also detected in Zellweger brain, and to a much lesser extent in normal neonatal brain (< 0.2%) (Fig. 1). The ratios of peak heights of these compounds relative to docosanoic acid (22:0) increased at least 15-fold in Zellweger brain. These latter compounds were not in the saturated fatty acid fraction isolated by argentation t.l.c. but were found instead in the tetraene, pentaene, and hexaene fractions, indicating that they may have been polyenoic fatty acids containing at least four double bonds. The molecular $[M+H^+]$ ions of the methyl ester derivatives as determined by chemical ionization mass

spectrometry were 401, 429, 453, 455, 457, 481, 483, 485, 511 and 513, indicating that they were possibly 26:5, 28:5, 30:7, 30:6, 30:5, 32:7, 32:6, 32:5, 34:6 and 34:5 fatty acids, respectively (Fig. 2). At even higher temperatures (> 320 °C) two other components with molecular $[M+H^+]$ ions 539 and 567 were detected, suggesting that they were possibly 36:6 and 38:6 fatty acids respectively. Prominent $[M+H-32]^+$ fragments, thought to be formed by elimination of methanol from the methyl ester group of long chain fatty acids (Araki *et al.* 1976) were detected in all spectra. A further characteristic ion was noted in all chemical ionization spectra at $[M+29]^+$ and was probably the adduct $[M+C_2H_5]^+$. Electron impact mass spectrometric analyses of the various components showed the presence of high-abundance fragments at m/z 79 and 91. These ions are characteristic of highly unsaturated fatty acid methyl esters (Araki *et al.* 1976). The ion at m/z 74, which is normally produced in high abundance by electron impact mass spectrometry of saturated fatty acid methyl esters (Odhan & Stenhagen, 1972), and which has been reported to be greatly reduced in the spectra of the corresponding polyenoic derivatives, was barely detectable. Molecular (M^+) ions were detected in all spectra examined but were present in low abundance in electron impact spectra.

The combined tetra-, penta- and hexaenoic acid fraction isolated from Zellweger brain extracts after argentation t.l.c. was catalytically hydrogenated. The peaks in the gas chromatograms corresponding to the suspected unsaturated fatty acids were no longer present but were replaced by another set of peaks with retention indices identical to those of a mixture of even numbered, straight chain, saturated fatty acid methyl esters (owing to the increased retention times of the corresponding saturated derivatives we were unable to detect saturated fatty acids with carbon chain lengths in excess of 32:0). Additionally the molecular $[M+H^+]$ ions observed in the chemical ionization mass spectra of these peaks were identical to those obtained for the corresponding saturated standards.

At present it is not known why these compounds accumulate in the brains of this group of patients. It is known however that the oxidation of saturated VLCFA is reduced markedly in their tissues and it is thought that this is due to a deficiency in a peroxisomal system which is probably specific for the β-oxidation of VLCFA (Singh *et al.* 1984*a*; Poulos *et al.*, 1986). This hypothesis would appear to be supported by the recent finding that saturated VLCFA are oxidized almost exclusively in rat liver peroxisomes (Singh *et al.* 1984*a*). It should be emphasized that all studies thus far on VLCFA oxidation have been confined to the saturated lipids and, to our knowledge, there have been no reports as yet of a possible peroxisomal involvement in the oxidation of polyenoic VLCFA. However, in view of the reported ability of the peroxisomes to oxidize shorter chain polyenoic fatty acids, such as docosahexaenoic acid (22:6) (Dommes *et al.* 1981), it is likely that this organelle plays a role in the oxidation of all VLCFA species.

An alternative explanation is that there may be an overproduction of polyenoic VLCFA due to a biosythetic abnormality. Some support for this hypothesis is provided by the detection of these compounds in trace amounts in normal neonatal brain, indicating that they are not abnormal brain components (Fig. 1). Moreover, Tsuji *et al.* (1984) have reported that cultured skin fibroblasts from patients with adrenoleukodystrophy, an inherited neurodegenerative disease which is also characterized by an accumulation of VLCFA and a deficiency in oxidation (Singh *et al.* 1984*a*, *b*) show an increased production of VLCFA from exogenous stearic acid, indicating a possible biosynthetic defect. If this finding is confirmed as well in Zellweger's fibroblasts, then it may point to the deficiency of a cellular component(s) involved in the regulation of chain elongation. At present the biosynthetic pathway for polyenoic VLCFA in brain is not known. Rat testicular cells and human endocytes actively synthesize these lipids from 20:3 and 20:4 fatty acids (Grogan & Heath, 1983; Grogan, 1984; Rosenthal & Hill, 1984), presumably by a combination of desaturation and chain elongation. It seems likely, therefore, that there may be active synthesis as well in brain and, as in the testis, 20:3 and 20:4 fatty acids may be the precursors.

As yet the positions of various double bonds in these fatty acids is not known, although rat testis polyenoic VLCFA have been reported to be $\omega-6$ derivatives (Bridges & Coniglio, 1970). It is possible that brain polyenoic VLCFA may be $\omega-6$ derivatives as well, formed by the normal processes of desaturation and chain elongation from either arachidonic or γ-homolinolenic acids (Naughton, 1981). The apparent absence of VLCFA with greater than seven double bonds would therefore indicate a lack of specificity of brain Δ^4 desaturase towards VLCFA even though the chain elongation mechanism is clearly able to operate. Investigations aimed at determining the fine structure of these brain lipids and their mode of synthesis are necessary.

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