Reprinted from

Biochimica et Biophysica Acta, 424 (1976) 1—7

BBA 56700

NERVONIC ACID BIOSYNTHESIS BY ERUCYL-CoA ELONGATION IN NORMAL AND QUAKING MOUSE BRAIN MICROSOMES. ELONGATION OF OTHER UNSATURATED FATTY ACYL-CoAs (MONO AND POLY-UNSATURATED)

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(Received April 14th, 1975)
(Revised manuscript received September 19th, 1975)

Summary

Biosynthesis of nervonic acid by enzymatic elongation of erucyl-CoA has been studied in mouse brain microsomes. The substrate and cofactor requirements have been measured. Malonyl-CoA and reduced nicotine-adenine-dinucleotide phosphate are required, but not FMN, FAD or NADH. The effect of protein concentration, incubation time, ATP and CoA has been determined; the reaction products were checked by gas-liquid chromatography with automatic counting of the eluate. Very little activity was found in hydroxylated fatty acids. In the presence of phosphotransacetylase (which impedes the de novo microsomal system), the main reaction product was nervonic acid. It is concluded that nervonic acid is biosynthesised by elongation using a two-carbon unit from malonyl-CoA. The same enzyme biosynthesises saturated and mono-unsaturated very long chain fatty acids. the elongation capacity of "quaking" microsomes is reduced to 30% of the normal value with both erucyl-CoA and behenyl-CoA.

Elongation of trans isomer (brassidyl-CoA) and poly-unsaturated homologue (clupanodonyl-CoA) was compared to elongation of erucyl-CoA in both normal and mutant mice. Both unsaturated acyl-CoAs are elongated under the same conditions as erucyl-CoA in brain: the poly-unsaturated acyl-CoA is elongated more actively than the mono-unsaturated acyl-CoA in the mutant.

Introduction

Nervonic acid ($C_{24:1}$, n-9) is an important fatty acid of the central nervous system concentrated in myelin galactolipids [1—9].

Two different pathways can be proposed for nervonic acid biosynthesis:

desaturation of lignoceric acid or elongation of shorter chains. The first hypothesis is temporarily excluded for the desaturase seems to have a huge decrease in its activity when chain length is over 18 carbon atoms [10] and we have not detected any desaturation of behenyl-CoA in mouse brain microsomes [11]. So the elongation of mono-unsaturated chains seems to be the most probable pathway.

In vivo, studies have shown that the brain is able to synthesise nervonic acid by an elongation process [12,13]. In vitro, acetyl-CoA can elongate erucyl-CoA ($C_{22:1}$ acyl, *cis*, *n*-9) in brain mitochondria [14], but only low quantities of fatty acids with 24 carbon atoms [15,16] are synthesised suggesting that this mitochondrial system is not involved in the synthesis of myelin fatty acids. C_{24} fatty acids are mainly found in myelin and there is a precursor-product relationship between microsomes and myelin [17,18]. Therefore, long chain fatty acid-synthesising enzymes are found in microsomes (and not in mitochondria), besides other lipid-synthesising enzymes [19—22]. Malonyl-CoA is incorporated in very long chain fatty acids [14,23] and we have demonstrated that there are at least three different systems in mouse brain microsomes [24,25,26]. One is a de novo system, the second one synthesises fatty acids with 18 carbon atoms from palmityl-CoA and the third one builds very long chain fatty acids from stearyl-CoA. These results have been confirmed [27] and we have recently demonstrated that malonyl-CoA can easily elongate behenyl-CoA ($C_{22:0}$ acyl) [11].

Studies on fatty acid elongation have been helped by the comparison between normal and quaking mouse; the latter is a recessive autosomal mutant characterised by defective myelination of the central nervous system [28]. The very long chain fatty acids typical of myelin galactolipids are drastically reduced [6,7,29,30]; biosynthesis of lignoceric acid either from stearyl-CoA [26,31] or from behenyl-CoA [11] is impaired in the mutant mice.

The purpose of this work is to demonstrate that *n*-9 mono-unsaturated fatty acids are biosynthesised in mouse brain microsomes, and to demonstrate that the same enzyme complex biosynthesis nervonic acid and lignoceric acid by elongation of the direct precursors. In this purpose the elongation of *cis* and *trans* mono-unsaturated isomers and poly-unsaturated fatty acyl-CoAs is being compared in the normal and the quaking mutant.

Materials and Methods

Microsomes isolation

C57-black mice of either sex were used. Microsomal preparations from 18-day-old mice have been previously described [17]. Briefly, tissue is washed and homogenised in cold 0.32 M sucrose, phosphate 0.1 M pH 7, NaCl 0.9%. It is first centrifuged at 17 500 × *g* and the pellet containing cellular debris, mitochondria, synaptosomes and myelin is discarded. The supernatant is then spun at 100 000 × *g* and the resuspended pellet is respun under the same conditions. The purity was checked by electron microscopy and marker enzymes [17].

Incubation and extraction of fatty acids

Proteins were determined by Lowry's method [32]. Acyl-CoAs were syn-

thesised from fatty acids and CoA with slight modifications (Ailhaud, G.P., personal communication) of a previously described technique [33]; their purity was checked by thin-layer chromatography. The assay for unsaturated acyl-CoAs elongation was measured by determining the amount of radioactive malonyl-CoA incorporated in fatty acids. Except when indicated otherwise, incubation mixture contained routinely 15 μM acyl-CoA, 50 μM [1,3-^{14}C]-malonyl-CoA (2 Ci/mol), 500 μM NADPH, 1 mg microsomal proteins. 3 μg of phosphotransacetylase was added to block de novo biosynthesis [11,25,26] and the final volume was made up to 1 ml with phosphate/sucrose buffer. Fatty acids were extracted and methylated as previously described [26]. Identification of fatty acid methyl esters was accomplished by a combination of gas-liquid and thin-layer chromatography. Separation of hydroxy and non-hydroxy methyl-esters was performed on silica-gel thin-layer chromatography using as solvent petroleum ether/ether (80/20, v/v); $AgNo_3$ (7%)-impregnated plates separated saturated and unsaturated fatty acids using benzene/hexane (50/50, v/v). Each experiment was performed a minimum of three times. All these experiments needed about 300 normal 18 day old mice and 50 quaking mutants of the same age.

Results and Discussion

(A) Optimal conditions for elongation of erucyl-CoA; comparison with behenyl-CoA

The effect of concentration of NADPH, NADH, FMN and FAD on activity is illustrated in Fig. 1. The optimal level for NADPH was found to be about 100 μM. After 200 μM appears a plateau. The effect of increasing the amount of one of the other nucleotides (NADH, FAD, FMN) was studied both in the absence of NADPH or with a constant amount (500 μM). The addition of NADH, FAD or FMN do not produce any increase in the incorporation, with or without NADPH. The curve for behenyl-CoA is drawn from the values previously published [11].

Erucyl-CoA is elongated at an optimal rate when the concentration is 17 μM. The inhibition at higher concentration may be due to an inhibition by an excess of substrate; or, possibly, 17 μM is the critical micellar concentration and the micelles are not the actual substrates. The activity of the elongation enzyme complex is found to be not strictly dependent on the presence of erucyl-CoA: when this acyl-CoA is omitted from the incubation mixture, there is some activity presumably due to elongation of endogenous medium chain fatty acid, as reaction products are palmitate (22%), stearate (50%) and $C_{22:6}$ (23%). Reaction products in the presence of acyl-CoA are shown in Table I. Exactly the same results have been obtained with behenyl-CoA.

In the presence of erucyl-CoA, increasing concentration of malonyl-CoA produces larger amount of this substrate to be incorporated into fatty acids and the curves for either erucyl-CoA or behenyl-CoA are nearly the same. The effect of incubation time on the elongation of erucyl-CoA have been studied. The amount of malonyl-CoA incorporated appears to be linear for the first sixty minutes. There is no difference between erucyl-CoA and behenyl-CoA.

The effect of ATP was also measured. The addition of ATP to the elonga-

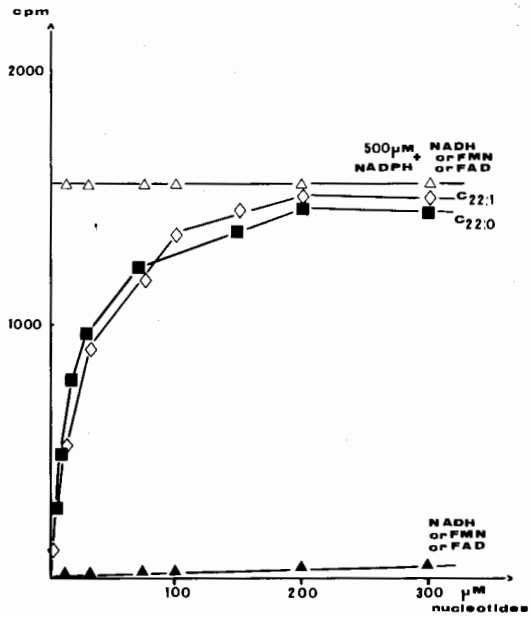

Fig. 1. Effect of various nucleotides on elongation of erucyl-CoA by radioactive malonyl-CoA. Effect of NADPH on incorporation of malonyl-CoA on erucyl-CoA ($C_{22:1}$ *cis* isomer: ◇) or behenyl-CoA ($C_{22:0}$: ■). NADH or FMN or FAD are not effective alone (▲) or in the presence of NADPH (△). Incubation medium as in methods. The radioactivity, given in cpm, per mg microsomal protein (specific activity), refers to the formation of total fatty acids (mainly C_{24} and C_{26}, little activity is found in C_{16} and C_{18}: see Table I), per mg microsomal protein.

tion reaction mixture did cause an increased incorporation of malonyl-CoA. In the presence of behenyl-CoA, the effect of ATP appears to be due to the activation of various endogenous fatty acids besides increased elongation of behenyl-CoA (unpublished results). With erucyl-CoA, the effect is probably the same. Increasing the amount of CoA does not produce any increase but a slight inhibition.

(B) Analysis of products of erucate elongation

More than 98% of the total labelled fatty acid methyl esters recovered on silica-gel thin-layer chromatography of the incubation mixture extracts mi-

TABLE I

REACTION PRODUCTS WITH AND WITHOUT PHOSPHOTRANSACETYLASE

The enzyme, when added, impedes de novo synthesis. mp: minor peaks, results expressed in percent.

Chain length	16:0	18:0	24:1	26:1	mp
Without phosphotransacetylase	32 ± 4	12 ± 3	40 ± 5	8 ± 3	5 ± 3
With phosphotransacetylase	6 ± 2	10 ± 3	64 ± 5	10 ± 3	10 ± 3

grated in the non-hydroxylated fatty acid area. Analysis on silver nitrate thin-layer chromatography shows that about 16% of the activity migrated within the saturated area (in the presence of phosphotransacetylase).

Reaction products are shown in Table I. Without phosphotransacetylase, about 44% of the activity is found in saturated C_{16} and C_{18} (due to de novo system and elongation of endogenous acceptors). Adding phosphotransacetylase decreases this percentage by impeding de novo system; so 74% of the total activity is found in very long chain monounsaturated fatty acids. (The minor peaks are mainly unsaturated C_{20}, and C_{22} with six double bonds).

(C) Elongation of monoenoic isomer and polyenoic isomer

Fig. 2 shows that either brassidyl-CoA or clupanodonyl-CoA are elongated by malonyl-CoA in mouse brain microsomes. The curves have the same shape as the one for erucyl-CoA.

(D) Comparison between normal and quaking mice

Fig. 3 shows the effect of amount of enzyme in normal and quaking mouse. The specific activity in quaking mouse is nearly 50% of normal, using *cis* or *trans* isomers of C_{22} acyl-CoA. Moreover, *cis* and *trans* isomers are elongated in the same way. It is unexpected to find that the poly-unsaturated seems to be elongated in a better way than the mono-unsaturated isomer; when using clupanodonyl-CoA, the difference between normal and mutant is about 40%. (Moreover the mutant brain has only 75% of the normal amount of microsomes [11]: therefore the synthesis of nervonic acid from erucyl-CoA is 35% normal in quaking mice; the elongation of clupanodonyl-CoA is 45% normal in the mutant).

(E) Conclusions

Nervonic acid biosynthesis is effective in mouse brain microsomes as erucyl-CoA is elongated by two carbon units. Malonyl-CoA is found to be the

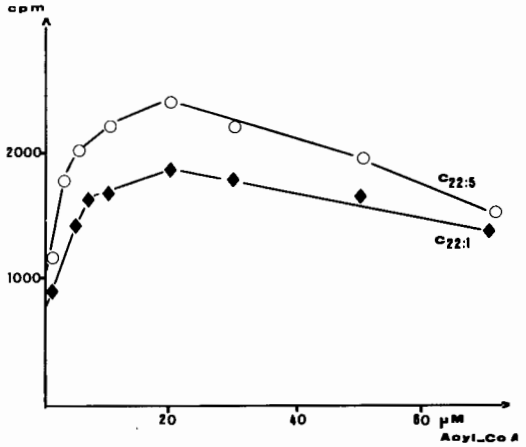

Fig. 2. Effect of various concentration of unsaturated acyl-CoAs; brassidyl-CoA ($C_{22:1}$ *trans*: ♦) and clupanodonyl-CoA ($C_{22:5}$: ○).

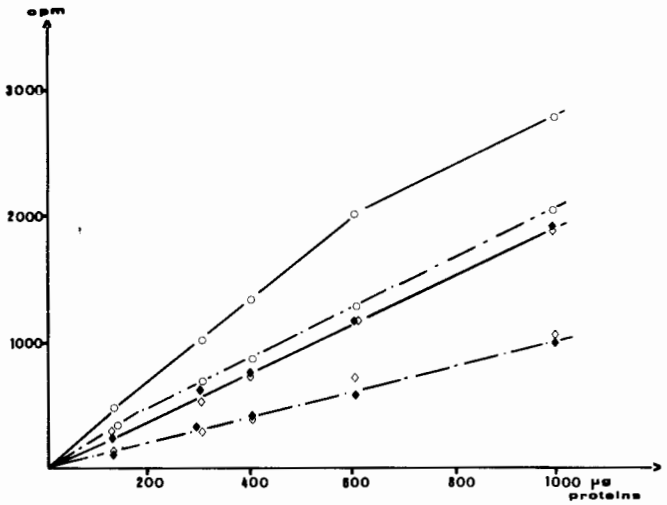

Fig. 3. Effect of varying concentration of microsomal protein on normal (full lines) and quaking (dotted lines) mice using erucyl-CoA ($C_{22:1}$ *cis*: ◇), brassidyl-CoA ($C_{22:1}$ *trans*: ◆), clupanodonyl-CoA ($C_{22:5}$: ○).

direct precursor in the presence of NADPH. So the conditions are the same for erucyl-CoA and behenyl-CoA, mainly providing respectively nervonate and lignocerate.

Thus, the *n*-9 mono-unsaturated series is made from elongation of pre-existing mono-unsaturated fatty acids. The first fatty acid being elongated is speculated to be oleic acid: in brain, it has been shown that stearic acid is easily desaturated [34,35,36] and we have shown that oleil-CoA and stearyl-CoA are elongated with the same kinetic (unpublished results). So the following pathway could be suggested.

Stearyl-CoA → (*n*-9), Δ^9 C_{18} → (*n*-9), Δ^{11} C_{20} → (*n*-9), Δ^{13} C_{22} → (*n*-9), Δ^{15} C_{24}

It is unexpected to find that *trans* isomer of erucyl-CoA (brassidyl-CoA) and poly-unsaturated fatty acyl-CoA with 22 carbon atoms (clupanodonyl-CoA) are elongated in the same way as erucyl-CoA in brain. Indeed, nervonic acid is found in brain, but *trans* isomer or poly-unsaturated homologues are not detectable. We propose that the enzyme is specific for the chain length and not for the degree of unsaturation; moreover the enzyme can work with any substrate which is available, and probably brassidic and clupanodonic acids are not. Or the enzymes which control fatty acid composition of complex lipids in brain can discriminate between the isomers on the basis of the double bond position, or between the homologues on the basis of the number of double bond. Unlike other membranes, the myelin membrane contains a high level of mono-enoic acyl group and a relatively low level of poly-unsaturated acyl group [37]. Their chain length may be necessary to give a more stable configuration to the myelin sheath; the mono-unsaturation preserves the integrity of the membrane as poly-unsaturated groups are highly susceptible to break down by peroxidase or lipoxygenase.

Acknowledgements

This work was supported by I.N.S.E.R.M., D.G.R.S.T. and Astra-Calvé.

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