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Formation of 22 and 24 carbon 6-desaturated fatty acids from exogenous deuterated arachidonic acid is activated in THP-1 cells at high substrate concentrations

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Abstract

Deuterated arachidonic acid (AA, [$^2H_8$]20:4 n-6) 1–25 μM, is converted to other fatty acids, as evaluated by gas chromatography-mass spectrometry, in THP-1 cells. The major products, in the 1 to 10 μM range, are 22:4 (elongated) and 20:3 (reduced in 5). At 25 μM, 24:4, 24:5 and 22:5 accumulate, with [$^2H_8$]/[$^2H_0$] ratios higher than in AA. At high AA concentration preferential conversion to elongated fatty acids with 5 unsaturations, through a 6 desaturase takes place and the 4-desaturated 22:5 appears to be formed through β-oxidation of 24:5.

Key words: THP-1 cell; Deuterated 20:4 n-6, GC-MS; 6 Desaturation of 24:4 n-6; 4 Desaturation in 22:5 n-6

1. Introduction

Formation of the 22 carbon Δ4 desaturated fatty acid (FA) in the n-3 series, docosahexaenoic acid (DHA, 22:6) has been assigned to a pathway involving peroxisomal β-oxidation of 24:6 n-3, in intact liver cells [1]. The corresponding Δ4 desaturated FA in the n-6 series, derived from arachidonic acid (AA, 20:4), i.e. 22:5 n-6, is present in relatively small concentrations in most tissues, except for the male gonads [2–4], and it accumulates in conditions of relative deficiency of the n-3 FA [5]. A pathway for the formation of 22:5 n-6 through an extra microsomal step involving β-oxidation of 24:5, in analogy to the synthesis of 22:6 n-3, has been proposed [6]. However, since the rates of conversion through sequential or alternative steps in FA metabolism and rates of acylation of individual FA in cell lipids are different [7], the substrate concentration may affect the pattern of formation and accumulation of individual products. Studies on the formation of 22:6 n-3 have used concentrations of the precursor (22:5 n-3) in ranges of 50 to 100 μM [1,6].

The use of deuterated FA, in which a given number of protium atoms (^1H) are replaced by deuterium (^2H) (> 98% enrichment), allows to evaluate the proportion of exogenous substrate incorporated in different cell lipid pools and/or entering various metabolic pathways. This is obtained through mass spectrometry (GC-MS) [8], which allows the distinct recognition and measurement of the two isotopes in individual FA isolated from biological samples and the determination of isotopic ratios. In addition GC-MS improves the detection of single products, due to the very high sensitivity and specificity of this technique.

We wish to report on the formation of FA with different chain length and unsaturation from [$^2H_8$]20:4, evaluated by GC-MS, in the human monocytic cell line THP-1 [9], with increasing substrate concentrations. The major finding is that $^2H_8$-labeling of 20:4, and formation of the elongated 22:4 and of the reduced 20:3 products increase up to 10 μM [$^2H_8$]20:4, whereas relative labeling and accumulation of 24:4, 24:5 and 22:5 increase only at higher substrate concentrations. The data indicate a concentration-dependent pattern in the conversion of 20:4, and suggest that at high 20:4 concentrations the major route leads to accumulation of 22 and 24 products, which is compatible with two consecutive elongations of 20:4 followed by a Δ6 desaturation.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; FA, fatty acids; PL, phospholipids; TL, total lipids; TG, triglycerides; PC, phosphatidyl choline; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; GC-MS, gas chromatography-mass spectrometry.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene, Arachidonic acid, FA-free BSA, and all PL standards were from Sigma Chemical Co. (St. Louis, MO, USA). Celite, filter agent was from Aldrich Chimica, S.r.l. (Milano, Italy). The

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solvents for HPLC and Silica gel 60 HR were from Merck Chemical Co. (Darmstadt, Germany). Arachidonic acid 5,6,8,9,11,12,14,15-[$^3H_8$] (specific activity 219 Ci/mmol) was from Amersham International, Buckinghamshire, UK; the radiochemical purity of the compound was assessed by HPLC and detection of radioactivity. Arachidonic acid -5,6,8,9,11,12,14,15-[$^2H_8$] (purity 98%) was from Biomol Research Laboratories Inc. (Plymouth, PA, USA). Fetal Calf Serum (FCS), RPMI 1640, and all compounds for cell growth were from Gibco (Paisley, UK).

2.2. Cell cultures

THP-1 cells were grown as previously described [9]. The medium contained 10% FCS and the total 20:4 n-6 content in FCS, measured by quantitative GLC analysis, was 85 nmol/ml.

The cell pellet obtained from several flasks of THP-1 cells was resuspended in fresh RPMI medium without FCS and the cell concentration was adjusted to 10^6 cells/ml. The preparation was subdivided into aliquots in flasks, which, after 24 h, were incubated in the presence of the [$^2H_8$] AA/BSA complex, prepared according to Spector and Hoak [10], at the final concentrations of 1, 5, 10 and 25 μM AA, for 24 h. This time of incubation gave maximal and stable incorporation of the FA. Two hours before the end of the incubation [$^3H_8$]20:4 was added (0.2 μCi/10^6 cells), in order to follow the incorporation in lipid classes. At the end of the incubation cells were centrifuged, resuspended in PBS + 0.5% BSA and after additional washings, they were counted.

2.3. Lipid extraction

Lipids were extracted [11], and the lipid concentration was measured by a microgravimetric method [12].

2.4. TLC and HPLC separations

Neutral lipids were separated by TLC, lipid classes were identified by the use of authentic compounds and the spots were scraped and used for further analyses. Separation of PL by HPLC was carried out essentially according to the method by Patton et al. [13], with slight modifications [9]. Individual PL were detected by monitoring absorption (205 nm) and radioactivity by an on-line detector (Flo-one beta, A-200, Radiomatic Instruments and Chemical Co., A Canberra Company, Tampa, FL, USA). Compounds were collected and used for GC-MS analysis of their FA.

2.5. Gas liquid chromatography (GC)

FA methyl esters were prepared from total lipids (TL) by acidic transesterification and analyzed by GC [9]. The FA were quantified by the use of an internal standard (nonadecanoic acid, C 19:0) and of calibration curves.

2.6. Gas chromatography-mass spectrometry

The PL isolated by HPLC, and triglycerides (TG) isolated by TLC were subjected to alkaline hydrolysis (2 M NaOH at 60°C, 2 h). After acidification at pH 3, C 19:0 was added as internal standard, FA were extracted and the pentafluorobenzyl (PFB) esters were obtained by treatment with PFB bromide (40 μl 1:9 (v:v) in acetonitrile) and 10 μl diiso-propylethanolamine (room temperature, 15 min). Derivatized FA were analyzed by GC-MS on an Omegawax 320 fused silica capillary column (30 m × 0.32 mm; Supelco Inc. Bellefonte, PA) connected with a HP 5988 mass spectrometer (Hewlett Packard, USA). Operating conditions were: temperature programming 160–300°C at 15°C/min increments, splitless injection mode, helium as gas carrier. Negative chemical inonization (NCI) was carried out with methane as reactant gas (source pressure of about 1 Torr), electron energy 220 eV and source temperature 200°C. For NCI-selected ion monitoring, carboxylated anions (M-181) were recorded. The concentration of each FA in samples was calculated on the basis of a standard curve.

2.7. Statistical analysis

Dunnet's test was applied to the evaluation of FA differences at various AA concentrations vs. non-supplemented cells.

3. Results

The supplementation of THP-1 cells with increasing [$^2H_8$]20:4 concentrations resulted in marked changes of the FA profiles of cell lipids (Table 1). An eight-fold elevation of 20:4 levels, which were very low in non-supplemented cells, occurred in the range of 1 to 25 μM 20:4 in the medium. This was associated with increments also of the elongation product 22:4, and with detectable accumulation of 22:5, in the presence of 25 μM 20:4. Some elevation of 20:3 n-6, the product of 20:4 retroconversion also occurred in the presence of 25 μM 20:4. Increment of substrate concentration in the medium resulted also in progressive reduction of 16:1 levels, which became statistically significant in the presence of 25 μM 20:4.

GC-MS analysis allows the detection and identifica-

Table 1
Weight percentage composition of FA of TL from THP-1 cells supplemented with different AA concentrations

FA	BSA	+AA 1 μM	+AA 5 μM	+AA 10 μM	+AA 25 μM
16:0	40.1 ± 1.5	37.0 ± 1.1	36.6 ± 0.8	37.0 ± 1.2	35.7 ± 5.8
18:0	13.2 ± 1.1	15.0 ± 0.6	12.9 ± 0.4	11.3 ± 0.7	8.8 ± 1.0*
16:1	13.0 ± 1.6	11.4 ± 1.0	8.6 ± 0.7	9.8 ± 0.6	6.6 ± 0.5*
18:1	25.7 ± 1.8	27.4 ± 1.2	28.7 ± 1.3	25.2 ± 0.9	20.6 ± 1.0
24:1	1.0 ± 0.4	1.2 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	0.8 ± 0.3
20:3n-9	1.3 ± 0.3	1.3 ± 0.2	1.1 ± 0.5	1.3 ± 0.3	0.7 ± 0.3
18:2n-6	0.9 ± 0.6	1.0 ± 0.5	1.4 ± 0.5	0.9 ± 0.3	1.5 ± 0.4
20:3	0.3 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.2	0.9 ± 0.4
20:4	1.4 ± 0.7	2.0 ± 0.5	5.2 ± 0.7	8.8 ± 1.2**	16.7 ± 3.2**
22:4	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.3	1.3 ± 0.9	4.7 ± 1.8**
22:5	–	–	–	–	0.5 ± 0.0
20:5n-3	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.1
22:5	0.7 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.2
22:6	1.5 ± 0.1	1.8 ± 0.1	1.5 ± 0.2	1.7 ± 0.2	1.1 ± 0.3

Values are the average ± S.E. of three measurements. Values with asterisks are significantly different from all the others at the following levels: *$P < 0.05$, **$P < 0.01$.

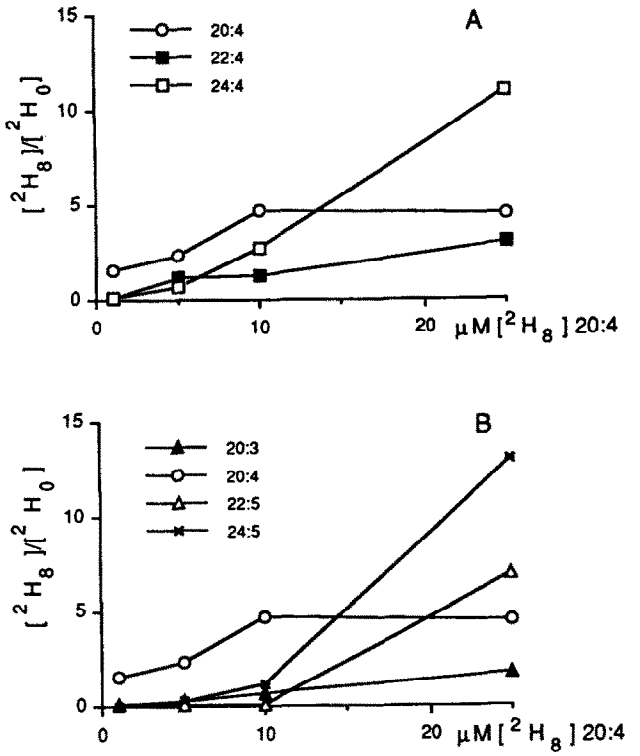

Fig. 1. $[^2H_8]/[^2H_0]$ ratios in 20:4 and other FA differing from 20:4 in chain length (Panel A), and in 20:4 and other FA differing from 20:4 in degree of unsaturation (Panel B), in the presence of increasing $[^2H_8]$20:4 concentrations in the medium. Values are the average of duplicate measurements on pools of three samples at each concentration point.

tion of FA with much greater sensitivity than conventional GC and the determination of the relative $[^2H_0]$ and $[^2H_8]$ isotopic contents. The $[^2H_8]/[^2H_0]$ ratio in the substrate was about 50 to 1 (> 98% enrichment) vs. a ratio close to 0 in the non-supplemented cells. The $[^2H_8]/[^2H_0]$ ratio in 20:4 (Fig. 1, Panel A) was already about 1 (50 percent deuterium) at 1 μM substrate vs. negligible values in the other FA. Then it increased progressively in supplemented cells up to 10 μM 20:4 in the medium, and it remained relatively constant up to 25 μM, reaching a value of around 4. In 22:4, the ratio increased up to the value of 1, from 1 μM to 5 μM exogenous 20:4, it remained constant up to 10 μM and increased again to about 3 at 25 μM 20:4. In 24:4, the isotopic ratio increased linearly from 5 to 25 μM 20:4, up to a value of about 11, quite higher than in 20:4. In products differing from 20:4 in the unsaturation (Fig. 1, Panel B), a small incorporation of $^2H_8$ occurred in 20:3, whereas in 22:5 and in 24:5, the isotopic ratios reached the values of 7 and 13, respectively, at 25 μM 20:4. When the amounts (nmol/mg of TL) of $[^2H_8]$FA were plotted versus increasing concentrations of $[^2H_8]$20:4 (Fig. 2), the accumulation of $[^2H_8]$20:4 was linear, and total 20:4 increased in parallel (Fig. 2, Panel A) up to a value of about 200 nmol/mg TL. 22:4 (Panel B) increased in the presence of 20:4 above 10 μM, reaching a substantial proportion of the amount of 20:4, at 25 μM. 20:3, a product of reduction of the 5 double bond, increased almost linearly, from 5 to 25 μM 20:4 (Panel C). 24:4, 24:5 and 22:5, present in few pmol/mg TL below 10 μM 20:4, reached the nmol/mg TL level, at 25 μM 20:4.

The isotopic ratios in individual lipid classes, in samples incubated with 25 μM 20:4 (Table 2) were highest, for 20:4, in diacyl PC, and TG, with values of 5.6 and 6, about double than in the other lipid classes (2.5–3.5). Different enrichments in single lipid classes took place for each FA. $[^2H_8]$20:3 was maximally enriched in diacyl PC, and to some extent in TG. In general, ratios for the

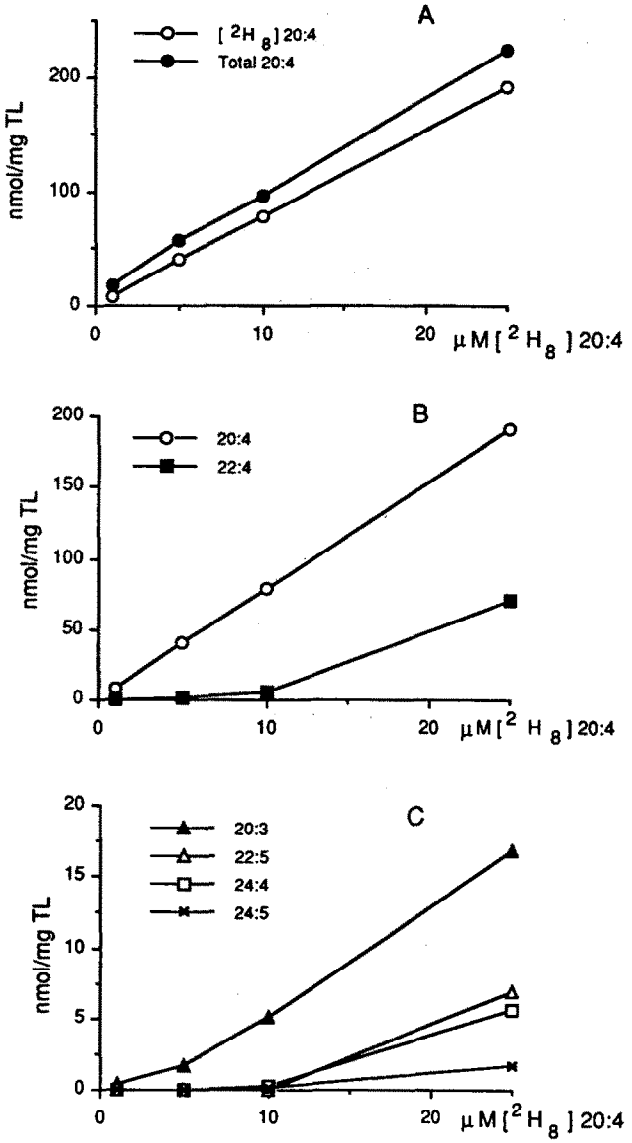

Fig. 2. Levels (nmol/mg of TL) of total 20:4 and $[^2H_8]$20:4 (Panel A), of $[^2H_8]$20:4 and $[^2H_8]$22:4 (Panel B), and of $[^2H_8]$FA differing from 20:4 in unsaturation (Panel C) in THP-1 cells in the presence of increasing $[^2H_8]$20:4 concentrations in the medium. Values are the average of duplicate measurements on pools of three samples at each concentration point.

24 C FA were higher in TG and diacyl PC than in the other fractions, the highest values being reached in 24:4 and 24:5 in TG. Among the PL, maximal isotopic ratios for 22:4, 24:4 and 24:5 were found in diacyl PC and phosphatidylserine (PS).

A general quantitative scheme of the steps leading to the formation of the FA derived from 20:4, in THP-1 cells incubated in the presence of 25 μM [$^2H_8$]20:4, is shown in Fig. 3. Quantitatively, elongation of 20:4 to 22:4 was the major step, followed by retroconversion to 20:3. The specific activities of these products remained lower than that of 20:4, even at 25 μM, indicating that a significant proportion of products of the first steps in 20:4 elongation and retroconversion was derived from the endogenous substrate. The proportionally higher incorporation of $^2H_8$ in 22:4, 24:4 and 22:5 indicated preferential formation from the exogenous 20:4.

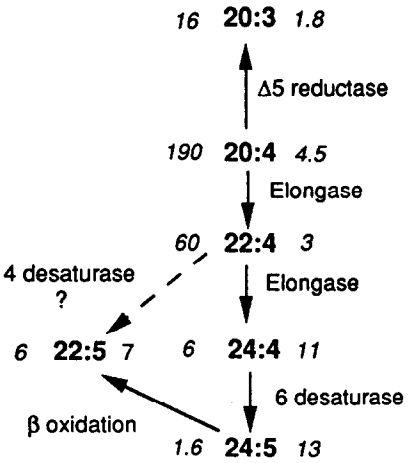

Fig. 3. Pathways for the conversion of [$^2H_8$]20:4 to the various FA. Numbers on the left of each FA indicate the amounts (nmol/mg total lipids) accumulated and those on the right the [$^2H_8$]/[$^2H_0$] ratios.

4. Discussion

GC-MS analysis and the use of deuterated 20:4 has allowed the measurement of 24 C products with 4 and 5 double bonds derived from exogenous vs endogenous 20:4. [$^2H_8$]24:4 and 24:5 were present in very small amounts, 5.5 and 2.7 pmol/mg TL, respectively, in the presence of 1 μM [$^2H_8$]20:4, and increased of about 1150- and 700-fold, respectively (up to 6.35 and 1.88 nmol/mg TL), when the exogenous 20:4 was increased 25-fold. Maximal increments occurred when [$^2H_8$] was raised from 10 to 25 μM. The increment of 22:5 n-6, the long-chain n-6 FA commonly detected in tissue lipid analysis, was quantitatively similar to that of 24:4. Accumulation of 20:3, formed through a $\Delta 5$ reductase, occurred with increasing [$^2H_8$]20:4. This reaction has been shown to be a prerequisite step in the β-oxidation of FA with their first double bond in position 5 [14].

The isotopic ratios in individual FA provide some indication of the preferential metabolic pathways at different substrate concentrations. The enrichment of 20:4 plateaued above 10 μM, suggesting that acylation reactions for this FA became saturated and that the additional substrate was preferentially elongated and desaturated. The specific acitivities of 20:4 and 24:4, however, followed a parallel trend up to 10 μM 20:4, indicating that elongation to the 24 C FA was an active pathway even at the lowest substrate concentrations.

At 25 μM 20:4, 24:4 and 24:5 had the highest specific activities, followed by 22:5, all being quite higher than that of 20:4; the deuterium enrichment of 20:3, was instead definitely lower. At 25 μM substrate concentration, [$^2H_8$]20:4 was thus preferentially elongated to 22:4 and 24:4. In addition, the identical isotopic ratios in 24:4 and 24:5, suggest that 24:5 was preferentially formed through a 6 desaturation of 24:4, rather than through elongation of 22:5. The progressive accumulation of 20:3, in contrast with the low $^2H_8$-labeling, indicates that it was preferentially produced from the endogenous pool(s), possibly displaced by the exogenous 20:4. The high labeling of 24:4 and 24:5, at 25 μM [$^2H_8$]20:4, in TG and diacyl PC suggests that the formed FA were incorporated in newly synthesized lipids. Measurements of TG levels in THP-1 cells at different 20:4 concentrations showed in fact accumulation of this lipid in cells, at 25 μM 20:4 (from 10.8 to 27 percent of cell TL). Since sequential elongation of 20:4 to 24 C FA and 6 desaturation to 24:5 occurred preferentially at 20:4 concentrations above 10 μM, when the 4 desaturated product 22:5 also accumulated, it is difficult to establish the preferential route of 20:4 conversion at physiological substrate concentrations. In our conditions, at 25 μM [$^2H_8$]20:4, the comparable $^2H_8$-labeling of 24:4 and 24:5, and the $^2H_8$ enrichement of 22:5 higher than that of 22:4, suggest that direct desaturation of 22:4 to 22:5 was not the major step, and that 4 desaturation was compatible with the pathway proposed for the n-3 series [1]. The 4 desaturation described

Table 2
[$^2H_8$]/[$^2H_0$] ratios for individual FA in individual lipid classes from THP-1 cells in the presence of increasing concentrations of [$^2H_8$]20 : 4 in the medium

Lipid class	20 : 3	20 : 4	22 : 4	22 : 5	24 : 4	24 : 5
PI	0.5	3.0	4.3	1.9	3.6	2.6
PE	0.5	2.4	5.9	4.2	12.4	8.0
PS	0.5	3.6	7.5	3.5	14.0	12.0
PC diacyl	7.2	5.6	10.3	4.7	12.0	9.3
PC alkylacyl	0.1	2.5	3.6	3.3	4.3	3.0
TG	1.9	6.0	9.3	7.6	15.0	14.6

Values are the average of three measurements. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; TG, triglycerides.

[1] in the formation 22 C n-3 FA involving two elongation steps and a 6 desaturation, however, has been reported in hepatocytes using 75 μM substrate (22:5 n-3). This concentration is presumably in excess over the endogenous substrate quite larger than, in our study, the $^2H_8$ 25 μM 20:4 concentration compared to that of the endogenous FA. Some desaturation of 22:4 n-6 to 22:5 has been reported in retinoblastoma cells over a long period of incubation, using relatively low concentrations of the substrate, which however is normally present as a minor FA in cell lipids [6].

Although it is difficult to compare metabolic pathways in different cells, it is temptative to speculate that concentration-dependent patterns of FA conversion may be generally present. It has been shown in addition, that at relatively high FA concentrations (above 50 μM) peroxisomal reactions are induced in cultured (endothelial) cells [15,16], and this could explain concentration-dependent (and time dependent) modifications of preferential patterns for FA conversion and product accumulation.

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