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Expression of fungal desaturase genes in cultured mammalian cells

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Abstract

Long-chain polyunsaturated fatty acids (LC-PUFA) are important components of cellular structure and function. Most of LC-PUFA are derived from linoleic acid and α-linolenic acid. In plants and fungi, these two acids can be synthesized from oleic acid via the action of two enzymes, Δ12 and Δ15-desaturases. Due to lack of these enzymatic activities and the ability to synthesize these two essential fatty acids, animals must obtain them from the diet. In this report, we demonstrated the expression of a fungal Δ12-desaturase gene in mouse L cells incubated in serum-free medium. The results showed a significant increase in the amount of linoleic acid with a concomitant decrease of oleic acid in cellular lipids. Most of the newly formed linoleic acid was incorporated into cellular phospholipids, particularly phosphatidylcholine. The increase of linoleic acid provided the substrate for the endogenous synthesis of (n-6) LC-PUFA, such as eicosadienoic acid (EDA), dihomo-γ-linoleic acid (DGLA) and arachidonic acid (AA). Prolonged incubation further increased the levels of linoleic acid derived from oleic acid by the action of Δ12-desaturase, and the levels of 20:2n-6 produced from linoleic acid by the action of the endogenous elongase. However, prolonged incubation suppressed significantly the formation of DGLA and AA. In a separate study, a fungal Δ6-desaturase gene has also been expressed in the mouse L cells incubated in serum-containing medium. The result shows a significant increase in levels of 20:3n-6 and 20:4n-6. These findings demonstrate that through genetic modification, it is possible to (1) generate cell lines which no longer require dietary 'essential' fatty acids and (2) alter the endogenous fatty acid metabolism to enhance the production of LC-PUFA and their derivatives. (Mol Cell Biochem **219**: 7–11, 2001)

Key words: cDNA, Δ12-desaturase, Δ6-desaturase, essential fatty acids, mouse L cells

Introduction

Fatty acyl desaturases are enzymes that are responsible for introducing double bonds into fatty acid chains to generate unsaturated fatty acids [1]. Both plant and animal cells have the ability to synthesize fatty acids up to 18 carbons in length and desaturate the fatty acid at carbon 9. However, they are significantly different in their ability to further elongate and desaturate fatty acids. Plant cells can insert double bonds at carbons 12 and 15 to form linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), but they generally lack the ability to elongate the polyunsaturated fatty acid chain past 18 carbons. In contrast, animal cells lack the Δ12- and Δ15-desaturase activities and hence cannot synthesize either linoleic acid or α-linolenic acid. However, once provided, animal cells have the ability to metabolize both linoleic and α-linolenic acids to form the (n-6) and (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) through an alternating series of desaturation and elongation. Since LC-PUFA are important components of cellular membrane structure, modifi-

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cation of cellular LC-PUFA levels has significant impact on membrane fluidity, cell-surface hormone receptors, generation of second messengers, and cellular signaling mechanisms which are necessary for normal cell functioning [2–5]. Hence, these essential fatty acids must be obtained from the diet [6].

In this study, we demonstrate the expression of a heterologous Δ12-desaturase cDNA in cultured mouse L cells. Expression of the Δ12-desaturase results in the generation of cells which no longer require exogenous sources of the essential fatty acid, linoleic acid (18:2n-6). We have also expressed a fungal Δ6-desaturase gene in mouse L cells and increased the cellular levels of n-6 LC-PUFA.

Materials and methods

Construction of Δ12-desaturase expression plasmid

A 1,209 bp *Eco*RI-*Xho*I DNA fragment encoding the *Mortierella alpina* Δ12-desaturase cDNA, (see ref. [7] and Genebank accession # AF110509), was ligated into plasmid pCMV-BGH-C which had previously cleaved with *Bgl*II and *Sma*I. The termini of all DNA molecules were made flush using Klenow polymerase prior to ligation. The resulting plasmid, pCMV-Δ2-bGH, utilizes the human Cytomegalovirus immediate early (CMV) transcriptional regulatory element to direct Δ12-desaturase expression and the bovine growth hormone (bGH) polyadenylation signal for proper mRNA processing [8].

Cell culture and generation of stable cell lines

Mouse L cells [thymidine kinase negative (TK-) and adenine phosphoribosyltransferase negative (APRT-)] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Nu-serum (Collaborative Research Inc., Bedford, MA, USA). Stably transformed L cells were generated using the Ca_2PO_4-precipitation method as previously described [9, 10]. Briefly, 1×10^6 of mouse L cells were seeded into 100 mm dishes for 18 h at 37°C. The selection plasmid, pDλAT3 (30 ng), high molecular weight L cell DNA (15 μg) and pCMV-Δ12-bGH (3 μg) were added to the cells as a Ca_2PO_4 precipitate and the cultures incubated for 4 h at 37°C. Following incubation, the solution was removed and the DNA introduced into the cells by a 90 sec DMSO osmotic 'shock'. The cells were then washed sequentially with 10 mL phosphate buffered saline, 10 mL serum-free DMEM and incubated with 10 mL culture medium. On the following day, the cells were passaged at a ratio of 1:3 into the selection medium.

Cells were first selected for the $APRT^+$ phenotype in DMEM containing 10% Nu-serum, 4 μg/mL azaserine and 15 μg/mL adenine. $APRT^+$ colonies were then grown in DMEM containing 10% Nu-serum, 15 μg/mL hypoxanthine, 1 μg/mL aminopterin and 5.15 μg/mL thymidine (HAT medium) to select for the TK^+ phenotype. Individual TK^+ clones were isolated from the HAT^+ pool by limited dilution of the cells into 96-well plates. Individual TK^+ clones were analyzed for the presence of integrated desaturase sequences by slot blot hybridization analysis using a [^{32}P]radiolabeled DNA probe containing sequences from bGH exon V and 3′-untranslated region. Mouse L cells containing Δ12-desaturase cDNA (Δ12-cloned cells) were thus generated. No morphological changes were noted in the transfected cells. There were also no change in the growth rates of the transfected cells compared to control L cells. Control cells (n = 6) and the Δ12-cloned cells (from 9 different stably transfected cells) were incubated in serum-free medium at 37°C for a period of 24 h. Separate studies were also carried out in which control and the Δ12 cloned cells were incubated in serum-free medium for different periods of time (up to 8 days).

Expression of Δ6-desaturase in cultured cells

To attempt to enhance the LC-PUFA synthetic pathway, we have also generated cultured cells expressing a heterologous Δ6-desaturase. A 1,382 bp *Eco*RI-*Xho*I DNA fragment encoding the *Mortierella alpina* Δ6-desaturase cDNA (see ref. [7], Genebank accession #AF110510) was ligated into plasmid pCMV-BGH-C to generate pCMV-Δ6-bGH plasmid. Similar to the method described above for generating Δ12-desaturase stable cell lines, pCMV-Δ6-bGH, was introduced into L cells. Seven transfected L cell clones containing the integrated Δ6-desaturase sequences were obtained. Since L cells expressing the Δ6-desaturase would be expected to still require essential fatty acids for normal growth, both control (L) and the Δ6-cloned cells in this experiment were incubated in serum-containing culture medium.

Fatty acid analysis

After incubation, the cells were harvested and cell pellets were analyzed for fatty acid composition as previously described [11]. Briefly, cell pellets were extracted with 15 mL of methanol and 30 mL of chloroform containing 100 μg of triheptodecanoin. After extraction, the total cell lipids were saponified, and the liberated fatty acids methylated. The distribution of fatty acid methyl esters were then analyzed by

gas chromatography (GC) using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and a fused silica capillary column (Suplecomega, 50 m × 0.25 mm i.d., Supleco, Belfonte, PA, USA). The identification of a given fatty acid was verified by GC-mass spectrometry using a Hewlett-Packard mass selective detector (model 5920) operating at an ionization voltage of 70 eV with a scan range of 20–500 Da. The mass spectra of new peaks were compared with those of authentic standards (Nu-Check Prep, Elysian, MN, USA) and those in the database NBS75K.L (National Bureau of Standards).

Results

Expression of Δ12-desaturase in cultured cells

Control and the Δ12-cloned cells were incubated in serum-free medium for 24 h. Thereafter, the cells were harvested, total cellular lipids extracted, and the levels of major fatty acids in the cells analyzed. In this study, the cellular levels of linoleic acid were used to reflect the activity of the Δ12-desaturase in the cells. Results in Table 1 show that the mean level of linoleic acid in the control cells was 3.6% (by wt of total cellular fatty acids), whereas in cells inoculated with the fungal Δ12-desaturase gene, the mean level of linoleic acid was significantly increased to 30.6%, a more than 8-fold increase. The increase in linoleic acid was at the expense of oleic acid (18:1n-9) which was reduced from 43.2 to 20.4%. Significant increases were also observed in levels of 18:3n-6 (the Δ6-desaturation product of linoleic acid), 20:2n-6 (the elongation product of linoleic acid), 20:3n-6 (the elongation product of γ-linolenic acid) and 20:4n-6 (the Δ5-desaturation product of 20:3n-6) in the Δ12-cloned cells. Among the n-3 LC-PUFA, only 22:5n-3 was slightly increased in cells inoculated with the fungal Δ12-desaturase gene.

Distribution of 18:1n-9 and its metabolite, 18:2n-6 in different cellular lipid fractions

The distributions of both 18:1n-6 and 18:2n-6 (the substrate and product of Δ12-desaturation) in cellular different lipid fractions and phospholipid subfractions are shown in Fig. 1. Over 90% of 18:1n-9 was incorporated into the phospholipid fraction (mostly phosphatidylcholine), followed by the triacylglycerol fraction. In cells expressing the fungal Δ12-desaturase gene, a significant proportion of 18:1n-9 was converted into 18:2n-6, which was incorporated into the phospholipid fraction, mainly the phosphatidylcholine fraction.

Table 1. Composition of major fatty acids in control and the Δ12-desaturase L cells incubated in the serum-free medium. Each clone contained a minimum of 5 copies of the Δ12-desaturase cDNA

Fatty acid	Control cells n = 6	12-desaturase-cloned cells n = 9
14:0	1.8 ± 0.3	1.6 ± 0.1
16:0	14.1 ± 1.8	14.2 ± 0.3
16:1n-7	6.4 ± 2.3	2.6 ± 0.1[a]
18:0	12.0 ± 1.5	13.7 ± 0.6
18:1n-9	43.2 ± 2.6	20.4 ± 0.4[a]
20:1n-9	3.5 ± 0.8	1.3 ± 0.2[a]
18:2n-6	3.6 ± 0.8	30.6 ± 3.8[a]
18:3n-6	n.d.	0.1 ± 0.04
20:2n-6	0.3 ± 0.1	3.7 ± 0.3[a]
20:3n-6	0.3 ± 0.1	0.4 ± 0.1
20:4n-6	1.3 ± 0.5	4.6 ± 2.1[a]
22:4n-6	0.1 ± 0.05	0.8 ± 0.5[a]
18:3n-3	0.2 ± 0.08	0.2 ± 0.04
20:5n-3	0.2 ± 0.1	0.2 ± 0.03
22:5n-3	0.4 ± 0.1	1.7 ± 0.6[a]
22:6n-3	0.3 ± 0.1	1.0 ± 0.5[a]

[a]significantly different from the control at $p < 0.05$. n.d. = not detected.

Time course study

In a separate study, the effect of varying incubation time on the levels of linoleic acid in the control and the Δ12-desaturase-cloned cells incubated in serum-free medium has also been examined. In control cells, the initial level of linoleic acid was around 3% of total fatty acid (Fig. 2). After 6 days of incubation, the level of linoleic acid fell to 0.8%. In contrast, the level of linoleic acid in the Δ12-desaturase-cloned cells increased within 24 h to 18.9% of the total fatty acids. The levels of eicosadienoic (20:2n-6), dihomo-γ-linolenic (20:3n-6) and arachidonic acid (20:4n-6) were also elevated by 12.3, 3.7 and 2.9-fold, respectively (Fig. 2). Prolonged incubation of the Δ12-cloned cells in serum-free medium further increased the cellular levels of 18:2n-6 and 20:2n-6. The levels of linoleic acid reached 27.4%, and 20:2n-6 reached 2.6% after 8 days of incubation. However, the levels of 20:3n-6 and 20:4n-6 fell progressively after 24 h of incubation (Fig. 2).

Expression of Δ6-desaturase in cultured cells

To examine whether the formation of long-chain n-6 fatty acids could be further enhanced, a fungal Δ6-desaturase gene has also been expressed in cultured mouse L cells. An increase in cellular levels of long-chain (n-6) fatty acids was used to demonstrate the successful expression of the Δ6-desaturase gene. When incubated in serum-free medium, the levels of 20:3n-6 and 20:4n-6 in the Δ6-desaturase cloned cells were

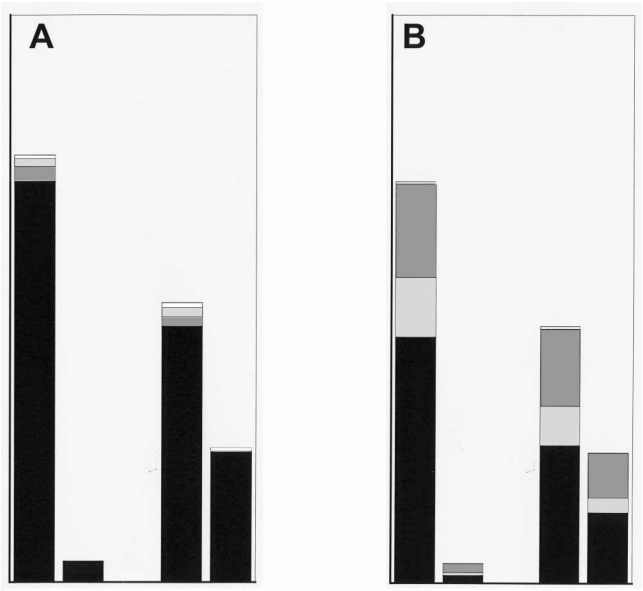

Fig. 1. Distribution of oleic (18:1n-9) and linoleic (18:2n-6) in different lipid fractions (A) and phospholipid subfractions (B) in control mouse L cells and the L cells transfected with the fungal Δ12-desaturase gene incubated in serum-free medium for 24 h. Panel A: cholesteryl esters (□), triacylglycerols (▨), diacylglycerols (▩), and total phospholipids (■). Panel B: phosphatidylcholine (PC, ■), phosphatidyl-serine and -inositol (PS+PI, ▨), phosphatidylethanolamine (PE, ▩), and lysophospholipids (LPL, □).

not significantly different from those in the control cells. Incubation in serum-containing medium increased only slightly the levels of n-6 fatty acids in the control cells but significantly elevated the levels of n-6 fatty acids in the Δ6-desaturase cloned cells (Fig. 3). There was a 2.8-fold elevation in the

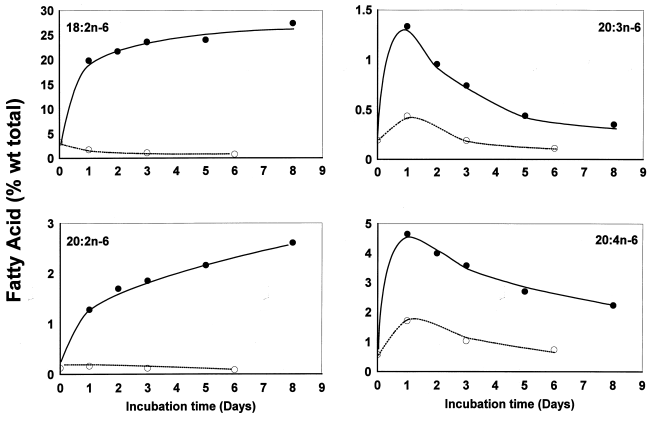

Fig. 2. Effect of varying incubation time on levels of linoleic acid (18:2n-6, panel A), eicosadienoic acid (20:2n-6, panel B), dihomo-γ-linolenic acid (20:3n-6, panel C), and arachidonic acid (20:4n-6, panel D) in control mouse L cells (O–O) and Δ12-desaturase cloned-cells (●–●) incubated in serum-free medium.

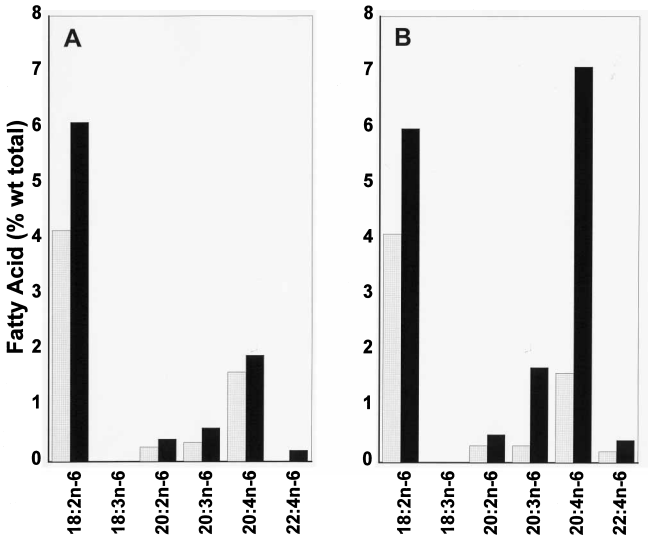

Fig. 3. Composition of n-6 fatty acids in control mouse L cells (□) and L cells transfected with the fungal Δ6-desaturase gene (■) incubated in serum-free medium (A) or serum-containing medium (B) for 24 h.

level of dihomo-γ-linolenic acid (20:3n-6) and a 3.8-fold increase in the levels of 20:4n-6 in cells that express the Δ6-desaturase gene.

Discussion

In mammalian tissues, linoleic acid is the precursor of many long-chain n-6 fatty acids that are important in regulating normal physiological functions [2–5]. However, mammalian tissues lack the Δ12-desaturase activity and can not synthesize linoleic acid. Therefore they must obtain this fatty acid from the diet [6]. In this report, we show that expression of a fungal Δ12-desaturase gene in the cultured mammalian cells resulted in a significant increase in cellular linoleic acid content. The increased 18:2n-6 provides substrate for the endogenous metabolic enzymes (Δ6-desaturase, Δ5-desaturase and elongase) as shown by an increase in levels of other long-chain n-6 fatty acids. The increase of 20:2n-6 suggests a direct elongation of 18:2n-6. The increase of 20:3n-6 and 20:4n-6 on the other hand, indicates that 18:2n-6 undergoes Δ6-desaturation to form 18:3n-6, which is subsequently elongated to form 20:3n-6. 20:3n-6 is then Δ5-desaturated to form 20:4n-6 [12]. It should be noted that L cells expressing the Δ12-desaturase could survive for a limited period in serum-free culture medium but could not be maintained indefinitely. These cells might be capable of extended culture if specific growth factors were added to the serum-free medium (i.e., use of delipidated serum). Nevertheless, our findings demonstrate for the first time that it is possible to generate cell lines and potentially animals which no longer required dietary essential fatty acids.

Interestingly, results in the prolonged incubation study show that while the level of 18:2n-6 is increasing, the activity of the endogenous metabolic enzymes (Δ6- and Δ5-desaturases) fell progressively. This could be due to an increase in cellular 18:2n-6 content which suppressed the activity of Δ6-desaturase and subsequently the formation of long-chain n-6 fatty acids such as 20:3n-6 and 20:4n-6. Evidence has shown that a high level of 18:2n-6 as substrate inhibits the activity of Δ6-desaturase [13]. We have also noted a progressive reduction in production of 20:4n-6 in the late passage cells than in the early passage cells (data not shown). It is not clear at the present time why the insertion of a fungal Δ12-desaturase gene would affect the activities of the endogenous metabolic enzymes in mouse L cells only at the late passage but not at the early passage.

In a separate study, we have also tested whether inserting a fungal Δ6-desaturase gene in the mouse L cells could raised the levels of 20:3n-6 and 20:4n-6. Results in Fig. 3 clearly show that an increase in these two long-chain n-6 fatty acids became significant only when the substrate 18:2n-6 was available in the medium. More, despite a lower cellular 18:2n-6 content, the increase of long-chain n-6 fatty acids was significantly greater in cells expressing the Δ6-desaturase gene than in cells expressing the Δ12-desaturase gene grown in serum-free medium (Table 1). This demonstrates that the introduced fungal Δ6-desaturase was active in addition to the endogenous Δ6-desaturase. Thus, by introducing exogenous genes encoding different fatty acid metabolic enzymes, the production of downstream long-chain polyunsaturated fatty acid can be modulated.

In summary, results in the present study demonstrate that it may be possible to generate cell lines which no longer require 'essential' fatty acids and that the endogenous PUFA synthetic pathways can be altered to enhance the production of specific PUFAs and their derivatives.

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