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# Mammalian Fatty Acid Elongases

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## Summary

Very long chain fatty acids confer functional diversity on cells by variations in their chain length and degree of unsaturation. Microsomal fatty acid elongation represents the major pathway for determining the chain length of saturated, monounsaturated, and polyunsaturated fatty acids in cellular lipids. The overall reaction for fatty acid elongation involves four enzymes and utilizes malonyl CoA, NADPH, and fatty acyl CoA as substrates. While the fundamental pathway and its requirements have been known for many years, recent advances have revealed a family of enzymes involved in the first step of the reaction, i.e., the condensation reaction. Seven fatty acid elongase subtypes (Elovl #1–7) have been identified in the mouse, rat, and human genomes. These enzymes determine the rate of overall fatty acid elongation. Moreover, these enzymes also display differential substrate specificity, tissue distribution, and regulation, making them important regulators of cellular lipid composition as well as specific cellular functions. Herein, methods are described to measure elongase activity, analyze elongation products, and alter cellular elongase expression.

### Keywords

Fatty acid elongase; Microsome; Reverse phase-high performance liquid chromatography (RP-HPLC); Recombinant adenovirus

## 1. Introduction

Fatty acid elongation occurs in three cellular compartments: the cytosol, mitochondria, and endoplasmic reticulum (microsomes). In the cytosol, fatty acid elongation is part of de novo lipogenesis and involves acetyl-CoA carboxylase and fatty acid synthase. Fatty acid synthase utilizes acetyl CoA and malonyl CoA to elongate fatty acids by two carbons. The primary end product of de novo lipogenesis is palmitate (16:0) and to a lesser extent myristate (14:0) and stearate (18:0). Fatty acid elongation also occurs in mitochondria and utilizes an enoyl-CoA reductase plus acetyl CoA and fatty acyl CoA as substrates. This site appears to be a minor pathway for fatty acid elongation in eukaryotic cells (1). Microsomal fatty acid elongation is considered the predominant pathway for elongating fatty acids 12-carbons and longer (2–10). This pathway utilizes fatty acids derived from endogenous pathways, like de novo lipogenesis, as well as exogenous fatty acids derived from the diet.

The overall pathway for microsomal fatty acid elongation involves four enzymes and fatty acyl CoA, malonyl CoA, and NADPH as substrates (Fig. 1). In step 1, a 3-keto acyl-CoA synthase catalyzes the condensation of malonyl CoA with a fatty acyl-CoA precursor. In step 2, a 3-keto acyl-CoA reductase reduces the resulting 3-keto intermediate. Step 3 involves the dehydration of the 3-hydroxy species; this reaction is catalyzed by a 3-hydroxy acyl-CoA dehydratase. In step 4, reduction of the step 3 product is catalyzed by *trans*-2,3-enoyl-CoA reductase (2,9). Cloning and genomic analysis have identified seven distinct 3-keto acyl-CoA synthases, i.e., fatty acid condensing enzymes, in mouse, rat, and human genomes (http://www.ensemble.org) (3,4). These enzymes are called elongases and have been given the designation Elovl for *elo*ngation of *v*ery *l*ong fatty acids (Elovl 1–7). The elongase subtypes, their mouse accession # and alias are: Elovl-1: NM_019422, SSC1; Elovl-2: AF170908, SSC2;

Elovl-3: BC016468, CIG30; Elovl-4: NM_148941, Elo4; Elovl-5: NM_134255, FAE1; Elovl-6: AY053453, LCE or FACE; Elovl-7: BAB31310, 9130013K24RIK. The two reductases, 3-keto acyl-CoA reductase and *trans*-2,3-enoyl-CoA reductase, have been cloned from human, mouse, and yeast (9,11). The dehydratase, however, has not been cloned.

Fatty acyl-CoA substrate specificity and rate of fatty acid elongation is determined by the first step in the pathway, i.e., the activity of the condensing enzyme and not the reductases or dehydratase (1,9). While Elovl-1, Elovl-3, and Elovl-6 elongate saturated and monounsaturated fatty acids (3,4,12). Elovl-2, Elovl-4, and Elovl-5 elongate polyunsaturated fatty acids. Elovl-5 also elongates some monounsaturated fatty acids, like palmitoleic acid (8). Elovl-5 specifically elongates γ-linolenoyl-CoA (18:3,n-6 CoA), while Elovl-2 specifically elongates 22-carbon PUFA (3,4,8). Substrates for Elovl-7 have not been described. The elongases (Elovl) are expressed differentially in mammalian tissues (3,4). For example, five elongases are expressed in rat and mouse liver, including Elovl-1, -2, -3, -5, -6 (6,7,13). In contrast, the heart expresses Elovl-1, -5, and -6, but not Elovl-2 (14). These enzymes are also regulated by diet, hormones, during development, and in chronic disease (3,7–9,12). As such, changes in their activity impact cellular lipid composition.

Fatty acid elongases function with fatty acid desaturases to generate many of the long chain mono and polyunsaturated fatty acid assimilated into cellular lipids. Elovl-6 and $\Delta^9$-desaturase generate 18-carbon monounsaturated fatty acids like oleic acid (8,12), while Elovl-2 and Elovl-5 along with $\Delta^5$ and $\Delta^6$ desaturases generate the end products of n-6 and n-3 synthesis, i.e., arachidonic and docosahexaenoic acid (3,4,8,15).

This chapter describes several methods to study elongase expression and function in cells and tissues. First is a detailed description of methods to measure fatty acid elongase activity in rat and mouse liver microsomal preparations. Second is an approach to measure fatty acid elongation in cultured cells, like rat primary hepatocytes. Third is an approach to modify the expression of fatty acid elongases in cultured cells and mouse liver.

## 2. Materials

### 2.1. Equipment

1. Wheaton Potter–Elvehjem tissue grinder (15 ml) with teflon pestel and a Wheaton overhead homogenizer (Fisher)
2. Nalgene 50 ml polypropylene centrifuge tubes (cat. #3110–0500)
3. 12-ml pyrex tubes with caps (#9825, Fisher)
4. 15-ml Kimble HS centrifugation tube (45500–15, Fisher)
5. 12 × 35-mm lipid storage vials with Teflon cap liners (cat.# 03–338AA, Fisher).
6. JA20.1 high-speed centrifuge rotor (Beckman Instruments)
7. SW55 TI ultracentrifuge rotor (Beckman Instruments)
8. Avanti high speed centrifuge (Beckman Instruments)
9. Optima L-90 ultracentrifuge (Beckman Instruments).
10. 25-mm cell scraper (Sarstedt, cat.# 83.1830)
11. Nitrogen evaporator: N-Evap 112 (Organomation Associates, Inc.)
12. Microcentrifuge evaporator: CentriVap Concentrator plus cold trap and organic solvent trap (Labconco)

13. Waters 600 controller (Waters Corporation)
14. Waters 2487 UV/Vis absorbance monitor
15. Waters 2420 evaporative light scatter detector
16. YMC J'Sphere ODS-H80, (4 μm, 8 nm; 250 × 4.6 mm I.D.) (Waters Corporation)
17. Flow-through β-scintillation counter, β-RAM, (IN/US Systems, Inc., Tampa, Fl)
18. Winflo software, (IN/US Systems, Inc., Tampa, Fl)

### 2.2. Reagents and Supplies

1. Protease inhibitors: Complete Mini, EDTA-free (Cat. # 11836170001, Roche Diagnostics, Indianapolis, IN)
2. Phosphatase inhibitors: β-glycerol phosphate, sodium pyrophosphate, $Na_3VO_4$ (Sigma-Aldrich)
3. Rotenone, malonyl CoA, NADPH, coenzyme A, butylated hydroxytoluene, porcine insulin, (Sigma-Aldrich)
4. $^{14}$C-malonyl CoA (40–60 Ci/mol) (Perkin Elmer)
5. Ultima Gold scintillation solution (Perkin Elmer)
6. Fatty acyl CoA (Avanti Polar Lipids, Alabaster, Al)
7. Probumin, fatty acid- and endotoxin-free bovine serum albumin (Celliance, Cat# 820473; Kankakee, Ill).
8. Non-esterified fatty acids, (Nu-Chek Prep, Elysian, MN)
9. HPLC-grade solvents: chloroform, methanol, acetonitrile, hexane, diethyl ether (Fisher)
10. Bio-Rad Protein assay (Quick start/Bradford, cat. # 500–0205).
11. BioCoat (type 1 collagen) cell culture plates (Beckon Dickinson, Belford, MA.)
12. Williams E medium, fetal bovine serum, glucose [dextrose], phosphate-buffered saline (Invitrogen).

## 3. Methods

### 3.1. Measuring Fatty Acid Elongase Activity in Microsomes

#### 3.1.1. Preparation of Mouse or Rat Liver Microsomes

1. Homogenize 0.2–0.5 g liver (freshly isolated liver or snap frozen liver) in 5 ml of Buffer A [250 mM sucrose + 10 mM Tris–Cl, pH 7.4, containing protease (Complete Mini, EDTA-free, Cat. # 11836170001, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (1-mM β-glycerol phosphate, 2.5-mM sodium pyrophosphate, 1-mM $Na_3VO_4$ (Sigma)] using a 15-ml Wheaton Potter–Elvehjem tissue grinder with a teflon pestle attached to a Wheaton overhead stirrer (Fisher). Homogenize at a low speed (setting of 3–4) for ~2–3 min while keeping the homogenizing flask on ice. Homogenize the tissue thoroughly.
2. Transfer the homogenate to 50 ml of Nalgene polypropylene centrifuge tubes (cat.# 3110–0500) and centrifuge at 12,000 rpm (18,547 × g) for 10 min using a JA20.1 rotor and a Beckman Avanti refrigerated centrifuge (4°C).

3. Recover supernatant and centrifuge the supernatant at 40,000 rpm (194,432 × g) using a Beckman SW55 TI rotor for 30 min in a Beckman Optima L-90 ultracentrifuge at 4°C.

4. Discard the supernatant and resuspend the pellet in Buffer A [0.25 M sucrose + 10 mM Tris–Cl, pH 7.4, plus protease and phosphatase inhibitors] at ~1 ml/g liver. The pellet can be difficult to resuspend and may need to be homogenized gently with a glass rod or Teflon pestle.

5. Determine protein concentration using the Bio-Rad Protein assay (Quick start/ Bradford, cat. # 500–0205).

6. Aliquot in labeled tubes and store frozen at −80°C. Microsomal fatty acid elongase activity is stable at least 1 month when stored at −80°C.

**3.1.2. Enzymatic Assay for Fatty Acid Elongation Activity in Rat Liver Microsomes—**The fatty acid elongase reaction is carried out in 1.5–1.8-ml screw-capped microfuge tubes (12).

| Components | Final Concentration | Volume/amount added |
|---|---|---|
| 100 mM $KH_2PO_4$, pH 6.5 | 50 mM | 50 µl |
| 1 mM rotenone (Sigma) | 5 µM | 0.5 µl |
| 10 mM malonyl CoA (Sigma) | 60 µM | 0.6 µl |
| 10 mCi/ml 14C-malonyl CoA (Perkin Elmer) | 15 nCi | 1.5 µl |
| 10 mM Fatty acyl CoA (in 50 mM $KH_2PO_4$, pH 5.8) (Avanti Polar Lipids) | 40 µM | 0.5 µl |
| 22 mM NADPH (Sigma) (made fresh each week in 50mM $KH_2PO_4$, pH 5.8) | 1 mM | 5 µl |
| 400-µM bovine serum albumin (fatty acid and endotoxin free in HOH, Probumin from Celliance, Cat# 820473; Kankakee, Ill). | 20 µM | 5 µl |
| Microsomal protein | 50 µg/0.1 ml | 50 µg |

Adjust final volume to 100 µl with water. Mix and incubate 37°C. for 20 min.

**3.1.3. Extraction and Analysis of Fatty Acid Elongation Reaction Products—**Stop the reaction with the addition of 100 µl of 5 M KOH-10% methanol. Incubate for 1 h at 65°C. This step will hydrolyze (saponify) the ester bond between the fatty acid and coenzyme A. Stop the saponification step by adding 100 µl of 5 M HCl and 100 µl of ethanol to each reaction. Extract fatty acids by adding 750 µl of hexane–acetic acid mix (98% hexane–2% glacial acetic acid), vortex vigorously for ~1 min. Separate the aqueous (lower) and organic (upper) phases by centrifugation in a microfuge (5 min at maximum speed). Transfer upper phase (hexane phase) to a scintillation vial. Repeat hexane extraction and combine the hexane supernatants. Add 5 ml of scintillation cocktail (Ultima-Gold, Perkin Elmer) and count radioactivity.

**3.1.4. Control Studies for Fatty Acid Elongation Assays—**Several important controls should be run for each study. First, NADPH is required for microsomal fatty acid elongation. In a control reaction, add all the reagents except NADPH. Radioactivity recovered in the hexane–acetic acid phase from this reaction represents background $^{14}C$ radioactivity. Second, add 1.5 µl of $^{14}C$-malonyl CoA directly to the scintillation vial. Once background subtractions are made, this will represent 100% of $^{14}C$. Since the reaction contains 6 nmol of malonyl CoA, a simple % calculation of total radioactivity appearing in the hexane–acetic acid phase will indicate the nmoles of malonyl CoA transferred to a fatty acid.

**3.1.5. Data Presentation for Fatty Acid Elongation Assays—**Express the results as nmoles/mg microsomal protein. When using 18:3(n-6) CoA as substrate and rat liver microsomes, expect ~3–5 nmol of substrate (malonyl CoA) converted to product (fatty acid)/ mg protein/20 min reaction.

**3.1.6. Follow-Up Studies—**With few exceptions, the product of the fatty acid elongase reaction is the result of the action of two or more fatty acid elongases. For example, 16:0-CoA is a substrate for Elovl1, Elovl-3, and Elovl-6, while 20:4(n6)-CoA is a substrate for Elovl-2 and Elovl-5. It is possible to measure Elovl-2 and Elovl-5 specifically using 22:5(n3)-CoA and 18:3(n6)-CoA as substrates, respectively. As such, an important step in elongase analysis is to establish the elongase subtype expression in cells under study. This can be accomplished using quantitative reverse transcriptase-polymerase chain reaction. Primers for several mouse, rat, and human elongases have been reported elsewhere (8).

**3.1.7. Alternative Substrates for Fatty Acid Elongation—**Because the commercial availability of fatty acyl CoAs is limited, nonesterified fatty acids can be substituted in the reaction. For details of this modification of the elongase assay, *see* **Note 1**.

### 3.2. Assessment of Fatty Acid Elongation in Cultured Cells

Addition of nonesterified fatty acids to cells leads to their rapid uptake, metabolism, and assimilation into complex lipids. Many fatty acids undergo elongation and desaturation. The products of these reactions can be monitored using a combination of $^{14}$C-labeled fatty acids, cell culture, total lipid extraction, and analysis of saponified fatty acids by reverse phase-high performance liquid chromatography (RP-HPLC) coupled to a flow-through β-scintillation counter (8,15,16).

**3.2.1. Studies with Rat Primary Hepatocytes—**Rat primary hepatocytes are treated with $^{14}$C-fatty acids to assess fatty acid elongation. Primary hepatocytes are prepared from Teklad chow-fed (*ad lib*) male Spraque–Dawley rats and cultured on 6-well BioCoat (type 1 collagen) plates (Beckon Dickinson, Belford, MA.) (16). Cells are incubated in Williams E medium (Invitrogen) containing 25 mM glucose, 1 microM insulin (Invitrogen, Carlsbad, CA); 25 μM bovine serum albumin (endotoxin and fatty acid free albumin from Celliance, Kankakee, Ill), and 100 μM $^{14}$C-fatty acid [1.6 Ci/mol] (16). See **Note 2** regarding fatty acid treatment of other cell types.

**3.2.2. Lipid Extraction and Analysis of Fatty Acid Metabolites—**Total lipid extraction and analysis of fatty acids is carried out after treating cells with $^{14}$C-fatty acids. At harvest, remove the media from cells and wash cells once with cold phosphate-buffered saline (PBS) containing 200 μM BSA followed by a wash with PBS alone. After removal of the PBS, resuspend cells in 0.5 ml of 40% methanol + 0.1N HCl; use a 25-mm cell scraper (Sardstedt) to remove cells from the plastic culture dish. Transfer the resuspended cells to 15-ml glass centrifuge (Kimble HS No. 45500.15) tube (*see* **Note 3**). If a protein assay is required, then 2 μl of the cell suspension can be taken for a protein assay (Bio-Rad, Hercules, CA). Add 2 ml of chloroform:methanol (2:1) plus 1 mM butylated hydroxytoluene (BHT) (Sigma-Aldrich) to the cell suspension and vortex carefully, avoid loss of sample. After thorough mixing, the sample is centrifuged in a JA20.1 rotor at 5,000 rpm (3220 × g) using a Beckman Avanti refrigerated (4°C) centrifuge. The organic (lower) phase is recovered and transferred to a 12-ml screw-cap tube (Fisher Pyrex #9825). The aqueous phase and protein interface is re-extracted with 2-ml chloroform as before. After centrifugation, the organic phase is transferred to the same 12-ml screw-capped tube. The organic solvents are removed by a constant stream of nitrogen using a nitrogen evaporator (Organomation Associates, Inc). All the moisture is removed using a microcentrifuge evaporator (CentriVap + cold and organic solvent traps, Labconco) overnight.

The next day, dissolve lipids in 500 μl of chloroform containing 1 mM BHT. Lipids are transferred to storage vials (12 × 35 mm, Fisher cat # 03338AA), labeled, and stored at −80° C. At this point, total lipid extracts can be fractionated by thin-layer chromatography to assess

the distribution of $^{14}C$ in complex lipids or saponified for analysis of fatty acid by RP-HPLC (15).

### 3.2.3. Saponification of Lipids

1. Transfer ~100 μl or >30,000 CPM of the $^{14}C$-label chloroform lipid extract to a clean 12-ml Pyrex tube with a teflon-lined screw cap. Remove the organic solvent with a stream of nitrogen.
2. Resuspend the dried lipid in 500-μl saponification buffer (0.4N NaOH in 80% methanol). Screw on the cap and heat the tube to 65°C for ~1 h.
3. Let the tubes cool and add 500 μl of 0.45 M HCl to neutralize. Add 2 ml of hexane containing acetic acid at 2%; cap and vortex vigorously.
4. Let the tube sit for ~3 min or until the aqueous and organic phases have separated. Recover the organic (upper) phase and transfer to a clean 12-ml pyrex tube.
5. Repeat the hexane–acetic acid extraction as before. Vortex, let sit for phase separation, and recover the upper phase; pool the organic phases.
6. Remove the organic solvent under a stream of nitrogen; dry the sample overnight in as above in a microcentrifuge evaporator.
7. Resuspend the saponified lipids in 100 μl of methanol containing 100 μM BHT. Transfer methanol soluble lipids to storage vials for −80°C storage or use for RP-HPLC. Quantify lipid recovery by β-scintillation counting.

**3.2.3. Alternative Methods to Examine Elongation Products—**An alternative method for analysis of elongation products involves treating cells with saponification buffer (0.4N NaOH in 80% methanol). In this method, cells grown in 6-well plates are resuspended in 500 μl of saponification buffer per well of a 6-well plate. Transfer the resuspended cells to a 12-ml pyrex tube, capped, and heated as described above **(Subheading 3.2.3**, **step 2)**. Follow **steps 2–7** to prepare fatty acids for RP-HPLC.

**3.2.5. High Performance Liquid Chromatography—**The equipment used for RP-HPLC includes a Waters 600 controller (Milford, MA), Waters UV/Vis absorbance monitor [model 2487], Waters evaporative light scatter detector [model 2420], and a β-RAM flow through β-scintillation counter (IN/US Systems, Inc., Tampa, Fl). The reverse phase column is a YMC J'Sphere (ODS-H80, 4 μm, 8 nm; 250 × 4.6 mm I.D.) (Waters Corporation). Eluate from the column passes through the UV detector, exits and is split 1:1 for flow into the evaporative light scatter detector and the flow through β-scintillation counter. The scintillation cocktail for the IN/US unit is Inflow 2:1 (IN/US Systems, Inc.). The ratio of eluant to scintillation cocktail is 2:1. The software for data collection and analysis is Winflo (IN/US Systems, Inc.).

Samples (20 microl) are injected and fatty acids are eluted from the column using a mobile phase consisting of a linear gradient starting at 77.5% acetonitrile + 22.4% water + 0.1% acetic acid and ending at 99.9% acetonitrile + 0.1% acetic acid over 90 min with a flow rate of 1.0 ml/min (*see* **Note 4**). Buffers are sparged with helium prior to and during HPLC runs. Fatty acids are detected using UV absorbance and evaporative light scatter (*see* **Note 5**). Waters 2487 dual absorbance monitor parameters are: 192 nm and a gain of 0.5. The parameters for the Waters 2420 light scatter detector are: gain = 100; PSI = 5 psi, ultrapure nitrogen; nebulizer tube temperature = 60% or 36°C; drift tube temperature = 45°C. While UV absorbance detects only unsaturated fatty acids, evaporative light scatter detects saturated and unsaturated fatty acids. The inline β-scintillation counter detects $^{14}C$-labeled fatty acids (8,16). In some cases, nonradioactive fatty acids can be used to treat cells (*see* **Note 6**). Additional verification of

fatty acid elongation products was established by gas chromatography–mass spectrometry at the mass spectrometry facility at Michigan State University (http://genomics.msu.edu/mass_spec/index.html). The equipment used in these studies was sufficient to establish the chain length and the number of double bonds (16). In addition, the chromatograms of $^{14}$C-label fatty acids were compared to the chromatograms of unlabeled fatty acid standards. Fatty acid standards used in our studies include: 14:0; 16:0; 18:0; 16:1,n-7; 18:1,n-9; 19:1,n- (recovery standard); 18:2,n-6; 18:3,n-3, 18:3,n-6; 20:3,n-6, 20:3,n-9, 20:4,n-6, 20:5,n-3; 22:5,n-3; 22:6,n-3. Each fatty acid is at 100 μM in methanol. All fatty acids are obtained from Nu-Chek Prep (Elysian, MN).

### 3. 3. Assessment of Fatty Acid Elongase Function in Vivo

**3.3.1. Loss-of- and Gain- of-Function Studies—**Mouse models of ablated expression of Elovl-3 and Elovl-6 genes have been reported (6,17). The phenotype of these animals indicates that Elovl-3 and Elovl-6 play important roles in skin lipid composition and whole body insulin action, respectively. Elovl-2 has been overexpressed in mouse adipocyte cell lines and appears to influence triglyceride metabolism (18). Elovl-5 has been over-expressed in mouse liver (15); elevated Elovl-5 expression affects multiple pathways involved in hepatic lipid and carbohydrate composition (15). Such studies indicated that fatty acid elongases have effects on cellular functions that go beyond effects on cellular lipid composition, per se.

**3.3.2. Adenoviral-Mediated Over Expression of Fatty Acid Elongases—**A key requirement for assessing elongase functions in cells and in vivo is to alter the expression of the enzyme in nearly all cells in culture or in a tissue. Our approach to alter elongase expression in cells and liver has used recombinant adenoviruses expressing specific elongases (condensing enzyme). This method allows for robust expression of the elongase in nearly every cell in culture and uniform expression in hepatic parenchyma cells in liver (15). Under these conditions, it is possible to assess the impact of these enzymes on cellular lipid composition and function. Methods to prepare recombinant adenoviruses expressing elongases and their use in cells or in vivo have been described elsewhere (8,15). Changes in cell/tissue lipid composition are assessed by the methods described above for monitoring elongase activity and fatty acid composition.

**3.3.3. Methods to Generate Recombinant Adenovirus—**Replication defective recombinant adenoviruses are a biohazard. As such, the use of these reagents require a biosafety level-2 (BSL2) for containment of both cell culture and animal infection studies. Adenoviruses generated in our laboratory are derivatives of the Ad-Easy recombinant adenovirus (Stratagene). Standard cloning protocols are followed to generate these viruses. Typically, coding regions of specific enzymes are generated by reverse transcriptase-polymerase chain reaction and inserted into the pShuttle-CMV vector (Stratagene). Cloning of cDNA for Elovl-2, Elovl-5, and Elovl-6 was described previously (7). The coding region for each transcript was ligated into Ad-Easy XL adenoviral vector system (Stratagene), recombined in BJ5183 cells, and propagated in XL10 Gold ultracompetent cells. Ad-DNA was packaged into adenoviral particles in Ad-293 cells. The resultant adenovirus was amplified in Ad-293 cells. Viral lysates are cesium purified twice and titered using the Adeno-X Rapid Titer Kit (Clontech).

**3.3.4. Infection of Primary Hepatocytes with Recombinant Adenovirus—**Rat primary hepatocytes are infected with recombinant adenovirus using the following protocol. Rat primary hepatocytes are prepared as described **(Subheading 3.2.1)**. Confluent primary hepatocytes are infected with virus at 5–10 plaque-forming units[PFU]/cell). When an adenovirus expressing green fluorescent protein (GFP) is used as a control for infection, greater than 80% of primary hepatocytes express functional protein at the 5–10 PFU/cell level. Under these infection conditions, fatty acid elongase activity increases twofold to sixfold (8,15).

Typically, cells are infected overnight; the next day cells are ready to assess the effects of altered elongase expression on fatty acid elongase activity **(Subheading 3.1)** or fatty acid elongation in cells **(Subheading 3.2)**.

**3.3.5. Infection of Mice with Recombinant Adenovirus—**C57BL/6 mice are infected with recombinant adenovirus using the following protocol. Male C57BL/6 (Charles River Laboratories or Jackson Labs) mice (20–22 g; ~2 months of age) are used for studies with recombinant adenovirus in vivo. Mice are maintained on a Harlan–Teklad (#8640) diet ad lib throughout all studies. Mice are injected with recombinant adenovirus by a retro-orbital route at a dose of $2 \times 10^{10}$ viral particles (cesium-purified)/mouse while under isoflurane anesthesia. A BD ultrafine II insulin syringe (short needle) is used for the injection. Mice injected with phosphate-buffered saline + 1.0% glucose served as a "Saline" control. Control virus infection uses a recombinant adenovirus expressing firefly luciferase (Luc) (15). In our experience, the Ad-Luc virus serves as an excellent control for infection. In contrast, Ad-GFP has been found to promote hepatotoxicity as reflected by elevated levels of plasma alanine aminotransferase activity. Body weight, food, and water intake is monitored daily. Typically animals displayed no adverse effects from the saline or adenovirus injection and gained weight equally for the duration of the study (5 days). Mice are euthanized on day 5 after infection for the collection of tissues and blood.

The advantage of in vivo studies is that it allows for a thorough examination of effects of altered hepatic elongase expression on liver and plasma lipid composition as well as multiple hepatic functions, like gene expression, cell signaling and glycogen, triglyceride, and cholesterol content. Such studies have provided novel insight into the impact altered elongase expression has on multiple pathways involved in lipid and carbohydrate metabolism (15). A limitation of this in vivo approach, however, is that recombinant adenoviruses infect hepatic cells, including Kupffer and parenchymal cells. Following infection of mice with recombinant adenovirus, we have found ample expression of the elongase in liver, but no change in elongase expression in nonhepatic tissues, like heart, kidney, and spleen (15). A second limitation is that expression of the transgene from the infecting adenovirus persists for only a week or so. Other methods for longer expression are available using other viral vectors, e.g., adeno-associated virus or helper-dependent adenovirus.

## 3.4. Results

An example of the elongation of a fatty acid in rat primary hepatocytes is illustrated in Fig. 2. In this study, primary hepatocytes were treated with $^{14}$C-20:4,n-6 as described in **Subheading 3.2**. After treating cells with the fatty acid, cells were harvested and lipids extracted. Saponified lipids were fractionated by RP-HPLC. The chromatogram in Fig. 2 illustrates the separation of $^{14}$C-fatty acids. Arachidonic acid ($^{14}$C-20:4,n-6) was added to the cells and both $^{14}$C-20:4,n-6 and its elongation product, adrenic acid ($^{14}$C-22;4,n-6) are recovered following lipid extraction and saponification. In this study, ~30% of the total $^{14}$C-fatty acid recovered from the cells was adrenic acid (22:4,n-6) indicating that the substrate, (20:4,n-6) was elongated in rat primary hepatocytes.

In an in vivo study, Elovl-5 was overexpressed in C57BL/6 mouse liver by using the recombinant adenovirus approach described in **Subheading 3.3.5**. Hepatic Elovl-5 enzyme activity was elevated threefold and a product of Elovl-5 action, i.e., di-homo-g-linolenic acid (20:3,n-6), was elevated greater than two-fold in both liver and plasma (15). In addition, the expression of several genes targeted by the fatty acid regulated transcription factor, PPARα, was suppressed by elevated Elovl-5 expression. The outcome of these studies indicates that changes in hepatic Elovl-5 activity affects both hepatic and plasma lipids as well as hepatic gene expression (15).

| Components | Final concentration | Amount/volume added |
|---|---|---|
| 50 mM CoASH | 100 μM | 0.5 μl |
| 42 mM ATP | 1 mM | 2.4 μl |
| 1 M $MgCl_2$ | 1 mM | 0.1 μl |
| 100 mM fatty acid (in Methanol) (Nu-Chek-Prep, Elysian, MN) | 20 μM | 0.25 μl |
| 100 mM NaOH | 20 μM | 0.25 μl |

## 4. Notes

1. Non-esterified fatty acids can replace fatty acyl CoAs as substrates. Delete fatty acyl CoA from the reaction mix and add the following components to the reaction mix described in **Subheading 3.2.1**.
2. When treating cells with fatty acids, it is important to establish the tolerance of cells for specific fatty acids. While hepatocytes and adipocytes can tolerate fatty acid levels as high as 1 mM, many cell types, like endothelial cells, cannot tolerate such high levels. As such, a titration experiment is required to establish conditions to minimize toxicity. It is also important to include bovine serum albumin (BSA) in the culture media. BSA has several binding sites for fatty acids (19), the mole ratio of fatty acid to BSA should not exceed 4 for in vitro studies. It is also important to establish the time course of fatty acid uptake by cells. In rat primary hepatocytes, >80% of the added $^{14}$C-fatty acid is assimilated into cellular lipids within 6 h (16).
3. Use glass for all lipid extractions and lipid storage. The exception is when pipetting small volumes; use plastic tips and the appropriate pipetting devices.
4. Use only HPLC-grade reagents for lipid extraction and analyses; including HPLC-grade water (Fisher) for preparing buffers.
5. Separation of fatty acids by RP-HPLC does not require *trans* methylation. The inclusion of acetic acid at 0.1% in the mobile phase is sufficient to protonate fatty acids, so separation and elution from the YMC J'sphere column is based on chain length and degree of unsaturation.
6. $^{14}$C-fatty acids are not always required to monitor cellular fatty acid elongation. Addition on nonradioactive 18:3,n-6, 20:5,n-3 or 22:5,n-3 results in the formation of 20:3,n-6, 22:5,n-3, and 24:5,n-3 in rat primary hepatocytes. These fatty acids are readily detected using either UV absorbance or evaporative light scatter.

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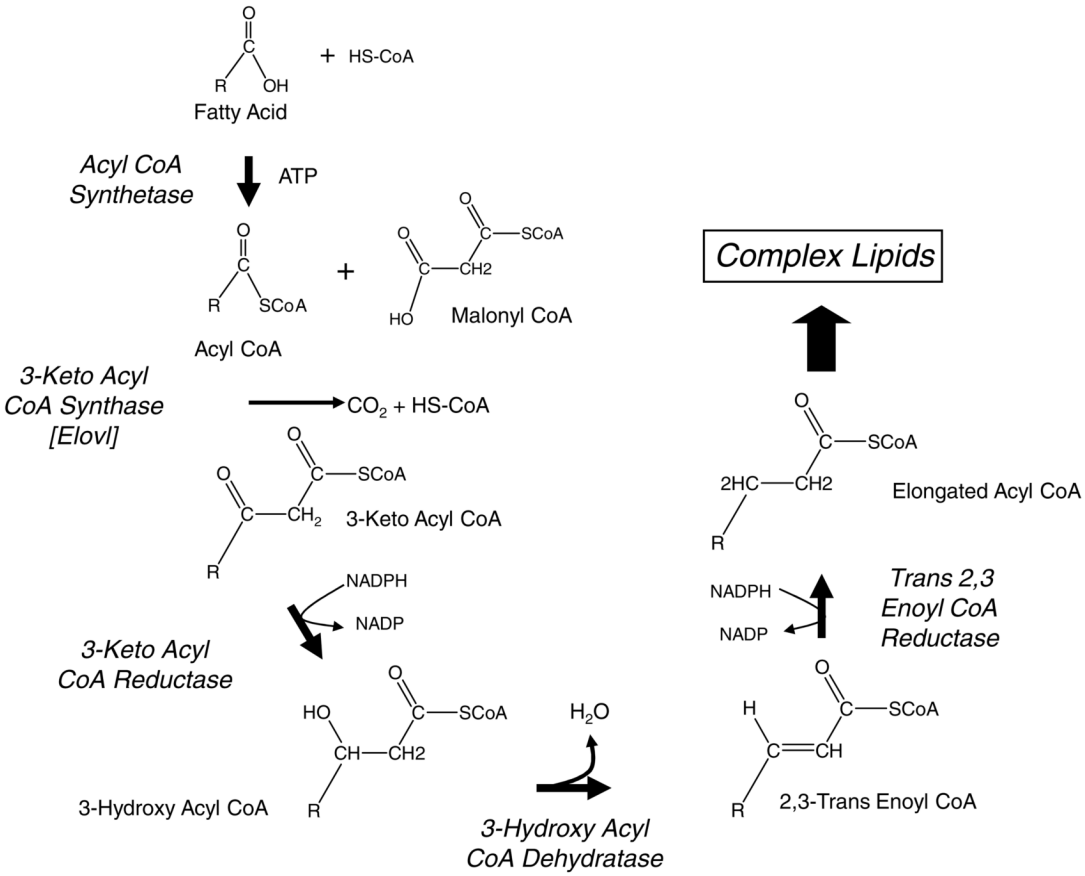


**Fig. 1.**

Proposed reaction sequence for hepatic microsomal fatty acid elongation. The reaction scheme described below is a modification of the one described by Cinti et al (2), Moon and Horton (12), and Denic and Weissman (10).

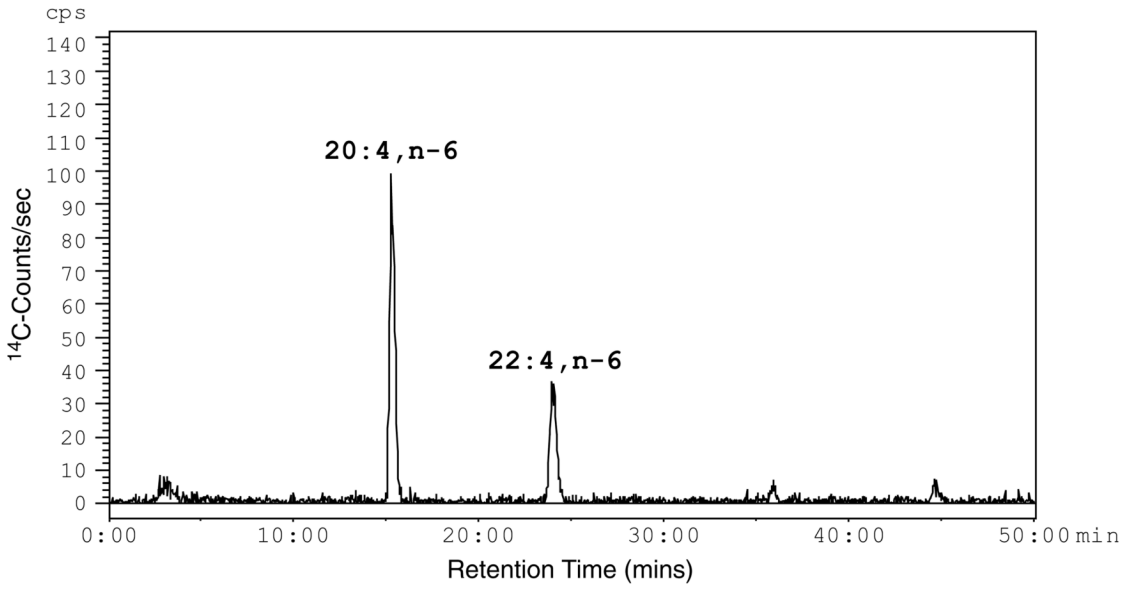


**Fig. 2.**

RP-HPLC analysis of fatty acid elongation products. Rat primary hepatocytes were treated with 100 μM 20:4,n-6 (1.6 Ci/mol) for 24 h. Lipids were extracted and saponified. Nonesterified fatty acids were separated by RP-HPLC. The distribution of $^{14}$C-fatty acids was determined by an inline β-scintillation counter (IN/US). Results are expressed as $^{14}$C-counts/sec (*Y*-axis) vs. retention time in minutes (*x*-axis). The figure illustrates the separation of arachidonic acid (20:4,n-6) and its elongation product, adrenic acid (22:4,n-6). Nonradioactive fatty acids are not shown in this figure.