Effects of exogenous C18 unsaturated fatty acids on milk lipid synthesis in bovine mammary epithelial cells

Supplementary Materials and Methods

Cell culture. Mammary tissues were thoroughly rinsed with PBS, finely minced with eye scissors and then incubated with DMEM/F12 medium (Gibco BRL, Grand Island, NY, USA) and an equal volume of type II collagenase (Gibco BRL, Grand Island, NY, USA) for 1 h at 37 °C in a 5% CO_2 incubator, following which the culture was filtered and centrifuged to collect BMECs. Cells were cultured in culture flasks (Corning, NY, USA) in basal culture medium at 37 °C in a 5% CO_2 incubator. The basal culture medium was composed of DMEM/F12 medium with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA), 100 μg/mL streptomycin (Sigma-Aldrich, USA), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 1 μg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 4 μg/mL prolactin (Sigma-Aldrich, St. Louis, MO, USA), 0.5% v/v insulin-transferrin-selenium-X supplement (Sigma-Aldrich, St. Louis, MO, USA), and 10 ng/mL EGF (Sigma-Aldrich, St. Louis, MO, USA). The BMECs were subcultured to 70-80% confluence, and cells from the third generation were used for the experiments.

Cell proliferation assay. A preliminary experiment showed that the suitable concentration ranges of the C18 UFAs were 50-400 μmol/L for oleic acid, 25-100 μmol/L for linoleic acid, and 0.5-2 μmol/L for linolenic acid. Cells were plated in 96-well plates (Corning, NY, USA) at 1×10^4 cells/well for 48 h and then cultured in BSA culture medium containing oleic acid, linoleic acid, and linolenic acid

(Sigma-Aldrich, St. Louis, MO, USA) at concentrations in the abovementioned ranges. Thereafter, 20 μL of 5 mg/mL MTT (AMRESCO, Inc., Solon, OH, USA) was added to each well, and the cells were incubated for another 4 h at 37 °C. Then, 100 μL of dimethyl sulfoxide (AMRESCO, Inc., Solon, OH, USA) was added to each well to dissolve the formazan crystals. The absorbance at 490 nm was read with a Multi-Mode Microplate Reader (Synergy^{TM} H4; BioTek, Winooski, VT, USA). The relative growth rate of the cells was calculated as follows: RGR = (treatment OD at 490 nm/control OD at 490 nm) × 100%. The results revealed that cell viability was increased by each LCFA at a low concentration and then inhibited when the concentration increased to higher levels (Suppl. Figure).

TAG accumulation. Cytosolic TAG accumulation was measured using an EnzyChrom Triglyceride Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Cells were lysed with 5% Triton X-100, and 10 μL of the lysed cell solution was added to a 96-well plate, following which 100 μL of assay buffer, 2 μL of enzyme mix, 5 μL of lipase, 1 μL of ATP and l μL of dye reagent were added to each well. The mixture was allowed to stand at room temperature for 30 min, and the absorbance of each well at 570 nm was read using a Multi-Mode Microplate Reader.

RNA extraction and real-time quantitative PCR. Cells were cultured in 6-well plates at 1×10^5 cells/well in DMEM/F12 medium for 48 h and stimulated by combination C18-UFAs treatment for 48 h; the optimum concentration of each FA had been determined by orthogonal experiments to determine cytosolic TAG accumulation.

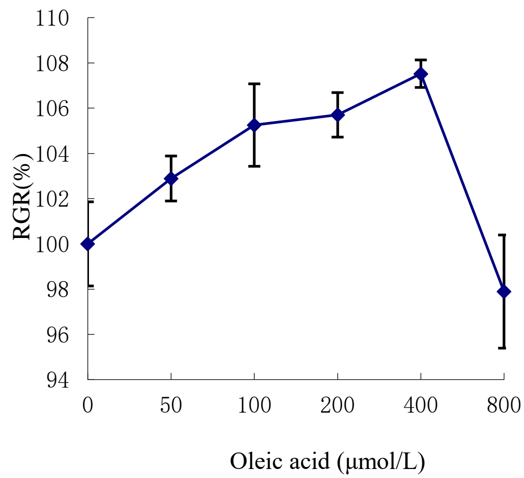
Total RNA was extracted from the cells using an RNAprep Pure Cell/Bacteria kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The RNA purity was determined by the 260/280 ratio, which was measured with a Multi-Mode Microplate Reader, and the mRNA integrity was assessed by 2% agarose gel electrophoresis. cDNA was synthesized using a PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). Real-time quantitative PCR was conducted on a LightCycler 480 instrument (Roche Holding AG, Basel, Switzerland) with a SYBR Premix Ex TaqTM II kit (TaKaRa, Dalian, China). The cycling conditions were as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 30 s, the annealing temperature for 20 s, and 72 °C for 30 s.

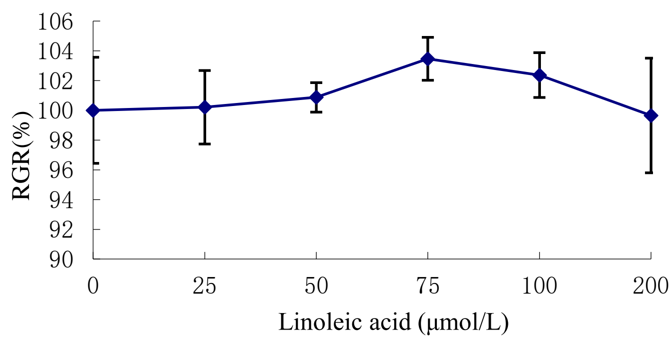
Measurement of the FA composition in cells. Cells treated with C18 UFAs were rinsed twice with ice-cold PBS and lysed in 2 mL of a 5% Triton X-100 solution for 15 min. The cells were then extracted in 4 mL of a hexane/isopropanol solution followed by 2 mL of a Na_2SO_4 solution, centrifuged at 5300 rpm for 10 min at room temperature, and dried with nitrogen. The FAs were transesterified with 2 mL of $NaOCH_3$ at 50 °C for 15 min, and 2 mL of hydrochloric acid/methanol was then added to the FAs, which were heated for 1.5 h at 80 °C. After cooling to room temperature, 3 mL of hexane was added. Samples were thoroughly vortexed and separated statically. The supernatant was dried with nitrogen. Finally, the samples were redissolved in hexane.

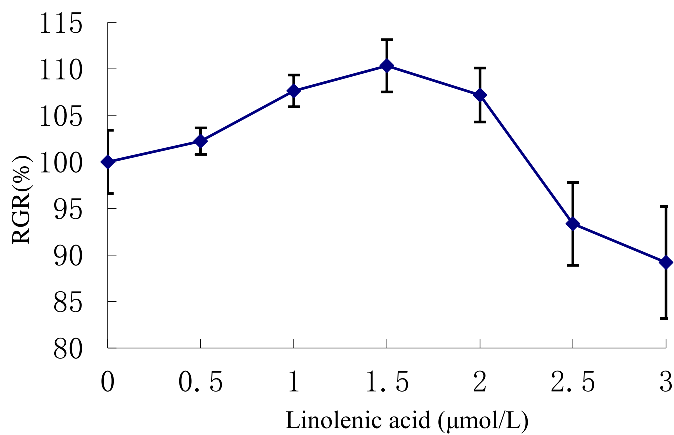
FA methyl esters were analyzed using a Varian 450-GC (Varian Medical Systems, CA, USA) gas chromatograph. Chromatographic separation was performed on an

SPTM-2560 column (Sigma-Aldrich, St. Louis, MO, USA) with an initial temperature of 120 °C for 5 min, followed by heating at 3 °C/min to 230 °C and then at 1.5 °C/min to 240 °C, incubation for 13 min, and heating at a rate of 20 °C/min until reaching 245 °C. The temperature of the FID detector was 260 °C, and the injected sample volume was 1 μL.

Supplementary Figure







Suppl Figure. The experiment to determine the cell viability when cells were treated with different concentrations of oleic acid, linoleic acid, and linolenic acid by methyl thiazolyl tetrazolium assay. Relative Growth Rate (RGR) = (Treatment OD 490/Control OD 490)×100%.

Supplementary Tables

Suppl Table 1. Orthogonal table of 3 long-chain fatty acids $L_9(3^3)$ in optimal concentration (μmol/L) selection

Treatments	Oleic acid	Linoleic acid	Linolenic acid
1	100	50	1
2	100	75	1.5
3	100	100	2
4	200	50	1.5
5	200	75	1
6	200	100	2
7	400	50	2
8	400	75	1.5
9	400	100	1

Suppl Table 2. Primer sequences and annealing temperatures used in real-time quantitative PCR

Genes[1]	GenBank no.	Primer sequences 5′ to 3′	Tm/°C	Length/bp
β-Actin	NM_173979.3	F. AACTCCATCATGAAGTGTGACG R. GATCCACATCTGCTGGAAGG	59	91
RPS9	NM_001101152	F. AAGCTGATCGGCGAGTATG R. GCATTACCTTCGAACAGACG	58	121
GAPDH	XM_001252479	F. GGGTCATCATCTCTGCACCT R. GGTCATAAGTCCCTCCACGA	59	177
ACACA	AJ132890	F. CATCTTGTCCGAAACGTCGAT R. CCCTTCGAACATACACCTCCA	58	101
FASN	CR552737	F. ACCTCGTGAAGGCTGTGACTCA R. TGAGTCGAGGCCAAGGTCTGAA	58	174
LPL	BC118091	F. ACACAGCTGAGGACACTTGCC R. GCCATGGATCACCACAAAGG	60	101
SCD	AY241933	F. TCCTGTTGTTGTGCTTCATCC R. GGCATAACGGAATAAGGTGGC	58	101
ACSL1	BC119914	F. GTGGGCTCCTTTGAAGAACTGT R. ATAGATGCCTTTGACCTGTTCAAT	57	120
SREBF1	NM_001113302	F. CCAGCTGACAGCTCCATTGA R. TGCGCGCCACAAGGA	61	334
PPARG	NM_181024	F. CCAAATATCGGTGGGAGTCG R. ACAGCGAAGGGCTCACTCTC	61	101

[1] ACACA = acetyl-coenzyme A carboxylase-α; FASN = fatty acid synthetase; LPL = lipoprotein lipase; SCD = stearoyl-coenzyme A desaturase; ACSL1 = acyl-CoA synthetase long chain 1; SREBF1 = sterol regulatory element binding factor 1; PPARG = peroxisome proliferator activated receptor-γ.

Suppl Table 3. Optimal concentrations of long-chain fatty acids obtained from triglyceride accumulation (mM) by analysis of orthogonal test

	Triglyceride content (μmol/L)		
	oleic acid	linoleic acid	linolenic acid
K1[1]	1.58	1.63	1.54
K2	1.68	1.57	1.65
K3	1.61	1.58	1.66
k1[2]	0.53	0.54	0.51
k2	0.56	0.52	0.55
k3	0.54	0.53	0.56
Optimal concentration(μM)	200	50	2
R^b	0.03	0.02	0.05

[1] Ki = the sum of triglyceride contents at Ai level

[2] ki = Ki/3

$Ri = \max\{ki\} - \min\{ki\}$