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Elongation and Desaturation Pathways in Mammary Gland Epithelial Cells Are Associated with Modulation of Fat and Membrane Composition

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ABSTRACT: The aim was to determine the relative role of each of the lactogenic hormones (insulin, prolactin and hydrocortisol) and their combinations in regulating elongation and desaturation of polyunsaturated fatty acids and subsequently on composition of cellular lipid compartments in mammary epithelia. Cultured cells of the mammary gland epithelial cell line HC11 were subjected to 48 h of hormonal treatment with different combinations of insulin, hydrocortisone and prolactin. Only the combination of all three hormones induced differentiation according to the marker β-casein gene expression. Inclusion of insulin in the treatment medium increased total fatty acid amount by 50% and increased the concentration of monounsaturated fatty acids by 12% while decreasing that of saturated fatty acids by 35%. Changes in the levels of fatty acids by chain length and saturation paralleled mRNA expression of the desaturases and elongases, whose expression levels were regulated again by inclusion of all three hormones in the treatment medium. Gene expression levels of the Δ6 desaturase and elongase 5 genes (Elovl 5) increased by approximately 1.5-fold, whereas expression of Elovl 4 decreased in the presence of all three hormones. Insulin was the main hormone inducing compositional differences in membrane lipids, increasing phosphatidylethanolamine and phosphatidylinositol and decreasing sphingomyelin and cholesterol. The results indicate that mammary gland epithelial cells express five out of the seven known elongase subtypes which are regulated primarily by the processes of differentiation and produce major compositional changes in mammary gland epithelial cells.

KEYWORDS: *mammary gland, triglyceride, phospholipid, elongase, desaturase*

1. INTRODUCTION

Differentiation of murine mammary gland cell lines is induced by the lactogenic hormones: prolactin, insulin and hydrocortisone.[1] The differentiation process produces multiple enzymatic and metabolic changes, facilitating high rates of fat, milk protein and carbohydrate synthesis (e.g., triglycerides, β-casein and lactose, respectively) by the mammary gland.[1] In vitro, mammary gland epithelial cell differentiation is mainly defined by the expression of the genes encoding milk's signature proteins, notably β-casein.[2−5] Thus, the lactogenic hormones' regulatory role is today defined primarily by their effects on the composition of milk proteins and their association with lactation process. Potential lipid-related changes during differentiation of mammary cells are not established.

In vitro studies have determined that the mammary gland epithelial cells obtain fatty acids for membrane and fat synthesis from two sources: short- and medium-chain fatty acids are considered the product of de novo synthesis, whereas long-chain fatty acids (C16 or longer) are preformed and absorbed from the circulation.[12,13] Many studies investigating mammary gland lipid metabolism distinguish between fatty acid origins based on these observations.[5,14,15] Independent of the fatty acid source, many mammalian tissues can modify fatty acid composition by elongation and desaturation processes facilitated by elongase and desaturase subtypes, respectively. In the mammary gland, Δ5 and Δ6 desaturase gene expression has been shown to correlate with that of sterol regulatory element binding protein,[16] and the activities of Δ9, Δ5 and Δ6 desaturases were shown to correlate with lactation progression.[17] Nonetheless, the regulation of desaturases by lactogenic hormones is still unclear.

Elongase regulation in the mammary gland is unknown, although this gland has been reported to be limited in its ability to elongate fatty acids.[15] Genotypic studies revealed seven elongase subtypes in mice, rats and humans genome (termed Elovl 1 to 7) with differential tissue distribution[18,19] and an expression pattern that is tightly associated with the stage of mammary developmental.[20] Each of the seven elongase isomers is characterized by substrate affinity that is determined by fatty acid chain length and level of unsaturation, although there is some degree of substrate overlap between the isomers.[20] It is still not known which of the elongases subtypes are expressed in the mammary gland and what is the nature of their expression regulation (developmental, differentiation, hormonal). In particular, the role of the lactogenic hormones in regulating the expression and activity of the elongases has not been studied.

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The enzymatic elongation and desaturation of fatty acids can direct them to different metabolic fates. For example, in liver it was shown that the fatty acids' length and saturation level affect their metabolism as precursors for energy generation or structural constituents of cellular membranes.[21,22] Fatty acid chain length and degree of unsaturation were shown to affect membrane physical properties which may in turn affect membrane functionality.[23] For example, it was postulated that the antilipogenic and anticarcinogenic effects of conjugated linoleic acid (CLA) can be attributed to its incorporation into the cellular membrane and the resulting changes in the membrane physical properties.[24] Lipid composition of the cellular membrane was found to vary with differentiation,[25] lipogenic enzyme activity[26] and hormone level.[27,28] These data imply that the lipid composition of various cellular compartments is subject to regulation of metabolic and developmental pathways and that elongation and desaturation processes contribute to these compositional variations. The question of whether mammary gland epithelial cell membrane lipid composition is associated with lipogenesis and differentiation, and which of the lactogenic hormones facilitate these alterations, has never been addressed.

In this study we investigated the compositional changes of membrane and fat of mammary gland epithelial cells exposed to insulin, prolactin and hydrocortisone. In parallel, the processes of elongation and desaturation and the expression of the genes supporting these pathways were determined.

2. MATERIALS AND METHODS

2.1. Cell Culture Materials. Dulbecco's modified Eagle medium (DMEM) and F-12 (HAM) 1:1, penicillin, streptomycin, amphotericin B, L-glutamate solution, Trypsin-EDTA Solution C and fetal bovine serum (FBS) were obtained from Biological Industries (product number 04−001−1A, Bet Haemek, Israel). Bovine insulin, hydrocortisone and ovine prolactin were purchased from Sigma Aldrich Israel Ltd. (Rehovot, Israel).

2.1.1. HC11 Culture: Experimental Design. Murine mammary gland cell line, HC11, was purchased from the American Type Culture Collection (ATCC). Cells were incubated at 37 °C with 5% CO_2 in DMEM/F-12 supplemented with 10% (v/v) FBS, penicillin (100 U/mL), streptomycin (50 ng/mL), amphotericin (0.25 ng/mL), L-glutamine (2 mM) and insulin (1 μg/mL), and the medium was replaced every 48 h. Once cells became confluent, the medium was replaced with serum and hormone-free medium for 48 h. Thereafter, cells were exposed to the treatment media for an additional 48 h. Treatments included the specified combinations of prolactin (P; 5 μg/mL), insulin (I; 5 μg/mL) and hydrocortisone (F; 0.5 μg/mL or 0.036 μg/mL). The hormone concentrations were determined according to preliminary dose−response experiments in our lab and in the literature.[1−5,11] Samples were obtained and analyzed for three biological replicates (n = 3 for every replicate), unless otherwise stated.

2.1.2. Sample Collection for Lipid Extraction. After the 48-h exposure to the lactogenic hormone combinations, cells were then washed with 4 mL of 0.05% Trypsin-EDTA in PBS, detached from the incubation plate and transferred to a new plastic vial. The mixture was centrifuged at 500g for 10 min. The supernatant was discarded and the cells were resuspended in 0.9 mL of saline. Washing was repeated once more by centrifuging at 500g for 5 min. The cells were resuspended in 400 μL of saline and stored at −20 °C until lipid extraction.

2.2. Lipid Extraction and Analysis: Chemicals and Reagents. For lipid extraction, analytical reagent grade methanol and chloroform were purchased from Bio-Lab Ltd. Laboratories (Jerusalem, Israel). For HPLC analysis, HPLC-grade chloroform, methanol and hexane were purchased from Bio-Lab. Triolein acyl glycerol (purity > 99%) was used as the triacylglycerol standard (Supelco, Bellefonte, PA). Cholesterol (purity > 99%) and phospholipid standards were supplied by Sigma Aldrich Israel, and consisted of phosphatidylethanolamine (PhE) (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, purity 99%), phosphatidylinositol (PhI) (L-α-phosphatidylinositol ammonium salt from bovine liver, purity 98%), phosphatidylserine (PhS) (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serinpuritye sodium salt, 95%), phosphatidylcholine (PhC) (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, purity ±99%) and sphingomyelin (SM) (from bovine brain, purity 97%). Heptadecanoic acid (C17:0) (Sigma Aldrich Israel) was used as a standard for free fatty acids in HPLC and as an internal standard for GC analysis. For fatty acid extraction and methylation, methanol (analytical reagent grade) was purchased from Bio-Lab, petroleum ether (analytical reagent grade) was from Gadot Lab Supplies (Netanya, Israel), and sulfuric acid was from Bet Dekel (Ra'anana, Israel). Retention times were determined by injection of commercial mixtures of two fatty acid methyl ester (FAME) standards: from C14:0 to C22:6n3 (PUFA-2, Animal Source) and FAME mix of C8:0−C24:0 (Supelco, no. 18919, 4-7015).

2.2.1. Extraction of Total Lipids. Lipids were extracted using cold extraction as previously described.[29] Briefly, 5 μg of heptadecanoic acid (C17:0) were added to 0.5 mL sample. Then, 10 mL of methanol−chloroform solution (2:1, v/v) was mixed in, 2 mL (0.2× volume) of double-distilled water was added and samples were incubated at 4 °C for 14 h. The upper phase was discarded and the lower phase containing total lipids was evaporated under 30 cm Hg vacuum (Sheldon's vacuum oven, Cornelius, OR) at 65 °C. For HPLC analysis, dried samples were diluted in 25 μL of chloroform−ethanol (3%, v/v) and stored at −20 °C. For GC analysis, FAME were generated by incubation of the total lipid extract with 2 mL of 5% (v/v) methanolic H_2SO_4 at 65 °C for 1 h in sealed vials. Samples were then cooled to room temperature and 1.5 mL of petroleum ether and 3 mL of double-distilled water were added. The upper phase was collected and evaporated in a vacuum oven under 30 cm Hg at 65 °C. Dried samples were dissolved in 30 μL of petroleum ether.

2.2.2. GC Analysis. Chromatographic analysis was performed with a 6890N gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with a fused-silica (60 m × 0.25 mm ID, 0.25 μm film) capillary column (DB-23, Agilent Technologies) under the following conditions: the oven temperature was programmed from 130 to 170 °C at a rate of 27 °C/min, from 170 to 215 °C at a rate of 2 °C/min, held at 215 °C for 8 min, from 215 to 250 °C at a rate of 40 °C/min, and held at 250 °C for 5 min. Run time was 37.9 min. Helium was used as the carrier gas at a flow rate of 2.21 mL/min. Flame-ionization detector temperature was 280 °C, and injector temperature was 270 °C. Air and hydrogen flows were adjusted to 40 mL/min H_2 and 450 mL/min air to give maximal detector response. The split ratio was set at 10:1 and 1 μL sample was injected. Peak identification was based on relative retention times of two external standards. The area of each fatty acid peak was recorded using ChemStation software (Agilent Technologies), and fatty acid weights were calculated using the internal standard's area under the peak.

2.2.3. Fatty Acid Classification. To better understand the lipid metabolism underlying differentiation of the mammary gland epithelial cells, fatty acids were grouped according to their chemical features into saturated (SFA; C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0), monounsaturated (MUFA; C16:1n7, C16:1n9, C18:1n7, C18:1n9, C20:1n9 and C22:1n9) and polyunsaturated (PUFA; C18:2n6, C18:3n6, C18:3n3, C20:4n6, C20:5n3, C22:4n6, C22:6n3).

2.2.4. Elongase and Desaturase Activity Markers. To evaluate elongase and desaturase activities, the ratio between amounts of substrate and product was determined and utilized to indicate activity:[20,30] the ratio between C18:0 and C16:0 was used as an activity marker for elongase 6 and C18:1n7/C16:1n7 was used as a marker for elongases 5 and 6 together. C16:1n7/C16:0 and C18:1n9/C18:0 were used as activity markers for Δ9desaturase. To evaluate the activities of elongases and desaturases under the regulation of the lactogenic hormones, the amount of product before cells were exposed to hormonal treatment (t_0) was deducted from the amount of the product at the end of the hormonal treatment period. This value was divided by the total amount of substrate, since all of the substrate in

the sample can be utilized by elongases and desaturases regardless of its stage of synthesis.

2.2.5. Separation of Polar and Neutral Lipids. Separation of polar and neutral lipid classes was performed by HPLC (HP 1200, Agilent Technologies) combined with a variable wavelength detector set at 205 nm,[21] and a gradient of hexane, ethanol and methanol (shown in Table 1). The column heated to 40 °C, flow was 1.2 mL/min,

Table 1. HPLC Solvent Gradient Method Used to Separate Cholesterol Ester, Triglycerides and Phospholipids Using Liquid Chromatography Equipped with Variable Wavelength Detector set at 205 nm

time (min)	% hexane	% ethanol	% methanol
0	99.5	0.5	
1	99.3	0.7	
10	99.3	0.7	
15	30	30	40
17	0	2	98
22	0	2	98
27	70	30	
34	99.5	0.5	
39	99.5	0.5	

injection volume was 20 μL. Three samples of lipid extracts were injected for each treatment. Separated neutral and polar lipid fractions were collected with a fraction collector (Teledyne Isco Inc., Lincoln, NE). The solvents were evaporated in a vacuum oven and prepared for GC analysis.

2.2.6. Phospholipid, Triglyceride and Cholesterol Quantification. Identification and quantification of cell lipids were performed by normal-phase liquid chromatography (HP 1200, Agilent Technologies) equipped with evaporating light-scattering detector (1200 series ELSD, Agilent Technologies).

The method employed for lipid separation consisted of dichloromethane, methanol and double-distilled water and is detailed in Table 2. This protocol induced the separation of triacyglycerol, two isomers of diacylglycerol, monoacylglycerol, cholesterol, free fatty acids, PhE, PhI, PhS, PhC and SM.

Table 2. HPLC Solvent Gradient Method Used for Quantifying Triglycerides, Cholesterol and Phospholipids Using Liquid Chromatography Equipped with ELSD Detector

time (min)	% dichloromethane	% methanol	% water
0	99	1	
8	93	7	
12	35	65	
22	35	64	1
26	35	60	5
40	35	60	5
45	80	20	
50	99	1	
55	99	1	

A column (Zorbax RX-SIL, 4.6 × 250 mm, Agilent Technologies) was heated to 50 °C, and flow was 1 mL/min. The ELSD was heated to 65 °C, nitrogen pressure was 3.9 bar, filter was 5, gain (sensitivity) was set to 7 for the first 11 min and then changed to 9 until the end of the run to enable detection of lower-abundance lipid components. Injection volume was 20 μL. Lipid extracts from four samples were injected. Quantification was based on areas under the standard curves of each lipid standard concentration. The power equations were triglyceride, $y = 0.0014x^{0.8695}$ ($r^2 = 0.995$); cholesterol, $y = 0.0245x^{0.581}$ ($r^2 = 0.9925$); PhE, $y = 0.1369x^{0.437}$ ($r^2 = 0.9908$); PhI, $y = 0.0103x^{0.7918}$ ($r^2 = 0.9898$); PhC, $y = 0.0408x^{0.5077}$ ($r^2 = 0.9986$), and sphingomyelin, $y = 0.0667x^{0.5287}$ ($r^2 = 0.9981$).

2.3. RNA Extraction and Analysis: Chemicals and Reagents. Tri reagent was purchased from Molecular Research Center Inc. (Cincinnati, OH). Absolute blue SYBR green ROX mix and Thermo Scientific Verso cDNA Synthesis kit were purchased from Thermo Scientific, ABgene (Epsom, UK). Turbo DNase kit was purchased from Ambion Inc. (Austin, TX). Fisher BioReagents Taq DNA polymerase was purchased from Fisher Biotec (Wembley, Australia). Primers were synthesized by Hy-laboratories (Rehovot, Israel) and from Syntezza (Jerusalem, Israel). Specific primers were designed for each gene using Primer Express software (Applied Biosystems, Foster City, CA).

2.3.1. RNA Extraction. Total RNA was isolated from HC11 cells by the acid guanidinium thiocyanate−phenol−chloroform extraction method.[31] The concentration and 260/280 nm optical density (OD) ratio of the RNA was determined by Nanodrop spectrophotometry (NanoDrop Technologies, Wilmington, DE). DNA contamination was reduced by DNase Stop Solution according to the manufacturer's guidelines. Extracted and purified RNA was kept at −80 °C until further analysis. The RNA was reverse-transcribed to produce cDNA using the Thermo Scientific Verso cDNA Synthesis kit according to the manufacturer's instructions.

2.3.2. Elongase Isomer Expression Analysis. To determine which of the elongase family members are expressed in the mammary gland epithelial cells, PCR analysis was conducted. Taq DNA polymerase and suitable primers were used to amplify the cDNA. The amplification parameters were: initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 45 s, annealing at 61 °C for 45 s, and extension at 72 °C for 2 min. Final extension was at 72 °C for 7 min. PCR products were size-separated by electrophoresis on a 3.5% agarose gel. The gels were stained with ethidium bromide.

2.3.3. Real-Time PCR. For gene-expression analysis, the StepOnePlus Real-Time PCR System (Applied Biosystems) was used. cDNA was mixed with primers (Table 3) and SYBR Green mix. Enzyme activation was at 95 °C for 15 min, followed by 40 cycles of 1 min denaturation at 95 °C, 2 min annealing at 60 °C, and 1 min extension at 72 °C. Analysis was performed by StepOne software v2.1 (Applied Biosystems). Dissociation-curve analysis was performed after each real-time experiment to confirm the presence of only one product and the absence of formation of primer dimers. For each gene, a standard curve was also generated to determine reaction efficacy of over 80%. The threshold cycle number (Ct) for each tested gene was used to quantify its relative abundance. Relative units were calculated as $2^{-\Delta(Ct)} = 2^{(Ct\ housekeeping\ gene - Ct\ target\ gene)}$.

2.4. Statistical Analysis. All statistical procedures were performed using JMP software version 7 (SAS Institute, Cary, NC). All reported data are means ± SEM. Comparisons by ANOVA were significant at $\alpha < 0.05$. When a significant difference was found, we performed the Tukey-Kramer HSD test. All dependent variables were checked for normality distribution by goodness of fit application and for homogenic variance by unequal variances in JMP software.

3. RESULTS

3.1. Hormone-Induced Differentiation. The expression of β-casein mRNA in confluent HC11 cell line was previously used as a differentiation marker.[2] In the present study β-casein expression was only detected when all three hormones were included in the treatment medium (PIF treatment; Figure S1).

3.2. Total Fatty Acids. Presence of insulin in the culture medium (IF, PI, PIF) increased total fatty acid amount/plate by 50 to 70% compared with the hormonal combination PF ($p < 0.0001$), and by 20 to 40% compared with the initial incubation time (t_0) ($p < 0.0001$) (Figure 1). Prolactin and hydrocortisone (PF) also increased total fatty acid amount by 1.4-fold compared with t_0. PIF treatment increased the amount of MUFA by 1.7-fold ($p < 0.0001$) and of SFA 40% compared with PF. The amount of PUFA was similar between all

Table 3. Primer Sequences Used for Real-Time PCR

gene	GenBank acc. no.	primer sequences[a]
Elovl 1	NM001039176	S: 5′ CCCTACCTTTGGTGGAAGAA 3′
		AS: 5′ ATCCAGATGAGGTGGATGATG 3′
Elovl 2	NM019423	S: 5′ ACGCTGGTCATCCTGTTCTT 3′
		AS: 5′ GCCACAATTAAGTGGGCTTT 3′
Elovl 3	NM007703	S: 5′ TCGAGACGTTTCAGGACTTAAGG 3′
		AS: 5′ GCTCTTCCGCGTTCTCATGT 3′
Elovl 4	NM148941	S: 5′ CTCAACGCCTTTCGATACAAAA 3′
		AS: 5′ ATGGGATCATACAACGCAGGAT 3′
Elovl 5	NM134255	S: 5′ GGTGGCTGTTCTTCCAGATT 3′
		AS: 5′ CCCTTCAGGTGGTCTTTCC 3′
Elovl 6	NM130450	S: 5′ ACAATGGACCTGTCAGCAAA 3′
		AS: 5′ GTACCAGTGCAGGAAGATCAGT 3′
Elovl 7	NM029001	S: 5′ CATCGAGGACTGTGCGTTTTT 3′
		AS: 5′ GAGGCCCAGGATGATGGTTT 3′
Δ5Desaturase (fads1)	NM146094	S: 5′ TGTGTGGGTGACACAGATGA 3′
		AS: 5′ GTTGAAGGCTGATTGGTGAA 3′
Δ6Desaturase (fads2)	NM019699	S: 5′ CCACCGACATTTCCAACA 3′
		AS: 5′ GGGCAGGTATTTCAGCTTCTT 3′
Δ9Desaturase (scd1)	NM009127	S: 5′ TCAACTTCACCACGTTCTTCA 3′
		AS: 5′ CTCCCGTCTCCAGTTCTCTT 3′
β-actin	NM007393	S: 5′ AGTGTGACGTTGACATCCGTA 3′
		AS: 5′ GCCAGAGCAGTAATCTCCTTCT 3′
β-casein	NM009972	S: 5′ AGAGGATGTGCTCCAGGCTA 3′
		AS: 5′ AATGACAGGCCCAAGAGATG 3′
18srRNA	NT111595	S: 5′ TTCCTTACCTGGTTGATCCTGCCA 3′
		AS: 5′ AGCCATTCGCAGTTTCACTGTACC 3′

[a]S, sense; AS, antisense.

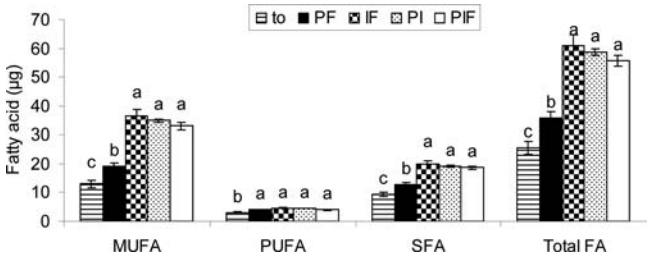

Figure 1. Hormone-dependent fatty acid amounts in mouse mammary epithelial cell line HC11. Confluent cells were cultured in DMEM/F12 for 2 days without hormones. Then media were replaced with serum-free treatment media containing combinations of insulin (I; 5 μg/mL), prolactin (P; 5 μg/mL) and hydrocortisone (F; 0.5 μg/mL), for 2 days. Cell lipid extracts were methylated and fatty acid amounts were analyzed by GC. t_0 represents values before the hormonal treatments. Values represent mean ± SEM (n = 4) of monounsaturated (MUFA), polyunsaturated (PUFA), saturated (SFA) and total (Total FA) calibrated fatty acids. Different letters indicate significant differences between treatment groups (p < 0.05).

hormonal treatments and differed only from t_0, which may be accounted for the shorter exposure to the treatment media. Fatty acid composition was not affected by prolactin (IF compared with PIF) or hydrocortisone (IP compared with PIF). Compared with t_0, prolactin and hydrocortisone (PF) increased the amounts of MUFA, PUFA and SFA by 45, 35 and 28%, respectively. The inclusion of insulin in the treatment medium altered cellular fatty acid composition (Table 4); MUFA concentration increased by 12%, while those of PUFA and SFA were decreased by 35% and 6%, respectively (p < 0.0001), between IF, PI and PIF and t_0.

3.3. Regulation of Fatty Acid Synthesis by Lactogenic Hormones. Fatty acids compositional changes in response to the hormonal treatment are illustrated as percentage point differences in Figure 2. Negative values indicate decreased concentration of the fatty acid at the end of the treatment relative to its concentration at t_0. As indicated above, the main fatty acids that were synthesized were monounsaturated fatty acids, most notably, c18;1n9 and c16;1n7. While c18;1n9 concentration increased by all treatments, that of c16;1n7 increased by treatment media which contained insulin, and its concentration decreased compared with t_0 when treatment medium contained prolactin and hydrocortisone alone. The elevation in monounsaturated fatty acid concentration resulted in a decreased concentrations of 16:0, 18:0 and 20:4n6, by 1.77 ± 0.16%, 1.21 ± 0.12%, and 2.46 ± 0.37%, respectively, at the end of the PIF treatment period compared with t_0. It should be noted that while c16;0 concentrations decreased by all treatments compared with t_0, that of c18;0 increased in PF compared with t_0.

3.4. Elongases and Desaturases: Fatty Acid Ratio As Activity Markers. To evaluate the effect of hormonal treatments on desaturation and elongation indices we utilized the amount of product synthesized only during the hormonal treatment phase of the experiment (de novo) and divided it by the total amount of the substrate available in the culture system. Insulin increased both Δ9desaturase activity markers (p < 0.0001 for C16:1n7/C16:0 and p = 0.0002 for C18:1n9/C18:0; Figure 3A). The activity marker for Δ9desaturase (C16:1n7/C16:0) was also affected by hydrocortisone (PI was significantly lower than IF and PIF). Insulin also increased value for elongase 6 activity marker by insulin (p < 0.001, Figure 3B).

Table 4. Fatty Acid Composition of Mammary Gland Epithelial Cells Exposed to Lactogenic Hormones[a]

	t_0	PF	IF	PI	PIF	*P*-value
C12:0	0.12 ± 0.06	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.03	0.08 ± 0.04	0.8379
C14:0	3.1 ± 0.14^{c}	3.1 ± 0.02^{c}	3.8 ± 0.04^{ab}	3.7 ± 0.05^{b}	4.1 ± 0.04^{a}	<0.0001
C16:0	18.4 ± 0.3^{a}	16.9 ± 0.3^{b}	15.3 ± 0.05^{c}	15.3 ± 0.02^{c}	15.6 ± 0.16^{c}	<0.0001
C16:1n7	7.4 ± 0.4^{c}	6.7 ± 0.02^{c}	11.4 ± 0.2^{a}	9.7 ± 0.1^{b}	11.0 ± 0.2^{a}	<0.0001
C18:0	13.9 ± 0.2^{a}	14.3 ± 0.1^{a}	12.4 ± 0.1^{b}	12.7 ± 0.1^{b}	12.7 ± 0.1^{b}	<0.0001
C18:1n9	27.4 ± 0.4^{c}	30.0 ± 0.06^{a}	29.4 ± 0.2^{ab}	30.3 ± 0.1^{a}	28.8 ± 0.1^{b}	<0.0001
C18:1n7	9.7 ± 0.1^{b}	9.5 ± 0.01^{b}	11.3 ± 0.2^{a}	11.6 ± 0.1^{a}	11.3 ± 0.1^{a}	<0.0001
C18:2n6	1.1 ± 0.02^{a}	0.7 ± 0.02^{b}	0.4 ± 0.01^{c}	0.5 ± 0.01^{c}	0.4 ± 0.01^{c}	<0.0001
C20:4n6	7.6 ± 0.22^{a}	8.1 ± 0.12^{a}	5.2 ± 0.04^{b}	5.4 ± 0.04^{b}	5.2 ± 0.04^{b}	<0.0001
C22:6n3	1.7 ± 0.06^{a}	1.3 ± 0.08^{b}	1.0 ± 0.01^{c}	1.0 ± 0.03^{c}	1.0 ± 0.05^{c}	<0.0001
MUFA	50.8 ± 0.7^{c}	53.1 ± 0.1^{b}	60.0 ± 0.2^{a}	59.5 ± 0.1^{a}	59.3 ± 0.4^{a}	<0.0001
PUFA	12.2 ± 0.2^{a}	11.1 ± 0.3^{b}	7.4 ± 0.1^{c}	7.6 ± 0.1^{c}	7.2 ± 0.1^{c}	<0.0001
SFA	37.1 ± 0.6^{a}	35.8 ± 0.3^{a}	32.7 ± 0.2^{b}	32.9 ± 0.1^{b}	33.5 ± 0.3^{b}	<0.0001

[a]Values are weight % means ± SEM, n = 3. Different letters (a, b, c) in a row indicate significant differences (p < 0.05). MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; t_0, before hormone treatments; I (insulin; 5 μg/mL); P (prolactin; 5 μg/mL); F (hydrocortisone; 0.5 μg/mL).

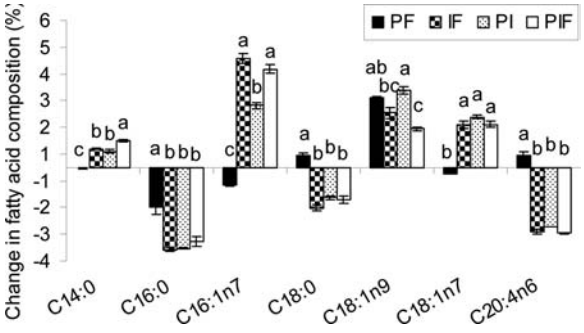

Figure 2. Compositional changes of HC11 cell line fatty acids profile induced by insulin prolactin and hydrocortisol. Cells were treated as described in the legend to Figure 1. Cell lipid extracts were methylated and fatty acid amounts were analyzed by GC. Percentage differences for each fatty acid relative to its concentration on t_0 are illustrated. Negative values indicate a lower concentration at the end of the hormonal treatment compared with t_0. Values represent fatty acid weight percentage point differences between the treatments and are expressed as means ± SEM (n = 3). Different letters indicate significant differences between treatment groups (p < 0.05).

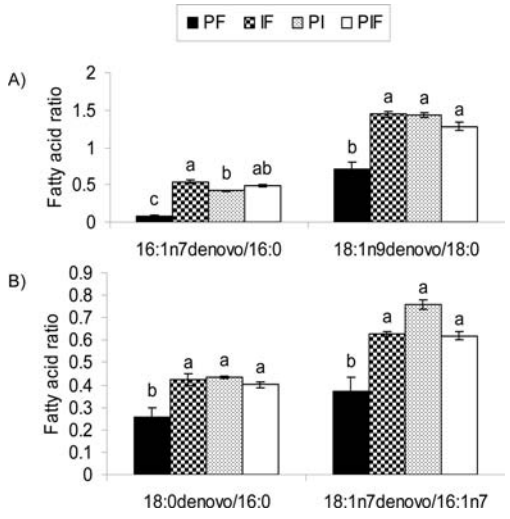

Figure 3. Hormone-dependent activity indicators of elongases and desaturases in mouse mammary epithelial cell line HC11. Cells were treated as described in the legend to Figure 1. Cell lipid extracts were methylated and fatty acid amounts were analyzed by GC. Values represent means ± SEM (n = 3) of the ratio between de novo-synthesized product (treatment-t_0) and total substrate. Δ9desaturase activity indicators 16:1n7/16:0 and 18:1n9/18:0 (A). Elongase 6 activity indicators 18:0/16:0 and 18:1n7/16:1n7 (B). Different letters indicate significant differences between treatment groups (p < 0.05).

3.5. Lactogenic Hormones Regulate Desaturase and Elongase Gene Expression. The mRNA expression levels of Δ5, Δ6 and Δ9 desaturase were higher than those of the elongase isomers, with Δ9desaturase mRNA levels being the highest of all elongases and desaturases. PIF increased Δ5desaturase mRNA expression 1.3-fold compared with PF (p = 0.0278, Figure 4). Differentiation medium (PIF) increased Δ6desaturase mRNA expression 1.68-, 1.32-, and 1.44-fold compared with PF, IF and PI, respectively (p < 0.0002). Differentiation also increased Δ9desaturase mRNA expression by 1.44- and 1.41-fold compared with PF and PI, respectively (p < 0.0004), but did not differ from the IF.

Elovl 1, 4, 5, 6, and 7 genes were expressed in HC11 cells. Elovl 2 and 3 were below detectable levels, whereas Elovl 1 showed the highest expression level (Figure 4). Expression of almost all of the elongase genes was regulated by the differentiation process. Under differentiation conditions (PIF), Elovl 1 gene expression was 1.43- and 1.7-fold higher than with PF and PI, respectively (p < 0.009). Elovl 4 gene expression decreased by differentiation medium compared with all other treatments (PF, PI, IF; p < 0.0001). Elovl 5 gene expression increased by 1.2−1.38 fold in the differentiation medium compared with all other treatments (p = 0.0002). Elovl 7 gene expression was increased by 1.69- and 2.3-fold in differentiation medium compared with PF and PI, respectively (p < 0.0023). These differences were attributed to the inclusion of all three lactogenic hormones in the treatment medium and since this was the only treatment that induced β-casein gene expression could be attributed to differentiation.Insulin increased Elovl 6 gene expression compared to PF (p = 0.0046).

3.7. Lactogenic Hormones Modulate Fatty Acid Composition of Membranes and Fat (Phospholipids and Triglycerides, Respectively). Lactogenic hormones modulated fatty acid composition in the fat and membrane cellular compartments (Tables 5 and 6, respectively): C12:0 and C14:0 wt % were higher in the triglycerides (more than

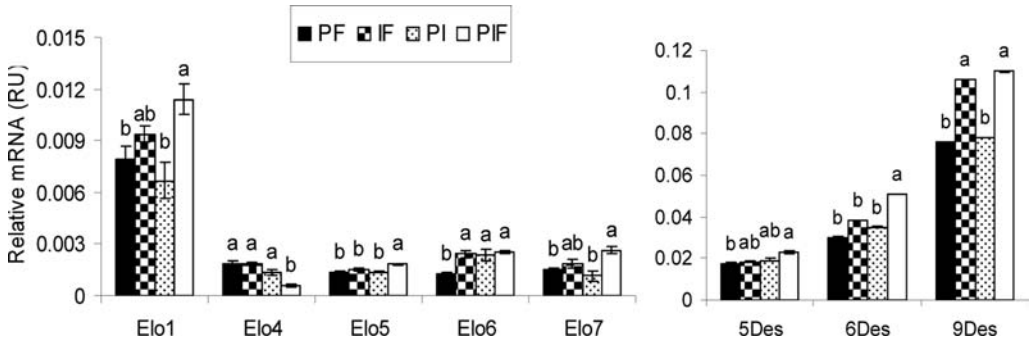

Figure 4. Hormone-dependent gene expression of elongases and desaturases in mouse mammary epithelial cell line HC11. Cells were treated as described in the legend to Figure 1. Total RNA was extracted and gene expression of the elongases and desaturases expressed in HC11 cells was determined using real-time PCR. Elongase (Elo) 1, 4, 5, 6, 7 and Δ5D, Δ6 and Δ9 desaturase (5, 6, 9Des) are shown normalized to β-actin. Values represent means ± SEM (n = 4). Different letters indicate significant differences between treatment groups ($p < 0.05$).

Table 5. Fatty Acid Composition of Mammary Gland Epithelial Cell Triglycerides[a]

	t_0	PF	IF	PI	PIF	P-value
C12:0	3.3 ± 0.3^{b}	6.0 ± 0.7^{a}	3.5 ± 0.3^{b}	2.0 ± 0.1^{b}	2.7 ± 0.2^{b}	0.0002
C14:0	8.7 ± 0.8^{a}	6.2 ± 0.3^{b}	7.4 ± 0.8^{ab}	6.7 ± 0.2^{ab}	7.5 ± 0.1^{ab}	0.0579
C16:0	24.8 ± 1.4	23.5 ± 1.3	21.0 ± 0.4	21.6 ± 0.5	23.6 ± 1.4	0.1573
C16:1n7	3.2 ± 0.1^{b}	4.1 ± 0.7^{b}	6.9 ± 0.2^{a}	6.4 ± 0.4^{a}	6.6 ± 0.4^{a}	0.0002
C18:0	10.0 ± 0.5	10.1 ± 0.4	8.6 ± 0.3	9.1 ± 0.1	9.0 ± 0.5	0.0783
C18:1n9	20.6 ± 0.6^{c}	21.4 ± 1.2^{bc}	25.0 ± 0.4^{a}	25.9 ± 0.4^{a}	24.2 ± 0.4^{ab}	0.0008
C18:1n7	6.1 ± 0.1^{b}	5.4 ± 0.4^{b}	7.9 ± 0.2^{a}	8.2 ± 0.1^{a}	7.2 ± 0.2^{a}	<0.0001
C18:2n6	2.9 ± 0.03^{a}	2.8 ± 0.21^{a}	1.7 ± 0.04^{b}	2.0 ± 0.21^{b}	1.5 ± 0.05^{b}	<0.0001
C20:4n6	3.6 ± 0.2^{a}	2.7 ± 0.2^{b}	2.1 ± 0.1^{bc}	2.0 ± 0.1^{c}	1.5 ± 0.1^{c}	<0.0001
MUFA	42.2 ± 1.8^{b}	42.5 ± 1.3^{b}	52.2 ± 0.3^{a}	53.7 ± 0.3^{a}	50.5 ± 1.1^{a}	<0.0001
PUFA	9.7 ± 0.3^{a}	10.8 ± 0.6^{a}	6.5 ± 1.1^{b}	5.9 ± 0.4^{b}	5.3 ± 0.3^{b}	0.0002
SFA	48.1 ± 2.1^{a}	46.6 ± 1.1^{ab}	41.3 ± 0.8^{bc}	40.5 ± 0.7^{c}	44.2 ± 1.3^{abc}	0.0085

[a]Values are weight % means ± SEM, n = 3. Different letters (a, b, c) in a row indicate significant differences ($p < 0.05$). MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; t_0, before hormone treatment; I (insulin; 5 μg/mL); P (prolactin; 5 μg/mL); F (hydrocortisone; 0.5 μg/mL).

Table 6. Fatty Acid Composition of Mammary Gland Epithelial Cell Phospholipids[a]

	t_0	PF	IF	PI	PIF	P-value
C12:0	0.3 ± 0.02	0.2 ± 0.06	0.2 ± 0.02	0.2 ± 0.03	0.2 ± 0.02	0.1329
C14:0	1.6 ± 0.1^{c}	2.2 ± 0.2^{b}	2.7 ± 0.1^{ab}	3.0 ± 0.1^{a}	3.1 ± 0.1^{a}	<0.0001
C16:0	19.5 ± 0.5^{a}	15.7 ± 0.4^{b}	15.2 ± 0.2^{b}	16.0 ± 0.2^{b}	16.0 ± 0.2^{b}	<0.0001
C16:1n7	2.8 ± 0.1^{b}	3.6 ± 0.2^{b}	7.1 ± 0.3^{a}	7.2 ± 0.1^{a}	7.6 ± 0.2^{a}	<0.0001
C18:0	19.1 ± 0.2^{a}	17.0 ± 0.2^{b}	15.2 ± 0.3^{c}	15.2 ± 0.2^{c}	15.0 ± 0.2^{c}	<0.0001
C18:1n9	25.3 ± 0.4^{a}	28.2 ± 0.1^{b}	29.6 ± 0.04^{a}	29.7 ± 0.2^{a}	28.8 ± 0.2^{ab}	<0.0001
C18:1n7	6.9 ± 0.1^{b}	7.0 ± 0.1^{b}	7.9 ± 0.2^{a}	7.4 ± 0.4^{ab}	7.9 ± 0.1^{a}	0.0073
C18:2n6	0.9 ± 0.02^{a}	0.8 ± 0.06^{a}	0.5 ± 0.02^{b}	0.4 ± 0.01^{b}	0.5 ± 0.0^{b}	<0.0001
C20:4n6	11.6 ± 0.4^{a}	12.7 ± 0.2^{a}	8.6 ± 0.2^{b}	7.9 ± 0.3^{b}	7.9 ± 0.1^{b}	<0.0001
C22:6n3	2.2 ± 0.13^{a}	2.0 ± 0.01^{ab}	1.7 ± 0.16^{bc}	1.5 ± 0.03^{c}	1.4 ± 0.03^{c}	0.0003
MUFA	41.1 ± 0.3^{c}	46.1 ± 0.5^{b}	53.6 ± 0.5^{a}	53.7 ± 0.1^{a}	53.8 ± 0.2^{a}	<0.0001
PUFA	16.8 ± 0.7^{a}	17.6 ± 0.3^{a}	12.4 ± 0.4^{b}	11.1 ± 0.3^{b}	11.2 ± 0.1^{b}	<0.0001
SFA	42.1 ± 0.9^{a}	36.3 ± 0.3^{b}	34.0 ± 0.1^{c}	35.1 ± 0.3^{bc}	35.0 ± 0.1^{bc}	<0.0001

[a]Values are weight % means ± SEM, n = 3. Different letters (a, b, c) in a row indicate significant differences ($p < 0.05$). MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; t_0, before hormone treatments; I (insulin; 5 μg/mL); P (prolactin; 5 μg/mL); F (hydrocortisone; 0.5 μg/mL).

10%) than in the phospholipids (less than 3.5%). Insulin increased MUFA, especially C18:1 and C16:1, in both triglycerides and phospholipids. Insulin increased fatty acids with a chain length of 18 carbons in the triglyceride fraction to at least 40% of total fatty acids (p = 0.0157, data not shown). In the phospholipid fraction, these fatty acids made up 52 to 53% of total fatty acids and were not changed by insulin (p = 0.4577). Insulin decreased the weight % of fatty acids with a chain length of 16 carbons or more (long-chain fatty acids), and these were found at a much higher percentage in phospholipids. For example, C20:4n6 made up 12.7 ± 0.2% in phospholipids when cells were cultured with PF, and decreased to 7.9 ± 0.1% with PIF treatment ($p < 0.0001$). In triglycerides, the reduction was from 2.7 ± 0.2% to 1.5 ± 0.2% ($p < 0.0001$).

3.8. Lactogenic Hormones Modulate Membrane Lipid Composition: Phospholipids and Cholesterol. The

amount of all phospholipids was significantly increased by insulin ($p < 0.01$), whereas only a tendency toward increase was found for the cholesterol amount ($p = 0.0556$, Figure 5A). A

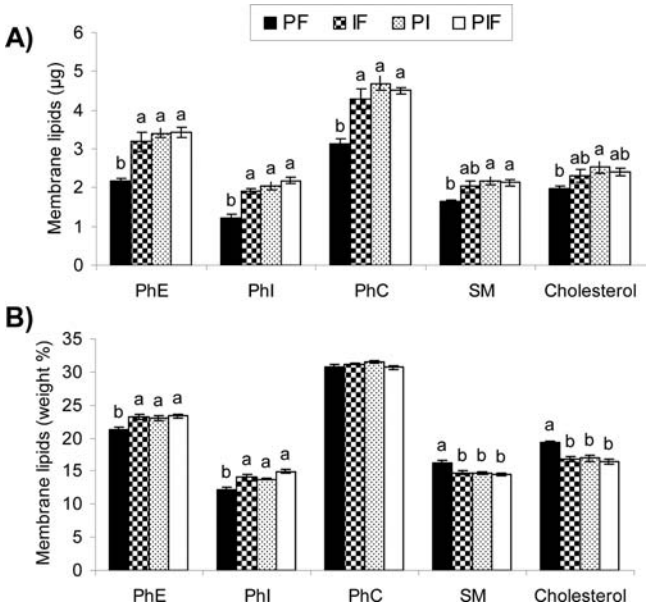

Figure 5. Hormone-dependent membrane lipid composition in mouse mammary epithelial cell line HC11. Cells were treated as described in the legend to Figure 1. Cell lipid extracts were separated by HPLC equipped with an ELSD to quantify main membrane compounds including phosphatidylethanolamine (PhE), phosphatidylinositol (PhI), phosphatidylcholine (PhC), sphingomyelin (SM) and cholesterol. The amount of membrane lipids (phospholipids and cholesterol) was determined (μg) (A). The membrane lipid composition (phospholipids and cholesterol) is illustrated as weight % (B). Values represent mean ± SEM ($n = 4$). Different letters indicate significant differences between treatment groups ($p < 0.05$).

determination of membrane lipid composition revealed that the insulin increased PhE ($p = 0.0061$) and PhI ($p = 0.0018$) weight %, but decreased those of SM (p = 0.0009) and cholesterol ($p = 0.0004$) (Figure 5B). The ratio between SM and cholesterol remained similar under all treatments ($p = 0.7429$). PhC weight % was constant for all treatments, although a tendency to increase was found when insulin was included in the medium ($p = 0.0765$).

4. DISCUSSION

In the present study, murine mammary gland epithelial cells were exposed to hydrocortisone, insulin and prolactin to induce functional differentiation and to determine their effects on membrane composition, and in parallel the expression patterns of genes encoding the fatty acid elongases and desturases subtypes were determined. The results revealed that insulin, and not the mammary cell stage of differentiation, is the main factor influencing fatty acid composition of membrane and fat cellular compartments. In addition, membrane phospholipid composition was modulated primarily by insulin and not the differentiation process. Nonetheless, elongase and desaturase gene expression was strongly associated with differentiation status, since a combination of the three hormones was required to induce changes in the gene expression of these enzymes, except for Elovl 6 which was influenced primarily by insulin, and Δ9desaturase which was influenced primarily by the combination of insulin and hydrocortisone.

When mammary gland lipogenesis is studied, the intraspecies differences in terms of biochemical regulatory mechanisms and synthesis pathways should be considered. In the present study, murine mammary gland cell line was used, and therefore the expected fatty acid composition was of very low concentration of short chain fatty acids,[16] unlike bovine or ovine fatty acid composition, which is expected to be rich in short and medium chain fatty acids, respectively.[15] The choice of cell line as a model for this study over other possible models like tissue explants was made to enable compositional analysis of only the epithelial cells and to avoid misinterpretation of the results in case of contamination by other mammary cell types. Furthermore, that cells in this model did not secrete their lipogenesis products facilitated quantification of the overall lipid synthesis\degradation balance in response to the hormonal treatments.

Insulin increased the synthesis of fatty acids, mainly MUFA, and resulted in changes in the fatty acid profile of the cells: a higher concentration of MUFA and lower concentrations of PUFA and SFA were found when the cells were exposed to insulin. These results are supported by similar changes in Δ9desaturase gene expression and activity indicators, all of which were markedly increased by insulin. Inclusion of hydrocortisone to the treatment media had a small influence that was additive to that of insulin on Δ9desaturase activity indicators, especially when the substrate used for the indicator calculation was C16:0.

According to the results of the present study utilizing mammary epithelial cells, approximately 60% of the fatty acids synthesized during the hormonal treatment phase of the experiment were long-chain fatty acids with chain lengths of more than 16 carbons. These results are in contrast with previous studies that found that the mammary gland synthesizes primarily fatty acids with carbon chain length of less than 16 atoms.[15,16] These inconsistencies most probably result from the use of different models: while the present study made use of an undifferentiated cell line, previous work examined the effect of various hormones on differentiated cell lines or tissue explants.[15,16] Mammary explants consist of differentiated epithelial cells and adipose cells, which can influence the activity of the epithelial cells as well as availability of fatty acids for lipid synthesis.[32,33] Moreover, previous studies supplemented the treatment media with serum[15,16] which contains long-chain unsaturated fatty acids that depress fat synthesis and elongation,[15] whereas the cells in the present study were not exposed to serum during the hormonal treatments, and hence, the only source for fatty acids was de novo synthesis. These results demonstrate that during functional differentiation and in the absence of long-chain fatty acids in the medium, the mammary gland epithelial cells synthesize long-chain fatty acids. The fact that a mammary gland epithelial cell line can synthesize long-chain fatty acids under lactogenic hormone regulation is supported by the variety of elongation enzymes expressed in the mammary gland cell line. Five of the seven known elongase subtypes were expressed in mammary gland epithelial cells. The inclusion of insulin, prolactin and hydrocortisone in the treatment medium increased the expression level of all elongase and desaturase genes except for Elovl 4, whose expression was reduced by this hormonal combination. Elovl 4 is suspected to elongate very long fatty acids (26−38 carbons).[34] Although not determined

in the present study, these fatty acids are rare but may have a functional role in developing but not differentiated tissues. Elovl 4 is also unique in that it is not expressed by hepatocytes,[25,34] and therefore, the elongation of very long fatty acids may be supported by local rather than systemic expression of this enzyme.

The fatty acid flux through elongation and desaturation pathways can be investigated by elongation and desaturation activity markers.[30] Since the aims of the study were to determine the products synthesized under the regulation of lactogenic hormones, we used the amount of product synthesized only during the hormonal treatment divided by the total substrate in the system to evaluate the enzymatic activity. This approach yielded results that correlated with the changes in gene expression levels found for desaturases and elongase and were in agreement with the effect of insulin these enzymes activity in rat liver.[25,34]

The results of the present study demonstrate that fatty acids are incorporated differently into phospholipids and triglycerides (Tables 5 and 6); for example, the concentration of C14:0 fatty acid did not change in the triglyceride fraction, whereas its concentration increased in the phospholipid fraction in the presence compared with absence of insulin in the treatment media. The different incorporation of fatty acids into the various cellular lipid fractions was also illustrated in human erythrocytes.[35]

Insulin was also found to modulate cellular membrane lipid composition. Inclusion of insulin in the treatment media increased Phe and PhI concentrations and decreased those of SM and cholesterol. Since SM is composed of very long SFA-like C22:0, C23:0 and C24:0,[36] a shortage of availability of these fatty acids might reduce SM synthesis. It should be noted that since the membrane composition is illustrated in weight percentage, changes in composition do not necessarily indicate changes in production of specific class of polar lipids. It has been reported previously that cholesterol membrane concentration can be modulated by SM and sphingomyelinase pathways,[37] which is in agreement with the results of the present study findings that SM-to-cholesterol ratio was kept constant by mammary epithelial cells under the various treatments. The importance of membrane's lipid composition is still under investigation. Previous studies clearly illustrate that the cell's ability to respond to environmental signals, as well as its lipogenic status, are influenced by membrane composition.[38,39] Although the intensive lipogenic activity of the epithelial cells of the mammary gland and the active role of the epithelial membrane in the secretion process of milk fat, the role of polar lipid composition of the membrane of mammary epithelial cells is rarely discussed. Here we provide the first evidence for the association between lipogenic activity and the membrane composition of mammary epithelial cells.

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Author Contributions

K.M. drafted the manuscript, generated, analyzed and interpreted the data. A.S. provided laboratory space and consulted regarding study design. N.A.A. designed and interpreted the data, drafted the manuscript and revised the article for intellectual content. All authors approved the submitted version.

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Notes

The authors declare no competing financial interest.

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