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The Effects of a Hyperinsulinemic-Euglycemic Clamp on Milk Fat Synthesis and the Expression of Fat Synthesis-Related Genes in the Mammary Gland Tissues of Lactating Goats

Xueyan Lin*, Guimei Liu*, Yabin Zhang, Zhengui Yan, Qiuling Hou, Kerong Shi, Yun Wang, Zhonghua Wang#

College of Animal Science, Shandong Agricultural University, Taian, China
Email: linxueyan@sdau.edu.cn

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Abstract

To determine whether insulin exerts an effect on milk fat yield through the direct regulation of milk fat synthesis in the mammary gland, the hyperinsulinemic-euglycemic clamp procedure was performed in lactating goats in the present study. The effects of the hyperinsulinemic-euglycemic clamp on milk yield, milk composition, milk fatty acid yield and the expression levels of mRNAs of milk fat synthesis-related genes were examined. The results revealed that the hyperinsulinemic-euglycemic clamp had no significant effect on the milk yield, the milk protein yield, the yield and content of lactose or the yield and content of solids-not-fat (SNF) ($P > 0.05$). In contrast, the milk fat percentage and milk fat yield were decreased by 35.3% and 33.6%, respectively ($P < 0.01$). Among the 19 fatty acids examined, the yields of 9 fatty acids were significantly reduced ($P < 0.05$) following the clamp procedure, including C16:0 (hexadecanoic acid), 3 fatty acids derived from blood (>C16) and 5 fatty acids synthesized de novo in the mammary gland (<C16). In addition, the hyperinsulinemic-euglycemic clamp significantly enhanced the degree of unsaturation in the C14 and C16 fatty acids ($P < 0.05$). The hyperinsulinemic-euglycemic clamp had no significant effects on the mRNA expression levels of 6 milk fat synthesis-related genes in the mammary gland tissues ($P > 0.05$), including acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), fatty acid-binding protein (FABP), lipoprotein lipase (LPL), stearoyl-CoA desaturase (SCD) and glycerol-3-phosphate acyltransferase (GPAT). However, the expression level of the SCD gene was significantly reduced during the post-procedure period ($P < 0.05$) but returned to a normal level at 48 h after termination of the clamp procedure. It was concluded that the hyperinsulinemic-euglycemic

*The authors have equal contribution to this article.
#Corresponding author.

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clamp exerted a direct effect on milk fatty acid desaturation.

Keywords

Insulin, Milk Fat, Goat, Lactation

1. Introduction

Milk fat depression (MFD) refers to the diet-induced inhibition of milk fat synthesis in ruminants. During MDF, the milk fat yield and percentage may be reduced to 50% of the normal values, while the milk yield and other milk components remain unaltered [1] [2].

Two main theories have been proposed to unravel the mechanisms underlying MFD: the biohydrogenation (BH) theory and the glucogenic-insulin theory. The BH theory is supported by sufficient experimental evidence and has been widely recognized. The BH theory states that under certain dietary conditions, the BH of polyunsaturated fatty acids by ruminal microorganisms results in increased yields of a number of intermediate metabolites. The intermediate metabolites enter the mammary gland via circulating blood. Once accumulated in the mammary gland to a certain critical level, the intermediate metabolites inhibit milk fat synthesis. It has been found that trans-10, cis-12 conjugated linoleic acid (t10c12CLA) inhibits milk fat synthesis [3]. Feeding high-concentrate diets or diets supplemented with unsaturated fat enhance the ruminal production of the fatty acid t10c12CLA. An abomasal infusion study has demonstrated that the critical level of t10c12CLA for the inhibition of milk fat synthesis is 0.045 g/kg of body weight (BW), at which the milk fat yield can be reduced by 45%. At 2 - 3 d after the termination of infusion, the milk fat yield returns to normal [4]. t10c12CLA upregulates the expression of fat synthesis-related genes in body tissues, while in mammary gland tissues, this fatty acid downregulates the expression of fat synthesis-related genes [5]-[7].

The glucogenic-insulin theory speculates that feeding a high-concentrate diet results in an increased yield of propionic acid in the rumen, which stimulates insulin secretion, and the mammary gland tissues are thought to be insulin-insensitive. When insulin secretion is elevated, increased amounts of nutrients are utilized for the synthesis of body fat, which suppresses milk fat synthesis [8]. Sufficient experimental evidence has indicated that insulin promotes body fat synthesis and inhibits body fat mobilization [9] [10]. However, there is no experimental evidence demonstrating that the mammary gland tissues are insensitive to insulin. In fact, mouse studies have shown that the mammary gland is insulin-insensitive only during the dry period. During the lactation period, the mammary gland becomes extremely sensitive to insulin [11]-[13]. If fat synthesis in the mammary gland is truly insulin-insensitive, increased amounts of nutrients will be utilized for body fat synthesis. However, no direct experimental evidence has been obtained to support this hypothesis. The stimulation of insulin secretion through the postruminal infusion of glucose or propionic acid or the enhancement of blood insulin levels via the hyperinsulinemic-euglycemic clamp procedure results in a reduced milk fat yield. However, the extent of the decrease in milk fat yield varies greatly (from 5% to 20%) among different experiments [1]. This discrepancy may be related to the amount of mobilizable body fat reserved in the experimental animals. Hyperinsulinemic-euglycemic clamp procedure in early-lactation dairy cows leads to a 27% reduction in milk fat yield [14]. In contrast, the hyperinsulinemic-euglycemic clamp procedure results in a 5% - 15% reduction in milk fat yield in mid-lactation and late-lactation dairy cows. These results suggest that insulin inhibits milk fat synthesis primarily through the suppression of body fat mobilization.

In the present study, lactating goats were selected as the experimental animals. The milk fat yield and the expression of fat synthesis-related genes in the mammary gland tissues were examined before the hyperinsulinemic-euglycemic clamp procedure, during the procedure and after the procedure. The purpose of the study was to determine whether insulin directly regulates fat synthesis in mammary gland tissues.

2. Materials and Methods

2.1. Experimental Animals and Diets

Three Laoshan dairy goats in mid-lactation [days in milk (DIM): 120 ± 32 d] were selected for the present study. The goats had similar body weights of 37 ± 3 kg and were fed a diet that was designed according to the Energy

and Protein Requirements of Ruminants (AFRC, 1993), which is an advisory manual prepared by the Agricultural and Food Research Council [15]. The diet consisted of roughage and concentrate feeds. While dried sweet potato vine was the sole constituent of the roughage, the concentrate feeds contained flaked corn, wheat bran and soybean meal. Pelleted feeds were prepared with roughage to concentrate feed ratio of 1:1 (**Table 1**).

2.2. Experimental Design and Methods

The experimental goats were housed individually and given ad libitum access to feed and drinking water. During the experimental period, the goats were kept in metabolic cages and subjected to restricted feeding. The amount of feed met the nutritional requirements for maintenance and lactation. The feed was divided into 12 parts and was given to the goats every 2 h using an automatic feeding machine. The goats had free access to water. The goats were milked twice daily at 07:30 and 19:30.

The hyperinsulinemic-euglycemic clamp procedure is detailed below. The hyperinsulinemic-euglycemic clamp experiment was conducted according to the protocols developed by Cheng *et al.* [16] and Bequette *et al.* [17] with modifications. An insulin (insulin was purchased from Sigma-Aldrich, product of Brazil) stock solution was prepared at 1 mg/mL in 1% bovine serum albumin (BSA, purchased from Sigma-Aldrich, St. Louis, Missouri, USA) and stored at −20˚C. The insulin solution was thawed immediately prior to use, diluted with normal saline to 12 μg/mL (pH 7.0 - 7.4) and sterilized using a 0.22-μm membrane filter. A 10% glucose solution and a 50% glucose solution (purchased from Shandong Lukang Chenxin Pharmaceuticals Co., Ltd.) were mixed at a ratio of 5:3 and the resulting 25% glucose solution was used for the infusion.

At 2 d prior to the hyperinsulinemic-euglycemic clamp experiment, catheters (for infusion and blood sampling) were inserted into the jugular veins on either side of the windpipe of the experimental goats. At 1 d prior to the experiment, venous blood samples were collected every 2.5 h between 07:30 and 20:30. A total of 6 batches of blood samples were collected, and blood glucose concentrations were measured. The basal blood glucose concentration was determined by averaging the glucose concentrations of the collected blood samples. On the day of the experiment, the intravenous infusion of insulin and glucose was conducted after the morning milking. Insulin was continuously infused at a rate of 10 mL/h (120 μg/h) with an injection pump (W0109-1B, Baoding Lange Constant Flow Pump Co., Ltd.), while glucose was infused at a rate of 70 mL/h with a constant flow pump (HL-2, Shanghai Huxi Analytical Instrument Factory). The insulin and glucose were infused into one of the jugular vein catheters through a 3-way connecting tube. During the infusion, blood samples were collected at 1-h intervals through the jugular vein catheter on the opposite side of the neck, and blood glucose concentrations were immediately determined with a blood glucose meter (OneTouch® Ultra, Johnson & Johnson, USA). The speed of the glucose infusion was adjusted constantly according to the test results to maintain a blood glucose concentration close to the baseline level (3 mmol/L). Backup blood samples were prepared for a laboratory retest (glucose oxidase assay). The hyperinsulinemic-euglycemic clamp experiment was conducted for 12 h.

Table 1. Composition and nutrient level of the experimental diet (DM basis).

Ingredients	Composition, %	Nutrient	Level
Pachyrhizus vine	53.33	ME, MJ/kg DM	15.7
Corn	25.28	MP, g/kg DM	72.5
Wheat bran	4.4	NDF, g/kg DM	36.2
Soybean meal	14.8	ADF, g/kg DM	22.8
Salt	0.49		
*Premix	1.1		
$CaHPO_4$	0.6		
Total	100		

Note: Per kg, the premix contains: Vitamin A 160K IU, Vitamin D3 30K IU, Vitamin E 300 mg, Vitamin K 30 mg, Cu 400 mg and Fe 1800 mg. ME, metabolizable energy; DM, dry matter; MP, microbial protein; NDF, neutral-detergent fiber; ADF, acid-detergent fiber.

2.3. Sample Collection and Analysis

Prior to the hyperinsulinemic-euglycemic clamp experiment, the amount of milk produced by the mammary glands on one side was recorded continuously for 3 d, and milk samples were collected. At 1 d prior to the experiment, mammary gland tissue samples were collected via fine needle aspiration of the mammary glands on the other side. At 12 h after the initiation of the hyperinsulinemic-euglycemic clamp procedure, the goats were milked. The amount of milk produced by the mammary glands on one side was recorded, and milk samples were collected. Meanwhile, tissue samples were collected from the mammary glands on the other side. The unilateral milk yield was recorded every 12 h for 48 h after termination of the procedure, and milk samples were collected. Mammary gland tissue samples were again collected from the mammary glands on the other side. The mammary gland tissue samples were washed with normal saline in an ice-bath, wrapped in aluminum foil and gauze bags and stored in liquid nitrogen. Each milk sample was divided into 2 aliquots and stored at −20˚C. The milk samples were used to analyze the milk composition and milk fatty acids.

During the hyperinsulinemic-euglycemic clamp procedure, 10-mL blood samples were collected hourly in heparinized blood collection tubes, and plasma samples were prepared by centrifugation at 3000 rpm. Each plasma sample was divided into 2 aliquots and stored at −20˚C. One aliquot was used in the glucose oxidase assay to determine the blood glucose concentration, while the other aliquot was used in the radioimmunoassay to determine the plasma insulin level.

Milk composition was determined with a milk composition analyzer (FOSS, type: 78110). Milk fatty acids were analyzed via gas chromatography. The mRNA expression levels of acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), lipoprotein lipase (LPL), fatty acid-binding protein (FABP), glycerol-3-phosphate acyltransferase (GPAT) and stearoyl-CoA desaturase (SCD) in the mammary gland tissues were examined using the reverse transcription polymerase chain reaction (RT-PCR) method. The primer sequences for the RT-PCR were designed according to a report published by Nan *et al.* [18] and were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The relative mRNA expression levels were determined via the $2^{-\Delta\Delta Ct}$ method. β-actin was used as the reference housekeeping gene. The mammary gland tissue samples collected during d 1 of the hyperinsulinemic-euglycemic clamp experiments were used as reference samples.

2.4. Statistical Analysis

The experimental data were subjected to analysis of variance (ANOVA) using the general linear model procedure of the SAS software (V9.1, 2003). The following statistical model was chosen: $Y_{ijk} = \mu + T_i + G_k + e_{ijk}$. Y_{ijk} was the observed value; μ was the mean value; T_i was the effect of the treatment; G_k was the effect on individual animals; and e_{ijk} was the random error. Multiple comparisons were performed using Duncan's method. $P \leq 0.05$ indicated a statistically significant difference. $P \leq 0.10$ indicated a tendency of change.

3. Results

3.1. Milk Yield and Composition

During the hyperinsulinemic-euglycemic clamp procedure, the blood glucose level of the experimental goats remained stable, while the insulin level was elevated (**Figure 1**).

The plasma insulin level was significantly elevated during the hyperinsulinemic-euglycemic clamp procedure compared to the time period prior to the procedure ($P < 0.01$). However, the difference in blood glucose levels was not statistically significant ($P > 0.10$) (**Table 2**).

The hyperinsulinemic-euglycemic clamp exerted no significant effect on the milk yield, the lactose yield and content or the solids-not-fat (SNF) yield and content ($P > 0.10$), while this procedure markedly reduced the milk fat content and yield and the milk protein content ($P < 0.01$) (**Table 3**). A comparison of the milk protein yields before and after the hyperinsulinemic-euglycemic clamp procedure revealed a tendency toward a decline ($P = 0.13$). The milk protein yields were markedly reduced at 0-12 h after terminating the procedure compared to the time period prior to the procedure ($P < 0.05$). However, the milk protein yields at 0 - 12 h after terminating the procedure were not notably different from those in the other time periods. At 36 h after terminating the hyperinsulinemic-euglycemic clamp procedure, the milk fat content and yield returned essentially to the levels prior to the procedure, and the differences were not statistically significant ($P > 0.05$). In contrast, the milk protein content at 48 h after the termination of the procedure did not return to the normal level and was significantly lower than that observed in the time period prior to the procedure ($P < 0.01$).

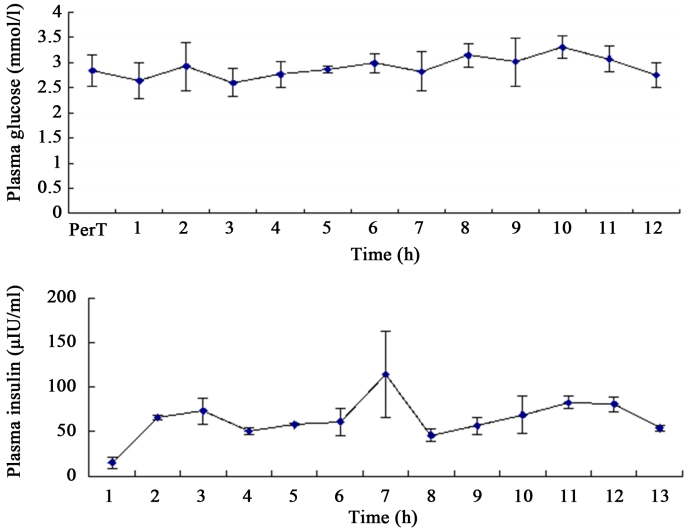

Figure 1. Plasma glucose and insulin levels of the experimental goats at different time points during the hyperinsulinemic-euglycemic clamp.

Table 2. Sequence of the primers for the RT-PCR determination of the targeted and reference genes.

Gene	Primer	Primer Sequence (5'-3')
ACC	F	AAGGTCGAGACCGAAAGAAAATAG
	R	GGCTTTCAGGTCTTCAGGTGTG
FAS	F	GCACTACCACAACCCAAACCC
	R	CGTTGGAGCCACCGAAGC
LPL	F	ACGGGCTCAGCGGTTCTA
	R	ACAGGTGGCAAGTGTCCTCA
H-FABP	F	AAGACCACGGCAGATGACAGG
	R	CGTCAACTATTTCCCGCACAAG
GPAT	F	TCAGGCAATAATCTCAACATCC
	R	TGTGCCTTCCAGGAAGATCTC
SCD	F	CCGCCCTGAAATGAGAGATG
	R	AGGGCTCCCAAGTGTAACAGAC
β-actin	F	CACTGGCATTGTCATGGACTCTG
	R	CCTTGATGTCACGGACGATTTC

Table 3. Effects of the hyperinsulinemic-euglycemic clamp on plasma insulin, glucose, milk yield and milk composition in lactating goats.

	Pre-treatment	Treatment	Post-treatment				SEM	*P*	
			12 h	24 h	36 h	48 h		Period	Animal
Plasma glucose, mmol/L	2.83	2.90	-	-	-	-	0.25	0.87	0.57
Plasma insulin, μ IU/ml	15.26^{A}	67.60^{B}	-	-	-	-	2.02	<0.01	0.10
Milk yield, g/12 h	263.19	273.57	253.03	265.10	282.03	273.30	17.54	0.47	<0.01
Milk fat, %	3.99^{A}	2.58^{D}	2.72^{CD}	3.20^{BCD}	3.33^{ABC}	3.467^{AB}	0.36	<0.01	0.15
g/12 h	10.55^{A}	7.00^{C}	6.92^{C}	8.46^{BC}	9.19^{AB}	9.44^{AB}	0.87	<0.01	<0.01
Milk protein, %	3.86^{A}	3.27^{B}	3.28^{B}	3.40^{B}	3.47^{B}	3.48^{B}	0.13	<0.01	<0.001
g/12 h	10.15^{a}	8.97^{ab}	8.33^{b}	9.09^{ab}	9.76^{ab}	9.51^{ab}	0.74	0.13	<0.01
Lactose %	4.20	4.09	4.27	4.31	4.28	4.25	0.21	0.83	0.06
g/12 h	11.07	11.14	10.80	11.46	12.04	11.64	0.97	0.68	<0.01
SNF, %	9.16	8.51	8.69	8.85	8.90	8.94	0.29	0.22	<0.001
g/12 h	24.10	23.23	22.01	23.57	25.01	24.46	1.84	0.47	<0.01

3.2. Fatty Acid Yields in Milk

The effects of the hyperinsulinemic-euglycemic clamp on milk fatty acid yields are displayed in **Table 4**. The yields of various milk fatty acids during the hyperinsulinemic-euglycemic clamp procedure were compared with those prior to the procedure. Among the 19 fatty acids examined, the yields of 9 fatty acids were markedly reduced during the procedure ($P < 0.05$); the yields of 6 fatty acids were numerically decreased ($P > 0.1$), and the yields of 4 fatty acids were slightly elevated ($P > 0.1$). Among the 9 fatty acids of which the yields were significantly reduced by the hyperinsulinemic-euglycemic clamp procedure, the yields of 5 fatty acids (C14:0, C16:0, t9C18:1, c9C18:1 and C20:1) recovered to the levels observed prior to the procedure at 24 h after terminating the procedure. The yield of 1 fatty acid (C8:0) was recovered at 36 h after terminating the procedure, while the yields of 3 fatty acids (C4:0, C6:0 and C10:0) were recovered at 48 h afterward. The yields of 2 linoleic acids

Table 4. Effects of the hyperinsulinemic-euglycemic clamp on yields of milk fatty acids in lactating goats.

	Pre-treatment	Treatment	Post-treatment				SEM	*P*	
			12 h	24 h	36 h	48 h		Period	Animal
Fatty acid Yields g/12 h									
C4:0	0.1783^{A}	0.1010^{D}	0.1069^{CD}	0.1371^{BC}	0.1379^{BC}	0.1675^{AB}	0.0097	<0.01	<0.001
C6:0	0.2062^{A}	0.1110^{C}	0.1178^{C}	0.143^{BC}	0.1553^{BC}	0.1873^{AB}	0.0141	<0.01	<0.0001
C8:0	0.2790^{a}	0.1416^{c}	0.1528^{c}	0.1815^{bc}	0.2056^{abc}	0.2442^{ab}	0.0252	<0.05	<0.001
C10:0	1.0029^{B}	0.6713^{F}	0.7096^{E}	0.8334^{D}	0.9038^{C}	1.0891^{A}	0.0109	<0.01	<0.0001
C12:0	0.7025^{ab}	0.6494^{b}	0.7622^{ab}	0.8670^{ab}	0.8194^{ab}	0.9145^{a}	0.0633	0.14	<0.0001
C14:0	1.1265^{a}	0.8419^{b}	0.8987^{b}	1.0395^{ab}	1.1286^{a}	1.2214^{a}	0.0584	<0.05	<0.01
C14:1	0.0355	0.0415	0.0459	0.0400	0.0359	0.0449	0.0067	0.84	0.29
C16:0	2.4463^{a}	1.8201^{b}	1.6929^{b}	1.9656^{ab}	2.1058^{ab}	2.2439^{ab}	0.1646	0.09	0.06
C16:1	0.0897	0.0916	0.0995	0.0923	0.0880	0.1034	0.0081	0.80	<0.0001
C18:0	0.6627	0.1535	0.1626	0.2981	0.4309	0.2639	0.1870	0.45	0.18
t9C18:1	0.0172^{ab}	0.01^{b}	0.0122^{b}	0.0163^{ab}	0.0204^{a}	0.0235^{a}	0.0020	<0.05	<0.0001
t11C18:1	0.0321	0.0431	0.0503	0.0649	0.0634	0.0642	0.0110	0.30	<0.0001
c9C18:1	2.5532^{a}	0.7579^{b}	0.8197^{b}	1.1053^{ab}	1.3647^{ab}	1.2647^{ab}	0.4246	0.12	0.02
c9c12C18:2	0.1463^{a}	0.1887^{ab}	0.1766^{ab}	0.2002^{ab}	0.2340^{ab}	0.2932^{b}	0.0340	0.21	<0.001
c9t11CLA	0.0564^{a}	0.0703^{a}	0.0769^{ab}	0.0901^{ab}	0.0962^{ab}	0.1235^{b}	0.0136	0.12	<0.0001
C18:3	0.0218^{abc}	0.0181^{bc}	0.0161^{c}	0.0192^{bc}	0.0248^{ab}	0.0269^{a}	0.0019	<0.05	<0.0001
C20:0	0.0102^{ab}	0.0086^{b}	0.0087^{b}	0.0116^{ab}	0.0153^{a}	0.0131^{ab}	0.0018	0.15	<0.0001
C20:1	0.0062^{a}	0.0032^{b}	0.0037^{b}	0.0049^{ab}	0.0059^{a}	0.0070^{a}	0.0006	<0.05	<0.0001
C22:0	0.0050	0.0035	0.0043	0.0043	0.0077	0.0062	0.0016	0.52	<0.001
C4-C14	3.5309^{A}	2.5578^{C}	2.7938^{C}	3.2414^{A}	3.3865^{A}	3.8688^{B}	0.0955	<0.001	<0.0001
C16:0 + C16:1	2.5360^{a}	1.9117^{b}	1.7924^{b}	2.0579^{ab}	2.1938^{ab}	2.3472^{ab}	0.1704	0.11	0.04
>C16	3.5111^{a}	1.2569^{b}	1.3313^{b}	1.8150^{ab}	2.2631^{ab}	2.0862^{ab}	0.5526	0.14	<0.01
C14:1/C14	0.0320^{a}	0.0503^{b}	0.0509^{b}	0.0385^{ab}	0.0306^{b}	0.0365^{ab}	0.0048	0.05	0.10
C16:1/C16	0.0372^{D}	0.0509^{AB}	0.0576^{A}	0.0467^{BC}	0.0404^{CD}	0.0438^{BCD}	0.0027	<0.01	<0.001
C9C18:1/C18:0	4.762	5.438	5.400	4.012	3.627	4.468	0.8850	0.65	0.73

(c9c12C18:2 and c9t11CLA) during the hyperinsulinemic-euglycemic clamp procedure were not notably different from those observed prior to the procedure. The yields of these 2 linoleic acids increased during the post-procedure stage and were significantly higher at 48 h after terminating the procedure compared to the time period prior to the procedure ($P < 0.05$). The hyperinsulinemic-euglycemic clamp enhanced the degree of unsaturation in the C14 and C16 fatty acids. The C14:1/C14:0 and C16:1/C16:0 ratios were significantly elevated during the hyperinsulinemic-euglycemic clamp procedure compared to the time period prior to the procedure ($P < 0.05$). The ratios gradually returned to normal after the procedure. The C14:1/C14:0 ratio at 24 h after terminating the procedure was not significantly different from that prior to the procedure, while the C16:1/C16:0 ratio at 36 h was not obviously different from that observed prior to the procedure.

Highly significant differences were detected in the yields of the C4-C14 fatty acids between the different time periods ($P < 0.01$). The yields were markedly reduced during the hyperinsulinemic-euglycemic clamp procedure and at 0-12 h after the termination of the procedure compared to the time period prior to the procedure ($P < 0.01$). In contrast, the yields were significantly elevated at 36 - 48 h after terminating the procedure compared to the time period prior to the procedure ($P < 0.01$). The yields of the fatty acids C16:0 + C16:1 and the fatty acids >C16 at different time periods had tendencies toward a decline ($P = 0.11$ and $P = 0.14$, respectively). The yields were considerably reduced during the hyperinsulinemic-euglycemic clamp procedure and at 0-12 h after the termination of the procedure compared to the time period prior to the procedure, while no significant differences were observed between the other time periods.

3.3. mRNA Expression Levels of Fat Synthesis-Related Genes in the Mammary Gland Tissues

During the hyperinsulinemic-euglycemic clamp procedure, the mRNA expression levels of the ACC, FAS, LPL, H-FABP and GPAT genes were slightly reduced compared with the time period prior to the procedure, whereas the expression level of the SCD gene was slightly elevated. However, the differences were not significant ($P > 0.1$, **Table 5**). The mRNA expression levels of the SCD gene were markedly reduced at 12 h, 24 h and 36 h after terminating the hyperinsulinemic-euglycemic clamp procedure compared to the time period prior to the procedure ($P < 0.05$). At 48 h, the mRNA expression level of the SCD gene recovered to a level not notably different from the level prior to the procedure. The mRNA expression levels of the ACC, FAS, LPL and H-FABP genes were somewhat low at 12 h, 24 h and 36 h after terminating the procedure but recovered to higher levels at 48 h. However, the differences among various time points were not statistically significant. The mRNA expression level of the GPAT gene declined steadily after the procedure. Again, the differences among various time points were not statistically significant.

4. Discussion

4.1. Effects of the Hyperinsulinemic-Euglycemic Clamp Procedure

Hyperinsulinemic-euglycemic clamping is among the most stringent experimental methods that are utilized to examine insulin functions in *in vivo* animal studies. During the hyperinsulinemic-euglycemic clamp procedure,

Table 5. Effects of the hyperinsulinemic-euglycemic clamp on the relative mRNA expression (2ΔΔCt) of milk fat synthesis-related genes in the mammary glands of lactating goats.

	Pre-treatment	Treatment	Post-treatment				SEM	*P*	
			12 h	24 h	36 h	48 h		Period	Animal
ACC	1.00	0.69	0.37	0.32	0.34	0.60	0.42	0.37	0.63
FAS	1.00	0.85	0.31	0.23	0.33	0.54	0.39	0.17	0.34
LPL	1.00	0.85	0.30	0.21	0.21	0.65	0.49	0.28	0.29
H-FABP	1.00	0.94	0.53	0.44	0.44	0.66	0.32	0.19	<0.01
GPAT	1.00	0.58	0.67	0.37	0.42	0.32	0.35	0.26	<0.05
SCD	1.00[a]	1.06[a]	0.42[b]	0.26[b]	0.33[b]	0.71[ab]	0.30	<0.05	0.22

exogenous insulin is injected into the animals to enhance the blood insulin level. Concurrently, glucose is injected to maintain stability of the blood glucose at basal levels. A significant elevation in the insulin level and the maintenance of the blood glucose level are the keys to a successful experiment. In the present study, the blood glucose concentration during the hyperinsulinemic-euglycemic clamp procedure was not significantly different from that present prior to the procedure ($P > 0.05$). The blood glucose concentrations were essentially stable at each time point during the procedure. The blood glucose concentrations detected in the 3 experimental goats were in the range of 2.25 - 3.40 mmol/L. The insulin concentration during the hyperinsulinemic-euglycemic clamp procedure was increased by an average of 4.43-fold compared to the basal insulin concentration, and the difference was statistically significant ($P < 0.01$). The plasma insulin concentrations in the 3 experimental goats measured at different time points during the clamp procedure were relatively stable and were all more than 2 times the baseline values. At 6 h after the initiation of the procedure, the plasma insulin concentration increased more than 5-fold compared to the baseline value. The results indicated that the hyperinsulinemic-euglycemic clamp achieved the expected effect.

4.2. Milk Yield and Composition

Milk yield is primarily affected by the lactose yield. The present study revealed that the hyperinsulinemic-euglycemic clamp had no significant effect on the lactose and milk yields in the lactating goats, which is consistent with previously published results of clamp experiments conducted in lactating goats and dairy cows [14] [16] [19].

Previous studies have demonstrated that the clamp procedure leads to varying degrees of decline in milk fat percentage and yield. McGuire *et al.* and Gariinari *et al.* have conducted clamp procedures in lactating dairy cows and observed a slight decrease in milk fat percentages and yields [20] [21]. The differences between the treatment group and the control group were not statistically significant ($P > 0.05$). Corl *et al.* have shown that the clamp procedure reduces milk fat yield by 27% in lactating dairy cows [14]. In addition, Bequette *et al.* and Argov-Argaman *et al.* have reported a 27% and a 17% decrease, respectively, in milk fat yields in lactating dairy goats that underwent the clamp procedure [17] [22]. The present study revealed that the milk fat percentage and yield in the lactating goats decreased 35.3% and 33.6%, respectively, during the hyperinsulinemic-euglycemic clamp procedure. After comparing the research studies, it was concluded that there are two main reasons for the varying degrees of reduction in milk fat yields/milk fat percentages following the clamp procedures. One reason is that there are differences in the lactation stages between the experimental animals. The dairy cows selected by Gariinari *et al.* were in mid-lactation (average DIM: 21 weeks), whereas the cows selected by Corl *et al.* were in early lactation (average DIM: 2 weeks) [14] [21]. Mashek *et al.* have conducted clamp procedures in dairy cows in 4 weeks and 17 weeks of lactation and observed a 12% and a 5% decrease in milk fat yields, respectively [19]. These results strongly indicate that the lactation stage has an impact on the effects of the clamp procedures. Dairy cows in early lactation have high levels of mobilizable body fat reserves. A negative energy balance during early lactation leads to a rapid decrease in mobilizable body fat. Therefore, the effect of lactation stage on the outcome of the clamp procedure may be related to the status of body fat reserves. The second reason lies in the plasma insulin levels induced by the clamp procedures. Bequette *et al.* have reported that the clamp procedure results in a 4-fold increase in plasma insulin levels [17]. while the milk fat yield declines greatly. In contrast, a study in which the clamp procedure only induced a small decrease in milk fat yield has indicated that the insulin concentration reached a level approximately 2 times the normal value. In the present study, the milk fat percentages and yields in the goats returned to normal at 36 h after terminating the clamp procedure, indicating that the delayed effects of hyperinsulinemia on milk fat synthesis are short-lived.

Under normal feeding conditions, enhancing blood insulin levels by increasing the amount of concentrate feed generally results in an elevated milk protein yield. The present study demonstrated that the hyperinsulinemic-euglycemic clamp induced a significant decrease in the milk protein percentage in lactating dairy goats ($P < 0.01$). In addition, the milk protein yield had a tendency to decrease ($P = 0.13$). These results are consistent with the findings of Bequette *et al.* [17]. The promoting effect of insulin on milk protein synthesis may require synergy between insulin and glucose, which may explain why the concentrate feed-induced enhancement of insulin levels promotes milk protein synthesis, whereas an inhibition of milk protein synthesis was observed in the present study and in the study of Bequette *et al.* [17]. In a number of studies, clamp procedures fail to induce changes in milk protein percentages and yields [14] [15] [20]-[22]. However, the clamp procedures conducted in

the above studies only resulted in an approximately 2-fold increase in the concentration of insulin, which is lower than the extent of the increase in insulin concentrations in the present study and in the study of Bequette *et al.* (4-fold increase) [17]. In addition, the results may be affected by the fact that the clamp procedures induce a decrease in DMI. To eliminate the effect of an altered DMI, the goats were subjected to restricted feeding in the present study. Therefore, the discrepancy regarding the effect of the clamp procedure on milk protein synthesis among the different studies may be related to the extent of the increase in insulin levels and changes in glucose metabolism, which requires further investigation.

4.3. Milk Fatty Acid Yields

In the present study, the yields of 19 milk fatty acids were examined. The results indicated that the yields of 9 milk fatty acids were significantly reduced during the hyperinsulinemic-euglycemic clamp procedure ($P < 0.05$). Among the 9 fatty acids, 5 fatty acids (<C16) were synthesized de novo in the mammary gland; 1 of the fatty acids (C16: 0) was derived either from de novo synthesis in the mammary gland or was absorbed from the blood, and the other 3 (>C16) were absorbed from the blood. The clamp procedure exerted no significant effect on the yields of the other fatty acids that were examined. These results are consistent with the findings of Bequette *et al.* [17] and do not support the hypothesis that insulin promotes the de novo synthesis of fatty acids in the mammary gland.

Gariinari *et al.* have enhanced the concentration of blood insulin through postruminal infusions of glucose and found that the yields of the C18 fatty acids in milk fat were reduced, while the yields of the C8-C16 fatty acids were elevated [21]. Other postruminal infusion studies have also indicated that the proportion of the fatty acids in milk fat that are synthesized de novo in the mammary gland is increased, while the proportion of the fatty acids absorbed from the blood is reduced [23] [24]. However, the yields of the fatty acids were not examined in the above studies. The hyperinsulinemic-euglycemic clamp experiments conducted by Corl *et al.* and Argov-Argaman *et al.* have also demonstrated an increase in the proportion of the fatty acids synthesized de novo in the mammary gland and a decrease in the proportion of the fatty acids derived from the blood [14] [22]. Again, the yields of the fatty acids were not determined in those two studies. Therefore, the two studies fail to provide direct evidence whether a hyperinsulinemic-euglycemic clamp promotes the de novo synthesis of fatty acids in the mammary gland. The present study and the study conducted by Bequette *et al.* have demonstrated that the clamp procedure results in a decrease in the yields of the de novo synthesized fatty acids in the mammary gland [17]. In contrast, the postruminal infusion study performed by Gariinari *et al.* has indicated that the yields of the C8-C16 fatty acids are significantly elevated [21]. In the postruminal infusion study, both the blood insulin level and the blood glucose level were elevated. Therefore, the discrepancy in the yields of the fatty acids synthesized de novo in the mammary gland between the two types of studies is likely due to the synergistic effect of insulin and blood glucose.

In the present study, the inhibitory effect of insulin on the fatty acid yield disappeared almost completely 48 h after terminating the hyperinsulinemic-euglycemic clamp procedure. However, differences were observed among the different fatty acids. Among all the fatty acids examined, 2 linoleic acids, c9c12C18:2 and c9t11CLA, behaved distinctly. The yields of the 2 fatty acids during the procedure were not significantly different from those prior to the procedure. However, the yields of the 2 fatty acids increased steadily after the clamp procedure. The yields were significantly elevated at 48 h after terminating the procedure compared to the time period prior to the procedure ($P < 0.05$). We are not yet able to provide a reasonable explanation for this phenomenon. The present study also revealed that the hyperinsulinemic-euglycemic clamp markedly enhanced the C14:1/C14:0 and the C16:1/C16:0 ratios ($P < 0.05$), indicating an increased degree of unsaturation in the two types of fatty acids. The two ratios gradually returned to normal after the clamp procedure. The two ratios reached levels that were not significantly different from those prior to the clamp procedure at 24 h and 36 h after the termination of the procedure, respectively.

4.4. mRNA Expression Levels of the Key Genes Involved in Milk Fat Synthesis

Short-chain fatty acids (C4-C14) are synthesized de novo in the mammary gland tissues [25]. ACC and FAS are key enzymes that control de novo fatty acid biosynthesis. In mammary gland tissues, FABP and LPL are the two key proteins that are involved in the absorption of fatty acids from the blood. SCD and GPAT play important roles in fatty acid desaturation and triglyceride synthesis in mammary gland tissues, respectively. Argov-Arga-

man *et al.* have demonstrated that a hyperinsulinemic-euglycemic clamp has no significant effect on the mRNA expression levels of the ACC, FAS and SCD genes in the mammary gland tissues of lactating goats [21]. Similarly, the present study found that the hyperinsulinemic-euglycemic clamp had no notable effect on the mRNA levels of 6 genes related to milk fat synthesis, including the ACC, FAS, FABP, LPL, SCD and GPAT genes. The present study and the study conducted by Argov-Argaman *et al.* [21] indicate that insulin exerts no direct effect on milk fat synthesis. Insulin may play a role in milk fat synthesis by regulating the metabolic distribution of milk fat synthesis precursors between the body tissues and the mammary gland tissues.

In the present study, no significant differences were found between the mRNA expression levels of the SCD gene prior to and during the hyperinsulinemic-euglycemic clamp procedure. However, SCD mRNA expression was significantly reduced after the termination of the procedure ($P < 0.05$) and then recovered to essentially a normal level 48 h after terminating the procedure. This result was inconsistent with the finding that the C14:1/C14:0 and C16:1/C16:0 ratios were enhanced during the hyperinsulinemic-euglycemic clamp procedure and then gradually returned to normal after the termination of the procedure. One possible explanation is that insulin directly inhibited SCD activity, resulting in an increased degree of fatty acid unsaturation during the procedure. The simultaneous recovery of the C14:1/C14:0 and C16:1/C16:0 ratios and the decrease in SCD gene expression after terminating the clamp procedure may be related to the short action time of insulin on SCD gene expression and the non-synchronization of gene expression and changes in enzyme activity.

5. Conclusion

In the present study, the hyperinsulinemic-euglycemic clamp procedure is performed to explore the mechanisms by which insulin inhibits milk fat synthesis. The present study supports the hypothesis that insulin affects milk fat yield primarily through altering the metabolic distribution of the precursors for fat synthesis between the body tissues and the mammary gland tissues. In addition, the results reveal that insulin directly affects fatty acid desaturation in the mammary gland tissues, which increase the degree of unsaturation of milk fatty acids.

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