Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy

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ORIGINAL RESEARCH

Palmitoleic acid reduces intramuscular lipid and restores insulin sensitivity in obese sheep

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Abstract: Obese sheep were used to assess the effects of palmitoleic (C16:1 *cis*-9) acid infusion on lipogenesis and circulating insulin levels. Infusion of 10 mg/kg body weight (BW)/day C16:1 intravenously in obese sheep reduced ($P<0.01$) weight gain by 77%. Serum palmitoleic levels increased ($P<0.05$) in a linear manner with increasing levels of C16:1 infusion. *Cis*-11 vaccenic (C18:1 *cis*-11) acid, a known elongation product of palmitoleic acid, was also elevated ($P<0.05$) in serum after 14 days and 21 days of infusion. Plasma insulin levels were lower ($P<0.05$) (10 mg/kg BW/day C16:1) than controls (0 mg/kg BW/day C16:1) at 14 days and 28 days of infusion. Infusion of C16:1 resulted in linear increases in tissue concentrations of palmitoleic, *cis*-11 vaccenic, eicosapentaenoic, and docosapentaenoic acids in a dose-dependent manner. Total lipid content of the semitendinosus (ST) muscle and mesenteric adipose tissue was reduced ($P<0.01$) in both 5 mg/kg and 10 mg/kg BW C16:1 dose levels. Total lipid content and mean adipocyte size in the longissimus muscle was reduced ($P<0.05$) in the 10 mg/kg BW C16:1 dose level only, whereas total lipid content and adipocyte size of the subcutaneous adipose tissue was not altered. Total lipid content of the liver was also unchanged with C16:1 infusion. Palmitoleic acid infusion upregulated ($P<0.05$) acetyl-CoA carboxylase (*ACC*), fatty acid elongase-6 (*ELOVL6*), and Protein kinase, AMP-activated, alpha 1 catalytic subunit, transcript variant 1 (*AMPK*) mRNA expressions in liver, subcutaneous adipose, and ST muscle compared to the controls. However, mRNA expression of glucose transporter type 4 (*GLUT4*) and carnitine palmitoyltransferase 1b (*CPT1B*) differed between tissues. In the subcutaneous adipose and liver, C16:1 infusion upregulated ($P<0.05$) *GLUT4* and *CPT1B*, whereas these genes were downregulated ($P<0.05$) in ST muscle with C16:1 infusion. These results show that C16:1 infusion for 28 days reduced weight gain, intramuscular adipocyte size and total lipid content, and circulating insulin levels. These changes appear to be mediated through alterations in expression of genes regulating glucose uptake and fatty acid oxidation specifically in the muscles.

Keywords: adipocytes, longissimus muscle, lipogenesis, insulin level, serum, fatty acid

Introduction

Palmitoleic (C16:1 *cis*-9) acid is a unique monounsaturated fatty acid that is produced almost exclusively through desaturation of palmitic acid via stearoyl-CoA desaturase-1 (*SCD1*). Dietary sources of palmitoleic acid are very limited with natural sources being macadamia oil and sea buckthorn oil, which contain about 19%–28% of palmitoleic acid. Palmitoleic acid has recently been named a lipokine that regulates lipogenesis and coordinates systemic metabolism.[1] These researchers found that infusing tripalmitolein versus tripalmitin for 6 hours into dietary-induced obese mice with homozygous mutations for fatty acid binding proteins (aP2, mal1) decreased *SCD1*, fatty acid synthase (*FASN*), and fatty acid elongase-6 (*ELOVL6*) expression in the

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liver compared to control. In addition, Cao et al[1] suggest that palmitoleic acid acts as an insulin-sensitizing hormone that improves glucose metabolism. Knockout of *SCD1* in mice reduces adipose tissue deposition, increases insulin sensitivity, and makes animals resistant to diet-induced weight gain.[2] In our laboratory, exogenous administration of palmitoleic acid to bovine adipocytes in vitro downregulates de novo lipogenesis and upregulates fatty acid oxidation to direct fatty acids toward energy expenditure and away from storage.[3,4] The objective of this experiment was to assess the effects of palmitoleic (C16:1) acid infusion at varying dose levels on lipogenesis and circulating insulin levels in obese sheep.

Materials and methods

Animals and sampling

Southdown sheep (n=15; 95 kg body weight [BW]) were used to assess the effects of palmitoleic (C16:1 *cis*-9) acid infusion on lipogenesis and circulating insulin levels in obese sheep. Omega-7 enriched oil (Provinal™; Tersus Pharmaceuticals) was infused, twice daily for 28 days via indwelling jugular catheter, at three levels of palmitoleic acid: 0 mg C16:1/kg BW/d, 5 mg C16:1/kg BW/d, or 10 mg C16:1/kg BW/d. Provinal™ is an omega-7 enhanced oil with 50% of total fatty acids as palmitoleic acid and less than 2.5% as palmitic acid. The oil was solubilized in 40% ethanol and immediately injected into the catheter at 8 am and 4 pm for each lamb. All lambs received the same amount of 40% ethanol per dose regardless of the oil level. A 16-gauge indwelling jugular catheter (Abbocath; Abbott Laboratories, Abbott Park, IL, USA) was inserted and sutured to the skin 1 day prior to initiation of experiment for substrate administration and blood sampling. Catheters were kept patent with 4% sodium citrate in sterile saline (Jorgensen Laboratories, Inc., Loveland, CO, USA). On days 0, 7, 14, and 21, blood samples were obtained from each lamb at 5 minutes postinfusion. Whole blood was placed into serum and heparinized vacuum tubes, and processed according to Long et al.[5]

Lambs were fed at National Research Council[6] recommendations and divided into three pens with equal representation of treatments per pen. At 28 days, lambs were slaughtered. Live weight was also collected on a weekly basis, and average daily gain was calculated. At slaughter, weights of kidney, omental and mesenteric adipose tissues, as well as hot carcass weight were collected. Samples of subcutaneous adipose tissue at the 12th rib, semitendinosus (ST) muscle, and liver were collected at slaughter and immediately frozen in liquid nitrogen for storage at −80°C until subsequent RNA extraction. At 24 hours postmortem, carcass data were collected and samples obtained from subcutaneous and intramuscular adipose tissues of the longissimus muscle for cell size determination. Samples of subcutaneous adipose, mesenteric adipose, longissimus muscle, ST muscle, and liver were collected for total lipid and fatty acid composition. One side of each carcass from 0 mg C16:1/kg BW/d and 10 mg C16:1/kg BW/d treatments was used to assess body composition analyses by physical dissection and proximate analyses.

Glucose and insulin

Glucose and insulin levels were measured as described by Long and Schafer.[7]

Proximate analyses

Dissected muscle and adipose tissues for body composition analyses were ground and mixed, and samples were obtained for proximate analyses according to Duckett et al.[8]

Adipocyte size and distribution

Adipose tissue samples were collected from intramuscular (dissected from longissimus muscle at the 12th rib) and subcutaneous (at the 12th rib) depots for determination of adipocyte cell size according to Etherton et al.[9] Adipocytes were counted and sized using a particle sizing and counting analyzer (Multisizer 4 Coulter Counter; Beckman Coulter Inc., Brea, CA, USA).

Lipids

Adipose, muscle samples, and liver were frozen, lyophilized, and ground in a food processor. Serum samples (1 mL) were also lyophilized. Total fat content and fatty acid composition were determined according to Duckett et al.[8]

For determination of lipid fractions, total lipids were extracted from ST muscle samples via Folch et al[10] method. The neutral lipid, phospholipid (PhL), and free fatty acid (FFA) fractions were separated according to Ruiz et al.[11] Each fraction was then dried under nitrogen, transmethylated, and analyzed by gas chromatography according to Duckett et al.[8]

RNA isolation

RNA was extracted from subcutaneous adipose tissues, ST muscles, and liver as described in Duckett et al.[12]

Quantitative PCR

Primers were designed using Primer3 software (http://primer3plus.com/) and are reported in Table 1. qPCR was conducted according to Duckett et al[12] using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Normalized delta CT values were calculated for each sample and analyzed as described in the Statistical analyses section.

Western blots

Proteins were extracted from liver, ST, and subcutaneous adipose tissues of control (0 mg/kg BW C16:1) and high (10 mg/kg BW C16:1) levels using T-PER solution containing Halt Phosphatase Inhibitor according to the manufacturers' recommendations. Protein concentrations were measured using Bradford method (Pierce Coomassie; Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were diluted in sample reducing buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 minutes. Proteins were separated using 12% polyacrylamide gels at 60 V for 30 minutes. Blotting of protein was performed on polyvinylidene difluoride membranes using a Trans-Blot (BioRad) apparatus at 350 mA for 1 hour at 4°C. Membranes were blocked with 5% fat-free milk in 0.1% Tween-20 phosphate buffered saline (PBS-Tween) at room temperature. Primary antibodies for AMPKα1 (PA1-2109, 1:5,000; Thermo Fisher Scientific) and p-AMPKα1 (PA5-17831, 1:1,000; Thermo Fisher Scientific) were incubated overnight at 4°C. Blots were washed with PBS several times and then incubated with secondary antibody (goat anti-rabbit IgG-HRP, 1:6,000 and 1:2,000; Thermo Fisher Scientific) for 1 hour at room temperature. Blots were washed with PBS several times and then SuperSignal West Femto substrate (34095; Thermo Fisher Scientific) was added for 5 minutes. Chemiluminescence was detected using an Alpha Innotech Imager (ProteinSimple, Santa Clara, CA, USA). Density of the bands was quantified using the Alpha Innotech software.

Statistical analyses

Data were analyzed in a completely randomized design using general linear models (GLM) procedures of SAS (SAS Institute Inc., Cary, NC, USA) with palmitoleic acid dose level, tissue, and two-way interaction in the model. For serum data, palmitoleic acid dose level, time for experiment, and the two-way interaction were included in the model. Orthogonal contrasts were used to assess linear or quadratic changes in variables because of dose level of palmitoleic acid infusion. For gene expression, delta CT values were analyzed using GLIMMIX procedure with treatment, tissue, and two-way interaction in the model. Gene expression levels were calculated using the $\Delta\Delta C_T$ methods[13] and are presented as fold-change in gene expression for experiment (10 mg/kg lipid weight (LW)/d C16:1) versus controls (0 mg C16:1/kg LW/d).

Results

Infusion of 10 mg/kg BW/d palmitoleic (C16:1 *cis*-9) acid intravenously in obese sheep reduced ($P<0.01$) weight gain

Table 1 Primer sequences (5′–3′) for quantitative real-time PCR

Gene abbreviations	Forward	Reverse	Efficiency
ACC	GCTGCTGGTGTTCCTAAAGC	GTCCTCAAATTGTGCGTGAA	1.01
AKT1	AAGAGGCAGGAGGAGGAGAC	CCCAGCAGCTTCAGGTACTC	1.01
AMPK	GTCAAAGTCGGCCAAATGAT	CCTCCGAACACGCAAATAAT	1.03
CPT1a	CCACCTCTTCTGCCTCTACG	TTGAACAGTTCCACCTGCTG	1.04
CPT1b	GGTCGACTTCCAGCTCAGTC	CAGGAGGAACCCACTGTTGT	1.05
ELOVL5	CGCCACACTTAACAGCTTCA	AGCTGGTCTGGATGATGGTC	1.10
ELOVL6	AGTGGATGCAGGAAAACTGG	AAGGGTCAGAGACCAGAGCA	1.05
FABP4	AATTGGGCCAGGAATTTGAT	GCACCAGCTTATCATCCACA	1.00
FASN	TCATCCCCCTGATGAAGAAG	GCAGTGGTCCACCAGGTAGT	1.09
GAPDH	GGGTCATCATCTCTGCACCT	GGTCATAAGTCCCTCCACGA	1.01
GLUT4	CTCTCCACTTGCCCAGAGAC	CTGCCTAGCCACAACACAAA	1.04
HSL	GGCACTCCTGGATGCTGATT	CACTGTCCGCGGAGATACTC	0.98
IR	CTGACTTCCGAGACCTCCTG	GTTCTGTGACTTGGGGTCGT	0.95
IRS	TCCGCCTTTCCTCAAGTTCC	AGCTGTGTCCACCTTTCGAG	0.99
PI3KR1	AAGTCATGGCCATTGTAGGC	ACCTCTTGCTCTTGCGGTAA	0.96
SCD1	CGACGTGGCTTTTTCTTCTC	GATGAAGCACAACAGCAGGA	0.96

Abbreviations: PCR, polymerase chain reaction; ACC, acetyl-CoA carboxylase; AKT1, v-akt murine thymoma viral oncogene homolog 1; AMPK, protein kinase, AMP-activated, alpha 1 catalytic subunit, transcript variant 1; CPT1a, carnitine palmitoyltransferase 1a; CPT1b, carnitine palmitoyltransferase 1b; ELOVL5, fatty acid elongase-5; ELOVL6, fatty acid elongase-6; FABP4, fatty acid binding protein 4; FASN, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; HSL, hormone-sensitive lipase; IR, insulin receptor; IRS, insulin receptor substrate; PI3KR1, phosphoinositide-3-kinase, regulatory subunit 1 (alpha); SCD1, stearoyl-CoA desaturase-1.

Table 2 Gain, carcass traits, and body composition of lambs infused with varying levels of palmitoleic (C16:1) acid

	mg C16:1/kg LW/d			SEM	Contrasts	
	0	5	10		Linear	Quadratic
Daily weight gain, kg/d	0.30	0.29	0.07	0.051	0.01	0.18
Carcass weight, kg	58.82	56.68	56.18	2.02	0.38	0.75
Ribeye area, cm^2	22.3	22.4	20.3	1.29	0.31	0.48
Fat thickness, cm	1.98	1.93	1.93	0.25	0.88	0.94
Body wall thickness, cm	4.72	4.98	4.93	0.37	0.70	0.74
Visceral fat depots, kg						
Perirenal fat	2.30	2.15	2.41	0.29	0.78	0.58
Omental fat	2.57	3.16	2.68	0.37	0.83	0.26
Mesenteric fat	0.99	0.87	0.83	0.073	0.15	0.67
Body composition, % of carcass weight						
Moisture	31.49	–	30.54	0.90	0.48	–
Lipid	39.18	–	40.44	2.25	0.70	–
Protein	14.94	–	14.58	0.78	0.75	–
Bone	14.39	–	14.43	1.09	0.98	–

Note: –, not measured.
Abbreviations: LW, lipid weight; d, days; SEM, standard error of the mean.

by 77% (Table 2). Carcass traits, visceral adipose depots, and body composition were not altered ($P>0.05$) by palmitoleic acid infusion for 28 days. The lambs from this experiment averaged 1.95 cm of fat thickness with a body composition of 40% of total lipid, which would be considered obese. Sheep are more similar to humans in fat distribution patterns than other animal models. Sheep deposit more fat within the abdominal cavity (about 33% of total fat) than pigs or rodents.[13]

Serum palmitoleic (C16:1 *cis*-9) levels increased ($P<0.05$) in a linear manner with increasing levels of palmitoleic acid infusion when sampled at 5 minutes postinfusion (Table 3). In addition, palmitoleic acid infusion increased ($P<0.001$) arachidonic (C20:4) and eicosapentaenoic (C20:5) acid contents in the serum in a linear manner. Myristoleic (C14:1) and linoleic (C18:2) acid contents in the serum tended ($P<0.10$) to be increased in a linear manner with palmitoleic acid infusion.

Table 3 Serum fatty acid content from lambs infused with varying levels of palmitoleic (C16:1) acid

Fatty acids, µg/mL	mg C16:1/kg LW/d			SEM	Contrasts	
	0	5	10		Linear	Quadratic
C14:0	3.47	3.28	3.35	0.17	0.62	0.55
C14:1	3.93	3.68	3.30	0.26	0.09	0.84
C16:0	107.94	102.68	110.53	5.72	0.75	0.35
C16:1 *cis*-9	8.93	11.69	14.28	1.16	0.001	0.95
C18:0	150.92	124.35	154.18	9.50	0.81	0.02
C18:1 *trans*	13.00	16.21	15.66	1.30	0.15	0.24
C18:1 *cis*-9	147.07	132.18	151.00	9.04	0.76	0.13
C18:1 *cis*-11	5.05	5.89	7.66	0.39	0.001	0.32
C18:2 *cis*-9,12	201.21	198.33	236.55	13.71	0.07	0.22
C18:3 *cis*-9,12,15	7.15	7.29	9.69	1.12	0.11	0.41
C20:4 *cis*-5,8,11,14	28.73	28.15	38.58	2.00	0.001	0.02
C20:5 *cis*-5,8,11,14,17	3.78	4.16	4.94	0.27	0.001	0.56
C22:5 *cis*-7,10,13,16,19	8.76	8.12	9.22	0.49	0.51	0.15
C22:6 *cis*-4,7,10,13,16,19	8.69	9.17	9.02	0.64	0.71	0.38
Total, µg/mL	757.60	716.29	837.11	43.45	0.22	0.10
Saturated	262.33	230.31	268.06	15.13	0.79	0.06
Monounsaturated	12.86	15.37	17.58	1.26	0.01	0.92
Polyunsaturated n-6	229.94	226.48	275.13	14.95	0.04	0.16
Polyunsaturated n-3	28.38	27.74	32.87	2.09	0.13	0.26
Ratio n-6:n-3	8.43	8.77	8.98	0.41	0.34	0.90
C16:1–C16:0	0.083	0.12	0.13	0.01	0.001	0.48
C18:1–C18:0	0.99	1.12	0.99	0.04	0.86	0.01
C16:1–C18:1c11	0.83	1.06	0.98	0.11	0.35	0.28

Abbreviations: LW, lipid weight; d, days; SEM, standard error of the mean.

These changes in individual serum fatty acid content resulted in linear ($P<0.05$) increases in total monounsaturated and polyunsaturated n-6 content in the serum. The ratio of palmitoleic to palmitic acid in the serum was linearly ($P<0.001$) increased in the sheep infused with palmitoleic acid. The ratio of oleic (C18:1 *cis*-9) to stearic (C18:0) acid was increased ($P<0.01$) in a quadratic manner. All interactions between treatment and days of treatment were nonsignificant except for *cis*-11 vaccenic acid. *Cis*-11 vaccenic (C18:1 *cis*-11) acid, a known elongation product of palmitoleic acid,[3,4] was also elevated ($P<0.05$) in serum after 14 days and 21 days of the experiment (Figure 1).

Plasma insulin levels were higher ($P<0.05$) in the 10 mg/kg BW/d C16:1 treatment than controls at the start of the experiment (Figure 2A). However, after 14 days and 28 days on treatment, insulin concentrations were lower ($P<0.05$) for 10 mg/kg BW/d C16:1 than controls (0 mg/kg BW). For the 5 mg/kg LW/d C16:1 dose level, circulating insulin levels were similar to controls at days 0 and 14 but were intermediate at day 28. Plasma glucose levels were unchanged with palmitoleic acid infusion and averaged 95±19.0 mg/dL. There was a treatment by time interaction ($P<0.05$) for Homeostatis Model Assessment-Insulin Resistance (HOMA-IR) level. HOMA-IR levels decreased ($P<0.05$) over time in 5 mg/kg LW/d and 10 mg/kg LW/d treatments compared to control, which did not change ($P>0.05$) over time (Figure 2B). Homeostatis Model Assessment-beta cell function (HOMA-B) and Homeostatis Model Assessment-insulin sensitivity (HOMA-S) values did not differ between palmitoleic acid treatment levels.

The fatty acid concentration of longissimus muscle, ST muscle, subcutaneous adipose, mesenteric adipose, and liver tissues is shown in Table 4. There were no interactions ($P>0.05$) between treatment and tissue, and the data are presented as the average fatty acid concentration for that treatment across all tissues. Palmitoleic, *cis*-11 vaccenic, eicosapentaenoic, and docosapentaenoic acid concentrations increased in a linear manner ($P<0.05$) with increasing level of palmitoleic acid infusion. Myristic (C14:1), palmitoleic, *trans*-octadecenoic, and margaric acid concentrations increased ($P<0.05$) in a quadratic manner with palmitoleic acid infusion level. Stearic (C18:0) acid decreased ($P<0.05$) in a quadratic manner with palmitoleic acid infusion level. Concentrations of total polyunsaturated n-6 and n-3 fatty acids were increased in a linear manner ($P<0.05$) with palmitoleic acid infusion. Saturated and odd-chain fatty acid concentrations were increased in a quadratic manner ($P<0.05$) with palmitoleic acid infusion. Other fatty acid concentrations were not altered by palmitoleic acid infusion. The ratio of palmitoleic to palmitic acid was linearly increased, whereas the ratio

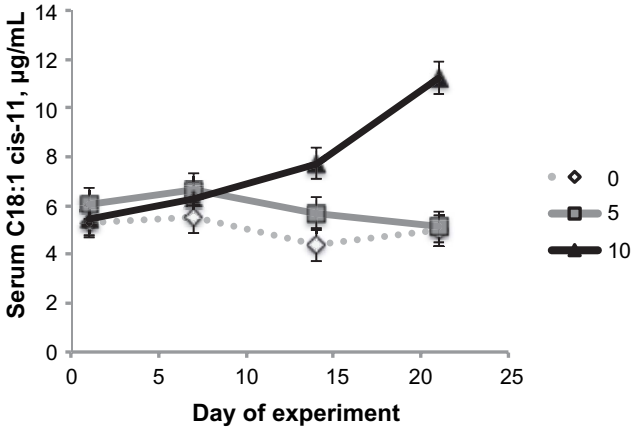

Figure 1 Effects of palmitoleic acid infusion on serum cis-11 vaccenic acid levels.
Notes: Changes in serum *cis*-11 vaccenic (C18:1 *cis*-11) acid concentration (µg/mL) by palmitoleic acid infusion level (0 mg/kg LW/d, 5 mg/kg LW/d, or 10 mg/kg LW/d) at 5 minutes post-dosing by time of the experiment.
Abbreviations: LW, lipid weight; d, days.

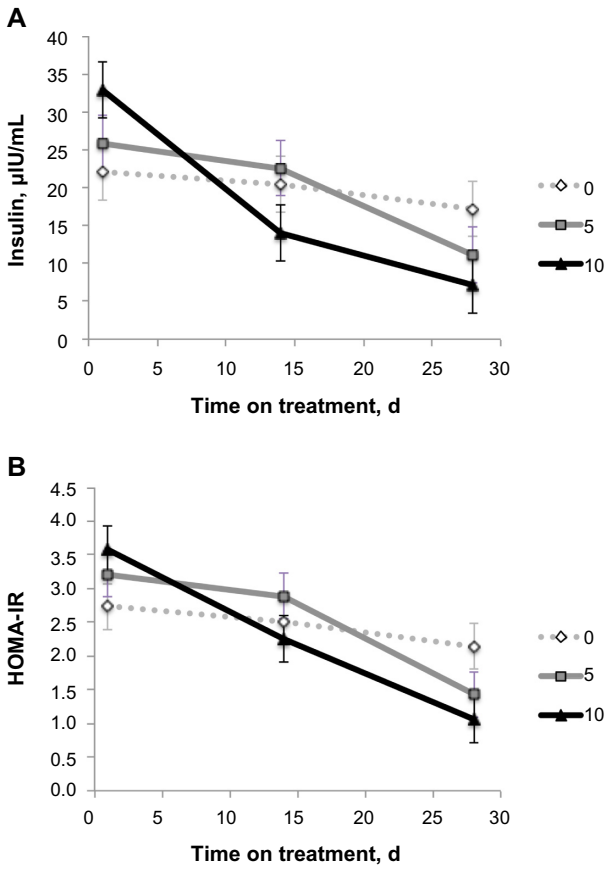

Figure 2 Changes in plasma insulin concentration and HOMA-IR values with palmitoleic acid infusion at three levels.
Notes: Plasma insulin concentrations (µIU/mL) (**A**) and HOMA-IR (**B**) in lambs infused with varying levels of palmitoleic (C16:1) acid levels (0 mg/kg LW/d, 5 mg/kg LW/d, or 10 mg/kg LW/d) by day of experiment.
Abbreviations: LW, lipid weight; d, days; HOMA-IR, Homeostatis Model Assessment-Insulin Resistance.

Table 4 Fatty acid composition of tissues (subcutaneous adipose tissue, mesenteric adipose tissue, liver, longissimus muscle, ST muscle) from lambs infused with varying levels of palmitoleic (C16:1) acid

Fatty acid, % of total	mg C16:1/kg LW/d			SEM	Contrasts	
	0	5	10		Linear	Quadratic
C14:0	1.50	1.68	1.45	0.04	0.35	0.001
C15:0	0.31	0.36	0.35	0.03	0.32	0.29
C16:0	20.74	21.35	20.08	0.28	0.11	0.01
C16:1 *cis*-9	0.89	1.13	1.02	0.04	0.04	0.01
C17:0	1.31	1.43	1.31	0.04	0.96	0.01
C18:0	26.01	23.15	25.09	0.45	0.16	0.001
C18:1 *trans*	2.69	3.87	3.24	0.26	0.15	0.01
C18:1 *cis*-9	33.71	33.68	33.24	0.39	0.41	0.67
C18:2 *cis*-9,12	4.22	4.40	4.63	0.15	0.06	0.91
C18:3 *cis*-9,12,15	0.45	0.48	0.54	0.02	0.02	0.57
C20:4 *cis*-5,8,11,14	2.76	2.68	2.88	0.12	0.52	0.40
C20:5 *cis*-5,8,11,14,17	0.14	0.22	0.31	0.041	0.001	0.90
C22:5 *cis*-7,10,13,16,19	0.90	0.92	1.09	0.061	0.03	0.35
C22:6 *cis*-4,7,10,13,16,19	1.05	1.11	1.24	0.11	0.25	0.81
Saturated fatty acids, %	48.25	46.18	46.62	0.52	0.03	0.05
Odd chain fatty acids, %	1.62	1.79	1.66	0.05	0.60	0.01
Monounsaturated fatty acids, %	34.60	34.81	34.27	0.40	0.57	0.45
Polyunsaturated fatty acids, n-6, %	6.42	6.55	6.93	0.16	0.04	0.55
Polyunsaturated fatty acids, n-3, %	2.12	2.26	2.65	0.10	0.001	0.38
Ratio, n-6:n-3	4.06	4.53	3.97	0.25	0.79	0.09
C16:1–C16:0 ratio	0.046	0.057	0.057	0.003	0.01	0.11
C18:1–C18:0 ratio	1.53	1.71	1.55	0.047	0.69	0.01
C16:1–C18:1c11 ratio	1.42	1.84	1.73	0.072	0.001	0.001

Abbreviations: ST, semitendinosus; LW, lipid weight; d, days; SEM, standard error of the mean.

of oleic to stearic acid was quadratically increased ($P<0.01$) with palmitoleic acid infusion. The ratio of palmitoleic acid to *cis*-11 vaccenic acid also increased in a linear and quadratic manner ($P<0.001$) with palmitoleic acid infusion.

Total lipid content of the longissimus muscle, ST muscle, and liver is shown in Figure 3A, and subcutaneous and mesenteric adipose tissues is shown in Figure 3B. Total lipid content of the ST muscle and mesenteric adipose tissue was reduced ($P<0.01$) in both 5 mg/kg BW C16:1 and 10 mg/kg BW C16:1 dose levels. Total lipid content of the longissimus muscle was reduced ($P<0.05$) in the 10 mg/kg BW C16:1 dose level only. The total lipid content of the liver and subcutaneous adipose tissues was not altered ($P>0.05$) with C16:1 infusion. In order to determine if the palmitoleic acid and its elongation product, *cis*-11 vaccenic acid, were partitioned into PhL or triacylglyceride fraction, lipid separation was conducted on the ST muscle samples. Results showed that 94% of the total palmitoleic acid in the muscle was present in the neutral (triacylglyceride) fraction with only about 3% present as FFA or PhL, regardless of treatment. For *cis*-11 vaccenic acid, it was also predominately (90%) located in the neutral lipid fraction with only 2% and 7% as FFAs or PhL, respectively, regardless of treatment. Mean adipocyte size was reduced ($P<0.05$) in intramuscular adipocytes from the longissimus muscle for 10 mg/kg BW C16:1 group compared to controls (Figure 4A). Adipocyte distribution showed a reduction ($P<0.05$) in the amount of 90–140 μm-sized adipocytes and an increase ($P<0.05$) in the amount of 20–80 μm-sized intramuscular adipocytes for the 10 mg/kg LW/d palmitoleic acid-treated group compared to controls (Figure 4B). Mean adipocyte size was not altered ($P>0.05$) in subcutaneous adipose tissues. These results show that palmitoleic acid infusion for 28 days reduced intramuscular lipid content of the longissimus and ST muscle with increasing levels of palmitoleic acid infusion. These reductions appear to be because of reductions in intramuscular triglyceride content and adipocyte size.

Changes in lipogenic gene expression for subcutaneous adipose, ST muscle, and liver with palmitoleic acid infusion are shown in Figure 5. Palmitoleic acid infusion upregulated ($P<0.05$) acetyl-CoA carboxylase (*ACC*) and *ELOVL6* mRNA expression. Expression of other genes involved in lipogenesis (*FASN*, fatty acid elongase-5 [*ELOVL5*], and carnitine palmitoyltransferase 1a [*CPT1A*]) was unchanged ($P>0.05$) with palmitoleic acid infusion. Palmitoleic acid infusion upregulated ($P<0.05$) protein kinase, AMP-activated, alpha 1 catalytic subunit, transcript variant 1 (*AMPK*) mRNA expression in all three tissues (liver, subcutaneous adipose, and

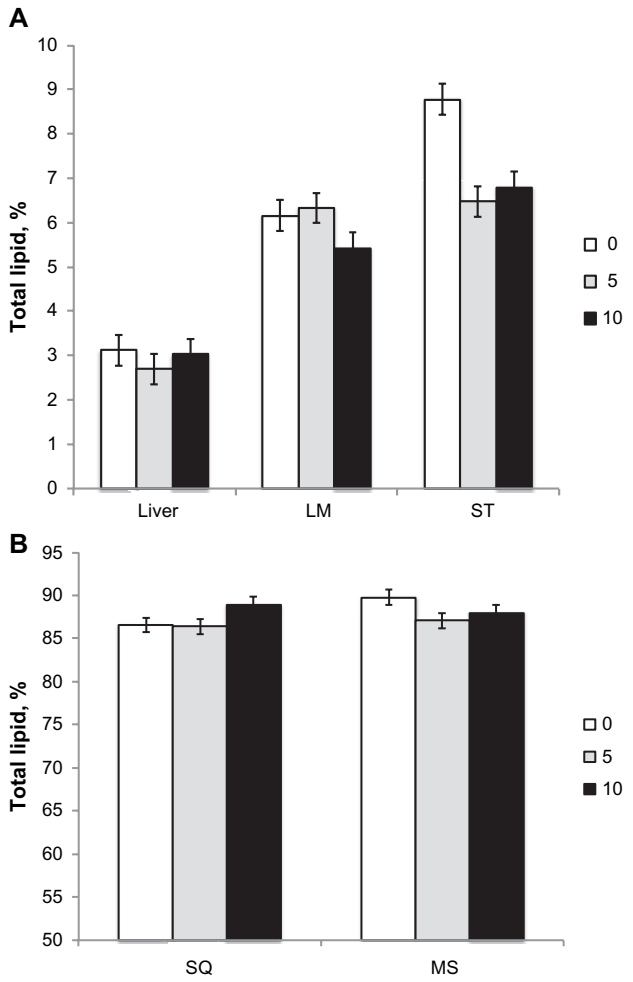

Figure 3 Effects of palmitoleic acid infusion on intramuscular lipid content.
Notes: Total lipid content (g/100 g of tissue) of liver, LM, and ST muscle (**A**), and SQ and MS fat (**B**) in lambs infused with varying levels of palmitoleic (C16:1) acid levels (0 mg/kg LW/d, 5 mg/kg LW/d, or 10 mg/kg LW/d) for 28 d.
Abbreviations: LM, longissimus muscle; ST, semitendinosus; SQ, subcutaneous; MS, mesenteric; LW, lipid weight; d, days.

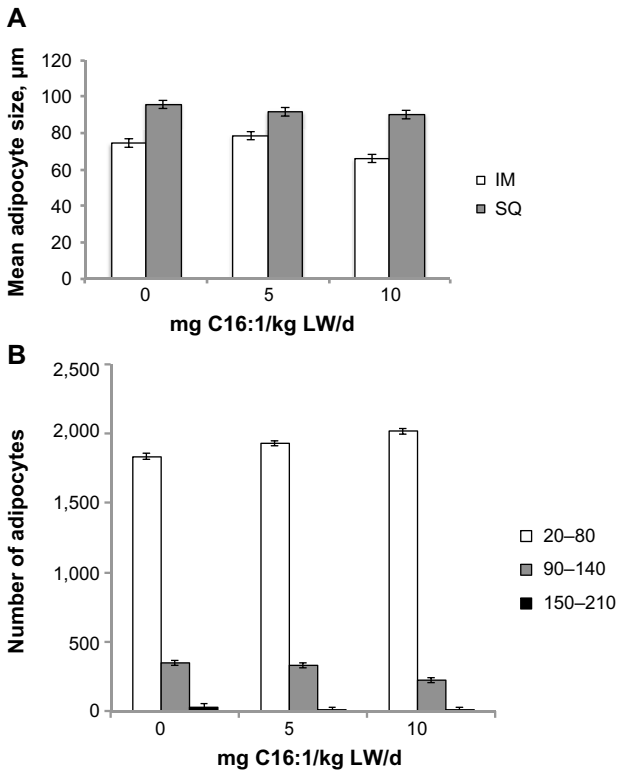

Figure 4 Effects of palmitoleic acid infusion on mean adipocyte size and distribution.
Notes: Mean adipocyte size (µm) (**A**) for both IM and SQ adipose tissues, and adipocyte size distribution (µm) (**B**) for IM adipose tissues by palmitoleic acid infusion level. IM adipocyte size was smaller ($P<0.05$) in palmitoleic acid infusion for dose level 10 mg/kg BW/d.
Abbreviations: LW, lipid weight; d, days; IM, intramuscular; SQ, subcutaneous; BW, body weight.

ST muscle) examined compared to the controls (Figure 6A). Protein levels of AMPKα1 did not differ ($P>0.05$) in the three tissues (liver, subcutaneous adipose, and ST muscle) of palmitoleic acid-treated group compared to controls (Figure 6B). Protein level of p-AMPKα1 and the ratio of p-AMPKα1 to AMPKα1 in all three tissues (liver, subcutaneous adipose, and ST muscle) were higher ($P<0.05$) for palmitoleic acid-treated group compared to controls (Figure 6C and D). Expression of other genes involved in glucose and insulin regulation (phosphoinositide-3-kinase, regulatory subunit 1 [alpha] [*PI3KR1*], insulin receptor [*IR*], v-akt murine thymoma viral oncogene homolog 1 [*AKT1*], and insulin receptor substrate 1 [*IRS1*]) was unchanged with palmitoleic acid infusion. The interaction between palmitoleic acid infusion level and tissue (subcutaneous [SQ], semitendinosus [ST], and liver) was significant ($P<0.05$) for glucose transporter type 4 (*GLUT4*) and carnitine palmitoyltransferase 1b (*CPT1BI*) (Figure 7). In the subcutaneous adipose and liver tissues, palmitoleic acid infusion upregulated ($P<0.05$) *GLUT4*, and *CPT1B*, whereas these genes were downregulated ($P<0.05$) with palmitoleic acid infusion in ST muscle.

Discussion

Infusion of palmitoleic acid increased serum palmitoleic acid levels in a dose-dependent manner. Previous research has shown that infusion of palmitoleic acid results in immediate uptake of the fatty acid into circulation.[5] In addition, *cis*-11 vaccenic (C18:1 *cis*-11) acid also increased with palmitoleic acid infusion over time. Based on the results, it took about 14 days of continuous palmitoleic acid infusion for *cis*-11 vaccenic acid to increase in serum above controls in palmitoleic acid-infused lambs. Using stable isotopes in vitro, Burns et al[3] confirmed that *cis*-11 vaccenic acid is an elongation product of palmitoleic acid. *ELOVL6* is involved with elongation of saturated fatty acids of 12–18 carbon length but cannot elongate past C18.[14] *ELOVL5* appears to be involved in elongation of polyunsaturated fatty acids but cannot elongate past

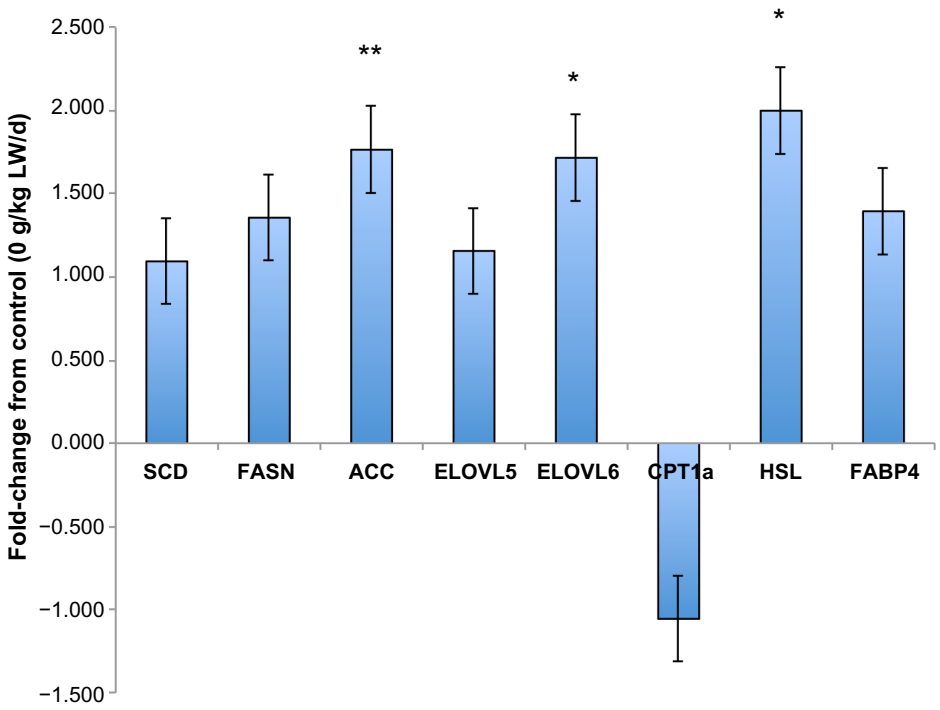

Figure 5 Fold-change in lipogenic gene expression for 10 mg C16:1/kg LW/d versus control (0 mg/kg BW/d) averaged over the three tissues (subcutaneous adipose tissue, ST muscle, and liver).
Notes: *$P<0.05$. **$P<0.01$.
Abbreviations: BW, body weight; LW, lipid weight; d, days; ST, semitendinosus; SCD, stearoyl-CoA desaturase; FASN, fatty acid synthase; ACC, acetyl-CoA carboxylase; ELOVL5, fatty acid elongase-5; ELOVL6, fatty acid elongase-6; CPT1a, carnitine palmitoyltransferase 1a; HSL, hormone-sensitive lipase; FABP4, fatty acid binding protein 4.

C22. Green et al[15] reported that *ELOVL6* elongates c16–c18, whereas *ELOVL5* elongates C16:1 *cis*-9–C18:1 *cis*-11 in rat insulinoma cell line. In this study, relative mRNA expression level of *ELOVL6* was upregulated because of palmitoleic acid treatment (10 mg/kg LW/d) in intramuscular adipose tissues. The relative mRNA expression level of *ELOVL5* did not change with palmitoleic acid infusion. Results from our in vitro studies also showed that exogenous palmitoleic acid addition to bovine adipocytes increased *cis*-11 vaccenic acid to 50% or greater of total fatty acids and altered *ELOVL6* mRNA expression with no change in *ELOVL5*.[3,4] These results would suggest that in ruminant species (ovine and bovine), ELOVL6 may be responsible for the elongation of palmitoleic acid to *cis*-11 vaccenic acid, differing from Green et al's[15] findings in rat cell culture.

Daily weight gain was reduced by 77% in the high-dose level of palmitoleic acid. Yang et al[16] found that palmitoleic acid administered orally to rats decreased food intake in a dose–response manner, which was accompanied by increased circulating and mRNA expression of cholecystokinin levels, a satiety-related hormone. Overall, body composition did not differ with palmitoleic acid treatment and averaged about 40% of total lipid. The sheep used in this study were obese and therefore, a short-term (28 days) treatment period would likely not be long enough to detect differences in overall body composition. Rates of lipolysis for sheep in a fed state are about 40% lower than rates of lipogenesis,[17] and rates of subcutaneous fat accretion are estimated at 0.0085 cm/d;[18] therefore, it would take approximately 114 days or more to reduce subcutaneous fat thickness by 20%. However, palmitoleic acid treatment did reduce intramuscular adipocyte size and total lipid content in the muscle tissues. Intramuscular fat is a late-developing fat depot,[19] which may make it more susceptible to short-term treatments prior to slaughter. Infusion of palmitoleic acid increased concentrations of palmitoleic and *cis*-11 vaccenic acids in all tissues. Because the palmitoleic acid response on total lipid was observed in the muscle, we further examined which fraction (triacylglyceride, PhL, or FFA) these fatty acids were partitioned into. Palmitoleic and *cis*-11 vaccenic acids were predominately (90% or greater) located in the triacylglycerol fraction with only minor portions in FFA or PhL fractions. Thus, it appears that exogenous palmitoleic acid is deposited in the intramuscular triacylglycerol fraction in the muscle where it reduces adipocyte size through reductions in total lipid content. These reductions in intramuscular adipocyte size and lipid content are also accompanied by a reduction in circulating insulin levels and appear to restore insulin sensitivity.

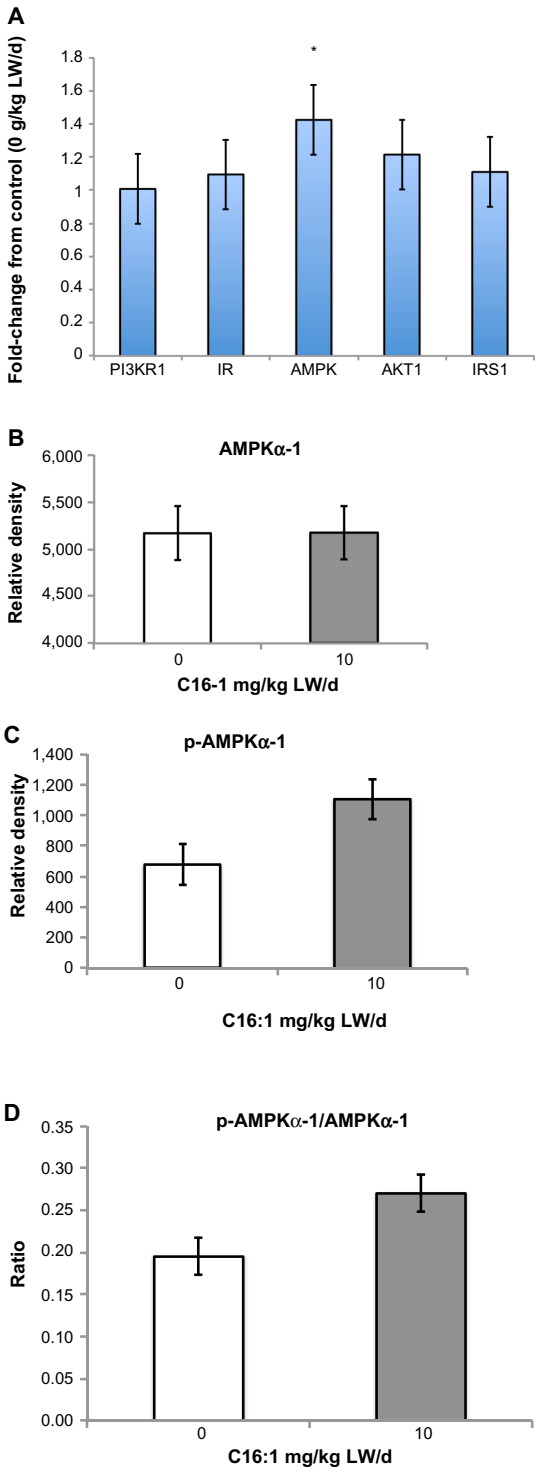

Figure 6 Effects of palmitoleic acid infusion on gene expression and protein content of tissues.

Notes: Fold-change in glucose/insulin-related gene expression (**A**) for 10 mg C16:1/kg LW/d versus control (0 mg/kg BW/d) averaged over the three tissues (subcutaneous adipose tissues, ST muscle, and liver). Density of AMPKα1 protein density (**B**), pAMPKα1 protein density (**C**), and ratio of pAMPKα1 to AMPKα1 for 10 mg C16:1/kg LW/d versus control (0 mg/kg BW/d) averaged over the three tissues (subcutaneous adipose tissues, ST muscle, and liver). *$P<0.05$.

Abbreviations: LW, lipid weight; d, days; BW, body weight; ST, semitendinosus; AMPK, protein kinase, AMP-activated, alpha 1 catalytic subunit, transcript variant 1; PI3KR1, phosphoinositide-3-kinase, regulatory subunit 1 (alpha); IR, insulin receptor; AKT1,v-akt murine thymoma viral oncogene homolog 1; IRS1, insulin receptor substrate 1.

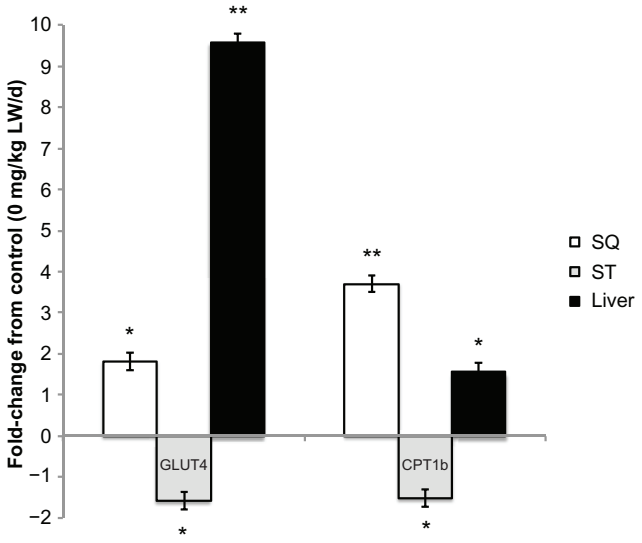

Figure 7 Palmitoleic acid infusion alters gene expression differentially by tissue.

Notes: Fold-change in gene expression for 10 mg C16:1/kg LW/d versus control (mg/kg LW/d) by tissue (subcutaneous adipose, liver, and ST muscle). The interaction between palmitoleic acid infusion and tissue (ST, SQ, liver) was significant ($P<0.05$) for GLUT4 and CPT1b. *$P<0.05$. **$P<0.01$.

Abbreviations: LW, lipid weight; d, days; ST, semitendinosus; SQ, subcutaneous; GLUT4, glucose transporter type 4; CPT1b, carnitine palmitoyltransferase 1b.

Research has shown that high levels of intramuscular lipid are negatively correlated to insulin sensitivity.[20,21] Perseghin et al[22] found through the use of nuclear magnetic resonance (NMR) spectroscopy that intramuscular triglyceride content of the soleus and plasma FFAs predicted whole body insulin sensitivity. Although the exact mechanism is not understood, it appears that skeletal muscle oxidation rates and/or the release of biologically active lipids or fatty acids that signal downstream molecules such as *IRS1*, protein kinase B/Akt, or protein tyrosine phosphatase 1B (*PTP1B*) are involved in restoring insulin sensitivity.[23–25] In this study, infusion of palmitoleic acid reduced circulating insulin levels without altering circulating glucose levels. Previous research has shown that plasma glucose and insulin levels were elevated immediately after a single pulse dose of palmitoleic acid infusion in a dose–response manner.[5] Insulin resistance, as determined using the HOMA calculator, is defined by a value of 2.71 or greater.[26] At the beginning of this study, the HOMA-IR levels for sheep used in this study were at or above this level indicating insulin resistance. By the end of the 28-day study, HOMA-IR levels declined ($P<0.05$) to 1.42 and 1.05 in the 5 mg/kg LW/d and 10 mg/kg LW/d palmitoleic acids, respectively. HOMA-IR levels in the controls (0 mg C16:1/kg LW/d) did not differ over the 28-day treatment period. These results indicate that palmitoleic acid treatment improved insulin resistance in obese sheep during a 28-day treatment period.

Relative mRNA expression of *AMPKα1* was upregulated in subcutaneous adipose, ST muscle, and liver with palmitoleic acid infusion. Protein expression of p-AMPKα1 and the ratio of p-AMPKα1 to AMPKα1 were also higher in subcutaneous adipose, ST muscle, and liver with palmitoleic acid infusion compared to control. Expression of other genes involved in glucose/insulin regulation (*IR*, *PI3KR1*, *AKT1*, *IRS*) was unchanged with palmitoleic acid infusion in these tissues. Hoffman et al[27] have shown that chromium supplementation activates *AMPK* in insulin-dependent skeletal muscle cells. These authors propose that *AMPK* activity in skeletal muscle is critical to determining fuel used for energy in cells and could prevent or delay the onset of insulin resistance. In a review by Coughlan et al,[28] they suggest that activation of AMPK will improve insulin sensitivity. AMPK appears to play a role in regulating skeletal muscle glucose and fatty acid utilization.[29,30] Locher et al[31] reported higher levels of AMPK phosphorylation in dairy cows during early lactation when cows are typically in a negative energy balance. Lipolysis appears to induce AMPK phosphorylation in 3T3L1 adipocytes in vitro.[32]

Relative mRNA expression of *GLUT4* and *CPT1B* showed a differential regulation depending on tissue. For the subcutaneous adipose and liver, palmitoleic acid upregulated *GLUT4* and *CPT1B* mRNA levels. However, palmitoleic acid infusion downregulated *GLUT4* and *CPT1B* mRNA levels in the ST muscle. The differential regulation of these genes suggests a possible mechanism by which palmitoleic acid may exert tissue-specific effects on intramuscular adipocytes to reduce triacylglyceride content and their size. The results in the subcutaneous adipose tissue gene expression are similar to what we observe in bovine adipocytes in culture with increases in *CPT1B* and reductions in *SCD1*.[3,4] However, the downregulation of *CPT1B* in the ST muscle would seem contrary to the results observed for reduced intramuscular adipocyte size and triacylglycerol content. Others[33] have also observed a reduced oxidative capacity in mice with improved whole body glucose utilization and insulin-stimulated glucose uptake. Other factors, like reactive oxygen species production in the mitochondria, may be more involved in insulin sensitivity.[23,34,35] Knockout of the *SCD1* gene in mice upregulated lipid oxidation and downregulated lipogenic genes to protect them from diet-induced obesity.[2] Previous research evaluating a pulse dose infusion of palmitoleic acid resulted in immediate elevations in circulating glucose and insulin that appeared to be related to glucose uptake into the peripheral cells. This longer term infusion study with palmitoleic acid shows that *GLUT4* mRNA expression is reduced in skeletal muscle, and indicates that this may be related to changes in insulin sensitivity when administered for 28 days. Additional research is needed to examine the mechanism of action as to how exogenous palmitoleic acid reduces intramuscular adipocyte size and triacylglyceride content to restore insulin sensitivity.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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