AMPKα, C/EBPβ, CPT1β, GPR43, PPARγ, and SCD Gene Expression in Single- and Co-cultured Bovine Satellite Cells and Intramuscular Preadipocytes Treated with Palmitic, Stearic, Oleic, and Linoleic Acid

S. H. Choi1,2,a, S. K. Park3,a, B. J. Johnson4, K. Y. Chung5, C. W. Choi6, K. H. Kim7,8, W. Y. Kim2, and S. B. Smith1,3,a

1 Department of Animal Science, Texas Agricultural Experiment Station, Texas A&M University, College Station, TX 77843, USA

ABSTRACT: We previously demonstrated that bovine subcutaneous preadipocytes promote adipogenic gene expression in muscle satellite cells in a co-culture system. Herein we hypothesize that saturated fatty acids would promote adipogenic/lipogenic gene expression, whereas mono- and polyunsaturated fatty acids would have the opposite effect. Bovine semimembranosus satellite cells (BSC) and intramuscular preadipocytes (IPA) were isolated from crossbred steers and cultured with 10% fetal bovine serum (FBS)/Dulbecco’s Modified Eagle Medium (DMEM) and 1% antibiotics during the 3-d proliferation period. After proliferation, cells were treated for 3 d with 3% horse serum/DMEM (BSC) or 5% FBS/DMEM (IPA) with antibiotics. Media also contained 10 μg/mL insulin and 10 μg/mL pioglitazone. Subsequently, differentiating BSC and IPA were cultured in their respective media with 40 μM palmitic, stearic, oleic, or linoleic acid for 4 d. Finally, BSC and IPA were single- or co-cultured for an additional 2 h. All fatty acid treatments increased (p = 0.001) carnitine palmitoyltransferase-1 beta (CPT1β) gene expression, but the increase in CPT1β gene expression was especially pronounced in IPA incubated with palmitic and stearic acid (6- to 17-fold increases). Oleic and linoleic acid decreased (p = 0.001) stearoyl-CoA desaturase (SCD) gene expression over 80% in both BSC and IPA. Conversely, palmitic and stearic acid increased SCD gene expression three fold in co-cultured in IPA, and stearic acid increased AMPKα gene expression in single- and co-cultured BSC and IPA. Consistent with our hypothesis, saturated fatty acids, especially stearic acid, promoted adipogenic and lipogenic gene expression, whereas unsaturated fatty acids decreased expression of those genes associated with fatty acid metabolism. (Key Words: Bovine, Co-culture, Fatty Acids, Gene Expression, Preadipocytes, Satellite Cells)

INTRODUCTION

The primary objective of this study was to demonstrate the effects of saturated fatty acids (palmitic and stearic acid) and unsaturated fatty acids (oleic and linoleic acid) on specific gene expression in bovine satellite cells (BSC) and intramuscular preadipocytes (IPA). The secondary objective was to document the effects of brief co-culture of BSC and IPA on adipogenic and lipogenic gene expression. We hypothesized that palmitic and stearic acid would increase expression of genes associated with lipid accumulation in BSC and IPA during differentiation. We also tested the effects of oleic acid and linoleic acid, as we predicted that they would depress adipogenic and/or lipogenic gene expression. Stearic acid is the most abundant fatty acid to pass from the rumen to the small intestine in cattle, although palmitic acid also is abundant in the small intestine of cattle (Ekeren et al., 1992; Chung et al., 2006b). Oleic acid is absorbed from the gastrointestinal tract of cattle (Ekeren et al., 1992) and is produced endogenously...
(St. John et al., 1991; Chang et al., 1992; Archibeque et al., 2005; Kadegowda et al., 2013), and linoleic acid is the most abundant polyunsaturated fatty acid in the diet and tissues of beef cattle (Chung et al., 2006b).

To accomplish these objectives, we documented the effects of exposure to fatty acids during culture on the expression of genes associated with adipocyte differentiation (CCAAT/enhancer-binding protein beta [C/EBPβ] and peroxisome proliferator-activated receptor gamma [PPARγ]), lipid metabolism (carnitine palmitoyltransferase-1 beta [CPT1β] and stearoyl-CoA desaturase [SCD]), and the regulation of triacylglycerol turnover (G protein-coupled protein receptor 43 [GPR43] and AMP-activated protein kinase alpha-1 [AMPKα]). In the present study, we hypothesized that co-culture of preadipocytes with myoblasts would modify the effects of saturated and unsaturated fatty acids on adipogenic and lipogenic gene expression.

**MATERIALS AND METHODS**

**Chemicals**

Fatty acids were purchased from Nu-Chek prep, Inc (Elysian, MN, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA), Calbiocem (La Jolla, CA, USA), Gibco (Grand Island, NY, USA) or Applied Biosystems (Foster City, CA, USA).

**Bovine satellite cell and preadipocyte isolation**

Muscle-derived BSC and IPA were isolated from semimembranosus muscle of 14-mo-old crossbred steers raised at Texas Tech University. Steers were killed with a captive bolt stunner followed by exsanguination. Using sterile techniques, approximately 500 g of the semimembranosus muscle was incubated with 0.1 g pronase (Calbiochem) in Earl’s Balanced Salt Solution (Sigma-Aldrich, USA) for 1 h at 37°C with frequent mixing. The dissected IPA was incubated with 0.1% type IV collagenase (Sigma-Aldrich, USA) in Earl’s Balanced Salt Solution for 1 h at 37°C with frequent mixing (Suryawan and Hu, 1997; Ohyama et al., 1998). After incubation, both mixtures were centrifuged at 1,500×g for 4 min, the pellets were suspended in phosphate buffered saline (Gibco) (140 mM NaCl, 1 mM KH₂PO₄, 3 mM KCl, 8 mM Na₂HPO₄), and the suspensions were centrifuged at 500×g for 10 min. The supernatants were centrifuged at 1,500×g for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated twice. The resulting cells preparations were suspended in cold (4°C) Dulbecco’s Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 10% (vol/vol) dimethylsulfoxide (Sigma-Aldrich, USA) and frozen. Cells were stored frozen in liquid nitrogen.

**Cell culture**

The BSC and IPA were obtained from a single batch and experiments were replicated in three independent incubations (Choi et al., 2013a). Cells were plated at a density of 1×10⁴ cells per well and grown at 37°C under a humidified atmosphere of 5% CO₂. Upon reaching confluence after 3 d, the growth medium was replaced with 3% horse serum/DMEM plus antibiotics (BSC) or 5% FBS/DMEM plus antibiotics (IPA). Differentiation medium also contained 10 μg/mL insulin and 10 μM pioglitazone (Kang et al., 2003; Chung and Johnson, 2009), and BSC and IPA were allowed to differentiate for 3 d. Subsequently, no fatty acids (control cells) or 40 μM fatty acids (palmitic, stearic, oleic, or linoleic acid) were added to the media, and the differentiating IPA and BSC were incubated for an additional 4 d. Fatty acids were dissolved in ethanol prior to addition to the cell culture (Satory and Smith, 1999; Chung et al., 2006a). Finally, differentiated IPA and BSC were single-cultured and co-cultured for an additional 2 h in DMEM plus antibiotics in the presence of the fatty acids.

**Real-time polymerase chain reaction**

RNA was extracted with Tri Reagent (Sigma Chemicals, St. Louis, MO, USA) as reported previously (Smith et al., 2012). The concentration of RNA was quantified with a NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Washington, DE, USA). The 260:280 ratio for all samples was greater than 1.85. Quantitative real time polymerase chain reaction (qRT-PCR) was used to analyze the expression of C/EBPβ, CPT1β, GPR43, PPARγ, AMPKα, and SCD (primers listed in Table 1). Commercially available eukaryotic 40S ribosomal protein S9 (RPS9) RNA (Applied Biosystems; GeneBank Accession #X03205) was used as the endogenous control. Other studies in bovine adipose tissue explants (Hosseini et al., 2010) and bovine liver (Baxa et al., 2010) demonstrated that RPS9 mRNA expression was stable and suitable as a housekeeping gene under their conditions. Additionally, RPS9 was used as a housekeeping gene for the expression genes in bovine muscle (Chung and Johnson, 2009; Baxa et al., 2010).

Complementary DNA was produced from 1 μg RNA using Taq- Man Reverse Transcriptase Reagents (Applied Biosystems, USA) by the protocol recommended by the manufacturer. Random hexamers were used as primers in cDNA synthesis. Measurement of the relative quantity of the cDNA of interest was carried out using TAMRA PCR.
Master Mix (Applied Biosystems, USA), appropriate forward and reverse primers, and 1 μL of the cDNA mixture. Assays were performed in duplicate in the GeneAmp 5700 Sequence Detection System (Applied Biosystems, USA) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min at 60°C). Cycle threshold (CT) values were means of duplicate measurements. The comparative CT values were employed to determine expression levels for target genes; fold change was determined as \(2^{-\Delta\Delta CT}\) with RPS9 as the endogenous control. Titration of the target mRNA primers against increasing amounts of cDNA gave linear responses with slopes between –2.8 and –3.0. In order to reduce the effect of assay-to-assay variation in the PCR assay, all values were calculated relative to a calibration standard run on every qRT-PCR assay. The ABI Prism 7000 detection system (Applied Biosystems, USA) was used to perform the assay utilizing the thermal cycling variables recommended by the manufacturer (50 cycles of 15 s at 95°C and 1 min at 60°C).

### Statistical analysis

Data were analyzed by analysis of variance as a three-factor design with cell type (adipocytes, myoblasts), culture method (single-culture, co-culture), and fatty acids (stearic, oleic, and linoleic acids) as the main effects (SuperAnova, Abacus Concepts, Berkeley, CA, USA). The model also tested all possible two- and three-way interactions among main effects. Means were separated by Fisher’s Protected LSD (SuperAnova, USA) if their respective F-test indicated significant differences (p<0.05).

### RESULTS

#### Cell type, culture method, and fatty acid treatment main effects

There were highly significant differences (p<0.001) between IPA and BSC for AMPKa, C/EBPβ, CPT1β, PPARγ, and SCD gene expression (Table 2). The mRNA levels for AMPKa, C/EBPβ, and PPARγ were higher in IPA than in BSC after differentiation, whereas CPT1β and SCD gene expression was higher in BSC than in IPA after differentiation. The mRNA levels for GPR43 did not differ between cell types (p = 0.303).

The culture method main effect (single or co-culture) was significant (p<0.001) for AMPKa, C/EBPβ, GPR43, PPARγ, and SCD gene expression (Table 2). The mRNA levels for AMPKa, C/EBPβ, GPR43, PPARγ, and SCD were greater in co-cultured cells than in single-cultured cells. Culture method had no effect on CPT1β gene expression (p = 0.197).

Fatty acid treatments affected the expression of all genes (p<0.001) except GPR43 and PPARγ (p = 0.136 and 0.165, respectively). Expression of AMPKa, C/EBPβ, and SCD was highest in cells incubated with stearic acid, whereas oleic and linoleic acid depressed SCD gene expression relative to control samples (Table 2). All fatty acids
Recent studies demonstrated that inhibition of SCD catalytic activity reduced the activities of genes associated with de novo fatty acid synthesis, such as fatty acid synthase while increasing expression of genes associated with fatty acid oxidation, such as CPT1β (Kim et al., 2011; Kadegowda et al., 2013). Acetate and propionate stimulate fatty acid oxidation, and a co-culture system (Hausman et al., 2006; Choi et al., 2013a) most likely allows for fatty acid oxidative metabolism 

The cell type-culture method-fatty acid three-way interactions

There were significant cell type-culture method-fatty acid interactions for AMPKa, C/EBPβ, PPARγ, and SCD gene expression (p<0.052) (Figures 1 and 2). Relative to the control cells, palmitic acid increased AMPKa gene expression in single-cultured IPA and both palmitic and stearic acid increased AMPKa gene expression in co-cultured IPA (Figure 1A). Similarly, palmitic and stearic acid increased C/EBPβ gene expression in single cultured IPA and stearic acid increased C/EBPβ gene expression more than palmitic acid in co-cultured IPA (Figure 1B). Linoleic acid increased PPARγ gene expression in single cultured BSC and stearic, oleic, and linoleic acid increased PPARγ gene expression in single cultured IPA (Figure 2A). None of the fatty acids treatments affected PPARγ gene expression in co-cultured IPA. Relative to the control cells, palmitic acid decreased SCD gene expression in single- and co-cultured BSC but increased SCD gene expression in co-cultured IPA (Figure 2B).

Culture method-fatty acid, cell type-fatty acid, and culture method-cell type two-way interactions for CPT1β and GPR43

The cell type-culture method-fatty acid interactions for CPT1β and GPR43 gene expression were not significant (p = 0.312 and 0.113, respectively) (Table 3). Nor was the culture method-fatty acid interaction significant for CPT1β and GPR43 gene expression (p = 0.341 and 0.125, respectively). The cell type-fatty acid interaction was significant for CPT1β (p<0.001) but not for GPR43 gene expression (p = 0.141). Also, the culture method x cell type was not significant for CPT1β (p = 0.141) but tended to be significant for GPR43 gene expression (p = 0.073) (Table 3).
increases lipid accumulation in adipocytes and promotes metabolism of fatty acids and glucose in other tissues (Kimura et al., 2013). These effects may be attenuated by AMPKα (Yin et al., 2003). Expression of AMPKα promotes fatty acid oxidation by upregulating PPAR gamma coactivator-1alpha (PGC-1α) gene expression (Wan et al., 2013). In our previous study (Choi et al., 2013a), co-culture of subcutaneous (SC) preadipocytes with BSC increased GPR43 gene expression nearly fivefold, which would antagonize the actions of AMPKα if activated by appropriate ligands (i.e., volatile fatty acids). In this study, co-culture increased GPR43 gene expression two- to five fold. Although culture method had no effect on CPT1β gene expression, the elevated GPR43 gene expression induced by co-culture suggests that the presence of IPA may increase lipid accumulation in BSC.

Figure 1. AMPKα gene expression (A) and C/EBPβ gene expression (B) in bovine satellite cells (BSC) and intramuscular preadipocytes (IPA). Cells were plated at a density of 1x10⁴ cells per well and grown at 37°C under a humidified atmosphere of 5% CO₂. Upon reaching confluence after 3 d, the growth medium was replaced with 3% horse serum/DMEM plus antibiotics (BSC) or 5% FBS/DMEM plus antibiotics (IPA). Differentiation medium also contained 10 μg/mL insulin and 10 μM pioglitazone, and BSC and IPA were allowed to differentiate for 3 d. Subsequently, no fatty acids (control cells) or 40 μM fatty acids (palmitic, stearic, oleic, or linoleic acid) were added to the media, and the differentiating IPA and BSC were incubated for an additional 4 d. Differentiated IPA and BSC were single-cultured and co-cultured for an additional 2 h in DMEM plus antibiotics in the presence of the 40 μM fatty acids. A. AMPKα: The cell type×culture method×fatty acid three-way interaction was significant (p = 0.029). Relative to the control cells, palmitic acid increased AMPKα gene expression in single cultured IM (intramuscular) preadipocytes and both palmitic and stearic acid increased AMPKα gene expression in co-cultured IPA. B. C/EBPβ: The cell type×culture method×fatty acid three-way interaction was significant (p = 0.003). Relative to the control cells, palmitic and stearic acid increased C/EBPβ gene expression in single cultured IPA and stearic acid increased C/EBPβ gene expression more than palmitic acid in co-cultured IPA. All data are the means±SEM for 3 independent incubations. abc Means within a cell type and culture method with common superscripts are not different (p>0.05). AMPKα, AMP-activated protein kinase alpha; CCAAT/enhancer-binding protein beta; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; SEM, standard error of the mean.
Co-culture generally increased PPARγ gene expression in BSC and IPA, which we did not observe previously in SC preadipocytes co-cultured with BSC (Choi et al., 2013a). However, PPARγ gene expression was profoundly higher in SC preadipocytes in our previous study than in the IPA of the current study. Conversely, C/EBPβ gene expression was hardly measurable in IPA in the current study. The expression of C/EBPβ occurs soon after preadipocytes are induced to differentiation (Darlington et al., 1998). These data suggest that IPA still were in early stages of differentiation even after 7 d of differentiation.

In an early study, we demonstrated high levels of SCD gene expression in bovine longissimus dorsi and SC adipose tissue (Cameron et al., 1994). In a recent study (Choi et al., 2014), intramuscular (IM) adipose tissue
Table 3. Culture method×fatty acid and cell type×fatty acid interaction means for gene expression in single- and co-cultured bovine satellite cells and intramuscular preadipocytes incubated in the absence and presence of 40 μM palmitic, stearic, oleic, or linoleic acid.

| Gene/culture method | Treatment | SEM  | p-values |
|---------------------|-----------|------|----------|
|                     | Control   | Palmitic | Stearic | Oleic | Linoleic |      |          |
| CPT1β               | Single culture | 178 | 1,069 | 980 | 1,582 | 959 | 141 | 0.341 |
|                     | Co-culture | 217 | 1,123 | 882 | 1,318 | 887 |      |          |
| GPR43               | Single culture | 0.58 | 0.34 | 0.50 | 0.78 | 0.46 | 0.11 | 0.125 |
|                     | Co-culture | 1.19 | 1.01 | 2.24 | 1.29 | 1.64 |      |          |
| CPT1β               | BSC       | 392  | 2,165 | 1,835 | 2,889 | 1,832 | 141 | <0.001 |
|                     | IPA       | 3.32 | 27.91 | 26.79 | 11.93 | 14.13 |      |          |
| GPR43               | BSC       | 0.69 | 0.46 | 1.72 | 0.93 | 0.78 | 0.11 | 0.141 |
|                     | IPA       | 1.08 | 0.89 | 1.02 | 1.14 |      |      |          |
| CPT1β               | BSC       | 1,883 | 1,738 |      | 141 | 0.141 |
|                     | IPA       | 12 | 22 |      |      |          |
| GPR43               | BSC       | 0.29 | 1.54 |      | 0.11 | 0.073 |
|                     | IPA       | 0.77 | 1.41 |      |      |          |

SEM, standard error of the mean; CPT1β, carnitine palmitoyltransferase-1 beta; GPR43, G protein-coupled protein receptor 43; BSC, bovine satellite cells; IPA, intramuscular preadipocytes.

a Relative CPT1β and GPR43 mRNA levels in total RNA isolated from BSC, IPA single- or co-cultured with insulin (10 μM), and pioglitazone (10 μM).

b Data are means for 6 observations, pooled over culture method.

c Data are means for 6 observations, pooled over cell type.

d Data are means for 30 observations, pooled over fatty acid treatment.

eq0.05 Means within a gene with common superscripts are not different (p>0.05).

explants were removed from cattle at 12, 14, or 16 mo of age and cultured for 48 h in the absence and presence of 40 μM α-linolenic acid, trans-10, cis-12 conjugated linoleic acid (CLA), oleic acid, stearic acid, and trans-vaccenic acid. The mRNA levels for SCD increased over threefold between 12 and 16 mo of age in IM adipose tissue, similar to the pattern of SCD gene expression in bovine SC adipose tissue (Martin et al., 1999). However, whereas unsaturated fatty acids strongly depressed SCD gene expression in both BSC and IPA in this study, they had no effect on SCD gene expression in IM adipose tissue explants (Choi et al., 2014). We previously demonstrated that trans-10, cis-12 CLA strongly depressed SCD expression in IM adipose tissue (Choi et al., 2014). Of the fatty acids tested in this study, stearic acid elicited the most consistent increases in adipogenic and lipogenic gene expression. The pronounced effects of stearic acid on PPARY, C/EBPβ, and SCD gene expression in co-cultured IPA. Dietary palmitate, fed in the form of palm oil, increased de novo fatty acid synthesis, enzymes associated with fatty acid synthesis, and adipocyte volume in bovine SC adipose tissue (Choi et al., 2013b). Elevated palmitic acid in plasma or muscle of young cattle may promote trans-differentiation of satellite cells, leading to muscle steatosis. This observation certainly warrants further investigation into the effects of palmitic acid on myogenic gene expression.

The saturated fatty acids, palmitic and stearic acid are noteworthy for their strong effects on gene expression. Palmitic acid increased C/EBPβ gene expression in both BSC and IPA (although C/EBPβ gene expression remained barely detectable in BSC). Palmitic acid also increased AMPKa and SCD gene expression in co-cultured IPA. Dietary palmitate, fed in the form of palm oil, increased de novo fatty acid synthesis, enzymes associated with fatty acid synthesis, and adipocyte volume in bovine SC adipose tissue (Choi et al., 2013b). Elevated palmitic acid in plasma or muscle of young cattle may promote trans-differentiation of satellite cells, leading to muscle steatosis. This observation certainly warrants further investigation into the effects of palmitic acid on myogenic gene expression.

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these genes in IPA.

Finally, the results of this study confirm our earlier report (Choi et al., 2013a) that co-culture of BSC and IPA alters the expression of genes associated with adipogenesis and lipogenesis. These data suggest that the coexistence of BSC and IPA within intact muscle would promote IPA differentiation and may even cause trans-differentiation of BSC to IM adipocytes.

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