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Oleic acid Stimulates the Formation of Adipocyte-Like Cells from Bovine Satellite Cells via *G-Protein Coupled Receptor* 43 and *CCAAT/Enhancer-Binding Protein Beta*

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Abstract

Numerous physiological and pathological processes are controlled by free fatty acids, which act as signaling molecules in mammals. We hypothesized that oleic acid (Ole) might stimulate the formation of satellite-cell-derived intramuscular adipose tissue. The role of Ole as a ligand of *G-protein-coupled receptor* 43 (*GPR*43) was previously identified. Thus, the objective of the current study was to determine the effect of Ole on *GPR*43 and factors related to the adipogenic differentiation of bovine satellite cells (BSC). Treatments of 100 μM and 500 μM Ole tended to induce greater ($P < 0.10$) mRNA expression of *CCAAT/enhancer-binding protein β* (*C/EBPβ*) compared to all other doses. The mRNA abundance of *peroxisome proliferator-activated receptor γ* (*PPARγ*) was not altered ($P > 0.10$) by treatment. The addition of 100 μM and 500 μM of Ole upregulated ($P < 0.05$) *GPR*43 mRNA expression. Protein level of GPR43 was increased ($P < 0.05$) by 100 μM of Ole treatments. Addition of Ole to BSC cultures induced transformation of myogenic cells into adipocyte-like cells that formed cytoplasmic lipid droplets. Increased expression of *C/EBPβ* in response to Ole might suppress myogenic differentiation. After the treatment of cells with Ole, increased expression of GPR43 could lead to phosphorylation of 5' AMP-activated protein kinase *α* (AMPK*α*). Altogether, the results indicated that increased Ole might stimulate adipose tissue accumulation within the skeletal muscle of cattle by promoting adipogenic differentiation and activation of GPR43 in satellite cells.

*Kim and Chung made an equal contribution.

Keywords

Bovine Satellite Cells, GPR43, Intramuscular Adipose Tissue, Oleic Acid

1. Introduction

The biology of intramuscular (IM) adipose tissue is widely discussed in meat-producing animals due to its economic value [1] [2] [3] [4]. Beef steaks with greater IM fat deposition are recognized by consumers in the U.S. as more palatable [5]. Adipose tissue within the skeletal muscle is derived from the various lineages. During fetal growth, progenitor cells of IM fat, muscle fibers, satellite cells, and fibroblasts are derived from a shared pool of mesenchymal stem cells [6]. These mononucleated myogenic cells play a pivotal role in postnatal muscle hypertrophy, and muscle regeneration by mesenchymal stem cell differentiation has been demonstrated in various animal models [7]. Since myonuclei of skeletal muscle are post-mitotic, satellite cells (the primary stem cells in adult skeletal muscle) are responsible for postnatal muscle hypertrophy and muscle regeneration [8] [9] [10]. In addition to the role as myogenic progenitor cells, satellite cells are multipotent [11]. This is supported by Asakura, Rudnicki and Komaki [12], who reported that satellite cells have the ability to differentiate into adipogenic and osteogenic cell types. Additionally, Joe, Yi, Natarajan, Le Grand, So, Wang, Rudnicki and Rossi [13] suggested that the subpopulation of the mononuclear mesenchymal cells called fibro/adipogenic progenitors within myogenic progenitors is possibly the primary source of fibroblasts and adipocytes in adult tissue.

The mechanism by which cells gain adipocyte-like characteristics has been investigated in livestock species, specifically with respect to increasing IM adipose tissue. The lipid filling process could be partially controlled by G-protein-coupled receptor 43 (GPR43), an adipocyte-specific membrane receptor that is activated by short and long-chain fatty acids; the receptor may regulate lipid accumulation by intracellular signals that limit lipolysis [14] [15] [16]. It is also reported that oleic acid (Ole), one of the most abundant unsaturated fatty acids in the animal body, could upregulate GPR43 expression in cultured bovine intramuscular adipocytes [14]. Therefore, the objective of the study was to determine the effect of Ole, a long-chain fatty acid, on adipogenic differentiation, lipid-filing, and GPR43 activity in bovine satellite cells.

2. Materials and Methods

Bovine Satellite Cell Isolation and Culture

The isolation and cultivation of bovine satellite cells were conducted as previously reported by Johnson, Halstead, White, Hathaway, DiCostanzo and Dayton [17] and Kim, Wellmann, Smith and Johnson [18]. Bovine satellite cells were isolated from 3 16-month-old crossbred steers (predominantly Angus, 474.5 ±

50.2 kg). Cattle were harvested under USDA inspection at the Gordon W. Davis Meat Sciences Lab at Texas Tech University (Lubbock, TX). Approximately 800g of *semimembranosus* muscle tissue was collected upon harvest and immediately transported to the laboratory.

Satellite cell isolation procedures were then conducted under sterile conditions. Connective tissue, blood vessels, and adipose tissue were dissected from the muscle samples before passing the muscle pieces through a sterile meat grinder. The ground muscle was aliquoted into sterile tubes and incubated in Earl's Balanced Salt Solution (Sigma Aldrich, St. Louis, MO) with 0.1% Pronase (Calbiochem, LaJolla, CA) for 1 hour at 37˚C, mixing the samples every 10 min. After incubation, the mixture was centrifuged at 1500 × g for 4 min at room temperature. The resulting pellet was suspended in phosphate-buffered saline (PBS; Sigma Aldrich), and the suspension was centrifuged at 500 × g for 10 min at room temperature. The supernatant was collected and centrifuged at 1500 × g for 10 min at room temperature to pellet the mononucleated cells. The resulting mononucleated-cell preparation was suspended in warm (37˚C) Dubecco's Modified Eagle's medium (DMEM; Gibco, Waltham, MA) supplemented with 10% Fetal Bovine Serum (Thermo Fisher science, Waltham, MA), and 1 × Antibiotic-Antimycotic (Gibco). Cells were incubated at 37˚C with a humidified atmosphere of 95% air and 5% CO_2. Once cells are reached 80% to 90% confluence, the growth medium was replaced with differentiation media. The differentiation cocktail was composed of DMEM supplemented with 2% horse serum, 10 μg/mL insulin, 10 μg/mL hydrocortisone, 10 μM ciglitizone, and 1 × Antibiotic-Antimycotic. Treatments of 0, 1, 10, 100, or 500 μM of Ole (Sigma Aldrich) were included in the differentiation cocktail. An additional treatment of 100 μM of stearic acid (St, Sigma Aldrich) was evaluated to compare the effect of saturated fatty acid (St) and unsaturated fatty acid (Ole) on lipid filling. After 96 h of incubation, cells were harvested and prepared for mRNA and protein analysis.

Fluorescence Microscopy

Isolated satellite cells were grown on three-well microscopy glass slides (Cat. #: 80381, Ibidi, Fitchburg, WI, USA) for 96 h after inducing differentiation. Slides were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, USA) for 10 min at room temperature. In order to prevent nonspecific background staining, fixed cells were incubated in 1% bovine serum albumin for 30 min. Fixed cells were incubated with BODIPY (493/503, Thermo Fisher Scientific) and diluted in 1 mg/ml of dimethyl sulfoxide (DMSO, Sigma-Aldrich) for 30 min to stain intracellular neutral lipid green. Glass slides were then rinsed 3 times with PBS for 5 min at room temperature. Fixed cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for 5 min to stain nuclei blue.

In order to stain GPR43, satellite cells were grown on three-well microscopy glass slides (Ibidi, Fitchburg) for 24 h after inducing differentiation. Slides were then fixed and incubated overnight at 4˚C in the anti-GPR 43 primary antibodies (mouse polyclonal, ab12571, Abcam, Cambridge, MA). After 3 rinses with

PBS for 5 min, samples were dark-incubated for 30 min at 25˚C in the secondary antibody (1:1000 goat anti-mouse, IgG1, Alexa-Fluor 633 (Invitrogen). Slides were then incubated with 1 μg/mL of 4'6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific) for 1 min, rinsed twice briefly in PBS, and then covered with AquaMount mounting media (Lerner Laboratories, Pittsburgh, PA) and thin glass coverslips (VWR International). The slides were cured at 4˚C for 24 h in the dark. The slides were imaged at 200× using an inverted fluorescence microscope (Nikon Eclipse, Ti-E; Nikon Instruments Inc., Mellville, NY) equipped with a UV light source (Nikon Intensilight Inc.; C-HGFIE).

RNA Isolation and Real-Time Quantitative PCR

The protocol for the isolation of mRNA from bovine satellite cells was conducted as described by Chomczynski and Sacchi [19]. Cellular mRNA was isolated with acid guanidinium thiocyanate-phenol-chloroform extraction reagent (TRI reagent; Sigma, St. Louis, MO). The concentration and purity of RNA were determined by absorbance at 260 nm and 280 nm using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). An acceptable range of 1.76 to 2.05 was established for the 260/280 ratio. Genomic DNA removal and cDNA synthesis were performed using the QuantiTect reverse transcription kit (Qiagen, Germantown, MD) according to manufacturer recommendations. Real-time quantitative polymerase chain reaction (RT-qPCR; 7900HT Real-Time PCR System, Applied Biosystems, Foster City, CA) was used to measure the quantity of the genes of interest (**Table 1**) relative to the quantity of *ribosomal protein subunit* 9 (*RPS*9) mRNA in total RNA. Since the expression of *RPS*9 has previously not differed across bovine tissues [18] [20] [21], *RPS*9 was used as the endogenous control to normalize gene expression. Measurement of the relative quantity of the cDNA of interest was carried out using TAMRA PCR Master Mix (Applied Biosystems, Foster City, CA). Assays were performed using a 7900HT Real-Time PCR System (Applied Biosystems) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95˚C and one min at 60˚C). Titration of mRNA primers against increasing amounts of cDNA gave linear responses with slopes between −2.8 and −3.0. RQ values based on ΔΔCT were analyzed by the RQ manager (Applied Biosystems).

Western Blotting

Cultured satellite cells were isolated with 200 μl of ice-cold buffer containing M-PER (Fisher Scientific, Fair Lawn, NJ), a protease inhibitor (Roche, Switzerland), and 2 mM Na_3VO_4 (Fisher Scientific). Cell homogenate was mixed with an equal volume of 2× standard SDS sample loading buffer (Invitrogen). The concentration of samples was determined with Pierce BCA protein assay following the manufacturer's guidance (Thermo Fisher Science) using a NanoDrop 1000 (NanoDrop products), with absorbance at 562 nm. Samples were loaded onto Novex 4% - 12% Bis-Tris gels (Invitrogen, Grand Island, NY) and separated by gel electrophoresis for 35 min. Proteins were then transferred to a nitrocellulose membrane (Invitrogen). Membranes were then incubated overnight

Table 1. Sequence for bovine PCR primers and TaqMan probes for adipogenic genes[1].

Item	Sequence (5' to 3')
AMPKα (accession no. NM_001109802)	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTTTGGATTTCTG
TaqMan Probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
CEBPβ (accession no. NM_176788)	
Forward	CCAGAAGAAGGTGGAGCAACTG
Reverse	TCGGGCAGCGTCTTGAAC
TaqMan Probe	6FAM-CGCGAGGTCAGCACCCTGC-TAMRA
GPR43 (accession no. FJ562212)	
Forward	GGCTTTCCCCGTGCAGTA
Reverse	ATCAGAGCAGCCATCACTCCAT
TaqMan Probe	6FAM-AAGCTGTCCCGCCGGCCC-TAMRA
PPARγ (accession no. NM_181024)	
Forward	ATCTGCTGCAAGCCTTGGA
Reverse	TGGAGCAGCTTGGCAAAGA
TaqMan Probe	6FAM-CTGAACCACCCCGAGTCCTCCCAG-TAMRA
RPS9 (accession no. DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA
SCD (accession no. AB075020)	
Forward	TGCCCACCACAAGTTTTCAG
Reverse	GCCAACCCACGTGAGAGAAG
TaqMan Probe	6FAM-CCGACCCCCACAATTCCCG-TAMRA

[1]AMPKα (adenosine monophosphate-activated protein kinase-α); C/EBPβ (CCAAT/enhancer-binding protein β); GPR43 (G protein couple receptor 43); PPARγ (Peroxisme proliferated activate receptor γ); RPS9 (ribosomal protein subunit 9); SCD (stearoyl CoA desaturase).

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at 4˚C at a dilution of 1:1000 of the following rabbit polyclonal primary antibodies: anti-AMPKα (Cell Signaling, Danvers, MA), anti-phosphorylated AMPKα (Cell Signaling), anti-GPR43 (ab12571, Abcam, Cambridge, MA), anti-GPR41 (ab103718, Abcam), and anti-GPR120 (ab230869, Abcam). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; epr16891, Abcam) served as the loading control.

Membranes were then incubated with a secondary antibody, Alexa-Fluor 633, goat anti-rabbit, at 1:2000 dilution for 2 h at room temperature. After 3 washes, membranes were visualized using an enhanced chemiluminescent substrate, Western blotting reagents (Amersham Bioscience), and exposure to film (MR, Kodak, Rochester, NY). The density of bands was quantified using Imager

Scanner II and ImageQuant TL software. Tissue lysates of all groups were run in a single gel to reduce variation. Band density was normalized by GAPDH content.

Statistical Analysis

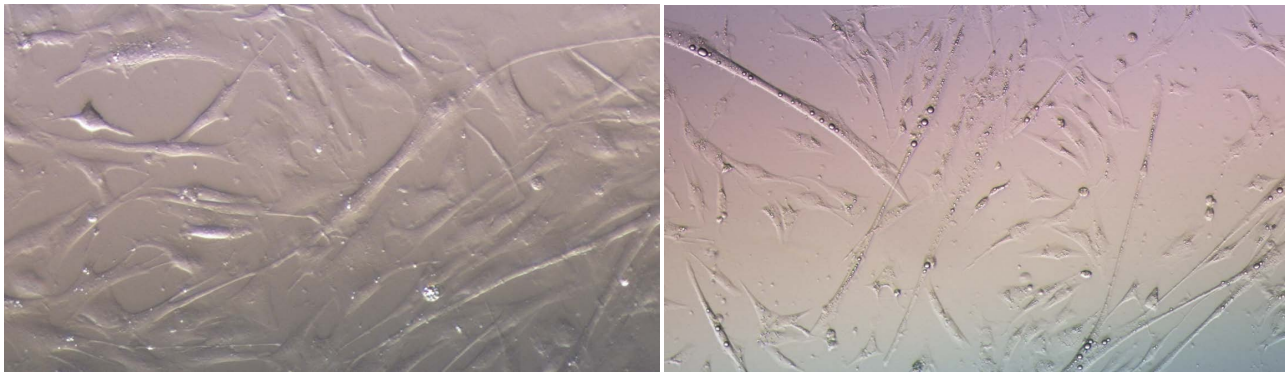
Cells used in this experiment were isolated from three steers; all six treatments were applied to cells from each steer, and the resulting cultures were for both mRNA and protein analysis. Data were analyzed using the MIXED procedure (PROC MIXED) of SAS (SAS Institute, Cary, NC) as appropriate for completely randomized designs. When a significant preliminary F-test was detected, the least-square means of each Ole dose were separated and denoted to be different using the pairwise comparison option of SAS. An α level of 0.05 was used to determine significance; tendencies were identified by *P*-value between 0.05 and 0.10.

3. Results and Discussion

Morphological Changes

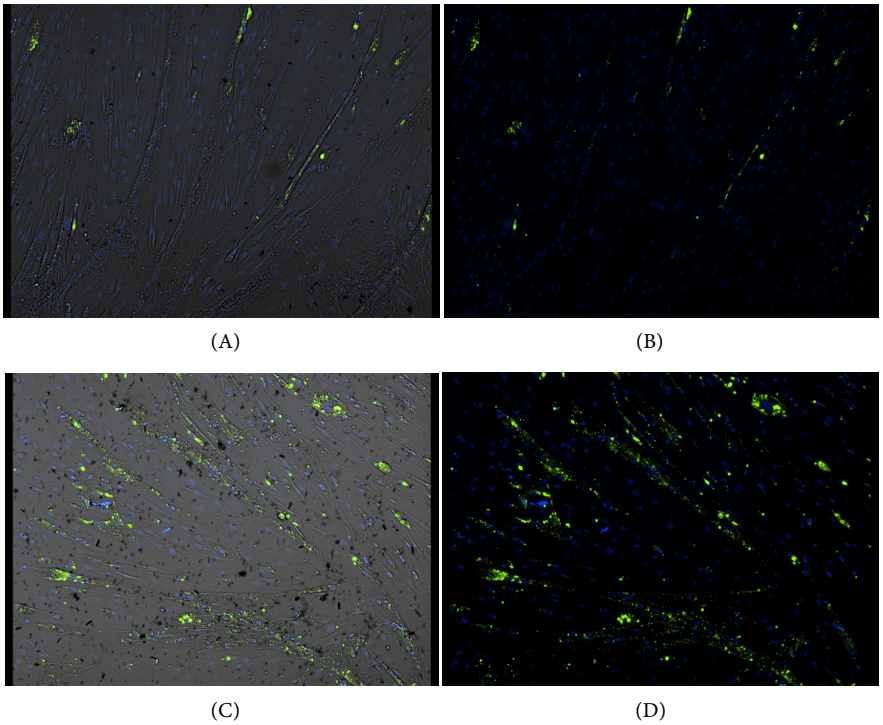
It has been reported that satellite cells have the ability to differentiate into adipocytes in the presence of adipogenic inducers in cattle [22] [23], mice [12], swine [24], and humans [25]. In our current study, the major morphological change observed during adipogenic differentiation was the accumulation of lipid droplets within cells (**Figure 1**). After 24 h of incubation with the myogenic differentiation media (2% horse serum in DMEM) supplemented with adipogenic differentiation factors (insulin and ciglitizone), the succession of primary myotubes was observed. After myotube formation, treatments of Ole (100 μM and 500 μM) resulted in the creation of lipid droplets within the myotubes (approximately 96 h after differentiation); visually, Ole promoted accumulation of neutral lipid (**Figure 2**). Given the morphological changes, the results suggested that satellite cells treated with Ole were committed to exhibiting adipogenic characteristics. This result is in agreement with Asakura, Rudnicki and Komaki [12], who demonstrated that myoblasts were able to transdifferentiate into adipocytes with differentiation media containing myogenic and adipogenic inducers. Teboul, Gaillard, Staccini, Inadera, Amri and Grimaldi [26] also observed a homogenous monolayer of lipid-containing cells when C2C12N cells were exposed to rosiglitazone, an adipogenic differentiation factor. Starkey, Yamamoto, Yamamoto and Goldhamer [27] suggested that satellite cells accumulated cytoplasmic lipid while expressing myogenic genes under the adipogenesis-inducing conditions applied to murine primary cell cultures. Using bovine satellite cells [28] found ciglitizone and Ole treatment increased the accumulation of triglyceride and lipid droplet area. Based on the morphological characteristics observed in the present study, adipogenic induction of muscle satellite cells might generate lipid droplets or promote lipid infiltration from supplementary lipid sources while simultaneously forming muscle fibers.

mRNA Gene Expression of Adipogenic and Myogenic Factors



(A) (B)

Figure 1. Images of bovine satellite cells incubated in differentiation medium containing 2% horse serum, 10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone (Image A) and differentiation medium with 100 μM of oleic acid (Image B). Images were taken 96 h after inducing differentiation medium. Images taken at 20× maginification.



(A) (B)

(C) (D)

Figure 2. Visualization of neutral lipid (green, Bodipy 493/503) and nuclei (blue, DAPI) of bovine satellite cells in differentiation medium containing 2% horse serum, 10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone (Image A and B) and differentiation medium with 100 μM of oleic acid (Image C and D). Images were taken 96 h after inducing differentiation. Images taken at 20× maginification.

Further analysis by RT-qPCR partially indicated that muscle cells simultaneously express adipogenic genes and myogenic genes (**Figure 3**). The addition of 100 μM and 500 μM Ole tended to increase ($P < 0.10$) mRNA gene expression of *C/EBPβ* compared to all other doses. *C/EBPβ* belongs to a family of basic leucine zipper transcription factors and acts as a commitment factor during adipogenic differentiation [29]. As adipogenic differentiation proceeds, the expression

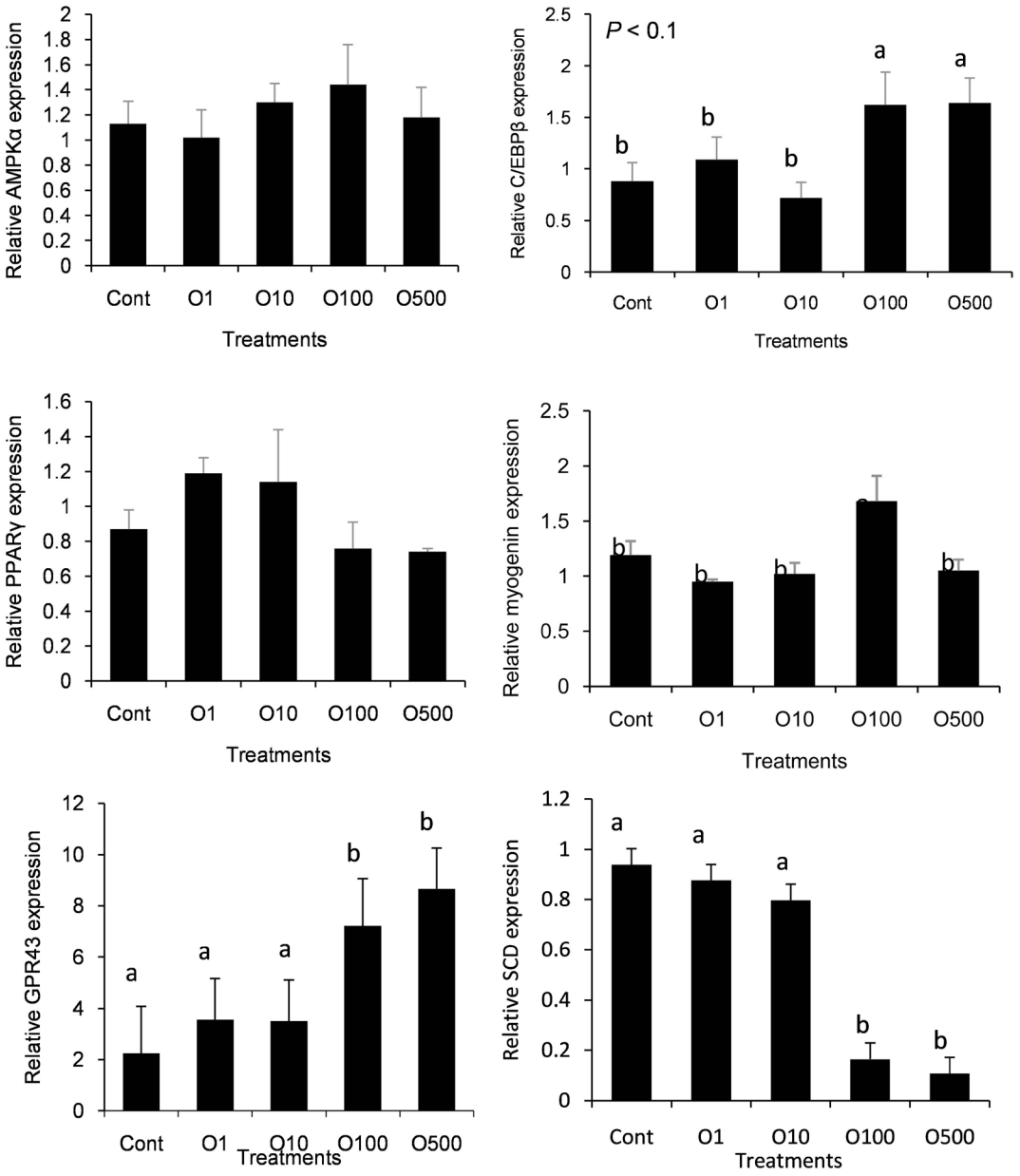


Figure 3. Expression of genes of AMPK*α*, C/EBP*β*, PPAR*γ*, myogenin, GPR43, and SCD when bovine satellite cells were incubated for 96 h in differentiation medium (10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone) with varying doses of oleic acid including 0 μM (Cont), 1 μM (O1), 10 μM (O10), 100 μM (O100), 500 μM (O500). An alpha level of 0.05 was used for mean separations. A tendency ($P < 0.10$) was observed for increased C/EBP*β* expression in O100 and O500 compared to all other treatments.

of *C/EBPβ* follows the expression of *PPARγ* [30]. However, the mRNA expression of *PPARγ* was not altered ($P > 0.10$) by treatment. This is partially in agreement with the findings of Chung and Johnson [23] that bovine satellite cells, in the presence of melengestrol acetate in the adipogenic cocktail with 100 μM of Ole acid, increased mRNA expression of *C/EBPβ* and *PPARγ*. Another study conducted using primary satellite cells from Yanbian yellow cattle found that Ole treatment with an absence of *PPAR* activator inducers, such as thiazoli-

dinedione (TZD), upregulated gene expression of *C/EBPβ* and *C/EBPα* [28]. The results of the current study concur with the findings of Teboul, Gaillard, Staccini, Inadera, Amri and Grimaldi [26] that mice satellite cell cultures supplemented with TZD and fatty acid formed adipogenic-like cells without upregulating *PPARγ* and *C/EBPα*.

While C/EBP*β* has a pivotal role in adipose cells, the protein is also detected in other cell types, including the liver, smooth muscle, and skeletal muscle [31]. Potentially, C/EBP*β* expression by skeletal muscle cells suppresses the expression of myogenic factors. Lala-Tabbert, AlSudais, Marchildon, Fu and Wiper-Bergeron [32] demonstrated that downregulation of *C/EBPβ* resulted in larger muscle fiber cross-sectional area and improved muscle repair. Marchildon, Lala, Li, St-Louis, Lamothe, Keller and Wiper-Bergeron [33] suggested that *C/EBPβ* inhibited MyoD protein expression and myogenesis in both primary myoblasts and C2C12 cells. However, results of the present study indicated that mRNA expression of myogenin was increased by treatment of 100 μM of Ole while *C/EBPβ* tended to be upregulated by both 100 μM and 500 μM of Ole ($P < 0.10$). Myogenin is one of four myogenic regulatory factors that bind to regulatory regions of DNA in order to regulate transcription during myogenic differentiation of satellite cells [34]. Expression of myogenin has previously been identified during myogenic differentiation and in the formation of myofibers; in adult animals, however, myogenin was not identified in the maintenance or development of satellite cells [35]. In the present study, bovine satellite cells likely retained some myogenic characteristics, while adipogenic differentiation was progressing. Adipogenic gene expression, lipid droplet formation, myogenic gene expression, and myofiber formation occurred simultaneously.

Contrary to the *C/EBPβ* expression, mRNA abundance of *stearoyl CoA desaturase* (*SCD*) was downregulated ($P < 0.05$) by high levels of Ole treatments (100 and 500 μM). Stearoyl CoA desaturase is an enzyme which catalyzes the formation of monounsaturated fatty acids, specifically Ole, from saturated fatty acid such as St [36]. The addition of unsaturated fatty acids to various cell types seems to reduce the expression of SCD consistently. Jones, Maher, Banz, Zemel, Whelan, Smith and Moustaid [37] reported that the addition of polyunsaturated fatty acid reduced mRNA expression of *SCD* in 3T3-L1 adipocytes. Others also detected similar results in bovine subcutaneous and IM adipocytes; linoleic and *α*-linoleic acid treatment decreased *SCD* expression [14] [38]. This may be due to the feedback inhibition by monounsaturated fatty acids. The expression of *SCD* might be inhibited or controlled by the product of its catalysis, especially Ole.

G-protein Coupled Receptor 43

G-protein coupled receptor 43 (GPR43) is expressed in mammalian tissues including adipose tissues [14] [39], inflammatory cells [40], gastrointestinal tract tissue [40], and myogenic-derived satellite cells [23]. The primary activator of GPR43 is known to be short-chain fatty acids. Acetic and propionic acid bind to

GPR43 with high affinity and regulate intracellular signaling pathways [41]. Previous findings have demonstrated that select long-chain fatty acids could also act as ligands of GPR43 in bovine adipose and satellite cells [14] [23]. The current study confirmed previous results demonstrating that Ole treatment increased mRNA expression and protein level of *GPR*43 (**Figures 3-5**). The addition of 100 μM and 500 μM of Ole increased ($P < 0.05$) mRNA gene expression of *GPR*43 and protein level ($P < 0.05$; **Figure 3** and **Figure 4**).

The role of GPR43 in lipid accumulation of adipose tissue has long been discussed from a diagnostic perspective. In essence, the expression of GPR43 is closely related to body fat mass and is highly regulated by nutrient intake in various animal models. Bjursell, Admyre, Göransson, Marley, Smith, Oscarsson and Bohlooly-Y [42] reported that *GPR*43-deficient mice fed a high-fat diet reduced fat mass but increased muscle mass. [43], Canfora, Jocken and Blaak [44] reported that GPR43 suppressed intracellular lipolysis via decreased phosphorylation of hormone-sensitive lipase, leading to increased PPARγ-mediated adipogenesis. This resulted in increased triglyceride accumulation in adipose tissue and reduced lipolysis. From these factors, it appears that lipid filling of bovine muscle cells might be regulated through the GPR43 pathway. In the current study, satellite cells incubated with Ole expressed both myogenic and adipogenic characteristics. In other words, cells accumulated lipid droplets within the multinucleated muscle fibers. Since adipogenic gene expression was limited, especially *PPARγ*, the role of *GPR*43 might be critical during the lipid filling process. Satellite cells have been considered a precursor of IM adipose tissue due to their multipotent characteristics and adipogenic potential. However, this lipid filling phenomenon seems to be attributed to lipid infiltration of formed muscle fibers, not because of adipogenic-differentiation of mononucleated satellite cells.

***Fatty Acid Sources and GPR*43**

In order to investigate the effect of other sources of long-chain fatty acids on *GPR*43 expression, St was also tested with BSC. The addition of St did not alter ($P > 0.10$) the protein level of *GPR*43, and only Ole increased ($P < 0.05$) *GPR*43 level (**Figure 6**). This was also confirmed by Chung, Smith, Choi and Johnson [14] who reported that Ole increased mRNA expression of *GPR*43 in bovine IM preadipocytes but did not have an influence on subcutaneous preadipocytes; however, the addition of St decreased *GPR*43 expression in both bovine IM and subcutaneous preadipocytes.

Oleic Acid and Phosphorylation of AMPKα

The addition of 100 μM of Ole increased ($P < 0.05$) protein level of phosphorylated AMPKα (**Figure 4**). This might be the consequence of activated GPR43. Supplied long-chain fatty acids have previously assimilated intracellularly and were converted to acetyl-CoA for incorporation into the tricarboxylic acid cycle [45]. As AMP in the cytosol is increased during the activity of acetyl-CoA, the increase in AMP/ATP ratio results in the phosphorylation of AMPK in the skeletal muscle [44]. In the early stages of AMPK activation, fatty acid oxidation is

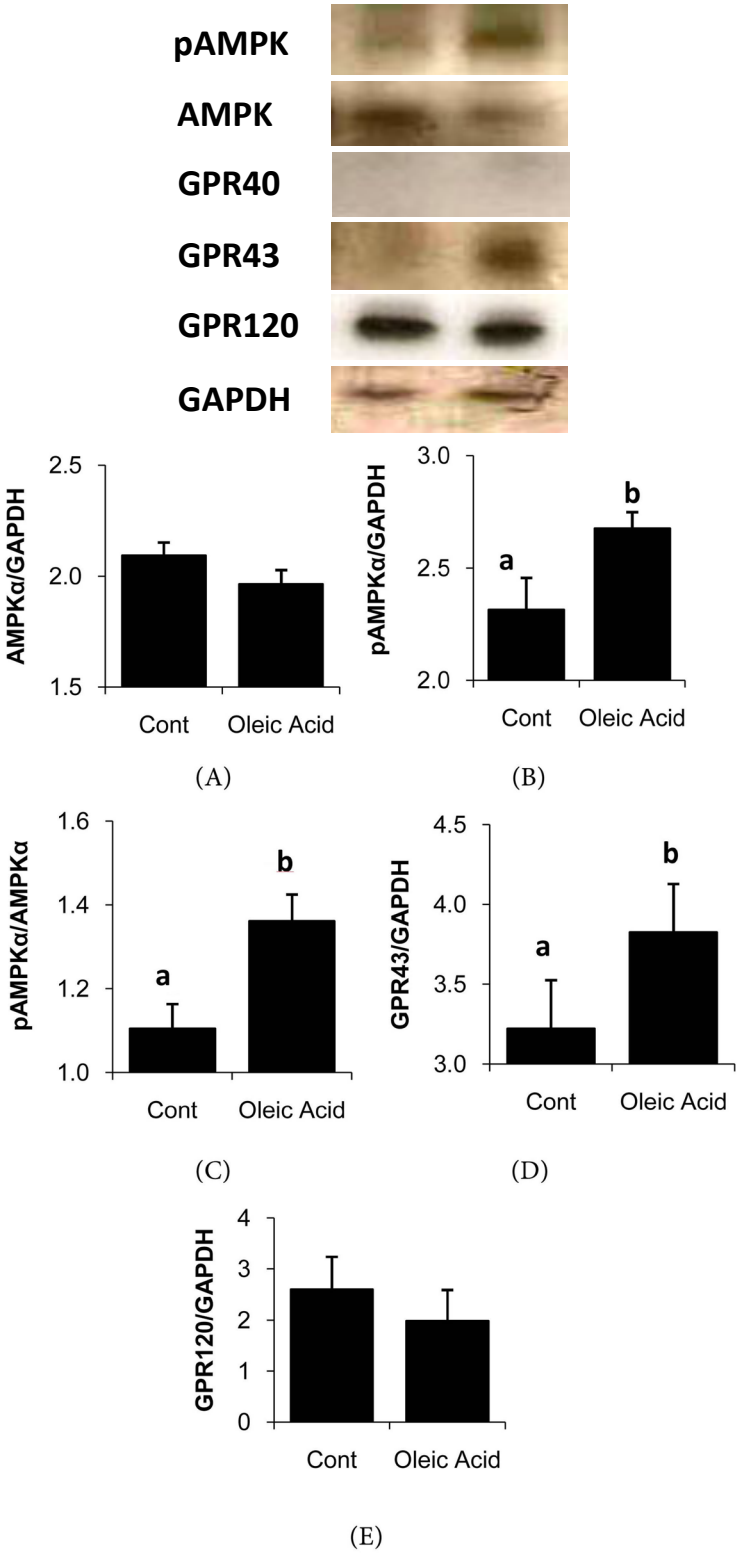
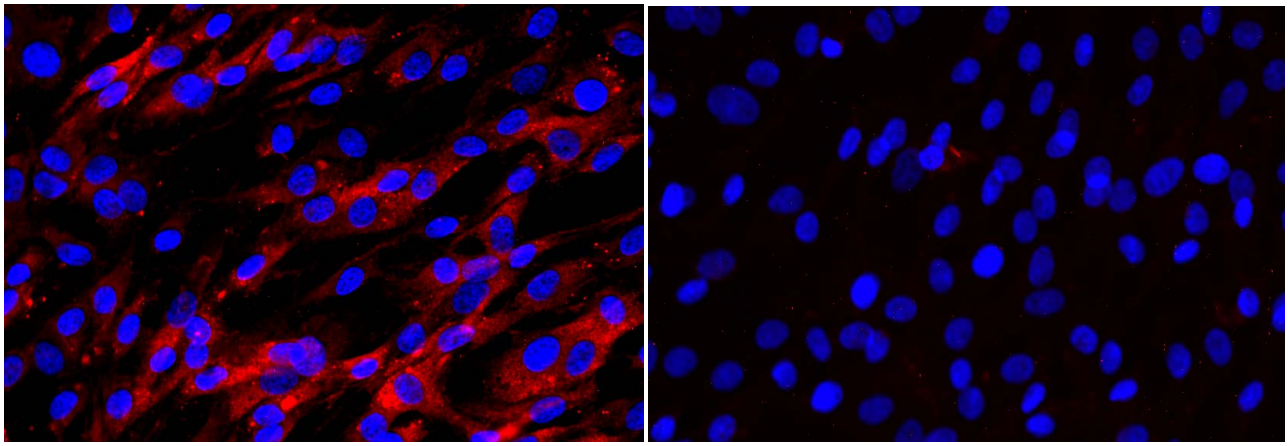


Figure 4. The protein level of AMPK*α* (A), phosphorylated AMPK*α* (pAMPK*α*, B), GPR43 (D), GPR120 (E) relative to GAPDH level, and pAMPK*α*/AMPK*α* ratio (C) when bovine satellite cells were incubated in differentiation medium (10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone) with 100 μM of oleic acid or without oleic acid for 96 h. An alpha level of 0.05 was used for mean separations.



(A) (B)

Figure 5. Visualization of GPR43 (red) and nuclei (blue, DAPI) of bovine satellite cells incubated in differentiation media containing 2% horse serum, 10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone (Image A) and differentiation medium with 100 μM of oleic acid (Image B). Imaging was performed using an inverted fluoresce microscope with a 40× objective 24 h after inducing treatment media.

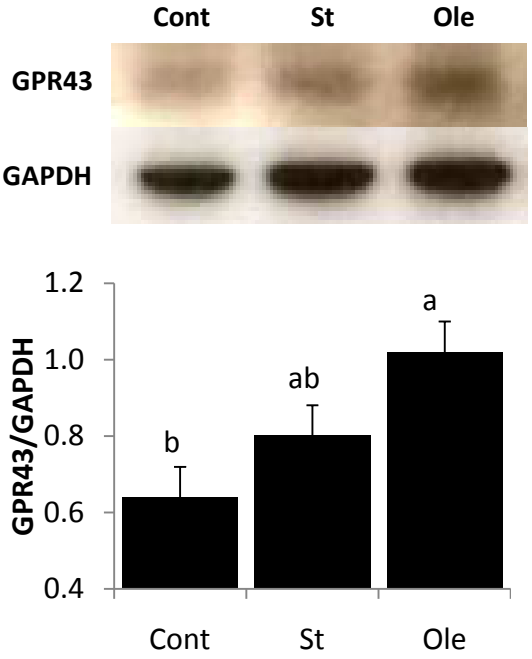


Figure 6. The protein level of GPR43 relative to GAPDH when bovine satellite cells were incubated in the medium containing adipogenic differentiation inducers (10 μg/ml of insulin, 10 μM of ciglitizone, and 25 nM of hydrocortisone) for 96 h. Cells were added one of three treatments: no fatty acid (Cont), 100 μM of stearic acid (St), or 100 μM of oleic acid (Ole). An alpha level of 0.05 was used for mean separations.

inhibited, but prolonged AMPK activation eventually stimulates fatty acid oxidation [46].

4. Conclusion

The addition of Ole in BSC cultures induced intramyocellular lipid accumula-

tion in skeletal muscle cells derived from satellite cells. The expression of *C/EBPβ*, one of the early markers of adipogenic differentiation, was observed in response to high levels of Ole supplementation, but myogenin was simultaneously upregulated by 100 μM of Ole treatment. This result could indicate that muscle cells containing intramyocellular lipid express muscle-like characteristics along with characteristics of adipose cells. The addition of Ole also increased GPR43 and seemingly activated AMPK*α*; however, the long-term response to Ole is unclear. It is likely that long-chain fatty acids act as potential ligands for GPR43 and control the intracellular signaling pathways promoting lipid filling. This finding indicates that intramuscular lipid accumulation could be induced in steers by long-chain fatty acids. Future studies should examine the effect of long-chain fatty acids on lipid metabolism of bovine cells for short- and long-term periods.

Funded

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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