All-trans retinoic acid increases the expression of oxidative myosin heavy chain through the PPAR δ pathway in bovine muscle cells derived from satellite cells¹

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ABSTRACT: All-trans retinoic acid (ATRA) has been associated with various physiological phenomenon in mammalian adipose tissue and skeletal muscle. We hypothesized that ATRA may affect skeletal muscle fiber type in bovine satellite cell culture through various transcriptional processes. Bovine primary satellite cell (BSC) culture experiments were conducted to determine dose effects of ATRA on expression of genes and protein levels related to skeletal muscle fiber type and metabolism. The *semimembranosus* from crossbred steers ($n = 2$ steers), aged approximately 24 mo, were used to isolate BSC for 3 separate assays. Myogenic differentiation was induced using 3% horse serum upon cultured BSC with increasing doses (0, 1, 10, 100, and 1,000 nM) of ATRA. After 96 h of incubation, cells were harvested and used to measure the gene expression of *protein kinase B (Akt)*, *AMP-activated protein kinase alpha (AMPK)*, *glucose transporter 4 (GLUT4)*, *myogenin*, *lipoprotein lipase (LPL)*, *myosin heavy chain (MHC) I*, *MHC IIA*, *MHC IIX*, *insulin like growth factor-1 (IGF-1)*, *Peroxisome proliferator activated receptor gamma (PPAR γ)*, *PPAR δ* , and

Smad transcription factor 3 (SMAD3) mRNA relative to *ribosomal protein subunit 9 (RPS9)*. The mRNA expression of *LPL* was increased ($P < 0.05$) with 100 and 1,000 nM of ATRA. Expression of *GLUT4* was altered ($P < 0.05$) by ATRA. The treatment of ATRA (1,000 nM) also increased ($P < 0.05$) mRNA gene expression of *SMAD3*. The gene expression of both *PPAR δ* and *PPAR γ* were increased ($P < 0.05$) with 1,000 nM of ATRA. Protein level of PPAR δ was also affected ($P < 0.05$) by 1,000 nM of ATRA and resulted in a greater ($P < 0.05$) protein level of PPAR δ compared to CON. All-trans retinoic acid (10 nM) increased gene expression of *MHC I* ($P < 0.05$) compared to CON. Expression of *MHC IIA* was also influenced ($P < 0.05$) by ATRA. The mRNA expression of *MHC IIX* was decreased ($P < 0.05$) with 100 and 1,000 nM of ATRA. In muscle cells, ATRA may cause muscle fibers to transition towards the MHC isoform that prefers oxidative metabolism, as evidenced by increased expression of genes associated with the MHC I isoform. These changes in MHC isoforms appeared to be brought about by changing PPAR δ gene expression and protein levels.

Key words: all-trans retinoic acid, bovine satellite cells, cell culture, PPAR δ

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INTRODUCTION

Vitamin A is involved in various functions such as vision, mammalian cell developments, cell proliferation, differentiation, and embryonic development (Villarroya et al., 1999; Marill et al.,

2003; Sporn, 2012). The isoform, retinoic acid (RA), is known to be involved in energy balance control mechanisms and adipose biology (Bonet et al., 2003; McDowell, 2008). All-trans RA (ATRA) has blocked adipogenesis in numerous cell culture studies (Schwarz et al., 1997; Suryawan and Hu, 1997; Ohyama et al., 1998; García-Rojas et al., 2010). To induce adipogenic-related gene expression, PPAR γ , an adipogenic differentiation regulator, must form a heterodimer with retinoid X receptor (RXR). The dimerization can be inhibited by the ATRA activation of retinoic acid receptor (RAR) (Villarroya et al., 1999; Bonet et al., 2003).

In skeletal muscle, ATRA appears to act via PPAR δ and is closely related to oxidative metabolism (Muio et al., 2002). Amengual et al. (2008) reported that ATRA increased gene expression linked to fatty acid oxidation. Tanaka et al. (2003) demonstrated that PPAR δ played an important role in fatty acid catabolism through the regulation of gene expression related to fatty acid transportation, β -oxidation, and mitochondrial respiration in L6 myotubes.

A variety of muscle fiber types exist consisting of 11 different myosin heavy chain isoforms. Of the 11 isoforms, bovine skeletal muscle only expresses 3, MHC I, IIA, and IIX, while other species also express MHC IIB (Chikuni et al., 2004). Muscle fiber types are broadly classified as type I (slow-twitch) and type II (fast-twitch), based on the heterogeneity of myosin heavy chain (MHC) expression. Type I muscle fibers possess an increased quantity of mitochondria to support oxidative metabolism, compared to type IIX muscle fibers that prefer to use glycolytic metabolism, while type IIA are intermediate (Booth and Thomason, 1991; Olson and Williams, 2000). The transition between fiber types is mediated by several factors such as peroxisome PPAR γ coactivator (PGC)-1 α and PPAR δ (Naya et al., 2000; Lin et al., 2002; Wang et al., 2004). We hypothesized that ATRA may change the muscle fiber type through the PPAR δ pathway. Therefore, this study was designed to investigate how different doses of ATRA impact gene expression and protein level related to muscle growth, energy metabolism, and muscle fiber types.

MATERIALS AND METHODS

Bovine Satellite Cell Isolation

Bovine satellite cells were isolated from cross-bred steers ($n = 2$ steers), aged approximately 24

mo, for 3 separate assays. Cattle were harvested in the Texas Tech University Meat Science laboratory (Lubbock, TX) under USDA inspection. The animal was rendered unconscious via captive bolt stunning, followed by exsanguination. The protocol for the isolation and cultivation of bovine satellite cells was conducted in reference to Johnson et al. (1998). Approximately 800 g of semimembranosus muscle tissue were collected using a sterile knife and immediately transported to the Texas Tech University Muscle Biology laboratory (Lubbock, TX). The satellite cell isolation procedure was conducted under aseptic conditions. Connective tissue, blood vessels, and adipose tissue were removed from the semimembranosus muscle, which was then passed through a sterile meat grinder. The ground muscle was placed into tubes and incubated with 0.1% of Pronase (Calbiochem, LaJolla, CA) in Earl's Balanced Salt Solution (EBSS; Sigma Aldrich, St. Louis, MO) in a shaking water bath for 1 h at 37°C. After incubation, the mixture was centrifuged at $1,500 \times g$ for 4 min at room temperature. The resulting pellet was suspended in phosphate buffered saline (PBS; Sigma Aldrich), and the suspension was centrifuged at $500 \times g$ for 10 min at room temperature. The supernatant was removed and then centrifuged at $500 \times g$ for 10 min at room temperature to form a pellet of mononucleated cells. The PBS wash and centrifugations were repeated twice more. The resulting cells were seeded in 6-well plates (growth area = 9.5 cm²) at a density of 1×10^4 in warm (37°C) Dulbecco's Modified Eagle's medium (DMEM; Gibco, Waltham, MA) with 10% fetal bovine serum (FBS; Thermo Fisher science, Waltham, MA), and $1 \times$ Antibiotic-Antimycotic (Gibco) at 37°C under a humidified atmosphere of 95% O₂ and 5% CO₂.

Bovine Satellite Cell Culture

Upon reaching 80% to 90% confluence, the growth medium was replaced by differentiation medium composed of DMEM, 2% horse serum and $1 \times$ Antibiotic-Antimycotic with doses of: 0, 1, 10, 100, and 1,000 nM of ATRA. Once the incubation media was changed from proliferation media (10% FBS with DMEM) to differentiation media (2% HS with DMEM), satellite cells immediately withdrew from the cell cycle and initiated myogenic differentiation. Typically, the process of myogenic differentiation from satellite cells is completed after 72 h of incubation with differentiation media and many myotubes are apparent (Will et al., 2015). Therefore, the cells were harvested 96 h after

differentiation for RNA and protein analysis. Cells used in this experiment were isolated from 2 steers, and cells from each steer were used in 3 separate assays; therefore, the assay was performed 6 times for each dose of ATRA.

Real-Time PCR Sample Preparation and RNA Isolation

The RNA from cultured bovine satellite cells was isolated with 500 μ l of iced buffer containing acid guanidinium thiocyanate–phenol–chloroform extraction reagent (Sigma, St. Louis, MO). The concentration and purity of RNA was determined with a spectrophotometer at an absorbance of 260 nm and 280 nm using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). An acceptable range of 1.76 to 2.05 was used for the 260/280 ratio. Genomic DNA removal and cDNA synthesis were performed using QuantiTect reverse transcription kit (Qiagen, Germantown, MD) according to manufacturer recommendations. Real-time quantitative polymerase chain reaction (PCR) (7900HT Real-Time PCR System, Applied Biosystems, Foster city, CA) was used to measure the quantity of the genes of interest relative to the quantity of *ribosomal protein subunit 9* (*RPS9*) mRNA in total RNA (Table 1). Expression of *RPS9* was not different across bovine tissues and widely used as a housekeeping gene (Bionaz and Loo, 2007; Janovick-Guretzky et al., 2007). Therefore, *RPS9* was used as the endogenous control in order to normalize the expression of genes. Measurement of the relative quantity of the cDNA of interest was carried out using TAMRA PCR Master Mix (Applied Biosystems) using the appropriate forward and reverse primers, and cDNA mixture (Table 1). Assays were performed in triplicate and using the thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min at 60°C). Titration of mRNA primers against increasing amounts of cDNA yielded linear responses with slopes between -2.8 and -3.0 . Real-time quantitative (RQ) values, based on $\Delta\Delta CT$, were analyzed by RQ manager (Applied Biosystems).

Western Blotting

At 96 h of treatment, cultured bovine satellite cells were isolated using 200 μ L of iced buffer containing M-PER (Fisher Scientific, Fair Lawn, NJ), protease inhibitor (Roche, Switzerland), and 2 mM Na_3VO_4 (Fisher Scientific). Samples were diluted with PBS to determine concentration via Pierce BCA protein assay (Thermo Fisher Science). Protein

concentration was determined using a NanoDrop 1000 spectrophotometer (NanoDrop products) at 562 nm. Samples were then loaded onto Novex 4–12% Bis-Tris gels (Invitrogen, Grand Island, NY) and separated by gel electrophoresis for 35 min. Following separation, the proteins were transferred to a nitrocellulose membrane (Invitrogen). Membranes were incubated with blocking solution containing non-fat dry milk (BIO RAD, Hercules, CA) and $1\times$ Tris-buffered saline (TBS) to prevent nonspecific binding. The membranes were then incubated with primary antibodies: anti-PPAR δ , rabbit polyclonal, dilution of 1:1,000 (Abcam Inc., Cambridge, MA), anti-PPAR γ , rabbit polyclonal, dilution of 1:1,000 (Abcam Inc.), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rabbit polyclonal, dilution of 1:1,000 (Cell Signaling Tech., Danvers, MA) for 20 h at 4°C. For the long incubation western blot analysis, GAPDH was used as a control protein (Dittmer and Dittmer, 2006). The secondary antibody Alexa-Fluor 633, goat anti-rabbit, dilution of 1:1,000 was added and incubated for 1 h at room temperature in the absence of light. Band density was visualized and quantified using Imager Scanner II and Image Quant TL software (GE healthcare, UK). A molecular weight standard (Precision Plus Protein, All Blue Standards, BIO RAD, Hercules, CA) was used for reference to quantify density of proteins. Band density was normalized according to the GAPDH content.

Immunohistochemistry

Cells were grown on 3-well microscopy glass slides (Ibidi, Fitchburg, WI) for 96 h after inducing differentiation with previously described differentiation media and treatment doses. Slides were washed with PBS and then fixed with 4% paraformaldehyde (Thermo Fisher Scientific) for 10 min at room temperature. Cells were then washed 3 times with PBS. In order to prevent nonspecific background staining, fixed cells were incubated in the blocking solution containing 2% bovine serum albumin (Thermo Fisher Scientific), 5% of horse serum (Thermo Fisher Scientific), and 0.2% triton X-100 in PBS for 30 min at room temperature. Fixed cells were incubated with the primary antibody at 4°C overnight. The following primary antibodies were used: anti-PPAR δ (rabbit polyclonal, dilution 1:100; Abcam Inc.), anti-PPAR γ (rabbit polyclonal, dilution 1:100; Abcam Inc.).

Bodipy (558/568) phalloidin along with appropriate secondary antibody (both Thermo Fisher

Table 1. Primer and probe sequences for the gene expression analysis

Item ¹	Sequence (5'–3')
<i>Akt</i>	
Forward	TTGCCCATAACTAAGCCTACATCTC
Reverse	CATGCGTGCCATTTGTTGAC
TaqMan Probe	6FAM- TGCCCCAGCAACAC-TAMRA
<i>AMPKα</i>	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTTTGGATTTCTG
TaqMan Probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
<i>GLUT4</i>	
Forward	CCTCGGCAGCGAGTCACT
Reverse	AAACTGCAGGGAGCCAAGAA
TaqMan Probe	6FAM-CCTTGGTCCTTGGCGTATTCTCCGC-TAMRA
<i>LPL</i>	
Forward	CGGACTCCAACGTCATCGT
Reverse	GCTTGGTGTACCCTGCAGACA
TaqMan Probe	6FAM-TCACGGGCCCAGCAGCATTATCC-TAMRA
<i>MHC I</i>	
Forward	CCCCTTCTCCCTGATCCACTAC
Reverse	TTGAGCGGGTCTTTGTTTTTCT
TaqMan Probe	6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA
<i>MHC IIA</i>	
Forward	GCAATGTGGAACGATCTCTAAAGC
Reverse	GCTGCTGCTCCTCCTCCTG
TaqMan Probe	6FAM-TCTGGAGGACCAAGTGAACGAGCTGA-TAMRA
<i>MHC IIX</i>	
Forward	GGCCCACTTCTCCCTCATTC
Reverse	CCGACCACCGTCTCATTCA
TaqMan Probe	6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA
<i>Myogenin</i>	
Forward	AGAAGGTGAATGAAGCCTTCGA
Reverse	GCAGGCGCTCTATGTACTGGAT
TaqMan Probe	6FAM-CCCAACCAGAGGCTGCCCAAAGT-TAMRA
<i>PPARγ</i>	
Forward	ATCTGCTGCAAGCCTTGGA
Reverse	TGGAGCAGCTTGGCAAAGA
TaqMan Probe	6FAM-CTGAACCACCCGAGTCCTCCCAG-TAMRA
<i>PPARδ</i>	
Forward	GCCCTTCAGTGACATCATTGAG
Reverse	CAGGTCACGTGCATCAAGTTCCA
TaqMan Probe	6FAM-CCAAGTTCGAGTTTGCCGTCAAGTTCAA –TAMRA
<i>RPS9</i>	
Forward	GAGCTGGGTTTGTGCGAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA
<i>SMAD3</i>	
Forward	CGTCCATCCTGCCTTTTCACT
Reverse	TTCTGCTCGCCCTTTTTC
TaqMan Probe	6FAM-CCCATCGTGAAGCGCCTGCT-TAMRA

¹Akt = Akt/Protein kinase B (PKB); AMPK α = adenosine monophosphate-activated protein kinase- α ; GLUT4 = glucose transporter 4; LPL = lipoprotein lipase; MHC I = myosin heavy chain I; MHC IIA = myosin heavy chain IIA; MHC IIX = myosin heavy chain IIX; PPAR γ = peroxisome proliferated activate receptor gamma; PPAR δ = peroxisome proliferated activate receptor delta; RPS9 = ribosomal protein subunit 9; SMAD3 = mothers against decapentaplegic.

Scientific) were used for labeling of F-actin, PPAR δ and γ in bovine satellite cells. Slides were then rinsed 3 times for 5 min using PBS at room temperature.

The slides were imaged at a magnification of 200 $\times$ using an inverted fluorescence microscope (Nikon Eclipse, Ti-E; Nikon Instruments Inc., Melville,

NY) equipped with a UV light source (Nikon Intensilight Inc.; C-HGFIE). The NIS Elements imaging software was used to color all images.

Statistical Analysis

Data were analyzed as a completely randomized design via the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Each level of ATRA was replicated 6 times. When a significant preliminary *F*-test was detected, ATRA doses were separated and denoted to be different using the pairwise comparisons option of SAS. All results are reported as least-squares means. An α level of 0.05 was used to determine significance, with tendencies discussed at *P*-values between 0.05 and 0.10.

RESULTS

Within 24 h of incubation in differentiation media, a succession of primary myotubes was observed. After 72 h of incubation, satellite cell-derived myogenic cells formed thicker myotubes. No differences were detected for mRNA expression of *Akt* ($P > 0.20$) or *AMPK α* ($P > 0.20$) (Fig. 1A and B). The mRNA expression of *LPL* was altered ($P < 0.05$) with increasing quantities of ATRA (Fig. 1D). The use of 100 nM and 1,000 nM ATRA doses resulted in greater ($P < 0.05$) mRNA expression of *LPL* compared to all other doses. The mRNA expression of *GLUT4* was altered ($P < 0.05$) by treatment (Fig. 1C). The use of 1 nM ATRA resulted in greater ($P < 0.05$) mRNA expression of *GLUT4* over 100 nM ATRA, while all other doses were intermediate ($P > 0.20$). The mRNA expression of *SMAD3* increased ($P < 0.05$) with ATRA (Fig. 1E). The use of 1,000 nM ATRA resulted in greater ($P < 0.05$) mRNA expression of *SMAD3* over CON, while all other doses of ATRA were intermediate and did not differ ($P > 0.20$) from CON or 1,000 nM ATRA.

The mRNA expression of *PPAR δ* increased ($P < 0.05$) with ATRA (Fig. 2A). The use of 1,000 nM ATRA resulted in the greatest ($P < 0.05$) mRNA expression of *PPAR δ* compared to all other doses. Additionally, the use of 100 nM ATRA resulted in greater ($P < 0.05$) mRNA expression of *PPAR δ* compared to CON, 1 nM, and 10 nM ATRA doses. Expression of *PPAR γ* mRNA was affected ($P < 0.05$) by treatment (Fig. 2B). The use of 1,000 nM ATRA resulted in the greatest ($P < 0.05$) mRNA expression of *PPAR γ* compared to all other doses. Protein level of *PPAR δ* was affected ($P < 0.05$) by ATRA (Fig. 2C and E). The use of 1,000 nM ATRA resulted in a greater ($P <$

0.05) protein level of *PPAR δ* compared to CON. However, no difference ($P > 0.20$) in protein level of *PPAR γ* (Fig. 2D) was detected in the 1,000 nM ATRA dose vs. control.

Immunostaining of PPAR isoforms was performed to determine the effect of 1,000 nM ATRA. Cells for immunohistochemistry of *PPAR δ* (Fig. 3) and *PPAR γ* (Fig. 4) were harvested and fixed after 96 h of incubation in differentiation media or with 1,000 nM ATRA.

No difference was detected ($P > 0.20$) for mRNA expression of *myogenin* (Fig. 5D) due to ATRA treatment.

The mRNA expression of *MHC I* was affected ($P < 0.05$) by treatment with ATRA (Fig. 5A). The use of 10 nM ATRA resulted in greater ($P < 0.05$) mRNA expression of *MHC I* compared to CON, while 1 nM, 100 nM, and 1,000 nM did not differ ($P > 0.20$) from CON or 10 nM doses. The mRNA expression of *MHC IIA* was influenced ($P < 0.05$) by treatment with ATRA (Fig. 5B). The use of 10 nM ATRA resulted in greater ($P < 0.05$) mRNA expression of *MHC IIA* compared to 100 nM ATRA, while CON, 1 nM, and 1,000 nM did not differ ($P > 0.20$) from 10 nM or 100 nM doses. The mRNA expression of *MHC IIX* was modified ($P < 0.05$) by ATRA treatment (Fig. 5C). The use of 100 nM and 1,000 nM ATRA resulted in reduced ($P < 0.05$) mRNA expression of *MHC IIX* compared to CON. The addition of 100 nM ATRA resulted in a decrease ($P < 0.05$) in mRNA expression of *MHC IIX* compared to 1 nM and 10 nM ATRA doses. The 1,000 nM ATRA dose resulted in decreased ($P < 0.05$) mRNA expression of *MHC IIX* compared to CON, while 1 nM and 10 nM ATRA doses were intermediate, not differing ($P > 0.20$) from CON or 1,000 nM ATRA.

DISCUSSION

According to USDA, maturity and intramuscular adipose tissue (marbling) are 2 important factors used to determine quality grade (USDA, 1996). Intramuscular adipose tissue in cattle is located between muscle bundles within perimysial connective tissue and closely related to overall meat palatability (Smith et al., 2004). Vitamin A is known as an inhibitor of intramuscular adipose tissue accumulation in beef cattle (Oka et al., 1998; Pyatt and Berger, 2005; Arnett et al., 2008). In countries where marbling is the most critical selection criteria in beef for consumers, like Japan and South Korea, cattle are commonly fed grain-based diets without added vitamin A or β -carotene (Smith and Lunt, 2007). Vitamin A isoforms, especially ATRA, have

Figure 1. Expression of energy metabolism-related genes relative to RPS9. All-trans retinoic acid (ATRA) did not affect gene expression of (A) protein kinase B (*Akt*; $P < 0.05$) or (B) AMP-kinase α (*AMPK α* ; $P < 0.05$). (C) A receptor responsible for cellular glucose uptake, *GLUT4*, was not altered ($P > 0.20$) by ATRA treatment. Gene expression related to fatty acid uptake, (D) *lipoprotein lipase* (*LPL*) ($P < 0.05$) was increased with high doses (100 and 1,000 nM) of ATRA. (E) Expression of *SMAD3* was the greatest in 1,000 nM ($P < 0.05$) of ATRA.

been reported to inhibit adipocyte differentiation in various studies including swine (Suryawan and Hu, 1997), cattle (Ohyama et al., 1998), and rodents (Schwarz et al., 1997; Mercader et al., 2006).

Two families of nuclear receptors, the RAR and the retinoid X receptor (RXR) regulate biological effects of RA. All-trans RA (ATRA), 9-cis RA, and 13-cis RA are known to be its natural ligands (Marill et al., 2003). Once RAs activate receptors, they form RAR/RXR heterodimers and control RA-responsive genes through binding DNA target sequences (Chambon, 1996; Marill et al., 2003). The inhibitory mechanism of RA on intramuscular adipose tissue accumulation occurs through

the down-regulation of transcription factor activity, such as PPAR γ , which is critical during adipogenesis. To regulate the expression of genes related to the terminal differentiation of adipose cells, PPAR γ must heterodimerize with another nuclear hormone receptor, RXR (Marill et al., 2003). Once ATRA binds to RAR with a high affinity and promotes the RAR/RXR heterodimer formation, PPAR γ cannot form the PPAR/RXR heterodimer. Therefore, ATRA is considered to play an antagonistic role in terminal differentiation of adipogenesis. The majority of PPAR γ studies have evaluated adipocytes; however, there is evidence that PPAR γ is present in and plays a significant role in other tissue

Figure 2. Expression of genes and Protein level of PPAR δ and PPAR γ when bovine satellite cells were incubated with 10 nM of all-trans retinoic acid (ATRA). (A) The greatest PPAR δ mRNA levels were detected ($P < 0.05$) in 1,000 nM followed by 100 nM ATRA. (C) ATRA increased ($P < 0.05$) PPAR δ protein level relative to glyceraldehyde 3-phosphate dehydrogenase. (B) The greatest effect ($P < 0.05$) on PPAR γ was detected with 1,000 nM of ATRA. (D) No significant difference ($P > 0.20$) in the protein level of PPAR γ was identified.

types such as skeletal muscle (Park et al., 1997; Zierath et al., 1998; Amin et al., 2010). In the current study, ATRA increased relative mRNA expression of PPAR γ , but did not change protein level in bovine skeletal muscle cells. Loviscach et al. (2000) reported that PPAR γ is present in human skeletal muscle, but has an abundance that is usually 5% or less of adipocytes. Synthetic PPAR γ ligands used in human medicine as antidiabetic compounds, such as troglitazone, rosiglitazone, and pioglitazone, stimulate glucose uptake and improve insulin sensitivity in adipocytes, hepatocytes, and skeletal

muscle cells (Tan, 2000; Deeg and Tan, 2008; Janani and RanjithaKumari, 2015). In another study, PPAR γ mRNA expression in skeletal muscle rapidly increased in response to insulin treatment (Park et al., 1997). However, ATRA acts through another PPAR isoform, PPAR δ in muscle cells. Unlike PPAR γ , both PPAR α and PPAR δ are predominant PPAR isoforms in cell types other than adipocytes (Grygiel-Górniak, 2014). Shaw et al. (2003) reported that PPAR δ plays a critical role in energy homeostasis of skeletal muscle, and ATRA has been reported as a high affinity ligand for PPAR δ .

Figure 3. Immunohistochemistry of PPAR δ expression in bovine skeletal muscle fibers. Bovine satellite cells were incubated in 2% horse serum/Dulbecco's Modified Eagle's Medium without (A) or with (B) all-trans retinoic acid. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). PPAR δ was stained with a rabbit polyclonal anti-PPAR delta (green). F-actin was visualized with Bodipy 558/568 phalloidin (yellow).

Once ATRA binds to PPAR δ , it activates transcription, promoting nuclear receptor coactivator-1 (SRC-1), but other isoforms, PPAR α and PPAR γ are not activated by ATRA. In a human myocyte culture study, a PPAR δ agonist increased fatty acid uptake and utilization, which lead to an increase in the AMP:ATP ratio in the cytosol. Because β -oxidation results in a slower rate of ATP production compared to glycolytic metabolism, the AMP:ATP increase is relative. Activation of AMPK occurred,

followed by an increase of PPAR δ in human skeletal muscle cells (Krämer et al., 2007).

Amengual et al. (2008) also demonstrated that RA treatment dose-dependently increased the capacity of skeletal muscle oxidation, via the upregulation of the gene expression of enzymes and transcription factors involved in fatty acid metabolism, including carnitine palmitoyltransferase 1 (CPT-1), acetyl-CoA carboxylase 2 (ACC2), and PPAR δ in the mice model. Similarly, a decrease

Figure 4. Immunohistochemistry of PPAR γ expression in bovine skeletal muscle fibers. Bovine satellite cells were incubated in 2% horse serum/Dulbecco's Modified Eagle's Medium without (A) or with (B) all-trans retinoic acid. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). PPAR γ was stained with a rabbit polyclonal anti-PPAR γ (red). F-actin was visualized with Bodipy 558/568 phalloidin (yellow).

in hepatic glucose output and an increase in glucose disposal indicated a shift toward oxidative metabolism in mice as a result of a PPAR δ agonist used in a study by Lee et al. (2006). In this study, ATRA upregulated the mRNA expression of PPAR δ . An increase in the level of PPAR δ protein was also detected with 10 nM of ATRA. Most genes regulated by PPAR δ are either directly, or indirectly, associated with fatty acid oxidation and lipid metabolism, including fatty acid binding protein 3 (*FABP3*), *LPL*, fatty acid transporter (*FAT*), carnitine O-palmitoyl transferase-I (*CPT-I*), long chain acylCoA dehydrogenase and uncoupling protein (*UCP-I*) (Bedu et al., 2005).

In the present study, *LPL* gene expression linearly increased with ATRA concentration, and expression was greater in doses up to 1,000 nM. Lipoprotein lipase, a tissue-bound triglyceride hydrolase in plasma lipoproteins, increases fatty acid abundance in the cytosol of muscle cells (Tan et al., 1977). The increase in *LPL* mRNA abundance augments the probability of fatty acid uptake, resulting in the cellular use of fatty acids as the primary substrate for oxidative metabolism. The gene expression of GLUT4, a membrane transporter involved in skeletal muscle glucose uptake, decreased with the addition of 100 nM of ATRA compared to 1 nM, indicating a potential

Figure 5. Expression of muscle fiber type-related genes relative to RPS9. (A) Expression of *MHC I* increased ($P < 0.05$) with all-trans retinoic acid (ATRA) treatment and the greatest effect ($P < 0.05$) was detected in 10 nM of ATRA treatment. (B) Gene expression of *MHC IIA*, associated with intermediate muscle fiber type, decreased ($P < 0.05$) with ATRA treatment. (C) Gene expression of *MHC IIX* ($P < 0.05$), associated with the most glycolytic muscle fiber type in bovine skeletal muscle, decreased with ATRA treatment. Addition of 100 and 1,000 nM decreased *MHC IIX* ($P < 0.05$) gene expression. (D) *Myogenin* expression did not change ($P > 0.05$) with ATRA treatment but can be an index of myogenic differentiation for satellite cells.

downregulation in glycolytic metabolism. These data indicated the possibility that ATRA activated fatty acid-associated metabolism in the cell and decreased glycolytic metabolism.

The protein Smad3, a member of the nine-Smad protein family, has been known to mediate downstream signaling of transforming growth factor- β (TGF- β) and other factors influencing tissue homeostasis (Kingsley, 1994; Brown et al., 2007). The specific role of Smad3 in skeletal muscle is still controversial (Ge et al., 2012). In the current study, ATRA upregulated the expression of Smad3 in bovine skeletal muscle cell cultures. Ge et al. (2011) reported that Smad3-null mice exhibited characteristics of muscle wasting, decreased satellite cell numbers, and impaired proliferation and differentiation of myoblasts. Lamarche et al. (2015) demonstrated that administration of RA promoted myogenesis through Smad 3-C/EBP β pathway in C2C12 myoblast. The transcription factor, C/EBP β , was shown to inhibit myogenic differentiation. RA increased expression of Smad 3, which binds in the

bzip region of C/EBP β , and it lead to the inhibition of C/EBP β binding to DNA. Halevy and Lerman (1993) also reported that ATRA induced differentiation of mouse myogenic C2 cells and primary chicken satellite cells into myotubes. Therefore, the ATRA-induced overexpression of Smad3 may enhance skeletal muscle regeneration.

Muscle fibers have been classified as slow-, intermediate-, or fast-twitch based on physiological characteristics determined by myosin heavy chain isoforms. In mammalian skeletal muscle, several different myosin heavy chain types (MHC I, IIA, IIX, IIB) exist, but bovine skeletal muscles consist of only 3 fiber types, MHC I, IIA, and IIX (Schiaffino et al., 1989; Chikuni et al., 2004; Choi and Kim, 2009). Type I muscle, red fibers, correspond to slow twitch, thin diameter, oxidative fibers that prefer to utilize fatty acids as a fuel source. In contrast, MHC IIX, white fibers are fast twitch, large diameter, glycolytic muscle fibers that prefer to use glucose as a fuel substrate. While MHC I and MHC IIX use either fatty

acids or glucose as primary fuel sources, MHC IIA expresses an intermediate phenotype, utilizing both fatty acids and glucose. Previous studies have demonstrated that stimulation of oxidative metabolism through PPAR δ increased the formation of oxidative muscle fibers in the mouse model (Grimaldi, 2003; Luquet et al., 2003; Feige et al., 2006). The current study indicated that ATRA treatment increased MHC I expression compared to CON, with the greatest expression resulting from 10nM of ATRA. Decreased expression of MHC IIA and MHC IIX muscle fiber types was also detected in response to ATRA treatment. The high levels of ATRA (100 nM and 1,000 nM) most effectively downregulated *MHC IIX* expression. It is reported that MHC I fibers are positively correlated with marbling content in the *longissimus* muscle of beef cattle, which directly affects palatability (Calkins et al., 1981). Oxidative muscle fibers tend to store more lipid as a fuel source for aerobic metabolism, compared to glycolytic muscle fibers (Hintz et al., 1984). Seidman and Crouse (1986) also reported that fiber type and meat tenderness have a strong correlation within the *longissimus* muscle. Glycolytic muscle fibers (MHC IIX positive) resulted in decreased tenderness of meat. Therefore, administration of ATRA may stimulate the development of more oxidative muscle fibers and potentially suppress glycolytic muscle fiber formation via MHC gene expression.

The inhibitory effect of high levels of vitamin A on animal adipose cells is controversial. Previous studies using species-diverse adipose cells indicated that RA, or its isoforms, blocks adipogenic differentiation via downregulation of adipogenic transcription factors, particularly, PPAR γ (Suryawan and Hu, 1997; Ohyama et al., 1998; Song et al., 2011). However, a considerable number of recent feeding studies indicated that utilizing various sources of vitamin A does not always negatively affect marbling accumulation in beef cattle. Studies supplementing vitamin A at 2,700 IU/kg DM (Gorocica-Buenfil et al., 2007), 2,200 IU/kg of dietary DM (Gorocica-Buenfil et al., 2008), and 3,750 IU/kg dietary DM (Pickworth et al., 2012) to feedlot steers detected no differences in intramuscular adipose tissue accumulation of the *semitendinosus*. Additionally, no differences in marbling scores were detected in those studies or studies using between 1,103 and 8,820 IU/kg of dietary DM of retinyl palmitate (Gorocica-Buenfil et al., 2007; Gorocica-Buenfil et al., 2008; Bryant et al., 2010; Pickworth et al., 2012). These studies indicated that the inhibitory activity of vitamin A on adipogenic differentiation is inconsistent between cell and animal models. Cell culture may better mimic prenatal muscle development, while animal models utilize muscles with a fixed fiber number. However, the accumulation of protein for postnatal muscle hypertrophy is dependent on the incorporation of satellite cells

Figure 6. Proposed schematic illustration of changes in skeletal muscle metabolism as a result of all-trans retinoic acid (ATRA). All-trans retinoic acid binds to PPAR δ with high affinity, activating it. Activation of PPAR δ provokes oxidative metabolism by stimulating fatty acid uptake via lipoprotein lipase (LPL). This potentially upregulates the oxidative muscle fiber formation but decreases the glycolytic muscle fiber formation.

into the muscle (Cheek, 1985). Therefore, satellite cell culture experiments provide a relevant in vitro opportunity to evaluate postnatal muscle changes at the cellular level. In skeletal muscle, vitamin A upregulated activity of PPAR δ , which is closely related to oxidative metabolism and subsequently increased MHC I positive, oxidative muscle fibers (Luquet et al., 2003; Feige et al., 2006). This may indicate that vitamin A shifts MHC toward oxidative metabolism (Fig. 6).

CONCLUSION

Our current study showed that ATRA potentially alters the profile of muscle fiber types through the PPAR δ pathway. Overexpression of PPAR δ stimulates a shift of muscle fibers towards oxidative metabolism, which in turn utilizes more fatty acids as a fuel source in muscle cells. Expression of MHC I was upregulated, while MHC IIX mRNA expression decreased. Although the inhibitory action during adipogenic differentiation manifested in other studies, ATRA may affect meat tenderness through a change in muscle fiber type. To see a practical response in animal skeletal muscle, techniques known to substantially increase circulating levels vitamin A, such as injection or encapsulated vitamin A should be utilized. If an increase in oxidative fiber type is sustained in vivo, the use of RAR antagonists should be considered to potentially increase lipid content within muscle. Further research is needed to investigate the ideal amount of vitamin A supplementation for the optimization of the production of economically valuable muscle fibers and marbling.

Conflict of interest statement. None declared.

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