CORPUS PUBLISHERS

Corpus Journal of Dairy and Veterinary Science (CJDVS)

Volume 2 Issue 2, 2021

Article Information

Received date: Sep 01, 2021

Published date: Sep 16, 2021

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DOI 10.54026/CJDVS1024

Research Article

AMP-Activated Protein Kinase Abundance and Activation is increased with the β-Adrenergic agonist Zilpaterol Hydrochloride in Muscle from Feedlot Cattle

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Abstract

The objective of this research was to evaluate the effect of Zilpaterol Hydrochloride (ZH) on myogenic or adipogenic gene and protein expression in skeletal muscle. Two feeding trials and one cell culture experiments were conducted. Semimembranosus muscle tissue was collected from steers that had been fed a diet containing 8.3 mg of ZH/kg DM for the last 0 or 20 d of the finishing period with a 3 d withdrawal period. To test the mode of action an *in vitro*model was used with, isolated bovine satellite cells isolated from muscle tissue. Real Time-QPCR (RTQPCR) was used to measure the relative mRNA abundance of Adenosine Monophosphate Protein Kinase α (AMPK), Myosin Heavy Chain (MHC) I, MHCIIA, MHCIIX, Insulin-like Growth Factor I (IGF-I), β-adrenergic receptor (βAR) 1 and 2, peroxisome proliferator-activated receptor gamma (PPARγ), and Stearoyl-CoA Desaturase (SCD). Western blotting was used to measure the relative protein abundance of AMPK and Phosphorylated-AMPK (pAMPK). No differences were detected in relative mRNA abundance of AMPK, MHCIIA, IGF-I, βAR1 and βAR2. However, MHCI, SCD, and PPARγ mRNA expression was decreased and MHCIIX mRNA increased from ZH fed cattle compared to non-ZH. For one experiment, AMPK protein expression increased, while in another experiment, AMPK phosphorylation increased with ZH fed animals. The increase in MHCIIX mRNA with ZH fed cattle indicated the start of a fiber type shift towards larger diameter fibers. This shift may have been due to increased expression and phosphorylation of AMPK. These data suggest that the shift increase in MHCIIX was likely due to the ZH administration.

Keywords: AMPK; β-adrenergic receptors;Skeletal muscle; Zilpaterol HCl

Introduction

Zilpaterol Hydrochloride (ZH) is a β_2-Adrenergic Agonist (βAA) and activates the same receptors as endogenous catecholamines [1]. Effects on energy, lipid, and protein metabolism within skeletal muscle have been reported when βAA was fed to cattle [2]. Additionally, supplementation with ZH has also been shown to cause shifts in skeletal muscle fiber type from slower-twitch more oxidative fibers to fast twitch glycolytic fibers [3]. According to Park et al. [4], the AMPK activator has been shown to cause a shift from more oxidative MHCIIA to glycolytic MHCIIX muscle fibers in pig skeletal muscle. Adenosine Monophosphate-Activated Protein Kinase α (AMPK) is an enzyme that plays a key role in energy use in mammalian cells [5]. The primary role of AMPK is to maintain energy homeostasis. Phosphorylation of AMPK is brought on by physiological stress such as glucose deprivation, ischemia, hypoxia, and oxidative stress [6]. The enzyme is a metabolic master switch and is activated when the amount of AMP is elevated and the amount of ATP is low [7]. When AMPK is activated via enzymatic phosphorylation, it causes a rapid decrease in energy storage and an increase in energy production.Examples of this are the inhibition of acetyl-CoA carboxylase, the enzyme responsible for converting acetyl-CoA into malonyl-CoA, as well as an increase in cellular glucose concentration through translocation of GLUT-4 to the cell membrane and inhibition of glycogen synthesis [8]. Additionally, it has been suggested that AMPK activation may cause a shift in the proportion of different skeletal muscle fiber types. Park et al. [4] reported that by utilizing the AMPK activator 5-Aminoimidazole-4-Carboxamide-1-b-D-Ribofuranoside (AICAR) the abundance of Myosin Heavy Chain (MHC) IIA fibers decreased and MHCIIX fibers increased.There is some evidence that βAA may stimulate the activation of AMPK through crosstalk between a secondary messaging system, activated by a βAA binding its receptor, and upstream activators of AMPK [9]. The objective of this study was to examine an increase in either AMPK or the activation of AMPK when ZH was fed to cattle. We were also interested in examining whether a shift in AMPK expression, or phosphorylation, would correspond with a shift in muscle fiber type from more oxidative to glycolytic fibers.

Materials and Methods

The experiment was conducted in Lubbock, Texas, United States of America (33°34'58"N; 101°53'18"W). Experimental procedures with cattle were in compliance with the guidelines stated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [10]. Experiments one and two were conducted using calf-fed Holstein steers. Steers from experiment one and two were fed either 0 or 8.3 mg ZH/kg DM for either 0 or 20 d, with a three d withdrawal. Experiment three consisted of cell culture studies with bovine muscle satellite cells isolated from four different crossbred steers. Pre-rigor semimembranosus muscle samples (experiment one; control (4), ZH (4) n=8, experiment two; control (7), ZH ((8) n=15) were collected (< 20 minutes post mortem), flash frozen in liquid nitrogen, and stored in dry ice. Upon arrival, semimembranosus samples were stored at-80^{0}C until protein and RNA extraction took place.

Preparation of protein samples and western blotting for experiment one

Muscle protein (n=8) was extracted using approximately 0.5 g of tissue in 2.5 mL of ice-cold extraction buffer containing the Tissue Protein Extraction Reagent (T-PER; Pierce Biotechnology, Rockford, IL), with proteinase inhibitor (Roche, Manheim, Germany), and 2 mM Na_3VO_4 (Fisher Scientific, Fair Lawn, NJ). Each sample was homogenized for 5 min with a tissue homogenizer to ensure complete tissue lysis, then transferred to micro-centrifuge tubes and centrifuged for 5 min at 14,000 x g.Supernatants were collected and protein concentrations were determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Average protein concentration of samples was 8 mg/mL. Tissue homogenates were mixed with an equal volume of 2X Standard Sodium Dodecyl Sulfate (SDS) sample loading buffer. Protein samples were denatured using equal volumes of SDS-β-mercaptoethanol and boiled for 2 min. Total protein (30 µg) was then separated by gel electrophoresis using Novex 10-20% Tris-Glycine Gels (Invitrogen, Carlsbad, CA).Gels were electrophoresed for 100 min at 125 V and 130 mA.The protein was then transferred onto a Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The PVDF membranes were blocked using the XCellSureLock™ Mini-Cell (Invitrogen, Carlsbad, CA) for 120 min at 4°C. Primary antibodies binding AMPKα (#2532, 1:1000, Cell Signaling Technology, Inc., Danvers, MA) and Glyceraldehyde 3-phosphate dehydrogenase (#9484, 1:1000, GAPDH; Abcam, Inc., Cambridge, MA) were added to the membrane in blocking buffer and incubated overnight at 4°C.Following overnight incubation, the membrane was washed three times with PBS-Tween (PBST) .The membrane was then incubated with secondary antibodies (Donkey anti rabbit HRP conjugate for AMPKα, 1:15,000, Invitrogen, Carlsbad CA; Goat anti mouse HRP conjugate for GAPDH, 1:15,000, Abcam, Inc., Cambridge, MA) for 2h.Blots were washed three times with PBST and exposed to X-ray film (Carestream Health, Rochester, NY) detection. Film was developed with enhanced chemiluminescence substrates (ELC) for HRP.The peak heights of AMPKα and GAPDH bands were quantified using a BioRad Imaging System (BioRad, Hercules, CA).

Preparation of protein samples for SDS page electrophoresis and western blotting for experiment two

Muscle protein (n=15) was extracted using approximately 1 g of tissue in 3 mL of ice-cold extraction buffer containing T-PER (Fisher Scientific, Fair Lawn, NJ), Protein Inhibitor (Roche, Manheim, Germany), and 2mM Na_3VO_4 (Fisher Scientific, Fair Lawn, NJ). Each sample was homogenized for 30 seconds with a tissue homogenizer. Homogenized samples were then centrifuged at 14,000 x g for 10 minutes at Room Temperature (RT). The aqueous layer was moved to 1.7 mL micro-centrifuge tubes and stored at-80°C before for protein quantification. Protein quantification was conducted using the Pierce BCA Assay Kit (Thermo Fisher Scientific, Waltham, MA) and absorbance was measured against a standard curve on a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Proteins from all gels were diluted 10:1 for measurement within the standard curve range of the assay. The original, undiluted protein samples were diluted to like concentrations, and average tissue concentration was approximately 11 mg/mL. Protein samples were denatured using equal volumes of SDS-β-mercaptoethanol and boiled for 2 min. Tissue protein was mixed with SDS Sample Loading Buffer (Invitrogen, Carlsbad, CA) at a ratio of 1:5 and the mixture was loaded onto gradient gels. Gradient gels (4-12%) were used for SDS-Page separation of proteins. Protein from all gels was transferred to PVDF membranes for Western blotting (BioRad). The membranes were blocked with non-fat dry milk (NFDM) base blocking agent, then primary antibodies for AMPK (Cell Signaling Technology, Inc., Danvers, MA) and pAMPK (rabbit mouse antibody, #2535, Cell Signaling Technology, Inc., Danvers, MA) were applied at a dilution of 1:1000 in TBS-Tween and incubated overnight at 4°C. Each gels run in parallel to use pan- and phos-AMPK analysis. Membranes were rinsed three times for 10 min TBS-Tween at RT, then secondary antibodies for AMPK and pAMPK were applied at a dilution of 1:2000 for 1 hr RT. Membranes were again rinsed three times for 10 min in TBS-Tween at RT, then dried and visualized to measure peak absorbance on a BioRad Imaging system with Quantity-One software (BioRad, Hercules, CA).

Muscle RNA was isolated from 1 g of tissue in 3 mL of ice-cold buffer containing TRI Reagent (Sigma, St. Louis, MO) for both experiments (experiment one; n=8, experiment two; n=15). Concentration of total RNA was determined by absorbance at 260 nm using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). TaqMan Reverse Transcription Reagents and Multiscribe (Applied Biosystems, Foster City, CA) were used to reverse transcribe 1 µg of total RNA into first strand cDNA.For cDNA synthesis, random hexamers were used as primers.The GeneAmp PCR System 9700 set according to manufacturer's instructions (25°C for 10 min, 37°C for 60 min, and 95.5°C for 5 min) was used for cDNA synthesis. One µg of cDNA was constructed from each sample and Real-Time Quantitative PCR (RTQPCR) was used to evaluate gene expression. The common genes of interest across both experiments were AMPKα, β1 adrenergic receptor (β1AR), β2AR, β3AR, MHCI, MHCIIA, MHCIIX, and insulin like growth factor-1(IGF-I). For experiment one, genes of interest also included CCAAT/Enhancer binding protein beta (C/EBPβ), peroxisome proliferator-activated receptor gamma (PPARγ), and Stearoyl-CoA Desaturase (SCD). The DNA sequence list for the primer probes used for each gene was provide (Table 1). The mRNA quantity was calculated for all genes relative to the amount of Ribosomal Protein Subunit 9 (RPS9) mRNA. Relative expression was quantified by using the 2-ΔΔCt method. All sample values were normalized against RPS9 and expressed in arbitrary units

Table 1: Sequence for bovine PCR primers and TaqMan probes for myogenic genes.

Item	Sequence (5' to 3')
β_1-adrenergic receptor (accession no. AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
β_2-adrenergic receptor (accession no. NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
β_3-adrenergic receptor (accession no. XF86961)	
Forward	AGGCAACCTGCTGGTAATCG
Reverse	GTCACGAACACGTTGGTCATG
TaqMan probe	6FAM-CCCGGACGCCGAGACTCCAG-TAMRA
AMPK-α (accession no. NM_001109802)	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTTTGGATTTCTG
TaqMan probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
MHC I (accession no. AB059400)	
Forward	CCCACTTCTCCCTGATCCACTAC
Reverse	TTGAGCGGGTCTTTGTTTTTCT
TaqMan probe	6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA
MHC IIA (accession no. AB059398)	
Forward	GCAATGTGGAAACGATCTCTAAAGC
Reverse	GCTGCTGCTCCTCCTCCTG
TaqMan probe	6FAM-TCTGGAGGACCAAGTGAACGAGCTGA-TAMRA
MHC IIX (accession no. AB059399)	
Forward	GGCCCACTTCTCCCTCATTC
Reverse	CCGACCACCGTCTCATTCA
TaqMan probe	6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA
PPARγ (accession no. NM_181024)	
Forward	ATCTGCTGCAAGCCTTGGA
Reverse	TGGAGCAGCTTGGCAAAGA
TaqMan probe	6FAM-CTGAACCACCCCGAGTCCTCCCAG-TAMRA
SCD (accession no. AB075020)	
Forward	TGCCCACCACAAGTTTTCAG
Reverse	GCCAACCCACGTGAGAGAAG
TaqMan probe	6FAM-CCGACCCCCACAATTCCCG-TAMRA
IGF-I (accession no. X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan probe	6FAM-TGCCCATCACATCCTCCTCGCA-TAMRA
RPS9 (accession no. DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

Bovine satellite cell isolation

Satellite cell isolation procedure was conducted as described previously by Johnson et al.[11]. Cattle were sacrificed by captive bolt stunning followed by exsanguination

according to standard industry procedures. Using sterile techniques, approximately 500 g were dissected from the semimembranosus muscle and transported to the cell culture laboratory. Subsequent procedures were conducted in a sterile field under a tissue culture hood. After removal of connective tissue the muscle was passed through a sterile meat grinder.The ground muscle was incubated with 0.1% pronase (CalBiochem, La Jolla, CA) in Earl's Balanced Salt Solution (EBSS) for 1 h at 37°C with frequent mixing. Following incubation, the mixture was centrifuged at 1,500 x g for 4 min, the pellet was suspended in phosphate buffered saline (PBS: 140 mM NaCl, 1 mM KH_2PO_4, 3 mM KCl, 8 mM Na_2HPO_4), and the suspension was centrifuged at 500 x g for 10 min. The supernatant fraction was centrifuged at 1500 x g for 10 min to pellet the mononucleated cells. The PBS wash and differential centrifugation was repeated two more times. The resulting mononucleated cell preparation was suspended in 4*C Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 10% (v/v) dimethylsulfoxide (DMSO) and frozen in liquid nitrogen. Cells from four different animals were stored frozen in liquid nitrogen.

Bovine satellite cell differentiation

Frozen bovine satellite cells (n=4 separate preparations) were plated on 9.62-cm^2 collagen coated culture plates for differentiation studies. At 48 h, cultures were rinsed three times with serum-free DMEM and antibiotics were added. At 96 h, cells were rinsed three times with serum-free DMEM and 3% horse serum (HS)/DMEM fusion media was added. After approximately 216 h in culture, cells fused into multinucleated myotubes.

RNA extraction and analysis for treatment cells

Bovine satellite cells were plated 2 x 10^4 cells / mL in a culture flask 6well plates (#3335, Corning, NY 14831). Relative mRNA abundance of genes in bovine satellite cell were analyzed with treatment of control and AICAR. After 96-h incubation, cells were rinsed three times with DMEM and 3% HS/DMEM was added. Zilpaterol hydrochloride, AICAR, and beta 2-agrenergic antagonist (ICI 118,551 ; 0, 0.1μM ZH, 1μM ZH, 0.1μM ICI, and 0.1μM ZH+ICI) were added to the cultures at 144 h.Total RNA was isolated using the Absolutely RNA Micro-prep Kit (Stratagene, La Jolla, CA). AICAR (0.1μM ICI) and control (non-treat AICAR) were used for MHCI, IIA, IIX analysis. TaqMan Reverse Transcription Reagents and Multiscribe (Applied Biosystems, Foster City, CA) were used to reverse transcribe 1 μg of total RNA into first strand cDNA.The concentration of RNA and cDNA synthesis was measured as previously described [12].

Statistical analysis

Data were analyzed using the MIXED procedure in SAS (SAS Institute, Cary, NC). The treatment levels were considered to be the fixed effects. LSMEANS and PDIFF functions were used to separate out the treatment means and P-values. Mean differences were considered to be significant when $P \leq 0.05$ and tendency when $0.1<P<0.05$.

Results

The mRNA levels of β1AR, β2AR, IGF-I, C/EBPβ, and MHCIIA were unaffected by ZH administration, as compared to the control (Table 2). PPARγ ($P<0.01$), SCD ($P = 0.014$), and MHCI ($P = 0.022$), mRNA levels were down regulated in response to ZH feeding, while AMPKα and MHCIIX mRNA levels tended to increase in the skeletal muscle of steers, as compared to the control (P=0.12 and 0.12, respectively).In another experiment, there was no change ($P> 0.05$) in the mRNA abundances of AMPKα, IGF-I β1AR, β2AR, MHCI or MHCIIA due to ZH feeding (Table 3). However, similar to our observations in the previous experiment, MHCIIX mRNA tended to increase with ZH administration (P=0.08). In addition to the effects on gene expression reported with ZH administration, changes to protein expression (Figures 1) and activation (Figure 2) were also observed in the current study. In experiment 1, AMPKα was increased within ZH-supplemented cattle, as compared to the control ($P < 0.05$). While for experiment 2, there was no change detected with AMPKα protein abundance for ZH and control treated cattle there was an increase in phospho-AMPKα from ZH-fed cattle as compared to control ($P < 0.05$). Bovine satellite cells treated with 0.1 and 1.0 μM ZH had increased mRNA level of IGF-I and MHCIIX ($P < 0.05$). We used the βAR antagonist ICI-118,551 to observe if the effects of ZH in BSC cultures were being mediated directly through the βAR. When BSC were treated with the ICI, the effects on IGF-I and MHCIIX gene expression observed with 0.1μM ZH were attenuated (Table 4). There were no effects on AMPKα, β1AR, β2AR, and β3AR mRNA levels in BSC cultures. Additionally, treatment with 0.1 μM ICI did not change β1AR, β2AR, and β3AR mRNA levels compare to control treatment. The ZH treatment of 20 d significantly increased muscle AMPK abundance as compared to control cattle ($P < 0.05$, Figure 1). There was numerically changed but no significance ($P > 0.05$) in AMPK protein expression between treatments (Figure 2). Zilpaterol hydrochloride had greater phosphorylation of AMPK as compared to control

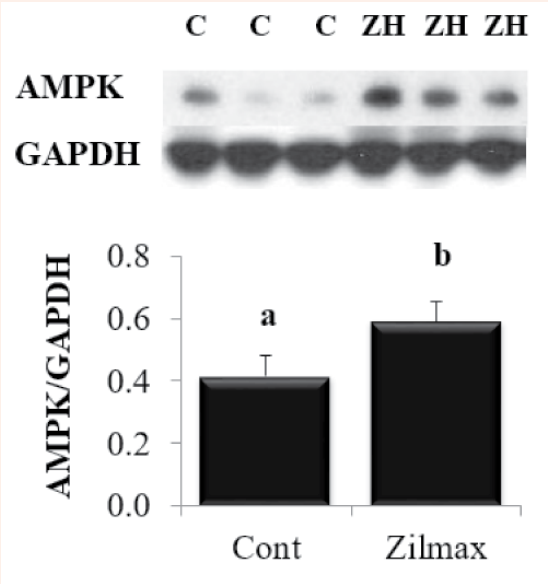

Figure 1: Western blots for Adenosine monophosphate protein kinase α (AMPK) protein abundance in skeletal muscle samples from cattle fed either 0 or 8.3 mg/kg of zilpaterol hydrochloride for 20 d (Exp. 1). Zilpaterol hydrochloride significantly increased muscle AMPK abundance as compared to control cattle ($P < 0.05$).

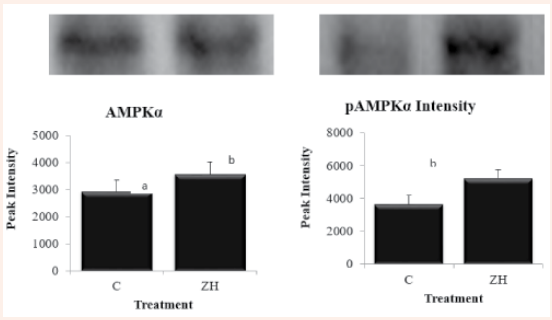

Figure 2: Muscle protein Adenosine monophosphate protein kinase α (AMPK) and phosphorylated-AMPK expression from cattle treated with either zilpaterol hydrochloride (ZH) or control (C) (Exp. 2).

($P < 0.05$), which suggested an increase in AMPK activation with ZH feeding. Dose dependent treatment of ZH increased phospho-AMPK protein abundance in myotube cultures (Figure 3). These data revealed the protein content of phospho-AMPK in ZH-treated myotube cultures was dose-dependently increased (P=0.05) relative to control. We were unable to detect differences in the total AMPK amount in myotube cultures, but there was an increase in phospho-AMPK protein.Satellite cell cultures were exposed to treatments for 48 h to determine if AICAR worked in a similar manner to MHC in bovine satellite cells (Figure 4). Relative mRNA abundance of MHCI, MHCIIA, and MHC IIX in bovine satellite cells were analyzed with treatment of control and AICAR. When bovine satellite cells reached 80% confluence cells were rinsed three times with serum-free DMEM and 3% hose serum/DMEM and 1.5μg/mL of BSA-linoleic acid plus treatments were added to the cells. The treatment of AICAR increased ($P < 0.05$) the MHC IIA and MHC IIX but not MHC I ($P > 0.05$) mRNA abundance compared to the control.

To further investigate the mechanism by which activation of β2AR was impacting skeletal muscle fiber types in bovine skeletal muscle, the extent of phosphorylation of AMPKα protein due to ZH addition in cultured bovine myotube cultures was measured. Addition of ZH to cultured myotubes at 168h in culture had a dose-dependent increase in extent of phospho-AMPKα abundance. These findings suggested that ZH was indeed impacting the extent of phosphorylation of AMPK protein and this in turn was mediating

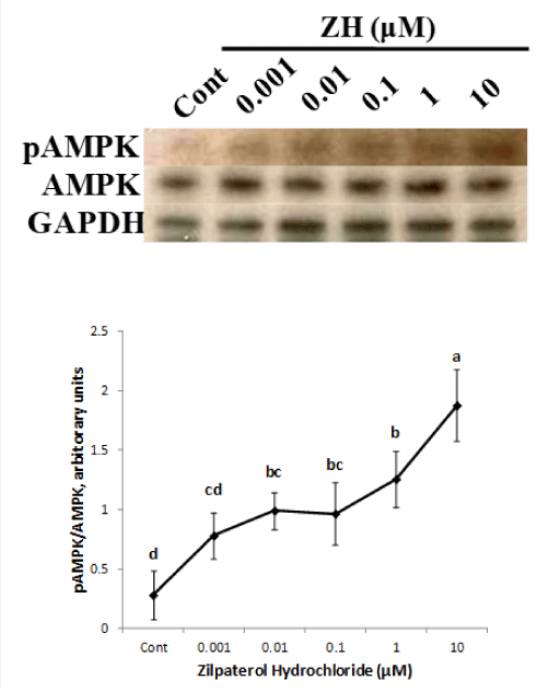

Figure 3: Western blot image of zilpaterol hydrochloride increased phospho-Adenosine Monophosphate Protein Kinase (AMPK) in myotube cultures (Exp. 3). Zilpaterol increased phospho-AMPK protein abundance in myotube cultures.

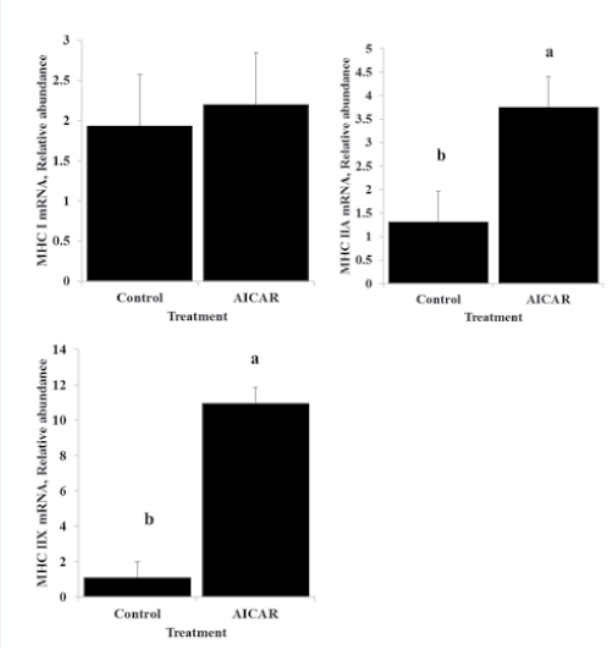

Figure 4: Relative mRNA abundance ofmyosin heavy chain (MHC)I, MHCIIA, and MHC IIX in bovine satellite cell treatment with control and 5'-aminoimidazole-4carboxamide-1-β-D-ribofuranoside (AICAR: 0.1μM). Cells were plated in 10% fetal bovine serum/Dulbecco's Modified Eagle Medium (DMEM). When bovine satellite cells reached 80% confluence cells were rinsed three times with serum-free DMEM and 3% hose serum/DMEM and 1.5μg/mL of BSA-linoleic acid plus treatments were added to the cells. Bars are mean values relative to the control ± standard error of the mean (SEM).

Table 2: Relative mRNA abundance of target genes in skeletal muscle samples from cattle fed either 0 or 8.3 mg/kg of zilpaterol hydrochloride for 20 d with 3 d withdrawal (Exp. 1).
a-b Within a row, means that do not have a common superscript differ, P<0.05.

	Treatment*			
Item†	Control	ZH	SEM	P-Value
AMPK	0.89	1.78	0.25	0.118
β1AR	1.66	1.05	0.51	0.432
β2AR	1.01	1.11	0.17	0.928
IGF-I	0.65	0.79	0.13	0.308
MHCI	0.69[a]	0.42[b]	0.07	0.022
MHCIIA	10.82	8.91	3.26	0.886
MHCIIX	0.7	1	0.18	0.121
C/EBPβ	0.69	0.46	0.16	0.504
PPARγ	2.5[a]	1.3[b]	0.46	0.009
SCD	0.79[a]	0.35[b]	0.16	0.014

*ZH – Zilpaterol hydrochloride, SEM – Standard error of the mean
†AMPK- Adenosine monophosphate kinase alpha
β1AR- Beta adrenergic agonist receptor type I
β2AR- Beta adrenergic agonist receptor type II
IGF-I- Insulin-like growth factor I
MHCI- Myosin heavy chain isoform type I
MHCIIA- Myosin heavy chain isoform type IIA
MHCIIX- Myosin heavy chain isoform IIX
C/EBPβ - CCAAT/Enhancing0binding protien beta
PPARγ - Perxisome proliferator-activated receptor gamma
SCD -Stearoyl-CoA desaturase.

Table 3: Relative mRNA abundance of target genes in skeletal muscle samples from cattle fed either 0 or 8.3 mg/kg of zilpaterol hydrochloride for 20 d with 3 d withdrawal (Exp. 2).
*ZH – Zilpaterol hydrochloride, SEM – Standard error of the mean
†AMPK- Adenosine monophosphate kinase alpha
IGF-I- Insulin-like growth factor I
βAR- Beta adrenergic agonist receptor
MHC- Myosin heavy chain isoform.

	Treatment*			
Gene†	Control	ZH	SEM	P-Value
AMPK	9.24	9.63	3.05	0.891
IGF-I	2.99	2.36	0.62	0.323
β2AR	3.19	4.11	1.42	0.532
β1AR	80.3	63.5	25.3	0.516
MHCI	1.77	1.5	0.55	0.632
MHCIIA	5.97	3.33	2.25	0.25
MHCIIX	2.79	5.03	1.22	0.08

the change of skeletal muscle fiber type.

Discussion

Little research has been conducted examining the effects of βAAs on AMPK. Adenosine monophosphate-activated protein kinase activates, in response to metabolic stress, has been well documented [4,13]. Increased AMP:ATP causes the activation of AMPK as both bind AMPK; AMP binding causes a conformational shift and presumably making it susceptible to phosphorylation by upstream kinases leading to activation. Adenosine monophosphate protein kinase α activities energy producing pathways and inactivates energy consuming pathways resulting in a net increase in cellular ATP [7]. Adenosine monophosphate protein kinase α works to increase the amount of substrate available for energy production through multiple mechanisms [14]. However, no clear pathway has been identified to explain how βAAs affect AMPK, if in fact they do. Based on the current research, it appears that feeding ZH causes an increase in both the abundance and activation of AMPK. Adenosine monophosphate-activated protein kinase phosphorylation at the Thr-172 residue increased due to supplementation with the β2AA, isoproterenol [14]. Although a different tissue type was examined, Yin et al. [14] reported

Table 4: Relative mRNA abundance of target genes in bovine satellite cell treatment with zilpaterol hydrochloride and β2 adrenergic antagonist, ICI-118,551 (Exp. 3).
*ZH – Zilpaterol hydrochloride, SEM – Standard error of the mean
†AMPK- Adenosine monophosphate kinase alpha
IGF-I- Insulin-like growth factor I
βAR- Beta adrenergic agonist receptor
MHC- Myosin Heavy Chain isoform.

	Treatment						
Gene†	Control	0.1μM ZH	1μM ZH	0.1μM ICI	0.1μM I+Z	SEM	P-Value
AMPK	1.01	1.09	1.54	1.05	0.92	0.3	0.179
IGF-I	0.84^{b}	3.69^{a}	3.04^{a}	0.93^{b}	0.88^{b}	0.68	0.001
MHCIIX	0.80^{b}	1.86^{a}	1.23^{ab}	0.77^{b}	0.83^{b}	0.32	0.008
β1AR	0.74	0.51	2.16	1.08	0.88	1.17	0.527
β2AR	0.81	0.82	1.11	0.83	0.51	0.23	0.109
β3AR	0.51	0.26	0.49	0.42	0.99	0.29	0.169

similar results in the 3T3-L1 adipocyte cell line related to the phosphorylation of AMPK. AMP-activated protein kinase phosphorylation at the Thr-172 residue increased due to supplementation with the β2AA, isoproterenol. Yin et al. [14]postulated that in order for isoproterenol to elicit maximal effects on lipolysis, AMPK had to be activated. Moule and Denton [15]reported a similar finding, stating that AMPK was activated by isoproterenol independent of cAMP activation. Additionally, Henriksson et al. [16]showed that SIK2, a member of the AMPK family, was activated by cAMP, and that activation played a role in lipid metabolism in adipocytes. These findings were similar to Berggren et al. [17] who found that PKA, which is activated by cAMP, phosphorylated SIK3 and may play a key role as a secondary messenger within adipocytes.

The role βAAs play in muscle is still unclear. Xu et al. [18] reported that β-1AAs (and not β2AAs) exert effects on AMPK in cardiac muscle. There seems to be a link between βAAs and the activation of AMPK, although, at this point, the link is not well understood. It is known thatβAAs, like ZH, bind to the β adrenergic receptor (βAR), which then activates the cAMP PKA signaling pathway [1]. The cAMP PKA signaling pathway was one of the first documented intracellular signaling pathways and is the pathway utilized by most Gs-protein coupled receptors. All of the βARs are G-protein coupled receptors [19]. Recently, Manglik et al., [20] suggested that dynamics of the βAR regulates a loose allosteric coupling of binding site and G-protein-coupling sites. The weak dynamic of varying degree of domains regulates the shapes downstream signal pathways. Although structural investigation indicated better explain for physical regulation of βAA, further investigation needs to be conducted in order to understand the relationship, and/or crosstalk between, cAMP and AMPK. While using a cell culture model, our data indicated that the combination of βAR agonist and antagonist had no effect in Bovine Satellite Cell (BSC) cultures. However, when BSC's were treated with βAR agonist, mRNA expression of MHCIIX and IGF-I were increased, while βAR antagonists actually blocked the effect of the agonist. During BSC differentiation, the expression of transcriptional factors increased at early phase but enzymatic proteins increased at the later phase of differentiation (48 h after treatment). The time difference during differentiation of BSC may increase the level of MHCIIX and IGF-I but did not affect the level of desensitized βARs. In a similar manner, a ractopamine hydrochloride (RH) triggered increase in cell number, protein, and DNA concentrations were blocked when treated with ICI [21]. Another study indicated that the treatment of ZH reduced level of βAR mRNA and protein in BSC culture [12]. In our cell culture study (Figure 4), the results indicate the induction in fast glycolytic MHCIIX and IGF-I by ZH was mediated through the βAR due to the ability of ICI-118,551 to block these effects.Muscle fiber type is linked with the myosin heavy-chain isoform expressed. Within a muscle there are multiple fiber types. The proportion of specific fiber types is driven by the purpose of that particular muscle, but muscle fiber type is not fixed and can change [22]. The characteristics that enable the classification of muscle fiber types include slow oxidative (MHCI), fast oxidative (MHCIIA) and fast glycolytic (MHCIIX, IIB) phenotypes [19]. It should be noted that bovine skeletal muscle does not express the MHCIIB muscle fiber [23]. Zilpaterol hydrochloride has been reported to cause a shift in muscle fiber type from an oxidative phenotype to more glycolytic muscle fibers in cattle [3,24]. The specific mechanism causing this shift is not well understood, but current research implicates AMPK as a major player in the mechanism of the shift. The AMPK activator, AICAR, has been shown to cause a shift from more oxidative MHCIIA to glycolytic MHCIIX muscle fibers in pig skeletal muscle [4]. According to the pig study, we hypothesized that MHC IIX mRNA levels in beef cattle would increase with the treatment of AICAR. In experiment four, increased MHC IIX mRNA abundance was observed with AICAR treatment.This is consistent with [4], who reported that MHC IIX mRNA increased in swine administered AICAR. Green et al. [25] and Suwa et al. [26] found a shift from slower oxidative fiber types to faster glycolytic fiber types types with the administration of AICAR. Park et al. [4] proposed that the shift in MHC isoforms was caused in part by AICAR activating AMPKα, a major regulator of cellular energy, through phosphorylation. The shift in MHC isoforms was especially noticed in the white fibers from the semitendinosus muscle. In the present study, the administration of AICAR was increased mRNA level of MHC IIA and MHC IIX but not affect in MHC I.

The shift observed in experiments one and two is consistent with those reported by Park et al. [4], although Park employed an AMPK activator. In the current study, the effects of ZH administration on the phosphorylation of AMPK suggest that ZH is causing an increase in AMPK activation, which could be the cause of the shift in MHC isoforms within skeletal muscle. Muscle fiber size has also been reported to increase with ZH feeding in cattle [27]. Again, the mechanism behind the hypertrophic effects of ZH is not well understood and requires further investigation. Stearoyl-CoA Desaturase (SCD) catalyzes the rate-limiting step in the biosynthesis of mono-unsaturated fatty acids from saturated fatty acids, and is expressed by both adipose tissue and skeletal muscle [28]. Stearyl-CoA desaturase mRNA expression was down regulated in muscle from ZH-treated cattle in the current study. This finding suggests that lipogenic genes are either being turned off, or down regulated due to ZH administration. The level of AMPK expression and activation via phosphorylation has previously been linked to the down regulation of SCD [29]. When SCD is down regulated, it can have an anti-steatotic effect in muscle tissue, and lead to an increase e in beta-oxidation of fatty acids [30]. This report provides further evidence to support the idea that the mode of action for βAA's, such as ZH, includes the activation of AMPK in order to elicit the observed effects on not only adipose tissue, but also on the flow and use of energy by muscle tissue. If lipid filling is reduced and β-oxidation in increased, the flow of energy is transitioning away from lipid storage, toward energy expenditure.

Further evidence for the energy repartitioning-associated effects of ZH are observable through changes in PPARγ. Peroxisome proliferator-activated receptor-γ is one member of a super family of receptors called thiazolidinediones. Peroxisome proliferator-activated receptor-γ is abundant in adipose tissue and plays a key role during cellular differentiation and maturation [31]. Activation of these receptors leads to gene transcription in many insulin target tissues including muscle [32]. Elevated PPARγ levels cause an increase in lipid storage though lipogenic gene expression [33]. Inhibition of PPARγ is known to be associated with AMPK activation via phosphorylation, and Jiang et al. [34] observed that AMPK expression and/or phosphorylation attenuated the effects of PPARγ, thereby reducing lipid synthesis. In experiment one of the present study, we observed an increase in pAMPK protein abundance and a simultaneous decrease in both PPARγ and SCD mRNA abundance after treatment with ZH. These data support the conclusion that ZH causes energy repartitioning in skeletal muscle via AMPK expression and subsequent activation. Our current findings also suggest that AMPK activation is unique to ZH. Other data from our lab indicated that RH fed at a rate of 200 mg/hd on each d did not affect AMPK or phosph-AMPK protein abundance as compared to control cattle (data not published). Little research has been conducted comparing the specific effects to MHC isoforms between RH and ZH. Avendaño-Reyes et al. [35] did compare performance and carcass changes between the two βAA. The authors findings included an advantage in loin muscle area with ZH feeding and no increase with RH supplementation. If ZH does indeed cause and activation of AMPK and through this, activation and change to the larger, glycolytic fiber types, we would expect ZH to outperform RH in loin eye area, and overall muscle growth. According to previous studies shown ZH increases loin eye area, these data support the conclusion that ZH causes energy repartitioning in skeletal muscle via AMPK expression and subsequent activation.

Conclusion

Zilpaterol hydrochloride administration in cattle was shown to increase AMPK protein abundance as well as activation. The specific mechanisms behind this activation are still not well understood and require further investigation. Also, ZH was shown to cause a shift in gene expression related to muscle fiber type from oxidative to more glycolytic fibers. This shift is consistent with the activation of AMPK. Our findings suggest that ZH is causing a change in AMPK expression, and activation.

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