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Interactive Effects of Zinc and Zilpaterol Hydrochloride on Bovine β-Adrenergic Receptors

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Abstract

The objective of this study was to determine if the addition of zinc (Zn) in combination with zilpaterol HCL (ZH) affected the interaction of ZH with the beta$_2$-adrenergic receptor (β-AR) by altering cAMP production, gene expression, and protein abundance in cultured skeletal muscle cells. Cultures of muscle bovine satellite cells were established and treated at 120 h with: 1) 0 μM Zn/zilpaterol hydrochloride (ZH; **CON**); 2) 0 μM Zn/10 μM ZH (**ZH**); 3) 1 μM Zn from Zn chloride/0 μM ZH (**Zn**); 4) 1 μM Zn from Zn chloride/10 μM ZH (**ZN/ZH**) in differentiation media for an additional 0, 6, 24, 48 and 96 h. Protein and mRNA were isolated and quantified at 24 and 96 h, and cAMP was measured at 0, 6, 24, 48 and 96 h. At 0, 24, 48 and 96 h, no differences ($P > 0.05$) were detected in cAMP production. At 6 h, Zn cells had the greatest concentration of cAMP ($P < 0.05$) compared to ZH treatments. No differences ($P > 0.05$) were detected in mRNA abundance at 24 h. At 96 h, 0 μM Zn/10 μM ZH cells had an increased abundance of myosin heavy chain (MHC)-I mRNA ($P < 0.05$) compared to CON. Furthermore, ZH had a greater abundance of MHC-IIX mRNA ($P < 0.05$) and a tendency for a greater abundance of IGF-1 mRNA ($P < 0.15$) compared to CON and ZN/ZH. No differences ($P > 0.05$) were detected in the protein abundance of β1AR and the β2AR. These results indicated Zn and ZH in combination did not have an additive effect on β_2-AR function as indicated by cAMP concentrations.

Keywords

β-Adrenergic Receptor, Zilpaterol Hydrochloride, Zinc

1. Introduction

Beta-adrenergic agonists (β-AA) are commonly used in the beef cattle feedlot

industry to improve growth performance and carcass characteristics through increased protein synthesis and decreased protein degradation [1]. Beta-adrenergic agonists have also been reported to increase lipolysis and decrease lipogenesis in adipose tissue [1] [2] [3]. These β-AA work through an interaction with the beta-adrenergic receptors (β-AR) [4] [5]. Zilpaterol HCl (ZH), a β-AA used in cattle, primarily binds with the β_2-AR, which is the most predominant β-AR found in cattle muscle and adipose tissue [1] [2]. Via a secondary messenger signal cascade event, cyclic adenosine monophosphate (cAMP) is activated thereby resulting in protein accretion and lipid catabolism [1] [2] [3].

Overstimulation of the β-ARs by β-AA has been reported to result in receptor desensitization [6] [7]. Receptor desensitization results in a down regulation of adenylate cyclase catalytic activity resulting in a reduction of cAMP synthesis [8]. When the β-ARs become desensitized, they are sequestered within an intracellular vesicle, thus losing the ability to induce signal transduction [6] [7].

Research has shown that the β_2-AR potentially have multiple allosteric binding sites for zinc (Zn) [1] [9]. Swaminath, Lee and Kobilka [10], suggested there are two main binding sites for Zn on the β-AR; one affects the agonist's ability to bind to the receptor, while the other affects the antagonist's ability to bind to the receptor thus increasing cAMP production. Zinc also regulates adenylate cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE) which are involved in the synthesis and degradation of cAMP after the β-AR is activated [11]. Several studies have reported that the catalytic activity of AC is inhibited by Zn; however, the mechanism responsible for this phenomenon is still unknown [12] [13] [14]. von Bülow, Rink and Haase [15] reported the addition of Zn to cellular lysate inhibits cyclic nucleotide degradation, signifying increases in cellular Zn will block PDE activity.

Little is known about how the combination of ZH and Zn might influence the β-AR's ability to produce cAMP, and its regulation of mRNA and protein synthesis. Thus, the objective of the present research was to determine if utilizing Zn in combination with ZH would affect the downstream signal transduction of cascade events commonly associated with β-AA thus altering cAMP activation, and mRNA and protein abundance.

2. Materials and Methods

2.1. Experimental Design and Treatments

This experiment was conducted as a 2 × 2 factorial, and each replicate (n = 4) was plated and cultured simultaneously. These experiments were conducted in 2017 in the Department of Animal and Food Sciences at Texas Tech University. The cells were treated with a laboratory grade Zn chloride (Acros Organics, Fisher Scientific, Fair Lawn, NJ), ZH, or a combination of the two. Each well was randomly assigned to one of four treatments: 1) 0 μM Zn/0 μM ZH (**CON**); 2) 0 μM Zn/10 μM ZH (ZH); 3) 1 μM Zn/0 μM ZH (**Zn**); 4) 1 μM Zn/10 μM ZH (**ZN/ZH**).

2.2. Satellite Cell Isolation

Satellite cell isolation was performed following procedures outlined by Johnson *et al.* [6]. Muscle tissue samples were extracted from the semimembranosus muscle of market age cattle at harvest. Tissue was then subjected to satellite cell extraction procedures, to isolate satellite cells from muscle tissue. Under a sterile hood, adipose and connective tissue were removed from muscle. The muscle was ground through a sterile grinder sterilized in 70% ethanol for 24 h prior to use. Ground muscle was then incubated in a solution consisting of 0.1% pronase (Calbiochem, La Jolla, CA) and Earl's Balanced Salt Solution (EBSS; Sigma, St. Louis, MO) for 1 h at 37˚C. At 10 min intervals, the samples were shaken vigorously. Following incubation, differential centrifugation at 1500 × *g* for 4 min at 25˚C was performed on the sample. The resulting supernatant was removed, and the pellet suspended in phosphate buffered saline (PBS; Invitrogen, Grand Island, NY; 140 mM NaCl, 3 mM Na_2-H-PO_4). The resulting pellet was then centrifuged at 500 × *g* for 10 min at 25˚C. The supernatant was transferred into another container and centrifuged at 15,000 × *g* for 10 min at 25˚C to form a pellet consisting of mononucleated cells. The differential centrifugation and the PBS wash steps were repeated twice. Total mononucleated cells were then suspended in cold Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Invitrogen) and 10% dimethylsulfoxide (DMSO; Sigma). The cell solution was then aliquoted into 1.8 mL nunc cryo tube vials (Fisher Scientific). Cells were then placed in a −80˚C freezer for 24 h and stored in liquid nitrogen until needed.

2.3. Satellite Cell Culture

Bovine satellite cells were cultured in 6-well plates (RNA and Protein analysis) or 24-well plates (cAMP analysis). Plates were coated with reduced factor matrigel (Matrigel; BD Biosciences, Bedford, MA) at least 1 h prior to plating cells and kept at 37˚C. Cells were plated and placed in a 37˚C incubator for 24 h in 10% Fetal Bovine Serum (FBS; GIBCO®; Invitrogen)/DMEM-3X antibiotic antimycotic (Invitrogen) 0.3X gentamycin (Sigma) media. Cells were rinsed and allowed to proliferate for 120 h in 10% FBS/DMEM-3X antibiotic antimycotic 0.3X gentamycin at 37˚C. At 120 h media was changed from proliferation to differentiation media (3% Horse Serum; GIBCO®; Invitrogen)/DMEM-3X antibiotic antimycotic 0.3X gentamycin). The treatment substrates were added to the differentiation media. Cells that were designated for mRNA and protein quantification were treated and incubated for either 24 or 96 h in differentiation media, while cells destined for cAMP analysis were treated and incubated for a total of 0, 6, 24, 48, or 96 h in differentiation media.

2.4. RNA Isolation and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

At 24 or 96 h of treatment, cells from 6-well plates were harvested for mRNA

analysis. The cells were rinsed 3 times in PBS, and total mRNA isolated with ice-cold buffer containing TRI Reagent® (Sigma, St. Louis, MO). Approximately 200 mL of TRI Reagent® were added to each well, and then incubated for 5 min at 25˚C. The wells were then scraped to ensure release of all cells from the bottom of the well. Homogenate was pipetted into a microcentrifuge tube, 100 µL of chloroform were added, vortexed for 30 s and incubated for 5 min at 25˚C. The sample was then centrifuged at 15,000 × *g* for 15 min that causing the samples to separate into 3 layers. The top supernatant layer was pipetted off and placed into a new microcentrifuge tube. Ice cold isopropyl alcohol (250 µL) was added to the supernatant, shaken, and incubated for 10 min at 25˚C. Samples were centrifuged at 15,000 × *g* for 10 min. The supernatant was poured off, the RNA pellet at the bottom of each tube air dried, and 500 µL of 75% ethanol was added to each tube to rinse and suspend the RNA pellet. Samples were then placed in a −80˚C freezer until needed (no longer than 3 months). Samples were removed from the freezer and thawed on ice and centrifuged at 250 × *g* for 10 min, ethanol poured off, and the pellet air dried. Nuclease free water (30 µL) was added to dissolve the RNA pellet. The concentration of RNA was determined with a spectrophotometer at an absorbance of 260 nm using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). Samples were treated with DNAse to remove any DNA contaminants using a DNA-free kit (Life Technologies). The RNA was then subjected to reverse-transcription to produce cDNA. The resulting cDNA was used for real-time quantitative reverse transcription-PCR (RT-qPCR) to measure the abundance of AMP-activated protein kinase alpha (AMPKα), beta-1 adrenergic receptor (β1AR), beta-2 adrenergic receptor (β2AR), insulin-like growth factor-I (IGF-I), myosin heavy chain (MHC)-I, MHC-IIA, MHC-IIX, C-enhancer binding protein beta (CEBPβ), G-protein coupled receptor 43 (GPR43), peroxisome proliferator-activated receptor gamma (PPARγ), and stearoyl-CoA desaturase (SCD) mRNA relative to the abundance of ribosomal protein subunit 9 (RPS9) mRNA in total RNA isolated from cells. Bovine primers and probes for AMPKα, β1AR, β2AR, IGF-I, MHC-I, MHC-IIA, MHC-IIX, GPR43, SCD, CEBPβ, and PPARγ are presented in Table 1. Assays were performed in the GeneAmp 7900HT Sequence Detection System (Applied Biosystems, Life Technologies) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95˚C and 1 min at 60˚C).

2.5. Protein Extraction and Western Blots

At 24 or 96 h of treatment, cells from 6-well plates were harvested for protein analysis. The cells were rinsed 3 times with PBS. Protein from cells was isolated with ice-cold buffer containing mammalian protein extraction reagent (M-PER; Fisher Scientific, Fair Lawn, NJ), protein inhibitor (Roche, Branchburg, NJ), and 2 mM Na_3VO_4 (Fisher Scientific). Approximately 500 µL of M-PER was added to each well and incubated for 5 min at 25˚C while shaking. The wells were then scraped to ensure all cells were released from the bottom of the well. Samples

Table 1. Sequence of bovine-specific PCR primers and TaqMan probes to be used for determination of expression of mRNA of AMPK*α*, MHC-I, MHC-IIA, MHC-IIX, IGF-I, *β*1AR, *β*2AR, *β*3AR, CEBP*β*, GPR43, GPR41, Glut4, PPAR*γ*, SCD and RPS9*.

Primer	Sequence (5' to 3')
AMPk*α* (accession #NM_001109802)	
Forward	ACCATTCTTGGTTGCTGAAACTC
Reverse	CACCTTGGTGTTTGGATTTCTG
TaqMan probe	6FAM-CAGGGCGCGCCATACCCTTG-TAMRA
MHC-I (accession #AB059400)	
Forward	CCCACTTCTCCCTGATCCACTAC
Reverse	TTGAGCGGGTCTTTGTTTTTCT
TaqMan probe	6FAM-CCGGCACGGTGGACTACAACATCATAG-TAMRA
MHC-IIA (accession #AB059398)	
Forward	GCAATGTGGAAACGATCTCTAAAGC
Reverse	GCTGCTGCTCCTCCTCCTG
TaqMan probe	6FAM-TCTGGAGGACCAAGTGAACGAGCTGA-TAMRA
MHC-IIX (accession #AB059399)	
Forward	GGCCCACTTCTCCCTCATTC
Reverse	CCGACCACCGTCTCATTCA
TaqMan probe	6FAM-CGGGCACTGTGGACTACAACATTACT-TAMRA
IGF-I (accession #X15726)	
Forward	TGTGATTTCTTGAAGCAGGTGAA
Reverse	AGCACAGGGCCAGATAGAAGAG
TaqMan probe	6FAM-GCCCATCACATCCTCCTCGCA-TAMRA
*β*1AR (accession #AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
*β*2AR (accession #NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
CEBP*β* (accession #NM_176788)	
Forward	CCAGAAGAAGGTGGAGCAACTG
Reverse	TCGGGCAGCGTCTTGAAC
TaqMan probe	6FAM-CGCGAGGTCAGCACCCTGC-TAMRA
GPR43 (accession #FJ562212)	
Forward	GGCTTTCCCCGTGCAGTA
Reverse	ATCAGAGCAGCCATCACTCCAT

Continued

TaqMan probe	6FAM-AAGCTGTCCCGCCGGCCC-TAMRA
PPARγ (accession #NM_181024)	
Forward	ATCTGCTGCAAGCCTTGGA
Reverse	TGGAGCAGCTTGGCAAAGA
TaqMan probe	6FAM-CTGAACCACCCCGAGTCCTCCCAG-TAMRA
SCD (accession #AB075020)	
Forward	TGCCCACCACAAGTTTTCAG
Reverse	GCCAACCCACGTGAGAGAAG
TaqMan probe	6FAM-CCGACCCCCACAATTCCCG-TAMRA
RPS9 (accession #DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

*AMPKα = AMP-activated protein kinase alpha, MHC-I = myosin heavy chain-I, MHC-IIA = myosin heavy chain-IIA, MHC-IIX = myosin heavy chain-IIX, β1AR = beta 1 adrenergic receptor, β2AR = beta 2 adrenergic receptor, β3AR = beta 3 adrenergic receptor, CEBPβ = C-enhancer binding protein beta, GPR43 = G-protein coupled receptor 43, GPR41 = G-protein coupled receptor 41, Glut4 = glucose transporter type 4, PPARγ = peroxisome proliferator-activated receptor gamma, SCD = stearoyl-CoA desaturase and RPS9 = ribosomal protein S9.

were centrifuged at 1500 × *g* for 15 min., separating the sample into 3 layers. The middle supernatant layer was removed and placed into a microcentrifuge tube. Protein samples were then diluted with either M-PER to determine protein concentration using the Pierce™ BCA™ protein assay (Thermo Fisher Scientific, Fairlawn, NJ). Protein concentration was then determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 562 nm. All samples were then diluted to the same concentration. Modified Wangs tracking dye was added to samples for western blot analysis. Samples were denatured with β-mercaptoethanol and incubated for 2 min at 95˚C. Samples were then loaded onto Novex 4% - 12% Bis-Tris gels (Invitrogen), and protein was separated by gel electrophoresis run for approximately 35 min at 165V and 27 mA. Proteins were transferred onto a nitrocellulose membrane (Invitrogen) for 7 min. Following transfer, the membrane were incubated with non-fat dry milk (BIO RAD, Hercules, CA), 10% 10 × Tris-buffered saline (TBS) in NanoPure water for 1 h at 25˚C to block non-specific antibody binding. The blocking solution was then removed from the membrane. The appropriate primary antibody: 1:1000 α-beta 1 AR, rabbit, IgG (abcam®, Cambridge, MA); 1:1000 α-beta 2 AR, goat, IgG (abcam) was mixed into 1 × TBS-Tween solution, added to the membrane and allowed to incubate for 2 h (β1AR) or 1 h (β2AR) at 25˚C. The membrane was then rinsed 3 times for 10 min in TBS-Tween. The appropriate Alexa fluorescent antibodies: goat α-rabbit, IgG, Alexa-Fluor 633 (Invitrogen); donkey α-goat, IgG, Alexa-Fluor 633 (Invitrogen) were then added at a dilution of

1:2000 in TBS-Tween to the membrane and incubated for 1 h at 25˚C in the absence of light. The membranes were then rinsed 3 times for 10 min in TBS-Tween in unlighted conditions. The membranes were then dried and visualized using Imager Scanner II and ImageQuant TL software. Densitometry measurements were made on the bands corresponding to β1AR and β2AR using a molecular weight standard for reference (Precision Plus Protein™ All Blue Standards; BIO RAD).

2.6. cAMP Isolation and ELISA

After 0, 6, 24, 48, and 96 h, cells from 24-well plates were harvested for cAMP analysis. Cells were rinsed 3 times in PBS. Then 100 µL of 0.1 M HCl was used to lyse the cells. Cells were incubated for 5 min at 25˚C while shaking. The wells were then scraped to ensure all cells were lysed and released from the bottom of the well. The sample was taken from the wells and placed into microcentrifuge tubes. An enzyme-linked immunosorbent assay (ELISA; Sigma, St. Louis, MO) was performed on samples to determine cAMP concentration, following instructions provided by the manufacturer. The results were read with a Spectra max 380pc plate reader and Softmax Pro software.

2.7. Statistical Analysis

Data were analyzed using the GLIMMIX procedure of SAS (v.9.3, SAS Institute; Carey, NC). The model included treatment as the fixed effect, and the Kenward-Roger adjustment was used to correct degrees of freedom. Means were separated using the LSMEANS procedure PDIFF option and considered different when $P \leq 0.05$. Tendencies for differences among treatment means were declared when $0.05 < P \leq 0.15$.

3. Results and Discussion

At 0, 6, 24, 48, and 96 h of incubation, cAMP was measured with no difference observed between treatments at 0, 24, 48, and 96 h ($P > 0.05$; Table 2). However,

Table 2. Relative cAMP concentration[1] changes in bovine skeletal muscle satellite cells treated with zinc (Zn) and zilpaterol hydrochloride (ZH).

	Treatment[2]					
Hour	Control	ZH	Zn	Zn/ZH	SEM[3]	*P*-Value
0	0.228	0.233	0.225	0.225	0.009	0.857
06	0.336[ab]	0.322[b]	0.354[a]	0.327[b]	0.010	0.028
24	0.265	0.241	0.266	0.248	0.014	0.231
48	0.211	0.201	0.203	0.198	0.009	0.590
96	0.202	0.198	0.206	0.204	0.014	0.955

[a,b]Means in the same row having different superscripts are significant at $P = 0.05$. [1]Picomoles of cAMP/ml. [2]Control = 0 µM ZH/0 µM Zn chloride, ZH = 10 µM ZH/0 µM Zn chloride, Zn = 0 µM ZH/1 µM Zn chloride, Zn/ZH = 1 µM Zn chloride/10 µM ZH. [3]Pooled standard error of the mean.

at 6 h, the ZN cells had a greater concentration of cAMP compared to ZH treatments ($P < 0.05$; **Table 2**). This is in contrast to that reported by [16], who reported no difference in cAMP concentration at 6 h between bovine satellite cells treated with Zn and ractopamine HCl (RH). In the Harris [16] study, using Zn and RH, cells treated with 1 μM Zn/10 μM RH exhibited the greatest cAMP concentration at 24 h and by 96 h the control group had a greater concentration of cAMP compared to the cells treated with RH only [16]. Ractopamine HCl is β-AA used in beef and pork production that primarily binds to β1AR [17]. Ractopamine HCl does not affect bovine cells to the extent as ZH because the majority of the β-AR are β2AR [1]. Klein, Sunahara, Hudson, Heyduk and Howlett [13] reported decreased concentrations of cAMP in N18TG2 Neurblastoma cells treated with 300 μM Zn^{2+} and forskolin or PGE_1 for 2 h. In the study, cells treated only with Zn^{2+}, resulted in no effect on cAMP concentration [13]. Swaminath, Steenhuis, Kobilka and Lee [9] reported Zn binds to the β2AR, causing increased agonist affinity and a greater production of cAMP. Swaminath, Lee and Kobilka [10] further reported multiple binding sites on the β2AR for Zn, with the most prominent binding site for Zn causing an increase in agonist binding affinity and a decrease in antagonist affinity.

Relative mRNA abundance of β1AR, β2AR, AMPKα, IGF-1, MHC-I, MHC-IIA, MHC-IIX, GPR43, SCD, CEBPβ, and PPARγ yielded no difference between treatments at 24 h ($P > 0.05$; **Table 3**). At 96 h, ZH cells tended to increase the abundance of MHC-I mRNA ($P < 0.10$; **Table 3**) compared to CON. Furthermore, ZH cells had a greater abundance of MHC-IIX mRNA ($P < 0.05$; **Table 3**) and a tendency for greater abundance of IGF-I mRNA ($P < 0.15$; **Table 3**) compared to CON and Zn/ZH. Harris [16] reported no differences in β1AR, β2AR, AMPKα, IGF-1, MHC-I, MHC-IIA, MHC-IIX mRNA abundance of bovine cells treated with Zn and RH at 24 and 96 h. Miller, Chung, Hutcheson, Yates, Smith and Johnson [2] however, reported a decrease in β1AR and β2AR mRNA abundance compared to control bovine cells, when cells were treated with 1 μM ZH. Tokach [18] found bovine cells treated with ZH increased the abundance of IGF-I mRNA; however, ZH decreased MHC-I mRNA abundance and increased MHC-IIX mRNA abundance compared to control cells at 120 h. In the current study, ZH increased the abundance of MHC-IIX mRNA ($P < 0.05$) and tended ($P < 0.15$) to increase the abundance of MHC-I mRNA compared to control cells at 96 h.

Protein abundance of β1AR and β2AR showed no difference between treatments at either 24 or 96 h ($P > 0.05$; **Table 4**). Our data support that of [16], who reported no change in β1AR and β2AR protein abundance of bovine cells treated with Zn and RH for 24 and 96 h. Miller, Chung, Hutcheson, Yates, Smith and Johnson [2] reported a decrease in β2AR protein when bovine cells were treated with varying levels of ZH compared to control cells.

Just as Zn and ZH affected protein expression in the cells, the combination also affected product signal transduction events downstream of binding. The increased binding affinity of ZH to the β2AR resulting from binding of Zn may

Table 3. Relative alterations of mRNA concentrations of AMPKα, IGF-I, MHC-I, MHC-IIA, MHC-IIX, β1AR, β2AR, GPR43, SCD, CEBPβ, and PPARγ genes in bovine skeletal muscle satellite cells treated with zinc (Zn) and zilpaterol hydrochloride (ZH).

	Treatment[2]					
Gene*[1]	Control	ZH	Zn	Zn/ZH	SEM[3]	*P*-Value
24 *Hour*						
AMPkα	0.792	0.819	1.134	0.862	0.206	0.328
IGF-I	2.394	18.566	5.474	10.172	8.642	0.339
MHC-I	34.292	40.735	46.494	43.229	36.289	0.843
MHC-IIA	92.801	208.030	81.721	59.259	84.236	0.458
MHC-IIX	39.623	76.555	31.411	36.516	55.969	0.676
β1AR	0.431	1.014	0.808	0.497	0.557	0.681
β2AR	0.611	0.342	0.613	0.583	0.219	0.550
GPR43	0.037	4.179	0.836	0.455	2.300	0.168
SCD	1.921	1.830	2.366	1.869	1.122	0.789
CEBPβ	1.051	1.421	1.528	1.836	0.648	0.944
PPARγ	0.133	1.699	0.403	0.393	0.760	0.148
96 *Hour*						
AMPkα	1.074	1.232	1.522	1.120	0.621	0.511
IGF-I	54.439	89.368	73.977	59.397	16.307	0.053
MHC-I	54.265	106.870	74.478	58.965	23.594	0.054
MHC-IIA	62.873	115.700	75.909	26.646	93.129	0.403
MHC-IIX	31.955b	90.481a	52.485ab	17.720b	18.407	0.003
β1AR	0.372	0.375	0.888	0.819	0.715	0.941
β2AR	0.295	0.253	0.418	0.331	0.216	0.876
GPR43	0.906	2.522	3.351	5.837	2.731	0.814
SCD	2.579	3.239	1.841	2.154	2.347	0.913
CEBPβ	1.626	1.523	1.529	2.235	1.108	0.593
PPARγ	0.454	0.993	1.166	1.435	0.636	0.755

[a,b]Means in the same row having different superscripts are significant at $P = 0.05$. *AMPKα = AMP-activated protein kinase alpha, IGF-1 = insulin like growth factor-1, MHC-I = myosin heavy chain-I, MHC-IIA = myosin heavy chain-IIA, MHC-IIX = myosin heavy chain-IIX, β1AR = beta 1 adrenergic receptor, β2AR = beta 2 adrenergic receptor, GPR43 = G-protein coupled receptor 43, SCD = stearoyl-CoA desaturase, CEBPβ = C-enhancer binding protein beta and PPARγ = peroxisome proliferator-activated receptor gamma. [1]Relative abundance of the AMPKα, MHC-I, MHC-IIA, MHC-IIX, β1AR, β2AR, GPR43, SCD, CEBPβ, and PPARγ genes were normalized with the RPS9 endogenous control by using the change in cycle threshold (ΔCT). [2]Control = 0 μM ZH/0 μM Zn chloride, ZH = 10 μM ZH/0 μM Zn chloride, Zn = 0 μM ZH/1 μM Zn chloride, Zn/ZH = 1 μM Zn chloride/10 μM ZH. [3]Pooled standard error of the mean.

consequently result in an inhibition of the synthesis of cAMP. The cAMP data indicated that 1μM Zn/10 μM ZH may be inhibiting the production or accelerating the degradation of cAMP. Lynch, Patson, Goodman, Trapolsi and Kimball [19] reported that Zn became inhibitory to cell growth at concentrations over

Table 4. Relative protein concentration changes of beta$_1$ and beta$_2$-adrenergic receptors (βAR) in bovine skeletal muscle satellite cells treated with zinc (Zn) and zilpaterol hydrochloride (ZH).

	Treatment[1]					
	Control	ZH	Zn	Zn/ZH	SEM[2]	*P*-Value
24 *Hour*						
β1AR[3]	22,521	22,256	21,880	21,575	2266	0.976
β2AR[3]	27,676	27,173	26,441	26,462	2741	0.961
96 *Hour*						
β1AR[3]	21,120	20,173	20,942	21,012	2582	0.980
β2AR[3]	26,203	25,588	25,514	29,001	3672	0.760

[1]Control = 0 μM ZH/0 μM Zn chloride, ZH = 10 μM ZH/0 μM Zn chloride, Zn = 0 μM ZH/1 μM Zn chloride, Zn/ZH = 1 μM Zn chloride/10 μM ZH. [2]Pooled standard error of the mean. [3]The values shown are the ratio of relative light units per second based on the intensity of the sample's protein band.

100 μM. When β-AAs bind to a β-AR, intrinsic Zn is released. With ZH having a high affinity to bind to β2ARs, which is the predominant β-AR found in beef cattle muscle and adipose tissue, and the β2AR potentially having multiple allosteric binding sites for Zn [9], the cell may be flooded with Zn from intrinsic and free sources of Zn. This may in part cause Zn to become inhibitory towards AC thus reducing the amount of cAMP produced. Since cAMP is a secondary messenger in the β-AR pathway that leads to an increase in myogenic mRNA transcription and ultimately muscle protein accretion, this could possibly explain the decreased myogenic activity we observed. However, large concentrations of Zn increase the uptake of glucose and de novo lipogenesis [19], possibly partially elucidating the reason for increased adipogenic activity observed in this study.

Based on the results of this study, we can conclude that independently, Zn and ZH positively impact myogenic synthesis; however, cAMP production, β-AR protein and mRNA abundance may not be affected by the combination of the two compounds. Increasing Zn supplementation may increase the concentration of extracellular free Zn; possibly increasing the binding affinity of the β-AA, therefore amplifying the signal transduction associated with β-AA. This amplified affect may result in over stimulation of the β-AR, thereby activating AC causing an increased release of intracellular Zn, which could negatively impact cAMP. While there is conflicting evidence on the implications between the interactions of Zn, β-AA and β-AR, these mechanisms are not fully understood, and future research should be conducted to further elucidate the molecular mechanisms that impact cellular muscle metabolism in biological processes involving Zn. Caution should be used extrapolating these *in vitro* results to expected results of feeding ZH to beef cattle.

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Conflicts of Interest

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

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