Investigation of the skeletal muscle transcriptome in lambs fed β adrenergic agonists and subjected to heat stress for 21 d[1]

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INTRODUCTION

Dietary β-adrenergic agonists (**β-AA**) are used in livestock to increase muscle protein accretion and decrease adipose deposition during the last 20 to 40 d of the finishing period (Johnson et al., 2014). These β-AA act through specific seven transmembrane receptors and are classified by the receptor isoform to which they primarily bind (Mersmann, 1998). Two β-AA are approved for use in beef cattle in the United States: ractopamine HCl (**RAC**, β1 agonist) and zilpaterol HCl (**ZH**, β2 agonist) (Johnson et al., 2014). Supplementation of β-AA increases efficiency of the animal and results in a leaner carcass (Elam et al., 2009). However, the skeletal muscle's genomic response to these treatments is not well understood. Heat stress (**HS**) has long been a major concern in the livestock industry. HS occurs when an animal's body temperature rises above its thermoneutral zone, at which point the heat load exceeds the animal's capacity for heat dissipation (Bernabucci et al., 2010), resulting in decreased feed intake and poor performance (Marai et al., 2007). Therefore, growth and production decrease during HS, affecting economically important carcass and reproductive traits. As a result, millions of dollars are lost each year due to HS (Renaudeau et al., 2012). Individually, HS and β-AA supplementation have antagonistic effects on muscle growth. However, there is a gap in understanding of the genomic mechanisms through which animals respond to these factors individually and in concert. The purpose of this study is to investigate the effects of β-AA, HS, and their interaction in skeletal muscle using transcriptomic analyses.

MATERIALS AND METHODS

Animals and experimental design

This study was approved by the Institutional Animal Care and Use Committee at the University of Nebraska–Lincoln, an AAALAC International accredited institution. Forty-nine crossbred (Columbia × Suffolk) wether lambs were acclimated to individual pens, environment, and diet. Animals were randomly assigned to one of three dietary supplements: RAC, ZH, or no supplement (control). In addition, they were assigned to one of two environmental conditions: thermoneutral (**TN**; 25 °C, 15% relative humidity) or HS (40 °C, 35% relative humidity). In total, eight lambs were subjected to each environmental condition × dietary supplement treatment, with the exception of one additional lamb in the TN control

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group. Lambs were fed for 21 d. On day 3, a biopsy (5 g) was taken from the semitendinosus muscle of the left hindlimb of each animal and flash frozen in liquid nitrogen. On d 22, all lambs were harvested at the Loeffel Meat Lab at the University of Nebraska—Lincoln.

Tissue Collection and RNA Isolation

RNA was isolated from each muscle sample following the Direct-zol RNA MiniPrep Plus kit (Zymo Research) with the addition of chloroform precipitation after homogenization in Trizol. RNA was quantified, and integrity evaluated using the Agilent Bio-analyzer. RNA samples were sent to the University of California, Davis Genome Center (Davis, CA) for QuantSeq 3′ mRNA library prep and sequencing on an Illumina HiSeq 4000.

Bioinformatic Analysis

Sequence quality was accessed (FastQC; Andrews, 2010) and poly-A tails and adapters trimmed (BBMap; Bushnell, 2016). The trimmed sequences were pseudoaligned to the Ovine Oar_v3.1 reference genome incorporating the flag—noLengthCorrection, and quantified in Salmon (Patro et al., 2017). After the removal of lowly expressed transcripts (count < 8), differential expression (**DE**) analysis was performed using limma-voom (Ritchie et al., 2015; Law et al., 2014). Transcripts with an adjusted $P \leq 0.05$ were considered significant and analyzed using Ingenuity Pathway Analysis (QIAGEN Inc.; IPA) to predict altered pathways. For transcripts without annotated gene IDs, human or mouse orthologs were input when possible.

RESULTS

An average of 4 million reads per sample was obtained. After quality control and trimming, 10,644 transcripts were observed in the data set. No interaction was found between temperature and supplement, therefore only main effects (HS vs. TN; RAC, ZH vs. control) were evaluated. Analyses showed that 435 transcripts were DE ($P \leq 0.05$) between HS and TN lambs (Table 1), and 48 were DE ($P \leq 0.05$) between ZH and control lambs (Table 2). No transcripts were DE between RAC and control lambs. In total, 78 pathways were identified as differently impacted between HS and TN lambs, and 12 pathways were identified as differently impacted between ZH and control lambs (Table 3).

Table 1. Top 10 transcripts (by Adj *P* value) altered in lambs fed ZH compared with those not supplemented

Gene name	LogFC	Adjusted *P* value
METTL21E	1.56	0.0002
PDE4B	1.19	0.0003
SLC25A25	0.98	0.0004
ENHO	1.30	0.0006
ADCK3	−0.97	0.0007
NRAP	−0.62	0.0015
PALLD	0.68	0.0015
TTC7A	1.06	0.0015
SMTNL1	−1.07	0.0015
CXADR	0.70	0.0015

Table 2. Top 10 transcripts (by Adj *P* value) altered in lambs due to heat stress

Gene name	LogFC	Adjusted *P* value
RBM3	−1.20	1.63E-7
POLR1D	0.56	3.5E-5
AHSA1	0.56	3.5E-5
CENPF	−1.28	0.0001
CCDC117	0.55	0.0001
ACOT9	−0.80	0.0004
ZBED5	−0.56	0.0004
MCM4	−0.84	0.0004
REPIN1	−1.01	0.0004
NUSAP1	−1.18	0.0004

DISCUSSION

The purpose of this study was to evaluate the interaction between β-AA and HS on lambs fed feedlot diets. These findings indicate there were no interacting effects on the semitendinosus, a widely studied hindlimb skeletal muscle. Moreover, it is worth noting that the most substantial impacts were due to HS. Interestingly, analyses of semitendinosus at harvest show only 13 of the 435 DE transcripts at day 3 remained DE after 21 d, indicating the acute response due to HS after 3 d is transient (R.M. Kubik, unpublished data) potentially due to acclimation to the environment. Substantial changes in the semitendinosus transcriptome were also observed in ZH lambs, but no changes were observed in RAC lambs. The presence of skeletal muscle response to ZH but not to RAC reflects metabolic studies in these same lambs that show skeletal muscle glucose oxidation is increased in ZH animals, but RAC did not affect muscle metabolism (Barnes

Table 3. Top five canonical pathways altered when lambs are subjected to heat stress (left) and when lambs are fed ZH compared with a control diet (right)

HS vs. TN	ZH vs. control
Cholesterol biosynthesis	Cellular effects of sildenafil
NRF2-mediated oxidative stress response	Catecholamine biosynthesis
Cell cycle: DNA damage regulation	Serotonin and melatonin biosynthesis
Protein ubiquitination pathway	Glutaryl-CoA degradation
Stearate biosynthesis	Cardiac β-adrenergic signaling

et al., 2017). Activator of HSP90 ATPase Activity (*AHSA1*) was DE in HS vs. TN, and *HSP90AA1* (activated by *AHSA1*) is a chaperone that is involved in structural maintenance, maturation, and regulation of target proteins involved in cell-cycle control. Additionally, Vihervaara et al., (2013) showed *AHSA1* increased 3-fold in cell cultures exposed to HS, which would coincide with our findings in this study. Pathway analysis shows cholesterol biosynthesis is decreased in heat-stressed animals, indicating a possible decrease in steroidogenesis. An increase in NRF2-mediated oxidative stress response was also observed. Oxidative stress can impair the response to HS and ultimately cause a delay in protein recovery (Adachi et al., 2009). Protein ubiquitination removes damaged protein within cells, a need for which increases during HS. Transcripts for genes within the ubiquitination pathway (*HSPB1, UBB, USB25, USP43*), which increase monoubiquitination and polyubiquitination, were increased in lambs under HS. Interestingly, in chronically heat-stressed lambs, there was a decrease in monoubiquitination and polyubiquitination, suggesting possible acclimation to the environment.

Of the transcripts DE in ZH vs. control, those of the greatest interest include *METTL21E*, *PDE4B*, and *SLC25A25*. *METTL21E* is a protein–lysine methyltransferase, and in cattle, it encodes a domain similar to members of the S-adenosylmethionine family (Marchler-Baueret al., 2007). Although the effect of β-AA on *METTL21E* is unstudied, it was upregulated in hypertrophied muscle associated with the callipyge mutation (Yu, 2013). *METTL21E* was upregulated in our lambs, which supports a possible functional role of *METTL21E* in muscle growth. *PDE4B* is a cAMP-specific, cyclic nucleotide phosphodiesterase that regulates the cellular concentration of cyclic nucleotides and plays a role in signal transduction (Smith et al., 2005). In a preliminary study, cultured myoblasts from steers treated with ZH showed upregulated *PDE4B* relative to controls (J.L. Petersen, unpublished data); this was also observed in the present study and points to a possible role of *PDE4B* in response to β2 supplementation. Finally, *SLC25A25*, a mitochondrial membrane solute transporter that possibly controls ATP homeostasis as a calcium-regulated shuttle, has been hypothesized to play a role in metabolic efficiency linked to muscle function (Anunciado-Koza et al., 2011). In mice lacking *SLC25A25*, ATP production required for skeletal muscle function was decreased. In our lambs fed ZH as well as in biceps femoris of cattle fed ZH (R.M. Kubik, unpublished data), *SLC25A25* was increased. These three genes are candidates for identifying specific mechanisms through which lean mass is increased due to ZH supplementation. With the transcript *DCC* DE, catecholamine biosynthesis is predicted to be downregulated in ZH fed lambs. β-AA bind to the same receptor as catecholamines. An increase in β-AA could signal a decrease in catecholamine biosynthesis. With DE in the transcripts *PDE4B*, *MYH3,* and *PRKG1*, there is a decrease in the pathway associated with the cellular effects of sildenafil (Viagra). β2-AA, as well as Viagra, are known vasodilators. In terms of muscle growth, an increase in vasodilation results in an increase in nutrient delivery and waste removal. These properties could contribute to altered muscle metabolism (Barnes et al., 2017).

IMPLICATIONS

β-AA supplementation is common in livestock production. Further, HS is both costly to the industry and impacts animal wellbeing. While no interaction between environment and supplement was identified, differentially expressed transcripts due to HS or to ZH supplementation revealed potential mechanisms through which animals respond to these treatments. Surprisingly, no impact of RAC was observed, indicating it has an alternative mode of action compared with ZH and reinforcing the need for additional molecular studies to elucidate its specific impacts. A greater

understanding of how animals respond to these supplements and environmental conditions has the potential to improve management practices and may lead to means in which to select for more efficient animals.

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