Heat stress (HS) markedly affects postabsorptive energetics and protein metabolism. Circulating urea nitrogen increases in multiple species during HS and it has been traditionally presumed to stem from increased skeletal muscle proteolysis; however, this has not been empirically established. We hypothesized HS would increase activation of the calpain and proteasome systems as well as increase degradation of autophagosomes in skeletal muscle. To test this hypothesis, lactating dairy cows (~139 d in milk; parity ~2.4) were exposed to thermal neutral (TN) or HS conditions for 7 d (8 cows/environment). To induce HS, cattle were fitted with electric blankets for the duration of the heating period and the semitendinosus was biopsied on d 7. Heat stress increased rectal temperature (1.3°C) and respiratory rate (38 breaths per minute) while it decreased dry matter intake (34%) and milk yield (32%). Plasma urea nitrogen (PUN) peaked following 3 d (46%) and milk urea nitrogen (MUN) peaked following 4 d of environmental treatment and while both decreased thereafter, PUN and MUN remained elevated compared with TN (PUN: 20%; MUN: 27%) on d 7 of HS. Contrary to expectations, calpain I and II abundance and activation and calpain activity were similar between groups. Likewise, relative protein abundance of E3 ligases, muscle atrophy F-box protein/atrogin-1 and muscle ring-finger protein-1, total ubiquitinated proteins, and proteasome activity were similar between environmental treatments. Finally, autophagosome degradation was also unaltered by HS. Counter to our hypothesis, these results suggest skeletal muscle proteolysis is not increased following 7 d of HS and call into question the presumed dogma that elevated skeletal muscle proteolysis, per se, drives increased AA mobilization.

Key words: calpains, ubiquitin, proteasome, autophagy, hyperthermia

INTRODUCTION

Frequently caused by environmental conditions, heat stress (HS) occurs when internal heat production and external heat accumulation exceeds the capacity for removal and results in a pathologic elevation of core temperature (Farag and Alagawany, 2018). Herein, HS is described as the collective symphony of physiological responses caused directly and indirectly by environment-induced hyperthermia. The US dairy industry loses approximately $1.5 billion (Key and Sneeringer, 2014) annually due to HS, which is largely driven by decreased milk quality and yield, seasonal infertility, and elevated veterinary costs (Rhoads et al., 2013). It has been previously established that HS increases blood, urine, and MUN (Shwartz et al., 2009a; Wheelock et al., 2010; Gao et al., 2017), which is generally accepted to be reflective of increased AA mobilization stemming from increased skeletal muscle proteolysis (Kamiya et al., 2006; Shwartz et al., 2009a; Wheelock et al., 2010); ostensibly to provide the substrates for gluconeogenesis and the synthesis of acute phase proteins (Baumgard and Rhoads, 2013).

Skeletal muscle makes up approximately 40% of mass in most production animals and serves as a reservoir for AA that can be mobilized in cases of nutrient deprivation largely by the calpain and ubiquitin-proteasome systems. Calpains are Ca^{2+}-dependent cysteine proteases (Goll et al., 2003) activated in response to a variety of muscle stresses including atrophy (Bartoli and Richard, 2005; Powers et al., 2007), disease (Goll et al., 2003; Bartoli and Richard, 2005; Selsby et al., 2010), tissue injury (Goll et al., 2003), aging (Powers et al., 2007), cachexia (Bartoli and Richard, 2005; Powers et al., 2007), burn injury (Murton et al., 2008), and sepsis (Smith et al., 2008). Under in vitro conditions, calpain-1 (μ-calpain) and calpain-2 (m-calpain) are activated in response to increasing Ca^{2+} concentrations and are inhibited by the endogenous calpain inhibitor, calpastatin (Hudson and Price, 2013). Calpain substrates include a variety of cytoskeletal and sarcomeric proteins and its products are frequently polypeptides...
that then become substrates for the ubiquitin-proteasome system (Goll et al., 2003). These polypeptides are ultimately tagged with ubiquitin, via a series of enzymatic steps, which culminate with ubiquitin ligases (E3s) covalently linking ubiquitin or a poly-ubiquitin chain to a target polypeptide (Goll et al., 2003; Fanzani et al., 2012). In skeletal muscle, the E3 ligases, muscle ring-finger protein-1 (Murf-1) and muscle atrophy F-box protein (MAFbx)/atrogin-1, are commonly induced during increased muscle proteolysis caused by a variety of stresses (e.g., disuse, burn injury, and so on; Bodine et al., 2001; Bartoli and Richard, 2005; Lang et al., 2007; Murton et al., 2008; Fanzani et al., 2012) and are transcriptionally regulated (Foletta et al., 2011; Bodine and Baehr, 2014). The ubiquitin tag routes the polypeptide to the proteasome for further degradation (Goll et al., 2003; Bell et al., 2016). The proteasome is a barrel-like protein complex, which identifies and degrades polypeptides with a variety of enzymatic activities (Voges et al., 1999; Reid, 2005; Bell et al., 2016). In pathological situations net mobilization of AA released from muscle can be used for hepatic acute phase protein synthesis, protein synthesis in other organs, and to support gluconeogenesis (Attaix et al., 2003).

Macroautophagy, commonly known as autophagy, is also a major proteolytic pathway used by skeletal muscle. While the ubiquitin-proteasome system is largely responsible for the degradation of short-lived proteins, the autophagy-lysosome pathway principally regulates levels of long-lived proteins and organelles (Fanzani et al., 2012). In addition, autophagy efficiently removes damaged proteins, protein aggregates, as well as dysfunctional cellular organelles (Levine and Kroemer, 2008; Sandri, 2010). During autophagy, these materials are packaged in autophagosomes, autophagosomes fuse with lysosomes, and contents are degraded (Sandri, 2010). Stressors such as hypoxia, nutrient depletion, oxidative stress, and shorter-term HS increase autophagic activity to promote cell survival (Vainshtein and Hood, 2016; Ganesan et al., 2017b; Summers and Valentine, 2020). Previous work from our laboratory indicates dysfunctional autophagy in skeletal muscle from 12 h through 7 d of HS (Brownstein et al., 2017; Ganesan et al., 2017a, 2018). Hence, increased autophagy may support proteolysis during HS, but autophagic dysregulation may not only limit skeletal muscle proteolysis but also contribute to HS-mediated skeletal muscle dysfunction.

Heat stress causes changes in circulating factors suggestive of increased skeletal muscle proteolysis or metabolic shift (Rhoads et al., 2013), which may, themselves, be reflective of underlying skeletal muscle dysfunction. For example, increased circulating lactate concentrations has been widely reported with HS (Collins et al., 1980; Kinnunen et al., 2009; Rhoads et al., 2011; White et al., 2012) and may reflect increased reliance on anaerobic glycolysis for ATP production in skeletal muscle. Likewise, circulating 3-methyl-histidine is increased with HS in poultry (Yunianto et al., 1997), rabbits (Marder et al., 1990), pigs (Pearce et al., 2013), and lactating cows (Schneider et al., 1988; Abeni et al., 2007). Circulating 3-methyl-histidine is commonly used as a biomarker of skeletal muscle proteolysis because a large proportion is packaged in skeletal muscle actin and myosin. Importantly though, 3-methyl-histidine can also be released from various cell types in the gut (Rennie and Millward, 1983), and even though under basal conditions this release may be negligible, HS-induced-intestinal remodeling and hyperpermeability (Abuajamieh et al., 2018; Mayorga et al., 2020) raise the possibility of increased release from the gut. Additional suggestive evidence of increased skeletal muscle proteolysis is the common finding of increased plasma urea nitrogen (PUN) with HS (Shwartz et al., 2009b; Pearce et al., 2013). Definitive interpretation of circulating urea is difficult and this is especially true for ruminants because rumen-derived ammonia and ammonia stemming from AA deamination are both substrates for hepatic ureagenesis (Parker et al., 1995; Milano et al., 2000; Lapierre and Lobley, 2001). Given that HS is accompanied by a catabolic endocrine footprint (i.e., increased cortisol, epinephrine, and so on; Follenius et al., 1982; Mete et al., 2012; Zheng et al., 2021) as well as metabolic stress and nutrient restriction there is a compelling rationale to suspect that hyperuricemia stems from skeletal muscle-mobilized AA. Importantly though, decreased protein synthesis or increased proteolysis in skeletal muscle from dairy cattle during HS have not been empirically established. Therefore, the objective of this investigation was to determine the extent to which HS promoted activation of the calpain and ubiquitin-proteasome systems and autophagy, common proteolytic pathways, in skeletal muscle of dairy cattle. We hypothesized that HS would activate the calpain and ubiquitin-proteasome systems and increase degradation of autophagosomes in skeletal muscle and that this activation would correspond with elevated and blood and MUN levels.

MATERIALS AND METHODS

Animal Treatment and Experimental Design

All animal work was approved by the Institutional Animal Care and Use Committee at Iowa State University. A detailed description of animal treatments
Tissue Collection and Protein Extraction

Muscle biopsies were obtained on d 7 of HS following the morning milking as previously described (Horst et al., 2019). In brief, the biopsy site was shaved and sterilized by scrubbing with betadine followed by a 70% ethanol spray. A caudal epidural was administered to each cow immediately before incision. The appropriate dosage of xylazine and lidocaine hydrochloride was based on body weight of each cow under direction of a veterinarian (0.05 mL/kg of BW of xylazine and 0.22 mL/kg of BW for lidocaine). Following confirmation of an effective epidural, an incision (~5 cm) was made to expose the semitendinosus in line with the posterior udder. A sterile biopsy punch (8 mm diameter, Miltex Inc.) was used for collection of 1–2 tissue cores. Following collection, biopsy sites were sutured, cleaned with 70% ethanol, and covered with an aerosol bandage (Allushield, Valley Vet). Muscle samples were frozen in liquid nitrogen and then stored at −80°C.

Western Blot

Whole homogenates were diluted to 4 mg/mL in 2× Laemmli buffer (Bio-Rad) containing 50 μL/mL β-mercaptoethanol and heated for 5 min at 95°C. Gels were loaded in a random order within each group by a trained technician. Ten microliters of protein (40 μg) was loaded randomly within groups into each well on 4% to 20% Mini-Protean TGX precast gels (Bio-Rad) and were separated for 30 min at 60 V at room temperature, followed by 75 min at 120 V at room temperature. Gels were transferred to a nitrocellulose membrane (Bio-Rad) for 1 h at 100 V at 4°C. Gels were stained with Ponceau S stain to ensure equal loading between samples and imaged using an Azure Biosystems c600 imaging system. The Ponceau S images were objectively quantified using total lane densitometry and both groups were similar to each other for all membranes. Membranes were washed twice in TTBS (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween20, pH 7.4) for 2 min and blocked for 1 h in 5% nonfat dehydrated milk in TTBS. Membranes were washed twice in TTBS for 2 min and primary antibodies were added to membranes rocking overnight at 4°C. Membranes were incubated in primary or secondary antibodies in TTBS or nonfat dry milk diluted in TTBS. Antibodies were diluted as follows (arranged in alphabetical order; all antibodies from Cell Signaling Technologies unless otherwise noted):

Adenosine monophosphate activated protein kinase (AMPKα; primary 1:1,000, #5832, secondary 1:2,000), phospho-AMPKα (Thr172; primary 1:1,000, #2535, secondary 1:2,000), autophagy related 16-like 1 (ATG16L1; primary 1:1,000, #8089, secondary 1:2,000 1% milk), autophagy related 12 (ATG12–5; primary 1:750, secondary 1:1,000), beclin-1 (primary 1:750 5% milk, #3495, secondary 1:1,000), calpain (primary 1:1,000, #14717, secondary 1:1,000), calpain 1 large subunit (Mu-type; primary 1:1,000, #2556, secondary 1:2,000 1% milk), calpain 2 large subunit (M-type; primary 1:000, #2539, secondary 1:2,000 1% milk), cathepsin B (primary 1:1,000, #31718, secondary 1:2,000), lysosome-associate membrane protein 2 (Lamp2; primary 1:1,000, #49067, secondary 1:2,000), microtubule-associated protein light chain (LC3 A/B; primary 1:500, #12741, secondary 1:1,000), MAFAx/atrogin-1 (Santa Cruz, primary 1:1,000 3% milk, #33782, secondary 1:2,000 5% milk), MuRF1 (Santa Cruz, primary 1:1,000, #398608, secondary 1:2,000), phosphatidylinositol 3-kinase (PI3K) class III (primary 1:1,000, #3358, secondary 1:3,000 5% milk), PTEN-induced kinase 1 (PINK1; primary...
**Protease Activity**

Calpain activity was measured using a fluorometric calpain activity assay kit (ab65308, Abcam) according to manufacturer’s instructions on a Tecan Spark fluorescent plate reader. Upon cleavage of substrate by calpain, the fluorogenic portion (7-amino-4-trifluoromethylcoumarin) was detected at a wavelength of 505 nm following excitation at 400 nm. Results are expressed as relative fluorescence units per milligram of lysate protein. Proteasome activity was measured using a 20S Proteasome Assay Kit (APT280, Millipore Sigma) according to manufacturer’s instructions. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. The free AMC fluorescence was quantified using a 380/460 nm excitation/emission filter set.

**Statistical Analysis**

For end point measures and baseline and final temperature, groups were compared using a Student’s *t*-test on GraphPad Prism 8.4.0 statistical software. Data are shown as mean ± standard error of the mean. For PUN and MUN, data were statistically analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc.). Each animal’s respective parameter was analyzed using repeated measures (represented as day within the experiment) with an autoregressive covariance structure. The random effect was cow within treatment. The model included treatment, day, treatment × day, and replication as fixed effects and periods 1 (acclimation) and 2 (environmental treatment) were analyzed separately from each other. Data were reported as least squares means ± standard error of the mean. Significance was established a priori as *P* < 0.05.

**RESULTS**

To induce HS, dairy cattle were fitted with an electric heat blanket for 7 d. A detailed report of this intervention has been previously published (Abeyta et al., 2023). In brief, baseline rectal temperature for TN and HS groups was 38.1 ± 0.1°C and 38.3 ± 0.1°C, respectively, and final temperature of TN and HS cattle following the 7-d environmental treatment was 38.2 ± 0.1°C and 39.5 ± 0.2°C, respectively, representing an approximate 1.3°C increase on d 7 of HS (*P* < 0.01). In addition, HS increased d 7 respiration rate by 38 breaths per minute (*P* < 0.01) and decreased d 7 DMI (34%; *P* < 0.01) and milk yield (30%; *P* < 0.01) relative to TN cows. Plasma urea nitrogen and MUN increased in HS cows while remaining relatively stable in TN (*P* < 0.01; Figures 1A and B), resulting in peaks on d 3 and 4 of HS for PUN and MUN (46 and 44%, respectively; *P* < 0.01). Following peak concentrations, PUN and MUN progressively decreased over time though remained elevated in HS relative to TN cows through d 7 of HS (20 and 27%, respectively; *P* = 0.04).

To determine the extent to which increased skeletal muscle proteolysis could support or drive increased AA mobilization during HS, we probed how HS altered several common proteolytic pathways in skeletal muscle. We discovered relative protein abundance of calpain I and calpain II, including activated forms, were similar between groups (Figure 2A). Calpain activity is inhibited by the endogenous inhibitor, calpastatin. Calpastatin is, itself, degraded by its client proteins, calpains I and II, and can also maintain calpain-inhibitory effects provided the inhibitory group is intact (Goll et al., 2003). We found that the banding pattern of calpastatin was similar between TN and HS groups (Figure 2A). Finally, using a fluorometric activity assay, and consistent with assessment of calpain activation via western blot, we discovered that total calpain activity was similar between groups (Figure 2B).

Calpain products are often ubiquitinated and targeted for degradation by the proteasome (Smuder et al., 2010). In skeletal muscle this is accomplished, in part, by the E3 ligases, MAFbx/atrogin-1 and Murf-1. Relative protein abundance of MAFbx/atrogin-1 and Murf1 (Figure 3A) were similar between groups as was total ubiquitinated proteins (Figure 3A). Finally, 20S proteasome activity was also similar between TN and HS groups (Figure 3B).

Autophagy is another means by which cells can degrade protein; commonly large protein aggregates and
even malfunctioning organelles. We measured markers of upstream activation of autophagy and discovered relative protein abundance of total AMPKα, pAMPKα (Thr172), and ratio of pAMPKα/total AMPKα were similar between groups (Figure 4A). Likewise, total ULK1, pULK1 (Ser555), and ratio of pULK1/total ULK1 protein abundance were similar between groups (Figure 4A). We measured markers of autophagosome nucleation and elongation and discovered that relative protein abundance of total Beclin-1, pBeclin-1, ratio of pBeclin-1/total Beclin-1, PI3 kinase class III, ATG16L1, and ATG12/5 were also similar between groups (Figure 4B). Finally, relative abundance of LC3A/B I, LC3A/B II, ratio of LC3A/B II/I, and p62, an inverse collagen of autophagosome degradation (Lippai and Löw, 2014), were similar between groups (Figure 4C). Lyosomal markers Lamp2 and Cathepsin B were also similar between groups (Figure 4C).

**DISCUSSION**

Prolonged exposure to environmental heat can lead to HS in dairy cattle. Heat stress increases circulating and MUN, which has traditionally been presumed to stem from mobilization of skeletal muscle AA via increased proteolysis. Loss of skeletal muscle AA could be caused by one of 3 biochemical scenarios: (1) increased proteolysis, (2) reduced reincorporation of AA into skeletal muscle due to decreased protein synthesis, or (3) some combination thereof. Skeletal muscle AA that are not (re)used for skeletal muscle protein synthesis can be released into circulation and used to support other systemic functions (e.g., liver-mediated synthesis of acute phase proteins; hepatic gluconeogenesis). Increased proteolysis in skeletal muscle and subsequent mobilization of AA commonly accompanies feed restriction, oxidative stress, and endotoxemia (Moylan and Reid, 2007) as occurs during HS. Proteolysis in skeletal muscle is largely driven by increased activities of the calpain system and the ubiquitin-proteasome system as well as autophagy (Smuder et al., 2018). Given increased BUN and MUN and cumulative stressors caused by HS we hypothesized that environment-induced HS would increase activation of the calpain and ubiquitin/proteasome systems and increase degradation of autophagosomes. Surprisingly, we discovered that these common mechanisms of skeletal muscle protein degradation were not activated following 7 d of HS in dairy cattle.

The calpains are cysteine proteases that are activated in response to increased cytosolic Ca\(^2+\) and inhibited by calpastatin (Goll et al., 2003). Calpains frequently degrade sarcomeric and cytoskeletal proteins, among others, that are then acted on by E3 ligases, ubiquitinated, and routed to the proteasome for more complete degradation. To achieve ubiquitination a series of enzymes, termed E1–E3 work in sequence to attach a polyubiquitin chain. Although there are a variety of these enzymes, the E3 ligases Murf1 and MAFbx/atrogin-1 are generally elevated in muscle wasting conditions (de Palma et al., 2008; Furrer and Handschin, 2019). Given the prominent role of the calpains and the ubiquitin-proteasome systems in skeletal muscle protein degradation and the common observation of increased circulating urea nitrogen during HS in dairy cattle and other species, we expected these pathways

**Figure 1.** (A) Plasma urea nitrogen (PUN) and (B) MUN during heat stress in dairy cattle. Heat stress increased PUN following 3 d of heat stress compared with a thermoneutral group. Plasma urea nitrogen remained elevated compared with a thermoneutral group through 7 d of heat stress, albeit lower than the 3-d peak. Likewise, MUN followed a similar pattern with a peak following 4 d of heat stress followed by a lower, but elevated, concentration through 7 d of heat stress compared with a thermoneutral group. *Indicates significantly (P < 0.05) different from thermoneutral group. Data are shown as LSM ± SEM.
to be strongly induced. Counter to our hypothesis, our data demonstrate that following 7 d of HS neither the calpain system nor the ubiquitin-proteasome system appear activated compared with muscle from TN animals. Of interest, 12 h of HS failed to increase calpain I transcript abundance in pectoralis muscle from broilers (Furukawa et al., 2016), though conversely, 3 w of HS increased Murf1 and MAFbx/atrogin-1 transcript in thigh muscle from broilers (Zuo et al., 2015).

In contrast to our previous results demonstrating cellular dysfunction following 7 d of HS in skeletal muscle from pigs (Ganesan et al., 2018), 7 d of HS did not promote or impair degradation of autophagosomes in this investigation. Upstream activation of autophagy was not stimulated by HS, similar to previous studies (Brownstein et al., 2017; Ganesan et al., 2018) in pigs. Autophagosome nucleation and elongation markers were similar between groups in this investigation, however, these were generally increased in skeletal muscle taken from heat-stressed pigs (Brownstein et al., 2017; Ganesan et al., 2018). Finally, in contrast to findings in porcine skeletal muscle, degradation of autophagosomes appeared to proceed normally as autophagosomes did not appear to accumulate in skeletal muscle (Brownstein et al., 2017; Ganesan et al., 2018). Lysosomal abundance was similar between groups in this investigation, and is in good agreement with results from previous studies (Brownstein et al., 2017; Ganesan et al., 2018). Conversely, autophagy was stimulated in ovaries collected from gilts following 5 d of cyclical HS (Hale et al., 2017). In total, these data indicate that activation of autophagy, autophagosome formation, and degradation of autophagosomes were not promoted following 7 d of HS in dairy cattle.

**Figure 2.** Assessment of calpain proteolysis pathway following 7 d of heat stress in skeletal muscle. (A) Calpain I, calpain II, and calpastatin were measured via western blot from whole homogenate of semitendinosus muscle (n = 8/group). Data were similar between groups. A Ponceau S stain (PonS) was used as a loading control. (B) Calpain activity was measured using a fluorometric calpain activity assay kit (ab65308, Abcam); data were similar between groups. Data are shown as LSM ± SEM.

**Figure 3.** Assessment of calpain products following 7 d of heat stress in skeletal muscle. (A) MAFbx/atrogin-1, Murf1, and total ubiquinated (UB) proteins were measured via western blot (n = 8/group). A Ponceau S stain (PonS) was used as a loading control. (B) Proteasome activity was measured using a 20S Proteasome Assay Kit (APT280, Millipore Sigma). TN = thermoneutral; HS = heat stress. Data are shown as LSM ± SEM.
Given that we were unable to detect increased activation of major proteolytic systems in heat-stressed skeletal muscle, these data call into question dogma regarding increased skeletal muscle proteolysis as the primary driver of skeletal muscle AA mobilization (O’Brien et al., 2010; Wheelock et al., 2010; Koch et al., 2016) and thus increased BUN in dairy cattle during HS. We view this as an important step toward elucidating metabolic dysregulation during HS. Importantly, our heating model increased rectal temperature and respiratory...
rate, along with a host of other changes, supporting our conclusion that animals were meaningfully heat-stressed. A limitation of our approach, however, is that we did not collect muscle temperature during this investigation allowing for the possibility, while remote, that despite elevated rectal temperature, muscle temperature remained similar to thermoneutral conditions. It is important to note that our model induced HS (increased rectal temperature, decreased feed intake, and so on) and increased PUN and MUN. Given this scenario, PUN and MUN would still be presumed to originate from muscle regardless of muscle temperature. We do note, however, that even though PUN and MUN were increased following 7 d of HS, circulating nitrogen was lower following 7 d of HS compared with 3 d of HS, though still elevated compared with thermoneutral controls. The persistent elevation of PUN and MUN is supportive of a urea nitrogen source; however, we cannot eliminate the possibility that skeletal muscle proteolysis was increased at an earlier time point and had returned to baseline conditions following 7 d of HS. Indeed, restoration of skeletal muscle proteostasis may be an early sign of acclimation. Alternatively, urea nitrogen could originate from the rumen via reduced incorporation of rumen ammonia into microbial crude protein (Baumgard and Rhoads, 2013; Cowley et al., 2015). Recent evidence indicates that HS can alter the rumen microbiome (Zhao et al., 2019); however, how this alteration may affect ammonia handling is less clear and we recognize this as an important area of future investigation. Further, milk protein synthesis is markedly reduced during HS, thus less AA are incorporated by mammary tissue, which creates a larger AA pool that needs deamination. We also cannot eliminate the possibility that a subtle change in proteolytic variables measured herein via western blot or activity assay persisted below our level of detection and contributed to increased urea nitrogen. Finally, we detected the possibility that HS changes the fate of freed AA following protein degradation in skeletal muscle without altering the rate of protein degradation, per se. In this scenario AA that might be recycled in skeletal muscle (i.e., reincorporated back into proteins) are exported and used to support other physiological processes and are detected as increased plasma, urine, or MUN following deamination in the liver. As a corollary, given undeniable alterations in PUN and the large AA content stored in skeletal muscle it is reasonable to suggest that a decreased rate of protein synthesis without a change in degradation could support increased release of AA from muscle without a change in the rate of degradation as reported herein. Utilization of labeled AA or other techniques are needed to thoroughly probe the balance of protein synthesis and degradation as well as identify AA fate during HS. Regardless, information generated herein clearly indicate that our interpretation and knowledge of postabsorptive protein metabolism during HS is inadequate. This key knowledge gap is surprising considering how important animal products are to the global protein economy.

CONCLUSIONS

Our discoveries indicate that HS did not cause increased activation of the calpain and ubiquitin-proteasome systems in skeletal muscle following 7 d of HS. Furthermore, HS did not increase degradation of autophagosomes in skeletal muscle. In total, these data do not support our hypothesis that HS increased protein degradation in skeletal muscle and do not point directly to increased skeletal muscle proteolysis as the source of increased blood, urine, and MUN observed in heat-stressed cattle. This outcome is startling considering the large pool of AA stored in skeletal muscle and may indicate that skeletal muscle release of AA is driven by decreased incorporation of AA into protein rather than an increased rate of degradation. Alternatively, increased urea nitrogen could originate from alimentary ammonia, particularly in ruminants. With aforementioned limitations in mind, we are eager to more thoroughly probe skeletal muscle proteostasis by exploring synthesis and proteolysis over the course of a long-term HS event.

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