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Short communication: Effect of heat stress on markers of autophagy in the mammary gland during the dry period

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ABSTRACT

Heat stress (HT) during the dry period compromises mammary gland (MG) growth, thus negatively affecting subsequent milk yield. Cooling during the late dry period, when mammary tissue proliferates, is a common management practice. However, it neglects MG involution during the early dry period, a process that is accomplished by both apoptosis and autophagy. Our objective was to evaluate the effect of HT on MG autophagy during the early dry period. Holstein cows were dried off ~45 d before expected calving and randomly assigned to 1 of 2 treatments: HT or cooling (CL). All cows were housed in the same free stall barn during the dry period, but only the stall area for CL cows was equipped with soakers and fans. Rectal temperature and respiration rate were measured daily during the dry period. Mammary gland biopsies were collected from each cow 3 d before dry-off and on d 3, 7, 14, and 22 ± 2 after dry-off. Autophagy in the MG was determined by measuring protein expression of 2 autophagic markers, autophagy-related protein 7 and microtubule-associated protein light chain 3 (LC3). The average temperature–humidity index during the dry period was 77.7, which indicated that HT and CL cows were exposed to significant heat stress. However, the cooling system effectively alleviated heat strain in CL cows by decreasing the rectal temperature (39.0 vs. 39.4°C) and respiration rate (47.3 vs. 71.2 breaths per minute) relative to HT cows. Protein expression of autophagy-related protein 7, a marker for early autophagosome formation, did not change within or between groups. In contrast, protein expression of LC3-II, a marker of autophagosomes, and its precursor LC3-I showed a dynamic expression pattern in MG from CL cows during the early dry period. Relative to HT cows, MG from CL cows displayed higher expression of LC3-I and LC3-II on d 7 and lower expression of LC3-II on d 14 and 22 after dry-off. Collectively, our data provide a possible mechanistic explanation for the impairment of MG capacity in HT dairy cows. Heat stress–related perturbations of autophagic activity may compromise the regenerative MG involution that is necessary for optimal cell proliferation.

Key words: autophagy, dry period, heat stress, mammary gland

Short Communication

With temperatures rising approximately 0.2°C per decade since 1980 (IPCC, 2013: Summary for Policymakers), more and more animals are at risk of being exposed to high ambient temperatures outside their thermal comfort zone for longer periods of time, especially in the southeastern United States. For dairy cows, one of the well-recognized negative effects of increased environmental temperatures and humidity is the decrease in milk production (West, 2003; Collier et al., 2006; Tao et al., 2011). One possible explanation for the detrimental effect of heat stress on lactation performance is the impaired renewal of mammary cells between lactations. We (do Amaral et al., 2009, 2011; Tao et al., 2011) and others (Wolfenson et al., 1988) have reported that heat stress abatement during the entire dry period increases subsequent milk yield, and that mammary cell proliferation is increased in parallel with cooling during the late dry period (Tao et al., 2011). Thus, it appears that environmental heat stress affects mammary gland (**MG**) remodeling, but the underlying mechanisms are not known.

During the initial phase of the dry period, the MG involutes and senescent cells are removed, which is followed by mammary cell proliferation during the late dry period in preparation for the next lactation, a process that has been reviewed in several publications (Capuco and Akers, 1999; Zarzynska and Motyl, 2008; Capuco and Ellis, 2013). Mammary involution is accomplished by both programmed cell death, namely apoptosis (Capuco et al., 1997), and autophagy (Zarzynska and Motyl, 2008). In contrast to apoptosis, autophagy,

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which literally means "self-eating," can lead to either cell death or cell survival (Codogno and Meijer, 2005). Autophagy is a major homeorhetic mechanism in which intracellular materials, such as proteins, protein aggregates, or organelles, are isolated into double-membrane vesicles called autophagosomes, which are delivered to lysosomes for degradation (Parzych and Klionsky, 2014). However, the importance of autophagy in the involuting bovine MG is still not clear but may play different and important roles depending on the stage of MG development. During mammary involution in the early dry period, autophagy increases dramatically (Zarzynska et al., 2007; Teplova et al., 2013), which may support removal of senescent cells; rescue of mammary cells, including progenitor cells, from destruction; or both (Zarzynska et al., 2007; Gajewska et al., 2013). On the other hand, during the mammary proliferation phase in the late dry period, mammary autophagy returns to baseline levels (Zarzynska et al., 2007) to facilitate the enhanced cell proliferation. Indeed, enhanced autophagy inhibits cell proliferation (Wang and Levine, 2010), whereas decreased autophagy can stimulate mammary cell proliferation (Qu et al., 2003).

Environmental heat stress can cause autophagic dysregulation. Indeed, in vitro studies using short-term heat shock demonstrated a stimulation of autophagy in a variety of cell types (Nivon et al., 2009; Zhao et al., 2009; Hsu et al., 2013). On the other hand, the disturbed hormonal profile caused by heat stress in late gestation dairy cows could result in blunted MG autophagy. For example, estrogen has a stimulatory effect on autophagy in the mammary epithelial BME-UV1 cells (Sobolewska et al., 2009). A heat stress–induced reduction in estrogen (Collier et al., 1982) could negatively affect MG autophagy during the early dry period. However, it is still unknown how heat stress affects autophagy in both mammary involuting and proliferating stages of MG development during the dry period, and this deserves further investigation.

Therefore, the present study aimed to investigate autophagic activity in bovine MG tissue during the early dry period and the effect of chronic environmental heat stress. We compared autophagic activity in MG biopsies taken before and during the dry period from cows that were either heat stressed (**HT**) or actively cooled (**CL**). Our hypothesis was that chronic heat stress perturbs autophagic activity during the dry period, thereby negatively affecting MG remodeling.

The Institutional Animal Care and Use Committee of the University of Florida approved all procedures. The study was conducted during the summer of 2014 at the University of Florida Dairy Unit in Hague, Florida. Multiparous Holstein cows were dried off approximately 45 d before the expected calving date and randomly assigned to 1 of 2 treatments, HT or CL. During the dry period, all cows were housed in the same freestall barn, but CL cows were cooled with shade, soakers, and fans, whereas HT cows were provided with shade only. Fans in CL pens were always on, and soakers were activated for 1.5 min at 5 min intervals when ambient temperature reached 21.1°C. Air temperature and relative humidity of each pen in the dry cow barn were recorded every 15 min by Hobo Pro series Temp probes (Onset Computer Corp., Pocasset, MA). The temperature–humidity index was calculated as described previously (Tao et al., 2011). Rectal temperature was measured and respiration rate was counted daily (1430 h) during the dry period. Mammary biopsies were collected from alternate rear quarters (HT, n = 8; CL, n = 8) 3 d before dry-off and 3, 7, 14, and 22 d after dry-off. The mammary biopsy procedure was previously described in detail (Wall et al., 2005; Tao et al., 2011). The biopsies were quickly rinsed with PBS, fat was trimmed off, and then samples were snap frozen in liquid nitrogen before storage at −80°C.

Autophagy in MG tissue was assessed by determining protein expression of 2 autophagic markers, autophagy-related protein 7 (**Atg7**) and microtubule-associated protein light chain 3 (MAP-LC3, subsequently referred to as **LC3**), in tissue homogenates. Over 30 proteins contribute to the highly regulated process of autophagy, during which cytoplasmic constituents are engulfed in membrane-bound autophagosomes, which ultimately fuse with a lysosome containing the degradative enzymes. The autophagy protein LC3 is essential for the expansion of the early autophagosome (Abeliovich et al., 2000) and has commonly been used as a marker for autophagic activity in the context of cellular housekeeping and autophagic cell death (Tanida et al., 2004). The precursor pre-LC3 is processed to its cytosolic form, LC3-I, subsequently activated by the autophagy protein Atg7, and lipidated to its membrane-bound form, LC3-II, which localizes to the developing autophagosome. Briefly, tissue samples were homogenized in PBS at 4°C using a Polytron PT 2100 homogenizer (Kinematica, Littau-Lucerne, Switzerland), kept on ice for 1 h, and then centrifuged (3,000 × g, 10 min, 4°C). The pellet was discarded, and the protein concentration was determined in the supernatant (Bradford Reagent, BioRad, Hercules, CA). The sample was then diluted with equal volume of Laemmli sample buffer (BioRad) and boiled for 5 min. Sodium dodecyl sulfate PAGE, electro-transfer of proteins, and immunoblotting were performed as previously described (Wohlgemuth et al., 2010). Antibodies used for immunoblotting were anti-LC3 (anti-rabbit LC3, Thermo Scientific, Waltham,