Effects of diacylglycerol O-acyltransferase 1 (DGAT1) on endoplasmic reticulum stress and inflammatory responses in adipose tissue of ketotic dairy cows

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ABSTRACT

Adipose tissue of ketotic dairy cows exhibits greater lipolytic rate and signs of inflammation, which further aggravate the metabolic disorder. In nonruminants, the endoplasmic reticulum (ER) is a key organelle coordinating metabolic adaptations and cellular functions; thus, disturbances known as ER stress lead to inflammation and contribute to metabolic disorders. Enhanced activity of diacylglycerol O-acyltransferase 1 (DGAT1) in murine adipocytes undergoing lipolysis alleviated ER stress and inflammation. The aim of the present study was to investigate the potential role of DGAT1 on ER stress and inflammatory response of bovine adipose tissue in vivo and in vitro. Adipose tissue and blood samples were collected from cows diagnosed as clinically ketotic (n = 15) or healthy (n = 15) following a veterinary evaluation based on clinical symptoms and serum concentrations of β-hydroxybutyrate, which were 4.05 (interquartile range = 0.46) and 0.52 mM (interquartile range = 0.14), respectively. Protein abundance of DGAT1 was greater in adipose tissue of ketotic cows. Among ER stress proteins measured, ratios of phosphorylated PKR-like ER kinase (p-PERK) to PERK and phosphorylated inositol-requiring enzyme 1 (p-IRE1) to IRE1, and protein abundance of cleaved ATF6 protein were greater in adipose tissue of ketotic cows. Furthermore, treatment with EPI led to greater ratios of p-PERK to PERK, p-IRE1 to IRE1, p-RELA to RELA, and cleaved ATF6 protein, whereas EPI stimulation inhibited protein abundance of NFKBIA. Overexpression of DGAT1 in EPI-treated adipocytes attenuated ER stress, the activation of NF-κB and JNK signaling pathways, and the secretion of inflammatory cytokines. In contrast, silencing DGAT1 further aggravated EPI-induced ER stress and inflammatory responses. Overall, these data indicated that activation of DGAT1 may act as an adaptive mechanism to dampen metabolic dysregulation in adipose tissue. As such, it contributes to relief from ER stress and inflammatory responses.

Key words: DGAT1, bovine adipocytes, endoplasmic reticulum stress, inflammatory response

INTRODUCTION

Development of physiological imbalance due to alterations in DMI and energy demands in dairy cows during the transition period renders them more susceptible to metabolic disorders (Drackley, 1999; Loor et al., 2013). Ketosis is a major metabolic disorder of high-yielding dairy cows during the transition period, characterized by elevated blood concentrations of BHB and fatty acids resulting from intense lipolysis (Loor et al., 2013).
et al., 2007; van der Drift et al., 2015). This lipolytic activation is an adaptive response to regulate energy homeostasis and help meet the deficit in energy intake. However, the persistent efflux of fatty acids from adipocytes to the bloodstream and other tissues contributes to lipotoxicity and endoplasmic reticulum (ER) stress (Chitraju et al., 2017).

The ER functions to synthesize, fold, and transport proteins, but under stress conditions cells activate the unfolded protein response (UPR), which includes transcriptional induction of 3 primary transducers: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and transcription factor 6 (ATF6; Harding et al., 2002; Zhao and Ackerman, 2006; Ron and Walter, 2007). The UPR is essential to clear unfolded proteins and restore ER homeostasis. Recent work in nonruminants has demonstrated that metabolic disorders including fatty liver and obesity can cause ER stress in both liver and adipose tissue (Jiao et al., 2011; Bogdanovic et al., 2015). Profiles of genes involved in the ER stress pathway, along with inflammation-related targets, are altered in liver and adipose tissue of peripartal cows (Loor, 2010; Minuti et al., 2020). More recent studies among cows experiencing ketosis or fatty liver have identified the activation of UPR-responsive genes in the liver (Zhu et al., 2019; Shi et al., 2021). Thus, we hypothesized that ER stress might also be induced in adipose tissue during ketosis.

Activation of ER stress is linked to major inflammatory networks via several distinct mechanisms, including the activation of c-jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) signaling pathways, all of which can induce a proinflammatory response (Gregor and Hotamisligil, 2007). In various nonruminant cell types, activation of the UPR causes upregulation of inflammatory genes including interleukin-6 (IL-6) and tumor necrosis factor (TNF; Gregor and Hotamisligil, 2007). Peripartal cow adipose tissue also experiences an upregulation in mRNA abundance of TNF and IL6 (Sadri et al., 2009; Schmitt et al., 2011; Mann et al., 2016). More importantly, a lipolysis-induced adipose tissue inflammatory phenotype has been identified in transition cows, including ketogenic cows (Contreras et al., 2015).

Diacylglycerol acyltransferase 1 (DGAT1) is a member of the membrane-bound O-acyltransferase gene family and is a multifunctional ER membrane protein with its likely active site on the luminal side of the ER membrane (Yen et al., 2008; McFie et al., 2010). The recent demonstration that DGAT1 functions in protecting ER from lipotoxicity and adipose tissue inflammation during stimulated lipolysis in murine adipocytes (Chitraju et al., 2017) underscores a previously-unrecognized function for this enzyme beyond esterification. Thus, the present study was undertaken to examine changes in DGAT1 abundance, ER stress, and inflammation signaling markers in adipose tissue of ketogenic cows and the associations among DGAT1 activity, ER stress, and inflammatory responses during lipolysis in bovine adipocytes. We tested the hypothesis that DGAT1 reduces lipolysis-induced ER stress and inflammatory responses in bovine adipocytes.

MATERIALS AND METHODS

Animals

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of Heilongjiang Bayi Agricultural University (Daqing, Heilongjiang, China). The Animal Ethics Committee of Heilongjiang Bayi Agricultural University approved the study protocol. Holstein dairy cows were selected from a 7,000-cow dairy farm with a freestall dairy housing facility (Nenjiang, Heilongjiang, China). A total of 160 multiparous (parity: median = 3, range = 2–4) lactating Holstein cows (DIM: median = 17 d, range = 12–21 d) were selected for screening. All cows were examined to ensure that no other complications such as hypocalemia or mastitis existed. Cows testing positive for sodium nitroprusside ketone bodies in milk were classified by veterinarians as suspected clinically ketogenic. Body condition was scored by the same herd veterinarian using a 5-point scale with 0.25-point increments (Ferguson et al., 1994). Based on clinical symptoms and serum BHB concentration, 15 clinically ketogenic cows with serum BHB concentrations above 3 mM and 15 cows with serum BHB concentrations below 1.2 mM (Vanholder et al., 2015) were randomly selected as the healthy control group. Blood samples without anticoagulant were collected before feeding, between 0700 and 0800 h, using jugular vein puncture. After clotting at room temperature for 2 h, blood samples were centrifuged at 1,900 × g and 4°C for 15 min to obtain serum. Blood variables were determined in an automatic analyzer (#7170, Hitachi), and serum concentrations of BHB (#RB1008, Randox Laboratories), fatty acids (#FA115, Randox Laboratories), and glucose (#GL3815, Randox Laboratories) detected with commercially available kits. Physiological parameters of ketogenic and healthy cows are presented in Table 1. All cows were fed the same diet during the dry period, formulated to meet NRC recommendations (NRC, 2001). During lactation, all animals were fed the same diet (NRC, 2001). Nutrient composition of dry and lactating cow diets is listed in Supplemental Table S1 (https://doi.org/10.7910/DVN/NPL9C9; Xu, 2022).
Adipose Tissue Collection and Processing

Subcutaneous adipose tissue was collected at 17 (±4) days postpartum from the tail-head depot of all cows on the same day as blood samples, using methods described previously (Xu et al., 2019b). In brief, one side of the tail-head and the pelage on the tail-head region were scrubbed thoroughly with surgical soap. Local anesthesia was administered in the area between the ischium and the tailbone. A scalpel incision of about 5 cm was made and the skin pulled with sterile hemostats before tissue collection. Adipose tissue samples (1 to 2 g) were collected using sterile forceps and surgical scissors by blunt dissection. Compression hemostasis was applied with sterile gauze to prevent any external bleeding after tissue collection. The sampling incision was sutured with 6 to 8 surgical staples (Henry Schein). Adipose tissue samples were washed with RNase-free PBS solution. Adipose tissue samples for total protein extraction were weighed and stored in cryovials under liquid nitrogen. Adipose samples for preadipocyte isolation were stored in warm culture medium bottles with sterile PBS solution containing 2,500 U/mL penicillin and 2,500 mg/mL streptomycin.

Isolation of Primary Cultured Bovine Preadipocytes

Isolation of preadipocytes from healthy cows was performed according to published protocols (Zhang et al., 2018; Sun et al., 2019; Xu et al., 2021a). In brief, adipose tissue (1 g) was digested with 5 mL of collagenase type I digestion solution (1 mg/mL; Sigma-Aldrich) and then filtered through a 40-μm cell filter (Solarbio). The filtrate was then centrifuged at 800 x g for 10 min at room temperature to separate stromal vascular cells. Residual erythrocytes in the resulting cell pellet were eliminated by ammonium-chloride-potassium lysis buffer (Solarbio) and then sequentially centrifuged at 800 x g for 10 min at room temperature.

After discarding supernatant, the resulting pellet was resuspended in basic culture medium (BCM) containing Dulbecco’s modified Eagle medium (DMEM)/F12 (cat. no. SH30023.01, HyClone), 10% fetal bovine serum (cat. no.10091148, Gibco), and 1% penicillin and streptomycin, and incubated at 37°C and 5% CO2 for 24 h. The medium was then replaced to remove non-adherent cells and tissue debris. Finally, 1.8 x 10⁵ cells were seeded per 6-well plate in BCM, and the culture medium was replaced every 48 h.

Cell Culture and Treatment

To differentiate primary preadipocytes, 5-d post-confluent cells were incubated in differentiation culture medium 1, containing 1% penicillin-streptomycin, 0.5 mM 3-isobutyl-1-methylxanthin (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), and 1 μg/mL insulin (Sigma-Aldrich) supplemented in BCM. After 4 d, differentiation culture medium 1 was replaced by differentiation culture medium 2, containing 1 μg/mL insulin supplemented in BCM. Two days later, the differentiation culture medium was replaced with BCM for an additional 2 d (Xu et al., 2019a, 2021a).

After differentiation, the total amount of mature adipocytes was 3.9 to 4.0 x 10⁵ in a 6-well plate, as determined by Trypan blue staining after trypsin digestion of 2 wells in each 6-well plate. Cells used for counting were not used for subsequent experiments. The remaining mature adipocytes were treated with epinephrine (EPI, Sigma-Aldrich) at concentrations of 1 μM for 2 h (Xu et al., 2021b) to examine the involvement of DGAT1 in lipolysis. To overexpress DGAT1, mature adipocytes were transfected with 2 μL of DGAT1 overexpression adenovirus or empty vector of adenovirus for 48 h in 2 mL of DMEM/F12 (HyClone), and then treated with 1 μM EPI for 2 h. Both DGAT1 overexpression adenovirus and the empty adenovirus vector were designed and constructed by Hanbio Biotechnology Co. Ltd.

Table 1. Baseline productive and physiological parameters of cows with and without ketosis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 15)</th>
<th>Ketosis (n = 15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity</td>
<td>3</td>
<td>3</td>
<td>0.102</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>611.97; 36.47</td>
<td>625.70; 24.95</td>
<td>0.063</td>
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<tr>
<td>BCS</td>
<td>2.67; 0.25</td>
<td>2.75; 0.25</td>
<td>0.001</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>21.26; 0.47</td>
<td>19.23; 1.28</td>
<td>0.001</td>
</tr>
<tr>
<td>Milk production (kg/d)</td>
<td>37.80; 1.32</td>
<td>27.97; 1.41</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum BHB (mM)</td>
<td>0.52; 0.14</td>
<td>4.05; 0.46</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum fatty acids (mM)</td>
<td>0.31; 0.06</td>
<td>1.04; 0.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum glucose (mM)</td>
<td>3.65; 0.30</td>
<td>2.10; 0.25</td>
<td>0.001</td>
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1Data were analyzed using the Wilcoxon test.

2IQR = interquartile range.
silence DGAT1, adipocytes were transfected with 100 pmol of DGAT1 small interfering RNA (si-DGAT1) or a negative control vector of siRNA with RNAfit Transfection reagents (cat. no. HB-RF-1000, Hanbio Biotechnology) in 2 mL of DMEM/F12 (HyClone) for 6 h. The medium was then replaced with BCM for 48 h. The si-DGAT1 was synthesized by Hanbio Biotechnology. The si-DGAT1 sequences were as follows: sense 5′ACGUAGUGAGUGUCUAGTT3′ and antisense 5′CUAGAGAACCUCAUCAAGUATT3′. The siRNA negative control vector was synthesized by Hanbio Biotechnology, and its sequences were as follows: sense 5′UUCUCGGAAACGUUCAGCUGTTTT′ and antisense 5′ACGUGACACGUUCGGAGATT′.

ELISA-Based Assay of Inflammatory Cytokines

Cell supernatant concentrations of TNF-α (SEA-133Bo) and IL-6 (SEA079Bo) were determined using commercially available ELISA kits according to the manufacturer’s instructions (USCN Life Science Inc.; Fan et al., 2021), and detected using multimode microplate readers (Thermo Fisher Scientific) at 450 nm.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted from adipocytes using Roche-TriPure isolation reagent (Roche) according to the manufacturer’s instructions. The quantity of RNA was measured via Nanophotometer (Thermo Fisher Scientific), and the optical density ratio at 260 and 280 nm was used as an indication of nucleic acid purity. The optical density ratio of RNA samples ranged from 1.9 to 2.0, indicating high purity (Bustin et al., 2009). Agarose gel electrophoresis was performed to determine the integrity of RNA samples using 1% agarose gels by visualization of the 18S and 28S ribosomal RNA bands. Bands of 18S and 28S were clear, and the 28S band appeared twice as intense as the 18S band. Reverse transcription was performed using the GoScript Reverse Transcription System (Promega), and quantitative real-time PCR analysis was performed with SYBR Green (Roche) using a qTower3G Real-Time PCR System (Analytik Jena). Reverse transcription was performed at 42°C for 60 min with 2 μg of total RNA in 25-μL volume. Conditions used for real-time PCR were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. All reactions were run in triplicate. Relative abundance of each target gene was normalized to ACTB and GAPDH. The fold change for each target gene relative to the control group was calculated using the 2−ΔΔCt method (Ma et al., 2019). Information on the primer sequences is summarized in Supplemental Table S2 (https://doi.org/10.7910/DVN/NPL9C9; Xu, 2022).

Western Blotting

Western blotting was performed using published methods (Xu et al., 2021a). In brief, total protein (including cell membrane, nucleus, nuclear matrix, and cytoplasmic proteins) was extracted from adipose tissue and adipocytes using a Total Protein Extraction Kit (C510003, Sangon Biotech) according to the manufacturer’s instructions. Adipose tissue (200 mg) was dissolved in lysis buffer supplemented with phenylmethylsulfonyl fluoride and phosphatase/protease inhibitors. Tissue lysates were then centrifuged at 4°C and 15,000 × g for 15 minutes to obtain total protein. For adipocytes, cells were resuspended in the lysis buffer described previously and centrifuged at 4°C and 12,000 × g for 5 minutes to obtain total protein. Protein concentrations of adipose tissue and adipocytes were determined using a bicinchoninic acid assay reagent kit (C503051-0500, Sangon Biotech). A total of 30 μg of protein from each sample was separated by 12% SDS-PAGE with known molecular weight markers (Sangon Biotech). Subsequently, protein was transferred onto 0.45-μm polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were incubated with primary antibodies against DGAT1 (1 μg/mL; ab189994, Abcam), phosphorylation (Ser724) of IRE1 (p-IRE1; 1:2,000; ab48187, Abcam), IRE1 (1 μg/mL; ab37073, Abcam), phosphorylation (Thr980) of PERK (p-PERK; 1:500; no. 3179, Cell Signaling Technology), PERK (1:500; no. 3192, Cell Signaling Technology), cleaved ATF6 (1 μg/mL; ab83504, Abcam), phosphorylation (Ser724) of RELA subunit of NF-κB (p-RELA; 1:1,000; no. 3033, Cell Signaling Technology), RELA subunit of NF-κB (REL; 1:1,000; no. 4764, Cell Signaling Technology), NF-κB inhibitor α (NFKBIA; 1:1,000; no. 4814, Cell Signaling Technology), phosphorylation (Thr183/Tyr185) of JNK (p-JNK; 1:1,000; no. 9251, Cell Signaling Technology), JNK (1:1,000; no. 9252, Cell Signaling Technology), and ACTB (1:2,000; ab8226, Abcam) at 4°C overnight. The membrane was then washed with Tris-buffered saline with Tween and incubated with the following secondary antibodies: horseradish peroxidase-conjugated Affinipure rabbit anti-goat IgG (H’+L; SA00001-4, 1:5,000; Proteintech), horseradish peroxidase-conjugated Affinipure goat anti-mouse IgG (H’+L; SA00001-1, 1:5,000; Proteintech), or horseradish peroxidase-conjugated Affinipure goat anti-rabbit IgG (H’+L; SA00001-1, 1:5,000; Proteintech) at room temperature for 1 h. The membrane was then washed with Tris-buffered saline with Tween. Immuno-reactive bands were then made visible by an enhanced
chemiluminescence solution (Millipore). Finally, quantifications of each band were performed using the ImageJ image analysis software (version 1.8.0; National Institutes of Health). The specificity of the antibodies used in this study is depicted in Supplemental Figure S1 (https://doi.org/10.7910/DVN/NPL9C9; Xu, 2022). We detected no difference in the expression of ACTB between groups. Thus, phosphorylated proteins including IRE1, PERK, RELA, and JNK were expressed as phosphorylated:total protein, and the remaining target proteins were normalized to ACTB (Xu et al., 2021a).

**Statistical Analysis**

Statistical analysis was conducted using SPSS software version 25.0 (IBM Corp.) and GraphPad Prism program (Prism 8.3.0, GraphPad Software). All data were tested for normality and homogeneity of variance and residuals using the Shapiro-Wilk and Levene tests, respectively. For basic characteristics of dairy cows with skewed distribution, nonparametric statistical analysis was performed using the Wilcoxon test (Zhu et al., 2019). Parametric statistical analysis was performed for other data with Gaussian distribution using the independent-sample t-test for 2 groups. Basic characteristics of dairy cows are expressed as the median and interquartile range. Other data with normal distribution are expressed as means ± standard error of the mean. One-way ANOVA was performed for multiple comparisons with Bonferroni correction. A P-value <0.05 was considered statistically significant, and a P-value <0.01 was considered highly significant.

**RESULTS**

**DGAT1 Abundance, ER Stress, and Inflammatory Status in Adipose Tissue**

Compared with controls, protein abundance of DGAT1 was greater in adipose tissue of ketotic cows (P < 0.01, Figure 1A, B). In addition, phosphorylation of IRE1 and PERK (leading to greater p-IRE1/IRE1 and p-PERK/PERK; P < 0.01, Figure 1C–E), and protein abundance of cleaved ATF6 (P < 0.01, Figure 1C–F) were greater in adipose tissue of ketotic cows. Protein and mRNA abundance of factors related to inflammation in adipose tissue are shown in Figure 2. The data indicated that protein abundance of NFKBIA was lower (P < 0.01, Figure 2A, B), whereas ratios of p-RELA/RELA and p-JNK/JNK were greater in adipose tissue of ketotic cows (P < 0.01, Figure 2A–D). In addition, mRNA abundance of proinflammatory cytokines including TNF and IL-6 was greater in adipose tissue of ketotic cows (P < 0.01, Figure 2E, F).

**Effects of EPI on DGAT1 Abundance, ER Stress, and Inflammatory Response in Bovine Adipocytes**

Compared with the control, protein abundance of DGAT1 was greater with 1 μM EPI (P < 0.01, Figure 3A, B). Likewise, a similar effect was observed for the ratios of p-IRE1/IRE1 and p-PERK/PERK and protein content of cleaved ATF6 (P < 0.01, Figure 3A–E). Compared with the control, treatment of EPI at 1 μM downregulated protein abundance of NFKBIA (P < 0.01, Figure 3F, G). In contrast, treatment of EPI led to greater ratios of p-RELA/RELA and p-JNK/JNK in bovine adipocytes (P < 0.01, Figure 3H, I). In addition, treatment of EPI upregulated the proinflammatory cytokines including TNF-α and IL-6 (P < 0.01, Supplemental Figure S2; https://doi.org/10.7910/DVN/NPL9C9; Xu, 2022).

**Effects of DGAT1 Overexpression on ER Stress and Inflammatory Response During EPI-Induced Lipolysis**

Compared with the empty adenovirus vector group, overexpression of DGAT1 upregulated mRNA and protein abundance of DGAT1 (P < 0.05, Figure 4A–C). Overexpression of DGAT1 mitigated the increase in phospho-IRE1 and phospho-PERK induced by EPI when compared with the EPI group (P < 0.05, Figure 4B–E). A similar response was observed for the protein abundance of cleaved ATF6 (P < 0.01, Figure 4B–F).

Treatment with EPI downregulated protein abundance of NFKBIA, whereas overexpression of DGAT1 inhibited the degradation of NFKBIA during stimulated lipolysis induced by EPI (P < 0.05, Figure 5A, B). Compared with the EPI group, overexpression of DGAT1 led to a decrease in ratios of p-RELA/RELA and p-JNK/JNK that were detected after stimulation with EPI (P < 0.05, Figure 5A–D). In addition, overexpression of DGAT1 reversed the EPI-induced secretion of TNF-α and IL-6 (P < 0.05, Figure 5E, F).

**Effects of DGAT1 Silencing on ER Stress and Inflammatory Response During EPI-Induced Lipolysis**

Compared with the negative control group, si-DGAT1 treatment downregulated mRNA and protein abundance of DGAT1 (P < 0.05, Figure 6A–C). Compared with the EPI group, silencing DGAT1 exacerbated the upregulation of phospho-IRE1 and cleaved ATF6 in EPI-treated adipocytes (P < 0.05, Figure 6B–F).

Compared with the EPI group, treatment of si-DGAT1 further upregulated the phosphorylation level of JNK, and aggravated the degradation of NFKBIA
induced by EPI ($P < 0.05$, Figure 7A–D). In addition, compared with treatment with EPI alone, the silencing of DGAT1 further exacerbated the secretion of TNF-α and IL-6 in EPI-treated bovine adipocytes ($P < 0.05$, Figure 7E, F).

**DISCUSSION**

There is growing recognition that adipocyte lipolysis rates in ketotic dairy cows trigger local lipotoxicity, which may further augment adipose tissue inflammation (Contreras and Sordillo, 2011; Vailati-Riboni et al., 2017). In nonruminants, it is well established that ER function serves to buffer fatty acids liberated from intracellular triacylglycerol hydrolysis, which naturally causes an ER stress response (Chitraju et al., 2019). Although studies have provided evidence for the existence of ER stress and inflammation during the transition period (Ringseis et al., 2015; McNamara and Huber, 2018), potential therapeutic targets during ketosis are not well known. Thus, in the present study, we sought to study changes in protein abundance of DGAT1, ER stress factors, and inflammatory signaling pathways in adipose tissue of healthy and ketotic dairy cows. Results indicated increased abundance of DGAT1 along with pronounced ER stress and inflammatory responses in adipose tissue of ketotic cows. Further, in vitro data indicated that activity of DGAT1 alleviates adipocyte ER stress and inflammatory responses during lipolysis.

A growing body of evidence suggests that mRNA and protein abundance of UPR chaperones are upregulated in liver and adipose tissue of peripartal dairy cows (Loor, 2010; Gessner et al., 2014; Zhu et al., 2019). Furthermore, elevated ER stress has also been identified in the liver of ketotic cows (Shi et al., 2021). Thus, together, these studies underscore a relationship between ER stress and ketogenesis during the peripartal period (Khan et al., 2015). In the present study, the

**Figure 1.** Abundance of DGAT1 and endoplasmic reticulum (ER) stress proteins in adipose tissue of healthy (n = 15) and clinically ketotic (n = 15) cows. (A) Representative western blots of DGAT1. (B) Protein abundance of DGAT1. (C) Representative western blots of DGAT1, phosphorylated inositol-requiring enzyme 1 (p-IRE1), IRE1, phosphorylated PKR-like ER kinase (p-PERK), PERK, and cleaved ATF6. (D–F) Quantification of p-IRE1/IRE1, p-PERK/PERK, and cleaved ATF6 in adipose tissue. For all bar plots shown, data are expressed as means ± SEM. **$P < 0.01$; statistical differences assessed by t-test.
upregulated phosphorylation levels of PERK and IRE1 and cleavage level of ATF6 confirmed the activation of ER stress in adipose tissue of ketotic dairy cows.

A link between ER stress and lipolysis was studied using bovine adipocytes upon stimulation of lipolysis with EPI. Epinephrine is among the most effective regulators of adipose tissue lipolysis working through β-adrenergic receptors (Lafontan, 2012). Our previous study detected an increase in the release of glycerol in bovine adipocytes after incubation with EPI (Xu et al., 2021b). Thus, consistent with our in vivo data from ketotic cows, lipolysis stimulated by EPI activated the 3 protein sensors of ER stress (IRE1, PERK, and ATF6) in bovine adipocytes. These results further underscored the central role of ER stress in the pathogenesis of ketosis in transition dairy cows.

In nonruminants, excessive adipocyte lipolysis is known to generate lipid mediators and trigger inflammation in adipose tissue (Gartung et al., 2016). Recent studies have consistently demonstrated that ketotic cows display systemic inflammation partly due to the lipotoxic effects of fatty acids released during lipolysis (Sordillo and Raphael, 2013; Zhang et al., 2018; Xu et al., 2019b). Crucial inflammatory pathways such as JNK and NF-κB signaling are upregulated in adipose tissue undergoing lipolysis, leading to increased production of downstream cytokines such as TNF-α and IL-6 (Laurencikiene et al., 2007; Gartung et al., 2016; Fan...
Figure 3. Effects of epinephrine (EPI) on DGAT1 abundance, endoplasmic reticulum (ER) stress, and inflammatory response in bovine adipocytes. Bovine adipocytes were treated with 1 μM EPI for 2 h. (A) Representative western blots of DGAT1, phosphorylated inositol-requiring enzyme 1 (p-IRE1), IRE1, phosphorylated PKR-like ER kinase (p-PERK), PERK, and cleaved ATF6. (B–E) Quantification of DGAT1, p-IRE1/IRE1, p-PERK/PERK, and cleaved ATF6. (F) Bos taurus adipocytes were pretreated with 1 μM SB203580, a JNK inhibitor, or 1 μM MG132, an inhibitor of NF-κB, followed by treatment with EPI for 2 h. (G) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (H) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (I) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (J) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (K) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (L) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (M) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (N) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (O) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (P) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (Q) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (R) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (S) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (T) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (U) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (V) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (W) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (X) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (Y) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h. (Z) Bos taurus adipocytes were treated with EPI (1 μM) for 2 h.
Figure 4. Effects of DGAT1 overexpression on endoplasmic reticulum (ER) stress during epinephrine (EPI)-induced lipolysis. Bovine adipocytes were treated with overexpression adenovirus of DGAT1 (Ad-DGAT1) for 48 h and further incubated in the absence or presence of 1 μM EPI for 2 h. (A) Relative mRNA abundance of DGAT1 after transfection of adipocytes with empty adenovirus vector (EV) or Ad-DGAT1 for 48 h. (B) Representative western blots of DGAT1, phosphorylated inositol-requiring enzyme 1 (p-IRE1), IRE1, phosphorylated PKR-like ER kinase (p-PERK), PERK, and cleaved ATF6. (C–F) Quantification of DGAT1, p-IRE1/IRE1, p-PERK/PERK, and cleaved ATF6 in bovine adipocytes. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. For all bar plots shown, data are expressed as mean ± SEM. Different lowercase letters denote significant differences (P < 0.05). All experiments were repeated 3 times in duplicate (6 wells/group), n = 3.
et al., 2021). Thus, in the present study, upregulated phosphorylation of NF-κB and JNK signaling, together with elevated expression of proinflammatory cytokines, further confirmed the occurrence of inflammatory responses in adipose tissue of ketotic cows.

Based on these facts, we inferred that inflammatory responses may participate in EPI-induced lipolysis in bovine adipocytes by regulating NF-κB and JNK inflammatory pathways. In accordance with this idea, EPI-induced lipolysis activated NF-κB and JNK signaling and increased mRNA abundance of inflammatory cytokines in bovine adipocytes. The present experimental results further confirm our previous data and underscore the link between inflammation and lipolysis (Zhang et al., 2018; Xu et al., 2019b; Fan et al., 2021).

Figure 5. Effects of DGAT1 overexpression on inflammatory response during epinephrine (EPI)-induced lipolysis. Bovine adipocytes were treated with overexpression adenovirus of DGAT1 (Ad-DGAT1) for 48 h and further incubated in the absence or presence of 1 μM EPI for 2 h. (A) Representative western blots of NF-κB inhibitor α (NFKBIA), phosphorylated RELA subunit of NF-κB (p-RELA), RELA, phosphorylated c-jun N-terminal kinase (p-JNK), and JNK. (B–D) Quantification of NFKBIA, p-RELA/RELA, and p-JNK/JNK in bovine adipocytes. (E, F) Content of TNF-α and IL-6 in bovine adipocytes. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. For all bar plots shown, data are expressed as mean ± SEM. Different lowercase letters indicate significant differences (P < 0.05). All experiments were repeated 3 times in duplicate (6 wells/group), n = 3.
Figure 6. Effects of DGAT1 silencing on endoplasmic reticulum (ER) stress and inflammatory response during epinephrine (EPI)-induced lipolysis. Bovine adipocytes were treated with DGAT1 small interfering RNA (Si-DGAT1) for 48 h and further incubated in the absence or presence of 1 μM EPI for 2 h. (A) Relative mRNA abundance of DGAT1 after transfection with negative control of siRNA (NC) or overexpression adenovirus of DGAT1 (Ad-DGAT1) for 48 h. (B) Representative western blots of DGAT1, phosphorylated inositol-requiring enzyme 1 (p-IRE1), IRE1, phosphorylated PKR-like ER kinase (p-PERK), PERK, and cleaved ATF6. (C–F) Quantification of DGAT1, p-IRE1/IRE1, p-PERK/PERK, and cleaved ATF6 in bovine adipocytes. Comparisons among groups were calculated using one-way ANOVA with subsequent Bonferroni correction. For all bar plots shown, data are expressed as mean ± SEM. Different lowercase letters indicate significant differences (P < 0.05). All experiments were repeated 3 times in duplicate (6 wells/group), n = 3.
In mammals, the last and only committed step of triacylglycerol synthesis is catalyzed by DGAT1 and DGAT2 (Yen et al., 2008; Liu et al., 2012). Both DGAT enzymes catalyze the same reaction, utilizing diacylglycerol and fatty acyl CoA as substrates, but are evolutionarily unrelated (Smith et al., 2000; Stone et al., 2006). DGAT1 is localized exclusively to the ER and (at least in nonruminants) has broader substrate specificity than DGAT2 (Wilfling et al., 2013). Previous studies have shown that DGAT1 and DGAT2 are required for triacylglycerol synthesis and lipid droplet formation in adipocytes (Harris et al., 2011). In addition to the well-known regulatory role in lipid metabolism, recent studies have shown that DGAT1 functions...
to maintain ER homeostasis in adipocytes (Chitraju et al., 2017, 2019). The fact that protein abundance of DGAT1 was upregulated in ketotic cows prompted us to further investigate its role during lipolysis-induced ER stress in bovine adipocytes.

In the present study, overexpression of DGAT1 alleviated lipolysis-induced ER stress, whereas inhibition of DGAT1 further aggravated ER stress induced by EPI-stimulated lipolysis, underscoring a negative association between DGAT1 and the activation of ER stress in bovine adipocytes. In agreement with these data, adipocyte-specific deletion of DGAT1 in mice causes chronic ER stress and cellular dysfunction during lipolysis, and DGAT1 mRNA levels in human adipose tissue exhibit an inverse correlation with many genes associated with ER stress (Chitraju et al., 2017). Additionally, these findings are consistent with in vitro analyses of DGAT1 in mouse embryonic fibroblasts and in murine 3T3-L1 adipocytes (Harris et al., 2011). The selective deletion of DGAT1 in several mouse tissues also predisposes them to lipotoxicity (Liu et al., 2014; Vujic et al., 2016). Thus, the present data demonstrating increased abundance of DGAT1 as a function of elevated ER stress in adipose tissue of ketotic dairy cows are suggestive of a survival adaptation to control metabolic stress induced during hyperketonemia.

Mounting evidence suggests that sensing and adaptive measures are integrated in the ER, and failure of the ER to adapt leads to aberrant metabolism, organelle dysfunction, and inflammation (Lemmer et al., 2021). For instance, activation of JNK by IRE-1 during ER stress is one key pathway that increases inflammation. In the nucleus, JNK upregulates the abundance of inflammatory genes (Gregor and Hotamisligil, 2007). IRE-1 can also activate the NF-κB pathway, which is critical in the induction of multiple inflammatory genes such as TNF and IL-6 and is also implicated in insulin resistance (Shoelson et al., 2006). The NF-κB pathway may also be activated through PERK signaling during the UPR. PERK-mediated phosphorylation of eukaryotic translation initiation factor 2 results in the inhibition of translation of the NFKBIA protein, the major negative regulator of NF-κB, thus allowing the activation of NF-κB and the induction of its proinflammatory targets (Gregor and Hotamisligil, 2007).

In vitro, induction of the UPR in murine adipocytes has been reported to cause increased release of pro-inflammatory cytokines, including IL-2, IL-8, MCP-1, and TNF-α (Longo et al., 2016). Because our data suggested that abundance and activity of DGAT1 regulate ER stress in bovine adipocytes, we speculate that this enzyme participates in the regulation of inflammatory responses. The fact that overexpression of DGAT1 alleviated lipolysis-induced inflammatory responses through downregulating NF-κB and JNK signaling supports this idea. Furthermore, in agreement with our results, adipose tissue inflammation has been observed in adipocytes from mice lacking DGAT1 when subjected to lipolysis-stimulating conditions (Hotamisligil, 2010; Montane et al., 2014; Keestra-Gounder et al., 2016). Taking all data together, we conclude that DGAT1 may ameliorate lipolysis-induced inflammation by mitigating ER stress in bovine adipocytes.

Although the focus on metabolic mechanisms of adipose tissue in ketotic cows dealt primarily with a lipolysis model in primary cultured bovine adipocytes, a recent review of the literature emphasizes the fact that even clinically healthy dairy cows experience adipose tissue mobilization in early lactation (Zachut and Contreras, 2022). Although our in vitro data suggest that DGAT1 may affect ER homeostasis and inflammation, an in vivo study is warranted to further demonstrate these effects in peripartal cows. In addition, specific causal mechanisms by which DGAT1 controls ER stress and inflammation could not be discerned in the present study. Thus, further research should be conducted to evaluate the potential mechanistic links between ER stress and inflammation in ketotic dairy cows.

**CONCLUSIONS**

Compared with healthy dairy cows, greater DGAT1 expression, ER stress state, and activation of inflammatory responses were observed in adipose tissue of ketotic cows. In vitro, activation of DGAT1 relieved EPI-induced ER stress and inflammatory responses in primary bovine adipocytes. As such, control of the abundance and activity of DGAT1 in response to ER stress and inflammation may be a central mechanism within adipose tissue that is triggered by extensive lipolysis in ketotic cows. Therapeutic strategies or management approaches that can enhance DGAT1 before and during onset of ketosis could help control ER stress and inflammatory responses within adipose tissue.

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