Role of diacylglycerol O-acyltransferase (DGAT) isoforms in bovine hepatic fatty acid metabolism

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ABSTRACT

Fatty acid accumulation in hepatocytes induced by high concentrations of fatty acids due to lipolysis and the associated oxidative damage they cause occur most frequently after calving. Because of their role in esterification of fatty acids, diacylglycerol acyltransferase isoforms (DGAT1 and DGAT2) could play a role in the susceptibility of dairy cows to develop fatty liver. To gain mechanistic insights, we performed in vivo and in vitro analyses using liver biopsies or isolated primary hepatocytes. The in vivo study ($n = 5$ cows/group) involved healthy cows [average liver triacylglycerol (TAG) = 0.78%; 0.58 to 0.93%, ratio of triglyceride weight to wet liver weight] or cows diagnosed with fatty liver (average TAG = 7.60%; 5.31 to 10.54%). In vitro, hepatocytes isolated from 3 healthy female calves (1 d old, 44 to 53 kg) were challenged with (fatty acids) or without (control) a 1.2 mM mixture of fatty acids in an attempt to induce metabolic stress. Furthermore, hepatocytes were treated with DGAT1 inhibitor or DGAT2 inhibitor for 2 h followed by a challenge with (DGAT1 inhibitor + fatty acids or DGAT2 inhibitor + fatty acids) or without (DGAT1 inhibitor or DGAT2 inhibitor) the 1.2 mM mixture of fatty acids for 12 h. Data analysis of liver biopsies was compared using a 2-tailed unpaired Student's *t*-test. Data from calf hepatocyte treatment comparisons were assessed by one-way ANOVA, and multiplicity for each experiment was adjusted by the Holm's procedure. Data indicated that both fatty liver and in vitro challenge with fatty acids were associated with greater mRNA and protein abundance of SREBF1, FASN, DGAT1, and DGAT2. In contrast, mRNA and

protein abundance of CPT1A and very low-density lipoprotein synthesis-related proteins MTTP and APOB were markedly lower. However, compared with fatty acid challenge alone, DGAT1 inhibitor + fatty acids led to greater mRNA and protein abundance of CPT1A and APOB, and greater mRNA abundance of *SREBF1* and *MTTP*. Furthermore, this treatment led to lower mRNA abundance of *FASN* and *DGAT2* and TAG concentrations. Compared with fatty acid challenge alone, DGAT2 inhibitor + fatty acids led to greater mRNA and protein abundance of CPT1A, MTTP, and APOB, and lower mRNA and protein abundance of SREBF1 and FASN. In addition, compared with control and fatty acids, there was greater protein abundance of GRP78 and PERK in both DGAT1 and DGAT2 inhibitor with or without fatty acids. Furthermore, compared with control and fatty acids, reactive oxygen species concentrations in the DGAT1 inhibitor with or without fatty acid group was greater. Overall, data suggested that DGAT1 is particularly relevant in the context of hepatocyte TAG synthesis from exogenous fatty acids. Disruption of both DGAT1 and DGAT2 altered lipid homeostasis, channeling fatty acids toward oxidation and generation of reactive oxygen species. Both DGAT isoforms play a role in promoting fatty acid storage into TAG and lipid droplets to protect hepatocytes from oxidative damage.

Key words: dairy cow, fatty liver, lactation, metabolism

INTRODUCTION

Negative energy balance (NEB) is a state commonly experienced by periparturient dairy cows due to insufficient DMI to sustain increased energy requirements for milk production (Grummer, 2007; Loo et al., 2013). Depending on the severity of NEB, fatty acids produced during adipose tissue mobilization could cause increased triacylglycerol (TAG) accumulation in the liver and lead to the occurrence of fatty liver (Murondoti et al., 2004;

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Shahsavari et al., 2016). Despite extensive research over the past decades, cellular mechanisms controlling the fate of fatty acids flowing into the liver during conditions such as NEB or fatty liver are not fully known.

Within the liver, transport of incoming fatty acids into the mitochondria via CPT1 for β -oxidation is a key pathway that reduces esterification of fatty acids, which leads to formation of TAG that can be used to synthesize very low-density lipoprotein (VLDL) for export (van den Top et al., 1996; Zhu et al., 2019). Available research indicates that accumulation of TAG is linked primarily to the inherently low rate of VLDL secretion in ruminants (Bauchart et al., 1996; Coleman et al., 2019). However, recent molecular data also suggest that a lipogenic program can be activated during conditions in which fatty acid supply to the liver increases markedly (Zhu et al., 2019).

The lipoprotein VLDL is the main route of endogenous TAG transport from the liver and plays an important role in lipid homeostasis in humans, rodents, and cattle (Katoh, 2002; Yli-Jokipii et al., 2003). First, the endoplasmic reticulum synthesizes TAG and transfers it to microsomal triglyceride transfer protein (MTTP) and then to nascent apolipoprotein B (APOB) as it is being translated within the lumen of the rough endoplasmic reticulum (Sturley and Hussain, 2012). After these nascent VLDL molecules are fully lipidated with TAG, they exit the endoplasmic reticulum and travel to the Golgi apparatus where the final steps of VLDL formation are completed (Sturley and Hussain, 2012). As an important raw material for VLDL synthesis, synthesis of TAG is regulated partly by the terminal enzymes in the pathway, DGAT1 and DGAT2 in mouse (Yu et al., 2002). Although DGAT2 is mainly expressed in hepatocytes where they catalyze TAG synthesis, DGAT1 is also expressed and can play an important role in TAG synthesis and VLDL assembly (Takahashi et al., 2007; Ying et al., 2017).

In nonruminants, it has been proposed that DGAT1 and DGAT2 differ in their respective preferences for pools of a fatty acid substrate (i.e., exogenous versus endogenous) with DGAT1 utilizing exogenous fatty acids and DGAT2 de novo synthesized fatty acids for TAG synthesis (Qi et al., 2012; McLaren et al., 2018; Zoni et al., 2021). Simultaneous inhibition of DGAT1 and DGAT2 led to a loss of cardiac TAG synthesis ability and enhanced lipotoxicity (Cheng et al., 2020). Thus, one aim of the present study was to elucidate expression characteristics of DGAT1, DGAT2, and related pathways in liver from dairy cows diagnosed with fatty liver. Another aim was to use primary calf hepatocytes to investigate specific functions of DGAT1 and DGAT2 in the context of fatty acid metabolism.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee for the Use and Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, China).

Liver Tissue Collection

Twenty-six lactating multiparous Holstein cows (8 ± 2 d postpartum, parity 2.5 ± 0.50 , BCS 3.06 ± 0.11) from a commercial dairy farm (Heilongjiang Province, China), housed in freestall barn with concrete stalls bedded with sawdust, fed ad libitum (Supplemental Table S1, <https://figshare.com/s/ee162ac77b6746511f41>), received a routine physical examination by the attending veterinarian before selection for the study to ensure blood BHB <1.2 mmol/L ($n = 10$ cows) or ≥ 3.0 mmol/L ($n = 16$ cows) and there were no other clinical disorders. Briefly, blood samples of each cow were collected on the morning before feeding by venipuncture of the caudal vein and heparin sodium was used as an anticoagulant. Blood BHB were immediately measured by cow blood ketone meter (TNN-2, Yicheng Bioelectronics Technology Co. Ltd.). Then, blood samples were centrifuged at $1,400 \times g$ for 10 min at 4°C . The plasma parameters, including fatty acids, glucose, and aspartate aminotransferase, were measured by a biochemical kit (Beijing Jiuqiang Biotechnology Co. Ltd.) with an automatic clinical analyzer (Synchron DXC800; Beckman Coulter Inc.). After blood collection, liver biopsies were conducted with an 18-gauge biopsy probe (EMENT-1815, Shenzhen Yiman Technology Co., Ltd.) as described previously (Vailati Riboni et al., 2015; Coleman et al., 2019). The liver biopsy specimens were immediately stored at -80°C for TAG analysis using an enzymatic kit (Applygen Technologies Inc.). Cows with liver TAG content lower than 1% (expressed as the ratio of triglyceride weight to wet liver weight) were classified as healthy and those with liver TAG content greater than 2% as mild fatty liver (Bobe et al., 2004). Milk yield was obtained from the farm milk recording system. Subsequently, 5 cows with average liver TAG = 7.60% (5.3 to 10.5%), milk yield 29.3 kg (21.3 to 38.6 kg), BHB = 3.66 mmol/L (3.1 to 4.2 mmol/L), serum fatty acids = 1.24 mmol/L (0.91 to 1.48 mmol/L), serum glucose = 2.11 mmol/L (1.90 to 2.43 mmol/L), and serum aspartate aminotransferase = 199.4 U/L (117 to 319 U/L) were chosen as the fatty liver group. Another 5 cows with average liver TAG = 0.78% (0.58 to 0.93%), milk yield 36.6 kg (31.5 to 42.5 kg), blood BHB = 0.90 mmol/L (0.7 to 1.1 mmol/L), serum fatty acids = 0.534 mmol/L (0.43 to 0.62 mmol/L), serum glucose = 3.19 mmol/L (2.81 to 3.69 mmol/L), and

serum aspartate aminotransferase = 88.4 U/L (82 to 94 U/L) were chosen as healthy controls.

Isolation and Culture of Primary Calf Hepatocytes

Three newborn healthy female Holstein calves (1 d old, 44 to 53 kg) were anesthetized with thiamylal sodium (50 mg/kg) followed by intravenous heparin (1,500 IU/kg) injection. Primary hepatocytes were isolated using a 2-step collagenase perfusion method as previously described (Du et al., 2018b; Zhang et al., 2020a). Briefly, the caudate lobe was isolated and washed with perfusion solution. The perfusion solution A (140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA; pH 7.4; 37°C; 50 mL/min for 10–15 min) preheated at 37°C was used to rinse the blood surface of the caudate process surface, and vascular intubation for perfusion. The liver was then perfused with the same flow rate of 37°C pre-warmed perfusion solution B (140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM CaCl₂; pH 7.4; 37°C; 50 mL/min for 5 min) through blood vessels until the liquid became clear. The liver was then perfused with perfusion solution B with 0.0002 g/mL collagenase IV (Type IV Collagenase, Gibco) through blood vessels until the liquid became turbid. Fetal bovine serum (FBS; Hyclone Laboratories) was added to terminate the digestion. Upon removal of the liver capsule, blood vessels, fat, and connective tissue, the shredded liver tissue was resuspended in 4°C pre-cooled RPMI1640 medium and filtered sequentially with 100 mesh (150 µm) and 200 mesh (75 µm) cell sieves. The obtained hepatocytes were washed twice with RPMI-1640 basal medium (Hyclone Laboratories) and centrifuged for 5 min at 500 × *g* at 4°C. Primary hepatocytes (1 × 10⁶ cells/mL) were seeded into a 6-well tissue culture plate using adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 10⁻⁶ mol/L of insulin, 10⁻⁶ mol/L of dexamethasone, 10 µg/mL of vitamin C) and incubated at 37°C in 5% CO₂ for 4 h. The medium was replaced with growth medium (RPMI-1640 with 10% FBS), and growth medium was replaced every 24 h.

After 44 h of culture, hepatocytes (n = 6 replicates per group) were starved for 12 h in serum-free RPMI-1640 basal medium. Subsequently, hepatocytes were maintained in RPMI-1640 basal medium containing 2% BSA and treated with or without 1.2 mM fatty acids (*cis*-9–18:1, 18:2, 16:0, 18:0, and *cis*-9–16:1 at 43.5, 4.9, 31.9, 14.4, and 5.3% of total fatty acids, respectively) for 12 h. The mixing ratio and concentration of fatty acids are based on previous studies on fatty acid com-

position of cows in ketosis and other in vitro studies (Yamdagni and Schultz, 1970; Li et al., 2012, 2021).

Inhibition of DGAT1 and DGAT2

Before inhibition of DGAT1 and DGAT2, hepatocytes were cultured in 6-well plates (1 × 10⁶) with growth medium for 44 h. Cells (n = 6 replicates per group) were divided into 6 groups: control, fatty acids, DGAT1 inhibitor, DGAT1 inhibitor + fatty acids, DGAT2 inhibitor, DGAT2 inhibitor + fatty acids. In the DGAT1 inhibitor group (DGAT1i) and DGAT1 inhibitor + fatty acid group (DGAT1i + fatty acids), 0.3 µM DGAT1 inhibitor (DGAT1 inhibitor 2, HY-50670, MCE) was used for 2 h before incubation with or without fatty acids. In the DGAT2 inhibitor group (DGAT2i) and DGAT2 inhibitor + fatty acid group (DGAT2i + fatty acids), 0.3 µM DGAT2 inhibitor (PF06424439 methanesulfonate, HY-108341A, MCE) was used for 2 h before culture with or without fatty acids.

mRNA Extraction and Reverse-Transcription Quantitative PCR

Total mRNA was extracted from liver tissue (n = 5 cows per group) and hepatocytes (n = 6 replicates per group) using Trizol according to the manufacturer's protocol (Invitrogen Corporation). Concentration of RNA was determined with a K5500 microspectrophotometer (Beijing Kaiao Science and Technology Development Co. Ltd.), the ratio of the optical density at 260/280 nm was 1.9, and the 28S ribosomal RNA band was approximately twice that of the 18S band in the total RNA sample, indicating that isolated RNA was of high quality (Skrypina et al., 2003). One microgram of total RNA was transcribed into cDNA using Reverse Transcriptase M-MLV (RNase H-; RR047A, TaKaRa Biotechnology Co. Ltd.). The SYBR green plus reagent kit (Roche) was used to prepare a 20-µL mixture and mRNA abundance was detected with a 7300 Real-Time PCR System (F. Hoffmann-La Roche AG). The reaction conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s; and extension at 72°C for 5 min. Evaluated target genes were *SREBF1*, *FASN*, *APOB*, *MTTP*, *DGAT1*, *DGAT2*, *CPT1A*, *GRP78*, and *PERK*. Data were normalized using the geometric mean of *ACTB* and *GAPDH*. Relative abundance was calculated using the 2^{-ΔΔCT} method. Gene primers were referenced in our previous work (Dong et al., 2019; Zhang et al.,

Table 1. Sequences of primers used for real-time PCR amplification

Gene	GenBank number	Primer (5' to 3')	Length (bp)
<i>ACTB</i>	NM_173979.3	Forward: GCTAACAGTCCGCTAGAAGCA Reverse: GTCATCACCATCGGCAATGAG	403
<i>GAPDH</i>	NM_001034034.2	Forward: GTCTTCACTACCATGGAGAAGG Reverse: TCATGGATGACCTTGGCCAG	197
<i>SREBF1</i>	NM_001113302.1	Forward: GCAGCCCATTCATCAGCCAGACC Reverse: CGACACCACCAGCATCAACCACG	119
<i>FASN</i>	NM_001012669.1	Forward: ACAGCCTCTTCTAGTTTAGACG Reverse: CTCTAGCACGATCAGCTCGA	144
<i>APOB</i>	XM_024999521.1	Forward: CAGCAGGCGGGAGGGAGAC Reverse: AACAGCAGCAGCAGCAGCAG	82
<i>MTTP</i>	NM_001101834.1	Forward: CGTAGATAGTACCTAGCTAGGCTCTAG Reverse: GGCGGCAAGGTAGGCTAATAGG	201
<i>DGAT1</i>	XM_025001414.1	Forward: CCACTAGGGACCTAGAGGTAGTC Reverse: GCATCACCACACACCAATTCA	101
<i>DGAT2</i>	NM_205793.2	Forward: CTAGGCTAGGTAGTTTAGACTAGGA Reverse: TAGCCAGGGAACCTTCTTAGCTT	239
<i>PERK</i>	XM_010810067.3	Forward: GCCGCTCAGCTCTCCTAGTCC Reverse: TGGCTCTCGGATGAACTGGTCTG	165
<i>GRP78</i>	XM_024998380.1	Forward: GCATCGACCTGGGTACCACCTA Reverse: CCCTTCAGGAGTGAAGCCACA	122
<i>CPT1A</i>	NM_001304989.2	Forward: ACGCCGTAGAAGTATAACCCT Reverse: CCAAAAATCGCTTAGTCCCTT	119

2020b) or designed using Applied Biosystems Primer Express software and are shown in Table 1.

Protein Extraction and Western Blotting

Liver tissue ($n = 5$ cows per group) and hepatocytes ($n = 6$ replicates per group) were lysed using RIPA buffer (Beyotime Biotechnology) containing protease inhibitors, incubated on ice for 30 min, then centrifuged at $14,000 \times g$ for 5 min at 4°C , and protein measured using the BCA protein assay kit (Beyotime). Aliquots of 30 μg total protein were loaded on 8% SDS polyacrylamide gels with known molecular weight markers (Solarbio Life Sciences). Subsequently, the protein was transferred onto 0.45- μm polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were blocked with 5% skim milk powder in Tris-buffered saline (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. Membranes were cut into corresponding fragments according to the molecular weight of the protein and exposed to rabbit anti-SREBF1 (75 kDa, 1:1,000, NB100-2215; Novus), rabbit anti-FASN (273 kDa, 1:1,000, C2065; Cell Signaling), goat anti-DGAT1 (55 kDa, 1:1,000, ab122924, Abcam), rabbit anti-DGAT2 (39 kDa, 1:2,000, A13890, Abclonal), rabbit anti-CPT1A (88 kDa, 1 $\mu\text{g}/\text{mL}$, ab83862, Abcam), rabbit anti-MTTP (99 kDa, 1 $\mu\text{g}/\text{mL}$, ab186446, Abcam), goat anti-APOB (460 kDa, 1:500, ab7616, Abcam), mouse anti-GRP78 (78 kDa, 1:250, sc-376768; Santa Cruz), rabbit anti-PERK (140 kDa, 1:1,000, C33E10; Cell Signaling), and mouse anti- β -actin (internal control; 43 kDa, 1:1,000, sc-47778; San-

ta Cruz) overnight at 4°C , respectively. After washing and incubation with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat antibody (Beyotime Biotechnology), respectively, for 40 min at room temperature, immunoreactive bands were detected by enhanced chemiluminescence (Beyotime) solution using a protein simple imager (ProteinSimple), and Image-Pro Plus 6.0 software (Media Cybernetics Inc.) was used to quantify the band strength. The target protein expression was normalized to β -actin expression.

TAG Content and Lipid Droplet Fluorescence Assay

Content of TAG in liver tissue ($n = 5$ cows per group) and primary hepatocytes ($n = 6$ replicates per group) was determined using an enzymatic assay kit (Applygen Technologies Inc.) in accordance with the manufacturer's instructions. Briefly, liver tissues (about 15 mg) or primary hepatocytes were added to 0.1 mL of lysis solution and homogenized by polytron homogenizer. After standing for 30 min and centrifuging at $14,000 \times g$ for 5 min at 4°C , an appropriate amount of supernatant was taken for subsequent experiments. The remaining liquid concentration of primary hepatocyte samples was determined using the BCA protein assay kit (Beyotime Biotechnology) for TAG content correction. For lipid droplet fluorescence, hepatocytes were washed with PBS and fixed at room temperature with 4% paraformaldehyde for 30 min. Cellular staining was performed using BODIPY 493/503 (Invitrogen Corporation). The nucleus was stained with Hoechst (Beyotime Biotechnology) at room temperature for 8

min. Fluorescence was determined using an inverted microscope (IX73, Olympus).

Cell Culture Supernatant APOB Lipid Moiety Content Assay

Hepatocyte culture supernatant from all treatments was collected and content of APOB lipid moiety was determined using an bovine APOB ELISA assay kit (Lengton Bioscience Co. Ltd.) in accordance with the manufacturer's instructions.

Cellular Oxidative Stress Indices

Content of malondialdehyde (MDA; Yamdagni and Schultz) in primary hepatocytes ($n = 6$ replicates per group) was determined with commercial kits following the manufacturer's instructions (MDA assay kit, S0131S, Beyotime Biotechnology). Changes in reactive oxygen species (ROS) in hepatocytes were determined with a commercial ROS assay kit (S0033S, Beyotime). A total of 1×10^5 cells were incubated with $10 \mu\text{M}$ carboxy-2',7'-dichloro-dihydro-fluorescein diacetate probe in PBS for 15 min at 37°C . Fluorescence was measured at 488 nm (excitation) and 525 nm (emission) by a Beckman CytoFLEX FCM (Beckman Coulter).

Statistical Analysis

The results are reported as means $\pm$ standard error of the means. The data were analyzed by using SPSS 22.0 (IBM Corp.) or Prism 7 statistical software (GraphPad InStat Software). Data generated with liver tissue was compared using a 2-tailed unpaired Student's *t*-test. Data from calf hepatocyte treatment comparisons were assessed by one-way ANOVA, and multiplicity for each experiment was adjusted by the Holm's procedure to control for type I error rate at 0.05. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

mRNA and Protein Abundance in Healthy Cows or Cows with Fatty Liver

Relative mRNA abundance of lipid synthesis-related genes (*SREBF1*, *FASN*, *DGAT1*, and *DGAT2*; $P < 0.01$) and endoplasmic reticulum stress-related genes (*GRP78* and *PERK*) was greater in cows with fatty liver compared with healthy control cows ($P < 0.01$; Figure 1A). In contrast, mRNA abundance of fatty acid oxidation- (*CPT1A*) and VLDL synthesis-related (*MTTP* and *APOB*) genes was lower in cows with fatty

liver compared with healthy control cows ($P < 0.01$; Figure 1A).

Protein abundance of lipid synthesis-related proteins (*SREBF1*, *FASN*, *DGAT1*, and *DGAT2*) and endoplasmic reticulum stress-related proteins (*GRP78* and *PERK*) was greater in cows with fatty liver compared with healthy control cows ($P < 0.01$; Figure 1C). In contrast, protein abundance of fatty acid oxidation (*CPT1A*; $P < 0.01$) and VLDL synthesis-related [*MTTP* ($P < 0.01$) and *APOB* ($P < 0.01$)] proteins was lower in cows with fatty liver compared with healthy control cows (Figure 1C).

Effect of Inhibition of DGAT1 and DGAT2 on Lipid Synthesis

Compared with the control, relative mRNA and protein abundance of *SREBF1*, *FASN*, *DGAT1*, and *DGAT2* were greater in response to 1.2 mM fatty acid treatment ($P < 0.01$; Figure 2A–C), whereas mRNA and protein abundance of *CPT1A*, *MTTP*, and *APOB* were lower ($P < 0.01$; Figure 2A–C).

As expected, inhibition of *DGAT1* and *DGAT2* led to lower mRNA and protein abundance of *DGAT1* and *DGAT2* ($P < 0.01$; Figure 2A–C). Compared with the control, relative mRNA abundance of *SREBF1* ($P < 0.01$) and *FASN* ($P < 0.01$) was greater in cells with the *DGAT1* inhibitor (Figure 2A–C). In contrast, mRNA abundance of *APOB* ($P < 0.01$) and *CPT1A* ($P < 0.01$) and protein abundance of *MTTP* ($P < 0.01$), *APOB* ($P < 0.01$), and *CPT1A* ($P = 0.047$) were lower in cells with *DGAT1* inhibitor compared with the control (Figure 2A–C).

Compared with the control, relative mRNA abundance of *FASN* was greater in cells with *DGAT2* inhibitor ($P < 0.01$; Figure 2A), whereas mRNA abundance of *SREBF1* ($P = 0.020$), *MTTP* ($P < 0.01$), and *CPT1A* ($P < 0.01$) and protein abundance of *MTTP*, *APOB*, and *CPT1A* were lower in cells with *DGAT2* inhibitor compared with the control ($P < 0.01$; Figure 2A–C).

Compared with the fatty acid group, mRNA abundance of *SREBF1* ($P < 0.01$), *CPT1A* ($P < 0.01$), *MTTP* ($P < 0.01$), and *APOB* ($P < 0.01$) and protein abundance of *APOB* ($P < 0.01$) and *CPT1A* ($P < 0.01$) were greater in cells with *DGAT1* inhibitor + fatty acids (Figure 2A–C). In contrast, mRNA abundance of *FASN* ($P < 0.01$) and *DGAT2* ($P < 0.01$) was lower in cells with *DGAT1* inhibitor + fatty acids compared with the fatty acid group (Figure 2A–C).

Compared with the fatty acid group, mRNA and protein abundance of *CPT1A* ($P < 0.01$), *MTTP* ($P < 0.01$), and *APOB* ($P < 0.01$) was greater in cells from the *DGAT2* inhibitor + fatty acid group (Figure

2A–C), whereas mRNA abundance of *SREBF1* ($P < 0.01$) and *FASN* ($P < 0.01$) and protein abundance of *FASN* ($P < 0.01$) were lower in cells with DGAT2 inhibitor + fatty acids compared with the fatty acid group (Figure 2A–C).

Effect of Inhibition of DGAT1 and DGAT2 on Lipid Accumulation and APOB Lipid Moiety Secretion

Concentration of TAG was greater in hepatocytes from cells incubated with fatty acids compared with the control ($P < 0.01$; Figure 3A). However, hepatocytes with DGAT1 inhibitor had lower TAG concentration compared with the control ($P < 0.01$; Figure 3A).

Concentration of TAG was lower in hepatocytes from cells with DGAT1 inhibitor + fatty acids compared with the fatty acid group ($P < 0.01$; Figure 3A), whereas TAG concentration in hepatocytes from DGAT2 inhibitor + fatty acids did not differ compared with the fatty acid group (Figure 3A).

Accumulation of lipid droplets in hepatocytes was consistent with changes observed in TAG concentration (Figure 3B), and APOB lipid moiety concentration was lower in supernatant from the fatty acid compared with the control group ($P = 0.014$; Figure 3C). However, there was no change in APOB lipid moiety concentration in supernatant from cells with DGAT1 and DGAT2 inhibitor compared with the control (Figure 3C).

Figure 1. The effect of fatty liver on the abundance of key molecules in lipid synthesis. Liver tissue from healthy cows and cows with fatty liver was collected and decomposed. (A) Relative mRNA abundance of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *APOB*, *MTTP*, *CPT1A*, *GRP78*, and *PERK*; (B) representative western blots of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *APOB*, *MTTP*, *CPT1A*, *GRP78*, and *PERK*; and (C) relative protein abundance of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *APOB*, *MTTP*, *CPT1A*, *GRP78*, and *PERK*. Comparisons among groups were calculated using a one-way ANOVA with a Duncan correction. The data presented are the mean $\pm$ SEM; ** $P \leq 0.01$.

Figure 2. The effect of DGAT1 and DGAT2 inhibitor on lipid synthesis in calf primary hepatocytes. Treatments were a control group (cells cultured in RPMI-1640 basic medium for 12 h), 1.2 mM fatty acids (cells treated with 1.2 mM fatty acids for 12 h), DGAT1 inhibitor (cells treated with 0.3 μ M DGAT1 inhibitor 2 for 12 h), DGAT1 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μ M DGAT1 inhibitor and 1.2 mM fatty acids for 12 h), DGAT2 inhibitor (cells treated with 0.3 μ M PF-06424439 for 12 h), DGAT2 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μ M PF-06424439 and 1.2 mM fatty acids for 12 h). (A) Relative mRNA abundance of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *CPT1A*, *MTTP*, and *APOB*; (B) representative western blots of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *MTTP*, and *APOB*; and (C) relative protein abundance of *SREBF1*, *FASN*, *DGAT1*, *DGAT2*, *MTTP*, and *APOB*. The data of the control were used to normalize other treatments. Treatment comparisons were assessed by one-way ANOVA and Holm's multiple comparison test to determine individual group differences. The data presented are the mean $\pm$ SEM; * $P \leq 0.05$, ** $P \leq 0.01$ indicate differences from control. # $P \leq 0.05$, ## $P \leq 0.01$ indicate differences from fatty acids alone.

Effect of Inhibition of DGAT1 and DGAT2 on Oxidative Stress and Endoplasmic Reticulum Stress

Relative mRNA and protein abundance of GRP78 and protein abundance of PERK in cells with both

DGAT1 and DGAT2 inhibitor with or without fatty acids were greater compared with the control and fatty acid groups ($P < 0.01$; Figure 4A–C).

Concentration of MDA was greater in hepatocytes receiving fatty acids compared with the control group ($P = 0.020$; Figure 4D). However, there were no marked

Figure 3. The effect of DGAT1 and DGAT2 inhibitor on triacylglycerol (TAG) concentration, APOB lipid moiety secretion, and immunofluorescence of lipid droplets in calf primary hepatocytes. Treatments were control group (cells cultured in RPMI-1640 basic medium for 12 h), 1.2 mM fatty acids (cells treated with 1.2 mM fatty acids for 12 h), DGAT1 inhibitor (cells treated with 0.3 μ M DGAT1 inhibitor 2 for 12 h), DGAT1 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μ M DGAT1 inhibitor 2 and 1.2 mM fatty acids for 12 h), DGAT2 inhibitor (cells treated with 0.3 μ M PF-06424439 for 12 h), and DGAT2 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μ M PF-06424439 and 1.2 mM fatty acids for 12 h). (A) TAG content in hepatocytes; (B) immunofluorescence results of lipid droplets; and (C) APOB lipid moiety content in the cell culture supernatant of primary hepatocytes. Immunofluorescence of lipid droplets, scale bar = 25 μ m. Treatment comparisons were assessed by one-way ANOVA and Holm's multiple comparison test to determine individual group differences. The data presented are the mean $\pm$ SEM; * $P \leq 0.05$, ** $P \leq 0.01$ indicate differences from control. ## $P \leq 0.01$ indicate differences from fatty acids alone.

Figure 4. The effect of DGAT1 inhibitor 2 and PF-06424439 on oxidative stress and mitochondrial damage in calf primary hepatocytes culture supernatant. Treatments were a control group (cells cultured in RPMI-1640 basic medium for 12 h), 1.2 mM fatty acids (cells treated with 1.2 mM fatty acids for 12 h), DGAT1 inhibitor (cells treated with 0.3 μM DGAT1 inhibitor 2 for 12 h), DGAT1 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μM DGAT1 inhibitor and 1.2 mM fatty acids for 12 h), DGAT2 inhibitor (cells treated with 0.3 μM PF-06424439 for 12 h), and DGAT2 inhibitor + 1.2 mM fatty acids (cells treated with 0.3 μM PF-06424439 and 1.2 mM fatty acids for 12 h). (A) Relative mRNA abundance of *GRP78* and *PERK*; (B) representative western blots of *GRP78* and *PERK*; (C) relative protein abundance of *GRP78* and *PERK*. (D) malondialdehyde (MDA) content in hepatocytes; (E) results of reactive oxygen species (ROS) flow cytometry; and (F) ROS content in hepatocytes. Treatment comparisons were assessed by one-way ANOVA and Holm's multiple comparison test to determine individual group differences. The data presented are the mean $\pm$ SEM; * $P \leq 0.05$, ** $P \leq 0.01$ indicate differences from control. # $P \leq 0.05$, ## $P \leq 0.01$ indicate differences from fatty acids alone.

changes in MDA concentration in hepatocytes with DGAT1 inhibitor and DGAT2 inhibitor compared with the control (Figure 4D).

Overall, compared with the control, ROS concentration was greater in hepatocytes with DGAT1 inhibitor ($P = 0.030$; Figure 4E–F). In contrast, compared with the control, concentration was lower in hepatocytes with DGAT2 inhibitor ($P < 0.01$; Figure 4E–F). In addition, compared with the fatty acid group, ROS concentration was greater in hepatocytes with DGAT1 ($P < 0.01$) and DGAT2 inhibitor + fatty acids ($P < 0.01$; Figure 4E–F).

DISCUSSION

Dairy cows are often afflicted by disorders related to fatty acid metabolism due to the state of NEB they face around parturition, which causes fatty acid mobili-

zation from adipose tissue and liver lipid accumulation (van den Top et al., 1995; Du et al., 2018a; McFadden and Rico, 2019). In nonruminants, de novo fatty acid synthesis along with uptake of fatty acids from the circulation both contribute to TAG synthesis in the liver (Steenenson et al., 2020). Recently studies with rodents, humans, and nonhuman primates reported that DGAT1 and DGAT2 are the major TAG synthesis regulatory proteins in hepatocytes (Hung et al., 2017; McLaren et al., 2018). Although differences among species might exist, the enzyme DGAT1 appears to prefer exogenous fatty acids for esterification, whereas DGAT2 uses mainly de novo synthesized fatty acids (McLaren et al., 2018). Unlike nonruminant species, mechanisms and specific roles of DGAT isoforms on lipid synthesis and metabolism in liver of dairy cows are still unclear. Thus, the present study evaluated characteristics of DGAT1 and DGAT2, and their related pathways in liver of

Figure 5. Schematic model illustrating the function of DGAT isoforms in regulating fatty acid metabolism in calf primary hepatocytes. Red and green = high and low expression, respectively, in the fatty acid group compared with control group; upward and downward blue arrows = high and low expression, respectively, with DGAT1 inhibition; upward and downward orange arrows: high and low expression, respectively, with DGAT2 inhibition. Mt = mitochondrion; DAG = diacylglycerol; ER = endoplasmic reticulum; FFA = free fatty acids; VLDL = very low-density lipoprotein; ROS = reactive oxygen species.

dairy cows with fatty liver. Furthermore, primary calf hepatocytes were used to investigate more directly the regulation of DGAT1 and DGAT2.

Compared with healthy control cows, the markedly greater abundance of DGAT1 and DGAT2 in cows with fatty liver agrees with a previous study and indicated that *de novo* fatty acid synthesis and fatty acid esterification to form TAG promote lipid accumulation in dairy cows that developed fatty liver (van den Top et al., 1996). The fact that inhibition of DGAT1 markedly increased abundance of SREBF1, FASN, and DGAT2, whereas the opposite occurred when DGAT2 was inhibited, underscored the link between this DGAT and *de novo* fatty acid synthesis. Similar results were reported recently when DGAT2 was inhibited in hepatocytes from rhesus primates (McLaren et al., 2018). Clearly, data seem to suggest that DGAT2 and a lipogenic response can compensate in situations where DGAT1 activity is diminished (Figure 5).

It was noteworthy that, whereas inhibition of DGAT1 reduced TAG concentrations during the fatty acid challenge, the same effect was not observed when DGAT2 was inhibited. Indeed, inhibition of DGAT1 by antisense oligonucleotides prevented development of fatty liver in response to feeding a high-fat diet in mice (Chitraju et al., 2019). This approach to control liver lipid accumulation reportedly had multiple beneficial effects concerning obesity and diabetes phenotypes in mice (Smith et al., 2000). Contrary to these results, research in rodents and primates indicated that DGAT2 plays a more dominant role in hepatic TAG synthesis in rodents compared with primary human, rhesus, or cynomolgus hepatocytes following selective inhibition of DGAT2 (McLaren et al., 2018). Those data led us to suggest that (similar to humans), compared with the response of DGAT2 to the loss of DGAT1, DGAT1 activity might be able to compensate for the loss of DGAT2 activity in dairy cow liver.

It is well established that, compared with nonruminants, the VLDL (initial form of plasma APOB lipid moiety secreted by hepatocytes) secretion rate in ruminants is markedly lower, and especially during conditions that favor accumulation of TAG in the liver (Bauchart, 1993). Thus, the fact that APOB and MTTP abundances were markedly lower in liver tissue of cows with fatty liver explains in part the accumulation of TAG when fatty acids in the circulation increase. It is not entirely surprising that inhibition of DGAT1 and DGAT2 unenhanced APOB lipid secretion in response to exogenous fatty acids, but is important to note that inhibition of DGAT2 was particularly effective in increasing APOB and MTTP abundance after fatty acid challenge. The binding and lipid transfer

activity of APOB and MTTP are one of the major determinants of VLDL secretion (Liu et al., 2014). In normal conditions, APOB synthesis is thought to far exceed its secretion, and when lipid binding decreases (e.g., in the absence of TAG), VLDL assembly decreases and APOB degradation increases (Cano et al., 2011). The reason for a lack of increase in APOB lipid secretion when DGAT2 was inhibited and abundance of APOB increased is not clear. It could be possible other events such as increased APOB degradation or other aspects of VLDL assembly and secretion in response to exogenous fatty acids were negatively affected by lack of DGAT2.

Fatty acid oxidation is a key biological process within the liver that helps regulate not only energy availability to liver cells, but also water-soluble fuels in the form of ketone bodies that peripheral tissues can use (Zhao et al., 2020). Activity of CPT1A regulates the entry of fatty acids into mitochondria before entry into β -oxidation (Li et al., 2013). Previous studies with cows experiencing moderate fatty liver and calf primary hepatocytes demonstrated that high concentration of fatty acids downregulated abundance of CPT1A (Dong et al., 2019). The fact that inhibition of DGAT1, not DGAT2, alleviated the upregulation in abundance of CPT1A induced by exogenous fatty acids suggested that it may play a regulatory role in the channeling of fatty acids among cellular compartments. Studies in mouse liver and isolated hepatocytes reported that inhibition of DGAT1 resulted in greater flow of fatty acids into mitochondria for oxidation, leading to the generation of high levels of ROS, mitochondrial damage, cytochrome release, and apoptosis (Yamaguchi et al., 2008; Cheng et al., 2020). In the present study, inhibition of DGAT1 also increased markedly fatty acid-induced ROS concentrations, suggesting that that DGAT1-regulated TAG synthesis is a major pathway that competes with CPT1A for utilization of fatty acids for FA oxidation and ROS production. The aggravation of ER stress resulting from inhibition of DGAT1 and DGAT2 during the fatty acid challenge suggested that both enzymes (and especially DGAT1) play a dominant role in reducing the concentrations of free fatty acids to protect cells from oxidative damage.

The current studies on the liver metabolic mechanism of adult ruminants mostly rely on calf hepatocytes (Graulet et al., 1998; Fu et al., 2012; Weld et al., 2019; Zhang et al., 2020a). However, study has also shown that lipid metabolism efficiency is related to the age of nonruminant animals (Hahn et al., 2017). Therefore, caution should be exercised when attempting to infer animal liver metabolism in perinatal cows from hepatocytes of 1-d-old calves. Although *in vitro* data sug-

gest that DGAT may affect lipid metabolism and TAG accumulation, the effects of DGAT on perinatal cows metabolism remain to be determined.

CONCLUSIONS

The process of TAG formation in hepatocytes especially when faced with high concentrations of fatty acids entails upregulating both DGAT1 and DGAT2 enzymes, which catalyze the last step in the esterification pathway. Inhibiting these enzymes, especially DGAT1, disrupts lipid homeostasis and results in channeling of fatty acids into mitochondria for oxidation, leading to generation of high levels of ROS along with mitochondrial damage. Thus, the high expression of DGAT1 in hepatocytes from cows experiencing fatty liver seems to play a dominant role in promoting fatty acid storage into TAG and lipid droplets to protect hepatocytes from oxidative damage.

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