High concentrations of fatty acids and β -hydroxybutyrate impair the growth hormone-mediated hepatic JAK2-STAT5 pathway in clinically ketotic cows

Xiliang Du, Yiwei Zhu, Zhicheng Peng, Yuanxu Cui, Qiang Zhang, Zhen Shi, Yuan Guan, Xueying Sha, Taiyu Shen, Yuchen Yang, Xiaobing Li, Zhe Wang, Xinwei Li,¹ and Guowen Liu¹

Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, 5333 Xi'an Road, Changchun, 130062, Jilin, China

ABSTRACT

The hepatic growth hormone (GH)–insulin-like growth factor (IGF)-I axis is essential for regulating intrahepatic lipid metabolism. Ketotic cows are characterized by high blood concentrations of fatty acids and β -hydroxybutyrate (BHB), which display lipotoxicity. The aim of this study was to investigate changes in the hepatic GH–IGF-I axis in ketotic cows and to determine the effects of fatty acids and BHB on the GH–IGF-I axis in calf hepatocytes. Liver and blood samples were collected from healthy ($n = 15$) and clinically ketotic ($n = 15$) cows. Hepatocytes were isolated from calves and treated with various concentrations of GH, fatty acids, and BHB. The results showed that clinically ketotic cows displayed a high blood concentration of GH, a low blood concentration of IGF-I, and decreased hepatic GHR1A expression as well as impaired hepatic Janus kinase 2 (JAK2)–signal transducer and activator of transcription 5 (STAT5) signaling. In vitro, GH treatment induced activation of the JAK2-STAT5 pathway to increase the mRNA expression and secretion of IGF-I in calf hepatocytes. More importantly, treatment with fatty acids or BHB significantly inhibited *GHR1A* mRNA and JAK2 protein expression, as well as the STAT5 phosphorylation level and phospho-STAT5 nuclear translocation; these effects markedly reduced *IGF1* mRNA expression and secretion in calf hepatocytes. In summary, these results indicate that high blood concentrations of fatty acids or BHB can impair the intrahepatic GH-mediated JAK2-STAT5 pathway and downregulate IGF-I expression and secretion in ketotic cows.

Key words: ketotic cows, growth hormone, insulin-like growth factor-I

INTRODUCTION

During the early lactation period, most dairy cows experience a negative energy balance (NEB) caused by decreased DMI and increased demand for energy to support milk production (Xu et al., 2015a,b). Severe NEB initiates fat mobilization and a subsequent increase in the blood concentrations of fatty acids and BHB (Liu et al., 2014), which may result in the development of ketosis and fatty liver. Neuroendocrine responses involving the hypothalamus-pituitary-liver axis may result in significant changes in the secretion of several hormones, including prolactin and growth hormone (GH; Elsasser et al., 2000). These hormones are believed to have a profound effect on the adaptation to NEB (Herd, 2000). Interestingly, postpartum dairy cows with NEB display GH resistance, high blood concentration of GH, and low blood concentration of IGF-I (Radcliff et al., 2006).

Growth hormone is a pituitary hormone that directly or indirectly affects numerous aspects of animal lactation, growth, and reproduction (Lucy et al., 2001; Douglas et al., 2016; Geiger et al., 2016). Hepatocytes are the target cells of GH and are the main source of IGF-I (Thissen et al., 1994; Sheehy et al., 2017). Growth hormone binds growth hormone receptor (GHR), thereby activating Janus kinase 2 (JAK2)–signal transducer and activator of transcription 5 (STAT5) signaling pathways and increasing the expression and synthesis of IGF-I in hepatocytes (Barclay et al., 2011). Furthermore, IGF-I acts as an endocrine factor that controls GH secretion through a negative feedback loop (Le Roith et al., 2001). Therefore, low hepatic expression of GHR could lead to decreased liver IGF-I production and less GH negative feedback (Silva et al., 2017).

The hepatic GH–IGF-I axis is essential in the regulation of intrahepatic lipid metabolism and mammary milk production (Piechotta et al., 2012; Mense et al., 2015; Silva et al., 2015). Studies have shown that dairy cows display downregulated hepatic GHR, increased blood GH concentration, and decreased blood IGF-I

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¹Corresponding authors: liuguowen2008@163.com and lixinwei100@126.com

Table 1. Nutrient composition of the diets

Item	Measurement
Ingredient (%)	
Corn silage	40.00
Corn	35.00
Wheat bran	8.00
Soybean meal	5.00
Sunflower	8.00
NaCl	1.00
Premix ¹	1.80
NaHCO ₃	1.20
Total	100.00
Nutrient composition (% of DM)	
NE _L (MJ/kg)	6.70
CP	15.20
NDF	33.45
ADF	17.20
NFC	40.40
Ca	0.70
P	0.50

¹The premix provided the following per kg of diet: vitamin A 200,000 IU, vitamin D 70,000 IU, vitamin E 1,000 IU, Fe 2,000 mg, Cu 600 mg, Zn 2,400 mg, Mn 1,300 mg, I 6 mg, Co 7 mg.

concentration during the first week of lactation compare with prepartum period (Lucy et al., 2001; Bernier-Dodier et al., 2011; Gross et al., 2011). Additionally, Fenwick et al. (2008) demonstrated that plasma protein and hepatic mRNA for IGF-I were reduced in cows with severe NEB induced by feed restriction on the second day of lactation compared with cows with mild NEB (Fenwick et al., 2008). Thus, these studies demonstrated that the GH-IGF-I axis is uncoupled in early-lactating cows.

Lipotoxicity is defined as lipid- and lipid metabolite-induced lean tissue dysfunction (Erikci and Hotamisligil, 2016). Dairy cows with clinical ketosis display high blood concentrations of fatty acids and BHB. Lucy et al. (2001) reported that GH resistance further promoted lipolysis in adipose tissue and was associated with an increase in fatty acid concentration in the blood during lactation (Lucy et al., 2001). Our previous studies indicated that fatty acids and BHB displayed lipotoxicity in liver tissues (Shi et al., 2014; Song et al.,

2014; Deng et al., 2015; Song et al., 2016); however, the effects of fatty acids and BHB lipotoxicity on the GH-IGF-I axis of calf hepatocytes were not characterized. Therefore, the aim of the current study was to investigate (1) changes in the hepatic GH-IGF-I axis in ketotic cows and (2) the effects of fatty acids and BHB on the GH-IGF-I axis in calf hepatocytes, as well as the mechanisms underlying these effects.

MATERIALS AND METHODS

Animals

The Ethics Committee on the Use and Care of Animals of Jilin University approved the study protocol (Changchun, China; 2015 clinical trial [2015–121]). To ensure that the cows used in this study had no comorbidities, all cows received a routine physical examination. A TMR that met the animals' nutritional requirements was fed ad libitum once per day at 1100 h; the basal diet formulation is shown in Table 1. We chose lactating Holstein cows with similar numbers of lactations (median = 3, range = 2 to 4) and DIM (median = 6 d; range = 3 to 9 d) from a 1,000-cow dairy farm located in Changchun, Jilin Province, China. The cows were classified as suspected clinical ketosis by veterinarians if feed intake, milk yield, or both were reduced and a nitroprusside test for ketone bodies in milk was positive (Duffield et al., 2009; Piechotta et al., 2012); subsequently, the blood concentration of BHB in these cows was measured. According to the clinical symptoms and serum BHB concentration (Oetzel, 2004), 15 clinically ketotic cows whose serum BHB concentration were higher than 3 mM and 15 healthy cows whose serum BHB concentration were less than 0.6 mM were chosen for the experiments. Characteristics of the ketotic and healthy cows are presented in Table 2.

The milk yield was recorded on 3 consecutive days at 0530 and 1500 h. Blood samples were withdrawn from the jugular vein between 0800 and 0900 h and centrifuged at $3,500 \times g$ for 15 min at 4°C. Blood was

Table 2. The basic description of healthy and ketotic cow (data were analyzed using non-parametric tests)

Item	Ketosis (n = 15)		Control (n = 15)		P-value
	Median	Interquartile range	Median	Interquartile range	
BW (kg)	657	648, 665	625	589, 639	0.0002
Milk production (kg of milk/cow per day)	25.6	24.3, 26.4	30	29.3, 31.2	<0.0001
DMI (kg/d)	20.1	19, 20.5	21.8	20.2, 22.6	0.0020
BCS	3.16	3.05, 3.2	2.71	2.54, 2.74	<0.0001
Glucose (mM)	2.17	2.05, 2.34	4.13	4.01, 4.27	<0.0001
Fatty acids (mM)	1.29	1.11, 1.47	0.27	0.18, 0.3	<0.0001
BHB (mM)	3.87	3.6, 4.04	0.36	0.27, 0.38	<0.0001

collected from all of the cows in the study at the same time. The serum was obtained and stored at -80°C until analysis. Liver tissue samples were taken from the 11th or 12th right intercostal space by liver puncture needle (Shanghai Surgical Equipment Factory, Shanghai, China) after blood collection (Radcliff et al., 2003). Prior to liver biopsy, the intercostal space was shaved, sanitized with iodine scrub and 75% alcohol, and anesthetized by subcutaneous injection of 2% lidocaine HCl (Sigma-Aldrich Co., St. Louis, MO). A scalpel blade was used to make a 3-mm stab incision in the skin. The puncture needle was then inserted through the intercostal muscle and into the liver. The liver tissue biopsies (approximately 150 mg) were immediately frozen in liquid nitrogen. During the experimental work, the cows were housed in a climate-controlled barn in individual tiestalls.

Determination of Blood Parameters

The concentration of BHB in the blood was measured using a Medisense Optium (Abbott Diabetes Care Inc., Alameda, CA; farm assay) with BHB test strips (Abbott Diabetes Care Inc.). The serum concentrations of glucose, BHB, and fatty acids were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) and a commercially available kit [glucose: Cat. No. GL3815; BHB: Cat. No. RB1008; fatty acids: Cat. No. FA115 (Randox Laboratories, Crumlin, UK)] according to the manufacturer's instructions (Silva et al., 2017). The concentrations of GH and IGF-I in serum and medium were determined by RIA (Hammon and Blum, 1997; Egli and Blum, 1998). The antibody against bovine GH was purchased from Abcam (Cambridge, UK; ab31496). The intra- and interassay coefficients of variation for the GH and IGF-I assays were less than 5%.

Hepatocyte Isolation and Culture

Calf hepatocytes were isolated using a modified 2-step collagenase perfusion method as previously described (Liu et al., 2014; Du et al., 2017a,b). Five calves (1 d old, female, 30–40 kg, healthy, and fasting) were used for hepatocyte isolation in this experiment. Hepatocytes were isolated from each calf separately and used in independent experiments. The caudate liver lobes of Holstein calves were obtained through surgical liver excision according to the method used in a previous study (Parker and Gaughan, 1988). Blood was removed from the surface of the caudate lobe by rinsing with perfusion solution A (140 mM NaCl, 10 mM HEPES, 6.7 mM KCl, 0.5 mM EDTA, and 2.5 mM glucose, pH 7.2–7.4). A blood vessel of moderate size was intubated,

and the liver was perfused with perfusion solution A (37°C) at a flow rate of 50 mL/min for 12 min. The liver was then perfused with solution B (140 mM NaCl, 30 mM HEPES, 6.7 mM KCl, 5 mM CaCl_2 , and 2.5 mM glucose, pH 7.2–7.4) at a flow rate of 50 mL/min for 3 min until the perfusate was clear. Subsequently, the liver was perfused with a collagenase IV solution (0.1 g of collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2–7.4) at a flow rate of 20 mL/min for 15 to 20 min. The liver was then placed on a sterile flat plate and 100 mL of fetal bovine serum was added to terminate the collagenase digestion. The liver was cut open and the liver capsule, blood vessels, fat, and connective tissue were removed using forceps and scissors. The liver parenchyma was filtered sequentially through 100- (150- μm) and 200-mesh (75- μm) cell sieves. The hepatocyte suspension was washed twice in RPMI-1640 basic medium (Hyclone Laboratories, Logan, UT) and centrifuged for 5 min at $500 \times g$ at 4°C . The hepatocyte suspension was seeded into a 6-well tissue culture plate (2 mL/well) at 1×10^6 cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% fetal bovine serum, 10^{-6} mol/L of insulin, 10^{-6} mol/L of dexamethasone, and 10 $\mu\text{g/mL}$ of vitamin C). The hepatocytes were incubated at 37°C in 5% CO_2 . After 4 h, the medium was replaced with growth medium containing 10% fetal bovine serum. The growth medium was replaced every 24 h.

Hepatocyte Treatments

Prior to treatment, the hepatocytes were cultured for 6 h without serum. Hepatocytes were maintained in RPMI-1640 basic medium and treated with 0, 5, 10 or 20 ng/mL GH (ProSpec, East Brunswick, NJ) for 3 h. The concentrations of fatty acids and BHB used in the treatments in our study were based on the concentrations of fatty acids and BHB in the blood of clinically ketotic cows (Rukkwamsuk et al., 2000; Oetzel, 2004). The fatty acid components (oleic acid, linoleic acid, palmitic acid, stearic acid, and palmitoleic acid; Sigma-Aldrich) used in this study were chosen based on the fatty acids found in the blood of ketotic cows (Yam-dagni and Schultz, 1970). The fatty acids were diluted in 0.1 M KOH at 60°C and the pH of the fatty acid solution was adjusted to 7.4 with hydrochloric acid (1 M). The stock fatty acid (52.7 mM) solution included oleic acid (22.9 mM), linoleic acid (2.6 mM), palmitic acid (16.8 mM), stearic acid (7.6 mM), and palmitoleic acid (2.8 mM). When hepatocytes were co-treated with GH and fatty acids, hepatocytes were treated with 10 ng/mL of GH for 3 h and the medium was renewed, and then hepatocytes were maintained in RPMI-1640 basic

medium containing 2% BSA and treated with 0, 0.6, 1.2, or 2.4 mM fatty acids for 12 h. The BHB was used in its sodium salt form as sodium β -hydroxybutyrate (Sigma-Aldrich); BHB powder was dissolved in distilled water and sterilized by filtration. When hepatocytes were co-treated with GH and BHB, hepatocytes were treated with 10 ng/mL of GH for 3 h and the medium was renewed, and then hepatocytes were maintained in RPMI-1640 basic medium and treated with 0, 1.2, 2.4, or 4.8 mM BHB for 12 h. The cell treatments and detailed groupings are described in the figure legends.

Quantitative Real-Time PCR Assay

The total RNA of hepatocytes and liver tissues was extracted using RNAiso Plus (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions (<http://www.takarabiomed.com.cn/Download/9108-9109.pdf>). The RNA concentration and quality were measured using a K5500 MicroSpectrophotometer (Beijing Kaiuo Technology Development Ltd., Beijing, China) and by electrophoresis (1% agarose gels). One microgram of the total RNA in each sample was then reverse-transcribed to cDNA in 20- μ L reactions using a reverse transcription kit (TaKaRa Biotechnology Co. Ltd.) according to the supplier's protocol (<http://www.takarabiomed.com.cn/Download/RR036A.pdf>). We evaluated mRNA expression levels using quantitative real-time (qRT)-PCR technology with the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co. Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems Inc., Waltham, MA). The qRT-PCR was conducted with initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s), and extension at 72°C for 5 min. The PCR efficiency (E) and correlation coefficient were determined based on the slope of the standard curves generated using serial 10-fold dilutions of sample cDNA. The efficiency was calculated as $E (\%) = (10^{-1/\text{slope}} - 1) \times 100$ (Kubista et al., 2006). The relative expression of each target gene was normalized to β -actin and calculated by a mathematical model, which includes an efficiency correction for PCR efficiency (Pfaffl, 2001). The PCR reaction was performed in triplicate for each cow and 15 cows were included in per group; PCR reaction was performed 15 times in each group. The primers used for GHR, IGF-I, and β -actin are shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13234>); β -actin was stably expressed in different groups (Supplemental Figure S1, <https://doi.org/10.3168/jds.2017-13234>; Morey et al.,

2011; Li et al., 2012). The efficiency of used primers was added in the Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-13234>).

Western Blotting Assay

Western blotting was performed as previously described (Dessauge et al., 2011). Briefly, the protein concentrations in extracts of hepatocytes and liver tissues were determined using a protein assay kit (Sangon Biotech Co. Ltd., Shanghai, China) after extraction of the hepatocytes and tissues according to the manufacturer's instructions (http://www.sangon.com/productImage/DOC/C510003/C510003_ZH_P.pdf). A total of 30 μ g of protein from each sample was separated by 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 3% BSA/Tris-buffered saline/Tween (TBS-T) buffer for 4 h. The blocked membranes were incubated overnight at 4°C with primary antibodies against GHR1A (1:200; Cat. sc-20748; Santa Cruz Biotechnologies, Santa Cruz, CA), JAK2 (1:1,000; Cat. 4040), phospho (p)-STAT5 (Tyr694; 1:1,000; Cat. 4322; Cell Signaling Technology, Danvers, MA), STAT5 (1:500; Cat. ab68465), and β -actin (1:2,000; Cat. ab8226; Abcam). The membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (ECL, Beyotime Biotechnology Inc., Shanghai, China). Finally, the bands were imaged using a ProteinSimple imager (ProteinSimple, Santa Clara, CA). The phosphorylation levels of STAT5 were calculated as p-STAT5/total STAT5.

Immunofluorescence Assay

An immunofluorescence assay was performed as previously described (Song et al., 2016). Hepatocytes were divided into 4 groups: control group, GH treatment group (hepatocytes were treated with 10 ng/mL of GH for 3 h), GH+fatty acids group (hepatocytes were treated with 10 ng/mL GH for 3 h and the medium was renewed, and then hepatocytes were treated with 1.2 mM fatty acids for 12 h), and GH+BHBA group (hepatocytes were treated with 10 ng/mL of GH for 3 h and the medium was renewed, and then hepatocytes were treated with 4.8 mM BHB for 12 h). The hepatocytes were fixed with 10% formalin in 0.1% PBS for 20 min. Antigen retrieval was performed using EDTA- Na_2 at 95°C for 5 min. The hepatocytes were permeabilized us-

ing 0.1% Triton X-100 (Sigma-Aldrich) and incubated with an antibody to p-STAT5 (diluted 1:100 with 0.1% PBS containing 5% goat serum) overnight at 4°C. The hepatocytes were then incubated for 30 min at room temperature with secondary antibodies conjugated to Cy3 (Beyotime, Beyotime Institute of Biotechnology, Jiangsu, China). We stained the hepatocyte nuclei with Hoechst 33258 (Beyotime) for 20 min. The coverslips were then sealed with glycerol and the samples were imaged using laser confocal microscopy (Fluoview FV1200, Olympus, Japan). The immunofluorescence experiments were repeated 3 times; each time, at least 3 zones were selected for measurement. Image-Pro Plus software (Media Cybernetics, Rockville, MD) was used to quantify the immunofluorescent signals.

Statistical Analysis

In the *in vivo* studies, data on baseline characteristics and data from Western blots and qRT-PCR were analyzed using nonparametric tests and paired *t* tests, respectively. In the *in vitro* studies, comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. All analyses were performed using GraphPad Prism 5.0 (Graph Pad Software, San Diego, CA) or SPSS (Statistical Package for the Social Sciences) 19.0 software (IBM, Armonk, NY). Baseline characteristics are expressed as the median and interquartile range, and other data are expressed as the mean $\pm$ SEM; *P* < 0.05 was considered statistically significant.

RESULTS

Ketotic Cows Displayed Uncoupled GH-Mediated JAK2-STAT5 Pathway

As shown in Table 2, the milk production, DMI, and blood concentration of glucose were significantly lower in ketotic cows than in control cows. Conversely, the blood concentrations of fatty acids and BHB were significantly higher in ketotic cows than in control cows.

We found that the blood concentration of GH was significantly higher in clinically ketotic cows than in control cows (Figure 1A). Conversely, the blood concentration of IGF-I was significantly decreased in clinically ketotic cows compared with control cows (Figure 1B). To further investigate the hepatic GH-IGF-I axis, the GH-mediated JAK2-STAT5 signaling pathway in the livers of clinically ketotic and healthy cows was measured. The mRNA and protein expression of hepatic *GHR1A* were significantly lower in clinically ketotic cows than in control cows (Figure 1C, D and

E). The protein level of JAK2 and the phosphorylation level of STAT5 were also significantly decreased in the livers of clinically ketotic cows (Figure 1D, F and G); consequently, the hepatic *IGF1* mRNA level was lower in clinically ketotic cows than in control cows (Figure 1H). Taken together, these results indicate that the GH-mediated JAK2-STAT5 signaling pathway is less sensitive in ketotic compared with healthy cows.

GH Activated JAK2-STAT5 Signaling Pathway and Increased Production of IGF-I

Calf hepatocytes were treated with GH at 0, 5, 10, or 20 ng/mL. The cell viability was not decreased by GH treatment (Supplemental Figure S2; <https://doi.org/10.3168/jds.2017-13234>). Following treatment with GH, the *GHR1A* mRNA level increased gradually in a GH dose-dependent manner; it was significantly higher in the 10 and 20 ng/mL GH treatment groups than in the control group (Figure 2A). Treatment with GH also significantly increased the JAK2 protein level and the STAT5 phosphorylation level in the hepatocytes (Figure 2B, C and D). More importantly, the *IGF1* mRNA level in the hepatocytes and the content of IGF-I in the medium also increased gradually, and both were markedly higher in the 10 and 20 ng/mL GH treatment groups than in the control group (Figure 2E and F). Together, these data suggest that GH activated the JAK2-STAT5 signaling pathway and subsequently increased the production of IGF-I in calf hepatocytes.

High Concentrations of Fatty Acids Inhibited GH-IGF-I Axis in Calf Hepatocytes

To investigate the effect of high concentrations of fatty acids on the GH-IGF-I axis, calf hepatocytes were treated with GH and fatty acids. We also performed the control experiments using different volumes of fatty acid solvent (0.01 M KCl; 0, 22.78, 45.54, and 91.08 μ L), and the results showed that different solvent volumes had no effects on cell viability or mRNA expression of *GHR1A* and *IGF1* (Supplemental Figure S3; <https://doi.org/10.3168/jds.2017-13234>). The *GHR1A* mRNA level was significantly decreased in the fatty acid and GH co-treatment groups compared with the GH treatment group (Figure 3A). Similarly, the levels of JAK2 protein expression and STAT5 phosphorylation were markedly decreased in the fatty acid and GH co-treatment groups (Figure 3B, C and D). To confirm the effect of fatty acids on the GH-mediated JAK2-STAT5 pathway in calf hepatocytes, the expression and nuclear translocation of p-STAT5 were determined using an immunofluorescence assay. Treatment with GH

significantly increased the fluorescence intensity and the nuclear translocation of p-STAT5 in calf hepatocytes (Figure 3G and H). Importantly, fatty acid treatment significantly decreased the fluorescence intensity and the nuclear translocation of p-STAT5 (Figure 3G and H). More importantly, fatty acids also significantly decreased the *IGF1* mRNA level in the hepatocytes and the content of IGF-I in the medium (Figure 3E and F). Taken together, these results show that high concentration of fatty acids significantly inhibit the GH-mediated JAK2-STAT5 signaling pathway and further downregulate the expression of IGF-I.

High Concentrations of BHB Inhibited the GH-IGF-I Axis in Calf Hepatocytes

Calf hepatocytes were treated with GH and BHB. The level of *GHR1A* mRNA was significantly decreased in the BHB and GH co-treatment groups compared with the GH treatment group (Figure 4A). The JAK2 protein level and the STAT5 phosphorylation level were also markedly decreased in the BHB and GH co-treatment groups (Figure 4B, C and D). Importantly, BHB treatment significantly decreased the fluorescence intensity and the nuclear translocation of p-STAT5

Figure 1. Changes in the growth hormone (GH)-IGF-I axis and the hepatic activating janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) signaling pathway in ketotic and healthy cows. (A) Blood concentration of GH in ketotic ($n = 15$) and healthy ($n = 15$) cows. (B) Blood concentration of IGF-I in ketotic ($n = 15$) and healthy ($n = 15$) cows. (C) Relative growth hormone receptor 1A (*GHR1A*) mRNA levels in ketotic ($n = 15$) and healthy ($n = 15$) cows. (D) Hepatic protein levels of *GHR1A*, JAK2, phospho (p)-STAT5, STAT5, and β -actin in ketotic ($n = 15$) and healthy ($n = 15$) cows. Representative blots are shown. (E, F, G) Quantification of hepatic protein levels of *GHR1A*, JAK2, and p-STAT5. (H) Relative *IGF1* mRNA levels in ketotic ($n = 15$) and healthy ($n = 15$) cows. The mRNA expression levels of the ketosis group were normalized to control group. Panels A and B were analyzed using nonparametric tests; panels C, E, F, G, and H were analyzed using paired t tests. The data are presented as mean $\pm$ SEM.

Figure 2. Effects of growth hormone (GH) on the activating janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) signaling pathway in calf hepatocytes. Calf hepatocytes were treated with various concentrations of GH (0, 5, 10, and 20 ng/mL). (A) Relative levels of growth hormone receptor 1A (*GHR1A*) mRNA in calf hepatocytes. (B) Protein levels of JAK2, phospho (p)-STAT5, STAT5, and β-actin in calf hepatocytes. (C and D) Quantification of protein levels of JAK2 and p-STAT5. (E) Relative levels of *IGF1* mRNA in calf hepatocytes. (F) IGF-I content of the medium. The mRNA expression levels of the hepatocyte treatment groups were normalized to control group. Data were analyzed using a 1-way ANOVA with subsequent Bonferroni correction. The data are presented as mean ± SEM. * $P < 0.05$; ** $P < 0.01$.

(Figure 3G and H). In addition, the *IGF1* mRNA level and the IGF-I content of the medium was significantly lower in hepatocytes co-treated with GH and BHB than in hepatocytes treated with GH alone (Figure 4E and F). These results indicate that high concentrations of BHB also significantly inhibited the GH-mediated JAK2-STAT5 pathway and further downregulated the expression of IGF-I.

DISCUSSION

The change in nutrient metabolism required to support lactation in high-producing dairy cows is controlled by hormones that coordinate a variety of processes, including the mobilization of fatty acids from adipose tissue and the synthesis of glucose in the liver (Radcliff et al., 2003). Therefore, dramatic endocrine changes occur during the transition period in dairy cows to regulate the required metabolic changes (Gross et al., 2011). Previous studies have shown that blood GH concentration increases shortly after parturition and that this increase coincides with a precipitous decline in blood IGF-I concentration (Hammon and Blum, 1997; Kobayashi et al., 1999; Radcliff et al., 2006; Piechotta et

al., 2012). The increase in blood GH promotes lipolysis in adipose tissue (Radcliff et al., 2003). Results obtained in mice showed that impairment of the GH-IGF-I axis was the response for liver steatosis and that inhibition of endogenous GH signaling could induce disorders in hepatic lipid metabolism (Fan et al., 2014). Interestingly, Radcliff et al. (2006) reported that postpartum dairy cows with NEB induced by feed restriction on the first day of lactation displayed high blood concentrations of GH and low blood concentrations of IGF-I (Radcliff et al., 2006). We also found that the blood concentration of GH was significantly higher and the blood concentration of IGF-I was significantly lower in clinically ketotic cows than in control cows. A decrease in blood IGF-I level reduces negative feedback for GH synthesis and secretion (Romero et al., 2012), and the loss of negative feedback results in an increase in blood GH concentration (Lundberg et al., 2015). The increase in blood GH promotes lipolysis in adipose tissue and further increases blood fatty acid concentration (Corbit et al., 2017). Moreover, a high blood concentration of fatty acids is a pathological factor of ketosis; fatty acids absorbed by the liver from plasma are metabolized into ketone bodies (BHB, acetoacetate, and acetone) in

hepatocytes. Therefore, uncoupling of the somatotrophic axis may be a cause of ketosis.

The actions of GH are mediated via the GHR, and at least 3 alternative forms of *GHR* mRNA exist in cattle (GHR1A, 1B, and 1C; Radcliff et al., 2003). The liver-specific *GHR1A* mRNA is believed to give rise to the

bulk of functionally active GHR in the liver (Radcliff et al., 2003). One study reported that *GHR1A* mRNA expression decreased significantly between d 0 and 1 and then decreased further from d 1 to 2 after isolation of adult primary bovine hepatocytes (Ehrhardt and Schmicke, 2016). Our results showed that *GHR1A*

Figure 3. Effects of fatty acids on the activating janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) signaling pathway and on IGF-I production in calf hepatocytes. Hepatocytes were treated with 10 ng/mL of growth hormone (GH) for 3 h and the medium was renewed, and then hepatocytes were maintained in RPMI-1640 (Hyclone Laboratories, Logan, UT) basic medium containing 2% BSA and treated with 0, 0.6, 1.2, or 2.4 mM for 12 h. (A) Relative levels of growth hormone receptor 1A (*GHR1A*) mRNA in calf hepatocytes. (B) Protein levels of JAK2, phospho (p)-STAT5, STAT5, and β -actin in calf hepatocytes. (C and D) Quantification of protein levels of JAK2 and p-STAT5. (E) Relative levels of *IGF1* mRNA in calf hepatocytes. (F) IGF-I content of the medium. (G and H) Fluorescence intensity and nuclear translocation of p-STAT5 in calf hepatocytes. The mRNA expression levels of the hepatocyte treatment groups were normalized to control group. Data were analyzed using a 1-way ANOVA with subsequent Bonferroni correction. The data are presented as mean $\pm$ SEM. $^{**}P < 0.01$. Color version available online.

Figure 4. Effects of BHB on the activating janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) signaling pathway and on IGF-I production in calf hepatocytes. Hepatocytes were treated with 10 ng/mL of growth hormone (GH) for 3 h and the medium was renewed, and then hepatocytes were maintained in RPMI-1640 (Hyclone Laboratories, Logan, UT) basic medium and treated with 0, 1.2, 2.4, or 4.8 mM BHB for 12 h. (A) Relative levels of growth hormone receptor 1A (*GHR1A*) mRNA in calf hepatocytes. (B) Protein levels of JAK2, phospho (p)-STAT5, STAT5, and β -actin in calf hepatocytes. (C and D) Quantification of protein levels of JAK2 and p-STAT5. (E) Relative levels of *IGF1* mRNA in calf hepatocytes. (F) IGF-I content of the medium. The mRNA expression levels of the hepatocyte treatment groups were normalized to control group. (G) Proposed model of the GH-IGF-I axis and the JAK2-STAT5 signaling pathway in calf hepatocytes. Excess fatty acids and BHB in the liver decrease *GHR1A* expression, which in turn decrease JAK2 expression and the expression and nuclear translocation of p-STAT5, resulting in reduced IGF-I synthesis and secretion. Data were analyzed using a 1-way ANOVA with subsequent Bonferroni correction. The data are presented as mean $\pm$ SEM. ** $P < 0.01$. Color version available online.

mRNA expression in calf hepatocytes decreased significantly between d 0 and 1 and then remained constant from d 1 through 3 (Supplemental Figure S4; [https://](https://doi.org/10.3168/jds.2017-13234)

doi.org/10.3168/jds.2017-13234). The discrepancies between the results obtained in our study and previous studies may have been caused by differences between

calf hepatocytes and adult bovine hepatocytes. Besides, several differences exist between cow and calf hepatocytes, including cell viability, cell function, and hormone sensitivity. Therefore, to investigate the effects of GH on adult primary bovine hepatocytes, additional studies will be necessary.

Growth hormone binds GHR, resulting in activation of JAK2 and phosphorylation of STAT5. Phosphorylated STAT5 translocates to the nucleus, where it activates transcription of the *IGF1* gene (Hanson et al., 2017). Our experiments also showed that calf hepatocytes treated with GH exhibited a significant increase in *GHR1A* mRNA expression, JAK2-STAT5 pathway activation, and IGF-1 secretion. Furthermore, Silva et al. (2017) also demonstrated that treatment of postpartum cows with 125 mg of recombinant bST upregulated hepatic *GHR1A* mRNA expression and increased IGF-I concentration by approximately 30% (Silva et al., 2017). Therefore, our data and the data reported in the previous study both suggest that GH regulates IGF-I expression and secretion. However, the GH-mediated JAK2-STAT5 signaling pathway in the liver tissue of ketotic cows has not been investigated. In our study, hepatic *GHR1A* mRNA and protein expression as well as the IGF-I concentration in blood were significantly decreased in clinically ketotic cows. More importantly, the hepatic JAK2 protein level and the STAT5 phosphorylation level were also markedly decreased. These data indicate that the hepatic GH-IGF-I axis was uncoupled and that the JAK2-STAT5 signaling pathway was impaired in clinically ketotic cows.

Dairy cows with ketosis are characterized by high blood concentrations of fatty acids and BHB. Our previous studies demonstrated that high levels of fatty acids and BHB induce hepatic oxidative stress, apoptosis, and inflammation (Shi et al., 2014; Song et al., 2014, 2016; Deng et al., 2015). Therefore, we speculated that high blood concentrations of fatty acids and BHB might impair the GH-induced JAK2-STAT5 pathway and further affect the expression and synthesis of IGF-I in hepatocytes. To address this question, calf hepatocytes were cultured and treated with GH, fatty acids, or BHB. Our results showed that treatment of hepatocytes with high concentrations of fatty acids or BHB markedly downregulated *GHR1A* expression, JAK2 protein level, and STAT5 phosphorylation. Importantly, we found that treatment with fatty acids or BHB significantly inhibited the nuclear translocation of p-STAT5. The decreased expression and nuclear translocation of p-STAT5 caused reduced expression of IGF-I in calf hepatocytes. The inhibitory effects of fatty acids and BHB on hepatic IGF-I expression are consistent with the low blood concentration of IGF-I in ketotic cows.

In a previous study, it was reported that SFA (palmitic acid and myristic acid) inhibit the activity of the *GHR* gene promoter and that this effect is mediated by direct action of the fatty acids (Thimmarayappa et al., 2006). Palmitic acid is an important component of fatty acids and the specific mechanism of BHB-reduced *GHR1A* expression has never been reported. Taken together, our results and those of previous studies indicate that high concentrations of fatty acids and BHB inhibit *GHR1A* expression and JAK2-STAT5 pathway both directly and indirectly in the hepatocytes of ketotic cows and subsequently decrease the expression and secretion of IGF-I. The results also demonstrate that ketosis leads to impairment of the GH signal transduction pathway. Low concentrations of IGF-I attenuate the negative feedback for GH secretion and increase GH release, which further promotes lipolysis of adipose tissue and augments fatty acids and BHB concentrations in blood. This vicious cycle further promotes the development of ketosis in dairy cows.

In summary, ketotic cows displayed high blood concentrations of fatty acids, BHB, and GH and low blood concentrations of IGF-I. The hepatic GH-mediated JAK2-STAT5 pathway was uncoupled in ketotic cows. In vitro, treatment of hepatocytes with high concentrations of fatty acids or BHB downregulated *GHR1A* expression and impaired the JAK2-STAT5 pathway, and these events subsequently inhibited the mRNA expression and secretion of IGF-I in calf hepatocytes (Figure 4G). These findings provide valuable information that increases our understanding of the molecular mechanisms of ketosis in dairy cows.

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