Hepatic nuclear factor kappa B signaling pathway and NLR family pyrin domain containing 3 inflammasome is over-activated in ketotic dairy cows

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ABSTRACT

Ketosis is an important metabolic disease that can negatively affect the production efficiency of dairy cows. Earlier studies have revealed metabolic and inflammatory alterations in the blood associated with ketosis; however, a link between ketosis and hepatic inflammation has not been well documented. The objective of this study was to investigate whether the nuclear factor kappa B (NF- κ B) signaling pathway and NLR family pyrin domain containing 3 (NLRP3) inflammasome were activated in the liver of ketotic cows. Liver and blood samples were collected from healthy ($n = 15$, control group) and ketotic ($n = 15$, ketosis group) cows that had a similar number of lactations (median = 3, range = 2 to 4) and days in milk (median = 6 d, range = 3 to 9 d). Results showed that serum levels of fatty acids, β -hydroxybutyrate (BHB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were higher and glucose was lower in ketotic cows. Concentrations of serum proinflammatory cytokines IL18, tumor necrosis factor (TNF)- α , and IL1B were greater and the anti-inflammatory cytokine IL10 was lower in the ketosis group. Cows with ketosis had triacylglycerol accumulation in the liver. Upregulation of phosphorylated (p)-NF- κ B and p-inhibitor of κ B (I κ B) α protein abundance in cows with ketosis indicated that the hepatic NF- κ B signaling pathway was overactivated. The mRNA abundance of *TNFA*, inducible nitric oxide synthase (*NOS2*), *IL18*, and *IL1B* were greater and *IL10* was lower in ketotic cows. More importantly, the mRNA and protein abundance of NLRP3 and caspase-1 (CASP1) along with CASP1 activity were greater in the liver of cows with ketosis.

Overall, the data indicate that the onset of ketosis is accompanied by activation of the NF- κ B signaling pathway and NLRP3 inflammasome, resulting in a state of inflammation.

Key words: ketosis, inflammation, nuclear factor- κ B signaling, NLR family pyrin domain containing 3 inflammasome (NLRP3) inflammasome

INTRODUCTION

During the transition from late gestation to early lactation, the vast majority of high-yielding cows suffer from metabolic stress resulting in large part from negative energy balance (Looor et al., 2013; Xu et al., 2016). Mobilization of adipose tissue due to negative energy balance leads to fatty acid release into blood and uptake primarily by the liver (Yang et al., 2018). A large portion of fatty acids in the liver are incompletely oxidized to ketone bodies, with BHB and acetoacetic acid being the most quantitatively important. Excessive ketone body accumulation in the blood leads to the onset of ketosis (Zhang et al., 2018), a disorder that occurs with high frequency during the transition period (Shi et al., 2015). Recent data revealed that serum concentrations of inflammatory biomarkers including haptoglobin, serum amyloid A, and tumor necrosis factor (TNF)- α were elevated in ketotic cows (Abuajamieh et al., 2016; El-Deeb and El-Bahr, 2017). Thus, systemic inflammation seems to be another feature of the ketotic state. Whether tissues such as the liver experience a state of inflammation during ketosis is not well known.

Nuclear factor kappa B (NF- κ B) is known for its function in immunity, inflammation, and metabolic diseases (Taniguchi and Karin, 2018). In its inactive form, NF- κ B is sequestered in the cytoplasm bound to the inhibitor of κ B (I κ B), whose phosphorylation is dependent on the activity of an inhibitor of κ B kinase (IKK β , Oeckinghaus et al., 2011). Once stimulated, NF- κ B unit p65 separates from I κ B and translocates into the nucleus, where NF- κ B can regulate the tran-

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scription of several inflammatory genes such as *TNFA* and pro-interleukin *IL1B* (Taniguchi and Karin, 2018; Cui et al., 2019). High-fat feeding in mice and non-alcoholic fatty liver disease (NAFLD) in humans are associated with overactivation of the NF- κ B signaling pathway in the liver (Arkan et al., 2005; Cai et al., 2005; Farrell and Larter, 2006). We recently demonstrated that a high concentration of fatty acids activated NF- κ B signaling and induced lipid accumulation in dairy cow hepatocytes (Li et al., 2015). These data indicate that activation of NF- κ B signaling might aggravate hepatic lipid metabolism in ketotic conditions but the activity of this pathway has not been evaluated in cows with ketosis.

The activation of NF- κ B not only upregulates numerous inflammatory cytokines and cytokine precursors but also primes NLR family pyrin domain containing 3 (NLRP3), which is important for inflammasome activation (Zhong et al., 2016). The NLRP3 inflammasome contains NOD-like receptor NLRP3, the adaptor molecule apoptosis-associated speck-like protein containing CARD (ASC), and the effector molecule pro-caspase-1 (pro-CASP1, Meng et al., 2019). An increase in hepatic NLRP3 inflammasome activation was detected in mice and humans with metabolic disorders such as type 2 diabetes mellitus, NAFLD, and obesity (De Nardo and Latz, 2011; Stienstra et al., 2011; Vandanmagsar et al., 2011). Inhibition of the NLRP3 inflammasome in mice fed a high-fat diet prevented the development of NAFLD (Yang et al., 2016). Together, these studies demonstrate that NLRP3 inflammasome activation is involved in the development of disorders of hepatic lipid metabolism. Whether the NLRP3 inflammasome is associated with ketosis in dairy cows is unknown.

Because of the high prevalence of ketosis in periparturient cows and given the role of NF- κ B signaling and NLRP3 inflammasome in hepatic metabolism, the objective of this study was to investigate the activation

status of these pathways via mRNA and protein abundance analyses of liver tissue from healthy and ketotic cows.

MATERIALS AND METHODS

Animals

Use of cows in this study was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China, 2017; clinical trial 201711039). The cows in the present study underwent a thorough clinical examination by the same veterinarian to ensure that the cows had no comorbidities with ketosis. All of the cows were housed in a tiestall barn and offered a TMR at 0700 and 1330 h daily for ad libitum intake, with free access to tap water. The basal diet formulation is given in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-16706>). Cows with similar numbers of lactations (median = 3, range = 2 to 4) and DIM (median = 6 d, range = 3 to 9 d) were selected from a 1,000-cow dairy farm located in Changchun, Jilin Province, China. Cows were preliminarily classified based on the degree of feed intake, milk yield, and positive ketone bodies in milk, as assessed by the nitroprusside test. Subsequently, the blood concentration of BHB was measured in order to select cows for further work. According to clinical symptoms and BHB level in the serum, 15 cows with serum BHB content >3 mM were classified as ketotic and 15 cows with serum BHB content <1.2 mM were classified as healthy (McArt et al., 2012; Vanholder et al., 2015). The basic description of the cows used is shown in Table 1.

Sample Collection

Milk yield was recorded on 3 consecutive days at 0530 and 1500 h. Milk samples (50 mL) were collected

Table 1. Basic description of the control and ketotic cows; data were analyzed using Wilcoxon signed-rank test and expressed as mean $\pm$ SEM

Item	Control (n = 15)	Ketosis (n = 15)	P-value
BW (kg)	637.53 $\pm$ 6.52	678.50 $\pm$ 6.84	0.0001
BCS	2.87 $\pm$ 0.03	3.18 $\pm$ 0.08	<0.0001
Milk yield (kg)	30.80 $\pm$ 0.34	24.62 $\pm$ 0.35	<0.0001
DMI (kg)	22.40 $\pm$ 0.39	19.24 $\pm$ 0.29	<0.0001
BHB (mM)	0.35 $\pm$ 0.01	3.49 $\pm$ 0.11	<0.0001
Fatty acids (mM)	0.29 $\pm$ 0.02	1.19 $\pm$ 0.06	<0.0001
Glucose (mM)	4.03 $\pm$ 0.07	2.23 $\pm$ 0.03	<0.0001
AST ¹ (U/L)	72.00 $\pm$ 1.59	156.40 $\pm$ 3.52	<0.0001
ALT ¹ (U/L)	18.80 $\pm$ 1.18	35.53 $\pm$ 1.87	<0.0001
Milk fat (%)	3.41 $\pm$ 0.23	4.13 $\pm$ 0.21	0.006
Milk protein (%)	3.31 $\pm$ 0.08	2.90 $\pm$ 0.07	<0.0001
Milk lactose (%)	5.08 $\pm$ 0.08	4.64 $\pm$ 0.18	0.039

¹AST = aspartate aminotransferase; ALT = alanine aminotransferase.

at each milking from each cow on 3 consecutive days, and then stored at 4°C with preservative (1 mg/mL of potassium dichromate) until analyzed for milk composition by using an infrared analyzer with a 4-channel spectrophotometer (Sun et al., 2019; Foss MilkoScan, Foss Food Technology Co., Eden Prairie, MN). Blood samples were collected between 0700 and 0900 h (before the morning feeding) from a coccygeal vein and immediately centrifuged at $3,500 \times g$ for 15 min at 4°C. 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. The intercostal space was shaved before the liver biopsy, sanitized with iodine scrub and 75% alcohol, and anesthetized with a 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 (~200 mg) were immediately frozen in liquid nitrogen or fixed with 4% paraformaldehyde or OCT compound (4583; Sakura Finetek Co., Torrance, CA).

Determination of Blood Parameters

Serum concentrations of glucose, BHB, and fatty acids were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercially available kits (BHB: cat. no. RB1008; fatty acids: cat. no. FA115; glucose: cat. no. GL3815; Randox Laboratories, Crumlin, UK). The activities of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined using an automatic biochemistry analyzer (Sekisui Medical Co. Ltd., Tokyo, Japan) with commercially available kits (AST: AS3804; ALT: AL3801; Randox Laboratories).

ELISA

The concentrations of TNF- α , IL18, IL1B, and IL10 were assessed with the following commercially available ELISA kits: Bovine TNF- α (Brodzki et al., 2019, SEA133Bo; USCN Life Science Inc., Wuhan, China); Bovine IL18 ELISA kit (SEA064Bo; USCN Life Science Inc.), IL1B ELISA kit (SEA563Bo; USCN Life Science Inc.), and IL10 ELISA kit (Brodzki et al., 2019, SEA056Bo; USCN Life Science Inc.) according to the manufacturer's instructions. The intra- and interassay coefficients of variation for all of the examined cytokines were <10 and 12%, respectively. The analyzed sensitivity of TNF- α , IL10, IL1B, and IL18 was less than 3.1, 6.4, 6.5, and 6.3 pg/mL, respectively. Every

serum sample was analyzed in triplicate, and absorbance values were read at 450 nm using a spectrophotometer (51119100; Thermo Fisher Scientific, Shanghai, China).

Liver Histology

Liver tissue was fixed in 10% formaldehyde neutral buffer solution, embedded in paraffin, and cut into 8- μ m slices, floated on a water bath, picked up onto glass slides, and placed in slide racks. Slides were dewaxed with xylene, rehydrated through descending concentrations of alcohol, and stained with hematoxylin and eosin (H&E).

For Oil-red O staining, liver tissue was frozen in OCT compound (4583; Sakura Finetek Co.), sectioned into 8- μ m slices at -18°C, and fixed with 75% alcohol at room temperature for 15 min. Next, slides were stained with Oil red O (O0625; Sigma-Aldrich Co.) and counterstained with hematoxylin. Representative photomicrographs were captured at 400 $\times$ magnification using a system incorporated in the microscope.

Triacylglycerol Content Determination

Liver tissue (~20 mg) was homogenized with lysate provided by the Applygen Kit (E1013; Applygen Technologies Inc., Beijing, China). A portion of the supernatant was used to determine total protein concentration using the bicinchoninic acid (BCA) assay (P1511; Applygen Technologies Inc.). The remaining supernatant was heated in a water bath (70°C) for 10 min. After cooling the samples at room temperature, they were vortexed and centrifuged at $2,000 \times g$ for 5 min at 4°C. The supernatant was collected and used for a triacylglycerol (TG) assay using an enzymatic kit (E1013; Applygen Technologies Inc.) following the manufacturer's instructions.

Western Blotting

Liver tissue (~20 mg) was lysed in ice-cold RIPA lysis buffer (C1053; Applygen Technologies Inc.) for 30 min before centrifugation at $12,000 \times g$ for 15 min at 4°C. The supernatant was collected and the protein concentrations were quantified using the BCA Protein Assay Kit (P1511; Applygen Technologies Inc.). Forty micrograms of protein was separated by 10% or 12% SDS-PAGE and then electrophoretically transferred onto polyvinylidene fluoride membranes, which were blocked for 4 h at room temperature with 3% BSA/Tris-buffered saline (TBS)-Tween buffer. The blocked membranes were incubated overnight at 4°C with diluted primary antibody NF- κ B p65 (1:1,000; 4764s; Cell Signaling Technology Inc., Danvers, MA), phos-

phorylated (p)-I κ B α (1: 1,000; 9246; Cell Signaling Technology Inc.), I κ B α (1: 1,000; 4814; Cell Signaling Technology Inc.), p-NF- κ B p65 (1: 2,000; ab86299; Abcam, Cambridge, MA), NLRP3 (1: 1,000; ab210491; Abcam), CASP1 (1: 5,000; ab201476; Abcam), and β -actin (1: 2,000; ab8226; Abcam). After washing 4 times with TBS containing 0.1% Tween 20 for 7 min at room temperature with shaking, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody for 45 min at room temperature with shaking, and then washed 4 times for 7 min. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL, WBKLS0500; Millipore, Bedford, MA). All bands were quantified by using Image-Pro Plus (Media Cybernetics Inc., Rockville, MD). In this study, β -actin used as a reference protein, the phosphorylation level of NF- κ B was calculated as p-NF- κ B/total NF- κ B, and the phosphorylation level of I κ B α was calculated as p-I κ B α / β -actin.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from liver tissue using RNAiso Plus (9109; TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instruction, and then quantified using a K5500 Micro-Spectrophotometer (Beijing Kaihao Technology Development Ltd., Beijing, China) and by electrophoresis (1% agarose gels). Complementary (c)DNA was generated from total RNA using a reverse transcription kit (RR047A; TaKaRa Biotechnology Co. Ltd.) accord-

ing to the manufacturer's instructions. We evaluated mRNA expression levels using quantitative real-time (qRT)-PCR technology with the SYBR Green QuantiTect RT-PCR Kit (RR420A; TaKaRa Biotechnology Co. Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems Inc., Waltham, MA). The qRT-PCR was conducted with an 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 (R^2) were calculated by the slope of the standard curves of using serial 10-fold dilutions of sample cDNA. [Note that when calculating the PCR amplification efficiency, R^2 represents the correlation coefficient (Bustin et al., 2009). Therefore, R^2 is the correlation coefficient in this study.] The efficiency was calculated as follows: $E (\%) = (10^{-1/\text{slope}} - 1) \times 100$ (Kubista et al., 2006). We calculated the relative gene expression by using a mathematical model, which includes an efficiency correction for PCR efficiency (Pfaffl, 2001). The relative expression of each target gene was normalized to 2 reference genes, *ACTB* and *GAPDH*. The qRT-PCR reaction was performed in triplicate for each of the 15 cows per group. The primers pairs used in this study are listed in Table 2; *ACTB* and *GAPDH* were stably expressed in different groups (Supplemental Figure S1, <https://doi.org/10.3168/jds.2019-16706>; Komatsu et al., 2005; Graber et al., 2010; Du et al., 2018a). The efficiency of primers used is given in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-16706>).

Table 2. Primer sequences (For, forward; Rev, reverse)

Gene	Sequence number	Primer sequence (5'-3')	Length (bp)	Annealing temperature (°C)
<i>TNFA</i>	NM_173966.3	For TCTACCAGGGAGGAGTCTTCCA Rev GTCCGGCAGGTGATCTCA	68	60
<i>IL10</i>	NM_174088.1	For GCCTTGTCGGAAATGATCCA Rev TCAGGCCCGTGGTTCTCA	68	60
<i>MCP1</i>	NM_174006.2	For TTAACCTCCCAAGTCGCCTGC Rev ACCCATTTCTGCTTGGGGTC	166	60
<i>NOS2</i>	XM_024979646.1	For ATCTGCAGACACGTGCGTTA Rev GTTCCAGACCCGGAAGTCAT	105	60
<i>IL18</i>	NM_174091.2	For GGCTGCCGTCTTCTGTAAG Rev GATCTGATTCCAGGTCTTCATCAT	200	60
<i>IL1B</i>	NM_174093.1	For GAATAACCCCGAGGACTGGC Rev GTCGGGCATGGATCAGACAA	82	60
<i>NLRP3</i>	NM_001102219.1	For CTTTCTGGACTCTGACCGGG Rev ATGCCTTCTCTTCCCCGTTG	229	60
<i>CASP1</i>	XM_024975700.1	For CAGTGGTCCCTCCTTTTCCAG Rev TCCACCTTGATCCCAGACCT	286	60
<i>ACTB</i>	NM_173979.3	For GCCCTGAGGCTCTCTTCCA Rev GCGGATGTGACGTCACA	101	60
<i>GAPDH</i>	NM_001034034.2	For GGCGTGAACCACGAGAAGTATAA Rev CCTCCACGATGCCAAAGTG	118	60

¹*TNFA*, tumor necrosis factor- α ; *IL10*, interleukin-10; *MCP1*, chemokine monocyte chemoattractant protein-1; *NOS2*, nitric oxide synthase; *IL18*, interleukin-18; *IL1B*, interleukin-1B; *NLRP3*, NLR family pyrin domain containing 3 inflammasome; *CASP1*, caspase-1; *ACTB*, β -actin.

CASP1 Activity Determination

The enzymatic activity of CASP1 was assayed using a CASP1 Activity Kit according to the manufacturer's protocol (C1102; Beyotime Institute of Biotechnology, Beijing, China). Briefly, liver tissue was homogenized in lysis buffer and centrifuged for 15 min at $16,000 \times g$ at 4°C. The supernatant was collected, and protein concentration determined by using the Bradford Protein Assay Kit (P0006; Beyotime Institute of Biotechnology). A volume of 50 μ L of tissue extract (containing 30 μ g of protein) was incubated in a 96-well plate with reaction buffer and acetyl-tyrosyl-valyl-alanyl-aspartic acid p-nitroanilide (Ac-YVAD- ρ NA) for 2 h at 37°C. The absorbance values of ρ -nitroaniline (ρ NA) at 405 nm were measured using a 96-well plate reader, recorded every 1 min for 5 consecutive min (51119100; Thermo Fisher Scientific). One unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-YVAD- ρ NA per hour at 37°C under saturated substrate concentrations.

IKK β Activity Determination

The IKK β activity in the tissue extracts was measured using an IKK β Kinase Assay Kit (GMS50162.4; GenMed Scientifics Inc., Wilmington, DE) according to the manufacturer's instructions. Briefly, liver tissue (~20 mg) was washed in pre-cold GenMed clean buffer (reagent A) and homogenized in an ice bath with lysis buffer (reagent B). After fully mixing, samples were incubated in an ice bath for 30 min. Then, the samples were centrifuged for 10 min at $10,000 \times g$ at 4°C. The supernatant was collected and the protein concentrations were determined using the BCA Protein Assay Kit (P1511; Applygen Technologies Inc.). Total IKK β activity was measured as follows: 130 μ L of GenMed buffer (reagent C), 20 μ L of GenMed enzymatic solution (reagent D), 20 μ L of GenMed reaction solution (reagent E), and 20 μ L of GenMed substrate (reagent F) were combined into a 96-well plate followed by incubation at 30°C for 3 min; then, 10 μ L of GenMed negative solution (reagent G) or sample was added, and absorbance values were read immediately at 450 nm using a spectrophotometer (51119100; Thermo Fisher Scientific). The nonspecific IKK β activity was measured as follows: 20 μ L of reagent C in a 1.5-mL centrifuge tube was combined with 10 μ L of GenMed reagent H and sample and then incubated at 30°C for 30 min. Then, 100 μ L of reagent C, 20 μ L of reagent D, 20 μ L of reagent E, and 20 μ L of reagent F were combined in a 96-well plate and incubated at 30°C for 3 min. Last, each sample was aliquotted into a corresponding well and absorbance was read immediately at 450 nm

using a spectrophotometer (51119100; Thermo Fisher Scientific). The specific IKK β activity was calculated as total IKK β activity – nonspecific IKK β activity.

Statistical Analysis

Data from Western blotting and qRT-PCR were normally distributed and analyzed with paired *t*-tests; other data were non-normally distributed and analyzed using the Wilcoxon signed-rank test. All analyses were performed using GraphPad Prism 5.0 (Graph Pad Software, San Diego, CA) or SPSS 23.0 software (IBM Corp., Armonk, NY). Data are expressed as the mean $\pm$ standard errors of the mean; $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly significant.

RESULTS

Blood Parameters

As shown in Table 1, BW ($P < 0.01$) and BCS ($P < 0.01$) were greater and DMI ($P < 0.01$) and milk yield were lower ($P < 0.01$) in cows with ketosis than in control cows. Blood concentrations of fatty acids and BHB and serum activities of ALT and AST were all greater ($P < 0.01$) in cows with ketosis. Milk protein ($P < 0.01$) and milk lactose ($P < 0.05$) contents were both lower in the cows with ketosis, and milk fat content was higher ($P < 0.01$) in the ketotic cows.

Liver TG Content

Data from H&E and Oil-red O staining indicated greater lipid accumulation in cows with ketosis. A considerable mass of fat vacuoles was detected in the liver of ketotic cows (Figure 1A), in accordance with the greater ($P < 0.01$) hepatic TG content (Figure 1B).

Systemic Inflammation Biomarkers

The serum levels of TNF- α ($P < 0.01$), IL18 ($P < 0.05$), and IL1B ($P < 0.01$) were all greater (Figure 2A, B and C), and concentration of IL10 lower ($P < 0.01$) in ketotic cows (Figure 2D).

Activation of NF- κ B Signaling in the Liver

Hepatic activity of IKK β ($P < 0.01$) and abundance of p-NF- κ B ($P < 0.05$) and p-I κ B α ($P < 0.01$) proteins were greater in cows with ketosis (Figure 3A, B, and C). Furthermore, hepatic mRNA abundance of proinflammatory factors *TNFA*, *NOS2*, and chemokine monocyte chemoattractant protein-1 (*MCP1*) was all greater (P

Figure 1. Liver histology and triglyceride (TG) content. (A) Representative images of hematoxylin and eosin (H&E; original magnification 400×; scale bar 20 µm) and Oil-red O (original magnification 400×; scale bar 20 µm) staining of liver sections. (B) Hepatic TG content in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$); TG contents were non-normally distributed and analyzed with Wilcoxon signed-rank test. Data were expressed as the mean $\pm$ SEM.

< 0.01) in cows with ketosis (Figure 4A, B, and C). The mRNA abundance of hepatic anti-inflammatory factor *IL10* was lower ($P < 0.01$) in cows with ketosis (Figure 4D).

Activation of Hepatic NLRP3 Inflammasome

The protein abundance of NLRP3 and CASP1 (Figure 5A, B) along with mRNA abundance of *NLRP3*, *CASP1*, *IL18*, and *IL1B* were all greater ($P < 0.01$) in cows with ketosis (Figure 5C). Furthermore, the activity of CASP1 in the liver of cows with ketosis was greater ($P < 0.01$, Figure 5D).

DISCUSSION

Periparturient dairy cows experience sudden changes in diet, metabolic, and immune responses, all of which render them susceptible to metabolic disorders such as fatty liver and ketosis (Bobe et al., 2004; Xu et al., 2015; Du et al., 2018b). Ketotic cows often display high blood concentrations of fatty acids and BHB (Deng et al., 2015). High levels of fatty acids and BHB can induce lipotoxicity (Shi et al., 2014; Song et al., 2014; Deng et al., 2015) and, at least in nonruminants, lead to metabolic dysfunction, cell death, and chronic inflammation (Ertunc and Hotamisligil, 2016). In human and mouse, metabolic disorders are accompanied by chronic inflammation (Dela Peña et al., 2005; Henao-Mejia et al., 2012; Esser et al., 2014). Serum levels of proinflammatory cytokines such as TNF- α and IL1B are elevated in cows with ketosis (Zhang et al., 2016; El-Deeb and El-Bahr, 2017). Thus, data from the present study confirmed that ketosis is associated with systemic in-

Figure 2. Serum concentrations of pro-inflammatory cytokines tumor necrosis factor (TNF)- α (A), IL18 (B), and IL1B (C), and anti-inflammatory cytokine IL10 (D) in ketotic ($n = 15$) and control ($n = 15$) cows. Data were non-normally distributed and analyzed with Wilcoxon signed-rank test. Data were expressed as the mean $\pm$ SEM.

Figure 3. The expression of nuclear factor (NF)-κB signaling proteins and activity of inhibitor of NF-κB (IKKβ). (A) Hepatic IKKβ activity and (B) expression of NF-κB signaling proteins in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$). Representative blots are shown. (C) Quantification of hepatic protein levels of phosphorylated (p)-NF-κB, NF-κB, p-IκBα, and IκBα, with β-actin as reference. Data in (A) were analyzed with Wilcoxon signed-rank test and expressed as mean $\pm$ SEM; data in C were analyzed with paired t -tests and expressed as the mean $\pm$ SEM.

inflammation and overactivation of hepatic NF-κB and NLRP3 inflammasome signaling, potentially contributing to liver damage and steatosis.

In human and mouse, a clear link exists between NF-κB signaling and fatty liver, and some evidence in cows suggests that this pathway may be relevant to the development of ketosis (Ribeiro et al., 2004; Dela Peña et al., 2005; Li et al., 2015). Thus, the greater phosphorylation levels of NF-κB and IκB along with upregulation

of *MCP1*, *NOS2*, and *TNFA*, downstream targets of the NF-κB signaling, are evidence that the hepatic NF-κB signaling pathway was overactivated during ketosis and might have played a role in the development of TG accumulation (Liedtke and Trautwein, 2012; Van Beek et al., 2012).

Previous data underscored the fact that exposure of hepatocytes to high levels of fatty acids and BHB could induce NF-κB signaling and upregulation of TNF-α synthesis (Shi et al., 2014, 2015). In turn, TNF-α and IL1B can further activate the NF-κB inflammation pathway (Liedtke and Trautwein, 2012; Van Beek et al., 2012). Taken together, we speculate that the high levels of fatty acids and proinflammatory cytokines all contributed to hepatic NF-κB signaling overactivation during ketosis.

An activation of NF-κB signaling could induce the priming step of NLRP3 inflammasome leading to upregulation of *NLRP3*, *IL18*, and *IL1B* (Esser et al., 2014) and oligomerization of the NLRP3 complex and CASP1 activation (Shao et al., 2015). Activated CASP1, in turn, cleaves pro-IL1B and pro-IL18 into their mature forms: IL1B and IL18 (Jo et al., 2016). Thus, the greater activity of CASP1 and upregulation of hepatic *IL1B* and *IL18* mRNA in the present study are suggestive of hepatic NLRP3 inflammasome overactivation during ketosis.

In nonruminants, palmitic acid but not oleic acid induces activation of the NLRP3 inflammasome, causing IL1B and IL18 production from immune cells (Wen et al., 2011). Murine hepatocytes also undergo activation of the NLRP3 inflammasome in response to a greater supply of palmitic acid (Yang et al., 2016). The concentration of reactive oxygen species is an efficient activator of NLRP3 inflammasome, and the fact that excessive concentrations of fatty acids impair mitochondrial function underscores the linkages between excessive

Figure 4. Relative hepatic mRNA expression of inflammatory cytokines *TNFA* (A), *NOS2* (B), *MCP1* (C), and *IL10* (D) in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$). Data were analyzed with paired t -tests and expressed as the mean $\pm$ SEM.

Figure 5. The mRNA and protein levels of NLR family pyrin domain containing 3 (NLRP3) inflammasome and the activity of caspase-1 (CASP1). (A) Hepatic protein levels of NLRP3 and CASP1 in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$). Representative blots are shown. (B) Quantification of hepatic protein levels of NLRP3 and CASP1. (C) Relative hepatic mRNA expression of *NLRP3*, *CASP1*, *IL18*, and *IL1B* in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$). (D) Activity of CASP1 in control cows ($n = 15$) and dairy cows with ketosis ($n = 15$). Data in (B) and (C) were analyzed with paired t -tests and expressed as the mean $\pm$ SEM; data in (D) were analyzed with Wilcoxon signed-rank test and expressed as the mean $\pm$ SEM.

lipolysis after calving and susceptibility for NLRP3 activation in the liver (McKeegan and Sturme, 2011; Lee et al., 2013; Putman et al., 2018). Clearly, the present data reveal a complex cascade of events that triggers hepatic NLRP3 inflammation with a consequent lipotoxic effect and accumulation of TG.

Besides compromising lipid metabolism in the liver, data from the mouse indicate that activation of NF- κ B signaling and NLRP3 inflammasome along with high levels of TNF- α could impair hepatic insulin sensitivity and contribute to lipid accumulation (Ham et al., 2016). A direct link between NLRP3 inflammasome, NF- κ B pathway, and altered liver metabolism has been reported in the context of high-fat feeding; that is, silencing of these pathways abrogates the negative effects of high dietary fat on hepatic lipid metabolism and insulin sensitivity (Zhao et al., 2017). Although the ruminant liver is not a lipogenic organ under normal conditions (Hanson and Ballard, 1967), evidence indicates that nutrition (Khan et al., 2014) could induce molecular changes in the liver conducive to lipid accumulation. Moreover, Bradford et al. (2009) reported that a higher TNF- α level could result in a lipid meta-

bolic disorder in the liver of cows. Thus, we speculated that an increase in the concentration of TNF- α during ketosis could further exacerbate the progression of hepatic lipid accumulation. Taken together, current and previous data indicate that activation of inflammatory pathways exacerbates disorders of hepatic lipid metabolism.

Consistent with previous studies (Bogin et al., 1988; Herdt, 2000; Du et al., 2017a), the greater blood levels of ALT and AST, indicators of liver damage, confirmed the existence of liver injury during ketosis. Hepatic injury can result in fatty acid-induced lipotoxicity, which accelerates hepatocyte death (Cazanave and Gores, 2010; Du et al., 2017b). At least in mice, overt inflammation can cause liver injury; for example, TNF- α and IL1B produced during activation of the NF- κ B and NLRP3 signaling pathways can lead to mitogen-activated protein kinase (MAPK) apoptosis (Wang et al., 2009). There is evidence that hepatic apoptosis rate via MAPK activation is greater in ketotic liver (Du et al., 2017b). From a mechanistic standpoint, there is a link between NF- κ B and NLRP3 and systemic concentrations of ALT and AST. In rodents, knockdown of

NF- κ B and NLRP3 was associated with a decrease in blood concentrations of ALT and AST during feeding of high fat diets or diets deficient in methionine-choline (Henao-Mejia et al., 2012). Therefore, our data and that of previous studies indicate that overactivation of NF- κ B and NLRP3 signaling pathway partly mediated and aggravated the pathological injury of the liver in ketotic cows.

CONCLUSIONS

The hepatic NF- κ B signaling pathway and NLRP3 inflammasome are overactivated in ketotic cows and contribute to hepatic injury and lipid accumulation. Accordingly, these pathways have important implications in the context of lipidosis. They represent a target for potential therapeutic applications in the field of periparturient cow management.

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