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BHBA Influences Bovine Hepatic Lipid Metabolism via AMPK Signaling Pathway

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ABSTRACT

β-hydroxybutyric acid (BHBA), an important metabolite in β-oxidation, is involved in the development of ketosis in dairy cows. It is known that AMP-activated protein kinase (AMPK) signaling pathway plays an important role in the regulation of lipid metabolism in hepatocytes. In the present study, bovine hepatocytes were treated with BHBA at variable concentrations and Compound C (Cpd C, an AMPK inhibitor) to investigate the effects of BHBA on the AMPK signaling pathway. The results showed that when the concentration of BHBA reached 1.2 mM, the AMPK signaling pathway was activated and the expression of sterol regulatory element binding protein-1c (SREBP-1c) as well as its target genes were significantly decreased. And these decreases were blocked by Cpd C. The binding activity and nucleus translocation of SREBP-1c showed a similar trend. The expression of peroxisome proliferator activated receptor-α (PPARα), carbohydrates response element binding protein (ChREBP) and their target genes were significantly increased while they were negatively suppressed by the Cpd C. The content of triglyceride (TG) had no obviously change in the BHBA and Cpd C-treated groups. These results indicate that BHBA can activate AMPK signaling pathway and regulate lipid synthesis and lipid oxidation genes of AMPK but showed no effect on TG in bovine hepatocytes. J. Cell. Biochem. 116: 1070–1079, 2015.

KEY WORDS: BHBA; AMPK SIGNALING PATHWAY; DAIRY COWS; HEPATOCYTES

Abbreviations: ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; ACSL-1, long-chain fatty acyl-COA synthetase 1; AMPK, AMP-activated protein kinase; BHBA, β-hydroxybutyric acid; ChREBP, carbohydrates response element binding protein; CPT-I, carnitine palmitoyl transferase I; CPT-II, carnitine palmitoyl transferase II; Cpd C, Compound C; EMSA, electrophoretic mobility shift assay; FAS, fatty acid synthase; IL-6, interleukin 6; IL-1β, interleukin 1 beta; L-FABP, liver fatty acid-binding protein; NEFA, non-esterified fatty acid; NF-κB, nuclear factor kappa B; PPARα, peroxisome proliferator activated receptor-α; SCD-1, stearoyl-CoA desaturase 1; SREBP 1c, sterol regulatory element binding protein-1c; TG, triglyceride; TNF-a, tumor necrosis factor alpha; VFA, volatile fatty acids.
Conflicts of interest: None.

Qinghua Deng and Guowen Liu contributed equally to this study.

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The hepatic lipid metabolism in dairy cows is different from that of monogastric animals such as humans and mice, and resulting in high incidence of ketosis and fatty liver in perinatal dairy cows. Firstly, dairy cows experienced a series of physiological stress pregnancy, delivery and starting milk during the lactation period. Secondly, most dairy cows displayed negative energy balance (NEB) caused by decreased dry matter intake and increased demand for energy to support milk production. This NEB initiates fat mobilization and a subsequent increase in non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHBA) blood concentration. BHBA is the main form of ketone body in dairy cows, and is resulted from incomplete oxidation of NEFAs in the liver cells. Furthermore, the ability of using BHBA is limited in the liver, resulting in the accumulation of excess BHBA in the liver and blood, which will finally lead to ketosis. Therefore, it is necessary to define the characteristic of fatty acid oxidative metabolism in bovine liver and seek the key point to control fatty acid oxidative metabolism disorder, which will provide valuable information to reveal the pathogenesis of ketosis in dairy cows.

BHBA is the golden marker for the diagnosis of ketosis in dairy cows [Oetzel, 2004; Xia et al., 2012]. The concentration of BHBA in blood of subclinical ketosis cows is from 1.2 to 1.4 mM, while higher than 1.4 mM is considered as clinical ketosis [Mahrt et al., 2014]. Studies demonstrated that the incidence of infectious diseases in dairy cows with ketosis was higher than that in the health cows [Grummer, 1993; Herdt, 2000; Oetzel, 2004], which increased enormous economic loss in dairy industry.

Liver is the vital organ to regulate lipid metabolism and maintain lipid homeostasis. AMP-activated protein kinase (AMPK) is a "sensor" and "regulator" of energy in hepatocytes [Kahn et al., 2005]. It can sense some stimulate factors such as metabolic signals, neuroendocrine hormone or cytokines to make response and then regulate lipid metabolism in hepatocytes [Woo et al., 2014]. AMPK modulates hepatic lipid metabolism by regulating several lipid metabolism-related transcription factors such as peroxisome proliferator activated receptor-α (PPARα), sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrates response element binding protein (ChREBP), all of which govern the expression of lipid metabolic enzymes [Bronner et al., 2004].

AMPK is a critical pathway to regulate lipid metabolism in liver [Li et al., 2013]. Studies demonstrated that BHBA could activate AMPK signaling pathway in GT1–7 hypothalamic cells [Laeger et al., 2012]. Dairy cows with ketosis displayed lipid metabolism disorder and accompanied with high blood level of BHBA. We speculate that high level of BHBA will impair AMPK signaling pathway and then result in hepatic lipid metabolism disorder. Therefore the bovine hepatocytes were cultured and treated with BHBA to evaluate the effect of BHBA on the hepatic lipid metabolism through AMPK signaling pathway in bovine hepatocytes.

MATERIALS AND METHODS

MATERIALS

β-hydroxybutyricacid was purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum, collagenase IV, RPMI-1640 were purchased from Gibco (Grand Island, NY). Dexamethasone acetate, vitamin C and other chemicals were provided by Baoman Biotechnology (Shanghai, China). PPARα antibody, L-FABP antibody and ACCα antibody were purchased from Abcam (Cambridge, England), FAS antibody, AMPKα antibody, phosphor-AMPKα (p-AMPKα) antibody and Histone antibody were purchased from Cell Signaling Technology (Berverly, MA), ChREBP antibody was purchased from LSBio (Life Span Bio Sciences, Seattle, WA), SREBP-1c antibody was purchased from Novus (Saint Louis, Missouri), ACSL-1 antibody and β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Compound C (an AMPK inhibitor) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bovine Serum Albumin V was purchased from Roche (Basel, Switzerland).

CELL CULTURE

The study protocol was approved by the Ethics Committee on the Use and Care of Animals, Jilin University (Changchun, China). The caudate lobe of liver was obtained through surgery liver excision from a newborn calf. Hepatocytes were isolated by a modified two-step collagenase perfusion method established by our laboratory [Li et al., 2012; Zhang et al., 2012]. The liver was perfused with perfusion solution to wash away the blood until the perfusion solution became clear (Perfusion solution A comprised 140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA. Perfusion solution B comprised 140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM $CaCl_2$. The pH of solution A and B was adjusted to 7.4.). The liver was then perfused with a collagenase IV solution (Digestion solution: 0.1 g collagenase IV was dissolved in 0.5 L of perfusion solution B.) to digest the liver tissue. The liver capsule was cut off after digestion. Then we used 100 mL RPMI-1640 culture medium containing 0.2% bovine serum albumin that pre-cooling to terminate the digestion (The RPMI-1640 basic culture medium was prepared according to the manufacturer's protocol, and comprised 26 mM $NaHCO_3$, 10 mM HEPES, and 20 mM NaCl. The solution pH was adjusted to 7.2.). Then we discarded the liver capsule, blood vessels, fat, and other parts of the liver caudate lobe. The hepatocytes suspension were filtered sequentially with 100 mesh (150 μm), and 200 mesh (75 μm) cell sieves. Cell density was adjusted to 2×10^6 cells/mL with adherent RPMI-1640 culture medium. The hepatocytes were seeded into a 6-well tissue culture plate (2 mL per well) and incubated at 37 °C in 5% CO_2. Every 24 h, the medium was replaced with fresh medium and the hepatocytes' shape and growth conditions were observed.

BHBA TREATMENT

The doses of BHBA were based on the blood BHBA concentration in dairy cows with ketosis. After 48 h of culture, the hepatocytes were serum-starved overnight and subsequently treated with 1.2 mM BHBA for 0, 1, 3, 6, 12 and 24 h. For the dose-response experiments, the hepatocytes were divided into six groups: a control group (0 mM BHBA), a low-dose group (0.6 mM BHBA), a medium-dose group (1.2 mM BHBA), a high-dose group (2.4 mM BHBA), a Cpd C group (10 μM Compound C) and a Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). These groups were then treated for 6 h before collecting samples.

QUANTITATIVE REAL-TIME RT-PCR

The total RNAs of cells were extracted using Trizol according to manufacturer's instructions (Invitrogen Corp, Carlsbad, CA). The RNA was reverse transcribed into cDNA using a reverse transcription kit (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. All primers were synthesized from Sangon (Sangon Biotech Co., Ltd., Shanghai, China). The mRNA expression levels were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis using the SYBR Green QuantiTect RT-PCR Kit (Roche, Basel, Switzerland). qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems/Life Technologies, Grand Island, NY). The gene primers were designed using Primer Express software 5.0. And the primers were showed in Table I. The mRNA expression levels were normalized to the house-keeping gene–β-actin. Real-time PCR was conducted under the following conditions: initial denaturation at 95 °C for 3 min, 45 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Hepatocytes were lysed in lysis buffer (50 mM Tris-HCl pH 8.0; 0.15 mM NaCl; 1% Triton X-100; 100 μg/ml PMSF), and the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL). Prepared proteins were performed by standard SDS-PAGE with 10% (w/v) polyacrylamide gels (0.1% SDS). Then electrotransfer of proteins from polyacrylamide gel onto PVDF membranes (Roche) using a semi-dry blotting apparatus. After washing membranes several times the membranes were incubated in blocking solution (3% Bovine Serum Albumin V in Tris-HCl buffer solution) for 4 h at room temperature. After blocking, membranes were hybridized overnight at 4 °C with antibodies AMPKα (diluted 1:500), phosphor-AMPKα (diluted 1:500), SREBP-1c (diluted 1:500), PPARα (diluted 1:500), ChREBP (diluted 1:500), Histone (diluted 1:500), ACCα (diluted 1:500), FAS (diluted 1:500), L-FABP (diluted 1:500), ACSL-1 (diluted 1:400), and β-actin (diluted 1:500). Next, the membranes were washed three times with TBS containing 0.1% Tween 20 for 5 min with shaking, and then incubated with appropriate peroxidase-conjugated secondary antibodies (Protein technology, Chicago, IL) for 45 min with shaking at room temperature and washed four times for 5 min. And the resulting bands were detected by ECL kit (Bioer, Hangzhou, China). The relative expression levels of above proteins were normalized to β-actin and Histone levels.

TABLE I. List of Primers Used for PCR

Genes	Primers used for PCR (5′ - 3′)
PPARα	F: GGCAACATCTGGACACATC
	R: CCCGCAGATCCTACACT
SREBP-1c	F: CGACACCACCAGCATCAACCACG
	R: GCAGCCCATTCATCAGCCAGACC
ChREBP	F: ATCCGCCTCAACAACGC
	R: TCCCTCCAAGACGACG
β-actin	F: GCTAACAGTCCGCCTAGAAGCA
	R: GTCATCACCATCGGCAATGAG
ACCα	F: TGCTGAATATCCTCACGGAGCT
	R: CGACGTTTCGGACAAGATGAGT
ACSL-1	F: TCGGAACTGAAGCCATCACC
	R: GCCTCGTTCCAGCAGATCAC
CPT-I	F: ACGCCGTGAAGTATAACCCT
	R: CCAAAAATCGCTTGTCCCTT
CPT-II	F: TGAACATCCTCTCCATCTGG
	R: GGTCAACAGCAACTACTACG
L-FABP	F: AAGTACCAAGTCCAGACCCAG
	R: CACGATTTCCGACACCC
FAS	F: ACAGCCTCTTCCTGTTTGACG
	R: CTCTGCACGATCAGCTCGAC

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear proteins were extracted using a nuclear protein extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The special probe recognition sequences for SREBP-1c, ChREBP and PPARα are shown in Table II. The probes were labeled with biotin for 30 min at 37 °C. The binding reaction was performed using the Light shift EMSA Optimization and Control Kit (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol. EMSA/gel-shift binding buffer containing 6 μg of nuclear extract was incubated with the biotin-labeled probe for 20 min at room temperature. DNA-protein complexes were separated by electrophoresis on non-denaturing 6.5% polyacrylamide Tris/borate/EDT (TBE) gels and were transferred onto PVDF membranes. Then, the membranes were cross-linked using a UV cross-linker (Cany Precision Instruments Co., Ltd., Shanghai, China). The biotin-labeled probe was detected with a chemiluminescence solution (Piece Biotechnology, Inc.). The blots were exposed to X-ray film.

IMMUNOFLUORESCENCE

We placed a round cover glass (diameter is 13 mm) into the 24-well tissue culture plate before cells were seeded, and the concentration was 1×10^5 cells/mL (0.5 mL per well). After incubation for 72 h, hepatocytes were treated with BHBA and Cpd C. The experiment was designed with a control group (0 mM BHBA), a medium-dose group (1.2 mM), a Cpd C group (10 μM Compound C) and a Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). After incubation with BHBA and Cpd C for 6 h, we washed the cells with the basic medium for three times, and then we used 4% triformol to fix the hepatocytes for 20 min and washed with 0.01% phosphate buffer solution (PBS) for three times. Antigen retrieval was performed with EDTA·Na_2 for 5 min in 95 °C, and after the temperature down to the room temperature we washed it with 0.01% PBS for three times. We used 0.1% Triton-100 (300 μl) to punching for 10 min and washed it with 0.01% PBS for three times. Then the cells were incubated overnight at 4 °C with antibodies PPARα (diluted 1:50 with goat serum),

TABLE II. Probe Sequences for PPARα, SREBP-1c, and ChREBP Used in the Electrophoretic Mobility Shift Assay (EMSA)

Transcription factor	Sequence (5′ - 3′)
PPARα	CAAAACTAGGTCAAAGGTCA
SREBP-1c	GGAGGCATCACCCCACCGAC
ChREBP	TCCTGCATGTGCCACAGGCGTGTCACC

SREBP-1c (diluted 1:50 with goat serum) and ChREBP (diluted 1:50 with goat serum), then washed it with 0.01% PBS for three times. The cells were incubated with appropriate peroxidase-conjugated secondary antibodies (Beyotime, Beyotime Institute of Biotechnology, Jiangsu, China) which labeled by Cy3 for 30 min keeping in dark place at room temperature and then washed it with 0.01% PBS for three times. Then we used DAPI nuclear dyes (200 μl, Hoechst 33258, Beyotime, Beyotime Institute of Biotechnology, Jiangsu, China) to dye hepatocytes for 7 min in dark place and then washed it with 0.01% PBS for three times. At last we observed the hepatocytes by fluorescence confocal microscope.

DETECTION OF TG CONTENT

Hepatocytes were collected after incubation with BHBA and Cpd C for 6 h. The cells were broken by ultrasonic processor to collect TG. The TG content was detected using the triglyceride detection kit (Nanjing Jiancheng Bioengineering Institute, Jiancheng, Nanjing, China).

STATISTICS

All results were expressed as the means ± standard error (SE). Statistical analysis was carried out using the statistical analysis program SPSS 13.0 (SSPS, Chicago, IL). $P < 0.05$ was considered to be significant.

RESULTS

Effects of BHBA treatment duration on AMPKα phosphorylation in bovine hepatocytes. The phosphorylation level of AMPKα was the highest at 6 h of BHBA treatment (Fig. 1).

Effects of different concentrations of BHBA on the phosphorylation of AMPKα in bovine hepatocytes. As shown in Figure 2, the level of AMPKα phosphorylation (p-AMPKα/AMPKα) was increased in the low-dose (0.6 mM BHBA) and medium-dose (1.2 mM BHBA) groups compared with the control group, and was significantly lower in the high-dose and Cpd C + BHBA groups than in the medium-dose group, it was lower in the Cpd C group than in the control group (Fig. 2).

The mRNA and protein expression levels of PPARα, SREBP-1c and ChREBP in hepatocytes. The mRNA and protein levels of PPARα were significantly increased in the medium-dose group compared with the control group. The mRNA level of PPARα was lower in the high-dose group than in the medium-dose group, but the protein level was high. In the Cpd C + BHBA group, the mRNA and protein levels of PPARα were significantly lower than in the medium-dose group, but were higher than the control group (Fig. 3A and D; $P < 0.05$).

The mRNA and protein levels of SREBP-1c were significantly decreased in the BHBA treatment groups than in the control group, and were higher in the Cpd C group than in the control group. In the Cpd C + BHBA group, the mRNA and protein levels were apparently higher than the medium-dose group (Fig. 3B and D; $P < 0.05$).

The mRNA and protein levels of ChREBP were obviously increased in the BHBA treatment groups compared with the control group, and were significantly decreased in the high-dose group compared with the medium-dose group. The mRNA level of ChREBP in the Cpd C group was significantly lower than the control group. The mRNA and protein levels of ChREBP in the Cpd C + BHBA group were obviously lower than the medium-dose group (Fig. 3D; $P < 0.05$).

The binding activities of PPARα, SREBP-1c, and ChREBP. The binding activities of PPARα and ChREBP were increased in the BHBA treatment groups. In the Cpd C + BHBA group, the binding activities were lower than the medium-dose group (Fig. 4A and B).

The binding activity of SREBP-1c was decreased in the BHBA treatment groups. In the Cpd C + BHBA group, the binding activity was higher than the medium-dose group (Fig. 4C).

The immunofluorescence results of PPARα, SREBP-1c and ChREBP translocate into the cell nucleus. BHBA increased PPARα and ChREBP translocated into cell nucleus, but the situations were opposite in Cpd C group (Fig. 5A and C). However, BHBA decreased SREBP-1c translocated into nucleus, but opposite in Cpd C group (Fig. 5B).

The mRNA and protein expression levels of downstream genes: ACSL-1, L-FABP, CPT-I, CPT-II, ACCα and FAS. The mRNA levels of the PPARα target genes long-chain fatty acyl-CoA synthetase (ACSL-1), carnitine palmitoyl transferase I (CPT-I), carnitine palmitoyl transferase II (CPT-II), and liver fatty acid-binding protein

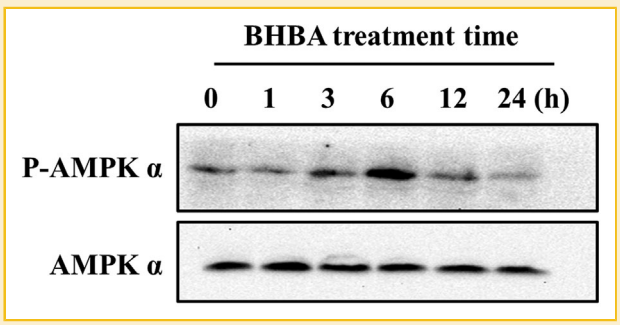

Fig. 1. Influence of BHBA treatment duration on the AMPKα phosphorylation in bovine hepatocytes. Hepatocytes were treated with 1.2 mM BHBA for 0, 1, 3, 6, 12, 24 h, respectively. 160x80mm (300 × 300 DPI).

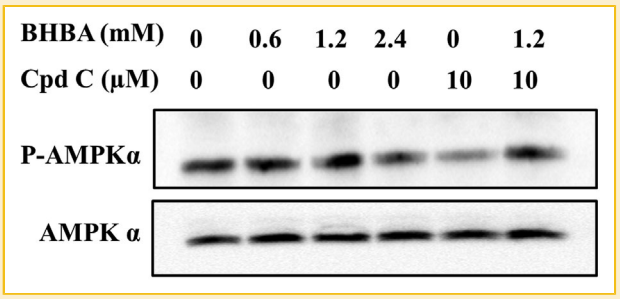

Fig. 2. Different concentrations of BHBA impact the phosphorylation of AMPKα in bovine hepatocytes. Hepatocytes were treated with BHBA and Cpd C and were divided into a control group (0 mM BHBA), a lowdose group (0.6 mM BHBA), a medium-dose group (1.2 mM BHBA), a high-dose group (2.4 mM BHBA), a CpdC group (10 μM Compound C), and a Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). The figure is western blotting results of p-AMPKα and AMPKα. 217 × 101 mm (300 × 300 DPI).

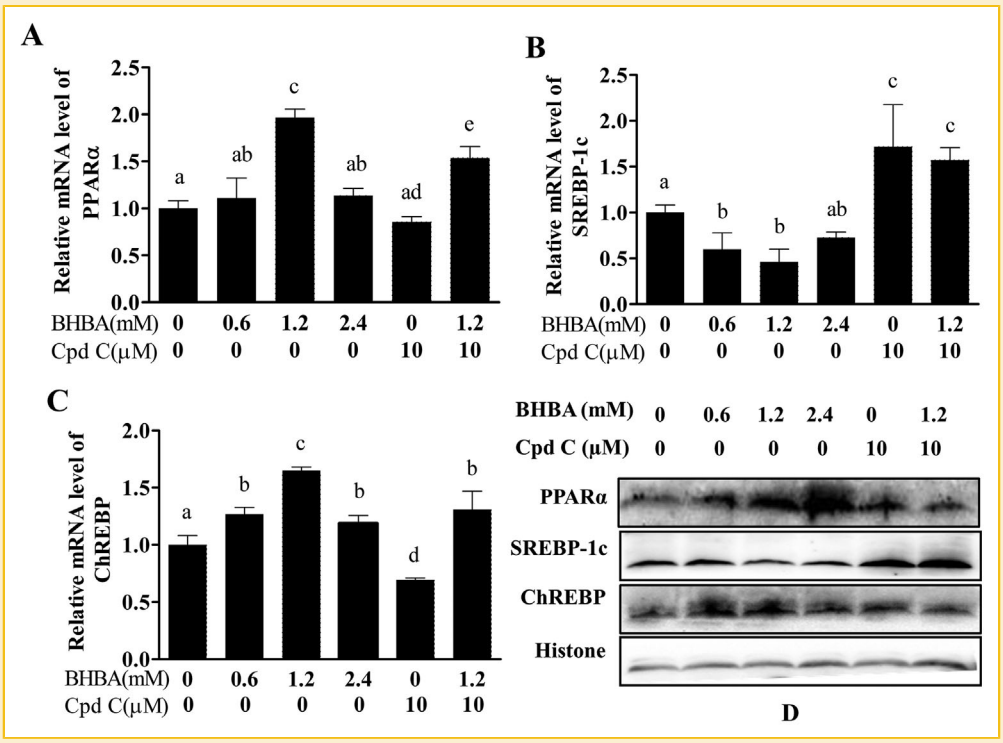

Fig. 3. Influence of BHBA and Cpd C on the mRNA and protein levels of nuclear transcription factors PPARα, SREBP-1c and ChREBP in hepatocytes. The hepatocytes were treated with BHBA and Cpd C and were divided into a control group (0 mM BHBA), a low-dose group (0.6 mM BHBA), a medium-dose group (1.2 mM BHBA), a high-dose group (2.4 mM BHBA), a Cpd C group (10 μM Compound C), and a Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). A–C: mRNA expression levels of PPARα, SREBP-1c and ChREBP respectively. Values are the means ± SE ($n = 6$), different letters are statistically different, $P < 0.05$. D: Protein expression levels of PPARα, SREBP-1c, and ChREBP. 282 × 204 mm (300 × 300 DPI).

(L-FABP) tended to increase in the BHBA treatment groups compared with the control group, but lower in the high-dose group than in the medium-dose group. The mRNA levels of above genes were lower in the Cpd C group than in the control group. In the Cpd C + BHBA group, the mRNA levels of above genes were significantly lower than the medium-dose group (Fig. 6A–D; $P < 0.05$).

In contrast, the mRNA levels of acetyl-CoA carboxylase α (ACCα) and fatty acid synthase (FAS) were decreased in the BHBA treatment groups compared with the control group. The mRNA level of ACCα in high-dose group was higher than that of medium-dose group. In the Cpd C group, the mRNA levels of ACCα and FAS were higher than those in control group. In the Cpd C + BHBA group, the mRNA level of ACCα was significantly higher than the medium-dose group (Fig. 6E and F; $P < 0.05$).

The protein levels of ACSL-1 and L-FABP in low-dose and medium-dose groups were increased compared with the control group, but were lower in Cpd C group than the control group. The protein level of L-FABP was lower in the high-dose group than the medium-dose group. The protein levels of ACSL-1 and L-FABP were lower in the Cpd C + BHBA group than the medium-dose group (Fig. 6G).

The protein levels of ACCα and FAS were significantly decreased in the BHBA treatment groups compared with the control group. The protein levels of ACCα and FAS were lower in the high-dose group than those in medium-dose group. In the Cpd C + BHBA treatment group, the protein level of FAS was markedly higher than the medium-dose group (Fig. 6G).

TRIGLYCERIDE CONTENT

The content of TG has no obviously change in the BHBA and Cpd C-treated groups (Fig. 7; $P > 0.05$).

DISCUSSION

Ketosis is a nutritional and metabolic disease induced by NEB and is characterized by hyperketonemia and hypoglycemia, especially occurs in high yielding cows [Wathes et al., 2011]. BHBA is the main form of ketone bodies in cows with ketosis. The blood concentration and the biological function of BHBA in cows are different from humans and mice. The blood concentration and the biological function of BHBA in cows are different from humans and mice. In dairy cows, BHBA is an important milk fat precursor that involved in fatty acid synthesis of milk fat. The plasma concentrations of BHBA in normal dairy cows are less than 1.2 mM. However, if subclinical or clinical ketosis develops, BHBA levels will exceed 1.2 mM or 1.4 mM, respectively [Iwersen et al., 2013]. Furthermore, the ability of using BHBA is limited in the liver of cows, resulting in the accumulation of excess BHBA in the liver and blood, which will finally lead to ketosis

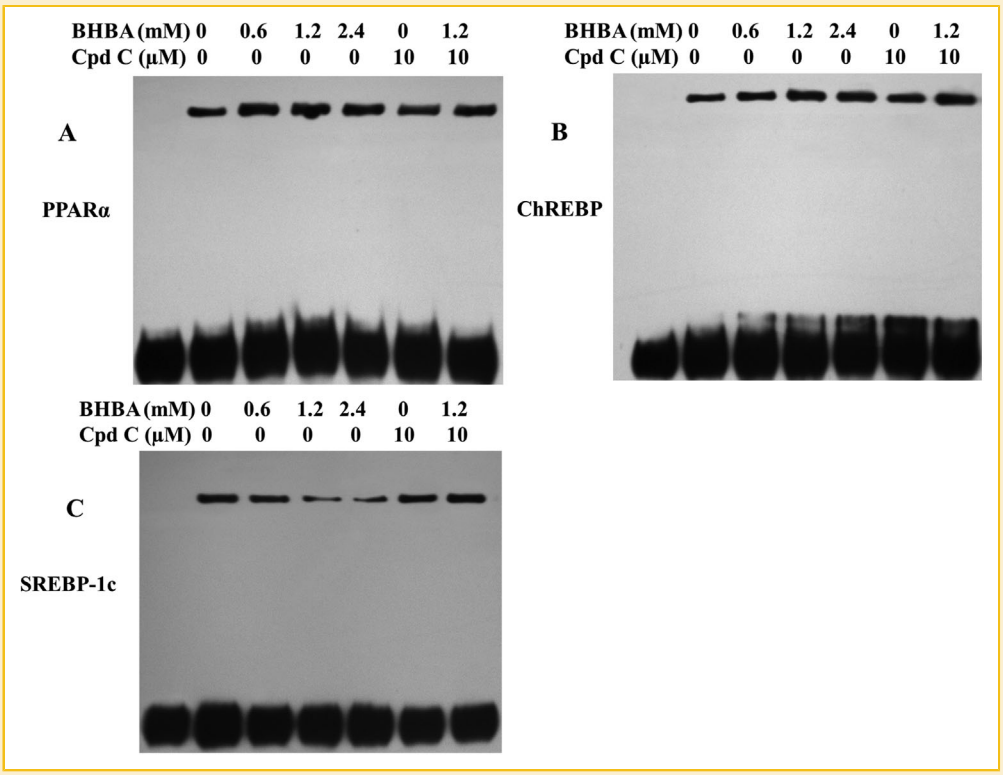

Fig. 4. The transcription activities of PPARα, SREBP-1c, and ChREBP. The hepatocytes were treated with BHBA and Cpd C and were divided into a control group (0 mM BHBA), a low-dose group (0.6 mM BHBA), a medium-dose group (1.2 mM BHBA), a high-dose group (2.4 mM BHBA), a Cpd C group (10 μM Compound C), and a Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). A: The EMSA result of PPARα. B: The EMSA result of ChREBP. C: The EMSA result of SREBP-1c. 181 × 138 mm (300 × 300 DPI).

[Grummer, 2008]. However, in human, BHBA can be used as energy molecule to provide energy, and BHBA blood concentration is 0.03–0.3 mM. It is not clear that the effects of BHBA on the lipid metabolism in hepatocytes of cows. We all have known that AMPK signaling pathway is important in liver which could regulate energy metabolism. However, there is limited information on whether BHBA could regulate lipid metabolism by this pathway. In this study we found that the phosphorylation level of AMPKα was increased in low and medium-dose BHBA treatment groups, but decreased in high-dose and Cpd C + BHBA groups (see Fig. 2). These results demonstrate that low and medium-dose BHBA can activate AMPKα pathway, while high level of BHBA suppress AMPK phosphorylation.

AMPK is an important lipid metabolism regulator that affects the lipid metabolism transcription factors such as PPARα, SREBP-1c and ChREBP [Bronner et al., 2004]. PPARα influences the intracellular lipid and carbohydrate metabolism by regulating the expression of genes involved in fatty acid transport, activation, and β-oxidation such as L-FABP, ACSL-1, CPT-1, and acyl-CoA oxidase (ACO) [Evans et al., 2004; Lefebvre et al., 2006]. Mice that lack of PPARα could induce fatty acid β-oxidation attenuation and result in the increase of free fatty acid and TG accumulation and then cause fatty liver [Djouadi et al., 1998]. CPT-I is an integral outer membrane protein that converts activated fatty acids into acylcarnitines [Lee et al., 2011]. CPT-II plays an essential role in the transfer of long-chain fatty acids inside mitochondria for β-oxidation. It binds acyl residues and help in their elimination [Dobrzyn et al., 2004; Longo et al., 2006]. In the process of fatty acid β-oxidation, ACSL-1 catalyzes long-chain fatty acids to long-chain fatty acyl-CoA and plays an important role in channeling fatty acids either toward complex lipid synthesis and storage or toward oxidation [Liu et al., 2013b]. L-FABP regulates the transport of fatty acids in the hepatocytes [Huang et al., 2002].

In this study, we demonstrated that BHBA increased the protein expression, nucleus translocation, binding activity of PPARα (see Fig. 3A, 3D, 4A and 5A). Moreover, the mRNA and protein levels of PPARα target genes, including L-FABP, ACSL-1, CPT I, and CPT II were in accordance with PPARα (see Fig. 6A–D and 6G). However, high level of BHBA treatment attenuated the above genes expression compared with the medium-dose BHBA group. Our data demonstrated that BHBA increased lipid oxidation in bovine hepatocytes. It is considerable that an increase of PPARα expression induced by BHBA increases the NEFA incomplete oxidation in hepatocytes, resulting in hyperketonemia in perinatal cows with NEB.

SREBP-1c and ChREBP are the most important inducers of the de novo hepatic lipogenesis by modulating lipogenic enzymes, such as ACCα and FAS [Postic and Girard, 2008; Tappy and Le, 2010]. Mice that overexpressed SREBP-1c had a 4-fold increase in the rate of fatty acid synthesis, and cause lipid accumulation in the liver.

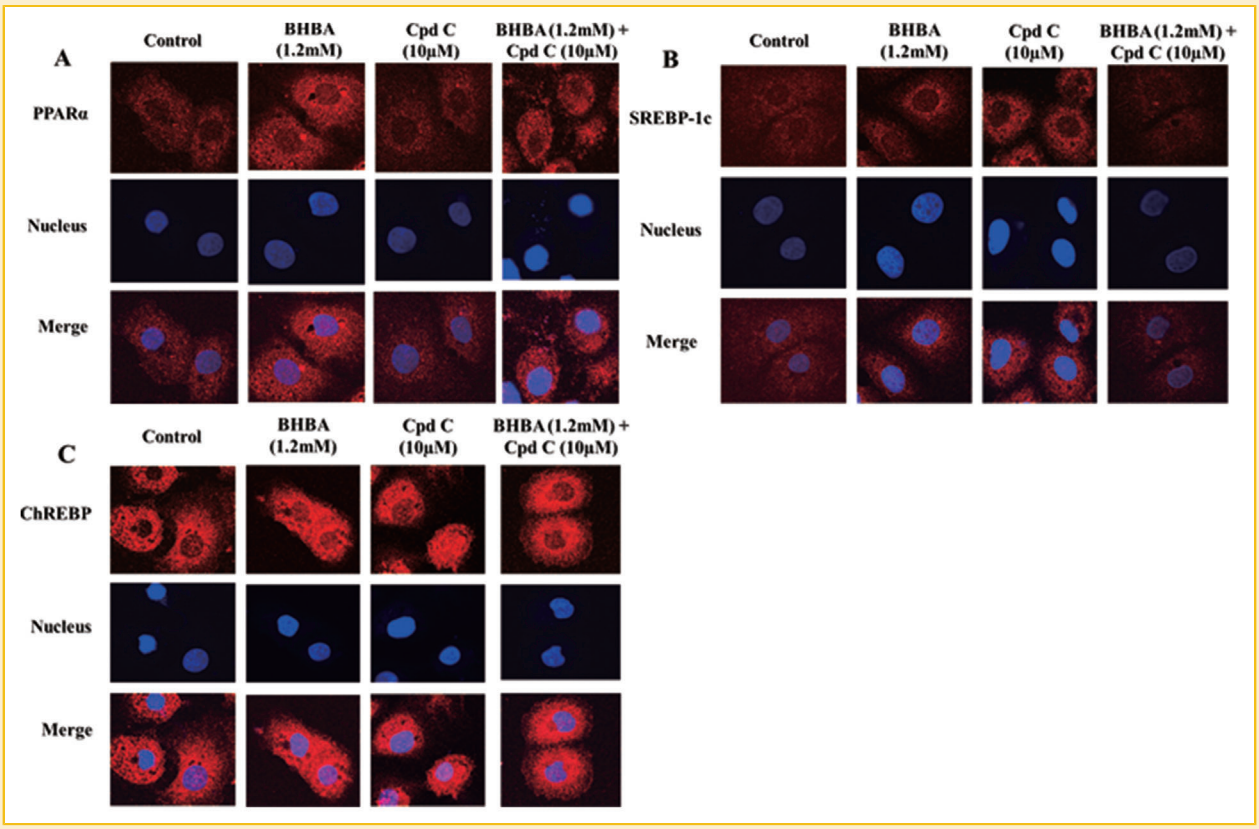

Fig. 5. The immunofluorescence results of PPARα, SREBP-1c, and ChREBP in bovine hepatocytes (800×). The hepatocytes were treated with BHBA and Cpd C and were divided into several groups: control group (0 mM BHBA), medium-dose group (1.2 mM BHBA), Cpd C group (10 μM Compound C) and Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). 65 × 42 mm (300 × 300 DPI).

Corresponding 2–6-fold increases were measured in mRNA levels for the lipogenic genes ACC and FAS [Shimomura et al., 1999]. Furthermore, Iizuka et al. (2004) reported that ChREBP regulated 50% of hepatic de novo lipogenesis [Iizuka et al., 2004]. The incidence of fatty liver was decreased when silencing the expression of SREBP-1c, and its target genes FAS and ACCα also significantly decreased in ob/ob mice [Yahagi et al., 2002; Tappy and Le, 2010]. ACCα is the most important enzyme during fatty acid synthesis [Choi et al., 2013], and is enriched in the liver, adipose and lactating mammary gland, where it catalyzes the biosynthesis of long-chain fatty acids [Liu et al., 2013a]. FAS is a determinant of the maximal capacity of the liver to synthesize fatty acids by de novo lipogenesis [Postic and Girard, 2008]. Caffeine activated AMPK and then decreased the mRNA levels of lipogenesis-associated genes (SREBP-1c and FAS) and increased lipolysis [Quan et al., 2013]. The DNA-binding activity of ChREBP significantly decreased in the liver of rats that fed a high fat diet [Dentin et al., 2005]. But the effect of BHBA on SREBP-1c and ChREBP has not well understood, particularly in bovine hepatocytes.

In this study, we demonstrated that the expression, binding and transcriptional activity of SREBP-1c were significantly decreased in BHBA treatment groups. Furthermore, we found that the nucleus translocation of SREBP-1c was also decreased in the BHBA treatment groups but increased in the Cpd C + BHBA groups (see Fig. 3B, 3D, 4C, and 5B). Nevertheless, the result of ChREBP was opposite with SREBP-1c (see Fig. 3C, D, 4B, and 5 C). Interestingly, the expression of their downstream genes FAS and ACCα were consistent with the change of SREBP-1c (see Fig. 6E–G). Many studies demonstrated that AMPK activation could down-regulated the lipogenesis-associated genes such as FAS and ACCα and then inhibited lipogenesis [Wu et al., 2013]. In this study, the expression of SREBP-1c was decreased but ChREBP was increased, so we speculated that BHBA can decrease the expression of FAS and ACCα mainly induced by SREBP-1c. High-dose BHBA treatment increased SREBP-1c expression and decreased ChREBP expression compared with medium-dose BHBA treatment groups, and expressions of lipid synthesis genes were consistent with SREBP-1c. Taken together, these results indicate that the effects of BHBA on bovine hepatic lipid metabolism are in a dose-dependent manner in vitro.

BHBA activated AMPK/PPARα signaling pathway and up-regulated the expressions of lipid oxidation genes, thereby increasing lipid oxidation in hepatocytes. In addition, activation of AMPK inhibited the expression, binding and transcriptional activity of SREBP-1c but increased ChREBP, and decreased lipid synthesis genes expression. BHBA activates AMPK to inhibit SREBP-1c, and maybe activate other signaling pathway in an AMPK-independent manner to increase the ChREBP. However, we found that the content of TG has no obviously change in the BHBA and Cpd

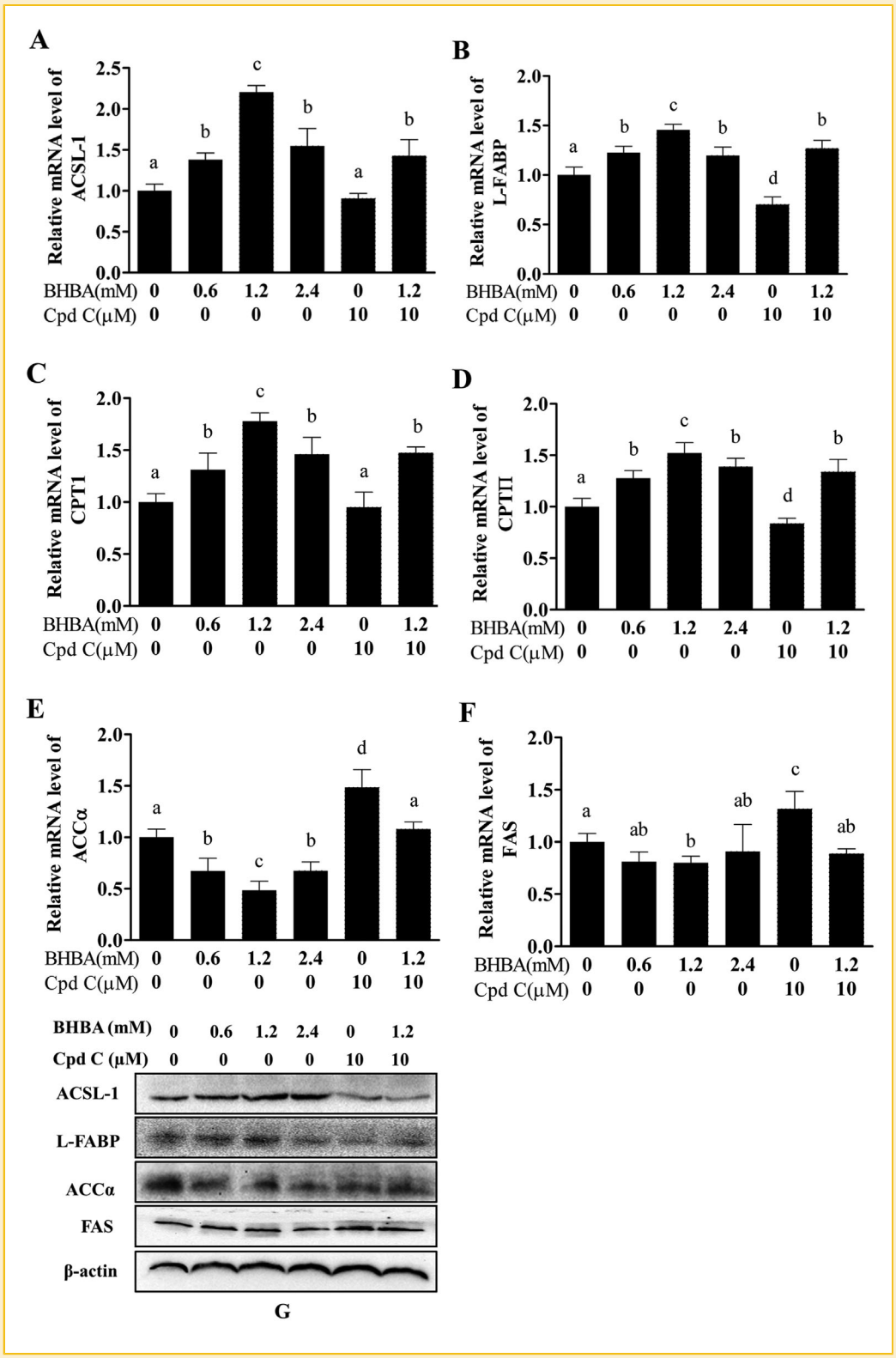

Fig. 6. The effect of BHBA on the expression levels of ACSL-1, L-FABP, CPT-I, CPT-II, ACCα, and FAS in bovine hepatocytes. The hepatocytes were treated with BHBA and Cpd C and were divided into several groups: control group (0 mM BHBA), low-dose group (0.6 mM BHBA), medium-dose group (1.2 mM BHBA), highdose group (2.4 mM BHBA), Cpd C group (10 μM Compound C), and Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). A–F: mRNA expression levels of ACSL-1, L-FABP, CPT-I, CPT-II, ACCα and FAS, respectively. Values are the means ± SE (n = 6), different letters are statistically different, $P < 0.05$. G: The protein levels of ACSL-1, L-FABP, ACCα, and FAS. 160 × 245 mm (300 × 300 DPI).

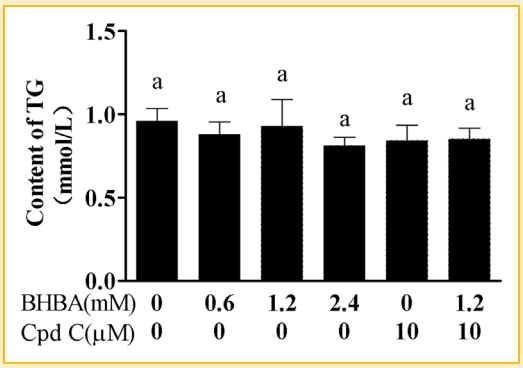

Fig. 7. Triglyceride contents in bovine hepatocytes. The hepatocytes were treated with BHBA and Cpd C and were divided into several groups: control group (0 mM BHBA), low-dose group (0.6 mM BHBA), medium-dose group (1.2 mM BHBA), high-dose group (2.4 mM BHBA), Cpd C group (10 μM Compound C), and Cpd C + BHBA group (10 μM Compound C + 1.2 mM BHBA). Values are the means ± SE ($n = 3$), $P > 0.05$. 103 × 71 mm (300 × 300 DPI).

C treatment groups compared with the control group (see Fig. 7). Clinical investigations have demonstrated that high levels of BHBA and NEFA could lead to hepatic lipid deposition in perinatal cows with NEB. Our data demonstrates that BHBA does not induce TG lipidosis in bovine hepatocytes in vitro. Therefore, we considered that high blood concentration of NEFA plays a dominant role in the induction of hepatic lipidosis. Because BHBA is a mesostate, the regulation of BHBA to lipid metabolism may be weaker than NEFA. However, Shi et al. reported that high level of BHBA could activate nuclear factor kappa B (NF-κB) signaling pathway to increase the synthesis of inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-a), and interleukin 1 beta (IL-1β), and induce inflammatory injury [Shi et al., 2014]. This demonstrates that accumulation of TG in hepatocytes may occur through NF-κB pathway. Taken together, BHBA activated AMPK signaling pathway, but there was no significant change in the TG content.

AUTHOR'S CONTRIBUTIONS

Conceived and designed the experiment: Qinghua Deng, Guowen Liu and Xiaobing Li. Performed the experiments: Qinghua Deng, Liheng Yin, Yuming Zhang, Wei Yang, Yu Li, Lihui Guo and Xue Yuan. Analyzed the data: Jianguo Wang, Renhe Zhang, Lei Liu, Xiaoxia Shi and Guoquan Sun. Wrote the paper: Qinghua Deng, Xinwei Li and Zhe wang.

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