Acetic Acid Activates the AMP-Activated Protein Kinase Signaling Pathway to Regulate Lipid Metabolism in Bovine Hepatocytes

Xinwei Li, Hui Chen, Yuan Guan, Xiaobing Li, Liancheng Lei, Juxiong Liu, Liheng Yin, Guowen Liu*, Zhe Wang*

Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, Jilin, China

Abstract
The effect of acetic acid on hepatic lipid metabolism in ruminants differs significantly from that in monogastric animals. Therefore, the aim of this study was to investigate the regulation mechanism of acetic acid on the hepatic lipid metabolism in dairy cows. The AMP-activated protein kinase (AMPK) signaling pathway plays a key role in regulating hepatic lipid metabolism. In vitro, bovine hepatocytes were cultured and treated with different concentrations of sodium acetate (neutralized acetic acid) and BML-275 (an AMPKα inhibitor). Acetic acid consumed a large amount of ATP, resulting in an increase in AMPKα phosphorylation. The increase in AMPKα phosphorylation increased the expression and transcriptional activity of peroxisome proliferator-activated receptor α, which upregulated the expression of lipid oxidation genes, thereby increasing lipid oxidation in bovine hepatocytes. Furthermore, elevated AMPKα phosphorylation reduced the expression and transcriptional activity of the sterol regulatory element-binding protein 1c and the carbohydrate responsive element-binding protein, which reduced the expression of lipogenic genes, thereby decreasing lipid biosynthesis in bovine hepatocytes. In addition, activated AMPKα inhibited the activity of acetyl-CoA carboxylase. Consequently, the triglyceride content in the acetate-treated hepatocytes was significantly decreased. These results indicate that acetic acid activates the AMPKα signaling pathway to increase lipid oxidation and decrease lipid synthesis in bovine hepatocytes, thereby reducing liver fat accumulation in dairy cows.

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* E-mail: liguowen2008@163.com (GL); wangzhe500518@sohu.com (ZW)

Introduction
The mechanism of carbohydrate digestion and nutrient metabolism in ruminants differs significantly from that in monogastric animals. Cellulose and starch are the main carbohydrates for ruminants. Ruminal micro-organisms convert cellulose and starch to volatile fatty acids (VFAs, including acetic acid, propionic acid, and butyric acid), carbon dioxide, and methane. VFAs are the main precursors of lipid and glucose synthesis in ruminants [1]. Acetic acid, which accounts for 70–75% of VFAs, is absorbed by the rumen wall and neutralized by conversion to acetate in the blood. The blood acetate concentration in dairy cows is 3.6 mM, which is dozens of times higher than that in monogastric cows [2]. The biological function of acetic acid in dairy cows is also different from that in humans and mice. Acetic acid is mainly used for milk fat synthesis in dairy cows [2]. However, in humans and mice, acetic acid is mainly used to generate energy through tricarboxylic acid cycle in hepatocytes [3]. In recent years, it has become evident that acetic acid can also act as a signaling molecule to regulate gene expression in the liver [4].

AMP-activated protein kinase (AMPK) is a phylogenetically conserved serine/threonine protein kinase that has been proposed to act as a “metabolic master switch” that modulates hepatic lipid metabolism to adapt to environmental or nutritional stress factors [5,6]. 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 carbohydrate responsive element-binding protein (ChREBP), all of which govern the expression of lipid metabolic enzymes [7–9]. Therefore, the AMPK signaling pathway plays a central role in hepatic lipid metabolism. AMPKα is activated in response to an increase in the ratio of AMP to ATP within the cell [10]. Furthermore, the liver kinase B1 (LKB1), a known tumor suppressor, is the upstream kinase in the AMP-activated protein kinase cascade [11]. Activation of AMPKα by LKB1 depends on the AMP/ATP ratio. Deleting LKB1 in the liver results in a proportional decrease in AMPKα phosphorylation at Thr172 [12]. Sirtuin1 (SIRT1) is also a fuel-sensing molecule that plays an important role in the regulation of cell energy metabolism. Study demonstrated that AMPK and SIRT1 regulated each other [13]. Price et al. [14]...
reported that resveratrol activated AMPK in a SIRT1-dependent manner through deacetylation of LKB1.

Kondo et al. [15] demonstrated that acetic acid-treated mice had lower triglyceride (TG) content in the liver and increased expression of lipid oxidation genes. Furthermore, Sakakibara et al. [16] reported that acetic acid activated hepatic AMPKα in diabetic KK-A(y) mice. Consequently, genes downstream of AMPKα that are involved in lipid oxidation were upregulated, increasing lipid oxidation. These studies demonstrated that acetic acid could reduce liver fat accumulation by activating the AMPK pathway. The hepatic lipid metabolism of dairy cows is different from that of monogastric animals such as humans and mice. And the effect of acetic acid on the hepatic lipid metabolism in dairy cows is also significantly different from that in humans and mice. However, in ruminants, it is unclear the mechanism of acetic acid on the regulation of hepatic lipid metabolism. Therefore, the objective of this study was to investigate the molecular mechanism by which acetic acid regulates lipid metabolism in bovine hepatocytes. The results of this study should provide insights into the physiological function of acetic acid in hepatic lipid metabolism in ruminants.

Results

Effect of the duration of acetic acid treatment on AMPKα phosphorylation and activity in hepatocytes

The phosphorylation level of AMPKα (p-AMPKα/AMPKα) and AMPKα activity in hepatocytes were highest at 3 h of acetate treatment (Fig. 1A–1C; p<0.01). At 3 h, the phosphorylation level of AMPKα in the acetic-acid-treated hepatocytes was 2.25-fold higher than in the control hepatocytes and AMPKα activity was 2.50-fold higher.

Acetic acid activates AMPKα in hepatocytes

To determine the effect of acetic acid on the AMPKα, we determined the AMP and ATP content, the AMPKα phosphorylation and activity, and the LKB1 protein expression. As shown in Figure 2, the ATP content was significantly lower in the medium- and high-dose treatment groups than in the control group (Fig. 2A; p<0.01). However, the AMP content was significantly increased in the acetate-treated groups (Fig. 2B; p<0.01). The AMP/ATP ratio increased from 2.00- to 10.00-fold from the low-dose acetate treatment group to the high-dose group (Fig. 2C; p<0.01). The phosphorylation level of AMPKα and AMPKα activity was also significantly higher in the acetate-treated groups than in the control group and was significantly lower in the BML-275 (an AMPKα inhibitor) and BML-275+acetate groups than in the control group (Fig. 2G, 2H; p<0.05 and p<0.01). Overall, these results demonstrate that acetic acid converts to acetyl-CoA with the consumption of ATP, resulting in a significant increase in the AMP/ATP ratio, which induces an increase of AMPK phosphorylation and activity. The protein levels of SIRT1 were significantly higher in the high-dose acetate treatment group than in the control group (Fig. 2E; p<0.05). However, there was no significant change in the protein levels of LKB1 (Fig. 2F). SIRT1 activates AMPKα dependent on LKB1. These results indicate that high levels of acetic acid increase SIRT1 expression. However, acetic acid does not significantly affect the protein expression of LKB1.

The expression and transcriptional activity of PPARα, SREBP-1c, and ChREBP in hepatocytes

The mRNA and protein expression levels of PPARα were significantly higher in the medium- and high-dose acetate treatment groups than in the control group and were significantly lower in the BML-275 and BML-275+acetate groups than in the control group (Fig. 3B, 3G; p<0.05 and p<0.01). However, the SREBP-1c and ChREBP showed the opposite results. The SREBP-1c mRNA levels were significantly lower in the medium- and high-dose treatment groups than in the control group (Fig. 3D; p<0.05 and p<0.01), and the SREBP-1c protein levels were significantly lower in the high-dose treatment group (Fig. 3E; p<0.01). The SREBP-1c mRNA and protein levels were significantly higher in the BML-275 and BML-275+acetate groups than in the control group (Fig. 3D, 3E; p<0.05 and p<0.01). The mRNA and protein levels of ChREBP were significantly lower in the high-dose treatment group than in the control group and were significantly higher in the BML-275 and BML-275+acetate groups than in the control group (Fig. 3F, 3G; p<0.05 and p<0.01). Taken together, these findings suggest that acetic acid increases the expression of PPARα and decreases the expression of SREBP-1c and ChREBP in bovine hepatocytes.

The transcriptional activity of PPARα was significantly higher in the medium- and high-dose acetate treatment groups than in the control group and was significantly lower in the BML-275 and BML-275+acetate groups than in the control group (Fig. 4A, 4B; p<0.05 and p<0.01). The transcriptional activity of SREBP-1c and ChREBP displayed the opposite trend. The transcriptional activity of SREBP-1c was significantly lower in the medium- and high-dose groups than in the control group and was markedly higher in the BML-275 and BML-275+acetate groups than in the control group (Fig. 4C, 4D; p<0.05 and p<0.01). The transcriptional activity of ChREBP was significantly lower in the medium- and high-dose groups (Fig. 4E, 4F; p<0.01). Taken together with the data presented in Fig. 4, these results strongly suggest that acetic acid increases the transcriptional activity of PPARα and inhibits the transcriptional activity of SREBP-1c and ChREBP in bovine hepatocytes.

The mRNA expression levels of the PPARα, SREBP-1c, and ChREBP target genes in hepatocytes

The mRNA levels of the PPARα target genes acyl-CoA oxidase (ACO), carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), and liver fatty acid-binding protein (L-FABP) tended to increase in the acetate-treated groups. The
mRNA levels of ACO, CPT1, and L-FABP were markedly higher in the medium- and high-dose acetate treatment groups than in the control group (Fig. 5A-5C; \( p < 0.05 \) and \( p < 0.01 \)). The mRNA levels of CPT2 were significantly higher in the high-dose group than in the control group (Fig. 5D; \( p < 0.01 \)). However, the mRNA levels of ACO, CPT1, and L-FABP were significantly lower in the BML-275 and BML-275 + acetate groups than in the control group (Fig. 5A-5C; \( p < 0.05 \) and \( p < 0.01 \)). Overall, these results demonstrate that acetic acid upregulates the mRNA expression of lipid oxidation genes by increasing the expression and transcriptional activity of PPAR\( \alpha \) in bovine hepatocytes.

In contrast, the mRNA expression levels of the SREBP-1c and ChREBP target genes, including acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1), tended to decrease in the acetate-treated groups. The mRNA levels of ACC1, FAS, and SCD-1 were significantly lower in the medium- and high-dose groups than in the control group and were markedly higher in the BML-275 and BML-275 + acetate groups than in the control group (Fig. 5A-5C; \( p < 0.05 \) and \( p < 0.01 \)). Overall, these results demonstrate that acetate upregulates the mRNA expression of lipid oxidation genes by increasing the expression and transcriptional activity of PPAR\( \alpha \) in bovine hepatocytes.

The phosphorylation level and enzyme activity of ACC1 in hepatocytes

The phosphorylation level of ACC1 (p-AC1/AC1) increased in an acetate dose-dependent manner and was significantly higher in the acetate-treated groups than in the control group. It was significantly lower in the BML-275 and BML-275 + acetate groups than in the control group (Fig. 6B; \( p < 0.01 \)). ACC1 activity was significantly lower in the medium- and high-dose groups than in the control group and was markedly higher in the BML-275 and BML-275 + acetate groups than in the control group (Fig. 6C; \( p < 0.01 \) and \( p < 0.05 \)). These results indicate that acetate-activated AMPK\( \alpha \) can directly phosphorylate ACC1 and inhibit its activity, which inhibits lipid synthesis in bovine hepatocytes.

Triglycerides content

Acetate decreased hepatocyte TG content in a dose-dependent manner. The TG content was 35% lower in the high-dose acetate treatment group than in the control group (Fig. 7; \( p < 0.01 \)). Taken together, our \( \textit{in vitro} \) data demonstrate that acetic acid activates AMPK\( \alpha \) signaling pathway to reduce TG content in bovine hepatocytes.

Discussion

In dairy cows, the liver is the main organ responsible for modulating lipid metabolism and maintaining lipid homeostasis.
through responses to nutrient signals [17]. In recent years, studies have shown that acetic acid can act as a signaling molecule that modulates the expression of lipid metabolism genes in hepatocytes [9,18]. An in vitro HepG2 cell study demonstrated that acetic acid activates AMPKα, which in turn upregulates the expression of lipid oxidation genes in the liver to reduce fat accumulation [16]. A liver-specific AMPKα deletion in mice leads to increased plasma TG content and hepatic lipogenesis [19].

The blood acetate concentration is dozens of times higher in dairy cows than that in humans and mice. Furthermore, the biological function of acetic acid in ruminants is different from that in humans and mice. However, it is not clear whether acetic acid activates the AMPK signaling pathway in the ruminant liver. In this study, we observed that the AMP/ATP ratio increased 2.00- to 10.00-fold in acetate-treated hepatocytes. The phosphorylation level of AMPKα and AMPKα activity were significantly increased in acetate-treated groups and were significantly lower in the BML-275+acetate groups than in the control group. abundant acetate was converted to acetyl-CoA with the consumption of ATP in hepatocytes, resulting in a significant increase in the AMP/ATP ratio. The high AMP/ATP ratio increased AMPKα phosphorylation with the help of LKB1. These results demonstrate that acetic acid activates AMPKα in bovine hepatocytes. SIRT1 and AMPK is the cell metabolism regulator that regulates the cell energy metabolism [13]. An in vivo study demonstrated that SIRT1 activated AMPK dependent on the LKB1 [20]. In this study, the protein levels of SIRT1 were significantly increased in the high-dose acetate treatment group. However, there was no significant change in the protein levels of LKB1. These results suggest that acetic acid does not significantly affect the SIRT1 and LKB1 in bovine hepatocytes, which may be due to the difference of energy metabolism in the bovine hepatocytes. The hepatic energy metabolism of dairy cows is different from that of monogastric animals such as humans and mice. Taken together, these results indicate that acetic acid activates AMPK signaling pathway mainly through consumption of the intracellular ATP.

AMPK acts as a key metabolic “masters witch” by regulating target transcription factors involved in lipid metabolism, including PPARα, SREBP-1c and ChREBP. PPARα is a ligand-activated transcription factor that plays a key role in the regulation of the expression of lipid oxidation genes, including ACO, CPT1, L-FABP, and CPT2 [21,22]. ACO is a rate-limiting enzyme in fatty acid β oxidation [23]. CPT1 and CPT2 transfer long-chain acyl-CoA into the mitochondria for β oxidation [20]. L-FABP regulates the intake and transport of fatty acids in the cell [24]. ACO, CPT1, L-FABP, and CPT2, which are regulated by PPARα, are the key enzymes of lipid oxidation in hepatocytes. In an in vitro study, HepG2 cells treated with 100, 200, or 500 μM acetate displayed significantly increased expression of PPARα and its target genes, including ACO and CPT1 [15]. However, in mice treated with acetic acid, Sakakibara et al. [16] demonstrated that

Figure 3. The mRNA and protein expression levels of PPARα, SREBP-1c, and ChREBP in the hepatocytes. Hepatocytes were treated with acetate and BML-275 and divided into a control group (0 mM acetate), a low-dose acetate treatment group (1.8 mM acetate), a medium-dose acetate treatment group (3.6 mM acetate), a high-dose acetate treatment group (7.2 mM acetate), a BML-275 group (10 μM BML-275), and a BML-275+acetate group (10 μM BML-275+3.6 mM acetate). Acetate (sodium acetate) was used in the form of neutralized acetic acid to avoid changing the pH of the medium. A: Western blotting results of PPARα, SREBP-1c, and ChREBP. B and C: mRNA and protein levels of PPARα. D and E: mRNA and protein levels of SREBP-1c. F and G: mRNA and protein levels of ChREBP. *p<0.05, **p<0.01 versus the control group. doi:10.1371/journal.pone.0067880.g003
Acetic acid did not affect the transcription of PPARα, and that ACO mRNA levels were not significantly increased. In this study, we demonstrated that acetic acid could activate AMPKα. The expression levels and transcriptional activity of PPARα were significantly increased in the medium- and high-dose acetate treatment groups and were significantly lower in the BML-275 and BML-275 + acetate groups than in the control group. These results indicate that acetic acid-activated AMPKα promotes the expression and transcriptional activity of PPARα. Moreover, the mRNA expression levels of PPARα target genes, including ACO, CPT1, L-FABP, and CPT2, were significantly upregulated in the acetate-treated groups. Acetic acid activates PPARα, which increases the expression of lipid oxidation genes, thereby increasing lipolysis in bovine hepatocytes. The blood concentration of acetic acid is dozens of times higher in dairy cows than in mice, and this high concentration of acetic acid promotes lipolysis in the hepatocytes of dairy cows. The variations in the effect of acetic acid on lipolysis may be due to differences in the treatment concentrations of acetate and animal species among experiments.

SREBP-1c and ChREBP govern lipogenesis through the transcriptional regulation of lipogenic genes, including ACC1, FAS, and SCD-1 [25,26]. The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis, and the enzyme that catalyzes this reaction, ACC1, is the major regulatory site in fatty acid synthesis [27]. FAS and SCD-1 catalyze fatty acid elongation and desaturation steps, respectively. FAS is a determinant of the maximal capacity of the liver to synthesize fatty acids by de novo lipogenesis [28]. SCD1 catalyzes the synthesis of monounsaturated fatty acids, particularly oleate (C18:1n-9) and palmitoleate (C16:1n-7), which are the major components of TG [28]. ACC1, FAS, and SCD-1 are the key rate-controlling enzymes in lipid synthesis. The DNA-binding activity of ChREBP is significantly decreased in the livers of rats fed a high fat diet [29]. Sakakibara et al. [16] reported that SREBP-1c mRNA

Figure 4. Transcriptional activity of PPARα, SREBP-1c, and ChREBP in hepatocytes. Hepatocytes were treated with acetate and BML-275 and divided into a control group (0 mM acetate), a low-dose acetate treatment group (1.8 mM acetate), a medium-dose acetate treatment group (3.6 mM acetate), a high-dose acetate treatment group (7.2 mM acetate), a BML-275 group (10 μM BML-275), and a BML-275 + acetate group (10 μM BML-275 + 3.6 mM acetate). Acetate (sodium acetate) was used in the form of neutralized acetic acid to avoid changing the pH of the medium. A and B: EMSA results for PPARα. C and D: EMSA results for SREBP-1c. E and F: EMSA results for ChREBP. * p<0.05, ** p<0.01 versus the control group. doi:10.1371/journal.pone.0067880.g004
expression levels were significantly decreased in rat primary hepatocytes treated with 200 μM acetate. Furthermore, an in vivo study demonstrated that the administration of acetic acid to rats decreased the expression of lipogenic genes such as ACC1 and FAS [30]. However, Kondo et al. [15] did not observe changes in the expression of SREBP-1c and its target genes in acetic acid-treated rats. Taken together, these conflicting results demonstrate that the effect of acetic acid on the expression and transcriptional activity of SREBP-1c and ChREBP is not well understood, particularly in ruminants.

In this study, we demonstrated that the expression and transcriptional activity of SREBP-1c and ChREBP were significantly decreased in the acetate-treated groups but were markedly increased in the BML-275 and BML-275 + acetate groups. These results indicate that acetic acid inhibits the expression and transcriptional activity of SREBP-1c and ChREBP. Furthermore, the mRNA expression levels of the SREBP-1c and ChREBP target genes ACC1, FAS, and SCD-1 were significantly lower in the acetate-treated groups than in the control group. Taken together, these results indicate that the effect of acetic acid on the expression and transcriptional activity of SREBP-1c and ChREBP is not well understood, particularly in ruminants.

In conclusion, the current study indicates that acetic acid can act as a signaling molecule to significantly increase lipolysis and decrease lipid synthesis in bovine hepatocytes. A potential mechanism in which acetic acid is metabolized to acetyl-CoA in hepatocytes, with the consumption of ATP, is shown in Figure 8. An elevated AMP/ATP ratio increases the phosphorylation and activity of AMPKα. Activated AMPKα promotes the expression and transcriptional activity of PPARα, thereby increasing the expression of lipolytic genes. Furthermore, activated AMPKα inhibits the expression and transcriptional activity of SREBP-1c and ChREBP, thereby reducing the expression of lipogenic genes. In addition, activated AMPKα directly phosphorylate and inhibit ACC1. Consequently, acetic acid increases lipolysis and decreases lipid synthesis in bovine hepatocytes, which reduces hepatic fat accumulation in dairy cows. The current study identifies a biochemical mechanism for the regulation of hepatic lipid metabolism by acetic acid in dairy cows.
Materials and Methods

Materials

Fetal bovine serum, collagenase IV, HepatoZYME medium and RPMI-1640 medium were purchased from Gibco (Grand Island, NY, USA). Insulin and HEPES were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Dexamethasone acetate, vitamin C, ascorbic acid, penicillin, streptomycin, and other chemicals were obtained from Baoman Biotechnology (Shanghai, China). BML-275 (an AMPKα inhibitor) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

Hepatocytes were isolated using the collagenase perfusion method and were obtained as previously described [33,34]. The study protocol was approved by the Ethics Committee on the Use and Care of Animals, Jilin University (Changchun, China). Briefly, the caudate lobe of the liver was obtained through surgical excision from a female Holstein calf anesthetized with thiamylal sodium under sterile conditions. The liver was perfused with perfusion solution to wash away the blood until the perfusion solution became clear. The liver was then perfused with a collagenase IV solution to digest the liver tissue. After digestion, the liver capsule was cut off. A total of 100 mL basic medium containing 0.2% bovine serum albumin was added to terminate digestion. The liver capsule, blood vessels, fat, and any parts of the liver caudate lobe that were incompletely digested were removed. The hepatocyte suspension was filtered sequentially with 100 mesh (150 μm), 200 mesh (75 μm), and 500 mesh (30 μm) cell sieves. Then, the hepatocyte suspension was washed twice with basic medium. The cell density was adjusted to 1×10^6 cells/mL with adherent culture medium. The hepatocyte suspension was seeded

Figure 6. The phosphorylation level and enzyme activity of ACC1. Hepatocytes were treated with acetate and BML-275 and divided into a control group (0 mM acetate), a low-dose acetate treatment group (1.8 mM acetate), a medium-dose acetate treatment group (3.6 mM acetate), a high-dose acetate treatment group (7.2 mM acetate), a BML-275 group (10 μM BML-275), and a BML-275+acetate group (10 μM BML-275+3.6 mM acetate). Acetate (sodium acetate) was used in the form of neutralized acetic acid to avoid changing the pH of the medium. A: Western blotting results for p-ACC1 and ACC1. B: The phosphorylation level of ACC1. C: Enzyme activity of ACC1. * p<0.05, ** p<0.01 versus the control group.

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Figure 7. TG content in hepatocytes. Hepatocytes were treated with acetate and BML-275 and divided into a control group (0 mM acetate), a low-dose acetate treatment group (1.8 mM acetate), a medium-dose acetate treatment group (3.6 mM acetate), a high-dose acetate treatment group (7.2 mM acetate), a BML-275 group (10 μM BML-275), and a BML-275+acetate group (10 μM BML-275+3.6 mM acetate). Acetate (sodium acetate) was used in the form of neutralized acetic acid to avoid changing the pH of the medium. * p<0.05, ** p<0.01 versus the control group.

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Figure 8. Acetic acid activates the AMP-activated protein kinase signaling pathway to regulate lipid metabolism in bovine hepatocytes. Acetic acid is metabolized to acetyl-CoA with consumption of ATP in bovine hepatocytes, resulting in a significant elevation of the AMP/ATP ratio and the phosphorylation and activation of AMPKα. Activated AMPKα increases the expression and transcriptional activity of PPARα and ChREBP, thereby increasing the expression of lipolytic genes, including ACO, CPT1, CPT2, and L-FABP. AMPKα activation inhibits the expression and transcriptional activity of SREBP-1c and ChREBP, thereby reducing the expression of lipogenic genes, including ACC1, FAS, and SCD-1. In addition, activated AMPKα directly phosphorylates and inhibits ACC1. Consequently, acetic acid increases lipolysis and reduces lipid synthesis in bovine hepatocytes.

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Acetic acid treatment

Cells were serum-starved overnight and then treated with sodium acetate (acetate) in the form of neutralized acetic acid to avoid changing the pH of the medium. The concentration of acetate was chosen according to the normal hematology standards of dairy cows. The hepatocytes were subjected to the following treatments. For time course experiments, hepatocytes were treated with 3.6 mM acetate for 0, 1, 3, 6, 10, 16, and 24 h. For dose-response experiments, hepatocytes were treated with acetate and BML-275 (BML-275 is an AMPK activator) in the form of neutralized acetic acid to avoid changing the pH of the medium. The hepatocytes were treated with acetate and BML-275 (BML-275 is an AMPK activator) in the form of neutralized acetic acid to avoid changing the pH of the medium. The concentration of acetate or BML-275 was replicated 24 times. Each treatment was normalized to β-actin levels.

AMP and ATP level determination

Hepatocytes were harvested with a cell scraper and collected directly into 0.5 mL ice-cold 6% (v/v) perchloric acid. The cells were lysed by pipetting up and down repeatedly. The lysate was centrifuged for 5 min at 12000 g at 4°C. The supernatant was used to determine the content of AMP and ATP by reverse-phase HPLC analysis (Thermo Fisher Scientific, Waltham, MA, USA).

AMPK and ACC1 activity determination

Hepatocytes were harvested with a cell scraper and transferred into a centrifuge tube. The cells were washed twice with ice-cold PBS and lysed with lysis buffer (Shanghai Bluegene Biotech Co., Ltd., Shanghai, China) in an ice bath for 30 min. The lysate was then centrifuged for 5 min at 12000 g at 4°C, and the supernatant was used to determine the activity of AMPK and ACC1 using a biochemical kit (Shanghai Bluegene Biotech Co., Ltd.) according to the supplier’s protocol.

Triglyceride content determination

Hepatocytes were harvested with a cell scraper and transferred into a centrifuge tube. The cells were washed twice with ice-cold PBS and lysed with an SL-1000D ultrasonic cell disruption apparatus (Shunliu Instrument Company, Nanjing, China). The lysate was centrifuged for 5 min at 12000 g at 4°C, and the supernatant was used to determine the triglyceride content using an automatic biochemical analyzer (Shenyang EKSV Medical Equipment Co., Ltd., China).

RNA extraction and real-time RT-PCR

Hepatocyte RNA was extracted with a Takara RNA extraction kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer’s instructions. RNA integrity was determined by electrophoresis on 1% agarose gels. The RNA was analyzed by spectrophotometry at 260 and 280 nm using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK). Samples with an optical density ratio of RNA at 260/280 nm >1.8 were used for further analyses. The RNA was transcribed into cDNA using a reverse transcription kit (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Gene primers were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and shown in Table 1.

The mRNA expression levels were evaluated by quantitative polymerase chain reaction (qRT-PCR) analysis using a SYBR Green QuantiTect RT-PCR Kit (Takara Biotechnology Co., Ltd.). qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression of each gene was normalized to β-actin levels.

Western blotting

Hepatocytes were harvested and washed twice in cold-PBS. Total cellular proteins and nuclear proteins were extracted using a protein extraction kit and a nuclear protein extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer’s instructions. Protein concentrations were measured with the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany). Proteins were separated on polyacrylamide gels and electrophoresed on 1% agarose gels. The RNA was analyzed by spectrophotometry at 260 and 280 nm using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK). Samples with an optical density ratio of RNA at 260/280 nm >1.8 were used for further analyses. The RNA was transcribed into cDNA using a reverse transcription kit (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Gene primers were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and shown in Table 1.

The mRNA expression levels were evaluated by quantitative polymerase chain reaction (qRT-PCR) analysis using a SYBR Green QuantiTect RT-PCR Kit (Takara Biotechnology Co., Ltd.). qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression of each gene was normalized to β-actin levels.
ferred onto PVDF membranes. The membranes were blocked in bovine serum albumin/TBST buffer for 4 h and hybridized with antibodies specific for SIRT1, LKB1, AMPKα, phosphorylated AMPKα (p-AMPKα), ACC1, phosphorylated ACC1 (p-ACC1), PPARγ, SREBP-1c, and ChREBP (Cell Signaling Technology, Inc., Danvers, MA, USA; Santa Cruz Biotechnology) overnight at 4°C. The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected with an enhanced chemiluminescence solution (ECL, Beyotime Biotechnology Inc., China). The blots were exposed to X-ray film, and the band intensity was measured using BandScan software version 5.0 (Glyco).

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay (EMSA) was used to detect the transcriptional activity of PPARγ, SREBP-1c, and ChREBP. Nuclear proteins were extracted using a nuclear protein extraction kit (Sangon Biotech Co., Ltd, Shanghai, China) according to the manufacturer’s instructions. Protein concentrations were measured with the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany). The special probe recognition sequences were exposed to X-ray film, and the band intensity was measured using BandScan software version 5.0 (Glyco).

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**References**

1. Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70:567–590.
2. Xiong BH, Liu DX, Zhang ZY (2002) Effect of changing the molar ratio of acetate to propionate in rumen fluid on rumen fermentation and some blood indexes. Acta Veterinaria et Zootechnica Sinica 33:537–543.
3. Fushimi T, Sato Y (2005) Effect of acetic acid feeding on the circadian changes in glycogen and metabolites of glucose and lipid in liver and skeletal muscle of rats. Br J Nutr 94:714–719.
4. Fushimi T, Suruga K, Yoshifumi O, Momoiko F, Yoshinori T, et al. (2006) Dietary acetic acid reduces serum cholesterol and triacylglycerols in rats fed a cholesterol-rich diet. British Journal of Nutrition 95:916–924.
5. Kahn BB, Alquier T, Carling D, Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metabolism 1:15–25.
6. Viollet B, Foyet M, Guigas B, Horman S, Denini R, et al. (2006) Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. J Physiol 574(Pt 1):41–53.
7. Bronner M, Hertz R, Bar-Tana J (2004) Kinase-independent transcriptional co-activation of peroxisome proliferator-activated receptor α by AMP-activated protein kinase. Biochem J 384:295–305.
8. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X et al. (2011) AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metabolism 13:376–380.
9. Kawaguchi T, Otatomi K, Yamashita H, Kabashima T, Ueda K (2002) Mechanism for fatty acid “sparring” effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. J Biol Chem 277:3029–3035.
10. Harde DG (2003) The AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology 144:5179–5183.
11. Harde DG (2004) The AMP-activated protein kinase pathway: new players upstream and downstream. J Cell Sci 117:5479–5407.
12. Shaw RJ, Lamia KA, Vyas D, Koo SH, Bardeesy N, et al. (2005) The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 310:1642–1646.
13. Ruderman NB, Xu XJ, Nelson L, Caccide JM, Saha AK, et al. (2010) AMPK and SIRT1: a long-standing partnership. Am J Physiol Endocrinol Metab 298:751–760.
14. Price NL, Gomes AP, Lang AJ, Duarte FV, Martin-Montalvo A, et al. (2012) SIRT1 is required for AMPK activation and the beneficial effects of reovatored mitochondrial function. Cell Metab 15:675–690.
15. Kondo T, Kishi M, Fushimi T, Kaga T (2009) Acetic acid upregulates the expression of genes for fatty acid oxidation enzymes in liver to suppress body fat accumulation. J Agric Food Chem 57:5982–5986.
16. Sakukihara S, Yamashita T, Oshima Y, Tsukamoto Y, Kadowaki T (2006) Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-Ay mice. Biochem Biophys Res Commun 344:597–604.
17. van Dorland HA, Sadri H, Morel I, Bruckmaier RM (2012) Coordinated gene expression in adipose tissue and liver differs between cows with high or low NEFA concentrations in early lactation [J]. Journal of Animal Physiology and Animal Nutrition 96:137–147.
18. Fushimi T, Sato Y (2005) Effect of acetic acid feeding on the circadian changes in glycogen and metabolites of glucose and lipid in liver and skeletal muscle of rats. British Journal of Nutrition 94:714–719.
19. Andreelli F, Foyet M, Kaoua C, Cani PD, Perrin C, et al. (2006) Liver adenosine monophosphate-activated kinase-alpha-2 catalytic subunit is a key target for the control of hepatic glucose production by adiponectin and leptin but not insulin. Endocrinology 147:2432–2441.
20. Hou X, Xu S, Mainland-Toolan KA, Sato K, Jiang B, et al. (2008) SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase, J Biol Chem 283:20015–20026.
21. Qin L, Wu X, Chao JP, Sonoy T, Yani WY, et al. (2008) Aldose reductase regulates hepatic peroxisome proliferator-activated receptor γ phosphorylation and activity to impact lipid homeostasis. J Biol Chem 283:17175–17183.
22. Sugden MC, Bulmer K, Gibbons GF, Knight BL, Holness MJ (2002) Peroxisome-proliferator-activated receptor-α (PPARα) deficiency leads to dysregulation of hepatic lipid and carbohydrate metabolism by fatty acids and insulin. Biochem J 364:361–368.
23. Eaton S (2002) Control of mitochondrial β-oxidation flux. Prog Lipid Res 41:197–239.
24. Huang H, Starodub O, McIntosh A, Schroeder F (2002) Liver fatty acid-binding protein targets fatty acids to the nucleus. Real time confocal and multiphoton fluorescence imaging in living cells. J Biol Chem 277:29139–29146.
25. Portoce M, Grazi N, Chia D, Boschetti F, Checcarelli G, et al. (2005) PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene 24:6465–6481.
26. Postic C, Dentin R, Denoueclad PD, Girard J (2007) ChREBP, a transcriptional regulator of hepatic glycerol and lipid metabolism. Amnu Rev Nutr 27:179–192.
27. Waki S, Stoops JK, Joshi VC (1983) Fatty acid synthesis and its regulation. British Journal of Nutrition 95:916–924.
28. van Dorland HA, Sadri H, Morel I, Bruckmaier RM (2012) Coordinated gene expression in adipose tissue and liver differs between cows with high or low NEFA concentrations in early lactation [J]. Journal of Animal Physiology and Animal Nutrition 96:137–147.
29. Fushimi T, Sato Y (2005) Effect of acetic acid feeding on the circadian changes in glycogen and metabolites of glucose and lipid in liver and skeletal muscle of rats. British Journal of Nutrition 94:714–719.
30. Andreelli F, Foyet M, Kaoua C, Cani PD, Perrin C, et al. (2006) Liver adenosine monophosphate-activated kinase-alpha-2 catalytic subunit is a key target for the control of hepatic glucose production by adiponectin and leptin but not insulin. Endocrinology 147:2432–2441.
31. Wang X, Xu S, Mainland-Toolan KA, Sato K, Jiang B, et al. (2008) SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase, J Biol Chem 283:20015–20026.
32. Qin L, Wu X, Chao JP, Sonoy T, Yani WY, et al. (2008) Aldose reductase regulates hepatic peroxisome proliferator-activated receptor γ phosphorylation and activity to impact lipid homeostasis. J Biol Chem 283:17175–17183.
33. Sugden MC, Bulmer K, Gibbons GF, Knight BL, Holness MJ (2002) Peroxisome-proliferator-activated receptor-α (PPARα) deficiency leads to dysregulation of hepatic lipid and carbohydrate metabolism by fatty acids and insulin. Biochem J 364:361–368.
34. Eaton S (2002) Control of mitochondrial β-oxidation flux. Prog Lipid Res 41:197–239.
35. Huang H, Starodub O, McIntosh A, Schroeder F (2002) Liver fatty acid-binding protein targets fatty acids to the nucleus. Real time confocal and multiphoton fluorescence imaging in living cells. J Biol Chem 277:29139–29146.
36. Portoce M, Grazi N, Chia D, Boschetti F, Checcarelli G, et al. (2005) PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene 24:6465–6481.
37. Postic C, Dentin R, Denoueclad PD, Girard J (2007) ChREBP, a transcriptional regulator of hepatic glycerol and lipid metabolism. Amnu Rev Nutr 27:179–192.
38. Waki S, Stoops JK, Joshi VC (1983) Fatty acid synthesis and its regulation. British Journal of Nutrition 95:916–924.
39. Fushimi T, Sato Y (2005) Effect of acetic acid feeding on the circadian changes in glycogen and metabolites of glucose and lipid in liver and skeletal muscle of rats. British Journal of Nutrition 94:714–719.
Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Biosci Biotechnol Biochem 71:1236–1243.

31. Ruderman NB, Saha AK, Vavvas D, Witters LA (1999) Malonyl-CoA, fuel sensing, and insulin resistance. Am J Physiol Endocrinol Metab 276:1–18.

32. Grummer RR (1993) Etiology of lipid-related metabolic disorders in periparturient dairy cows. J Dairy Sci 76:3882–3896.

33. Zhang ZG, Li XB, Gao L, Liu GW, Kong T, et al (2012) An updated method for the isolation and culture of primary calf hepatocytes. Vet J 191:323–326.

34. Li X, Li X, Bai G, Chen H, Deng Q, et al (2012) Effects of non-esterified fatty acids on the gluconeogenesis in bovine hepatocytes. Mol Cell Biochem 359:385–394.

35. Kim EK, Miller I, Aja S, Landree LE, Pinn M, et al (2004) C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J Biol Chem 279:19970–19976.