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Acetoacetic acid induces oxidative stress to inhibit the assembly of very low density lipoprotein in bovine hepatocytes

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Dairy cows with fatty liver or ketosis exhibit hyperketonemia, oxidative stress, and a low rate of very low density lipoprotein (VLDL) assembly, and there may be a potential link among these characteristics. Therefore, the objective of this study was to determine the effect of acetoacetic acid (AcAc) on the assembly of VLDL in cow hepatocytes. Cultured cow hepatocytes were treated with different concentrations of AcAc with or without N-acetylcysteine (NAC, an antioxidant). AcAc treatment decreased the mRNA expression and activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and significantly increased malondialdehyde (MDA) content, indicative of oxidative stress. Furthermore, AcAc treatment significantly down-regulated the mRNA expression of apolipoprotein B100 (ApoB100), apolipoprotein E (ApoE), and low density lipoprotein receptor (LDLR), which thus decreased VLDL assembly and increased triglyceride (TG) accumulation in these bovine hepatocytes. Importantly, NAC relieved AcAc-induced oxidative stress and increased VLDL assembly. In summary, these results suggest that AcAc-induced oxidative stress affects the assembly of VLDL, which increases TG accumulation in bovine hepatocytes.

Keywords: Bovine hepatocytes, acetoacetic acid, oxidative stress, very low density lipoproteins.

Fatty liver and ketosis are common metabolic diseases for dairy cows in the transition period, a time when most dairy cows undergo negative energy balance (NEB) caused by increased requirement for energy to support milk synthesis (Fatima et al. 2014; Schulz et al. 2014). This NEB initiates lipid mobilisation and subsequent high plasma concentrations of non-esterified fatty acids (NEFAs; McCabe et al. 2012). Large quantities of NEFAs are metabolised into ketones, such as acetoacetic acid (AcAc), acetone and β-hydroxybutyrate (BHBA), or synthesised into triglycerides (TG) in hepatocytes, thereby inducing ketosis and/or fatty liver (Karcagi et al. 2010; McArt et al. 2013). NEFAs are first metabolised by hepatocytes via β-oxidation to produce AcAc, and subsequently to acetone and BHBA, which finally leads to ketosis (Hayirli, 2006; Turk et al. 2013). Unfortunately, clinical investigation has shown that ketosis can in turn further aggravate the development of fatty liver. The possible mechanism of this interaction is an interesting topic for further research.

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It has been proposed that AcAc could act as a signalling molecule and be involved in the development of human diabetes, which is in some respects similar to ketosis and fatty liver in dairy cows (Abdelmegeed et al. 2004; Kanikarla-Marie & Jain, 2015). Elevated levels of AcAc may increase lipid peroxidation in people with type 1 diabetes (Jain & McVie, 1999), and high levels of AcAc constitute a risk factor for the oxidative modification of VLDL in diabetic patients (Jain et al. 1998). Similarly, levels of VLDL are significantly decreased in dairy cows with ketosis (Sun et al. 2014). It is known that fatty liver occurs when the rate of hepatic TG synthesis exceeds the rate of TG disappearance through either hydrolysis or exported via VLDL (Gessner et al. 2014). The ability of dairy cows to secrete hepatic TG as VLDL is extremely low compared with nonruminant animals (McCabe et al. 2012). The main structural proteins apolipoprotein B100 (ApoB100) and apolipoprotein E (ApoE), and the regulatory proteins microsomal triglyceride transfer protein (MTP) and low density lipoprotein receptor (LDLR), are all involved in the synthesis and assembly of VLDL in liver (Mason, 1998; Larsson et al. 2004; Sacks, 2015). It is unclear whether AcAc can regulate the expression of ApoB100, ApoE, MTP

and LDLR, and hence influence the synthesis and assembly of VLDL in bovine hepatocytes.

We have previously shown that the ketone body, BHBA, can induce oxidative stress in bovine hepatocytes (Shi et al. 2014). In this study, we hypothesised that AcAc could also influence the synthesis and assembly of VLDL through a mechanism involving oxidative stress in bovine hepatocytes.

Materials and methods

Ethical approval of the study protocol

All the experiments were performed in accordance with the Guiding Principles in the Use of Animals adopted by the Chinese Association for Laboratory Animal Sciences. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University (Changchun).

Materials

RPMI-1640 medium, heparin sodium, Collagenase IV and foetal bovine serum were purchased from Gibco (Grand Island, NY, USA). AcAc, HEPES, insulin, and NAC (N-acetylcysteine, an antioxidant) were provided by Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone acetate, vitamin C, penicillin, streptomycin and other chemicals were purchased from Baoman Biotechnology (Shanghai, China). Six-well plates and filters were purchased from Corning Incorporated (Corning, NY, USA).

Isolation of cow primary hepatocytes and AcAc treatment

Primary bovine hepatocytes were isolated and cultured as previously described (Shi et al. 2015). The concentrations of AcAc used in this study were based on the serum AcAc concentrations in dairy cows with ketosis or fatty liver. N-acetylcysteine (NAC) was an antioxidant in liver diseases. The hepatocytes were starved of serum overnight before AcAc treatment. Then, the hepatocytes were treated with 0 mM AcAc (control group, GC), 0·6 mM AcAc (low-dose group, GL), 2·4 mM AcAc (medial-dose group, GM), and 4·8 mM AcAc (high-dose group, GH), and 10 mM NAC + 4·8 mM AcAc (NAC + GH) for 24 h, respectively. Each treatment was replicated nine times.

The determination of the oxidative stress markers superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA)

After treatment with different concentrations of AcAc with or without NAC for 24 h, the hepatocytes were collected and washed twice in ice-cold phosphate-buffered saline (PBS). Then, the cells were lysed using P0013D (Beyotime, Jiangsu, China). Subsequently, the lysate was centrifuged at 12 000 ***g*** for 5 min at 4 °C. The supernatant was used to determine the content of MDA and the activities of GSH-Px, SOD and CAT using the appropriate biochemical kits (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

Real-time PCR

Total RNA from hepatocytes was isolated with TRIzol reagent (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan) according to the supplier's protocol. The concentration of RNA was determined using a K5500 Micro-Spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China). Approximately 5 μg of total RNA was reverse-transcribed to cDNA in 20-μl reactions using PrimeScript Reverse Transcriptase (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. All primers were designed using Primer Express software (PE Applied Biosystems, Inc., Foster City, CA, USA; sequences are given in online Supplementary Table S1) and synthesised from Sangon (Sangon Biotech Co., Ltd., Shanghai, China). 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/Life Technologies, Grand Island, NY, USA). The relative expression of each gene was calculated by the $2^{-\Delta\Delta CT}$ method and was normalised to β-actin levels.

Determination of VLDL content

After the hepatocytes were treated with AcAc, the medium from the 6-well plates was collected in 1·5 ml EP tubes. The VLDL content synthesised and secreted by hepatocytes was determined using ELISA kits (Shanghai Bluegene Biotech Co. Ltd., Shanghai, China; cat. no. E11V0006), according to the manufacturer's protocol.

Determination of TG content

After the cell medium was removed, hepatocytes were harvested and transferred into a centrifuge tube. Then, the cells were washed twice with ice-cold PBS and lysed using lysis buffer (Shanghai Bluegene Biotech Co. Ltd.) in ice bath conditions for 30 min. Finally, the lysate was centrifuged for 5 min at 12 000 ***g*** at 4 °C, and the supernatant was used to determine the content of triglycerides with a commercial kit (Applygen Technologies Inc., Beijing, China) using an automatic biochemical analyser (Shenyang EKSV Medical Equipment Co. Ltd., Shenyang, China).

Statistical analysis

The results are expressed as the mean ± standard error (SEM). The data were analysed by a one-way ANOVA using the SPSS software program (SPSS Inc., Chicago, IL). The differences were considered significant at $P < 0{\cdot}05$.

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Table 1. The different measures of oxidative stress in bovine hepatocytes

Indicator Group	MDA (mmol/mg)	GSH-Px mRNA	CAT	Mn-SOD	Cu/Zn-SOD
GC	6·323 ± 0·563	1·000 ± 0·090	1·000 ± 0·075	1·000 ± 0·111	1·000 ± 0·078
GL	6·667 ± 0·362	0·547 ± 0·081*	0·942 ± 0·042	0·583 ± 0·042*	0·588 ± 0·081*
GM	8·436 ± 0·237*	0·548 ± 0·065*	0·674 ± 0·063*	0·639 ± 0·131*	0·702 ± 0·071*
GH	10·660 ± 0·370*	0·456 ± 0·026*	0·690 ± 0·040*	0·502 ± 0·100*	0·529 ± 0·026*
NAC + GH	8·026 ± 0·204**				

Hepatocytes were treated with 0 mM AcAc (control group, GC), 0·6 mM AcAc (low-dose group, GC), 2·4 mM AcAc (medial-dose group, GM), and 4·8 mM AcAc (high-dose group, GH), and 10 mM NAC + 4·8 mM AcAc (NAC + GH), respectively. The data are shown as the mean ± SEM. Asterisks denote significance ($P < 0·05$) from the GC (*) or from the GH (**).

Table 2. The levels of ApoB100, ApoE, MTP, LDLR, VLDL, and TG in bovine hepatocytes

Indicator Group	ApoB100 mRNA	ApoE	MTP	LDLR	VLDL (mg/ml)	TG (mmol/l)
GC	1·000 ± 0·114	1·000 ± 0·168	1·000 ± 0·558	1·000 ± 0·067	0·423 ± 0·016	0·140 ± 0·014
GL	0·848 ± 0·111	0·761 ± 0·148	0·934 ± 0·129	1·004 ± 0·055	0·395 ± 0·015	0·160 ± 0·016
GM	0·711 ± 0·048*	0·277 ± 0·070*	0·929 ± 0·038	0·776 ± 0·029*	0·233 ± 0·024*	0·205 ± 0·011*
GH	0·604 ± 0·038*	0·228 ± 0·044*	0·895 ± 0·118	0·708 ± 0·035*	0·203 ± 0·015*	0·283 ± 0·012*
NAC + GH	1·196 ± 0·151**	0·697 ± 0·112**	1·079 ± 0·084	1·175 ± 0·112**	0·314 ± 0·034**	0·190 ± 0·014**

Hepatocytes were treated as described in Table 1. The data are shown as the mean ± SEM. Asterisks denote significance ($P < 0·05$) from the GC (*) or from the GH (**).

Results

The effect of AcAc on oxidative stress in bovine hepatocytes

Table 1 shows the effect of AcAc on different measures of oxidative stress in bovine hepatocytes. MDA content was increased by AcAC to a peak in the GH group which was partially reversed by the antioxidant, NAC. The antioxidant indicators SOD, CAT, and GSH-Px were detected by real-time PCR and enzyme activity. The mRNA expression of Mn SOD, Cu/Zn SOD, and GSH-Px were all decreased in the AcAc-treated groups compared to the GC group. The CAT mRNA expression level had no change in the GL group, but was significantly lower in the GM and GH groups when compared with the GC group. Enzyme activity data confirmed that all activities were significantly decreased in the GM and GH groups (data are presented in online Supplementary Table S2).

The effect of AcAc on ApoB100, ApoE, MTP, and LDLR in bovine hepatocytes

The mRNA expression of ApoB100, ApoE, MTP, and LDLR (important molecules involved in the hepatic synthesis and assembly of VLDL) are shown in Table 2. With the exception of MTP, all were significantly lower in the GM and GH groups when compared to the GC group, but higher in the NAC + GH group when compared with the GH group. MTP mRNA expression was not altered by AcAc.

The effect of AcAc on the content of VLDL and TG in bovine hepatocytes

VLDL and TG contents were detected using ELISA kits (Table 2). VLDL content was gradually decreased in the GM and GH groups when compared to the GC group, but remarkably increased in the NAC + GH group when compared with the GH group. The content of TG changed in reverse to VLDL, increased in the GM and GH groups when compared with the GC group, but decreased in the NAC + GH group when compared with the GH group.

Discussion

Ketosis and fatty liver are closely related metabolic diseases in high-production dairy cows (Hayirli, 2006; González et al. 2011). Dairy cows with ketosis or fatty liver are characterized by high blood levels of NEFA and AcAc (Gross et al. 2013; Wang et al. 2015). A previous study has shown that high concentrations of NEFAs could inhibit the synthesis and assembly of VLDL, leading to TG accumulation in bovine hepatocytes (Liu et al. 2014). In this study, we showed that high concentrations of AcAc treatment could result in dramatically elevated TG content, and significantly decreased VLDL content in bovine hepatocytes. Therefore, we conclude that high levels of AcAc in the blood may be an important factor for the development of fatty liver. Furthermore, it also partly explains why dairy cows with ketosis are more susceptible to fatty liver during the transition period.

It is well known that VLDL serves as the main pathway to eliminate TG in liver (Mason, 1998; Li et al. 2014). Thus, abnormal assembly of VLDL is an important factor in the induction of fatty liver in dairy cows. ApoB100, the TG-binding protein, is the structural backbone for the formation and secretion of VLDL (Larsson et al. 2004; Liu et al. 2014). VLDL cannot be produced at sufficient amounts in dairy

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cows with fatty liver because of a low ApoB100 expression (Gessner et al. 2014). Our results showed that high levels of AcAc could significantly inhibit the expression of Apo B100 in bovine hepatocytes. Additionally, ApoE is also present as a structural constituent of VLDL, and plays a vital role in the regulation of hepatic VLDL-triglyceride secretion (Kuipers et al. 1997). ApoE-deficient mice had impaired VLDL-triglyceride secretion, accumulated large amounts of TG, and developed fatty liver (Kuipers et al. 1997; Tsukamoto et al. 2000). Our results demonstrate that AcAc may reduce the structural constituents of VLDL by suppressing the expression of ApoB100 and ApoE genes in hepatocytes. Furthermore, some studies have indicated that MTP could regulate the expression of ApoB and ApoB-containing lipoprotein (Tietge et al. 1999; Borradaile et al. 2003). MTP, an important regulatory molecule, is required for transport TG to be incorporated into lipoprotein (Mason, 1998; Wang et al. 2012). However, AcAc had no effect on the expression of MTP in this study. Indeed, a clinical test reported that MTP expression was not significantly changed in cases of dairy cows with fatty liver, which further support our results (Bremmer et al. 2000). Importantly, another crucial regulatory molecule, LDLR, was down-regulated in AcAc-treated bovine hepatocytes. LDLR is a key receptor that is involved in the hepatic VLDL assembly, and ApoE is a high-affinity ligand for the LDLR (Larsson et al. 2004; Sacks, 2015). Moreover, the secretion of ApoB100 was found to be inhibited by LDLR from hepatocytes (Twisk et al. 2000; Larsson et al. 2004). These data provides evidence that AcAc may inhibit LDLR expression, which increases the secretion of ApoB100 from hepatocytes and affects ApoE combined with LDLR in the assembly of VLDL.

It is well documented that elevated oxidative stress is implicated in the secretion of TG-VLDL in some metabolic diseases (Anderson et al. 2001; Pan et al. 2004; Bloomer et al. 2015). Moreover, cows with ketosis or fatty liver displayed oxidative stress. MDA is considered the classical oxidative stress marker in cells (Rybka et al. 2013; Zhang et al. 2015). In this study, we found that the content of the oxidative stress marker MDA was significantly increased in hepatocytes treated with AcAc, and that this was reversible by the antioxidant molecule NAC. Previous studies showed that decreasing MDA production mainly depended on three important antioxidant enzymes SOD, CAT, and GSH-Px in cells (Rybka et al. 2013; Sangwan et al. 2015; Shi et al. 2015), all of which were decreased by AcAc treatment. Therefore, we propose that high concentrations of AcAc can induce oxidative stress in bovine hepatocytes. This agrees with previous observations of AcAc-induced oxidative stress in rat hepatocytes, human erythrocytes and vascular endothelial cells (Jain et al. 1998; Jain & McVie, 1999; Abdelmegeed et al. 2004; Kanikarla-Marie & Jain, 2015). Our previous studies have demonstrated that oxidative stress was a common mechanism for inflammatory injury of liver in dairy cows (Shi et al. 2014, 2015). Here, we can conlude that high levels of AcAc can induce oxidative stress and influence the synthesis and assembly of VLDL, thereby resulting in TG accumulation in bovine hepatocytes. This may explain why ketosis can further aggravate the development of fatty liver, due to the possible mechanism of high blood levels of AcAc inhibiting the hepatic VLDL assembly through induction of oxidative stress.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0022029916000546

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