Effects of nonesterified fatty acids on the synthesis and assembly of very low density lipoprotein in bovine hepatocytes in vitro

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ABSTRACT

High serum concentrations of nonesterified fatty acids (NEFA), which may affect the synthesis and assembly of very low density lipoproteins (VLDL), are associated with fatty liver during the early lactation period. Therefore, the objective of this study was to investigate the effects of NEFA on the synthesis and assembly of VLDL in bovine hepatocytes. Bovine hepatocytes were cultured and treated with different concentrations of NEFA. The mRNA expression of apolipoprotein B100 (*ApoB100*) and apolipoprotein E (*ApoE*) was significantly lower in the NEFA treatment groups than in the control group (0 mM NEFA). The abundance of mRNA from microsomal triglyceride transfer protein (*MTP*) and the low density lipoprotein receptor (*LDLR*) was significantly lower in the medium- and high-dose NEFA treatment groups. The protein expression of ApoB100, ApoE, MTP, and LDLR was found to be significantly and dose-dependently decreased in groups of NEFA-treated hepatocytes. The VLDL content was also significantly decreased in the NEFA-treated hepatocytes. Large amounts of triglycerides accumulated in the hepatocytes. These results indicate that NEFA significantly inhibits the expression of *ApoB100*, *ApoE*, *MTP*, and *LDLR*, thereby decreasing the synthesis and assembly of VLDL and inducing TG accumulation in bovine hepatocytes.

Key words: bovine hepatocyte, nonesterified fatty acids, very low density lipoprotein, in vitro

INTRODUCTION

Fatty liver is a major lipid metabolic disorder of dairy cows in early lactation and is associated with decreased

health status and reproductive performance. In dairy cows, fatty liver occurs primarily in the first 4 wk after calving (Grummer, 1993), when up to 50% of all dairy cows exhibit some accumulation of triglycerides (TG) in the liver (Jorritsma et al., 2000, 2001). The natural incidence of fatty liver is significantly higher in ruminant animals than in monogastric animals, which is due to the special metabolic characteristics of ruminants. During the early lactation period, most dairy cows experience a negative energy balance (NEB) caused by the increased demand for energy to support milk production (Friggens et al., 2007; Xu et al., 2008). This NEB initiates fat mobilization and a subsequent increase in NEFA blood concentration (Grummer, 2008). In addition, dairy cows have a very slow rate of secretion of hepatic very low density lipoproteins (VLDL) compared with most species (Pullen et al., 1990).

A high concentration of blood NEFA plays a pivotal role in the pathogenesis of fatty liver syndrome (Jorritsma et al., 2001; Zhang et al., 2011). Nonesterified fatty acids are esterified into TG when uptake exceeds oxidation capacity for NEFA in the hepatocytes. Therefore, fatty liver occurs when the rate of hepatic TG synthesis exceeds the rate of TG disappearance through either hydrolysis or secretion via VLDL (van Dorland et al., 2011). Apolipoprotein B100 (*ApoB100*), apolipoprotein E (*ApoE*), microsomal triglyceride transfer protein (*MTP*), and low density lipoprotein receptor (*LDLR*) are the main structural and regulatory proteins for the synthesis and assembly of VLDL (Mason, 1998; Greenow et al., 2005).

Several studies have demonstrated that NEFA can act as signaling molecules involved in regulating the expression of lipid metabolism genes (Duplus et al., 2000; Jump et al., 2005). High serum concentrations of NEFA are associated with the fatty liver of dairy cows. Therefore, we hypothesized that NEFA could affect the synthesis and assembly of VLDL in bovine hepatocytes. The objective of this study was to investigate the effects of NEFA on the synthesis and assembly of VLDL in bovine hepatocytes cultured in vitro.

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MATERIALS AND METHODS

Materials

Fetal bovine serum, collagenase IV, and RPMI-1640 medium were purchased from Gibco (Grand Island, New York, NY). Oleic acid, linoleic acid, palmitic acid, stearic acid, palmitoleic acid, insulin, and HEPES were purchased from Sigma Aldrich (St. Louis, MO). Dexamethasone acetate, vitamin C, ascorbic acid, penicillin, streptomycin, and other chemicals were provided by Baoman Biotechnology (Shanghai, China).

Reagents and Solutions

Perfusion solution A comprised 140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA. The solution pH was adjusted to 7.4. Perfusion solution B comprised 140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM CaCl_2 . The solution pH was adjusted to 7.4. For digestion, 0.1 g of collagenase IV was dissolved in 0.5 L of perfusion solution B. 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. The adherent medium consisted of RPMI-1640 basic medium supplemented with 10% fetal calf serum, 10^{-6} M insulin, 10^{-6} M dexamethasone, 10 $\mu\text{g/mL}$ vitamin C, 100 U/mL benzylpenicillin, and 100 $\mu\text{g/mL}$ streptomycin. The growth medium consisted of RPMI-1640 basic medium supplemented with 10% fetal calf serum, 100 U/mL benzylpenicillin, and 100 $\mu\text{g/mL}$ streptomycin. Phosphate buffer solution comprised 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 . The pH of PBS solution was adjusted to 7.4. Tris-buffered saline and Tween buffer solution comprised 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20. The solution pH was adjusted to 7.4.

Cell Culture

The study protocol was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). The caudate liver lobe of a dairy cow was obtained through surgical liver excision according to a previous study (Parker and Gaughan, 1988). The dairy cow was positioned in left lateral recumbency. General anesthesia was maintained with thi-amylal sodium. A 20-cm right abdominal incision was made from the costal margin. The skin, muscle layers, and peritoneum were incised. Self-retaining retractors were placed in the incision. The caudate lobe of the liver was excised. Subsequently, fibrin glue was sprayed

on the hepatic cross section. The hepatic cross section was sutured immediately. The incision was closed in 3 layers (peritoneum, abdominal muscle, and skin). After surgery, the dairy cow was carefully nursed to prevent inflammation and infection.

The hepatocytes were isolated by the collagenase perfusion method. The caudate lobe of the liver was quickly moved to a super-clean bench. The bloodstains on the liver surface were removed with perfusion solution A (37°C). Blood vessels were intubated, and the liver was perfused with perfusion solution A at a flow rate of 50 mL/min for 15 min. The liver was then perfused with perfusion solution B (37°C) at the same flow rate until the liquid became clear. Then, the liver was perfused with digestion solution to dissociate liver tissue structure over 15 min until the liquid became muddy. The liver was moved to a sterile flat plate after digestion, and 100 mL of basic medium (4°C) containing 0.2% BSA was added into the flat plate to terminate the digestion. The liver was cut open, and the liver capsule, blood vessels, fat, and connective tissue were removed using forceps and scissors. The liver parenchyma was cut into pieces and filtered sequentially with 100 mesh (150 μm), 200 mesh (75 μm), and 400 mesh (37.5 μm) cell sieves. The hepatocyte suspension was washed twice with basic medium and centrifuged for 10 min at $500 \times g$ at 4°C . The Trypan blue dye exclusion method was used to assess cell viability. The percentage of viable cells was almost 99%. The cell density was adjusted to 1×10^6 cells/mL using adherent medium. The hepatocyte suspension was seeded into a 6-well tissue culture plate (2 mL per well). The hepatocytes were incubated at 37°C in 5% CO_2 . After 4 h, the adherent medium was replaced with growth medium containing 10% fetal bovine serum. The growth medium was replaced every 24 h.

NEFA Preparation and Treatment

The composition and concentration of NEFA used in this study were chosen according to the hematology standards of dairy cows with fatty liver (Bertics et al., 1992). Stock NEFA solution was prepared as previously described (Li et al., 2012). Briefly, oleic acid (10.875 mmol), linoleic acid (1.225 mmol), palmitic acid (7.975 mmol), stearic acid (3.6 mmol), and palmitoleic acid (1.325 mmol) were dissolved in 100 mL of potassium hydroxide solution (1 M) at 60°C . The pH of the NEFA solution was adjusted to 7.6 with hydrochloric acid (1 M), and then the solution was diluted to 500 mL with distilled water. Therefore, the concentration of stock NEFA solution was 50 mM, containing 21.75 mM oleic acid, 15.95 mM palmitic acid, 7.2 mM stearic acid, 2.65 mM palmitoleic acid, and 2.45 mM linoleic acid.

Table 1. Primer sequences (For = forward; Rev = reverse) of the genes

Gene	Primers used for PCR	Length of fragment (bp)	Annealing temperature (°C)
<i>ApoB100</i>	For: GATACTCAGAACGGAGCAAT Rev: GCACCAATCAGATAACAGGA	223	60
<i>ApoE</i>	For: TCCTGAATGACCTGGGTGTTG Rev: TCTGTGGGTTGCCGTGGTG	217	60
<i>MTP</i>	For: CAGTTTGCAGCCTTGGTTCTG Rev: TCTGTGGGTTGCCGTGGTG	201	60
<i>LDLR</i>	For: GCT GTT CTG CCT TTC TCC TT Rev: ACT TTC TCC CCT GAC CCT TG	228	60
<i>ACTB</i>	For: GCCCTGAGGCTCTCTTCCA Rev: GCGGATGTGACGCTACA	101	60

The basic medium was supplemented with 3.8% BSA. Before treatment with NEFA solvent, the cells were starved of serum overnight. The stock NEFA solution was added to the basic medium. The hepatocytes were treated with 0 mM NEFA (control group), 0.5 mM NEFA (low-dose group), 1.0 mM NEFA (medium-dose group), or 1.5 mM NEFA (high-dose group) for 24 h. Hepatocytes were treated with different concentrations of NEFA and with different concentrations of KCl. In an additional experiment, hepatocytes were treated with 0, 2, 4, or 6 mM KCl and treated for 24 h. Each treatment concentration of NEFA or KCl was replicated 12 times.

RNA Extraction and PCR

Total hepatic RNA was extracted using TRIzol (TaKaRa Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions. Total RNA was dissolved in diethylpyrocarbonate water. Five microliters of RNA solution was used to detect its concentration using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK). The RNA in all samples was diluted to the same concentration using diethylpyrocarbonate water. Approximately 5 µg of RNA in each sample was reverse transcribed to cDNA in 20-µL reactions using a reverse transcription kit (TaKaRa Biotechnology Co. Ltd.), according to the supplier's protocol. The gene primers were designed using Primer 5.0 (PE Applied Biosystems Inc., Foster, CA) and are shown in Table 1. The concentration of the primer was 10 µM. The annealing temperature of *ApoB100*, *ApoE*, *MTP*, *LDLR*, and *ACTB* were all 60°C. The mRNA expression was evaluated by quantitative reverse transcription-PCR (qRT-PCR) analysis using the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co. Ltd.). The qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method and was normalized to abundance of β -actin (Livak and Schmittgen, 2001).

Western Blotting

Hepatocytes were harvested and washed twice in ice-cold PBS. The total cell protein was extracted using a protein extraction kit (Sangon Biotech Co. Ltd., Shanghai, China) according to the manufacturer's instructions. The protein concentration was measured using BioRad protein assay reagent (Bio-Rad, München, Germany). The protein was separated in polyacrylamide gels and electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in BSA-Tris-buffered saline-Tween buffer for 4 h. The concentrations of BSA and Tween were 5 and 0.1%, respectively. The membranes were hybridized with antibodies specific for ApoB100, ApoE, MTP, and LDLR at dilutions of 1:500, 1:500, 1:500, and 1:1,000, respectively, overnight at 4°C. The ApoB100 and ApoE antibodies are goat polyclonal antibodies and react with ApoB100 and ApoE of bovine origin (Abcam, Cambridge, UK; cat. no. ab7616 and ab20261). The MTP and LDLR antibodies are affinity-purified goat polyclonal antibodies raised against a peptide mapping at the N-terminus of MTP and C-terminus of LDLR of bovine origin, respectively (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. H1407 and L3008). The PVDF membranes were incubated with appropriate peroxidase-conjugated secondary antibodies at dilutions of 1:5,000, 1:5,000, 1:4,000, and 1:4,000. Immunoreactive bands were detected with enhanced chemiluminescence solution (Beyotime Biotechnology Inc., Haimen, China). The blots were exposed to x-ray film for radiography of the bands, and the gray values of the bands were measured using BandScan software version 5.0 (ProZyme Inc., San Leandro, CA).

Determination of VLDL Content

The medium from the 6-well plates was collected after NEFA treatment. The content of VLDL synthesized 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. All the samples were assayed in duplicate.

Determination of TG Content

The cell medium was removed using a micropipettor. Hepatocytes were harvested using a cell scraper and transferred into a centrifuge tube. Then, the cells were washed twice with ice-cold PBS. The cells were lysed using lysis buffer (Shanghai Bluegene Biotech Co. Ltd.) in ice bath conditions for 30 min. Subsequently, the lysate was centrifuged for 5 min at $12,000 \times g$ at 4°C , and the supernatant was used to determine the content of triglycerides with a commercial kit (TOOS0929; Applygen Technologies Inc., Beijing, China) using an automatic biochemical analyzer (Shenyang EKSV Medical Equipment Co. Ltd., Shenyang, China).

Statistical Analysis

The results are expressed as the mean $\pm$ standard deviation (SD). The data were analyzed by a one-way ANOVA followed by Duncan's multiple range test (SPSS 13.0 software; SPSS Inc., Chicago, IL). A P -value of < 0.05 was considered statistically significant, and values < 0.01 were considered markedly significant.

RESULTS

Expression Abundance of Genes Involved in VLDL Assembly

We observed no significant difference in the mRNA expression of *ApoB100*, *ApoE*, *MTP*, or *LDLR* between KCl-treated groups and the control group. In addition, we observed no difference in VLDL content among the treatment groups and no statistical significance between groups. These results demonstrated that KCl did not markedly affect the synthesis and assembly of VLDL in bovine hepatocytes (Figure 1).

The mRNA abundance of *ApoB100* and *ApoE* in NEFA-treated groups was significantly lower than that of the control group [$P < 0.05$ and $P < 0.01$ for *ApoB* (Figure 2A); $P < 0.01$ for *ApoE* (Figure 2B)]. The mRNA abundance of *MTP* and *LDLR* in the medium- and high-dose NEFA treatment groups was significantly lower than that of the control group (Figure 2C, 2D; $P < 0.01$ for both *MTP* and *LDLR*). The protein expression of *ApoB100*, *ApoE*, *MTP*, and *LDLR* was found to be significantly and dose-dependently decreased in all groups of NEFA-treated hepatocytes (Figure 2E).

VLDL and TG Content

The VLDL content was found to be significantly and dose-dependently decreased in all groups of NEFA-treated hepatocytes compared with the control hepatocytes ($P < 0.05$ and $P < 0.01$ for VLDL content; Figure 3). The TG content was found to be significantly and dose-dependently increased in all groups of NEFA-treated hepatocytes compared with the control hepatocytes ($P < 0.05$ and $P < 0.01$ for TG content; Figure 4).

DISCUSSION

Fatty liver is usually associated with a pronounced NEB at the beginning of lactation in high-yielding dairy cows. A high concentration of plasma NEFA has many pathological consequences. Cameron et al. (1998) reported that a high plasma concentration of NEFA was a risk factor for the induction of ketosis and fatty liver in dairy cows. Fatty liver may develop when the rate of TG synthesis exceeds the rate of TG export as VLDL (Bernabucci et al., 2004). Sevinc et al. (2003) reported that the serum content of VLDL was significantly decreased in dairy cows with fatty liver. Therefore, in the present study, hepatocytes were cultured and treated with different concentrations of NEFA. The stock NEFA solution included KCl. The results of the additional experiment showed that KCl did not affect expression of *ApoB100*, *ApoE*, *MTP*, or *LDLR*, or VLDL assembly. The effect of NEFA on VLDL assembly was investigated in hepatocytes treated with different concentrations of NEFA.

Triglycerides are combined with apolipoprotein and exported from hepatocytes as VLDL. A clinical study demonstrated that synthesis and secretion of VLDL are significantly decreased in the liver of dairy cows with fatty liver (Kato, 2002), which may due to the high concentration of NEFA. The proteins *ApoB100* and *ApoE* are the most important structural components of VLDL; *ApoB100* is the TG-binding protein in VLDL and is necessary for stabilizing the VLDL particles (Bernabucci et al., 2004). A study revealed that the nascent VLDL particles acquired *ApoE* during the assembly cascade (Mensenkamp et al., 2001). Uchida et al. (1992) demonstrated that the plasma concentration of *ApoB100* in the dairy cows with fatty liver was significantly lower than that of healthy cows. In addition, Mensenkamp et al. (2001) reported that the secretion of VLDL-TG was severely impaired in *ApoE*-deficient mice. In the present study, the mRNA and protein expression of *ApoB100* and *ApoE* were significantly lower in NEFA-treated groups than in the control group.

Figure 1. The mRNA expression of *ApoB100*, *ApoE*, *MTP*, and *LDLR* and very low density lipoprotein (VLDL) content in the KCl-treated bovine hepatocytes. The hepatocytes were treated with 0, 2, 4, or 6 mM KCl. Each treatment concentration of KCl was replicated 12 times.

These results indicate that a high concentration of NEFA inhibits the expression of *ApoB100* and *ApoE* in bovine hepatocytes. Dairy cows experience a negative energy balance, which subsequently induces a high concentration of blood NEFA. Large amounts of NEFA were esterified into TG in hepatocytes. Furthermore, high concentrations of NEFA affected the synthesis of VLDL. Our in vitro study also demonstrated that NEFA inhibited the expression of *ApoB100* and *ApoE* in hepatocytes, thereby decreasing the structural components of VLDL.

Both MTP and LDLR are important regulatory proteins for the synthesis of VLDL; MTP is responsible for the transport of TG into the growing VLDL particles, resulting in the formation of nascent lipoprotein particles (Sundaram and Yao, 2010), whereas LDLR

inhibits the secretion of *ApoB100* and regulates the assembly of VLDL (Read et al., 2000). Bremmer et al. (2000) found no relationship between the degree of fatty liver and the activity of MTP in dairy cows. However, our in vitro data demonstrated that the abundance of MTP was significantly lower in the NEFA-treated hepatocytes than in the control hepatocytes. A deficiency of MTP reduced the combination between TG and apolipoprotein. The discrepancy between our results and those of previous studies may be due to the effects of the energy metabolic hormones and nutrition metabolic signals such as insulin, glucagon, and BHBA in vivo. To our knowledge, no data are available on the changes of mRNA and protein expression of *LDLR* in dairy cows with fatty liver. In this study, the mRNA and protein expression of *LDLR* was significantly lower

Figure 2. The mRNA and protein expression of *ApoB100*, *ApoE*, *MTP*, and *LDLR* in the NEFA-treated bovine hepatocytes. The hepatocytes were treated with 0 mM NEFA (control group), 0.5 mM NEFA (low-dose group), 1.0 mM NEFA (medium-dose group), and 1.5 mM NEFA (high-dose group). (A) mRNA expression of *ApoB100*; (B) mRNA expression of *ApoE*; (C) mRNA expression of *MTP*; (D) mRNA expression of *LDLR*; (E) Western blot results of ApoB100, ApoE, MTP, and LDLR. Each treatment concentration of NEFA was replicated 12 times. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

in the NEFA-treated groups than in the control group, which indicates that NEFA inhibits the expression of *LDLR*. A low abundance of LDLR results in an increase of ApoB100 secretion from hepatocytes, thereby decreasing the structural components of VLDL. Taken together, with the decreased expression of ApoB100, ApoE, MTP, and LDLR, the synthesis and assembly of VLDL were significantly decreased in the NEFA-treated hepatocytes. Consequently, a large amount of TG accumulated in the hepatocytes and the TG content was significantly higher in the NEFA-treated hepatocytes than in the control hepatocytes.

Our results confirm that a high concentration of NEFA is an important pathogenic factor for accumulation of TG in hepatocytes; NEFA inhibited the synthesis and assembly of VLDL. Unfortunately, dairy cows have a very slow rate of VLDL export from the liver; thus, the capacity of that route of NEFA disposal is easily exceeded. Therefore, a high concentration of NEFA accentuates the accumulation of TG in hepatocytes. In clinical application, increasing VLDL export is the most effective strategy to treat fatty liver in dairy cows. Choline, methionine, and glucagon are used for the prevention and treatment of fatty liver. An in

Figure 3. Content of very low density lipoprotein (VLDL) in the NEFA-treated hepatocytes. The hepatocytes were treated with 0 mM NEFA (control group), 0.5 mM NEFA (low-dose group), 1.0 mM NEFA (medium-dose group), and 1.5 mM NEFA (high-dose group). Each treatment concentration of NEFA was replicated 12 times. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

vivo study demonstrated that choline and methionine could enhance VLDL export from the liver (Grummer, 1993). Cooke et al. (2007) reported that choline deficiency could increase the accumulation of TG in

liver. Choline serves as a substrate for the synthesis of phosphatidylcholine, a constituent of VLDL (Cooke et al., 2007). Methionine is required for the synthesis of protein (a constituent of VLDL) and as a methyl donor for phosphatidylcholine synthesis (Bauchart et al., 1998). Glucagon stimulates hepatic glycogenolysis, gluconeogenesis, and insulin production. Glucagon has an effect of reducing liver TG content in dairy cows with fatty liver (Nafikov et al., 2006). Therefore, an increase of VLDL synthesis is critical in preventing fatty liver in dairy cows.

CONCLUSIONS

A high concentration of NEFA significantly inhibited the expression of *ApoB100*, *ApoE*, *MTP*, and *LDLR*, thereby decreasing the synthesis and assembly of VLDL and inducing TG accumulation in bovine hepatocytes.

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Figure 4. Content of triglycerides (TG) in the NEFA-treated hepatocytes. The hepatocytes were treated with 0 mM NEFA (control group), 0.5 mM NEFA (low-dose group), 1.0 mM NEFA (medium-dose group), and 1.5 mM NEFA (high-dose group). Each treatment concentration of NEFA was replicated 12 times. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

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