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Effects of insulin-like growth factor-1 on the assembly and secretion of very low-density lipoproteins in cow hepatocytes *in vitro*

Xinwei Li [a], Yuan Guan [b], Ying Li [a], Dianjun Wu [a], Lei Liu [a], Qinghua Deng [c,*], Xiaobing Li [a], Zhe Wang [a], Guowen Liu [a,*]

[a] Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, 5333 Xi'an Road, Changchun, Jilin 130062, China
[b] College of Animal Science, Jilin University, 5333 Xi'an Road, Changchun, Jilin 130062, China
[c] College of Animal Science and Technology, Inner Mongolia National University, Tongliao 028042, China

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ABSTRACT

Fatty liver is a major metabolic disorder of dairy cows. One important reason is that hepatic very low-density lipoproteins (VLDL) assembly was significant decreased in dairy cows with fatty liver. In addition, the impairment of insulin-like growth factor (IGF)-1 synthesis was involved in the development of fatty liver. Therefore, the objective of this study was to investigate the effects of IGF-1 on the VLDL assembly in cow hepatocytes. In this study, cow hepatocytes were cultured and then transfected with Ad-GFP-IGF-1 (inhibited the IGF-1 expression) and Ad-GFP (negative control), and treated with different concentrations of IGF-1, respectively. The results showed that IGF-1 increased the mRNA abundance of apolipoprotein B100 (ApoB100), apolipoprotein E (ApoE), microsomal triglyceride transfer protein (MTTP), and low-density lipoprotein receptor (LDLR) and then increased the VLDL assembly in cow hepatocytes. Nevertheless, impairment of IGF-1 expression by Ad-GFP-IGF-1 could inhibit above genes expression and VLDL assembly in hepatocytes. Taken together, these results indicate that IGF-1 increases the VLDL assembly and impairment of IGF-1 expression decreases the VLDL assembly in cow hepatocytes.

1. Introduction

Fatty liver is a metabolic disorder that can affect up to 50% of the high producing cows during the transition period, potentially compromising health, production and reproduction (Grummer, 2008). The natural incidence of fatty liver is significantly higher in dairy cows than in mice or humans, which is due to the special metabolic characteristics (Liu et al., 2014). Firstly, perinatal dairy cows were subjected to a period of negative energy balance (NEB), which is due to the decreased dry matter intake and increased energy demand. Excessive NEB initiates fat mobilization and a subsequent increase in blood non-esterified fatty acids (NEFAs) concentration. Large amounts of NEFAs could not be completely oxidized and metabolized into ketones. Furthermore, non-esterified fatty acids are esterified into triglycerides (TG) when uptake exceeds oxidation capacity for NEFA in the hepatocytes (Bobe et al., 2003). Liver lipid accumulation occurs when the rate of TG synthesis exceeds the rate of TG disappearance through either hydrolysis or secretion via very low-density lipoproteins (VLDL) (van Dorland et al., 2011). In addition, dairy cows with fatty liver have a low rate of hepatic VLDL assembly relative to healthy cows (Bobe et al., 2004). Collectively, these reasons resulted in a high incidence of fatty liver in dairy cows, especially in high producing cows.

Growth hormone (GH) activated Janus kinase (JAK)-2-signal transducer and activator of transcription (STAT)-5 signaling pathway to increase the synthesis and secretion of insulin-like growth factor (IGF)-1 in hepatocytes (Barclay et al., 2011). Data suggested that part of the regulation effects of GH on hepatic lipid metabolism were mediated by IGF-1 (Vijayakumar et al., 2010). Clinical investigation and animal study demonstrated that the impairment of IGF-1 signaling or low physiological concentration of IGF-1 was closely linked to liver steatosis in patients with non-alcoholic fatty liver disease (NAFLD) (Vijayakumar et al., 2010).

Abbreviations: VLDL, very low-density lipoproteins; NEFA, nonesterified fatty acids; IGF-1, insulin-like growth factor 1; ApoB100, apolipoprotein B100; ApoE, apolipoprotein E; MTTP, microsomal triglyceride transfer protein; LDLR, low-density lipoprotein receptor; NEB, negative energy balance; TG, triglycerides; GH, growth hormone; JAK2-STAT5, Janus kinase (JAK)-2-signal transducer and activator of transcription (STAT)-5; PBS, phosphate buffer solution; NAFLD, non-alcoholic fatty liver disease; MAPK/JNK, mitogen-activated protein kinase/c-Jun-N terminal kinase; MAPK/ERK, mitogen-activated protein-kinase/extracellular signal-regulated kinase; PI3-K, phosphoinositide 3-kinase.

* Corresponding author. Fax: +86 0431 87836160.
E-mail addresses: 18747500536@126.com (Q. Deng), liuguowen2008@163.com (G. Liu).

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Apolipoprotein B100 (ApoB100), apolipoprotein E (ApoE), microsomal triglyceride transfer protein (MTTP), and low-density lipoprotein receptor (LDLR) are the main structural and regulatory proteins for the assembly of VLDL (Bobe et al., 2004). Dairy cow with fatty liver exhibited a lower VLDL assembly compared with healthy cow (Sevinç et al., 2003). Lindén et al. (2000) reported that GH/IGF-1 axis could promote the lipid export from hepatocytes as form of VLDL. Concentration of IGF-1 in the serum of cows is decreased during restriction of dietary energy and during the NEB associated with early lactation (Vicini et al., 1991; Sharma et al., 1994; Kobayashi et al., 1999). Furthermore, there are several studies described that GH–IGF-1 axis was uncoupled around parturition in liver. GH concentration was increased whereas IGF-1 was decreased in blood, which resulted from a down-regulation of GH-receptor in liver (Vicini et al., 1991; Sharma et al., 1994; Vandehaar et al., 1995; Kobayashi et al., 1999). Interestingly, Fenwick et al. (2008) have demonstrated that severe NEB further decreased hepatic IGF-I synthesis in dairy cows. The pathological basis of fatty liver in dairy cows is NEB. The degree of NEB was significantly higher in cows with fatty liver than in perinatally healthy cows. We therefore speculated that decreased IGF-1 may further affect hepatic VLDL assembly. Collectively, these results triggered our interest in exploring the relationship between IGF-1 and VLDL assembly in cow hepatocytes. Therefore, the aim of this study was to investigate the effects of IGF-1 on the assembly of VLDL in cow hepatocytes cultured *in vitro*.

2. Materials and methods

2.1. Materials and reagents

Fetal bovine serum, collagenase IV, and RPMI-1640 medium were purchased from Gibco (Grand Island, New York, USA). Insulin and HEPES were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Dexamethasone acetate, vitamin C, ascorbic acid, penicillin, streptomycin, and other chemicals were provided by Baoman Biotechnology (Shanghai, China). IGF-1 was purchased from PROSPEC company (Israel).

2.2. Adenovirus silencing vector construction

Construction of IGF-1 adenovirus vector: An effective shRNA, an interference sequence, was subcloned into the multicloning site of the pMD-18T vector (TaKaRa, Otsu, shiga, Japan). The vector was then digested by EcoRI/BamHI restriction enzymes (Beyotime Institute of Biotechnology, Nantong, China) and subcloned into the multicloning site of a pBHAd-U6-GFP vector (Hanbio Biology, Shanghai, China), resulting in the pAd-GFP-IGF-1 adenovirus plasmid, which inhibited the expression of IGF-1. Furthermore, we also constructed the adenovirus-green fluorescent protein (Ad-GFP) as a negative control. The pAd-GFP and pAd-GFP-IGF-1 was transfected into Human Embryonic Kidney 293 (HEK293) cells. After packaging, collection, and amplification, the viral titer was 1.2×10^{10} PFU/mL. Green fluorescent protein (GFP) is a green fluorophore. The Ad-GFP-IGF-1 and Ad-GFP was transfected into cow hepatocytes, and the multiplicity of infection (MOI) of Ad-GFP and Ad-GFP-IGF-1 is 100.

2.3. Cell culture and treatment

The study protocol was approved by the Ethics Committee on the Use and Care of Animals, Jilin University (Changchun, China). In this study, the experimental animal is a multiparous healthy Holstein cow and in the stage of lactation (5 years old, 3 parity and 401 kg). Approximate 200 g caudate liver lobe was obtained through surgical liver excision according to previous studies performed by an experienced veterinary surgeon (Parker and Gaughan, 1988; Liu et al., 2014).

The hepatocytes were isolated by collagenase IV perfusion method as previously described (Zhang et al., 2012; Li et al., 2014; Shi et al., 2014). The hepatocyte was seeded into a six-well tissue culture plate (2 mL per well) at 1×10^6 cells/mL and incubated at 37 °C in 5% CO_2. The medium was replaced with growth medium every 24 h. After 3 days of culture, cells were serum-starved overnight and then treated with different concentrations of IGF-1 (Kobayashi et al., 1999), and transfected with Ad-GFP and Ad-GFP-IGF-1, respectively. The transfection process was as follows. Briefly, cell culture medium was removed and cell was washed with PBS. Then, cell was incubated with new medium at 37 °C in 5% CO_2 for 1 h. Cells were transfected with Ad-GFP and Ad-GFP-IGF-1 at multiplicity of infection of 50 and incubated for 45 min respectively, and then cell medium was added to 3 mL per well. The hepatocytes were divided into a control group, an Ad-GFP transfection group (negative control group), an Ad-GFP-IGF-1 transfection group, a 25 ng/mL IGF-1 treatment group, and a 50 ng/mL IGF-1 treatment group. Ad-GFP transfection group acts as a negative control group. The hepatocytes were treated with IGF-1 and adenovirus for 12 h and 24 h, respectively, and each treatment was replicated 12 times.

2.4. RNA extraction and real-time RT-PCR

The total RNA in hepatocytes was extracted using TRIzol kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The RNA concentration was determined using a K5500 Micro-Spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China). RNA integrity was tested by gel electrophoresis. Furthermore, total RNA was treated with RNase-free DNase I (Takara Shuzo Co., Ltd., Kyoto, Japan) and analyzed by spectrophotometry at 260 and 280 nm using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK). Only samples with an optical density ratio at 260/280 nm > 1.8 were used in further analyses. Approximately 5 μg 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 manufacturer's protocol. The gene primers and annealing temperature are shown in Table 1. The abundance of mRNA was quantitated by using SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co., Ltd.) on an ABI Prism 7500 (Applied Biosystems, Foster City, USA). Each 25 μL mix was prepared in triplicate and was composed of 10 μL of ddH_2O, 12.5 μL of SYBR Premix Ex Taq (2×), 0.5 μL of Rox Referfece Dye II (50×), 0.5 μL of forward primer, 0.5 μL of reverse primer and 1 μL of cDNA template. The relative expression of genes was normalized

Table 1
The primers sequences of the genes.

Genes	Primers used for PCR	Length of fragment (bp)
IGF-1	For:GATCTGAGGAGGCTGGAGATGTAC Rev: GTTTCCTGCACTCCCTCTACTTGT	144
ApoB100	For: GATACTCAGAACGGAGCAAT Rev: GCACCAATCAGATAACAGGA	223
ApoE	For: TCCTGAATGACCTGGGTGTTG Rev: TCTGTGGGTTGCCGTGGTG	217
MTTP	For: CAGTTTGCAGCCTTGGTTCTG Rev: TCTGTGGGTTGCCGTGGTG	201
LDLR	For: GCT GTT CTG CCT TTC TCC TT Rev: ACT TTC TCC CCT GAC CCT TG	228
GAPDH	For: GGCGTGAACCACGAGAAGTATAA Rev: CCTCCACGATGCCAAAGTG	118