The effects of non-esterified fatty acids and β-hydroxybutyrate on the hepatic CYP2E1 in cows with clinical ketosis

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SUPPLEMENTARY FILE

Supplementary Materials and Methods

Isolation of cow primary hepatocytes

Three 1-day-old calves (Holstein calf, female, 30-40 kg, health, fasting) were sacrificed by bleeding from the carotid artery following intravenous injection with sodium pentobarbital. The liver caudate lobe was obtained through surgical liver excision. The hepatocytes were isolated using the collagenase Ⅳ perfusion method as described by Li et al. (2016b) and detailed in online Supplementary File . The liver was perfused with perfusion solution A (140 m*M* NaCl, 10 m*M* HEPES, 6.7 m*M* KCl, 0.5 m*M* EDTA and 2.5 m*M* glucose, pH 7.2-7.4, 37°C) at a flow rate of 50 mL/min for 12 min. Then, the liver was perfused with solution B (140 m*M* NaCl, 30 m*M* HEPES, 6.7 m*M* KCl, 5 m*M* $CaCl_2$ and 2.5 m*M* glucose, pH 7.2-7.4, 37°C) at a flow rate of 50 mL/min for 3 min until the liquid became clear. Subsequently, the liver was perfused with a collagenase IV solution (0.1 g collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2-7.4, 37°C) at a flow rate of 20 mL/min for 15-20 min. One hundred milliliters of basic medium (4°C) containing 10% fetal bovine serum bovine serum albumin (BSA) were added to terminate the digestion. The liver was cut

open, and the liver capsule, blood vessels, fat, and connective tissue were removed. The liver parenchyma filtered sequentially with 100 mesh (150 μm) and 200 mesh (75 μm) cell sieves. The hepatocyte suspension was washed twice and centrifuged for 5 min at 800 *g* at 4°C. The hepatocyte suspension was seeded into a six-well tissue culture plate (2 mL per well) at 1×10^6 cells/mL. The hepatocytes were incubated at 37°C in 5% CO_2. After 4 h, the adherent medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 10^{-6} *M* insulin, 10^{-6} *M* dexamethasone, 10 μg/mL vitamin C) was replaced with growth medium containing 10% fetal bovine serum.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

The total RNA was extracted from liver and hepatocytes using RNAiso Plus (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The RNA concentration and quality were measured using K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd., Beijing, China) and electrophoresis (1% agarose gels). Then, one microgram total 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 mRNA expression levels were evaluated using a 7500 Real-Time PCR System (Applied Biosystems Inc., CA, USA) with the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co., Ltd.). The primer sequences (5′-3′) of CYP2E1 are For-CATCGCTGTGACTGTGGC and Rev-TTCGT GGAGCAGATTGGA, and the length of the target fragment is 249 bp. The relative expression of CYP2E1 was normalized to β-actin, and analyzed by the $2^{-\Delta\Delta CT}$ method.

The qRT-PCR was conducted under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s) and extension at 72°C for 5 min.

Western blotting

The total protein of the liver tissue and hepatocytes were extracted using a protein extraction kit (Sangon Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The target protein was separated by polyacrylamide gel electrophoresis (12% percent) and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Then, the PVDF membranes were blocked in 3% BSA–Tris-buffered saline–Tween buffer for 4 h at room temperature. The blocked membranes were hybridized with CYP2E1 polyclonal antibody (Abcam, Cambridge, MA, USA; ab28146) overnight at 4 °C. After being washed 3 times, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Beyotime Biotechnology Inc., shanghai, China) at dilutions of 1:5,000 at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Beyotime Biotechnology Inc.). Beta actin was used a reference protein in this study and each band was related to β-actin. All bands were analyzed using Image-pro Plus (Media Cybernetics, Rockville, MD).

Supplementary Table S1

The basic description of the clinical ketosis cows and control cows

	Ketosis (n = 6)		Control (n = 8)		P
	Median	IQR	Median	IQR	
Body weight (kg)	517.65	507.7, 529.68	520.5	499.2, 540.1	0.9497
Milk production (kg of milk/cow per day)	22.85	18.08, 24.63	22.8	20.9, 28	0.4908
BHB (*mM*)	3.88	3.52, 4.55	0.45	0.28, 0.48	0.0007
NEFA (*mM*)	1.25	1.15, 1.4	0.27	0.18, 0.33	0.0024
Glucose (*mM*)	2.28	1.94, 2.4	3.86	3.72, 4.08	0.0044

Supplementary Table S2

Composition of the diet

Ingredient (% of DM)	
Corn silage	40.0
Corn	35.0
Wheat bran	8.0
Soybena meal	5.0
Sunflower	8.0
NaCl	1.0
Premix*	1.8
$NaHCO_3$	1.2
Total	**100**

Nutrient composition (% of DM)	
NEL (MJ/Kg)	6.7
CP	15.2
NDF	33.4
ADF	17.2
NFC	40.4
Ca	0.7
P	0.5

*One kg of premix contained the following: Vitamin A 200,000 IU, Vitamin D 70,000 IU, VitaminE 1,000 IU, Fe 2,000 mg, Cu 600 mg, Zn 2,400 mg, Mn 1,300 mg, I 6 mg, Co 7 mg.

DM, Dry Matter; NEL, Net energy for lactation; CP, Crude protein; NDF, Neutral detergent fibre; ADF, Acid detergent fibre; NFC, Non-fibre carbohydrate.

Supplementary Figure S1