Supplementary material

The regulation of PTEN on insulin and lipid metabolism in bovine hepatocytes in vitro

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MATERIALS AND METHODS

Cell culture

The study protocol was approved by the Ethics Committee on the Use and Care of Animals, Jilin University (Changchun, China). The caudate lobe was obtained through surgery liver excision from a newborn calf. Hepatocytes were isolated by a modified two-step collagenase perfusion method (Deng *et al.*, 2014; Zhang *et al.*, 2012). The liver was perfused with perfusion solution to wash away the blood until the perfusion solution became clear (Perfusion solution A comprised 140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA. Perfusion solution B comprised 140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM $CaCl_2$. The pH of solution A and B was adjusted to 7.4). The liver was then perfused with a collagenase IV solution (0.1 g collagenase IV was dissolved in 0.5 L of perfusion solution B) to digest the liver tissue until the liquid became muddy. The liver capsule was cut off after digestion. We used 100 mL RPMI-1640 culture medium containing 0.2% bovine serum albumin that pre-cooling to terminate the digestion (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 liver capsule, blood vessels, fat, and other parts of the liver caudate lobe were then discarded. The hepatocyte suspension was filtered sequentially with 100 mesh (150 μm), and 200 mesh (75 μm) cell sieves. Cell density was adjusted to 2×10^6 cells/mL with adherent 1640 culture medium. The hepatocytes were seeded into a 6-well tissue culture plate (2 mL per well) and incubated at 37 °C in 5% CO_2. Every 24h, the medium was replaced with fresh medium and the hepatocytes' shape and growth conditions were observed daily.

Quantitative real-time PCR

The total RNAs of cells were extracted using Trizol according to manufacturer's instructions (Invitrogen Corp, Carlsbad, CA, USA). The RNA was reverse transcribed into cDNA using a reverse transcription kit (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. All primers were synthesized from Sangon (Sangon Biotech Co., Ltd., Shanghai, China). The mRNA expression levels were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis using the SYBR Green QuantiTect RT-PCR Kit (Roche, Basel, Switzerland). qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems/Life Technologies, Grand Island, NY, USA). The gene primers (Table 1) were designed using Primer Express software 5.0. The mRNA expression levels were normalized to the house-keeping gene–β-actin. Real-time PCR was conducted under the following conditions: initial denaturation at 95 °C for 3 min, 45 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min). The relative

expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method and was normalized to abundance of β-actin.

Protein extraction and western blot analysis

Hepatocytes were lysed with lysis buffer, and the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Prepared proteins were performed by standard SDS-PAGE with 10% (w/v) polyacrylamide gels (0.1% SDS), and electrotransferred onto PVDF membranes (Roche, Basel, Switzerland) using a semi-dry blotting apparatus. After washing several times, the membranes were incubated in blocking solution (3% Bovine Serum Albumin Ⅴ in Tris-HCl buffer solution) for 4h at room temperature and incubated overnight at 4°C with antibodies against phosphor-IRS1, IRS1, PI_3K (Abcam, Cambridge, England), p-AKT, AKT, SREBP-1c, FAS (Cell Signaling Technology, Berverly, MA, USA), p-GSK, GSK (Millipore, Boston, Massachusetts, USA), β-actin (Santa Cruz, CA, USA). The membranes were then washed three times with TBS containing 0.1% Tween 20 for 5 min with shaking, and then incubated with appropriate peroxidase-conjugated secondary antibodies (Protein technology, Chicago, IL, USA) for 45 min with shaking at room temperature and washed four times for 5 min. The resulting bands were detected by ECL kit (Millipore, Boston, Massachusetts, USA). The relative expression levels of the proteins were normalized to β-actin level.

References

Deng Q, Li X, Fu S, Yin L, Zhang Y, Wang T, Wang J, Liu L, Yuan X, Sun G, Wang Z, Liu G & Li X 2014 SREBP-1c gene silencing can decrease lipid deposits in bovine hepatocytes cultured in vitro. Cell Physiology and Biochemistry **33** 1568-1578

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Table 1

List of primers used for PCR

Genes	Primers used for PCR (5’ - 3’)
β-actin	F: GCTAACAGTCCGCCTAGAAGCA
	R: GTCATCACCATCGGCAATGAG
G6-Pase	F: AGCAAGTGGTTCCCGTTTC
	R: ACCCAGGCGAGGCAGTA
PEPCK	F: AAGTACCTTGAGGAGCAAGTGAA
	R: GGTGCGTTGTATGGATTGGA
SREBP-1c	F: CGACACCACCAGCATCAACCACG
	R: GCAGCCCATTCATCAGCCAGACC
FAS	F: ACAGCCTCTTCCTGTTTGACG
	R: CTCTGCACGATCAGCTCGAC
PTEN	F: AAGCTTATGAGAGACGGCGGCGG
	R:GGATCCTCAGACTTTTGTAATTTGTGTATGC