Short communication: High insulin concentrations inhibit fatty acid oxidation-related gene expression in calf hepatocytes cultured in vitro

P. Li,*†¹ C. C. Wu,†¹ M. Long,* Y. Zhang,* X. B. Li,†² J. B. He,* Z. Wang,† and G. W. Liu†²

*College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110161, Liaoning, China

†Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun 130062, Jilin, China

ABSTRACT

In dairy cows, ketosis is an important disease associated with negative energy balance, which leads to low blood glucose levels and high blood nonesterified fatty acid levels. The liver is the most active organ in cows for the metabolism of nonesterified fatty acids. Insulin is an anabolic hormone that plays numerous roles in the metabolism of carbohydrates, lipids, and proteins, as well as being a potent regulator of fatty acid oxidation. In this study, using fluorescent quantitative reverse-transcription PCR, ELISA, and primary hepatocytes cultured in vitro, we examined the effect of insulin (0, 5, 10, 20, 50, and 100 nmol/L) on fatty acid oxidation by monitoring mRNA and protein expression levels of key enzymes: long-chain acyl-coenzyme A synthetase, carnitine palmitoyltransferase I, and long-chain acyl-coenzyme A dehydrogenase. The results showed that the mRNA and protein expression of long-chain acyl-coenzyme A synthetase, carnitine palmitoyltransferase I, and long-chain acyl-coenzyme A dehydrogenase was markedly decreased when the concentration of insulin in the media was increased. These findings indicate that high levels of insulin significantly inhibit the expression of genes related to fatty acid oxidation and consequently results in a decreased level of fatty acid oxidation in calf hepatocytes.

Key words: fatty acid oxidation, insulin, hepatocyte, dairy cow

Short Communication

Ketosis is an important disease connected with negative energy balance (NEB) in dairy cows during the periparturient period. Negative energy balance can be induced through the decreased intake of DM during lactation, leading to low blood glucose levels and body fat mobilization (Bobe et al., 2003; Loores et al., 2007),

which increases blood NEFA levels. During NEB, the low blood glucose is insufficient to provide enough energy to restore a positive energy balance (Meikle et al., 2004); therefore, FA may be the main energy substrates during this condition. The liver is the most active metabolic organ in cows to metabolize NEFA (Melendez et al., 2006), and the oxidative metabolism of NEFA in the liver of NEB cows may be important in providing enough energy to restore a positive energy balance.

Concentrations of hormones such as insulin, glucagon, and estrogens in plasma are altered at the end of gestation and may influence the metabolic fate of FA within the liver of dairy cows. In vitro data in nonruminants have indicated that estrogens increase the incorporation of media FA into hepatic triglyceride (TG), and in vivo data for ruminants have shown that estrogens accelerate fat infiltration of the liver during starvation (Grummer et al., 1990). Insulin deficiency has been shown to enhance ketogenesis in monogastrics and ruminants, whereas exogenous insulin is known to promote TG synthesis (Heitmann and Fernandez, 1986; Duerden and Gibbons, 1990). However, the effect of insulin on FA oxidation in the liver, especially in ruminants, is unknown. Long-chain acyl-CoA synthetase (ACSL) is a key enzyme that activates FA to acyl-CoA, carnitine palmitoyltransferase I (CPT I) is a membrane transporter of FA (acyl-CoA) from the cytoplasm into the mitochondrial matrix and is a primary regulatory enzyme involved in mitochondrial β -oxidation, and long-chain acyl-CoA dehydrogenase (ACADL) mainly catalyzes the first step of the β -oxidation of long-chain FA in the mitochondria. These 3 key enzymes are involved in the FA metabolism pathway in the liver, especially in the activation, translocation, and β -oxidation of FA. The aim of the present study was to determine the effect of insulin on the mRNA and protein expression of ACSL, CPTI, and ACADL in bovine hepatocytes, which may provide insight into how insulin affects FA metabolism in the liver of dairy cows.

Hepatocytes were cultured as described previously (Li et al., 2012b; Zhang et al., 2012). The cells were cultured in 6-well plates at a density of 10^6 cells/cm²

Received September 15, 2012.

Accepted February 22, 2013.

¹P. Li and C. C. Wu contributed equally to this study.

²Corresponding authors: liuguowen2008@163.com and lxbvip@139.com

Table 1. Primers used for the analysis of expression of fatty acid oxidation-related genes

Gene name	Sequence of primer (5'-3')	Length of amplicon (bp)	GenBank accession no.	Annealing temperature (°C)
ACSL	Forward: TCGGAACTGAAGCCATCAC Reverse: GCCTCGTTCCAGCAGATCAC	173	NM_001076085	58
CPT I	Forward: CAAAACCATGTTGTACAGCTTCCA Reverse: GCTTCCTTCATCAGAGGCTTCA	111	BF039285	54
ACADL	Forward: CCCAGGTTTTGGTCTTCATTCA Reverse: GGCTCTGTCATTGCTATGGCA	132	NM_001076936	58
β -actin	Forward: GTCATCACCATCGGCAATGAG Reverse: GCTAACAGTCCGCCTAGAAGCA	403	NM_173979	57

(Costar; Corning Inc., New York, NY). After a 4-h attachment period, the media and unattached cells were aspirated and replaced with RPMI 1640 culture medium (Gibco, New York, NY) containing 10% fetal bovine serum. The cells were then cultured at 37°C and 5% CO₂. Every 24 h, the medium of each culture was aspirated and refreshed. At 72 h, the cells were cultured with RPMI 1640 culture medium free of fetal bovine serum. Insulin was then added to the medium of each well to give a final concentration of insulin of 0, 5, 10, 20, 50, or 100 nmol/L.

After 24 h of incubation, the total RNA and protein of the hepatocytes was extracted. Total RNA was isolated from hepatocytes using RNAiso Plus [TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China] according to the manufacturer's instructions and quantified by spectrophotometry with a BioPhotometer (Vaudaux-Eppendorf AG, Basel, Switzerland). Then, 1 µg of total RNA was reverse transcribed with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase RNase H Minus, Point Mutant (Promega, Madison, WI) using 100 pmol of random hexamer primers (Invitrogen, Leek, the Netherlands). The cDNA (25 ng/µL) was amplified by fluorescent quantitative reverse-transcription PCR using SYBR Premix Ex Taq II [TaKaRa Biotechnology (Dalian) Co. Ltd.] according to the manufacturer's instructions. A master mix of the following reaction components was prepared: 6 µL of water, 0.8 µL of forward primer (10 µmol/L), 0.8 µL of reverse primer (10 µmol/L), 10 µL of SYBR Premix Ex Taq II (2×), 0.4 µL of ROX reference dye [50×; TaKaRa Biotechnology (Dalian) Co. Ltd.]. Then, 18 µL of master mix (containing SYBR Premix Ex Taq II, ROX reference dye, primers, and water) and 2 µL of sample volume (containing 50 ng of cDNA) were used in the following 2-step PCR program: denaturation for 30 s at 95°C, 40 cycles of amplification (each consisting of 5 s at 95°C and 30 s at 60°C), and finally a melting curve program (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). Expression of ACSL, CPT I, ACADL, and β -actin genes was evaluated on the basis of mRNA copy number per 1 µg of total RNA. The β -actin gene was also analyzed

(in addition to the target genes of interest) to monitor reverse-transcription PCR efficiencies and to provide an internal reference. Target gene mRNA abundance was displayed as the target gene mRNA copy number per 1 µg of total RNA divided by the β -actin mRNA copy number. Primers for CPT I were identical to that of van Dorland et al. (2009), and primers for ACSL, ACADL, and β -actin were designed using Primer Express version 2.0 software (PE Applied Biosystems, Foster City, CA; Table 1).

Cells were harvested from the culture dishes by scraping, recovered by sedimentation, and stored at -80°C before analysis. Frozen cell pellets containing approximately 25×10^6 cells/treatment group (3 dishes) were homogenized in a 0.1 M concentration of potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol with a Dounce manual homogenizer (Wheaton Industries Inc., Millville, NJ). Particulate matter was removed by centrifugation for 2 min at $10,000 \times g$ and stored in aliquots at -80°C. Total protein was assayed using the Bradford protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA) with BSA as the standard.

Long-chain acyl-CoA synthetase protein was measured using the bovine acyl-CoA synthase ELISA kit (catalog no. CSB-E12165B; Cusabio Biotech Co. Ltd., Wuhan, China) with a minimum detectable concentration of 0.39 ng/mL. Carnitine palmitoyltransferase I protein was measured using the bovine CPT 1 ELISA kit (catalog no. CSB-E12136B; Cusabio Biotech Co. Ltd.) with a minimum detectable concentration of 0.31 ng/mL. Long-chain acyl-CoA dehydrogenase protein was measured using the bovine long chain ACADL ELISA kit (catalog no. CSB-E12138B; Cusabio Biotech Co. Ltd.) with a minimum detectable concentration of 3.12 ng/mL. All kits were used according to the manufacturer's instructions, and each value was measured in triplicate. Protein levels of ACSL, CPT I, and ACADL were displayed as the target protein content/total protein content.

Statistical analysis of the data was conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL) with the

significance level set at $P < 0.05$. The values were expressed as the mean $\pm$ standard deviation. Parameters were compared by one-way ANOVA using Duncan's test and $P < 0.05$ was considered significant.

The agarose gel electrophoresis of total RNA shown in Figure 1 indicates that 3 distinct bands of total RNA were extracted, corresponding to 28S, 18S, and 5S RNA. The A_{260}/A_{280} ratio (representing the ratio of absorbance at 260 nm to absorbance at 280 nm) of total RNA was >1.9 and met the specified purity requirements. As shown in Figure 2, the mRNA and protein levels of ACSL decreased gradually with increasing concentrations of insulin. The ACSL mRNA and protein levels were markedly decreased when the insulin concentrations exceeded 20 nM ($P < 0.05$). Low insulin concentrations (≤ 10 nM) did not significantly affect the mRNA and protein levels of ACSL in hepatocytes. As shown in Figure 3, the mRNA and protein levels of CPTI decreased gradually with increasing insulin concentrations. The CPTI mRNA and protein levels were markedly decreased when the insulin concentrations exceeded 5 nM ($P < 0.05$; mRNA) and 10 nM ($P < 0.05$; protein). As shown in Figure 4, the mRNA and protein levels of ACADL decreased gradually with increasing concentrations of insulin. The ACADL mRNA and protein levels were markedly decreased when the insulin concentrations exceeded 10 nM ($P < 0.05$; mRNA) and 20 nM ($P < 0.05$; protein). Low concentrations of insulin (≤ 5 nM) did not significantly affect the mRNA and protein levels of ACADL in hepatocytes.

Insulin is an anabolic hormone that plays numerous roles in the metabolism of carbohydrates, lipids, and proteins and acts to preserve nutrient availability. It is also a potent regulator of FA metabolism. The main role of insulin in muscle and fat (by acting through the cell surface insulin receptor) is to stimulate glucose uptake into cells and to maintain blood sugar homeostasis (Biddinger and Kahn, 2006; Vikram and Jena, 2010; Zhang et al., 2011). Furthermore, insulin is also known to regulate gluconeogenesis and FA metabolism in the liver. The results of Hamel et al (2001) indicate that insulin inhibits peroxisomal FA oxidation by

Figure 1. Agarose gel electrophoresis of hepatocyte total RNA.

Figure 2. Effects of insulin (INS) on long-chain acyl-CoA synthetase (ACSL) mRNA and protein expression. Values are means $\pm$ SD. Different small letters (a–c) indicate significant differences between the parameters of ACSL mRNA or ACSL protein ($P < 0.05$), and the same small letters indicate no significant differences between different concentration sets in the same line ($P > 0.05$). Color version available in the online PDF.

insulin-degrading enzyme in isolated rat hepatocytes. Liu et al. (2009) reported that transcript levels of key genes involved in β -oxidation (including CPTI and ACADL) were decreased by incubation with insulin in rat hepatocytes. In the present study, the mRNA and protein levels of ACSL, CPTI, and ACADL were all significantly inhibited by insulin.

Figure 3. Effects of insulin (INS) on carnitine palmitoyltransferase I (CPTI) mRNA and protein expression. Values are means $\pm$ SD. Different small letters (a–d) indicate significant differences between the parameters of CPTI mRNA or CPTI protein ($P < 0.05$), and the same small letters indicate no significant differences between different concentration sets in the same line ($P > 0.05$). Color version available in the online PDF.

Figure 4. Effects of insulin (INS) on long-chain acyl-CoA dehydrogenase (ACADL) mRNA and protein expression. Values are means $\pm$ SD. Different small letters (a–c) indicate significant differences between the parameters of ACADL mRNA or ACADL protein ($P < 0.05$), and the same small letters indicate no significant differences between different concentration sets in the same line ($P > 0.05$). Color version available in the online PDF.

Long-chain acyl-CoA synthetase activates FA conversion to fatty acyl-CoA for either FA oxidation or for TG synthesis in the liver (Li et al., 2012a). Carnitine palmitoyltransferase I is a potent regulator of translocation of long-chain FA from the cytoplasm into the mitochondria (where they partition for esterification and oxidation). Long-chain acyl-CoA dehydrogenase is a key enzyme during the regulation of FA β -oxidation in mitochondria (Dann and Drackley, 2005; Liu et al., 2009). In addition to inhibiting ACSL expression (resulting in decreased production of acyl-CoA for esterification and oxidation), insulin also inhibits CPTI expression and limits the transfer of acyl-CoA into mitochondria for oxidative metabolism. Furthermore, insulin can promote TG accumulation in the liver by increasing the expression of ACC and FAS (Han et al., 2009), thus suggesting that acyl-CoA synthesized by ACSL may be driven toward TG synthesis (rather than FA oxidation). However, the esterification process of FA was not evaluated in the present study, and additional research is needed to confirm our findings.

Nonesterified FA levels in the liver are mainly metabolized via 3 pathways: (1) complete oxidation, producing H_2O , CO_2 , and ATP; (2) incomplete oxidation, producing ketones; and (3) esterification to TG. Therefore, FA metabolism may be the main energy source to provide enough energy for dairy cows experiencing NEB. Brockman (1979) reported the antiketogenic effects of insulin, which include decreasing liver NEFA uptake (through stimulation of lipogenesis and inhibition of lipolysis in adipose tissue), enhancing peripheral

tissue ketone utilization, and altering enzyme activities and availability of substrates that are involved in ketogenesis in the liver. Insulin is administered to alleviate severe NEB, and its administration in combination with dextrose is an effective treatment for hepatic lipodosis and ketosis in dairy cows. The results of Hayirli et al. (2002) indicated that plasma BHBA concentrations decreased when cows were injected with slow-release insulin and considered the inhibition of CPTI by insulin to play a key role in BHBA production. Our data suggested that insulin has the same effect on CPTI in bovine hepatocytes. Furthermore, the inhibitory effect of insulin on ketogenesis is also related to its inhibitory effect on ACSL and ACADL. Considering that the concentration of insulin used in our study was higher than the physiological concentration in dairy cows (Brockman, 1979; Hayirli et al., 2002) and that these high levels of insulin inhibit mitochondrial FA metabolism (by regulating the expression of key genes and decreasing BHBA production) in dairy cows, it is possible that the inhibitory effect mentioned above may lower the concentration of ketogenesis substrates and prevent ketosis in dairy cows. In summary, the results of this study indicate that high levels of insulin significantly inhibit the expression of FA oxidation-related genes and decrease FA oxidation in bovine hepatocytes.

ACKNOWLEDGMENTS

This work was supported by the Program for New Century Excellent Talents in University (NCET-11-0199), the National Key Technology R&D Program (grant no. 2012BAD12B03), the National Natural Science Foundation of China (Beijing, China; grant no. 30600441, 30972212, 30871897, 31072178, 31201961, and 31272621), and the Science Fund for Distinguished Young Scholars of Jilin University (grant no. 201100009).

REFERENCES

- Biddinger, S. B., and C. R. Kahn. 2006. From mice to men: Insights into the insulin resistance syndromes. *Annu. Rev. Physiol.* 68:123–158.
- Bobe, G., R. N. Sonon, B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003. Metabolic responses of lactating dairy cows to single and multiple subcutaneous injections of glucagon. *J. Dairy Sci.* 86:2072–2081.
- Brockman, R. P. 1979. Roles for insulin and glucagon in the development of ruminant ketosis. *Can. Vet. J.* 20:121–126.
- Dann, H. M., and J. K. Drackley. 2005. Carnitine palmitoyltransferase I in liver of periparturient dairy cows: Effects of prepartum intake, postpartum induction of ketosis, and periparturient disorders. *J. Dairy Sci.* 88:3851–3859.
- Duerden, J. M., and G. F. Gibbons. 1990. Storage, mobilization and secretion of cytosolic triacylglycerol in hepatocyte cultures. *Biochem. J.* 272:583–587.

- Grummer, R. R., S. J. Bertics, D. W. Lacount, J. A. Snow, M. R. Dentine, and R. H. Stauffacher. 1990. Estrogen induction of fatty liver in dairy cattle. *J. Dairy Sci.* 73:1537–1543.
- Hamel, F. G., R. G. Bennett, J. L. Upward, and W. C. Duckworth. 2001. Insulin inhibits peroxisomal fatty acid oxidation in isolated rat hepatocytes. *Endocrinology* 142:2702–2706.
- Han, C., J. Wang, L. Li, Z. Zhang, L. Wang, and Z. Pan. 2009. The role of insulin and glucose in goose primary hepatocyte triglyceride accumulation. *J. Exp. Biol.* 212:1553–1558.
- Hayirli, A., S. J. Bertics, and R. R. Grummer. 2002. Effects of slow-release insulin on production, liver triglyceride, and metabolic profiles of Holsteins in early lactation. *J. Dairy Sci.* 85:2180–2191.
- Heitmann, R. N., and J. M. Fernandez. 1986. Autoregulation of hepatic and alimentary ketogenesis in sheep. *J. Dairy Sci.* 69:1270–1281.
- Li, P., X. B. Li, S. X. Fu, C. C. Wu, X. X. Wang, G. J. Yu, M. Long, Z. Wang, and G. W. Liu. 2012a. Alterations of fatty acid β -oxidation capability in the liver of ketotic cows. *J. Dairy Sci.* 95:1759–1766.
- Li, X., X. Li, G. Bai, H. Chen, Q. Deng, Z. Liu, L. Zhang, G. Liu, and Z. Wang. 2012b. Effects of non-esterified fatty acids on the gluconeogenesis in bovine hepatocytes. *Mol. Cell. Biochem.* 359:385–388.
- Liu, H.-Y., E. Yehuda-Shnaidman, T. Hong, J. Han, J. Pi, Z. Liu, and W. Cao. 2009. Prolonged exposure to insulin suppresses mitochondrial production in primary hepatocytes. *J. Biol. Chem.* 284:14087–14095.
- Loor, J. J., R. E. Everts, M. H. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. *Physiol. Genomics* 32:105–116.
- Meikle, A., M. Kulcsar, Y. Chilliard, H. Febel, C. Delavaud, D. Cavestany, and P. Chilibroste. 2004. Effects of parity and body condition at parturition on endocrine and reproductive parameters of the cow. *Reproduction* 127:727–737.
- Melendez, P., J. P. Goff, C. A. Risco, L. F. Archbald, R. Littell, and G. A. Donovan. 2006. Incidence of subclinical ketosis in cows supplemented with a monensin controlled-release capsule in Holstein cattle, Florida, USA. *Prev. Vet. Med.* 73:33–42.
- van Dorland, H. A., S. Richter, I. Morel, M. G. Doherr, and N. Castro. 2009. Variation in hepatic regulation of metabolism during the dry period and in early lactation in dairy cows. *J. Dairy Sci.* 92:1924–1940.
- Vikram, A., and G. Jena. 2010. S961, an insulin receptor antagonist causes hyperinsulinemia, insulin-resistance and depletion of energy stores in rats. *Biochem. Biophys. Res. Commun.* 398:260–265.
- Zhang, Z., X. Li, G. Liu, L. Gao, C. Guo, T. Kong, H. Wang, R. Gao, Z. Wang, and X. Zhu. 2011. High insulin concentrations repress insulin receptor gene expression in calf hepatocytes cultured in vitro. *Cell. Physiol. Biochem.* 27:637–640.
- Zhang, Z. G., X. B. Li, L. Gao, G. W. Liu, T. Kong, Y. F. Li, H. B. Wang, C. Zhang, Z. Wang, and R. H. Zhang. 2012. An updated method for the isolation and culture of primary calf hepatocytes. *Vet. J.* 191:323–326.