

The Effects of Steroid Hormones on Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase (LCHAD) Enzyme Expression in LCHAD Deficient Cells and Induction of Long-Chain Fatty Acid Binding Proteins on Long-Chain Fatty Acid-Induced Hepatotoxicity

Galia Pollock, Jessica Lezen, Victor Perez-Alvarez, Jordyn Lerner, Frank Burczynski, Julia Uhanova, Gerald Yosel Minuk

Galia Pollock, Jessica Lezen, Victor Perez-Alvarez, Jordyn Lerner, Julia Uhanova, Section of Hepatology, Department of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada
Frank Burczynski, Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada

Frank Burczynski, Gerald Yosel Minuk, Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada

Correspondence to: Gerald Yosel Minuk, Morberg Family Chair in Hepatology, University of Manitoba, John Buhler Research Centre, 715 McDermot Ave, Winnipeg, MB, R3E 3P4, Canada.

Email: gerald.minuk@umanitoba.ca

Telephone: +1-204-789-3204 Fax: +1-204-789-3987

Received: July 29, 2015 Revised: September 12, 2015

Accepted: September 16, 2015

Published online: September 21, 2015

ABSTRACT

BACKGROUND: Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency has been implicated in the pathogenesis of acute fatty liver of pregnancy (AFLP) and the Hemolysis Elevated Liver Enzyme, Low Platelet (HELLP) syndrome. Yet not all pregnant women with LCHAD deficiency develop these conditions and whether induction of long-chain fatty acid binding proteins (L-FABP) might be of therapeutic value remains to be determined.

OBJECTIVES: To document: (1) the effects of pregnancy associated steroid hormones on LCHAD expression and (2) induction of L-FABP on long-chain fatty acid (LCFA)-induced hepatotoxicity.

METHODS: LCHAD deficient B2325 cells were exposed to various concentrations of β -estradiol and/or progesterone in vitro and LCHAD mRNA and protein expression documented by RT-PCR and Western Blot analysis for 24-72 hours thereafter. In

addition, Huh-7 hepatocytes exposed to toxic concentrations of LCFA (a mixture of oleic and palmitic acid) for 24 hours were treated with L-FABP inducers clofibrate or simvastatin (6.25-100 mM) for a subsequent 48 hours prior to documenting cell toxicity.

RESULTS: Neither β -estradiol, progesterone or a combination thereof consistently decreased LCHAD mRNA or protein expression. Moreover, hepatocyte survival was not altered by either clofibrate or simvastatin.

CONCLUSIONS: Increases in steroid hormones associated with pregnancy are unlikely to contribute to LCFA-induced hepatotoxicity in LCHAD deficient women. Induction of L-FABP does not hold promise as a therapeutic strategy for pregnant women with AFLP or HELLP.

© 2015 ACT. All rights reserved.

Key words: LCHAD; AFLP; HELLP; Long-chain fatty acids; Pregnancy; Liver failure; Hepatitis

Pollock G, Lezen J, Perez-Alvarez V, Lerner J, Burczynski F, Uhanova J, Minuk GY. The Effects of Steroid Hormones on Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase (LCHAD) Enzyme Expression in LCHAD Deficient Cells and Induction of Long-Chain Fatty Acid Binding Proteins on Long-Chain Fatty Acid-Induced Hepatotoxicity. *Journal of Gastroenterology and Hepatology Research* 2015; 4(9): 1768-1771 Available from: URL: <http://www.ghrnet.org/index.php/joghr/article/view/1222>

INTRODUCTION

Acute fatty liver of pregnancy (AFLP) and the Hemolysis Elevated Liver Enzymes and Low Platelet (HELLP) Syndrome are rare but serious conditions that tend to occur in the third trimester of pregnancy^[1]. The incidence of AFLP has been estimated to be

1:10,000-15,000 and HELLP 1:200-1,000 pregnancies^[1]. However, these figures likely represent underestimates as women with AFLP and HELLP are often misdiagnosed with other liver conditions that share similar presentations such as pregnancy induced hypertension.

Recent molecular advances suggest that AFLP and to a lesser extent HELLP, may result from mitochondrial dysfunction in maternal and fetal livers^[2,3]. Compared to healthy newborn controls, long-chain fatty acid (LCFA) oxidation defects are 50 times more common in the fetuses of mothers with AFLP^[4]. Of particular importance is a defect in LCFA oxidation which has been linked to a deficiency in the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) enzyme^[5]. LCHAD is one of four enzymes responsible for metabolizing LCFA within the mitochondria of hepatocytes. A deficiency in its activity results in increased accumulation of LCFA and associated toxic metabolites in the fetal followed by maternal circulation. Because AFLP mothers are heterozygote for this enzyme deficiency, the mother's ability to metabolize LCFAs is impaired and reactive oxygen species-induced hepatotoxicity ensues^[6-9]. The most common mutation responsible for LCHAD deficiency is a G to C mutation at nucleotide position 1528 (E474Q amino acid substitution). This mutation exists on chromosome 2 and results in altered mitochondrial trifunctional complex activity. To date, AFLP has been documented in 30-80% of pregnancies where the fetus was found to have LCHAD deficiency^[10]. Approximately 10% of HELLP mothers are also LCHAD deficient^[11].

Given the above proposed pathogenesis and the development of AFLP or HELLP in some but not all LCHAD deficient mothers, the question arises as to whether increases in circulating estrogen and progesterone levels associated with pregnancy contribute to the severity of AFLP or HELLP by further downregulating LCHAD expression and whether treatment with agents that increase LCFA binding proteins (L-FABP) such as clofibrate or statins might prevent or attenuate hepatocyte injury in this setting^[12-14].

MATERIALS AND METHODS

B2325 skin fibroblasts heterozygote for the E474Q mutation were kindly provided by Dr. R. Boriack at the Children's Medical Centre, Dallas, TX and Huh-7 cells by Dr. Y. Gong at the University of Manitoba, Winnipeg, Canada. Polyclonal rabbit IgG LCHAD antibody was purchased from Protein-Tech Group, Inc. (Chicago, IL). β -estradiol, progesterone, clofibrate, simvastatin, oleic acid and palmitic acid were purchased from Sigma-Aldrich (St. Louis, MO) and WST-1 reagent from Roche Diagnostics (Indianapolis, IN).

Steroid Hormones and LCHAD expression

In time dependent experiments, B2325 cells (1×10^4 cells/well) were incubated with β -estradiol alone (50 nM), progesterone alone (550 nM) or a combination of β -estradiol (50 nM) and progesterone (550 nM) for 24, 48 or 72 hours. In concentration dependent experiments, B2325 cells were exposed to β -estradiol at concentrations of 5-500 nM for 72 hours. The concentrations of β -estradiol and progesterone approximated those reported in the serum of women in their third trimester of pregnancy^[15].

Following 24, 48 and 72 hours of exposure to steroid hormones, B2325 cell mRNA was extracted by TRIZOL for LCHAD mRNA expression by RT-PCR and LCHAD protein by Western blot analysis as described by Spiekerkoetter *et al.*^[16].

Effect of L-FABP Inducers on Cell Survival

Human Huh-7 hepatocytes (1×10^4 cells/well) were seeded in 96

well plates and pre-treated with a 1:1 mixture of the LCFA oleic acid and palmitic acid (0.55 nM) for 24 hours. Thereafter, clofibrate or simvastatin (6.25-100 nM) were added (with continued exposure to LCFA) for an additional 48 hours prior to documenting cell viability by WST-1 analysis. Wells with no clofibrate or simvastatin served as controls.

Data Analyses

All experiments were performed on at least three occasions and the results provided represent the mean \pm SEM unless otherwise indicated. Statistical analyses consisted of a Student's T-test for parametric data and Wilcoxon Rank-Sum test for non-parametric data. *P* values <0.05 were considered significant.

RESULTS

The results of LCHAD mRNA expression in B2325 cells following exposure to β -estradiol alone, progesterone alone and a combination of the two steroids for 0-72 hours are provided in figure 1. Although β -estradiol markedly inhibited LCHAD mRNA expression at 24 hours, by 48 and 72 hours, expression levels had returned to baseline values. Moreover, there was no concentration-dependent effect of β -estradiol on LCHAD mRNA expression (Figure 2). As with β -estradiol, there was a trend towards progesterone inhibiting LCHAD mRNA expression at 24 and 48 hours but this effect was no longer apparent at 72 hours. Of note, these effects at 24 hours for β -estradiol and 24-48 hours for progesterone were not apparent when the combination of β -estradiol and progesterone was employed.

In keeping with a lack of significant and consistent inhibition of LCHAD mRNA by either steroid hormone alone or combination thereof, were the results of Western blotting for LCHAD protein expression. Here, at no time following exposure to β -estradiol and/or progesterone was LCHAD protein expression consistently decreased (data not shown).

Figure 3 provides the results of experiments documenting the toxicity of LCFAs on human Huh-7 hepatocytes. Survival rates were approximately 50% those of controls (no exposure to LCFAs) following 24 hours of exposure to LCFAs. However, the addition of the L-FABP inducers; clofibrate and simvastatin at concentrations of 6.5-100 μ M had no concentration-dependent effect on cell survival following exposure to LCFAs (Figures 4A and 4B respectively). Indeed, the only significant (*p*<0.05) finding in these experiments was a decrease rather than increase in hepatocyte survival following exposure to 12.5 μ M of clofibrate.

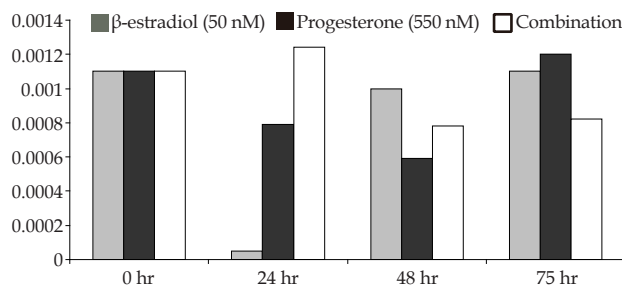


Figure 1 Effect of β -estradiol (50 nM), progesterone (550 nM) and a combination of both agents on long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) mRNA expression by RT-PCR in LCHAD deficient B2325 human fibroblasts after 24, 48 and 72 hours of exposure. The low level of expression with β -estradiol at 24 hours was not sustained nor evident when the combination of β -estradiol and progesterone were employed.

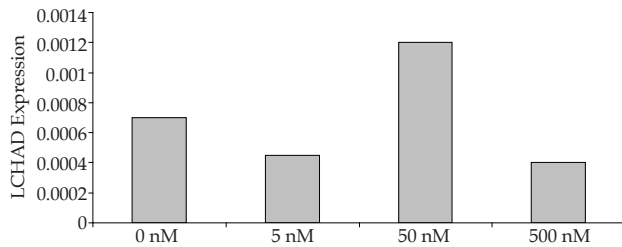


Figure 2 Effect of various concentrations (5-500 nM) of β -estradiol on long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) mRNA expression after 48 hours of exposure. There was no concentration-dependent effect of β -estradiol on LCHAD expression.

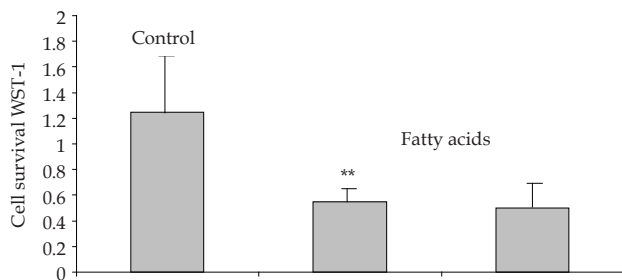


Figure 3 Effect of a mixture of long-chain fatty acids (LCFA) oleic and palmitic acid (0.55 mM) on Huh-7 hepatocyte survival. Cell viability was decreased by approximately 50% after 24 hours of exposure to LCFA.

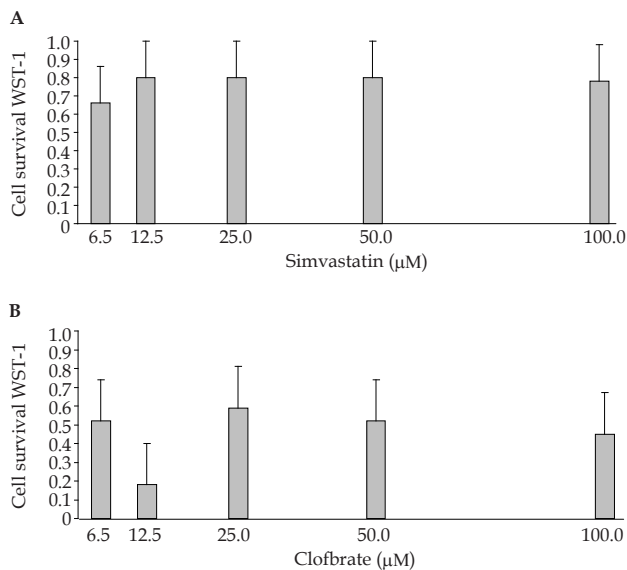


Figure 4 Huh-7 cell viability after exposure to a mixture of oleic and palmitic acid (0.55 mM) for 72 hours with 0.5-100 μ M of simvastatin (Figure 4A) or clofibrate (Figure 4B) being added for the final 48 hours. There was no concentration-dependent effect of either agent on cell viability relative to untreated controls (standardized to 1.0 cell survival).

DISCUSSION

The results of this study indicate that at concentrations relative to those reported during the third trimester of pregnancy in humans, steroid hormones do not alter the expression of LCHAD, a key enzyme responsible for oxidative phosphorylation of LCFAs in the liver. The results also indicate that L-FABP inducers such as clofibrate and simvastatin do not attenuate LCFA-induced hepatocyte injury.

Previous studies have demonstrated that steroid hormones and/

or their receptor antagonists alter hepatic mitochondrial enzyme activity and oxidative phosphorylation of LCFAs^[17,18]. Thus, we hypothesized that the increased sensitivity of some pregnant women with AFLP or HELLP to LCFA-induced hepatotoxicity results from a down regulation of LCHAD and/or other mitochondrial tri-functional proteins involved in LCFA β -oxidation. The results of the present study suggest that is not the case. Although it is possible that tri-functional proteins other than LCHAD are down regulated by steroid hormones, in the absence of data documenting heterozygosity for deficiencies with these enzymes, such an alternative explanation appears unlikely.

Both clofibrate and simvastatin upregulate L-FABP expression 5-10 fold^[19-21]. Clofibrate has also been reported to alter LCFA β -oxidation independent of L-FABP induction^[15]. These properties support the possibility that clofibrate and/or simvastatin might prevent or attenuate LCFA-induced hepatotoxicity. However, despite employing concentrations in keeping with those reported previously and allowing for a sufficient period of time (48 hours) for L-FABP upregulation to occur, neither agent protected hepatocytes from LCFA-induced injury^[20].

There are a number of limitations to this study that warrant emphasis. First, the experiments were performed *in vitro* rather than in animal models or humans with AFLP or HELLP. The lack of an acceptable animal model for these disorders and absence of *in vitro* evidence to support clinical trials in humans were the explanations for this limitation. Second, whereas the effects of estrogen and progesterone on LCHAD expression were documented in B2325 cells heterozygote for the E474Q mutation; clofibrate and simvastatin protection experiments were performed in Huh-7 cells. Here, it was felt that any potential therapeutic benefit of pharmacologic intervention should be demonstrated in hepatocytes (Huh-7) rather than skin fibroblasts (B2325). Third, the effects of steroid hormones on LCHAD mRNA and protein expression were documented but functional enzymatic activity was not ascertained. Finally, both clofibrate and simvastatin were added to Huh-7 cells 24 hours following exposure to LCFA. Thus, the possibility remains that these agents could provide protection against the development of AFLP and/or HELLP if administered prior to the onset of injury. However, the majority of such cases appear after the clinical onset of the condition and recurrent disease with subsequent pregnancies is relatively uncommon^[23].

In conclusion, the results of this study argue against steroid hormone induced suppression of LCHAD expression as contributing to the pathogenesis of AFLP and HELLP in pregnant women. The results also argue against upregulation of L-FABP as a therapeutic strategy for these conditions.

ACKNOWLEDGMENTS

This research was supported by a grant from the Canadian Liver Foundation. The authors wish to thank Ms R. Vizniak for her prompt and accurate typing of the manuscript.

CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

REFERENCES

- 1 Schutt VA, Minuk GY. Liver diseases unique to pregnancy. *Best Pract Res Clin Gastroenterol* 2007; **21**(5): 771-792
- 2 Ibdah JA, Yang Z, Bennett MJ. Liver disease in pregnancy and fe-

- tal fatty acid oxidation defects. *Mol Genet Metab* 2000 Sep-Oct; **71(102)**: 182-189
- 3 Treem WR, Shoup ME, Hale DE, Bennett MJ, Rinaldo P, Millington DS, Stanley CA, Riely CA, Hyams JS. Acute fatty liver of pregnancy, hemolysis, elevated liver enzymes, and low platelets syndrome, and long chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. *Am J Gastroenterol* 1996 Nov; **91(11)**: 2293-300
 - 4 Browning MF, Levy HL, Wilkins-Haug LE, Larson C, Shih VE. Fetal fatty acid oxidation defects and maternal liver disease in pregnancy. *Obstet Gynecol* 2006 Jan; **107(1)**: 115-120
 - 5 Strauss AW, Bennett MJ, Rinaldo P, Sims HF, O'Brien LK, Zhao Y, Gibson B, Ibdah J. Inherited long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency and a fetal-maternal interaction cause maternal liver disease and other pregnancy complications. *Semin Perinatol* 1999 Apr; **23(2)**: 100-112
 - 6 Haglind CB, Stenlid MH, Ask S, Alm J, Nemeth A, Dobein U, Nordenstrom A. Growth in Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency. *JIMD Rep* 2013; **8**: 81-90
 - 7 Wojtczak L and Schonfeld P. Effect of fatty acids on energy coupling processes in mitochondria. *Biochim Biophys Acta* 1993; **1183**: 41-57
 - 8 Kramer JH and Weglicki WB. Inhibition of sarcolemmal Na⁺-K⁺-ATPase by palmitoyl carnitine: Potentiation by propranolol. *Am J Physiol* 1985; **248**: H75-H81
 - 9 Mak IT, Kramer JH and Weglicki WB. Potentiation of free radical-induced lipid peroxidative injury to sarcolemmal membranes by lipid amphiphiles. *J Biol Chem* 1986; **261**: 1153-1157
 - 10 Ibdah JA. Acute fatty liver of pregnancy: an update on pathogenesis and clinical implications. *World J Gastroenterol* 2006; **12(46)**: 7397-7404
 - 11 Egerman RS, Sibai BM. HELLP syndrome. *Clin Obstet Gynecol* 1999; **42**: 381-389
 - 12 Rajaraman G and Burczynski FJ. Effect of dexamethasone, 2-bromopalmitate and clofibrate on L-FABP mediated hepatoma proliferation. *J Pharm Pharmacol* 2004; **56(9)**: 1155-1161
 - 13 Rajaraman G, Wang GQ, Yan J, Jiang P, Gong Y and Burczynski FJ. Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment. *Mol Cell Biochem* 2007; **295(1-2)**: 27-34
 - 14 Seo M, Inoue I, Ikeda M, Nakano T, Takahashi S, Katayama S, Komoda T. Statins Activate Human PPARalpha Promoter and Increase PPARalpha mRNA Expression and Activation of HepG2 Cells. *PPAR Res* 2008; **2008**: 316306
 - 15 Boroditsky RS, Reyes FI, Winter JS, Faiman C. Maternal serum estrogen and progesterone concentrations preceding normal labor. *Obstet Gynecol* 1978 Jun; **51(6)**: 686-691
 - 16 Spiekerkoetter U, Sun B, Khuchua Z, Bennett MJ, Strauss AW. Molecular and phenotypic heterogeneity in mitochondrial trifunctional protein deficiency due to beta-subunit mutations. *Hum Mutat* 2003 Jun; **21(6)**: 598-607
 - 17 Kelly DM, Nettleship JE, Akhtar S, Muraleedharan V, Sellers DJ, Brooke JC, McLaren DS, Channer KS, Jones TH. Testosterone suppresses the expression of regulatory enzymes of fatty acid synthesis and protects against hepatic steatosis in cholesterol-fed androgen deficient mice. *Life Sci* 2014 Jul 30; **109(2)**: 95-103
 - 18 Cardoso CM, Moreno AJ, Almeida LM, Custodio JB. Comparison of the changes in adenine nucleotides of rat liver mitochondria induced by tamoxifen and 4-hydroxytamoxifen. *Toxicol In Vitro* 2003 Oct-Dec; **17(5-6)**: 663-670
 - 19 Bai X, Lin X, Drayton J, Liu Y, Ji C, Odle J. Clofibrate increases long-chain Fatty Acid oxidation by neonatal pigs. *J Nutr* 2014 Nov; **144(11)**: 1688-1693
 - 20 Yan J, Gong Y, Wang G, Gong Y, Burczynski FJ. Regulation of liver fatty acid binding protein expression by clofibrate in hepatoma cells. *Biochem Cell Biol* 2010 Dec; **88(6)**: 957-967
 - 21 Landrier JF, Thomas C, Grober J, Duez H, Percevault F, Souidi M, Linard C, Staels B, Besnard P. Statin induction of liver fatty acid-binding protein (L-FABP) gene expression is peroxisome proliferator-activated receptor-alpha-dependent. *J Biol Chem* 2004 Oct 29; **279(44)**: 45512-44518
 - 22 Luxon BA, Milliano MT, Weisiger RA. Induction of hepatic cytosolic fatty acid binding protein with clofibrate accelerates both membrane and cytoplasmic transport of palmitate. *Biochim Biophys Acta*. 2000 Sep 27; **1487(2-3)**: 309-318
 - 23 Bacq Y, Assor P, Gendrot C, Perrotin F, Scotto B, Andres C. Recurrent acute fatty liver of pregnancy. *Gastroenterol Clin Biol* 2007 Dec; **31(12)**: 1135-1138
- Peer reviewer:** Mortada El-Shabrawi, Professor, 3 Nablos Street, Off Shehab Street, Mohandesseen, 12411, Cairo, Egypt.