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

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
1:10,000-15,000 and HELLP 1:200-1,000 pregnancies[11]. 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 fetus followed by maternal circulation. Because AFLP mothers are heterozygote for this enzyme deficiency, the mother’s ability to metabolize LCFA 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 may influence the severity of AFLP or HELLP in some but not all LCHAD deficient mothers. 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 LCFA on human Huh-7 hepatocytes. Survival rates were approximately 50% those of controls (no exposure to LCFA) following 24 hours of exposure to LCFA. However, the addition of the L-FABP inducers; clofibrate and simvastatin at concentrations of 6.5-100 µM had no concentration-dependent effect on cell survival following exposure to LCFA (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 µM of clofibrate.

Steroid Hormones and LCHAD expression

In time dependent experiments, B2325 cells (1×10⁴ 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[11].

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 Spierkerkoetter et al[10].

Effect of L-FABP Inducers on Cell Survival

Human Huh-7 hepatocytes (1×10⁴ 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 ± 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.

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.
Previous studies have demonstrated that steroid hormones and/or simvastatin do not attenuate LCFA-induced hepatocyte injury. Liver steroid hormones do not alter the expression of LCHAD, a key functional enzymatic activity was not ascertained on LCHAD mRNA and protein expression were documented but functional enzymatic activity was not ascertained. Thus, the possibility remains that these factors 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. 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

2. Ibdah JA, Yang Z, Bennett MJ. Liver disease in pregnancy and fe-

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


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


**Peer reviewer:** Mortada El-Shabrawi, Professor, 3 Nablos Street, Off Shehab Street, Mohandesseeen, 12411, Cairo, Egypt.