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Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase☆

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ABSTRACT

While nicotine replacement therapy is assumed to be a safer alternative to smoking during pregnancy, the long-term consequences for the offspring remain elusive. Animal studies now suggest that maternal nicotine exposure during perinatal life leads to a wide range of adverse outcomes for the offspring including increased adiposity. The focus of this study was to investigate if nicotine exposure during pregnancy and lactation leads to alterations in hepatic triglyceride synthesis. Female Wistar rats were randomly assigned to receive daily subcutaneous injections of saline (vehicle) or nicotine bitartrate (1 mg/kg/day) for two weeks prior to mating until weaning. At postnatal day 180 (PND 180), nicotine exposed offspring exhibited significantly elevated levels of circulating and hepatic triglycerides in the male offspring. This was concomitant with increased expression of fatty acid synthase (FAS), the critical hepatic enzyme in *de novo* triglyceride synthesis. Given that FAS is regulated by the nuclear receptor Liver X receptor (LXRα), we measured LXRα expression in both control and nicotine-exposed offspring. Nicotine exposure during pregnancy and lactation led to an increase in hepatic LXRα protein expression and enriched binding to the putative LXRE element on the *FAS* promoter in PND 180 male offspring. This was also associated with significantly enhanced acetylation of histone H3 [K9,14] surrounding the *FAS* promoter, a hallmark of chromatin activation. Collectively, these findings suggest that nicotine exposure during pregnancy and lactation leads to an increase in circulating and hepatic triglycerides long-term *via* changes in the transcriptional and epigenetic regulation of the hepatic lipogenic pathway.

Introduction

It is well established that smoking during pregnancy is associated with numerous adverse obstetrical outcomes including an increased risk of spontaneous abortions (Ness et al., 1999), placental complications (Ananth et al., 1999), impaired fetal growth (Andres and Day, 2000; Meyer et al., 1976) and perinatal mortality Andres and Day, 2000; Meyer et al., 1976). Although rates of smoking during pregnancy have declined, approximately 9–20% of mothers world-wide continue to smoke during pregnancy (Al-Sahab et al., 2010; Dhalwani et al., 2013; Paterson et al., 2003; Rogers, 2009; Tong, et al., 2013). This translates into approximately ~75,000 babies born each year in Canada alone who were exposed to first hand smoke *in utero* (Andres and Day, 2000; Paterson et al., 2003). Furthermore, almost half of the women who are able to quit smoking during pregnancy relapse within four months of delivery (Tong et al., 2009). This is of great concern considering that a recent meta-analysis of thirty prospective studies found that babies born to women who smoked regularly during pregnancy have a 47% increase in the odds of becoming overweight (Weng et al., 2012). Moreover, the association between smoking and a predisposition of children being overweight was demonstrated to be largely unaffected by the socioeconomic status of the mother, fetal growth and maternal weight (Oken et al., 2008). This suggests that it is the direct and long-term effect of intrauterine exposure to the chemicals in cigarette smoke and not lifestyle factors associated with smoking that accounts for the increased risk of obesity in the offspring of women who smoke in pregnancy.

Nicotine replacement therapy (NRT) has been widely developed as an effective therapy for smoking cessation (Okuyemi et al., 2000; Oncken and Kranzler, 2003). NRT provides a substitute source of nicotine that significantly reduces the symptoms of nicotine withdrawal and leads to pleasurable experiences such as mood modulation and stimulation (Benowitz, 2010; Benowitz and Jacob, 1990). Yet, often

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due to low adherence there is limited clinical data available on the efficacy and safety of NRT use in pregnancy (Coleman et al., 2012; Pollak et al., 2007; Wisborg, et al., 2000). However, animal studies now demonstrate that nicotine may be the single most important component of cigarette smoke leading to long-term adverse metabolic outcomes (Bruin et al., 2007; Gao et al., 2005; Gao et al., 2008; Holloway et al., 2005; Newman, et al., 1999; Pausova et al., 2003; Williams and Kanagasabai, 1984). Indeed, animals exposed during fetal and/or neonatal life to nicotine have increased adiposity (Gao et al., 2008), abnormal glucose homeostasis (Bruin et al., 2007; Holloway et al., 2005; Somm et al., 2008) and elevated blood pressure (Fox et al., 2012; Gao et al., 2008). Although NRT drastically reduces the number of chemicals that both mother and fetus are exposed to, the long-term risk of nicotine exposure alone still remains elusive (Dempsey and Benowitz, 2001; Osadchy et al., 2009).

Numerous clinical studies have found that adults exposed to smoking *in utero* have increased plasma triglycerides, a characteristic often linked with obesity and an independent risk factor significantly associated with cardiovascular (CV) disease (Bansal et al., 2007; Bosello and Zamboni, 2000; Cupul-Uicab et al., 2012; Nordestgaard et al., 2007; Power et al., 2010; Riediger and Clara, 2011). Given that nearly one-third of Canadian children and youth (5– to 17-year old) are either overweight or obese (Roberts et al., 2012), and the risk that elevated triglycerides pose (Bansal et al., 2007; Nordestgaard et al., 2007), it is clear that strategies are warranted for the prevention or reduction of hypertriglyceridemia in these children. We have previously reported that in rats, fetal and neonatal exposure to nicotine increases circulating triglyceride levels in adult male offspring (Holloway et al., 2005). There are three main sources of free fatty acids that contribute to increased triglycerides; dietary, circulating, and *de novo* synthesis (Jensen-Urstad and Semenkovich, 2012). In brief, *de novo* lipogenesis in the liver begins with the carboxylation of acetyl-coA to malonyl-coA through the actions of acetyl-coA carboxylase (ACCα) (Kim, 1997). Fatty acid synthase (FAS) then converts malonyl-coA to its major product, palmitic acid (Jensen-Urstad and Semenkovich, 2012). Stearoyl-CoA-1 (SCD-1) catalyzes the conversion of this saturated fatty acid into a monounsaturated fatty acid, which subsequently undergoes desaturation and elongation reactions (Miyazaki and Ntambi, 2003). Finally, acyl CoA:diacylglycerol acyltransferase (DGAT) catalyzes the final step of triglyceride synthesis (Cases et al., 1998; Smith et al., 2000). These synthetic enzymes play critical roles in the *de novo* synthesis pathway as animal models of FAS, SCD-1 and ACCα ablation all lead to the disruption of triglyceride homeostasis (Chakravarthy et al., 2005; Chu et al., 2006; Mao et al., 2006).

The liver X receptor (LXR) is a key nuclear receptor involved in the transcriptional regulation of *de novo* triglyceride synthesis (Horton et al., 2003; Schultz et al., 2000). In mice, the oral administration of an LXRα agonist, T0901317 led to elevated plasma triglyceride levels concomitant with increased gene activity of key synthetic enzymes ACCα, FAS and SCD-1 (Schultz et al., 2000). Similarly, the sterol regulatory element-binding protein-1c (SREBP-1c) is able to regulate these fatty acid synthetic genes. However, studies involving LXRα/$\beta^{-/-}$ mice treated with T0901317 did not lead to an increase in triglyceride levels (Horton et al., 2003). These studies suggest that LXRs play a critical role in *de novo* hepatic lipogenesis (Schultz et al., 2000).

Given its role in regulating fatty acid homeostasis (Schultz et al., 2000), cholesterol homeostasis (Repa et al., 2000) and gluconeogenesis (Mitro et al., 2007), LXRα has become an attractive candidate in elucidating the molecular mechanisms underlying metabolic derangements following early life insults. Several studies from our laboratory have implicated that alterations in LXR activity during adverse perinatal development led to symptoms of metabolic syndrome including hypercholesterolemia and impaired glucose tolerance/homeostasis in adulthood (Osumek et al., 2013; Sohi et al., 2011; Vo et al., 2013). However to date, the effects of *in utero* nicotine exposure on LXRα activity and LXR-mediated hepatic lipogenesis in postnatal life are unknown. This study was designed to test the hypothesis that hypertriglyceridemia in offspring exposed to nicotine during fetal and neonatal life (Holloway et al., 2005) involves transcriptional and epigenetic regulation of LXR-target genes involved in hepatic *de novo* triglyceride synthesis.

Materials and methods

Animals and dietary regime. All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous female Wistar rats (200–250 g, Harlan, Indianapolis, IN, USA) were randomly assigned to receive daily subcutaneous injections of saline (vehicle) or nicotine bitartrate (1 mg/kg per day, Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks prior to mating, during pregnancy until weaning (postnatal day 21) as previously described (Bruin et al., 2008a; Holloway et al., 2006). This dose of nicotine has been previously shown to lead to cotinine levels in maternal serum that are similar to "moderate" female smokers (80–163 *n*g/ml) and in nicotine-exposed offspring serum at birth, that are comparable to infants nursed by smoking mothers (Eskenazi and Bergmann, 1995; Holloway et al., 2006). Dams were allowed to deliver normally and at postnatal day 1 (PND 1) all litters were culled to eight. After weaning offspring were caged as sibling pairs and at PND 21 a subset of male offspring were fasted overnight and sacrificed by CO_2 inhalation for liver tissue collection. A second subset of animals was allowed to develop naturally. At PND 180 male rat offspring were fasted overnight and sacrificed by CO_2 inhalation for body weight measurements and blood and liver tissue collection. All animals were weighed at necropsy. Liver samples were snapped frozen in liquid nitrogen and stored at −80 °C until further molecular analysis. Blood was collected, allowed to clot, spun and serum was stored at −80 °C for analysis.

Plasma and hepatic lipid measurements. Total cholesterol, triglyceride and glucose measurements from blood and hepatic tissue samples were automatically detected using Cobas® Mira S analyzer at the Metabolic Phenotype Laboratory at Robarts Research Institute (London, Ontario, Canada). For triglyceride measurements, triglycerides were hydrolyzed by lipoprotein lipase to glycerol and fatty acids. Glycerol was then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate was catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 alters the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red-colored quinoneimine dye, which was measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample. For cholesterol measurements, cholesterol esterase cleaved cholesterol esters, which then were converted to choleste-4-en-3-one and H_2O_2 by cholesterol oxidase. Cholesterol levels were quantified using a colorimetric assay that measured the breakdown of H_2O_2 *via* the Trinder reaction as previously described (Sohi et al., 2011). Glucose measurements were determined using a glucose assay kit from Roche Diagnostics (Roche, Misissauga, Ontario, Canada) that was run on the analyzer.

Quantitative real-time PCR analysis. Total RNA from male liver tissue at all ages (PND 1, PND 21, and PND 180) was extracted by the one-step method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). RNA was treated with deoxyribonuclease to remove any contaminating DNA. 4 μg of the total RNA was reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primer sets directed for the genes of interest (FAS, ACCα, SCD-1, and DGAT) were generated using OligoPerfect™Designer (Invitrogen, Carlsbad, CA, USA) (Table 1). The Bio-Rad CFX384 Real Time System was employed to determine quantitative mRNA expression using the DNA binding dye SsoFast™ EvaGreen® Supermix (Bio-Rad, Mississauga, Ontario, Canada). The