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Omega-3 Fatty Acid Deficiency Augments Risperidone-Induced Hepatic Steatosis in Rats: Positive Association with Stearoyl-CoA Desaturase

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Abstract

Psychiatric patients frequently exhibit long-chain *n*-3 (LC*n*-3) fatty acid deficits and elevated triglyceride (TAG) production following chronic exposure to second generation antipsychotics (SGA). Emerging evidence suggests that SGAs and LC*n*-3 fatty acids have opposing effects on stearoyl-CoA desaturase-1 (SCD1), which plays a pivotal role in TAG biosynthesis. Here we evaluated whether low LC*n*-3 fatty acid status would augment elevations in rat liver and plasma TAG concentrations following chronic treatment with the SGA risperidone (RSP), and evaluated relationships with hepatic SCD1 expression and activity indices. In rats maintained on the *n*-3 fatty acid-fortified (control) diet, chronic RSP treatment significantly increased liver SCD1 mRNA and activity indices (18:1/18:0 and 16:1/16:0 ratios), and significantly increased liver, but not plasma, TAG concentrations. Rats maintained on the *n*-3 deficient diet exhibited significantly lower liver and erythrocyte LC*n*-3 fatty acid levels, and associated elevations in LC*n*-6/LC*n*-3 ratio. In *n*-3 deficient rats, RSP-induced elevations in liver SCD1 mRNA and activity indices (18:1/18:0 and 16:1/16:0 ratios) and liver and plasma TAG concentrations were significantly greater than those observed in RSP-treated controls. Plasma glucose levels were not altered by diet or RSP, and body weight was lower in RSP- and VEH-treated *n*-3 deficient rats. These preclinical data support the hypothesis that low *n*-3 fatty acid status exacerbates RSP-induced hepatic steatosis by augmenting SCD1 expression and activity.

Keywords

Omega-3 fatty acids; Risperidone; Atypical antipsychotic; Stearoyl-CoA desaturase-1; Oleic acid; Polyunsaturated fatty acids; Triglycerides; Glucose; Liver; Rat

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1. Introduction

Treatment-emergent elevations in serum triglyceride (TAG) levels and weight gain are frequently observed in psychiatric patients following chronic exposure to second generation antipsychotic (SGA) medications, including clozapine, quetiapine, olanzapine, and risperidone (RSP)[1-3]. SGA-induced elevations in TAG levels has significant health consequences because it increases risk for metabolic syndrome, cardiovascular disease, obesity, and non-alcoholic fatty liver disease (NAFLD), a syndrome characterized by excessive hepatic TAG accumulation (i.e., hepatic steatosis) and increased vulnerability to hepatotoxicity [4]. This is particularly problematic in view of epidemiological data indicating that there has been a 6-fold increase in SGA prescription rates in pediatric and adolescent patients during the past decade [5,6], and prospective studies indicate that youth are highly vulnerable to SGA-induced TAG increases and weight gain [7-10]. Moreover, in clinical practice most children starting SGA medications do not receive recommended regular glucose or lipid screening/monitoring [11]. These data highlight the urgent need to identify modifiable risk and protective factors associated with adverse cardiometabolic effects of SGA medications.

One mechanism that may increase risk for SGA-induced hyperlipidemia is a deficiency in long-chain omega-3 (LCn-3) fatty acids, including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)[12]. Specifically, increasing LCn-3 fatty acid status through dietary supplementation is efficacious for reducing elevated TAG levels [13], and LCn-3 fatty acids are approved by the United States FDA for the treatment of hypertriglyceridemia. Preclinical feeding studies have found that low LCn-3 fatty acid status is associated with greater liver TAG content and secretion in different rodent models [14-19]. Clinical studies have found that red blood cell (RBC) and liver LCn-3 fatty acid levels are positively correlated [20], and patients with obesity and NAFLD exhibit significantly lower liver and RBC LCn-3 fatty acid levels [20-22]. Importantly, schizophrenic [23-25] and bipolar disorder [26-28] patients treated with SGA medications also exhibit significantly lower RBC LCn-3 fatty acid levels compared with healthy controls. Lastly, increasing LCn-3 fatty acid status through dietary supplementation decreased elevated serum TAG levels in SGA-treated patients [29,30]. Together, these data suggest that low LCn-3 fatty acid status may represent a modifiable risk factor for SGA-induced elevations in TAG production and weight gain.

Translational evidence further suggests that stearoyl-CoA desaturase-1 (*SCD1*, Δ9-desaturase) plays a pivotal role in regulating lipid and metabolic homeostasis [31]. Preclinical studies have demonstrated that mutation of the *SCD1* gene impairs constitutive and diet-induced TAG production [32-34], increases insulin sensitivity [35], and reduces visceral adiposity and increases resistance to diet-induced obesity [36]. Selective pharmacological inhibition of the *SCD1* enzyme reduces elevated TAG and glucose levels in rodent metabolic disease models [37,38]. Feeding high-carbohydrate diets is associated with the up-regulation of hepatic *SCD1* mRNA expression and elevated fasting and postprandial TAG levels in rodent models [39]. In human subjects, elevations in the plasma product-precursor ratio of oleic acid to stearic acid (18:1/18:0), an index of *SCD1* activity (the 'desaturation index'), is positively correlated with human liver biopsy *SCD1* mRNA expression [40], plasma TAG levels [41], and obesity [39]. Together, this body of evidence suggests that *SCD1* plays a pivotal role in regulating lipid and metabolic homeostasis.

Converging evidence suggests that LCn-3 fatty acids and SGA medications both converge on *SCD1* expression and activity. Specifically, *in vitro* studies have found that different SGA medications up-regulate *SCD1* mRNA expression in human cell lines and rat hepatocytes [42-45], and that chronic SGA treatment increases adipose *SCD1* mRNA

expression and *SCD1* activity indices in rats [46,47]. Schizophrenic patients treated with the SGA olanzapine exhibit greater *SCD1* mRNA expression in peripheral blood cells compared with drug-free patients [48]. In contrast to SGA medications, LC n -3 fatty acids down-regulate *SCD1* mRNA expression at the level of transcription and mRNA stability [49], and dietary LC n -3 fatty acid supplementation decreases *SCD1* activity in rat liver microsomes *ex vivo* [50] and *SCD1* activity indices in human subjects [51,52]. We recently reported that dietary-induced n -3 fatty acid deficiency robustly up-regulated *SCD1* mRNA expression and activity in rat liver, and that this effect was positively correlated with postprandial plasma TAG levels and prevented by prior normalization of n -3 fatty acid status [15]. Together, these data suggest that SGA medications and LC n -3 fatty acid have opposing effects on *SCD1* mRNA expression and activity.

These translational data suggest that the low LC n -3 fatty acid status may increase vulnerability to elevated TAG production following chronic exposure to SGA medications by augmenting hepatic *SCD1* expression and activity. Evaluation of these relationships in clinical populations is complicated by difficult-to-control variables that influence lipid and metabolic homeostasis, including medications and dietary carbohydrate and fatty acid intake, and definitive evaluation requires the use of an animal model so that these variables can be systematically manipulated. The primary objectives of the present study were to evaluate the hypothesis that low LC n -3 fatty acid status is associated with greater TAG elevations in response to chronic RSP treatment, and to evaluate relationships with hepatic *SCD1* expression and activity. We additionally investigated relationships with body weight gain and plasma glucose levels. Based on the evidence reviewed above, our specific prediction was that low LC n -3 fatty acid status would be associated with greater RSP-induced elevations in liver and plasma TAG concentrations, and associated increases in liver *SCD1* expression and activity.

2. Materials and methods

2.1. Diets

The compositions of the α -linolenic acid (ALA, 18:3 n -3)-fortified (control, ALA+, TD. 04285, Harlan-TEKLAD, Madison, WI) and ALA-(TD.04286) diets are presented in Table 1. Diets were vacuum packaged and stored at 4°C. Both diets provided 3.8 Kcal/g (15.9 kJ/g), and were matched for percent Kcal from protein (19.2%), carbohydrate (64.4%), and fat (16.5%). The control diet was fortified with flaxseed oil (0.6%), which contains the short-chain n -3 fatty acid precursor ALA (18:3 n -3). Analysis of diet fatty acid composition by gas chromatography confirmed that both diets were closely matched in saturated fatty acids, monounsaturated fatty acids, and the short-chain n -6 fatty acid precursor linoleic acid (18:2 n -6)(Table 1).

2.2. Animals

Male offspring were bred in-house to nulliparous female Long-Evans hooded rats. To generate rats with low LC n -3 fatty acid status, dams were fed the ALA-diet for 1 month prior to mating through weaning, and male offspring were maintained on the ALA-diet post-weaning (P21) to adulthood (P100)(n =20). Controls (i.e., normal LC n -3 fatty acid status) were born to dams maintained on the ALA+ diet, and received the ALA+ diet post-weaning (P21) to adulthood (P100)(n =20). Rats were housed 2 per cage with food and water available *ad libitum*, and maintained under standard *vivarium* conditions on a 12:12 h light:dark cycle. Food (g/kg/d) and water (ml/kg/d) intake, and body weight (kg) were recorded over the course of the experiment. Rats were euthanized by decapitation on P100-101 between 9:00-12:00 am in a counter-balanced manner relative to the common removal of food hoppers at 9:00 am. Trunk blood was collected into EDTA-coated tubes and

plasma isolated by centrifugation at 4°C, and erythrocytes (red blood cells, RBC) washed 3x with 4°C 0.9% NaCl. Liver samples were uniformly harvested and flash frozen in liquid nitrogen. All biological samples were stored at -80°C. All experimental procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee, and adhere to the guidelines set by the NIH.

2.3. Drug administration

On P60, one half of control (n=10) and *n*-3 deficient (n=10) rats were randomly assigned to receive chronic treatment with drug vehicle (n=10, 0.1 M acetic acid diluted in deionized water) or risperidone (n=10, 3.0 mg/kg/day; Ortho-McNeil Janssen Pharmaceuticals) through their drinking water, as previously described [47,53]. The 3.0 mg/kg/day dose was selected based on our prior studies finding that it produces therapeutically-relevant risperidone concentrations in rat [53]. We have previously found that low LC*n*-3 fatty acid status does not alter plasma RSP levels or pharmacokinetics (i.e., 9-OH-RSP/RSP ratio)[53]. For three days prior to drug delivery, 24 h water consumption was determined for each cage using bottle weights (1 g water = 1 ml water), and ml water intake/mean body weight (kg) calculated. Risperidone was dissolved in 0.1 M acetic acid in deionized water to prepare a stock solution (stored at 4°C) which was added to tap water in a volume required to deliver the targeted daily dose. To maintain intake of the targeted daily dose, drug concentrations were adjusted to mean daily fluid intake and mean body weight (ml/kg/day) every 3 days. Dehydration was routinely monitored over the course of the study using the skin tenting method. Red opaque drinking bottles were used to protect drug from light degradation. Rats were maintained on drug until being euthanized on P100 (i.e., 40 days of treatment).

2.4. Liver and plasma TAG concentrations

Liver tissue was homogenized, lipids extracted according to Folch et al. [54], and TAG concentrations (mg/g) assayed with a manual colorimetric assay [55]. Plasma TAG concentrations were determined using commercially available kits according to the manufacturer's instructions (GPO-PAP, RANDOX Laboratories Ltd., Antrim UK). All analyses were performed by a technician blinded to treatment.

2.5. Plasma glucose concentrations

Plasma glucose concentrations were determined using commercially available kits according to the manufacturer's instructions (GOD-POD, Genzyme Diagnostics P.E.I. Inc., Charlottetown, PE, Canada). All analyses were performed by a technician blinded to treatment.

2.6. Fatty acid composition

The gas chromatography procedure used to determine RBC and liver total fatty acid composition has been described in detail previously [15, 47,53]. Briefly, total fatty acid composition was determined with a Shimadzu GC-2010 (Shimadzu Scientific Instruments Inc., Columbia MD). The column is a DB-23 (123-2332): 30m (length), I.D. (mm) 0.32 wide bore, film thickness of 0.25 μ M (J&W Scientific, Folsom CA). The carrier gas was helium with a column flow rate of 2.5 ml/min. Analysis of fatty acid methyl esters was based on area under the curve calculated with Shimadzu Class VP 7.4 software. Fatty acid identification was based on retention times of authenticated fatty acid methyl ester standards (Matreya LLC Inc., Pleasant Gap PA). Analyses were performed by a technician blinded to treatment. Data are expressed as weight percent of total fatty acids (mg fatty acid/100 mg fatty acids). We also determined the composition of the principal substrates (16:0, 18:0) and products (16:1*n*-7, 18:1*n*-9) of *SCD1*, and calculated the liver 16:1/16:0 and 18:1/18:0 ratios as indices of liver *SCD1* activity ('destauration index')[41]. We additionally calculated the

liver 18:3*n*-6/18:2*n*-6 (delta6-desaturase), 20:4*n*-6/20:3*n*-6 (delta5-desaturase), and 16:0/18:2 ('*de novo* lipogenesis')[56-58] ratios.

2.7. Liver mRNA expression

Frozen liver was homogenized (BioLogics Model 300 V/T ultrasonic homogenizer, Manassas, VA) in Tri Reagent (MRC Inc., Cincinnati, OH), and total RNA isolated and purified using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was treated to remove potential DNA contamination using RNase-free DNase (Qiagen, Valencia, CA), and RNA quantified using a Nanodrop instrument (Nanodrop Instruments, Wilmington, DE). RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA was prepared from 1 µg total RNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) along with no RT controls to confirm lack of genomic DNA contamination. Liver mRNA levels of stearoyl-CoA desaturase (*SCD1*, Rn00594894_g1) were measured by real-time quantitative PCR using an ABI 7900HT500 Real Time PCR System (Applied Biosystems, Foster City, CA). The nucleotide sequences of primer/probe sets can be obtained from www.appliedbiosystems.com. Samples were run as quadruplicates in Microamp Fast 96 well reaction plates using 20 µl reaction volumes consisting of 10 µl of 2X TaqMan Fast Universal PCR Mastermix (Applied Biosystems, Foster City, CA), 1 µl of TaqMan gene expression assay, 4 µl of cDNA and 5 µl of DEPC-treated H₂O. No template controls (NTC) substituting DEPC-treated water for cDNA were also run with each plate to verify lack of cross contamination. Thermal cycling conditions were: 95°C for 10 min followed by 95°C for 1 sec denaturing step and 60°C for 20 sec, annealing step for 40 cycles. Pilot studies found that the conventional housekeeping genes GAPDH, β-actin, and 18S rRNA were all significantly and selectively lower in the livers of RSP-treated *n*-3 deficient rats, whereas ribosomal protein L32 (Rn00820748_g1)[59,60] was stable across groups and was used as the reference gene.

Data were analyzed by both the standard curve and delta delta CT methods. For the standard curve method, serial dilutions of pooled sample reference cDNA were used to generate 5 point standard curves run in duplicate on each plate for both L32 and SCD1. Relative quantity values (RQ values) were generated by the Taqman 7900 HT software (7900 HT Sequence Detection System, version 2.3, Applied Biosystems, Foster City, Ca.) based on these curves. The relative expression of SCD1 for each sample was then calculated based on its RQ data normalized to the relative expression of the corresponding reference gene values. This analysis was confirmed by comparing cycle thresholds for both the target and housekeeping genes using the Relative Expression Software Tool (REST Version 2.0.13, Qiagen, Valencia, CA)[61], which employs the delta delta CT method [62] and also calculates efficiency of the reactions based on standard curves.

2.8. Statistical analysis

Group effects in fatty acid composition, product/precursor ratios, and liver gene expression data were evaluated with a two-way ANOVA, with diet (control, deficient) and treatment (vehicle, risperidone) as the main factors. Individual group differences were determined using *t*-tests (2-tail, α=0.05). The distribution of all dependent variables was examined for normality with Bartlett's test. Liver *Scd1* mRNA and TAG levels, and plasma TAG levels, were log transformed. Parametric (Pearson) correlation analyses were performed to determine relationships between primary outcome measures (2-tail, α=0.05). All analyses were performed with GB-STAT (V.10, Dynamic Microsystems, Inc., Silver Springs MD).

3. Results

3.1. Food/water intake and body weight

For food intake (g/kg/d), the main effect of diet ($p=0.35$) and treatment ($p=0.19$), and the diet x treatment interaction ($p=0.50$), were not significant (Fig. 1A). For fluid intake (ml/kg/d), the main effect of diet ($p=0.91$) and treatment ($p=0.15$), and the diet x treatment interaction ($p=0.35$), were not significant (Fig. 1B). For baseline (P60) body weight (CON+VEH: 349.9 ± 8.6 ; CON+RSP: 349 ± 12 ; DEF+VEH: 345 ± 10 ; DEF+RSP: 345 ± 9.2 kg), the main effect of diet ($p=0.88$) and treatment ($p=0.68$), and the diet x treatment interaction ($p=0.85$), were not significant. For endpoint (P100) body weight, there was a significant main effect of diet ($p=0.004$), and the main effect of treatment ($p=0.58$) and the diet x treatment interaction ($p=0.94$) were not significant. Compared with CON+VEH, DEF+VEH ($p=0.01$) and DEF+RSP ($p=0.01$), but not CON+RSP ($p=0.97$), rats exhibited significantly lower endpoint body weight (Fig. 1C).

3.2. Liver and plasma TAG concentrations

For liver TAG concentrations (mg/g), the main effects of diet ($p=0.0001$) and treatment ($p=0.0009$) were significant, and the diet x treatment interaction was not significant ($p=0.16$). Compared with CON+VEH, CON+RSP ($p=0.005$), DEF+VEH ($p=0.05$), and DEF+RSP ($p=0.0002$) groups exhibited significantly greater liver TAG concentrations, and DEF+RSP exhibited significantly greater liver TAG concentrations compared with CON+RSP ($p=0.006$) and DEF+VEH ($p=0.001$) (Fig. 2A). For plasma TAG concentrations (mg/dL), the main effect of diet was significant ($p=0.003$), and the main effect of treatment ($p=0.53$) and the diet x treatment interaction ($p=0.49$) were not significant. Compared with CON+VEH, DEF+VEH ($p=0.04$) and DEF+RSP ($p=0.01$), but not CON+RSP ($p=0.93$), exhibited significantly greater plasma TAG concentrations, and DEF+RSP ($p=0.01$), but not DEF+VEH ($p=0.39$), exhibited significantly greater plasma TAG concentrations compared with CON+RSP (Fig. 2B).

3.3. Plasma glucose concentrations

For plasma glucose concentrations (mg/dL), the main effects diet ($p=0.15$) and treatment ($p=0.44$), and the diet x treatment interaction ($p=0.94$), were not significant (Fig. 2C).

3.4. Liver mRNA expression

For *L32* mRNA expression, the main effects of diet ($p=0.69$) and treatment ($p=0.19$), and the diet x treatment interaction ($p=0.81$), were not significant. For log transformed *SCD1/L32* mRNA expression, the main effects of diet ($p=0.0001$) and treatment ($p=0.001$), and the diet x treatment interaction ($p=0.04$), were significant (Fig. 3A). Compared with CON+VEH, CON+RSP ($p=0.003$), DEF+VEH ($p=0.0001$) and DEF+RSP ($p=0.0001$) exhibited significantly greater liver *SCD1/L32* mRNA expression. Liver *SCD1/L32* mRNA expression in the DEF+RSP group was greater than the CON+RSP group ($p=0.01$).

3.5. Liver and RBC fatty acid composition

Group differences in liver total fatty acid composition are presented in Table 2. Mead acid (20:3 $n-9$) was detected in the livers, but not RBCs, of CON+RSP ($0.25\pm 0.02\%$) and DEF+RSP ($0.24\pm 0.02\%$, $p=0.84$) groups. For the liver 18:1/18:0 ratio (an index of delta9-desaturase activity), the main effects of diet ($p=0.0001$) and treatment ($p=0.0001$) were significant, and the diet x treatment interaction was not significant ($p=0.11$) (Fig. 3B). For the 16:1/16:0 ratio (an index of delta9-desaturase activity), the main effects of diet ($p=0.0001$) and treatment ($p=0.0001$), and the diet x treatment interaction ($p=0.0002$), were significant (Fig. 3C). For the 18:3/18:2 ratio (an index of delta6-desaturase activity), the

main effects of diet ($p = 0.0001$) and treatment ($p = 0.0002$) were significant, and the diet x treatment interaction was not significant ($p = 0.18$) (Fig. 3D). For the 20:4/20:3 ratio (an index of delta5-desaturase activity), the main effect of treatment ($p = 0.002$) was significant, and the main effect of diet ($p = 0.07$) and the diet x treatment interaction ($p = 0.25$), were not significant (Fig. 3E). For the 16:0/18:2 ratio (an index of DNL), the main effects of diet ($p = 0.0001$) and treatment ($p = 0.0001$) were significant, and the diet x treatment interaction was not significant ($p = 0.07$) (Fig. 3F). Group differences in RBC total fatty acid composition are presented in Supplemental Table 1, and correlations between relevant RBC and liver fatty acids and fatty acid ratios are presented in Supplemental Table 2.

3.6. Correlations among primary outcome measures

Among all rats ($n = 40$), liver *SCD1/L32* mRNA expression was positively correlated with liver 18:1/18:0 ($r = +0.63$, $p = 0.0001$) and 16:1/16:0 ($r = +0.48$, $p = 0.002$) ratios. LC *n*-3 fatty acid composition was inversely correlated with *Scd1/L32* mRNA expression ($r = -0.65$, $p = 0.0001$), the liver 18:1/18:0 ($r = -0.71$, $p = 0.0001$) and 16:1/16:0 ($r = -0.86$, $p = 0.0001$) ratios, as well as liver ($r = -0.44$, $p = 0.01$) and plasma ($r = -0.48$, $p = 0.001$) TAG concentrations. Plasma TAG concentrations were positively correlated with liver *SCD1/L32* mRNA expression ($r = +0.52$, $p = 0.0007$) (Fig. 4A), the liver 18:1/18:0 ratio ($r = +0.61$, $p = 0.0001$) (Fig. 4B), and the liver 16:1/16:0 ratio ($r = +0.49$, $p = 0.001$) (Fig. 4C). Liver TAG concentrations were positively correlated with liver *SCD1/L32* mRNA expression ($r = +0.54$, $p = 0.002$) (Fig. 4D), the liver 18:1/18:0 ratio ($r = +0.81$, $p = 0.0001$) (Fig. 4E), and the liver 16:1/16:0 ratio ($r = +0.42$, $p = 0.01$) (Fig. 4F). Liver TAG concentrations were also positively correlated with plasma TAG concentrations ($r = +0.35$, $p = 0.05$) and the liver 18:3/18:2 ratio ($r = +0.61$, $p = 0.0003$), but not the liver 20:4/20:3 ratio ($r = -0.23$, $p = 0.19$). Plasma TAG concentrations were positively correlated with the liver ($r = +0.62$, $p = 0.0001$) and RBC ($r = +0.59$, $p = 0.0001$) 16:0/18:2 ratio, and liver TAG concentrations were positively correlated with the liver ($r = +0.76$, $p = 0.0001$) and RBC ($r = +0.37$, $p = 0.03$) 16:0/18:2 ratio. Liver and plasma TAG concentrations, liver *SCD1/L32* mRNA expression, and the liver 18:1/18:0 and 16:1/16:0 ratios were not significantly correlated with plasma glucose concentrations or body weight.

4. Discussion

This preclinical study tested the hypothesis that low LC *n*-3 fatty acid status would augment treatment-emergent elevations in TAG levels and body weight in male rats following chronic RSP treatment, and investigated relationships with *SCD1* expression and activity indices (16:1/16:0, 18:1/18:0). In support of our hypothesis, RSP-treated *n*-3 deficient rats exhibited significantly greater plasma and liver TAG levels compared with RSP-treated controls, as well as greater liver *SCD1* mRNA expression and activity indices. Contrary to the hypothesis, RSP-treated *n*-3 deficient rats exhibited lower body weight compared with RSP-treated controls. In agreement with prior studies [15,18], VEH-treated *n*-3 deficient rats exhibited greater plasma and liver TAG levels, and greater *SCD1* mRNA expression and activity compared with VEH-treated controls. Together, these preclinical data provide proof-of-concept evidence that low *n*-3 fatty acid status is a risk factor for greater RSP-induced hepatic steatosis, but not body weight gain or elevated glucose levels, and suggest a strong association with *SCD1* expression and activity.

This study has three notable limitations. First, only male rats were employed, precluding evaluation of potential gender differences. However, male rats were selected to obviate potential interactions with rhythmical variations with ovarian hormones which were previously found to influence fatty acid biosynthesis [63]. Second, only one dose of RSP was investigated precluding evaluation of dose-response effects. However, the 3.0 mg/kg/day dose was specifically selected based on our prior studies finding that it produces

therapeutically-relevant RSP concentrations in rat plasma [53], and produces only moderate increases in plasma TAG levels [47], thereby permitting evaluation of hypothesized augmenting effects of low LC n -3 fatty acid status. Third, only one type and class of antipsychotic was investigated, and other antipsychotic medications may be associated with different results. It is notably, however, that our prior study found that similar to RSP other SGA medications, including olanzapine and quetiapine, increase the plasma 18:1/18:0 ratio in control rats [47].

Consistent with prior studies finding that male rats do not exhibit treatment-emergent elevations in body weight following chronic exposure to atypical antipsychotic medications including RSP [47,64-66], the present study found that chronic RSP treatment did not significantly increase body weight gain in either control or n -3 deficient rats. Indeed, we found that chronic RSP treatment was associated with lower body weight in n -3 deficient rats. While the present study did not evaluate visceral adiposity, it may be relevant that a previous study found that chronic olanzapine treatment increased visceral adiposity in male rats despite reduced body weight, an effect that may be attributable to reductions in lean muscle mass [67]. However, in the present study RSP-treated rats maintained on the control diet did not exhibit body weight reductions, and VEH-treated rats maintained on the n -3 deficient diet exhibited a reduction in body weight, suggesting the effect can not be solely attributed to RSP-induced reductions in cage activity (i.e., sedation). Our results further suggest that elevated TAG levels are not associated with greater body weight gain. This finding is congruent with a recent study finding that semi-chronic treatment with olanzapine increased serum TAG levels but did not significantly increase body weight in pair-fed female rats [46], as well as clinical observations of elevated TAG levels but not body weight in SGA-treated patients [2,68,69].

Consistent with our prior dose-ranging study [47], the present study found that chronic treatment with RSP (3 mg/kg/d) did not significantly alter postprandial plasma TAG or glucose levels in rats maintained on the n -3 fatty acid-fortified diet. However, in the present study we found that chronic RSP treatment did increase liver TAG levels. Also consistent with our prior report [47], we found that chronic RSP treatment increased liver and RBC eicosapentaenoic acid (EPA, 20:5 n -3) composition in rats maintained on the n -3 fatty acid-fortified diet (ALA+). Because this effect was not observed in rats maintained on the n -3 deficient diet (ALA-), this observation indicates that RSP augments ALA-EPA biosynthesis [53]. It is relevant, therefore, that prior studies have found that selectively increasing EPA intake is associated with reductions in liver TAG content and SCD1 mRNA and activity indices in mice fed a high-carbohydrate diet [70], and we previously found that EPA status was inversely correlated with plasma TAG levels in SGA-treated rats [47]. Moreover, EPA supplementation dose-dependently decreased elevated plasma TAG levels in clozapine-treated patients [30]. Therefore, elevations in ALA-EPA biosynthesis in RSP-treated rats maintained on the n -3 fatty acid-fortified diet may have counteracted increases in plasma TAG concentrations, despite greater liver TAG concentrations. These findings further suggest that augmentation of ALA-EPA biosynthesis may have contributed to the blunting of RSP-induced elevations in liver TAG concentrations in RSP-treated controls compared with RSP-treated n -3 deficient rats.

Feeding high-carbohydrate diets leads to significant elevations in the ‘*de novo* lipogenesis’ pathway, which converts excess carbohydrates to lipids for storage as fatty acids and TAG [57], and is associated with elevated hepatic *SCD1* mRNA expression and TAG levels in rodents [33,34,39]. Clinical studies have found that feeding a high-carbohydrate diet is associated with a significant reduction in plasma TAG 18:2 n -6 levels, and associated elevations in plasma TAG 16:0 levels and the 16:0/18:2 ratio [56-58]. In the present study, we similarly found that liver 18:2 n -6 levels were significantly reduced, and liver 16:0 levels

and the 16:0/18:2 ratio significantly increased, in RSP-treated rats despite equivalent carbohydrate intake. Moreover, we found that the liver 16:0/18:2 ratio was positively correlated with liver and plasma TAG levels as well as SCD1 expression and activity. Additionally, the present study found that 18:2 n -6 levels were significantly reduced, and the 18:3/18:2 ratio significantly increased, in the liver of RSP-treated rats despite similar 18:2 n -6 intake, a finding that is consistent with elevated delta-6 desaturase activity [71,72]. It is relevant, therefore, that prior *in vitro* and *in vivo* studies have found that 18:2 n -6 is a potent inhibitor of SCD1 transcription [49], suggesting that RSP- and diet-induced elevations in delta-6 desaturase-mediated 18:2 n -6 consumption may contribute to the augmentation of SCD1 expression and activity. These data may also be relevant to understanding prior clinical evidence that delta-6 desaturase activity indices are positively associated with TAG levels [73].

The present observation that chronic dietary LC n -3 fatty acid deficiency is sufficient to cause excessive hepatic TAG accumulation (i.e., hepatic steatosis) is consistent with prior rodent studies [17,18], as well as the clinical observation that liver biopsies from patients with hepatic steatosis exhibit significantly lower LC n -3 fatty acid composition and a greater LC n -6/LC n -3 fatty acid ratio compared with healthy controls [20,22]. A novel finding of the present study is that prior dietary-induced reductions in liver LC n -3 fatty acid composition significantly augmented the development of hepatic steatosis in response to chronic RSP exposure. The reduction in liver arachidonic acid composition in RSP-treated rats irrespective of diet is also consistent with the finding that liver arachidonic acid composition is lower in patients with hepatic steatosis [22], and may reflect shunting away from fatty acid biosynthesis to cyclooxygenase-2-mediated prostaglandin synthesis. This is supported in part by the finding that the fatty acid elongate product of arachidonic acid, docosatetraenoic acid (22:4 n -6), was also significantly lower in RSP-treated rats irrespective of diet. It is also relevant that hepatic steatosis increases vulnerability to hepatotoxicity [4]. Although a prior histological study did not observe reductions in hepatocyte number following 42-day RSP treatment in rats maintained on n -3 fatty acid-fortified diet [74], additional histological studies are warranted to investigate whether low LC n -3 fatty acid status additionally increases vulnerability to RSP-induced hepatotoxicity.

In summary, the present study provides preclinical evidence that low n -3 fatty acid status augments RSP-induced hepatic steatosis independent of alterations in body weight or plasma glucose levels, as well as RSP-induced increases in liver SCD1 mRNA expression and activity. This preclinical finding, and the clinical observations that psychiatric patients treated with SGA medications exhibit low RBC LC n -3 fatty acid status [23-28] and increasing LC n -3 fatty acid status reduces TAG levels in patients treated with clozapine [29,30], suggest that low LC n -3 fatty acid status may represent a modifiable risk factor for SGA-induced hepatic steatosis. Indeed, together these data suggest that increasing LC n -3 fatty acid status may represent a viable adjunctive intervention to protect against the development of SGA-induced hepatic steatosis in psychiatric patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effects of chronic treatment with VEH or RSP on (A) food intake (g/kg/d), (B) fluid intake (ml/kg/d), and (C) endpoint (P100) body weight (kg) in rats maintained on the control (CON, ALA+) or *n*-3 fatty acid deficient (DEF, ALA-) diet. Values are group mean $\pm$ S.E.M. ## $p < 0.001$ vs. same-treatment (VEH, RSP) in the control diet group.

Figure 2. Effects of chronic treatment with VEH or RSP on (A) liver TAG concentrations (mg/g), (B) postprandial plasma TAG concentrations (mg/dL), and (C) postprandial plasma glucose concentrations (mg/dL) in rats maintained on the control (CON, ALA+) or *n*-3 fatty acid deficient (DEF, ALA-) diet. Values are group mean $\pm$ S.E.M. # *p* 0.05, ## *p* 0.01 vs. same-treatment (VEH, RSP) in the control diet group, ** *p* 0.01, *** *p* 0.0001 vs. VEH-treated controls within each diet group. Note that statistical analyses were performed on log transformed liver and plasma TAG data.

Figure 3.

Effects of chronic treatment with VEH or RSP on liver (A) *SCD1/L32* mRNA expression, (B) the 18:1/18:0 ratio (C) the 16:1/16:0 ratio, (D) the 18:3/18:2 ratio, (E) the 20:4/20:3 ratio, and (F) the 16:0/18:2 ratio in rats maintained on the control diet (CON, ALA+) or *n*-3 fatty acid deficient (DEF, ALA-) diet. Values are group mean ± S.E.M. **p* 0.05, ***p* 0.01, ****p* 0.0001 vs. VEH-treated controls within each diet group, #*p* 0.05, ##*p* 0.01, ###*p* 0.0001 vs. same-treatment (VEH, RSP) in the control diet group. Note that statistical analyses were performed on log transformed *SCD1/L32* mRNA data.

Figure 4. Relationships between plasma (A-C) and liver (D-F) TAG concentrations and liver *Scd1*/*L32* mRNA expression (A,D), the liver 18:1/18:0 ratio (an index of *SCD1* activity)(B,E), and the liver 16:1/16:0 ratio (an index of *SCD1* activity). Pearson correlation coefficients and associated p-values (two-tailed) are presented.

Table 1**Diet Compositions**

Ingredient¹	ALA+	ALA-
Casein, vitamin free	20	20
Carbohydrate		
Cornstarch	20	20
Sucrose	27	27
Dextrose	9.9	9.9
Maltose-dextrin	6	6
Cellulose	5	5
Mineral mix	3.5	3.5
Vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat		
Hydrogenated coconut oil	4.5	5.1
Safflower	1.9	1.9
Flaxseed	0.6	0
Fatty acid composition ²		
C8:0	4.3	5.0
C10:0	3.8	4.2
C12:0	29	32.8
C14:0	11	12.5
C16:0	8.3	8.7
C18:0	9.4	10.2
18:1n-9	6.7	4.7
18:2n-6	22.7	21.9
20:4n-6	nd	nd
18:3n-3	4.6	nd
22:6n-3	nd	nd

¹
g/100 g diet

²
wt % of total fatty acids

nd = not detected

Table 2

Liver fatty acid composition

Fatty Acid ¹	CON+VEH	CON+RSP	DEF+VEH	DEF+RSP
Myristic acid (14:0)	0.5 ± 0.03	1.1 ± 0.09 ***	1.6 ± 0.07 ###	2.4 ± 0.11 *** ###
Palmitic acid (16:0)	18.3 ± 0.24	22.6 ± 0.79 ***	22.7 ± 0.84 ###	28.2 ± 0.79 *** ###
Stearic acid (18:0)	18.1 ± 0.37	17.3 ± 0.51 ***	15.5 ± 0.52 ###	12.7 ± 0.58 *** ###
Total SFA	36.9 ± 0.20	41.1 ± 0.48 ***	39.8 ± 0.62 ###	43.4 ± 0.64 *** #
Palmitoleic acid (16:1 <i>n</i> -7)	1.0 ± 0.10	2.9 ± 0.31 ***	4.7 ± 0.41 ###	5.8 ± 0.23 * ###
Oleic acid (18:1 <i>n</i> -9)	7.1 ± 0.25	15.2 ± 0.97 ***	15.0 ± 1.18 ###	21.9 ± 0.94 *** ###
Vaccenic acid (18:1 <i>n</i> -7)	3.1 ± 0.14	2.9 ± 0.11 ***	4.7 ± 0.24 ###	3.6 ± 0.25 * #
Total MUFA	11.2 ± 0.42	18.6 ± 1.96 **	24.4 ± 1.44 ###	30.9 ± 0.98 ** ###
Linoleic acid (18:2 <i>n</i> -6)	18.1 ± 0.78	12.1 ± 0.83 ***	7.3 ± 0.60 ###	5.3 ± 0.28 * ###
γ-Linoleic acid (18:3 <i>n</i> -6)	0.2 ± 0.01	0.2 ± 0.00	0.2 ± 0.02	0.2 ± 0.01
Homo-γ-linoleic acid (20:3 <i>n</i> -6)	0.7 ± 0.04	0.9 ± 0.05 *	0.6 ± 0.05	0.5 ± 0.05 ###
Arachidonic acid (AA, 20:4 <i>n</i> -6)	23.1 ± 0.51	17.4 ± 0.89 ***	20.2 ± 1.14 #	13.5 ± 0.81 ***
Docosatetraenoic acid (22:4 <i>n</i> -6)	0.4 ± 0.03	0.2 ± 0.02 ***	0.5 ± 0.04	0.3 ± 0.02 ** ##
Docosapentaenoic acid (22:5 <i>n</i> -6)	0.2 ± 0.02	0.2 ± 0.02	4.7 ± 0.23 ###	3.2 ± 0.19 *** ###
Total LC <i>n</i> -6	24.6 ± 0.54	18.8 ± 0.95 ***	26.1 ± 1.30	17.7 ± 0.99 ***
α-Linolenic acid (18:3 <i>n</i> -3)	0.5 ± 0.05	0.4 ± 0.05	nd ###	nd ###
Eicosapentaenoic acid (EPA, 20:5 <i>n</i> -3)	0.3 ± 0.02	0.4 ± 0.02 **	nd ###	nd ###
Docosapentaenoic acid (22:5 <i>n</i> -3)	0.9 ± 0.03	0.7 ± 0.06 **	nd ###	nd ###
Docosahexaenoic acid (DHA, 22:6 <i>n</i> -3)	6.6 ± 0.36	6.1 ± 0.23	0.5 ± 0.03 ###	0.3 ± 0.02 * ###
Total LC <i>n</i> -3	7.8 ± 0.32	7.2 ± 0.27	0.5 ± 0.03 ###	0.3 ± 0.02 * ###
AA/DHA	3.5 ± 0.12	2.8 ± 0.10 ***	42.9 ± 0.71 ###	41.7 ± 0.98 ###
AA/EPA	83.1 ± 6.57	46.2 ± 3.27 ***	202.0 ± 9.21 ###	135.1 ± 8.10 *** ###
AA/EPA+DHA	3.4 ± 0.11	2.7 ± 0.09 ***	35.2 ± 0.39 ###	31.6 ± 0.73 *** ###
LC <i>n</i> -6/LC <i>n</i> -3	3.1 ± 0.08	2.6 ± 0.06 ***	55.8 ± 1.20 ###	54.7 ± 1.30 *** ###

¹Data are expressed as mean ± S.E.M. weight percent total fatty acids nd = not detected (below the limit of detection)

* *P* 0.05

** *P* 0.01

*** *P* 0.001 vs. VEH-treated controls within each diet group

P 0.05

P 0.01

P 0.001 vs. same-treatment (VEH, RSP) in the control diet group