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The Effects of Trans-nonachlor on Hepatic Lipid Metabolism in Immortalized Rat Hepatoma Cells vs. Rat Primary Hepatocytes

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The Effects of Trans-nonachlor on Hepatic Lipid Metabolism in Immortalized Rat Hepatoma Cells vs. Rat Primary Hepatocytes

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Abstract: A highly pertinent cause of hepatic disfunction and metabolic disorders such as type 2 diabetes are persistent organic pollutants, namely organochlorine (OC) pesticide metabolites such as trans-nonachlor. Several studies have found positive correlations with OC pesticides and the development of the hepatic component of metabolic syndrome: non-alcoholic fatty liver disease (NAFLD), also known as hepatic steatosis. This condition is characterized by the accumulation of lipids within liver cells. Work in the Howell Lab at Mississippi State University's College of Veterinary Medicine explored the cellular mechanisms behind this association in parallel studies investigating the effects of the organochlorine pesticide, trans-nonachlor, on hepatic lipid metabolism in two different cell types: immortalized rat hepatoma (McA) cells and rat primary hepatocytes. The purpose of this study was to compare the results from these two aforementioned studies, highlighting similarities in the results while also discussing potential reasons for differences. The following results were the same across both cell types following exposure to trans-nonachlor: intracellular lipid accumulation increased, de novo lipogenesis increased, and oxidative stress and fatty acid uptake were unchanged. Fatty acid oxidation increased in McA cells but decreased in primary hepatocytes. The results for VLDL/triglyceride secretion were inconclusive. Taken together, the results indicate that trans-nonachlor promotes intracellular neutral lipid accumulation primarily via increased de novo lipogenesis, with decreased fatty acid oxidation as another potential contributor.

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1. Introduction

Before any particular pesticide-induced alterations in normal metabolic processes can be discussed, it is important to first have a general understanding of normal hepatic lipid metabolism. The liver is the largest organ in the human body, accounting for about 2% of body weight. Fat can either be stored or mobilized for energy. The storage form of fat is triglyceride, which consists of three fatty acid chains attached to a glycerol backbone. The mobilized form of fat is fatty acid. While adipose tissue is the main storage site for fat, the liver is not a fat storage depot; in fact, it normally harbors very low levels of triglycerides. The liver is a main site of fat metabolism, for which it has three main functions: breakdown of fatty acids for energy, synthesis of fat from carbs and proteins, and synthesis of cholesterol, phospholipids, and lipoproteins. When the body needs energy, triglycerides are mobilized from adipose tissue, transported as free fatty acids in the blood, and then taken up by target organs, where fat is either metabolized for energy production or stored as triglycerides. Fats, carbohydrates, and proteins that are consumed can also be converted to fat in the liver and then sent off to be stored in adipose tissue. The liver also manufactures lipoproteins, which serve as the carriers of lipids as they pass through the bloodstream.[1]

In certain cases, the normal physiological state of the liver can be adversely affected. Metabolic syndrome was defined by the World Health Organization in 1999 as a pathological manifestation of metabolic dysfunction characterized by several risk factors, including abdominal obesity, insulin resistance, hypertension, and hyperlipidemia. Metabolic syndrome is not itself a disease, but rather a collection of these risk factors.[2] As the liver is a primary organ involved in systemic metabolism, its normal functions are adversely affected as part of the syndrome's pathogenesis. Non-alcoholic fatty liver disease (NAFLD), also known as hepatic

steatosis, is heavily interconnected with the risk factors of metabolic syndrome and is thus considered to be the "hepatic component" of the condition and related conditions such as type 2 diabetes (T2D).[3] NAFLD is the most common cause of liver disease in industrialized countries such as the U.S. It is typically associated with obesity and T2D.[4] A strong correlation exists between NAFLD and diabetes: someone with NAFLD has 5x the risk of developing the latter disease.[5] NAFLD is characterized by excess fat accumulation in liver cells. In other words, there is neutral lipid accumulation (primarily as triglyceride) in hepatocytes. Lipids can accumulate within hepatocytes for several reasons, such as increased fatty acid uptake, increased de novo lipogenesis (DNL), decreased fatty acid oxidation, or overwhelmed very low density lipoprotein (VLDL) secretion.[6] As lipid accumulation leads to liver inflammation (hepatitis), the condition progresses to non-alcoholic steatohepatitis (NASH), a more severe form of NAFLD. Liver inflammation destroys parenchymal cells, which are replaced with fibrous scar tissue (fibrosis) that contracts around blood vessels, impeding blood flow. Fibrosis of the liver is known as cirrhosis.[4] The subsequent damage to the liver's hepatocytes is detrimental to its function.

A potential cause of hepatic disfunction and metabolic disorders such as T2D is exposure to persistent organic pollutants (POPs), such as organochlorine pesticide metabolites. POPs, which are lipophilic and hydrophobic, accumulate in the body's fatty tissues and reside there for many years because they are resistant to metabolic degradation.[7] Epidemiological studies have shown strong positive correlations with T2D and elevated concentrations of POPs, such as OC pesticides, in the blood.[8,9] In vivo studies in rodents show that chronic exposure to a mixture of POPs, including OC pesticides, via a diet high in fats promotes T2D and NAFLD. POPs in diets with fats derived from sources like farmed fatty fish, dairy products, and meat are of particular interest because animal products are one of the main sources of POP exposure in people.[7,10]

Studies in male C57BL/6H mice indicate that both subacute and chronic exposure to the organochlorine pesticide metabolite dichlorodiphenyldichloroethylene (DDE) promotes fasting hyperglycemia, a physiological alteration associated with changes in glucose homeostasis in T2D, without concurrent insulin resistance.[11,12] In the ob/ob mouse, a genetic model of obesity and T2D, exposure to OC pesticides has been shown to promote hepatic steatosis.[13] The Howell Lab at Mississippi State University's College of Veterinary Medicine explored the cellular mechanisms behind this association in parallel studies investigating the effects of the organochlorine pesticide, trans-nonachlor, on hepatic lipid metabolism in two different cell types.[14,15]

Two different cell types were used as models to answer these questions: McArdle-RH7777 (McA) rat hepatoma cells, and rat primary hepatocytes. The key difference between these cell lines is that McA cells, which are immortalized, replicate indefinitely in culture, whereas primary hepatocytes do not multiply once cultured following harvest. Frozen McA cell stocks were obtained from a private company (American Type Culture Collection; ATCC), while rat primary hepatocytes were surgically harvested from rats and perfused on-site, then immediately plated without ever being frozen. McAs are good models for studying NAFLD for several reasons, one of which being that they can be made steatotic when exogenous fatty acids are introduced to the media. They also secrete lipoproteins like VLDL and HDL in a way that's similar to primary hepatocytes.[16] In addition, secretion of ApoB100 protein for VLDL is suppressed by insulin in McA cells via a process similar to rat primary hepatocytes.[17] Whereas most immortalized hepatoma cell lines express Srebp-1a, McA cells express insulin-sensitive lipogenic mediator sterol response element binding protein-1c (Srebp-1c), which is the same type carried by primary hepatocytes.[18] Srebp is the master transcriptional regulator for the lipogenic

gene cascade. For example, the presence of glucose stimulates insulin secretion, which in turn promotes expression of Srebp1 leading to increased de novo lipogenesis.[19] But McAs do have major drawbacks, including increased fatty acid uptake and fatty acid oxidation compared to primary hepatocytes.[20] Furthermore, in serum-rich culture conditions, McA cells develop insulin resistance and subsequently secrete more ApoB and VLDL.[21] Differences in serum used in the two cell types' culture media may also potentially cause differences in experimental results.[16] The initial set of experiments was carried out on McA cells, as they are more cost effective and easier to work with than primary hepatocytes. Because primary cells are a superior model, the same set of experiments was performed on those next to strengthen the validity of the results. The purpose of this study is to compare the results from these two aforementioned studies, highlighting similarities in the results while also discussing potential reasons for differences.

2. Methods

2.1 Reagents

McA rat hepatoma cells were purchased from ATCC. Their normal growth media consisted of high-glucose DMEM, 20% Fetal Bovine Serum (FBS), sodium pyruvate, glutamine, penicillin, and streptomycin. Normal growth media for primary hepatocytes was RPMI-1640 supplemented with fatty acid-free bovine serum albumin (BSA), sodium pyruvate, glutamine, penicillin, streptomycin, and insulin. Trans-nonachlor was purchased from Accu-Standard. Oleic acid, palmitic acid, Oil Red O stain, and Janus Green stain were obtained from Sigma Aldrich. Pesticide metabolites were put in solution with the solvent dimethylsulfoxide (DMSO) for a final DMSO concentration of 0.025%. BODIPY-FA for fatty acid uptake experiments was obtained from Life Technologies. ^{14}C-acetate for DNL experiments was obtained from Perkin Elmer. β-hydroxybutyrate (β-HB), glutathione, TBARS, and triglyceride assay kits were obtained from Cayman Chemical.

2.2 Primary hepatocyte isolation and culture

Livers were surgically harvested from rats and then perfused with collagenase solution. Cell suspensions were filtered through 100 micron, then 70 micron filters and centrifuged to purify the homogenate. Cell concentration and viability via trypan blue exclusion were measured via hemocytometer. Cell suspensions with 75% viability or greater were subsequently plated at a concentration of 1×10^6 cells/mL on cell culture plates coated with rat tail collagen to help with cell adherence. They were left to sit in normal growth media for 3-4 h to allow for sufficient cell attachment. Adhered cells were washed with PBS and then subjected to various experimental protocols.

2.3 Intracellular lipid accumulation

Both cell types were exposed to trans-nonachlor in normal growth media to determine its effects on intracellular neutral lipid accumulation. Cells were exposed to vehicle (dimethylsulfoxide, DMSO, 0.025%) or various concentrations of OC pesticide (0.02, 0.2, 2.0, 20, or 80 μM). In all assays tested, results from no-vehicle controls were not significantly different than vehicle treated cells, suggesting that the DMSO vehicle itself does not adversely affect metabolic activity of the cells. McA cells and primary hepatocytes were exposed to trans-nonachlor for 24 h. Afterwards, cells were washed with PBS and underwent Oil Red O staining to measure intracellular neutral lipid accumulation. A commercially-available triglyceride assay (Cayman Chemical) was used to measure intracellular triglyceride levels to confirm that most of the intracellular neutral lipid accumulation was composed of triglycerides.

2.4 Oil red O staining

Oil Red O stain selectively dyes intracellular neutral lipids, mainly triglyceride and cholesterol ester. Cells were stained with Oil Red O for 15 min at room temperature. Then, the stain was washed off with deionized water so that any remaining stain was present intracellularly. The cells were then allowed to air dry overnight. Intracellular stain was extracted with 100% isopropanol and the absorbance at 520 nm was measured with a cell plate reader. The concentration of Oil Red O stain is proportional to the concentration of intracellular neutral lipids. To normalize for cell number, cells were then stained with Janus Green for 5 min. This stain was extracted with 0.5 N HCl and absorbance was measured at 595 nm using the same plate reader. The concentration of Janus Green stain is proportional to the cell number. Final data were obtained by dividing Oil Red O absorbance by Janus Green absorbance to calculate the intracellular neutral lipid accumulation per cell concentration.

2.5 Fatty acid uptake

Fatty acid uptake assay with Bodipy-labeled fatty acid markers offered a superior method of measuring fatty acid uptake following free fatty acid induced neutral lipid accumulation in the McA cells. The extent to which trans-nonachlor exposure induced fatty acid uptake was determined in both McA cells and primary hepatocytes using Bodipy-labeled 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY 500/510 C12; BODIPY-FA; Molecular Probes). Bodipy is a fluorophore, a fluorescent chemical that re-emits light upon excitation, while dodecanoic acid is a 12-carbon fatty acid commonly used to track fatty acid flux. Cells were exposed to either vehicle (DMSO 0.025%) or trans-nonachlor (20 µM in McA; 0.2, 2, or 20 µM in primary hepatocytes) for 24 h. Following exposure, cells were washed with PBS and placed in their respective serum free medias (DMEM for McA; RPMI 1640 for primary hepatocytes) with BSA, then exposed to Bodipy-labeled dodecanoic acid for 1 min and 60 min. Fatty acid uptake occurs through multiple mechanisms, including rapid transporter mediated uptake and diffusion. The 1 min exposure was performed to measure rapid transporter uptake, and the 60 min exposure was performed to measure the combination effect of rapid transporter uptake and diffusion as well as the net effect of uptake and breakdown during that time period. Following Bodipy-labeled dodecanoic acid exposure, cells were washed with PBS and homogenized in sodium dodecyl sulfate (SDS). Subsequently obtained fluorescence intensity of the cell homogenate was a proportional measure of Bodipy, and thus fatty acid, uptake by the cells. Protein concentration of cell homogenates was determined using a Bradford assay. Data was expressed as the fluorescence per unit protein concentration, or RFU per µg protein.

2.6 Fatty Acid Oxidation

Fatty acid oxidation is essentially the breakdown of fat into a form that can be used for energy. In the liver, fatty acids are oxidized into a two-carbon compound called acetyl-CoA. This can be used to make ATP, an energy molecule, or make ketones such as β-hydroxybutyrate. In experimental applications, the levels of cellular β-hydroxybutyrate have been widely used as an index of hepatic fatty acid oxidation. Both cell types were treated with vehicle (DMSO 0.025%) or trans-nonachlor (20 µM in McAs; 0.2 and 20 µM in primary hepatocytes) for 24 h in serum-free media, then either 400 µM OA or 0.5% BSA was added for the last 8 h. Following exposure, levels of β-hydroxybutyrate were measured via manufacturer assay (Cayman Chemical).

2.7 VLDL/Triglyceride Secretion

Both cell types were treated using the exposure procedure outlined in Section 2.6. Following this, the triglycerides were extracted. 500 µl of media from the cell culture was extracted using the Folch method and the organic phase was dried under a stream of nitrogen gas. The resulting dried lipid layer was resuspended in 100% methanol and these extracted triglycerides were quantified using a colorimetric triglyceride assay (Cayman Chemical).

2.8 De Novo Lipogenesis

^{14}C-acetate was introduced into the cells to measure de novo lipogenesis. By radiolabeling the acetate with radioactive ^{14}C, it is possible to track the production of new fatty acids, specifically de novo lipogenesis. Both cell types were exposed to vehicle (DMSO 0.025%) or trans-nonachlor (20 µM in McAs; 0.2, 2, and 20 µM in primary hepatocytes) in serum free, fatty acid free media containing 0.5% BSA. In the last 8 h of exposure, the cells were stimulated with insulin (100 nM). Following exposure, ^{14}C-acetate was added to the cells and incubated for 3 h.

Then, the cells were washed with, and resuspended in, PBS. The lipid portion of the cell suspension was extracted in either 3:2 hexane:isopropanol (primary hepatocytes) or 2:1 chloroform:methanol (McAs), then centrifuged to separate the organic (cells and lipids) and aqueous phases. ^{14}C-acetate that was not taken up by the cells for de novo lipogenesis was in the aqueous phase, whereas the ^{14}C-acetate that was used for de novo lipogenesis was present in the organic phase. The radioactivity of organic phase was quantified by liquid scintillation counting.

2.9 Oxidative stress (Lipid peroxidation/cellular glutathione)

Lipid peroxidation is an index of oxidative stress levels throughout the cell. Oxidative stress is free radical formation within the cell i.e. free oxygen. If the levels of free radicals get too high, these reactive oxygen species begin binding to lipids and proteins to create adducts, which cause cell damage. Cells normally employ anti-oxidant mechanisms to effectively dispose of these free radicals before they form adducts. However, if there are simply too many free radicals and adducts to break down, then the cell will undergo apoptosis, essentially a self-destructive process. Environmental toxins and exposure to pesticides can induce the production of reactive oxygen species. Malondialdehyde (MDA) is a free radical, specifically an aldehyde formed as a product during lipid peroxidation. It is widely used as a biomarker for lipid peroxidation of fatty acids and it reacts well with thiobarbituric acid, the main chemical constituent of peroxidation assays. Glutathione is the major antioxidant in most cells. It catalyzes the reduction of peroxides into water and alcohol for filtration and excretion from the body.[22] If trans-nonachlor causes oxidative stress on hepatocytes, this could be a potential factor that explains this pesticide's induction of intracellular neutral lipid accumulation. Both McA cells and primary hepatocytes were exposed to vehicle (DMSO 0.025%) or trans-nonachlor (20 µM in McAs; 0.2 and 20 µM in primary hepatocytes). Thiobarbituric acid reactive substances (TBARS) assay was performed to

quantify cellular levels of MDA and another assay was used to determine levels of glutathione (Cayman Chemical).

2.10 SDS-PAGE and Western Blot

SDS-PAGE and Western Blot were used to measure quantities of several different proteins in the cells. Fatty acid synthase (FAS), ApoB, and β-actin were measured in both cell types. β-actin is a cytoskeletal protein. As a housekeeping protein, it was used to normalize the values of the other proteins that were measured. McA cells were exposed to trans-nonachlor (0 and 20 μM) for 24 h in serum-free media with fatty acid free BSA. For the last 8 h, they were treated with OA (400 μM) and then whole cell lysates were made. Primary hepatocytes were exposed for DNL or VLDL/triglyceride secretion and then cell homogenates were made. For both cell types, cellular proteins were separated from the cell lysates by SDS-PAGE, transferred to PVDF membranes, and then underwent Western Blot procedure. Proteins were pictured with chemiluminescence and digital images were recorded using Bio-Rad. Band densities were measured with ImageJ software. Data are presented as a normalized ratio of FAS or ApoB integrated densities to β-actin integrated density.

2.11 Statistical Analysis

Data are expressed as the mean ± the standard error of the mean (SEM). Each individual animal's duplicate measurements were averaged, with the average subsequently treated as the individual animal's final value. For experiments with one independent treatment but three or more groups, such as concentration response studies, data were analyzed using a one-way analysis of variance (ANOVA) along with a Tukey's post hoc test (SigmaPlot 12.5). For experiments with only two groups, data were analyzed using a Student's *t*-test in order to

compare results between groups. A two-way ANOVA with a Tukey's post hoc test was performed for experiments with two independent treatment groups, such as trans-nonachlor with or without insulin stimulation. The threshold for statistical significance was set as $P \leq .05$.

3. Results

3.1 Trans-nonachlor exposure significantly increases intracellular lipid accumulation in McA cells and primary hepatocytes

Both McA cells and primary hepatocytes were initially exposed to trans-nonachlor (20 and 80 μM) in normal growth media for 24 h to quantify levels of intracellular neutral lipid accumulation, the hallmark of hepatic steatosis. In both McA cells and primary hepatocytes, trans-nonachlor exposure significantly increased intracellular neutral lipid accumulation. In both cell types, exposure to both concentrations (20 and 80 μM) of trans-nonachlor for 24 h caused significant increases in neutral lipid accumulation compared to vehicle. McA cells exposed to the lower trans-nonachlor concentration (20 μM) showed increased staining of neutral lipids by 61% compared to vehicle. The higher concentration (80 μM) increased staining of neutral lipids by 78% compared to vehicle. In primary hepatocytes, the lower trans-nonachlor concentration (20 μM) increased neutral lipid staining by 37% compared to vehicle, while the higher concentration (80 μM) increased staining by 41% compared to vehicle. In both cell types, both the higher and lower concentrations of trans-nonachlor had a similarly-sized effect on neutral lipid accumulation. There was no significant difference between their effects, which suggests that after a certain concentration, the "steatotic effect" levels out.

3.2 The trans-nonachlor induced neutral lipid accumulation is mainly due to build-up of triglycerides

To prove that the staining results were, in fact, due to intracellular accumulation of triglycerides cellular triglyceride levels were also measured. In both cell types, there was a positive association found between 24 h trans-nonachlor exposure (20 μM) and increased cellular

triglyceride concentrations compared to vehicle. In McA cells, there was a significant, 71% increase in triglyceride levels following trans-nonachlor exposure (20 μM) compared to vehicle. Primary hepatocytes exposed to the metabolite (20 μM) showed a significant, 13% increase in triglyceride levels compared to vehicle. These data indicate that the Oil Red O results appear to be due to intracellular accumulation of triglycerides in particular.

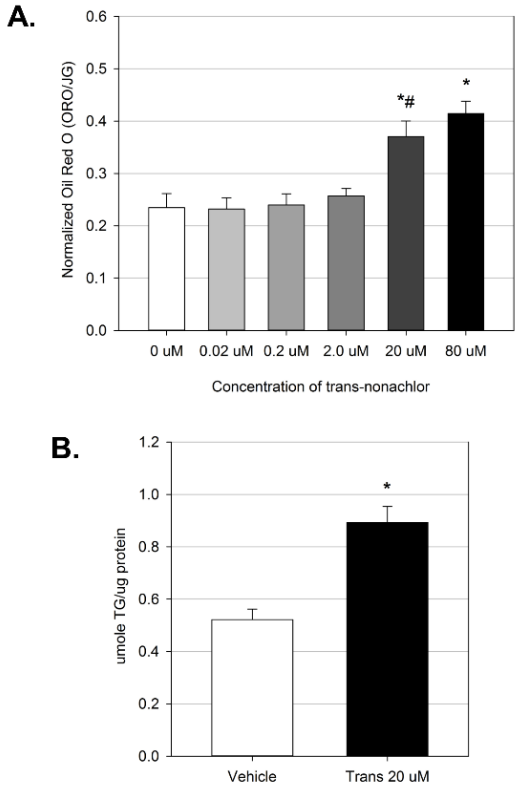

Fig. 1. Trans-nonachlor exposure significantly increases intracellular lipid accumulation in McA cells. After being exposed for 24 hours, (A.) neutral lipid accumulation and (B.) cellular triglyceride levels were measured. Data represent the average ± SEM of n = 8/group for Oil Red O and n = 5/group for triglyceride measurement. *P < 0.05 vs. vehicle; #P < 0.05 vs. 2 μM.

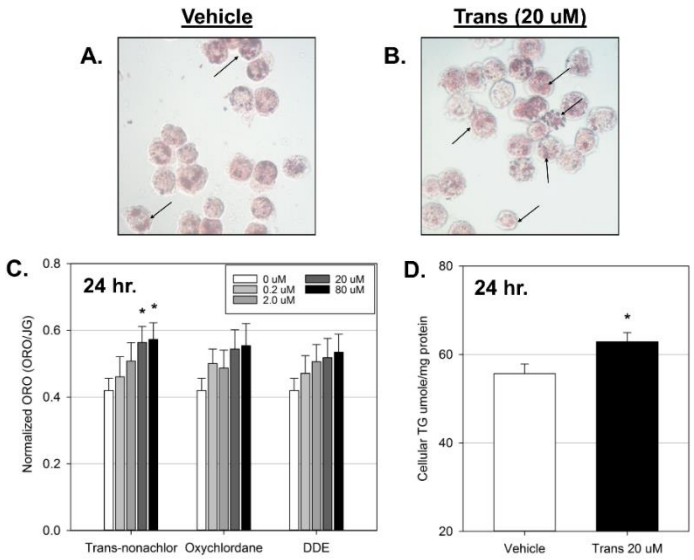

Fig. 2. Trans-nonachlor exposure significantly increases intracellular lipid accumulation in primary hepatocytes. After being exposed for 24 hours, (C.) neutral lipid accumulation and (D.) cellular triglyceride levels were measured. Data represent the mean ± SEM for n = 5-6 animals/group for ORO staining and n = 4 animals/group for triglyceride measurement. *P ≤ .05 versus vehicle (0 μM). Micrographs demonstrate Oil Red O staining in primary hepatocytes treated with (A.) vehicle and (B.) trans-nonachlor.

3.3 Fatty acid uptake is not increased in McA cells or primary hepatocytes following trans-nonachlor exposure

Free fatty acid uptake is a major contributor to the development of hepatic steatosis. The experiment investigated the effects of trans-nonachlor exposure on Bodipy labeled dodecanoic acid. At 1 min following addition of the Bodipy labeled dodecanoic acid, neither McA cells nor primary hepatocytes exposed to trans-nonachlor (20 µM in McA; 0.2, 2, or 20 µM in primary hepatocytes) for 24 h had significantly altered free fatty acid uptake. At 60 min following addition of the Bodipy labeled dodecanoic acid, treated McA cells did show a significant increase in accumulation of free fatty acid to vehicle-treated cells. However, treated primary hepatocytes did not have a significant increase at this 60 min timepoint. In McA cells, it appears that exposure to trans-nonachlor does not cause direct fatty acid uptake by rapid transporter proteins but does cause increased fatty acid accumulation overall. In primary hepatocytes, exposure to trans-nonachlor does not increase fatty acid uptake or accumulation.

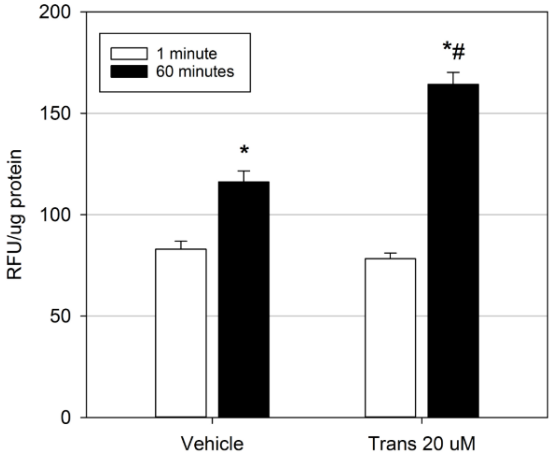

Fig. 3. Fatty acid uptake (1 min) is not increased in McA cells, however there is a significant increase in free fatty acid accumulation (60 min). *P < 0.05 vs. vehicle @ 1 min and #P < 0.05 vs. vehicle @ 60 min.

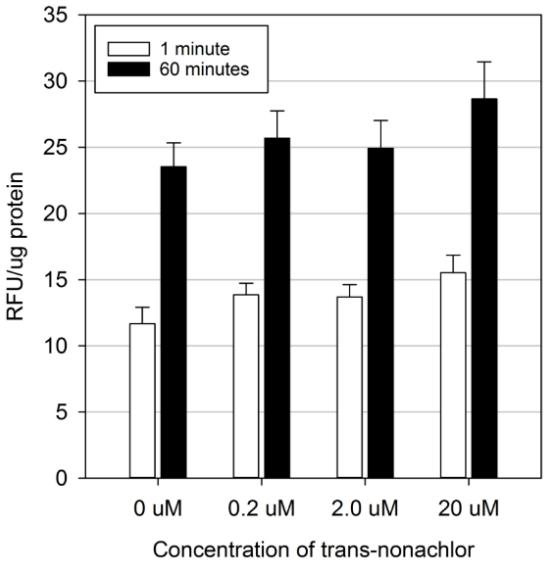

Fig. 4. Neither fatty acid uptake (1 min) nor fatty acid accumulation (60 min) is significantly increased in primary hepatocytes. Data represent the mean ± SEM for 8-9 animals/group.

3.4 Exposure to trans-nonachlor increases fatty acid oxidation in McA cells and decreases oxidation in primary hepatocytes

Both cell types were exposed to vehicle and 20 µM trans-nonachlor for 24 h in serum-free media (BSA) with or without addition of fatty acids (OA) for the last 8 h of incubation. β-HB is a commonly used indicator of fatty acid oxidation and it was used as such in this experiment. In McA cells, trans-nonachlor exposure causes increased fatty acid oxidation. As expected, in vehicle-treated cells, the addition of fatty acids (OA) significantly increased β-HB levels by 41% compared to BSA-treated vehicle cells. Trans-nonachlor exposure in BSA-treated cells led to a significant 62% increase in β-HB levels compared to BSA-treated vehicle. Trans-nonachlor exposure in fatty acid-treated cells significantly increased β-HB levels by 42% compared to fatty-acid treated vehicle. In a normal physiological state, the presence of increased serum fatty acids leads to increased fatty acid oxidation to maintain homeostasis. This is what happened in this case. In primary hepatocytes, trans-nonachlor exposure causes decreased fatty acid oxidation under both basal and steatotic conditions. Trans-nonachlor (20 µM) significantly decreased both basal and fatty acid (OA) induced cellular β-HB levels by 42% and 49% compared to vehicle exposed cells.

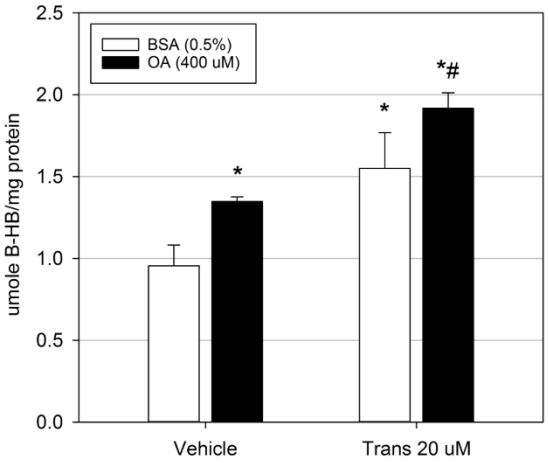

Fig. 5. Fatty acid oxidation is significantly increased in McA cells following trans-nonachlor exposure (20 μM) for 24 h in serum free media with or without OA for the final 8 h. Cellular β-HB levels were determined as an index of fatty acid oxidation. Data represent the mean ± SEM of 5–6/group. *P < 0.05 vs. vehicle + BSA; #P < 0.05 vs. vehicle + OA.

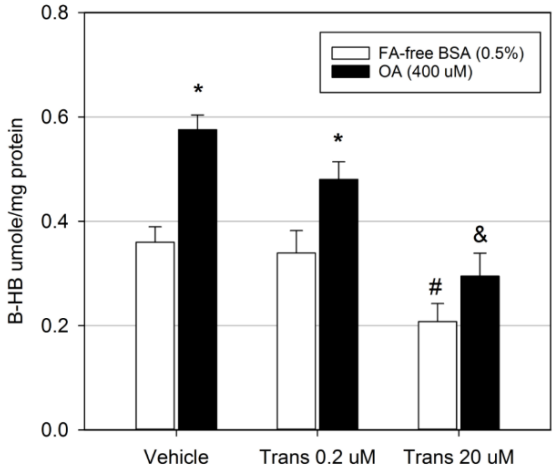

Fig. 6. Fatty acid oxidation is significantly decreased in primary hepatocytes following trans-nonachlor exposure (0.2 and 20 μM) for 24 h in serum free media with or without OA for the final 8 h. Cellular β-HB levels were determined as an index of fatty acid oxidation. Data represent the mean ± SEM of n = 4-6 animals/group. *P ≤ .05 versus matching trans-nonachlor concentration without OA; #P ≤ .05 versus vehicle without OA; &P ≤ .05 versus vehicle and trans-nonachlor (0.2 μM) with OA.

3.5 Trans-nonachlor does not affect VLDL secretion in McA cells, but increases VLDL secretion in primary hepatocytes under steatotic conditions

Triglyceride secretion was quantified by measuring media levels of triglycerides. Both cell types were treated using the same culture protocol as outlined in the fatty acid oxidation experiment. This experiment investigated whether decreased VLDL/triglyceride secretion could be a contributing factor to the trans-nonachlor-induced increase in intracellular neutral lipid accumulation. In McAs, trans-nonachlor exposure does not affect triglyceride secretion. No significant effects were noted as a result of the exposure. In primary hepatocytes, trans-nonachlor exposure increases triglyceride secretion under steatotic conditions. Under basal conditions (BSA without added OA), trans-nonachlor exposure (0.2 or 20 μM) did not cause significant

increases in triglyceride secretion. However, the higher concentration (20 µM) did significantly increase triglyceride secretion under steatotic (OA-stimulated) conditions by 49% and 40% compared to BSA-treated and OA-treated vehicle, respectively. The data suggest that trans-nonachlor does not impair triglyceride secretion under conditions of elevated serum fatty acids. However, levels of VLDL secretion may not be able to compensate for the increased accumulation of triglycerides within the hepatocytes.

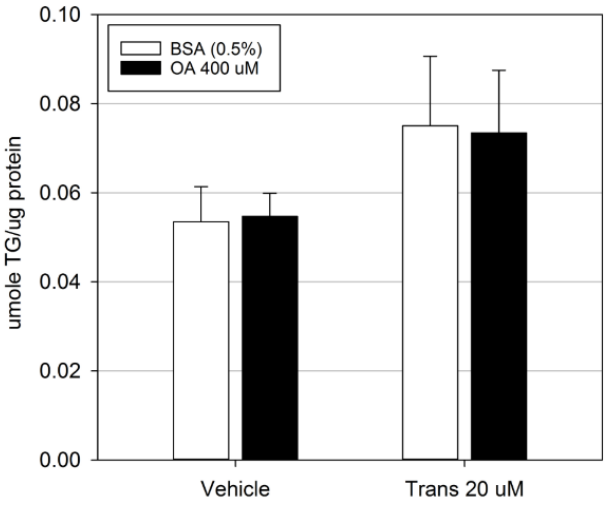

Fig. 7. VLDL/TG secretion in McA cells is not affected following trans-nonachlor exposure (20 µM) for 24 h in serum free media with or without OA for the final 8 h. Following exposure, media triglyceride levels were determined. Data represent the mean ± SEM of n = 6/group.

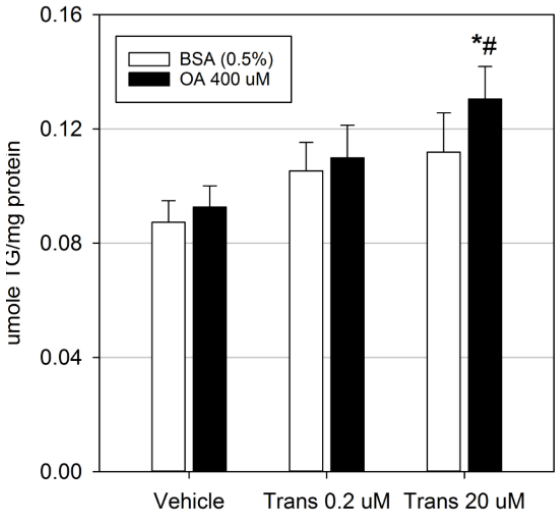

Fig. 8. VLDL/TG secretion in primary hepatocytes is significantly increased under steatotic conditions. Cells were exposed to trans-nonachlor (0.2 and 20 µM) for 24 h in serum free media with or without OA for the final 8 h. Following exposure, media triglyceride levels were determined. Data represent the mean ± SEM of n = 8-9 animals/group. *P ≤ .05 versus vehicle without OA; #P ≤ .05 versus vehicle with OA.

3.6 Cellular ApoB levels in McA cells are significantly decreased under steatotic conditions following trans-nonachlor exposure

Cellular production levels of ApoB100 as an indicator of pre-secretion VLDL in hepatocytes were investigated. In McA cells, trans-nonachlor exposure without additional fatty acid (BSA

instead of OA) did not show significant changes in ApoB100 levels compared to vehicle. However, trans-nonachlor exposed McA cells treated with OA significantly decreased ApoB100 levels by 100% compared to equivalently treated vehicle. While not conclusive, these data suggest that exposure to trans-nonachlor may potentially have a deleterious effect on cellular production of ApoB100, and thus on the cells' ability to secrete VLDL/triglyceride. Trans-nonachlor exposed primary hepatocytes produced more conclusive results. While the pesticide did increase cellular levels of ApoB100, these increases were not statistically significant. In primary hepatocytes, trans-nonachlor exposure does not appear to have an effect on the production of ApoB protein within the cells.

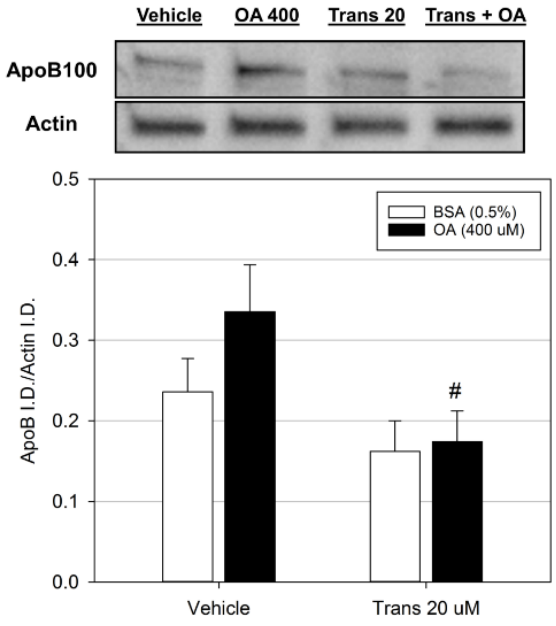

Fig. 9. Cellular ApoB levels in McA cells are significantly decreased under steatotic conditions following trans-nonachlor exposure (20 µM). Cells were exposed for 24 h in serum free media with or without OA for the final 8 h. Following exposure, cellular ApoB levels were determined using Western Blot. Data represent the mean ± SEM of n=6/group. #P < 0.05 vs. vehicle + OA.

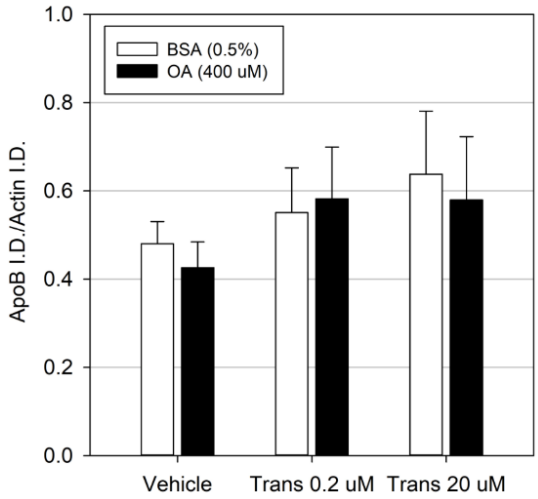

Fig. 10. Cellular ApoB levels in primary hepatocytes are not significantly affected by trans-nonachlor exposure (0.2 and 20 µM). Cells were exposed for 24 h in serum free media with or without OA for the final 8 h. Following exposure, cellular ApoB levels were determined using Western Blot. Data represent the mean ± SEM of n = 8-9 animals/group.

3.7 Trans-nonachlor exposure significantly increases basal de novo lipogenesis in McA cells and significantly increases insulin-stimulated de novo lipogenesis in primary hepatocytes

DNL is the second largest source of intracellular free fatty acid which can be subsequently taken up and stored in hepatocytes in the form of triglycerides. Cells were exposed to vehicle and trans-nonachlor (20 µM) in serum free media with or without insulin stimulation (100 nM) during the last 8 h of incubation. Following the full 24 h treatment, cells underwent a lipogenic assay in which ^{14}C-acetate was used as an index of de novo lipogenesis. In McA cells, trans-nonachlor exposure significantly increases DNL under basal conditions. Insulin stimulation normally increases DNL, and this trend was seen with the increased DNL in insulin-stimulated vehicle vs. vehicle without insulin. Trans-nonachlor significantly increased basal, but not insulin-stimulated, ^{14}C-acetate integration into hepatocyte lipids by 36% compared to vehicle. These data indicate that DNL may be a contributing factor to the neutral lipid accumulation resulting from trans-nonachlor exposure. In primary hepatocytes, trans-nonachlor exposure significantly increases DNL under conditions of elevated insulin. While trans-nonachlor exposed cells in BSA showed concentration-dependent increases in DNL compared to vehicle, these were not statistically significant. However, insulin-stimulated ^{14}C-acetate incorporation was significantly increased in trans-nonachlor (20 µM) exposed cells by 41% compared to vehicle. In primary hepatocytes, increased de novo lipogenesis may be a contributing factor towards trans-nonachlor induced accumulation of intracellular neutral lipids.

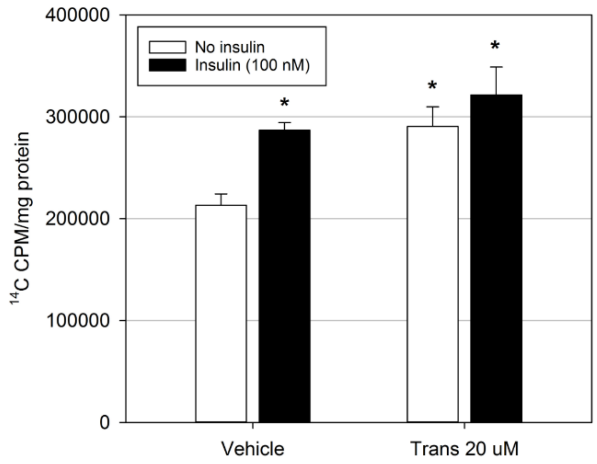

Fig. 11. Trans-nonachlor exposure significantly increases basal de novo lipogenesis in McA cells. Cells were exposed to trans-nonachlor (0 or 20 μM) for 24 h in serum free media with or without insulin stimulation for the final 8 h then lipogenesis was determined by ^{14}C-acetate incorporation into total cellular lipids. Data represent the mean ± SEM of n = 4/group. *P < 0.05 vs. vehicle without insulin.

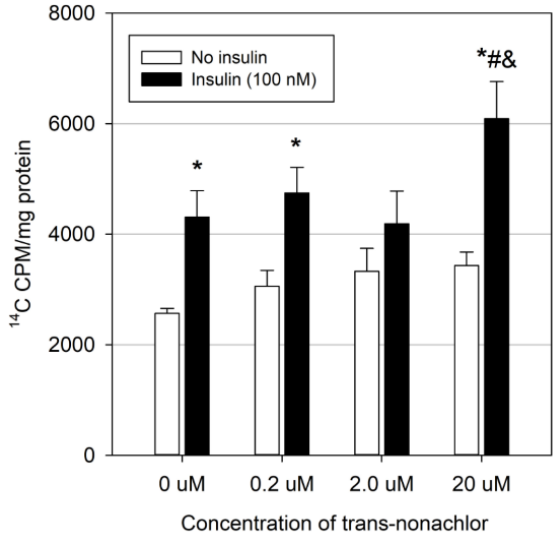

Fig. 12. Trans-nonachlor exposure significantly increases insulin-stimulated de novo lipogenesis in primary hepatocytes. Cells were exposed to trans-nonachlor for 24 h in serum free media with or without insulin stimulation for the final 8 h then lipogenesis was determined by ^{14}C-acetate incorporation into total cellular lipids. Data represent the mean ± SEM for 5 animals/group. *P ≤ .05 versus matching trans-nonachlor concentration without insulin; #P ≤ .05 versus trans-nonachlor (0 μM) with insulin; &P ≤ .05 versus trans-nonachlor (2.0 μM) with insulin.

3.8 Trans-nonachlor significantly increases FAS in primary hepatocytes under basal conditions

The effect of trans-nonachlor on FAS, a major protein involved in the control of lipogenesis, was investigated using SDS-PAGE and Western Blot techniques. While trans-nonachlor did cause an increase in FAS protein levels under basal and insulin-stimulated conditions in McA cells, they were not statistically significant. In primary hepatocytes, trans-nonachlor exposure causes significant increases in FAS protein levels. As expected, insulin stimulation in vehicle-treated cells caused a significant increase in FAS levels by 260%. Primary hepatocytes exposed to trans-nonachlor (20 μM) for 24 hours without insulin stimulation showed a significant 340% increase

in FAS levels compared to vehicle. Trans-nonachlor exposure with concurrent insulin stimulation failed to cause a significant increase in FAS.

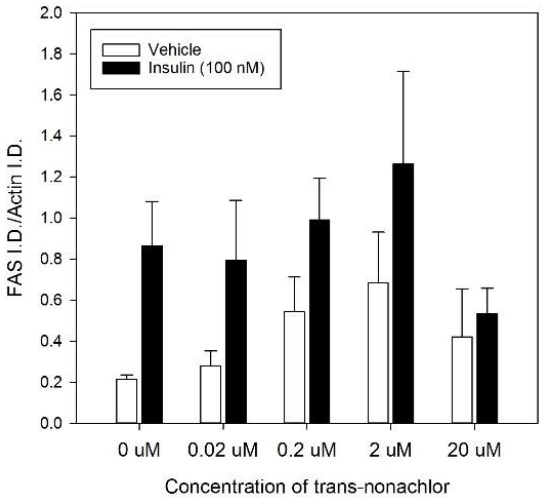

Fig. 13. Trans-nonachlor exposure does not significantly affect FAS in McA cells. Cells were exposed for 24 hours with insulin-stimulation for the final 8 hours then lysates were subjected to western blotting for FAS expression. Data represent the mean ± SEM of n = 5/group.

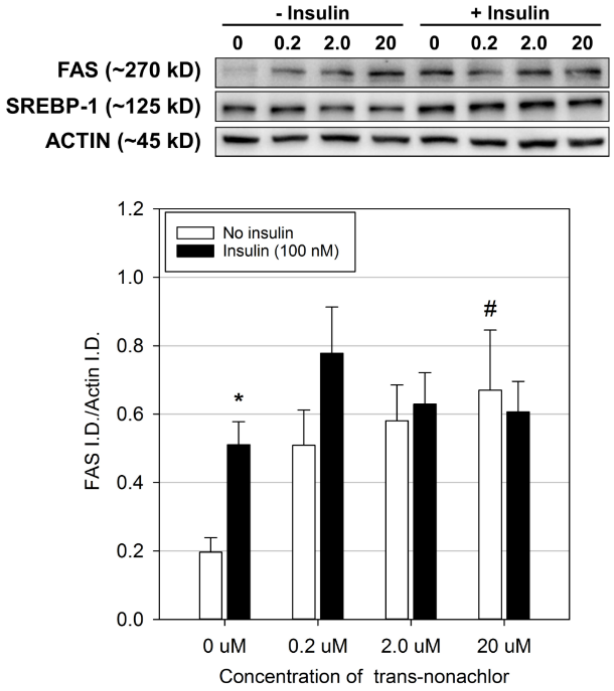

Fig. 14. Trans-nonachlor exposure significantly increases FAS in primary hepatocytes. Cells were exposed for 24 hours with insulin-stimulation for the final 8 hours then lysates were subjected to western blotting for FAS expression. Representative western blots are shown above the graphs. Data represent the mean ± SEM of n = 6-7 animals/group. *P ≤ .05 versus matching trans-nonachlor concentration without insulin; #P ≤ .05 versus trans-nonachlor (0 µM) without insulin.

3.9 Trans-nonachlor did not have any effects on oxidative stress in either cell type

Changes in levels of oxidative stress is a known contributor to lipid accumulation in hepatocytes and subsequent hepatic steatosis. McA cells and primary hepatocytes were exposed to vehicle and transnonachlor (20 µM in McA; 0.2 and 20 µM in primary hepatocytes) for 24 h and then MDA levels and glutathione levels were measured. There were no significant changes in either one. Oxidative stress is unlikely a mechanism through which trans-nonachlor induces neutral lipid accumulation.

4. Discussion

4.1 Intracellular neutral lipid accumulation

Intracellular neutral lipid accumulation increased in both cell types when exposed to trans-nonachlor, under both basal and steatotic conditions. However, the trans-nonachlor (20 μM) induced lipid accumulation was slightly greater in McA cells than in primary hepatocytes (41% vs 37%). Additionally, both cell types showed increases in intracellular triglyceride levels following exposure to trans-nonachlor, suggesting that the accumulated neutral lipid profile is comprised mainly of triglycerides. Again, however, the results for the triglyceride levels in McA cells were more extreme than in the primary hepatocytes (71% vs 13%). The fact that McA cells replicate in culture may have contributed to the greater levels of neutral lipid accumulation compared to the primary hepatocytes, which do not multiply in culture.

4.2 Fatty acid uptake

The primary source of lipids that accumulate within hepatocytes is free fatty acids that are taken up into the cell via rapid transporter proteins.[15] So, the most obvious explanation for the accumulation of neutral lipids within hepatocytes would hypothetically be an increase in fatty acid uptake. However, in both McA cells and primary hepatocytes, trans-nonachlor exposure does not appear to affect fatty acid uptake. At 1 min, any Bodipy labeled dodecadoic acid that is taken up by the cells is reflective of direct uptake of free fatty acids by rapid transporter proteins embedded in the cellular membrane. At this timepoint, neither cell showed increases in labeled fatty acids, indicating that no fatty acid uptake had taken place via transporter proteins. At 60 min, the concentration of Bodipy labeled dodecadoic acid is indicative of an overall accumulation of free fatty acids owing to the combination of several mechanisms, including fatty

acid uptake, oxidation, and secretion.[23] Trans-nonachlor exposed McA cells did show an increase in labeled fatty acids after 60 min. Thus, although trans-nonachlor causes increased accumulation of free fatty acids within the cells, this increase is not due to the direct uptake of fatty acids but may be a product of decreased oxidation or secretion. In primary hepatocytes, trans-nonachlor had no significant effects on free fatty acid accumulation at 60 min. Since neither cell type showed trans-nonachlor induced increases in fatty acid uptake, it is unlikely to be a contributing factor to the intracellular neutral lipid accumulation that has been associated with this pesticide.

4.3 De Novo Lipogenesis

DNL is the second largest source of lipids that accumulate within liver cells. DNL is typically increased in cases of increased serum insulin concentrations, as is the case with T2D. In both McA cells and primary hepatocytes, trans-nonachlor caused increased de novo lipogenesis. However, this increase only occurred in McA cells under basal conditions, while it occurred in primary hepatocytes only upon stimulation with insulin. The results from the McA cells suggest that trans-nonachlor increases de novo lipogenesis in normal physiological conditions. The primary hepatocyte data somewhat contradict these results, as they suggest that trans-nonachlor exposure increases the formation of new lipids only in states of hyperinsulinemia. However, McA cells do have a higher intrinsic lipogenic drive because they are immortalized, which may account for the differing results. Levels of FAS were also measured to track changes in lipogenesis. FAS is a lipogenic protein and is the key rate limiting enzyme in fatty acid synthesis.[19] An increase in FAS would point towards increased lipogenesis. FAS levels in McA cells responded to trans-nonachlor exposure under basal and insulin stimulation with general increases, but these were not statistically significant. However, this particular experiment may have had different results, had there been more repetitions per group. In primary hepatocytes,

trans-nonachlor exposure without concurrent insulin stimulation increased FAS levels. However, exposure with insulin had no significant effects. Together, these results indicate that trans-nonachlor promotes increased lipogenesis. Despite the differences in the cells' reactions to trans-nonachlor regarding insulin stimulation, the general trend in both cell types indicates an increase in lipogenesis. Thus, de novo lipogenesis appears to be a contributing factor to trans-nonachlor induced neutral lipid accumulation within liver cells.

4.4 Fatty acid oxidation

Decreased fatty oxidation is another potential explanation for trans-nonachlor's associated lipid accumulation. In primary hepatocytes, exposure to the pesticide metabolite significantly decreased β-HB levels, thereby decreasing fatty acid oxidation, in both basal and fatty acid stimulated media. The results from the primary hepatocyte experiment indicate that decreased fatty acid oxidation may contribute to a higher availability of fatty acids for lipid accumulation. However, in McA cells, exposure to trans-nonachlor caused a significant increase in fatty acid oxidation under both basal and steatotic (OA-stimulated) conditions. Because trans-nonachlor did not reduce basal or fatty acid stimulated oxidation, this suggests that oxidation is not a mechanism through which trans-nonachlor causes neutral lipids to accumulate within McA cells. One possible explanation for this discrepancy is the fact that McA cells tend to exhibit stronger fatty acid oxidation activity compared to primary hepatocytes.[20] Additionally, since the McA cells exhibited a larger increase in trans-nonachlor induced intracellular neutral lipid accumulation and triglyceride levels than primary hepatocytes, this may have indirectly caused a dramatic increase in fatty acid oxidation. Even if the pesticide did cause a direct reduction in fatty acid oxidation, it is possible that the extreme upsurge in neutral lipid accumulation caused a more significant, albeit indirect, escalation of oxidation, thus resulting in a net increase.

Considering that primary hepatocytes are widely considered to be a better model than McA cells, it is likely that the decreased fatty acid oxidation seen in this cell type is a more accurate reflection of general trans-nonachlor induced alterations. Thus, it is likely that decrease fatty acid oxidation is a contributory mechanism towards the development of hepatic steatosis.

4.5 VLDL/triglyceride secretion

Decreased triglyceride laden VLDL secretion poses another possible explanation for intracellular neutral lipid accumulation caused by trans-nonachlor exposure. In McA cells, trans-nonachlor exposure did not affect triglyceride secretion. However, in primary hepatocytes, exposure to the pesticide metabolite caused increased VLDL secretion under steatotic (increased fatty acid) conditions. These results cannot be used to conclusively say whether or not sub-optimal VLDL secretion is a mechanism through which intracellular neutral lipid accumulation occurs. In addition to secreted triglyceride levels, ApoB was measured as an indicator of pre-secretion VLDL stability. ApoB100 is the main protein constituent for VLDL particles. Triglycerides are transported out of the liver in the form of very low density lipoproteins (VLDL). For every VLDL particle, there is one molecule of ApoB.[24] When triglycerides attach themselves to ApoB, it forms a new VLDL particle. If newly-produced ApoB is not lipidated, the hepatocyte will break it down, thus controlling VLDL secretion.[24] While ApoB levels in primary hepatocytes were not affected by trans-nonachlor exposure, cellular ApoB levels in McA cells were significantly decreased following trans-nonachlor exposure under steatotic conditions. In the McA cells, decreased affinity of triglycerides to ApoB100 proteins indicates impaired VLDL stability. Research has pointed towards both similarities and differences in ApoB secretory mechnisms in McA cells vs. primary hepatocytes. Although it has been shown that secretion of ApoB100 is suppressed by insulin in McA cells via a process similar to rat primary hepatocytes,

McA cells react differently to serum-rich culture conditions by developing insulin resistance and subsequently secreting more ApoB and VLDL.[17,21] Thus, there may be additional undiscovered differences regarding ApoB secretion in McA cells that have not yet been brought to light. Given the lack of available information regarding ApoB secretion in McA cells, again these results cannot conclusively say whether alterations in ApoB secretion contribute to potentially sub-sufficient levels of VLDL following trans-nonachlor exposure.

4.6 Oxidative stress

Oxidative stress was investigated as another potential factor in trans-nonachlor induced neutral lipid accumulation. In both cell types, trans-nonachlor had no effects on lipid peroxidation (MDA levels) or levels of cellular glutathione, suggesting that oxidative stress is unlikely to be a mechanism contributing to the intracellular accumulation of lipids.

5. Conclusion

The purpose of this study was to elucidate the similarities and differences in the results of the two parallel studies conducted on immortalized rat hepatoma (McA) cells and rat primary hepatocytes. These two studies investigated the effects of the organochlorine pesticide metabolite, trans-nonachlor, on cellular mechanisms governing hepatic lipid metabolism. Following exposure, both cell types showed significant increases in triglyceride-dominated intracellular neutral lipid accumulation. These results implicate exposure to trans-nonachlor in the promotion of non-alcoholic fatty liver disease (also known as hepatic steatosis), the hepatic component of metabolic syndrome and related conditions such as T2D. From parallel results seen in both cell types, the most likely contributor to this hepatic steatosis is increased de novo lipogenesis. Even though fatty acid oxidation was increased in McA cells, it was, in fact, decreased in primary hepatocytes. Given the fact that the latter cell type is widely considered to be a more physiologically relevant model, it is likely that decreased fatty acid oxidation may play a role in the development of fatty liver. Neither cell type had altered oxidative stress or fatty acid uptake, so it is improbable that these mechanisms are responsible for the hepatic steatosis. The results for VLDL/triglyceride secretion were unfortunately inconclusive, but sub-optimal VLDL secretion should not be discounted as a potential reason for the accumulation of lipids within hepatocytes; further experiments should be conducted to clarify the association.

Over the course of this study, it has become increasingly evident that there is a definite need for the physiological differences between immortalized rat hepatoma cells and rat primary hepatocytes to be investigated. While some studies have revealed differences in cellular mechanisms and genetics, there have been no experiments designed for the sole purpose of

investigating these contradictions. Such information would greatly benefit all future research related to pathologies of the liver.

This investigation has brought to light several likely cellular mechanisms by which trans-nonachlor induces neutral lipid accumulation. However, further studies, particularly those involving *in vivo* experiments, are necessary to help clarify how these physiological alterations are truly manifested in mammals. Most importantly, this study adds to the mounting evidence implicating organochlorine pesticides in the development of metabolic syndrome in humans, a connection which is steadily becoming more difficult to refute.

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