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Inhibition of cholinergic and non-cholinergic targets following subacute exposure to chlorpyrifos in normal and high fat fed male C57BL/6J mice

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Abstract

The effects of obesity on organophosphate pesticide-mediated toxicities, including both cholinergic and noncholinergic targets, have not been fully elucidated. Therefore, the present study was designed to determine if high fat diet intake alters the effects of repeated exposure to chlorpyrifos (CPS) on the activities of both cholinergic and noncholinergic serine hydrolase targets. Male C57BL/6J mice were placed on either standard rodent chow or high fat diet for four weeks with CPS exposure (2.0 mg/kg) for the last 10 days of diet intake. Exposure to CPS did not alter acetylcholinesterase in the central nervous system, but it did significantly inhibit circulating cholinesterase activities in both diet groups. CPS significantly inhibited hepatic carboxylesterase and fatty acid amide hydrolase and this inhibition was significantly greater in high fat fed animals. Additionally, CPS exposure and high fat diet intake downregulated genes involved in hepatic de novo lipogenesis as well as cytochrome P450 enzymes involved in hepatic xenobiotic metabolism. In summary, the present study demonstrates that high fat diet intake potentiates CPS mediated inhibition of both carboxylesterase and fatty acid amide hydrolase in the liver of obese animals following subacute exposure and suggests obesity may be a risk factor for increased non-cholinergic hepatic CPS toxicity.

Keywords

Organophosphate; Chlorpyrifos; High fat diet; Acetylcholinesterase; Carboxylesterase; Endocannabinoid

1. Introduction

Recent epidemiological studies have implicated exposure to organochlorine and organophosphate (OP) pesticides, including chlorpyrifos (CPS), in the progression of

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Conflicts of interest

None of the authors have any conflicts of interest with the present study.

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metabolic dysfunction commonly associated with obesity and type 2 diabetes (Lee et al., 2006a, 2006b, 2007a, 2007b, 2011; Malekiran et al., 2013; Montgomery et al., 2008; Raafat et al., 2012). The potential contribution of OP exposure to the increasing prevalence of obesity and type 2 diabetes is significant given that OP pesticide usage comprises nearly fifty percent of all pesticide usage in the United States and CPS is the most widely utilized among the OP pesticides for agriculture applications (Grube et al., 2011; Solomon et al., 2014). The most widely recognized acute OP toxicity is central nervous system (CNS) toxicity resulting from inhibition of acetylcholinesterase (AChE) in the brain following high levels of OP exposure. However, the roles of other serine hydrolases in OP-mediated toxicities are garnering attention and may mediate potential chronic toxicities following lower levels of OP exposure. Some OP compounds, including CPS following its bioactivation to CPS-oxon, have been shown to be inhibitors of non-cholinergic serine hydrolases such as carboxylesterase (CES), monoacylglycerol lipase (MAGL), and fatty acid amide hydrolase (FAAH) (Quistad et al., 2006). CES encompasses a family of enzymes with different isoforms and tissue distributions that play a primary role in xenobiotic metabolism as well as lipid metabolism through hydrolysis of substrates containing amide and ester bonds (Ross et al., 2010). Major functions of MAGL and FAAH are the control of endogenous endocannabinoid signaling where they metabolize 2-arachidonoylglycerol (2-AG) and anandamide (AEA), respectively (Nomura et al., 2008a, 2008b). Hydrolysis of these two endocannabinoids tightly regulates their availabilities for signaling via cannabinoid receptors (Nomura et al., 2008b).

While the afore mentioned studies have implicated OP pesticide exposure as a potential risk factor for the development of obesity, Lee et al. (2014) recently demonstrated increased BMI is a predictive indicator of severity of systemic intoxication following acute OP exposure in humans, especially highly lipophilic OP pesticides such as CPS (Lee et al., 2014). Obese patients had a longer stay in the intensive care unit, had to be maintained on mechanical ventilation for a longer duration, and stayed in the hospital for a longer period of time when compared to lean patients. This indicated that the metabolic phenotype associated with obesity may augment OP-induced toxicity following high exposures. Recent studies from Kondakala et al. (2017) demonstrated that acute exposure to CPS, which was devoid of inhibitory effects on AChE activity in the CNS, inhibited hepatic CES activity in lean animals and this inhibitory effect was potentiated in high fat fed, obese animals (Kondakala et al., 2017). Additionally, hepatic FAAH activities were significantly inhibited in high fat fed, obese animals, whereas there was no effect on FAAH activity in lean animals. Thus, these studies indicate body composition may adversely affect cholinergic and non-cholinergic toxicities following acute CPS exposure, which may be dependent on level of exposure.

Given these previous studies implicating body composition as a potential factor in CPS-mediated toxicity following acute exposure, the present study was designed to determine if body composition altered cholinergic and non-cholinergic serine hydrolase activities following repeated exposure to CPS where repeated exposure may promote cumulative toxicity. Cholinergic targets were central (CNS) and peripheral (RBC and plasma) AChE activities. Non-cholinergic targets were assessed via effects on CES, MAGL, and FAAH activities primarily in the liver given its vital role in first pass metabolism and bioactivation

of CPS as well as its central role in systemic lipid metabolism and glucose homeostasis. However, effects of CPS exposure on CES activity in the adipose tissue as well as MAGL and FAAH activities in the CNS were also determined given the high prevalence and functional significance of these serine hydrolases in the individual tissues.

2. Materials and methods

2.1. Animal care and use

Male C57BL/6J mice (n = 40) were purchased from Jackson Laboratory at 4 weeks of age and allowed to acclimate for one week prior to experimental manipulation. All animals were housed at the Mississippi State University (MSU) College of Veterinary Medicine in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved animal facility. Animals were group housed on a 12-h light/dark cycle with access to food and water ad libitum. All animal protocols were approved by the MSU Institutional Animal Care and Use Committee (IACUC).

2.2. Experimental design

Following a one-week acclimation period, animals were divided into two groups with one group being maintained on standard rodent chow (n = 20; Teklad 18% protein rodent diet) and the other group being placed on a high fat diet (60% kcal from lard; Research Diets). Animals were maintained on these dietary regimens for a total of 28 days (4 weeks). For the last 10 days of standard or high fat diet intake, animals were treated with either vehicle (n = 10/group; corn oil; 1 ml/kg) or CPS (n = 10/group; 2.0 mg/kg) via oral gavage once per day. Previous studies in our lab and by others have shown that acute exposure to CPS (2.0 mg/kg) via oral gavage significantly inhibits both red blood cell (RBC) AChE and plasma cholinesterase (ChE; a mixture of butyrylcholinesterase and AChE) activities without significant inhibition of CNS AChE (Kondakala et al., 2017; Zheng et al., 2000). Therefore, the present dosage was utilized to be devoid of inhibitory effects on CNS AChE and to allow comparison between our previous studies using acute CPS exposure in high fat fed animals. Following the last dose of CPS, animals were fasted for 12 h prior to euthanasia by CO₂ asphyxiation. Blood was collected via cardiac puncture in heparinized vacutainers and separated via centrifugation to obtain RBC and plasma fractions then stored at -80 °C. Tissues (brain, liver, and adipose tissue) were collected and immediately snap frozen in liquid nitrogen then stored at -80 °C for subsequent analysis.

2.3. Metabolic phenotyping

Physical and biochemical metabolic phenotyping was performed as previously described (Howell et al., 2014; Kondakala et al., 2017; Mulligan et al., 2017). Immediately prior to sacrifice, animals were weighed then fasting blood glucose was determined via handheld glucometer (Alphatrak II; Bayer Animal Health) in whole blood obtained via tail nick. Fasting blood levels of insulin (Ultrasensitive Mouse Insulin ELISA; Crystal Chem), triglyceride (Cayman Chemical), and total cholesterol (Amplex Red Total Cholesterol Assay; Life Technologies) levels were determined from the isolated plasma fraction. Hepatic steatosis was assessed via measurement of hepatic triglyceride content (Cayman Chemical) and expressed as micromoles (μmoles) of triglyceride per milligram (mg) of protein.

2.4. Tissue cholinesterase activities

Plasma ChE as well as RBC and CNS AChE activities were determined via a modification of the Ellman method as previously described (Chambers and Chambers, 1989, 1990; Ellman et al., 1961; Kondakala et al., 2017). Briefly, RBCs were diluted to a final concentration of 0.0005 μ l RBC/ml in sodium phosphate buffer (0.1 M, pH 7.4) and absorbance monitored continuously after addition of substrates for 60 min at 412 nm. Brain tissue was homogenized in 0.05 M Tris-HCl (pH 7.4) and homogenates were then pre-incubated at 37 °C for 15 min. Acetylthiocholine (1 mM final concentration; Sigma Aldrich) was then added as a substrate and the reaction continued for an additional 15 min. At the end of 15 min, the reaction was terminated by addition of 250 μ l of a 4:1 (v:v) mixture of 5% SDS:5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) then absorbance measured at 412 nm. Protein concentrations were measured by Bradford assay and data expressed as nanomoles (nmoles) per minute per mg protein.

2.5. Tissue CES activities

Total CES activities in both liver and adipose tissue were determined by continuous CES assay as previously described (Crow et al., 2007; Kondakala et al., 2017). Briefly, hydrolysis reactions were carried out with 100 μ g tissue homogenate protein/ml using *para*-nitrophenyl valerate (Sigma Aldrich) as substrate at a final concentration of 500 μ M. Reactions were pre-incubated for 5 min at 37 °C then initiated by addition of diluted tissue homogenates in 50 mM Tris-HCl (pH 7.4) buffer. The reaction was continued for 5 min with continuous measurement at 405 nm to determine the rate of *para*-nitrophenol formation. An extinction coefficient of 13 cm⁻¹ mM⁻¹ was used for calculation and data were normalized to enzyme activity units then expressed as μ mole per minute per mg protein (Morgan et al., 1994).

2.6. Endocannabinoid hydrolysis activities

The activities of MAGL and FAAH were determined by hydrolysis reactions utilizing their primary endogenous endocannabinoid substrates 2-AG and AEA, respectively, as previously described (Carr et al., 2013, 2014; Kondakala et al., 2017). Briefly, tissue homogenates were diluted with Tris-HCl (50 mM, pH 7.4) to a final concentration of 0.5 mg/ml then pre-incubated at 37 °C for 5 min. Following pre-incubation, homogenates were spiked with either 2-AG (50 μ M; Cayman Chemical), AEA (50 μ M; Cayman Chemical), or ethanol (2% v/v final concentration) as a blank, then reactions were incubated for 30 min at 37 °C to determine MAGL and FAAH activities, respectively. Reactions were terminated with cold acetonitrile containing deuterated arachidonic acid (2.5 μ M; AA-d8; Cayman Chemical). Samples were centrifuged for 10 min at 16,000 $\times$ g and supernatant transferred to LC-MS vials for measurement of arachidonic acid (AA). Hydrolytic activities of MAGL and FAAH are expressed as the ratio of peak areas for AA/AA-d8 following background subtraction of endogenous AA levels.

2.7. Hepatic gene expression analysis

Expression of key genes regulating hepatic lipid and xenobiotic metabolism were examined by real-time polymerase chain reaction (PCR) as previously described (Mulligan et al., 2017). Genes governing lipid hepatic lipid metabolism include the lipogenic genes sterol

response element binding protein-1c (*Srebp-1c*), fatty acid synthase (*Fas*), and acetyl-CoA carboxylase-1 (*Acc-1*), the fatty acid translocase *Cd36*, and genes governing fatty acid oxidation including carnitine palmitoyl transferase-1a (*Cpt-1a*) and peroxisomal acyl-coenzyme A oxidase-1 (*Acox-1*). Genes governing xenobiotic metabolism include cytochromes 3a11 (*Cyp3a11*), 1a2 (*Cyp1a2*), 2b10 (*Cyp2b10*), and 2c29 (*Cyp2c29*). These cytochromes are murine orthologues of the prominent human cytochrome P450 enzymes (Nelson et al., 2004). To measure gene expression, total RNA was isolated (Genejet RNA Isolation kit; Thermo Fisher) then cDNA was transcribed (Verso cDNA Synthesis Kit; Thermo Fisher) from 1 µg of total RNA. cDNA amplification was measured by Sybr Green detection (Sybr Select Master Mix; Thermo Fisher) with beta-actin as housekeeping gene. Alterations in target gene expression were determined by the Ct method as previously performed (Howell et al., 2009; Mulligan et al., 2017). Data are expressed as the fold change from the average of standard diet fed vehicle-treated animals.

2.8. Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM) for each group. Statistical analysis was performed by two-way ANOVA with Tukey's post hoc test for pairwise comparisons (SigmaPlot v12.5) to determine effects of diet and CPS treatment. A P-value of less than or equal to 0.05 was deemed to be the threshold for statistical significance. For real-time PCR data, statistical analysis was performed on Ct values and not fold change from standard diet fed vehicle-treated values.

3. Results

3.1. Effects of subacute exposure to CPS on physical and biochemical indices of metabolic function

As anticipated, consumption of high fat diet for 4 weeks induced weight gain, dyslipidemia, and hepatic steatosis (Table 1). Specifically, animals on the high fat diet had significantly greater body weights when compared to corresponding standard chow fed animals with increases of 12.5% in vehicle-treated animals and 12.4% in CPS-treated animals. This increase in body weight was accompanied by increased liver triglyceride accumulation and hypertriglyceridemia. Hepatic triglyceride content was significantly increased by 79% in high fat fed animals in the vehicle-treated group and by 75% in high fat fed animals in the CPS-treated group compared to corresponding controls. Increased hepatic triglyceride content was associated with a significant increase in plasma triglyceride content by 30% in high fat fed animals versus standard chow fed animals in the vehicle-treated group but not in the CPS-treated group (14% increase compared to standard chow fed) which may suggest a decrease in VLDL secretion following CPS exposure. Plasma total cholesterol levels were significantly increased following CPS exposure in high fat fed animals by 22% compared to standard chow fed animals. However, cholesterol levels were not significantly elevated in vehicle-treated high fat fed animals suggesting CPS exposure increases high fat diet-induced hypercholesterolemia. Fasting glucose and insulin levels were not significantly altered following high fat diet feeding and CPS exposure indicating a lack of significant effect of diet or CPS treatment on glucose homeostasis.

3.2. Subacute CPS exposure inhibits RBC and plasma cholinesterase activities

Subacute exposure to CPS in both feeding groups did not significantly inhibit AChE activity in the CNS (Fig. 1A). However, peripheral indices of cholinergic toxicity including RBC AChE and plasma ChE were significantly inhibited by CPS. RBC AChE activity was significantly inhibited by 42% in standard chow fed animals and by 40% in high fat fed animals following CPS exposure (Fig. 1B). Interestingly, there was a significant increase in overall RBC AChE activity by 64% in vehicle-treated animals following high fat diet intake. However, this increase in RBC AChE activity was inhibited by CPS to a comparable extent in high fat fed animals compared to standard chow fed animals. Plasma ChE activities displayed a similar pattern of diet effect and CPS-mediated effects compared to RBC AChE activities (Fig. 1C). Exposure to CPS significantly inhibited plasma ChE activities by 57% in standard chow fed animals and by 76% in high fat fed animals. As observed with RBC AChE activities, high fat feeding significantly increased plasma ChE activities. Taken together, the present data indicate subacute exposure to CPS significantly inhibits peripheral, circulating ChE activities and not CNS AChE activity.

3.3. CPS-mediated inhibition of hepatic CES is increased in high fat fed animals

CPS-mediated inhibition of hepatic CES activity was determined as a potential non-cholinergic target of CPS given that CES plays a major role in CPS detoxification as well as a major role in hepatic lipid metabolism. Exposure to CPS significantly inhibited hepatic CES activities in both standard chow and high fat diet fed animals by 33% and 61%, respectively, compared to their corresponding vehicle-treated controls (Fig. 2A). Additionally, CPS-mediated inhibition of CES activity was significantly greater in high fat fed animals when compared to CP-treated standard chow fed animals. Interestingly, high fat feeding significantly decreased basal hepatic CES activities by 17% in vehicle-treated animals indicating an inhibitory effect of diet. Thus, these data indicate a significant interaction between diet and CPS exposure with high fat feeding potentiating CPS-mediated inhibition of hepatic CES activity. These effects of subacute CPS exposure on CES activity appear to be primarily isolated to the liver given CPS exposure actually increased CES activity in the adipose of animals on standard chow diet (Fig. 2B). Interestingly, high fat diet intake significantly decreased adipose CES activity by 76% in vehicle-treated animals and this decrease was not significantly altered by CPS exposure.

3.4. Hepatic MAGL and FAAH are inhibited by CPS in high fat fed animals

Although CES is a major serine hydrolase involved in CPS detoxification, MAGL and FAAH have been shown to be inhibited by CPS exposure and are endocannabinoid metabolizing enzymes. Thus, they represent potential non-cholinergic targets of CPS. Hepatic MAGL and FAAH activities in liver homogenates were determined by measuring the rates of hydrolytic metabolism of their endocannabinoid substrates (2-AG and AEA, respectively) to arachidonic acid (Fig. 3). Exposure to CPS did not significantly alter MAGL activity in standard chow fed animals. However, MAGL activity was significantly increased by 31% in vehicle-treated high fat fed animals compared to vehicle-treated high fat fed animals and this increase was significantly inhibited by CPS exposure (Fig. 3A). Thus, CPS only inhibited MAGL activity in high fat fed animals.

In contrast to hepatic MAGL activity, hepatic FAAH activity was more sensitive to the inhibitory effect of CPS and were more akin to the effects of CPS on hepatic CES activity. CPS exposure significantly inhibited hepatic FAAH activities in standard chow and high fat fed animals by 29% and 69%, respectively, compared to their corresponding vehicle-treated controls (Fig. 3B). Thus, the extent of CPS-mediated inhibition of hepatic FAAH was over 2-fold greater in high fat fed animals compared to standard chow fed animals. These data indicate that CPS inhibited FAAH activity in both standard chow and high fat diet fed animals. Like CES activity, the degree of CPS-induced suppression was enhanced in high fat fed animals. Interestingly, MAGL (Fig. 3C) and FAAH (Fig. 3D) activities in the CNS were not significantly inhibited following CPS exposure regardless of dietary regimen.

3.5. Exposure to CPS and high fat diet intake independently decrease hepatic lipogenic gene expression

The effects of subacute exposure on key genes governing hepatic lipid metabolism was explored given the potential roles of CES, FAAH, and MAGL in altering hepatic lipid metabolism. Exposure to CPS significantly decreased the expression of *Fas* and *Acc-1*, mediators of lipogenesis, as well as *Acox-1*, a mediator of peroxisomal fatty acid oxidation, in standard chow fed animals (Fig. 4). This inhibitory effect of CPS exposure on *Fas* and *Acc-1* expression was also observed in high fat fed animals, whereas the effects of CPS on *Acox-1* were restricted to standard chow fed animals. High fat intake also significantly decreased the expression of *Fas* and *Acc-1* in vehicle-treated animals indicating an inhibitory effect of high fat diet on these lipogenic enzymes. However, the inhibitory effect of high fat diet on *Fas* and *Acc-1* expression was not altered by CPS exposure.

3.6. Hepatic cytochrome P450 expression is decreased by diet and CPS exposure

Metabolism of CPS, including its desulfuration and dearylation, is governed by hepatic P450 isoforms. Thus, expression of the murine isoforms, *Cyp3a11*, *Cyp1a2*, *Cyp2b10*, and *Cyp2c29*, which are orthologues of the known human and rat P450 isoforms that govern CPS metabolism were explored. Overall, exposure to CPS in the standard chow fed animals elicited marked reductions in P450 expression with significant decreases in *Cyp3a11* and *Cyp2b10* compared to vehicle treated standard chow fed animals (Fig. 5). High fat diet intake in vehicle treated animals also significantly decreased expression of all measured P450 isoforms compared to standard chow fed vehicle treated animals. Additionally, CPS exposure in high fat fed animals significantly reduced *Cyp1a2*, *Cyp2b10*, and *Cyp2c29* expression compared to CPS exposure in standard chow fed animals. Taken together, there was an overall depressive effect of high fat feeding on expression of each P450 isoform and this depressive effect exacerbated the CPS-mediated inhibition of *Cyp1a2*, *Cyp2b10*, and *Cyp2c29* when compared to standard chow fed animals.

4. Discussion

The goal of the present study was to examine the effects of repeated exposure to CPS on both cholinergic and non-cholinergic endpoints of OP toxicity in both lean and high fat fed, obese animals to determine whether alterations in body composition, i.e. increased adiposity, alters CPS toxicity as indicated by inhibition of target serine hydrolases. As noted, there is a

relative lack of human and animal studies examining the effects of body composition on OP toxicities. Lee et al. (2014) determined that increased BMI has a detrimental effect on OP toxicity, especially CPS, by increasing severity of intoxication and thus requiring more intensive medical treatment (Lee et al., 2014). Recently, our group demonstrated that acute exposure to a single dose of CPS via oral gavage, at a concentration that does not cause inhibition of AChE in the CNS, significantly inhibited hepatic CES and FAAH in high fat fed animals with a significant interaction between CPS exposure and high fat diet intake (Kondakala et al., 2017). On the basis of this study, it appears that high fat diet intake significantly exacerbates the inhibitory effects of acute CPS exposure on hepatic CES and FAAH activities. However, the effects of repeated CPS exposure were not examined, which is of significance given the reported elimination half-life of 21 h following dermal exposure in mice and 27 h following oral exposure in humans (Nolan et al., 1984; Shah et al., 1981). This prolonged half-life would be expected to promote CPS bioaccumulation and increase CPS-mediated effects over time. This bioaccumulative effect is evident in previous studies demonstrating repeated dosing of CPS promotes significant RBC AChE inhibition at lower doses when compared to acute exposure in rats (Marty et al., 2012; Zheng et al., 2000).

The current data indicate that repeated exposure to CPS (2.0 mg/kg) via oral gavage for 10 days did not significantly alter AChE activity in the CNS in either standard chow or high fat fed mice. This is in agreement with our previous observations following acute exposure to CPS (2.0 mg/kg) in animals fed standard chow or high fat diet for four weeks (Kondakala et al., 2017). This lack of significant effect of CPS on CNS AChE with acute or subacute administration of toxicant was not unexpected given that previous studies have demonstrated CPS (3.12 mg/kg; oral gavage) exposure for 5 days produces only 10% AChE inhibition in the brain (Cometa et al., 2007). Further, the calculated no observable effect limit (NOEL) for brain AChE activity following 30-day exposure to CPS in mice via oral gavage is 1.56 mg/kg/day (Cometa et al., 2007). In the current study, both plasma ChE and AChE in RBCs were more sensitive than CNS AChE to CPS treatment, as previously reported following subacute CPS exposure in both standard chow and high fat fed rats (Zheng et al., 2000). Interestingly, the currently observed inhibitory effects of CPS on RBC AChE activity were comparable to those previously observed following acute CPS exposure in animals receiving the same dietary regimen and did not indicate increased inhibition following repeated exposures (Kondakala et al., 2017). This may indicate that CPS-oxon in the circulatory system has reached a steady-state with the current 10-day dosing regimen. However, plasma ChE activity in the vehicle-treated high fat fed animals was significantly greater than that in vehicle-treated standard chow fed animals in the current study whereas plasma ChE activity in the vehicle-treated high fat fed animals was significantly lower than in vehicle-treated standard chow fed animals in our previous study (Kondakala et al., 2017). This alteration in plasma ChE activity in the high fat fed group may be due to an interaction between the high fat diet and repeated corn oil administration given that there did not appear to be a marked alteration in plasma ChE in the standard chow fed animals during acute or subacute experimental paradigms.

Previous studies have revealed that acute oral exposure to CPS (1 or 2.0 mg/kg) do not significantly alter RBC AChE activities in humans which is in contrast to our previous studies demonstrating marked inhibition of RBC AChE activity following oral exposure to

CPS (2.0 mg/kg) by 39% in C57BL/6J mice (Griffin et al., 1999; Kondakala et al., 2017; Timchalk et al., 2002). This difference may be due to differences in oral absorption of CPS between species with approximately 70% absorption in humans and 90% absorption in mice (Ahdaya and Guthrie, 1981; Nolan et al., 1984). With regards to repeated exposures of CPS as used in the current study, a NOAEL of 0.1 mg/kg/day for humans in terms of RBC AChE inhibition has been reported which is greater than ten-fold lower than the presently utilized concentration of CPS (Coulston et al., 1972). Occupational exposure would be the most likely type of exposure to receive elevated doses of CPS on a routine basis. Phung et al. (2012) compared CPS exposure levels between farmers and pesticide workers with USA farmers being the lowest with a median exposure of 2.5 µg/kg/day and USA pesticide workers being the highest with a median exposure of 53.2 µg/kg/day and a maximum exposure of 210 µg/kg/day in the USA pesticide worker group, which is above the NOAEL for repeated CPS exposures (Phung et al., 2012). Additional studies by Singleton et al. (2015) demonstrated significant inhibition of blood AChE in Egyptian pesticide applicators following CPS application indicating repeated CPS exposures above the NOAEL for RBC AChE inhibition (Singleton et al., 2015). While our currently utilized repeated dose of 2.0 mg/kg/day is above the NOAEL for RBC AChE inhibition, in terms of risk assessment using applied safety factors of 4x for intraspecies variation and 10x for Food Quality Protection Act (FQPA) adjustment, a calculated dose of 50 µg/kg/day could have applicability given current occupational exposure levels. Additionally, intentional ingestion of CPS has been shown to result in a dose of 300 mg/kg which is 150-fold greater than the currently employed dose for repeated exposure (Lotti et al., 1986).

Based on the literature, OP pesticides can promote alterations in glucose homeostasis and systemic lipid metabolism. Specifically, fasting hyperglycemia and hypertriglyceridemia have been noted following acute exposure to CPS (50 mg/kg) via subcutaneous injection in rats (Acker and Nogueira, 2012). In our previous study, acute exposure to CPS (2 mg/kg) via oral gavage produced hypoglycemia in animals fed a high fat diet (Kondakala et al., 2017). The current data indicate a comparable pattern to our previous study, with a decrease in fasting blood glucose in high fat fed mice following subacute exposure to CPS. With regards to effects on plasma triglycerides, our current data indicate that subacute exposure to CPS did not significantly alter plasma triglyceride levels in animals fed the standard chow diet. This is in contrast to our previous observations where acute exposure to CPS caused significant hypertriglyceridemia in standard chow fed animals (Kondakala et al., 2017). It should be noted that the significant increase in plasma triglycerides induced by high fat feeding was not observed in subacute CPS exposure group. Thus, the possibility arises that subacute exposure to CPS significantly decreases very low density lipoprotein (VLDL) secretion in high fat fed animals.

Of the currently examined serine hydrolases, the most sensitive noncholinergic targets of CPS or CPO is CES with ranking sensitivities of CES > FAAH > MAGL (Casida and Quistad, 2004, 2005; Quistad et al., 2006). As mentioned, CES is a diverse family of enzymes with tissue specific distribution patterns and well-known roles in xenobiotic metabolism including xenobiotic detoxification (Potter and Wadkins, 2006; Ross et al., 2010; Satoh and Hosokawa, 1998; Satoh et al., 2002). Our current data indicate subacute exposure to CPS inhibits hepatic CES activity and this inhibition is potentiated by high fat

diet feeding. These data are in agreement with our previous studies with acute CPS exposure where CPS-mediated inhibition of hepatic CES activity was also potentiated in high fat fed animals (Kondakala et al., 2017).

This effect of CPS treatment on hepatic CES activity may alter hepatic lipid metabolism given the emerging role of CES isoforms in hepatic lipid metabolism. Specifically, global and liver specific deletion of *Ces1d* has been shown to improve dyslipidemia by lowering plasma VLDL triglyceride presumably by decreasing hepatic VLDL secretion (Lian et al., 2012a, 2012b). This observed decrease in VLDL resulting from *Ces1d* deletion may be due to decreased fatty acid availability resulting from decreased de novo lipogenesis or increased fatty acid oxidation (Lian et al., 2012a, 2012b). Our current gene expression data demonstrate a CPS-mediated decrease in *Fas* and *Acc-1* expression, which may indicate a decrease in hepatic de novo lipogenesis. However, another CES isoform, *Ces1g*, was shown to have a beneficial effect in terms of hepatic lipid metabolism in that ablation of *Ces1g* promotes hepatic steatosis and dyslipidemia, which could be rescued with restoration of hepatic *Ces1g* expression/activity (Bahitham et al., 2016; Quiroga et al., 2012). In the present study, CPS exposure in combination with high fat feeding did not significantly increase plasma triglycerides, whereas vehicle-treated high fat fed animals displayed significant elevations in plasma triglycerides. Thus, inhibition of hepatic *Ces1d* may partially explain this reduction. Future studies will be necessary to dissect which CES isoforms are inhibited following CPS exposure in both standard chow and high fat fed mice.

One of the most interesting findings of the current study is that, similar to acute CPS exposure, subacute CPS exposure mediated inhibition of hepatic CES and FAAH was potentiated in high fat fed animals. A possible explanation may lie in the overall reduction of cytochrome P450 expression in the present study. In this scenario, CPS detoxification may not balance with CPS bioactivation. The high fat diet-induced potentiation of CPS inhibition of non-cholinergic serine hydrolases appeared to be selective for the liver because there was a lack of MAGL or FAAH inhibition in the CNS and a lack of CES inhibition in the adipose. Given that CPS was administered via oral gavage, the possibility arises CPS is sequestered into the lipid droplets of the steatotic livers of high fat fed animals and slowly leaches out to produce a more pronounced hepatic effect. This would be in line with a hypothesis proposed by Chambers and Carr (1993) where the lipophilic CPS may form a storage depot in lipid rich tissues (Chambers and Carr, 1993). Further toxicokinetic studies with CPS are warranted to determine if high fat fed obese animals display prolonged cholinergic and non-cholinergic toxicities following cessation of CPS exposure and to determine the effects of chronic exposures which may promote more pronounced CPS accumulation compared to acute and subacute exposures.

In summary, the present study demonstrated a significant interaction between high fat diet and subacute CPS exposure with regards to inhibition of hepatic CES and FAAH activities. High fat diet intake significantly potentiated CPS-mediated inhibition of these two serine hydrolases. Additionally, repeated exposure to CPS significantly inhibited hepatic MAGL activity in high fat fed animals. These effects of CPS appeared to be restricted to the periphery given CPS did not significantly alter AChE, MAGL, or FAAH activities in the CNS. Overall, the present data indicate that alterations in body composition such as

increased adiposity and hepatic steatosis may potentiate CPS-mediated inhibition of hepatic serine hydrolases that play key roles in lipid metabolism, indicating obesity may be a relatively unexplored risk factor for CPS toxicity. As mentioned, future studies are warranted to determine the effects of high fat diet and resulting obesity on the toxicokinetics of CPS as well as on the toxicity of chronic CPS exposure in terms of cholinergic and non-cholinergic targets.

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Fig. 1. Effects of CPS exposure on CNS, RBC, and plasma ChE activities. Twelve hours following CPS exposure, AChE activities in brain (A.), RBCs (B.), or cholinesterase (ChE) activity in plasma (C.) were determined. Data are expressed as mean $\pm$ SEM of $n = 8$ animals/group. * $P < 0.05$ vs. vehicle-treated SD; # $P < 0.05$ vs. vehicle-treated high fat diet (HFD).

Fig. 2.

Hepatic CES activity was significantly inhibited by CPS in standard and high-fat diet fed animals. Hepatic (A.) and adipose (B.) CES activities were determined in tissue homogenates using *para*-nitrophenyl valerate as substrate. Data represent the mean $\pm$ SEM of $n = 8$ animals/group for liver and mean $\pm$ SEM of $n = 6$ animals/group for adipose. * $P < 0.05$ vs. vehicle-treated SD; # $P < 0.05$ vs. vehicle-treated HFD; & $P < 0.05$ vs. CPS-treated SD.

Fig. 3. Hepatic 2-AG and AEA hydrolysis was significantly inhibited following CPS exposure in high-fat fed animals. Following sacrifice, hepatic 2-AG (A.) and AEA (B.) hydrolysis and CNS 2-AG (C.) and AEA (D.) hydrolysis were determined as an index of MAGL and FAAH activities, respectively. Data represent the mean $\pm$ SEM of $n = 5-6$ animals/group. * $P < 0.05$ vs. vehicle-treated SD; # $P < 0.05$ vs. vehicle-treated HFD; & $P < 0.05$ vs. CPS-treated SD.

Fig. 4.

CPS exposure and high fat diet intake decrease hepatic lipogenic gene expression.

Expression of genes governing key processes in hepatic lipid metabolism was determined by real-time PCR following sacrifice. Data are expressed as the fold change from standard diet (SD), vehicle-treated controls and represent the mean $\pm$ SEM of $n = 5-6$ animals/group. * $P < 0.05$ vs. SD vehicle-treated animals.

Fig. 5.

High-fat diet intake decreases hepatic p450 expression. Expression of major hepatic p450 isoforms was determined by real-time PCR following sacrifice. Data are expressed as the fold change from SD vehicle-treated controls and represent the mean $\pm$ SEM of $n = 5-6$ animals/group. * $P < 0.05$ vs. vehicle-treated SD; & $P = 0.05$ vs. CPS-treated SD.

Table 1

Physical and biochemical measurements following CPS exposure. Mice were fed standard rodent chow or high fat diet for four weeks with administration of CPS (2.0 mg/kg; oral gavage) for the final 10 days. Data represent the mean $\pm$ SEM of n = 8–10 animals/group.

Measurement @ sacrifice	CPS 2.0 mg/kg		
	Vehicle	Standard diet	High fat diet
Glucose (mg/dL)	226.9 (4.8)	215.4 (10.2)	219.3 (13.2)
Insulin (ng/ml)	0.47 (0.04)	0.40 (0.05)	0.49 (0.05)
Triglyceride (mg/dL)	121.6 (9.6)	158.3 (9.8) *	125.3 (7.5)
Total cholesterol (mM)	1.35 (0.06)	1.48 (0.06)	1.28 (0.07)
Liver triglyceride (μ mole/mg)	7.1 (0.6)	12.7 (1.8) *	7.1 (0.4)
Body weight (g)	24.7 (0.7)	27.8 (0.8) *	24.9 (0.3)
			28.0 (0.6) * $\&$

* P 0.05 vs. vehicle-treated Standard diet (SD)

$\&$ P 0.05 vs. CPS-treated SD.