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Permethrin and ivermectin modulate lipid metabolism in steatosis-induced HepG2 hepatocyte

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ABSTRACT

Recent studies have reported the positive association between exposure to insecticides and increased risk of obesity and type 2 diabetes, which are closely associated with non-alcoholic fatty liver disease (NAFLD). However, it is not known if insecticide exposure can contribute to NAFLD. Thus, the goal of the current study was to determine if insecticide exposures can exacerbate the physiological conditions of NAFLD by modulating hepatic lipid metabolism. The effects of 12 insecticides on triglycerides (TG) accumulation were tested using palmitic acid (PA)-induced HepG2 hepatoma steatosis model. Results showed that among tested insecticides, permethrin and ivermectin significant interacted with palmitic acid to potentiate (permethrin) or decrease (ivermectin) TG accumulation. Further study showed that permethrin significantly promoted fatty acid synthesis, while suppressed lipid oxidation-related genes only under steatosis conditions. In comparison, ivermectin inhibited lipogenesis-related genes and promoted farnesoid X receptor, which upregulates fatty acid oxidation. Results in this study suggested that hepatic lipid metabolism may be more susceptible to insecticide exposure in the presence of excessive fatty acids, which can be associated with the development of NAFLD.

1. Introduction

Recent studies have reported that insecticide exposures may disrupt lipid and glucose metabolism and contribute to the development of obesity and type 2 diabetes (Sun et al., 2017a; Swaminathan, 2013; Xiao et al., 2017a). Specifically, exposures to various insecticides induced triglyceride (TG) accumulation in adipocytes (Kim et al., 2014, 2017; Park et al., 2013; Sun et al., 2016a) and interacted with dietary fat, leading to disrupted lipid metabolism and insulin resistance in mice (Sun et al., 2016b, 2017c; Xiao et al., 2017b, 2018).

Non-alcoholic fatty liver disease (NAFLD) exhibits excessive TG accumulation in the liver without routine alcohol intake and is considered the most common liver disease, with approximately 25% of adults affected worldwide (Younossi et al., 2016). There are several factors that could contribute to the development of NAFLD, including lifestyle, bodyweight and diet (Neuschwander-Tetri, 2010; Postic and Girard, 2008). NAFLD starts benignly but can progress to hepatitis, fibrosis or liver cancer due to liver injury (Argo and Caldwell, 2009). NAFLD treatments are currently geared to dietary restriction and weight loss, with limited standard treatment (Centis et al., 2013; Cheng et al., 2016). It is known that metabolic disorders, such as obesity and type 2 diabetes, are significantly correlated to the pathogenesis of NAFLD (Gaggini et al., 2013; Krishan, 2016; Marchesini et al., 2001). Additionally, it has been reported that the exposure to insecticide increased hepatic fat accumulation via altered hepatic lipid metabolism that may lead to the development of NAFLD (Yang and Park, 2018; Xiao et al., 2017a). Thus, it is important to determine how insecticides modulate hepatic lipid metabolism. In this study, 12 commonly used insecticides from the groups of avermectin (ivermectin), carbamate (aldicarb), neonicotinoid (imidacloprid), organochlorine (4,4 ′-dichlorodiphenyldichloroethylene, DDE, and 4,4′-dichlorodiphenyltrichloroethane, DDT), organophosphorus (malathion), phenylpyrazole (fipronil), pyrethroids (cypermethrin, permethrin, and bifenthrin), and ryanoid (chlorantraniliprole) were selected based on their popularities and availabilities to evaluate their effect on TG accumulation in HepG2 hepatocytes. The insecticides used and their mode of actions have been summarized in Supplementary Table S1.

Based on the knowledge that (i) the liver is the primary organ for

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metabolisms of xenobiotics and (ii) insecticides may potentiate fat accumulation in the liver, the goal of this study was to determine how insecticides, particularly permethrin and ivermectin, influence hepatic lipid metabolism, and if insecticide exposure can aggravate the symptoms of NAFLD using palmitic acid (PA)-induced steatosis in HepG2 hepatocytes.

2. Materials and methods

2.1. Materials and chemicals

HepG2 human hepatocytes were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and Trypan Blue stain were purchased from Thermo Fisher Scientific (Waltham, MA). Fetal bovine serum (FBS) and fatty acid free bovine serum albumin (BSA, purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin was from GE Healthcare (Marlborough, MA). The insecticides, cypermethrin (> 98%), DDT (> 98.7%), DDE (> 99.7%), and permethrin (> 98.1%, a mixture of 38.7% *cis* and 59.4% *trans* isomers) were purchased from Fluka Chemical Corp. (Milwaukee, WI). Ivermectin (> 98%) was purchased from Alfa Aesar (Haverhill, MA). Deltamethrin (> 99%), fipronil (> 98%), imidacloprid (> 99.5%), and malathion (> 99.5%) were purchased from Chem Service (West Chester, PA). Aldicarb (> 98%), bifenthrin (> 98%), and chlorantraniliprole (> 99.7%) were obtained from National Pesticide Standard Repository, Environmental Protection Agency (Washington, DC). Palmitic acid (> 99%) was from Nu-Chek Prep, Inc. (Elysian, MN). Primary antibodies used to detect protein expression by western blot analysis included rabbit antibodies against phosphorylated and non-phosphorylated eukaryotic initiation factor 2 α (p-eIF2 α and eIF2 α), phosphorylated and non-phosphorylated 5′ adenosine monophosphate-activated protein kinase α (p-AMPKα and AMPKα), phosphorylated and non-phosphorylated acetyl-CoA carboxylase (p-ACC and ACC), inositol-requiring enzyme 1 α (IRE1α), and binding immunoglobulin protein (BiP) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit antibody against phosphorylated IRE1α (p-IRE1α) was purchased from Abcam (Cambridge, UK). Rabbit antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and secondary goat horseradish peroxidase (HRP)-conjugated antibodies against rabbit were obtained from Santa Cruz Biotechnology (Dallas, TX).

2.2. Cell culture and treatment

HepG2 hepatocytes were cultured in DMEM with 10% FBS, 10,000 U/mL penicillin and 10 mg/mL streptomycin and kept at 37 °C with 5% CO_2 and 95% air, as previously described (Kim et al., 2013). All insecticide samples were dissolved in dimethyl sulfoxide (DMSO) as vehicles and further diluted to achieve final concentrations of 1–50 μM. The final DMSO concentration in cell culture media was maintained at 0.1% v/v through the entire study. Cell viability was evaluated via direct viable cell count. HepG2 cells were treated with insecticides for 24 h and subjected to viable cell count using a hemocytometer (Thermo Fisher Scientific, Waltham, MA) and the dye exclusion method with 0.4% Trypan Blue stain. There was no interaction between PA and these insecticides with respect to cell viability (Suppl. Figs. S1A–S1B). PA decreased total cell count when treated with permethrin ($P < 0.0001$, Suppl. Fig. S1A), which is consistent with a previous observation that 200 μM PA induced about 20% lipoapoptosis in HepG2 cells after 24 h treatments (Malhi et al., 2006; Ricchi et al., 2009). By comparison, either 50 μM of permethrin or 10 μM of ivermectin had no effect on the total cell count (Suppl. Figs. S1A and S1B). Based on these results, the highest concentrations of PA, permethrin, and ivermectin used for further experiments were 200 μM, 50 μM, and 10 μM, respectively. In previous studies, the serum level of permethrin in humans was reported at 2.2 μM after oral intoxication (Gotoh et al., 1998), while the serum level of ivermectin in humans after oral administration has been recorded at 92 nM (Canga et al., 2008). Although the concentrations of permethrin and ivermectin used in this study were significantly higher than in previous human cases, insecticides with lipophilic properties can accumulate in hydrophobic areas, such as in adipose tissue (Jaga and Dharmani, 2003). In fact, DDE has been observed as high as 2542 ng/g lipids in serum, which is equivalent to 7.3 μM (Sun et al., 2017b).

2.3. Membrane potential determination

Cell membrane depolarization was determined by measuring the relative membrane potential using a bis-(1,3-Dibutylbarbituric Acid) trimethine oxonol (DiBAC(4)3)-based method (Miao and Joyner, 1994). DiBAC(4)3 stock solution was prepared in DMSO and diluted in a HEPES buffer to make a fresh working solution before each use. Fluorescence intensity was measured at excitation 485nm/emission 516 nm to obtain the membrane potential.

2.4. Triglycerides content determination

HepG2 cells were treated with serum-free DMEM containing 200 μM palmitic acid, 0.5% BSA and insecticides (with 0.1% DMSO) for 24 h. A palmitic acid (PA)-BSA complex (molar ratio = 2.66:1) was prepared by mixing 40 mM palmitic acid in 0.1 M KOH and 10% BSA in a PBS buffer overnight at room temperature as previously described with slightly modification (Cousin et al., 2001). The pH of the PA-BSA complex stock (20x) was adjusted to 7.2 and sterilized by filtration before use. After treatment, cells were lysed with PBS-containing 0.1% Triton X-100 and the TG content measured using the Infinity Triglycerides reagent kit (Thermo Fisher Scientific, Waltham, MA, US) following the manual's instructions. Protein contents were measured by the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA).

2.5. Immunoblotting

Immunoblotting was performed as previously described (Kim et al., 2013). HepG2 cells were homogenized in a Radioimmunoprecipitation assay (RIPA) buffer by the ultrasonic cell disruptor (Fisherbrand™ Model 50, Thermo Fisher Scientific, Waltham, MA) to extract whole-cell proteins. Protein extracts were blotted to a polyvinylidene fluoride membrane (Immobilon-P, 0.45 μM pore size, EMD Millipore, Burlington, MA) after separated by 8 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (200 V, 60 min). Primary and horseradish peroxidase (HRP)-conjugated secondary antibodies were then applied to detect and visualize target proteins. A chemiluminescence signal was determined using Image Station 4000 MM (Kodak, Rochester, NY) for quantification.

2.6. mRNA expression assays

The expression levels of mRNA of selected genes were determined using a real-time polymerase chain reaction (PCR). mRNA was extracted using the TRIzol Reagent kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Reverse transcriptase quantitative real-time PCR (RT-qPCR) was performed according to manufacturer's instructions to determine the mRNA. Taqman RT-qPCR assay reagents for mRNA expressions included: 18s RNA housekeeping gene (catalog No. 4319413E) for control, sterol regulatory element-binding protein 1 (SREBP1, Hs01088679_g1), acetyl-CoA carboxylase (ACC, Hs01046047_m1), fatty acid synthase (FAS, Hs00236330_m1), peroxisome proliferator-activated receptor α (PPARα, Hs00947536_m1), Farnesoid X receptor (FXR, Hs01026590_m1), carnitine palmitoyltransferase 1α (CPT1α, Hs00912671_m1), cluster of differentiation 36 (CD36, Hs00354519_m1), diacylglycerol O-acyltransferase 2 (DGAT2,

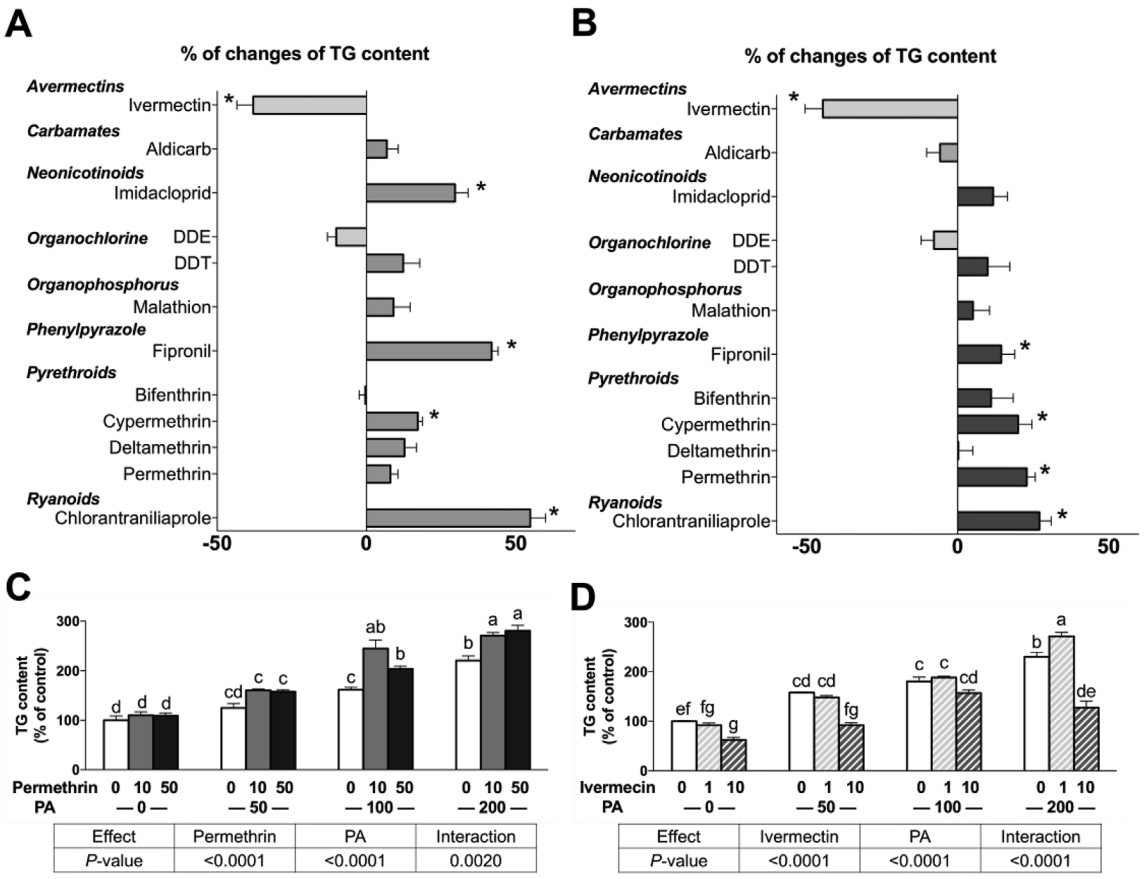


Fig. 1. Effects of insecticides on TG accumulation in non-induced and steatosis-induced HepG2 hepatocytes. TG accumulation with insecticides in non-induced (A) and steatosis-induced HepG2 cells (B). TG accumulation with permethrin (C) and ivermectin (D). HepG2 cells were incubated with 10 μM of 12 insecticides with or without 200 μM of palmitic acid (PA). Interactions were examined following 24 h treatments of 10, 50 μM of permethrin or 1, 10 μM of ivermectin with or without 50, 100, and 200 μM of palmitic acid. Numbers are mean ± S.E.M. (n = 4–6). *Statistically different from the control group at $P < 0.05$. Means with different letters are statistically different at $P < 0.05$. TG; triglycerides.

Hs01045913_m1) and DNA damage-inducible transcript 3 (DDIT3, Hs00358796_g1), which were purchased from Applied Biosystems (Foster City, CA).

2.7. Statistical analysis

Results in Fig. 1A and B were analyzed by one-way analysis of variance (ANOVA). Results in Fig. 1C and D were analyzed by two-way ANOVA. Since insecticide (either permethrin or ivermectin) were not used together in the same experiment of Figs. 2–7 and Supplementary Fig. S1, two separate 2-way ANOVAs (one for permethrin vs. control and the other for ivermectin vs. control) were used. All analyses were done by SAS Software (version 9.4, SAS Institute, Cay, NC). If there was no significant interaction between an insecticide and palmitic acid (PA), the overall effects of the insecticide and PA were discussed including multiple comparison for doses analyzed by Tukey's honest significance test. If there was significant interaction between an insecticide and PA, Tukey's honest significance test for multiple comparison was completed and letters were applied to indicate significant difference between treatments. Note that, regardless of the presence of significant interaction, all results were presented per treatment group for visual presentation purposes only. Even though the same controls were used for permethrin and ivermectin, due to two separate analyses and the absence/presence of significant interaction between PA and insecticide, trends for PA or controls may be different between permethrin and ivermectin. Comparisons with a *P*-value of less than 0.05 were considered statistically different.

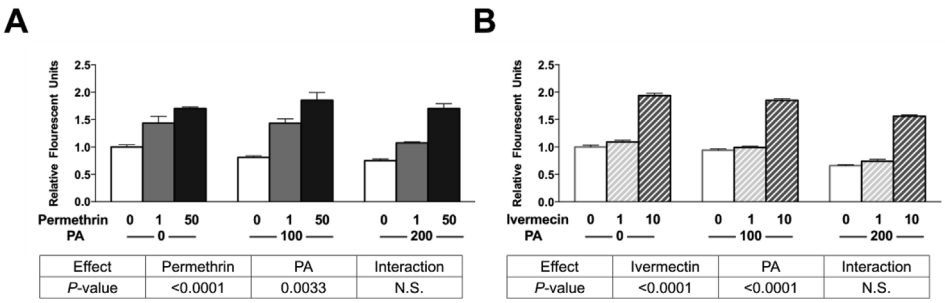
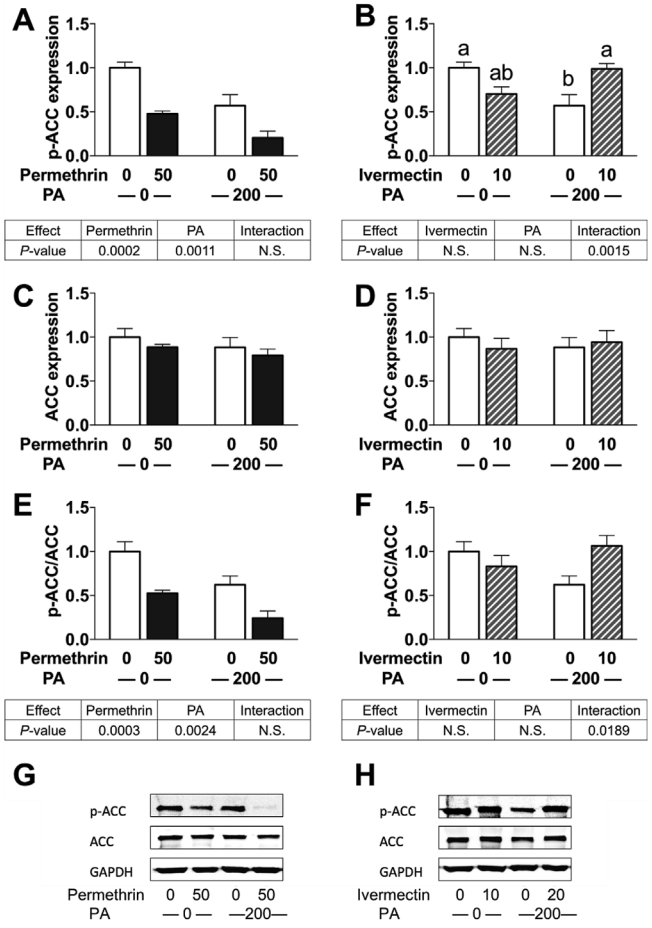


Fig. 2. Effects of permethrin and ivermectin on membrane potentials with palmitic acids (PA). Membrane potential after treatment of permethrin (A) or ivermectin (B). Cells were treated with 10 and 50 μM of permethrin or 1 and 10 μM of ivermectin in combination of 0, 100 and 200 μM of palmitic acid (PA). Membrane potentials were assayed after 40 min by combining treatments along with 250 μM DiBAC(4)3 in HEPES buffer. Numbers are mean ± S.E.M. (n = 6–8). Means with different letters are statistically different at $P < 0.05$. DiBAC(4)3; bis-(1,3-Dibutylbarbituric Acid) trimethine oxonol; N.S., not significant.



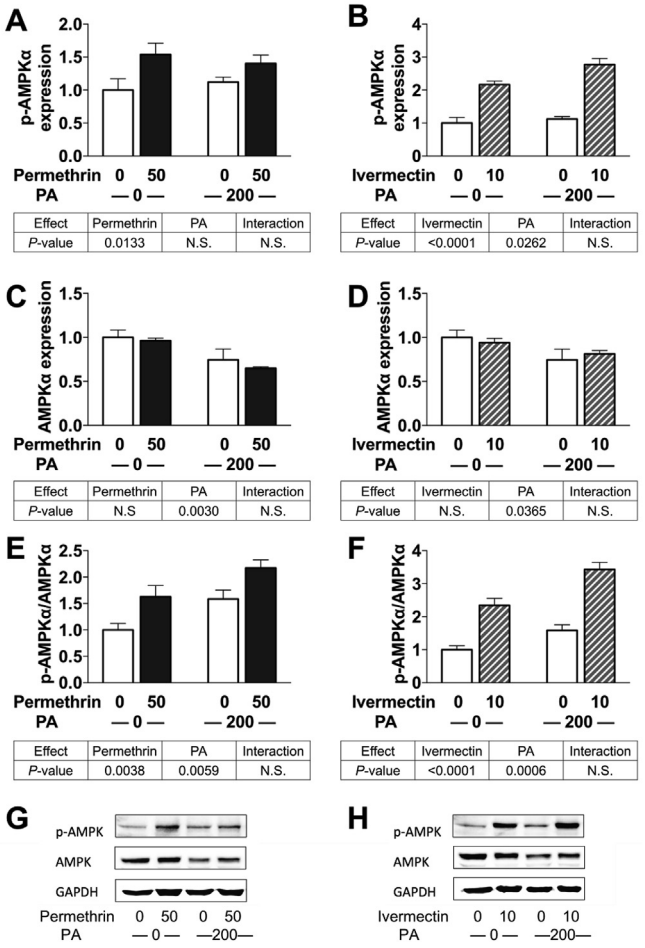
Effect	Permethrin	PA	Interaction
P-value	0.0002	0.0011	N.S.

Effect	Ivermectin	PA	Interaction
P-value	N.S.	N.S.	0.0015

Effect	Permethrin	PA	Interaction
P-value	0.0003	0.0024	N.S.

Effect	Ivermectin	PA	Interaction
P-value	N.S.	N.S.	0.0189

Fig. 3. Effects permethrin and ivermectin on phosphorylation of ACC in HepG2 cells. p-ACC (phosphorylated acetyl-CoA carboxylase) with permethrin (A) and ivermectin (B). Expression of ACC with permethrin (C) or ivermectin (D). Ratio of p-ACC/ACC with permethrin (E) or ivermectin (F). Representative pictures with permethrin (G) or ivermectin (H). Cells were treated with 50 μM of permethrin or 10 μM ivermectin with or without 200 μM palmitic acid (PA) for 24 h. Protein expressions were analyzed by western blot. Numbers are mean ± S.E.M. (n = 4). Means with different letters are statistically different at $P < 0.05$. N.S., not significant.



Effect	Permethrin	PA	Interaction
P-value	0.0133	N.S.	N.S.

Effect	Ivermectin	PA	Interaction
P-value	<0.0001	0.0262	N.S.

Effect	Permethrin	PA	Interaction
P-value	N.S	0.0030	N.S.

Effect	Ivermectin	PA	Interaction
P-value	N.S.	0.0365	N.S.

Effect	Permethrin	PA	Interaction
P-value	0.0038	0.0059	N.S.

Effect	Ivermectin	PA	Interaction
P-value	<0.0001	0.0006	N.S.

Fig. 4. Effects permethrin and ivermectin on phosphorylation of AMPKα in HepG2 cells. p-AMPKα (phosphorylated 5′ adenosine monophosphate-activated protein kinase α) with permethrin (A) or ivermectin (B). Expression of AMPKα with permethrin (C) or ivermectin (D). Ratio of p-AMPKα/AMPKα with permethrin (E) or ivermectin (F). Representative pictures with permethrin (G) or ivermectin (H). Cells were treated with 50 μM of permethrin or 10 μM ivermectin with or without 200 μM palmitic acid (PA) for 24 h. Protein expressions were analyzed by western blot. Numbers are mean ± S.E.M. (n = 4). Means with different letters are statistically different at $P < 0.05$. N.S., not significant.

3. Results

3.1. Effects of insecticides on TG accumulation in non-induced and steatosis-induced HepG2 hepatocytes

To understand the role of insecticides in hepatic lipid metabolism, the effects of 12 insecticides on TG accumulation in HepG2 cells were tested in the presence or absence of 200 μM of PA. Compared to other fatty acids reported to induce steatosis, this concentration of PA was selected based on a previous report that this would result in relatively moderate induction of TG accumulation with minimum cytotoxicity in HepG2 hepatocytes (Ricchi et al., 2009). Thus, this condition would allow us to evaluate the effect of insecticides in potentially increasing TG accumulation in HepG2. Results in Fig. 1A showed that several insecticides significantly increased TG accumulation: the pyrethroid, cypermethrin (17%); the neonicotinoid, imidacloprid (30%), the phenylpyrazole, fipronil (42%), and the ryanoid, chlorantraniliprole (55%), while an avermectin, ivermectin, significantly decreased TG (38% reduction) compared with their respective controls. The organochlorines, DDT and DDE; the organophosphorus, malathion; the carbamate, aldicarb; and the pyrethroids, bifenthrin, deltamethrin, and permethrin, did not alter the TG content in non-induced HepG2 cells.

When treated with 200 μM PA, the effects of certain insecticides were enhanced (as shown in Fig. 1B): permethrin (8%–23%), cypermethrin (17%–20%) and ivermectin (−38% to −47%) compared with their respective controls. Because of their distinctive effects on TG accumulation and enhancement in the presence of PA, permethrin, and ivermectin were selected for further study.

Results in Fig. 1C show a significant interaction between permethrin and PA ($P = 0.0020$). Permethrin at 10 and 50 μM significantly enhanced TG accumulation when co-treated with 100 or 200 μM of PA compared to the respective controls (Fig. 1C). By contrast, 10 μM of ivermectin significantly decreased TG accumulation, without or with 50 and 200 μM of PA (Fig. 1D), with a significant interaction between ivermectin and PA ($P < 0.0001$); There was a 38% TG reduction without PA, 42% reduction with 50 μM of PA, and 47% reduction with 200 μM of PA, respectively, compared to the respective controls.

3.2. Effects of permethrin and ivermectin on membrane potential

Since permethrin and ivermectin both act by altering the membrane potential of cells, the effects of different concentrations of PA on

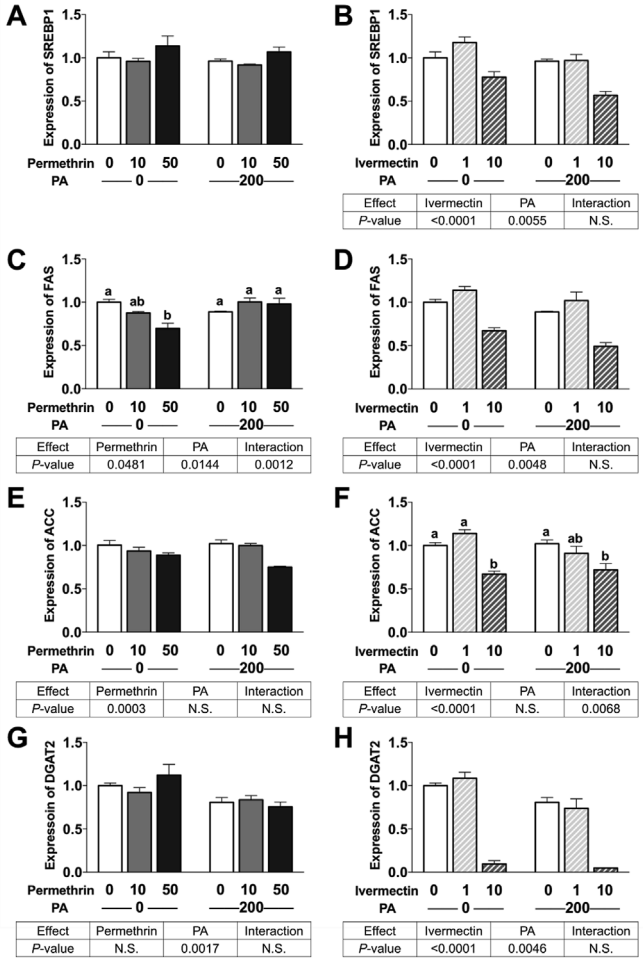


Fig. 5. Interactions of permethrin, ivermectin and palmitic acid on lipogenesis pathways in HepG2 cells. SREBP1 (sterol regulatory element-binding protein 1) with permethrin (A) or ivermectin (B). FAS (fatty acid synthase) with permethrin (C) or ivermectin (D). ACC (acetyl-CoA carboxylase) with permethrin (E) or ivermectin (F). DGAT2 (diglyceride acyltransferase 2) with permethrin (G) or ivermectin (H). Cells were treated with 10, 50 μM of permethrin or 1, 10 μM ivermectin with or without 200 μM palmitic acid (PA) for 24 h mRNA expression analyzed by RT-qPCR. Numbers are mean ± S.E.M. (n = 4). Means with different letters are statistically different at $P < 0.05$. N.S., not significant.

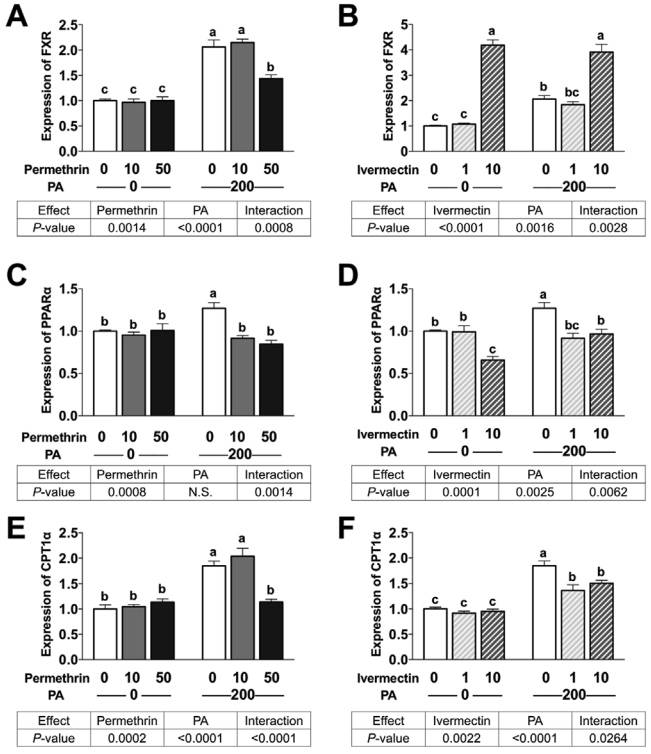


Fig. 6. Interactions of permethrin, ivermectin and palmitic acid on expression of fatty acid oxidation pathways. FXR (farnesoid X receptor) with permethrin (A) or ivermectin (B). PPARα (peroxisome proliferator-activated receptor α) with permethrin (C) or ivermectin (D). CPT1α (carnitine palmitoyltransferase I α) with permethrin (E) or ivermectin (F). Cells were treated with 10, 50 μM of permethrin or 1, 10 μM ivermectin with or without the 200 μM palmitic acid (PA) for 24 h mRNA transcription levels were analyzed by RT-qPCR. Numbers are mean ± S.E.M. (n = 4). Means with different letters are statistically different at $P < 0.05$. N.S., not significant.

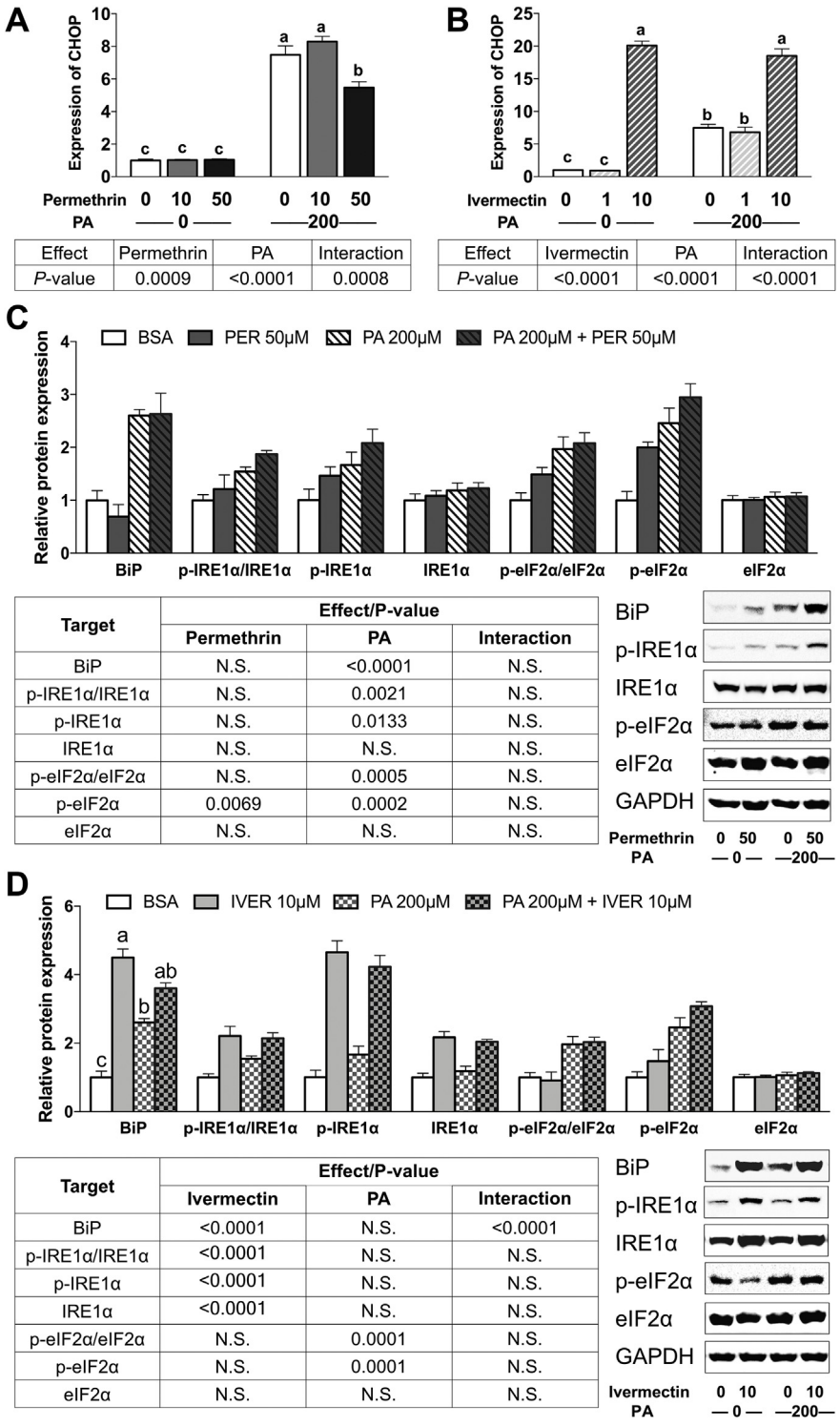
membrane potential were tested with and without permethrin or ivermectin. There was no interaction between the insecticides and PA on membrane potentials (Fig. 2A and B). PA significantly caused hyperpolarization of HepG2 compared to the control (overall $P = 0.0033$ with significant difference between the control and both 100 μM and 200 μM PA, $P < 0.05$ for both, Fig. 2A). Permethrin significantly caused membrane depolarization ($P < 0.0001$) regardless of the presence of PA (Fig. 2A). Ivermectin also induced membrane depolarization regardless of PA ($P < 0.0001$, Fig. 2B). Ivermectin is generally considered a hyperpolarizing insecticide that decreases membrane potential, but several studies also reported it as a depolarizing agent since it causes the efflux of chloride ions and elevates membrane potential when it targets the glutamate-gated chloride channel (Ozkucur et al., 2015; Pemberton et al., 2001; Sabin et al., 2015).

3.3. Effects of permethrin and ivermectin on AMPK-ACC pathway in steatosis-induced HepG2 hepatocytes

Acetyl-CoA carboxylase (ACC) is one of the two rate-limiting enzymes for lipogenesis (Bechmann et al., 2012). In order to determine the mechanisms involved in permethrin- and ivermectin-enhanced TG accumulation in HepG2 cells, the activation of ACC by phosphorylation was measured. There were significant effects of permethrin on p-ACC (57% reduction compared to the controls with $P = 0.0002$) and PA ($P = 0.0011$) without interaction between permethrin and PA (Fig. 3A). There was no effect of permethrin or PA on ACC (Fig. 3C), resulting in significant effects of permethrin and PA on the ratio of p-ACC/ACC (53% reduction by permethrin and 43% reduction by PA compared to the controls with $P = 0.0003$ and $P = 0.0024$, respectively) without interaction between permethrin and PA (Fig. 3E).

Since there was significant interaction between ivermectin and PA on p-ACC ($P = 0.0015$, Fig. 3B), all treatment groups were separately compared. Ivermectin non-significantly decreased p-ACC when treated without PA, but significantly increased it with PA (72% increase compared to the PA-treated control, $P = 0.0256$, Fig. 3B). Neither ivermectin nor PA changed the protein expression level of ACC (Fig. 3D). There was a significant interaction between ivermectin and PA ($P = 0.0189$) on the ratio of p-ACC/ACC without any significant differences between groups (Fig. 3F).

5′-Adenosine monophosphate-activated protein kinase α (AMPKα) is a cellular energy modulator that regulates energy expenditure. It is also one of the upstream regulators of phosphorylation of ACC. Permethrin significantly increased the protein expression level of p-AMPKα (39% increase compared to the controls with $P = 0.0133$) without any effects of PA or interaction between permethrin and PA (Fig. 4A). Permethrin had no significant effects on AMPK, while PA significantly reduced AMPK without interaction with permethrin (Fig. 4C), which resulted in a significant increase in the ratio of p-AMPKα/AMPKα (47% increase by permethrin compared to the controls



Effect	Permethrin	PA	Interaction
P-value	0.0009	<0.0001	0.0008

Effect	Ivermectin	PA	Interaction
P-value	<0.0001	<0.0001	<0.0001

Target	Effect/P-value		
	Permethrin	PA	Interaction
BiP	N.S.	<0.0001	N.S.
p-IRE1α/IRE1α	N.S.	0.0021	N.S.
p-IRE1α	N.S.	0.0133	N.S.
IRE1α	N.S.	N.S.	N.S.
p-eIF2α/eIF2α	N.S.	0.0005	N.S.
p-eIF2α	0.0069	0.0002	N.S.
eIF2α	N.S.	N.S.	N.S.

Target	Effect/P-value		
	Ivermectin	PA	Interaction
BiP	<0.0001	N.S.	<0.0001
p-IRE1α/IRE1α	<0.0001	N.S.	N.S.
p-IRE1α	<0.0001	N.S.	N.S.
IRE1α	<0.0001	N.S.	N.S.
p-eIF2α/eIF2α	N.S.	0.0001	N.S.
p-eIF2α	N.S.	0.0001	N.S.
eIF2α	N.S.	N.S.	N.S.

Fig. 7. Effects of permethrin and ivermectin on ER stress pathways. CHOP (CCAAT-enhancer-binding protein homologous protein) with permethrin (A) or ivermectin (B). Protein expression of ER stress pathways with permethrin (C) or ivermectin (D); BiP (binding immunoglobulin protein); IRE1α (inositol-requiring enzyme 1 α); eIF2α (eukaryotic initiation factor 2 α); and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Cells were treated with 10 and 50 μM permethrin or 1 and 10 μM ivermectin (A and B), 50 μM permethrin or 10 μM ivermectin (C and D) with or without the 200 μM palmitic acid (PA) for 24 h mRNA transcript levels were analyzed by RT-qPCR (A and B). Protein expressions analyzed by western blot (C and D). Numbers are mean ± S.E.M. (n = 4). Means with different letters are statistically different at $P < 0.05$. N.S., not significant.

with $P = 0.0038$) and significant effects of PA ($P = 0.0059$) without interactions between permethrin and PA (Fig. 4E).

There was a significant effect of ivermectin on p-AMPKα (133% increase compared to the controls with $P < 0.0001$) and significant effects of PA ($P = 0.0262$) without interaction between ivermectin and PA (Fig. 4B). This significant effect of PA is, however, not consistent with results in Fig. 4A due to two separate analyses, one for permethrin vs. control and the other for ivermectin vs. control. No significant effect of ivermectin was observed on AMPKα but a significant PA effect was observed ($P = 0.0365$) without interaction between ivermectin and AMPK (Fig. 4D). There was a significant increase in the ratio of p-AMPKα/AMPKα by ivermectin (124% increase compared to the controls with $P < 0.0001$) and PA ($P = 0.0006$) without interaction between ivermectin and PA (Fig. 4F). These results show that the phosphorylation of ACC may not be regulated by AMPK in this model.

3.4. Effects of permethrin and ivermectin on lipogenesis pathways

In order to verify the roles of permethrin and ivermectin in hepatic lipid metabolism, as well as the interaction between these insecticides and PA, the mRNA expression levels of lipogenesis-related genes were tested in both non-induced and steatosis-induced HepG2 cells. SREBP1

is a nuclear transcription factor that promotes lipogenesis (Bechmann et al., 2012). Permethrin or PA treatment did not affect the transcript level of SREBP1 nor was there any interaction between permethrin and PA (Fig. 5A). However, there was significant interaction with permethrin and PA on the transcript level of FAS ($P = 0.0012$, Fig. 5C). Without PA, 50 μM of permethrin significantly decreased the transcript level of FAS compared to the control (30% reduction, $P = 0.0012$), while this effect of permethrin on FAS was eliminated when co-treated with PA (Fig. 5C). Expression of ACC was decreased by permethrin treatment ($P = 0.0003$) without any effects of PA or their interaction (Fig. 5E); 50 μM permethrin treatments significantly decreased ACC expression compared to the controls ($P = 0.0003$) and 10 μM permethrin treatments ($P = 0.0038$, Fig. 5E). Expression of DGAT2 was not changed by permethrin, but significantly influenced by PA alone ($P = 0.0017$) without interaction between permethrin and PA (Fig. 5G).

By contrast, ivermectin significantly decreased the transcript level of SREBP1 by 11% compared to the controls ($P < 0.0001$, Fig. 5B); the expression of SREBP1 by10 μM ivermectin treatments was significantly lower than the controls and 1 μM ivermectin ($P < 0.0001$ for both, Fig. 5B). PA had significant effects on SREBP1 regardless of the effect of ivermectin ($P = 0.0055$ for PA and $P > 0.05$ for interaction, Fig. 5B). Ivermectin significantly decreased the transcript levels of FAS (12% reduction compared to the controls, $P < 0.0001$) and significant PA effects were observed ($P = 0.0048$) independent of the effect of ivermectin (Fig. 5D). There was significant interaction between ivermectin and PA on ACC expression ($P = 0.0068$, Fig. 5F). ACC expression was significantly decreased by ivermectin at 10 μM; 39% reduction compared to the no PA control, $P = 0.0033$, and 30% reduction compared to the PA control, $P = 0.0280$, respectively (Fig. 5F). For expression of DGAT2, there were significant effects of ivermectin (46% reduction compared to the controls, $P < 0.0001$) and PA ($P = 0.0046$) without interaction between ivermectin and PA (Fig. 5H). Treatments of 10 μM ivermectin were significantly different from other treatments on DGAT2 expression ($P < 0.0001$ for all, Fig. 5H).

3.5. Effects of permethrin and ivermectin on FXR, PPARα, and CPT1α

In addition to the lipogenesis pathway, hepatic TG accumulation also depends on energy expenditure. FXR promotes lipid utilization, including fatty acid (FA) oxidation and hepatic lipid secretion (Kalaany and Mangelsdorf, 2006), and it is also one of the upstream regulators of PPARα, which regulates FA oxidation via CPT1α, a rate-limiting enzyme in the mitochondrial FA β-oxidation pathway (Terada and Motojima, 2011). Since there was significant interaction between permethrin and PA ($P = 0.0008$) in the expression of FXR, all groups were compared separately (Fig. 6A). Permethrin did not affect FXR without PA, but 50 μM of permethrin decreased FXR (34% decrease compared to the PA-treated control, $P = 0.0007$, Fig. 6A). PA significantly increased the transcript level of FXR ($P < 0.0001$, Fig. 6A). Similarly, there was significant interaction between permethrin and PA on PPARα ($P = 0.0014$, Fig. 6C). Permethrin did not affect PPARα without PA, but decreased PPARα when co-treated with PA (28% and 33% reductions with 10 and 50 μM permethrin with $P = 0.0012$ and $P = 0.0002$ compared to the PA-treated control, respectively, Fig. 6C). PA treatment alone significantly increased expression of PPARα ($P = 0.0147$, Fig. 6C). Additionally, there was significant interaction between permethrin and PA on transcription of CPT1α ($P < 0.0001$, Fig. 6E). The transcript level of CPT1α was unchanged by permethrin without PA, but was decreased by 38% compared to the PA-treated control when co-treated with 50 μM permethrin and PA ($P < 0.0001$, Fig. 6E). PA alone significantly increased expression of CPT1α (84% increase, $P < 0.0001$, Fig. 6E). Overall, FA oxidation-related genes were decreased when permethrin was co-treated with PA compared to the respective controls.

There was significant interaction between ivermectin and PA on the expression of FXR ($P = 0.0028$, Fig. 6B), PPARα ($P = 0.0062$, Fig. 6D), and CPT1α ($P = 0.0264$, Fig. 6F). The transcription of FXR was increased by 10 μM of ivermectin in non-PA treated cells (318% increase) but was partially attenuated when co-treated with PA (90% compared to the PA-treated control, Fig. 6B). Ivermectin also significantly altered the transcription of PPARα; without PA, only the 10 μM concentration of ivermectin decreased PPARα (34% reduction compared the control, $P = 0.0098$), while both 1 and 10 μM of ivermectin decreased PPARα when co-treated with PA (28% and 24% reductions with 1 and 10 μM ivermectin, $P = 0.0037$ and $P = 0.0137$, compared to the PA-treated control, respectively, Fig. 6D). Additionally, CPT1α transcription was not affected by ivermectin without PA, but was significantly decreased by both 1 and 10 μM of ivermectin when co-treated with PA (26% reduction by 1 μM ivermectin with $P = 0.0022$, and 19% reduction by 10 μM ivermectin with $P = 0.0347$ compared to the PA-treated control, respectively, Fig. 6F).

3.6. Effects of permethrin and ivermectin on ER stress pathways

Endoplasmic reticulum (ER) stress results from the accumulation of unfolded or misfolded proteins and is one of the modulators in lipogenesis pathways (Lee et al., 2012). Thus, the effects of permethrin and ivermectin on ER stress in HepG2 cells were determined. CCAAT-enhancer-binding protein homologous protein (CHOP) is a biomarker for the activation of ER stress, which is initiated by BiP and then branched into 3 major pathways, including phosphorylation of IRE1α and eIF2α, where they both activate CHOP (Oslowski and Urano, 2011). There was significant interaction between permethrin and PA ($P = 0.0008$, Fig. 7A). The transcript level of CHOP was not changed by permethrin without PA, but was suppressed by 50 μM permethrin when co-treated with PA (27% reduction compared to the PA-treated control, $P = 0.0024$, Fig. 7A). PA also significantly increased levels of CHOP ($P < 0.0001$, Fig. 7A). For ER stress upstream markers, there were no interactions between permethrin and PA for all markers tested (Fig. 7C). The only significant effect of permethrin was for p-eIFα, which was significantly increased (43% compared to the controls, $P = 0.0069$, Fig. 7C). PA had significant effects on BiP, p-IRE1α/IRE1α, p-IRE1α, p-eIF2α/eIF2α, and p-eIF2α compared to no PA treatments (Fig. 7C). Overall, these findings suggest that ER stress may have minimal influence on permethrin-altered lipid metabolism in this model.

There was significant interaction between ivermectin and PA on CHOP ($P < 0.0001$, Fig. 7B). Ivermectin significantly increased the transcription level of CHOP (> 20 fold without PA, while 147% increase with PA treatments compared to the controls, $P < 0.0001$ for both, Fig. 7B). For ER stress-related proteins, there was interaction between ivermectin and PA on BiP ($P < 0.0001$), but not others (Fig. 7D). Without PA treatments, ivermectin increased the protein expression level of BiP, 350% increase compared to the no-PA control ($P < 0.0001$), while the effect of ivermectin on BiP was attenuated when co-treated with PA (40% increase compared with the PA control, $P = 0.0118$, Fig. 7D). In addition, ivermectin also increased the protein expression level of p-IRE1α (230% increase, $P < 0.0001$) and IRE1α (92% increase, $P < 0.0001$), and the ratio of p-IRE1α/IRE1α (72% increase, $P < 0.0001$) compared to the respective controls without the interaction with PA (Fig. 7D). Additionally, no significant effect of ivermectin was observed on the protein expression levels of p-eIF2α or eIF2α, and on the ratio of p-eIF2α/eIF2α. These findings suggested that ER stress caused by ivermectin might be partially involved in ivermectin-altered lipid metabolism in HepG2 cells.

4. Discussion

The liver is one of the major organs for lipid and glucose metabolism (Scollon et al., 2009) and alteration of hepatic lipid and glucose metabolism were observed in the presence of excessive FA (Ghose et al., 2011) and NAFLD (Leung and Nieto, 2013; Woolsey et al., 2016). Base

on the correlation of insecticide exposure and alteration of lipid and glucose metabolism (Sun et al., 2017a, 2017b; Xiao et al., 2017a), the current study investigated the significance of insecticides on hepatic lipid metabolism. This study is the first to report the effect of insecticides and FA overloads in HepG2 hepatocytes.

Permethrin is the single most used synthetic pyrethroid worldwide (Council, 1999), and is a member of one of the major classes of insecticide commercially available (Sparks, 2013). Permethrin acts as an agonist in voltage-sensitive sodium channels both in invertebrates and vertebrates, including humans. In this study, permethrin increased lipogenesis and decreased FA oxidation in HepG2 cells. The current results also suggested that the post-translational regulation of ACC might be important in permethrin-induced lipogenesis as permethrin only had minimum effects on the expression of other lipogenesis-related genes, SREBP1, ACC and DGAT2 in HepG2. The current results also suggested that permethrin regulated ACC independent of AMPK, which is one of the post-translational regulators that inactivates ACC. This result is consistent with a previous *in vivo* study finding that permethrin activates AMPK in mice liver (Xiao et al., 2018). However, other studies reported that permethrin induced lipogenesis, dependent on the AMPK pathway in adipocytes (Kim et al., 2014) and adipose tissues (Xiao et al., 2018), by decreasing the ratio of pAMPK to AMPK. Similarly, deltamethrin promoted adipogenesis in 3T3-L1 adipocytes and *C. elegans* via the AMPK-dependent pathway (Shen et al., 2017), but had no effects on TG accumulation in this study. Another pyrethroid, cypermethrin, significantly increased TG accumulation in the current study, but it is not clear if cypermethrin targeted AMPK differently in hepatocytes versus other cells as it is known to activate AMPK in neuroblastoma cells (Mishra et al., 2018). These findings suggest that the effects of pyrethroids on AMPK may be tissue-specific, and further studies are needed to elucidate the mechanism of the permethrin-induced post-translational regulation of ACC, as well as the effects of other pyrethroids on the hepatic lipogenesis pathway. Additionally, the previous study showed that permethrin-induced adipogenesis in 3T3-L1 adipocytes is associated with ER stress (Xiao et al., 2017c), while deltamethrin was reported to cause ER stress in neuroblastoma cells (Hossain and Richardson, 2011). As permethrin showed a minimum effect on the ER stress in HepG2 cells in this study, further investigations are also needed to elucidate the mechanism of the permethrin in lipogenesis independent of the ER stress pathway.

In addition to increased lipogenesis, the current results suggest that permethrin suppressed FA oxidation in part by downregulating the FXR-PPARα-CPT1α pathway. These results are consistent with a previous study, where permethrin reduced hepatic PPARα in the liver of high-fat diet-fed male mice (Xiao et al., 2018). In addition, another pyrethroid tested in the current study, bifenthrin, was reported to decrease CPT1α in HepG2 cells (Xiang et al., 2018) though it did not change TG in the current study. These findings suggest that reduced FA oxidation may be involved in the effect of permethrin on hepatic lipid metabolism.

Ivermectin is a macrocyclic lactone avermectin, which is used for controlling parasites or insects by acting on the glutamate-gated chloride channel (Bloomquist, 1993). Avermectins, including ivermectin, also are known agonists of FXR, which regulates hepatic lipid and glucose metabolism by suppressing lipogenesis and gluconeogenesis (Jin et al., 2013, 2015) and have been suggested as potential therapeutic agents for NAFLD (Cariou, 2008; Carr and Reid, 2015; Mudaliar et al., 2013; Trauner et al., 2010). Upregulation of FXR is known to decrease lipogenesis and increase fatty acid oxidation. The current results were consistent with the above in that ivermectin upregulated FXR, leading to the down-regulation of SREBP, FAS, and ACC, potentially acting as an agonist of FXR. However, ivermectin significantly reduced PPARα and CPT1α, which were inconsistent effects for an FXR agonist (Torra et al., 2003). Overall, these results suggest that ivermectin suppressed lipogenesis via FXR-mediated mechanisms, while the effects of ivermectin on fatty acid oxidation may be independent of FXR. Alternatively, AMPK is also known as an important regulator of lipogenesis and fatty acid oxidation, where the current results showed that activation of AMPK by ivermectin did not lead to post-translational changes of ACC, although this may lead to increased fatty acid oxidation by reducing the level of malonyl-CoA, the intermediate of lipogenesis that directly inhibits CPT1α (Lage et al., 2008). Additionally, ER stress is known to play a significant role in increased lipogenesis (Lee et al., 2012; Özcan et al., 2004). However, the current results indicate that ivermectin induced ER stress while reducing lipogenesis. Thus, further study is required to elucidate the role of ivermectin on the PPARα pathway and the correlation between ivermectin-associated ER stress and alteration of lipogenesis.

In addition to alteration of lipid metabolism, alteration of glucose metabolism, dyslipidemia, and other liver functions, such as hepatic oxidative stress and liver injury, can also contribute to the development of NAFLD (Albano et al., 2005; Argo and Caldwell, 2009; Gaggini et al., 2013). Altered glucose metabolism can result from insulin resistance, which is known to increase hepatic FA uptake, lipogenesis and blocked lipid secretion, and the degree of insulin resistance is related to the degrees of NAFLD (Bugianesi et al., 2005; Gaggini et al., 2013; Yki-Jarvinen, 2010). Several pyrethroids including permethrin, were previously reported to disturb glucose metabolism. Permethrin was reported to interrupt insulin signaling in myotubes (Kim et al., 2014; Sun et al., 2017b). Cismethrin (Cremer and Seville, 1982), cypermethrin (Veerappan et al., 2012), and deltamethrin (Ray and Cremer, 1979; Yousef et al., 2006) also increased blood glucose level in rats.

Dyslipidemia can also promote hepatic FA uptake, which results in increased hepatic TG and development of NAFLD (Gaggini et al., 2013). It has been reported previously that the pyrethroids, allethrin, prallethrin, cypermethrin, and deltamethrin, induced dyslipidemia in rodents and humans (Ince et al., 2012; Narendra et al., 2008; Veerappan et al., 2012; Yousef et al., 2006). Hepatic oxidative stress can also induce inflammation and cause liver injury, which triggers liver regeneration, leading to reduced liver function and eventually resulting in fibrosis and cirrhosis, the irreversible stages of NAFLD (Albano et al., 2005; Argo and Caldwell, 2009). As critical factors in the progression of NAFLD, several pyrethroids have been demonstrated to cause hepatic oxidative stress in rodents, including bifenthrin (Zhang et al., 2015), cyhalothrin (Al-Sarar et al., 2014), cypermethrin (Aldana et al., 2001; Atessahin et al., 2005; Giray et al., 2001; Yavasoglu et al., 2006), deltamethrin (Abdel-Daim et al., 2013; Abdou and Abdel-Daim, 2014; Chargui et al., 2012; Tuzmen et al., 2008; Yousef et al., 2006), and permethrin (Yang et al., 2018). In addition, avermectins were also found to induce oxidative stress in the liver (Zhu et al., 2013).

In summary, the current results suggest that permethrin promoted lipogenesis and suppressed FA oxidation, while ivermectin suppressed lipogenesis in HepG2 cells. A limitation of the current study was focusing only on the effects of insecticides on hepatic lipid metabolism. Moreover, although HepG2 cells exhibit many features of normal liver cells, it may still represent the potential implication of these chemicals in hepatocyte functions. Further studies, including testing gene-modified cells (i.e. applying RNA interference) and *in vivo* studies, would be needed to expand the significance of the current research, as well as to elucidate the roles of the tested insecticides in the development of NAFLD and their effects on glucose metabolism, dyslipidemia, and oxidative stress.

5. Conclusions

This study suggests that both permethrin, a pyrethroid insecticide, and ivermectin, an avermectin insecticide, can alter hepatic lipid metabolism and interact with administered palmitic acid to enhance their impacts. The results are significant in that FA overload, one of the most important factors in NAFLD development, may leave the liver more susceptible to environmental chemicals such as insecticides. However, the detailed mechanisms by which permethrin and ivermectin interact

with FAs to affect hepatic lipid metabolism still require further investigation.

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Appendix A. Supplementary data

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Transparency document

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