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Original Research Article

Cytochrome P450 expression-associated multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD) in HepG2 cells

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Abstract

Purpose: *To establish a free fatty acid (FFA)-induced non-alcoholic fatty liver disease (NAFLD) model in HepG2 cells.*
Methods: *HepG2 cells were incubated with 0.1, 1, or 5 mM oleic acid (OA) or palmitic acid (PA) for 24 h. Histological features were examined by oil-red-O staining. Expression levels of metabolic genes (peroxisome proliferator activated receptors α/γ, sterol regulatory element binding proteins 1a/1c, acetyl-CoA carboxylase, acyl-CoA oxidase, and fatty acid synthase), antioxidative genes (catalase and superoxide dismutases 1/2), and cytochrome P450 genes (CYP1A2, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11) were determined by reverse transcription-real time polymerase chain reaction (RT-qPCR).*
Results: *Intracellular lipid storage was observed in cells treated with 1 mM OA or PA while cell shrinkage was present at 5 mM concentrations of both. Expression of all metabolic genes were elevated by 1 mM PA and 5 mM OA and PA. Expression of all antioxidative genes were diminished by 5 mM OA whereas 5 mM PA only reduced superoxide dismutase-2 expression. Expression of CYP1A2, CYP2D6, and CYP3A4 genes were down-regulated by both FFAs, CYP2C19 was induced by PA, while CYP2E1 and CYP4A11 were up-regulated in a concentration-dependent manner.*
Conclusion: *PA was the more potent steatogenic agent in an OA- or PA- induced NAFLD model in HepG2 cells. Increase in intracellular hepatic lipid and expression of metabolic genes, suppression of antioxidative genes, suppression of CYP1A2, CYP2D6, and CYP3A4, and induction of CYP2E1 and CYP4A11 correlated with the multiple-hit pathogenesis model of NAFLD. These findings suggest that PA-induced NAFLD model in HepG2 cells is a suitable in vitro model for studying novel therapeutic approaches to NAFLD treatment.*

Keywords: *NAFLD, Multiple-hit pathogenesis, Free fatty acid, Oleic acid, Palmitic acid*

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a major chronic liver disease affecting around 30 % of Western and Asian populations [1]. To date, fatty liver disease patients have been given life-style modification advice comprised of recommendations on diet and encouragement to

exercise as no effective treatment has been reported. The development of NAFLD is associated with excess fat consumption and metabolic syndromes [1]. Fatty acids are involved in lipogenesis and development of hepatic fat accumulation. Oleic acid (OA) and palmitic acid (PA) are the most common dietary fatty acids. OA is the most abundant fatty acid in triglyceride (TG) stored in human adipose tissue followed by PA [2]. Monounsaturated OA (C18:1 n-9) is found in vegetable oils including olive, sunflower, safflower, and canola and in animal fats from beef, pork, camel, and krill [3]. Saturated PA (C16:0) is found in beef, palm oil, lard, and unsalted butter [4]. Previous studies in primary hepatocytes, immortalized hepatic cell lines, mice, geese, and humans have suggested induction of NAFLD by OA, PA, and their combinations occurs through cytotoxic effects, disruption of the lipid metabolic and oxidant-antioxidant systems, and alteration of cytochrome P450 (CYP) profiles [5].

CYP is a superfamily of mono-oxygenase enzymes that are highly abundant in the liver and play a key role in metabolism of drugs, xenobiotics, and toxic chemicals [6]. CYPs have been implicated in the pathogenesis of fatty liver disease by promoting oxidative stress and inflammation, however the metabolic pathways have not been sufficiently described [6].

This study aimed to establish an i*n vitro* NAFLD model in HepG2 cells to investigate the modulation of metabolic systems during steatohepatitis. NAFLD pathogenesis is believed to occur through multiple-hit including insulin resistance, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, oxidant-antioxidant imbalance, and inflammation, all of which are targets for NAFLD therapies. Therefore, hepatosteatosis was induced in HepG2 cells with OA and PA to examine the association of CYP450 regulation with multiple-hit pathogenesis of NAFLD.

EXPERIMENTAL

Chemicals

Dulbecco's modified Eagle medium (DMEM) [+] 1 g/L D-glucose [+] L-glutamine [+] 110 mg/mL sodium pyruvate, and fetal bovine serum (FBS) were purchased from Gibco® (Life Technologies™, MA, USA). Oleic acid (OA; O1008), palmitic acid (PA; P0500), oil red O were obtained from Sigma-Aldrich (Missouri, USA). All other laboratory chemicals were of the highest purity from commercial suppliers.

Experimental design and treatment

HepG2 cells (ATCC® HB-8065, Manassas, USA) were cultured in DMEM supplemented with 10% FBS and 10,000 units/mL penicillin/streptomycin at 37ºC under 95% humidity and 5% CO_2. The cells were seeded in a 6-well plate at a density of 5×10^5 cells/well until reaching to 80% confluence. Stock solutions (1 M) of OA and PA were prepared by dissolving in isopropanol and diluted with medium to 0.1, 1, and 5 mM before incubating with the cells for 24 h. Then the medium and the cells were collected for further analysis.

Examination of intracellular fat by oil red O staining

Oil red O solution (ORO; 0.18 %) was freshly prepared in 60 % isopropanol. At 24 h after the last treatment, the medium was removed, and the monolayer cells were washed with phosphate buffered saline (PBS). The cells were fixed by immersing in 10% neutral-buffered formalin and washed twice with distilled water, followed by immersing in 60% isopropanol. Then the fixed cells were stained with ORO. The background was cleaned via immersing in 60% isopropanol, followed by washing with distilled water. The histological features were evaluated using a Motic AE2000 inverted microscope at 10× magnification (Motic, Kowloon, Hong Kong). The image was recorded and analyzed on screen using a Motic image plus 3.0 software [7].

Determination of mRNA expression by reverse transcription/real-time polymerase chain reactions (RT/qPCR)

Total RNA was prepared by guanidine-thiocyanate-phenol-chloroform method [8] and the concentration was determined by NanoDrop 2000c UV-spectrophotometer (NanoDrop Technologies, Inc., Thermo Fisher Scientific, MA, USA). Total RNA was converted to cDNA using ReverTraAce® (Toyobo Co., Ltd., Osaka, Japan) at 25 ºC for 10 min, 42 ºC for 60 min, and 95 ºC for 5 min. Expressions of metabolic genes, i.e. PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c, ACC, COX, and FAS, antioxidative genes, CAT, SOD1, and SOD2, CYPs, i.e. CYP1A2, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11, and β-actin were subjected to qPCR with the specific primers (Table 1) under conditions recommended by the supplier and calculated as described [8]. The mRNA levels were normalized to that of a reference gene, β-actin, and expressed as the fold differences to control.

Table 1: Specific primers for determination of mRNA expressions using Qpcr

Gene		Forward and reverse primers (5' → 3')	Annealing temperature (°C)
PPAR-α	*Forward*	GGT GGA CAC GGA AAG CCC AC	60.6
	Reverse	GGA CCA CAG GAT AAG TCA CC	
PPAR-γ	*Forward*	GGC GAG GGC GAT CTT GAC AGG	57.4
	Reverse	TGC GGA TGG CCA CCT CTT TGC	
SREBP-1a	*Forward*	TCA GCG AGG CGG CTT TGG AGC AG	57.4
	Reverse	CAT GTC TTC GAT GTC GGT CAG	
SREBP-1c	*Forward*	GGA GGG GTA GGG CCA ACG GCC	57.4
	Reverse	CAT GTC TTC GAA AGT GCA ATC C	
ACC	*Forward*	GAA TGT TTG GGG ATA TTT CAG	60.7
	Reverse	TTC TGC TAT CAG TCT GTC CAG	
ACOX	*Forward*	GGG CAT GGC TAT TCT CAT TGC	60.2
	Reverse	CGA ACA AGG TCA ACA GAA GTT AGG	
FAS	*Forward*	CGG AAA CTG CAG GAG CTG TC	60.0
	Reverse	CAC GGA GTT GAG CCG CAT	
CAT	*Forward*	AGT GAT CGG GGG ATT CCA GA	60.8
	Reverse	GAG GGG TAC TTT CCT GTG GC	
SOD1	*Forward*	GGT GTG GCC GAT GTG TCT AT	60.6
	Reverse	TGG GCG ATC CCA ATT ACA CC	
SOD2	*Forward*	GCA CTA GCA GCA TGT TGA GC	60.1
	Reverse	CCG TTA GGG CTG AGG TTT GT	
CYP1A2	*Forward*	ACA AGG GAC ACA ACG CTG AA	60.0
	Reverse	AGG GCT TGT TAA TGG CAG TG	
CYP2C19	*Forward*	ACC CAA AGG ACC TTG ACA CA	61.1
	Reverse	AGA TAG TGA AAT TTG GAC CAG AGG A	
CYP2D6	*Forward*	AGC TTT CTG GTG ACC CCA TC	61.1
	Reverse	GGA CCC GAG TTG GAA CTA CC	
CYP2E1	*Forward*	AAT GGA CCT ACC TGG AAG GAC	60.0
	Reverse	CCT CTG GAT CCG GCT CTC ATT	
CYP3A4	*Forward*	GCC TGG TGC TCC TCT ATC TA	54.7
	Reverse	GGC TGT TGA CCA TCA TAA AAG	
CYP4A11	*Forward*	CCC CTT GTG GCC TTT GG	60.0
	Reverse	GCG TCA GGG TAG CCT TCC A	
β-actin	*Forward*	TCC GCA AAG ACC TGT ACA CC	61.1
	Reverse	GAG TAC TTG CGC TTG GGA GG	

RESULTS

Histomorphology of OA and PA-induced NAFLD in HepG2 cells

The histological features of ORO-stained HepG2 cells are shown in Figure 1. The control HepG2 cells demonstrated regular cell characteristics (Figure 1 A) while those exposed to OA and PA free fatty acids (FFA) showed increasing storage of intracellular lipid in a concentration-dependent manner. The lowest concentration (0.1 mM) of OA (Figure 1 C) or PA (Figure 1 F) caused limited accumulation of lipid droplets with otherwise unchanged histomorphology compared to the controls. At the higher concentration (1 mM) large numbers of lipid droplets (in red) were observed for both OA (Figure 1 D) and PA (Figure 1 G). The droplets appeared fused with evidence of cell shrinkage (black arrows) in cells treated with PA (1 and 5 mM; Figure 1 G and H). Large amounts of lipid were present in cells treated with 5 mM OA (Figure 1 E) and with 1 and 5 mM PA. At these higher concentrations of PA, the cytoplasmic materials appeared clumped, the cells were condensed and the nuclei had disappeared (Figure 1 G and H). These observations revealed that both the FFA type and concentration contributed to the amount of lipid accumulation in HepG2 cells.

Effect of OA- or PA-induced NAFLD on the mRNA expression of metabolic genes in HepG2 cells

Expression of the PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c, ACC, ACOX, and FAS metabolic genes were changed in the FFA-loaded HepG2 cells (Table 2 and Table 3). PPAR-α and ACOX mRNAs were significantly induced by OA and PA at higher concentrations. Expression of PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c, and ACC mRNAs were elevated by OA (5 mM) and PA (0.1 - 5 mM). Expression of FAS mRNA was increased by OA (5 mM) and PA (1 and 5 mM).

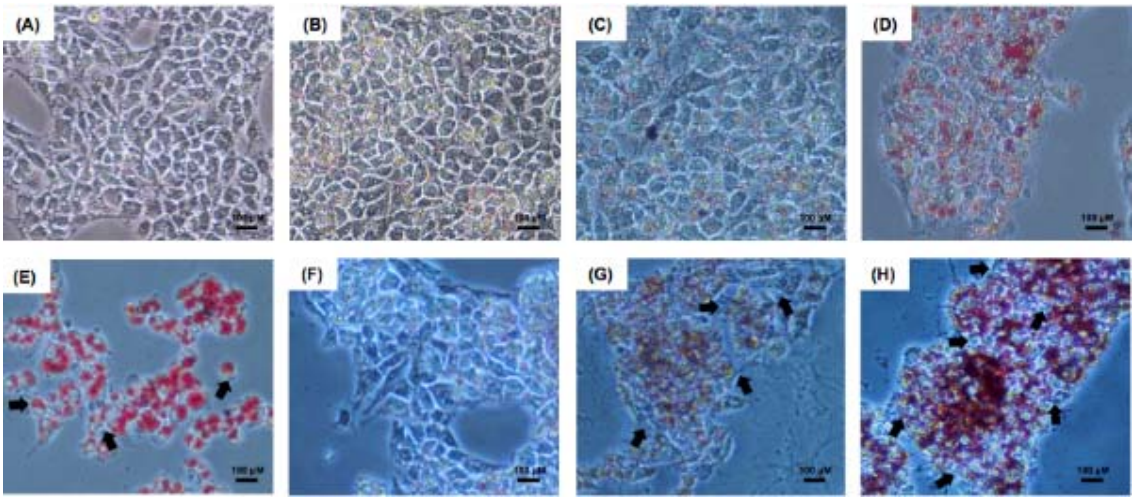

Figure 1: Histomorphology of NAFLD-induced HepG2 cells using oleic acid and palmitic acid. HepG2 cells were treated with (A) non-treatment, (B) control, 0.5% isopropanol, (C-E) oleic acid in isopropanol at concentrations of 0.1, 1, and 5 mM, respectively, and (F-H) palmitic acid in isopropanol at concentrations of 0.1, 1, and 5 mM, respectively

Effect of OA- or PA-induced NAFLD on mRNA expression of antioxidative genes in HepG2 cells

Expression of all antioxidative genes (CAT, SOD1, and SOD2) in the FFA-loaded HepG2 cells were significantly suppressed at the highest concentration (5 mM) of OA, while PA only down-regulated SOD2 (Table 4)

Table 2: Induction of metabolic genes, PPAR-α, PPAR-γ, SREBP-1a, and SREBP-1c, in OA- and PA-induced NAFLD in HepG2 cells

Treatment	Fold difference (metabolic genes/β-actin)			
	PPAR-α	***PPAR- γ***	***SREBP-1a***	***SREBP-1c***
Control	1.00 ± 0.16	1.00 ± 0.14	1.00 ± 0.14	1.06 ± 0.18
0.5% Isopropanol	0.94 ± 0.11	0.94 ± 0.16	0.93 ± 0.16	0.99 ± 0.13
Oleic acid				
0.1 mM	0.81 ± 0.16	1.03 ± 0.09	1.03 ± 0.06	0.88 ± 0.07
1 mM	1.16 ± 0.18 *	1.20 ± 0.07	1.26 ± 0.07	1.10 ± 0.19
5 mM	1.23 ± 0.14 *	1.71 ± 0.13 *	1.70 ± 0.10 *	1.70 ± 0.31 *
Palmitic acid				
0.1 mM	1.03 ± 0.10	2.01 ± 0.06 *	2.00 ± 0.06 *	1.81 ± 0.07 *
1 mM	1.32 ± 0.11 *	2.57 ± 0.20 **	2.55 ± 0.16 *	2.73 ± 0.30 *
5 mM	1.57 ± 0.04 *	3.20 ± 0.06 **	3.15 ± 0.06 **	4.14 ± 0.12 **

*$p < 0.05$, **$p < 0.001$ vs. control using one-way ANOVA with Tukey *post hoc* test

Table 3: Induction of metabolic genes, ACC, ACOX, and FAS, in OA- and PA-induced NAFLD in HepG2 cells

Treatment	Fold difference (metabolic gene/β-actin)		
	ACC	***ACOX***	***FAS***
Control	1.09 ± 0.18	1.02 ± 0.20	1.01 ± 0.16
0.5% Isopropanol	0.94 ± 0.12	1.07 ± 0.15	0.99 ± 0.17
Oleic acid			
0.1 mM	1.00 ± 0.04	0.83 ± 0.14	0.89 ± 0.17
1 mM	1.21 ± 0.16	1.45 ± 0.28 *	1.10 ± 0.18
5 mM	1.44 ± 0.18 *	1.89 ± 0.23 *	1.37 ± 0.20 *
Palmitic acid			
0.1 mM	1.35 ± 0.15 *	1.33 ± 0.14 *	0.91 ± 0.01
1 mM	1.62 ± 0.39 *	1.43 ± 0.30 *	1.59 ± 0.07 **
5 mM	2.22 ± 0.11 **	2.26 ± 0.28 **	2.39 ± 0.06 **

*$p < 0.05$, **$p < 0.001$ vs control using one-way ANOVA with Tukey *post hoc* test

Table 4: Modification of antioxidative genes, CAT, SOD1, and SOD2, in OA- and PA-induced NAFLD in HepG2 cells

Treatment	Fold difference (antioxidative genes/β-actin)		
	CAT	SOD1	SOD2
Control	1.10 ± 0.10	1.00 ± 0.08	1.05 ± 0.21
0.5% Isopropanol	1.19 ± 0.15	0.98 ± 0.17	1.04 ± 0.04
Oleic acid			
0.1 mM	0.84 ± 0.03	1.11 ± 0.16	1.19 ± 0.06
1 mM	0.67 ± 0.10	0.85 ± 0.16	0.75 ± 0.14
5 mM	0.19 ± 0.03 **	0.44 ± 0.05 *	0.45 ± 0.02 *
Palmitic acid			
0.1 mM	1.32 ± 0.13 **	1.09 ± 0.17	0.47 ± 0.09 *
1 mM	0.95 ± 0.07	0.90 ± 0.02	0.44 ± 0.04 *
5 mM	0.69 ± 0.23	0.84 ± 0.21	0.42 ± 0.02 *

*p < 0.05, **p < 0.001 vs. control using one-way ANOVA with Tukey *post hoc* test

Table 5: Modification of CYPs, CYP1A2, CYP2C19, CYP2D6, and CYP3A4, in OA- and PA-induced NAFLD in HepG2 cells

Treatment	Fold difference (CYPs/β-actin)			
	CYP1A2	***CYP2C19***	***CYP2D6***	***CYP3A4***
Control	1.10 ± 0.16	1.00 ± 0.11	1.05 ± 0.05	1.05 ± 0.16
0.5% Isopropanol	0.97 ± 0.18	1.02 ± 0.17	1.08 ± 0.04	1.00 ± 0.12
Oleic acid				
0.1 mM	0.96 ± 0.19	1.06 ± 0.01	1.15 ± 0.10	0.62 ± 0.08 **
1 mM	0.75 ± 0.05 *	0.69 ± 0.10 **	1.05 ± 0.00	0.59 ± 0.17**
5 mM	0.62 ± 0.00 *	0.31 ± 0.02 **	0.96 ± 0.12	0.41 ± 0.02 **
Palmitic acid				
0.1 mM	0.77 ± 0.04 *	1.79 ± 0.03 **	1.30 ± 0.02 *	0.39 ± 0.04 **
1 mM	0.77 ± 0.04 *	1.64 ± 0.02 **	0.65 ± 0.05 *	0.14 ± 0.01 **
5 mM	0.52 ± 0.01 **	1.03 ± 0.07	0.59 ± 0.07 *	0.07 ± 0.07 **

*p < 0.05, **p < 0.001 vs. control using one-way ANOVA with Tukey *post hoc* test

Effect of OA- or PA-induced NAFLD on mRNA expression of CYPs in HepG2 cells

Expression of CYP1A2 mRNA was significantly suppressed by all OA or PA treatments, except OA at the concentration of 0.1 mM (Table 5). Expression of CYP2C19 mRNA was suppressed by OA (1 and 5 mM) but induced by PA (0.1 and 1 mM) (Table 5). OA did not change expression of CYP2D6, while PA induced CYP2D6 at the lowest concentration but suppressed it at the two higher concentrations (1 and 5 mM, Figure 5 A). The expression of CYP3A4 was suppressed by OA and PA in a concentration-dependent manner (Table 5). The expression of CYP2E1 and CYP4A11 mRNA were increased by OA (5 mM) and all PA treatments (Table 6).

Table 6 Modification of CYPs, CYP2E1 and CYP4A11, in OA- and PA-induced NAFLD in HepG2 cells

Treatment	Fold-difference (CYPs/β-actin)	
	CYP2E1	***CYP4A11***
Control	1.00 ± 0.13	1.01 ± 0.20
0.5% Isopropanol	1.03 ± 0.23	1.00 ± 0.05
Oleic acid		
0.1 mM	1.01 ± 0.14	0.99 ± 0.06
1 mM	1.23 ± 0.16	1.29 ± 0.06
5 mM	2.03 ± 0.17 *	1.47 ± 0.28 *
Palmitic acid		
0.1 mM	2.46 ± 0.03 *	1.70 ± 0.25 *
1 mM	3.15 ± 0.03 *	3.96 ± 0.20 *
5 mM	4.98 ± 0.05 **	4.97 ± 0.10 **

*p < 0.05, **p < 0.001 vs. control using one-way ANOVA with Tukey *post hoc* test

DISCUSSION

This study optimized an *in vitro* model of NAFLD in HepG2 cells and explored the effect of OA- and PA-induced NAFLD on intracellular lipid levels and mRNA expression of metabolic, antioxidative, and CYP genes. This model was developed using HepG2 cells at the density of 5 × 10^5 cells/well with OA and PA at various concentrations that correlated to human diets ranged from 0.1 to 1 mM [9]. The advantages of HepG2 cells are that this cell type carries an adiponutrin variant responsive to hepatic fat accumulation, they are easy to handle and culture with high reproducibility, low cost, and relatively stable in gene expression profiles. The HepG2 adiponutrin variant $Ile^{148}Met$ is emphatically associated with liver fat content, especially TG, the main fat accumulated in the liver and the major cause of fatty liver disease. This HepG2 adiponutrin variant has been shown

to increase intracellular TG content and TG concentrations in media [10]. In the current study, accumulation of intracellular lipid was demonstrated by changes to histomorphological features. HepG2 cells treated with PA at 1 mM and OA and PA at 5 mM demonstrated fat overloading with karyopyknotic cell shrinkage and the presence of apoptotic bodies indicating up-regulation of metabolic genes. The findings that PA provided more steatogenic effects and apoptotic induction was in agreement with previous studies [11].

One of the markers of multiple-hit pathogenesis of NAFLD comprises induction of FFA synthetic pathways through PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c, ACC, ACOX, and FAS, which not only directly affect lipogenesis but also influence insulin resistance [12,13]. In our hands, OA or PA dramatically increased expression of those metabolic genes, and PA at 1 mM was the optimal concentration for NAFLD induction in the HepG2 model.

Regarding NAFLD, fat accumulates in the liver as TG by esterification of FFAs and glycerol. Once FFAs enter hepatocytes, they form fatty acyl-CoAs through acyl-CoA synthase. Fatty acyl-CoAs could then pass into either the β-oxidation pathway or esterification, which results in hepatic fat accumulation [14]. The highest dose of OA and all doses of PA induced SREBP-1C, resulting in up-regulation of hepatic *de novo* lipogenesis and hepatic fat accumulation. Moreover, upregulation of ACC, ACOX, and FAS prompt insulin resistance.

Insulin resistance inhibits β-oxidation of FFAs, promoting fat accumulation in the liver [13]. This supports PA activating insulin resistance more than OA. At the same concentration, OA stimulated only ACOX expression while PA triggered increased ACC, ACOX, and FAS expression. High dose of FFAs in hepatocytes induces FAS to activate serine-kinase to induce a defect in insulin signaling pathways, resulting in insulin resistance. Insulin suppresses adipose tissue lipolysis; hence, increased insulin resistance diminishes this repression, leading to increased supply of FFAs to the liver [15]. Although OA at 5 mM induced FAS, PA demonstrated more specificity increasing expression at the lowest concentration.

The observed reductions of CAT and SOD in our model indicated oxidative stress. SOD consists of 3 subtypes [16]. SOD1 is located in the cytoplasm while SOD2 is in the mitochondria and SOD2 polymorphisms are significantly correlated with liver injury. SOD3 is found extracellularly. Alteration of mitochondrial or peroxisomal function impairs a cells ability to handle an increase in lipid flux, resulting in destruction of fat homeostasis and generation of toxic metabolites via overproduction of lipid and reactive oxygen species (ROS), finally causing hepatocyte necroinflammation and exacerbation of mitochondrial damage [14]. The highest concentration of OA led to down-regulation of CAT, SOD1, and SOD2, while PA suppressed only SOD2, but it did so from the lowest concentration. Suppression of SOD2 enhances oxidative stress via mitochondrial dysfunction. Since this occurred even at the lowest concentration of PA, this suggests PA was a greater pro-oxidant than OA.

The modulation of CYP expression observed in our model correlated to the degree of steatosis. While the highest concentration of OA up-regulated CYP2E1 and CYP4A11, the lowest concentration of PA was able to significantly increase expression of CYP2E1 and CYP4A11. These results correlate with previous studies that showed OA and PA increase the level of CYP2E1 and CYP4A11 in rodents, human hepatocytes, and differentiated human cells [17,18]. Induction of CYP2E1 and CYP4A11 impairs both the ER and mitochondria. Mitochondrial impairment leads to electron leakage during the mitochondrial respiratory chain process and metabolism phase, resulting in lipid peroxidation, ROS overproduction, and oxidative stress. ER impairment activates the unfolded protein response (UPR) and activates c-Jun N-terminal kinase (JNK), which trigger inflammation, apoptosis, and insulin resistance [13,19].

Furthermore, increased CYP2E1 and CYP4A11 expression and concomitant exposure to their substrate drugs can lead to severe cellular injury due to the over-production of toxic metabolites, such as acetone from CYP2E1 and ketone bodies from CYP4A11 [20,21]. These findings support that CYP2E1 induces fatty liver disease whether it is induced by alcohol, or not [22,23]. Previous investigations have demonstrated a relationship between NAFLD progression and decreased activity of CYP1A2, CYP2D6, and CYP3A4 [18,24]. In our model, PA reduced expression of CYP1A2, CYP2D6, and CYP3A4, while OA down-regulated CYP1A2 and CYP3A4. The expression of CYP2C19 has been reported either as induction or inhibition in humans [18,25]. Concentrations of 1 and 5 mM OA decreased CYP2C19 expression while 0.1 and 1 mM PA induced CYP2C19. These findings

suggest that modulation of CYP2C19 expression was dependent on the structure of the FFA.

The present model revealed the relevance of the FFA-concentration to the degree of steatosis. The activation of PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c by fat accumulation in the liver causes lipotoxicity, mitochondrial dysfunction, and ER stress. Furthermore, the induction of ACC, ACOX, and FAS, which are down-stream of the carbohydrate-responsive element-binding protein (ChREBP) and SREBP, indicates the development of insulin resistance while the down-regulation of CAT and SOD2 mRNA expression causes oxidative stress. Taken together, the increases in CYP2E1 and CYP4A11 expression, decreases in CYP1A2, CYP2D6, and CYP3A4 expression and changes in CYP2C19 expression affect the progression of NAFLD via ER and mitochondrial injury and potentially through an increase in toxic products from clinical drug metabolism and the associated imbalance in the expression of metabolic and anti-oxidative genes potentially authorizes gene mutations. This correlation of multiple changes to CYP450 regulatory profiles and metabolic and anti-oxidative gene expression support the multiple-hit pathogenesis model of NAFLD progression.

CONCLUSION

This study has established a novel *in vitro* NAFLD model in HepG2 cell derived multiple-hit pathogenesis associated with regulation of CYP450s. PPAR-α, PPAR-γ, SREBP-1a, SREBP-1c, ACC, ACOX, and FAS were induced while CAT and SOD2 were suppressed. CYP2E1 and CYP4A11 were elevated while CYP1A2, CYP2C19, CYP2D6 and CYP3A4 were attenuated. PA was a more potent steatogenic agent with less apoptotic effects than OA. PA at a concentration of 1 mM was optimal for induction of NAFLD in HepG2 cell.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

The authors declare that this work was done by the authors named in this article and that all liabilities pertaining to claims relating to the content of this article will be borne by them. NS carried out the experiments and statistical analysis, and drafted the manuscript. WC supervised the experimental work, verified the data, and proved the draft of manuscript. KJ designed the conceptual framework and revised the manuscript. The manuscript was comprehensively read and approved for publication by all authors.

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