2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)-ELICITED STEATOSIS: THE ROLE OF ARYL HYDROCARBON RECEPTOR (AHR) IN LIPID UPTAKE, METABOLISM, AND TRANSPORT IN *SCD1*$^{+/+}$, *SCD1*$^{-/-}$, AND C57BL/6 MICE

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ABSTRACT

TCDD-ELICITED STEATOSIS: THE ROLE OF AHR IN LIPID UPTAKE, METABOLISM AND TRANSPORT IN *SCD1*$^{+/+}$, *SCD1*$^{-/-}$, AND C57BL/6 MICE

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Metabolic syndrome (MetS) and its associated disorders such as obesity, type II diabetes, non-alcoholic fatty liver disease (NAFLD), and hypertension are epidemic in Western countries including the United States. Conventional thought holds excess energy consumption accountable for MetS phenotypes, and although Western diet and culture is characterized by too many calories consumed and too few calories burned, environmental endocrine disrupting chemicals (EDCs) have emerged into the spotlight for their role in positive energy balance. Dioxin-like compounds (DLCs) including 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) are environmentally ubiquitous and persistent EDCs that alter energy balance and lipid metabolism in animals and humans. TCDD elicited hepatic steatosis involves aryl hydrocarbon receptor (AhR) activation and is marked by increased triglycerides, free fatty acids, inflammatory cell infiltration, and increased serum alanine aminotransferase levels. Hepatic steatosis in the absence of alcohol consumption is a cryptic, yet significant manifestation of MetS and its associated diseases and may precede cirrhosis as well as other extrahepatic effects. Stearoyl-CoA desaturase 1 (Scd1) catalyzes the rate-limiting step in monounsaturated fatty acid (MUFA) biosynthesis. Its deficiency protects mice from diet-induced steatosis, and the enzyme is a target for the treatment of metabolic related disorders. In this report the role of AhR regulation of lipid uptake,

metabolism, and transport in TCDD-elicited steatosis was characterized using *Scd1* null mice, diet, and ^{14}C-lipid uptake studies. Collectively, these studies showed that 1) AhR regulation of Scd1 contributes to the hepatotoxicity of TCDD, 2) dietary fat is the primary source of lipid in TCDD-elicited steatosis, 3) TCDD increases the uptake of dietary lipids, and 4) AhR mediates not only altered hepatic lipid composition, but also systemic lipid composition. This work indicates that AhR activation results in a systemic response that involves coordinated interactions between the digestive tract, circulatory system, and liver, with important health implications for individuals at risk for metabolic disease.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AhR	Aryl hydrocarbon receptor
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic-helix-loop-helix
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
CHOL	Cholesterol
ChREBP	Carbohydrate response element binding protein
CM	Chylomicron
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DBD	DNA binding domain
DLC	Dioxin like chemical
DRE	Dioxin response element
EDC	Endocrine disrupting compound
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
FA	Fatty acid

FFA	Free fatty acid
FAME	Fatty acid methyl ester
Foxo1	Forkheadbox transcription factor 1
GC-MS	Gas chromatography-mass spectrometry
HDL	High-density lipoprotein
HFD	High fat diet
HLH	Helix-loop-helix
HNF4α	Hepatocyte nuclear factor alpha
LDL	Low-density lipoprotein
LBD	Ligand binding domain
LXR	Liver X receptor
MCD	Methionine choline deficient
MetS	Metabolic syndrome
MSS	Matrix similarity score
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
N/A	Not available
NC	No change
ND	Not detected
NEFA	Non-esterified fatty acids
NLS	Nuclear localization signal
NR	Nuclear receptor

OVX	Ovariectomized
PAH	Polyaromatic hydrocarbon
PAS	Per-Arnt-Sim
PBDF	Polybrominated dibenzofuran
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
Per	Period
PGC1α	Peroxisome proliferator activated receptor γ coactivator 1 α
PND	Postnatal day
PPARα	Peroxisome proliferator activated receptor alpha
PUFA	Polyunsaturated fatty acid
PWM	Position weight matrix
PVDF	Polyvinylidene flouride
PXR	Pregnane X receptor
QRTPCR	Quantitative real time polymerase chain reaction
RAR	Retinoic acid receptor
RLW	Relative liver weight
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid X receptor
Scd1	Stearoyl-CoA desaturase 1

SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acid
Sim	Single-minded
TAD	Transcriptional activation domain
TAG	Triglyceride
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalents
TFA	Total fatty acid
TiPARP	TCDD-inducible poly(ADP-ribose) polymerase 7, PARP7
TLC	Thin layer chromatography
TR	Thyroid receptor
VLDL	Very low-density lipoprotein

CHAPTER 1

CHAPTER 1

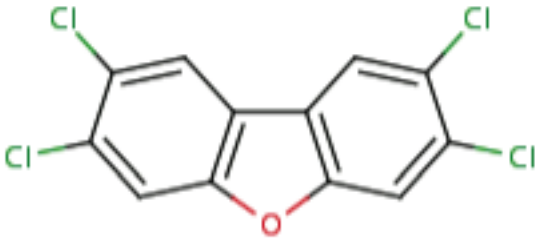
REVIEW OF THE LITERATURE: DIOXINS, AHR, LIVER, AND SCD1

INTRODUCTION

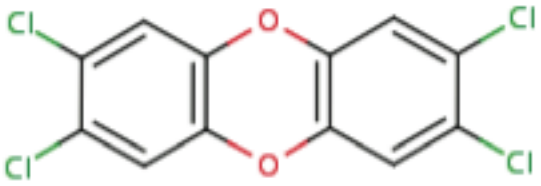
The AhR is a basic helix-loop-helix (bHLH) Period (Per)/ aryl hydrocarbon receptor nuclear translocator (ARNT)/ single minded (Sim) (PAS) transcription factor family member that mediates a wide-range of species- and tissue-specific effects in response to ligand activation [1]. Polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) are the best-characterized high-affinity AhR ligands that are ubiquitously distributed throughout the environment. HAHs describe a family of polychlorinated dibenzo-ρ-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated dibenzofurans (PBDFs) and polychlorinated biphenyls (PCBs). PCDD/Fs and PBDFs with chlorine or bromine substitutions in the 2, 3, 7, and 8 positions and PCBs that have four or more coplanar chlorine atoms are termed “dioxin-like chemicals” (DLCs) due to their structural and chemical similarity (Figure 1.1). DLCs elicit common, species- and tissue-specific toxic effects including wasting syndrome, immunotoxicity, reproductive abnormalities, tumor promotion, induction of gene expression, hepatotoxicity, and endocrine disruption [2-4]. Due to their resistance to degradation and lipophilicity, DLCs bioaccumulate in animal fat stores, persist in the environment, and pose a risk to wildlife and human health [5].

Persistent AhR activation significantly alters hepatic function with the potential to negatively affect metabolism and influence the development of metabolic disease. AhR

Figure 1.1. Chemical Structures of TCDD and dioxin-like chemicals.
For interpretation of the references to color in this and all other figures the reader is referred to the electronic version of this dissertation.



2,3,7,8-tetrachlorodibenzofuran
(TCDF)



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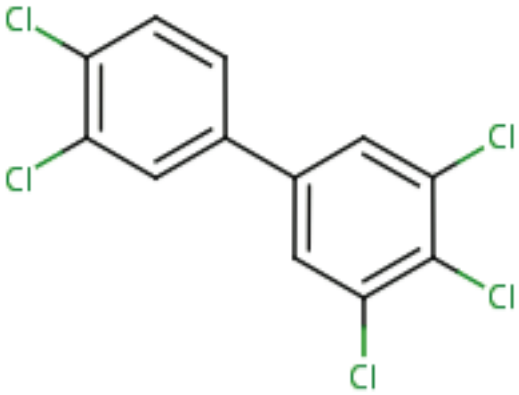
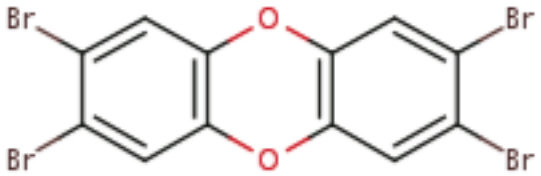
(TCDD)



3,3',4,4',5-pentachlorobiphenyl
(PCB126)



2,3,7,8-tetrabromodibenzo-4-dioxin
(TBDD)

ligands elicit lipid abnormalities such as fatty liver (steatosis) [6-9] that is also the hepatic manifestation of MetS [10, 11], a term used to describe a group of multi-factorial risk factors that include dyslipidemia, obesity, insulin resistance or type II diabetes, and hypertension [12]. Examination of ligand-activated, AhR-mediated alterations in global lipid uptake, metabolism, and transport can significantly expand our current understanding of AhR physiological function, as well as etiology of metabolic disease.

DIOXIN AND DIOXIN-LIKE COMPOUNDS: SOURCES AND TOXICITY

DLCs represent a class of toxic chemicals predominantly produced as by-products of combustion, chemical processes involving chlorine, metals smelting, refining, and process sources, environmental reservoirs, and natural sources such as forest fires and volcanoes [13]. The term dioxin is often used to describe not only TCDD, the most widely studied HAH, but also complex mixtures of TCDD and related chemicals. TCDD is one of the most toxic dioxins with a toxic equivalency factor (TEF) = 1 and therefore used as the reference compound for this class of chemicals. The TEF approach [14, 15] was developed to simplify risk assessment and extrapolate the toxicity of complex dioxin mixtures into a single value expressed as toxic equivalents (TEQs). For the TEF approach, each dioxin-like congener is assigned a TEF by scientific experts based on available data relative to TCDD, ranging from 0.000001 to 1 [16]. The TEFs for each congener in a mixture are multiplied by their respective mass concentration to identify a TEQ representative of a mixture's toxicity.

According to a 2003 draft report, the U.S. Environmental Protection Agency estimated average U.S. adult TEQ intake at 66 pg/day, well above the 2012 EPA non-cancer reassessment reference dose of 0.7 pg/kg/day or 49 pg/day for an average 70 kg adult. Human exposure to

dioxin is primarily through the food supply, mainly consumption of animal fats including meat, fish, and dairy. Dioxins bioaccumulate in animal fat depots and in humans, persist with a half-life of ~7 years [17]. Therefore, although strict industrial regulations have led to a ~90% reduction in nationwide emission over the past 40 years, food safety issues have become an increasing public concern [18].

Numerous human effects documented from direct dioxin contamination incidents have emphasized the clinical impact of dioxin exposure. Cross-sectional and longitudinal studies have evaluated 1) U.S. chemical workers exposed to dioxin after a TCP reactor explosion in West Virginia [19], 2) German BASF employees exposed to TCDD after an uncontrolled reactor decomposition reaction [20], 3) U.S. Air Force Ranch Hand personnel exposed to the herbicide Agent Orange during the Vietnam war [21], 4) residents of communities in Missouri exposed to dioxin contaminated waste oil [22], and 5) residents of Seveso, Italy exposed to industrial emissions caused by an explosion of a trichlorophenol reactor [23, 24]. These studies have linked exposure in adults and children to chloracne [25, 26], impaired immune function [20, 22], impaired nervous system and reproductive development [27], altered liver function [20-22, 28], incidences of dyslipidemia [20, 25, 26, 28], and Type II diabetes [29-31]. The fetus and newborn infants, with rapidly developing organ systems, are subgroups particularly sensitive to dioxin exposure. In particular, Dutch postnatal studies identified an association between maternal exposure and elevated TSH and T4 levels in nursing infants [32] that suggest dioxin exposure modulates the hypothalamic-pituitary-thyroid axis, an essential metabolic regulator.

Recently, environmental contaminants have received increasing attention for their roles in disrupting energy balance and homeostasis [33-36]. In humans, dioxin's metabolic disruptive effects are further highlighted by epidemiological studies that frequently link dioxin exposure to

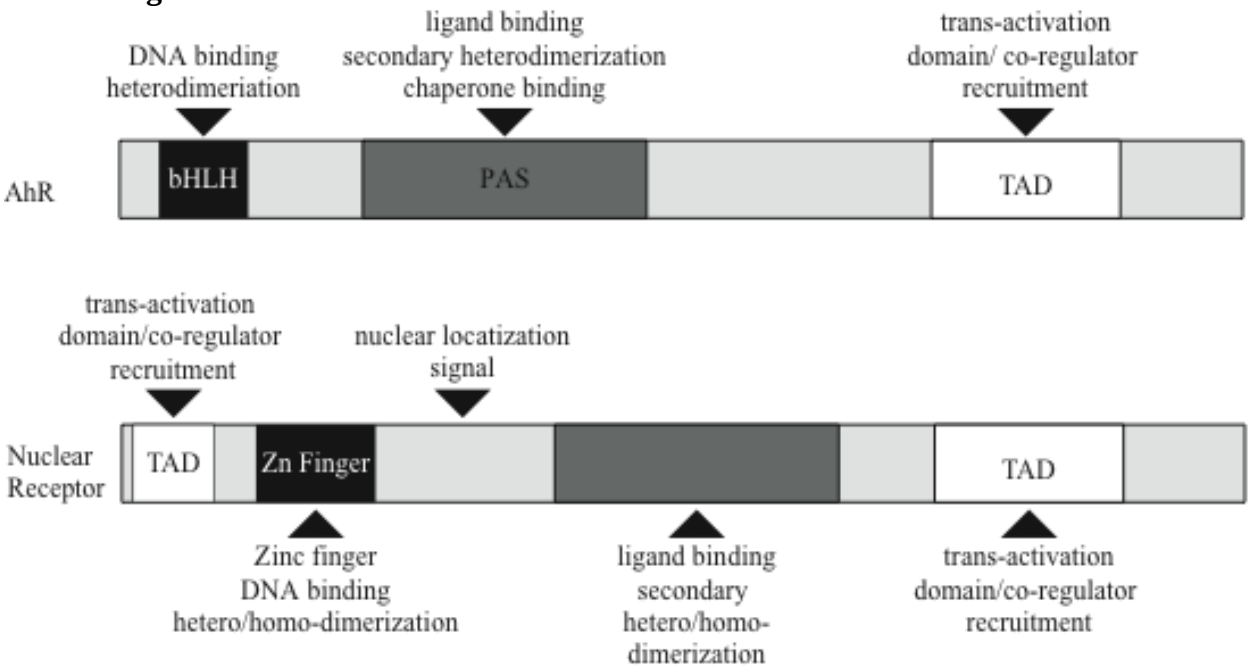
dyslipidemia, insulin resistance, and cardiovascular abnormalities [30, 37-44]. In rodents, dioxin elicits similar effects [45] including reduced gluconeogenesis [46], inhibited adipogenesis [47], and decreased glucose transport activity *in vitro* [48, 49]. Collectively, these studies emphasize the potential for dioxin exposure to adversely affect human health and strongly imply endocrine-disruption. Considering that the AhR mediates most, if not all effects elicited by dioxin, the receptor has been extensively studied and will be discussed below.

ARYL HYDROCARBON RECEPTOR (AHR): MOLECULAR MECHANISM AND PHYSIOLOGICAL FUNCTION

The AhR is a bHLH-PAS transcription factor that mediates most, if not all, effects elicited by dioxins. The AhR bHLH domain is strictly conserved, allows contact with DNA, and functions as the primary heterodimerization surface [50] (Figure 1.2). The PAS domain serves as a secondary heterodimerization surface that promotes interactions with other PAS protein family members and also functions as a ligand binding domain (LBD) [50-52]. The C-terminal transcriptional activation domain (TAD) is essential for AhR mediated gene transcription and recruits transcriptional co-regulators and other basal transcription machinery [50].

PAS family transcription factors, such as AhR, are structurally and functionally distinct from nuclear receptors (NR). All NR are grouped into a large superfamily of homologous proteins based on their conserved DNA binding domain (DBD) that consists of two highly conserved zinc finger motifs [53]. NRs are classified as either Type I or Type II depending on their ability to homo- or heterodimerize and bind to inverted or direct repeat DNA half sites, respectively. Estrogen receptor (ER) is an example of a Type I NR. Peroxisome proliferator activated receptor (PPAR), retinoid X receptor (RXR), retinoic acid receptor (RAR) and thyroid

Figure 1.2. General schematic representation of AhR (top) and nuclear receptors (bottom) domain organization.



receptor (TR) are all Type II receptors [53]. The C-terminal conserved LBD contains a ligand-dependent activation domain, which interacts with chaperone proteins and transcriptional co-regulators. The N-terminus also contains a transcriptional activation surface that recruits coregulators [53].

Unlike nuclear receptors, the AhR resides in the cytoplasm. The classical mechanism of AhR activation involves ligand diffusion into the cytoplasm where it is bound by the AhR (Figure 1.3). Ligand binding is presumed to induce a conformational change exposing the AhR nuclear localization signal (NLS) and induce dissociation of chaperone proteins hsp90, p23, and XAP2 [54, 55]. The liganded AhR translocates to the nucleus where it forms a heterodimer with ARNT, another member of the bHLH-PAS family. This high-affinity AhR:ARNT heterodimer binds specific DNA recognition sites with the invariant core sequence GCGTG that are termed dioxin response elements (DREs) [56, 57]. AhR:ARNT DNA binding leads to chromatin remodeling, transcriptional co-activator recruitment, and altered rates of gene transcription [54, 58]. *Cytochrome P450 1a1* and *1a2* are the best characterized and prototypical AhR activated genes whose transcriptional activation is often used as litmus test for AhR induction and ligand potency [3, 59-62]. AhR:ARNT-dependent gene transcription terminates after heterodimer dissociation from the DRE followed by ubiquitin-mediated 26S proteasome pathway AhR degradation [63].

Accumulating evidence indicate a polymorphic cytochrome P450 response to TCDD toxicity across species that is dependent on AhR binding affinities [64]. Specifically, inbred mouse strains have inter-individual genetic susceptibility to dioxin toxicity due to four distinct *Ahr* alleles (Ahr^{b1}, Ahr^{b2}, Ahr^{b3}, and Ahr^{d}, Table 1.1) with 10-20-fold differences in ligand binding affinity that renders them either responsive (b alleles, $K_d \leq 1$ nM) or unresponsive d

Figure 1.3. The ligand-activated AhR signaling pathway.
Ligand passively diffuses into the cell and is bound by the aryl hydrocarbon receptor (AhR). Ligand binding induces a conformational change leading to dissociation of chaperone proteins (hsp90, p23, and XAP2) and exposing the AhR nuclear localization signal. The liganded AhR translocates to the nucleus and heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR:ARNT heterodimer binds to dioxin response elements (DRE) located in the promoters of target genes to activate gene transcription.

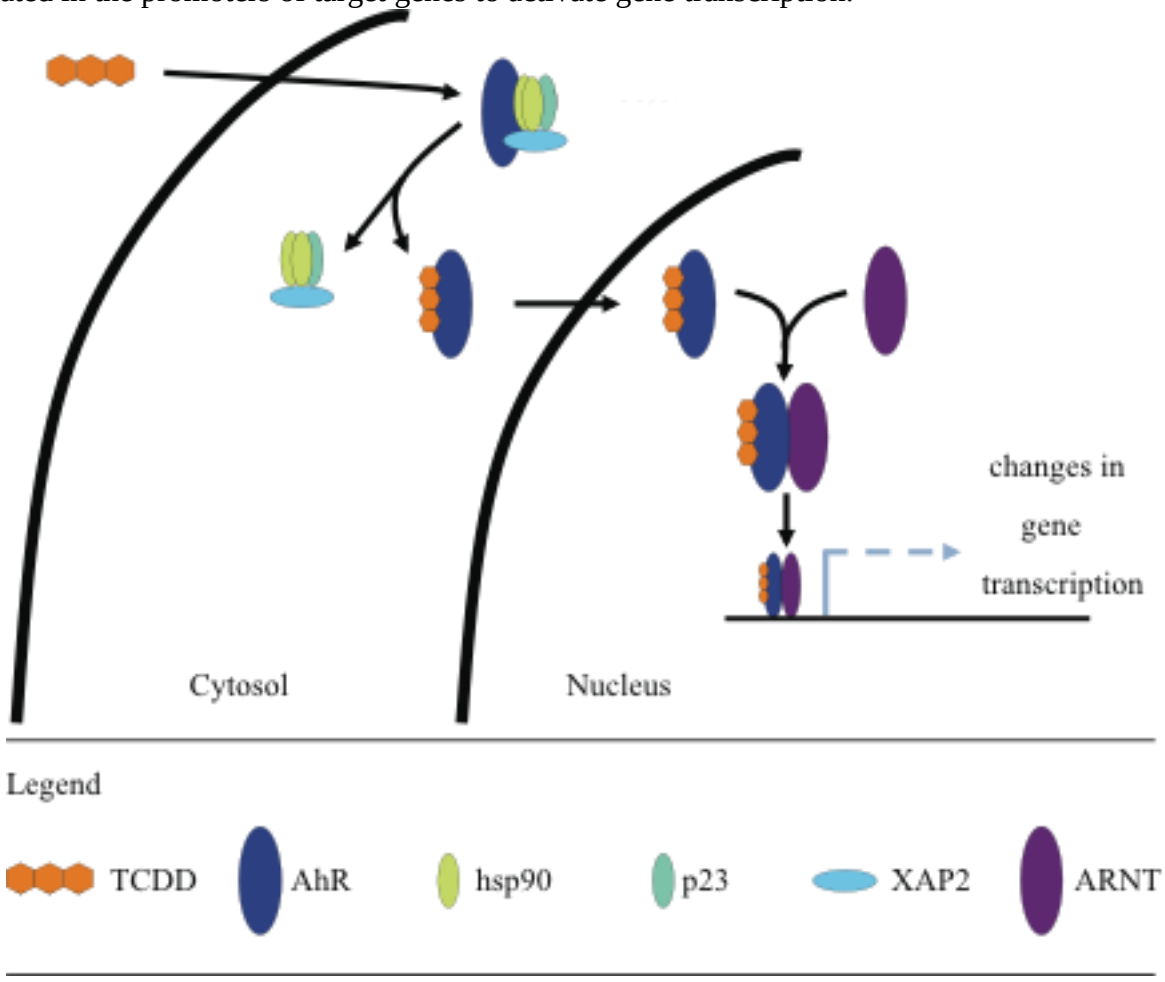


Table 1.1. Summary of AhR Alleles Across Mouse Strains

AhR Allele	AhR^{d}	AhR^{b1}	AhR^{b2}	AhR^{b3}	Unknown
Affinity for TCDD	Low	High	High	High	---------
Strain	AKR/J	MA/MyJ	A/J	**MOLF/Ei**	SM/J
	DBA/2J	C57BL/6J	BALB/cByJ	SPRETUS/Ei	**WSB/EiJ**
	I/LnJ	C57BLKS/J	C3H/HeJ		RIIIS/J
	LG/J	C57BR/cdJ	CBA/J		FVB/NJ
	LP/J	C57L/J	PERA/EiJ		NOD/LtJ
	NZB/BlNJ	C58/J	PL/J		NON/LtJ
	129S1/SvImJ		SEA/GnJ		**PWD/PhJ**
	SWR/J		BUB/BnJ		
	SJL/J		CE/J		
	CAST/Ei				

Bold – ancestral wild-type derived strains. Data were summarized from Thomas et al., 2002 [66] and the mouse phenome database (http://phenome.jax.org).

Allele (K_d = 16 nM) [65-67]. Studies using transgenic mice harboring the b allele have highlighted the importance of the AhR nuclear signaling pathway in mediating the toxicities of dioxins, with a particular focus on TCDD [68, 69]. Transgenic mice carrying mutations in the NLS or DBD are resistant to TCDD toxicity, including hepatic steatosis. However, TCDD-elicited DRE-independent and/or ARNT-independent effects have also been reported [70-72].

Despite generation of the AhR knock-out [73, 74] and transgenic animal models [69, 75], the physiological function of AhR, beyond xenobiotic metabolism and hepatic growth and development, remains elusive. AhR conservation among all vertebrates [76] as well as similar cytochrome P450 up-regulation in fish, rodents, and birds [77, 78] suggests a fundamental role for the AhR. Yet it is unlikely that ancient exposure to POPs (that have been introduced into the environment along with the industrial revolution [79]) provided selective pressure on AhR during vertebrate evolution. No high affinity endogenous AhR ligand has been identified although the AhR is activated by diverse lipophilic endobiotics (indoles, tetrapyrroles, and arachidonic acid metabolites) and naturally derived molecules (vegetable-, fruit-, and tea-derived indoles and flavonoid metabolites) [1, 80]. Collectively, the developmental phenotypes in AhR null mice and AhR evolutionary conservation suggest a role for AhR in cellular physiology other than metabolism of foreign chemicals, although it remains to be elucidated. Nonetheless, AhR ablation is accompanied by several liver defects, a metabolic organ coincidentally targeted by TCDD toxicity.

LIVER: A METABOLIC ORGAN AND DIOXIN TARGET

The liver is a primary target of TCDD with steatosis, hepatomegaly, and elevated liver enzymes (alanine aminotransferase and glutathione S-transferase) representing significant

hepatotoxic endpoints [45, 81-84]. Strategically situated between the portal and systemic circulation, the liver, which has first access to absorbed nutrients as well as other exogenous substances, is the site for primary metabolism. The principal function of the liver is to metabolize, detoxify, inactivate, and convert both endogenous and exogenous substance that are either excreted into bile or returned to the systemic circulation [85]. Another important liver function is synthesis and degradation of protein, carbohydrates, and lipids for distribution to extrahepatic tissues depending on energy needs. Finally, the liver regulates whole body cholesterol balance via biliary excretion of cholesterol (CHOL), CHOL conversion to bile acids, and by regulating CHOL synthesis [85]. Consequently, the liver is an essential regulator of whole body metabolism and energy homeostasis that is disrupted by TCDD.

Studies suggest that TCDD exposure in rodents and humans disrupts hepatic energy balance by altering normal anabolic and catabolic pathways. For example, one of the most important metabolic functions performed by the liver is maintenance of blood glucose levels between meals. When insulin levels are high and glucagon levels are low (after a meal), the liver takes-up glucose and either stores it as glycogen or breaks it down into pyruvate. Conversely, when glucose and insulin levels are low and glucagon levels are high, hepatic gluconeogenesis and glycogenolysis prevail [85]. During periods of energy excess, the liver can convert glucose into fatty acids (FAs) that are esterified into triglycerides (TAGs). TAGs are either stored or packaged with cholesterol and apolipoprotein b100 into very low-density lipoproteins (VLDL) for export into the blood. TAGs within circulating VLDL are hydrolyzed by circulating lipases that release FA for peripheral tissue (primarily muscle and adipose) uptake [86]. During periods of negative energy balance, FAs metabolized by β-oxidation generate acetyl CoA and ketone bodies. Dioxin exposure disrupts liver metabolism by decreasing gluconeogenesis [87], FA and

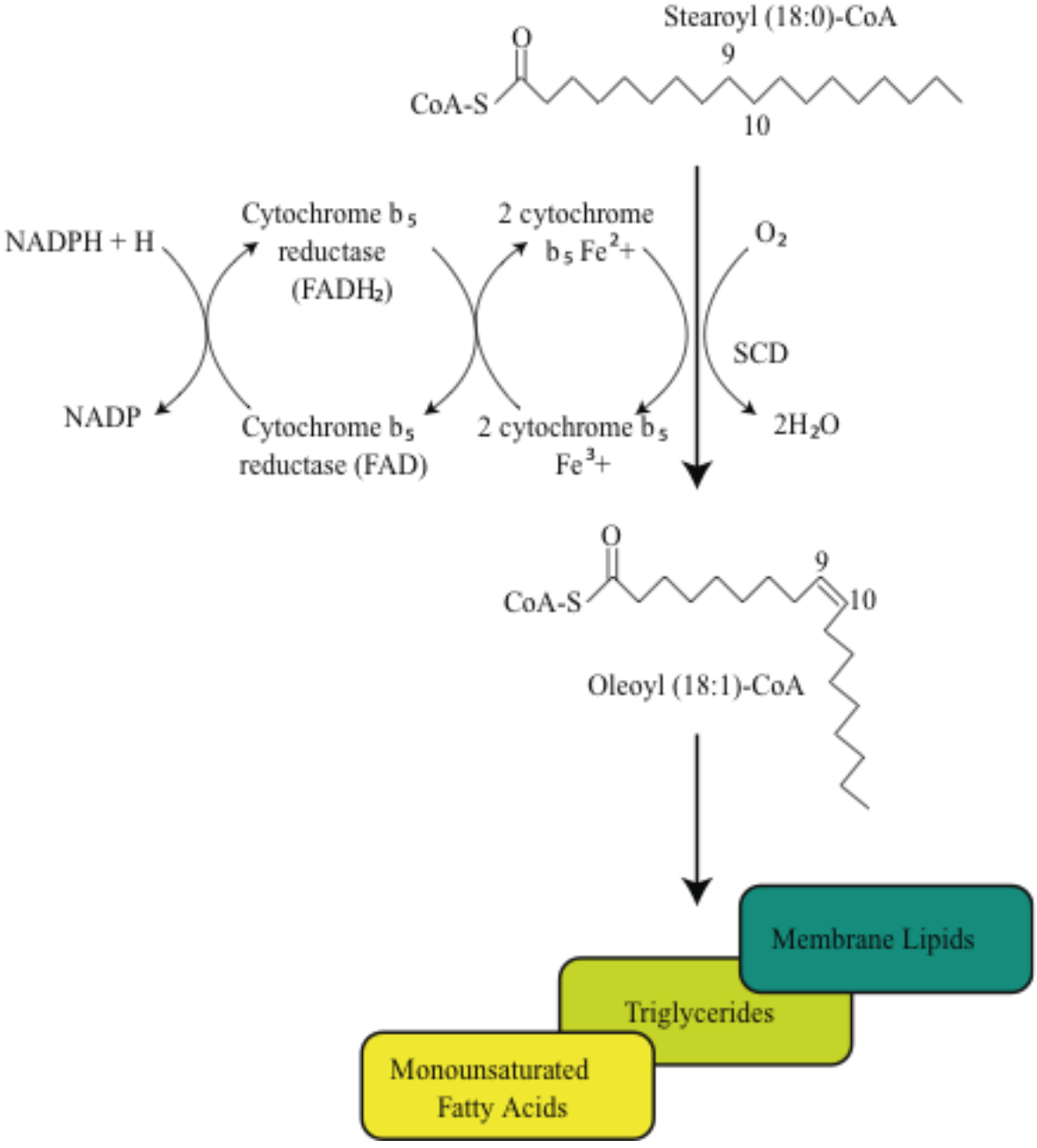
CHOL synthesis [88, 89], and mitochondrial beta-oxidation [9, 90], while promoting hepatic steatosis [6, 7, 91-93] regardless of the hepatic energy state. These effects collectively net altered energy balance with implications for global metabolic disruption.

Importantly, TCDD-elicited steatosis and lipid abnormalities are also observed in humans [8]. Hepatic steatosis in the absence of alcohol consumption constitutes NAFLD, the hepatic manifestation of MetS, with insulin resistance as the primary pathogenic route [11]. Epidemiological studies have not only linked human dioxin exposure to incidences of steatosis, but also other MetS associated risk factors including dyslipidemia, insulin resistance/diabetes [8, 38, 39, 94, 95], and hyperglycemia [96]. Collectively, these studies in combination with TCDD-dependent alterations in hepatic lipid and glucose metabolism, further suggest TCDD and similar compounds may underlie AhR-mediated alterations in global lipid metabolism and the etiology of metabolic disease.

STEAROYL COA DESATURASE 1 (SCD1): ROLE IN LIPID METABOLISM AND METABOLIC DISEASE

Interestingly, several toxicities elicited by AhR activation, including steatosis and insulin resistance, are common effects observed in Scd1 mouse models [97-99]. Scd1 is an endoplasmic reticulum bound trans-membrane Δ9 desaturase that catalyzes the rate-limiting step in MUFAs synthesis. MUFAs are critical components of complex lipids including membrane lipids, phospholipids, TAGs, and CHOL esters [100]. Scd1 together with cofactors NADH, b_5 reductase, cytochrome b_5, and oxygen introduce a double bond into fatty acyl-coenzyme A [101] (Figure 1.4). Palmitate (16:0) and stearate (18:0) are the

Figure 1.4. The desaturation of fatty acids by stearoyl-CoA desaturase 1 (Scd1).
The oxidative reaction catalyzed by Scd1 introduces a double bond into the Δ9 position between carbons 9 and 10. Electrons flow from NADPH to cytochrome b_5 reductase to cytochrome b_5 to activate O_2 that is reduced to H_2O. Steatate is the preferred substrate for Scd1 and desaturated to oleate, a major constituent of monounsaturated fatty acids, triglycerides, and membrane lipids.



preferred substrates for SCD and are desaturated into palmitoleate (16:1n7) and oleate (18:1n9), respectively.

Multiple SCD isoforms have been identified in mice (*Scd1-Scd4*) [102-105] and humans (SCD1 and SCD5) [106-108] and these genes exhibit tissue-specific expression. In mice, *Scd1* and *Scd2* are primarily expressed in liver and adipose, but also other lipogenic tissues. *Scd2* is expressed in neonates, while *Scd1* is the predominant form expressed in adults. Unlike other *Scd* isoforms that confer activity towards stearoyl-CoA [99], *Scd3* exhibits substrate preference for palimtoyl-CoA [109] and is primarily expressed in skin, Harderian gland and preputial gland [105]. *Scd4* is exclusively expressed in heart [102]. Human SCD1 is primarily expressed in adipose and liver and shares ~85% amino acid similarity with *Scd1-Scd4*. SCD5 is unique to primates and abundantly expressed in brain and pancreas [108].

Scd1 is transcriptionally regulated by a variety of nutrients (glucose, fructose, CHOL, polyunsaturated fatty acids (PUFAs)), hormones (insulin, glucagon, thyroid hormone, estrogen) and environmental factors (temperature, light, cadmium) [99, 110, 111] and their cognate transcription factors. Specifically, nutrient sensing by liver X receptor (LXR) [112], sterol regulatory element binding protein-1c (SREBP-1c) [113, 114], carbohydrate response-element binding protein (ChREBP) [115, 116], pregnane -X receptor (PXR) [117, 118] and PPARs [119] positively regulate *Scd1* transcription. Scd1 activity is also controlled by post-translational ubiquitin-pathway mediated degradation [120] that is inhibited by PPARα activation [121].

Evidence from knockout and transgenic studies suggests that Scd1, required for TAG, phospholipid, CHOL ester, and VLDL synthesis [122, 123], is important for the development of diet-induced metabolic disorders. Specifically, mice with targeted disruption of the *Scd1* gene are protected from high-fat and high-carbohydrate diet-induced steatosis and obesity [113, 124-126],

and exhibit improved insulin sensitivity [97, 127]. In humans, increased Scd1 expression is associated with insulin resistance [128] and an increased 18:1n9/18:0 plasma ratio (an indirect measure of Scd1 activity) that positively correlates with increased TAGs [129]. Furthermore, several single nucleotide polymorphisms located within intron, exon, and 3'UTR SCD1 genic regions are associated with body fat distribution and insulin sensitivity [130]. Consequently, Scd1 has become a pharmacological target for the treatment of MetS risk factors including obesity and diabetes [97].

CONCLUSION

TCDD and similar chemicals bioaccumulate and persist in the food chain to elicit adverse health effects in humans and animals. Accumulating evidence suggests these chemical effects superimpose upon a Western lifestyle to initiate or exacerbate disruption of energy metabolism [131]. Hepatic steatosis is the consequence of TCDD-mediated AhR activation and also linked with metabolic disease. Hepatic steatosis is a significant complication associated with obesity and other MetS phenotypes [132], such as insulin resistance, however the underlying pathophysiological mechanism in the absence of alcohol consumption remains unknown [133]. Several Scd1 phenotypes (steatosis, increased TAG levels, insulin resistance) overlap with TCDD-elicited toxicities, suggesting plausible interactions between AhR and Scd1 regulation of energy metabolism. Furthermore, the liver performs a pivotal role in nutrient sensing, uptake, and efflux. Therefore AhR regulation of lipid uptake, metabolism, and transport in an Scd1 knockout model may further our understanding of steatosis pathogenesis with important implications for MetS risk factor etiology.

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REFERENCES

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CHAPTER 2

CHAPTER 2

RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

RATIONALE

Hepatic steatosis in the absence of alcohol consumption is considered the hepatic manifestation of MetS [1]. Human exposure to TCDD and related compounds have been implicated in the development of MetS [2, 3] and its associated risk factors including insulin resistance [4, 5], hypertriglyceridemia [6-8], and steatosis [9]. In rodents Scd1 performs a critical role in lipid homeostasis and when deleted, protects mice from diet-induced hepatic lipid accumulation and improves insulin sensitivity [10]. TCDD and DLC exposure in mice elicits hepatotoxicity marked by the differential expression of genes involved in lipid metabolism, including Scd1 [11], and increased hepatic vacuolization due to lipid accumulation [11-14]. In summary, these results suggest that AhR-mediated effects on Scd1 regulation, lipid metabolism, and transport may underlie DLC-induced steatosis and other related pathophysiologies.

HYPOTHESIS

TCDD-elicited, AhR mediated steatosis involves Scd1 dependent and independent alterations in lipid uptake, metabolism, and transport.

SPECIFIC AIMS

Specific aim 1 will test the subhypothesis that Scd1 deficiency protects from TCDD-elicited steatosis. AhR regulation of Scd1 and TCDD-induced steatosis in Scd1 wild-type and null mice will be characterized.

Specific aim 2 will test the subhypothesis that diet is the primary source of lipid in TCDD-elicited steatosis. Dietary fat, carbohydrate, and ^{14}C-oleate will be examined as hepatic lipid sources in TCDD-elicited steatosis.

Specific aim 3 will test the subhypothesis that TCDD not only alters liver lipid composition, but also parametrial adipose and serum lipid composition. TCDD effects on liver, parametrial fat pad (PFP), and serum fatty acid levels and composition will be examined by gas chromatography-mass spectrometry (GC-MS). In addition serum lipid profiles (total CHOL, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TAG) and VLDL) will be examined.

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CHAPTER 3

Angrish MM, Jones AD, Harkema JR, Zacharewski TR: **Aryl Hydrocarbon Receptor-Mediated Induction of Stearoyl-CoA Desaturase 1 Alters Hepatic Fatty Acid Composition in TCDD-Elicited Steatosis.** *Toxicol Sci* 2011, **124:**299-310.

CHAPTER 3

ARYL HYDROCARBON RECEPTOR-MEDIATED INDUCTION OF STEAROYL-COA DESATURASE 1 ALTERS HEPATIC FATTY ACID COMPOSITION IN TCDD-ELICITED STEATOSIS

ABSTRACT

TCDD induces hepatic dyslipidemia mediated by the AhR. Scd1 performs the rate-limiting step in MUFA synthesis, desaturating 16:0 and 18:0 into 16:1n7 and 18:1n9, respectively. To further examine the role of Scd1 in TCDD-induced hepatotoxicity, comparative studies were performed in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice treated with 30 μg/kg TCDD. TCDD induced Scd1 activity, protein, and mRNA levels ~2-fold. In *Scd1*$^{+/+}$ mice, hepatic effects were marked by increased vacuolization and inflammation, and a 3.5-fold increase in serum ALT levels. Hepatic TAGs were induced 3.9-fold and lipid profiling by GC-MS measured a 1.9-fold increase in FA levels, consistent with the induction of lipid transport genes. Induction of Scd1 altered FA composition by decreasing saturated fatty acid (SFA) molar ratios 8% and increasing MUFA molar ratios 9%. Furthermore, ChIP-chip analysis revealed AhR enrichment (up to 5.7-fold) and computational analysis identified 16 putative functional DREs within *Scd1* genomic loci. Band shift assays confirmed AhR binding with select DREs. In *Scd1*$^{-/-}$ mice, TCDD induced minimal hepatic vacuolization and inflammation, while serum ALT levels remained unchanged. Although Scd1 deficiency attenuated TCDD induced TAG accumulation, overall FA

levels remained unchanged compared to *Scd1*$^{+/+}$ mice. In *Scd1*$^{-/-}$ mice, TCDD induced SFA ratios 8%, reduced MUFA ratios 13%, and induced polyunsaturated fatty acid ratios 5% relative to treated *Scd1*$^{+/+}$ mice. Collectively, these results suggest AhR regulation of Scd1 not only alters lipid composition, but also contributes to the hepatotoxicity of TCDD.

INTRODUCTION

TCDD and related compounds elicit a broad spectrum of biological responses ranging from effects on development to pathologies affecting specific organ functions as well as the immune and nervous system. These effects are mediated by the AhR a cytosolic ligand-activated bHLH PAS family transcription factor [1, 2]. The proposed mechanism involves ligand binding to the cytosolic AhR, leading to dissociation of chaperone proteins and translocation of the AhR to the nucleus [3]. The activated AhR then heterodimerizes with the ARNT [4] to bind DREs located within regulatory regions of target genes [5] recruiting chromatin remodeling complexes and transcriptional co-regulators that modulate gene transcription [6].

Although the range of endogenous functions regulated by the AhR remains uncertain, studies in null mice show it is necessary for proper liver development and mediates the toxicity of TCDD [7-9]. TCDD elicits hepatomegaly [2] and liver pathologies that include hepatocellular neoplasms [10], inflammation, necrosis, steatosis, and the differential expression of lipid metabolism and transport genes in mice [11-14]. DNA binding by AhR is compulsory for TCDD-induced hepatic steatosis [15].

Scd1 catalyzes the rate-limiting step MUFA biosynthesis and is a target for the treatment of metabolic related disorders [16, 17]. Scd1 desaturates palmitate (16:0) and stearate (18:0) into

palmitoleate (16:1n7) and oleate (18:1n9), respectively, which can be metabolized further to other MUFAs and PUFAs, the primary constituents of membrane lipids, triglycerides, phospholipids and cholesterol esters [18, 19]. Dietary, hormonal and environmental factors regulate Scd1 mRNA expression and protein stability, emphasizing the importance of Scd1 in lipid metabolism [18, 20]. Furthermore, *Scd1*$^{-/-}$ mice are resistant to diet-induced steatosis and exhibit impaired triglyceride synthesis [19, 21]. In humans, the Scd1 activity index (serum ratios of 16:1/16:0 or 18:1/18:0) correlates with hypertriglyceridemia and insulin resistance [22, 23] and is predictive of metabolic syndrome [24]. Furthermore, single nucleotide polymorphisms have been identified in human *Scd1* that are associated with obesity and insulin sensitivity [25].

In this study, we examined AhR regulation of Scd1 and TCDD elicited steatosis in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice. Hepatic fatty acid profiling with complementary histopathology, enzyme activity assays, and gene expression were assessed in immature *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ female mice and integrated with AhR ChIP-chip, band shift, and computational data. Our results suggest that the regulation of hepatic lipid transport and metabolism genes by the AhR, including *Scd1*, is involved in TCDD-induced steatosis in the mouse.

MATERIALS & METHODS

ANIMAL BREEDING AND GENOTYPING

B6.129-*Scd1*^{tm1Myz}/J heterozygous mice were obtained from the Jackson Laboratory (Ben Harbor, Maine) and bred at the Michigan State University Laboratory Animal Care facility. Mice were maintained on a 12h light/dark cycle and housed in autoclaved polycarbonate cages with microisolator lids containing aspen woodchips and nesting material. Animals were allowed free access to Harlan Teklad irradiated F6 rodent diet 7964 (Madison, WI) and autoclaved

deionized water throughout the study. On postnatal day (PND) 21 mice were genotyped by ear punch and weaned. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee.

In Vivo Treatment

On PND 28 mice were gavaged with 0.1 ml of sesame oil for a nominal dose of 0 (vehicle control) or 30 μg TCDD per kg body weight. The immature mouse was used to facilitate comparisons with other data sets as well as avoid potential interactions with estrogens produced by developed ovaries. Doses were chosen to elicit moderate hepatic effects while avoiding overt toxicity in long term studies. Litters were combined with no more than five animals per cage. Animals were sacrificed at 24, 72 and 168 h post dose. Mice were weighed and blood was collected via submandibular vein puncture before sacrifice. Tissue samples were removed, weighed, flash frozen in liquid nitrogen and stored at -80°C. The right lobe of the liver was fixed in 10% neutral buffer formalin for histological analysis.

Histopathology and Clinical Chemistry

Fixed liver tissues were processed as previously described [14] at the Michigan State University Investigative Histopathology Laboratory, Division of Human Pathology, using a modified version of previously published procedures [26]. Serum alanine aminotransferase levels (ALT) were measured by the Michigan State University Diagnostic Center for Population and Animal Health Clinical Laboratory.

Hepatic Triglyceride Levels

Frozen liver samples (~100 mg) were homogenized (Polytron PT2100, Kinematica) in 1 ml of 1.15% KCl. Triglycerides were extracted from 200 μl of hepatic homogenate with 800 μl of isopropyl alcohol by vortex mixing for 10 min. The samples were centrifuged for 5 min at

800xg at room temperature and the supernatant was collected into separate vials. The concentration of hepatic triglycerides was determined by spectrophotometry from 20 µl supernatant with a commercial L-Type Triglyceride M kit (Wako Diagnostics, Richmond, VA) with Multi-Calibrator Lipids as a standard (Wako Diagnostics) according to manufacturer's protocol. Final results were normalized to the initial weight of the liver sample.

GC-MS Fatty Acid Methyl Ester (FAMES) Hepatic Lipid Profiling

Liver samples (~100 mg, n=5/group) were homogenized in 40% methanol and acidified with concentrated HCl (~34µL). Lipids were extracted with chloroform: methanol (2:1) containing 1 mM 2,6-di-tert-butyl-4-methylphenol. The organic phase was removed and protein and aqueous phases were re-extracted with chloroform. The organic phases were pooled and solvents evaporated under nitrogen. The samples were resuspended in 3 N non-aqueous methanolic HCl and held at 60ºC overnight. The next day samples were cooled to room temperature and 0.9% (w/v) NaCl and hexane was added. The organic phase was separated by centrifugation, collected, dried under nitrogen and resuspended in hexane. Samples were separated and analyzed with an Agilent 6890N GC with a DB23 column (30 meter length, 0.25 mm id, 0.25 µm film thickness) interfaced to an Agilent 5973 MS. 19:1n9 free fatty acid (FFA) and 19:0 TAG were added as extraction efficiency controls and 17:1n1 fatty acid methyl ester was spiked in as a loading control (Nu-chek, Elysian, MN). GC/MS data files were converted to Waters MassLynx file format and were analyzed with MassLynx software and are reported as µmol/g liver tissue or mol%. Fatty acid levels are based upon peak areas from total ion chromatograms and µmol/g is obtained from a linear calculation of a calibration curve normalized to sample weight. Principal component analysis of fatty acid abundance was performed in R V2.6.0.

SCD1 ACTIVITY ASSAY

Scd1 activity assays were performed as previously described [27]. Microsomal fractions were isolated by differential centrifugation, and protein was quantified by using the Bradford assay (BioRad). Assays were performed at 37°C for 30 min with 100 μg microsomal protein, 0.03 μCi ^{14}C-stearoyl-CoA (ARC 0756, 50-60 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO) 2 mM NADH, 0.03 mM cold stearoyl-CoA in 0.1 M phosphate buffer at pH 6.8. The reaction was quenched with 2.5 M KOH and saponified at 80°C for 45 min. Fatty acids were acidified with formic acid, extracted with hexane and dried under nitrogen. FAs were resuspended in 50 μL hexane and separated by 100 g/L $AgNO_3$ impregnated TLC using $CHCl_3$:MeOH:acetic acid:water (90:8:1:0.8 v/v) as a developing solvent. TLC plates were dried and exposed to autoradiography film, bands scraped into scintillation fluid, and measured by liquid scintillation counting. Scd1 activity is expressed as nmol/(mg protein*min).

WESTERN BLOTTING

Microsomal fractions isolated for activity assays (10 μg each) were separated in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene flouride (PVDF) membrane (Millipore Billerica, MA). Scd1 protein was immunoblotted with an Scd1 antibody (SC-14719; Santa Cruz Biotechnology Santa Cruz, CA). Conventional western blot loading controls such as Gapdh and Actb are cytosolic proteins, and therefore removed during microsome purification. Epoxide hydrolase (Epxh1, ab76226; Abcam, MA) is a microsomal protein and was used as a reference for loading control. Immunoreactive bands were visualized by chemiluminescnce with the Pierce ECL Western blotting substrate (Thermo Scientific Rockford, IL) [21]. Immunoreactive bands were quantified by densitometry (ImageJ) and normalized to the loading control.

AhR ChIP-chip and DRE Motif Comparisons

The genomic locations of AhR enrichment were previously determined by ChIP-chip from hepatic tissue of immature female ovariectomized C57BL/6 mice orally gavaged with 30 μg/kg TCDD [28]. The genomic locations of the 5'-GCGTG-3' DRE core sequences were previously determined in mouse [29]. The DRE core along with the flanking upstream and downstream 7 bp were compared to a position weight matrix and matrix similarity scores (MSS) were calculated [29]. Regions of AhR enrichment were compared against DRE core sequences across *Scd1*, *Scd2*, *Scd3*, and *Scd4* loci. Associated mouse genomic annotation (mm9) was downloaded from the UCSC Genome Browser [30].

Band Shift Assays

Putative DREs with a MSS > 0.8 located from -10 kb upstream from the *Scd1* transcriptional start site (TSS) were targeted for band shift assays that were performed as previously described [31]. Equimolar complimentary DNA oligonucleotides (Table A.1) were combined and annealed by heating at 95°C for 5 min and cooling to room temperature. The double-stranded DNA oligonucleotide was labeled with T4 polynucleotide kinase (M0201S; New England Biolabs) and [y-32P]ATP (0135001; MP Biomedicals, OH). Each reaction contained 3 μg hepatic guinea pig cytosol extract* mixed with either 20 nM TCDD or an equivalent volume of DMSO and was incubated for 1 hr at room temperature. Guinea pig AhR is readily transformed *in vitro* and binds DREs with high affinity [32]. The activated cytosols were combined with HEDG buffer (25 mM Hepes, pH 7.7/1 mM EDTA/1 mM dithiothreitol/10% glycerol) and 1.7 μg polyd(I-C) (Invitrogen) and incubated at room temperature for an additional 15 minutes. ^{32}P-labeled double-stranded DNA oligonucleotide (10,000 cpm, 0.1-0.3 ng in HEDG buffer) was added to the pre-incubation mixture and incubated for an additional 15

minutes. For supershift assays, either 1 μg AhR (ab2769; Abcam, MA) or ARNT (SC-5580; Santa Cruz Biotechnology Santa Cruz, CA) antibody was added to the pre-incubation mixture. Samples were resolved by non-denaturing polyacrylamide gel electrophoresis and imaged by autoradiography. In competition experiments unlabeled competitor DNA was added to the pre-incubation mixture at 100-fold molar excess.

RNA ISOLATION

RNA was isolated from frozen liver samples with 1.3 mL TRIzol (Invitrogen) according to the manufacturer's protocol and an additional acid phenol:chloroform extraction as previously described [11]. Total RNA was resuspended in RNA storage solution, quantified by spectrophotometery at A_{260} and quality assessed by gel electrophoresis.

QRTPCR

Quantitative real-time PCR (QRTPCR) of *Scd1*, *Scd2*, *Scd3*, *Scd4*, *Cyp1a1*, *Nqo1*, *Tiparp*, *Elovl5*, *Cd36*, *LdlR*, *Fabp4*, *Vldlr*, *Acaca*, and *Fasn* expression was performed as previously described [11]. The copy number of each sample was standardized to the geometric mean of *Gapdh*, *Hprt*, and *Actb* to control for differences in RNA loading, quality, and cDNA synthesis [33]. Data are reported as the fold change of standardized treated over standardized vehicle.

STATISTICAL ANALYSIS

Data were analyzed by analysis of variance (ANOVA) followed by Tukey's *post hoc* test in SAS, unless otherwise stated. Differences between treatment groups were considered significant when $p<0.05$.

RESULTS

TCDD EFFECTS ON BODY WEIGHTS, LIVER HISTOPATHOLOGY, AND CLINICAL CHEMISTRY

Consistent with previously reported changes in body weights and liver histopathology changes in mice exposed to TCDD [11], *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ intact female mice gavaged with 30 μg/kg TCDD had increased relative liver weight (RLW) with the greatest increases observed at 168 h (Table A.3). No significant alterations in body weight or body weight gain were detected throughout the study. Histopathological changes were marked by cytoplasmic vacuolization in the periportal and midzonal regions that decreased with time (not shown). In *Scd1* wild-type mice, hepatic vacuolization was accompanied by cellular inflammation that increased by 168 h, while minimal cellular inflammation was observed at only 168 h in *Scd1* null mice. Further analysis of serum ALT levels, a marker of liver damage, identified a 3.5-fold increase in treated wild-type mice at 168 h (Figure A.1), while ALT levels remained unchanged in *Scd1* nulls. Increased ALT levels suggest liver damage in treated wild-types only, however longer term exposure may be necessary to differentiate histopathological differences between the two strains.

TCDD EFFECTS ON HEPATIC LIPID CONTENT IN SCD1$^{+/+}$ *MICE*

In *Scd1*$^{+/+}$ mice, TCDD induced hepatic TAGs and hepatic total fatty acid (TFA) content 3.9- and 1.9-fold, respectively, compared to vehicle controls (Figure 3.1, panel A). Analysis of fatty acid composition by GC-MS identified TCDD-induced alterations in the SFA/MUFA/PUFA molar proportions (Figure 3.1, panel A). Overall, TCDD reduced SFA by 8%, increased MUFA by 9%, and had no effect on PUFA proportions. Further analysis of individual fatty acid species revealed that palmitate (16:0), stearate (18:0), and lignoceric acids

(24:0) represented more than 95% of the total SFA content (Table 3.1). Interestingly, palmitate (67%) and stearate (16%) are the major SFA constituents of the rodent chow diet (Harlan Teklad rodent diet 7964).

Increases in absolute hepatic levels of MUFAs were primarily due to a 3-fold increase of oleic acid (18:1n9), representing >85% of all MUFAs (Table 3.1). Increases in accumulation of palmitoleic acid (16:1n7, increased 1.9-fold), eicosenoic acid (20:1n9, increased 7.2-fold), erucic acid (22:1n9, increased 2-fold), and nervonic acid (24:1n9, increased 6-fold) accounted for the remaining 15% of MUFAs. ,

Although TCDD did not alter PUFA levels expressed as mol%, absolute hepatic levels were increased 1.8-fold by TCDD (Table 3.1). In general, TCDD treatment increased all PUFAs (absolute hepatic levels) examined except for timnodonic acid (20:5n3). Linoleic acid (18:2n6) and arachidonic acid (20:4n6, AA) were the dominant PUFAs, representing ~60% and ~25% of PUFAs, respectively. The n-3 and n-6 PUFAs exhibit anti- and pro-inflammatory effects, respectively [34]. The n3/n6 ratio was induced by TCDD and accompanied by the depletion of AA (Figure 3.1, panel A).

TCDD EFFECTS ON HEPATIC LIPID CONTENT IN SCD1$^{-/-}$ MICE

Scd1 performs the rate-limiting step in MUFA synthesis and its deficiency has been reported to impair triglyceride synthesis and protect from diet-induced hepatic steatosis. In *Scd1*$^{-/-}$ mice, TCDD increased TAGs 2.5-fold and TFA content 1.6-fold compared to vehicle controls. However, TAG levels were 42% lower in *Scd1*$^{-/-}$ mice when compared to treated wild-type mice, yet there was no difference in TFA levels between genotypes (Figure 3.1, panel B).

Figure 3.1. Hepatic lipid composition at 168 h in vehicle and 30 µg/kg TCDD treated *Scd1*$^{+/+}$ (Panel A) and *Scd1*$^{-/-}$ (Panel B) mice.

Total triglycerides (TAGs) were extracted from mouse liver and quantified using a commercial L-Type Triglyceride M kit (Wako Diagnostics). Data are reported as mg/dL TAG per gram of liver tissue. **Absolute total hepatic fat** was extracted from *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mouse liver and analyzed by GC-MS and are reported as µmol/g tissue. **Hepatic fat compositions** (saturated fatty acid (SFA)/ monounsaturated fatty acid (MUFA)/ polyunsaturated fatty acid (PUFA) ratios) are reported as mol%. SFA (dark grey)/ MUFA (light grey)/ PUFA (medium grey) proportions are represented. **n3/n6 fatty acid ratios** are reported as mol %. **Arachidonic acid (20:4n6)** levels are reported as mol%. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test. Bars represent mean ± SEM, n=5 biological replicates, (*) represents $p<0.05$ for TCDD compared to Vehicle within a genotype, (**) represents $p<0.05$ for *Scd1*$^{-/-}$ TCDD compared to *Scd1*$^{+/+}$ TCDD, and (***) represents $p<0.05$ for *Scd1*$^{-/-}$ Vehicle compared to *Scd1*$^{+/+}$ Vehicle.

Figure 3.1 (cont'd)

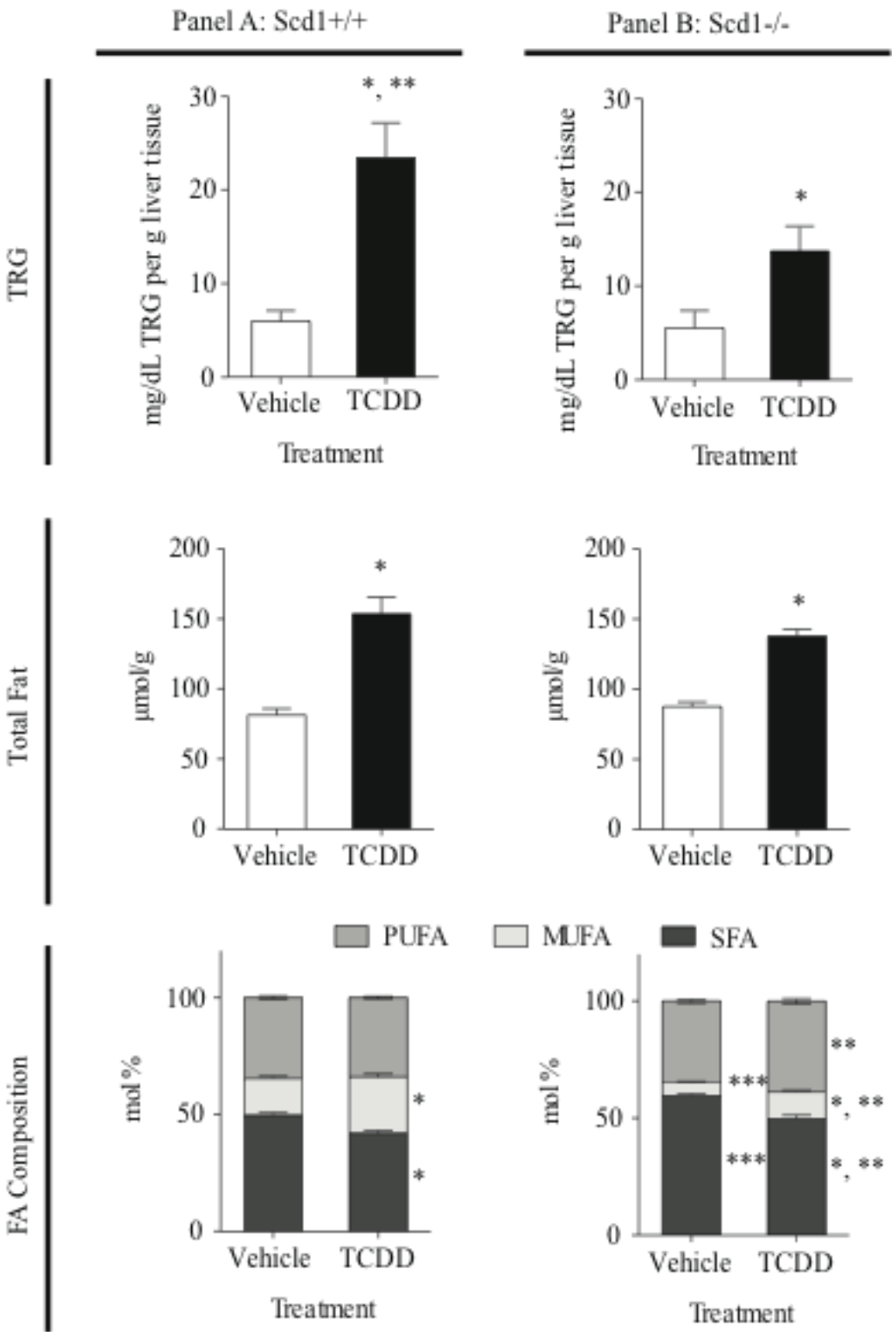
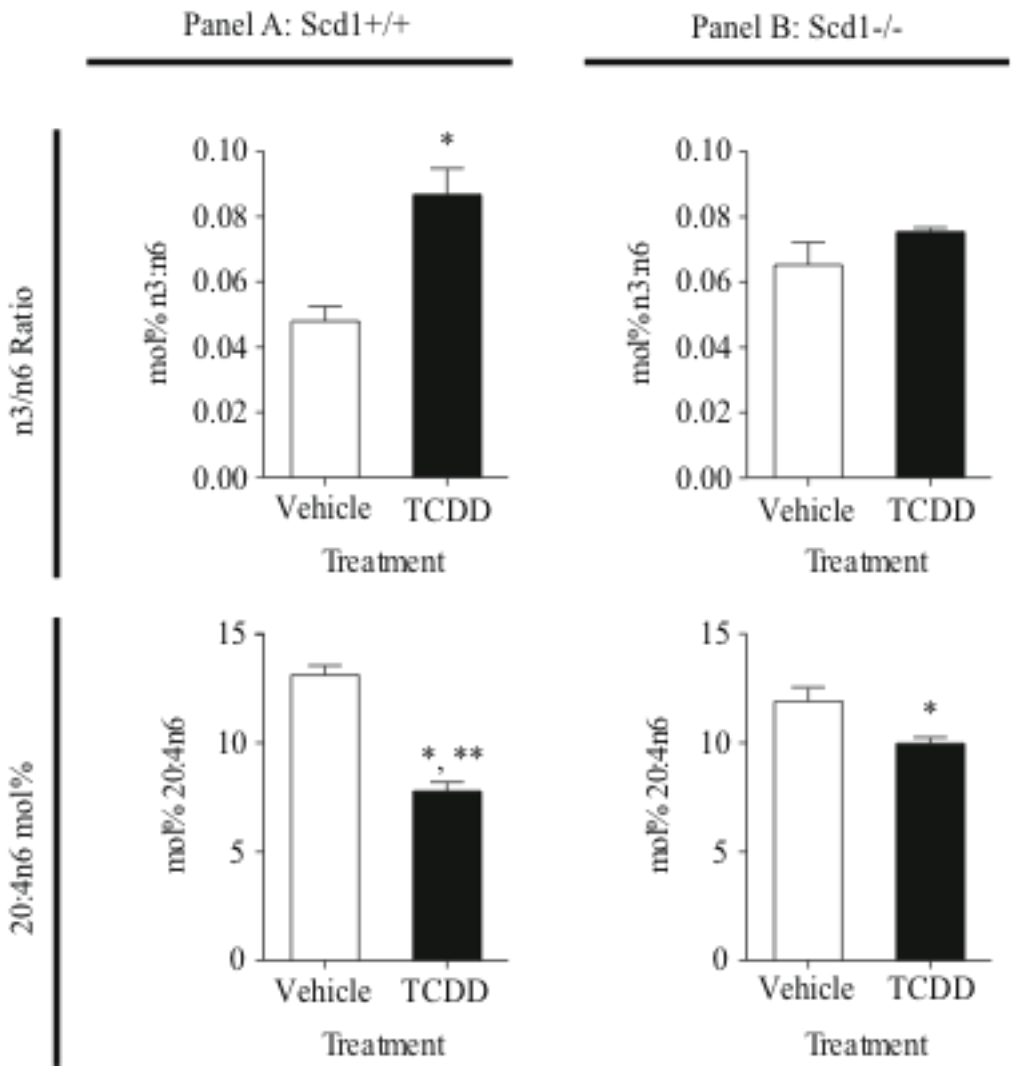


Figure 3.1 (cont'd)



Examination of the SFA/MUFA/PUFA ratios in *Scd1*$^{-/-}$ mouse liver revealed that TCDD reduced SFA proportions and increased relative MUFA and PUFA levels compared to vehicle controls. Scd1 deficiency also induced SFA by 8%, reduced MUFA by 13%, and induced PUFA by 5% compared to treated wild-type mice, consistent with loss of Scd1 activity (Figure 3.1, panel B).

Further analysis of individual fatty acid species identified increases of 18:0, while 16:1n7, 18:1n9, and 20:1n9 were reduced in treated null mouse livers compared to treated wild-type mice (Table 3.1). Surprisingly, MUFA increases in *Scd1* null mice were primarily due to a 3.2-fold induction of 18:1n9. Similar to *Scd1*$^{+/+}$ mice, overall PUFA levels were induced by TCDD in *Scd1*$^{-/-}$ mice. In contrast, the n3/n6 ratios were not altered and the molar ratio of AA [(nmol/g AA)/(nmol/g total FA detected)] was higher compared to treated wild-type mice (Figure 3.1, panel B).

PRINCIPAL COMPONENT ANALYSIS

Principal component analysis (PCA) was performed to characterize the trends exhibited by GC/MS-FAMES data. PCA of hepatic TFAs indicated a clear time-, treatment-, and Scd1 dose-dependent effect on hepatic fatty acid composition (Figure 3.2). PC1 and PC2 accounted for 95% of the cumulative proportion of the variance with vehicles clustering along PC1, treated groups separating along PC1, and genotype separating along PC2. All treated animals exhibited a similar temporal trajectory.

Table 3.1. Total Hepatic Lipid Composition (μmol/g) in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ Mice 168 h Post 30 μg/kg TCDD Dose.

Lipid	*Scd1*$^{+/+}$ Vehicle	*Scd1*$^{+/+}$ TCDD	*Scd1*$^{-/-}$ Vehicle	*Scd1*$^{-/-}$ TCDD
Total fatty acids	81.4 ± 10.4	153.6 ± 26.7*	87.6 ± 7.3	137.8 ± 11.1*
Saturated fatty acid	40.6 ± 6.5	64.6 ± 11.1*	52.3 ± 5.3	68.8 ± 7.1*
Palmitic acid (16:0)	19.9 ± 2.6	29.2 ± 3.7*	19.8 ± 2.6	27.8 ± 2.8*
Stearic acid (18:0)	17.0 ± 3.1	20.2 ± 2.2*, **	19.6 ± 2.1	27.1 ± 3.2*
Arachidic acid (20:0)	0.064 ± 0.006	0.066 ± 0.010	0.117 ± 0.076	0.093 ± 0.011
Behenic acid (22:0)	0.269 ± 0.044***	0.175 ± 0.017	0.425 ± 0.140	0.292 ± 0.056
Lignoceric acid (24:0)	3.33 ± 1.01***	14.9 ± 7.3*	12.4 ± 1.9	13.4 ± 1.9
Monounsaturated fatty acid	12.7 ± 1.5***	36.8 ± 7.8*, **	5.1 ± 0.5	15.8 ± 0.01*
Palmitoleic acid (16:1n7)	1.24 ± 0.26***	2.39 ± 0.64*, **	0.20 ± 0.03	0.46 ± 0.08
Oleic acid (18:1n9)	11.2 ± 1.4***	32.9 ± 6.9*, **	4.68 ± 0.44	14.7 ± 1.8*
Eicosenoic acid (20:1n9)	0.10 ± 0.015***	0.72 ± 0.22*, **	0.040 ± 0.008	0.27 ± 0.056*
Erucic acid (22:1n9)	0.028 ± 0.005	0.056 ± 0.009*	0.031 ± 0.005	0.064 ± 0.009*
Nervonic acid (24:1n9)	0.135 ± 0.043	0.802 ± 0.353*, **	0.189 ± 0.047	0.277 ± 0.040
Polyunsaturated fatty acid	28.1 ± 3.4	52.2 ± 10.1*	30.1 ± 2.4	53.2 ± 5.3*
Total n3 fatty acids	1.30 ± 0.37	4.23 ± 1.56*	1.85 ± 0.49	3.70 ± 0.36*
Total n6 fatty acids	26.7 ± 3.09	47.6 ± 8.5*	28.2 ± 2.0	49.2 ± 4.9*

Table 3.1 (cont'd)

Lipids	***Scd1*$^{+/+}$ Vehicle**	***Scd1*$^{+/+}$ TCDD**	***Scd1*$^{-/-}$ Vehicle**	***Scd1*$^{-/-}$ TCDD**
Linoleic acid (18:2n6)	14.8 ± 1.83	31.7 ± 5.67*	16.7 ± 1.65	31.7 ± 3.53*
Eicosadienoic acid (20:2n6)	0.31 ± 0.042	1.26 ± 0.26*	0.30 ± 0.040	1.20 ± 0.25*
Dihomo-ϒ-linolenic acid (20:3n6)	0.95 ± 0.10	2.60 ± 0.69*	0.786 ± 0.111	2.52 ± 0.37*
Arachidonic acid (20:4n6)	10.6 ± 1.38	12.0 ± 2.66	10.4 ± 0.88	13.7 ± 1.06*
Docosapentaenoic acid (22:2n6)	0.015 ± 0.007	0.035 ± 0.012*	0.018 ± 0.004	0.042 ± 0.008*
Eicosatetraenoic acid (20:4n3)	0.033 ± 0.005	0.162 ± 0.057*	0.032 ± 0.006	0.153 ± 0.046*
α-Linolenic acid (18:3n3)	0.458 ± 0.101	1.36 ± 0.30*	0.045 ± 0.088	1.09 ± 0.25*
Timnodonic acid (20:5n3)	0.614 ± 0.240	0.593 ± 0.231	0.629 ± 0.234	0.694 ± 0.146
Docosapentaenoic acid (22:5n3)	0.193 ± 0.078	2.12 ± 1.04*	0.743 ± 0.232	1.76 ± 0.24*

Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test, n=5 biological replicates. ([a]) $p<0.05$ for TCDD vs. Vehicle within the same genotype; ([b]) $p<0.05$ for +/+ TCDD vs. -/- TCDD; ([c]) $p<0.05$ for +/+ Vehicle vs. -/- Vehicle

TCDD Effects on the Scd1 Desaturation Index

Scd1 is the primary hepatic Δ9 desaturase, metabolizing 16:0 and 18:0 into 16:1n7 and 18:1n9, respectively. TCDD induced the hepatic 18:1n9/18:0 ratio in *Scd1*$^{+/+}$ mice compared to vehicles (Figure 3.3A). In humans, positive correlations between the Scd1 desaturation index and triglyceride levels have been reported [35]. Similarly, TAG levels increased with the 18:1n9/18:0 ratio in TCDD treated *Scd1*$^{+/+}$ mice (Pearson's $r = 0.825$, $p = 0.0003$) (Figure 3.3B).

TCDD Effects on Hepatic Lipid Metabolism Gene Expression

To further examine TCDD's effects on hepatic lipid composition in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice, QRTPCR was used to quantify mRNA levels for genes involved in lipid metabolism. Consistent with TCDD-induced increases in hepatic lipid levels, the fatty acid transport genes *Cd36*, *Ldlr*, *Vldlr* and *Fabp4*, were induced 3-5-fold in both genotypes (Figure 3.4A-D). Furthermore, *Cd36*, *Vldlr* and *Fabp4* were significantly increased in treated *Scd1*$^{-/-}$ mice compared to *Scd1*$^{+/+}$ mice and may explain increases in 18:1n9 levels, the primary MUFA (81.3% of all MUFAs) in Harlan Teklad rodent diet 7964, in null mice.

Four isoforms, *Scd1-4*, exist in the mouse and may underlie increases in 18:1n9. However, *Scd3* is reported to be expressed by sebocytes in skin, the preputual gland, and the Harderian gland [36] while *Scd4* expression is limited to the heart [37]. Neither *Scd3* or *4* were detected in the liver of either TCDD-treated genotype. *Scd2* is primarily expressed in adipose tissue, but also in the neonatal mouse liver. *Scd2* was expressed in wild-type mice, but not induced by TCDD (Figure 3.4E). Surprisingly, *Scd2* mRNA levels were scarce (100-fold lower) in null mice compared to wild-types. The lack of *Scd2* mRNA expression in nulls suggests *Scd1* deletion also affected *Scd2* expression.

Figure 3.2. Principal component analysis (PCA) of GC-MS lipid profiles from TCDD (T) or Vehicle (V) treated *Scd1*$^{+/+}$, *Scd1*$^{+/-}$, and *Scd1*$^{-/-}$ mice 24, 72 and 168 h post-dose.

PCA was performed in R as described in the Materials and Methods. PC1 and PC2 accounted for 95% of the cumulative proportion of the variance with vehicles clustering along PC1, treated groups separating along PC1 and genotype separating along PC2. Dashed lines (*Scd1*$^{-/-}$) dotted lines (*Scd1*$^{+/-}$) solid lines (*Scd1*$^{+/+}$), n=5 biological replicates.

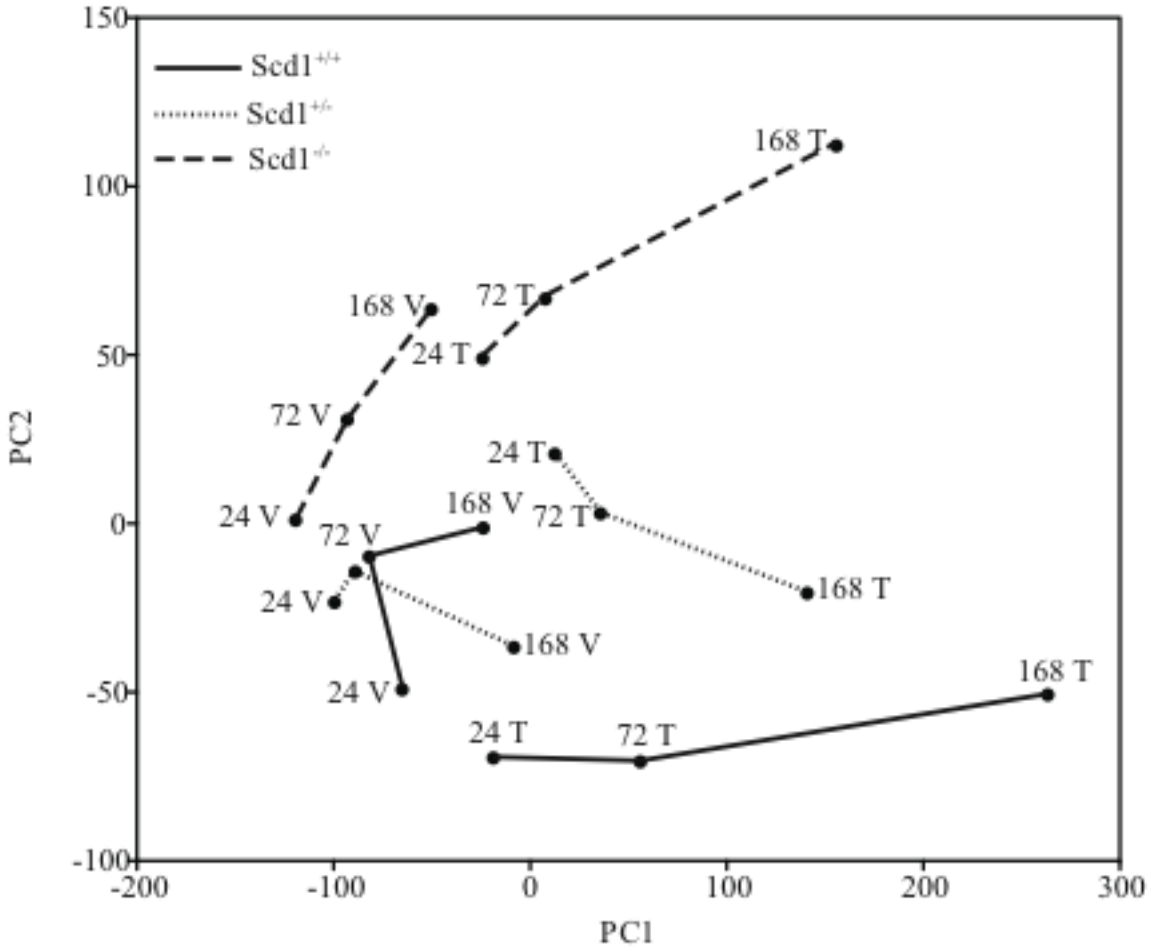


Figure 3.3. Scd1 activity (desaturation) index and Correlation with Hepatic Triglyceride Levels.

(A) Scd1 desaturation index in female TCDD (T) or vehicle (V) treated *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice. The desaturation index is the ratio of palmitoleic (16:1n7) or oleic acid (18:1n9) to the precursors palmitic (16:0) or stearic acids (18:0), respectively. Bars represent mean ± SEM, n=5 biological replicates, * represents $p<0.05$ for TCDD compared to vehicle within a genotype, ** represents $p<0.05$ for *Scd1*$^{-/-}$ TCDD compared to *Scd1*$^{+/+}$ TCDD, and *** represents $p<0.05$ for *Scd1*$^{-/-}$ vehicle compared to *Scd1*$^{+/+}$ vehicle. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test. (B) Correlation analysis was performed in Graphpad Prism 5.0a between the hepatic triglyceride (TAG) levels and the 18:1n9/18:0 desaturation index in *Scd1*$^{+/+}$ mice gavaged with 30μg/kg with TCDD or vehicle for 168 h.

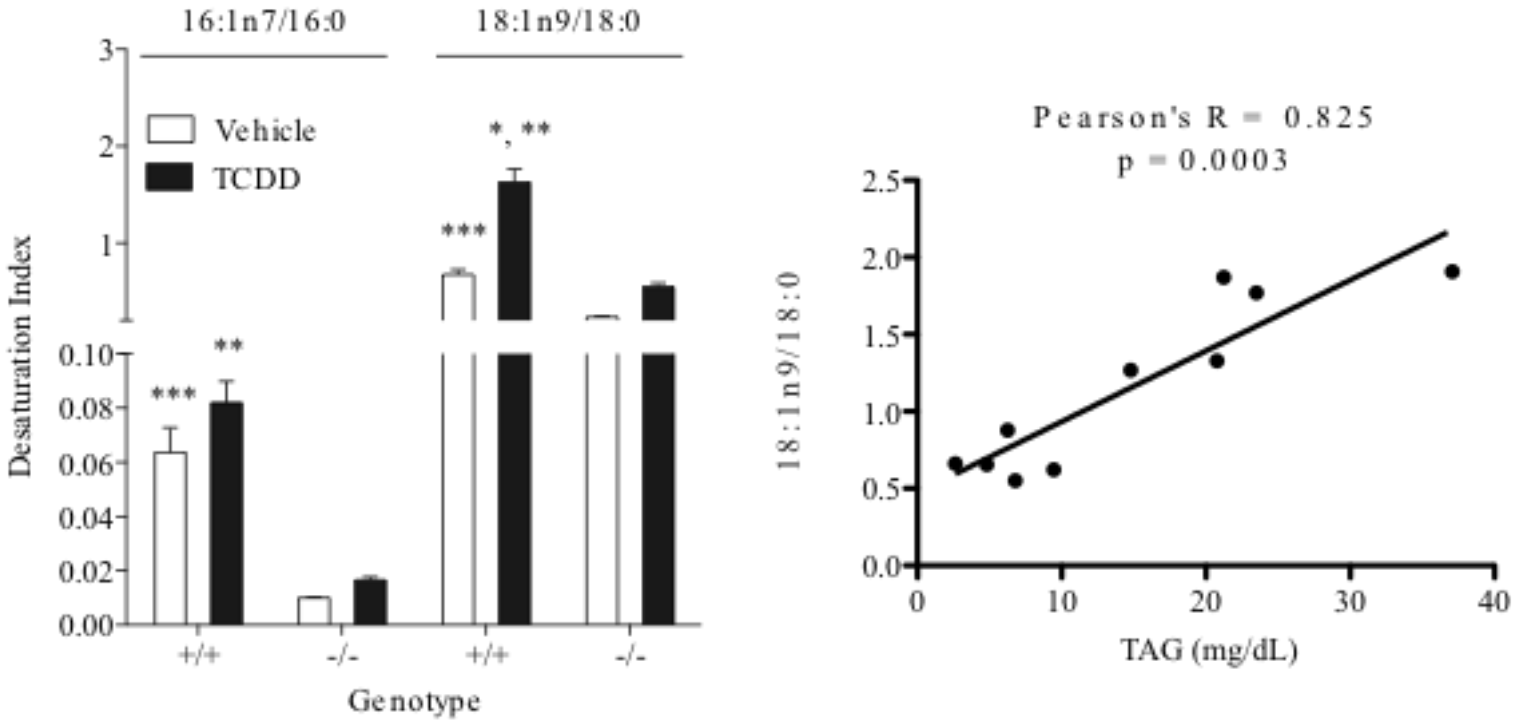
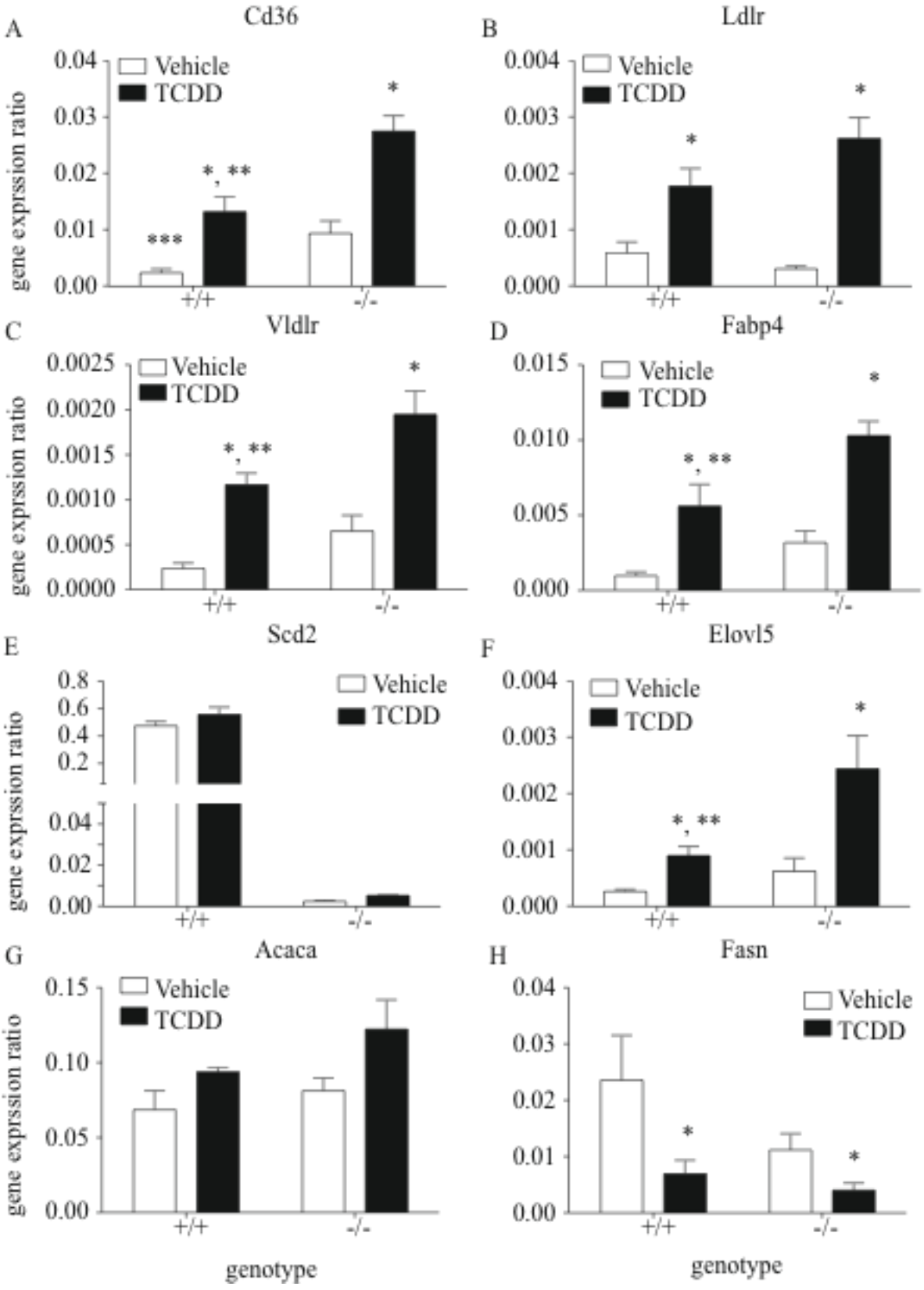


Figure 3.4. QRTPCR of hepatic lipid transport, modification, and biosynthesis genes in *Scd1*$^{+/+}$ (+/+) and *Scd1*$^{-/-}$ (-/-) mice gavaged with 30 μg/kg TCDD or sesame oil vehicle for 24 h.

The gene expression ratio is the total quantity normalized to the geometric mean of *Hprt*, *Actb*, and *Gapdh*. Genes are indicated by official gene symbols. Error bars represent the standard error mean (SEM), n=5, * represents $p<0.05$ for TCDD compared to vehicle within a genotype, ** represents $p<0.05$ for *Scd1*$^{+/+}$ TCDD compared to *Scd1*$^{-/-}$ TCDD and *** represents $p<0.05$ for *Scd1*$^{+/+}$ vehicle compared to *Scd1*$^{-/-}$ vehicle. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test.

Figure 3.4 (cont'd)



TCDD-mediated increases in the PUFAs eicosatetraenoic acid (20:4n3) and docosapentaenoic acid (22:5n3) were consistent with the 2-fold induction of *Elovl5* (Figure 3.4F) an elongase that catalyzes the elongatation very long chain PUFAs [38]. The lipogenic gene *Acaca*, which provides malonyl-CoA for fatty acid biosynthesis, was not altered by TCDD (Figure 3.4G). *Fasn*, the long-chain fatty acid synthetase was reduced 2.5-3-fold by TCDD in both genotypes (Figure 3.4H), suggesting that increases in hepatic lipid content are not due to *de novo* lipogenesis. Finally, the well-characterized TCDD-inducible genes *Cyp1a1*, *Nqo1*, and *Tiparp* exhibited comparable induction in wild-type and null mice (Figure A.2).

TCDD EFFECTS ON SCD1 ACTIVITY, MRNA AND PROTEIN

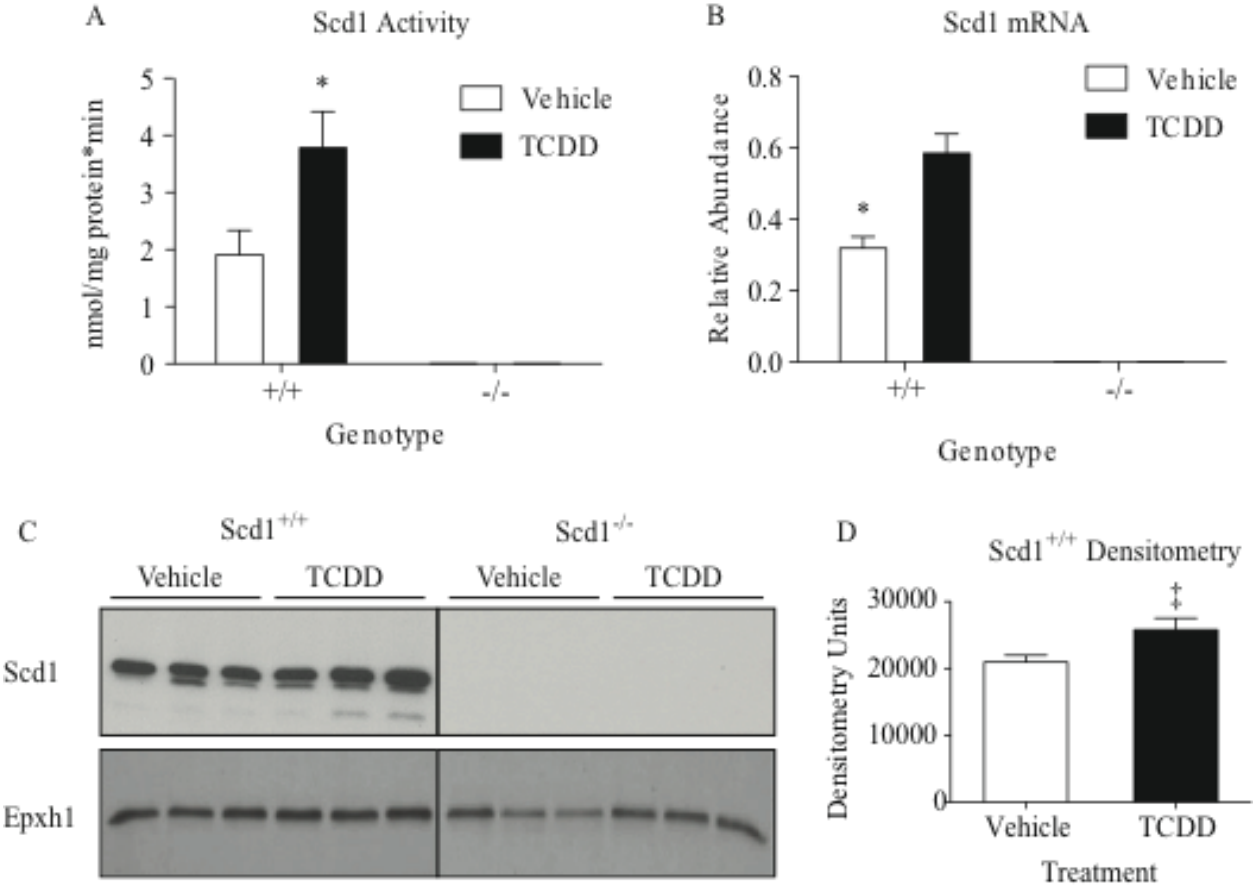
The effects of TCDD on Scd1, activity, mRNA, and protein levels were examined. Scd1 enzyme activity was measured by following the conversion of ^{14}C18:0 to ^{14}C18:1n9. Consistent with increases in the 18:1n9/18:0 desaturation index, TCDD induced Scd1 activity 2-fold in wild-type mice compared to vehicles at 24 h (Figure 3.5A). QRTPCR also showed that TCDD induced *Scd1* mRNA levels 1.5-fold in *Scd1*$^{+/+}$ mice (Figure 3.5B). Furthermore, western blots showed an increase in Scd1 immunoreactivity in treated *Scd1*$^{+/+}$ microsomal fractions (Figure 3.5C and 3.5D). Induction in Scd1 activity, mRNA, and protein were not detected in control or TCDD-treated *Scd1*$^{-/-}$ mice.

PUTATIVE DRE DISTRIBUTION AND AHR ENRICHMENT AT SCD1 LOCI

To further examine AhR regulation of *Scd1*, Figure 3.6A summarizes the ChIP-chip analysis of AhR enrichment at *Scd1-4* genomic loci induced by TCDD [28]. The moving average (MA) value visualizes the enriched genomic regions within the *Scd1-4* loci while the $\log_2$

Figure 3.5. Scd1 activity, mRNA, and protein levels in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice treated with 30 μg/kg TCDD (T) or sesame seed oil (V) 24 h post-dose.

(A) Scd1 activity. Hepatic microsomes (100 μg, n=5) isolated from mice were incubated with 0.03 μCi ^{14}C-stearoyl-CoA (^{14}C 18:0), NADH and stearoyl-CoA. ^{14}C 18:0 was separated from ^{14}C 18:1 by silver ion chromatography and radioactivity measured by scintillation counting. Scd1 activity is expressed as nmol ^{14}C 18:0 converted to ^{14}C 18:1 per mg Scd1 protein per min. (B) QRTPCR of Scd1 mRNA (n=5). Expression is represented as a ratio of the total quantity of *Scd1* normalized to the geometric mean of *Hprt*, *Actb*, and *Gapdh*. (C) Scd1 Western blot. Hepatic Scd1 protein (n=3) was detected in 10 μg of microsomes. Epoxide hydrolase (Epxh1) was used as a microsomal protein reference for loading control. (D) Densitometry. Densitometry was determined with ImageJ from Scd1 bands and normalized to Ephx1 bands. ‡ for $p = 0.08$. For A, B, and D bars represent mean ± SEM, * indicates $p<0.05$ for T compared to V within a genotype. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test.



enrichment illustrates the fold change for each Affymetrix probe. Six AhR enriched regions (up to 5.7-fold, FDR < 0.01, red bar) ranging from 70 to 4200 bp were associated with *Scd1* (Figure 3.6A). AhR enrichment was located within 10 kb upstream region through the 3'-UTR. In contrast, the adjacent ~150 kb genomic region spanning *Scd2*, *Scd3*, and *Scd4* exhibited only two, AhR-enriched regions (2- to 2.7-fold).

AhR is proposed to regulate gene expression through DNA binding at DREs containing the core sequence 5'-GCGTG-3'. DREs with matrix similarity scores (MSS) ranging from 0.69 to 0.93 were identified within the genomic region spanning *Scd* genomic loci [29]. Of the 39 DREs possessing a putative functional (high scoring) MSS (> 0.8; track 4, horizontal line), 16 were located 10 kb upstream of the *Scd1* TSS through the *Scd1* 3'-UTR (Figure 3.6B). Five of these 16 high scoring *Scd1* DREs overlapped with regions of AhR enrichment (track 4, yellow shading). Notably, 7 DREs that did not overlap with regions of significant AhR enrichment coincided with regions lacking tiling probes (track 4, purple shading). One DRE core fell within a 2-fold AhR enriched region that failed to meet the FDR cut-off of 0.01 (track 4, green shading). Moreover, three AhR enriched regions lacked any DRE cores (track 5, orange shading) consistent with promoter- and genome-wide ChIP-chip studies reporting 50% overlap between DRE cores and AhR enrichment as well as other studies suggesting AhR interaction with DNA independent of ARNT [28, 29, 39-41].

BAND SHIFT ASSAYS

Two DRE cores (Figure 3.6B, track 4, indicated by*) within 10 kb upstream of the *Scd1* TSS and with a MSS > 0.8 exhibited band shifts with TCDD-activated guinea pig cytosol (Figure 3.6C). The addition of AhR or ARNT antibodies to the incubation resulted in a supershift,

Figure 3.6. Dioxin response element (DRE) distribution and TCDD-inducible AhR enrichment within *Scd* loci, and band shift assays.
(A) Genomic region spanning *Scd1*, *Scd2*, *Scd3*, and *Scd4*. (B) Scd1 genomic region only. Genomic DRE distributions and regions of AhR enrichment induced by TCDD were previously determined [28, 29]. Track 1: scale and chromosome position. Track 2: probe tiling across the Affymetrix 2.0R mouse array. Track 3: gene organization including transcriptional start site (TSS) (closed arrow), exons (closed boxes), introns and direction of transcription (solid arrowhead line). Track 4: location of DRE cores (5'-GCGTG-3'). Height of vertical bars indicate matrix similarity score (MSS) for the 19 bp DRE sequence [29]. The horizontal line indicates the MSS = 0.8. MSSs greater than 0.8 are considered putative functional DREs. The asterisk (*) denotes 19 bp DRE sequences that bound TCDD-activated AhR in band shift assays. Track 5: regions of significant (FDR<0.01) AhR enrichment in genome-wide ChIP-chip assays. Tracks 6 and 7: histograms depicting the signal intensities for the moving average (MA; blue, track 6) and $\log_2$ fold enrichment (green, track 7) values for regions exhibiting AhR enrichment in genome-wide ChIP-chip assays. The above tracks were modified from the UCSC genome browser. (B) Scd1 genomic region only. Track 4: yellow shading, putative DRE-AhR enrichment overlap; purple shading, putative DREs lacking affymetrix tiling probes; grey shading, putative DREs that do not overlap with AhR enrichment; green shading, putative DRE in an AhR region that failed to meet the FDR cut-off of 0.01. Track 5: orange shading, AhR enriched regions that do not overlap with putative DREs. (C) Representative band shift assays with putative, functional DREs (track 4, asterisks (*)). The DRE position is numerically indicated relative to the *Scd1* TSS. Solid arrows indicate a TCDD-inducible band shift. Hatched or dashed arrows indicate an AhR or ARNT supershift, respectively.

Figure 3.6 (cont'd)

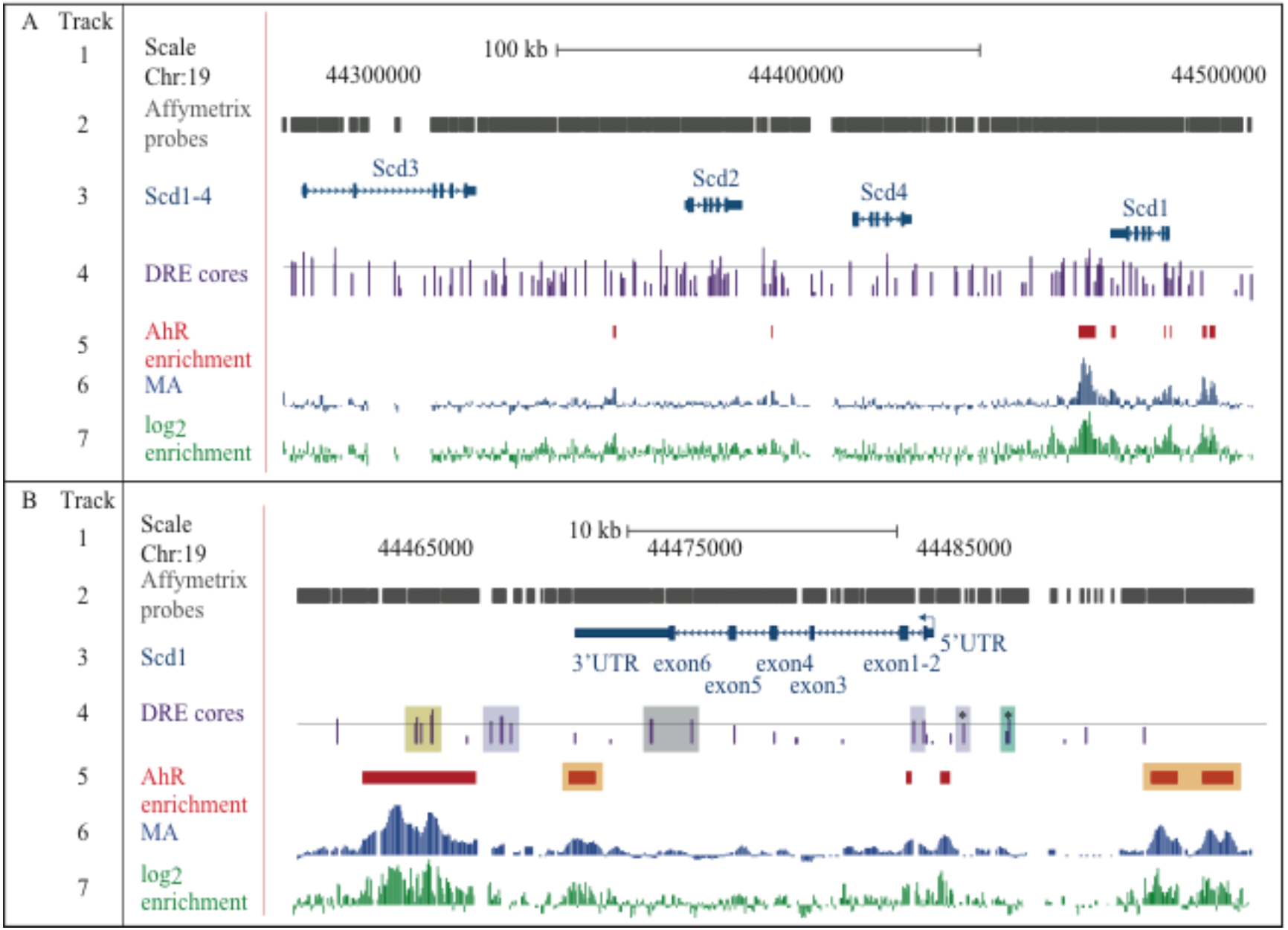
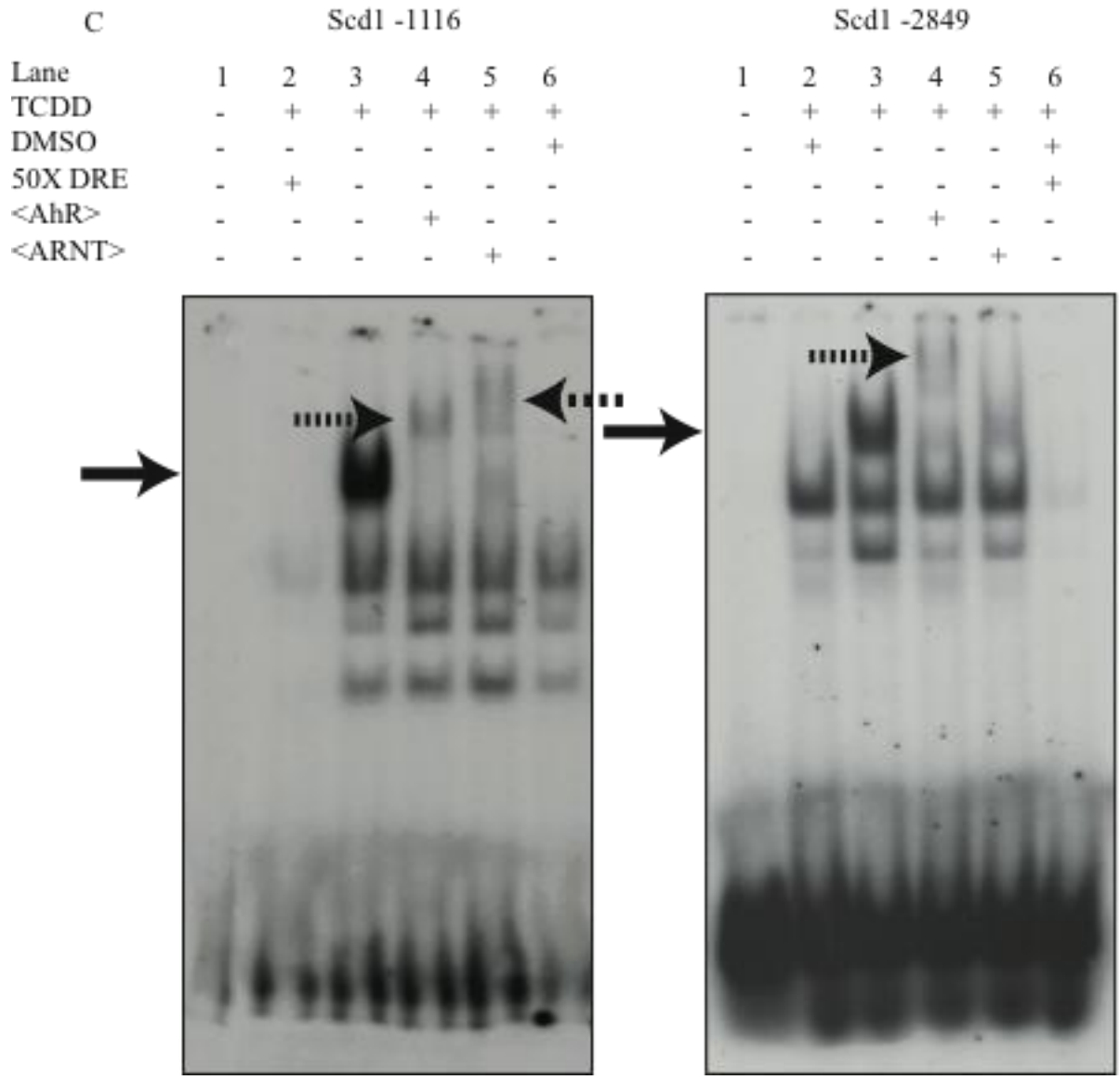


Figure 3.6 (cont'd)



confirming AhR binding to these putative DREs. Nucleotides flanking the DRE core sequence binding affinity and co-activator recruitment and may underlie differences in the banding pattern between the two DREs [42-45]. These results, in addition to mRNA, protein and activity levels, support AhR recruitment to and regulation of *Scd1* by TCDD.

DISCUSSION

Previous studies examining the role of Scd1 in hepatic diseases have focused on dietary or genetic modulation of lipid metabolism [25, 35, 46-48], but not the effects of environmental contaminants. In this study, AhR mediated induction of Scd1 by TCDD and subsequent effects on hepatic FA composition were examined in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice. Here we report that TCDD induced hepatic TRG and FA accumulation, elicited differential gene expression of lipid metabolism and transport, and increased Scd1 mRNA, protein, and activity, as a result of AhR recruitment to *Scd1* genic regions. Collectively, these studies suggest that AhR-regulation of Scd1 alters hepatic fatty acid composition, which influences TCDD-induced hepatotoxicity.

TCDD altered hepatic gene expression associated with lipid transport, partitioning and metabolism in mice [11, 14, 49]. For example, TCDD induced the lipolytic genes lipoprotein lipase (*Lpl*), phospholipase A2, group XIIA (*Pla2g12a*), monoglyceride lipase (*Mgll*) and pancreatic lipase-related protein (*Pnliprp1*) that hydrolyze hepatocellular TRG stores into FFAs and monoglycerides [50]. It also induced low-density lipoprotein receptor (*Ldlr*), very low-density lipoprotein receptor (*Vldlr*), Cd36 antigen (*Cd36*), and fatty acid binding protein (*Fabp4*) in *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ mice. Ldlr and Cd36 are membrane-associated proteins that facilitate the uptake of chylomicron and VLDL remnants as well as long-chain fatty acids [51, 52]. Cd36 has been implicated in the etiology of obesity and diabetes, and Cd36 null mice exhibit minimal

TCDD-elicited steatosis [53-56]. Fabps are cytosolic, high-affinity long-chain fatty acid binding proteins that target lipids to intracellular compartments [57]. Fabp4, which mediates lipid trafficking to the nucleus and may provide ligands for PPARs [58, 59], was also induced. Furthermore, inhibition of VLDL secretion has been reported in mice following TCDD treatment [56]. These changes likely underlie effects that contribute to TCDD-elicited hepatic fatty acid accumulation.

ChIP-chip analysis identified six *Scd1* genic regions with AhR enrichment. Two proximal AhR enriched regions were within 2 kb of the TSS [28]. The 3' regions also exhibited AhR enrichment and high-scoring DREs, suggesting AhR regulation by distal enhancer sites. Although distal regulation remains largely uninvestigated, studies suggest transcription factor binding at these sites promotes chromatin looping and structural modifications that facilitate gene expression [60-62]. Two 5' AhR enriched regions at distal sites lacked DRE cores, providing further evidence of DRE-independent AhR-DNA interactions, which may involve tethering to other DNA interacting transcription factors [63]. However, the arrays do not have uniform tiling across the genome and several genomic regions lack probe coverage in areas containing high-scoring DREs that may confound mapping AhR enrichment to regions containing DREs.

AhR enrichment at *Scd1* genic regions and the induction of Scd1 mRNA, protein, and activity by TCDD provided compelling evidence for AhR regulation of *Scd1*. The induction of Scd1 activity increased MUFA levels, decreased SFA levels and increased MUFA:SFA and PUFA:SFA ratios. Scd1 deficiency did not affect TCDD induced hepatic lipid accumulation (as measured by GC-MS analysis of FAMES). However, null mice had fewer TAGs, reduced MUFA levels and exhibited less hepatic injury relative to treated wild-type mice. More

specifically, *Scd1*$^{-/-}$ mice exhibited no increase in serum ALT, less hepatic vacuolization and reduced immune cell infiltration compared to *Scd1*$^{+/+}$ mice suggesting lower overall TCDD elicited hepatotoxicity.

In addition to *Scd1,* hepatic FA profiles indicate that AhR regulates other lipid modifying enzymes in addition to Scd1. Increases in n-6 and n-3 pathway intermediates (e.g. 20:2n6, 20:3n6, 20:4n3, 22:5n3) suggests the induction of Elovl5 activity [64]. QRTPCR, band shift and ChIP-chip assays (Appendix A Figure 3A-C) verified AhR mediated induction of Elovl5 by TCDD. The n-3 and n-6 PUFAs, particularly eicosapentanoic acid (EPA, 20:5n3) and arachidonic acid (AA, 20:4n6) metabolites, exhibit anti- and pro-inflammatory activities, respectively [34, 65-67]. EPA is an Elovl5 substrate, therefore excess 20:5n3 may be elongated into 22:5n3, which is increased in both genotypes. AA is not an Elovl5 substrate, but is rapidly metabolized by TCDD-inducible prostaglandin-endoperoxide synthase 1 (Ptgs1), arachidonate 12-lipoxygenase (Alox12) [14, 28], and glutathione transferases [68] into pro-inflammatory ecosanoids. Additionally, AA is liberated from membrane phospholipids by Pla2g12a, another TCDD-inducible gene. AA levels were lower in treated *Scd1*$^{+/+}$ mice compared to vehicles and treated nulls, and, although we cannot rule-out their conversion into inflammatory ecosanoids, are consistent with the increased level of inflammation in wild-type mice compared to nulls. Inflammation in *Scd1*$^{+/+}$ mice may also be due to the induction of Scd1, which would sequester cytochrome b_5, uncouple P450 monoxygenases, and increase superoxide and ROS formation [69, 70]. However, P450 monoxygenase and xanthine dehydrogenase induction by TCDD are the primary ROS contributors [71, 72].

Our results differ from studies examining the protective effects of Scd1 deficiency from steatosis and exacerbated steatohepatitis elicited using *in vivo* dietary-induced models of liver injury. For example, high fat diets (HFD) induce steatosis in mice. *Scd1*$^{-/-}$ mice fed HFD exhibit no evidence of hepatomegaly or histological changes [73], yet hepatomegaly is observed in all animals exposed to TCDD (Poland and Knutson, 1982). Methionine choline deficient (MCD) diets induce steatohepatitis, but in contrast to TCDD, decrease Scd1 expression and induce Cyp4A expression, an enzyme involved in lipid peroxidation (Li et al., 2009, Yamaguchi et al., 2007, and Anstee & Goldin, 2006). Cyp4a is a peroxisome proliferator activated receptor (PPAR) target and administration of a PPAR agonist to MCD fed mice decreases liver damage (Nagasawa et al., 2006), suggesting a role for PPAR rather than AhR in MCD-elicited liver injury. Furthermore, our results are consistent with increased MUFA:SFA ratios in lipotoxic mechanisms of liver injury (Larter et al. 2008).

TCDD-induced steatosis is a significant hepatotoxic effect and AhR-mediated induction of Scd1 exacerbates TCDD hepatotoxicity by altering the composition of accumulated lipids. Scd1-mediated increases in unsaturated fatty acid levels may alter membrane fluidity, increase lipid peroxidation and reactive oxygen species formation, as well as propagate inflammatory responses through TRAIL-mediated cytotoxicity [74, 75]. Steatosis followed by a progressive inflammatory response poses a significant risk of progression to cirrhosis and may contribute to hepatocellular carcinoma development in rodents.

The induction of hepatic lipid accumulation in mice is consistent with the occurrence of dyslipidemia in humans following TCDD exposure at high doses [76]. Interestingly, the hepatic fatty acid composition induced by TCDD is similar to serum and lipid profiles (e.g. increase in TAGs, and 16:1n7 and 18:1n9 levels) described for human NAFLD patients [77, 78]. NAFLD is

considered the hepatic manifestation of metabolic syndrome, and precedes non-alcoholic steatosis, and cirrhosis. This report suggests that AhR regulation of lipid transport, metabolism, and modifying enzymes, including Scd1, alters lipid composition that contributes to the hepatotoxicity of TCDD.

FOOTNOTES

[1]Guinea pig cytosol was a gift from Dr. Michael Denison, Department of Environmental Toxicology, Meyer Hall, University of California, Davis, CA 95616, USA.

FUNDING

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APPENDIX A

APPENDIX A

Table A.1. Putative DRE Sequences

Gene	MSS	Position	Sequence
Scd1	0.812732	-2849	TCTCTCTGCGTGCCTTTAT
			AGAGAGACGCACGGAAATA
	0.803264	-1116	TGGCTAAGCGTGACCACAG
			ACCGATTCGCACTGGTGTC

Matrix Similarity Score (MSS), Position is relative to the transcriptional start site.

Table A.2. Terminal Body Weight, Body Weight Gain, Absolute Liver Weight and Relative Liver Weight for *Scd1*$^{+/+}$ and *Scd1*$^{-/-}$ Mice

Time (h)	Genotype	Treatment	Body Weight (g)	Body Weight Gain (g)	Liver Weight (g)	Relative Liver Weight
24	+/+	Vehicle	14.7 ± 1.0	1.4 ± 0.9	0.844 ± 0.064	0.057 ± 0.004
		TCDD	14.2 ± 0.5	0.8 ± 0.5	0.914 ± 0.079	0.064 ± 0.004*****
	-/-	Vehicle	13.1 ± 1.3	1.2 ± 0.4	0.771 ± 0.116	0.059 ± 0.005
		TCDD	13.2 ± 1.3	0.6 ± 0.5	0.905 ± 0.123	0.068 ± 0.003*****
72	+/+	Vehicle	13.2 ± 2.5	1.4 ± 0.9	0.728 ± 0.204	0.054 ± 0.008
		TCDD	13.0 ± 1.2	1.9 ± 0.7	0.818 ± 0.116	0.063 ± 0.006
	-/-	Vehicle	12.6 ± 1.5	2.2 ± 0.5	0.756 ± 0.095	0.060 ± 0.003
		TCDD	12.8 ± 1.6	1.9 ± 0.5	0.891 ± 0.102	0.070 ± 0.010*****
168	+/+	Vehicle	15.5 ± 1.2	2.6 ± 1.8	0.778 ± 0.135	0.050 ± 0.006
		TCDD	15.0 ± 1.3	2.0 ± 0.8	0.959 ± 0.114*****	0.064 ± 0.003***, ****
	-/-	Vehicle	15.8 ± 1.8	3.5 ± 0.7	0.926 ± 0.097	0.059 ± 0.004
		TCDD	15.3 ± 1.5	3.3 ± 0.7	1.080 ± 0.111	0.071 ± 0.005*****

Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test, n=8 biological replicates. * $p<0.05$ for TCDD vs. Vehicle within the same genotype, ** $p<0.05$ for +/+ TCDD vs. -/- TCDD

Table A.3. TCDD Effects on Liver Histopathology in Scd1 Wild-Type and Null Mice

Time (hr)		24		72		168	
Genotype		+/+	-/-	+/+	-/-	+/+	-/-
Vacuolization	**V**	0.5	0.2	0.3	0.3	0.1	0.0
	T	3.0	2.3	2.3	1.5	0.9	1.6
Inflammation	**V**	0.3	0.0	0.0	0.0	0.4	0.0
	T	0.4	0.0	0.4	0.0	1.5	0.4
Hypertrophy	**V**	0.0	0.0	0.0	0.0	0.0	0.0
	T	1.4	0.0	0.8	0.0	1.3	1.0

The severity of vacuolization, inflammation and hypertrophy are scored as 1-minimal, 2-mild, 3-moderate, 4-marked. N=8, TCDD (T), Vehicle (V).

Figure A.1. Serum alanine aminotransferase levels in *Scd1*+/+ and *Scd1*-/- mice treated 168 h with either vehicle or 30 μg/kg TCDD.

Serum alanine amino transferase (ALT) levels in *Scd1*+/+ and *Scd1*-/- mice treated with 30 μg/kg TCDD or sesame seed oil vehicle 24 h post-dose. Error bars represent the standard error mean (SEM), n=3, (*) represents $p<0.05$ for TCDD compared to vehicle within a genotype. Data were analyzed by Dunnett's t-test.

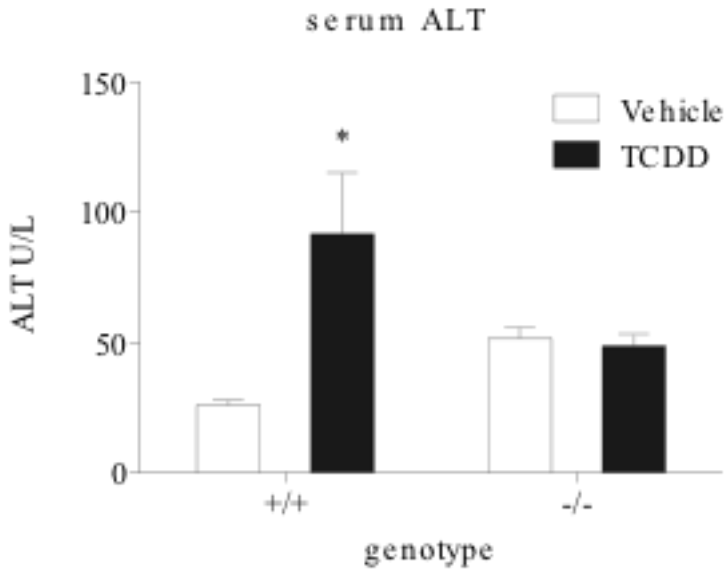


Figure A.2. QRTPCR of the TCDD-inducible genes *Cyp1a1*, *Nqo1*, and *Tiparp* in *Scd1*$^{+/+}$ (+/+) and *Scd1*$^{-/-}$ (-/-) mice gavaged with 30 μg/kg TCDD or sesame oil vehicle for 24 h.
The gene expression ratio is the total quantity normalized to the geometric mean of *Hprt*, *Actb*, and *Gapdh*. Genes are indicated by official gene symbols. Error bars represent the standard error mean (SEM), n=5, * represents $p<0.05$ for TCDD compared to vehicle within a genotype and ** represents $p<0.05$ for *Scd1*$^{+/+}$ TCDD compared to *Scd1*$^{-/-}$ TCDD. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test.

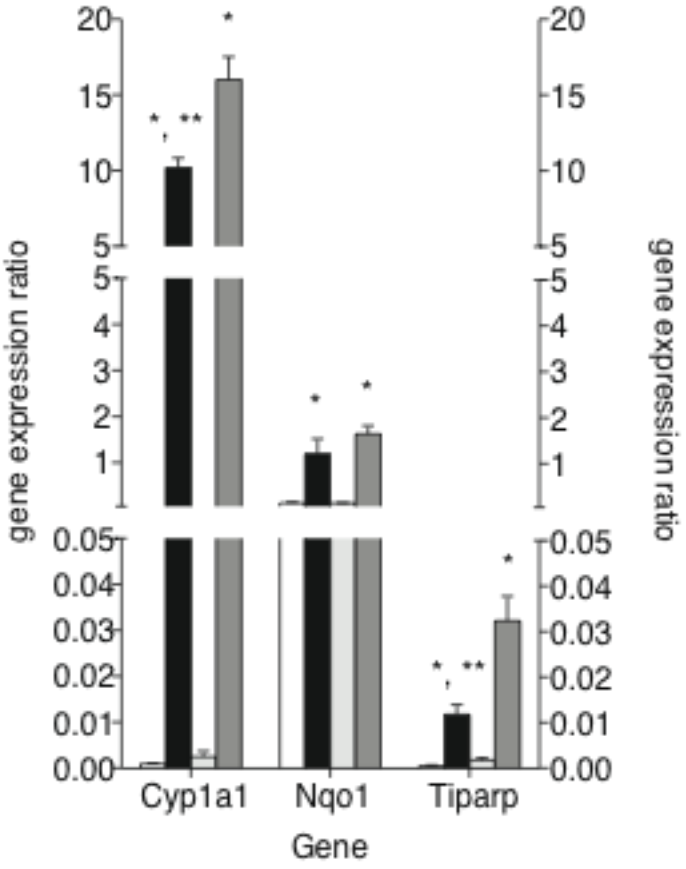


Figure A.3. QRTPCR of *Elovl5* mRNA (A), Elovl5 band shift assays with putative, functional DREs (B), and DRE distribution and TCDD-inducible AhR enrichment within the *Elovl5* locus (C).

(A) Elovl5 mRNA expression is represented as a ratio of the total quantity of *Elovl5* normalized to the geometric mean of *Hprt*, *Actb*, and *Gapdh*. Bars represent mean ± SEM, * indicates $p<0.05$ for T compared to V within a genotype and ** represents $p<0.05$ for $Scd1^{-/-}$ T compared to $Scd1^{+/+}$ T within a time point. Data were analyzed by factorial ANOVA followed by Tukey's *post hoc* test, n=5. (B) The DRE position is numerically indicated relative to the *Elovl5* TSS. Solid arrows indicate a TCDD-inducible band shift. Hatched or dashed arrows indicate an AhR or ARNT supershift, respectively. (C) DRE distributions and regions of AhR enrichment induced by TCDD were previously determined [28, 29]. Track 1: scale and chromosome position. Track 2: probe tiling across the Affymetrix 2.0R mouse array. Track 3: gene organization including transcriptional start site (TSS) (closed arrow), exons (closed boxes), introns and direction of transcription (solid arrowhead line). Track 4: location of DRE cores (5'-GCGTG-3'). Height of vertical bars indicate matrix similarity score (MSS) for the 19 bp DRE sequence [29]. The horizontal line indicates the MSS = 0.8. MSSs greater than 0.8 are considered putative functional DREs. The asterisk (*) denotes 19 bp DRE sequences that bound TCDD-activated AhR in band shift assays. Track 5: regions of significant (FDR<0.01) AhR enrichment in genome-wide ChIP-chip assays. Tracks 6 and 7: histograms depicting the signal intensities for the moving average (MA; blue, track 6) and $\log_2$ fold enrichment (green, track 7) values for regions exhibiting AhR enrichment in genome-wide ChIP-chip assays. The above tracks were modified from the UCSC genome browser.

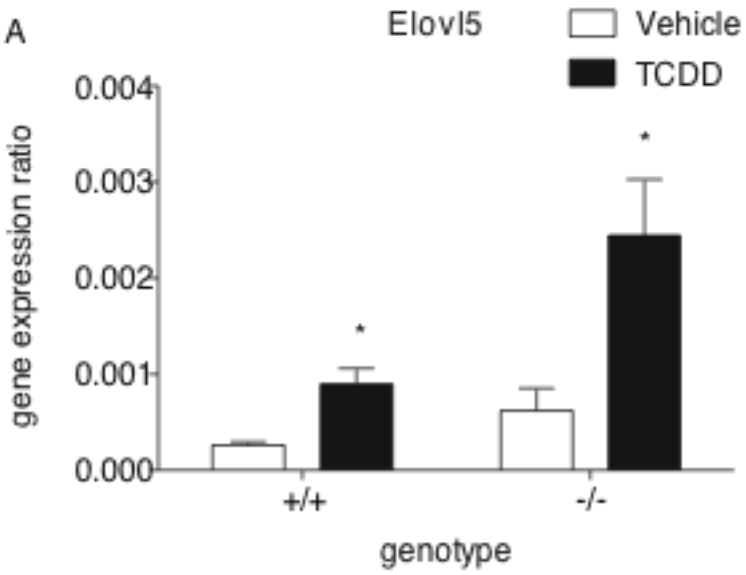


Figure A.3 (cont'd)

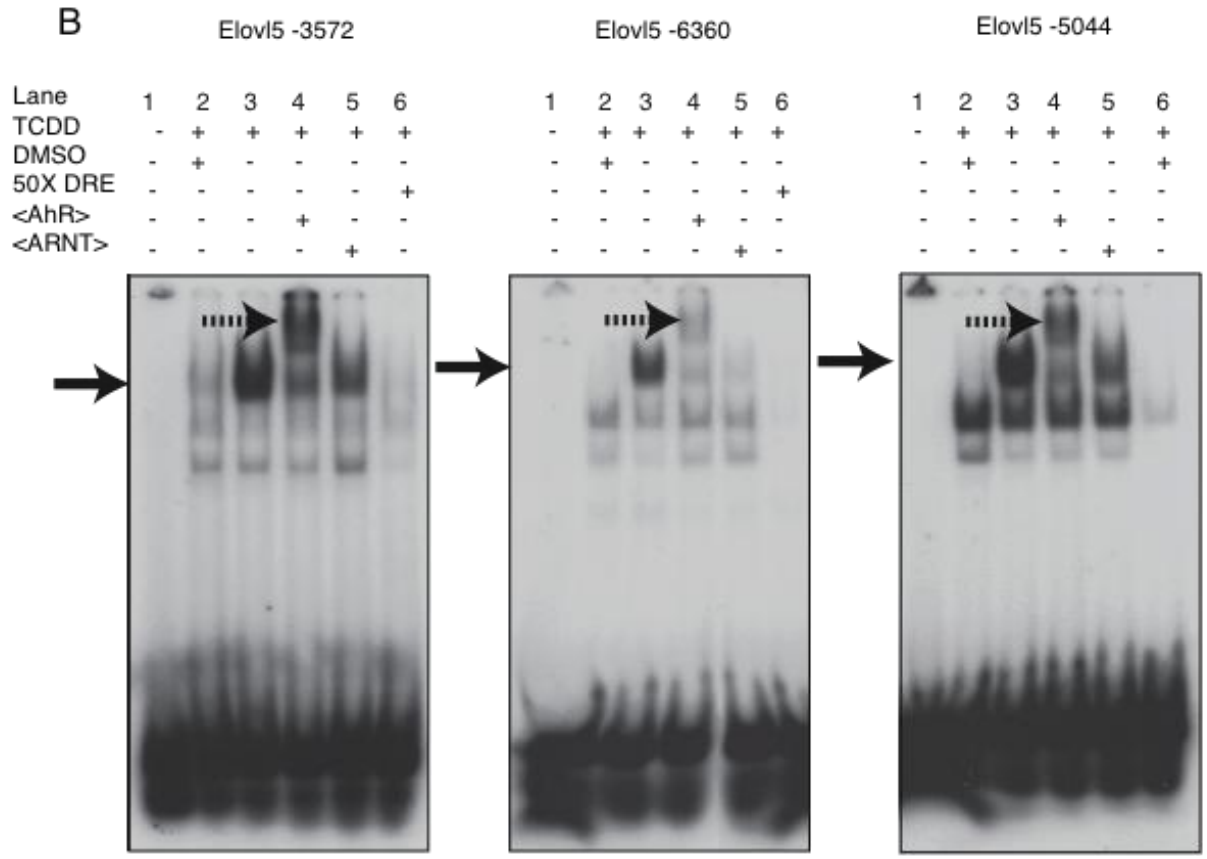
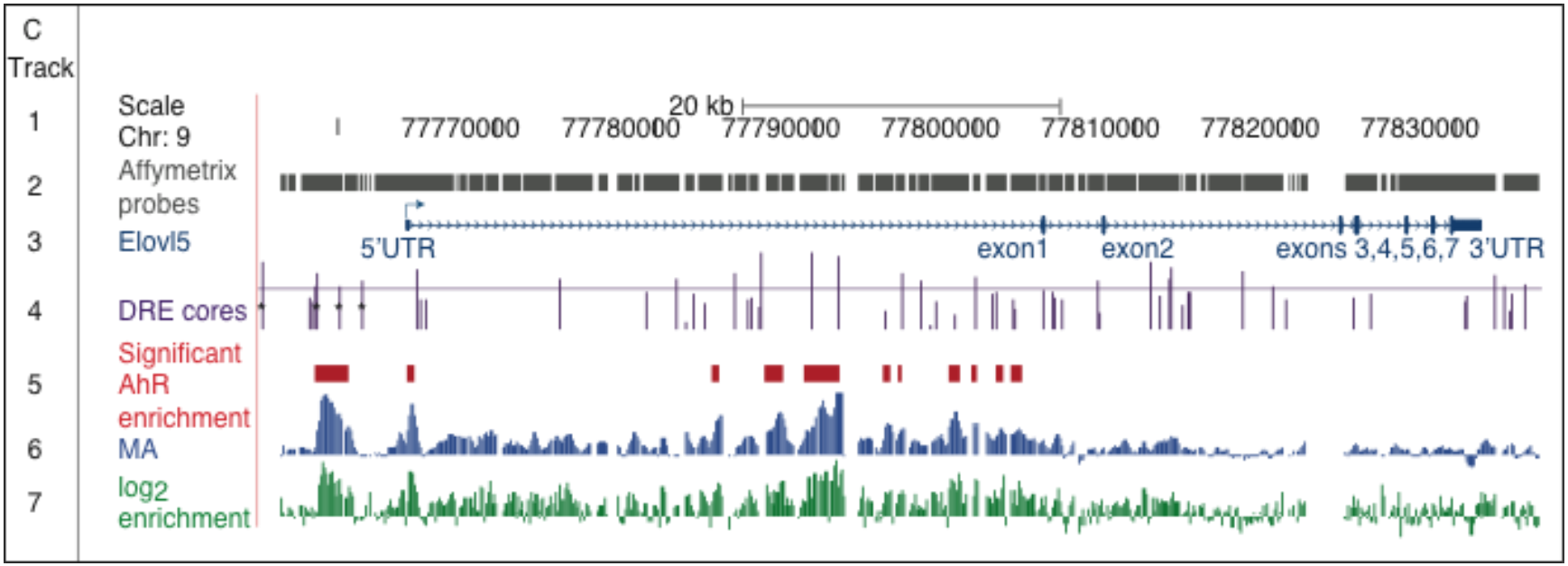


Figure A.3 (cont'd)



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CHAPTER 4

Angrish MM, Mets, BD, Jones, AD, Zacharewski TR: **Dietary Fat is a Lipid Source in 2,3,7,8-Tetrachlorodibenzo-ρ-dioxin (TCDD)-Elicited, Hepatic Steatosis in C57BL/6 Mice**. (accepted, Toxicol Sci).

CHAPTER 4

DIETARY FAT IS A LIPID SOURCE IN 2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)-ELICITED, HEPATIC STEATOSIS IN C57BL/6 MICE

ABSTRACT

TCDD increases FA transport and FA levels, resulting in hepatic steatosis in mice. Diet as a source of lipids was investigated using customized diets, *Scd1* null mice, and ^{14}C-oleate (18:1n9) uptake. C57BL/6 mice fed 5%, 10%, or 15% fat or 50%, 60%, or 70% carbohydrate diets exhibited increased relative liver weight following gavage with 30 µg/kg TCDD for 168 h. Hepatic lipid extract analysis from mice fed 5%, 10% and 15% fat diets identified a dose-dependent increase in total FAs induced by TCDD. Fat-diet fed mice also exhibited a dose-dependent increase in the dietary essential linoleic (18:2n6) and α-linolenic (18:3n3) acids. No dose-dependent FA increase was detected on carbohydrate diets, suggesting dietary fat as a source of lipids in TCDD-induced steatosis as opposed to *de novo* lipogenesis. TCDD also induced oleate levels 3-fold in *Scd1* null mice that are incapable of desaturating stearate (18:0). This finding is consistent with oleate representing >90% of all MUFAs in rodent chow. Moreover, TCDD increased hepatic ^{14}C-oleate levels 2-fold in wild-type and 2.4-fold in Scd1 null mice concurrent with the induction of intestinal and hepatic lipid transport genes (*Slc27a*, *Fabp*, *Ldlr*, *Cd36*, and *Apob*). In addition, computational scanning identified putative DREs and *in vivo* ChIP-chip analysis revealed regions of AhR enrichment proximal to lipid transport genes

differentially regulated by TCDD. Collectively, these results suggest the AhR mediates increased uptake of dietary fats that contribute to TCDD-elicited hepatic steatosis.

INTRODUCTION

Activation of the AhR, a bHLH PAS transcription factor, elicits a broad spectrum of species-specific effects [1]. Epidemiological and rodent studies have linked exposure to TCDD and related compounds to dyslipidemia and disrupted energy balance [2, 3]. Briefly, ligands bind to the cytosolic AhR causing a conformational change, dissociation of chaperone proteins, and translocation to the nucleus where it heterodimerizes with ARNT [4, 5]. The complex binds DREs to modulate gene transcription, although DRE-independent binding to DNA has also been reported [6, 7].

The role of the AhR in hepatotoxicity and lipid metabolism has not been fully elucidated. TCDD induced hepatic steatosis is characterized by increases in TFAs, TAGs, vacuolization, inflammatory cell infiltration, and serum ALT levels [8, 9] that are absent in *AhR*, null mice The AhR also mediates mobilization of peripheral fat [10-12], inhibition of FA oxidation [10, 13], repression of VLDL secretion [13], and hepatic lipid and composition alterations [14].

In this report, we examine diet as a lipid source in TCDD-elicited hepatic steatosis. Dose-dependent increases in hepatic fat accumulation, including essential dietary FAs, suggest dietary fat rather than carbohydrate is an important lipid source in TCDD-elicited steatosis. Increases in hepatic MUFA levels in *Scd1* null mice, and hepatic ^{14}C levels, provide further evidence that AhR activation increases dietary fat processing that contributes to TCDD-elicited hepatic steatosis. Complementary gene expression analysis integrated with computational DRE search [15] and ChIP-chip [6] data indicate the AhR mediates intestinal and hepatic responses that enhanced dietary fat processing and transport, resulting in hepatic steatosis. Collectively, these

results suggest continuous AhR activation may contribute to diseases associated with hepatic steatosis.

MATERIALS AND METHODS

ANIMAL HANDLING

C57BL/6 ovariectomized (ovx) female mice were obtained from Charles Rivers Laboratories (Portage, MI) on postnatal day (PND) 25 with body weights within 10% of the mean body weight upon arrival. B6.129-*Scd1*^{tm1Myz}/J heterozygous mice (Jackson Laboratory, Ben Harbor, ME) had free access to chow or custom diets and water upon arrival and throughout the study. On PND 21 mice were genotyped and weaned. Mice were maintained on a 12 h light/dark cycle, housed in standard cages containing aspen woodchips. B6.129-*Scd1*^{tm1Myz}/J heterozygous mice were fed Harlan Teklad 7964 F6 Rodent diet (chow). All procedures were carried out with All-University Committee on Animal Use and Care approval.

DIET COMPOSITIONS

Custom diets consisted (by weight) of 19.6% protein, 5%, 10%, and 15% fat with decreasing carbohydrate (68%, 56%, and 44%) for an isocaloric intake of 3.7 kcal/g (Table 4.1). Carbohydrate adjusted diets consisted (by weight) of constant fat and protein (6.6% and 19.6%, respectively) with increasing total carbohydrate content of 50%, 60% and 70% for total caloric intakes of 3.3, 3.7, and 4.1 kcal/g, respectively (Table 4.1). Custom diets use different ingredients compared to standard chow, which confounds comparisons to other studies. Specifically, custom

Table 4.1. Nutrient and Energy Composition of Custom Diets

	Chow	Isocaloric, Fat Adjusted Diets			Constant Fat, Carbohydrate Adjusted Diets		
		% Fat			% Carbohydrate		
		5%	10%	15%	50%	60%	70%
Metabolizable Energy (kcal/g)	3.1	3.7	3.7	3.7	3.3	3.7	4.1
% by Weight							
Fat	6.4	5	10	15	6.6	6.6	6.6
Protein	24.3	18.3	18.3	18.3	18.3	18.3	18.3
Carbohydrate	38.7	63.6	52.4	41.1	48.6	59	68.6
Fiber	11.3	3	10.5	18	18	7	---

diet formulations use purified ingredients. In contrast, chow (31% protein, 19% fat, and 50% carbohydrate calories by weight) consists of a proprietary blend of soybean meal, ground corn, wheat, fishmeal, soybean oil, whey, brewers yeast, and other vitamins and minerals.

DIET STUDY IN VIVO TREATMENT

On PND 28, mice fed custom diets (n=5) were gavaged with 0.1 ml of sesame oil (vehicle control) or 30 μg TCDD (Dow Chemical Company, Midland, MI) per kg body weight. Immature ovx mice were used to facilitate comparisons with other data sets, as well as minimize potential interactions with estrogens from maturing ovaries. The dose was chosen to elicit moderate hepatic effects while avoiding overt toxicity. Animals were sacrificed at 24 and 168 h post dose, weighed, and blood was collected via submandibular vein puncture before sacrifice. Tissue samples were removed, weighed, flash frozen in liquid nitrogen, and stored at -80°C.

GC-MS FATTY ACID METHYL ESTER (FAMES) HEPATIC LIPID PROFILING

Hepatic lipid analysis was performed as previously described [14]. Briefly, liver lipids were extracted by Folch method, dried down under nitrogen, converted to methyl esters and resuspended in hexane. Samples were separated and analyzed by GC-MS. 19:1n9 FFA and 19:0 TAG were added as extraction efficiency controls and 17:1n1 FAME (Nu-chek, Elysian, MN) was spiked in as a loading control. Data were analyzed with QuanLynx software and reported as μmol/g liver tissue. FA levels are based on peak areas from total ion chromatograms and μmol/g is obtained from a linear calculation of a calibration curve normalized to sample weight.

^{14}C-OLEATE STUDIES

On PND 28, *Scd1* wild-type and null mice (n=5) were gavaged with sesame oil or 30 μg/kg TCDD. 4 h before sacrifice at 120 h, mice were gavaged with 2 μCi ^{14}C-oleate (0.1 mL of 20 μCi/mL in sesame oil; ARC 0297; American Radiolabeled Chemicals, St. Louis, MO).

Blood was collected from the saphenous vein at 0.5, 1 and 2 h after ^{14}C-oleate gavage or the submandibular vein at 4 h. Tissues were harvested, weighed, flash frozen in liquid nitrogen, and stored at -80°C. Duodenum (~3.5 cm) and jejunum (~6 cm) sections were collected, flushed with phosphate buffered saline, and cut longitudinally. Intestinal epithelium were scraped into vials containing ~1.0 ml of TRIzol (Invitrogen, Carlsbad, CA), snap-frozen in liquid nitrogen, and stored at -80°C. For fecal pellet analysis, mice were gavaged with 2 μCi ^{14}C-oleate 120 h post TCDD dose and all fecal matter was collected until 48 h post ^{14}C-oleate gavage.

Liver, parametrial adipose, and muscle samples were homogenized in Folch solution (2:1 chloroform:methanol), 0.2 mL 40% methanol was added, vortexed, and centrifuged at 10,000 x g for 5 min. The organic phase was dried under nitrogen and resuspended in hexane. Samples were directly added to 10 mL liquid scintillation fluid (Safety-Solve, RPI, Mount Prospect, IL) and ^{14}C levels counted on a Packard Tri Carb Liquid Scintillation Counter (PerkinElmer; Waltham, MA). Each sample was spiked with 17:1n1 FAME (Nu-chek; Elysian, MN) to control for extraction efficiency and quantified by GC-MS. Liver and adipose samples were normalized to sample weight x whole organ weight. Muscle samples were normalized to sample weight. For ^{14}C levels in fecal pellets, ~30 μg dried pellets were ground to a fine powder with mortar and pestle and added to 10 mL liquid scintillation fluid. Fecal samples were normalized to total dry fecal pellet weight. For ^{14}C levels in serum, 5 μL of serum was added directly to scintillation cocktail. Samples were normalized to the average body weight of each mouse.

QUANTITATIVE REAL TIME PCR (QRTPCR)

RNA was isolated from frozen liver samples and intestinal scrapings and QRTPCR expression was performed as previously described [8]. The copy number of each sample was standardized to the geometric mean of *Gapdh*, *Hprt*, and *Actb* to control for differences in RNA loading, quality, and cDNA synthesis [16]. Data are reported as the fold change of standardized treated over standardized vehicle values.

STATISTICAL ANALYSIS

Data were analyzed by ANOVA followed by Tukey's *post hoc* test in SAS V9.2 (SAS Institute, Cary, NC). Differences between treatment groups were considered significant when $p<0.05$.

RESULTS

BODY AND LIVER WEIGHTS

Mice fed fat adjusted diets had increased RLWs (Appendix B, Table 1) at 24 and 168 h following a single oral gavage of 30 μg/kg TCDD. In contrast, mice fed carbohydrate adjusted diets exhibited increased RLW at 168 h only. There were no significant alterations in body weight or body weight gain throughout the study, suggesting treatment had no effect on feed consumption.

HEPATIC LIPID CONTENT IN MICE FED AN ISOCALORIC, FAT ADJUSTED DIET

Vehicle treated mice exhibited a dose-dependent decrease in TFAs primarily due to decreases in MUFAs (Table 4.2A). However, TCDD increased TFAs 1.4-, 1.7-, and 2.0-fold in mice fed 5%, 10%, and 15% fat diets, respectively (Figure 4.1A), compared to diet-matched vehicles. More specifically, absolute levels of SFA MUFA, and PUFA

Table 4.2. Hepatic Lipid Levels in Mice Fed Fat or Carbohydrate Adjusted Diets 168 h Post 30 μg/kg TCDD Dose

A. Adjusted Fat Diet		**% Fat**		
	Treatment	**5%**	**10%**	**15%**
Total FA	**Vehicle**	135.6 ± 16.7	113.5 ± 9.5	96.3 ± 7.1††
	TCDD	195.0 ± 14.0*	194.3 ± 16.4*	188.0 ± 15.6*
Total SFA	**Vehicle**	40.9 ± 3.3	41.9 ± 3.8	36.6 ± 2.0
	TCDD	53.9 ± 3.3*, **	53.8 ± 1.4*	47.7 ± 3.8*
Total MUFA	**Vehicle**	54.1 ± 12.1	31.0 ± 4.8††	18.2 ± 2.8††
	TCDD	92.6 ± 12.0*, **	66.0 ± 11.0*	54.8 ± 7.3†
Total PUFA	**Vehicle**	33.2 ± 2.7	40.6 ± 2.6	41.5 ± 2.9
	TCDD	47.7 ± 2.8*, **, †	74.4 ± 4.4*, **	85.5 ± 7.2*
B. Adjusted Carbohydrate Diet		**% Carbohydrate**		
	Treatment	**50%**	**60%**	**70%**
Total FA	**Vehicle**	108.8 ± 24.6	113.0 ± 4.7	135.9 ± 9.8
	TCDD	156.8 ± 7.6*	148.6 ± 22.3**	193.0 ± 34.8*
Total SFA	**Vehicle**	45.1 ± 7.74	46.7 ± 3.2	49.7 ± 4.9
	TCDD	54.3 ± 2.7	51.0 ± 5.4**	64.5 ± 8.7*
Total MUFA	**Vehicle**	28.4 ± 11.6	31.0 ± 2.6	51.2 ± 6.1
	TCDD	50.7 ± 5.5**	51.3 ± 12.8	79.8 ± 26.0*
Total PUFA	**Vehicle**	35.4 ± 5.6	35.3 ± 3.3	35.0 ± 2.4
	TCDD	51.8 ± 2.5*	46.3 ± 4.9*	48.7 ± 5.0*

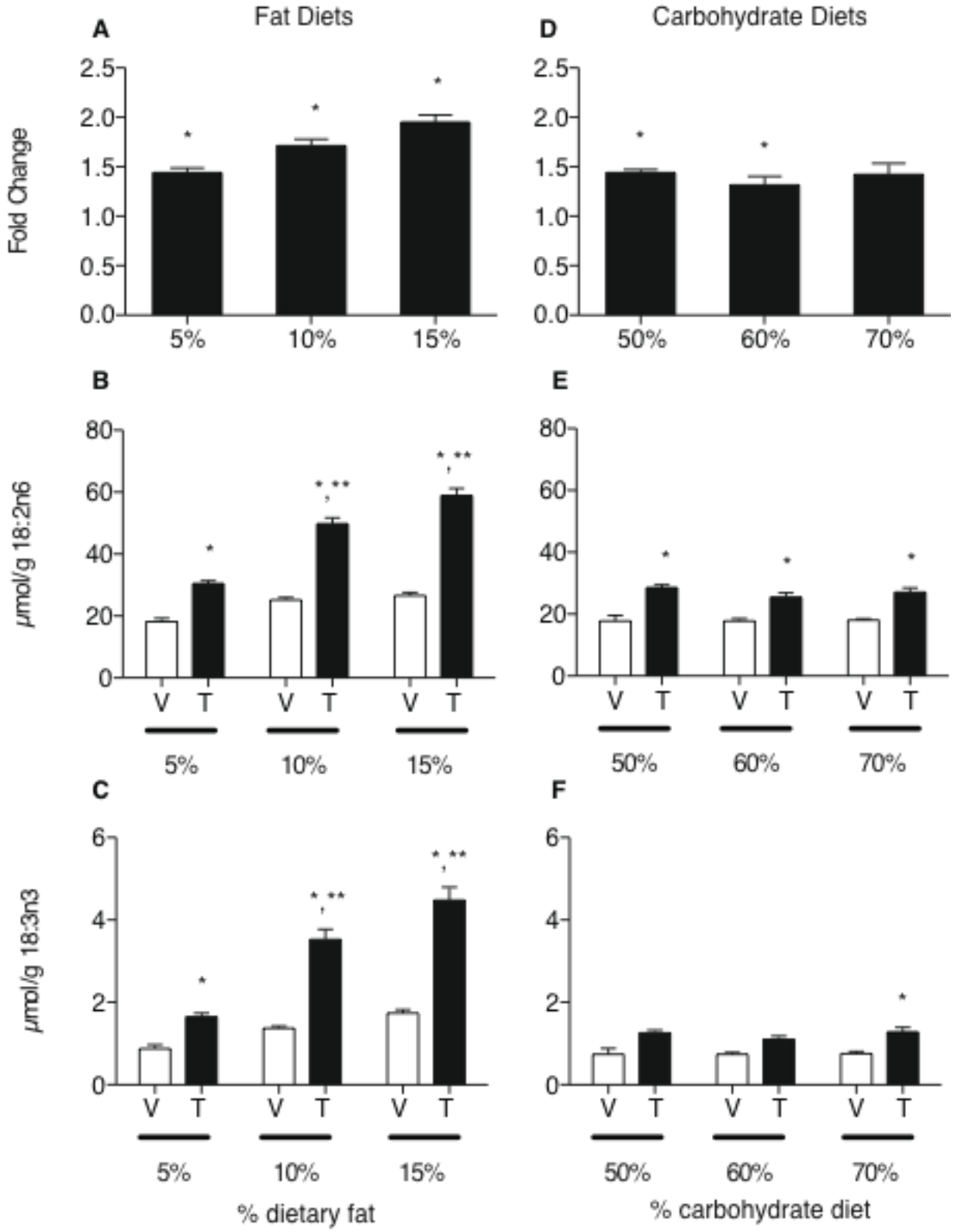
**A. Adjusted Fat Diet : ***p<0.05 for TCDD compared with Vehicle within a diet, **p<0.05 for TCDD 15% fat compared with TCDD 5% or 10%, † p<0.05 for TCDD 5% fat compared with TCDD 10%, ††p<0.05 for Vehicle 5% compared with Vehicle 10% or 15%,

Table 4.2 (cont'd)

n=5. **B. Adjusted Carbohydrate Diet: *** $p<0.05$ for TCDD compared with Vehicle within a diet,** $p<0.05$ for TCDD 70% carbohydrate compared with TCDD 50% or 60% carbohydrate, n=5.

Figure 4.1. Hepatic absolute and essential fatty levels in mice fed increasing fat or carbohydrate diets treated with sesame oil vehicle (V) or 30 μg/kg TCDD (T) for 168 h. (**A-C**) 5%, 10%, and 15% fat diet fed mice, (**A**) total fatty acids (TFA), (**B**) α-linoleic acid (18:2n6), and (**C**) α-linolenic acid (18:3n3) levels. * $p<0.05$ for TCDD compared with Vehicle within a diet, ** $p<0.05$ for TCDD 15% fat or 10% fat compared with TCDD 5% fat. (**D-F**) 50%, 60%, and 70% carbohydrate diet fed mice, (**D**) TFAs, (**E**) 18:2n6, (**F**) 18:3n3. ***** $p<0.05$ for TCDD compared with Vehicle within a diet; ** $p<0.05$ for TCDD 70% carbohydrate compared with TCDD 50% carbohydrate. (**A-F**) Bars represent mean ± standard error of the mean (SEM), n=5.

Figure 4.1 (cont'd)



increased with palmitic (16:0) and oleic (18:1n9) acids representing 80-90% of all hepatic SFAs and MUFAs, respectively (Table B.2). Note that palmitic and oleic acids represent >66% and >98% of dietary SFAs and MUFAs, respectively, in the fat adjusted diets (Table B.4A), with absorption efficiencies of >90% [17].

Hepatic PUFAs also exhibited a dietary fat dose-dependent increase (Table 4.2A) primarily due to linoleic acid (18:2n6) accumulation (Figure 4.1B). 18:2n6, which represents ~65% of all hepatic PUFAs, is an essential FA that can only be acquired from the diet with a reported absorption efficiency of >95% [17]. α-Linolenic acid (18:3n3), another dietary essential FA, exhibited similar hepatic increases with increasing dietary fat content that was further induced by TCDD (Figure 4.1C). 18:2n6 and 18:3n3 represent >99% of the PUFA content in isocaloric, fat adjusted diets (Table B.4A). These results suggest that AhR activation enhances dietary fat processing and/or transport that contributes to TCDD elicited hepatic steatosis.

HEPATIC LIPID CONTENT IN MICE FED A CONSTANT FAT, CARBOHYDRATE ADJUSTED DIET

Mice fed 50%, 60%, or 70% carbohydrate diets did not exhibit a dose-dependent increase in hepatic TFA following TCDD treatment. TCDD induced TFAs ~1.4-fold across all carbohydrate adjusted diets (Figure 4.1D). Absolute SFA and MUFA levels increased 1.3- and 1.6-fold, respectively, in mice fed the 70% carbohydrate diet compared to controls (Table 4.2B). 16:0, 18:1n9, and 18:2n6 were the predominant hepatic SFA, MUFA, and PUFA species, respectively, similar to the composition in mice fed fat adjusted diets (Table B.4). However, 18:2n6 and 18:3n3 levels, the essential dietary FAs, remained constant across TCDD treated mice on carbohydrate diets (Figure 4.1E-F), suggesting dietary carbohydrate is not a significant contributor to AhR-mediated steatosis.

^{14}C-OLEATE STUDIES

Lipid accumulation in the liver was investigated in *Scd1* wild-type and null mice gavaged with 2 μCi ^{14}C-oleate 5 days post-dose with 30 μg/kg TCDD. Scd1 performs the rate-limiting step in MUFA synthesis. Therefore, null mice are incapable of desaturating 18:0 to 18:1n9, yet 18:1n9 is still available via the diet (Table B.4). ^{14}C-levels in hepatic lipid extracts increased 2- and 2.4-fold in TCDD treated wild-type and *Scd1* null mice, respectively (Figure 4.2A). This increase is consistent with the ~2-fold oleate increase in mice fed fat or carbohydrate adjusted diets (Appendix B Tables 2 and 4), and the ~3-fold increase in 18:1n9 levels in TCDD-treated Scd1 wild-type and null mice (Figure 4.2B) [14]. The absorption efficiency of oleate is reported to be >95% efficient [17]. Furthermore, oleate represents >90% of total MUFAs in treated mouse livers (Figure 4.2C) [14]. TCDD increased serum (1.4-fold) and muscle (1.2-fold) and decreased adipose (-1.3-fold) and stool (-1.5-fold) ^{14}C levels, although statistical significance was not achieved (Figure B.1A-D).

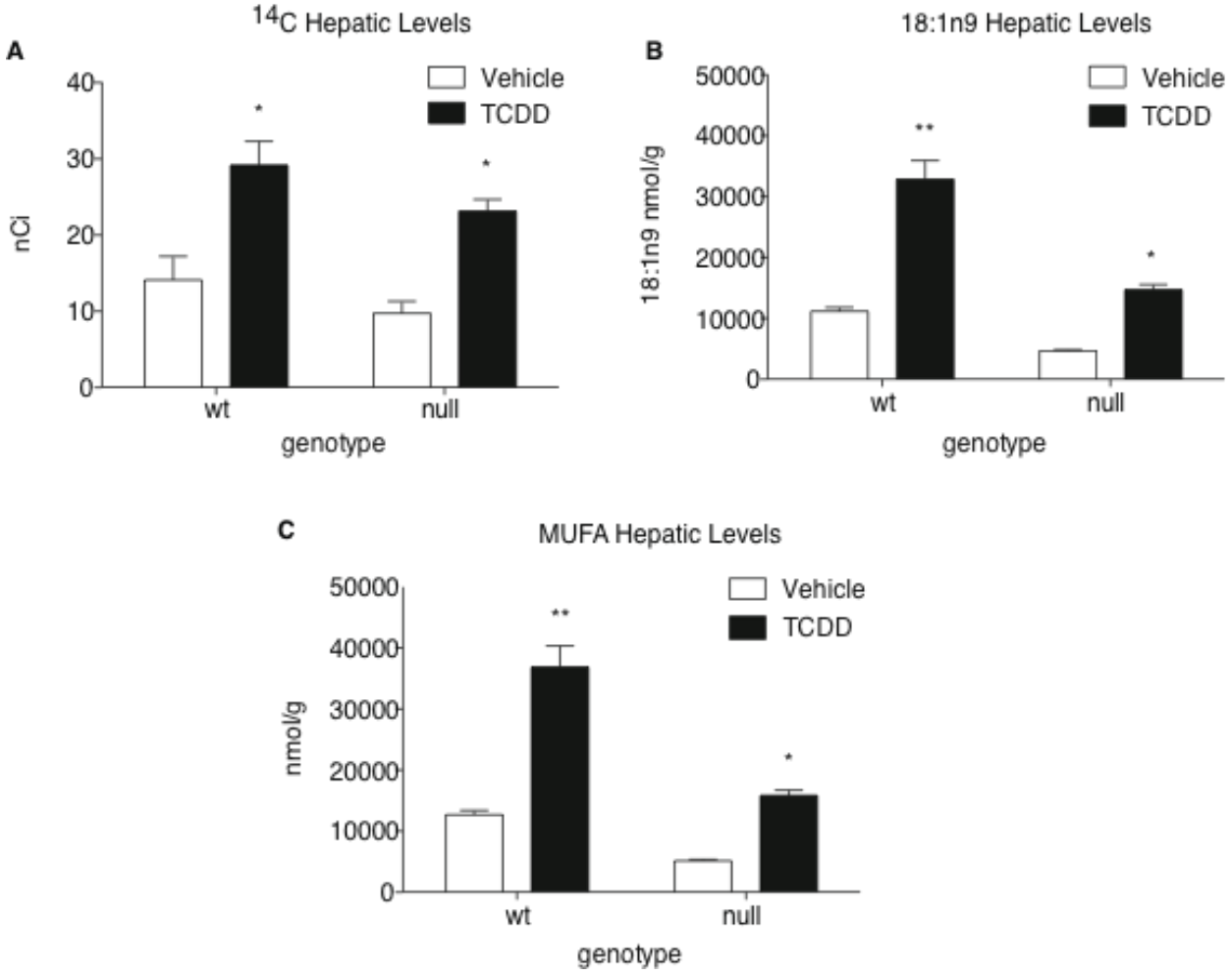
DIFFERENTIAL INTESTINAL AND HEPATIC GENE EXPRESSION

To further examine the effect of AhR activation on dietary lipid uptake gene expression, intestinal and hepatic mRNA was examined. Of the genes examined, >80% contained a putative, functional DRE (matrix similarity score > 0.847) and/or had hepatic AhR enrichment in ChIP-chip analysis (Table 4.3) [6, 15].

Dietary FA (>16C) hydrolyzed from TAG by gastric and pancreatic lipases in the intestinal lumen are actively transported into enterocytes before export into the lymphatic and systemic circulation [18]. In the duodenum, TCDD induced *Ldlr* (2.3-fold), *Cd36* (2.7-fold), and *Slc27a4* (1.4-fold), as well as *Fabp1* (1.4-fold), and *Fabp4* (1.4-fold) (Table 4.3). Although implicated in

Figure 4.2. Hepatic lipid levels in *Scd1* wild-type (wt) and null mice 120 h post-dose with 30 μg/kg TCDD dose.

(**A**) ^{14}C-levels were measured in lipid extracts by liquid scintillation counting after gavage with 2 μCi ^{14}C-oleate 4 h prior to sacrifice. GC-MS analysis of hepatic oleate (18:1n9) (**B**) and monounsaturated fatty acid (**C**) levels (expressed in nmol/g) in *Scd1* wild-type and null mice 168 h after oral gavage with 30 μg/kg TCDD [14]. * $p<0.05$ for TCDD compared with Vehicle, ** $p < 0.05$ for *Scd1* wt TCDD compared with *Scd1* null TCDD. Bars represent mean ± SEM, n=5.



mitochondrial β-oxidation [19, 20] and insulin sensing [21], *Slc27a1* expression (2.3-fold in duodenum, -5.8-fold in jejunum) occurs primarily in muscle and adipose tissue making its role in other tissues uncertain.

Endothelial lipases hydrolyze serum lipids absorbed by the intestine. The resulting products are taken up by the liver via facilitated transport or receptor-mediated endocytosis. TCDD induced hepatic long-chain FA uptake family members *Cd36* (4.4-fold), *Slc27a3* (2.7-fold), *Slc27a4* (3.0-fold), and *Ldlr* (3.2-fold) (Table 4.3). Hydrolytic cleavage of TAG by cytosolic lipases further adds FAs to the hepatic pool. TCDD induced *Lpl* (3.2-fold), *Clps* (2.6-fold), *Pnlprp1* (3.7-fold), and *Mgll* (1.4-fold), but repressed *Pnpla3* (3.3-fold) (Table 4.3). Interestingly, sequence variations in *PNPLA3* are associated with hepatic TAG content and NAFLD in humans [22].

Intracellular FAs are directed to TAG biosynthetic and β-oxidation pathways by Fabps. *Fabp1* and *4* were induced 2.9- and 1.4-fold, respectively. However, TCDD repressed hepatic mitochondrial acyl-CoA synthetase genes (*Acsm1-4* and *Acsl1*, *3-4*, repressed 1.3 to 2.3-fold) that activate FAs for transport into the inner membrane space for subsequent β-oxidation (Table 4.3). These gene expression changes are consistent with the reported inhibition of mitochondrial β-oxidation by TCDD [10, 13]. Furthermore, TCDD induced the expression of hepatic triglyceride biosynthesis genes (*Mogat1, Mogat2*, *Dgat1*, and *Dgat2* induced 3.6-, 3.0-, 1.6-, and 1.3-fold, respectively), consistent with hepatic TAG accumulation [9, 14].

Hepatic differential gene expression is also consistent with TCDD elicited disruption of carbohydrate catabolism (Table 4.3). TCDD induced hexokinase (*Hk3*) 2.2-fold, which catalyzes the irreversible phosphorylation of glucose to glucose-6-phosphate (G6P). In contrast, genes involved in gluconeogenesis (*Pcx* -1.3-fold, *Pck1* -2.0-fold, *G6pc* -2.7-fold) and glycogen

Table 4.3. DREs[1] and Regions of AhR Enrichment[2] in TCDD Responsive Genes Associated with Lipid and Carbohydrate Transport and Metabolism.

Gene ID	Gene Symbol	Liver	Duodenum	Jejunum	# of DREs[1]	ChIP peaks 2h[2]	Function[3]	Regulated by[3]
Xenobiotic Metabolism								
13076	*Cyp1a1*	5799*	222*	22.7*	7	4	xenobiotic metabolism	AhR
Fatty Acid and Triglyceride Synthesis								
14104	*Fasn*	-2.8*	NC	NC	4	1	fatty acid synthesis	SREBP, TR, LXR, cAMP, AMPK
153674	*Acly*	-2.6**	nd	nd	0	0	citrate metabolism	oxaloacetate, ATP
107476	*Acaca*	-1.4**	nd	nd	3	5	malonyl CoA acylation	glucagon
68393	*Mogat1*	3.6*	NC	NC	1	0	triglyceride synthesis	PPAR, CEBP
233549	*Mogat2*	3.0*	NC	1.4*	4	3		
13350	*Dgat1*	2.0	1.4	1.6*	4	0		
67800	*Dgat2*	1.3	1.4	1.6	2	7		
Fatty Acid Transport								
238055	*Apob*	2.5*	NC	1.8*	2	0	VLDL and chylomicron assembly	APOBEC-1
16835	*Ldlr*	3.2*	2.3*	NC	2	4	lipoprotein uptake	LXR

Table 4.3 (cont'd)

Gene ID	Gene Symbol	Liver	Duodenum	Jejunum	# of DREs[1]	ChIP peaks 2h[2]	Function[3]	Regulated by[3]
12491	*Cd36*	4.4*	2.7*	NC	0	1	fatty acid uptake	PPAR, CEBPα, AMPK
26457	*Slc27a1*	-2.9*	2.3*	-5.8†	2	0	mitochondrial β-oxidation	PPARα, PPARγ
26568	*Slc27a3*	2.7*	NC	NC	1	0	unknown	
26569	*Slc27a4*	3.0*	1.4†	1.4†	0	0	peroxisomal β-oxidation, CHOL-ester synthesis	PPARγ, SREBP1c
14080	*Fabp1*	2.9*	1.4*	1.7*	2	4	TAG synthesis, β-oxidation	PPARα, HNF4α
11770	*Fabp4*	1.4†	1.5	2.5	0	0	chylomicron assembly	cJun, PPARγ
117147	*Acsm1*	-2.0**	nd	nd	3	0	medium chain fatty acid transport (mitochondrial β-oxidation)	acetyl-CoA, malonyl-CoA, NADPH, NADH
233799	*Acsm2*	-1.5**	nd	nd	0	0		
20216	*Acsm3*	-1.6**	nd	nd	2	0		
233801	*Acsm4*	-1.2**	nd	nd	0	0		
14081	*Acsl1*	-1.4**	nd	nd	6	5	long chain fatty acid transport (mitochondrial β-oxidation)	
74205	*Acsl3*	-2.3**	nd	nd	0	0		
50790	*Acsl4*	-1.3**	nd	nd	0	2		

Table 4.3 (cont'd)

Gene ID	Gene Symbol	Liver	Duodenum	Jejunum	# of DREs[1]	ChIP peaks 2h[2]	Function[3]	Regulated by[3]
Fatty Acid Metabolism								
16956	*Lpl*	3.2**	nd	nd	2	0	TAG metabolism of lipoproteins & chylomicrons	Insulin, glucagon, epinephrine
109791	*Clps*	2.6**	nd	nd	0	0		
18946	*Pnliprp1*	3.7**	nd	nd	0	0		
11343	*Mgll*	1.4**	nd	nd	9	0	monoglyceride metabolism	PPARα
116939	*Pnpla3*	-3.3**	nd	nd	0	1	TAG metabolism	
Glycolysis/ Gluconeogenesis/ Glycogen Synthesis								
18534	*Pck1*	-2.0**	nd	nd	0	1	gluconeogenesis	insulin, glucagon, cAMP
14377	*G6pc*	-2.7**	nd	nd	1	5		insulin, glucose
18563	*Pcx*	-1.3**	nd	nd	5	4		ATP
103988	*Gck*	-1.5**	nd	nd	3	2	glucose metabolism	G6P, insulin, glucagon
212032	*Hk3*	2.2**	nd	nd	1	0		
232493	*Gys2*	-1.5**	nd	nd	2	4		

* $p<0.05$ or † $p<0.01$ for TCDD compared with Vehicle, QRT PCR data at 24h post-dose. QRTPCR data were analyzed by Dunnett's t-test, n-=5. ** for P1(t) ≥ 0.999 for microarray data at 168 h post-dose [15]. [1]DRE distributions were previously determined [15]. Only DREs satisfying a matrix similarity score of $\geq$ 0.85 were included. [2]AhR enrichment was previously determined [6]. [3]Data from [19, 20, 51]. NC, no change; nd, not detected.

synthesis (*Gck*, -1.5-fold, *Gys2* -1.5-fold) were repressed. Similarly, genes that provide FA synthesis substrates, such as ATP-citrate lyase (*Acly* -2.6-fold) and acetyl-CoA carboxylase (*Acaca* -1.4-fold), and fatty acid synthetase activity (*Fasn*, -2.8-fold) were repressed (Table 4.3). These changes are consistent with the lack of a dose-dependent increase in hepatic TFAs through *de novo* lipogenesis in mice fed carbohydrate adjusted diets. Furthermore, reported changes in hepatic gene expression are consistent with hepatic TCDD reported in the same model [8].

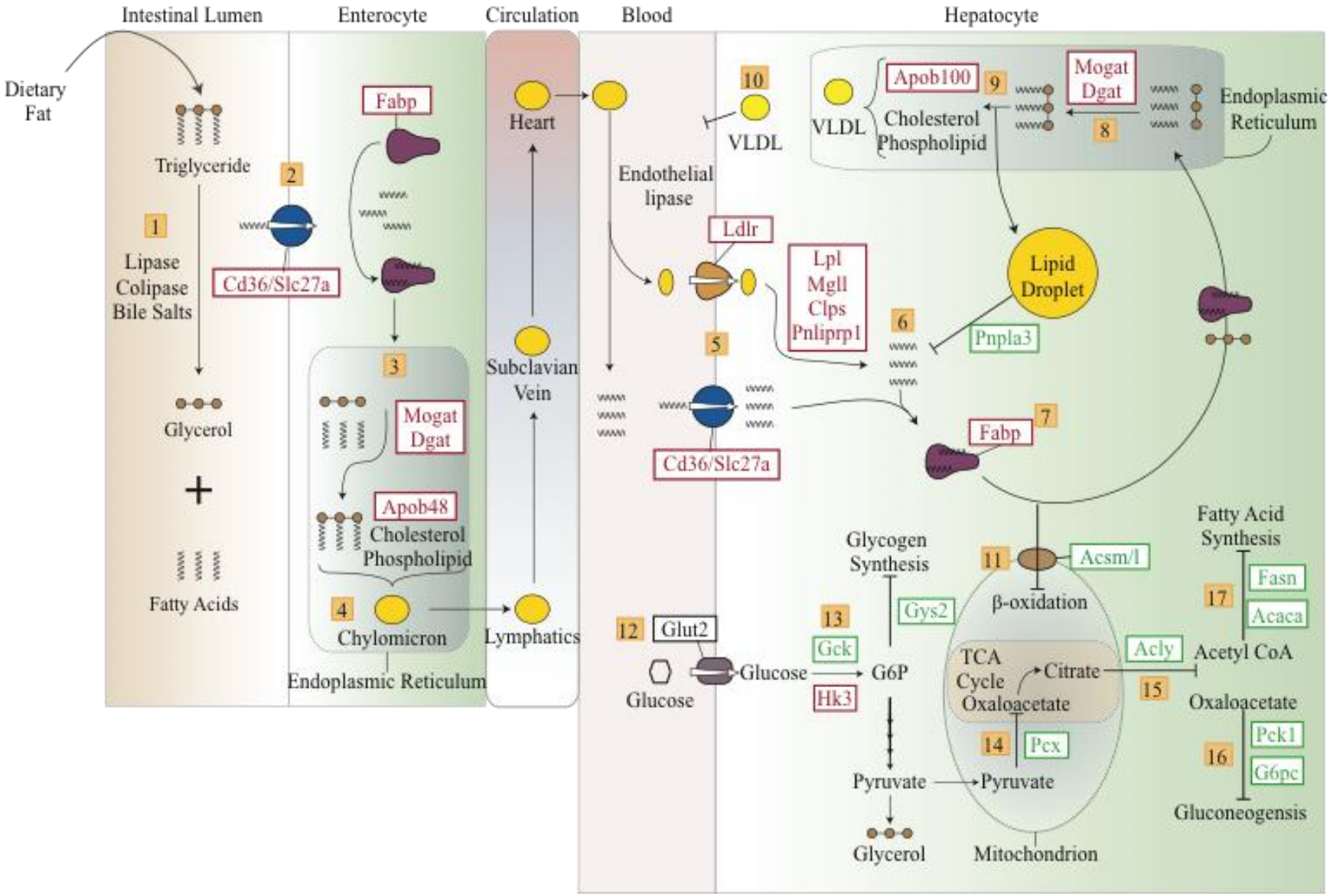
DISCUSSION

Hepatic steatosis can result from the disruption of multiple processes involved in lipid and carbohydrate uptake, metabolism and efflux. Our studies provided evidence that dietary fat, rather than carbohydrate, is an important lipid source in TCDD-elicited steatosis in mice. Computational DRE search, ChIP-chip, and gene expression [6] data indicate AhR activation results in a coordinated response involving the digestive, circulatory and hepatic systems (Figure 4.3). This suggests any ligand (e.g., chemical, drug, endogenous substance, natural product) capable of activating the AhR may enhance dietary fat processing and transport, although continuous exposure may be required.

Increases in hepatic ^{14}C levels clearly demonstrated diet as a source of lipids in TCDD-elicited hepatic steatosis. Previous studies have implicated the mobilization of peripheral adipose tissue based on increased serum 16:0, 18:1, 18:2, and 18:3 free FA levels and their abundance in adipose tissue [10-12]. However, these FAs also represent the primary lipids in chow (Table B.4). Furthermore, increases in oleate, the primary MUFA in rodent chow, and

Figure 4.3. AhR-mediated increase in dietary lipid in TCDD elicited hepatic steatosis.
Step 1 - Dietary fat is hydrolyzed in the intestinal lumen by pancreatic lipase, colipase, and bile salts into glycerol and free fatty acids (FFAs). **Step 2 -** FFA are transported into enterocytes by fatty acid transport proteins and sequestered by cytosolic fatty acid binding proteins (Fabps). **Step 3 -** Fabps deliver FAs to the endoplasmic reticulum (ER) where they are synthesized into triglycerides (TAGs) with glycerol. **Step 4 -** TAGs are packaged with apolipoprotein b48, cholesterol, and phospholipids into chylomicrons that are secreted into the systemic circulation. **Step 5 -** In the blood, endothelial lipases hydrolyze TAGs associated with chylomicrons and lipoproteins into FFAs and remnant lipoprotein products that are actively transported into hepatocytes via fatty acid transport proteins and receptor-mediated endocytosis. **Step 6 -** Intracellular lipoprotein TAGs are further hydrolyzed by cytosolic lipases into free fatty acids. **Step 7 -** Fabp binds FFA and transports them to the endoplasmic reticulum for TAG biosynthesis. **Step 8 -** In the ER, Mogat and Dgat synthesize TAG from FFA and glycerol. **Step 9** - TAGs synthesized in the ER are stored in cytosolic lipid droplets and/or packaged with apolipoprotein b100 (Apob100), cholesterol, and phospholipids into very low-density lipoproteins (VLDL). **Step 10 -** TCDD is reported to inhibit VLDL secretion thus contributing to hepatic fat accumulation [25]. **Step 11** - Acyl-CoA synthetase is required for FA acylation, transport into the mitochondria, and subsequent β-oxidation. **Step 12** - Glucose is transported into hepatocytes via the insulin-independent glucose transporter Glut2. **Step 13 -** Glucose is phosphorylated to glucose-6-phosphate by glucokinase (Gck) or hexokinase (Hk3). **Step 14** - The mitochondrial gluconeogenic enzyme pyruvate carboxylase (*Pcx*) converts pyruvate to oxaloacetate that can enter the TCA cycle for conversion to citrate. **Step 15** – Cytosolic citrate is converted by ATP citrate lyase (*Acly*) to acetyl CoA and oxaloacetate. **Step 16** - Oxaloacetate may be converted to glucose via gluconeogenic enzymes phosphoenolpyruvate carboxykinase (*Pck1*) and glucose 6 phosphatase (*G6pc*). **Step 17 –** Acetyl-CoA carboxylase (*Acaca*) converts acetyl-CoA to malonyl-CoA, a substrate for fatty acid synthesis that is catalyzed by fatty acid synthase (*Fasn*). Lines with arrowheads, reaction/pathway direction; lines with blunted ends, reaction/pathway inhibition; red boxes, induced gene expression; green boxes, repressed gene expression.

Figure 4.3 (cont'd)



hepatic ^{14}C levels in Scd1 null mice provide further evidence of a role for dietary fat in AhR-mediated steatosis.

Complementary gene expression analysis is consistent with a role for the AhR in mediating hepatic accumulation of dietary lipids (Figure 4.3). Free FAs hydrolyzed by pancreatic and gastric lipases, colipases and bile salts passively diffuse (FA<16C) and are actively transported (FA>16C) into enterocytes by *Cd36* and *Slc27a4* that were induced by TCDD (steps 1-3). A role for Cd36 in intestinal lipid clearance and FFA uptake has been demonstrated in null mice [23], while Slc27a4 (Fatp4) is associated with obesity in humans [24]. Once intracellular, fatty acid binding proteins (Fabps) sequester FAs to prevent their transport back into the intestinal lumen and targets them to specific organelles [25]. TCDD inducible *Fabp1*, unlike other family members, binds two rather than one FA, as well as other small hydrophobic ligands [26], and is involved in intestinal FA processing and chylomicron maturation (step 4) [27, 28]. Although intestinal lipid absorption is highly efficient [17, 29], AhR activation may enhance intestinal lipid processing and efflux, consistent with TCDD induced increases in serum FFAs and TAGs [8].

The concurrent induction of several hepatic genes associated with lipid transport, processing and metabolism further promotes steatosis (Figure 4.3). *Ldlr*, *Cd36*, and *Slc27a* actively transport increased circulating FFAs, chylomicrons and lipoprotein remnants into the liver (step 5). TCDD also induced lipoprotein lipase (*Lpl*), monoglyceride lipase (*Mgll*), pancreatic lipase-related protein 1 (*Pnliprp1*), and pancreatic colipase (*Clps*) that hydrolyze intracellular lipoprotein remnants to further increase the intracellular FA pool (step 6). Induced *Fabp1* binds and sequesters intracellular FAs and targets them for TAG synthesis [30]. Mogat1/2 and Dgat1/2, induced by TCDD, then facilitate hepatic TAG biosynthesis (steps 7-8). TAGs are

stored in lipid droplets (step 9), or incorporated into VLDLs (step 10). However, TCDD inhibits VLDL secretion (step 10) [13], consistent with AhR-mediated increases in hepatic TAG and vacuolization [14, 31].

Fabp1 also targets FAs for mitochondrial β-oxidation [30]. TCDD inhibits FA oxidation [10, 13] possibly by inhibiting transport into mitochondria, further adding to hepatic FA accumulation. More specifically, TCDD inhibits medium and long chain FA mitochondrial acyl-CoA synthetase gene expression (*Acsm1-4*, *Ascl1 and 3-4*) (step 11) that is required for transport across the mitochondrial matrix via carnitine for subsequent β-oxidation. Yet, TCDD induced ketone body accumulation *in vitro* [10], suggesting an intact carnitine pathway. Nonetheless, plasma ketones do not increase in response to TCDD exposure *in vivo* [32] and requires additional investigation.

The inability of carbohydrate diets to enhance hepatic steatosis appears to involve TCDD dysregulation of carbohydrate metabolism gene expression (step 12). For example, anabolic pathways typically dominate in fed animals, yet TCDD decreased glucokinase (*Gck*) and glycogen synthase (*Gsy2)*, suggesting suppression of hepatic glycogen synthesis (step 13). Glucokinase is the predominant enzyme regulating hepatic glucose metabolism in response to nutritional states such as refeeding and insulin stimulation [33]. Although TCDD induced hexokinase (*Hk3*), this enzyme is expressed at low levels in hepatocytes, yet exhibits compensatory induction following glucokinase repression, as found in liver cirrhosis [34]. TCDD also inhibited mitochondrial pyruvate carboxylase (*Pcx*) that converts pyruvate into oxaloacetate, suggesting flux towards glycerol production to support hepatic TAG production leading to greater sequestration of hepatic FA. Metabolomic studies also report TCDD increases

hepatic glycerol levels [35] and that TCDD treatment prevents glycerol ketogenesis without affecting esterification to TAG [10].

TCDD suppressed phosphoenolpyruvate carboxykinase (*Pck1*) and glucose-6 phosphatase (*G6pc*) expression, key regulators of gluconeogenesis (steps 14 and 16). These genes are commonly regulated by peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) [36] that is functionally impaired by AhR-mediated induction of *TiPARP* (TCDD-inducible poly(ADP-ribose) polymerase 7, PARP7) [37]. TCDD also repressed ATP-citrate lyase (*Acly*), which converts citrate to oxaloacetate and acetyl-CoA (step 15). Acetyl CoA is critical for *de novo* FA synthesis by *Fasn* (step 17), which was dose-dependently repressed by TCDD, TCDF, and PCB126 [38].

These gene expression changes are consistent with TCDD-mediated inhibition of gluconeogenesis [39], *de novo* lipogenesis [40] and FA oxidation, and may be partially explained by AhR interactions with other signaling pathways involved in hepatic glucose and FA metabolism regulation including PPARs (Table 4.3), Pgc1α [37], Forkhead box O1 (Foxo1) [41] and hepatocyte nuclear factor 4, alpha (HNF4α) [19]. Evidence suggests AhR and PPAR signaling pathways interact [42-44] to alter PPAR expression [45]. Other studies identified overrepresentation of PPAR and HNF4α binding motifs in ChIP-chip regions of AhR enrichment that lack DRE cores suggesting AhR binding to DNA independent of DREs [6, 7]. For example, AhR interacts with chicken ovalbumin upstream promoter transcription factor (COUP-TF) [46], and COUP-TF is reported to antagonize HNF4α-mediated responses by binding to HNF4α response elements [47]. Consequently, AhR-COUP-TF complexes binding to HNF4α response elements may inhibit HNF4α-regulated lipid transport and metabolism gene

expression and contribute to TCDD-elicited steatosis [6]. Interestingly, hepatic steatosis has been reported in HNF4α null mice [48].

Collectively, our data indicate that TCDD-mediated hepatic steatosis involves enhanced uptake of dietary fat suggesting a novel endogenous role for the AhR. Other ligands including endogenous metabolites (indoles, tetrapyrroles, and arachidonic acid metabolites), and natural products (e.g. vegetable-, fruit-, and tea-derived indole and flavonoid metabolites) [49, 50] also activate the AhR providing a possible selective evolutionary advantage that optimizes fat absorption to maximize energy intake. Interactions with other nuclear receptors and transcription factors can further impact energy homeostasis and lipid metabolism, transport and deposition. However, persistent AhR activation in combination with the consumption of a high fat diet may also have adverse health implications for fatty liver and its associated diseases including NAFLD, metabolic syndrome and diabetes. Preliminary studies indicate AhR-activation increases TFA levels in human primary hepatocytes (data not shown), although further studies are needed to elucidate species-specific differences in AhR-mediated effects including steatosis.

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APPENDIX B

APPENDIX B

Table B.1. Terminal Body Weight, Body Weight Gain, Absolute Liver Weight, and Relative Liver Weight

Time (h)	% by weight	Treat-ment	Terminal Body Weight (g)	Body Weight Gain (g)	Liver Weight (g)	Relative Liver Weight
A. Fat Adjusted Diets						
24	5%	Vehicle	14.26 ± 0.71	0.84 ± 0.57	0.82 ± 0.10	0.058 ± 0.005
		TCDD	14.70 ± 0.78	1.08 ± 0.41	1.04 ± 0.07	0.071 ± 0.002*
	10%	Vehicle	14.68 ± 0.66	1.20 ± 0.43	0.89 ± 0.06	0.060 ± 0.002
		TCDD	14.68 ± 0.73	1.24 ± 0.22	0.99 ± 0.05	0.067 ± 0.002*
	15%	Vehicle	14.92 ± 0.41	1.04 ± 0.21	0.87 ± 0.06	0.058 ± 0.004
		TCDD	14.40 ± 0.60	0.70 ± 0.31	0.94 ± 0.05	0.065 ± 0.003*
168	5%	Vehicle	17.28 ± 0.87	3.50 ± 1.37	1.00 ± 0.09	0.058 ± 0.003
		TCDD	16.78 ± 1.13	3.30 ± 0.74	1.29 ± 0.11	0.077 ± 0.002*
	10%	Vehicle	15.88 ± 1.01	2.58 ± 0.89	0.84 ± 0.05	0.053 ± 0.001
		TCDD	15.72 ± 0.61	2.02 ± 0.82	1.05 ± 0.08	0.067 ± 0.003*
	15%	Vehicle	17.12 ± 0.90	3.56 ± 0.75	0.90 ± 0.06	0.052 ± 0.002
		TCDD	17.14 ± 0.75	3.46 ± 0.33	1.14 ± 0.07	0.067 ± 0.004*
B. Carbohydrate Adjusted Diets						
24	50%	Vehicle	16.02 ± 1.11	0.18 ± 0.13	0.86 ± 0.09	0.053 ± 0.004
		TCDD	15.92 ± 0.35	0.12 ± 0.16	0.94 ± 0.02	0.059 ± 0.001
	60%	Vehicle	16.22 ± 0.32	0.58 ± 0.37	0.89 ± 0.21	0.055 ± 0.012
		TCDD	15.80 ± 0.66	0.10 ± 0.19	0.94 ± 0.10	0.060 ± 0.004
	70%	Vehicle	15.90 ± 0.49	0.52 ± 0.16	0.96 ± 0.04	0.060 ± 0.004

Appendix B, Table 1 Cont'd

Time (h)	% by weight	Treat-ment	Terminal Body Weight (g)	Body Weight Gain (g)	Liver Weight (g)	Relative Liver Weight
		TCDD	15.94 ± 0.47	0.56 ± 0.27	1.09 ± 0.12	0.068 ± 0.007
168	50%	Vehicle	17.40 ± 0.60	1.96 ± 0.71	0.99 ± 0.08	0.057 ± 0.004
		TCDD	17.34 ± 0.64	1.88 ± 0.42	1.14 ± 0.05	0.066 ± 0.002*
	60%	Vehicle	18.14 ± 1.01	2.30 ± 0.64	1.04 ± 0.08	0.058 ± 0.003
		TCDD	17.06 ± 0.56	0.98 ± 0.64	1.14 ± 0.04	0.067 ± 0.001*
	70%	Vehicle	18.38 ± 0.46	2.50 ± 0.84	1.11 ± 0.11	0.060 ± 0.005
		TCDD	17.24 ± 0.51	1.54 ± 0.62	1.18 ± 0.12	0.068 ± 0.006

* $p<0.05$ for TCDD vs. Vehicle within a diet.

Table B.2. Hepatic Lipid Levels (μmol/g) in Fat Adjusted Diet-Fed Mice 168 h Post 30 μg/kg TCDD Dose

	Treat-ment	5% Fat	10% Fat	15% Fat
Saturated Fatty Acids				
Palmitic acid (16:0)	Vehicle	27.50 ± 3.63	21.62 ± 1.97†	17.59 ± 1.19†
	TCDD	33.79 ± 3.27*, **	29.79 ± 2.51*	24.94 ± 3.27*
Behenic acid (22:0)	Vehicle	0.26 ± 0.03	0.28 ± 0.03	0.30 ± 0.01
	TCDD	0.15 ± 0.01*	0.22 ± 0.02*	0.23 ± 0.01*
Monounsaturated Fatty Acids				
Palmitoleic acid (16:1n7)	Vehicle	5.69 ± 1.88	2.58 ± 0.31†	1.31 ± 0.36†
	TCDD	7.50 ± 1.54**	5.42 ± 1.20*	3.91 ± 1.02*
Oleic acid (18:1n9)	Vehicle	47.41 ± 10.19**	27.71 ± 4.37**	16.42 ± 2.52
	TCDD	81.90 ± 10.29*, **, ††	58.30 ± 9.52*, **	48.95 ± 6.10*
Eicosenoic acid (20:1n9)	Vehicle	0.61 ± 0.13	0.37 ± 0.08†	0.23 ± 0.05†
	TCDD	2.72 ± 0.34*, **	1.87 ± 0.35*, ††	1.66 ± 0.25*
Polyunsaturated Fatty Acids				
α-Linolenic acid (18:3n3)	Vehicle	0.88 ± 0.21	1.37 ± 0.12†	1.74 ± 0.19*
	TCDD	1.65 ± 0.19*, **, ††	3.53 ± 0.55*	4.48 ± 0.70*
Eicosatrienoic acid (20:3n3)	Vehicle	0.04 ± 0.01	0.06 ± 0.01†	0.07 ± 0.01†
	TCDD	0.25 ± 0.03*, **, ††	0.53 ± 0.07*	0.65 ± 0.07*
Eicosatetraenoic acid (20:4n3)	Vehicle	0.06 ± 0.02	0.08 ± 0.02	0.09 ± 0.01
	TCDD	0.17 ± 0.03**, ††	0.42 ± 0.03*	0.61 ± 0.16*
Timnodonic acid (20:5n3)	Vehicle	0.40 ± 0.04	0.08 ± 0.02	0.40 ± 0.03
	TCDD	0.30 ± 0.03*, ††	0.42 ± 0.02*	0.76 ± 0.15*
Docosapentaenoic acid (22:5n3)	Vehicle	0.24 ± 0.03	0.41 ± 0.02	0.31 ± 0.06
	TCDD	0.68 ± 0.04*, **, ††	0.60 ± 0.02*	1.49 ± 0.33*

Table B.2 (cont'd)

	Treat-ment	5% Fat	10% Fat	15% Fat
Linoleic acid (18:2n6)	Vehicle	18.21 ± 2.46	25.19 ± 1.80†	26.55 ± 2.25†
	TCDD	30.50 ± 1.90*, **, ††	49.83 ± 4.24*, **	58.89 ± 5.18*
Eicosadienoic acid (20:2n6)	Vehicle	0.45 ± 0.02	0.49 ± 0.05†	0.49 ± 0.04
	TCDD	1.43 ± 0.1*, **, ††	2.42 ± 0.28*, **	2.94 ± 0.18*
Dihomo-ϒ-linolenic acid (20:3n6)	Vehicle	1.33 ± 0.02	1.08 ± 0.14	0.98 ± 0.08
	TCDD	2.24 ± 0.1*, **, ††	3.26 ± 0.10*, **	3.98 ± 0.48*
Arachidonic acid (20:4n6)	Vehicle	11.29 ± 0.39	11.27 ± 0.75	10.59 ± 0.75
	TCDD	9.71 ± 1.22*, ††	11.61 ± 0.71	10.62 ± 0.11

The following fatty acids were detected, but not significantly altered by TCDD: adrenic acid (22:4n6), nervonic acid (24:1n9), stearic acid (18:0), and behenic acid (22:0). Data are reported as μg fatty acid methyl ester/g liver tissue. N=5, * $p<0.05$ for TCDD vs. Vehicle within a diet, ** $p<0.05$ for TCDD 15% fat vs. TCDD 5% or 10%, † $p<0.05$ for Vehicle 5% fat vs. Vehicle 10% or 15%, †† $p<0.05$ for TCDD 5% fat vs. TCDD 10%.

Table B.3. Lipid Composition (μmol) in Fat Adjusted (A) and Carbohydrate Adjusted (B) Diets

A. Fat Diets	**% Fat**		
	5%	**10%**	**15%**
Saturated Fatty Acids			
Palmitic Acid (16:0)	23.91 ± 3.01	43.92 ± 4.44	59.18 ± 9.10
Stearic Acid (18:0)	9.18 ± 1.09	18.39 ± 1.71	26.34 ± 4.08
Arachidic Acid (20:0)	0.54 ± 0.06	1.72 ± 0.12	1.72 ± 0.26
Behenic Acid (22:0)	0.50 ± 0.05	1.05 ± 0.11	1.59 ± 0.24
Lignoceric Acid (24:0)	0.11 ± 0.02	0.23 ± 0.03	0.36 ± 0.06
Monounsaturated Fatty Acids			
Palmitoleic Acid (16:1)	0.19 ± 0.02	0.33 ± 0.04	0.48 ± 0.08
Oleic Acid (18:1)	39.17 ± 4.76	80.08 ± 6.81	113.47 ± 16.74
Gondoic Acid (20:1)	0.24 ± 0.03	0.51 ± 0.06	0.76 ± 0.12
Polyunsaturated Fatty Acids			
Linoleic Acid (18:2n6)	94.51 ± 11.47	180.39 ± 16.52	248.93 ± 37.16
α-Linolenic Acid (18:3n3)	16.95 ± 2.10	47.74 ± 3.00	47.74 ± 7.10

Table B.3 (cont'd)

B. Carbohydrate Diets	% Carbohydrate		
	50%	60%	70%
Saturated Fatty Acids			
Palmitic Acid (16:0)	28.33 ± 2.48	31.47 ± 1.42	26.01 ± 5.73
Stearic Acid (18:0)	12.25 ± 0.95	11.47 ± 0.1	11.47 ± 1.91
Arachidic Acid (20:0)	0.81 ± 0.06	0.76 ± 0.04	0.76 ± 0.09
Behenic Acid (22:0)	0.78 ± 0.05	0.75 ± 0.08	0.75 ± 0.06
Lignoceric Acid (24:0)	0.22 ± 0.03	0.19 ± 0.02	0.19 ± 0.03
Monounsaturated Fatty Acids			
Palmitoleic Acid (16:1)	0.25 ± 0.03	0.27 ± 0.00	0.23 ± 0.04
Oleic Acid (18:1)	53.01 ± 4.22	56.91 ± 1.41	46.48 ± 1.82
Gondoic Acid (20:1)	0.34 ± 0.03	0.33 ± 0.02	0.33 ± 0.04
Polyunsaturated Fatty Acids			
Linoleic Acid (18:2n6)	115.13 ± 9.59	126.43 ± 2.75	106.73 ± 20.02
α-Linolenic Acid (18:3n3)	19.86 ± 1.63	21.81 ± 0.23	18.54 ± 3.44

[1]GC-MS-FAMES hepatic lipid profiling was performed as described in the materials and methods from 100 mg ground rodent diet.

Table B.4. Hepatic Lipid Levels (μmol/g) in Carbohydrate Adjusted Diet-Fed Mice 168 h Post 30 μg/kg TCDD Dose

	Treatment	50% Carbohydrate	60% Carbohydrate	70% Carbohydrate
Saturated Fatty Acids				
Palmitic acid (16:0)	Vehicle	22.64 ± 11.18	24.46 ± 1.86	26.92 ± 3.21
	TCDD	26.67 ± 1.04**	25.83 ± 3.67**	35.72 ± 7.18*
Stearic acid (18:0)	Vehicle	14.29 ± 1.63	14.69 ± 1.10	15.04 ± 1.20
	TCDD	17.05 ± 1.27	15.97 ± 1.49	19.14 ± 2.24*
Lignoceric acid (24:0)	Vehicle	7.80 ± 0.90	7.18 ± 0.89	7.32 ± 1.10
	TCDD	10.20 ± 0.59*	8.86 ± 1.10	9.34 ± 0.85*
Monounsaturated Fatty Acids				
Palmitoleic acid (16:1n7)	Vehicle	2.72 ± 1.16	2.71 ± 0.42	4.00 ± 0.54
	TCDD	4.26 ± 0.62**	4.03 ± 1.23*, **	6.72 ± 2.15*
Oleic acid (18:1n9)	Vehicle	24.80 ± 10.31	27.40 ± 2.37	45.80 ± 5.50
	TCDD	44.68 ± 4.71**	45.51 ± 11.31**	70.95 ± 23.23*
Eicosenoic acid (20:1n9)	Vehicle	0.75 ± 0.16	0.43 ± 0.05	0.89 ± 0.20
	TCDD	1.18 ± 0.17*	1.24 ± 0.30*	1.67 ± 0.65*
Polyunsaturated Fatty Acids				
α-Linolenic acid (18:3n3)	Vehicle	0.74 ± 0.32	0.74 ± 0.74	0.75 ± 0.11
	TCDD	1.26 ± 0.14*	1.10 ± 1.10	1.28 ± 0.26*
Eicosatrienoic acid (20:3n3)	Vehicle	0.05 ± 0.02	0.05 ± 0.05	0.05 ± 0.01
	TCDD	0.18 ± 0.03*	0.16 ± 0.16*	0.13 ± 0.02*

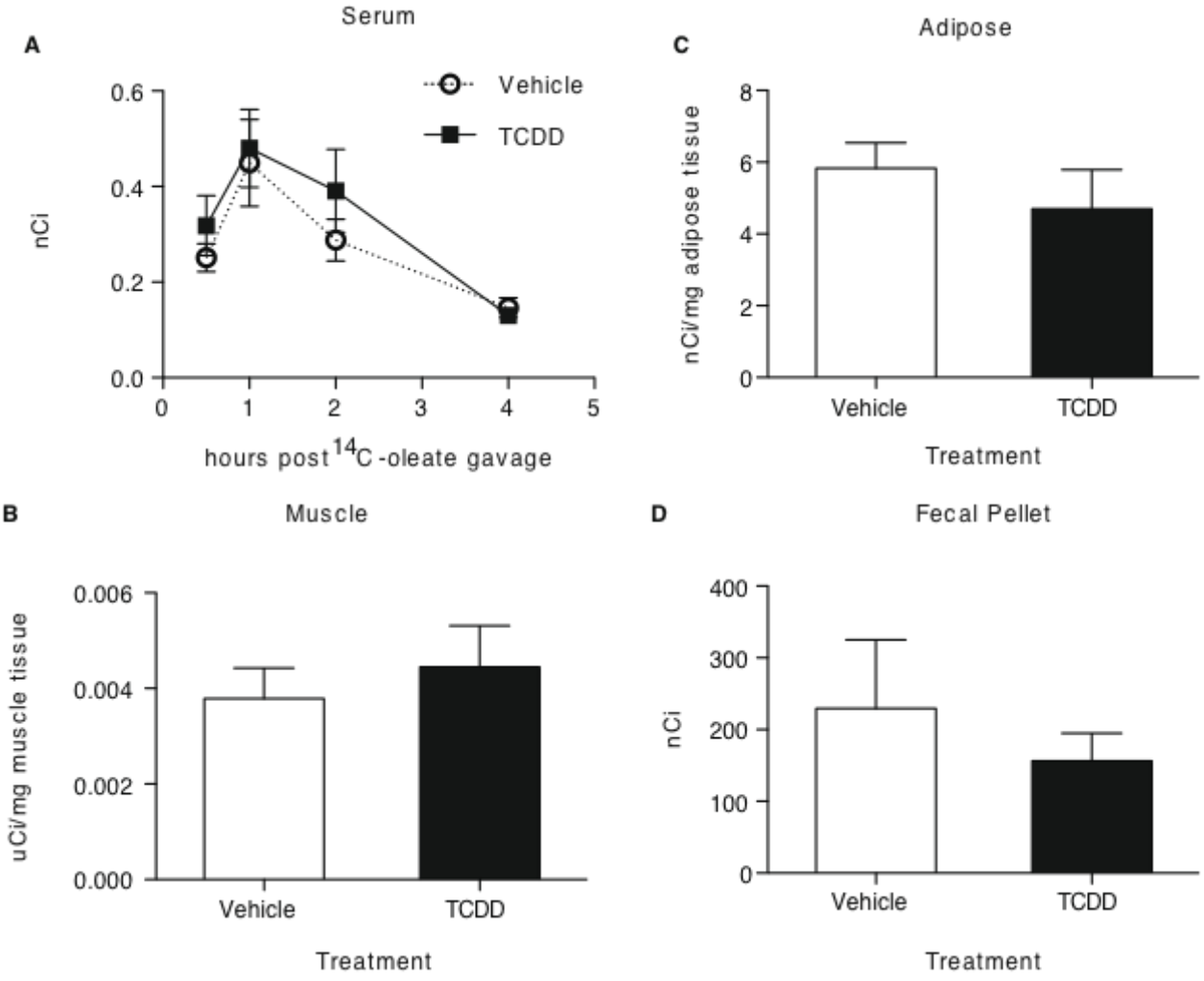
Table B.4 (cont'd)

	Treatment	50% Carbohydrate	60% Carbohydrate	70% Carbohydrate
Timnodonic acid (20:5n3)	Vehicle	0.42 ± 0.12*	0.33 ± 0.33*	0.34 ± 0.02
	TCDD	0.46 ± 0.03	0.36 ± 0.36	0.42 ± 0.06*
Linoleic acid (18:2n6)	Vehicle	17.76 ± 4.13	17.79 ± 17.79	18.07 ± 0.97
	TCDD	28.55 ± 2.18*	25.47 ± 25.47*	27.06 ± 3.28*
Eicosadienoic acid (20:2n6)	Vehicle	0.48 ± 0.09	0.48 ± 0.48	0.59 ± 0.05
	TCDD	1.11 ± 0.13*	1.01 ± 1.01*	0.94 ± 0.14*
Dihomo-ϒ-linolenic acid (20:3n6)	Vehicle	1.23 ± 0.21	1.26 ± 1.26	1.49 ± 0.13
	TCDD	2.46 ± 0.13*	2.32 ± 2.32*	2.43 ± 0.36*
Arachidonic Acid (20:4n6)	Vehicle	14.03 ± 1.30	13.97 ± 13.97	13.14 ± 1.52
	TCDD	16.71 ± 0.73*	14.88 ± 14.88	15.40 ± 1.42

The following fatty acids were detected, but not significantly altered by TCDD: adrenic acid (22:4n6), nervonic acid (24:1n9), behenic acid (22:0), docosapentaenoic acid (22:5n3), and eicosatetraenoic acid (20:4n3). Data are reported as μg fatty acid methyl ester/g liver tissue. N=5, * $p<0.05$ for TCDD vs. Vehicle within a diet, ** $p<0.05$ for TCDD 70% carbohydrate vs. TCDD 50% or 60% carbohydrate.

Figure B.1. ^{14}C levels in Scd1 wild-type (wt) and null mice gavaged with 30 μg/kg TCDD for 120 h (A-C) or 168 h (D).

^{14}C levels were measured from (**A**) serum, (**B**) muscle, and (**C**) adipose 4 h after gavage with 2 μCi ^{14}C-oleate. (**D**) Fecal ^{14}C levels were measured from animals gavaged with 2 μCi ^{14}C-oleate 120 h post TCDD dose. Total ^{14}C levels were measured from all fecal matter excreted at ^{14}C-oleate gavage until 48 h post ^{14}C-oleate gavage. (**A**-**D**) Bars represent mean ± SEM, n=5.



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CHAPTER 5

Angrish MM, Dominici CY, Jones AD, Zacharewski TR: **2,3,7,8-Tetrachlorodibenzo-ρ-dioxin (TCDD)-Elicited Effects on Liver, Serum, and Adipose Lipid Composition in C57BL/6 Mice.**

CHAPTER 5

TCDD-ELICITED EFFECTS ON LIVER, SERUM, AND ADIPOSE LIPID COMPOSITION IN C57BL/6 MICE

ABSTRACT

The AhR mediates alterations in hepatic lipid composition elicited by TCDD. In order to further investigate the effects of TCDD, liver, serum and parametrial fat pad (PFP) FAMEs and lipids were examined in fasted 4-week-old female mice orally gavaged with 30 µg/kg TCDD and sacrificed at 24, 72, and 168 h. Mean (µmol/g) hepatic FAME levels (Vehicle/TCDD: 236.7 compared with 392.2) increased, but did not change in PFP or serum. Hepatic levels of SFAs (16:0, 18:0, 20:0, and 22:0) decreased, while TCDD increased MUFAs (16:1n7, 18:1n9, and 20:1n9) consistent with AhR-mediated induction of Scd1. In addition, TCDD induced levels of selected PUFAs (20:2n6, 20:3n6, 18:3n3, and 22:5n3) while others (20:4n6, 22:6n3) decreased. There were modest increases in PFP 20:2n6 and 20:3n6 levels, while 18:0 decreased and 18:1n9 increased in serum. TCDD also lowered total CHOL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) by 25% in serum. Decreases in serum CHOL, LDL, and HDL levels were consistent with the differential expression of hepatic CHOL metabolism and transport genes *Hmgcs1* (-2.1-fold), *Hmgcr* (-2.3-fold), *Apoa1* (-1.7-fold), *Lcat* (2.0-fold), and *Ldlr* (3.6-fold). Moreover, TCDD decreased serum Apob100 (4.4-fold) and Apob48 (2.2-fold) levels, consistent with serum lipid clearance and decreased hepatic efflux. Collectively, these results suggest TCDD-elicited, AhR-mediated decreases in circulating lipid levels are consistent with the induction of hepatic steatosis.

INTRODUCTION

TCDD and similar chemicals bioaccumulate in food supplies, mainly animal fats, and elicit adverse hepatotoxic effects in animals and humans that are mediated by the AhR. The AhR is a ligand activated PAS transcription factor family member [1] that binds structurally diverse environmental contaminants (halogenated aromatic hydrocarbons and non-halogenated polycyclic aromatic hydrocarbons) with high affinity [2]. Although the AhR also binds endogenous endobiotics (indoles, tetrapyrroles, and arachidonic acid metabolites), and natural products (vegetable-, fruit-, and tea-derived indoles and flavonoid metabolites) [3, 4], no known endogenous high affinity ligand has been identified. The classical AhR pathway involves ligand activation, translocation to the nucleus, and heterodimerization with ARNT [5, 6]. The AhR:ARNT heterodimer binds DREs in the promoters of target genes and recruits transcriptional co-activators to positively regulate gene transcription [7, 8].

TCDD-elicited, AhR-mediated disruption of hepatic energy balance results in steatosis accompanied by increased triglycerides, inflammation and the differential expression of hepatic genes involved in lipid metabolism [9-11]. These gene expression changes include induction of hepatic lipid transporters *Cd36* [12], *Vldlr*, *Ldlr*, *Fabp1*, and *Slc27a3-4* concurrent with decreased *Fasn* expression [13, 14] and enzyme activity [15]. Diet and ^{14}C-oleate studies strongly suggest dietary fat as a lipid source in TCDD-elicited steatosis [16] and, collectively with gene expression data, imply hepatic lipid accumulation is due to TCDD enhanced hepatic uptake of dietary lipid rather than *de novo* synthesis. It is also believed that adipose lipolysis can contribute to ectopic fat accumulation, including hepatic steatosis. However, data regarding the importance of adipose lipolysis in the promotion of TCDD-elicited hepatic steatosis are lacking

since conclusions were based on serum FA measurements [17-19]. No experiments have reported TCDD effects on adipose tissue FA composition.

Excessive hepatic lipid accumulation frequently results in non-alcoholic steatohepatitis (NASH), a widespread condition linked to insulin resistance [20] and dyslipidemia that is characterized by altered serum lipid profiles [21]. Animal and human epidemiological studies collectively provide conflicting data relating serum lipid levels to TCDD exposure, even from the same lab [22, 23]. However hepatic steatosis and insulin resistance are conditions associated with TCDD exposure in humans [9, 10, 24, 25]. Considering that the liver partitions lipids for hepatocellular storage as well as storage in peripheral adipose tissue via the circulatory system [26], it is appreciated that TCDD effects on hepatic lipids are complex. Therefore, TCDD alterations in FA composition in the liver, PFP, and serum of fasted mice were investigated. Furthermore, serum lipid panels, which are routine tests used to identify alterations in metabolism, were also examined. Collectively, the reported changes in systemic lipid profiles provide insight into hepatic lipid metabolism with potential implications for novel biomarkers of TCDD-elicited hepatotoxicity and steatosis.

MATERIALS AND METHODS

ANIMAL HUSBANDRY AND IN VIVO TREATMENT

Heterozygous B6.129-*Scd1*^{tm1Myz}/J mice (Jackson Laboratory, Ben Harbor, Maine) were bred, genotyped and weaned as previously described [27]. On PND 28 mice (n=5) were gavaged with 0.1 ml of sesame oil (vehicle control) or 30 μg/kg TCDD (Dow Chemical Company, Midland, MI). Wild-type, immature mice were used to facilitate comparisons with other data sets and to minimize potential interactions with estrogens produced by developing ovaries. The dose

was chosen to elicit moderate hepatic effects while avoiding overt toxicity. Animals were fasted 4 h prior to sacrifice at 24, 72, and 168 h post-dose. Mice were weighed and blood was collected via submandibular vein puncture before sacrifice. Liver and PFP tissue were weighed and samples flash frozen. All procedures were carried out with Michigan State University Institutional Animal Care and Use Committee approval.

Serum Lipid Panels

Serum total CHOL, free CHOL, LDL, high density HDL, FFA, and TAG were measured according to the manufacturer's microtiter protocol (CHOL E; Free CHOL E, L-Type LDL, L-Type HDL, NEFA-HR(2), L-Type TG M, Wako Diagnostics).

GC-MS Fatty Acid Methyl Ester (FAMES) Lipid Profiling

Lipid analysis was performed as previously described [27]. Lipids extracted from liver (~100 mg), PFP (~30 mg), and serum (50 μL) were separated and analyzed with an Agilent 6890N GC with a 30mDB23 column interfaced to an Agilent 5973 MS. 19:1n9 FFA and 19:0 TAG were added as extraction efficiency controls and 17:1n1 FAME (Nu-chek, Elysian, MN) was spiked in as a loading control. GC-MS data files were converted to Waters MassLynx file format, analyzed with MassLynx software and reported as μmol/g liver or adipose tissue and nmol/mL serum. FA levels are based on peak areas from total ion chromatograms, and μmol is obtained from a linear calculation of a calibration curve and normalized to sample weight (liver and PFP) or volume (serum). Mol% is the ratio of a particular FA relative to TFA.

Western Blot

Apolipoprotein B (ApoB) Western blot was performed from serum. Briefly, serum was diluted 1:5 in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA). Diluted serum was separated in 5% SDS-PAGE and transferred to a

PVDF membrane (Millipore Billerica, MA). ApoB protein was immunoblotted with an ApoB antibody (SC-11795; Santa Cruz Biotechnology Santa Cruz, CA). Immunoreactive bands were visualized by chemiluminescence with the Pierce ECL Western blotting substrate (Thermo Scientific Rockford, IL) and quantified by densitometry (ImageJ).

RNA ISOLATION

RNA was isolated from frozen liver and PFP samples with ~1.0 mL TRIzol (Invitrogen) according to the manufacturer's protocol and an additional acid phenol:chloroform extraction as previously described [13]. Total RNA was resuspended in RNA storage solution, quantified by spectrophotometery at A_{260} and quality assessed by gel electrophoresis.

QRTPCR

Quantitative real-time PCR (QRTPCR) was performed as previously described [13]. The copy number of each sample was standardized to the geometric mean of *Gapdh*, *Hprt*, and *Actb* to control for differences in RNA loading, quality, and cDNA synthesis [28]. Data are reported as the fold change of standardized treated over standardized vehicle.

STATISTICAL ANALYSIS

Data were analyzed by ANOVA followed by Tukey's *post hoc* test in SAS 9.2 (SAS Institute, Cary, NC), unless otherwise stated. Differences between treatment groups were considered significant when $p<0.05$.

RESULTS

TCDD EFFECTS ON BODY, LIVER, AND ADIPOSE WEIGHTS

Fasted mice gavaged with 30 μg/kg TCDD had increased absolute and relative (whole liver weight normalized to whole body weight at sacrifice) liver weights at 72 and 168 h

Table 5.1. Effect of TCDD on Body, Liver, and Parametrial Fat Pad (PFP) Weights 24, 72, and 168 h Post-Dose

Time (h)	Treatment	Body Weight (g)	Body Weight Gain (g)	Absolute Liver Weight (g)	Relative Liver Weight	Absolute PFP Weight (g)	Relative PFP Weight
24	Vehicle	12.1 ± 1.9	0.5 ± 0.6	0.74 ± 0.103	0.061 ± 0.002	0.04 ± 0.02	0.0035 ± 0.0011
	TCDD	12.8 ± 0.9	-0.2 ± 0.8	0.80 ± 0.095	0.063 ± 0.007	0.05 ± 0.01	0.0040 ± 0.0009
72	Vehicle	14.5 ± 0.9	1.6 ± 0.2	0.79 ± 0.051	0.055 ± 0.001	0.08 ± 0.02	0.0055 ± 0.0013
	TCDD	14.0 ± 0.9	1.4 ± 0.1	0.94 ± 0.074*	0.067 ± 0.003*	0.06 ± 0.03	0.0039 ± 0.0019
168	Vehicle	15.3 ± 0.7	3.3 ± 1.1	0.85 ± 0.070	0.055 ± 0.002	0.06 ± 0.01	0.0041 ± 0.0006
	TCDD	15.2 ± 0.6	3.4 ± 1.0	1.04 ± 0.060*	0.068 ± 0.002*	0.07 ± 0.02	0.0044 ± 0.0010

* $p<0.05$ for TCDD compared with Vehicle.

post-dose (Table 5.1). There were no significant alterations in body weight, body weight gain, or adipose weight throughout the study suggesting treatment had no effect on food consumption.

TOTAL LIVER, ADIPOSE, AND SERUM LIPID CONTENT

We have previously reported TCDD- and *Scd1* genotype-dependent alterations on hepatic lipid composition in fed mice [27]. Similar to the livers of fed TCDD treated mice, analysis of FA composition by GC-MS in fasted mice identified increased mean (μmol/g) TFAs (1.7-fold), SFAs (1.25-fold), MUFAs (3.1-fold), and PUFAs (1.5-fold) (Table 5.2). In contrast to liver, TFA levels were unaffected in adipose and serum.

FAME LEVELS IN LIVER, PFP AND SERUM

As reported in fed mice, TCDD decreased hepatic SFA levels relative to vehicle (Figure 5.1A). Palmitate (16:0) and stearate (18:0), precursors for long chain FA synthesis and the Scd1 desaturation reaction, were significantly depleted. However, palmitate (16:1n7), oleate (18:1n9), and eicosanoic acid (20:1n9), downstream products of SFA desaturation and elongation, increased with treatment.

The n-3 and n-6 PUFAs are derived from the diet or via modification of the essential FAs linoleic (18:2n6) and α-linolenic (18:3n3) acids [29]. Both n-3 and n-6 pathway intermediates exhibited a mixed response to TCDD (Figure 5.1A). Specifically, 18:2n6 levels were not altered, while levels of its derivatives, eicosadienoic acid (20:2n6) and dihomo-γ-linolenic acid (20:3n6), were increased. Arachidonic acid (AA, 20:4n6), another downstream product of linoleic acid that can be further metabolized into bioactive products promoting pro-inflammatory conditions [30], exhibited a significant decrease in TCDD-treated mice.

N-3 derivatives are highly regarded for their health benefits, including improved insulin

Table 5.2. Liver, Parametrial Fat Pad, and Serum Total Lipid Content

	Treatment	Liver (µmol/g)	PFP (µmol/g)	Serum (nmol/mL)
Total FA	**Vehicle**	237 ± 26	3408 ± 1280.9	413 ± 104
	TCDD	392 ± 58*	3928 ± 945.2	411 ± 100
Total SFA	**Vehicle**	89 ± 6.8	1251 ± 435.3	147 ± 37
	TCDD	113 ± 10*	1476 ± 413.2	137 ± 39
Total MUFA	**Vehicle**	34 ± 4.2	901 ± 338.2	63 ± 17
	TCDD	106 ± 22*	1033 ± 214.6	74 ± 19
Total PUFA	**Vehicle**	114 ± 15	1257 ± 513.0	204 ± 53
	TCDD	173 ± 28*	1420 ± 323.6	200 ± 47
20:4n6/18:2n6	**Vehicle**	0.8 ± 0.1*	0.022 ± 0.010	0.24 ± 0.04
	TCDD	0.5 ± 0.1	0.027 ± 0.004	0.21 ± 0.05
20:5n3/18:3n3	**Vehicle**	1.0 ± 0.03*	nd[1]	nd[2]
	TCDD	0.4 ± 0.1	nd[1]	nd[2]

* $p<0.05$ for TCDD compared with Vehicle; nd, not detected. [1]20:5n3 was not detected in PFP. [2]20:5n3 and 18:3n3 were not detected in serum.

resistance and plasma lipid profiles [30]. The precursor for all n-3 FAs, α-linolenic acid (18:3n3), was increased by TCDD relative to vehicles (Figure 5.1A). The n-3 intermediate timnodonic acid (20:5n3) was decreased, while docosapentaenoic acid (22:5n3) was induced. Docosahexaenoic acid (DHA, 22:6n3), required for proper infant growth and neurodevelopment [29], was decreased by TCDD.

Increased 20:2n6, 20:3n6, and 22:5n3 were consistent with *Elovl5* induction [27]. Although lower 20:4n6 and 20:5n3 levels suggest decreased Δ5 desaturase activity [31], Δ5 destaturase mRNA levels were unaffected by TCDD (data not shown). Therefore, decreased n-3 and n-6 pathway product/precursor ratios (20:5n3/18:3n3, decreased 57% and 20:4n6/18:2n6, decreased 40%, Figure 5.2) are the possible result of TCDD-mediated metabolic PUFA conversions into anti- and pro-inflammatory signaling molecules [32].

TCDD had minimal effects on PFP FAME levels with no alterations in SFAs or MUFAs (Figure 5.1B). Only 20:2n6 and 20:3n6 PUFAs were increased by treatment. In serum, 16:0, 18:0, 18:1n9, 18:2n6, and 20:4n6 (Figure 5.1C) were detected. These FAs are the predominant species in Harlan Teklad diet F6 rodent diet 7964 as well as lipoprotein glycerolipids, cholesteryl esters, and phospholipids [33]. The SFA 18:0 was decreased, while the MUFA 18:1n9 increased. No alterations in serum PUFAs were detected.

TCDD INDUCTION OF THE SCD1 DESATURATION INDEX

We previously reported AhR-mediated increases in Scd1 activity [27]. The 18:1n9/18:0 ratio is an indirect measure of Scd1 activity, and its increase is associated with hyperlipidemia in humans [34]. The Scd1 activity index was induced 2.9- and 1.4-fold in TCDD compared with vehicle in liver and serum, respectively (Figure 5.2). The adipose Scd1 activity index was not affected by treatment.

Figure 5.1. Total fatty acid composition in liver (A), parametrial fat pad (PFP, B), and serum (C) of mice 168 h post 30 µg/kg TCDD or sesame oil vehicle dose.

Total fatty acids were extracted by Folch method and quantitated by GC-MS as described in the materials and methods. * $p<0.05$ for TCDD compared with vehicle. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Bars represent mean + standard error of the mean (SEM). Liver, n=4; adipose and serum, n=5.

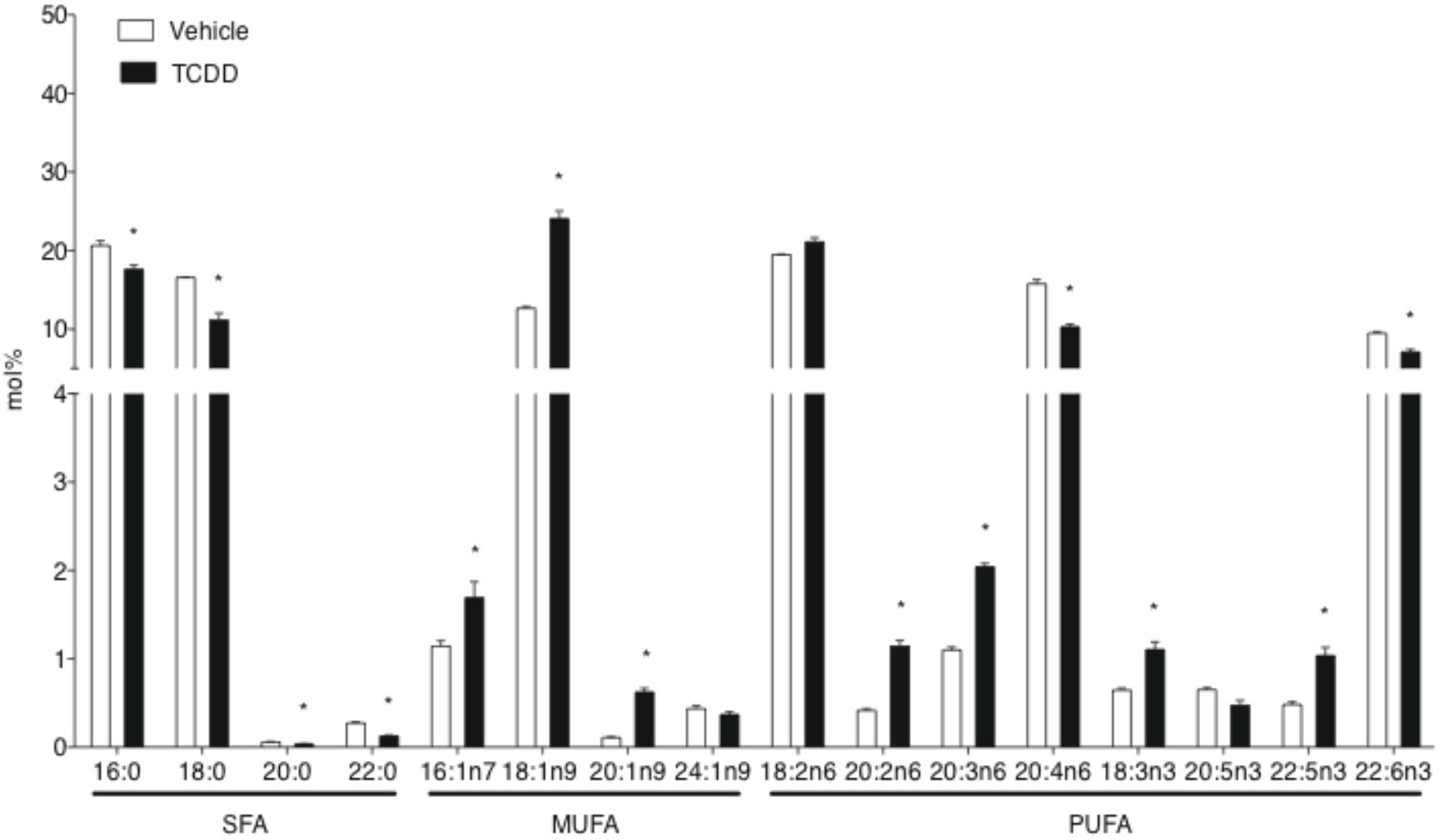
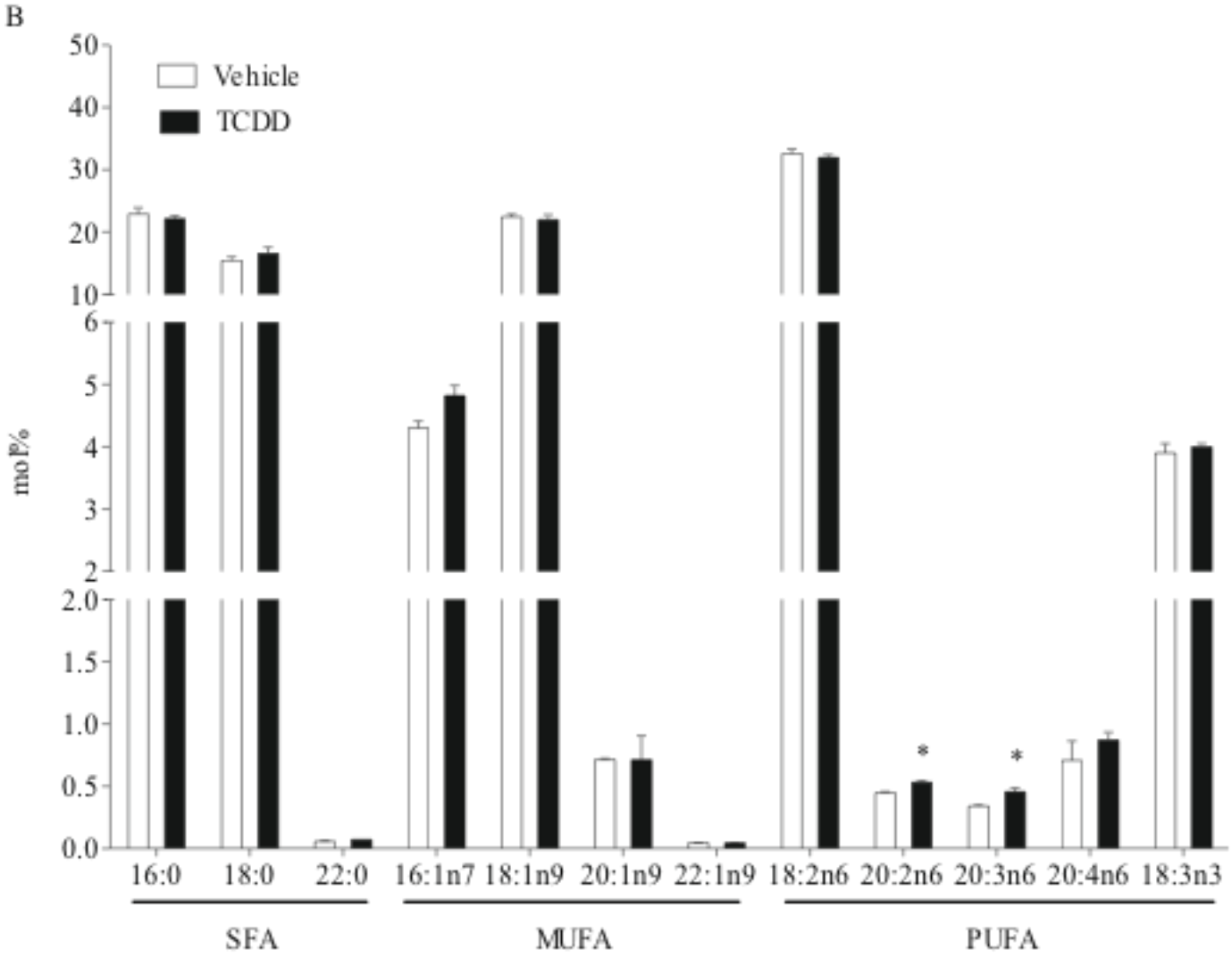


Figure 5.1 (cont'd)



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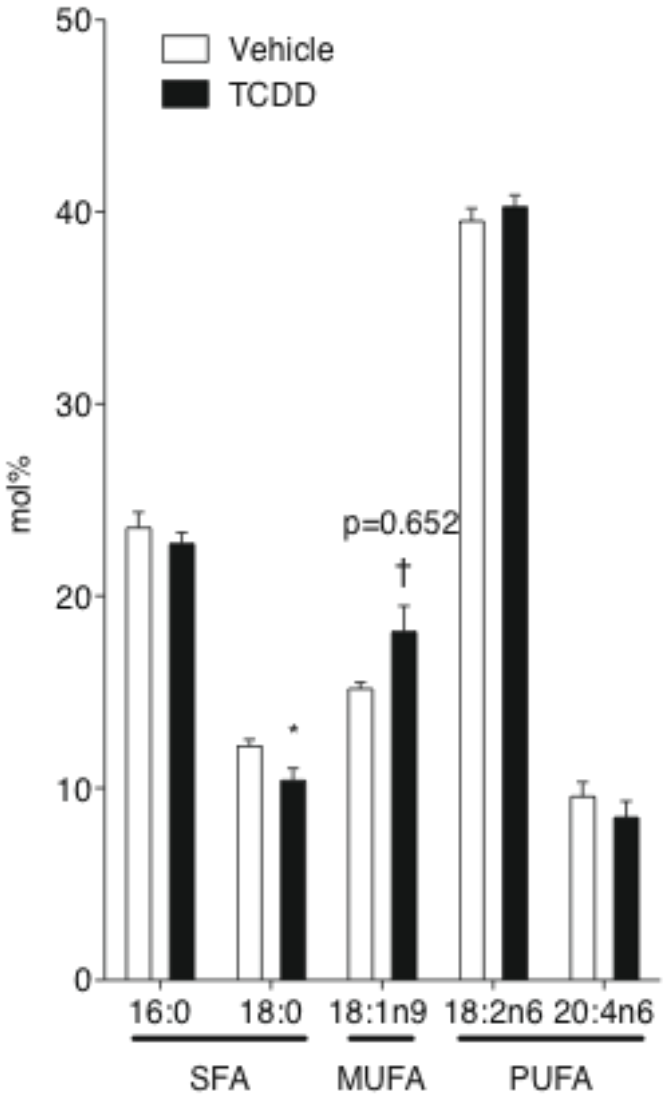
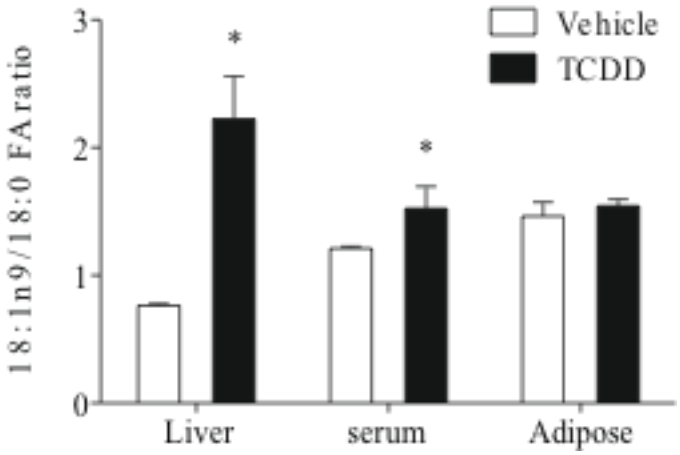


Figure 5.2. The 18:1n9/18:0 desaturation index in liver, serum, and parametrial fat pad (PFP) tissue of mice treated with 30 µg/kg TCDD or sesame oil vehicle 168 h post-dose.
The desaturation index is the ratio of oleic acid (18:1n9) to the precursors stearic acids (18:0) and an indirect measure of Scd1 activity. * $p<0.05$ for TCDD compared with vehicle. Bars represent mean + SEM, n=5.



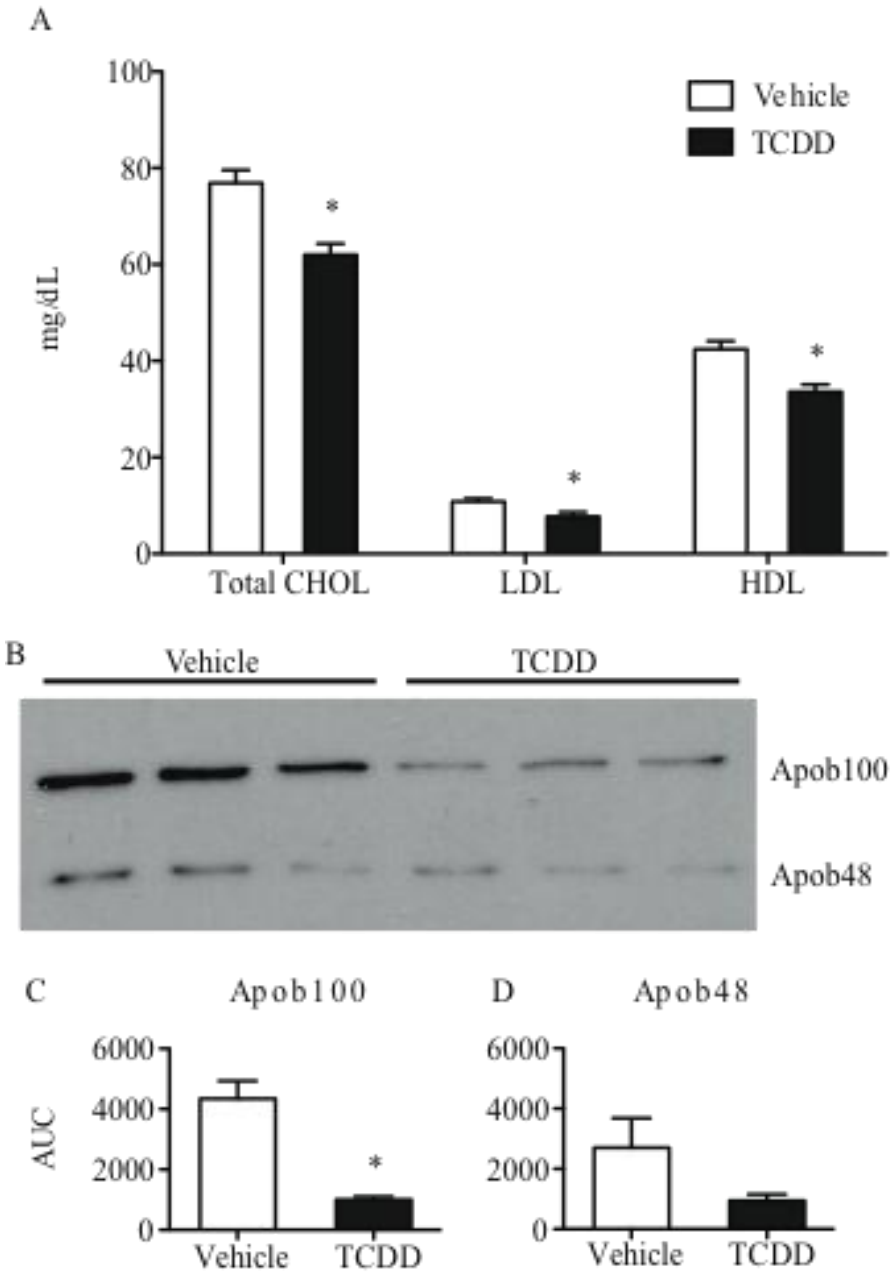
ALTERATIONS IN SERUM LIPIDS

A 20% decrease in total CHOL, a measure of free CHOL (unchanged by TCDD, data not shown), LDL CHOL (decreased 30%), and HDL CHOL (decreased 20%) was detected in TCDD mice compared with vehicles (Figure 5.3A). Decreased LDL, a modification product of VLDL [33], suggests decreased VLDL secretion that was further supported by a 4.3-fold decrease in serum Apob100 protein levels (Figure 5.3B-C). Apob100 is the major non-exchangeable lipoprotein found in VLDL and LDL particles produced by the liver. Apob48 is a truncated form of Apob100 and the major scaffold protein of chylomicrons produced exclusively by the small intestine of mammals and also the livers of mice [33]. TCDD decreased serum Apob48 protein levels 2.8-fold compared to vehicles (Figure 5.3B & D). Serum free FA, free CHOL, and triglycerides (TAG) were not affected by treatment in fasted mice (data not shown).

DIFFERENTIAL HEPATIC AND ADIPOSE GENE EXPRESSION BY TCDD

To further examine the effect of TCDD on lipid metabolism, QRTPCR was performed from liver and adipose mRNA extracts. Decreases in serum CHOL levels suggested TCDD-elicited alterations in hepatic CHOL biosynthesis. QRTPCR analysis identified a 2.1- and 2.3-fold decrease in hepatic *Hmgcs1* and *Hmgcr* (Figure 5.4A-B) whose gene products catalyze the initial condensation and reduction reactions, respectively, in the cholesterol biosynthetic pathway [33]. Apolipoprotein A1 (Apoa1) is the primary HDL apolipoprotein expressed in the liver, and to a lesser extent in the intestine. Apoa1 is also a cofactor for lecithin cholesterolacyltransferase (Lcat), an enzyme that produces the bulk of plasma CHOL esters. In the liver, TCDD decreased *Apoa1* 1.7-fold, while *Lcat* was increased 2-fold (Figure 5.4C-D). The lipoprotein transporter *Ldlr*, involved in lipoprotein endocytosis and reverse CHOL transport, was increased 3.6-fold by TCDD (Figure 5.4E). Increased *Lcat* and *Ldlr* expression are consistent with TCDD-mediated

Figure 5.3. Serum cholesterol and apolipoprotein b (Apob) 100 and Apob48 levels in Scd1 wild-type mice treated with 30 µg/kg TCDD or sesame oil vehicle.
(A) Total cholesterol (CHOL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were decreased 25% by TCDD at 168 h post-dose. Serum lipids were measured by commercial assay (WAKO Diagnostics) (n=8) and are presented as mg/dL. (**B**) Apob100 and Apob48 protein levels were detected by Western blot at 72 h from serum diluted 1:5 (n=3). (**C-D**) Densitometry (determined with ImageJ) identified a TCDD-dependent 4.4- and 2.2-fold decrease in Apob100 (**C**) and Apob48 (**D**) bands, respectively. * $p<0.05$ for TCDD compared with Vehicle. Bars represent mean + SEM.



increases in hepatic CHOL levels, while decreased CHOL synthesis gene expression suggests feedback inhibition.

In addition to the marginal changes in PFP lipid composition, TCDD altered the expression of several genes involved in FA metabolism and transport. For example, *Slc27a1*, a fatty acid transporter that translocates to the plasma membrane in response to insulin [35], was induced 7.5-fold, and triglyceride lipase *Pnpla3* was repressed 2.4-fold (Figure 5.4F-G). Interestingly, TCDD induced *Adipoq* 1.6-fold, whose gene product adiponectin exhibits hormonal regulation of lipid and glucose metabolism (Figure 5.4H). Furthermore, TCDD induced the prototypical TCDD-inducible gene *Cyp1a1* mRNA levels (Figure 5.4I), indicating adipose tissue is responsive to TCDD treatment. TCDD also induced *Scd2*, the predominant adipose Δ9 desaturase, 2.1-fold, consistent with TCDD-dependent AhR-mediated regulation of Δ9 desaturase activity (Figure 5.4J) [27].

DISCUSSION

The liver's role in lipid storage and lipid partitioning is a fundamental process regulating whole body energy metabolism [36]. Altered hepatic and systemic lipid homeostasis is a pathophysiological sign of liver disease [21, 37] that is also observed after TCDD exposure. By examining lipogenic tissue FA levels and composition along with serum lipid panels, the current study provides direct insight into the effects of acute TCDD exposure on the state of whole-body FA and CHOL metabolism, with implications for AhR-mediated hepatic steatosis.

The current study corroborated existing evidence that TCDD increases hepatic lipid accumulation and alters hepatic FA composition [27, 38, 39]. Specifically, TCDD increased MUFA levels, particularly 18:1n9 consistent with AhR mediated induction in Scd1 activity [27].

Figure 5.4. Differential expression of hepatic genes involved in cholesterol metabolism (A-E) and parametrial fat pad genes involved in lipid metabolism and transport (F-J) in mice gavaged with 30 μg/kg TCDD or sesame oil vehicle for 24 h.

The relative abundance is the total mRNA quantity normalized to the geometric mean of *Hprt*, *Actb*, and *Gapdh*. Genes are indicated by official gene symbols. * Represents $p < 0.05$ for TCDD compared to vehicle. Bars represent mean + SEM, n=5 biological replicates.

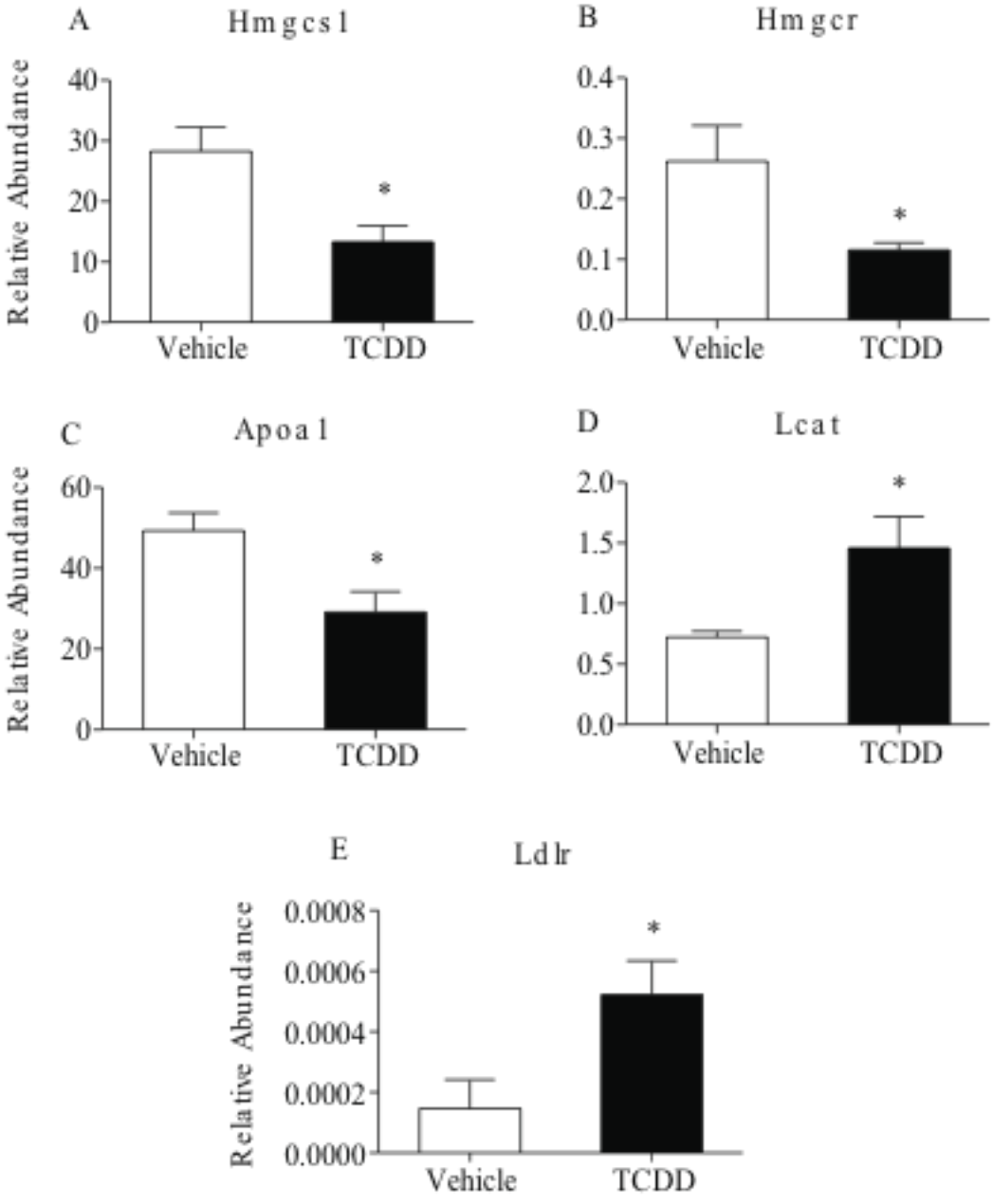
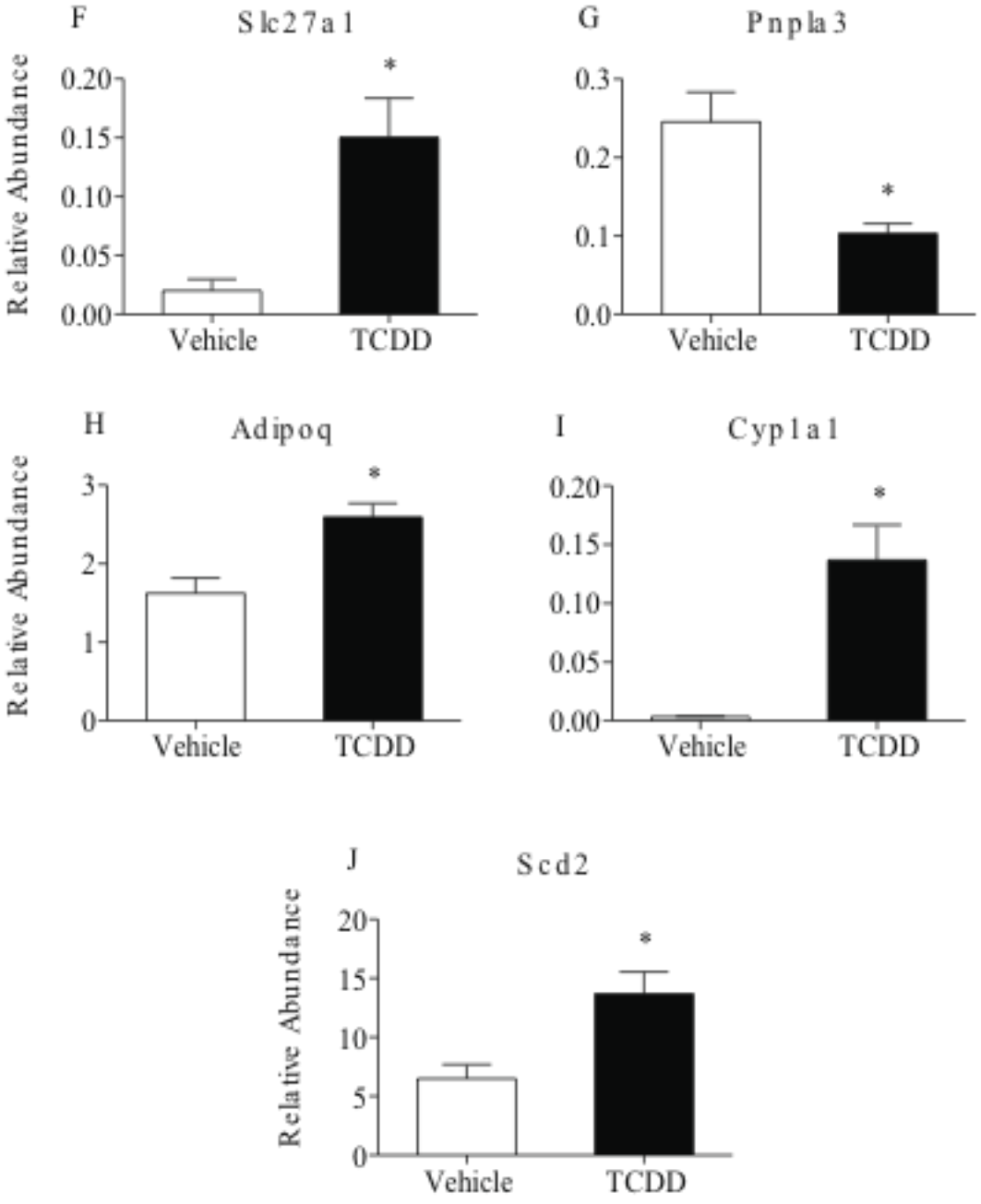


Figure 5.4 (cont'd)



TCDD also increased PUFA levels marked by increased 20:3n6 and decreased 20:4n6 (AA) and 22:6n3 (DHA), indicators of increased Elovl5 activity [40]. These TCDD-elicited FA changes are common effects observed during excessive hepatic lipid accumulation including NAFLD, a condition considered the hepatic manifestation of MetS risk factors, including obesity and other pathophysiological conditions of hepatic FA overload such as insulin resistance [41, 42].

Insulin resistance is a condition shown to be associated with TCDD exposure in humans [43, 44] as well as adipose tissue fatty acid mobilization [36]. Furthermore, adipose lipolysis is a reported FA source in TCDD-elicited steatosis [17-19]. However, the current study challenges TCDD-mediated adipose lipolysis by the observation that adipose FA levels were not affected by treatment. This was not completely surprising since studies attributed adipose lipolysis to increased serum 16:0, 18:1, 18:2, and 18:3 levels. Not only are these FAs predominant in adipose tissue [45-48], but also in rodent chow [16]. Furthermore, TCDD-treated animals examined from these studies were not fasted and it has been demonstrated that dietary fat is an important lipid source in TCDD-elicited steatosis [16].

The current study further supports existing evidence that diet is a source of lipids in TCDD-elicited steatosis. In the fasted state, serum reflects lipid origins and analysis of serum FAMEs, FFAs, and TAGs from fasted mice identified no change after TCDD treatment. In contrast, FFAs [13, 49] and TAGs [23, 50, 51] were increased by TCDD in fed animals, suggesting diet is necessary for TCDD-elicited hyperlipidemia. In the fasted state, serum lipids also reflect lipid metabolic transformations [26]. Specifically, the 18:1n9/18:0 product/precursor ratio, a functional measure of Scd1 activity increased in the liver, was also increased in serum. Elevated 18:1n9/18:0 ratios are associated with hyperlipidemic humans [34] raising the possibility that 18:1n9/18:0 ratios are a potential biomarker for hepatic steatosis.

Alterations in CHOL, lipoproteins, and apolipoproteins provided further insight into TCDD-dependent alterations in lipid metabolism. TCDD decreased serum Apob48 and Apob100 protein levels, the primary apolipoproteins in CM and VLDL particles, suggesting enhanced clearance from serum and/or efflux from liver. However, unlike humans that exclusively synthesize Apob48 in the intestine and Apob100 in liver, post-transcriptional editing in mouse liver produces Apob48 and Apob100 protein [52]. Therefore, Apob levels in treated, fasted mice suggest decreased hepatic efflux and are supported by TCDD-mediated decreases in VLDL secretion [12]. Decreased serum Apob levels may be explained by TCDD-dependent activation of IRS-PI3K-AKT pathway [53] and/or promotion of ER stress that assists Apob degradation [42, 54]. Specifically, microsomal triglyceride transfer protein (Mttp), a lipogenic mediator required for VLDL assembly [54-56], is regulated by Foxo1, a transcription factor inhibited by the IRS-PI3K-AKT signaling [57]. Furthermore, Foxo1 is regulated by PGC1α, a transcriptional coactivator deactivated by TCDD induction of TiPARP [58]. However, Mttp is minimally repressed by TCDD (-1.2-fold, [59]) and the exact relationship between intracellular signaling, Mttp function, and Apob production/degradation requires further investigation.

Decreased Apob and VLDL levels are also consistent with TCDD-mediated decreases in LDL particles, products of metabolic VLDL transformation in the serum [33], and decreased serum total CHOL and HDL. These changes in conjunction with increased hepatic expression of reverse CHOL transport genes *Ldlr* and *Lcat*, decreased hepatic CHOL biosynthesis gene expression, and increased hepatic CHOL levels [14] strongly imply TCDD-dependent dysregulation of liver CHOL metabolism. Increased reverse CHOL transport further adds to the hepatic CHOL pool enhancing feedback inhibition of CHOL biosynthesis. Furthermore, increased hepatic CHOL may initially stimulate bile acid production, that is later feedback

inhibited, netting hepatic hypercholesterolaemia and exacerbated hepatic steatosis. These changes are consistent with differential expression of Cyp7a1 (induced at 24 h, repressed at 72-168 h post-TCDD dose) the rate-limiting enzyme in bile acid synthesis that is also feedback inhibited by CHOL [59]. Given these data, it would be interesting to further investigate DRE-independent AhR-mediated regulation of the CHOL biosynthetic pathway [60] in the absence of direct CHOL regulation.

Lipids are not only important biological components, but also potent signaling molecules, metabolic regulators and transcription factor ligands [33] that when altered, effect diverse biological processes. The present study provides several examples of TCDD-elicited alterations in systemic lipid composition that may be of consequence in the pathophysiology of AhR-mediated hepatic steatosis.

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CHAPTER 6

CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

The preceding reports examined the role of AhR-mediated regulation of lipid uptake, metabolism, and transport in TCDD-elicited steatosis using Scd1 null mice, diet, ^{14}C-lipid uptake, and GC-MS lipid analysis. Collectively, these studies showed that AhR regulation of Scd1 contributed to the hepatotoxicity of TCDD (Chapter 3), dietary fat is the primary source of lipid in TCDD-elicited steatosis (Chapter 4), and AhR mediated not only altered hepatic lipid composition, but also systemic lipid composition (Chapter 5). Collectively, this work provides compelling evidence that TCDD activation of the AhR coordinates interactions between the digestive tract, circulatory system and liver to evoke hepatic steatosis, the hepatic manifestation of MetS. Considering that TCDD and related chemicals are ubiquitous environmental contaminants, the data suggest a pathophysiological relationship between DLC exposure and adverse health effects such as metabolic disease.

Although the toxicological effects of AhR activation have been extensively studied, this research illustrates a complex and equivocal function for the AhR in biological and physiological processes effecting lipid metabolism. The data generated advances knowledge on how TCDD-dependent AhR activation produces steatosis, which is a significant hepatotoxic effect of TCDD. It furthers our understanding of the mode-of-action and events that may be linked to cancer. Specifically, the novel findings that AhR regulation of Scd1 promotes inflammation and increased MUFA levels may be key events linked to cryptic cirrhosis and hepatocellular carcinoma observed in numerous rodent bioassays [1]. Cancer cell growth and proliferation requires energy and the production of new lipids for membrane synthesis. MUFAs are the major

fatty acid species in mammals and fundamental constituents of diacylglycerols (DAGs), phospholipids, TAGs, and CHOL esters vital for membrane structure and energy storage [2]. Furthermore, Scd1 performs the rate-limiting step in MUFA formation and its inhibition slows the rate of cell proliferation and decreases cell survival [3-5]. Therefore, the understudied involvement of Scd1 in mechanisms of TCDD-elicited carcinogenesis warrants further examination. However, establishing that Scd1 is not only necessary, but also sufficient for the observed TCDD-elicited increase in inflammation and MUFAs may be an important step prior to conducting carcinogenicity bio-assays.

Notably, studies in *Scd1* null mice identified increased hepatic MUFAs even though no Scd1 activity was detected (Chapter 3). Diet and ^{14}C studies demonstrated for the first time that these MUFA increases were due to TCDD enhanced lipid uptake from the diet. Yet, it remains unclear whether increased hepatic lipid content resulted from enhanced intestinal absorption, hepatic clearance from the serum, or both. To clarify, intestinal lipoprotein secretion rate *in vivo* could be measured [6-8] by tracking ^{14}C-oleate gavage immediately followed by injection with Triton WR-1339 (500 mg/kg), a detergent that forms micelles around lipid particles preventing their hydrolysis and absorption [9]. Serum ^{14}C secretion rates would indicate whether TCDD increases dietary lipid absorption with implications for dioxin as an emerging factor in the obesity epidemic [10, 11].

Lipid balance is tightly regulated since its alteration influences cellular functions. GC-MS analysis conducted throughout Chapters 3-5 identified significant treatment effects on FA composition linked to gene expression changes. It is not known, however, whether these TCDD-elicited FA changes affect the lipid signatures of free FA, TAG, DAG, CHOL ester, and

phospholipids, important complex lipids regulating signaling, membrane integrity, and lipid secretion and storage. Future comprehensive lipidomic analyses coupled with gene expression, ChIP-chip, and computational DRE distribution data [12] should provide the appropriate framework for hypothesis generation and confirmation by focused studies.

In conclusion, although several epidemiological [13-20] and rodent [21-24] studies have linked dioxin exposure to lipid abnormalities and increased risk of metabolic disease, a variety of factors obscure their relevance and impact on human health. Exposures rarely occur in isolation and seldom affect a genetically homogenous population. Strong genetic components are believed to underlie xenobiotic-induced adverse effects with inter-individual variation and idiosyncratic reactions representing common regulatory concerns [25]. Therefore, future studies using genetically diverse recombinant inbred mouse populations can model the heterogeneous human population [26] for the systemic identification of complex environmental and genetic combinations that may underlie etiologic interactions.

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