EFFECTS OF INTER-STRAIN DIFFERENCES IN THE METABOLISM OF TRICHLOROETHYLENE ON LIVER AND KIDNEY TOXICITY

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ABSTRACT

Hong Sik Yoo: Effects of Inter-strain Differences in the Metabolism of Trichloroethylene on Liver and Kidney Toxicity
(Under the direction of Ivan Rusyn)

Trichloroethylene (TCE) is an environmental and occupational health hazard which is characterized as 'carcinogenic to humans' by the IARC and U.S. EPA. However, several issues critical for assessing human health risks from TCE remain unresolved, such as (1) the amount of metabolites formed in various tissues, and possible inter-individual differences; and (2) the mode of action involved in toxicity in organs of concern. In this study, we tested a hypothesis that amounts of metabolites of TCE in mouse liver and kidney are associated with tissue-specific toxicity by evaluating the quantitative relationship between strain-, dose-, and time-dependent levels of trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), *S*-(1,2-dichlorovinyl)glutathione (DCVG), and *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) in serum, liver, and kidney and various toxicity phenotypes in tissues using a panel of inbred mouse strains. Sub-acute (600 mg/kg/d of TCE for 5 days) and sub-chronic (100 or 400 mg/kg/d of TCE for 1, 2, or 4 weeks) designs were used. In addition, this study investigated relationship between oxidative TCE metabolism and tissue-specific toxicity in the context of PPARα status (wild-type, *Ppara*-null, and humanized *Ppara*). In specific aim 1, we demonstrated the inter-strain differences and the decreasing trend of metabolism over time in TCE metabolism in liver. Across varying genetic background, levels of TCA and DCA in liver were correlated with PPARα activation but not with hepatocellular proliferation. In specific aim 2, we found a significant correlation

between renal levels of TCA and kidney injury molecule-1 expression, both of which decreased over time. However, the significant increase in cellular proliferation in proximal tubular epithelium was evident only in NZW/LacJ strain treated for 4 weeks, which may characterize the sub-chronic toxicity in kidney as cytotoxicity followed by compensatory proliferation. In specific aim 3, it was shown that TCA may be associated with oxidative stress and liver enlargement through PPARα-independent pathway. Overall, this body of work makes a novel and significant contribution to the field of environmental health science, providing the quantitative data on time-, tissue-, and strain-dependent variations in TCE metabolism and the experimental evidence regarding relationship between metabolism and toxicity.

To my mentor, friends, and family, I could not have done this without you.
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CHAPTER 1: INTRODUCTION

I. TRICHLOROETHYLENE (TCE): A HIGH PRODUCTION VOLUME INDUSTRIAL CHEMICAL

I.A. Chemical characteristics of TCE

TCE is a chlorinated alkene compound that is a clear, colorless, and non-flammable liquid at room temperature. It has been used for a variety of purposes such as industrial, commercial, medical, and consumer applications. Its widespread use is largely attributable to powerful solvent action on various lipophilic compounds derived naturally or synthetically. TCE is practically insoluble in water but is readily miscible with a number of organic solvents including ether, chloroform, and alcohol without chemical change (Powell 1947). TCE is highly volatile with vapor pressure of 7.8 kPa at 20 °C (Mackay and Shiu 1981), which is a reason of vapor intrusion or inhalational exposure. However, unlike other volatile compounds, TCE is neither flammable nor explosive at room temperature, and at higher temperatures it is only moderately flammable, which is why TCE has been more preferred in many occupational settings than other organic solvents (ATSDR 1997).

The water-air distribution coefficient of TCE was given as 3.0 at 20°C and 1.6 at 37°C, for concentrations in air between 0.26 and 15.8 mg/100 mL (Powell 1947). The distribution coefficient between blood and air was 18-21 at 20°C and 8-10 at 37°C (Powell 1947). The fat-gas partition coefficient was calculated as 960 at 37°C, confirming its strong lipophilic nature (Mapleson 1963).

TCE is spontaneously oxidized in presence of oxygen and/or UV radiation and forms acidic products including hydrogen chloride. It reacts with metals such as magnesium or aluminum at 300-600°C to form an extremely toxic compound, phosgene that has long been an occupational health concern because this condition is often found in the vicinity of welding and degreasing operations (Waters et al. 1977).

I.B. Production and Consumption

There are no known natural sources of TCE. Emil Fischer discovered TCE while the preparation of tetrachlorethane in 1864 (Fischer 1864); however, it was not commercially utilized for industrial purpose until over 40 years later, and the massive production began in the 1920s (Cotter 1950). It is estimated that the production of TCE in 1990 was approximately 131,000,000 kg in Western Europe, 79,000,000 kg in the U.S., and 57,000,000 kg in Japan, and its annual consumption in these areas was estimated to be up to 100% of production levels (WHO 2010). In 2005, global consumption of TCE was estimated to 430,000,000 kg, while global production capacity of 667,000,000 kg (The Dow Chemical Company 2008). In the U.S., two major chemical companies, Dow Chemical and PPG industries manufacture TCE, and their combined production capacity was estimated to 150,000,000 kg in 2005 (ICIS 2005). Global consumption of TCE in 2011 was estimated at 429,000,000 kg, of which 116,000,000 kg was in the U.S. (Glauser 2012).

I.C. Use

Currently, TCE is mainly used as chemical intermediate in the production of fluoro-chemicals, as process solvent in the manufacturing of pharmaceuticals and other chemicals, and as solvent for degreasing processes, particularly in the metal fabricating and aircraft/aerospace industry (ICIS 2005). Other than the use of TCE for manufacturing

secondary products, TCE is predominantly used as a degreasing agent, an application for which it is remarkably suitable due to its solvent action, non-corrosivity, rapid evaporation, lack of flammability, and ease of recycling. It is used as such in a variety of industries, including electronics, defense, aerospace, aviation, rail, shipbuilding, and automotive. Among a number of TCE's cleaning applications is the flushing of liquid hydrogen tanks, liquid oxygen tanks, and associated piping systems in the aerospace industry. During the process of vapor degreasing, oily compounds are removed and accumulated in the TCE reservoir at the bottom of the degreaser. As the proportion of these impurities in the solvent increases, the efficiency of the solvent decreases. Thus, TCE is required to be periodically replaced with fresh solvent, or distilled to remove impurities. And the used TCE and accumulated materials must be discarded for recycling or disposal. However, inappropriate discarding practices of these wastes may cause the introduction of TCE into soil and ground water (Hargarten et al. 1961).

In addition to its primary use as a degreasing agent, TCE has also been used to a lesser degree as a heat transfer medium; a refrigerant; a cleaner for optical lenses and film; and an ingredient in paint strippers, printing inks, lacquers, pesticides, lubricants, paints, rust preventers and adhesives (Doherty 2000).

TCE, along with carbon tetrachloride, was one of the first chlorinated solvents that were used in dry cleaning as a substitute for petroleum-based cleaners. In 1930, TCE was introduced as a dry cleaning solvent to the U.S. Although it was partly replaced in the 1950s by tetrachloroethylene, it is still widely used as a dry-side pre-cleaning or spotting agent (Linn 2010).

I.C. Historical impacts of TCE on environment and associated regulations

The widespread use of TCE in degreasing coupled with poor disposal practices prevalent during most of the twentieth century resulted in substantial releases of TCE into the environment. According to the U.S. EPA (2011b), TCE is one of the most common contaminants found at Federal Superfund sites, having been detected at 338 of 1,299 sites on the National Priority List as of 2011. The presence of TCE in soil, groundwater, and vapor at these locations may result in prolonged human exposure via both inhalation and ingestion routes. Due to its characteristic high persistence in soil, it will likely continue to be present in these media for a longer period.

In the U.S., the 1970 Clean Air Act regulated TCE as an ambient air pollutant and set emission limits on TCE. In addition to the act, a few other regulations influenced the use of TCE. On October 21, 1976, the Resource Conservation and Recovery Act (RCRA) was passed. One of the aims in the act is to prevent the contamination of ground waters from substances leaching from disposal sites.

TCE was one of the 65 priority pollutants in the 1977 Clean Water Act that was amended to ensure better regulation of disposals of hazardous chemicals. A tax on the chemical and petroleum industries and broad Federal authority to respond directly to releases or threatened releases of hazardous substances were established under the 1980 Comprehensive Environmental Response, Compensation & Liability Act (CERCLA), which created the “Superfund” for the cleanup of the nation's uncontrolled hazardous waste sites. To comply with the 1974 Safe Drinking Water Act’s requirement to set up regulations for public water supplies, the U.S. EPA proposed non-enforceable Maximum Contaminant Level Goals (MCLGs) for TCE and seven other chemicals on June 12, 1984. Enforceable

Maximum Contaminant Levels (MCLs) for the eight chemicals including TCE were suggested on November 13, 1985, and became effective January 9, 1989. The MCL for TCE was set at 5 parts per billion (ppb), and remains at the level to this day (Gilbert 2014).

The national impact of TCE contamination and following regulation is best exemplified by the Honoring America's Veterans and Caring for Camp Lejeune Families Act of 2012 (H.R. 1627, P.L. 112-154, enacted on August 6, 2012). As noted in the National Research Council's 2009 report "Contaminated Water Supplies at Camp Lejeune: Assessing Potential Health Effects," it was found in the 1980s that the water-supply systems on the Marine Corps Base Camp Lejeune had been contaminated with the industrial solvents TCE and perchloroethylene (PCE) for several decades. Enlisted-family housing, barracks for unmarried service personnel, base administrative offices, schools, recreational areas, the base hospital and other drinking water supplies were affected. As a result of this, an estimated 750,000 Marine veterans and family members throughout the U.S. may have been exposed to toxic chemicals including TCE while spending time at the North Carolina Marine Corps base. The H.R. 1627 is one of the unique examples of the Federal Government taking responsibility for health care coverage of those who may have suffered adverse health effects as a consequence of exposures at Camp Lejeune, NC,

II. HUMAN HEALTH HAZARDS OF TCE

II.A. Non-cancer adverse health effects

Central nervous system. One of the primary effects of acute and high dose of TCE exposure occurs in the central nervous system. Numerous cases have been reported regarding occupational poisoning associated with neurologic effects (McCarthy and Jones 1983), which is inevitable, given the historical use of TCE for analgesia or anesthesia in dental and obstetrics clinics (Barry and O'Connor 1946; Galley 1945).

In terms of chronic effects, there is strong evidence, based on multiple human and experimental animal studies, that the exposure to TCE adversely affects the central nervous system (U.S. EPA 2011b). Especially, changes in trigeminal nerve function or morphology and impairment of vestibular function have been reported (Chiu et al. 2013). As TCE can target dopaminergic neurons, a possible relationship between human exposure to this agent and Parkinson's disease has been suggested (Guehl et al. 1999). Although a recent epidemiologic study on twins indicated possible etiologic relations between Parkinsonism and TCE (Goldman et al. 2012), many other epidemiologic studies yielded mostly weak associations (Lock et al. 2013).

Kidney. A number of studies reported the adverse effects of TCE on non-cancer endpoints in kidney using non-specific urinary markers including β2-microglobulin, N-acetyl-beta-D-glucosaminidase, α1-microglobulin, GST-alpha, or total protein (U.S. EPA 2011b). Urinary kidney injury molecule-1 (KIM-1) was also demonstrated to be a sensitive marker of kidney injury in humans exposed to TCE (Vermeulen et al. 2012). While National Research Council concluded that humans exposed to TCE have tubular proteinuria (National Research Council 2006), its underlying mechanism is yet to be understood, partly due to

experimental difficulties to prove association between glutathione (GSH)-conjugated metabolites and cytotoxicity in the kidney of humans. Green et al. (2004) suggested an alternative mechanism focusing on oxidative metabolite-induced and formic acid-mediated renal toxicity; however, counter-evidence was also reported (Lock et al. 2007).

Liver. Studies on TCE and liver injury markers in serum have shown both positive (Rasmussen et al. 1993) and negative (Nagaya et al. 1993) associations. In a severe case accompanied by hypersensitivity dermatitis, high elevation of serum injury markers was detected in several subjects (Jung et al. 2012; Kamijima et al. 2007; Liu 2009). Chronic liver disease cases, including non-alcoholic steatosis and cirrhosis, have been reported (Liu 2009; Schuttmann 1970; Thiele et al. 1982); however, evidence in epidemiological studies is limited.

Autoimmune-related toxicity. As reviewed by (Cooper et al. 2009), there is strong evidence indicating that TCE exposure increased the risk of autoimmune disease including systemic lupus erythematosus, systemic sclerosis and fasciitis, both from occupational and environmental exposure. The effects of TCE exposure on the immune system were reported as early as in 1957 (Reinl 1957), and recently a prospective cohort study has further implicated TCE in the development of systemic sclerosis (Marie et al. 2014). Extensive research on immunotoxicity has been conducted as it is related with other disease domains such as developmental, hepatic, and neurotoxicity (Gilbert 2014).

II.B. Cancer hazard

II.B.1. Kidney

The IARC and U.S. EPA concluded that there was convincing evidence of a causal association between TCE and kidney cancer in humans (Chiu et al. 2013; IARC 2013), based largely on a comprehensive meta-analysis of 15 independent epidemiologic studies (Scott and Jinot 2011). From the meta-analysis, it was observed that the relative risk estimates consistently increased across the 15 studies regardless of study design and population. Another meta-analysis suggested that significant and stronger risk estimates were observed only in studies where TCE exposure was scientifically assessed (Karami et al. 2012). A recent epidemiological study also demonstrated increased risk of kidney cancer from the U.S. Marine Corps base Camp Lejeune cohort (hazard ratio = 1.35, 95% CI: 0.84-2.16), albeit not significant (Bove et al. 2014).

There is evidence that TCE can cause kidney cancer in rodents. Especially, it is noteworthy that TCE-induced kidney tumors were found in multiple strains of male rats exposed by gavage (National Toxicology Program 1990). Although the increases in incidence were low, it was still considered biologically significant given the very low historical incidence of renal tumors in rats. In an inhalation study, TCE was not associated with increase in kidney tumor incidence in mice or hamsters (Henschler et al. 1980), but increased renal adenocarcinomas were detected in male rats at the high dose (600 ppm) after 2 years of exposure (Maltoni et al. 1988). Thus, TCE has been shown to provoke neoplasms in the kidney of rats, treated via both inhalation and ingestion. Although the TCE-induced increase in renal cancer was low, because of the rarity of these tumors in historical controls

and the reproducibility of this result, it was reached to a consensus that the finding is biologically significant (Chiu et al. 2013).

II.B.2. Liver

The U.S. EPA published a TCE risk assessment document that concluded that the weight of evidence for liver cancer was not sufficient to classify TCE as carcinogenic to humans (U.S. EPA 2011b). The EPA provided a main reason that although meta-analysis showed significant increase of risk in TCE-induced liver cancer, "only cohort studies are available and most of these studies have small numbers of cases." Likewise, IARC's review on one case-control study and nine cohort studies stated that the results were inconsistent and insufficient in terms of adjusting confounding factors (IARC 2013).

In animal models, there is clear evidence of TCE hepato-carcinogenicity in both male and female mice (Maltoni et al. 1988; National Toxicology Program 1990). However, the human relevance of these findings was questioned because of the quantitative differences in TCE metabolism between species as well as difference in sensitivity of PPARα receptor to oxidative metabolites of TCE.

II.B.3. Lymphatic and hematopoietic system

The IARC and U.S. EPA concluded that epidemiologic evidence regarding association between TCE and non-Hodgkin lymphoma (NHL) is strong but less convincing than liver or kidney cancer (IARC 2013; U.S. EPA 2011b). Recent epidemiologic analyses have shown conflicting results. Cocco et al. (2013) demonstrated an increase in the risk of specific NHL subtypes associated with occupational exposure to TCE in a pooled analysis of four international case-control studies. In addition, a meta analysis supported an association between occupational TCE exposure and NHL after applying a strengthened exposure

assessment (Karami et al. 2013). Contrary to these finding, other studies reported insignificant association between TCE and acute myeloid leukemia (Talibov et al. 2014) or non-Hodgkin's lymphoma (Hansen et al. 2013).

III. MECHANISMS OF TCE CARCINOGENESIS

III.A. TCE metabolism

One of the most critical problems in TCE risk assessment is the complex metabolism of TCE. Except in rare cases, TCE toxicity is considered to be mediated by metabolites rather than TCE itself. TCE is rapidly metabolized through either oxidation by cytochrome P450 or GSH conjugation (Chiu et al. 2006).

III.A.1. CYP-dependent oxidation

The overall scheme of CYP-dependent oxidation is illustrated in Figure 1. TCE metabolism is catalyzed by CYP450 enzymes. In addition to CYP2E1, a primary enzyme involved in this step, several enzymes including CYP1A1/2, CYP2A6, and CYP3A4 have been reported to have some activity (Lash et al. 2000). The initial step yields a reactive intermediate, TCE-O-P450 to produce chloral or TCE-epoxide. Chloral may undergo dehydration to produce trichloroethanol (TCOH) or trichloroacetic acid (TCA) by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH), respectively. Dichloroacetic acid (DCA) can be formed from dechlorination of TCA or oxidation of TCE-epoxide (Lash et al. 2014).

III.A.2. GSH conjugation

The overall scheme of GSH conjugation is described in Figure 2. TCE metabolism by GSH conjugation begins with the action of GSH *S*-transferase (GST). The first step is an S_N2 substitution reaction between TCE and GSH, producing *S*-(1,2-dichlorovinyl)glutathione

(DCVG). DCVG is further metabolized predominantly in kidney by gamma-glutamyl transpeptidase and then cysteinyl-glycine dipeptidase to yield *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) which has three possible fates: (1) mercapturation to N-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC); (2) formation of reactive thiol, *S*-(1,2-dichlorovinyl)thiol (DCVT) by beta-lyase; (3) oxidation to *S*-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS) by flavin-containing monooxygenase (Lash et al. 2014). Relatively small amounts of GSH conjugation metabolites have been detected in systemic circulation (Kim et al. 2009b), which may be explained by active excretion of GSH conjugation metabolites to bile followed by enterohepatic recirculation (Davidson and Beliles 1991). Despite the small flux relative to oxidative metabolites, DCVG and DCVC have been extensively studied regarding their effects on renal toxicities (Anders and Dekant 1998; Lash et al. 1998; Mally et al. 2006).

III.A.3. Variability in TCE metabolism

Inter-individual differences. Inter-strain difference in TCE metabolism was well-demonstrated in mice (Bradford et al. 2011). A population variability analysis revealed that the metabolic flux through glutathione conjugation was more variable (10-fold range) than that through CYP-mediated oxidation (5-fold range) in mice (Chiu et al. 2014). In humans, very limited data exist to characterize the inter-individual difference. However, the available data (Lash et al. 1999a; Lash et al. 1999b) suggest that there is significant variation in GSH conjugation in humans. In particular, variations in the rate of GSH conjugation of TCE were measured as 2.4-fold in human liver cytosol and 6.5 fold in liver microsomes.

Sex difference. Overall, metabolism capacity is thought to be higher in males than females in rodents (U.S. EPA 2011b). However, in guinea pigs, the amount of TCA in urine

was significantly greater in females than males, while there was no sex difference in the total amount of TCA and TCOH (Hibino et al. 2014). In humans, there are conflicting results regarding gender differences in pharmacokinetics of TCE. Sato et al. (1993) reported that there is a sex difference in the pharmacokinetic profiles of TCE. Although retention of TCE in the body was longer in men than in women, the blood level of TCE in women was greater than in men 16 hours after exposure. However, another PBPK study concluded that gender-related pharmacokinetic differences in the uptake and metabolism of trichloroethylene are minor (Fisher et al. 1998).

Species difference. Higher metabolic capacity in mice than in rats was suggested as deduced from the amount of exhalation of unchanged TCE (U.S. EPA 2011b). In line with the analysis above, the rate of metabolism was faster in mice than in rats or humans in terms of enzyme kinetics parameter (V_{MAX}/Km) related with CYP-mediated oxidation (Elfarra et al. 1998; Lipscomb et al. 1996; Lipscomb et al. 1997). However, percentage of urinary excretion of TCA was much higher in non-human primates compared to mice and rats (Fisher et al. 1991).

III.B. Genotoxicity

TCE and its metabolites have been extensively studied for potential genotoxicity and mutagenicity. Available evidence indicates that TCE itself is not prone to inducing gene mutation in most standard assays (Rusyn et al. 2014). There is some evidence that TCE may be a weak inducer of mutations, however the observed activity was generally weak, even at very high doses. In addition, any mutagenic potential of TCE itself is likely to be resulted from one or more of its metabolites (Moore and Harrington-Brock 2000).

For oxidative metabolites of TCE, IARC (2013) concluded that there is weak to moderate evidence indicating that DCA is associated with genotoxicity; and that TCA is not genotoxic; and that strong evidence supports that chloral hydrate causes genotoxicity. TCOH was negative in the Salmonella TA100 assay (DeMarini et al. 1994), but has not been evaluated in the other recommended screening assays.

Data associating GSH-conjugated metabolites with mutagenicity is sufficient to conclude a mutagenic mode of action (MoA) is operative (Chiu et al. 2013). DCVG has shown direct-acting mutagenicity that was enhanced with kidney mitochondria, cytosol, or microsomes. In the contrary, addition of liver subcellular fractions did not enhance the mutagenicity of DCVG, which strongly suggests that *in situ* metabolism plays a significant role in the genotoxicity in kidney (Rusyn et al. 2014). DCVC exhibited a strong, direct-acting mutagenicity regardless of the presence of mammalian enzymes (Dekant et al. 1986b; Irving and Elfarra 2013). The genotoxicity of DCVC is further proved by the predominantly positive results in other available *in vitro* and *in vivo* assays (Clay 2008; Jaffe et al. 1985).

III.C. Non-genotoxic mechanisms: Kidney

III.C.1. α2u-Globulin nephropathy

Given that TCE-induced renal cancer is observed only in male rats among rodents, the role of α2u-globulin nephropathy has been investigated. Green et al. (2003) reported an increase in hyaline droplets and immunostaining for α2u-globulin in the kidneys of male rats exposed to TCOH in the drinking water for 52 weeks. However, the increase in α2u-globulin was transient, being observed after 28 and 40, but not 52, weeks of exposure. Overall, evidence supports that not only are TCE and its metabolites genotoxic, but also the

characteristic histopathology, α2u-accumulation or reversible binding to α2u-globulin is not associated with exposure to TCE (Goldsworthy et al. 1988; Rusyn et al. 2014).

III.C.2. Cytotoxicity and regenerative proliferation

Available data consistently shows that TCE causes cytotoxicity, contributing to renal carcinogenesis in rodents; however, the evidence is stronger for a mutagenic mode of action (Chiu et al. 2013). Several human studies demonstrated that exposure to TCE is associated with cytotoxicity in kidney (Bolt et al. 2004; Green et al. 2004; Vermeulen et al. 2012). Likewise, there is clear evidence that TCE is nephrotoxic in rodents (Green et al. 1997; National Toxicology Program 1990). *In vitro* studies provide strong evidence that DCVC or its metabolite is nephrotoxic in primary human (Lash et al. 2005; Lock et al. 2006) and rodent (Stevens et al. 1986) cells. However, data linking TCE-induction of proliferation with clonal expansion is lacking.

III.C.3. Oxidative metabolites-mediated formic acid excretion

It has been postulated that there is an association between oxidative metabolites (TCA and TCOH) and elevated formation of formic acid by a disruption of the vitamin B(12) dependent methionine salvage pathway (Green et al. 2003; Green et al. 2004). Exposure to formic acid has been associated with adverse renal effects in humans (Liesivuori et al. 1992). However, a critical problem in the hypothesis is that chloral, an upstream metabolite of TCA and TCOH, does not induce the increase of formic acid (Dow and Green 2000).

III.C.4. PPARα activation

No study investigated PPARα activation in human kidneys following exposure to TCE. In rodents, only one *in vivo* study showed the increases in renal palmitoyl-CoA oxidation activity in both rats and mice (Goldsworthy and Popp 1987). Given the observation

that the enzyme activity in mice is stronger than in rats, the authors concluded that PPARα activation may not be associated with species-specific renal carcinogenesis in rodents treated with TCE.

Overall, evidence suggests that DCVC or its downstream reactive electrophile metabolites may be responsible for kidney tumors in humans by a mutagenic MoA, as well as an MoA that involves cytotoxicity and compensatory cell proliferation (Rusyn et al. 2014). An integration of mutagenicity and cytotoxicity, with mutagenicity increasing the rate of mutation and regenerative proliferation promoting the survival or clonal expansion of mutated cells, while biologically plausible, has not been experimentally tested.

III.D. Non-genotoxic mechanisms: Liver

III.D.1. PPARα activation

PPARα activation is one of the most studied MoA hypotheses that explains how TCE causes liver tumors in mice. The hypothesis that TCE causes tumors via PPARα-activation is based on the observation that TCA is a major oxidative metabolite which activates PPARα and provokes hepatocyte proliferation. However, there has been increasing evidence undermining the confidence in the conclusion that PPARα activation is a primary mechanism. First, whereas TCA induces PPARα activation in both rats and mice, exposure to TCA resulted in tumorigenesis in B6C3F1 mice but not F344 rats (DeAngelo et al. 1997). Second, it has been reported that the tumor phenotype of TCA-induced mouse liver tumors shows different pattern of H-ras mutation frequency from DCA (Bull et al. 2002).

III.D.2. Cytotoxicity and secondary oxidative stress

Several cohort studies in humans described elevations in serum liver function markers, or changes in plasma or serum bile acid in workers exposed to TCE (U.S. EPA 2011b). Case

studies of liver injury and cirrhosis (Kamijima et al. 2008; Thiele et al. 1982) have also been reported. However, the overall evidence of this mechanism in humans is not sufficient. A number of animal studies have revealed that TCE is hepatotoxic in terms of the increases in serum enzymes and bile acids (Hamdan and Stacey 1993; Nunes et al. 2001). Data on TCE-induced oxidative stress in humans is limited. Several studies measured urinary 8-hydroxy-deoxyguanosine (8-OHdG) related with TCE exposure (Abusoglu et al. 2014); however, Rusyn et al. (2006) noted that 8-OHdG may not adequately reflect genomic damage.

III.D.3. Epigenetic alterations

Several studies suggested that DNA hypomethylation may be associated with the carcinogenicity of TCA and DCA in mice. In an initiation (i.p. injection of N-methyl-N-nitrosourea)-promotion (administration of TCA or DCA in drinking water) study design, the decreased level of 5-methylcytosine was observed in hepatocellular adenomas and carcinomas compared to non-involved tissue from the same animal (Tao et al. 1998). Sub-chronic drinking-water exposure to TCA or DCA also caused a decreased total liver DNA methylation. In addition, Ge et al. (2001) demonstrated an association between hypomethylation and cell proliferation in the liver of TCA- or DCA-treated mice. However, no data from human or experimental animal studies are available specifically testing this hypothesis for TCE.

IV. GAPS IN KNOWLEDGE

One of the most critical problems in TCE risk assessment is the complex metabolism of TCE. While the mode of action involved in species-specific differences in toxicity has not been fully understood, it is well accepted that “knowledge of TCE metabolism is critical for determining susceptibility, target organ specificity and gender and species differences”

(National Research Council 2006). However, data on tissue-specific levels of metabolites is very limited.

Another critical problem is that the mode of action through which TCE causes cancer is not clear. The causal relationship between TCE and liver cancer in humans has been a source of debate since a National Cancer Institute (NCI) study revealed that TCE causes a significant increase in liver tumors in mice (National Cancer Institute 1976). However, despite the extensive data that have been accumulated in the field, the epidemiological association between TCE and liver cancer in humans is still inconclusive. With regard to mode of action, besides PPARα agonism, several hypotheses, including mutagenicity, disturbance of glycogenesis, hypomethylation, and oxidative stress, have been suggested and examined (Rusyn et al. 2014). However, little attention has been given to integrating PPARα activation with non-cancer endpoints that result from various toxicity pathways such as perturbation of glycogen synthesis, elevation of serum bile acids, epigenetic alterations, and immune responses. The liver is the primary site for first-pass metabolism, and it has various functions that are critical for detoxification and elimination of xenobiotics. Therefore, the responses to TCE in liver are diverse. In this regard, it is likely that the model that includes multiple cellular pathways will increase our ability to predict the toxicity of TCE and identify human sub-populations that are particularly vulnerable to TCE.

It is likewise controversial which mode of action is operational in kidney cancer. Although the U.S. EPA characterized TCE as ‘carcinogenic to humans’ in kidney based on weight-of-evidence approach including convincing epidemiologic data and supporting toxicokinetic data, there has been evidence undermining the mutagenic mode of action involving activation of GSH conjugation metabolite. For instance, whereas DCVC may be

more nephrotoxic in mice than in rats, a significant increase in TCE-induced renal cancer in mice has never been demonstrated (Eyre et al. 1995; Green et al. 1997). The important point is that the dispute over mode of action is closely related to the way to understand and describe the metabolism of TCE. The U.S. EPA (2011b) stated that one of the reasons why it is hard to resolve the issues regarding modes of action is that "the metabolic yield of GSH conjugation metabolites following exposure to TCE remains uncertain."

Lastly, in the field of TCE hazard assessment, little attention has been given to population-based approach. One of the biggest gaps in cancer risk assessment as identified by the National Research Council is the fact that inter-individual variability is not being addressed at all (in animal studies) or incompletely (in epidemiological studies). Indeed, the uncertainty that is associated with intra-species variability in both laboratory animals and humans is now based on default uncertainty factors rather than science. Although rodent models have been widely used in TCE research, existing animal models are not population-based, and therefore it is difficult to understand the variation in response to TCE. Bradford et al. (2011) demonstrated that a consideration of variation is useful to differentiate between strain-dependent and -independent effects.

V. SPECIFIC AIMS

In this regard, the comparison between variation of metabolism and variation of response sheds light on understanding how TCE causes toxicity. Hence, the purpose of the dissertation research is to test the overall hypothesis: TCE-induced toxicities in liver and kidney are associated with the tissue-specific formation of metabolites and the variation in metabolism described by population-based approach.

Specific Aim 1: Inter-Strain Variability in the Metabolism of TCE: Role in Liver Toxicity

One of the key components to understand TCE-induced hepatotoxicity is the creation of a reliable model encompassing metabolism, gene expression and toxicity pathway. This aim tested the hypothesis that genetically-controlled differences in oxidative metabolism of TCE between strains will lead to quantifiable biologically important differences in terms of PPARα activation and hepatocyte proliferation. We produced data regarding: correlation between oxidative metabolites; effects of the metabolism on PPARα activation; correlation between metabolism, PPARα activation, and liver toxicity; variation of metabolism over time and its consequence.

Specific Aim 2: Inter-Strain Variability in the Metabolism of TCE: Role in Kidney Toxicity

The important point is that the dispute over mode of action in TCE-induced renal carcinogenesis is closely related to the way to understand and describe the metabolism of TCE. This aim tested the hypothesis that genetically-controlled differences in metabolism of TCE between individuals will lead to quantifiable biologically important differences in formation of kidney-selective metabolites thus potentially predisposing individuals to organ-

specific toxicity. We produced data regarding: metabolites profile; association between metabolites and cytotoxicity; association between metabolites and cell proliferation.

Specific Aim 3: Effects of PPARα on Metabolism and toxicity of TCE

One of the most contentious issues in TCE-induced hapatotoxicity is the role of PPARα agonism. This aim is designed to investigate the relationship between metabolism and PPARα agonism and its effect on toxicity in liver. This aim tested the hypothesis that both TCE metabolism and PPARα activation will affect liver toxicity, and that their relationship will be explained using gene expression analysis and phenotyping. We produced data regarding: difference of metabolism; association between gene expression and genotype; relationship between genotype and toxicity end points.

VI. FIGURES

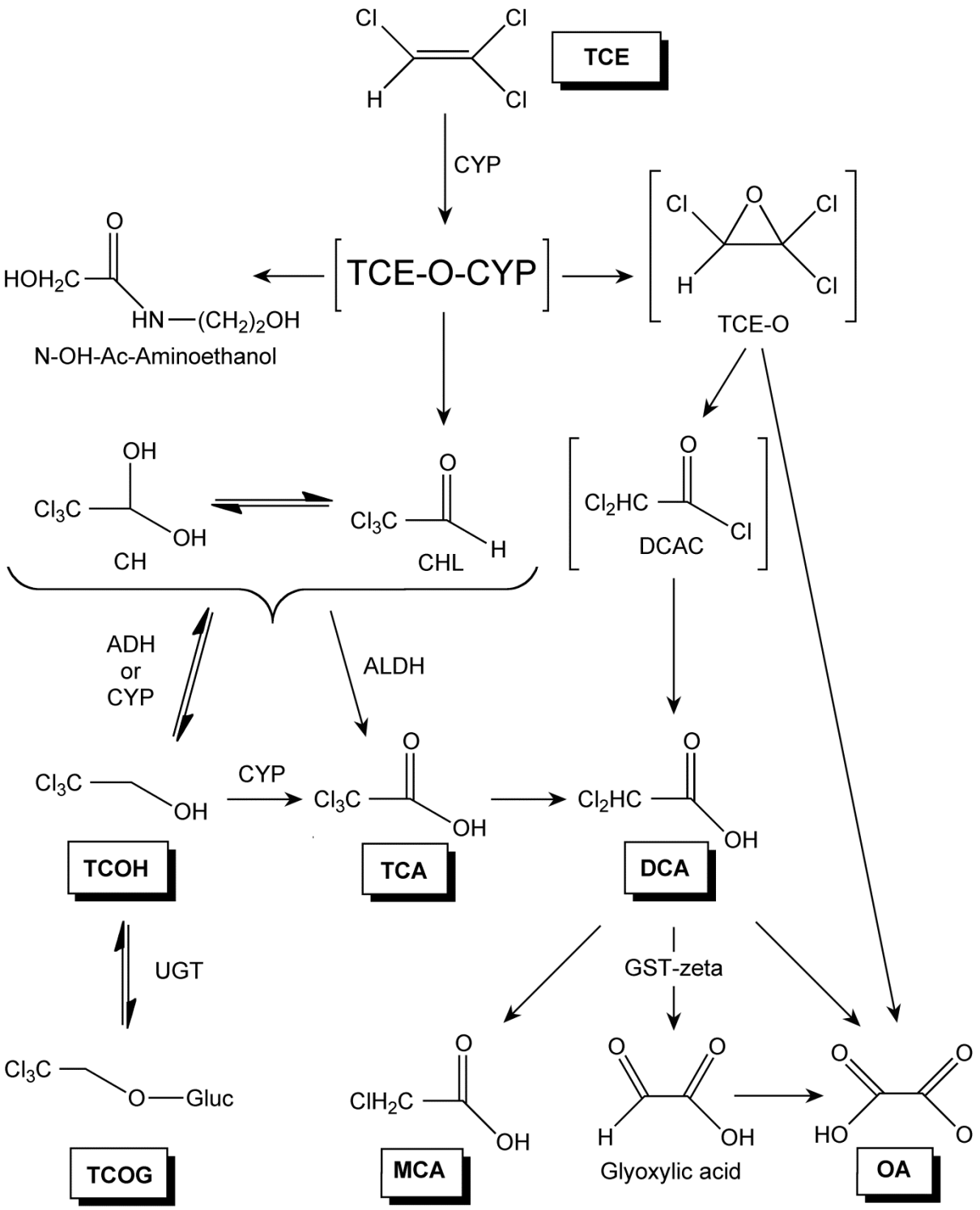


Figure 1.1. Scheme for biotransformation of TCE by oxidative metabolism. Reproduced from (Lash et al. 2014).

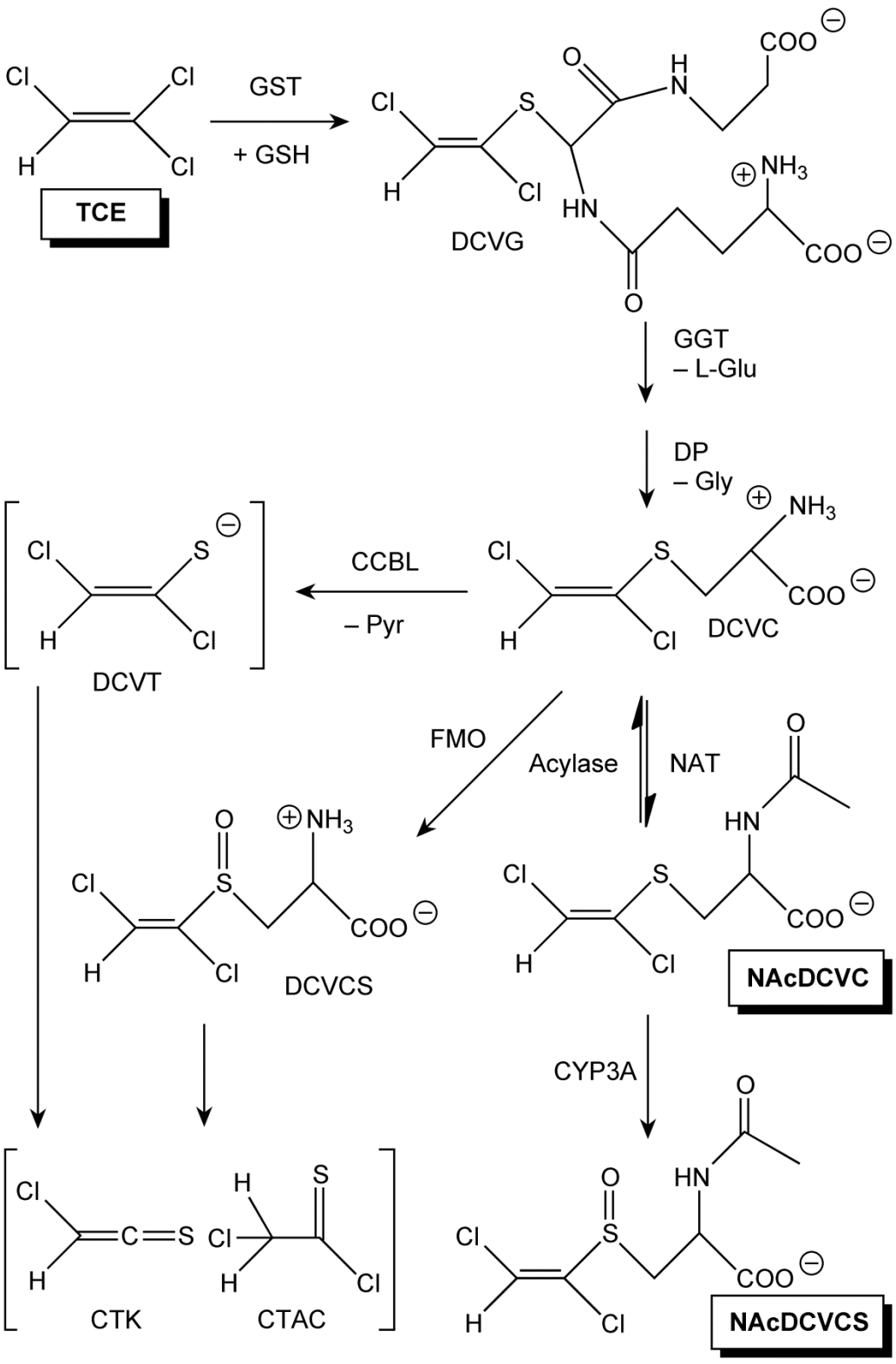


Figure 1.2. Scheme for biotransformation of TCE by the GSH conjugation pathway. Reproduced from (Lash et al. 2014).

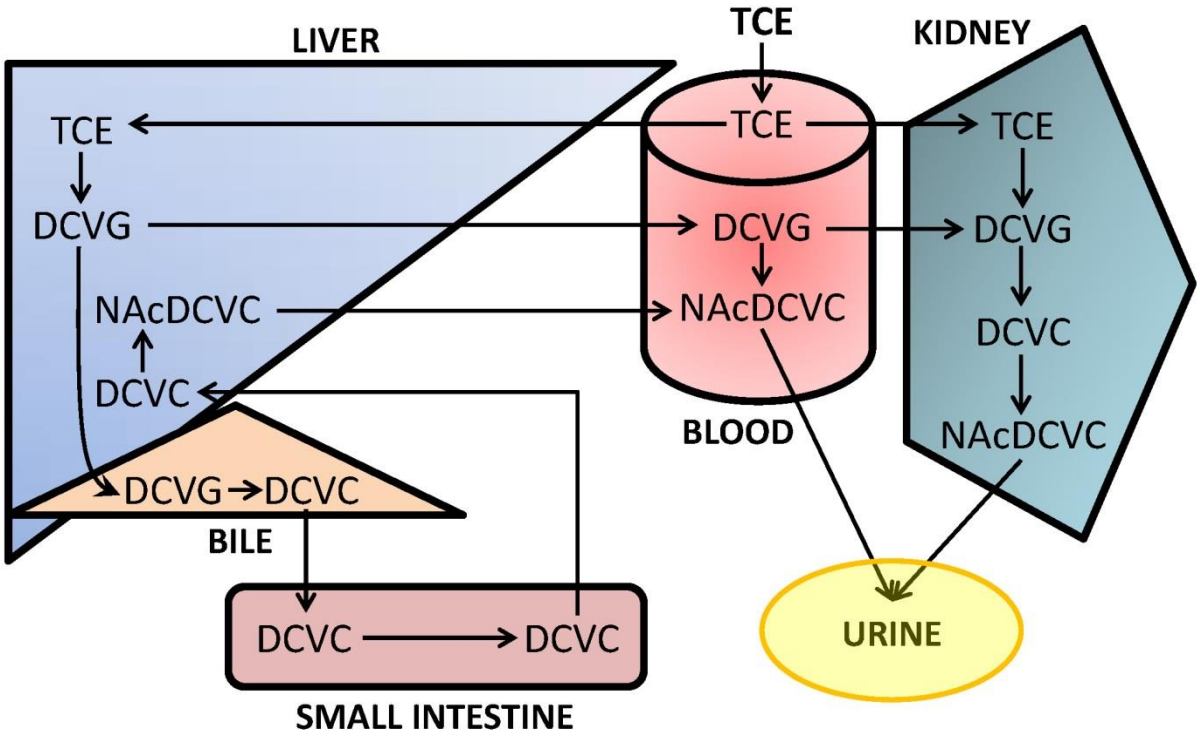


Figure 1.3. Inter-organ pathways for metabolism of TCE by the GSH conjugation pathway. The scheme summarizes the tissue localization of metabolic reactions and membrane transporters involved in whole body metabolism of TCE by the GSH conjugation pathway. Most of the DCVG formation occurs in the liver, which is very efficient at excreting it into bile or plasma. Biliary DCVG is processed to yield the cysteine conjugate DCVC, which then returns to the liver by enterohepatic recirculation. Most of the hepatic DCVC is N-acetylated to form the mercapturate NAcDCVC, which is efficiently excreted into plasma. Plasma DCVG and NAcDCVC move through the blood and are extracted by the kidneys. DCVG formation also occurs, although to a lesser extent, within the kidneys themselves. Reproduced from (Lash et al. 2014).

CHAPTER 2: INTER-STRAIN VARIABILITY IN THE METABOLISM OF TCE: ROLE IN LIVER TOXICITY

I. INTRODUCTION

Trichloroethylene (TCE) is a chlorinated organic solvent widely used as a feedstock material in chemical manufacturing, as well as in various industrial applications including dry cleaning and vapor degreasing. Since the beginning of its wide-spread production dating back to the 1920s (NICNAS 2000), it has been recognized as an occupational and environmental health concern due to high human exposure and its potential to be a health hazard (NRC 2006). The number of workers in occupations with likely TCE exposure has declined in the developed countries in the past 20 years (von Grote et al. 2003). Still, TCE is one of the major contaminants found in the Superfund hazardous waste sites across the United States (U.S. EPA 2011b) and is ranked 16th on the Substance Priority List by the US Agency for Toxic Substances and Disease Registry (ATSDR 2011).

TCE poses a potential human health hazard for non-cancer toxicity to the central nervous system, kidney, liver, immune system, male reproductive system, and the developing fetus (Chiu et al. 2013). In addition, TCE is classified as carcinogenic to humans by IARC (Guha et al. 2012) and the U.S. EPA (U.S. EPA 2011b) based on the evidence in humans of a causal relationship between kidney cancer and exposure to TCE. Positive, although less consistent, associations with TCE were reported in epidemiological studies of liver cancer and non-Hodgkin lymphoma (Scott and Jinot 2011).

Liver toxicity and carcinogenicity remain largely unresolved with regard to the human health hazard of TCE (Rusyn et al. 2014). There is clear evidence that TCE is a liver carcinogen in rodents (Anna et al. 1994; National Toxicology Program 1990); however, the relationship between occupational TCE exposure and risk of liver cancer in humans is still inconclusive, given that epidemiologic studies have observed both positive (Hansen et al. 2013) and negative associations (Radican et al. 2008; Vlaanderen et al. 2013). A recent meta-analysis reported a meta-relative risk of 1.3 (95% CI 1.1-1.6) for the overall TCE exposure and liver cancer based on nine human cohorts (Scott and Jinot 2011).

There are also uncertainties regarding the potential mode of action for TCE tumorigenesis in liver (Rusyn et al. 2014). TCE is metabolized through both cytochrome P450-dependent oxidation and glutathione conjugation to form a number of toxic species (Lash et al. 2014). Metabolites of the oxidative pathway, trichloroacetic (TCA) and dichloroacetic (DCA) acids, are widely considered to be primary mediators of the toxicity and carcinogenicity of TCE in the liver via peroxisome proliferator-activated receptor (PPARα) activation. The latter is one of the most studied mechanisms of action for TCE-induced liver cancer in rodents (Keshava and Caldwell 2006; Klaunig et al. 2003). Studies *in vitro* have shown that human PPARα is activated by TCA and DCA, but relatively inactive to TCE itself (Maloney and Waxman 1999; Zhou and Waxman 1998). In addition, human hepatocytes transfected with mouse PPARα displayed increased expression of PPARα, and increased peroxisome proliferator response element-reporter activity when treated with TCA and DCA (Walgren et al. 2000).

Even though it is widely assumed that tissue-specific formation of TCE metabolites is one of the critical determinants for the plethora of its adverse health effects, most studies of

TCE toxicokinetics were performed in blood. Few studies evaluated TCE metabolism in tissues, however one study in rats did not find a dose-response relationship in formation of TCE metabolites in liver and kidney (Lash et al. 2006). Thus, we tested a hypothesis that formation of oxidative metabolites of TCE in mouse liver is associated with liver-specific toxicity by evaluating the quantitative relationship between strain-, dose-, and time-dependent formation of TCA and DCA in serum and liver, and various liver toxicity phenotypes in a panel of mouse inbred strains.

II. MATERIALS AND METHODS

Animals and treatments. Male mice (aged 6-7 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in polycarbonate cages on Sani-Chips (P.J. Murphy Forest Products Corp., Montville, NJ) irradiated hardwood bedding. Animals were fed an NTP-2000 (Zeigler Brothers, Inc., Gardners, PA) wafer diet and water *ad libitum* on a 12 h light-dark cycle. All studies were approved by the UNC Institutional Animal Care and Use Committee.

Two study designs were utilized in this work. First, we performed a sub-acute study where vehicle (10 mL/kg, 5% Alkamuls EL-620 in saline) or TCE (600 mg/kg/d, in vehicle) was administered by gavage to mice from 7 inbred strains (129S1/SvImJ, A/J, BTBR T+tf/J, C57BL/6J, CAST/EiJ, NOD/ShiLtJ, and NZW/LacJ) for 5 consecutive days. These strains were selected to maximize inter-strain differences in metabolism of TCE based on the previous study of TCE metabolism in a panel of inbred strains (Bradford et al. 2011) and the results of the statistical modeling of the effect of time and strain on TCE metabolite concentrations which supports the sample size used in this study (Chiu et al. 2014). Second, based on the data from the sub-acute study, we selected two inbred strains (C57BL/6J and

NZW/LacJ) that represented widely varying degrees of formation of oxidative metabolites of TCE for a subsequent sub-chronic study. Specifically, animals of each strain were randomly assigned to one of the three groups (5% Alkamuls EL-620 in saline vehicle, 100, or 400 mg/kg/d of TCE) and were dosed by oral gavage daily for 5 days, 2 weeks, or 4 weeks (the latter two were dosed for 5 days/week).

In all studies, mice were given drinking water containing 0.2 g/L of 5-bromo-2'-deoxyuridine (BrdU) for 72 hrs prior to sacrifice for subsequent proliferation assay. Blood, liver tissues, and a section of a duodenum were collected 2 hrs after the last treatment. The timing of sacrifice was selected based on the toxicokinetic studies of TCE in the mouse (Bradford et al. 2011; Kim et al. 2009b) showing that concentrations of both oxidative and glutathione conjugation metabolites of TCE peak around 2 hrs after dosing. Blood was drawn from vena cava and centrifuged to prepare serum using Z-gel tubes (Sarstedt, Germany) according to the manufacturer's instructions. Body and organ weights were recorded. Liver and duodenum sections were fixed in neutral buffered formalin for 24 hrs, and the remainder of the liver tissue was frozen in liquid nitrogen. All serum and tissue samples were stored at -80°C until analyzed.

Quantification of TCE metabolites. Concentrations of TCA, DCA, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and *S*-(1,2-dichlorovinyl) glutathione (DCVG) in serum and liver were determined using HPLC-ESI-MS/MS as detailed elsewhere (Kim et al. 2009a) with slight modifications. Quantification of trichloroethanol (TCOH) in liver was performed by GC-MS using the method of (Song and Ho 2003). The configuration of the instruments was identical to that in the above mentioned references, but the extraction methods were optimized for each tissue (liver or serum) and metabolite as follows.

TCA and DCA: Serum (50 μL) was mixed with 100 μL of the internal standards (difluoroacetic acid (DFA) and trifluoroacetic acid (TFA), 50 nmol/mL each). Serum proteins were then removed by filter-centrifugation (Microcon YM-10; Millipore, Billerica, MA) at 14,000×g for 1 hr. Liver samples (100 mg) were homogenized in 500 μL of 0.01 M PBS (pH 7.4) with 20 μL of internal standards (DFA and TFA, 20 nmol/mL each) using Tissuelyser (Qiagen, Valencia, CA) for 1 min. The homogenates were filter-centrifuged (Amicon Ultra Centrifugal Filters 10K; Millipore) at 14,000×g for 1 hr. After the filtrate was acidified with 100 μL of 3% (v/v) sulfuric acid, 2 mL of diethyl ether was added and solutions were vortexed vigorously for 1 min. The upper ether layer was transferred to another vial, reduced in volume to less than 300 μL under continuous stream of N_2, then transferred to a glass vial insert containing 5 μl of double-distilled water and dried completely. The residue was reconstituted in 20 μL of HPLC mobile phase consisting of 70% acetonitrile, 30% 1 mM ammonium citrate in double-distilled water. The lower limits of quantification (LLOQ) were: 0.04 nmol/mL in serum and 0.1 nmol/g in liver for DCA, 5 nmol/mL in serum and 8 nmol/g in liver for TCA.

DCVG and DCVC: Serum (50 μL) was mixed with 100 μL of the internal standard solution ([$^{13}C_2$,^{15}N]DCVG and [$^{13}C_3$,^{15}N]DCVC, 5 nmol/mL each). Serum proteins were then removed by filter-centrifugation (Microcon YM-10; Millipore) at 14,000×g for 1 hr. Liver tissue (100 mg) was homogenized in 500 μL of 0.01 M PBS (pH 7.4) with 20 μL of internal standards ([$^{13}C_2$,^{15}N]DCVG and [$^{13}C_3$,^{15}N]DCVC, 25 nmol/mL each) using Tissuelyser (Qiagen) for 1 min. The homogenates were filter-centrifuged (Amicon Ultra Centrifugal Filters 10K; Millipore) at 14,000×g for 1 hr. From each prepared sample, DCVG and DCVC were extracted using a solid phase extraction cartridge (StrataTM X-AW, 30mg 96-well plate;

Phenomenex, Torrance, CA). The cartridges were conditioned with 300 μL of methanol, followed by equilibration with 300 μL of water. Samples were loaded, washed with 300 μL of water, and eluted with 250 μL of basic methanol (pH adjusted to 10.8 by 29.1% NH_4OH). The final eluent was collected in 300 μL glass vial inserts and dried in a SpeedVac Concentrator before reconstitution with 20 μL of 4:1 water/methanol containing 0.1% acetic acid. The LLOQ were: 0.5 pmol/mL in serum and 2 pmol/g in liver for DCVG, 1 pmol/mL in serum and 20 pmol/g in liver for DCVC.

TCOH: Liver tissue (30 mg) was homogenized in 500 μL of sodium acetate buffer (pH 4.6) with 1,000 units of β-glucuronidase (Sigma [G0751], St. Louis, MO) using Tissuelyser (Qiagen) for 1 min, followed by overnight incubation at 37°C. After centrifugation at 14,000×g for 5 min, the supernatant was transferred to a new tube, then mixed with 20 μL internal standard (DCA, 10 mM in methanol) and 550 μL of water/0.1 M sulfuric acid/methanol (6:5:1). The mixture was heated at 70°C for 20 min. After cooling to room temperature, 2.5 mL hexane was added, the mixture vortexed for 10 min and centrifuged at 2,500×g for 2 min. The upper layer was concentrated under a stream of N_2 to less than 20 μL and used for GC-MS analysis as detailed in (Song and Ho 2003). The LLOQ was 5 nmol/g in liver.

Gene expression analysis by real-time PCR. Total RNA was isolated from liver samples using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2000 Bioanalyser, respectively. Total RNA was reverse transcribed using random primers and the high capacity complementary DNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The

following gene expression assays (Applied Biosystems) were used for quantitative real-time PCR: PPARα (*Ppara*, Mm00440939_m1); palmitoyl acyl-Coenzyme A oxidase 1 (*Acox1*, Mm01246831_m1); cytochrome P450, family 4, subfamily a, polypeptide 10 (*Cyp4a10*, Mm01188913_g1); and beta glucuronidase (*Gusb*, Mm00446953_m1). Reactions were performed in a 96-well plate, and all samples were plated in duplicate using a LightCycler® 480 instrument (Roche Applied Science, Indianapolis, IN). The cycle threshold (Ct) for each sample was determined from the linear region of the amplification plot. The ΔCt values for all genes relative to the control gene *Gusb* were determined. The ΔΔCt were calculated using treated group means relative to strain-matched control group means. Fold change data were calculated from the ΔΔCt values.

Protein level measurements. Proteins were extracted from 20 mg of frozen liver samples using T-PER® Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) and Halt™ Protease Inhibitor Cocktail (Pierce Biotechnology) according to the manufacturer's instructions. Protein concentration was measured using Pierce® BCA Protein Assay Kit (Pierce Biotechnology) and a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA). Extracts containing 30 μg of protein were resolved on 12% polyacrylamide sodium dodecyl sulfate-containing gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in Odyssey® Blocking Buffer (LI-COR, Lincoln, NE) and probed with 1:5000 diluted anti-Cytochrome P450 2e1 antibody (Abcam, Cambridge, MA), anti-Aldh2 antibody (Abcam), or anti-Adh antibody (Abcam) overnight at 4°C. Blots were washed in 0.1% Tween20 in 0.01 M PBS, incubated with 1:20,000 IRDye® 680LT donkey anti-rabbit IgG (LI-COR), and detected using an Odyssey Infrared Imaging System (LI-COR). Equal protein loading was confirmed by total protein staining with 0.1% (w/v)

naphthol blue black in 7% (v/v) acetic acid in water for 10 min. The signal intensity was analyzed by Image Studio Software (LI-COR).

Statistical analysis. The significance of inter-strain effect on metabolism was assessed by ANOVA modeling. Given the small sample size, the exact permutation test was used to determine significant differences between control and TCE-treated groups (α=0.05). The Spearman (rank) correlation analysis across all variables was conducted to account for the difference in scale of the variables. In correlation analyses, false discovery rate correction (Storey and Tibshirani 2003a) was applied to all *p*-values to correct for multiple comparisons. The resultant significant (<0.1) *q*-values are reported in the Supplemental Tables. All statistical analyses were performed using SAS software ver. 9.3 (SAS Institute, Cary, NC).

III. RESULTS

Sub-acute (5 days) study of strain-dependent effects of TCE in mouse liver

We evaluated serum and liver levels of TCE metabolites from P450-mediated oxidation (TCA, DCA, TCOH) and glutathione conjugation (DCVC and DCVG) in seven mouse inbred strains (Figure 1). Mice were treated for 5 days by oral gavage of 600 mg/kg of TCE in aqueous vehicle. Across all strains, serum levels (Figure 1, left panel) of TCA were on average 1,000-fold greater than those for DCA, and the amounts of DCA were about 100-fold higher than those of either DCVC or DCVG. Higher levels of TCA (about 2-fold) and DCA (about 10-fold) were found in liver (Figure 1, right panel) than in serum, but the relative ratio of TCA to DCA was similar. The levels of TCOH, also a major TCE metabolite in liver, were comparable to those of TCA. Levels of DCVG were much higher in liver than in serum (about 100-fold) and only about 10-fold lower than those of DCA. We observed a substantial difference between the concentrations of DCVG and DCVC in liver. While the

amounts of DCVG and DCVC in serum were comparable (<2-fold difference), the concentration of DCVG in liver was at least 50 to 100-fold higher than that of DCVC, which was below the LLOQ in most samples. We found a significant strain effect (p<0.05) in both serum and liver for every metabolites examined (TCA, DCA, TCOH, DCVG, and DCVC).

We examined the relationships between TCE metabolites in either serum or liver (Supplemental Table 1). In serum, we found no significant correlations among 5 metabolites. In liver, levels of TCA and DCA were significantly correlated (ρ=0.74, q=0.001). Although both TCA and TCOH are major oxidative metabolites, we found no correlation between their levels in liver. Inter-tissue (liver *vs* serum) correlation of TCE metabolites was also evaluated. We found significant correlation between liver and serum levels for TCA (ρ=0.62, q=0.020), serum TCA and liver DCA (ρ=0.78, q=0.001), and serum DCVC and liver DCVG (ρ=0.67, q=0.017).

To determine whether inter-strain differences in TCE metabolism may be due to strain-dependent variability in expression of Cyp2e1, we measured the levels of liver Cyp2e1 protein in vehicle- and TCE-treated animals (Figure 2). A significant strain effect (p<0.05) was found in baseline liver Cyp2e1 levels. However, treatment with TCE was without effect on liver Cyp2e1 across all strains. Interestingly, the background liver levels of Cyp2e1 were not correlated (ρ=0.74, p=0.055) with the amount of TCA in liver of TCE-treated animals.

Liver size (relative to body weight) and hepatocellular proliferation were examined in vehicle- and TCE-treated mice (Figures 3A-B). We observed significant effects of sub-acute treatment with TCE on liver enlargement in 129S1/SvlmJ, NOD/ShiLtJ and BTBR T+tf/J, and on hepatocyte proliferation in NOD/ShiLtJ, BTBR T+tf/J, and NZW/LacJ strains. Because peroxisome proliferation has been suggested as contributing to hepatomegaly in

rodents (Marsman et al. 1988), we evaluated expression of *Cyp4a10* and *Acox1*, marker genes for this mechanistic event, in mouse liver. Expression of the transcription factor *Ppara*, was not affected by TCE (data not shown); however, expression of *Cyp4a10* and *Acox1* was markedly elevated in all strains, except for CAST/EiJ (Figures 3C-D), with TCE-induced effect being greater for *Cyp4a10* than for *Acox1*.

Because inter-strain differences in both TCE metabolism and liver effects were observed in this study, we examined the associations among these endpoints in liver (Supplemental Table 2). Levels of TCA and DCA were strongly correlated with expression of *Acox1* and *Cyp4a10* (Figures 4A-B). However, neither the expression of *Acox1* and *Cyp4a10,* nor the level of TCA, was correlated with hepatocellular proliferation (Figures 4C and 4D, respectively).

Sub-chronic (up to 4 weeks) study of strain-dependent effects of TCE in mouse liver

Based on the differences in TCE metabolism observed in the sub-acute (5 days) TCE exposure studies, we selected C57BL/6J and NZW/LacJ strains to further test our hypothesis that inter-strain differences in amounts of oxidative metabolites of TCE in the liver are associated with liver-specific toxicity. In these studies, we examined the time-dependent (1, 2 and 4 weeks) and dose-dependent (100 and 400 mg/kg/day, *i.g.*) effects of TCE.

As expected, the difference in TCE metabolism between two strains was also observed at lower doses and in longer-term studies (Figure 5). Serum and liver levels of TCA and DCA were higher in NZW/LacJ mice, and liver levels of DCVG were higher in C57BL/6J mice. Similar to the findings in the sub-acute study, levels of TCA and TCOH in liver were 100 to 1,000-fold greater than those of DCA, and levels of DCA were 10 to 100-fold higher than those of DCVG. Levels of DCVC in serum and liver were below the LLOQ.

Amounts of all measured TCE metabolites in liver and serum generally trended downward over time. Specifically, the levels of TCA in serum and liver decreased over time in both strains, while the levels of TCOH in liver decreased over time only in C57BL/6J strain. Likewise, the level of DCVG in liver was highest after 5 days of treatment.

Among TCE metabolites that were measured in this study, serum and liver levels of TCA and DCA were highly correlated ($q<0.001$) across all animals in the study (Supplemental Table 3). Liver levels of TCOH exhibited similar strong correlation with the levels of TCA and DCA in either serum or liver. No correlation was found among the levels of DCVG in liver and other TCE metabolites.

To examine whether strain-dependent changes in TCE-metabolizing enzymes could account for the observed decrease in metabolite formation over time, we examined the levels of Cyp2e1, Aldh2, and Adh proteins in liver, and found that expression of these enzymes was not affected by TCE treatment (Figure 6).

We also examined the effects of sub-chronic treatment with TCE on liver weight and the marker genes of hepatocellular proliferation and peroxisome proliferator expression. Even though the amount of oxidative metabolites of TCE trended downward over time, the increased liver to body weight ratio was still observed in mice treated with 2 week or 4 week in as strain-dependent manner. In case of hepatocellular proliferation, most prominent effects were observed at the 4 week time-point (Figures 7A-B). Histopathological evaluation of the liver sections revealed concordant increases in relative size of the hepatocytes, as well as hemosiderin deposits in the high-dose 4 week treatment groups of both strains (Supplemental Figure 1). A prominent effect of TCE on peroxisome proliferation in the liver was observed in the sub-chronic study. Although TCE did not affect expression of *Ppara* (data not shown),

expression of *Acox1* and *Cyp4a10* was strongly induced in a dose-dependent manner in both strains of mice treated with TCE for 5 days (Figures 7C-D). Interestingly, the magnitude of upregulation of *Acox1* and *Cyp4a10* by TCE diminished over time. In the 2 and 4 week treatment groups, there was either no difference in gene expression between vehicle and TCE-treated animals, or differences were less prominent.

Similar to the result of the sub-acute study, the levels of TCA and DCA were significantly correlated with *Acox1* and *Cyp4a10* expression in liver regardless of TCE dose or treatment duration (Figures 8A-B). The *Cyp4a10* expression was significantly correlated with liver-to-body weight ratio (ρ=0.68, q<0.001, Figure 8C); however, hepatocellular proliferation did not correlate with other variables (Figure 8D, Supplemental Table 4).

IV. DISCUSSION

The challenge of addressing the variability in susceptibility to environmental exposures is frequently one of the most contentious issues in human health assessments. Because of the heterogeneity of the human population, it is generally expected that there will be a broad range of toxicokinetic and toxicodynamic responses to chemicals or drugs (Zeise et al. 2013). Traditionally, the inter-individual differences in the toxicokinetics are accounted for by default assumptions and only in rare cases, are based on human toxicokinetic data. Seldom is there sufficient data to evaluate the extent of variability in toxicodynamics.

Because TCE metabolism to form chloroacetic acids and GSH-conjugates is widely accepted as the mechanism leading to toxicity in various organs (Lash et al. 2001), the interplay between toxicokinetics and toxicodynamics is a critically important consideration in the evaluation of human health hazard of TCE. Some understanding of the human population variability in toxicokinetics of TCE is available based on the limited data from clinical

studies and Bayesian modeling (Chiu et al. 2009). The metabolism of TCE across species (*e.g.*, rodents and humans) is qualitatively similar (Lash et al. 2014), and thus inter-species and –individual variability in TCE toxicity is likely due to the variability in TCE metabolism (Chiu et al. 2013; Green 1990). TCE toxicity is also dose-dependent, which suggests the link between toxicokinetics and toxicodynamics (Chiu et al. 2013). It is yet to be experimentally demonstrated, however, that inter-individual variability in TCE metabolism, not exposure (*i.e.*, dose), will result in quantitative differences in its toxicity. In this regard, an examination of the relationship between the variability of metabolism and the variability in toxicity may shed light on our understanding of human health hazard of TCE. Therefore, the purpose of this study was to use a population-based approach to test the hypothesis that TCE-induced toxicity in liver is associated with the liver-specific formation of oxidative TCE metabolites.

In our previous work, we demonstrated that both oxidative metabolism and GSH conjugation of TCE vary considerably among inbred mouse strains (Bradford et al. 2011), and that such variability was associated with strain-specific differences in gene expression in mouse liver. While these results allowed for a quantitative evaluation of the relationship between metabolism and TCE-induced gene expression in the liver, the focus of the study was on toxicokinetic profiling over a 24 hour time period and the use of one high dose of TCE (2.1 g/kg, *i.g.*). To further explore the time- and dose-relationships between TCE metabolism and toxicity in the context of inter-strain variability, we conducted a series of studies that aimed to quantitate the levels of TCE metabolites in serum and liver. We also evaluated cell proliferation and peroxisome proliferation, two widely accepted liver toxicity phenotypes reflective of the major mechanistic events considered to play a role in liver carcinogenesis of TCE in rodents (Rusyn et al. 2014).

The first important and novel outcome of this study is quantitative data on a broad range of metabolites produced via oxidative metabolism and GSH conjugation of TCE in serum and liver in the context of inter-strain variability in TCE metabolism. While many published reports provide quantitative information on serum levels of TCA along with a few reports of DCA after treatment with TCE in rodents and humans, little information exists on the formation of these metabolites in the liver. Similar to our findings in the single-dose studies in mouse serum (Bradford et al. 2011; Kim et al. 2009b), there is up to 5 orders of magnitude difference in the relative flux of TCE through CYP450-dependent oxidation (primarily TCA) compared to GSH conjugation. The similarities in the levels of TCE metabolites formed through oxidation and GSH conjugation in this and our previous studies confirm that GSH conjugation of TCE is a minor pathway in mice, regardless of the dose or duration of treatment.

In the mouse liver, TCA was also the predominant metabolite formed (about 2 orders of magnitude greater than DCA); however, while the difference in relative flux of TCE through CYP450-dependent oxidation compared to GSH conjugation was still large, it was smaller than that in serum, about 3 orders of magnitude. In the rat liver, TCA and TCOH are also predominant TCE metabolites and there is about 100-fold difference between CYP450-dependent oxidative metabolism and GSH-conjugation (Lash et al. 2006). TCE metabolism by CYP450-dependent pathway is known to occur at a faster rate in mice than in rats (Prout et al. 1985), and our data are in line with these observations.

Serum levels of TCA and DCA were not correlated with each other, which is consistent with our previous study (Kim et al. 2009b) that postulated that DCA formation is not occurring exclusively from TCA but also from dechlorination of dichloroacetyl chloride.

However, we found that liver levels of TCA and DCA did correlate significantly, which supports a hypothesis that TCA is the major precursor for the formation of DCA in this organ (Ketcha et al. 1996). A detailed liver toxicokinetic study of oxidative metabolites of TCE may be necessary to further characterize the metabolic fate of TCA and DCA.

Our finding that the concentration of DCVG in liver was much higher than that in serum and higher than the concentration of DCVC in either serum or liver further demonstrates that TCE conjugation with GSH to form DCVG occurs predominantly in the liver (Lash et al. 2014). Given that DCVG is rapidly excreted into the bile (van Bladeren 2000), the relative flux of TCE through GSH conjugation in the liver may be even greater than that observed in our study. As it has been suggested that GSH conjugation may be much greater in humans than in rodents (Lash et al. 1999b), careful estimation of the biliary excretion of GSH conjugates of TCE may be needed to completely understand the kinetics of this metabolic pathway.

Based on the levels of TCA and DCA observed in the sub-acute study (Figure 1), two strains were chosen, C57BL/6J and NZW/LacJ, which represent low or high levels of CYP450-dependent oxidation of TCE, respectively. Time-course analysis of TCE metabolites in liver showed a decreasing trend of TCA and TCOH concentration, especially in the high dose (400 mg/kg/day) group. Previous studies of TCE metabolism showed that under conditions of acute exposure in mice, the induction of the monooxygenase system results in greater liver metabolism of TCE (Dekant et al. 1986a) and no apparent saturation of metabolism is observed (Prout et al. 1985). However, saturation of TCE metabolism does occur in the rat liver at high doses (Dekant et al. 1986a; Prout et al. 1985). A long-term study

with TCE in rats and mice showed that daily dosing for 180 days did not induce the overall metabolism of TCE, but did double the urinary excretion of TCA (Green and Prout 1985). The time-dependent change in kinetics may be explained by either auto-induction (Chaudhry et al. 2010) or depletion of co-substrates such as NADPH, NADH, and NAD^+ (Lipscomb et al. 1996). Auto-induction may not explain our findings because Cyp2e1, Aldh2, and Adh, major enzymes involved in oxidative metabolism of TCE, were not affected by TCE dose or by the duration of exposure. In addition, it is possible that a decrease in TCE metabolism may be due to insufficient availability of co-substrates that are affected by not only by the extent of metabolism, but also by the redox status of the liver. Oxidative stress, as a secondary event that follows cytotoxicity and peroxisome proliferation in the liver, is one such mechanistic event. Several studies that examined TCA- and DCA-induced hepatic oxidative stress demonstrated small, albeit significant, increases in lipid peroxidation and oxidative DNA damage (Austin et al. 1996; Parrish et al. 1996).While we reason that the time-dependent decrease in TCE metabolism observed herein may plausibly result from the saturation of co-substrate supply or from oxidative stress, these mechanisms need further study.

The second important and novel outcome of our work is the investigation of the quantitative relationships among TCE metabolite levels and liver toxicity phenotypes. Because significant inter-strain variability was observed in TCE metabolism and liver toxicity, this study offers a unique opportunity to provide a scientific data-driven rationale for some of the major assumptions in human health assessment of TCE and other chlorinated solvents. Specifically, we examined major metabolizing enzymes responsible for oxidative

biotransformation of TCE, as well as markers of cell proliferation and peroxisome proliferation in liver.

Inter- and intra-species differences in oxidative metabolism of TCE have been examined in relation to the expression levels of key xenobiotic metabolism enzymes, primarily CYP2E1 (Lash et al. 2014). We observed a correlation between the amounts of TCA produced in liver and a baseline Cyp2e1 protein level across strains. This finding may be attributable to the previously reported observation that not only is CYP2E1 involved in the first step of TCE oxidation, but CYP2E1 also catalyzes the transformation of TCOH to TCA (Stenner et al. 1997). CYP2E1 is not the only CYP450 enzyme that may act on TCE. For example, liver Cyp2e1 content was found to be similar in rats and mice (Nakajima et al. 1993) even though major differences in TCE metabolism are known to exist between mice and rats. Human CYP1A1/2, CYP2A6, and CYP3A4 are also known to oxidize TCE, with CYP1A2 being the major alternative to CYP2E1 (Lash et al. 2014).

A recent study using a mouse model of the human population showed that TCE metabolism was strongly associated with induction of PPARα-mediated lipid and nucleic acid metabolism pathways in mouse liver regardless of the genetic background (Bradford et al. 2011). In our study, we found that under conditions of sub-acute and sub-chronic treatment with TCE, significant up-regulation of *Acox1* and *Cyp4a10* expression occurred in 6 out of 7 strains and the extent of gene expression was correlated with the liver levels of TCA and DCA. Strong correlation between TCA and DCA levels in liver makes it difficult to interpret their relative contributions to the induction of peroxisome proliferation response. However, DCA is widely regarded as a weaker ligand for PPARα activation compared to

TCA (Corton 2008), and the amount of DCA detected in this study in the liver is orders of magnitude lower than that of TCA.

Similar to an observed time-dependent decrease in TCE metabolite formation, the expression levels in *Acox1* and *Cyp4a10* decreased over time in the sub-chronic study, which indicates a close association between peroxisome proliferation and the oxidative metabolism of TCE. Contrary to the decrease in the oxidative metabolism and peroxisome proliferation over time, liver enlargement and hepatocellular proliferation effects were most prominent in mice treated with TCE for 4 weeks. Neither TCA nor DCA in liver was correlated with hepatocellular proliferation in the sub-chronic study, which may suggest that multiple metabolites or pathways are likely to be involved in liver toxicity due to TCE (Rusyn et al. 2014).

V. FIGURES AND TABLES

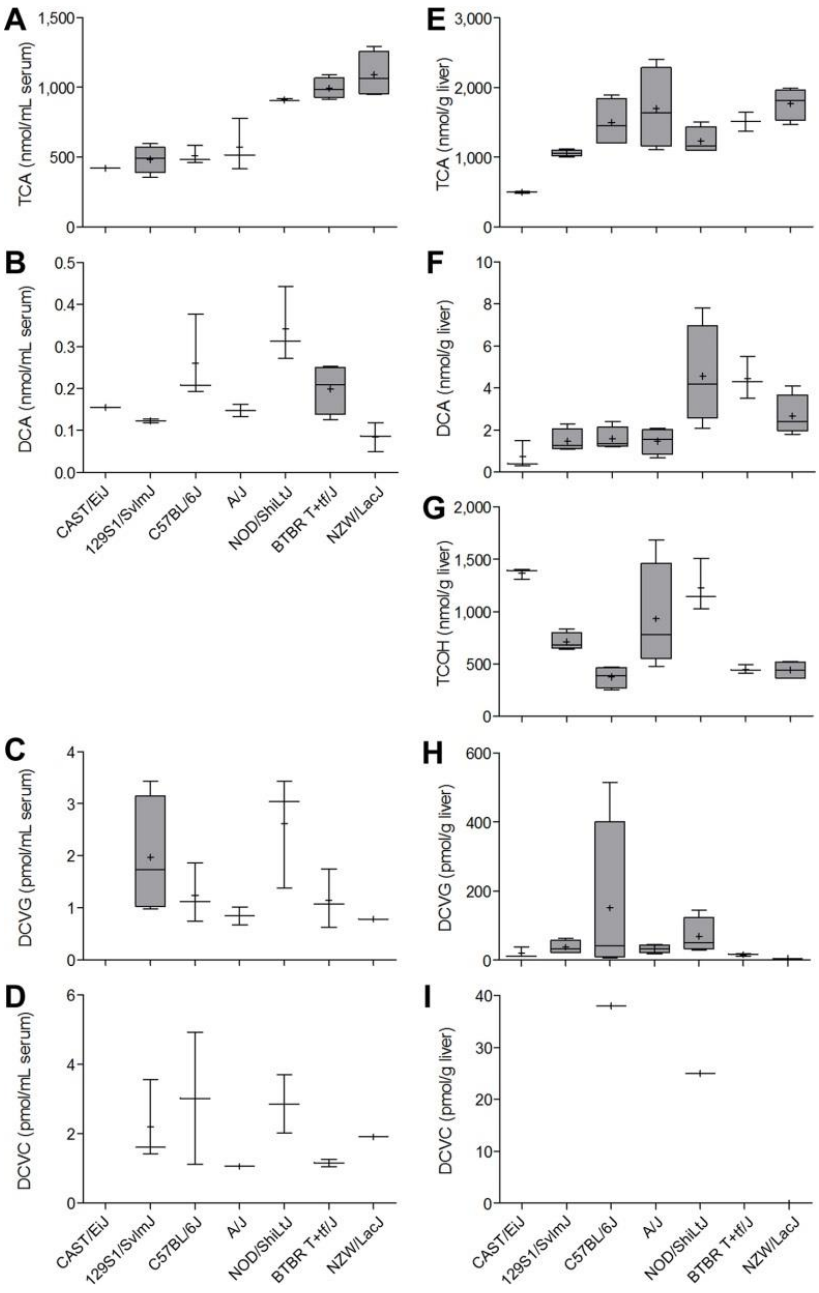


Figure 2.1. Inter-strain variability in TCE metabolism in the mouse in a sub-acute study. Serum (A-D) and liver (E-I) levels of metabolites were assessed 2 h following the last of 5 daily doses (600 mg/kg) of TCE. Box and whisker plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group.

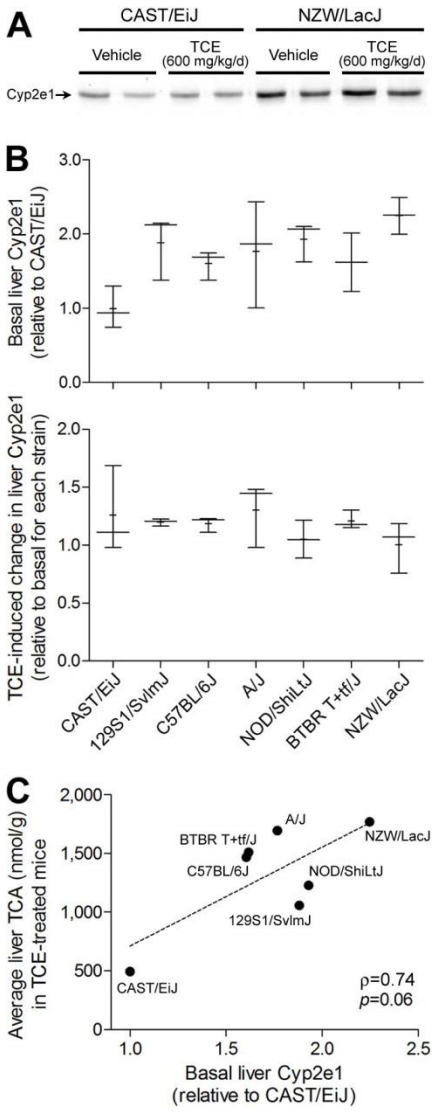


Figure 2.2. Relationships between TCE metabolism in a sub-acute study and liver Cyp2e1 protein levels. (A) A representative Western blot of Cyp2e1 protein expression in liver of vehicle- and TCE-treated mice from CAST/EiJ and NZW/LacJ strains. (B) Inter-strain differences in basal and TCE-induced liver expression of Cyp2e1. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). There were 3 animals per group. (C) Correlation between basal liver expression of Cyp2e1 and liver TCA amounts in TCE-treated mice of 7 strains. Each dot represents a mouse strain. Spearman rank (ρ) correlation is shown.

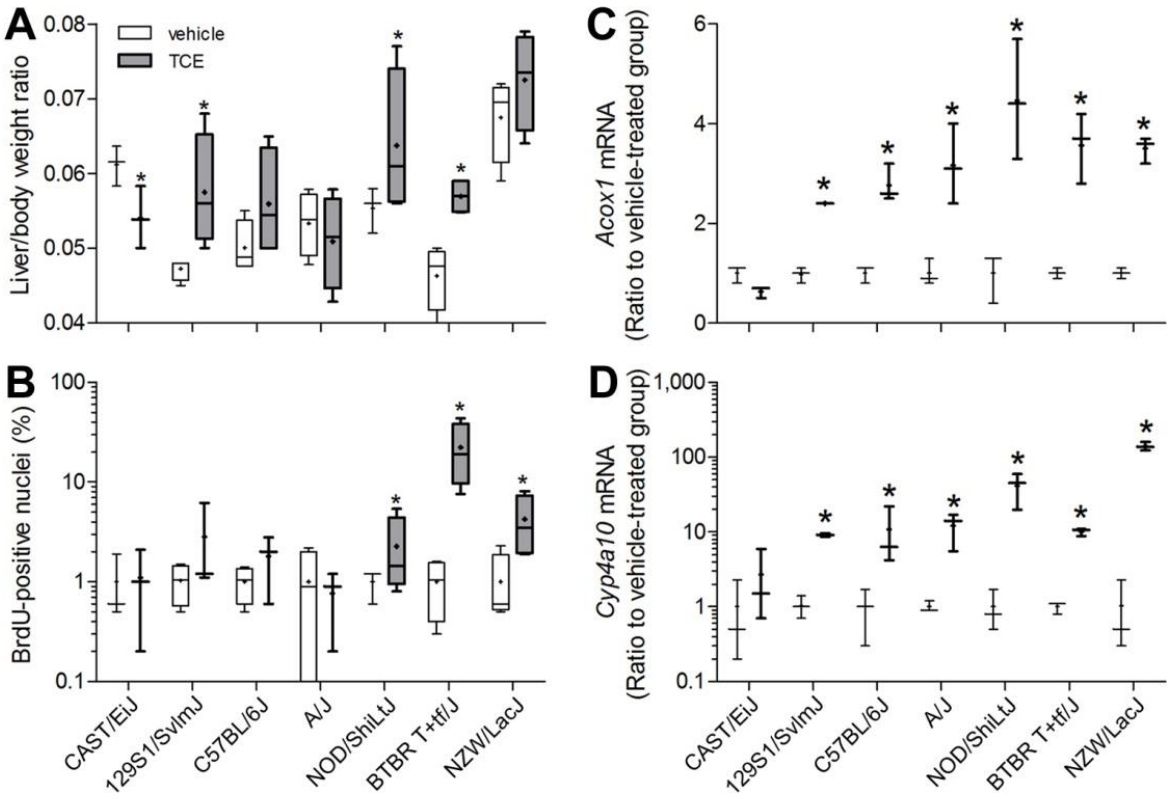


Figure 2.3. Inter-strain differences in liver toxicity of TCE in a sub-acute study. Liver to body weight ratios (A), percent BrDU-positive hepatocyte nuclei (B), and liver expression of peroxisome proliferation marker genes *Acox1* (C) and *Cyp4a10* (D) were evaluated in mice treated with vehicle (white) or TCE (black; 600 mg/kg) for 5 days. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group. Asterisk (*) denotes a significant difference ($p<0.05$) compared to vehicle-treated group within same strain.

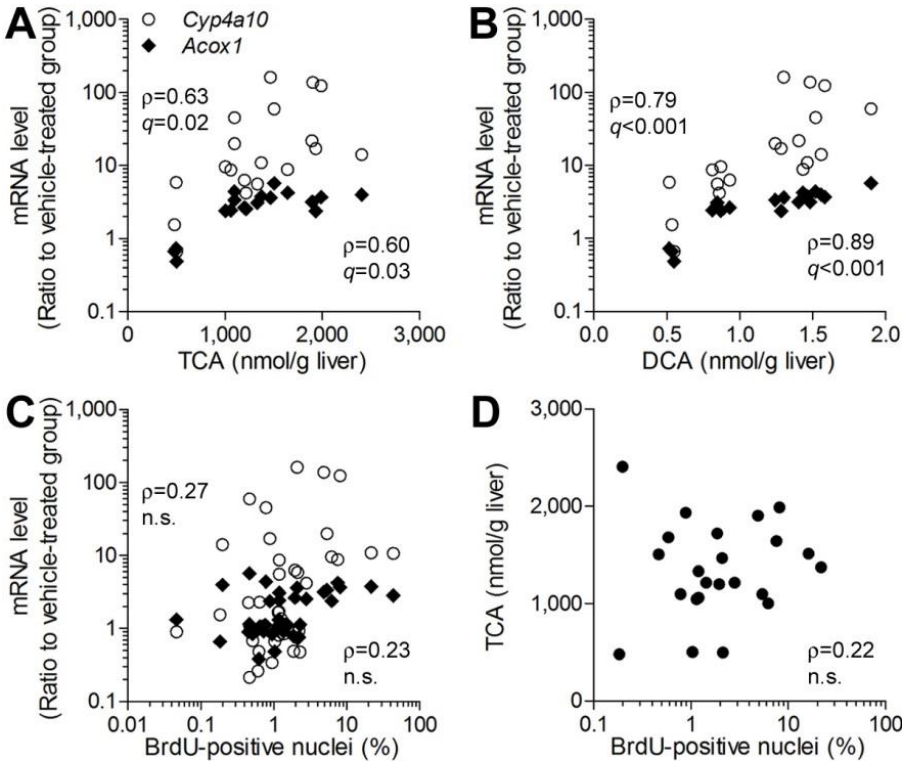


Figure 2.4. Correlation between TCE metabolites and liver toxicity phenotypes. TCE-induced increase in *Cyp4a10* and *Acox1* expression in the mouse liver was significantly correlated with liver levels of TCA (A) and DCA (B), and hepatocellular proliferation (C). Weak correlation was observed between TCA concentration in the liver and hepatocellular proliferation (D). Each symbol represents an individual animal in the study. Spearman rank (ρ) correlations and false discovery rate-corrected significance values (q<0.1) are shown.

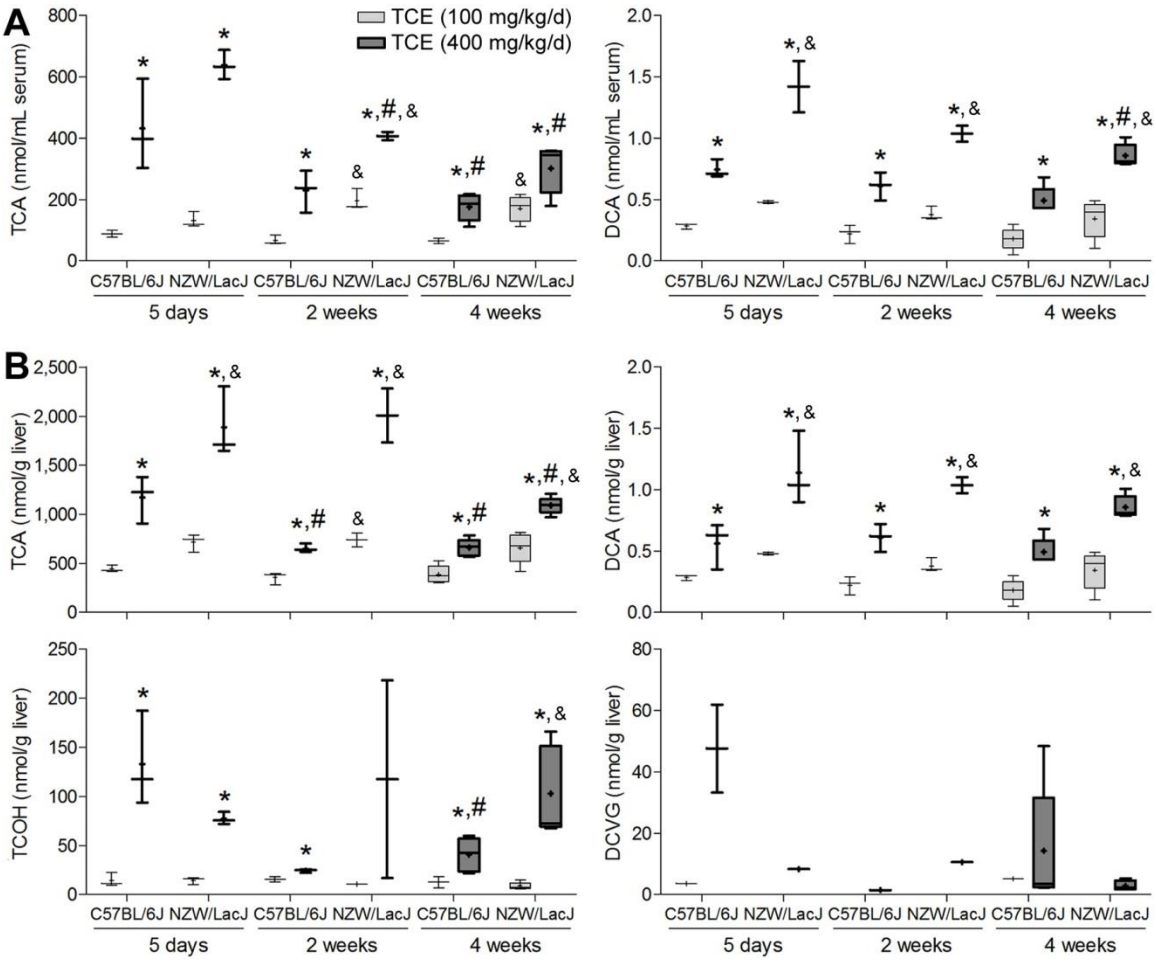


Figure 2.5. Time-course analysis of TCE metabolism in C57BL/6J and NZW/LacJ mice in a sub-chronic study. Serum (A) and liver (B) levels of metabolites were assessed 2 h following the last dose after 1, 2 or 4 wks (100 or 400 mg/kg/d) of TCE. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). Light-gray, 100 mg/kg/d groups; dark gray, 400 mg/kg/d groups. When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group. Asterisks denote a significant ($p<0.05$) difference as compared to (*) the group dosed with 100 mg/kg/d (same strain and time point), (#) the 5 day treatment group (same strain and dose), or (&) the values in C57BL/6J strain (same dose and time point).

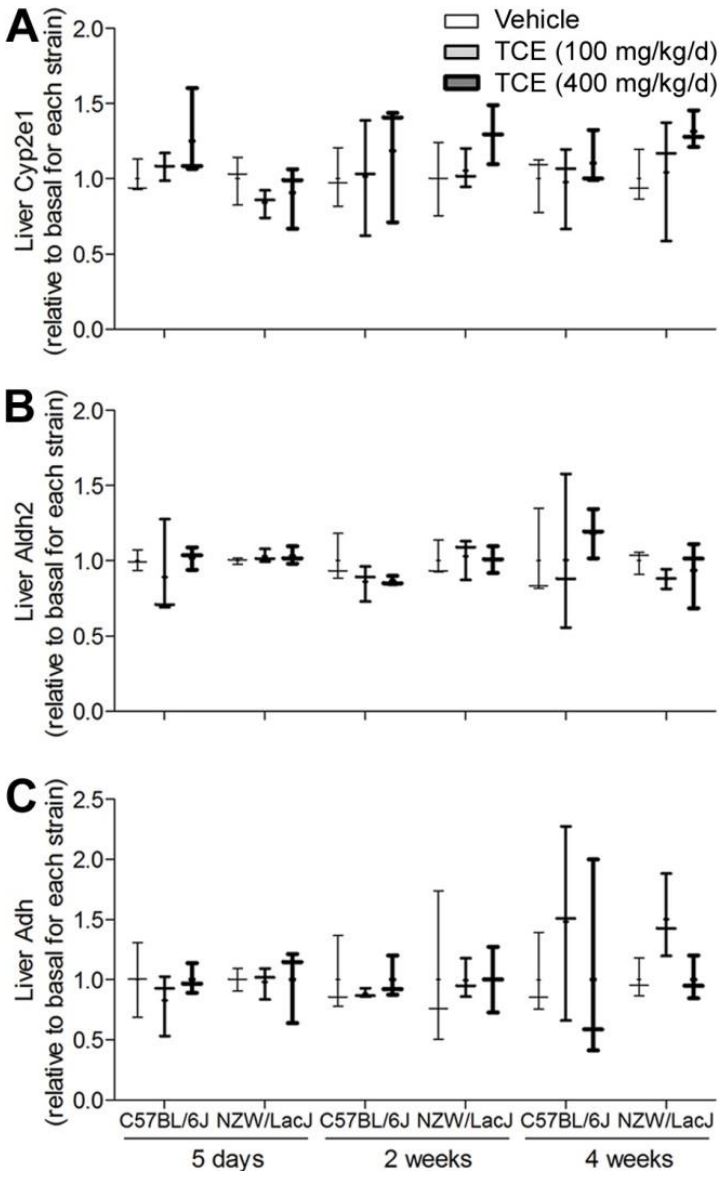


Figure 2.6. The effect of a sub-chronic treatment with TCE on liver Cyp2e1 (A), Aldh2 (B) and Adh (C) protein levels in C57BL/6J and NZW/LacJ mice. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). Thickness of the line corresponds to the vehicle, 100 and 400 mg/kg/d groups. There were 3 animals per group.

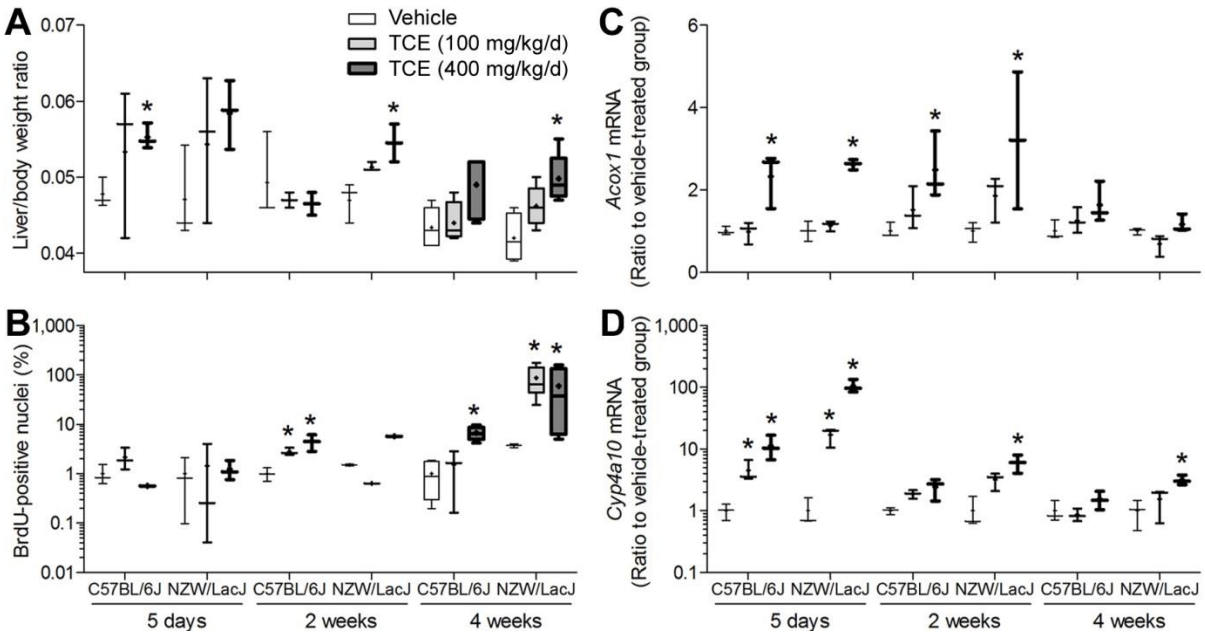


Figure 2.7. Differences in liver toxicity of TCE in C57BL/6J and NZW/LacJ mice in a sub-chronic study. Liver to body weight ratios (A), percent BrDU-positive hepatocyte nuclei (B), and liver expression of peroxisome proliferation marker genes *Acox1* (C) and *Cyp4a10* (D) were evaluated in mice treated with TCE (100 or 400 mg/kg) for 1, 2 or 4 wks. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). White, vehicle-treated groups; light-gray, 100 mg/kg/d groups; dark gray, 400 mg/kg/d groups. When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group. Asterisk (*) denotes a significant ($p<0.05$) difference as compared to vehicle-treated group (same strain and time point).

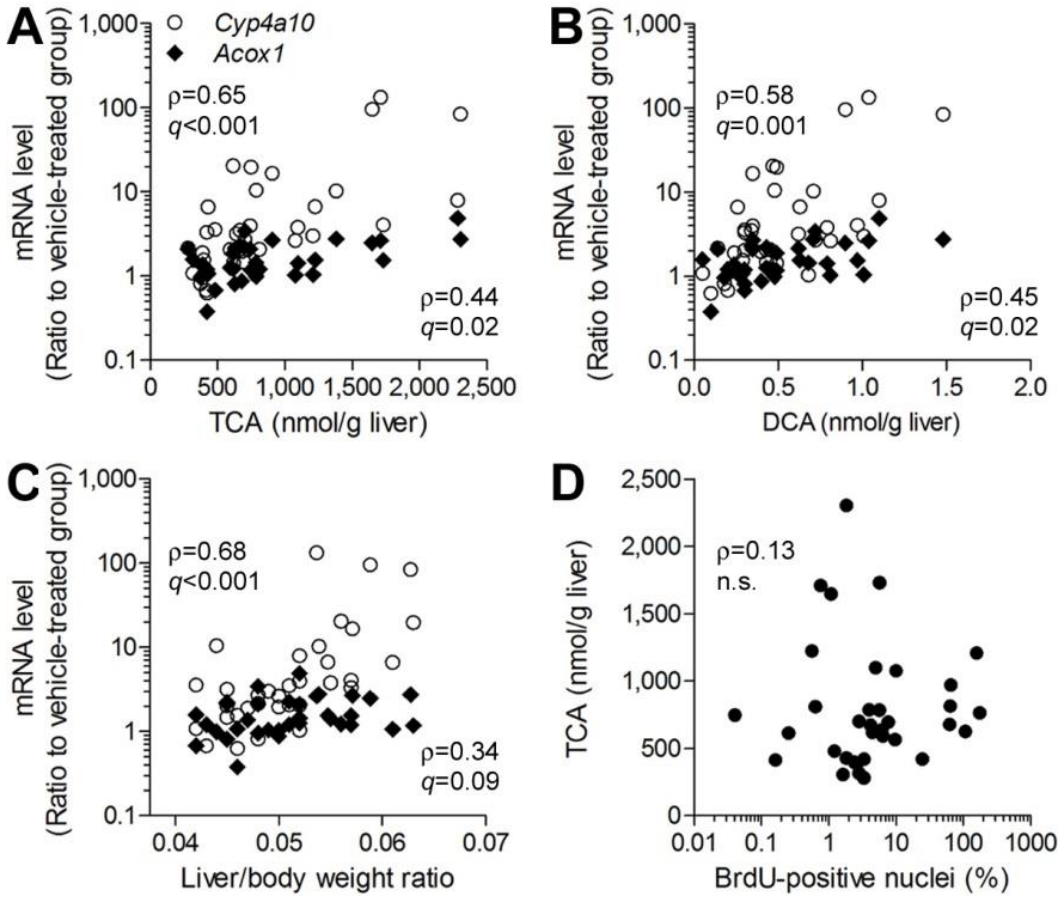


Figure 2.8. Correlation between TCE metabolites and liver toxicity phenotypes in C57BL/6J and NZW/LacJ mice in a sub-chronic study. TCE-induced increase in *Cyp4a10* and *Acox1* expression in the mouse liver was significantly correlated with liver levels of TCA (A) and DCA (B), and liver to body weight ratio (C). (D) No correlation was observed between TCA concentration in the liver and hepatocellular proliferation. Each symbol represents an individual animal in the study. Spearman rank (ρ) correlations and false discovery rate-corrected significance values (q<0.1) are shown.

Supplementary Table 1. Correlations among TCE metabolites in serum and liver in TCE-treated mice in a sub-acute study (600 mg/kg/d for 5 days).

		TCA in serum	DCA in serum	DCVG in serum	DCVC in serum	TCA in liver	DCA in liver	TCOH in liver	DCVG in liver
DCVG in liver	ρ	-0.49	0.54	0.77	0.67	-0.10	0.09	0.40	1
	q	-	-	-	**0.017**	-	-	-	
	n	21	17	6	19	26	26	25	26
TCOH in liver	ρ	-0.28	0.13	-0.1	0.26	-0.28	-0.12	1	
	q	-	-	-	-	-	-		
	n	20	16	5	18	25	25	25	
DCA in liver	ρ	0.78	0.19	0.37	0.00	0.74	1		
	q	**0.001**	-	-	-	**0.001**			
	n	21	17	6	19	26	26		
TCA in liver	ρ	0.62	-0.24	-0.2	-0.09	1			
	q	**0.020**	-	-	-				
	n	21	17	6	19	26			
DCVC in serum	ρ	-0.48	0.03	0.54	1				
	q	-	-	-					
	n	19	15	7	20				
DCVG in serum	ρ	-0.29	-0.31	1					
	q	-	-						
	n	7	6	7					
DCA in serum	ρ	-0.11	1						
	q	-							
	n	18	18						
TCA in serum	ρ	1							
	q								
	n	22							

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 2.2. Correlations among TCE metabolites and toxicity phenotypes in the liver of TCE-treated mice in a sub-acute study (600 mg/kg/d for 5 days). Data from vehicle-treated samples were not included in the analyses.

		TCA	DCA	TCOH	DCVG	Liver/Body weight ratio	*Acox1*	*Cyp4a10*	Hepato-cellular proliferation
Hepatocellular proliferation	ρ	0.22	0.30	-0.43	-0.39	0.16	0.23	0.27	1
	q	-	-	-	-	-	-	-	
	n	23	23	22	23	24	20	20	24
Cyp4a10	ρ	0.63	0.79	-0.13	0.00	0.35	0.68	1	
	q	**0.022**	**<0.001**	-	-	-	**0.006**		
	n	19	19	18	19	20	20	20	
Acox1	ρ	0.60	0.89	-0.15	0.11	0.09	1		
	q	**0.031**	**<0.001**	-	-	-			
	n	19	19	18	19	20	20		
Liver/Body weight ratio	ρ	-0.03	0.15	-0.42	-0.38	1			
	q	-	-	-	-				
	n	26	26	25	26	27			
DCVG	ρ	-0.10	0.09	0.40	1				
	q	-	-	-					
	n	26	26	25	26				
TCOH	ρ	-0.28	-0.12	1					
	q	-	-						
	n	25	25	25					
DCA	ρ	0.74	1						
	q	**<0.001**							
	n	26	26						
TCA	ρ	1							
	q								
	n	26							

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 2.3. Correlations among TCE metabolites in serum and liver in TCE-treated mice in a sub-chronic study (100 or 400 mg/kg/d for 1, 2 or 4 wks).

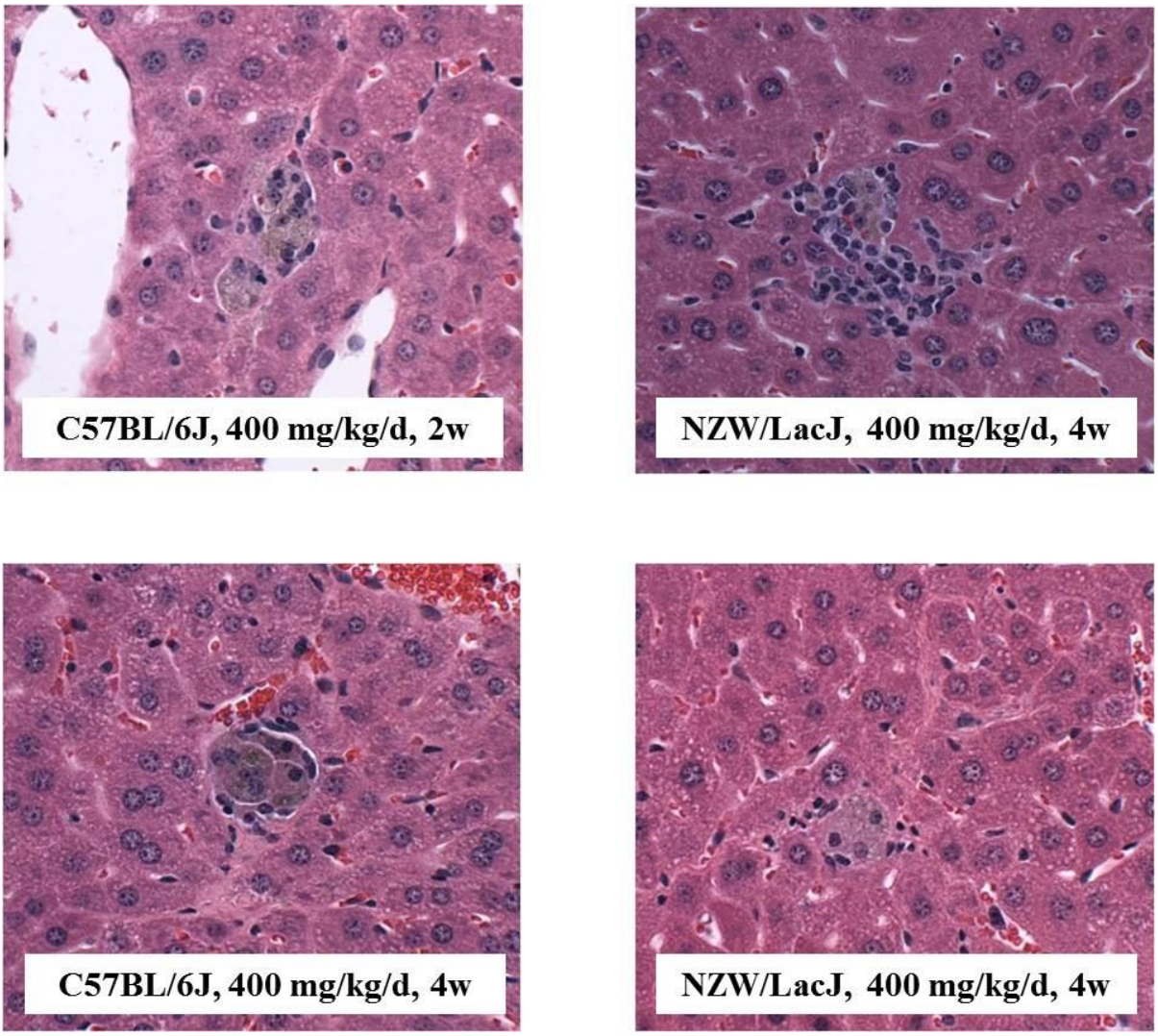
		TCA in serum	DCA in serum	TCA in liver	DCA in liver	TCOH in liver	DCVG in liver
DCVG in liver	ρ	0.04	-0.09	0.20	-0.13	0.41	1
	q	-	-	-	-	-	
	n	15	15	16	16	16	16
TCOH in liver	ρ	0.65	0.72	0.60	0.65	1	
	q	**<0.001**	**<0.001**	**<0.001**	**<0.001**		
	n	36	36	37	37	37	
DCA in liver	ρ	0.83	0.97	0.90	1		
	q	**<0.001**	**<0.001**	**<0.001**			
	n	40	42	43	43		
TCA in liver	ρ	0.90	0.91	1			
	q	**<0.001**	**<0.001**				
	n	40	42	43			
DCA in serum	ρ	0.87	1				
	q	**<0.001**					
	n	39	42				
TCA in serum	ρ	1					
	q						
	n	40					

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 2.4. Correlations among TCE metabolites and toxicity phenotypes in the liver of TCE-treated mice in a sub-chronic study (100 or 400 mg/kg/d for 1, 2 or 4 wks). Data from vehicle-treated samples were not included in the analyses.

		TCA	DCA	TCOH	DCVG	Liver/Body weight ratio	*Acox1*	*Cyp4a10*	Hepato-cellular proliferation
Hepatocellular proliferation	ρ	0.13	0.09	-0.09	-0.24	-0.25	-0.26	-0.43	1
	q	-	-	-	-	-	-	**0.0515**	
	n	35	35	32	14	34	28	28	35
Cyp4a10	ρ	0.65	0.58	0.36	0.50	0.68	0.39	1	
	q	**<0.001**	**0.001**	**0.089**	-	**<0.001**	**0.045**		
	n	35	35	31	12	34	35	35	
Acox1	ρ	0.44	0.45	0.48	0.11	0.34	1		
	q	**0.022**	**0.020**	**0.020**	-	**0.092**			
	n	35	35	31	12	34	35		
Liver/Body weight ratio	ρ	0.47	0.43	0.34	0.16	1			
	q	**0.007**	**0.017**	**0.083**	-				
	n	41	41	36	16	41			
DCVG	ρ	0.20	-0.13	0.41	1				
	q	-	-	-					
	n	16	16	16	16				
TCOH	ρ	0.60	0.65	1					
	q	**<0.001**	**<0.001**						
	n	37	37	37					
DCA	ρ	0.90	1						
	q	**<0.001**							
	n	43	43						
TCA	ρ	1							
	q								
	n	43							

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).



Supplementary Figure 2.1. Hemosiderin depositions observed in liver from TCE-treated group. Deposition of yellowish-brown pigments was a result of phagocytozed red blood cells in macrophages in liver.

CHAPTER 3: INTER-STRAIN VARIABILITY IN THE METABOLISM OF TCE: ROLE IN KIDNEY TOXICITY

I. INTRODUCTION

Trichloroethylene (TCE) is classified as 'group 1 carcinogen' by IARC (Guha et al. 2012) and as 'carcinogenic to humans' by the U.S. EPA (Chiu et al. 2013). These cancer classifications relied on human epidemiologic evidence linking exposures to TCE with renal cell carcinoma (Karami et al. 2012; Scott and Jinot 2011). In addition, the mutagenic mode-of-action (MoA) provides the biological plausibility to the human epidemiological findings (U.S. EPA 2011b). The mechanistic and animal data that strengthens human evidence for the increased risk of renal cell carcinoma associated with exposures to TCE includes observations of an increase in kidney cancer incidence in male rats (National Toxicology Program 1988, 1990), qualitative similarities in TCE metabolism between rodents and humans (Chiu et al. 2006), genotoxic and mutagenic properties of glutathione (GSH)-conjugated metabolites of TCE (Moore and Harrington-Brock 2000), and nephrotoxicity observed in mice treated with GSH conjugation metabolites (Shirai et al. 2012). Overall, evidence suggests that *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), or its downstream reactive electrophile metabolites, may be responsible for kidney tumors in humans by a mutagenic MoA, as well as an MoA that involves cytotoxicity and compensatory cell proliferation (Rusyn et al. 2014).

The key role for GSH conjugation-derived TCE metabolites in the kidney effects of this toxicant is also supported by several mechanistic studies in humans. These include

observation of a certain *VHL* tumor suppressor gene mutation pattern in TCE-exposed renal cell carcinoma cases (Brauch et al. 1999), an association between polymorphisms of GSTM1/GSTT1 and risk of renal cell cancer among workers exposed to TCE (Bruning et al. 1997), and detection of *S*-(1,2-dichlorovinyl)glutathione (DCVG), a precursor of DCVC (Lash et al. 2014), in the blood of humans exposed to TCE (Lash et al. 1999b). However, there are still many unresolved issues and uncertainties that reduce the confidence in these inferences. First, it is still debated whether or not the *VHL* mutation is an early event leading to TCE carcinogenesis. Induction of *VHL* mutation by TCE or its metabolites has not been experimentally demonstrated (Mally et al. 2006), and the association between *VHL* mutation and TCE exposure was not reproduced in human studies with similar design (Charbotel et al. 2007; Moore et al. 2011). Although a recent study provides mechanistic evidence that the P81S missense mutation in *VHL* region can initiate various cellular responses required for tumor growth (Desimone et al. 2013), it remains unclear whether this mutation can result from exposure to TCE or its metabolites.

A number of non-genotoxic mechanisms have been suggested as playing a role in TCE-associated kidney cancer, but they lack evidence as strong as that for genotoxicity and mutagenicity of GSH conjugation metabolites of TCE. Proposed non-genotoxic mechanistic events include α2u-globulin-associated nephropathy, cytotoxicity not associated with α2u-globulin-associated nephropathy, and peroxisome proliferator-activated receptor alpha (PPARα) activation (Rusyn et al. 2014). In addition, there is very limited data regarding the formation of GSH conjugation metabolites of TCE in tissues. Quantification of the metabolites is crucial not only for the toxicokinetic model-based extrapolation from rodents to humans, but also for addressing tissue/gender/species-specific toxicity of TCE.

Metabolism is closely associated with the mode of action in TCE-induced cancer and non-cancer effects as it was noted that the "knowledge of TCE metabolism is critical for determining susceptibility, target organ specificity and gender and species differences" (National Research Council 2006).

In this study, we tested a hypothesis that levels of GSH conjugation metabolites of TCE in the kidney are associated with kidney-specific toxicity in mice by evaluating the quantitative relationship between strain-, dose-, and time-dependent formation of DCVG and DCVC, as well as various kidney toxicity phenotypes, in a panel of mouse inbred strains.

II. MATERIALS AND METHODS

***Animals and treatments*.** Male mice (aged 6-7 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in polycarbonate cages on Sani-Chips (P.J. Murphy Forest Products Corp., Montville, NJ) irradiated hardwood bedding. Animals were fed NTP-2000 (Zeigler Brothers, Inc., Gardners, PA) wafer diet and water *ad libitum* on a 12 h light-dark cycle. All studies were approved by the UNC Institutional Animal Care and Use Committee.

Two study designs were utilized in this work. First, we performed a sub-acute study where vehicle (10 mL/kg, 5% Alkamuls EL-620 in saline) or TCE (600 mg/kg/d, in vehicle) was administered by gavage to mice from 7 inbred strains (129S1/SvImJ, A/J, BTBR T+tf/J, C57BL/6J, CAST/EiJ, NOD/ShiLtJ, and NZW/LacJ) for 5 consecutive days. These strains were selected to maximize inter-strain differences in metabolism of TCE based on the previous study of TCE metabolism in a panel of inbred strains (Bradford et al. 2011) and the results of the statistical modeling of the effect of time and strain on TCE metabolite concetnrations which supports the sample size used in this study (Chiu et al. 2014). Second,

based on the data from the sub-acute study, we selected two inbred strains (C57BL/6J and NZW/LacJ) that represented widely varying degrees of formation of oxidative metabolites of TCE for a subsequent sub-chronic study. Specifically, animals of each strain were randomly assigned to one of the three groups (5% Alkamuls EL-620 in saline vehicle, 100, or 400 mg/kg/day of TCE) and were dosed by oral gavage daily for 5 days/week for 1, 2, or 4 weeks.

In all studies, mice were given drinking water containing 0.2 g/L of 5-bromo-2'-deoxyuridine (BrdU) for 72 hrs prior to sacrifice. Blood, kidney and a section of a duodenum were collected 2 hrs after the last treatment. The timing of sacrifice was selected based on previous toxicokinetic studies of TCE in the mouse (Bradford et al. 2011; Kim et al. 2009b) showing that concentrations of both oxidative and glutathione conjugation metabolites of TCE peak around 2 hrs after dosing. Blood was drawn from *vena cava* and centrifuged to prepare serum using Z-gel tubes (Sarstedt, Germany) according to the manufacturer's instructions. Blood urea nitrogen (BUN) and serum creatinine were quantified using QuantiChrom Urea Assay (BioAssay Systems, Hayward, CA) and Creatinine (serum) Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), respectively. Body and organ weights were recorded. Kidney and duodenum sections were fixed in neutral buffered formalin for 24 hrs, and the remainder of the kidney tissue was frozen in liquid nitrogen. All serum and tissue samples were stored at -80°C until analyzed.

***Quantification of TCE metabolites*.** Concentrations of trichloroacetic acid (TCA), dichloroacetic acid (DCA), DCVC, and DCVG in kidney were determined using HPLC-ESI-MS/MS as detailed elsewhere (Kim et al. 2009a) with slight modifications. Quantification of trichloroethanol (TCOH) in kidney was performed by GC-MS using a method of (Song and

Ho 2003). The configuration of the instruments was identical to that in the above mentioned references, but the extraction methods were optimized for each metabolite as follows.

TCA and DCA: Kidney samples (20 mg) were homogenized in 500 μL of 0.01 M PBS (pH 7.4) with 20 μL of internal standards (difluoroacetic acid and trifluoroacetic acid, 20 nmol/mL each) using Tissuelyser (Qiagen, Valencia, CA) for 1 min. The homogenates were filter-centrifuged (Amicon Ultra Centrifugal Filters 10K; Millipore) at 14,000×g for 1 hr. After the filtrate was acidified with 100 μL of 3% (v/v) sulfuric acid, 2 mL of diethyl ether was added, and solutions were vortexed vigorously for 1 min. The upper ether layer was transferred to another vial, reduced in volume to less than 300 μL under continuous stream of N_2, then transferred to a glass vial insert containing 5 μL of double-distilled water and dried completely. The residue was reconstituted in 20 μL of HPLC mobile phase consisting of 70% acetonitrile, 30% 1 mM ammonium citrate in double-distilled water. The lower limit of quantification (LLOQ) was 0.02 nmol/g kidney for DCA and 5 nmol/g kidney for TCA.

DCVG and DCVC: Kidney tissue (50 mg) was homogenized in 500 μL of methanol with 20 μL of internal standards ([$^{13}C_2$,^{15}N]DCVG and [$^{13}C_3$,^{15}N]DCVC, 25 nmol/mL each) using Tissuelyser (Qiagen) for 1 min at 30/s. After adding 200 μL of chloroform, the homogenate was placed in shaker at 300 rpm for 10 min at 4°C, followed by centrifugation at 1,000×g for 5 min. The supernatant was then transferred to a new tube in which 200 μL of chloroform and 200 μL of water were added before centrifugation at 1,000×g for 5 min. From the upper aqueous phase, DCVG and DCVC were extracted using solid phase extraction (SPE) cartridge (StrataTM X-AW, 30mg 96-well plate; Phenomenex, Torrance, CA). After conditioning with 300 μL of methanol, followed by equilibration with 300 μL of water, the samples were loaded, washed with 300 μL of water, and finally eluted with 250 μL

of basic methanol (pH adjusted at 10.8 by 29.1% NH_4OH). The final eluent was collected into 300 μL glass vial inserts and dried in a Speed Vac Concentrator before reconstitution with 20 μL of 4:1 water/methanol containing 0.1% acetic acid. The LLOQ was 1 pmol/g kidney for DCVG and 10 pmol/g kidney for DCVC.

TCOH: Kidney tissue (30 mg) was homogenized in 500 μL of acetate buffer (pH 4.6) with 1,000 units of β-glucuronidase (cat. #G0751, Sigma, St. Louis, MO) using Tissuelyser (Qiagen) for 1 min, followed by overnight incubation at 37°C. After centrifugation at 14,000×g for 5 min, the supernatant was transferred to a new tube, then mixed with internal standard (DCA, 10 mM in methanol, 20 μL) and 550 μL of water/0.1 M sulfuric acid/methanol (6:5:1). The mixture was heated at 70°C for 20 min. After cooling to room temperature, 2.5 mL of hexane was added, the mixture was vortexed for 10 min and centrifuged at 2,500 g for 2 min. The upper layer was concentrated under N_2 to less than 20 μL and used for GC-MS analysis as detailed in (Song and Ho 2003). The LLOQ for TCOH was 15 nmol/g kidney.

***Gene expression analysis by real-time PCR*.** Total RNA was isolated from kidney samples using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2000 Bioanalyser, respectively. Total RNA was reverse transcribed using random primers and the high capacity complementary DNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The following gene expression assays (Applied Biosystems) were used for quantitative real-time PCR: peroxisome proliferator–activated receptor alpha (*Ppara*, Mm00440939_m1); palmitoyl acyl-Coenzyme A oxidase 1 (*Acox1*, Mm01246831_m1); cytochrome P450, family

4, subfamily a, polypeptide 10 (*Cyp4a10*, Mm01188913_g1); and beta glucuronidase (*Gusb*, Mm00446953_m1). Reactions were performed in a 96-well plate, and all samples were plated in duplicate using LightCycler® 480 instrument (Roche Applied Science, Indianapolis, IN). The cycle threshold (Ct) for each sample was determined from the linear region of the amplification plot. The ΔCt values for all genes relative to the control gene *Gusb* were determined. The ΔΔCt were calculated using treated group means relative to strain-matched control group means. Fold change data were calculated from the ΔΔCt values.

Determination of proximal tubule cell proliferation. Deparaffinized and rehydrated kidney sections were immersed in 4N HCl and subsequently pepsin solution (Dako, Carpinteria, CA) for antigen retrieval and then incubated in peroxidase blocking reagent (Dako). Dako EnVision System HRP kit was used for the detection of BrdU-incorporated nuclei (monoclonal anti-bromodeoxyuridine antibody, Dako, 1:200 dilution). Data were presented as a fraction of BrdU staining-positive nuclei in the tubular epithelium of the renal cortex (no fewer than 1,000 nuclei counted per a kidney section).

***Determination of KIM-1 expression in kidney*.** Detection of KIM-1 was accomplished by modifying a published method (Humphreys et al. 2011). Formalin-fixed and paraffin-embedded kidney sections were deparaffinized and rehydrated. Antigens were retrieved by 4N HCl and pepsin solution (Dako) afterward. After peroxidase blocking, immunohistochemical detection was conducted using Dako Liquid DAB Substrate Chromogen System with primary anti KIM-1 antibody (2 ug/mL in PBS) (R&D Systems, Minneapolis, MN) and secondary goat IgG HRP-conjugated Antibody (1:100 in PBS) (R&D Systems). The proportion of positive-stained proximal tubules in outer medulla was determined under light microscopy. Data were presented as a fraction of proximal renal

tubules staining positive for KIM-1 (no fewer than 200 proximal renal tubules counted per a kidney section).

***Statistical analyses*.** The significance of inter-strain effect on metabolism was assessed by ANOVA modeling. Given the small sample size, the exact permutation test was used to determine significant differences between control and TCE-treated groups (α=0.05). The Spearman (rank) correlation analysis across all variables was conducted to account for the difference in scale of the variables. In correlation analyses, false discover rate correction (Storey and Tibshirani 2003b) was applied to all *p*-values to correct for multiple comparisons. The resultant significant (<0.1) *q*-values are reported in the Supplemental Tables. All statistical analyses were performed using SAS software ver. 9.3 (SAS Institute, Cary, NC).

III. RESULTS

Sub-acute (5 days) study of strain-dependent effects of TCE in mouse kidney

We measured kidney levels of TCE metabolites from CYP450-mediated oxidation (TCA, DCA, TCOH) and GSH conjugation (DCVC and DCVG) in mice from seven inbred strains treated with an aqueous suspension of TCE (600 mg/kg/d) for 5 days (Figure 1). Across all strains, kidney levels of TCA were 100 to 1,000-fold greater than those for DCA, and the amounts of DCA were about 100 to 1,000-fold higher than those of either DCVG or DCVC. Mouse kidney levels of TCOH, also a major oxidative metabolite of TCE, were 2 to 4-fold higher than those of TCA. The concentration of DCVC in kidney was at least 50 to 100-fold higher than that of DCVG, which was below the LLOQ in most samples. We found a significant strain effect (p<0.05) for each metabolite. Liver and serum concentrations of these metabolites are reported in Chapter 2.

We examined the correlation between TCE metabolites in mouse kidney, liver, and serum (Supplementary Table 1). We observed no significant correlation among metabolites in the kidney. However, we found an inter-organ correlation for GSH conjugation-derived TCE metabolites. Although DCVG and DCVC were not correlated with each other in either liver or kidney, we found a significant correlation between liver DCVG, and kidney and serum DCVC, as well as with kidney DCVG, even after correction for multiple comparisons. Liver DCA correlated significantly with serum, liver and kidney TCA.

We examined effects of repeat dosing with TCE on kidney weight (relative to body weight), proximal tubule cell proliferation, and BUN (Figures 2A-C). We observed significant effect on kidney enlargement in response to sub-acute treatment with TCE in 129S1/SvImJ and BTBR T+tf/J strains; however, proximal tubule cell proliferation, as evaluated by BrdU immunohistochemistry, was not affected by TCE in any strain. BUN levels did not change in response to TCE treatment.

Given the large amounts of CYP450-mediated oxidative metabolites and the strain-dependent kidney enlargement, we evaluated kidney expression of *Ppara*, as well as *Cyp4a10* and *Acox1*, marker genes for PPARα activation (Figures 2D-F). We observed significant exposure-related increases in expression of the transcription factor *Ppara* in NOD/ShiLtJ and NZW/LacJ strains. TCE had a significant effect of expression of *Acox1* only in NOD/ShiLtJ, while expression of *Cyp4a10* was significantly elevated in four strains including C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, and NZW/LacJ. Where induced by TCE, the magnitude of the effect on *Cyp4a10* was found to be much greater than that on *Acox1*.

Histopathological evidence of kidney injury was previously observed in mice exposed to TCE sub-acutely or sub-chronically only at very high doses (in excess of 3,000 mg/kg)

(National Cancer Institute 1976; National Toxicology Program 1990). Recently, KIM-1 was qualified as a sensitive and early biomarker of acute kidney injury (Ozer et al. 2010). Therefore, we examined KIM-1 expression and found that TCE significantly induced expression of KIM-1 on the apical membrane of the proximal tubular S3 segment in the outer medulla in 129S1/SvImJ, C57BL/6J, A/J, and BTBR T+tf/J strains (Figure 3).

Because inter-strain differences in both TCE metabolism and kidney toxicity were observed in this study, we examined correlations among these endpoints in kidney (Supplementary Table 2). Liver DCVG and kidney DCVC are significantly correlated (Figure 4A). Also, *Acox1* and *Cyp4a10* were significantly correlated with each other (Figure 4B). We found no significant correlation between kidney levels of DCVC and expression of either *Acox1* or *Cyp4a10* (Figure 4C). KIM-1 expression was not significantly correlated with any TCE metabolite, including DCVC (Figure 4D), or other endpoints such as markers of PPARα activation.

Sub-chronic (up to 4 weeks) study of strain-dependent effects of TCE in mouse kidney

Based on the differences in TCE metabolism observed in the sub-acute (5 days) TCE exposure study, we selected C57BL/6J and NZW/LacJ strains to further test our hypothesis that inter-strain differences in formation of GSH-conjugation metabolites of TCE in the kidney are associated with kidney-specific toxicity. We examined time- (1, 2 and 4 weeks) and dose-dependent (100 and 400 mg/kg/d, *i.g.*) effects of TCE in these two strains.

We found that strain-dependent differences in TCA level in kidney are dose-dependent (Figure 5). Overall, kidney levels of TCA and TCOH were higher in NZW/LacJ mice, similar to the sub-acute study result (Figure 1). Also, concordant with the findings in the sub-acute study, levels of TCA and TCOH in kidney were 100 to 1,000-fold greater than

those of DCA. Levels of DCVC and DCVG in kidney were below the LLOQ; however, the doses of TCE used herein were lower than those in the sub-acute study. Amounts of CYP450-depedent oxidative metabolites of TCE in the kidney generally trended downward over time. Specifically, the levels of TCA in kidney decreased over time in both strains, while the level of TCOH in kidney decreased over time only in C57BL/6J strain, which was consistent with the findings in the liver. We found no dose-, time-, or strain-dependent differences in kidney levels of DCA.

Among CYP450-dependent oxidative metabolites that were quantified in this study, kidney levels of TCA and TCOH were highly correlated (Supplemental Table 3) across both strains in the sub-chronic study. Interestingly, kidney levels of TCA and TCOH were significantly correlated with liver levels of TCA, DCA, and TCOH, which may reflect the strong decreasing trend in formation of CYP450-depedent oxidative metabolites of TCE over time that was observed both in the liver and kidney.

We also examined the effects of sub-chronic treatment with TCE on the kidney toxicity markers and the marker genes of PPARα activation. We found no effect of sub-chronic TCE treatment on kidney in terms of traditional biomarkers such as kidney-to-body weight ratio, BUN, and serum creatinine (Figures 6A-C). However, we observed a prominent effect of TCE on the marker genes of PPARα activation in the kidney. Expression of *Acox1* and *Cyp4a10* significantly increased in a dose-dependent manner in both strains of mice treated with TCE for 5 days (Figures 6E-F). *Ppara* was also significantly induced by TCE in NZW/LacJ mice at the highest dose at 5 days. Interestingly, the extent of up-regulation of *Acox1* and *Cyp4a10* by TCE decreased over time. In the 2 and 4 weeks treatment groups, we found little to no differences in gene expression between vehicle and TCE-treated animals,

except for significant induction of *Ppara* and *Acox1* in C57BL/6J mice treated with 400 mg/kg/d TCE for 2 weeks.

Furthermore, we observed that effects of TCE on proximal tubule cellular proliferation and KIM-1 expression were treatment-, time- and strain-dependent (Figure 7). Whereas we found that the proximal tubule cellular proliferation was significantly elevated only in NZW/LacJ strain after 4 weeks of treatment with TCE, a significant increase in KIM-1 expression was observed in both strains only after 5 days of treatment with TCE.

We found that kidney levels of TCA were significantly correlated with TCOH (Figure 8A), and *Acox1* and *Cyp4a10* expression in the kidney regardless of TCE dose or treatment duration (Figure 8B). We also observed that kidney levels of TCA were significantly correlated with expression of KIM-1 (Figure 8C), but not with proximal tubule cellular proliferation (Figure 8D).

IV. DISCUSSION

This study is first to report the levels of CYP-mediated oxidative and GSH-conjugation metabolites of TCE in the kidney of exposed mice. The estimation of the relative flux to each of these two pathways of TCE metabolism has been subject to debate (Rusyn et al. 2014). Although recent human cancer hazard assessments concluded that GSH conjugation pathway is primarily responsible for TCE-induced kidney effects (Chiu et al. 2013; Guha et al. 2012), there are considerable uncertainties regarding the extent of the flux to GSH conjugation pathway between species and among individuals. Thus, we aimed to provide quantitative estimates of dose-, time-, and strains-specific differences in TCE metabolite burden in the mouse kidney, and to determine whether TCE-associated toxicity phenotypes correlate with TCE metabolite levels. Not only does this study provide for the

first time comprehensive quantitative estimates of TCE metabolism and toxicity in kidney of mouse as species, but also it explored inter-strain variability in these phenotypes. Indeed, a mouse model of the human population is an effective tool for understanding the molecular underpinnings of the population variability in response to toxic agents (Rusyn et al. 2010), including studies of adverse health effects of chlorinated solvents (Bradford et al. 2011).

Species comparison of TCE metabolite levels in the kidney

In the sub-acute study, similar with the findings in the liver, we found up to 5 orders of magnitude difference in the relative flux of TCE through CYP450-dependent oxidation (primarily TCA) as compared to GSH conjugation in mouse kidney. These findings are consistent with the TCE metabolite data in mouse serum (Bradford et al. 2011; Chiu et al. 2014; Kim et al. 2009b). However, our findings differ from those reported in one rat study where kidney levels of TCE metabolites were evaluated (Lash et al. 2006). Lash et al. (2006) reported kidney levels of DCVG in female rats to be 100-fold higher than those of TCA and TCOH. Furthermore, kidney levels of DCVC in female rats in Lash et al. (2006) were similar to those observed in our study in male mice, but the levels of DCVG reported in female rat kidneys were about 10-fold greater. No DCVG or DCVC were detected in male rat kidneys by Lash et al. (2006).

A criticism of the technical issues that accompany the methodology for detection of GSH conjugation metabolites of TCE used in the studies of Lash and co-workers has been detailed in (U.S. EPA 2011b), and it was argued that the technique of (Fariss and Reed 1987) may overestimate the amounts of DCVG. The major differences in the relative flux through the oxidative and GSH conjugation pathways are, however, due to much lower amounts of TCA reported by Lash et al (2006). Furthermore, these results are not in accord with

published reports of blood or plasma levels of TCE-derived DCA and TCA in rat and mouse (Abbas and Fisher 1997; Kim et al. 2009b; Larson and Bull 1992; Merdink et al. 1998). Thus, the toxicological implications of the quantitative difference among studies and species should be interpreted with caution. We reason that our results on TCE metabolite levels in mouse kidneys provide valuable new data that fill a gap in organ-specific health assessments of TCE and related solvents.

Intra- and inter-organ metabolism of TCE

We also found that the effect of the first-pass metabolism of TCE in the liver may still be sustained in the kidney. TCA and TCOH are two major TCE metabolites to be excreted, and we found that kidney levels of TCOH were 2 to 3-fold higher than those of TCA, which is in accord with the observation that cumulative urinary excretion of TCOH was about 2-fold higher than that of TCA in humans exposed to chloral hydrate (Merdink et al. 2008). A significant correlation between liver DCVG and kidney DCVC also supports a hypothesis that DCVG is primarily formed in the liver and then translocates to the kidney, where subsequent metabolism to DCVC involves γ-glutamyltransferase and cysteinylglycine dipeptidase (Lash et al. 1998).

With respect to inter-organ differences in CYP450-dependent oxidation, we found no relationship between liver and kidney, other than significant correlation between liver DCA and kidney TCA in a sub-acute study, possibly due to strain-difference in metabolism and transport. However, in a sub-chronic study, liver levels of TCA, DCA, and TCOH were significantly correlated with kidney levels of TCA, DCA, and TCOH. This finding suggests effective inter-organ transport of the oxidative metabolites of TCE. Among GSH conjugation metabolites of TCE, the only significant correlation found was between liver DCVG and

kidney DCVC. This correlation, combined with the observations that DCVG is detected in much greater concentrations in the liver than DCVC, and that the opposite is true in the kidney, strongly supports the dependence of the downstream metabolism of DCVG on kidney-specific enzymes (Lash et al. 2000).

For the sub-chronic study, C57BL/6J and NZW/LacJ strains were selected to maximize the strain differences in the extent of TCE metabolism. One of the notable differences between these two strains is the relative difference in flux to each pathway. As expected from the data in the sub-acute study, kidney levels of TCA and TCOH were found to be higher in NZW/LacJ than in C57BL/6J mice. However, DCVC and DCVG, which were expected to be higher in C57BL/6J, were not detected in the sub-chronic study where the doses used were lower than those in the sub-acute study. The sensitivity of the analytical methods, combined with low extraction recoveries, presents a continued limitation to our ability to quantify the levels of these critically important TCE metabolites in studies that use environmentally-relevant exposures to TCE. Therefore, further improvements are needed to fill the data gaps.

TCE-induced kidney toxicity

Our study provides important information on the TCE-associated increase in relative kidney weight and acute kidney injury. The finding that relative kidney weight and KIM-1 expression, an indicator of both damage and regeneration (Ichimura et al. 2004), were increased in response to sub-acute TCE treatment in some, but not all mouse strains, provides additional direct evidence that TCE can cause nephrotoxicity in the mouse, but that this effect is strain-dependent. Another sub-acute (10 days) study with TCE found no significant increases in kidney/body weight ratios in B6C3F1 mice (Goldsworthy and Popp 1987).

Depletion of GSH in the renal cortex of NMRI mice treated with a single *i.p.* dose of TCE was also shown (Cojocel et al. 1989). Mild to moderate cytomegaly and karyomegaly of the renal tubular epithelial cells of the inner cortex were observed in some, but not all, male B6C3F1 mice treated for 13 weeks with 3000 or 6000 mg/kg/d TCE (National Toxicology Program 1990). In our study, an increase in KIM-1 expression was observed in 4 out of 7 strains treated with TCE (600 mg/kg/d) for 5 days, and confirmed in the two strains treated with TCE (400 mg/kg/d) for 5 days in the sub-chronic study. We found no positive KIM-1 signal with or without TCE treatment in NOD/ShiLtJ strain in which relatively higher amount of kidney DCVC was detected; however, NOD/ShiLtJ mice appear to be uniquely resistant to kidney injury as even upon treatment with a known renal toxicant DB289 no increase in urinary KIM-1 was detected, while in C57BL/6J mice a 10-fold increase was observed (Harrill et al. 2012).

Interestingly, at both 2 and 4 week treatment time points, KIM-1 expression was not significantly increased, albeit it was somewhat higher in TCE-treated NZW/lacJ mice. This dampening of the nephrotoxicity under conditions of the continuous exposure may be due to the compensatory responses of the kidney. Indeed, tubular epithelial cells can undergo compensatory proliferation and adaptation following injury (Duffield et al. 2005; Lin et al. 2005).

TCE effects on PPARα-associated pathways

Limited evidence exists to suggest that TCE exposure induces peroxisome proliferation in the kidney of exposed rodents. Peroxisome proliferation in the rat and mouse kidney has been evaluated in only one other study (Goldsworthy and Popp 1987) where animals were exposed to TCE (1000 mg/kg/d) for 10 days. Increases in renal palmitoyl–CoA

oxidation activity were observed in both rats and mice treated with TCE. We show that treatment with a comparable dose of TCE (600 mg/kg/d) for 5 days resulted in induction of *Cyp4a10* that was significant in 4 out of 7 strains tested. *Acox1* and *Ppara* were also markedly induced in NOD/ShiLtJ strain, in which *Cyp4a10* induction was almost 100-fold. Marked strain-dependence of the TCE effect on PPARα-mediated signaling in mouse kidney is similar to that in the liver, as it was found that PPAR-mediated pathway was one of the most pronounced strain-dependent molecular effects of TCE in mouse liver (Bradford et al. 2011).

In the sub-chronic study, not only did we observe dose- and strain-specific effects on the same endpoints, but we also found a decreasing trend of *Acox1* and *Cyp4a10* expression over time in the kidney. There is a similar observation in a time-course study with a model peroxisome proliferator di-2-ethylhexyl phthalate (Ohno et al. 1982). The authors counted the number of peroxisomes in the kidney cortex of rats exposed to DEHP and found that the number of peroxisomes peaked at 1 week and decreased at 2 and 4 weeks. Similar to the effects of TCE in the liver, it is likely that this dampening of an effect is related to a decrease in the amount of CYP450-mediated oxidative metabolites of TCE. Indeed, there was a significant correlation among kidney levels of TCA and expression of both *Acox1* and *Cyp4a10*.

TCE metabolite levels and kidney toxicity phenotypes

In addition, the data collected in this study allows for a detailed quantitative investigation of the relationships between TCE metabolite levels and kidney toxicity phenotypes. While CYP450-mediated oxidative metabolites of TCE are known to activate PPARα signaling, and the GSH conjugation metabolite, DCVC has been shown to be

nephrotoxic in mice (Darnerud et al. 1989; Jaffe et al. 1985), no previous study examined the correlation between metabolism and toxicity. Indeed, a number of confirmatory and novel observations were drawn from such comparisons in the work presented herein.

In the sub-acute study, the strain-dependent minimal to mild nephrotoxicity, as evidenced by KIM-1 immunohistochemistry, did not correlate with any measured TCE metabolites, including DCVC. In the sub-chronic study, KIM-1 staining in proximal tubules, a marker of both cytotoxicity and regeneration was correlated with kidney amounts of TCA. These findings suggest that multiple metabolites and /or pathways are likely to be involved in TCE-induced kidney cytotoxicity in mice. In fact, evidence of cytotoxicity in the rat kidney was observed not only in animals treated with DCVC, but also those treated with TCA and TCOH (Dow and Green 2000; Green et al. 2003). Renal toxicity in long-term studies in rats has been attributed to stimulation of formic acid excretion by oxidative metabolites of TCE (Green et al. 1998). The reported connection between formic acid excretion, acidosis, renal toxicity and renal cancer in rats exposed to TCE for 2 years has not been explored yet in mice and our data suggest that this mechanism may also be plausible.

In the sub-acute study, we did find a similar strain- and treatment-dependent pattern between the level of DCVC and expression of *Cyp4a10,* although the correlation was not significant in part due to the small number of data points. This is a noteworthy finding because CYP450-mediated oxidative, but not GSH conjugation, metabolites are regarded as responsible for PPARα activation, although the data in the kidney is limited. TCA, but not DCVC, was strongly correlated with *Acox1* and *Cyp4a10* in the sub-chronic study; however, this correlation may primarily reflect the decreasing trend over time observed simultaneously in the levels of metabolites and the extent of PPARα activation. When the data is stratified by

strain, the levels of TCA and TCOH were higher in NZW/LacJ, while the extent of PPARα target gene expression was higher in C57BL/6J in both sub-acute and sub-chronic studies. This result questions a direct relationship between formation of oxidative metabolites and PPARα activation in the mouse kidney. PPARα activation has important roles in renal function, such as maintenance of energy balance through regulating β-oxidation of fatty acids (Portilla et al. 2000), blood pressure regulation linked with CYP450 4A enzyme (Guan and Breyer 2001), and peroxisomal respiration associated with oxidative stress (Zaar 1992). Just as the perturbation of PPARα activity may result in various adverse effects, so a number of endogenous and exogenous factors can induce PPARα activation (Peters et al. 2012).

We also note that the correlation analyses performed herein, while highly informative, have limitations as they do not take into consideration potential differences in clearance between the different metabolites, across strains, and effects of TCE treatment. In addition, the concentration-time profiles of different metabolites in serum vary greatly (Kim et al. 2009b) and our study collected metabolite data only at one time point, two hours prior to sample collection, whereas the toxicity effects are likely to be the cumulative result of treatments on the previous days, as well as that prior to sample collection.

V. FIGURES AND TABLES

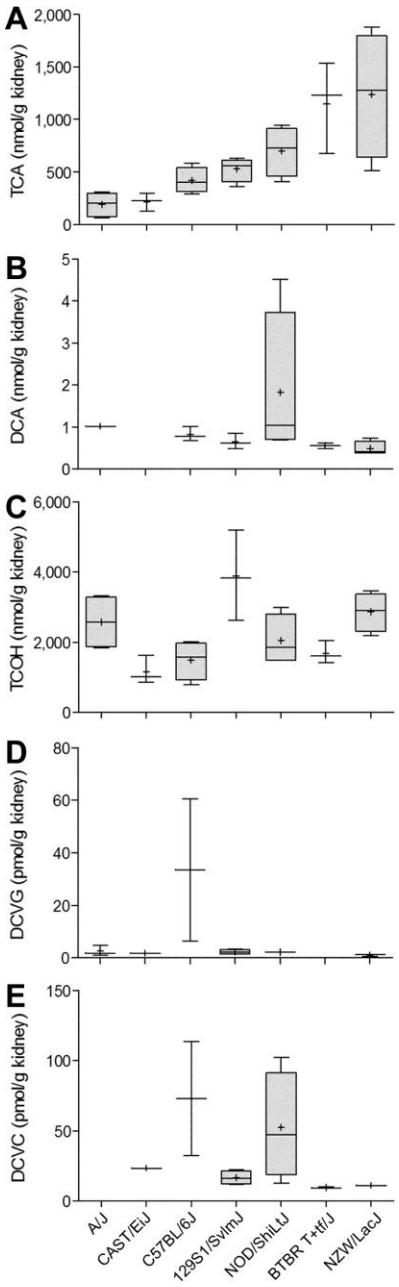


Figure 3.1. Inter-strain variability in TCE metabolism in the kidney of mouse in a sub-acute study. Kidney levels of metabolites were assessed 2 h following the last of 5 daily doses (600 mg/kg/d) of TCE. Box and whisker plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group.

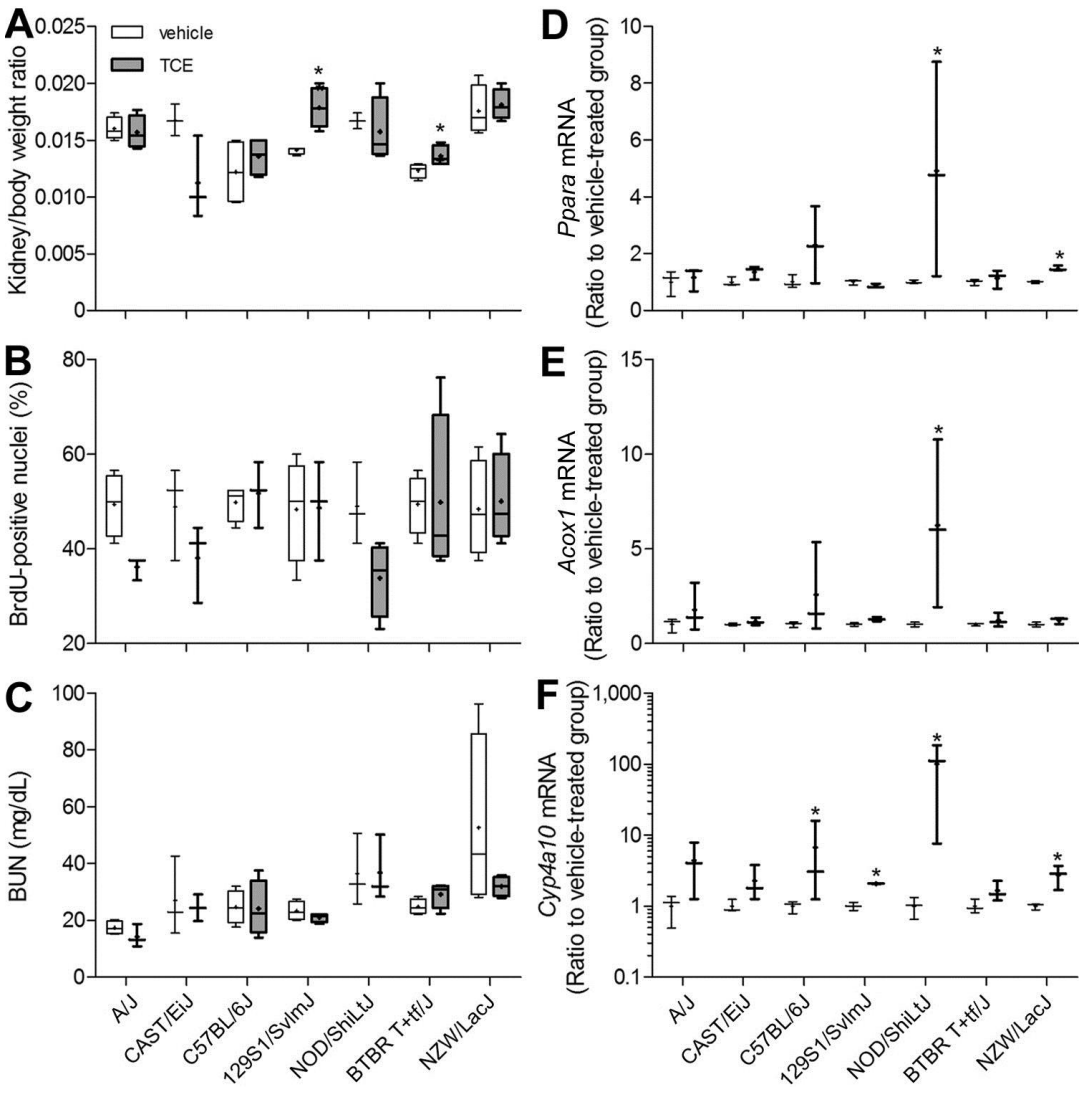


Figure 3.2. Inter-strain differences in kidney toxicity of TCE in a sub-acute study. (A) Kidney to body weight ratios, (B) percent BrdU-positive proximal tubule cell nuclei, (C) blood urea nitrogen, and kidney expression of peroxisome proliferation marker genes *Ppara* (D), *Acox1* (E), and *Cyp4a10* (F) were evaluated in mice treated with vehicle (white) or TCE (gray; 600 mg/kg) for 5 days. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). Asterisk (*) denotes a significant difference ($p<0.05$) compared to vehicle-treated group within same strain. When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group.

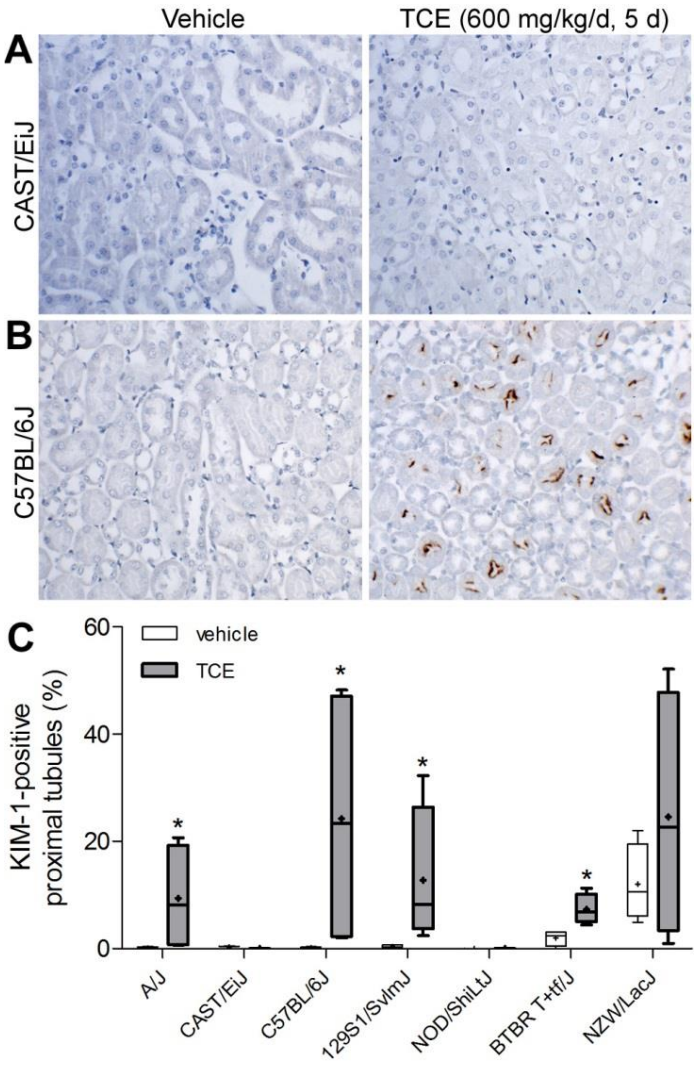


Figure 3.3. Inter-strain differences in KIM-1 expression in mouse kidney in a sub-acute study. Immuno-histochemical detection of KIM-1 (brown deposits on the apical membrane side of kidney proximal tubules in the outer medulla) was performed. Representative images from vehicle (left) and TCE (600 mg/kg/d for 5 days)-treated CAST/EiJ (A) and C57BL/6J strains are shown. (C) Quantitative analysis of KIM-1-positive proximal tubules in the kidneys of mice treated with vehicle (white) or TCE (gray). Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). Asterisk (*) denotes a significant difference ($p<0.05$) compared to vehicle-treated group within same strain. There were 4 animals per group.

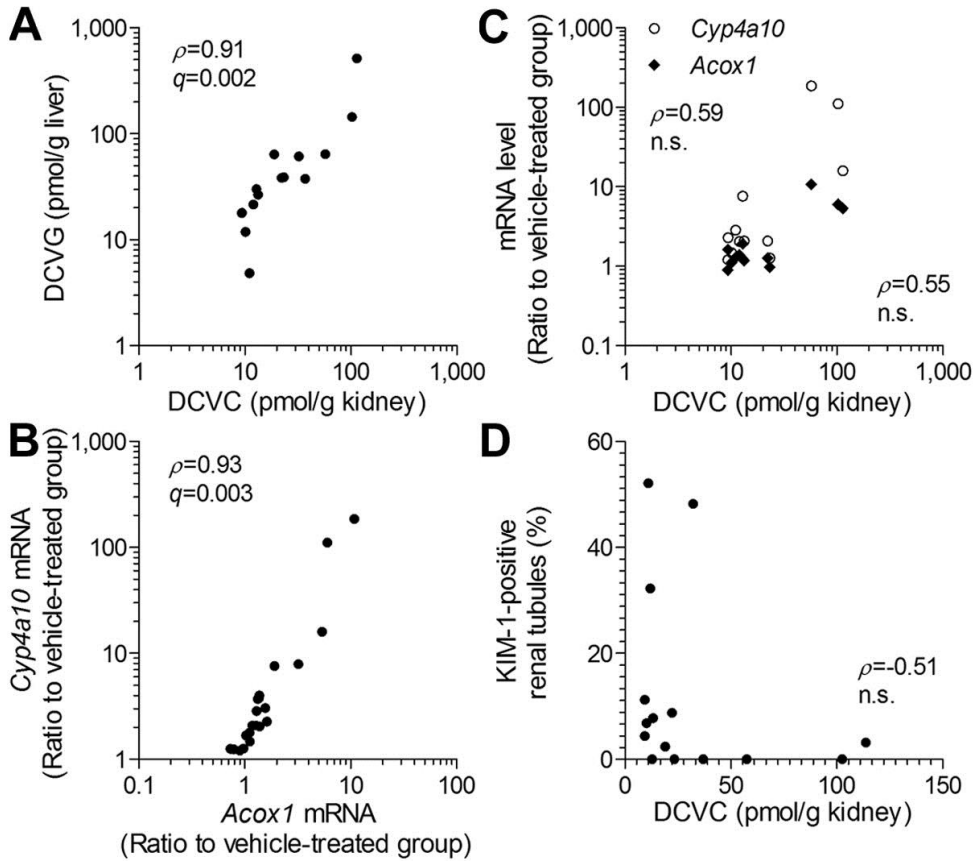


Figure 3.4. Correlation among kidney DCVC and toxicity phenotypes in a sub-acute study. (A) DCVG in the liver was significantly correlated with the kidney levels of DCVC. (B) Kidney expression of *Cyp4a10* and *Acox1* were significantly correlated. (C) TCE-induced increase in *Cyp4a10* (open circle) and *Acox1* (filled rhombus) expression in the mouse kidney was not significantly correlated with kidney level of DCVC. (D) No significant correlation was observed between DCVC concentration in the kidney and KIM-1 expression. Each dot represents an individual TCE-treated animal in the study. Spearman rank (ρ) correlations and false discovery rate-corrected significance values (q<0.1) are shown.

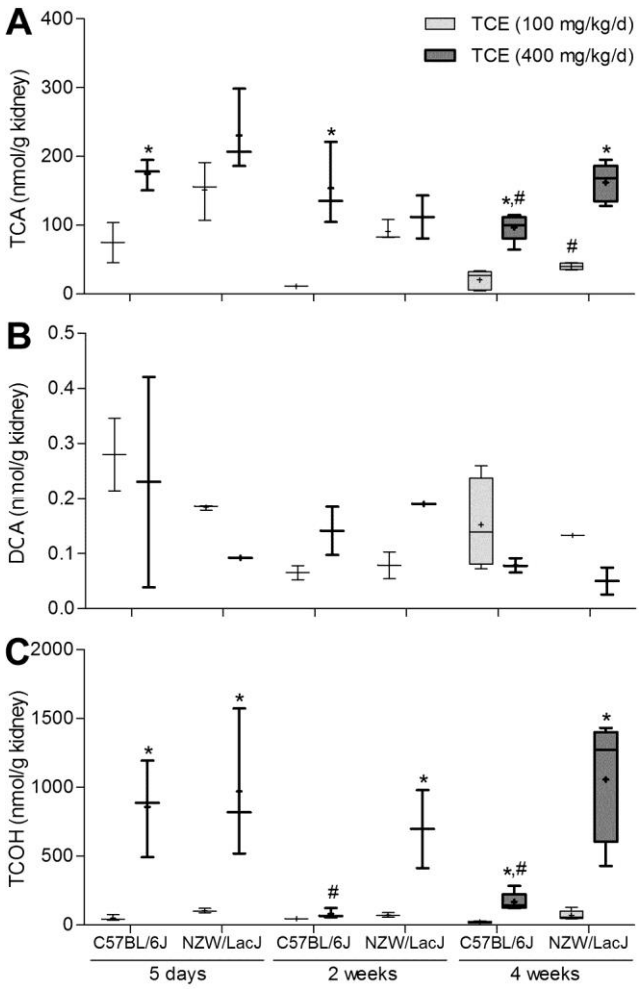


Figure 3.5. Time-course analysis of TCE metabolism in C57BL/6J and NZW/LacJ mice in a sub-chronic study. TCA (A), DCA (B), and TCOH (C) levels in the kidney were assessed 2 h following the last dose after 1, 2 or 4 wks (100 or 400 mg/kg/d) of TCE. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). Light-gray, 100 mg/kg/d groups; dark gray, 400 mg/kg/d groups. Asterisks denote a significant ($p<0.05$) difference as compared to (*) the group dosed with 100 mg/kg/d (same strain and time point), or (#) the 5 day treatment group (same strain and dose). Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 5 animals per group were available. Otherwise, there were 3 animals per group.

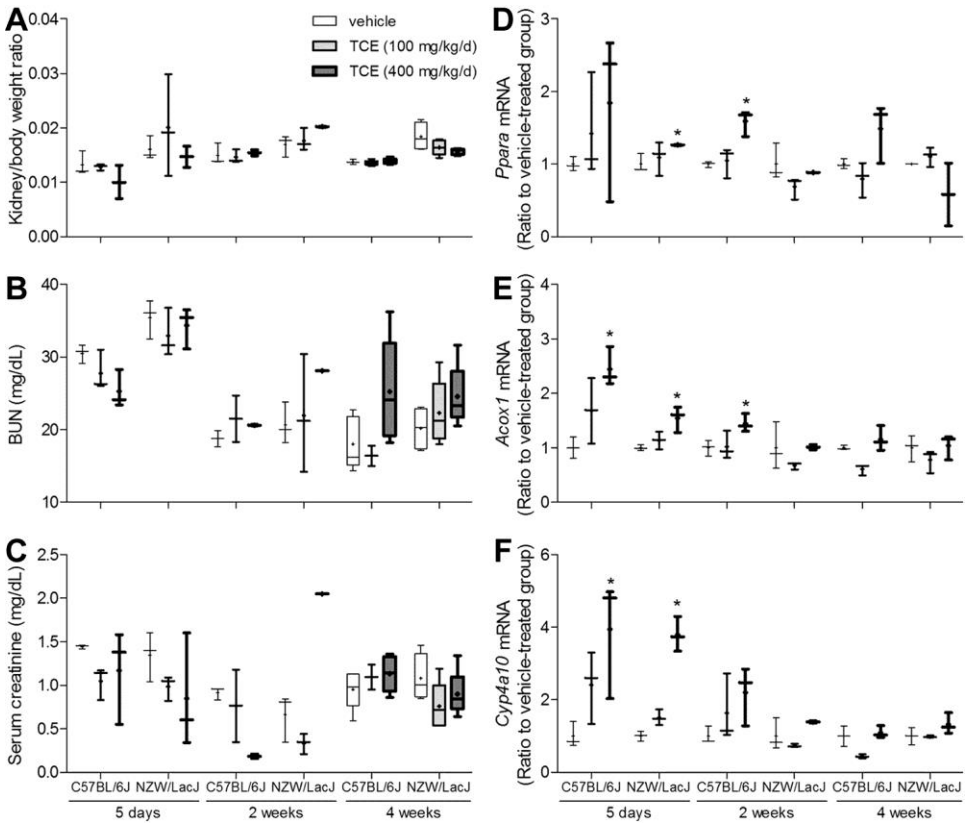


Figure 3.6. Differences in kidney toxicity of TCE in C57BL/6J and NZW/LacJ mice in a sub-chronic study. Kidney to body weight ratios (A), blood urea nitrogen (B), serum creatinine (C), and kidney expression of peroxisome proliferation marker genes *Ppara* (D), *Acox1* (E), and *Cyp4a10* (F) were evaluated in mice treated with TCE (100 or 400 mg/kg) for 1, 2 or 4 wks. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). White, vehicle-treated groups; light-gray, 100 mg/kg/d groups; dark gray, 400 mg/kg/d groups. Asterisk (*) denotes a significant ($p<0.05$) difference as compared to vehicle-treated group (same strain and time point). Thickness of the line corresponds to the vehicle, 100 and 400 mg/kg/d groups. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 5 animals per group were available. Otherwise, there were 3 animals per group.

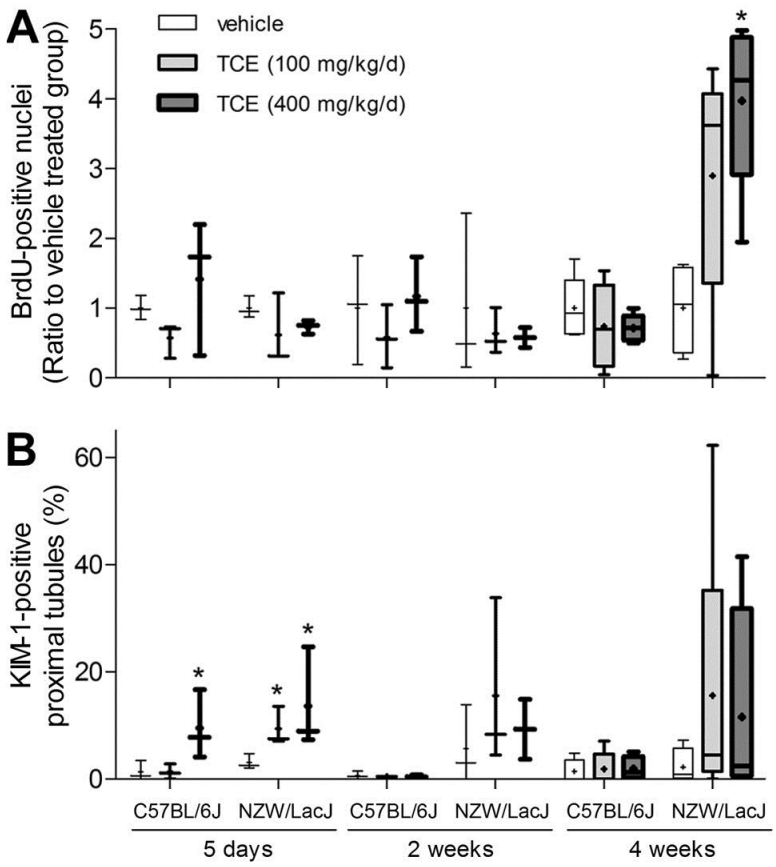


Figure 3.7. Differences in kidney toxicity of TCE in C57BL/6J and NZW/LacJ mice in a sub-chronic study. Percent BrdU-positive proximal tubule cell nuclei (A), and kidney expression of KIM-1 (B) were evaluated in mice treated with TCE (100 or 400 mg/kg/d) for 1, 2 or 4 wks. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). White, vehicle-treated groups; light-gray, 100 mg/kg/d groups; dark gray, 400 mg/kg/d groups. Asterisk (*) denotes a significant ($p<0.05$) difference as compared to vehicle-treated group (same strain and time point). Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 5 animals per group were available. Otherwise, there were 3 animals per group.

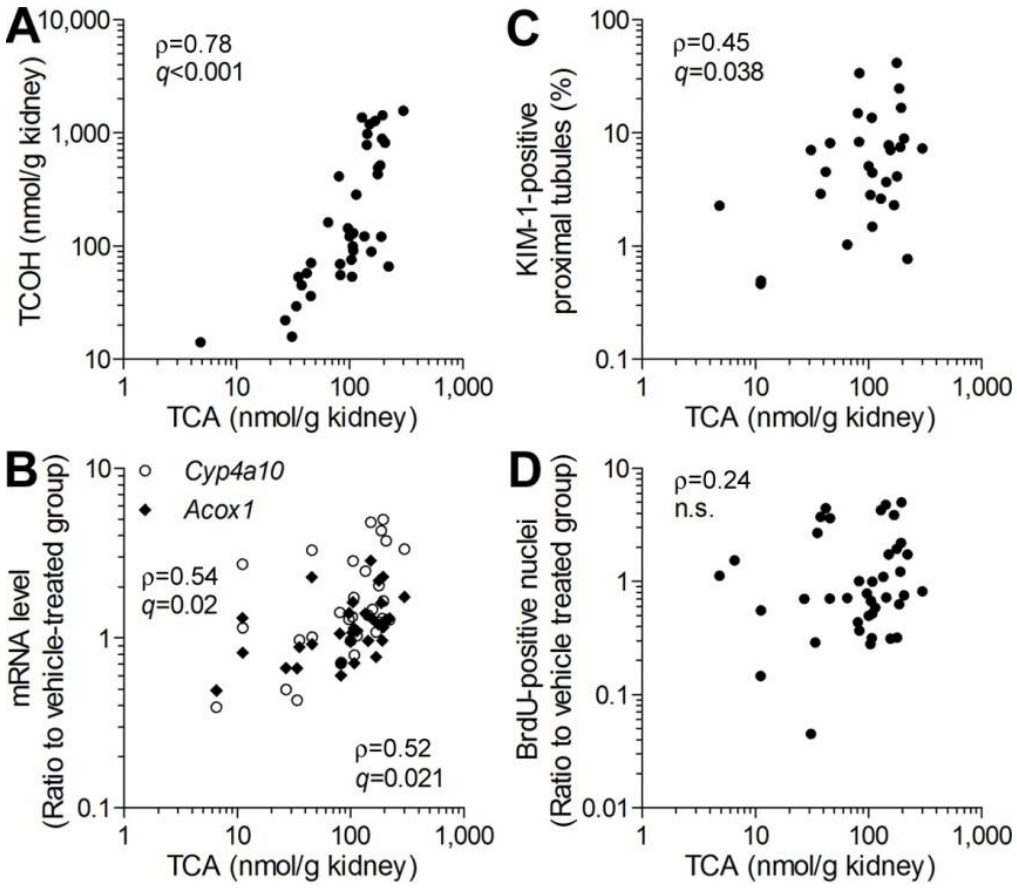


Figure 3.8. Correlation between TCE metabolites and kidney toxicity phenotypes in C57BL/6J and NZW/LacJ mice in a sub-chronic study. (A) Levels of TCA and TCOH in kidney were significantly correlated with each other. (B) TCE-induced increase in *Cyp4a10* (open circle) and *Acox1* (filled rhombus) expression in the mouse kidney was significantly correlated with kidney levels of TCA. (C) Levels of TCA in the kidney were significantly correlated with KIM-1 expression in the proximal tubules. (D) No correlation was observed between TCA concentrations in the kidney and proximal tubule cellular proliferation. Each dot represents an individual TCE-treated mouse. Spearman rank (ρ) correlations and false discovery rate-corrected significance values (q<0.1) are shown.

Supplementary Table 3.1. Correlations among TCE metabolites in serum, liver, and kidney in TCE-treated mice in a sub-acute study (600 mg/kg/d for 5 days).

			TCA			DCA			TCOH			DCVG			DCVC	
			serum	liver	kidney	serum	liver	kidney	liver	kidney	serum	liver	kidney	serum	liver	kidney
DCVC	kidney	ρ	-0.56	0.02	-0.34	0.43	0.13	0.64	0.46	-0.36	0.6	0.91	0.72	0.62		
		q	-	-	-	-	-	-	-	-	-	**0.002**	-	-		
		n	12	15	15	10	15	12	14	14	6	15	9	12	2	15
	liver	ρ														
		q														
		n	0	2	2	0	2	2	2	2	0	2	1	0	2	
	serum	ρ	-0.48	-0.09	0.12	0.03	0.00	0.32	0.26	-0.09	0.54	0.67	0.5			
		q	-	-	-	-	-	-	-	-	-	**0.023**	-			
		n	19	19	19	15	19	13	18	18	7	19	11	20		
DCVG	kidney	ρ	-0.17	0.04	-0.21	0.52	0.2	0.22	0.11	-0.43	-0.7	0.83				
		q	-	-	-	-	-	-	-	-	-	**0.002**				
		n	14	15	15	10	15	10	14	14	5	15	15			
	liver	ρ	-0.49	-0.10	-0.30	0.54	0.09	0.52	0.40	-0.19	0.77					
		q	-	-	-	-	-	-	-	-	-					
		n	21	26	26	17	26	17	25	25	6	26				
	serum	ρ	-0.29	-0.2	0.31	-0.31	0.37	0.09	-0.1	0.2						
		q	-	-	-	-	-	-	-	-						
		n	7	6	6	6	6	6	5	5	7					
TCOH	kidney	ρ	0.02	0.09	0.04	-0.39	-0.02	-0.21	-0.10							
		q	-	-	-	-	-	-	-							
		n	20	25	25	16	25	16	24	25						
	liver	ρ	-0.28	-0.28	-0.30	0.13	-0.12	0.47								
		q	-	-	-	-	-	-								
		n	20	25	25	16	25	16	25							
DCA	kidney	ρ	-0.54	-0.10	-0.37	0.69	0.27									
		q	-	-	-	-	-									
		n	14	17	17	12	17	17								
	liver	ρ	0.78	0.74	0.62	0.19										
		q	**0.002**	**0.002**	**0.011**	-										
		n	21	26	26	17	26									
	serum	ρ	-0.11	-0.24	-0.24											
		q	-	-	-											
		n	18	17	17	18										
TCA	kidney	ρ	0.61	0.34												
		q	**0.033**	-												
		n	21	26	26											
	liver	ρ	0.62													
		q	**0.031**													
		n	21	26												
	serum	ρ														
		q														
		n	22													

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 3.2. Correlations among TCE metabolites and toxicity phenotypes in kidneys of TCE-treated mice in a sub-acute study (600 mg/kg/d for 5 days). Data from vehicle-treated samples were not included in the analyses.

		TCA	DCA	TCOH	DCVG	DCVC	Kidney/ Body weight ratio	BUN	Proximal tubule cell proliferation	*Acox1*	*Cyp4a10*	KIM-1
KIM-1	ρ	0.21	-0.48	0.29	-0.31	-0.51	0.19	-0.19	0.56	-0.29	-0.35	
	q	-	-	-	-	-	-	-	**0.086**	-	-	
	n	26	17	25	15	15	27	24	24	21	21	27
Cyp4a10	ρ	0.06	0.42	-0.02	0.64	0.59	0.15	0.46	-0.24	0.93		
	q	-	-	-	-	-	-	-	-	**0.003**		
	n	21	13	20	10	12	21	18	20	21	21	
Acox1	ρ	0.22	0.40	-0.09	0.55	0.55	0.07	0.50	-0.27			
	q	-	-	-	-	-	-	-	-			
	n	21	13	20	10	12	21	18	20	21		
Proximal tubule cell proliferation	ρ	0.14	-0.34	0.06	-0.21	-0.36	0.19	0.12				
	q	-	-	-	-	-	-	-				
	n	23	15	22	12	13	24	21	24			
BUN	ρ	0.54	-0.13	-0.04	-0.09	-0.07	-0.04					
	q	-	-	-	-	-	-					
	n	23	15	22	15	13	24	24				
Kidney/ Body weight ratio	ρ	0.12	-0.27	0.80	-0.30	-0.14						
	q	-	-	**0.003**	-	-						
	n	26	17	25	15	15	27					
DCVC	ρ	-0.34	0.64	-0.36	0.72							
	q	-	-	-	-							
	n	15	12	14	9	15						
DCVG	ρ	-0.21	0.22	-0.43								
	q	-	-	-								
	n	15	10	14	15							
TCOH	ρ	0.04	-0.21									
	q	-	-									
	n	25	16	25								
DCA	ρ	-0.37										
	q	-										
	n	17	17									
TCA	ρ											
	q											
	n	26										

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 3.3. Correlations among TCE metabolites in serum, liver, and kidney in TCE-treated mice in a sub-chronic study (100 or 400 mg/kg/d for 1, 2 or 4 wks).

			TCA			DCA			TCOH		DCVG
			serum	liver	kidney	serum	liver	kidney	liver	kidney	liver
DCVG	liver	ρ	0.04	0.20	0.16	-0.09	-0.13	-0.08	0.41	0.22	
		q	-	-	-	-	-	-	-	-	
		n	15	16	16	15	16	9	16	15	16
TCOH	kidney	ρ	0.74	0.79	0.78	0.82	0.78	-0.13	0.86		
		q	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**		
		n	38	40	37	39	40	23	35	40	
	liver	ρ	0.65	0.60	0.71	0.72	0.65	-0.34			
		q	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-			
		n	36	37	34	36	37	21	37		
DCA	kidney	ρ	-0.15	-0.18	0.02	-0.20	-0.28				
		q	-	-	-	-	-				
		n	22	25	24	25	25	25			
	liver	ρ	0.83	0.90	0.76	0.97					
		q	**<0.001**	**<0.001**	**<0.001**	**<0.001**					
		n	40	43	40	42	43				
	serum	ρ	0.87	0.91	0.80						
		q	**<0.001**	**<0.001**	**<0.001**						
		n	39	42	39	42					
TCA	kidney	ρ	0.60	0.73							
		q	**<0.001**	**<0.001**							
		n	37	40	40						
	liver	ρ	0.90								
		q	**<0.001**								
		n	40	43							
	serum	ρ									
		q									
		n	40								

Table displays Spearman rank correlation (r), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

Supplementary Table 3.4. Correlations among TCE metabolites and toxicity phenotypes in kidneys of TCE-treated mice in a sub-chronic study (100 or 400 mg/kg/d for 1, 2 or 4 wks). Data from vehicle-treated samples were not included in the analyses.

		TCA	DCA	TCOH	Kidney /Body weight ratio	SCr	BUN	Proximal tubule cell proliferation	*Acox1*	*Cyp4a10*	KIM-1
KIM-1	ρ	0.45	-0.06	0.35	0.13	0.03	0.33	-0.11	0.10	0.25	
	q	**0.038**	-	-	-	-	-	-	-	-	
	n	37	23	37	39	35	35	40	34	34	40
Cyp4a10	ρ	0.54	0.10	0.48	-0.24	0.22	0.40	0.07	0.91		
	q	**0.020**	-	**0.051**	-	-	-	-	**0.002**		
	n	32	21	32	34	30	30	35	35	35	
Acox1	ρ	0.52	0.05	0.34	-0.33	0.09	0.30	0.11			
	q	**0.021**	-	-	-	-	-	-			
	n	32	21	32	34	30	30	35	35		
Proximal tubule cell proliferation	ρ	0.24	0.02	0.26	0.19	-0.14	-0.19				
	q	-	-	-	-	-	-				
	n	40	25	40	42	37	37	43			
BUN	ρ	0.42	0.30	0.33	-0.06	0.21					
	q	**0.095**	-	-	-	-					
	n	35	20	35	36	37	37				
SCr	ρ	-0.01	0.44	0.31	-0.25						
	q	-	-	-	-						
	n	35	20	35	36	37					
Kidney/Body weight ratio	ρ	0.09	-0.14	0.12							
	q	-	-	-							
	n	39	24	39	42						
TCOH	ρ	0.78	-0.13								
	q	**0.002**	-								
	n	37	23	40							
DCA	ρ	0.02									
	q	-									
	n	24	25								
TCA	ρ	1									
	q										
	n	40									

Table displays Spearman rank correlation (ρ), false discovery rate-corrected significance value (q), and the number of data points with matching values in the same animal (n). Empty cell indicates that no correlation value was computed. Dash (-) indicates that the correlation was not significant (q>0.1).

CHAPTER 4: EFFECTS OF PPARα ON METABOLISM AND TOXICITY OF TRICHLOROETHYLENE

I. INTRODUCTION

The role of peroxisome proliferator-activated receptor alpha (PPARα) activation in liver cancer in mice exposed to trichloroethylene (TCE) and its human relevance remain to be contentious issues in TCE hazard assessment (Keshava and Caldwell 2006; Klaunig et al. 2007). Activation of PPARα signaling and associated molecular events has been observed to occur in rodent liver upon administration of not only TCE, but also a number of xenobiotics such as industrial chemicals, herbicides, and some pharmaceuticals (Klaunig et al. 2003). Important species differences (e.g., induction of peroxisomes and liver enlargement observed in rodent, but not human liver) and similarities (e.g., activation of lipid catabolism and hypocholesterolemia) have been reported in the action of PPARα-activating chemicals (Peters et al. 2012).

Much of the debate with respect to the role of PPARα in adverse health effects of TCE has focused on the toxicodynamic aspects of the mechanism as it is assumed that the PPARα pathway affects enzymatic pathways that do not play a role in TCE metabolism. Specifically, it is well recognized that PPARα activation by TCE metabolites plays a role in the hepatocarcinogenesis. It has been hypothesized that the key mechanistic events for TCE-induced carcinogenesis in liver constitute the following: a TCE oxidative metabolite, trichloroacetic acid (TCA), after being produced in the liver, activates the PPARα receptor, which then leads to alterations in cell proliferation and apoptosis resulting in clonal

expansion of initiated cells (Corton et al. 2014). While *in vivo* administration of TCE causes activation of PPARα in murine models (Elcombe et al. 1985; Nakajima et al. 2000) and TCA and DCA are reasonably potent ligands of murine PPARα (Zhou and Waxman 1998), the complexity of the molecular responses to TCE suggest that PPARα may not be the exclusive mode of action in TCE hepatocarcinogenesis in rodents (Guyton et al. 2009; Rusyn et al. 2014). Furthermore, recent research has shown that PPARα activation and the resulting biological events are not sufficient to induce hepatocarcinogenesis (Yang et al. 2007). Moreover, it was demonstrated that a potent PPARα agonist, bis(2-ethylhexyl) phthalate leads to the induction of liver tumors in PPARα-null mice (Ito et al. 2007), which indicates that the hypothesized PPARα mode of action may not be necessary for liver tumor induction.

In addition, it should be noted that there are differences between TCE-induced and oxidative metabolites-induced toxicity. TCA-induced tumors have molecular features similar to those induced by typical PPARα activators, whereas TCE-induced tumors may occur by modes of action different from that of TCA, based largely on dissimilarities in molecular markers found in TCE versus TCA-induced tumors (Bull et al. 2002; Corton 2008). Moreover, the liver tumor formation due to dichloroacetic acid (DCA) occurs at a much lower exposure level than that which induces PPARα activation (Laughter et al. 2004; Stacpoole 2011). Hence, it is reasonable that multiple modes of action including PPARα activation may be involved in liver carcinogenesis.

In both proponents and opponents of PPARα activation MoA, an important hypothesis is that difference in internal dose caused by different metabolism capacity may explain the observed disparities described above and elsewhere (National Toxicology Program 1990), however there is no study associating levels of metabolites in tissues with

toxicity in the context of PPARα. Although it is known that TCA, a major metabolite of TCE, may activate PPARα, uncertainties remain regarding the association between the extent of oxidative TCE metabolism, PPARα activation, and toxicity pathways that may be independent of PPARα. In this regard, the present study is designed to investigate the effect of PPARα status (wild-type, *Ppara*-null, and humanized-*Ppara*) on liver and kidney toxicity in the context of TCE metabolism in mice.

II. MATERIALS AND METHODS

***Animals and treatments*.** Male and female mice from 3 different genotypes were utilized. Wild-type (129S1/SvImJ) and *Ppara*-null (129S4/SvJae-*Ppara*^{tm1Gonz}/J) mice of 9-10 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME), and humanized-*Ppara* mice on an Sv/129 genetic background were provided by Dr. Gonzalez at the Laboratory of Metabolism, the National Cancer Institute (Cheung et al. 2004). All mice were housed in polycarbonate cages on Sani-Chips (P.J. Murphy Forest Products Corp., Montville, NJ) irradiated hardwood bedding. Animals were fed an NTP-2000 (Zeigler Brothers, Inc., Gardners, PA) wafer diet and water *ad libitum* on a 12 h light-dark cycle. All studies were approved by the UNC Institutional Animal Care and Use Committee.

Two study designs were utilized in this work. First, we performed a sub-chronic study where TCE (400 mg/kg/day, in 5% Alkamuls EL-620 in saline) was administered by gavage to male and female mice from the 3 different genotypes for 4 weeks (5 days/week). Mice were also given drinking water containing 0.2 g/L of 5-bromo-2'-deoxyuridine (BrdU) for 72 hrs prior to sacrifice. Blood, liver, kidney and a section of a duodenum were collected 5 hrs after the last TCE treatment. The timing of sacrifice was selected based on the toxicokinetic studies of TCE in the mouse (Kim et al. 2009b). Second, we conducted a toxicokinetic study

where wild-type, *Ppara*-null, and h*Ppara* mice received a single dose (400 mg/kg) of TCE in 5% Alkamuls EL-620 in saline by gavage and sacrificed 2, 5, and 12 hrs after TCE treatment followed by the collection of liver, kidney and blood. Blood was drawn from vena cava and centrifuged to prepare serum using Z-gel tubes (Sarstedt, Germany) according to the manufacturer's instructions. Body and organ weights were recorded. Liver, kidney, and duodenum sections were fixed in neutral buffered formalin for 24 hrs, and the remainder of the liver and kidney tissues were frozen in liquid nitrogen. All serum and tissue samples were stored at -80°C until analyzed.

Quantification of TCE. Prior to extraction, liver (100 mg) and kidney (30 mg) samples were homogenized with two volumes of deionized water (w/v) using a finger pestle. Tissue homogenate (200 µL) were transferred in auto sampler vials containing 200 µL of ammonium sulfate solution, then 1 µL of internal standards (TCE-deuterated) were added using microsyringe. After putting a magnetic lid on a sample using vial capper, the vials were vortexed for 30 sec and placed into the autosampler for analysis. The analyses were carried out on an Agilent 7890 gas chromatograph (GC) coupled with a 5975C mass selective detector. The GC was equipped with a 0.75 mm i.d. Solid Phase Microextraction (SPME) liner. Separation of the analytes was obtained on a DB-5MS column (Phenomenex, 30 m x 0.25 mm i.d., 0.25 µm film thickness) using helium as a carrier gas (flow rate, 1 mL/min). The GC injection port and interface transfer line were maintained at 200 ℃ and 280 ℃, respectively. During the fiber desorption process, the splitless mode of injection was operated. After 2 min, the split vent valve opened to sweep any residual vapors from the liner. The oven temperature was initially held at 35 ℃ for 3min, then increased to 70 ℃ at 10 ℃/min. The mass spectrometer was operated positive electron ionization (EI) mode with an electron

energy of 71eV. Quantitation of TCE was performed using selected-ion monitoring (SIM) mode by quantitation ion monitoring m/z 130. [131 for TCE-d]. GC-HS-SPME was performed using a 100 μm polydimethylsiloxane (PDMS) fiber mounted on a Combi-Pal system autosampler. Fibers were conditioned at 200 ℃ for 30 min prior to use. Sample vials were preheated in the agitator for 5 min before analysis, and the SPME fiber was then exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 15 min at 30 ℃ under agitation, the fiber was withdrawn into the needle and immediately desorbed at 200 ℃ for 2 min into the GC injection port.

***Quantification of TCE metabolites*.** In order to measure the levels of TCA, DCA, *S*-(1,2-dichlorovinyl)glutathione (DCVG), and *S*-(1,2-dichlorovinyl)-l-cysteine (DCVC) in liver and kidney tissues, 2 mL Eppendorf Safe Lock Tubes™ containing one stainless steel ball each with 300 μL of chloroform and 60 μL of water were incubated on dry ice for 10 min in a cooling block. Liver (100 mg) or kidney (50 mg) tissue was then incised from frozen tissue samples and placed into each of these tubes. Mixture of internal standards (20 μL; [$^{13}C_5$, ^{15}N] DCVC and [$^{13}C_4$, ^{15}N] DCVG, 25 nmol/mL each; trifluoroacetic acid and difluoroacetic acid, 40 nmol/mL) was added to make 100 μL of final aqueous volume. The tubes were then homogenized at 30 Hz for 3 min using TissueLyser (Qiagen, Valencia, CA). After homogenization, the tubes were centrifuged at 14,000×g for 30 min at 4°C. Aqueous liver or kidney extract was transferred to a centrifugal filter unit (Amicon® Ultra 0.5, Millipore, MA, USA) and centrifuged at 14,000×g for 60 min at 4°C. After centrifugation, the filtrate was transferred to a glass vial containing 300 μL vial insert and was stored at -80°C until injection to HPLC-ESI-MS/MS with an Aquity UPLC® system (Waters, Milford, MA) coupled to a TSQ Quantum Ultra triple-quadrupole mass analyzer (Thermo Fisher

Scientific, Waltham, MA) using a heat-assisted electrospray ionization source. The lower limits of quantification (LLOQ) in this study were: 8 nmol/g in liver and 15 nmol/g in kidney for TCA, 0.5 nmol/g in kidney for DCA, 2 pmol/g in liver and 8 pmol/g in kidney for DCVG, 20 pmol/g in kidney for DCVC.

***Determination of triglyceride content in liver*.** Triglycerides were extracted by homogenizing 20 mg of frozen liver tissue in 500 µL of isopropyl alcohol, and 4 µL of the extract was used in subsequent analysis. The level of triglycerides was determined by using L-type Triglyceride-M Assay Kit (Wako Chemicals, Richmond, VA) according to the manufacturer's instructions.

***Quantification of glutathione, cysteine, and nicotinamide adenine dinucleotide phosphate (NADP) redox status*.** The concentrations of free reduced (GSH) and oxidized glutathione (GSSG) and cellular methylation biomarkers, *S*-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-homocysteine (SAH) were determined as measures of redox/metabolic status in liver and kidney by using the high performance liquid chromatography (HPLC) with colorimetric electrochemical detection (HPLC-ED) system (MCM, Inc., Tokyo, Japan). The methodological details for the detection of GSH and GSSG (Melnyk et al. 1999), SAM and SAH (Melnyk et al. 2000) by HPLC have been described previously. NADPH/NADP+ ratio in liver was measured using a NADP/NADPH Quantification Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

***Gene expression analysis by real-time PCR*.** Total RNA was isolated from liver and kidney samples using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2000 Bioanalyser, respectively.

Total RNA was reverse transcribed using random primers and the high capacity complementary DNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The following gene expression assays (Applied Biosystems) were used for quantitative real-time PCR: peroxisome proliferator–activated receptor alpha (*Ppara*, Mm00440939_m1); palmitoyl acyl-Coenzyme A oxidase 1 (*Acox1*, Mm01246831_m1); cytochrome P450, family 4, subfamily a, polypeptide 10 (*Cyp4a10*, Mm01188913_g1); and beta glucuronidase (*Gusb*, Mm00446953_m1). Reactions were performed in a 96-well plate, and all samples were plated in duplicate using LightCycler® 480 instrument (Roche Applied Science, Indianapolis, IN). The cycle threshold (Ct) for each sample was determined from the linear region of the amplification plot. The ΔCt values for all genes relative to the control gene *Gusb* were determined. The ΔΔCt were calculated using treated group means relative to strain-matched control group means. Fold change data were calculated from the ΔΔCt values.

***Determination of hepatocyte and proximal tubule cell proliferation*.** Deparaffinized and rehydrated liver and kidney sections were immersed in 4N HCl and subsequently pepsin solution (Dako, Carpinteria, CA) for antigen retrieval and then incubated in peroxidase blocking reagent (Dako). Dako EnVision System HRP kit was used for the detection of BrdU-incorporated nuclei (monoclonal anti-bromodeoxyuridine antibody, Dako, 1:200 dilution). Data for liver tissues were presented as a fraction of BrdU staining-positive nuclei in the centrilobular region (no fewer than 1,000 nuclei counted per liver section). Data for kidney tissues were presented as a fraction of BrdU staining-positive nuclei in the tubular epithelium of the renal cortex (no fewer than 1,000 nuclei counted per kidney section).

***Determination of KIM-1 expression in kidney*.** Detection of KIM-1 was accomplished by modifying a published method (Humphreys et al. 2011). Formalin-fixed

and paraffin-embedded kidney sections were deparaffinized and rehydrated. Antigens were retrieved by 4N HCl and pepsin solution (Dako) afterward. After peroxidase blocking, immunohistochemical detection was conducted using Dako Liquid DAB Substrate Chromogen System with primary anti KIM-1 antibody (2 ug/mL in PBS) (R&D Systems, Minneapolis, MN) and secondary goat IgG HRP-conjugated Antibody (1:100 in PBS) (R&D Systems). The proportion of positive-stained proximal tubules in outer medulla was determined under light microscopy. Data were presented as a fraction of proximal renal tubules staining positive for KIM-1 (no fewer than 200 proximal renal tubules counted per kidney section).

***Statistical analysis*.** Given the small sample size, the exact permutation test was used to determine significant differences between control and TCE-treated groups (α=0.05). All statistical analyses were performed using SAS software ver. 9.3 (SAS Institute, Cary, NC).

III. RESULTS

Sub-chronic (4 weeks) study of PPARα-dependent effects of TCE in mouse liver and kidney

Levels of TCE in liver 5 hrs after last dose were in the range of 0.1-6.7 nmol/g liver (Figure 1A). There was no difference in TCE concentration by genotype and sex. The level of TCA in liver at the same time point was 100 to 200-fold higher than that of TCE in liver (Figure 1B), which is indicative of efficient CYP450-mediated oxidative metabolism of TCE in liver. Interestingly, levels of TCA in liver were significantly different by PPARα genotype even though mice of different sexes and genotypes were treated under identical conditions with the same solution of TCE or vehicle. The highest mean liver levels of TCA were observed in male and female wild-type mice.

TCE was present in detectable amounts in kidney at the levels comparable to those in liver (Figure 1C). In contrast to the finding in liver, amounts of TCA were not different by genotype in the kidney (Figure 1D), with the TCA amount being 100-200 fold greater than those for TCE. In both liver and kidney, levels of TCA were higher in male than in female mice. Levels of DCA in kidney, DCVG in liver and kidney, DCVC in kidney were below their respective LLOQ.

Liver size (relative to body weight), hepatocellular proliferation, and triglyceride content in liver were examined in vehicle- and TCE-treated mice (Figures 2A-C). We observed significant effects of sub-chronic treatment with TCE on liver enlargement in wild-type and *Ppara*-null males. In these two groups, the hepatocellular proliferation was increased, albeit not statistically significant. A modest but significant increase in hepatic levels of triglycerides was observed only in TCE-treated wild-type males. Basal levels of triglycerides in *Ppara*-null mice were higher compared to wild-type and h*Ppara* mice, an observation previously attributed to the disruption of fat metabolism pathway in livers of *Ppara*-null mice.

Treatment with TCE also resulted in the decrease of fGSH/fGSSG ratio, an indicator of oxidative stress, in the liver of wild-type and *Ppara*-null male group (Figure 2D). However, SAM/SAH ratio was not affected by TCE treatment across genotypes and sexes (Figure 2E). The NADPH/NADP+ ratio in liver, another indicator of oxidative stress, was significantly decreased in male and female wild-type mice in response to TCE (Figure 2F).

Because PPARα activation has been suggested as contributing to liver enlargement in rodents (Marsman et al. 1988), we evaluated expression of *Ppara*, *Acox1*, and *Cyp4a10*, marker genes for this mechanistic event. In liver (Figures 3A-C), expression of the

transcription factor *Ppara* was not affected by TCE except in wild-type males; however, expression of *Cyp4a10* and *Acox1* was markedly elevated not only in wild-type but also in h*Ppara* mice, with TCE-induced effect being much greater for *Cyp4a10* than for *Acox1*.

For the evaluation of kidney toxicity phenotypes, we measured kidney to body weight ratio, proximal tubule cell proliferation, and KIM-1 expression, but none of these were affected by TCE treatment (Figures 4A-C). In addition, fGSH/fGSSG and SAM/SAH ratios did not change upon exposure to TCE (Figures 4D-E).

Given the large amounts of a CYP450-mediated oxidative metabolite detected in kidney, we evaluated kidney expression of *Ppara*, *Cyp4a10* and *Acox1* (Figures 5A-C). Although we did not observe TCE-induced effect on kidney in terms of apical endpoints, the up-regulation of gene *Cyp4a10*, associated with PPARa activation, was observed in the kidney of wild-type and h*Ppara* males.

Toxicokinetic study of PPARα-dependent effects on TCE metabolism

One of the novel findings in the sub-chronic study was that the levels of TCA concentration in liver were highly dependent on the status of PPARα. As the association between PPARα activation and TCE metabolism has not been investigated in detail, we conducted a toxicokinetic study where levels of TCE and its metabolites were quantified in the liver and kidney of mice at 2, 5, and 12 h after treatment with single-dose of TCE (400 mg/kg) in the aqueous vehicle by oral gavage.

Levels of TCE in male liver were at the highest at 2 h and gradually decreased over time in all strains (Figure 6A). Mean levels of TCE in liver at 5 h was comparable with that observed in the sub-chronic study. Levels of TCE in male kidney showed similar time-course

profile with that in liver (Figure 6B). In females, kinetic profile of TCE concentration in liver and kidney is in line with that in males (Figures 6C-D).

In the analysis of TCA in male liver, the observed difference in liver TCA from the sub-chronic study was reproduced (Figure 7A). Mean levels of TCA in male liver were at the highest in wild-type. In case of kidney level of TCA in males, wild-type and h*Ppara* groups exhibited similar levels compared with the data from the sub-chronic study; however, the mean level in *Ppara*-null group was lower than that observed in the sub-chronic study (Figure 7B). In all strains of female group, mean levels of TCA at 5 hrs in both liver and kidney were comparable with the levels observed in the sub-chronic study (Figures 7C-D). While TCE concentration decreased after 2 h time point regardless of tissue, genotype, and sex, TCA analysis revealed an increasing trend over time, which was evident in wild-type males. Levels of DCA in kidney, DCVG in kidney, and DCVC in kidney were below the LLOQ.

In order to determine the acute effect of TCE treatment on PPARα activation, we evaluated liver expression of *Cyp4a10* and *Acox1*, marker genes for the mechanistic event (Figures 8A-B). A significant up-regulation of gene, *Acox1* was observed both in male and female wild type groups. Meanwhile, *Cyp4a10* was markedly elevated not only in wild-type but also in h*Ppara* group. In line with the observation in the sub-chronic study, TCE-induced effect was much greater for *Cyp4a10* than for *Acox1*.

IV. DISCUSSSION

Association between exposure to TCE and PPARα activation in the liver of mice is well-established (Klaunig et al. 2003). PPARα activation in response to TCE is likely to be mediated by CYP450-dependent oxidative metabolism (Ramdhan et al. 2008). Indeed, TCA

and DCA has been reported to be capable of activating mouse PPARα as evidenced *by in vitro* receptor activation assays (Issemann and Green 1990; Zhou and Waxman 1998) and *in vivo* mouse study (Laughter et al. 2004). Likewise, *in vitro* transactivation study has demonstrated that human PPARα is activated by either TCA or DCA, while the parent compound, TCE is relatively inactive (Maloney and Waxman 1999).

Three previous experimental studies examined the effect of PPARα genotype on TCE-induced hepatotoxicity using wild-type and *Ppara*-null (and h*Ppara*) mice (Laughter et al. 2004; Nakajima et al. 2000; Ramdhan et al. 2010). It was consistently observed that the expression of enzymes regulated by PPARα, including CYP4A, increased in wild-type, but not in *Ppara*-null mice. Nakajima et al. (2000) evaluated volume density of peroxisomes in the pericentral area of liver and found that hepatic peroxisome proliferation increased by TCE treatment in wild-type but not in *Ppara*-null mice. However, the observations on hepatomegaly were not consistent. Nakajima et al. (2000) and Ramdhan et al. (2010) [750 mg/kg/d of TCE for 2 weeks and 2,000 ppm of TCE (equivalent to 1,600 mg/kg/d) for 7 days (8 hrs/day), respectively] reported the increase in liver to body weight ratio in both wild-type and *Ppara*-null groups, while the other study where 1,500 mg/kg/d of TCE was dosed for 3 weeks, observed liver enlargement only in wild-type (Laughter et al. 2004).

The present study described the levels of TCE and TCA in the liver and kidney of mice sub-chronically treated with TCE. As expected, TCA concentration was 100 to 200-fold higher than TCE concentration in liver and kidney, which may indicate extensive metabolism of the parent compound. Although no sex difference was found in levels of TCE in liver and kidney, TCA levels tended to be higher in males than in females, which is not surprising as reviewed by the U.S. EPA (U.S. EPA 2011b) that the metabolism capacity is thought to be

higher in male than female in rodents. The most intriguing result of the TCA analysis may be that levels of TCA were significantly different between wild-type, *Ppara*-null, and h*Ppara* in liver. Data on differential TCE metabolism among the strains is limited. Ramdhan et al. (2010) measured urinary TCA and trichloroethanol (TCOH) and found that TCA levels are significantly lower and TCOH levels tended to be higher in *Ppara*-null compared to wild-type and h*Ppara*. They quantified protein expression of CYP2E1 and ALDH2 enzymes, both of which are involved in oxidative metabolism of TCE but did not observe significant differences among controls.

A single-dose toxicokinetic study was also conducted to evaluate a possibility that toxicity reversely affects metabolism in ways of mutual activation or negative feedback. Even after a single dose of TCE, the differences in liver levels of TCA by PPARα status, observed in the sub-chronic study, were closely replicated. This may suggest that there are intrinsic differences in oxidative metabolism of TCE between wild-type, *Ppara*-null, and h*Ppara*.

Liver enlargement was found not only in wild-type but also in *Ppara*-null males, which is in line with the previous observations (Nakajima et al. 2000; Ramdhan et al. 2010). Hepatomegaly can be implicated by many factors including hypertrophy, cellular proliferation, fat/glycogen depositions, abscess, and tumor (Barka and Popper 1967). In the present study, the increase in liver to body weight ratio in wild-type may be caused by PPARα activation-mediated hepatocellular hypertrophy. However, it is not clear which pathway contributed to the liver enlargement in *Ppara*-null. In a previous study, *Ppara*-null mice did not show increased hepatocyte proliferation after administration of peroxisome proliferators; however, hyperplasia, measured by BrdU labeling, significantly increased after

48 h post-hepatectomy in liver of both wild-type and *Ppara*-null mice (Rao et al. 2002). While we observed TCE-induced increase in hepatocellular proliferation in *Ppara*-null, albeit not significant, no evident necrosis in microscopic examination was found. Ramdhan et al. (2010) suspected steatosis as a cause of hepatomegaly based on dose-dependent increase in lipid accumulation in *Ppara*-null group; however, hepatic triglyceride levels increased only in wild-type male mice treated with TCE in our study. The PPARα-dependent and -independent effects could have been mediated by TCE metabolism. Interestingly, it appears that the extent of liver enlargement is associated with TCA concentration in liver.

Exposure to TCE has been associated with oxidative stress in the liver, as determined by various indicators such as superoxide dismutase, GSH/GSSG, malondialdehyde (MDA), NAD+/NADH, or hydrogen peroxide levels in rats (Khan et al. 2009; Tabrez and Ahmad 2011) and mice (Gilbert et al. 2009). Free GSH/GSSG ratios in plasma were also significantly lower in TCE-treated mice (Blossom et al. 2012). A decrease in the active reduced form of GSH and increase in the inactive oxidized disulfide form (GSSG) is a strong indicator of oxidative stress leaving the cell vulnerable to oxidative damage from pro-oxidant environmental exposures. The present study demonstrated that free GSH/GSSG ratio in liver significantly decreased by TCE treatment in wild-type and *Ppara*-null male mice. As peroxisome is a source of reactive oxygen species (Schrader and Fahimi 2004), it is reasonable that PPARα activation is followed by oxidative stress in wild-type. However, the finding from *Ppara*-null mice may suggest alternative pathway independent of PPARα activation. Interestingly, the increase in free GSH/GSSG ratio in liver was considerably associated with TCA levels in liver. Indeed, a few studies reported TCA and DCA-induced oxidative stress in liver in terms of lipid peroxidation and oxidative DNA damage (Austin et

al. 1996; Parrish et al. 1996). Moreover, the authors reported a compelling result that increase in 8-OHdG in nuclear DNA extracted from liver, induced by administration of TCA, occurs too early to be dependent on peroxisome proliferation.

Hence, we suggest that TCA may induce oxidative stress in terms of decreased ratio of free GSH/GSSG independent of PPARα activation. However, it is not clear how oxidative stress is associated with liver enlargement, partly due to the complex nature of the relationship between oxidative stress and cellular physiology. It should be stated that oxidant-mediated effects are associated with both stimulated proliferation (Burdon 1995) and the onset (Slater et al. 1996) and effector phase (Buttke and Sandstrom 1994) of apoptosis. While we reason that the TCA-induced oxidative stress plays a role in liver enlargement, these mechanisms need further study.

In kidney analyses, no TCE-induced effect was observed in terms of proliferation, tubular injury, and oxidative stress. Evidence regarding effect of sub-chronic exposure to TCE on apical endpoints is very limited in mice. With regard to oxidative stress, it was reported that TCE exposure causes the dose-related increases in MDA-/hydroxynonenal (HNE)-protein adducts and the decreases in GSH/GSSG ratio in the kidneys at 24 and 36 weeks, with greater changes at 36 weeks of exposure (Wang et al. 2012).

Gene expression analysis for *Acox1* and *Cyp4a10*, both of which are regulated by PPARα, revealed that both wild-type and h*Ppara* mice are responsive to exposure to TCE in relation with PPARα activation. Especially, *Cyp4a10* expression in h*Ppara* was as strong as in that of wild-type. Ramdhan et al. (2010) reported increased levels of mRNA and protein expression that are involved in PPARα activation in h*Ppara* mice compared to control. However, it is not true that h*Ppara* mice are less responsive than wild-type to all PPARα

activator. Perfluorooctanoic acid-induced increase in *Cyp4a10* expression was stronger in h*Ppara* than in wild-type (Albrecht et al. 2013).

We observed an increase in liver inflammation in TCE-treated h*Ppara* mice, similar to the findings of Ramdhan et al (2010); however, the human relevance of this finding should be interpreted with caution because of the possibility that the interactions between humanized PPARα and mouse-specific signaling pathways may not adequately simulate those in humans.

V. FIGURES AND TABLES

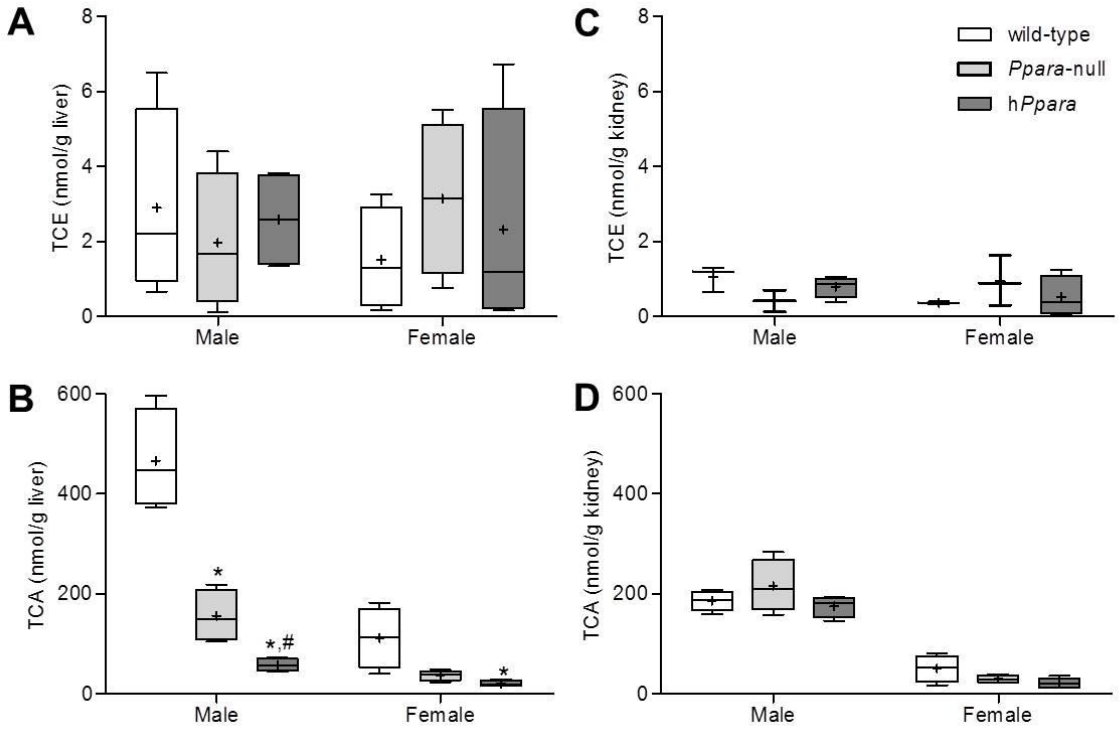


Figure 4.1. Concentration of TCE and TCA in the liver and kidney of mouse in a sub-chronic study. Liver and kidney levels were assessed 5 h following the last of 5 daily doses of TCE per week for 4 weeks (600 mg/kg/d). Box and whisker plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group. Asterisk (*) denotes a significant difference (p<0.05) compared to wild-type group within same sex. Hashtag (#) denotes a significant difference (p<0.05) compared to *Ppara*-null group within same sex.

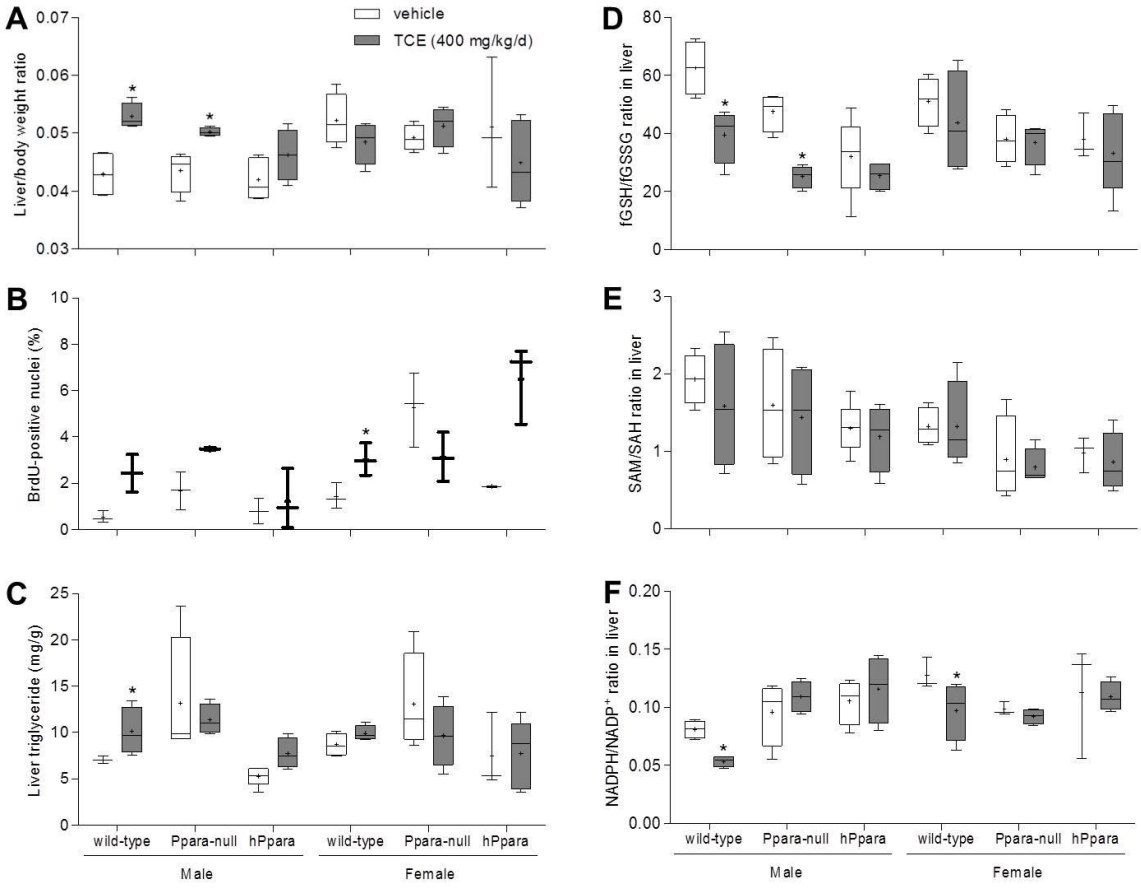


Figure 4.2. PPARα-dependent effects of TCE on liver toxicity in a sub-chronic study. Liver to body weight ratios (A), percent BrdU-positive hepatocyte nuclei (B), triglyceride concentration (C), fGSH/fGSSG ratio (D), SAM/SAH ratio (E), NADPH/NADP+ ratio (F) were evaluated in mice treated with vehicle (white) or TCE (dark gray; 400 mg/kg/d) for 4 weeks. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group. Asterisk (*) denotes a significant difference (p<0.05) compared to vehicle-treated group within same strain and sex.

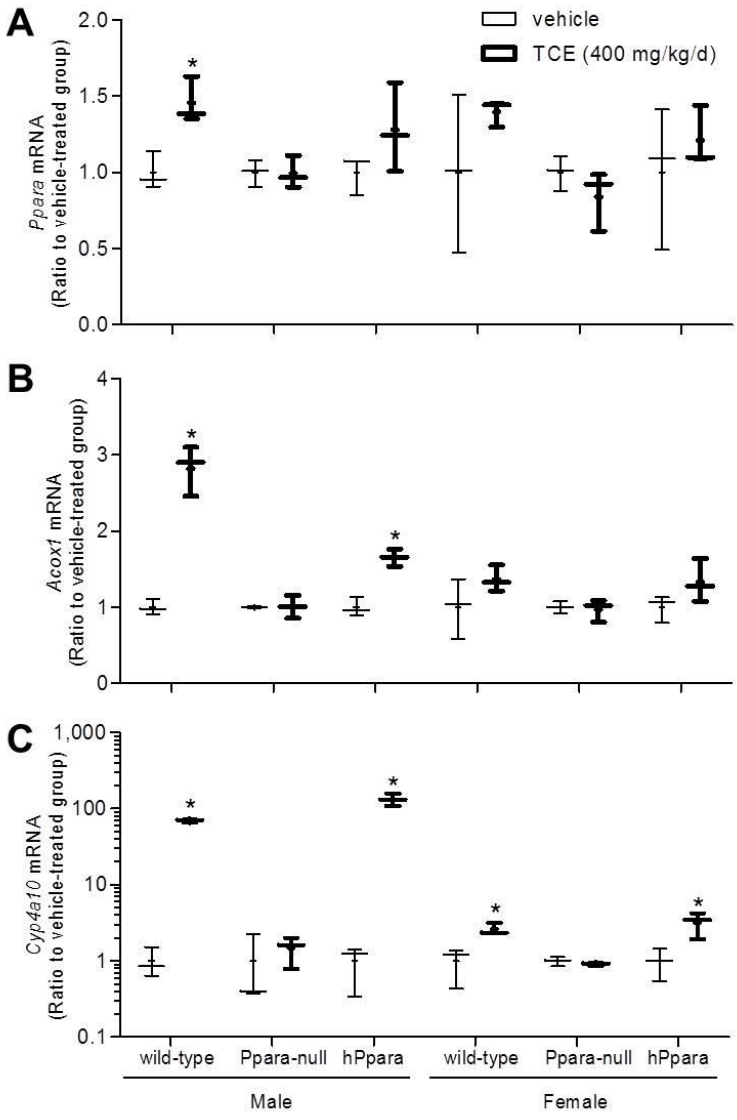


Figure 4.3. Liver expression of PPARα activation marker genes *Ppara* (A), *Acox1* (B), and *Cyp4a10* (C) were evaluated in mice treated with vehicle or TCE for 4 weeks. Whiskers plots are shown (+, mean; line, median; whiskers, min to max). Thickness of the line corresponds to the vehicle and 400 mg/kg/d groups. There were 3 animals per group. Asterisk (*) denotes a significant difference (p<0.05) compared to vehicle-treated group within same strain and sex.

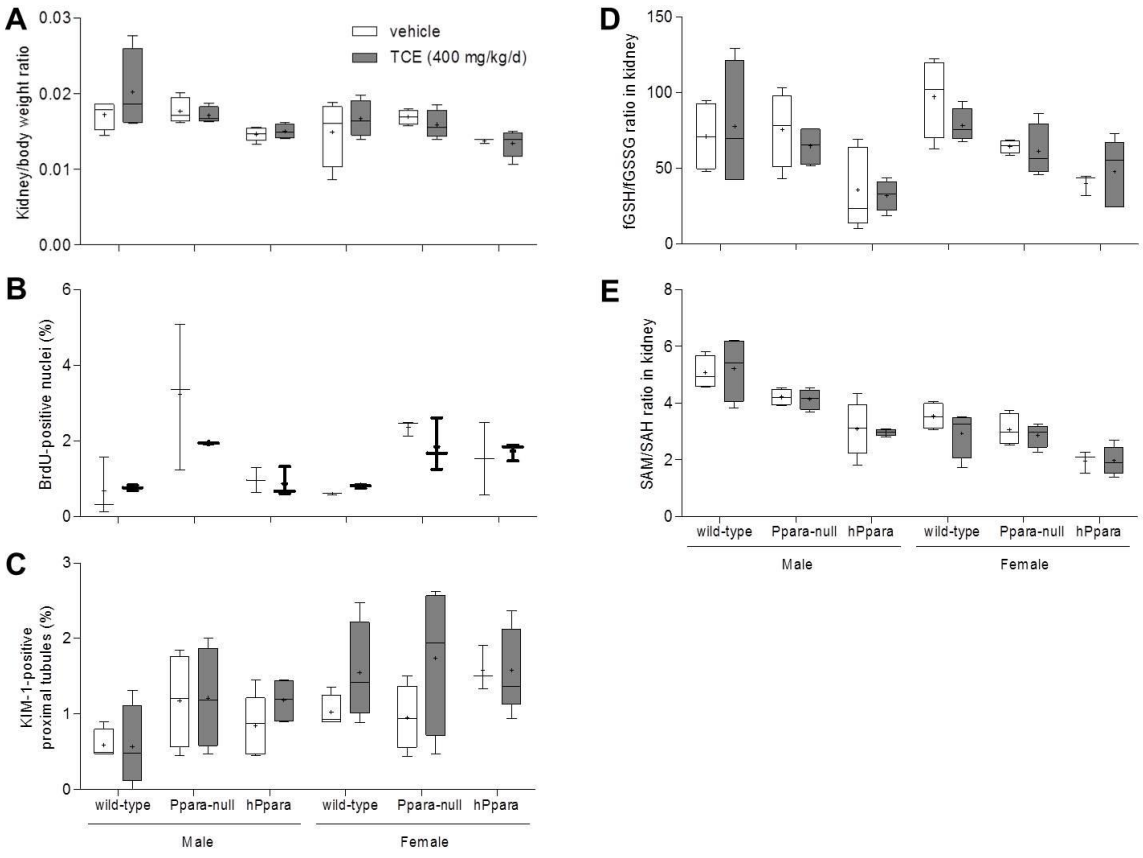


Figure 4.4. PPARα-dependent effects of TCE on kidney toxicity in a sub-chronic study. Kidney to body weight ratios (A), percent BrdU-positive proximal tubule cell nuclei (B), KIM-1 expression (C), fGSH/fGSSG ratio (D), SAM/SAH ratio (E) were evaluated in mice treated with vehicle (white) or TCE (dark gray; 400 mg/kg/d) for 4 weeks. Box and whiskers plots are shown (+, mean; line, median; box, inter-quartile range; whiskers, min to max). When box is shown, 4 animals per group were available. Otherwise, there were 3 animals per group.

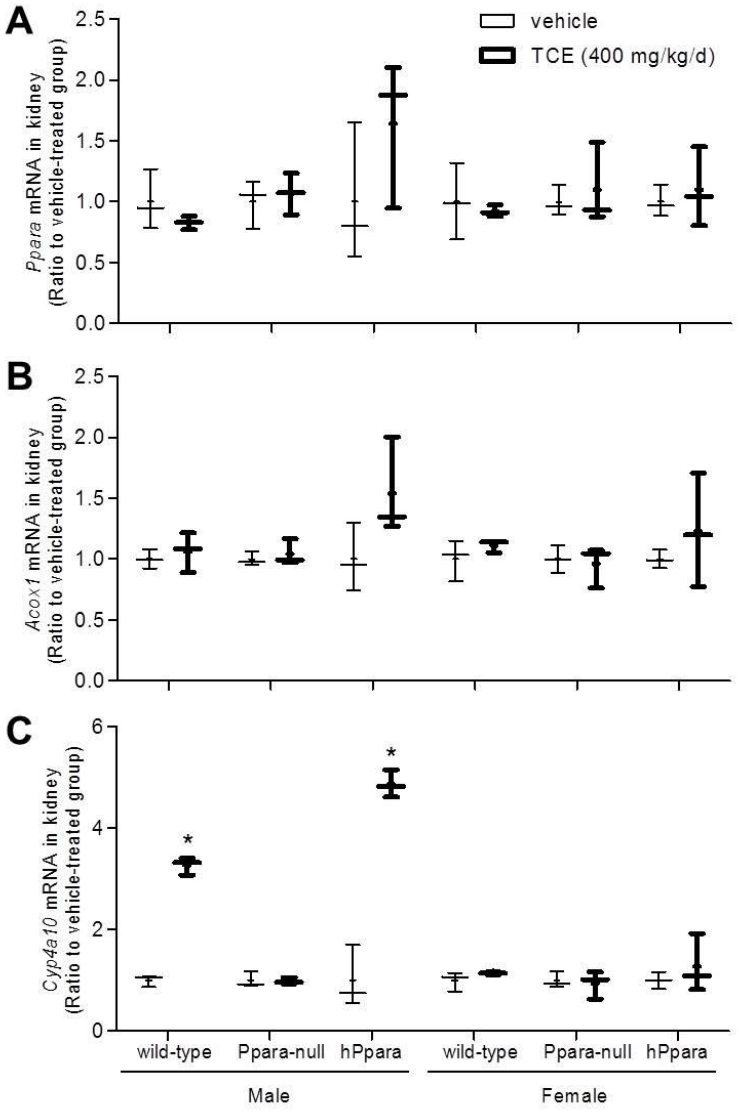


Figure 4.5. Kidney expression of PPARα activation marker genes *Ppara* (A), *Acox1* (B), and *Cyp4a10* (C) were evaluated in mice treated with vehicle or TCE for 4 weeks. Whiskers plots are shown (+, mean; line, median; whiskers, min to max). Thickness of the line corresponds to the vehicle and 400 mg/kg/d groups. There were 3 animals per group. Asterisk (*) denotes a significant difference (p<0.05) compared to vehicle-treated group within same strain and sex.

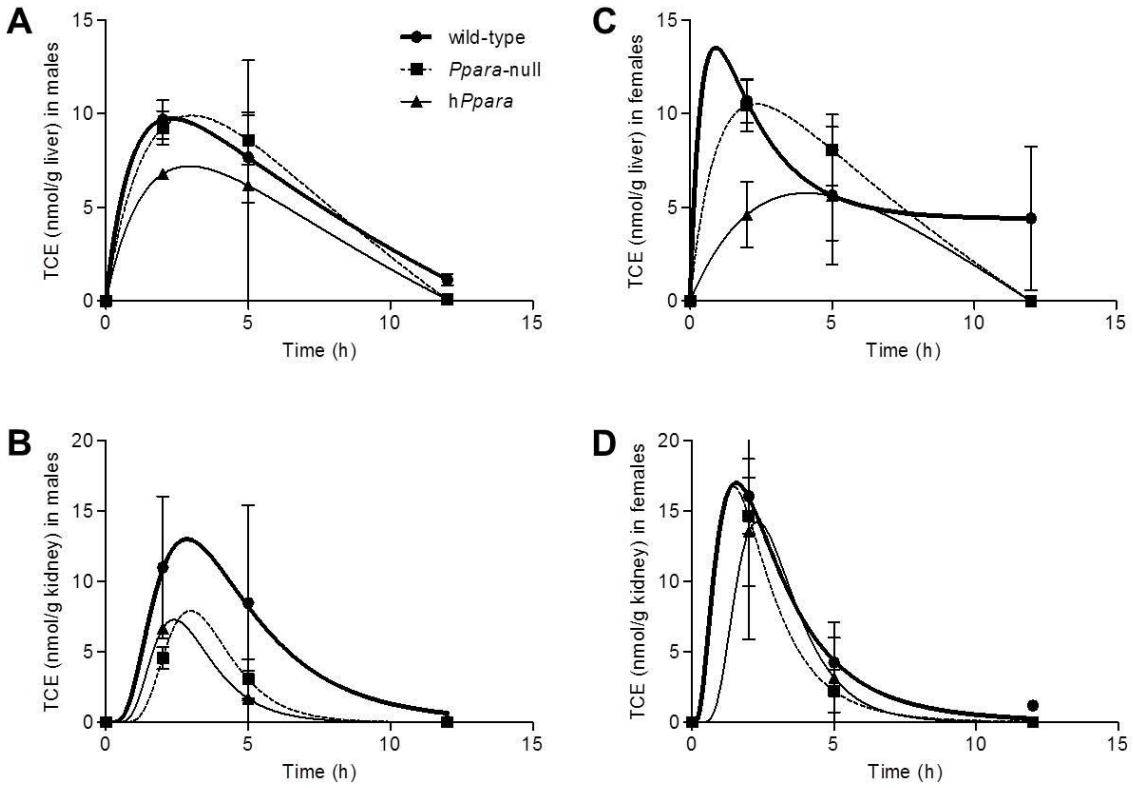


Figure 4.6. Time-course profiles of liver TCE in males (A), kidney TCE in males (B), liver TCE in females (C), kidney TCE in females (D) were determined at 2, 5, and 12 h after single dose of TCE (400 mg/kg).

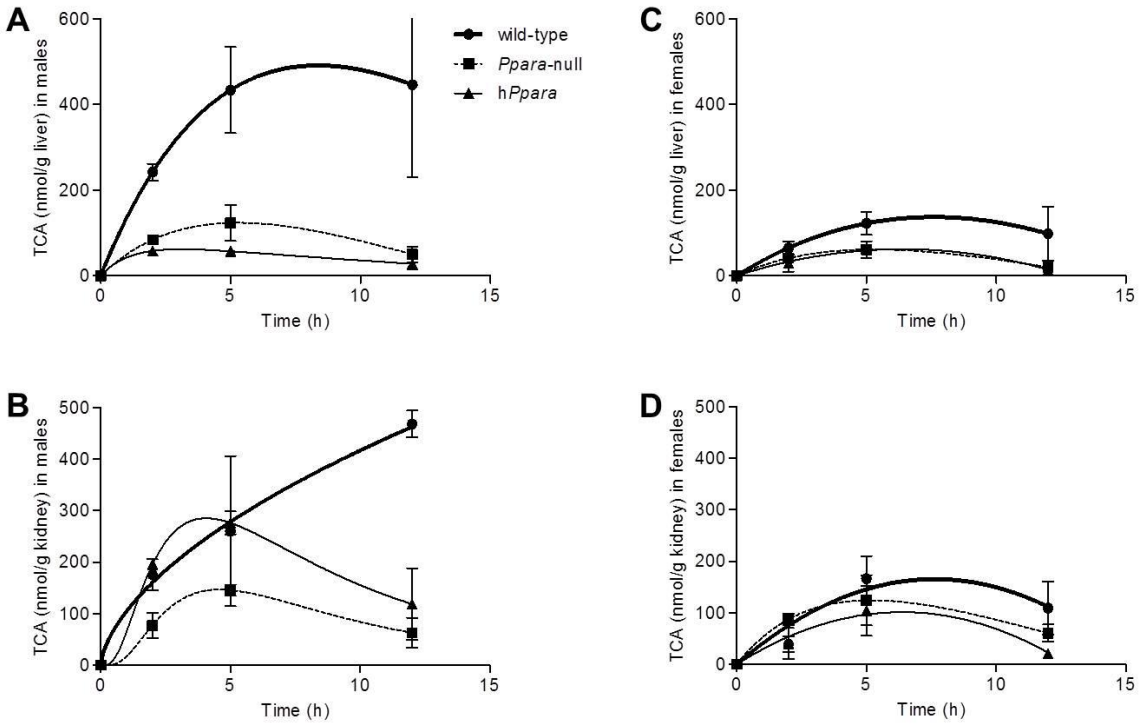


Figure 4.7. Time-course profiles of liver TCA in males (A), kidney TCA in males (B), liver TCA in females (C), kidney TCA in females (D) were determined at 2, 5, and 12 h after single dose of TCE (400 mg/kg).

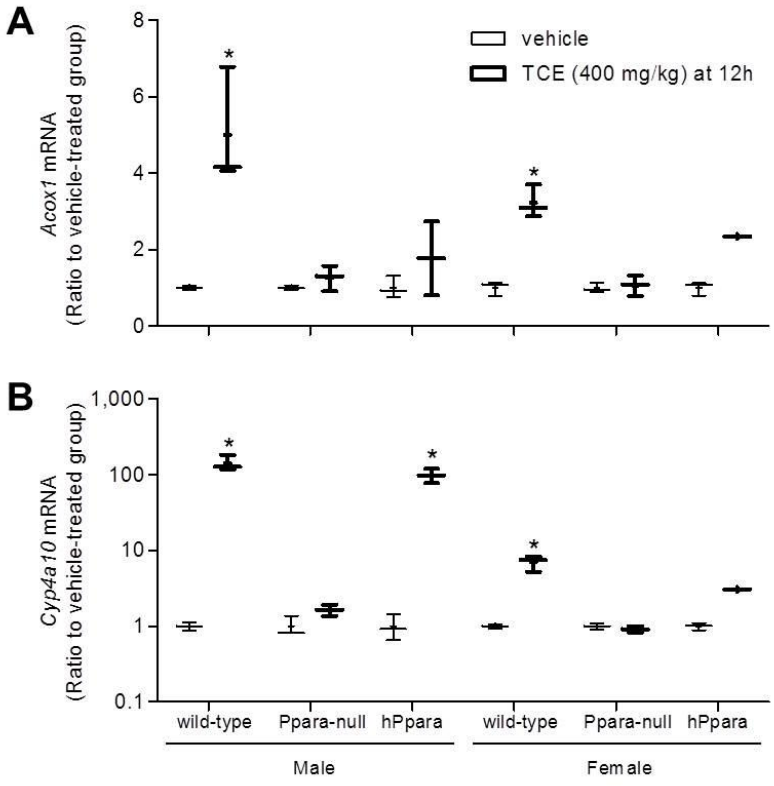
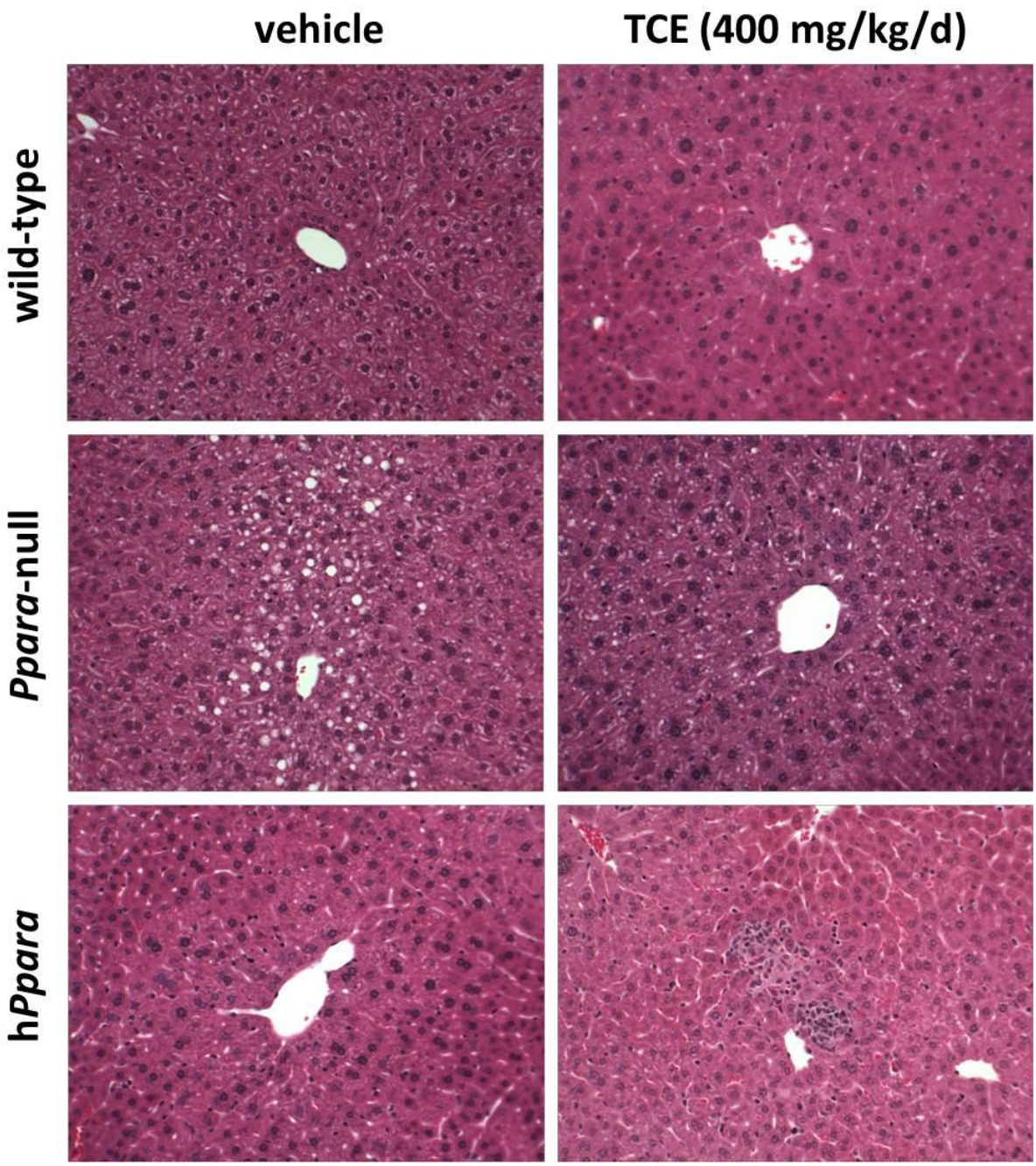


Figure 4.8. Liver expression of PPARα activation marker genes *Acox1* (A) and *Cyp4a10* (B) were compared in mice between vehicle- and TCE-treated (single dose of 400 mg/kg) group. Whiskers plots are shown (+, mean; line, median; whiskers, min to max). Thickness of the line corresponds to the vehicle and 400 mg/kg groups. Asterisk (*) denotes a significant difference (p<0.05) compared to vehicle-treated group within same strain and sex.



Supplementary Figure 4.1. Representative images of hematoxylin & eosin-stained liver sections (×200). Hypertrophy around central vein in TCE-treated wild-type was observed (upper right). As expected, spontaneous steatosis was found in centrilobular region of vehicle-treated *Ppara*-null (center left). In h*Ppara*, minimal to mild inflammations were observed (bottom right).

Sex	Tissue	Genotype	AUC^{last} (nmol/1h)	
			TCE	TCA
Male	Liver	wild-type	66.6	4337.0
		Ppara-null	66.3	1002.0
		h*Ppara*	48.1	530.5
	Kidney	wild-type	70.0	3380.0
		Ppara-null	26.7	1142.0
		h*Ppara*	25.1	2288.0
Female	Liver	wild-type	70.4	1119.0
		Ppara-null	66.5	478.7
		h*Ppara*	39.6	428.8
	Kidney	wild-type	65.6	1312.0
		Ppara-null	47.8	1052.0
		h*Ppara*	49.7	696.2

Supplementary Table 4.1. Areas under the curve for TCE and TCA were calculated from 0 to12 hrs in the toxicokinetic study

CHAPTER 5: GENERAL DISCUSSION

I. CONCLUSIONS

I.A. Specific Aim 1

We demonstrated the inter-strain differences and the decreasing trend of TCE metabolism over time in liver, which may be indicative of the role of genetic factors beyond already established metabolism enzymes (CYP2E1, ADH, ALDH, and etc.). Given that the levels of enzymes were not affected by TCE treatment, the decreasing trend suggests a possibility that the metabolism may be reversely affected by toxicity. The correlation analysis between TCA, DCA, PPARα activation, and liver toxicity suggests that the inter-individual differences in TCE metabolism may be associated with the liver effects. TCA and DCA were strongly correlated with PPARα activation in terms of associated gene expression such as *Cyp4a10* and *Acox1*. However, neither the metabolites nor the PPARα activation correlated with hepatocellular proliferation, which may indicate that multiple pathways may be involved in liver toxicity. The results from the sub-chronic time-course study provided additional clue about the origination of hepatocellular proliferation. Contrary to the decreases in oxidative metabolism and PPARα activation over time, mice dosed with TCE for 4 weeks showed the most prominent liver adverse effects in terms of hepatocellular proliferation, which strongly suggests that the proliferation is independent of PPARα activation.

I.B. Specific Aim 2

We demonstrated inter-strain variability in TCE metabolism in kidney, which exhibited a different pattern compared to the metabolism in liver. Analyses of metabolites

and cross-tissue comparison provided data for long-lasting questions, such as the relative flux to each pathway in tissues and the quantitative fate of metabolites. Across all strains, renal levels of TCA and TCOH were on average 1,000-fold greater than those of DCA, and levels of DCA were 100-fold higher than those of DCVC. In the sub-acute study, we observed strain-dependent increase in the expression of PPARα-dependent genes and the minimal to mild renal injury measured by KIM-1 expression. However, these effects were not correlated with any individual TCE metabolites, which may suggest that multiple metabolites or pathways are likely to be involved in kidney toxicity due to TCE. In the sub-chronic study, we found a significant correlation between renal levels of TCA and KIM-1 expression, both of which decreased over time. However, the significant increase in proliferation in proximal tubular epithelium was evident only in NZW/LacJ strain treated for 4 weeks, which may characterize the sub-chronic toxicity in kidney as cytotoxicity followed by compensatory proliferation.

I.C. Specific Aim 3

In the sub-chronic study applying a mouse model (wild-type, *Ppara*-null, and h*Ppara*), the levels of TCA in liver were significantly different depending on the presence/absence of the PPARα receptor, which may result from intrinsic differences in TCE metabolism rather than toxicities based on the result from toxicokinetic study. The difference in levels of TCA in liver between wild-type, *Ppara*-null, and h*Ppara* tended to be associated with liver enlargement and oxidative stress. This observation suggests PPARα-independent effects of TCA on liver toxicity. Liver and kidney gene expression changes indicative of PPARα activation in h*Ppara* mice were comparable to those in wild-type, albeit the liver levels of TCA were much lower in h*Ppara* than in wild-type mice. In addition, we observed

an increase in liver inflammation in TCE-treated h*Ppara* mice; however the human relevance of this finding should be interpreted with caution because of the possibility that the interactions between humanized PPARα and mouse-specific signaling pathways may not adequately simulate those in humans.

II. SIGNIFICANCE

The metabolic transformations of TCE are complex and involve formation of many metabolites that vary in their toxicity and organ-specific effects (Lash et al. 2014). P450-dependent oxidation and GSH conjugation are key primary pathways, and liver and kidney are primary targets. The complexity of TCE metabolism and apparent existence of many potential modes of action make it equally challenging for research, risk assessment and policy making.

One of the biggest gaps in risk assessment, as recognized by the NRC (2006), is that inter-individual variability is investigated incompletely (in epidemiological studies) or not at all (in animals). There is a critical necessity for the development of ways to estimate the quantitative influence of human inter-individual variability on personal risk from exposure to chemicals (Zeise et al. 2013). Although a number of computational and statistical tools are available (Dorne et al. 2012), there are few experimental data with which to derive such population distributions for most toxicants. One possible way to fill this knowledge gap is to estimate the nature and quantitative extent of human variability by the mouse model of the human population (Rusyn et al. 2010).

The present study is the first to quantify the levels of TCA, DCA, TCOH, DCVG, and DCVC in liver, serum, and kidney of mice. These data may be available to validate current PBPK models that were constructed largely based on parameters of serum concentration and

in vitro enzyme kinetics (Evans et al. 2009). In addition, data on the inter-strain variability distribution in mice may be further extrapolated to humans using PBPK model, and the resulting human variability distribution may be compared with available data on the variability of the human pharmacokinetics of TCE to determine whether the mouse-derived population model is useful to estimate human variability (Chiu et al. 2014).

In addition, we reported the quantitative differences between metabolites, which would significantly influence future research on toxicities or combined effects between metabolites. First, genotoxicity of DCVC has been regarded as primary MoA for kidney cancer due to exposure to TCE; however, one of the arguments against the conclusion is that there is no reliable data regarding the level of DCVC in tissue of concern. We detected DCVC in kidney and described its strain-dependent quantitative variance, which may support the DCVC-mediated MoA. Second, many studies challenged the combined effect of metabolites, especially TCA and DCA. However, in most cases, the quantitative difference between TCA and DCA was not adequately considered in their experimental designs (Hassoun et al. 2014; Laughter et al. 2004), applying similar dose or concentration of DCA with TCA. Our results may be a guideline for future research on combined effects of metabolites.

We investigated the relationship between TCE metabolism and tissue specific toxicity among seven inbred mouse strains in a time and dose-dependent fashion from which a number of novel findings were drawn. The multi-strain approach provided critical information for understanding genetic background-dependent and -independent components of TCE's mode of action which enables identification of biomarkers of toxicity. We demonstrated a strong correlation between TCA, DCA, and PPARα activation in liver across

varying genetic background, which suggests that we cannot rule out the role of DCA in TCE-induced liver toxicity.

The present study raised an important question whether the observed hepatocellular proliferation is PPARα-mediated or not. Apparently, PPARα activation plays a role in proliferation in liver of mice exposed to TCE; however the evidence undermines the hypothesis that the proliferation is entirely caused by the receptor activation. Indeed, various factors are involved in cell proliferation. In normal circumstances, cell proliferation depends on (1) availability of tissue-type specific growth factors, (2) interaction of transmembrane proteins called integrins with extracellular matrix components, (3) contact inhibition of movement, or (4) limited proliferation capacity of individual cells (Duronio and Xiong 2013; Studzinski 1974). Likewise, abnormal increase of cell proliferation may be caused by a number of factors including (1) mutations in cell cycle-control genes, (2) epigenetic change over expression of cytokines, (3) compensatory regeneration following injury, (4) redox status, or (5) increase in growth factors by activation of innate immunity (Chiu and Dawes 2012; Wu and Hua 2008).

It has been known that renal injury in rodents is not evident until at least 3 months of exposure to TCE (National Toxicology Program 1990), which is partly true because the conclusion was drawn based on somewhat insensitive markers. We have set up a sensitive immunohistochemistry method to detect KIM-1, adapted from recent effort to develop sensitive kidney injury markers (Ozer et al. 2010), and successfully applied the result in association analysis with metabolites, and found the positive relationship between KIM-1 and TCA.

Mouse model using wild-type, *Ppara*-null, and h*Ppara* has been widely utilized to investigate PPARα-dependent effects of a number of PPARα activators including TCE. However, it has been raised that intrinsic differences among the strains may hinder the identification of causal relationship between exposure and outcome. In TCE research, the variance in metabolism is a critical intrinsic factor affecting phenotypes. The analyses of pathologic end points coupled with quantification of TCA shed light on how TCA affect liver toxicity while distinguishing between PPARα-dependent and –independent effects.

III. LIMITATIONS

From a study design point of view, one of the limitations is that a small number of animals (n=3 to 5 per group) is applied to each group. Thus, there were cases that we cannot demonstrate statistical significance in spite of seemingly different data between control and treated group.

Sensitivity of quantification method for metabolites is a major limitation of the study. We were not able to detect DCA, DCVG, and DCVG especially in low-dose group, and many measurements of DCA, DCVC, and DCVG were near the limit of quantification, where analytical errors are wider, so the precision was limited by experimental variation. The sensitivity of the analytical methods, combined with low extraction recoveries, presents a continued limitation to our ability to quantify the levels of these critically important TCE metabolites in studies that use environmentally-relevant exposures to TCE. Therefore, further improvements are needed to fill the data gaps.

The present study mainly focused on apical endpoints rather than toxicity pathways. This type of analysis may not be powerful to find subtle difference in effect between metabolites that are sharing similar toxicological properties. As our results showed that an

apical endpoint may be associated with multiple metabolites, pathway-based in-depth analysis is recommended to dissect the relationship between exposure to TCE and disease outcome, as discussed in IV.3.

We applied a panel of 7 inbred mouse strains which enabled us to differentiate genetic background-dependent from –independent effects. However, this model does not provide enough variance for genome wide association study to find genetic determinants of inter-individual variability in toxicokinetics and toxicodynamics of TCE. Genetic resources with publicly available dense SNP database across dozens of strains have been developed (Frazer et al. 2007; Szatkiewicz et al. 2008). Still, traditional *in vivo* toxicity studies are often carried out in a single strain in order to fix as many variables as possible. This provides information in a single strain, yet the reality of human toxicity is more complex. Data interpretation with respect to the population effects is plagued by the largely inaccurate inference from a single genome; inability to distinguish small yet important changes from background; ineffective use of reproducible genetic variation; and inefficient use of defined genotypes to model particular humans phenotypes. To address these limitations, novel animal models are required.

IV. FUTURE DIRECTIONS

IV.1. Parent compound vs. metabolites

A comparative study between TCE and a metabolite may provide better understanding on the role of individual metabolites. For instance, one can design a dose-response study comprising TCE- and DCVC-treated group separately, then find a dose level at which concentration of DCVC in kidney are similar between TCE- and DCVC-treated

groups, followed by phenotyping in kidney between two groups. This type of comparative study will help to identify a metabolite responsible for certain toxicity.

IV.2. Comparative study between TCE and tetrachloroethylene (PCE)

In a similar way, a comparative study on toxicokinetics and toxicodynamics between TCE and tetrachloroethylene (PCE) will increase our knowledge on the association between metabolism and toxicity because there is big overlap in metabolism between TCE and PCE in spite of the observed difference in toxicodynamics between TCE and PCE (Buben and O'Flaherty 1985). The U.S. EPA has set 0.0005 mg/kg/day for TCE and 0.006 mg/kg/d for PCE as reference doses for chronic oral exposure (U.S. EPA 2011a, b). Obviously, the conclusions were derived from available evidence for each compound rather than direct comparison. The studies where TCE and PCE are directly compared would provide the best assessment of relative toxicity to the endpoints and species of interest.

IV.3. Pathway-based analysis

Given that the adverse effect of TCE on an apical endpoint can be derived from multiple metabolites through multiple pathways, a pathway-based analysis will shed light on dissecting molecular underpinning by which toxicity of TCE is caused. During the past decade, the effect of epigenetic alteration in etiology and pathogenesis of disease has been increasingly investigated (Feinberg 2007). These epigenetic mechanisms are crucial for the proper maintenance of homeostasis in cells. Thus, the disruption of this balance causes the development of a number of human disease related with environmental exposure (Baccarelli and Bollati 2009). However, the effect of epigenetic alterations on the liver toxicity due to TCE and the cause of the inter-individual variation in sensitivity to TCE are largely unknown.

In addition, sequencing of RNA has long been recognized as an effective method for discovery of gene and remains the gold standard for annotation of both coding and noncoding genes (Haas and Zody 2010). Dose-, time-, genetic background-dependent transcript differences in response to TCE can be identified with RNA sequencing-enabled gene expression analysis.

IV.4. Investigation of genetic determinants

The Collaborative Cross (CC), a new population-based genetic resource, satisfies 4 essential criteria (Chesler et al. 2008) for an optimal laboratory animal experimental model that can support systems genetic studies: 1) genome-wide variation; 2) randomization; 3) infinite reproducibility; and 4) large size to afford statistical power. The overall design of CC consists of 8 founder strains and "funnel" breeding to mix the genomes before inbreeding. The founders capture ~90% of the known allelic diversity across the entire mouse genome (Roberts et al. 2007). The CC strains have a population structure that randomizes existing genetic variation and provides unparalleled power to assign causality and understand biological networks underlying disease (Broman 2005; Valdar et al. 2006). The types, distribution and frequency of SNPs are close to those in human populations and the genetic diversity captured in CC lines is unmatched. Importantly, preliminary phenotypic characterization of pre-CC strains indicates that a very large variability exists following changes in environmental conditions. The CC provides a platform for systems genetics that integrates classical genetics and systems biology tools to identify genetic networks that underlie complex phenotypes. The use of this resource enables translational studies by creating a population-based model which can be used to uncover genetic causes of inter-individual variability and solve a critical gap in the scientific basis for risk assessment.

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