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The effect of Trichloroethylene metabolites on the hepatic vitamin B_{12}-dependent methionine-salvage pathway and its relevance to increased excretion of formic aciduria in the rat.

Noreen Yaqoob, Katarzyna M Bloch, Andrew R Evans and Edward A Lock*

The industrial solvent trichloroethylene (TCE) and its two major metabolites trichloroethanol (TCE-OH) and trichloroacetic acid (TCA) cause formic aciduria in male F344 rats. Prior treatment of male F344 rats with 1-aminobenzotriazole a cytochrome P450 inhibitor, followed by TCE (16mk/kg, po), completely prevented formic aciduria, but had no effect on formic acid excretion produced by TCA (8 or 16mg/kg, po), suggesting TCA may be the proximate metabolite producing this response. Dow and Green reported an increase in the concentration of 5-methyltetrahydrofolate (5-MTHF) in the plasma of rats treated with TCE-OH, suggesting a block in the cycling of 5-MTHF to tetrahydrofolate (THF). This pathway is under the control of the vitamin B_{12} –dependent methionine salvage pathway. We therefore treated rats with three daily doses of methylcobalamin (CH_3Cbl) or hydroxocobalamin (OHCbl), a cofactor for methionine synthase, or L-methionine, followed by TCE (16mg/kg) to determine if they could alleviate the formic aciduria. These pre-treatments only partially reduced the excretion of formic acid in the urine. While prior treatment with S-adenosyl -L-methionine had no effect on formic acid excretion. Consistent, with these findings the activity of methionine synthase in the liver of TCE-treated rats was not inhibited. Transcriptomic analysis of the liver identified nine differential expressed genes, of note was down regulation of Lmbrd1 involved in the conversion of vitamin B_{12} into methylcobalamin (CH_3Cbl) a cofactor for methionine synthase. Our findings indicate that the formic aciduria produced by TCE-OH

[a] *School of Pharmacy and Biomolecular Sciences,*
[b] *Liverpool John Moores University,*
[c] *Byrom Street,*
[d] *Liverpool, L3 3AF, UK*
[e]
[f]
[g]

* corresponding author.
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and TCA may be the result of a block in the recycling of 5-MTHF to THF, the effect on the methionine salvage pathway being a secondary response following acute exposure.

1. Introduction

1, 1, 2-Trichloroethylene (TCE) has been used commercially for over 85 years, primarily as a non-flammable solvent for degreasing metal parts but also as a general purpose solvent.[1] From the 1970's onward TCE became of environmental concern, due to evaporation while handling, chemical processing and leakage from chemical waste sites, leading to its contamination of ground water and air.[2-4]

Extensive studies with TCE in experimental animals have shown that the primary target organs for toxicity are the liver and lungs in mice and kidneys in rats. In longer-term studies an increase in tumour incidence occurs in these tissues, see reviews.[5-7] TCE is primarily metabolised in rodent liver via cytochrome P4502E1[8,9] and to a lesser extent by other cytochromes P450.[10] to form an unstable epoxide which re-arranges to form chloral. Chloral is converted to trichloroethanol (TCE-OH) and its glucuronide and trichloroacetic acid (TCA), which are the major metabolites in the urine of experimental animals and humans.[11,12] TCE can also undergo metabolism via glutathione conjugation producing the cysteine conjugate S-(1, 2-dichlorovinyl)-L-cysteine (DCVC) which is converted to a reactive metabolite by the enzyme cysteine conjugate β-lyase in the kidney see reviews.[5,12] This route of metabolism also generates several other glutathione derived metabolites, known to be genotoxic, and is currently seen as the most likely mechanistic event that may lead to cancer. [5,9,12]

Green *et al*.,[13] reported that single or multiple oral doses of TCE (1000mg/kg) or inhalation exposure for 28-days, 6h/day at 250 and 500ppm produced formic aciduria in male F-344 rats. They also reported that TCE-OH and TCA, but not DCVC, produced formic aciduria.[14] Dose-response studies with TCE in male F344 rats showed that 4mg/kg produced a small increase in urinary formic acid, which was statistically significantly increased at 8 and 12mg/kg being maximal at 16mg/kg and above, at about 10mg formate excreted/24h. Female F-344 rats showed a similar profile but formic acid excretion was lower than in males.[15] Most importantly, the formic acid excreted was not a metabolite of TCE, most likely coming from perturbation of endogenous metabolic pathways.[13] Green and co-workers postulated that sustained exposure of rat renal tubule cells to formic acid, following high dose exposure, could lead to renal tubule necrosis and regeneration, which may account for the small increase in renal cancer in male rats.[13,16] However, TCE-induced formic aciduria occurs at much lower doses than those that produce renal cancer in rats and mice suggesting these events are unrelated. Rusyn *et al.,* [5] concluded

that oxidative metabolites, TCE-OH and TCA, do not appear sufficient to explain the range of renal effects observed after TCE exposure.

The mechanism by which TCE produces formic aciduria is not known. Studies by Dow and Green[14] reported an increase in 5-MTHF in the plasma, and suggested TCE may be interfering with folate metabolism which is under the control of the vitamin B_{12} –dependent methionine salvage pathway. In this paper we have:

1. Examined the effect of inhibiting oxidative metabolism of TCE on formic aciduria.
2. Studied the effect of supplementing rats with vitamin $B_{12,}$ L-methionine or S-adenosyl-L-methionine on TCE produced formic aciduria.
3. Measured the activity of methionine synthase in the liver of control and TCE dosed rats.
4. Performed transcriptomic analysis on the liver and kidney of rats exposed to a dose of TCE which produced formic aciduria.

2. Materials and Methods

2.1 *Materials*

Deuterium oxide (D_2O, 99.9%) from Cambridge Isotope Laboratories, Inc. Andover, Massachusetts, USA. Trichloroethylene, reagent grade, 98%, stabilised with about 1% 1-2-epoxybutane, trichloroethanol, trichloroacetic acid, 1-aminobenzotriazole, hydroxocobalamin, methylcobalamin, L-methionine, S-adenosyl-L-methionine p-toluenesulphonate, and Dowex 1 x 8-200 ion exchange resin were from Sigma Aldrich, Poole, UK. 5-[^{14}C] Methyl-tetrahydrofolic acid, barium salt 55µCi, 1.85 M Bq . from GE Healthcare, Amersham, UK. Pure corn oil, low in saturates and high in polyunsaturates from Tesco Supermarket, Liverpool, UK. Protein assay kit, Bio-Rad Detergent Compatible from Bio-Rad Laboratories, UK. Norell 5mm NMR tubes with round bottom and 178mm length from Glass Precision Engineering Scientific Limited, Leighton Buzzard, UK. All other materials were of the highest grade available commercially.

2.2 Animals

The Ethics Committee at Liverpool John Moores University approved this project which was conducted in accordance with a license issued under the UK, Scientific Procedures Act, 1986. Male F-344 rats were from the breeding colony the Life Science Support Unit, Liverpool John Moores University. All rats were housed in North Kent Plastic cages on Beta bed sawdust (Grade 5, Datesand Ltd, Manchester, UK) which was changed daily. The animal rooms were kept at a constant temperature of 20ºC ± 2ºC and humidity of 50% ± 5% with a 12h light-dark cycle starting at 04.00h. Rats were allowed rat expanded diet (Bantin and Kingman, Hull, UK.), and water *ad libitum*. Rats, 190-240g body weight between 7-9 weeks of age were placed in metabolic cages for 24h to acclimatize prior to dosing and urine collected (day 0). Urine was collected at 4^0C in containers to which was added 0.1ml of 10% sodium azide to prevent bacterial growth. Urine volume and pH was measured and a sample taken for ^{1}H NMR and the remainder frozen at -80^0C.

2.3 *Dosing of animals and urine collection*

Twelve metabolism cages were available, so either two animals/group or three animals/group were used. The study was then repeated to increase the numbers which are stated in the legend to the Figures. For the initial study, rats were dosed orally for 3 days as follows: TCE in corn oil at 16mg/kg/day at 5ml/kg; TCE-OH in corn oil at 16mg/kg/day at 5ml/kg; TCA in 2mM phosphate buffer pH 7.4 at 16 or 8mg/kg/day at 5ml/kg. Controls received corn oil alone or 2mM phosphate buffer pH 7.4 at 5ml/kg/day. Urine was collected 24h before dosing (Day 0) and after dosing at 24h (day1), 48h (day 2) and 72h (day 3) as described above and analyzed for formic acid.

In another study rats were pre-treated with 1-aminobenzo-triazole a broad-spectrum cytochrome P450 inhibitor.[17] at 100mg/kg ip at 5ml/kg in isotonic saline followed 4h later by TCE 16mg/kg or TCA at 8mg/kg or 16mg/kg. Controls received ABT alone followed by corn oil or 2mM phosphate buffer pH 7.4 at 5ml/kg/day. Urine was collected prior to dosing and 24h after dosing either TCE or TCA. In a preliminary study, a group of three control and three ABT–treated rats were killed 24h after dosing and the livers removed for the determination of cytochrome P-450 activity.

Another group of rats were given a single oral dose of TCE 16mg/kg in corn oil and 2h later given methylcobalamin or hydroxocobalamin a cofactor for methionine synthase at 12.5 or 100µg/kg/day for 3 days at 1ml/kg, sc in isotonic saline. Controls were dosed with corn oil followed 2h later by

methylcobalamin or hydroxocobalamin daily for 3 days or TCE followed 2h by isotonic saline daily for 3 days. Urine collected on day 0 and 24, 48 and 72h after TCE administration.

In the next study, rats were given a single oral dose of TCE 16mg/kg and 0.5h later given L-methionine, a product of methionine synthase, at 100mg/kg/day ip for 3 days at 1ml/kg in sterile water. Controls were dosed with corn oil followed 0.5h later by L-methionine daily for 3 days, or TCE followed by sterile water daily for 3 days.

S-adenosyl-L-methionine is unstable when administered to rats so we used a stable form, S-adenosyl-L-methionine-*p*-toluenesulfonate that releases S-adenosyl-L-methionine once inside the cell. Rats were given a single oral dose of TCE at 16mg/kg, in corn oil at 5ml/kg followed 0.5h later by S-adenosyl-L-methionine-*p*-toluenesulfonate at 10mg/kg/day i.p.[18] daily for 3 days in sterile water. Controls were dosed with corn oil followed 0.5h later by S-adenosyl-L-methionine-*p*-toluenesulfonate daily for 3 days, or TCE alone followed by sterile water daily for 3 days. Urine was collected on day 0 and 24, 48 and 72h after TCE administration. For the transcriptomic study, three rats were dosed orally each day with TCE in corn oil at 16mg/kg and 5ml/kg/day for three days. Three Control rats received corn oil 5ml/kg/day orally for 3 days.

2.4 *Collection of tissues*

Twenty- four hours after the last dose of TCE or corn oil rats were killed by exposure to a rising concentration of carbon dioxide. A portion about 50mg of liver and renal-cortex was rapidly removed from three rats given 16mg/kg/day TCE and three receiving corn oil alone and immersed in RNA later, for transcriptomic analysis. Samples of liver from 16 mg/kg/day TCE and corn oil treated rats were stored at -80^{0}C for analysis of methionine synthase activity.

2.5 *Measurement of metabolic changes in rat urine.*

^{1}H-NMR spectroscopy was used to measure metabolic changes in urine following multiple dosing. Aliquots of urine (500µl) were mixed with 0.2M phosphate buffer pH 7.4 in D_2O (250µl) containing sodium-3(trimethylsilyl) propionate-2,2,3,3-d4 (TSP; 0.5mg TSP/ml buffer) and then centrifuged at 14,000g for 10 min at 4ºC. The supernatants (600µl) were placed into a 5mm NMR tube and ^{1}H-NMR spectra acquired using a Bruker 300MHz instrument (Bruker Analytik GmbH, Germany). The standard 'noesypr1d' pulse sequence was utilised for data acquisition on urine, which efficiently suppresses the large water signal. Bruker software quantitated the signal intensities and spectra, which were baseline

corrected using Mestrec software and normalised to TSP. Urinary metabolites were quantified with reference to TSP, the peak height of TSP being set at 0ppm. Formic acid had a resonance at 8.44ppm, which is in agreement with the publish spectra for this compound. The accuracy of the determination of formic acid was determined by spiked addition and found to be 98%. The detection of formic acid using the 3σ method [19] gave an LOD of 0.004 mg/ml and using the 10σ method an LOQ of 0.04 mg/ml.

2.6 *Preparation of liver microsomal fraction and cytochrome P450 assays.*

The livers of 3 control and 3 ABT-treated rats were perfused *in situ* with ice-cold 0.9% saline, rapidly excised, blotted dry, weighed and then homogenized (1:4 w/v) in ice-cold buffer (0.154 M KCl, 50mM Tris-HCl, pH 7.4). The microsomal fraction was then prepared as described by Taylor *et al.,* [20] and re-suspended in ice-cold storage buffer (0.154M KCl, 10mM HEPES, 1mM EDTA, 20 % (w/v) glycerol, pH 7.6) and stored in 500ul aliquots at -80°C.

4-Nitrophenol hydroxylase activity indicative of cytochrome P450 2E1 was measured in the microsomal fraction by monitoring the increase in the formation of 4-nitrocatechol from 4-nitrophenol as described by Reinke and Moyer.[21] Ethoxycoumarin-O-deethylase activity indicative of cytochrome P450 2B1/2 was monitored by the formation of 7-hydroxycoumarin from 7-ethoxycoumarin.[22] Results are expressed as nmol 4-nitrocatechol or 7-hydroxycoumarin formed min^{-1} mg $protein^{-1}$. Protein concentration was determined using a Bio-Rad protein assay kit, using bovine serum albumin as a standard.

2.7 *Measurement of methionine synthase activity in rat liver*

The activity of methionine synthase was determined using radiolabelled ^{14}[C] methyl tetrahydrofolate as described by Banerjee *et al.,*[23] in the liver of rats given TCE in corn oil at 16 mg/kg/day for 3 days. Controls given corn oil alone daily for three days. Radioactivity was measured using a liquid scintillation counter (Packard liquid scintillation counter, Model 2100, UK). Specific activity was reported as pmol methionine formed min^{-1} mg $protein^{-1}$.

2.8 *RNA isolation and microarray*

Trizol reagent was used for RNA isolation. Total RNA was purified using the RNeasy Total RNA Mini Kit (Qiagen) according to manufacturer's instructions. RNA was checked for purity and integrity using Agilent 2001 Bioanalyzer (Agilent Technologies GmbH, Germany) before processing. Transcriptomics data was generated using GeneChip Rat Genome 230 2.0 Arrays. cDNA was prepared using the Affymetrix IVT express

kit (Affymetrix, Santa Clara). cDNA synthesis and labelling were performed according to the manufacturer's procedures. Subsequent labelling of the samples was conducted by synthesis of Biotin-labelled complementary RNA (cRNA) using the GeneChip IVT labelling kit (Affymetrix). Purified cRNA was quantified using a spectrophotometer, and un-fragmented samples were checked on the Bioanalyzer. Subsequently, cRNA samples were fragmented for target preparation according to the Affymetrix manual and checked on the Bioanalyzer. Samples were stored at −20 °C until ready to perform hybridization. cRNA targets were hybridized on high-density oligonucleotide gene chips GeneChip Rat Genome 230 2.0 arrays according to the Affymetrix Eukaryotic Target Hybridization manual. The gene chips were washed and stained using the Affymetrix Fluidics Station 450 and Genechip Operating Software and scanned by means of an Affymetrix GeneArray scanner.

2.9 *Microarray analysis*

Analysis and quality controls were done in R using: simpleaffy [24], affycoretools [25] and affyPLM [26] all BioConductor packages. The raw data from CEL files were read into the R environment using the package affy [27] Data was normalized using GCRMA method. [28] To identify the differentially expressed genes (DEGs) limma package[29] was used. Comparisons were made between control, corn oil-treated (n=3) and TCE-treated, 16mg/kg/day (n=3) samples. Genes with adjusted p-value < 0.05 (FDR) were considered significant.

2.10 Data deposition

MIAMI-compliant microarray data obtained from rat *in vivo* studies are deposited in the NCBI GEO database under accession number GSE 122664.

2.11 Statistical analysis

All other data is expressed as mean ± standard error of the mean (SEM). Significant differences were determined between control and treated groups at each dose or time using one-way ANOVA with Bonferroni correction. Study differences between control and treated where determined using a two-tailed *t*-test. Statistical analysis used InStat Graphpad with a p-value < 0.05 considered statistically significant.

3 Results

3.1 The effect of TCE, TCE-OH and TCA on formic acid excretion

^{1}H-NMR analysis of urine showed that the most marked difference in the spectra between control (corn oil-treated) and TCE-treated rats was the appearance of a resonance at 8.44ppm which was attributed to formic acid (compare Figure 1A, 1B and 1C). Spiking urine samples with formic acid, increased the same spectra as in the samples. In the spectra for Figure 1C resonances for the glucuronide metabolite of TCE-OH are also present. The concentration of formic acid in male rat urine 24h before dosing and following TCE, TCE-OH or TCA at 16 mg/kg/day for three days is shown in Figure 2. Urinary formic acid excretion is statistically significantly increased compared to control with all 3 chemicals, 24, 48 and 72h after dosing. However, TCA produced a 2-fold higher concentration of formic acid compare to TCE-OH and TCE (Figure 2). These finding support the view that TCE requires metabolism to produce formic aciduria. This finding is supported by prior treatment of rats with 1-aminobenzotriazole (ABT) a broad-spectrum suicide inhibitor of cytochrome P450[17] that totally prevented the urinary excretion of formic acid produced by TCE (Figure 3A). We showed that ABT 100mg/kg inhibited hepatic cytochrome P450 4-nitrophenol hydroxylase activity (CYP2E1) by about 70%, 24h after dosing, using 4-nitrophenol as substrate, (control activity, 1.1 ± 0.1nmol/min/mg protein; treated, 0.30 ± 0.05mnol/min/mg protein, $P<0.05$). The activity of 7-ethoxycoumarin deethylase (CYP 2B1/2) using 7-ethoxycoumarin as substrate was also reduced 24h after dosing by 56% (control, 0.30 ± 0.02nmol.min/mg/protein; treated, 0.13 ± 0.05nmol/min/mg protein, $P<0.05$). A similar study using TCA in place of TCE showed that prior ABT treatment did not prevent the formic aciduria following a single dose of TCA at either 16mg/kg or 8mg/kg (Figure 3B) suggesting TCA is the proximate metabolite producing this response.

3.2 The effect of vitamin B_{12} and L-methionine on TCE – Induced formic acid excretion

It has been postulated that TCE metabolites TCA and TCE-OH may inhibit enzymes where cobalmin is a co-factor Dow and Green.[14] Methionine synthase is a vitamin B_{12} –dependant enzyme, involved in methyl group transfer. We examined the effect of supplementing components of this pathway on formic acid excretion. Rats were given methylcobalmin (CH_3Cl) or hydroxocobalamin (OHCbl) following single dose of TCE 16mg/kg. Two hours after dosing TCE, CH_3Cbl at 12.5µg/kg/day or 100µg/kg/day or OHCbl 12.5µg/kg/day was dosed daily for 3 days. Controls were dosed with corn oil followed 2h later by OHCbl or CH_3Cbl daily for 3 days. Following a single dose of TCE at 16mg/kg urinary formic acid was increased 1 day after dosing, peaked on day 2 and then started to decline on day 3 (Figure 4). Treatment with 12.5µg/kg/day CH_3Cbl or OHCbl had no effect on formic excretion after one dose, but following two and

3 doses there was a statistically significant reduction compared to TCE alone (Figure 4). Increasing the dose of CH_3Cbl to 100µg/kg/day resulted in a reduction of formic acid excretion on all 3 days (Figure 4). Consistent with these findings rats given 100mg/kg/day L-methionine, 0.5h after a single dose of TCE 16mg/kg also reduced the total excretion of formic acid in urine over 3 days, which was more marked after 2 and 3 doses (Figure 5). We also treated rats with S-adenosyl-L-methionine-*p*-toluenesulfonate, a biological methyl donor, to see if it could alleviate formic acid excretion induced by 16mg/kg TCE. No reduction in formic acid excretion was observed using S-adenosyl-L-methionine- *p*-toluenesulfonate (Figure 6). We also measured the activity of methionine synthase in the liver of rats dosed with 16mg/kg/day TCE for three days or corn oil alone. Corn oil treated control, 2.0 ± 0.11(6) pmol/ min/mg protein, TCE 16mg/kg/day, 1.99 ± 0.09 (6) pmol/min/mg protein, indicating no effect on enzyme activity at this dose of TCE.

3.3 Transcriptomics analysis of liver and kidney exposed to TCE

To understand more about the perturbation of formic acid metabolism, we used transcriptomic analysis to determine transcripts altered in the liver or kidney of rats following three daily doses of TCE at 16mg/kg/day compared to corn oil treated controls. Nine differential expressed genes were identified, *Hsdl2, Lmbrd1, Mt1, Per3, Atp1a1, Slc20a2, Arl6ip5, Copz1* and *Me1* in liver and none *in* the renal-cortex (Table 1, Supplementary Materials). Of particular interest is the down regulation in the liver of *Lmbrd1*, involved in the conversion of vitamin B_{12} into one of two molecules, S-adenosylcobalamin (AdoCbl) or methylcobalamin (CH_3Cbl). CH_3Cbl is a cofactor for methionine synthase, which converts the amino acid homocysteine to methionine, which is relevant to our studies. Also of note is the up regulation of *Mt-1,* a member of the metallothionein family, which act as anti-oxidants, protecting against hydroxyl free radicals. Also of interest is the down regulation of *Me1* which encodes a cytosolic, NAD^+/$NADP^+$-dependent enzyme that generates NADH/NADPH required for the biosynthesis of fatty acids and 5-MTHF.

4. Discussion

4.1 Formic aciduria produced by TCE and related chemicals

Male F334 and male Wistar strain of rats excrete large amounts of formic acid in their urine following exposure to TCE either as a single oral dose [13,15] or by inhalation[13] which is associated with acidification

of the urine. For ^{1}H-NMR analysis, the urine is adjusted to pH 7.4 in 0.2M phosphate buffer to rule out any pH effects on analysis. Excretion of formic acid has a half-life of between 4-5 days, and hence remains elevated at high levels throughout a 28-day inhalation study[13] and a 12-week oral study.[30] Sex and strain difference in response to chlorinated solvents are common, TCE producing a lower formic aciduria in female F344 rats, compared to male F344 rats,[15] which may be related to differences CYP2E1 expression. Male C_{57}Bl/6 mice exposed to TCE or TCA caused formic aciduria,[31] we are not aware of studies with TCE, looking for formic acid excretion in female mice. Several other halogenated solvents also produced this response; bromodichloro- methane in male F344 rats and male B6C3F1 mice[32]; carbon tetrachloride, dichloroacetic acid and chloroform in male F344 rats. [14,15] The major oxidative metabolites of TCE, TCE-OH and TCA also produce formic aciduria.[13-16, 30,31] Early studies used high doses of TCE (1000mg/kg) however, formic aciduria occurs at >100-fold lower dose, 8mg/kg/day TCE for 3 days in male F344 rats.[15] Green and co-workers suggested that a marked and sustained excretion of formic acid in male rats might lead to cytotoxicity in renal tubular cells, with a subsequent regeneration which if sustained could lead to renal tubule tumours[13] We reported that a dose of TCE 100-fold lower than a dose which caused a small increase in renal tumours, produced the same magnitude of formic aciduria as the large dose of TCE[15]. Indicating that the increased excretion of formic acid is not linked to the production of renal tumours in male rats. In contrast, TCE metabolism via, glutathione conjugation to form S-1,2-dichlorovinyl-L-cysteine, produced no increase in formic acid excretion,[14-15] but is universally accepted as the route of metabolism (Figure 7) responsible for producing renal toxicity.[5,9,12]

TCA is a strong acid, therefore dosing solutions were adjusted to pH 7.4 when given orally to male F344 rats where it produced a more marked excretion in formic acid than either TCE or TCE-OH (Figure 2) suggesting it may be the proximate metabolite. Support comes from studies inhibiting cytochromes P450 prior to dosing TCE, which completely prevented formic acid excretion (Figure 3A) while this treatment had no effect on TCA produced excretion of formic acid (Figure 3B). Whether inhibition of TCE-OH metabolism to TCA prevents formic aciduria is an interesting question, as this could mean both compounds have a common mechanism via TCA. Since the formate excreted is not derived from TCE[13] it must come from a large pool of formate available in the rat for intermediary metabolism.

The common structural feature of the chemicals that cause formic aciduria is the trichloro(bromo)methyl group, and the property they have in common is the ability to generate free radicals. Carbon tetrachloride and chloroform can generate a trichloromethyl radical *in vitro* and *in vivo*

[33-35] which is responsible for liver injury.[36] TCE-OH and TCA induce lipid peroxidation by a free radical mechanism.[37,38] TCE-OH also produced free radicals using spin-trapping techniques.[39] Thus, generation of a radical metabolite may be essential for the perturbation in formic acid metabolism. Could these chemically reactive radicals interfere with folate metabolism and thereby reduce formate utilisation?

4.2 Mechanism for trihalogenated chemicals disruption of formic acid metabolism

Tetrahydrofolic acid (THF) is the coenzyme that plays a central role in the metabolism of one-carbon compounds. The vitamin folic acid is a precursor of THF that comes from an external source. This requirement is met by a balanced diet; in addition, bacteria in the intestine synthesize folic acid and some is absorbed. When a folate deficiency develops, this pathway can no longer utilize formic acid and the excess is excreted. A connection between TCE-induced formic acid excretion and the folic acid-dependent pathway was show when the diet of rats, was supplemented with folic acid, resulting in a decrease in urinary formic acid excretion when dosed with TCE-OH or TCA.[14] In addition, there was an increase of 5-methyl tetrahydrofolate (5-MTHF) in the plasma suggesting a reduction in the cycling back o THF. The vitamin B12–dependent methionine salvage pathway [40] controls regulation of tissue concentrations of THF. Recovery of THF by this salvage pathway involves the enzyme methionine synthase where vitamin B_{12} acts as a coenzyme in the transfer of the methyl group from CH_3Cbl onto homocysteine to form methionine. The cobalamin in the Co^{1+} oxidation state then reacts with 5-MTHF to regenerate CH_3Cbl and produce THF. Formic acid then reacts with THF to form 5-formyl-THF then via a series of steps to produce 5-MTHF (Figure 7).

If the methionine salvage pathway is impaired by TCE metabolites, administration of the coenzyme for methionine synthase or its product might restore enzyme function and reduce the formic aciduria. Administration of CH_3Cbl or OHCbl did partially reduce formic acid excretion following the second and third doses (Figure 4). A small reduction in formic aciduria was also seen after dosing L-methionine to TCE treated rats (Figure 5).This response suggests some relief on the methionine salvage pathway. However, provision of S-adenosyl-L-methionine had no effect on this pathway (Figure 6). Overall, administration of methyl group donors or the addition of CH_3Cbl did not prevent the excretion of formic acid, it reduced it somewhat but this does indicate that the methionine salvage pathway is not the primary target leading to this response. Consistent with this we found in no effect on the activity of hepatic methionine synthase in rats dosed with 16mg/kg/day TCE for 3 days. However, in a 12-week study with TCE-OH (100mg/kg/day) and TCE (500mg/kg/day) we reported a 43% and 46% respectively

reduction in hepatic methionine synthase activity.[30] Indicating down regulation of enzyme activity at higher doses and longer times. Transcriptomic analysis on the liver of rats given TCE 16mg/kg/day for 3 days detected only nine transcripts that were statistically different from control rats given corn oil alone. No altered transcripts were found in the renal-cortex. With only three animals/group variability may explain the low number of transcripts detected as may the low dose of TCE used. Hendriksen *et al.,*[39] reported studies with TCE at 500 and 1500mg/kg/day for 14 days using male F344 rats and the same platform we used. They reported up-regulation of genes involved in phase1 and phase 2 drug metabolising enzymes and we detected three transcripts in common, *Me1*, malic enzyme; *Mt1*, metallothionein and *hsdi2*, hydroxysteroid 17β dehydrogenase 2. We also detected a reduction in the *Lmbrd1* transcript, which is involved in the conversion of vitamin B_{12} into CH_3Cbl and involved in the methionine salvage pathway. The decrease is small 15% compared to control. In contrast, many transcriptomic studies using the liver of mice exposed to TCE. For example, Bradford *et al.,*[40] used a large number of different strains, dosed with TCE at 2100mg/kg for up to 24h and did not find changes in *Lmbrd1.* Our finding in the F344 rat need confirmation.

What effect formic aciduria has on rats during a lifetime of exposure to these solvents is not clear. Rodents do not develop pernicious anaemia, but minor changes in red blood cell volume and reduced haemoglobin concentration following TCE-OH exposure for 28 days.[14] The relevance of impaired folate metabolism to humans following TCE exposure is unknown, humans are generally less folate deficient in comparison to rodents and therefore less susceptible to this mechanism. Other factors need to be considered, the exposure of rats at relatively low doses in this study, is much higher than that found in the workplace. Human metabolism of TCE is estimated to be about 20-fold lower than in the rat.[41] In fact, workers exposed to TCE at a concentration range of 32(0.5-252) ppm over several years had < 2-fold increase in urinary formic acid, the latter correlating with the excretion of TCA. This concentration of formic acid in the urine of exposed workers is unlikely to have come from inhibition of the methionine salvage pathway.[42]

Conclusions

We have shown that treatment of rats with vitamin B_{12} or L-methionine produced a small reduction in the excretion of formic acid caused by TCE and TCA. However, S-adenosyl-L-methionine had no effect on formic acid excretion, suggesting the problem may be more related to the inability of 5-MTHF to recycle

back to THF. Metabolites of TCE are proposed to interfere with the recycling of 5-MTHF probably by a free radical mechanism, inhibiting the utilisation of formate, which is excreted in the urine.

There are no conflicts of interest to declare.

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Figure 1. ^{1}H NMR spectra of male rat urine following daily dosing with corn oil (A) or 16 mg/kg/day TCE (B) for 3 days.

The urine was analyzed using ^{1}HNMR spectroscopy with sodium-3(trimethylsilyl) propionate-2,2,3,3-d4 (TSP, 0.5mg/ml) as reference at 0ppm. Formic acid appears at 8.44ppm. A and B show typical spectra from one rat in each group. TMAO, trimethylamine-N-oxide.

Figure 2. Urinary excretion of formic acid in male F-344 rats given daily oral doses of TCE, TCE-OH and TCA for three days.

Urine was collected 24h before dosing (day 0), and days 1,2 and 3 after dosing. Urine samples were analyzed using ^{1}H NMR spectroscopy with TSP as reference signal at 0ppm. Formic acid appears at 8.44ppm. Values are mean, ± SEM. The study had 3 animals/group and was repeated n=6. *P<0.05 statistically different from control day 0. +P<0.05 statistically different from TCE on respective days.

Figure 3. Urinary excretion of formic acid in male F-344 rats given 1-aminobenzotriazole (ABT) a suicide inhibitor of cytochromes P450, followed by a single of TCE or TCA.

Rats were given single ip dose of ABT (100mg/kg) followed 4h later by TCE, 16mg/kg po (A) or TCA, 8 or 16mg/kg po (B). Controls received saline followed by corn oil or TCE 16mg/kg in corn oil or TCA 8 or 16mg/kg in buffer or ABT followed by corn oil. Urine was collected 24h before dosing day 0 and 24h after dosing day 1. Urine samples were analyzed using 1H NMR spectroscopy with TSP asreference signal at 0 ppm. Formic acid appears at 8.44ppm. Values are mean, ± SEM. Part A) had 3 animals/group and was repeated giving n=6. Part B) had 2 animals /group and was repeated giving n=4. *P<0.05 statistically different from control day 0. +P<0.05 statistically different from TCE.

Figure 4. Urinary excretion of formic acid in male F-344 rats given one dose of 16mg/kg TCE followed by daily doses of methyl or hydroxocobalamin for three days.

Rats were given a single oral dose of TCE 16mg/kg followed 2h later by OHCbl or CH3Cbl at 12.5µg/kg or 100µg/kg, sc on day 1 and then Cbl alone on day 2 and 3. Control rats received either corn oil alone followed by Cbl or saline, TCE (16mg/kg) alone followed 2h later by saline and then daily doses of saline day 2 and day 3. Urine was collected 24h before dosing on day 0 and 24h after daily dosing on day 1, 2 and 3. Urine samples were analyzed using ^{1}H NMR spectroscopy with TSP as reference signal at 0ppm. Formic acid appears at 8.44ppm. Values are mean, ± SEM. Corn oil and Cbl animals were 2/group and was repeated n=4. The TCE treated animals were 3/group and repeated giving n=6. *P<0.05 statistically different from control on respective days. + P<0.05 statistically different from 16mg/kg TCE on respective days.

Figure 5. Urinary excretion of formic acid in male F-344 rats given a single dose of 16mg/kg TCE followed by daily doses of L-methionine for three days.

Rats were given a single oral dose of TCE 16mg/kg) followed 0.5h by L-methionine (100mg/kg), ip on day 1 and then L-methionine alone on day 2 and 3. Other rats received TCE followed 0.5h later by sterile water on day 1 and sterile water alone on days 2 and 3. Controls received corn oil, followed 0.5h later by either L-methionine or sterilized water alone which was repeated on days 2 and 3. Urine was collected 24h before dosing on day 0 and after dosing on day 1, 2 and 3. Urine samples were analyzed using ^{1}H NMR spectroscopy with TSP as reference signal at 0ppm. Formic acid appears at 8.44ppm. Values are mean, ± SEM. The study had 3 animals/group and was repeated giving n=6. *P<0.05 statistically different from control on respective days. +P<0.05 statistically different from 16mg/kg TCE on respective days.

Figure 6. Urinary excretion of formic acid in male F-344 rats given one dose of 16mg/kg TCE followed by daily doses of 10mg/kg S-adenosyl-L-methionine-p-toluenesulfonate (SAM) for three days.

Rats were given a single oral dose of 16mg/kg TCE followed 0.5h later by SAM 10mg/kg ip on day 1 and then SAM again on day 2 and 3. Another group were give TCE 16mg/kg followed 0.5h later by sterilized water Day 1 and then sterile water again on day 2 and 3. Controls received corn oil followed 0.5h later by either SAM or sterilized water on day 1, and then SAM or sterile water again on day 2 and 3. Urine was collected 24h before dosing on day 0 and 24h after dosing on day 1, 2 and 3. Urine samples were analyzed using ^{1}H NMR spectroscopy with TSP as reference signal at 0ppm. Formic acid appears at 8.44ppm. Values are mean, ± SEM. The study had 3 animals/group and was repeated giving n=6. *P<0.05 statistically different from control on respective days.

Table 1. Differentially expressed genes identified in rat liver following three daily doses of trichloroethylene at 16mg/kg/day, compared with corn oil treated control rats.

Results are from three trichloroethylene treated rats and three control rats.

Figure 7. The metabolism of TCE, showing the pathway of renal toxicity and the proposed mechanism for the interaction of TCE metabolites with the methionine salvage pathway leading tetrahydrofolate deficiency and increased excretion of formic acid in rat urine.