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Lack of formic acid production in rat hepatocytes and human renal proximal tubule cells exposed to chloral hydrate or trichloroacetic acid

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Abstract

The industrial solvent trichloroethylene (TCE) and its major metabolites have been shown to cause formic aciduria in male rats. We have examined whether chloral hydrate (CH) and trichloroacetic acid (TCA), known metabolites of TCE, produce an increase in formic acid *in vitro* in cultures of rat hepatocytes or human renal proximal tubule cells (HRPTC). The metabolism and cytotoxicity of CH was also examined to establish that the cells were metabolically active and not compromised by toxicity. Rat hepatocytes and HRPTC were cultured in serum-free medium and then treated with 0.3–3mM CH for 3 days or 0.03–3mM CH for 10 days respectively and formic acid production, metabolism to trichloroethanol (TCE-OH) and TCA and cytotoxicity determined. No increase in formic acid production in rat hepatocytes or HRPTC exposed to CH was observed over and above that due to chemical degradation, neither was formic acid production observed in rat hepatocytes exposed to TCA. HRPTC metabolised CH to TCE-OH and TCA with a 12-fold greater capacity to form TCE-OH versus TCA. Rat hepatocytes exhibited a 1.6-fold and 3-fold greater capacity than HRPTC to form TCE-OH and TCA respectively. CH and TCA were not cytotoxic to rat hepatocytes at concentrations up to 3mM/day for 3 days. With HRPTC, one sample showed no cytotoxicity to CH at concentrations up to 3mM/day for 10 days, while in another cytotoxicity was seen at 1mM/day for 3 days. In summary, increased formic acid production was not observed in rat hepatocytes or HRPTC exposed to TCE metabolites, suggesting that the *in vivo* response cannot be modelled *in vitro*. CH was toxic to HRPTC at millimolar concentrations/day over 10 days, while glutathione derived metabolites of TCE were toxic at micromolar concentrations/day over 10 days (Lock et al., 2006) supporting the view that glutathione derived metabolites are likely to be responsible for nephrotoxicity.

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Keywords

Trichloroethylene; chloral hydrate; trichloroethanol; trichloroacetic acid; formic acid; human renal proximal tubule cells; rat hepatocytes

1. Introduction

The industrial solvent 1,1,2-trichloroethylene (TCE) has been reported to interfere with folic acid metabolism in male rats leading to excretion of large quantities of formic acid in urine (Green et al., 1998; Dow and Green, 2000). Formic aciduria has been suggested to account for the renal injury observed following chronic exposure in the rat. Metabolites of TCE, trichloroethanol (TCE-OH), trichloroacetic acid (TCA) and to a lesser extent CH also cause formic aciduria in the rat (Dow and Green, 2000). While workers occupationally exposed to TCE excrete formic acid in their urine, the amount is small and unlike that observed in the male rat (Green et al., 2004).

It is well established that TCE undergoes metabolism by hepatic cytochrome P450 in experimental animals and man to produce chloral hydrate (CH) which is then further metabolised to TCE-OH and TCA (Davidson and Beliles, 1991; Elfarra et al., 1998; Lash et al., 2000; Bloemen et al., 2001; Lock and Reed, 2006). The target organs for toxicity and carcinogenicity following exposure of rats or mice to TCE are the liver, kidney and lung (Bull, 2000; Green, 2000; Lock and Reed, 2006). TCE has been reported to undergo metabolism to CH in rat liver (see references above) and rat kidney (Cummings et al., 2001) primarily by cytochrome P450 2E1, although other cytochromes P450 such as 2B1 and 2C11 can also convert TCE to CH. Human kidney contains little or no cytochrome P450 2E1 (Amet et al., 1997), hence studies with human renal microsomes failed to detect CH formation in three out of four samples, and in the fourth only a very small amount was observed at the highest substrate concentration (Cummings & Lash, 2000). Thus, exposure of human kidneys to oxidative metabolites of TCE is largely due to extra-renal metabolism.

The aim of this current work was to: 1) ascertain if rat hepatocytes exposed to CH or TCA in culture produce formic acid, like that observed in the whole animal; 2) determine that rat hepatocytes under our culture conditions can metabolise CH to TCE-OH and TCA; 3) examine the cytotoxicity of CH and TCA to rat hepatocytes in culture; and 4) compare the findings in rat hepatocytes with those in human renal proximal tubule cells (HRPTC).

2. Materials and methods

2.1. Materials

Collagen, dexamethasone, gentamicin sulfate, insulin, chloral hydrate (CH), trichloroethanol (TCE-OH), trichloroacetic acid (TCA) and William's Medium E were obtained from Sigma Chemical Company (St. Louis, MO). Collagenase Type 2 was obtained from Worthington Biochemicals (Freehold, NJ); Hank's balanced salt solutions and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO). Sodium 3-trimethylsilylpropionate (TMSP-2,2,3,3-d4 [D,98%]) was obtained from Cambridge Isotope Laboratories (Andover, MA).

2.2. Animals

Adult male Sprague-Dawley rats (100–150g) were purchased from Harlan Lab Animals (Indianapolis, IN). They were housed in AAALAC-accredited animal facilities and maintained

on a 12-h light:12-h dark cycle with free access to food and water for at least 1 week prior to use.

2.3. Hepatocyte isolation, culture and treatment

Hepatocytes were isolated from rats by collagenase perfusion as previously described (McMillan and Jollow, 1992). Hepatocytes were separated from other liver cell types by centrifugation at $50 \times g$ for 2 min at 4 °C. Viability of the hepatocytes was determined by trypan blue exclusion; preparations with viability >90% were used. The cells were plated on collagen-coated 6-well tissue culture plates at confluency (1.25×10^6 cells/well) in William's Medium E containing 2 mM l-glutamine, 34 ng/ml insulin and 0.1 mg/ml gentamicin sulfate, and 10% FBS. After a 2 h attachment period, medium and unattached cells were removed and the medium was replaced with serum-free, insulin-free William's Medium E containing 1 μ M dexamethasone, 2 mM l-glutamine, and 0.1 mg/ml gentamicin sulfate. Twenty-four hours later, hepatocytes were treated with CH or TCA (0.3–3.0 mM). Stock solutions of TCA and CH were prepared in serum-free culture medium and neutralized with NaOH. A small volume of the neutralized stock solutions was added to the cell cultures to achieve the desired concentration. Twenty-four hours after exposure (Day 1) the medium was removed and stored at –70°C for analysis of CH metabolites and formic acid. The cells were then exposed to CH or TCA in fresh medium for a further 24h and the medium removed (Day 2), and the process was then repeated (Day 3). After the last dose the medium was removed and stored for subsequent analysis, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) activity was determined in the cells as described in section 2.4.

2.4. Renal proximal tubule cell isolation, culture and treatment

HRPTC were isolated from whole kidneys procured by International Bioresearch Solutions (Tucson, AZ). MUSC Institutional Review Board approved this protocol. Kidneys were obtained from 2 donors with the following characteristics: HK 1#; 18 year old, Hispanic, male; HK2#; 24 year old African American, male. Cells were isolated from slices of renal cortex as described by Lock et al., (2006). The isolated cells were diluted to 1×10^6 cells/ml, resuspended in Dulbecco's modified Eagle's medium: Ham's F12 nutrient mix without phenol red, glucose or pyruvate (DMEM:F12 [1:1]) and plated at 1ml/well in 6-well polystyrene culture plates. The basal medium was DMEM:F12 (described above) with the following supplements; 15mM Hepes, 20mM NaHCO₃, 100units penicillin/ml + 100 μ g/ml streptomycin on days 0–4 and 0.25 μ g/ml amphotericin B/ml, bovine insulin 5 μ g/ml, human transferrin 5 μ g/ml, sodium selenite 5 μ g/ml, hydrocortisone 100ng/ml, epidermal growth factor 30ng/ml, 3,3',5-triiodo-DL-thyronine 7.5pg/ml and L-ascorbic acid phosphate (500 μ M) at pH 7.4 and 295 mosmol/kg. The basal medium was then supplemented with glucose at 1 mM.

One ml of two-times supplemented medium was added to the cells and they were left to stand in a humidified incubator under an atmosphere of 5%CO₂/95% air for 48h. The media was then changed daily and the cells grown at 37°C in a humidified incubator with orbital shaking at 70 rpm under an atmosphere of 5%CO₂/95% air until confluent, generally 8–10 days. Confluent monolayers of HRPTC were treated with CH (0.03–3mM) every day for up to 10 days. The medium from each day was removed and stored at –70°C for analysis of CH metabolites and formic acid. Cell viability was determined 1, 3, 7 and 10 days after exposure using MTT. The CH medium was removed and 1ml of MTT (0.5mg/ml) was added to the individual culture wells. Cells were incubated at 37°C for 15–30min, the medium removed and the tetrazolium released by the addition of dimethylsulfoxide (1ml). The optical density was then determined at 560nm and normalized to solvent treated cultures.

2.5 NMR analysis of formic acid

Aliquots of culture medium from HRPTC or rat hepatocytes (500µl) were mixed with 250µl of phosphate buffer (0.2M; pH 7.4; prepared in D₂O) containing sodium 3(trimethylsilyl) propionate-2,2,3,3-d₄ (TSP; 0.5mg TSP/ml buffer) (Holmes et al. 1998) and centrifuged at 14,000 x g for 10 min at 4°C. The supernatants (700µl) were placed into a 5mm NMR tube (Wilmad). ¹H NMR spectra were acquired using a Varian UNITYINOVA spectrometer (Varian, Inc., Palo Alto, CA) operating at 400MHz proton. The standard two pulse proton sequence with presaturation for water suppression was utilised for data acquisition. Sixty four free induction decays were accumulated into 64k data points with a spectral width of 8012.82Hz, all spectra were phase and baseline corrected. Formic acid was quantitated by reference to the internal quantitation standard TSP.

2.6 Chloral hydrate metabolism

The major metabolites of CH, TCA and TCE-OH, were determined in culture medium from HRPTC and hepatocytes by gas chromatography headspace analysis as described previously (Bronley-DeLancey *et al.*, 2006; Muralidhara and Bruckner 1999). Samples were analyzed on a PerkinElmer Autosystem XL gas chromatograph fitted with a headspace autosampler and an electron-capture detector (PerkinElmer, Inc., Wellesley, MA). Metabolites were resolved using a 10 in × 1/8 in outer-diameter stainless-steel column packed with 10% OV-17 on Supelcoport (Supelco, Bellefonte, PA). The temperatures for analyses were as follows: the column was run isothermal at 150°C, injector at 200°C, transfer line at 120°C, and detector at 360°C. Nitrogen was used as the carrier gas at 60 ml/min with a headspace pressure of 20 psi. The samples were heated at 110°C in the autosampler chamber for 30 min before injection into the GC; run time was 8 min. The metabolites were quantified against a standard curve using authentic TCE-OH and TCA (10–1,000 ng).

2.7 Statistical analysis

The data are expressed as mean ± standard error of the mean. Significant differences between the experimental groups were determined for each measurement using a one-way ANOVA followed by the Bonferroni test. A corrected p-value < 0.05 was considered statistically significant.

3. Results

3.1 Determination of formic acid production by rat hepatocytes or human renal proximal tubule cells

The presence of formic acid in both rat hepatocytes and HRPTC was determined using ¹H-NMR spectroscopy. We determined the concentration of formic acid (¹H-NMR peak at 8.45ppm) in the medium from rat hepatocytes exposed to CH (0.3–3mM) over 3 days. At concentrations of 0.3mM CH the concentration of formic acid was below the limits of detection, while at 1 and 3mM CH there was a dose-related increase in formic acid in the media (Figure 1). No difference in formic acid production was seen between days 1 and 3 of exposure (Figure 1). Inactivation of enzyme activity by either fixing the hepatocytes in methanol or inactivation at 100°C did not prevent formic acid production, suggesting the process was not enzyme mediated. This was confirmed by incubation of CH alone in media for 1, 2 or 3 days which resulted in a concentration-related increase in formic acid (Figure 1). Exposure of hepatocytes to neutralised TCA (0.3–3mM) over 3 days did not lead to an increase in formic acid content in the media (Figure 1) suggesting the reaction was CH specific.

HRPTC were exposed to CH (0.03–3mM) over 10 days. At 0.03 and 0.1mM CH the concentration of formic acid was below the limits of detection, while at doses of CH of 0.3mM

and above there was a clear concentration-related increase in formic acid in the medium of both HRPTC samples (Figure 2). No difference in formic acid production was seen between days 1 and 10, therefore the data was pooled. In Figure 2 the error bars represent the mean and SE of analyses conducted on days 1, 3, 7 and 10 of exposure to CH. Importantly, incubation of CH alone in media for 24h also led to a concentration-related increase in formic acid indicating that like in rat hepatocytes cultures, the process was not enzyme-mediated. The higher concentration of formic acid observed in the presence of cells compared to medium alone indicates the cellular presence of formate in the absence of CH (Figure 2). While the higher concentration of formate in the 3mM exposed cells in sample HK2# may reflect cytotoxicity.

3.2 Metabolism of chloral hydrate by rat hepatocytes and human renal proximal tubule cells

As no enzyme-mediated formation of formic acid was observed in these cultures we decided to check that they were metabolically active under the conditions we were using. Rat hepatocytes readily metabolized CH to TCE-OH and TCA, the rate of metabolism being similar over the 3 days (Figure 3). The extent of metabolism to TCE-OH expressed as a percentage of the concentration was $97 \pm 8\%$ at 0.3mM, $71 \pm 4\%$ at 1mM and $27 \pm 1\%$ at 3mM. Metabolism to TCA accounted for $9 \pm 1\%$ at 0.3mM, $8 \pm 1\%$ at 1mM and $6 \pm 1\%$ at 3mM. The rate of metabolism of CH to TCE-OH appeared to become saturated at a concentration above 1mM; the formation of TCA did not appear to be rate limiting (Figure 4). Heat treatment and methanol fixation of rat hepatocytes completely prevented metabolism of CH to TCE-OH and TCA, indicating an enzyme-mediated metabolism (Figure 3).

HRPTC also readily metabolized CH to TCE-OH and TCA, the rate of metabolism being the same on day 1 and day 10 of exposure (Figure 5). Analysis was only conducted at 0.3–3mM CH with the error bar showing the range for duplicate analyses. In HK 1# the extent of CH metabolized to TCE-OH each day over 10 days expressed as a percentage of the concentration was $44 \pm 6\%$ and $45 \pm 5\%$ at 0.3 and 1mM respectively, and $20 \pm 3\%$ at 3mM. Metabolism to TCA was $3.6 \pm 1.2\%$ and $3.2 \pm 0.3\%$ at 0.3mM and 1mM respectively, and $2 \pm 0.4\%$ at 3mM. Thus, metabolism of CH by HRPTC appeared to become saturated at concentrations greater than 1mM. The extent of metabolism in HK 2# was less than in HK 1#, with only $22 \pm 3\%$ of the concentration metabolised to TCE-OH at 0.3mM, $15 \pm 5\%$ at 1mM and 1.9 ± 0.2 at 3mM. Metabolism to TCA was also reduced, being $1.2 \pm 0.3\%$ at 0.3mM CH, $0.8 \pm 0.2\%$ at 1mM CH and 0.13 ± 0.03 at 3mM. The lower metabolism in HK 2# may reflect a polymorphism in the rate of metabolism of CH (see Discussion) while at the high dose of 3mM CH the reduction is due to cytotoxicity (see below).

3.3 Cytotoxicity of chloral hydrate to rat hepatocytes and human renal proximal tubule cells

The aim of these studies was to determine whether CH or TCA were cytotoxic under the conditions used to look for formic acid production. Rat hepatocytes exposed to CH at concentrations up to 3mM showed no signs of toxicity over 3 days of exposure, in fact MTT activity increased up to 140% of control suggestive of an increase in cell number (Figure 6). Similarly rat hepatocytes exposed to neutralised TCA over 3 days also showed no signs of toxicity there being a progressive increase in MTT activity up to 140% of control at 3mM (Figure 6). Treatment of rat hepatocytes with methanol or heat inactivation at 100°C followed by exposure to 1mM CH or TCA for 3 days completely abolished MTT activity (Figure 6).

CH at concentrations up to 3mM produced little or no toxicity to HRPTC from HK 1# over the 10day dosing period. In contrast, in HK 2# signs of cytotoxicity were seen at 1mM CH after 3 days exposure, with about 50% loss of MTT activity which had increased to 70% loss by days 7 and 10 (Figure 7). At 3mM CH the extent of cytotoxicity was more marked, with

60% loss of activity after 3 days of exposure and almost complete loss by days 7 and 10 (Figure 7).

4. Discussion

We report three important findings from our studies; 1) the increase in formic acid production seen in rat hepatocytes and HRPTC exposed to CH is due to chemical degradation of CH. Exposure of rat hepatocytes to TCA did not lead to increased formic acid production, unlike that observed in rats given TCA. 2) HRPTC can metabolise CH to TCE-OH and TCA, as has been reported for human liver and lung (Lipscomb et al., 1997; Elfarra et al., 1998; Forkert et al., 2005), and 3) CH has a low toxicity to rat hepatocytes but appears to be more toxic to HRPTC.

Male rats excrete large quantities of formic acid in their urine following exposure to TCE, up to 10mg/day compared to <0.5mg/day in control. Similar findings are seen in male rats treated with the TCE metabolites, TCE-OH, TCA and, to a lesser extent, CH (Dow and Green, 2000). Our aim was to ascertain if formic acid could be produced *in vitro* in culture in male rat hepatocytes analogous to that seen in the male rat *in vivo*. A small concentration of formic acid was observed in the medium but was shown to be the result of chemical degradation of CH to formate, as it occurred in the absence of cells. This finding is supported by the studies of Newman and Wackett, (1991) showing that CH undergoes degradation at neutral and alkali pH to chloroform and formic acid, the reaction being temperature dependent (Figure 8). TCA which also causes a marked formic aciduria in rats, also showed no increase in formic acid in cultures of rat hepatocytes. The concentration of CH we used *in vitro* is much higher than that likely to be formed *in vivo* following exposure to TCE. For example, in rats given a single large oral dose of 1g/kg TCE peak plasma concentrations were about 2µg/ml ~0.012mM (Prout et al. 1985), presumably due to rapid conversion of CH to TCE-OH and TCA. In contrast, the concentration of TCA used *in vitro* is similar to that formed *in vivo*. TCA accumulates in the plasma, being cleared very slowly, reaching concentrations of about 1mM in rats given 1g/kg TCA (Prout et al., 1985). Humans exposed to TCE in the workplace will have plasma concentrations very much lower than we used in these studies, even though TCA accumulates in human plasma (see Davison and Beliles, 1991). The human kidney is exposed to CH, mainly formed extra-renal from TCE so we exposed HRPTC to CH daily for up to 10 days. As with the hepatocytes we saw no increase in enzyme-mediated formic acid production. Thus, the perturbation of formate metabolism that occurs in rats given CH or TCA cannot be reproduced *in vitro* in either rat hepatocytes or HRPTC. This finding suggests that the mode of action leading to formic acid production is complex, requiring integrated organs systems for expression.

In order to show that our cells in culture were metabolically active we examined the metabolism of CH. We observed metabolism of CH to TCE-OH and TCA in rat hepatocytes in agreement with the findings of others using rats or *in vitro* systems (Ikeda et al., 1980; Miller and Guengerich, 1983; Elcombe, 1985; Davidson, and Beliles, 1991; Lipscomb et al., 1996; Elfarra et al., 1998; DeSmet et al., 2000; Bloemen et al., 2001). Rat hepatocytes readily metabolised CH to TCE-OH with an apparent K_m of 1.2 ± 0.2 mM and V_{max} of 1.45 ± 0.09 nmol/min/ 10^6 cells. Metabolism of CH to TCA occurred with an apparent K_m of 4.3 ± 0.3 mM and V_{max} of 0.85 ± 0.09 nmol/min/ 10^6 cells. Derivation of 'intrinsic clearance' or 'capacity factors' (V_{max}/K_m) illustrated a 6-fold difference in efficacy of TCE-OH formation versus TCA formation by these hepatocytes. These findings are in broad agreement with those reported by Lipscomb et al., (1996) who compared CH metabolism in human and rat liver. The metabolism of CH to TCE-OH by rat hepatocytes became saturated at high substrate concentration while metabolism to TCA did not (Figure 5). These findings are in good

agreement with those reported by Lipscomb et al., (1996) for rat and human liver fractions and also closely resemble that seen *in vivo* in rats exposed to TCE (Prout et al., 1985).

We report for the first time that HRPTC can metabolise CH to TCE-OH and TCA. HRPTC readily metabolized CH to TCE-OH with an apparent K_m of 6.7mM and V_{max} of 5.1nmol/min/ 10^6 cells. Metabolism of CH to TCA occurred with an apparent K_m of 1.9mM and V_{max} of 0.12nmol/min/ 10^6 cells. Derivation of 'intrinsic clearance' or 'capacity factor' (V_{max}/K_m) illustrated a 12-fold difference in efficacy of TCE-OH formation versus TCA formation by these HRPTC. Our findings in human kidney are very similar to those of Lipscomb et al., (1996) for human liver *in vitro* who reported a 10-fold greater TCE-OH formation versus TCA formation from CH. CH undergoes metabolism via alcohol dehydrogenase to TCA, with NAD^+ as the cofactor (Figure 8). CH is not a substrate for cytosolic or mitochondrial rat liver aldehyde dehydrogenase (Sharpe and Carter, 1991). CH can also undergo metabolism via alcohol reductase, with NADH as the cofactor, to form TCE-OH (Figure 8). Metabolism of CH to TCE-OH and TCA by HK 2# was about 50% less compared to HK1#, metabolism being reduced at all time points at 3mM/day reflecting the cytotoxicity observed. Although we have only examined two samples, others have reported marked genetic polymorphisms in alcohol dehydrogenase and aldehyde reductase (Mizo et al., 1994; Li et al., 2001; Yokoyama and Omori, 2005; Wall, 2005). Recent studies using 13 samples of cryopreserved human hepatocytes, and CH as substrate, reported a large variability in alcohol dehydrogenase and aldehyde dehydrogenase activity among these samples (Bronley-DeLancey *et al.*, 2006). These findings may explain the difference we have observed. Rat hepatocytes showed a greater capacity, about 1.6 and 3-fold to produce TCE-OH and TCA respectively than HRPTC, reflecting a higher rate of CH metabolism by rat hepatocytes compared to HRPTC. Thus both rat hepatocytes and HRPTC were able to metabolise CH under the culture conditions we used to look for formic acid production suggesting that biotransformation of CH, if required, was not a limiting factor.

Neither CH nor its metabolite TCA were cytotoxic to rat hepatocytes at concentrations of 3mM/day for 3 days; these findings are consistent with studies by Bruschi and Bull, (1993); Lash et al., (1995); Walgren et al., (2004 and 2005). There was a tendency for MTT activity to be higher than controls for both CH and TCA treated cells, this was not however statistically significant and may just reflect slight differences in plating density, hepatocyte adherence or a compound related alteration in mitochondrial dehydrogenase activity. HRPTC HK 1# CH showed little or no cytotoxicity at concentrations up to 3mM/day for 10 days however HRPTC HK 2# was more sensitive to CH, cytotoxicity being observed at 1mM following three doses (Figure 6) although no cytotoxicity was seen at 0.3mM CH over 10 days exposure. The basis for the difference in sensitivity is not known but may be related to human polymorphisms in CH metabolising enzymes, as discussed above. Thus cytotoxicity cannot explain the lack of formic acid production from CH or TCA in rat hepatocytes or in one sample of HRPTC. Overall our findings on cytotoxicity are in agreement with the work of others (see Bloeman et al., 2000; Lash et al; 2000, Lock and Reed, 2006) supporting the view that oxidative metabolism of TCE to CH is not responsible for the renal toxicity observed in rodents. Metabolism via glutathione conjugation forming S-1,2-dichlorovinyl-L-cysteine (DCVC) is considerably more toxic to HRPTC (Lash et al., 2001; Lock et al., 2006).

In summary, we have been unable to detect an increase in formic acid in the culture medium following exposure of rat hepatocytes to CH or TCA and HRPTC to CH, unlike that observed in rats given these chemicals (Green et al., 1998; Dow and Green, 2000). The cells can metabolise CH to TCE-OH and TCA, suggesting biotransformation is not a limiting factor and further that cytotoxicity was not an issue in rat hepatocytes or in one sample of HRPTC. We only had access to two human kidney samples and additional studies are needed to increase the number of samples, especially with the known human polymorphism for CH metabolism.

Further studies are also needed to understand the mode of action leading to formic aciduria in rats exposed to TCE and its metabolites; this may then enable us to model the *in vivo* findings in isolated cellular systems.

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Figure 1.

Formic acid production from chloral hydrate and trichloroacetic acid in the presence of rat hepatocytes or in culture medium alone.

Hepatocytes were incubated with CH or TCA at the concentrations shown, each day for 3 days. CH or TCA were also incubated with culture medium alone. Some samples were inactivated either by heating to 100°C or fixing in methanol and then incubating with media containing CH or TCA. On days 1, 2 and 3 samples of medium was taken to determine formic acid content using ^1H -NMR spectroscopy. Results are expressed as mean $\pm$ SE with 3–4 different cultures per concentration. * Significantly different from appropriate control, $P < 0.05$. ----- limit of detection.

Figure 2.

Formic acid production from chloral hydrate in the presence of HRPTC or in culture medium alone

HRPTC were incubated with CH at the concentrations shown each day for 10 days or CH was incubated with culture medium alone (n=4). On days 1, 3, 7 and 10 media was taken to determine formic acid content using ^1H -NMR spectroscopy. Values are shown as mean $\pm$ SE of the four days examined for two separate human kidney samples.

* significantly different from medium alone.

Figure 3.

Metabolism of chloral hydrate to trichloroethanol and trichloroacetic acid by rat hepatocytes in culture.

Hepatocytes were incubated with CH or TCA at the concentrations shown each day for 3 days. Samples of media were taken on days 1, 2, and 3 for analysis of TCE-OH and TCA by gas chromatography. Results are expressed as mean $\pm$ SE with 3–4 different cultures per concentration & time. Values are expressed as μ mol product formed/24h/ 10^6 cells.

* significantly different from 0.3mM CH on days 1, 2 and 3.

significantly different from 1mM CH on day 3.

Figure 4.

Concentration-dependent formation of trichloroethanol and trichloroacetic acid by rat hepatocytes exposed to chloral hydrate in culture.

Hepatocytes were incubated with CH at the concentrations shown for one day and samples of medium taken for analysis of TCE-OH and TCA by gas chromatography. Results are expressed as mean $\pm$ SE with 3–4 different cultures per concentration. Values are expressed as μmol product formed/24h/ 10^6 cells

Figure 5.

Metabolism of chloral hydrate to trichloroethanol and trichloroacetic acid by human renal proximal tubules cells in culture.

HRPTCs were incubated with CH at the concentrations shown each day for 10 days. On days 1, 3, 7 and 10, media from a single well/concentration & time, was taken for analysis of TCE-OH and TCA by gas chromatography. Two separate human kidney samples were examined. Values are expressed as μmol product formed/24h as the mean and range of duplicate analyses

Figure 6.

Cytotoxicity of chloral hydrate and trichloroacetic acid to rat liver hepatocytes in culture. Hepatocytes were incubated with CH or TCA at the concentrations shown each day for 3 days. Some samples were inactivated either by heating to 100°C or fixing in methanol and then incubating with media containing CH or TCA. On day 3 cells were taken to determine cytotoxicity using MTT. Results are expressed as mean $\pm$ SE of 3–4 different cultures per concentration. * Significantly different from appropriate control, $P < 0.05$. Values are expressed as percentage of the control MTT activity which was for CH, 0.346 OD 560nm/15min/well and for TCA, 0.402 OD 560nm/20min/well.

Figure 7.

Cytotoxicity of chloral hydrate to human renal proximal tubules cells in culture.

HRPTC were incubated with CH at the concentrations shown each day for 10 days. On days 1, 3, 7 and 10, cells from a single well, were taken to determine cytotoxicity using MTT. Two separate human kidney samples were examined. Values are expressed as percentage of the control MTT activity which was for HK1#; day 1, 1.65; day 3, 1.26; day 7, 1.29; day 10, 1.39 OD 560nm/15min/well and for HK2#; day 1, 0.28; day 3, 0.58; day 7, 1.38; day 10, 1.02 OD 560nm/30min/well.

Figure 8. Biotransformation of chloral hydrate by rat hepatocytes and human renal proximal tubules cells and chemical transformation of chloral hydrate to formic acid.