Covalent Binding of the Carcinogen Trichloroethylene to Hepatic Microsomal Proteins and to Exogenous DNA in Vitro

Sipra Banerjee and Benjamin L. Van Duuren

ABSTRACT

Studies were carried out on the in vitro covalent binding of the carcinogen trichloroethylene (TCE) to liver microsomal preparations and to exogenous DNA. The binding of TCE to liver microsomal proteins of male C57BL/6 × C3H/He F1 (hereafter called B6C3F1) hybrid mice, a species and strain susceptible to TCE-induced liver tumorigenesis, was 46% higher than that of [14C]TCE to microsomal proteins from male Osborne-Mendel rats, a species and strain resistant to TCE-induced hepatocellular carcinoma. The in vitro binding of [14C]TCE to liver microsomal proteins was 37% higher for male B6C3F1 mice; female B6C3F1 mice that have been reported to show a lower incidence of TCE-induced hepatocellular carcinoma than do males. Microsomal proteins from the lung, stomach, and kidney of B6C3F1 hybrid mice also metabolized TCE, as indicated by the covalent binding of [14C]TCE to microsomal proteins from these organs. For rats the binding of TCE to liver microsomal proteins of Sprague-Dawley animals was higher than that of Osborne-Mendel and Fischer 344 rats. Incubation of [14C]TCE with salmon sperm DNA in the presence of microsomal preparations from B6C3F1 hybrid mice resulted in covalent binding of [14C]TCE to DNA. This binding was much higher in the presence of microsomal proteins from male rather than female mice. The binding to DNA and protein was enhanced by in vivo phenobarbital administration. The effects of 1,2-epoxy-3,3,3-trichloropropane on the covalent binding of [14C]TCE to protein and DNA were also examined.

INTRODUCTION

TCE is extensively used as an industrial organic solvent for metal degreasing, dry cleaning, and food extraction and as an ingredient in paints, adhesives, varnishes, and printing ink. It has also been widely used as an anesthetic. Previously, we suggested that TCE could be a potential carcinogen, particularly in the liver. Ref. 24 and earlier publications cited therein). Later, TCE was found to cause hepatocellular carcinoma in C57BL/6 × C3H/He F1 (hereafter called B6C3F1) hybrid mice, and the incidence of tumors was much higher in males than in females. However, TCE failed to induce tumors in Osborne-Mendel rats. TCE is mutagenic after metabolic activation.

Indirect-acting chemical carcinogens undergo metabolism to activated carcinogenic intermediates through enzymatic oxidation by cytochrome P450-dependent oxygenases. Experimental evidence supporting the formation of epoxide intermediates has been obtained for both VC and TCE, as had been suggested earlier on structural grounds. The activated carcinogenic intermediates react with nucleophilic moieties of cellular proteins and nucleic acids. VC epoxide rearranges spontaneously to chloroacetaldehyde, which should be considered as a potential activated carcinogenic intermediate; TCE epoxide, on the other hand, is stable at room temperature in the absence of water but hydrolyzes rapidly at pH 7.4 (37°) to dichloroacetic acid with a half-life of 1.3 min (12).

The metabolism and biological activity of most carcinogens vary with respect to species, strain, sex, and organ. Therefore, it was important to compare the metabolism of TCE for species of animals that developed liver tumors after exposure to TCE and those that were resistant to such tumor induction. This report presents comparative studies on the in vitro covalent binding of [14C]TCE to microsomal proteins of different strains, species, sexes, and organs of mice and rats. In addition, studies are presented on the in vitro binding of [14C]TCE to exogenous DNA in the presence of liver microsomal preparations.

MATERIALS AND METHODS

Chemicals. Custom-labeled [1,2-14C]TCE (specific activity, 1 mCi/mmol) and [14C]toluene were supplied by New England Nuclear, Boston, Mass. The radiochemical purity of [14C]TCE, as determined by gas chromatography-mass spectrometry, was >99%; this material was used as such. Highly polymerized salmon sperm DNA and bovine pancreas ribonuclease were purchased from Calbiochem, San Diego, Calif. 3MC and TCPO were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. PB was purchased from Elkins-Sinn, Inc., Cherry Hill, N. J., and diphenylamine was obtained from Eastman Kodak Chemical Co., Rochester, N. Y.

Animals. B6C3F1 hybrid mice and Osborne-Mendel and Fischer 344 rats were kindly provided by the National Cancer Institute, Bethesda, Md. Sprague-Dawley rats were obtained from A. R. Schmidt, Madison, Wis.

Preparation of Microsomes. Hepatic microsomes from...
rats and mice and microsomes from lung, stomach (after being cut lengthwise and washed thoroughly), and kidney from mice were prepared as described previously (25). The microsomes from all sources were enzymatically characterized for determination of their purity. The microsomal preparations were devoid of mitochondrial or plasma membrane contamination as determined by the absence of marker enzymes, succinic acid dehydrogenase, or phosphodiesterase and 5'-nucleotidase, respectively. These enzymes were assayed as reported elsewhere (26). The microsomal pellet was suspended in 0.05 M potassium phosphate buffer, pH 7.4, at a protein concentration of 8 mg/ml. Protein was determined by the method of Lowry et al. (14). The microsomes were used immediately after isolation in the incubation experiments.

In Vivo Treatment. For induction of the microsomal hydroxylation enzyme system, PB in 0.15 M NaCl and 3MC in trioctanoin (0.15 M NaCl; trioctanoin, 1:2.6) at dosages of 100 and 30 mg/kg body weight, respectively, were administered i.p. once daily for 3 days to male B6C3F1 mice weighing 15 to 20 g. Control mice received either 0.15 M NaCl or trioctanoin only. Animals were killed 24 hr after the last injection. Livers were excised, and microsomes were prepared as described earlier (25).

Covalent Binding of TCE to Microsomes. The incubation system used for covalent binding of [14C]TCE to microsomal protein was reported previously (25). For incubation for 60 min at 37°, microsomal protein was precipitated with trichloroacetic acid and purified as described earlier (25).

Binding of Metabolically Activated TCE to DNA and Microsomal Proteins. Salmon sperm DNA was incubated with [14C]TCE at 37°for 60 min in a Dubnoff shaking bath in the presence of microsomes prepared from male B6C3F1 mice and a NADPH regenerating system. The incubation system consisted of 6.67 µmoles of [14C]TCE, 8.75 mg of DNA, 10.0 µmoles of glucose 6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase, 2.0 µmoles of NADP+, 5.0 µmoles of MgCl2, 1.8 to 2.0 mg of microsomal protein, and 12.5 µmoles of potassium phosphate buffer, pH 7.4, for a total volume of 3.25 ml. After incubation the microsomes were centrifuged at 35,000 × g for 45 min at 4°. Protein was precipitated from the microsomal pellet with 5% trichloroacetic acid and purified as reported previously (25), and radioactivity was counted. The purified microsomal protein was checked for DNA contamination with Burton's method (7). The supernatant containing labeled DNA and free TCE was treated with sodium dodecyl sulfate (final concentration, 1.0%) for 15 min. The resulting solution was then deproteinized by stirring vigorously for 30 min with an equal volume of a saturated solution of phenol in water. The resulting emulsion was separated into 2 layers by centrifuging at 10,000 rpm in a RC-5 Sorvall refrigerated centrifuge. The upper aqueous phase containing DNA was carefully transferred into a flask, and 2 volumes of cold ethanol were gently layered on top of the solution. The fibrous form of DNA was then spooled on a rod, removed, and dissolved in 7 to 8 ml of 0.015 M NaCl:0.0015 M trisodium citrate, pH 7.0. This DNA solution was incubated with RNase (final concentration, 50 µg/ml) for 30 min at 37°. The RNase used was preheated at 100° for 15 min to destroy any DNase present. After hydrolysis of the RNA, the mixture was deproteinized again with phenol and centrifuged. The upper aqueous layer was pipetted into a flask, and the DNA was precipitated with cold ethanol as before. The mixture was then stored overnight at −20°. After collection of the precipitated DNA, it was washed with ethanol once at 70° and then once more at room temperature to remove unbound TCE. Finally, the DNA pellet was washed with ether and air-dried. The DNA residue was checked for protein and RNA contamination (14, 21).

For determination of whether TCE can bind to DNA without metabolic activation, [14C]TCE was incubated with DNA and a NADPH regenerating system without microsomes. After incubation, the DNA was separated and purified as described above.

Quantitation of Macromolecules and Measurement of Radioactivity. DNA was hydrolyzed with 0.5 n perchloric acid at 95° for 15 min, and the nucleoside content was determined with the diphenylamine reaction (7) with hydrolyzed salmon sperm DNA as standard. The protein residue was dissolved in 0.2 n NaOH by heating at 90° for 15 min, and the protein contents were determined by the procedure of Lowry et al. (14). The radioactivity of the DNA and protein samples was determined by counting in a Packard liquid scintillation spectrometer. Samples of 2 to 4 mg of TCE-bound DNA were used for counting. Quenching was determined with [14C]toluene, radioactivity values were obtained for the DNA, and protein samples were corrected accordingly (85 and 94% for DNA and protein, respectively, in Aquasol). Results were expressed as pmoles of TCE bound per mg of DNA and nmoles of TCE bound per mg of protein.

RESULTS

Binding of TCE to Hepatic Microsomal Protein of Rodents. In these experiments microsomes were incubated with TCE for 60 min since previous work had shown that the binding of TCE to microsomal protein had reached a maximum at this length of time (25). As indicated in Table 1, hepatic microsomes from male B6C3F1 mice bound 46% more TCE than did microsomes from Osborne-Mendel rats. This binding to protein was extremely strong and irreversible, as trichloroacetic acid precipitation and vigorous washing in methanol:ether followed by ether did not dissociate the radioactivity from the microsomal protein. Therefore, it was unlikely that the binding was not covalent in nature. Further evidence for the covalent nature of the binding was obtained by digestion of the washed protein residue with pronase at 37° for 4 hr (25) to hydrolyze the primary

<table>
<thead>
<tr>
<th>Species</th>
<th>[14C]TCE bound to protein (nmoles/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6C3F, mouse</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Osborne-Mendel</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

*Results are the average ± S.D. of 9 mice and 5 rats. Each animal constituted a separate experiment, and 3 separate analyses were carried out for each animal. This result is statistically significant (p < 0.01).*
structure of the proteins into amino acids and small peptides. This digest was then extracted with 4 volumes of ether that could not extract water-soluble products, indicating that TCE metabolites were covalently bound to amino acids derived from microsomal proteins.

**Strain and Sex Variations in the Binding of TCE to Rat Microsomal Protein.** The covalent binding of [14C]TCE to microsomal protein varied with respect to the strain and age of rats, as shown in Table 2. Thirty % more TCE was bound to the microsomes from mature male Sprague-Dawley rats than was bound to those of immature rats of the same strain. This difference is statistically significant (p < 0.01). A strain difference in the binding was also observed; the binding of TCE to microsomal protein was 44 and 63% more in 5- to 9-week-old Sprague-Dawley rats than it was in Osborne-Mendel and Fischer 344 rats of same age, respectively. This result also is statistically significant (p < 0.001). The sex variation in the binding of TCE to protein was observed only in Osborne-Mendel rats, which was statistically significant (p < 0.05), but not in other strains. Twenty-nine % more binding was associated with protein of male compared to female Osborne-Mendel rats.

**Binding of TCE to Microsomes from Extrahepatic Tissues.** For determination of whether organs other than liver were capable of metabolizing TCE, microsomes were isolated from the stomach, lung, and kidney of male B6C3F, mice and incubated with [14C]TCE. As shown in Table 3, TCE bound to the microsomal protein of all tissues studied as efficiently as it did to that of liver. Kidney microsomes bound the lowest amount of TCE. Microsomes from the lung of female mice bound 18% more TCE than did those from male mice. There was no significant sex difference in the binding of TCE to microsomal protein isolated from the stomach or kidney.

**Microsome-dependent Binding of TCE to DNA.** The amount of TCE bound to DNA increased with increasing concentration of microsomes (Table 4). The TCE-DNA product did not contain any protein or RNA contamination, showing the purity of DNA. DNA, after repeated dissolving in 0.015 M NaCl:0.0015 M trisodium citrate buffer (pH 7.0), reprecipitation by cold ethanol, and extractions in ethanol and ether, retained its radioactivity, which indicates a stable and probably covalent linkage formed between TCE and DNA. When DNA was incubated with [14C]TCE without microsomes, no radioactivity was associated with this macromolecule.

**Sex Differences in the Binding of TCE to Macromolecules of B6C3F, Mice.** The amount of TCE bound to hepatic protein and salmon sperm DNA incubated in the presence of microsomes from male and female mice is shown in Table 5. Thirty-seven % more TCE was bound to microsomal protein from male rather than female mice. This sex difference was more striking when [14C]TCE was incubated with DNA in the presence of microsomes prepared from both sexes. The covalent binding of TCE to DNA was 160%

<table>
<thead>
<tr>
<th>Organ</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Lung</td>
<td>2.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*The values are the averages from 2 groups of animals with 3 animals in each group.*

**Microsome-dependent binding of TCE to DNA in vitro**

Salmon sperm DNA was incubated with [14C]TCE for 60 min in the presence of hepatic microsomes isolated from male B6C3F, mice. After the incubation, DNA was isolated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Microsome concentration (mg of protein)</th>
<th>[14C]TCE bound to DNA (pmoles/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No detectable radioactivity</td>
</tr>
<tr>
<td>0.6</td>
<td>67.3</td>
</tr>
<tr>
<td>1.2</td>
<td>79.9</td>
</tr>
<tr>
<td>2.5</td>
<td>102.0</td>
</tr>
</tbody>
</table>

*Results are the average of 2 animals. Each animal constituted a separate experiment, and 3 analyses were carried out for each animal.*

**Sex differences in the covalent binding of [14C]TCE to macromolecules**

Microsomes from 5- to 9-week-old male and female B6C3F, mice were incubated with [14C]TCE and salmon sperm DNA as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Sex</th>
<th>nmoles/mg of protein</th>
<th>% change compared to female pmoles/mg of DNA</th>
<th>% change compared to female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>1.9 ± 0.1</td>
<td>35.7 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2.6 ± 0.3 +37</td>
<td>93.2 ± 11.2 +161</td>
<td></td>
</tr>
</tbody>
</table>

*The results for TCE protein binding are the average ±S.D. of 10 male and 7 female animals; the results for TCE-DNA binding are the average ±S.D. of 6 male and 4 female animals. Each animal constituted a separate experiment, and 3 analyses were carried out for each animal. The female to male difference is statistically significant (p < 0.001).*
Higher when the DNA was incubated in the presence of microsomes isolated from male rather than female mice.

Macromolecule-bound TCE in the Presence of PB and 3MC-treated Hepatic Microsomes. Table 6 illustrates the effects of in vivo administration of PB and 3MC on the binding of TCE to protein and DNA. Hepatic microsomes were isolated from PB and 3MC-treated male B6C3F1 mice and incubated with [14C]TCE. The covalent binding of TCE to microsomal protein and DNA was enhanced by 58 and 41% (p < 0.001 and <0.05), respectively, by PB over the control group.

3MC, a carcinogen and inducer of cytochrome P-448 (15), increased the binding to protein by 31% compared with the control. This difference is statistically significant (p < 0.05). For 3MC administration to the animals, trioctanoin was used as the solvent. The same amount of TCE was bound to microsomal protein of the trioctanoin-treated animals as was bound to that of the 0.15 M NaCl control. In the presence of microsomes prepared from mice treated with trioctanoin, the amount of TCE bound to DNA, however, was 163 ± 40.7 pmol/mg of DNA, an increase of 84% (p < 0.01) compared to the 0.15 M NaCl group (not shown in Table 6).

TCPO, a known inhibitor of epoxide hydrolase, enhanced the binding of TCE to DNA compared to the control. The amount of TCE bound to DNA also increased with increasing TCPO concentration (14 to 120%). At a concentration of 1.2 mM, TCPO caused an increase of 15% in the binding to protein and to DNA. When the concentration of TCPO was increased above 1.2 mM, the amount of TCE bound to protein decreased, compared to a constant increase in the binding of TCE to DNA.

DISCUSSION

Earlier studies from this laboratory showed that TCE is metabolized in vitro by rat liver microsomes and that the reactive metabolite(s), possibly an epoxide or another electrophilic species, bind(s) covalently to microsomal protein (2, 25).

These studies indicate that the in vitro covalent binding of TCE to liver microsomal protein is significantly higher for B6C3F1 hybrid mice than for Osborne-Mendel rats. In addition, the results show that microsomal protein from male B6C3F1, hybrid mice binds more TCE than does that of female mice of the same strain. These findings correlate with the observed carcinogenesis bioassays (23).

The covalent binding of TCE to liver microsomal protein of Sprague-Dawley rats was higher than that of Osborne-Mendel and Fischer 344 rats. Microsomal proteins from male Osborne-Mendel rats bound more TCE than did that of the corresponding females. These sex, strain, species, and age differences are most probably due to the differences in the mixed-function oxidase level (6). Similar observations have been reported earlier (10, 29).

All extrahepatic tissues examined (i.e., lung, stomach, and kidney) metabolized TCE at rates comparable to that found in the liver, as judged by the amount of TCE bound to microsomal protein from these tissues. Similar results have been reported for VC, which showed significant irreversible binding to protein from kidney, lung, and intestine (5).

These studies indicate that TCE also binds covalently to exogenous DNA in vitro and that metabolic activation mediated by microsomes is required for this binding, as was found also for the carcinogen benzo(a)pyrene (9). A highly significant amount of binding of TCE to DNA in the presence of microsomes from male mice rather than from female mice was observed, which correlates with the carcinogenesis bioassays (23).

The trioctanoin effect on TCE binding to DNA discussed in "Results" is at present difficult to explain. However, the use of organic solvents in in vivo and in vitro systems influences the metabolism of a number of compounds (1, 20, 28).

Addition of TCPO, a potent inhibitor of microsomal epoxide hydrolase (18), to the incubation system enhanced the

### Table 6

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmoles/mg of protein</th>
<th>% change over the control</th>
<th>pmoles/mg of DNA</th>
<th>% change over the control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.6 ± 0.3</td>
<td>92.5 ± 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>2.6 ± 0.1</td>
<td>89.0 ± 11.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB in 0.15 M NaCl</td>
<td>4.1 ± 0.4</td>
<td>+58</td>
<td>125.3 ± 20.2</td>
<td>+41</td>
</tr>
<tr>
<td>Trioctanoin</td>
<td>2.6 ± 0.3</td>
<td>+31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3MC in trioctanoin</td>
<td>3.4 ± 0.2</td>
<td>+31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4</td>
<td>81.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCPO</td>
<td>1.2 mM</td>
<td>+15</td>
<td>93.0</td>
<td>+14</td>
</tr>
<tr>
<td></td>
<td>2.4 mM</td>
<td>-32</td>
<td>115.0</td>
<td>+41</td>
</tr>
<tr>
<td></td>
<td>4.8 mM</td>
<td>-39</td>
<td>179.0</td>
<td>+120</td>
</tr>
</tbody>
</table>

* Results are the average ±S.D. of 6 animals in both the 0.15 M NaCl control and the PB-treated group and of 4 animals in the trioctanoin control and the 3MC-treated group. The data for the TCPO experiment are the average of 2 animals with 3 analyses for each animal. The p values are discussed in the text.
binding of TCE to DNA compared to the amount of TCE bound to DNA in the absence of TCPO. This effect increased with increasing TCPO concentration. Based on these studies and earlier observations (24, 25) the metabolic scheme shown in Chart 1 is proposed. Chart 1 accounts for the known metabolites of TCE (24) and the TCPO findings reported in this paper. However, other activated carcinogenic intermediates of TCE cannot be excluded, based on the findings reported here and those reported earlier (5, 19, 24) for the human carcinogen VC (16).

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REFERENCES

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