Effects of Chronic Ethanol Consumption on Benzo(a)pyrene Metabolism and Glutathione S-Transferase Activities in Syrian Golden Hamster Cheek Pouch and Liver

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ABSTRACT

The metabolism of benzo(a)pyrene (BaP) by hepatic or cheek pouch epithelium microsomes obtained from Syrian golden hamsters which had been consuming an ethanol-containing liquid diet for 4 wk and from pair-fed controls was measured. Glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene or (±)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene as substrates was measured in cytosol obtained from the liver or cheek pouch epithelium of the same animals. Cytosolic hepatic glutathione levels were measured in both ethanol-consuming and control animals. The metabolism of BaP to 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene (BaP-4,5-diol), 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (BaP-7,8-diol), 9-hydroxybenzo(a)pyrene (9-OH-BaP), and 3-hydroxybenzo(a)pyrene (3-OH-BaP) by hepatic microsomes from ethanol-consuming hamsters was significantly reduced (40-52%) (P < 0.05) compared to control microsomes. However, a 2-fold increase (P < 0.05) in the metabolism of BaP to BaP-7,8-diol and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene was measured with microsomes from the cheek pouch epithelium of ethanol-consuming animals. There was no significant change in the production of BaP-4,5-diol, 9-OH-BaP, or 3-OH-BaP by cheek pouch epithelium microsomes of ethanol-consuming hamsters compared to controls. No difference in glutathione S-transferase activity of hepatic or cheek pouch epithelial cytosol between control and ethanol-consuming hamsters towards 1-chloro-2,4-dinitrobenzene or (±)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene was observed. Hepatic glutathione content was significantly (P < 0.05) decreased after 2 wk (23%) and 4 wk (33%) of ethanol consumption. The results suggest a mechanism by which ethanol might enhance BaP tumorigenesis in the hamster cheek pouch.

INTRODUCTION

Epidemiological studies demonstrate that the combination of chronic alcohol consumption and tobacco smoking is a major composite risk factor for the development of oral cavity cancer (1). It has been suggested that as much as 50% of oral cavity cancer in the United States is associated with heavy drinking (2). It has been suggested that as much as 50% of oral cavity cancer in the United States is associated with heavy drinking (2).

The ability of ethanol to induce cytochrome P-450-dependent mixed-function oxidases has been recognized for some time (4, 5). BaP is metabolized by these microsomal enzymes to a number of metabolites, including the presumed ultimate carcinogen, BPDE. It has been demonstrated that microsomes prepared from the intestine of ethanol-consuming rats have increased BaP hydroxylase activity as well as an increased capacity to activate BaP to a mutagen in the Salmonella typhimurium test system (6). In the study reported here, we have directly measured the metabolites of BaP produced by hepatic and cheek pouch epithelium microsomes of ethanol-consuming and control hamsters.

In parallel to this investigation, we have determined the effect of chronic ethanol consumption on the glutathione-dependent detoxification of BPDE. The detoxification of BaP metabolites by glutathione has been established in both microsomal and whole cell systems (7, 8). In isolated hepatocytes, depletion of glutathione markedly enhanced DNA binding of BPDE with a concurrent decrease in the amount of glutathione conjugates formed (9). A large (5-g/kg body weight) dose of ethanol administered to rats, mice, or baboons caused a significant decrease in hepatic glutathione levels (10–12). The chronic administration of ethanol, in a dose comparable to that consumed by alcoholics, also produced a decrease in the hepatic glutathione content of rats (10) and baboons (12). The level of glutathione in the liver of alcoholics with cirrhosis was depressed compared to that of patients with liver disease unrelated to alcohol (13, 14).

The conjugation of glutathione with BPDE is catalyzed by GST (15). In rats (16, 17), chronic ethanol consumption induced a significant increase in the specific activity of GST when CDNB, DCNB, p-nitrobenzyl chloride, and ENPP were used as substrates, and in mice (18, 19) with CDNB, DCNB, and p-nitrobenzyl chloride as substrates. However, the effect of ethanol on the specific activity of GST with BPDE as a substrate has not been measured.

MATERIALS AND METHODS

Chemicals. BaP and BaP metabolite standards were obtained from the National Cancer Institute Carcinogen Standard Reference Reposi-
The average daily consumption of diet was 28 ml/day/100 ethanol diets were essentially those described by Lieber and DeCarli (21). Ethanol-consuming animals were allowed access to control liquid diet No. 711 (Bioserv, Frenchtown, NJ) and the second group was divided into two groups. One group remained on the control diet, and the second group were sacrificed at 9 a.m. by CO2, and the liver and cheek pouches were removed. The squamous epithelial tissue of the cheek pouch was isolated (3), and microsomal and cytosolic fractions were prepared by differential centrifugation as previously described (20). Microsomes were resuspended in 0.5 ml 50 mM Tris, pH 7.4. Liver microsomes and cytosol were prepared in the same manner. All samples were frozen at −70°C in several aliquots until assayed. Microsomal and cytosolic protein concentrations were determined by the method of Lowry et al. (23).

**BaP Metabolism.** For determination of BaP metabolism by hamster cheek pouch microsomes, [3H]BaP (1 μM, 60–87 Ci/mmol) 2 mM NADPH, and 8 mM MgCl2 were incubated with 0.3–0.5 mg microsomal protein in 0.5 ml 50 mM Tris (pH 7.4) for 30 min. Metabolites were analyzed by the dual label HPLC assay described previously (20). In this assay, the reaction mixture was quenched with ethanol containing [4C]BaP metabolites, as markers and internal standards. Samples were then extracted 3 times with ethyl acetate containing 0.1 mM vitamin E. The organic layer was dried, filtered, and evaporated under N2. The residue was dissolved in hexane:benzene (1:1, v/v) and applied to a silica column. Unreacted [3H]BaP was eluted with hexane:benzene, and [3H]-BaP metabolites were eluted with methanol containing vitamin E (0.1 mM). This fraction was evaporated to dryness under a stream of N2 and redissolved in 100 μl of methanol. BaP metabolites were separated by HPLC on a Hilar Lichrosorb RP 18 (10 μm) column (American Scientific Products, Edison, NJ), which was eluted with a methanol:H2O gradient from 80–80% methanol in 40 min, 1.5 ml/min. The effluent was monitored at 254 nm, and 1.5-min fractions were collected. The fractions collected were mixed with 5.5 ml scintillation fluid (Monofluor; National Diagnostics, Somerville, NJ) and counted using a Beckman LS-6800 liquid scintillation system.

Liver microsomes (0.7–0.8 mg protein) were incubated with [14C]BaP (80 μM, 3.3 Ci/mmol), 2 mM NADPH, and 8 mM MgCl2 in 2 ml 50 mM Tris (pH 7.4) for 4 min. BaP metabolites were extracted and analyzed by HPLC as described above, except that silica gel column chromatography was not used.

**Glutathione Levels.** The total glutathione, oxidized and reduced, of liver cytosol was determined by the method of Teitz (24). Cytosol was prepared for analysis as follows: 50 μl 50% trichloroacetic acid were added to a 0.5-ml aliquot of cytosol; the protein precipitate was removed by centrifugation; and the supernatant was extracted with ether until the pH was greater than 2.5. The glutathione assay mixture contained: 0.1 unit glutathione reductase; 0.25 μmol NADPH; 0.1 μmol DTNB; 1 μmol EDTA; and 0–100 μl sample in 1.0 ml 0.1 mM sodium phosphate (pH 7.5). The change in absorbance at 412 nm was monitored, and the glutathione content was calculated by comparison to a standard curve.

**GST Activity.** GST activity with CDNB as a substrate was measured according to the method of Habig (25).

The glutathione conjugate of BPDE was measured as its FDNB derivative (9). BPDE was stored in 5% triethylamine in dry tetrahydrofuran until immediately prior to use in this assay. The required aliquot of BPDE was evaporated under N2 and redissolved in 10 μl dimethyl sulfoxide less than 1 min before use. Cytosol, containing 0.2–0.4 mg protein, was incubated at 37°C with 0.1 μmol BPDE and 0.2 μmol glutathione in a final volume of 0.2 ml 50 mM Tris (pH 7.4). The incubation was terminated after 2 min by the addition of 20 μl 50% trichloroacetic acid. Protein was removed by centrifugation, and the supernatant was neutralized with excess NaHCO3. The sample was derivatized with iodoacetic acid and FDNB according to the method of Reed (26). Iodoacetic acid (0.8 μmol in 10 μl H2O) was added to 90 μl sample. After 1 h, 0.1 ml 0.1 mM FDNB in ethanol was added. After a minimum of 4 h, the sample was analyzed by HPLC. Typically, 50 μl sample were injected on a Waters μBondapac:NH2 carbohydrate analysis column and eluted with an ammonium acetate:acetic acid:methanol gradient (26). The composition of Solvent A was methanol:H2O (4:1) (v/v). Solvent B was prepared as follows: 154 g ammonium acetate, 122 ml H2O, and 378 ml acetic acid were mixed; and 200 ml resulting solution were added to 800 ml Solvent A. The gradient used was a 30-min linear gradient from 100% Solvent A to 85% Solvent A, 15% Solvent B, followed by a 10-min isocratic period, and then a 10-min linear gradient to 10% Solvent A, 90% Solvent B. The flow rate was 1 ml/min, and the effluent was monitored at 365 nm.

The quantitation of the BPDE:glutathione conjugate required the enzymatic synthesis of a tritium-labeled conjugate for use as a standard. BPDE (0.25 μmol) was incubated with 0.5 μmol [3H]glutathione (2 μCi/mmol) and 0.7 mg purified GST (Sigma; 65 units/mg activity with CDNB) in 0.8 ml 50 mM Tris (pH 7.4). The incubation was terminated by the addition of trichloroacetic acid, and the [3H] BPDE:glutathione conjugate formed was derivatized and separated by HPLC as described above. The conjugate peak was collected, the amount of radioactivity present was measured, and the concentration of product was calculated. The UV absorbance spectrum of this conjugate was characteristic of the BPDE:glutathione conjugate as described by Cooper (15). This material was used to construct a standard curve of peak area versus conjugate concentration.

**Statistical Analysis.** The statistical significance of the data was determined by use of the t-test. Error bars in Charts 2 and 3 equal 95% confidence intervals.

**RESULTS**

**Metabolism of BaP.** The effect of chronic ethanol consumption on the metabolism of BaP by both hepatic and cheek pouch microsomes of the Syrian hamster was studied. The major metabolites of BaP formed by microsomes obtained from control hamsters and from hamsters which had been pair fed a 36% isocaloric ethanol-containing diet for 4 wk were compared. A dual label HPLC assay developed in this laboratory (20) was used to quantitate each metabolite produced by cheek pouch epithelial microsomes. A typical HPLC trace of ethyl acetate-extracted 3 times with ethyl acetate containing 0.1 mM vitamin E. The conjugate peak was collected, the amount of radioactivity present was measured, and the concentration of product was calculated. The UV absorbance spectrum of this conjugate was characteristic of the BPDE:glutathione conjugate as described by Cooper (15). This material was used to construct a standard curve of peak area versus conjugate concentration.

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extractable BaP metabolites produced by microsomes from the cheek pouch of a control hamster is illustrated in Chart 1. Most of the unreacted \([3H]\)BaP, which accounted for greater than 99% of the tritium in the quenched reaction mixture, was removed prior to HPLC analysis. The major metabolite peaks formed coeluted with BaP-9,10-diol, BaP-4,5-diol, BaP-7,8-diol, 9-OH-BaP, and 3-OH-BaP. The two small peaks eluting between 36 and 42 min coeluted with the 1,6- and 3,6-quinones of BaP. Since these peaks were quite variable and were partially present in control reactions using heat-treated enzyme, they were not quantitated.

The concentration of BaP chosen to assay cheek pouch epithelium microsomal metabolism was based on kinetic studies by Mass and Kaufman with hamster tracheal microsomes (27), in which they measured the \(K_m\) to be 1.4 \(\mu M\). In preliminary experiments with cheek pouch microsomes, we found no increase in metabolism when the BaP concentration was increased from 1–2 \(\mu M\); therefore, all incubations were with 1 \(\mu M\) BaP for 30 min. This time period was chosen since, in an earlier study (20), it was shown that all five metabolites were detectable, and their production was approximately linear for this time. The results of these studies with cheek pouch epithelial microsomes are presented in Chart 2. The totals of the five BaP metabolites measured were 6.6 ± 5.3 (SD) pmol/mg protein for control animals and 11.9 ± 8.4 (SD) pmol/mg protein for animals on the ethanol diet. This observed difference was not statistically significant (\(P < 0.05\)), but the amounts of certain individual metabolites were significantly elevated in the ethanol-treated animals. Of particular interest was the more than 2-fold increase in production of the proximate carcinogen of BaP, BaP-7,8-diol. BaP-9,10-diol was also significantly increased.

Chart 2. BaP metabolites produced by cheek pouch microsomes of ethanol-consuming (\(\bullet\)) \((n = 15)\) and control (\(\square\)) \((n = 13)\) hamsters measured by HPLC analysis. * significant differences \((P < 0.05)\). The assay was performed as described in "Materials and Methods." Bars, 95% confidence limits.

<table>
<thead>
<tr>
<th>BaP metabolites (pmol/min/g tissue)</th>
<th>Cheek pouch</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ((n = 13))</td>
<td>Ethanol ((n = 15))</td>
<td>Control ((n = 4))</td>
</tr>
<tr>
<td>9-OH-BaP</td>
<td>0.28 ± 0.13</td>
<td>0.61 ± 0.26</td>
</tr>
<tr>
<td>BaP-4,5-diol</td>
<td>0.18 ± 0.12</td>
<td>0.26 ± 0.21</td>
</tr>
<tr>
<td>BaP-7,8-diol</td>
<td>0.40 ± 0.19</td>
<td>1.12 ± 0.34</td>
</tr>
<tr>
<td>9-OH-BaP</td>
<td>0.27 ± 0.14</td>
<td>0.51 ± 0.22</td>
</tr>
<tr>
<td>3-OH-BaP</td>
<td>0.44 ± 0.48</td>
<td>0.95 ± 0.77</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± SD.  
\(^{b}\) Significantly \((P < 0.05)\) different from control.

Chronic ethanol consumption is known to increase hepatic microsomal protein (5). The same effect was observed in cheek pouch epithelial tissue. The average microsomal protein per g tissue was 9.3 ± 1.9 (SD) in control animals and 11.4 ± 0.7 (SD) in ethanol-consuming animals. When the formation of BaP metabolites is expressed as pmol metabolite per g cheek pouch epithelial tissue, the ethanol effect on metabolism of BaP is even more pronounced (Table 1).

The opposite effect was observed in liver microsomes from the same two groups of animals (Chart 3). These incubations were carried out with 60 \(\mu M\) BaP for 4 min, conditions demonstrated by Prough (28) to result in the linear production of BaP-7,8-diol. All BaP metabolites measured, except BaP-9,10-diol, were decreased in the microsomes from the chronic ethanol-consuming hamsters compared to controls. The decreases ranged from 40% for BaP-4,5-diol to 52% for BaP-7,8-diol. In this experiment, the microsomal protein was increased an average of 30% per g liver. When the effect of ethanol on hepatic microsomal metabolism of BaP is expressed as product formed per g tissue, there was still a slight decrease in all metabolites measured, but only 9-OH-BaP and 3-OH-BaP were significantly decreased \((P < 0.05)\) (Table 1).

**Hepatic Glutathione Content.** The total hepatic cytosol glutathione content was measured after hamsters had been maintained on ethanol-containing liquid diets or control liquid diets for various lengths of time. As can be seen by the data in Chart 4, there was no difference in hepatic glutathione content in animals...
maintained on an ethanol diet as compared to control animals after 3 or 7 days. After 14 days of ethanol consumption, hepatic glutathione content was decreased by 23% and after 28 days by 33%.

**GST Activity.** GST activity was measured using either CDNB or BPDE as the substrate. The CDNB:glutathione conjugate was measured spectrophotometrically and quantitated using its known extinction coefficient (25). A typical HPLC trace of a liver cytosol BPDE:GST assay mixture is illustrated in Chart 5. The peak eluting at 34 min coeluted with standard conjugate and increased with protein concentration and time. The peak in this particular trace contained 1.4 nmol conjugate. A heat-treated enzyme control contained a small peak at 34 min. The area of this small peak corresponded to 27 pmol nonenzymatically formed conjugate and was subtracted when calculating enzymatic conjugate formation. The other peaks present in this trace were present in the control reaction.

The GST activity measured in hamster liver cytosol and in cheek pouch epithelial cytosol obtained from either control or ethanol-consuming hamsters is presented in Table 2. All enzyme assays were conducted using conditions under which conjugate production was linear with time and substrate concentration. There was no significant difference (P < 0.05) in the activity of GST, in either tissue type or with either substrate, between hamsters which were consuming ethanol for 4 wk and pair-fed controls. It is interesting to note that hepatic GST activity was approximately 5 times greater than the activity in cheek pouch with CDNB as the substrate (P < 0.01), but when BPDE was the substrate, the activity was only slightly higher in the cytosol of liver compared to cheek pouch.

**DISCUSSION**

Microsomes obtained from the cheek pouch of hamsters which were consuming an ethanol-containing diet for 4 wk metabolized BaP to BaP-7,8-diol and BaP-9,10-diol to a greater extent than did microsomes from pair-fed controls. It is interesting to note that hepatic GST activity was approximately 5 times greater than the activity in cheek pouch with CDNB as the substrate (P < 0.01), but when BPDE was the substrate, the activity was only slightly higher in the cytosol of liver compared to cheek pouch.

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control diet</th>
<th>Ethanol-containing diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDNB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheek pouch</td>
<td>1000 ± 50(3)</td>
<td>1020 ± 215 (3)</td>
</tr>
<tr>
<td>Liver</td>
<td>5330 ± 840 (4)</td>
<td>4720 ± 630 (3)</td>
</tr>
<tr>
<td>BPDE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheek pouch</td>
<td>7.5 ± 1.3 (4)</td>
<td>8.2 ± 1.9 (4)</td>
</tr>
<tr>
<td>Liver</td>
<td>12.8 ± 4.9 (6)</td>
<td>14.0 ± 4.8 (5)</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Numbers in parentheses, number of hamsters.
some of ethanol-consuming animals suggests the possibility of increased BPDE production. If the oral tissues of ethanol-consuming animals have an increased ability to metabolize BaP to BPDE in vivo, this might result in an increase in carcinogenicity upon exposure to BaP. Bioassays for BaP carcinogenicity in ethanol-consuming and control hamsters have not been reported.

Hepatic microsomes obtained from chronic ethanol-consuming hamsters metabolize BaP to BaP-7,8-diol and BaP-9,10-diol to a lesser extent than microsomes from pair-fed control animals, when the results are expressed per g microsomal protein. When the results are expressed as product per g tissue, this decrease is not statistically significant. This suggests that the decrease in metabolism per g microsomal protein is the result of an increase in total microsomal protein, not necessarily a decrease in enzyme activity. In contrast, chronic ethanol consumption has apparently increased the levels of specific microsomal enzyme(s) responsible for BaP metabolism in the cheek pouch.

It has been demonstrated in previous studies that the effects of ethanol consumption on BaP metabolism are dependent on tissue (30). In the intestine, ethanol increased the BaP hydroxylase activity of male rats, but no change was observed in the liver or lung. These studies only measured BaP hydroxylase activity and not the ability of these microsomes to produce other BaP metabolites.

The hepatic glutathione content of hamsters was depressed after 2 wk and 4 wk of chronic ethanol exposure. The glutathione-depleting effect induced in the liver by an acute dose of ethanol has been reproduced by several investigators. However, varying results have been reported in studies (10, 12, 16) on the effects of chronic ethanol consumption on glutathione content, depending on the length and time of ethanol exposure. The animals in our study were allowed access to diet from 5 p.m. to 9 a.m. and sacrificed at 9 a.m. Therefore, the exposure to ethanol may have been immediately prior to sacrifice. The depleting effect observed is not likely to be only an acute effect, since no change was observed until after 2 wk of chronic ethanol consumption. However, chronic ethanol consumption may be potentiating an acute effect, increasing the susceptibility of the liver to ethanol’s acute glutathione-depleting effect. The combination of acute and chronic ethanol exposure and the normal diurnal variations in glutathione complicate the interpretation of ethanol’s effect on glutathione levels. Further studies are required to elucidate the mechanism by which ethanol consumption can affect hepatic and cheek pouch glutathione levels.

Chronic ethanol consumption for 4 wk by Syrian golden hamsters did not induce hepatic or cheek pouch epithelial GST activity with either CDNB or BPDE as substrate. A small 23% increase in GST activity with CDNB as substrate was demonstrated in mice administered an ethanol-containing diet for 8 days (19). However, no induction of GST with the epoxide substrate, ENPP, was measured after 14 days of ethanol consumption (18). In rats, an increase in GST activity towards both CDNB (20%) and ENPP (28%) was measured after 3 wk of chronic ethanol consumption (16). These variable results indicate that the extent of inducibility of GST is dependent on the species as well as the substrate being assayed.

Based on the results reported here, several speculative hypotheses are possible with respect to ethanol’s potential as a cocarcinogen in the hamster cheek pouch model. The increased metabolism by cheek pouch epithelial microsomes of BaP to BaP-7,8-diol may result in an increase in exposure to BPDE of this tissue in vivo. This could, in turn, increase DNA binding and carcinogenicity of BaP in the cheek pouch of ethanol-consuming animals. At the same time, an ethanol-induced decrease in hepatic BaP metabolism might result in increased exposure of extrahepatic tissues to this tobacco smoke carcinogen. Also, a decrease in hepatic glutathione with no change in GST activity might result in a decrease in BaP detoxification and possibly increased extrahepatic exposure. All of these possibilities require further study, particularly in vivo studies.

In a previous study, we demonstrated that human oral tissue microsomes can metabolize BaP (20). Hepatic glutathione levels of alcoholics are depressed (13, 14). In addition, rats exposed nose only to cigarette smoke 10–20 min/day for 17 wk showed a small but significant decrease in hepatic glutathione content (31). An increase in BaP metabolism by oral tissues and a decrease in its detoxification by glutathione after chronic ethanol consumption might be contributing factors in the increased risk of oral cavity cancer among alcoholics who smoke.

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