Enhanced Metabolism and Mutagenesis of Nitrosopyrrolidine in Liver Fractions Isolated from Chronic Ethanol-consuming Hamsters

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ABSTRACT

The effect of chronic ethanol consumption on the ability of isolated liver fractions to metabolize the carcinogen N-nitrosopyrrolidine (NPY) was examined. Microsomal fractions of treated animals exhibited increased rates of \( \alpha \)-hydroxylation of NPY. Similar increases in the specific activities of aniline hydroxylase, reduced nicotinamide adenine dinucleotide phosphate cytochrome \( \epsilon \) reductase, and the specific content of cytochrome P-450 were also observed. In contrast, no differences in the specific activities of benzo(\( \alpha \))pyrene hydroxylase or glucose-6-phosphatase were observed. Liver postmitochondrial supernatants from ethanol-consuming animals were able to produce 5 times more mutants than did control preparations. It is concluded that \( \alpha \)-hydroxylation of NPY is probably the mechanism by which NPY is converted to a mutagen and that this pathway can be induced by ethanol.

INTRODUCTION

Chronic alcohol and tobacco consumption in combination have long been recognized as the 2 major risk factors for cancer of the oral cavity, esophagus, larynx, hypopharynx, and vocal cords in man (36-38). The data obtained in the United States for cancer at these sites indicate that neither alcohol nor tobacco consumption, individually, significantly increases risk. In addition, an association of chronic alcohol consumption with cancer of the liver has been observed (15-18, 30, 34).

In view of the extensive literature available on the effect of chronic ethanol consumption on the oxidative capacity of isolated liver microsomes, it was important to determine if the metabolic activation of a potential environmental carcinogen could be enhanced in microsomal fractions isolated from ethanol-consuming animals. In order to undertake studies of the metabolic consequences of both alcohol and tobacco consumption, a suitable animal model is a necessary prerequisite. The Syrian hamster appears to be ideally suited for these types of studies: (a) because of a low incidence of URT infections as well as low rates of spontaneous tumor formation in the URT, the Syrian golden hamster was selected as a test animal for chronic tobacco inhalation studies (12, 27, 28); (b) the hamster is quite susceptible to cyclic nitrosamine carcinogenesis and develops URT tumors when treated with NNN, a tobacco-specific carcinogen (13); (c) the hamster readily consumes ethanol when it is administered either as part of a Lieber-DeCarli liquid diet (25) or as an unsweetened ethanol-water mixture in preference to drinking water (3).

There were several reasons that led to the selection of NPY for these investigations. NPY has a widespread occurrence in the environment with particularly high concentrations being found in both mainstream and sidestream cigarette smoke (5). NPY can be considered a model compound for the tobacco-specific carcinogen NNN, since the latter is a 3-pyridyl analog of NPY. The mechanisms by which NPY and NNN are metabolized have been studied in detail, and the data suggest that hydroxylation of the \( \alpha \) position of the pyrroline ring is the initial step in the formation of active metabolites of both compounds (4, 6, 7, 11). NPY has been shown to be mutagenic in 2 separate microbial bioassay systems following metabolic activation (9, 10). Finally, p. o. administration of NPY to rats results in the formation of hepatocellular carcinomas (20, 32).

The recent development of a reproducible assay for the \( \alpha \)-hydroxylation of NPY has made it possible to examine the effects of environmental modifiers on the metabolism of this cyclic nitrosamine (8). It is the purpose of this communication to document the effect of chronic ethanol consumption on the \( \alpha \)-hydroxylation and mutagenicity of NPY.

MATERIALS AND METHODS

Chemicals. Cytochrome \( \epsilon \) (type III), NADPH (type X), N-tris(hydroxymethyl)methylglycine, Tris, glucose 6-phosphate monosodium salt, and glucose-6-phosphate dehydrogenase (type XII) were obtained from Sigma Chemical Co., St. Louis, Mo. NPY was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; 2,4-dinitrophenylhydrazine was obtained from Eastman Kodak Co., Rochester, N. Y.; and NNN was synthesized as described previously (14). Sucrose, special enzyme grade, was from Schwarz/Mann, Orangeburg, N. Y.

Animals. Male Syrian golden hamsters were obtained from ARS/Sprague-Dawley, Madison, Wis. Animals were housed 3/cage and allowed free access to water. At 8 weeks of age, hamsters were placed on control Lieber-DeCarli Liquid Diet 711 plus added fiber (Bio-Serv, Inc., Frenchtown, N. J.). At 9 weeks of age, animals were either continued on control diet or placed on the ethanol-containing diet, where ethanol isocalorically replaced carbohydrate. Thirty-five % of the total caloric intake was ethanol. Diet intake was restricted to approximately 40 ml/animal. Diet intake per cage was monitored daily, and the amount received by control animals was adjusted to match the intake of ethanol-consuming animals. Animals were sacrificed by CO\(_2\) anesthesia after 4 weeks of ethanol consum-
tion. All animals were housed in the Research Animal Facility of the Naylor Dana Institute.

Subcellular Fractionation. Homogenization of livers was performed as previously described using a Polytron homogenizer (Wilhems type; Kinematica, Lucerne, Switzerland) (24). The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant from this step was then centrifuged at 105,000 × g for 60 min and decanted. The surface of the pellet was carefully rinsed with 0.15 M KCl, and microsomes were resuspended in a final volume of 1 to 2 ml.

Biochemical Analysis. Microsomal fractions were assayed as follows: aniline hydroxylation as described by Mazel (22); NADPH cytochrome c reductase by the method of Phillips and Langdon (31); benzo(a)pyrene hydroxylation activity as described by Nebert and Gelboin (29); glucose-6-phosphatase as described by Aronson and Touster (2); and cytochrome P-450 according to the method of McLean and Day, using an extinction coefficient of 91 mm⁻¹ for Amax=490 (26). Substrate-induced spectral changes were analyzed according to the method of Remmer et al. (33). Protein was determined by the method of Lowry et al. (21).

The standard assay conditions for determination of the α-hydroxylation of NPY consisted of 40 μmol NPY; 0.6 μmol NADPH; 10 μmol glucose-6-phosphate; 10 units glucose-6-phosphate dehydrogenase; 1 to 3 mg microsomal protein; and 70 μmol Tris-HCl, pH 7.4, in a final volume of 2.0 ml. Reactions were initiated by the addition of microsomes. Following incubation for 60 min at 37°, reactions were quenched by the addition of 2.0 ml ice-cold ethanol.

After removal of protein by centrifugation, 3 ml of the supernatant were treated with 0.2 ml of 0.15 M KCl, 0.5 ml of 70% methanol. A 0.04 ml aliquot of this sample was analyzed by high-pressure liquid chromatography. A 50-min gradient from 30% methanol:70% water to 100% methanol was used. Gradient elution followed a concave curve (Curve 7 on the Waters programmer). The flow rate was 2 ml/min. The formation of the dinitrophenylhydrazone of the major reaction product, 4-hydroxybutyraldehyde, was determined by comparison of peak heights with those obtained with authenticated standards (8).

Spectral interaction of aniline, NPY, and NNN with isolated hamster liver microsomes Microsomal protein concentration was 1.3 mg/ml. Spectra were run in the presence of 2 mm substrate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption</th>
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<tbody>
<tr>
<td></td>
<td>Max.</td>
</tr>
<tr>
<td>Aniline</td>
<td>425</td>
</tr>
<tr>
<td>NNN</td>
<td>425</td>
</tr>
<tr>
<td>NPY</td>
<td>425</td>
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</tbody>
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Effect of 4-week ethanol consumption on isolated hamster liver microsomes

<table>
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<tr>
<th>Cytochrome P450-associated activities</th>
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<tbody>
<tr>
<td>Control</td>
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<td>----------</td>
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<tr>
<td>Microsomal protein (mg protein/g liver)</td>
</tr>
<tr>
<td>Cytochrome P450 content (nmol/mg protein)</td>
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<tr>
<td>Aniline hydroxylase (nmol/min/mg protein)</td>
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<tr>
<td>NADPH cytochrome c reductase (μmol/min/mg protein)</td>
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<tr>
<td>Benzo(a)pyrene hydroxylation (nmol/min/mg protein)</td>
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<tr>
<td>NPY α-hydroxylase (nmol/min/mg protein)</td>
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<tr>
<td>Glucose-6-phosphatase (μmol/min/mg protein)</td>
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a Means ± S.D. of 6 separate preparations.
increases in cytochrome P-450 content and aniline hydroxylase activity can be seen. NADPH-cytochrome c reductase is increased to a lesser degree. The increase in NPY \( \alpha \)-hydroxylation activity is of a magnitude similar to that observed for aniline hydroxylase. In contrast, the activities of glucose-6-phosphatase and benzo(\( \alpha \))pyrene hydroxylase are not affected by chronic ethanol consumption.

The effect of chronic ethanol administration on the ability of postmitochondrial supernatants to convert NPY to a mutagen is presented in Chart 1. The dose-response curve appears hyperbolic when either control or ethanol postmitochondrial supernatants are used for metabolic activation. Increasing the amount of NPY to 100 \( \mu \)mol did not result in any further increase in the number of mutants (data not shown). The postmitochondrial supernatants from ethanol-consuming animals were able to induce mutation with equivalent concentrations of NPY 4 to 5 times better than those from control preparations.

**DISCUSSION**

The association of chronic alcohol usage as a risk factor for cancers of the head and neck area (36–38) as well as for cancer of the liver (15–18, 34) is one of the strongest epidemiological leads for the etiology of cancer in man, and it is surprising, therefore, that little effort has been made to reproduce these observations in an animal model where the effect of alcohol exposure can be studied so as to acquire information on the underlying mechanisms. Our detailed working hypothesis for the etiology of alcohol-related cancers has been presented elsewhere (23). Briefly stated, we have suggested that alcohol could increase the risk of cancer by increasing the rate of metabolic activation of environmental carcinogens. The data presented in this report represent our initial attempt to test this hypothesis. These data demonstrate that, in the Syrian golden hamster, chronic ethanol consumption results in increased rates of \( \alpha \)-hydroxylation of NPY by isolated liver microsomes as well as enhanced capacity of postmitochondrial supernatants to convert NPY to a mutagen; in addition, these data suggest one mechanism by which chronic ethanol consumption could result in increased risk for cancer.

The facts that the spectral interaction of NPY is similar to that observed for aniline and that the in vitro rates of hydroxylation of both compounds are similarly increased as a result of chronic ethanol consumption suggest that the metabolism of both compounds may involve similar initial pathways.

The parallel increases in the rates of \( \alpha \)-hydroxylation and mutagenicity of NPY produced by liver fractions from ethanol-consuming hamsters provide further evidence that \( \alpha \)-hydroxylation may be an activation step for this cyclic nitrosamine. Whether or not these observed in vitro increases in \( \alpha \)-hydroxylation and mutagen formation by isolated hamster liver fractions indicate that ethanol consumption can lead to increased tumorigenicity of cyclic nitrosamines must await the outcome of full carcinogenicity studies with hamsters which are currently in progress. The extent to which these ethanol-associated alterations in liver microsomal metabolism occur in the microsomal fractions of target tissues is also under investigation.

**REFERENCES**


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**Metabolism and Mutagenesis of NPY**
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