Metabolic Activation of Arylhydroxamic Acids by N-O-Acyltransferase of Rat Mammary Gland

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ABSTRACT

The lactating mammary glands of rats contain an arylhydroxamic acid N,O-acyltransferase that catalyzes the formation of an arylamine-substituted nucleic acid on incubation with N-hydroxy-N-2-acetylaminofluorene or N-hydroxy-N-4-acetylamino-nobiphenyl and transfer RNA. The acyltransferase activity migrates as a single component with a molecular weight of 28,000 on gel filtration on Sephadex G-100.

Acyltransferase activities of the lactating mammary glands of Sprague-Dawley animals are approximately twice those of the less susceptible Fischer strain as determined by assay with either hydroxamic acid. The fluorene substrate was 15 times as efficient as the biphenyl compound in promoting adduct formation.

Ribosomal RNA adducts formed in vivo after administration of N-hydroxy-N-2-acetylaminofluorene were consistent with an acyltransferase mechanism of activation in that the adducts did not retain the acetyl group.

INTRODUCTION

Tumors of the mammary gland can readily be induced on administration of carcinogenic aromatic amines to female Sprague-Dawley rats (5). Systemic exposure to amines, amides, hydroxamic acids, and other derivatives are effective in producing mammary tumors. Single applications of the compound directly to the mammary gland of 6- to 8-week-old Sprague-Dawley females has shown that the hydroxamic acid, N-hydroxy-AAF,3 is a more active local carcinogen than are the amide, hydroxylamine, or nitroso derivatives (18).

Theoretical considerations and experimental evidence support the proposition that chemicals induce tumors as a result of the modification of DNA. Tumors arising from exposure to aromatic amines are believed to result from the formation of metabolites that are capable of reaction with DNA and RNA, since the parent compounds do not have the capacity to react with nucleic acids (5). The reactivity of activated metabolites may also be regarded as a form of instability, since reaction may occur extracellularly or with cellular molecules not involved in the carcinogenic process. This inherent instability limits the distance through which the activated metabolites may exert their effects, and, as a consequence, makes it likely that the final metabolic activation step occurs in the tissue in which tumors are formed. Accordingly, studies directed towards clarification of the role of metabolic activation in carcinogenesis require examination of both the potential and actual metabolic characteristics of the target tissue.

Metabolic activation of arylhydroxamic acids has been demonstrated to occur as a result of conjugation with sulfate (6, 14) or of N,O-acyltransfer (3, 11, 14). Studies in 2 laboratories have failed to detect enzymes capable of conjugation of N-hydroxy-AAF with sulfate in preparations from mammary glands of rats (2, 8). Activation of N-hydroxy-AABP, but not N-hydroxy-AAF, has been reported in experiments with extracts of rat mammary glands (2).

In this paper, we present data demonstrating that (a) the lactating mammary gland of the rat contains an acyltransferase that activates both of these arylhydroxamic acids; (b) the level of this enzyme is higher in Sprague-Dawley animals than in the less susceptible Fischer strain; and (c) RNA adducts formed in vivo after administration of N-hydroxy-AAF are consistent with an acyltransferase mechanism of activation.

MATERIALS AND METHODS

Chemicals. N-Hydroxy-[14C,3H]AAF and N-hydroxy-N-[carbonyl-14C]acetyl-4-aminofluorene (N-2-fluorenylacetylnobiphenyl) were synthesized, and the radiochemical purity was established as described previously (4).

Animals. Female rats (Sprague-Dawley, ARS/Sprague-Dawley, Madison, Wis.; Fischer 344, Charles River Breeding Laboratories, Wilmington, Mass.) made pregnant as a result of the caging of 3 females with one male were transferred to individual stainless steel cages prior to delivery of their litters. They were maintained with their litters on dust-free softwood shavings. Temperature was held at 24°; food (Purina laboratory Chow; Ralston Purina Co., St. Louis, Mo.) and distilled water were available continually. Litter size was reduced to 8 at the time of delivery.

Tissue Preparations. Mammary glands were obtained from animals 7 to 10 days postpartum. The inguinal glands were removed, rinsed with pyrophosphate-NaCl buffer (pH 7.0, 0.05 M) that contained 1 mm dithiothreitol, weighed, minced, and homogenized with 4 volumes of buffer in a Teflon-glass homogenizer at ice-bath temperatures. Cytosols were prepared by centrifugation at 105,000 x g (average) for 1 hr at 4°.

Assay for Acyltransferase. Acyltransferase activity was estimated by determining the amount of arylamine-substituted nucleic acids formed on incubation of radiochemically labeled N-hydroxy-AAF or N-hydroxy-AABP with tRNA and enzyme...
preparations as described previously (11). Average values of duplicate assays are given.

**Gel Filtration.** Chromatography of cytosols prepared from homogenates of mammary glands with 1.5 volumes of buffer was carried out as described previously using pyrophosphate-NaCl buffer (pH 7.0, 20 mM) that contained 1 mM diethiothreitol (20); flow rates of 60 to 70 mL/hr were maintained with a roller pump. In order to minimize enzyme losses through oxidation, the buffer reservoir and fraction collector were maintained under an argon atmosphere. Columns and the effluent fractions were kept at 4°C. Effluents were monitored for absorption at 280 nm by use of a flow monitor (Instrument Specialty Co., Lincoln, Nebr.) and for acyltransferase activity as described above.

**rRNA Adduct Formation in Vivo.** N-Hydroxy-[14C, 3H]AAF (2.1 mCi 14C per mmol; 10.7 mCi 3H per mmol) was administered i.p. as an ethanol solution (10 mg N-hydroxy-AAF per ml 95% ethanol per kg body weight) to lactating Sprague-Dawley rats 7 to 10 days postpartum. The inguinal mammary glands were removed 3 hr later and used to prepare rRNA by repeated phenol extraction and precipitation of the rRNA with potassium acetate according to the method of Irving and Veazey (9). These rRNA preparations were comparable to those obtained from liver, gastrointestinal tissues, and kidney as judged by UV characteristics (A258 nm; A260 nm/A280 nm = 2.2 to 2.3; and A170 nm/A200 nm = 2.1 to 2.2) and their sedimentation in 5 to 20% sucrose gradients. For assay of 3H and 14C content, the rRNA was precipitated from 0.01 M sodium chloride:0.0015 M sodium citrate (pH 7.0) solutions by addition of 2 volumes of 95% ethanol that contained 2% potassium acetate. The precipitate was washed with 95% ethanol, and the rRNA was then solubilized (NCS; Amersham Corp., Arlington Heights, Ill.) and counted in a liquid scintillation counter (Isocap-300; Searle Analytic, Des Plaines, III.) using an automatic external standardization technique to determine counting efficiencies (15).

**RESULTS**

Incubation of N-hydroxy-AAF or N-hydroxy-AABP with tRNA and cytosols from mammary glands of lactating Sprague-Dawley rats resulted in the incorporation of the labeled rings into the nucleic acid (Table 1). Little of the acetyl group was associated with the RNA; similar small quantities of ring and acetyl groups were associated with the nucleic acid (Table 1). Little of the acetyl group was associated with the RNA; similar small quantities of ring and acetyl groups were associated with the nucleic acid (Table 1). The ability of cytosols of mammary glands from lactating Sprague-Dawley rats to catalyze the reaction of N-hydroxy-(14C,3H)AAF (19.1 mCi 14C per mmol; 6.25 mCi 3H per mmol) and N-hydroxy-N-(carbonyl-14C)acetyl-4-amino(3H)biphenyl into tRNA on incubation with cytosols from mammary glands of Sprague-Dawley rats is given as the nmol of N-hydroxy-(14C,3H)AAF bound to tRNA per ml of effluent in the 20-mm acyltransferase assay. The relative molar quantities of the ring and acetyl moieties were calculated from the isotopic contents of the nucleic acid adducts and the specific activities of the substrates. Experiments 1 and 2 were carried out on different days with cytosols from 2 different animals.

**Table 1**

<table>
<thead>
<tr>
<th>Experi-&lt;br/&gt;ment</th>
<th>Cytosol incu-&lt;br/&gt;bated</th>
<th>Substrate</th>
<th>Isotope bound to tRNA (dpm)</th>
<th>Ring/ace-&lt;br/&gt;tyl bound to tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 N-hydroxy-AAF</td>
<td>492 a</td>
<td>106 b</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.4 N-hydroxy-AAF</td>
<td>485 a</td>
<td>1850 b</td>
<td>11.8</td>
</tr>
<tr>
<td>2</td>
<td>0.2 N-hydroxy-AAF</td>
<td>593 a</td>
<td>3606 b</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>0.4 N-hydroxy-AAF</td>
<td>69 a</td>
<td>16 a</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>0.2 N-hydroxy-AABP</td>
<td>743 b</td>
<td>59 a</td>
<td>5.0</td>
</tr>
</tbody>
</table>
|                 | 0.4 N-hydroxy-AABP    | 2130 b    | 32 b                        | 26.0                        | ① Acetyl label.<br/> ② Ring label.

*Acetyl label.<br/> Ring label.*

![Chart 1](https://example.com/chart1.png)

**Chart 1.** Gel filtration profile of acyltransferase activity of cytosols from rat mammary gland. Cytosols from 40% homogenates of mammary glands of lactating Sprague-Dawley (15 ml) or Fischer rats (20 ml) were chromatographed on columns (5 x 88 cm) of Sephadex G-100 as described in "Materials and Methods." Recovery of acyltransferase ranged from 90 to 100%; the activity is given as the nmol of N-hydroxy-AAF bound to tRNA per ml of effluent in the 20-min acyltransferase assay.
Covered from these animals disclosed that less than 10% of cinogenesis of the mammary gland of female Sprague-Dawley rats was, however, 15 times greater than with use of the biphenyl derivative. The mammary gland acyltransferase of the Sprague-Dawley strain of rat possesses an arylhydroxamic acid N,O acyltransferase that is capable of activating both fluorene and biphenyl substrates. Unlike the small intestinal enzyme of the Sprague-Dawley rat, which has 2 species of acyltransferase (20), the mammary gland has only one acyltransferase as determined by gel filtration chromatography, and this enzyme is comparable in size to the liver acyltransferase (11) with a molecular weight of 28,000.

Cytosols from mammary glands of Sprague-Dawley rats have approximately twice the acyltransferase activity of comparable cytosols from Fischer animals. Similar strain differences have been noted in assays of cytosols of lung and gastrointestinal tissues from 1-month-old animals (13). While the induction of extrahepatic tumors by aromatic amines in tissues of Fischer animals was relatively less common than in Sprague-Dawley rats (18, 19), there was little difference between the susceptibility of the mammary gland of these 2 strains to the directly acting carcinogen N-nitrosomethylurea (7). It is not known, however, whether a 2-fold difference in the level of an enzyme capable of metabolic activation could account for these differences in sensitivity. Previous reports have not detected mammary gland acyltransferase that was capable of utilizing N-hydroxy-AAF as a substrate (2). The techniques used in the present study differ from the previous experiments in that dithiothreitol and argon have been used to protect the sulfhydryl groups of the enzyme (11), and tRNA, rather than methionine, has been used as the trapping agent of the reactive metabolite.

Several lines of evidence support the conclusion that acyltransferase may be involved in the induction of mammary tumors by aromatic amines. The observation that aminofluorene substitution of nucleic acids occurs in mammary gland is consistent with the activation of N-hydroxy-AAF by acetyltransfer in this tissue. No other enzyme capable of generating nucleic acid adducts has been demonstrated in rat mammary gland. Assays in vitro show conclusively that the tissue possesses the enzyme. Carcinogenicity tests have previously demonstrated that the substrate for acyltransferase, N-hydroxy-AAF, induced tumors when applied directly to the mammary gland of Sprague-Dawley rats, whereas the amide, acetylaminofluorene, and the hydrolysis product of N-hydroxy-AAF, the hydroxylamine, did not induce tumors when administered by this technique (18). This technique has also shown that Sprague-Dawley animals are more susceptible to N-hydroxy-AAF than are Fischer animals, which is in agreement with the difference in acyltransferase levels in these 2 strains (18, 19).

Perhaps the strongest evidence implicating acyltransferase in mammary tumor induction comes from a recent study in which the N-formyl, N-acetyl, and N-propionyl derivatives of N-2-fluorenylhydroxylamine were injected directly into the mammary glands of Sprague-Dawley rats (1). The number of tumors that formed at the site of injection paralleled the ability of partially purified rat liver acyltransferase to catalyze the formation of nucleic acid adducts (12); the N-acetyl derivative was more carcinogenic than either of the other 2 hydroxamic acids.

These data support the concept that the induction of mammary gland tumors by aromatic amines involves the metabolic activation of arylhydroxamic acids by N,O acyltransfer. Further detailed experiments will be required to establish whether this hypothesis is in fact true for mammary gland and whether this metabolic activation mechanism is of importance in other tissues that have this enzymatic capability.

### Table 2

**Arylhydroxamic acid acyltransferase of the mammary gland of lactating rats**

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>N-hydroxy-AAF</th>
<th>N-hydroxy-AABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley</td>
<td>100 ± 20*</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>Fischer</td>
<td>45 ± 20 (p &gt; 0.05)</td>
<td>2.9 ± 0.7 (p &gt; 0.05)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

### Table 3

**Incorporation of N-hydroxy-AAF into rRNA in mammary glands of lactating Sprague-Dawley rats**

<table>
<thead>
<tr>
<th>Sample size (A260 units)</th>
<th>Isotope content (dpm)</th>
<th>N-hydroxy-AAF moiety bound to rRNA (pmol/100 A2600 unit)</th>
<th>% of acetyl group retained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ring-14C Acetyl-13H</td>
<td>rRNA</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>150</td>
<td>182</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>340</td>
<td>956</td>
<td>117</td>
</tr>
<tr>
<td>3</td>
<td>143</td>
<td>672</td>
<td>0</td>
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</tbody>
</table>
REFERENCES


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