ABSTRACT

The activities of enzymes which synthesize and metabolize catecholestrogens were studied in biopsy samples of human breast neoplasms. Estrogen 2-hydroxylase, a cytochrome P-450-dependent enzyme, was present in both benign and malignant neoplasms but not in normal breast tissue. Catechol O-methyltransferase activity was present in all samples examined and was significantly higher in malignant tumors [549 ± 31 (S.E.) pmol/20 min/mg protein] than in benign neoplasms (226 ± 41 pmol/20 min/mg protein) or in normal breast tissue (133 ± 28 pmol/20 min/mg protein). There was no correlation, however, between estrogen 2-hydroxylase and catechol O-methyltransferase activities. The enzymes responsible for the synthesis and metabolism of catecholestrogens are present in some breast tumor specimens, suggesting that in such tissues these metabolites may be formed in vivo.

INTRODUCTION

The formation of catecholestrogens via 2-hydroxylation of estradiol and estrone is now considered a major pathway of estrogen metabolism in both laboratory animals and humans (7). The enzyme catalyzing this reaction, estrogen 2-hydroxylase, is a cytochrome P-450-dependent monooxygenase localized primarily in liver (11) but also found in varying amounts in brain (18, 20), kidney, testes, adrenal, lung, pituitary, heart, and placenta (4, 10). The catecholestrogens are further metabolized to their O-methylated derivatives by COMT (9), a ubiquitous soluble enzyme.

Although the exact physiological significance of the catecholestrogens is unknown, earlier work demonstrating their marked affinity for estrogen receptors of brain (6), uterus (13), and liver (22), coupled with recent work from our laboratory demonstrating a similar affinity for estrogen receptors of the MCF-7 human breast cancer cell line, suggests that these metabolites may be biologically active. In contrast, their O-methylated derivatives have no affinity for estrogen receptors and appear to lack biological activity (14). While uterotrophic activity has been observed with chronic administration of 2-hydroxyestradiol (14), increased uterine weight cannot be induced by 2-hydroxyestrone, the principal circulating catecholestrogen, unless very high concentrations are achieved (23). In other systems (8, 19), catecholestrogens have been shown to inhibit the effects of estrogens. These results suggest that the catecholestrogens may act as weak estrogens or as endogenous antiestrogens and thereby influence the growth of estrogen-sensitive neoplasms.

Since a high percentage of human breast tumors are estrogen sensitive (15) and because both estrogen receptors (15) and estrogen synthesis (21) have been reported in such tumors, we have studied the major enzymes involved in the formation and metabolism of the catecholestrogens in human breast tumors. We now report that estrogen 2-hydroxylase is present in human breast neoplasms and confirm a previous report (1) that COMT activity is higher in malignant tumors than in benign tumors or normal breast tissue.

MATERIALS AND METHODS

Chemicals

17β-Estradiol, NADPH, S-adenosyl-L-methionine iodide, 2-hydroxyestrone, 2-methoxyestrone, and 2-methoxyestradiol were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol was obtained from Bethesda Research Laboratories, Inc., Rockville, Md. 2-Hydroxyestradiol was a gift of Schering AG, Berlin, Germany. L-Ascorbic acid and n-heptane were from Fisher Scientific Co., Fair Lawn, N. J. [methyl-3H]-S-Adenosylmethionine (specific activity, 11.2 Ci/mmol) was purchased from New England Nuclear, Cambridge, Mass. Tris base was purchased from Schwarz/Mann, Orangeburg, N. Y. SKF-525A was a gift of Dr. S. Nelson, NIH, Bethesda, Md.

Preparation of COMT

COMT was purified from rat liver according to the procedure of Axelrod and Tomchick (3) as modified by Nikodejvic et al. (17). The enzyme preparation was stored at −20⁰ with no loss of activity for at least 1 year.

Breast Tissue

Samples of malignant and benign breast tumors and normal breast tissue were obtained from the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. Tumor samples weighing between 0.4 and 20 g were obtained from the National Cancer Institute through a cooperative program of numerous hospital centers throughout the United States. Following biopsy, tumor specimens were rapidly frozen on dry ice and stored in vacuum-sealed containers at −70⁰. All samples were assayed within 3 months of collection. Histological diagnoses as well as demographic data on all patients were provided by the attending pathologists at the participating hospitals. All tumor specimens used in this study were from women, ages 24 to 78. Specimens
diagnosed as malignant were carcinomas of the breast, while benign tumors included fibroadenoma, fibrocystic disease, and hyperplasia. Tissues diagnosed as histologically normal breast were obtained from women who had no evidence of malignant tumor in that breast. The MCF-7 human breast cancer cells were kindly provided by Dr. Marc Lippman of the National Cancer Institute.

Preparation of Tumors Prior to Assay

All tumor specimens were thawed, trimmed of adipose tissue, and weighed. The tissue was homogenized in 3 to 4 volumes of ice-cold isotonic sucrose, and the microsomal and soluble fractions were prepared by differential centrifugation at 105,000 × g for 60 min. The microsomal pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4). The COMT activity was stable for at least 6 months, but the microsomal estrogen 2-hydroxylase activity declined with repeated freezing and thawing. However, the estrogen 2-hydroxylase activity remained stable for at least 4 months in the frozen breast samples. Therefore, estrogen 2-hydroxylase activity was measured in tissue samples thawed only once.

MCF-7 cells were washed 3 times with ice-cold 10 mM sodium phosphate (pH 7.4) in 0.9% NaCl solution. Two ml of 10 mM Tris buffer (pH 7.4) were then added to the culture flask, and the cells were gently scraped from the bottom of the flask and disrupted with a Brinkmann Polytron for 30 sec. The membranes and nuclear debris were separated by differential centrifugation at 20,000 × g for 20 min, and the supernatant fraction was then used for measuring COMT activity.

Enzyme Assays

COMT. COMT was assayed by a modification of the method of Axelrod et al. (2). Incubations were performed in 15-ml, glass-stoppered centrifuge tubes. The incubation reaction consisted of the following: 20 μl of 1 M Tris-HCl buffer (pH 7.4); 10 μl of 1 M MgCl2; 5 μl of S-[3H]adenosylmethionine; 10 μl of 0.1 mM S-adenosylmethionine iodide; 100 μl of 2 mM dithiothreitol; substrate consisting of 20 nmol of 2-hydroxyestradiol or 2-hydroxyestradiol in 5 μl of absolute ethanol; and 40 to 400 μg of supernatant protein in a total volume of 200 μl. Reactions were started by the addition of substrate, followed by placing the tubes in a shaking water bath at 37°. Blanks consisted of tubes lacking substrate or containing heat-treated enzyme; these blanks were not significantly different from one another. The reactions were stopped after 20 min by the addition of 0.5 ml of 0.5 M borate buffer (pH 10). The radiolabeled O-methylated catecholestrogens were extracted into 6 ml of n-heptane as previously described (18). The organic phase was separated by centrifugation, and 2 ml were placed in scintillation vials, evaporated to dryness in a chromatography oven (80°), and counted for radioactivity directly in Aquasol (New England Nuclear).

Estrogen 2-Hydroxylase. This enzyme was assayed by the method of Paul et al. (18), in which the hydroxylation of estradiol is coupled with rapid O-methylation by partially purified COMT, which is added in excess amount. This assay is exceedingly sensitive and can detect as little as 150 fmol of product. The specificity of this method has been established by thin-layer chromatography (18), recrystallization to constant specific activity (4), and direct-probe mass spectrometry (18).

In the present study, the incubation mixture consisted of: 50 μl of 10 M Tris buffer (pH 7.4); 10 μl of 1 M MgCl2; 5 μl of S-[3H]adenosylmethionine; 50 μg of partially purified COMT; 5 μl of 10 mM L-ascorbic acid; substrate consisting of 50 nmol of 17β-estradiol in 2 μl of absolute ethanol; 0.06 μmol of NADPH; and 40 to 350 μg of microsomal protein in a total volume of 150 μl. Mixtures lacking substrate or containing heat-treated enzyme served as blanks. There was no significant difference between the 2 types of blanks. The reaction was stopped after 10 min, and the products were extracted as described previously. Proteins were measured by the method of Lowry et al. (12).

Product identification was confirmed in this study by subjecting the remaining organic phase containing the reaction product (approximately 4 ml reduced by evaporation under N2) to thin-layer chromatography in 2 different solvent systems. Authentic 2-methoxyestrone and 2-methoxyestradiol were added as nonradioactive carriers. TLC was performed on silica gel 125-mm 60 F-254-precoated plates (Merck, Darmstadt, Germany). The solvent systems used were: (a) chloroform:acetone (96:3:1); and (b) benzene:ethanol (9:1). Following development and drying of the plates, 1-cm sections were scraped individually into 15-ml conical glass-stoppered centrifuge tubes, and the radiolabeled products were extracted into 3 ml of methanol. One-ml aliquots were then transferred to scintillation counting vials containing 10 ml of Aquasol.

RESULTS

Estrogen 2-hydroxylase activity could be demonstrated in some, but not all, samples of neoplastic breast tissue, both malignant and benign (Table 1). No detectable activity was found in normal breast tissue. Product identification was confirmed by demonstrating that greater than 80% of the reaction products comigrated with authentic 2-methoxyestradiol or 2-methoxyestrone in 2 different solvent systems. Furthermore, the enzymatic reaction was inhibited by approximately 50% by 1 mM SKF-525A [2078 ± 22 (S.D.) cpm versus 1165 ± 36 cpm; n = 3; p < 0.01 by Student’s t test], suggesting that this enzyme may also be a P-450-dependent monooxygenase similar to that found in liver. The estrogen 2-hydroxylase activity was higher in the malignant than in the benign neoplasms, but the difference was not significant because of the wide range of enzyme activity in the malignant tissue.

In contrast to estrogen 2-hydroxylase activity, COMT activity

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of samples</th>
<th>No. of samples with detectable activity</th>
<th>Estrone 2-hydroxylase activity (pmol 2-methoxyestradiol formed/10 min/mg microsomal protein)</th>
<th>Range of activity (pmol 2-methoxyestradiol formed/10 min/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12</td>
<td>0</td>
<td>1.7 ± 0.4a</td>
<td>0.5–5.2</td>
</tr>
<tr>
<td>Benign tumor</td>
<td>22</td>
<td>7</td>
<td>1.19 ± 5.5b</td>
<td>0.5–57.7</td>
</tr>
<tr>
<td>Malignant tumor</td>
<td>21</td>
<td>10</td>
<td>11.9 ± 5.5b</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.

b Not significantly different from the activity in benign tumors.
was found in all breast samples. COMT activity in benign tumors was not significantly greater than in normal tissue, but it was several times higher in malignant tumors (Table 2). Cells from the MCF-7 human breast tumor line were found to have extremely high COMT activity, comparable to that observed in many malignant specimens (1304 pmol of product formed per 20 min per mg protein). There was no apparent correlation (r = 0.01) between estrogen 2-hydroxylase and COMT levels in neoplastic tissues. Although the samples were not matched for age, no correlation between age and the activity of either enzyme was noted.

DISCUSSION

These experiments show that human mammary neoplasms can enzymatically synthesize and metabolize catecholestrogens. While estrogen 2-hydroxylase activity is present in some neoplastic tissue, there appears to be no significant difference in activity between benign and malignant tumors. In addition, no correlation between catecholestrogen-synthesizing and -metabolizing activities could be found. Although the catecholestrogen-synthesizing capacity of most tumors studied is low, it is possible that the estrogen 2-hydroxylase activity in these breast tumors is being underestimated, since its activity may decline during the varying interval between the biopsy and the freezing of the specimen. Nevertheless, our results support an earlier report of the presence of 2-methoxysterone following the incubation of breast tumor tissue with estrogen (5). Taken together with the relatively high concentration of estrogen reported in some malignant tumors (16), it is possible that hydroxylation takes place in vivo.

Our findings also confirm previous work (1) that malignant tissue has greatly increased COMT activity, and we now show that high activity is maintained in the MCF-7 cell line in long-term culture. Lymph nodes invaded by breast cancer metastases have also been shown to have very high COMT activity (1). The COMT assay described in this paper, using a catecholestrogen as substrate, is a simple, rapid, and highly reproducible method that takes advantage of the facts that catecholestrogens have an extremely low Km for COMT (9) and that their O-methylated products can be selectively extracted into nonpolar solvents.

In our study, there was considerable overlap in COMT activity among normal tissue, benign neoplasms, and malignant tumors. We did not, however, divide the malignant tumors on the basis of histological grade as was done by Assicot et al. (1). These investigators found that the most differentiated malignant tumors had COMT activities within the normal range, while the more poorly differentiated tumors had markedly increased enzyme activities. The wide range in enzyme activities might be a result of variations in cell number between the normal and the neoplastic tissues. No differences between protein contents on the basis of wet weight were noted. Cell population differences could be normalized by expressing enzyme activity as a function of DNA content.

As major metabolites of estradiol that also bind to estrogen receptors, the catecholestrogens may influence the growth of hormone-sensitive neoplasms. The 2-hydroxyestrogens may act as weak estrogens that are still capable of stimulating growth in estrogen-sensitive tissues. However, catecholestrogens can antagonize the action of estrogens in several systems (8, 19), and thus may act as endogenous antiestrogens. The dramatically elevated COMT activity seen in some malignant tumors may reflect a compensatory detoxifying mechanism whereby the estrogenic properties of the catecholestrogens are abolished by methoxylation. On the other hand, the increased COMT activity may enhance tumor growth by depleting the pool of impeding estrogens. Studies to elucidate the effect of the catecholestrogens on breast tumor growth using the MCF-7 tumor line are in progress.

REFERENCES


Catecholestrogen Synthesis and Metabolism by Human Breast Tumors \textit{in Vitro}

Andrew R. Hoffman, Steven M. Paul and Julius Axelrod


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