Uterine peroxidase as a marker for estrogen action
(steroid action/antiestrogen/enzyme induction)

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ABSTRACT Administration of a single dose of estradiol to immature rats gives rise to the appearance of substantial amounts of peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) enzyme activity in the uterus. This enzyme induction, which is inhibited by administration of actinomycin D and cycloheximide, can be detected at 4 hr after administration of estradiol, reaches a maximum level by 20 hr, and thereafter declines. The amount of uterine peroxidase seen at 20 hr after a single dose increases with dose from 0.1 to 100 μg of estradiol. Estrone and estriol also show dose-dependent induction of peroxidase, and the quantitative peroxidase responses to these steroids follow their uterotropic capacities. The antiestrogen C1628, capable of low levels of enzyme induction by itself, can inhibit the induction due to estrogen.

Solubilization of the uterine enzyme with divalent cations, especially calcium, results in a substantially increased yield of peroxidase. This extraction method provides an enzyme of about 50,000 molecular weight in distinction to the large aggregated form obtained by the usual extraction with sodium chloride.

The significant progress of the last 15 years toward a better understanding of the role of steroid hormones in controlling target cell function has been possible to a large extent by the elucidation of the steroid–receptor interaction pathway (1–3), coupled with investigations of biochemical parameters whose expression is regulated by these steroids (3–5). Studies on the mechanism of estrogen action have used two main model systems. The atrophic rodent uterus, demonstrating dramatic growth response to estrogen, has been widely studied regarding the estrogen–receptor interaction pathway as correlated with tissue growth and control of RNA synthesis (4, 6, 7). The chick oviduct, after primary steroid stimulation to produce a differentiated, functional organ, responds to estrogen by producing large quantities of the specific protein, ovalbumin (3, 5). There has been considerable increase in our knowledge of the nature of the hormone regulation of specific protein synthesis resulting from studies with the oviduct model in recent years (8–10).

However, in the uterine model no analogous specific gene product, characteristically a marker for estrogen action, has been available to allow comparable, detailed studies in this more complex, mammalian steroid target organ. There have been numerous reports of uterine proteins, in particular enzymes, whose concentration are increased after estrogen administration. However, even with those showing significant increases in proportion to the uterus after estrogen stimulation, the extent of the steroid induction is only 5–10-fold (11). Considerable interest has been shown in IP or “induced-protein,” an acidic uterine protein first described by Notides and Gorski (12). Recent studies have shown that this protein, inductive in otrò (13), is an early response, detectible by 15 min, and declining in amount after 2 hr (14). While such a time course of induction might be consistent with a role of “induced protein” as an early key intermediate protein (15) in estrogen action, it would obviously put expression of this protein prior to the observed increases in macromolecular synthesis involved in the actual growth response of the uterus.

Increases in uterine peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) content after estrogen administration were reported over 20 years ago by Stotz and coworkers (16) but attributed to an influx of eosinophils into the tissue (17). More recent evidence, both biochemical (18, 19) and histochemical (20–22), clearly indicates a uterine origin for the enzyme. Although the capability of uterine peroxidase and H2O2 to participate in the metabolism of [14C]estradiol has been the basis for one of the frequently used assays of uterine peroxidase (23), it has not been established that the enzyme actually participates in the uterine metabolism of estradiol in vivo. Histochemical studies suggested that the estrogen-induced peroxidase accumulation occurred in only those tissues, such as uterus, vagina, and carcinoen-induced mammary tumor (24), whose growth is effected by estrogen and not other estrogen target tissues, like the pituitary and hypothalamus, where the response is primarily functional. We suggested therefore that peroxidase induction may be a useful marker for estrogen-effectuated growth responses (22).

This paper reports the development of a modified extraction procedure using calcium chloride which more effectively and completely solubilizes uterine peroxidase and shows that the sensitivity of the guaiacol assay of the enzyme enables quantitation of the peroxidase activity of individual uteri of immature rats. The quantitative data presented, both regarding the time course of estradiol induction of uterine peroxidase and the sensitivity of uterine peroxidase to modulation by various estrogens and antiestrogen, confirm that this enzyme is a useful gene product for assessing effects of hormones on uterine growth.

MATERIALS AND METHODS

Rats and Injection. Female Sprague–Dawley rats were obtained from ABR/Sprague–Dawley, Madison WI at 20 days of age, allowed free access to food and water and, as immature rats, used by age 23 days. Castrate rats were of the same strain, subjected to bilateral ovariecotomy under ether anesthesia when 23–35 days old and used 1–3 weeks after surgery. Estradiol-17β, estrone, and estriol (Sigma Chemical Co.) and the antiestrogen C1628, 1:2-(pα(p-methoxyphenyl)-β-nitrostyrlyl(phenoxy)ethyl) pyrroldidine, mononocrate, obtained by the courtesy of J. Reel, Parke–Davis Co., were administered subcutaneously in saline, ethanol solution, or in sesame oil. Actinomycin D and cycloheximide were administered by intraperitoneal injection.

Extraction of Peroxidase Activity. At the indicated times the rats (four to eight per group) were sacrificed by decapitation. The entire uterus from oviduct to cervix was removed,
quickly dissected free of fat and connective tissue, nicked, blotted on hard filter paper to excise luminal fluid, and weighed. Each uterus was minced with a scissors into a 12-ml polycarbonate tube and homogenized, 10 sec with ice cooling in 10 mM Tris-HCl, pH 7.2, buffer (25 mg of tissue per ml unless otherwise stated), with a polytron PT 10ST homogenizer at setting 6. After removal of aliquots for DNA analysis (25), the homogenates were centrifuged for 45 min at 39,000 × g at 2°C. The hypothic supernatant fraction contains no peroxidase activity. The peroxidase activity was solubilized by rehomogenizing the 39,000 × g sediment with 10 mM Tris-HCl, pH 7.2, buffer containing 0.6 or 1.2 M NaCl, or more effectively with 0.5 M CaCl₂. The extract was collected by centrifugation for 45 min at 39,000 × g at 2°C.

Peroxidase Assay. Assays were performed by modification of the method of Himmelhoch et al. (26); the rate of oxidation of guaiacol was measured at 25°C. The assay mixture, 3.0 ml total volume, contained 13 mM guaiacol and 0.3 mM H₂O₂ in the extraction buffer. The reaction was started by the addition of enzyme (extract), and initial rates of guaiacol oxidation were determined from the increase in absorbance at 470 nm for the first minute. An enzyme unit was defined as the amount of enzyme required to produce an increase of 1 absorbance unit/min under the assay conditions.

RESULTS

Extraction of Uterine Peroxidase. Since rat uterine peroxidase activity is associated with the endoplasmic reticulum (21) and vesicles (19) and is not found in particulate-free cytosols of hypothic homogenates, some extraction or solubilization method is necessary. The usual method, based on the work of Klabanoff (17), effected solubilization by extraction with NaCl. The resultant enzyme shows a very large molecular size, as indicated in Fig. 1, being excluded from Sephadex G-200 along with the major part of the proteins of the NaCl extract. Furthermore, since the enzyme is also excluded from Sepharose 4B, the approximate size of several million daltons would suggest an appreciably larger enzyme than that has been reported for other mammalian peroxidase enzymes (for example, 150,000 molecular weight for myeloperoxidase (25) and 300,000 for thyroid peroxidase (27)). Investigating alternative solubilization conditions, we discovered that calcium chloride was a definitely superior extractant (Table 1). The total amount of enzyme obtained by calcium chloride extraction of an aliquot of pooled rat uterus was considerably larger than that extracted with sodium chloride. Moreover, further extraction of the 1.2 M NaCl pellets with either 0.5 M CaCl₂ or 1.2 M NaCl did not provide any significant additional amounts of peroxidase. It therefore appeared that extraction with calcium chloride provided a more active enzyme preparation. Gel filtration of the CaCl₂ extract on Sephadex G-200 (Fig. 2) clearly indicated a different molecular size for the peroxidase activity. Now the enzyme was separated from the majority of the 280 nm absorbing materials and eluted as a somewhat broad peak of activity in the 50,000 molecular weight region. Peroxidase solubilization with calcium chloride shows a distinct concentration dependence (Fig. 3). Little enzyme activity is solubilized with 0.2 M CaCl₂ while essentially all the peroxidase appears in the 0.3 M extract. Such a sharp extraction profile may suggest that a specific solubilization is occurring.

Peroxidase Induction with Estradiol. The uterus of the immature or castrate rat has little detectable peroxidase activity. After a single dose of 10 μg of estradiol was given (Fig. 4), peroxidase was first detected at 4 hr, continued to increase in amount until about 20 hr, and declined thereafter. A similar time course is seen in both the castrate and immature rat and after 1 μg of estradiol is given, as well as after the 10-μg dose shown in Fig. 4. The appreciable standard deviation seen near the peak induction times are believed to result from animal variability in the time at which the peak of activity occurs.

Table 1. Comparison of solubilization of uterine peroxidase activity with NaCl and CaCl₂

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Peroxidase activity*</th>
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<tbody>
<tr>
<td>0.6 M NaCl</td>
<td>1.4</td>
</tr>
<tr>
<td>1.2 M NaCl</td>
<td>1.1</td>
</tr>
<tr>
<td>0.5 M CaCl₂</td>
<td>5.3</td>
</tr>
<tr>
<td>2.5 M CaCl₂</td>
<td>5.9</td>
</tr>
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* Peroxidase activity is given as enzyme units per ml of extract of aliquots of a minced pool of uterus of rats 20 hr after administration of 5 μg of estradiol. Extracts of the 39,000 × g sediment corresponded to 50 mg of uterus per ml.

![Fig. 1. Gel chromatography of uterine peroxidase in 0.6 M NaCl extract.](image1)

![Fig. 2. Gel chromatography of uterine peroxidase in 0.5 M CaCl₂ extract.](image2)
assay replication errors are small compared to the differences seen between individual animals.

The estradiol induction of uterine peroxidase appears to require both RNA and protein synthesis (Table 2). Cycloheximide given 2 hr after 1 μg of estradiol prevents the induction of enzyme otherwise seen 20 hr after estrogen is given. Actinomycin D given the same time as or either 2 or 4 hr after estradiol also prevented the appearance of peroxidase at 20 hr. When this inhibitor of RNA synthesis was given 6 hr after estradiol, the increase in peroxidase activity seen in the uterus 20 hr after estradiol would suggest that RNA produced during the first 6 hr gives rise to some subsequent enzymatically assayable peroxidase.

The amount of uterine peroxidase present 20 hr after estradiol administration is clearly dose dependent (Fig. 5). Although the control animals had detectable activity, 0.1 μg of estradiol effected a significant increase in uterine peroxidase activity. Interestingly, continued increases in peroxidase were seen with 1, 10, and 100 μg of estradiol. However, it would appear that this wide range dose response may be due to the fact that only a single dose of estradiol was used. Indeed, on extended daily injections, saturation of the response of uterine peroxidase occurs at lower doses.

**Peroxidase and Other Estrogens and Antiestrogen.** Dose-dependent responses of uterine peroxidase are seen after injection of estrone and estriol also (Fig. 6). Consistent with the comparable effects of estrone and estradiol on uterine growth and their relative affinities for the cytosol estrogen receptor, about 10 times as much estrone is required to obtain a comparable peroxidase induction seen with estradiol. Estriol is

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**Table 2.** Actinomycin D and cycloheximide inhibition of estradiol induction of uterine peroxidase activity

<table>
<thead>
<tr>
<th>Treatment, hr*</th>
<th>Peroxidase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act. D</td>
<td>Cyclo.</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
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* Time at which inhibitors [150 μg of actinomycin (Act. D) or 250 μg of cycloheximide (Cyclo.), intraperitoneally] were given is hours after administration of 1 μg of estradiol; uteri were assayed 20 hr after estradiol injection.
† Peroxidase activity of calcium chloride extract (see Materials and Methods) is given as the mean enzyme units per mg of DNA ± SEM.
somewhat less dose-effective, especially at the higher dose levels. It is noteworthy that appreciable uterine peroxidase is induced by each of these estrogens.

Antiestrogens, like the Parke-Davis compound CI628 used here, frequently act as weak estrogens themselves. As shown in Table 3, both 50 and 500 \( \mu \)g of CI628 gave rise to small amounts of uterine peroxidase, each less than that seen after administration of 0.1 \( \mu \)g of estradiol. However, the simultaneous administration of CI628 with either 1 or 10 \( \mu \)g of estradiol effected an appreciable reduction in the peroxidase levels compared to animals given the respective doses of estradiol alone.

**DISCUSSION**

A number of desirable qualities can be described for an optimal, or at least useful, biologic marker for estrogen action in the uterus. In the first place, sensitive methods should be available for its detection and quantitation so that meaningful changes in marker levels in an individual uterus or even in individual uterine cells should be possible. Second, the marker should clearly be a uterine product that is absent, or alternatively present only in insignificant amounts, in the atrophic tissue. Administration of estrogen should effect induction of the marker in a dose-dependent manner. The half-life of the marker should be sufficient so that its degradation does not complicate measurement of the induction. Third, modulation of marker expression should follow closely the regulation of uterine growth by various estrogens, steroids, and antagonists. It would furthermore be advantageous if the marker were a well characterized product, or if not, that the marker were present in sufficient amounts in the target organ to allow purification of the marker and isolation and quantitation of its mRNA.

To an appreciable extent peroxidase activity would appear to be an appropriate marker for estrogen action in the uterus. As presented in this paper, using the guaiacol assay the enzyme is readily quantitated in individual uteri with a sensitivity that allows its detection as early as 4 hr after estrogen administration.

**Table 3. Antiestrogen blockade of estrogen induction of uterine peroxidase activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \mu )g estradiol</th>
<th>( \mu )g CI628</th>
<th>Peroxidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>—</td>
<td>—</td>
<td>2.38 ± 1.04</td>
</tr>
<tr>
<td>1.0</td>
<td>—</td>
<td>—</td>
<td>29.0 ± 9.6</td>
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<tr>
<td>10.0</td>
<td>—</td>
<td>—</td>
<td>42.1 ± 9.2</td>
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<tr>
<td>—</td>
<td>50</td>
<td>—</td>
<td>0.38 ± 0.24</td>
</tr>
<tr>
<td>—</td>
<td>500</td>
<td>—</td>
<td>0.58 ± 0.29</td>
</tr>
<tr>
<td>1.0</td>
<td>500</td>
<td>—</td>
<td>6.03 ± 3.33</td>
</tr>
<tr>
<td>10.0</td>
<td>500</td>
<td>—</td>
<td>7.61 ± 3.84</td>
</tr>
</tbody>
</table>

*Peroxidase activity (mean enzyme units per g of tissue ± SEM) of five rat uteri per group 20 hr after subcutaneous administration of estradiol and/or CI628 in 0.2 ml of sesame oil, or of vehicle alone.

Previous histochemical studies detected uterine peroxidase 12 hr after 200 \( \mu \)g of estradiol was given (20), but using more physiologic dose levels no peroxidase stain was evident 6 hr after estradiol; weak activity was seen 24 hr (22) after a single dose of estrogens. This would suggest that the histochemical stain for peroxidase is probably less sensitive than the biochemical assay by guaiacol oxidation. Probably a more serious defect associated with histochemical detection of peroxidase is its essentially qualitative nature. Hence, previous studies detected CI628 induction of uterine peroxidase but could not differentiate peroxidase levels among animals given estrone alone, CI628 alone, or the two in competition. Our present results clearly indicate that although CI628 effects low levels of uterine peroxidase induction such levels are significantly less than those seen after estrogen, and the combination of estrogen and CI628 gives intermediate amounts. On the other hand, the capability of the histochemical stain to identify individual cell types containing peroxidase provides a valuable kind of information not readily available from guaiacol assay of uterine extracts. The assay measuring the conversion of \( [14C] \)estradiol to water soluble products, like the guaiacol assay, is sufficiently sensitive to detect increased peroxidase activity prior to 12 hr after estradiol administration (18, 23). The higher baseline seen with the assay for water-soluble products and the maximum conversion of about 60% of the estradiol tend to minimize the apparent extent of the stimulation of uterine peroxidase. The evidence that actinomycin D and cycloheximide prevent the steroid-induced appearance of peroxidase confirms evidence from previous histochemical (20) and biochemical (18) reports that macromolecular synthesis is required for uterine expression of the marker. More detailed studies will be needed to establish the timing of required RNA and protein synthesis.

All the available evidence concerning uterine peroxidase levels under various hormonal conditions is consistent with the proposed marker function. In addition to the reported absence in the uterus of the immature or ovariectomized host and its induction with estradiol, the variation with the estrus cycle (18, 20, 22) in rats as well as in human beings (28) has been documented. In this report we have shown that the relationship of efficacies of estradiol, estrone, and estril for induction of uterine peroxidase relates to their previously documented uterotrophic activities (29) and with each a dose dependency is observed. Furthermore the quantitative peroxidase results using the antiestrogen, CI628, indicate, as previously suggested, that, while weakly estrogenic itself (30), CI628 is inhibitory to estrogen action when given with estradiol (31, 32). Another antiestrogen, nafoxidine or U 11100A, has been recently reported by McNabb and Jellinck (33) to show a similar effect on uterine

**FIG. 6.** Comparison of estradiol (E2), estrone (E1), and estriol (E3) induction of uterine peroxidase activity in the immature rat. Procedure is as described for Fig. 5, 20 hr after administration of steroid or vehicle alone, but with results presented based on DNA assay of uterine homogenate (25). SEM as shown.
peroxidase activity as determined by the assay for water-soluble product. The nonsteroidal estrogen diethylstilbestrol is highly potent for uterine peroxidase induction, while substantial amounts of progesterone and testosterone are ineffective (22).

More detailed studies regarding the macromolecular nature of the hormonal regulation of the uterine peroxidase gene would be enhanced by isolation of purified uterine peroxidase and its mRNA. As suggested by the magnitude of the peroxidase induction, the estrogen-stimulated rat uterus is an appropriate source for purification of this enzyme marker.

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