Review of Studies on Estrogen Biosynthesis in the Human

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Abstract

While the gonads and placenta are usually thought of as the principal sites of estrogen synthesis, many other tissues are now known to contain the aromatase enzyme. Despite a wealth of information, the functional significance, if any, of this widespread distribution is not yet clear. Nonetheless, elevated estrogen production resulting from either an apparent increase in enzyme activity or increased substrate availability in blood can have important effects on target tissues such as the breast, uterus, and bone. Our early studies suggesting that aromatase inhibitors can effectively reduce the impact of peripheral estrogen synthesis have been amply confirmed by the elegant studies of Santen and others. Many previous studies have suggested that aromatase activity may be present in breast tumors. The profound implications for growth of estrogen-sensitive tumors led us to reevaluate this question. Using a sensitive modification of our original radiometric aromatase assay, we have found detectable activity in only about 40% of human breast tumors (n = 100). Only rarely (1/100) does the aromatase activity exceed 1.0 pm/hr/g tissue or 0.001% of that found in the human placenta. These quantitative results contradict several published reports which suggest that most breast tumors contain biologically significant levels of aromatase activity.

The mammalian aromatase (estrogen synthetase) enzyme catalyzes the formation of aromatic C_{18} estrogenic steroids from C_{19} androgens containing the Δ^+ 3-ketone grouping in Ring A of the steroid nucleus. This enzyme has received considerable attention because of the complexity of the chemical reaction (Chart 1) and also because of the central importance of estrogens in many reproductive and metabolic processes. The uniquely large capacity of the human placenta to synthesize estrogens provided not only convenient starting material (urine) for the isolation of steroid hormones in the early dawn of endocrinology but also a readily available tissue for biochemical studies of the aromatase enzyme. As became evident during these conference proceedings, interest in aromatase now extends far beyond human pregnancy to diverse areas such as its role in sexual behavior and its pharmacological inhibition in treatment of estrogen-dependent cancer. In view of the many comprehensive reviews that are available (7, 10, 40, 42), I will confine this article to a brief account of my own interests in aromatase and its products over the past 20 years.

Following completion of my Ph.D. work with Seymour Lieberman at Columbia University, I joined Paul MacDonald in the Department of Obstetrics and Gynecology at Southwestern Medical School in Dallas in 1962. We set up a laboratory and immediately began to work on the problem of estrogen production and metabolism in pregnant women. While still in New York, I had conceived a new approach to the difficult problem of isolating estrogens from the urine of pregnant women by gradient elution chromatography on Celite using ethylene glycol and mixtures of isooctane and ethyl acetate. This system, which was adapted to microcolumn purification of steroids prior to radioimmunoassay by Abraham et al. (1), made it possible for us to isolate estrone, estradiol, and estriol in pure form from 5 to 10 liters of urine in about 5 days. However, when we hit upon the idea that placental estrogens may be derived from circulating DS, it took only about 48 hr to obtain the answer from the urine of the first subject who had received 3H-labeled DS and 14C-labeled estradiol. When both 3H and 14C channels of the scintillation counter lit up, our shouts and whoops of excitement went unheard, as it was about 4 a.m. We quickly confirmed this discovery and published both the methodology and preliminary findings in December 1963 (36, 38). The unique experimental design, using 2 isotope-labeled tracers, which allows quantitation of conversion and production rates of precursor and product, proved to be a powerful tool that has solved many problems during the ensuing 18 years.

We continued our studies of pregnancy and established the following major points: (a) placent al conversion of maternal DS increases from about 1 to 2% at 6 to 7 weeks to 30 to 40% at term of normal gestation and accounts for about 50% of total estradiol production (38, 39); (b) estradiol is formed by 16α-hydroxylation of DS prior to aromatization, and only about 10% of the total is derived from maternal precursors (39); (c) estrogen production during anencephalic pregnancy is derived almost exclusively from maternal DS and is proportional to the level of adrenal secretory activity (24); (d) trophoblastic tissues in women pregnant with hydatidiform moles or in patients with choriocarcinoma retain the capacity to aromatize DS (20, 25); and (e) the conversion of DS to estrogens is negligible in nonpregnant individuals (25). This work firmly established the important principle of hormone production from inactive precursor molecules in or near target tissues which is now recognized in many other endocrine systems.

In about 1966, we began to study the human placental aromatase system in vitro. We were particularly interested in the kinetics of the overall aromatization process but were initially discouraged by the painfully tedious assays involving isolation of radiolabeled estrogenic products by chromatography. After reading several papers in which 1,2-3H-labeled androgens were used in metabolic studies and yields of estrogen had to be corrected for losses, it occurred to me that this loss could be used to advantage in assaying aromatase activity. Our first description of a tritium release (radiometric) assay was presented at the 1969 meeting of the Society for Gyn-
colonic Investigation, when we also reported the observation that aminoglutethimide is a potent aromatase inhibitor (6). We were not aware of an earlier report describing a similar tritium release assay published by Frieden et al. (12) in 1968, which I found only recently. Using the tritium release assay, 2 graduate students, Aubrey Thompson and Susan Bolton, began biochemical work on the aromatase enzyme with the goal of establishing the stoichiometry of the overall process.

Measurements of O_2 consumption during aromatization of androstenedione gave totally unexpected results. The initial rates of O_2 consumption were far in excess of the rate of estrogen appearance and were linear for only a short period of time (48–50). After eliminating several possible explanations such as the initiation of lipid peroxidation by O_2 radicals generated during the aromatization reaction, we tested the effects of various oxidase inhibitors and found that 1 to 10 mM KCN markedly reduced the rate of oxygen consumption but had little effect on the rate of estrogen formation. The rate of NADPH consumption was equal to the rate of O_2 utilization under all conditions. Under these conditions, the stoichiometry of the reaction agreed with the postulated mechanism (Chart 1). Others had shown earlier that 19-OHA and 19-OXOA were isolaible products of placental aromatase action on androstenedione and that they could also be converted to estrogens (28). By quantifying all these products, we were able to show that the total amount of oxygen consumed in the absence of KCN could be accounted for by production of 19-OHA (1 mol O_2), 19-OXOA (2 mol O_2), and estrogens (3 mol O_2). Kinetic studies by Wilcox and Engel (54) demonstrating that 19-OHA appeared before estrogens and then disappeared during aromatization of androstenedione suggested to them that 19-hydroxylation was the rate-limiting step. However, our studies indicated that this conclusion was untenable since large amounts of 19-OHA and 19-OXOA were formed with high concentrations of an aromatase nonenzymatically and that it could be trapped during incubation of labeled androstenedione with placental microsomes (43). Nonetheless, the potential importance of competition between cellular 5a-reductase and aromatase for Delta4, 3-keto substrates became obvious. Stimulation of 5a-reductase activity by luteinizing hormone in ovarian cells, for example, would very effectively reduce estrogen synthesis (44). In 1975, we also reported that Delta1-testolactone (Teslac) inhibited aromatization both in vitro and in vivo and suggested that the clinical effectiveness of this and perhaps other modified androgens in breast cancer may be due to inhibition of peripheral estrogen production rather than to direct androgenic effects (44). Subsequent studies demonstrated inhibition of estrogen production in breast cancer patients in vivo (5).

Following the demonstration that placental estrogen synthesis arises from circulating precursors, it was natural to ask whether similar processes are of physiological significance in nonpregnant individuals. Many studies over the past 15 years in women, men, and children (15, 22, 40, 45) have provided abundant affirmative evidence. Studies of estrogen production under various conditions in women revealed that increased estrone formation from circulating androstenedione occurs in conditions such as obesity, hyperandrogenism, and liver disease which are known to increase risk for endometrial cancer (40, 45). This association led to a flurry of reports indicating that use of exogenous estrogens increases the risk of endometrial cancer in postmenopausal women. However, when studies of postmenopausal patients and appropriately weight-matched controls were carried out, no differences in either the extent of conversion (21) or plasma estrogens (17) were found. Although these results have been interpreted by some to mean that endogenous estrogens play no role in the development of endometrial cancer, a more likely interpretation is that a certain level of estrogen production is necessary, but not sufficient, to cause cancer. The latter view is in accord with the generally held opinion that estrogens act as promoters rather than as carcinogens in development of cancer of their target organs.

Early studies suggested that the conversion of androstenedione to estrone was more efficient in cancer patients in vivo and also in adipose tissue studied in vitro (33). The latter finding has been confirmed recently in an extensive in vitro study (11). The authors offered no explanation for the apparent discrepancy with the earlier in vivo studies. More recent studies have suggested that aromatase resides primarily in stromal cells rather than in the adipocytes of adipose tissue (3) and further
that the enzyme activity appears to be inducible by glucocorticoids (46). Coupled with the observation that estrogens stimulate proliferation of adipocyte precursor cells in culture (32), these findings suggest that adrenal hormone secretion may regulate adipose mass through both the level of aromatase activity and substrate availability. The failure of weight loss to reduce the conversion of androstenedione to estrone (45) is consistent with the stromal cells being the principal site of the aromatase enzyme.

Aromatase Activity in Tumor Cells

Many studies have shown that human mammary tumors possess a variety of steroid-metabolizing enzymes including sulfokinase, sulfatase, 3β-hydroxysteroid dehydrogenase, 3-and 17β-dehydrogenases, and aromatase. Except for the latter, most activities are sufficiently high that their demonstration has been straightforward and unequivocal. Breast tumor aromatase activity, however, is extremely low in most specimens, and the assay methods have been used at the limits of detection to demonstrate activity (2, 4, 18, 27, 31). The extent of conversion of labeled androgens to estrone and/or estradiol during tissue incubation has commonly been observed to be about 0.01%. Even with the most rigorous purification, derivatization, and crystallization techniques, it is extremely difficult if not impossible to rule out the presence of one part of estrogen or very similar impurity in 10,000 parts of the added radiolabeled substrate. Indeed, few studies have demonstrated increasing product formation with time of incubation using the classical isotope methods.

We have chosen to use the [1β-3H]androstenedione radiometric assay for detecting aromatase activity in human breast and other tumors. We recognize that this alternative method can give misleading results if either 1- or 2-hydroxylations of substrate occur. However, if used properly, i.e., with complete removal of unstable tritium in position 2 by base-catalyzed enolization prior to assay followed by rigorous purification and removal of steroids from the incubation mixture, this assay can set an upper limit for aromatase activity. Of course, we make the reasonably sound assumption that the mechanism of aromatization in tumors involves loss of the 1β- and 2β-hydrogens as in the placenta. This is to be expected if the appearance of aromatase in tumors is an indication of dedifferentiation of normal cells so that they produce fetal or trophoblastic products such as human chorionic gonadotrophin. Furthermore, there is little evidence to indicate that normal breast epithelial cells contain aromatase activity. In a preliminary study (45), we found that about one-third of breast and colon tumors contained aromatase activity. More recently, we have assayed 100 consecutive breast tumor specimens that were prepared for estrogen receptor assay. In brief, 1 part tissue was homogenized in 10 parts of 10 mM Tris buffer containing 0.01 mM EDTA, 10% glycerol, and 10 mM dithiothreitol and centrifuged at 105,000 x g for 1 hr as described previously (16). The pellets were resuspended in the original volume of buffer and 1 ml aliquots incubated with [1β-3H]androstenedione (10-7 M) and a NADPH generating system as described (50). Control experiments with placental microsomes showed that the addition of glycerol or dithiothreitol had no effect on aromatase activity. Following incubation for 1 hr at 37°C, the tubes were extracted with 4 ml of chloroform to remove the bulk of radioactive steroids. Aliquots of the aqueous phase were passed through Pasteur pipets containing a layer of charcoal supported by a bed of Celite. The steroid-free aqueous phase was counted.

Double isotope (estrone + estradiol) 0.35
Estrone 3H:14C (C: 36, 4.9, 2.9, 2.3; X: 2.2, 1.6, 1.2, 0.9)

Aromatase Activity in Nonendocrine Tissues

Several recent studies have examined various tissues or cells for the presence of aromatase activity. We have recently reexamined the liver in this regard (41). These studies demonstrated that the aromatase activity in human fetal liver is similar to that found in the placenta (Table 1). The level of aromatase activity in adult human liver, however, is still not certain. In unpublished studies, we have failed to establish the presence of the aromatase enzyme in adult liver from humans.

Table 1

<table>
<thead>
<tr>
<th>Aromatization of androstenedione by human liver</th>
<th>nmol/hr/g</th>
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</thead>
<tbody>
<tr>
<td>Fetal 1β-3H assay</td>
<td>9.22</td>
</tr>
<tr>
<td>Double isotope (estrone + estradiol)</td>
<td>8.12</td>
</tr>
<tr>
<td>Estrone 3H:14C (C: 472, 437, 452, 426; X: 374, 381, 352, 393)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

1 C, isotope ratios following 4 steps of chromatographic purification; X, successive values following crystallization with carrier steroid.
or other species such as mouse, rat, guinea pig, or monkey. High yields are obtained with the radiometric assay, but we have been unable to confirm the formation of estrogens by product isolation (Table 1). At least 4 explanations of this discrepancy are possible. As suggested previously (37), the presence of much more active steroid-metabolizing enzymes (e.g., $5\alpha$- and $5\beta$-reductases) in adult as compared to fetal liver may limit access of the added substrate to the aromatase enzyme. Alternatively, estrogens formed by hepatic cells may be immediately hydroxylated to form catechol estrogens which are then further metabolized. This appears unlikely since we have not been successful in trapping 2-, 4-hydroxy- or 2-methoxyestrone or estradiol. It is possible that hepatic aromatase activity declines following birth. Total loss of activity seems unlikely, however, in view of the experiments of Smuk and Schwerts (48). Finally, the discrepancy between the radiometric and isolation assays may be due to the presence of $C_2$, 2-hydroxylase activity known to be present in adult liver as suggested by Osaka.\footnote{Y. Osawa, personal communication.} Introduction of the 2-hydroxyl group would labilize the 1/2 tritium of substrates used for radiometric assay of aromatase and yield indistinguishable kinetics. We are presently testing this hypothesis.

It is now evident that aromatase is not restricted to classical endocrine tissues such as the placenta, gonads, or adrenals. With the recognition that significant estrogen production continues in the absence of ovaries or adrenal glands (23), many studies have attempted to identify other tissues that are active. Because of our studies demonstrating increased peripheral conversion of androstenedione to estrone in obesity, adipose tissue was the first tissue examined in detail. The first positive report by Schindler et al. (33) detailing experiments that were initiated in my laboratory has been confirmed by others (30). The work of Longcope et al. (19) using an in vivo model indicates that muscle, in addition to adipose tissue, appears to be active. The important studies of Schweikert et al. (35) have demonstrated that human fibroblasts and hair follicles (34) also contain aromatase. The latter observation assumed greater importance with the extended demonstration that the Sebright bantam hen feathering trait is due to excessive aromatase activity (13) rather than to androgen resistance as first postulated by Fuller Albright. The extent to which this phenomenon occurs in humans is not known although a similar situation in a feminized young boy was described earlier (15). The importance of brain and, in particular, hypothalamic aromatase activity has been demonstrated in many species by Naftolin et al. (28) and others.

Contrary to what was believed only 10 years ago, it would seem that many tissues are involved in the synthesis of the aromatic Ring A estrogens. The full biological significance of the widespread tissue distribution of aromatase activity remains to be determined. Many exciting new findings such as the ability of prolactin to inhibit follicle-stimulating hormone induction of ovarian aromatase activity (53) and the development of highly effective "suicide" inhibitors (9) suggest that future studies of aromatase will be highly rewarding. They also suggest caution to those who are interested in aromatase inhibitors for treatment of estrogen-dependent cancer or as infertility agents since many other normal functions may be disturbed by inhibition of peripheral estrogen synthesis.

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References

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