In Vitro Assays of Aromatase and Their Role in Studies of Estrogen Formation in Target Tissues¹

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Abstract

In recent years, there has been increasing recognition of the importance of steroid aromatization in organs besides the steroidogenic ones. Estrogens formed from C-19 precursors in peripheral tissues can clearly contribute to the levels of estrogens in the circulation, while aromatization in target cells may be an important determinant of the concentration of estrogens to which a particular population of target cells is exposed. Assays sufficiently sensitive and simple enough to permit quantification and characterization of aromatase activity in peripheral and target tissues are now available. One of these, based on the quantification of ³H₂O formed from [1-¹³C]androstenedione, has, in our hands, a sensitivity of 15 fmol, is highly reproducible, and is relatively simple. It has been validated for stoichiometry between amounts of ³H₂O and [6,7-³H]estradiol formed from [1-¹³C]androstenedione and [6,7-³H]androstenedione, respectively. Using this assay, we have been able to quantify aromatase activity in discrete brain nuclear regions dissected from Vibratome sections of fetal rat brains and to identify in discrete areas sex differences and temporal changes during development that are obscured when larger tissue specimens are used. However, aromatase activity in brain nuclei as well as other target organs, including breast tissue, is likely to be concentrated in specialized cell populations. This heterogeneity limits the interpretation that can be placed on data obtained from standard "test tube" assays on homogenized tissue. We have used quantitative cytochemical assays for enzymes and cytochrome P-450 to identify regional specialization in the membrana granulosa of preovulatory follicles and localize cells that may be involved in steroidogenesis, including aromatization. Our findings underscore the need for new quantitative cytochemical and immunocytochemical assays to localize and to measure the amount and activity of aromatase in situ within identified populations of target cells.

The primary role of steroid aromatase in steroidogenic glands in determining levels of circulating estrogens is self-evident. But steroid aromatase is now recognized to be distributed widely in tissues outside steroidogenic glands. The cumulative effect of these so-called peripheral sites on estrogen production rates and on circulating hormone levels can be gauged from in vivo studies such as those discussed by others at this conference (9). Clearly, extraglandular tissues and organs with a capacity to aromatize C-19 precursors can have a measurable effect on the amount of estrogen to which target organs are exposed. The importance of this in normal females during the reproductive years may be debated. There is, however, little doubt that extraglandular aromatization as a source of circulating estrogens assumes significance when the contribution made by the ovaries is diminished or eliminated through either the normal process of aging or use of drugs that arrest cyclic ovarian function and that this contribution may be increased by obesity (4, 8).

The significance of aromatization in peripheral tissues, however, may not be limited to the contribution made to circulating estrogen levels. At least some of the peripheral tissues or organs in which conversion of C-19 precursors to estrogens has been demonstrated, notably the brain and mammary gland, are also important targets for the action of estrogen. Fat cells must also be considered in this category, since they not only aromatize steroids (2, 13, 17) but also possess estrogen receptors, concentrate estrogens in their nuclei, and form progesterone receptors in response to stimulation by estrogens (6, 7, 25). Aromatization in target organs therefore must be considered as part of the general phenomenon of formation of steroid metabolites within target cells, metabolites with actions different from those of the circulating steroids from which they are derived. This phenomenon, first identified in relation to 5α-reduction (1, 28), may be viewed as a mechanism for diversification and amplification of hormone action. This mechanism should allow for the generation at discrete sites of concentrations of active metabolites different from those in the general circulation and for controlling these local concentrations by modulating the activity of the enzymes responsible for the formation of the metabolites.

The brain is one target organ in which the significance of in situ estrogen formation has been investigated in some detail over the last decade. There is evidence now that estrogen formation in this organ can play a crucial role in both neuronal differentiation during development and neuronal function in the differentiated organism (11). It may be useful, therefore, to examine some of the principles and concepts that are evolving from studies of brain and their relevance to the significance of aromatization in breast tissue.

The level of aromatization in the hypothalamus and amygdala, as in the breast, is low. Hypothalami of adult rats were found to convert in vitro only about 0.02% of a C-19 substrate to estrogen during a 1-hr incubation. The corresponding figures for hypothalami of neonatal rats are greater but still less than 1% of the substrate (26). Yet, as discussed at this symposium (11), there is good evidence to support the thesis that, at least in this species, aromatization in the CNS² plays an essential role in...
role in such vital functions as the masculinization of the male brain during the critical perinatal period. The reason why such apparently small amounts of estrogen formed in situ can have such major effects is to be sought in the heterogeneous nature of the hypothalamus. The ability to form estrogens from C-19 precursors is not uniformly distributed but is concentrated in a few nuclear regions (10, 18). It is reasonable to assume that even within these anatomically defined regions, the capacity to aromatize is possessed by only certain populations of specialized cells. That these cell populations may themselves be anatomically segregated within a brain nucleus is suggested by the elegant autoradiographic studies of Sheridan (19, 20). This investigator compared the distribution of radioactivity in different regions of the telencephalon and diencephalon of rats in autoradiograms prepared following the injection in vivo of either 1α- or 1β-tritiated testosterone. Since the label from the 1β position is lost during aromatization, cell populations showing concentrations of radioactivity over cell nuclei only after an injection of [1α,2-3H]testosterone can be assumed to be cells in which the steroid translocated to the cell nucleus is an estrogen derived from the injected androgen. Particularly convincing are the findings in immature rats (20). Such rats have a high level of circulating α-,-fetoprotein that binds estrogens with high affinity and which would impede any estrogens formed outside the CNS from entering target cells. Thus, any labeled estrogen seen associated with a nucleus of a neuron in these immature rats was likely to have been formed in situ in the cytoplasm of the same neuron.

The above studies confirm the notion that the low levels of aromatase activity measured in whole hypothalami are a consequence of tissue dilution. Such tissue dilution will occur inevitably whenever enzyme activity is assayed in vitro in large tissue samples composed of functionally heterogeneous cell types. Tissue dilution can also obscure important changes in enzyme activity. This can be seen when our findings on the ontogeny of steroid aromatase in the CNS of male and female rat fetuses, measured in large diencephalic-telencephalic specimens, are compared with data obtained on enzyme activity in individual nuclear areas dissected from the same regions of brain.

The study of the ontogeny of steroid aromatase was motivated by our interest in determining the role of in situ aromatization in neuronal differentiation, particularly masculine differentiation. Under experimental conditions, estrogens have been shown to influence axonal differentiation as well as dendritic organization both in vivo and in vitro (12, 24). Such mechanisms could clearly modify the organization of certain neuronal circuits and imprint on them permanent biochemical, morphological, and functional features that characterize the brain of the male rat. It is, however, not yet clear whether this is, in fact, how estrogens formed from testosterone in brain during the critical period influence sexual differentiation. It is within this context that we set out to determine first whether, in the developing rat, aromatizing capacity of male hypothalami and limbic system was higher than that of females, as suggested by Reddy et al. (14), and, if such a sex difference existed, where in the CNS and when during perinatal development such differences were maximal. This information could then be a guide to when and where to look for morphological and biochemical correlates of aromatization in situ during the normal course of sexual differentiation of brain.

A prerequisite for such a study is an assay for steroid aromatase in a heterogeneous target organ with low levels of enzyme activity that is sensitive enough to measure enzyme activity without having to use an inordinately large number of animals to obtain a single value and, also, simple and rapid enough to allow for the generation of a sufficient number of individual values to provide a basis for statistical analysis.

The assay based on the measurement of the amount of tritiated water formed from androstenedione or testosterone tritiated in the 1β position fulfills these criteria (15, 22). In our hands, the sensitivity of the assay is of the order of 10 to 15 fmol (blank between 0.006 and 0.01% of the substrate). The low blank, and therefore high sensitivity, is achieved by eliminating the labile tritium at position 2 from the [1,2-3H]androstenedione substrate using mild alkali treatment. The predominately 1β-labeled substrate is then chromatographed, and water is removed from it using sodium sulfate. To maintain low blanks, it is necessary to use the substrate within a week of its preparation. The assay is highly reproducible with an interassay coefficient of variation of less than 15%.

When the assay was applied to quantify aromatase activity in combined hypothalamic-amygdaloid specimens from rat fetuses, no sex difference was detected (Chart 1). The period of development covered, from Day 17 to 21 PC, encompasses the initial intranatal phase of masculine differentiation. Based on our findings of circulating testosterone levels in perinatal rats, we have proposed that androgen-dependent masculine differentiation of CNS begins between Days 17 and 18 PC, since this is the first time when a sex difference in circulating levels of testosterone can be detected (27). This difference results from a surge of testosterone secretion in male fetuses lasting for 2 days. We have hypothesized that this testosterone surge may induce aromatase activity in the CNS of males and thereby sensitize the brain to the masculinizing action of the much lower levels of testosterone found in the circulation of males after the testosterone surge. A prerequisite for such a mechanism of sensitization would seem to be necessary because, after Day 19 and therefore during the rest of the perinatal period known to be critical for masculine differentiation, testosterone levels in males are only slightly higher than those of female littermates (28). However, as can be seen in Chart 1, there was no evidence of either a sex difference in aromatase activity or of any stimulatory effect of testosterone on this function.

A very different pattern emerged when aromatase activity was measured in the 2 sexes in selected, anatomically defined nuclear regions of the diencephalon and telencephalon of perinatal rat brains. These nuclear regions were dissected under a microscope from 300-μm coronal sections, cut from fresh tissue with a Vibratome. Because of the sensitivity of the aromatase assay, it was not necessary to pool samples from more than one fetus. A comparison of aromatase activity of 6 discrete regions from male and female rats between Day 21 PC through Day 5 after birth has been completed. In 4 of the 6 regions, aromatase activity on Day 20.5 PC was found in a pilot study to be significantly higher than the mean values found on the same day in the much larger hypothalamic-amygdaloid specimens shown in Chart 1. These 4 areas were the ventro-
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Chart 1. Steroid aromatase activity in vitro of combined hypothalamic-preoptic region of fetal rat brain. Homogenates of brain tissue from male and female fetuses between Days 17 and 21.5 PC were incubated for 1 hr at 30°C in 200 μl of the following incubation medium: 1 μCi [1-3H]androstenedione (0.5 μM), 15 mM potassium phosphate (pH 8.0), 30-1 mM EDTA-5 mM glucose-6-phosphate-0.5 mM NADP-glucose-6-phosphate dehydrogenase (1.25 units/ml). The incubation was arrested with 0.4 μl of a 20-mg/ml suspension of charcoal in 5% trichloroacetic acid. After centrifugation at 4°C, the supernatant was placed in a scintillation vial, and the radioactivity was counted at 25% efficiency. Numbers in parentheses, number of samples analyzed at each age group. Brains from the fetuses were obtained at half-day intervals with expected time of ovulation being counted as Time 0 of pregnancy. The brain specimens from fetuses in the middle of the dark phase (-topic) versus males and females, respectively) were obtained from mothers that had been kept on a reversed light-dark schedule. Analysis of variance did not indicate any sex difference.

A sex difference in aromatase activity was evident in 2 of the 4 regions. In the ventromedial hypothalamic area, males had slightly higher levels of enzyme activity than did females throughout the period studied (p < 0.008). More unexpected and intriguing was the finding of significantly higher levels of enzyme activity in females than males in the adjacent anterior hypothalamic area on Day 20.5 PC (mean levels for females, 1054 pmol per protein per hr versus 500 pmol per protein per hr in males, p < 0.002). This sex difference disappeared as a result of a very much more rapid decline in enzyme activity in females than males over the next 2 days. It should be noted that, on Day 20.5 PC, the mean level of aromatase activity per mg protein in the anterior hypothalamic area of females was nearly 10 times and in males 5 times higher than mean values on the same day for larger tissue specimens (Chart 1). This underscores the difficulty of interpreting values for enzyme activity obtained from target organs as a whole when what is likely to be crucial is the level of enzyme activity in a selected population of specialized target cells.

Determining levels and characterizing the pattern of changes in enzyme activity in a target organ, such as the CNS, however, clearly represent only the first step towards achieving the ultimate goal of defining the local consequences of in situ estrogen formation. The fundamental questions that need to be answered are: why should there be certain cells within the target organ that go into the business of making estrogen? what is the effective sphere of influence of the estrogen formed in a target cell? and what are these influences? In practical terms, this means identifying the cells that aromatize steroids and examining consequences of aromatization on these cells and on their neighbors.

In the case of the developing CNS, there are various aspects of neuronal differentiation, both morphological and biochemical, that could be influenced by the estrogens formed in situ (10). The usefulness of our finding is in helping to select conditions under which it might be profitable to look for morphological and biochemical correlates of in situ aromatization, that is, where to look and when to look. For example, in our studies, a comparison of various morphological and biochemical parameters in the anterior hypothalamic area of the 2 sexes on Day 20.5 PC, when aromatizing capacity of females is double that in males, may provide clues on the local effects of estrogen on neuronal differentiation. The area of brain involved is certainly small enough to reduce the chances of tissue dilution obscuring sex differences in biochemical functions that could be related to local sex difference in estrogen formation. These areas are also small enough to allow for detailed comparison to be made of the morphology of the region in the 2 sexes, at least at the light microscopic level. However, even within this small area, only a proportion of the cells is likely to have the capacity to form estrogens and to be responsible for the relatively high levels of aromatase activity measured. Ultimately, to determine the role of in situ aromatization will require that these cells be identified so that their biochemical and morphological characteristics can be defined. These are questions that can now be approached by cytochemical techniques, that is, techniques which allow for the localization of enzymes and for quantification of their amounts and activity in individual cells in tissue sections in which intracellular organization and cellular integrity are preserved. With respect to aromatase, once antibodies to the enzyme become available, it should be possible to localize the enzyme immunocytochemically. The use of labeled suicide inhibitors combined with autoradiography may provide another approach to localizing aromatase in target cells. The techniques of immunocytochemistry and autoradiography can be used quantitatively to provide at least a relative index of the amount of enzyme present (21). The possibility of quantifying the activity of the enzyme in situ in intact cells hinges on developing an agent which couples with estrogen to yield a colored product. This would permit measuring enzyme activity in situ in intact cells using microspectrophotometry, an approach by which colored reaction products of biochemical reactions can be quantified accurately and with a high degree of sensitivity in individual cells in cryostat sections.

The potential of quantitative cytochemical approaches for...
measuring enzyme activity and cell constituents is illustrated by our studies of the membrana granulosa of the preovulatory follicle of the rat (29, 30). The aim of these studies was to demonstrate that the membrana granulosa is functionally heterogeneous. Specifically, we wished to demonstrate that steroidogenesis, including aromatization, was likely to be a specialized function of the peripheral pseudostratified granulosa cells that abut on the basement lamina. The 2 functions examined by quantitative cytochemistry, the activity of glucose-6-phosphate dehydrogenase and cytochrome P-450, though not specific for aromatization, are essential to the process (5). Furthermore, in the case of glucose-6-phosphate dehydrogenase, it is possible to differentiate at the cytochemical level between the generation of 2 types of reducing equivalents by the enzyme (3, 23). The first of these, type I hydrogen, appears to be the one available for steroid hydroxylating reactions, including those associated with aromatization. The second, type II hydrogen, is likely to be utilized for more general cellular biosynthetic reactions, e.g., lipid synthesis (3, 23). Using microspectrophotometry made it possible to demonstrate during the last 2 days of the estrous cycle an abrupt increase in type I hydrogen generation that was 3-fold greater in the peripheral than the paraanal cells of the membrana granulosa of the preovulatory follicles (30). The spectral shift that characterizes the binding of carbon monoxide to reduced cytochrome P-450 was also shown to be associated with the peripheral but not the paraanal granulosa cells of such preovulatory follicles (29). More direct evidence that aromatization in the preovulatory follicle is restricted to a specialized subpopulation of peripheral cells will have to await the development of appropriate reagents, as suggested above. However, as demonstrated by these studies, quantitative cytochemistry can offer an in vitro assay technique with a high-enough resolution to allow biochemical characterization of individual cell types and thereby help evaluate the significance of aromatization in target organs. The methodology is sufficiently sensitive to be applied to biopsy specimens, which is an obvious advantage in studies to biopsy specimens, which is an obvious advantage in studies

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References


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