

# Aromatase inhibitors and enzyme stability

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## Abstract

The effects of two steroidal (4-hydroxyandrostenedione and atamestane) and three non-steroidal (fadrozole, vorozole, and pentozole) aromatase inhibitors on the levels of aromatase mRNA and protein were examined *in vitro* and *in vivo*. Immunocytochemistry revealed increased quantities of immunoreactive aromatase in human choriocarcinoma-derived JEG-3 cells in response to pretreatment with the non-steroidal inhibitors. To elucidate this effect in detail, aromatase protein in JEG-3 cells after treatment with various inhibitors was quantified using an enzyme-linked immunosorbent assay (ELISA). A time-dependent increase in aromatase protein in the cells was observed with all the aromatase inhibitors except 4-hydroxyandrostenedione, whereas aromatase mRNA levels in the cells remained unchanged during the inhibitor treatment. The three non-steroidal agents caused an approximately fourfold increase in aromatase protein in the cells 24 h after the treatment, as compared with untreated controls. The increase in aromatase protein in the cells was not blocked by treatment with cycloheximide, an inhibitor of protein synthesis. The inhibitors also appeared to block the rapid degradation observed in JEG-3 cells after induction by forskolin. *In vivo*, daily injection of the inhibitors into adult female mice caused increases in levels of both aromatase mRNA and protein in the ovary. The increase in aromatase mRNA in this *in vivo* study could be explained by an increase in gonadotropin concentrations in response to decreased plasma concentrations of estrogens. In conclusion, we suggest that aromatase inhibitors increase aromatase protein through stabilization and reduced protein turnover.

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## Introduction

Aromatase, also called estrogen synthetase, catalyzes aromatization of androgens to estrogens and is a key enzyme in estrogen biosynthesis. It is known to play an important role through estrogen production in various physiological functions. This enzyme has been shown to be present in breast (Abul-Hajj *et al.* 1979, Santner *et al.* 1984, Miller & O'Neill 1987) and endometrial cancer tissues (Noble *et al.* 1996), in addition to various gonadal and extragonadal tissues. In the case of breast cancer, aromatase protein and mRNA were reported to be localized in adipose stromal cells proximal to tumors (Bulun *et al.* 1993, Santen *et al.* 1994, Sasano *et al.* 1994, Harada *et al.* 1995) and intratumoral stromal cells (Esteban *et al.* 1992, Lu *et al.* 1996). Estrogens are known to function as mitogenic factors in certain tissues, suggesting that local production of estrogens by aromatase may play an essential role in the pathogenesis of estrogen-dependent breast and endometrial cancers, maintaining

proliferation in a paracrine or autocrine fashion. Therefore, one approach to therapy is to reduce or eliminate continuous stimulation by circulating and locally produced estrogens, and a number of aromatase inhibitors capable of causing cancer regression in patients have been introduced for such endocrine therapy (Brodie 1994).

Initially, aminoglutethimide found a clinical application as a possible therapeutic aromatase inhibitor for breast cancer (Santen & Misbin 1981). However, it also inhibits the cholesterol side-chain cleavage reaction by P450<sub>scc</sub>, resulting in a deficiency of glucocorticoids and mineralocorticoids in addition to sex steroids. 4-Hydroxyandrostenedione (4-OHA) was subsequently utilized as a potent and specific inhibitor (Marsh *et al.* 1985), functioning as a mechanism-based inhibitor or a suicide substrate and causing time-dependent inactivation in the presence of cofactors (Brodie *et al.* 1981*b*). More recently, more potent and selective non-steroidal inhibitors of

imidazole, triazole, and tetrazole derivatives have been developed and clinically examined (Brodie 1994).

There have been many reports concerning development and clinical trials of new aromatase inhibitors for breast cancer patients. However, the effects of aromatase inhibitors on synthesis and degradation of aromatase mRNA and protein remain unclear. In this study, we examined these parameters in cultured cells and in experimental animals. The results suggested that some aromatase inhibitors stabilize the enzyme and prevent its degradation, probably through formation of tightly associated aromatase-inhibitor complexes.

## Materials and Methods

### Reagents

Atamestane (1-methyl-androsta-1,4-diene-3,17-dione), 4-OHA, fadrozole (4-(5,6,7,8-tetrahydroimidazo-[1,5a]-pyridine-5-yl)-benzotrile monohydrochloride), vorozol (6-[ (4-chlorophenyl) (1H-1,2,4-triazol-1-yl) methyl] 1-methyl-1H-benzotriazole), and pentrozole (5-[cyclopentylidene-(1-imidazolyl)-methyl] thiophene-2-carbonitrile monohydrochloride) were kindly synthesized and provided by the laboratories of Schering AG (Berlin, Germany).

### Cell culture

Human choriocarcinoma-derived JEG-3 cells were maintained in Minimum Essential Medium Alpha Modification supplemented with 10% fetal calf serum at 37°C in a 95% air-5% CO<sub>2</sub> humidified atmosphere.

### Preparation of total RNA and microsomal fractions

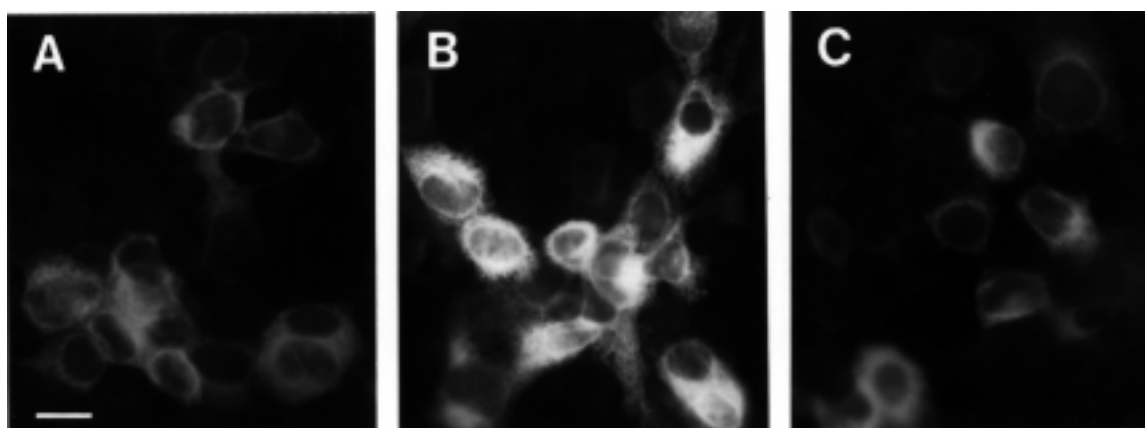
Microsomal fractions were prepared by successive centrifugation (Harada & Omura 1980). Total RNA fractions were isolated from cultured cells and mouse ovaries using the Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions.

### Immunocytochemical study

The rabbit polyclonal anti-human aromatase antibody used in this study has been confirmed to be monospecific by biochemical and immunological tests (Harada 1988) and has been validated for the detection of aromatase by immunocytochemical staining in several laboratories (Balthazart *et al.* 1990, Jakab *et al.* 1993, Santen *et al.* 1994, Sasano *et al.* 1994). The immunocytochemical procedures have been described previously (Hatano *et al.* 1994). Immunocytochemical staining of aromatase was carried out using rabbit anti-human aromatase and fluorescein isothiocyanate (FITC)-labeled donkey anti-rabbit immunoglobulin antibodies as primary and secondary antibodies, respectively.

### Enzyme-linked immunosorbent assay (ELISA) of aromatase

We first determined optimal conditions for the ELISA. High concentrations of detergent and glycerol were found to be significantly inhibitory for the immunological reaction and a high concentration of proteins disturbed the



**Figure 1** Immunocytochemical staining of aromatase in JEG-3 cells treated with aromatase inhibitors. JEG-3 cells were cultured in the presence of 10  $\mu$ M aromatase inhibitors at 37°C for 24 h, and then subjected to immunocytochemical staining using primary rabbit anti-human aromatase and secondary FITC-labeled donkey anti-rabbit immunoglobulin antibodies. Immunoreactive aromatase in the control (A), vorozole-treated (B), and atamestane-treated (C) cells was observed by fluorescent microscope. Scale bar indicates 20  $\mu$ m.

accuracy of quantitation. Microsomal fractions (0.5 mg/ml) were solubilized with 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl, 0.1% Tween 20, and 0.2% sodium cholate and then centrifuged to obtain the solubilized supernatants. Microtiter wells were precoated with 2 µg/ml anti-aromatase antibody at 37°C for 2h. After washing with phosphate-buffered saline (PBS), blocking with Dulbecco's PBS containing 1% BSA at room temperature for 30 min, and again washing with PBS, 200 µl aliquots of the solubilized supernatants of microsomal fractions were added to the wells, and incubated at room temperature for 2 h. Wells for blanks and aromatase standards were also included, with addition of serial dilutions (0-50 ng/ml) of purified human aromatase in place of solubilized supernatant. After washing with PBS, 200 µl biotin-labeled anti-aromatase antibody (5 µg/ml in PBS containing 1% BSA) were added to each well, followed by incubation at room temperature for 1 h. After washing with PBS, 200 µl alkaline phosphatase-streptavidin were added to each well, and incubated for 30 min. After washing with PBS, 200 µl aliquots of 2 mg/ml *p*-nitrophenylphosphate in 0.1 M diethanolamine (pH 9.8) containing 1 mM MgCl<sub>2</sub> were added to the wells. After further incubation for 40 min in the dark, the reactions were stopped by adding 50 µl 2 M NaOH. Absorbance was measured at 405 nm on a Microplate Reader MTP-32 (Corona Electric; Katsuta, Japan).

#### Quantitative analysis of aromatase mRNA

The aromatase mRNA levels in total RNA fractions were fluorimetrically determined by reverse transcriptase-polymerase chain reaction (RT-PCR) using a fluorescent dye, FAM (Perkin Elmer; Foster City, CA, USA)-labeled primer in the presence of an internal standard RNA, as previously described (Harada & Yamada 1992, Utsumi *et al.* 1996). The fluorescent RT-PCR products were analyzed on a 2% agarose gel with a Gene Scanner 362 Fluorescent Fragment Analyzer (Perkin-Elmer). The amount of aromatase mRNA in the total RNA was calculated from the peak areas of the fluorescent products by the internal standard method.

#### Other methods

Plasma follicle-stimulating hormone (FSH) was determined by enzyme immunoassay using an FSH EIA kit (Amersham; Amersham, Buckinghamshire, UK). Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce; Rockford, IL, USA) using BSA as a standard.

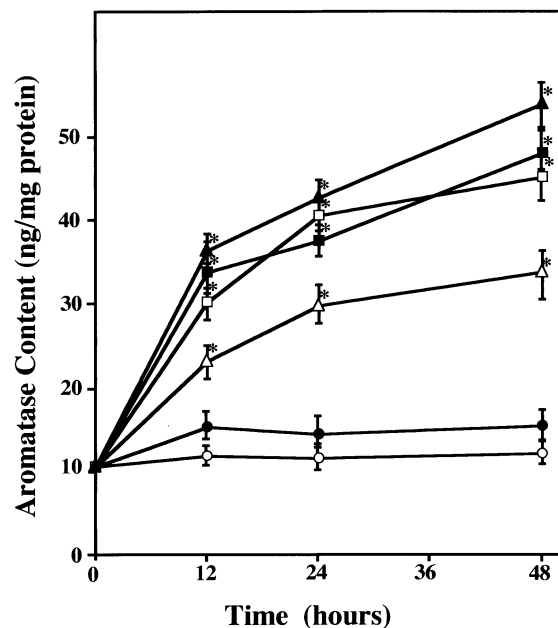
#### Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Scheffé's test. A *P* value <0.05 was considered to be significant.

## Results

#### Immunocytochemical analysis in JEG-3 cells treated with aromatase inhibitors

Changes in the protein concentrations of aromatase in JEG-3 cells by treatment with aromatase inhibitors were first examined by immunocytochemical staining. Vorozole, a non-steroidal aromatase inhibitor, markedly increased immunoreactive aromatase protein in the JEG-3 cells (Fig. 1B), compared with untreated controls (Fig. 1A). Only a slight increase in aromatase protein was observed with atamestane, a steroidal aromatase inhibitor (Fig. 1C). Similar increases were observed with the other non-steroidal aromatase inhibitors, fadrozole and pentozole, whereas the steroidal aromatase inhibitor,



**Figure 2** Time-course of increase of aromatase protein in JEG-3 cells treated with various aromatase inhibitors. JEG-3 cells were cultured in the absence (○) or presence of 10 µM 4-OHA (●), atamestane (△), pentozole (▲), vorozole (◻), or fadrozole (■) at 37°C. The cells were collected 12, 24, and 48 h after addition of the inhibitors to the culture medium, and the contents of aromatase protein in the cells were determined by ELISA. Results are the means ± S.E.M. of three experiments.

\**P* < 0.05 compared with the respective control values.

**Table 1** Effects of aromatase inhibitors on aromatase protein concentrations in JEG-3 cells. After pretreatment with 10  $\mu$ M forskolin for 48 h, the cells were washed with PBS to remove forskolin completely and cultured for 24 h without forskolin in the presence of 10  $\mu$ M aromatase inhibitors or in the absence of the inhibitors as a control. The aromatase protein contents were then determined by ELISA. Results are the means  $\pm$  S.E.M. of three experiments

Treatment	Aromatase content (ng/mg microsomal protein)
(After pretreatment	121.5 $\pm$ 5.1)†
Control	55.7 $\pm$ 1.7
Atamestane	99.6 $\pm$ 6.9*
4-OHA	58.9 $\pm$ 2.8
Fadrozole	202.1 $\pm$ 9.3*
Vorozole	192.4 $\pm$ 7.8*
Pentrozole	196.1 $\pm$ 9.1*

† At the end of the 48-h pretreatment with forskolin.

\*  $P < 0.05$  compared with control.

4-OHA, produced few effects within the cells (data not shown).

#### Time-dependent changes of aromatase protein concentrations in JEG-3 cells treated with aromatase inhibitors

To measure the increase in aromatase protein of JEG-3 cells by aromatase inhibitors, a quantitative ELISA was developed. Although aromatase protein in untreated control cells remained essentially unchanged during the 48-h observation period, all non-steroidal aromatase inhibitors, fadrozole, vorozole, and pentrozole, caused time-dependent increases in aromatase protein to about fourfold the control value after 24 h, as shown in Fig. 2. A time-dependent increase in aromatase protein was also observed with the steroidal aromatase inhibitor, atamestane, but to a much lesser extent, whereas no significant effects of the other steroidal aromatase inhibitor, 4-OHA, were noted.

#### Effects of aromatase inhibitors on aromatase protein concentrations after forskolin induction in JEG-3 cells

The effects of aromatase inhibitors on protein concentrations of aromatase in JEG-3 cells were further investigated after induction of aromatase by forskolin. Aromatase was increased about tenfold (121.5  $\pm$  5.1 ng/mg microsomal protein) 48 h after treatment of the cells with forskolin. As shown in Table 1, 24 h after removal of forskolin, the induced concentration decreased by about

50%. This reduction was not affected by 4-OHA treatment, but was prevented by atamestane. In contrast, addition of the non-steroidal aromatase inhibitors to the cells caused significant increases in aromatase protein, in spite of the absence of the inducer.

#### Effects of aromatase inhibitors on the levels of aromatase mRNA in JEG-3 cells

The levels of aromatase mRNA in JEG-3 cells treated with aromatase inhibitors were fluorimetrically determined by a quantitative RT-PCR method using an internal standard RNA and a fluorescent dye-labeled primer. As shown in Table 2, no significant intergroup differences were observed, indicating that none of the aromatase inhibitors produced substantial effects on the levels of aromatase mRNA in the cells.

#### Effects of cycloheximide on the inhibitor-associated increase in aromatase protein in JEG-3 cells

The present study indicated that the increase in aromatase protein in JEG-3 cells treated with aromatase inhibitors was not due to increased mRNA levels, but rather to increased translation or decreased degradation. To assess these possibilities, cycloheximide was added as an inhibitor of protein synthesis, simultaneously with aromatase inhibitors, and changes in aromatase protein concentrations were again observed after 24 h. As shown in Table 3, the concentrations of aromatase protein in the cells treated with cycloheximide were decreased compared with the values without cycloheximide (Fig. 2). However, all the non-steroidal aromatase inhibitors still caused an approximately threefold increase, and

**Table 2** Effects of aromatase inhibitors on the levels of aromatase mRNA in JEG-3 cells. JEG-3 cells were cultured in the presence or absence of 10  $\mu$ M aromatase inhibitors at 37°C for 24 h, and total RNA fractions were recovered from the cells. The levels of aromatase mRNA were determined by quantitative RT-PCR using an internal standard RNA and a fluorescent dye-labeled primer. Results are the means  $\pm$  S.E.M. of three experiments

Treatment	Aromatase mRNA (amol/ $\mu$ g total RNA)
Control (untreated)	6.07 $\pm$ 0.32
Atamestane	6.31 $\pm$ 0.20
4-OHA	5.98 $\pm$ 0.05
Fadrozole	6.04 $\pm$ 0.15
Vorozole	5.95 $\pm$ 0.19
Pentrozole	6.14 $\pm$ 0.24

**Table 3** Effects of cycloheximide on the increase of aromatase protein in JEG-3 cells caused by aromatase inhibitors. JEG-3 cells were cultured in the presence of 20 µg/ml cycloheximide together with 10 µM aromatase inhibitors at 37°C for 24 h. The contents of aromatase protein in the cells were then determined by ELISA. Results are the means ± S.E.M. of three experiments

Treatment	Aromatase content (ng/mg microsomal protein)
Control (untreated)	4.24±0.41
Atamestane	7.16±0.98
4-OHA	4.41±0.31
Fadrozole	13.57±0.95*
Vorozole	12.73±0.80*
Pentrozole	14.61±1.13*

\* $P < 0.05$  compared with control.

atamestane also exerted some apparent effect. 4-OHA, in contrast, did not cause any change in the level of aromatase protein. As essentially the same pattern of increase of aromatase protein by aromatase inhibitors was observed with and without cycloheximide, the results indicate that the increase in aromatase protein associated with aromatase inhibitors is mediated by stabilization of the enzyme and prevention of protein degradation.

### Effect of daily injection of aromatase inhibitors to adult female mice on the expression levels of ovarian aromatase

Fadrozole was administered daily to adult female mice for 8 days. Blood and ovaries were collected from the mice 12 h after the final dose, and plasma FSH and ovarian aromatase mRNA were determined by enzyme immunoassay and quantitative RT-PCR, respectively. Aromatase protein in the ovaries was increased about three- to fourfold by this treatment (data not shown). Simultaneously, fadrozole caused a significant increase in plasma FSH and ovarian aromatase mRNA levels, compared with controls (Table 4).

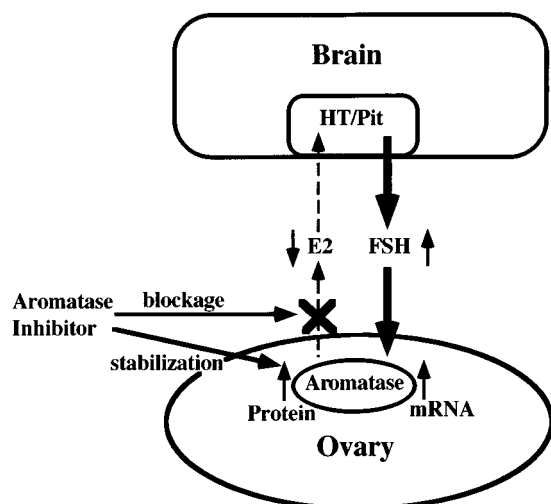
### Discussion

The present study showed first that the non-steroidal aromatase inhibitors, fadrozole, vorozole, and pentrozole, increased the immunoreactive aromatase protein in human choriocarcinoma-derived JEG-3 cells. Furthermore, a time-dependence of the inhibitor influence was demonstrated by quantitative analysis using ELISA. The rapid degradation of forskolin-induced aromatase in the cells after removal of the inducer also appeared to be prevented by most of the inhibitors. Additional evidence that a decrease in the turnover of enzyme protein was responsible for the observed increase was provided by the apparent lack of change in aromatase mRNA level and by the fact that cycloheximide did not cause a significant

**Table 4** Effects of *in vivo* administration of fadrozole on the concentrations of serum FSH and ovarian aromatase mRNA in mice. Fadrozole, a non-steroidal aromatase inhibitor, was injected i.i.p. to five adult female mice with a daily dose of 10 mg/kg body weight for 8 days, and then serum FSH and ovarian aromatase mRNA levels were determined. Untreated adult female mice in estrous were also examined as controls

Treatment	Samples	FSH (ng/ml)	Aromatase mRNA (amol/ng RNA)
Control	1	17.4	3.03
	2	16.8	3.40
	3	16.2	5.93
	4	15.9	7.41
	5	17.1	3.22
Mean±S.D.		16.7±0.6	4.69±1.97
Fadrozole	1	24.0	10.54
	2	23.4	14.42
	3	20.4	23.88
	4	21.2	17.06
	5	23.1	12.37
Mean±S.D.		22.4±1.5*	15.65±5.20*

\* $P < 0.05$  when compared with the control.



**Figure 3** Effects of aromatase inhibitors on ovarian aromatase in adult female mouse. Ovarian aromatase is increased at the levels of the mRNA and the protein in response to treatment with aromatase inhibitors. The inhibitors protect aromatase from protein degradation, by means of stabilization. Simultaneously, they suppress production of estrogens (E2) by ovarian aromatase, resulting in an increase in FSH through the hypothalamic-pituitary (HT/Pit) axis and a consequent increase in ovarian aromatase mRNA.

alteration in the pattern of increase. Thus the experimental results using cultured cells strongly suggest that aromatase inhibitors exert their action on aromatase protein through protection against protein degradation, rather than through increases in transcription or translation, or mRNA stabilization. In contrast, interpretation of the results from the *in vivo* experiments is more complex. The *in vivo* administration of aromatase inhibitors to adult female mice caused a significant increase in both aromatase protein and mRNA in the ovaries (Fig. 3). In this case, the inhibitors were able to suppress the production of estrogen by aromatase in the ovaries and other tissues, causing a decrease in plasma concentrations of estrogens and a consequent increase in plasma concentrations of FSH through the hypothalamic-pituitary-ovarian axis. As a result, aromatase in the mouse ovaries is increased both through increased levels of aromatase mRNA in response to the enhanced FSH stimulus, and through increased concentrations of aromatase protein as a result of stabilization due to the formation of a tight aromatase-inhibitor complex. Miller & Mullen (1993) showed that, after aromatase inhibitor (aminoglutethimide) treatment, aromatase activities in the tumors of patients with breast cancer were significantly greater than those in same

tumors before the treatment. This result may also support the clinical significance of our observation. It is necessary to consider clinically whether, because the inhibitor therapy induces aromatase, such therapy would necessitate the use of greater doses, to inhibit the enzyme activity.

There are several possible explanations for the distinct differences in the effects of the various aromatase inhibitors used. First, the capacities of aromatase inhibitors to increase aromatase protein in cells may reflect their binding affinities ( $K_i$  values) for aromatase. The  $K_i$  values of 4-OHA and atamestane are reported to be about 250 nM (Henderson *et al.* 1986), whereas those of fadrozole and vorozole are 1.6 (Steele *et al.* 1987) and 0.7 nM (Vanden Bossche *et al.* 1990), respectively. Judging from this difference, non-steroidal inhibitors would be expected to be more tightly associated with the aromatase molecules in stable complexes that may be more resistant to proteolytic cleavage. Secondly, the steroidal aromatase inhibitors, 4-OHA and atamestane, are known to be mechanism-based inhibitors or suicide inhibitors (Brodie *et al.* 1981b, Henderson *et al.* 1986). They promote time-dependent inactivation of aromatase through production of a reactive intermediate by the catalytically active enzyme, and probably time-dependent degradation as a result of increasing sensitivity of the inactive form to proteolytic cleavage. It is likely that 4-OHA is a stronger mechanism-based inhibitor than atamestane, as their inactivation rates of aromatase are reported to be  $4.5 \times 10^{-3}$  (Brodie *et al.* 1981b) and  $1.8 \times 10^{-3} \text{ s}^{-1}$  (Henderson *et al.* 1986), respectively. Consequently, this may explain why 4-OHA did not cause an appreciable increase in aromatase protein, in contrast to atamestane.

Aromatase inhibitors and substrates are believed to bind competitively to the same binding sites within aromatase molecules and to form conformationally tight complexes, so that they could be expected to be resistant to proteolytic degradation. Stabilization and increased content caused by substrates or inhibitors has been found for many enzymes: for example, the cellular concentrations of arginase (Schimke 1964) and tryptophan pyrrolase (Schimke *et al.* 1965) in rat liver are known to become increased as a result of decreased protein degradation dependent on the substrates, arginine and tryptophan. Furthermore, steroidogenic cytochrome P450<sub>11 $\beta$</sub>  and aromatase were successfully purified in the presence of deoxycorticosterone (Takemori *et al.* 1975) and testosterone (Harada 1988) or androstenedione (Tan & Muto 1986) used as substrate stabilizers to prevent inactivation of the enzyme. More recently, immunoreactive aromatase in the quail brain was found to be increased by the specific inhibitors, fadrozole and vorozole (Foidart *et al.* 1994). These results suggest that

tight aromatase-inhibitor complexes are also relatively resistant to proteolytic degradation and, consequently, cause increases in aromatase protein, although it might be possible that binding of the inhibitors to the active site of aromatase induced a conformational change in the enzyme and opened up binding sites available to the antibody used in the ELISA, resulting in an apparent, not real, increase in the content of the enzyme.

For the purposes of the present study, we developed an ELISA for quantitative analysis of aromatase protein in the cells, while Kitawaki *et al.* (1989) previously introduced a similar method for quantitation of catalytically active aromatase in human placenta. As the content of aromatase in microsomes of JEG-3 cells is quite low (about 10 ng/mg protein) compared with that of the placenta (about 15 µg/mg protein) (Kitawaki *et al.* 1989), we solubilized microsomes with low concentrations of Tween 20 and sodium cholate and performed the ELISA in the absence of glycerol, to avoid any unnecessary interference with the immunological reaction. Under these conditions, protein recovery was improved, accurate quantitation was confirmed, and aromatase was determined as a more stable P420 form (Omura & Sato 1964), which is catalytically inactive.

The results obtained in this study suggest the conclusion that blockage of the degradation of aromatase by the inhibitors of the enzyme causes a marked increase in immunoreactive aromatase protein in cultured cells and other experimental systems.

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