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 pentrozole) 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.

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 P450sc, 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. 1981b). 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)-benzonitrile monohydrchloride), vorozole (6-[(4-chlorophenyl) (1H-1,2,4-triazol-1-yl) methyl]-1-methyl-1H-benzotriazole), and pentrozole (3-[cyclopentylidene-(1-imidazolyl)-methyl] thiophene-2-carbonitrile monohydrchloride) 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% CO₂ 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 μM 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 µm.
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 MgCl₂ 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 pentrozole, whereas the steroidal aromatase inhibitor,
Harada et al.: Stabilization of aromatase by its inhibitors

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±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 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aromatase content (ng/mg microsomal protein)</th>
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<tbody>
<tr>
<td>(After pretreatment)</td>
<td>121.5±5.1†</td>
</tr>
<tr>
<td>Control</td>
<td>55.7±1.7</td>
</tr>
<tr>
<td>Atamestane</td>
<td>99.6±6.9*</td>
</tr>
<tr>
<td>4-OHA</td>
<td>58.9±2.8</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>202.1±9.3*</td>
</tr>
<tr>
<td>Vorozole</td>
<td>192.4±7.8*</td>
</tr>
<tr>
<td>Pentrozole</td>
<td>196.1±9.1*</td>
</tr>
</tbody>
</table>

† At the end of the 48-h pretreatment with forskolin.  
* P < 0.05 compared with control.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aromatase mRNA (amol/µg total RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>6.07±0.32</td>
</tr>
<tr>
<td>Atamestane</td>
<td>6.31±0.20</td>
</tr>
<tr>
<td>4-OHA</td>
<td>5.98±0.05</td>
</tr>
<tr>
<td>Fadrozole</td>
<td>6.04±0.15</td>
</tr>
<tr>
<td>Vorozole</td>
<td>5.95±0.19</td>
</tr>
<tr>
<td>Pentrozole</td>
<td>6.14±0.24</td>
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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 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 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.

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

*P < 0.05 compared with control.

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.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.

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

*P<0.05 when compared with the control.
The stabilization. In contrast, interpretation of the results from increases in transcription or translation, or mRNA protection against protein degradation, rather than through inhibitors exert their action on aromatase protein through 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 axis and a consequent increase in ovarian aromatase mRNA.

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 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 (Ki values) for aromatase. The Ki 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 $1.8 \times 10^{-3}$ 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, steroidaligeneric cytochrome P450$_{11B}$ 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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