Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R

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Abstract

A number of conditions related to sex-reversal in boys and men and precocious puberty in girls are caused by estrogen-secreting adrenal tumors. In these tumors, cytochrome P450 aromatase (aromatase) that is encoded in the CYP19 gene is expressed at high levels. To investigate the molecular mechanism of aromatase expression in these adrenal tumors, we characterized the activity, gene transcript and genomic promoter region of aromatase in the human adrenocortical carcinoma cell line H295R. Aromatase activity and the transcript of the CYP19 gene were highly up-regulated by forskolin, but not by dexamethasone. The results from exon I-specific reverse transcriptase (RT)-PCR and the transfection of reporter constructs suggested that promoter I.3 and promoter II were activated in H295R. Deletion and mutation analysis suggested that cAMP response element-like sequence (CLS) and steroidogenic factor-1 (SF-1) motif, were critical for the activation of promoter II. The results of this work should provide the basis for the molecular analysis of aromatase expression in adrenocortical cells.

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Introduction

Estrogen-secreting adrenal tumors may cause precocious puberty in girls and sex-reversal in boys and men (Young et al. 1996, Watanabe et al. 2000, Phornphutkul et al. 2001). Abnormally high serum concentrations of estrogen cause gynecomastia in boys and men and very early menarche in girls. In these adrenal tumors, cytochrome P450 aromatase (aromatase) that is encoded in the CYP19 gene is highly expressed, whereas normal adrenal tissues have no detectable aromatase activity.

Aromatase is the final and rate-limiting enzyme of estrogen biosynthesis. Multiple signaling pathways regulate expression of aromatase activity and CYP19 gene transcripts. In ovary, aromatase activity can be stimulated by an increase in the intracellular cAMP concentration in response to tropic stimulation by follicle-stimulating hormone (Simpson et al. 1997). In human osteoblasts, aromatase activity can be stimulated by glucocorticoids (Shozu & Simpson 1998). In human adipose stromal cells aromatase activity can be stimulated by both cAMP and glucocorticoids (Zhao et al. 1995, Simpson et al. 1997). In response to the multiple signals, multiple promoters are activated and multiple species of exon I are selected as a consequence of alternative splicing (Fig. 1) (Simpson et al. 1997, Sasano & Harada 1998, Sebastian & Bulun 2001); for example, exon I.3 and exon II are selected in the ovary. Proximal promoters of these species of exon I (promoter I.3 and promoter II), which have a cAMP response element (CRE)-like sequence (CLS) or CREaro, are activated in a cAMP-dependent manner. In human osteoblasts, exon I.4 is selected as the 5’-untranslated region of the CYP19 gene transcript. Its proximal promoter region (promoter I.4), which has the glucocorticoid response element (GRE), is activated by glucocorticoids. In adipose stromal cells, exon I.3, exon II and exon I.4 are selected. Central nervous system-specific CYP19 exon I (exon 1f) was identified in mice and humans (Honda et al. 1996, Sasano et al. 1998). Studies on estrogen-secreting adrenal tumors have revealed that exon I.3 and/or exon II are selected in the CYP19 gene transcript (Young et al. 1996, Watanabe et al. 2000).

Precise characterization of the response to hormones and chemicals, as well as the molecular mechanisms involved, is needed for the development of treatment for estrogen-secreting adrenal tumors. For this characterization work, an adrenal tumor cell line that has the same profile in vivo as estrogen-secreting adrenal tumors would be a useful tool.

In the present study, we characterized the expression of aromatase activity and CYP19 gene transcript in the human adrenocortical carcinoma cell line H295R. H295R is a well-known steroidogenic cell line that synthesizes adrenal androgens and cortisol (Gell et al. 1998, Ohno et al. 2002). Expression of aromatase activity has been reported in some studies (Sanderson et al. 2000, 2001a,b, 2002), but...
its precise molecular mechanism is still not clear. We also characterized the profile of the activity of the CYP19 promoter region. The results of this work should provide the basis for the molecular analysis of aromatase expression in malignant adrenocortical cells.

Materials and Methods

Materials

Forskolin was purchased from Wako Pure Chemical Industries (Osaka, Japan) and dexamethasone was obtained from Sigma. Both compounds were dissolved in ethanol, and the final concentration of ethanol in the medium was 0.1% (v/v). Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium and the mixture of penicillin (5000 U/ml) and streptomycin (5000 µg/ml) were obtained from In vitrogen. DMEM and BSA F-V (BSA) were obtained from Sigma and Nacalai Tesque (Kyoto, Japan) respectively. Other reagents and materials used included: ITS plus (insulin, transferrin, selenium; BD Biosciences, Bedford, MA, USA); Ultrasor G (Biopepra S.A., Cergy-Saint-Christophe, France); fetal calf serum (FCS; Sanko Junyaku, Tokyo, Japan); ISOGEN (Nippongene, Toyama, Japan); oligonucleotide primers (Sigma Genosys Japan, Hokkaido, Japan); Fugene 6 transfection reagent (Roche); avian myeloblastosis virus (AMV) reverse transcriptase, Taq DNA polymerase, pGL3-Basic, phRL-TK, Dual-Luciferase reporter assay system, and restriction enzymes (Promega); human placenta ribonuclease inhibitor (Takara Shuzo, Shiga, Japan); [1β,3H]androstenedione, ACS II aqueous counting scintillant and Oligo dT primer (pd(T)12–18) (Amersham); BCA protein assay kit (Pierce, Rockford, IL, USA); Quikchange site-directed mutagenesis kit and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA); Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany).

Cells

The H295R human adrenocortical carcinoma cell line was a kind gift from Prof. J I Mason (University of Edinburgh, Edinburgh, UK). H295R cells were maintained in DMEM/F-12 medium supplemented with 2% (v/v) Ultrasor G, 1% (v/v) ITS plus, penicillin (25 U/ml) and streptomycin (25 µg/ml). The human granulosa-like tumor cell line, KGN, that was established by Nishi et al. (2001) was obtained from Riken Cell Bank (Tsukuba, Japan). KGN cells were cultured in DMEM/F-12 medium supplemented with 10% (v/v) FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml). The SV-HFO human osteoblastic cells were a kind gift from Dr Hideki Chiba (Sapporo Medical University, Sapporo Japan). SV-HFO cells were maintained in DMEM supplemented with 10% (v/v) FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained as monolayer cultures in 10 cm dishes at 37°C in an atmosphere of 5% CO₂-95% air.

Aromatase assay

Aromatase activity in H295R cells was measured by the tritiated-water method based upon Ackerman et al. (1981). H295R cells were seeded at 2.5 × 10⁴ cells/well on 24-well plates. After 24 h of culturing, the medium was replaced with the treatment medium (DMEM/F-12 with BSA (0.01 mg/ml), 1% (v/v) ITS plus, penicillin (25 U/ml) and streptomycin (25 mg/ml)). After a 24-h incubation, the medium was replaced with the treatment medium containing the chemical compounds. After treatment for 24 h, the medium was replaced with the assay medium (DMEM/F-12 with penicillin (25 U/ml) and streptomycin (25 µg/ml)) containing 30 pmol [1β,3H]androstenedione. After a 2-h incubation at 37°C, 200 µl of incubation media was mixed with 200 µl of 30% trichloroacetic acid and the mixture was vortexed
vigorously. After centrifugation, 200 µl of the upper aqueous phase was mixed with 400 µl DCC (5% (w/v) charcoal, 0.5% (w/v) dextran) and the mixture was vortexed vigorously. After centrifugation, 200 µl of supernatant was mixed with 1 ml ACS II aqueous counting scintillant. The count of $[^{3}H]$OH that was released during the aromatization of $[1^{3}H]$androstenedione was measured. The amount of protein in the H295R cells in each well was measured with a BCA protein assay kit after protein extraction with 0.1% SDS. The $[^{3}H]$HOH count was normalized using the amount of protein.

**RT-PCR**

H295R cells were seeded at the population of $5 \times 10^6$ cells/dish on 10-cm dishes. After a 24-h culture, the medium was replaced with the treatment medium. After incubation for 24 h, the medium was replaced with the treatment medium containing the chemical compounds. After another 24-h incubation, total RNA was extracted using ISOGEN. First-strand cDNA was prepared from total RNA using Oligo dT primer (pd(T)$_{12-18}$), human placenta ribonuclease inhibitor and AMV reverse transcriptase according to the manufacturer's instructions. RT-PCR of the gene transcripts of CYP19 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on 1 µl of first-strand cDNA using Taq polymerase and oligonucleotide primers. The cycles were: 94°C for 30 s; 17 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 10 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 15 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. RT2 and RT8 primer were used to amplify the 395 and 289 (truncated) base pairs consisting of partial sequences of exon II and exon III of the CYP19 gene. The cycles were: 94°C for 5 min; 10 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s; 10 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s; 12 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. RT3 and RT8 primer were used to amplify the 305 base pairs consisting of partial sequences of exon PII and exon III. The cycles were: 94°C for 5 min; 10 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; 10 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 15 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. RT2 and RT8 primer were used for the amplification of 306 base pairs consisting of partial sequences of human GAPDH mRNA. The cycles were: 94°C for 5 min; 25 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 30 s. The PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gel.

**Reporter vectors**

Several lengths of the genomic sequence of promoter I.3 and promoter II of human CYP19 gene were amplified by PCR using Pfu turbo DNA polymerase and one of the following: HAPII-AS1 primer (5′-TCCCCCGGGCT CCTGTTCCTTCAAGGG-3′), HAPII-S1 primer (5′-CGGGGTATCCGGACTCC ACCTCTGGGAATGACG-3′), HAPII-S2 primer (5′- CGGGGTATCCGGCTTTCAATTGGGAATGCACG TCAC-3′), HAPII-S3 primer (5′-CGGGGTACCCACCC ACTCAAGGGCAAGATG-3′) or HAPII-S4 primer (5′- CGGGGTACCCGCTGAATTCCAAAG-3′). PCR products were digested with KpnI and SmaI, and subcloned into KpnI–SmaI-digested pGL3-Basic. Mutations of the CLS and SF-1 motifs were generated according to Michael et al. (1997) using a Quikchange site-directed mutagenesis kit and oligonucleotide primers. CLSmut (+) primer (5′-GGCTTTCCAATTGGGAAATG GAATTTGGTAGAGTCTCAGGTTCC-3′) and CLSmut (-) primer (5′-CCCTTGAGTAGGTTAGAGTGA ATTCATTCGAATGGGAAAGC-3′) were used to prepare the reporter construct that had the mutation in the CLS motif. To prepare the SF-1-mutated reporter construct, SF-1 mut (+) primer (5′-GAATTCCAGTACCTCCTACCCACTCAAGGG-3′) or SF-1 mut (-) primer (5′-CCCTTGAGTAGGTTAGAGTGA ATTCATTCGAATGGGAAAGC-3′) and SF-1-TTCmut (+) primer (5′-CAGAAGGGACTTAAGAGTCTGCA 63-3′) or SF-1-TTCmut (-) primer (5′-GAAGAGCTTTTGCTTTTCTGCAG CATTTGACCTTG-3′).

**Transfection and luciferase assay**

H295R cells were seeded at $2.5 \times 10^5$ cells/well on 24-well plates. After a 24-h culture, firefly luciferase reporter construct and seapansy luciferase internal control vector (phRL–KT) were transfected using Fugene 6 transfection reagent according to the manufacturer's instructions. After the 24-h transfection, the medium was replaced with treatment medium containing the chemical compounds and incubated for 8 h. Luciferase activity was measured using a Dual-Luciferase reporter assay system and Sirius luminometer.
The aromatase assay and transfection data for various treatments were compared with the data for vehicle treatments using Student’s *t*-test.

**Results**

**Aromatase activity in H295R**

A tritiated-water assay method was used to verify the aromatase activity in H295R. We also checked whether two well-known aromatase stimulators (forskolin (FSK) and dexamethasone (Dex)) could stimulate aromatase activity in H295R. FSK is a well-known inducer of intracellular cAMP concentration through its activation of adenylate cyclase. In the ovary, the expression of aromatase activity and *CYP19* gene transcript is primarily regulated by cAMP (Simpson et al. 1997). Dex is a synthetic glucocorticoid, and in human adipose tissue and osteoblasts, aromatase activity was stimulated by 100 nM Dex (Zhao et al. 1995, Shozu & Simpson 1998). Glucocorticoids are mainly secreted from the adrenal gland and H295R cells also synthesize cortisol; we therefore examined the possibility that glucocorticoids affected aromatase activity in an autocrine fashion. As shown in Fig. 2, aromatase activity in H295R was detected by the tritiated-water method (2·91 pmol/mg per 2 h). Aromatase activity was increased 15-fold (44·0 pmol/mg per 2 h) by 5 µM FSK. On the other hand, aromatase activity could not be stimulated by Dex. These results suggest that a signaling pathway involving cAMP, but not glucocorticoids, regulates the expression of aromatase activity in H295R.

**CYP19 gene transcript in H295R**

To determine if the up-regulation of aromatase in H295R was regulated at the stage of gene transcription, we performed RT-PCR on first-strand cDNAs from H295R after treatment with FSK or Dex. As shown in Fig. 3, RT-PCR, using the primers that correspond to exon II and exon III of the human *CYP19* gene, detected the increase in *CYP19* gene transcript after stimulation with FSK but not Dex. This result suggests that the expression of aromatase activity in H295R is regulated largely at the stage of gene transcription.

![Figure 2 Aromatase activity in H295R. H295R cells were treated with 0.1% ethanol (vehicle), or the indicated concentration of FSK or Dex for 24 h. After incubation, aromatase activity was measured as described in the Materials and Methods section. Experiments were performed in triplicate. Error bars represent the s.e.m.; asterisks indicate the up-regulation compared with vehicle control; ***P < 0.001.](image)
Promoter analysis

From the RT-PCR results, we verified that promoter I.3 and promoter II were activated in H295R by transfection of the firefly luciferase reporter vector into H295R cells. Promoter I.3 and promoter II are located within 717 base pairs upstream of exon PII/exon II (Fig. 4). Promoter II exists within exon I.3. As shown in Fig. 5, -278/+23 Luc that harbors the entire sequence of promoter II was activated in a dose–dependent manner by FSK in H295R.

Figure 3 CYP19 gene transcript in H295R. H295R cells were incubated with 25 μM FSK or 100 nM Dex for 24 h. After incubation, total RNA was extracted and first-strand cDNA was prepared. RT-PCR using exon I-specific primers was performed. See Materials and Methods section for details. Total RNA from the human ovary-derived cell line KGN and the human osteoblastic cell line SV-HFO was used as the positive control.

Figure 4 Position of exon I and putative transcription factor binding sites around exon II of the human CYP19 gene. The positions of exon I (top) and transcription factor binding sites (middle: CREα, TATA, CLS, SF-1, S1) in the partial human genomic sequence of the CYP19 gene are indicated. The position of genomic sequences inserted into firefly luciferase reporter vector (bottom) is also indicated.
Because the profile of the forskolin dose-dependency is similar to that of aromatase activity, we concluded that aromatase expression in H295R is regulated at the stage of gene transcription.

The human CYP19 gene has several sequences that respond to the increase in intracellular cAMP concentration in the promoter I.3/II region. Although a consensus CRE does not exist, CREaro (Zhou & Chen 1999) and the CLS (Michael et al. 1997) motifs have some homology to consensus CRE and have important roles in the responses of promoter I.3 and promoter II to cAMP. To verify the importance of these cAMP responsive sequences, we transfected the 5′-deleted series of the promoter I.3/II sequence and stimulated with 25 µM FSK. As shown in Fig. 6, −717/+23 Luc that has the full sequence of promoter I.3/II activity increased about sixfold in response to FSK. −278/+23 Luc has half the activity compared with −717/+23 Luc. The deleted 5′ sequence has two CREaro motifs and therefore this result reflects the importance of these motifs. −227/+23 Luc, which has the full sequence of promoter I.3/II activity, was also activated by FSK. The cAMP sensitivity of −717/+23 Luc and −227/+23 Luc is in good agreement with the result of the RT-PCR experiment that suggested that promoter I.3 and promoter II are activated in H295R cells. Another 29 base deletions (−198/+23 Luc) caused almost complete abolishment of FSK sensitivity. This 29-base sequence has CLS, suggesting that this motif is critical for the cAMP response of the promoter region. The reporter activity of −119/23 Luc also has no FSK sensitivity. These two constructs have almost the same activity profile as the empty firefly luciferase vector pGL3-Basic (Basic). There is another important sequence for the expression of steroidogenic P450 enzymes (steroidogenic factor-1 motif; SF-1) between −198 and −119. However, CLS appears to be the most important motif for the expression of CYP19 in H295R.

The results of 5′ deletion analysis suggest that the CLS motif may be critical for the response of promoter II to FSK. Furthermore, the SF-1 motif is an important sequence for the regulation of steroidogenic P450 enzyme gene expression. In addition, the silencer element (S1) abuts the SF-1 motif. An S1 mutation increases promoter II activity in human adipose tissue (Zhou & Chen 1998). Based on this information, we prepared mutated promoter II reporter vectors that have mutations in the CLS, SF-1 or S1 motifs. As shown in Fig. 7, a mutation in CLS abolished the response to FSK. Mutation of the SF-1 motif also abolished the response to FSK. These results seem to support the importance of these two motifs. The S1-mutated promoter II sequence has some FSK sensitivity, but diminished promoter activity, suggesting that the role of this motif in H295R would be different to its role in human adipose tissue.

**Figure 5**  Activation of promoter II sequence of human CYP19 gene in H295R. −278/+23 Luc firefly luciferase vector was transfected into H295R cells with a seapansy luciferase internal control vector (pRL-TK) using Fugene 6 transfection reagent. After 24 h of transfection, the cells were treated with 25 µM FSK for 8 h. Luciferase activity in the cell lysates was measured. Experiments were performed in triplicate. Error bars represent the S.E.M.; asterisks indicate up-regulation compared with the vehicle control; **P<0.01, ***P<0.001.
adrenocortical carcinoma cell line H295R. Aromatase activity and gene transcripts were up-regulated by an intracellular signaling pathway involving cAMP, but not by glucocorticoids; this suggests that ACTH or other agents that increase intracellular cAMP concentration are stimulators of aromatase activity in estrogen-secreting adrenal tumors \textit{in vivo}. Further experiments are needed to identify the true stimulator \textit{in vivo}. Exon I.3 and exon II were selected as the 5' untranslated regions. These findings are in good agreement with results from adrenal tumors (Young \textit{et al.} 1996, Watanabe \textit{et al.} 2000). Dependency on cAMP and exon I usage indicate that the molecular mechanism of aromatase expression in adrenal tumors is similar to that in the ovary. Promoter I.3 and promoter II are also activated in human adipose tissue, however, the mutation of the S1 motif did not increase promoter activity but rather decreased it in H295R cells. Therefore, the mechanism of activation of promoter I.3/II in H295R

![Deletion analysis of promoter I.3/II region in the CYP19 gene.](image)

**Figure 6** Deletion analysis of promoter I.3/II region in the CYP19 gene. Firefly luciferase reporter vectors harboring 5'-deleted series of promoter I.3/II region in human CYP19 gene were transfected into H295R cells with a seapansy luciferase internal control vector (pRL-TK) using Fugene 6 transfection reagent. After 24 h of transfection, the cells were treated with 25 \mu M FSK for 8 h. Luciferase activity in the cell lysates was measured. Experiments were performed in triplicate. Error bars represent the S.E.M.; asterisks indicate up-regulation compared with the vehicle control; \(*P<0.05, **P<0.01\).

![Mutation analysis of promoter II region in the CYP19 gene.](image)

**Figure 7** Mutation analysis of promoter II region in the CYP19 gene. -278/+23 Luc and its mutants that have mutations in CLS, SF-1 and S1 motifs were transfected into H295R with a seapansy luciferase internal control vector (pRL-TK) using Fugene 6 transfection reagent. After 24 h of transfection, the cells were treated with 25 \mu M FSK for 8 h. Luciferase activity in the cell lysates was measured. Experiments were performed in triplicate. Error bars represent the S.E.M.; asterisks indicate up-regulation compared with the vehicle control; \(*P<0.01, **P<0.001\).
would appear to be different to that in adipose tissue. The molecular mechanism of CYP19 gene expression may even be different to that in ovary. For example, CRE-binding protein (CREB), which is reported to be a binding protein for the CLS motif in promoter II (Michael et al. 1997), is not expressed in H295R cells; elevated expression of CRE modulator (CREM) may compensate for the lack of CREB (Groussin et al. 2000). Further characterization of the molecular mechanism, including the binding protein for CLS and S1 motifs, may clarify the specific mechanism of CYP19 gene expression in adrenal tumors.

The H295R cell line was established as NCI-H295 from a 48-year-old woman (Gazdar et al. 1990) whose menses had ceased. This patient had no symptoms caused by high concentrations of estrogen. In addition, radioimmunoassay (RIA) analysis of the culture media of this cell line failed to detect either estrone or estradiol. This could be because of the very low concentrations of aromatase substrates. R1A also failed to detect testosterone and the concentration of androstenedione was very low (dehydroepiandrosterone was 75 900 ng/10⁸ cells per 24 h, but androstenedione was 28 ng/10⁸ cells per 24 h). Taking into consideration the high expression of aromatase activity in H295R cells, the loss of estrogen excess in the donor patient was caused by the low concentration of aromatase substrates, at least around the tumor. Boys would have adequate concentrations of androstenedione and testosterone, most likely from the testes. In girls, theca cells in the ovaries would secrete sufficient levels of aromatizable androgens. Further investigation is needed of the systemic or local conditions that cause estrogen excess, including aromatase substrate and up-regulators of aromatase expression in adrenal tumors. H295R cells may be a powerful tool with which to clarify the mechanism underlying this phenomenon.

Some adrenal tumors have high aromatase activity whereas normal adrenal tissues have no detectable aromatase activity. Thus, some specific molecular mechanism that makes adrenal cells transform into a tumor may be involved in expression of the CYP19 gene in adrenal tumors. Comparison of the molecular mechanism of promoter I/II activation between normal adrenal cells and H295R should provide some useful information about the molecular mechanism of transformation into an adrenal tumor.

Aromatase inhibitors may be potent tools with which to treat precocious puberty in patients with estrogen-secreting adrenal tumors. Unfortunately, the inhibition of aromatase activity throughout the entire body inhibits aromatase activity in peripheral tissues, including bone. Inhibition of aromatase activity in bone tissue would affect bone growth and the maintenance of bone mineral density. To avoid this unwanted effect, adrenal-specific inhibition of aromatase expression is required. The development of an adrenal-specific inhibitor would be based upon an adrenal-specific molecular mechanism of CYP19 expression. H295R cells would appear to be a useful tool for the development of this type of inhibitor.

In summary, H295R cells that have similar profiles of aromatase expression would be a powerful tool with which to investigate the effects of hormones and chemicals, as well as their molecular mechanisms, upon aromatase in adrenal tumors.

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References


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