Retinoic acids promote the action of aromatase and 17β-hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17β-estradiol in placental cells

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Abstract

The biosynthesis of 17β-estradiol (E2) in human placenta involves the actions of aromatase and 17β-hydroxysteroid dehydrogenase type 1 (17HSD1). Aromatase, an enzyme complex comprised of P450aromatase (P450arom) and NADH-cytochrome P450 reductase, converts androgens to estrogens, whereas 17HSD1 catalyzes the reduction of estrone to E2.

In the present study, the effects of retinoic acids (RAs) on P450arom and 17HSD1 expression in placental cells were investigated. Treatment with all-trans-RA (at-RA) or 9cis-RA increased E2 production in JEG-3 choriocarcinoma cells and cytotrophoblast (CTB) cells isolated from normal early placentas. Meanwhile, the activity of aromatase and expression of P450arom mRNA were induced by at-RA in JEG-3 cells. Northern blot analysis showed that the effect on P450arom mRNA expression occurs in a dose- and time-dependent fashion. Similar to at-RA and 9cis-RA, Ro40–6055, the retinoic acid receptor α (RARα)-selective activator, increased the expression of P450arom and 17HSD1 mRNA in JEG-3 cells. On the other hand, Ro41–5253 (Ro41), the RARα-selective antagonist, blocked the stimulatory effect of RAs on P450arom expression. Surprisingly, Ro41 induced the activity and mRNA expression of 17HSD1 in JEG-3 cells, which is in contrast to the expected inhibitory effect and, moreover, remarkably potentiated the induction by at-RA and 9cis-RA. However, reporter gene analysis revealed that the influence of Ro41 on the transcription of the HSD17B1 gene, which encodes 17HSD1, is considerably milder in JEG-3 cells, and it only additively enhanced the effect of at-RA. Finally, it was found that at-RA and 9cis-RA increased the expression of P450arom and 17HSD1 mRNA in CTB cells, but to a lesser extent.

The data suggest that RAs may play a role in promoting the biosynthesis of E2 in the placenta. In addition, Ro41 has divergent effects on gene expression in JEG-3 cells.


Introduction

The placenta is a unique organ of the feto-maternal unit. It not only anchors the fetus physically to the uterus and co-ordinates the dialogue between the embryo and the maternal body, but it also acts as an endocrine unit for the production of the hormones needed by the maternal body and the fetal organs. In the human, the functions of the ovary gradually decline after fertilization and, concurrently, the placenta becomes the primary site of estrogen biosynthesis during pregnancy. By 7 weeks of gestation, nearly all estrogens in the circulation are produced by the placenta (Simpson & MacDonald 1981). Unlike the ovary, the human placenta is not capable of de novo estrogen synthesis from cholesterol because of the absence of the 17α-hydroxylase/C17–20Lyase, which hydroxylates C21 steroids at position C17. Alternatively, it uses fetal-derived androgens, such as dehydroepiandrosterone (DHEA) and DHEA sulfate, as precursor molecules to synthesize estrogens (Albrecht & Pepe 1990, Conley & Mason 1990).

The biosynthesis of estrogens in the human placenta occurs in trophoblast cells, involving the action of two steroidogenic enzymes: aromatase and 17β-hydroxysteroid dehydrogenase type 1 (17HSD1). Aromatase catalyzes the aromatization of androgens to estrogens, for example, androstenedione (A-dione) to estrone (E1) and testosterone to 17β-estradiol (E2), the active form of estrogens, while 17HSD1 is responsible for the conversion of E1 to E2. Both enzymes are abundantly expressed in syncytiotrophoblast (STB) cells, the most active endocrine cell type in the placenta (Fournet-Dulguerov et al. 1987).

Aromatase is an enzyme complex that consists of two microsomal proteins: P450aromatase (P450arom) and 17HSD1.
and NADH–cytochrome P450 reductase. The non-
steroidalogenic cells transfected with the full-length
P450arom cDNA are capable of aromatization of various
C19 androgens to estrogens (Corbin et al. 1988, Pompon
et al. 1993). Also, the dynamic changes in the P450arom
mRNA level correlate well with aromatase activity (Evans
et al. 1987). On the other hand, the reductase component
of the aromatase complex is only modestly regulated
(Steinkampf et al. 1987). These results indicate that
aromatase activity is primarily determined by P450arom.

P450arom is encoded by the CYP19 gene, which
contains 10 exons and 9 introns (Means et al. 1989).
Compared with the other members of the P450 gene
superfamily, the CYP19 gene is exceptionally large, over
250 kb in size. In the human, P450arom is expressed in
various tissues, such as the ovary, placenta and adipose
tissue (Means et al. 1991, Jenkins et al. 1993, Kilgore et
Selective use of distinct tissue-specific promoters and the
subsequent post-transcriptional process using alternative
polyadenylation signals at the 3′-ends of transcripts result
in tissue-specific expression of a number of P450arom
mRNA species (Simpson et al. 1994).

The gene encoding 17HSD1 is HSD17B1 (previously
also called EDH17B2) containing two transcription start
sites, which generate 1.3 and 2.3 kb mRNA species
(LuuThe et al. 1990, Peltoketo et al. 1992). Of these two
transcripts, only the 1.3 kb mRNA is evidently believed to
be translated into 17HSD1 protein (Miettinen et al. 1996).
Our recent studies demonstrated that the transcription of
the HSD17B1 gene is controlled by a promoter, a silencer
and an enhancer located in the 5′-flanking region (Piao
et al. 1995, 1997a, Leivonen et al. 1999). The cell
specificity of the enhancer determines the cell-specific
expression of the HSD17B1 gene (Piao et al. 1995).

In addition to being nutritional signals, derivatives of
vitamin A called retinoids have been suggested to a
function of placental trophoblast cells (Matsuo &
Strauss 1994). A number of studies have shown that
retinoids regulate the biosynthesis of several placental
hormones, such as progesterone, human chorionic gona-
dotropin (hCG) subunits, and placental lactogen (Kato &
Braunstein 1991, Matsuo & Strauss 1994, Stephanou &
Handwerger 1995, Guibourdenche et al. 1999a,b). Also,
retinoids induce leptin synthesis and secretion in STB cells
(Guibourdenche et al. 1999a,b). Our previous work demon-
strated that retinoic acids (RAs) induce 17HSD1 expres-
sion in choriocarcinoma cells (Piao et al. 1999). In
addition, a recent report showed that they have a stimu-
atory effect on aromatase activity in breast cancer cells
(Mu et al. 2000). These data prompted us to further
examine whether RAs affect E2 biosynthesis in placental
cells. In the present study, we examined the effects of RAs
on E2 biosynthesis in cultured human cytotrophoblast
(CTB) cells and the JEG-3 choriocarcinoma cell line
derived from human trophoblast. JEG-3 cells retain the
capability of converting C19 steroids to estrogens (Bahn
et al. 1981) and have been widely used for the study of the
production of placental hormones. We have focused on
how RAs, either independently or in combination with
other factors, affect the expression of P450arom and
17HSD1. Our data suggest that RAs may promote the
biosynthesis of E2 in the placenta.

Materials and Methods

Chemicals and reagents

The isotopes [2,4,6,7-3H]E1 (101 Ci/mmol), [2,4,6,7-
3H]E2 (98 Ci/mmol) and [1β-3H]A-dione (24 Ci/mmol)
were obtained from Amersham Life Science (Little
Chalfont, Bucks, UK). [3H]Acetyl coenzyme A
(200 mCi/mmol, CAT assay grade) was from Du Pont
NEN (Boston, MA, USA). [α-32P]dCTP (3000 Ci/
mmol) was purchased from Yahui Biomedical Engineering
Inc. (Beijing, China). Epidermal growth factor (EGF),
8-bromo-adenosine-3′,5′-monophosphate, cyclic, free
acid (8Br-cAMP), N-[(1-2,3-dioleoyloxy)propyl]-
N,N,-trimethyl-ammoniummethylsulfate (DOTAP) and
chloramphenicol acetyl transferase (CAT) were the pro-
ducts of Boehringer Mannheim (Mannheim, Germany).
Trypsin, DNase I, fetal calf serum (FCS), bovine serum
albumin (BSA), all-trans RA (at-RA), interleukin-1β
(IL-1β), bovine insulin, DHEA, A-dione, E1 and E2 were
from Sigma Chemical Co. (St Louis, MO, USA). 12-O-
tetradecanoylphorbol-13-acetate (TPA) was supplied by
Calbiochem-Novabiochem Inc. (La Jolla, CA, USA).
AMV reverse transcriptase, Taq DNA polymerase,
RNasin and restriction enzymes were obtained from
Promega Co. (Madison, WI, USA). TRIZol reagent, nix
translation system and media for cell culture were pur-
chased from Gibco BRL-Life Technologies (Grand Island,
NY, USA). Plates for cell culture were obtained from
Nunc Inter Med (Roskilde, Denmark). 9cis-RA, the
selective retinoic acid receptor (RARα) activator Ro40–
6055 (Ro40) and inhibitor Ro41-5253 (Ro41) were gifts from
F Hoffmann-La Roche Ltd (Basel, Switzerland). The
antiserum against E2 was raised in this laboratory and has
been used in previous studies (Zhuang & Li 1991,
Rong-Hao et al. 1996). The plasmid containing hu-
man P450arom cDNA was provided by Professor Olavi
Pelkonen (Department of Pharmacology, University of
Oulu, Finland).

Cell culture

JEG-3 choriocarcinoma and T47D breast cancer cell
lines were obtained from the American Type Culture
Collection (Rockville, MA, USA). JEG-3 cells were
maintained in Dulbecco’s Modified Eagle’s Medium
(DMEM) supplemented with 10% FCS, 2 mM glutamine,
1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulfate. T47D cells were cultured in RPMI 1640 medium containing 10% FCS, 1 mM non-essential amino acids and 0.2 IU insulin/ml. Before each experiment, the cells were detached with 0.025% trypsin–0.02% EDTA. JEG-3 cells were applied to 24-well plates (2 × 10^5 cells/well) for E_2 detection, 6-well plates (2.5 × 10^5 cells/well) for the measurement of aromatase and reductive 17HSD activities, and 10 cm plates (2.5 × 10^6 cells/plate) for Northern blot analysis. The cells were allowed to grow for 24 h in the presence of DMEM for JEG-3 cells and F12:DMEM=1:1) containing 10 ng/ml EGF, 10 µg/ml insulin, 2 mM glutamine and 0.1% BSA. 

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### Analysis of the conversion of DHEA/A-dione to E_2 in cultured cells

After treatment with various stimuli for 24 h, JEG-3 and CTB cells cultured in 24-well plates were washed twice with serum-free media (DMEM for JEG-3 cells and FD for CTB cells). Then, 1 ml of the same medium containing 500 nM DHEA or A-dione was added to each well. The cells were incubated for 1 h at 37 °C. E_2 was detected by adding 0.5 ml 30% trichloroacetic acid to the media and then the plates were placed in an ice bath for 10 min. After that, the media were extracted with 5 ml diethyl ether–ethyl acetate (9:1) twice, for 15 min each time. A 900 µl aliquot of the aqueous phase was measured in a scintillation counter.

### Reductive 17HSD activity

Reductive 17HSD activity was determined by calculating the conversion rate of E_2 to E_1. After JEG-3 cells had been stimulated for 24 h, the media were removed from the 6-well plates and 2 ml serum-free medium containing 500 nM unlabeled E_1 and 2.5 × 10^5 c.p.m. of [2,4,6,7-3H]E_1 was added to each well. The cells were then incubated for 1 h at 37 °C in cell culture conditions and the subsequent steps followed the method described previously (Miettinen et al. 1996).

### RNA isolation and Northern blot analysis

Total RNAs from cultured cells were isolated using TRIzol reagent according to the manufacturer’s instruction. Fifteen micrograms of each sample were resolved on a 1% agarose/formaldehyde gel and then transferred onto a Hybond nylon membrane (Amersham International plc, Amersham, Bucks, UK) by pressure blotting. The subsequent steps followed a method described previously (Xu et al. 2000). The same membrane was separately probed by the 32P-labeled 1.0 kb EcoRI–SacI fragment of human 17HSD1 cDNA (Piao et al. 1997b), 1.4 kb SacI fragment of human P450arom cDNA (Ghersevich et al. 1994) and glyceraldehyde–3-phosphate dehydrogenase (GAPDH) cDNA. The autoradiographic signals were quantified by densitometry analysis with a Molecular Dynamics 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

### Transfection and CAT assay

The construct pBL(−661/−392)CAT2 containing 17HSD1 enhancer has been used in our previous study (Piao et al. 1995). The enhancer is located at the region from −661 to −392 in the gene, with respect to the transcription start site for the 1.3 kb 17HSD1 mRNA. Transient transfection experiments were performed as described previously (Piao et al. 1995). Briefly, the cells were applied to 6 cm plates in amounts of 7.5 × 10^5 cells/plate (JEG-3) or 1.5 × 10^8 cells/plate (T47D) and cultured for 24 h before transfection. Five micrograms of each plasmid construct were transfected into the cells using the transfection reagent DOTAP (5-µg/ml). After 18 h, the media were replaced and the cells were cultured for a further 48 h in the presence of various stimuli.
After transfection, the cells were collected and subjected to four freeze–thaw cycles (freezing in dry ice/ethanol for 5 min and thawing at 37 °C for 3 min) in 150 µl 0·25 M Tris–HCl (pH 7·8). The treatment was followed by heat inactivation at 65 °C for 20 min, after which the CAT activity of the samples was measured by fluor diffusion assay (Neumann et al. 1987). Protein concentration was measured by a Bio–Rad protein assay system (Bio–Rad Laboratories, Richmond, CA, USA). CAT expression is measured by a Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA, USA). CAT expression is described as ng/mg protein resulting from comparison of the CAT activity in samples with the CAT standard curve.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The primers used for RT-PCR analysis of retinoid X receptors (RXRs) and RARs are summarized in Table 1. Total RNA (1·0 µg) of JEG-3 cells was reverse transcribed using an oligo(dT) primer in a total volume of 20 µl. For PCR amplification, 2 µl of RT products was added to a mixture (50 µl) containing 20 mM Tris–HCl (pH 8·4), 50 mM KCl, 1·5 mM MgCl₂, 0·25 mM dNTPs, 1 µM sense and antisense primers, and 5 U Taq DNA polymerase. The reaction was performed in 30 sequential cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, using the GenAmp PCR System 2400 (Perkin-Elmer Corporation, Norwalk, CT, USA).

Statistics

All experiments were done at least twice, and statistical significance was determined by analysis of variance or Student's t-test where appropriate.

Results

Actions of RAs on E₂ biosynthesis in cultured JEG-3 and CTB cells

JEG-3 and CTB cells were treated with 100 nM at-RA or 9cis-RA for 24 h, and the capacity of treated cells to convert DHEA to E₂ was examined by measuring the E₂ concentration in the media. In JEG-3 cells, both at-RA and 9cis-RA stimulated E₂ production over 2·2-fold (Fig. 1A). In CTB cells, at-RA and 9cis-RA caused 2·0- and 2·8-fold increases in E₂ synthesis respectively. Also, the conversion of A-dione to E₂ in JEG-3 cells was increased over 2·0-fold by the same administration of at-RA or 9cis-RA (Fig. 1B).

Induction of aromatase activity by at-RA, 8Br-cAMP, TPA and IL-1β in JEG-3 cells

Since aromatase is involved in E₂ biosynthesis, aromatase activity was analyzed after the treatment of JEG-3 cells with 0·1–1000 nM at-RA for 48 h. Increased aromatase activity (2·0-fold) was detected after treatment with 0·1 nM at-RA, and the activity was further enhanced dose-dependently, reaching 3·8-fold at 1000 nM. On the other hand, when the cells were stimulated with 100 nM at-RA, significantly increased aromatase activity (4·0-fold) could be observed at 6 h, and the induction remained for up to 48 h (Fig. 2).

In addition to at-RA, 8Br-cAMP, TPA (the protein kinase C activator) and IL-1β increased aromatase activity to different extents in JEG-3 cells. Furthermore, simultaneous administration of at-RA with 8Br-cAMP or TPA potentiated the stimulatory effect of at-RA on aromatase activity, while IL-1β merely enhanced the induction in an additive manner (Fig. 3).

Effect of at-RA, 8Br-cAMP and TPA on the expression of P450arom mRNA in JEG-3 cells

To elucidate whether the transcription of the CYP19 gene is affected, JEG-3 cells were treated for 6–48 h with different doses of at-RA, ranging from 0·1 to 1000 nM, and P450arom expression was determined by Northern blot analysis. Similar to the effect of at-RA on aromatase activity, an increased expression of P450arom mRNA was

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Positions (nt)</th>
<th>Product size (bp)</th>
</tr>
</thead>
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<tr>
<td>RARα</td>
<td>Sense: CTGCCGACACCTGGGGGC 123–141</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: GTGTGGTAGGCTGGTGG 264–282</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RARβ</td>
<td>Sense: TTCAATCGGGGGCTGGGAAA 322–345</td>
<td>154</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: CGTTCCTCTAGTACAAAC 457–475</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RARγ</td>
<td>Sense: GACTCCTGCTGCTGGT 434–452</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: GCTGCCGGGACCGACCTGC 577–595</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RXRα</td>
<td>Sense: ATGGACACAAACATTTCTGC 76–97</td>
<td>268</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: GGGACCCGAAGTCTGGAC 325–343</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RXRβ</td>
<td>Sense: ATGTCTTGGCCGCTGGCC 118–136</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: GCTGCCGGGACCGACCTTAG 232–250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RXRγ</td>
<td>Sense: GAGGCTCTCCTGTCACACAC 74–92</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: GGTGCCGAGACTCACTGC 184–202</td>
<td></td>
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</tr>
</tbody>
</table>
observed after treatment of the cells with 0.1 nM at-RA, and the stimulation was further increased with the elevation of the at-RA concentration tested, reaching a 4.8-fold maximal value compared with the control level (Fig. 4, left panel). Unlike the influence on the enzyme activity, the induction on P450arom mRNA expression appeared to be time-dependent. Treatment of cells with 100 nM at-RA caused a 1.4-fold increase in the mRNA level at 6 h, and the stimulation continued to rise in parallel with the time-course, reaching 2.7-fold at 48 h (Fig. 4, right panel).

The influence of at-RA on CYP19 gene expression in JEG-3 cells was also examined in the presence of 8Br-cAMP and TPA. The expression of 3.0 and 2.4 kb P450arom mRNA was remarkably increased by treatment of the cells with 20 nM TPA and especially 1.5 mM 8Br-cAMP. Both compounds further enhanced the effects of at-RA (Fig. 5).
Effects of the selective RARα agonist Ro40 and antagonist Ro41 on the expression of P450arom and 17HSD1 mRNA in JEG-3 cells

The effects of the selective RARα agonist Ro40 and antagonist Ro41 on P450arom and 17HSD1 expression in JEG-3 cells were investigated by Northern analysis for further elucidation of the regulatory mechanisms of RAs (Fig. 6). Treatment of cells with 100 nM Ro40 for 24 h resulted in a stronger induction of P450arom expression than treatment with 100 nM at-RA or 9cis-RA. This stimulation, as expected, was blocked by simultaneous administration of 1·0 µM Ro41, which also decreased the effects of at-RA and 9cis-RA. Unexpectedly, in addition to being induced by at-RA (9·6-fold), 9cis-RA (8·5-fold) and Ro40 (6·8-fold), 17HSD1 expression was also increased by Ro41 (3·6-fold). Furthermore, Ro41 in combination with at-RA and 9cis-RA caused 28- and 24-fold induction respectively. On the other hand, co-treatment with Ro40 and Ro41 resulted in an additive increase in 17HSD1 expression.

Effects of Ro40 and Ro41 on reductive 17HSD activity in JEG-3 cells

To confirm the stimulatory influence of Ro40 and Ro41 on 17HSD1 expression, the reductive 17HSD activity of cultured JEG-3 cells was determined after the same treatment of the cells as for mRNA analysis. Stimulation with 100 nM at-RA, 9cis-RA and Ro40 as well as 1 µM Ro41 led to 2·5-, 2·5- 2·3- and 1·7-fold increases in reductive 17HSD activity respectively. After the cells had been treated with 1 µM Ro41 in combination with 100 nM at-RA, 9cis-RA and Ro40, the induction was potentiated to 5·4-, 5·2- and 3·4-fold respectively (Fig. 7).

Effect of Ro41 on HSD17B1 enhancer

Reporter gene analysis, using the construct pBL(−661/−392)CAT2 and the control vector pBLCAT2, was conducted to further understand how Ro41 affects the transcription of the HSD17B1 gene. In the construct pBL(−661/−392)CAT2, the HSD17B1 enhancer containing an RA response element (RARE) at the position from −512 to −479 is linked to a thymidine kinase (TK) promoter, whereas pBLCAT2 merely has a TK promoter (Piao et al. 1995). Reporter gene analysis showed that the TK promoter alone has almost no activity and cannot be induced by at-RA treatment in both T47D and JEG-3 cells. When it was connected with the HSD17B1 enhancer, however, the TK promoter displayed significant transcriptional activity (Fig. 8). In T47D cells, Ro41 (1 µM) did not affect pBL(−661/−392)CAT expression, but blocked the effect of at-RA (100 nM) remarkably. In JEG-3 cells, treatment with 100 nM at-RA and 1 µM Ro41 enhanced pBL(−661/−392)CAT expression to 260% and 130% respectively. After the cells were stimulated with both at-RA and Ro41, the CAT expression was additively increased to 300% (Fig. 8).
Figure 3 Interaction of at-RA with various factors on aromatase activity in JEG-3 cells. Aromatase activity was examined after the cells had been incubated for 24 h with (1) vehicle, (2) 100 nM at-RA, (3) 1.5 mM 8Br-cAMP (cAMP), (4) 20 nM TPA, (5) 25 pg/ml IL-1β, (6) 100 nM at-RA + 1.5 mM 8Br-cAMP, (7) 100 nM at-RA + 20 nM TPA, and (8) 100 nM at-RA + 25 pg/ml IL-1β. The data show the means ± range of two independent experiments with duplicate samples in each. **P<0.01, ***P<0.001.

Figure 4 Dose- and time-effect of at-RA on P450arom mRNA expression in JEG-3 cells. The cells were treated with 0.1–1000 nM at-RA for 48 h (left panel), or 100 nM at-RA for 6–48 h (right panel). Northern blot analysis was performed using total RNA isolated from the treated cells. The positions of the 3.0 kb and 2.4 kb P450arom transcripts are indicated by the arrows. The numbers below the P450arom signals represent the intensity ratio (3.0 kb P450arom/GAPDH) compared with the values (defined as 1.0) of the control samples. Two independent experiments were done with similar results and representative figures are shown.
RAR and RXR transcripts in JEG-3 cells

In order to further investigate the possible mechanisms of RAs, especially the profile of retinoid receptors, RT-PCR analysis was performed using total RNAs isolated from JEG-3 cells as the template and the primer set specific to each subtype of RXR and RAR. As shown in Fig. 9, RAR/afii9825 and RXR/afii9825 were found whereas other members of RXR and RAR could not be detected.

Effects of RAs on the expression of 17HSD1 and P450arom mRNA in cultured CTB cells

Finally, the expression of 17HSD1 and P450arom in CTB cells was also examined. Upon stimulation with 100 nM at-RA or 9cis-RA for 24 h, the expression of the 3.0 kb mRNA of P450arom in CTB cells was increased to 130% by at-RA and 170% by 9cis-RA. 17HSD1 expression was also elevated by the same treatment, but to a lesser extent (Fig. 10).

Discussion

Estrogens play a crucial role during human pregnancy (Loriaux et al. 1972). Even though it is well known that the placenta is the major site of estrogen production in pregnant women, the primary factor(s) affecting estrogen biosynthesis are difficult to define because of the complexity of the placental endocrine environment. The present study showed that RAs promote E2 production not only in JEG-3 cells but also in CTB cells isolated from normal placenta. These results indicate that RAs may act as regulatory factors of E2 biosynthesis in the placenta.

P450arom and 17HSD1 play essential roles in placental E2 biosynthesis. We recently demonstrated that RAs stimulate 17HSD1 expression in JEG-3 cells, and the effect is mediated by an RARE in the enhancer region of the HSD17B1 gene, which encodes 17HSD1 (Piao et al. 1995, 1997b). In the present work, it was revealed that aromatase is also subject to regulation of RAs in JEG-3 cells at the levels of both enzyme activity and P450arom mRNA expression. The data further demonstrate that RAs play a role in regulating key steroidogenic enzymes in choriocarcinoma cells.

The expression of the CYP19 gene in the placenta and choriocarcinoma cells is controlled by a distal promoter I.1 located adjacent upstream of exon I.1 in the gene (Means et al. 1991). Recently, an imperfect palindromic sequence 5'-AGGTCA TGCCC-3', situated at the region from -183 to -172 within the promoter has been identified to be functional as an RARE. This sequence binds the
RXRα/VDRα (vitamin D receptor α) heterodimer and mediates transactivation of promoter I.1 by RXR- and VDR-selective agonists in JEG-3 cells (Sun et al. 1998). In the bandshift experiment, the complex formed by this element and nuclear extracts isolated from JEG-3 cells was not recognized by the specific antibody against RARα, indicating that the sequence is not bound by the RXRα/\ VDRα heterodimer. On the other hand, the RAR-selective ligand TTNBP induced the promoter activity as well as the aromatase activity, indicating that RXR/RAR heterodimer(s) are involved in regulating the expression of the CYP19 gene in JEG-3 cells (Sun et al. 1998). In the present study, RT-PCR analysis showed that JEG-3 cells express only RXRα and RARα. It was also found that at-RAL, the ligand for RARs, as well as Ro40, a specific ligand for RARα, induced P450arom mRNA expression and, further, this induction was blocked by Ro41, the selective inhibitor for RARα. Collectively, it is indicated that another element, which can interact with RXRα/\ VDRα heterodimer, may exist in the regulatory region in the CYP19 gene.

In this study, we observed that a short-period stimulation (6 h) with at-RA resulted in a maximal increase in aromatase activity in JEG-3 cells. On the other hand, the influence on the mRNA expression of P450arom appeared to be time-dependent, reaching the highest value at 48 h as examined. Also, the extent of induction on enzyme activity was higher than that on mRNA expression. Thus, the action of RAs on aromatase in choriocarcinoma cells may occur at translational or post-translational levels, in addition to increasing the transcription of the CYP19 gene or stabilization of P450arom transcripts.

P450arom expression in choriocarcinoma cells is regulated by the protein kinase A and protein kinase C pathways (Ritvos & Voutilainen 1992). Also, IL-1β stimulated the aromatase activity in CTB cells (Nestler 1993). The present study demonstrated that the induction of RAs on aromatase activity is enhanced by cAMP, TPA and IL-1β in JEG-3 cells. In addition, both cAMP and TPA elevated the RA-induced expression of P450arom mRNA. It should be noted that, compared with retinoids, cAMP and TPA appeared to be more potent inducers for P450arom expression. However, retinoids are nutritional molecules exclusively available in the placenta and therefore have the potential to play a significant role in the biosynthesis of E2. The above data, together with our recent finding that a number of factors, such as EGF, TPA, cAMP and calcium ion, interact with RAs in regulating the action of 17HSD1 in JEG-3 cells (Piao et al. 1997b), indicate that E2 biosynthesis in trophoblastic cells may be controlled by complicated mechanisms.

Apart from aromatase and 17HSD1, 3HSD1 also participates in placental E2 biosynthesis by catalyzing the conversion of DHEA to A-dione. Thus, the effect of RAs on the expression of 3HSD1, if any, may also count for...
increased production of E₂ from DHEA. However, the conversion of A-dione to E₂, which does not involve the action of 3 HSD1, was similarly induced by RAs in JEG-3 cells, implying that aromatase and 17HSD1 are the main targets for the action of RAs, at least in JEG-3 cells.

Ro41 has been identified to act as a selective antagonist for RARα (Apfel et al. 1992), but this does not readily explain the effect of Ro41 on 17HSD1 expression in JEG-3 cells. In a previous report, Ro41 was demonstrated to inhibit the stimulation of at-RA on 17HSD1 expression in breast cancer T47D cells (Reed et al. 1994). Consequently, this study also showed that Ro41 blocks the activation of at-RA on the transcription of the HSD17B1 gene in the same cells. In contrast, Ro41 induced the expression and activity of 17HSD1 in JEG-3 cells and, moreover, potentiated the effects of RAs. However, the same phenomenon was not identically observed in reporter gene analysis showing that, although Ro41 was able to increase the enhancer activity in JEG-3 cells, the extent was noticeably less than its influence on the abundance of 17HSD1 mRNA. Moreover, the combined effect of Ro41 and at-RA on HSD17B1 enhancer in JEG-3 cells was only additive, not as synergistic as was found in mRNA analysis of 17HSD1. Therefore, the effect of Ro41 on the expression of 17HSD1 in JEG-3 cells seems...
to occur at both transcriptional and post-transcriptional levels, especially the mRNA stabilization. Alternatively, Ro41 may activate a subtype of retinoid receptors, which is targeted to an unknown regulatory area in the HSD17B1 gene rather than the RARE located at the region from –512 to –479. Nevertheless, this phenomenon is probably specific to JEG-3 cells, because Ro41 did block the stimulatory effect of at-RA on 17HSD1 expression in breast cancer cells (Reed et al. 1994).

Accumulating evidence suggests that retinoids regulate the production of key placental hormones and steroidogenic enzymes in JEG-3 cells (Kato & Braunstein 1991, Matsuo & Strauss 1994, Stephanou & Handwerger 1995, Piao et al. 1997b, Guibourdenche et al. 1998a, Tremblay et al. 1999). It was recently reported that RXRa and RARα are expressed in JEG-3 cells, and RXRa mediates the biological effects of retinoids on cell function, i.e. hCG secretion. On the other hand, neither Western blot nor Northern blot analyses could detect the presence of RXRB, RXRγ, RARβ and RARγ in the JEG-3 cells (Guibourdenche et al. 1998a). In agreement with these data, our RT-PCR analysis revealed that only RARα and RXRa are present in JEG-3 cells. Meanwhile, we found that RARα is also a mediator of the action of retinoids on JEG-3 cells, since Ro40, the selective ligand for RXRa and at-RA, the ligand for RARα, stimulated the expression of P450arom and 17HSD1 mRNA. It can also be speculated that the effects are mediated by RXRα/RARα, which is the only form of heterodimer available in JEG-3 cells.

It is agreed that STB cells, which are differentiated from CTB cells, are mainly responsible for hormone production in the placenta. Correspondingly, P450arom and 17HSD1 are exclusively expressed in STB cells in vivo (Fournet-Dulguerov et al. 1987). However, cultured CTB cells isolated directly from the placenta cannot be used as a model for in vitro studies because of their lack of viability under cell culture conditions. On the other hand, in vitro cultured CTB cells possess some features of STB cells, such as the ability to produce hCG, E2 and progesterone (Kliman et al. 1991, Zhuang & Li 1991). They could therefore be employed as a cell model to investigate the mechanisms of steroidogenesis in the placenta. In this study, it was clearly shown that CTB cells cultured in vitro express both P450arom and 17HSD1, and are capable of producing E2 from DHEA. In situ hybridization and immunohistochemical staining also confirmed 17HSD1 expression in CTB cells during the first trimester (our unpublished data).

In the human placenta, RARα and RXRa are the major forms of retinoid receptors expressed in trophoblastic cells, and RXRa has been proved to be involved in regulating the production of hCG and leptin (Roulier et al. 1994, Guibourdenche et al. 1998b,c). In line with these observations, the present study demonstrated that at-RA, the ligand for RARα and especially 9cis-RA, the ligand for RXRs, can promote E2 production as well as the expression of P450arom and 17HSD1 mRNA in cultured CTB cells. On the other hand, the extent of the induction of RAs on P450arom and 17HSD1 expression is less than that on the conversion of DHEA to E2 in CTB cells, suggesting that RAs may also act on the translational or post-translational levels of these two enzymes.

In brief, the results of the present study demonstrate that RAs promote the biosynthesis of E2 in placental cells by increasing the expression of aromatase and 17HSD1 gene, but the mechanisms of the divergent effects of Ro41 on gene expression in JEG-3 cells remain to be elucidated.

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