Effect of epidermal growth factor and prostaglandin on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells

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Abstract

We investigated the effects of epidermal growth factor (EGF) and prostaglandins (PG) on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells. EGF significantly increased aromatase activity and *CYP19* gene transcript in NCI-H295R cells. Exon PII was selected from among several tissue-specific exon I regions. Promoter II that abuts on exon PII was activated by EGF. PGE₂ also significantly increased aromatase activity, *CYP19* gene transcript, and promoter II activity. The results of experiments using protein kinase (PK) inhibitors suggest that the cAMP-PKA signaling pathway is involved in the up-regulation of

aromatase expression by EGF. PGE₂ activated promoter II activity in 4 h, while 12 h was required for its activation by EGF. In addition, PGE₂ was secreted from NCI-H295R cells in response to EGF. Selective agonists for prostaglandin receptors EP₁ and EP₂ significantly increased aromatase activity, which was decreased by the corresponding antagonists. Finally, antagonists for EP₁ and EP₂ inhibited the up-regulation of aromatase expression following EGF. These results suggest that PGE₂ secondarily acts as an autocrine signal in the up-regulation of aromatase expression by EGF in NCI-H295R cells.

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Introduction

There have been reports indicating that tumors which secrete abnormally high levels of estrogen cause gynecomastia in boys and men and precocious puberty in girls. In these cases, cytochrome P450 aromatase (CYP19), the key enzyme of estrogen biosynthesis, is highly expressed (Young *et al.* 1996, Watanabe *et al.* 2000, Phornphutkul *et al.* 2001). In another case, a mutation in the promoter region of the *CYP19* gene that codes for aromatase caused excess expression of aromatase and progression of gynecomastia (Shozu *et al.* 2003).

CYP19 gene is composed of multiple untranslated exon I and coding regions (exon II to exon X) (Bulun *et al.* 2004). A special feature of the *CYP19* gene is its multiple exon I. At least ten exon I regions are selected in a tissue-specific fashion. After alternative splicing, mRNA with a tissue-specific 5'-untranslated region is formed. In this tissue-specific selection of exon I, promoter regions that abut on the 5'-end of each exon I are activated by a distinct signal pathway. For example, promoter II and promoter I.3 that abut on the 5'-end of exon PII and exon I.3 respectively, are activated by the signaling pathway that includes the elevation of intracellular cAMP concentration. On the other hand, promoter I.4 that abuts on the 5'-end of exon I.4 is activated by glucocorticoid.

We found previously that forskolin largely up-regulates aromatase expression in NCI-H295R cells, a human adrenocortical carcinoma cell line (Watanabe & Nakajin 2004). Promoter I.3 and promoter II are activated in NCI-H295R cells. From these findings, we were interested in identifying physiological factors that induce aromatase expression in NCI-H295R cells in order to clarify the mechanism of aromatase expression in adrenal tumors.

Epidermal growth factor (EGF) increases aromatase activity and expression in MCF-7 and adipose stromal cells and induces expression of cyclooxygenase 2 (COX-2) in adipose stromal cells (Richards *et al.* 2002). In breast tumors, prostaglandin (PG) E₂ increases intracellular cAMP levels and stimulates estrogen biosynthesis (Zhao *et al.* 1996); furthermore, it up-regulates aromatase activity and expression in adipose stromal cells (Richards & Brueggemeier 2003). EGF affects the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) type II and CYP17 in NCI-H295R cells (Doi *et al.* 2001). Also in NCI-H295R cells, up-regulation of aromatase expression by PGE₂ has been reported (Heneweer *et al.* 2004). However, the mechanisms of the effects of EGF and PGE₂ on aromatase expression in NCI-H295R cells have not been examined in detail at the molecular biological level. Therefore, we conducted detailed studies on the effects

of EGF and prostaglandins on aromatase expression in NCI-H295R cells.

Materials and Methods

Materials

EGF was purchased from Cosmo Bio Co. Ltd (Tokyo, Japan). EGF was dissolved in 10 mM acetic acid containing 0.1% BSA. Prostaglandin A₁ (PGA₁) and PGF_{2α} were obtained from Cayman Chemical (Ann Arbor, MI, USA). PGE₁ and PGE₂ were purchased from Sigma Aldrich Japan (Tokyo, Japan). Prostaglandins were dissolved in ethanol. H-89 was purchased from Seikagaku Corporation (Tokyo, Japan) and PD98059 and KN-93 from Cosmo Bio Co. Ltd. Protein kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO). Butaprost, sulprostone, AH6809 and SC-19220 were obtained from Cayman Chemical. ONO-DI-004 and ONO-AE1-259-01 (Suzawa *et al.* 2000, Narumiya & FitzGerald 2001) were generous gifts provided by Ono Pharmaceutical Co. Ltd (Osaka, Japan). Butaprost, ONO-DI-004 and ONO-AE1-259-01 were dissolved in ethanol. Sulprostone, AH6809 and SC-19220 were dissolved in DMSO. The concentration of each solvent was 0.1% (v/v). D-MEM/F-12, D-MEM/F-12 without phenol red and a mixture of penicillin (5000 U/ml) and streptomycin (5000 µg/ml) were purchased from Invitrogen Japan K.K. (Tokyo, Japan). ITS plus was purchased from Nippon Becton Dickinson Company, Ltd (Tokyo, Japan). Nu-Serum I was purchased from Cosmo Bio Co. Ltd and fetal calf serum (FCS) was from Sanko Junyaku (Tokyo, Japan).

Cells

NCI-H295R human adrenocortical carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). NCI-H295R cells were cultured in D-MEM/F-12 supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 1% (v/v) ITS plus and 2.5% (v/v) Nu-Serum I. The human granulosa-like cell line KGN (Nishi *et al.* 2001) was obtained from Riken Cell Bank (Tsukuba, Japan). KGN cells were cultured in D-MEM/F-12 medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and FCS (10% v/v). Cells were maintained as monolayer cultures in 10-cm dishes at 37 °C in an atmosphere of 5% CO₂-95% air.

Aromatase assay

NCI-H295R cells were seeded at a density of 5.0×10^5 cells/well on 24-well plates. After 48-h culture, the medium was replaced with the treatment medium (D-MEM/F-12 without phenol red with 1% (v/v) ITS plus, penicillin (50 U/ml) and streptomycin (50 mg/ml)).

After incubation for 24 h (serum starvation), the cells were treated with treatment medium that contained peptide or chemical compounds. After incubation, aromatase activity in the NCI-H295R cells was measured by the tritiated water method described previously (Watanabe & Nakajin 2004).

RT-PCR

After serum starvation, the NCI-H295R cells were treated with EGF for 24 h or PGE₂ for 4 h. Then, total RNA was extracted using ISOGEN (Nippongene, Toyama, Japan). First-strand cDNA was prepared from total RNA using Oligo (dT)₁₅ primer (Promega, Madison, WI, USA), human placenta ribonuclease inhibitor (Takara Shuzo, Shiga, Japan) and AMV reverse transcriptase (Promega) according to the manufacturers' instructions. The RT-PCR of the gene transcripts of EGF receptor (EGFR), CYP19 (coding region (exon IX-X) and exon PII-including 5' region), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and prostaglandin receptors (EP₁, EP₂, EP₃ and EP₄) were based on published data (Moroni *et al.* 2001, Schlotzer-Schrehardt *et al.* 2002, Iwasaki *et al.* 2003, Richards & Brueggemeier 2003, Watanabe & Nakajin 2004). The PCR products were visualized with ethidium bromide after electrophoresis on 1.5% agarose gel, and quantified by calculating the intensity of the band of the PCR product of CYP19 (coding region (exon IX-X) and exon PII-including 5' region) relative to the intensity of the band of the PCR product of GAPDH.

Transfection and luciferase assay

NCI-H295R cells were seeded at a density of 5.0×10^5 cells/well on 24-well plates. After culturing for 24 h, firefly luciferase reporter vectors harboring various lengths of promoter I.3/II or empty firefly luciferase vector (pGL3-Basic) and sea pansy luciferase internal control vector (phRL-TK) were transfected using Eugene 6 transfection reagent (Roche Diagnostics K.K., Tokyo, Japan). After 24-h transfection, the cells were serum starved for 4 h. The cells were then treated with peptides and chemical compounds. After treatment, the cells were lysed and luciferase activities in the lysate were measured using a Dual-Luciferase reporter assay (Promega) and a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany).

Enzyme immunoassay of PGE₂

The concentration of PGE₂ in the conditioned media of NCI-H295R cells that were treated with EGF for

Figure 1 EGF increases aromatase activity in NCI-H295R cells. (A) Result of RT-PCR for EGF receptor gene transcript in NCI-H295R cells. RT – and + indicate cDNA preparation without and with the reverse transcriptase. (B) Increase in aromatase activity in NCI-H295R cells by EGF. After serum starvation for 24 h and then EGF treatment for 24 h, aromatase activity was measured. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/mL); * $P<0.05$; ** $P<0.01$.

24 h was measured using Prostaglandin E₂ EIA Kit – Monoclonal (Cayman Chemical).

Statistical analysis

The statistical significance of experimental data was determined by one-way ANOVA followed by the Dunnett, Tukey, or Bonferroni/Dunn tests for multiple comparisons.

Results

Effect of EGF on aromatase expression in NCI-H295R cells

Before evaluation of the effect of EGF, we tested the expression of EGF receptor (EGFR) gene transcript in NCI-H295R cells by RT-PCR. As shown in Fig. 1A,

the EGFR gene transcript was expressed in NCI-H295R cells. We then tested the effect of EGF on aromatase expression in NCI-H295R cells. As shown in Fig. 1B, aromatase activity in the cells was significantly increased by EGF in a concentration-dependent fashion. We also tested the effect of EGF on the expression of the *CYP19* gene. As shown in Fig. 2A and B, one of the *CYP19* gene transcripts corresponding to the coding region and the other gene transcript which includes exon PII were both up-regulated by EGF in a concentration-dependent manner. These results suggest that EGF up-regulates aromatase expression at the level of *CYP19* gene expression in NCI-H295R cells.

Effect of EGF on promoter I.3/II activity of the *CYP19* gene

There are two cAMP-responsive promoter regions upstream of exon PII in the *CYP19* gene. Promoter II abuts on the 5'-end of exon PII and this promoter includes the sequence of exon I.3. Promoter I.3 abuts on exon I.3. To clarify the mechanism of *CYP19* gene expression, we transfected the firefly luciferase reporter constructs that harbor several 5'-deleted promoter I.3/II sequences as shown in Fig. 3. The results in Fig. 4 show that the reporter activity (promoter activity) of –717/+23 Luc was activated significantly and in a concentration-dependent fashion by treatment with EGF for 12 h. This result suggests that activation of the promoter region that abuts on the 5'-end of exon PII is involved in the up-regulation of aromatase expression by EGF.

To clarify the sequence that is important for the activation of promoter I.3/II, we transfected the deletion constructs into NCI-H295R cells, treated the cells with 100 ng/ml EGF, and then measured promoter activity. As shown in Fig. 5, –717/+23 Luc and –278/+23 Luc had the same promoter activity. However, the promoter activity of –227/+23 Luc, which has a deletion sequence that includes TATA of promoter I.3, was less than half that of –278/+23 Luc. This result suggests that the 51 base pair sequence that includes TATA of promoter I.3 is important for the promoter activity. In addition, –198/+23 Luc was still activated significantly by EGF, while –119/+23 Luc was not significantly activated. This result suggests that the 79 base pair sequence between –198 and –119 in promoter II is critical for the activation of promoter I.3/II by EGF.

Effects of prostaglandins on aromatase expression in NCI-H295R cells

We tested the effects of prostaglandins (prostaglandin A₁ (PGA₁), PGB₂, PGD₂, PGE₁, PGE₂ and PGF_{2α}) on aromatase expression in NCI-H295R cells. As shown in Fig. 6, all the prostaglandins except PGF_{2α} increased aromatase activity significantly. The increases in aromatase activity by PGE₁ and PGE₂ were particularly large.

Figure 2 EGF up-regulates *CYP19* gene transcripts in NCI-H295R cells. (A) Results of RT-PCR for coding region and exon PII of *CYP19* gene transcripts and GAPDH gene transcript. Total RNA was extracted after serum starvation for 24 h and then EGF treatment for 24 h. Then, cDNAs were prepared. cDNA prepared from total RNA of the human ovary-derived cell line KGN and treated with forskolin (FSK) was used as the positive control. RT- and RT+ indicate cDNA preparation without and with the reverse transcriptase. (B) Quantification of expression level of coding region and exon PII of *CYP19* gene transcript. The intensities of the bands of RT-PCR corresponding to coding region and exon PII were normalized with the bands of GAPDH. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); * $P<0.05$; ** $P<0.01$.

Based on these findings, we concentrated our efforts on the effect of PGE₂. As shown in Fig. 7A, four subtypes of prostaglandin receptors (EP₁, EP₂, EP₃ and EP₄) were expressed in NCI-H295R cells. Furthermore, as shown in Fig. 7B, aromatase activity in NCI-H295R cells was increased by PGE₂ significantly and in a concentration-dependent manner. As shown in Fig. 8A and B, *CYP19* gene transcripts which include the coding region and exon PII sequences were both up-regulated in response to PGE₂ in a concentration-dependent fashion. These results suggest that PGE₂ up-regulates aromatase expression at the level of *CYP19* gene expression.

Effect of PGE₂ on the promoter I.3/II activity of *CYP19* gene

As was the case for EGF, we tested the effect of PGE₂ on promoter I.3 and promoter II. As shown in Fig. 9,

the promoter activity of -717/+23 Luc was activated significantly and in a concentration-dependent fashion. PGE₂ activated promoter activity in 4 h whereas up-regulation with EGF required 12 h. We also tested the effect of PGE₂ on a deletion construct of promoter I.3/II. As shown in Fig. 10, reporter activity decreased as the promoter length was shortened. The deletion construct -198/+23 Luc was still activated by PGE₂ significantly, while -119/+23 Luc was not activated significantly. This result suggests that the 79 base pair sequence between -198 and -119 in promoter II includes an important sequence, as was the case for EGF.

Effects of EP receptor-specific agonists and an antagonist

To elucidate which PGE₂ receptor (EP₁, EP₂ and EP₃) is involved in the induction of aromatase expression,

Figure 3 Position of multiple exon I and putative transcription factor binding sites around exon II of the human *CYP19* gene. The positions of multiple exon I (top) and transcription factor binding sites (middle: CREaro, TATA, CLS, SF-1, S1) in the partial genomic sequence of the *CYP19* gene are indicated. The positions of genomic sequences inserted into firefly luciferase reporter vector (bottom) are also indicated.

we compared aromatase activity in the presence of some agonists and an antagonist. As shown in Fig. 11, aromatase activity was induced by ONO-DI-004 (selective agonist for EP₁), ONO-AE1-259-01 (selective agonist for EP₂) and butaprost (selective agonist for EP₂). These inductions were completely inhibited by AH6809 (antagonist for EP₁ and EP₂). It was not possible to obtain specific agonists and antagonists for EP₄. These data suggest that EP₁ and EP₂ are involved in the induction of aromatase in NCI-H295R cells. The fact that there was no induction by sulprostone (selective agonist for EP₃) suggests that EP₃ is not involved in the induction.

Effects of protein kinase inhibitors

We tested the effects of several protein kinase (PK) inhibitors to elucidate the intracellular signaling pathways

Figure 4 EGF activates promoter I.3/II region in NCI-H295R cells. -717/+23 Luc firefly luciferase reporter vector or empty firefly luciferase vector (Basic) was transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Eugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with EGF for 12 h. Then, luciferase activity in the cell lysate was measured. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); $*P<0.05$.

that up-regulate aromatase expression. As shown in Fig. 12, up-regulation of aromatase activity by EGF was significantly inhibited to varying degrees by PD98059 (inhibitor of MAP kinase kinase), KN-93 (inhibitor of calcium-calmodulin kinase II) and H-89 (inhibitor of PKA). Aromatase activity was down-regulated to 35.3% of the value with EGF alone by PD98059. Aromatase activity was below the control level in the presence of KN-93. Interestingly, aromatase activity was down-regulated to 21.1% by H-89 (Fig. 12A). This result suggests that the cAMP-PKA pathway is involved in the up-regulation of aromatase in response to EGF. The aromatase activity in response to PGE₂ was down-regulated to 65.9% of the value with PGE₂ alone by

Figure 5 Effect of EGF on the activity of the deletion series of promoter I.3/II sequence. 5'-deleted series of firefly luciferase reporter vector that were shown in Fig. 3 or empty firefly luciferase vector (Basic) were transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Eugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with EGF for 12 h. Then, luciferase activity in the cell lysate was measured. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Bonferroni/Dunn's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (-); $*P<0.05$.

Figure 6 Effect of various prostaglandins (PGs) on aromatase activity in NCI-H295R cells. After serum starvation for 24 h, cells were treated with PGs for 4 h. Then, aromatase activity was measured. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences compared with the vehicle control; * $P<0.05$; ** $P<0.01$.

PD98059 and to 42.4% by KN-93. H-89 almost completely diminished aromatase activity (Fig. 12B). These results suggest that the cAMP–PKA pathway is the main signaling pathway in the up-regulation of aromatase in response to PGE₂. These results also suggest that the cAMP–PKA pathway evoked in response to PGE₂ would be involved in the up-regulation of aromatase expression in response to EGF in NCI-H295R cells.

EGF induces PGE₂ secretion from NCI-H295R cells

The results of the experiments using several protein kinase inhibitors suggest that the PGE₂–cAMP–PKA pathway may be involved in the up-regulation of aromatase expression in response to EGF. PGE₂ secreted from NCI-H295R cells in response to EGF may stimulate the cells in an autocrine fashion. Based on this, we attempted to determine whether PGE₂ is secreted from NCI-H295R cells in response to EGF. As shown in Fig. 13, the secretion of PGE₂ in conditioned media increased in a concentration-dependent manner in response to EGF, but the secretion of PGD₂ and PGE₁ did not respond to EGF (data not shown). This result suggests that PGE₂ acts partly as a secondary factor in the up-regulation of aromatase expression by EGF.

EP receptor antagonists down-regulate aromatase activity which is induced by EGF

To confirm that PGE₂ is the secondary factor in up-regulation of aromatase expression by EGF, we tested if EP receptor antagonists inhibit the induction of aromatase expression by EGF. As shown in Fig. 14, induction of aromatase activity with EGF was inhibited with AH6809 (antagonist for EP₁ and EP₂) and SC-19220 (selective antagonist for EP₁). This result suggests that the PGE₂

Figure 7 PGE₂ increased aromatase activity in NCI-H295R cells. (A) Results of RT-PCR for gene transcripts of PGE₂ receptors in NCI-H295R cells. RT – and + indicate cDNA preparation without and with the reverse transcriptase. Arabic numerals on the right indicate the length of base pairs of RT-PCR product. (B) Increase in aromatase activity in NCI-H295R cells by PGE₂. Each column represents the mean with S.E.M. ($n=3$). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); * $P<0.05$; ** $P<0.01$.

signaling pathway (at least EP₁ and EP₂) is involved in the induction of aromatase expression in NCI-H295R cells by EGF.

Discussion

Aromatase is not expressed in normal adrenal cortex, but it is expressed after carcinogenesis. This fact suggests that there would be common molecular mechanisms between carcinogenesis and aromatase expression in adrenocortical carcinoma. Therefore, investigation of the mechanism of CYP19 gene expression in estrogen-secreting adrenocortical carcinomas may supply helpful data for determining the mechanism of carcinogenesis in the adrenal cortex. An adrenocortical carcinoma itself or its primary culture would be suitable tools for this kind of investigation.

Figure 8 PGE₂ up-regulates CYP19 gene transcripts in NCI-H295R cells. (A) Results of RT-PCR of coding region and exon PII of CYP19 gene transcripts and GAPDH gene transcript. After serum starvation for 24 h and PGE₂ treatment for 4 h, total RNAs were extracted. Then, cDNAs were prepared. cDNA prepared from total RNA of the human ovary-derived cell line KGN and treated with forskolin (FSK) was used as the positive control. RT – and RT+ indicate cDNA preparation without and with the reverse transcriptase. (B) Quantification of expression level of coding region and exon PII of CYP19 gene transcripts. The intensities of the bands of RT-PCR corresponding to coding region and exon PII were normalized with the bands of GAPDH. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); **P<0.01.

However, because of the shortage of estrogen-secreting adrenocortical carcinomas and the difficulty associated with molecular investigation (e.g. transfection experiments), these tools are not suitable for studying the molecular biological mechanism. On the other hand, a cell line that is established from an adrenal tumor is easy to culture and stock, and the cell line is easily transfected. Taking into consideration these features, the human adrenocortical carcinoma cell line NCI-H295R can be a useful model of estrogen-secreting adrenocortical carcinoma.

The effects of EGF and PGE₂ on aromatase activity and expression have been well studied in human adipose stromal cells (Richards *et al.* 2002, Richards & Brueggemeier 2003). The up-regulation of aromatase activity and gene transcript with PGE₂ in H295R cells has been reported (Heneweer *et al.* 2004).

In this study, we investigated the molecular biological mechanism of the up-regulation of aromatase expression

in NCI-H295R cells in response to EGF and PGE₂. Both EGF and PGE₂ up-regulated aromatase expression at the level of CYP19 gene transcription. The results of transfection experiments using a deletion construct of promoter I.3/II suggest the existence of an important sequence in the 79 base pairs between –198 and –119 in promoter II. The binding site for steroidogenic factor 1 (SF-1) is in this sequence. Since the mutation in SF-1 site abolished the response of promoter II to forskolin (Watanabe & Nakajin 2004), SF-1 or another transcription factor that binds to this site must have an important role.

EGF, a ubiquitous hormone, would exert its effect on the adrenal cortex in an endocrine fashion. Furthermore, transforming growth factor- α , which has a similar structure to EGF and is able to bind to EGFR, is expressed in adrenal cortex (Sasano *et al.* 1994). Based on these observations, downstream of the EGFR signaling pathway could be evoked in adrenocortical carcinomas.

Figure 9 PGE₂ activates promoter 1.3/II region in NCI-H295R cells. – 717/+23 Luc firefly luciferase reporter vector or empty firefly luciferase vector (Basic) was transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Eugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with PGE₂ for 4 h. Luciferase activity in the cell lysate was then measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); *P<0.05; **P<0.01.

Prostaglandins can be synthesized in an adrenocortical carcinoma, and they can work in an autocrine or paracrine fashion. In rabbit chondrocyte and human squamous carcinoma cell lines, EGF induced the secretion of PGE₂ via up-regulation of the activities of phospholipase A₂ (PLA₂) and COX-2 (Sato *et al.* 1997, Huh *et al.* 2003). This may suggest that PGE₂ acts as a secondary factor

Figure 10 Effect of PGE₂ on the activity of the deletion series of promoter 1.3/II sequence. 5’-deleted series of firefly luciferase reporter vector that were shown in Fig. 3 or empty firefly luciferase vector (Basic) were transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Eugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with PGE₂ for 4 h. Then, luciferase activity in the cell lysate was measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Bonferroni/Dunn’s multiple comparison test. Asterisks denote significant differences compared with the vehicle control (–); *P<0.05; **P<0.01.

Figure 11 Effects of several EP agonists and an antagonist on aromatase expression. After serum starvation for 24 h, the cells were treated with PGE₂ or several agonists and/or an antagonist for EP receptors as shown for 24 h and then the aromatase activities were measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks denote significant differences compared with the vehicle control (first column); **P<0.01.

to EGF in the up-regulation of aromatase expression. Therefore, we checked whether PGE₂ was secreted from NCI-H295R cells in response to EGF. In this study, NCI-H295R cells secreted PGE₂ in response to EGF (Fig. 13), and PGE₂ increased aromatase activity to a greater extent than other prostaglandins (Fig. 6). The inhibition of EGF-induced aromatase expression with PGE₂ receptor antagonists confirmed that PGE₂ is the secondary factor of aromatase expression with EGF (Fig. 14). PGE₁ also increased aromatase activity to a degree similar to that of PGE₂, but EGF could not stimulate NCI-H295R cells to secrete a sufficient concentration of PGE₁ (data not shown) to increase aromatase activity. These results suggest that several prostaglandins are secreted in response to EGF, and that these prostaglandins evoke some intracellular signaling pathways. According to the experiments using several protein kinase inhibitors (Fig. 12), the intracellular signaling pathways that include MAP kinase, and calcium-calmodulin kinase are important for up-regulation of aromatase by EGF. In response to EGF, EGF receptors (receptor-type tyrosine kinase) activate the MAP kinase pathway through phosphorylation of Ras protein. It is also well known that EGF receptors increase the intracellular calcium concentration. Therefore, it would be reasonable to conclude that inhibition of MAP kinase kinase and calcium-calmodulin kinase II down-regulate aromatase expression in NCI-H295R cells. Interestingly, a PKA inhibitor (H-89) down-regulated aromatase activity. This result suggests that the cAMP–PKA pathway is involved in the up-regulation of aromatase

Figure 12 Effect of several protein kinase inhibitors on the increase in aromatase activity in NCI-H295R cells with EGF (A) or PGE₂ (B). After pretreatment with protein kinase inhibitors for 1 h, cells were treated with protein kinase inhibitors and EGF for 24 h (A) or protein kinase inhibitors and PGE₂ for 4 h (B), following which aromatase activity was measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Asterisks denote significant differences compared with the vehicle control; *P<0.05; **P<0.01; ***P<0.001. PD98059, inhibitor of MAP kinase kinase; KN-93, inhibitor of calcium-calmodulin kinase; H-89, inhibitor of PKA.

by EGF. Probably a second factor induced by EGF would increase the intracellular cAMP concentration. PGE₂ is known to increase the intracellular cAMP concentration. In addition, PGE₂ is secreted from NCI-H295R cells in response to EGF (Fig. 13). Furthermore, the induction of aromatase expression with PGE₂ might depend mainly on the PKA pathway (Fig. 12B). Thus, PGE₂ would be a second factor involved in the up-regulation of aromatase expression by EGF. Up-regulation of aromatase expression with PGE₂ appears to depend partially on MAP kinase kinase and calcium-calmodulin kinase II (Fig. 12B). These phenomena could be explained by the existence of EP₁, which has some relationship with the signaling pathways involving calcium and the transactivation of EGF receptors with PGE₂ (Pai *et al.* 2002). This signaling pathway would

Figure 13 NCI-H295R cells secrete PGE₂ in response to EGF. Cells were treated with EGF for 24 h, then the concentrations of PGE₂ in conditioned media were measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks denote significant differences from the vehicle control (0 ng/ml); **P<0.01.

Figure 14 EP receptor antagonists down-regulate aromatase activity in NCI-H295R cells induced with EGF. After serum starvation for 24 h, the cells were treated with antagonists and/or EGF for 24 h. Then, aromatase activities were measured. Each column represents the mean with S.E.M. (n=3). The differences were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. Asterisks denote significant differences compared with EGF only (column B); ***P<0.001.

act synergistically with other signals in response to EGF to up-regulate aromatase expression.

The result of the experiments using several agonists and antagonists of EP receptors suggest that EP₁ and EP₂ are involved in the up-regulation of aromatase expression but that EP₃ is not involved.

In the present study, we showed that EGF and prostaglandins up-regulate aromatase expression in NCI-H295R cells. The autocrine and paracrine system of aromatase

up-regulation in adrenal tumors is similar to the system in human adipose stromal cells surrounding breast cancer (Richards *et al.* 2002, Richards & Brueggemeier 2003). In addition, we investigated the molecular mechanism of the up-regulation of aromatase expression by EGF and PGE₂ using promoter analysis and protein kinase inhibitors. However, the up-regulated aromatase activity is lower than the aromatase activity in adrenocortical carcinoma (Watanabe *et al.* 2000). Therefore, multiple factors, not only EGF and prostaglandins, would synergistically up-regulate aromatase expression at a high level. The search for such factors would help to clarify the mechanism of high level aromatase expression in adrenocortical carcinomas. Finally, it is hoped this search will assist in the development of drugs that down-regulate aromatase expression specifically in adrenocortical carcinomas.

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