# Differential regulation of 3β-hydroxysteroid dehydrogenase type II and 17α-hydroxylase/lyase P450 in human adrenocortical carcinoma cells by epidermal growth factor and basic fibroblast growth factor

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## Abstract

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are pluripotent growth factors that stimulate both the proliferation and steroidogenesis of adrenocortical cells. Here we demonstrate that EGF and bFGF specifically induce mRNA of 3β-hydroxysteroid dehydrogenase type II (3βHSD II) and suppress that of 17α-hydroxylase/lyase P450 (CYP17) in human adrenocortical H295R cells. The induction of 3βHSD II mRNA did not occur until 6 h after the growth factor treatment and was completely abolished in the presence of a protein synthesis inhibitor, cycloheximide (CHX), suggesting that the induction required *de novo* protein synthesis. The CYP17 mRNA suppression began at almost the same time as the induction of the 3βHSD II mRNA. Interestingly, the CYP17 mRNA level was increased by the CHX treatment. Both the 3βHSD II and CYP17 mRNAs were repressed by treatment with a calmodulin kinase II (CaMK II) inhibitor, KN-93, and were enhanced by a mitogen-activated protein kinase (MAPK) inhibitor, PD98059. The PD98059-mediated induction of the 3βHSD II mRNA was completely blocked by the CHX treatment. Interestingly, treatment with EGF in the presence of both PD98059 and CHX produced a greater increase in the CYP17 mRNA than did treatment in the presence of PD98059 alone. These results suggest that CHX-sensitive factor(s) and CaMK II- and MAPK-signaling pathways may have important roles in both induction of 3βHSD II and suppression of CYP17 by EGF or bFGF in H295R cells.

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## Introduction

Numerous investigators have reported that epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) influence steroidogenesis in adrenocortical cells (Gospodaeowicz & Handley 1975, 1986, McAllister & Hornsby 1987*a*, Fisher & Lakshmanan 1990, Mesiano *et al.* 1993, Mesiano & Jaffe 1997). Singh and coworkers reported that EGF stimulated cortisol secretion from cultured bovine (Singh & Waters 1983) and sheep (Singh *et al.* 1985) adrenal cortical cells. The stimulation was abolished when inhibitors of cholesterol biosynthesis, compactin and AY9944, were added. Further studies showed that the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the rate of cholesterol synthesis were increased in the EGF-treated cells. In contrast, the rates of conversion of radioactive pregnenolone to steroid intermediates were little influenced by the growth factor. On the basis of these results, they concluded that EGF activated the biosynthesis of the steroid hormone precursor, rather than the steroidogenic enzymes themselves.

bFGF has been suggested as a physiological regulator of adrenocortical functions both *in vivo* (Mesiano *et al.* 1991, Basile & Holzwarth 1994) and *in vitro* (Crickard *et al.* 1981, Hornsby *et al.* 1983, Hotta & Baird 1986, Ho & Vinson 1995). It was purified from extracts of bovine adrenals, and a large amount was detected in the zona fasciculata and the medulla (Basile & Holzwarth 1993). It was also detected immunochemically and by northern blot analysis in bovine (Gospodarowicz *et al.* 1986), rat (Basile & Holzwarth 1993) and human (Mesiano *et al.* 1991) adrenal cortex. Basile & Holzwarth (1993) observed that, in a unilaterally adrenalectomized rat, the remaining adrenal gland underwent compensatory hypertrophy, and they suggested that bFGF might be involved in this phenomenon. Recently, Thomas *et al.* (1997) showed that transplantation of bovine adrenocortical cells with


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bFGF-overproducing mouse fibroblasts into immunodeficient mice resulted in both cell proliferation and activation of steroidogenesis of the transplants. Receptors for bFGF were also shown to exist in the rat zona glomerulosa (Basile & Holzwarth 1994). These previous reports strongly suggest that EGF and bFGF act as paracrine effectors in the adrenal cortex and may modulate steroidogenesis in adrenocortical cells.

H295R cells were originally isolated from human adrenal carcinoma cells (Gazdar *et al.* 1990, Rainey *et al.* 1994). The cells were reported to possess five steroidogenic P450s – namely, side-chain cleavage P450 (CYP11A), 17α-hydroxylase/lyase P450 (CYP17), 21-hydroxylase P450 (CYP21), 11β-hydroxylase P450 (CYP11B1) and aldosterone synthase P450 (CYP11B2) – and 3β-hydroxysteroid dehydrogenase type II (3βHSD II) (Bird *et al.* 1996). Because of this, these cells have often been used for studying the molecular mechanism underlying human adrenocortical steroidogenesis. In the present study, we investigated the effects of EGF and bFGF on gene expression of steroidogenic enzymes in H295R cells.

## Materials and Methods

### *Cell culture and treatment with EGF and bFGF*

H295R cells were generous gifts from J Ian Mason at University of Edinburgh, UK. The cells were maintained in Dulbecco's Modified Eagle's/Ham's F-12 medium (DMEM/F-12; Gibco BRL, Grand Island, NY, USA) containing 1% ITS plus (insulin/transferrin/selenium/linoleic acid; Becton Dickinson Labware, Lincoln Park, NJ, USA), 2% Ultroser G (BioSepra Inc., France) and antibiotics, at 37 °C under an atmosphere of 5% $CO_2$–95% air, as described before (Bird *et al.* 1996). The cells ($5 \times 10^5$) were cultured in 100 mm dishes for 48 h and then incubated for further 24 h in serum-free medium (DMEM/F-12 containing antibiotics and 0·01% BSA). To initiate the experiments, the medium was exchanged for one containing EGF (Austral Biochemicals, San Ramon, CA, USA) or bFGF (Austral Biochemicals) at the final concentration of 10 ng/ml. After the 24 h incubation, the cells were washed with PBS, and the total RNA was extracted. Protein kinase inhibitors (bisindolylmaleimide I, H-8, H-89, PD98059 and KN-93) and a protein synthesis inhibitor (cycloheximide; CHX) were purchased from Calbiochem Ltd (La Jolla, CA, USA) and Sigma, respectively, dissolved in dimethylsulfoxide, and stored at −20 °C until required for use. Cells that had been maintained in the serum-free medium for 24 h, were first incubated with or without bisindolylmaleimide I, H-8, H-89, PD98059 or KN-93 for 1 h, and then stimulated with EGF or bFGF dissolved in the freshly prepared inhibitor-containing medium.

### *RNA extraction*

Isolation of the total RNA was performed according to the method of Chomczynski & Sacchi (1987) with some modifications (Takemori *et al.* 1997). Briefly, the cells were washed with 1 ml PBS, and then dissolved in 1·6 ml guanidine isothiocyanate solution (Trizol reagent; Gibco BRL). The cell lysates were transferred into microcentrifuge tubes and mixed with 0·4 ml chloroform. After the centrifugation, an aqueous phase was taken, to which ethanol was added to precipitate total RNAs. The RNAs were washed with 70% ethanol, dried and dissolved in 200 μl distilled water. DNA contaminant in the RNA preparation was digested by 1 U DNase I (Gibco BRL) using the buffer supplied by the manufacturer. The RNAs were again extracted by phenol–chloroform (1:1) and reprecipitated by ethanol.

### *Northern blot analysis*

To prepare a cDNA probe for 3βHSD II, a DNA fragment of the exon 3 was amplified by PCR using primers shown in Table 1 and ligated into the *Sma*I site of pUC18. cDNA fragments of CYP11A and CYP21 were also prepared, using RT-PCR with sets of primers listed in Table 1, and introduced into pT7R (Novagen, Madison, WI, USA). A *Nde*I-*Hind*III cDNA fragment of CYP17 was excised from pCW (Katagiri *et al.* 1995) that had been kindly provided by M Katagiri at Osaka Kyoiku University, Japan. We utilized a rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA fragment (Halder *et al.* 1998) as the control probe. Total RNAs (5 μg) were separated by electrophoresis in 1% agarose gel in the presence of 6% formaldehyde and then transferred onto nylon membrane (Hybond-N+; Amersham, Arlington Heights, IL, USA) by capillary action in 20 × SSPE (0·2 M phosphate buffer, pH 7·7, containing 3·6 M NaCl and 0·02 M EDTA). The RNA on the membrane was hybridized with [$^{32}$P]-labeled DNA fragments in 5 × SSPE containing 50% formamide, 0·5% SDS, 0·1% polyvinylpyrrolidone, 0·1% Ficoll and 0·1% BSA at 42 °C for 16 h. After the hybridization, the membrane was washed sequentially in 2 × SSPE/0·1% SDS, 1 × SSPE/0·1% SDS, and 0·2 × SSPE/0·1% SDS at 60 °C, and then exposed to X-ray film at −80 °C with an intensifying screen. The radioactivity in hybridized signals was quantified with a phosphoimager (BAS2000; Fuji-film, Tokyo, Japan).

### *Determination of mRNAs for CYP11B1 and CYP11B2 by RT-PCR followed by Southern blot analysis*

Total RNAs (2 μg) were reverse-transcribed by 200 U reverse transcriptase (Super Script II; Gibco BRL) in 20 μl reaction mixture containing 150 ng random hexamer. An aliquot (5 μl) of the reaction mixture was used for PCR

**Table 1** Sequence of oligonucleotides used in the experiments

| Enzyme | Forward primers (5′→3′) | Reverse primers (5′→3′) |
|---|---|---|
| 3βHSD type II | GCA CAT GGA TCT GTG CAT GTG GTT GCA G† | GAC CTG GGC TTG TGC CCC TGT TGC C† |
| 3βHSD type I-specific | TGG TCC GCC TGT TGG TGG AA‡ | CTA CCT CTA TGC TAC TGG TGT AG‡ |
| 3βHSD type II-specific | TCA TCC GCC TCT TGG TGA AG‡ | (common to 3βHSD types I and II) |
| CYP11A | TTG CCT TTG AGT CCA TCA CT† | GAG CAG GAC TTG GGA CAG AC† |
| CYP21 | CTC AGC TGC CTT CAT CAG TTC† | CAC CCC TTG GAG CAT GTA GT† |
| Common to CYP11B and CYP11B2 | CAA ATG TGG CGT GTT CTT GT‡ | AGT TGC TGG CTT CTA TGG‡ |
| CYP11B1-specific | CCC AA**C** GC**T** GTG CA§ | |
| CYP11B2-specific | CCC AA**G** GC**C** GTG CA§ | |

†Primers to generate cDNA probes for northern blot analysis.
‡Primers for RT-PCR analysis.
§Oligonucleotide probes for Southern blot analysis after RT-PCR.

amplification with 20 pmol primer sets (common primer) shown in Table 1 in 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The amplified cDNA fragments were separated in 1·5% agarose gel and transferred onto a nylon membrane. To distinguish the cDNA of CYP11B1 from that of CYP11B2, the membrane was subjected to Southern blot analyses using [$^{32}$P]-labeled oligonucleotide probes specific to the respective P450s (Table 1). The membrane was hybridized in 5 × SSPE containing 0·5% SDS, 0·1% polyvinylpyrrolidone, 0·1% Ficoll and 0·1% BSA at 40 °C for 16 h, and washed three times in 0·2 × SSPE containing 0·1% SDS at room temperature for 20 min.

## Results

### *Effect of EGF and bFGF on expression of steroidogenic mRNAs in H295R cells*

Northern blot analyses were performed on the growth-factor-treated H295R cells to examine the level of mRNAs of 3βHSD, CYP17, CYP11A and CYP21. To distinguish between CYP11B1 and CYP11B2 mRNAs, both were first amplified by RT-PCR using primers common to the two mRNAs and then the respective cDNAs were detected by Southern blot analysis using specific oligonucleotide probes. As shown in Fig. 1A and C, the level of mRNA of 3βHSD markedly increased in the EGF- or bFGF-treated cells, whereas that of CYP17 was decreased substantially by the EGF and bFGF treatment. The maximal stimulation of 3βHSD expression and the strongest suppression of CYP17 were found with a concentration of 10 ng/ml of either growth factor (data not shown). In contrast, the mRNA levels of the other steroidogenic P450s – CYP11A, CYP21, CYP11B1 and CYP11B2 – showed little change with the growth factor treatment (Fig. 1A, B and C). The RT-PCR analyses using the primers specific to either the type I or the type II isoform of 3βHSD revealed that only the type II cDNA was clearly amplified from the total RNAs extracted from H295R cells (Fig. 1D).

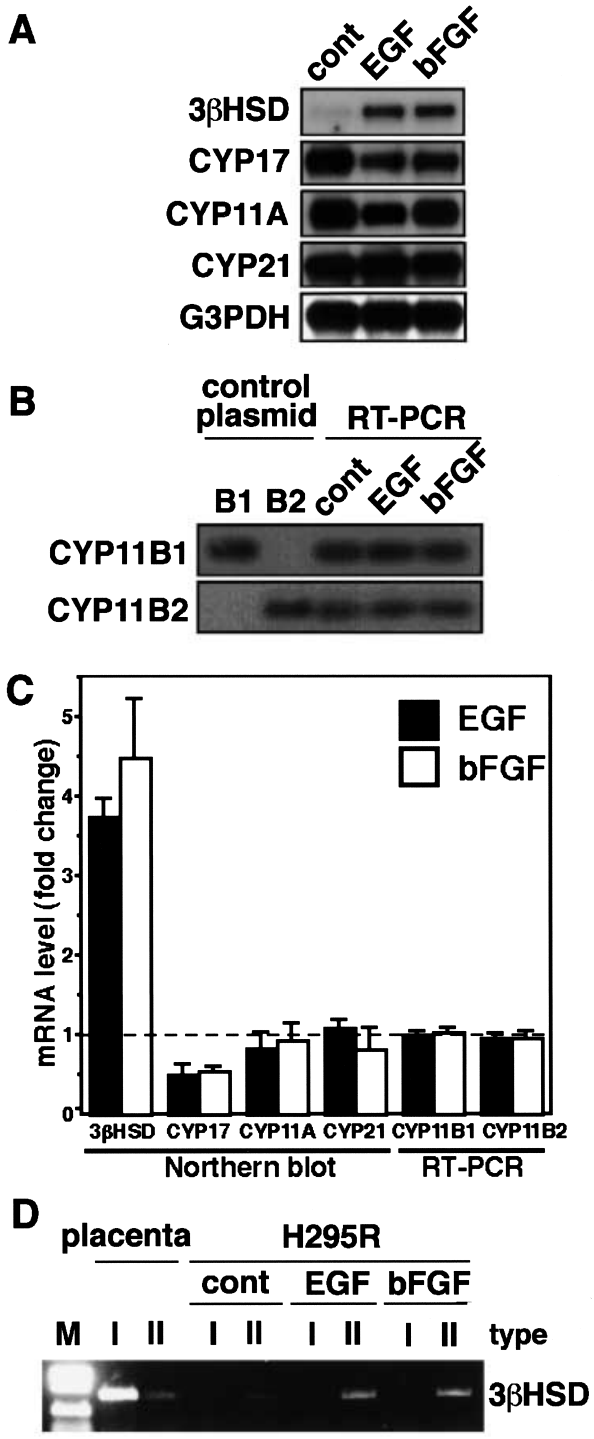
### *Time courses of 3βHSD and CYP17 mRNA expression*

Time courses of the EGF- and bFGF-mediated induction of 3βHSD mRNA and suppression of CYP17 mRNA were examined (Fig. 2). A distinct increase in 3βHSD mRNA was seen after the 6 h incubation with EGF, its level becoming maximal after 12 h and gradually diminishing thereafter. When bFGF instead of EGF was used as the stimulator, the mRNA level continued to increase after 12 h. In contrast, EGF or bFGF decreased the level of CYP17 mRNA, which reached the lowest point after 24 h. There was no significant change in the cell number under these growth factor treatments.

### *Effect of various protein kinase inhibitors on EGF- and bFGF-mediated gene expression*

Numerous investigators have reported that protein kinases are involved in the signal transduction system of *3βHSD* and *CYP17* gene expression (McAllister & Hornsby 1987*b*, Brentano *et al.* 1990, Chris *et al.* 1990, Bird *et al.* 1996). Therefore, several protein inhibitors were tested to explore the signaling pathway occurring in the EGF- and bFGF-treated H295R cells. As shown in Fig. 3, protein kinase A inhibitors, H-8 (15 μM) and H-89 (20 μM), or a protein kinase C (PKC) inhibitor, bisindolylmaleimide I (10 μM), seemed to have no effect on the levels of 3βHSD and CYP17 mRNAs in both EGF- and bFGF-treated cells. A mitogen-activated protein kinase (MAPK) inhibitor, PD98059 (20 μM), in contrast, further increased the growth factor-dependently increased 3βHSD mRNA level. PD98059 appeared to restore the growth factor-mediated suppression of CYP17 mRNA, but had little effect on the basal levels of expression of both genes (data not shown).

Next, the inhibitor of $Ca^{2+}$/calmodulin-dependent kinase II (CaMK II), KN-93, was tested. KN-93 (5 μM) markedly decreased the level of 3βHSD mRNA in the EGF- and bFGF-treated cells. It exerted little effect on the basal mRNA expression in the non-treated cells (data not



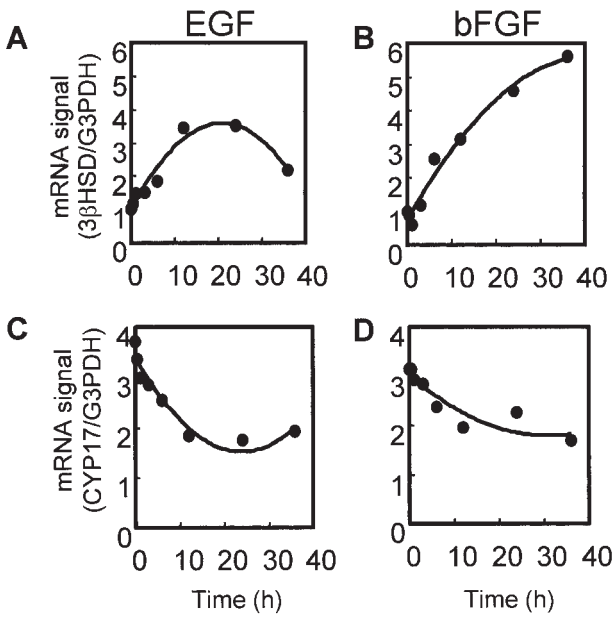




**Figure 2** Time course of 3βHSD and CYP17 mRNA expression stimulated by growth factors. Total RNAs (10 μg) were isolated from H295R cells at the indicated times after the addition of EGF (10 ng/ml: A, C) and bFGF (10 ng/ml: B, D). The levels of mRNA of 3βHSD (A, B) and CYP17 (C, D) were determined by a phosphoimager (BAS 2000) after northern blot analysis, and then normalized by those of G3PDH. Each point represents a mean value of duplicate experiments.

**Figure 1** Effect of EGF and bFGF on mRNA expression of steroidogenic enzymes. (A) Total RNAs (10 μg) were prepared from H295R cells that had been incubated without or with EGF (10 ng/ml) and bFGF (10 ng/ml) for 24 h. mRNAs of 3βHSD, CYP17, CYP11A and CYP21 were subjected to northern blot analyses. mRNA of G3PDH was visualized as the internal standard. (B) The RNAs were reverse-transcribed and the cDNA fragments amplified by PCR using primers hybridizable with both CYP11B1 and CYP11B2 cDNAs. The PCR products were then subjected to Southern blot analyses using oligonucleotide probe specific to the respective enzymes. Plasmid DNAs inserted with CYP11B1 or CYP11B2 cDNA fragments (two lanes on the left) were used for the positive controls of the specific hybridization. Each blot (A and B) is representative of three independent experiments. (C) The levels of mRNAs of 3βHSD, CYP17, CYP11A and CYP21 (in A) and CYP11B1 and CYP11B2 (in B) were determined by a phosphoimager (BAS 2000) after northern blot analysis and Southern blot analysis coupled with RT-PCR, respectively. Each mRNA level was first normalized to the level of G3PDH mRNA and then expressed as the fold change (means ± S.E.M.; $n=3$). (D) The cDNA fragments were amplified with 3βHSD type I- or type II-specific primers. The DNA fragments were separated on an agarose gel, and visualized by ethidium bromide staining. Total RNAs of placenta were used as the positive control for 3βHSD type I and type II mRNAs. cont, Control; M, molecular markers.

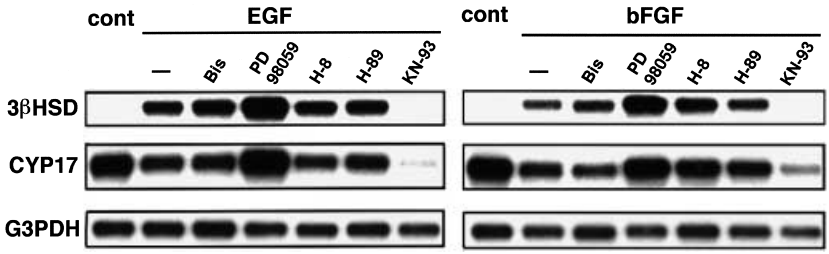



**Figure 3** Effect of various protein kinase inhibitors on the EGF and bFGF treatments. (A) H295R cells were pretreated with or without protein kinase inhibitors (bisindolylmaleimide I (10 μM), PD98059 (20 μM), H-8 (15 μM), H-89 (20 μM) and KN-93 (5 μM)) for 1 h, and then stimulated with EGF (10 ng/ml) or bFGF (10 ng/ml) for 24 h. Total RNAs were isolated and subjected to northern blot analysis as described in Materials and Methods. The figure is representative of three independent experiments. cont, control.

shown). Another CaMK II inhibitor, KN-62 (10 μM), or a calmodulin antagonist, W-7 (30 μM), also inhibited the 3βHSD expression data (not shown). It should be noted that KN-93 further decreased the growth-factor-dependent repressed mRNA level of CYP17.

*Effect of CHX on EGF- and bFGF-mediated gene expression*

Because the induction of 3βHSD and suppression of CYP17 appeared to be late events of growth factor treatment, we examined whether *de novo* protein biosynthesis was required for this phenomenon. An inhibitor of protein synthesis, CHX (10 μg/ml), was added to the culture medium together with EGF and bFGF. As shown in Fig. 4A, CHX completely suppressed the EGF- and bFGF-mediated induction of 3βHSD mRNA, suggesting that *de novo* protein synthesis is required for the induction of 3βHSD mRNA. The CHX treatment also completely abolished the PD98059-mediated enhancement of 3βHSD mRNA in EGF-stimulated H295R cells (Fig. 4B). To our surprise, CHX seemed to prevent the EGF- and bFGF-mediated repression of CYP17 mRNA. The treatment with EGF in the presence of both PD98059 and CHX produced a more increased level of CYP17 mRNA than did treatment in the presence of PD98059 alone (Fig. 4B). Similar results were obtained by treatment with the two inhibitors in the bFGF-stimulated cells (data not shown).

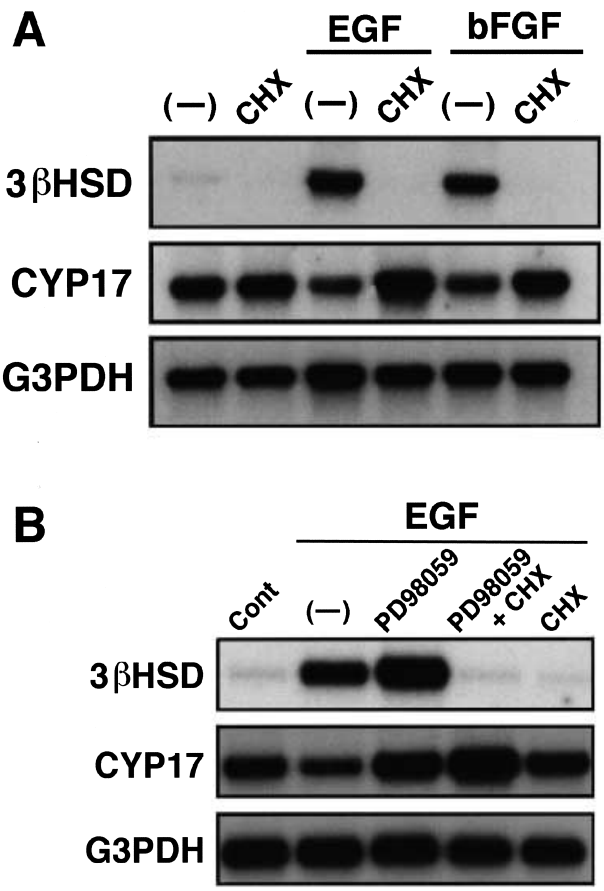



**Figure 4** Effect of CHX on the EGF and bFGF treatment. (A) H295R cells were stimulated with EGF (10 ng/ml) or bFGF (10 ng/ml) in the presence or absence of CHX (10 μg/ml) for 24 h. Total RNAs were isolated and subjected to northern blot analysis as described in Materials and Methods. The panel is representative of three independent experiments. (B) H295R cells were treated with PD98059 (20 μM), CHX (10 μg/ml) or both PD98059 (20 μM) and CHX (10 μg/ml) in the presence of EGF (10 ng/ml). The panel is a representative of duplicated experiments. Cont, control.

## Discussion

Previous studies of bovine (Singh & Waters 1983) and sheep (Singh *et al.* 1985) adrenal cortical cells demonstrated that the addition of EGF to culture media resulted in stimulated cortisol secretion from and cholesterol synthesis in the cells, without significant effect on the production rate of steroid intermediates. Abolition of the EGF-mediated stimulation of cortisol secretion by inhibitors of cholesterol synthesis led to the idea that HMG-CoA

reductase, the rate-limiting enzyme of cholesterol synthesis, may be the major target of EGF action in the cells. The presence of EGF receptor has been reported in human normal, tumor (Sasano *et al.* 1994), and fetal (Smikle *et al.* 1996) adrenocortical cells. Coulter *et al.* (1996*b*) reported that the administration of EGF increased the volume of the definitive zone of fetal adrenals of rhesus monkeys in late gestation. This zone-specific increase seemed to be the result of cellular hypertrophy, not of cell proliferation. It was also found that EGF treatment induced 3βHSD II protein in the definitive and transitional zones without significant effect on CYP17. Similar induction of 3βHSD II occurred in fetal rhesus monkeys in which endogenous adrenocorticotrophic hormone (ACTH) secretion was stimulated by administration of an inhibitor of CYP11B1, metyrapone (Coulter *et al.* 1996*a*). Coulter and colleagues were unable to determine whether the effect on adrenocortical function that was produced by the EGF administered to the animals had occurred as the result of the activation of the hypothalamic–pituitary–adrenal axis, as described by Luger *et al.* (1988) and Polk *et al.* (1987), or as the result of the direct action of EGF on adrenocortical cells. Our present study with H295R cells, however, demonstrated that treatment of the cells with EGF or bFGF stimulated transcription of the *3βHSD II* gene in the absence of ACTH or cAMP, the second messenger of ACTH.

Interestingly, the EGF or bFGF treatments resulted in a decrease in CYP17 mRNA levels in H295R cells. Welsh & Hsueh (1982) also reported that gonadotropin-stimulated production of testosterone, androstenedione and 17α-hydoxyprogesterone in cultured testicular cells was inhibited by EGF. Because EGF also inhibited the conversion of exogenous 17α-hydoxyprogesterone to androstenedione, they proposed that the EGF effect occurred via inhibition of 17α-hydoxylase and 17,20-lyase. These results suggested that EGF and bFGF could directly modulate the gene expression of steroidogenic enzymes in steroidogenic cells.

Hornsby *et al.* (1983) reported that bFGF acted as a mitogen in human fetal adrenal cortex, and that the mitogenic effect of bFGF was greater in the definitive zone than in the fetal zone. The mRNA of bFGF in cultured human adrenocortical cells was increased by about threefold by the addition of ACTH. These results suggest that bFGF may be one of the autocrine factors of adrenocortical cells, in which secretion is regulated by ACTH. Li *et al.* (1998) reported that bFGF treatment increased the level of CYP17 mRNA in cultured bovine fasciculata cells. Our results, in contrast, showed that both bFGF and EGF decreased the level of CYP17 mRNA in H295R cells. This discrepancy may have resulted from the species difference or from the difference in the zonal origin of the cells.

Membrane-associated receptor tyrosine kinases such as EGF/TGFα receptor and bFGF receptor are believed to stimulate several protein-kinase-mediated signaling pathways, such as those involving protein kinase C (PKC) and MAPK (Kim & Muller 1999, Rowan *et al.* 2000). Several investigators (Leers *et al.* 1997, Bird *et al.* 1998) have demonstrated that a PKC agonist, phorbol-12-myristate-13-acetate (PMA), increased 3βHSD II mRNA expression. MAPK was immunochemically detected in the rat zona glomerulosa, and MAPK seemed to be involved in gene expression of 3βHSD II, and thus activate the production of aldosterone (McNeil *et al.* 1998). These findings indicate that PKC- and MAPK-mediated signaling pathways may be involved in regulating *3βHSD II* gene expression, suggesting the possibility that the effect of EGF and bFGF on *3βHSD II* gene expression described here could be mediated via these signaling systems. The PKC inhibitor, bisindolylmaleimide I, however, did not block the EGF- or bFGF-mediated induction of 3βHSD II mRNA in a concentration of 10 μM – a concentration sufficient to block the PMA-mediated induction of 3βHSD II mRNA (data not shown). Moreover, the level of 3βHSD II mRNA was further increased, not inhibited, by the addition of the MAPK inhibitor PD98059 in the presence of EGF or bFGF. These results suggest that a signaling system mediated by a member(s) other than PKC or MAPK may be important for the EGF- and bFGF-stimulated induction of 3βHSD II mRNA. A possible candidate signal would be CaMK II, because KN-93, an inhibitor of CaMK II, markedly inhibited the EGF- and bFGF-stimulated induction of 3βHSD II mRNA. However, this inhibitor also inhibited the expression of CYP17 mRNA. This indicates that the CaMK II-mediated pathway may be essential for the gene expression of 3βHSD II, but this signaling system could not account for the repressive effects of EGF and bFGF on the expression of the *CYP17* gene.

It took several hours for EGF or bFGF to increase the 3βHSD II mRNA level in H295R cells (Fig. 2). As in the PKC-mediated induction of 3βHSD II mRNA (Leers *et al.* 1997), CHX blocked the effect of EGF and bFGF on 3βHSD II mRNA expression (Fig. 4), indicating that *de novo* protein synthesis is required for the EGF- and bFGF-mediated *3βHSD II* gene expression. In contrast, EGF or bFGF decreased the level of CYP17 mRNA in H295R cells (Fig. 1). Interestingly also, the EGF- or bFGF-mediated suppression of CYP17 mRNA was abolished by CHX treatment (Fig. 4). These results may suggest the presence of a CHX-sensitive protein factor(s) that is induced in H295R cells by the growth factor treatment, and which stimulates the expression of the *3βHSD II* gene but inhibits that of the *CYP17* gene.

Treatment of the cells with EGF in the presence of both CHX and PD98059, however, revealed the complicated involvement of the MAPK signaling pathway in the expression of these genes. The incubation with both CHX and PD98059 resulted in further increase in the level of CYP17 mRNA in EGF-stimulated H295R cells, whereas

the presence of PD98059 seemed not to influence the blocking effect of CHX on *3βHSD II* gene expression (Fig. 4B). These results indicate that more than one factor – one sensitive to the CHX-treatment and the other related to the MAPK signaling pathway – is involved in the regulation of *3βHSD II* and *CYP17* gene expression. In the EGF-mediated activation of the *3βHSD II* gene, the CHX-sensitive factor may act more strongly than that involved in the MAPK pathway, because treatment with both CHX and PD98059 completely abolished expression of the gene. In contrast, in the EGF-mediated suppression of the *CYP17* gene, the CHX-sensitive factor and the factor involved in the MAPK signaling pathway seemed to repress the gene independently. To date, we have failed in our attempts to search the database of the promoter regions of the *3βHSD II* and *CYP17* genes for sequences typical of *cis* elements of transcription factors that may act downstream of either the MAPK or CaMK II-mediated signaling pathway.

Further investigation is in progress in our laboratory to elucidate the precise mechanism underlying the enhancement of 3βHSD II mRNA and suppression of CYP17 mRNA by EGF and bFGF.

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