Role of glucocorticoid receptor and CCAAT/enhancer-binding protein α in the feed-forward induction of 11 β -hydroxysteroid dehydrogenase type 1 expression by cortisol in human amnion fibroblasts

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Abstract

The amount of cortisol available to its receptors is increased by the pre-receptor enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) which converts cortisone to cortisol. We examined the molecular mechanisms of the feedback effect of cortisol on 11 β -HSD1 mRNA expression in human amnion fibroblasts. Our data showed that cortisol-induced 11 β -HSD1 mRNA expression dose dependently in amnion fibroblasts, which could be completely blocked both by the mRNA transcription inhibitor 5,6-dichlorobenzimidazole riboside and by the glucocorticoid receptor (GR) antagonist RU486, and partially blocked by global inhibition of CCAAT/enhancer-binding proteins (C/EBPs) with transfection of C/EBP-specific dominant-negative expression CMV500 plasmid (AC/EBP) into the cells. Likewise, the induction of the promoter activity by cortisol could also be completely blocked by RU486 and

partially by AC/EBP transfection. Progressive 5' deletion of the 11 β -HSD1 promoter located the region responsible for cortisol's induction within -204 bp upstream to the transcription start site. Specific nucleotide mutations of the putative glucocorticoid responsive element or CCAAT in this promoter region attenuated the induction by cortisol. Moreover, chromatin immunoprecipitation assay and electrophoretic mobility shift assay showed that GR and C/EBP α but not C/EBP β could bind this promoter region upon cortisol stimulation of amnion fibroblasts. In conclusion, we demonstrated that GR and C/EBP α were involved in cortisol-induced 11 β -HSD1 mRNA expression via binding to 11 β -HSD1 promoter in amnion fibroblasts, which may cast a feed-forward production of cortisol in the fetal membranes at the end of gestation.

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Introduction

The amount of cortisol available to glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) is largely dependent upon the pre-receptor enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD; Seckl 1993, Draper & Stewart 2005). There are two isozymes of 11 β -HSD in the body (Seckl 1993, Draper & Stewart 2005). 11 β -HSD1 is ubiquitously distributed in glucocorticoid (GC) target organs where it enhances the binding of cortisol to the relatively low-affinity GR by converting biologically inactive cortisone into active cortisol (Seckl 1993, Tomlinson *et al.* 2004, Draper & Stewart 2005), while the distribution of 11 β -HSD2 is mostly limited to mineralocorticoid target organs where it protects the relatively high-affinity MR from cortisol binding by converting cortisol to cortisone, thus conferring the specific binding of MR by aldosterone (Seckl 1993).

GCs are important hormones in pregnancy. The placenta, fetal membranes, and the fetus are all GC targets during

pregnancy. GCs have been shown to stimulate rather than inhibit prostaglandin and corticotrophin-releasing hormone production in human fetal membranes and placenta (Casey *et al.* 1985, Potestio *et al.* 1988, Karalis *et al.* 1996, King *et al.* 2002, Sun *et al.* 2003). These paradoxical effects of GCs on human fetal membranes and placenta are believed to be part of the positive feedback loops involved in human parturition (Whittle *et al.* 2001). It is well known that GCs are indispensable hormones for cell differentiation and organ maturation in the fetus (Gonzales *et al.* 1986), but excess of GCs impedes fetal growth (Shams *et al.* 1998, Seckl *et al.* 2000). In consideration of all these above effects of GCs in pregnancy, understanding the metabolism of GCs by 11 β -HSD could undoubtedly have an important impact on the outcome of pregnancy.

Our previous studies have shown that 11 β -HSD1 and 11 β -HSD2 are differentially distributed in human fetal membranes and placenta, with 11 β -HSD1 mainly distributed in the fetal membranes and 11 β -HSD2 exclusively distributed in the placenta (Sun *et al.* 1997a,b). 11 β -HSD2 in the placenta

provides a barrier for maternal cortisol entering the fetal circulation (Seckl *et al.* 2000). Although the physiological functions of 11 β -HSD1 in the fetal membranes are less clear, it is believed that 11 β -HSD1 in the fetal membranes may either provide an extra-adrenal source of cortisol for the fetus or simply amplify the local actions of cortisol in the fetal membranes (Murphy 1977, Alfaidy *et al.* 2003).

Human amnion fibroblasts are enriched not only in 11 β -HSD1 but also in prostaglandin-synthesizing enzymes (Sun *et al.* 2003). Therefore, it is particularly of interest to examine the regulation of 11 β -HSD1 expression in these cells in terms of prostaglandin synthesis. Our previous study has shown that dexamethasone, the synthetic GC, could concurrently increase the expressions of 11 β -HSD1 and the key enzymes involved in prostaglandin synthesis such as cytosolic phospholipase A₂ and cyclooxygenase 2 in human amnion fibroblasts (Sun & Myatt 2003, Sun *et al.* 2003). However, the feedback effect of cortisol, the endogenous product of 11 β -HSD1-catalyzed reaction, on 11 β -HSD1 expression has not been characterized in human amnion fibroblasts.

Sequence analysis revealed a putative GC-response element (GRE) and several consensus CCAAT boxes in human 11 β -HSD1 promoter (Tannin *et al.* 1991), which may bind either GR or CCAAT/enhancer-binding proteins (C/EBPs) respectively. However, we do not know whether the putative GRE and CCAAT boxes are truly involved in the regulation of 11 β -HSD1 expression by GCs. C/EBPs have been reported to be closely related to the actions of GCs in a number of tissues (Rudiger *et al.* 2002, Roth *et al.* 2004, Yang *et al.* 2005a,b, Woltje *et al.* 2006). Although C/EBPs have been shown to be involved in basal expression of human or mouse 11 β -HSD1 gene transcription (Williams *et al.* 2000, Gout *et al.* 2006), it is not known whether C/EBPs play any roles in the regulation of 11 β -HSD expression by GCs. To address the above issues, we examined the feedback regulation of 11 β -HSD1 mRNA expression by cortisol in cultured human amnion fibroblasts and explored the underlying molecular mechanisms.

Materials and Methods

Human amnion fibroblast preparation and treatments

Human fetal membranes were collected from patients undergoing elective cesarean section at term according to the guidelines set forth in a protocol that is in compliance with the Institutional Review Board of Fudan University and University of Cincinnati. Amnion was peeled off chorion and washed thoroughly in cold PBS (pH 7.5) to get rid of the residual blood. Amnion was then digested with 0.125% trypsin (Sigma) and 0.02% DNase (Sigma) twice for 30 min at 37 °C. The digestion media were discarded, and the remaining amnion tissue was washed vigorously with PBS to get rid of residual epithelial cells. The remaining tissue was then subject to digestion with 0.1% collagenase (Roche) at 37 °C for 1 h. The digestion medium was collected and centrifuged at 2300 r.p.m. Cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM;

Sigma) and loaded onto a discontinuous Percoll (Amersham Biosciences) gradient column (5, 20, 40, and 60%). The gradient column was centrifuged at 2500 *g*, and a single band of cells around 20% Percoll concentration was collected. Cells were plated at a density of 1.5×10^6 cells per well in a six-well plate in DMEM without phenol red containing 10% fetal calf serum (FCS) and antibiotic-antimycotic solution. The cell identity prepared as described above has been verified previously and more than 90% of the cells are fibroblasts (Sun *et al.* 2003). The amnion fibroblasts were cultured for 2–3 days at 37 °C and 5% CO₂ in air. On the day of treatments, the medium was changed to FCS-free medium and the cells were then treated with cortisol (0.01, 0.1, and 1 μ M) for 24 h with and without the GR antagonist RU486 (1 μ M), or in the presence of transfection of C/EBP-specific dominant-negative expression CMV500 plasmid (AC/EBP) or empty CMV500 plasmid (courtesy of Dr Vinson C, National Cancer Institute, Center for Cancer Research, NIH). AC/EBP has been shown to have global inhibition on all C/EBPs (Olive *et al.* 1996). The cells were transfected with 0.5 μ g/well CMV500-AC/EBP or empty CMV500 plasmids using lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco) 12 h before cortisol treatment. In another set of experiments, the cells were also treated with the mRNA transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB, 75 μ M; Sigma) 1 h prior to cortisol treatment and then DRB treatment was continued with cortisol treatment for another 24 h. At the end of the above treatments, cells were collected for total RNA extraction using RNeasy kit (Qiagen). RNA (1.0 μ g) was reverse transcribed with oligo(dT)_{12–18} primers using Superscript II kit (Life Technologies Inc.) for subsequent measurement of 11 β -HSD1 mRNA level with real-time PCR (RT-PCR).

Measurements of 11 β -HSD1 mRNA levels with RT-PCR

To measure 11 β -HSD1 mRNA levels in response to the above treatments, quantitative RT-PCR (qRT-PCR) analysis was performed with the primer sequences as follows: sense 5'-CTCAACCACATCACCAACAC-3' and antisense 5'-TCTGATGGAGGAGAAGAACC-3'. To control sampling errors, qRT-PCR for housekeeping gene β -actin was routinely performed on each sample. The primer sequences for human β -actin were as follows: sense 5'-GGGAAATCGTGCGTGACATTAAG-3' and antisense 5'-TGTGTTGGCGTACAGG-TCTTTG-3'. Reaction solution of qRT-PCR consisted of 2.0 μ l diluted RT-PCR product, 0.2 μ M of each paired primers and power SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The annealing temperature was set at 61 °C, and amplification cycles were set at 45 cycles. The absolute mRNA levels in each sample were calculated according to a standard curve set up using serial dilutions of known amounts of specific templates against corresponding cycle threshold (C_t) values. Then, the ratio of the target gene over β -actin in each sample was obtained to normalize the expression of the target gene. The specificity of the primers has been verified previously (Sun & Myatt 2003).

Construction of 5' deleted 11 β -HSD1 gene promoters into pGL3 enhancer plasmid carrying luciferase reporter gene

Cloned human 11 β -HSD1 gene promoter, 1178 bp upstream to the translation start site, was originally cloned from human DNA sequence of clone RP1-28O10 on chromosome 1q32.2-41 (Genbank no. AL031316) in Dr Kaiping Yang's laboratory (University of Western Ontario, Ontario, Canada). Progressive 5' deletion of the cloned 11 β -HSD1 gene promoter was produced with PCR using a combination of a common antisense primer (located immediately before the translation start site) and a series of 5' end sense primers (progressing towards the transcription start site) as paired primer sets. The sequences and annealing positions of these primers are shown in Fig. 1. Restriction sites for KpnI and XhoI are designed into the sense and antisense primers respectively, so that the amplified products carried restriction sites on the 5' and 3' ends (Fig. 1). Correspondingly various lengths of 5' deleted 11 β -HSD1 gene promoters were produced as follows: -1085, -1005, -857, -467, -204, -84, and -64 bp upstream to the transcription start site. All the sub-cloned promoters also carried 93 bp upstream to the translation start site (Fig. 1). Specific nucleotide mutation was introduced into the predicted sequence responsible for the effect of cortisol by designing the mutation sites into the sense primers (Fig. 1, underlined nucleotides). After obtaining the PCR products, the PCR products were cut with KpnI and XhoI and ligated into the polycloning sites of pGL3 enhancer vector upstream to the firefly luciferase reporter gene (Promega). All the above sub-cloned sequences and mutation sites were verified by examining the size of PCR products upon gel electrophoresis as well as by sequencing.

Transient transfection of WISH cells with pGL3 enhancer plasmid carrying 11 β -HSD1 promoter-driven luciferase reporter gene and measurement of promoter activity

Amnion epithelium-derived WISH cells were obtained from the American Type Culture Collection (Rockville, MD, USA) with the intention of overcoming relatively poor transfection efficiency in primary cells. Passage of WISH cells was carried out with trypsin digestion and the cells were subsequently grown in a 24-well plate in DMEM containing 10% FCS and antibiotic-antimycotic solution and were allowed to grow to about 85–90% confluence at 37 °C and 5% CO₂ in air before the start of transfection and treatments. On the day of transfection, the cells were washed with PBS and the medium was changed to antibiotic-free DMEM containing 10% FCS. Then, the cells were co-transfected with 0.5 μ g/well of empty pGL3 enhancer plasmid or pGL3 enhancer plasmid carrying 11 β -HSD1 promoter-driven firefly luciferase gene and 0.05 μ g/well of phRL vector carrying renilla luciferase gene using lipofectamine 2000 in Opti-MEM. The phRL vector was used for transfection efficiency control. In another set of experiment, the cells were co-transfected with 0.5 μ g/well CMV500-AC/EBP plasmid or with 0.5 μ g/well empty CMV-500 plasmid along with the constructed pGL3 enhancer plasmid. All the above transfection were carried out for 12 h.

After 12 h of transfection, the culture medium was changed to serum-free medium and the cells were treated with cortisol (final concentration: 0.001, 0.01, 0.1, and 1 μ M) in the presence and absence of RU486 (1 μ M) for 24 h. The culture media were then discarded and the cells were washed with PBS and lysed with lysis buffer (Promega). The cellular debris was pelleted by centrifugation (12 000 *g*, 1 min at 4 °C).

Figure 1 Illustration of the positions and sequences of primers used for sub-cloning 11 β -HSD1 promoter. All the sub-cloned promoters carried KpnI and XhoI sites and were cloned into the corresponding sites upstream to luciferase (Luc) reporter gene of pGL3 enhancer plasmid. Boxed nucleotides are putative glucocorticoid-response element and C/EBP-binding site and the underlined nucleotides were mutated for both reporter gene study and EMSA study.

The supernatant was used for luciferase activity assay using Dual-Luciferase assay system (Promega) measuring firefly and renilla luciferase of the same samples with a luminometer. The ratio of 11 β -HSD1 promoter-driven firefly luciferase activity against renilla luciferase activity was obtained in order to correct differential transfection efficiency in each well and to express the promoter activity.

Demonstration of GR expression in WISH cells with PCR and western blotting analysis

Nuclear protein and total RNA were extracted from some of the WISH cells treated with cortisol (1 μ M) overnight using an extraction kit from Active Motif (Carlsbad, CA, USA) and an RNeasy kit from Qiagen respectively. The expression of GR protein was examined following a standard western blotting protocol. Briefly, 50 μ g protein of each sample were electrophoresed in 12% Tris-glycine gel (Invitrogen) and transferred to the nitrocellulose blot. The blot was blocked with nonfat milk solution and incubated with 1:1000 dilution of rabbit antibody against GR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. After washing, the blot was incubated with secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz) for 1 h. The enhanced chemiluminescence detection system (Amersham) was used to detect the bands with peroxidase activity. For measurement of GR mRNA level, total RNA was reverse transcribed with oligo(dT)₁₂₋₁₈ primers using Superscript II kit (Life Technologies Inc.) and subsequent standard PCR protocol was followed with the primer sequences as follows: sense 5'-GGCAATACCAGGTTTCAGGAACCTTACA-3' and antisense 5'-ATTTCACCATCTACTCTCCCATCACTG-3'. Reaction solution of PCR consisted of 2.0 μ l diluted RT-PCR product, and 0.2 μ M of each paired primers. The annealing temperature was set at 60 °C and amplification cycles were set at 45 cycles. The PCR product was electrophoresed in 1.5% agarose gel.

Demonstration of GR and C/EBP α binding to 11 β -HSD1 promoter with chromatin immunoprecipitation (ChIP) assay in human amnion fibroblasts

Primary human amnion fibroblasts were prepared as described above and plated in 10-cm Petri dishes at a density of 10⁷ cells/dish. The cells were treated with cortisol (1 μ M) for 12 h before the start of ChIP assay using a kit from Upstate Biotechnology (Temecula, CA, USA) and a method modified from the manufacturer's protocol. Upon termination of treatment, the cells were fixed with 1% formaldehyde to cross-link the transcription factors to chromatin DNA. After washing with PBS, the cells were incubated with glycine (125 mM) for 30 min and then scraped off the dish in cold PBS containing protease inhibitor cocktail. The cells were spun down at 720 g at 4 °C and were resuspended with ice-cold lysis buffer supplemented with protease inhibitor cocktail and incubated on ice for 30 min. The cells were then gently broken

up on ice using a Dounce homogenizer to aid nuclei release. The nuclei were spun down at 2400 g, 4 °C and were resuspended in digestion buffer supplemented with protease inhibitor cocktail. Enzymatic shearing of the chromatin DNA was then carried out at 37 °C. The shearing time was optimized according to the pilot study to produce an optimized size of input DNA around 500 bp. The shearing was stopped by adding ice-cold EDTA and the sheared DNA was centrifuged at 2500 g at 4 °C. The supernatant containing sheared DNA was collected for subsequent immunoprecipitation. The diluted sheared DNA was incubated by shaking with GR antibody (Santa Cruz) or C/EBP α antibody (Santa Cruz) or C/EBP β antibody (Santa Cruz) or normal serum as negative control for 12 h at 4 °C. Protein A agarose/salmon sperm DNA was then added to the above solution according to the animal source the antibody was derived from and incubated for 2 h at 4 °C. The antibody/protein/DNA/agarose complex was then spun down at 2500 g and resuspended in low salt buffer and allowed to pass through a filter column (0.45 μ m). The column was washed repeatedly with low and high salt buffers, LiCl and TE buffer in order to minimize the nonspecific background. After washing, the filter column was eluted with elution buffer (1% SDS, 0.1 M NaHCO₃) and the flow through was collected for subsequent reverse cross-linking with 5 M NaCl at 65 °C for 4 h. At the end of reverse cross-linking, 0.5 M EDTA, 1 M Tris-HCl (pH 6.5), and proteinase K were added and incubated for 1 h at 45 °C. The sheared DNA recovered from reverse cross-linking was extracted with DNA extraction kit for further PCR analysis.

PCR primers for amplifying DNA fragments immunoprecipitated by GR, C/EBP α and C/EBP β antibodies were designed according to the results of 11 β -HSD1 promoter analysis study to ensure the primers spanned the predicted promoter region responsible for the regulation by cortisol. The positions and sequences of these primers are illustrated in Fig. 8. Real-time PCR was performed on the DNA fragments. Some of the PCRs were stopped at 23 cycles and PCR products were analyzed with 2% agarose gel electrophoresis. Some of the PCRs were continued to obtain C_t values. The relative level of DNA fragments was calculated according to a standard curve set up using serial dilutions of the PCR product.

Electrophoretic mobility shift assay (EMSA)

In order to further confirm the putative GRE- and C/EBP-binding sites in 11 β -HSD1 promoter, EMSA was performed using biotin end-labeled double-stranded oligonucleotides designed according to the 11 β -HSD1 promoter-driven reporter gene study. The sense oligonucleotide sequence for putative GRE is as follows: 5'-GAATCCAGTCCTGTA-CAGTCATGAGCTT-3', which corresponds to the promoter region from -207 to -178 bp spanning the predicted GRE (underlined). The sense oligonucleotide sequence for putative C/EBP-binding sites is as follows: 5'-CCACCCAAAGCCAATCGCTGCTCTGA-3', which corresponds to the promoter region from -86 to -61 bp

spanning the predicted C/EBP-binding site (underlined). To verify the specific binding to the predicted GR and C/EBP sites by the transcription factors, oligonucleotide sequences carrying the same mutations (bold letters) in the predicted GRE and CCAAT as in the reporter gene study were also utilized in the binding reaction. Nuclear protein was extracted from cultured human amnion fibroblasts stimulated by cortisol (1 μ M) for 12 h using an extraction kit from Active Motif. The binding reaction of nuclear protein (3 μ g) and biotin-labeled oligonucleotides with or without mutations (15 fmol) was carried out overnight at 4 °C in the presence of binding buffer (10 mM Tris, 50 mM KCl, pH 7.5), poly (dI-dC; 50 ng/ μ l) with or without 200-fold molar excess of unlabeled specific competitor sequences or GR antibody (1 μ g, Santa Cruz) or C/EBP α antibody (1 μ g, Santa Cruz) or C/EBP β antibody (1 μ g, Santa Cruz). The binding solution was then electrophoresed in a 6% DNA retardation gel (Invitrogen) in 0.5 $\times$ TBE at 100 V and transferred to a nylon membrane using a semi-dry transfer unit at 380 mA for 30 min. The transferred DNA was cross-linked to the membrane under u.v.-light. The biotin-labeled DNA on the membrane was detected using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA) according to a protocol provided by the manufacturer. Briefly, after blocking, the membrane was incubated with streptavidin-horseradish peroxidase conjugate/blocking buffer. The membrane was then washed and incubated in substrate equilibration buffer. After discarding the substrate equilibration buffer, the membrane was incubated with substrate (luminol/enhancer/stable peroxide solution) for streptavidin-horseradish peroxidase for 1 min and finally the membrane was exposed to X-ray film.

Statistical analyses

All data are reported as mean $\pm$ S.E.M. Paired Student's *t*-tests or two-way ANOVA followed by the Student-Newman-Keuls test were used where applicable to assess significant differences between groups. Significance was set at $P < 0.05$. The values for *n* refer to the number of experiments performed with primary cells prepared from different patients or from different passages of WISH cells.

Results

Induction of 11 β -HSD1 mRNA expression by cortisol in human amnion fibroblasts

Treatment of human amnion fibroblasts with cortisol for 24 h caused an average of 3.6- and 7.9-fold increases in 11 β -HSD1 mRNA expression respectively at 0.1 and 1 μ M, but not at 0.01 μ M cortisol (Fig. 2). Treatment of the cells with the mRNA transcription inhibitor DRB (75 μ M) completely blocked the induction of 11 β -HSD1 mRNA expression by cortisol (0.1 μ M; Fig. 3, top panel), suggesting that this induction was dependent on the ongoing transcription.

Figure 2 Concentration-dependent effect of cortisol on 11 β -HSD1 mRNA levels in human amnion fibroblasts. $n=3$, * $P < 0.05$ versus no cortisol treatment control group.

The induction of 11 β -HSD1 mRNA expression by cortisol (0.1 μ M) could also be completely blocked by the GR antagonist RU486 (1 μ M, Fig. 3, middle panel) and partially blocked by global inhibition of C/EBPs with transfection of AC/EBP into the cells (Fig. 3, bottom panel).

Basal and cortisol-induced 11 β -HSD1 promoter activity in WISH cells transfected with pGL3 enhancer plasmid carrying 11 β -HSD1 promoter-driven reporter gene

In order to overcome the relative difficulties in transfecting primary amnion fibroblasts, a human amnion epithelium-derived immortalized cell line, WISH cells, was used for promoter analysis study. Analysis with PCR and western blot revealed GR mRNA and protein expression in WISH cells (Fig. 4, top panel). All the plasmid constructs containing different lengths of 11 β -HSD1 promoter exhibited basal promoter activity when transfected into WISH cells, while the empty pGL3 enhancer plasmid bearing no promoter exhibited mostly background activity (Fig. 4, open columns). The basal promoter activity was significantly increased when the 5' end of -1085 bp was deleted to -1005 bp and was further dramatically increased when deleted to -857 bp (Fig. 4 open columns). There was no further change with deletion from -857 to -467 bp (Fig. 4, open columns). However, the basal activity was dramatically decreased with deletion from -467 to -204 bp (Fig. 4, open columns). Further modest decrease was observed with deletion from -204 to -84 bp, but there was no further change with deletion from -84 to -64 bp (Fig. 4, open columns). These observations suggest that repressors for promoter activity might exist in the promoter region upstream to -857 bp, while enhancers for promoter activity might exist in the promoter region between -857 and -204 bp.

Cortisol (1 μ M) treatment of the cells transfected with the above constructed plasmids significantly increased all the promoter activities including -1085, -1005, -857, -467, -204, and -84 bp except the -64 bp promoter (Fig. 4). Moreover, similar fold of cortisol-induced increases was

Figure 3 Effect of DRB (75 μ M, top panel, $n=6$), RU486 (1 μ M, middle panel, $n=4$) and transfection of C/EBP-specific dominant-negative expression CMV-500 plasmid (AC/EBP, bottom panel, $n=4$) on cortisol (F, 0.1 μ M)-induced changes of 11 β -HSD1 mRNA levels in human amnion fibroblasts. ** $P<0.01$ versus control (ctr), * $P<0.05$, ## $P<0.01$ versus cortisol + DRB or cortisol + AC/EBP respectively.

observed among the promoters of -204 , -1005 , and -1085 bp, which was significantly greater than the fold of increases induced by cortisol in promoters of -467 and -857 bp ($P<0.05$). These observations suggest that the high basal activity of -467 and -857 bp might impede further increases upon cortisol stimulation. In consideration of the

dramatic decrease in cortisol-induced promoter activity with deletion from -204 to -84 bp (Fig. 4) and the similar fold of cortisol-induced increases among the promoters of -204 , -1005 , and -1085 bp, we inferred that the promoter region downstream to -204 bp and upstream to -84 bp might bear a crucial cortisol-response element, although the involvement of other promoter regions both upstream to -204 bp and downstream to -84 bp in cortisol's induction could not be ruled out. Such an element was shown to be present in the promoter region between -84 and -64 bp, though appeared to be less important, as cortisol caused a modest increase of promoter activity of the -84 bp (Fig. 4).

Effect of GR antagonist RU486 and co-transfection of AC/EBP plasmid on cortisol-induced 11 β -HSD1 promoter activity in WISH cells

Sequence analysis of the promoter region downstream to -204 bp and upstream to -64 bp with transcription element search system (TESS) revealed a putative GRE and several CCAAT boxes in this region. Based on this analysis, we further studied the effect of cortisol in this region in relation to the roles of GR- and CCAAT-binding proteins, C/EBPs. Results showed that cortisol induced the promoter activity of -204 bp in a dose-dependent manner (Fig. 5, top panel). Co-treatment of the cells with cortisol (0.1 μ M) and the GR antagonist RU486 (1 μ M) completely blocked the induction of the promoter activity of -204 bp by cortisol (Fig. 5, bottom panel), suggesting that the induction was fully dependent on GR activation. Despite this, the induction of the promoter activity of -204 bp by cortisol (0.1 μ M) could also be partially blocked by global inhibition of C/EBPs with co-transfection of AC/EBP plasmid into the cells (Fig. 6, top panel), suggesting that in addition to the dominant role of GR, C/EBPs might also be involved in the induction of promoter activity by cortisol. However, the involvement of C/EBPs was more likely to be secondary to GR activation as GR antagonist completely blocked the induction by cortisol. Nevertheless, inhibition of C/EBPs with AC/EBP plasmid transfection completely inhibited cortisol (0.1 μ M)-induced promoter activity of -84 bp (Fig. 6, bottom panel), suggesting that C/EBPs, possibly secondary to GR activation, were the transcription factor responsible for cortisol's induction in the region downstream to -84 bp and upstream to -64 bp.

Effect of specific nucleotide mutations in the putative GRE and C/EBP-binding site (CCAAT box) on cortisol-induced 11 β -HSD1 promoter activity

Based upon the above promoter studies, we introduced nucleotide mutations (underlined and bold letters) into the putative GRE (CTG**TACAG**) at -197 to -190 bp of the -204 bp promoter and a C/EBP-binding site (CCAAT box, C**CAATC**) at -76 to -71 bp of the -84 bp promoter (Fig. 1). Although single nucleotide mutation of the putative GRE at position -192 bp (from C to T) did not affect the

Figure 4 Top panel: PCR and western blotting analysis showing glucocorticoid receptor (GR) mRNA and protein expression in WISH cells ($n=4$). Bottom panel: The basal (top panel, $n=4-12$) and cortisol ($1 \mu\text{M}$)-induced (bottom panel, $n=4-12$) 11 β -HSD1 promoter activity in WISH cells. * $P<0.05$, ** $P<0.01$, comparison between basal activities of various lengths of promoters; # $P<0.05$, comparison between cortisol-induced activity with respective basal activity. pGL3e: empty pGL3 enhancer plasmid.

induction by cortisol (data not shown), two nucleotide mutations at positions -194 and -193 bp (from TA to CG) partially inhibited the induction of the promoter activity of -204 bp by cortisol (Fig. 7, top panel), while two nucleotide mutations of the CCAAT box at positions -75 and -73 bp (from C and A to T and G respectively) almost completely blocked the induction of the promoter activity of -84 bp by cortisol (Fig. 7, bottom panel). These observations further confirmed the involvement of the predicted GRE and CCAAT in cortisol-induced 11 β -HSD1 promoter activity on the one hand and also suggested that in addition to GRE, other elements such as CCAAT were also involved in cortisol's induction on the other hand.

Binding of GR and C/EBP α but not C/EBP β to 11 β -HSD1 promoter upon cortisol stimulation in human amnion fibroblasts

ChIP assay showed that treatment of human amnion fibroblasts with cortisol ($1 \mu\text{M}$) for 12 h caused association of GR and C/EBP α to 11 β -HSD1 promoter as reflected by the abundance of PCR products amplified using primers

spanning the predicted promoter sequences (Fig. 8, middle panel). Analysis with qRT-PCR also revealed that the amount of DNA precipitated by GR or C/EBP α antibody was more in cortisol stimulation group than that of without cortisol stimulation group (Fig. 8, bottom panel). However, there were no obvious differences between cortisol and without cortisol-treated groups using DNA precipitated by C/EBP β antibody (data not shown), suggesting that cortisol caused no obvious binding of C/EBP β to 11 β -HSD1 promoter.

EMSA revealed that nuclear protein extracted from amnion fibroblasts stimulated by cortisol for 12 h could bind the oligonucleotides at positions of -207 to -178 bp spanning the predicted GRE producing distinguishable shifted band upon gel electrophoresis (Fig. 9, upper). Incubation with GR antibody but not C/EBP α antibody supershifted the band (Fig. 9, upper left). Although there was some binding of the mutated oligonucleotides bearing the putative GRE with the nuclear protein, incubation of GR antibody caused no obvious supershift of the binding (Fig. 9, upper right). Likewise, nuclear protein derived from amnion

Figure 5 Concentration-dependent effect of cortisol on 11 β -HSD1 promoter activity ($n=3$, top panel) and effect of RU486 (1 μ M) on cortisol (F, 0.1 μ M)-induced 11 β -HSD1 promoter activity ($n=3$, bottom panel) in WISH cells. * $P<0.05$, ** $P<0.01$ versus no cortisol treatment control group, ## $P<0.05$ versus cortisol (F) group.

fibroblasts simulated by cortisol could also bind the oligonucleotides spanning the predicted CCAAT box at positions of -86 to -61 bp and produced distinguishable shifted band upon gel electrophoresis (Fig. 9, bottom). Incubation of the CCAAT oligonucleotides with C/EBP α antibody but not C/EBP β antibody supershifted the band (Fig. 9, bottom left), while incubation of the mutated oligonucleotides bearing CCAAT with C/EBP α antibody produced no obvious supershifted band (Fig. 9, bottom right). These observations were consistent with the results of ChIP assay, suggesting C/EBP α rather than C/EBP β was involved in the induction of 11 β -HSD1 promoter activity by cortisol. In addition, there were no obvious shifts observed in incubation without nuclear protein (far left lane in each gel picture of Fig. 9) and all the shifts and supershifts produced by nuclear protein and antibodies were abolished when excessive nonlabeled-specific oligonucleotides were included in the incubation (far right lane in each gel picture of Fig. 9), suggesting that the binding of nuclear protein to the oligonucleotides was specific.

Figure 6 Effect of co-transfection of C/EBP-specific dominant-negative expression CMV500 plasmid (AC/EBP) on the induction of 11 β -HSD1 promoter (-204 bp, top panel; -84 bp, bottom panel) activity by cortisol (0.1 μ M) in WISH cells. $n=4$; * $P<0.05$, ** $P<0.01$ versus respective controls; # $P<0.05$, ## $P<0.01$ versus respective AC/EBP groups.

Discussion

Previous studies in humans and animals have shown that GCs increase 11 β -HSD1 expression in a number of tissues and cells (Hammami & Siiteri 1991, Bujalska *et al.* 1997, Whorwood *et al.* 2002, Sun & Myatt 2003, Li *et al.* 2006). In this study, we demonstrated that cortisol, the endogenous GC as well as the end product of 11 β -HSD-catalyzed reduction reaction, could positively feedback on 11 β -HSD1 mRNA expression in human amnion fibroblasts. This feed-forward regulation could be achieved at cortisol concentration around 0.1 μ M, which may be reached at the end of pregnancy both in maternal plasma and amniotic fluid (Blankstein *et al.* 1980). Although 11 β -HSD1 is a relatively low-affinity enzyme for the substrate with a K_m value in the micromolar range (Seckl 1993, Tomlinson *et al.* 2004, Draper & Stewart 2005), cortisone, like cortisol, rises steadily with gestational age in the amniotic fluid, and reaches the micromolar range at the end of pregnancy (Blankstein *et al.* 1980). In consideration of

Figure 7 Nucleotide mutations in the predicted glucocorticoid-response element (GRE, $n=5$, top panel) and C/EBP-binding site (CCAAT, $n=4$, bottom panel) on the induction of 11 β -HSD1 promoter (-204 and -84 bp) activity by cortisol ($0.1 \mu\text{M}$) in WISH cells. * $P<0.05$, ** $P<0.01$ versus respective controls; # $P<0.05$, ## $P<0.01$ versus respective mutation groups.

these facts, we speculate that the reaction catalyzed by 11 β -HSD1 in the fetal membranes could become feed forward at the end of pregnancy. Nevertheless, *Alfaidy et al. (2003)* also demonstrated that the expression of 11 β -HSD1 in the fetal membranes increased with gestational age. This feed-forward expression of 11 β -HSD1 at the end of gestation may be required both for the full activation of GR in the fetal membranes and for fetal maturation (*Murphy 1977*). Cortisol derived from fetal membranes might be supplemental to the function of fetal adrenal glands, as the adrenal glands of the human fetus mainly produce dehydroepiandrosterone rather than cortisol during gestation (*Mesiano & Jaffe 1997*). The finding that the homozygous mice with a targeted disruption of 11 β -HSD1 gene have impaired surfactant protein production in the lung, lends further support for a crucial role of 11 β -HSD1 in fetal maturation (*Hundertmark et al. 2002*).

Tannin et al. (1991) isolated a human cDNA clone encoding 11 β -HSD1 from a human testis cDNA library. Their primer

extension analysis using liver RNA indicated that transcription starts 93 bp upstream from the translation start site. Their data also showed that there was no TATA box in the 5' untranslated region, but there were a consensus CCAAT box at -76 bp upstream from the start of transcription and a palindromic sequence resembling part of a GRE at -188 bp upstream from the start of transcription. In this study, we found that cortisol induced similar extent of increases in the promoter activities of -204 , -1005 , and -1085 bp, and the induction was greatly decreased when deletion progressed from -204 to -84 bp and disappeared when deletion progressed to -64 bp. These observations suggested that a crucial GRE might be present in the promoter region between -204 and -84 bp, although other promoter regions could also participate, such as the one in between -84 and -64 bp as demonstrated in this study. Coincidentally, the promoter region between -204 and -84 bp contains the putative GRE and the promoter region between -84 and -64 bp contains CCAAT box respectively, as speculated by *Tannin et al. (1991)*.

GCs act through binding to their cytosolic GR, serving as a transcription factor. Once activated by GCs, GRs forms homodimers and translocate into the nucleus, where they ultimately bind to specific GRE on gene promoters to regulate transcription (*Hayashi et al. 2004*, *Smoak & Cidlowski 2004*). However, this mechanism does not explain all the actions of GCs. Several studies demonstrated that interaction of GR with other transcription factors could provide an alternative pathway for GC actions (*Hayashi et al. 2004*, *Smoak & Cidlowski 2004*). GCs have been found to induce C/EBP α or C/EBP β expression in a number of tissues or cells (*Zilberfarb et al. 2001*, *Hernandez et al. 2003*, *Yang et al. 2005a,b*). Furthermore, interaction of C/EBP α and GR has been found to regulate cell proliferation (*Rudiger et al. 2002*, *Roth et al. 2004*). *Williams et al. (2000)* found that C/EBP α was an important regulator of mouse basal 11 β -HSD1 promoter activity in hepatoma cells. In a recent study, C/EBP β was also found to regulate human 11 β -HSD1 promoter activity in mouse adipose cells (*Gout et al. 2006*). All these findings pointed to a role of C/EBPs in the regulation of 11 β -HSD1 expression at least at the basal status. In this study, we found that the induction of 11 β -HSD1 mRNA expression and promoter activity by cortisol involves not only GR but also C/EBP α .

The observation that the induction of 11 β -HSD1 expression could be completely blocked by a GR antagonist suggests a predominant role of GR in this induction. Studies of GRE mutation, ChIP assay, and EMSA not only confirmed the putative GRE in 11 β -HSD1 promoter but also provided evidence for a direct interaction of GR with GRE in 11 β -HSD1 promoter. However, in addition to the direct interaction of GR with GRE, this study also provided evidence for an indirect role of GR in the induction of 11 β -HSD1 promoter activity by cortisol, as the induction of both 11 β -HSD1 mRNA expression and promoter activity by cortisol could also be partially blocked by C/EBP inhibition. A number of studies (*Zilberfarb et al. 2001*, *Hernandez et al.*

Figure 8 Chromatin immunoprecipitation (ChIP) assay showing glucocorticoid receptor (GR) and C/EBPα bound to 11β-HSD1 promoter in human amnion fibroblasts upon cortisol (1 μM, F) stimulation for 12 h. Top panel illustrates the positions and sequences of primers used in PCR amplifying the DNA fragments immunoprecipitated by GR antibody or C/EBPα antibody. Middle panel: representative gel picture showing the PCR products electrophoresed in 2% agarose gel. Input: sheared DNA; F(+Ab): with cortisol treatment and with antibody precipitation; Ctrl: without cortisol treatment but with antibody precipitation; F(-Ab): with cortisol treatment but without specific antibody precipitation. Bottom panel shows the three separate results of RT-PCR analysis of DNA precipitated by GR and C/EBPα antibodies respectively.

2003, Yang *et al.* 2005a,b) have demonstrated that GCs induced C/EBP expression in various tissues. These observations were in favor of the participation of C/EBPs in the induction of 11β-HSD1 expression by cortisol. Furthermore, the ChIP assay and EMSA in this study revealed that C/EBPα rather than C/EBPβ was involved in the induction by cortisol. This observation was supported by the study of Williams *et al.* (2000) showing that C/EBPα was a powerful transcription factor for the induction of basal 11β-HSD1 mRNA expression in the mouse, while C/EBPβ functioned mainly as a relative inhibitor of basal 11β-HSD1 expression.

However, it should also be kept in mind that there are several CCAAT-like sequences both upstream and downstream to -84 bp as revealed by TESS analysis. Using footprint analysis,

Williams *et al.* (2000) also found multiple C/EBPα-binding sites scattered along the -812 bp of mouse 11β-HSD1 promoter. Thus, in addition to the CCAAT box downstream to -84 bp as demonstrated in this study, other CCAAT-like sequences upstream to -84 bp may also participate in the induction of 11β-HSD1 expression by cortisol.

In conclusion, we demonstrated in this study that the feed-forward regulation of 11β-HSD1 gene expression by cortisol involves both direct binding of GR to GRE in the promoter and indirect activation of C/EBPα binding to the CCAAT box in the promoter in human amnion cells. This feed-forward regulation of 11β-HSD1 by cortisol may be required for the full local actions of cortisol in the fetal membranes as well as for the fetal maturation at the end of gestation.

Figure 9 Electrophoretic mobility shift assay showing nuclear protein extract from human amnion fibroblasts caused shifts of the oligonucleotides bearing putative glucocorticoid-response element (GRE) or C/EBP-binding site (CCAAT). Glucocorticoid receptor antibody or C/EBP α but not C/EBP β antibody caused supershifts of the respective shifted nuclear protein–oligonucleotide complex. Lane nnp: no nuclear protein; lane no Ab: with nuclear protein but without antibody; lane GR: with nuclear protein and GR antibody; lane C/EBP α : with nuclear protein and C/EBP α antibody; lane C/EBP β : with nuclear protein and C/EBP β antibody; lane NLO: with nuclear protein, antibody and 200-fold nonlabeled oligonucleotides. Wild: oligonucleotide sequence without mutations; mut: oligonucleotide sequence with mutations.

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