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ORIGINAL ARTICLE

Stimulation of 11β-HSD1 expression by IL-1β via a C/EBP binding site in human fetal lung fibroblasts

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Abstract Proinflammatory cytokines, just like glucocorticoids (GCs), have been reported to upregulate 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) expression in many cell types. This concerted regulation of 11β-HSD1 by interleukin-1β (IL-1β) and GCs is in marked contrast to their antagonistic effects on inflammation. Further, the molecular mechanisms underlying the induction of 11β-HSD1 by IL-1β are not very well understood. In this study, we demonstrated that IL-1β dramatically stimulated 11β-HSD1 expression and enzyme activity as well as promoter activity including the −64 bp fragment upstream to the transcription start site in human fetal lung fibroblasts (HFL-1). Nucleotide mutations of the proximal CCAAT box within this region abolished the induction of 11β-HSD1 promoter activity by IL-1β. Western blotting analysis demonstrated that IL-1β induced the expression of C/EBPβ dramatically while C/EBPα was barely detectable in HFL-1 cells. Global inhibition of CCAAT/enhancer-binding proteins (C/EBPs) with transfection of C/EBP-specific dominant-negative expression plasmid (CMV500-A-C/EBP) significantly attenuated the induction of 11β-HSD1 by IL-1β, whereas over-expression of C/EBPβ enhanced the expression of 11β-HSD1. Chromatin immunoprecipitation assay revealed the recruitment of C/EBPβ to the promoter region containing the C/EBP binding site. In conclusion, IL-1β induces the expression of 11β-HSD1 mRNA in the fetal lung tissue through mechanisms that involve C/EBPβ binding to the promoter. This impact of IL-1β on the expression of 11β-HSD1 in human fetal lung cells may explain the alternate mechanism for the lung maturation that appears to occur when there is a risk of premature delivery of the fetus due to the presence of infection.

Keywords Glucocorticoid · 11β-HSD1 · IL-1β · Fetal lung development · C/EBPβ

Introduction

Significant amounts of prospective clinical and experimental data have been amassed to demonstrate that a reduction in respiratory distress syndrome (RDS) in preterm infants is associated with antenatal treatment with glucocorticoids (GCs) as well as with intrauterine infection and/or inflammation such as chorioamnionitis that often occurs in preterm delivery [1, 2].

Glucocorticoids exert their biological actions through binding to the glucocorticoid receptor (GR), member of nuclear hormone receptor transcription factor superfamily [3]. The amount of GCs available to GR is largely dependent on the amount of 11β-hydroxysteroid dehydrogenase (11β-HSD) activity [4]. Two isoforms of 11β-HSD, the products of distinct genes, have been recognized. 11β-HSD2, a NAD-dependent dehydrogenase that converts biologically active GCs into inactive metabolites, is expressed mainly in mineralocorticoid target tissues [5] as well as in the placenta and the developing fetus [6]. In contrast, 11β-HSD1 is a NADP(H)-dependent enzyme, which acts mainly as a 11-ketosteroid reductase in intact cells that convert inactive GC metabolites into biologically active GCs [7]. 11β-HSD1 is ubiquitously distributed in GC

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target tissues including the lungs [7, 8]. Hundermark et al. [9] showed that inhibition of 11β-HSD1 activity or knockout of the 11β-HSD1 gene severely impaired lung maturation in mice. This observation indicates that 11β-HSD1 plays a vital role in the process of fetal lung maturation. Proinflammatory cytokines, like GCs, upregulate 11β-HSD1 expression in many cell types [10, 11]. This concerted regulation of 11β-HSD1 by IL-1β and GCs is in marked contrast to their antagonistic effects on inflammation [12]. We now know that CCAAT-enhancer binding protein (C/EBP) mediates the regulation of 11β-HSD1 expression by GCs [13, 14]. Previously we have demonstrated the induction of 11β-HSD1 expression by IL-1β could also be attenuated by transfection with C/EBP dominant negative expression plasmid in human fetal lung fibroblasts (HFL-1) [11]. However, the cis-element of the promoter and the specific transactivator involved in the induction by IL-1β is not well understood. Understanding this mechanism may provide an alternate mechanism for the maturation of fetal mammalian lungs when the fetus is at risk of preterm delivery due to infection.

Results

Regulation of 11β-HSD1 and 11β-HSD2 by IL-1β in HFL-1 cells

Treatment of HFL-1 cells with IL-1β at 0.1, 1, and 10 ng/ml for 12 h increased the expression of 11β-HSD1 mRNA in a concentration-dependent manner by an average of 13.2, 19.3, and 26.1 fold, respectively (Fig. 1a). Consistent with these changes in mRNA expression, an apparent increase in 11β-HSD reductase activity was observed after HPLC analysis of the relative amounts of the exogenous substrate, cortisone, and the resulting metabolite, cortisol, in medium from incubations of HFL-1 cells in vitro (Fig. 1b). The expression of 11β-HSD2 mRNA was much less than that of 11β-HSD1 mRNA in HFL-1 cells (Fig. 1a). In fact, inclusion of IL-1β in the incubation medium significantly reduced the expression of 11β-HSD2 mRNA in HFL-1 cells (Fig. 1a).

Induction of 11β-HSD1 mRNA by IL-1β requires de novo protein synthesis

Treatment of the HFL-1 cells with cycloheximide (CHX, 10 μM), a general translation inhibitor, significantly attenuated the expression of 11β-HSD1 mRNA by IL-1β (10 ng/ml) (Fig. 2), which indicates that the induction of IL-1β on 11β-HSD1 mRNA expression requires the synthesis of at least one other protein.

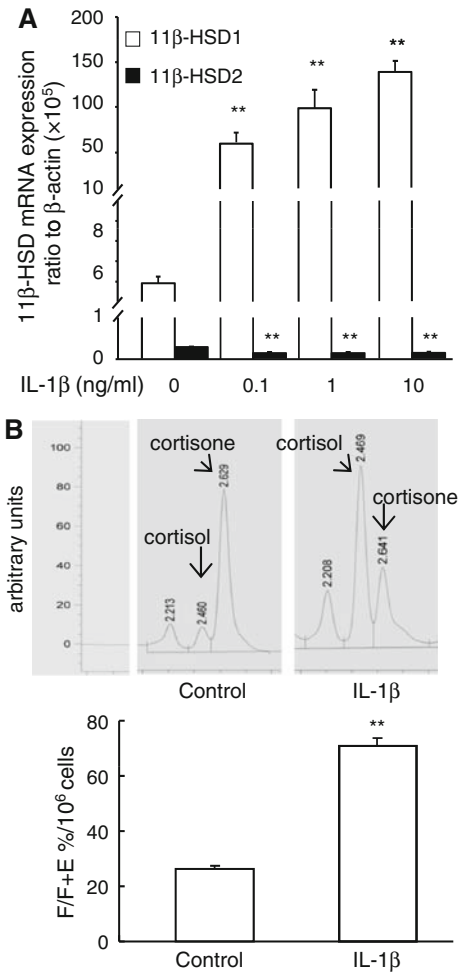

Fig. 1 Impact of IL-1β on the expression of 11β-HSD1 and 11β-HSD2 mRNAs and 11β-HSD activity in HFL-1 cells. **a** Impact of increasing doses of IL-1β on expression of mRNA for 11β-HSD1 and 11β-HSD2 mRNAs as assessed by real time PCR (n = 3–5 independent experiments). **b** Impact of a single dose of IL-1β (10 ng/ml) on the relative conversion of cortisone to cortisol in HFL-1 cells. Upper panel **b**: representative HPLC chromatograms; lower panel **b**: Mean + SEM of four independent experiments. E: cortisone, F: cortisol. ** $P < 0.01$ versus vehicle control group

Induction of 11β-HSD1 promoter activity by IL-1β via the CCAAT consensus sequence

IL-1β (10 ng/ml) treatment of the HFL-1 cells transfected with constructed plasmids carrying various lengths of 11β-HSD1 promoter and reporter gene significantly increased all the promoter activities including −1005, −204, −84, and −64 bp promoters (Fig. 3b). Bioinformatic analysis of the promoter region within −64 bp with transcription element search system (TESS) revealed a putative CCAAT box at −52 to −47 bp (Fig. 3a). Nucleotide mutations of this CCAAT box (from AAT to GGC) (boxed bold letters) completely blocked the induction of the promoter activity of −64 bp by IL-1β (Fig. 3b).

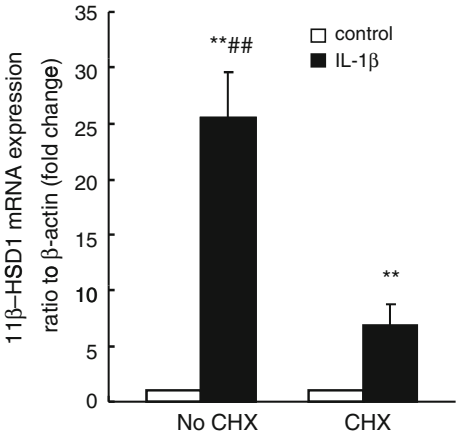

Fig. 2 Impact of protein synthesis inhibitor cycloheximide (CHX, 10 μM) on IL-1β (10 ng/ml)-induced expression of 11β-HSD1 mRNA in HFL-1 cells. $n = 4$ independent experiments. ** $P < 0.01$ versus respective control groups, $^{\#\#}$ $P < 0.01$ versus group incubated with IL-1β in the presence of CHX

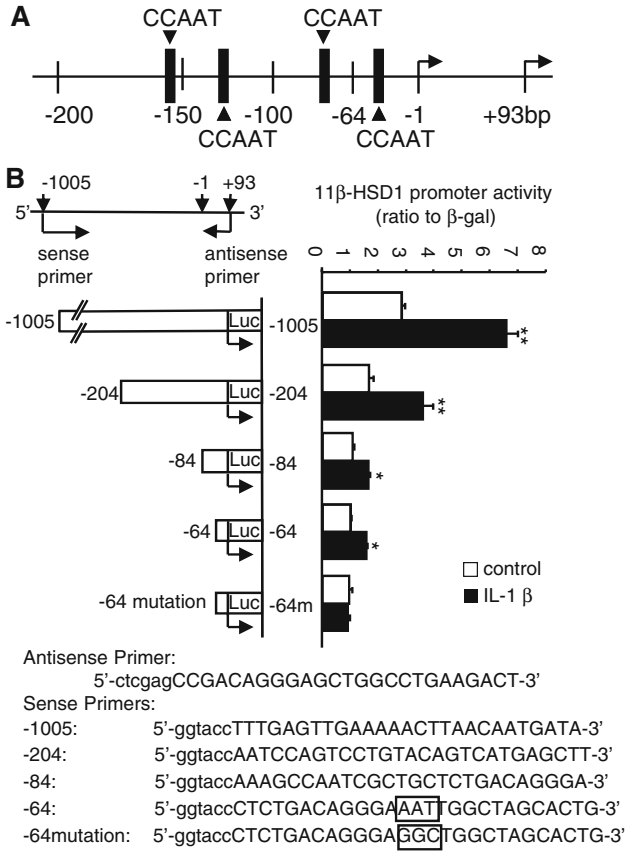

Fig. 3 Impact of IL-1β on 11β-HSD1 promoter activity in HFL-1 cells. **a** Schematic illustration of the putative CCAAT boxes in 11β-HSD1 gene promoter. **b** Impact of progressive 5′ deletions of 11β-HSD1 promoter on the induction by IL-1β (10 ng/ml) in HFL-1 cells. Upper panel **b**: Mean + SEM of 11β-HSD1 promoter activity in the presence or absence of IL-1β, $n = 3$–6 independent experiments; lower panel **b**: the primer sequences used for subcloning of 11β-HSD1 gene promoter. *Small case letters* indicate the sequences of restrictive enzymes. * $P < 0.05$, ** $P < 0.01$ versus respective vehicle control groups

Involvement of C/EBPs in the induction of 11β-HSD1 expression by IL-1β in HFL-1 cells

Based upon the above studies, we investigated the role of C/EBPs in the regulation of 11β-HSD1 mRNA expression by IL-1β. Global inhibition of C/EBPs with transfection of C/EBP-specific dominant-negative expression (CMV500-A-C/EBP) vector into the cells could attenuate the induction of 11β-HSD1 promoter (both −1005 and −64 bp) activities by IL-1β (10 ng/ml) (Fig. 4a). This observation suggests the C/EBPs are involved in IL-1β-induced expression of 11β-HSD1 in HFL-1 cells. Further, the basal activities of both −1005 and −64 bp 11β-HSD1 promoters were reduced by the transfection of CMV500-A-C/EBP plasmid (Fig. 4a). This suggests the involvement of C/EBPs in maintaining the basal expression of 11β-HSD1.

The role of CEBPβ in the induction of 11β-HSD1 expression by IL-1β in HFL-1 cells

We have demonstrated, previously, that C/EBPα is involved in the induction of the expression of 11β-HSD1 gene by cortisol in human amnion fibroblasts [13]. Our present observation that human amnionic fibroblasts express high levels of C/EBPα with very low expression of C/EBPβ (Fig. 4b) supports and extends our previous observation. In marked contrast, we now clearly demonstrate that HFL-1 cells express high levels of C/EBPβ that can be stimulated dramatically by exposure to IL-1β (10 ng/ml) for 12 h (Fig. 4b), where only barely detectable amounts of C/EBPα were observed in the presence or absence of IL-1β (Fig. 4b). Chromatin immunoprecipitation (ChIP) assay demonstrated that IL-1β (10 ng/ml) increased the binding of C/EBPβ to the 11β-HSD1 promoter fragment containing the putative C/EBP binding site in HFL-1 cells (Fig. 4d). Transfection of HFL-1 cells with pMSV-C/EBPβ over-expression plasmid significantly induced the activity of −64 bp 11β-HSD1 promoter (Fig. 4c). Together, these observations support the concept of tissue specific roles for the C/EBP isoforms in regulating the induction of 11β-HSD1; providing a strong evidence that C/EBPβ is the isoform that mediates the induction of 11β-HSD1 by IL-1β in HFL-1 cells.

Discussion

There is convincing evidence in the literature that demonstrates that normal maturation of fetal lung is dependent on increased amount of endogenous GCs [1, 15]. However,

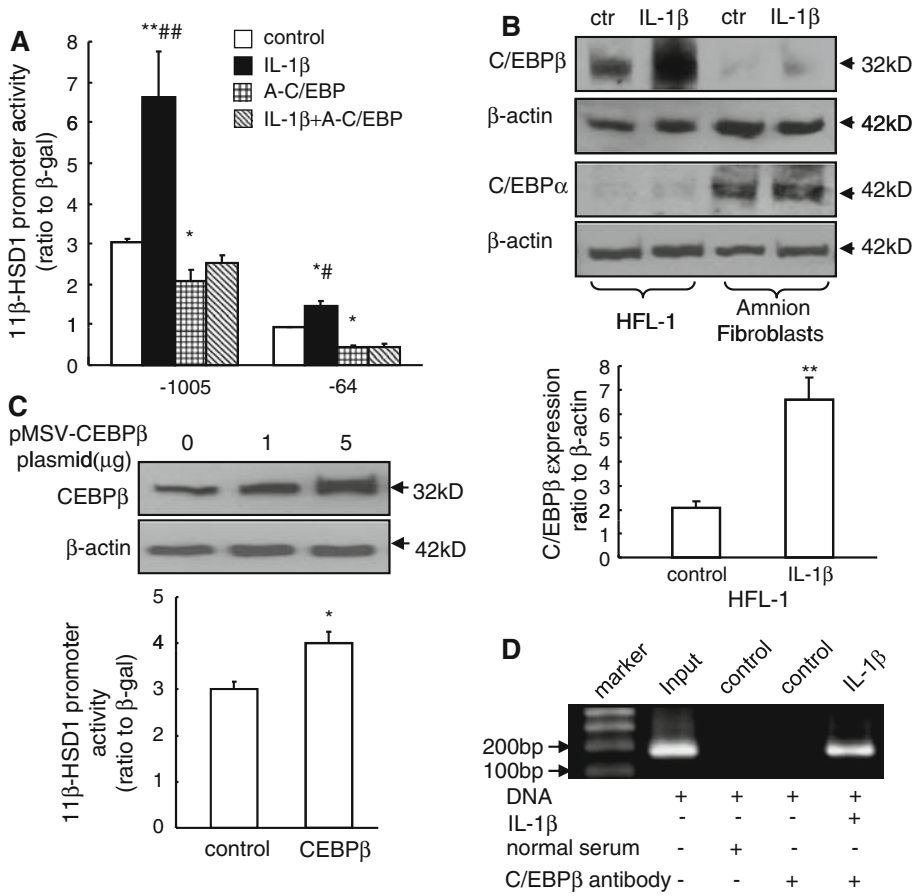

Fig. 4 Involvement of C/EBPβ in the induction of 11β-HSD1 expression by IL-1β in HFL-1 cells. **a** Effect of transfection with C/EBP-specific dominant-negative expression plasmid (CMV500-A-C/EBP) or empty CMV500 plasmid on IL-1β-induced11β-HSD1 promoter activities (−1005 and −64 bp fragments), $n = 5$ independent experiments. * $P < 0.05$, ** $P < 0.01$ versus respective control groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus groups transfected with CMV500-A-C/EBP in the presence of IL-1β. **b** Impact of IL-1β on the expression of C/EBPα and C/EBPβ protein in HFL-1 cells and human amnion fibroblasts. Upper panel **b**: representative western blots; lower panel **b**: Mean + SEM of 3 independent experiments. ** $P < 0.01$ versus control group. **c** Effect of transfection with pMSV-C/EBPβ expression plasmid on 11β-HSD1 promoter activity (−64 bp fragment) in HFL-1 cells. Upper panel **c**: representative Western blots showing C/EBPβ over-expression in HFL-1 cells transfected with pMSV-C/EBPβ exression plasmid; lower panel **c**: Mean + SEM of three independent experiments. * $P < 0.05$ versus control group. **d** Representative chromatin immunoprecipitation (ChIP) gel image of the PCR products amplified from the DNA fragments immunoprecipitated by C/EBPβ antibody in HFL-1 cells

intracellular GC concentrations can differ greatly from blood levels due to the action of 11β-HSDs in adult mammals [16]. We now have demonstrated that HFL-1 cells can express considerable amount of 11β-HSD1 in response to exposure to IL-1β, while the expression of 11β-HSD2 by these cells remain quite limited. The abundant expression of 11β-HSD1 mRNA in HFL-1 cells exposed to IL-1β in vitro, and the resulting increased relative ratio of cortisone-to-cortisol in incubations of such cells with exogenous cortisone, indicates that the reductase activity of 11β-HSD1 also increases in these cells in response to IL-1β in vitro.

It has been long known that intrauterine infection/chorioamnionitis is an important cause of preterm labor. In the in vivo experimental model, intra-amniotic endotoxin caused chorioamnionitis and elevated proinflammatory cytokine expression in the amnion/chorion, and led to lung maturation in preterm infants at the same time [17, 18]. Although proinflammatory cytokines may promote fetal lung maturation by increasing alveolar saturated phosphatidylcholine pool size and surfactant protein expression [17], enhancement of the local concentration of GCs via induction of 11β-HSD1 within the lung tissue appears to be another important mechanism, which may provide a self-protective mechanism for a fetus that is under the threat of an impending infection-induced preterm birth.

Although IL-1β is an important mediator of inflammation and activates the NFκB pathway, which is antagonized by GCs [12], both GCs and IL-1β have the ability to induce the expression of 11β-HSD1 [13, 14]. This observation suggests that IL-1β and GCs use a common pathway for enhancing the expression 11β-HSD1 that is distinct from

the pathway(s) they use to modulate inflammatory responses. Although the transcriptional regulation of the 11β-HSD1 gene can be highly tissue-specific, accumulating evidence indicates a crucial role of the C/EBP family in both basal and inducible transcription of 11β-HSD1 [16]. Analysis of the 11β-HSD1 promoter revealed several CCAAT consensus sequences [19]. We demonstrated previously that C/EBPα mediates the induction of 11β-HSD1 by GCs in human amnion cells [13], while Sai et al. [14] demonstrated that C/EBPβ is involved in the induction of 11β-HSD1 by GCs in A549 cells.

These observations, and our present observations that IL-1β can induce a dramatic increase in the expression of C/EBPβ while the expression of C/EBPα remains barely detectable, and the recruitment of C/EBPβ to 11β-HSD1 gene promoter upon IL-1β stimulation in HFL-1 cells, agree with our concept that IL-1β induces the expression of 11β-HSD1 via the induction of C/EBPβ in HFL-1 cells. The high level of C/EBPα and low level of C/EBPβ in human amnion fibroblast cells might also explain, at least in part, the predominant role of C/EBPα rather than C/EBPβ in the regulation of 11β-HSD1 expression by GCs in this cell type [14]. However, there are at least six isoforms of C/EBPs that have been reported [20]; the role of other isoforms in the induction of 11β-HSD1 expression by IL-1β awaits further study. We postulate that tissue-specific expression patterns of C/EBPα and C/EBPβ might determine which isoform of C/EBPs participates in the regulation of 11β-HSD1 expression in different tissues [13, 14].

In conclusion, in this study we have demonstrated that C/EBPβ mediates the induction of 11β-HSD1 by IL-1β in HFL-1s. These observations support the concept that the induction of 11β-HSD1 in fetal lung cells by IL-1β may provide an important alternate mechanism to enhance fetal lung maturation when the fetus is threatened by infection-induced preterm labor.

Materials and methods

Cell culture

HFL-1 cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in minimum essential medium alpha medium (MEMα) (Invitrogen, Grand Island, NY, USA). For C/EBP expression control study, human amnion fibroblasts were prepared from the fetal membranes collected from pregnant women undergoing elective cesarean sections at term under a protocol approved by the ethics committee of the School of Life Sciences, Fudan University. The culture methods have been described previously [21]. Briefly, human fetal membranes were collected from patients undergoing elective cesarean section at term. Amnion was peeled off the chorion, washed thoroughly in cold PBS (pH 7.5), and digested with 0.125% trypsin (Sigma, St. Louis, MO, USA) twice for 30 min at 37°C. The trypsin digestion medium was discarded, and the amnion tissue was washed vigorously with PBS to get rid of the residual epithelial cells. The remaining amnion tissue was further digested with 0.1% collagenase type 1 (Worthington, NJ, USA) at 37°C for 1 h. The digestion medium was then collected and centrifuged at 2300 rpm for 15 min. Cell pellets were collected and the resuspended cells were loaded onto pre-prepared discontinuous Percoll (GE Healthcare, Uppsala, Sweden) gradients (5, 20, 40, and 60%, respectively). The gradients were centrifuged at 2500 rpm for 20 min. A single band of cells around 20–40% Percoll concentration was collected and cultured in complete DMEM containing 10% Newborn bovine serum (NBS) (Invitrogen, Auckland, New Zealand) and antibiotic–antimycotic (Invitrogen, Grand Island, NY, USA). The identity of cells has been previously verified, and more than 90% of the cells are fibroblasts [22].

Quantitative real time–polymerase chain reaction (qRT–PCR)

The cells were treated with different concentrations of interleukin-1β (IL-1β, 0.1, 1, and 10 ng/ml; Biosource, Camarillo, CA, USA) in serum free medium for 12 h. To examine whether the effect of IL-1β on 11β-HSD1 expression was dependent on de novo protein synthesis, the cells were treated with IL-1β (10 ng/ml) in the presence or absence of protein synthesis inhibitor cycloheximide (CHX, 10 μM, Sigma). At the end of the above treatments, total RNA was extracted from HFL-1 cells using an UNIQ-10 RNA extraction kit (Sangon, Shanghai, China). Total RNA was reverse-transcribed to complementary DNA (cDNA) with oligo (dT) 12–18 primers using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and cDNA was used for subsequent measurement of 11β-HSD1 and 11β-HSD2 mRNA levels with qRT–PCR.

Primers for amplifying 11β-HSD1 were 5′-GAATTC AGACCAGAGAGGCGC-3′ (sense) and 5′-AACTGAGG AAGTTGACTTCCA-3′ (antisense) and primers for amplifying 11β-HSD2 were 5′-GACATGCCATATCCG TGCTT-3′ (sense) and 5′-GCTGGATGATGCTGAC CTTG-3′ (antisense). 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 (Ct) values. To control sampling errors, qRT–PCR for housekeeping gene β-actin was routinely performed on each sample. Primers for β-actin amplification were

5′-GGGAAATCGTGCGTGACATTAAG-3′ (sense) and 5′-TGTGTTGGCGTACAGGTCTTTG-3′ (antisense). 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 was confirmed by examining both the sizes of the PCR products after electrophoresis in 2% agarose gel and the sequences of PCR products.

High performance liquid chromatography (HPLC)

For measurement of the relative conversion of cortisone to cortisol, HFL-1 cells were washed twice with PBS after IL-1β treatment for 24 h and replaced with serum free media with 2 μM cortisone (Sigma) for 1 h. After incubation, medium was collected. Cortisol and cortisone in the culture medium were extracted using ethyl acetate and dried with an evaporator immediately. The steroids were dissolved in the mobile phase and then separated by HPLC using a mobile phase consisting of methanol and H_2O (7:3, v/v) on a C18 column at a flow rate of 1.0 ml/min. The column oven was maintained at 40°C. The UV wavelength for detection was 246 nm. The relative amounts of cortisone and cortisol in these samples were calculated from the corresponding peak areas according to the standard curve. Steroid standards, viz. cortisol and cortisone (Sigma, St. Louis, MO) were dissolved at a consecutive concentration of 0.025, 0.05, 0.1, 0.5, 1, and 2 μM in the mobile phase used for HPLC analysis. Standard curve was drawn from the corresponding UV peak areas of the different steroid concentrations. Recoveries of cortisol and cortisone from the samples were 81.3 ± 5.6 and 77.8 ± 3.6% (mean ± SEM), respectively.

Protein extraction and western blotting

The whole-cell protein was extracted using a kit from Active Motif (Carlsbad, CA, USA) according to the manufacturer's instruction. In order to measure C/EBPα and C/EBPβ protein levels 12 h after IL-1β treatment, western blotting was conducted using a standard protocol. After blocking, the blot was incubated with primary antibodies against human C/EBPα (1:200, Santa Cruz), C/EBPβ (1:200, Cell signaling), and β-actin (1:1000, Sigma), and then with appropriate secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG) coupled to horseradish peroxidase (1:5000, Sigma). The enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA) was used to detect the protein bands on the blot. The relative variability in sample loading and transferring efficiency was assessed by monitoring β-actin protein bands on the blot.

Construction of 11β-HSD1 gene promoter fragments into pGL3 enhancer plasmid carrying luciferase reporter gene

Cloned human 11β-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 11β-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 toward the transcription start site) as paired primer sets. The sequences and annealing positions of these primers are shown in Fig. 3. Restriction enzyme sites for KpnI and XhoI were designed into the sense and antisense primers, respectively (Fig. 3). The correspondingly 5′ deleted 11β-HSD1 gene promoter fragments were produced as follows: −1005, −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. 3). Specific nucleotide mutations were introduced into the predicted sequence responsible for the effect of IL-1β by designing the mutated nucleotides into the sense primers (Fig. 3, bold nucleotides). 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 subcloned sequences and mutations were verified by examining the size of PCR products upon gel electrophoresis as well as by sequencing.

Transient transfection of HFL-1 cells with constructed plasmids

The cells were co-transfected with 0.5 μg/well of pGL3 enhancer plasmid carrying 11β-HSD1 promoter-driven firefly luciferase gene and 0.05 μg/well of β-galactosidase (β-gal) vector using lipofectamine 2000 in Opti-MEM® (Invitrogen). The β-gal vector was used for transfection efficiency control. In some of the above transfection experiments, the cells were co-transfected either with 0.5 μg/well C/EBP-specific dominant-negative expression (CMV500-A-C/EBP) plasmid (courtesy of Dr. C Vinson, National Cancer Institute, Center for Cancer Research, NIH, USA) to inhibit C/EBP function globally, or with empty CMV500 plasmid as control [19]. In another set of experiments, HFL-1 cells were transfected with pMSV-C/EBPβ [23] (courtesy of Dr. Alan Friedman, Johns Hopkins University, USA) to over-express C/EBPβ, and then 11β-HSD1 promoter activity was assayed. All of the above

transfection was carried out for 12 h. After 12 h of transfection, the culture medium was changed to serum-free medium in the presence or absence of IL-1β (10 ng/ml) for 24 h before the luciferase activity assay.

The cells were lysed with lysis buffer (Promega). After centrifugation, the supernatant was used for firefly luciferase activity assay using Luciferase assay system (Promega) with a luminometer according to the protocol provided by the manufacturer. β-Galactosidase activity of the same samples was measured using β-galactosidase Enzyme Assay System (Promega) with a spectrophotometer according the protocol provided by the manufacturer. The ratio of 11β-HSD1 promoter-driven firefly luciferase activity against β-gal enzyme activity was obtained in order to correct differential transfection efficiency in each well and to express the promoter activity.

Chromatin immunoprecipitation assay

HFL-1 cells were treated with IL-1β (10 ng/ml) for 12 h. Upon termination of treatment, ChIP assay was conducted using a kit from Upstate Biotechnology (Temecula, CA, USA) and a method modified from the manufacturer's protocol. Briefly, 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 and then scraped off the dish in PBS containing protease inhibitor cocktail. After spinning down, the cells were resuspended with lysis buffer supplemented with protease inhibitor cocktail and broken up using a Dounce homogenizer to aid nuclei release. After spinning down, the nuclei were resuspended in digestion buffer supplemented with protease inhibitor cocktail. The shearing of chromatin DNA was carried out by sonication to produce an optimized size of input DNA around 500 bp. The sheared DNA was collected for subsequent immunoprecipitation with C/EBPβ antibody (Santa Cruz) or normal serum as negative control. The immunoprecipitate was then incubated with protein A agarose/salmon sperm DNA and the antibody/protein/DNA/agarose complex was washed adequately and collected for subsequent reverse cross-linking. The sheared DNA recovered from reverse cross-linking was extracted with DNA extraction kit for further PCR analysis. The positions and sequences of the primers used for PCR are the same as used in cloning the −64 bp promoter fragment spanning the predicted C/EBP binding site in promoter region (Fig. 3a, b). Real time PCR was performed on the sheared DNA fragments and was stopped before saturation according to the observation of the amplification curve. PCR products were analyzed with 2% agarose gel electrophoresis.

Statistics

All data are reported as mean ± SEM. All experiments were repeated at least three times. Paired Student's *t*-test or One-way ANOVA test followed by the Student Newman–Keuls test was used to assess significant differences where appropriate. Significance was set at $P < 0.05$.

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