

Note

Mechanisms of CYP3A Induction by Glucocorticoids in Human Fetal Liver Cells

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Summary: Human fetal liver (HFL) cells express major drug metabolic enzymes CYP3A4, CYP3A5 and CYP3A7. In the fetal hepatocytes, betamethasone and dexamethasone (DEX) markedly enhanced the expression levels of CYP3A4 and CYP3A7 mRNAs and slightly increased the expression level of CYP3A5 mRNA. Interestingly, a high correlation between the CYP3A induction ability and the intensity of anti-inflammatory effect was observed. Human glucocorticoid receptor (GR)–small interfering RNA clearly attenuated the expression level of GR mRNA, and diminished the DEX-stimulated CYP3A4, CYP3A5 and CYP3A7 expression in HFL cells. These findings indicate that GR mediates the induction of CYP3A4 and CYP3A7 expression in human fetal hepatocytes as well as the CYP3A5.

Keywords: CYP3A; induction; glucocorticoid; human fetal liver cells; glucocorticoid receptor; small interfering RNA; specific small interfering RNA

Introduction

Cytochrome P450 (CYP) comprises a gene superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products. One of them, the human CYP3A subfamily, contains mainly four isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43. In particular, CYP3A4, CYP3A5 and CYP3A7, highly expressed in liver, associate with the metabolism of many compounds. The expression levels of CYP3A isoforms are enhanced by treatment with various agents, such as rifampicin (RIF), phenobarbital, clotrimazole, and dexamethasone (DEX).^{1–3} We previously clarified that CYP3A4 and CYP3A7 mRNA expression levels were markedly up-regulated by DEX, but not by RIF, in human fetal liver (HFL) cells.⁴ These data suggested that the mechanisms of CYP3A induction in HFL differed from those in adult liver.

The glucocorticoid-induced CYP3A5 expression is mediated by glucocorticoid receptor (GR) signaling.^{5,6} As previously described, transcriptional activation of the *CYP3A4* gene by glucocorticoids is also known to occur through two distinct mechanisms involving GR: first by controlling the expression of pregnane X receptor (PXR) under physiological conditions through the classical GR pathway, and second by activating PXR under bolus or stress conditions.^{7,8} The role of GR in CYP3A4 regulation is, however, unclear, and an accurate assessment of whether GR plays a direct and/or indirect role remains obscure.⁹ We have reported that concomitant treatment with RU486, a GR antagonist, suppressed DEX-mediated induction of CYP3A4, CYP3A5, and CYP3A7 expression completely in HFL cells.⁴ These data suggested that GR was required in the CYP3A4 induction.

Introduction of specific small interfering RNA (siRNA) in cells has been shown to specifically knock down the target

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gene expression.¹⁰⁾ Recently, Matsubara *et al.*¹¹⁾ reported that adenovirus vector expressing human PXR-siRNA (AdhPXR-siRNA) was a potent tool to discern the role of PXR in the chemical-mediated activation of the *CYP3A4* gene. The system could be useful for assessment of a variety of nuclear receptor functions *in vivo* and *in vitro*.

In the present study, to further characterize whether GR is involved in DEX-mediated activation of *CYP3A4* and *CYP3A7* genes as well as the *CYP3A5* gene, we investigated the effect of glucocorticoids on the *CYP3A* expression and the role of GR in the induction of the *CYP3A* gene expression in HFL cells using the AdhGR-siRNA system.

Materials and Methods

Materials: SuperScript II first-strand synthesis system for reverse transcription-polymerase chain reaction (RT-PCR) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA); DEX, hydrocortisone, prednisolone and betamethasone were obtained from Wako Pure Chemicals (Osaka, Japan); fludrocortisone and methylprednisolone were obtained from MP Biomedicals (Costa Mesa, CA, USA); cortisone and Williams' medium E were obtained from Sigma Chemical Co. (St. Louis, MO, USA); *TaKaRa* EX Taq was obtained from Takara-Bio (Otsu, Japan); and PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). All other reagents used were of the highest quality available.

Human fetal liver cells: HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA, USA). The cell culture was initiated from a pool of six normal human liver tissues (average gestation 13 weeks) by elutriation following dispase digestion of tissue. The cell culture was cryopreserved at -150°C until use after proliferation.

Cell culture and drug treatment: HFL cells were incubated on dishes coated with type I collagen. Williams' medium E (Sigma Chemical Co.) containing 10% (v/v) fetal bovine serum, antibiotics (50 $\mu\text{g}/\text{mL}$ penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 100 $\mu\text{g}/\text{mL}$ neomycin), and 2 mM L-glutamine was used for culture of HFL cells under the condition of 5% CO_2 at 37°C . The medium was exchanged every 24 h.

Induction of *CYP3A* mRNA by representative glucocorticoids: HFL cells were cultured for 7 days after seeding of 5,000 cells/well onto 6-well culture plates, and then treated with 10 nM cortisone, hydrocortisone, prednisolone, methylprednisolone, fludrocortisone, betamethasone or DEX for 72 h. The compounds were dissolved in dimethyl sulfoxide (DMSO), which was added to the culture medium at a final concentration of 0.1%. The medium was replaced daily with fresh medium containing either test compounds dissolved in vehicle or vehicle alone. After treatment, total RNA was prepared from the cells.

Construction of siRNA-expressing adenovirus: Human H1 RNA gene promoter was used for the expression

of siRNA. AdhGR-siRNA was constructed with AdEasy™ System (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's protocol. The hGR-specific siRNA, designed by Takara-Bio, was amplified by PCR with primers 5'-CGCGTCGACATGACCCTACTGCAGTACTTTCAAGA-GAAG-3' and 5'-CGCAAGCTTAAAAAATGACCCTACTGCAGTACTTCTCTTGAAAG-3'. PCR product was digested with *Sal* I and *Hind* III, and ligated into the same restriction sites of pShuttle-H1. AdCont (AxCALacZ), which expresses β -galactosidase, was provided by Dr. Izumi Saito (Tokyo University). The titer of adenovirus, 50% titer culture infections dose (TCID_{50}), was determined in HEK293 cells. The value of TCID_{50} was reported to be almost equivalent to that of plaque-forming units.¹²⁾ Multiplicity of infection (MOI) was calculated by dividing TCID_{50} by the number of cells.

Effects of GR knockdown by adenoviral hGR-siRNA expression on *CYP3A* induction by DEX: HFL cells were cultured for 1 day after seeding 100,000 cells/well onto 6-well culture plates, and then were infected with AdhGR-siRNA (MOI of 10 or 50). The HFL cells cultured for 3 days after infection were incubated with Williams' medium E containing vehicle (0.1% DMSO) or 100 nM DEX for 2 days. We used AdCont as a control for RNA knockdown experiments. Thereafter, total RNA was prepared from HFL cells and *CYP3A* mRNA was analyzed by semiquantitative RT-PCR.

RNA extraction and semiquantitative RT-PCR analysis: Total RNA was extracted from the treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated from 2 μg total RNA. Reverse-transcription reaction was performed using a SuperScript II (Invitrogen) according to the manufacturer's instructions. With the cDNA obtained, PCR was carried out using a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) and PCR Express thermal cycler (Hyaid, Middlesex, UK). The primers and amplification conditions used are summarized in **Table 1**. The amplified products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The levels of these mRNAs were quantified from their band densities on the agarose gels using Printgraph AE-6914 and Scion Image Software (by Dr. W. Rasband, <http://www.scioncorp.com/>), and were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Results and Discussion

Effects of representative glucocorticoids on expression of *CYP3A* mRNA: Anti-inflammatory effects of glucocorticoids are caused by binding of GR homodimers to glucocorticoid response elements in the promoter region of steroid-sensitive genes, which may encode anti-inflammatory proteins.¹³⁾ If GR mediates the *CYP3A* induction, the glucocorticoid-mediated change in *CYP3A* expression could be highly correlated with the anti-inflam-

Table 1. PCR primers and conditions

Primer	Length/ annealing temperature	Sense primer 5'→3'	Antisense primer 5'→3'
CYP3A4	626 bp/60°C	CTGTGTGTTTCCAAGAGAAGTTAC	ACCTCATGCCAATGCAGTTT
CYP3A5	239 bp/62°C	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTGTC
CYP3A7	475 bp/54°C	CTATGATACTGTGCTACAGT	TCAGGCTCCACTTACGGTCT
GR	557 bp/54°C	ACACAGGCTTCAGGTATCTT	ACTGCTTCTGTTGCCAAG
PXR	442 bp/68°C	CAAGCGGAAGAAAAGTGAACG	CTGGTCTCGATGGCAAGTC
GAPDH	307 bp/54°C	CATCACCATCTCCAGGAGC	CATGAGTCCTCCACGATACC

Table 2. Relative anti-inflammatory potencies of representative glucocorticoids and their ability to induce CYP3As

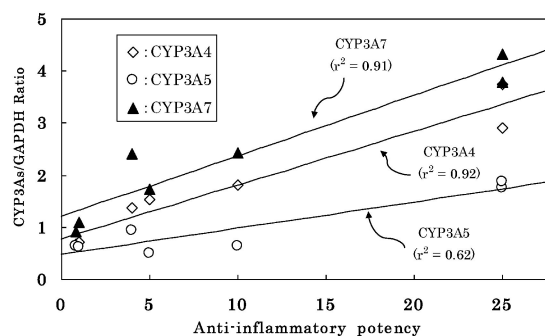
Compound	Anti-inflammatory potency ^a	Induction ability (CYP3As/GAPDH) ^b		
		CYP3A4	CYP3A5	CYP3A7
Control (DMSO)	—	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
Cortisone	0.8	0.67 ± 0.05	0.63 ± 0.05	0.91 ± 0.26
Hydrocortisone	1	0.71 ± 0.05	0.62 ± 0.02	1.10 ± 0.18
Prednisolone	4	1.38 ± 0.23	0.93 ± 0.03	2.41 ± 0.65
Methylprednisolone	5	1.53 ± 0.12	0.49 ± 0.05	1.73 ± 0.54
Fludrocortisone	10	1.82 ± 0.30	0.64 ± 0.08	2.43 ± 0.57
Betamethasone	25	2.90 ± 0.51	1.75 ± 0.16	4.33 ± 0.80
Dexamethasone	25	3.75 ± 1.27	1.87 ± 0.09	3.79 ± 1.60

^aRelative to hydrocortisone, which is assigned a value of 1.¹³⁾

^bHFL cells were cultured for 7 days after seeding 5,000 cells/well onto 6-well culture plates, and then treated with 10 nM glucocorticoids for 72 h. After treatment, total RNA was prepared from the cells. The mRNA levels were calculated using semi-quantitative RT-PCR analysis as described in Materials and Methods. The values are expressed as the mean ± standard deviation of three experiments.

matory effect of the glucocorticoids. Thus we investigated whether representative glucocorticoids used in clinical medicine induce CYP3A4, CYP3A5 and CYP3A7 expression in HFL cells (Table 2). The levels of CYP3A4 and CYP3A7 mRNAs were markedly enhanced by treatment with 10 nM of betamethasone or DEX, which are classified as the most potent glucocorticoids. The expression level of CYP3A5 mRNA was also enhanced, but the induction of CYP3A5 expression was slight, compared to that of the CYP3A4 and CYP3A7. Generally, a good correlation has been found between the affinity for the cytosolic GR and anti-inflammatory potencies of glucocorticoids.¹⁴⁾ Interestingly, a high correlation between the inducibility and the anti-inflammatory potencies of glucocorticoids¹⁵⁾ was observed, especially for CYP3A4 ($r^2 = 0.92$) and CYP3A7 ($r^2 = 0.91$) (Fig. 1). These results may indicate that GR mediates the induction of CYP3As in HFL cells. Glucocorticoids might induce the expression of CYP3As by complexing with GR, undergoing nuclear translocation, interacting with DNA *cis* sequences and modulating gene transcription.

Effects of adenoviral hGR-siRNA expression on CYP3A induction by DEX: Expression levels of CYP3A4, CYP3A5 and CYP3A7 mRNAs were increased 6.9-, 1.6- and 5.3-fold, respectively, of the control by treatment with 100 nM of DEX (Figs. 2A and 2C).

**Fig. 1. Correlation between the anti-inflammatory potencies of glucocorticoids and the CYP3A-induction ability**

The molecular mechanism of induction is best understood for CYP3A4. The first 1 kb of the 5'-flanking regions of CYP3A4 and CYP3A7 share 91% sequence similarity.¹⁶⁾ However, the 5'-flanking region of CYP3A5 (-1 to -1,434 bp) shares 60 and 59% sequence similarity to that of CYP3A4 and CYP3A7, respectively.¹⁷⁾ The low homology might be one of the factors of weak induction of CYP3A5.

To discern the involvement of GR in the induction process of CYP3As by DEX, AdhGR-siRNA was introduced to specifically knock down the target gene expression in the cells (Fig. 2). GR mRNA was detected in HFL cells and the expression was suppressed to less than 50% of that in the control by the expression of hGR-siRNA (Figs. 2A and 2B). Introduction of AdhGR-siRNA almost completely inhibited the DEX-mediated induction of CYP3A4 and CYP3A5 mRNAs (Fig. 2C). On the other hand, the induction of CYP3A7 mRNA was suppressed to about 60% of AdCont used as a control for RNA knockdown experiments. We do not know the reason why the induction of CYP3A7 mRNA was not completely suppressed by hGR-siRNA (Fig. 2C). This phenomenon might be caused by the different structure of the 5'-flanking regions of CYP3A4 and CYP3A7, including GR responsive elements.

Chemical-induced expression of the CYP3A4 gene can be mediated by PXR heterodimerized with retinoid X receptor through binding to the CYP3A4 5'-flanking region.¹⁸⁻²⁰⁾ In the present study, both 10 and 100 nM of DEX showed clear induction of CYP3A4 and CYP3A7 expression in HFL cells (Table 2 and Fig. 2C), concentrations sufficient to activate GR but not PXR.⁷⁾ We have reported that PXR mRNA is

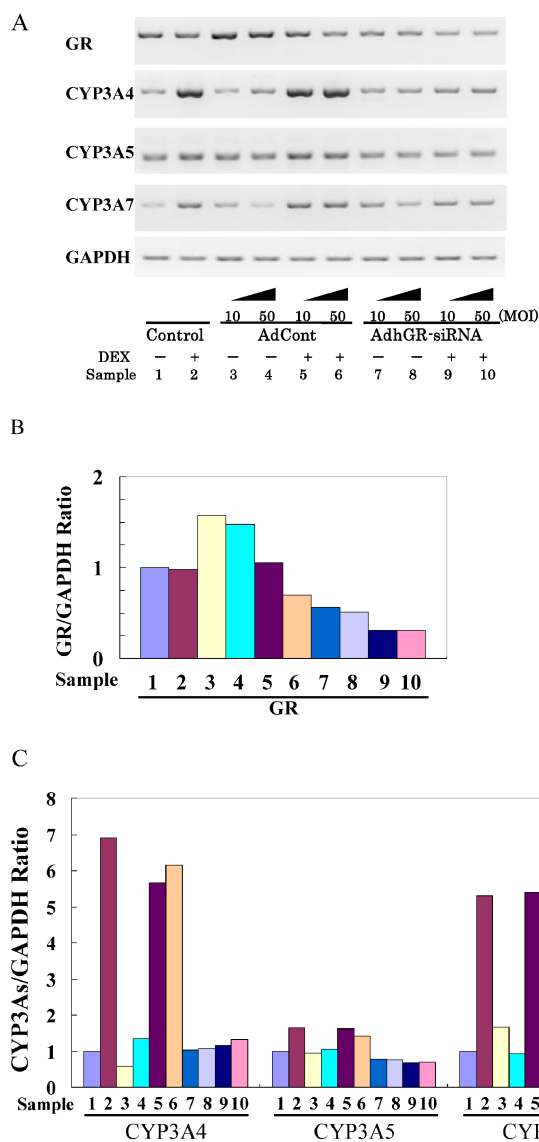


Fig. 2. (Color online) Effects of adenoviral GR-siRNA expression on GR, CYP3A4, CYP3A5 and CYP3A7 mRNA levels

HFL cells were cultured for 1 day after seeding 100,000 cells/well onto 6-well culture plates, and then were infected with AdhGR-siRNA (MOI of 10 or 50). We used AdCont as a control for RNA knockdown experiments. The HFL cells cultured for 3 days after infection were incubated with Williams' medium E containing vehicle (0.1% DMSO) or 100 nM DEX for 2 days. Then, total RNA was prepared from HFL cells and GR, CYP3A4, CYP3A5 and CYP3A7 mRNAs were analyzed by semiquantitative RT-PCR as described in Materials and Methods. A: An image of ethidium bromide-stained agarose gel is shown. Data presented are the ratio of B: GR, and C: CYP3A4, CYP3A5, and CYP3A7 mRNA to GAPDH normalized at 1.0 for DMSO treatment alone in the absence of adenovirus (Sample 1). The columns present the mean of two individual experiments.

not detected by RT-PCR in HFL cells.²¹⁾ Furthermore, insufficient RIF-mediated CYP3A4 induction was observed in HFL cells with only PXR overexpression, maybe because of lower expression of some crucial transcription factors

such as hepatocyte nuclear factor 4 α (HNF4 α) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1 α) in the HFL cells.²²⁾ More recently, Pang *et al.* reported that glucocorticoids, including dexamethasone, cortisol, corticosterone, and cortisone, all induced the expression of CYP3A7 mRNA, whereas RIF had no effect on CYP3A7 expression in double-transgenic mice expressing human PXR and CYP3A4/7. They suggested that CYP3A7 is developmentally regulated in mouse liver primarily by glucocorticoids through the GR.²³⁾ These results strongly support a view that DEX induces CYP3A expression through GR, but not PXR, in human fetal liver.

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