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Regular Article

Effects of Hypoxia-inducible Factor-1α Chemical Stabilizer, $CoCl_2$ and Hypoxia on Gene Expression of CYP3As in Human Fetal Liver Cells

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Summary: Distinctive response patterns of CYP3A4 and CYP3A7 to cobalt chloride ($CoCl_2$) in human fetal liver (HFL) cells were observed and compared with those under hypoxic conditions. The expression levels of CYP3A4 and CYP3A7 mRNAs were decreased by $CoCl_2$ and hypoxia, although significance could not be determined in HFL cells cultured under 3% O_2. The hypoxia-inducible factor-1α (HIF-1α) protein content in HFL cells was significantly increased by $CoCl_2$ and 3% O_2. Transcriptional activities of CYP3A4 and CYP3A7 were not altered by 3% O_2 when reporter plasmids containing the promoter region ranging up to about 10 kb and 12 kb upstream, respectively, were transfected into HFL cells, although the activity was significantly suppressed by $CoCl_2$. These results suggested that the mechanisms controlling CYP3A gene expression of HIF-1α chemical stabilizer in fetal hepatocytes might be different from those in adult hepatocytes, and that HIF-1α is not directly involved in regulation of CYP3A4 or CYP3A7 expression.

Keywords: CYP3A4; CYP3A7; cobalt chloride; human fetal liver cell; hypoxia; HIF-1α

Introduction

Cytochrome P450 (CYP) is a superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products.[1)] Among them, the human CYP3A subfamily includes four isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43.[2)] The expression patterns of members of the CYP3A subfamily, especially CYP3A4 and CYP3A7, are characterized by marked interindividual variability and distinct developmental profiles. CYP3A4 is a major isoform that accounts for about 30% of the total amount of CYP in the adult human liver and is responsible for approximately 50% of the metabolism of known drugs by CYPs in human.[3,4)] CYP3A7 is predominantly expressed in prenatal tissues, accounting for 50% of the total CYP content in the fetal liver.[5)] CYP3A7 expression is detected as early as 50–60 days of gestation and persists until after birth, when it decreases over the first few months of life.[5,6)]

Hypoxia activates transcription factors, which regulate the adaptive response of the cells to hypoxia.[7,8)] Cells adapt to the low oxygen partial pressure by upregulating the transcription of multiple genes, such as vascular endothelial growth factor (VEGF), erythropoietin, and several glycolytic enzymes. Many molecular and cellular responses to hypoxia are mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer consisting of a constitutively expressed subunit (HIF-1β) and an oxygen-regulated subunit (HIF-1α).[9)] In the presence of oxygen and iron, HIF-1α is rapidly degraded *via* the prolyl-hydroxylase/von Hippel–Lindau pathway.[10)] Hypoxia stabilizes HIF-1α, allowing formation of the heterodimer HIF-1α/β, which binds to hypoxia responsive elements (HRE) within the promoters of several genes involved in cell proliferation, differentiation, survival, cell migration, angiogenesis, and energy metabolism.[7,11)] The hypoxic state can be mimicked

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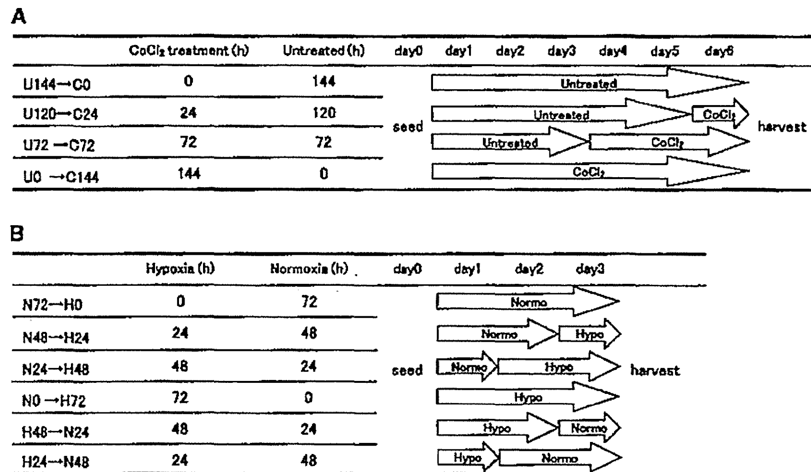

Fig. 1. Experimental protocols for (A) $CoCl_2$ treatment group and (B) hypoxia group

by chemical stabilizers of HIF-1α, such as cobalt chloride ($CoCl_2$) and the iron chelator deferoxamine.[12–16]

Early *in vivo* and *in vitro* studies suggested that acute hypoxia reduces the activity of drug metabolism.[17] Legendre *et al.* reported that drug-metabolizing enzymes, including CYP3A4, were downregulated by hypoxia and treatment with $CoCl_2$ in highly differentiated human hepatoma HepaRG cells.[18] They suggested that HIF-1α plays a central role in the repression of CYP3A4 in cells treated with $CoCl_2$.[18] We have reported previously that the expression mechanisms of CYP3A in human fetal liver (HFL) differ from those in the adult liver.[19] To our knowledge, the effects of HIF-1α chemical stabilizers and hypoxia on the expression of CYP3A enzymes in HFL have not been investigated, even at the mRNA level. The present study was performed to clarify the effects of HIF-1α stabilizer, $CoCl_2$ and hypoxia, and the involvement of HIF-1α on CYP3A4 and CYP3A7 expression in HFL cells.

Materials and Methods

Materials: Dulbecco's phosphate-buffered saline (PBS) was purchased from DS Pharma Biomedical Co. (Osaka, Japan). Penicillin-streptomycin-neomycin antibiotic mixture, SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR), and Lipofectamine 2000 were from Invitrogen Corp. (Carlsbad, CA). Williams' medium E, $CoCl_2$, and protease inhibitor cocktail were from Sigma-Aldrich Co. (St. Louis, MO). Cell Banker was from Wako Pure Chemicals (Osaka, Japan). Collagen (type I) was from Koken (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). SYBR Premix Ex Taq and LA PCR *in vitro* Cloning kit were obtained from TaKaRa Bio (Shiga, Japan). The Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI). The Great EscAPe™ SEAP Chemiluminescence Kit 2.0 was from Clontech (Palo Alto, CA). The BCA Protein Assay Kit was from Thermo Fisher Scientific (Waltham, MA). All other reagents used were of the highest quality available.

Cells: HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA). Cell culture was initiated from a pool of six normal human liver tissues (average 13 weeks of gestation) by elutriation following dispase digestion of tissue. The cells were subcultured when the culture reached 90–100% confluence, and suspended in Cell Banker after proliferation. Aliquots of the suspensions were cryopreserved at −150°C until use.

Cell culture and $CoCl_2$ treatment: HFL cells were seeded onto dishes coated with type I collagen and cultured in Williams' medium E supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, and antibiotics (50 µg/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL neomycin) under an atmosphere of 5% CO_2 in air at 37°C. The medium was replaced daily with fresh medium. In the $CoCl_2$ treatment group, HFL cells were cultured for 144 h after seeding of 1×10^5 cells onto 6-well tissue culture plates (BD Biosciences, Heidelberg, Germany), and then treated with 200 µM $CoCl_2$ for 24 h, 72 h, and 144 h after cultivation for 120 h, 72 h, and 0 h (**Fig. 1A**). $CoCl_2$ was dissolved in sterile water and sterilized by membrane filtration. The $CoCl_2$ solution was added to culture medium at a ratio of one-thousandth to the medium. In the hypoxia groups, HFL cells were cultured for 72 h after seeding of 1×10^5 cells onto 6-well tissue culture plates, and then cultured after 72 h of normoxia (N) or normoxia followed by different periods (h) of hypoxygenation (N → H), at 72 h of hypoxia (H) or hypoxia followed by different periods (h) of reoxygenation (H → N) (**Fig. 1B**). The control group was cultured for 72 h under normoxic conditions. For hypoxia, HFL cells were incubated under atmospheric conditions of 3% O_2/5% CO_2/92% N_2 at 37°C.

RNA extraction and reverse transcription reaction: Total RNA was extracted from the treated cells using

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Table 1. Sequences of primers and real-time PCR conditions

Primer name	Forward primer sequence (5′ to 3′)	Revers primer sequence (5′ to 3′)	Product size (bp)	Annealing temp.	Number of cycles	GenBank accession number
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG	298	60	40	AF182273
CYP3A7	AGATTTAATCCATTAGATCCATTCG	AGGCGACCTTCTTTTATCTG	148	60	40	AF315325
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185	60	40	BC013310
VEGFA	TGCTTCTGAGTTGCCCAGGA	TGGTTTCAATGGTGTGAGGACATAG	176	60	40	NM001025366.1

an illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK). First-strand cDNA was generated from 4 µg of total RNA. The reverse transcription reaction was performed using the SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions (Invitrogen Corp.).

Real-time RT-PCR analysis: For detection of expression levels, CYP and VEGF mRNAs were analyzed by SYBR Green real-time quantitative RT-PCR. The levels of these mRNAs were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. All PCR procedures were performed using the 7300 Fast Real-time PCR System (Applied Biosystems) according to each manufacturer's instructions. PCR was performed using diluted cDNA template in 12.5-µL reaction mixtures containing 0.20 µM of each primer and 6.25 µL of SYBR Premix Ex Taq. The primers used are summarized in **Table 1**.

Western blotting analysis: HFL cells were lysed in lysate solution (20 mM Tris-HCl, pH 6.8, 50 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail after rinsing in Dulbecco's PBS. Western blotting analysis was carried out according to the method reported previously.[20] The proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto Immun-Blot PVDF Membranes (Bio-Rad, Hercules, CA). The blotted membrane was probed with anti-HIF-1α mouse monoclonal antibody (BD Transduction Laboratories, San Jose, CA). The immunoreactive proteins were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare) and light emission was quantified with an Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Luciferase and secreted alkaline phosphatase assays: The respective promoter fragments were linked to the firefly luciferase reporter gene in the pGL3-basic vector (Promega) or the secreted alkaline phosphatase (SEAP) reporter gene in the pSEAP2-basic vector (Clontech, Palo Alto) to produce reporter constructs. The *Renilla* luciferase gene (Promega) under the control of the thymidine kinase promoter (phRL-TK) was used as an internal control for firefly luciferase reporter gene expression. The 5′ untranslated region of the *CYP3A4* gene was isolated by PCR from a human genomic DNA library. The promoter region of the *CYP3A4* gene was prepared using 5′-ACGCGTGATGAACACATGCTACAGAATGGATAGC-3′ and 5′-CTCGAGTGCACAGCAGTGATTCAGTGAGG-3′ with *Mlu*I and *Xho*I restriction sites at the 3′-ends of the primers, respectively. The PCR product was digested with *Mlu*I and *Xho*I, and inserted into the *Mlu*I and *Xho*I sites of the pSEAP2-basic vector. The final product was named p3A4-10k (including from −9,532 to +12 bp). The 5′ untranslated region of the *CYP3A7* gene was isolated by PCR from a human genomic DNA library. The promoter region of the *CYP3A7* gene was prepared using 5′-ACGCGTGCTTGTTATCTGTGTACATGAGAGTC-3′ and 5′-GTCGACTGCACAGCAGTGATTCAGTGAGG-3′ with *Mlu*I and *Sal*I restriction sites at the 3′-ends of the primers, respectively. The PCR product was digested with *Mlu*I and *Sal*I and inserted into the *Mlu*I and *Sal*I sites of the pGL3-basic vector. The final product was named p3A7-12k (including from −12,688 to +13 bp). PCR was performed using an LA PCR *in vitro* Cloning kit according to the manufacturer's instructions (TaKaRa Bio).

HFL cells were transfected with pSEAP2-basic vector or the reporter plasmid p3A4-10k. In evaluation of CYP3A7 promoter activity, HFL cells were cotransfected with pGL3-basic vector and control vector phRL-TK or the reporter plasmid p3A7-12k using Lipofectamine 2000 in complete medium. After 4–6 h, medium was replaced with fresh medium, and the cells were exposed to $CoCl_2$ (200 µM) or hypoxia (3% O_2) for 48 h. After the specified exposure times, the cells transfected with pGL3-basic or p3A7-12k vector plasmids were harvested in 10 mM PBS (pH 7.5) containing 0.5 mM EDTA and frozen for subsequent assays. The reporter assay was performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). After assay, the ratio of firefly luciferase activity to *Renilla* luciferase activity (relative luciferase activity) was used as the normalized luciferase activity. SEAP activity in culture medium was measured using a Great EscAPe™ SEAP Chemiluminescence Kit 2.0. The luminescence obtained from reactions of the luciferase and SEAP assays was then monitored using a Sirius Luminometer (Berthold, Pforzheim, Germany) and Glomax™ 96 Microplate Luminometer (Promega), respectively. SEAP activity was normalized relative to protein concentration.

Protein concentration: Protein concentration was determined using a BCA protein Assay Kit in accordance with the manufacturer's instructions, using bovine serum albumin as a standard.

Statistical analysis: All values are expressed as means ± standard deviation (SD). All data were analyzed by the unpaired Student's t test or Dunnett's multiple compari-

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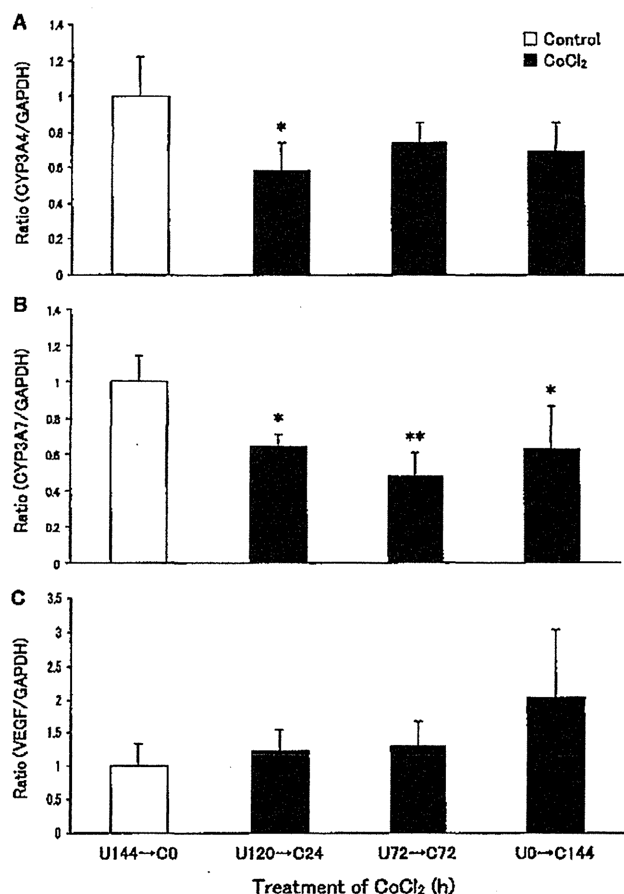

Fig. 2. Effects of $CoCl_2$ on expression of CYP3As and VEGF mRNA in HFL cells

(A) CYP3A4 mRNA level, (B) CYP3A7 mRNA level, (C) VEGF mRNA level. HFL cells were treated with $CoCl_2$ (200 μM) for 24 h (U120 → D24), 72 h (U72 → D72), and 144 h (U0 → D144), after cultivation for 120 h, 72 h, and 0 h, respectively. Total RNA was extracted from HFL cells cultured for 144 h. The levels of expression of CYP3As and VEGF mRNAs were analyzed by SYBR Green real-time RT-PCR as described in the Materials and Methods. Data are presented as the ratios of CYP3As and VEGF to GAPDH and normalized relative to 1.0 for the untreated group. Values are expressed as the means ± SD ($n = 3$). Significantly different from control (* $p < 0.05$, ** $p < 0.01$).

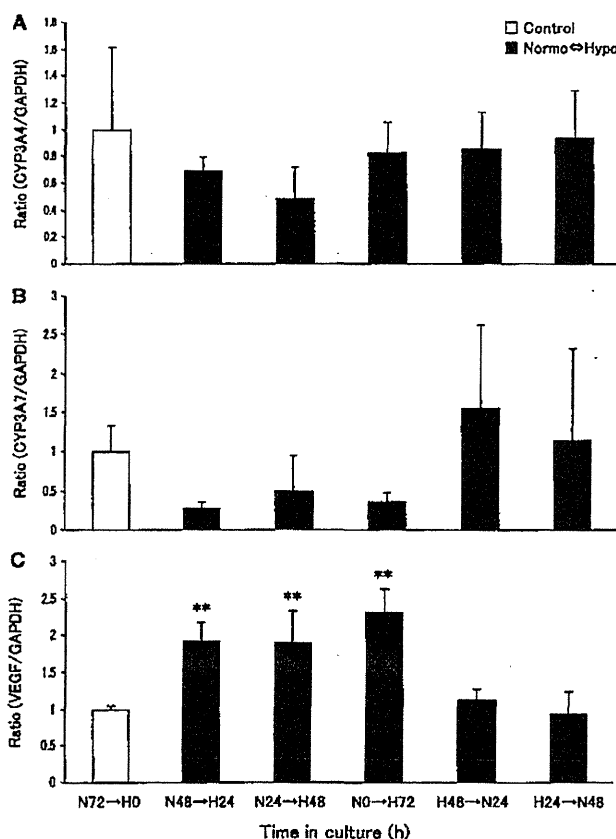

Fig. 3. Effects of hypoxia on expression of CYP3As and VEGF mRNA in HFL cells

(A) CYP3A4 mRNA level, (B) CYP3A7 mRNA level, (C) VEGF mRNA level. HFL cells cultured for 72 h of normoxia (N) or normoxia followed by different periods (h) of hypoxygenation (N → H), at 72 h of hypoxia (H) or hypoxia followed by different periods (h) of reoxygenation (H → N). Total RNA was extracted from HFL cells cultured for 72 h. CYP3As and VEGF mRNA expression were analyzed by SYBR Green real-time RT-PCR as described in the Materials and Methods. Data are presented as the ratios of CYP3As and VEGF to GAPDH and normalized relative to 1.0 for normoxia. Values are expressed as the means ± SD ($n = 3$–5). Significantly different from control (** $p < 0.01$).

son test for significance of differences between the mean values for each group. In all analyses, $p < 0.05$ was taken to indicate statistical significance. All experiments were repeated more than three times to confirm reproducibility.

Results

Effects of $CoCl_2$ on expression of CYP3As and VEGF mRNAs: To evaluate the effects of HIF-1α chemical stabilizer on the expression of CYP3A4 and CYP3A7 mRNAs in HFL cells, the cells were treated with $CoCl_2$ (200 μM) for 24, 72, and 144 h. VEGF mRNA expression was monitored as a marker of HIF-1α accumulation in cells, because VEGF gene expression was induced by an increase in HIF-1α level. As shown in **Figure 2**, the levels of CYP3A4 and CYP3A7 mRNA expression in HFL cells were decreased by 59% and 48%, respectively, compared to the control by treatment with $CoCl_2$ for 24 h and 72 h, although the level of VEGF mRNA expression was not significantly altered by this compound.

Effects of hypoxia on expression of CYP3As and VEGF mRNAs: To evaluate the effects of hypoxia on the expression of CYP3A4, CYP3A7, and VEGF mRNAs in HFL cells, the cells were cultured under 3% O_2 for 72 h. As shown in **Figure 3**, the expression levels of CYP3A4 and CYP3A7 mRNAs tended to be reduced by hypoxia. In contrast, the level of VEGF mRNA expression was increased

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by about 2-fold in HFL cells cultured under normoxic conditions for 48 h followed by hypoxia for 24 h, normoxia for 24 h followed by hypoxia for 48 h and hypoxia for 72 h, although the level remained unchanged in HFL cells cultured under hypoxia for 48 h followed by normoxia for 24 h or hypoxia for 24 h followed by normoxia for 48 h (**Fig. 3C**).

Accumulation of HIF-1α by $CoCl_2$ treatment or hypoxia: To confirm whether HIF-1α protein was accumulated by $CoCl_2$ or hypoxia, we harvested the lysates of HFL cells cultured in the presence of 200 μM $CoCl_2$ or under 3% O_2 for 24 and 72 h, and subjected them to Western blotting analyses (**Fig. 4**). A protein immunologically related to HIF-1α was detected. When HFL cells were cultured in medium containing $CoCl_2$ for 72 h, the contents of HIF-1α protein were significantly increased by 3.3-fold compared to controls, although the protein level was not changed by $CoCl_2$ treatment for 24 h. The contents of HIF-1α protein in HFL cells were significantly increased by 2.7-fold and 2.1-fold, respectively, compared to control by hypoxia for 24 h and 72 h.

Transcriptional activities of *CYP3A4* and *CYP3A7* genes: The SEAP reporter plasmid containing the CYP3A4 promoter up to about −10 kb (p3A4-10k) and the luciferase reporter plasmid containing the CYP3A7 promoter up to about −12 kb (p3A7-12k) were transfected into HFL cells treated with 200 μM $CoCl_2$ and cultivated under 3% O_2 (**Fig. 5**). There were no significant changes in either reporter activity with 3% O_2. On the other hand, $CoCl_2$ significantly suppressed the reporter activities of both p3A4-10k and p3A7-12k.

Discussion

Legendre *et al.* reported that $CoCl_2$, hypoxia, or HIF-1α overexpression led to CYP3A4 downregulation in HepaRG cells.[18] They suggested that HIF-1α was one of the most important factors in CYP3A4 repression and might regulate CYP3A4 expression by an indirect mechanism or by interacting with a DNA regulatory sequence localized somewhere else in the CYP3A4 gene.[18] In the present study, the effects of $CoCl_2$ on CYP3A4 and CYP3A7 mRNAs in HFL cells were observed and compared to those seen under hypoxic conditions. The expression levels of CYP3A4 and CYP3A7 mRNAs were significantly decreased by $CoCl_2$, although hypoxia tended to decrease these expression levels.

VEGF induction has been demonstrated in endothelial cells exposed to cobalt and hypoxia.[21–24] It has been reported that the upregulation of VEGF expression is mediated through an increase in HIF-1α level.[24] Immuno-

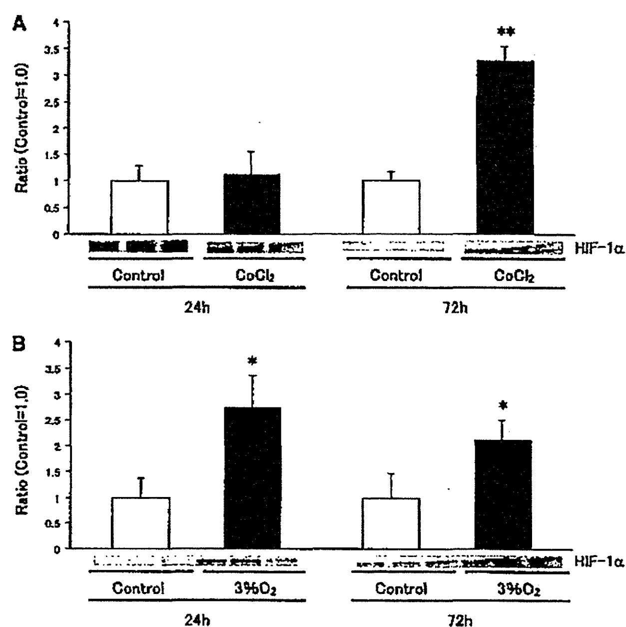

Fig. 4. Western blotting analysis of HIF-1α in HFL cells treated with $CoCl_2$ and hypoxia
HFL cells treated with $CoCl_2$ (A) and cultured in 3% O_2 (B) for 24 h and 72 h after seeding were subjected to SDS-PAGE and electrophoretically transferred onto membranes. The proteins were reacted with anti-HIF-1α mouse monoclonal antibody. Each lane contained 45 μg of protein. Values are expressed as the means ± SD ($n = 3$). Significantly different from control (*p < 0.05, **p < 0.01).

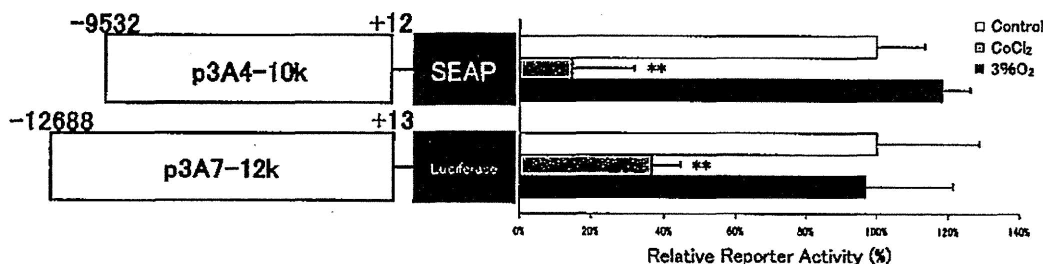

Fig. 5. Transcriptional activities of CYP3A4 and CYP3A7 in HFL cells
HFL cells were transfected with pSEAP2-basic vector or the reporter plasmid p3A4-10k. In the evaluation of CYP3A7 promoter activity, HFL cells were cotransfected with pGL3-basic vector and control vector phRL-TK or the reporter plasmid p3A7-12k. After 4–6 h, medium was replaced with fresh medium, and the cells were exposed to $CoCl_2$ (200 μM) or 3% O_2 for 48 h. The ratio of firefly luciferase activity to *Renilla* luciferase activity was used as the normalized luciferase activity. SEAP activity was normalized relative to protein concentration. Values are expressed as the means ± SD ($n = 8–12$). Significantly different from control (**p < 0.01).

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logically related proteins detected by the antibody raised against human HIF-1α were detected in HFL cells on Western blotting analysis (**Fig. 4**). We have confirmed that the effects of treatment with 200 μM $CoCl_2$ for 24 h and 72 h on CYP3A4 and CYP3A7 were almost the same as those of 200 μM $CoCl_2$ for 24 h and 72 h, respectively, after cultivation for 120 h and 72 h (data not shown). The content of HIF-1α was increased by 2–3-fold compared to controls by $CoCl_2$ and hypoxia (**Fig. 4**). The expression of VEGF mRNA was induced by $CoCl_2$ and hypoxia in HFL cells, although the change in the cells treated with $CoCl_2$ was not significant (**Figs. 2C** and **3C**). VEGF mRNA levels may not be any more susceptible to HIF-1α chemical stabilizers than HIF-1α protein levels. These results of $CoCl_2$ and hypoxia on HIF-1α protein were consistent with the expression patterns of VEGF mRNA in HFL cells. HIF-1α is rapidly degraded under normoxia, as oxygen-dependent HIF-1α stabilization requires an oxygen tension of <2–3% O_2.[25,26] The switch back to normoxia from hypoxia caused rapid degradation of HIF-1α, even if it had been accumulated in HFL cells by cultivation under hypoxia. The switch back to normoxia from hypoxia caused no obvious changes in VEGF mRNA level compared with controls incubated for 72 h under normoxic conditions.

HIF-1 DNA binding activity is induced when mammalian cells are subjected to hypoxia.[27,28] We searched for the consensus sequence of HRE in the promoter regions of CYP3A4 and CYP3A7 up to 10 kb and 12 kb upstream of the respective transcriptional start codons and identified 6 and 7 canonical mammalian HREs, respectively, in the promoter regions of CYP3A4 and CYP3A7. However, the transcriptional activities of CYP3A4 and CYP3A7 were not decreased by hypoxia when the reporter plasmids containing the promoter region up to about 10 kb and 12 kb upstream, respectively, were transfected into HFL cells (**Fig. 5**). These results indicated that HIF-1α is not directly involved in the hypoxia-induced downregulation of CYP3A7. On the other hand, the transcriptional activities of *CYP3A4* and *CYP3A7* genes were significantly suppressed by $CoCl_2$. These results were consistent with the effects of $CoCl_2$ on the expression of CYP3A4 and CYP3A7 mRNAs. However, we could not clarify the mechanisms underlying the suppressive effects of $CoCl_2$ on the expression of CYP3A4 and CYP3A7 in HFL cells.

The results of the present study indicated that the expression levels of CYP3A4 and CYP3A7 mRNAs were significantly decreased by $CoCl_2$ in HFL cells. The content of HIF-1α was increased by $CoCl_2$ and hypoxia. In conclusion, these results suggest that the mechanisms by which the HIF-1α chemical stabilizer controls CYP3A gene expression in fetal hepatocytes may be different from those in adult hepatocytes, and that HIF-1α is not directly involved in regulation of CYP3A4 and CYP3A7 expression.

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Regular Article

Lower Expression of HNF4α and PGC1α Might Impair Rifampicin-mediated CYP3A4 Induction under Conditions Where PXR Is Overexpressed in Human Fetal Liver Cells

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Summary: Pregnane X receptor (PXR) mRNA was detected in HepG2 cells by RT-PCR, but not in human fetal liver (HFL) cells. CYP3A4 was induced by rifampicin (RIF), mifepristone (RU486), clotrimazole (CTZ), and dexamethasone (DEX) in HepG2 cells, while these PXR ligands with the exception of DEX did not induce CYP3A4 mRNA expression in HFL cells. Ad-PXR infection increased mRNA levels of PXR and CYP3A4 in both cells despite the absence of PXR ligands. Similar results were observed in reporter gene assays. However, in HFL cells, RIF-mediated CYP3A4 induction was insufficient compared with HepG2 cells, despite PXR overexpression. The expression levels of five coactivators (HNF4α, PGC1α, SRC1, CBP, and P300) related to CYP3A4 expression in HepG2, HFL cells, and human adult liver were analyzed by RT-PCR. Expression levels of HNF4α and PGC1α in HFL cells were downregulated to 20% of those in the human adult liver. On the other hand, the expression level of HNF4α in HepG2 cells was higher than that in HFL cells, although PGC1α expression level was almost the same as that in HFL cells. HNF4α mRNA expression level in HepG2 cells was 57% of that in human adult liver, and the level in HFL cells was 30% of that in HepG2 cells. These results suggested that lower expression of HNF4α and PGC1α may impair RIF-mediated CYP3A4 induction under conditions of PXR overexpression in HFL cells.

Keywords: PXR; drug-mediated induction; CYP3As; fetal liver; HNF4α; PGC1α

Introduction

Cytochrome P450 (CYP) is a superfamily of heme proteins that play a critical role in the oxidative metabolism of endogenous and xenobiotic substances.[1] The human CYP3A subfamily includes four functional members, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, which are expressed at different levels in the liver, kidney, and gastrointestinal tract.[2,3] CYP3A4 is the predominant isoform expressed in the adult human liver and was reported to be responsible for the metabolism of more than 60% of therapeutic drugs.[4,5] In contrast, CYP3A7 is the major CYP isoform in the human fetal liver.[6]

CYP expression is modulated by a large variety of endogenous and exogenous substances, including drugs, and induction of CYP expression has been analyzed extensively in experimental animals. Toxicological and pharmacological studies designed to evaluate the safety and efficacy of candidate drugs and chemicals in the human fetus are largely dependent on extrapolation from animal models. However, it is difficult to estimate alterations of drug metabolism by inducers of drug-metabolizing enzymes in the human fetus due to the problems of differences among species.[7,8] Species differences in the inducibility of drug-metabolizing enzymes result in important differences in the metabolism of drugs and potential carcinogens. Therefore, primary cultures of hepatocytes and human hepatoma HepG2 cells have been widely used to study drug metabolism.[7,9] On the other hand, there have been few reports regarding the expression and induction of drug-metabolizing enzymes in human fetal hepatocytes in culture.

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Previous studies of the induction of CYP3As by rifampicin (RIF) and glucocorticoids have elucidated a mechanism of regulation in which orphan nuclear receptors, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) and steroid receptors, such as the glucocorticoid receptor (GR), play central roles in regulation of gene expression.[10–15] Upon ligand binding, PXR forms a heterodimer with retinoid X receptor α (RXRα) and transactivates ER-6 (everted repeat separated by six nucleotides) and DR-3 (direct repeat separated by three nucleotides) elements upstream of CYP3A4.[16–20] We reported previously that CYP3A4 and CY3A7 mRNA expression levels were markedly upregulated by dexamethasone (DEX), but not by RIF, in human fetal liver (HFL) cells. We considered that CYP3A4 and CYP3A7 mRNA expression were not induced by RIF because PXR expression level was very low or absent in HFL cells.[11,21]

Recently, some transcription factors (coactivators and corepressors) that mediate CYP3As expression have been reported. Hepatocyte nuclear factor 4α (HNF4α) is one of the most important liver-enriched transcription factors for hepatocyte differentiation.[22] HNF4α plays critical roles not only in specification of the hepatic phenotype during liver development but also in the transcriptional regulation of genes involved in glucose, cholesterol, fatty acid, and xenobiotic metabolism and in the synthesis of blood coagulation factors.[23,24] Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) is a key metabolic regulator and was originally identified as a peroxisome proliferator-activated receptor γ interacting coactivator in brown adipose tissue.[25] Recent studies showed that PGC1α is a versatile coactivator for numerous nuclear receptors and has been implicated in diverse biological activities, including lipid and carbohydrate metabolism.[26,27] PGC1α has been shown to increase the HNF4α-mediated transactivation of CYP7A1, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase genes.[27–29] PXR promotes CYP3A4 gene transcription by interacting with HNF4α, steroid receptor coactivator 1 (SRC1), and PGC1α. Moreover, RIF strongly stimulates PXR and HNF4α interaction and CYP3A4 promoter activity, which are further enhanced by PGC1α and SRC1 but inhibited by the small heterodimer partner (SHP).[13,30]

In the present study, we investigated the effects of PXR overexpression on drug-mediated induction of CYP3As mRNA expression in HFL cells and the expression levels of other transcription factors that were reported to be related to CYP3As expression.

Materials and Methods

Materials: Dulbecco's modified Eagle's medium and Williams' medium E were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin–streptomycin–neomycin antibiotic mixture and SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) were from Invitrogen Corp. (Carlsbad, CA). Cellbanker, clotrimazole (CTZ), DEX, dimethyl sulfoxide (DMSO), mifepristone (RU486), RIF, and human normal adult liver total RNA (male, 64 years old) were from Wako Pure Chemicals (Osaka, Japan). An illustra RNAspin Mini RNA Isolation Kit was obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, UK). The Luciferase Assay System was from Promega Co. (Madison, WI). ExTaq and PrimeSTAR HS DNA Polymerases were from Takara Bio Inc. (Otsu, Japan). Collagen (type I) was from Koken (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Ishikari, Japan). All other reagents used were of the highest quality available.

Cells: HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA). 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 cells were subcultured when the culture reached 90–100% confluence, and suspended in Cellbanker after proliferation. Aliquots of the suspensions were cryopreserved at −150°C until use. HepG2 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University, Japan.

Cell culture and adenovirus infection: HepG2 and HFL cells were seeded onto dishes coated with collagen (type I) and cultured in Dulbecco's modified Eagle's medium and Williams' medium E, respectively, supplemented with 10% FBS, 2 mM glutamine, and antibiotics (50 µg/ml penicillin, 50 µg/ml streptomycin, and 100 µg/ml neomycin) under an atmosphere of 5% CO_2 in air at 37°C. The medium was replaced every 24 h. The recombinant adenoviruses, Ad-*lacZ*, Ad-PXR, and Ad-CYP3A4-362-7.7k-Luc, were provided by Yamazoe and Nagata. HepG2 and HFL cells were subcultured for 3 or 7 days after seeding of 4×10^4 cells onto 6- or 24-well culture plates. When subculture was performed, the cells were infected with Ad-*lacZ* (50 MOI), Ad-PXR (50 MOI), and Ad-CYP3A4-362-7.7k-Luc (25 MOI) solution for 1 h. In the drug treatment groups, HepG2 and HFL cells were treated with DEX (100 µM), RIF (40 µM), RU486 (10 µM), and CTZ (10 µM) for 48 h. The above compounds were dissolved in DMSO (vehicle), which was added to the culture medium at a final concentration of 0.1%. The medium was replaced daily with fresh medium containing either the test compounds dissolved in vehicle or vehicle alone. After treatment with the test compounds for 48 h, the cells were harvested using the cell lysis solution for RT-PCR and reporter gene assays.

RNA extraction and reverse transcription reaction: Total RNA was extracted using an illustra RNAspin Mini RNA Isolation Kit according to the manufacturer's instructions. First-strand cDNA was generated from 1–2 µg of total RNA. Reverse transcription reaction was performed using a SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions.

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Table 1. Sequences and semiquantitative RT-PCR conditions

Primer name	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Product size (bp)	Annealing temp.	Number of cycles	GenBank accession number
PXR	CAAGCGGAAGAAAAGTGAACG	CTGGTCCTCGATGGGCAAGTC	442	68	32	NM_022002
RXRα	CAAACAGCTTTTCACCCTG	AGTAGGCCTCCAAGGAC	392	56	32	NM_002957
HNF4α	GCCCTACCTCAAAGCCATCAT	GACCCTCCCAGCAGCATCTC	273	60	35	NM_000457
PGC1α	TCAGTCCTCACTGGTGGACA	CTGCTTCGTCGTCAAAAACAGC	352	64	33	NM_013261
SRC1	ATGGTGAGCAGAGGCATGACA	AAACGGTGATGCTCATGTTG	349	64	33	Linja et al.[54]
P300	CCTGAGTAGGGGCAACAAGA	GTGTCTCCACATGGTGCTTG	353	64	32	Linja et al.[54]
CBP	GATCGCCACGTCCCTTAGTA	CCCCAAGTGTCCCTGATCTA	202	64	36	U47741
SMRT	AAGTCCATCCTCACGTCCAC	TGAAGCACACTGGGTCTCTG	203	60	34	U37146
SHP	GCTGTCTGGAGTCCTTCTGG	GAAAGAAGAGGTCCCCAAGCAG	261	60	34	AB058644
NcoR1	CAAGTTTCCTCGCAGACTCC	CTCCTCTCTGGGGATTTTCC	221	64	34	NM_006311
ApoCIII	ATGAAGCACGCCACCAAGAC	TGGGGTAGGAGAGCACTGAGA	317	58	32	AY422951
PEPCK	CTGGGCGATGGGGAGTTTGTCA	GATTGTGTTCTTCTGGATGGTCTTG	519	58	32	BC023978
GAPDH	CATCACCATCTTCCAGGAGC	CATGAGTCCTTCCACGATACC	307	59	22	BC013310

Table 2. Sequences and SYBR Green real-time RT-PCR condition

Primer name	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Product size (bp)	Annealing temp.	Number of cycles	GenBank accession number
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG	298	60	45	AF182273
CYP3A5	CTCTCTGTTTCCAAAAGATACC	TGAAGATTATTGACTGGGCTG	194	60	45	NM_000777
CYP3A7	AGATTTAATCCATTAGATCCATTCG	AGGCGACCTTCTTTTATCTG	148	60	45	AF315325
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185	60	45	BC013310

Semiquantitative RT-PCR: The mRNA expression levels of nuclear receptors, transcription factors, and target genes of HNF4α and PGC1α were analyzed by semiquantitative RT-PCR. PCR was carried out with the cDNAs thus obtained using a MyCycler thermal cycler (Bio-Rad, Hercules, CA) and PCR Express thermal cycler (Hybaid Ltd., Teddington, Middlesex, UK). The primers and amplification conditions used are summarized in **Table 1**. The levels of these mRNAs were quantified from their band densities on agarose gels using Printgraph AE-6914 and Scion Image Software (by W. Rasband), and were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Real-time RT-PCR analysis: CYP3As mRNA expression levels were analyzed by SYBR Green real-time RT-PCR. PCR was carried out with the cDNAs thus obtained using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers and amplification conditions used are summarized in **Table 2**. The levels of these mRNAs were normalized relative to that of GAPDH mRNA.

Reporter gene assay: Reporter gene assay was performed according to the manufacturer's instructions using a Sirius Luminometer (Berthold Japan, Tokyo, Japan). The activity was expressed as relative light units (RLU)/mg of protein. The effects of drugs are presented as the ratio of treatment to no treatment.

Statistical analysis: Statistical comparisons were performed with Student's t-test.

Results

PXR and RXRα mRNA expression levels in HepG2 and HFL cells: PXR and RXRα are nuclear receptors that play important roles in RIF-mediated CYP3As induction in the human adult liver. First, RT-PCR was used to analyze mRNA expression levels of PXR and RXRα in HepG2 and HFL cells. RXRα mRNA was detected in HepG2 and HFL cells. RXRα mRNA expression level in HFL cells was similar to that in HepG2 cells. PXR mRNA was detected in HepG2 cells, but not in HFL cells. Therefore, for overexpression of PXR, both cell lines were infected with Ad-PXR. Adenovirus-mediated transfection of PXR caused significant increases in PXR mRNA expression level in HepG2 and HFL cells (**Fig. 1**).

CYP3As mRNA expression levels in HepG2 and HFL cells: RIF is a highly effective inducer of CYP3A enzymes in adult human liver cells in primary culture.[1] Here, we evaluated the effects of PXR ligands including RIF on CYP3A4, CYP3A5, and CYP3A7 mRNA expression in HepG2 and HFL cells by real-time RT-PCR. CYP3A4,

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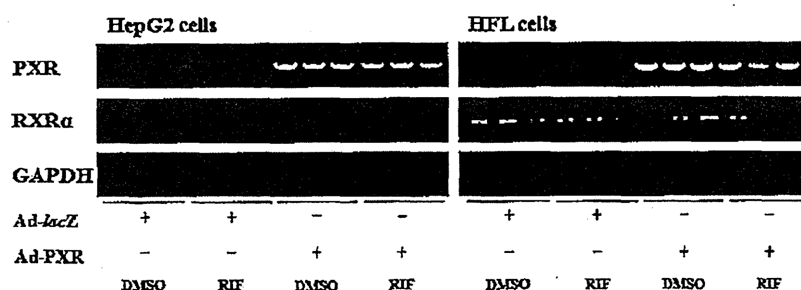

Fig. 1. Nuclear receptor mRNA expression in HepG2 and HFL cells
Total RNA was extracted from HepG2 and HFL cells infected with Ad-*lacZ* or Ad-PXR at 50 MOI for 1 h. mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown.

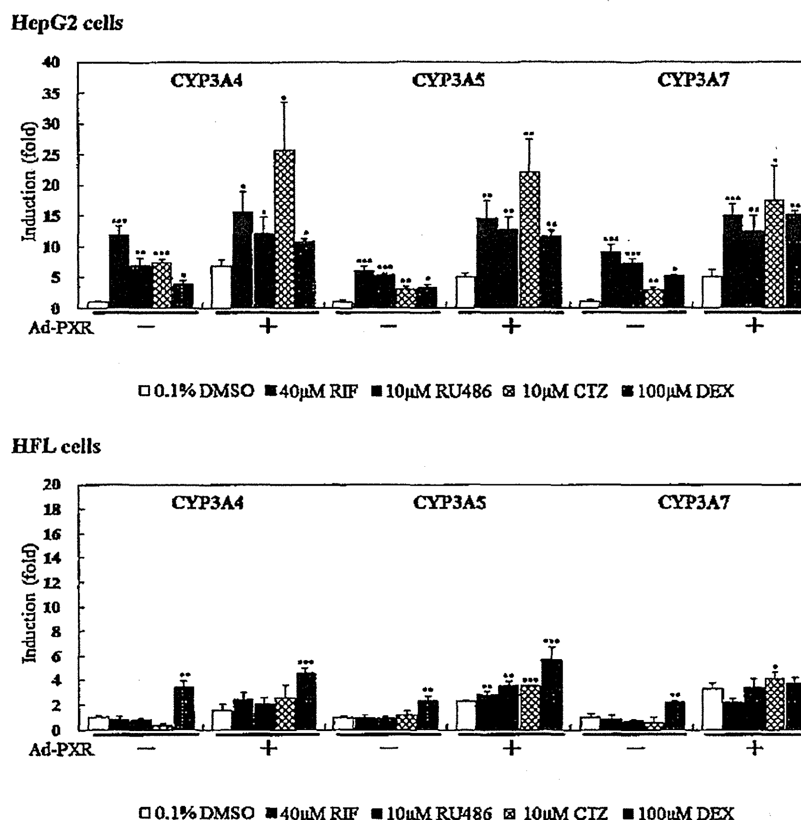

Fig. 2. Effects of inducers and PXR infection on CYP3As mRNA expression in HepG2 and HFL cells
HepG2 and HFL cells were infected with Ad-*lacZ* or Ad-PXR at 50 MOI and treated with vehicle (DMSO), 40 μM RIF, 10 μM RU486, 5 μM CTZ, or 100 μM DEX for 48 h. After treatment, mRNA was analyzed by SYBR Green real-time RT-PCR as described in Materials and Methods. Data are presented as the ratios of CYP3A4, CYP3A5, or CYP3A7 to GAPDH and normalized at 1.0 for Ad-*lacZ* infection and DMSO treatment alone. Columns and vertical bars represent means and SD ($n = 4$). Significantly different from DMSO group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

CYP3A5, and CYP3A7 mRNA expression levels in HepG2 cells were increased from 6- to 11-fold by RIF (**Fig. 2**). The other PXR ligands, RU486, CTZ, and DEX, induced an increase in CYP3A4, CYP3A5, and CYP3A7 mRNA expression by 3- to 7.4-fold in HepG2 cells. In contrast, RIF, RU486, and CTZ did not appreciably induce CYP3A4, CYP3A5, or CYP3A7 mRNA expression in HFL cells, although the expression levels of CYP3A4, CYP3A5, and CYP3A7 were increased to 3.4-, 2.3-, and 2.2-fold, respectively, by DEX.

Next, we examined the effects of PXR overexpression on PXR ligand-mediated CYP3As mRNA expression. Adenovirus-mediated transfection of PXR caused significant increases in CYP3A4, CYP3A5, and CYP3A7 mRNA expres-

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sion in HepG2 and HFL cells without PXR ligands (**Fig. 2**). Under conditions of PXR overexpression, PXR ligand-mediated CYP3A4, CYP3A5, and CYP3A7 mRNA expression levels were increased to a greater extent than in HepG2 cells uninfected with Ad-PXR. Regardless of PXR overexpression in HFL cells, PXR ligands except DEX did not appreciably induce expression of CYP3A4, CYP3A5, or CYP3A7 mRNAs compared to HepG2 cells (**Fig. 2**).

CYP3A4 promoter activity in HepG2 and HFL cells: We used a luciferase promoter construct (Ad-CYP3A4-362-7.7k-Luc) to investigate the effects of PXR-mediated transactivation and PXR ligands on CYP3A4 promoter activity in HFL cells. Ad-CYP3A4-362-7.7k-Luc construct contained the core promoter (−362/+11) and the enhancer region (−7.2k/−7.8k) including ER-6 and DR-3 motifs.

CYP3A4 transactivation was detected without overexpression of PXR in HepG2 cells by PXR ligands, while no transactivation was detected in HFL cells (**Fig. 3**). Overexpression of PXR enhanced the transactivation in HepG2 and HFL cells. The activity was also increased to a greater extent by PXR ligands (RIF, RU486, and CTZ) in HepG2 cells. However, appreciable RIF-induced transactivation was not observed in HFL cells, even after overexpression of PXR. Similarly, other PXR ligands except CTZ did not show sufficient transactivation in HFL cells. However, CTZ significantly increased CYP3A4 transactivation after overexpression of PXR in HFL cells (7.2-fold, $p < 0.01$) (**Fig. 3**).

Transcription factor expression levels in HFL cells: We measured the expression levels of five coactivators in HepG2 and HFL cells and in human adult liver by RT-PCR. The expression levels of HNF4α and PGC1α mRNAs in HFL cells were about 20% of those in human adult liver. The expression level of PGC1α mRNA in HepG2 cells was similar to that in HFL cells. On the other hand, HNF4α mRNA level in HepG2 cells was 57% of that in human adult liver. The expression levels of CBP and P300 were 60–70% of those in the human adult liver, while SRC1 mRNA expression level in HFL cells was higher than those in HepG2 cells and human adult liver (1.3-fold) (**Fig. 4**). We also examined the expression of three corepressors, SMRT, NCoR1, and SHP. The expression levels of SMRT and NCoR1 in HFL cells were similar to those in HepG2 cells and 70–90% of those in human adult liver. SHP mRNA expression was not detected in HFL cells (**Fig. 4**). Ad-PXR infection did not affect mRNA levels of the above coactivators or corepressors in HepG2 or HFL cells (data not shown).

HNF4α and PGC1α target gene expression in HFL cells: To confirm the functions of HNF4α and PGC1α in HFL cells, we examined mRNA expression levels of ApoCIII and PEPCK, which are target genes of HNF4α and PGC1α. ApoCIII mRNA was detected in HepG2 cells, although the expression level was about 10% of that in human adult liver. On the other hand, ApoCIII mRNA was not detected

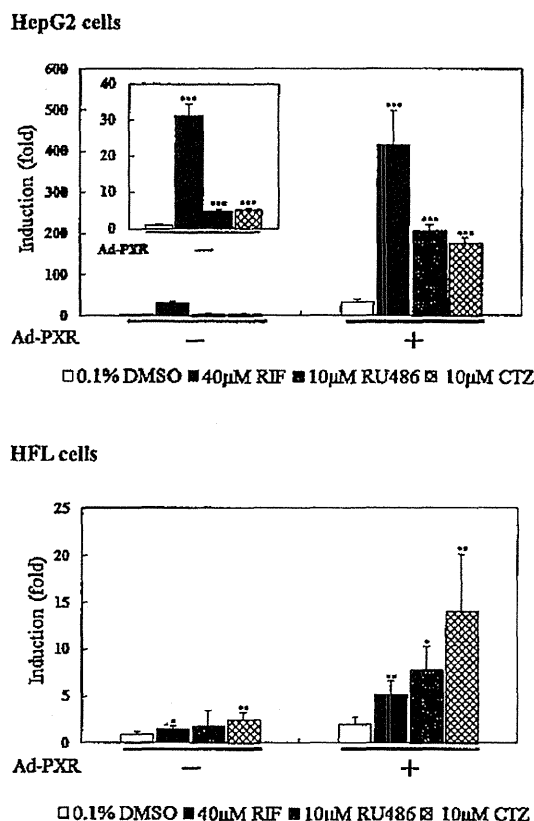

Fig. 3. Effects of inducers and PXR infection on CYP3A4 promoter activity in HepG2 and HFL cells
HepG2 and HFL cells were coinfected with Ad-CYP3A4-Luc (30 MOI) and Ad-*lacZ* (50 MOI) or Ad-PXR (50 MOI) and treated with vehicle (DMSO), 40 µM RIF, 10 µM RU486, or 5 µM CTZ for 48 h. After treatment, CYP3A4 promoter activity was analyzed by luciferase gene assay as described in Materials and Methods. Data are presented as the ratios of CYP3A4 promoter activity as relative light units (RLU)/mg to protein and normalized at 1.0 for Ad-*lacZ* infection and DMSO treatment alone. Columns and vertical bars represent means and SD ($n = 4-6$). Significantly different from DMSO group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

in HFL cells under the present analytical conditions. PEPCK mRNA was not detected in HFL or HepG2 cells (**Fig. 5**).

Discussion

CYP3As gene expression is modulated by a large variety of endogenous and exogenous substances, including drugs, and induction of CYP3As expression has been analyzed extensively using experimental animals. However, it has been difficult to estimate alterations in drug metabolism by inducers of drug-metabolizing enzymes in the human fetus due to the problems of differences among species. Recently, primary cultures of human hepatocytes, HepG2 and HepaRG cells, have been used to study drug metabolism and enzyme induction. On the other hand, there have been few reports regarding the expression and induction of drug-metabolizing enzymes in human fetal hepatocytes in culture.

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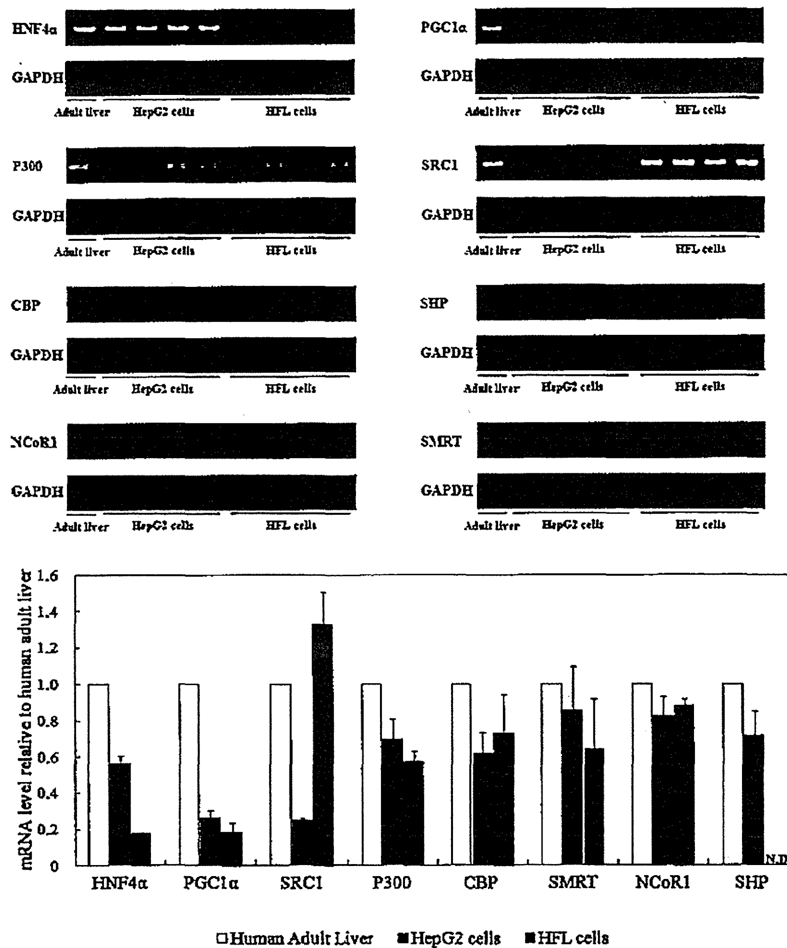

Fig. 4. Expression of coactivators and corepressors mRNA in HepG2 and HFL Cells
mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown. Data are presented as ratios of coactivators or corepressors to GAPDH and normalized at 1.0 for human adult liver. Columns and vertical bars represent means and SD ($n = 1$ or 4).

We reported previously that CYP3A4 and CY3A7 mRNA expression levels were markedly upregulated by DEX in HFL cells.[11,21] We showed that GR plays a critical role in DEX-mediated CYP3As induction in HFL cells. However, RIF-mediated CYP3As induction was not observed, and PXR mRNA was not detected in HFL cells by RT-PCR.[11] We considered that the very low level or absence of PXR expression may impair RIF-mediated CYP3As induction.

Upon ligand binding, PXR forms a heterodimer with RXRα and transactivates ER-6 and DR-3 regions upstream of CYP3A4. Takada *et al.* performed reporter gene assays in HepG2 cells using a CYP3A4-luciferase fusion vector (pCYP3A4-362-7.7k) containing ER-6 and DR-3 regions and concluded that these regions were important in PXR-mediated CYP3A4 transactivation. Furthermore, they described the possibility of another *cis*-element mediating the basal transactivation by PXR in addition to ER-6 and DR-3 regions.[20] Accordingly, PXR and RXRα are essential for PXR ligand-mediated CYP3A4 induction. RXRα expression was confirmed in HFL cells, but PXR was not detected (**Fig. 1**). The present study was performed to examine the effects of PXR overexpression on PXR ligand-mediated CYP3As induction in HFL cells. The HepG2 cell line was established from a liver tumor biopsy specimen from a 15-year-old white male subject.[31] The induction of CYP in HepG2 cells has been studied as a model of human liver.[32,33] This cell line, however, has features of hepatoblast-like cells, because the cells express CYP3A7[34] and secrete AFP[31] into the cell culture medium, both proteins that are found predominantly in human fetal hepatocytes.[35] PXR mRNA was not detectable in HFL cells, although it was expressed in HepG2 cells. PXR has been identified as the major receptor responsible for transcriptional activation of CYP3A gene expression.[36] Another cellular factor, GRα,[37,38] is known to influence xenobiotic-mediated transcription induction, through cooperative activation of PXR expression. The level of GRα mRNA expression was almost identical in HepG2 and HFL cells. These CYP enzymes in HFL cells were

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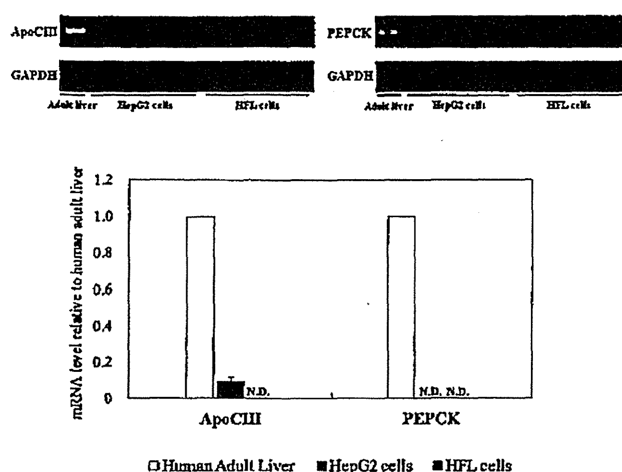

Fig. 5. Expression of HNF4α and PGC1α target gene mRNA in HepG2 and HFL cells

mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods. Images of ethidium bromide-stained agarose gels are shown. Data are presented as ratios of ApoCIII or PEPCK to GAPDH and normalized at 1.0 for human adult liver. Columns and vertical bars represent means and SD ($n = 1$ or 4). ApoCIII and PEPCK mRNA were not detected in HFL cells.

significantly induced by DEX. We suggested previously that one reason why CYP3A4 and CYP3A7 mRNA expression were not induced by RIF in HFL cells may be that PXR expression level is very low in these cells as compared with HepG2 cells.[11] In this study, we used HepG2 cells as a positive control for HFL cells.

PXR overexpression, without PXR ligands, caused increases in CYP3As expression in HepG2 and HFL cells (**Fig. 2**). Similar results were observed in the reporter gene assays (**Fig. 3**). Support for this observation was provided in a report by Zang *et al.*[39] These results indicated that PXR plays an important role in basal CYP3As expression. RIF, RU486, CTZ, and DEX are known to be PXR ligands. These ligands caused the induction of CYP3As with or without PXR overexpression in HepG2 cells (**Figs. 2** and **3**) and the expression levels were further increased by PXR overexpression in HepG2 cells (**Figs. 2** and **3**).

Previously, we suggested that PXR underexpression impaired RIF-mediated CYP3A4 induction in HFL cells. However, these results suggested that factors other than PXR underexpression may control RIF-mediated CYP3A4 induction in HFL cells. DEX, which is known to be a GR ligand, increased CYP3As mRNA expression, unrelated to PXR overexpression. Interestingly, in HFL cells CTZ-mediated CYP3A4 induction was not detected by RT-PCR (**Fig. 2**), while CTZ markedly increased CYP3A4 transactivation under conditions where PXR was overexpressed (**Fig. 3**). In addition, CTZ-mediated CYP3A4 induction was lower than that induced by RIF in HepG2 cells (**Fig. 2**). Furukawa *et al.* reported that CTZ strongly activated CYP3A4 promoter activity while RIF did not in HepG2 cells using a CYP3A4-luciferase reporter construct (−362/+11).[40] These results indicated that the mechanisms of RIF- and CTZ-mediated CYP3A4 induction may be different.

Recently, transcription factors that mediate CYP3As expression have been identified. Various studies have shown that HNF4α interacts strongly with the p160 family coactivators (SRC1, 2, and 3) and that HNF4α activity is enhanced by the action of CBP/P300.[41–45] On the other hand, SHP has been shown to decrease PXR-mediated transactivation and another corepressor-silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), nuclear receptor corepressor 1 (NCoR1), inhibits PXR transactivation.[13,30,46] HNF4α is one of the most important liver-enriched transcription factors involved in hepatocyte differentiation. HNF4α increases PXR-mediated CYP3A4, CYP2C8, and CYP2C9 transactivation.[47–50] Furthermore, PGC1α has been shown to increase the HNF4α-mediated transactivation of CYP7A1, PEPCK, and glucose-6-phosphatase genes.[27–29]

Interaction with PXR, HNF4α, SRC1, and PGC1α has been shown to enhance RIF-mediated CYP3A4 transactivation in HepG2 cells.[50] Martinez-Jiménez *et al.* demonstrated that in HepG2 cells, PGC1α and SRC1 expression levels were lower to allow maximal function of HNF4α. HNF4α was expressed at levels as high as in human liver but its activity on target genes (ApoCIII, ApoAV, PEPCK, AldoB, OTC, and CYP7A1) was very low or absent in HepG2 cells. They concluded that PGC1α and SRC1 are key coactivators for the proper function of HNF4α.[51] We found similar results in HepG2 cells, in that PGC1α and SRC1 expression levels were 20−25% of those in the human adult liver (**Fig. 4**). HNF4α mRNA was detected in both HepG2 and HFL cells, but its expression level was lower in HFL cells than in HepG2 cells (**Fig. 4**). PGC1α mRNA expression level in HFL cells was similar to that in HepG2 cells, but lower than that in the human adult liver (**Fig. 4**). Corepressor SMRT and NCoR1 mRNA expression levels in HFL cells were similar to those in HepG2 cells, but SHP was not detectable in HFL cells (**Fig. 4**). These results suggested that lower levels of HNF4α and PGC1α expression may impair RIF-mediated CYP3A4 induction under conditions where PXR is overexpressed in HFL cells. The expression levels of the HNF4α target genes ApoCIII and PEPCK are indirectly enhanced by PGC1α,[26,27,52,53] and ApoCIII and PEPCK mRNA expression levels were very low or below the limit of detection in HepG2 cells (**Fig. 5**). Similarly, neither ApoCIII nor PEPCK mRNA expression was detected in HFL cells (**Fig. 5**). These results suggested that low levels of HNF4α and PGC1α expression may impair HNF4α target gene expression in HFL cells.

The PXR promoter has been reported to contain the HNF4α binding site that is required for expression of PXR in fetal hepatocytes. Further, HNF4α regulates the expression of xenobiotic-related PXR target genes through control of PXR in fetal liver development.[49] These reports suggested

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that HNF4α is important for the expression of PXR and PXR target genes.

The above results suggested that low levels of HNF4α and PGC1α expression may be responsible for PXR dysfunction as RIF-mediated CYP3As induction was insufficient in HFL cells compared with HepG2 cells.

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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 µg/mL penicillin, 50 µg/mL streptomycin, and 100 µg/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′-CGCGTCGACATGACCCTACTGCAGTACTTTCAAGAGAAG-3′ and 5′-CGCAAGCTTAAAAAAATGACCCTACTGCAGTACTTCTCTTGAAAG-3′. PCR product was digested with *Sal* I and *Hin*d III, and ligated into the same restriction sites of pShattle-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 (Hybaid, 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-

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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	TGAAGAAGTCCTTGCGTGTC
CYP3A7	475 bp/54°C	CTATGATACTGTGCTACAGT	TCAGGCTCCACTTACGGTCT
GR	557 bp/54°C	ACACAGGCTTCAGGTATCTT	ACTGCTTCTGTTGCCAAG
PXR	442 bp/68°C	CAAGCGGAAGAAAAGTGAACG	CTGGTCCTCGATGGGCAAGTC
GAPDH	307 bp/54°C	CATCACCATCTTCCAGGAGC	CATGAGTCCTTCCACGATACC

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

[a]Relative to hydrocortisone, which is assigned a value of 1.[13)]
[b]HFL 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 semiquantitative RT-PCR analysis as described in Materials and Methods. The values are expressed as the mean ± standard deviation of three experiments.

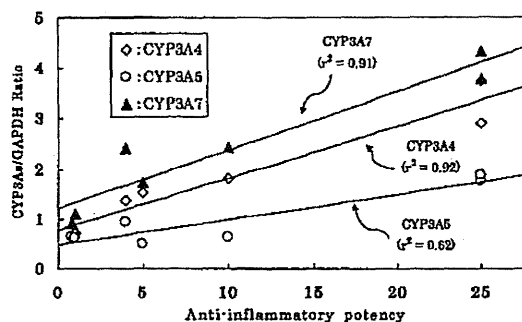

Fig. 1. Correlation between the anti-inflammatory potencies of glucocorticoids and the CYP3A-induction ability

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 glucocorticoids[15)] 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**). 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

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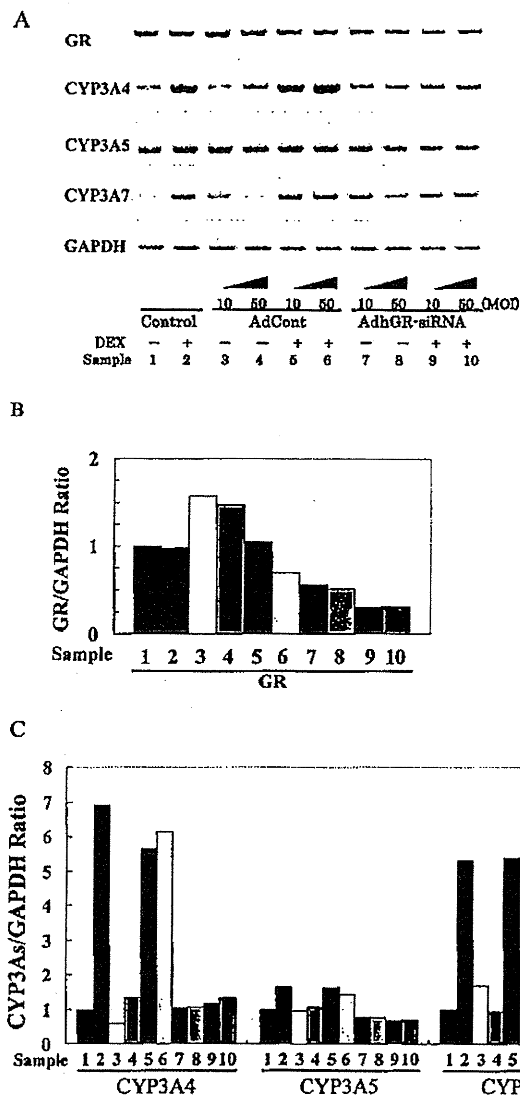

Fig. 2. (Color online) Effects of adenoviral GR-siRNA expression on GR, CYP3A4, CYP3A5 and CYP3A7 mRNA levels
HFL cells were cultured for 1 day after seeding 100,000 cells/well onto 6-well culture plates, and then were infected with AdhGR-siRNA (MOI of 10 or 50). We used AdCont as a control for RNA knockdown 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 4α (HNF4α) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) 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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