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Drug Metab. Pharmacokinet. 27 (2): 200–206 (2012).

Regular Article

Polycyclic Aromatic Hydrocarbons Activate CYP3A4 Gene Transcription through Human Pregnane X Receptor

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Aryl hydrocarbon receptor (AhR) activators have been shown to induce members of the cytochrome P450 (P450) 1 family. Here we demonstrate that the AhR activators induce CYP3A4 through human pregnane X receptor (PXR). AhR activators, polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increased CYP3A4 reporter activity and CYP3A4 mRNA expression in HepG2 cells. The CYP3A4 reporter activity was also increased by treatment with cigarette tar. The increased CYP3A4 reporter activity was clearly knocked down by the introduction of human PXR-small interfering RNA, but not by that of human AhR-small interfering RNA. The CYP3A4 reporter activity enhanced by overexpression of human PXR was further increased by treatment with PAHs and TCDD as well as by treatment with rifampicin. These results suggest that PAHs contained in cigarette smoke induce CYP3A4 in human liver.

Keywords: AhR activator; polycyclic aromatic hydrocarbons; TCDD; CYP3A4 induction; PXR

Introduction

Members of the cytochrome P450 (CYP) supergene family of monooxygenases play an important role in efficient detoxification, by converting pollutants, plant toxins, carcinogens, and drugs to products that can be excreted in urine or bile.[1,2] Human CYP3A4 is of particular significance in this respect because it is involved in the metabolism of approximately two-thirds of clinically relevant drugs.[3] A number of compounds, including pesticides, herbal supplements, vitamins, and drugs, activate *CYP3A4* gene transcription both in the liver and in the small intestine.[4,5] This process of induction constitutes the molecular basis for a number of important drug interactions in patients taking multiple medications.

Pregnane X receptor (PXR; NR1I2) is the principal regulator of *CYP3A4* gene expression and binds as a heterodimer with retinoid X receptor α (RXRα) to regulatory DNA sequences. These sequences include AG(G/T)TCA-like direct repeats spaced by 3 bases (DR3) located at −8 kb upstream from the transcription start point and identified as distal nuclear receptor-binding element 1 (dNR1) and the everted repeats separated by 6 bases (ER6) located in the CYP3A4 proximal promoter (prER6).[6] Recently, we identified a distinct PXR response element as an essential distal nuclear receptor-binding element (eNR3A4) for CYP3A4 induction.[7] PXR is activated by a number of structurally and chemically diverse ligands such as various xenobiotics [rifampicin (RIF) and clotrimazole],[8] pesticides (pyributicarb and endosulfan),[9,10] natural and synthetic

Received August 22, 2011; Accepted October 20, 2011
J-STAGE Advance Published Date: November 10, 2011, doi:10.2133/dmpk.DMPK-11-RG-094
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This work was supported in part by a Grant-in Aid for Scientific Research (C) from the Japan Society for the Promotion Science, by the High-Tech Research Center Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Program for the Promotion of Studies in Health Sciences of the Ministry of Health, Labor and Welfare of Japan.

steroids (dexamethasone),[8] bile acids (lithocholic acid)[11] and herbal medicines (St. John's wort, *Ginkgo biloba*, and *Sophora flavescens*).[12–14] As a result, CYP3A4 induction by these PXR activators leads to the accelerated metabolism of the drugs themselves and the efficacy of concomitant drugs is decreased. Interestingly, there are significant species differences in the activation by PXR ligands between humans and rodents.[15] Drugs such as RIF and clotrimazole activate human PXR but are weak activators of rodent PXR. In contrast, dexamethasone and pregnenolone 16α-carbonitrile activate rodent PXR but are weak activators of human PXR.

Cigarette smoke contains thousands of chemical compounds including a number of carcinogenic polycyclic aromatic hydrocarbons (PAHs) and is known to affect drug therapy in both pharmacokinetic and pharmacodynamic events.[16,17] PAHs induce CYP1A1 and CYP1A2, which mediate the rate-limiting step in the metabolism of many drugs, including theophylline and clozapine, as well as in the bioactivation of procarcinogens. These P450 inductions are also expected to cause drug interaction in this respect. In fact, it was reported that cigarette smoking reduced the therapeutic efficacy of theopylline.[18] The molecular mechanisms by which PAHs or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) transcriptionally activates the *CYP1A1* and *CYP1A2* genes involve the binding of the ligand to aryl hydrocarbon receptor (AhR).[19]

Diazepam (DZ) is a drug that relieves anxiety and controls agitation; it has long been controversial whether cigarette smoking reduces its therapeutic efficacy. Norman *et al.* reported that cigarette smoking strongly decreased the elimination half-life and AUC of DZ, but Klots *et al.* reported no effect.[20,21] DZ is mainly metabolized to 3-hydroxydiazepam and *N*-desmethyldiazepam by CYP3A4 and CYP2C19, respectively. However, there have been no reports about the induction of CYP3A4 and/or CYP2C19 by AhR activators, PAHs or TCDD. Recently, we found a novel event in CYP3A4 induction by AhR activators.

In the present study, we investigated the effect of the AhR activators on transcriptional activation of the *CYP3A4* gene and clearly demonstrated that the AhR activators induced CYP3A4 through PXR pathways in HepG2 cells.

Materials and Methods

Materials: 3-Methylcholanthrene (3-MC) and RIF were purchased from Sigma-Aldrich (St. Louis, MO). Benzo[*a*]-pyrene (B[*a*]P) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TCDD was purchased from Wako Pure Chemicals (Osaka, Japan). Tar was provided from Japan Tobacco Inc. (Tokyo, Japan). The chemicals used for this study were dissolved in dimethylsulfoxide (DMSO). All other reagents used were of the highest quality available. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hyclone Laboratories (Logan, UT). MEM Non-Essential Amino Acids and Antibiotic-Antimycotic were purchased from Invitrogen (Paisley, UK).

Cell culture: HepG2 human hepatoma cells and Huh7 human hepatoma cells were obtained from American Type Culture Collection (Manassas, VA). LS174T human colon adenocarcinoma cells were obtained from the Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). HepG2-derived cell lines stably expressing the *CYP3A4*-luciferase reporter gene, 3-1-20 cells, were as reported previously.[22] These cells were cultured in DMEM supplemented with 10% fetal bovine serum, MEM Non-Essential Amino Acids, and Antibiotic-Antimycotic. The cells were seeded at the appropriate cell density for each experimental condition. The next day, the cell medium was exchanged for the medium containing chemicals (0.1% DMSO) and cultured for 48 h. Adenovirus infection and small interfering RNA (siRNA) transfection were carried out as described previously.[23]

Construction of recombinant adenovirus: Construction of the *CYP3A4* gene reporter adenovirus (AdCYP3A4-362-7.7k) and human PXR-expressing adenovirus (AdhPXR) was performed previously.[9] Control adenovirus, β-galactosidase-expressing adenovirus (AdCont; AxCALacZ), was provided by Dr. Izumi Saito (Tokyo University, Tokyo, Japan). The titer of adenoviruses, 50% titer culture infectious dose (TCID50), was determined as reported previously.[9] Multiplicity of infection (MOI) was calculated by dividing the $TCID_{50}$ by the number of cells.

Small interfering RNA-mediated protein knockdown: Double-stranded siRNAs (25-mer) targeting human AhR (hAhR), human PXR (hPXR), and control siRNA were obtained from Invitrogen (Carlsbad, CA). The corresponding target mRNA sequences for the siRNAs were as follows: hAhR-siRNA, 5′-uuaagucggucucuaugccgcuugg-3′; hPXR-siRNA, 5′-uuucaucugagcguccaucagcucc-3′; control siRNA 5′-uagucauugcacacugcacaguagc-3′. Cells were transfected with each siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Luciferase gene reporter assay: The cells were washed with Dulbecco's phosphate buffered saline (D-PBS) obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and suspended in Passive Lysis Buffer from Promega (Madison, WI) in a microcentrifuge tube. The cell suspension was centrifuged at 12,000 × *g* for 5 min at 4°C, and the cell extract was used for luciferase assay. Luciferase assay was performed according to the manufacturer's instructions using the Luciferase Assay System and GloMax™ 96 Microplate Luminometer (Promega). Resulting data are presented as ratio of luminescence of treated cell samples to that of control. Luminescence of each sample was normalized by its protein concentration determined with the Protein Assay Kit from Bio-Rad Laboratories (Hercules, CA).

Table 1. Sequences of the oligonucleotide primers used for PCR amplification

	Primer sequence
Human CYP3A4	
sense	5′-GATTGACTCTCAGAATTCAAAAGAAACTGA-3′
antisense	5′-GGTGAGTGGCCAGTTCATACATAATG-3′
Human CYP1A1	
sense	5′-ACTGCTTAGCCTAGTCAACCTG-3′
antisense	5′-CAATCAGGCTGTCTGTGATGTC-3′
Human GAPDH	
sense	5′-GAGTCAACGGATTTGGTCGT-3′
antisense	5′-TTGATTTTGGAGGGATCTCG-3′

RNA isolation and quantitative real-time polymerase chain reaction analysis: Total RNA was isolated from 3-1-20 cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. cDNA was prepared from 0.5 μg of total RNA with MMLV reverse transcriptase (Promega) using oligo (dT) primer (Greiner Japan, Tokyo, Japan) and porcine RNase inhibitor (Takara Bio, Shiga, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using Permix Ex Taq (Perfect Real Time, Takara Bio) in a Thermal Cycler Dice Real Time System (Takara Bio). All samples were quantified using a comparative Ct method for relative quantification of gene expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers are shown in **Table 1.**

Statistical analysis: Data were evaluated by the paired Student's *t*-test. When the p value was less than 0.05, the difference was considered to be significant.

Results

Transcriptional activation of the CYP3A4 reporter gene by tar: We investigated whether tar as a major component of cigarette smoke activates the CYP3A4 transcription using HepG2-derived cells stably expressing the *CYP3A4* gene reporter (3-1-20 cells) as reported previously.[22] The cells were treated with 1 to 200 μg/ml tar for 48 h and reporter activities were determined. As shown in **Figure 1**, tar increased *CYP3A4* gene reporter activity in a dose-dependent manner. In contrast, nicotine, which is one of the major components of cigarette smoke, had no effect (data not shown).

Transcriptional activation of the CYP3A4 reporter gene by PAHs: Tar, a by-product of the gasification process, is a complex mixture consisting of a wide range of different compounds in which PAH is one of the major compounds.[24] Therefore, we examined the effect of typical PAHs and TCDD on CYP3A4 reporter activity, which are known as ligands for AhR. RIF was used as a positive control. 3-1-20 cells were treated with RIF (10 μM), 3-MC (1 μM), B[*a*]P (1 μM), and TCDD (10 nM) for 48 h. As shown in **Figure 2**, RIF, 3-MC, B[*a*]P, and TCDD increased CYP3A4 reporter activity by 18.6 ± 2.5-fold,

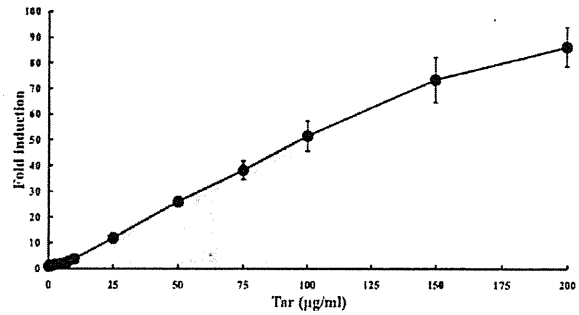

Fig. 1. Effect of tar on CYP3A4 reporter activity in 3-1-20 cells
Clone 3-1-20 was seeded at 1 × 10⁴ cells in 96-well tissue culture plates with 0.1 ml of DMEM 1 day before tar treatment. The cells were treated with 1–200 μg/ml tar for 2 days and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments.

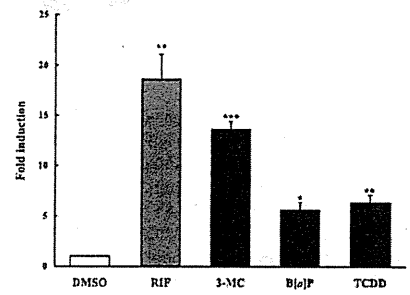

Fig. 2. Effects of 3-MC, B[*a*]P and TCDD on CYP3A4 reporter activity in 3-1-20 cells
Clone 3-1-20 was seeded at 3 × 10⁴ cells in 48-well tissue culture plates with 0.2 ml of DMEM 24 h before RIF, 3-MC, B[*a*]P, or TCDD treatment. The cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$, difference from the vehicle-treated cells based on unpaired Student's *t*-test.

13.6 ± 0.8-fold, 5.57 ± 0.8-fold, and 6.3 ± 0.8-fold, respectively. Furthermore, we investigated the effect of PAHs on endogenous CYP3A4 mRNA expression in 3-1-20 cells. As a result, all chemicals used in this study increased CYP3A4 mRNA expression (**Fig. 3A**). The CYP3A4 mRNA expressions of 3-MC and B[*a*]P were higher than that of RIF. On the other hand, CYP3A4 mRNA expression induced by TCDD was equivalent to that by RIF. In addition, 3-MC, B[*a*]P, and TCDD strongly increased endogenous CYP1A1 mRNA expression, whereas RIF had no effect (**Fig. 3B**). In particular, 3-MC and TCDD increased CYP1A1 mRNA expression by 365.7 ± 39.3-fold and 378.3 ± 37.0-fold, respectively. These results suggest that PAHs induce

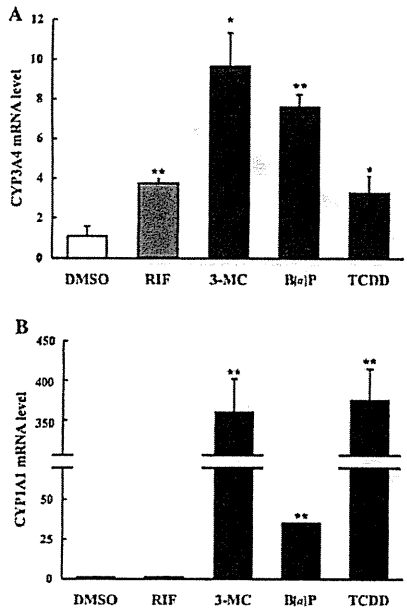

Fig. 3. Effects of 3-MC, B[*a*]P and TCDD on CYP mRNA expression in 3-1-20 cells

Clone 3-1-20 was seeded at 3 × 10^4 cells in 48-well tissue culture plates with 0.2 ml of DMEM 24 h before RIF, 3-MC, B[a]P, and TCDD treatment. The cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and then assayed using real-time RT-PCR. CYP3A4 mRNA or CYP1A1 mRNA expression was normalized by expression of the GAPDH housekeeping gene and presented as fold increase compared with that of vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. (A) CYP3A4 mRNA level, (B) CYP1A1 mRNA level. *, $p < 0.05$, **, $p < 0.005$, difference from the vehicle-treated cells based on unpaired Student's *t*-test.

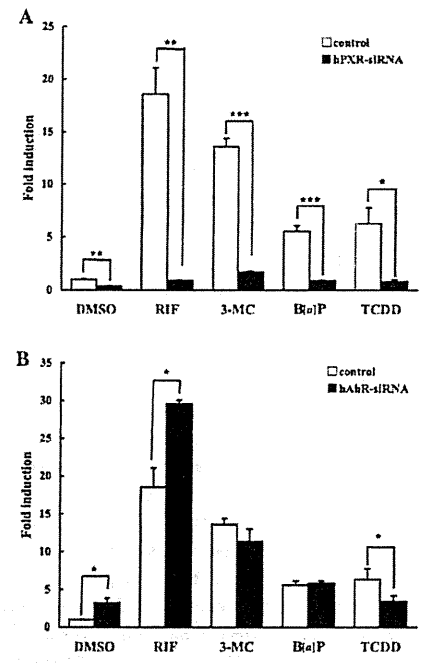

Fig. 4. Effects of hPXR-siRNA and hAhR-siRNA on CYP3A4 reporter activity in 3-1-20 cells

Clone 3-1-20 was seeded at 3 × 10^4 cells in 48-well tissue culture plates and the cells were treated with hPXR-siRNA (A) or hAhR-siRNA (B). After 48 h, the cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells transfected with the control siRNA. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$, difference from the control siRNA-transfected cells based on unpaired Student's *t*-test.

CYP3A4 expression in 3-1-20 cells. In addition, these compounds also increased CYP3A4 mRNA expression in HepG2 cells (data not shown).

Effects of PXR and AhR on activation of CYP3A4 reporter gene by PAHs: PXR is widely known as a major transcription factor mediating CYP3A4 induction, whereas AhR is well known as a transcription factor mediating CYP1A1 induction by PAHs. Therefore, to clarify whether PXR or AhR mediates the CYP3A4 activation by PAHs, we utilized knockdown of PXR or AhR using hPXR-siRNA or hAhR-siRNA and overexpression of PXR using AdhPXR, hPXR-expressing adenovirus. CYP3A4 reporter activities induced by 3-MC, B[*a*]P, and TCDD were significantly decreased by the introduction of hPXR-siRNA (**Fig. 4A**). In contrast, knockdown of the AhR using hAhR-siRNA did not affect the CYP3A4 reporter activity induced by 3-MC or B[*a*]P, whereas CYP3A4 reporter activities induced by TCDD were slightly decreased by the introduction of hAhR-siRNA (**Fig. 4B**). Then, we examined the effect of overexpression of PXR using AdhPXR on CYP3A4 reporter activity induced by PAHs in 3-1-20 cells. As shown in **Figure 5**, we found that the CYP3A4 reporter activation treated with 3-MC, B[*a*]P, and TCDD as well as RIF was increased by the introduction of AdhPXR in 3-1-20 cells.

Effect of PAHs on CYP3A4 reporter activity in Huh7 cells and LS174T cells: Next, we investigated whether the PAHs induce CYP3A4 reporter activity in different cell lines. Huh7 cells and LS174T cells were used, which are *in vitro* models of hepatic and intestinal cells, respectively. As shown in **Figure 6**, the CYP3A4 reporter activation upon treatment with 3-MC, B[*a*]P, and TCDD as well as RIF was increased by the introduction of AdhPXR in Huh7 cells. On the other hand, RIF increased CYP3A4 reporter activity, whereas 3-MC, B[*a*]P, and TCDD had no effect on the activity in LS174T cells.

Discussion

In this study, we investigated the effect of PAHs on transactivation of the *CYP3A4* gene using 3-1-20 cells. 3-MC

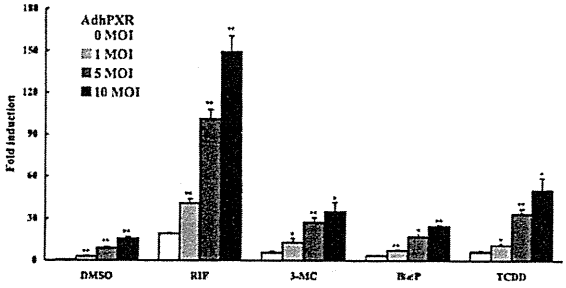

Fig. 5. Effect of AdhPXR on CYP3A4 reporter activity in 3-1-20 cells

Clone 3-1-20 was seeded at 3×10^4 cells in 48-well tissue culture plates pre-incubated for 24 h and then treated with AdhPXR (MOI of 0, 1, 5, 10). Forty-eight hours after infection, these cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells uninfected with AdhPXR. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.005$, difference from the uninfected cells based on unpaired Student's *t*-test.

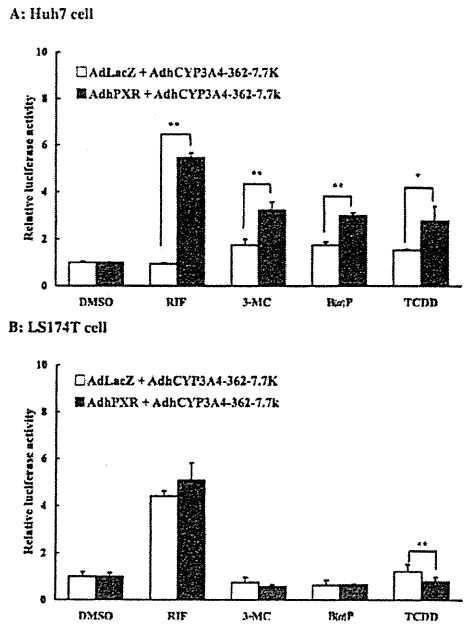

Fig. 6. Effect of AdhPXR on CYP3A4 reporter activity in Huh7 cells and LS174T cells

Huh7 cells (A) and LS174T cells (B) were seeded at 2×10^4 cells in 24-well tissue culture plates pre-incubated for 24 h and then were treated with AdLacZ (10 MOI) or AdhPXR (10 MOI) and Ad-CYP3A4-362-7.7k (5 MOI). Forty-eight hours after infection, these cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[a]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold change compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.005$, difference from the corresponding AdLacZ-infected cells based on unpaired Student's *t*-test.

and B[*a*]P, which are typical PAHs, activated the *CYP3A4* reporter gene (**Fig. 2**). The expression of endogenous CYP3A4 mRNA was also induced by PAHs in 3-1-20 cells (**Fig. 3**). These results indicate that PAHs are potent inducers of CYP3A4. However, the CYP3A4 induction profiles by RIF and PAHs were different between CYP3A4 reporter activity and CYP3A4 mRNA expression level. It is reported that the distal promoter region, from −7836 nt to −7200 nt, and the proximal promoter region, from −362 nt to +11 nt, of the *CYP3A4* gene play important roles for CYP3A4 induction.[25] Recently, Matsumura *et al.* discovered that a novel enhancer region from −11.4 to −10.5 kb, designated the constitutive liver enhancer module of CYP3A4 (CLEM4), is involved in the constitutive activation of the *CYP3A4* gene in HepG2 cells.[26] Furthermore, Liu *et al.* identified a functional nuclear receptor responsive element (F-ER6 at −11368 to −11351) in Huh7 cells.[27] Taking these reports together, for intrinsic CYP3A4 induction, all these *cis*-elements might be required in human liver. However, the cell line 3-1-20 was established by Norachartiyapot *et al.* by insertion of the luciferase reporter plasmid, pGL3-CYP3A4-362-7.7k, which includes both −7836 to −7200 of the distal promoter region and −362 to +11 of the proximal promoter region of the *CYP3A4* gene, the elements reported to be necessary for high response to RIF, into HepG2 cell chromosome.[22] Therefore, the differences of these CYP3A4 activation patterns by RIF and PAHs might be attributable to other enhancer regions not included in pGL3-CYP3A4-362-7.7k. In addition, CYP1A1 plays critical roles in the metabolism of PAHs.[28] Thus, 3-MC and B[*a*]P were initially metabolized by CYP1A1 induced through AhR activation in this culture condition. Subsequently, the metabolites of 3-MC and B[*a*]P might cause CYP3A4 induction. On the other hand, the induction profile was different among AhR ligands. Although CYP3A4 induction potency is similar between 3-MC and B[*a*]P, the CYP1A1 induction potency of B[*a*]P was far lower than that of 3-MC. This result may suggest that AhR is not directly involved in the *CYP3A4* gene activation by 3-MC and B[*a*]P.

CYP3A4 induction by xenobiotics and hormones is mediated by PXR,[29-31] constitutive androstane receptor (CAR, NR1I3),[32] vitamin D receptor (VDR, NR1I1),[33] and glucocorticoid receptor-α (GRα, NR3C1)[34] in the liver. In particular, chemical-induced activation of the *CYP3A4* gene is mainly mediated by PXR through binding to the *CYP3A4* 5′-flanking region,[29,31,35] whereas PAHs cause transactivation of *CYP1A1* and *1A2* genes *via* AhR.[19] Therefore, we knocked down hPXR expression using hPXR-siRNA. When hPXR-siRNA was introduced into the 3-1-20 cells, the activation of the *CYP3A4* reporter gene by PAHs as well as RIF was significantly decreased (**Fig. 4A**), whereas the knockdown of AhR by hAhR-siRNA had no effect on the activation of the *CYP3A4* reporter gene by RIF and PAHs (**Fig. 4B**). The same results were observed when hPXR-

siRNA was introduced by adenovirus (data not shown). Furthermore, the overexpression of PXR using AdhPXR increased CYP3A4 reporter activity by PAHs as well as RIF in an infection dose-dependent manner (**Fig. 5**). Therefore, these results strongly suggest that 3-MC and B[*a*]P enhance the transactivation of the *CYP3A4* gene through PXR activation but not through AhR activation. On the other hand, knockdown of AhR by hAhR-siRNA decreased the CYP3A4 reporter activity by TCDD (**Fig. 4**). This reason for this is unclear. The *CYP3A4* gene might be transactivated partially by TCDD through the AhR pathway owing to its strong AhR activation potency. Further studies are needed to resolve this issue.

It is known that the drug-induced expression of the *CYP3A4* gene in the liver is predominantly regulated through PXR.[36] In contrast, VDR controls the CYP3A4 transactivation with the secondary bile acid, lithocholic acid (LCA), in the intestine.[37,38] In addition, recently, a novel PXR functional *cis*-acting PXR-binding element designated eNR3A4 has been discovered to be an essential element for RIF-inducible CYP3A4 transactivation in human liver.[7] This element is located approximately 7.6 kb upstream from the transcription initiation site of the *CYP3A4* gene, to which hPXR binds as a heterodimer with human RXRα. Furthermore, Pavek *et al.* reported that eNR3A4 has negligible or no effect on CYP3A4 transactivation through VDR.[39] Thus, these findings indicate that eNR3A4 is a key regulatory element for the xenobiotic induction of CYP3A4 through hPXR in the liver. In this study, we show that PAHs increased CYP3A4 reporter activity in PXR-overexpressing hepatoma cells, whereas this phenomenon was not observed in PXR-overexpressing intestinal cells (**Fig. 6**). These results indicate that eNR3A4 might have been involved in the activation of the *CYP3A4* gene through hPXR by PAHs in the liver. Since the difference of the HepG2 cells and the LS174T cells in an induction mechanism of the *CYP3A4* gene by PAHs is unknown, further analysis is still in progress.

Numerous studies of induction by treatment with PAHs have been carried out to date. PAHs as well as TCDD have been believed to strongly induce CYP1A1 and CYP1A2 through the AhR pathway. However, there are no data about CYP3A induction by them in experimental animals. Recently, it was reported that 3-MC induces CYP3A4 in HepG2 cells.[40] In addition, when the reporter assay was measured in pGL3-CYP3A4-362-7.7k-transformed rat hepatoma Reuber H35 cells, PAHs and TCDD did not increase the reporter activity (data not shown). Together with these results, it is suggested that PAHs and TCDD activate human PXR, but not rat or mouse PXRs. Further studies are needed to clarify this issue.

In conclusion, we have demonstrated that PAHs activate *CYP3A4* gene transcription through the activation of hPXR in HepG2 cells. Thus, PAHs may contribute to CYP3A4 induction in human liver. More detailed study on the molecular mechanism behind the CYP3A4 induction by PAHs may provide important information on the drug-drug interaction.

Acknowledgments: We thank Mr. Shinpei Kobayashi and Ms. Rie Shoji for excellent technical assistance.

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Xenobiotica, 2012, 1–10, Early Online

ISSN 0049-8254 print/ISSN 1366-5928 online
DOI: 10.3109/00498254.2011.648670

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healthcare

ORIGINAL ARTICLE

Dexamethasone-mediated transcriptional regulation of rat carboxylesterase 2 gene

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Abstract

1. Rat carboxylesterase 2 (rCES2), which was previously identified as a methylprednisolone 21-hemisuccinate hydrolase, is highly inducible by dexamethasone in the liver. In the present study, we investigated the molecular mechanisms by which this induction occurs.
2. Injection of dexamethasone (1 mg/kg weight) into rats resulted in increases in the expression of rCES2 mRNA in a time-dependent manner with a peak at 12 h after injection. In primary rat hepatocytes, the expression level of rCES2 mRNA was increased by treatment with 100 nM dexamethasone, and the increase was completely blocked in the presence of 10 μM mifepristone (RU-486), a potent inhibitor of glucocorticoid receptor (GR), or 10 μg/mL cycloheximide, a translation inhibitor. Luciferase assays revealed that 100 nM dexamethasone increased *rCES2* promoter activities, although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. The increased activities were completely inhibited by treatment of the hepatocytes with 10 μM RU-486.
3. Based on these results, it is concluded that dexamethasone enhances transcription of the *rCES2* gene via GR in the rat liver and that the dexamethasone-mediated induction of rCES2 mRNA may be dependent on *de novo* protein synthesis. Our results provide clues to understanding what compounds induce rCES2.

Keywords: rCES2, induction, glucocorticoid receptor

Introduction

Carboxylesterases (CESs, EC 3.1.1.1) belong to the α,β-hydrolase-fold family (Bencharit et al., 2003). CESs catalyse hydrolytic reactions in a variety of xenobiotic and endobiotic substrates because of their ability to hydrolyse a broad spectrum of ester, amide, thioester, and carbamate compounds (Sanghani et al., 2004). CESs are classified into five families (CES1-5) (Satoh and Hosokawa, 2006). The major two CES families, CES1 and CES2, are well characterised compared with others. The two families have different substrate specificities. CES1 mainly hydrolyses a substrate that can be converted to a small alcohol moiety and a large acyl moiety. This is in contrast to CES2, which mainly hydrolyses a substrate that can be converted to a large alcohol moiety and a small acyl moiety. A number of chemical compounds are known to induce expression of CESs, yet there is little information about the molecular mechanisms of induction of CESs.

CESs are prominently involved in many pharmaceutical agents. Many esterified drugs such as cocaine and meperidine are metabolised by CESs into inactive products (Potter and Wadkins, 2006). On the other hand, a number of ester-containing prodrugs, whose pharmacological activities are generally masked, are hydrolysed by CESs and then exert their activities. Examples of prodrugs are the anticancer drug CPT-11 (irinotecan), which is metabolised by CES1 and CES2 to the active metabolite SN-38 (Humerickhouse et al., 2000), and the anti-influenza drug

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(Received 07 November 2011; revised 05 December 2011; accepted 07 December 2011)

oseltamivir, which is metabolised by CES1 to Ro 64-0802 (Shi et al., 2006). Thus, CESs play an important role in determining the metabolic fate of many drugs.

Dexamethasone is a synthetic glucocorticoid and is used to treat many different conditions such as skin diseases, asthma, and rheumatoid arthritis. Dexamethasone alters expression levels of a large number of genes including tyrosine aminotransferase (Schmid et al., 1987), glutamine synthetase (Gaunitz et al., 2002) and Na^+-K^+-ATPase (Celsi et al., 1991) and also many drug-metabolizing enzymes such as CYP2C9 (Gerbal-Chaloin et al., 2002), CYP3A4 (Pascussi et al., 2001) and human CES1 (Zhu et al., 2000). With respect to gene regulation by dexamethasone, some molecular mechanisms are known. In many cases, homodimers consisting of glucocorticoid receptors (GRs) activated by ligands such as dexamethasone directly bind to glucocorticoid response elements (GREs) and regulate gene expression. At high concentrations (more than 10 μM), dexamethasone can activate pregnane X receptor as well as GR, leading to alteration of gene expression (Huss and Kasper, 2000; Pascussi et al., 2001). In addition, GR activated by dexamethasone contributes to induction of the *CYP2A6* gene by interacting with hepatocyte nuclear factor-4α (Onica et al., 2008). Increased or decreased drug-metabolizing enzymes can change drug potency *in vivo* and influence the incidence of adverse effects. Therefore, the molecular mechanisms by which dexamethasone regulates *CES* gene expression need to be understood to accurately predict the potency of drugs metabolised by CESs.

The expression levels of several rat *CES* genes are altered by dexamethasone treatment. Zhu et al. (2000) reported that the expression of rat CES1 (hydrolase A, B, and S) in the liver was suppressed after intraperitoneal injection of dexamethasone. GR is involved in the molecular mechanisms of the suppression (Shi et al., 2008). In contrast, rat CES2 (rCES2) (GenBank ID, AB191005), previously called CES RL4, is markedly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms underlying the induction of rCES2 have remained unknown. Dexamethasone-mediated induction of rCES2 is known to cause a drug interaction. Since rCES2 hydrolyses methylprednisolone 21-hemisuccinate (MPHS), which is prescribed for various conditions including systemic lupus erythematosus, hemorrhagic shock and rejection episodes in renal transplant recipients, to the active metabolite methylprednisolone, treatment with dexamethasone increases MPHS hydrolase activity in the rat liver. Drug-mediated induction or repression of rat *CES* gene expression should be noted as long as rats continue to be used commonly in non-clinical studies.

The goal of this study was to determine the molecular mechanisms underlying dexamethasone-mediated induction of rCES2. In the present study, treatment of primary rat hepatocytes with mifepristone (RU-486), a potent antagonist of GR, resulted in inhibition of both increase in rCES2 mRNA and elevation of *rCES2* promoter activity caused by dexamethasone. These results suggested that GR plays a critical role in dexamethasone-mediated transcriptional activation of the *rCES2* gene. Moreover, results obtained by using cycloheximide indicated the possibility that *de novo* protein synthesis is necessary for the induction of rCES2 mRNA by dexamethasone.

Materials and methods

Materials

Dexamethasone-water soluble, triamcinolone acetonide, cycloheximide, RU-486, MPHS, and methylprednisolone were purchased from Sigma-Aldrich (St. Louis, MO). Dexamethasone (used with corn oil), corn oil, collagenase, a Ligation-Convenience kit, and prednisolone were purchased from Wako Pure Chemical Industries (Osaka, Japan). Trypsin inhibitor from soybean (>7000 BAEE units/mg), Williams' medium E without phenol red, Opti-MEM I, DNase I, and a Zero Blunt TOPO PCR Cloning kit were purchased from Invitrogen Life Technologies (Carlsbad, CA). Pentobarbital sodium (somnopentyl) was purchased from Schering-Plough Corp. (Kenilworth, NJ). Human recombinant insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals Inc. (Princeton, NJ). A nylon mesh filter was purchased from Sefar Inc. (Heiden, Switzerland). AteloCell (native collagen bovine dermis) was purchased from Koken Co. Ltd. (Tokyo, Japan). FuGENE HD transfection reagent was purchased from Roche Diagnostics Corp. (Indianapolis, IN). BD Matrigel (phenol red-free) was purchased from BD Biosciences (Bedford, MA). ISOGEN was purchased from Nippon Gene (Toyama, Japan). A ReverTra Ace qPCR RT kit, THUNDERBIRD Probe qPCR Mix, KOD -plus- DNA Polymerase, and *Kpn* I were purchased from Toyobo (Osaka, Japan). *Xho* I was purchased from Takara Shuzo (Kyoto, Japan). TaqMan Gene Expression Assays (probe and primer sets) for rCES2 (AssayID: Rn00592205_m1) (FAM) and rat tyrosine aminotransferase (TAT) (Rn01431532_m1) (FAM) and Pre-Developed TaqMan Assay Reagents for eukaryotic 18S rRNA (VIC) were purchased from Applied Biosystems (Foster, CA). Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). *Dpn* I was purchased from New England BioLabs (Hitchin, Hertfordshire, UK).

Intraperitoneal injection of dexamethasone

Male Sprague-Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) of 5-6 weeks (150-200 g) of age were used in this experiment. Dexamethasone in corn oil (0.25 mg/mL) was intraperitoneally injected into rats at a dose of 1 mg/kg body weight, or only corn oil was injected as a control in the same way. Rats were put under anesthesia with diethyl ether at 3, 6, 12, and 24 h after injection of dexamethasone or at 24 h after injection of only corn oil for the control, and the livers were removed for relative quantification of mRNA expression and protein expression. One

rat per experiment was used at each time point and the experiment was repeated thrice ($n=3$/time point).

Isolation of hepatocytes

Rat hepatocytes were isolated using a collagenase two-step perfusion method described by Seglen (1976) with some modifications. Rats were anesthetised by intraperitoneal injections of pentobarbital (100 mg/kg body weight) and the abdominal cavity was incised. An indwelling needle consisting of an inner needle and a flexible cover needle was inserted into the portal vein, and the inner needle was removed from the indwelling needle. The flexible cover needle and portal vein were immediately bound using a clamp, and the cover needle was connected to a tube with a pump. A pre-perfusate (137 mM NaCl, 5.37 mM KCl, 1.05 mM $MgCl_2 \cdot 6H_2O$, 0.832 mM $MgSO_4 \cdot 7H_2O$, 0.500 mM $NaH_2PO_4 \cdot 2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 0.500 mM EGTA, 4.17 mM $NaHCO_3$, and 5.00 mM D-glucose; pH 7.2 and 37°C) was flowed through the portal vein at a flow rate of ~24 mL/min. The inferior vena cava was immediately cut to make an exit site for the pre-perfusate. About 6 min later, the pre-perfusate was replaced by flowing a collagenase solution (137 mM NaCl, 5.37 mM KCl, 5.05 mM $CaCl_2$, 0.500 mM $NaH_2PO_4 \cdot 2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 4.17 mM $NaHCO_3$, 1 g/L collagenase, and 100 mg/L trypsin inhibitor; pH 7.5 and 37°C). The collagenase solution was flowed for ~6 min at the same flow rate. After perfusion of the collagenase solution, the digested liver was resected and washed briefly with ice-cold Hanks' balanced salt solution, HBSS (137 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.336 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 5.55 mM D-glucose, and 4.17 mM $NaHCO_3$; pH ~7.3). Cells were dispersed from the digested liver in 50 mL of ice-cold HBSS and the cellular suspension was filtrated through a nylon mesh filter (pore size, 150 μm). The filtrated cells were centrifuged for 2 min at 4°C and the supernatant was aspirated. For removal of non-parenchymal hepatocytes, the cells containing hepatocytes were suspended in 15 mL of cold HBSS and centrifuged for 2 min at 4°C, and the supernatant was removed (This series of steps for removal was performed twice.). Finally, the obtained hepatocytes were suspended in 20 mL of an ice-cold standard culture medium (Williams' medium E without phenol red, containing 0.25 U/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin) and the viability of hepatocytes was assessed by 0.4% trypan blue exclusion. Hepatocytes for which viability exceeded 85% were used in the present study.

Primary cultures of rat hepatocytes for mRNA expression analysis

Primary cultures of rat hepatocytes were performed using the method previously described by Kocarek and Reddy (1996) with some modifications to extract total RNA and perform the ensuing real-time polymerase chain reaction (PCR). Hepatocytes were isolated from SD rats of 5–7 weeks (~160–250 g) of age by the method described above. The cells were suspended in the standard culture medium and were seeded at 7.5×10^5 cells/mL × 2 mL/well onto 6-well plates coated with 0.5 mg/well (for example, 3.57 mg/mL × 140 μL/well) of matrigel. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Twenty-four hours after seeding, the culture medium in the plates was replaced with 2 mL fresh medium. Dexamethasone (water-soluble) was dissolved in Milli-Q (MQ) water to make a 100-μM dexamethasone solution as a stock solution. RU-486 or cycloheximide was dissolved in ethanol to make a 10-mM RU-486 solution or 1-mM and 10-mg/mL cycloheximide solutions as stock solutions. The culture medium in plates was again replaced with 2 mL fresh medium 48 h after seeding, and reagents (drugs and vehicles) were added to the culture medium. The drug solutions or solvents were added at 0.1% (v/v). Extraction of total RNA from hepatocytes in each well was performed 24 h after treatment with reagents.

Relative quantification of mRNA expression

Relative quantification of target transcripts was performed essentially as described previously (Hori and Hosokawa, 2010). Total RNA was extracted from rat liver pieces and primary hepatocytes using an ISOGEN and treated with DNase I to prevent contamination by DNA. The treated RNA and a ReverTra Ace qPCR RT kit were used to synthesise first-strand cDNA. The expression level of rCES2 mRNA was analysed using cDNA (1 μg/sample), a THUNDERBIRD Probe qPCR Mix, gene-specific TaqMan probe and primer sets, and an Applied Biosystems 7500 Real-Time PCR System. Rat CES2 mRNA expression was normalised with 18S rRNA expression. The $\Delta\Delta C_t$ method was used for analysis of data on rCES2 mRNA expression. The conditions of real-time PCR were as follows: 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of rat TAT mRNA was analysed in the same way as that for rCES2 mRNA.

Western blot analysis

The expression level of rCES2 protein in the rat liver was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot essentially as previously described (Furihata et al., 2005). Rat liver pieces obtained from rats that were injected with or without dexamethasone were homogenised with 1.15% KCl. The 20% (w/v) homogenates were centrifuged at 9000*g* for 20 min, and the supernatants were centrifuged at 105,000*g* for 1 h. After replacement of the supernatants with fresh KCl buffer, the samples were again centrifuged at 105,000*g* for 1 h. The microsomal pellets were suspended in SET buffer (0.25 M sucrose, 1 mM EDTA·2Na, and 10 mM Tris-HCl; pH 7.4). SDS-PAGE was performed using 10% polyacrylamide gels and 10 μg/well of microsomal protein, and Western blot was performed using polyclonal anti-rCES2 antibodies, which were previously prepared (Derbel et al., 1996). The density of bands was measured

using ImageJ 1.44o software (National Institutes of Health, Bethesda, MD).

MPHS hydrolase activity assay

MPHS hydrolase activity was determined essentially according to the method described previously (Furihata et al., 2005). Rat liver microsomes were preincubated in citrate-phosphate buffer (pH 5.5) for 5 min at 37°C. The reaction was initiated by adding MPHS dissolved in 1% dimethylformamide water to the mixture. The mixture (50 mM citrate-phosphate buffer, 0.04–0.3 mg/mL microsomal protein, and 400 μM MPHS) was incubated for 15 min at 37°C. The reaction was terminated by adding 100 μL of acetonitrile containing 40 μM prednisolone, which was used as an internal standard, to 100 μL of the mixture. After the removal of protein, the amount of methylprednisolone formed from MPHS was determined by HPLC. Methylprednisolone was detected at a wavelength of 254 nm. HPLC system consisted of a LC-20AD pump unit (Shimadzu Corp., Kyoto, Japan), a SIL-20A autosampler (Shimadzu), a CTO-10AS VP column oven (Shimadzu), a SPD-20A UV/VIS detector (Shimadzu), a SCL-10A VP system controller (Shimadzu), and a Mightysil RP-18 GP 150 mm × 4.6 mm column (Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (pH 7.0)/acetonitrile (65:35, v/v) and was delivered at a flow rate of 0.9 mL/min.

Reporter constructs

The 5′-flanking region from −2957 to +51 (−2957/+51), when the transcription start site of the *rCES2* gene is +1, was amplified by PCR with KOD -plus- DNA Polymerase, rat genomic DNA as a template, and primers listed in Table 1. The primers were designed on the basis of the sequence obtained from a search by basic local alignment search tool (BLAST) with the sequence of AB191005. The amplified fragment was cloned into pCR -Blunt II-TOPO vector provided in the Zero Blunt TOPO PCR Cloning kit following the manufacturer's instructions, and this plasmid was designated pCR-rCES2 -2957/+51. After sequencing, pCR-rCES2 -2957/+51 was digested by the two restriction enzymes, *Kpn* I and *Xho* I. After purification, the digested fragment containing the sequence of −2957/+51 was ligated using a Ligation-Convenience kit to pGL3-Basic vector, a luciferase reporter vector, digested by the same restriction enzymes. After amplification using competent *E. coli* JM109 and the ensuing purification, this plasmid was designated pGL3-rCES2 -2957/+51 for luciferase assays. A variety of deletion plasmids (pGL3-rCES2 −1955/+51, −1569/+51, −991/+51, −662/+51,−195/+51, and −73/+51) were made in the same manner as that described above, except that these deletion plasmids were made using primers listed in Table 2 and pCR-rCES2 -2957/+51 as a template. The plasmid of pGL3-rCES2 -6/+51 was made by a site-directed mutagenesis method described below. First, PCR was performed using pGL3-rCES2 -195/+51 as a template and primers that were designed to anneal to the same sequence on opposite strands and to carry a *Kpn* I site (Table 3). The PCR product, plasmid, was electrophoresed and the target plasmid was extracted. The plasmid was treated with *Dpn* I to digest template plasmid, pGL3-rCES2 -195/+51. Following transformation of JM109 and purification of the plasmid, sequencing was performed to confirm the existence of the *Kpn* I site in the plasmid. Then the plasmid was digested by *Kpn* I and the fragment containing the sequence of −6/+51 was self-ligated using a Ligation-Convenience kit. In this way, pGL3-rCES2 -6/+51 plasmid was made.

Table 1. Specific primers in the 5′-flanking region of the *rCES2* gene for genomic cloning and luciferase assays.

Position	Sequence
Forward primer	
-2957/-2933	5′-GATGGCTGCGTGATACTTCTTCTGG-3′
Reverse primer	
+23/+51	5′-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3′

Table 2. Specific primers in the 5′-flanking region of the *rCES2* gene for luciferase assays.

Position	Sequence
Forward primer	
-1955/-1936	5′-ATCTTGGTGCCTTCTAACTG-3′
-1569/-1549	5′-TCTTTGACTAGCGAAATGGTG-3′
-991/-968	5′-TTTTTTTTTCTGGTGATGGATTCG-3′
-662/-642	5′-CCTCTGGAGACACTTCAGACA-3′
-195/-173	5′-AGTCCACACTGTGCCTTTCCAGG-3′
-73/-51	5′-TTCACCCACGACATCATGTTCCC-3′
Reverse primer	
+23/+51	5′-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3′

Table 3. Mutant primers for site-directed mutagenesis to make *Kpn* I site (small letter).

Position	Sequence
Forward primer	
-25/+10	5′-CCTGCCTGGGCAAggtaccCGGTTTATTCTTCCTG-3′
Reverse primer	
-25/+10	5′-CAGGAAGAATAAACCGggtaccTTGCCCAGGCAGG-3′

Transient transfection and luciferase assay

Transient transfection into primary rat hepatocytes was performed using the method previously described by Runge-Morris et al. (1999) with some modifications. Hepatocytes were isolated from SD rats of 5–7 weeks (~150–250 g) of age by the method described above. Hepatocytes were suspended in a cold standard culture medium which was supplemented with 100 nM triamcinolone acetonide (TA). The cells were seeded at 3×10^5 cells/mL × 0.5 mL/well onto 24-well plates coated thinly with collagen. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Following ~6 h of seeding, the medium was replaced with 0.5 mL of Opti-MEM I, and plasmids were transfected to the cells as follows: 500 ng/well pGL3 plasmid (12.5 ng/μL), 50 ng/well phRL-TK

plasmid (12.5 ng/μL), and 1.4 μL/well FuGENE HD. Five hours after transfection, the medium was replaced with 0.5 mL of the standard culture medium without TA, and hepatocytes were overlaid with 0.05 mg/well (for example, 3.57 mg/mL × 14 μL/well) of matrigel. Thirty hours after addition of matrigel, hepatocytes were treated with either 100 nM dexamethasone or MQ water in 0.5 mL of fresh medium without TA and again overlaid with 0.05 mg/well of matrigel. Simultaneously with dexamethasone or MQ water, in an experiment for which results are shown in Figure 5C, hepatocytes were exposed to RU-486 or ethanol. Twenty-four hours later, hepatocytes were rinsed once with PBS (-), and dual-luciferase reporter assays were performed according to the manufacturer's instructions.

Statistical Analysis

Multiple groups were compared by one-way analysis of variance (one-way ANOVA) followed by Dunnett's (Figure 1A and 1C) or Tukey's (Figures 2, 3, and 5C) multiple comparison test. Two groups were compared by Student's *t*-test (Figures 4 and 5A). These statistical analyses were performed using the free software R version 2.13.0 (R Development Core Team, 2011). A value of $P < 0.05$ was considered statistically significant.

Results

Dexamethasone-mediated increases in TAT and rCES2 mRNA expression and alterations of rCES2 protein expression in the rat liver

Dexamethasone was injected into rats to investigate temporal changes in TAT and rCES2 mRNA expression. The expression levels of rCES2 mRNA peaked at 12 h after injection of dexamethasone and the maximum level was ~400-fold higher than that of the control (dexamethasone 0 h) (Figure 1A). The level of TAT mRNA, which is well known to be induced by dexamethasone, was also increased by dexamethasone. The increased level of TAT mRNA was highest at 6 h after injection, and this change was in agreement with the results of a previous study (Shi et al., 2008). Significant increases of rCES2 protein and MPHS hydrolase activity were observed at 12 and 24 h after injection (Figure 1B and 1C).

Comparison between rat primary hepatocytes and livers in expression of TAT and rCES2 mRNA

The expression levels of TAT or rCES2 mRNA in rat hepatocytes and livers were compared. Hepatocytes as a control were treated with both water and ethanol. When based on the average ΔC_t value obtained from

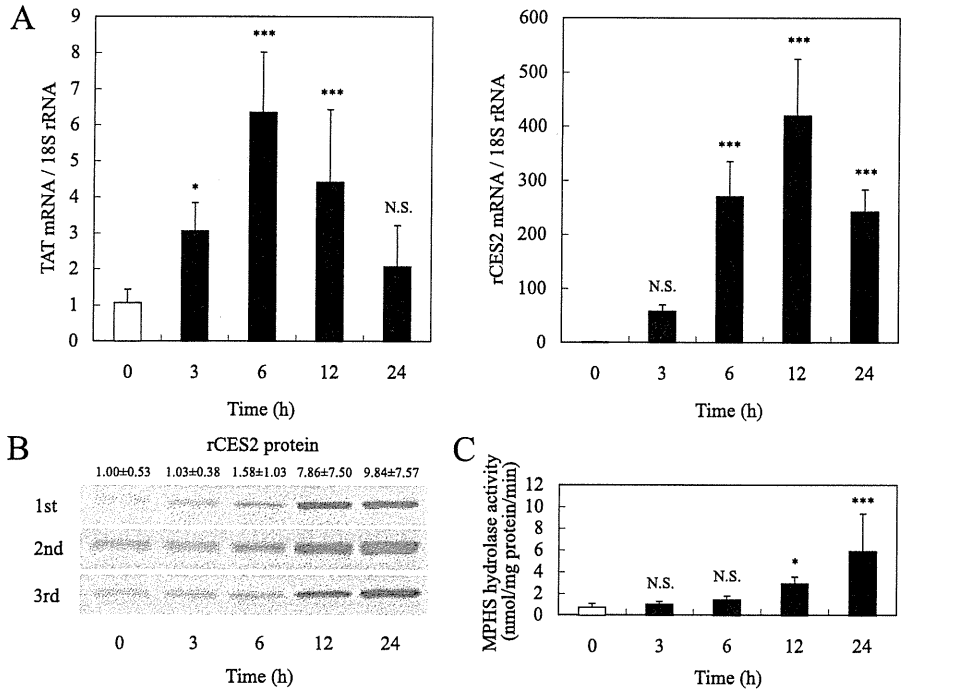

Figure 1. (A) Time course of increases in TAT and rCES2 mRNA by dexamethasone. Rats were sacrificed at 3, 6, 12, and 24 h after injection of dexamethasone, and liver pieces were used to determine the expression levels of TAT and rCES2 mRNA by real-time PCR. Each value is shown as the mean ± standard deviation (SD) of three independent experiments ($n = 3$/group), which were performed in triplicate. In analysis of TAT mRNA, the C_t (threshold cycles) values (mean ± SD) of a control (dexamethasone 0 h) were 23.1 ± 1.04 (TAT) and 12.6 ± 0.813 (18S rRNA). In analysis of rCES2 mRNA, the C_t values of the control were 27.8 ± 0.905 (rCES2) and 12.5 ± 0.535 (18S rRNA). N.S. indicates not statistically significant. Statistically significant difference; $^*P < 0.05$ and $^{***}P < 0.001$. (B) Alterations of rCES2 protein expression by dexamethasone. Relative expression levels of rCES2 protein (mean ± SD of three independent experiments) were estimated using a standard curve that generated by a microsomal sample obtained form a rat injected with dexamethasone. (C) Temporal changes in MPHS hydrolase activity in liver microsomes after injection of dexamethasone. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Statistically significant differences; $^*P < 0.05$ and $^{***}P < 0.001$.

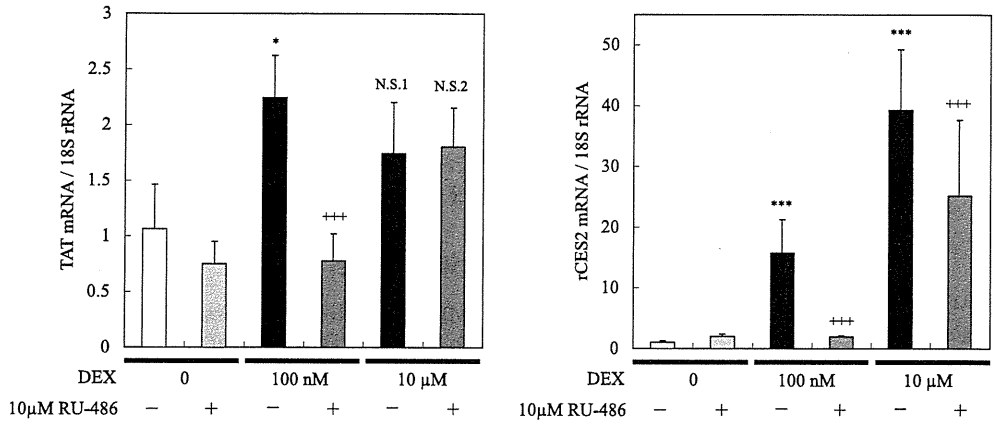

Figure 2. Effects of dexamethasone and RU-486 on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone or water in the presence of RU-486 or ethanol. The expression levels of TAT and rCES2 mRNA were analysed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Hepatocytes as a control (white bars) were treated with both water and ethanol. In analysis of TAT mRNA, the average C_t values (mean ± SD) of the control were 21.9±0.410 (TAT) and 11.5±0.189 (18S rRNA). In analysis of rCES2 mRNA, the average C_t values of the control were 23.9±0.433 (rCES2) and 11.7±0.247 (18S rRNA). DEX indicates dexamethasone. N.S.1 indicates not statistically significant (control versus DEX treatment). N.S.2 indicates not statistically significant (DEX treatment versus DEX+RU-486 treatment). Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P<0.05$ and $^{***}P<0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+++}P<0.001$.

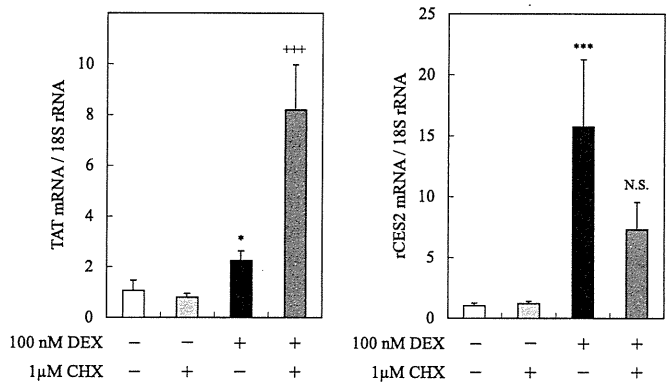

Figure 3. Effect of 1 μM cycloheximide on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone (100 nM) or water in the presence of cycloheximide (1 μM) or ethanol. The expression levels of TAT and rCES2 mRNA were analysed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Data analysis was performed in combination with data used to make Figure 2. DEX and CHX indicate dexamethasone and cycloheximide, respectively. N.S. indicates not statistically significant (DEX treatment versus DEX+CHX treatment) and we retain the null hypothesis because P value is 0.0748. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P<0.05$ and $^{***}P<0.001$. Statistically significant differences (DEX treatment versus DEX+CHX treatment); $^{+++}P<0.001$.

the control hepatocytes, rCES2 mRNA expression ratio (rCES2 mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.02±0.235 and 0.126±0.0491, respectively. Likewise, TAT mRNA expression ratio (TAT mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.06±0.400 and 0.946±0.328, respectively.

Alterations of TAT and rCES2 mRNA expression by dexamethasone, RU-486, and cycloheximide in primary rat hepatocytes

The effects of dexamethasone, RU-486, and cycloheximide on TAT and rCES2 mRNA expression were investigated. Dexamethasone (100 nM and 10 μM) dose-dependently caused an elevation of rCES2 mRNA in primary rat hepatocytes (Figure 2). The increased rCES2 mRNA by 100 nM dexamethasone was completely inhibited by treatment with 10 μM RU-486 (Figure 2). Treatment with 1 μM cycloheximide for 24 h resulted in repression, in part, of the increase in rCES2 mRNA by 100 nM dexamethasone (Figure 3), while the elevation of rCES2 mRNA was completely inhibited by treatment with 10 μg/mL (~36 μM) cycloheximide for 24 h (Figure 4). In the case of TAT mRNA, 100 nM dexamethasone increased the expression, and the effect of 10 μM dexamethasone was weaker than that of 100 nM dexamethasone (Figure 2). Note that the expression of 18S rRNA was decreased by only ~2- to 3-fold at 10

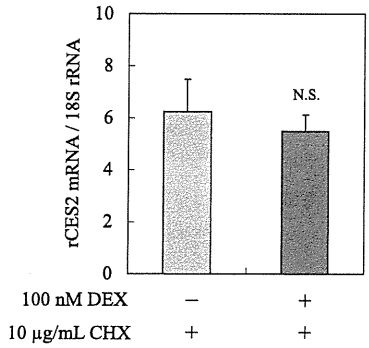

Figure 4. Effect of 10 μg/mL cycloheximide on the expression of rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone (100 nM) or water in the presence of cycloheximide (10 μg/mL) or ethanol. The expression levels of rCES2 mRNA were analysed by real-time PCR. The vertical axis indicates the ratio (rCES2 mRNA/18S rRNA) based on the same control as shown in Figures 2 and 3. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate. The average C_t values (mean ± SD) of the samples obtained from the hepatocytes treated with 10 μg/mL cycloheximide without dexamethasone were 22.7 ± 0.422 (rCES2) and 13.1 ± 0.227 (18S rRNA). N.S. indicates not statistically significant.

μg/mL cycloheximide and that the values of the ratio (rCES2 mRNA/18S rRNA) were seemingly increased by treatment with 10 μg/mL cycloheximide. Therefore, we separated data obtained from samples with 10 μg/mL cycloheximide from the other data on mRNA expression and performed analysis as described in Statistical Analysis and the legend to Figure 4.

Activation of *rCES2* promoters by dexamethasone and inhibition of the activation by RU-486

Nucleotide sequences necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were investigated using transient transfections of deletion plasmids consisting of a variety of lengths of the *rCES2* promoter and dual-luciferase assays. Dexamethasone treatment, however, tended to decrease values of *Renilla* luciferase activity (Figure 5A and C). The decreased levels appeared to vary among cell populations that have pGL3 plasmids harbouring different *rCES2* promoter regions, implying that phRL-TK plasmid is unsuitable for an internal control in the present study. Therefore, to determine the effect of dexamethasone on *rCES2* promoter activity, we analysed data on *Firefly* luciferase activity instead of data on relative luciferase activity (*Firefly*/*Renilla*). Basal transcriptional activities were almost the same among the plasmids including the region of −195/+51 (Figure 5A). The basal transcriptional activity of the plasmid including the region of −195/+51 was decreased by more than half by deletion of the sequence from −195 to −74 and was abolished by additional deletion of the sequence from −73 to −7. Dexamethasone treatment resulted in an ~2- to 3-fold increase in promoter activity in the region of −2957/+51 (Figure 5A and C). Ten micromolars of RU-486 repressed the elevation of the promoter activation by 100 nM dexamethasone in the region of −2957/+51 (Figure 5C). The *rCES2* promoter activity tended to be increased by dexamethasone associating with the sequences of −73/−7 and −991/−663 (Figure 5B). The dexamethasone-mediated promoter activation was abolished by RU-486 treatment in the region of −73/+51 as well as in the region of −2957/+51 (Figure 5C).

Discussion

MPHS is hydrolysed to methylprednisolone in rat hepatic microsomes (Hattori et al., 1981). MPHS hydrolase activity is increased following each injection of various glucocorticoids including dexamethasone and also methylprednisolone (Hattori et al., 1992a; Hattori et al., 1992b). We previously identified an MPHS hydrolase as rCES2 that is strongly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms by which the induction occurs have remained unknown. In the present study, we demonstrated that GR contributes to dexamethasone-mediated transcriptional activation of the *rCES2* gene.

First, we confirmed an increase in rCES2 mRNA at a lower concentration of dexamethasone than that employed in our previous study. Our previous study using reverse transcription-PCR showed that the expression of rCES2 mRNA was markedly increased when rats were injected with dexamethasone at a dose of 5 mg/kg body weight for 4 consecutive days (Furihata et al., 2005). Consistent with this observation, when rats were injected with dexamethasone at a single does of 1 mg/kg body weight in the present study, more than 200-fold increases in rCES2 mRNA were observed in the liver from 6 to 24 h after injection (Figure 1A). Hattori et al. (1992b) previously showed that when a suspension of dexamethasone in sesame oil was intraperitoneally administered to rats as a single dose of 60 μmol/kg (~23.5 mg/kg), MPHS hydrolase activity in rat microsomes rapidly increased and plateaued between 20 and 40 h after a 4-h lag period. Consistent with their results for hydrolase activity, the expression level of rCES2 mRNA increased with time until 12 h after injection of dexamethasone and the increase of rCES2 mRNA was followed by strong increases of rCES2 protein and MPHS hydrolase activity (Figure 1B and C). Hansen et al. (1999) reported that the mean of the maximum concentrations (C_{max}) in plasma was 682 ng/mL (~1.7 μM) after a single subcutaneous injection of 0.8 mg/kg dexamethasone into pregnant rats. Thus, our results indicate that treatment with ~2 μM of dexamethasone for 6 to 24 h is probably sufficient for a significant increase in rCES2 mRNA in the liver.

In rat primary hepatocytes, the level of rCES2 mRNA was increased by treatment with 100 nM dexamethasone (Figure 2). This result implies that the responsiveness of the primary hepatocytes used in the present study to dexamethasone was restored and that the elevation of rCES2 mRNA occurred in hepatic parenchymal cells. Dexamethasone-mediated increase in TAT mRNA,

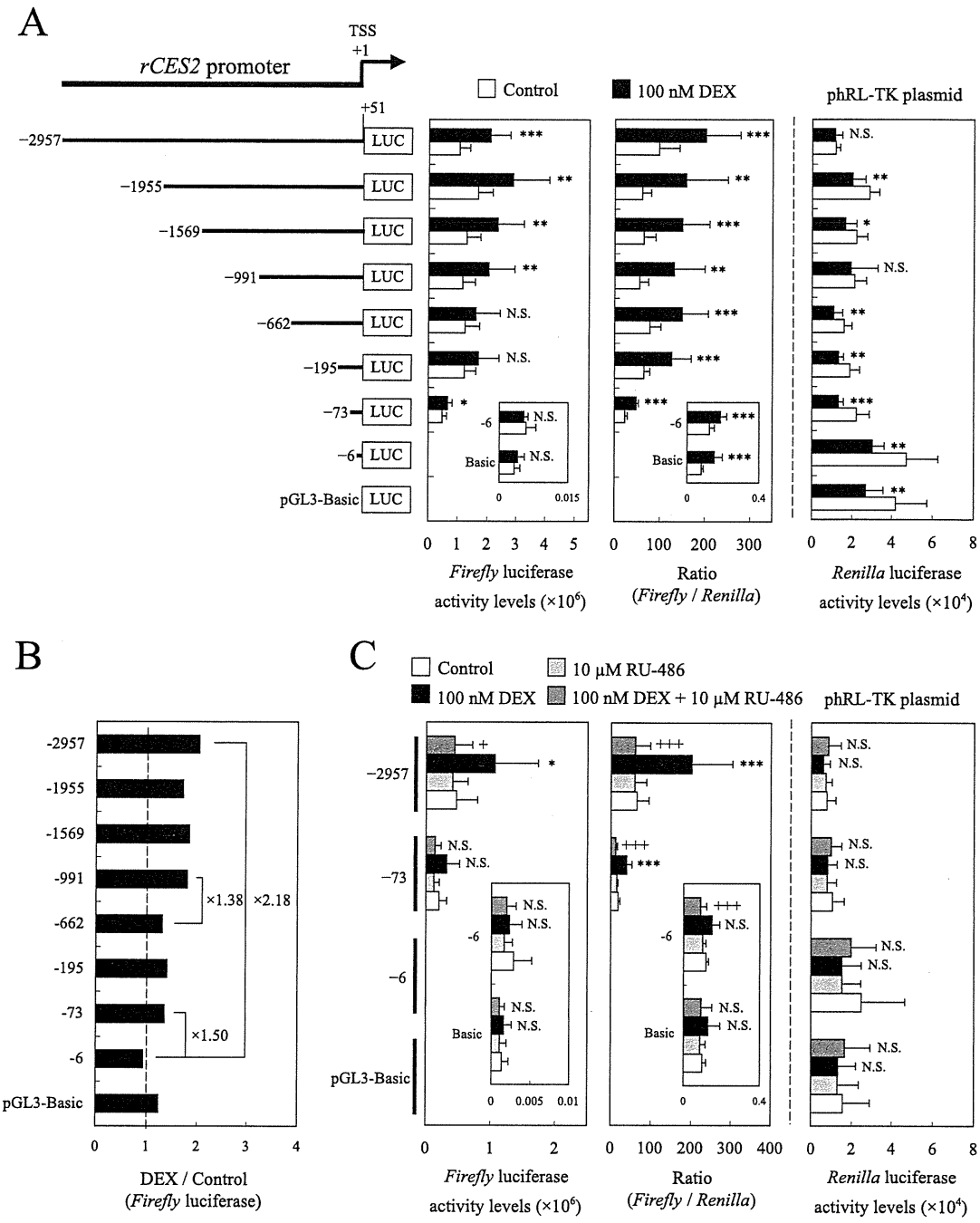

Figure 5. Analysis of the 5′-flanking region of the *rCES2* gene. (A) Deletion analysis of the 5′-flanking region of the *rCES2* gene. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid (*Firefly* luciferase) and 50 ng/well phRL-TK plasmid (*Renilla* luciferase) were treated for 24 h with dexamethasone (100 nM) or water, and dual-luciferase assays were performed. TSS indicates the transcription start site of the *rCES2* gene. Hepatocytes as a control were treated with water (white bars). DEX indicates dexamethasone. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. N.S. indicates not statistically significant. Four independent experiments were performed in triplicate. (B) Promoter regions necessary for response to dexamethasone. The ratios (DEX/control) were based on the results of *Firefly* luciferase activity levels shown in Figure 5A. (C) Effect of RU-486 on dexamethasone-mediated activation of *rCES2* promoters. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid and 50 ng/well phRL-TK plasmid were treated for 24 h with dexamethasone (100 nM) or water in the presence of RU-486 (10 μM) or ethanol, and dual-luciferase assays were performed. Three independent experiments were performed in triplicate. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P<0.05$ and $^{***}P<0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+}P<0.05$ and $^{+++}P<0.001$.

which serves as a positive control for GR-mediated up-regulation (Courtois et al., 1999), was also observed in primary hepatocytes. In addition, 10 μM RU-486, which is known to antagonise the effect of dexamethasone on TAT mRNA expression (Runge-Morris et al., 1996), completely repressed the increase in rCES2 mRNA by 100 nM dexamethasone, similar to TAT mRNA (Figure 2). Since 100 nM dexamethasone can activate GR (Runge-Morris et al., 1999), these results suggest that dexamethasone-mediated increase in rCES2 mRNA occurs through GR. Stress-induced glucocorticoid hormones would increase the expression level of rCES2 mRNA in the rat liver, since 10 μM dexamethasone was more effective than 100 nM dexamethasone.

Promoter regions necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were examined on the basis of data from luciferase assays. The results of luciferase assays showed that basal transcriptional activity was almost lost by truncation of the sequence of −73/−7, indicating that the region of −73/−7 is essential for binding of general transcription factors (Figure 5A). In the region of −2957/+51, dexamethasone treatment resulted in activation of the *rCES2* promoter to a level ~2- to 3-fold higher than that of the controls (Figure 5A and 5C), although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. RU-486 treatment was highly effective in repressing the promoter activation by dexamethasone in the region of −2957/+51 (Figure 5C). Taken together with the results of real-time PCR, these results strongly suggest that GR-mediated transcriptional activation participates in the dexamethasone-mediated increase in rCES2 mRNA in rat hepatocytes. The results of luciferase assays showed that responsivity to dexamethasone was altered in the regions of −73/−7 and−991/−663 (Figure 5B). The response in the region of −73/−7 appeared to be inhibited by RU-486, although the response to dexamethasone was not confirmed by statistical analysis in the experiment using both dexamethasone and RU-486 (Figure 5C). Therefore, nucleotide sequences necessary for dexamethasone-mediated activation of the *rCES2* promoter may be located in the regions of −73/−7 and −991/−663.

It appears that dexamethasone-mediated induction of rCES2 mRNA requires ongoing protein synthesis. When hepatocytes were exposed to 100 nM dexamethasone in the presence of 1 μM cycloheximide, an inhibitor of protein synthesis, the expression level of rCES2 mRNA was repressed by ~50% of that in cells exposed to 100 nM dexamethasone in the absence of cycloheximide, although the difference was not supported by statistical analysis (Figure 3). In contrast, the expression of TAT mRNA was markedly increased by cycloheximide in combination with dexamethasone, in accordance with results of previous studies using cortisol or hydrocortisone acetate (Hofer and Sekeris, 1978; Chesnokov et al., 1990). Although the augmentation of TAT mRNA is not attributed to the inhibition of protein synthesis *per se* (Ernest, 1982), it is conceivable that protein synthesis in rat hepatocytes was somewhat inhibited by 1 μM cycloheximide in the present study, considering that 1 μM cycloheximide treatment for 2 h decreased *de novo* protein synthesis by ~50% in primary rat hepatocytes (Sidhu and Omiecinski, 1998). At 10 μg/mL, cycloheximide completely inhibited dexamethasone-mediated induction of rCES2 mRNA (Figure 4). Cycloheximide at this concentration is known to inhibit *de novo* protein synthesis by more than 90% in human hepatocytes (Gerbal-Chaloin et al., 2002). Therefore these findings suggest that the striking induction of rCES2 mRNA by dexamethasone may require the presence of a protein (s) produced by *de novo* synthesis. A protein(s) necessary for dexamethasone-mediated induction of rCES2 mRNA may be induced in response to dexamethasone if the protein(s) is not short-lived.

In the present study, we demonstrated that dexamethasone enhances transcription of the *rCES2* gene and that GR contributes significantly to dexamethasone-mediated induction of rCES2 mRNA. We also found that another as-yet-unidentified factor(s) may play an essential role in the induction. Thus, we propose that therapeutic compounds that can activate GR induce rCES2.

Declaration of interest

This work was supported in part by grants from the Chiba Institute of Science and the Chiba University.

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