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Biochemical Pharmacology 69 (2005) 1287–1297
Biochemical Pharmacology
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Dexamethasone-induced methylprednisolone hemisuccinate hydrolase: Its identification as a member of the rat carboxylesterase 2 family and its unique existence in plasma

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Received 4 November 2004; accepted 25 January 2005

Abstract

Carboxylesterases (CESs) play important roles in the metabolism of many ester-drugs. In the present study, we identified and characterized dexamethasone-induced methylprednisolone hemisuccinate (MPHS) hydrolase in rat liver microsomes. Intraperitoneal injection of dexamethasone resulted in a significant increase in the level of MPHS hydrolase activity accompanied by induction of a specific CES isozyme. Since the biochemical characteristics of the induced CES isozyme were very similar to those of rat CES RL4, we hypothesized that these were the same enzymes. The results of nano-electrospray ionization tandem mass spectrometry analysis revealed that both dexamethasone-induced CES isozyme and CES RL4 possessed identical peptide fragments to those of AB010635, a rat CES2 isozyme, supporting our hypothesis. Furthermore, the results of reverse transcription-polymerase chain reaction showed that the amount of AB010635 mRNA in dexamethasone-treated liver was greater than that in control liver. To confirm that AB010635 encodes dexamethasone-induced CES isozyme, cDNA cloning was performed and the obtained cDNA was expressed in Sf9 cells by using a baculovirus-mediated expression system. The recombinant CES protein could hydrolyze MPHS and exhibited biochemical characteristics similar to those of CES RL4. Collectively, the results indicated that dexamethasone-induced MPHS hydrolase in liver microsomes is a rat CES2 isozyme. Interestingly, the results also showed that this rat CES2 isozyme exists in plasma and that the amount of this protein is increased by dexamethasone. These findings, together with the findings described above, provide important information for the study of phramacokinetics and pharmacodynamics of ester-drugs as well as for the study of CESs.

Keywords: Carboxylesterase; Dexamethasone; Methylprednisolone hemisuccinate; Induction; Baculovirus-mediated expression system; Microsomes

1. Introduction

Carboxylesterases (CESs, EC.3.1.1.1) are members of a α,β-hydrolase-fold family [1,2] and they show ubiquitous tissue expression profiles with the highest levels of CES activity present in liver microsomes in many mammals [1]. CESs are categorized as phase-I drug-metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs, such as angiotensin-converting enzyme inhibitors (temocapril, cilazapril, quinapril, and imidapril) [3,26], anti-tumor drugs (CPT-11 and capecitabin) [4,5] and narcotics (cocaine, heroin, and meperidine) [6,7]. In this regard, it is thought that CESs are one of the major determinants for pharmacokinetics and pharmacodynamics of ester-(pro)drugs. Actually, it has been shown that dog CES1 isozyme was involved in a pulmonary first-pass effect in the disposition of a propranolol ester prodrug [8]. It has also been shown that the expression level of human CES isozyme was correlated with the conversion ratio of CPT-11 to SN-38, the active metabolite, which is thought to be a key step for the chemotherapeutic action of this anti-tumor drug [9,10].

Several CES isozymes have been identified in mammals, and it has been proposed that they can be classified into

Abbreviations: CES, carboxylesterase; CES RH1, rat carboxylesterase RH1; CES RL1, rat carboxylesterase RL1; CES RL4, rat carboxylesterase RL4; Dex, dexamethasone; ESI/MS/MS, electrospray ionization tandem mass spectrometory; MPHS, methylprednisolone hemisuccinate; Ms, microsomes; PNPA, *p*-nitrophenylacetate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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doi:10.1016/j.bcp.2005.01.017

four families according to homologies of their amino acid sequences [1]. Each CES isozyme shows distinct tissue expression profiles and preferential substrate specificities [1,2]. In this regard, when analyzing the involvement of CESs in drug metabolism, each CES isozyme should be individually examined.

Much interest has been shown by both clinicians and researchers in the induction of expression of drug-metabolizing enzymes by chemicals since it is one of the main reasons for drug–drug interaction causing adverse effects and for the reduction in pharmacological potencies of drugs [11,12]. As for CESs, it has been shown that rodent CES isozyme(s) was induced by phenobarbital [13], aminopyrine [14], or peroxisome proliferators (clofibrate, di-(2-ethylhexyl)phthalate, and perfluorinated fatty acids) [15–17]. Zhu et al. [18] reported that dexamethasone caused a slight increase in human CES isozymes. Among the inducers, dexamethasone possesses a potent and interesting ability to affect CES expression in the rat liver. Hattori et al. [19,20] reported that methylprednisolone hemisuccinate (MPHS) was hydrolyzed to methylprednisolone via CES in rat liver microsomes and that several clinically used glucocorticoids, including dexamethasone, caused a remarkable increase in the level of this hydrolytic activity. In contrast to the report of induction of CES activity, some researchers have shown that the level of microsomal *p*-nitrophenylacetate hydrolase activity was significantly decreased in rat liver microsomes [18,21,22]. This apparent contradiction in the same animals is probably due to the different methods for determination of CES activity by using different CES substrates; thus, it is hypothesized that the CES isozyme contributing to *p*-nitrophenylacetate hydrolysis in rat liver microsomes is different from the one contributing to MPHS hydrolysis. However, rat CES isozymes responsible for MPHS hydrolysis in rat liver microsomes have not been identified.

The purpose of this study was to identify the dexamethasone-induced CES isozyme that can hydrolyze MPHS in the rat liver. The results revealed that the dexamethasone-induced CES isozyme belongs to the CES2 family (rCES2) and possesses different biochemical properties from those of known rat CES1 isozymes. We also identified the gene encoding rCES2 by cDNA cloning and functional expression in Sf9 cells. Furthermore, we found that rCES2 was present in plasma and that the amount of rCES2 protein in plasma was increased by dexamethasone accompanying an increase in the level of MPHS hydrolase activity.

2. Materials and methods

2.1. Animals, dexamethasone treatment and preparation of tissue samples

Adult male Sprague–Dawley rats (Japan SLC Inc., Shizuoka, Japan) of 7 weeks in age were used in this study. The rats were housed in a 18:00–06:00 h dark/06:00–18:00 h light cycle with free access to chow and water. One group of rats ($n = 3$) was given dexamethasone (Wako Pure Chemicals, Osaka, Japan) in corn oil by intraperitoneal injection at a dose of 5 mg/kg body weight for 4 consecutive days, and another group ($n = 3$) was given corn oil in the same way. The rats were sacrificed on day 5 after injection, and the livers were removed, weighed and perfused with 1.15% KCl. Microsomes were isolated by differential centrifugation as described previously [13] and were resuspended in SET buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris, pH 7.4). Blood was also collected from the aorta of each rat with a syringe under diethyl ether anesthesia, and plasma was obtained by centrifugation (1500 × *g* for 10 min) of the blood specimens. These samples were stored at −80 °C until used. Control (corn oil) and dexamethasone-treated rat liver microsomes are referred to here as Control Ms and Dex Ms, respectively, and, similarly, control (corn oil) and dexamethasone-treated rat plasma are referred to here as Control Plasma and Dex Plasma, respectively. Protein concentrations were determined by using a Bio-Rad Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Glutamate–oxaloacetate transaminase (GOT) and glutamate–pyruvate transaminase (GPT) activities were measured by using a GOT-UV TEST Wako and a GPT-UV TEST Wako (Wako Pure Chemicals), respectively. All subsequent procedures were performed at 0–4 °C.

2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and esterase activity staining after non-denaturing PAGE

SDS-PAGE was performed with 10% acrylamide gel. After the electrophoresis, either coomassie brilliant blue staining (Quick-CBB kit; Wako Pure Chemicals) or Western blotting (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) was performed. In both cases, the procedure was performed according to the manufacturer's directions. Specific antibodies used in Western blotting were anti-CES RH1 antibodies, anti-CES RL1 antibodies and anti-CES RL4 antibodies, which were previously prepared [13,23]. Anti-β-actin antibodies and secondary antibodies for them were purchased from Sigma (St. Louis, MO, U.S.A.).

Esterase activity staining after non-denaturing PAGE was performed as previously described [17]. α-Naphtylacetate was obtained from Tokyo Kasei (Tokyo, Japan).

In these experiments, purified rat CES isozymes were used as positive controls [13,23].

2.3. Determination of hydrolase activity

MPHS and methylprednisolone (MP) were purchased from Sigma. MPHS hydrolase activity was determined essentially according to the method of Hattori et al. [19]. MPHS was dissolved in 1% dimethylformamide

water. MPHS hydrolase activity was assayed in 50 mM citrate–phosphate buffer (pH 5.5), and MP, a metabolite of MPHS, was analyzed by using the following HPLC method. The HPLC system consisted of a model L7100 pump (Hitachi, Tokyo, Japan), a model L7400 UV detector (Hitachi), a model L7200 autosampler (Hitachi), a model D7500 integrator (Hitachi), and a COSMOSIL 5C18-MS-II 4.6 mm × 250 mm column (Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 0.05 M phosphate buffer (pH 7.0)–acetonitrile (65:35, v/v) and was delivered at a flow rate of 0.9 ml/min. MP was detected at a wavelength of 254 nm. A calibration curve was generated from 0.25 to 75 μM by processing an authentic standard substance through the entire procedure. Fluoxymesterone (Sigma) was used as an internal standard. The substrate concentration of MPHS for kinetic study ranged from 5 to 400 μM.

p-Nitrophenylacetate (PNPA) was from Nacalai Tesque. Hydrolysis of PNPA was determined colorimetrically in 50 mM Tris–HCl buffer (pH 8.0) at 30 °C by measuring the amount of *p*-nitrophenol released as previously described [17]. The substrate concentration of PNPA for determination of hydrolytic activity was 500 μM.

Specific activities of CES towards two substrates used were expressed in terms of the amount of substrate hydrolyzed in 1 min under specified conditions. Enzyme kinetic parameters were estimated using a computer program (DeltaGraph ver 4.5, SPSS Inc., Chicago, IL) designed for nonlinear regression analysis as described previously [17]. Each mean kinetic value was the average of three individual experiments with ±S.D. Student's *t*-test was performed to determine significance of difference between two groups. *P* values less than 0.05 were taken to be significant.

2.4. Inhibition assay by using specific IgG

The IgG fractions of rabbit anti-serum or control serum were purified from whole antibodies by using a HiTrap Protein A HP (Amersham Bioscience, Piscataway, NJ, U.S.A.) and a PD-10 column (Amersham Bioscience) according to manufacturer's protocols.

Inhibition of hydrolase activity by using specific IgG was performed according to the procedure described previously [17] with slight modification. Control Ms ($n = 3$) and Dex Ms ($n = 3$) were pooled and solubilized with 0.5% cholic acid in 10 mM Tris–HCl buffer (pH 8.0). The mixture was centrifuged at 10,000 × *g* for 30 min, and the supernatant was removed and used for inhibition assays. Solubilized Control Ms and Dex Ms were incubated with control or anti-CES RL4 IgG (0.1 mg) for 30 min at 37 °C, and the mixtures were left for 24 h at 4 °C. Then nProtein A Sepharose 4 Fast Flow (Amersham Bioscience) was added to the mixture. After incubation on ice for 1 h, the mixture was centrifuged for 10 min at 20,000 × *g*. The supernatant was removed and used for a determination of MPHS hydrolase activity as described above. Inhibition assay was also performed for Control Plasma and Dex Plasma in the same way except for the solubilization by cholic acid.

2.5. Immunoprecipitation and determination of amino acid sequence of dexamethasone-induced carboxylesterase by nano-electrospray ionization tandem mass spectrometory (nano-ESI/MS/MS) analysis

Solubilized Dex Ms and Dex Plasma were incubated with anti-CES RL4 IgG for 12 h at 4 °C and centrifuged at 18,000 × *g* for 30 min. After washing with 10 mM Tris–HCl buffer, the precipitates were dissolved in solubilization buffer containing SDS. The samples were incubated in a water bath for 60 min at 60 °C and subjected to SDS-PAGE. Then, the separated proteins were visualized by coomassie brilliant blue as described above. The single band separated was taken out from the gel. Peptide sequence of the protein was determined by nano-ESI/MS/MS analysis (consigned to Exigen; Tokyo, Japan).

2.6. Determination of amino acid sequence of purified CES RL4

Purified CES RL4 (100 μg) was digested with endoproteinase Glu-C (V8 protease) (Sigma) in 100 mM Tris–HCl buffer (pH 8.0) containing 0.1% SDS. The mixture was incubated for 3 h at 37 °C and then subjected to reverse-phase HPLC described above. The column used was a TSK octadecyl-4 PW (TOSOH, Tokyo, Japan). An eluted peptide was sequenced by automated Edman degradation using a model 470A gas-phase sequencer (Applied Biosystems, Foster City, CA) with an on-line Spectra-Physics Model SP8100 PTH amino acid analyzer (Applied Biosystems) as described previously [24].

2.7. Total RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from each liver sample (control liver, $n = 3$; dexamethasone-treated liver, $n = 3$) using TRIzol reagent (Invitrogen, Calsbad, CA). To prevent contamination with genomic DNA, the extracts were treated with DNase I (Takara Shuzo, Shiga, Japan). Subsequently, first-strand cDNA was synthesized from 2 μg of each RNA by Ready-To-Go RT-PCR Beads with an oligo (dT) primer (Amersham Bioscience). For certification of synthesis of equal amounts of cDNA, PCR was performed (94 °C for 15 s, 49 °C for 20 s, 68 °C for 40 s and 35 cycles) using a set of primers for detection of *rat glyceraldehyde-phosphate dehydrogenase* (GAPDH) gene expression: sense, 5′-TGCACCACCAACTGCTTA-3′; anti-sense, 5′-GGATGCAGGGATGATGTTC-3′. Another set of primers (sense, 5′-CAAGAGTGGAGCAGA-GATTC-3′; anti-sense, 5′-GTACTTCATCATCCGCCT-AC-3′) was used for PCR (94 °C for 15 s, 55 °C for

30 s, 68 °C for 45 s and 36 cycles) to detect AB010635 gene expression. The PCR product was purified by using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). The nucleotide sequences of the products were determined by using a Dye Terminator Cycle Sequencing-Quick Start Kit and CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, CA) and were confirmed by sequencing at least twice in each direction.

2.8. cDNA cloning of AB010635

PCR amplification was carried out to determine the coding region of AB010635 cDNA using rat liver cDNA described above as a template. The following primers were used: sense, 5′-TAGCCCGACGAACTGAGAACTG-3′; anti-sense, 5′-CCACACTCTGTTCCTTTCACCA-3′. The amplified DNA fragments were subcloned into the pCR-Blunt II TOPO vector (Invitrogen). The nucleotide sequences were determined as described above and were confirmed by sequencing at least twice in each direction.

2.9. Expression of AB191005/AB010635 in Sf9 cells

Expression of AB191005/AB010635 by using a BAC-TO-BAC Baculovirus Expression System (Invitrogen) was carried out in accordance with the directions of the manufacturer as described previously [17]. The cDNA in the cloning vector was sub-cloned into the pFAST-BAC1 vector using *Eco*RI and alkaline phosphatase treatment. The pFAST-BAC1 vector containing rat CES cDNA was transformed into DH10Bac cells, and this was followed by transposition of the inserts into bacmid DNA. Non-recombinant bacmid DNA (mock) was also prepared by the same procedures. The recombinant and mock bacmid DNAs were separately transfected into Sf9 cells with CELL FECTIN Reagent (Invitrogen), and the virus was harvested 72 h later. The cells were centrifuged at 1700 × *g* for 10 min to separate cells and virus. The supernatant containing the virus was stored at 4 °C in the dark with 5% fetal bovine serum. Cells were routinely harvested 96 h after infection, washed twice with phosphate-buffered saline, and stored −80 °C until used. Lysates were prepared by disrupting the cells with a sonicator until the cells had been totally lysed as determined by microscopy. Cytosolic fractions of Sf9 cells transfected with mock baculovirus and those expressing AB191005/AB010635 were prepared by subjecting the cell lysate to centrifugation (105,000 × *g* for 60 min at 4 °C) and were used for the assays.

3. Results

3.1. Effects of dexamethasone on carboxylesterase activity in rat liver microsomes

Intraperitoneal injection of dexamethasone for 4 consecutive days resulted in a marked increase in the level of MPHS hydrolase activity (24.5 fold increase, $P < 0.005$), while the level of hydrolase activity towards PNPA was decreased ($P < 0.005$) (Fig. 1). These changes are in good agreement with previously reported results [19,21]. In respect to physiological effects of dexamethasone, rats given dexamethasone exhibited hepatomegaly accompanying increases in GOT and GPT activity levels, although these increases were not statistically significant and were not correlated with the level of increase in MPHS hydrolase activity in microsomes (data not shown).

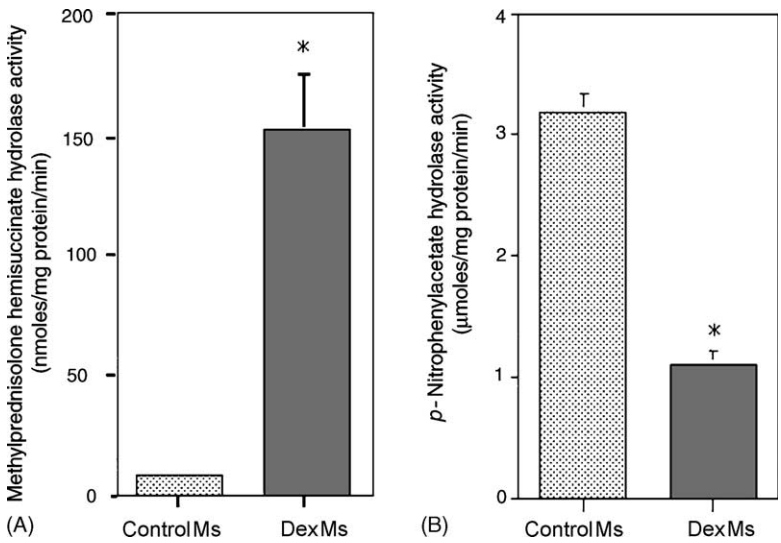

Fig. 1. Effects of dexamethasone on microsomal CES activities in rat liver. (A) MPHS hydrolase activity and (B) PNPA hydrolase activity. The sample number in each group was three. Each value is the mean of three independent assays (±S.D.). Statistically significant difference compared with Control Ms ($^{*}P < 0.005$).

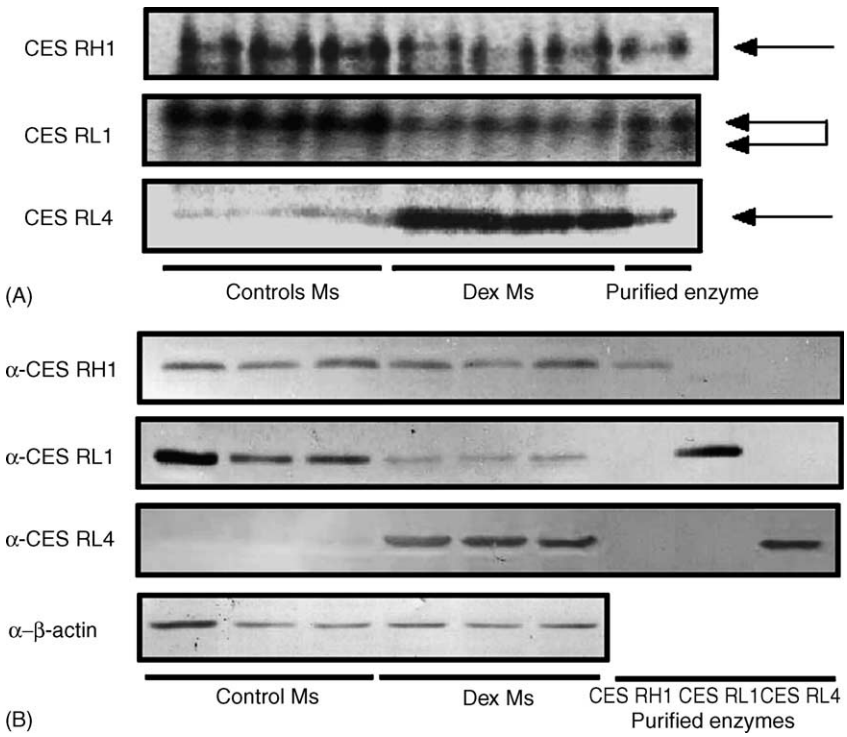

Fig. 2. Identification of dexamethasone-induced CES isozyme in rat liver microsomes. The sample loaded in each lane is indicated below the panel in (A) and (B). (A) Esterase activity staining after non-denaturing PAGE. The amount of microsomal proteins loaded in each lane was 5 μg. The name of the CES isozyme detected in each panel is shown on the left side of the panel, and an arrow(s) indicates the corresponding activity band(s). Purified CES isozymes (CES RH1, CES RL1, and CES RL4, 100 ng each) were used as positive controls. (B) Western blotting. The amount of microsomal proteins loaded in each panel was 2.5 μg. Anti-CES RH1 antibodies, anti-CES RL1 antibodies, and anti-CES RL4 antibodies were used to detect the expression of the corresponding CES isozyme as indicated on the left side of each panel. Anti-β-actin antibodies were used to detect β-actin protein for verification that a similar amount of protein was loaded in each lane. Purified CES isozymes (CES RH1, CES RL1, and CES RL4, 100 ng each) were used as positive controls.

3.2. Identification of carboxylesterase induced by dexamethasone in rat liver microsomes

To identify the dexamethasone-induced CES isozyme, esterase activity staining after non-denaturing PAGE was performed. As shown in Fig. 2A, induced esterase activity was detected at the same migrated position as that of purified CES RL4. The protein induced by dexamethasone was also detected by Western blotting using CES RL4 specific antibodies at the same migrated position as that of purified CES RL4 (Fig. 2B). These results indicated that dexamethasone induced a CES isozyme that was closely related to CES RL4 in terms of molecular weight of a subunit, isoelectronic point and immunocrossreactivity. It was also found that the level of expression of the dexamethasone-induced CES isozyme was very low in normal conditions. As for other CES isozymes, the results showed that expression of both CES RH1 (also named ES-10, pI6.1 or hydrolase A) and CES RL1 (also named ES-4 or hydrolase B) were repressed, in consistent with previous results. The activity band of CES RL1 appeared as a doublet in Fig. 2A. Sanghani et al. [25] obtained the same results and concluded that both bands were originated from ES-4 by liquid chromatography ESI/MS analysis.

The results described above implied that the dexamethasone-induced CES isozyme was identical to CES RL4. To determine this possibility, amino acid sequences of both proteins were analyzed. A peptide fragment containing 25 amino acid residues of CES RL4 (including two undefined residues, N-QHAPXYFKNVRPXHVKADHADEVPF-C) was identified, and several peptide fragments derived from the dexamethasone-induced CES isozyme were identified. A database search revealed that the amino acid sequence of these peptide fragments obtained from CES RL4 and dexamethasone-induced CES isozyme were identical to that of AB010635 (Fig. 5A). These findings indicated that CES RL4 and dexamethasone-induced CES isozyme are probably the same protein. The dexamethasone-induced CES isozyme is, therefore, hereafter named CES RL4.

3.3. Inhibition of MPHS hydrolase activity in rat liver microsomes by anti-CES RL4 IgG

Inhibition assays were performed using anti-CES RL4 IgG to determine whether CES RL4 is responsible for the increase in the level of MPHS hydrolase activity in rat liver microsomes induced by dexamethasone. As shown in Fig. 3, anti-CES RL4 IgG significantly inhibited MPHS hydrolase activity in both Control and Dex Ms (decreases to 53.8% and 21.8% of that of control IgG, respectively). Collectively, the results were in good agreement with those obtained by Western blotting and esterase activity staining. The findings

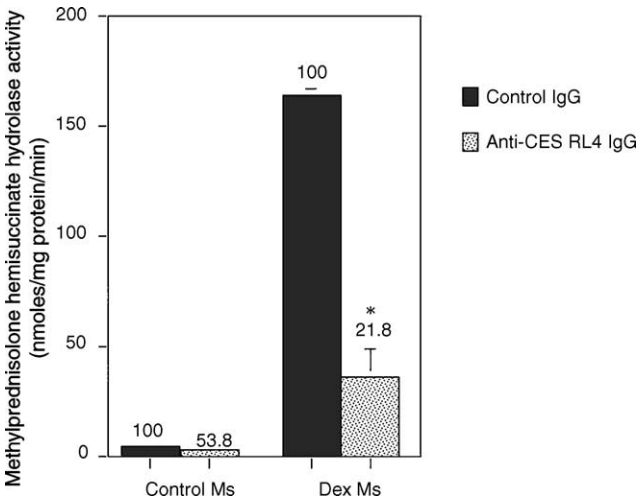

Fig. 3. Effects of anti-CES RL4 IgG on MPHS hydrolase activity in rat microsomes. Control IgG (0.1 mg) and anti-CES RL4 IgG (0.1 mg) were used in the assays for which results are shown by a solid bar and a dotted bar, respectively. The sample number of Control Ms or Dex MS was three. Each value is the mean of three independent assays (±S.D.). The number above each bar is relative percentage of activity when that of control IgG in each group (Control Ms or Dex Ms) is set to 100%. Statistically significant difference compared with the results of control IgG in each group ($^{*}P < 0.005$).

showed that the increase of the level of MPHS hydrolase activity in Dex Ms resulted from the induction of CES RL4. We also determined the contribution of CES RL4 to hydrolysis of PNPA in Dex Ms. In contrast to the finding of strong inhibition of MPHS hydrolase activity, the effect of anti-CES RL4 IgG on microsomal PNPA hydrolase activity was small (decrease to 82.7% of that of control IgG).

3.4. Induction of AB010635 mRNA expression by dexamethasone in rat liver

The results of protein analysis described above presented the possibility that expression of AB010635 mRNA was induced in dexamethasone-induced rat liver. RT-PCR is not a completely quantitative method for evaluation of mRNA expression; nevertheless, the results of RT-PCR using specific primers for AB010635 showed that the amount of AB010635 mRNA was obviously greater in dexamethasone-treated liver than in control liver (Fig. 4B). Both dexamethasone-treated liver cDNA and control liver cDNA are free from genomic DNA contamination (Fig. 4A) and are synthesized in equal amounts (Fig. 4C). The nucleotide sequences of PCR products were confirmed as fragments of AB010635 cDNA by direct DNA sequence. In this confirmation, one nucleotide of the products was found to be mismatched to that of AB010635 (at position +1093).

3.5. cDNA cloning of AB191005/AB010635 and its expression in Sf9 cells

The protein of which partial amino acid sequence is identical to that of AB010635 has been detected in rat liver

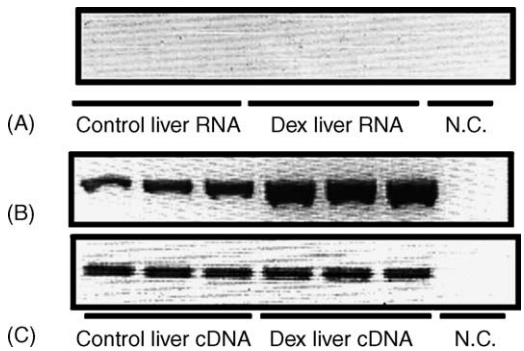

Fig. 4. Induction of AB010635 mRNA expression by dexamethasone in rat liver. RT-PCR analysis was performed by using total liver RNA from each rat in panel A and liver cDNA from each rat in panel B. The name of the sample used in each lane is indicated below the panel. N.C. indicates non-template control. (A) Detection of genomic DNA of the *GAPDH* gene and (B) detection of AB010635 cDNA, and (C) detection of GAPDH cDNA.

microsomes [25], but it has not been characterized. There has been no report of AB010635 cDNA being expressed in eukaryotes. Thus, to confirm that AB010635 encodes CES RL4, cDNA cloning was performed. The nucleotide sequence of the obtained cDNA clone showed perfect homology to AB010635 except for two nucleotides (C1093T and C1548T). The former one was also found in the results of RT-PCR analysis as described above. These substitutions were also found in the data of the Rat Genome Project (http://www.sanger.ac.uk/). We submitted the nucleotide sequence of our cDNA clone to GenBank, EMBL and DDBJ (accession no. AB191005). These nucleotide changes are not accompanied by amino acid alteration.

The deduced amino acid sequence of AB191005/AB010635 is shown in Fig. 5A with comparison to human CES2 isozyme (hCE-2, [7]) and mouse CES2 isozyme (mCES2, [17]). The sequence contained several conserved amino acid residues among CES isozymes and an endoplasmic reticulum (ER) retention signal at the carboxyl-terminal, but no glycosylation motif was found. (Details are given in the legend of Fig. 5A.) The sequence of AB191005/AB010635 showed high similarity to those of other CES2 isozymes, especially to that of mCES2, suggesting that AB191005/AB010635 belongs to the CES2 family (Fig. 5B). Thus, AB191005/AB010635 is hereafter called rCES2.

When the recombinant bacmid DNA was transfected into Sf9 cells, the cells expressed the recombinant protein as determined by Western blotting and esterase activity staining after non-denaturing PAGE (Fig. 6A and B). The recombinant rCES2 exhibited the same biochemical properties as those of purified CES RL4 in terms of molecular weight of a subunit, p*I*, and immunocrossreactivity. The recombinant proteins were recovered from the cytosolic fraction as in the case with other CES isozymes expressed in Sf9 cells [17,26]. The hydrolase activity level of recombinant protein towards PNPA was very low (1.6 fold

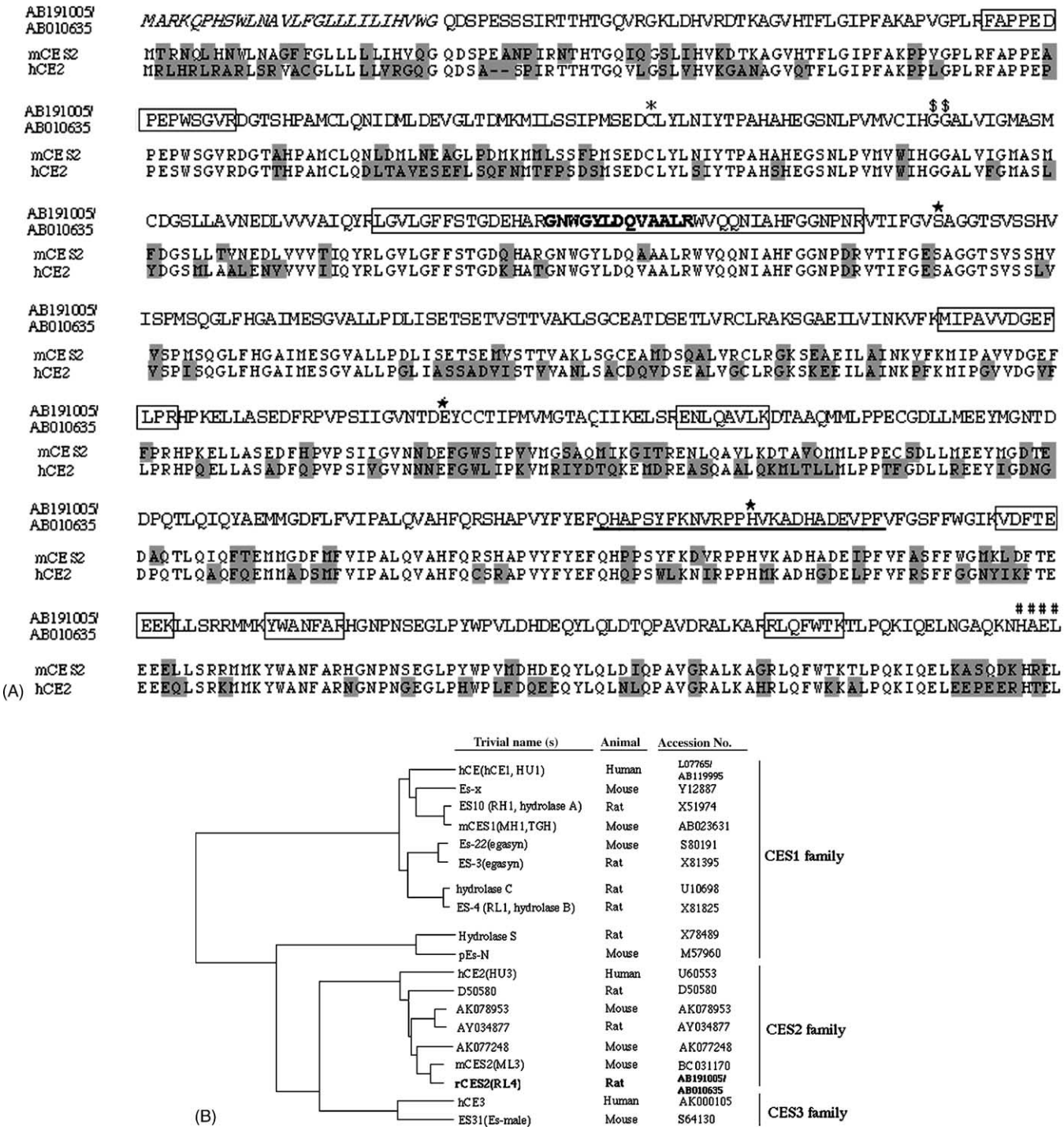

Fig. 5. Classification of AB191005/AB010635 as a member of the CES2 family. (A) Comparison of the deduced amino acid sequence of AB191005/AB010635 with those of human and mouse CES2 isozymes. Shadows indicate differences in amino acid residues. Italic letters indicate the signal peptide (1–26) predicted by computer analysis (Signal IP 3.0 Server; http://www.cbs.dtu.dk/services/SignalP/). The symbol (*) indicates conserved Cys (125) residue, and stars indicate three amino acids composing a catalytic triad, Ser (230), Glu (347), and His (459); symbol ($) indicates two Gly residues (150 and 151) involved in an oxyanion hole loop, and four residues at the carboxyl-terminal (His-Ala-Glu-Leu, 557–561), indicated by hash symbol (#), are thought to function as an endoplasmic reticulum retention signal. No putative glycosylation site was found. Numbers in parentheses indicate the relative position of the corresponding amino acid residue with respect to the first Met residue. An underline indicates the peptide identified by amino acid sequence analysis of purified CES RL4. Boxes indicate the peptides identified by amino acid sequence analysis of dexamethasone-induced CES isozyme in liver microsomes, and bold letters indicate the peptide of the dexamethasone-induced CES isozyme in plasma. (B) Phylogenic tree of human and rodents CES isozymes. Trivial names, animals, and accession numbers are shown from left to right.

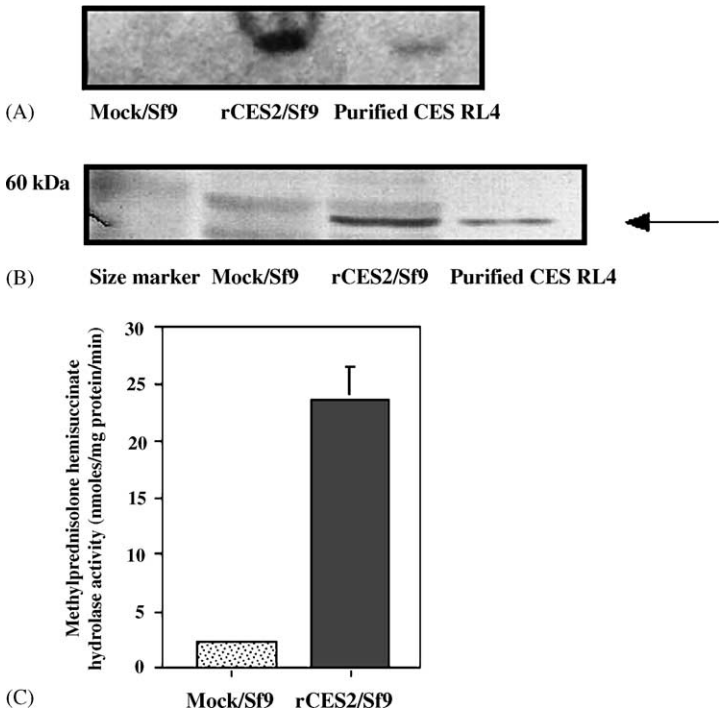

Fig. 6. Characterization of recombinant rat CES expressed in Sf9 cells.Mock/Sf9, cytosolic fraction of Sf9 cells transfected with empty baculovirus. rCES2/Sf9, cytosolic fraction of Sf9 cells expressing rCES2. (A) Esterase activity staining. Cytosolic proteins of Sf9 cells (10 μg) were loaded in each lane. Purified CES RL4 (100 ng) was used for comparison. (B) Western blotting using anti-CES RL4 antibodies. Cytosolic proteins of Sf9 cells (15 μg) were loaded in each lane. Purified CES RL4 (100 ng) was used for comparison. An arrow indicates the band recognized by anti-CES RL4 antibodies. (C) Determination of MPHS hydrolase activity. Each value is the mean of three independent assays (±S.D.).

compared to that of mock/Sf9, results not shown) according to the results of inhibition assays. The recombinant rCES2 possessed a high level of MPHS hydrolase activity (Fig. 6C), and the kinetic parameters (K_m and V_{max}) of the activity were 76.6 ± 0.02 μM and 28.8 ± 3.0 nmol/mg protein/min, respectively, these values being comparable to those of purified CES RL4 ($K_m = 120.0 \pm 2.0$ μM and $V_{max} = 4.3 \pm 0.4$ μmol/mg protein/min).

Together with other results, these data show that rCES2 encodes CES RL4. CES RL4 should be called rCES2 in accordance with the nomenclature system suggested by Satoh and Hosokawa [1], although the system is currently under establishment.

3.6. Detection of rCES2 in rat plasma

Hattori et al. [19] reported that treatment of rats with dexamethasone caused induction of MPHS hydrolase activity not only in liver microsomes but also in plasma, although the nature of the induced protein was not determined. In our experiments, accordingly, the level of MPHS hydrolase activity in Dex Plasma was increased to 15.5 fold ($P < 0.005$) compared with that in Control Plasma (Fig. 7A). Esterase activity staining after non-denaturing PAGE and Western blotting showed that, as in the case of microsomes, the induced protein migrated to the same position as rCES2 (Fig. 7B and C). On the other hand, other CES members, CES RH1 and CES RL1, did not exist in either Control Plasma or Dex Plasma. The activity bands (indicated by an asterisk in Fig. 7B) observed in plasma samples were attributed to plasma CES isozyme (hydrolase S), since it has been reported that mRNA expression of this plasma CES isozyme was suppressed by dexamethasone [18]. When an inhibition assay using anti-CES RL4 IgG was performed, the MPHS hydrolase activities in Control Plasma and Dex Plasma were strongly inhibited (decrease to 10% of control IgG, $P < 0.005$, and to 17.5% of control IgG, $P < 0.005$, respectively) (Fig. 7A). To identify the CES isozyme that exists in plasma, immunoprecipitation and amino acid sequence analysis was performed by using Dex Plasma as describe above. The amino acid sequence of a peptide fragment originating from dexamethasone-induced plasma CES isozyme was identical to that of rCES2 (Fig. 5).

4. Discussion

Identification of the enzyme that metabolizes a drug is important to understand the biological fate of the drug after administration, and induction of expression of the enzyme strongly influences the pharmacokinetics and pharmacodynamics of the drug. From these points of view, this study, in which we identified dexamethasone-induced MPHS hydrolase as a member of the CES2 family, provides three major topics for discussion: (1) biochemical properties of

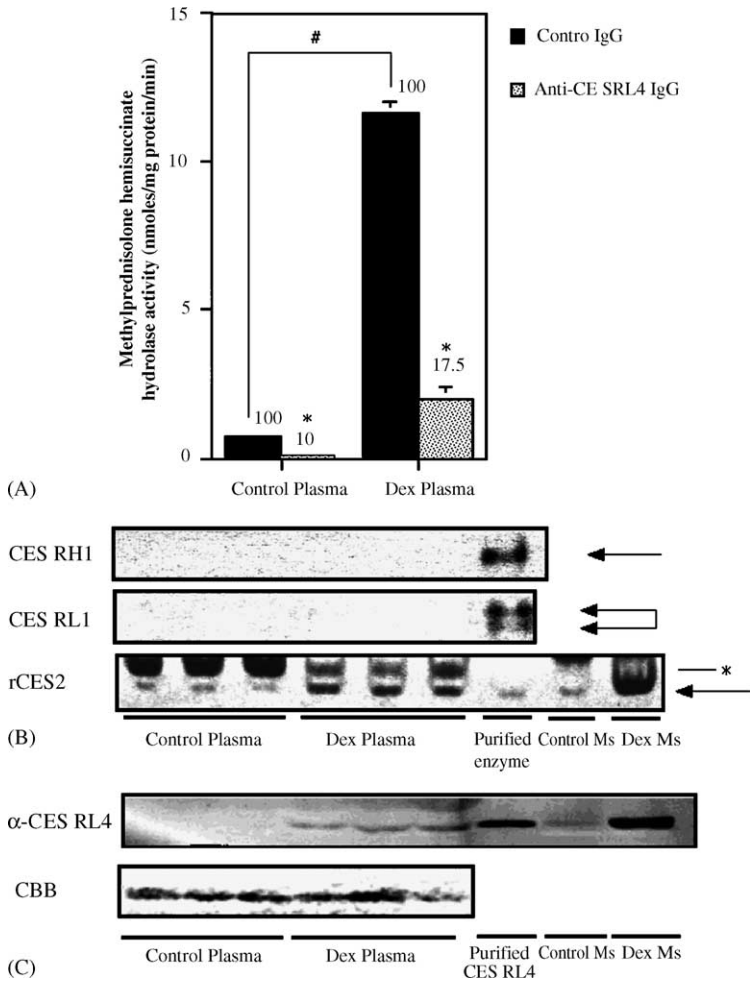

Fig. 7. Detection of rCES2 protein in rat plasma and increase in its amount by dexamethasone. (A) Determination of MPHS hydrolase activity and its inhibition by anti-CES RL4 IgG. Control IgG (0.1 mg) and anti-CES RL4 IgG (0.1 mg) were used in the assays for which results are shown by a solid bar and a dotted bar, respectively. The sample number of Control Plasma or Dex Plasma was three. Each value is the mean of three independent assays (±S.D.). The number above each bar is relative percentage of activity when that of control IgG in each group (Control Plasma or Dex Plasma) is set to 100%. Statistically significant difference compared with the results of control IgG in each group ($^{*}P < 0.005$). Statistically significant difference compared between the results of Control Plasma and Dex Plasma ($^{\#}P < 0.005$). (B) Esterase activity staining after non-denaturing PAGE. The sample loaded in each lane is indicated below each panel. Plasma proteins (10 μg) were used, and purified CES isozymes (CES RH1, CES RL1, and CES RL4, 100 ng each) were used as positive controls. Pooled Control Ms and Dex Ms (3.0 μg) were also used as references. (C) Western blotting. Plasma proteins (20 μg) were loaded in each panel. Anti-CES RL4 antibodies were used to detect rCES2 protein as indicated on the left side of the upper panel. An approximately 80-kDa plasma protein was visualized by coomassie brilliant blue staining for verification that a similar amount of protein was loaded in each lane. Purified rCES2 (100 ng) was used as a positive controls. Pooled Control Ms and Dex Ms (3.0 μg) were also used as references.

rCES2 as a drug-metabolizing enzyme, (2) rCES2 existence in plasma, and (3) the mechanism by which rCES2 is induced.

MPHS is a widely used drug for the treatment of systemic lupus erythematosus and in renal transplantation and medical emergencies, and the hydrolysis of MPHS is critical for this drug to exert its pharmacological actions. To the best of our knowledge, rCES2 is the first enzyme catalyzing this hydrolysis to be identified. Hattori et al. [19] reported that MPHS hydrolase activity was induced by several glucocorticoids. Such induction of rCES2 may significantly affect pharmacokinetic parameters of its substrate, such as MPHS or CPT-11 (CPT-11 is a substrate for rCES2, unpublished observation, A. Fujii, M. Hosokawa, T. Furihata, and K. Chiba). There are currently no experimental data that support this idea, but it is important to test the idea since it has been reported that MPHS pulse treatment resulted in a decrease in the area under the curve and an increase in clearance of cyclosporin through induction of hepatic cytochrome P450 (CYP) 3A activity in the rat [27].

A comparison of the biochemical properties of rCES2 and those of rat CES1 isozymes shows that they are strikingly different. Regarding the preferential substrates, CES RH1 and CES RL1 do not seem to be involved in the hydrolysis of MPHS in the rat liver. On the other hand, the contribution of rCES2 to PNPA hydrolysis is very low. In addition, temocapril is a substrate for CES RH1 but not for

rCES2 (unpublished observation, T. Furihata, M. Hosokawa, A. Fujii, and K. Chiba). This difference is probably due to the different structure of rCES2 from those of CES1 isozymes, as the results of comparison of their deduced amino acid sequences, immuno-crossreactivity, and esterase activity staining after non-denaturing PAGE indicate. The results obtained in this study, together with the findings discussed below, provide important information for study of pharmacokinetics of ester-(pro)drugs that can be substrates for rCES2, including MPHS, as well as for study of prodrug design.

The next issue to discuss is how rCES2 exists in plasma. Needless to say for the case of dexamethasone treatment, the results of both esterase activity staining after non-denaturing PAGE and inhibition assays indicate that the active form of rCES2 is present in plasma even under normal conditions, although rCES2 protein was hardly detected by Western blotting probably due to the very small amount of rCES2. What makes rCES2 exist in plasma? As shown in Fig. 5, the deduced amino acid sequence of rCES2 has a HXEL motif in the C-terminal region, which is a variant of the consensus eukaryotic ER retention signal of KDEL [28]. Therefore, rCES2 is firstly thought to exist in the lumen of the ER as other CESs do. Together with the fact that the levels of plasma GOT and GPT activities were increased by dexamethasone treatment, one possible explanation of how rCES2 can exist in plasma is that hepatocyte necrosis, exacerbated by dexamethasone, ultimately results in the leakage of rCES2 protein. However, this is unlikely since other CES isozymes did not leak into Dex plasma even if they were still present in the dexamethasone-treated liver and the degree of hepatotoxity caused by dexamethasone in each rat was not correlated with the level of the corresponding plasma rCES2 activity. Another possible explanation of the existence of rCES2 in plasma is its active release from hepatocytes. Although we did not perform any experiments to determine this possibility, it has been shown that the release of rCES2 into plasma was significantly inhibited by monensin, which could inhibit the release of secretory proteins by disrupting the Golgi apparatus [19,29]. If this hypothesis is true, at least in part, the active release of rCES2 would be of biochemical interest as well as clinical interest. Since the possibility of passive release of rCES2 resulting from hepatocyte necrosis cannot be ruled out, further studies are needed to determine how rCES2 can exist in plasma.

Finally, we focus on the question of how rCES expression is induced by dexamethasone. Given the fact that the amount of rCES2 mRNA in the liver was apparently increased when dexamethasone was administered, there are likely to be two mechanisms for rCES2 induction. One is enhanced intracellular stability of rCES2 mRNA since it has been demonstrated that dexamethasone can stabilize mRNA of the several genes [30,31], and the other is the enhanced transcription of the *rCES2* gene triggered by specific transcription factors. For the latter case, the involvement of glucocorticoid receptor (GR) in rCES2 induction was likely to occur. We cannot determine which is the major mechanism accounting for rCES2 induction at present. However, several experiments to examine this are in progress in our laboratory, and we expect that results of those experiments will shed light on the question.

In conclusion, we identified a dexamethasone-induced CES isozyme that can hydrolyze MPHS in the rat liver and plasma as a member of the CES2 family, rCES2. The different biochemical properties of rCES2 from those of known rat CES1 isozymes, including its unique existence in plasma, will be useful information for studies aimed at elucidation of functions of CESs in drug metabolism. In addition, we also identified the gene encoding rCES2 by cDNA cloning and functional expression in Sf9 cells. Since we demonstrated that the level of the corresponding mRNA expression was markedly increased, the identification of the coding gene is valuable for studies aimed at elucidation of the molecular mechanisms by which dexamethasone induces rCES2 expression.

Acknowledgment

This work was supported in part by a grant from the Ministry of Education, Sciences, Sports and Culture of Japan (grant numbers 14572090 and 16590081).

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