Involvement of glucocorticoid receptor and pregnane X receptor in the regulation of mouse CYP3A44 female-predominant expression by glucocorticoid hormone

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d) Non-standard abbreviations: CAR, constitutive androstane receptor; EC50, a half-maximal effective concentration; GAPDH, glyceraldehyde-3-phosphate; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; PCN, pregnenolone-16α-carbonitrile; PXR, pregnane X receptor; PXRE,
PXR-responsive element; RT-PCR, reverse transcriptional polymerase chain reaction; RU486, 11β-[4-dimethylamino]phenyl-17β-hydroxy-17-[1-propynyl] estra-4,9-diene-3-one; RXR, retinoid X receptor; TAT, tyrosine aminotransferase.
Abstract

The role of glucocorticoid receptor (GR) and pregnane X receptor (PXR) in the regulation of female-predominant expression of mouse CYP3A44 by glucocorticoid hormones was evaluated using a primary culture of female mouse hepatocytes, since the expression was suppressed in adrenalectomized female mice, restored by dexamethasone (DEX) treatment, and was not detected in male mouse livers. Glucocorticoid hormones, such as DEX, hydrocortisone and corticosterone, RU486, antagonists for GR and an agonist for PXR, and rifampicin, an agonist for PXR were chosen to investigate the relationship of GR/PXR activation and Cyp3a44 gene expression. Glucocorticoid-inducible expression of CYP3A44 was not suppressed, but rather increased by RU486. Treatment of GR expression plasmid-transfected hepatocytes with DEX concentration-dependently enhanced the expression of PXR as well as CYP3A44 mRNAs. The synergistic effect of DEX at submicromolar concentration and rifampicin is observed. Furthermore, transfection of PXR and retinoid X receptor-α (RXRα) also showed prominent induction of CYP3A44 mRNA by DEX. These results suggest that DEX plays a dual role in CYP3A44 expression; first, direct activation of Cyp3a44 gene by PXR-RXRα complex, and second, indirect activation of Cyp3a44 gene through the induction of PXR gene expression by the GR pathway.
Cytochrome P450 (CYPs) proteins form a superfamily of heme-containing enzymes involved in the oxidative metabolism of both endogenous and exogenous compounds; the former include steroids, fatty acids, retinoids, bile acids etc., and the latter, drugs and environmental pollutants (Gonzalez, 1991). The CYP3A subfamily represents the most abundant cytochrome P450s in adult human liver, comprising approximately 30% of the total content. The human CYP3A subfamily comprises 4 isoenzymes, CYP3A4, CYP3A5, CYP3A7 and CYP3A43, which show variable expressions in the population. Among them, the CYP3A4 isoform is the most prevalent in adults. It is estimated that about 50% of currently marked drugs are metabolized by CYP3A4 (Bertz and Granneman, 1997).

Recently, several members of the nuclear hormone receptor superfamily such as constitutive androstane receptor (CAR), pregnane X receptor (PXR) (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998), vitamin D receptor (VDR) (Drocourt et al., 2002) as well as the glucocorticoid receptor (GR) (Pereira et al., 1998; Schuetz et al., 1996) have been revealed to be responsible for endobiotic- and xenobiotic-mediated expression of CYP3A genes. GR is activated upon binding of glucocorticoids and then regulates gene transcription either actively or repressively (Beato et al., 1995; Gupta and Lalchhandama,
2002). Several lines of evidence support the role of GR in CYP gene regulation. For instance, the human CYP3A5 gene promoter contains two glucocorticoid response elements (GRE), separated by 160 bp, which confer the responsiveness of the reporter gene to glucocorticoid in HepG2 cell (Schuetz et al., 1996). Furthermore, it has been reported that GR binds to GRE present in the rat CYP3A1 gene, suggesting that cooperation of the upstream GRE and downstream elements may be required for the maximal response of CYP3A to glucocorticoids (Pereira et al., 1998). In addition to GR, the increased expression of CYP3A mRNA is also mediated via PXR. On activation by a xenobiotic ligand, PXR dimerizes with retinoid X receptor-α (RXRα) and the formed heterodimer binds to their respective response elements to induce CYP3A expression (Bourguet et al., 2000; Mangelsdorf et al., 1995). Functional cross talk between the GR- and PXR-signalling pathways has been reported in human CYP3A4 and rat CYP3A23 gene expression (Pascussi et al., 2000; Pascussi et al., 2001; Huss and Kasper, 2000); however, the identity of virtual controlling of the activity of PXR at low ligand concentration is still unclear. If a similar regulation pathway were found in other laboratory animal species, the model would be valuable for more comprehensive understanding of orthologous CYP in humans.

With regard to the CYP3A subfamily in mice, CYP3A44, female-
predominant CYP3A mRNA, was isolated, and it was found that the expression is dependent on the feminine plasma growth hormone profile (Sakuma et al., 2002). Furthermore, we also found that glucocorticoids increased CYP3A44 expression in cultured hepatocytes (our unpublished results); however, the role of nuclear hormone receptors in the regulation of Cyp3a44 gene expression has not been extensively determined. Observation suggests that pregnenolone-16α-carbonitrile (PCN) and dexamethasone (DEX) induced CYP3A44 mRNA expression is PXR-dependent in male mice. On the other hand, PCN and DEX down-regulated CYP3A44 expression in female PXR null mice (Anakk et al., 2007). Given these findings, it is suggested that gender, the xenobiotic activator and the nuclear receptor comprehensively act to control Cyp3a44 gene expression; therefore, the role of GR or PXR in the overall regulation of mouse Cyp3a44 gene expression was independently explored.

In the present study, we investigated the role of GR and PXR in the regulation of Cyp3a44 gene expression using a primary culture of mouse hepatocytes. The results suggest that DEX plays a dual role in CYP3A44 expression; first, direct activation of the Cyp3a44 gene by PXR-RXRα complex, and second, indirect activation of the Cyp3a44 gene through induction of PXR gene expression by the GR pathway.
Materials and methods

Materials

Materials for culturing hepatocytes were purchased from Wako Pure Chemical (Osaka, Japan), Gibco-BRL (Grand Island, NY), and Sigma Chemicals (St. Louis, MO). Percoll was obtained from Amersham-Pharmacia Biotech (Uppsala, Sweden). Transpass™ D1 Transfection Reagent was supplied by New England Biolabs (Hercules, CA). Dexamethasone, hydrocortisone, corticosterone and 11β-[4-dimethylamino] phenyl-17β-hydroxy-17-[1-propynyl] estra-4, 9-diene-3-one (RU486) were obtained from Sigma Chemicals (St. Louis, MO). Rifampicin was obtained from Wako Pure Chemical (Osaka, Japan). TaKaRa RNA PCR Kit (AMV) version 3.0 was obtained from TaKaRa Shuzo (Kyoto, Japan). All other laboratory chemicals were of the highest grade commercially available.

Animals

Mice were housed in the University of Toyama’s Animal Center facility under the supervision of certified laboratory veterinarians and were treated according to a research protocol approved by the University’s Institutional Animal Care and Use Committee. Animals were allowed food and water ad libitum and were subjected to a 12-h light/dark cycle.
C57BL/6 mice of both sexes were purchased from Sankyo Experimental Animals (Tokyo, Japan). Four-week-old C57BL/6 mice were adrenalectomized or sham-operated and killed 5 days later. Some adrenalectomized or sham-operated mice were subcutaneously administered DEX at 10 mg/kg/day for the last 3 days. The liver was excised immediately after death and used for the preparation of total RNA.

**Preparation of primary hepatocyte cultures**

Eight-week-old female ddY mice were purchased from Japan SLC, Inc (Shizuoka, Japan). The livers were perfused with collagenase-containing Hanks’ solution and viable hepatocytes were isolated by Percoll isodensity centrifugation as described (Nemoto and Sakurai, 1995) and seeded in dishes at a density of 2x10^6 cells/4ml/60mm. The Waymouth medium did not contain phenol red, a pH indicator, to exclude estrogen-like action. The culture dishes were maintained at 37°C in a CO₂-humidified incubator. The medium was renewed 24 hours after seeding and treatment with either DEX, hydrocortisone, corticosterone, RU486 or rifampicin was started 1 day after the medium change. Each chemical was dissolved in DMSO to 0.1% final concentration. The cells were harvested 24 h later to prepare the total RNA fraction.
Plasmids

The GR expression plasmid was generated by replacing the DNA fragment between NheI and XbaI sites containing the coding sequence of Renilla luciferase of pRL-SV40 vector (Promega) with the 2,385 bp cDNA fragment involving the entire coding region (2,379 bp) and 6 bp 3’-noncoding regions of mouse GR. Both expression plasmids of PXR and the RXRα were constructed using the same strategy. The PXR expression plasmid contains the 1,312 bp cDNA fragment with the entire coding region (1,296 bp) and both 6 bp 5’- and 10 bp 3’-noncoding regions of mouse PXR. The RXRα expression plasmid contains the 1,409 bp cDNA fragment with the entire coding region (1,404 bp) and 5 bp 5’-noncoding regions of mouse RXRα.

Transfection of the nuclear receptor expression plasmid into hepatocytes in cultures

Mouse hepatocytes were cultured in Waymouth medium and transfected using Transpass™ D1 Transfection Reagent (Biolabs, Hercules, CA). Transfection mixtures consisted of Waymouth medium, empty plasmid or nuclear receptor expression plasmid and Transpass™ D1 at 2ml, 5 μg and 5 μl, respectively. Transfection continued for 3 h and the medium was changed. The cells were treated with DEX at various concentrations.
after a further 24 h incubation. Total RNA was prepared from other 24 h-treated cells.

**Real-time RT-PCR**

Hepatic total RNA was prepared from hepatocytes as described previously (Nemoto and Sakurai, 1995). Semiquantitative RT-PCR of CYP3A44 and GAPDH with \(^{32}\)P radio-labeled primers was performed using a TaKaRa RNA PCR Kit (AMV) version 3.0 as described previously (Sakuma et al., 2002; Sakuma et al., 2000). Quantitative real-time RT-PCR was performed using a TaKaRa RNA PCR Kit (AMV) version 3.0, in combination with a gene-specific TaqMan MGB Gene Expression Detection kit or SYBR Green reagent. The forward primer, reverse primer and the TaqMan MGB probe of the TaqMan MGB Gene Expression Detection kit for CYP3A44 designed by ourselves with the assistance of Primer Express software were 5’-GAAACTGCAAGCCAGGAGCATA-3’, 5’-TTTCTTACAGACTCTCTCTCTCAAGTCTAGTAAACAAT-3’, and 5’-FAM-AATAAGGAACTCCCACCTG-MGB-3’, respectively. For CYP3A41, the forward primer, reverse primer and TaqMan MGB probe of the TaqMan MGB Gene Expression Detection kit designed by ourselves were 5’-GCCAAAGGGATTTTAAGAGTTGACT-3’, 5’-GGTGTCAGGAAATGGAAAAAGTACA-3’, and
5’-FAM-ATCCTTGTTCCTCTCAG-MGB-3’, respectively. PXR or GAPDH cDNA was detected with SYBR Green reagent and gene-specific primer sets. The forward and reverse primers for PXR were 5’-GCCAAAGGGATTTTAAGAGTTGACT-3’ and 5’-GGTGTCAGGAAATGGAAAAAGTACA-3’, respectively, and for GAPDH were 5’-TCCACTCACGGCAAATTCAACG-3’ and 5’-TAGACTCCACGACATACTCAGC-3’, respectively. PCR conditions were as follows: for Cyp3a44, initial denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and extension at 62°C for 1 min were performed. PCR, denaturation, and extension were repeated for 60 cycles. For Cyp3a41, initial denaturation at 95°C for 4 min, denaturation at 95°C for 15 s, and extension at 60°C for 1 min were performed. PCR, denaturation, and extension were repeated for 50 cycles. For PXR, initial denaturation at 95°C for 4 min, denaturation at 95°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 1 min were performed. PCR, denaturation, annealing, and extension were repeated for 40 cycles. For GAPDH, initial denaturation at 95°C for 4 min, denaturation at 95°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 1 min were performed. PCR, denaturation, annealing, and extension were repeated for 40 cycles. The mRNA quantitation of CYP3A44, CYP3A41 or PXR was normalized to GAPDH mRNA and expressed as fold induction, and
compared with control mRNA expression as 1. Amplification and detection were performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) with ABI Prism® 7000 SDS software.
Results

To investigate the role of glucocorticoids in regulation of the Cyp3a44 gene in vivo, adrenalectomy and then treatment with DEX were carried out. Fig. 1 shows CYP3A44 mRNA expression in male and female mouse livers. In females, adrenalectomy drastically decreased the expression and DEX restored it; however, CYP3A44 mRNA expression was not detected in males, consistent with our previous report (Sakuma et al., 2002).

The effect of adrenalectomy and restoration by synthetic glucocorticoid treatment suggests the role of glucocorticoids in the control of Cyp3a44 gene expression in mouse liver. In the next experiment, the effect of glucocorticoids on CYP3A44 mRNA expression in cultured hepatocytes was investigated. Hepatocytes were treated with DEX, hydrocortisone or corticosterone at 10^{-5} \text{M} or 10^{-7} \text{M}, either in the absence or presence of 10^{-5} \text{M} RU486, which functions as an antagonist for GR (Cadepon et al., 1997) and an agonist for PXR (Kliewer et al., 1998). As shown in Fig. 2A, the expression of CYP3A44 mRNA was induced by DEX at both 10^{-7} \text{M} and 10^{-5} \text{M} and by hydrocortisone and corticosterone at 10^{-5} \text{M}; however, combined treatment with RU486 did not suppress glucocorticoid-induced CYP3A44 mRNA expression, which was a different result for RU486 with other mouse female-predominant
CYP3A41 mRNA expression (Fig. 2B). On the other hand, treatment with RU486 alone significantly induced the mRNA expression of CYP3A44, and combined treatment of hydrocortisone at $10^{-5}$M with RU486 significantly enhanced CYP3A44 mRNA expression over the level attained by hydrocortisone alone.

As DEX and natural glucocorticoids act as a common ligand for GR in various animal species, we next investigated the role of these glucocorticoids in the induction of CYP3A44 mRNA by a GR-mediated pathway. In our cell culture system, the expression of GR mRNA declined to 10% in the liver during the initial 24 h cultivation (data not shown); therefore, experiments were carried out in primary cultured mouse hepatocytes transfected with GR expression plasmid in the presence of $10^{-7}$M to $10^{-5}$M DEX or corticosterone. As shown in Fig. 3, in the absence of an expressed receptor, concentration-dependent effects of DEX on CYP3A44 mRNA were observed. As expected, when GR expression plasmid was transfected, DEX treatment at $10^{-6}$M up to $10^{-5}$M caused an increase in CYP3A44 expression over the level attained by empty plasmid; however, no significant increase in expression was seen at $10^{-7}$M DEX in the existence of GR expression plasmid. Similarly, induction by corticosterone was enhanced by transfection of the GR expression plasmid, but it was observed only at $10^{-5}$M. These results demonstrate the role of
DEX and corticosterone for GR in the induction of *Cyp3a44* gene expression at supramicromolar concentration.

It is known that a submicromolar concentration of DEX results in the glucocorticoid receptor-mediated expression of human *PXR* gene, which, in turn, is able to transactivate the human *CYP3A4* gene, although the identity of the virtual activator of PXR at a submicromolar concentration of DEX is unclarified (Pascussi et al., 2001). We wondered whether the expression of mouse PXR also could be increased by DEX in primary cultured mouse hepatocytes. We anticipated that, if this was the case, one mechanism of DEX induction of the *Cyp3a44* gene might act through GR-mediated expression of PXR. The results reported in Fig. 4 show that endogenous mouse PXR mRNA is induced significantly by DEX treatment at $10^{-5}$M. Furthermore, transfection of GR-expression plasmid considerably induced PXR mRNA expression up to 2,500-fold.

These findings, when considered with an activation of GR caused an increase in endogenous PXR mRNA at low concentration of DEX, the next experiment was carried out. To observe the potentiation of PXR transactivation in expression of *Cyp3a44* gene at low concentration of DEX, rifampicin, which is a ligand for PXR was used in the absence or presence of $10^{-7}$M DEX, a concentration sufficient to activate GR but not PXR and also at $10^{-5}$M DEX, a concentration which activate both GR and PXR.
(Lehmann et al., 1998). As shown in Fig. 5, DEX at $10^{-7}$M or rifampicin alone did not induce CYP3A44 mRNA. Concomitant addition of DEX at $10^{-7}$M and rifampicin, however, enhanced Cyp3a44 gene expression. As aspected, DEX at $10^{-5}$M in association with rifampicin increased Cyp3a44 expression over the level attained by DEX at $10^{-5}$M alone. These observations serve as evidence for the synergistic effect of DEX at low concentration, and PXR activator on CYP3A44 induction. Taken together with DEX increasing PXR expression via a GR-mediated mechanism and this synergistic effect, the possibility that GR/PXR-dependent regulation for basal Cyp3a44 gene expression is suggestible; however, no significant increase in Cyp3a44 expression was seen at $10^{-7}$M DEX in the existence of GR expression plasmid (Fig. 4). This observation might be resulted from the lack of some PXR ligand(s) in primary cultured mouse hepatocytes, which is necessary for the full induction.

DEX-induced expression of the Cyp3a44 gene was not antagonized by the addition of RU486, and the induction of Cyp3a44 also occurred by RU486 alone (Fig. 2A). This suggests that DEX-induced CYP3A44 mRNA might be a PXR-dependent process; thus, we examined the effects of transfection of expression plasmids for PXR and RXRα on the expression of the Cyp3a44 gene. Primary cultured mouse hepatocytes were transfected with these plasmids in the presence of $10^{-7}$M to $10^{-5}$M
DEX. As shown in Fig. 6, when PXR and RXRα were transfected, treatment with DEX at $10^{-6}$M to $10^{-5}$M caused a significant increase in Cyp3a44 gene expression over the level attained by an empty plasmid. If increased response of the Cyp3a44 gene to glucocorticoids after transfection of the GR expression plasmid is due to an increased level of GR, not of PXR, it is anticipated that transfection of PXR and RXRα expression plasmids does not cause the increased response to DEX treatment. This result strongly suggests that CYP3A44 mRNA induction with a supramicromolar concentration of DEX is mediated through a direct PXR-dependent mechanism.
Discussion

We have investigated the mechanism by which DEX produces a dual role in CYP3A44 expression. First, direct activation of the Cyp3a44 gene by PXR-RXRα complex at supramicromolar concentration and, second, indirect activation of the Cyp3a44 gene through the induction of PXR gene expression by the GR pathway.

CYP3A44 has been identified as a female-predominant gene in the livers of C57BL/6 and ddY mice, and its expression was also dependent on the feminine plasma growth hormone profile (Sakuma et al., 2002). A recent study using transgenic human CYP3A mice also revealed the importance of the GH profile in modulating the sex-dependent expression of the mouse Cyp3a44 gene (Cheung et al., 2006). Furthermore, the role of nuclear receptors PXR and CAR in the regulation of Cyp3a44 gene expression has been defined as showing that gender also influences the critical impact of PXR- and CAR-mediated effects on CYP3A44 expression (Anakk et al., 2007). Anakk et al. reported that DEX induced CYP3A44 mRNA expression in male 129sv/C57Bl6 mixed background mice, which failed to be observed in our study (Fig. 1). The difference in the CYP3A44 expression profile might be due to the mouse strains examined in different laboratories.

In the present study, we demonstrated that the expression of
Cyp3a44 gene in mouse liver is under the control of glucocorticoids (Fig. 1). We propose that, in the absence of a xenobiotic inducer, glucocorticoids at the physiological level control the basal expression of CYP3A44 whereas, in the presence of an inducer, Cyp3a44 gene expression is induced.

The results using GR expression plasmid (Figs. 3, 4) suggest that regulation of the Cyp3a44 gene might have occurred in a GR-dependent manner; however, contradictory observations demonstrated that CYP3A44 had a different expression profile from CYP3A41 and TAT, a prototypical target gene of GR (Grange et al., 1989), in which its expression peaked at lower concentrations of DEX (10^{-7}M) (Fig. 2, Sakuma et al., 2004). CYP3A44 showed maximum induction by DEX at a higher concentration (10^{-5}M), and the profile was similar to that of CYP3A11 (Sakuma et al., 2004), a target of PXR (Kliewer et al., 1998). Furthermore, RU486, an antagonist for GR (Cadepon et al., 1997) and an agonist for PXR (Kliewer et al., 1998), did not show any suppressive effect on DEX-induced CYP3A44 mRNA expression, different from CYP3A41 expression, in which its expression is mediated by GR (Fig. 2B). Based on these observations, DEX, hydrocortisone or corticosterone—induction of CYP3A44 may not be directly involved in GR activation.

However, the present data clarified a certain role of GR in
CYP3A44 mRNA expression. We suggest that GR indirectly controls DEX induction of CYP3A44 mRNA by increasing the expression of PXR. The following observations support this hypothesis: (a) increased accumulation of endogenous mouse PXR mRNA was observed after transfection of GR expression plasmid in the presence of DEX (Fig. 4); (b) transfection of GR expression plasmid in primary hepatocytes in the presence of DEX significantly activated CYP3A44 mRNA expression. Nevertheless, no GR-transfection effect was observed at 10^{-7} M DEX, the concentration at which GR-target genes such as the TAT gene is efficiently inducible (Fig. 3, Sakuma et al., 2004); (c) increased accumulation of endogenous PXR mRNA was observed only in the presence of DEX, whereas other glucocorticoids, such as hydrocortisone and corticosterone, did not affect the one (data not shown). Therefore, it is suggested that DEX may act through the indirect GR-mediated activation of CYP3A44 in the present culture system.

The involvement of PXR might be confirmed for Cyp3a44 gene expression by DEX (Fig. 6); however, the possibility that DEX only acts at supramicromolar concentration through direct PXR-mediated activation of the Cyp3a44 gene is strongly suggested. The prototypical model of PXR-mediated gene induction at high concentrations of DEX has been well documented for the expression of the human CYP3A4 gene (Pascussi et al.,
2001) or rat glutathione S-transferase A2 gene (Falkner et al., 2001). Studies by Pascussi et al. (2001) and Falkner et al. (2001) have shown that both genes are induced by supramicromolar concentrations of DEX-mediated PXR activation. Recent studies indicate the possibility that the expression of human CYP3A4 in the liver is relatively higher in women than in men (Dhir et al., 2006), suggesting that CYP3A44 has more similar regulation properties than CYP3A11, because the latter shows no significant difference between males and females. Taking those studies and the present findings together, Cyp3a44 could be considered as a relevant murine CYP3A model gene of human CYP3A4 with respect to PXR-mediated induction and sexually dimorphic expression.

Considering that the dose of DEX used in this in vivo study was higher than those of therapeutic treatments, resulting higher concentration of glucocorticoid in plasma than that in physiological status. Furthermore, we recognize that since DEX is a synthetic derivative of glucocorticoids, the inductive effect observed in adrenalectomized mice must be reflected by both pharmacological, i.e. the reaction caused by high concentration of drug, and physiological effect, i.e. the response caused by glucocorticoid hormones at physiological concentration. Since the expression of the Cyp3a44 gene was decreased after adrenalectomy, it is expected that glucocorticoid has a physiological role in the regulation of the Cyp3a44
gene. However, a contradictory observation in an *in vitro* study showed no effect of natural glucocorticoids at submicromolar concentration (physiological condition) (Fig. 3). The reason for this contradiction is not clear at present, but it is likely that it may result from the lack of some factor(s) in primary cultured mouse hepatocytes, which is necessary for the high level expression of the *Cyp3a44* gene *in vivo*. Although the *in vivo* studies were undertaken in C57BL/6 mice and the cultured hepatocytes (*in vitro*) were undertaken in ddY mice, our unpublished results revealed no strain differences in *Cyp3a44* gene expression between these two strains.

From the above findings, a GR/PXR-dependent regulation mechanism model of CYP3A44 expression is presented in Fig. 7. First, DEX at high concentration activates PXR resulting in *Cyp3a44* gene expression (direct PXR-dependent mechanism). Second, DEX increases PXR expression via a GR-mediated mechanism (indirect GR-dependent mechanism). At low DEX concentration, the same mechanism might be possible (Fig.4, 5); however, induction of the *Cyp3a44* gene by the direct PXR-dependent mechanism is limited to a very low level, because of the relatively low potential of DEX activating PXR (EC$_{50}$=10 µM) (Lehmann et al., 1998).
References


Footnotes

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Legends for figures

Fig. 1: Expression of CYP3A44 mRNAs after adrenalectomy and DEX treatment in the liver of male and female C57BL/6 mice. Four-week-old male and female mice were adrenalectomized (ADX) or sham-operated (Sham). Some animals were administered dexamethasone (DEX) subcutaneously at 10 mg/kg/day for 3 days. Representative result of semiquantitative RT-PCR analysis is shown using a CYP3A44-selective primer set (Sakuma et al., 2002). The 5’-labeled sense primer for CYP3A44 was used and gel-exposed X-ray film is shown. Amplification of GAPDH cDNA was carried out with the unlabeled primer set, and an image of ethidium bromide-stained polyacrylamide gel is shown.

Fig. 2: Effects of glucocorticoids and RU486 on the expression of CYP3A44 and CYP3A41 mRNA in primary cultured mouse hepatocytes. (A) Hepatocytes isolated from female ddY mice were cultured for 2 days and treated with 10^{-7}M or 10^{-5}M DEX, hydrocortisone or corticosterone, respectively, either in the absence or presence of 10^{-5}M RU486 for another 24 h. Expression of CYP3A44 mRNAs was evaluated by quantitative real-time RT-PCR analysis. CYP3A44 mRNA was normalized to the level of GAPDH and is shown relative to the level in untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined
using Student’s $t$ test, $^*p<0.05$ compared with untreated hepatocytes, $^{**}p<0.005$ compared with untreated hepatocytes, $^{***}p<0.001$ compared with untreated hepatocytes, and $^{##}p<0.005$ compared with $10^{-5}$M hydrocortisone-treated hepatocytes.

(B) Hepatocytes isolated from female ddY mice were cultured for 2 days and treated with $10^{-7}$M or $10^{-5}$M DEX, hydrocortisone or corticosterone, respectively, either in the absence or presence of $10^{-5}$M RU486 for another 24 h. Expression of CYP3A41 mRNAs was evaluated by quantitative real-time RT-PCR analysis. CYP3A41 mRNA was normalized to the level of GAPDH and is shown relative to the level in untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using Student’s $t$ test, $^*p<0.05$ compared with untreated hepatocytes, $^{**}p<0.005$ compared with untreated hepatocytes, $^{*}$p<0.05 compared with $10^{-5}$M DEX-treated hepatocytes, $^{**}$p<0.005 compared with $10^{-7}$M DEX-treated hepatocytes, and $^{#}$p<0.01 compared with $10^{-5}$M hydrocortisone-treated hepatocytes.

Fig. 3: Effects of GR, DEX and corticosterone on the expression of CYP3A44 mRNA in primary cultured mouse hepatocytes. Hepatocytes isolated from female ddY mice were cultured for 2 days and transfected with control vector plasmid (empty plasmid) or GR expression plasmid.
Twenty-four hours later, cells were treated with DEX at $10^{-7}$ M to $10^{-5}$ M for another 24 h. Expression of CYP3A44 mRNA was evaluated by quantitative real-time RT-PCR analysis. Each mRNA was normalized to the level of GAPDH and is shown relative to those in empty plasmid-transfected hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using Student’s $t$ test, $^*p<0.05$ compared with transfected with empty plasmid treated with $10^{-6}$ M DEX hepatocytes, $^#p<0.05$ compared with transfected with empty plasmid treated with $10^{-5}$ M DEX hepatocytes and $^$p$<0.05$ compared with transfected with empty plasmid treated with $10^{-5}$ M corticosterone hepatocytes. Multiple groups after various treatments were compared using one-way analysis of variance (one-way ANOVA), $^\triangle p<0.05$ compared with transfected with empty plasmid treated with $10^{-7}$ M DEX hepatocytes, $^\triangle\triangle p<0.01$ compared with transfected with empty plasmid treated with $10^{-7}$ M DEX hepatocytes, and $^&p<0.01$ compared with transfected with empty plasmid untreated hepatocytes.

Fig. 4: Effects of GR and DEX on the expression of endogenous mouse PXR mRNA in primary cultured mouse hepatocytes. Hepatocytes isolated from female ddY mice were cultured for 2 days and transfected with control vector plasmid (empty plasmid) or GR expression plasmid.
Twenty-four hours later, cells were treated with DEX at $10^{-7}$ M to $10^{-5}$ M for another 24 h. Expression of endogenous mouse PXR mRNAs was evaluated by quantitative real-time RT-PCR analysis. Each mRNA was normalized to the level of GAPDH and is shown relative to those in empty plasmid-transfected hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using Student’s $t$-test, *$p<0.05$ compared with transfected with empty plasmid untreated hepatocytes, and #$p<0.05$ compared with transfected with empty plasmid treated with $10^{-5}$M DEX hepatocytes.

Fig. 5: Effects of DEX and rifampicin on the expression of CYP3A44 mRNA in primary cultured mouse hepatocytes. Hepatocytes isolated from female ddY mice were cultured for 2 days and treated with $10^{-7}$M or $10^{-5}$M DEX, either in the absence or presence of $10^{-7}$M rifampicin for another 24 h. Expression of CYP3A44 mRNAs was evaluated by quantitative real-time RT-PCR analysis. Each mRNA was normalized to the level of GAPDH. Each column represents the mean ± SD (n=3). ND: not detected. Multiple groups after various treatments were compared using one-way analysis of variance (one-way ANOVA), *$p<0.01$ compared with untreated hepatocytes, #$p<0.01$ compared with $10^{-7}$M DEX-treated hepatocytes, and #*$p<0.01$ compared with rifampicin-treated hepatocytes.
Fig. 6: Effects of PXR, RXRα and DEX on the expression of CYP3A44 mRNA in primary cultured mouse hepatocytes. Hepatocytes isolated from female ddY mice were cultured for 2 days and transfected with control vector plasmid (empty plasmid) or PXR and RXRα expression plasmid. Twenty-four hours later, cells were treated with DEX at $10^{-7}$ M to $10^{-5}$ M for another 24 h. Expression of CYP3A44 mRNA was evaluated by quantitative real-time RT-PCR analysis. Each mRNA was normalized to the level of GAPDH. Each column represents the mean ± SD (n=3). ND: not detected. Significance was examined using Student’s $t$ test, $^5p<0.05$ compared with transfected with empty plasmid treated with $10^{-5}$M DEX hepatocytes, and $^#p<0.001$ compared with transfected with empty plasmid treated with $10^{-6}$M DEX hepatocytes.

Fig. 7: Model summarizing the proposed role of nuclear receptors in the regulation of $Cyp3a44$ gene expression in cultured hepatocytes. First, PXR is activated by DEX at high concentration resulting $Cyp3a44$ gene expression (direct PXR-dependent mechanism). Second, DEX increases PXR levels via a GR-mediated mechanism (indirect GR-dependent mechanism) which, in turn, is able to activate $Cyp3a44$ gene expression.
Figure 1

The image shows a gel electrophoresis result comparing male and female mice under Sham and ADX conditions with or without DEX treatment. The bands indicate the expression levels of CYP3A44 and GAPDH genes.
Figure 3

The figure shows a bar graph comparing the relative expression of CYP3A44 mRNA under different conditions.

- **Left Panel:**
  - X-axis: DEX Concentration (-logM)
  - Y-axis: Relative CYP3A44 mRNA expression
  - Comparisons: Empty plasmid vs. GR expression plasmid
  - Significant differences indicated by asterisks (*), triangles (△), and a symbol (#).

- **Right Panel:**
  - X-axis: Corticosterone Concentration (-logM)
  - Y-axis: Relative CYP3A44 mRNA expression
  - Significant differences indicated by dollar signs ($).
Figure 4

The figure shows a bar graph depicting the relative PXR mRNA expression levels in response to different concentrations of DEX. The graph compares two conditions: the empty plasmid and the GR expression plasmid. DEX concentrations are indicated on the x-axis, ranging from 10^{-7} to 10^{-5} M, while the y-axis represents the relative PXR mRNA expression levels. The bars for GR expression plasmid are significantly higher compared to the empty plasmid, especially at the highest DEX concentration.
Figure 5

![Graph showing relative CYP3A44 mRNA expression](image-url)

- **Relative CYP3A44 mRNA expression**
  - ND
  - ND
  - ND
  - *
  - $\#$

- **Conditions**
  - **DEX (10^{-7}M)**
    - ND
    - +
    - ND
    - +
    - ND
    - +
    - ND
    - +
  - **DEX (10^{-5}M)**
    - ND
    - ND
    - +
    - +
    - ND
    - ND
    - +
  - **Rifampicin (10^{-7}M)**
    - ND
    - ND
    - +
    - +
    - ND
    - ND
    - +
Figure 6

The figure shows a bar graph comparing the relative CYP3A44 mRNA expression levels between two conditions: Empty plasmid and PXR+RXR expression plasmid. The x-axis represents different concentrations of DEX (in units of -logM), ranging from 7 to 5. The y-axis represents the relative mRNA expression levels, ranging from 0 to 1.4. The graph includes error bars to indicate variability. The legend notes that ND stands for 'not detectable'.