Synergism of Glucocorticoid Hormone with Growth Hormone for Female-Specific Mouse Cyp3a44 Gene Expression

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ABSTRACT:

CYP3A44 and CYP3A41 are female-specific CYP3A in the mouse liver. In primary cultured mouse hepatocytes, dexamethasone concentration-dependently induced CYP3A44 mRNA, and the highest response was seen at $10^{-6}$ M. In contrast, CYP3A41 mRNA expression was highest at lower concentrations ($10^{-7}$ or $10^{-6}$ M). At submicromolar concentration ($10^{-7}$ M), the induction of CYP3A44 mRNA was very slight, but strongly enhanced induction was observed by the simultaneous addition of growth hormone (GH). Similar enhancement was also observed in CYP3A41 mRNA expression. Continuous exposure to GH, which mimics female-type secretion from the pituitary gland, was effective to enhance the expression of both mRNAs, but discontinuous exposure (male-type) was not. This synergistic induction of CYP3A44 mRNA was further enhanced by the transfection of glucocorticoid receptor (GR) expression plasmid or by the cotransfection of pregnane X receptor (PXR) and retinoid X receptor (RXR) expression plasmids. Similar synergistic induction was seen in CYP3A41 mRNA by the transfection of GR expression plasmid but was not enhanced by cotransfection of PXR and RXR expression plasmids. These observations suggest that functional cross-talk between signaling pathways of female-type GH secretion and glucocorticoid hormone might be involved in the female-predominant expression of both genes. Additionally, one or more nuclear receptors mediating induction by glucocorticoid hormone are employed for collaboration with GH.

Several clinical studies have suggested that oxidative metabolism of drugs and steroids is disturbed after the therapeutic use of glucocorticoids (Watkins et al., 1985, 1989) or growth hormone (GH) (Levitsky et al., 1989; Cheung et al., 1995; Jurgens et al., 2002) as well as in disease states associated with altered concentrations of circulating hormones. The changes in drug and steroid metabolism observed in these studies suggest that the expression of P450 enzymes may be greatly influenced by extensive and complex hormonal regulation in humans.

With respect to the regulation of CYP genes by glucocorticoid hormones, extensive studies have focused on control of the pregnane X receptor (PXR; NR1I2) as well as the glucocorticoid receptor (GR; NR3C1). For instance, the human CYP3A4 gene, the most abundant P450 isoform in adult livers, could be induced by dexamethasone via a two-step mechanism involving sequential low- and high-concentration components, in which GR and PXR participate in each step, respectively (Pascussi et al., 2000, 2001).

Female-specific expression of rodent CYP genes, including rat CYP2C12 (MacGeoch et al., 1984; Kamataki et al., 1985) and CYP3A9 (Kawai et al., 2000) and mouse Cyp3a41 (Sakuma et al., 2002) and Cyp3a44 (Sakuma et al., 2002), demonstrated that the sex-specific pattern of GH secretion from the pituitary gland is a critical determinant of sexually dimorphic expression. Sex differences in plasma GH profiles are most marked in rodents, but significant male-female differences in the regulation of pituitary GH release also exist in humans (Gatford et al., 1998). Interestingly, recent experiments using the primary culture of human hepatocytes indicated that the CYP3A4 gene is controlled by GH (Liddle et al., 1998; Dhir et al., 2000). Additionally, several lines of evidence suggested that the metabolism of several clinical drugs, mainly oxidized by CYP3A4, is more efficient in women than in men (Greenblatt et al., 1980; Watkins et al., 1985, 1989; Harris et al., 1995; Gorski et al., 1998; Wolbold et al., 2003). It is therefore interesting whether glucocorticoid-dependent and GH-dependent mechanisms can interact.

Recently, GH-mediated expression of the CYP3A4 gene has been demonstrated to be female-specific in the livers of transgenic mice carrying a bacterial artificial chromosome clone, which contains the complete CYP3A44 gene sequence, including 5'- and 3'-flanking regions (Cheung et al., 2006). Additionally, the expression profile and responsiveness to GH treatment of the human CYP3A44 gene were identical to those of mouse Cyp3a44 gene. These findings strongly suggest that both human CYP3A4 and mouse Cyp3a44 genes share

ABBREVIATIONS: GH, growth hormone; PXR, pregnane X receptor; GR, glucocorticoid receptor; PCR, polymerase chain reaction; rh, recombinant human; MSG, monosodium L-glutamate; DEX, dexamethasone; RU486, 11β-[4-dimethylaminophenyl]-17β-hydroxy-17-[1-propynyl]estra-4,9-dien-3-one; RXR, retinoid X receptor; RT, reverse transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; m, mouse; STAT, signal transducer and activator of transcription; ANOVA, analysis of variance.
functionally common intrinsic cis-elements and that these elements function in response to GH signaling in the liver of transgenic mice. We have previously reported that the mouse Cyp3a41 gene is controlled by GH and glucocorticoid hormones and that both pathways synergistically interact (Sakuma et al., 2002, 2004). Cyp3a44 and Cyp3a41 genes share 95.3% identity in their nucleotide sequence of the coding region and show similar expression profiles, such as sexual specificity and tissue distribution. Thus, both genes are thought to have evolved from a common ancestral gene; there are, however, differences in their expression details. For example, mRNA expression of CYP3A44 is relatively constant during the developmental stage from fetus to adult, whereas the expression of CYP3A41 is markedly changed (Sakuma et al., 2002); however, it has not been demonstrated whether the expression of the Cyp3a44 gene is synergistically controlled by GH and glucocorticoid hormones and whether the control mechanism is identical between the two genes. Therefore, in this report, we investigated the role of GH and glucocorticoid hormones in the regulation of Cyp3a44 gene and compared it with that of the Cyp3a41 gene.

Materials and Methods

Materials. Materials for culturing hepatocytes were purchased from Wako Pure Chemicals (Osaka, Japan), Invitrogen (Carlsbad, CA), and Sigma-Aldrich (St. Louis, MO). Percoll was obtained from GE Healthcare (Buckinghamshire, UK). Takara RNA PCR Kit (AMV) version 2.1 was obtained from Takara (Kyoto, Japan). TaqMan MGB Gene Expression Detection kits were products of Applied Biosystems (Foster City, CA). Recombinant human GH (rhGH) and monosodiumL-glutamate (MSG) were obtained from Wako Pure Chemicals, and an Alzet micro-osmotic pump was from Durect (Cupertino, CA). DEX, hydrocortisone, corticosterone, and RU486 were obtained from Sigma-Aldrich. Other chemicals were of the highest grade commercially available.

Animals. Animals were housed in the Division of Animal Resources and Development, Life Science Research Center, University of Toyama under the supervision of certified laboratory veterinarians and were treated according to the research protocol approved by the University’s Institutional Animal Care and Use Committee. At all times, the mice were housed on paper chip bedding in plastic cages, with water and commercial mouse diet supplied ad libitum. The housing room was air conditioned (20–23°C) and had a 12-h light/dark cycle. Adult C57BL/6Ncrj mice of both sexes were mated in the center under the supervision of certified laboratory veterinarians. Within 24 h after birth and on alternate days for the first 9 days of life, the pups were s.c. injected with MSG at 2 mg/kg body weight five times. The pups were weaned at about 28 days of age. GH replacement by rhGH of mice neonatally treated with MSG began at 8 weeks of age. rhGH was administered by either s.c. injection at a dose of 50 μg/injection twice daily (mimicking male-type GH secretion) or continuous infusion using a s.c. implanted micro-osmotic pump at a rate of 1.5 μg/h (mimicking the female type) for 7 days to male or female mice neonatally treated with MSG, respectively. The effects of MSG treatment were confirmed by verifying growth retardation. GH replacement was checked by examining body weight gain. The mice were sacrificed 1 day after the last day of treatment, and the liver was excised immediately for preparation of total RNA.

Preparation of Primary Hepatocyte Cultures. The livers of female or male ddY mice were perfused with collagenase-containing Hanks’ solution, and viable hepatocytes were isolated by Percoll isodensity centrifugation as described (Nemoto et al., 1989). Standard culture conditions were as follows: cells were dispersed in Waymouth MB 752/1 medium containing bovine serum albumin (2 g/l), insulin (0.5 mg/l), transferrin (0.5 mg/l), and selenium (0.5 μg/l) and seeded in uncoated dishes at a density of 2 × 10^6 cells/ml/60 mm. Waymouth medium did not contain phenol red, a pH indicator, to exclude estrogen-like action. Culture dishes were maintained at 37°C in a CO₂humidified incubator.

Construction of Expression Plasmids for GR, PXR, or RXRα. The GR expression plasmid was generated by replacing the DNA fragment between NheI and XhoI sites containing the coding sequence of Renilla luciferase of the pRL-SV40 vector (Promega, Madison, WI) with the 2385-bp cDNA fragment involving the entire coding region (2379 bp) and 6-bp 3’-noncoding regions of mouse GR. Both expression plasmids of PXR and RXRα were constructed using the same strategy. The PXR expression plasmid contains the 1312-bp cDNA fragment with the entire coding region (1296 bp) and both 6-bp 5’-noncoding and 10-bp 3’-noncoding regions of mouse PXR. The RXRα expression plasmid contains the 1409-bp cDNA fragment with the entire coding region (1404 bp) and 5-bp 5’-noncoding region of mouse RXRα.

Transfection of the Nuclear Receptor Expression Plasmid into Hepatocytes in Cultures. Mouse hepatocytes were cultured for 24 h in Waymouth medium and then transfected using TransfAccel D1 Transfection Reagent (New England Biolabs, Ipswich, MA). Transfection mixtures consisted of Waymouth medium, empty plasmid, or nuclear receptor expression plasmid and TransfAccel D1 at 2 ml, 5 μg, and 5 μl, respectively. Transfection continued for 3 h, and then the medium was changed. Cells were treated with DEX at various concentrations and/or GH (71 ng/ml) after a further 24-h incubation. Total RNA was prepared from other 24-h-treated cells.

Quantitative RT-PCR Analysis. Total RNA was prepared from liver tissues or hepatocytes of mice using TRIzol reagent (Invitrogen). The expression of CYP3A44 mRNA was analyzed by real-time RT-PCR using a Takara RNA PCR Kit (AMV) version 2.1 with the TaqMan MGB probe. The forward primer, reverse primer and TaqMan MGB probe for CYP3A44, designed by ourselves with assistance from Primer Express software, were 5’-GAACT-GACGCCAGATGCCATA-3’, 5’-TTTTCTACAGTCCTCTCTCAATGTAACAAT-3’, 5’-FAM-AATAAGCACCTCACCCTG-MGB-3’, respectively. This primer and probe set was designed to distinguish CYP3A44 cDNA from those of CYP3A11, 3A13, 3A16, 3A25, 3A41, 3A41A (Zaphiropoulos, 2003), 3A57, and 3A59. The expression of CYP3A41 mRNA was analyzed by real-time RT-PCR with a TaqMan MGB probe as described previously (Jarkajornjorn et al., 2006a). The expression of CYP2D9 mRNA was analyzed by real-time RT-PCR with TaqMan Gene Expression assay for CYP2D9 (Mm00651731_m1). GADPH cDNA amplification was detected using Takara RNA PCR Kit (AMV) version 2.1, SYBR Green reagent and the primer set specific to mouse GADPH cDNA (Jarkajornjorn et al., 2006a). PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with ABI Prism 7000 SDS software.

Results

Eight mouse Cyp3a genes have been identified to date, and their products show high amino acid sequence homology (e.g., 90.7% identity between CYP3A41 and CYP3A44). Additionally, the substrate specificity of each mouse CYP3A has not yet been analyzed; therefore, an isoform-specific antibody or substrate is not available at present. Thus, in this report, we investigated the expression of Cyp3a44 and Cyp3a41 genes at mRNA level using real-time RT-PCR, which is able to distinguish the target gene from other highly related but differently controlled genes. It is known that MSG treatment in the neonatal period produces a profound but rather selective GH deficiency in adults (Olney and Ho, 1970; Pampori et al., 1991). Thus, to confirm control of the expression of the Cyp3a44 gene by GH, mRNA expression in the liver of neonatally MSG-treated mice was analyzed. As shown in Fig. 1, the expression of CYP3A44 mRNA was detected in 3-week-old mice of both sexes. At 9 weeks of age, mRNA expression in female mice increased, whereas it declined in male livers, resulting in female-specific expression, as previously reported (Sakuma et al., 2002). Neonatal treatment with MSG suppressed the developmental increase of mRNA expression in females. Continuous administration of GH using an osmotic minipump, which mimics female-type secretion, clearly induced mRNA expression of CYP3A44 over the level of untreated female mice. In contrast, s.c. injection of GH twice a day, which mimics male-type secretion, did not show any effects on expression in male mice. These findings support the idea that GH secretion is a major determinant for the female-specific expression of CYP3A44 mRNA.
In addition to GH, we demonstrated that glucocorticoid hormone also mediates the regulation of Cyp3a44 gene expression in our previous study (Bhadhprasit et al., 2007). Therefore, to examine synergistic interaction between GH- and glucocorticoid hormone-mediated pathways in vitro, we analyzed the effects of GH and glucocorticoid hormone treatment on the expression of the Cyp3a44 gene using mouse hepatocytes in primary culture and compared them with those of the Cyp3a41 gene.

As shown in Fig. 2A, the inductive effects of GH, a submicromolar concentration (10⁻⁷ M) of DEX, hydrocortisone, or corticosterone on Cyp3a44 mRNA expression were small; however, concomitant addition of GH and a glucocorticoid showed strong inductive effects in female hepatocytes. Additionally, RU486 alone at supramicromolar concentration (10⁻⁵ M) induced CYP3A44 mRNA expression, and concomitant treatment with GH and RU486 showed further inductive effects. In contrast, DEX at submicromolar concentration clearly induced CYP3A41 mRNA, and the concomitant addition of GH showed further induction in female hepatocytes (Fig. 2B). RU486 alone or concomitant addition with GH did not have any effect on CYP3A41 mRNA expression. In accordance with Cyp3a44, submicromolar concentration of hydrocortisone or corticosterone did not show a strong inductive effect on CYP3A41 mRNA, whereas concomitant addition of GH clearly had an inductive effect. Additionally, basal expression and response to these hormone treatments were observed only in cells isolated from female mice, suggesting that the intrinsic cellular factor in female hepatocytes might determine the ability to express and respond to these hormones.

It is known that the different secretion pattern of GH is one of the key determinants of the sexually dimorphic expression of several genes; therefore, we examined whether a different profile of GH exposure can cause a distinct response of two genes even with synergistic action with DEX. Primary mouse hepatocytes were exposed to DEX and GH with different protocols for 49 h. One protocol involved continuous exposure to both hormones, regarded as female-type exposure, and the other was discontinuous exposure to GH; hepatocytes were exposed to both GH and DEX for 1 h, and then hepatocytes were exposed to DEX alone for 11 h. This successive treatment was repeated for four cycles (48 h) and concluded with an additional treatment with GH and DEX for 1 h (total 49 h). We regarded the latter protocol as mimicking male-type secretion. As shown in Fig. 3A, mRNA expression of male-predominant Cyp2D9, which is known to be induced by male-type GH secretion (Noshiro and Negishi, 1986; Jarukamjorn et al., 2006b), is enhanced by discontinuous exposure to GH. In contrast, synergistic induction of CYP3A44 and CYP3A41 mRNAs in female hepatocytes was observed in cells treated with DEX and continuous exposure to GH but not in cells treated with DEX and discontinuous exposure to GH, even though cumulative GH exposure during the initial 48 h was the same between the two protocols, and the concentration of GH in the additional final 1-h exposure was 12-fold higher than for continuous exposure during the same period (Fig. 3B).

We have observed a rapid decrease in the expression of glucocorticoid-related receptors such as GR and PXR after transferring hepatocytes to a primary culture from the body (approximately 10% of the liver level, our unpublished observation). To intensify the cell response and to identify nuclear receptors involved in glucocorticoid-mediated induction of two Cyp3a genes, we examined the transfection effect of nuclear receptor expression plasmids on synergistic interaction of GH and glucocorticoids (Figs. 4 and 5). DEX induced CYP3A44 mRNA expression concentration-dependently up to 10⁻⁵ M (Fig. 4A, empty). The addition of GH increased CYP3A44 mRNA expression approximately 10-fold at 10⁻⁷ M DEX (Fig. 4A, empty + GH). Transfection of mouse (m) GR expression plasmid enhanced this synergistic interaction approximately 4-fold, resulting in a 40-fold induction compared with the control group (Fig. 4A, GR + GH). This result suggests that GR might be involved in synergistic interaction between GH and glucocorticoid at a submicromolar concentration of glucocorticoid. Additionally, the level of enhanced expression by GH increased DEX concentration-dependently up to 10⁻⁷ M. With CYP3A41, similar synergistic interaction and enhancement by mGR were also observed; however, the highest expression was seen either in the presence or absence of GH at 10⁻⁷ M DEX (Fig. 4B).

In our previous report, we demonstrated that glucocorticoid-induced expression of the Cyp3a44 gene can be mediated by PXR at supramicromolar concentration (Bhadhprasit et al., 2007). Thus, we also analyzed the effect of the transfection of expression plasmids for PXR and RXRα. Transfection of mouse (m) PXR and mRXRα expression plasmids enhanced this synergistic interaction on the Cyp3a44 gene approximately 1.9- to 2.7-fold (Fig. 5A). Enhancement was not restricted at supramicromolar concentration but was also observed at submicromolar concentration. This result suggests that PXR/RXRα might be involved in the synergistic interaction between GH and glucocorticoids on the Cyp3a44 gene at both submicromolar and supramicromolar concentrations of glucocorticoids. On the other hand, transfection of mPXR and mRXRα expression plasmids did not show a significant effect on synergistic interaction on the Cyp3a41 gene at all DEX concentrations examined (Fig. 5B). This result is in agreement with our previous study that the participation of PXR in the expression of the Cyp3a41 gene was not indicated by the use of RU486, a PXR agonist and a GR antagonist (Sakuma et al., 2004).
Discussion

In this study, we examined whether the GH signaling pathway for Cyp3a44 gene expression interacts with the glucocorticoid signaling pathway. Experimental data using MSG-treated mice were fundamentally consistent with those of the experiment using hypophysectomized mice (Sakuma et al., 2002), supporting the idea that GH secretion is one of the major determinants for female-specific expression of CYP3A44 mRNA. Interestingly, continuous administration of GH (1 μg/h) alone into MSG-treated mice clearly induced CYP3A44 expression over the level of untreated female mice, whereas restoration after the same treatment in hypophysectomized mice was 20% of control female mice (Sakuma et al., 2002). It is reported that serum glucocorticoid hormone levels declined after hypophysectomy (Pandak et al., 1997). On the other hand, it was reported that mice neonatally treated with MSG showed increased serum corticosterone levels under resting conditions (Magarinos et al., 1988). Additionally,
when hepatocytes were cultivated in medium containing both DEX and GH, synergistic induction of CYP3A44 mRNA by GH was seen only in cells exposed to GH continuously. Taken together with other data obtained in in vitro experiments, it is possible to predict that interaction between GH signaling by female-type exposure and glucocorticoid hormone signals functions in vivo as well as in vitro and is involved in female-predominant expression in the liver.

One possible mechanism of female-specific expression is repression by putative male-specific suppressor(s). A highly probable mechanism involves GH pulse-activated STAT5b. It is expected that GH
pulse-activated STAT5b could induce the expression of a male-specific repressor, which in turn could inhibit the expression of downstream female-specific genes (Waxman and O’Connor, 2006). In fact, it is highly likely that the male-specific repression of Cyp2b9 and Cyp2a4 gene expression in mouse liver is based on this mechanism. In the case of CYP3A44, mRNA expression was not observed in neonatally MSG-treated adult male mice (Fig. 1), and practically the same observation was made in hypophysectomized male mice (Sakuma et al., 2002). This observation supports the idea that female-specific expression of CYP3A44 is not due to repression by masculine GH secretion in males. This hypothesis, based on our studies, agrees with a recent report that CYP3A44 mRNA expression is not induced in STAT5b-deficient mice (Holloway et al., 2006).

In Fig. 2, RU486 alone or the concomitant addition of GH induced CYP3A44 mRNA expression. In contrast, these treatments did not influence CYP3A41 mRNA expression. These different responses to RU486 could be explained by the fact that RU486 has both PXR agonist and GR antagonist activities. Therefore, CYP3A44 is induced by the PXR agonist activity of RU486 via a PXR-mediated mechanism, whereas CYP3A41 is not. This explanation is supported by the results of Figs. 4 and 5, in which expression plasmids of GR and PXR/RXR, respectively, were utilized.

The inducible expression of CYP3A44 by submicromolar concentration of DEX or natural glucocorticoids alone is particularly low in primary cultured mouse hepatocytes (Fig. 2). Thus, the participation of this induction mechanism in constitutive expression in the liver was disputable, whereas involvement in the induction by pharmacological dosing was highly possible. However, the concomitant addition of GH caused strong induction of the expression, suggesting that glucocorticoid signaling participates in constitutive expression by means of cooperative interaction with GH. Based on the result in Fig. 5, it is predicted that PXR/RXRα could act as a mediator of glucocorticoid signaling at physiological concentration; however, Anakk et al. (2007) reported that the expression of CYP3A44 mRNA in female PXR-disrupted mice is unchanged. Taken together, it is likely that the participation of PXR/RXRα in constitutive expression is partial and can be compensated by GR. Indeed, it is possible for PXR/RXRα to act as a mediator at the pharmacological dose.

In this study, it was revealed that both GR and PXR could be involved in glucocorticoid hormone-mediated and GH-cooperative expression of Cyp3a44 gene. This is in contrast to Cyp3a41; virtually only GR participates in the induction by glucocorticoid hormones as well as in cooperation with GH signaling. The consequence of this difference is not clear at present but might cause different biological responses since ligand selectivity of these nuclear receptors is not identical. Similarly, different DEX concentration dependence of the two Cyp3a genes was seen in induction by DEX alone and was maintained even in synergistic interaction with GH and DEX. This suggests that factors directly interacting with DEX are identical in both induction mechanisms, and that GH can simply intensify the level of expression induced by glucocorticoid alone. At present, the detailed mechanism of the synergistic interaction between GH and glucocorticoid hormone is unclear, and further investigation is required. In conclusion, the present study found the following: 1) hepatic expression of the Cyp3a44 gene is under the control of GH, and synergistic interaction with glucocorticoid hormones and female-type GH secretion is necessary for higher expression; and 2) in contrast to the Cyp3a41 gene, in which GR is involved in the control by glucocorticoid hormones alone or in synergistic control by glucocorticoid hormones and GH, both PXR and GR could be involved in synergistic control by glucocorticoid hormones and GH, as well as by glucocorticoid hormones of the Cyp3a44 gene alone.

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References


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