DMD Fast Forward. Published on February 6, 2008 as DOI: 10.1124/dmd.107.019935
DMD Fast Forward. Published on February 6, 2008 as doi:10.1124/dmd.107.019935

DMD 019935

Synergism of glucocorticoid hormone with growth hormone for female-specific mouse *Cyp3a44* gene expression

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a) Running title: GH regulation of mouse *Cyp3a44* gene expression

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c) Text: 30 pages

Tables: 0

Figures: 5

References: 36

Abstract: 210 words

Introduction: 616 words

Discussion: 851 words

d) Non-standard abbreviations: CYP, cytochrome P450; DEX, dexamethasone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GH, growth hormone; GR, glucocorticoid receptor; MSG, monosodium L-glutamate; PCR, polymerase chain reaction; PXR, pregnane X receptor; RT, reverse transcription; RU486, 11β-[4-dimethylamino]phenyl-17β-hydroxy-17-[1-propynyl]estra-4,9-dien-3-one; STAT, signal transducer and activator of transcription.

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Abstract

CYP3A44 and CYP3A41 are female-specific CYP3A in the mouse liver. In primary cultured mouse hepatocytes, dexamethasone (DEX) concentration-dependently induced CYP3A44 mRNA, and the highest response was seen at 10^{-5}M. In contrast, CYP3A41 mRNA expression was highest at lower concentrations (10^{-7}M 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 co-transfection of pregnane X receptor (PXR) and retinoid X receptor alpha (RXRα) expression plasmids. Similar synergistic induction was seen in CYP3A41 mRNA by the transfection of GR expression plasmid, but was not enhanced by co-transfection of PXR and RXR expression plasmids. These observations suggest that functional crosstalk 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.

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Several clinical studies have suggested that oxidative metabolism of drugs and steroids is disturbed after the therapeutic use of glucocorticoids (Watkins et al., 1985; Watkins et al., 1989) or 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 by 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; Pascussi et al., 2001).

Female-specific expression of rodent *CYP* genes, including rat *CYP2C12* (MacGeoch et al., 1984; Kamataki et al., 1985), *CYP3A9* (Kawai et al., 2000) and mouse *Cyp3a41* (Sakuma et al., 2002), *Cyp3a44* (Sakuma et al., 2002) demonstrated that the sex-specific pattern of growth hormone (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

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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., 2006). 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 (Watkins et al., 1985; Watkins et al., 1989; Greenblatt et al., 1980; 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 BAC clone, which contains the complete *CYP3A4* gene sequence, including 5' and 3' flanking regions (Cheung et al., 2006). Additionally, the expression profile and responsiveness to GH treatment of the human *CYP3A4* gene were identical to those of mouse *Cyp3a44* gene. These findings strongly suggest that both human *CYP3A4* and mouse *Cyp3a44* genes share 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; Sakuma et al., 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

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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, while the expression of CYP3A41 is markedly changed (Sakuma et al., 2002). Their responsiveness to glucocorticoid hormones also differs between the two genes (Bhadhprasit et al., 2007); 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 to that of the *Cyp3a41* gene.

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Materials and Methods

Materials

Materials for culturing hepatocytes were purchased from Wako Pure Chemicals (Osaka, Japan), Invitrogen Corp. (Carlsbad, CA), and Sigma-Aldrich (St. Louis, MO). Percoll was obtained from GE Healthcare UK Ltd. (Buckinghamshire, England). TaKaRa RNA PCR Kit (AMV) version 2.1 was obtained from TaKaRa Shuzo (Kyoto, Japan). TaqMan MGB Gene Expression Detection kits were products of Applied Biosystems (Branchburg, NJ). Recombinant human GH (rhGH) and monosodium L-glutamate (MSG) were obtained from Wako Pure Chemicals (Osaka, Japan) and an Alzet micro-osmotic pump from Durect (Cupertino, CA). DEX, hydrocortisone, corticosterone and 11 β-[4-dimethylamino] phenyl-17 β -hydroxy-17- [1-propynyl] estra-4, 9-dien-3-one (RU486) were obtained from Sigma Chemicals. 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 paperchip bedding in plastic cages, with water and commercial mouse diet supplied *ad libitum*. The housing

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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 subcutaneously injected with MSG at 2 mg/g 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 subcutaneous injection at a dose of 50μg/injection twice daily (mimicking male-type GH secretion) or continuous infusion using a subcutaneously 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 sacrified 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 microgram/L), and seeded in uncoated dishes at a

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density of 2 x 10^6 cells/ 4 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 CO_2-humidified incubator.

Construction of expression plasmids for GR, PXR or RXRα

The GR expression plasmid was generated by replacing the DNA fragment between *Nhe* I and *Xba* I sites containing the coding sequence of *Renilla* luciferase of pRL-SV40 vector (Promega, Madison, WI) 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 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 RXR α expression plasmid contains the 1,409 bp cDNA fragment with the entire coding region (1,404 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 Transpass™ D1 Transfection Reagent (New England Biolabs, Hercules, CA). Transfection mixtures consisted of Waymouth medium, empty plasmid or nuclear receptor expression plasmid and Transpass ™ D1 at 2ml, 5 μg

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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 (71ng/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 Corp, Carlsbad, CA). The expression of CYP3A44 mRNA was analyzed by real-time RT-PCR using a TaKaRa RNA PCR Kit (AMV) version 2.1 with 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'-GAAACTGCAGGCAGAGATCCATA-3', 5'-TTTCTTACAGACTCTCTC TCAAGTCTAGTAACAAT -3' and 5'-FAM-AATAAGGCAACTCCCACCTG-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, 3A59. The expression of CYP3A41 mRNA was analyzed by real-time RT-PCR with a TaqMan MGB probe as described previously (Jarukamjorn et al., 2006). The expression of CYP2D9 mRNA was analyzed by real-time RT-PCR with TaqMan Gene Expression assay for CYP2D9 (Mm00651731_m1). GAPDH cDNA amplification was detected using TaKaRa RNA PCR Kit (AMV) version 2.1, SYBR Green reagent, and the primer set specific to mouse GAPDH cDNA (Jarukamjorn

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et al., 2006). PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with ABI Prism 7000 SDS software.

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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, in order 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 mini-pump, which mimics female-type secretion, clearly induced mRNA expression of CYP3A44 over the level of untreated female mice. In contrast, subcutaneous injection of GH twice a day, which mimics male-type secretion,

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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, RU-486 alone at supramicromolar concentration (10^{-5}M) induced CYP3A44 mRNA expression, and concomitant treatment with GH and RU-486 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

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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 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 11h. This successive treatment was repeated for 4 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., 2006), 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

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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 1h 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). In order 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 GR (mGR) 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^{-5}M. With CYP3A41, similar synergistic interaction and enhancement by mGR were also observed; however, the highest expression was seen either in the

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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 PXR (mPXR) and RXRα (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).

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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, while 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

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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 DJ 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, RU-486 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, while 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

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mouse hepatocytes (Fig. 2). Thus, the participation of this induction mechanism in constitutive expression in the liver was disputable, while 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. reported that the expression of CYP3A44 mRNA in female PXR-disrupted mice is unchanged (Anakk et al., 2007). 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 dependency 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

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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; 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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Acknowledgements

We would like to acknowledge Ms Yukako Ishino and Ms Kaori Kitajima for their assistance with experiments.

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Footnotes

This work was partly supported by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Smoking Research Foundation.

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Figure legends

Fig. 1 Expression profile of CYP3A44 mRNA in the liver of C57BL/6 mice neonatally treated with MSG.

Male and female mice neonatally treated with MSG were killed at 3 and 9 weeks of age. Adult male and female mice neonatally treated with MSG were subcutaneously injected with rhGH (50 μg/mouse) every 12 h for 7 days (GH(i)) or received an continuous infusion (1.5 μg/h) of rhGH (GH(sc)). Hepatic total RNA (20 ng) was reverse-transcribed and cDNA was amplified using a specific TaqMan MGB Gene Expression Detection kit for *Cyp3a44*, and the SYBR Green PCR Master Mix for GAPDH. The mRNA expression of CYP3A44 was normalized to that of GAPDH. The normalized mRNA level of CYP3A44 is shown relative to the level in untreated female mice of 9 weeks of age (=1). Each column represents the mean ± S.D. (n=4). Significance was examined using ANOVA and the Bonferroni test (* $P<0.01$).

Fig. 2 Effects of GH, glucocorticoid hormones and anti-glucocorticoid RU486 on the expression of CYP3A44 and CYP3A41 mRNAs in mouse hepatocytes in primary culture.

Hepatocytes isolated from female or male ddY mice were cultured for 2 days and treated with GH, DEX, hydrocortisone, corticosterone and RU486 for another 24 h. Expression of each mRNA was evaluated by quantitative real-time RT-PCR analysis. Each mRNA level was normalized to the level of GAPDH and is shown

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relative to those in untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using ANOVA and the Dunnett test (* $P<0.05$). (A), CYP3A44; (B), CYP3A41.

Fig. 3 Distinct effect of continuous exposure from discontinuous exposure of GH on the expression of CYP3A44 and CYP3A41 mRNA in female hepatocytes in primary culture.

Hepatocytes isolated from female or male ddY mice were cultured for 2 days, and then cells were treated with DEX and GH by two different protocols for 49 h: continuous exposure to both DEX (10^{-7}M) and rhGH (20 ng/ml) for 49 h, and discontinuous exposure to GH. Hepatocytes were cultivated in medium containing both GH (240 ng/ml) and DEX (10^{-7}M) for 1 h, and then the medium was changed to that containing DEX (10^{-7}M) alone and incubated for 11 h. This treatment was repeated 4 times during 48 h, and concluded with an additional treatment with GH and DEX for 1 h (total 49 h). Expressions of CYP2D9 mRNA in male hepatocytes (A) or CYP3A44 and CYP3A41 mRNAs in female hepatocytes (B) were evaluated by quantitative real-time RT-PCR. mRNA expression levels were normalized to those of GAPDH and are shown relative to those in control plasmid-transfected untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using ANOVA and the Bonferroni test. *, ** Significantly different from DEX (10^{-7}M)-treated cells ($P<0.05$, $P<0.01$, respectively).

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Fig. 4 Enhancement of GH- and DEX-induced synergistic expression of CYP3A44 and CYP3A41 mRNAs by glucocorticoid receptor in mouse hepatocytes in primary culture.

Hepatocytes isolated from female ddY mice were cultured for 2 days and transfected with control (empty vector) or mGR expression plasmid. Twenty-four hours later, cells were treated with DEX at 10^{-7}M to 10^{-5}M and/or rhGH (71ng/ml) for another 24 hours. Expression of CYP3A44 or CYP3A41 mRNAs was evaluated by quantitative real-time RT-PCR. mRNA expression levels were normalized to those of GAPDH and are shown relative to those in control plasmid-transfected untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using ANOVA and the Bonferroni test (* $P<0.05$; ** $P<0.01$).

Fig. 5 Enhancement of GH- and DEX-induced synergistic expression of CYP3A44 or CYP3A41 mRNAs by pregnane X receptor and retinoid X receptor alpha in mouse hepatocytes in primary culture.

Hepatocytes isolated from female ddY mice were cultured for 2 days and transfected with control plasmid (empty vector) or mPXR and mRXRα expression plasmids. Twenty-four hours later, cells were treated with DEX at 10^{-7}M to 10^{-5}M and/or rhGH (71ng/ml) for another 24 hours. Expression of CYP3A44 or CYP3A41 mRNAs mRNA was evaluated by quantitative real-time RT-PCR

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analysis. mRNA levels were normalized to those of GAPDH and are shown relative to those in control plasmid-transfected untreated hepatocytes. Each column represents the mean ± SD (n=3). Significance was examined using ANOVA and the Bonferroni test (* $P<0.01$).

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Figure 1

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Figure 2

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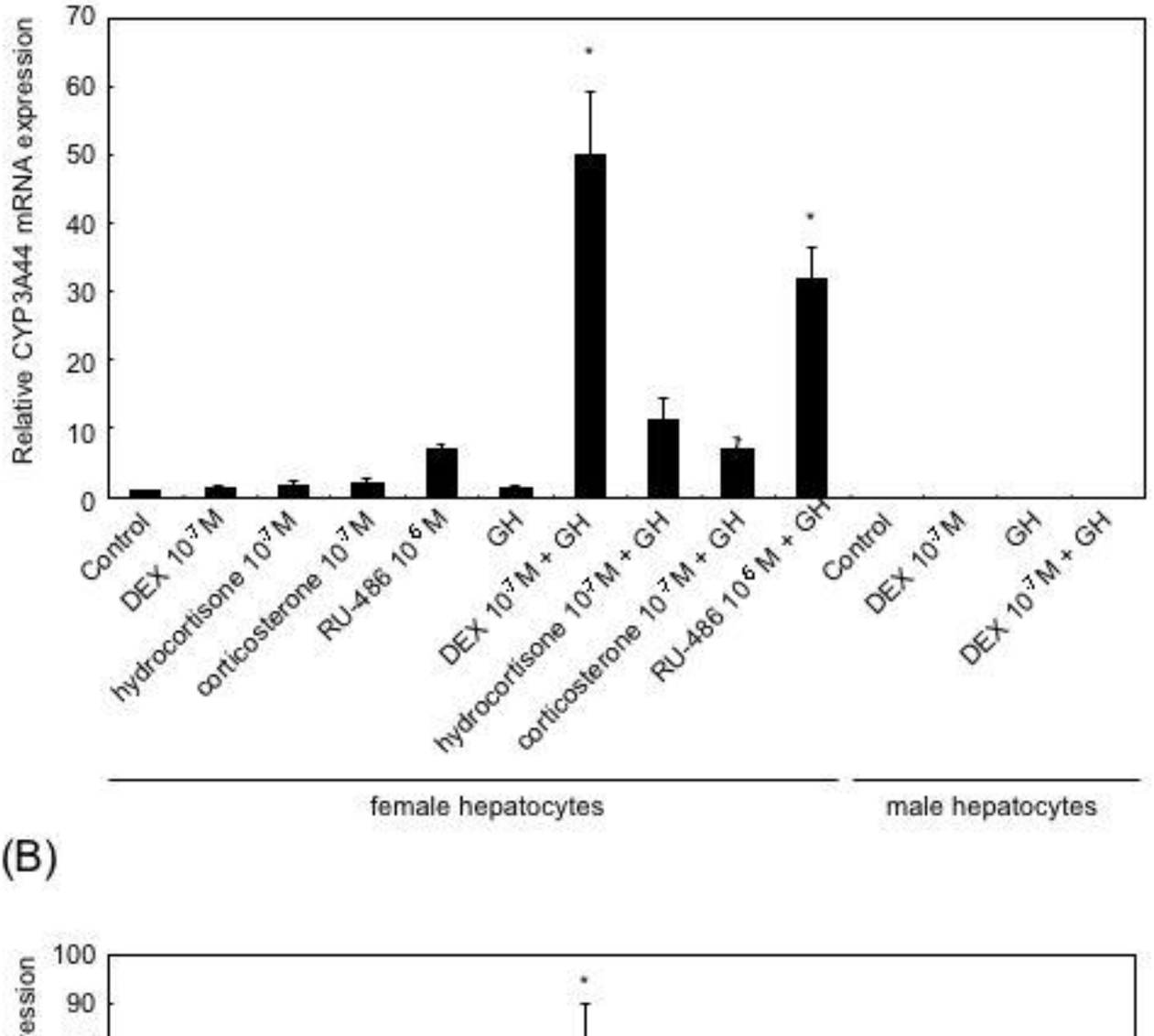
(A)



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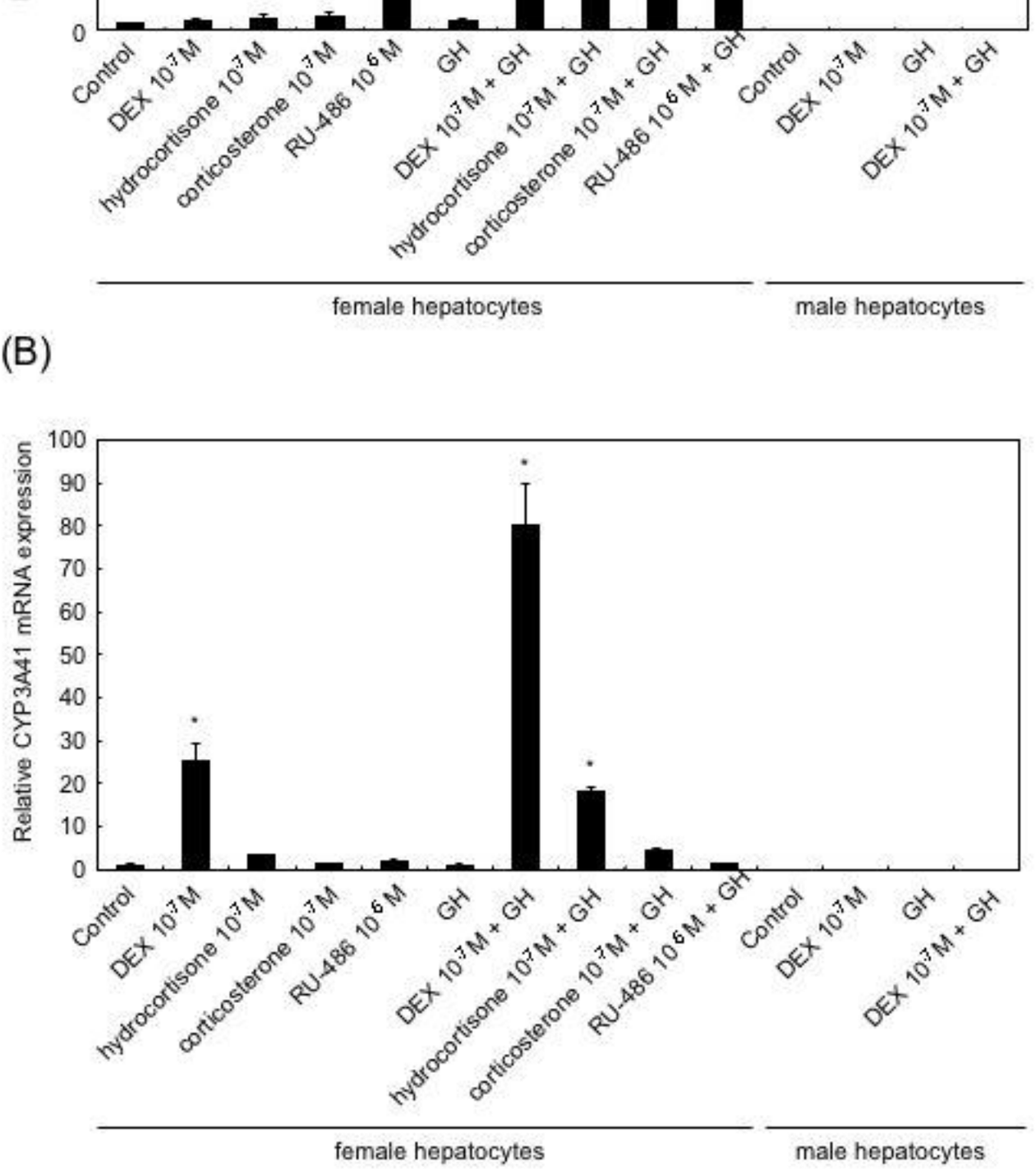

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Figure 3

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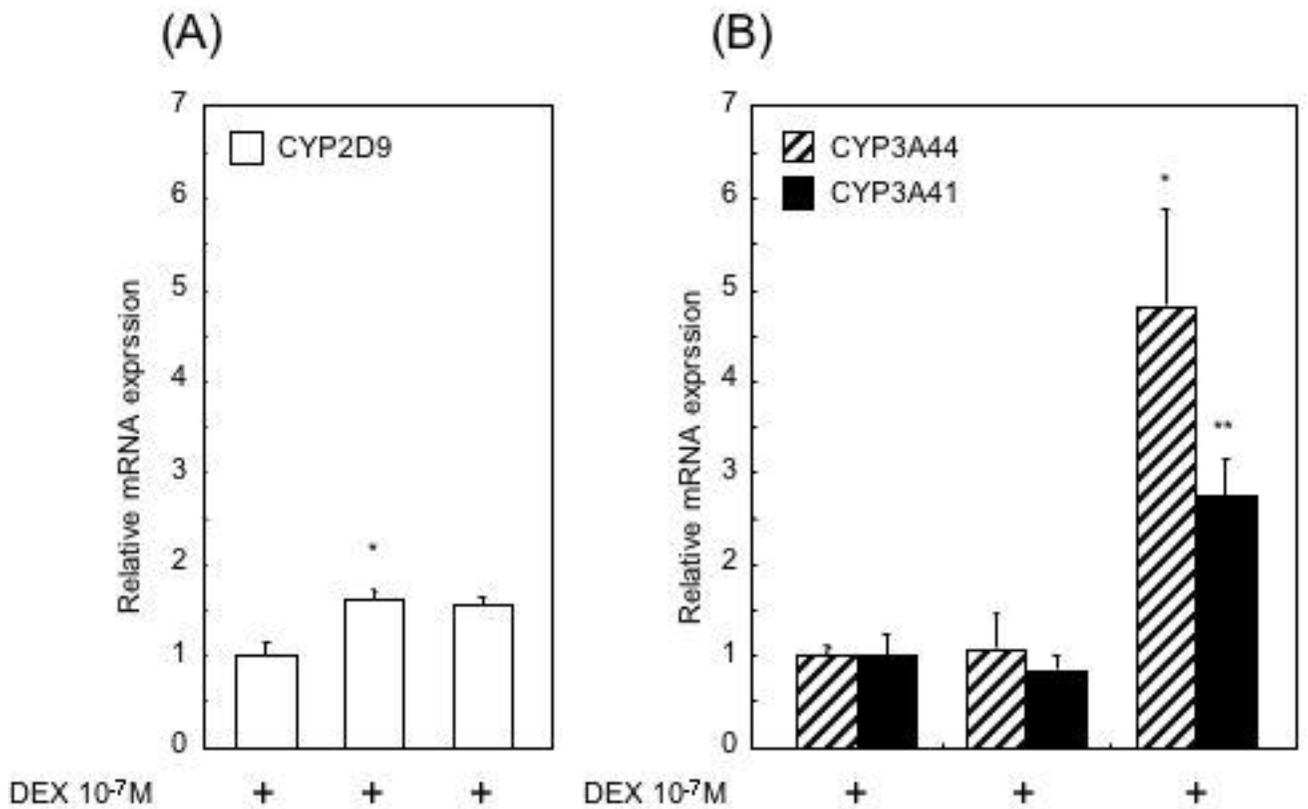

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Figure 4

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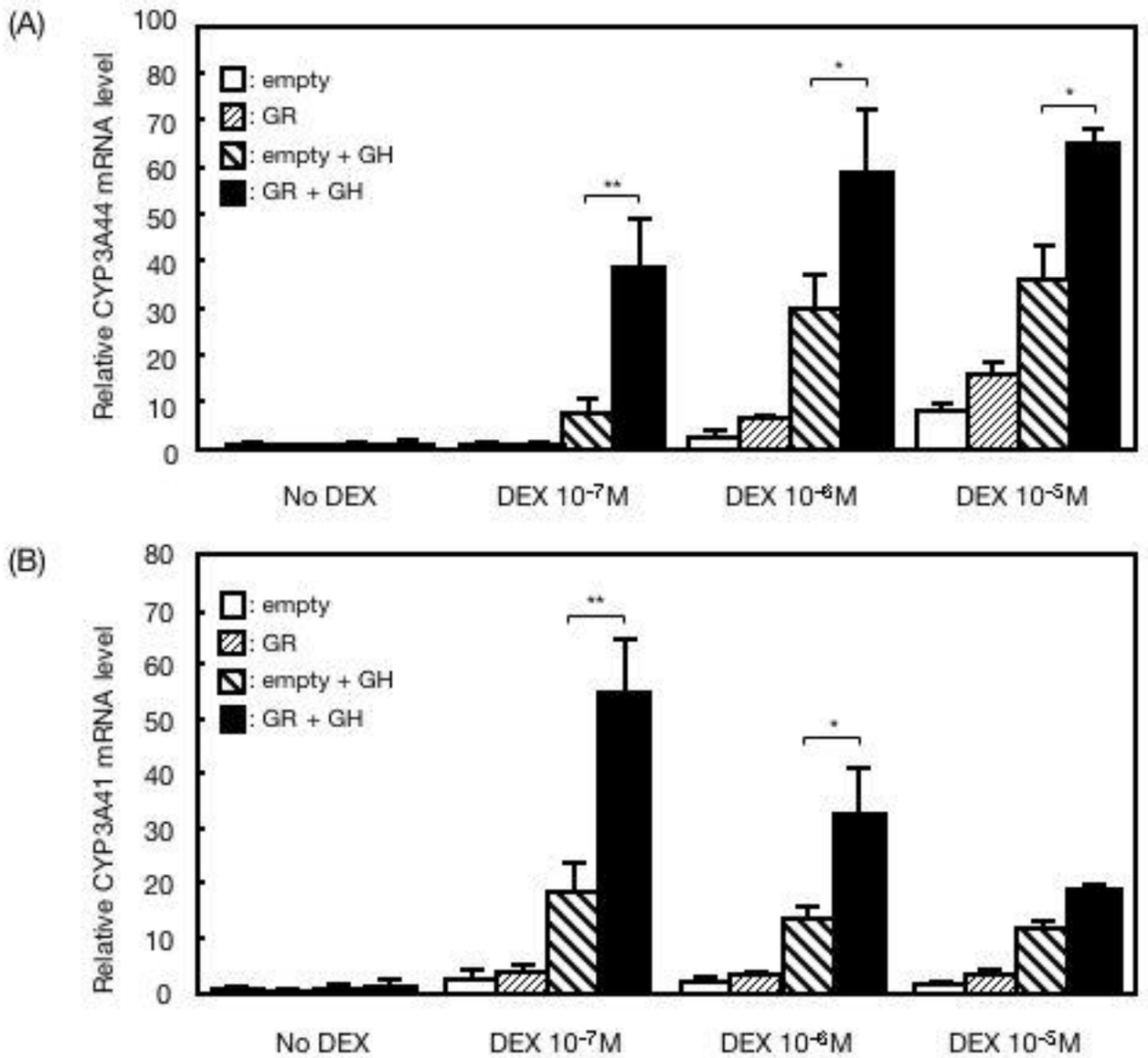

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Figure 5

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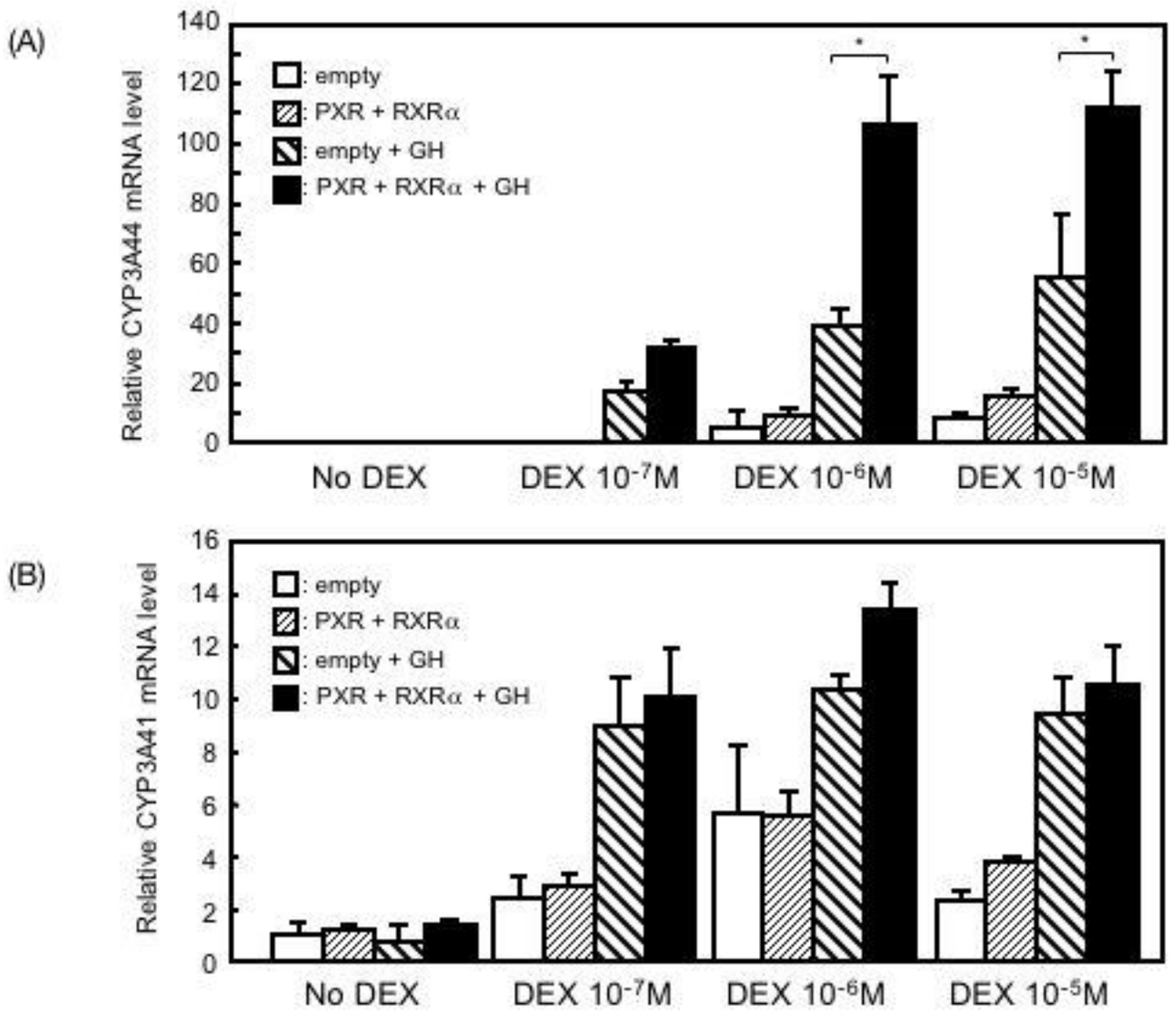

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