Modified expression of cytochrome P450 mRNAs by growth hormone in mouse liver

Kanokwan Jarukamjorn\textsuperscript{a,b}, Tsutomu Sakuma\textsuperscript{b}, Atika Jaruchotikamol\textsuperscript{b}, Yukako Ishino\textsuperscript{b}, Miki Oguro\textsuperscript{b}, Nobuo Nemoto\textsuperscript{b,*}

\textsuperscript{a} Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
\textsuperscript{b} Department of Toxicology, Faculty of Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

Received 11 October 2005; received in revised form 5 November 2005; accepted 7 November 2005
Available online 27 December 2005

Abstract

The expression of eight mouse hepatic cytochrome P450s (P450s) genes was investigated at the mRNA level in relation with the pattern of growth hormone (GH) administration. The constitutive expression of five sex-dependent P450s was sexually dimorphic, namely female > male for CYP2A4, CYP2B9, CYP2B10, and CYP3A41, and male > female for CYP2D9. In mice neonatally treated with monosodium \textit{l}-glutamate to produce GH-deficiency, GH was found to be an essential factor with GH archetype as a determinant in the regulatory mechanism of hepatic CYP2D9 and CYP3A41 expression, and GH was shown to be a repressive factor for the constitutive expression in females. Implantation with micro-osmotic pump containing GH (to yield a constant release of GH to mimic the plasma GH profile in females) to male mice increased CYP2A4, CYP2B9, CYP2B10, and CYP3A41, but decreased CYP2D9 expression to female levels, while conversely, twice-daily administration of GH (to produce the so-called male pattern of plasma GH levels) to female mice resulted in the repression of female-specific, CYP2B9 and CYP3A41, as well as female-predominant, CYP2A4 and CYP2B10, expression, and induction of male-specific CYP2D9 expression. Thus, the sex-dependent plasma GH profile (referred to hereafter as the GH archetype) was a decisive factor for the expression of sex-specific P450 genes in adult mouse liver. On the other hand, the regulation of CYP1A2, CYP2C29, and CYP3A11 expression was either sex-independent or GH archetype-independent, considering the comparable levels between sexes of the constitutive expression and GH-inducible expression of these isoforms. Moreover, the observations suggested for the first time that the expression of CYP2B9 and CYP2A4 was not entirely GH-independent, but rather involved an imprinting GH-related factor that participated in the regulatory mechanism of P450 expression in females.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Growth hormone; Cytochrome P450; CYP1A2; CYP2A4; CYP2B9; CYP2B10; CYP2C29; CYP2D9; CYP3A11; CYP3A41; Monosodium glutamate

1. Introduction

Growth hormone (GH) is a pituitary-derived polypeptide hormone that is released into the circulation in an intermittent or pulsatile pattern and exhibits multiple physiological effects (Lobie and Waxman, 2003). GH release is ultimately regulated by the action of gonadal hormones on the hypothalamus, which gives rise to

Abbreviations: GH, growth hormone; P450s, cytochrome P450s; MSG, monosodium \textit{l}-glutamate; rh, recombinant human; NSS, 0.9% normal saline solution; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; STAT, signal transducer and activator of transcription
* Corresponding author. Tel.: +81 76 434 7520; fax: +81 76 434 5048.
E-mail address: nemoto@ms.toyama-mpu.ac.jp (N. Nemoto).
imported sex-differences in the temporal pattern of circulating GH (Janss0n et al., 1985). Sex-differences in plasma GH profiles are most dramatic in rodents, but significant male–female differences in the regulation of pituitary GH release also exist in humans (Veldhuis et al., 2001). Sex-dependent differences in plasma GH profiles first emerge at puberty but are set, and ultimately regulated, by gonadal steroid imprinting during the neonatal period (Jansson and Frohman, 1987). The sexually dimorphic plasma GH profiles dictate the sex-dependent effects that GH imparts on body growth rates at puberty and on the sex-dependent expression of cytochrome P450s (P450s) and other liver enzymes (Shapiro et al., 1995; Waxman, 2000a). However, how GH differentially influences the expression of sex-dependent and -independent P450s is an important question that is still under investigation.

P450s are heme-containing membrane-bound enzymes that play important roles in the metabolism of endogenous steroids and fatty acids, as well as detoxification of foreign compounds, including many drugs and environmental chemicals (Negishi et al., 1983). Hepatic P450 genes are regulated at the level of transcription, particularly contributed to regulating the expression of sex-specific and -nonspecific P450s. neonatal administration of monosodium L-glutamate (MSG) to rats, mice, and possibly other species produces a profound, but rather selective growth hormone deficiency, resulting in a well-defined syndrome characterized by stunted growth and obesity (Olney and Ho, 1970; Shapiro et al., 1989). Affected adults exhibit abnormalities in both the secretion of GH and the expression of P450s (Kaukhold et al., 2002; Agrawal and Shapiro, 1997). Hypothalamic lesions induced by neonatal administration of MSG permanently and rather selectively block GH secretion, resulting in dramatic changes in hepatic monoxygenase activities, reflecting in part the abnormal modulation of some sex-dependent P450s in the rats (Pampori et al., 1991; Waxman et al., 1990a). Since neonatal exposure to MSG has been proven to induce GH-depletion in rats, providing a useful model for studying the regulation by GH of hepatic monoxygenases (Agrawal and Shapiro, 1997; Pampori et al., 1991; Waxman et al., 1990a; Shapiro et al., 1993), it is worth attempting to use a similar model in mice to examine the effect of GH on the expression of mouse hepatic P450 enzymes.

The present study investigated whether GH differently contributed to regulating the expression of sex-dependent genes Cyp2a4, Cyp2b9, Cyp2c10, Cyp2c49, and Cyp3a41, as well as sex-independent Cyp1a2, Cyp2c29, and Cyp3a11 genes in mouse liver. Our observations suggest that the sex-dependent plasma profile of GH (GH archetype) is one of the important determinants for the differential patterns of constitutive expression of mRNAs of P450s. Administration of GH on schedules mimicking the female and male GH archetype to mice provided evidence supporting the role of GH archetype on the modification profile of P450s mRNA expression.

2. Materials and methods

2.1. Materials

An Alzet® micro-osmotic pump, model 1007D, was obtained from Durect Corporation (Cupertino, CA). Recombinant human growth hormone (rGH) and monosodium L-glutamate (MSG) were supplied by Wako Pure Chemical (Osaka, Japan). The TaqMan® Gold RT-PCR kit, TaqMan® Gene Expression Assays, and SYBR® Green PCR Master Mix were products of Applied Biosystems (Branchburg, NJ). All other laboratory chemicals were of the highest available purity from commercial suppliers.

2.2. Animals

C57BL/6N(C) mice (Charles River, Japan) 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. At all times, the mice were housed on paper chip bedding in plastic cages, with water and commercial mouse diet supplied ad libitum. The mice quarters were air conditioned (20–23 °C) and had a 12-h light/dark cycle. Adult mice of both sexes were subcutaneously injected with rhGH (50 μg/mouse) at a dose of 50 μg/l (suggested by the supplier). The cDNAs were synthesized under the conditions recommended by the supplier (Applied Biosystems, Branchburg, NJ) of the TaqMan® Gold RT-PCR kit using a specific TaqMan® Gene Expression Assay (Inventoried) for Cyp2d9 (Mm00725580_q1), Cyp2c29 (Mm00725580_q1), Cyp2a4 (Mm00651731_q1), and GAPDH, in which the forward and reverse primers for Cyp2a4 were 5'-AGG CAC GTG TAT CAG AGC ACC A-3' and 5'-ATG CTT TGG TCT TCT CAG-3', respectively, and those for GAPDH were 5'-TCC ACT CAC GGC AAA TTC AAC G-3' and 5'-TAG ACT CCA CGA CAT ACT CAG C-3', respectively. The specificity of amplification of CYP2A4 and GAPDH cDNAs was confirmed by polyacrylamide gel electrophoresis and the dissociation curve of each product. PCR was performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems) with ABI Prism® 7000 SDS software. The PCR conditions were as follows: activation of AmpliTag UNG and AmpliTaq Gold at 50 °C for 2 min and 95 °C for 10 min, respectively, and then amplification with denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The amplified products of CYP1A2, CYP2B9, CYP2B10, CYP2C29, CYP2D9, CYP3A11, and CYP4A11 were detected directly by monitoring the fluorescence of the reporter dye (FAM), for which an increase in fluorescent signal was detected only if the target sequence was complementary to the probe and amplified by the PCR. The amplified PCR products of CYP2A4 and GAPDH were monitored directly by measuring the increase in SYBR® Green that was bound to double-stranded DNA amplified by the PCR.

3. Results

3.1. Impact of GH on hepatic mRNA expression of P450s in mice neonatally treated with MSG

To investigate the physiological role of GH archetype on the expression of P450 genes, pups were treated with MSG during the early period of life to become profoundly growth retarded. The expression levels of P450s in mice neonatally treated with MSG were relatively low as compared to those in the intact mice of the same sex, respectively (Fig. 1A and B). The administration of GH on schedules mimicking the respective archetypes restored the sexual dimorphism of the mRNA expression of these two genes in the adult mouse neonatally treated with MSG as the constitutive expression in the intact mice: the male-type GH administration significantly elevated the CYP2D9 mRNA expression in the adult males, while the female-type GH administration markedly raised that of CYP3A41 mRNA in the adult females. These findings suggest that GH is an essential factor for sexual dimorphism of the constitutive expression of the Cyp3a41 and Cyp2d9 genes in mouse liver.

Unlike CYP2D9 and CYP3A41 expression, CYP2B9 and CYP2A4 mRNA expression strongly increased
Fig. 1. Expression profile of sex-specific and -nonspecific P450 mRNAs in the liver of C57BL/6 mice neonatally administered 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/kg) every 12 h for 7 days or received an osmotic infusion (1.5 μg/h) of rhGH. Hepatic total RNA (4 ng) was reverse-transcribed and cDNA was synthesized using a specific TaqMan® Gene Expression Assay (Inventoried) for Cyp1a2, Cyp2b9, Cyp2b10, Cyp2c29, Cyp2d9, and Cyp3a11, and a specific TaqMan® MGB Gene Expression Detection kit for Cyp3a41, and the SYBR® Green PCR Master Mix for Cyp2a4 and GAPDH. The mRNA levels of these P450s were normalized to that of GAPDH. The normalized mRNA level of CYP2D9 is shown relative to the level in un-treated male mice, while those of CYP1A2, CYP2A4, CYP2B9, CYP2B10, CYP2C29, CYP3A11, and CYP3A41 are shown relative to the level in un-treated female mice. Each column represents the mean ± S.D. (n=4), 3, 3 weeks of age; 9, 9 weeks of age; NT, no treatment; MSG, neonatal administration of MSG; GH (i), continuous infusion of rhGH using a micro-osmotic pump; GH (sc), twice daily subcutaneous injection of rhGH. Significance was examined using the Student’s t-test; *p < 0.01 and **p < 0.001 (vs. 3-week-old intact mice of each sex); #p < 0.01 and ##p < 0.001 (vs. same age of each sex); $p < 0.01 and $$p < 0.001 (vs. 3-week-old mice of each sex with neonatal exposure to MSG); ∆p < 0.01 and ∆∆p < 0.01 (vs. 9-week-old mice of each sex with neonatal exposure to MSG).

The constitutive expression of CYP2B10 mRNA in adult mice neonatally treated with MSG was female-predominant, like that in the intact mice, though an increase of expression was clearly observed (Fig. 1E). The female-type administration of GH greatly repressed the CYP2B10 mRNA expression in the females neonatally treated with MSG. These observations suggested a repressive role of GH on the expression of this mRNA in females. Unexpectedly, the male-type GH administration increased the CYP2B10 mRNA expression in the males neonatally treated with MSG.

The constitutive expression of sex-nonspecific CYP1A2, CYP2C29, and CYP3A11 mRNAs was comparable between the sexes in intact mice (Fig. 1F–H).
Neonatal administration of MSG did not change the mRNA expression levels of these sex-nonspecific genes in adult mice, although a decrease of CYP1A2 and CYP3A11 mRNA expression was observed at 3 weeks of age (Fig. 1F–H). Continuous infusion of GH in mice neonatally treated with MSG clearly suppressed the expression of CYP2C29 and CYP3A11 mRNAs, but not CYP1A2 mRNA, while no significant change of the expression of these mRNAs was detected after the male-type administration of GH. The observation that GH did not modulate the CYP3A11 mRNA expression in males neonatally treated with MSG, in accord with the observations about the expression in hypophysectomized mice (Sakuma et al., 2002) implied that the expression of sex-nonspecific CYP3A11 mRNA was GH-independent in male mice.

3.2. Hepatic mRNA expression of P450s by GH archetype in intact mice

To investigate the role of exogenously administered GH on the gene expression of hepatic female-specific Cyp2b9 and Cyp3a41, as well as hepatic female-predominant Cyp2ad and Cyp2b10 genes, in vivo the secretion pattern of GH was mimicked by appropriate administration (see Section 2) of rhGH to mice of both sexes. The constitutive expression of CYP2A4, CYP2B9, CYP2B10, and CYP3A41 mRNAs was sexually dimorphic, namely female > male: CYP2A4 (female to male mRNA expression ratio = 3.3), CYP2B9 (16.9-fold), CYP2B10 (9.6-fold), and CYP3A41 (>1000-fold sex difference) (Fig. 2A–D). Repetition of GH (mimicking the respective GH archetypes) consistently authorized expression profiles of female-specific genes similar...
to those in the intact mice. Continuous infusion of rhGH (mimicking the female GH archetype) significantly promoted the expression of these four P450 mRNAs in the males, whereas twice daily injections of rhGH (mimicking the male GH archetype) markedly diminished the expression of CYP2A4, CYP2B9, and CYP3A41, but not of CYP2B10, in the females. Impressively, exogenous GH administration on a schedule mimicking that of intact circulating GH did not disrupt the GH archetype’s regulation of the expression of these mRNAs; the expression of CYP2A4, CYP2B9, and CYP3A41 mRNAs was not repressed in the males or inducible in the females by the male-type or female-type schedule of administration of GH, respectively. On the other hand, the CYP2B10 mRNA expression was impacted by sex, not by the profile of GH administration. The administration of GH on either the male- or female-type schedule noticeably elevated the expression of CYP2B10 mRNA only in the males, but it did not modulate it in the females.

The expression of the hepatic male-specific Cyp2d9 gene, as well as hepatic sex-nonspecific Cyp2c29, Cyp3a11, and Cyp1a2 genes, was examined after administration of GH to intact mice of both sexes. The level of CYP2D9 mRNA expression was constitutively high in the males, while it was low in females (Fig. 2E, 12.3-fold sex difference) as in previous reports (Sueyoshi et al., 1999; Sakuma et al., 2004b). Twice daily injection of rhGH significantly promoted CYP2D9 mRNA expression in female mice, whereas continuous infusion of rhGH markedly diminished the expression in the males. The results suggested that the GH archetype’s regulation of CYP2D9 mRNA expression was disrupted by exogenously administered GH, corresponding to the findings of previous studies (Sakuma et al., 2004b). The constitutive expression of CYP2C29, CYP3A11, and CYP1A2 mRNAs was comparable between the sexes (Fig. 2F–H). Administration of GH on the male-type schedule significantly increased the expression of these sex-nonspecific P450s mRNA in both sexes, but modulation of the expression of these genes was not observed after administration of GH on the female-type schedule.

### 4. Discussion

The present study investigated the effect of the sex-specific pattern of GH secretion on the expression of eight sex-dependent and sex-independent P450 genes (Table 1). The observations demonstrated that the GH archetype was a decisive factor for the expression of sex-dependent P450 genes in the adult mouse liver. The mouse neonatally treated with MSG is an experimental model which allows for the non-surgical suppression of
the adult GH level with largely unchanged levels of other pituitary-dependent hormones, such as testosterone and estradiol (Waxman et al., 1990a), to examine the imprinting role of GH archetype on the developmental profile of expression of P450s. Furthermore, the GH-deficient Little mouse, a mutant mouse strain which has an autosomal recessive inheritance of growth defect (Eicher and Beamer, 1976), is another experimental model to study the effect of GH after birth on the regulation of P450s. Another model, primarily based on hypophysectomy with GH replacement (Sakuma et al., 2004a, b), was utilized to investigate P450 regulation by GH in adult animals. In agreement with the finding of experiments in GH-deficient Little mice (Noshiro and Negishi, 1986) and hypophysectomized mice (unpublished result), we found here that the repression of CYP2D9 mRNA by GH-depletion was counteracted by exogenous pulsatile GH replacement, but not by continuous infusion of GH (Fig. 1A). This finding revealed that GH is an essential factor in association with GH archetype as a determinant in the regulatory mechanism of the male-specific Cyp2d9 gene in the mouse liver.

GH exhibited the same pattern in the regulation of CYP3A41 mRNA expression in mice neonatally treated with MSG (Fig. 1B) and hypophysectomized mice (Sakuma et al., 2002, 2004a). In addition to the regulation by GH, adrenalectomy resulted in partial repression of both CYP2D9 and CYP3A41 (Sakuma et al., 2004a) mRNA expression in mouse liver. Furthermore, administration of dexamethasone, a synthetic glucocorticoid hormone, to adrenalectomized mice significantly elevated the mRNA expression levels of these two genes (Sakuma et al., 2004a; unpublished result). Therefore, the mechanism of the regulation of the Cyp2d9 gene by GH might also apply to the regulation of the Cyp3a41 gene. However, some different features of the regulation of these two genes by other endocrine hormones, such as sex hormones, need to be clarified further to fully elucidate the regulatory mechanisms of these genes.

The strong increase of CYP2A4 mRNA expression during development together with the suppression of the expression by pulsatile GH in mice neonatally treated with MSG (Fig. 1D) were in accord with the findings of experiments using GH-deficient Little mice (Noshiro and Negishi, 1986). For the Cyp2b10 gene, the same mRNA expression pattern was seen in mice neonatally treated with MSG (Fig. 1C): the CYP2B9 mRNA expression was markedly elevated in mice neonatally treated with MSG, in which the expression was suppressed by GH treatment. The changes of CYP2A4 and CYP2B9 mRNA expression in mice neonatally treated with MSG suggested the GH-dependent expression of these genes in females. Similar phenomena, in which GH suppressed the expression in both sexes, were observed in the cases of rat CYP3A2 (Waxman et al., 1990b; Pamporti et al., 2001) and rat CYP2B1 and CYP2B2 (Murayama et al., 1991). These findings revealed that GH is a repressive factor for the constitutive expression in females and suggested for the first time that CYP2B9 and CYP2A4 expression in females was not entirely GH-independent. Furthermore, these findings suggested the existence of a GH-related imprinting factor that participates in the regulatory mechanism of the expression of P450s in females.

Replacement of GH in male mice on a female-type schedule increased the expression of female-specific CYP2B9 and CYP3A41 mRNAs, as well as female-predominant CYP2A4 and CYP2B10 mRNAs, but repressed the expression of male-specific CYP2D9 mRNA to female levels. Conversely, induction of CYP2D9 expression and suppression of CYP2A4, CYP2B9, and CYP3A1 expression, but not of CYP2B10 expression, was noted after replacement of GH on a male-type schedule in female mice. Based on the mechanisms for the feminization of the sex-specific P450s proposed by Negishi’s group (Sueyoshi et al., 1999) and the critical importance of STAT5b protein for sex-specific P450 expression (Waxman et al., 1995; Udy et al., 1997; Teglund et al., 1998; Davey et al., 1999; Park et al., 1999), the following regulatory pathway has been proposed: the nuclear translocation of STAT5b in female hepatocytes might be initiated by the pharmacological status of GH from pulsatile GH treatment, resulting in activation of male-specific gene or repression of female-specific genes in females. Vice versa, continuous GH treatment might mask the physiological level of GH secretion in male mice, resulting in a lack of nuclear translocation of STAT5b in hepatocytes; consequently, the expression of female- or male-specific genes in the males was up- or down-regulated, respectively. Alternatively, exogenous GH treatment might have conveyed pharmacological or toxicological action of GH in the circulation, and consequently disguised the physiological regulation of the expression of P450s by GH. In addition, partial repression of CYP2D9 mRNA (unpublished data) or induction of CYP2B9 mRNA in males after adrenalectomy (Jarukamjorn et al., 1999) suggested the contribution of glucocorticoid hormone and/or sex-specific secretion of endocrine hormones in the sex-specific regulation of the expression of these genes. However, this explanation did not apply to the female-predominant Cyp2b10 gene, whose GH-regulated expression was apparent only in the males (Fig. 2D). These observations suggested...
the possibility that the expression of CYP2B10 mRNA was fully constitutive in the females, so no induction resulted from GH treatment in the females. Alternatively, the mechanism of regulation of the Cyp2b10 gene by GH might be sex-independent, but concentration-dependent. A previous study, showing that not only the GH secretion profile, particularly the duration for which the GH level was detectable, but also the mean concentration of GH was one of the important determinants for the expression of P450s (Pampori et al., 2001; Pampori and Shapiro, 1994), strongly supported the proposed mechanism. Continuous GH administration allowed the GH level to be maintained relatively stably in the circulation compared with pulsatile GH administration. Consequently, the expression of CYP2B10 mRNA was higher in females than in males, i.e. was female-predominant.

The observations that the constitutive expression and GH-inducible expression of the Cyp2c29, Cyp3a11, and Cyp2a2 genes was comparable between the sexes revealed that the regulatory mechanism of these genes was either sex-independent or GH-independent. Regarding this possibility, the pulsatile GH-inducible expression of these genes conceivably resulted from the responsiveness of these genes to GH as an exogenous chemical or xenobiotic. Pulsatile GH treatment via multiple subcutaneous injections in principle results in peak-trough fluctuations of GH higher than those resulting from continuous infusion, which causes relatively low and quite stable plasma levels of GH (Wilkinson, 2001).

In conclusion, the present findings, regarding both the expression of P450 genes in intact and MSG-neonatally treated mice, strongly support the notion of a decisive role of GH archetype in controlling the expression of sexually dimorphic P450 genes. The physiological expression profile of sex-dependent P450 genes was modified by exogenous GH repetition as pharmacologically or toxicologically responsive recognition. The expression change of some sex-independent P450s was noted in mice neonatally treated with MSG, though. Moreover, GH-regulated expression is not restricted to males, but further studies are needed to fully elucidate the regulatory mechanisms of P450 genes. Whether adrenal or sex hormone(s) play roles in the regulatory mechanisms of sexually dimorphic P450 genes or whether interactions among these hormonal factors dictate these sex-dependent actions are interesting questions that must be addressed in future studies.

Acknowledgements

This work was partly supported by the Tokyo Biochemical Research Foundation (TBRF), Grants-in-Aid from the Japanese Ministry of Education, Culture, Sport, and Science, and the Smoking Research Foundation.

References


