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SUBNORMAL CONCENTRATIONS IN THE FEMININE PROFILE OF CIRCULATING GROWTH HORMONE ENHANCE EXPRESSION OF FEMALE-SPECIFIC CYP2C12

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Abstract—When newborn female rats were treated with monosodium glutamate, 4 mg/g body weight, on days 1 and 3 of life, circulating growth hormone concentrations were permanently reduced 75–85% in adulthood, whereas the feminine secretory profile characterized by frequent growth hormone pulses, separated by short-lived, measurable troughs, persisted. Associated with this reduction in growth hormone secretion was a mild obesity and a slight depression in peripubertal body weight. In contrast, expression of growth hormone-dependent, female-specific CYP2C12 was increased by almost 100% when measured at both its protein and mRNA levels. In agreement, this supraphysiological expression of CYP2C12 was reflected at a pharmacologic level by a simultaneous elevation in *in vitro* and *in vivo* hexobarbital metabolism. When growth hormone secretion was pulsatile (i.e. masculine) or was eliminated from the circulation (i.e. hypophysectomy), hepatic CYP2C12 protein and mRNA were undetectable. The present findings suggest that the normal levels of plasma growth hormone found in female rats are not necessarily optimum for the expression of female-specific CYP2C12.

Key words: growth hormone-sexual profiles; monosodium glutamate; cytochrome P450 2C12; cytochrome P450 2C12—growth hormone regulation; drug metabolism—sexual dimorphism; CYP2C12

Studies have shown that the female pattern of constant growth hormone secretion [1] stimulates hepatic expression of female-specific CYP2C12 and fully suppresses male-specific CYP2C11, 2A2 and 3A2 [2–5]. Whereas the masculine pattern of pulsatile growth hormone release characterized by episodic bursts every 3–4 hr with undetectable hormone levels between the pulses [1, 6] suppresses CYP2C12 expression, it stimulates the expression of the 2C11 form of cytochrome P450 [2–5]. In the absence of growth hormone (i.e. hypophysectomy), neither CYP2C11 nor 2C12 is expressed [2–4]. In contrast, the levels of male-specific CYP2A2 and 3A2 are greatest in the hypophysectomized rat, but while the enzymes are only partially suppressed under the influence of pulsatile growth hormone, they disappear when the hormone is secreted constantly [3, 5]. In a somewhat similar manner, expression of CYP2A1 is female-predominant because constant growth hormone secretion is less repressive than is pulsatile release of the hormone [3, 5]. Thus, in females it is the continuous secretion of growth hormone that helps to regulate the gender's characteristic profile of hepatic microsomal sex-specific cytochromes P450.

To determine how the liver discriminates between a pulsatile and a continuous pattern of circulating growth hormone, it is important to identify the essential component(s) of the secretory rhythm that represents the signalling element(s) [7]. That is, how important are the amplitudes of the growth hormone peaks and troughs, or for that matter their duration in signalling the induction or suppression of sex-specific cytochromes? In this regard, we have used an animal model that allows us to dissect out some of the salient components of the growth hormone ultradian rhythm. Adult male rats previously treated as neonates with MSG†, 4 mg/g body weight, on alternate days for the first 9 days of life, have no measurable levels of circulating growth hormone [7, 8], whereas the concentrations of other pituitary hormones remain relatively normal [9–12]. In the absence of a pulsatile pattern of growth hormone secretion, the animals are unable to express male-specific CYP2C11 [7, 8]. Male rats treated during the same neonatal time period with half of the dose of MSG (i.e. 2 mg/g) exhibit typical masculine patterns of growth hormone release except that the amplitudes of the ultradian pulses are reduced to 10–20% of normal male levels. In spite of this 80–90% reduction of growth hormone pulse heights in 2 mg MSG-treated rats, their hepatic cytochromes P450 profile remains masculinized [7, 8]. Thus, it appears that for males neither the amplitude of the pulse nor its total content of growth hormone is critical for the expression of normal male levels of CYP2C11, 2A2 and 3A2 and their associated drug- and steroid-metabolizing activities. Furthermore, we have also found that the number of daily growth hormone pulses is not critical, *per se*, in regulating male-specific CYP2C11 expression [13]. That is, the normal 6, or even 4 or 2 daily growth hormone pulses per day are equally effective in inducing

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† Abbreviation: MSG, monosodium glutamate.

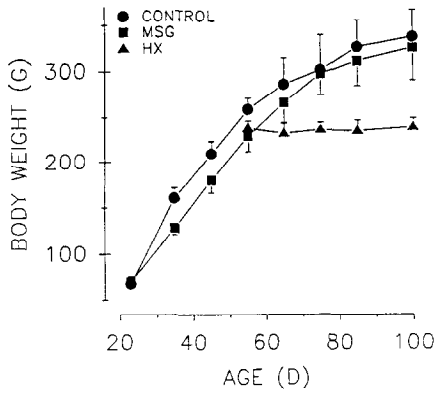

Fig. 1. Body weight gain of control, hypophysectomized (HX) and monosodium glutamate (MSG)-treated female rats. Newborn female rats were injected within 24 hr of birth and on postnatal day 3 with either MSG (4 mg/g body weight) or an equivalent amount of diluent (i.e. controls). Rats were hypophysectomized by the vendor at 8 weeks of age, the first recorded body weights. Results are presented as the means ± SD of at least 6 rats/data point.

CYP2C11 expression. What is critical, however, for CYP2C11 expression is that there be a minimal period (i.e. interpulse period) during which time the hepatocytes do *not* experience growth hormone. In the present study, using a slight modification of our previous MSG regimen, we have examined the importance of the characteristic female profile of growth hormone secretion in regulating expression of female-specific CYP2C12.

MATERIALS AND METHODS

Animals. Animals were housed in the University of Pennsylvania Laboratory Animal Resources facility, under the supervision of certified Laboratory Animal Medicine veterinarians, and were treated according to a research protocol approved by the University's Institutional Animal Care and Use Committee. Newborn male and female Sprague–Dawley rats [Crl:CD(SD)BR] were treated within 24 hr of birth and on postnatal day 3 with either MSG (4 mg/g body weight) (Sigma Chemical Co., St. Louis, MO) or an equivalent amount of 1.97 M NaCl diluent (0.012 mL/g body weight) by s.c. injection. Additional female rats [Crl:CD(SD)BR] were hypophysectomized by the vendor (Charles River Laboratories, Wilmington, MA) at 8 weeks of age and were maintained in our facilities for 5 weeks. The effectiveness of the surgery was verified by the lack of weight gain over this period (see Fig. 1) and the absence of pituitaries or fragments when necropsied at the end of the study (i.e. 102–107 days of age). Details regarding animal husbandry and breeding procedures, as well as serial blood collections using our chronic indwelling right atrial catheterization apparatus, have been reported [7, 8, 14]. Plasma growth hormone determination by radioimmunoassay [7, 8, 14] and hexobarbital sleep time measurements [7, 8] were described previously.

Drug- and steroid-metabolizing enzymes. Hepatic microsomes were prepared by our previously described method [15]. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [15] of the radioenzyme procedure of Kupfer and Rosenfeld [16]. The activities of 7-ethoxyresorufin *O*-deethylase [17] and pentoxyresorufin dealkylase [18] were measured spectrofluorimetrically. Microsomal fractions were assayed for testosterone hydroxylases [19] using 4.6 mm × 15 cm reverse phase C18 columns and Diode Array Detector-HPLC (Perkin Elmer, Norwalk, CT). The hydroxylated steroids were separated by a concave gradient solvent system (2 mL/min) of methanol : water : acetonitrile, using gradient-4 from 38:58:4 to 55:35:10 on Perkin–Elmer columns. The products were verified further with a solvent system (1.5 mL/min) of water : tetrahydrofuran (85:15) on Zorbax (MacMod, Chaddsford, PA) columns. To verify the HPLC results, [4-^{14}C] testosterone was used to assay these enzymes, and the products were resolved by TLC and quantified by previously reported methods [20].

CYP2C12 determinations. Isolated liver microsomes were assayed by western blotting, using monoclonal antibodies reactive with cytochrome P450 form 2C12 [21], as previously described [22]. Ten micrograms of microsomal protein was electrophoresed on 0.75 mm thick SDS–polyacrylamide gels [23] containing 12% polyacrylamide. To obtain better separation of CYP2C12 from its cross-reacting protein, the electrophoresis was carried out on 12-cm long gels until the interested proteins were 2 cm above the lower edge; this was monitored by the pre-stained marker proteins. The proteins were electroblotted onto 0.22 μm nitrocellulose filters [24]. The filters were incubated with PBS containing 2% bovine serum albumin for 2 hr, before probing with monoclonal anti-rat P450 2C12. The primary antibody was located with an alkaline phosphatase-conjugated goat anti-mouse IgG kit (Bio-Rad Laboratories, Richmond, CA). Quantitation of relative P450 levels was determined by laser densitometry of the nitrocellulose blots, as previously reported [25].

Total hepatic RNA was isolated by using a single-step guanidinium-thiocyanate method [26] and quantitated spectrophotometrically [27]. RNA (10 μg) was electrophoresed on formaldehyde gels and transferred onto Nylon membranes (NEN Research Products, Boston, MA), and these northern blots were probed for CYP2C12 mRNA by using ^{32}P-labeled oligonucleotides [22]. Relative mRNA levels were determined on slot blots by quantifying at least five concentrations of RNA specific for CYP2C12 on laser densitometry [22].

Chemicals. Unless specified otherwise in Materials and Methods and Acknowledgements, materials for the drug- and steroid-metabolizing enzyme assays were purchased from the Sigma Chemical Co., materials for the western, northern and slot blot procedures were purchased from Bio-Rad Laboratories, and general laboratory chemicals and solvents (analytical or HPLC grade) were purchased from Fischer Scientific, Pittsburgh, PA.

Statistics. Data were subjected to analysis of

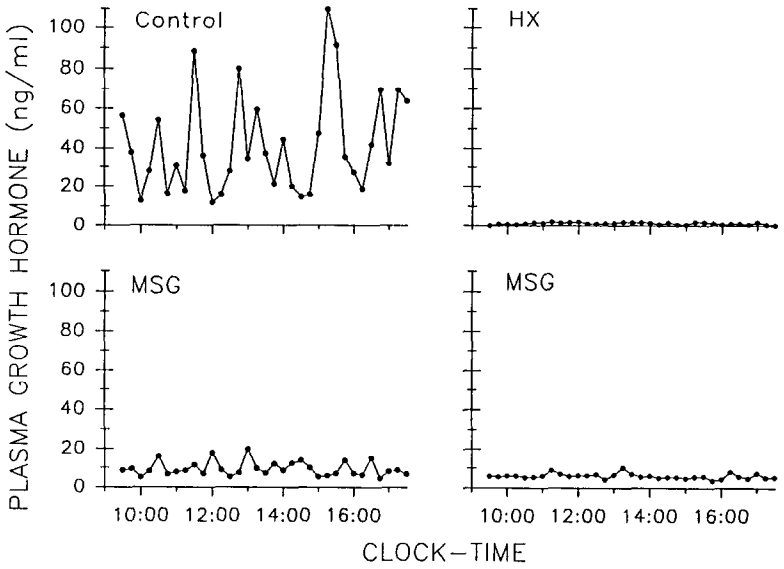

Fig. 2. Plasma levels of circulating growth hormone obtained from 14-week-old, individual, undisturbed catheterized control, hypophysectomized (HX), and MSG-treated female rats at 15 min intervals for 8 consecutive hours. See Fig. 1 for details of animal protocols. Growth hormone profiles from two different MSG-treated rats are presented. Similar growth hormone profiles were obtained from 3 to 4 additional animals in each treatment group.

variance, and differences among pairs of means were determined using "*t*" statistics and the Bonferroni procedure for multiple comparisons.

RESULTS

Growth. In contrast to the hypophysectomized rats who exhibited zero body weight gain following pituitary ablation, neonatal treatment with MSG had only a small inhibitory effect on body weight gain that appeared to be limited to the peripubertal period (Fig. 1). By 65 days of age, the body weights of the control and MSG-treated females were statistically indistinguishable. Nevertheless, the seemingly normal body weights of the MSG females were due, in some part, to an abnormal deposition of carcass fat as evident by a significant ($P < 0.01$) increase in the Lee index [28], a calculated measure of obesity (control, 0.31 ± 0.01; MSG, 0.33 ± 0.01; mean ± SD).

Growth hormone. The established female-dependent profile of plasma growth hormone secretion was found in our control female rats (Fig. 2). Growth hormone was released in a rather constant pattern characterized by frequent pulses producing circulating hormone peaks of around 50–90 ng/mL, followed by short-lived troughs that were always measurable, and generally remained above 15–20 ng/mL. In contrast, there were no statistically detectable (<2–3 ng/mL) levels of growth hormone in any of the plasma samples obtained during 8 continuous hr of serial blood collection from hypophysectomized rats. Although the mean concentration of growth hormone within the 8 hr collection period was reduced 75–85% in the females neonatally treated with MSG, the basic feminine profile of growth hormone secretion persisted (Fig. 2). In spite of a dramatic reduction in both pulse heights (10–20 ng/mL) and interpulse baselines (5–10 ng/mL), growth hormone secretion in the females injected with MSG was still characterized by numerous plasma peaks, followed by short-lived troughs that were always measurable.

CYP2C12 expression. Expression of hepatic CYP2C12 at both the protein (Fig. 3) and mRNA (Fig. 4) levels were repressed completely in control males where growth hormone secretion was pulsatile, and in hypophysectomized females where growth hormone was eliminated from the circulation. In contrast, treatment with MSG resulted in an enhanced expression of the P450 form in adulthood. That is, there was a 70% increase in the expression of CYP2C12 mRNA, which concurred with the 90% increase in the CYP2C12 protein.

Drug- and steroid-metabolizing enzymes. Females treated neonatally with MSG exhibited an almost 60% increase in hepatic microsomal hexobarbital hydroxylase (Table 1), a monooxygenase comprising several forms of P450, of which CYP2C12 is a major constitutive contributor in the female [29]. This elevation in hexobarbital hydroxylase activity was reflected in a significant decrease in hexobarbital-induced sleeping times (i.e. *in vivo* hexobarbital metabolism). To monitor MSG-induced alterations in other forms of P450, we measured various monooxygenase activities that are known to represent or, in some cases, are specific catalytic activities for several induced and constitutive forms of hepatic cytochromes P450 in the rat (Table 1). Ethoxyresorufin *O*-deethylase, CYP1A1 and 2C11 [30, 31];

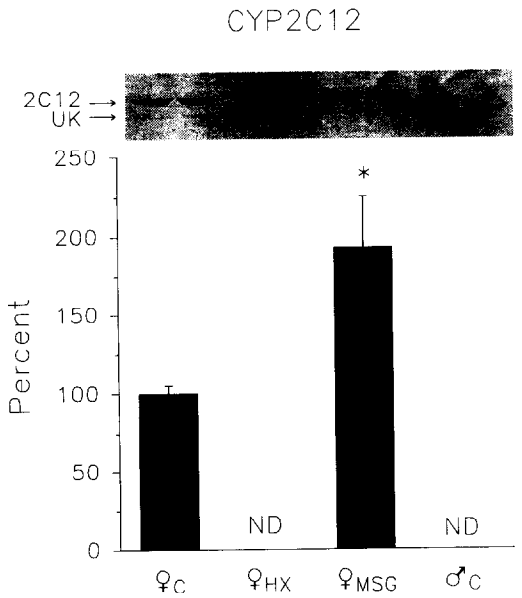

Fig. 3. Western blot analysis of CYP2C12 proteins in liver microsomes isolated from diluent-treated, control male (♂C), control female (♀C), hypophysectomized female (♀HX) and MSG-treated female (♀MSG) rats. See Fig. 1 for details of animal protocol. The top panel is a computer scan of the western blots analyzed with monoclonal antibody to CYP2C12 and a cross-reactive unknown (UK) microsomal polypeptide which is probably male-specific CYP2C13 (Agneta Mode, personal communication, cited with permission). The bottom panel is a graphic presentation of the relative CYP2C12 levels determined by laser densitometry of the nitrocellulose blots. Key: (*) $P < 0.01$ when compared with the control females.

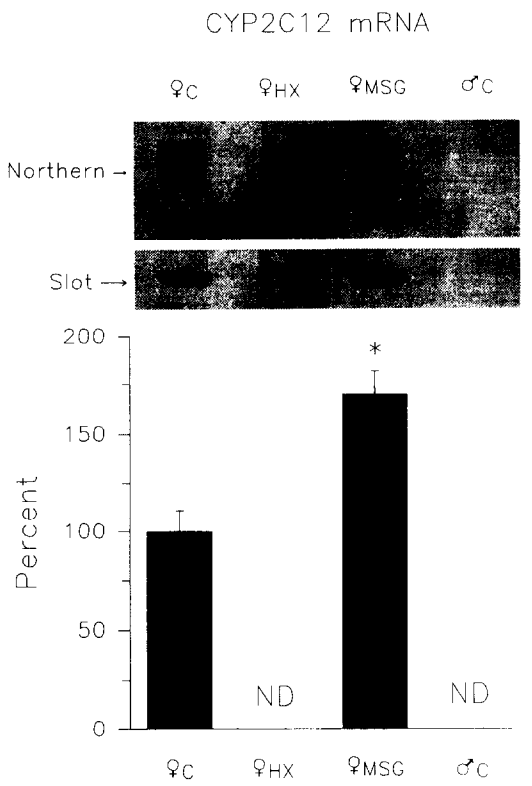

Fig. 4. CYP2C12 mRNA analysis of liver from diluent-treated control male (♂C), control female (♀C), hypophysectomized female (♀HX) and MSG-treated female (♀MSG) rats. See Fig. 1 for details of animal protocol. The top panel is a computer scan of a northern blot analyzed with a ^{32} P-labeled oligonucleotide probe specific for CYP2C12. The bottom panel is a graphic presentation of the relative CYP2C12 mRNA levels determined by laser densitometry of actual slot blot films at five different RNA concentrations, but presented here as a computer scan of the slot blots at a single concentration (middle panel). Key: (*) $P < 0.01$ when compared with the control females.

pentoxyresorufin *O*-dealkylase, CYP2B1, 2B2 and 2C11 [30, 31]; testosterone 2 α-hydroxylase, CYP2C11 [3, 20]; testosterone 6 β-hydroxylase, CYP1A2, 2A2, 2C13, 3A1 and 3A2 [32]; testosterone 7 α-hydroxylase, CYP2A1 [3, 20]; and testosterone 16 α-hydroxylase, CYP2B1, 2B2, 2C6, 2C11 and 2C13, [3, 32] were all unaffected by MSG treatment. [The possibility of a minimal MSG-induced expression of CYP2C11 (see testosterone 2 α-hydroxylase in Table 1) was excluded by the absence of any detectable CYP2C11 mRNA in the livers of the MSG-treated, as well as control and hypophysectomized females (northern blots are not presented).]

DISCUSSION

In agreement with previous reports [2–5], we have found that CYP2C12 expression is dependent upon the constant-like secretion of growth hormone characteristic of the female rat*, whereas episodic (i.e. pulsatile) secretion of the hormone found in the male rat, or the elimination of growth hormone seen in the hypophysectomized rat, results in the complete suppression of CYP2C12 at both its protein and mRNA levels. Moreover, the present study indicates that the normal circulating levels of growth hormone found in the female rat are not necessarily optimum for the expression of female-dependent CYP2C12. In fact, a 75–85% reduction in the mean concentration of plasma growth hormone in an otherwise feminine profile characterized by numerous, randomly generated pulses separated by short-lived, measurable troughs, results in an almost doubling in the concentration of hepatic microsomal CYP2C12. Since liver CYP2C12 protein levels are regulated primarily through transcriptional mechanisms [33, 34], it is not surprising that

*Although the female profile of plasma growth hormone secretion is characterized by frequent pulse amplitudes exceeding background by 3- to 5-fold, the effectiveness of growth hormone secretion with barely detectable pulses to induce CYP2C12 in some of the MSG-treated females (Fig. 2) suggests that, irrespective of the pulses, it is simply the constant exposure to growth hormone, *per se*, that signals the hepatocytes to express CYP2C12.

Table 1. Hepatic microsomal monooxygenase activities and hexobarbital-induced sleeping times in adult female rats treated neonatally with MSG*

	Hexobarbital hydroxylase	Sleep time	Ethoxyresorufin *O*-deethylase	Pentoxyresorufin *O*-dealkylase	Testosterone			
					2 α-OH	6 β-OH	7 α-OH	16 α-OH
Control	100 ± 11	100 ± 6	100 ± 13	100 ± 9	100 ± 16	100 ± 14	100 ± 15	100 ± 38
MSG	158 ± 22†	69 ± 8†	107 ± 18	103 ± 18	133 ± 39	117 ± 13	107 ± 18	101 ± 30

* Newborn female rats were injected with either MSG (4 mg/g body weight) or an equivalent amount of vehicle (controls) on days 1 and 3 of life and euthanized at 102–107 days of age. Sleep time and enzyme levels (mean ± SD, $N \geq 4$) for the MSG-treated rats are presented as a percent of the values of the control (=100%). The control (i.e. 100%) microsomal enzyme values were 1192 ± 132, 128 ± 17, 33 ± 3, 25 ± 4, 349 ± 49, 1494 ± 224 and 46 ± 17 pmol/min/mg protein for hexobarbital hydroxylase, ethoxyresorufin *O*-deethylase, pentoxyresorufin *O*-dealkylase, and testosterone 2 α-, 6 β-, 7 α- and 16 α-hydroxylases, respectively. Control sleep time was 180 ± 11 min. Testosterone metabolites are listed according to hydroxylation sites. Hexobarbital-induced sleeping times were determined at 75 days of age.

† $P < 0.01$ compared with controls.

the increased CYP2C12 was accompanied by a commensurate increase in CYP2C12 mRNA. This supraphysiological expression in CYP2C12 in the adult females neonatally treated with MSG is reflected at a pharmacologic level by an apparent increase in the contribution of the cytochrome to the multi-P450-dependent *in vitro* and *in vivo* metabolism of hexobarbital.

Our finding that dramatically subnormal levels of circulating growth hormone are capable of regulating CYP2C12 expression is not unique. In fact, we have reported that an 80–90% reduction in the plasma concentration of growth hormone in male rats has no effect on the masculine-like expression levels of male-dependent CYP2C11, 2A2 and 3A2 [7, 8]. The effectiveness of very low levels of growth hormone to regulate CYP2C12 expression may be explained by the high affinity of the growth hormone receptor for its ligand, $K_d = 10^{-10}$ M [35], which corresponds to a half-maximal saturation of the receptor at a plasma concentration of only 2 ng/mL. However, the ability of subnormal concentrations of growth hormone to induce supraphysiologic levels of CYP2C12 is unlikely to be understood until the membrane transduction and intracellular signalling mechanisms mediating growth hormone regulation of the cytochromes P450 are identified. Nevertheless, the fact that female-specific CYP2C12 can be expressed at almost 2-fold its normal level with as little as 15–25% of physiologic plasma concentrations of growth hormone suggests an inherent inefficiency in the production and secretion of growth hormone in the female rat. However, unlike many hormones with specific target tissues, growth hormone has a global effect on the body and appears to regulate functions in most cell types. Thus, while remarkably reduced levels of plasma growth hormone are capable of maintaining normal or above normal levels of CYP2C12, we have also found that these hormone concentrations are insufficient to maintain normal body growth, fat metabolism and some organ weights [7, 36]. Similarly, restoration of normal male body growth in hypophysectomized rats can be achieved when physiologic growth hormone pulses are administered six or seven times per day [13, 37], whereas only the six pulse regimen is effective in inducing male-specific CYP2C11 expression [37]. Perhaps the secretory profiles and concentrations of growth hormone normally found in rats represent a compromise that assures an adequate response by all growth hormone responsive tissues.

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