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EFFECTS OF NEONATALLY ADMINISTERED MONOSODIUM GLUTAMATE ON THE SEXUALLY DIMORPHIC PROFILES OF CIRCULATING GROWTH HORMONE REGULATING MURINE HEPATIC MONOOXYGENASES

NISAR A. PAMPORI and BERNARD H. SHAPIRO*

Laboratories of Biochemistry, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, PA 19104-6048, U.S.A.

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Abstract—Neonatal male and female mice were treated with monosodium glutamate (MSG) at either 2.0 or 4.0 mg/g body weight on alternate days during the first 9 days of life. As adults, mice were catheterized to obtain unstressed, serial blood samples for the determination of ultradian profiles of circulating growth hormone. In addition, monooxygenase levels (i.e. steroid hydroxylases and drug-metabolizing enzymes) were measured in hepatic microsomes. Generally, both doses of MSG produced the same developmental defects. Mice neonatally exposed to the amino acid developed a syndrome characterized by retarded growth, obesity and reduced organ weights. While vehicle-treated mice secreted growth hormone in sexually dimorphic patterns defined by pulse frequency (i.e. F > M), hormone concentrations in plasma samples obtained during 8 continuous hr of serial blood collections from both male and female MSG-treated mice were barely detectable at best, and exhibited no pulsatility. Approximately 15% of the measured monooxygenases were male-predominant, 35% were female-predominant and 50% had no sex differences. The enhanced expression of the hepatic monooxygenases in response to MSG-induced depletion of plasma growth hormone indicates that the hormone basically functions as a suppressor of the murine enzyme system.

Key words: growth hormone, murine; growth hormone, gender differences; glutamate, monosodium; monooxygenases, murine hepatic; drug metabolism, murine

Neonatal administration of MSG† to rats, mice and possibly other species produces a profound, but rather selective growth hormone deficiency, resulting in a well-defined syndrome of stunted body growth and obesity [1–3]. Coincidently, the sexually dimorphic, ultradian rhythms in circulating growth hormone have been shown to regulate the gender-dependent expression of rat hepatic monooxygenases [4, 5]. In male rats, growth hormone is secreted in episodic bursts every 3.5 to 4 hr. Between the peaks, growth hormone levels are undetectable. In female rats, the hormone pulses are more frequent and are of lower magnitude than in males, whereas the interpeak concentrations of growth hormone are always measurable [3, 6, 7]. Apparently, exposure to the more constant feminine secretory profile of growth hormone produces the lower level of hepatic drug metabolism found in female rats. Conversely, the ultradian rhythm in growth hormone secretion characterized as masculine is responsible for the occurrence of a 3- to 5-fold higher level of hepatic drug metabolism [4, 5]. In this regard, we have reported that hypothalamic lesions induced by neonatal administration of 4 mg MSG/g body weight (consistently, the most commonly used dose) permanently, and rather selectively, block growth hormone secretion, resulting in a severe reduction in hepatic monooxygenase activities, reflected in part by the complete suppression of sex-dependent hepatic cytochromes P450 2C11, 2A2 and 3A2 [3, 8, 9]. Neonatal treatment with half of this dose (i.e. 2 mg/g) produces an 80–90% reduction in the pulse amplitudes of plasma growth hormone, but the sexually dimorphic profiles in hormone secretion (i.e. male pulsatile; female constant-like) remain distinct in both sexes [8, 9]. In spite of subnormal pulse heights, hepatic monooxygenase activities are not decreased, demonstrating the unimportance of the physiologic growth hormone pulse amplitudes in regulating the expression of cytochromes P450 [8, 9].

In contrast to the rat, mice exhibit a reversed sexual dimorphism in hepatic monooxygenases [10–12]. It is the female mouse that metabolizes drugs more quickly than the male, and the pulsatile profile of plasma growth hormone that induces hepatic monooxygenases in the rat represses these enzymes in the mouse [13, 14]. Since the MSG-treated rat has proven to be a very useful model in which to study growth hormone regulation of rat hepatic monooxygenases [3, 8, 9, 15], we now have examined the effects of MSG-induced growth hormone

* Corresponding author: Bernard H. Shapiro, Ph.D., Laboratories of Biochemistry, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104-6048. Tel. (215) 898-1772; FAX (215) 898-9923.

† Abbreviations: MSG, monosodium glutamate; and RIA, radioimmunoassay.

depletion on the expression of murine monooxygenases.

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. Animals were housed on hardwood bedding in plastic cages, with water and commercial mouse diet supplied *ad lib.* The animal quarters were air-conditioned (20–23°) and had a photoperiod of 12 hr of light, 12 hr of darkness (lights on at 07:00 hr). After a 2- to 3-week acclimation period in our facilities, the animals were bred by randomly housing two adult female Swiss mice [Crl:CD-1 (ICR)BR] with an individual adult male of the same strain. On the day of parturition all litters were reduced to ten pups, with a sex ratio of 1:1 or as close to that as possible. Starting within 24 hr after birth, and on alternate days for the first 9 days of life, the pups were injected s.c. with either monosodium L-glutamate (2.0 or 4.0 mg/g body weight; Sigma Chemical Co., St. Louis, MO) or an equivalent volume of 1.97 M NaCl diluent for a total of five injections. The pups were weaned at 25 days of age.

Blood collections. Three or four animals (4–5 months of age) from each treatment group, and representing all litters in the group, were implanted with chronic indwelling right atrial catheters [16] anchored to the skin with a sewing snap. Use of our mobile catheterization apparatus permitted repetitive blood sampling from unrestrained, unstressed, and completely conscious animals [16, 17]. Collections began 5–7 days after surgery. Blood samples were obtained from mice once every 15 min for 8 continuous hr. A 12-μL volume of blood was withdrawn into a heparinized microsyringe. The sample was mixed with 108 μL of buffer (pH 7.6) containing KPO_4 (0.01 mol/L), NaCl (0.15 mol/L), NaN_3 (3.1 mmol/L), EDTA (3.8 mmol/L), 0.2% (w/v) bovine serum albumin and 0.4% (v/v) normal monkey serum (Cooper Biomedical, Malvern, PA) and centrifuged at 10,000 *g*. Following centrifugation, a 100-μL aliquot of cell-free supernatant was removed and stored at −80° for later growth hormone quantification. Plasma growth hormone profiles monitored a second time, 5–7 days later, were indistinguishable from the first collection. On non-collection days, the catheters were flushed twice with 50 μL of heparinized (10 IU/mL) saline (0.15 mol/L) followed by 50 μL of heparinized (50 IU/mL) saline (0.15 mol/L) containing 0.05 mg gentamicin sulfate/mL.

Growth hormone. Plasma growth hormone concentrations were determined in duplicate by standard RIA techniques [3, 18] using mouse-specific materials supplied by Dr. A. F. Parlow and the NIDDK (Bethesda, MD). Iodinated mouse growth hormone tracer (AFP-10783-B) was prepared with chloramine-T and then purified using gel filtration chromatography. A 10-point standard curve was prepared with serial dilutions of mouse growth hormone (AFP-10783-B) and corresponded to plasma concentrations in unknown samples ranging from 0.4 to 200 ng/mL. The primary (monkey) anti-mouse growth hormone antiserum (NIDDK No. 35) was used at a final tube dilution of 1:400,000 in a total 500-μL reaction mixture. After a 24-hr incubation at 22–24°, 100 μL of a 1:5 dilution of P4 goat antimonkey immunoglobulin G (Antibodies, Inc., Davis, CA) were added simultaneously with 100 μL polyethylene glycol 8000 (30%, w/v) and centrifuged for 30 min at 2000 *g* to separate bound from free growth hormone. To correct for non-specific binding, all growth hormone concentrations in unknown samples were normalized to background values (2–3 μg/L) obtained from a single pooled supply of blood collected from confirmed hypophysectomized mice. Statistical validation of the assay has been reported elsewhere [18].

Sleeping times. At 50 days of age, barbiturate-induced sleeping times were measured in some of the offspring after an i.p. injection of hexobarbital (125 mg/kg body weight). Recovery from unconsciousness was indicated by the full restoration of the righting response, defined as the ability of the animal when placed on its back on a flat surface to snap over on its paws three times within 15 sec [19].

Microsomes. Animals were killed by decapitation at 150 days of age, and hepatic microsomes were prepared by our previously described method [3].

Drug-metabolizing enzymes. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [19] of the radioenzyme procedure of Kupfer and Rosenfeld [20]. Microsomal 7-ethoxycoumarin *O*-deethylase was measured spectrofluorimetrically [21]. NADPH–cytochrome P450 reductase activity was assayed as previously described [3].

Steroid hydroxylase assays. Microsomal fractions were assayed for steroid hydroxylase activities by incubating a 1-mL reaction mixture containing 155 μmol HEPES buffer, pH 7.4, 3 μmol $MgCl_2$, 0.1 μmol EDTA, 20 μL glycerol, 50 nmol testosterone or androstenedione (with or without 1×10^5 dpm of the respective [4-^{14}C]androgen in 1 μL methanol), 200 μg microsomal protein and an NADPH-generating system (10 μmol glucose-6-phosphate, pH 7.4, 0.3 μmol NADPH, 0.5 U glucose-6-phosphate dehydrogenase) for 10 min at 37°. Using either androgen substrate, product formation was linear with respect to time, substrate and microsomal protein concentrations. The reaction was initiated by addition of the generating system. Hydroxylated metabolites were extracted with 6 mL of ethyl acetate, dried under nitrogen, and separated on 4.6 mm × 15 cm reverse phase C18 columns and Diode Array Detector-HPLC (Perkin–Elmer, Norwalk, CT), by a concave gradient solvent system (2 mL/min) of methanol:water:acetonitrile, using gradient-4 from 38:58:4 to 55:35:10 (for hydroxytestosterones) or 36:60:4 to 55:35:10 (for hydroxyandrostenediones) on Perkin–Elmer columns (Fig. 1, a and b). The 6β- and 16β-hydroxyandrostenedione products that were not separable on this system were identified with a solvent

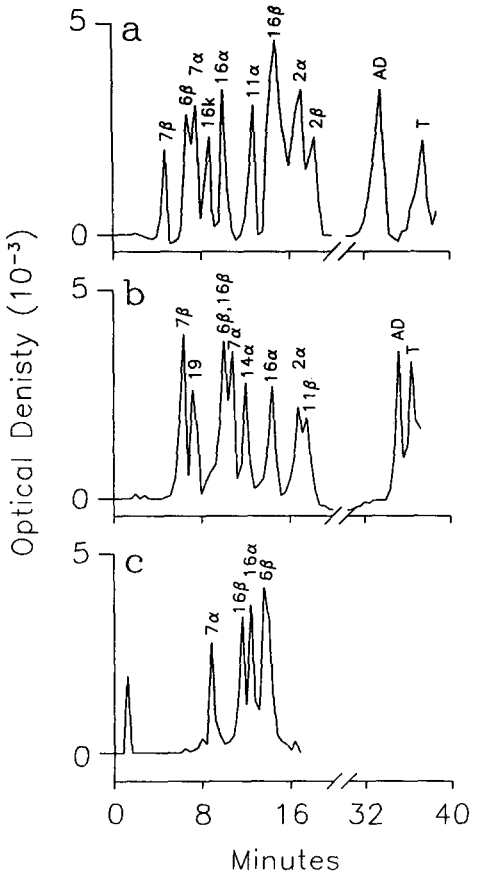

Fig. 1. HPLC chromatograms of standard hydroxylated and/or keto "K" derivatives of testosterone "T" (a) and androstenedione "AD" (b and c). Fifty to one hundred picomoles of each steroid in a mixture of about 20 μL were separated either on Perkin–Elmer (a and b) or Zorbax (c) columns, as described in the text. Absorbance was measured at 245 nm with a sensitivity of 0.005.

system (1.5 mL/min) of water:tetrahydrofuran (85:15) on Zorbax (MacMod, Chaddsford, PA) columns (Fig. 1c). To verify the HPLC results, [4-^{14}C]testosterone and [4-^{14}C]androstenedione were used to assay these enzymes, and the products were resolved by TLC and quantified by previously reported methods [22].

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

RESULTS

Body and organ weights. The increased Lee indices in all of the MSG-treatment groups indicate that the elevated body weights of these animals were due, at least in part, to an abnormal deposition of carcass fat (Table 1). Although there were significant sex differences in the body and organ weights of the control mice, neonatal exposure to MSG in both sexes resulted in a similar degree of obesity and reduction in organ weights. In general, there was no dose-dependent response, as the lower 2 mg dose of MSG produced similar detrimental effects on growth and body organs as 4 mg of MSG. The pituitary, however, was a clear exception. The 2 mg dose of the amino acid resulted in a 40–60% reduction in pituitary weight and the 4 mg dose caused an additional 40–70% decline in the weight of the gland (Table 1).

Growth hormone. As we had reported previously [18], control mice secreted growth hormone in a sexually dimorphic, ultradian pattern (Fig. 2). Male mice exhibited a regular periodicity of growth hormone peaks which occurred about every 2.5 hr with interpulse stable baseline concentrations that were significantly longer in duration than in females. That is, we found a greater growth hormone pulse frequency in females that reduced cycle length to an average of 1.0 to 1.5 hr and significantly increased the overall mean hormone concentration.

Regardless of sex or dose, pulsatile secretion of growth hormone ceased in adult mice neonatally treated with MSG. Growth hormone concentrations in the plasma samples obtained during 8 continuous hr of serial blood collections from both male and female mice hovered around the sensitivity of the assay, 2–3 ng/mL. There were no significant differences in circulating growth hormone levels between the 2 and 4 mg dose of MSG.

Hepatic monooxygenases. Of the twenty or so hepatic enzymes measured, four were male-predominant (Table 2). The microsomal concentrations of androstenedione 7α- and 7β-hydroxylases and testosterone 16α-hydroxylase were greater in male liver than in female liver. Whereas growth hormone depletion resulting from neonatal administration of MSG had no statistical effect on the activities of these three enzymes in males, the same MSG treatment in females increased the enzyme concentrations to male-like levels. In contrast to all other enzyme activities measured in this study, the male-predominant conversion of androstenedione to testosterone (a non-monooxygenase activity) was reduced by neonatal exposure to MSG in both sexes.

The concentrations of five steroid hydroxylases, i.e. androstenedione 11β- and 19-hydroxylases as well as testosterone 2α-, 6α- and 15α-hydroxylases, were greater in female liver than in male liver. The microsomal drug-metabolizing enzymes ethoxycoumarin *O*-deethylase and hexobarbital hydroxylase (measured *in vitro* and *in vivo*) were also more active in female liver (Table 3). In the case of the males, MSG-induced depletion of growth hormone secretion was associated with an increase in the female-predominant enzymes to levels equal to, or somewhat greater than that found in control female mice. In general, the activities of these hepatic monooxygenases in females remained unchanged or were increased moderately by MSG treatment. Both doses of MSG had similar effects. The 100% increase in activity of hepatic androstenedione 11β-hydroxylase in both adult males and females neonatally treated with MSG was more representative of the magnitude of change found in the sex-independent enzymes (Table 4). In this regard, the

Table 1. Body dimensions and organ weights of adult mice treated neonatally with MSG

Sex	MSG treatment (mg/g)	Body weight (g)	Lee index* ($\times 10^{-3}$)	Liver (mg/g body weight)	Kidneys (mg/g body weight)	Pituitary (μg/g body weight)	Adrenals (μg/g body weight)
Male	0	34 ± 4†	334 ± 4‡	51.0 ± 1.1‡	16.7 ± 1.6‡	48.6 ± 8.5‡	158 ± 28†
	2	40 ± 3	357 ± 13	33.8 ± 4.2	10.7 ± 1.0	27.4 ± 8.6	120 ± 16
	4	40 ± 4	365 ± 15	31.8 ± 4.3	7.0 ± 0.7‡	7.9 ± 2.8‡	121 ± 27
Female	0	29 ± 2‡§	332 ± 9‡	45.8 ± 3.0‡	13.7 ± 1.4‡‖	61.1 ± 13.0‡	283 ± 33‡§
	2	40 ± 1	359 ± 8	33.7 ± 1.5	8.2 ± 0.3	22.5 ± 4.0	180 ± 19
	4	37 ± 4	368 ± 12	31.8 ± 2.5	7.2 ± 0.3‡	13.7 ± 3.0‡	130 ± 26†

Pups were injected with either 2.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life and euthanized at 150 days of age. Results are means ± SD of at least 7 mice/group.

* Lee index: [$\sqrt[3]{\text{body weight (g)}}$/naso-anal length (cm)] determined at 50 days of age.

†‡ Significantly different from 2 mg MSG-treated mice of the same sex: †$P < 0.05$; and ‡$P < 0.01$.

§‖ Compares diluent-treated males with diluent-treated females: §$P < 0.05$; and ‖$P < 0.01$.

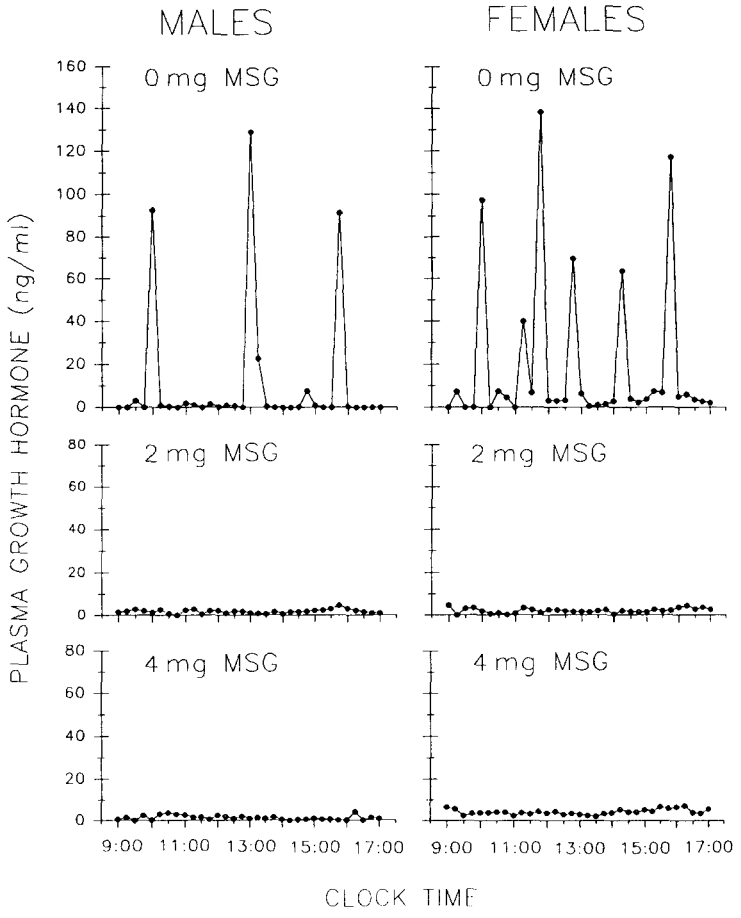

Fig. 2. Plasma levels of circulating growth hormone obtained from individual, undisturbed catheterized control and MSG-treated mice at 15-min intervals for 8 consecutive hr. Similar findings were obtained from two to three additional animals in each treatment group. Plasma was assayed for each mouse from two collections obtained 5–7 days apart.

Table 2. Male-predominant hepatic microsomal monooxygenase activities of adult mice treated neonatally with MSG

Sex	MSG treatment (mg/g)	Androstenedione			Testosterone
		7α-OH*	7β-OH	Testosterone	16α-OH
		(pmol/min/mg protein)			
Male	0	373 ± 90	18 ± 4	479 ± 25	397 ± 56
	2	272 ± 73	17 ± 6	140 ± 41‡	362 ± 70
	4	336 ± 112	17 ± 3	98 ± 27‡	413 ± 72
Female	0	197 ± 84‖	12 ± 3§	261 ± 38‖	236 ± 29‖
	2	434 ± 29‡	21 ± 4‡	117 ± 16‡	304 ± 48‡
	4	576 ± 49‡	18 ± 5†	125 ± 79‡	421 ± 87‡

Pups were injected with either 2.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life and euthanized at 150 days of age. Results are means ± SD of at least 7 mice/group.

* With the exception of the testosterone-metabolite of androstenedione (a non-monooxygenase reaction), metabolites are listed under their substrates according to hydroxylation sites.

†‡ Significantly different from diluent-treated mice of the same sex: †$P < 0.05$; and ‡$P < 0.01$.

§‖ Compares diluent-treated males with diluent-treated females: §$P < 0.05$; and ‖$P < 0.01$.

very small sex difference in the mean concentrations of this enzyme suggests a statistical aberration that may have incorrectly classified androstenedione 11β-hydroxylase as female-predominant instead of the more appropriate sex-independent. The inconsistent response to testosterone 6α-hydroxylase to MSG treatment (increased, decreased and unchanged) may be due to the fact that the product of the reaction is not 6α-hydroxytestosterone, and thus, may not represent a monooxygenase activity (see footnote in Table 3).

Half of the measured hepatic enzymes were sex-independent, meaning that there were no gender differences in their activities (Table 4). In contrast to the sex-dependent enzymes in which the inductive effects of MSG treatment were basically limited to the gender with the lower monooxygenase activities (Tables 2 and 3), the concentrations of the sex-independent hepatic enzymes were increased from 50 to over 100% in both males and females exposed to MSG as newborns. With the exception of testosterone 2β-, 6β- and 7α-hydroxylases in male liver, the two doses of MSG produced similar effects on the sex-independent microsomal monooxygenases.

Lastly, we measured the levels of hepatic NADPH–cytochrome P450 reductase, an essential element in the monooxygenase system in catalyzing the electron transfer from NADPH to cytochrome P450, and found that the enzyme concentration in females (511 ± 35 nmol/min/mg protein) was significantly higher ($P < 0.001$) than in males (363 ± 22 nmol/min/mg protein). In agreement with the response of other female-predominant enzymes (Table 3), MSG-induced growth hormone depletion had no effect on the concentration of NADPH–cytochrome P450 reductase in the female, but significantly ($P < 0.01$) increased the activity of the enzyme by 20% in the male. Again, we found no dose-dependent response of MSG on the concentration of the reductase.

DISCUSSION

In agreement with previous rat and murine studies [1–3], we have found that mice neonatally exposed to MSG develop a syndrome characterized by retarded growth (as a measured component of the Lee index), obesity and reduced organ weights. Although never before reported, our finding of an almost complete depletion of circulating growth hormone in the MSG-treated mouse may explain the many growth abnormalities attributed to the syndrome. In our rat studies, we have reported that the 2 mg dose of MSG, as well as 1 and 0.5 mg, had graded effects that were less detrimental on growth and development than the 4 mg dose [3, 9]. In contrast, the present experiments indicated that in the mouse, 2 mg of MSG generally produces the same type and magnitude of defects as the 4 mg dose. The only outstanding exception would be the dose effect on pituitary weights, which was similar to the dose–response found in rats [3]. Irrespective of the pituitary effects, 2 mg MSG was as effective as 4 mg MSG in blocking pituitary secretion of growth hormone. Since we have reported a clear dose–response in the rat to MSG from 0.5 to 4.0 mg [9], the present findings suggest that the mouse may be more sensitive to the developmental effects of the amino acid. It is possible that a dose–response may only be demonstrable in mice at considerably lower doses than 2 mg MSG/g body weight [1].

Attempts to compare the present enzyme findings in the mouse to what is known in the rat are limited by our meager knowledge of the murine hepatic monooxygenase system. In contrast to the rat where some two-dozen or so constituent isoforms of hepatic cytochrome P450 have been characterized extensively at a molecular and regulatory level [4, 5, 23, 24], only a couple of constituent forms of cytochrome P450, with overlapping catalytic activities, and representing a small fraction of the total cytochrome P450 pool, have been identified in

Table 3. Female-predominant hepatic microsomal monooxygenase activities and hexobarbital-induced sleeping times of adult mice treated neonatally with MSG

Sex	MSG treatment (mg/g)	Androstenedione 11β-OH* (pmol/min/mg protein)	Androstenedione 19-OH (pmol/min/mg protein)	Testosterone 2α-OH (pmol/min/mg protein)	Testosterone 6α-OH (pmol/min/mg protein)	Testosterone 15α-OH (pmol/min/mg protein)	Ethoxycoumarin O-deethylase (pmol/min/mg protein)	Hexobarbital hydroxylase (pmol/min/mg protein)	Sleeping time (min)
Male	0	60 ± 5	78 ± 14	151 ± 16	285 ± 39	370 ± 37	2286 ± 395	1134 ± 87	91 ± 25
	2	124 ± 9‡	119 ± 11‡	286 ± 17‡	502 ± 87‡	620 ± 36‡	3927 ± 583‡	2056 ± 125‡	27 ± 6‡
	4	129 ± 20‡	116 ± 21‡	284 ± 46‡	306 ± 72	665 ± 119‡	3887 ± 649‡	2031 ± 203‡	37 ± 12‡
Female	0	70 ± 3§	117 ± 13‖	242 ± 36‖	604 ± 48‖	563 ± 90‖	3028 ± 367‖	1653 ± 165‖	56 ± 10‖
	2	114 ± 19‡	130 ± 5	302 ± 29†	484 ± 46‡	680 ± 80†	3801 ± 346‡	1999 ± 142‡	29 ± 4‡
	4	138 ± 30‡	122 ± 19	276 ± 28	382 ± 34‡	684 ± 98†	4320 ± 582‡	2181 ± 92‡	24 ± 5‡

Pups were injected with either 2.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life and euthanized at 150 days of age. Hexobarbital-induced sleeping times were determined at 50 days of age. Results are means ± SD of at least 7 mice/group.

* Androgen metabolites are listed under their substrates according to hydroxylation sites. Having no standard, 6α-hydroxytestoserone (6α-OH) has been tentatively identified by comparison with published HPLC retention times and TLC R_f values.

†‡ Significantly different from diluent-treated mice of the same sex: †$P < 0.05$; and ‡$P < 0.01$.

§‖ Compares diluent-treated males with diluent-treated females: §$P < 0.01$; and ‖$P < 0.01$.

Table 4. Sex-independent hepatic microsomal monooxygenase activities of adult mice treated neonatally with MSG

Sex	MSG treatment (mg/g)	Androstenedione 6β-OH* (pmol/min/mg protein)	Androstenedione 15α-OH (pmol/min/mg protein)	Androstenedione 16α-OH (pmol/min/mg protein)	Testosterone 2β-OH (pmol/min/mg protein)	Testosterone 6β-OH (pmol/min/mg protein)	Testosterone 7α-OH (pmol/min/mg protein)	Testosterone 11α-OH (pmol/min/mg protein)	Testosterone 16β-OH (pmol/min/mg protein)	Testosterone ASD (pmol/min/mg protein)
Male	0	1015 ± 82	509 ± 27	521 ± 83	44 ± 8	761 ± 85	173 ± 35	138 ± 25	378 ± 78	1194 ± 181
	2	1455 ± 185‡	775 ± 104‡	737 ± 84‡	49 ± 6	789 ± 55	141 ± 23	302 ± 101‡	634 ± 126‡	2267 ± 212‡
	4	1558 ± 112‡	770 ± 35‡	810 ± 144‡	73 ± 8‡	869 ± 69†	312 ± 84‡	270 ± 30‡	630 ± 142‡	2582 ± 390‡
Female	0	973 ± 52	476 ± 35	507 ± 70	43 ± 10	772 ± 84	199 ± 72	108 ± 29	384 ± 49	1425 ± 226
	2	1450 ± 59‡	645 ± 68‡	756 ± 85‡	74 ± 10‡	887 ± 79†	434 ± 59‡	236 ± 43‡	531 ± 54‡	2284 ± 132‡
	4	1698 ± 135‡	794 ± 77‡	894 ± 49‡	80 ± 9‡	1051 ± 231‡	453 ± 87‡	293 ± 87‡	680 ± 104‡	2887 ± 610‡

Pups were injected with either 2.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life and euthanized at 150 days of age. Results are means ± SD of at least 7 mice/group.

* With the exception of the androstenedione (ASD)-metabolite of testosterone, metabolites are listed under their substrates according to hydroxylation sites.

†‡ Significantly different from diluent-treated mice of the same sex: †$P < 0.05$; and ‡$P < 0.01$.

the mouse liver [23]. Thus, while it is known that increased activities of 7-ethoxyresorufin *O*-deethylase, testosterone 6β-, 2α- and 15α-hydroxylases and androstenedione 16β-hydroxylase are specific indicators of enhanced expression of rat cytochromes P450 1A1, 3A, 2C11, 2A2 and 2B1, respectively [5, 23, 24], there are far fewer specific murine catalytic markers. Testosterone 15α-hydroxylase appears to be specific for the female-predominant, constituent murine cytochrome P450 2a-4, while testosterone 16α-hydroxylase is representative of a constituent, male-specific isoform(s), as yet unclassified [23, 25]. In addition, testosterone is also hydroxylated at the 16α-position by at least two female-specific, inducible isoforms of cytochrome P450 in the 2b subfamily [23], but are unlikely to be expressed at more than minimal levels in our study [25].

In spite of the paucity of information regarding the molecular composition of the murine hepatic monooxygenase system, several conclusions pertaining to gender differences and growth hormone regulation can be derived from our results. Our findings are in agreement with previous studies reporting that regardless of strain, female mice exhibit the higher levels of hepatic hexobarbital hydroxylase [10–14] and testosterone 15α-hydroxylase [25–27], whereas males have greater concentrations of testosterone 16α-hydroxylase [25–28]. Since P450 2a-4 (i.e. testosterone 15α-hydroxylase) is a selective metabolizer of ethoxycoumarin [28], our finding of greater ethoxycoumarin *O*-deethylase activity in female liver than in male liver was not unexpected. Several other regio- and stereospecific testosterone hydroxylation sites have been reported [26, 27] and, depending upon strain, testosterone 6α- and 7α-hydroxylases have been found to be either female-predominant or sex-independent. (Unlike previous studies which limited their measurements to testosterone hydroxylations [25–28], we have also examined regio- and stereospecific androstenedione hydroxylations. Although the enzymatic reactions with both steroid substrates are not necessarily comparable [5, 22], they do expand our definition of sexually dimorphic monooxygenase activities in the mouse liver.)

Disregarding specific hydroxylation sites, the present results indicate that there are considerably more female-predominant than male-predominant hepatic monooxygenase activities, and that the number of sex-independent activities are somewhat greater than female-predominant enzyme reactions. These results support earlier studies demonstrating that drugs are metabolized in female mice more quickly than in males [10–12, 19], but unlike the unique and exaggerated differences in the rat where sexual dimorphisms can be greater than 600% [3–5], murine drug-metabolizing enzyme concentrations, depending upon strain, are only 40–100% greater in females than in males [12]. The magnitude of this smaller, but consistent sexual dimorphism in murine drug metabolism is reflected in our findings of a less than 1-fold gender difference in the sex-dependent drug and androgen hydroxylations (compared to many 10- to 20-fold sex differences in the rat [3–5, 9]), and by the large number of microsomal monooxygenases (almost half of those measured) that are sex-independent.

It is possible that the 40% gender difference in hepatic microsomal NADPH–cytochrome P450 reductase (F > M) may account, in part, for the 40–60% sex difference in the female-predominant monooxygenases. However, it should be noted that the concentration of the reductase is 500- to 1000-times greater than any individual monooxygenase activity; previously studied sex differences in rat and murine hepatic monooxygenases have been shown to be solely due to gender differences in cytochrome P450 isoforms [4, 5, 9]; and the greater concentration of hepatic NADPH–cytochrome P450 reductase in females does not explain the presence of a large number of sex-independent monooxygenases, not to mention the male-predominant enzymes.

Previously, we had shown that gender differences in murine drug metabolism were regulated by growth hormone [13, 14], and then subsequently identified the sexual dimorphisms in the secretory growth hormone profiles of the mouse [18]. Our present results expand our list of sex-dependent drug-metabolizing enzymes to include cytochrome P450-dependent steroid hydroxylases, and present gender differences in plasma growth hormone that are indistinguishable from our earlier study. We have proposed that the interpulse periods, when growth hormone levels are undetectable, are the actual signaling elements in the sexually dimorphic growth hormone profiles that regulate the gender differences in murine monooxygenases [18]. Thus, the twice-as-long interpulse periods in the male mouse are responsible for the enhanced expression of the male-predominant hepatic monooxygenases and/or suppression of the female-predominant enzymes. Likewise, the shorter interpulses in the female increase the expression of the female-predominant monooxygenases and/or suppress the male-predominant hepatic enzymes. Recently, we demonstrated the existence of a similar growth hormone regulatory mechanism in the rat where we were able to measure both the sex-dependent isoforms of cytochrome P450 and their specific mRNAs [29].

Assuming, as we already have shown in the rat [3, 8, 9, 15], that it is the depletion in circulating growth hormone that is responsible for the abnormal expression of hepatic monooxygenases in the MSG-treated mice, then the present studies suggest that, under most circumstances, growth hormone normally represses the expression of murine hepatic monooxygenases. Since the levels of the sex-independent monooxygenases in both males and females are elevated by MSG treatment, it would appear that these enzymes are equally inhibited by either the masculine or feminine patterns of growth hormone secretion, but the absence of the hormone (i.e. MSG treatment) allows for their full expression. Furthermore, while the feminine pattern of frequent growth hormone pulses suppresses male-predominant monooxygenases and the masculine profile of extended growth hormone interpulses inhibits female-predominant monooxygenases, the elimination of circulating growth hormone by MSG is equally effective in enhancing expression of the sex-

dependent monooxygenases as are the sexually homologous growth hormone profiles.

The concept that growth hormone may normally suppress monooxygenase activities has support from both rat and murine studies. In the case of the rat, the levels of male-specific cytochromes P450 2A2 (testosterone 15α-hydroxylase) and 3A (testosterone 6β-hydroxylase) are greatest in hypophysectomized animals, but whereas the enzymes are partially suppressed, and thus expressed at reduced levels, under the influence of the male pattern of growth hormone secretion, they disappear when the hormone is secreted in the feminine profile [4, 5, 29]. Moreover, female-specific cytochrome P450 2C12 is fully suppressed by the male rhythm of plasma growth hormone, but is optimally expressed in the presence of the female hormone profile or in the absence of any growth hormone [8]. Analogous experiments using hypophysectomized mice [13, 14] and isolated growth hormone deficient "little" mice [25, 30] have demonstrated that the levels of the female-dependent testosterone hydroxylases and drug-metabolizing enzymes are suppressed the most by the masculine plasma growth hormone profile, are less suppressed by the feminine hormone profile, but are most fully expressed when plasma growth hormone is eliminated.

Why then the existence of male and female plasma growth hormone profiles to regulate sex-dependent levels of murine hepatic monooxygenases when the enzymes can be fully expressed in the absence of growth hormone? In response, it should be noted that without the sexual dimorphism in growth hormone secretion there would be no gender differences in cytochrome P450-dependent metabolism of such endogenous compounds as steroids, bile acids, thyroid hormones, prostanoids and fatty acids. Since untold numbers of sexual dimorphisms occur in all eukaryotes, it is likely, that in this case, the full and equal expression in both sexes of all cytochrome P450-dependent monooxygenases might not be beneficial to the species. Perhaps it is important that male and female mice metabolize endogenous metabolites, like sex steroids, at different rates and by different monooxygenase-dependent pathways.

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REFERENCES

1. Olney J and Ho O-L, Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. *Nature* **227**: 609–611, 1970.
2. Olney JW, Excitotoxic food additives: Functional teratological aspects. *Prog Brain Res* **73**: 283–294, 1988.
3. Shapiro BH, MacLeod JN, Pampori NA, Morrissey JJ, Lapenson DP and Waxman DJ, Signalling elements in the ultradian rhythm of circulating growth hormone regulating expression of sex-dependent forms of hepatic cytochrome P450. *Endocrinology* **125**: 2935–2944, 1989.
4. Legraverend C, Mode A, Wells T, Robinson I and Gustafsson J-Å, Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J* **6**: 711–718, 1992.
5. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
6. Tannenbaum GS and Martin JB, Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* **98**: 562–570, 1976.
7. Edén S, Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology* **105**: 555–560, 1979.
8. Waxman DJ, Morrissey JJ, MacLeod JN and Shapiro BH, Depletion of serum growth hormone in adult female rats by neonatal monosodium glutamate treatment without loss of female-specific hepatic enzymes P450 2d (IIC12) and steroid 5α-reductase. *Endocrinology* **126**: 712–720, 1990.
9. Pampori NA, Agrawal AK, Waxman DJ and Shapiro BH, Differential effects of neonatally administered glutamate on the ultradian pattern of circulating growth hormone regulating expression of sex-dependent forms of cytochrome P450. *Biochem Pharmacol* **41**: 1299–1309, 1991.
10. Catz C and Yaffe SJ, Strain and age variations in hexobarbital response. *J Pharmacol Exp Ther* **155**: 152–156, 1966.
11. Vesell ES, Factors altering the responsiveness of mice to hexobarbital. *Pharmacology* **1**: 81–97, 1968.
12. MacLeod JN, Sorensen MP and Shapiro BH, Strain independent elevation of hepatic mono-oxygenase enzymes in female mice. *Xenobiotica* **17**: 1095–1102, 1987.
13. MacLeod JN and Shapiro BH, Growth hormone regulation of hepatic drug-metabolizing enzymes in the mouse. *Biochem Pharmacol* **38**: 1673–1677, 1989.
14. Shapiro BH, Pituitary-dependent masculinization of hepatic hexobarbital hydroxylase in Crl:CD-1 (ICR)BR mice. *Life Sci* **36**: 1169–1174, 1985.
15. Shapiro BH, Pampori NA, Ram PA and Waxman DJ, Irreversible suppression of growth hormone-dependent cytochrome P450 2C11 in adult rats neonatally treated with monosodium glutamate. *J Pharmacol Exp Ther* **265**: 979–984, 1993.
16. MacLeod JN and Shapiro BH, Repetitive blood sampling in unrestrained and unstressed mice using a chronic indwelling right atrial catheterization apparatus. *Lab Anim Sci* **38**: 603–608, 1988.
17. Pampori NA, Agrawal AK and Shapiro BH, Renaturalizing the sexually dimorphic profiles of circulating growth hormone in hypophysectomized rats. *Acta Endocrinol (Copenh)* **124**: 283–289, 1991.
18. MacLeod JN, Pampori NA and Shapiro BH, Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. *J Endocrinol* **131**: 395–399, 1991.
19. Shapiro BH and Szczotka SM, Androgenic repression of hexobarbitone metabolism and action in Crl:CD-1(ICR)BR mice. *Br J Pharmacol* **81**: 49–54, 1984.
20. Kupfer D and Rosenfeld J, A sensitive radioactive assay for hexobarbital hydroxylase in hepatic microsomes. *Drug Metab Dispos* **1**: 760–765, 1973.
21. Aitio A, A simple and sensitive assay of 7-

ethoxycoumarin deethylation. *Anal Biochem* **85**: 488–491, 1978.
22. Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
23. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K and Nebert DW, The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trival names of enzymes, and nomenclature. *DNA Cell Biol* **12**: 1–51, 1993.
24. Schenkman JB, Steroid metabolism by constitutive cytochromes P450. *J Steroid Biochem Mol Biol* **43**: 1023–1030, 1992.
25. Noshiro M and Negishi M, Pretranslational regulation of sex-dependent testosterone hydroxylases by growth hormone in mouse liver. *J Biol Chem* **261**: 15923–15927, 1986.
26. Ford HC, Lee E and Engel LL, Circannual variation and genetic regulation of hepatic testosterone hydroxylase activities in inbred strains of mice. *Endocrinology* **104**: 857–861, 1979.
27. Hawke RL and Neims AH, Maturation of various testosterone hydroxylase activities in hepatic microsomes from AKR/J mice. *Prog Clin Biol Res* **135**: 25–35, 1983.
28. Harada N and Negishi M, Mouse liver testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$). Purification, regioselectivity, stereospecificity, and immunochemical characterization. *J Biol Chem* **259**: 12285–12290, 1984.
29. Waxman DJ, Pampori NA, Ram PA, Agrawal AK and Shapiro BH, Interpulse interval in circulating growth hormone patterns regulate sexually dimorphic expression of hepatic cytochrome P450. *Proc Natl Acad Sci USA* **88**: 6868–6872, 1991.
30. MacLeod JN and Shapiro BH, Elevated hepatic hexobarbital hydroxylase activity in growth hormone deficient C57BL/6J "little" mice. *Fedn Proc* **45**: 406, 1986.