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DIFFERENTIAL EFFECTS OF NEONATALLY ADMINISTERED GLUTAMATE ON THE ULTRADIAN PATTERN OF CIRCULATING GROWTH HORMONE REGULATING EXPRESSION OF SEX-DEPENDENT FORMS OF CYTOCHROME P450

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Abstract—Neonatal male rats were treated with monosodium glutamate (MSG) at either 0.5, 1.0, 2.0, 3.0 or 4.0 mg/g body weight on alternate days during the first 9 days of life. As adults, rats were catheterized to obtain unstressed, serial blood samples for the determination of ultradian patterns of circulating growth hormone. In addition, the levels of drug-metabolizing enzymes (i.e. hexobarbital hydroxylase, cytochromes P450 and b_5, NADPH-cytochrome P450 reductase and ethoxyresorufin *O*-deethylase) as well as sex-dependent forms of cytochrome P450 [i.e. male-dependent cytochromes P450 2c (IIC11), 2a (IIIA2) and RLM2 (IIA2) and female-dependent cytochromes P450 2d (IIC12) and 3 (IIA1)] and/or their catalytic activities were measured in the hepatic microsomes of the treated rats. The results demonstrated a dose-dependent, graded response to MSG treatment. As the dose of MSG increased from 0.5 to 4.0 mg, there was a concurrent decline in the amplitudes of the characteristically masculine, episodic bursts of growth hormone, until at the highest dose (4 mg), the pulses were no longer detectable. Associated with this dose-dependent alteration in the ultradian pattern of growth hormone secretion was a measurable change in the activities of the sex-dependent hepatic enzymes. As the pulse heights of the hormone declined to 10–20% of their normal amplitudes, the levels of the male-dependent enzymes (i.e. the drug-metabolizing enzymes, as well as the male forms of cytochrome P450 and their specific steroid hydroxylases) were maintained, and in some cases, exceeded the levels normally found in males. However, as the hormone pulse heights declined, there appeared an accompanying increase in the activities of some of the female-dependent enzymes. Finally, with the loss of all detectable levels of circulating growth hormone, the normal masculine profile of hepatic enzymes was reversed to an apparently normal (with the exception of cytochrome P450 2d) feminine profile. Summarizing, the results indicate that (1) neonatal administration of MSG can produce dose-dependent, graded, long-term developmental defects in the ultradian rhythm of circulating growth hormone and associated sex-dependent hepatic enzymes, and (2) while the male-dependent hepatic enzymes can be maintained at normal or even higher levels in the face of an up to 90% reduction in the pulse heights of plasma growth hormone, the activities of the female-dependent enzymes may begin to increase.

Neonatal administration of monosodium glutamate (MSG) to rats and mice as well as other species [1, 2] produces a well-defined syndrome characterized by neuroendocrine deficiencies causing stunted body growth and obesity [1–4]. Whereas serum levels of corticotrophin [5], prolactin [6, 7], thyroid-stimulating hormone (TSH) [8] and gonadotrophins [9] are usually normal, or perhaps only slightly depressed, serum concentrations of growth hormone are reduced profoundly in MSG-treated rats [6–8].

MSG produces lesions in the arcuate nuclei, the circumventricular organs, and the retinae when administered during the critical neonatal period of brain development [1, 2, 6, 7]. Morphological manifestations of the lesions are apparent within 15–20 min after MSG treatment, and neuronal necrosis is complete within 2–4 days [2, 10]. Effective doses can destroy 80–90% of the neurons in the arcuate nucleus [1, 7, 10] whereas adjacent areas, such as the ventromedial nucleus, are spared. This selective effect of MSG on hypothalamic centers explains why growth hormone is the most profoundly affected pituitary hormone. That is, neonatal administration of MSG causes almost complete disappearance of growth hormone-releasing factor (GRF)-immunoreactive cell bodies in the arcuate nucleus and GRF-immunoreactive fibers in the median eminence [11], while apparently having no effect on somatostatin levels [7]. Thus, circulating concentrations and pulsatile release of growth hormone are reduced severely by MSG treatment [4, 6, 7].

Coincidentally, the ultradian rhythms in circulating growth hormone have been shown to mediate sex steroid regulation of the sexually dimorphic

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expression of hepatic monooxygenases [12, 13]. In male rats, growth hormone is secreted in episodic bursts every 3–4 hr. Between the peaks, growth hormone levels are extremely low or undetectable. In females, peaks are of lower magnitude than in males and occur irregularly, whereas the troughs between the peaks are elevated considerably compared to those in males [14, 15]. Apparently, exposure to the more tonic feminine secretory pattern 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 allows for the occurrence of a 3- to 5-fold higher level of hepatic drug metabolism [12, 13].

Recently, we reported [16, 17] that neonatal administration of 4 mg MSG/g body weight (consistently, the most commonly used dose) results in the total loss of all measurable levels of circulating growth hormone in adulthood. Neonatal treatment with half this dose (i.e. 2 mg/g) causes an 80–90% reduction in the pulse amplitudes of plasma growth hormone, but the sexually dimorphic, ultradian patterns of hormone secretion remain distinct in both sexes. Not unexpectedly, the dose-dependent alterations in circulating growth hormone had different effects on drug metabolism and the activities and concentrations of various forms of hepatic cytochrome P450.*

These results, using just two doses of the amino acid, suggested a dose dependency producing measured effects on growth hormone secretion and associated hepatic cytochrome P450. In the present investigation, we examined the long-term effects of neonatal treatment with five different doses of MSG to determine if early exposure to the amino acid could produce dose-dependent, graded, teratological defects in growth hormone secretion and associated liver function.

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 rat 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 0800 hr). After a 2- to 3-week acclimation period in our facilities, the animals were bred by randomly housing two adult female Sprague–Dawley rats [Crl:CD(SD)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 (0.5, 1.0, 2.0, 3.0, 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 [19] anchored to the skin with a sewing snap. Use of our recently developed mobile catheterization apparatus permitted repetitive blood sampling from unrestrained, unstressed, and completely conscious animals [16, 19]. Blood collections began 5–7 days after surgery. During sampling, 50 μL blood was removed every 15 min for 6 consecutive hr. Blood was centrifuged, and plasma was stored at −80° for future assay. Seven to ten days later, blood was collected a second time from each animal following the same procedures. To maintain extended patency, catheters were flushed two or three times per day with heparinized (10 I.U./mL) saline.

Growth hormone. Plasma growth hormone concentrations were determined in duplicate by standard radioimmunoassay (RIA) techniques [20, 21] using rat-specific materials supplied by the NIDDK (Bethesda, MD). Iodinated rat growth hormone tracer (NIDDK rGH I-5) was prepared with chloramine-T and then purified using gel filtration chromatography. A 10-point standard curve was prepared with serial dilutions of rat growth hormone (NIDDK rGH RP-2) and corresponded to plasma concentrations in unknown samples ranging from 0.8 to 400 ng/mL. The primary (monkey) antiserum (NIDDK anti-rGH S-5) was used at a final tube dilution of 1:30,000 in a total 500-μL incubate. 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) was added simultaneously with 100 μL polyethylene glycol 6000 (30%, w/v) and centrifuged for 30 min at 2000 *g* to separate bound from free growth hormone.

Sleeping times. At about 3 months of age, barbiturate-induced sleeping times were measured in some of the offspring after an i.p. injection of hexobarbital (150 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 [22].

Microsomes. Animals were killed by decapitation at 6 months of age and hepatic microsomes were prepared by our previously described method [16].

Drug-metabolizing enzymes. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [22] of the radioenzyme procedure of Kupfer and Rosenfeld [23]. Kinetic data for hexobarbital hydroxylase were obtained with eight different concentrations of hexobarbital (0.034 to 0.34 mM). Cytochrome P450 was determined from the reduced carbon monoxide difference spectrum

* P450 protein designations used in this study were summarized previously [13]. The systematic nomenclature for individual P450 gene products (classified according to gene families) is indicated by Roman numerals as reported previously [18].

Table 1. Body dimensions and organ weights of 6-month-old rats treated neonatally with MSG

Sex	MSG treatment (mg/g)	Body weight (g)	Lee index*	Liver (mg/g body weight)	Kidneys (mg/g body weight)
Male	0	588 ± 70	0.332 ± 0.031	34.1 ± 2.2	6.61 ± 0.77
	0.5	573 ± 96	0.311 ± 0.017	30.3 ± 0.8†	5.91 ± 0.53
	1.0	525 ± 87	0.338 ± 0.036	26.8 ± 1.3†	5.80 ± 0.62
	2.0	556 ± 36	0.348 ± 0.035	28.6 ± 1.4†	5.14 ± 0.35†
	3.0	470 ± 74‡	0.373 ± 0.037†	27.8 ± 2.9†	5.14 ± 0.30†
	4.0	417 ± 45†	0.404 ± 0.029†	28.7 ± 4.4‡	5.06 ± 0.23†
Female	0	307 ± 14†	0.333 ± 0.020	35.4 ± 3.0	7.84 ± 0.42†

Pups were injected with 0.5, 1.0, 2.0, 3.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life. Results are means ± SD of at least five rats/group.

* Lee index: [$\sqrt[3]{\text{body weight (g)}}$/naso-anal length (cm)] determined at about 3 months of age with at least fifteen rats/group.

†‡ Significantly different compared to diluent-treated males: †$P < 0.01$, and ‡$P < 0.05$.

according to the method of Omura and Sato [24]. Cytochrome b_5 was measured as described previously [24]. The activity of ethoxyresorufin *O*-deethylase was measured fluorimetrically by the method of Burke and Mayer [25]. NADPH–cytochrome P450 reductase activity was assayed by monitoring the rate of cytochrome *c* reduction at 550 nm [26].

Immunoquantitation of cytochromes P450. Individual rat hepatic cytochromes P450 were assayed in isolated liver microsomes by Western blotting, using antibodies reactive with cytochrome P450 forms 2a (IIIA2), 3 (IIA1), 2d (IIC12) and RLM2 (IIA2) as previously described [27, 28].

Steroid hydroxylase and reductase assays. Microsomal fractions were assayed for steroid hydroxylase and reductase activities using [4-^{14}C]androstenedione or [4-^{14}C]testosterone, with the products resolved by TLC and quantified by our previously reported methods [27, 29].

Kinetic analysis and statistics. Under the conditions of all of the assays, product formation was linear with respect to time and microsomal protein concentrations. Apparent Michaelis constants (K_m) and maximum velocities (V_{max}) were determined from linear regression models of the data using the method of Hofstee [30]. The correlation coefficients for all Hofstee plots were positive, exceeded 0.95, and were statistically significant ($P < 0.01$).

Experimental groups were compared for statistically significant differences by analysis of variance and the Duncan procedure for multiple comparisons [31].

RESULTS

Body and organ weights. Neonatal treatment with either the 3 or 4 mg dose of MSG resulted in subnormal body, liver and kidney weights as well as a demonstrative obesity (as measured by an elevated Lee index [32]) in adulthood (Table 1). The lower 0.5, 1.0 and 2.0 mg doses of MSG produced no long-lasting effects on body weights or Lee indices. Irrespective of dose, the relative liver weights of all MSG-treated rats were significantly less than that of control males and females.

Hepatic drug-metabolizing enzymes. The sexual dimorphisms in hepatic drug-metabolizing enzyme activities were evident in the present study (Table 2). Total microsomal cytochrome P450 levels were greater in the livers of control males than in control females. Similarly, the V_{max} for hepatic hexobarbital hydroxylase was four times greater in males than in females. This sexual difference in enzyme activity was reflected in hexobarbital-induced sleeping times that were three times longer in females than in males. In addition, the female K_m for hexobarbital hydroxylase was 65% greater than the value found for males. No sexual differences in the activities of cytochrome b_5, ethoxyresorufin *O*-deethylase or NADPH–cytochrome P450 reductase were observed in the present study.

Adult males, neonatally treated with 4 mg MSG/g body weight had dramatically feminized (or demasculinized) levels of hepatic drug-metabolizing enzymes (Table 2). Their total cytochrome P450 and hexobarbital hydroxylase (V_{max} and K_m) activities were similar to those of control females. In this regard, hexobarbital-induced sleeping times were increased 90% in males treated with the 4 mg MSG dose, although these sleep times were still shorter ($P < 0.001$) than those in control females. With the exception of cytochrome P450, which was unaffected by the 3 mg dose of MSG, hexobarbital hydroxylase kinetics and sleep times were similarly feminized by both the 3 mg and 4 mg dose of the amino acid. While there was no sexual difference in the activity of ethoxyresorufin *O*-deethylase, neonatal treatment with the two largest doses of MSG resulted in a subsequent decrease in the activity of the enzyme.

In contrast to the 3 and 4 mg doses of MSG, the lower 0.5, 1.0 and 2.0 mg doses did not alter the masculine levels of cytochrome P450, hexobarbital hydroxylase (V_{max} and K_m) or hexobarbital-induced sleeping times. In fact, some of these lower doses appeared to supermasculinize the hepatic drug-metabolizing enzymes, i.e. microsomal levels of total cytochrome P450 and hexobarbital hydroxylase were greater in the 1 mg MSG-treated males than in control males.

Table 2. Hepatic microsomal drug-metabolizing enzymes and hexobarbital-induced sleeping times of 6-month-old rats treated neonatally with MSG

Sex	MSG treatment (mg/g)	Cytochrome P450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)	NADPH P450 reductase (nmol/min/mg protein)	Hexobarbital hydroxylase V_{max} (nmol/min/mg protein)	Hexobarbital hydroxylase K_m (10^{-4} M)	Sleeping time (min)	Ethoxyresorufin O-deethylase (pmol/min/mg protein)
Male	0	1.07 ± 0.11	0.73 ± 0.10	334 ± 39	7.44 ± 1.71	1.13 ± 0.14	41.2 ± 3.8	77.9 ± 8.4
	0.5	0.99 ± 0.17	0.68 ± 0.06	299 ± 18	7.47 ± 2.09	1.12 ± 0.10	40.8 ± 2.6	77.1 ± 4.5
	1.0	1.26 ± 0.08*	0.83 ± 0.02	325 ± 20	9.23 ± 0.50*	1.26 ± 0.06	39.5 ± 4.8	80.2 ± 8.4
	2.0	1.22 ± 0.15	0.69 ± 0.09	350 ± 75	7.90 ± 1.73	1.25 ± 0.08	36.9 ± 3.6†	70.9 ± 8.7
	3.0	0.93 ± 0.14	0.65 ± 0.11	352 ± 37	1.34 ± 0.58†	1.83 ± 0.42†	63.2 ± 9.4†	53.7 ± 2.6†
	4.0	0.87 ± 0.14*	0.65 ± 0.06	295 ± 33	1.53 ± 0.19†	2.21 ± 0.25†	78.3 ± 11.3†	58.9 ± 7.6†
Female	0	0.86 ± 0.06†	0.72 ± 0.04	351 ± 29	1.69 ± 0.26†	1.87 ± 0.29†	126.9 ± 7.7†	72.8 ± 2.4

Pups were injected with 0.5, 1.0, 2.0, 3.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life. Results are means ± SD of at least five rats/group, except for sleeping times which had at least fifteen rats/group and were determined when the rats were about 3 months of age.

*† Significantly different compared to diluent-treated males: *$P < 0.05$, and †$P < 0.01$.

Growth hormone. The established sexual dimorphism in ultradian patterns of plasma growth hormone was found in our control male and female rats (Fig. 1). In male rats, growth hormone was released in pulses every 3–3.5 hr, resulting in short-lived peaks of around 200–300 ng/mL, followed by approximately 2 hr of virtually undetectable trough levels (<5 ng/mL). In contrast, growth hormone was released in female rats in a more constant pattern. Numerous low amplitude pulses of the hormone resulted in peaks of 75–100 ng/mL, followed by short-lived troughs that were always measurable and rarely fell below 25 ng/mL.

There were no detectable levels of growth hormone in any of the plasma samples obtained during 6 continuous hours of serial blood collections from the adult males treated neonatally with 4 mg MSG/g body weight.* Males exposed to the 3 mg dose of MSG exhibited one of two identifiable patterns of circulating growth hormone. Like the 4 mg MSG-treated rats, two of the 3 mg treated rats had no detectable levels of plasma growth hormone in either of two serial collections obtained from each rat. In contrast, a single pulse (10–20 ng/mL) of growth hormone was detected in all of the 6 hr serial blood collections of two other catheterized, 3 mg MSG-treated rats (Fig. 1).

Male rats treated with either the 1 mg or 2 mg dose of MSG exhibited typical masculine patterns of growth hormone release, except that the amplitudes of the ultradian pulses were reduced to 10–20% of normal male levels (Fig. 1). Otherwise, like normal males, the peaks occurred about every 3–4 hr and the intervening 2-hr troughs had generally undetectable levels of growth hormone. Rats injected with the lowest 0.5 mg dose of MSG also maintained a masculine ultradian profile of growth hormone secretion. The pulse amplitudes of the hormone in two of the four catheterized animals appeared to be normal, while in the other two rats the pulses were not as great as that found in control males, but significantly higher than the growth hormone pulses in the 1 and 2 mg MSG-treated males (Fig. 1). (The occurrence of a growth hormone pulse composed of low-multiple peaks as depicted in Fig. 1 for the 0.5 mg MSG male is in no way unique to the treatment and has been reported in normal males [7, 33]. We have found that the total contents of hormone under low-multiple or high-single peak pulses are generally similar in the same rat.)

Hepatic forms of cytochrome P450. Quantitation of individual microsomal forms of cytochrome P450 by Western blot analyses and indirect measures of the cytochrome levels by microsomal androstenedione and testosterone hydroxylase activities were in agreement with the results of the more general assays for multicytochrome P450-dependent drug-metabolizing enzymes shown in Table 2. The adult male-specific

* Circulating growth hormone levels measured in serial blood samples from hypophysectomized rats with atrial catheters were similar to those found in 4 mg MSG-treated males. In both cases, the hormone concentrations were near or below the sensitivity of the assay, i.e. 1–3 ng/ml. (unpublished observations).

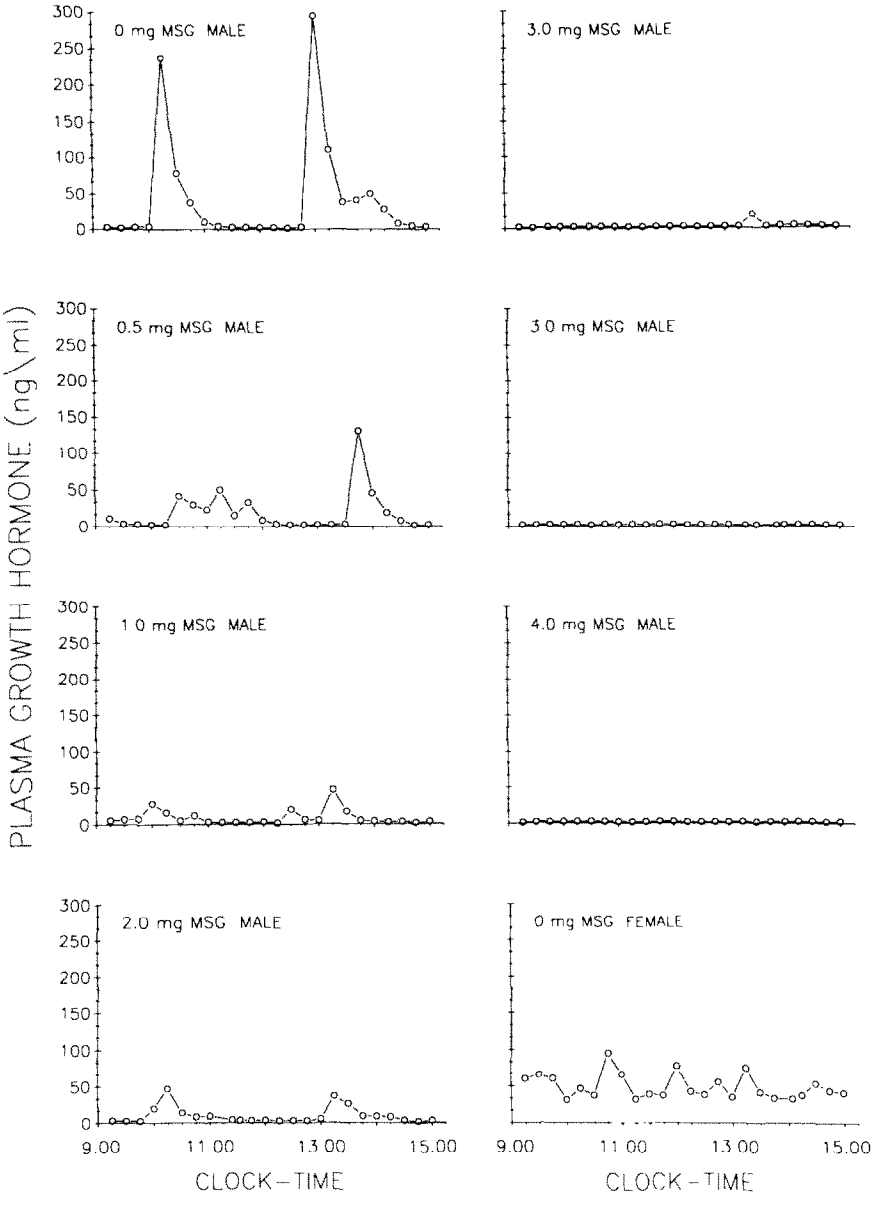

Fig. 1. Plasma levels of circulating growth hormone obtained from individual, undisturbed catheterized control and MSG-treated rats at 15-min intervals for 6 consecutive hr. Except for the 0.5 mg and 3.0 mg MSG-treated rats, similar findings were obtained from two to three additional animals in each treatment group. (Two of the 0.5 mg MSG-treated males had ultradian growth hormone patterns similar to control males, whereas two other rats in the treatment group had patterns like that depicted in the figure. Both growth hormone patterns for the 3.0 mg MSG treatment were obtained from two animals, each. See "Results" for further details.)

cytochrome P450 2c (IIC11)-dependent steroid 16α-hydroxylase and 2α-hydroxylase activities in the 4 mg MSG-treated males were about 10% of control male levels (Tables 3 and 4). This feminization was consistent with a profound decline in the concentrations of the male-specific cytochromes P450 2a (IIIA2) and P450 RLM2 (IIA2) (Fig. 2) and their respective androgen 6β- and 15α-hydroxylase activities (Tables 3 and 4). Along with a decrease in the levels of the male-specific forms of cytochrome P450 was a concomitant increase in the levels of female-predominant enzymes. Cytochrome P450 3 (IIA1) (Fig. 2) and its catalytic androgen 7α-hydroxylase activities were increased to female-like levels in the hepatic microsomes of 4 mg MSG-treated males. Similarly, testosterone 5α-reductase activity was increased 20-fold by neonatal administration of the 4 mg dose of MSG. In contrast, there was no detectable induction of female-specific cytochrome P450 2d (IIC12), as probed on Western blots, in the 4 mg MSG-treated males (Fig. 2).

With a few exceptions, early exposure to the 3 mg

Table 3. Hepatic microsomal testosterone hydroxylase and reductase activities of 6-month-old rats treated neonatally with MSG

Sex	MSG treatment (mg/g)	Hydroxylase					Reductase
		2α-	16α-	6β-	15α-	7α-	5α-
		(pmol/min/mg protein)					
Male	0	503 ± 136	803 ± 228	208 ± 45	256 ± 74	354 ± 64	61 ± 89
	0.5	548 ± 132	726 ± 208	207 ± 28	201 ± 85	442 ± 42	159 ± 214
	1.0	599 ± 124	786 ± 97	212 ± 18	307 ± 116	449 ± 97	192 ± 291
	2.0	814 ± 393	1133 ± 268*	426 ± 107†	362 ± 109	809 ± 118†	526 ± 331*
	3.0	93 ± 31†	142 ± 62†	118 ± 20†	103 ± 43†	1609 ± 143†	465 ± 352*
	4.0	79 ± 19†	107 ± 58†	76 ± 11†	112 ± 72*	1612 ± 312†	1222 ± 209†
Female	0	65 ± 16†	55 ± 25†	52 ± 19†	49 ± 17†	1508 ± 158†	2744 ± 1353†

Pups were injected with 0.5, 1.0, 2.0, 3.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life. Results are means ± SD of at least five rats/group.
*† Significantly different compared to diluent-treated males; *$P < 0.05$, and †$P < 0.01$.

Table 4. Hepatic microsomal androstenedione hydroxylase activities of 6-month-old rats treated neonatally with MSG

Sex	MSG treatment (mg/g)	Hydroxylase		
		16α-	6β-	7α-
		(pmol/min/mg protein)		
Male	0	515 ± 85	362 ± 40	519 ± 88
	0.5	627 ± 195	348 ± 9	628 ± 104
	1.0	808 ± 111*	377 ± 156	546 ± 117
	2.0	728 ± 122†	516 ± 103†	990 ± 244*
	3.0	140 ± 87*	197 ± 66*	1553 ± 184*
	4.0	40 ± 10*	58 ± 11*	1462 ± 78*
Female	0	70 ± 16*	77 ± 25*	1405 ± 134*

Pups were injected with 0.5, 1.0, 2.0, 3.0 or 4.0 mg MSG/g body weight or an equivalent volume of diluent on days 1, 3, 5, 7 and 9 of life. Results are means ± SD of at least five rats/group.
*† Significantly different compared to diluent-treated males: *$P < 0.01$, and †$P < 0.05$.

dose of MSG produced the same sex-dependent alterations in hepatic enzyme activities as the larger 4 mg dose. As regards the exceptions, however, while neonatal administration of 3 mg MSG clearly feminized (i.e. increased) testosterone 5α-reductase activity, it remained at only one-third the level found in the 4 mg MSG-treated males. Similarly, male-dependent androgen 6β-hydroxylase activity was completely feminized in the 4 mg MSG-treated rats, but was reduced by only 45% in the livers of the 3 mg MSG-treated males (Tables 3 and 4). In agreement, the microsomal concentration of cytochrome P450 2a (i.e. the 6β-hydroxylase protein) appeared to be somewhat greater in the livers of the 3 mg than 4 mg MSG-treated rats (Fig. 2).

In spite of a significant decline in the pulse amplitudes of circulating growth hormone in some 0.5 and all 1.0 mg MSG-treated males, the masculine profile of the sexually dimorphic hepatic cytochromes P450 and their specific catalytic activities was unaffected. Rats treated with the 2 mg dose of MSG had similar ultradian patterns of plasma growth hormone as the 1 mg MSG-treated rats but showed a significant hypermasculinization of several male-specific forms of cytochrome P450 and an intermediate feminization of some female-predominant enzymes. Male-dependent androgen 16α- and 6β-hydroxylase activities and the concentrations of cytochrome P450 2a (IIIA2) were greater in the 2 mg MSG-treated rats than in control males. Furthermore, cytochrome P450 3 (IIIA1) and its catalytic 7α-hydroxylase activity as well as testosterone 5α-reductase activity were feminized (i.e. increased) in the 2 mg MSG-treated males, although not to the same degree as found in the 4 mg treated males. [The 1 mg MSG-treated rats exhibiting the greatest degree of hypermasculinization of hepatic monooxygenases (Table 2) also had higher than male levels of androstenedione 16α-hydroxylase (Table 4) and cytochrome P450 2a (Fig. 2), but unlike the 2 mg MSG-treated males, no induction of female-dependent enzyme activities.]

DISCUSSION

Previous studies of the dose–response effects of

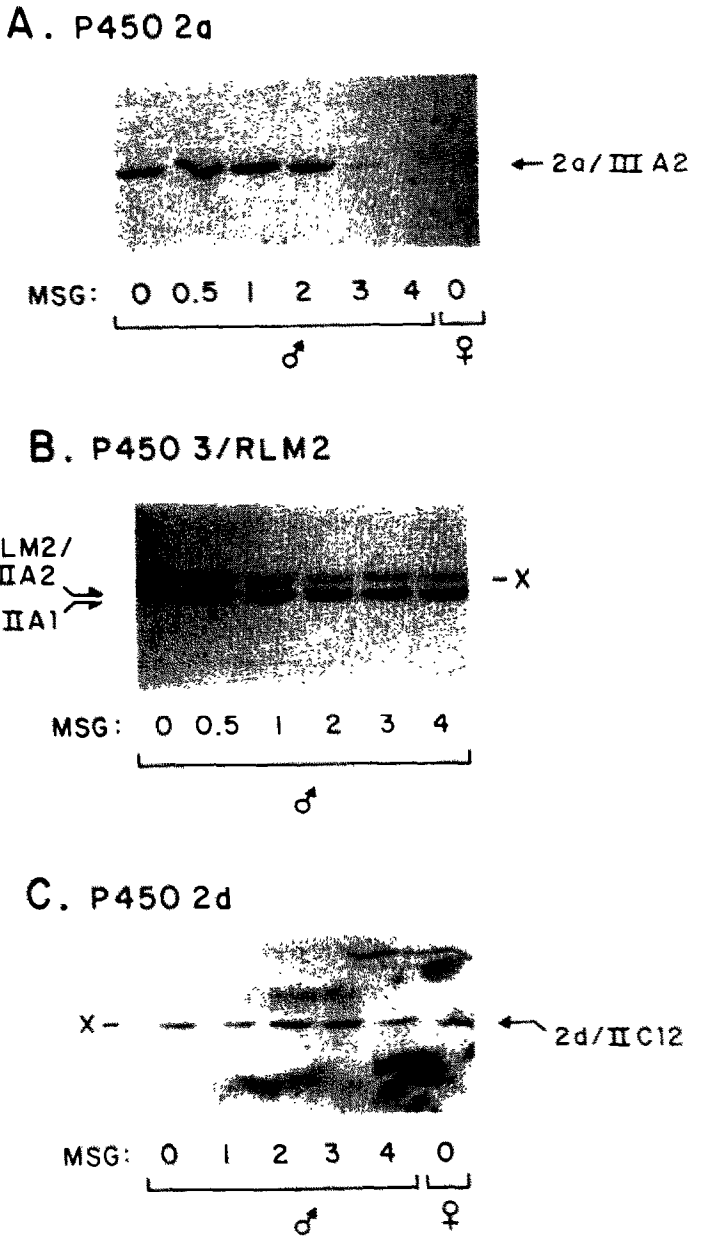

Fig. 2. Western blot analysis of cytochrome P450 proteins in liver microsomes isolated from MSG-treated rats. Liver microsomes were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose sheets, and then stained with antibody to P450 2a (IIIA2) (panel A), antibody to P450 3 (IIA1) that crossreacts with P450 RLM2 (IIA2) and an unidentified microsomal polypeptide designated × (panel B) or with antibody to the female-specific P450 2d (IIC12) marked by an arrow on the right side of panel C that is crossreactive with a male-predominant polypeptide (named × in panel C). Cytochrome P450 2d migrated slightly before polypeptide × that was present in all the male samples.

neonatally administered MSG have usually been conducted on mice. At a MSG dose of 0.5 mg/g body weight, about 50% of the mice develop histologically detectable hypothalamic lesions with an associated growth retardation. For the most part, treated animals without lesions appear to be normal. As the dose of the amino acid is increased, a larger percentage of the mice develop lesions, and it is these animals that exhibit stunted growth and obesity [34, 35]. Thus, results based upon these growth parameters have indicated an "all-or-none" dose–response. This apparent lack of a measured response proportional to the dose may explain why the vast majority of studies with MSG have used the same maximally effective dose [3, 4, 6, 9].

Our studies differ from the earlier dose–response

reports in two ways: (1) we used rats instead of mice, and (2) we measured more subtle biochemical effects of the MSG treatment rather than gross morphological alterations (i.e. body weight, obesity, and hypothalamic lesions). As a result of these experimental differences, we were able to find a dose–response for MSG that produced measured defects in proportion to the dose, and not simply an "all-or-none" response.

The present results both confirm and expand our previous observation that neonatal administration of 4 mg MSG/g body weight produces different effects on the ultradian pattern of plasma growth hormone and the levels of sex-dependent cytochrome P450 enzymes in adults than a 2 mg dose of the amino acid [16]. In half of the treated animals, as small a dose as 0.5 mg MSG caused a substantial decline in the pulse amplitudes of circulating growth hormone. When the dose of MSG was increased to 1 mg or 2 mg, the pulse heights of the hormone were further reduced to only 10–20% of their control levels. Otherwise, the masculine profile of episodic bursts (albeit at reduced levels) of growth hormone every 3–4 hr with undetectable concentrations between peaks were maintained in the 0.5, 1.0 and 2.0 mg MSG-treated males. Reflecting this dose-dependent reduction in the pulse heights of plasma growth hormone was a measured alteration in the activities of the hepatic cytochrome P450 enzymes. The more modest decline in growth hormone pulse heights observed in some of the 0.5 mg MSG-treated rats appeared to have no effect on the masculine profiles of hepatic enzymes. When the pulse amplitudes of the hormone were reduced by 80–90% of their normal levels in the 1 mg MSG-treated males, there was evidence of hypermasculinization (increased activity) of some cytochrome P450-dependent enzymes [i.e. total cytochrome P450, hexobarbital hydroxylase (V_{max}), androstenedione 16α-hydroxylase and possibly cytochrome P450 2a (IIIA2)]. The other male-dependent forms of cytochrome P450 were expressed at usual masculine levels, while the female-dependent enzymes remained at normally low male-like levels. When the dose of MSG was increased to 2 mg/g, there appeared to be no change in the ultradian pattern of plasma growth hormone from that seen in the 1 mg treated males. Similarly, there was a hypermasculinization of several male-dependent forms of cytochrome P450 [i.e. cytochrome P450 2c (IIC11)-dependent androgen 16α-hydroxylase and cytochrome P450 2a (IIIA2)-dependent androgen 6β-hydroxylase]. Along with this hypermasculinization was the first indication of some enzyme feminization. Neonates treated with 2 mg MSG had above normal levels of female-dependent testosterone 5α-reductase and cytochrome P450 3 (IIA1)-dependent androgen 7α-hydroxylase as adults. Although these enzyme activities were not as great as those found in females, they were elevated in comparison to normal males. Considering the similarities in their ultradian patterns of plasma growth hormone, it is not clear why the 2 mg dose of MSG would produce some feminization of liver enzymes, while the 1 mg dose of the amino acid did not. Careful examination of the circulating profiles of growth hormone in the 1 mg and 2 mg MSG-treated males suggests slightly (10–30%) greater pulse amplitude in the rats treated with the lower dose. However, any biological significance of this finding would be questionable since successive pulse heights can normally vary 100–200% within the same animal [6, 7, 15, 33]. Clearly, a much larger cohort than used in this study would have to be examined to establish a difference in the growth hormone secretory patterns of the 1 mg and 2 mg MSG-treated rats. It is also possible that the hepatic feminization seen in the 2 mg MSG-treated rats is independent of any changes in growth hormone secretion, but is a result of an effect on some additional, but unmeasured regulatory factor, such as thyroxine [36–38].

A dramatic change in the sexually dimorphic composition of hepatic enzymes occurred at the 3 mg MSG dose. Hexobarbital metabolism [i.e. hexobarbital hydroxylase (V_{max} and K_m) and hexobarbital-induced sleep times] became completely feminized. Male-specific cytochromes P450 2c (IIC11)-dependent androgen 2α- and 16α-hydroxylase, P450 2a (IIIA2)-dependent androgen 6β-hydroxylase and P450 RLM2 (IIA2)-dependent testosterone 15α-hydroxylase, as well as their actual enzyme concentrations estimated from Western blots, were reduced to female-like levels in the adult males, neonatally treated with 3 mg MSG. Concurrently, female-dependent testosterone 5α-reductase and cytochrome P450 3 (IIA1) and its catalytic androgen 7α-hydroxylase activities were increased towards female levels. Administration of the 4 mg dose produced similar enzymatic effects as the 3 mg dose, with the exception that female-specific testosterone 5α-reductase was three times greater in the 4 mg MSG-treated males than in the 3 mg treated animals, and male-dependent cytochrome P450 2a (IIIA2) and its catalytic androgen 6β-hydroxylase as well as total cytochrome P450 were less repressed in the 3 mg treated males. An explanation for the greater degree of enzymatic feminization seen in the 4 mg MSG-treated rats may lie in differences in the secretory patterns of growth hormone. Adult rats, neonatally treated with 4 mg MSG/g body weight, had no detectable concentration of growth hormone in any of the plasma samples obtained during 6 continuous hours of blood collection. In contrast, half the catheterized males exposed to the 3 mg dose displayed a small (10–20 ng/mL), but measurable burst of growth hormone within the 6-hr collection period. Perhaps this small secretory pulse was enough to prevent the more complete hepatic feminization seen in the 4 mg MSG-treated male rats. It should be noted, however, that in no case did the amino acid completely feminize the liver. None of the doses induced any observable expression of female-specific cytochrome P450 2d (IIC12). In agreement, Yamazoe *et al.* [39] reported no expression of this cytochrome in adult males treated with the 4 mg dose while we found a modest 20% of female levels in similarly treated males [16]. The secretory pattern of growth hormone required for the expression of cytochrome P450 2d is presently unresolved [17].

Recent studies have shown that the female pattern of relatively constant growth hormone secretion

stimulates hepatic expression of female-dependent cytochrome P450 3 and steroid 5α-reductase and fully suppresses male-specific cytochromes P450 2c, 2a, and RLM2 [27, 28, 40]. In contrast, the masculine pattern of pulsatile growth hormone release stimulates the expression of the 2c form of cytochrome P450 [27, 40, 41]. Although the levels of male-specific cytochromes P450 2a and RLM2 are greatest in the hypophysectomized rat, the enzymes are partially suppressed, and thus expressed at reduced levels, under the influence of pulsatile growth hormone, but disappear when the hormone is secreted constantly, provided that thyroid hormone is also present [27, 38]. These models of growth hormone regulation of hepatic cytochromes P450 expression have been derived largely from studies involving the ablation of growth hormone together with all other pituitary-dependent hormones after hypophysectomy, in conjunction with defined though incomplete, hormone replacement regimens. In contrast, the present studies, in agreement with our previous report [17], demonstrate that when adult growth hormone levels are selectively eliminated as a consequence of the hypothalamic lesions induced by neonatal MSG treatment, the resulting effects on growth hormone regulated hepatic P450 enzymes do not completely parallel the changes in cytochrome P450 profiles after hypophysectomy.

Possible explanations for the inconsistencies between the effects of hypophysectomy and MSG-induced loss of growth hormone on the sexually dimorphic expression of hepatic cytochromes P450 have been discussed in detail elsewhere [17]. Basically, the inconsistencies may be due to the fact that the hypophysectomized animal is a model of multiple primary hormone deficiencies (i.e. LH, FSH, prolactin, TSH, ACTH, growth hormone, etc.) resulting in multiple secondary hormone deficiencies (i.e. thyroxine, corticosterone, testosterone, etc.) which contrasts with the more selective growth hormone deficiency induced by MSG. In addition, the hypophysectomized animal experiences growth hormone ablation as an adult, while the neonatally MSG-treated rat is probably lacking growth hormone during the critical period of neuroendocrine differentiation. This early growth hormone deficiency may affect normal development of the hepatic cytochromes P450.

In addition to demonstrating the dose-dependent teratogenic properties of MSG, it is possible to use the affected animals as models to study essential components of the ultradian patterns of plasma growth hormone that regulate sex-dependent hepatic enzymes. Regardless of the fact that the hypophysectomized animal is a model of multiple hormone deficiencies, in relation to the present study, it is also *totally* deficient of growth hormone. In contrast, we have found that we can induce selective aberrations in the ultradian profile of plasma growth hormone in male as well as female [17] rats by varying the neonatal dose of MSG. Thus, in some of the 0.5 mg MSG-treated rats we have seen that a possible 50% reduction in the secretory pulse heights of growth hormone has no effect on maintaining the masculine profile of hepatic enzymes. When the amplitudes are further reduced by up to 90% in the 1 mg and 2 mg MSG-treated males, not only are the male-dependent enzymes maintained, but in some cases they exceed normal levels. Concurrently, the levels of certain female-predominant enzymes (i.e. cytochrome P450 3 and testosterone 5α-reductase) begin to increase. Thus, it would appear that neither the amplitude of the pulse nor its total content of growth hormone is critical for the expression of male-dependent forms of cytochrome P450. However, there seems to be a critical amplitude, below which the pulse of growth hormone cannot as thoroughly suppress the expression of female-predominant enzymes. The hepatic feminization of the 3 mg and 4 mg MSG-treated males indicates that the growth hormone troughs must be interrupted by periodic pulses, albeit greatly reduced from normal, for expression of the masculine profile of hepatic enzymes. (It is possible that as small a pulse as 10–20 ng/mL within a 6-hr period, as found in some of the 3 mg MSG-treated males, is sufficient to suppress the more complete feminization observed in the 4 mg MSG-treated males.) In agreement with our previous findings [17], we see that high levels of female-dependent cytochrome P450 3 and androgen 5α-reductase, characteristic of the gender, can be expressed in the complete absence of growth hormone. In summary then, the MSG-treated rat can serve as a useful model to identify salient components of the growth hormone ultradian rhythms regulating hepatic functions.

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