GENDER, AGE AND DOSE EFFECTS OF NEONATALLY ADMINISTERED ASPARTATE ON THE SEXUALLY DIMORPHIC PLASMA GROWTH HORMONE PROFILES REGULATING EXPRESSION OF THE RAT SEX-DEPENDENT HEPATIC CYP ISOFORMS

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ABSTRACT:
Newborn male and female rat pups were injected with either 2 mg or 4 mg monosodium aspartate (MSA)/g body weight or diluent on alternate days for the first 9 days of life. Both doses of the amino acid had profound effects on the sexually dimorphic growth hormone secretory profiles in adulthood. There were no measurable levels of growth hormone in any of the plasma samples obtained during 8 continuous hr of serial blood collections from the adult males and females treated neonatally with 4 mg of MSA. Male rats treated with half the dose of the amino acid (i.e., 2 mg MSA/g) exhibited typical masculine profiles of growth hormone release, except that the amplitudes of the ultradian pulses were reduced to 10–20% of normal male levels. Otherwise, like normal males, the peaks occurred about every 3–4 hr and the intervening 2.5-hr troughs had undetectable levels of growth hormone. In a similar sense, females treated with 2 mg of MSA maintained their sexually dimorphic pattern of plasma growth hormone, i.e., frequent pulses of hormone followed by short-lived troughs. However, the peaks rarely exceeded 20 ng/ml and the troughs usually fell to a measurable 8 to 10 ng/ml resulting in an approximate 75% reduction in the mean plasma concentration. Growth hormone- and gender-dependent expression of CYP2C7, 2C11, 2C12, 2C13, 2A1, 2A2, and 3A2 (mRNAs, proteins, and catalytic activities) were generally unaffected by neonatal exposure to 2 mg of MSA. In contrast, the higher 4-mg dose of the amino acid completely or near completely suppressed male-specific CYP2C11, 2C13, 2A2, and 3A2 expression while inducing small increases in female-specific CYP2C12 and female-predominant CYP2A1 in the treated males. Females exposed to the 4 mg MSA dose exhibited less severe isoform changes characterized by small reductions in CYP2C12 and 2C7 levels. Whereas expression levels of most of the CYP isoforms in both sexes were lowest in the pubertal (47-day-old) rats, and occasionally higher in the adults (207-day-old) as compared with the early postpubertal (70-day-old) rats, the effects of neonatal MSA were the same at all ages studied. Since each of the CYP isoforms are regulated by different “signaling elements” in the sexually dimorphic plasma growth hormone profiles, it is possible to correlate MSA-induced alterations in CYP expression levels to specific changes in the gender-dependent growth hormone profiles.

Neonatal administration of the ubiquitous food additive monosodium glutamate (MSG)1 to rats and mice, and possibly other species, can produce a well-defined syndrome characterized by stunted body growth and obesity (1). These defects are a result of glutamate-produced lesions in the developing brain that are generally limited to growth hormone-releasing hormone (GRH) secretory neurons in the arcuate nuclei and circumventricular organs of the hypothalamus (1–3). In the absence of GRH release, the pituitary does not secrete growth hormone and the affected animals exhibit obesity and retarded growth (1, 4). MSG-induced defects, however, are not limited to abnormalities in growth patterns. We have found that the activities of hepatic drug metabolizing enzymes are dramatically depressed in adult rats neonatally exposed to MSG (4–6). Like the regulation of growth, the expression of sex-dependent2 isoforms of CYP, major components in the drug metabolizing enzyme system, is also regulated by growth hormone (7–9). CYP2C11, the predominant isoform in adult male rat liver comprising up to 50% of the total CYP content (10), is dependent upon the presence of the masculine episodic growth hormone profile (7–9). The elimination of this profile from the circulation, and subsequent suppression of CYP2C11, helps to explain the 80–90% decline in drug metabolizing capacity of MSG-treated male rats.

The developmental defects produced by early exposure to glutamate do not appear to be unique to this amino acid, but can be induced by other excitotoxic amino acids like aspartate, one of the two constituent amino acids in the artificial sweetener aspartame (1, 11). As with MSG, neonatal treatment (oral or parenteral) of monosodium aspartate (MSA) has been reported to produce hypothalamic lesions (1, 2) resulting in a permanent growth hormone deficiency and associated subnormal hepatic hexobarbital hydroxylase activity (12). However, hexobarbital hydroxylation is dependent upon the contributing activities of several CYPs (13), and it is unknown which of the growth hormone-dependent isoforms of CYP are affected by MSA. In the present study, we have examined the dose-dependent effects of neonatally administered MSA on the sexually dimorphic growth hormone secretory profiles regulating the gender-dependent expression.

1 Abbreviations used are: MSA, monosodium aspartate; CYP, cytochrome P450; GRH, growth hormone-releasing hormone; MSG, monosodium glutamate.
2 The terms sex-dependent, sex-predominant or dominant, and sex-specific are often used indiscriminately. We use sex- or gender-dependent to imply that expression levels are dependent upon the existence of gender; sex- or gender-predominant indicates expression levels, regardless of magnitude, are consistently greater in one gender; and sex- or gender-specific implies that expression is basically restricted to only one gender.
of hepatic CYP isoforms in pubertal, early postpubertal, and adult male and female rats.

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. At all times animals were housed on hardwood bedding in plastic cages, with water and commercial rat diet supplied ad libitum. The animal quarters were air conditioned (20–23°C) 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 mixed and randomly assigned to the dams at 10 pups per litter, 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, all the pups in a litter were injected sc with either 2 or 4 mg microsomal protein of monosodium-L-aspartate (>99.9% pure, Sigma Chemical Co., St. Louis, MO) or an isosmotic equivalent of NaCl diluted for a total of five injections (i.e., all pups in a litter received the same treatment). The pups were weaned at 24 days of age. At around 7, 10, and 30 weeks of age, five to six rats in each treatment group were decapitated. Livers were quickly removed and perfused with ice-cold saline. Each liver was quickly minced; a portion for mRNA determinations was plunged into liquid nitrogen and subsequently stored at −70°C. The remaining liver mince was used for microsome preparation.

Blood Collection. Three to four animals (4 to 5 months of age) from each treatment group, and representing all litters in the group, were implanted with chronic indwelling right atrial catheters (14). Use of our mobile catheterization apparatus permitted repetitive blood sampling from unrestrained, unstressed, and completely conscious animals. Blood collections began 5–7 days after surgery. During sampling, 25 μl blood was removed every 15 min for 8 consecutive hr. Blood was centrifuged, and 10 μl plasma aliquots were stored at −70°C for future assay. Seven to 10 days later, blood was collected a second time from each animal following the same procedure. To maintain extended patency, catheters were flushed twice per day with heparinized (10 IU/ml) saline.

Growth Hormone. Eight-hour plasma growth hormone profiles were determined by using a homologous radioimmunoassay with a sensitivity of 2–3 ng/ml. All values were normalized by subtracting values obtained from hypophysectomized rats. Procedural details and statistical validation of the assay have been reported by us elsewhere (4, 12).

Western Blots. Hepatic microsomes were isolated by our reported method (12) and assayed by western blotting for the presence of CYP2C7, 2C11, 2C12, 2C13, and 3A2 proteins (15). Ten micrograms of microsomal protein were electrophoresed on 0.75-mm-thick SDS-polyacrylamide gels containing 7.5% acrylamide and electro-blotted onto nitrocellulose filters. The blots were probed with monoclonal anti-rat CYP2C11 (Oxford Biomedical Research Inc., Oxford, MI) and anti-rat CYP2C12/13 (kindly provided by Dr. Marika Rönnholm, Huddinge University Hospital, Sweden) mouse IgG, polyclonal anti-rat CYP2C7 (kindly provided by Dr. Stelvio M. Bandiera, University of British Columbia, Canada) and anti-rat CYP3A1/2 (XenoTech, Kansas City, KS). The characterization and high specificity of the individual antibodies to the CYP isoforms have been presented elsewhere (16).

The primary antibody was located with horseradish peroxidase conjugated to anti-mouse or anti-rabbit IgG and detected with an enhanced chemiluminescence kit (Amersham, Des Plaines, IL). Quantification of relative CYP levels was done by laser densitometry of the X-ray films.

Northern Blots. Total RNA was isolated from −0.5 g of individual rat liver by a single-step guanidinium-thiocyanate method (17). RNA samples from individual livers were fractionated by electrophoresis under denaturing conditions in 1.2% agarose gels containing 1x MOPS buffer and 1.28% formaldehyde. The RNA was transferred to GeneScreen nylon membranes (NEN DuPont, Boston, MA) by capillary transfer in 10x standard saline-citrate and then fixed to the filters by UV cross-linking. Prehybridizations and hybridizations in Rapid-hyb buffer (Amersham Corp.) using a 32P-labeled 2C11 oligonucleotide probe were performed with high stringency washings. The washed blots were wrapped in clear plastic and exposed to X-ray films with 2 intensifying screens at −70°C for 1–3 days. Northern blots were then successively stripped and reprobed with selective 32P-labeled 2A1, 2A2, 2C6, 2C7, 2C12, 2C13, and 3A2 oligonucleotide probes whose nucleotide sequences have been reported elsewhere (18–20). Their high degree of specificity has been demonstrated (18–20) and was further confirmed by routine competitive northern blotting analysis (21, results not presented). Evidence that RNA was equally loaded and transferred was obtained by equivalent intensity of ethidium bromide staining of 18S and 28S rRNA bands (22). Further, the rat 18S rRNA oligonucleotide probe was used as a control to verify the consistency and integrity of RNA loading (23). Quantitation of the mRNA by laser densitometry of the X-ray films was kept within the linear range as established by slot blot hybridizations and normalized to the 18S rRNA signals in each lane.

Testosterone Metabolism. In vitro testosterone 2α-, 6β-, and 1α-5α-reductase, and 7-ethoxycoumarin O-deethylase activities, indicative of activity levels of CYP2C11, 3A2/2C13, 2A1 and 2A2 proteins, respectively, and female-specific testosterone 5α-reductase, as well as 3α-hydroxysteroid dehydrogenase activity levels of CYP2C12 concentrations (cf. 18, 24), were assayed by our published HPLC (25) and TLC (26) methods.

Drug Metabolism. The activities of 7-ethoxycoumarin O-deethylase (27), pentoxyresorufin dealkylase (28), coumarin 7-hydroxylase, and 7-ethoxycoumarin O-deethylase (29) were measured spectrophotometrically. NADPH-cytochrome P450 reductase activity was assayed by monitoring the rate of cytochrome c reduction at 550 nm in 0.3 M KPi, pH 7.7 at 25°C in 1 ml incubations containing 10 μg microsomal protein (30).

Statistics. All data were subjected to analysis of variance, and differences were determined with s statistics and the Bonferroni procedure for multiple comparison.

Results

Growth Hormone. The established sexual dimorphisms in the ultradian patterns of plasma growth hormone were found in our control male and female rats (fig. 1). Generally in male rats growth hormone was released in pulses approximately every 3–4 hr, resulting in short-lived peaks of around 175 ng/ml, followed by approximately 2.5 hr or more of virtually undetectable trough levels (<3 ng/ml). In contrast, growth hormone was released in female rats in a more continuous pattern. Numerous low amplitude pulses of the hormone resulted in peaks of 50–75 ng/ml followed by short-lived troughs that were always measurable and rarely fell below 15–20 ng/ml.

There were no measurable levels of growth hormone in any of the plasma samples obtained during 8 continuous hr of serial blood collections from the adult males and females treated neonatally with 4 mg of MSA (fig. 1). Male rats treated with half the dose of the amino acid (i.e., 2 mg MSA/g) exhibited typical masculine profiles of growth hormone release, except that amplitudes of the ultradian pulses were reduced to 10–20% of normal male levels. Otherwise, like normal males, the peaks occurred about every 3.5 hr and the intervening 2.5-hr troughs had undetectable levels of growth hormone. In a similar sense, females treated with 2 mg of MSA maintained their sexually dimorphic pattern of plasma growth hormone, i.e., frequent pulses of hormone followed by short-lived troughs. However, the peaks rarely exceeded 20 ng/ml and the troughs usually fell to a measurable 8 to 10 ng/ml resulting in an approximate 75% reduction in the mean plasma concentration. Thus, while neonatal administration of the 2-mg dose of MSA did not disrupt the normal sex-dependent periodicity of growth hormone secretion in males and females, it did result in a dramatic reduction in the levels of circulating growth hormone.

CYP Proteins. The gender-dependent expression levels of the hepatic microsomal CYP proteins were in agreement with previous reports (7–9). That is, CYP2C11, 2C13, and 3A2 were all male specific, whereas CYP2C12 was female specific and CYP2C7 was female predominant (fig. 2). Although there were some age-dependent
effects, in general neonatal exposure to the 2 mg/g dose of MSA resulted in either a small or no reduction in the levels of the measured CYP proteins. In contrast, expression levels of the isoforms were dramatically affected by the 4-mg dose of the amino acid. CYP2C11 and 3A2 were suppressed in male liver to the point of being undetectable, whereas CYP2C13 in males and CYP2C12 in females were reduced by 60 to 70% and CYP2C7 measured in female liver was reduced 30 to 50%. Although the 4-mg dose of MSA had a small suppressive effect on the already low concentrations of hepatic CYP2C7 levels in males, it actually induced measurable concentrations (i.e., 10% of female levels) of female-specific CYP2C12 in male rats (fig. 2).

**Testosterone Metabolites.** MSA-induced effects on the in vitro catalytic activities of the male-dependent testosterone hydroxylases reflected similar changes in male-specific CYP protein levels. Whereas CYP2C11-dependent testosterone 2α-hydroxylase, CYP3A2 and 2C13-dependent testosterone 6β-hydroxylase and CYP2A2-dependent testosterone 15α-hydroxylase were unaffected by neonatal exposure to 2 mg/g of MSA, the 4-mg dose produced a long lasting 80% or more reduction in the CYP-dependent enzyme activities (fig. 3). Female-dependent testosterone metabolites were much less affected by the neonatally administered MSA. Like the male-dependent CYP catalytic enzymes, the activities of female-predominant CYP2A1-dependent testosterone 7α-hydroxylase, and testosterone 5α-reductase [a non-CYP-dependent enzyme whose activities generally reflect CYP2C12 levels (16, 31)] were unaltered by the 2 mg dose of MSA. Doubling the dose of MSA to 4 mg/g had no suppressive effect on the 7α-hydroxylase and 5α-reductase activities, but in most cases induced a small but significant elevation in the hepatic activities of these enzymes (fig. 3).

MSA-induced changes in testosterone metabolism could not be explained by similar changes in the activity of hepatic NADPH-cytochrome P450 reductase whose levels at all ages studied were unaltered in both male and female rats by neonatal exposure to MSA (data not presented).

Earlier, we reported an up to 100% difference in the induction levels of hepatic CYP2B1 and 2B2 between peripubertal and adult rats (32). With this observation in mind, in the present study we examined gender-dependent CYP levels in peripubertal (47 days of age), recent postpubertal (70 days of age) and mature adult (207 days of age) rats. Although CYP levels tended to be lower in the 47-day-old animals and were occasionally higher in the 207-day-old rats, the effects of MSA were the same at each age studied (fig. 2). That is, if neonatal MSA suppressed a CYP isoform at 47 days of age, the enzyme remained suppressed, and at near the same level at 70 and 207 days of age. Accordingly, we limited our present CYP mRNA mea-
Drug Metabolism. Neonatal exposure to MSA seemed to have its greatest effect on the drug metabolizing activities of pentoxyresorufin O-dealkylase and coumarin 7-hydroxylase (fig. 5). Since the major hepatic CYP isoform contributing to pentoxyresorufin O-dealkylase is CYP2C11 in male rats (33) and CYP2C12 in female rats (34), it is not surprising that the MSA-induced reduction in the levels of this drug metabolizing enzyme very much reflected a similar decline in CYP2C11 and CYP2C12 (figs. 2 and 4). Furthermore, the MSA-induced increase in hepatic coumarin 7-hydroxylase activity was in agreement with the increase in CYP2A1-dependent testosterone 7α-hydroxylase (fig. 3) and CYP2A1 mRNA (fig. 4); the major isoform contributing to coumarin 7-hydroxylase activity (35).

Discussion

In all species studied (9), growth hormone is secreted in sexually dimorphic ultradian profiles. In the case of the rat, males secrete growth hormone 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 more frequently, whereas the troughs between the peaks are considerably elevated compared with those of males. It is these gender differences in the growth hormone secretory profiles that regulate the sexually dimorphic expression of nearly a dozen constitutive and inducible isoforms of hepatic CYP. That is, expression of the major female-specific CYP2C12 (as well as the non-CYP 5α-reductase) is dependent upon the feminine profile of continuous growth hormone secretion. Exposure to the masculine profile of episodic hormone release as well as the absence of the hormone from the circulation (e.g., hypophysectomy) results in the complete suppression of CYP2C12 (7, 31). In a somewhat similar vein, female-predominant CYP2C7 expression is also dependent upon the feminine growth hormone profile and is completely suppressed in the hypophysectomized rat. However, exposure to the masculine profile allows for expression of CYP2C7 at 25–40% of normal female levels (7, 31). Expression of the major male-specific CYP2C11 requires the episodic “on-off” masculine profile of growth hormone secretion. Whereas the feminine pattern of continuous hormone secretion blocks CYP2C11 expression, total growth hormone depletion from the circulation allows for CYP2C11 expression at 15 to 25% of intact male levels (7, 10, 16). After hypophysectomy, female-predominant CYP2A1 (1:3; M:F) concentrations decline, but remain above male levels, and are restored to intact female-like levels by continuously administered growth hormone (16, 36). Although the expression levels of CYP2C7, 2C11, 2C12, and 2A1 are greatest when exposed to their gender-dependent growth hormone profiles, other isoforms may be optimally expressed in the absence of growth hormone. Male-specific CYP2A2 and 3A2 are maximally expressed in the hypophysectomized rat, disappear when growth hormone is secreted constantly, but are only partially suppressed, relative to the high levels observed in hypophysectomized rats, under the influence of episodic growth hormone (37, 38). Male-specific CYP2C13 is optimally expressed when exposed to the masculine profile of episodic hormone release as well as the absence of the hormone from the circulation (e.g., hypophysectomy). Male-specific CYP2C13 is optimally expressed when exposed to the masculine hormone profile or under conditions of no growth hormone, whereas the feminine growth hormone profile completely suppresses CYP2C13 (7, 16). Although there are additional examples, it becomes clear that the expression and/or suppression of each isoform of CYP is likely to be regulated by a different “signal” in the sexually dimorphic growth hormone profile. These “signals” may be recognized by the hepatocyte in the frequencies and/or durations of the pulse and interpulse periods. Or perhaps the hepatocyte can monitor the mean plasma concentration of the hormone (9).

Neonatal exposure to MSA produced a long-lasting and profound reduction in the circulating levels of growth hormone that was associated, at the 4 mg dose, with a suppression of major CYP isoforms. Although CYP levels tended to be lowest in the pubertal rats, the

![Fig. 3. Long lasting effects of neonatally administered MSA on the catalytic activities of hepatic microsomal CYP isoforms.](Image)
effects of the amino acid were the same at all ages studied. In contrast, the dose of MSA had a dramatic effect on growth hormone secretion and CYP expression. Whereas adult male and female rats treated neonatally with 4 mg/g body weight of MSA on alternate days for the first 9 days of life had no measurable plasma growth hormone, male rats treated with half the dose of the amino acid (i.e., 2 mg MSA) exhibited typical masculine profiles of growth hormone release, except that the amplitudes of the pulses were reduced to 10 to 20% of normal male levels. Similarly, females treated with 2 mg MSA maintained their sexually dimorphic pattern of plasma growth hormone secretion, but as a result of a decline in both the pulse and interpulse amplitudes, the mean plasma concentration was reduced.

In spite of the major reduction in circulating growth hormone levels by 2 mg MSA, expressions of male-specific CYP2C11, 2C13, 2A2, and 3A2, as well as female-specific CYP2C12 and female-predominant CYP2C7 and 2A1 (mRNA, protein and catalytic activities), were generally unaffected or slightly reduced in both males and females. Although seemingly inconsistent with the role of growth hormone regulation of CYP, the results are actually in agreement with studies of CYP2C11 and 2C12 expression in incompletely growth hormone-depleted rats and hypophysectomized-growth hormone replaced rats (16, 20, 39) which demonstrated the effectiveness of “mini” amplitude pulses and subnormal concentrations of growth hormone in maintaining normal, and even supranormal levels of CYP2C11 and 2C12, respectively. In agreement with the present findings, we have reported that the critical signaling element in the masculine episodic plasma growth hormone profile that simultaneously induces CYP2C11 and suppresses CYP2C12 is the approximate 2.5 hr growth hormone-devoid interpulse periods necessarily separated by pulses of inconsequential heights (40). Similarly, the signaling element in the feminine growth hormone profile that induces CYP2C12 and represses CYP2C11 is the continuous presence of the hormone in the circulation at levels as low as 3% of normal (6, 16). Thus, it seems reasonable to conclude that the mini pulses of growth hormone secreted in the 2-mg MSA-treated male rats were sufficient to maintain normal-like male levels of CYP2C11, 2C13, 2A2, and 3A2 while suppressing female-dependent CYP2C12, 2C7, and 2A1. Moreover, the continuous secretion of subnormal concentrations of growth hormone in the 2-mg
Hepatic drug metabolizing enzyme levels were determined in livers from 47-, 70-, and 207-day-old male and female rats neonatally treated with either 2 mg or 4 mg monosodium aspartate (MSA) or diluent (0 mg MSA) on alternate days for the first 9 days of life. Each data point is a mean ± SD with a N ≥ 5 and expressed as a percentage of the mean value of the 70-day-old diluent-treated male or female group with the greater enzyme activity. *p < 0.01; compared with the diluent-treated control animals of the same age and sex, †p < 0.01; compared with the 70-day-old rats of the same sex and MSA treatment.

MSA-treated female rats were effective in maintaining feminine levels of CYP2C12, 2C7, and 2A1 while completely suppressing male-specific CYP2C11, 2C13, 2A2, and 3A2. In contrast to the 2-mg dose of MSA, the long-term consequences of the 4-mg dose on CYP expression are more obscure. The complete suppression of CYP2C11 in adult males neonatally exposed to 4 mg of MSA can be explained by the loss in episodic growth hormone secretion, a requisite for CYP2C11 expression (7–10, 40). However, male-specific CYP2C13, 2A2, and 3A2, which are normally regulated by the masculine episodic growth hormone profile, can be maximally expressed even in the absence of the hormone (i.e., hypophysectomy). It is the continuous presence of growth hormone in the circulation (i.e., the feminine growth hormone profile) that completely suppresses CYP2C13, 2A2, and 3A2 expression (16, 37). The present findings of complete (CYP2A2 and 3A2) or near complete (CYP2C13) suppression of these male-specific CYP isoforms in 4-mg MSA-treated male rats secreting no detectable levels of growth hormone appear to contradict observations obtained from hypophysectomized rats.
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