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Gender-Related Expression of Rat Microsomal Epoxide Hydrolase during Maturation: Post-transcriptional Regulation

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SUMMARY

Expression of microsomal epoxide hydrolase (mEH), levels of mEH mRNA, and the rate of mEH mRNA transcription were examined in hepatic and renal tissues at 4, 24, 44, and 56 weeks of age in male and 4, 14, 24, 34, and 44 weeks of age in female Sprague-Dawley rats. Immunoblot analyses revealed that hepatic mEH levels in males increased in an age-dependent manner, with a maximal increase (~3-fold) being noted at 44 weeks of age, whereas the expression of hepatic mEH in females decreased significantly at 14 weeks of age or older, by ~70%, compared with that of 4-week-old rats. Microsomes from kidney tissue failed to exhibit an age-dependent change in either sex. mEH mRNA levels were measured in total and poly(A)$^+$ RNA isolated from hepatic and renal tissues. RNA blot hybridization analyses, probed with a 1.3-kilobase mEH cDNA, revealed that the levels of hepatic mEH mRNA in total RNA isolated from males were elevated ~1.5-, 2.8-, and 2.3-fold at 24, 44, and 56 weeks of age, respectively, relative to those at 4 weeks of age, whereas the levels of hepatic mEH mRNA in poly(A)$^+$ RNA from males failed to change in an age-dependent manner. In contrast, the levels of hepatic mEH mRNA in either total or poly(A)$^+$ RNA from female animals were dramatically decreased from 4 to 14 weeks of age, by ~90%. The suppressed levels of mEH mRNA in females were maintained at 24, 34, and 44 weeks of age (~80%). However, the levels of renal mEH mRNA failed to change in an age-dependent manner in either sex, which was consistent with there being no change in the levels of mEH protein in kidney. In order to examine whether the gender-related maturational changes in hepatic mEH mRNA levels could result from transcriptional regulation, nuclear run-on assays were performed. The rate of hepatic mEH gene transcription failed to change in either males or females at the ages that exhibited significant changes in both mRNA levels and protein expression, suggesting that transcriptional regulation is not associated with the gender-dependent modulation of mEH mRNA levels during maturation. These results provide evidence that the expression of hepatic mEH protein increases with age in males, whereas the levels of hepatic mEH protein remarkably decrease in postpubescent females, with no change in renal mEH levels in either sex, and that these changes in protein levels are associated with modulation of the levels of mEH mRNA, in the absence of transcriptional regulation.

mEH is active in catalyzing the hydration of reactive epoxide intermediates that are formed by cytochromes P450 (1-3). The expression of mEH is primarily associated with detoxication and protection of cellular macromolecules from metabolic intermediates (1, 2), whereas induction of this enzyme by certain xenobiotics, such as barbiturates, correlates with tumorigenesis and tumor-promoting ability (4). The specific activity and the mRNA level of mEH have been reported to be inducible by phenobarbital, 3-methylcholanthrene, *N*-acetylaminofluorene, *trans*-stilbene oxide, and alkylnitrosamines (5-9). The levels of mEH protein and its mRNA are also increased in hepatic preneoplastic nodules and neoplastic lesions (10).

Developmental changes in mEH activity have been reported (11-14). An ~4-fold increase in mEH activity was observed from the 5-day-old male rat to adult, and mEH activity in both males and females is equivalent at 4 weeks of age in Sprague-Dawley and other strains of rats (e.g., Fischer F-344 and CFN rats) (11, 12, 14, 15). Simmons and Kasper (14) have shown that this developmental increase in mEH protein is associated with an elevation in mRNA, using Holtzmann rats. Changing hormonal levels may play a role in determining adult levels of this enzyme, thereby modulating the developmental pattern. Nutritional parameters, such as starvation, also stimulate mEH activity by 20% in both sexes (12).

Age- and sex-dependent changes in the activities of drug-metabolizing enzymes have also been examined previously (15-18). In particular, the activity of hepatic mEH in males has been reported to be increased with age in rats and mice (17, 18), whereas the activities of hepatic microsomal monooxygenases generally decline with age and glutathione *S*-transferase activity remains unchanged (15). Thus, it has been established that adult males exhibit ~3-fold higher mEH activity than adult females (11, 12, 17). However, no information is available

ABBREVIATIONS: mEH, microsomal epoxide hydrolase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; pp63, rat insulin receptor inhibitor phosphoprotein (63 kDa); kb, kilobase(s); SSC, standard saline citrate.

on the molecular and regulatory basis for age- and sex-related changes in mEH.

This study was designed to examine whether the gender-related changes in mEH activity during maturation occur as a result of changes in protein and mRNA levels in both hepatic and renal tissues and whether transcriptional regulation is responsible for the modulation of mEH mRNA levels. Because gender-dependent differential induction of mEH has been reported with a variety of chemical inducers (6, 7, 19), this information will be essential to determine the appropriate animal model for studying the induction of mEH and to understand the molecular regulatory mechanisms of mEH induction. Tremendous interindividual variations in mEH activity have been reported from human liver biopsies, supporting the position that a variety of factors may affect mEH levels (20). This information on the mEH expression and molecular regulation in a rat model may assist in the understanding of age- and gender-related changes in the levels of human mEH.

The present study establishes that the levels of hepatic mEH in Sprague-Dawley rats change in a gender-dependent manner during maturation and that the mechanism is associated with modulation of mEH mRNA levels, with no transcriptional regulation of this gene being involved. mEH protein and mRNA levels, as well as the transcriptional rate, were quantified by immunoblot, RNA blot hybridization, and nuclear run-on analyses.

Experimental Procedures

Materials. [α-^{32}P]dATP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 mCi/mmol), and [α-^{32}P]UTP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Biotinylated goat anti-rabbit IgG, streptavidin-conjugated horseradish peroxidase, and random prime labeling and 5′-end labeling kits were purchased from BRL (Gaithersburg, MD). Rabbit anti-rat mEH antibody was kindly provided by Dr. C. B. Kasper, McArdle Laboratory for Cancer Research, University of Wisconsin (Madison, WI). Both sense and antisense pp63 DNAs cloned in an M13mp19 phage were kindly provided by Dr. A. S. Goustin, Center for Molecular Biology, Wayne State University (Detroit, MI). Insert DNA size for antisense or sense pp63 clones was 865 base pairs.

Animals. Male and female Sprague-Dawley rats from Harlan (Indianapolis, IN) were used. Nine groups of animals (6–12 rats in each age group) were utilized in this series of experiments and fed *ad libitum*. The animals were fasted 16–18 hr before sacrifice. The fasting did not affect mEH expression significantly. The groups of animals with different ages were selected based on both previous reports on age- and sex-dependent mEH activity (i.e., the ages that exhibit consistent increases in mEH enzyme activity in males) and the availability of animals from the supplier. Body weights of rats used in this series of experiments were as follows: at 4, 24, 44, and 56 weeks of age in males, 88 ± 7, 448 ± 18, 423 ± 24, and 423 ± 56 g, respectively; at 4, 14, 24, 34, and 44 weeks of age in females, 62 ± 9, 218 ± 21, 266 ± 32, 255 ± 26, and 290 ± 30 g, respectively. Body weights of female rats were significantly less than those of males, by ~40%. This is consistent with previously published observations (17).

Isolation of microsomal proteins. Hepatic or renal microsomes prepared by differential centrifugation were washed in pyrophosphate buffer and stored in 50 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol at −80°, until used. Protein was assayed by the method of Lowry *et al.* (21), using lysozyme as a protein standard.

Gel electrophoresis and immunoblot analysis. SDS-PAGE analysis was performed according to the method of Laemmli (22), and immunoblot analysis was carried out as described previously (23, 24).

Isolation of total RNA and poly(A)$^+$ RNA. Total RNA was isolated using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction, according to the methods of Cathala *et al.* (25) and Chomczynski and Sacchi (26), as modified by Puissant and Houdebine (27). Poly(A)$^+$ RNA was isolated from the total RNA by using an oligo(dT)-cellulose column, according to the method of Jacobson (28).

mEH cDNA synthesis and cloning in an M13mp19 phage vector. cDNA derived from hepatic poly(A)$^+$ RNA obtained from rats treated with pyrazine was amplified using the polymerase chain reaction technique.[1] Polymerase chain reactions were performed using first-strand cDNAs as a template, in a Perkin Elmer Cetus thermocycler, for 30 cycles, using the following parameters: denaturing at 94° for 1 min, annealing at 50° for 1 min, and elongation at 72° for 3 min. Sense primer 5′-d(AGCAGGCACTTCTGTT)-3′ was constructed from region 108–123 in the 5′ untranslated region, and antisense primer 5′-d(CCACAGTTGGTATCC)-3′ was complementary to the region 1375–1390 (29). The amplified DNA (~1.3 kb) was cloned in an M13mp19 phage vector, and both sense and antisense clones were selected.

DNA sequence analysis. Both cloned sense and antisense mEH DNAs were analyzed by DNA sequencing, which was carried out by a modified dideoxy chain-termination method (30). DNA sequence analyses revealed that the DNA sequences of sense and antisense mEH clones match the published cDNA sequence for mEH (29).

RNA slot blot hybridization. RNA slot blot analysis was performed as described previously (24). Briefly, the blot was incubated with hybridization buffer containing 50% deionized formamide, 5 × Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine, and 0.1% bovine serum albumin (Pentex fraction V)], 0.1% SDS, 200 μg/ml sonicated salmon sperm DNA, and 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4), at 42° for 1 hr without probe. Hybridization was performed at 42° for 18 hr with heat-denatured mEH cDNA (1.3 kb), which was random prime-labeled with [α-^{32}P]dATP. Filters were washed in 2 × SSC, 0.1% SDS, for 10 min twice at room temperature and in 0.1 × SSC, 0.1% SDS, for 10 min twice at room temperature. Filters were finally washed in the solution containing 0.1 × SSC and 0.1% SDS for 60 min at 60°.

Northern blot hybridization. Northern blot was carried out according to the procedures described previously (25). The same samples used for Northern blot analyses were also applied to slots, and slot-blotted membranes were hybridized with mEH cDNA probe. The stripped membranes were hybridized with ^{32}P-end labeled poly(dT)$_{16}$, to quantitate the amount of mRNA loading (24). Films were exposed at −80° for 1–6 hr, using DuPont intensifying screens. Duplicate slot and Northern blot analyses were performed on two or three different mRNA samples from each age group in both sexes.

Isolation of nuclei. Nuclei were isolated at 4°, as described previously (31, 32). Isolated nuclei were aliquoted after being counted microscopically, and concentrations were normalized. Resuspended nuclei were quickly frozen in liquid nitrogen and stored at −80° until used.

Nuclear run-on transcription assay. Nuclei (5 × 10^7) were resuspended in 10 mM Tris·HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 80 mM KCl, 0.5 mM dithiothreitol, 20% glycerol, 1.25 mM nucleotides (ATP, CTP, and GTP), and 200 μCi of [α-^{32}P]UTP (3000 Ci/mmol). Reactions were performed at 30° for 30 min with shaking, and the nuclei were then digested with RNase-free DNase I and proteinase K, as described previously (33). Labeled nuclear RNA was isolated as described for total RNA isolation. Approximately 2 × 10^7 cpm of ^{32}P-labeled RNA were hybridized to 3 μg of single-stranded antisense or sense mEH DNA cloned in M13mp19 phage, which was immobilized on nitrocellulose membranes. Hybridizations were carried out for 24 hr at 42° in 5 × SSPE, 0.5% SDS, 50% formamide, 1 × Denhardt's solution, 0.5 mg/ml yeast tRNA.

Filters were washed with 1 × SSC, 0.1% SDS, for 20 min three times

[1] Kim, S. G., G. L. Kedderis, R. Batra, and R. F. Novak, manuscript in preparation.

at 42° and subsequently washed with 0.1 x SSC, 0.1% SDS, for 1 hr at 60°. Both antisense and sense cloned pp63 DNAs were used as internal controls.

Scanning densitometry. Scanning densitometry was performed with a Molecular Dynamics computing densitometer. The volume of each slot or dot was integrated using ImageQuant software (Molecular Dynamics, version 3.0), followed by background subtraction. The quantitation of the mRNA loaded on the slot blot was accomplished by hybridization of stripped membranes with ^{32}P-end labeled poly(dT)$_{16}$, and the relative change in mEH mRNA was determined from normalization of hybridization signal to the mRNA loaded onto the slots (24). The quantitation of hybridization signal in the nuclear run-on assays was accomplished by normalization of mEH signal intensity to pp63 hybridization intensities.

Results

Immunoblot analyses. The SDS-PAGE of hepatic microsomes of male rats at 4, 24, 44, and 56 weeks of age revealed an apparent age-dependent increase in intensity of a band migrating in the region of mEH. A polyclonal antibody that detected mEH was used to examine the change in mEH protein levels in hepatic microsomes. The expression of hepatic mEH in males increased ~2-, 3-, and 3-fold at 24, 44, and 56 weeks, respectively, compared with the levels at 4 weeks of age (Fig. 1A).

Immunoblot analyses of hepatic microsomes from female rats at 4, 14, 24, 34, and 44 weeks of age were performed to examine age-related levels of mEH protein. The level of female hepatic mEH at 4 weeks of age was ~1.5-fold higher than that of male hepatic mEH at the same age. The expression of hepatic mEH in females at different ages was in sharp contrast to that in males, exhibiting a profound decrease in mEH levels from 4 to 14 weeks of age or older, of ~70% (Fig. 1B); additional immunoblot experiments were performed with the hepatic microsomes isolated from 44-week-old female rats (data not shown).

mEH expression in kidney remained constant in both sexes at the ages examined, as detected by immunoblot analyses (Fig. 1C).

RNA hybridization analyses. To determine whether the gender-dependent changes in mEH levels during maturation are accompanied by changes in mRNA levels, mEH mRNA levels were measured in total and poly(A)$^+$ RNA isolated from hepatic and renal tissues. Northern blot analyses were primarily performed, and two serially diluted RNA samples obtained for each age were also applied onto slots for quantitation in order to obtain the optimal linear range for the autoradiographic film exposure.

Hepatic mEH mRNA levels in total RNA isolated from males were elevated ~1.5-, 2.8-, and 2.3-fold at 24, 44, and 56 weeks of age, respectively, relative to the levels of mEH RNA at 4 weeks of age, as assessed by both Northern and slot blot hybridization analyses (Fig. 2, A, B, and C). mEH mRNA levels in total RNA from 4-week-old females were ~1.5-fold higher than those in 4 week-old males (Fig. 2, A and B). mEH mRNA levels in females, however, decreased dramatically at 14 weeks of age, by ~90%, relative to those at 4 weeks of age (Fig. 2, A, B, and D). Thereafter, the hepatic mEH mRNA levels in females tended to increase slightly at 24, 34, and 44 weeks of age (Fig. 2, D and E). The levels of mEH mRNA in total RNA from kidney, however, remained constant over the ages examined in both sexes (data not shown; some of the data for females

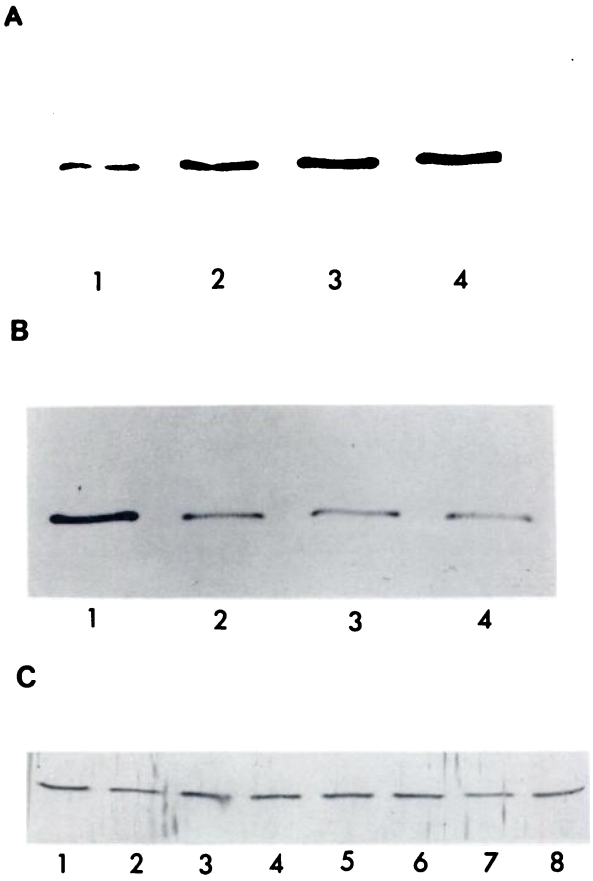

Fig. 1. Immunoblot analyses of hepatic and renal microsomes, isolated from male and female rats, with rabbit anti-rat mEH antibody. A, Western immunoblot analysis shows the levels of hepatic mEH protein in microsomes isolated from male rats at 4, 24, 44, and 56 weeks of age (*lanes 1* through *4*, respectively). B, Immunoblot analysis shows the levels of hepatic mEH protein in microsomes isolated from female rats at 4, 14, 24, and 34 weeks of age (*lanes 1* through *4*, respectively). C, This blot represents the levels of renal mEH protein in microsomes isolated from male rats at 4, 24, 44, and 56 weeks of age and from female rats at 4, 14, 24, and 34 weeks of age (*lanes 1* through *8*, respectively). Lanes were loaded with 0.5 μg of liver microsomes or 1.2 μg of kidney microsomes. These immunoblots are representative blots among the two or three analyses performed for the two different samples from each age group, and identical results were obtained from each analysis. These blots show the significant age-related increases of hepatic mEH protein in males and the profound decrease of hepatic mEH protein in females from 4 weeks to 14 weeks or older; the microsomes from kidney tissue failed to exhibit a maturational change in either sex.

are shown in Fig. 2E). These results are consistent with the changes in mEH protein levels in both males and females.

Gender-related maturational changes in mEH mRNA expression were also examined in poly(A)$^+$ RNA. The ^{32}P-labeled mEH cDNA probe hybridized to mEH message, as evidenced by the appearance of a single band in the Northern blot analyses (~1.8 kb) (Fig. 3). When poly(A)$^+$ RNA was used for quantitation of mEH mRNA levels, the age-related change in hepatic mEH mRNA levels in males observed for total RNA was abolished. This result might suggest that two different pools of mEH mRNA either exist or develop as male rats age [i.e., RNA with long and short poly(A)$^+$ tails] (Fig. 3, A and C). The female-specific suppression in mEH mRNA levels at age 14 weeks or older, relative to those at 4 weeks of age (by ~80–90%), was confirmed with poly(A)$^+$ RNA (Figs. 2E and 3, B

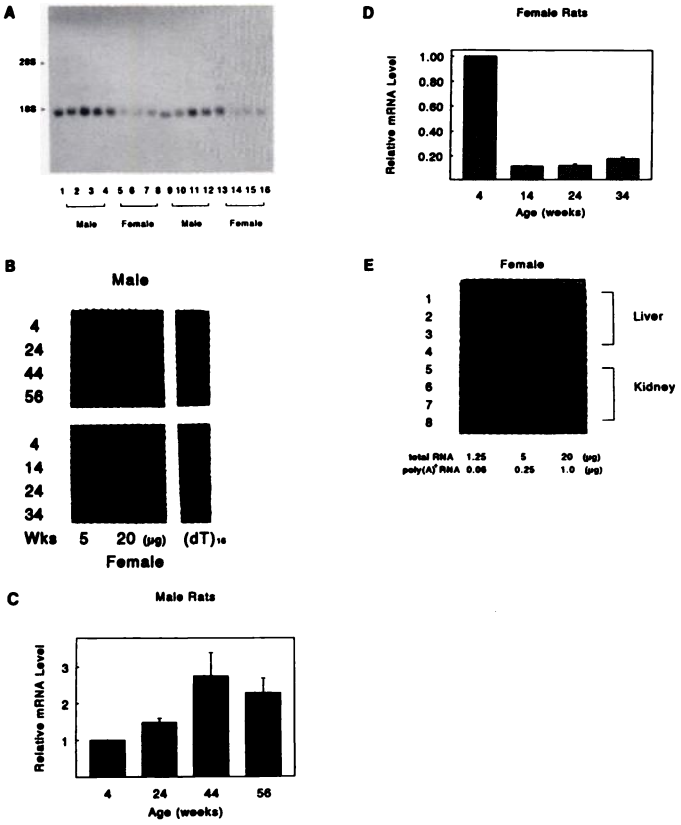

Fig. 2. Northern and slot blot analyses of hepatic mEH mRNA in total RNA. A, Hepatic total RNA (15 μg) isolated from male rats at 4, 22, 44, and 56 weeks of age (*lanes 1* through *4* and *9* through *12*, respectively) and from female rats at 4, 14, 24, and 34 weeks of age (*lanes 5* through *8* and *13* through *16*, respectively) was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled mEH cDNA probe. Duplicate Northern blot analyses were performed on different mRNA samples. B, Hepatic total RNA (5 and 20 μg, respectively) isolated from the same groups of animals as used in A was blotted onto slots. After hybridization with the mEH cDNA probe, the membrane was stripped and rehybridized with ^{32}P-labeled poly(dT)$_{16}$, for quantitation of mRNA loading. C, Relative increase in hepatic mEH mRNA levels in male rats. D, Relative decrease in hepatic mEH mRNA levels in females. The change in mEH mRNA, relative to the levels of 4-week-old animals, was quantitated by densitometric scanning of slot blots. Each point represents the mean ± standard deviation of at least three determinations from three to six pooled animals. These results were reproducible in another set of experiments using the equivalent age groups. E, Slot blot analysis of hepatic mEH mRNA in total RNA (*rows 1, 2, 5,* and *6*) and poly(A)$^+$ RNA (*rows 3, 4, 7,* and *8*) from female rats at 4 (*odd numbers*) and 44 (*even numbers*) weeks of age. Female rats at 44 weeks of age also exhibited a significant decrease in hepatic mEH mRNA levels in total RNA fractions (~80%), compared with those at 4 weeks of age. A similar decrease was noted in poly(A)$^+$ RNA fractions. In contrast, no significant change in mEH mRNA levels was observed in kidney between 4 and 44 weeks of ages. This experiment was repeated once using the same age groups.

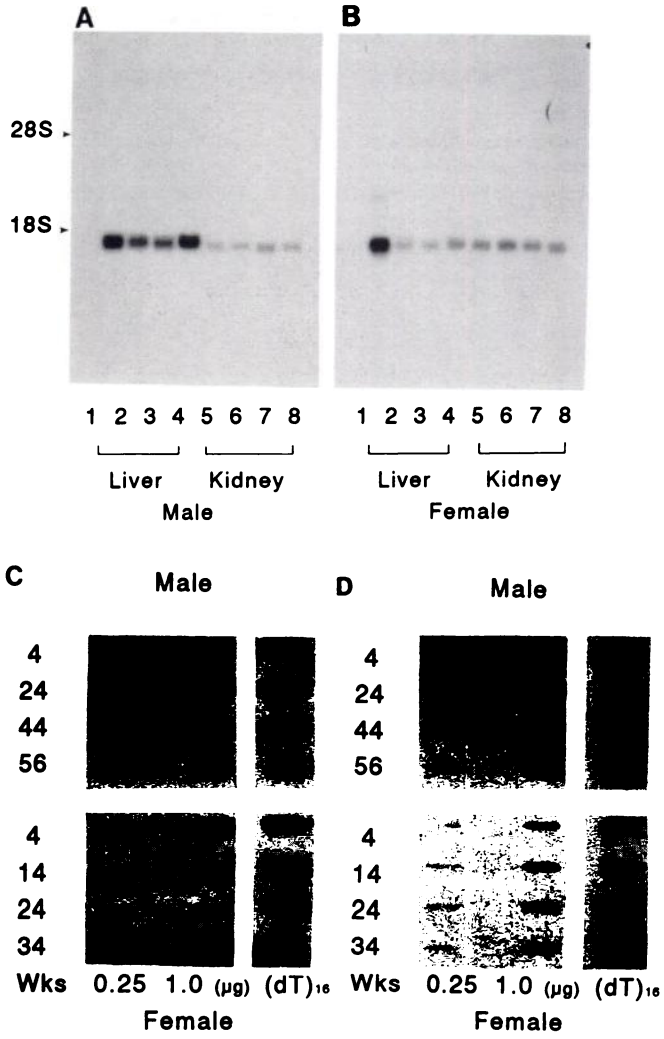

Fig. 3. Northern and slot blot analyses of mEH mRNA levels in hepatic and renal poly(A)$^+$ RNA. A, Two micrograms of poly(A)$^+$ RNA isolated from male rats at 4, 22, 44, and 56 weeks of age (*lanes 1* through *4*, respectively, for hepatic mEH mRNA levels; *lanes 5* through *8*, respectively, for renal mEH mRNA levels) were used for Northern blot analysis. B, Two micrograms of poly(A)$^+$ RNA isolated from female rats at 4, 14, 24, and 34 weeks of age (*lanes 1* through *4*, respectively, for hepatic mEH mRNA levels; *lanes 5* through *8*, respectively, for renal mEH mRNA levels) were analyzed as described in Experimental Procedures. C, Hepatic poly(A)$^+$ RNA (0.25 and 1.0 μg, respectively) was blotted and probed with mEH cDNA probe. The same hepatic samples used for Northern blot analyses were applied to slots and hybridized with a ^{32}P-labeled cDNA probe. The levels of hepatic mEH mRNA in poly(A)$^+$ RNA from males exhibited no significant change at 24, 44, and 56 weeks of age, relative to the levels at 4 weeks of age, whereas the levels of hepatic mEH mRNA in poly(A)$^+$ RNA from female animals were dramatically decreased at 14 weeks or older, by ~90%, compared with those at 4 weeks of age. D, Renal poly(A)$^+$ RNA (0.25 and 1.0 μg, respectively) isolated from the same groups of animals was blotted, and the levels of mEH mRNA were examined. The levels of renal mEH mRNA failed to change in either sex.

and C). The levels of mEH mRNA in kidney appeared to be significantly less than those in liver at 4 weeks of age and remained constant over the ages examined in either males or females (Fig. 3, A and B). Thus, in contrast to the results obtained with liver, the levels of mEH mRNA in kidney failed to change in a gender-dependent manner during maturation, consistent with there being no change in the levels of mEH protein in kidney (Figs. 2E and 3D). The stripped membranes were reprobed with ^{32}P-end labeled poly(dT)$_{16}$, to quantitate the amount of mRNA loaded onto the slots and to ensure that equal amounts of poly(A)$^+$ RNA were loaded onto the agarose gel and transferred to the nitrocellulose paper (Fig. 3C).

Nuclear run-on analyses. In order to examine whether the gender-dependent changes in mEH mRNA levels during maturation result from changes in the transcriptional rate, nuclear run-on transcription assays were performed. The results of the mEH mRNA age-related profile suggested that maximal changes in the rate of transcription could be obtained between

4 and 44 weeks of age in males. Thus, nuclei were isolated from the livers of males between 4 and 44 weeks of age, whereas nuclei were isolated at 4, 14, 24, and 34 weeks of age from females. Newly synthesized radiolabeled RNA was isolated and hybridized to cloned antisense or sense single-stranded mEH DNA. As internal controls, the ^{32}P-labeled transcripts synthesized in nuclei were also hybridized to rat pp63 antisense or sense DNA. The intensities of the autoradiographic signals from these hybridization experiments yielded an estimate of the relative sense and antisense transcription rates of the mEH and pp63 genes in rat hepatic tissue. As shown in Fig. 4, nuclear run-on analyses revealed that no significant changes in the rates of hepatic mEH gene transcription were observed in either males or females at the ages at which significant changes in both mRNA levels and protein expression occurred. These data suggest that transcriptional regulation is not associated with the gender-related modulation of mEH mRNA levels during maturation.

Discussion

A number of laboratories have studied gender-related changes in drug-metabolizing enzymes in rodent species (11–18). Chengelis (16, 17) established that age- and sex-related differences occur in the activities of cytochrome P450 and phase II drug-metabolizing enzymes, including mEH, in Sprague-Dawley rats. Most of the studies examining mEH assessed catalytic activity of mEH toward styrene oxide. However, small organic molecules, such as benzil and benzimidazole, stimulate mEH activity *in vitro*, due to an interaction with the enzyme (34). The levels of mEH may be variably assessed with the substrates used in different studies. Thus, the amount of enzyme should be quantitatively determined immunochemically, in conjunction with biochemical assays.

Results of Chengelis' study (17) demonstrated that mEH activity consistently increases as males age up to 80 weeks. In females, mEH activity slightly decreases at 14 weeks of age,

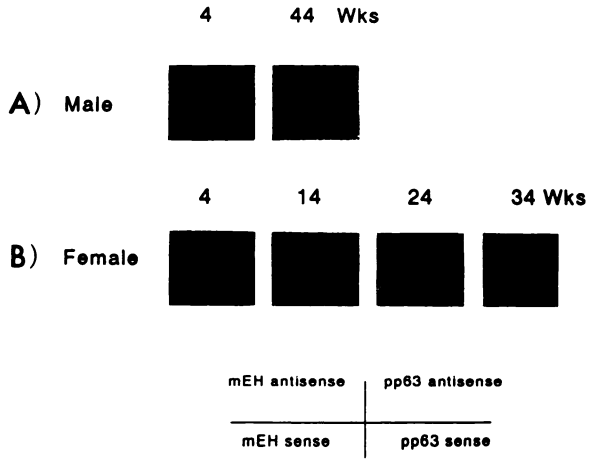

Fig. 4. Nuclear run-on analyses. Hepatic nuclei (5×10^7) isolated from the livers of male rats at 4 and 44 weeks of age (A) and from female rats at 4, 14, 24, and 34 weeks of age (B) were incubated in the presence of [α-^{32}P]UTP. Newly synthesized RNA was hybridized to both antisense and sense strands of mEH DNA cloned in an M13mp19 phage. Both antisense and sense strands of pp63 DNA clones were used as internal controls. Duplicate assays were performed, and the results had <10% variation in integrated intensity. The rates of hepatic mEH gene transcription failed to change in either males or females at the ages where significant changes in both mRNA levels and protein expression occur.

relative to that at 4 weeks of age, and gradually returns to the prepuberty levels of activity up to 80 weeks (17). The aim of the present study was to examine the molecular basis and regulatory mechanism(s) of mEH expression during maturation in both sexes. The results of immunoblot analyses in the present study are consistent with previous observations, which showed age- and sex-specific changes in mEH activity. Whereas the difference in mEH activity between 4 and 14 weeks of age was ~2-fold in females according to Chengelis (17), the expression of hepatic mEH protein in females decreased by ~70% from 4 to 14 weeks of age or older in the present study. Microsomes from kidney tissue, however, failed to exhibit an age-related change in either sex, providing evidence that there is, indeed, organ-dependent regulation in the expression of mEH protein. Sheehan *et al.* (35) showed that lead acetate administration resulted in the tissue-specific transcriptional activation of the mEH gene in kidney, whereas no change in mRNA level was noted in liver. Thus, mEH gene regulation in liver and kidney appeared to be differentially modulated by xenobiotics. The present results, showing organ-dependent expression of mEH protein in different age groups, support the idea that there might be tissue-specific factors responsible for regulating the expression of this enzyme. In addition, whether the differential rates of protein turnover play a role in tissue-dependent expression of the protein remains to be established.

The present study delineates the regulatory mechanism involved in the expression of mEH protein at the molecular level. mEH mRNA levels measured in total RNA isolated from hepatic and renal tissues are consistent with the expressed protein levels in both sexes. However, the levels of hepatic mEH mRNA in poly(A)$^+$ RNA from males failed to increase in an age-dependent manner. These results suggest that pools of mEH mRNA with different sizes of poly(A)$^+$ tails either exist or develop as male rats age. In contrast to the gradual increase in mEH protein and mRNA levels in males, the levels of hepatic mEH mRNA in either total or poly(A)$^+$ RNA from female animals were remarkably decreased at 14 weeks of age or older, by ~80–90%, compared with those at 4 weeks of age. This suppression of hepatic mEH mRNA levels in female correlates with the suppression of hepatic mEH protein levels in this sex, suggesting that neither translational efficiency nor stabilization of protein is primarily involved in the expression of mEH protein. This is further supported by the fact that the levels of renal mEH mRNA failed to change in an age- or sex-related manner, which is consistent with there being no change in the levels of mEH protein.

The rates of hepatic mEH gene transcription failed to change in either males or females at the ages that exhibited significant changes in both mRNA levels and protein expression, as assessed by nuclear run-on assays. These results, along with significant changes in mRNA levels, strongly suggest that transcriptional regulation is not associated with the gender-dependent modulation of mEH mRNA and protein levels during maturation. Rather, post-transcriptional regulation is responsible for the changes in mRNA levels. Cytoplasmic regulation of mEH mRNA (e.g., mRNA destabilization in females after puberty) may contribute to the overall levels of mEH mRNA and protein. There are reports that support the role of cytoplasmic regulation of mEH mRNA (36, 37). For example, pregnenolone-16α-carbonitrile treatment of animals causes marked reduction of transcriptional activity of the mEH gene,

and the levels of nuclear pre-mRNA are accordingly suppressed. Despite transcriptional down-regulation, slight elevation of the mEH polyribosomal mRNA levels occurs (36). The exact mechanism(s) for this post-transcriptional regulation associated with age- and sex-related changes in mEH mRNA levels should be further addressed.

Given the fact that the onset of puberty begins at ~40–60 days of age in rats (38), female-specific suppression in the levels of both mEH protein and mRNA from 4 to 14 weeks of age might be interpreted as either the development of specific regulatory element(s) and/or factor(s) for mEH mRNA destabilization or the lowering of the levels of stabilization factors in livers of female rats after puberty. This may reflect production of endogenous substances, including sex and/or steroid hormones and growth factors. Discovery of the specific factors that modulate mEH mRNA levels will provide valuable information on the extent of mEH expression in the sexes and on induction by xenobiotics. In addition, mEH protein and mRNA levels in postpubertal female rats may vary, depending on the stage in the estrous cycle. However, consistent results were obtained in the present study using multiple groups of female animals at 14, 24, 34, and 44 weeks of age, although animals were possibly at different estrous cycle stages.

This information on the gender- and organ-dependent expression of mEH protein and mRNA levels during maturation may be exploited to provide additional information for mechanistic studies of mEH gene expression and regulation. For example, these findings are important in light of gender-dependent xenobiotic induction of drug-metabolizing enzymes, given the fact that significant differences in gender-dependent expression of mEH induced by xenobiotics in rats have been reported. 2-Acetylaminofluorene increases the mEH activity in liver by 7.5-fold in male rats, whereas this compound fails to induce mEH in female animals (8). However, studies performed in this laboratory revealed that imidazole antimycotic agents increase mEH mRNA levels significantly in both male and female rats.[2] Such differences between sexes in the effects of different xenobiotics may be addressed in conjunction with organ- and age-related expression and regulation of this enzyme. In particular, Chengelis (17) has shown that mEH is the only enzyme that exhibits significant sex- and age-related changes in enzymatic activity among the phase II drug-metabolizing enzymes, including glutathione *S*-transferases, UDP-glucuronosyltransferases, and 3′-phosphoadenosine-5′-phosphosulfate sulfotransferases.

In summary, these results provide evidence that the levels of hepatic mEH protein increase with age in males, whereas the levels of hepatic mEH protein remarkably decrease in postpubescent females, with no change in renal mEH levels in either sex, and that these changes in protein levels are associated with modulation of the levels of mEH mRNA in the absence of transcriptional regulation.

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