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EXPRESSION OF RAT MICROSOMAL EPOXIDE HYDROLASE DURING PREGNANCY

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Abstract—Microsomal epoxide hydrolase (mEH) protein and messenger ribonucleic acid (mRNA) levels were assessed in maternal rats during pregnancy and mEH gene expression was compared with cytochrome P450 expression. Immunoblot analysis using goat anti-rat mEH antibody showed that hepatic mEH levels in adult rats at day 12 of gestation were comparable to those in virgin female rats at the same age, whereas mEH protein levels were substantially decreased by ~70% at day 20 of pregnancy. Northern and slot blot analyses revealed that hepatic mEH mRNA levels failed to be modulated at day 12 of pregnancy, whereas mEH mRNA levels were decreased to ~20% of virgin control in late pregnancy (*i.e.* day 20), which was consistent with the result of mEH immunoblot analysis. Hepatic cytochrome P4502E1 levels were also diminished at day 12 and day 20 of gestation by ~50% and ~70%, respectively, relative to virgin control rats, as supported by immunoblot analysis. Hepatic P4502E1 mRNA levels at day 12 and day 20 of pregnancy were also significantly decreased to 30% and 8% of virgin rats at 17 weeks of age, respectively, demonstrating that suppression in P4502E1 protein levels accompanied decreases in its mRNA expression. The levels of cytochrome P4501A2 mRNA at either day 12 or day 20 of pregnancy was decreased by ~50% relative to virgin female rats, which was less than that observed in mEH or P4502E1 expression. Pregnancy failed to affect P4501A1 mRNA levels, which were not detectable in female rats at 17 weeks of age. Renal mEH mRNA levels in maternal rats at day 20 of gestation were also decreased to 25% of virgin control. These results demonstrated that levels of mEH and P4502E1 proteins decreased significantly in late pregnancy with concomitant decreases in their mRNA levels.

Key words: epoxide hydrolase; pregnancy; gene expression; cytochrome P4502E1; cytochrome P4501A1; cytochrome P4501A2

Epoxide hydrolase catalyzes the hydration of epoxide reactive intermediates formed by phase I metabolizing enzymes [1]. Among the epoxide hydrolases identified, microsomal epoxide hydrolase (mEH)* has broad substrate specificity and is primarily responsible for xenobiotics, including therapeutic agents. Thus, the expression of mEH is believed to be mainly associated with detoxication and protection of cellular macromolecules from metabolic intermediates, although certain dihydrodiols formed by mEH, such as benzo[a]pyrene-7,8-diol, are metabolized by cytochrome P450 to highly reactive diol epoxides [1, 2].

In previous studies, expression of mEH protein, levels of mEH mRNA and the rate of mEH mRNA transcription during maturation were studied in hepatic and renal tissues of male and female Sprague-Dawley rats [3]. Immunoblotting analysis revealed that hepatic mEH protein increased in males in an age-dependent manner during maturation, whereas the expression of hepatic mEH in females decreased significantly from 4 to 14 weeks of age or in older animals (*i.e.* postpubescent age), demonstrating that the expression of mEH is highly associated with age and gender. The changes in mEH protein levels during maturation involved modulation of mEH mRNA levels, as shown by RNA hybridization analysis [3].

Studies showed that pregnancy led to decreases in cytochrome P450 content [4, 5]. In particular, the expression of cytochrome P4502E1 (P4502E1) decreased during pregnancy, as assessed by the metabolism of toluene and trichloroethylene as well as immunoblot analysis using monoclonal antibodies [5]. Borlakoglu *et al.* also reported that cytochrome P450 and cytochrome b5 were reduced by 50% at day 10 of gestation [6]. However, information on the expression of mEH during pregnancy is not yet available. Given the importance of this enzyme in detoxification and in potential association with teratogenicity in response to xenobiotics, including antiepileptic agents, the present study was initiated to examine the levels of hepatic mEH protein and mRNA levels during early and late terms of pregnancy. mEH expression was compared with the expression of certain forms of cytochrome P450 including P4502E1, 1A2, and 1A1 [7].

MATERIALS AND METHODS

Materials

Poly(dT)$_{16}$ was purchased from Pharmacia-LKB (Piscataway, NJ, U.S.A.). Supported nitrocellulose transfer membranes were obtained from Schleicher & Schuell (Keene, NH, U.S.A.). [α^{32}P]ATP and [γ^{32}P]ATP (>110 TBq/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.). 5′-End labelling kits were purchased from Life Technologies (Gaithersburg, MD, U.S.A.).

Animals

Pregnant and virgin Sprague-Dawley rats at the age of 4, 14, and 17 weeks were purchased from Harlan (Indianapolis, IN, U.S.A.). Subcellular fractions and poly(A)$^+$ RNA were isolated from the pooled samples from 3 rats in each group. Results were confirmed using different groups of rats.

* Abbreviations: mEH, microsomal epoxide hydrolase; P4501A1, cytochrome P4501A1; P4501A2, cytochrome P4501A2; P4502E1, cytochrome P4502E1; pp63, rat insulin receptor inhibitor phosphoprotein (63kDa).

Isolation of microsomal proteins

Animals were fasted 16 hr before sacrifice. Hepatic 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, and stored at −80°C until use. Protein was assayed by the method of Lowry *et al.* using lysozyme as a protein standard [8].

Gel electrophoresis and immunoblotting analysis

SDS-PAGE analysis (7.5%) was performed, as described by Laemmli [9]. Immunoblotting analysis was carried out according to the previously published procedures [10, 11].

Isolation of total 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.* [12] and Chomczynski and Sacchi [13], as modified by Puissant and Houdebine [14]. Poly(A)$^+$ RNA was isolated from the total RNA using oligo(dT)-cellulose column according to the method of Jacobson [15].

RNA blot hybridization

mEH, P4502E1, P4501A2, and P4501A1 cDNA probes were produced by polymerase chain reaction (PCR), as described previously [16, 17]. The sizes for mEH, P4502E1, P4501A2, and P4501A1 cDNA fragments used were 1.4 kb, 0.8 kb, 1.1 kb, and 0.6 kb, respectively. Poly(A)$^+$ RNA isolated from rat livers and kidneys was resolved by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose paper by capillary transfer. The blot was incubated with hybridization buffer containing 50% deionized formamide at 42°C with a labeled cDNA probe. Following hybridization, the membranes were washed twice in 2× SSC (1× SSC, standard saline citrate: 150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS for 10 min at room temperature, twice in 0.1× SSC and 0.1% SDS for 10 min at room temperature and once for 60 min at 60°C. Filters were autoradiographed with Kodak X-Omat AR film at −80°C in intensifying screens.

Scanning densitometry

Scanning densitometry was performed with a Soft Laser Scanning Densitometer (Model, SLR-1D/2D, Bio-Med Instrument Incorporation, Fullerton, CA, U.S.A.). The area of each lane or slot was integrated using Laser and Camera Hard Disk Program, 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 mRNA determined from normalization of the hybridization signal to the mRNA loaded onto the slots. Serially diluted samples were employed in conjunction with scanning densitometry for both Western and RNA blot analyses to avoid saturation in development.

RESULTS

mEH immunoblot analysis

Immunoblot analysis using goat anti-rat mEH antibody showed that hepatic mEH levels in adult rats at day 12 of gestation were comparable to those in virgin control rats (Fig. 1A). The SDS-PAGE of hepatic microsomes isolated from pregnant rats at day 20 of gestation showed an apparent decrease in intensity of a band migrating in the region of mEH. Western immunoblot analyses revealed that mEH levels were decreased from day 12 to day 20 of pregnancy by ~70% (Fig. 1A).

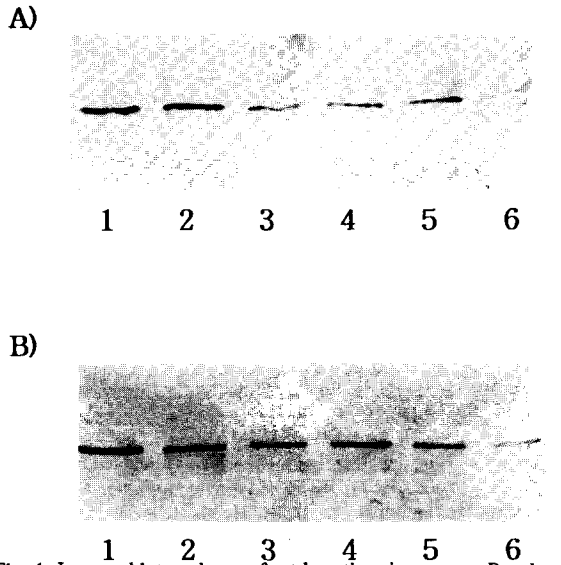

Fig. 1. Immunoblot analyses of rat hepatic microsomes. Panel (A) This blot exhibits levels of hepatic mEH protein in microsomes isolated from pregnant rats. Shown are the lanes containing hepatic microsomes from pregnant rats as compared to virgin controls (lanes 1 and 4, 4 μg and 2 μg, respectively) at day 12 of gestation (lanes 2 and 5, 4 μg and 2 μg, respectively) and at day 20 of gestation (lanes 3 and 6, 4 μg and 2 μg, respectively). This immunoblot is a representative blot among the 4 analyses performed. Panel (B) Identical samples were employed to assess P4502E1 levels in the hepatic microsomes. Lanes are associated with the same hepatic microsomes as described above, except for the protein loading: lanes 1–3 and 4–6 contained 20 μg and 10 μg of proteins, respectively.

Studies have shown that immunochemically-detectable P4502E1-catalytic activities decrease in rats during pregnancy [5, 18]. In this study, P4502E1 levels were assessed for a comparative purpose in the same hepatic microsomal preparations by immunoblotting. P4502E1 levels were also diminished at day 12 and day 20 of gestation by ~50% and ~70%, respectively, as shown in Fig. 1B.

mEH RNA hybridization analysis

To determine whether or not a decrease in mEH protein expression in late pregnancy was accompanied by a change in mRNA level, RNA blot hybridization analysis was carried out with poly(A)$^+$ RNA fractions isolated from 4 week or 17-week-old virgin female rats or from 17-week-old pregnant rats at day 12 and day 20 of gestation. Northern blot hybridization was chosen for primary analysis and serially diluted RNA samples were also blotted for slot blot hybridization analyses for optimal quantitation of mRNA changes (Fig. 2A and 2B). RNA blot analyses showed that relative hepatic mEH mRNA levels at 17 weeks of age were significantly less than those of female rats at 4 weeks of age, which was consistent with the previous demonstration in this laboratory [3]. No significant changes were noted in pregnant rats at day 12 of gestation, as compared with virgin

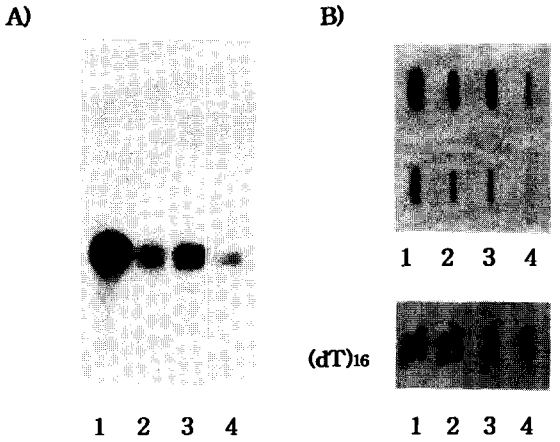

Fig. 2. RNA blot analyses of hepatic mEH mRNA in pregnant rats. Panel (A) Northern blot analysis. Three μg of hepatic poly(A)$^+$ RNA isolated from maternal rats of gestation days 12 and 20 was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with a mEH cDNA probe. The autoradiography shows the levels in mEH mRNA in virgin female rats of 4 (lane 1) and 17 (lane 2) weeks of age and in pregnant rats at gestation days 12 (lane 3) and 20 (lane 4). Panel (B) slot blot analysis of mEH mRNA levels. The poly(A)$^+$ RNA (1.0 and 0.25 μg) isolated from the same samples employed in Northern blot analysis was blotted to nitrocellulose membranes and hybridized with a 1.3 kb mEH cDNA probe. A stripped slot-blotted membrane was hybridized with ^{32}P-labeled poly(dT)$_{16}$ for confirmation of mRNA loading on the slots. Shown is an upper row of the blot.

females at the same age as assessed by both Northern and slot blot analyses (Fig. 2A and 2B). Although no change in mEH mRNA levels at this midgestation time was observed, hepatic mEH mRNA levels were decreased at day 20 of gestation to 20% of those in 17-week-old virgin rats. Equal loading of mRNA to slots was confirmed by rehybridization of the blots with poly(dT)$_{16}$ (Fig. 2). The mEH mRNA levels during early and late gestation were consistent with those in mEH protein, indicating that mEH expression failed to change until late pregnancy and that the suppression in mEH levels was associated with a decrease in its mRNA levels (*i.e.* pretranslational regulation), presumably through transcriptional regulation.

In a subsequent study, expression of certain cytochrome P450 genes during pregnancy was assessed for comparative purposes by Northern and slot RNA hybridization analyses using PCR-amplified cDNA fragments.

P4502E1 mRNA hybridization analysis

Hybridization analysis was performed in this study to examine the levels of hepatic P4502E1 mRNA at the gestational days as above. P4502E1 mRNA levels were significantly decreased to 30% and 10% of those in virgin females at 17 weeks of age at day 12 and day 20 of gestation, respectively (Fig. 3A and 3B). These results strongly support the hypothesis that suppression in P4502E1 protein levels is highly associated with mRNA expression. These results demonstrated that expression of both mEH and P4502E1 was suppressed during late pregnancy.

P4501A2 mRNA hybridization analysis

Although a great portion of nucleotide sequences overlap between P4501A1 and P4501A2, no substantial

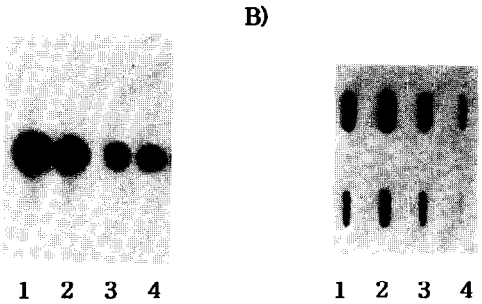

Fig. 3. RNA blot analyses of P4502E1 mRNA during pregnancy. The same time points and amounts of the samples as described in Figure 2 were used to assess P4502E1 mRNA levels using a P4502E1 cDNA probe. Panel (A) Northern blot analysis of hepatic P4502E1 mRNA levels. Panel (B) Slot blot analysis of P4502E1 mRNA levels.

cross-hybridization appeared to occur under the stringent conditions and with the probes employed, as observed by the differences in hybridization intensity between the blots (Fig. 4 and Fig. 5). The expression of P4501A2 mRNA at either day 12 or 20 of gestation was decreased by 50% to 60% (Fig. 4A and 4B), which was less significant than the pregnancy-associated changes in either mEH or P4502E1 mRNA expression.

P4501A1 mRNA hybridization analysis

Developmental changes in hepatic P4501A1 expression have been reported [19]. P4501A1 was not detectable at 14 weeks of age or in older animals. Pregnancy failed to affect P4501A1 expression as shown in Northern blot analysis (Fig. 5A). Neither rats at gestational day 12 nor day 20 exhibited changes in P4501A1 mRNA levels. Given no significant expression of P4501A1 mRNA in untreated animals, marginal changes in intensity, if any, in slot blot analysis were within the variations (Fig. 5B). Equal loading of RNA in the lanes was confirmed by quantifying pp63 mRNA levels (Fig. 5A).

Renal expression of mEH mRNA

Previous studies revealed that microsomes from kidney tissue failed to exhibit an age-dependent change in either males or females: no decrease in mEH protein nor mRNA was noted in female rats during maturation [3]. To determine whether or not the suppression in hepatic mEH expression in late pregnancy is tissue-specific, re-

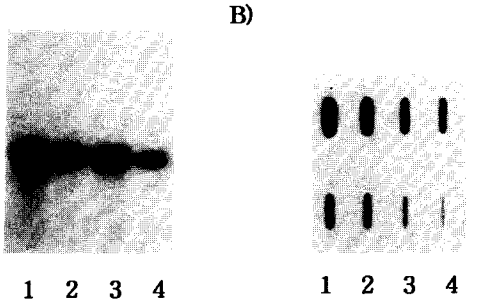

Fig. 4. RNA blot analysis of hepatic P4501A2 mRNA during pregnancy. The lanes represent the same conditions as in Figure 3. Panel (A) Northern blot analysis. Panel (B) Slot blot analysis of P4501A2 mRNA levels.

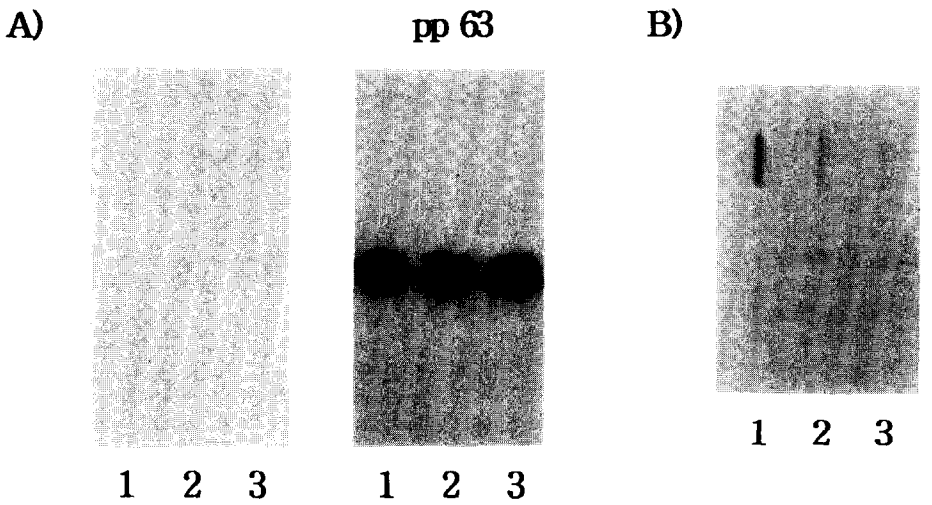

Fig. 5. RNA blot analysis of hepatic P4501A1 and pp63 mRNA during pregnancy. Panel (A) Northern blot analysis. Panel (B) Slot blot analysis. Lanes 1 through 3 represent the levels in hepatic P4501A1 mRNA in virgin female rats at 17 weeks of age and in pregnant rats at gestation days 12 and 20, respectively.

nal mEH mRNA levels were monitored. Renal mEH mRNA levels were decreased at day 20 of gestation to 25% of unpregnant animals and no change was observed at day 12 of gestation. Thus, the results were consistent with those in the liver, although the intensity of hybridization in kidney was much weaker than that in the liver (Fig. 6A and 6B). Because of the low sensitivity, the changes in protein levels were not assessed.

DISCUSSION

Studies have shown that levels of mEH are useful as a biomarker in determining which infants are at increased risk for congenital malformations induced by xenobiotics, including anticonvulsant drugs [20, 21]. Because the antiepileptic drugs phenytoin and phenobarbital readily distribute to the rat fetus, changes in maternal plasma concentrations of these agents are accompanied by parallel changes in fetal tissue [22]. Thus, alterations in maternal metabolism during pregnancy may affect fetal metabolism and development in response to xenobiotics. The implications of mEH in drug-induced teratogenicity prompted us to study further the expression of this metabolizing enzyme during pregnancy.

The present study was performed to compare hepatic and renal mEH expression during mid and late terms of pregnancy with that in virgin females. The aim of this study was to establish whether mEH protein levels change during pregnancy and whether changes in these levels involve the modulation of its mRNA levels. In addition, the mRNA levels of P4502E1, P4501A1, and P4501A2 were quantified for comparative purposes. This study establishes that hepatic and renal expression in mEH protein and mRNA substantially decreases in late pregnancy in parallel with certain cytochrome P450 expression. These changes in the expression of mEH and other P450 enzymes may represent alterations in circulating hormone levels, such as progesterone, in gestation and/or placental hormones.

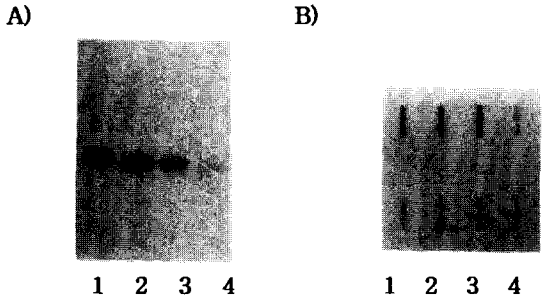

Fig. 6. RNA blot analysis of mEH mRNA in kidney during pregnancy. The lanes are associated with the renal poly(A)$^+$ RNA (3 μg each) derived from the rats as described above. Panel (A) Northern blot analysis for renal mEH mRNA levels. Panel (B) Slot blot analysis.

The critical period that affects fetal development in humans is considered to be 3–5 weeks after conception, which is likely to be the period of organogenesis [23]. The incidence of malformations was shown to be increased in children born to mothers with epilepsy. As mentioned above, a further increase in the incidence of cleft lip and palate was observed when the mother received anticonvulsant drugs [24]. The absence of maternal mEH suppression during the early gestation period (*i.e.* organogenesis), as demonstrated in this study, supports the hypothesis that malformations such as fetal hydantoin syndrome may not be directly associated with endogenous changes in maternal hepatic mEH levels during pregnancy. Teratogenicity may be, rather, associated with other metabolizing enzymes including cytochrome P450, which might be actively involved with the production of reactive metabolic intermediates. Roy and Snodgrass suggested that protein thiol groups are important sites for *in vitro* covalent binding of a reactive intermediate of phenytoin [25]. Preliminary studies accomplished in this laboratory indicated that phenytoin at daily doses of 50 and 100 mg/kg failed to induce mEH in rats, whereas treatment of animals with phenytoin at a dose of 200 mg/kg resulted in a 20-fold elevation in mEH mRNA levels (unpublished data). Thus, a teratogenic dose of 65 mg/kg phenytoin failed to stimulate mEH gene expression, which suggests that mEH expression need not be stimulated at this dose.

A number of other studies have also demonstrated that

the expression of cytochrome P450 (*e.g.* P4502C11) are substantially modulated in a gender-specific manner. An immunochemical inhibition study on the conversion of toluene to benzyl alcohol has also shown that both P4502E1 and P4502C11/6 are slightly decreased during gestation. The results of the present study show that the decreases in P4502E1 mRNA during pregnancy were ~70% and 90% at gestation days 12 and 20, respectively. The suppression in P4502E1 mRNA levels was more distinct than the decrease in its protein levels. Furthermore, a detectable decrease in renal P4502E1 mRNA was also observed at day 20 of gestation (data not shown). Borlakoglu *et al.*, however, observed that P4501A1, 1A2, 2A1, 2B1, 2E1, and 4A1 were not immunochemically detectable in pregnant rats at the protein loading of 3 μg per well and that their mRNA levels were detected at variable intensities [6]. The decreases in P4502E1 protein and mRNA levels in the present study are in agreement with the immunoblot data, which showed significant and progressive reduction in hepatic P4502E1 in both untreated and acetone-treated rats during pregnancy (and Northern mRNA blot analysis), as shown by Casazza *et al.* [18]. According to their study, the reduced level of P4502E1 mRNA returned to the control level after parturition.

A previous study has shown that the total cytochrome P450 content in mice declines along with some catalytic activities [4]. Nakajima *et al.* suggested that levels of P4501A1 and 1A2 failed to change during pregnancy, based on their observations for toluene metabolism and immunochemical inhibition using monoclonal antibodies [5]. The fact that no change in P4501A1 mRNA levels was observed in the present study is consistent with the results of metabolic activities, confirming that P4501A1 mRNA expression failed to change during pregnancy. However, a 50% decrease in P4501A2 mRNA level was observed here. This minor discrepancy between immunochemical and RNA blot hybridization analyses may result from the differences in specificity and sensitivity of the assays.

In summary, this study shows that the expression of hepatic and renal mEH as well as P4502E1 decreases in late pregnancy and that suppression is associated with decreases in their mRNA levels. The information in this study may increase our understanding of pregnancy-associated physiological and biochemical changes and the possible role of certain hormones.

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