0026-895X/93/050722-04$03.00/0

MOLECULAR PHARMACOLOGY, 43:722-725

Marked Endogenous Activation of the *CYP1A1* and *CYP1A2* Genes in the Congenitally Jaundiced Gunn Rat

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Received September 23, 1992; Accepted March 1, 1993

SUMMARY

The homozygous recessive jaundiced Gunn rat lacks expression of bilirubin UDP-glucuronosyltransferase and serves as a model for Crigler-Najjar syndrome type I, in which high and toxic plasma levels of bilirubin result from this genetic defect in bilirubin conjugation. Both rats and humans dispose of this heme waste product by an alternate metabolic route that involves oxidation of the compound, followed by biliary excretion of the more polar metabolites. To determine the role of cytochrome P450 in this process, hepatic levels of cytochrome P450 mRNA and protein were measured in jaundiced and nonjaundiced Gunn rats as a function of age and sex. The mRNA and protein levels of cytochrome P450(CYP) 1A1 and CYP1A2 were markedly elevated in the jaundiced rats at the age of 10 days, compared with their nonjaundiced littermates. Levels of CYP2E1 mRNA and protein did not differ between these rats, indicating that the CYP1A P450 genes were specifically induced. CYP1A1 mRNA and protein levels increased further in the jaundiced animals between 10 days and 1 month of postnatal life but remained undetectable in the nonjaundiced littermates. On the other hand, CYP1A2 mRNA and protein content increased during this time period in both jaundiced and nonjaundiced rats, but at the age of 1 month there were no major differences between the two groups. CYP1A2 mRNA and protein levels were indistinguishable in 3-month-old jaundiced and nonjaundiced Gunn rats, whereas CYP1A1 could not be detected in either group. These data suggest that young jaundiced Gunn rats cope with the degradation of toxic bilirubin by increasing hepatic levels of CYP1A1 and CYP1A2. On the other hand, normal developmental activation of CYP1A2 may provide the alternative pathway for bilirubin degradation in adult animals. This is the first demonstration of the induction of cytochrome P450 gene expression to permit the elimination of an endogenously generated neurotoxic chemical in a genetic disease in which the normal excretory mechanism is impaired.

The superfamily of cytochrome P450 genes encodes a variety of proteins that catalyze the oxidation of endo- and xenobiotics into both biologically inactive and, in some cases, reactive metabolites (1). Among the endogenous substrates of these enzymes are steroids, fatty acids, and prostaglandins.

Kapitulnik and Ostrow (2) suggested that bilirubin, the neurotoxic end-product of hemoglobin catabolism, could be an additional endogenous substrate for cytochrome P450. Bilirubin is normally excreted in the bile after being conjugated with glucuronic acid. Patients with CNS-I are not able to conjugate bilirubin and thus develop a lifelong and severe unconjugated hyperbilirubinemia.

The jj Gunn rat is the animal model of the human syndrome. These rats lack a functional bilirubin UDP-glucuronosyltransferase gene (3, 4). Plasma bilirubin levels increase in the jj Gunn rat rapidly after birth, but this increase levels off during the third week of extrauterine life, suggesting that an alternate metabolic pathway is recruited to dispose of the neurotoxic bilirubin. This "salvage" system arrests the increase in plasma levels of the pigment by converting it into polar oxidation products, which can then be excreted in the bile without the need for conjugation (5). Inducers of CYP1A1 and CYP1A2, such as TCDD, 3-MC, and β-naphthoflavone, markedly decreased plasma bilirubin levels in jj Gunn rats (2, 6, 7), whereas inducers of other forms of cytochrome P450 failed to do so (8, 9). In addition, specific constitutive increases in the microsomal content of CYP1A1 and CYP1A2 were documented in jj Gunn rat liver (6), suggesting that these cytochrome P450 forms could be involved in bilirubin homeostasis in the jj Gunn rat. Recently, De Matteis *et al.* (10, 11) provided additional evidence for the presence of a TCDD-inducible microsomal system in jj Gunn rat liver that degrades bilirubin via cytochrome P450-mediated oxidation.

The present report demonstrates that CYP1A1 and CYP1A2 are constitutively increased in jj Gunn rat liver as a result of the marked elevation of their respective mRNAs.

This work was supported in part by grants from the State of Niedersachsen, Germany, and the Israel Ministry of Health.

ABBREVIATIONS: CNS-I, Crigler-Najjar syndrome type I; jj, homozygous jaundiced; Jj, heterozygous nonjaundiced; CYP1A1, cytochrome P450 1A1; CYP1A2, cytochrome P450 1A2; CYP2E1, cytochrome P450 2E1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene.

Materials and Methods

Animals. Female and male Gunn rats were obtained from the Animal Facility of the Hebrew University Medical School. This colony originated from breeders obtained from the Skin and Cancer Hospital, Temple University (Philadelphia, PA). The rats were maintained in an environment free of known inducers of cytochromes P450 and were fed a breeder rat diet, containing 11% fat (Weizmann Institute, Rehovot, Israel), and water *ad libitum*.

Protein blots. Microsomes were prepared from pooled livers of 10-day-, 1-month-, and 3-month-old jj and Jj Gunn rats by differential centrifugation (12). Each sample was pooled from livers of three to eight animals. Microsomes were electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (13). Samples were then transferred to nitrocellulose sheets and treated with rabbit polyclonal antibodies to CYP1A1/CYP1A2 and CYP2E1 (14), as described by Towbin *et al.* (15). The blots were stained with alkaline phosphatase-labeled goat anti-rabbit IgG (KPL Laboratories, Gaithersburg, MD).

RNA blots. Total RNA was obtained by the method of Chirgwin *et al.* (16) and was subjected to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde (17), followed by Northern blot hybridization. The filters were hybridized with ^{32}P-labeled cDNA probes prepared by nick translation and were washed according to the method of Church and Gilbert (18). The mouse CYP1A1 and CYP1A2 cDNAs (19) and rat CYP2E1 cDNA (20) have been described.

Results

Bilirubin levels increased in the jj animals after birth, peaked at 2 weeks of age, decreased slightly thereafter, and reached a plateau at about 1 month (Fig. 1). There were no differences in plasma bilirubin levels between male and female jj rats (data not shown).

Both CYP1A1 and CYP1A2 proteins were constitutively increased postnatally in the livers of jj animals (Fig. 2). This increase was already evident at the age of 10 days, compared with Jj animals, in which CYP1A1 was absent and CYP1A2 was barely detectable. Both proteins were markedly expressed in jj animals at the age of 1 month. However, whereas CYP1A1 was still absent in Jj rat livers, CYP1A2 was clearly present at this age in the latter livers and its levels were similar to those found in the livers of jj animals (Fig. 2). CYP1A1 was not detected in either jj or Jj adult animals, and there were no differences between their hepatic CYP1A2 levels (Fig. 2). Hepatic CYP2E1 protein contents were similar for jj and Jj rats among all age groups (Fig. 2).

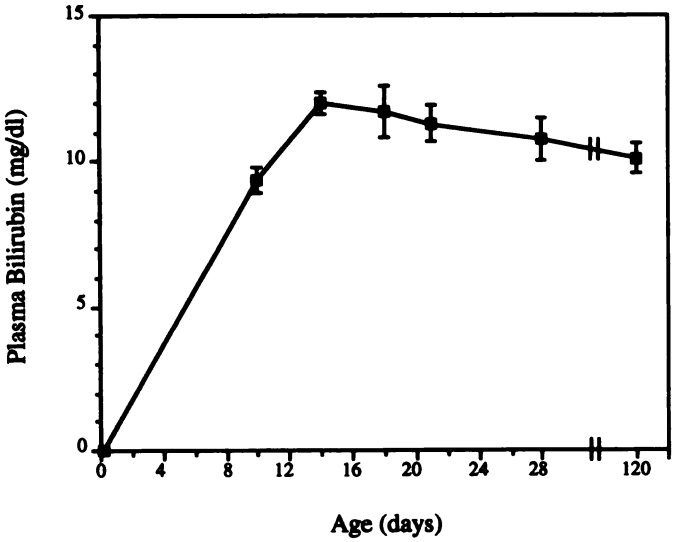

Fig. 1. Levels of plasma bilirubin in jj Gunn rats as a function of age. Values from male and female animals were combined for each age group and are presented as mean ± standard deviation (three to eight animals per age group).

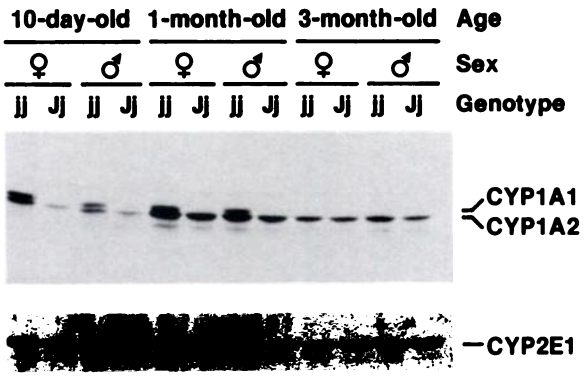

Fig. 2. Western immunoblot analysis of CYP1A1, CYP1A2, and CYP2E1 in livers of jj and Jj male and female rats of various ages. Ten micrograms of microsomal protein were electrophoresed on sodium dodecyl sulfate-containing 10% polyacrylamide gel and were electrotransferred to nitrocellulose paper. The filters were treated with rabbit anti-rat CYP1A1 or rabbit anti-rat CYP2E1 and alkaline phosphatase-labeled goat anti-rabbit IgG. Alkaline phosphatase precipitates were generated using developing reagent.

CYP1A1 and CYP1A2 mRNAs were also examined in jj and Jj rats of different ages. CYP1A1 mRNA levels were significantly elevated in both male and female 10-day-old jj animals, compared with their Jj littermates (Fig. 3). Similarly, but less markedly, CYP1A2 mRNA was increased in the jj animals. The increased expression of CYP1A1 peaked at 1 month of age and disappeared in the adult animals. On the other hand, the CYP1A2 gene was maximally expressed at 1 month of age and remained expressed during adulthood, but the mRNA levels were similar for the jj and Jj animals (Fig. 3). There were no differences between the jj and Jj rat livers in their contents of CYP2E1 and β-actin mRNAs (Fig. 3).

Discussion

The congenital unconjugated hyperbilirubinemia of Gunn rats has been shown here to be associated with a specific stimulation of the synthesis of CYP1A1 and CYP1A2 mRNAs, resulting in an increased hepatic content of the respective proteins.

Three lines of evidence suggest that these two cytochromes P450 might be involved in the alternate pathways of bilirubin catabolism that are responsible for the elimination of bilirubin in jj Gunn rats. First, in the absence of bilirubin UDP-glucuronosyltransferase, bilirubin is converted to water-soluble metabolites that are readily excreted into the bile. Some of these metabolites are hydroxylated derivatives of bilirubin that resemble characteristic products of cytochrome P450-mediated oxidation (5). These bilirubin metabolites include a hydroxy derivative at the exovinyl side chain and several monohydroxy and dihydroxy derivatives at the methene bridges connecting the pyrrole rings. A glutathione conjugate at the exovinyl side chain of bilirubin was found in bile of jj Gunn rats, suggesting a role for cytochrome P450 in generating the corresponding epoxide, which is then conjugated with glutathione (21).

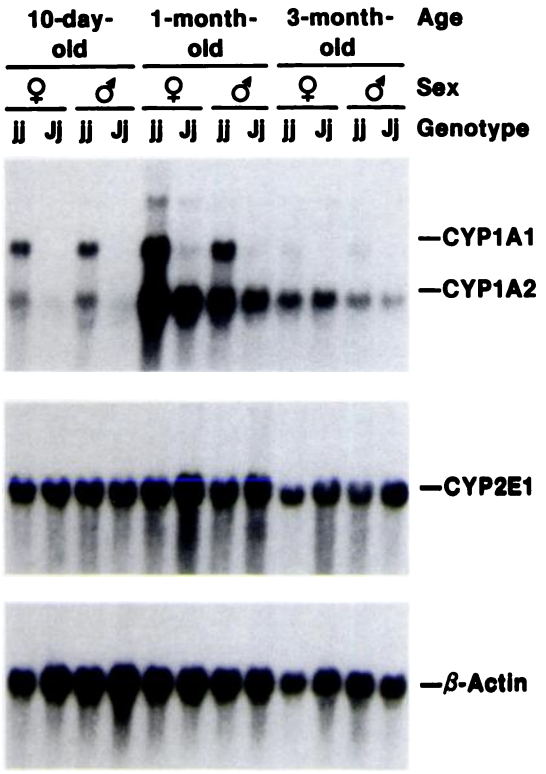

Fig. 3. Northern blot analysis of mRNAs encoding CYP1A1, CYP1A2, CYP2E1, and β-actin. Ten micrograms of total liver RNA from jj and Jj male and female rats of various ages were denatured and subjected to electrophoresis. The nucleic acids were transferred to nylon membranes and hybridized to the respective cDNA probes that had been labeled with ^{32}P by nick translation. The filters were washed and subjected to autoradiography for 24 hr, with the aid of a DuPont Lighting Plus intensifying screen.

Second, specific inducers of CYP1A1 and CYP1A2, such as TCDD, 3-MC, β-naphthoflavone, and the clinically used phenothiazines (2, 6, 7, 22), markedly reduced plasma bilirubin levels, accelerated the fractional turnover of bilirubin, decreased the total pigment pool, and increased the biliary excretion of hydroxylated bilirubin metabolites in jj Gunn rats. In contrast, inducers of other forms of cytochrome P450, such as phenobarbital and clofibrate, did not affect the plasma bilirubin levels in these rats (8, 9). Interestingly, phenobarbital also did not ameliorate the jaundice of the totally conjugation-deficient patients with CNS-I, whereas it was successful in reducing effectively the plasma bilirubin levels in the partially deficient Crigler-Najjar Syndrome type II patients (23). The negative results obtained with clofibrate suggest also that peroxisomal oxidases are not involved in the alternate pathways of bilirubin catabolism.

Third, in *in vitro* experiments with microsomes from TCDD-treated animals it was shown that bilirubin degradation requires NADPH and O_2 and can be inhibited by an antibody that recognizes both CYP1A1 and CYP1A2 (10, 11).

Our present results tend to support the role of both CYP1A1 and CYP1A2 in the alternate pathways of bilirubin catabolism in jaundiced Gunn rats. CYP1A1 expression is markedly increased throughout the first month of life in jj rats, whereas it is absent in Jj rat liver. The expression of the CYP1A2 gene parallels that of CYP1A1 but exhibits less marked quantitative differences between jj and Jj rats. It will be of interest to demonstrate whether a causal relationship exists between the changes in expression of CYP1A1 and CYP1A2 in jj Gunn rats as a function of age. Nevertheless, it is possible that in the immediate postnatal period both constitutively expressed cytochromes P450 intervene in bilirubin oxidation, whereas only CYP1A2 remains involved in this pathway in the adult animals.

The cytochrome P450-mediated elimination of bilirubin appears to be a much less efficient process than normal hepatic glucuronidation, and therefore plasma bilirubin levels remain elevated, although constant, in both adult jj Gunn rats and patients with CNS-I. Recently, it was suggested that adult jj Gunn rats eliminate bilirubin via direct transfer from blood to the intestinal lumen, where the bilirubin is degraded to urobilinogen and excreted in the feces (24). It is not clear whether such an excretory route is operative in 1-month-old jj rats and whether it is inducible by TCDD or 3-MC in the adult jj animals.

It remains to be established what endogenous compounds mediate the specific accumulation of CYP1A1 and CYP1A2 mRNAs and whether this increase results from transcriptional activation of the CYP1A1 and CYP1A2 genes or mRNA stabilization. Nevertheless, it is of interest that the most prominent increases in CYP1A1 and CYP1A2 mRNA levels in jj rats were observed at the age of 1 month, that is, after the postnatal peak in plasma bilirubin levels that occurs during the third week of life, thus suggesting a role for bilirubin in the regulation of CYP1A1 and CYP1A2 expression.

This is the first demonstration of induction of cytochrome P450 gene expression by an endogenous substance, which permits the excretion of an endogenously generated neurotoxic chemical in a genetic disease in which the normal excretory pathway of this chemical is impaired. The search for a safe, nontoxic, specific inducer of CYP1A1 and/or CYP1A2 could be of great value in improving the treatment of patients with CNS-I, because the available therapeutic measures are very limited, cumbersome, and not effective on a long term basis.

References

1. Gonzalez, F. J. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40**:243–288 (1988).
2. Kapitulnik, J., and J. D. Ostrow. Stimulation of bilirubin catabolism in jaundiced Gunn rats by an inducer of microsomal mixed-function monooxygenases. *Proc. Natl. Acad. Sci. USA* **75**:682–685 (1978).
3. Iyanagi, T., T. Watanabe, and Y. Uchiyama. The 3-methylcholanthrene-inducible UDP-glucuronosyltransferase deficiency in the hyperbilirubinemic rat (Gunn rat) is caused by a −1 frameshift mutation. *J. Biol. Chem.* **264**:21302–21307 (1989).
4. Roy-Chowdhury, J., T. Huang, K. Kesari, M. Lederstein, I. M. Arias, and N. Roy-Chowdhury. Molecular basis for the lack of bilirubin-specific and 3-methylcholanthrene-inducible UDP-glucuronosyltransferase activities in Gunn rats: the two isoforms are encoded by distinct mRNA species that share an identical single base deletion. *J. Biol. Chem.* **266**:18294–18298 (1991).
5. Berry, C. S., J. E. Zarembo, and J. D. Ostrow. Evidence for conversion of bilirubin to dihydroxyl derivatives in the Gunn rat. *Biochem. Biophys. Res. Commun.* **49**:1366–1375 (1972).
6. Kapitulnik, J., J. P. Hardwick, J. D. Ostrow, C. C. Webster, S. S. Park, and H. V. Gelboin. Increase in a specific cytochrome P-450 isoenzyme in the liver of congenitally jaundiced Gunn rats. *Biochem. J.* **242**:297–300 (1987).
7. Kapitulnik, J., and F. J. Gonzalez. The role of cytochrome P-450 in the elimination of bilirubin in congenital jaundice (Crigler-Najjar Syndrome Type I). *J. Basic Clin. Physiol. Pharmacol.* **3**(suppl.):90–91 (1992).
8. Cohen, A. N., J. Kapitulnik, J. D. Ostrow, E. A. Zenone, C. Cochrane, L. Celic, and H. Cheney. Effects of phenobarbital on bilirubin metabolism and its response to phototherapy in the jaundiced Gunn rat. *Hepatology* **5**:310–316 (1985).
9. Zenone, E. A., and J. D. Ostrow. Oxidative enzyme systems and the catabolism of unconjugated bilirubin (UCB). *Gastroenterology* **72**:1180 (1977).
10. De Matteis, F., T. Trenti, A. H. Gibbs, and J. B. Greig. Inducible bilirubin-degrading system in the microsomal fraction of rat liver. *Mol. Pharmacol.* **35**:831–838 (1989).
11. De Matteis, F., S. J. Dawson, A. R. Boobis, and A. Comoglio. Inducible

bilirubin-degrading system of rat liver microsomes: role of cytochrome P450IA1. *Mol. Pharmacol.* **40**:686–691 (1991).
12. Lu, A. Y. H., and W. Levin. Partial purification of cytochromes P-450 and P-448 from rat liver microsomes. *Biochem. Biophys. Res. Commun.* **46**:1334–1339 (1972).
13. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
14. Song, B. J., T. Matsunaga, J. P. Hardwick, S. S. Park, R. L. Veech, C. S. Yang, H. V. Gelboin, and F. J. Gonzalez. Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat. *Mol. Endocrinol.* **1**:542–547 (1987).
15. Towbin, H., T. Staehlin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
16. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
17. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**:4743–4751 (1977).
18. Church, G. M., and W. Gilbert. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**:1991–1995 (1984).
19. Gonzalez, F. J., P. I. Mackenzie, S. Kimura, and D. W. Nebert. Isolation and characterization of full-length mouse cDNA and genomic clones of 3-methylcholanthrene-inducible cytochrome P_1-450 and P_3-450. *Gene* **29**:281–292 (1984).
20. Song, B.-J., H. V. Gelboin, S.-S. Park, C. S. Yang, and F. J. Gonzalez. Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450s: transcriptional and post-transcriptional regulation of the rat enzyme. *J. Biol. Chem.* **261**:16689–16697 (1986).
21. Shore, L. J., W. S. Mogilevsky, and G. B. Odell. The *in vitro* formation of glutathione conjugates of bilirubin dimethylester by hepatic microsomes of jaundiced Gunn rats. *Hepatology* **10**:615 (1989).
22. Kapitulnik, J., and J. D. Ostrow. Biphasic effect of phenothiazines on plasma bilirubin levels in congenitally jaundiced Gunn rats. *Hepatology* **7**:1072 (1987).
23. Arias, I. M., L. M. Gartner, M. Cohen, J. BenEzzer, and A. J. Levi. Chronic nonhemolytic hyperbilirubinemia with glucuronyl transferase deficiency: clinical, biochemical, pharmacologic, and genetic evidence for heterogeneity. *Am. J. Med.* **47**:395–409 (1969).
24. Kotal, P., and J. Fevery. Urobilinogen-i is a major derivative of bilirubin in bile of homozygous Gunn rats. *Biochem. J.* **268**:181–185 (1990).

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