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DEVELOPMENTAL CHANGES IN THE CATALYTIC ACTIVITY AND EXPRESSION OF CYP2D ISOFORMS IN THE RAT LIVER

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ABSTRACT:

Developmental changes in bufuralol 1′-hydroxylation activity, which is known as a typical activity of cytochrome P-450 (CYP)2D isoforms, in the liver of rats were investigated. The catalytic activities of hepatic microsomes increased with development especially from 3 to 7 weeks. Eadie–Hofstee plots of bufuralol 1′-hydroxylation were obtained for monophasic kinetics (K_m: 0.037 μM) at 1 week and for biphasic kinetics (K_m: 0.051 and 6.4 μM) at 7 weeks of age. Quinine completely inhibited bufuralol 1′-hydroxylation activity of hepatic microsomes of 1- and 7-week-old rats. These results indicated that at least two kinds of CYP2D isoforms, which differ markedly in their affinity for bufuralol, were present at 7 weeks of age and that the CYP2D isoform that had low affinity for bufuralol was expressed with development. To assess the affinity of CYP2D isoforms for bufuralol, the kinetic properties of CYP2D1, 2D2, 2D3, and 2D4 expressed in yeast cells were investigated. The K_m value of CYP2D2, 0.044 μM, was extremely small compared with that of the other rat CYP2D isoforms. We further investigated developmental changes of CYP2D isoform mRNA by reverse transcription–polymerase chain reaction. CYP2D3 mRNA increased with development although CYP2D1 and 2D2 mRNA were not changed. The CYP2D4 mRNA was not detected. These findings indicated that CYP2D2, which had high affinity for bufuralol, was expressed in immature and mature rats, but CYP2D3, which had low affinity for bufuralol, was expressed only in mature rats.

The cytochrome P-450 (CYP)[1] superfamily plays an important role in the metabolism of both xenobiotics and endogenous substances. The CYP2D subfamily, in which polymorphism has been reported in rats and humans (Gut et al., 1986; Matsunaga et al., 1989; Eichelbaum and Gross, 1990), participates in the metabolism of many drugs, including β-blocking agents, antiarrhythmic agents, antidepressants, and other clinically useful drugs (Dayer et al., 1986; Brosen et al., 1986; Wrighton and Stevens, 1992).

Five CYP2D isoforms, CYP2D1 through CYP2D5, have been found in the rat liver (Gonzalez et al., 1987; Matsunaga et al., 1990; Kawashima and Strobel, 1995), but the physiological roles, substrate specificity, and kinetic properties of each of these CYP2D isoforms presently are unknown. In humans, only one CYP2D isoform, CYP2D6, has been found. Which CYP2D isoform in the rat corresponds to human CYP2D6, or which physiological functions the multiple forms of CYP2D have in the rat are unknown.

The developmental expression of multiple forms of CYP has been studied extensively in the rat (Maeda et al., 1984; Waxman et al., 1985; Schenkman et al., 1989), but little is known about the CYP2D subfamily. Although it has been reported that the *CYP2D3* gene is active late in development (Matsunaga and Gonzalez, 1990), the developmental expression of CYP2D1 and CYP2D2 is not well known. The developmental change of the catalytic activity of the CYP2D isoforms and the relationship between the gene expression and the catalytic activity of the CYP2D isoforms are not clear. Little is known about the sex differences in the gene expression and the catalytic activity of CYP2D isoforms during development in the rat.

In this study, we investigated the developmental changes of catalytic activity, kinetic properties, and gene expression of the CYP2D isoforms using several groups of rats at different ages to clarify the regulation of individual CYP2D isoforms during the developmental process. In addition, we examined the kinetic properties of the CYP2D isoforms expressed in the yeast cells.

[1] Abbreviations used are: CYP, cytochrome P-450; RT-PCR, reverse transcription–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Materials and Methods

Materials. Bufuralol and 1′-hydroxybufuralol were obtained from Daiichi Pure Chemicals Co. (Tokyo, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP were purchased from Oriental Yeast Co. (Tokyo, Japan). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Male (1-, 3-, 7-, 14-, and 34-week-old) and female (1-, 3-, 7-, 14-, and 40-week-old) Wistar rats were obtained from Japan Clea (Tokyo, Japan).

Expression of CYP2D Isoforms in *Saccharomyces cerevisiae*. CYP2D1, 2D2, 2D3, and 2D4 were expressed in *S. cerevisiae* AH 22 strain as described previously (Wan et al., 1997). The culture of yeast was performed as described (Oeda et al., 1985; Sakaki et al., 1992). After centrifugation, the cells were washed twice with buffer A containing 10 mM Tris-HCl (pH 7.5), 2 M D-glucitol, 0.1 mM dithiothreitol (DTT), and 0.2 mM EDTA and digested with zymolyase in buffer A (0.7 mg/ml) for 2 h at 30°C by gently shaking. After centrifugation at 2000*g*, the supernatant was decanted and the cells were washed again with buffer A before being suspended in buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 mM DTT, and 0.65 M D-glucitol. The resultant suspension was sonicated on ice 10 times (30 s each time). The microsomal fraction was prepared by differential centrifugation and suspended in buffer containing 0.1 M sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM DTT, and 20% glycerol. Specific contents of yeast microsomes expressing CYP2D1,

2D2, 2D3, and 2D4 were 240, 7.0, 53, and 150 pmol/mg of microsomal protein, respectively.

Catalytic Activity. Bufuralol 1′-hydroxylation activity in hepatic microsomes was assayed as described previously (Kronbach et al., 1987) with some modifications. Briefly, 0.25 ml of a solution containing 10 μg of microsomal protein, 3.3 mM glucose 6-phosphate, 0.1 U of glucose 6-phosphate dehydrogenase, 1.3 mM $MgCl_2$, and 1.3 mM NADP in 0.1 M potassium phosphate buffer (pH 7.4) was used. After 3 min of preincubation at 37°C, the reaction was started by the addition of 0.25 or 0.0025 μmol of bufuralol (final concentration of 100 or 1 μM). The incubation continued with gentle shaking at 37°C for 10 min, and the reaction was stopped with 10 μl of 6 M perchloric acid. Denatured protein was precipitated by centrifugation at 8000*g* for 4 min, and 50 μl of the supernatant was applied to a reverse-phase column (TSKgel ODS-80Ts, 4.6 × 250 mm; TOSOH, Tokyo, Japan) using an autosampler (AS-8020, TOSOH). The column temperature was maintained at 50°C. The mobile phase consisted of 1 mM perchloric acid and acetonitrile (7:3) delivered at a flow rate of 1.0 ml/min. The excitation and emission wavelengths for fluorometric determination of 1′-hydroxybufuralol were 252 and 302 nm, respectively.

Kinetic Studies. To determine the initial velocity of the bufuralol 1′-hydroxylation, incubation time was fixed at 1 min. The substrate concentrations in the reaction mixture ranged from 0.05 to 100 μM. Other conditions, including those for high-performance liquid chromatography, were as described above. In the kinetic assay for the expressed CYP2D isoforms, 5 to 20 μl of yeast microsomes containing 5 pmol of each CYP2D isoform was added to the reaction mixture, and other conditions were the same as for rat hepatic microsomes. The enzyme kinetic parameters (K_m and V_{max}) were analyzed according to a nonlinear least-squares regression analysis. When Eadie–Hofstee plots were biphasic, we assumed that two enzymes were involved in the reaction. K_{m1} and V_{max1} and K_{m2} and V_{max2} denote the Michaelis constants and the maximum rates of the high- and low-affinity phases, respectively.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA from the rat liver was isolated using a total RNA Isolation kit IsoGen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. RNA concentration and purity were determined by using a spectrophotometer. The reverse transcription and amplification reactions were performed using an RNA PCR kit, AMV version 0.2.1 (Takara, Kyoto, Japan) according to the protocol described in the instructions that were included with the kit. First-strand cDNA was synthesized with 10 μg of RNA and 50 pmol of random primers in a total volume of 20 μl and amplified by PCR with a set of primers (10 pmol each in a total volume of 100 μl). The following primers were used: 5′-ATCGCTGGACTTCTCGCTAC-3′ (position 140–159) and 5′-GTCTTCTGACCTTGGAAGAC-3′ (position 778–797) for CYP2D1/5 (Gut et al., 1986), 5′-CTACTGCCCATCTATAATCA-3′ (position 366–385) and 5′-CCAAAGCTCTCCTTCAATGT-3′ (position 681–700) for CYP2D2 (Gonzalez et al., 1987), 5′-ACCAATGCTGTCATCCATGAGGT-3′ (position 1130–1152) and 5′-GCTGGACTAGAATTTCTTTCCTT-3′ (position 1599–1581) for CYP2D3 (Gut et al., 1986), 5′-ATTGAGAGGTGTTTC-CAAAG-3′ (position 152–171) and 5′-CCAGGAAGGCATCAGTCAGA-3′ (position 1015–1034) for CYP2D4/18 (Kawashima and Strobel, 1995), and 5′-TCGTCTCATAGACAAGATGG-3′ (position 56–75) and 5′-GTAGTT-GAGGTCAATGAAGGG-3′ (position 170–190) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985). The size of amplified nucleotides was 658 bp for CYP2D1/5, 335 bp for 2D2, 452 bp for 2D3, 883 bp for 2D4/18, and 136 bp for GAPDH.

A portion of 2 μl was used for PCR. The specificity of the primers in the PCR condition used in this study was investigated previously with CYP2D1 to 5 and 18 cDNAs (Hiroi et al., 1998). Because of the similarity of nucleotide sequences of CYP2D1 and CYP2D5, it was difficult to distinguish between the two isoforms. The reactions were performed for 30 cycles with a Takara PCR Thermal Cycler MP (Takara, Kyoto, Japan) with a 1.0-min denaturation at 94°C, a 1.0-min annealing at 56°C, and a 2.5-min extension at 72°C. The PCR products were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide. A linear correlation between band intensity of CYP2D nucleotides amplified by PCR and total RNA was obtained from 0.1 to 2 μg of total RNA. The image of the gel was taken by a gel-imaging system (Atto, Tokyo, Japan). The density of band amplified by PCR was analyzed with the NIH image program.

Other Methods. Hepatic microsomes were prepared as described previously (Funae and Imaoka, 1985). Total CYP content was assayed by a method described previously (Omura and Sato, 1964). The amount of CYP2D was determined by immunoblot analysis as described previously (Ohishi et al., 1993). Statistical significance was calculated by Student's *t* test or Dunnett's test.

Results

CYP2D Protein. The developmental changes in the amount of CYP2D protein in the rat hepatic microsomes were investigated by immunoblot analysis (Fig. 1). The anti-CYP2D antibody used in this study recognized all of the CYP2D isoforms. The amount of CYP2D increased from 3 to 14 weeks of age in both sexes. In comparison to the amount of CYP2D at 1 week, a significant increase was observed at 7 (1.3 times), 14 (1.5 times), and 34 weeks (1.3 times) in males and at 7 (1.5 times) and 14 weeks (1.7 times) in females. No sex difference in the amount of CYP2D was observed among the age groups. In the process of development, the amount of CYP2D in the rat liver increased significantly in a sex-independent manner.

Bufuralol 1′-Hydroxylation. The developmental changes in the catalytic activity of the CYP2D isoforms were investigated by using bufuralol, which is a specific substrate for CYP2D isoforms at a substrate concentration of 100 μM (Fig. 2A). The bufuralol 1′-hydroxylation activity in the rat hepatic microsomes increased as the rat developed and reached a maximum level at 14 weeks in males and 7 weeks in females. Compared with the activity at 1 week, a significant increase of the activity was observed at 7 (1.7 times), 14 (1.8 times), and 34 weeks (1.6 times) in males and at 7 (1.8 times), 14 (1.7 times), and 40 weeks (1.7 times) in females. No sex differences in this activity were observed in any age group.

The Eadie–Hofstee plots of bufuralol 1′-hydroxylation over the substrate-concentration range of 0.05 to 100 μM exhibited nearly monophasic kinetics for 1-week-old male rats, whereas biphasic kinetics were observed for 7-week-old male rats (Fig. 3). Similar kinetic curves also were observed in female rats at the corresponding ages (data not shown). For 7-week-old rats, low- and high-affinity phases

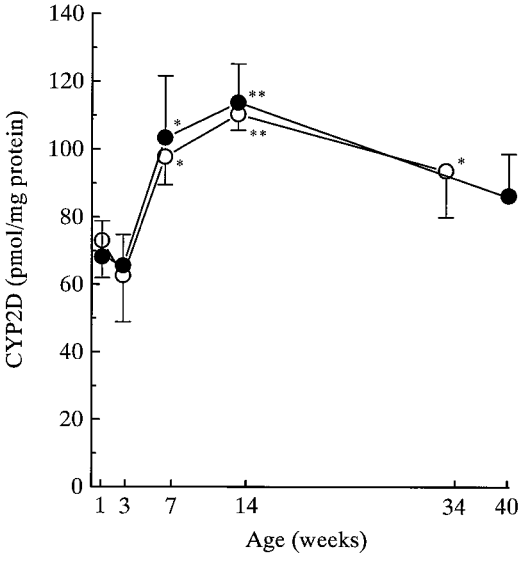

FIG. 1. *Levels of CYP2D protein in rat hepatic microsomes of various age groups.*

The levels of CYP2D protein in male (○) and female (●) rats were determined by immunoblot analysis. Hepatic microsomes (2 μg) were applied to an acrylamide gel, and immunoblotting was performed as described in *Materials and Methods*. Each point represents the mean ± S.D. from four different rats. *$P < .05$ and **$P < .01$, significantly different from 1-week-old rats (Dunnett's test).

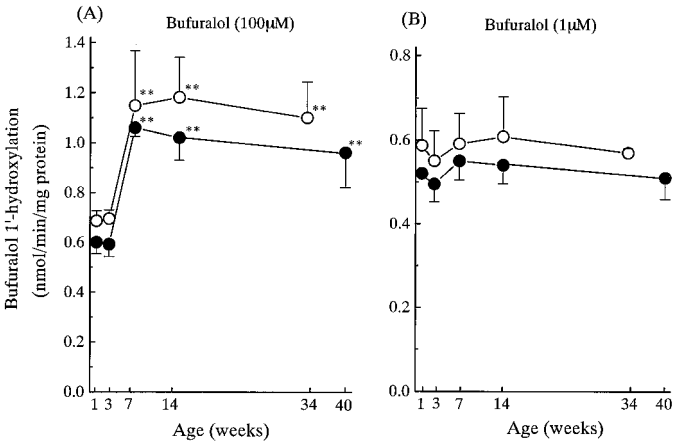

FIG. 2. *Bufuralol 1′-hydroxylation activity in rat hepatic microsomes of various age groups.*

A 0.25-ml aliquot of incubation mixture contained 10 μg of hepatic microsomal protein from male (○) and female (●) rats, and bufuralol was added to the incubation mixture at a final concentration of 100 μM (A) and 1 μM (B). The catalytic activities are expressed as nmol of product produced per min per mg of microsomal protein. Each point represents the mean ± S.D. from four different rats. $^{**}P < .01$, significantly different from 1-week-old rats (Dunnett's test).

were observed (Fig. 3B). The K_m values differed by approximately 100-fold between the two phases; the K_m values of the high-affinity (K_{m1}) and low-affinity (K_{m2}) phases in the male were 0.051 and 6.4 μM and in the female were 0.049 and 5.3 μM, respectively (Table 1). The V_{max} value of the low-affinity phase (V_{max2}, 1.4 nmol/min/mg protein) was 1.9 times that of the high-affinity one (V_{max1}, 0.74 nmol/min/mg protein) in the male, and in the female, V_{max2} (1.3 nmol/min/mg protein) was 1.6 times larger than V_{max1} (0.79 nmol/min/mg protein). The K_m value for bufuralol 1′-hydroxylation in 1-week-old male rats was 0.037 μM and was almost the same as that observed in the high-affinity phase in 7-week-old male rats. No sex differences were observed in the kinetic parameters of bufuralol 1′-hydroxylation in 1- and 7-week-old rats. These results suggest that at least two enzymes, which are responsible for bufuralol 1′-hydroxylation and differ in their affinity for this reaction, exist in the hepatic microsomes of the 7-week-old rats, whereas only the high-affinity enzymes exist in the microsomes of the 1-week-old rats.

The results of the kinetic analysis described above indicate that the CYP2D isoforms with a high affinity for bufuralol existed in the hepatic microsomes in both 1- and 7-week-old rats and that the V_{max} values of the high-affinity phase are similar at 1 and 7 weeks of age. It was thought that bufuralol 1′-hydroxylation activity at low substrate concentration was maintained at a constant level during development. To confirm this assumption, bufuralol 1′-hydroxylation activity at the substrate concentration of 1 μM was measured for all age groups (Fig. 2B). No significant difference in the catalytic activity was observed across the age groups in both sexes, suggesting that the CYP2D isoforms with a high affinity for bufuralol were expressed equally in the rat liver regardless of age or sex.

To confirm the involvement of CYP2D isoforms in bufuralol 1′-hydroxylation, we examined the effects of quinine, a selective inhibitor of CYP2D, on bufuralol 1′-hydroxylation activity in the rat hepatic microsomes at low (1 μM) and high (100 μM) substrate concentrations (Table 2). Quinine completely inhibited the bufuralol 1′-hydroxylation activity at both substrate concentrations in 1- and 7-week-old male rats (Table 2), and similar results were obtained for the corresponding ages of the female rats (data not shown). These results suggest that CYP2D isoforms are responsible for bufuralol 1′-hydroxylation at substrate concentrations of both 1 and 100 μM; namely, the high- and low-affinity enzymes for bufuralol belong to the CYP2D subfamily.

Kinetic Analysis of Expressed CYP2D Isoforms. To clarify which CYP2D isoforms correspond to the high- and low-affinity ones, a kinetic analysis of the rat CYP2D isoforms (2D1, 2D2, 2D3, and 2D4) expressed in yeast cells was done with bufuralol (Table 3). Among the CYP2D isoforms studied, the K_m value of CYP2D2 (0.044 μM) was much smaller than that of other CYP2D isoforms and was nearly equal to the K_m value observed in 1-week-old rats and in the high-affinity phase in 7-week-old rats. The K_m values of CYP2D1 and 2D3 were 9.5 and 17 μM, respectively, and these values corresponded well to those observed in the low-affinity phase in 7-week-old rats.

mRNA Levels of CYP2D Isoforms. Because it was difficult to immunologically distinguish CYP2D isoforms, we estimated the mRNA level of each isoform by RT-PCR to investigate the relationship between the catalytic activities and the mRNA levels of CYP2D isoforms. The mRNA levels of CYP2D1, 2D2, 2D3, and 2D4 were estimated by comparing each with that of GAPDH, which was constitutively and constantly expressed in the rat liver (Tso et al., 1985) and was expressed as a ratio of CYP2D isoform to GAPDH (Fig. 4). The mRNA levels of CYP2D1 and CYP2D2 were almost unaffected by age over the range of 1 to 34 weeks in males and 1 to 40 weeks in females. The mRNA level of CYP2D3 increased with development until 7 weeks in males and 14 weeks in females. The mRNA of CYP2D4 was not detected in the rat liver at any age group in this experiment.

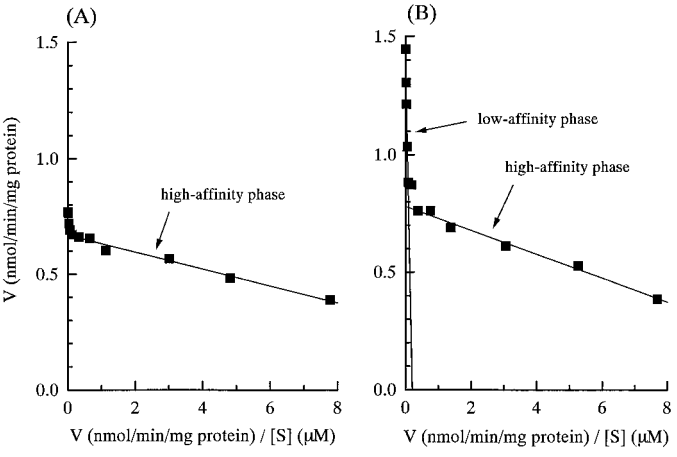

FIG. 3. *Eadie–Hofstee plots for bufuralol 1′-hydroxylation in rat hepatic microsomes.*

A 0.25-ml aliquot of incubation mixture contained 10 μg of hepatic microsomal protein from 1-week-old (A) and 7-week-old (B) male rats. The substrate concentrations in the reaction mixture ranged from 0.05 to 100 μM. Each plot represents a typical result for four rats.

TABLE 1

Kinetic parameters of bufuralol 1′-hydroxylation in rat hepatic microsomes

Rat	K_m		V_{max}	
	μM		*nmol/min/mg protein*	
1 wk, male	0.037 ± 0.005		0.67 ± 0.05	
1 wk, female	0.039 ± 0.004		0.68 ± 0.03	
	(K_{m1})	(K_{m2})	(V_{max1})	(V_{max2})
7 wk, male	0.051 ± 0.04	6.4 ± 0.6	0.74 ± 0.06	1.4 ± 0.2
7 wk, female	0.049 ± 0.05	5.3 ± 0.3	0.79 ± 0.04	1.3 ± 0.2

Each value represents the mean ± S.D. of four rats.

TABLE 2

Effect of quinine on bufuralol 1′-hydroxylation at high and low substrate concentrations in the hepatic microsomes of male rats

Quinine Concentration	Residual Activity (% of control)			
	Bufuralol, 1 μM		Bufuralol, 100 μM	
	1 wk	7 wk	1 wk	7 wk
μM				
0	100	100	100	100
1	19.6	23.8	N.T.[a]	N.T.
10	2.9	7.1	57.5	64.0
100	1.7	2.2	12.8	24.3
1000	N.T.	N.T.	1.6	2.9

Each value represents the mean of two samples.
[a] N.T., not tested.

TABLE 3

Kinetic parameters of bufuralol 1′-hydroxylation in expressed CYP2D isoforms

CYP2D	Km	V_{max}
	μM	*nmol/min/nmol P-450*
2D1	9.5 ± 0.8	2.9 ± 0.2
2D2	0.044 ± 0.004	6.3 ± 0.2
2D3	17 ± 1.2	8.0 ± 0.2
2D4	15 ± 0.7	8.5 ± 0.1

Each value represents the mean ± S.D. of three determinations.

Discussion

The amount of CYP2D protein in rat hepatic microsomes increased with development until 14 weeks of age. Among the CYP2D isoforms, the mRNA level of CYP2D3 significantly increased with age, and this result is consistent with a previous study (Matsunaga and Gonzalez, 1990). However, we found that the mRNA levels of CYP2D1 and CYP2D2 did not change significantly during the developmental process. These results suggest that the increase of the amount of CYP2D protein was mainly responsible for the increase of CYP2D3.

The kinetic studies on bufuralol 1′-hydroxylation and mRNA levels of the CYP2D isoforms suggested that the high-affinity CYP2D isoform observed in all age groups is CYP2D2. Because the affinity of the CYP2D2 for other substances has not been studied, physiological roles of the CYP2D2, which has an extremely high-affinity for bufuralol, are not known. The kinetic analysis of the expressed CYP2D isoforms also showed that the K_m and V_{max} values of CYP2D3 for bufuralol 1′-hydroxylation were the largest among the CYP2D isoforms whose mRNA were detected in the rat liver. From these kinetic data and the change in mRNA levels of CYP2D3 during development, it is considered that the increase of the catalytic activity of bufuralol 1′-hydroxylation during the developmental process was caused mainly by the increase of CYP2D3.

The expression of multiple forms of CYP during development has been studied in rats (Maeda et al., 1984; Waxman et al., 1985; Schenkman et al., 1989). The regulation of several CYP isoforms was reported to be sex-dependent; the expression of CYP2A2, CYP2C11, CYP2C12, CYP3A2, and CYP4A2 increased with development in a sex-specific manner (Imaoka et al., 1991; Kobliakov et al., 1991). However, we found no sex differences in the amount, bufuralol 1′-hydroxylation activity, kinetic property, or mRNA levels of the CYP2D isoforms. Accordingly, the regulation of the CYP2D isoforms during the developmental process in the rat is unaffected by sex.

In conclusion, the expression of each CYP2D isoform in the rat liver differs during the developmental process. CYP2D2, which has an extremely high affinity for bufuralol, is expressed in immature and mature rats, but CYP2D3, which has low affinity for bufuralol, is expressed only in mature rats.

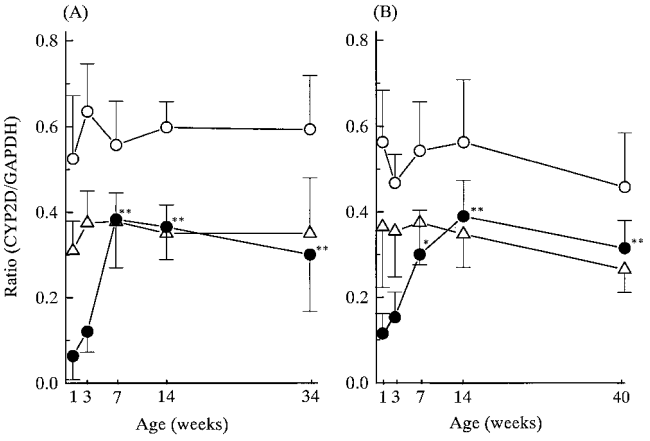

FIG. 4. *Expression of the CYP2D mRNA in the rat liver of various age groups.*

Expression of mRNA of CYP2D1 (△), CYP2D2 (○), and CYP2D3 (●) in the liver of male (A) and female (B) rats was estimated by RT-PCR and was expressed as the ratio of each CYP2D isoform to GAPDH. Each point represents the mean ± S.D. from four different rats. *$P < .05$ and **$P < .01$, significantly different from 1-week-old rats (Dunnett's test).

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