Pergamon

Biochemical Pharmacology, Vol. 47, No. 5, pp. 905–909, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0057-E

SHORT COMMUNICATIONS

Retinal dehydrogenation and retinoic acid 4-hydroxylation in rat hepatic microsomes: developmental studies and effect of foreign compounds on the activities

(*Received* 10 *August* 1993; *accepted* 9 *November* 1993)

Abstract—All-*trans*-retinoic acid (RA) regulates the transcription of a number of mammalian genes and is therefore important in the control of many cellular processes. RA is formed and deactivated within the cell so that biotransformation modulates RA availability. This study investigated the relationship between the formation of RA from retinal and its metabolism by 4-hydroxylation in rat hepatic microsomes. From kinetic studies the Michaelis constants for RA formation and 4-hydroxylation were 52 and 24 μM, respectively, and the maximal reaction velocities were 33 and 136 pmol/min/mg protein, respectively. Thus, 4-hydroxylation was the more efficient process. In microsomes from 1-week-old rats, RA formation was very low (~2 pmol/min/mg protein) but was several-fold greater in adults of both sexes (~10 pmol/min/mg protein). In contrast, 4-hydroxylation was quantitatively more significant at all ages examined between 1 and 15 weeks; by 10 and 15 weeks a sexual dimorphism was apparent (M > F). Thus, the ratio of RA 4-hydroxylation to RA formation was comparatively large in microsomes from 1-week-old rats and declined to a stable value around 4–6 weeks of age. With the exception of dexamethasone, which decreased the activity, administration of foreign compounds to male rats had little effect on RA formation. Both dexamethasone and phenobarbital induced RA 4-hydroxylation but DMSO and β-naphthoflavone were without effect. From these findings, 4-hydroxylation, particularly in very young animals, may be an effective means of controlling RA production. RA 4-hydroxylation, like other cytochrome P450 activities, was inducible in rat liver but no evidence was found for induction of the microsomal retinal dehydrogenase.

Key words: retinal dehydrogenation; retinoic acid 4-hydroxylation; cytochrome P450; hepatic microsomes

Most of the biological actions of vitamin A, including the regulation of morphogenesis and cellular differentiation, are mediated by RA* acting through a series of nuclear receptors [1]. RA is produced intracellularly from the enzymic dehydrogenation of the precursor retinal [2]. This process occurs in microsomal and cytosolic fractions of many retinoid-dependent tissues, such as the liver, lung and testis [3]. The duration of action of RA is also determined by its metabolic deactivation, for example by 4-hydroxylation [4], a reaction that is catalysed by microsomal cytochrome P450 enzymes [5]. Thus, the rates of RA formation (by retinal dehydrogenation) and metabolism (by 4-hydroxylation) are determinants of the intracellular concentration of RA and its availability for interaction with RA receptors. The present study was undertaken to characterize the relationship between microsomal RA formation and its 4-hydroxylation in rat liver. A kinetic analysis of the reactions was undertaken as well as an assessment of the effects of age, gender and exposure to typical lipophilic cytochrome P450 inducing agents on the activities.

Materials and Methods

Chemicals. RA, retinal, 13-*cis*-RA, DEX, βNF, NAD and NADPH, were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). PB was purchased from the Pharmacy Department, Westmead Hospital and DMSO was obtained from Ajax Chemicals (Auburn, NSW, Australia). 4-HydroxyRA was generously provided by Dr P. Sorter (Hoffmann La Roche, Nutley, NJ, U.S.A.). [15-^{14}C]RA was from Amersham Australia (Sydney, NSW). Chromatographic procedures used HPLC grade solvents and all other reagents were at least analytical grade.

Animals. Wistar rats were provided by the Department of Animal Care at Westmead Hospital. In the developmental study, rats of both sexes were killed at 1, 2, 4, 6, 10 and 15 weeks of age. Each liver was processed individually for the 4, 6, 10 and 15 week old rats but at earlier time points pools of microsomes were prepared from two to four livers. In induction studies, adult male rats (*ca.* 200 g) were administered either PB (100 mg/kg), DEX (100 mg/kg) or βNF (40 mg/kg) by intraperitoneal injection once daily on 3 consecutive days. DMSO (2 mL/kg) was administered intraperitoneally twice daily for the same period. Animals were killed by exsanguination under anaesthesia 24 hr after the final dose of inducer. The livers were removed, perfused with saline, homogenized as described elsewhere [6] and centrifuged at 10,000 *g* for 10 min. Washed hepatic microsomes were prepared by ultracentrifugation of the resultant supernatant at 105,000 *g* for 60 min, resuspension of the microsomal pellet in fresh buffer and recentrifugation at 105,000 *g* for 30 min.

Retinal dehydrogenation in rat hepatic microsomes. Retinal dehydrogenation to RA was measured in hepatic microsomes and was performed in amber borosilicate tubes essentially as described by Napoli and Race [3]. Incubations (0.4 mL) included 0.5 mg microsomal protein and, except in the estimation of kinetic parameters, 100 μM retinal in potassium phosphate buffer (0.1 M, pH 7.4, containing 1 mM EDTA). Reactions were initiated by NAD (1 mM),

* Abbreviations: RA, all-*trans*-retinoic acid; DEX, dexamethasone; PB, phenobarbital sodium; βNF, β-naphthoflavone.

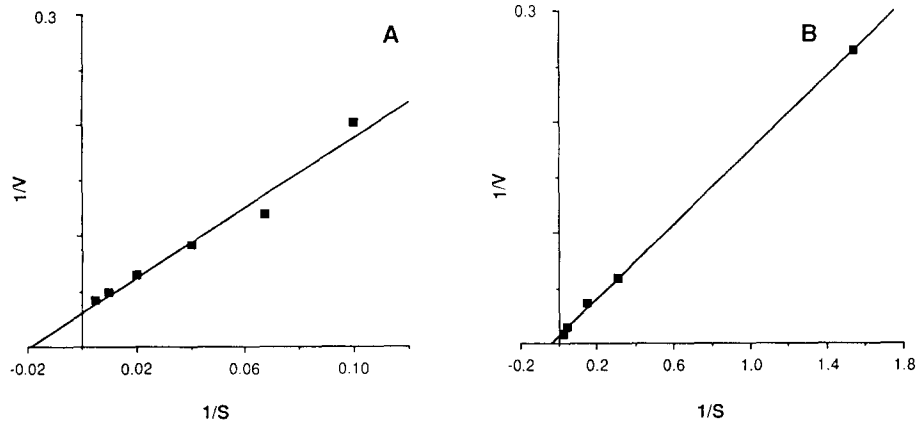

Fig. 1. Lineweaver–Burk plots of (A) retinal dehydrogenation and (B) RA 4-hydroxylation in untreated male rat hepatic microsomes. Units: substrate concentrations in μM and reaction velocities in pmol product/min/mg protein.

conducted at 37° for 60 min and terminated by the addition of cold ethanol, containing EDTA (0.25 mg) and ascorbic acid (0.25 mg), followed by the addition of 2 mL ethyl acetate. Under these conditions product formation was linear and substrate utilization in hepatic microsomes from adult male rats was 1%. After extraction, samples were reduced to dryness under N_2 and then dissolved in 50 μL methanol for application to an Ultrasphere ODS column (5 μM, 25 cm × 4.6 mm i.d., Beckman Instruments Inc., San Ramon, CA, U.S.A.) attached to a Waters Associates HPLC system. The mobile phase was 30 mM ammonium phosphate in methanol (5:95) and elution was at 1.5 mL/min with a detection wavelength of 340 nm. Retention times for RA and retinal were 3.1 and 5.2 min, respectively, under these conditions.

Rat hepatic microsomal RA 4-hydroxylation. RA 4-hydroxylation was assayed in amber borosilicate tubes essentially as described elsewhere [7]. With the exception of the kinetic experiments the RA concentration selected for study was 10 μM, because its physiological concentration is unlikely to be very high. Thus, [15-^{14}C]RA (2×10^5 dpm/1.0 mL incubation) was added to incubations in 5 μL DMSO (0.5% final concentration, which was found in preliminary studies to have no effect on the activity) that also contained 0.5 mg microsomal protein in 0.1 M phosphate buffer pH 7.4. Reactions (37°) were initiated by NADPH (1 mM) and were terminated after 30 min as described above for the assay of RA formation. Under these conditions, product formation was linear and substrate utilization in hepatic microsomes from adult male rats was 7%. After extraction, the samples were subjected to HPLC as outlined above for RA formation except that the mobile phase was methanol:acetonitrile:0.1 M ammonium acetate (73:12:15), pH 6.8, run at 1 mL/min, and the detection wavelength was 340 nm [8]. The eluates from each sample were collected into scintillation vials at 1-min intervals and counted for radioactivity (ACS II, Amersham Australia). 4-HydroxyRA and RA eluted at 3.3 and 9.6 min, respectively; 13-*cis*-RA eluted at 8.3 min in this system.

Other assays. Protein was estimated by the method of Lowry *et al.* [9] using BSA as the standard.

Statistics. All data are means ± SEM. Comparisons were made by one-way ANOVA and Student–Newman–Keuls testing.

Results

Kinetics of RA formation and RA 4-hydroxylation in hepatic microsomes. Hepatic microsomes have been shown previously to produce RA from both retinol and retinal although, under normal circumstances, extra-microsomal RA formation may be quantitatively more significant. The further metabolism of RA by 4-hydroxylation also occurs in microsomes and appears to represent a major step in the termination of RA action. As part of the present study, the kinetic characteristics of microsomal formation of RA from retinal and RA 4-hydroxylation were assessed. Thus, as shown in Fig. 1A, RA formation from retinal occurred over a wide range of substrate concentrations (10–200 μM) and the Michaelis constant (K_m) and maximal reaction velocity (V_{max}) were determined to be 52 μM and 33 pmol/min/mg protein, respectively. In contrast, the K_m and V_{max} estimates from RA 4-hydroxylation were 24 μM and 136 pmol/min/mg protein, respectively (Fig. 1B). The V_{max}/K_m ratios for retinal dehydrogenation and RA 4-hydroxylation were 0.64 and 5.7×10^{-6} L/min/mg protein, respectively, thus indicating that 4-hydroxylation was approximately 9-fold more efficient than retinal dehydrogenation in adult rat liver.

Age-related changes in microsomal retinal dehydrogenation and RA 4-hydroxylation in rat liver. The development and gender profile of microsomal retinal dehydrogenation and RA 4-hydroxylation activities were determined in hepatic microsomes from untreated rats of both sexes. As shown in Fig. 2A, at 1 and 2 weeks of age, retinal dehydrogenase activity was relatively low in hepatic microsomes from male (2.3 and 3.3 pmol/min/mg protein) and female (2.6 and 4.0 pmol/min/mg protein) rats. Between 4 and 6 weeks of age, the activity increased to around adult levels that were maintained thereafter. Overall, by 15 weeks of age, microsomal retinal dehydrogenation had increased approximately 5.5- and 3.1-fold in male and female rat liver, respectively. Apparent differences in activity between the sexes did not attain statistical significance. Thus, although retinal dehydrogenation underwent development-related increases in activity, sexual dimorphisms were not apparent.

Analogous measurements of RA 4-hydroxylation also revealed development-related increases in the activity but, in addition, the activity was greater in adult male than adult female rat liver microsomes (Fig. 2A). Thus, RA 4-hydroxylation activity was low in microsomes from 1-week-old animals (33 and 24 pmol/min/mg protein in male and female liver, respectively). In male rats the activity increased steadily and was 1.5-fold higher at 15 weeks than

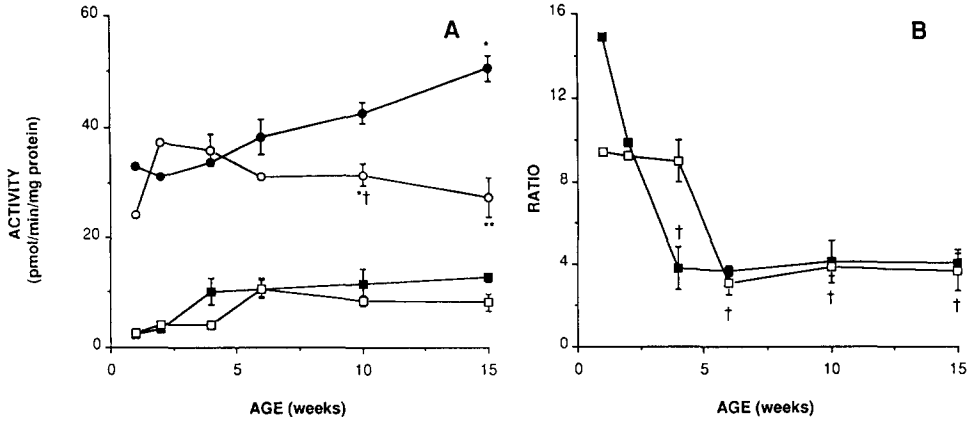

Fig. 2. Developmental profiles of (A) retinal dehydrogenation (squares) and RA 4-hydroxylation (circles) in hepatic microsomes from untreated male (closed symbols) and female (open symbols) rats. The ratios of RA 4-hydroxylation to retinal dehydrogenation were calculated separately for each microsomal fraction and are shown in (B). Significant difference: *between male and female rat liver at 4 weeks of age ($P < 0.001$), †from female rat liver at 4 weeks of age ($P < 0.005$), *†between male and female rat liver at 10 weeks of age ($P < 0.05$) and **between male and female rat liver at 15 weeks of age ($P < 0.001$).

Table 1. Effect of *in vivo* administration of foreign compounds on microsomal retinal dehydrogenation and RA 4-hydroxylation in male rat liver

Foreign compound	Retinal dehydrogenation	RA 4-hydroxylation	Ratio*
	(pmol/min/mg protein)		
None (control)	17 ± 2	44 ± 3	2.5 ± 0.3
PB	20 ± 1	157 ± 4†	8.1 ± 0.6†
DEX	9 ± 1‡	95 ± 6†	10.6 ± 1.3†
βNF	15 ± 2	39 ± 2	2.7 ± 0.3
DMSO	12 ± 2	39 ± 5	3.2 ± 0.1

Values are means ± SEM of three separate microsomal fractions in each group.
* Ratio = RA 4-hydroxylation/retinal dehydrogenation.
Significant difference from corresponding control group: †$P < 0.01$, ‡$P < 0.05$.

in 1-week-old liver. In contrast, the activity appeared optimal between 2 and 4 weeks of age in female rat liver and declined after puberty. In adult rats a sex dimorphism was apparent (M:F ratio 1.4 at 10 weeks, $P < 0.05$, and 1.9 at 15 weeks of age, $P < 0.001$). Figure 2B demonstrates that the ratio of 4-hydroxylation to retinal dehydrogenation was comparatively large in microsomes from 1-week-old rat liver (~9 in females and ~15 in males), but decreased to a value of ~4 by 4–6 weeks of age; the value remained essentially constant to at least 15 weeks of age.

Effect of in vivo *administration of foreign compounds on microsomal retinal dehydrogenation and RA 4-hydroxylation in rat liver.* Retinal dehydrogenation in hepatic microsomes was not influenced by the pretreatment of rats with PB, βNF or DMSO according to the standard dosage regimen employed in studies of cytochrome P450 induction (Table 1). However, DEX administration decreased the activity to about 53% of control (9 ± 1 vs 17 ± 2 pmol/min/mg protein, $P < 0.05$). Microsomal RA 4-hydroxylation was significantly increased to 3.6- and 2.2-fold of control by administration of PB and DEX to male rats. Two other P450 inducers, βNF and DMSO, did not significantly influence the rate of microsomal RA 4-hydroxylation in rat liver. Thus, administration of foreign compounds influenced the rate of microsomal RA formation and 4-hydroxylation and resulted in a shift in the ratio of the two activities. As shown in Table 1, the ratio of 4-hydroxylation to retinal dehydrogenation in untreated rat liver was 2.5 ± 0.3 under the experimental conditions selected. The ratio was increased markedly by exposure to PB and DEX, but not by βNF or DMSO, suggesting that foreign compounds may decrease the effective concentration of RA produced by microsomes by enhancing the rate of 4-hydroxylation. Apart from DEX, microsomal enzyme inducers had little effect on RA synthesis from retinal and no evidence of enhanced dehydrogenation was found under any of the conditions evaluated.

Discussion

It now appears clear that RA is significant in the regulation of gene transcription by ligand-activated receptors. The biotransformation of less active precursor retinoids to RA occurs intracellularly and these processes are likely to be important in the overall extent of RA receptor-mediated cellular control [10]. Retinal is the immediate precursor of RA and its dehydrogenation in liver may involve the participation of several aldehyde dehydrogenases at different subcellular sites, although the

identity of the most active enzyme(s) is yet to be established unequivocally. Once formed, RA may be deactivated by 4-hydroxylation and further conversion to 4-oxoretinoic acid [11]. These metabolic processes probably determine the effective concentration of RA present within the cell.

RA is crucial in limb and cranio-facial development in the embryo and cellular differentiation after birth [12, 13]. In the present study, it was found that the ratio of RA 4-hydroxylation to retinal dehydrogenation is large in microsomes from 1-week-old rats and decreases with age. Thus, it appears likely that 4-hydroxylation is important in the control of RA levels in young animals. It may be for this reason that the changes in RA 4-hydroxylation observed in the developmental profile are relatively minor and are somewhat different from any other P450 reactions in rat liver which tend to exhibit more pronounced increases in activity with age [14].

There have been reports that typical microsomal enzyme inducing agents also influence the rate of RA 4-hydroxylation in rat liver. Thus, administration of ethanol [15], PB [4], 3-methylcholanthrene [4] and 3,3',4,4',5,5'-hexabromobiphenyl [16] have been reported to enhance microsomal 4-hydroxylation activity. The present study also demonstrates that PB and DEX, but not βNF or DMSO, are effective inducers of the activity in male rat liver. These findings are largely accountable by the fact that RA 4-hydroxylation is a cytochrome P450-dependent reaction and has been described for a number of mammalian cytochrome P450s [5]. In contrast, little information exists on the xenobiotic inducibility of microsomal retinal dehydrogenation to RA. The present study demonstrates that, of the agents evaluated, only DEX exerted a significant effect on RA production from retinal.

It should be added that, under normal circumstances, the majority of cellular retinal oxidation appears to be catalysed by cytosolic enzymes. Thus, Napoli and Race [2] have suggested that 5–10% of the retinal dehydrogenase activity measured in liver homogenate may be catalysed by microsomal enzymes. Nevertheless, the unequivocal interpretation of such data derived from estimates of apparent enzyme activities would be strengthened by the use of inhibitory antibodies directed against the cytosolic and microsomal enzymes. It is also conceivable that exposure to certain chemicals *in vivo* may result in the preferential inhibition of the cytosolic retinal dehydrogenase. Thus, at a concentration of 10 μM, 4-methylpyrazole elicited 39% inhibition of the cytosolic activity [3], but in contrast, the microsomal activity was refractory to inhibition at concentrations up to 1 mM (unpublished data). The marked sensitivity of the cytosolic activity to inhibition by this agent could lead to the microsomal enzyme assuming greater importance in cellular RA synthesis after 4-methylpyrazole exposure.

Apart from enzymatic processes, it has emerged recently that cellular retinol and RA binding proteins, both members of the family of lipid binding proteins, can modulate the biotransformation of retinol and RA, respectively [10, 17]. Factors that modify the expression of these proteins may also be expected to influence retinoid metabolism *in situ*. Accordingly, it is of some interest that DEX administration reportedly decreases the mRNA species in rat liver that corresponds to cellular retinol binding protein [18]. This protein also has the capacity to bind retinal and to facilitate its interaction with cytosolic dehydrogenases, leading to RA formation [19]. Thus, DEX administration *in vivo* may have important consequences for RA production by the cell. The apparent induction of RA degradation by DEX could well exacerbate the effects of low intracellular RA concentrations.

Acknowledgements—This work was supported by a grant from the National Health and Medical Research Council of Australia. R.M. was the recipient of a Biomedical Postgraduate Research Scholarship from the Gastrointestinal Society of Australia. We are grateful to Dr Peter Sorter of Hoffmann-La Roche, Nutley, NJ for the geneous gift of 4-hydroxyretinoic acid.

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