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In-vitro Metabolism of Retinoic Acid by Different Tissues from Male Rats

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Abstract

Significant differences between the metabolism of retinoic acid by different tissues might be an important determinant of the effectiveness of a systemically administered inhibitor at a particular tissue site. Here the metabolism of retinoic acid has been studied in microsomal fractions from different tissues (liver, kidney, intestinal mucosa, lung, skin, brain) of the male rat to determine their relative metabolic activity.

Kinetic analysis revealed major differences between the activity of different tissue microsomes. This is shown by the V_{max} values for the metabolism of retinoic acid—liver ($102 \pm 39{\cdot}0$ pmol (mg protein)$^{-1}$ min^{-1}) was 100 times more active than the lung ($1 \pm 0{\cdot}03$ pmol (mg protein)$^{-1}$ min^{-1}), which was the least active. The range of K_m values for microsomes from the different tissues was narrow (0·48–1·40 μM). Taking into account the mass of the tissue, the gross activity ranking for metabolism of retinoic acid was liver $\gg$ skin = kidney > brain > intestinal mucosa $\gg$ lung.

It is concluded that metabolism of administered retinoic acid occurs mainly in the liver but that cellular retinoic acid levels in some other tissues (skin, kidney, brain) could be reduced (metabolized) to such an extent that higher levels might be observed after the use of inhibitors of retinoic acid metabolism.

Retinoic acid is a physiologically important metabolite of retinol capable of inducing epithelial differentiation and other systemic functions of vitamin A in-vivo (Raner et al 1996). In-vitro retinoic acid is a more potent inducer of differentiation than retinol (McCormick et al 1983), the most abundant retinoid in the circulation.

Inhibiting retinoic acid metabolism increases its biological potency (Napoli 1993, 1996) suggesting that retinoic acid acts directly and is not converted into an active metabolite (Van Wauwe et al 1994). Although the liver is recognized as an important site for P450-mediated oxidation of administered retinoic acid in the rat (Ahmad et al 1994), it is possible that other sites in the body might contribute to the metabolic clearance of retinoic acid. Levels of retinoic acid-metabolizing enzymes in different tissues could affect the duration of action of administered retinoic acid in these tissues.

A current strategy for development of potential anti-cancer drugs is the design of selective inhibitors of retinoic acid oxidation to enhance its beneficial effects (Van Wauwe et al 1992; Ahmad et al 1995). Significant variation of the metabolism of retinoic acid by different tissues might be an important determinant of the effectiveness of a systemically administered inhibitor at a particular tissue site, the inhibitor being most effective at elevating endogenous retinoic acid levels, or maintaining exogenous-derived cellular retinoic acid levels, where the activity of the metabolizing enzymes is highest. In this context this study compares the in-vitro metabolism of retinoic acid in the liver, skin, kidney, brain, intestinal mucosa and lung of adult male albino rats.

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Materials and Methods

Chemicals

[11,12-^{3}H]All-*trans* retinoic aid was purchased from Dupont, UK (Stevenage, Herts, UK). All-*trans* retinoic acid, NADPH, butylated hydroxyanisole, ketoconazole and protein standards were obtained from Sigma (Poole, Dorset, UK). Formic acid, ammonium acetate and Hisafe III scintillation fluid (optiphase III) were obtained from Fisons

(Leicestershire, UK). Solvents used for chromatography were HPLC grade from Rathburn Chemicals (Walkerburn, UK). Other laboratory reagents were of analytical reagent grade and were obtained from BDH (Poole, Dorset, UK).

Preparation of microsomes
Healthy male Wistar rats, 250 g, were fasted overnight and killed by stunning. The liver, scrotal skin, kidney, whole brain, intestinal mucosa and lungs were removed, washed with cold normal saline solution (if necessary), and weighed. The tissues were separately homogenized with three times their weight of phosphate buffer (50 mM, pH 7·4) containing 0·25 M sucrose. The homogenate was centrifuged for 20 min at 10 000 *g* and 3°C. The pellet was discarded and the supernatant spun for 1 h at 100 000 *g* and 3°C. The pellet (microsomal fraction) was suspended in phosphate buffer (pH 7·4) by use of a Potter-Elvejhem homogenizer. Samples (0·5 mL) were pipetted into capped plastic tubes, snap-frozen in liquid nitrogen and stored at −80°C until required for use.

Assay
Microsomes from different tissues (liver and kidney (protein 0·120 mg mL^{-1}, 10 μL), intestinal mucosa (protein 0·080 mg mL^{-1}, 150μL), lung (protein 0·6 mg mL^{-1}, 150 μL), scrotal skin (protein 0·08 mg mL^{-1}, 100 μL), and brain (0·16 mg mL^{-1}, 100 μL)) were incubated, in triplicate, with [11,12-^{3}H]retinoic acid (3·0 μM, 10 μL), NADPH (2 mM, 50 μL), ethanol (10 μL), and phosphate buffer (50 mM, pH 7·4, final volume 400 μL) at 37°C for either 15 min (liver and kidney) or 60 min (intestine, lung, skin and brain). The reaction was terminated by addition of formic acid (1% (v/v), 0·1 mL) and then extracted into ethyl acetate containing 0·05% (v/v) butylated hydroxyanisole (2 × 2 mL).

The extract was evaporated to dryness in-vacuo at room temperature and the residue dissolved in the mobile phase used for reversed-phase HPLC (high-performance liquid chromatography).

The HPLC method used was based on that of Roberts et al (1979, 1992) and Van Wauwe et al (1992) but was less time-consuming. Compounds were separated on a 300 mm × 3·9 mm i.d., 10-μm particle, C_{18} μBondapak column (Millipore) with acetonitrile–water–formic acid, 75 : 25 : 0·05 (v/v), containing 10 mM ammonium acetate, as mobile phase. The flow rate was 1·2 mL min^{-1} and eluted ^{3}H compounds were detected on-line by use of a Reeve model 970 detector, by use of Hisafe II

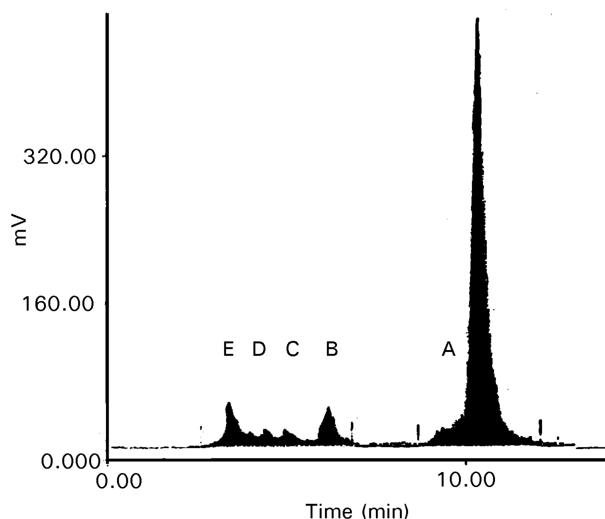

Figure 1. Chromatogram of metabolites of retinoic acid from male rat liver microsomal enzymes: A. retinoic acid; B. 4-hydroxyretinoic acid; C, D. unknown metabolites; E. 4-ketoretinoic acid.

scintillation fluid (optiphase). Under these conditions the retention times of [11,12-^{3}H] retinoic acid, 4-hydroxyretinoic acid and 4-ketoretinoic acid were 10, 6 and 3·3 min, respectively; the retention times of two minor unknown metabolites were 4·8 and 4·3 min. 4-Hydroxyretinoic acid and 4-ketoretinoic acid were identified by use of the appropriate standard compounds (Figure 1). The percentage metabolism of retinoic acid was calculated from peak areas by use of equation 1.

$$\text{metabolism (\%)} = [(\text{area of metabolite peaks})/(\text{area of metabolite and retinoic acid peaks})] \times 100 \quad (1)$$

Validation of the assay
By use of a range of protein concentrations the assays were shown to be linear for protein concentrations up to 0·240 mg mL^{-1} (liver), 0·6 mg mL^{-1} (kidney), 0·15 mg mL^{-1} (intestinal mucosa), 0·8 mg mL^{-1} (lung), 0·24 mg mL^{-1} (brain) and 0·120 mg mL^{-1} (skin). By use of a range of incubation times the assays were shown to be linear for time periods up to 20 min (liver), 40 min (kidney), 75 min (intestinal mucosa), 75 min (lung), 60 min (brain) and 75 min (skin).

The optimum pH for the assay was determined by use of phosphate buffer (50 mM), prepared from disodium hydrogen orthophosphate dihydrate and monosodium dihydrogen orthophosphate dihydrate and adjusted to pH 6·5–10·0. The optimum pH for rat liver microsomes was 7·4.

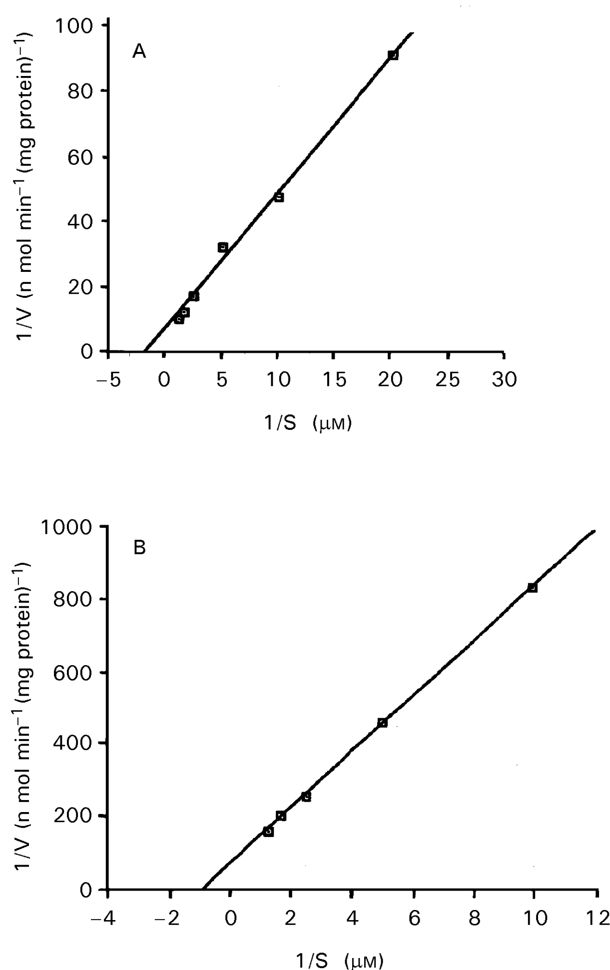

Figure 2. Representative Lineweaver-Burk plots for metabolism of retinoic acid by A. male rat liver microsomes and B. male rat brain microsomes. Each point was the mean from three determinations, each with triplicate tubes.

The optimum temperature for the assay was studied over the range 4–40°C and found to be 37°C.

Kinetic parameters

By use of substrate concentrations from 0·1 to 0·8 μM, V_{max} and K_m values were determined for the individual tissue microsomes from Lineweaver-Burk plots of 1/v against 1/s (Figure 2). Mean values for three determinations, each with triplicate tubes, are shown in Table 1.

Involvement of P450(s) in metabolism

Experiments were conducted, in triplicate tubes, by the general assay procedure, to show that the metabolism of retinoic acid (3 μM) required rat liver microsomes (0·120 mg mL^{-1}, 10 μL) and NADPH (2 mM, 50 μmL), and that the metabolism involved P450(s)—it was shown that metabolism was inhibited when carbon monoxide was bubbled through the mixture at the outset (Table 2).

Results

Involvement of P450 in metabolism

The requirements for metabolism of retinoic acid by rat liver microsomes and the extent of non-enzymatic production of retinoic acid metabolites during the assay were determined. Table 2 shows there was no detectable metabolite formation in the incubation mixture in the absence of enzyme and NADPH; only a very small amount of metabolism occurred in the absence of NADPH or with the heat-denatured enzyme. Carbon monoxide greatly reduced the capacity of the assay mixture to metabolize retinoic acid.

Cellular location of enzyme activity

The various sub-cellular fractions from rat liver were adjusted to a volume of 5 mL and a sample (10 μL) was assayed by standard methods. The amounts of metabolism by the 10 000 g supernatant, the 100 000 g supernatant and the 100 000 g pellet were 19·7, 3·7 and 27·6%, respectively.

Table 1. In-vitro metabolism of retinoic acid by microsomes from different rat tissues.

Source of microsomes	Weight of organ (g)[a]	Vmax (pmol (mg protein)$^{-1}$ min^{-1})	K_m (μM)
Liver	9·4	102 ± 39·0	0·51 ± 0·1
Skin	2·6	50·0 ± 2·8	1·4 ± 0·15
Kidney	2·5	17·0 ± 2·0	0·48 ± 0·05
Brain	2·0	14·5 ± 0·7	1·14 ± 0·08
Intestinal mucosa	1·8	4·0 ± 0·3	0·49 ± 0·07
Lung	1·6	1·0 ± 0·03	0·50 ± 0·05

[a]Average weight of rat 250 g. Results are means ± s.d., n = 3.

Table 2. Involvement of P450 in the metabolism of retinoic acid.

Incubation mixture	Amount of metabolites formed (%)
Retinoic acid + phosphate buffer	0
Retinoic acid + rat liver microsomes + phosphate buffer	2·96 ± 0·15
Retinoic acid + heat denatured rat liver microsomes + NADPH + phosphate buffer	2·43 ± 0·51
Retinoic acid + rat liver microsomes + NADPH + phosphate buffer (solution bubbled with carbon monoxide)	5·26 ± 0·25
Retinoic acid + rat liver microsomes + NADPH + phosphate buffer	26·03 ± 0·25

Results are means ± s.d., n = 3.

Table 3. Retinoic acid metabolites produced by microsomes from different rat tissues.

Tissue	Peak area as a percentage of the total		
	4-Hydroxyretinoic acid	Unknown metabolites	4-Ketoretinoic acid
Liver	38·0	24·5	37·5
Skin	40·5	15·5	44·0
Kidney	47·8	19·5	32·7
Brain	63·8	20·0	16·2
Intestinal mucosa	22·6	23·2	54·2
Lung	24·2	15·8	60·0

Results are means, n = 3.

These results show that retinoic acid metabolism in rat liver is mainly microsomal.

In-vitro metabolism by different rat tissues
Retinoic acid metabolism occurred in microsomes from all tissues examined although levels of activity varied (Table 1). Liver microsomes (V_{max} 102 ± 39 pmol (mg protein)$^{-1}$ min^{-1}) were the most active and lung microsomes the least active ($1 \pm 0·03$ pmol (mg protein)$^{-1}$ min^{-1}).

Comparison of the retinoic acid-metabolizing patterns of the different tissues
A quantitative comparison of the amounts of the retinoic acid metabolites 4-hydroxyretinoic acid, 4-ketoretinoic acid, and two unidentified metabolites, as determined by HPLC analysis, is presented in Table 3. The highest values for 4-ketoretinoic acid were obtained for lung (60%) and intestinal mucosa (54%); the lowest were obtained for brain microsomes (16·2%).

Discussion

It was shown (by the requirement for NADPH and the inhibition by carbon monoxide) that the oxidation of retinoic acid in rat liver microsomes is mediated by cytochrome P450; this is in accord with previous findings (Van Wauwe et al 1994; Napoli 1996).

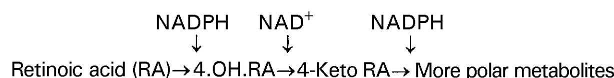

Figure 3. The metabolism of retinoic acid.

This study showed that retinoic acid was metabolized by all the tissues—all produced the same number of radioactive HPLC peaks. Quantitatively, however, there were noticeable differences (Table 3).

Roberts et al (1980), Frolik (1981) and Van Wauwe et al (1994) have suggested that metabolism by hamster liver microsomes proceeds as in Figure 3. Retinoic acid is initially hydroxylated at the C-4 position of the cyclohexenyl ring by an enzyme with properties similar to the P450-mediated monooxygenase system. According to Roberts et al (1980) the second step of the sequence, conversion of the 4-hydroxyl group to a 4-keto group, can be characterized as a dehydrogenase reaction in that NAD^+ is the required cofactor. The final reaction of the sequence, metabolism of the 4-keto derivative to metabolites that seem, in HPLC analysis, to be more polar, seems again to have the characteristics of a P450-type enzyme system (Van Wauwe et al 1994).

Rat liver microsomes isomerize all-*trans*-or 13-*cis*- or 9-*cis*-retinoic acid into the other two isomers, the all-*trans* form being preferentially converted to the 13-*cis* form. The relevant 4-oxo metabolites are also observed and are interconvertible (Marchetti et al 1997). In the assay method described here isomerization to other retinoic acid forms would not be included in the metabolite titre, because all three isomers would appear in the retinoic acid HPLC peak (cf. Marchetti et al 1997). The 4-oxo metabolites of the 13-*cis* and 9-*cis* forms would be included, because these occur on the chromatogram near the all- *trans* form. Another noted metabolite (Fiorella & Napoli 1994) is the 18-hydroxy compound. Although in rat lung and liver this constitutes only 9% and 7%, respectively, of the 4-hydroxy and 4-keto titre it occurs to a greater extent (25%) in the testis.

In this work quantitative analysis using the areas under HPLC peaks showed that greater amounts of 4-ketoretinoic acid were produced by lung tissue (60%) and intestinal mucosa (54·2%) than by the other tissues; the value for brain (16·2%) was the lowest. This might indicate that the dehydrogenase activity responsible for conversion of 4-hydroxyretinoic acid to 4-ketoretinoic acid is greater in the lung and the intestinal mucosa than in the other tissues, a conclusion supported by the smaller amounts of 4-hydroxyretinoic acid produced by these two tissues (24·2 and 22·6% respectively), and the higher level (63·8%) in the brain. The levels of the unknown metabolites were small and within a narrow range (15·8–24·5%) and it is unlikely that this P450-mediated step need be considered in the above context.

Liver microsomes seem to have the highest P450 activity, as might be expected from its important role in metabolism. Extrapolating this work to the in-vivo situation, activity (expressed as $V_{max} \times$ tissue weight; Table 1) suggests that the ranking for metabolism of retinoic acid is liver $\gg$ skin $=$ kidney $>$ brain $>$ intestinal mucosa $\gg$ lung.

Compounds that delay the in-vivo breakdown of retinoic acid, by inhibiting retinoic acid metabolizing enzymes, leading to enhancement of endogenous retinoic acid levels, are potentially valuable therapeutic agents in the control of the growth of cancer cells. This work indicates that for male rat microsomes cellular endogenous retinoic acid levels in skin, kidney and brain, or those apparent after retinoic acid administration, could be elevated or maintained, respectively, by specific inhibitors of retinoic acid metabolism.

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