IN VITRO METABOLISM OF THREE MAJOR ISOMERS OF RETINOIC ACID IN RATS
Intersex and Interstrain Comparison

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(Received August 2, 1996; accepted January 23, 1997)

ABSTRACT:
Cytochrome P450 expression in liver is influenced by several factors, including sex and strain. Whereas little is known about their metabolic capabilities, Hairless rats are widely used for the studies of topical agents. We compared Sprague-Dawley and Hairless rat metabolic behavior to validate the use of Hairless rats in pharmacokinetic and metabolic studies of topically applied drugs. Liver microsomes of male and female rats of both strains were used to investigate the in vitro metabolism of three retinoic acid (RA) isomers: all-trans-RA, 13-cis-RA, and 9-cis-RA. In all cases, a major isomerization of the tested isomer in the two others was observed. This process was independent of the presence of NADPH, but depended on the presence of microsomal proteins. In addition, we observed, to a lesser extent, the formation of 4-oxo metabolites of RA, an active metabolite of retinol (vitamin A) that plays a role in growth and differentiation of epithelial tissues (1, 2). However, RA potency is attenuated by its rapid metabolism and degradation (3–5).

RA is an active metabolite of retinol (vitamin A) that plays a role in growth and differentiation of epithelial tissues (1, 2). However, RA potency is attenuated by its rapid metabolism and degradation (3–5). One important route of RA metabolism consists of hydroxylation at position 4 of the cyclohexenyl ring to form 4-hydroxy-RA, which is readily oxidized to 4-oxo-RA. Other metabolic pathways of RA include isomerization, decarboxylation, and glucuronidation processes (6–11).

All-trans-RA, 13-cis-RA, and 9-cis-RA are three stereoisomers resulting from a direct interconversion that could be mediated by thiol groups of microsomal proteins (12–14). The structures of the three stereoisomers studied and their proposed metabolic pathways are shown in Figs. 1 and 2 (refs. 11, 15–19 contained therein).

In vitro, 4-hydroxylation of RA is mediated by a CYP-dependent monooxygenase system that requires NADPH and oxygen (8, 10, 19). RA hepatic 4-hydroxylation has been shown to be catalyzed in vitro by different CYP subfamilies according to the species studied. The main isozymes involved in humans, rabbits, and rats are, respectively, CYP2C8 (20), CYP2B4 and CYP1A2 (21), and CYP3A (22).

The present study was undertaken to investigate the potential difference in RA metabolism in rats according to sex and to strains studied. Because 9-cis-RA is a new retinoid that has yet to be commercialized, few studies are available on its metabolism, and none compares the biotransformation of the three isomers: 9-cis-RA, 13-cis-RA, and all-trans-RA. Sprague-Dawley rats are, with Wistar rats, the main strains used in vivo and in vitro to study the metabolism of xenobiotics. Due to their lack of hair, Hairless rats are widely used in dermatological studies, but little is known about their enzymatic equipment. The Fuzzy rat, which is derived from the Wistar Furth rat, has the appearance of a Hairless rat. Cherithandam et al. (23) examined the differences in monooxygenase activities between Fuzzy and Sprague-Dawley rats, and described variations in the catalytic activities and in the immunocinematic reactivities of the major forms of hepatic microsomal CYPs. Because hairless rats are mutants of the Sprague-Dawley strain, we compared their metabolic capabilities to validate their use in dermatological, pharmacological, and toxicological studies after topical treatment.

Materials and Methods

Chemicals. 13-cis-RA, all-trans-RA, 9-cis-RA, and the corresponding 4-oxo-RAs were kindly provided by Hoffmann-La Roche (Basel, Switzerland). NADPH (type I, sodium salt) and DMSO were purchased from Sigma Chemical Co. (St. Quentin Fallavier, France). Other chemicals were obtained from commercial sources and were of analytical or HPLC grade.

Animals and Preparation of Microsomal Fractions. Male Sprague-Dawley and Hairless rats weighing 240–259 g and female Sprague-Dawley and Hairless rats weighing 200–219 g were obtained from Iffa Credo (l’Arbresle, France). Five animals were used for each sex and strain. A...
standard diet and water were supplied *ad libitum*. After sacrifice of the animals, livers were removed, pooled, and immediately stored at −80°C until microsome preparation. Microsomal fractions were prepared in Tris buffer (0.1 M; pH 7.4) by differential ultracentrifugation as previously described (24, 25).

**Laboratory Precautions.** Because of retinoid photosensitivity, all experiments were performed under dim yellow light. Samples and reference compounds were stored at −20°C or +4°C.

**RA Isomer Metabolism.** Microsomes (1 mg microsomal protein/ml) were incubated at 37°C in 0.005 M MgCl₂:0.1 M Tris HCl buffer (pH 7.4, final volume of incubation = 500 μl) with two concentrations of the three RA isomers (1 and 100 μM) in 0.5% DMSO. This concentration of solvent had no effect on enzymatic activity. After a 5-min preincubation, the reaction was initiated by the addition of NADPH (final concentration = 1 mM). The reaction was terminated after 30 min with the addition of a 2 ml mixture of diethyl ether:ethyl acetate (50:50, v/v). Supernatant fluids were evaporated to dryness under a stream of nitrogen and then dissolved in 50–100 μl methanol for HPLC analysis. Three sets of duplicate experiments were performed.

**HPLC Analysis.** Analytical procedure was adapted from that of Disdier et al. (26). Reversed-phase HPLC analysis was performed on a Hewlett-Packard 1090 system equipped with an automatic injector and an UV-detector (HP 1050). Samples were analyzed on a Novapak C₁₈ steel cartridge column (4.6 × 250 mm i.d.) with 4 μm particles (Waters, Millipore S.A.). Elution was conducted at 1 ml/min by using a methanol:acetonitrile:tetrahydrofuran (65:35:5, v/v) acidified water (2% acetic acid) mixture delivered along a 25% to 12% water gradient over 25 min. Standards were detected at 350 nm. The chromatograms obtained in these conditions are shown in fig. 3.

**Enzymatic Activities.** Microsomal protein concentrations were determined by the method of Bradford (27), using bovine serum albumin as standard (Bio-Rad protein assay kit; Bio-Rad, Richmond, CA). Total CYP and β concentrations were measured according to Omura and Sato (28). EROD and PROD activities were performed on a SFM 25 Kontron spectrophuorimeter using a standard curve (29, 30). Para-nitrophenol hydroxylation was assayed by the modification of the Reinke and Moyer (31) and Tassaneeyakul et al. (32) methods. Aminopyrine and erythromycin N-demethylase activities were determined by colorimetric estimation of the formaldehyde formed. Experiments were performed in triplicate.

**Western Blot Analysis.** Microsomal samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel. The antibodies used and raised in rabbit or in sheep, were anti-rat CYP2B1/2, CYP2E1,
CYP3A (ECL Western blotting kits; Amersham, Arlington Heights, IL), and CYP1A2 (Valbiotech, Paris, France). Positive controls were used. Detection was performed using a chemiluminescence system. After autoradiography, the protein level was estimated by scan densitometry.

**Statistical Analysis.** Results are expressed as the mean \( \pm \) SD. The statistical significance of differences between male and female or Sprague-Dawley and Hairless rats was evaluated by the Kruskal-Wallis test. A \( p \) value of 0.05 or less was considered statistically significant.

**Results**

**RA Metabolism.** CYP-dependent metabolism of RA characterized by the formation of 4-hydroxy metabolites was measured by the presence of 4-oxo compounds in the incubation mixture. Indeed, 4-hydroxy-RA was readily oxidized to 4-oxo-RA in a non-P450-mediated process and was then not detected after HPLC analysis.

**Fig. 3.** HPLC profiles of (A) standard solutions of the three RA isomers and their corresponding 4-oxo metabolites and (B) compounds recovered after 1 \( \mu \)M incubation of 13-cis-RA with male Sprague-Dawley hepatic microsomes.

1, 13-cis-RA; 2, all-trans-RA; 3, 9-cis-RA; 4, 4-oxo-all-trans-RA; 5, 4-oxo-13-cis-RA; 6, 4-oxo-9-cis-RA; 7, 13-cis-acitretin (internal control); 8, acitretin (internal control).
Whatever compound we used (all-trans-RA, 13-cis-RA or 9-cis-RA), we detected the 4-oxo metabolites and the two isomers of the tested compound (figs. 4 – 6).

The isomerization process was studied: 1) in the absence of microsomes and NADPH; 2) with microsomes, but without NADPH; and 3) with microsomes and NADPH. As shown in fig. 7, for the three isomers tested, isomerization was negligible when the retinoid was incubated at 37°C without microsomes and NADPH. When the incubation was achieved in presence of microsomes, a strong isomerization was observed in the three cases, preferentially to the all-trans form when a cis-isomer was incubated and to the 13-cis form when all-trans-RA was incubated. Isomerization was quantitatively more important with, in descending order, 13-cis-RA, 9-cis-RA, and all-trans-RA. When NADPH was added in the incubation mixture, the same results were obtained.

In the presence of this cofactor, the oxidative process led to the formation of 4-oxo metabolites. All-trans-RA was the least metabolized isomer. When incubation was achieved at 100 μM, the main compound produced was its 13-cis isomer. Among the 4-oxo metabolites, the best represented was the 4-oxo-all-trans-RA, but the quan-

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FIG. 4. Metabolic profile of 13-cis-RA in hepatic microsomes of Sprague-Dawley (SD) and Hairless rats after incubation with (A) 100 μM and (B) 1 μM 13-cis-RA.

Values represent means ± SD from three sets of duplicate determinations. Significant difference from male values was estimated by the Kruskal-Wallis test (*p < 0.05; **p < 0.01).
tities obtained did not exceed 20 pmol/min/mg of microsomal proteins. At 1 μM, the metabolic profile was similar. There was a slight but statistically significant difference between the two sexes at 100 μM (p < 0.05). At 1 μM, 4-oxo-13-cis-RA, and 4-oxo-9-cis-RA were not detected in female rats. No significant difference was observed between the two strains of rats for the two retinoid concentrations studied.

13-cis-RA was the most metabolized isomer. After an incubation at 100 μM, the main 4-oxo metabolite found was the 4-oxo-9-cis-RA, but all-trans-RA was quantitatively the major compound recovered in the incubation mixture. There was a 10-fold factor between 13-cis-RA and all-trans-RA metabolism, because the rate of formation of the main 4-oxo metabolite was >200 pmol/min/mg protein. We observed an identical metabolic profile after 1 μM incubation. With this isomer, there seemed to be a marked sexually dimorphic metabolism, because values obtained for metabolites in male rats were higher (or even double) than those obtained in females. Similar results were obtained for Sprague-Dawley and Hairless rats.

When 9-cis-RA was incubated, we did not find all the 4-oxo metabolites. We again observed a quantitative difference in the me-

Fig. 5. Metabolic profile of 9-cis-RA in hepatic microsomes of Sprague-Dawley (SD) and Hairless rats after incubation with (A) 100 μM and (B) 1 μM 9-cis-RA.

Values represent means ± SD from three sets of duplicate determinations. Significant difference from male values was estimated by the Kruskal-Wallis test (*p < 0.05; **p < 0.01).
tabolism according to sex ($p < 0.01$), but not with rat strain. After 100 µM incubation, we detected the 4-oxo-9-cis and 4-oxo-all-trans metabolites. No 4-oxo-13-cis-RA was obtained neither in males nor in females. After 1 µM incubation, only the 4-oxo-9-cis-RA was recovered. In all of the cases, the main compound observed was all-trans-RA.

**Total CYPs and Cytochrome b$_5$ Levels.** Total CYP contents measured in Sprague-Dawley and Hairless rats were significantly higher in male than in female for both strains ($p < 0.01$). The same result was obtained for the cytochrome b$_5$ levels (table 1).

**Liver Enzymatic Activities.** As shown in table 1, for four of the five activities tested (PROD, erythromycin N-demethylase, aminopyrine N-demethylase and $p$-nitrophenol hydroxylase activities), the values obtained were statistically higher in males than in females of the two strains of rats ($p < 0.01$). In contrast, EROD was the only activity higher in female than in male rats of the two strains studied. In all cases, no major difference was observed between Sprague-Dawley and Hairless rats for the tested activities.

**Western Blot Analysis.** Western blot analysis performed on liver microsomes showed that three of the four CYP subfamilies studied

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*Fig. 6. Metabolic profile of all-trans-RA in hepatic microsomes of Sprague-Dawley (SD) and Hairless rats after incubation with (A) 100 µM and (B) 1 µM all-trans-RA.*

Values represent means ± SD from three sets of duplicate determinations. Significant difference from male values was estimated by the Kruskal-Wallis test ($^*p < 0.05; ^{**}p < 0.01$).
(CYP2B, CYP2E, and CYP3A) were more expressed in male livers than in female livers in both Sprague-Dawley and Hairless rats. For CYP1A, the opposite result was obtained (fig. 8). These results were in agreement with those obtained with liver enzymatic activities (table 1).

**Discussion**

One of the more significant pathways in the biotransformation of RA includes C4-hydroxylation and the further metabolism to 4-oxo-RA. Indeed, 4-oxo metabolites are more polar, less reactive, and more rapidly eliminated than RA.

However, we demonstrated that in vitro, after the incubation of one of the three RAs studied, the main compound recovered in the incubation mixture was one of the two other isomers and not the 4-oxo metabolites. The mechanism of isomerization remains relatively unknown, although several studies have been performed. It would seem that isomerization of RA could be a nonenzymatic process. Indeed, isomerization is still important if boiled microsomes are used for the incubation of RA (11). Moreover, the isomerization process is nonstereospecific, because 13-cis and 9-cis-RA are both obtained after all-trans-RA incubation (13). From our own results, we could only say that isomerization reactions are NADPH-independent, but require the presence of microsomes (fig. 7). We noted that, both in male and female Sprague-Dawley and Hairless rats, more RA was isomerized from the cis form to the all-trans form than vice versa. Sass et al. (11) obtained the same result with male Wistar rats. This is compatible
with the thermodynamic stability of retinoid isomers, with the all-
trans molecules being more stable than the corresponding cis-isomers (33, 34). Some authors advocate the hypothesis that isomerization is catalyzed by sulfhydryl groups present in the proteins of the micro-
somal preparation that are mostly oxidized during the heating process. Catalysis could occur by a free radical mechanism rather than a Michaelis addition of a thiolate anion (12–14).

RA metabolism is complicated by many isomerization reactions that occur at different stages of the biotransformation pathway. The natural metabolite of each RA isomer is the corresponding 4-oxo compound. But interconversion of all-trans-, 13-cis- and 9-cis-RA isomers leads to the formation of the two other 4-oxo metabolites. Furthermore, each 4-oxo compound can produce the two others by an isomerization process. This explains why, after incubation of one of the three RA isomers with hepatic microsomal fractions, we were able to recover the three 4-oxo metabolites in the incubation mixture.

RA metabolism characterized by C4-hydroxylation has been studied predominantly in hepatic microsomes from several mammalian species, including hamsters, rats, rabbits and humans. Martini and Murray (22) demonstrated that the CYP3A subfamily is involved in RA hydroxylation in untreated rat liver. But, the enzymatic activity is neither related to the rat CYP3A1 isozyme that is not expressed in untreated rat liver, nor to the rat CYP3A2 isozyme that is the constitutive male-specific steroid 6β-hydroxylase. They described a sex-
related difference in metabolism of all-trans-RA in Wistar rats (male > female) that is in agreement with our results in Sprague-
Dawley and Hairless rats. This sex difference in RA metabolism can be explained by the existence of a sexually dimorphic expression of P450 genes (35). Some of the hepatic CYP450 isofoms are expressed in age-and/or sex-related manners in the livers of experimental ani-
mals, especially in rats (36, 37). These differences seem to be gov-
erned by sex steroids and other hormones. Kato (36) showed that gonadal hormones are involved in the sex-related difference and that androgens play a major role in the sex-related expression of the drug-metabolizing activities. Growth hormone seems to be involved in gender-related difference in CYP450 expression, too. The male pituitary secretes growth hormone episodically, whereas the female pituitary secretes the hormone continuously (38). This sexually differ-
entiated secretory rhythm is proposed to be the mechanism that determines the phenotypes of the sex-specific P450.

Kato and Yamazoe (39) listed the sex-related differences in the hepatic levels of P450. They made a distinction between male-specific P450 isozymes (CYP2A2, CYP2C11, CYP2C11, CYP2C13, CYP2C22, and CYP3A2) with a hepatic level 10- to 20-fold higher in male than in female rats, male dominant P450 isozymes (CYP2B1, CYP2B2, and CYP3A1) whose expression is 2- to 5-fold higher in male rats than in female rats, female-specific P450 isoform (CYP2C12) with a hepatic level 20-fold higher in female than in male

### TABLE 1

**Liver enzymatic activities according to sex in Sprague-Dawley and Hairless rats**

<table>
<thead>
<tr>
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<th>Sprague-Dawley Rats</th>
<th>Hairless Rats</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>CYP P450s (nmol/mg protein)</td>
<td>0.59 ± 0.02</td>
<td>0.48 ± 0.01**</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</td>
<td>0.81 ± 0.001</td>
<td>0.61 ± 0.009**</td>
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<tr>
<td>EROD (pmol/min/mg protein)</td>
<td>101.1 ± 19.76</td>
<td>182.54 ± 6.05</td>
</tr>
<tr>
<td>PROD (pmol/min/mg protein)</td>
<td>23.04 ± 0.16</td>
<td>10.4 ± 0.41**</td>
</tr>
<tr>
<td>Aminopyrine N-demethylase (nmol/min/mg protein)</td>
<td>3.45 ± 0.14</td>
<td>2.49 ± 0.009**</td>
</tr>
<tr>
<td>Erythromycin N-demethylase (nmol/min/mg protein)</td>
<td>5.79 ± 0.26</td>
<td>4.4 ± 0.18**</td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase (pmol/min/mg protein)</td>
<td>0.87 ± 0.04</td>
<td>0.51 ± 0.05*</td>
</tr>
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</table>

Each point represents the mean ± SD of three independent experiments. Statistical significance was estimated by the Kruskal-Wallis test: *p < 0.05, compared with male rat group; **p < 0.01, compared with male rat group.
rats, and female dominant P450 isozymes (CYP1A2, CYP2A1, CYP2C7, and CYP2E1) whose level is 1.5- to 2-fold higher in female rats than in male rats.

We observed that enzymatic activities related to CYP2B, CYP2C, CYP2E, and CYP3A were significantly higher in male rats than in females in both strains of rats. This sex difference in enzymatic activity was related to a sex difference in CYP isoform expression (table 1, fig. 8). Our results are in agreement with those described by Kato and Yamazoe (39), except for CYP2E1 whose expression was found higher in both Sprague-Dawley and Hairless male rats. CYP3A, which is probably the most involved enzyme in the in vitro rat hepatic RA metabolism, is significantly more expressed in the livers of males than in the livers of female rats (p < 0.05). On the other hand, no statistically significant difference was observed for the enzymatic activity related to this isozyme nor for RA metabolism between the two strains of rats studied. CYP1A, which is more expressed in female rats than in male rats, was unlikely to be involved in RA biotransformation, which is greater in male rats than in female rats (table 1, fig. 8).

According to the results shown in figs. 4-6, the three RAs studied are not metabolized to the same extent. 13-cis-RA is the most hydroxylated isomer in the two strains of rats, both in males and females. When the incubation was achieved at 100 μM, the least metabolized isomer was the all-trans-RA. Why cis-isomers are more metabolized than trans-isomers is not well understood. The configuration of the molecules is certainly responsible for this difference in the in vitro metabolism. Perhaps the cis-position allows a stronger binding of the substrate to the active site of the P450 enzyme. Another explanation for this substrate configuration-dependent metabolism can be found in the nature of the isoform involved. The CYP450 isoform that metabolizes cis- and trans-RA-isomers can be different as shown with isomers of retinal. Indeed, Raner et al. (40) demonstrated that in vitro 4-hydroxylation of 13-cis- and all-trans-retinal was predominantly catalyzed by the CYP1A1 isoform, whereas CYP2B4 and CYP2C3 are most active in the metabolism of 9-cis-retinal. Further experiments will be needed to confirm one of these hypotheses about the oxidative metabolism of RA isomers.

Concerning the metabolic comparison between Sprague-Dawley and Hairless rats, we can say that because no statistical difference was observed in RA biotransformation and that no major difference was obtained in the enzymatic activities and in the immunochromatographic determination of CYP isoforms expression, the use of Hairless rats in studies involving metabolism of drugs and other chemicals after cutaneous exposure is justified. An opposite result was obtained by Cheriathundam et al. (23) with Sprague-Dawley and Fuzzy rats. They described remarkable variations in the catalytic activities and to some extent in the immunochromatographic reactivities of the major forms of hepatic microsomal CYPs. The explanation of the discrepancies observed in these two studies may be found in the genetic origin of the rats. Cheriathundam et al. compared Sprague-Dawley and Wistar mutant (i.e. Fuzzy rats) rats, whereas we compared Sprague-Dawley and Sprague-Dawley mutant (i.e. Hairless rats) rats.

In summary, the present study using Sprague-Dawley and Hairless rats shows that the metabolism of RA isomers varies with sex, but not within the two strains of rats. The demonstration that Sprague-Dawley and Hairless rats are comparable in terms of retinoid metabolism and expression of main CYP isoforms strengthens the relevance of the use of Hairless rats instead of Sprague-Dawley or Wistar rats whose use is complicated by the presence of hair. This provides some validation of the use of Hairless rats in pharmacokinetic studies after topical application or in metabolic studies during transcutaneous migration of drugs, including retinoids.

References


