Identification of 5,6-Epoxyretinoic Acid as an Endogenous Retinol Metabolite*

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Rats on a normal diet were administered physiological doses of [3H]retinyl acetate or [3H]retinol orally for 5 days to label endogenous retinoid pools. The kidney retinoids were extracted and separated by DEAE-Sephadex into neutral and acidic fractions. All-trans-retinoic acid and 5,6-epoxyretinoic acid were isolated and unequivocally identified by chromatographic analysis, chemical derivatization, and mass spectroscopy. The identities of retinol and retinyl palmitate were confirmed by high performance liquid chromatography and reactivity with trifluoroacetic acid. Control experiments showed that retinoid epoxidation truly occurred in vivo. The specific radioactivities of the recovered acidic retinoid metabolites were similar to those of the recovered neutral retinoids. Thus, retinoic acid and its metabolite 5,6-epoxyretinoic acid were endogenous rat kidney retinoids which are in the pathway of retinol metabolism under physiological conditions. The concentrations of retinyl palmitate (8.7 μM), retinol (4.8 μM), all-trans-retinoic acid (1.3 μM) and 5,6-epoxyretinoic acid (0.25 μM) measured indicate that acidic retinoids are comparatively significant vitamin A metabolites in kidney.

Retinoic acid is an intermediate in retinol metabolism which can satisfy the function of vitamin A in epithelial differentiation but not in vision or mammalian reproduction (1–4). In fact, retinoic acid is an order of magnitude more potent than retinol in suppressing keratinization and in reversing the squamous metaplastic lesions formed in cultured trachea of vitamin A-deficient hamsters (5). Retinoic acid is three orders of magnitude more potent than retinol in inducing differentiation of embryonal carcinoma cells (6). These data support the supposition that retinoic acid, or a retinoic acid metabolite, rather than retinol, modulates epithelial differentiation.

Progress in elucidating retinoic acid metabolism was accelerated by the introduction of HPLC systems capable of efficiently resolving retinoids (7–11). With the aid of these systems, 5,6-epoxyretinoic acid was the first target-tissue metabolite of retinoic acid to be identified (12–14). This metabolite was initially isolated from the intestinal mucosa of vitamin A-deficient rats given pharmacological doses of retinoic acid. Further work resulted in the detection of transient levels of tritiated 5,6-epoxyretinoic acid in the liver, kidney, and intestinal mucosa of vitamin A-deficient rats dosed with physiological amounts of tritiated retinoic acid (15). However, steady state levels of the metabolite were not observed. Since 5,6-epoxyretinoic acid possesses potent retinoid activity in vivo (16–19), a closer examination of its in vivo occurrence is important. This study will demonstrate that 5,6-epoxyretinoic acid, like retinoic acid, occurs as a metabolic product of retinol in kidney under physiological conditions. Moreover, the steady state concentrations of retinoic acid and 5,6-epoxyretinoic acid in kidney of rats fed a stock diet have been determined and compared to those of retinol and retinyl palmitate.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Identification of 5,6-Epoxyretinoic Acid—5,6-Epoxyretinoic acid was detected in each of the three experiments conducted. The isolated metabolite was homogeneous after chromatography on column 4 (Fig. 1). The height of the epoxide peak on column 4 was compared to the peak heights of six 5,6-epoxide standards, in the range of 25 to 250 ng, injected immediately after the biological materials were collected in each experiment. The relationship between peak height (centimeters) and mass of injected standards was linear with a slope of 20 and a correlation coefficient, r, of 0.99. The mean amount (±S.D.) of 5,6-epoxyretinoic acid recovered in the three isolation experiments was 105 ± 5 ng. Fifty nanograms of the metabolite isolated in experiment 2 were analyzed by mass spectroscopy. A molecular ion at m/e 316 and a fragmentation pattern characteristic of 5,6-epoxyretinoic acid was observed (14, 22).

The metabolite recovered from column 4 in experiment 3 was methylated and further purified by normal phase HPLC (column 5). Fifty nanograms of the methylated metabolite was analyzed by mass spectroscopy (Fig. 4). A typical methyl 5,6-epoxyretinolate mass spectrum was obtained (14). The molecular ion (M') at m/e 330 was the base peak and confirmed the molecular weight. The peaks at m/e 315 (M'-CH3) and 271 (M'-CO2CH3) are characteristic of methylated retinoid carboxylic acids (22).

Identification of all-trans-Retinoic Acid—The all-trans-retinoic acid peak obtained from column 2 (Fig. 1) was isolated and characterized. In both experiments, the mass of isolated...
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A)
control, the \([^{3}H]\)retinol introduced in the homogenate of
covered in the neutral fraction
clusive identity, however, remains to be determined. As
esters of retinol other than retinyl palmitate
experiment 3 was analyzed. Ninety-eight per cent was re-
second HPLC system (column
tral retinoid fraction
before the biological sample.

significant peaks at \(m/e 299\) and \(267\) with synthetic methyl retinoate obtained immediately
as expected, large peaks migrated with authentic retinol (peak

No peak corresponding to retinal was detected, However,
profile not shown). The

endogenous rat kidney

Steady state concentrations of endogenous retinoids in rat kidney
Each value represents the average of two determinations. The
concentrations calculated in each experiment agreed within 10%.

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Concentration (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinyl palmitate</td>
<td>8.6</td>
</tr>
<tr>
<td>Retinol</td>
<td>4.7</td>
</tr>
<tr>
<td>Retinal</td>
<td>N.D.</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>1.3</td>
</tr>
<tr>
<td>5,6-Epoxyretinoic acid</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* Not detected.

neutral fraction was applied to column 9, only \([^{3}H]\)retinol was observed.

Concentrations of Endogenous Kidney Retinoids—The
specific radioactivities of the retinol, retinoic acid, and 5,6-
epoxyretinoic acid isolated in experiments 1 and 2 were deter-
mmed (Table I). Notably, the degree of isotope dilution of the
three retinoids isolated was similar. The concentrations of
retinyl palmitate, retinol, all-trans-retinoic acid, and 5,6-epoxyretinoic acid were calculated (Table II). As expected, retinyl palmitate was the most predominant retinoid present during the steady state and retinol was the second most abundant (27). Unexpected, however, were the relatively high steady state levels of all-trans-retinoic acid and 5,6-epoxyretinoic acid in normal rat kidney. Micromolar concentrations of the two acidic metabolites were detected in two separate experiments. Retinoic acid was about 4-fold less concentrated than retinol and about 7-fold less abundant than retinyl palmitate. The 5,6-epoxide was approximately 19-fold and 34-fold less concentrated than retinol and retinyl palmitate, respectively.

**DISCUSSION**

The identifications of endogenous rat kidney retinoids are based on diverse evidence. Each isolated compound co-migrated with its synthetic standard on a minimum of two HPLC systems with different selectivities. In addition, the methyl esters of the acidic retinoids also co-migrated with the appropriate standards. The resolving power of these HPLC systems has been established (7, 9, 10). Moreover, the mass spectral data obtained for 5,6-epoxyretinoic acid, methyl 5,6-epoxyretinoic, and methyl retinato supported the assignments. Finally, chemical reactivity was used to substantiate identification of 5,6-epoxyretinoic acid which isomerized to the 5,8-oxide upon treatment with dilute hydrochloric acid. The identity of the neutral retinoids was confirmed by their ability to form a complex with trifluoroacetic acid yielding a chromophore with an intense absorbance at 616 nm (24, 25). Thus, the structural characterizations are firm.

The validity of the conclusion that 5,6-epoxyretinoic acid is a true *in vivo* retinol metabolite is supported by several observations. The dosed retinoids were judged to be radiochemically pure by HPLC, which eliminated 5,6-epoxidized retinoid contaminants as the source of the isolated metabolite. The 5,6-epoxide in kidney was diluted to the same specific radioactivity as retinoic acid. Thus, selective decomposition of the radiolabel can be excluded. Furthermore, tissue homogenates adulterated with titrated retinol and retinoic acid accumulated an extremely low amount of 5,6-epoxide (<1%) upon manipulation through the isolation procedure. Clearly, the majority of 5,6-epoxide detected did not arise from radiolabel contamination or experimental handling.

The specific radioactivities of retinol, retinoic acid, and 5,6-epoxyretinoic acid isolated from rats on a normal diet administered chronic, oral doses of [3H]retinol or [3H]retinyl acetate compared favorably. Each compound dosed was diluted to approximately the same degree by the kidney retinoid pool (50-60-fold). The acidic metabolites reflected the specific activities of their neutral precursors, although the pools of acidic retinoids were smaller than those of the neutral retinoids. This indicates that the titrated doses most likely equilibrated with endogenous vitamin A stores and entered the physiological route of vitamin A metabolism under normal dietary conditions. Thus, the isotope dilution results confirm the notion that retinoic acid is a normal intermediate in the pathway of retinol metabolism (28) and extend the realization to 5,6-epoxyretinoic acid.

Failure to detect 5,6-epoxyretinol implies vitamin A epoxidation *in vivo* is restricted to the metabolic pathway after retinoic acid formation. A suggested pathway of retinol metabolism in kidney, based on these data, involves oxidation of retinol to all-trans-retinoic acid followed by epoxidation to yield 5,6-epoxyretinoic acid. The question of whether alkene isomers of 5,6-epoxyretinoic acid are formed during retinoid acid metabolism, however, remains unanswered.

Recently, retinoic acid has been reported as an endogenous retinoid in blood using a gas chromatographic/mass spectral assay (29). However, the experiments were not well controlled. Indeed, subsequent work by others, using similar methodology, failed to verify the observation (30). This report demonstrates unequivocally that retinoic acid and 5,6-epoxyretinoic acid are endogenous retinoids in at least one vitamin A-target tissue under physiological conditions. Moreover, the steady state concentrations of retinoic acid and 5,6-epoxyretinoic acid measured were substantial, even when compared to the concentrations of total neutral retinoids in kidney. Therefore, our work has demonstrated that retinoic acid and its metabolite 5,6-epoxyretinoic acid are quantitatively significant *in vivo* under physiological conditions.

Unlike retinoic acid, 5,6-epoxyretinoic acid has poor growth-promoting activity in vitamin A-deficient rats (31). These results may depend upon the route of administration, the frequency of dosing, an inability of the 5,6-epoxide to accumulate in target tissues, or a short biological half-life. The brevity of the epoxide's half-life in vivo was suggested by its transient appearance after a pulse dose of [3H]retinoic acid to vitamin A-deficient rats (15). This perception is supported by the following observations. Three hours after intrajugular dosing of 1 μg of [3H]retinoic acid to vitamin A-deficient rats, 39% of the small intestinal mucosa radioactivity and 85% of the liver radioactivity is present as retinoic acid. In contrast, a similar experiment with 1 μg of [3H]epoxide showed that after 2 h, only 19% of the liver radioactivity and 3% of the intestinal radioactivity were present as unchanged epoxide. Growth rate also reflects a composite of vitamin A functions which may not be totally fulfilled by all retinoic acid metabolites (32). Therefore, it is probably imprudent to use growth-promoting ability as an exclusive measure of the vitamin A activity of 5,6-epoxyretinoic acid, and perhaps other retinoic acid metabolites as well.

In contrast to its poor growth-promoting activity, 5,6-epoxyretinoic acid stimulates embryonal carcinoma cell differentiation (17), promotes the cellular adhesion of transformed fibroblasts (16), maintains proper epithelial differentiation in cultured tracheal explants (33), and inhibits skin papilloma formation and the induction of ornithine decarboxylase activity by chemical carcinogens in mouse skin (19). However, further studies are necessary to establish what role 5,6-epoxyretinoic acid serves in vitamin A function.

This study has established that 5,6-epoxyretinoic acid is an endogenous rat kidney retinol metabolite under physiological conditions. It has also shown that retinoic acid occurs as a metabolic product of retinol in kidney under physiological conditions and has shown that the kidney concentrations of retinoic acid and 5,6-epoxyretinoic acid are substantial. This work has also demonstrated the feasibility of isolating and identifying endogenous retinoids from kidney. Similar methodology can now be applied to identifying and quantifying retinol metabolites in other tissues.

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**REFERENCES**


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EXPERIMENTAL PROCEDURES

Endogenous retinoic acid was measured as described previously (18) and modified for the measurement of 5,6-epoxyretinoic acid. The technique utilized the gas chromatographic method of Palmiter et al. (19) with the following modifications. A solution of 5,6-epoxyretinoic acid was prepared by acidification of a solution of 5,6-epoxyretinoic acid to pH 2 with 0.2 N HCl. The solution was then extracted with chloroform/methanol (1:3) and the methanol solution was dried under nitrogen. The dry residue was dissolved in hexane and the solution was then applied to a column of 5% 4,4'-diaminobenzidine on 2% DEAE-cellulose. The column was eluted with 0.1 M sodium acetate buffer (pH 5.0) and a gradient of 0.12 M sodium acetate and 0.12 M sodium citrate buffer (pH 5.0). The 5,6-epoxyretinoic acid fraction was then collected and analyzed by gas chromatography. The 5,6-epoxyretinoic acid fraction was then analyzed by gas chromatography.

The retinoic acid fraction obtained from column 2 was applied to column 3. In experiment 3, the entire retinoic acid fraction was isolated from column 2, and the total retinoic acid fraction recovered from column 2 was used for radioassay. In experiment 3, an aliquot (100 nCi) of the 5,6-epoxyretinoic acid fraction obtained from column 1 was isolated for radioassay. In experiment 2, an aliquot (100 nCi) from column 2 was isolated and the remainder was incubated with column 1. In each experiment, the aliquots applied to the chromatographic columns were counted by liquid scintillation spectrometry.

The radioactive retinoic acid fraction obtained from column 2 was applied to column 3. In experiment 1, the entire retinoic acid fraction was isolated from column 2, and the total retinoic acid fraction recovered from column 2 was used for radioassay. In experiment 2, an aliquot (100 nCi) of the 5,6-epoxyretinoic acid fraction obtained from column 1 was isolated for radioassay. In experiment 3, an aliquot (100 nCi) from column 2 was isolated and the remainder was incubated with column 1. In each experiment, the aliquots applied to the chromatographic columns were counted by liquid scintillation spectrometry.
Endogenous 5,6-Epoxyretinoic Acid

Figure 1. Isolation of endogenous 5,6-epoxyretinoic acid. Four separate experiments were performed. The animals in experiment 1 were dosed with [3H-5,6-epoxyretinoic acid]. The animals in experiment 2 were not dosed with radioactively-retinoic acid. Instead, radiotracers were added to the homogenates prepared from their kidneys.

Figure 2. HPLC analysis of isolated 5,6-epoxyretinoic acid and the 3,4-epimer. The samples were shipped from a radially-compressed reverse-phase column with 10 mM ammonium acetate in methanol/water (3/7), volume 6. The elution of 3,6-epoxyretinoic acid (100 µg isolated) from the second column (experiment 2) is indicated by the solid line. The profile of the species recovered from column 4, treated with acid and re-injected onto column 5, is represented by the dashed line. A and B indicate the elution positions of the major diastereomer of 3,6-epoxyretinoic acid and authentic 3,6-epoxyretinoic acid, respectively. The small peak which elutes prior to peak A co-migrates with the minor diastereomer of 3,6-epoxyretinoic acid.

Figure 3. HPLC analysis of the methyl ester of isolated 5,6-epoxyretinoic acid and the 3,4-epimer (hepatic). The samples were eluted from a normal-phase column with tert-butylmethylsilica (1000, column 7). The methyl ester of 3,6-epoxyretinoic acid (100 µg) isolated from liver of rats on a high dose diet (experiment 2) is indicated by the solid line. The profile of the acid-treated methyl ester water (100 µg) is indicated by the dashed line. A and B show the elution positions of authentic methyl 3,6-epoxyretinoic acid and methyl 3,6-epoxyretinoic acid (major diastereomer), respectively. The small peak which elutes after peak B co-migrates with the minor diastereomer of methyl 3,6-epoxyretinoic acid.
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