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Terminal-group oxidation of retinol by mouse epidermis

Inhibition *in vitro* and *in vivo*

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Locally applied retinol is metabolized to retinoic acid in mouse epidermis *in vivo*. To characterize the oxidation system we investigated the ability of soluble extracts of hairless-mouse epidermis to convert retinol and retinal into retinoic acid. The extracts oxidized retinol to retinoic acid in two steps catalysed by two NAD^+-dependent enzymes that were resolved on h.p.l.c. The first enzyme catalyses the reversible oxidation of retinol to retinal and is an alcohol dehydrogenase isoenzyme. The second enzyme oxidizes retinal to retinoic acid. Retinol oxidation by epidermal extracts was inhibited by the alcohol dehydrogenase inhibitor 4-methylpyrazole and by the polyene citral. The toxicity and relatively low potency at inhibiting the epidermal alcohol dehydrogenase isoenzyme curtailed the use of 4-methylpyrazole *in vivo*. However, citral significantly inhibited retinoic acid formation from retinol in the epidermis *in vivo*. The ability to inhibit the oxidation of retinol to retinoic acid in mouse epidermis provides a potential method to resolve the roles of retinol and retinoic acid in epithelial function.

INTRODUCTION

All-*trans*-retinoic acid (retinoic acid) is a vitamin A metabolite [1–3] that has received widespread attention in recent years. It exhibits almost as wide a spectrum of biological activity as all-*trans*-retinol (retinol) and has proved more potent than retinol in a variety of assay systems *in vivo* and *in vitro* [4]. Retinoic acid cannot fulfill all the requirements of the visual [5] and the reproductive [6] systems, which need retinal or retinol, but can fulfill the vitamin A requirements for growth and the maintenance of normal epithelial function [7], supporting a possible role for retinoic acid in such areas. Oxidation to retinoic acid may be required for retinol to induce the differentiation of mouse embryonal carcinoma cells [8]. However, with respect to the formation of several common metabolites from retinol and retinoic acid [9], the presence of retinoic acid glucuronides in the bile of rats fed retinyl acetate [9], and the more rapid elimination of retinoic acid from the body [7], retinoic acid behaves as a retinol elimination product. Thus retinoic acid formation may constitute the deliberate or obligatory synthesis of an active metabolite from retinol, or possibly the synthesis of a fortuitously active, more rapidly eliminated, detoxification product.

Retinoic acid is formed in mouse epidermis after local application of retinol to the skin [3]. The epidermis is a retinoid-sensitive tissue, and it and its appendages are currently the major target in the therapeutic use of retinoids. Epidermis contains high levels of cellular retinoic acid-binding protein (CRABP) [10,11], a potential mediator of retinoid action. Despite the abundance of CRABP, the endogenous epidermal retinoic acid level is very low, being less than 10 pmol/g [3,12]. We have proposed that, under such conditions, retinoic acid formation may be rate-limiting for any CRABP-mediated action in the epidermis [3].

One approach to discriminate between the roles of activation and elimination would be to utilize inhibitors of retinoic acid formation. Enzymes that can oxidize the terminal group of retinol and retinal have been found in several tissues [13], and retinol is oxidized to retinal by alcohol dehydrogenases *in vitro* [14]. In the present study we have used a simple h.p.l.c. method to resolve the oxidizing enzymes in mouse epidermis, using sensitive assays to detect the oxidizing activities based on established reverse-phase h.p.l.c. methodology to resolve the oxidation products from the substrates. We used these assays to screen potential inhibitors of retinol terminal-group oxidation. Active inhibitors were tested for their ability to inhibit retinol oxidation in mouse epidermis *in vivo*.

METHODS AND MATERIALS

Preparation of tissue extracts and assay of retinoid-oxidizing activities

Mice were killed by cervical dislocation, the dorsal skins dissected free, and the epidermis recovered by a brief heat treatment [3]. Tissues were homogenized mechanically at 4 °C with a Brinkman tissue homogenizer at setting 6 for 20 s in 10 vol. of 0.05 M-potassium phosphate buffer, pH 7.8, the homogenates centrifuged (30000 *g*, 30 min), and the supernatants (3–5 mg of protein/ml) recovered and stored at −70 °C until assayed.

Assays were performed in amber glass tubes and routinely included 0.1 ml of tissue extract, 0.05 M-potassium phosphate, pH 8, 5 mM-NAD^+, 1 mM-retinol or -retinal and bovine serum albumin (1 mg/ml) in a total volume of 0.30 ml. Reactions were initiated by adding substrate, prepared by dissolving the retinoid first in dimethyl sulphoxide and then into assay buffer containing bovine serum albumin such that the final concentration of dimethyl sulphoxide was 0.5%.

Abbreviation used: CRABP, cellular retinoic acid-binding protein.

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Inhibitor solutions were prepared in the same manner. Reaction mixtures were incubated at 37 °C for 1 h and the reaction terminated by adding 0.7 ml of ice-cold methanol containing 0.1% acetic acid. Product formation was measured by chromatographing portions on a Resolvex C_{18} column fitted with a guard column (Fisher Scientific), eluting isocratically at 3 ml/min with methanol/0.01 M-potassium acetate (4:1, v/v), pH 6.0, and detecting the retinoids from their absorbance at 340 nm [3].

Fractionation of enzyme activities and determination of M_r values

Epidermal extract (0.5 ml aliquots; 1.5–2.5 mg of protein) was applied to a TSK 125 gel-permeation column (300 mm × 7.5 mm) equipped with a guard column (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and eluted with 0.02 M-potassium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min, with eluate monitored at 280 nm. Fractions (0.5 ml) were collected and assayed for retinol- and retinal-oxidizing activity. The elution volumes of the activity peaks were used to obtain M_r values by reference to a standard curve of the elution volumes of known proteins plotted against the logarithm of their M_r values.

Metabolism studies *in vivo*

Retinol (25, 50 or 100 nmol in 0.1 ml of acetone) was applied to the dorsal skins 30 min after application of citral or 4-methylpyrazole. The mice were killed 2 h after retinol treatment, the epidermis was recovered and extracted twice with 20 vol. of chloroform/methanol (2:1, v/v) [3,19] containing butylated hydroxytoluene (0.1 mg/ml), and the retinol and retinoic acid contents determined by h.p.l.c. [3]. Extraction efficiencies, based on the recoveries of radioactivity from the epidermis of groups of four mice dosed with 100 nmol of retinol or retinoic acid to which had been added tracer amounts of the appropriate ^{3}H-labelled retinoids, were 98.4 ± 0.7% and 99.9 ± 0.2% respectively.

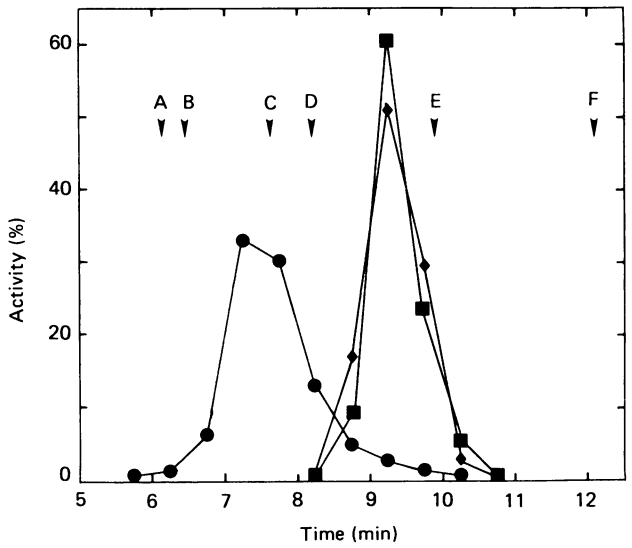

Fig. 1. H.p.l.c. of an epidermal extract on a TSK-125 column

A portion (0.5 ml) of epidermal extract (1.5 mg of protein) was eluted at a flow rate of 1 ml/min. Fractions (0.5 ml) were assayed for their ability to oxidize retinal to retinoic acid (●), to oxidize retinol to retinal (■) and to oxidize ethanol (◆). Activities are expressed as the percentage of that recovered. A, Dextran Blue (void volume); B, bovine γ-globulin (M_r 158000); C, bovine serum albumin (M_r 67000); D, ovalbumin (M_r 45000); E, carbonic anhydrase (M_r 29000); F, cytochrome *c* (M_r 12400).

Miscellaneous

Retinoids, proteins and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Skh/hrl (hairless) mice were obtained from Temple University (Philadelphia, PA, U.S.A.), [11,12-^{3}H(n)]retinol (sp. radioactivity 60 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.) and [11-^{3}H]retinoic acid (sp. radioactivity 1.51 Ci/mmol) was provided by the National Cancer Institute (Bethesda, MD, U.S.A.). Retinol was purified before use by reverse-phase h.p.l.c. on a Resolvex C_{18} column eluted with methanol/water (9:1, v/v). Retinoids were handled under darkroom lighting and their concentrations determined from their molar absorption coefficients in ethanol (for retinol, $\epsilon_{325} = 50900\ M^{-1}\cdot cm^{-1}$; for retinal, $\epsilon_{381} = 43450\ M^{-1}\cdot cm^{-1}$; for retinoic acid, $\epsilon_{351} = 45000\ M^{-1}\cdot cm^{-1}$. Alcohol dehydrogenase kinetics were determined spectrophotometrically [14]. Protein was determined by the Bradford method [16]. Because the substrates and inhibitors used are insoluble in water, data for inhibition of the retinoid-oxidizing enzymes are expressed as the inhibitor concentration decreasing product formation from 1 mM-substrate by 50% (I_{50}).

RESULTS

Product identification

Retinoic acid was identified by its co-elution on reverse-phase h.p.l.c. with an authentic standard at four different pH values [3,15], by its co-elution with the anticipated methylation product all-*trans*-methyl retinoate on methylation with diazomethane [3], and from the u.v.-absorbance spectrum of the h.p.l.c.-resolved product. Retinal and retinol were identified from their elution positions on h.p.l.c. and by their absorbance spectra.

Assay system characteristics

Product formation measured by the h.p.l.c. method was linear with time for at least 1 h and linear with amount of protein added (up to 3 mg/ml of assay mix), using crude or TSK-125-column-purified preparations. The ability of crude epidermal extracts to oxidize retinol to retinoic acid was markedly stimulated by the addition of NAD^+. Addition of 1 mM- and 5 mM-NAD^+ led to a 3.7- and 5.1-fold increase in product formed respectively. Addition of 1 mM- or 5 mM-$NADP^+$ or -FAD had no effect. The abilities of epidermis, dermis and liver extracts to form retinoic acid from 1 mM-retinol in the standard assay were: epidermis, 6.6 nmol/h per mg of protein (0.34 μmol/h per g of tissue); dermis, no activity detected; liver, 4.0 nmol/h per mg of protein (1.28 μmol/h per g of tissue). When the epidermal extract was assayed with 5 mM-ascorbate, 5 mM-mercaptoethanol or 5 mM-dithiothreitol, retinoic acid formation decreased to 82, 62 and 86% respectively. Triton X-100 had no effect at assay levels of 0.02%, decreased activity to 61% at 0.1%, and completely prevented retinol oxidation at 5%. Addition of ethanol to the assay

Table 1. Inhibition of retinoic acid formation by citral *in vivo*

Groups of three mice were dosed with 0.1 ml of acetone (control) or with 11 μmol of citral (treated) 30 min before retinol application. Epidermal retinoids were determined 2 h after retinol treatment, and are expressed as the mean $\pm$S.D. of the amounts/g of epidermis. Epidermal retinoic acid levels were significantly lower (P 0.05) than controls at all retinol doses.

	Control		Treated	
Dose of retinol (nmol/mouse)	Retinol (nmol/g)	Retinoic acid (pmol/g)	Retinol (nmol/g)	Retinoic acid (pmol/g)
25	36.4$\pm$1.0	519$\pm$95	35.9$\pm$8.4	243$\pm$18
58	105$\pm$22	2090$\pm$390	105$\pm$12	860$\pm$80
100	141$\pm$11.1	3343$\pm$1161	149$\pm$32	1342$\pm$298

mixture at 1% and 5% decreased the amount of retinoic acid formed to 82% and 66% respectively.

Enzyme fractionation and M_r values

Epidermal retinol- and retinal-oxidizing activities were resolved by h.p.l.c. on a TSK 125 column (Fig. 1). Recoveries of the activities from the column varied between 70 and 120%. The retinal-oxidizing enzyme had an M_r of about 76000, within the range (75000–80000) reported for the rat intestinal-mucosa enzyme [13]. Retinol- and ethanol-oxidizing activities were co-eluted at a position corresponding to an M_r of about 32000. This value was surprising, being lower than that reported for mouse alcohol dehydrogenase subunits (37000–47000) [17]. However, mouse liver alcohol dehydrogenase activity and commercially obtained horse liver alcohol dehydrogenase (M_r 80000) were also eluted at this position. The mouse and horse alcohol dehydrogenases were also eluted later than expected from a TSK 400 column and when the phosphate concentration of the elution solvent was increased to 0.5 M. Alcohol dehydrogenases thus undergo anomalous migration on these columns.

Properties of the retinal-oxidizing enzyme

The TSK-125-column-purified enzyme oxidized retinal to retinoic acid, with an apparent K_m for retinal of 19 μM (determined with 10 μM–1 mM-retinal). Retinal was not formed when portions of the same preparation were incubated with 1 mM-retinoic acid and 5 mM-NADH, indicating that the reaction is not reversible. The activity was inhibited by citral (I_{50} = 0.32 mM). Oxidizing activity increased with pH over the range pH 6–8, then reached a plateau.

Properties of the retinol-oxidizing and alcohol dehydrogenase activities

The apparent K_m for retinol was 89 μM, determined with 10 μM–1 mM-retinol, Retinol was formed when the enzyme preparation was incubated with 1 mM-retinal and 5 mM-NADH, and the elution profiles from the TSK 125 column were identical for the retinal-reducing and retinol-oxidizing activities. Thus the conversion of retinol into retinal was reversible. Retinal formation was inhibited by citral (I_{50} = 0.9 mM) and 4-methylpyrazole (I_{50} = 0.3 mM). The enzyme preparation oxidized ethanol (apparent K_m = 320 mM), and ethanol oxidation was inhibited by 4-methylpyrazole (apparent K_i = 0.85 mM), values similar to those reported for the type-C alcohol dehydrogenase isoenzyme (K_m = 230 mM, K_i = 1.5 mM respectively) isolated from the stomachs of CBA/H-strain mice [17]. By comparison the liver alcohol dehydrogenase of Skh/hrl mice had an apparent K_m for ethanol of 0.12 mM, with a K_i for the inhibition of ethanol oxidation by 4-methylpyrazole of 1.0 μM, values similar to those reported for the pure type-A isoenzyme from mouse liver (K_m for ethanol = 0.15 mM; K_i for 4-methylpyrazole = 2 μM) [17]. On the basis of the kinetic, substrate and inhibitory properties, it was concluded that the epidermal retinol-oxidizing enzyme is the type-C alcohol dehydrogenase isoenzyme.

Effect of citral and r-methylpyrazole on retinoic acid formation *in vivo*

Attempts to inhibit retinol oxidation *in vivo* by using 4-methylpyrazole were compounded by its toxicity. The relatively high K_i for the inhibition of the epidermal compared with the liver dehydrogenase suggested that systemic doses approaching the LD_{50} (that causing death in 50% of the mice so treated) (7.8 mmol/kg [18]) would be required to obtain significant inhibition *in vivo*. Doses of 10 mmol/kg intraperitoneally proved rapidly fatal, and doses of 2 mmol/kg induced comas. Topical application of 12 μmol of 4-methylpyrazole decreased the epidermal retinoic acid level found 2 h after retinol treatment by 17%; however, it also induced systemic and cutaneous toxicity. We concluded that the use of 4-methylpyrazole *in vivo* to inhibit the epidermal dehydrogenase is impractical. Citral treatment decreased the epidermal retinoic acid levels found 2 h after retinol application by at least 50% (Table 1) without causing any gross or histologically discernible toxicity. Since the epidermal retinol levels were the same in the citral-treated and vehicle-treated mice (Table 1), citral treatment did not appear to interfere with retinol uptake.

DISCUSSION

We have demonstrated that the retinol- and retinal-oxidizing enzymes present in mouse epidermis can be conveniently resolved by h.p.l.c. using a TSK 125 gel-permeation column. The separation of the two enzyme activities is due to the anomalous retardation of alcohol dehydrogenases on the TSK 125 column.

Three murine alcohol dehydrogenases are known, coded for by three separate gene loci [20], and their expression is tissue-specific [21]. The major alcohol dehydrogenase of mouse epidermis is clearly the type-C

isoenzyme, similar to the enzyme purified from stomach [17]. Our observation that retinol is readily oxidized by the epidermal enzyme is in keeping with the preference of the type-C isoenzyme for unsaturated medium-chain alcohols [17]. This isoenzyme has been detected in epithelial and reproductive tissues, including the stomach, epididymis, ovary, uterus and lungs [21]. In rat ocular tissues the major alcohol dehydrogenase present also shows kinetics for ethanol and retinol oxidation similar to those shown by the type-C isoenzyme [14]. The occurrence of this isoenzyme in a variety of retinoid-responsive and -dependent tissues suggests that this is the form of alcohol dehydrogenase active in retinol metabolism at the physiological level.

Having some knowledge of the oxidizing system, we were able to screen potential inhibitors of retinol oxidation. Since one of the enzymes is an alcohol dehydrogenase, the potent inhibitor of ethanol oxidation 4-methylpyrazole [18] was an obvious candidate. However, its toxicity made its use impracticable as an inhibitor in mouse epidermis *in vivo*. Its more marked potency at inhibiting type-A alcohol dehydrogenase suggests that 4-methylpyrazole is more likely to be useful when targeted at tissues containing the appropriately sensitive alcohol dehydrogenase isoenzyme. Citral (3,7-dimethylocta-2,6-dienal), first proposed as vitamin A antagonist over 30 years ago [22], proved to be effective at inhibiting retinol oxidation *in vitro* and *in vivo*. Citral is a mixture of *cis* (neral) and *trans* (geranial) isomers, the citral used in these studies being nominally 59% neral. We do not know whether one isomer is active or both are. Assuming that citral also inhibits retinoic acid formation from retinol in other tissues, our findings provide a biochemical basis to explain the earlier reports that citral acts as a vitamin A antagonist in epithelia [22,23]. If this is so, the ability of citral to inhibit biological effects of retinol indicates that retinoic acid formation is obligatory in at least some of the effects of vitamin A on epithelia. The epidermis is a retinoid-responsive tissue uniquely available to direct treatment with pharmacologically active agents *in situ*. The ability to inhibit retinoic acid formation in this tissue with citral provides a basis for further studies to distinguish more clearly between biological effects of retinoic acid and those of its parent molecule, retinol.

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