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*J Clin Invest.* 1992;90(4):1269-1274. <https://doi.org/10.1172/JCI115990>.

### Research Article

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# Human Skin Levels of Retinoic Acid and Cytochrome P-450-derived 4-Hydroxyretinoic Acid after Topical Application of Retinoic Acid In Vivo Compared to Concentrations Required to Stimulate Retinoic Acid Receptor-mediated Transcription In Vitro

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## Abstract

Metabolism of retinoic acid to a less active metabolite, 4-hydroxyretinoic acid, occurs via cytochrome P-450 isozyme(s). Effect of a pharmacological dose of retinoic acid on the level of retinoic acid in skin and on cytochrome P-450 activity was investigated. A cream containing 0.1% retinoic acid or cream alone was applied topically to adult human skin for four days under occlusion. Treated areas were removed by a keratome and a microsomal fraction was isolated from each biopsy. In vitro incubation of <sup>3</sup>H-retinoic acid with microsomes from in vivo retinoic acid treated sites resulted in a 4.5-fold increase ( $P = 0.0001$ ,  $n = 13$ ) in its transformation to 4-hydroxyretinoic acid in comparison to in vitro incubations with microsomes from in vivo cream alone treated sites. This cytochrome P-450 mediated activity was oxygen- and NADPH-dependent and was inhibited 68% by 5  $\mu$ M ketoconazole ( $P = 0.0035$ ,  $n = 8$ ) and 51% by carbon monoxide ( $P = 0.02$ ,  $n = 6$ ).

Cotransfection of individual retinoic acid receptors (RARs) or retinoid X receptor- $\alpha$  (RXR- $\alpha$ ) and a chloramphenicol acetyl transferase (CAT) reporter plasmid containing a retinoic acid responsive element into CV-1 cells was used to determine the ED<sub>50</sub> values for stimulation of CAT activity by retinoic acid and its metabolites. Levels of *all trans* and 13-*cis* RA in RA-treated tissues were greater than the ED<sub>50</sub> values determined for all three RARs with these compounds. Furthermore, the level of *all trans* RA was greater than the ED<sub>50</sub> for RXR- $\alpha$  whereas the 4-OH RA level was greater than the ED<sub>50</sub> for RAR- $\beta$  and RAR- $\gamma$  but less than for RAR- $\alpha$  and RXR- $\alpha$ . These data suggest that there are sufficient amounts of retinoic acid in treated skin to activate gene transcription over both RARs and RXR- $\alpha$ . (*J. Clin. Invest.* 1992. 90:1269–1274.) Key words: high performance liquid chromatography • P-450 inhibitors • reporter assay • ED<sub>50</sub> values

This work was presented in part at the annual meeting of the Society for Investigative Dermatology, Seattle, WA, 1–4 May 1991.

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Received for publication 21 October 1991 and in revised form 11 February 1992.

J. Clin. Invest.

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0021-9738/92/10/1269/06 \$2.00

Volume 90, October 1992, 1269–1274

## Introduction

Retinoids such as *all trans* retinol (ROL)<sup>1</sup> and *all trans* retinoic acid (RA) are important in epithelial proliferation and differentiation (1–3) and repair of photodamaged skin (4, 5). ROL is stored in tissues as retinyl esters (6–8) or bound to cellular retinol binding protein (CRBP) (3). Little or no RA is stored in tissues. Retinoids present in human skin are ROL, retinyl esters, 3,4-didehydroretinyl esters, and RA (9). RA and 3,4-didehydro RA (ddRA) act as important morphogens in the chick wing bud system either directly (2) or indirectly (10, 11). The major retinoic acid receptor (RAR) form expressed in the epidermis is RAR- $\gamma$  (12, 13). Expression of retinoid X receptor- $\alpha$  (RXR- $\alpha$ ) has also been demonstrated in human skin (14).

Metabolism of RA occurs via a cytochrome P-450 (P-450) mediated enzyme system in tissues such as liver (15–17), trachea (15), intestine (16), and rodent skin (18). The primary product formed is 4-OH RA which is further transformed to 4-oxo RA. Topical application of RA to skin of 4-d-old rats increases RA metabolism fourfold. In vitro ketoconazole inhibits conversion of RA to 4-OH RA by microsomes from rat epidermis (18). In liver the formation of 4-OH RA via P-450 isozyme(s) is inhibited both in vivo and in vitro by ketoconazole (19).

In this study a single topical dose of 0.1% RA cream or cream vehicle was applied to adult human skin and kept under constant occlusion. After 96 h vehicle- and RA-treated sites were tested for their capacity to metabolize RA and were analyzed for RA and RA metabolite content. RA and its metabolites were also tested for their capacity to induce RAR- $\alpha$ , RAR- $\beta$ , RAR- $\gamma$ , or RXR- $\alpha$ -mediated transcription of a reporter gene in a cotransfection system.

## Methods

Isocitrate, isocitrate dehydrogenase, NADPH, NAD, 13-*cis* RA, and RA were purchased from Sigma Chemical Co. (St. Louis, MO). CV-1 cells, African Green Monkey kidney cells, were purchased from American Type Culture Collection (Rockville, MD). <sup>3</sup>H-RA was obtained from DuPont NEN (Boston, MA) and [<sup>14</sup>C]chloramphenicol from Amersham Corp. (Arlington Heights, IL). RA metabolites 4-OH RA, 4-oxo RA, 13-*cis* 4-oxo RA, and 5,6-epoxy RA were gifts from Drs.

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CRBP, cellular retinol binding protein; ddRA, 3,4-didehydro retinoic acid; RA, *all trans* retinoic acid; RAR, RA receptor; ROL, *all trans* retinol; RXR- $\alpha$ , retinoid X receptor- $\alpha$ .

Michael Rosenberger and P. F. Sorter of Hoffmann-LaRoche (Nutley, NJ). Metabolite 3,4-ddRA was a gift from Drs. I. Kompis and M. Klaus of Hoffmann-LaRoche AG (Basel, Switzerland). The 0.1% RA (Retin-A®) and vehicle cream were supplied by Ortho Pharmaceutical Corp. (Raritan, NJ). HPLC grade acetonitrile and methanol were purchased from Burdick & Jackson Laboratories Inc., a division of Baxter Products (Romulus, MI). Spherisorb ODS1 column was purchased from Phase Separations Inc. (Norwalk, CT).

**RA application and biopsy procedure.** 0.5 g of RA (0.1% Retin-A® cream) and 0.5 g of vehicle cream were applied to separately marked 3 × 2 inch areas on the buttock region of adult volunteers and kept occluded for four days with Saran Wrap and secured with surgical tape (20). At four days test areas were evaluated for erythema, washed, and keratomed under 1% lidocaine local anesthesia (20). In experiments to determine RA and RA metabolite levels, all three RA-treated areas were washed with chlorhexadine soap solution. In addition, two sites were washed with ethanol and one of the ethanol plus soap-washed sites was tape-stripped to "glistening", i.e., for removal of stratum corneum, before keratomed. Epidermal samples were immediately frozen in liquid nitrogen and stored at -80°C until used. Skin punch biopsies (2 mm) were taken from tape-stripped sites and placed in 10% neutral buffered formalin. These biopsies were processed for hematoxylin and eosin histological analysis of adequacy of stratum corneum removal by stripping. All subjects gave informed, written consent. The study was approved by the University of Michigan Medical Center Institutional Review Board.

**RA metabolism assay.** Microsomal fractions were prepared as previously described (21). RA and RA metabolites were kept in amber glass in darkened rooms with yellow lighting whenever possible. The rate of RA metabolism was determined by incubating 100 µg microsomal protein in 0.01 M phosphate buffer (pH 7.4) containing an NADPH regenerating system (21), and <sup>3</sup>H-RA (1 µM, sp act 1 mCi/0.1 µmol). Samples were incubated for 15–45 min at 35°C. The addition of 100 µl methanol containing 100 µg/ml of butylated hydroxytoluene cooled to -20°C terminated the reaction. After centrifugation at 1,000 g for 10 min, the supernatant fractions were analyzed for RA metabolites by reverse-phase HPLC and liquid scintillation spectrometry.

Ketoconazole, an inhibitor of cytochrome P-450, was incubated at 35°C for 10 min with all components of the assay present except for <sup>3</sup>H-RA which was added to start the reaction. For incubations with CO as inhibitor, all components of the assay were present but tubes were kept on ice during 15 min flushing with CO. Tubes were sealed and transferred to a 35°C water bath for formation of RA metabolites. Control tubes were handled in the same manner except the atmosphere was not equilibrated with CO.

**HPLC separation of retinoids.** An HPLC system (1090 M; Hewlett Packard Co., Palo Alto, CA) containing a Spherisorb ODS1 column (Phase Separations Inc., 25 cm × 4.6 mm), a diode array detector, and chem workstation was used to separate retinoids. Mobile phases used for gradient elution of retinoids were those of Bugge et al. (22). Mobile phase A was acetonitrile:0.02 M ammonium acetate:acetic acid (1:1:0.01) and mobile phase B was acetonitrile:0.2 M ammonium acetate:acetic acid (19:1:0.008). At the start of the run solvent A was 100% followed by a linear gradient to 30% solvent B at 3 min, a shallow gradient to 81% solvent B at 38 min, and 100% solvent B at 40 min. The flow rate was 0.5 ml/min and the total time for the separation was 45 min. Effluent from the HPLC column flowed directly into a flow-through scintillation spectrometer (Beta-One; Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) with a computerized data capture system to determine radioactivity in each metabolite peak. The amount of metabolite formed was calculated based on the specific activity of added retinoic acid.

**Extraction of retinoids from biopsy samples.** Frozen keratome biopsies, each weighing ~ 120 mg, were powdered and immediately transferred into 20 vol of chloroform:methanol (2:1) containing tracer amounts of <sup>3</sup>H-RA. Retinoids were extracted with gentle mixing for 15 min followed by centrifugation at 1,000 g for 10 min. Supernatant fractions were removed and saved. Pellets were reextracted with 5 vol

of the same solvent. After centrifugation supernatant fractions were saved and pellets were extracted with methanol:water acidified to pH 3.2 (95:5). After centrifugation the three supernatant fractions were combined and were evaporated to dryness under a stream of nitrogen. Samples were resuspended in methanol for HPLC injection. Recovery of retinoids and assessment of nonenzymatic isomerization of *all trans* RA to 13-*cis* RA during extraction procedure were based on the amount of <sup>3</sup>H-RA recovered as <sup>3</sup>H-*all trans* or <sup>3</sup>H-13-*cis* RA in comparison to the amount initially added as *all trans* <sup>3</sup>H-RA.

**Cotransfection of CV-1 cells and reporter assay.** Procedures reported by Astrom et al. (23) were used to determine ED<sub>50</sub> values for chloramphenicol acetyl transferase (CAT) activity by RA and RA metabolites. CV-1 cells were cotransfected with RAR-α, RAR-β, RAR-γ, or RXR-α expression vectors and the reporter plasmid TRE<sub>3</sub>-*tk*-CAT. Human RXR-α cDNA (24) was cloned from human skin as described (14) and subcloned into the expression vector pSVL (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) for use in the transfection assay (14). The effect of various concentrations of RA and RA metabolites (10<sup>-11</sup>–5 × 10<sup>-4</sup> M) on CAT activity was determined as described (23).

**Other methods.** Statistical analyses for all assays were performed using a two-tailed paired *t* test. Protein content was determined by the method of Lowry et al. (25) with BSA as standard.

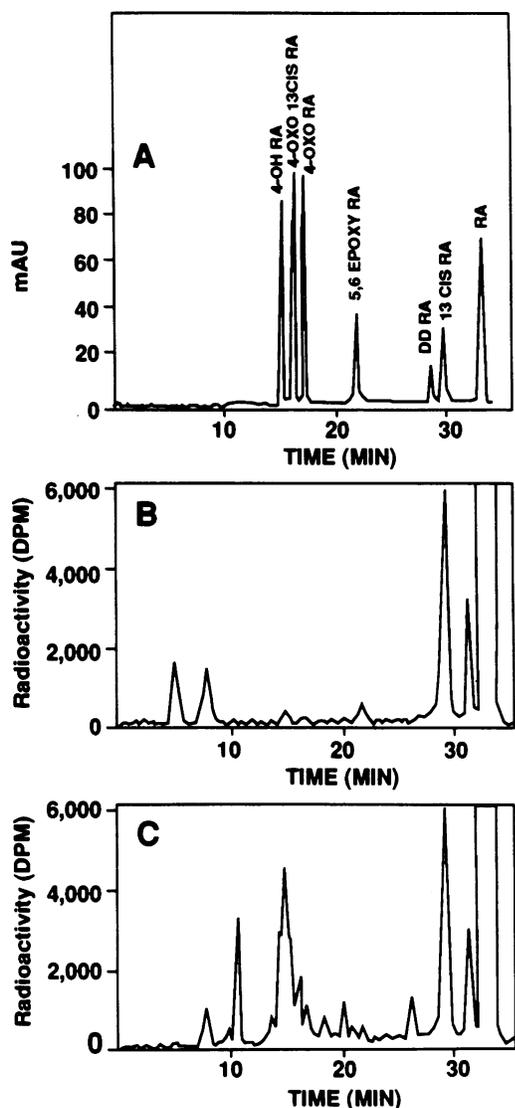
The concentrations of retinoids in skin were calculated as follows. The biopsies were weighed before pulverization. The recovery of unlabeled material was equated to the recovery of added <sup>3</sup>H-RA. Calibration curves for the retinoids were stored in the HP-1090 HPLC unit. The absorption (peak area) from each sample was converted to nanograms of retinoid based on the calibration curves. The nanograms of retinoid divided by the percent recovery gave the value for total retinoid. This value was divided by the wet weight in grams to give micrograms per gram skin. 1 g wet wt of tissue was equivalent to 1 ml of water for purposes of calculating molarity.

## Results

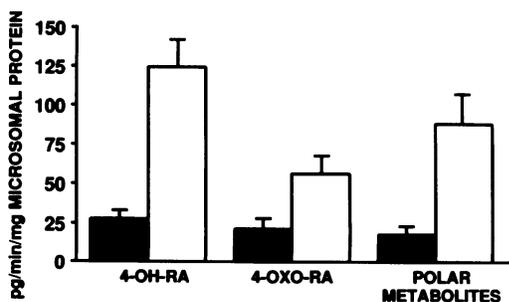
Retention times for nonradioactive retinoid standards are shown in chromatogram depicted in Fig. 1 A. RA metabolites separated by HPLC after *in vitro* incubation of <sup>3</sup>H-RA with microsomal fractions obtained from biopsies treated *in vivo* with vehicle cream or 0.1% RA cream are shown in chromatograms depicted in Fig. 1 B (vehicle) and Fig. 1 C (0.1% RA). Incubation of <sup>3</sup>H-RA with microsomes from RA-treated sites showed increased formation of 4-OH RA, 4-oxo-RA, and more polar metabolites in comparison to metabolites generated by microsomes from vehicle-treated sites.

Formation of RA metabolites was time (10–60 min) and protein concentration (100–400 µg) dependent (data not shown). Data showing increased P-450 mediated RA metabolism four days after a single topical application of 0.1% RA cream in comparison to vehicle cream for 13 normal volunteers are given in Fig. 2. There was a 4.5-fold (*P* = 0.0001) increase in formation of 4-OH RA (124.4 ± 17.4 pg/min per mg microsomal protein) by microsomes from RA-treated sites in comparison to formation of metabolites by microsomes from vehicle treated sites (27.7 ± 5.5 pg/min per mg microsomal protein). There was a 2.6-fold increase in 4-oxo RA formation (*P* = 0.0003) and a 4.8-fold increase in formation of more polar metabolites (*P* = 0.007) in RA-treated versus vehicle-treated areas.

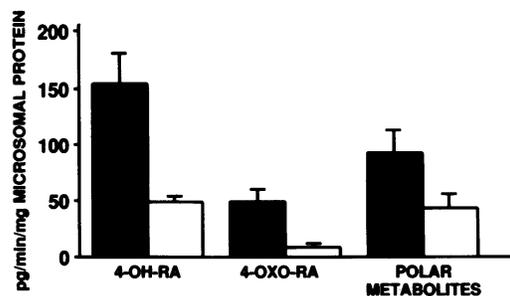
Data showing inhibition of P-450 mediated activity by ketoconazole are given in Fig. 3. With biopsies from RA-treated sites as source of microsomes there was a 68% decrease in 4-OH RA formation (*P* = 0.0035, *n* = 8) in incubations containing 5 µM ketoconazole and <sup>3</sup>H-RA (49.0 ± 5.5 pg/min per mg micro-



**Figure 1.** Reverse-phase HPLC separation of retinoids as detailed in Methods. (A) Chromatogram shows retention times for nonradioactive retinoid standards. (B) Depicts the chromatogram obtained after *in vitro* incubation of  $^3\text{H}$ -RA with microsomal fractions from vehicle-treated sites. (C) Depicts chromatogram obtained after *in vitro* incubation of  $^3\text{H}$ -RA with microsomal fraction from RA-treated sites.



**Figure 2.** Retinoic acid treatment of human skin *in vivo* causes cytochrome P-450 mediated increased retinoic acid metabolism. *In vitro* assay conditions and analytical procedures are detailed in Methods. Comparison of RA-treated versus vehicle-treated sites gave the following percent increases and *P* values: 4-OH RA (349%; 0.0001), 4-oxo RA (162%; 0.0003), and polar metabolites (381%; 0.007). *n* = 13. ■, VEH; □, RA.

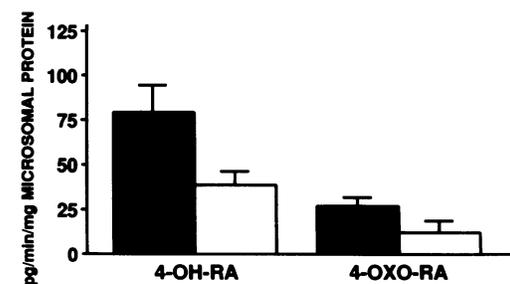


**Figure 3.** Ketoconazole inhibits retinoic acid-induced cytochrome P-450-mediated transformation of retinoic acid into 4-OH retinoic acid. Conditions for *in vitro* assay of the microsomal fraction from RA-treated sites and analytical procedures are detailed in Methods. Comparison of  $^3\text{H}$ -RA versus  $^3\text{H}$ -RA + 5  $\mu\text{M}$  ketoconazole gave the following percent decreases and *P* values: 4-OH RA (68%; 0.0035), 4-oxo RA (82%; 0.003), and polar metabolites (53%; 0.029). *n* = 8. ■,  $^3\text{H}$ -RA - 1  $\mu\text{M}$ ; □,  $^3\text{H}$ -RA + KETO-5  $\mu\text{M}$ .

somal protein) in comparison to  $^3\text{H}$ -RA alone ( $154.1 \pm 26.2$  pg/min per mg microsomal protein). There were significant decreases in formation of 4-oxo RA (82%,  $P = 0.003$ ,  $n = 8$ ) as well as more polar metabolites (53%,  $P = 0.029$ ,  $n = 7$ ) in incubations containing ketoconazole and  $^3\text{H}$ -RA in comparison to  $^3\text{H}$ -RA alone. As shown in Fig. 4, CO significantly decreased by 51% ( $P = 0.02$ ,  $n = 6$ ) formation of 4-OH RA.

Concentrations of retinoids required to obtain half-maximal stimulation ( $\text{ED}_{50}$ ) of CAT activity by RAR- $\alpha$ , RAR- $\beta$ , RAR- $\gamma$ , or RXR- $\alpha$  in cotransfected CV-1 cells are given in Table I. Data for RAR- $\gamma$ , the major receptor expressed in epidermal cells, show that RA and 3,4 ddRA have an  $\text{ED}_{50}$  of 1–2 nM while 4-oxo RA requires a 10-fold higher concentration (13 nM) and 4-OH RA a 20-fold higher concentration (30 nM). The 5,6 epoxy RA  $\text{ED}_{50}$  for RAR- $\gamma$  was 49 nM.

To determine whether concentrations of RA or its metabolites reached sufficient levels to potentially activate transcription by RARs or RXR- $\alpha$  after topical application of RA, levels of RA and its metabolites present in viable epidermis at four days were determined. Fig. 5 shows that washing the sites with soap and ethanol was not sufficient to remove RA remaining on the surface of skin. After tape stripping the amount of RA measured decreased 80% whereas 4-OH RA levels remained



**Figure 4.** CO inhibits cytochrome P-450-mediated retinoic acid metabolism into 4-OH retinoic acid. Microsomal fractions from sites treated *in vivo* with 0.1% RA cream were used in *in vitro* incubations. Assay conditions and analytical procedures are detailed in Methods. A comparison of incubation with RA + CO versus RA alone gave the following percent decreases and *P* values: 4-OH RA (51%; 0.02) and 4-oxo RA (53%; 0.07). *n* = 6. ■,  $^3\text{H}$ -RA - 1  $\mu\text{M}$ ; □,  $^3\text{H}$ -RA + CO - 1  $\mu\text{M}$ .

**Table I. Concentrations of Retinoids Required to Obtain Half-Maximal Stimulation ( $ED_{50}$ ) of  $TRE_3$ -tk-CAT Transcription by Retinoic Acid Receptors and RXR- $\alpha$  Cotransfected into CV-1 Cells**

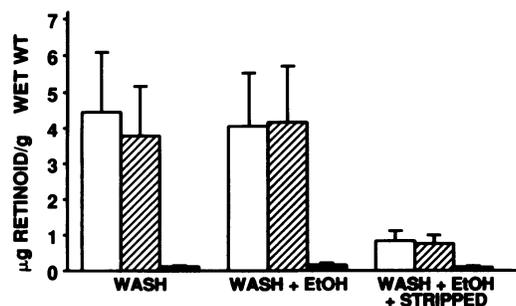
Compounds	$ED_{50}$			
	RAR- $\alpha$	RAR- $\beta$	RAR- $\gamma$	RXR- $\alpha$
	<i>nM</i>			
Retinoic Acid	24*	4*	1.3*	236
dd-Retinoic Acid	34	13	1.8	245
4-oxo-Retinoic Acid	79	28	13	377
4-OH Retinoic Acid	> 500	75	30	> 500
5,6-Epoxy Retinoic Acid	237	56	49	> 500

Data represent the average value for two to three titrations from  $10^{-11}$  to  $5 \times 10^{-4}$  M retinoid concentrations. \* These data points for RA were previously published (23) and are included to facilitate comparison to metabolite data.

the same regardless of the method of tissue preparation. *All trans* and 13-*cis* RA were present in almost equal amounts. The 13-*cis* RA present in tissue was not due to nonenzymatic isomerization during extraction since  $91.2\% \pm 1.7\%$  of the  $^3H$ -RA added as tracer to determine recovery of material remained as *all trans* and  $8.8\% \pm 1.1\%$  was 13-*cis* RA. As shown in Table II, *all trans* RA was present as 824 ng/gm wet wt or  $\sim 2.75 \mu M$  concentration (three log units higher than the  $ED_{50}$  for RAR- $\gamma$ ), 13 *cis*-RA as 745 ng/gm wet wt or  $\sim 2.5 \mu M$  ( $60 \times ED_{50}$  value for RAR- $\gamma$ ), and 4-OH RA as 93 ng/gm wet wt or  $\sim 0.3 \mu M$  concentration ( $10 \times ED_{50}$  value for RAR- $\gamma$ ). 4-Oxo RA was not detected in RA-treated biopsies even though epidermal microsomes from similar biopsies showed conversion to both 4-OH and 4-oxo RA.

## Discussion

The effects of daily application of RA without occlusion to photodamaged skin have been investigated in clinical studies (4, 5). The improvement seen is variable and dose dependent (4, 5). Pilot studies with 0.1% RA cream with or without occlusion used topically for various periods of time showed that RA under occlusion for four days gave a highly consistent erythematous reaction as well as characteristic histological (20) re-



**Figure 5.** Retinoid content in areas treated four days with 0.1% RA cream followed by removal of surface RA by the indicated methods. Extraction procedure detailed in Methods. Concentrations in stripped tissue samples were *all trans* RA ( $\approx 2.7 \mu M$ ), 13 *cis*-RA ( $\approx 2.5 \mu M$ ), and 4-OH RA ( $\approx 0.3 \mu M$ ).  $n = 5$  for each method of surface RA removal. □, *all trans* RA; ▨, 13-*cis* RA; ■, 4-OH RA.

**Table II. Comparison of Tissue Levels after Topical Application of All Trans Retinoic Acid to Human Skin In Vivo with  $ED_{50}$  Values for Transcriptional Activation of  $TRE_3$ -tk-CAT in a Cotransfection Assay**

	<i>all trans</i> RA	13- <i>cis</i> RA	4-OH RA
ng/g wet wt	824±281	745±24	93±36
nM	$\approx 2750$	$\approx 2480$	$\approx 310$
	Retinoid (nM)		
$ED_{50}$ RAR- $\alpha$	24*	190*	> 500
$ED_{50}$ RAR- $\beta$	4*	43*	75
$ED_{50}$ RAR- $\gamma$	1.3*	140*	30
$ED_{50}$ RXR- $\alpha$	236	ND	> 500

\* These data points were previously published (23) and are included for facilitating comparisons.

sponse in 9 of 10 patients. Because of this consistent retinoid response, the four day occluded patch was used in the current study. Routine nonoccluded clinical use of RA cream produced an inconsistent retinoid response too variable for experimentation.

In the present study a single topical application of RA with occlusion continuously for four days to adult human skin resulted in sufficient retinoic acid penetration into viable layers of the epidermis to induce RA metabolism. Our data and that of others (18) suggest that this increased metabolism is probably due to increased P-450 activity. Furthermore, induction of P-450 activity in response to RA has been reported in other tissues (15–19). In addition, 5,6 epoxy RA can be formed either by a lipid peroxidase (26) or a P-450-mediated activity (27). However, the P-450 isozyme(s) induced in human skin in response to topical RA is unknown.

P-450 enzymes purified from rabbit liver have been evaluated for their capacity to metabolize RA. IA2 and IIB4 isozymes were most effective in metabolizing RA to 4-OH RA while IIC3, IIE1, IIG2, and IIE2 were relatively ineffective, and isozymes IA1 and IIIA6 were ineffective (28).

Cultured human keratinocytes have been shown to contain the mRNA for P-450 isozyme IA1 (29). However, the IA1 isozyme was one of two forms from rabbit liver that did not metabolize RA. An immunohistochemical study of cultured human epidermal foreskin keratinocytes showed binding of monoclonal antibodies to isozymes belonging to families II and III (30). In neonatal rats topical application of 3-methylcholanthrene was more effective than topical application of RA in inducing RA metabolism by P-450 isozyme(s) (18). Phenobarbital was the least effective inducer of RA metabolism (18). In hamsters intraperitoneal injection of methylcholanthrene increased RA metabolism in liver twofold while orally given RA increased RA metabolism eightfold in liver (16). Thus metabolism of RA in response to inducers of P-450 activity may vary as a result of route of administration, species involved, or tissue studied.

Cytochrome P-450 mediated metabolism of RA may be a significant mechanism for regulation of pharmacological RA levels in vivo. In fact the conversion of RA to less active metabolites (i.e., 4-OH and 4-oxo RA) could limit the biological activity of pharmacological doses. Whether a P-450 activity

attenuates physiologically relevant, endogenous RA levels can not be addressed with the data presented here. However, our results do demonstrate that topical application of pharmacological doses of RA in humans induce a 4.5-fold increase in metabolism of RA to 4-OH RA and more polar metabolites. Ketoconazole, a topical antifungal agent currently in routine clinical use, is an inhibitor of some P-450 mediated activities. Ketoconazole at concentrations of  $7 \times 10^{-7}$  M to  $5 \times 10^{-6}$  M inhibits in vitro RA metabolism by rat epidermal microsomes. Similar concentrations are required to suppress certain P-450 dependent reactions in steroid synthesis (19). One possible side effect of ketoconazole use is increased RA levels due to decreased RA metabolism. This may be of clinical importance in as much as simultaneous use of topical ketoconazole and RA could decrease the amount of RA required to obtain clinical efficacy.

To determine the potency of ddRA and RA metabolites in comparison to RA and 13-*cis* RA in RAR- and RXR-mediated transcriptional activation, a reporter gene (TRE<sub>3</sub>-*tk*-CAT) was cotransfected together with receptor expression vectors into CV-1 cells and concentrations required to obtain half-maximal induction of CAT activity (ED<sub>50</sub>) were determined. Similar reporter assay systems have been established with cell lines other than CV-1 cells. The ED<sub>50</sub>s observed for RA in these systems vary with RAR receptor type rather than the cell line used for cotransfection procedure (23, 24). The CV-1 cells used in the reporter assay are epithelial cells but the appropriateness of any in vitro system to in vivo occurrences is always open to question.

Of the three known RARs, RAR- $\gamma$  is the major expressed form in human skin (12, 13) and in the reporter assay system used in these experiments RAR- $\gamma$  has the lowest ED<sub>50</sub> for *all trans* RA (1.3 nM) (23), *all trans* ddRA (1.8 nM), *all trans* 4-oxo-RA (13 nM), and *all trans* 4-OH RA (30 nM). The ED<sub>50</sub> values are two- to fourfold higher for RAR- $\beta$  than those determined for RAR- $\gamma$  except for *all trans* ddRA which is seven times higher. The ED<sub>50</sub> values for RAR- $\alpha$  were the highest obtained with any of the three RARs. The ED<sub>50</sub> values for *all trans* RA (23) and *all trans* ddRA with RAR- $\alpha$  was similar to the value obtained with 4-OH RA and RAR- $\gamma$  (30 nM). The ED<sub>50</sub> of 4-OH RA for RAR- $\alpha$  was similar to that of RXR- $\alpha$  (> 500 nM). RXR- $\alpha$  has been shown to be expressed in skin in vivo (14) but demonstrates higher ED<sub>50</sub> values for all compounds tested compared to RARs. The ED<sub>50</sub>s in the presence of RXR- $\alpha$  are 20 times higher for each compound in comparison to values for RAR- $\gamma$ . The lowest ED<sub>50</sub> for 13-*cis* RA is 43 nM with RAR- $\beta$  receptor (23). The value for 13-*cis* RA with RAR- $\gamma$  is 140 nM (23) (Table II).

To determine whether metabolites might play a significant role in retinoid effects observed following topical application of RA, treated sites were analyzed for presence of RA and RA metabolites. Washing with soap and ethanol was not sufficient to remove RA still present on the surface and within stratum corneum of skin as evidenced by  $\sim 4 \mu\text{g}$  RA per g wet wt (12  $\mu\text{M}$ ) in the specimen. Removal of stratum corneum and surface RA with tape stripping reduced these values to 1.5  $\mu\text{g}$  RA per g wet wt (5  $\mu\text{M}$ ). The method of preparing the site for biopsy did not change the level of 4-OH RA (93 ng/g wet wt, 0.3  $\mu\text{M}$ ). This suggests that the presence of 4-OH RA indicates increased RA content and increased metabolism of RA within living layers of epidermis without necessitating tape stripping of skin to determine RA metabolism.

In tape-stripped biopsy samples, 53% of RA extracted was *all trans* RA (2.7  $\mu\text{M}$ ) and 47% was 13-*cis* RA (2.5  $\mu\text{M}$ ). This concentration of *all trans* RA is 2,000 times higher than the ED<sub>50</sub> for RAR- $\gamma$  and 50-fold higher than the ED<sub>50</sub> for RXR- $\alpha$  in the cotransfection assay. The high levels of extracted 13-*cis* RA after topical application of *all trans* RA and apparent lack of significant nonenzymatic isomerization during retinoid extraction from tissue suggests that epidermis may contain an active RA isomerase. The amount of 13-*cis* RA in these samples is 10-fold higher than the ED<sub>50</sub>s in the reporter assay of 140 nM with RAR- $\gamma$  and 190 nM with RAR- $\alpha$  and 50-fold higher than the ED<sub>50</sub> of 43 nM for RAR- $\beta$  (23). The 4-OH RA levels were 10 times higher than the ED<sub>50</sub> determined for RAR- $\gamma$  and four times higher than the ED<sub>50</sub> for RAR- $\beta$  but less than the ED<sub>50</sub> of > 500 nM with RAR- $\alpha$  and RXR- $\alpha$  in the cotransfection assay.

The above comparisons are made on the assumption that concentrations of retinoids in skin have functional activity similar to that observed when the same retinoids are added to cell cultures containing genetically engineered retinoid response systems. However, there may be unrecognized differences between in vivo and in vitro cell penetration, distribution, and metabolism.

In conclusion, the effects observed clinically after topical application of pharmacological doses of *all trans* RA under occlusion for four days may be due in part to *all trans* RA and 13-*cis* RA as well as 4-OH RA. The large quantity of *all trans* RA as well as lesser but substantial amounts of 13-*cis* and 4-OH RA in relation to the concentration required for half-maximal activation of transcription in the cotransfection assay suggests that the biological response of skin (20) in response to RA under short-term occlusion may be mediated at least in part by both RARs and RXR- $\alpha$ . Whether molecular changes in skin in response to RA application can be mediated by RAR- and RXR- $\alpha$ -independent mechanisms remains unknown.

## Acknowledgments

We wish to thank Ulrika Pettersson for excellent technical assistance in the reporter gene assay, Robin Gardner for providing biopsy materials, and Ted Hamilton for statistical analyses.

This study was supported in part by a grant from R. W. Johnson Pharmaceutical Research Institute and the Babcock Endowment.

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