Unoccluded Retinol Penetrates Human Skin *In Vivo* More Effectively Than Unoccluded Retinyl Palmitate or Retinoic Acid

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The formation of all-*trans* retinoic acid is an oxidative process whereby retinol is converted to retinaldehyde and then to retinoic acid. Because retinol causes qualitative molecular changes similar to those produced by retinoic acid, we compared potency of retinol, retinaldehyde, and retinyl palmitate to retinoic acid and assessed the effects of occlusion. Retinoids were prepared in an experimental vehicle of 95% ethanol:propylene glycol (7:3) with anti-oxidant. Induction of retinoic acid 4-hydroxylase activity was the end point for comparison. Retinoic acid concentrations from 0.001% to 0.05% under occlusion produced a linear dose-response induction of 4-hydroxylase activity. The concentrations of the other retinoids under occlusion required to achieve significant induction of enzyme activity were 0.6% retinyl palmitate, 0.025% retinol, and 0.01% retinaldehyde. The linear dose-response was lost with retinoid concentrations in excess of 0.25% retinol or 0.5% retinaldehyde. Statistical analyses showed no difference in 4-hydroxylase activity between unoccluded and occluded retinol treated sites. By contrast, however, unoccluded sites treated with retinoic acid or retinyl palmitate had less induction of 4-hydroxylase activity than occluded sites. Retinol, retinaldehyde, and retinyl palmitate did not produce erythema but did increase epidermal thickness. Although retinol is a weaker retinoid than retinoic acid, the increased penetration of unoccluded retinol in comparison to unoccluded retinoic acid with this prototypic vehicle confers on retinol a more effective delivery of a retinoidal effect than unoccluded retinoic acid. Retinol at 0.25% may be a useful retinoid for application without occlusion because it does not irritate but does induce cellular and molecular changes similar to those observed with application of 0.025% retinoic acid. ***Key words: cytochrome P-450/4-hydroxylase/percutaneous absorption/vehicle. J Invest Dermatol 109:301–305, 1997***

Retinoids play an important role in control of proliferation and differentiation of tissues including skin. It is well established that all-*trans*-retinol (ROL) is converted to all-*trans*-retinoic acid (RA) in a tightly controlled two-step oxidative process (Ross, 1993; Napoli *et al*, 1995; Boerman and Napoli, 1996). The data of Kurlandsky *et al* (1994) showed that ROL must be converted to RA to function as a hormone in human keratinocyte cultures. Previously, Connor (1988) had demonstrated that ROL must be metabolized to RA to produce retinoid effects in hairless mice. Because this conversion is necessary for retinoid effects, ROL has been considered a much less potent retinoid than RA and little is known about the efficacy of retinyl palmitate (ROL Palm).

The cellular content of RA is also controlled by the induction of a cytochrome P-450-dependent RA 4-hydroxylase (RA 4-OHase) activity that metabolizes RA to a much less active metabolite, namely, 4-hydroxyretinoic acid (4-OH RA; Roberts *et al*, 1979). The *in vivo* topical application of RA on neonatal rat skin (Vanden Bossche *et al*, 1988) or adult human skin (Duell *et al*, 1992) induces RA 4-OHase activity in the epidermis. Measuring the induction of this activity provides a method for assessing retinoid activity in epidermis.

Our earlier studies in which retinoids were applied to human skin always included occlusion of treated sites (Fisher *et al*, 1991; Duell *et al*, 1992: Kang *et al*, 1995; Duell *et al*, 1996). The use of retinoids in the treatment of dermatologic diseases or in over-the-counter cosmetic preparations, however, is without occlusion. In this study the following areas were investigated: (i) Increases in RA 4-OHase activity in response to increases in the concentration of RA, ROL, all-*trans*-retinaldehyde (RAL), and ROL Palm applied under occlusion were determined, and (ii) the role of occlusion in the magnitude of RA 4-OHase activity was observed after topical application of RA, ROL, and ROL Palm.

MATERIALS AND METHODS

The following compounds were obtained from Sigma (St. Louis, MO): ROL, RAL, ROL Palm, NADPH, isocitrate, isocitrate dehydrogenase, 13-*cis*-retinol, 13-*cis*-retinaldehyde, and 13-*cis*-retinoic acid. 4-Hydroxy RA, 4-oxo-retinoic acid, 13-*cis*-4-oxo-retinoic acid, and 5,6-epoxy retinoic

Manuscript received August 12, 1996; revised April 25, 1997; accepted for publication May 13, 1997.

This work was presented in part at the annual meeting of the Society for Investigative Dermatology, Washington, DC, May 1–5, 1996.

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Abbreviations: RA, all-*trans*-retinoic acid; RA 4-OHase, retinoic acid 4-hydroxylase; 4-OH RA, 4-hydroxyretinoic acid; ROL, all-*trans*-retinol; RAL, all-*trans*-retinaldehyde; ROL Palm, retinyl palmitate.

0022-202X/97/$10.50 •

acid were gifts (from Drs. P. F. Sorter and M. Rosenberger, Hoffmann LaRoche, Nutley, NJ) and 9-*cis*-retinoic acid (from Drs. J. Grippo and A. Levin, Hoffmann LaRoche, Nutley, NJ). Tritiated RA was purchased from DuPont New England Nuclear (Boston, MA). Organic solvents used for extracting retinoids from tissue and for high-performance liquid chromatography (HPLC) were HPLC grade. Retinoids were separated on a Hewlett-Packard 1090M HPLC unit (Hewlett-Packard, Palo Alto, CA) containing a Spherisorb ODS1 reverse-phase column from Phase Separations (Norwalk, CT). To quantitate retinoid metabolites formed, the HPLC effluent was channeled directly into a Radiomatic model A295 flow-through scintillation spectrometer (Packard Instruments, Downers Grove, IL).

Topical Application of Retinoids to Adult Human Skin

Routine Procedure Retinoids were dissolved in a vehicle consisting of 95% ethanol:propylene glycol (7:3, vol/vol) containing 0.5 mg butylated hydroxytoluene per ml of solvent except for ROL Palm, which was dissolved in 95% ethanol plus butylated hydroxytoluene. All work with retinoids was carried out under subdued yellow light. Up to five different concentrations of retinoids were applied to the same individual. The areas were occluded with Saran Wrap until time for biopsy.

Modification for Occluded and Unoccluded Treatment Areas In this series one pair of application sites was treated by using the normal procedures; i.e., a single application of retinoid or vehicle was applied to the areas and occluded for 4 d. A second pair of application sites received applications of vehicle or retinoid once daily for 4 d. Each application was performed in the Department of Dermatology. These unoccluded areas were left to dry before covering with normal clothing. After 4 d, both pairs of application sites were biopsied.

All subjects gave informed written consent. These studies were approved by the University of Michigan Medical Center Institutional Review Board.

Biopsy Procedure, Microsomal Preparation, and RA 4-OHase Assay Keratome biopsies were obtained as previously described (Duell *et al*, 1992). The tissue was immediately frozen in liquid nitrogen and stored at −70°C until used. To prepare the microsomal fraction, tissue was powdered under liquid nitrogen in a mortar and pestle. The powder was transferred to a ten Brock homogenizer containing 0.1 M Tris(hydroxymethyl)aminomethane, pH 7.2, 0.5 M KCl, and 0.1 mM ethylenediamine tetraacetic acid and dispersed with six to eight strokes of the pestle. The microsomes were isolated by differential centrifugation. RA 4-OHase activity was determined in an *ex vivo* assay by using [^{3}H]*trans*-RA as substrate and an NADPH-regenerating system (Duell *et al*, 1992). The reaction was stopped with ice-cold methanol and the protein was pelleted by centrifugation. Each supernatant fraction was filtered into an HPLC vial and injected onto the HPLC column.

Extraction of Epidermis Treated with Retinoids Vehicle- and ROL Palm-treated sites were tape stripped to glistening to remove the stratum corneum and upper nonviable layers of the epidermis to remove trapped ROL Palm. The biopsy procedure and grinding procedure were as given above. The powdered tissue was added to a homogenizer containing organic solvents chloroform:methanol (2:1) with a tracer amount of tritiated RA to determine recoveries. After six to eight strokes of the pestle, the homogenized biopsy was transferred to a polypropylene screw cap tube. The samples were vortex-mixed intermittently for 10 min. The samples were centrifuged and the supernatant fractions were transferred to new tubes. The pelleted material was re-extracted and processed in a similar manner. The final extraction was with methanol:acidified water, pH 3.2 (85:15). The combined supernatants were evaporated to dryness, resuspended in methanol, filtered into HPLC vials, and evaporated to dryness. The samples were resuspended and injected onto the HPLC. The HPLC elution program was lengthened to 110 min for the ROL Palm-treated tissue samples as previously given (Kang *et al*, 1995) to quantitate the retinyl ester content of the samples.

Miscellaneous Procedures Protein determinations were carried out by the method of Lowry *et al* (1951) with bovine serum albumin as the standard. Statistical analyses for all assays were performed using either a two-tailed paired t test or the repeated measures analysis of variance and the Tukey procedure for multiple group comparisons.

RESULTS

Dose-Response Relationship between Retinoid Concentrations Applied with Occlusion and the Amount of RA 4-OHase Activity Induced The data given in **Fig 1** indicate that 24 h after topical application of RA with occlusion, there is a linear increase in RA 4-OHase activity with increasing RA concentrations up to the maximum level tested of 0.05% RA (796 ± 163 pg of 4-OH RA formed per min per mg of protein, n = 6). The lowest concentration tested, 0.001% RA, gave a statistically significant induction of RA 4-OHase activity (237 ± 44 pg of 4-OH RA formed per min per mg of microsomal protein) in comparison to induction of activity in vehicle-treated areas (105 ± 35, n = 6, $p < 0.01$).

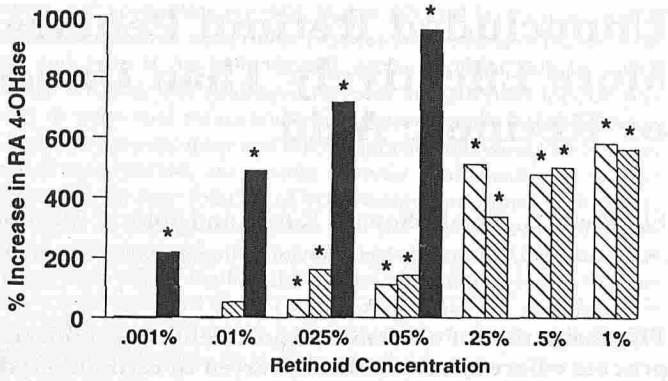

Figure 1. Percent increase in RA 4-OHase activity in human epidermis 24 or 48 h after application of retinoids with occlusion. Keratome biopsy specimen were obtained 24 or 48 h after a single application of different concentrations of retinoids under occlusion. Microsomal fractions from these specimens were used in the *ex vivo* assay with 2 μM [^{3}H]RA as substrate. Details are given in *Materials and Methods*. The mean value of RA 4-OHase activity in vehicle-treated sites (75 ± 12 pg of 4-OH RA formed per min per mg of microsomal protein, n = 23) from the dose-response series was used as baseline for calculating the percent increase in RA 4-OHase activity after retinoid application. Widely hatched bars, ROL-treated sites; thinly hatched bars, RAL-treated sites; solid bars, RA-treated sites. Six individuals were biopsied for all retinoid concentrations, except for 0.05% ROL and 0.5% ROL, where 12 individuals were biopsied. *$p < 0.05$ for the retinoid-treated sites *versus* vehicle.

ROL concentrations of 0.001–1.0% with occlusion were applied for 48 h to two groups of six individuals with vehicle and 0.05% and 0.5% concentrations included in both series to give an n = 12 for these three concentrations. Data given in **Fig 1** show that 0.001% and 0.01% concentrations did not give significant inductions of RA 4-OHase activity in comparison to activity in vehicle-treated areas. A statistically significant ($p < 0.02$) increase in RA 4-OHase activity, however, occurred for all remaining concentrations with a linear increase from 0.025% to 0.25%. Only a slight increase in enzyme activity occurred with the highest concentration of 1.0%. The greatest induction of enzyme activity after ROL applications was 510 ± 79 pg of 4-OH RA formed per min per mg of protein after application of 1.0% ROL for 48 h.

RAL was applied for 48 h with concentrations of 0.001–1.0% with occlusion. The increase in RA 4-OHase activity exhibited a linear dose-response relationship between concentration of RAL applied and induction of enzyme activity as shown in **Fig 1**. A statistically significant increase ($p < 0.05$) in activity occurred at all concentrations tested in comparison to activity in vehicle-treated areas except for 0.001% and 0.01%. In an earlier series of six individuals, 0.01% RAL gave a significant increase in enzyme activity after 24 h (data not shown). Apparently 0.01% RAL is the threshold concentration in some individuals for sufficient RAL penetration and sufficient conversion to RA to induce RA 4-OHase activity. At 1.0% RAL, the enzyme activity was 496 ± 85 pg of 4-OH RA formed per min per mg of protein; this activity is similar to the activity obtained with ROL at the same concentration.

Data in **Fig 1** indicate that the hormone RA is the best inducer of RA 4-OHase activity at all concentrations. Of the concentrations tested, RAL is a better inducer of enzyme activity at low concentrations (0.01% and 0.025%) than ROL, but at higher concentrations (0.05% to 1.0%) both ROL and RAL are equally effective inducers of the enzyme activity.

Retinoids Extracted from Epidermal Sites Treated with ROL Palm under Occlusion Because ROL and ROL Palm are included in cosmetic preparations, ROL Palm penetration and induction of 4-OHase activity were assessed. ROL Palm (0.6%) was applied to human epidermis for 48 or 72 h under occlusion and a similar site with vehicle alone. After tape stripping the sites, the biopsies were processed as in *Materials and Methods*. The results in **Fig 2** show that *trans*-ROL was the predominant retinoid extracted from the tissue at 48 or 72 h after application of ROL Palm. 13-*cis*-ROL was present in measureable quantities in the viable layers probably due to isomerization of *t*-ROL as it penetrated into the epidermis. ROL linoleate was the most prominent retinyl ester with little free ROL Palm detected in the viable layers. A small amount of 14-hydroxy-4,14-*retro*retinol was also extracted from the biopsy samples but RA was not detected.

Application of ROL Palm to Human Skin with Occlusion Induces RA 4-OHase Activity Two series of volunteers were involved in assessing the effects of ROL Palm and palmitate under occlusion on the induction of RA 4-OHase activity. In the first series, biopsy samples were obtained 4 d after a single application of vehicle or 0.1%, 0.3%, and 0.6% ROL Palm or 0.025% RA, n = 7. In the second series, biopsy samples were obtained 4 d after a single application of vehicle or 0.1%, 0.3%, and 0.6% palmitate or 0.6% ROL Palm. The data in **Fig 3** indicate that at a concentration of 0.6% ROL Palm there is a statistically significant induction of RA 4-OHase (247 ± 42 pg of 4-OH RA formed per min per mg of microsomal protein, $p < 0.01$, n = 7) in comparison to activity in vehicle-treated areas (19 ± 6, n = 7). The enzyme activity in 0.025% RA-treated sites was 354 ± 38 pg of 4-OH RA formed per min per mg of microsomal protein and was not significantly different from the activity in the 0.6% ROL Palm-treated sites. Palmitate itself did not induce RA 4-OHase activity. The 0.6% ROL Palm-treated sites were the positive control for this series. There was a significant increase in RA 4-OHase activity in ROL Palm-treated areas in comparison to activity in areas treated with palmitate alone, $p < 0.01$. Upon hydrolysis 0.6% ROL Palm would give approximately 0.3% ROL. The RA 4-OHase induced by 0.6% ROL Palm is 54% of the activity induced by 0.25% ROL shown in **Fig 1**.

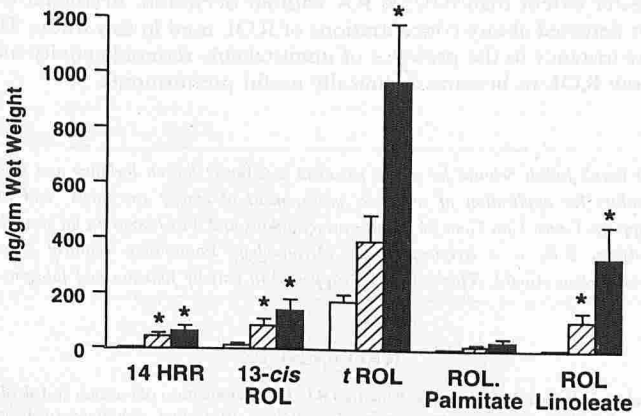

Figure 2. All-*trans*-ROL is the most abundant retinoid extracted after a single application of ROL Palm to human skin under occlusion for 48 or 72 h. ROL Palm (0.6% in 95% ethanol—the highest concentration that would go into solution) was applied to human skin. The stratum corneum was removed by tape stripping of the surface. The viable portion of the epidermis was keratomed and the biopsy was extracted as described in *Materials and Methods*. The HPLC run time was 110 min, which was required to separate the ROL esters. 14-HRR, 14 hydroxy-4,14-*retro*-retinol. Open bars, vehicle; hatched bars, 48-h ROL Palm; solid bars, 72-h ROL Palm. *$p < 0.05$ in comparison of data from ROL Palm-treated sites to vehicle-treated sites. *Error bars,* SEM (n = 8).

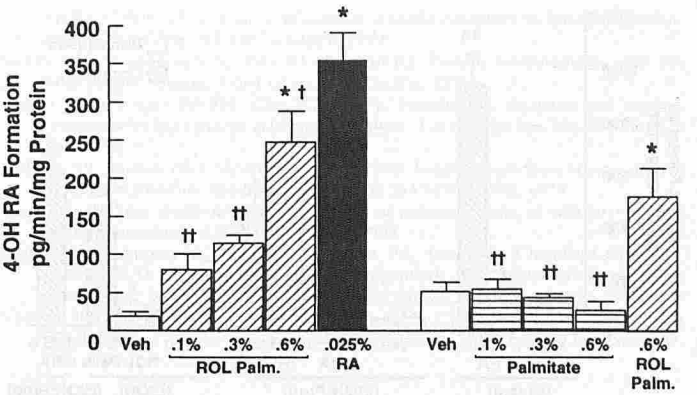

Figure 3. Increased RA 4-OHase activity was detected 4 d after a single occluded application of ROL Palm but not with the application of palmitate alone. Methods are as in **Fig 1**. To the left for ROL Palm, bars are as follows: open bars, vehicle; hatched bars, ROL Palm; solid bars, 0.025% RA. To the right for the palmitate series, bars are as follows: open bars, vehicle; horizontally strippled bars, palmitate; hatched bars, ROL Palm. *$p < 0.01$ for retinoid in comparison to vehicle; †p not significant in the comparison between 0.025% RA and ROL Palm; ††$p < 0.05$ when comparing data from 0.6% ROL Palm-treated sites with data from sites treated with other ROL Palm concentrations or with palmitate-treated sites. *Error bars,* SEM [n = 7 (for ROL Palm) and 6 (for palmitate)].

Occlusion of Treated Areas Enhances Induction of RA 4-OHase Activity for Retinoids Tested Except for ROL The data shown in **Fig 4** are the results obtained with three separate groups of volunteers. The initial retinoid and vehicle applied was Retin A, 0.1% RA in cream vehicle, and cream vehicle. Sites treated with RA and occluded showed a marked induction of RA 4-OHase activity of 927 ± 118 pg of 4-OH RA formed per min per mg of protein ($p = 0.0005$ in comparison to activity in areas treated with cream vehicle). The unoccluded sites, however, showed a much smaller induction of enzyme activity, only 16% of the activity induced in the occluded areas. A statistically significant induction of RA 4-OHase activity in the unoccluded RA-treated sites (150 ± 12 pg of 4-OH RA formed per min per mg of protein) in comparison to activity in unoccluded vehicle-treated areas (85 ± 8) was detected ($p = 0.008$). Activity in the unoccluded sites, however, was also significantly lower in a comparison with the activity in occluded sites ($p = 0.0007$).

In the second group, 0.025% RA and 0.25% ROL were prepared in a vehicle composed of 95% ethanol:propylene glycol (7:3) with the anti-oxidant butylated hydroxytoluene. There was no difference in enzyme activity observed in the unoccluded ROL-treated areas (455 ± 64 pg of 4-OH RA formed per min per mg of protein) in comparison with activity in occluded ROL treated areas (465 ± 54, n = 8, $p = 0.9$). Unoccluded areas treated with RA in this vehicle, however, induced a higher level of enzyme activity than had been observed in areas treated with RA in cream vehicle. The unoccluded RA-treated areas contained 42% of the enzyme activity measured in the occluded RA-treated areas (553 ± 99 pg of 4-OH RA per min per mg of protein, n = 8) but there was still a significant difference ($p < 0.005$) in enzyme activity when the two sites were compared. There was no significant difference between enzyme activity measured in occluded 0.025% RA-treated areas and the activity in occluded or unoccluded 0.25% ROL-treated areas ($p > 0.4$).

The data given in **Fig 4** also show the results obtained with application of ROL Palm. There is a significant induction of 4-OHase activity in tissue from sites treated with 0.6% ROL Palm without occlusion (87 ± 14 pg of 4-OH RA per min per mg of protein, n = 8) in comparison to activity in vehicle-treated areas (46 ± 9 pg of 4-OH RA per min per mg of protein, n = 8, $p < 0.05$). The OHase activity in occluded ROL Palm-treated areas was 371 ± 92 pg of 4-OH RA formed per min per mg of protein, n =

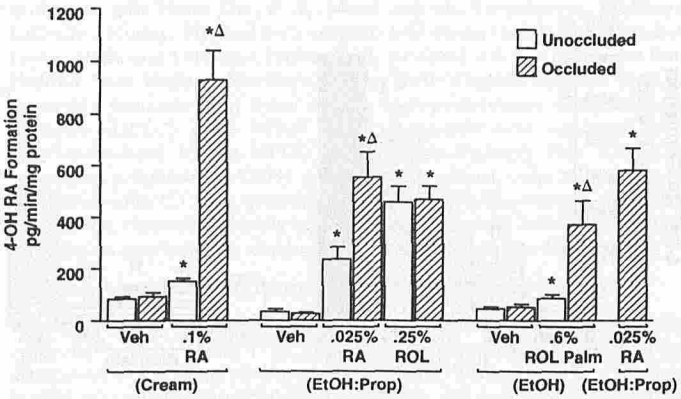

Figure 4. Occlusion enhances induction of RA 4-OHase activity in retinoid-treated epidermal sites except for ROL. To the left, one set of paired sites received a single application of RA cream or vehicle cream and these sites were occluded for 4 d. The other set of paired sites were left unoccluded and were treated daily for 4 d with 0.1% RA in cream or its vehicle cream as detailed in *Materials and Methods*. All other methods as given in **Fig 1**. *$p = 0.008$ for comparisons of enzyme activities in vehicle sites *versus* activity in RA-treated sites for both unoccluded or occluded areas. $\Delta p = 0.0007$ for the comparison of enzyme activity in unoccluded RA-treated sites *versus* activity in occluded RA-treated sites. *Error bars,* SEM ($n = 6$). In the middle, RA and ROL were prepared in vehicle composed of 95% ethanol:propylene glycol with anti-oxidant. Data for RA 4-OHase activity after application of retinoids or vehicle are shown. *$p < 0.001$ in a comparison of retinoid-treated areas to vehicle-treated areas. There was no significant difference in enzyme activity when the occluded ROL- and RA-treated areas were compared to unoccluded ROL (p = NS). $\Delta p < 0.005$ when induction of RA 4-OHase activity in RA-treated sites under occlusion was compared to activity in RA-treated sites left unoccluded. *Error bars,* SEM ($n = 8$). To the right, ROL Palm was prepared in a vehicle composed of 95% ethanol with anti-oxidant. Data for RA 4-OHase activity after application of retinoids or vehicle are shown for each compound applied. *$p < 0.05$ in a comparison of retinoid-treated areas to vehicle-treated areas; $\Delta p < 0.05$ when induction of RA 4-OHase activity in occluded ROL Palm sites were compared to activity in unoccluded ROL Palm-treated sites. *Error bars,* SEM ($n = 8$).

8, and was significantly increased in comparison to activity in occluded vehicle-treated sites of 54 ± 11 pg of 4-OH RA formed per min per mg of protein ($n = 8$, $p < 0.05$). There was also a statistically significant difference ($p < 0.05$) in the RA 4-OHase activity in the occluded ROL Palm-treated sites in comparison to the activity in unoccluded ROL Palm-treated sites. ROL Palm without occlusion gave results similar to 0.1% RA in cream vehicle without occlusion.

Clinical and Histologic Changes after Application of ROL, RAL, and ROL Palm The two common effects observed after application of RA to the epidermis are erythema and hyperplasia. A single application of 0.05% ROL, 0.05% RAL, or 0.3% ROL Palm produced a statistically significant ($p < 0.05$, $n = 8$) increase 4 d later in epidermal thickness of 58% (63 ± 6.6 μm) for ROL, 33% (52 ± 5.8 μm) for RAL, and 38% (55 ± 5 μm) for ROL Palm in comparison to epidermal thickness in vehicle-treated areas (40 ± 2.7 μm; data not shown). The erythema observed in response to RA applications, however, was not observed with these retinoids (data not shown).

DISCUSSION

In this study the effectiveness of ROL, RAL, and ROL Palm as retinoids in comparison to RA was investigated with and without occlusion. The induction of RA 4-OHase activity was the biochemical endpoint, and erythema and increased epidermal thickness were the clinical endpoints. With occlusion a linear dose-response increase in RA 4-OHase activity was obtained from the lowest (0.001%) to the highest (0.05%) concentrations of RA. It is reasonable to assume that a higher concentration of a precursor (ROL Palm, ROL, or RAL) would be required to achieve the same magnitude of response achieved with the hormone itself. The data presented herein are consistent with this assumption in that a higher concentration of ROL (25-fold) or RAL (10-fold) than RA is required to induce a significant increase in 4-OHase activity. Earlier reports had also shown that with occlusion higher ROL levels (0.01%) were required to increase cellular retinoic acid binding protein II mRNA (Kang *et al*, 1995) in comparison with the concentrations of RA (0.001%) needed to observed similar results (Elder *et al*, 1993).

In this study the induction of 4-OHase activity begins to plateau at a concentration of 0.25% ROL and 0.5% RAL. ROL may be shunted into retinyl ester formation to prevent further conversion of ROL to RA, which has been recently reported to occur in a human keratinocyte culture system by Kurlandsky *et al* (1996). Because RAL can be reduced to ROL as well as oxidized to RA, the plateauing of RA 4-OHase induction by high RAL concentrations probably reflects the same control mechanisms with an increased metabolism of RAL to ROL and ester formation. In a long-term study of Saurat *et al* (1994), the topical application of RAL increased epidermal thickness and cellular retinoic acid binding protein II mRNA content. These effects are the classical responses observed after topical application of RA. The lowest concentration of RAL used in their study was 0.05%.

Application of ROL Palm increased ROL but not ROL Palm content in the treated sites. This is consistent with the hydrolysis of ROL Palm to ROL and subsequent conversion to RA before the induction of RA 4-OHase activity occurs. The *in vitro* test system by Boehnlein *et al* (1994) produced similar results in that topical application of ROL Palm to guinea pig or human skin increased ROL concentration in the skin but not ROL Palm concentrations. Palmitate itself had no effect on induction of RA 4-OHase activity.

Both the composition of the vehicle and occlusion altered the induction of 4-OHase activity. RA in cream vehicle and ROL Palm in 95% ethanol vehicle required occlusion to achieve high levels of 4-OHase activity reflecting relatively high concentrations of RA in the viable areas of the epidermis. ROL penetration into the epidermis and induction of 4-OHase activity was substantial at high doses without occlusion and was not further increased by occlusion of the treated areas. Absence of a carboxyl group may permit penetration of ROL without occlusion. Topical application of 0.25% ROL without occlusion induces 4-OHase activity to a greater extent than 0.025% RA without occlusion. Erythema was not detected at any concentrations of ROL used in this study. The low irritancy in the presence of unmistakable retinoid activity may allow ROL to become a clinically useful prohormone.

We thank Judith Schmitt for expert technical assistance; Suzan Rehbine and Robin Gardner for application of retinoids, procurement of biopsy specimens, and tape stripping; Laura Van Goor for graphic presentations and Ted Hamilton for statistical analyses. S.K. is a recipient of the Dermatology Foundation Clinical Career Development Award. This work was supported in part by Johnson and Johnson.

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