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Metabolism of Topical Retinaldehyde

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Abstract
Objective: In order to circumvent the tolerance problems encountered with topical application of retinoic acid – a biologically active metabolite of vitamin A – we performed in various models a series of experiments aimed at assessing the bio-availability of topical retinaldehyde and its conversion into either retinoid stores or biologically active metabolites. ***Methods:*** (i) ^{3}H-retinaldehyde was used as a precursor of either ^{3}H-retinol or ^{3}H-retinoic acid in human skin extracts and human cultured keratinocytes; (ii) the concentration of various retinoids resulting from the metabolism of topical retinaldehyde was determined in mouse skin and in human plasma. Retinoids were quantified by reverse-phase HPLC with UV detection. ***Results:*** Human keratinocytes were shown to take up retinaldehyde and to convert it into retinoic acid in a differentiation-dependent manner, differentiating cells oxidising retinaldehyde more efficiently. In vivo models allowed us to demonstrate that retinaldehyde is taken up by the skin and is then predominantly converted into retinyl esters – a storage form of vitamin A – while delivering relatively low amounts of retinoic acid from a large reservoir. ***Conclusion:*** Topical retinaldehyde can be used as a precursor of endogenous retinoids, since it is converted into both storage and bioactive forms of vitamin A.

Introduction

In spite of the clinical benefits gained by the use of topical retinoic acid isomers in several skin diseases, the erythemogenic properties of these vitamin A metabolites often prevent their use in clinics and promoted a great deal of interest for a new class of vitamin A analogues which would be well tolerated, while preserving the therapeutic potential of natural ligands for nuclear retinoic acid and retinoid X receptors. Since irritation is probably not involved in the beneficial action of retinoids [1, 2], all-*trans*-retinaldehyde (RAL), which was shown to be well tolerated [3], was investigated for its use as an alternative for topical retinoic acid. In particular, we have conducted studies aimed at analysing the two metabolic pathways of RAL, i.e. (i) the oxidation into retinoic acid isomers – the ligands for nuclear receptors – and (ii) the reduction into all-*trans*-retinol (ROL), followed by esterification with long-chain fatty acids, leading to the storage form of retinoids. We have used ex vivo, in vitro and in vivo approaches.

Results

Ex vivo: Human Skin Extracts

Cytosolic extracts were prepared from keratomised normal human skin and incubated with 600 n*M* ^{3}H-RAL.

The metabolites formed depended on the redox co-factor co-incubated with ^{3}H-RAL: in the presence of 5 m*M* NADH, ^{3}H-ROL was formed at a rate of 1.0 ± 0.1 pmol/mg protein/h, whereas in the presence of 2 m*M* NAD, ^{3}H-all-*trans*-retinoic acid (RA) was formed at a rate of 1.5 ± 0.2 pmol/mg protein/h [4] (table 1).

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Table 1. Ex vivo: cytosolic extracts of human skin slices (180 µm)

Precursor	Analyte	Co-factor	Value pmol/mg protein/h	Reference
600 n*M* ^{3}H-RAL	^{3}H-ROL	NADH (5 m*M*)	1.0 ± 0.1	4
600 n*M* ^{3}H-RAL	^{3}H-RA	NAD (2 m*M*)	1.5 ± 0.2	4

Table 2. In vitro: human cultured keratinocytes

Fraction	Differentiated	Precursor	Co-factor	Analyte	Value	Unit	Reference
Cytosol	yes	600 n*M* ^{3}H-ROL	–	^{3}H-RA	4.49 ± 0.10	pmol/mg protein/h	4
	yes	600 n*M* ^{3}H-RAL	NAD (2 m*M*)	^{3}H-RA	51.6	pmol/mg protein/h	4
	no	600 n*M* ^{3}H-RAL	NAD (2 m*M*)	^{3}H-RA	14.4	pmol/mg protein/h	4
	yes	600 n*M* ^{3}H-RAL	NADH (5 m*M*)	^{3}H-ROL	8.2	pmol/mg protein/h	4
	no	600 n*M* ^{3}H-RAL	NADH (5 m*M*)	^{3}H-ROL	8.0	pmol/mg protein/h	4
Whole cells	yes	50 n*M* ^{3}H-ROL	–	^{3}H-dRA	6.1 ± 2.2	pmol/mg DNA	5
	no	50 n*M* ^{3}H-ROL	–	^{3}H-dRA	1.9 ± 0.5	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-ROL	–	^{3}H-RA	8.1 ± 3.3	pmol/mg DNA	5
	no	50 n*M* ^{3}H-ROL	–	^{3}H-RA	7.6 ± 4.3	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-ROL	–	^{3}H-dROL	0.7 ± 0.2	pmol/mg DNA	5
	no	50 n*M* ^{3}H-ROL	–	^{3}H-dROL	0.8 ± 0.1	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-ROL	–	^{3}H-ROL	17.2 ± 3.8	pmol/mg DNA	5
	no	50 n*M* ^{3}H-ROL	–	^{3}H-ROL	24.4 ± 7.6	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-ROL	–	^{3}H-RE	1,617 ± 695	pmol/mg DNA	5
	no	50 n*M* ^{3}H-ROL	–	^{3}H-RE	380 ± 132	pmol/mg DNA	5
Whole cells	yes	50 n*M* ^{3}H-RAL	–	^{3}H-dRA	27.6 ± 7.7	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-dRA	8.3 ± 2.2	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-RAL	–	^{3}H-RA	59.5 ± 15.4	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-RA	16.9 ± 5.0	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-RAL	–	^{3}H-dROL	0.8 ± 0.7	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-dROL	0.2 ± 0.1	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-RAL	–	^{3}H-ROL	11.5 ± 3.2	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-ROL	5.3 ± 1.3	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-RAL	–	^{3}H-RAL	2.8 ± 0.9	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-RAL	2.9 ± 1.1	pmol/mg DNA	5
	yes	50 n*M* ^{3}H-RAL	–	^{3}H-RE	914 ± 237	pmol/mg DNA	5
	no	50 n*M* ^{3}H-RAL	–	^{3}H-RE	288 ± 75	pmol/mg DNA	5

dRA = Dehydro-RA; dROL = dehydro-ROL.

In vitro: Human Cultured Keratinocytes

Cytosolic Extracts. Human keratinocytes were cultured in low or normal calcium concentrations in order to distinguish between non-differentiating (low calcium) and differentiating (normal calcium) keratinocytes.

In the presence of 5 m*M* NADH, 600 n*M* ^{3}H-RAL were reduced into ^{3}H-ROL at about 8 pmol/mg protein/h in both culture types; conversely, the oxidation into ^{3}H-RA, in the presence of 2 m*M* NAD, was more rapid in differentiating cultures (51.6 pmol/mg protein/h) than in non-differentiating ones (14.4 pmol/mg protein/h) [4] (table 2).

Whole Cells. Since some enzymes are found in microsomes rather than in the cytosol, we performed a new series of experiments with whole-cell extracts of differentiating and non-differentiating cultured human keratinocytes. In this study, cultures were incubated with 50 n*M* ^{3}H-RAL without addition of exogenous redox co-factor.

Table 3. In vivo: topical application of RAL or 9-*cis*-RAL (14 days) on C5BL/6 mouse tail skin, washed or unwashed (analysis of whole skin)

Application	Washing	Analyte	Value pmol/g wet weight	Reference
Vehicle	no	ROL	244 ± 42	7
	yes	ROL	206 ± 20	6
	no	RE	282 ± 55	unpublished results
	yes	RE	417 ± 36	unpublished results
	no	RA	<15	7
	yes	RA	<15	6
RAL	no	ROL	2,619 ± 118[a]	7
	yes	ROL	1,556 ± 81[a, b]	6
	no	RE	8,600 ± 2,053[a]	unpublished results
	xes	RE	4,101 ± 218[a]	unpublished results
	no	RA	43 ± 9	7
	yes	RA	25 ± 3	6
9-*cis*-RAL	no	ROL	916 ± 32[a]	unpublished results
	yes	ROL	958 ± 22[a]	6
	no	RE	1,269 ± 243	unpublished results
	yes	RE	1,507 ± 147[a]	unpublished results
	no	RA	29 ± 1	unpublished results
	yes	RA	30 ± 1	6

[a] $p < 0.001$: different from vehicle group.
[b] $p < 0.001$: different from unwashed samples.

Differentiating cultures did metabolise ^{3}H-RAL into RA, dehydro-RA, ROL, dehydro-ROL and retinyl ester (RE) at a higher rate than non-differentiating cultures, although RAL uptake was the same in both culture types. When using ^{3}H-ROL as retinoid precursor, differentiating cultures were shown to produce more RA and RE than non-differentiating ones, whilst the other metabolites were produced at the same rate in both culture types. Moreover, except for RE in differentiating cultures, the metabolism of ^{3}H-RAL was higher than that of ^{3}H-ROL [5] (table 2).

These in vitro studies allowed us to demonstrate that RAL can be metabolised into RA, ROL and RE by human cultured keratinocytes; this process is dependent on the differentiation stage of keratinocytes since it is higher in differentiating than in non-differentating cells. We next studied if this occurs in vivo in a mouse model.

In vivo: Metabolism of Topical Retinoids by Mouse Tail Skin

Topical Retinal Isomers in Washed and Unwashed Mouse Tail. The tail of C57BL/6 mice was treated for 14 days with either excipient, RAL 0.05% or 9-*cis*-RAL 0.05%, a potential precursor of 9-*cis*-RA which was shown to have biological activities in this mouse model [6]. In half of the samples, the skin was washed with a solution of 0.1% Triton X-100; the whole skin of all samples was analysed for retinoid determination.

In unwashed tail skin, topical RAL promoted a 10-fold increase in ROL and a 30-fold increase in RE as compared to vehicle; this increase was more moderate in 9-*cis*-RAL-treated skin. The RA concentration was under the detection limit of 15 pmol/g wet weight in vehicle-treated skin but was measurable following a topical treatment with either RAL or 9-*cis*-RAL. The washing of the skin removed about one half of the retinoid content in RAL-treated skin (ROL, RE and RA), whilst this operation had no effect on the retinoid content of vehicle- and 9-*cis*-RAL-treated skin (table 3).

Topical RAL on Hairless Mice. The back of hairless mice was treated for 7 days with vehicle or RAL 0.05%, then the skin was harvested and the epidermis was separated from the dermis by heat. The retinoid content was determined in the epidermis. Topical RAL promoted a 6-fold increase in ROL and a 13-fold increase in RE, as well as a higher concentration of RA in the epidermis (226 pmol/g wet weight) than that of whole tail skin of C57BL/6 mice (43 pmol/g wet weight) (table 4).

Table 4. In vivo: topical application (7 days) on hairless mouse back skin (unwashed): analysis of epidermis

Application	Analyte	Value (pmol/g ww)	Reference
Vehicle	ROL	152 ± 20	unpublished results
Vehicle	RE	1,102 ± 85	unpublished results
Vehicle	RA	< 15	unpublished results
RAL	ROL	947 ± 79***	unpublished results
RAL	RE	13,761 ± 2,336***	unpublished results
RAL	RA	226 ± 26***	unpublished results

*** Different from vehicle group ($p < 0.001$).

Systemic Effects of the Skin Metabolism of Topical RAL in Humans

To see if topical application of a large quantity of RAL on human skin is associated with detectable alteration of constitutive levels of plasma retinoids resulting from metabolism of RAL in the skin, plasma retinoids [ROL, RE (all-*trans*-retinyl oleate + palmitate), RAL, RA, 13-*cis*-RA, 4-oxo-13-*cis*-RA] were analysed by HPLC in 10 healthy male volunteers kept under a poor vitamin A diet before, during and after daily topical application for 14 days of 7 mg (25 µmol) of RAL to 40% of the body surface.

The introduction of a 1-week restricted vitamin A diet before RAL application resulted in a decrease in plasma levels of ROL, RA and RE. Topical RAL did not induce an alteration of the retinoid metabolite plasma levels. No RAL was detectable in any of the plasma samples.

These results indicated that the skin metabolism of topically applied RAL does not result in detectable alteration of constitutive levels of plasma retinoids in humans. Since there was no increase in plasma RE during topical RAL treatment (despite a previous reduction in plasma RE due to the diet), it is likely that the RE produced from the daily applied 7 mg of RAL remained stored in the epidermis. The proportion of RAL transformed into ROL is not likely to be delivered systemically since epidermal enzymes would metabolise it to RE. At any rate, the constitutive levels of plasma ROL (about 2 µ*M*) were not altered by the small amount that may originate from skin. In addition to providing important safety data pertinent to the potential use of RAL as a topical agent in humans, these results supported the concept of targeting vitamin A metabolites in the skin upon topical treatment with RAL.

Discussion

These observations indicate that RAL would fulfil one criterion in the concept that is to target multipotential vitamin A activity into distinct compartments of the epidermis. Indeed, topically applied RAL would (i) be a precursor of either ROL, RE or RA, (ii) bypass the first, rate-limiting step of ROL oxidation into RA and (iii) be handled only by the epidermal cells having enzymatic activities at a pertinent stage of differentiation resulting in a controlled delivery of vitamin A metabolites into the cells. RAL does not bind to retinoid nuclear receptors [8, 9]. Therefore its biological activity should result from its enzymatic transformation into RA by epidermal keratinocytes and should be qualitatively similar to that of RA. In order to exert a quantitatively comparable activity to that resulting from direct application of RA, topical RAL should generate enough amounts of RA to saturate nuclear retinoic acid receptors. As shown above, this is achieved in mouse skin where RAL concentrations reach 4–20 n*M*, a range of concentrations high enough to saturate nuclear receptors [8, 10], but much less than those (1–10 µ*M*) reached after topical RA [7, 11]. Finally, the modulation of retinoid metabolism induced by a topical RAL application would be restricted to the skin, since a 14-day topical treatment with RAL has no effect on the concentration of plasma retinoids.

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