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# Metabolic Conversion of Retinol to Retinoic Acid Mediates the Biological Responsiveness of Human Mammary Epithelial Cells to Retinol

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The biological effects of vitamin A are mediated in part by retinoic acid (RA) modulation of gene transcription. In this study, we examined whether normal human mammary epithelial cells (HMECs) are biologically responsive to retinol (ROH), the metabolic precursor of RA. While both ROH and tRA resulted in time- and dose-dependent decreases in total cell number, tRA was markedly more potent. Metabolically, treatment of HMECs with physiological doses of ROH resulted in rapid uptake and subsequent production of both retinyl esters and tRA. Although a comparatively minor metabolite, tRA levels peaked at 6 h and remained above endogenous levels for up to 72 h in proportion to cellular ROH concentrations. In HMECs transfected with an RA-responsive luciferase reporter gene, treatment with 3 μM ROH resulted in an increase in luciferase activity to a level intermediate between that observed with 0.001 and 0.01 μM tRA. Citral, an RA-synthesis inhibitor, was also used to examine the biological activity of ROH. Compared to ROH alone, ROH plus citral treatment resulted in three-fold less tRA synthesis and a >65% attentuation of RA-responsive reporter gene activity which persisted through 72 h. Citral also significantly attenuated the extent of ROH-mediated reductions in total HMEC number. Thus, treatment with physiological concentrations of ROH results in fewer total numbers of HMECs and this response is a consequence of cellular tRA synthesis which can induce RA-responsive gene expression. J. Cell. Physiol. 186:437–447, 2001. 

All-*trans* retinol (ROH) and its metabolites are important regulators of epithelial cell differentiation and growth, and are required for the maintenance of normal epithelial tissue integrity and function (De Luca et al., 1994; Gudas et al., 1994). The biological effects of vitamin A are thought to be mediated primarily by its oxidative metabolite retinoic acid (RA), although other metabolites may exert biological actions in certain cell types and tissues (Gudas et al., 1994; Napoli 1999; van der Saag, 1996).

Conceptually, ROH is part of a hormone-like signaling system which functions in an autocrine and/or paracrine manner. Cells take-up ROH from the circulation and convert it to retinyl esters for storage and/or generate active metabolites including all-trans retinoic acid (tRA) and its isomer 9-*cis* retinoic acid (9cRA) (Giguere, 1994; Napoli, 1999). Many of the effects of tRA and 9cRA are transduced by two distinct families of nuclear receptors which are members of the larger steroid/thyroid hormone receptor superfamily (Mangelsdorf, 1994; van der Saag, 1996). All-*trans* RA binds to and activates nuclear retinoic acid receptors (RARs), while 9cRA serves as an activating ligand for both RARs and the retinoid X receptors (RXRs) (Heyman et al., 1992; Levin et al., 1992). These receptors function principally as homo- or heterodimeric transcription factors that modulate gene transcription by binding to RA response elements (RAREs and RXREs) in the promoter region of target genes (Mangelsdorf, 1994). Thus, in responsive cells, transcription is regulated in part by the abundance of RA isoforms which favor specific homo- or heterodimer receptor activation (Mangelsdorf, 1994; Pfahl and Chytil, 1996).

In vivo, two sources of RA could contribute to the vitamin A requirements of epithelial tissues. Circulating plasma contains 4–14 nM of endogenous RA (Fex et al., 1996; Meyer et al., 1994; Takeda and Yamamoto,

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1994) which could be taken-up directly and utilized by target cells. Alternatively, cells could acquire ROH from plasma which contains approximately 2–2.5 μM ROH under normal physiological conditions (Looker et al., 1988; Nierenberg et al., 1997; Rogers et al., 1993). This cell-acquired ROH could then be used in situ to metabolically regulate RA generation. The relative contribution of each of these sources to cellular RA status is, however, unknown.

Studies using keratinocytes as a model system have shown that these cells have the ability to convert exogenously administered ROH to a number of metabolites including tRA (Kurlandsky et al., 1994; Randolph and Simon, 1993, 1995). Given the lower biological potency of ROH compared to tRA, and the reduction in ROH-induced cellular responses following treatment with agents that inhibit tRA production (Connor, 1988; Kurlandsky et al., 1994; Raner et al., 1996; Tanaka et al., 1996; Vandersea et al., 1998), it is reasonable to suggest that in responsive cells, ROH-induced biological effects are a function of the extent of conversion of ROH to tRA. Although a link between ROH-induced biological responses and the generation of RA has been shown in epidermal cells (Connor, 1988; Kurlandsky et al., 1994), this metabolic link has not been demonstrated in other cell types.

Mammary carcinoma cells are responsive to ROH (Halter et al., 1993; Lacroix and Lippman, 1980; Ueda et al., 1980) and RA (Agadir et al., 1999; Takatsuka et al., 1996), exhibiting dose-dependent reductions in proliferation. While it is known that tRA also inhibits the growth of normal mammary cells (Lee et al., 1995; Seewaldt et al., 1997), the role of ROH in growth regulation of normal mammary cells remains to be ascertained. Mammary epithelia is dependent on vitamin A, as exemplified by its role in normal glandular activity and development (Sankaran and Topper, 1982; Chew et al., 1985). This, as well as the presence of ROH in breast tissue (Rautalahti et al., 1990; Nierenberg and Nann, 1992; Zhu et al., 1995) and the presence of substantial quantities of ROH and retinyl esters in human milk (Barua et al., 1997; Canfield et al., 1997, 1998; Ortega et al., 1997), indicates that mammary cells can take-up, esterify, and secrete ROH during lactation. In addition, normal mammary epithelial cells in vitro have been shown to rapidly metabolize exogenous ROH to retinyl esters, indicating that esterification is an important aspect of normal mammary cell function at times other than lactation (Chen et al., 1997). Given that tRA is an active metabolite of ROH regulating gene expression, the purpose of this study was to establish whether the metabolic generation of RA from ROH is an obligatory part of ROH's biological activity in normal human mammary epithelial cells (HMECs).

## MATERIALS AND METHODS

### Retinoids

Retinoids were handled under reduced, indirect light to prevent photo-oxidation and were stored under inert gas. Stock solutions of ROH, tRA, 9cRA and 13-*cis* retinoic acid (13cRA) were obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and were prepared in dimethylsulfoxide (DMSO) in amber vials at known concentrations. The internal standard *p*-(E-2-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthalenyl-1-propenyl) benzoic acid (TTNPB) (Biomol Corp., Plymouth Meeting, PA) was prepared as described above. Concentrations of individual retinoid stock solutions were determined spectrophotometrically using their published wavelength maximums and corresponding molar extinction coefficients (Furr et al., 1994). Citral (3,7-dimethyl-2,6-octadienal) (Sigma Chemical Co., St. Louis, MO), used in metabolic inhibition studies, was also prepared as a stock solution in DMSO.

### Cells and cell culture

HMECs (Clonetics, San Diego, CA) were primary cultures derived from healthy women who had undergone reduction mammoplasties. All experiments were performed using cells between passages 6 and 12. Cells were grown and maintained in mammary epithelial basal medium (MEBM) supplemented with 13 mg/ml bovine pituitary extract, 0.5% serum, 5 μg/ml insulin, 10 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 μg/ml amphotericin-β (Clonetics, San Diego CA). Cells were maintained in a humidified environment at 37°C with 5% $CO_2$ in air.

### Cellular retinol uptake and metabolism

To assess ROH uptake and its subsequent cellular metabolism, HMECs were plated in 100-$mm^2$ dishes and grown in MEBM. At approximately 80% confluence, experiments were initiated by the addition of fresh media containing either 2.5 μM ROH or DMSO (vehicle control). Media aliquots were taken initially to verify ROH concentrations and at designated times (2, 6, 24, 48 and 72 h) for HPLC analysis. These media ROH concentrations were compared to those of dishes containing ROH-treated media but no cells.

At designated times following the initiation of retinoid treatment, the media was removed and the cells were rinsed twice with ice-cold phosphate-buffered saline (PBS). Following aspiration of the second PBS rinse, 2 ml of a 0.002% (v/v) sodium dodecyl sulfate solution was added to each dish to initiate cell lysis and facilitate the removal of the adherent monolayer. Cells were scraped with a plastic cell lifter and the cell suspensions were transferred into 15 ml tubes and stored at −80°C. For metabolic dose-response studies, cells were treated with 0.625, 1.25 or 2.5 μM ROH as above. Cells were then harvested, extracted and retinoids analyzed at 0.25, 6 and 24 h after initiation of treatment with the indicated dose of ROH. Additional experiments which assessed the ability of citral to inhibit the metabolism of ROH to RA were initiated as previously described. Once plated, cells were treated with either 1.25 μM ROH alone, 1.25 μM ROH plus 5 μM citral, or the vehicle (DMSO) alone. Cells were then harvested at the indicated times as described above.

### Retinoid extraction

Cell lysates were sonicated on ice (Vibra Cell Sonicator, Sonics and Materials Inc., Danbury, CT) and a small aliquot was removed for protein quantification. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL).

Following sonication, the internal standard TTNPB was added to the lysate. Retinoids were then extracted by sequential additions of 1.5 vol acetonitrile/butanol (1:1 v/v), 0.5 vol hexane/chloroform (2:1 v/v), followed by 0.2 vol saturated $K_2HPO_4$, and were mixed vigorously. Following centrifugation at 13,000*g* for 10 min, the organic phase was transferred to an amber tube and dried in the dark, under a vacuum. The resultant sample residue was reconstituted in 150 μl methanol/acetonitrile/isopropanol (3:1:1 v/v) and analyzed using HPLC for identification and quantitation of retinoids. Cell-associated retinoids are expressed as picomoles retinoid per milligram protein. Retinoids from media samples collected at designated time points were extracted by the addition of 300 μl of methanol/isopropanol/acetonitrile (3:1:1 v/v) and were frozen until analyzed. Samples were thawed, centrifuged at 13,000*g* for 15 min, and the supernate was analyzed directly using HPLC.

### HPLC analysis

Retinoid analysis and quantitation was performed using a reverse phase Nova-Pak C18 (8.0 cm × 10.0 cm) 4 μm pore size analytical column (Waters Associates, Milford, MA). Media ROH samples were eluted under isocratic conditions (1 ml/min) using an elution solvent (mobile phase) of methanol/TAI/0.005 M ammonium acetate (78:10:12 v/v). The TAI solution was comprised of tetrahydrofuran/acetonitrile/2-propanol (1:3:.02 v/v). Reconstituted cell extracts were analyzed for retinoids under isocratic conditions using an elution solvent mobile phase of methanol/TAI/0.005 M ammonium acetate (67:10:23 v/v). Retinoids were detected by UV absorbance at 324 nm for the media samples, and at 350 nm for the cell extracts. Under these conditions, the system has a lower limit of detection for RA of approximately 1.3 pmoles at a signal to noise ratio of 2.5; detection limits for ROH are approximately 3.1 pmoles. Retinyl esters were eluted using a nonaqueous mobile phase of acetonitrile/dichloromethane (80:20 v/v) (Furr et al., 1986).

For initial calibrations, pure retinoid standards were made in methanol and their concentrations were determined by UV absorbance using published maximal absorbance wavelengths and corresponding molar extinction coefficients (Furr et al., 1994). The elution position of matching retinoid standards was used to identify specific retinoid peaks which were then quantified by computer integration of the areas under the respective peaks. Accuracy of the method and calibration conditions were further verified using authenticated samples containing known quantities of retinoids (National Bureau of Standards). The purity of the retinoids (retinol and retinoic acid isomers) was verified by determining the absorption spectra of isolated peaks. Purity was also assessed on selected samples by evaluation of absorption spectra using photodiode array detection.

### Retinoid effects on cell number

To assess the effects of retinoids on total cell number, HMECs were seeded in 24-well culture dishes at $1.5 \times 10^3$ cells/well and allowed to attach and grow overnight. Fresh media containing varying concentrations (1 nM–10 μM) of either ROH, tRA, or DMSO was then added to the cells. In separate experiments, cells were treated with 13cRA, 9cRA or retinyl palmitate (0.01, 0.10 or 1.0 μM) as described above. To assess the effect of the metabolic inhibitor citral on cell number, HMECs were seeded as described above and were grown in media containing either 1.25 μM ROH alone, 5 μM citral alone, or 1.25 μM ROH plus 5 μM citral, and compared to cells treated with vehicle (DMSO) only as controls. Cell dishes were light protected for the duration of the experiment. Cells were treated once with no refeeding. The effect of the retinoids on cell number was assessed on days 3, 6, and 9, using a dye-binding assay (Serrano et al., 1997), based on the method initially described by Kueng et al. (1989). At designated times, the media was aspirated and the adherent monolayer was rinsed with PBS and fixed using a 1/10 volume of gluteraldehyde (11%) for 20 min. Fixed cells were rinsed thoroughly, air-dried and stained for 20 min in a 0.1% crystal violet solution in fresh 2 mM boric acid. Unbound dye was rinsed off, and the stain was solubilized in acetic acid and quantified on an ELISA plate reader (EAR 400 AT, SLT Lab Instruments, Austria) at 590 nm. Sample values were compared to those obtained from cells grown in media containing vehicle alone (0.1% DMSO). Four or five wells were used per treatment/time point. Since the cells are primary cultures from different donors with subtle, inherent differences in growth rates, and the dye used is made up fresh each time, there are slight, but measurable differences in actual OD readings between experiments. Thus, the data are presented as the mean ± standard deviation (SD) as a percent of controls to facilitate the use of values from at least three independent, replicate experiments.

### Transfection of HMECs and luciferase assays

Transient transfection studies using a luciferase-reporter construct were performed to assess RA-specific gene activation. The plasmid pGudLuc7rare (provided by Dr. Michael Denison and Jane Rogers), contains two tandem copies of the RA-response element sequence from the human RARβ gene ligated upstream of a minimal MMTV promoter and the firefly luciferase gene. The pGudLuc7rare plasmid, a carrier plasmid (PGL3.2; Promega Corp., Madison, WI) and renilla-reporter plasmid (pRL-CMVRenilla; Promega Corp., Madison, WI) containing the anthozoan coelenterate renilla reniformis gene, were introduced into HMECs by transient transfection using calcium-phosphate (Sambrook et al., 1989). Transfections were conducted in HMECs plated in 6-well tissue culture plates in media and were approximately 75% confluent at the time of transfection. Twenty-four hours after transfection, fresh media containing retinoids (tRA or ROH as indicated) either alone or in combination with citral, or DMSO vehicle alone, was then added. Following incubation for the indicated times (24, 72, 96 or 144 h.), cells were harvested and assayed for luciferase and renilla activity using the luciferase-renilla dual reporter assay kit (Promega Corp.). Luciferase-renilla reporter gene activities were determined using a luminometer (Turner Designs, Sunnyvale, CA). Treatments were conducted in duplicate or triplicate per experiment. Luciferase activity is presented following normalization to renilla-luciferase activity to account for variations in transfection efficiency. Each experiment was conducted

twice; data are presented as mean ± SD luciferase activity in arbitrary units.

### Statistical analysis

One-way analysis of variance (ANOVA) with subsequent post-hoc comparisons by Scheffe, were performed using Statview (Brainpower Inc., Calabazas, CA). A $P$ value of 0.05 was considered statistically significant. Values are presented as the mean ± standard deviation (SD) unless otherwise indicated.

## RESULTS

### Effects of ROH and tRA on cell number

Treatment of HMEC cultures with either ROH or tRA resulted in fewer cells and this response was both time- and dose-dependent (Fig. 1A and 1B). The effect of these retinoids on cell number was not apparent until after day 3, except at the highest dose tested (10 μM), for which significantly fewer cells were observed on day 3. That the effects on total cell number were not apparent until >3 days of exposure is not unexpected, given that the average doubling time of control HMECs was 40.24 ± 4.3 h. HMECs exhibited greater sensitivity to the anti-proliferative effects of tRA in comparison to its physiological substrate, ROH. Based on day 9 values, all doses of tRA >0.001 μM resulted in at least 25% fewer cells, while only doses of ROH exceeding 1.0 μM achieved this same extent of change in cell number. These and other data (not shown) indicate that an ROH concentration of ~1.25 μM was necessary for a 50% reduction in the total number of cells by day 9. In subsequent studies, cells were treated with the tRA isomers 13cRA and 9cRA. In comparison to an equivalent (1 μM) concentration of tRA, the isomer 9cRA was only about 28% as potent as tRA, while the 13-*cis* isomer was only about 22% as potent (data not shown). Retinyl palmitate, a prominent retinyl ester produced from incubation with retinol, did not effect cell numbers at the concentrations tested (data not shown). Thus, tRA was the dominant anti-proliferative retinoid.

### Cellular ROH uptake and metabolism

HMECs treated with 2.5 μM ROH exhibited rapid and substantial uptake of ROH. The extent of cell uptake is reflected in the marked decrease in the media ROH concentration which declined to 20% of that in dishes without cells by 24 h (Fig. 2). Media ROH levels then remained relatively constant through 72 h in HMEC-containing dishes. ROH disappearance from media in dishes without cells occurred at a fairly constant, slow rate (~0.008 μM ROH/h) through the 72-h time-course of the study. The rate of media ROH disappearance in the presence of HMECs was approximately 10 times faster (0.079 μM ROH/h) during the first 24 h of treatment. No other retinoids were detected in the media at any time.

Determination of cell-associated ROH concentrations substantiated the rapid and marked uptake pattern by the media analysis. HMECs contained a small quantity of endogenous ROH (~36 pmoles/mg protein). Incubation in media containing 2.5 μM ROH resulted in a rapid accumulation of ROH by 0.2 h, reaching a peak concentration at 2 h (~820 pmoles/mg protein) (Fig. 3A).

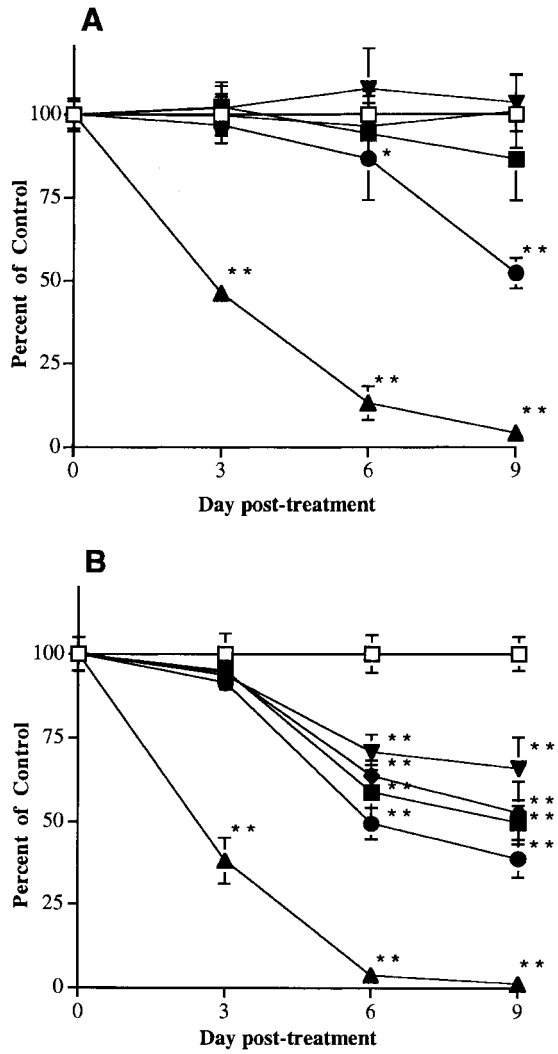


Fig. 1. Dose and time-dependent changes in relative cell number of HMECs treated on day 1 with (**A**) retinol at: 0.001 μM (▼), 0.01μM (◆), 0.1 μM (■), 1.0 μM (●) or 10 μM (▲) or (**B**) all-*trans* retinoic acid at: 0.001 μM (▼), 0.01μM (◆), 0.1 μM (■), 1.0 μM (●) or 10 μM (▲). Each symbol represents the mean ± SD cell number as a percent of the corresponding experiment time-point control from at least two independent experiments. Control cells exhibited a doubling time (mean ± SD) of 40.24 ± 4.6 h. *Significantly different than control ($P \leq .05$); **Significantly different than control ($P \leq .01$) by one-way ANOVA test.

Cell-associated ROH concentrations then decreased rapidly by 6 h before exhibiting a more gradual decline over the remaining incubation period. Even with this progressive decline, cell-associated ROH levels were still slightly greater at 72 h when compared to basal, pretreatment values.

Endogenous tRA was present in HMECs at low concentrations (~3.5 pmoles/mg protein). Upon ROH treatment, cell-associated levels of tRA increased by 0.2 h (~10 pmoles/mg protein), and continued to increase, reaching a peak concentration by 6 h (~43 pmoles/mg protein) (Fig. 3B). Thereafter, cellular tRA concentrations declined gradually. Even at 72 h, cellular tRA levels were still more than four-fold higher than the initial,

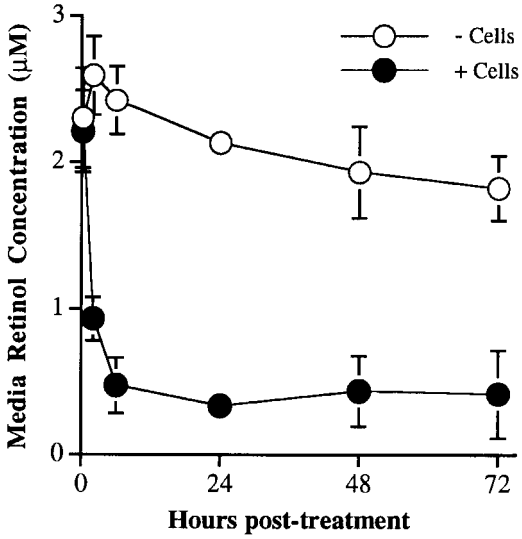


Fig. 2. Comparison of the retinol concentration of media following treatment with 2.5 μM retinol in either the presence or absence of cells. Each symbol represents the mean ± SD from two independent experiments each utilizing duplicate dishes per time point.

pretreatment value. Levels of tRA in DMSO treated cells did not change over the 72-h time-course (data not shown). While tRA was the predominant polar metabolite produced from ROH, both 13cRA and 9cRA were also detected. The temporal appearance of 13cRA was similar to that of tRA with a peak of ~6.5 pmoles/mg protein; 9cRA appearance, however, exhibited no discernable temporal pattern and never exceeded ~2.5 pmoles/mg protein (data not shown). Based on the temporal appearance of 13cRA and subsequent tests evaluating isomerization during sample preparation (data not shown), it is likely that a substantial proportion of the 13cRA is a result of nonenzymatic isomerization. Although it is less clear whether 9cRA was the product of similar events, it is likely that it is generated in part via nonenzymatic isomerization.

Incubation with 2.5 μM ROH also resulted in the production and accumulation of retinyl esters in HMECs. While initial concentrations of retinyl esters were negligible, they reached peak concentrations at 6 h (~370 pmoles/mg protein) and remained constant for the duration of the study (Fig. 3C). Retinyl oleate and palmitate were the predominant esters followed by linoleate, heptadecanoate, and stearate (data not shown). The relative proportions of these individual retinyl ester forms did not change during the experiment. Total retinyl ester concentrations remained unchanged in untreated cells over the time-course of the experiment (data not shown).

### Dose dependent ROH metabolism

Studies evaluating the response of HMECs to varying concentrations of ROH indicated both dose- and time-dependence for ROH uptake and tRA synthesis. As shown in Figure 4A, peak levels of cell-associated ROH were proportional to the concentration of ROH in the media. Each dose of ROH resulted in a similar temporal pattern of cellular ROH accumulation. Cell-associated levels of tRA were also proportional to the concentration of ROH in the cells (Fig. 4B). Quantitatively, tRA was a relatively minor metabolite. The peak concentration of tRA was only 5.5% of the peak level of ROH. By 24 h, however, cell-associated tRA as a proportion of the corresponding dose of ROH had increased to an average of 13%.

### Luciferase activity in HMECs treated with ROH or tRA

In HMECs transiently transfected with a RARE-luciferase reporter plasmid, treatment with either ROH or tRA resulted in a marked increase in luciferase activity, indicating RA-specific transcriptional induction of the reporter gene. As depicted in Figure 5, treatment with tRA resulted in a dose-dependent increase in luciferase activity at 24 h, with 1 μM tRA resulting in luciferase activity > 30-fold higher than vehicle only treated controls. The level of luciferase activity in response to lower concentrations of tRA was similar (between 8.5- and 10.6-fold higher than control) for both 0.01 and 0.10 μM tRA. Luciferase activity in response to 3 μM ROH was intermediate between that observed for the two lower doses of tRA.

### Effects of citral on ROH metabolism and cell number

To further examine the relationship between ROH conversion to tRA and the biological activity of ROH, studies were conducted using citral, an inhibitor of RA synthesis. In preliminary studies (data not shown), citral concentrations of ≥12 μM resulted in cytotoxicity with exposure longer than 6 days. Thus, in these experiments a lower, nontoxic citral dose was used. To further aid in comparisons, a dose of 1.25 μM ROH (the concentration resulting in a 50% reduction in cell number) was used in all experiments.

Treatment of HMECs with citral resulted in marked inhibition of tRA synthesis and an attenuation of biological responses to retinol. As shown in Figure 6, simultaneous treatment of HMECs with 1.25 μM ROH plus 5 μM citral resulted in 2.5 to 3.1-fold less cell-associated tRA at 4 and 6 h, respectively, compared to cells treated with only 1.25 μM ROH. As depicted in Figure 7, this citral inhibition of tRA synthesis markedly affected the ability of ROH to induce RA-specific luciferase reporter activity. Simultaneous treatment of reporter gene transfected HMECs with 1.25 μM ROH and 5 μM citral, resulted in a >80% attenuation of luciferase activity at 24 h compared to cells treated with 1.25 μM ROH alone. This effect persisted through 72 h, at which point luciferase activity was still >65% lower in cells treated with ROH plus citral compared to cells treated with ROH alone. A similar level of attenuation of retinol-induced luciferase activity by citral was observed up to 144 h (data not shown). Citral also attenuated the extent of the ROH-mediated reduction in HMEC number. As shown in Figure 8, incubation with 1.25 μM ROH resulted in 36% and 55% fewer cells on days 6 and 9, respectively, compared to controls. The combination of 5 μM citral and 1.25 μM ROH together, resulted in a >31% attenuation of the anti-proliferative effects observed with ROH alone. Citral alone (5 μM) did not affect cell number at any time.

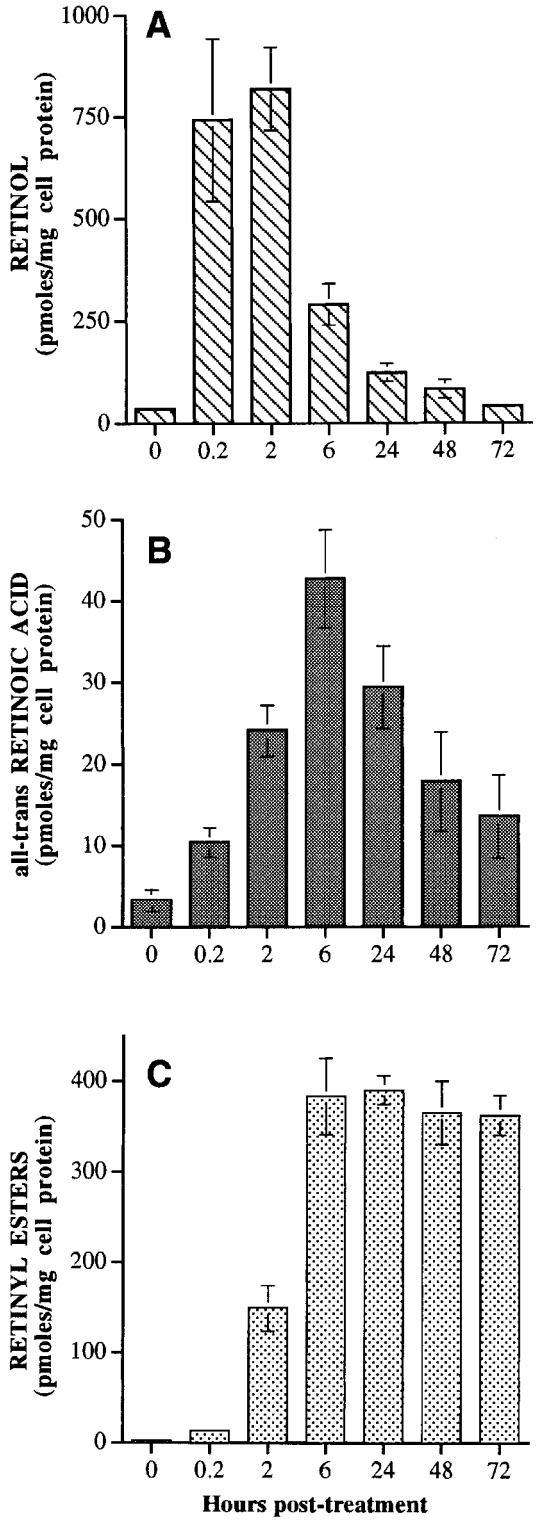


Fig. 3. Concentration of HMEC-associated retinoids over time following treatment with 2.5 μM retinol. Panel **A** shows the pattern of HMEC-associated retinol. Panel **B** shows the pattern of HMEC-associated all-*trans* RA. Panel **C** shows the pattern of HMEC-associated retinyl esters. Each bar represents the mean ± SD from two independent experiments each utilizing duplicate dishes per time point.

## DISCUSSION

Retinoic acid is a potent modulator of cell growth and differentiation. In our studies, we show that both all-*trans* retinoic acid (tRA), and its metabolic precursor retinol (ROH), exhibit anti-proliferative effects and that the lesser number of HMECs in response to ROH is mediated by the conversion to RA. In our normal HMEC model system, ROH reduced the number of cells with doses at or exceeding 1 μM, and resulted in 50% fewer cells at ~1.25 μM. Studies using melanoma (Lotan, 1979), mammary carcinoma (Halter et al., 1990), and airway epithelial cells (Rutten et al., 1988; Chopra et al., 1989; Halter et al., 1990) have demonstrated a similar effect on cell number with corresponding doses of ROH. Circulating levels of ROH in humans are approximately 2 μM (Rogers et al., 1993; Nierenberg et al., 1997), suggesting that ROH could regulate mammary epithelial cell growth in vivo. In comparison, tRA was much more potent, exhibiting a ≥ 25% reduction in total cell number at doses exceeding 0.001 μM and mediating a 50% decrease in cell number at ~0.05 μM. The extent of HMEC sensitivity with respect to decreases in total cell numbers in response to tRA is consistent with previous reports using both normal (Lee et al., 1995; Chen et al., 1997; Seewaldt et al., 1999a) and cancer-derived mammary cells (Takatsuka et al., 1996; Fontana et al., 1988; Chen et al., 1997; Cho et al., 1997; Agadir et al., 1999). Overall, our findings in HMECs are consistent with previous comparative studies demonstrating a greater biological potency of RA compared to ROH or other metabolic RA precursor retinoids (Lacroix and Lippman, 1980; Lotan et al., 1980; Ueda et al., 1980). Given that ROH serves as the metabolic precursor of tRA, these observations suggest that ROH-mediated decreases in cell number could be a result of the metabolic conversion of ROH to RA by the HMECs.

The manner by which tRA exerts its anti-proliferative effects is unknown. Although apoptosis was not assessed directly in our studies, visual observations did not indicate cell death (not shown). Recent studies using similar mammary cells have provided evidence that tRA inhibition of cell growth is irreversible and is a consequence of cell cycle arrest, not apoptosis (Seewaldt et al., 1997, 1999a,b) and that dysregulation of cyclin D1 may be a factor (Seewaldt et al., 1999b).

Examination of ROH metabolism by intact HMECs demonstrated that these cells have the capacity to take-up ROH and metabolically generate RA. Uptake of ROH by HMECs was rapid and substantial, occurring largely within 2 h of treatment. The introduction of a physiological dose of ROH into the media in an unbound state likely contributed to the observed rapidity of cell uptake. Although the kinetics of cellular ROH uptake are affected by its mode of delivery, the ratio of product to substrate has been shown to remain constant, irrespective of whether the ROH is presented in a free or protein-bound form (Hodam and Creek, 1998). Although the cellular ROH concentrations declined substantially by 6 h, they were still within physiologically relevant concentrations through 72 h.

Temporally, the peak concentration of cell-associated tRA coincided with the precipitous decline in cell-associated ROH. The cellular RA concentrations ob-

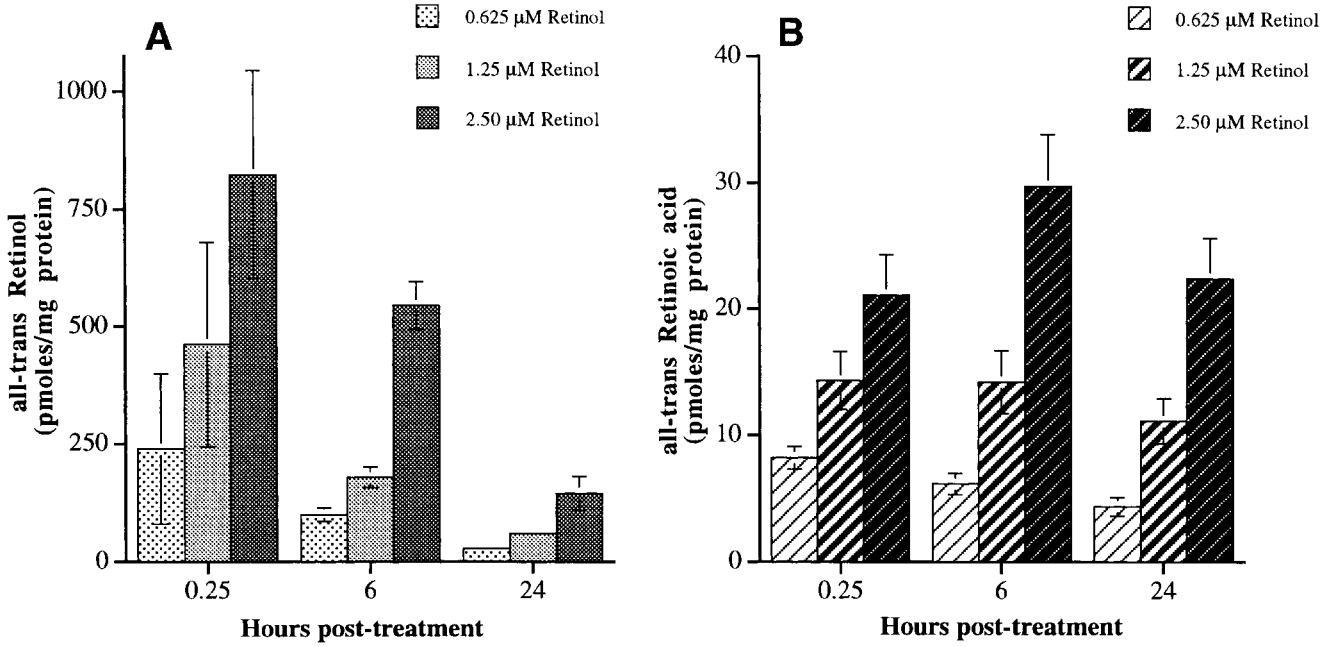


Fig. 4. Comparison of retinol uptake and retinoic acid synthesis by HMECs in response to varying media retinol concentrations. Cells were treated with media containing either 0.625, 1.25 or 2.50 μM retinol. At the indicated times following initiation of treatment, cells were harvested, extracted and retinoids analyzed and quantitated by HPLC as described in Methods. Shown are the levels of HMEC-associated retinol (panel **A**) and all-*trans* RA (panel **B**). Each bar represents the mean ± SD from two independent experiments each utilizing duplicate dishes per time point.

served in our studies at 6 h following ROH treatment are similar in magnitude to those obtained using keratinocytes (Randolph and Simon, 1993; Kurlandsky et al., 1994). While tRA was the predominant polar metabolite produced from the conversion of ROH to RA, both 13cRA and 9cRA were also detected. Although the temporal pattern of 13cRA was similar to that of tRA, cell-associated levels of the 13-*cis* isomer never exceeded 17% of that for tRA (data not shown). 9cRA, however, exhibited no specific temporal pattern. Although trace quantities of 9cRA were detected at 1 h, cell-associated levels of this isomer remained at ≤6% of that for tRA (data not shown). In other systems, evidence has been presented for both enzymatic and nonenzymatic generation of 13-*cis* and 9-*cis* RA (Shih et al., 1986, 1997; Urbach and Rando, 1994; Mertz et al., 1997; Lanvers et al., 1998; Romert et al., 1998). Although we cannot rule out the possibility of enzymatic 13-*cis* RA synthesis, a major proportion is likely the result of nonenzymatic oxidation. However, given that tRA can isomerize to 13cRA and 9cRA, these metabolites may be contributing to the cellular pool of tRA. Thus, the values reported herein for tRA would be even greater if the contribution of all isomers to cellular RA levels is considered.

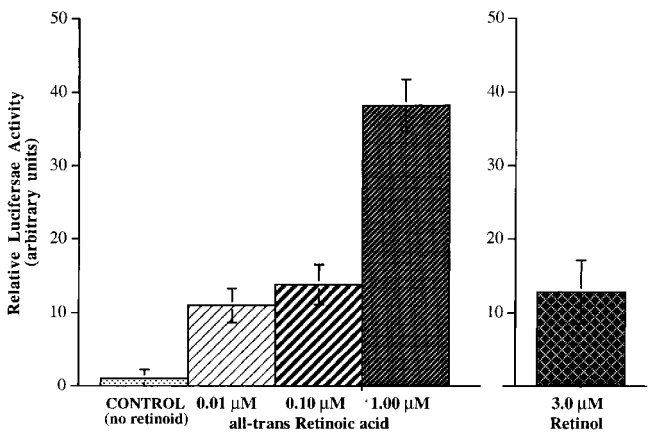


Fig. 5. Comparison of luciferase activity in transfected HMECs treated with either retionol or all-*trans* retinoic acid. Cells were transfected with an RA-specific reporter plasmid containing two tandom copies of the RA-response element from the human RARβ gene ligated upstream of the gene for luciferase, the plasmid pRL-CMV-Renilla, and carrier plasmid (pGL3.2) as described in the Methods. Twenty-four hours after transfection, cells were treated with fresh media containing the indicated retinoids or vehicle (DMSO) alone as a control. Following a 24-h treatment period, cells were assayed for luciferase and renilla activity as described in the Methods. Experiments were carried out twice, each using 2 lines–3 wells/treatment. Each bar represents the mean ± SD of renilla-normalized luciferase activity from triplicate dishes per time point from a representative experiment.

It should be noted that these HMECs contained a small quantity of endogenous ROH and a minute quantity of tRA. Since these cells are primary cultures used at early passages, the levels of endogenous retinoids likely reflect stores remaining from exposure to circulating levels in vivo, and thus would also correspond to some degree with the dietary intake of the individual donors. Since the 0.5% serum in the culture media results in a concentration of ≤0.015 μM ROH (unpublished observations), the growth media would likely make a negligible contribution to endogenous ROH levels.

Metabolic dose-response studies demonstrate that tRA production is dependent on the pool of cellular ROH, which in turn is proportional to the concentration of

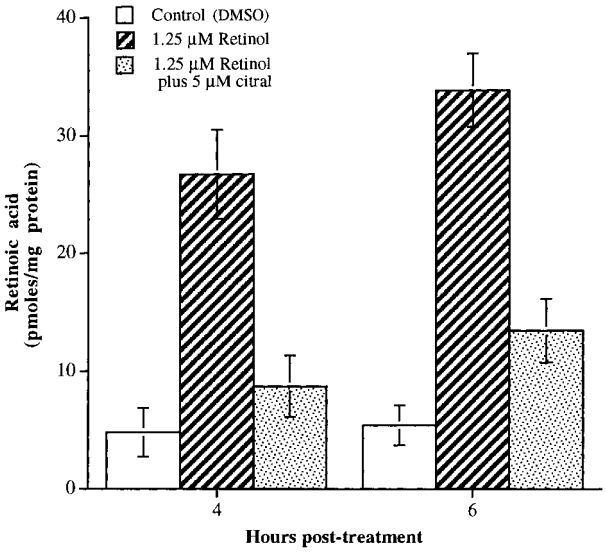


Fig. 6. Effect of citral on retinoic acid synthesis from retinol in HMECs. Cells were plated in standard MEBM. After 2 days (~80% confluency), media was replaced with fresh media containing either 1.25 μM retinol alone, 1.25 μM retinol together with 5 μM citral, or vehicle (DMSO) alone. At the indicated times cells were harvested, extracted and retinoids analyzed and quantitated by HPLC as described in Methods. Each bar represents the mean ± SD of HMEC-associated all-*trans* RA from two independent experiments each utilizing duplicate dishes/treatment/time point.

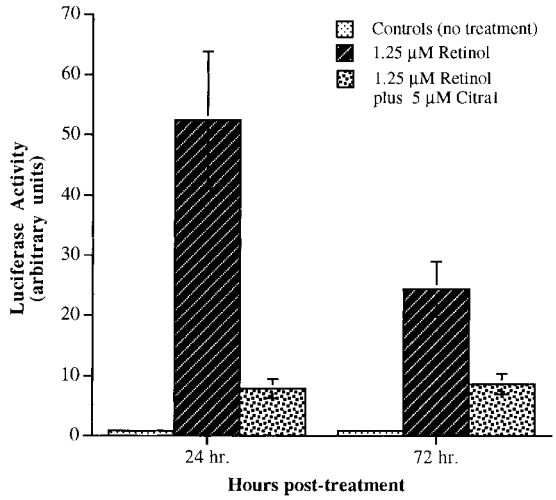


Fig. 7. Effect of citral treatment on retinol-induced luciferase activity in transfected HMECs. Cells were transfected with an RA-specific reporter plasmid (pGudLuc4RARE), the plasmid pRL-CMVRenilla, and a carrier plasmid (pGL3.2) as described in the Methods. At 24 h after transfection, cells were treated with fresh media containing either 1.25 μM retinol alone, 1.25 μM retinol together with 5 μM citral, or vehicle (DMSO) alone. Cells were then assayed for luciferase and renilla activity at the indicated times following effector addition as described in the Methods. Experiments were carried out twice, each using 2–3 wells/treatment. Each bar represents the mean SD of renilla-normalized luciferase activity from triplicate dishes per time point from a representative experiment.

ROH in the media. Peak-to-peak comparisons of substrate (ROH) and product (tRA) indicate that the same proportion of tRA was formed from ROH, irrespective of the concentration of ROH in the media. At any concentration, however, only a relatively small proportion of the administered ROH appears to be converted to tRA, (between 5% and 14%, depending on the time-point); a finding consistent with other reports of ratios of ROH to RA production (Wang et al., 1993; Kurlandsky et al., 1994; Napoli, 1999). Metabolically, the majority of ROH taken-up by HMECs was converted to retinyl esters. Unlike tRA, the pattern of retinyl ester synthesis remained relatively constant from 6 h following treatment, at which time retinyl esters were the predominant cell-associated retinoid. Similar results have been reported both in vivo and in vitro (Randolph and Simon, 1993; Chen et al., 1997; Yost et al., 1988). Retinyl oleate was the predominant ester in HMECs, with retinyl linoleate, palmitate and others also contributing to the total retinyl ester pool (data not shown). Retinyl oleate and palmitate were the predominant forms of esters in studies using human keratinocytes (Randolph and Simon, 1993; Kurlandsky et al., 1994). Although similar to the pattern of retinyl esters in keratinocytes, the predominance of retinyl oleate observed in HMECs is different from that observed in liver tissue and isolated hepatocytes wherein retinyl palmitate is the predominant retinyl ester (Furr et al., 1986; Furr, 1990). While retinyl palmitate can be the predominant ester form in milk, the composition of milk retinyl esters as well as that of milk fat, is determined by both the type of fatty acids in the diet and from diet-related differences in de novo synthesis of fatty acids within the mammary gland (Ross et al., 1985). In addition, the retinyl ester content of human milk has been shown to change over the course of the post-partum period (Chappell et al., 1985).

To further ascertain the biological significance of the production of tRA from ROH, a specific, RA-responsive (βRARE-luciferase) reporter gene construct was transfected into HMECs to assess whether RA-dependent transcriptional activation could occur in response to ROH treatment. Compared to controls, treatment of transfected cells with 3 μM ROH resulted in a 12-fold increase in luciferase activity. The magnitude of this response to ROH was intermediate between those obtained with 0.01 and 0.10 μM tRA, a concentration of tRA similar to that generated by HMECs in metabolism studies. Thus, the synthesis of tRA by HMECs from a physiological dose of ROH would likely affect endogenous expression of RA-responsive genes.

To determine if tRA is directly responsible for the reductions in total cell number, citral, an aldehyde dehydrogenase inhibitor previously shown to prevent the production of RA from ROH and retinal (Connor and Smit, 1987; Kurlandsky et al., 1994; Tanaka et al., 1996; Kikonyogo et al., 1999), was introduced into the cell system. Treatment of HMECs with 5 μM citral resulted in a 31% attenuation of the reduction in cell number compared to treatment with ROH alone. While studies using citral in other systems have demonstrated greater reductions in biological activity than we observed here, this is likely due to the use of higher doses of citral (as high as 100 μM) and/or a shorter experimental time-course (Connor, 1988; Schuh et al., 1993; Tanaka et al., 1996; Kurlandsky et al., 1994). In our 9-day time-course

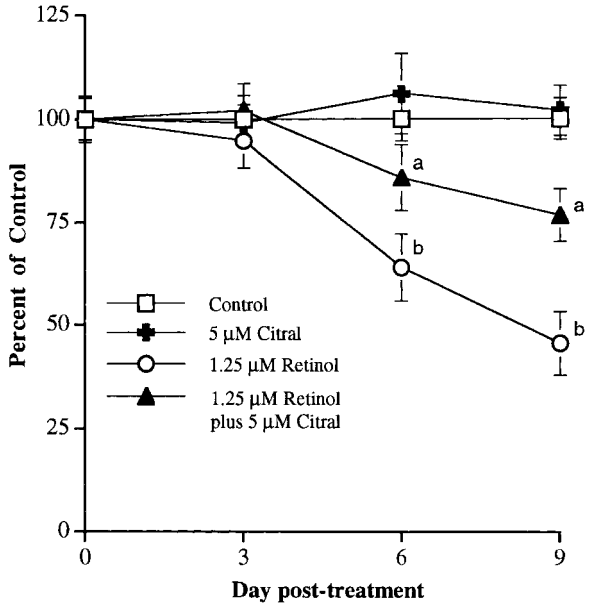


Fig. 8. Effect of citral on retinol-induced changes in cell number. At 24 h after plating, HMECs were treated with media containing either 1.25 μM retinol alone, 5 μM citral alone, 1.25 μM retinol together with 5 μM citral, or vehicle (DMSO) alone. At the indicated times, cell number was determined by the dye-binding assay as described in the Methods. Data are presented as the percent (mean ± SD) of control cells as each time-point from two independent experiments each utilizing 4 wells/treatment/time point. Different letter subscripts denote significant differences between groups ($P \leq .01$) by one-way ANOVA test.

HMEC system, doses of citral exceeding 12 μM were cytotoxic.

To substantiate that citral attenuation of the decrease in cell number in response to ROH was a direct result of altered tRA synthesis, we also examined the production of tRA in the presence of ROH and citral. Indeed, cells cultured in the presence of 5 μM citral and 1.25 μM ROH produced at least 53% less tRA than cells cultured with ROH alone, indicating that citral was significantly inhibiting tRA production. Citral inhibition of tRA synthesis also markedly affected the ability of ROH to induce RA-specific luciferase reporter activity. Simultaneous treatment of transfected HMECs with 1.25 μM ROH and 5 μM citral, resulted in a >67% attenuation of luciferase activity at 24 h compared to cells treated with 1.25 μM ROH alone. Of biological importance is the observation that the citral attenuation of luciferase activity persisted through 72 h.

Citral treatment also resulted in highly consistent effects on tRA synthesis (60–68% inhibition) and ROH-induction of RA-specific reporter gene activation ( ≥ 65% inhibition). However, citral resulted in a comparatively lower extent of attenuation of ROH-induced decreases in cell number (32%). This apparent discrepency may be related inherent differences in the cell population used for these particular assays (e.g., biological variability), the differences in time-points used to evaluated the effects, and/or be reflective of the nature of these biological endpoints. Measurable effects of citral on both tRA synthesis and reporter gene activity would likely occur much more rapidly (within hours) compared to effects on cell number. These normal cells have an average doubling time of ~40 h. Thus, the effect on cell number is likely a result of a cascade of events that would take days to culminate in the relative decrease in cells. There may also be a threshold level of RA necessary to cause a change in cell number. This 'threshold effect' could be due to the extent to which tRA is bound to cellular binding proteins and RA-receptors. Growth responsiveness of a variety of cell lines to tRA has been correlated with RARβ and/or RARα expression (Seewaldt et al., 1995; Shao et al., 1996; Nicke et al., 1999; Qiu et al., 1999; Shang et al., 1999). Although less tRA was present owing to citral inhibition of its synthesis, disproportionately more of it could be bound to RA-receptors versus sequestered by binding to cellular binding proteins, with the end result being a less than predicted effect on cell number. Metabolic clearance (catabolism) of tRA may also be a factor contributing to the biological activity or tRA. Accelerated RA catabolism can be induced with prolonged exposure to tRA (Martini and Murray, 1993; Muindi et al., 1994; Howell et al., 1998). Thus, even with a citral-induced reduction in RA synthesis, the induction of catabolic pathways could result in RA breakdown to a proportionally greater extent, reducing tRA half-life and attenuating longer term biological responses such as those affecting cell numbers. It is worth noting that the extent of cell number reduction observed with 1.25 μM ROH plus 5 μM citral, is similar to the extent of cell number reduction from exogenous 0.001 μM tRA alone, and that in metabolic studies, 1.25 μM ROH resulted in $\sim 10^{-9}$ M tRA production.

In aggregate, our studies demonstrate that HMECs have the capacity to take-up extracellular ROH and efficiently metabolize it to both the active and storage forms, (retinoic acid and retinyl esters), respectively. These findings are in accordance with studies examining the metabolism of retinoids in keratinocytes (Randolph and Simon, 1993; Kurlandsky et al., 1994). Our observation that concentrations of ROH typically found in human circulation exert anti-proliferative effects on normal HMECs, demonstrates the application of our model system and findings to in vivo conditions. In addition, we show that in HMECs, the ROH-mediated reduction in cell number is a direct consequence of the quantity of RA produced in situ. Thus, perturbations in the cellular capacity to generate RA from its precursor ROH may play a prominent role in processes affecting cell proliferation.

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## LITERATURE CITED

Agadir A, Chen G, Bost F, Li Y, Mercola D, Zhang X. 1999. Differential effect of retinoic acid on growth regulation by phorbol ester in human cancer cell lines. J Biol Chem 274:29779–29785.

Barua S, Tarannum S, Nahar L, Mohiduzzaman M. 1997. Retinol and alpha-tocopherol content in breast milk of Bangladeshi mothers under low socio-economic status. Int J Food Sci Nutr 48:13–18.

Canfield LM, Giuliano AR, Neilson EM, Blashil BM, Graver EJ, Yap HH. 1998. Kinetics of the response of milk and serum beta-carotene to daily beta-carotene supplementation in healthy, lactating women. Am J Clin Nutr 67:276–283.

Canfield LM, Giuliano AR, Neilson EM, Yap HH, Graver EJ, Cui HA, Blashill BM. 1997. beta-Carotene in breast milk and serum is increased after a single beta-carotene dose. Am J Clin Nutr 66:52–61.

Chappell JE, Francis T, Clandinin MT. 1985. Vitamin A and E content of human milk at early stages of lactation. Early Hum Dev 11:157–167.

Chen AC, Guo X, Derguini F, Gudas LJ. 1997. Human breast cancer cells and normal mammary epithelial cells: retinol metabolism and growth inhibition by the retinol metabolite 4-oxoretinol. Cancer Res 57:4642–4651.

Chew BP, Zamora CS, Luedecke LO. 1985. Effect of vitamin A deficiency on mammary gland development and susceptibility to mastitis through intramammary infusion with *Staphylococcus aureus* in mice. Am J Vet Res 46:287–293.

Cho Y, Tighe AP, Talmage DA. 1997. Retinoic acid induced growth arrest of human breast carcinoma cells requires protein kinase C alpha expression and activity. J Cell Physiol 172:306–313.

Chopra DP, Klinger MM, Sullivan JK. 1989. Effects of vitamin A on growth and differentiation of human tracheobronchial epithelial cell cultures in serum-free medium. J Cell Sci 93:133–142.

Connor MJ. 1988. Oxidation of retinol to retinoic acid as a requirement for biological activity in mouse epidermis. Cancer Res 48:7038–7040.

Connor MJ, Smit MH. 1987. Terminal-group oxidation of retinol by mouse epidermis. Inhibition in vitro and in vivo. Biochem J 244: 489–492.

De Luca LM, Darwiche N, Celli G, Kosa K, Jones C, Ross S, Chen LC. 1994. Vitamin A in epithelial differentiation and skin carcinogenesis. Nutr Rev 52:S45–52.

Fex GA, Andreasson S, Ehinger B. 1996. Serum retinoids in retinitis pigmentosa patients treated with vitamin A. Graefes Arch Clin Exp Ophthalmol 234 Suppl 1:S18–21.

Fontana JA, Hobbs PD, Dawson MI. 1988. Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. Exp Cell Biol 56:254–263.

Furr HC. 1990. Reversed-phase high-performance liquid chromatography of retinyl esters. Methods Enzymol 189:85–94.

Furr HC, Barua AB, Olson JA. 1994. Analytical methods. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids: biology, chemistry and medicine. New York, Raven Press, p 179–209.

Furr HC, Cooper DA, Olson JA. 1986. Separation of retinyl esters by non-aqueous reversed-phase high-performance liquid chromatography. J Chromatogr 378:45–53.

Giguere V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr Rev 15:61–79.

Gudas LJ, Sporn MB, Roberts AB 1994. Cellular biology and biochemsitry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids: biology, chemistry and medicine. New York: Raven Press, p 443–520.

Halter SA, Fraker LD, Parl F, Bradley R, Briggs R. 1990. Selective isolation of human breast carcinoma cells resistant to the growth-inhibitory effects of retinol. Nutr Cancer 14:43–56.

Halter SA, Winnier AR, Arteaga CL. 1993. Pretreatment with vitamin A inhibits transforming growth factor alpha stimulation of human mammary carcinoma cells. J Cell Physiol 156:80–87.

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 1992. 9-*cis* Retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68:397–406.

Hodam JR, Creek KE. 1998. Comparison of the metabolism of retinol delivered to human keratinocytes either bound to serum retinol-binding protein or added directly to the culture medium. Exp Cell Res 238:257–264.

Howell SR, Shirley MA, Ulm EH. 1998. Effects of retinoid treatment of rats on hepatic microsomal metabolism and cytochromes P450. Correlation between retinoic acid receptor/retinoid x receptor selectivity and effects on metabolic enzymes. Drug Metab Dispos 26:234–239.

Kikonyogo A, Abriola DP, Dryjanski M, Pietruszko R. 1999. Mechanism of inhibition of aldehyde dehydrogenase by citral, a retinoid antagonist. Eur J Biochem 262:704–712.

Kueng W, Silber E, Eppenberger U. 1989. Quantification of cells cultured on 96-well plates. Anal Biochem 182:16–19.

Kurlandsky SB, Xiao JH, Duell EA, Voorhees JJ, Fisher GJ. 1994. Biological activity of all-trans retinol requires metabolic conversion to all-trans retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes. J Biol Chem 269:32821–32827.

Lacroix A, Lippman ME. 1980. Binding of retinoids to human breast cancer cell lines and their effects on cell growth. J Clin Invest 65:586–591.

Lanvers C, Hempel G, Blaschke G, Boos J. 1998. Chemically induced isomerization and differential uptake modulate retinoic acid disposition in HL-60 cells. Faseb J 12:1627–1633.

Lee PP, Lee MT, Darcy KM, Shudo K, Ip MM. 1995. Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: a comparison of the retinobenzoic acid derivative RE80 with retinoic acid. Endocrinology 136:1707–1717.

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A. 1992. 9-cis Retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. Nature 355:359–361.

Looker AC, Johnson CL, Underwood BA. 1988. Serum retinol levels of persons aged 4–74 years from three Hispanic groups. Am J Clin Nutr 48:1490–1496.

Lotan R. 1979. Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res 39:1014–1019.

Lotan R, Ong DE, Chytil F. 1980. Comparison of the level of cellular retinoid-binding proteins and susceptibility to retinoid-induced growth inhibition of various neoplastic cell lines. J Natl Cancer Inst 64:1259–1262.

Mangelsdorf DJ. 1994. Vitamin A receptors. Nutr Rev 52:S32–44.

Martini R, Murray M. 1993. Participation of P450 3A enzymes in rat hepatic microsomal retinoic acid 4-hydroxylation. Arch Biochem Biophys 303:57–66.

Mertz JR, Shang E, Piantedosi R, Wei S, Wolgemuth DJ, Blaner WS. 1997. Identification and characterization of a stereospecific human enzyme that catalyzes 9-*cis*-retinol oxidation. A possible role in 9-*cis*-retinoic acid formation. J Biol Chem 272:11744–11749.

Meyer E, Lambert WE, De Leenheer AP. 1994. Simultaneous determination of endogenous retinoic acid isomers and retinol in human plasma by isocratic normal-phase HPLC with ultraviolet detection. Clin Chem 40:48–57.

Muindi JR, Young CW, Warrell RP, Jr. 1994. Clinical pharmacology of all-trans retinoic acid. Leukemia 8:1807–1812.

Napoli JL. 1999. Retinoic acid: its biosynthesis and metabolism. Prog Nucleic Acid Res Mol Biol 63:139–188.

Nicke B, Biecken EO, Rosewicz S. 1999. Induction of retinoic acid receptor beta mediates growth inhibition in retinoid resistant human colon carcinoma cells. Gut 45:51–57.

Nierenberg DW, Dain BJ, Mott LA, Baron JA, Greenberg ER. 1997. Effects of 4 y of oral supplementation with beta-carotene on serum concentrations of retinol, tocopherol, and five carotenoids. Am J Clin Nutr 66:315–319.

Nierenberg DW, Nann SL. 1992. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. Am J Clin Nutr 56:417–426.

Ortega RM, Andres P, Martinez RM, Lopez-Sobaler AM. 1997. Vitamin A status during the third trimester of pregnancy in Spanish women: influence on concentrations of vitamin A in breast milk. Am J Clin Nutr 66:564–568.

Pfahl M, Chytil F. 1996. Regulation of metabolism by retinoic acid and its nuclear receptors. Annu Rev Nutr 16:257–283.

Qiu H, Zhang W, El-Naggar AK, Lippman SM, Lin P, Lotan R, Xu XC. 1999. Loss of retinoic acid receptor-beta expression is an early event during esophageal carcinogenesis. Am J Pathol 155:1519–1523.

Randolph RK, Simon M. 1993. Characterization of retinol metabolism in cultured human epidermal keratinocytes. J Biol Chem 268:9198–9205.

Randolph RK, Simon M. 1995. Metabolic regulation of active retinoid concentrations in cultured human epidermal keratinocytes by exogenous fatty acids. Arch Biochem Biophys 318:6–14.

Raner GM, Vaz AD, Coon MJ. 1996. Metabolism of all-trans, 9-*cis*, and 13-*cis* isomers of retinal by purified isozymes of microsomal cytochrome P450 and mechanism-based inhibition of retinoid oxidation by citral. Mol Pharmacol 49:515–522.

Rautalahti M, Albanes D, Hyvonen L, Piironen V, Heinonen M. 1990. Effect of sampling site on retinol, carotenoid, tocopherol, and tocotrienol concentration of adipose tissue of human breast with cancer. Ann Nutr Metab 34:37–41.

Rogers EJ, Sabrah T, Hebert JR, Ausman LM, Hegsted DM, Nicolosi RJ. 1993. A comparison of serum retinol concentration between human and different species of normo and hypercholesterolemic nonhuman primates fed semi-purified diets with defined amounts of vitamin A. Comp Biochem Physiol Comp Physiol 106:749–754.

Romert A, Tuvendal P, Simon A, Dencker L, Eriksson U. 1998. The identification of a 9-*cis* retinol dehydrogenase in the mouse embryo reveals a pathway for synthesis of 9-*cis* retinoic acid. Proc Natl Acad Sci U S A 95:4404–4409.

Ross AC, Davila ME, Cleary MP. 1985. Fatty acids and retinyl esters of rat milk: effects of diet and duration of lactation. J Nutr 115: 1488–1497.

Rutten AA, Wilmer JW, Beems RB. 1988. Effects of all-*trans* retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture. I. A cell proliferation study. Virchows Arch B Cell Pathol Incl Mol Pathol 55:167–175.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Sankaran L, Topper YJ. 1982. Effect of vitamin A deprivation on maintenance of rat mammary tissue and on the potential of the epithelium for hormone-dependent milk protein synthesis. Endocrinology 111:1061–1067.

Schuh TJ, Hall BL, Kraft JC, Privalsky ML, Kimelman D. 1993. v-erbA and citral reduce the teratogenic effects of all-trans retinoic acid and retinol, respectively, in Xenopus embryogenesis. Development 119:785–798.

Seewaldt VL, Dietze EC, Johnson BS, Collins SJ, Parker MB. 1999a. Retinoic acid-mediated G1-S-phase arrest of normal human mammary epithelial cells is independent of the level of p53 protein expression. Cell Growth Differ 10:49–59.

Seewaldt VL, Johnson BS, Parker MB, Collins SJ, Swisshelm K. 1995. Expression of retinoic acid receptor beta mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. Cell Growth Differ 6:1077–1088.

Seewaldt VL, Kim JH, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ. 1997. All-trans-retinoic acid mediates G1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ 8:631–641.

Seewaldt VL, Kim JH, Parker MB, Dietze EC, Srinivasan KV, Caldwell LE. 1999b. Dysregulated expression of cyclin D1 in normal human mammary epithelial cells inhibits all-*trans*-retinoic acid-mediated G0/G1-phase arrest and differentiation in vitro. Exp Cell Res 249:70–85.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602.

Shang Y, Baumrucker CR, Green MH. 1999. Signal relay by retinoic acid receptors alpha and beta in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. J Biol Chem 274:18005–18010.

Shao Z, Yu L, Shen Z, Fontana JA. 1996. Retinoic acid nuclear receptor alpha(RAR alpha) plays a major role in retinoid-mediated inhibition of growth in human breast carcinoma cells. Chin Med Sci J 11:142–146.

Shih TW, Lin TH, Shealy YF, Hill DL. 1997. Nonenzymatic isomerization of 9-*cis*-retinoic acid catalyzed by sulfhydryl compounds. Drug Metab Dispos 25:27–32.

Shih TW, Shealy YF, Strother DL, Hill DL. 1986. Nonenzymatic isomerization of all-*trans*- and 13-*cis*-retinoids catalyzed by sulfhydryl groups. Drug Metab Dispos 14:698–702.

Takatsuka J, Takahashi N, de Luca LM. 1996. Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. Cancer Res 56:675–678.

Takeda N, Yamamoto A. 1994. Simultaneous determination of 13-*cis*- and all-*trans*-retinoic acids and retinol in human serum by high-performance liquid chromatography. J Chromatogr B Biomed Appl 657:53–59.

Tanaka M, Tamura K, Ide H. 1996. Citral, an inhibitor of retinoic acid synthesis, modifies chick limb development. Dev Biol 175:239–247.

Ueda H, Takenawa T. Millan JC, Gesell MS, Brandes D. 1980. The effects of retinoids on proliferative capacities and macromolecular synthesis in human breast cancer MCF-7 cells. Cancer 46:2203–2209.

Urbach J, Rando RR. 1994. Isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid. Biochem J 299:459–465.

van der Saag PT. 1996. Nuclear retinoid receptors: mediators of retinoid effects. Eur J Clin Nutr 50 Suppl 3:S24–28.

Vandersea MW, Fleming P, McCarthy RA, Smith DG. 1998. Fin duplications and deletions induced by disruption of retinoic acid signaling. Dev Genes Evol 208:61–68.

Wang XD, Krinsky NI, Russell RM. 1993. Retinoic acid regulates retinol metabolism via feedback inhibition of retinol oxidation and stimulation of retinol esterification in ferret liver. J Nutr 123:1277–1285.

Yost RW, Harrison EH, Ross AC. 1988. Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein. J Biol Chem 263:18693–18701.

Zhu ZR, Parviainen M, Mannisto S, Pietinen P, Eskelinen M, Syrjanen K, Uusitupa M. 1995. Vitamin A concentration in breast adipose tissue of breast cancer patients. Anticancer Res 15: 1593–1596.