Metabolism of the chemopreventive retinoid *N*-(4-hydroxyphenyl)retinamide by mammary gland in organ culture

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N-(4-Hydroxyphenyl)retinamide (4-HPR) is considered to be the most effective chemopreventive retinoid for chemically induced mammary carcinogenesis in rats. However, the mechanism of 4-HPR action in mammary cells is poorly understood. In the present study we examined the metabolism of 4-HPR in the mouse mammary gland in organ culture. Mammary glands excised from BALB/c mice were incubated with 4-HPR in the presence of insulin, prolactin and steroid hormones for 6 days. The glands were extracted with chloroform/methanol (2:1, v/v), and the metabolites were separated on a reversed-phase h.p.l.c. column. Three metabolites were separated in addition to 4-HPR; one of the metabolites, M_2 , was co-eluted with 13-*cis*-4-HPR, M_3 was co-eluted with *N*-(4-methoxyphenyl)retinamide (4-MPR) and M_1 remains unidentified. There appeared to be some hormonal regulation in the distribution of metabolites in the glands. Increased levels of 4-MPR and M_1 were observed in insulin-plus-prolactin-treated glands as compared with the glands incubated with steroid hormones. Furthermore, it was observed that M_1 isolated from the livers of 4-HPR-treated rats competed for the cellular retinoic acid-binding protein (CRABP) sites; however, 4-HPR did not bind to CRABP. These results indicate that mouse mammary gland can metabolize 4-HPR and that the metabolites which compete for CRABP sites may have physiological significance in the retinoid inhibition of mammary carcinogenesis.

INTRODUCTION

The chemopreventive role of retinoids against chemically induced carcinogenesis of several organs has been well established (Sporn & Newton, 1979; Moon & Itri, 1984). Of all the retinoids evaluated to date, for carcinogen-induced mammary cancers in rats, *N*-(4-hydroxyphenyl)retinamide (4-HPR) appears to be the most efficacious in decreasing the multiplicity of cancers and enhancing the latency of tumour appearance (Moon *et al.*, 1979). Not only does 4-HPR suppress the development of mammary cancers in rats, it inhibits the proliferation of the normal mammary gland (Moon *et al.*, 1983). In mouse mammary-gland organ cultures, 4-HPR inhibited prolactin-induced structural differentiation of the gland as well as carcinogen-induced development of hyperplastic alveolar nodule-like lesions (Chatterjee & Banerjee, 1982; Mehta *et al.*, 1983). These results suggest a good correlation in observed response to retinoids between '*in vivo*' and mammary-gland organ-culture systems. We have utilized this '*in vitro*' model system to study 4-HPR metabolism and the mechanism of 4-HPR action in mammary gland.

Although the mechanism for retinoid action is poorly understood, mediation of its action by retinoid-binding protein is considered as one of the possible mechanisms (Chytil & Ong, 1978; Sani *et al.*, 1984; Petkovich *et al.*, 1987). In recent years results both in support of (Chytil & Ong, 1978; Trown *et al.*, 1980; Sani *et al.*, 1984) and in opposition to (Libby & Bertram, 1982; LaCroix *et al.*, 1984) receptor-mediated retinoid action have been reported. The presence of retinoid-binding proteins in

normal and neoplastic tissues as well as hormonal regulation of cellular retinoic acid-binding protein (CRABP) in mammary gland *in vitro* have been reported from our laboratory (Mehta & Moon, 1985). Even though 4-HPR is an effective retinoid in suppressing the development of normal and neoplastic mammary gland, 4-HPR does not compete for CRABP sites. These results indicated that either CRABP does not have any role in 4-HPR action or 4-HPR has to be metabolized by the target organ and that one of the functional metabolites may have to bind to CRABP to initiate its action.

More recently, the pharmacokinetics of 4-HPR has been studied in mice and rats (Hultin *et al.*, 1986). It has been reported that the distribution of 4-HPR metabolites after 4-HPR treatment of the animals is qualitatively similar, but quantitatively different, among liver, mammary gland and urinary bladder, as well as between rats and mice. However, these results do not indicate whether 4-HPR is metabolized by each respective organ or is metabolized by the liver and the metabolites distributed to various organs.

In the present study we examined 4-HPR metabolism by mammary gland in organ culture and the ability of metabolites to compete for CRABP sites.

EXPERIMENTAL

Animals

Female BALB/c mice, 3–4 weeks of age, were obtained from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. Mice were housed in groups of five in

Abbreviations used: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; 4-MPR, *N*-(4-methoxyphenyl)retinamide; CRABP, cellular retinoic acid-binding protein; hormones: I, insulin; P, prolactin; A, aldosterone; F, cortisol; E, oestradiol; Pg, progesterone.

polycarbonate cages on Ab-Sorb-Dri (Garfield, NJ, U.S.A.) bedding and were allowed free access to water and food. The animals were given a daily subcutaneous injection of a suspension of 1 μ g of oestradiol-17 β and 1 mg of progesterone in saline (0.9% NaCl) for 9 days before organ cultures. This treatment is considered necessary for optimum growth of the mammary glands in organ culture.

Organ culture

The procedure for mammary-gland organ culture has been described in detail previously (Mehta *et al.*, 1984). Briefly, thoracic glands were dissected aseptically on sterile silk rafts and transferred to 60 mm-diameter culture dishes containing chemically defined vitamin A-free CMRL 1066 medium. The medium was supplemented with 100 units each of streptomycin and penicillin, 35 μ g of glutamine, 2.5 μ g of Fungizone (GIBCO, New Brunswick, NJ, U.S.A.) and appropriate hormone combinations. The concentrations of various hormones included in this study were insulin [(I) 5 μ g], prolactin [(P) 5 μ g], aldosterone [(A) 1 μ g], cortisol [(F) 1 μ g], oestradiol-17 β [(E) 0.001 μ g] and progesterone [(Pg) 1 μ g] all per ml of medium. When retinoids, 4-HPR or 4-MPR, were included in the medium, they were used at a concentration of 1 μ M and were present for the entire culture period. The glands were incubated at 37 °C under an atmosphere of O₂/CO₂/N₂ (10:1:9) for 7 days (unless mentioned otherwise). At the end of the culture period, pooled glands were frozen in liquid N₂ and stored at -70 °C in the freezer for biochemical analysis.

Extraction and h.p.l.c. analysis of retinoids

Methods for extraction of retinoids from tissues and h.p.l.c. analysis were described previously (Hultin *et al.*, 1985). Briefly, mammary glands were extracted twice with 2 vol. of chloroform/methanol (2:1, v/v) for 30 min each. The organic phase was separated by centrifugation and evaporated under N₂. The residue was redissolved in 0.2 ml of methanol. Retinyl acetate was used as an internal standard. Retinoids from the mammary-gland extracts were separated on a reversed-phase Whatman C₁₈ Partisil 10 ODS-2 column with a Spectra Physics 8700 h.p.l.c. instrument. The retinoids were eluted with a linear gradient of methanol/water (7:3, v/v) to 100% methanol in 30 min, with a flow rate of 1.2 ml/min, and monitored at 350 nm.

In order to generate the radioactivity profile of 4-HPR metabolism in mammary glands, the glands were incubated with 1 μ M unlabelled 4-HPR for 7 days in I+P+A+F-supplemented medium. Before termination of the culture on day 7, the glands were incubated with 1 μ Ci of [³H]4-HPR (sp. radioactivity 3.47 Ci/mmol)/ml for 6 h.

Retinoids from the glands were extracted with chloroform/methanol and the extract was subjected to h.p.l.c. analysis. Fractions (1 ml) of the eluate were collected and counted for radioactivity in a Tracor Analytical mark III liquid-scintillation counter.

CRABP assay

The procedure for the measurement of CRABP in the cytosol of cultured glands has been described previously (Mehta & Moon, 1985). Briefly, 20–30 glands incubated with growth-promoting hormones in the absence of retinoids were homogenized in 0.05 M-Tris/

HCl buffer, pH 7.0, containing 1.5 mM-EDTA and 1 mM-dithiothreitol. The cytosol was prepared by centrifuging the homogenate at 105 000 *g* for 30 min at 0–4 °C. Aliquots (0.5 ml) of cytosol were incubated with [³H]-retinoic acid (50 nM, sp. radioactivity 1.23 Ci/mmol), either alone or in the presence of excess unlabelled retinoids or retinoid metabolites, for 18 h in the dark at 0–4 °C. The retinoid metabolites were collected from h.p.l.c. analysis of liver extracts from 4-HPR-treated rats. After dextran-coated-charcoal treatment of the reaction mixtures, 0.2 ml samples were subjected to preformed linear sucrose density gradients (5–20% sucrose in Tris/EDTA buffer). The gradients were centrifuged for 2 h at 65 000 rev./min in a vertical-tube rotor, and fractionated into 10-drop fractions. The radioactivity was measured in each fraction.

Protein concentration in the cytosol was determined by Kute *et al.*'s (1980) modification of the original Waddell procedure.

RESULTS

In order to determine whether 4-HPR is metabolized by the mammary glands, 20–25 glands were incubated with a combination of hormones (I, P, A + F) for 7 days in the presence of 1 μ M-4-HPR. Incubation of the glands in the absence of 4-HPR served as a control. The tissue was extracted and subjected to h.p.l.c. analysis as described in the Experimental section. As Fig. 1(a) shows, 4-HPR was taken up by the mammary gland, and three metabolites, M₁, M₂, and M₃, were separated with a major peak of 4-HPR. The retention times for these metabolites were estimated as 13.7 min for M₁, 22.9 min for M₂, 23.7 min for 4-HPR, and 27.8 min for M₃. The metabolites M₂ and M₃ were co-eluted with 13-*cis*-4-HPR and 4-MPR respectively, whereas M₁ remains unidentified. Fig. 1(c) is an h.p.l.c. profile of the extract from control glands. None of the 4-HPR metabolites were observed in these tissues; however, some of the peaks were common for both control and 4-HPR-treated glands. An h.p.l.c. profile for retinoid extract of the medium containing 4-HPR is shown in Fig. 1(d). It is evident that 4-HPR was not degraded in the medium into any of the metabolites observed in the tissue. In a separate experiment, it was also determined that 4-HPR does not break down during the retinoid extraction procedure (results not shown). A similar metabolism pattern has also been observed for the extracts prepared from mammary gland, liver and urinary bladders after 4-HPR treatment of the animal *in vivo* (Hultin *et al.*, 1986).

Since two principal metabolites of 4-HPR were observed to be M₁ and 4-MPR, mammary glands were incubated with 1 $\times 10^{-6}$ M-4-MPR for 7 days in the medium containing I+P+A+F. The retinoids were extracted and the extract was subjected to h.p.l.c. analysis. As shown in Fig. 1(b), two metabolites, M₁ and 4-HPR, were separated. These results indicated that M₁ may be a common metabolite for both 4-HPR and 4-MPR in the mammary gland.

As shown in Fig. 1(a), the proportions of M₁ and M₃ were estimated to be very low as compared with 4-HPR in the extract. In order to demonstrate that M₁ was a true metabolite of 4-HPR, the mammary glands were incubated with 1 μ M-[³H]4-HPR on day 7 of culture for a 6 h period. The tissue extract was subjected to h.p.l.c.,

Fig. 1. Comparison of 4-HPR and 4-MPR metabolism in mammary gland in organ cultures

Mammary glands were incubated in the medium supplemented with IPAF for 7 days in the presence of either $1 \mu\text{M}$ -4-HPR (a), $1 \mu\text{M}$ -4-MPR (b) or in the absence of retinoids (c). (d) Represents the extract of the culture medium supplemented with hormones and 4-HPR. The glands were extracted with chloroform/methanol and subjected to h.p.l.c. analysis as described in the Experimental section.

and radioactivity was measured in 1 ml fractions. As Fig. 2 shows, two radioactivity peaks were detected; one was co-eluted with 4-HPR, whereas the other was observed at 15.6 min. Although this peak appeared nearly 2 min later than the designated M_1 peak, in our previous studies *in vivo* with [^3H]4-HPR the radioactive metabolite was usually co-eluted with the M_1 peak. Thus it is considered possible that the 15.6 min peak of radioactivity may be comparable with the designated M_1 peak.

Previous studies from our laboratory indicated that retinoids inhibit P-induced differentiation of the mammary gland, whereas there was very little effect of retinoids on I+P+A+F- or I+P+E+Pg-induced growth of the mammary glands. We examined the pattern of 4-HPR metabolism in the glands treated with three different hormone combinations. The glands were incubated with either I+P, I+P+A+F or I+P+E+Pg for 7 days with 4-HPR. H.p.l.c. analyses of the extracts

were performed and the proportions of various metabolite fractions were determined. Results are shown in Table 1. No difference in the metabolism pattern was observed between I+P+A+F- or I+P+E+Pg-treated glands; however, the glands incubated with I+P in the absence of steroids contained relatively increased proportion of both M_1 and 4-MPR.

We have previously reported that mammary glands cultured for 7 days in the presence of hormones contained CRABP and that 4-HPR does not compete for the CRABP sites (Mehta *et al.*, 1980). In order to evaluate whether the 4-HPR metabolites compete for CRABP sites, BALB/c mice were treated with 4-HPR (5 mg/kg intraperitoneally) daily for 5 days, and livers were removed. Retinoids were extracted in chloroform/methanol (2:1, v/v) and samples of the extract were subjected to h.p.l.c. The metabolites M_1 and 4-MPR were collected from two preparative h.p.l.c. runs and

Fig. 2. H.p.l.c. and radioactivity profiles of the retinoid extract of the organ cultured mammary glands

Mammary glands (30 in all) were incubated with I + P + A + F + 1 μ M-4-HPR for 7 days. On the last day of incubation, glands were pulsed with 1 μ Ci of [3 H]4-HPR (sp. radioactivity 3.46 Ci/mmol)/ml for 6 h before termination. The glands were extracted with chloroform/methanol, and a sample was subjected to h.p.l.c. Radioactivity was measured in 1 ml fractions.

Table 1. Hormonal regulation of 4-HPR metabolism in mammary gland *in vitro*

Hormones	No. of expts.	Percentage of 4-HPR or metabolites			
		M ₁ *	M ₂	M ₃	4-HPR
I + P	5	4.49 $\pm$ 1.8†	14.10 $\pm$ 2.2	12.62 $\pm$ 3.5‡	68.69 $\pm$ 9.5
I + P + A + F	13	2.67 $\pm$ 0.4	12.33 $\pm$ 1.2	2.59 $\pm$ 0.7	82.30 $\pm$ 2.4
I + P + E + Pg	5	1.98 $\pm$ 0.5	13.60 $\pm$ 3.2	2.07 $\pm$ 0.3	82.21 $\pm$ 6.4

* M₁, unidentified metabolite; M₂, 13-*cis*-4-HPR; M₃, 4-MPR.

† The values were calculated by dividing the area under the peak by the sum of area under all four peaks.

‡ Significantly different from I + P + A + F- or I + P + E + Pg-treated groups ($P < 0.01$).

evaluated for their ability to compete for CRABP sites. Results from sucrose-density-gradient analysis of CRABP are shown in Fig. 3. It was observed that M₁, the unidentified metabolite, competed for the CRABP sites. All-*trans*-retinoic acid at 10-fold excess showed 25% competition, whereas M₁ inhibited the binding in the 2 S region by approx. 45%. The exact concentration of M₁ present in the reaction mixture is not known, since the chemical nature of the metabolite is still unknown. However, if one assumes that the absorption maxima for 4-HPR and M₁ are similar, concentrations of M₁ can be calculated by comparing the peak heights of M₁ and 4-HPR. By using this comparison it was determined that the amount of M₁ present in the reaction mixture was 75 ng. Neither 4-MPR nor the authentic 4-HPR competed for the CRABP sites (results not shown).

DISCUSSION

The chemopreventive nature of 4-HPR against chemically induced carcinogenesis of various target organs has

been documented. This retinoid is of specific interest, owing to its low toxicity in rodents. It has been reported that 4-HPR does not accumulate in the liver, and thus produces lower toxicity in the animals. More recently, this has been confirmed by the pharmacokinetics of 4-HPR *in vivo*. The $t_{1/2}$ for 4-HPR from rat mammary gland was 43.6 h, whereas it was 9.4 h for liver (Hultin *et al.*, 1986). These results suggested that 4-HPR is delivered to the mammary gland; however, it is not known whether mammary glands are capable of metabolizing the retinoid. One of the questions asked in the present study was whether mammary gland can metabolize 4-HPR *in vitro*. The results obtained here indicated that the mammary cells do metabolize 4-HPR to three metabolites. All of these metabolites have also been recorded for liver and mammary-gland extracts of 4-HPR-treated animals *in vivo* (Hultin *et al.*, 1986). Thus the metabolism pattern observed *in vivo* and *in vitro* are very similar. When the proportions of the metabolites are compared between the mammary glands of BALB/c mice *in vivo* and *in vitro*, an increased concentration of 4-MPR (M₃) was observed

Fig. 3. Competition for CRABP sites by 4-HPR metabolites

Mammary glands cultured with I+P+A+F for 6 days were homogenized in Tris/EDTA buffer, and cytosol was prepared. Aliquots (0.5 ml) were incubated with either [3 H]retinoic acid (50 nM) (●), [3 H]retinoic acid (50 nM) + 1 μ M unlabelled retinoic acid (□), or [3 H]retinoic acid (50 nM) + approx. 75 ng of M_1 (○).

under treatment *in vivo* as compared with that *in vitro*; however, very little difference was observed in the proportions of M_1 and M_2 between these two treatments. These results from studies *in vivo* and *in vitro* suggest that 4-MPR may be a storage form in the mammary glands *in vivo*. In the present study it was also noted that the mammary glands are capable of metabolizing 4-MPR to M_1 (Fig. 1b). At present, it is not known whether 4-MPR can be metabolized by liver or mammary gland *in vivo*. The results described here suggest that M_1 may be a common metabolite of both 4-HPR and 4-MPR. There appears to be some interconversion of 4-HPR into 4-MPR and 4-MPR into 4-HPR *in vitro*. However, the accumulation of 4-MPR in 4-HPR-treated glands or 4-HPR in 4-MPR-treated glands is not very noticeable.

Mammary-gland differentiation and carcinogenesis are influenced by P and steroid hormones. 4-HPR inhibited the P-induced differentiation of mammary gland in organ cultures, but was without much effect on the proliferation induced by prolactin plus steroid hormones (Mehta *et al.*, 1983; Telang & Sarkar, 1983). In the same system 4-HPR was less effective against the carcinogen-induced lesions when E+P were present in the medium as opposed to I+P+A+F (Chatterjee & Banerjee, 1982). Furthermore, 4-HPR was found to be more effective against ovarian-hormone-independent cancers rather than -dependent ones (McCormick *et al.*, 1982). These results suggested the possibility that hormones may influence 4-HPR metabolism in the mammary gland. Experiments were designed to ask the question as to whether there is any difference in the 4-HPR metabolism pattern between mammary glands incubated with various hormone combinations. There was no apparent difference in the metabolism pattern of 4-HPR between the two steroid-hormone combinations tested; however, there was more M_1 and M_3 in the glands treated in the absence of steroid hormones as compared with the glands incubated in the presence of steroid hormones. It is not clear whether the increased concentration of M_1

and M_3 in these glands is due to increased metabolism of 4-HPR in the absence of steroid hormones or reduced clearance of M_1 and M_3 from the tissue. If it is assumed that the 4-HPR by itself is not the active component and that it has to be metabolized before its action manifests itself, then either M_1 or 4-MPR may be an effective chemopreventive compound and the 4-HPR action in the mammary gland may be regulated by hormones. However, owing to unavailability of the compounds, carcinogenesis studies have not been conducted with 4-MPR or M_1 to demonstrate conclusively their role in mammary carcinogenesis.

Although currently there is no conclusive evidence suggesting that the retinoid action is mediated via specific retinoid-binding proteins, the presence of CRABP is reported in numerous publications in the literature (Chytil & Ong, 1978). Nuclear interaction of CRABP has also been reported for several tissues and cell types, including normal and neoplastic mammary gland (Wiggart *et al.*, 1977; Sani & Donovan, 1979; Mehta *et al.*, 1982). More recently, analysis of cDNA encoding a protein that specifically binds retinoic acid has been reported (Gignere *et al.*, 1987; Petkovich *et al.*, 1987). This receptor protein is homologous with receptors for E and thyroid hormone. These new developments clearly indicate that the retinoid action may be mediated by the nuclear retinoid-binding proteins described previously. On the other hand, evidence also exists for certain cells responding to specific retinoids in tissue culture with no detectable levels of retinoid-binding protein (Libby & Bertram, 1982; LaCroix *et al.*, 1984). In this study it was observed that 4-HPR did not compete for CRABP in the mammary tissues; however, one of the metabolites, M_1 , competed effectively for the binding sites. These results suggest that 4-HPR action may be mediated by an interaction between a 4-HPR metabolite and retinoid-binding protein. However, the direct proof for the effectiveness of M_1 in inhibiting I+P-induced differentiation or 7,12-dimethylbenz[*a*]anthracene-induced mammary lesions, similar to that observed with 4-HPR or all-*trans*-retinoic acid, is lacking, owing to unavailability of M_1 in large enough quantities to define chemically its structure and to test its effectiveness in mammary-gland organ culture. Although M_1 is more polar in nature than 4-HPR, it is not co-eluted with all-*trans*-retinoic acid. However, it is quite likely that M_1 may have a carboxy end group, since it has been reported that the presence of a carboxy end group is essential for binding to CRABP sites (Trown *et al.*, 1980; Sani *et al.*, 1984).

In conclusion, the present data indicate that the mammary gland is capable of metabolizing 4-HPR and that the metabolism pattern is similar to that observed for liver and mammary gland under conditions *in vivo*. Secondly, there may be hormonal regulation of 4-HPR metabolism in this tissue; finally, the action of 4-HPR may require both the ability of the organ to metabolize the retinoid and the presence of retinoid-binding protein.

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