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SECRETION OF N-(4-HYDROXYPHENYL) RETINAMIDE-RETINOL-BINDING PROTEIN FROM LIVER PARENCHYMAL CELLS: EVIDENCE FOR REDUCED AFFINITY OF THE COMPLEX FOR TRANSTHYRETIN

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The synthetic retinoid 4-HPR has been shown to markedly lower the plasma concentration of both retinol and RBP in rats and humans. We have studied the effect of 4-HPR on the secretion of retinol-RBP from liver cells *in vivo* and *in vitro.* In rats maintained with a normal diet, a vitamin A-deficient diet or a normal diet supplemented with 4-HPR, chylomicrons [^{3}H]retinyl esters were rapidly cleared from the plasma. The secretion of chylomicron-derived [^{3}H]retinol from tissues to the circulation, however, was different. In control rats, the lymph-derived [^{3}H]retinol peaked after about 2 hr, whereas 4-HPR treatment effectively reduced this peak of [^{3}H]retinol. Our results suggest that 4-HPR inhibits secretion of retinol-RBP from the liver. Therefore, we decided to study the effect of 4-HPR on the secretion of RBP using the human hepatoma cell line HepG2. Retinol and 4-HPR were found to induce the secretion of RBP. The medium from cells treated with 4-HPR was immunoprecipitated with antibodies against human RBP. HPLC analysis of the precipitated RBP revealed the presence of 4-HPR. When the medium from cells incubated with either 4-HPR or retinol was applied to a TTR affinity column, we found that RBP from cells incubated with 4-HPR had a considerably reduced affinity for TTR. We conclude that 4-HPR binds RBP and thereby induces secretion of RBP in HepG2 cells, and that the secreted 4-HPR–RBP complex has a reduced affinity for TTR. This observation may explain the 4-HPR–induced reduction of plasma retinol and RBP observed in *in vivo* studies. *Int. J. Cancer* 71:654–659, 1997.
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Retinoids are important regulators of normal growth and differentiation (reviewed in Blomhoff, 1994), and both natural and synthetic retinoids are now used clinically in the treatment of cancer and in severe acne. However, retinoids used in high doses in clinical treatment have been shown to exhibit teratogenicity, severe hepatotoxicity and anti-keratinizing activity. Of the different retinoids tested, amides of all-*trans* retinoic acid exhibit minimal toxicity *in vivo* and high biological potency in several test systems (Moon *et al.,* 1979). In particular, the synthetic retinoid 4-HPR has been suggested to be a useful drug for preventing cancer of the mammary gland (Moon *et al.,* 1979; Welsch *et al.,* 1983) because it is effective in *in vivo* experiments and accumulates in this organ (Moon *et al.,* 1979). 4-HPR has been shown to inhibit cell proliferation and induce apoptosis in breast cancer cell lines and in several other cell lines (reviewed by Formelli *et al.,* 1996). 4-HPR does not appear to participate directly in the retinoid signalling pathway. Its activity, however, seems to be associated with the expression of the retinoic acid receptors in cells (Formelli *et al.,* 1996).

Initial clinical studies with 4-HPR at high doses (Kingston *et al.,* 1986; Kaiser-Kupfer *et al.,* 1986) were hampered by the fact that it has profound effects on the normal metabolism of vitamin A. It has been demonstrated that the regulation of RBP is greatly affected both in humans and experimental animals treated with 4-HPR (Formelli *et al.,* 1987, 1989). When doses of 100–200 mg of 4-HPR per day were administered to humans, plasma levels of retinol and RBP were reduced to approximately two thirds of the normal levels within 24 hr (Formelli *et al.,* 1989; Dimitrov *et al.,* 1990). This may explain the vitamin A deficiency symptoms, such as night blindness, that are observed after long-term treatment with 4-HPR (Formelli *et al.,* 1989).

Vitamin A in the form of retinol is absorbed in the small intestine and esterified to retinyl esters. It is then transported in chylomicrons; subsequently, chylomicron remnants are transported mainly to the liver. Most of the retinyl esters are then hydrolysed to retinol and transferred from the parenchymal cells to the liver stellate cells, which are the main body store for vitamin A (Blomhoff, 1994), or transferred to plasma as a complex bound to RBP. RBP is a well-characterized protein that consists of a single polypeptide chain of 21 kDa and binds one molecule of retinol. It is believed that binding of retinol to RBP triggers secretion of the complex as a consequence of a conformational change in RBP. In circulation, retinol-bound RBP is associated with the protein TTR. Structural data show that TTR interact with RBP close to the retinol binding site and with the hydroxyl group of bound retinol (Monaco *et al.,* 1995). Crystallographic studies with N-ethyl retinamide (Zanotti *et al.,* 1993) and 4-HPR (Zanotti *et al.,* 1994) showed that the substitution of the hydroxyl group of retinol with an amide group is compatible with a correct retinoid binding in the RBP-retinol binding site. In addition, Berni and Formelli (1992) and Zanotti (1993) showed that the 4-HPR–RBP and N-ethyl retinamide complexes formed *in vitro* did not bind to TTR.

The aim of the present study was to further characterize the interaction of 4-HPR with RBP. We thus performed a series of experiments using both an *in vivo* model and the human hepatoma cell line Hep G2 as a model system.

MATERIAL AND METHODS

Chemicals

4-HPR was kindly donated by Dr. R.C. Moon (Chicago, IL). Standards of retinol purchased from Sigma (St. Louis, MO) and 4-HPR were stored as stock solutions (2–8 mM) in ethanol at −20°C. 11,12-[^{3}H]all-*trans*-retinol (27 Ci/mmol) was purchased from New England Nuclear (Dreieich, Germany). [^{35}S] methionine/[^{35}S] cysteine was purchased from Dupont (Wilmington, DE). Rabbit anti-human RBP antibody was purchased from DAKO (Copenhagen, Denmark). TTR was purchased from Scigen (Sittingbourne, UK).

Animals and diet

Male Wistar rats (250–300 g) were used. The animals were fed an ordinary pellet diet (3155, AREX, Møllesentralen, Norway) that

Abbreviations: 4-HPR, N-(4-hydroxyphenyl)retinamide; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; RBP, retinol binding protein; TTR, transthyretin.

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contained 9.4 μmol of retinoids (50% retinyl acetate and 50% retinyl palmitate) per kg. Assuming that rats eat 20 g of this diet per day, the daily intake of retinoids is about 0.2 μmol. The 4-HPR group received the same diet plus 13 μmol 4-HPR/day (*i.e.*, 20 mg 4-HPR/kg body weight/day) for 8 days. 4-HPR was dissolved in ethanol:ground nut oil (1:5). The 4-HPR solution was given by ventricular tubing. Young Wistar rats (50–75 g) were fed a vitamin-A–free diet for 10–12 weeks, at which time they stopped gaining weight. The total retinol level in livers of the vitamin-A–deficient rats was less than 10 nmol/g tissue as determined by HPLC.

Collection of radioactively labeled lymph

The operation of the donor rats was performed under anesthesia (Dueland *et al.*, 1982). When acceptable lymph flow was obtained and within 2 hr after the operation, the donor rats were given 630 nmol of 11,12-[^{3}H] all-*trans* retinol (250 μCi) dissolved in ground nut oil through duodenal tubing, and lymph-containing labeled chylomicrons were collected for 10–12 hr. Lymph (200 μl) that contained chylomicrons with labeled retinyl esters ($0.5–2.0 \times 10^6$ dpm) was subsequently injected in the right femoral vein of the experimental rats. Two hundred microliters of lymph contained approximately 5–10 mg of triglycerides.

Quantitation of radioactivity in the blood

Two hundred microliters of the plasma were mixed thoroughly with 4 ml of 96% ethanol. After 15 min, 10 ml hexane and 4 ml H_2O were added for extraction of the lipids. The solution was centrifuged, and the hexane phase was evaporated under nitrogen. The extracted lipids were finally dissolved in scintilation liquid, and the radioactivity was determined.

Cultivation of HepG2 cells

HepG2 cells were purchased from the ATCC (Rockville, MD). The cells were cultured in DMEM (Bio Whittaker, Wokingham, UK), containing 10% fetal bovine serum, L-glutamine (0.6 mg/ml) penicillin (10 units/ml) and streptomycin (10 μg/ml). The medium was changed every second or third day. The cultures were trypsinized and reached confluency every week under these conditions.

Metabolic labeling of HepG2 cells and immunoprecipitation

Confluent dishes of HepG2 cells were incubated overnight in a serum-free medium (DMEM supplemented with 2 mM L-glutamine). The next day, the cells were incubated in methionine-free DMEM for 45 min at 37°C before labeling in the same medium containing 0.1 mCi/ml [^{35}S]methionine/[^{35}S] cysteine for 2 hr. The cells were subsequently washed twice in DMEM and pre-incubated for 10 min in DMEM containing 2 mM puromycin to inhibit protein synthesis. The cells were then incubated in the same medium in the presence of retinol or 4-HPR. The medium was collected after the incubations, and the cells were washed twice in ice-cold PBS (pH 7.4) before addition of lysis buffer (0.1 NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF [Sigma] and 15.2 TIU [trypsin inhibitor units]/L Aprotinin [Sigma]). The cells were incubated on ice for 30 min before being scraped off and then shaken vigorously at 4°C for 30 min. The media and lysates were centrifuged at 12,800 g for 5 min at 4°C in an Eppendorf centrifuge to remove cell debris and nuclei. Immunoprecipitation was performed by incubating the media and lysates for 1 hr at 4°C with 10 μl of the rabbit anti-human RBP antibody. Fifty microliters of Protein A-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated in PBS was then added, and the mixture was incubated for 1 hr with gentle rocking at 4°C. The Sepharose beads were washed twice in ice-cold lysis buffer, twice in ice-cold PBS and finally in ice-cold distilled water.

SDS-PAGE

The samples were resuspended in sample buffer and heated to 95°C for 3 min, after which they were briefly centrifuged. Proteins were separated by 10% SDS-PAGE and subsequently fixed in 10% acetic acid/40% methanol and dried before analysis of the gels by imaging using Phosphoimager II (Molecular Dynamics, Sunnyvale, CA). Radioactivity in the protein bands was then quantitated using the software ImageQuaNT (Molecular Dynamics).

HPLC

Following immunoprecipitation of RBP, the medium (0.5 ml) was extracted with 1 ml of isopropanol, mixed thoroughly and centrifuged (1.400 g for 15 min) at 4°C. The supernatant was injected onto the chromatographic system. Solid phase extraction of 4-HPR from 1 ml of isopropanol was performed on-line with a 2.1×10 mm poly ether ether ketone (PEEK) column (Jour Research, Onsala, Sweden) dry packed with Bondapack particles (Millipore, Milford, MA). The column was equipped with 5 μm pore PAT (teflon-treated PEEK) frits (Jour Research). The mobile phase carrying the sample consisted of pure methanol. A reduced elution strength of the mobile phase and high recovery of the analytes (>95%) were obtained by on-line dilution of the methanol through a T-piece with a second pump delivering 2% methanol in water. The 2 liquids were mixed in a $1.0 \text{ m} \times 1.0$ mm inner diameter tubing before they entered the solid phase extractor. Five minutes after sample injection, the combined mobile phase and the 2% methanol in water were directed to waste by turning the 6-port switching valve. This action automatically backflushes the extracted compounds of the PEEK column with acetonitrile/water/formic acid (87:10:3, v/v), which was delivered by a third pump. The analytical column was a 250×4.6 mm Supelcosil C-18 column (Supelco, Bellafonte, PA) protected by a 20×4.6 mm Pelliguard guard column (Supelco) and a 2-μm PEEK filter (F, Upchurch Scientific, Oak Harbor, WA). Monitoring of the eluting retinoids was performed with a SPD-M10A diode array UV detector set to 363 nm (Shimadzu, Duisburg, Germany). This detector also provided qualitative information in the form of UV spectra and purity of the eluting peaks. The entire chromatographic system was controlled from a personal computer with Shimadzu CLASS LC-10 HPLC software based on Microsoft Windows and MS-DOS.

TTR affinity chromatography

TTR was coupled to a CNBr activated Sepharose 4B column (Pharmacia) according to the manufacturer's instructions. HepG2 cells were metabolically labeled as described above. After preincubation for 10 min in DMEM containing 2 mM puromycin to inhibit protein synthesis, the cells were incubated in the same medium in the presence of 5 μM retinol or 4-HPR. Two or 4 ml of the cultured cell medium were then applied onto a TTR-Sepharose 4B affinity column. The column was washed with 10 ml of PBS and eluted with 25 ml of distilled water adjusted to pH 10.0 with NH_3. Fractions of approximately 1 ml were collected. Five hundred microliters of the fractions were subsequently immunoprecipitated and resuspended in 50 μl sample buffer. After heating at 95°C for 3 min, the Sepharose beads were spun down and the samples were analysed by SDS-PAGE.

Competition of retinol binding to RBP

Confluent cultures of HepG2 cells were incubated overnight in serum-free DMEM supplemented with 2 mM L-glutamine. The following day, the medium was replaced with the same medium containing 10 μl [^{3}H]retinol (0.01 mCi/ml; 0.3 μM) in the absence or presence of 5 μM 4-HPR or retinol. The cells were incubated for 4 hr, and the medium was collected. The medium (2 ml) was subsequently applied onto a TTR affinity column. Fractions from the TTR column were analyzed for the presence of [^{3}H]retinol by scintilation counting.

RESULTS

Effect of 4-HPR on secretion of lymph-derived [^{3}H]retinol in vivo

When lymph containing [^{3}H]retinyl ester-labeled chylomicrons is injected intravenously into rats, radioactivity is cleared rapidly from the circulation followed by a transient increase in plasma

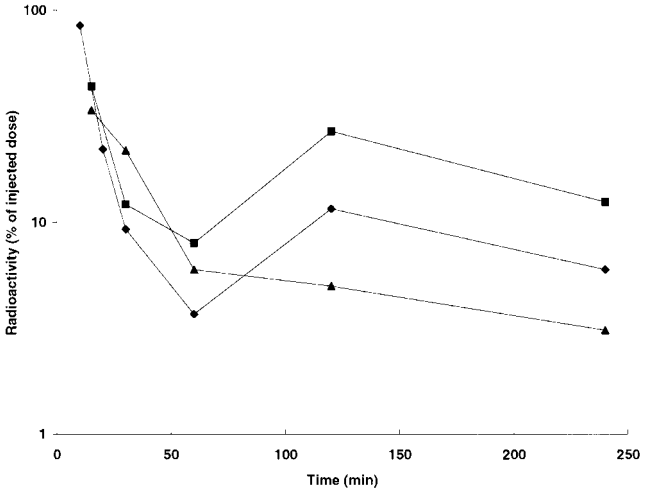

FIGURE 1 – Plasma radioactivity following injection of [^{3}H]retinyl ester labeled chylomicrons. Control rats (diamonds), vitamin-A–deficient rats (squares) and 4-HPR–treated rats (triangles) were injected with lymph-containing chylomicron [^{3}H]retinyl esters (0.5–2.0 $\times$ 10^6 dpm), and blood samples were taken at different time points. The radioactivity was measured by scintillation counting. The recovery of radioactivity in plasma after 2 hr was 12.3 $\pm$ 0.4% of injected dose (standard error of the mean [SEM], n = 3) in control rats, 20.5 $\pm$ 0.76% of injected dose (SEM, n = 3) in vitamin-A–deficient rats and 5.0 $\pm$ 0.98% of injected dose (SEM, n = 3) in 4-HPR–treated rats. The y-axis is in log-scale.

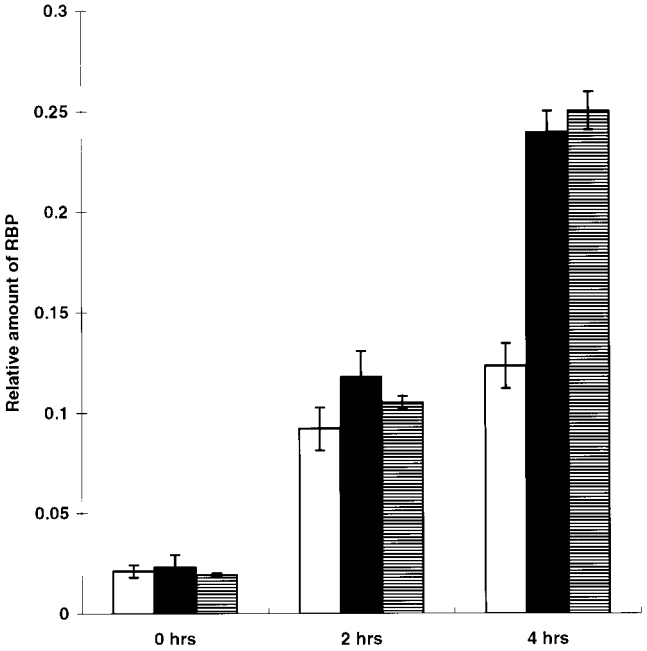

FIGURE 2 – Effect of 4-HPR on the secretion of RBP from cultured HepG2 cells. Confluent cultures of HepG2 cells (approximately 10 $\times$ 10^6 cells) were incubated overnight in serum-free DMEM before metabolic labeling using [^{35}S] methionine/[^{35}S] cysteine in methionine-free DMEM supplemented with 2 mM L-glutamine for 2 hr. The cells were pre-incubated in DMEM containing 2 mM puromycin for 10 min at 37°C, and the incubation continued in the same medium in the presence of 2 µM retinol, 2 µM 4-HPR or ethanol in an equivalent volume (less than 0.1%). At the indicated times, the medium and cell lysates were collected and immunoprecipitated using a polyclonal antibody against human RBP (1 hr at 4°C) and Protein-A Sepharose. The precipitated material was analysed by SDS-PAGE, and the gels were analysed by phosphoimaging. The radioactive bands corresponding to RBP were quantitated using the software ImageQuaNT and corrected for background. The relative amount of RBP secreted in the medium is represented as fractions of total RBP produced by the cell (*i.e.*, RBP in the medium and cell lysate). The white bars represent RBP in control medium, the black bars represent RBP in medium from retinol-treated cells, and the striped bars represent RBP in medium from 4-HPR–treated cells. The data presented are an average of 4 experiments (standard error of the mean [SEM] is indicated).

radioactivity. This increase represents radioactive retinol bound to RBP that has been secreted from the liver. We decided to test the effect of 4-HPR on this transient increase of plasma [^{3}H]retinol. For this purpose, rats were fed a vitamin-A–deficient diet, a normal diet or a normal diet supplemented with 4-HPR (13 µmol 4-HPR/d) for 1 week. The rats were injected with lymph containing chylomicron [^{3}H]retinyl esters, killed at subsequent times after injection and the radioactivity in the blood was measured. As shown in Figure 1, chylomicron [^{3}H]retinyl esters were rapidly cleared from plasma in all 3 groups. The appearance of lymph-derived [^{3}H]retinol in the circulation was, however, different. In control rats, the lymph-derived [^{3}H]retinol peaked after approximately 2 hr. 4-HPR effectively reduced this appearance of [^{3}H]retinol. As expected, the appearance of [^{3}H]retinol in the plasma was increased in vitamin-A–deficient animals compared with controls.

The effect of 4-HPR on the secretion of RBP from Hep G2 cells

The lower radioactivity recovered in plasma after 2 hr in the 4-HPR–treated rats suggests either that 4-HPR inhibits secretion of the retinol-RBP complex from the liver or that 4-HPR induces retention of both the retinol and RBP in the liver. Alternatively, because 4-HPR does bind to RBP *in vitro* (Berni and Formelli, 1992; Sani, 1993), one might hypothesize that in cells 4-HPR competes with retinol for the binding to RBP, so that the complex of RBP and 4-HPR is secreted while retinol is retained by the liver. To distinguish between these possibilities, we studied the effect of 4-HPR on the secretion of RBP by cultured hepatocytes using the human hepatoma cell line HepG2. This cell line has been shown to secrete RBP at an enhanced rate when incubated in the presence of retinol (Marinari *et al.*, 1987).

Dishes of confluent HepG2 cells were incubated overnight in a serum-free medium. The cells were then metabolically labeled with 0.1 mCi/ml [^{35}S] methionine/[^{35}S] cysteine and incubated with retinol or 4-HPR up to 4 hr. The amount of secreted RBP was then measured. The data presented in Figure 2, show that retinol and 4-HPR increase the secretion of RBP to the medium. Following 4 hr of incubation, the medium from retinol-treated cells contained 94% more RBP than medium from control cells, whereas the medium from 4-HPR–treated cells contained 103% more RBP. However, the retinol-induced secretion appears to occur between 2 and 4 hr of incubation with retinol or 4-HPR. We then studied the dose-response of retinol and 4-HPR on the secretion of RBP from HepG2 cells. The cells were labeled as described above and incubated with 0, 2 and 10 µM retinol or 4-HPR for 4 hr. The data presented in Figure 3 show that both retinol and 4-HPR induced a concentration-dependent secretion of RBP. At 2 and 10 µM, 4-HPR induced RBP secretion to the same extent as retinol.

To determine whether 4-HPR is able to compete with retinol for binding to RBP in HepG2 cells, confluent cultures of HepG2 cells were incubated in DMEM containing 0.3 µM [^{3}H]retinol in the absence or presence of 5 µM 4-HPR or retinol. The cells were incubated for 4 hr, and the medium was collected. The medium was subsequently applied onto a TTR affinity column, the bound material was eluted at low ionic strength and the radioactivity determined in the fractions. The results presented in Figure 4 show that both 4-HPR and retinol almost eliminated radioactivity in the fraction containing [^{3}H]retinol-RBP compared with the control experiment. These data therefore suggest that 4-HPR competes with [^{3}H]retinol for binding to RBP, implying that a complex of 4-HPR and RBP is secreted from these cells.

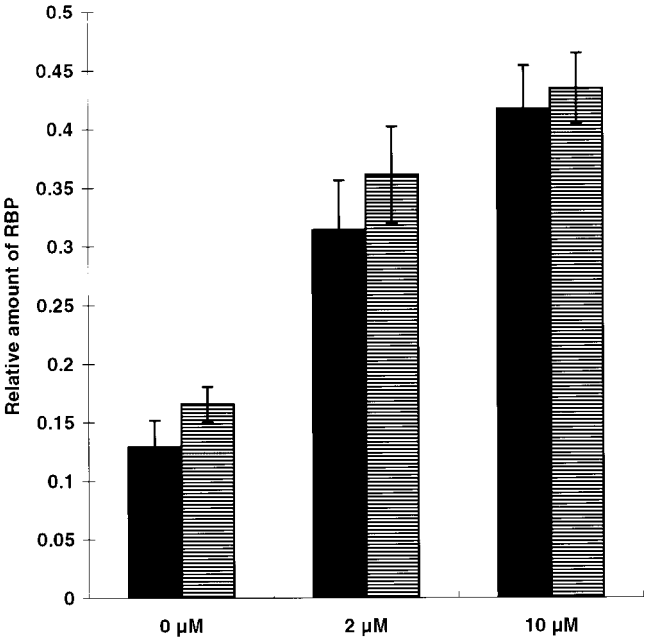

FIGURE 3 – Dose-response of retinol and 4-HPR. The cells were treated as described in Figure 2, except that the cells were treated with 0, 2, and 10 µM retinol or 4-HPR for 4 hr. The amount of RBP was quantitated as in Figure 2; the values represents an average of 3 experiments. The black bars represent RBP in medium from retinol-treated cells, and the striped bars represent RBP in medium from 4-HPR–treated medium. Standard error of the mean (SEM) is indicated.

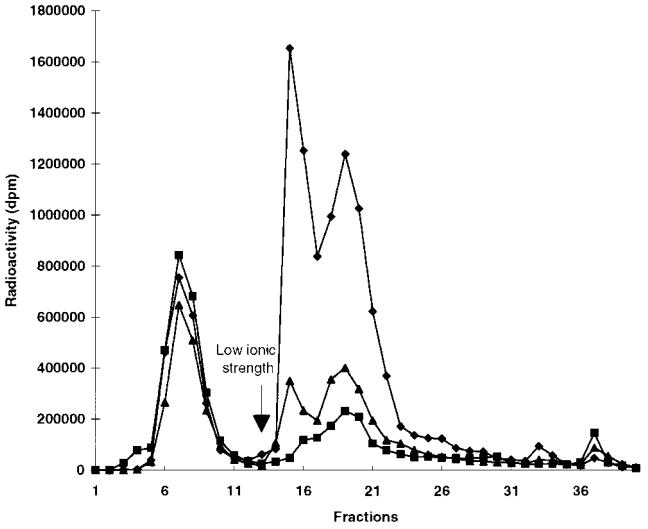

FIGURE 4 – Competition binding of retinol and 4-HPR. Confluent cultures of HepG2 cells were incubated overnight in serum-free DMEM supplemented with 2 mM L-glutamine. The following day, the medium was replaced with the same medium containing 10 µl [^{3}H]retinol (0.01 mCi/ml) in the absence (diamonds) or presence (squares) of 5 µM ROH or 4-HPR (triangles). The cells were incubated for 4 hr, and the medium was collected. The medium (2 ml) was subsequently applied to a TTR affinity column. The column was washed with 10 ml of PBS and eluted with 25 ml of distilled water adjusted to pH 10.0 with NH_3. Fractions of approximately 1 ml were collected. Ten microliters of the fractions were then counted in a scintillation counter, and the dpm were calculated.

HPLC analysis of the medium from 4-HPR–treated cells

To determine if 4-HPR was indeed bound to RBP, we collected the medium of 4-HPR–treated HepG2 cells. The medium was subsequently immunoprecipitated with rabbit anti-human RBP. This was extracted with isopropanol (1:2) and subsequently analyzed by HPLC. We detected a peak that co-eluted with the 4-HPR standard (Fig. 5), and the spectrum of this peak was identical to the spectrum of the 4-HPR standard (Fig. 5, insert). No peak co-migrated with the retinol standard. We conclude from these experiments that in HepG2 cells treated with 4-HPR, RBP is secreted as a complex with 4-HPR.

Effect of retinol and 4-HPR on the binding of RBP to TTR

Several studies have shown that 4-HPR reduces the level of RBP in plasma (Formelli *et al.,* 1987, 1989). One possible explanation is increased filtration by the kidneys due to reduced affinity of 4-HPR–RBP for TTR. We therefore tested whether RBP secreted from retinol and 4-HPR–treated HepG2 cells would bind to a TTR affinity column. HepG2 cells were metabolically labeled, and RBP secretion induced by retinol or 4-HPR. The medium from retinol-treated cells was applied to a TTR affinity column. As shown in Figure 6A, more than 90% of RBP eluted in 1 peak following the change to low ionic strength. When medium from the 4-HPR–treated HepG2 cells was applied to the TTR column, 80% of the RBP appeared in the flow-through, and only approximately 12% of the radioactivity was eluted at low ionic strength (Fig. 6B). The flow-through and the material released at low ionic strength from the 4-HPR–treated cells were re-chromatographed on the TTR column. More than 95% of the RBP in the flow-through still did not bind to the column, whereas more that 90% of the RBP in the bound material attached to the column in the second run (data not shown). These data show that RBP secreted from the 4-HPR–treated cells appears to have a reduced affinity for TTR compared with retinol-RBP.

DISCUSSION

4-HPR is currently being tested in clinical trials for treatment of breast cancer, prostate cancer, oral leukoplakia and bladder cancer (Costa *et al.,* 1995) with promising results. However, clinical studies have shown that treatment with 4-HPR exerts profound effects on the plasma concentration of vitamin A. Clinical trials thus require discontinuation of 4-HPR for a few days every month during the treatment period (Rotmensz *et al.,* 1991). The mechanism by which 4-HPR reduces plasma retinol has not been fully elucidated (Smith *et al.,* 1992; Berni and Clerici, 1992; Schaeffer *et al.,* 1993; Sani, 1993). The aim of our study was to investigate the cellular mechanisms for the reduced plasma retinol and RBP levels observed in patients receiving 4-HPR treatment.

We have studied the effect of 4-HPR on the secretion of retinol and RBP from liver cells *in vivo* and *in vitro.* In rats, we observed that the appearance of lymph-derived [^{3}H]retinol-RBP in the circulation was effectively reduced in 4-HPR–treated rats. Our data are in agreement with those of Smith *et al.* (1992) who have shown that concomitant with a reduction in plasma retinol and RBP concentrations, liver RBP was increased by 28% 5 hr following an intravenous injection of 4-HPR. However, the total vitamin A content in the liver was not significantly altered. In kidneys, no significant difference in the concentration of retinol and RBP was found between control and 4-HPR–treated rats. The reduced appearance of lymph-derived [^{3}H]retinol-RBP is most likely due to a reduced secretion of [^{3}H]retinol-RBP from liver cells. Based on these data, it has been concluded that secretion of both retinol and RBP from the liver is inhibited by 4-HPR. In a report by Formelli *et al.* (1993), it was found that 11 days of treatment with 4-HPR (20 mg/kg) did significantly lower the retinol content in rat kidneys.

Because Sani (1993) and Berni *et al.* (1993) have shown that 4-HPR can compete with retinol for the binding to RBP *in vitro,* an alternative explanation of the *in vivo* experiments may be that the 4-HPR–RBP complex is secreted to plasma while [^{3}H]retinol is

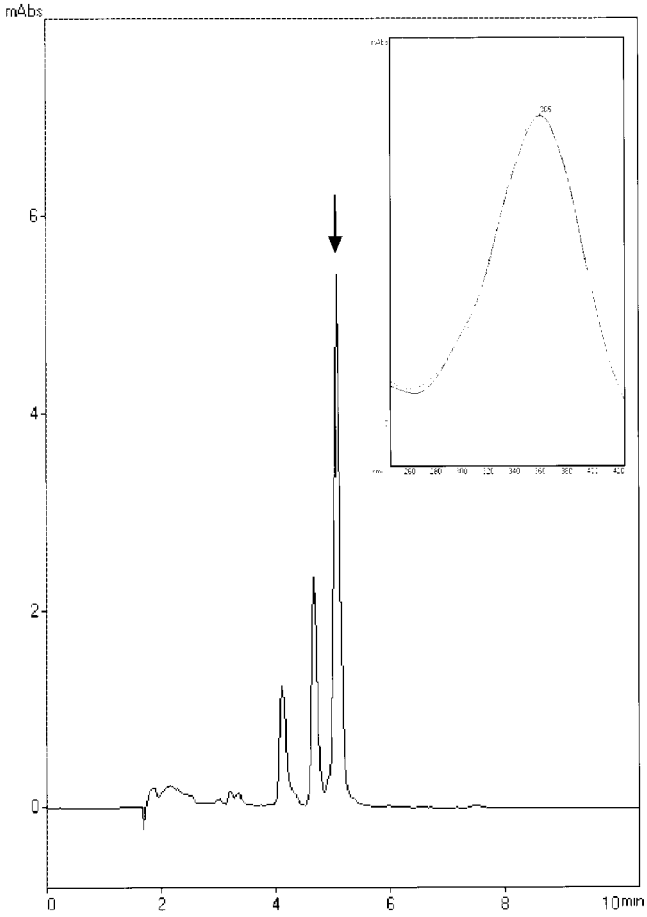

FIGURE 5 – HPLC analysis of the medium from the 4-HPR–treated cells. The medium from the 4-HPR–treated cells was immunoprecipitated as described in Material and Methods. The immunoprecipitated material was extracted with isopropanol and subsequently analysed by HPLC. The arrow indicates the migration of the 4-HPR standard. **Insert:** The UV spectrum of the 4-HPR peak. The continous line represent the sample, and the dotted line represents the spectrum of the 4-HPR standard.

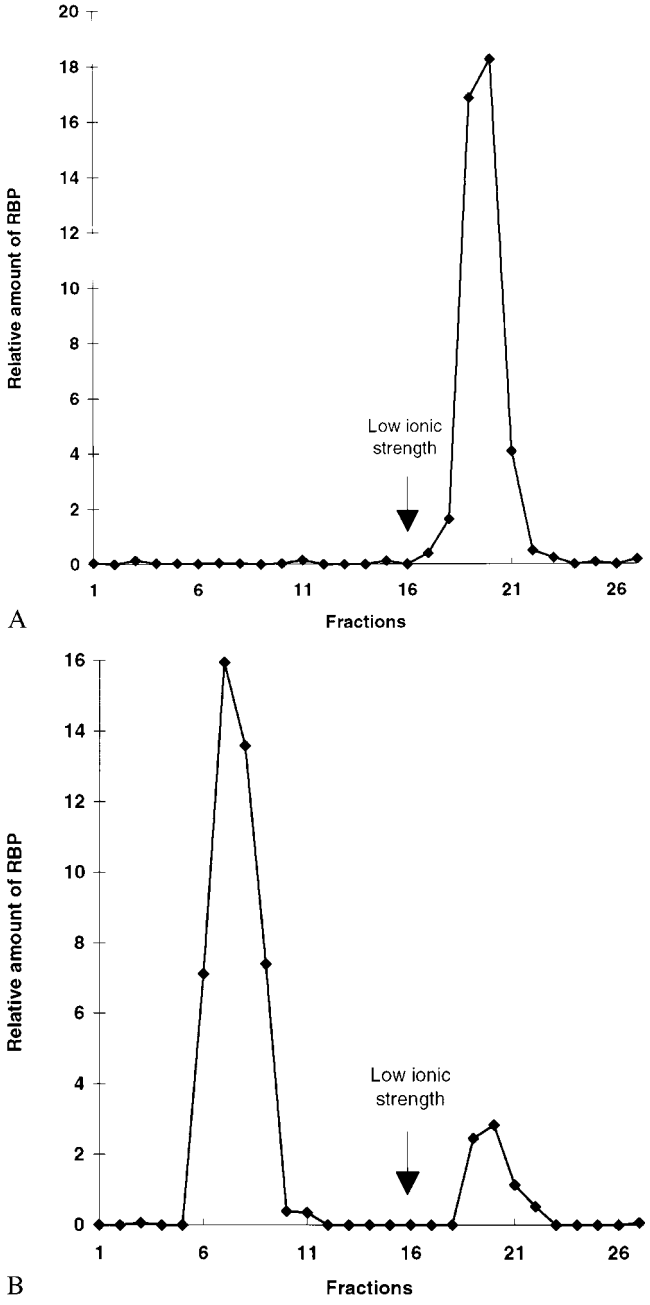

FIGURE 6 – Binding of RBP from the retinol (*a*) or 4-HPR–treated (*b*) cells to TTR affinity column. The cells were treated as described in Figure 2, except that the cells were incubated with 5 µM retinol or 4-HPR for 4 hr. Four milliliters medium from the retinol or 4-HPR–treated cells were applied onto a TTR affinity column. The column was washed with 10 ml of PBS and eluted with 25 ml of distilled water (adjusted to pH 10.0 with NH_3). Fractions of 1 ml were collected, and 500 µl of the fraction were immunoprecipitated with rabbit anti-human RBP as described in Material and Methods. The precipitated fractions were then analysed by SDS-PAGE, and the gels were analysed by phosphoimaging. The radioactive bands corresponding to RBP were quantitated using the software ImageQuaNT and corrected for background. The elution profile from a typical experiment is shown.

retained by the cells. This hypothesis is supported by the results of Schaeffer *et al.* (1993), who concluded that 4-HPR can induce secretion of RBP from the liver of rats *in vivo.*

We have studied the effect of 4-HPR on the secretion of RBP by cultured hepatocytes and the binding of the RBP-complex to TTR by using the human hepatoma cell line HepG2. HepG2 cells have previously been shown to secrete RBP at an enhanced rate in the presence of retinol (Marinari *et al.*, 1987). We find that retinol and 4-HPR (both added at a concentration of 2 µM) increase the secretion of RBP into the medium. These findings are consistent with the hypothesis that 4-HPR competes with retinol for the binding to RBP in the liver cells, and that a complex of 4-HPR-RBP is secreted while retinol is retained in the liver.

By co-incubating HepG2 cells with [^{3}H]retinol and retinol or 4-HPR, we demonstrated that both retinol and 4-HPR reduce binding of [^{3}H]retinol to RBP. Berni *et al.* (1993) have previously shown that when 2 µM 4-HPR was added to isolated retinol-RBP, it only displaced the natural ligand to a small extent when incubated for 1 hr. They concluded that the displacement of bound ligand appears to be an unfavorable process. Sani (1993) has previously observed a complete displacement of [^{3}H]retinol in the presence of a 100-fold molar excess of 4-HPR. In our experiments with HepG2 cells, a competition between 4-HPR and [^{3}H]retinol occured. However, this competition is observed only after 4 hr of co-incubation of cells with retinol or 4-HPR. Furthermore, we observed that the retinol and 4-HPR–induced secretion occured only after 4 hr (Fig. 2). This is in agreement with recent data from

our laboratory (V. Natarajan, unpublished results) showing that the formation of holo-RBP intracellularly is a rather slow process and appears to be rate-limiting in the secretion of RBP.

HPLC analysis of RBP immunoprecipitated from the medium from 4-HPR–treated cells was performed. We were able to detect a 4-HPR peak that co-eluted with the 4-HPR standard and exhibited a UV spectrum similar to that of the standard. We thus concluded that the RBP from the 4-HPR–treated cells was secreted as a 4-HPR–RBP complex.

Normally, retinol-RBP forms a complex with TTR in plasma, thus preventing its glomerular filtration. Studies with N-ethyl retinamide showed that the substitution of the hydroxyl group of retinol with an amide group results in the inhibition of binding of RBP to TTR (Zanotti *et al.,* 1993). Berni and Formelli (1992) showed that the 4-HPR–RBP complex formed *in vitro* did not bind to TTR. This is most likely because 4-HPR sterically inhibits binding to TTR, in accordance with the results of Malpeli *et al.* (1996). Monaco *et al.* (1995) showed that the hydroxyl group in retinol makes contact with the TTR and thereby stabilizes the retinol-RBP–TTR complex. Our data show that RBP secreted from 4-HPR–treated HepG2 cells binds to a lower extent to a TTR column than RBP secreted from retinol-treated cells, which is in agreement with these results.

In summary, we conclude that 4-HPR induces the secretion of RBP, and that the 4-HPR–RBP complex has a reduced affinity for TTR. The observed lowered plasma concentration of retinol and RBP in patients receiving 4-HPR treatment can thus be explained by reduced secretion of retinol from the liver and increased glomerular filtration of 4-HPR–RBP, respectively.

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