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In vitro interaction of fenretinide with plasma retinol-binding protein and its functional consequences

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The synthetic retinoid fenretinide (4-HPR: *N*-[4-hydroxyphenyl] all-*trans*-retinamide) interacts with plasma apo-retinol-binding protein (RBP) to form a tight complex (K'_d = 0.2 μM) which does not exhibit binding affinity to transthyretin (TTR). Therefore, a substantial modification of the retinol hydroxyl group does not appear to affect the interaction with RBP but does drastically interfere with the protein–protein recognition. The remarkable early reduction in plasma retinol level induced by fenretinide administration may be associated with the high binding affinity of this retinoid to RBP and to its interference with the RBP–TTR complex formation.

Retinol-binding protein; Fenretinide; *N*-Ethylretinamide; Retinoid binding; Transthyretin; Plasma retinol level

1. INTRODUCTION

Retinol-binding protein (RBP) is a well-characterized protein which transports retinol in plasma [1,2]. It consists of a single polypeptide chain of 21 kDa which binds one molecule of retinol. RBP interacts in plasma with thyroxine-binding transthyretin (TTR), a homotetramer of mol. wt. 55 kDa. The delivery of retinol to cell-surface receptors of target cells [3] is believed to induce conformational changes in the RBP molecule responsible for the reduction of its binding affinity to TTR [1]. Thus, owing to its small size, free uncomplexed plasma apo-RBP can undergo ready filtration and degradation in the kidney [4].

The retinol-binding site of RBP can accomodate a variety of analogs of the natural ligand [1]. The administration of pharmacologically active retinoids raises the question of their possible binding to RBP which might cause an interference with the secretion and/or transport of retinol in plasma. Such a mechanism may be involved in the substantial reduction in retinol and RBP plasma levels induced by fenretinide [5], a retinoid which is effective in inhibiting carcinogenesis in experimental animals [6]. In this work we have established that fenretinide interacts with RBP to form a tight complex which lacks binding affinity to TTR. These interactions might be related to the influence of fenretinide on plasma retinol level.

Abbreviations: RPB, retinol-binding protein; TTR, transthyretin.

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2. MATERIALS AND METHODS

2.1. *Materials*

Human and bovine holo-RBP and TTR were purified from fresh plasma as described [7–9] and were quantified at 280 nm using absorption coefficients $A^{1\%}_{1cm}$ of 18.6 and 14.3, respectively. Apo-RBP was prepared by extracting retinol from holo-RBP with diethylether [10]. Human TTR–Sepharose 4B affinity resin was prepared as described [11]. Fenretinide (4-HPR, *N*-[4-hydroxy- phenyl]all-*trans*-retinamide) and all-*trans*-*N*-ethylretinamide were gifts from R.W. Johnson Pharmaceutical Research Institute (Pa, USA) and from F. Hoffman-La Roche (Basel, Switzerland), respectively. CNBr-activated Sepharose 4B and Sephadex G-50 Fine were purchased from Pharmacia (Uppsala, Sweden).

2.2. *Fluorescence binding assay*

Fenretinide binding to human apo-RBP was monitored by following the quenching of protein fluorescence as described [10].

2.3. *Preparation of fenretinide-RBP and retinol-RBP complexes*

A 2× molar excess of fenretinide or retinol was incubated with human apo-RBP (50 nmol in 0.5 ml) in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7, at 20°C for 1 h. The retinoid–RBP complex was then separated from the excess retinoid by chromatography on a Sephadex G-50 Fine column.

2.4. *In vivo treatment and plasma retinol concentration assay*

Female Sprague–Dawley rats (150–170 g) (Charles River, Calco, Italy) were treated by intubation with fenretinide or *N*-ethylretinamide suspended in sesame oil at the dose of 20 mg/kg. Control rats received sesame oil. Three rats per group were used. Blood was drawn at time 0, 5 and 24 h after treatment. Plasma retinol concentrations were assayed by high performance liquid chromatography (HPLC) as previously described [5].

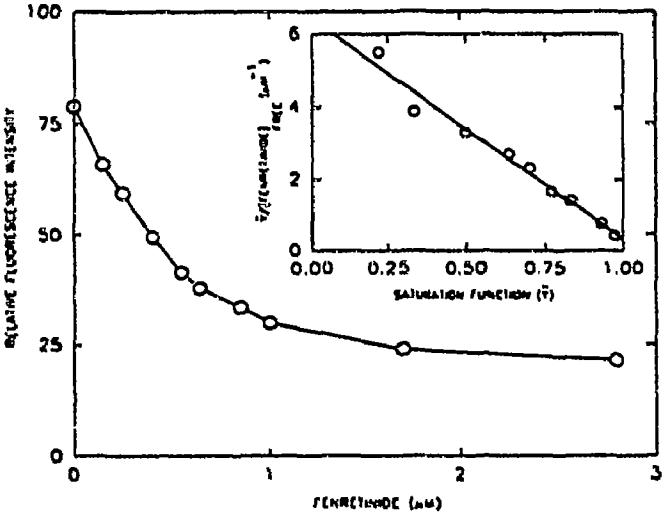

Fig. 1. Titration of human apo-RBP with fenretinide. The intensity of protein fluorescence is plotted as a function of fenretinide concentration. The inset shows the Scatchard plot of the data. Conditions: 0.5 μM RBP in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7, at 20°C. Protein fluorescence was monitored at 335 nm with excitation at 285 nm.

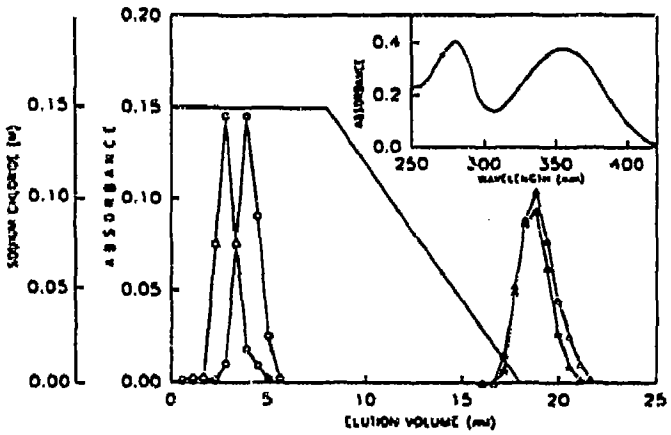

Fig. 2. Affinity chromatography of the fenretinide–RBP complex on TTR–Sepharose 4B. 10–20 nmol of fenretinide-(human)RBP or human retinol–RBP complexes were applied to the human TTR coupled to Sepharose 4B affinity column (1×5 cm) [11,13], equilibrated with 0.05 M sodium phosphate, 0.15 M NaCl, pH 7. Elution was performed at a 0.2 ml/min flow rate with the same buffer for the fenretinide–RBP complex, and with a linear gradient from this buffer to 0.001 M sodium phosphate, pH 7, for the retinol–RBP complex. The elution profiles were monitored by absorbances at 355 and 330 nm for fenretinide–RBP (○) and native retinol–RBP (△) complexes, respectively, and by absorbance at 280 nm for the control bovine serum albumin (□). The elution profile for the retinol–RBP complex obtained by the binding of retinol to apo-RBP, which is almost coincident with that of the native retinol–RBP complex, is also shown (▲). (Inset) Absorption spectrum of the fenretinide–RBP complex, in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.

3. RESULTS AND DISCUSSION

3.1. *Interaction of fenretinide with apo-RBP*

The addition of fenretinide to apo-RBP causes a marked quenching of tryptophan fluorescence, due to efficient energy transfer to the bound retinoid. A typical fluorescence titration curve of human apo-RBP with fenretinide is shown in Fig. 1. It is apparent from the Scatchard plot [12] that the binding data are well fitted on the basis of one binding site for fenretinide, with an apparent dissociation constant of 0.17 μM at 20°C (Fig. 1, inset). A binding affinity to apo-RBP similar to that previously found for retinol is consistent with the conclusion that the modification of the retinol hydroxyl group does not significantly affect the interaction of retinoids with RBP [10].

3.2. *Lack of interaction of the fenretinide–RBP complex with TTR*

The fenretinide–RBP complex, whose absorption spectrum is shown in the inset of Fig. 2, was subjected to chromatography on a human TTR–Sepharose 4B affinity column. Its elution volume was similar to that of the control, bovine serum albumin (Fig. 2), indicating the lack of a significant binding affinity to TTR at 'physiological' ionic strength, by contrast with the retention of native or reconstituted retinol–RBP complexes, which were eluted from the column only at very low ionic strength (Fig. 2) [13]. We have also verified that the interactions of fenretinide with the bovine RBP–TTR system are quite similar to those established for the human RBP–TTR system (data not shown). Therefore, the binding properties that we have presented presumably represent a general feature and may be extended to other mammalian RBP–TTR systems as well.

3.3. *The interaction of fenretinide with RBP in relation to its influence on plasma retinol concentration*

The administration of fenretinide induces a substantial early reduction in plasma retinol concentration both in rats (Table I) [14] and in humans [5]. A similar effect has also been found for *N*-ethylretinamide (Table I), whose binding properties in the interaction with the RBP–TTR system are very close to those of fenretinide (R. Berni, unpublished results). The lowering of plasma retinol levels might be associated with symptoms of

Table I

Effect of fenretinide and *N*-ethylretinamide on plasma retinol concentration in rats[a]

Treatment	Retinol plasma concentration (ng/ml)		
	0 h	5 h	24 h
None	324 ± 24	313 ± 29	317 ± 26
Fenretinide	297 ± 16	126 ± 14	94 ± 7
N-Ethylretinamide	364 ± 83	182 ± 34	224 ± 17

[a]Three rats for each groups; means ± S.D.

vitamin A deprivation observed after treatment with fenretinide [15]. We suggest that the reduction in plasma retinol levels is related to the high binding affinity of fenretinide and *N*-ethylretinamide to RBP, which may result in a competition with retinol for the RBP retinol-binding site. Accordingly, fenretinide- and *N*-ethylretinamide-RBP complexes might be formed in plasma. Due to the lack of binding affinity to TTR, they might be subsequently cleared by glomerular filtration. Even more relevant might be the competition of the two retinoids with retinol for RBP in the hepatocyte, before the secretion of RBP itself. After fenretinide administration plasma RBP decreases proportionally to retinol [5], thus suggesting that the competition results in an interference with the retinol–RBP secretion into the blood. This hypothesis is in accordance with the previous observations that the secretion of RBP is strictly dependent on the availability of retinol [1] and is effectively inhibited by several retinoids in cultured liver cells [16].

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