Biochemical Pharmacology, Vol. 43, No. 4, pp. 919–922, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Retinoyl β-glucuronide: lack of binding to receptor proteins of retinoic acid as related to biological activity

(*Received* 12 *August* 1991; *accepted* 4 *October* 1991)

Abstract—Retinoid β-glucuronides have emerged as biologically active, water-soluble, natural retinoids with relatively few toxic and teratogenic effects. The mechanism of action of these glucuronides in the control of epithelial differentiation, growth, and tumorigenesis is unknown. Since retinoyl β-glucuronide (RAG) contains a free carboxyl group, we studied the interactions of RAG with cellular retinoic acid-binding protein (CRABP) and nuclear receptors of retinoic acid (RARs), the possible mediators of the biological action of retinoic acid (RA). RAG did not exhibit any significant affinity to bind either CRABP or RARs. During 24- and 48-hr incubations of RAG in chick cytosol, detectable amounts of RA were generated which interacted with the RA receptors. In chick skin, the biological activity of RAG may be due to this slowly released RA. Other possible modes of action of RAG are suggested.

The glucuronic conjugates of retinol and retinoic acid (RA*) were first noted in the bile of rats over 20 years ago [1–3]. Although these water soluble compounds were initially considered as only detoxification and excretion products of vitamin A, they were later found to be subject to enterohepatic circulation and were detected in a variety of tissues [2, 4–7]. These glucuronides, retinoyl β-glucuronide (RAG) in particular, are active in the promotion of normal growth, induction of cellular differentiation and inhibition of neoplastic transformation [8–13]. In addition, these compounds are less toxic and teratogenic than other retinoids [14].

Little is known about the mechanism for intracellular transport and the molecular mechanism of action of RAG. It has been postulated that the action of retinol and RA in the control of epithelial differentiation, growth, and morphogenesis is mediated, respectively, by their specific cellular binding proteins, cellular retinol-binding (CRBP) and cellular retinoic acid-binding protein (CRABP) [15, 16], and/or by RA receptors (RARs) [17–19]. We therefore undertook a study on the interaction of retinoid β-glucuronides with these cellular binding proteins and nuclear receptors.

Materials and Methods

RA and retinol were purchased from the Sigma Chemical Co., St. Louis, MO. [11,12-^{3}H]RA (3.66 Ci/mmol) was supplied by the national Cancer Institute, Bethesda, MD. RAG was prepared by published procedures [20, 21].

CRABP-binding affinities of retinoids were determined by a technique involving sucrose-density-gradient sedimentation [22, 23]. Briefly, skin cytosol (2.5 mg protein) from 12- to 13-day-old chick embryos was incubated with 300 pmol of [11,12-^{3}H]RA in the presence or absence of 150-fold molar excesses of the unlabeled test compounds, followed by dialysis against 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl for 18 hr. The 2S CRABP peaks were determined from the radioactivity profiles obtained after sedimentation of the above preparations through 5–20% sucrose-gradients at 180,000 *g* for 18 hr [22, 23]. The relative binding affinities of the test compounds for CRABP were expressed on the basis that a 150-fold molar excess of unlabeled RA caused 100% inhibition of binding of [^{3}H]RA [22].

Binding affinities of retinoids for RAR were assessed by use of nuclear extracts prepared from 12- to 13-day-old chick embryo skin as described earlier [19]. The nuclear pellet was homogenized in 10 mM Tris–HCl, pH 8.5, 10 mM thioglycerol, 10% glycerol, 0.8 M KCl, and 10 mM sodium molybdate containing 1 mM phenylmethyl sulfonyl fluoride and 5 U/mL each of aprotinin and leupeptin, and centrifuged at 120,000 *g* for 60 min. The nuclear extracts (2 mg protein) were incubated overnight at 4° with 3 nM [^{3}H]RA in the presence or absence of 100-fold molar excess of RA or other test compounds. The free ligands were removed by treatment with dextran-coated charcoal [19]. The preparations were then analyzed for the presence of the RAR–[^{3}H]RA complex by chromatography through a Sepharose-12 size-exclusion column attached to a Pharmacia FPLC system. Bovine serum albumin (M_r 68,000), ovalbumin (M_r 44,000) and cytochrome *c* (M_r 12,400) were used as external markers for a molecular size.

For stability studies on RAG, the retinoid was incubated at a concentration of 10^{-4} M in chick embryo skin cytosol in 50 mM Tris–HCl, pH 7.4, or in the Tris-buffer alone for periods up to 48 hr at room temperature. The preparations, after extraction with *n*-butanol:methanol (95:5, v/v) in 0.005% butylated hydroxytoluene, were examined for hydrolytic or degradative products of RAG by passing through a Chromanetics Spherisorb ODS reverse-phase column attached to a Waters HPLC system. The solvent used was 80% acetonitrile and 20 mM ammonium acetate. These preparations were also examined for their capacity to bind to CRABP in competition with [^{3}H]RA in sucrose-density-gradient sedimentation experiments.

Results and Discussion

The sucrose-gradient sedimentation profiles of [^{3}H]RA in the presence or absence of unlabeled retinoids, after incubation with the CRABP-containing preparation from chick skin, are shown in Fig. 1A. The 2S radioactive peak which corresponds to the CRABP-[^{3}H]RA complex was eliminated completely by competition with a 150-fold molar excess of RA. However, a similar fold excess of RAG did not affect the 2S peak, indicating that the glucuronide does not compete for the RA binding site on CRABP.

Certain retinoids, e.g. retinobenzoic acids, Ch 55 and AM 80, which do not show affinity for CRABP, bind to RARs and possess high biological potency [24]. We tested whether RAG, a biologically potent retinoid, also functions in a similar manner by directly interacting with RAR. Figure 1B illustrates the resolution of RAR on a Superose-12 column. The radioactive peak corresponding to the molecular size of 50,000 is due to RAR–[^{3}H]RA complex [19]. Upon incubation of the preparations with a 100-fold molar excess of unlabeled RA, this radioactive peak was not present, indicating the specificity of RA binding to the

* Abbreviations: RA, retinoic acid; RAG, retinoyl β-glucuronide; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; and RAR, nuclear receptor of retinoic acid.

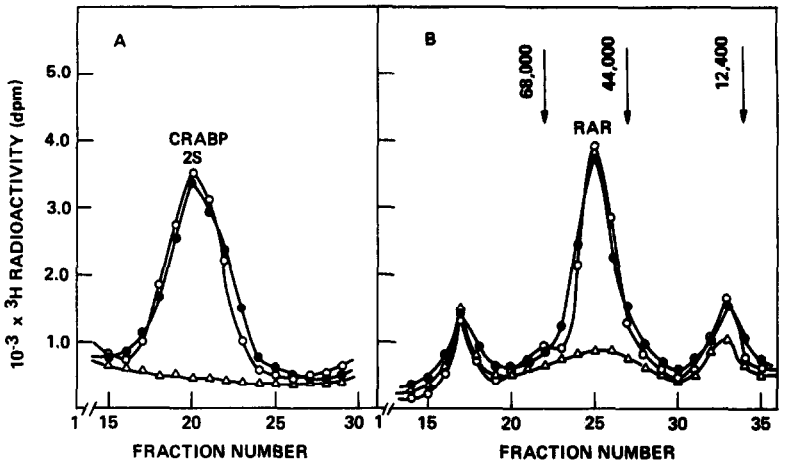

Fig. 1. (A) Sucrose-density-gradient centrifugation patterns of chick embryo skin cytosol (2.5 mg protein) + [^{3}H]retinoic acid (300 pmol) in the presence or absence of 150-fold molar excess of unlabeled test compounds. Key: (○) skin extract + [^{3}H]RA (control); (△) control + RA; and (●) control + RAG. (B) Radioactivity distribution patterns after Superose-12 column chromatographic resolution of nuclear extract (2 mg protein) after incubation with 3 nM [^{3}H]RA in the presence or absence of 100-fold molar excess of unlabeled retinoids. Key; (○) control; (△) control + RA; (●) control + RAG. The elution volumes of protein markers are shown by the arrows [bovine serum albumin (68,000); ovalbumin (44,000); cytochrome *c* (12,400)].

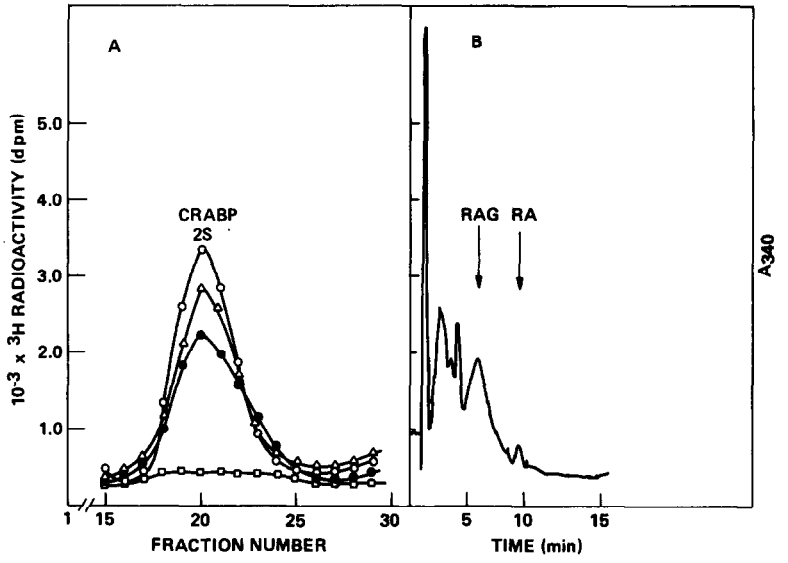

Fig. 2. (A) Sedimentation patterns through sucrose-gradients of chick embryo skin cytosol (2.5 mg protein) after incubation with 10^{-4} M RAG for 24 and 48 hr, and subsequent incubation with [^{3}H]RA (300 pmol). Key: (○) skin cytosol + [^{3}H]RA (control); (△) skin cytosol after incubation with RAG for 24 hr + [^{3}H]RA; (●) skin cytosol after incubation with RAG for 48 hr + [^{3}H]RA; and (□) skin cytosol + [^{3}H]RA + 150-fold excess of RA. (B) HPLC Spherisorb ODS column chromatographic resolution of RAG-derived products after incubation in chick skin cytosol for 48 hr. Elution of authentic samples of RAG and RA are shown by arrows.

nuclear receptors. Competition with 100-fold molar excess of RAG, however, did not produce any substantial inhibition in the [^{3}H]RA binding to RAR. Thus, we conclude that RA does not interact with either RAR or CRABP to display its biological action.

One of the possibilities for RAG to be biologically active is through generation of RA by its hydrolytic cleavage. To test such a possibility, RAG was incubated for up to 48 hr at room temperature in 50 mM Tris–HCl, pH 7.4, and in chick skin cytosol prepared in the same buffer. These preparations were tested for their capacity to bind to CRABP. Figure 2A represents the Sucrose density-gradient sedimentation patterns of the cytosol preparations in the presence of [^{3}H]RA. No competition was evident for [^{3}H]-RA binding to CRABP in preparations that were preincubated up to 12 hr. After 24 and 48 hr of incubation,

the preparations showed, respectively, 15 and 32% inhibition of [^{3}H]RA binding to CRABP, indicating that free RA may have been generated during the longer incubation periods. Two experiments performed under identical conditions yielded similar results. The preparations incubated in buffer alone, however, did not exhibit substantial inhibition of [^{3}H]RA binding (not shown). This suggests that the formation of RA from RAG is largely due to enzymatic hydrolysis.

The HPLC-column chromatographic resolution of retinoids is shown in Fig. 2B. RAG and RA eluted, respectively, at 6.0 and 9.7 min. After 48 hr of incubation in cytosol, a detectable amount of RA was generated from RAG. Under these conditions, however, RAG was predominantly converted into more polar metabolites, which eluted prior to RAG. The nature of these metabolites is not known, but 4-oxo and 4-hydroxy derivatives are likely to be generated under these conditions [21].

Our data indicate that RAG does not interact directly with either CRABP or RAR in the mediation of its biological activity in the modulation of differentiation and tumorigenesis [8–13]. The free carboxyl group on the glucuronide moiety does not substitute for the carboxyl group of RA for affinity binding to these protein receptors. RAG has been shown to stimulate granulocyte differentiation in HL-60 cells [12, 13, 25], which contains RARs but not detectable amounts of CRABP. On prolonged incubation (24–72 hr), release of RA from RAG in HL-60 cells has been reported [12, 13]. Short incubations for 1 hr did not cause such release of RA. Our findings on the generation of RA from RAG in the cytosol during prolonged incubation are in agreement with the above observations.

Concerning the biological activity of RAG, some questions that remain unanswered are: (a) For RAG-induced gene expression, does RAG function in a manner independent of CRABP and RAR, perhaps by serving as a natural ligand for RXR, the orphan receptor for retinoids [26]? (b) Does RAG function as a substrate to form retinoylated nuclear proteins, such as those observed in HL-60 cells [27]? (c) Is the RA generated from RAG responsible, at least in part, for the biological activity of RAG in tissues? Further studies on the mechanism by which RAG induces cellular differentiation are warranted.

Acknowledgements—This research was supported by NIH (PHS) Grants CA 34968, DK 32793 and 39733, and CR 46406.

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