HYDROCORTISONE RECEPTORS AND THEIR NUCLEAR ACCEPTOR SITES IN THE DEVELOPING CHICK RETINA

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SUMMARY: In the embryonic chick retina, hydrocortisone (HC) elicits an age-dependent induction of glutamine synthetase (GS). Cytoplasmic HC-receptors from retinas of both preinducible (7-day) and inducible (12-day) stages of embryonic development display similar chromatographic and sedimentation properties. Specific, saturable receptor-hydrocortisone (R-HC) acceptor sites are detectable in nuclei from retinas of both stages. The concentration of these sites in the chromatin from 7-day retina is greater than that in the 12-day retina. These results suggest that the development of GS inducibility in the retina is either due to ontogenic changes in the specificity of the receptor-acceptor interaction or due to developmental changes affecting the transcription or translation of GS mRNA.

In the embryonic chick retina, HC[1] and several other related corticosteroids elicit in-ovo and in-vitro an age-dependent, tissue-specific induction of GS (1). The embryonic retina shows three distinct phases of hormone-sensitivity (2). An early preinducible phase (6-7 day) when HC has virtually no effect on the low level of GS present, an intermediate inducible phase (8-14 day), during which period the sensitivity of the tissue to GS induction by HC markedly increases reaching a maximum between 11-13 day and a late induced phase (17-20 day) when endogenous steroids in-ovo increase the level of GS to a very high level and the tissue is essentially insensitive to additional exposure to hormone. Our initial investigations (3,4) on the effect of HC on GS induction in the isolated 12-day embryonic retina showed that the early events are very similar to the action of steroid hormones in various adult tissues (5,6). Following the association of HC with cytoplasmic receptors, the R-HC complexes migrate to nuclei and bind to nuclear acceptor sites. When retinas from 12-day embryos are incubated with progressively higher doses of HC, increases in the levels of GS induction correlates well with the amount of nuclear bound R-HC complex (4). More recently, we have demonstrated that treatment of 12-day retina with HC elevates the level of GS-mRNA in the polysomes from induced cells (7). However, the molecular basis of the lack of hormone-sensitivity at the preinducible stage is unknown. Previous reports (8,9) indicate that the lack of competence for GS induction in the preinducible retina is not due to insufficient quantity of cytoplasmic HC-receptors. The

[1]Abbreviations: HC - hydrocortisone; GS - glutamine synthetase; R-HC - Receptor-hydrocortisone complex; TMC - 0.02M Mg Cl_2, 0.001M $CaCl_2$, pH 7.8; TMCE - TMC plus 1.5mM EDTA.

purpose of this investigation was to compare the physicochemical and nuclear binding properties of the cytoplasmic HC-receptors in preinducible and inducible retinas and identify the factors responsible for the age-related development of HC-sensitivity.

MATERIALS AND METHODS: Materials. 1,2,6,7, ^{3}H-HC (91-93 Ci/mMole) was obtained from New England Nuclear or Amersham. Non-radioactive hormones, cytochrome C and Bovine serum albumin were from Sigma. Neural retinas from 7-day and 12-day embryonic chicks were uses as preinducible (insensitive to GS induction by HC) and inducible (sensitive to GS induction by HC) tissues respectively.

Preparation of Nuclei, Chromatin and Cytosol: Except for the following modification procedures for preparation of nuclei and cytosol were those as described before (3) for the 12-day retina. Because of their lower density, 7-day retina nuclei were purified by sedimentation through 1.85 M sucrose-TMC buffer instead of 1.95 M sucrose-TMC used for the 12-day nuclei. Nuclei from 7-day retinas have a protein: DNA ratio of about 3:1, whereas for the 12-day retina nuclei, this ratio is about 2.1:1.

Chromatin was prepared from purified nuclei using a modification of the procedure of Spelsberg and Hnilica (10). Nuclei were homogenized and washed respectively with 0.5% Nonidet-P40 (once), 0.08M NaCl-0.02M EDTA pH 6.3 (twice), 0.15M NaCl (twice) and TMC buffer (twice). After each wash, the nuclei were pelleted by centrifugation at 15,000 g for 10 min. Final nuclear pellets were sonicated in TMC buffer and used for the estimation of radioactivity, DNA and protein.

Following separation from the nuclei, EDTA (1.5mM final concentration) was added to the cytosols for improved stability of the HC-receptors. ^{3}Cytosols stored in ice in thepresence of EDTA and a saturating dose (50 nM) of ^{3}H-HC did not undergo any detectable loss in receptor activity for at least 24 hours.

Chromatographic and Sedimentation Analysis: Cytosols (8-16 ml containing 2-3 mg/ml protein), preincubated with 50 nM ^{3}H-HC for one hour, were added to 1.5 x 30 cm DEAE-sephadex-A-50 columns equilibrated with TMCE buffer. Following passage of 50 ml of TMCE and 25 ml of 0.1M NaCl-TMCE to remove the non-receptor proteins, the HC-binding proteins were eluted with a 0.1-0.3M NaCl gradient. Fractions representing the major peak of bound HC were pooled, concentrated by ultrafiltration in Amicon Cell using PM 30 filters. Aliquots (0.2 ml) of the concentrates were sedimented at 48,000 RPM for 16 hours in 4.8 ml gradients of 5-20% sucrose-TMCE using an SW 50.1 rotor in Spinco Model L-265 centrifuge. Cytochrome C and bovine serum albumin were used as markers.

Determination of Specifically Bound HC in Different Fractions in Retinas Cultured in Presence of ^{3}H-HC: Retinas (3 retinas per 10 ml for 12-day and 9 retinas per 10 ml for 7-day) were cultured at 37°C for the indicated times in the presence of varying concentrations of ^{3}H-HC in 99% Eagles Basal medium (with Earle's balanced salt solution) and 1% penicillin-streptomycin mixture as described before (4). For all ^{3}H-HC concentrations used, parallel cultures containing in addition to ^{3}H-HC, 200-fold non-radioactive HC as competitor, were run to determine the level of non-specific binding. Purified nuclei were washed twice with 0.5M Sucrose-TMC prior to assay of bound HC. The amount of specifically bound HC in the nuclei or chromatin were calculated by subtracting the amount bound in the presence of the competitor from that bound in its absence. The non-specific binding was approximately 10% of the total in all cases.

HC-receptor activity in the cytosols were determined by the charcoal assay procedure as described before (3.4).

Other Assays: Radioactivity was measured at 27% efficiency in a Beckmann LS200 counter using a mixture of Toluene-Triton-X 100 (3:1 v/v) containing

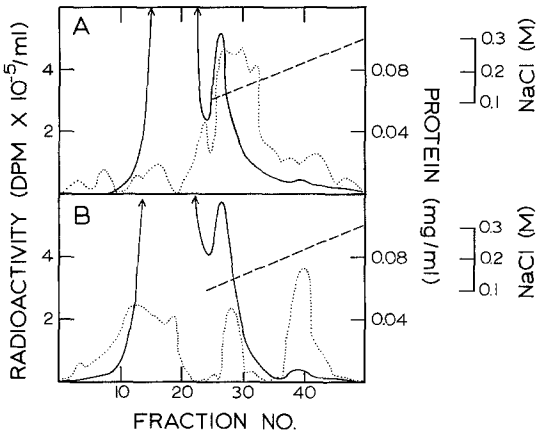

Fig. 1. DEAE-sephadex chromatography of HC-receptors of 7-day (A) and 12-day (B) retina cytosol. Cytosols were incubated with 50 nM ^{3}H-HC at 0°C for one hour and applied to the columns. _____, Radioactivity, ..., protein, - - - - - -, molarity of NaCl in the gradient. The initial radioactivity peak is unbound HC. The major peak of bound HC fractions (26-35) eluting at a salt concentration of about 0.12M were pooled, concentrated and used for sedimentation analysis.

4g Omnifluor (New England Nuclear) per litre. DNA and protein were estimated by the procedures of Burton (11) and Lowry et al., (12) respectively.

RESULTS: Fig. 1 illustrates that when cytosol from retinas of preinducible (7-day) or inducible (12-day) stages of embryonic development were incubated with 50 nM ^{3}H-HC and fractionated in DEAE-sephadex columns, the major HC-binding peak in both cases emerged at a salt concentration of about 0.12M. The specificity of binding of HC is indicated by the fact that in parallel samples incubated with 50 nM ^{3}H-HC plus 200-fold non-radioactive HC, little or no radioactivity is observed in this region (data not shown). Additional minor HC-binding components were present in both samples (Fig. 1), but their amounts are too small to evaluate quantitative differences or specificity of binding.

Fractions representing the major peak of bound HC were pooled, concentrated 50-fold by ultrafiltration in Amicon cell (PM 30 filter) and appied to sucrose gradients for sedimentation analysis. Two distinct peaks with sedimentation coefficients of about 3S and 5S were observed for the bound hormone from both 7-day and 12-day retina cytosol (Fig. 2). Although the ratios of the 3S and the 5S components appears to be similar, their exact proportions are difficult to evaluate since the rates of dissociation of HC from these components may not be identical. The relative binding affinities of the 3S and 5S components to HC and other GS-

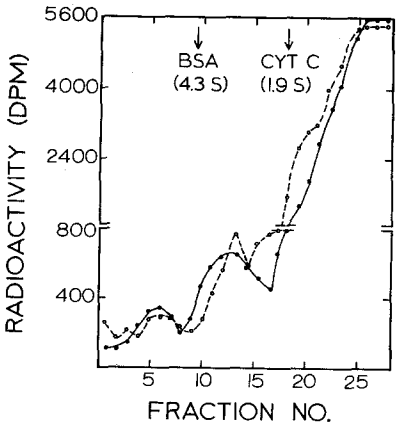

Fig. 2. Sucrose gradient centrifugation of HC-receptors of 7-day (0-0-0) and 12-day (●-●-●) retina cytosol. Fractions (26-35) from the DEAE-sephadex elution (Fig. 1) were pooled concentrated 50-fold by Amicon (PM 30 filter) and 0.2 ml samples were applied to 5-20% gradients. Cytochrome C (1.9S) and BSA (4.3S) were used as markers.

inducing and non-inducing steroids and the individual role of these components in GS induction is at present unknown.

To determine if the lack of response to GS induction by HC in the preinducible retina is due to the inability of the cytoplasmic receptors to interact with the nuclear acceptor sites, we compared the translocation of cytoplasmic receptors to nuclei and the binding of the R-HC complexes to nuclear acceptor sites in cultures of preinducible (7-day) and inducible (12-day) retinas Fig. 3 shows that when incubated with near saturating dose of ^{3}H-HC (9 nM), nuclear translocation of cytoplasmic receptors takes place effectively in the 7-day retina as in the 12-day retina. Over 90% of the cytosol receptors were translocated into nuclei in both cases and at the plateau level, the amount of nuclear bound hormone in the 7-day retina was higher than that in the 12-day retina. To examine this in more detail, retinas were incubated with increasing concentration of ^{3}H-HC in the presence or absence of unlabeled HC for one hour and the amount of specifically bound HC in the nuclei was determined at each concentration (see 'Methods'). The results (Fig. 4) showed that as in the case of 12-day retina, nuclear binding in the 7-day retina tends to reach saturation in the presence of higher doses (9-90 nM) of HC. At saturation, the estimated concentration of nuclear acceptor sites was significantly greater in the 7-day retina than in the 12-day retina (Table I). To determine if only part of the nuclear radioactivity represented chromatin-bound ^{3}H-HC and the rest was free in

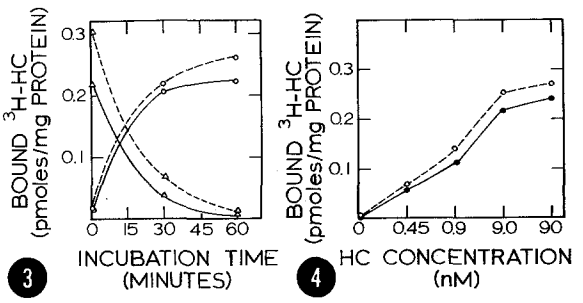

Fig. 3. Distribution of bound ^{3}H-HC in the nuclei (circles) and cytosol (triangles) in 7-day (---) and 12-day (——) retinas during incubation with a near saturating dose (9nM). Retinas from 7-day or 12-day embryos were incubated with ^{3}H-HC in the presence or absence of 200 fold non-radioactive HC and the amount of specifically bound ^{3}H-HC in the nuclei or cytosol was determined as described in 'Methods'. Zero time data points were obtained by addition of ^{3}H-HC (with or without the competing non-radioactive HC) to retina cultures at time zero and immediate fractionation of nuclei and cytosol.

Fig. 4. Concentration of bound HC in the nuclei of 7-day (- - -) and 12-day (——) retina following a 60 minute incubation with the indicated doses of ^{3}H-HC.

TABLE I

Concentration of Nuclear Acceptor Sites for R-HC complexes in the Nuclei and Chromatin from 7-day and 12-day Retina

Age of the Retina	Amount of specifically bound ^{3}H-HC at saturation (pmoles/mg DNA)				DNA per Nucleus (pgms)	Approximate No. of R-HC binding sites per nucleus*
	Nuclei			Chromatin		
	Expt.I	Expt.II	Av.			
7-day	0.81	0.78	0.795	0.78	7.6	3600
12-day	0.58	0.48	0.53	0.57	5.0	1600

*Calculated assuming a 1:1 association of the R-HC complex with the nuclear acceptor sites and equal distribution of acceptor sites in all cell types in the retina tissue. Protein: DNA ratios for the 7-day and 12-day retina nuclei are 3:1 and 2.1:1 respectively.

the nucleoplasm, chromatin was prepared from both 7-day and 12-day retinas which were preincubated with 9 nM ^{3}H-HC (in the presence and absence of 200-fold unlabeled HC) for 60 minutes and the amount of specifically bound ^{3}H-HC to chromatin was determined. The results (Table I) clearly indicate that in both 7-day and 12-day retinas, most of the radioactivity is bound to chromatin and that little or no

hormone was present in the nucleoplasm. Based on the higher DNA content of the 7-day nuclei, the estimated concentration of R-HC acceptor sites in the 7-day retina nuclei (3600 per nucleus) is about twice that in the 12-day retina nuclei (1600 per nucleus).

DISCUSSION: There have been several reports (13-16) that the mere presence of steroid hormone receptors in a tissue does not necessarily correlate with the sensitivity of the tissue to the hormone. Giannopoulus (15) has recently reported the presence of cytoplasmic receptors for dexamethasone as well as their nuclear acceptor sites in fetal rat liver which is insensitive to glucocorticoids. However, the level of nuclear bound dexamethasone, at saturation, was almost half in the nuclei from fetal liver compared to that in livers from post natal rats. The present results indicate that the lack of inducibility of GS by HC in the preinducible retina is neither due to inadequate amount of HC-receptors nor due to their insufficient interaction with the nuclei. The sedimentation, chromatographic and nuclear binding properties of the HC-receptors of preinducible and inducible retinas are very similar. However, despite these similarities, the difference with respect to GS inducibility between the 7-day and the 12-day retina may be due to the presence of multiple classes of HC-receptors binding to different chromatin acceptor sites at different stages of retina maturation. Hopefully, studies on the relative binding affinities of the individual receptor components to GS-inducing and non-inducing steroids will resolve this problem.

Alternatively, the difference in GS inducibility between the 7-day and 12-day retina may lie at a step distal to the binding of HC-receptors to retina chromatin, such as transcription or translation of GS mRNA. Since in the inducible (12-day) retina the interaction of HC with chromatin results in an elevated level of functional mRNA for GS (7), a comparison of the effect of HC on polysomal (functional) and non-polysomal (non-functional) levels of GS mRNA in the 7-day and 12-day retinas appears to be the next logical step.

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