GLUCOCORTICOID INHIBITION OF GLYCOSAMINOGLYCAN BIOSYNTHESIS: DECREASE OF PROTEIN ACCEPTOR

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Summary

The body weight and glycosaminoglycan content of 12 day old chick embryos are significantly reduced by hydrocortisone (0.5 mg/egg). The hormone effect appears to be an inhibition of glycosaminoglycan synthesis because this amount of hormone does not increase the rate of radioactive sulfate turnover in cartilage. Xylosyl-transferase activity is decreased in a crude preparation and experiments performed using Smith-degraded proteoglycan for the acceptor suggest that the enzyme itself is not rate limiting. By mixing enzyme and endogenous protein acceptor fractions from control and hormone treated embryos, it was shown that the available acceptor sites for xylose are decreased by glucocorticoid treatment.

Introduction

Glucocorticoids cause growth retardation in children (1, 2) and osteoporosis in adults (3). The biosynthesis of glycosaminoglycans, one of the essential components of the extra-cellular matrix required for normal skeletal growth, is inhibited by glucocorticoids (4, 5).

Neither the specific site of the glucocorticoid inhibition of glycosaminoglycan biosynthesis nor the mechanism of its effect on bone has been clearly defined. Postulated mechanisms include suppression of the growth hormone-dependent somatomedin generating system (6, 7) or a direct effect at the cartilage level (8).

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Glycosaminoglycans are synthesized by stepwise linkage of sugar residues to a preformed protein acceptor (9). The first step in this pathway is the transfer of xylose from UDP-xylose to the protein acceptor by the enzyme xylosyltransferase: [UDP-xylose-protein xylosyltransferase, (EC 2.4.2.26)] (10). Two galactose and one glucuronic acid residues are added in sequence and require specific glycosyltransferases (11). The level of xylosyltransferase correlates with the rate of sulfate incorporation into glycosaminoglycans and presumably the amount synthesized (12).

Glycosaminoglycan synthesis is inhibited by puromycin and cyclohexamide and the inhibition can be partially reversed by adding β-xylosides as acceptors (13, 14). These studies suggest the availability of acceptor may be the critical factor in the control of glycosaminoglycan biosynthesis rather than the activity of UDP-xylosyltransferase. The UDP-glycosyltransferase reactions are bi-substrate reactions with the endogenous acceptor (the second substrate) being present in the crude preparation with enzyme (9). Studies by Stoolmiller et al have shown that xylosyltransferase can be separated from the endogenous acceptor and that a Smith-degraded proteoglycan is a suitable acceptor for xylose in this reaction (12). Using these techniques, we have tried to distinguish between an effect of glucocorticoids on formation of the linkage region and an effect on the formation of the acceptor protein.

Materials and Methods

Fertile eggs were furnished by the Keith Smith Hatchery, Hot Springs, Arkansas. [U^{14}C]-leucine (250 Ci/mole); UPD [U^{14}C]-galactose (280 Ci/mole); USP [U^{14}C]-xylose (172 Ci/mole); and UDP [U^{14}C]-glucuronic acid (233 Ci/mole) were purchased from New England Nuclear Corporation, Boston, MA. Hydrocortisone phosphate was obtained from the Upjohn Co., Kalamazoo, MI., and pronase was obtained from Calbiochem, LaJolla, CA. All other chemicals were reagent grade and were obtained from Fisher Scientific Co., St. Louis, MO.

Eggs were incubated in a Humidaire egg incubator at 39°, with high humidity and rotated hourly. Injections were made onto the chorioallantoic membrane of 10 day old chick embryos with either hydrocortisone phosphate (0.5 mg), or 0.9% saline in a volume of 0.1 ml. The embryos were killed on day 12, weighed, and the wet and dry weight of the pelvic rudiment determined. The pelvic rudiments or long bone cartilage were removed from other embryos and pooled for assay of glycosyltransferase activities.

Assay of Glycosyltransferases: The glycosyltransferase activities were measured as described by Grebner et al (15) with minor modifications in our laboratory (16). Xylosyltransferase activity was assayed at pH 6.5 with 3.2 µM UDP [^{14}C] xylose and 6 mM $MnCl_2$. In studies using exogenous acceptor, xylosyltransferase was assayed as described by Stoolmiller et al (12) and modified in our laboratory using 250 µg of the exogenous acceptors plus 5 nmol of unlabeled UDP-xylose (17).

The exogenous acceptor was prepared by Smith degradation of bovine chondrotin sulfate-protein complex as described by Baker et al (17) and kindly given to us by Dr. A. L. Dorfman (University of Chicago).

Galactosyltransferase activity was assayed at pH 5.5, using 7.5 µM UDP [^{14}C] galactose and 4 mM $MnCl_2$. Glucuronyltransferase activity was assayed at pH 6.5 using 4.3 µM UDP [^{14}C] glucuronic acid and 2 mM $MnCl_2$. Incorporation is linear for at least 30 minutes and the assays were incubated for 20 minutes.

To estimate the number of available endogenous acceptor sites for xylose, the assay was carried out as described previously (17) using the 10,000 x g supernatant from control and hydrocortisone treated cartilage. Excess UDP [^{14}C] xylose (3.2 µM) was added at 30 minute intervals and aliquots were removed at 15 minute intervals. Controls contained enzyme which had been incubated without substrate for the same time interval and substrate added 15 minutes prior to stopping the reaction to assure that the enzyme remained active over the time course of the experiment.

Glycosaminoglycans were isolated from cartilage by a modification [Elders et al (16)] of the method of Antonopoulos et al (19) and uronic acids were determined by using the carbazole method of Bitter and Muir (20). Protein concentrations were determined by the method of Lowry et al, using bovine serum albumin as a standard (21). Radioactivity was measured in a liquid scintillation spectrometer using 10 ml of the xylene-dioxane cellosolve counting solution described by Bruno and Christian (22).

For estimation of the half-life of glycosaminoglycans in embryonic chick cartilage, pelvic rudiments were removed and incubated in Ham's F-10 nutrient mixture for 16 hours with 5 µCi of carrier free $Na_2{}^{35}SO_4$. Cartilages were then washed thoroughly and incubated at 37° C in 95% air, 5% CO_2 in Ham's F-10 nutrient mixture without ^{35}S and with or without 0.5 mM cortisol phosphate. The media was changed every 48 hours. Five rudiments were removed at various times during the incubation and the radioactivity of the ^{35}S relative to uronic acid was determined as described by Elders et al (16).

Results and Discussion

Treatment of 10 day old chick embryos with 0.5 mg of hydrocortisone phosphate per egg for 48 hours caused a reduction in body weight of the embryo from 3.84±0.23 to 2.37±0.09 grams and a decrease in the uronic acid content of cartilage from 32.8±2.2 to 17.8±1.2 micrograms/mg dry weight. This marked reduction in glycosaminoglycan content of the cartilage could be secondary to either a reduction in the rate of biosynthesis or an increase in rate of turnover. Labeling of the cartilage with ^{35}S and observing the rate of disappearance

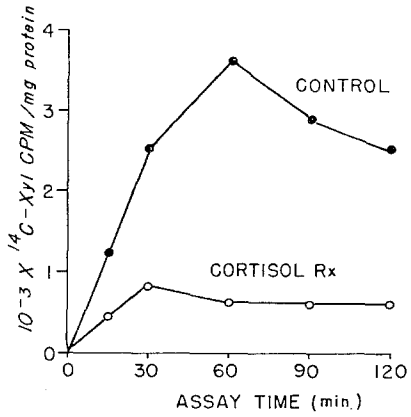

Figure 1: Glycosaminoglycan turnover in control and hydrocortisone (cortisol) treated chick cartilage. The radioactivity of ^{35}S relative to the uronic acid content of isolated glycosaminoglycan is plotted semilogarithmically against time. Each point represents the mean for 5 pelvic rudiments.

TABLE I

Glycosyltransferase Activities in
Control and Hydrocortisone Treated Cartilage

Enzyme Assayed	Control	Hydrocortisone Treated
	pmol/mg protein/hr	
Xylosyltransferase	7.6±0.5	4.1±0.3
Galactosyltransferase	23.2±2.0	20.8±1.6
Glucuronyltransferase	2.1±0.2	2.7±0.3

Cartilage was pooled from 6 dozen 12 day old embryos following injection on the 10th day of incubation. The control group received saline and the treated group 0.5 mg hydrocortisone phosphate in saline. The values represent the mean and standard error for 6 assays using 100 µl of the 10,000 x g supernatant fraction.

of ^{35}S from glycosaminoglycans (Figure 1) shows that turnover is not increased. The $t_{\frac{1}{2}}$ was 11 days for the hydrocortisone treated cartilage and 8 days for control cartilage.

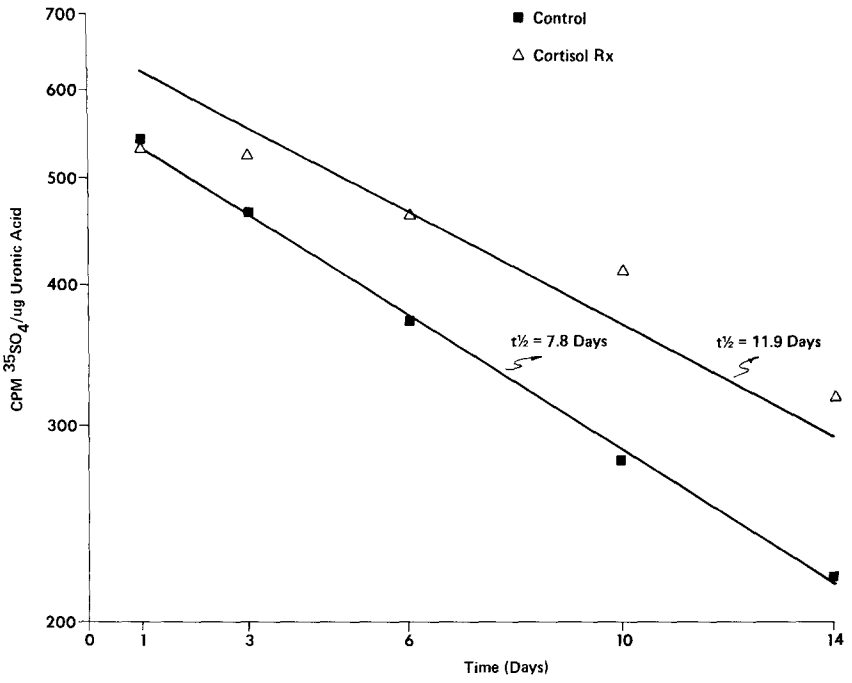

Figure 2: Effect of incubation time on the extent of xylose incorporation into a trichloroacetic acid precipitable protein in the 10,000 x g cartilage supernatant solution from 12 day old control and hydrocortisone treated chick embryos.

Using cartilage from 12 day embryos treated with 0.5 mg of hydrocortisone on day 10, the activities of the glycosyltransferases were measured. The activity of xylosyltransferase in the 10,000 x g supernatant prepared from these cartilages show a 50% reduction in activity compared to the saline-injected controls (Table I). There were only slight changes in galactosyl and glucuronyltransferase activity in these preparations. The addition of hydrocortisone phosphate to the xylosyltransferase assay, in vitro, in concentrations ranging from 25 μM to 25 mM had no effect on isotope incorporation.

The rate of xylose incorporation for both control and hydrocortisone treated cartilage was linear for 30 minutes but was reduced for the hydrocortisone treated cartilage (Fig. 2). When the incubation time was extended to 120 minutes to determine the extent of incorpotation of xylose, the

TABLE II

XYLOSYLTRANSFERASE ACTIVITY

A. Individual Fractions from Control and Hydrocortisone Treated Cartilage

Cartilage Fractions	Xylose incorporated pmol/mg protein/hour		
	Control	Hydrocortisone	% Decrease
10,000 g supernatant (Enzyme & Acceptor)	42.4±4.1	18.4±1.7	57%
105,000 g supernatant ("Enzyme")	12.4±1.1	8.4±1.1	32%
105,000 g pellet ("Acceptor")	28.2±2.3	6.2±1.4	78%

B. Mixed Fractions from Control and Hydrocortisone Treated Cartilage

Source of Fractions		Xylose incorporated pmol/mg protein/hr
Acceptor	Enzyme	
Control	Control	32.0±2.1
Hydrocortisone	Hydrocortisone	15.8±1.2
Control	Hydrocortisone	44.4±3.6
Hydrocortisone	Control	18.4±1.8

Cartilage from 6 dozen 12 day old control and 10 dozen hydrocortisone treated chick embryos (0.5 mg hydrocortisone/egg on day 10 of embryonic life) was used for preparing the fractions. The 10,000 x g supernatant solution was made 1 M with respect to KCl, frozen and thawed 6 times and centrifuged at 105,000 x g for one hour to obtain a supernatant solution and a pellet. The 105,000 x g pellet was reconstituted with buffer to the same volume as the supernatant solution. 100 µl of each fraction was used for assay of the individual fractions (A), and 50 µl of enzyme and 50 µl of acceptor was used for the mixed fraction assays (B). The values are the mean and the standard error for 6 assay tubes.

incorporation by control cartilage was 5-6 times greater than by the hydrocortisone treated cartilage. This suggests that hydrocortisone either decreases the number of acceptor sites available for xylose incorporation or inhibits xylose transfer.

These options were studied as follows: The enzyme was separated from the acceptor and each fraction was either used separately or mixed with another fraction to assay the xylosyltransferase activity (Table II). The rate of xylose incorporation by the 10,000 g supernatant of the hydrocortisone treated cartilage containing both enzyme and acceptor protein, was decreased 57%. The xylose incorporation rate in the 105,000 g supernatant ("enzyme" fraction) was decreased 32% and in the 105,000 x g pellet ("acceptor" fraction) was decreased 78% (Table IIA). When control "enzyme" fraction was mixed with hormone treated protein "acceptor" fraction, the rate of xylose incorporation was similar to that seen with the hydrocortisone treated enzyme and acceptor fractions (Table IIB). Conversely, when control acceptor fraction was mixed with hydrocortisone treated enzyme, the rate of xylose incorporation was greater than that of mixed control and acceptor, suggesting the acceptor rather than the enzyme was rate limiting.

To confirm this postulate, xylosyltransferase activity was determined using Smith-degraded proteoglycan as an exogenous acceptor (Table III). The rate of xylose incorporation was not significantly different for the two preparations: 235 pmoles/mg/hr for the control enzyme fraction, compared to 190 pmoles/mg/hr for the hydrocortisone treated fraction.

These observations provide a plausible explanation for the effects of the glucocorticoid hormones on skeletal growth and metabolism. Hydrocortisone appears to inhibit the synthesis of glycosaminoglycan acceptor protein or the transport of the acceptor to the glycosylation site in embryonic chick cartilage. These studies do not establish that the observed changes are a primary effect of the hormone on the acceptor protein. However, the data do suggest the cartilage acceptor protein content is depressed more than the enzyme protein

TABLE III

Effect of Hydrocortisone on Xylosyltransferase Activity with and without Smith-degraded Proteoglycan

"Enzyme" Preparation (105,000 x g supernatant)	Xylose Incorporated (pmole/hr per mg of protein)	
	Without Acceptor	With Acceptor
Control	6.2±0.4	235±18
Hydrocortisone	4.2±0.4	190±16

The enzyme fraction was from the same preparation used for the experiment presented in Table II. 250 µg Smith-degraded proteoglycan was used for the acceptor containing tubes and 5 nmol unlabeled UDP-xylose added with the labeled substrate to all tubes. The values represent the mean and standard error of the mean for 6 assay tubes.

content by this mode of treatment in this age embryo. Pharmacologic doses of glucocorticoid hormones decrease serum somatomedin concentration in nephrotic children (23) and the hormone effect observed here may be due to suppression of embryonic growth factors necessary for cartilage glycosaminoglycan biosynthesis An additional effect on the in vivo concentration of the UDP-sugars cannot be excluded with available data. Proof for these speculations will depend on direct measurement of the endogenous acceptor protein and UDP-xylose concentrations. The availability of acceptor has been postulated to be the rate limiting factor in glycosaminoglycan synthesis (24) rather than the rate of xylosylation (12). The data presented from this model agree with the point of view that the rate of synthesis of acceptor protein controls the rate of glycosaminglycan biosynthesis.

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