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Increased Synthesis of Hyaluronic Acid by Insulin in Embryonic Chick Skin[1] (37739)

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Insulin is known to be an anabolic hormone which may be instrumental in the regulation of a variety of biochemical systems. Our interest in insulin was initiated by the clinical observation that a large number of scleroderma patients exhibit hyperinsulinism (1). Scleroderma is a disease associated with accumulation of collagen in skin (2) and other internal organs. Stimulation of collagen synthesis in embryonic chick skin by insulin has been reported (3). The purpose of the present study was to find whether insulin has an effect on the biosynthesis of glycosaminoglycans (GAG). This report presents evidence that insulin specifically stimulates the synthesis of hyaluronic acid.

Material and Methods. Skin from 10-day-old chick embryo was removed and incubated in 35 × 10 mm Falcon tissue culture dishes at 37° in 95% O_2 + 5% CO_2. Usually skin from six embryos was combined in each incubation dish. The incubation medium (pH 7.4) consisted of BME Earle's base (1X) supplemented with glutamine (0.2 m*M*) and sodium bicarbonate (0.02 *M*) and also contained penicillin (100 units/ml) and streptomycin (100 μg/ml). Pork insulin twice crystallized (a gift from Dr. M. Root, Eli Lilly Research Laboratories) was dissolved in a small volume of 0.1 *N* HCl and brought to volume with the incubation medium. Insulin prepared in this manner was added to the appropriate incubation dishes at a final concentration of 386 μg/ml of media. The control dishes received an equal volume of incubation medium. Both insulin and control dishes were preincubated for 45 min before the addition of labeled precursor. 60 μCi of [6-^{3}H]glucosamine (1150 mCi/mmole) was added to each dish and incubation continued for 6 hr. A preliminary time course study had indicated that insulin had a stimulatory effect at 6 hr of incubation. At the end of the incubation period, the dermis were rinsed with ice-cold incubation medium and dehydrated with acetone. The tissues were defatted in chloroform–methanol (1:1, v/v) at room temperature for 3 days with at least six changes of the fat solvent. The tissues were dried in a desiccator until constant dry weight was obtained.

The dried, defatted dermis were digested with papain (4), and dialyzed in running tap water for 48 hr and then against distilled water. An aliquot of the retained material was used for determination of total hexosamine incorporation. Radioactivity was measured in a Beckman liquid scintillation equipped with external standardization. Radioactive GAG were isolated from the digested tissue by cetyl pyridinium chloride precipitation and further checked by assaying for both uronic acid and hexosamine.

Fractionation of GAG was carried out by electrophoresis on cellulose acetate strips. The buffer consisted of equal parts of 0.1 *M* calcium acetate–0.1 *M* copper acetate (pH 3.6, ionic strength 0.05). A mixture of standard GAG was added to each sample and duplicates were run simultaneously in a horizontal chamber at 2.0 mA/strip for 2 hr. One of each pair of strips was stained in Alcian

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TABLE I. *In Vitro* Incorporation of [6-^{3}H]Glucosamine into Hexosamine Containing Macromolecules and Glycosaminoglycans by Control and Insulin-Treated Chick Embryo Skin.[a]

	Hexosamine containing macromolecules[b]		Glycosaminoglycans[c]	
	(cpm/mg dry tissue)			
Sample	Control	Insulin	Control	Insulin
1	30,584	40,819	8830	16,219
2	31,528	39,765	10,808	17,015
3	27,207	37,530	10,923	16,931
4	24,128	35,115	12,902	15,724
5	21,099	—	12,922	—
Mean	26,909	38,307	11,277	16,472

[a] Both control and insulin-treated skins were incubated for 6 hr with 60 μCi of [6-^{3}H]glucosamine in a medium described in detail in the text.

[b] The dry, defatted tissues were subjected to papain digestion and then exhaustively dialyzed. The retentants in the dialysis bags were assayed for radioactivity.

[c] Glycosaminoglycans were isolated from dialyzed papain digested materials by the cetyl pyridinium chloride procedure.

blue to identify the individual GAG. This was used as a marker to cut the unstained sample GAG strip into several segments. Each segment of the cut acetate strip was counted in 15 ml of scintillation mixture. GAG were also separated by acetate paper electrophoresis using the acetic acid–pyridine buffer (pH 3.5) (5). Hexosamine was determined by modification of the Elson-Morgan reaction (6) after hydrolysis of the sample in 2 *N* HCl at 100° for 16 hr. Uronic acid was measured by the method of Bitter and Muir (7) with glucuronolactone as standard.

Results. Insulin stimulated glucosamine incorporation into hexosamine containing macromolecules (Table I). There is about 40% more radioactive glucosamine incorporation in the presence of insulin. On further isolation of GAG by the cetyl pyridinium procedure, insulin-treated dermis still maintained 40% more radioactivity than control GAG (Table I). Thus glucosamine incorporation and its specific biosynthesis into GAG are stimulated by insulin. Table II shows the radioactivity of the individual GAG fractions in control and insulin-treated dermis. Stimulation of GAG by insulin is primarily directed towards hyaluronic acid, where the radioactivity is increased by about 100%. The pooled insulin and control samples were electrophoretically run in both copper acetate and Gore buffers (5). In all runs, the pattern was reproducible and the stimulation of hyaluronic acid by insulin was well over 100% of the value for control. It should be noted that recovery of the radioactivity for both control and experimental dermis were similar.

Discussion The data presented in this communication indicates that insulin stimulates the *in vitro* biosynthesis of GAG and that this action is specific for hyaluronic acid. These findings gain additional support from preliminary studies in our laboratory showing that insulin had no effect on the incorporation of $Na_2{}^{35}SO_4$ into sulfate containing GAG in 10-day-old chick embryo dermis. Schiller and Dorfman (8) had previously observed a decrease in hyaluronic acid in alloxan diabetic rats which could be corrected by administration of insulin. Recently Berenson *et al* (9) using streptozotocin to induce diabetes in rats found a 20% decrease in total GAG content in the skin compared to the control animals; furthermore, this reduction was particularly noted in hyaluronic acid and dermatan sulfate B of the GAG.

No attempts have been made to elucidate the mechanism of stimulation of hyaluronic acid by insulin. A possible action of insulin may be on the first two steps of glucosamine metabolism, *viz,* transport and phosphorylation. Such a role of insulin has been established in the isolated diaphragm where insulin increased the levels of glucosamine 6-phosphate up to sixfold (10). Insulin may also stimulate hyaluronic acid synthesis by increasing the levels of various glucosyl transferases involved in the biosynthesis of GAG.

Our preliminary studies showing no influence of insulin on $Na_2{}^{35}SO_4$ incorporation in embryonic chick skin may also rule out a role of insulin on the final step of biosynthesis of sulfated GAG: transfer of sulfate from 3-phosphoadenosine-5-phosphosulfate

TABLE II. Effect of Insulin on Biosynthesis of Various GAG Fractions.[a]

	Control (cpm)	%	Insulin (cpm)	%
Hyaluronic acid	9362	59.8	18,042	74.2
Dermatan and chondroitin sulfate	3585	22.9	3972	16.3
Heparin	2712	17.3	2292	9.4
Total cpm	15,659		24,306	

[a] GAG were fractionated by electrophoresis on cellulose acetate paper in pyridine acetic acid buffer (pH 3.5) as described in the text. These are representative samples of the pooled dermis from control and insulin-treated embryonic tissues.

(PAPS) by sulfate transferases.

Summary. The *in vitro* effect of insulin on glycosaminoglycan synthesis was studied using D-[6-^{3}H]glucosamine in 10-day embryonic chick skin. Insulin stimulated both the total incorporation of glucosamine and glycosaminoglycan synthesis. Stimulation of glycosaminoglycan is specific for hyaluronic acid.

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