

The *in Vitro* Effect of Insulin on Collagen Synthesis in Embryonic Chick Tibia¹ (37196)

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(Introduced by R. H. Davis)

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It has been suggested that insulin may play a role in the incorporation of amino acids in protein synthesis (1-3). In 1958, Hay (4) observed larger fibroblast outgrowths from 7-day chick embryo bones when insulin was added to the culture medium. Gavin *et al.* (5) have reported the presence of specific insulin binding sites in cultured fibroblasts. Increased proliferation of cartilage cells from a fracture site of 14-day embryonic chick tibia *in vitro* in the presence of insulin has been noted (6). Clinical observations of a significant number of scleroderma and scleredema patients exhibiting hyperinsulinism showed increased collagen deposition in the skin (7, 8). The above data suggests the possible involvement of insulin as a regulator of collagen biosynthesis.

The purpose of this paper is (1) to see if insulin has an effect on collagen synthesis (2) if the effect is early or delayed and (3) if there is an effect on the glycosylation of hydroxylysine.

Material and Methods. Four to six pairs of tibia from 10-day Leghorn chick embryos were removed under a dissecting microscope, freed of all soft tissue and preincubated in 2.5 ml of incubation medium (BME-Earle's Base, TC-Glutamine (0.2 mM), Sodium bicarbonate solution (0.17%) Fetal calf serum, Type II (10%), Penicillin (100 U/ml), Streptomycin (100 µg/ml) and Mycostatin (80 U/ml) for 1 hr at 37° in Falcon 35 × 10 mm tissue culture dishes

in an atmosphere of 95% O₂ and 5% CO₂. Tibia obtained from the right side of the embryo were pooled and served as controls (minus insulin) for tibia from the left side of the embryo. Pork insulin was dissolved in 0.1 N HCl, brought to volume with incubation medium, added to the culture dishes (final concentration 154 µg/ml or 3.2 U/ml) and the preincubation continued for an additional 30 min. At the end of the preincubation period, 5 µCi of either L-[¹⁴C] proline (209 mCi/mole or L-[¹⁴C] lysine (225 mCi/mole) uniformly labeled (New England Nuclear) was added to each culture dish and the incubation continued for 2, 4, 6, 24, and 48 hr.

The method used for the study of [¹⁴C] lysine incorporation, hydroxylation and glycosylation was based on that described by Blumenkrantz and Prockop (9). The procedure of Kivirikko *et al.* (10) was used to assay for hydroxyproline content. The pork insulin used was a gift of Dr. M. Root of Eli Lilly and Co., Indianapolis, Indiana.

Tibias were also grown in the presence and absence of insulin for 7 days with media changes every 48 hr and assayed for protein (11) and hydroxyproline (12). Since an association between collagen and sulfated glycosaminoglycans has been postulated (13), hexosamine determinations (14) were also performed.

Results and Discussion. The rate of incorporation of both [¹⁴C] lysine and its subsequent conversion to the hydroxy form was found to be linear throughout the time periods studied. Insulin present at a concentration of 154 µg/ml was selected for use in the study since a 6-fold increase in concen-

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TABLE I. Time-Course Study of the *in Vitro* Effect of Insulin on [¹⁴C]Proline and [¹⁴C]Lysine Incorporation and Conversion to Hydroxy Derivatives by 10-Day Embryonic Chick Tibia.

Sample	Incubation time	Total [¹⁴ C] proline in-corporation (counts/min/bone) × 10 ⁻²	Total hydroxy [¹⁴ C]proline (counts/min/bone) × 10 ⁻²	Total [¹⁴ C] lysine in-corporation (counts/min/bone) × 10 ⁻²	Total hydroxy [¹⁴ C]lysine (counts/min/bone) × 10 ⁻²	Nonglycosylated [¹⁴ C]lysine (counts/min/bone) × 10 ⁻²	Glycosylated hydroxy [¹⁴ C]lysine (counts/min/bone) × 10 ⁻²
Control	2	187	25.0	130	16.3	12.3	4.0
Insulin		293	35.8	182	19.2	17.1	2.1
Control	4	654	74.8	187	28.8	18.0	10.8
Insulin		889	98.8	188	24.7	16.7	8.0
Control	6			227	34.5	20.2	14.3
Insulin				249	31.5	18.2	13.3
Control	24	2450	302.4	1707	176.7	130.7	46.0
Insulin		2608	273.6	1874	171.6	125.3	46.3
Control	48	5095	636.0	3401	395.4	230.2	165.2
Insulin		5453	537.6	3057	309.1	255.6	53.5

* The value in parenthesis represents the number of experiments performed. The data presented represents a single experiment from the group and is representative of the trend observed in the total group.

tration produced no additional effect on [^{14}C]proline incorporation. The addition of insulin to the culture medium promoted an early increase in the incorporation rate of both [^{14}C]proline and [^{14}C]lysine. There was a 57 and a 36% increase in [^{14}C]proline uptake after 2 and 4 hr incubation with insulin (Table I) with a concomitant increase in the conversion of proline to hydroxyproline of 43 and 32%, respectively. Others (2, 3) have reported a delayed effect (48 hr) of insulin on proline incorporation and hydroxyproline synthesis when newborn rat tibia were used.

The stimulatory effect of insulin on [^{14}C]lysine incorporation into bone protein was pronounced (40%) after only 2 hr incubation (Table I). Increased levels of hydroxylysine were observed (18%) only at the 2 hr period. On the other hand, there was a decrease in the glycosylation of the newly synthesized hydroxylysine.

It should be noted that there was an identical increase in both [^{14}C]proline and hydroxyproline content (hydroxyproline/proline ratio was similar for control and insulin treated) suggesting that the effect was solely on collagen biosynthesis. Comparing the proline and lysine data in Table I it would appear that insulin is stimulating collagen biosynthesis and at the same time slightly inhibiting the lysine hydroxylase. This is possible since it is known that two enzyme sites are involved in the hydroxylation of protocollagen proline and lysine (15).

No differences were noted in protein, hydroxyproline or hexosamine between control and insulin-treated tibia when grown for 7 days in organ culture. Similar observations have also been made by Prasad and Rajan (6) using 14-day embryonic chick tibia.

It is therefore evident that insulin has only

a slight effect on collagen synthesis in the model system employed; the effect is early; lysine hydroxylase and the glycosylating enzyme system appear to be slightly inhibited.

Summary. A slight increase in collagen synthesis by 10-day embryonic chick tibia in the presence of insulin was noted after two hours incubation (18% increase in hydroxy- ^{14}C]lysine and 43% increase in hydroxy- ^{14}C]proline) and four hours (32% increase in hydroxy- ^{14}C]proline). Insulin had no effect on collagen synthesis in long-term experiments.

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