Actions of Insulin and Growth Hormone on Collagen and Chondroitin Sulfate Synthesis in Bone Organ Cultures

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SUMMARY

1. The effects of insulin and growth hormone have been studied on collagen and chondroitin sulfate synthesis in bones growing in a steady state achieved by a continuous-flow bone culture system.

2. It is seen that insulin, but not growth hormone, has a significant effect on the synthesis of collagen and a marginal effect on the synthesis of chondroitin sulfate.

3. The significance of these findings is discussed. DIABETES *18*:280-84, May, 1969.

Insulin and growth hormone have been shown to influence a large number of cellular processes. These have been reviewed in recent years by Krahl,[1] Weil[2] and Wool.[3] Although a number of theories (Levine,[4] Bessman,[5] Randle[6] and Stirewalt et al.[7]) have been put forward, the mechanisms of action of these hormones have not yet been elucidated.

Understanding of the mechanisms involved is seriously handicapped by a relative lack of information on the synthesis of specific substances, especially defined proteins, although Goldstein and Reddy[8] and Walker and Roa[9] have studied the effects of insulin on the synthesis of different protein fractions. The former group examined insulin effects on the incorporation of C-14 leucine, during two-hour incubation periods, into various crude fractions of rat caudofemoralis muscle proteins, while the latter group found that insulin treatment of alloxan diabetic rats restores liver glucokinase levels to normal within twenty-four hours.

The synthesis of collagen and chondroitin sulfate may be readily followed in vitro, particularly in the case of bone tissue.[10-12] Little evidence has existed to connect insulin with bone metabolism; collagenous tissues have been considered insulin insensitive,[13] but recent work[14-17] has suggested that this view may not be correct. Both collagen and chondroitin sulfate metabolism are influenced by growth hormone in vivo,[11] but so far it has not been possible to reproduce equivalent effects in vitro.[11]

The purpose of the investigation to be described was to study the specific effects of insulin and growth hormone on collagen and chondroitin sulfate synthesis in bones growing in a steady state culture system. In this system, described elsewhere,[12] a chemically defined medium is constantly pumped through organ chambers, thus maintaining a constant level of all substrates and hormones throughout the experiment. A chemically defined medium was chosen in order to avoid the inclusion of any unidentified factors (such as are present in serum) which might interfere with the actions of individual hormones.

The results of this investigation indicate that connective tissue in vitro is sensitive to insulin but not growth hormone. In particular collagen synthesis was stimulated by insulin while chondroitin sulfate synthesis was relatively unaffected.

MATERIALS AND METHODS

1. *Bones.* Tibiae from twelve to forty-eight-hour postnatal albino rats of the WAG strain were used throughout.

2. *Culture medium.* The medium was essentially an Eagle's Basal Medium modified in the manner previously described[12] to include a full range of plasma amino acids, with the exception of hydroxyproline, at approximately physiologic concentrations. The five hormones listed below were also added. The concentrations given for these hormones are those employed in the basic hormone medium and are based on estimates of physiologic levels reported in the literature.[18-21] In experiments investigating the effects of individual hormones, control media were devoid of the hormone in

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question, whereas the experimental medium contained the hormone at a level ten times the value given below.

(a) Insulin. Three preparations of insulin were used: amorphous bovine insulin (25.2 U./mg.), a gift of Eli Lilly and Co., Indianapolis, Indiana, U.S.A.; crystalline bovine zinc insulin (24.2 U./mg.), a gift of Commonwealth Serum Laboratories (CSL), Melbourne, Australia; amorphous bovine insulin prepared from the CSL product by the picrate precipitation method.[26] The basic medium level of insulin was 0.001 U. per milliliter.

(b) Growth hormone. Bovine growth hormone was a gift of the National Institutes of Health, Bethesda, Maryland, U.S.A. The basic medium level of growth hormone was 0.1 μgm. per milliliter.

(c) Thyroid hormones. Sodium L-thyroxine and sodium triiodo-L-thyronine were gifts from Glaxo-Allenbury's Pty. Ltd., Melbourne, Australia. The basic medium levels used were 0.07 μgm. per milliliter and 0.001 μgm. per milliliter, respectively.

(d) Cortisol. The form used was Solu-Cortef (hydrocortisone sodium succinate), a product of the Upjohn Company, Kalamazoo, Michigan, U.S.A. The basic medium level was 0.08 μgm. per milliliter.

3. *Labeled precursors.* The following labeled substrates were used:

(a) H-3 (G)L-proline (Radiochemical Centre, Amersham, England) was purified by paper chromatography in a 1-butanol:glacial acetic acid:water (12:3:5, v:v:v) solvent system prior to use as an aqueous solution.

(b) Sulphur-35 carrier free sodium sulphate (Radiochemical Centre, Amersham, England).

(c) C-14 D-glucose (U) (Radiochemical Centre, Amersham, England).

4. *Dissection and culture technic.* The dissection technic, the culture system and the culture technic have been described previously.[12]

5. *Radioassays.* Bones were prepared and analyzed for H-3 proline, H-3 hydroxyproline and sulphur-35 activities as previously described.[12] C-14 labeled mucopolysaccharides were prepared by a method based on the principles of polyanion precipitation as reviewed by Scott.[22] The bones were partially digested in 2 ml. of papain-buffer solution (0.25 mg./ml. papain, 2.0 m*M* EDTA and 2.0 m*M* cysteine at pH = 6.5) for a period of thirty-six hours at 65° C. The pH of the digest was adjusted to 7.5-8.0 and the digestion was completed by adding 0.2-0.5 mg. trypsin per 2 ml. digest and incubating for a further three hours at room temperature. The residue was removed by centrifugation, and the supernatant, containing the mucopolysaccharides, was dialyzed overnight against running tap water. This dialyzed supernatant was made 0.05*M* with respect to NaCl and carrier chondroitin sulfate (2.0 mg.) was added. One per cent aqueous cetyltrimethylammonium bromide (CTAB) was then added dropwise until a flocculent precipitate appeared. The precipitate was spun down and the supernatant was again treated with CTAB. This procedure was repeated until no further precipitation occurred. The precipitate was then washed three times with 1.5 ml. of absolute ethanol saturated with potassium thiocyanate (to remove CTAB) and three times with 10 ml. of absolute ethanol (to remove thiocyanate ions). The precipitate was then dissolved in 1.0 ml. of water; an 0.25 ml. aliquot of this solution was added to 15 ml. of aqueous scintillator (200 ml. xylene, 600 ml. dioxane, 600 ml. ethoxyethanol, 14 gm. POP, 0.17 gm. dimethyl-POPOP, 112 gm. naphthalene) and counted in a Nuclear-Chicago Unilux liquid scintillation counter.

RESULTS

Table 1 shows the effects of insulin on the incorporation of tritiated proline as bone proline (total protein synthesis) and hydroxyproline (collagen synthesis), and of sulphur-35 sulfate and C-14 glucose (chondroitin sulfate and total mucopolysaccharides, respectively). It is seen that under the conditions of the experiment there is insulin stimulation of incorporation of proline, both as such and as hydroxyproline, into protein, whereas stimulations of sulphur-35 sulphate and C-14 glucose incorporation into mucopolysaccharides are relatively small (table 1).

Table 2 shows that growth hormone had no significant effect on any of the variables studied.

DISCUSSION

Incorporation of proline as proline and as hydroxyproline provides a measure of bone protein synthesis. In particular, incorporation into hydroxyproline provides an actual measure of collagen synthesis. This is because of the unique hydroxyproline content of collagen and the fact that this hydroxyproline is formed by the hydroxylation of peptide bound proline.[27] Our results, therefore, indicate that collagen synthesis is accelerated by insulin, thus supporting the data of Mikkonen et

TABLE 1

The effect of insuiin

Labeled bone substance	Expt. no.	Total activities (cpm./mm. final length) Control	Insulin	Per cent stimulation	Significance
H-3 OH Proline	1	465± 57 (8)	578± 86 (8)	+24	p<0.01
	2	228± 25 (17)	346± 70 (18)	+52	p<0.01
	3	558± 65 (17)	657± 69 (10)	+18	p<0.01
H-3 Proline	1	—	—	—	—
	2	656± 98 (17)	882±154 (18)	+34	p<0.01
	3	1,050±116 (17)	1,278±120 (10)	+22	p<0.01
S-35 Sulphate	1	1,976±187 (8)	2,103±246 (8)	+ 6	p>0.1
	2	1,586±125 (17)	1,631±160 (18)	+ 3	p>0.1
	3	4,643±565 (17)	5,068±418 (10)	+ 9	p<0.05
C-14 Mucopolysaccharide	4	202± 8 (12)	216± 15 (18)	+ 7	p<0.05

(In all experiments the test flasks contained basic hormone medium with the exception that the insulin concentration was raised to 0.01 U. per milliliter. The control medium had no insulin. Amorphous insulin prepared in our laboratory (see Materials) was used in Experiment 1, bovine crystalline Zn^{+2} insulin in Experiment 2 and amorphous bovine insulin prepared commercially (see Materials) in Experiments 3 and 4. Culture time in all cases was forty-eight hours. In Experiments 1-3, both sulfur-35 sulfate (50-100 μcurries/liter) and titriated proline (800-1,500 μcuries/liter) were present in the medium. In Experiment 4, C-14 glucose (30 μcuries/liter) was the only labeled precursor added to the medium. Results are expressed as means of total activities ± one SD; the number of bones contributing to each mean is shown in parentheses.)

al.[17] obtained using rat granuloma slices. The results reported here, however, are more significant in that Mikkonen's group used a concentration of 1 mg. insulin per milliliter of incubation medium, whereas in these experiments the much more physiological concentration of 0.0004 mg. per milliliter (0.01 U./ml.) was sufficient to produce an effect, thus indicating the superiority of the continuous-flow system in studying hormonal effects on the synthesis of connective tissue components.

It is of course possible that the insulin effect observed is secondary to an insulin stimulation of glucose metabolism. This possibility was reviewed by Krahl[23] in the case of the well-known insulin stimulation of protein synthesis in muscle. He concluded that only when the supply of energy or intermediates from carbohydrates becomes limiting (such as after long fasting) is the insulin effect on amino acid incorporation dependent on the transport of extracellular glucose into the muscle cell. It is reasonable to draw a similar conclusion for bone cells in this case, as the bones are taken from fed animals and glucose is freely provided during the forty-eight-hour incubation.

The marginal insulin effects observed in this system on incorporation of S-35 sulfate and C-14 glucose into mucopolysaccharides are in contradistinction with those reported by Salmon and his co-workers,[15,29,30] who observed significant effects of insulin on incorporation of sulfate into costal cartilage slices from hypophysectomized, normal fed and normal fasted rats. The differences in results may be due to differences in systems; however, it is interesting to observe that in spite of the

TABLE 2

The effect of growth hormone

(In both experiments the test flasks contained basic hormone medium with the exception that the growth hormone concentration was raised to 1.0 μgm./milliliter. Growth hormone was absent from the control medium. In both experiments sulfur-35 sulfate (80-100 μcuries/liter) and tritiated proline (800-1,500 μcuries/liter) were present in the medium. Culture time was forty-eight hours. Results are expressed as means of total activities ± one S.D.; the number of bones for each value is shown in parentheses.)

Labeled bone substance	Expt. no.	Total activities Control	Growth hormone	Significance
H-3 OH Proline	1	285± 22 (16)	314± 17 (16)	p>0.1
	2	659± 59 (6)	591± 89 (12)	p>0.1
H-3 Proline	1	769± 75 (16)	802± 84 (16)	p>0.1
	2	1,617± 84 (6)	1,544±171 (12)	p>0.1
S-35 Sulphate	1	4,924±606 (18)	4,722±350 (18)	p>0.1
	2	6,324±512 (6)	6,055±580 (12)	p>0.1

glucose requirement for chondroitin sulfate synthesis, and the established role of insulin in glucose utilization by various tissues (see review by Krahl[1]), the effect on collagen synthesis is far more significant.

Although it is generally accepted that growth hormone is essential to bone and cartilage development in vivo[11,13,16] and that collagen and chondroitin sulphate syntheses are influenced by variations in growth hormone levels,[11] attempts to demonstrate such effects in vitro have been generally unsuccessful.[11] Although lineal growth and net synthesis can be demonstrated in culture and have been in this system,[12] once again we were unable to demonstrate any in vitro effect of growth hormone, suggesting that all systems tried are deficient in one or more factors necessary for growth hormone action. One such factor could be the sulfation factor described by Salmon and Daughaday's group.[11,15,29,30] Another possible explanation for the failure of growth hormone to act in this system is that the growth of newborn rats may be insensitive to growth hormone. This is suggested by the work of Walker et al.,[28] who have shown that rats hypophysectomized at six days of age continue to grow (at reduced rates) for eighteen to twenty days.

Many complex interrelationships have been described between insulin and growth hormone in vivo, and much evidence is available that both are required for anabolism. In cases where active anabolism is proceeding, the circulating levels of both hormones appear to be raised.[11,24,25] However, we have demonstrated that, in vitro, insulin is effective but growth hormone is without action, thus raising the possibility that the in vivo effect of growth hormone (at least on collagen and chondroitin sulfate synthesis) may be mediated through insulin.

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REFERENCES

[1] Krahl, M. E.: The Action of Insulin on Cells. New York, Academic Press, 1961.

[2] Weil, R.: Pituitary growth hormone and intermediary metabolism. 1. The hormonal effect on the metabolism of fat and carbohydrates. Acta Endocrin. *49*: Suppl. 98, 1965.

[3] Wool, I. G., Rampersad, O. R., and Moyer, A. N.: Effect of insulin and diabetes on protein synthesis by ribosomes from heart muscle. Significance for theories of the hormones mechanism of action. Amer. J. Med. *40*:716-23, 1966.

[4] Levine, R.: The action of insulin at the cell membrane. Amer. J. Med. *40*:691-94, 1966.

[5] Bessmann, S. P.: A molecular basis for the mechanism of insulin action. Amer. J. Med. *40*:740-49, 1966.

[6] Randle, P. J.: Insulin. *In*: Mechanisms of Hormone Action, P. Karlson, ed., New York, Academic Press, 1965, pp. 94-97.

[7] Stirewalt, W. S., Wool, I. G., and Cavicchi, P.: The relationship of RNA and protein synthesis to the sedimentation of muscle ribosomes: Effect of diabetes and insulin. Proc. Nat. Acad. Sci. *57*:1885-92, 1967.

[8] Goldstein, S., and Reddy, W. J.: Effect of insulin on the incorporation of C-14 leucine into rat caudofemoralis protein. Biochim. Biophys. Acta *141*:310-18, 1967.

[9] Walker, D. G., and Roa, S.: The role of glucokinase in the phosphorylation of glucose by rat liver. Biochem. J. *90*: 360-68, 1964.

[10] Jeffrey, J. J., and Martin, G. R.: The role of ascorbic acid in the biosynthesis of collagen. 1. Ascorbic acid requirement by embryonic chick tibia in tissue culture. Biochim. Biophys. Acta *121*:269-80, 1966.

[11] Daughaday, W. H., and Kipnis, D. M.: The growth promoting and anti-insulin actions of somatotrophin. *In*: Recent Progress in Hormone Research, G. Pincus, ed., Vol. 22, New York, Academic Press, 1966, pp. 49-99.

[12] Schwartz, P. L., Wettenhall, R. E. H., and Bornstein, J.: The growth of newborn rat tibiae in a continuous-flow organ culture system. J. Exp. Zool. *168*:517-30, 1968.

[13] Asboe-Hansen, G.: The hormonal control of connective tissue. *In*: International Review of Connective Tissue Research, D. A. Hall, ed., Vol. 1, New York, Academic Press, 1963, pp. 29-61.

[14] Chen, J. M.: The effect of insulin on embryonic limb-bones cultivated in vitro. J. Physiol. *125*:148-62, 1954.

[15] Salmon, W. D., Jr., and Daughaday, W. H.: A hormonally controlled serum factor which stimulates sulphate incorporation by cartilage in vitro. J. Lab. Clin. Med. *49*:825-36, 1957.

[16] Vaes, G. M., and Nichols, G., Jr.: Metabolism of glycine-1-C-14 by bone in vitro: Effects of hormones and other factors. Endocrinology *70*:890-901, 1962.

[17] Mikkonen, L., Lampiaho, K., and Kulonen, E.: Effect of thyroid hormones, somatotrophin, insulin and cortico-steroids on synthesis of collagen in granulation tissue both in vivo and in vitro. Acta Endocrin. *51*:23-31, 1966.

[18] Korner, A.: Pituitary growth and lactogenic hormones. *In*: Hormones in Blood. C. H. Gray, and A. L. Bacharach, ed., New York, Academic Press, 1961, pp. 209-249.

[19] Batchelor, B. R.: Insulin-like activity. Diabetes *16*:418-34, 1967.

[20] Gray, C. H., Greenaway, J. M., and Holness, N. J.: The corticosteroids. *In*: Hormones in Blood. C. H. Gray, and A. L. Bacharach, ed., New York, Academic Press, 1961, pp. 439-514.

[21] Robbins, J., Rall, J. F., and Condliffe, P. G.: The thyroid stimulating hormones and the iodine-containing hormones. *In*: Hormones in Blood. C. H. Gray, and A. L. Bacharach, ed., New York, Academic Press, 1961, pp. 49-147.

[22] Scott, J. E.: Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. *In*: Methods of Biochemical Analysis, D. Glick, ed., Vol. VIII, London, Interscience Publishers Ltd., 1960, pp. 145-197.

[23] Krahl, M. E.: The Action of Insulin on Cells: Relations of amino acid incorporation to glucose and other sugars. New York, Academic Press, 1961, pp. 28-33.

[24] Glick, S. M., Roth, J., Yalow, R. S., and Berson, S. A.: The regulations of growth hormone secretion. *In*: Recent Progress in Hormone Research, G. Pincus, ed., Vol. 21, New York, Academic Press, 1965, pp. 241-284.

[25] Rabinowitz, D., Merimee, T. J., and Burgess, J. A.: Growth hormone-insulin interaction: Fact and speculation. Diabetes *15*:905-10, 1966.

[26] Fenton, E. L.: The assay of insulin solutions by paper chromatography. Biochem. J. *81*:570-75, 1961.

[27] Udenfriend, S.: Formation of hydroxyproline in collagen. Science *152*:1335-40, 1966.

[28] Walker, D. G., Asling, C. W., Simpson, M. E., Li, C. H., and Evans, H. M.: Structural alterations in rats hypophysectomized at six days of age and their correction with growth hormone. Anat. Rec. *114*:19-48, 1952.

[29] Salmon, W. D., Jr.: Importance of amino acids in the actions of insulin and serum sulphation factor to stimulate sulphate uptake by cartilage from hypophysectomized rats. J. Lab. Clin. Med. *56*:673-81, 1960.

[30] Salmon, W. D., Jr., DuVall, M. R., and Thompson, E. Y.: Stimulation by insulin in vitro of incorporation of (35-S) sulphate and (14-C) leucine into protein polysaccharide complexes, (H-3) uridine into RNA, and (H-3) thymidine into DNA of costal cartilage from hypophysectomized rats. Endocrinology *82*:493-99, 1968.

Body Odor and Metabolic Defects

. . . An odor described as fishy or resembling that of rancid butter or cooked or rotten cabbage has been noted in the urine, sweat, and breath at one to two months of age in hypermethioninemia, an unusual familial disorder described by T. L. Perry et al. (Pediatrics *36*:236, 1965). The smell in the urine was thought due to alpha-ketogamma-methiol butyric acid or a derivative of this compound. There was a fifty-fold elevation of serum methionine, and unusually large amounts of methionine, methionine sulfoxide, and tyrosine were excreted in the urine, which also contained amino acids containing sulfur such as homocystine and cystathionine, and abnormal amounts of histidine, glutamine, glycine, serine, threonine, ornithine, lysine, and citrulline.

The exact mechanism of hypermethioninemia was not revealed, but the major known pathway of methionine degradation was intact. In the three siblings described, death occurred at the age of eleven to twelve weeks following irritability, progressive drowsiness, convulsions, and hemorrhage. Renal tubular dilatation and calcification, islet cell hyperplasia, lymphoid atrophy, and liver cirrhosis were found at autopsy.

A metabolic abnormality should be suspected in infants with an unusual body odor. Recognition of the odor should be followed by identification of the nature of the metabolic defect present and early initiation of appropriate dietary therapy. Dietary therapy has been shown to be effective in preventing severe mental retardation and to result in objective clinical improvement in phenylketonuria, methionine malabsorption, and maple syrup urine disease.

In isovaleric acidemia clinical improvement has occurred following self-imposed protein restriction. Odor is a particularly important diagnostic clue in isovaleric acidemia, since, apart from the smell, no simple screening method is available for diagnosis of this metabolic defect, which cannot be detected by routine blood and urine amino acid screening or by ketoacid chromatography . . .

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