BRIEF NOTES AND COMMENTS

A Long-Term Effect of Insulin on Collagen Synthesis by Newborn Rat Bone in Vitro

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SUMMARY

In order to further characterize the effects of insulin on collagen synthesis by newborn rat bone in vitro, the time course of insulin action on incorporation of proline as proline and as hydroxyproline has been studied. In an incubation system, there was no stimulation by insulin at either three or six hours. In a flowing organ culture system, no significant effect of insulin could be observed at six hours; a small but significant effect on incorporation of proline as proline (but not as hydroxyproline) was seen at eighteen hours; and a highly significant effect on the incorporation of proline as both proline and hydroxyproline was observed at forty-two hours. Possible explanations for the delayed onset of insulin activity are discussed and the pattern contrasted with the rapid effect of insulin on collagen synthesis by skin of newborn rats. DIABETES *19*: 465-66, June, 1970.

Available evidence suggests that protein synthesis by various types of connective tissue in vitro is sensitive to insulin.[1-4] We have previously[5] described a flowing system for organ culture of tibiae from newborn rats in a chemically defined medium. In this system, low concentrations of insulin had a stimulatory effect on the incorporation of H-3 proline into bone matrix, both as H-3 proline and as H-3 hydroxyproline.[4] The labeling of hydroxyproline was taken as a measure of collagen synthesis.

In an attempt to learn something of the mechanism of the insulin effect on bone collagen synthesis, studies of the time course of insulin action were undertaken. The findings reported in this paper indicate that insulin stimulation becomes obvious only after a relatively long period of time.

MATERIALS AND METHODS

A simple incubation system was believed adequate for short-term studies, particularly as skin from newborn rats responds to insulin within ten minutes[6] in a system identical to the one described here. Accordingly, tibiae from newborn rats of the WAG strain were incubated for periods up to six hours in our complete medium (Eagle's Basal Medium modified to contain a full range of amino acids, and triiodothyronine, thyroxine, cortisol, and bovine growth hormone at approximately physiologic concentrations[4,5]). H-3 proline (25 to 50 μc./ml. of 645 mc./mmole) was added as tracer. Groups of four bones, each bone weighing 10 to 15 mg., were incubated with shaking at 37° C. in siliconized 25-ml. Erlenmeyer flasks, each containing 3 ml. of medium gassed with 95 per cent O_2-5 per cent CO_2. Where indicated, bovine crystalline zinc insulin, 0.1 U./ml., was added to the medium. At the end of the incubation, the bones were washed as described previously[5] except that in order to remove unincorporated H-3 proline five washes of nonradioactive proline (3 mg./ml.) were used in place of the five sulphate washes. The bones were then measured and hydrolyzed; proline and hydroxyproline were isolated and assayed for radioactivity by the method described previously.[4,5]

RESULTS

We were unable to demonstrate a stimulation of incorporation by insulin in these conditions at either three or six hours of incubation. Table 1 shows the results of one such three-hour incubation. Variation of the concentration of insulin (0.01 to 10.0 U./ml.) or of the concentration of total medium proline (30 to 80 μg./ml.) failed to induce a stimulation.

Because of these findings, we considered the possibility that the effect of insulin on newborn rat bone was solely a long-term one. We therefore returned to the flowing organ culture system and carried out cultures for periods of six to forty-two hours in the complete medium. The activity of H-3 proline was 0.6 to 3.6 μc./ml. of 645 mc./mmole. Penicillin was added to the media in these experiments (Carbenicillin 60 μg./ml. and Ampicillin 15 μg./ml.). Where indicated, insulin was added at a final concentration of 0.01 U./ml. of medium. After culture, the bones were washed and assayed as described above.

These experiments revealed that there was indeed no significant effect of insulin on the incorporation of H-3 proline as proline or hydroxyproline for a period of at least six hours (table 2). At eighteen hours, a small but significant stimulation of H-3 proline incorporation was observed. No significant effect of insulin upon the incorporation of H-3 proline as H-3 hydroxyproline was observed at eighteen hours. Never-

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TABLE 1

Effect of insulin on incorporation of H-3 proline into bone hydroxyproline and proline during three-hour incubation in complete medium. Numbers in parentheses indicate the number of bones studied. Values are the mean ± S.E.M. The activity of H-3 proline in the medium was 29 μc./ml.

	Counts/min./mm. length		Difference	
	No addition	Zn insulin, 0.1 U./ml.	(Per cent of control)	Significance
Hydroxyproline	1,820±80 (8)	1,865±45 (8)	+2.5	N.S.
Proline	5,800±245 (8)	5,960±155 (8)	+2.8	N.S.

TABLE 2

Effect of insulin on incorporation of H-3 proline into bone hydroxyproline and proline during flow-through culture in complete medium. Numbers in parentheses indicate the number of bones studied. Values are the mean ± S.E.M. The results are from five separate experiments, one each at six and forty-two hours, three at eighteen hours. The activities of H-3 proline in the media were approximately 3.6 μc./ml. (six-hour experiment), 2.8 μc./ml. (eighteen-hour experiments), and 0.6 μc./ml. (forty-two-hour experiment).

Time of culture hours		Counts/min./mm. length		Difference	
		No addition	Zn insulin, 0.01 U./ml.	(Per cent of control)	Significance
6	Hydroxyproline	335±9 (12)	342±11 (11)	+2.1	N.S.
	Proline	828±24 (11)	858±24 (11)	+3.6	N.S.
18	Hydroxyproline	675±12 (41)	689±17 (41)	+2.1	N.S.
	Proline	1,775±25 (38)	1,910±36 (38)	+7.6	$p<0.01$
42	Hydroxyproline	887±15 (18)	1,065±25 (16)	+20.1	$p<0.001$
	Proline	1,907±37 (18)	2,077±50 (16)	+8.9	$p=0.01$

theless, we again obtained at forty-two hours an insulin effect similar to that which we have previously reported.[4]

DISCUSSION

The results indicate that under our conditions, newborn rat tibiae are sensitive to insulin only after a long period of time. There is little likelihood that such a delayed effect could be due to an insulin-induced alteration to cell permeability. A primary effect on the synthesis of protein must be seriously considered. Also, because of the extreme delay before the onset of the insulin effect, there is the possibility that insulin is acting as a stabilizing influence, preventing the breakdown of protein rather than stimulating synthesis.

A further possible explanation for the delayed effect is that eighteen hours or more might be required to overcome the effects of any residual insulin. Salmon's[7] larger and more rapid effects on protein-polysaccharide were obtained with higher insulin levels and with cartilage from hypophysectomized rats. Such rats have low plasma insulin levels[8] and hence tissues which are likely to be more sensitive than tissues from normal animals to the hormone.

Whatever the explanation, the results of these time studies are in contrast to those of time studies with newborn rat skin, where a short-term effect (within ten minutes of the start of incubation) of insulin has been observed.[6] This contrast suggests that the protein synthesizing systems of various connective tissues respond differently to insulin in vitro. It also renders questionable the current practice of considering various types of connective tissue as a single entity when discussing or reviewing studies of the effects of insulin on such tissues.

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Status of Problem of Usage of Tolbutamide
Preliminary Statements

FDA STATEMENT

Friday, May 22, 1970

"The FDA said today it agrees with the recent study which indicated that in the treatment of mild, adult onset diabetes mellitus the use of the drug Orinase (tolbutamide) is no more effective than diet alone.

"In commenting on about-to-be released findings on a long term study of diabetic treatment, the agency said that in the types of patients studied, a regimen employing Orinase was no more effective than diet alone, and as far as death from heart disease and related conditions is concerned, may be less effective than diet or diet and insulin.

"The results of this study, conducted by the University Group Diabetes Program, were reviewed by the Food and Drug Administration and an expert advisory committee. Despite a number of limitations in the study, both the agency and its advisory committee agree with its stated conclusions which are: 'This study provides no evidence that the combination of diet and tolbutamide therapy as described and used for mild non-insulin dependent diabetes is more effective than diet alone. Moreover, the findings suggest that diet plus tolbutamide may be less effective, insofar as cardiovascular mortality is concerned, than diet alone or than diet and insulin combined.'

"The study was started in 1961 and conducted in 12 university medical schools. It is the largest prospective study of this sort and includes over 800 patients, most of whom have been followed for eight years.

"The study compared the results of various treatment regimens in patients with recently diagnosed adult onset mild diabetes who were not insulin dependent and who were expected to live at least five years after entry into the study. All patients were placed on a diabetic diet. Tolbutamide was given to one group in a fixed dosage, insulin was given in a fixed dosage to a second group and in varying dosage to a third and a control group was treated with diet and a placebo.

"The FDA emphasized that the conclusions of this study pertain only to patients with mild, adult onset, non-insulin dependent diabetes and to the specific type agents and dosage schedules used. Further studies and analysis of other ongoing studies will be necessary to determine if similar conclusions are warranted in regard to treatment of other diabetics with more or less severe disease and with other currently available agents or dosage schedules. A determination of the ultimate usefulness of oral antidiabetic agents must await further studies.

"Pending results of such studies the FDA recommends that Orinase (tolbutamide) and other sulfonylurea type agents Dymelor (acetohexamide), Diabinese (chlorpropamide), Tolinase (tolazamide) should be used only in patients with symptomatic adult onset diabetes mellitus who cannot be adequately controlled by diet alone and who are not insulin dependent (i.e., require insulin). In instances where these oral agents are deemed necessary by the physician their dosage should be tailored to the individual patient's needs as recommended in the labeling of these products.

"Other recent studies on this subject have also been analyzed by the FDA. These studies do not alter the validity of the conclusions of the University Group Diabetes study.

"Diabetic patients currently taking tolbutamide or other chemically related sulfonylurea agents who are under adequate medical supervision should continue on their current regimen until advised by their physicians.

"FDA said it will take the following actions:

"1. Require labeling changes for sulfonylurea drugs, to reflect the results of this study.
"2. Inform physicians of the findings of this study.
"3. Require the industry to institute long-term studies on the use of their products in various types of diabetic patients.
"4. Continue to monitor all studies pertaining to the use of anti-diabetic agents in patients with diabetes mellitus of varying severity.
"5. Continue an intensive examination of all new evidence in the field to be able to make prompt reevaluation of these decisions as necessary."

STATEMENT OF CHAIRMAN OF UGDP

Thursday, May 21, 1970

"Premature release of some of the information from reports which are to be presented to the American Diabetes Association in St. Louis on June 14 is most unfortunate, not only from a scientific standpoint, but for the thousands of diabetics in this country who are

vitally concerned about the value of various forms of therapy now in use," said Dr. Max Miller, professor of medicine at Case Western Reserve School of Medicine and chairman of the University Group Diabetes Program (UGDP).

"It subjects to debate in newspapers and other media very serious questions which have been under study for many years, which must be discussed and evaluated first by impartial investigators in the field. Naturally we have been concerned by our findings regarding the efficacy of certain hypoglycemic drugs. Final judgment of the relevancy and significance of the UGDP findings will rest on the detailed analyses by our peers and by comparison with other possibly related studies. Complete provision has been made for review and dissemination of the proceedings of the ADA meeting on June 14. Until that date it would be inappropriate to discuss the UGDP findings."

The following abstracts of the UGDP studies appeared in DIABETES:*19*, Supplement 1, the Program of the Thirtieth Annual Meeting of the American Diabetes Association:

"The University Group Diabetes Program: The Effects of Hypoglycemic Agents on Vascular Complications in Patients with Adult Onset Diabetes 1. Design and Methods," Martin G. Goldner, Brooklyn, N.Y., and Thaddeus E. Prout, Baltimore, Md., page 387.

"The University Group Diabetes Program: The Effects of Hypoglycemic Agents on Vascular Complications in Patients with Adult Onset Diabetes 2. Findings at Baseline," Thaddeus E. Prout, Baltimore, Md., and Martin G. Goldner, Brooklyn, N.Y., page 374.

"The University Group Diabetes Program: The Effects of Hypoglycemic Agents on Vascular Complications in Patients with Adult Onset Diabetes 3. Course and Mortality," Thaddeus E. Prout, Baltimore, Md., and Martin G. Goldner, Brooklyn, N.Y., page 375.

ABSTRACTS

Aharonson, Z.; Shani Mishkinsky, J.; and Sulman, F. G. (Dept. of Applied Pharmacology, Sch. of Pharmacy, Hebrew Univ., Jerusalem, Israel): HYPOGLYCAEMIC EFFECT OF THE SALT BUSH (ATRIPLEX HALIMUS)— A FEEDING SOURCE OF THE SAND RAT (PSAMMOMYS OBESUS). Diabetologia 5:379-83, 1969.

Verbatim summary. The fact that the so-called "sand rat" (Psammomys obesus) is highly susceptible to diabetes, and succumbs to it while changing its food from green leaves to laboratory pellets, puzzled us for a long time. Several hypotheses for this phenomenon were suggested, based on the idea that the sand rats are predisposed to diabetes, and that diabetes occurs when the rats are fed on a high caloric diet. In addition to this, it was noticed that the diabetic rats have high plasma insulin levels, which indicated an impairment in their peripheral utilization of glucose. Assuming that the green leaves which the sand rats find in nature prevent their becoming diabetic, we examined the main feeding source of the sand rats in Israel for possible hypoglycemic activity. Press juice from green leaves of Atriplex halimus, as well as their water extract and dialysate, were fed to normal and to alloxan diabetic albino rats, and showed a significant hypoglycemic effect without any decrease in appetite. Moreover, their food and water intake was increased by 50 to 800 per cent within five hours after treatment. The effect was also preserved in the ash of the dialysate. The composition of the hypoglycemic principle is now under study. It is not based on the presence of cations, since the active extracts contained K, Na, Ca, Mg and Al only.

Allison, S. P.; Chamberlain, M. J.; Miller, J. E.; Ferguson, R.; Gillett, A. P.; Bemand, B. V.; and Saunders, R. A. (Depts. of Med. and Experimental Path., Univ. of Birmingham, Birmingham, England): EFFECTS OF PROPRANOLOL ON BLOOD SUGAR, INSULIN AND FREE FATTY ACIDS. Diabetologia 5:339-42, 1969.

Verbatim summary. Three types of experiments were carried out in normal subjects to determine the effect of therapeutic doses of oral propranolol on (1) the blood sugar, plasma insulin and free fatty acids (FFA) during prolonged fasting and exercise, (2) intravenous glucose tolerance and the rise in insulin level after intravenous glucose, and (3) the intravenous glucose tolerance on exercise. Propranolol caused only slight lowering of the blood sugar in normals, even after twenty-four-hour fasting. This was most noticeable during exercise. There was no significant effect of propranolol on fasting insulin levels, on glucose tolerance at rest or exercise, or on the response of plasma insulin levels to intravenous glucose. Lowering of plasma FFA levels was found in all subjects when taking propranolol particularly during and after exercise. Possible mechanisms of hypoglycemia in those cases reported in the literature are discussed. It is concluded that hypoglycemia is not a major problem in propranolol therapy.

Andreani, Domenico; Menzinger, Guido; Fallucca, Francesco; Aliberti, Giuseppe; Tamburrano, Guido; and Cassano, Cataldo (Instituto di II. Clinica Medica, Università degli Studi di Roma and Cattedra di Terapia Medica Sistematica, Università Cattolica del S. Cuore, Rome, Italy): INSULIN LEVELS IN THYROTOXICOSIS AND PRIMARY MYXOEDEMA: RESPONSE TO INTRAVENOUS GLUCOSE AND GLUCAGON. Diabetologia 6: 1-7, 1970.

Verbatim summary. Glucose disappearance, insulin-like activity (ILA) and serum immunoreactive insulin (IRI) were studied after intravenous injection of glucose or glucagon in patients suffering from thyrotoxicosis or primary myxedema. A group of normal subjects was also investigated.