Effect of Insulin on the Conversion of Glucose-C-14 to C-14-0_2 by Normal and Diabetic Fibroblasts in Culture

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SUMMARY

In an attempt to elucidate the basic genetic defect(s) in diabetes, the acute effect of insulin on the conversion of glucose-C-14 to C-14-0_2 in cultured human diploid fibroblasts was measured under various conditions. Freshly trypsinized cells did not respond to insulin, but after more than sixteen hours in growth medium, a small but significant stimulation, requiring high concentrations of insulin (0.26 U. per ml.) was observed. Stimulation by insulin in various normal strains ranged from 5 to 45 per cent with a mean of 18 per cent ($p < .001$). C-14-0_2 production was approximately linear with time after an initial lag phase and proportional to the number of cells inoculated. Increasing the concentration of glucose in the medium increased C-14-0_2 production, with stimulation by insulin apparent at only very low concentrations and at 250 mg. per 100 ml., the highest concentration studied. Experiments comparing the oxidation of glucose-1-C-14 versus glucose-6-C-14 showed that substantial C-14-0_2 production occurred via the hexose monophosphate shunt, but that most of the increase in C-14-0_2 production in the presence of insulin occurred via the Krebs cycle.

A comparison of basal and insulin-stimulated C-14-0_2 production in normal and diabetic fibroblasts revealed no significant differences. Possible explanations for the failure to distinguish between the two groups are discussed. DIABETES *18*:545-49, August, 1969.

The genetics of diabetes mellitus are poorly understood.[1,2] For a rational approach to the problem it is necessary to elucidate the basic defect(s) and ascertain reliable biochemical markers. Cell culture is eminently suitable for this purpose because it provides the opportunity to study the genotype of human cell populations in a controlled environment several generations removed from the neurohumoral influences of the donor. The cell type which can regularly be grown in quantity from humans is the fibroblast; these cells remain diploid in chromosome number, and eventually enter a senescence phase.[3] We have undertaken the development of an in vitro system using human fibroblasts to study basal glucose oxidation and the response of this process to insulin. This report deals with the characterization of the system in normal cells, and a preliminary study in diabetic cells.

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MATERIALS AND METHODS

Source of tissue: All cultures were derived from skin biopsies obtained either at the time of elective surgery, or from the forearm with a 3 mm. punch biopsy. Biopsy material was stored in growth medium at 4° C. until use. Tissues from patients with diabetes mellitus were provided through the courtesy of Dr. Donald Barnett of the Joslin Clinic. All normals were individuals with negative family histories for diabetes; adult normals had been found to have a normal two-hour postprandial blood sugar.

Initiation of cultures: Tissues were explanted into 60 × 15 mm. petri dishes (Falcon Plastic Co.) within twenty-four hours of excision, by cutting with scalpel blades to pinhead size, followed by immobilization under a glass coverslip secured at one corner by pressing down over a small pat of silicone grease. Five ml. of growth medium were added to each dish, followed by incubation at 37° C. in a humidified 95 per cent air: 5 per cent CO_2 atmosphere. The growth medium used was Eagle's Minimum Essential Medium supplemented with "nonessential" amino acids, 15 per cent fetal calf serum, glucose (2.5 mg. per ml.), sodium pyruvate (0.11 mg. per ml.), penicillin (100 U. per ml.), streptomycin (100 U. per ml.), $Fe(NO_3)_3 \cdot 9H_2O$ (0.1 mg. per ml.), and sodium bicarbonate (2.2 mg. per ml.). After approximately four weeks, fibroblasts growing from the explants were released from the adherent monolayer as is customary, with 0.25 per cent trypsin (Difco) in phosphate buffered normal saline,

and then transferred to larger petri plates for subsequent culture.

Assay technic: All assays were carried out on the cells during the active growth phase in vitro.[3] Cells were freed from newly confluent petri plates by trypsinizing, followed by suspension in cold growth medium. An aliquot was counted in a Coulter electronic cell counter and 100,000 cells were inoculated into 10 ml. Erlenmeyer flasks (Kontes Glass Company) containing 2.5 ml. of growth medium. After eighteen hours of incubation at 37° C. the medium was removed and the cells rinsed with cold Krebs-Ringer phosphate buffer* free of glucose. 1.3 ml. of the same buffer containing glucose-C-14 was added, and the flasks were sealed with serum sleeve-type stoppers housing a polypropylene center well, followed by incubation under air with shaking at 37°C. Five-times recrystallized porcine insulin (Lot No. PJ-5589), generously provided by Dr. Mary Root of the Eli Lilly Company, was present in half of the flasks at a concentration of 0.26 U. per ml. After incubation for ninety minutes the flasks were chilled on ice; in rapid succession 0.4 ml. of hyamine (1 M. in methanol, Packard Instrument Company) was injected into the center well, and 0.2 ml. of 10 N sulfuric acid into the medium. Flasks were then shaken at room temperature for one hour to release and trap CO_2. Hyamine was mixed with 10 ml. of toluene-2, 5-diphenyloxazole-1, 4-bis-2 (4-methyl-5-phenyl-oxazolyl) benzene fluor solution in vials and counted in a liquid scintillation counter with about 60 per cent efficiency. Results are expressed as CPM per 100,000 inoculated cells per ninety minutes of incubation.

Isotopes: Glucose-C-14, labeled uniformly, or in the 1 or 6 position, was supplied by the New England Nuclear Company at specific radioactivities of 188, 10.0, and 4.58 millicuries per millimole respectively, and after dilution with unlabeled glucose used at a final concentration of 1 mg. per 100 ml. and a specific radioactivity of .025 μc. per μg., unless otherwise indicated.

RESULTS

In initial studies freshly trypsinized cells suspended in Krebs-Ringer medium were used. Cells assayed in this way oxidized glucose but did not respond to insulin. When cells were inoculated into growth medium and allowed to attach (which takes about one to two hours), followed then by rinsing and assay, they were found to respond to insulin with a maximum around sixteen to twenty-four hours (figure 1). In subsequent experiments cells inoculated eighteen hours previously were used. Under these conditions glucose oxidation was essentially proportional to the number of cells inoculated

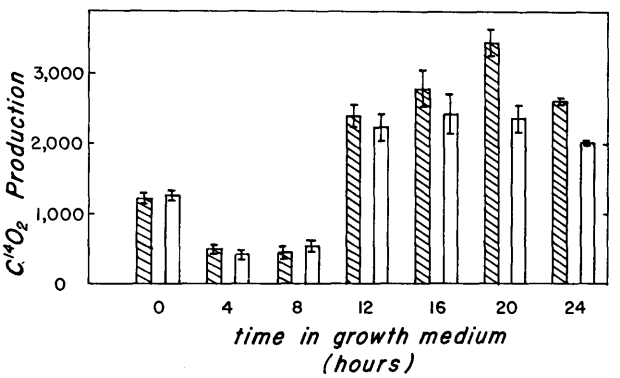

FIG. 1. Relationship of insulin effect to time in growth medium after treatment with trypsin for subculturing. 10^5 cells were inoculated into growth medium and assayed after the times shown. Zero time fibroblasts were rinsed, suspended in Krebs-Ringer phosphate medium and assayed directly. For all other time points, cells were assayed as described in Methods. Open bars, control; cross-hatched bars, insulin (± S.E.M.).

(figure 2), and approximately linear with time after an initial lag (figure 3). In figure 3 it is shown that insulin increased oxidation at each time point tested.

The average response of insulin in normal cell strains was to increase oxidation by 18 per cent (range 5 to 45 per cent in twenty-six experiments, each done in tripli-

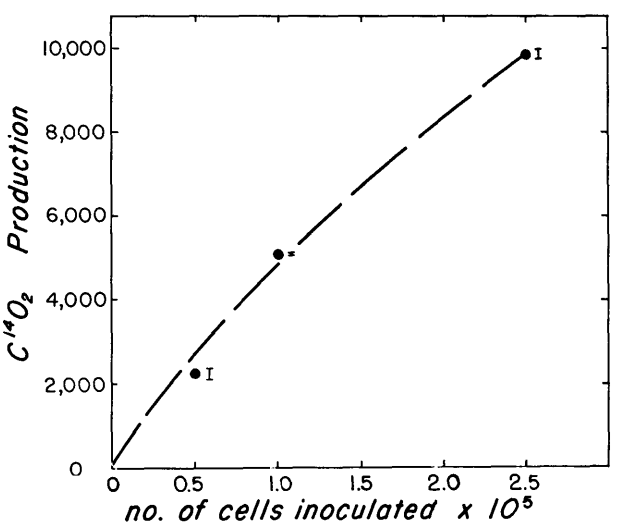

FIG. 2. Relationship between number of cells inoculated and C-14-O_2 production. 0.5, 1.0, and 2.5 × 10^5 cells were inoculated into growth medium and incubated at 37° C. for eighteen hours, followed by assay as usual. Each point represents the mean of three observations (± S.E.M.).

*Krebs-Ringer phosphate was modified by omission of Ca^{++}, which produced no apparent difference in results but facilitated preparation and handling.

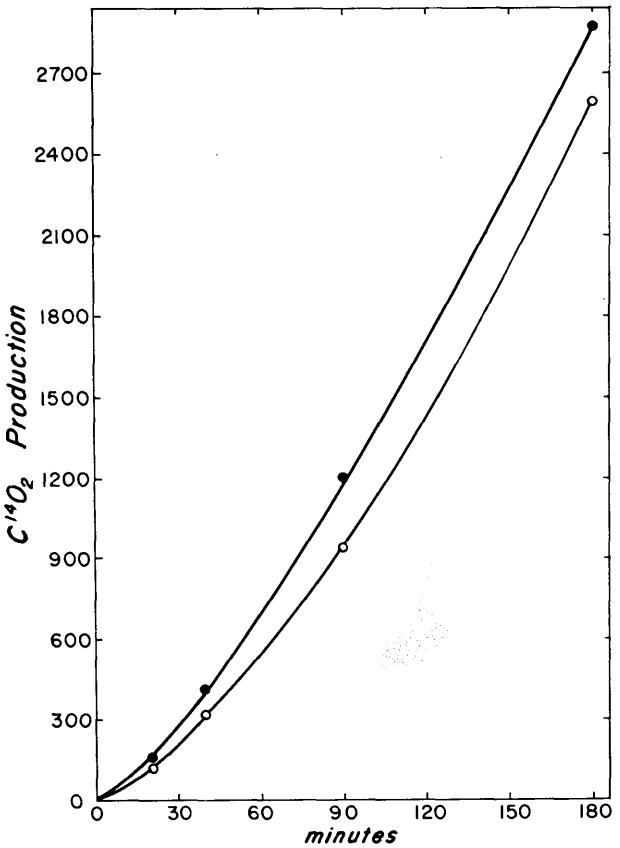

FIG. 3. Time study of insulin effect on glucose-C-14 conversion to C-14-O_2. 10^5 cells were assayed in the usual manner. Each point is the mean of three observations in one experiment which was representative of four carried out under identical conditions. S.E.M. are smaller than diameters of points and are not shown. Control, (o); insulin (•). Insulin stimulation $p < .02$ at 20 minutes; $p < .01$ at 40 and 90 minutes, and $p < .05$ at 180 minutes.

cate). This insulin response, although small, was highly significant ($p < .001$).

The substitution of various media in the assay (data not shown) failed to enhance basal or insulin-treated oxidation. The following were tried: Krebs-Ringer bicarbonate ± 4 per cent dialyzed albumin, Eagle's medium without fetal calf serum, and with 1, 5, and 10 per cent fetal calf serum, Krebs-Ringer phosphate buffer with 1, 5 and 10 per cent fetal calf serum. Altering the pH of Krebs-Ringer phosphate medium over a range of 6.8 to 7.4 had no significant affect on basal or insulin-treated CO_2 production. Attempts to reduce the insulin concentration below 0.1 U. per ml., even in the presence of gelatin or dialyzed albumin, led to erratic stimulation so that 0.26 U. per ml. were used thereafter.

Table 1 shows the effect on glucose oxidation of increasing the glucose concentration in the medium from 0.01 to 250 mg. per 100 ml. Glucose oxidation increased with each increment in glucose concentration up to 250 mg. per 100 ml. The addition of insulin stimulated glucose oxidation at very low glucose concentrations, the effect disappearing at intermediate levels, with a small stimulatory effect apparently reappearing at 250 mg. per

TABLE 1

Effect of glucose concentration on insulin stimulation of C-14-O_2 production. Glucose at different specific radioactivities was used in this experiment and then the results normalized to 0.025 μc. per μg. The values in the columns headed Control and Insulin represent the mean CPM from three experiments (± S.E.M.).

Glucose mg. per 100 ml.	Control	Insulin	Per cent stimulation	p
.01	.0180±.0003	.0205±.0005	13	< .01
1	995±70	1,268±50	27	< .02
10	2,808±110	3,003±120	7	N.S.
25	5,940±230	5,750±65	—3	N.S.
50	7,102±370	7,180±750	—1	N.S.
100	10,325±170	9,338±945	—9	N.S.
250	18,085±492	20,590±455	14	< .05

100 ml. Table 2 compares C-14-O_2 production from glucose-1-C-14 and glucose-6-C-14. CO_2 production was higher when the glucose was labeled in the 1 position; however, on a percentage basis a greater response to insulin was found when the label was restricted to the number 6 carbon. These data suggest that while there is substantial activity in the hexose monophosphate shunt, most of the increment in C-14-O_2 production due to insulin occurs via the Krebs cycle.

TABLE 2

Relationship of basal and insulin-stimulated glucose oxidation to position of C-14 label in glucose molecule. Both incubation media contained glucose at 1 mg. per 100 ml. and 0.025 μc. per μg. Results expressed as in table 1.

Position of C-14	C-14-O_2 production: Control	Insulin	Difference	Per cent stimulation	p
1	2,274±85	2,472±78	198	9	N.S.
6	307±19	442±20	135	44	<.01

Finally a comparison was made of both the basal and insulin-stimulated glucose oxidation of eight normals and seven diabetics. Some clinical information on the diabetic series is given in table 3. A wide range of basal CO_2 output and response to insulin was seen in both the normals and diabetics (figure 4). The mean per cent insulin stimulation in normals was 18.0 ± 3.9

TABLE 3
Clinical data on diabetic patients

Code no. & sex	Age at time of biopsy	Duration of diabetes (yrs.)	Clinical type	Current therapy
10 M	70	17	MD	Tolbutamide 0.5 gm. b.i.d.
15 F	40	5	JD	26U NPH
22 F	56	38	JD	10U CZI 16 NPH
24 M	63	10	MD	Tolbutamide 0.5 gm. b.i.d.
29 M	9	3	JD*	12U Lente
30 M	39	5	JD	4 CZI 38 NPH
31 F	5	3/12	JD	In remission

Abbreviations: JD = Juvenile diabetes
MD = Maturity onset diabetes
*Also 21 trisomy, and primary myxedema

per cent (S.E.M.) and in the diabetics, 15.3 ± 1.9 per cent. The difference between these two groups is not statistically significant.

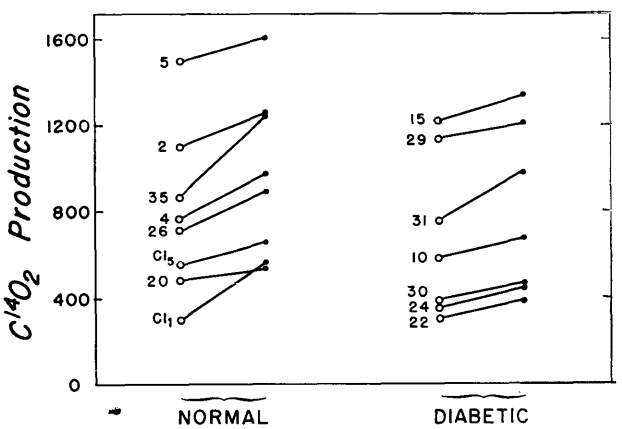

FIG. 4. Effect of insulin on C-14-O_2 production of normal and diabetic cells. 10^5 cells were assayed as usual. Each point represents the mean of six or more observations made in at least two experiments carried out under identical conditions. Control (o); insulin (•).

DISCUSSION

The increase in glucose oxidation when cultured fibroblasts are acutely exposed to insulin is small and requires a high concentration of the hormone, which raises the possibility of a nonspecific effect.[4] Earlier studies carried out on human cells in culture over a considerably longer period also showed that the effects of insulin on glucose metabolism were small when expressed in terms of nucleic acid or cell numbers.[6-8] Regarding a different metabolic parameter in a recent study of cultured primary chick fibroblasts,[5] insulin was found to stimulate protein synthesis with an order of magnitude similar to that observed here for glucose oxidation. In contrast to fibroblasts, isolated fat cells show as much as tenfold stimulation of glucose oxidation when exposed to equivalent insulin concentrations,[9,10] most of this stimulation being effected via the hexose monophosphate shunt.[11]

In other cell types, studies on mammary gland explants show that insulin stimulates epithelial cells from mature but not from immature mice to synthesize DNA and divide, while fibroblasts fail to respond in either case.[12-15] It is as if the ability to respond to insulin, presumably present in the genome of all cells, is acquired with time in epithelial cells, but is only partially expressed in the fibroblast. Nevertheless the fibroblast system has at least two advantages. The use of actively dividing cells permits repeated studies on a given donor and it especially provides that the composition of the cells reflects not the metabolic environment of the donor, but the uniform influences of the growth medium. Only with such a system can a valid analysis of the genotype be made.

The absence of an insulin effect on freshly trypsinized cells and the reappearance of the effect after several hours in growth medium is consistent with previous reports localizing the site of action of insulin to the cell membrane.[16-18] Perhaps cell membrane receptors are maximally stimulated or damaged by high concentrations of trypsin,[19-21] and then are restored during the recovery stage. While the difficulty in enhancing the insulin effect could be related to residual trypsin activity, this consideration is minimized by two factors. Serum proteins in the growth medium are present in great excess to neutralize the trypsin, and the attachment of cells to glass cannot occur in the presence of trypsin activity.

The initial lag in glucose oxidation (figure 3) followed by linearity may relate to the time required for equilibration of precursor pools with the isotope.

The dependence of oxidation of glucose on its concentration in the medium, and the different effects of insulin at various glucose concentrations has been seen in other in vitro studies.[9,22,23] Oxygen consumption of cultured mammalian cells also seems to be related to the glucose concentration.[24,25]

Stimulation by insulin at only the highest and the lowest glucose concentrations used suggests that fibroblasts may contain at least two hexokinases, each with a different Km. for glucose. Such evidence has been presented in a heteroploid line of cells cultured from human liver,[26] as well as other mammalian tissues.[27-29]

The failure to distinguish any difference between normal and diabetic fibroblasts in either basal or insulin-stimulated CO_2 production is a disappointment. A plausible explanation is that the difference exists at a more proximal site e.g., the β cell. On the other hand it may be that fibroblasts derived from diabetic skin explants have undergone selection so that only insulin-responsive cells grow to be assayed in this system; or the high insulin doses employed in these studies may fail to detect a subtle blunting of response to the hormone in diabetic cells. Finally, in choosing to study glucose oxidation, a parameter more suitable for this system may have been overlooked. Studies designed to examine these possibilities are in progress.

ACKNOWLEDGMENT

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