Effects of Insulin from Normal and Diabetic Human Pancreas on RNA Labeling in Fibroblast Cultures

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SUMMARY

The nutrient medium of a series of cultures inoculated simultaneously with mouse fibroblasts of the 3T6 strain was replaced by fresh medium containing Uridine-2-C-14 and 5,000 μunits of insulin from four normal and two diabetic pancreases. After a twelve-hour incubation period the cellular monolayer was subdivided by conventional chemical methods and the specific activity of the RNA fraction estimated. A consistent difference was observed between RNA specific activity of the cellular monolayers treated with diabetic pancreatic insulin and normal pancreatic insulin. These data constitute a further argument strengthening the case for an "abnormal insulin in diabetes mellitus." DIABETES *20*:10-14, January, 1971.

The suggestion that diabetes mellitus might, in some degree, be related to the biosynthesis of an "abnormal insulin" has been supported by three lines of evidence.[1-4] It was first shown that immunopurified serum insulin from untreated juvenile diabetics was abnormally resistant to degradation by a crude rat muscle insulinase.[1,2] Secondly, a difference in biological activity was demonstrated between partially purified normal pancreatic and diabetic pancreatic insulin when the capacity of insulin to stimulate the incorporation of glucose into rat diaphragm glycogen[3] was used as a discriminant. Finally, a significant decrease in the incorporation of Uracil-2-C-14 into the RNA fraction of mouse fibroblast cultures incubated with identical immunoassayable amounts of partially purified normal and diabetic pancreatic insulin was reported.[4] The present study confirms the difference in RNA labeling using Uridine-2-C-14 and highly purified normal and diabetic pancreatic insulin.

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METHODS

The well established 3T6 mouse fibroblast cell line was used.[5] The cells were employed in early generations and discarded after two months of continuous growth phase. Their mean doubling time was thirteen hours; a stationary phase was usually reached six days after inoculation or five days from time zero. The cells were grown in plastic petri dishes (Falcon Plastics, L.A.) at constant humidity in an atmosphere of 10 per cent CO_2 and 90 per cent air. All inoculations and transfers were made with trypsinized suspensions of log phase cells. Cellular morphology was assessed with each medium change. Cell counting and cultures for possible bacterial contamination were carried out in each experiment.

Studies with either crystalline human insulin (Connaught Medical Research Laboratories, Toronto, Canada Lot ET 210) or with normal and diabetic pancreatic insulin were carried out between day two and day four of the fibroblast growth cycle. The culture medium was then changed and Uridine-2-C-14 (New England Nuclear Corp., S.A. 51.5 mc/mMole) was added along with insulin. All insulin samples were immunoassayed[6] before and after dilution in pH 7.5 1 M phosphate buffer containing 2 per cent B.S.A. They were passed through millipore filters before use at a concentration of 5,000 microunits per cell culture plate.

Each experiment was terminated by the addition of 2 ml. of ice cold distilled water; the monolayers were removed by the application of a gentle shearing force using a rubber policeman. Extraction of the nucleic acid protein complex was carried out by a modified Schmit Tanhauser method.[7] RNA was assayed by the orcinol reaction[8] with use of a yeast RNA standard, (Cal Bio

Chem A Grade), the DNA determination by the diphenylamine reaction[9] with a calf thymus DNA standard (Mann Res. Lab., Inc., N.Y.) and the proteins by the Lowry method.[10] The C-14 activity of the RNA fractions was estimated in duplicate in a Packard Tricarb Scintillation Counter and expressed as CPM per mg. of RNA. The DNA and protein fractions contained less than 3 per cent of the RNA radioactivity.

I. *Studies with Connaught Human Pancreatic Insulin.*

A. Plastic tissue culture dishes (60 x 15 mm.) were seeded with 5 x 10^5 late log phase cells in 5 ml. of Minimum Essential Medium with L-glutamine (M.E.M. Hyland Lab., Inc., L.A., Calif.) and 10 per cent fetal calf serum (Hyland Lab., Inc., L.A., Calif.). Three days later, to each plate was added fresh medium, .25 μcurie of Uridine-2-C-14 and 1,000 or 5,000 μunits of insulin. At various intervals during the next twelve hours, four insulin-treated and two control plates were removed and the RNA specific activity estimated.

B. Seeding of 2.5 x 10^5 late log phase cells in 30 x 15 mm. petri dishes was made in 2.5 ml. M.E.M. with 10 per cent fetal calf serum. After twenty-four hours, the medium was changed to M.E.M. with 0.5 per cent Bacto-Peptone (Difco Lab., Detroit, Michigan). Two days later, .125 μcurie of Uridine-2-C-14 and 5,000 μunits of insulin were added. Five control and five insulin treated plates were harvested periodically during the following twenty-four hours; they were assayed for DNA, RNA, proteins and the RNA specific activity was calculated.

II. *Studies with normal and diabetic pancreatic insulin.*

A. Outline of extraction procedure:

The detailed procedure used will be reported elsewhere.[11] Briefly, the specimens of pancreas were obtained at the postmortem table, frozen and maintained at —20° C. until processed. After homogenization, each pancreas was extracted with 75 per cent ethanol at pH 2.[12] To each ml. of the clear extract, 6 ml. of 3:1 ether ethanol mixture was added. The resulting crude insulin precipitate was dissolved in 1 M acetic acid and then dialyzed against 1 M acetic acid.[13] The retentate was gel filtered with G50 Sephadex Superfine (Pharmacia, Uppsala) and the insulin containing fraction was lyophilized. Density gradient electrophoresis in an Ampholine column was carried out using Ampholyne pH 4-6 (L.K.P. Producter, Stockholm), all reagents being 8 M with urea. The insulin containing density gradient fraction was then gel filtered as before and the insulin peak lyophilized. Disc gel electrophoresis of identically prepared final insulin fractions showed a pattern identical to that obtained with both porcine and human crystalline insulin (Eli Lilly Co., Indianapolis, Indiana). No proinsulin or other contaminating protein bands were detectable. After hydrolysis, amino acid analysis carried out on the Beckman 120B revealed qualitative and quantitative amino acid patterns consistent with the composition of human insulin.[14]

TABLE 1

Control and diabetic postmortem pancreases for isolation of insulin. Microscopic pancreatic changes were minimal. They were limited to mild fibrosis in D1 and to scattered lymphocytic and neutrophilic infiltrates in C2.

	Age	Sex	Nature of Illness	Cause of Death
C1	75	F	Gastric carcinoma	Metastases
C2	47	F	Atherosclerosis	Mesenteric artery thrombosis
C3	35	M	Embryonal carcinoma of left testis	Metastases
C4	13	M	—	Accidental Death
D1	57	M	Adult onset diabetes treated with diet alone	Myocardial infarction
D2	71	F	Adult onset diabetes treated with Orinase	Myocardial infarction

B. Experimental protocol:

Three different experiments were carried out comparing insulins extracted from two diabetic to those isolated from four normal pancreases. Table I gives information concerning the age, sex, underlying illness of the patients from whom pancreatic tissue was obtained.

Late log phase cells at a concentration of 2.5 x 10^5 were seeded in 30 x 10 mm. plastic petri dishes containing 2 ml. M.E.M. with 10 per cent fetal calf serum. After twenty-four hours, the medium was replaced with M.E.M. containing 0.5 per cent Bacto-peptone. Forty-eight hours later, to new medium, was added 0.125 μcurie of Uridine-2-C-14 per plate and 5,000 μunits of insulin After a twelve-hour incubation period, the cells were harvested.

RESULTS

I. *Connaught Human Pancreatic Insulin*

A. In this initial experiment, two control plates and two plates treated with 1,000 or with 5,000 μunits of insulin were removed and the RNA specific activity (S.A.) determined after ½, 1, 2, 4, 8 and 12 hours of incubation. As seen in table 2, the duration of incubation of the cells with Uridine-2-C-14 exerts a significant influence on all three specific activity curves. The standard one way analysis of covariance with time, a quadratic covariate, shows that the plot obtained with the RNA S.A. of the plates treated with 5,000 microunits of insulin is significantly different from that of both the control plates ($P < .001$) and the plates added with 1,000 units ($P < .001$). Thus, there appears to be both a time and dose response to the insulin mediated

TABLE 2

Effect of duration of incubation and amount of Connaught human pancreatic insulin using a standard one way analysis of covariance with a quadratic covariate. There is no difference between the curves drawn from the RNA S.A. of the control and 1,000 microunit plates. However, statistical significance at a P level smaller than $< .001$ is achieved with the 5,000 microunit RNA S.A. values.

Time	Control Plates	Insulin	
		1,000 μU	5,000 μU
½ hour	40	54	54
	38	66	54
1 hour	98	119	96
	98	124	89
2 hours	174	224	205
	200	213	221
4 hours	329	329	407
	343	327	404
8 hours	499	471	632
	404	506	557
12 hours	533	585	607
	517	559	657

incorporation of Uridine-2-C-14 into RNA.

B. In the second study with Connaught human pancreatic insulin (table 3), five control plates and five plates treated with 5,000 μunits each of insulin were harvested after 4, 8, 12 and 24 hours of incubation. Analysis of covariance with time, a linear covariate shows that the two RNA S.A. slopes are different ($P < .01$). On the other hand, plotted DNA, RNA and protein determinations fail to distinguish between the control and the insulin treated plates. Cell counts at time 0 on five plates averaged 1.5×10^6. After twenty-four hours of incubation, the mean cell count was 2.4×10^6 and 2.5×10^6 for the five control and five insulin treated plates respectively. This small variation is within the accepted range of error for hemocytometer counting.

II. *Normal and diabetic pancreatic insulin.*

The three experiments illustrated in table 4 show significant differences between the RNA S.A. of plates treated with identical immunoassayable amounts of normal and diabetic pancreatic insulin. The labeling of RNA was much lower in experiment 1 than in experiments 2 and 3. This is not altogether surprising as the cells used were not synchronized. Experiments were carried out at different times as crystalline insulin samples become available and with cultures from cells of different generations. As in the experiments carried out with Connaught human insulin, there was no difference in cell counts, DNA, RNA or proteins between the normal and the diabetic insulin treated plates.

DISCUSSION

Virtually every known growth and developmental hormone as well as a few "metabolic" hormones exert a

TABLE 3

Each determination is the mean of five plates. Analysis of covariance using time as a linear covariate for the control and the insulin-treated plates fails to discriminate between the RNA, DNA and proteins. It elicits a significant difference ($P < .01$) between the slopes of the curves fitted with the RNA specific activity values.

	Duration of Incubation	Control Plates	Insulin 5,000 μunits
RNA S.A. (C.P.M./mg. RNA)	4 hours	43	47
	8 hours	79	100
	12 hours	127	158
	24 hours	221	266
RNA (μg./plate)	4 hours	55	51
	8 hours	53	53
	12 hours	58	64
	24 hours	66	67
DNA (μg./plate)	4 hours	7	6
	8 hours	—	—
	12 hours	9	12
	24 hours	15	18
PROTEINS (μg./plate)	4 hours	108	100
	8 hours	—	—
	12 hours	123	132
	24 hours	152	148

pronounced effect on the ability of the target tissue to synthesize RNA.[15] The insulin stimulated incorporation of precursors into RNA has been studied previously in vitro.[16,18] Relatively large concentrations of insulin were necessary compared to the amounts used in the current study with monolayered mouse fibroblast cultures. The small amounts of diabetic insulin available and the opportunity to look at a different parameter of biological activity[4] prompted the current approach.

We have previously demonstrated that small amounts of normal human insulin enhance the incorporation of a pyrimidine into the RNA fraction of mouse fibroblasts in tissue culture. In contrast, diabetic insulin in quantities comparable by immunoassay, mediated a sig-

TABLE 4

Experiments with chromatographically pure normal and diabetic pancreatic insulins. P values were determined by the Student two-tailed T-test. In Experiment 3, T's were calculated using a one way analysis of variance pooled mean square.

Expt. No.	Pancreas No.	No. of Plates	RNA S.A. $\bar{x}$	RNA S.A. S.E.	P
1	C1	15	63.1	2.2	$< .005$
	D1	15	54.3	1.9	
2	C2	10	180.4	2.3	$< .01$
	D1	10	168.0	3.5	
3	C3	15	160.5	2.5	$< .005$
	D2	10	147.0	3.6	
	C4	15	160.8	2.8	$< .005$

nificantly smaller incorporation of pyrimidine.[4] In our previous studies, Uracil-2-C-14 was employed as the precursor and insulin preparations were only partially purified. In the experiments reported here, the nucleoside, Uridine-2-C-14, was used as an RNA precursor, and only chromatographically pure normal and diabetic insulin was employed. The change from Uracil to Uridine was effected in the hope of reducing the incubation time of the cells with insulin and of shortening the time lag between the addition and measurable effects of insulin. In fact by using Uridine, a normal endogenous intermediate in RNA synthesis, we were able to shorten the incubation time from forty-eight to twelve hours.

The incorporation of the nucleoside uridine into its nucleotide was undoubtedly linked to the metabolism of fiibroblast cells. However, in view of the prolonged period of labeling, it was unwarranted to equate the extent of isotope incorporation with the RNA synthetic rate. Insulin might have altered isotope uptake into precursor pools leading to an increase in the RNA labeling independent of rate of synthesis. Furthermore, the rates at which the pools of nucleotides became radioactive probably reflected a change not only in the synthesis of RNA but possibly in its degradation rate.[19] The effect of insulin on RNA labeling related not only to the incubation time but also the amount of insulin added to each plate. On the other hand, cell counts, total RNA, DNA and proteins per plate were not significantly different at any one incubation time between control and insulin containing plates.

In the first phase of this study in which the effect of Connaught human pancreatic insulin on RNA labeling was assessed, it was elected to dispense with fetal calf serum and use bacto-peptone. This was done in order to avoid the "background" insulin present in fetal calf serum (10 microunits/ml.) and with the hope of augmenting the effect of insulin. This measure only led to a small percentage increase in the insulin effect at the expense of RNA specific activities which were lower in cell cultures grown in the absence of fetal calf serum (table 3 versus table 2). The precise reason for the lower RNA S.A. is not readily apparent from our studies. One possibility, however, is suggested by the difference in growth characteristics of the cells grown in the two media. In bacto-peptone supported cultures, the cells grew more slowly and reached their lag phase approximately thirty hours later than cells supported by fetal calf serum. The transport of labeled precursors may be enhanced in the more avidly growing cells. Moreover, in view of the prolonged logarithmic phase of cells grown in bacto-peptone, the timing of the introduction of labeled nucleoside provides substrate at different stages of cell differentiation. It is therefore reasonable, to attribute the difference in nucleoside incorporation (RNA S.A.) to critical timing of cell differentiation.

The earliest evidence for an abnormal insulin[1] was based on the resistance to insulinase of immunopurified serum insulin from juvenile diabetics. It could be criticized on the grounds of not distinguishing between an abnormal insulin and proinsulin. In fact, later studies[20] have shown that the insulinase resistance is a feature of the insulin peak from G 50 Sephadex. It was, therefore, necessary to pursue the experimental theme using highly purified insulin. Despite the limited scope of the current experiments, the results add to the evidence for an abnormal insulin.

Kimmel and Pollock[13] found no difference in amino acid composition between normal and diabetic insulins but did not carry out biological testing. Similar results were recently obtained with a group of elderly untreated diabetics but, in addition, identical immunoreactivity and hypoglycemic action was demonstrated.[11] A similar amino acid composition does not eliminate transpositions in sequence, amidation or deamidation. Likewise, it is consistent with the remote possibility of a different tertiary structure with an identical primary sequence.[21] It seems possible that diabetes has a number of etiologies and that in a certain part of the disease spectrum it is associated with either a heritable disorder of the insulin or, as mentioned by Blumenthal,[22] with an acquired somatic mutation of the beta cells. Confirmation of these suppositions awaits a more precise definition not only of the structure of abnormal insulin but also of its incidence in clinical diabetes.

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