STIMULATION WITH INSULIN IN VITRO OF PROTEIN SYNTHESIS BY DIABETIC HEPATIC MICROSOMES

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1. Introduction

In attempts to account for the diverse biological effects of insulin with a primary mechanism of action, several studies have implicated the cell membrane as containing an insulin-receptor site [1, 2]. Incubation of fat cells with trypsin abolishes the cellular response to insulin [2, 3] and treatment of adipocytes with *p*-chlormercuribenzene sulfonic acid (which reacts with protein sulfhydryl groups) mimics the actions of insulin on glucose transport and lipolysis [4]. Incubation of rat diaphragm with the tryptophan-labeling reagent 2-OH-5-nitrobenzyl bromide (HNB) inhibits the effect of insulin on protein synthesis, and it was proposed that this effect is mediated through an interaction between insulin and a membrane-bound, tryptophan- containing recognition site [5].

In contrast, the results of Wool and Cavicchi [6] do not support the theory that the effect of insulin on protein synthesis is mediated by an effect of the hormone on the cell membrane. Ribosomes isolated from skeletal muscles of alloxan diabetic rats are less active than those of normal animals in synthesizing protein in an in vitro system to which the necessary cofactors and ions are present in optimal amounts. Injection of insulin to diabetic animals 1 hr before preparation of the ribosomes stimulated incorporation to near-normal levels, demonstrating that this effect of insulin persists after the structure and organization of the cell have been destroyed [7]. However, they were unable to demonstrate an effect of insulin in vitro on protein synthesis by cell-free ribosomal preparations of diabetic animals.

In the experiments reported below, it was found that insulin in vitro had a definite effect on the incorporation of ^{3}H-phenylalanine by hepatic microsomes from diabetic pancreatectomized rats.

2. Materials and methods

White rats (Institute strain) were used for all experiments. Operated animals were sacrificed 4–6 months after a 95% pancreatectomy, when diabetes was well-established, and were matched with control animals of the same age and sex. Hepatic microsomes were prepared by the method of Grossi and Moldave [8], followed by resuspension and washing with 0.5 M KCl, recentrifugation at 105,000 *g* and suspension in Medium A of Moldave [9]. Soluble fraction, containing the pH 5 enzymes and the aminoacyl transferases, was prepared from normal rat liver homogenates. Protein was measured according to the method of Lowry et al. [10]. Microsomal concentration was determined by absorption ratios at 260 and 280 nm, using $A_{1\%}^{260}$ for RNA as 17.6 obtained from absorption concentration curves with purified RNA (Sigma Chemical Co.).

The standard incubation mixture consisted of the following: ATP 5 mM; GSH 5 mM; GTP 0.25 mM; $MgCl_2$ 5 mM; tris-HCl buffer, pH 7.4, 50 mM; KCl 40 mM, and soluble fraction 1 mg protein. The concentration of ^{3}H-phenylalanine substrate (7.2 Ci/mmole) was 1.5×10^{-5} M. Normal or diabetic microsomes were present at a concentration of 1 mg/ml. Insulin, when added, was present at a concentration of 1 or 100 μg/ml. The insulin used was a preparation of glucagon-free bovine insulin (Eli Lilly Co.) containing 27 U/mg. A stock solution of 1 mg/ml was prepared, adjusted to pH 7.0 with concentrated NaOH.

Incubation was performed in a total volume of 0.15 ml at 37° for 30 min. The reaction was terminated by chilling in ice, following which aliquots were pipetted on to numbered squares of dry Whatmann 3MM filter paper and dropped into 10% trichloroacetic acid. The filter papers were changed to a 5% trichloroacetic acid solution, heated at 90° for 15 min, and washed successively with 5% trichloroacetic acid, alcohol–ether (2:1), and ether [11]. After thoroughly drying, the papers were placed in Bray's solution for determinatio n of radioactivity in a liquid scintillation counter.

The concentration of soluble fractions used was chosen on the basis of earlier experiments in our laboratory in which it was found that labeled amino acid incorporation into microsomal protein was directly proportional to crude supernatant protein up to 1.0–1.5 mg, using the preparations described above [12]. Although the apparent amount of protein seems large, it should be remembered that labeled amino acid, and not aminoacyl-sRNA was used as substrate, and thus aminoacyl synthetase as well as transferase activity had to be provided in sufficient amounts for incorporation to occur. Furthermore, since these experiments were designed to determine the effect of insulin on protein synthesis by normal and diabetic microsomes, it was felt that all cofactors and soluble enzymes required for this process should be present in optimal or excess amounts.

When ATP or ATP and GTP were omitted from incubations, the incorporation of labeled amino acid was about 60% of that seen when these substances were present at the concentrations described. This is in agreement with results of other workers [13]. Since the microsomal and supernatant fractions were relatively crude, contaminating amounts of these nucleotides were undoubtedly still present in these preparations. However, ATP and GTP were added in optimum amounts to be certain that these factors were not rate-limiting in the studies performed.

3. Results and discussion

The incorporation of ^{3}H-phenylalanine into microsomal protein was significantly depressed in diabetic animals. While addition of insulin to incubations with microsomes from normal animals was without effect,

Table 1
Effect of insulin in vitro on ^{3}H-phenylalanine incorporation by normal and diabetic mircosomes.

Group	Condition	Insulin (μg/ml)	cpm		p of difference
A	Normal	0	6980±380	(3)	A–B 0.05
B	Diabetic	0	5450±490	(5)	A–C n.s.
C	Normal	1	6550±210	(3)	A–D n.s.
D	Normal	100	6140±200	(3)	A–E 0.001
E	Diabetic	1	13000±1500	(3)	A–F 0.001
F	Diabetic	100	10240±360	(3)	E–F n.s.

Standard assay mixture as described in text; incubate 30 min at 37°. Microsome concentration = 1 mg/ml; supernatant fraction = 1 mg; ^{3}H-phenylalanine = 1.5×10^{-5} M (7.2 Ci/mmole). Results expressed as mean ± S.E.M., determined by trichloroacetic acid precipitable protein on Whatmann 3MM filter papers. Number of observations parenthesis.

1 μg/ml of insulin produced a significant stimulation of incorporation by diabetic microsomes to values above normal levels. The insulin response in diabetic preparations was not proportional to concentrations, since 100 μg did not significantly stimulate values over those seen with 1 μg/ml. These results are seen in table 1.

The major differences between the preparations in the above experiments and those described by Wool include the following: a) animals were made chronically diabetic by pancreatectomy (4–6 months) instead of by acute alloxan treatment (2–10 days); b) hepatic tissue rather than skeletal muscle was used; c) substrate was labeled amino acid and not labeled aminoacyl-tRNA; and d) microsomes washed with KCl rather than deoxycholate-treated ribonucleoprotein particles were assayed.

Since the soluble fraction was always from normal animals in these experiments, an effect of insulin on amino activation seems unlikely. Nor do the results support the theory that insulin stimulates amino acid incorporation via an effect of the hormone on the cell membrane – or at least not in the chronic diabetic state – since disruption of cellular integrity and removal of cell membrane occurs during the microsomal preparation. However, it should be noted that in our preparation it is assumed that the ribosomes are still attached to intracellular membranous structures. Considering the potent effect of insulin on intracellular lipid synthesis and turnover, the chronic

diabetic animal, which is depleted of circulating insulin, theoretically lacks substrate for membrane phospholipid synthesis. Although there is no direct evidence to suggest a hormonal control of intracellular membrane formation or reorganization in order to facilitate translation of mRNA, the response of these membranes to insulin may be involved in the hormone's effect on translation in the chronically insulin-deficient state.

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