Treatment of Isolated Fat Cells with Trypsin Inhibits the Effects of Insulin on Incorporation of Leucine into Protein

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Summary. Incorporation of 0.1 mM ^3H -leucine into protein by isolated fat cells, with pyruvate as the only extracellular carbohydrate, was stimulated by: a) insulin, 0.1–4 ng/ml, or b) a mixture of 19 amino acids, 0.05–5 mM. Treatment of fat cells with trypsin, under conditions known to reduce binding of insulin by their plasma membranes, abolished stimulation of incorporation by the hormone but not by the amino acid mixture. These

results imply that the actions of insulin on incorporation of amino acids into protein are initiated by interaction with plasma membranes.

Key words: Isolated fat cells, insulin, trypsin treatment, plasma membranes, amino acid incorporation, amino acid mixture.

Receptors on fat cell plasma membranes are thought to mediate the effects of insulin on glucose metabolism and lipolysis [1–4]. Insulin also stimulates incorporation of amino acids into protein. We attempted to find out whether this hormonal effect, which does not depend on the aforementioned metabolic changes [5, 6], also begins at plasma membranes.

Materials and Methods

Twice-crystallized trypsin was obtained from Nutritional Biochemicals, crystalline soybean trypsin inhibitor from Worthington, and cycloheximide and puromycin dihydrochloride from Sigma. Single component porcine insulin [7] and other reagents [8] were as previously described.

During storage of ^3H -leucine¹ (New England Nuclear), there appeared a radiochemical impurity which would bind to the albumin of incubation media, thereby producing spurious “incorporation” in the absence of cells. Therefore, at intervals of two to three months the ^3H -leucine was repurified by paper chromatography (solvent, *n*-butanol/acetic acid/water, 4/1/1, v/v/v). This procedure reduced the impurity to 0.1% of the total radioactivity, corresponding to 10% of the true basal incorporation by fat cells.

Isolated rat fat cells were prepared and counted as previously described [8]. Incubation flasks contained 3×10^5 to 4×10^5 fat cells, 5×10^4 to 1×10^5 cpm ^3H -leucine, 50 nanomoles of L-leucine and additions, as described in the text, in albumin-bicarbonate medium [8], total volume 0.5 ml. Incubations were stopped

by addition of 3 ml Dole’s mixture [9], with vigorous mixing. Protein precipitates were collected by centrifugation, washed once with 5% trichloroacetic acid containing 0.01 M nonradioactive leucine, heated to 90° for 30 min, cooled, and trapped on glass-fibre discs (GF/A, Whatman). After further washing with 5% trichloroacetic acid, the discs were transferred to scintillation vials, and 1 ml “NCS” (Amersham/Searle) or “Protosol” (New England Nuclear) was added. The precipitates were dissolved at 55°, and counted at 25% efficiency in a toluene-based scintillator.²

Treatment of fat cells with trypsin, 1 mg/ml for 10–15 min, was by the method of Kono[3]. Control cells were carried through the same manipulations, with the omission of trypsin and inhibitor. Trypsin-treated cells had no more insulin-degrading activity than control cells, as determined by immunoassay of the media before and after incubation (data not shown).

Results

Incorporation of ^3H -leucine into the product was essentially abolished by inhibitors of protein synthesis (Table 1). When no substrate was added to the incubations, insulin did not significantly stimulate incorporation (data not shown). The substrate routinely employed was 0.5 mM pyruvate, which allowed a maximal response to insulin (Table 2). The half-maximal dose of insulin was about 0.4 ng/ml (Fig. 1).

Increasing concentrations of a mixture of all the natural amino acids except leucine, progressively augmented both basal and insulin-stimulated incorporation (Table 3). At a total concentration of 5 mM, the mixture appeared to supplant the effects of insulin. No single

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¹ Abbreviation: ^3H -leucine, L-leucine-4,5- ^3H .

² Composition: 2,5-diphenyloxazole (PPO), 4 g/L; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 50 mg/L in toluene.

Table 1. *Effects of inhibitors of protein synthesis on incorporation of leucine.*

Date of Experiment	Additions	Incorporation nanomoles/10 ⁶ cells/1 h
2/25/72	None	2.74 (2.59—2.93)
	Cycloheximide: 0.01 mM	0.26 (0.25—0.26)
	0.1 mM	0.16 (0.14—0.19)
2/28/72	None	2.29 (2.15—2.39)
	Puromycin: 0.1 mM	0.07 (0.06—0.08)

Fat cells were incubated for 1 h with 0.5 mM pyruvate and additions as noted in the table. Incorporation of ³H-leucine into protein is shown as the mean value from one experiment, with the range of triplicate incubations in parentheses.

sugar or amino acid could reproduce this phenomenon (data not shown). After fat cells were treated for 10 to 15 min with trypsin, 1 mg/ml, their incorporation of ³H-leucine was stimulated fully by the amino acid mixture, but their responsiveness to a supramaximal concentration of insulin was diminished (Table 4).

Fig. 1. *Dose-response curve for insulin.* Incubations were for 1 h, with 0.5 mM pyruvate added. Each point is the mean of quadruplicate incubations in one experiment, expressed relative to a value of 100 for incorporation without insulin. Mean absolute values for incorporation without insulin; 1.97 (experiment 1) and 2.49 (experiment 2) nanomoles/10⁶cells/1h

Table 2. *Glucose or pyruvate as energy sources for leucine incorporation*

Additions to Incubation medium	Incorporation nanomoles/10 ⁶ cells/1 h		Mean change produced by insulin
	No insulin	Insulin, 40 ng/ml	
Glucose, 5 mM (15)	2.22 ± 0.08	3.01 ± 0.10	+ 35%
Pyruvate, 0.5 mM (45)	2.46 ± 0.06	3.38 ± 0.10	+ 38%
Pyruvate, 5 mM (3)	2.88 ± 0.56	4.00 ± 0.81	+ 38%

Fat cells were incubated for 1 h, with additions as indicated in the table. Incorporation of ³H-leucine into protein is expressed as mean ± S.E. for the number of sets of triplicate incubations shown in parentheses.

Table 3. *Effects of amino acid mixture on incorporation of leucine*

Additions to Incubation medium	Incorporation nanomoles/10 ⁶ cells/1 h		Mean change produced by insulin
	No insulin	Insulin, 40 ng/ml	
Pyruvate, 0.5 mM	3.27 (3.15—3.36)	4.31 (4.21—4.40)	+ 32%
Pyruvate, 0.5 mM + Amino acid mixture:			
0.05 mM	3.51 (3.49—3.53)	4.61 (4.52—4.66)	+ 31%
0.5 mM	4.07 (4.02—4.11)	4.93 (4.74—5.04)	+ 21%
5 mM	5.24 (5.17—5.37)	5.55 (5.42—5.58)	+ 6%

Fat cells were incubated for 1 h, with additions as indicated in the table. The amino acid mixture consisted of equimolar amounts of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine, dissolved in incubation medium. The sum of the final concentrations of all the amino acids is indicated in the table. Incorporation of ³H-leucine into protein is expressed as the mean value from one experiment, with the range of triplicate incubations in parentheses.

Discussion

The abolition of incorporation by inhibitors of protein synthesis suggests that the product we measured was not formed by nonspecific binding of the label. The dose-response relationship and magnitude of stim-

ulation by insulin are in agreement with a previous report [5].

Exogenous metabolizable substrate was required for stimulation of incorporation by insulin. Previous investigators, who reported otherwise [5, 10], had in fact supplemented their incubation media with amino

acid mixtures. We have not explored in detail the characteristics which permit a substrate to support the effect of insulin. The provision of all the natural amino acids circumvented some rate-limiting step(s) on which insulin acts, presumably by virtue of properties of the constituents other than their ability to serve as fuel, since comparable amounts of carbohydrates or single amino acids did not supplant the effects of insulin.

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Table 4. Responses of trypsin-treated fat cells

	Basal incorporation* a.	Stimulation** by		"p" b vs. a	c vs. a
		Insulin, 40 ng/ml b.	Amino acid mixture, 5 mM c.		
1. Control	2.42 ± 0.34 (8)	+36.8 ± 4.9% (8)	+36.0 ± 2.3% (3)	< 0.001	< 0.01
2. Trypsin-treated	2.14 ± 0.33 (8)	+14.2 ± 3.1% (8)	+28.7 ± 3.7% (3)	< 0.01	< 0.02
"p" 1 vs. 2	N.S.	< 0.01	N.S.		

* nanomoles/10⁶ cells/1 h

** per cent change from basal incorporation

Fat cells were treated with trypsin as described in the text. They were then incubated with 0.1 mM ³H-leucine and 0.5 mM pyruvate. Results are expressed as mean ± S.E., for the number of sets of triplicate incubations shown in parentheses. Values for "p" were calculated by the two-tailed "t" test, based on paired comparisons [21].

Treatment of fat cells with trypsin is thought to modify the affinity [11] and/or capacity [12, 13] of membrane binding sites for insulin. The ability of trypsin-treated fat cells to respond to the amino acid mixture indicates that they retained the capacity to augment incorporation of leucine. We therefore infer that the receptors for the action of insulin on leucine incorporation are on cell surfaces and are accessible to trypsin.

Until now, the hypothesis that interaction of insulin with plasma membranes suffices to initiate all the effects of the hormone [14, 15] has been supported only by measurements of glucose metabolism and lipolysis [2, 3, 12]. Stimulation of amino acid incorporation, which may be mediated by an improved ability of fat cells to maintain intracellular amino acid concentrations [16, 17], does not depend on changes in glucose metabolism or lipolysis [5, 6]. Therefore, our results extend the support for the above hypothesis. Further confirmation will now require measurements of "intracellular" effects of insulin such as improvement in ribosomal function [18, 19] or induction of enzyme synthesis [20].

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