UCLA - 12 - 8 65

UNIVERSITY OF CALIFORNIA, LOS ANGELES

MASTER

LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY

CONTRACT NO. AT (04-1) GEN-12

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

INSULIN AND SEPHAROSE-INSULIN EFFECTS ON TYROSINE
TRANSAMINASE LEVELS IN CULTURED RAT LIVER CELLS

M.B. DAVIDSON, A.J. VAN HERLE & L.E. GERSCHENSON

LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY

900 VETERAN AVENUE

UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA 90024

DEPARTMENT OF MEDICINE AND THE DEPARTMENT OF PATHOLOGY

UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA 90024

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Atomic Energy Commission, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Acknowledgement: These studies were supported in part by the Department of Medicine, Department of Pathology, Contract AT(04-1) GEN-12 between the Atomic Energy Commission and the University of California and by Grants-in-Aid from the Diabetes Association of Southern California to M.B.D. and L.E.G.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

1

**Insulin and Sepharose-insulin Effects on Tyrosine Transaminase
Levels in Cultured Rat Liver Cells**

ABSTRACT

To determine if intracellular translocation of the intact insulin molecule was necessary for insulin stimulation of enzyme synthesis, the effect of crystalline insulin and insulin coupled to Sepharose on tyrosine transaminase levels in cultured rat liver cells was studied. After cells had been exposed to dexamethasone for 18 hours, the addition of crystalline insulin caused a marked increase in tyrosine transaminase levels over the next 2 hours. This increase in enzyme synthesis was blocked by cycloheximide but was not inhibited by Actinomycin D. Insulin was covalently linked to Sepharose beads via the B-29 lysine by the method of Cuatrecasas and washed extensively. Three separate preparations by three different people in two independent laboratories were made. Free insulin, measured by both immunoassay and bioassay, leaked off of the agarose beads in all three preparations, commencing shortly after the washing was completed and continuing progressively. In conclusion, insulin stimulates tyrosine transaminase synthesis in cultured rat liver cells at a point distal to the transcription step of protein synthesis. Sepharose-insulin is not a suitable model with which to investigate the metabolic effects of bound insulin.

INTRODUCTION

Insulin not only increases the transport of sugars and amino acids into cells, but also stimulates certain intracellular synthetic pathways independent of its effect on transport (1). Insulin binding to cell membranes is considered important in the stimulation of transport. However, it is uncertain whether the intact insulin molecule needs to be translocated into the cell or whether some insulin-mediated signal initiated at the cell membrane is the mechanism whereby insulin exerts its intracellular activity. Consequently, the effects of both crystalline insulin and insulin covalently linked to agarose polymers on tyrosine transaminase levels in cultured rat liver cells were studied in an attempt to answer this crucial question.

RESULTS

Beef or pork insulin was covalently linked via the B-29 lysine to large agarose polymers, Sepharose 4-B, whose particle size ranges between 60-300 microns. Three separate preparations were made by three different people in two independent laboratories utilizing the method of Cuatrecasas (2). These preparations were washed extensively from two to six days. The final washes of the first preparation of Sepharose-insulin were lyophilized and reconstituted in a small volume. No immunoassayable insulin nor biological activity on rat hemidiaphragms and fat pads were detectable. The Sepharose-insulin preparations themselves, however, were active on these tissues and their activity was blocked by anti-insulin antibody (Table 1). Sepharose beads alone (without insulin) had no effect on rat hemidiaphragm or epididymal fat pads (data not shown).

The effects of insulin and the three preparations of Sepharose-insulin on cultured rat liver cells are shown in Table 2. As reported previously (5), dexamethasone markedly stimulated the specific activity of tyrosine transaminase and crystalline insulin further increased the same enzyme in cells pretreated with the glucocorticoid (6). Sepharose-insulin also increased the level of this enzyme while Sepharose beads alone were without effect. Anti-insulin antibody completely blocked the effects of both insulin and Sepharose-insulin.

Although these data seemingly indicate that intracellular translocation of the intact insulin molecule is unnecessary to affect enzyme synthesis, this conclusion hinges on a stable bond between insulin and the agarose polymer with no leakage of free insulin. However, this was not the case. Immunoassayable insulin was found in the supernatants of all three Sepharose-

insulin preparations (Table 3). Tissue or cells were not responsible for the splitting off of free insulin since significant amounts were found in the supernatant after centrifugation but before incubation. Furthermore, the forces of centrifugation did not break the Sepharose-insulin bond since when the coupled beads were simply allowed to settle, the concentration of immunoassayable insulin in the supernatant was equivalent to the amount in the centrifuged supernatant.

This free insulin in the supernatants also showed activity in rat epididymal fat pads (Table 4) and in cultured rat liver cells (Table 5), both of which could be inhibited by anti-insulin antibody. Note that the supernatant from preparation III significantly increased tyrosine transaminase levels even though it was collected only fifteen minutes after the final washing of the Sepharose-insulin beads had been completed. The supernatant tested in Table 4 was collected after incubation and contained 42 uU/ml by immunoassay. Its lack of effect on rat diaphragm is not surprising since this bioassay system is usually not sensitive to insulin concentrations less than 50 uU/ml.

The figure shows the rate at which insulin dissociates from the Sepharose beads. At a 1:50 dilution, the Sepharose-insulin beads of preparation III originally contained 560 uU/ml as measured by immunoassay. Aliquots taken over the next fifty days revealed a considerable amount of insulin in the supernatant. At the first time interval sampled, which was several hours after the extensive washing was completed, 6% of the insulin was already present in the supernatant.

Although it seemed unlikely that the bond between the B-29 lysine of insulin and the Sepharose bead was stable and that small pieces of the polymer were breaking off, studies were carried out to test this possibility.

No agarose could be measured chemically in the supernatants (8). Furthermore, when Sepharose-insulin preparations were filtered through an EAWP millipore filter (which does not adsorb insulin), appreciable amounts of immunoassayable insulin appeared in the filtrate (Table 6). It should be noted that if an HAWP millipore filter (the usual one used which adsorbs insulin) had been utilized exclusively, it would have been concluded that little free insulin was leaking off of the Sepharose beads.

Finally, the effects of cycloheximide and Actinomycin D on insulin-mediated tyrosine transaminase synthesis were studied (Table 7). Cycloheximide reduced the specific activity of the enzyme almost to control levels while Actinomycin D did not inhibit the effect of insulin.

DISCUSSION

Insulin effects on specific metabolic pathways or on enzyme synthesis in isolated cell systems have recently been reported. In cultured rat liver cells, insulin stimulates glycogen synthesis (9,10), glycolysis (10), the formation of polysomes (11) and the synthesis of pyruvate kinase (12); in human diploid cells, WI-38, insulin increases glycolysis as well as protein and DNA synthesis (13); in chick embryo fibroblasts, insulin enhances protein synthesis by isolated ribosome (14); and in cultured hepatoma cells, insulin raises tyrosine transaminase levels (15). Gerschenson et al (6) found that insulin will increase the specific activity of tyrosine transaminase in cultured rat liver cells and that this stimulation is not inhibited by Actinomycin D. This last finding suggests that the site of this hormonal effect is distal to the transcription step which is similar to the postulated locus of insulin action on protein synthesis in muscle (16). Similar results are described here.

To answer the important question of whether the intact insulin molecule had to enter the cell in order to increase enzyme synthesis, the effect of

Sepharose-insulin on the tyrosine transaminase levels of these cultured rat liver cells was studied. Insulin coupled to this agarose polymer via the B-29 lysine residue had supposedly been shown to stimulate oxidation of glucose and inhibit lipolysis in isolated fat cells (2), to stimulate RNA synthesis (17) and α -aminoisobutyric acid transport (18) in isolated mammary gland cells and to activate glycogen synthetase in minced tadpole liver (19). However, in only the last study was an attempt made to prove that free insulin leaking off of the Sepharose-beads was not causing the effects being measured. These investigators (19) compared the activity of glycogen synthetase in tissues placed in two chambers separated by a millipore filter to one side of which Sepharose-insulin had been added. The activity of the enzyme in the tissue directly exposed to the coupled insulin was increased compared to the tissue on the other side of the filter. Although this was interpreted as proving no leakage of free insulin from the Sepharose beads, the more likely explanation is that the released insulin was simply adsorbed to the HAWP filter used (20) and never gained entrance to the other chamber. Crystalline insulin (0.48 U/ml) was used in the same system as a control and did stimulate glycogen synthetase activity in the other chamber. However, since at least 7 per cent of free insulin will pass across the millipore filters usually used (Table 6), a concentration of insulin of approximately 30 mU/ml would be attained in the other chamber and probably accounts for the similarity of enzyme activity on the two sides.

The results of our studies on three separate Sepharose-insulin preparations clearly demonstrate that immunoassayable and biologically active insulin leaks off of the agarose beads, commencing within minutes of the final washing and continuing progressively. While this manuscript was in preparation, Fritz (20) reported that the covalent bond between insulin and Sephadex G-100 via

an isothiocyanate linkage through the α -amino groups of the N-terminal residue was unstable. We do not maintain that all of the activity of Sepharose-insulin preparations is due to the free insulin present. There is some evidence that insulin coupled to agarose polymers does retain its activity. The supernatant, which was inactive on rat diaphragm (Table 4) and contained 42 uU/ml of insulin, was collected immediately after incubation of preparation I which contained 210 uU/ml and was active on muscle (Table I). Thus, the effect of preparation I was probably due to the insulin linked to Sepharose since the amount of free insulin in the supernatant was below the limits of sensitivity of the rat diaphragm assay. In addition, Cuatrecasas was able to show activity of Sepharose-insulin on isolated fat cells in concentrations as low as 1-3 uU/ml. However, the fact that free insulin leaks off of the beads clearly makes this preparation unsuitable to investigate the metabolic effects of bound insulin.

REFERENCES

1. M. B. Davidson and C. J. Goodner, *Diabetes* 15, 835 (1966).
2. P. Cuatrecasas, *Proc. Nat. Acad. Sci.* 63, 450 (1969).
3. M. B. Davidson and C. J. Goodner, *Diabetes* 15, 380 (1966).
4. M. B. Davidson, *Am. J. Physiol.* 25, 105 (1968).
5. L. E. Gerschenson, M. Andersson, J. Molson and T. Okigaki, *Science* 170, 859 (1970).
6. L. E. Gerschenson, M. Andersson and M. B. Davidson, Manuscript in preparation.
7. J. S. Soeldner and D. Slone, *Diabetes* 14, 771 (1965).
8. Method, E. Cabib, personal communication.
9. L. E. Gerschenson and D. Casanello, *Biochem. Biophys. Res. Comm.* 33, 584 (1968).
10. D. E. Casanello and L. E. Gerschenson, *Exp. Cell Res.* 59, 283 (1970).
11. L. E. Gerschenson, T. Okigaki, M. Andersson, J. Molson and M. B. Davidson, *Exp. Cell Res.* 71, 49 (1972).
12. L. E. Gerschenson and M. Andersson, *Biochem. Biophys. Res. Comm.* 43, 1211 (1971).
13. J. W. Griffiths, *J. Cell Sci.* 7, 575 (1970).
14. A. G. Schwartz and H. Amos, *Nature* 219, 1366 (1972).
15. T. D. Gelehrter and G. M. Tomkins, *Proc. Nat. Acad. Sci. U.S.* 66, 390 (1970); J. R. Reel, K. Lee and F. T. Kenney, *J. Biol. Chem* 245, 5800 (1970).
16. I. G. Wool, W. S. Stirewalt, K. Kurihara, R. B. Low, P. Bailey and D. Oyer, in *Recent Progress in Hormone Research*, Vol 24, E. B. Astwood, Ed. (Academic Press, New York, 1968), p. 139.
17. R. W. Turkington, *Biochem. Biophys. Res. Comm.* 41, 1362 (1970).
18. T. Oka and Y. J. Topper, *Proc. Nat. Acad. Sci.* 68, 2066 (1971).
19. L. M. Blatt and K-H Kim, *J. Biol. Chem.* 246, 4895 (1971).

20. K-H Kim, personal communication.
21. I. B. Fritz, in Biochemical Actions of Hormones, G. Litwack, Ed.
(Academic Press, New York, 1972) p. 186.
22. We thank M. Andersson and R. G. Karjala for technical help and Dr. J. Morris for advice in preparing Sepharose Insulin. Supported by contract AT(04-1) GEN-12 between the Atomic Energy Commission and the University of California and by grants-in-Aid from the Diabetes Association of Southern California to M.B.D. and L.E.G.

Figure 1. Immunoassayable insulin in supernatants of a Sepharose-insulin preparation versus time. A 1:50 final dilution of preparation III was stored in 50 ml of an antibiotic solution containing Penicillin, Polymyxin B and Streptomycin. The original concentration of insulin coupled to the beads was 560 uU/ml. One-half ml aliquots were removed after centrifugation at various time intervals for insulin determinations. No corrections were made for changes in volume. Neither uncoupled Sepharose beads stored identically nor its supernatants sampled at the same time intervals contained any measurable immunoassayable insulin.

Table 1. Effect of sepharose-insulin (S-I) preparations on glucose uptake by rat diaphragm (3) and glucose oxidation by rat epididymal fat pads (4).

Preparation	Dilution	No. of animals	Hemidiaphragms ($\mu\text{g}/\text{mg}/\text{hr} \pm \text{SE}$)		Epididymal Fat Pads ($\text{Cpm } \text{C}^{14}\text{O}_2/\text{mg}/2 \text{ hr} \pm \text{SE}$)	
			Basal	S-I	Basal	S-I
I	1:1250	6	*2.41 $\pm$ 0.16	*4.03 $\pm$ 0.29	17.9 $\pm$ 1.9	67.8 $\pm$ 5.4
**I	1:1250	2	*2.50 $\pm$ 0.05	*2.82 $\pm$ 0.09	20.8 $\pm$ 4.8	22.4 $\pm$ 0.4
II	1:200	4	0.42 $\pm$ 0.12	2.06 $\pm$ 0.34	18.0 $\pm$ 0.4	56.7 $\pm$ 7.3
III	1:200	3	1.45 $\pm$ 0.02	3.16 $\pm$ 0.26	16.7 $\pm$ 2.0	65.9 $\pm$ 9.8

*No preincubation period

**Anti-insulin antibody added to flasks in which tissues from these animals were incubated

Table 2. Effect of Sepharose-insulin preparations and crystalline insulin on tyrosine transaminase levels in cultured rat liver cells. Incubations were carried out as described previously (5). After 18 hours of exposure to dexamethasone, the media was removed and the cells were reincubated for 2 more hours with various additions. Three petri dishes of cells were used for each experimental condition. Tyrosine transaminase levels in cells from each dish were measured in triplicate. The results are expressed as specific activity; nanomoles of the complex p-hydroxyphenyl pyruvate:enol and borate formed per min/mg. of protein with the molar absorptivity of the complex taken to be 10,700.

Additions	Tyrosine transaminase (nM product/mg protein/min $\pm$ SE)	Additions	Tyrosine transaminase (nM product/mg protein/min $\pm$ SE)
-	116.0 $\pm$ 1.1	-	44.3 $\pm$ 0.4
D	364.0 $\pm$ 2.3	D	562.3 $\pm$ 3.4
D, S	357.8 $\pm$ 3.5	D, I	666.9 $\pm$ 2.8
D, I	698.8 $\pm$ 4.6	D, S-I**	772.4 $\pm$ 5.8
D, S-I*	659.0 $\pm$ 4.6	-	51.0 $\pm$ 0.6
D, anti-I	382.8 $\pm$ 2.3	D	208.0 $\pm$ 1.6
D, I, anti-I	405.3 $\pm$ 3.0	D, I	297.0 $\pm$ 2.2
D, S-I*, anti-I	338.7 $\pm$ 2.0	D, S-I***	249.0 $\pm$ 2.2

D - Dexamethasone phosphate (10^{-5} M)

S - Sepharose beads (1:200 final dilution)

I - Insulin (150 uU/ml)

S-I - Sepharose-insulin

Anti-I - 10x purified anti-insulin serum

*Preparation I (1:200 final dilution)

**Preparation II (1:40 final dilution)

***Preparation III (1:40 final dilution)

Table 3. Immunoassayable Insulin (uU/ml) in Supernatants (S/N) of Sepharose-insulin (S-I) Preparations. A double antibody immunoassay for insulin was used (7).

Preparation	I	II	III
S-I beads	210	156	280
After incubation S/N	42	--	172
Centrifuged S/N	23	54	17-200*
Settled S/N	--	50	--

*Depends on time of sampling

Table 4. Bioassay of a supernatant (S/N) from a Sepharose-insulin preparation on rat hemidiaphragms (3) and epididymal fat pads (4).

No. of animals	*Hemidiaphragms (ug/mg/2 hr $\pm$ SE)		Epididymal Fat Pads (Cpm $C^{14}O_2$ /mg/2 hr $\pm$ SE)	
	Basal	S/N	Basal	S/N
3	4.09 $\pm$ 0.48	3.93 $\pm$ 0.62	36.0 $\pm$ 4.8	61.3 $\pm$ 5.4
**3	3.51 $\pm$ 0.38	3.48 $\pm$ 0.28	18.9 $\pm$ 1.3	22.7 $\pm$ 1.8

*No preincubation period

**Anti-insulin antibody added to flasks in which tissue from these animals were incubated.

Table 5. Bioassay of supernatants (S/N) from Sepharose-insulin preparations on cultured rat liver cells. Experiments carried out as described in Table 2.

Additions	Tyrosine Transaminase (nM product/mg.protein/min $\pm$ SE)
D	364.0 $\pm$ 2.3
D, S/N*	581.3 $\pm$ 3.7
D, S/N, * anti-I	430.0 $\pm$ 2.7
D	562.3 $\pm$ 3.4
D, S/N**	594.0 $\pm$ 4.4
D	208.0 $\pm$ 1.6
D, S/N***	249.0 $\pm$ 1.8

D - Dexamethasone phosphate (10^{-5} M)

Anti-I - 10x purified anti-insulin serum

*S/N from preparation I tested at a 1:200 final dilution

**S/N from preparation II tested at a 1:40 final dilution

***S/N from preparation III tested at a 1:40 final dilution and collected only 15 minutes after completion of extensive washing of Sepharose-insulin beads.

Table 6. Millipore filtration of Sepharose-insulin (S-I) preparations

Preparation	I	I	II	III
S-I beads	*145	10,500	133	163
**HAWP filter	1	752	1	3
***EAWP filter	75	8,000	24	74

*Immunoassayable insulin (uU/ml)

**Insulin is adsorbed to this type of filter which is the kind routinely used.

***Insulin passes through filter

Table 7. Effect of Actinomycin D and cycloheximide on insulin-mediated tyrosine transaminase synthesis in cultured rat liver cells. Experiments carried out as described in Table 2.

Addition	Tyrosine Transaminase (nM product/mg protein/min $\pm$ SE)
-	222.7 $\pm$ 2.0
D	361.4 $\pm$ 2.4
D, I	547.8 $\pm$ 2.8
D, I, A	640.6 $\pm$ 4.6
D, I, C	260.1 $\pm$ 2.4
D, S-I*	473.1 $\pm$ 2.9
D, S-I*, A	600.3 $\pm$ 3.6
D, S-I*, C	247.8 $\pm$ 1.7

D - Dexamethasone phosphate (10^{-5} M)

I - Insulin (150 uU/ml)

S-I* - Sepharose-insulin, preparation I (1:200 final dilution)

A - Actinomycin D (0.25 ug/ml)

C - Cycloheximide (25 ug/ml)

Mayer B. Davidson
Department of Medicine
UCLA School of Medicine
Los Angeles, California 90024

A. J. Van Herle
Department of Medicine
UCLA School of Medicine
Los Angeles, California 90024

L. E. Gerschenson
Laboratory of Nuclear Medicine
and Radiation Biology and
Department of Pathology
UCLA School of Medicine
Los Angeles, California 90024