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Loss of Immunological and Biological Activity of Insulin With a Human Muscle Extract

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The isolation of a human muscle extract that degrades insulin to products incapable of any immunological or biological activity is reported. The extract K_m value for human insulin is 0.79, and 1.43 for porcine proinsulin. It does not degrade Human Growth Hormone at the concentrations tested, it is inactivated by N-ethyl-maleimide, Trasylol, and heat, and it does not require glutathione for its action. Human muscle, therefore can contain an enzyme similar to «insulin-specific-protease» present in rat muscle.

The catabolism of insulin *in vivo* has been a subject of interest recently. MIRSKY (10) isolated an insulinase from rat liver, and CHANDLER and VARANDANI (2) have isolated and purified an enzyme that splits the insulin molecule into the A and B chains by disulphide bond cleavage and subsequent proteolysis (12).

A protease from rat muscle that specifically hydrolyzes insulin to non-immunoassayable derivates has been isolated by BRUSH (1) and purified by DUCKWORTH *et al.* (4). This enzyme appears to be located in the cytoplasm, does not require glutathione for its action, is sulphydryl-dependent and has a low value for insulin.

In this work we have tried to find out whether a similar enzyme is present in human muscle, and to study some of its properties.

Materials and Methods

Materials. ^{125}I labelled and non-labelled human insulin, growth hormone and antibodies were supplied by CEA-CENSORIN (France). Porcine proinsulin was obtained through Dr. E. Lázaro (Madrid), from Dr. E. Chance (USA). Cristalline

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bovine insulin, was supplied by Sigma Chem. (St. Louis). Trasylol is a commercial preparation manufactured by Bayer (Germany), containing 10,000 kallikrein inhibitor units/ml. Other reagents were of the best quality available.

Isolation of human muscle extract. Human skeletal muscle was obtained from a patient submitted to amputation of a leg due to arteriosclerotic thrombosis. Only the proximal portion of the muscle well above the nechrotic zone was taken. The tissue was immediately frozen in liquid N_2. The muscle extract —as isolated according to BRUSH (1) with some modifications. The tissue was homogenized in 0.35 M sucrose (5 ml/g) and centrifuged at 38,000 × *g* for one hour. The supernatant was dialyzed against distiled deionized water and then lyophilized. The dry powder was dissolved in one-eighth volume of the supernatant and undissolved material removed by centrifugation at 10,000 × *g*. Sufficient $Ca(PO_4)_2$ gel was then added to obtain a ratio of gel to protein of 1:0.84; protein content was determined by the LOWRY method (9).

Enzymatics studies. Degradative activity was assayed by incubation of human or bovine insulin, porcine proinsulin and human growth hormone, dissolved in 100 µl of 40 mM phosphate buffer pH 7.5 with 100 µl of human muscle extract containing 2.13 mg/ml of protein, at 37° C for different periods of time. Reactions were stopped by the addition of 100 µl of 10 mM N-ethyl-maleimide; 100 µl of Trasylol were used to stop the reaction when the remaining insulin was to be assayed biologically on rat adipose tissue.

The effects of both N-ethyl-maleimide (10 mM) and Trasylol (10,000 kallikrein inhibitor units/ml) were compared by adding each one to the muscle extract-insulin system after 0, 10, 20 and 30 min of incubation, and the remaining insulin was measured by radioimmunoassay.

Glutathione, at a final concentration of 1 mM, was added to the muscle extract at time 0, and then incubated with insulin for 10 min, the reaction was stopped by N-ethyl-maleimide.

The muscle extract preparation was heated at 60° C for 10 min and then it was incubated with insulin for 30 min. The reaction was stopped by N-ethyl-maleimide.

Amounts of muscle extract enough to degrade insulin in a 10 min-period, and proinsulin in a 15 min-period, following the first kinetic order, were used for estimation of K_m values in both substrates.

Human growth hormone at three different concentration was incubated with the human muscle extract. After periods of 10, 20 and 30 min, the same volume of 10 mM N-ethyl-maleimide was added to each sample and the remaining hormone was measured by radioimmunoassay.

Radioimmunoassay of hormones. Immunoassayable insulin and proinsulin were determined in triplicate by the double antibody method, HALES and RANDLE (5). Human insulin and porcine proinsulin supplemented with the human muscle extract and N-ethyl-maleimide at time 0, were used as standards.

Human growth hormone was measured by the double antibody method, MOLINATTI *et al.* (11) in duplicate. The standards were supplemented with the muscle extract and N-ethyl-maleimide at time 0.

Incubation of adipose tissue. Bovine insulin at a final concentration of 5 mU/ml in 50 mM phosphate buffer pH 7.5 was incubated in the presence and in the absence of the human muscle extract at a final concentration of 2 mg protein/ml. The muscle extract in buffer, and buffer alone, were incubated simultaneously. After 30 min at 37° C, Trasylol (10,000 units/ml) was added to each tube in a volume equivalent to one third of the in-

cubation mixture. Aliquots of 100 μl were added to siliconized flasks containing small pieces of epididimal adipose tissue (aproximately 1880 mg), from nine fasted Wistar rats, and incubated in 1 ml of Krebs-Ringer-bicarbonate buffer pH 7.4 containing 2 % defated bovine albumin and 3 mM (U-^{14}C) glucose, 1 μCi/ml. The incubation was carried out at 37° C for 1 hour in flasks that were sealed with a rubber cap, from which a polyethylene cups were suspended. At the end of the incubation period, 0.2 ml of Hyamine hydroxide in metanol (1 M) was introduced into the cup through the rubber cap, and the reaction was stopped by injecting 0.2 ml of 5 M H_2SO_4 into the incubation medium. Radiactivity of CO_2 was measured in a liquid scintillation system, and glucose in the medium by the glucose oxidase method of HUGGETT and NIXON (6).

Results

Degradation of hormones. 50 % of insulin and 13% of proinsulin were degraded in 10 min, and 90 % of insulin and 40 % of proinsulin in 30 min (fig. 1).

Figures 2 and 3 show the reciprocal degradation plot of different concentrations of insulin by the human muscle extract and the proinsulin respectively. The K_m for both insulin and proinsulin estimated by this procedure had values of 0.79 nM and 1.43 nM.

Table I shows the lack of degradation of human growth hormone by the human extract.

Loss of biological activity of insulin. The biological activity of insulin was completely lost after its degradation with the human muscle extract (fig. 2) and it had no significant effect by itself. In a previous experiments, it was demonstrated, that Trasylol at the doses used did not interfere with glucose metabolism in rat adipose tissue *in vitro*.

Inactivation of the human extract muscle. Trasylol at the doses used was efficient as N-ethyl-maleimide in preventing

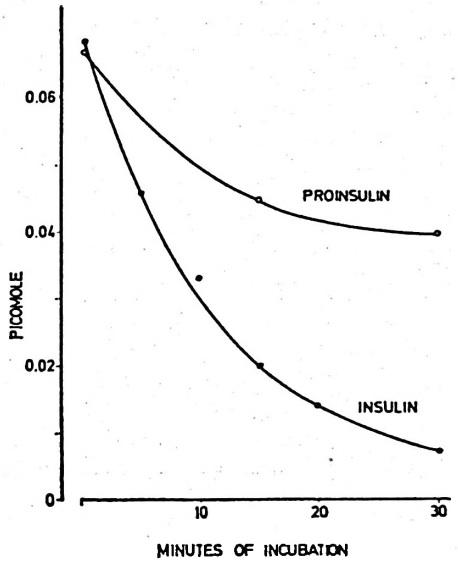

Fig. 1. *Degradation of insulin and proinsulin by Human muscle extract:*
0.0689 pmole of human insulin or porcine proinsulin (both in 0.04 M phosphate buffer pH 7.5) were incubated with 100 μl of human muscle extract (2.13 mg/ml) in 0.05 phosphate buffer pH 7.5 at 37° C. Reactions were stopped by addition of 10 mM N-ethyl-maleimide. The remaining insulin or proinsulin were determined by radioimmunoassay, using standards to which N-ethyl-maleimide and human muscle extract were added at time 0.

Table I. *Effect of human muscle extract on human growth hormone.*
Human Growth Hormone was dissolved in 40 mM phosphate buffer, pH 7.5 at three concentrations, and 100 μl of each were incubated in duplicate with 100 μl of human muscle extract (2.13 mg of protein/ml) at 37° C. Reactions were stopped at the indicated periods by N-ethyl-maleimide, and the remaining hormone measured by radioimmunoassay.

Initial (ng/ml)	Percent remaining after		
	10 min	20 min	30 min
2.5	104	108	112
5.0	100	104	102
10.0	100	92	102

the degradation of insulin when added to the muscle extract system (table II).

When the human muscle system was previously heated at 60° C for 10 min, its activity on insulin degradation was lost, even as it was when incubated for 30 min with insulin (0.3 % activity remained).

Glutathione at a final concentration of 1 mM did not change the activity of the human muscle extract on insulin degradation.

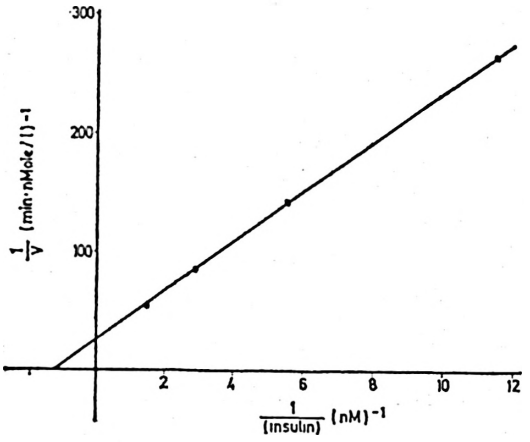

Fig. 2. *Reciprocal plot of the degradation of insulin:*
Human insulin (0.0689, 0.0344, 0.0172 and 0.0086 pmole) in a constant volume of 100 µl of phosphate buffer was incubated with 100 µl of human muscle extract preparation (0.85 mg of protein/ml) in triplicate. After 10 min at 37° C. The reaction was stopped by addition of 100 µl of 10 mM N-ethyl-maleimide, and the remaining insulin assayed by radioimmunoassay, using standards to which N-ethyl-maleimide and human muscle extract were added at time 0.

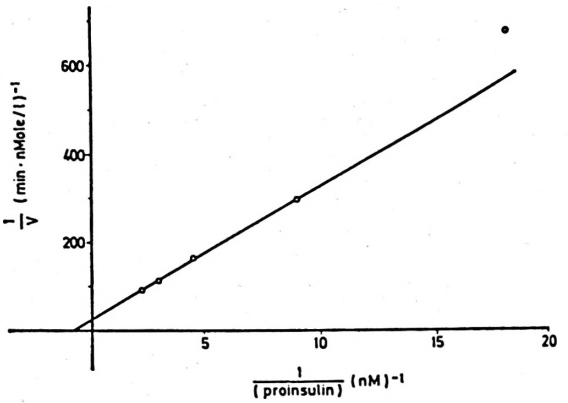

Fig. 3. *Reciprocal plot of the degradation of proinsulin:*
Porcine proinsulin (0.0689, 0.0344, 0.0172 and 0.0086 pmole) in a constant volume of 100 µl of phosphate buffer was incubated with 100 µl of human muscle extract preparation (1.07 mg of protein mg of protein/ml) in triplicate. After 15 min at 37° C, the reaction was stopped by addition of 100 µl of 10 mM N-ethyl-maleimide, and the remaining proinsulin determined by radioimmunoassay, using proinsulin standards to which N-ethyl-maleimide and human muscle extract were added at time 0.

Table II. *Loss of biological activity of insulin by degradation with human muscle extract.* Pieces of rat epididimal adipose tissue were incubated in Krebs-Ringer bicarbonate buffer containing (U-^{14}C) glucose. 100 µl of bovine insulin (375 µU.I.) were added, to each group of flasks previously incubated for 30 min in the presence (e), and the absence (b) of human muscle extract. Phosphate buffer was used as control (a) and human muscle extract (d) was incubated without insulin. Values are mean ± SD (n = 5).

	µMole of glucose/g tissue/h		Insulin detected Immunologically (µU/ml)*
	Taken up	converted to CO_2	
a) Control	6.77±1.28	1.91±0.38	
b) Insulin	10.51±0.98 **	2.95±0.33 **	230.0
c) Insulin + H. muscle extract	5.95±0.98	1.62±0.24	0.2
d) H. muscle extract	6.08±0.80	1.75±0.18	

* Measured in aliquots taken before addition to tissue and using human insulin as standard.
** $P < 0.001$.

Table III. *Inactivation of human muscle extract.*

100 μl of human insulin (100 μU/ml) were mixed with 100 μl of human muscle extract (2.13 mg/ml of protein) in triplicate and the reaction was allowed to proceed at 37° C. Inhibitor (100 μl) was added at the times indicated, and the remaining insulin measured by radioimmunoassay. Standards containing human muscle extract and either N-ethyl maleimide or Trasylol added at time zero.

	Percent insulin remaining	
Added at time (min)	N-ethyl-maleimide (10 mM)	Trasylol (10,000 units/ml)
0	100	100
10	54	62
20	30	30
30	19	28

Discussion

The ability of a human muscle extract to degrade the immunological and biological properties of insulin *in vivo*, suggest that a similar enzyme system to that described by BRUSH (1), KITABCHI and STENTZ (8) and DUCKWORTH *et al.* (4) in rat muscle is also present in man.

The preparation used, according to the results which we obtained would appear to contain an enzyme that shares some of the properties of its aquivalent in rat muscle, as its activity is blocked by N-ethyl-maleimide and it is not altered by glutation. However, the muscle extract has a K_m value for insulin lower than that estimated by BRUSH (1), DUCKWORTH *et al.* (4) and CRESTO *et al.* (3) in the rat enzyme. This fact could be explained by our use of substrate and «enzyme» obtained from the some species.

This human muscle extract shows greater affinity for insulin than for proinsulin, K_m (Proinsulin)/K_m (insulin) ratio being about 2. This relation is very similar to that found by CRESTO *et al.* (3) in the rat enzyme. We were unable to determine K_m for human proinsulin with this preparation, but since the rat enzyme increase its affinity for substrate whereas porcine proinsulin splits off to smaller intermediates (1, 7). K_m relation for porcine proinsulin and human insulin seems to be nearly identical in both enzymes, it may be assumed that the human muscle extract would attack human insulin to a greater degree than it would attack human proinsulin. Finally, our preparation did not degrade human growth hormone in the concentrations tested.

Resumen

Se estudia la presencia de una posible enzima en el músculo esquelético humano, que degrada insulina a productos sin actividad biológica ni inmunológica. El extracto muscular presenta una K_m de 0,79 para insulina humana y 1,43 para proinsulina porcina es inactivada por N-etil-maleimida, por trasilol y por el calor. No requiere la presencia de glutation para ejercer su acción.

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