The Effects of Trypsin and Phospholipase C on Insulin Binding and Action in the Isolated Adipocyte

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The effect of alterations to the insulin receptor on the insulin sensitivity of isolated adipocytes was studied. Receptor changes were induced by treatment of adipocytes with either phospholipase C or trypsin. After enzyme treatment, binding of insulin to insulin receptors and insulin-mediated glucose metabolism were examined. Exposure of adipocytes to phospholipase C (2 units/ml) significantly increased insulin binding to the cells, but destroyed the ability of the cells to oxidize glucose. After treatment with trypsin (500 µg/ml) for 5 min, insulin binding to the adipocytes was significantly increased. This was shown to be due to an increase in insulin-receptor affinity. Metabolic studies showed that trypsin treatment led to an increase in basal glucose transport but markedly decreased the response to insulin at all concentrations tested. Adipocytes treated with trypsin showed no significant difference in basal glucose oxidation rates when compared with controls, but were less sensitive to insulin at low insulin concentrations, and showed a decreased maximum response at high insulin concentrations. In conclusion, these findings indicate a dissociation between induced changes in binding of insulin to insulin receptors and subsequent hormone action. The importance of post-receptor events in the biological action of insulin is highlighted.

In recent years it has been shown that alterations in insulin sensitivity may be caused by changes occurring at the level of the insulin receptor. These changes may involve alterations in receptor affinity (Muggeo *et al.*, 1977; Soman *et al.*, 1978), receptor number (Soll *et al.*, 1975; Harrison *et al.*, 1976) or both (Bar *et al.*, 1976; Kahn *et al.*, 1977), associated with a variety of effects at later metabolic steps. The mechanisms involved in these alterations are not clearly understood.

The present study was designed to examine the effect of induced changes in binding of insulin to insulin receptors on the metabolic effects of the hormone. Our previous studies have shown that both phospholipase C and trypsin have significant effects on specific insulin binding to receptors on particulate placental membranes (Clark *et al.*, 1978). In the present paper these observations have been extended to an insulin-responsive tissue, the isolated adipocyte. Insulin binding and subsequent stimulation of both glucose transport and glucose oxidation have been well documented in this tissue (Cuatrecasas, 1971a; Kono & Barham, 1971; Gliemann *et al.*, 1975a). Here, the effects of phospholipase C and trypsin treatment of adipocytes on

insulin binding and on the subsequent metabolic response of the cells to insulin were determined.

Materials and Methods

Crude collagenase (EC 3.4.24.3) (125 units/mg) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Phospholipase C (EC 3.1.4.3) (400 units/mg; from *Bacillus cereus*) was purchased from Boehringer Mannheim (Australia) Pty. Ltd., Mount Waverley, Vic., Australia. Trypsin (EC 3.4.21.4) (200 Bz-Arg-OEt units/mg; from ox pancreas) was purchased from BDH Chemicals, Poole, Dorset, U.K. Soya-bean trypsin inhibitor (1 mg inhibited 10000 Bz-Arg-OEt units of trypsin activity) and bovine serum albumin (Cohn fraction V) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pig monocomponent insulin was a gift from the Novo Research Institute, Bagsvaerd, Denmark. All radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation and enzyme treatment of isolated adipocytes

Male Sprague-Dawley rats fed *ad libitum* and weighing 200–220 g were used for the preparation of

isolated adipocytes. Rats were killed by cervical spinal dislocation; the epididymal fat-pads were removed, rinsed and incubated at 37°C with shaking by the method of Rodbell (1964). The incubation medium was Dulbecco buffer, pH 7.4 (Dulbecco & Vogt, 1954), containing collagenase (3 mg/ml) and albumin (40 mg/ml). After 60 min the cells were filtered, centrifuged at 500g for 1 min and washed twice in buffer.

In the experiments involving enzyme treatment, adipocytes (50–75 mg of lipid/ml) were preincubated with phospholipase C (2 units/ml) or trypsin (500 µg/ml) at 37°C for 15 min and 5 min respectively. The reactions were stopped either by washing (phospholipase C) or by addition of soya-bean trypsin inhibitor (200 µg/ml) as described previously for the particulate placental membranes (Clark *et al.*, 1978). The adipocytes were then used in the various experiments as described below.

Insulin-binding studies

[¹²⁵I]Iodinated pig monocomponent insulin (100–150 µCi/µg) was prepared by the method of Roth (1975), as previously described (Clark *et al.*, 1978). Adipocytes suspended in Dulbecco buffer, pH 7.4, containing albumin (10 mg/ml) were incubated with a tracer amount of ¹²⁵I-labelled insulin (approx. 20 000 c.p.m., equivalent to 60 pg of insulin) in the presence or absence of an excess (10 µg) of unlabelled insulin in a total volume of 1 ml. After 30 min at 24°C, cell-bound ¹²⁵I-labelled insulin was separated from free ¹²⁵I-labelled insulin as described by Gammeltoft & Gliemann (1973); 300 µl of cell suspension was layered over 100 µl of the oil dinonyl phthalate, in plastic Microfuge tubes. After centrifugation for 30 s in a Beckman Microfuge, the tubes were cut through the oil layer and the bound and free radioactivities were measured in an Autogamma spectrometer (Packard Instrument Co.). The non-specific binding (¹²⁵I-labelled insulin bound in the presence of 10 µg of unlabelled insulin) was subtracted from the total binding to obtain the specifically bound ¹²⁵I-labelled insulin (De Meyts, 1975). Non-specific binding was always less than 20% of the total binding. The binding of ¹²⁵I-labelled insulin was assessed in the presence of unlabelled insulin over the concentration range 1–100 ng/ml. These results were plotted in accordance with the method of Scatchard (1949).

Glucose transport

Glucose transport was measured as described by Olefsky (1975). Briefly, control and trypsin-treated cells were incubated at 24°C with or without insulin (1–10 ng/ml) in Dulbecco buffer, pH 7.4, containing albumin (10 mg/ml). After 45 min 2-deoxy[1-³H]glucose was added at a total 2-deoxyglucose concentration of 0.14 mM, and transport was measured over

a 5 min period. The assay was terminated by centrifuging a sample of the cell suspension through dinonyl phthalate as described above. The cell layer was readily dissolved in the scintillation fluid [toluene/Triton (100:19, v/v) containing 0.4% 2,5-diphenyloxazole and 0.04% 1,4-bis-(5-phenyloxazol-2-yl)benzene] and its radioactivity counted in a liquid-scintillation spectrometer (Packard Instrument Co.). The amount of sugar trapped in the extracellular fluid was measured by using [³H]inulin by the method of Gliemann *et al.* (1972); this ranged from 1% to 5% of the total sugar uptake. All results have been corrected for this factor. L-[¹⁴C]Glucose was used to test the specificity of the transport system.

Glucose oxidation

The ability of control, phospholipase C- and trypsin-treated adipocytes to oxidize glucose was measured by the method of Rodbell (1964). The cells were incubated at 37°C for 2 h, with shaking, in Dulbecco buffer, pH 7.4, containing albumin (40 mg/ml), with or without insulin (0.04–10 ng/ml). D-[U-¹⁴C]Glucose was added at the start of the incubation in a final glucose concentration of 1 mM. The rate of glucose oxidation was constant over the 2 h period, and at the end of the incubation the generated CO₂ was collected in Hyamine 10X (Packard Instrument Co.) and its radioactivity counted in a liquid-scintillation spectrometer.

Insulin degradation

Degradation of ¹²⁵I-labelled insulin by control, phospholipase C- and trypsin-treated adipocytes was measured by incubating these with ¹²⁵I-labelled insulin at 24°C for various times in a total volume of 1 ml. Then 1 ml of 10% (w/v) trichloroacetic acid was added to the incubation mixtures and the resultant precipitate was separated by centrifugation. The radioactivity remaining in the supernatant was taken to represent degraded ¹²⁵I-labelled insulin.

Specific insulin binding and metabolic activity were measured quantitatively on the basis of the lipid content of the fat-cells as determined by the method of Bligh & Dyer (1959).

Results

Effect of phospholipase C

As shown in Table 1, phospholipase C (2 units/ml) increased specific binding of ¹²⁵I-labelled insulin to the adipocytes by 96% above the control value ($P < 0.001$). However, at this concentration of phospholipase C there was no metabolic activity of the cells either basally or with a maximally stimulating dose of insulin. Treatment with phospholipase C had no effect on degradation of ¹²⁵I-labelled insulin by the adipocytes (results not shown).

Table 1. *Effect of phospholipase C treatment on specific binding of ¹²⁵I-labelled insulin and glucose oxidation by the adipocytes*

Adipocytes were treated with phospholipase C (2 units/ml) for 15 min at 37°C. Control and phospholipase-treated cells were used in (i) a binding assay or (ii) a glucose oxidation assay in the presence or absence of a maximally effective insulin concentration (10 ng/ml), as described in the text. The values shown are the means ± s.e.m. for triplicate determinations.

	Specific insulin binding (pg/ml of lipid)	Glucose oxidation (nmol of CO ₂ /h per 100 mg of lipid)	
		Basal	Insulin (10 ng/ml)
Control	1.17 ± 0.09	14.1 ± 2.3	40.5 ± 4.7
Phospholipase C (2 units/ml)	2.29 ± 0.16	0.2 ± 0.2	0.4 ± 0.4

Fig. 1. *Scatchard plot derived from equilibrium studies with control (●) or trypsin-treated (▲) adipocytes* Adipocytes were treated with trypsin (500 µg/ml) for 5 min at 37°C; at this time an excess of soya-bean trypsin inhibitor was added and a binding assay was performed with increasing concentrations of unlabelled insulin as described in the text.

Effect of trypsin

Exposure of adipocytes to trypsin at a concentration of 500 µg/ml for 5 min at 37°C led to a significant increase in specific binding of ¹²⁵I-labelled insulin of 20% (*P* < 0.001) at tracer insulin concentrations. The data from competition curves with increasing amounts of unlabelled insulin were used to construct a Scatchard plot. Fig. 1 shows that the increased insulin binding observed with trypsin-treated cells was associated with an increased mean slope of the Scatchard plot, indicating an increase in the apparent affinity of the receptors (De Meyts, 1975). The intercept of a Scatchard plot with the abscissa gives a measure of the number of receptor sites (De Meyts, 1975). By extrapolation of the curves in Fig. 1 it can be seen that there is also a fall in the number of receptors on the trypsin-treated cells. Owing to the difficulties in defining affinity

Fig. 2. *2-Deoxyglucose uptake by control (●) and trypsin-treated (▲) adipocytes*

Adipocytes were treated with trypsin (500 µg/ml) for 5 min at 37°C; at this time an excess of soya-bean trypsin inhibitor was added. The cells were then preincubated in the absence or presence of insulin (1–10 ng/ml) for 45 min at 24°C. 2-Deoxyglucose transport was measured as described in the text. Each point represents the mean ± s.e.m. for quadruplicate determinations from three separate experiments. Differences between the mean values were evaluated by Student's *t* test.

constants from curvilinear Scatchard plots (Clark *et al.*, 1978) an operational index of receptor affinity has been used. This is determined as the concentration of unlabelled insulin that displaces 50% of the specifically bound ¹²⁵I-labelled insulin. For the control adipocytes this value was 6.8 nM-insulin, compared with the trypsin-treated cells, where such displacement was produced by an insulin concentration of 1.3 nM.

Fig. 2 shows the effect of increasing insulin concentration on 2-deoxyglucose transport by control and trypsin-treated adipocytes. Trypsin treatment led to a significant increase in basal 2-deoxyglucose transport, from 1.82 to 2.25 nmol/5 min per 100 mg of lipid (*P* < 0.02). However, trypsin treatment markedly decreased the cells' response to insulin at

all concentrations tested. At an insulin concentration of 10 ng/ml, transport in control cells showed an increase of 90% over basal values compared with an increase of only 23% in trypsin-treated cells.

There was no transport of L-[^{14}C]glucose by either the control or the trypsin-treated cells (results not shown). This indicates that the transport mechanism remained specific for the natural D-isomers even after the treatment with trypsin.

Trypsin treatment had no significant effect on basal glucose oxidation rates (Fig. 3). However, the trypsin-treated cells were less sensitive to very low insulin concentrations (0.04–0.4 ng/ml), as shown by the slight shift to the right in the dose-response curve. In addition to this decreased sensitivity to insulin, the response to maximal doses of insulin was significantly less in the trypsin-treated cells ($259 \pm 19\%$ of basal compared with $375 \pm 21\%$ for control cells; $P < 0.001$).

Fig. 4 shows the insulin-binding data for the control and trypsin-treated cells plotted as a function of the insulin concentration. On comparing this graph with Figs. 2 and 3, it can be seen that specific binding of ^{125}I -labelled insulin to the trypsin-treated adipocytes was significantly higher than to control cells at insulin concentrations up to approx. 4 ng/ml. This insulin range includes the complete concentration-response curve for both 2-deoxyglucose transport (Fig. 2) and glucose oxidation (Fig. 3), both of

which show a maximum response at an insulin concentration of approx. 2 ng/ml.

The results with trypsin cannot be explained on the basis of an effect of trypsin-treated cells on insulin degradation. Table 2 shows that over a 2 h time period there was no significant difference in degradation of ^{125}I -labelled insulin by control or trypsin-treated adipocytes.

Fig. 4. Effect of trypsin treatment on specific binding of ^{125}I -labelled insulin at various insulin concentrations. Adipocytes were treated with trypsin (500 $\mu\text{g}/\text{ml}$) for 5 min at 37°C , at which time an excess of soya-bean trypsin inhibitor was added. Control ($\bullet$) and trypsin-treated ($\blacktriangle$) adipocytes were incubated with increasing concentrations of unlabelled insulin (1–10 ng/ml) as described in the text. Each point represents the mean $\pm$ S.E.M. for triplicate determinations from two separate experiments. Differences between the mean values were evaluated by Student's t test.

Fig. 3. Ability of control ($\bullet$) and trypsin-treated ($\blacktriangle$) adipocytes to oxidize glucose

Adipocytes were treated with trypsin (500 $\mu\text{g}/\text{ml}$) for 5 min at 37°C , at which time an excess of soya-bean trypsin inhibitor was added. Incubations with D-[U- ^{14}C]glucose were performed for 2 h at 37°C in the absence or presence of insulin (0.04–10 ng/ml) as described in the text. Each point represents the mean $\pm$ S.E.M. for triplicate determinations for six separate experiments. Differences between the mean values were evaluated by Student's t test.

Table 2. Degradation of ^{125}I -labelled insulin by control and trypsin-treated adipocytes

Adipocytes were treated with trypsin (500 $\mu\text{g}/\text{ml}$) for 5 min at 37°C , at which time an excess of soya-bean trypsin inhibitor was added. Degradation of ^{125}I -labelled insulin by control or trypsin-treated cells was assessed by trichloroacetic acid precipitation of intact material at various times, as described in the text. The values shown are the means $\pm$ S.E.M. for triplicate determinations.

Time (min)	^{125}I -labelled insulin degraded (%)	
	Control	Trypsin-treated (500 $\mu\text{g}/\text{ml}$)
0	7.8 ± 1.2	8.8 ± 0.9
30	21.8 ± 2.3	19.7 ± 2.0
60	32.6 ± 2.5	29.3 ± 2.4
120	43.1 ± 2.9	42.1 ± 3.0

Discussion

The increases observed in specific insulin binding to adipocytes after either phospholipase C or trypsin treatment are qualitatively similar to those previously obtained for insulin binding to particulate placental membranes (Clark *et al.*, 1978). Moreover, the increase in binding of insulin to receptors on exposure of the adipocytes to trypsin has been shown to be due to an increase in the overall affinity of the receptor for insulin (Fig. 1); again this effect is the same as previously reported for the insulin receptor from particulate placental membranes. Although the affinity of the receptors is increased, giving rise to an increase in insulin binding at low insulin concentrations, the number of available receptors is decreased by about 50%.

Phospholipase C totally inhibited glucose oxidation by the adipocytes (Table 1), a result that has previously been demonstrated by Cuatrecasas (1971c). He found that the suppression of glucose oxidation occurs at very low phospholipase C concentrations that do not stimulate insulin binding; thus a complete dissociation of binding and biological action occurs. The mechanism of this action of phospholipase C is unclear.

Stimulation of glucose transport in the absence of insulin may reflect an interaction of trypsin with the insulin receptor, perhaps by an effect on the membrane surrounding the receptor, as postulated previously (Clark *et al.*, 1978). However, a direct effect of trypsin on the glucose-transport unit itself cannot be excluded. Recently it has been proposed that insulin acts on glucose transport by activating basally inactive transport units (Wardzala *et al.*, 1978). It is possible that the stimulation of glucose transport after trypsin treatment and in the absence of insulin may be due to a direct effect of trypsin on these basally inactive transport units. The decreased response to subsequent insulin stimulation suggests that trypsin leads to a dissociation between insulin binding and glucose transport.

Kono (1969) found that a very short incubation (1 min) of adipocytes with trypsin caused a marked stimulation of glucose oxidation in the absence of insulin, an insulin-like effect. Over a longer time period (15 min) basal glucose oxidation was decreased, as was insulin-stimulated oxidation. In the present study trypsin did not appear to have an insulin-like effect on glucose oxidation; although basal glucose transport was increased by treatment with trypsin, there was no significant effect on basal glucose oxidation. At low concentrations of insulin (<0.4 ng/ml) the trypsin-treated adipocytes were less sensitive to insulin, and at higher concentrations (>2 ng/ml) they showed a decreased maximal response compared with controls. Over both concentration ranges insulin binding to the trypsin-

treated cells was greater (Fig. 4), and thus a change in receptor number cannot explain the decreased maximal response. Theoretically, even at infinitely high concentrations that completely saturate the insulin receptors, and where binding is diminished in the trypsin-treated cells, the fall in receptor number on trypsin treatment is not sufficient to cause a decrease in the maximum glucose oxidation rate because of the implications of the spare receptor hypothesis (Gliemann *et al.*, 1975b). It is possible that the lowered maximum glucose oxidation rate may simply reflect the lowered maximum glucose transport rate.

Previous reports (Cuatrecasas, 1971b; Kono & Barham, 1971) on trypsin treatment of adipocytes have always shown a marked decrease in insulin binding to adipocyte receptors, but the activity of the trypsin used was not stated. Similar results have been obtained in the present study, with higher trypsin concentrations causing a decrease in the binding of tracer amounts of ¹²⁵I-labelled insulin to the receptor (results not shown). However, it should be noted that in trypsin-treated adipocytes all the alterations observed in both glucose transport and glucose oxidation occurred at insulin concentrations where the binding of the hormone to the trypsin-treated cells was significantly increased (Fig. 4).

From the overall results it can be seen that simply inducing an increase in insulin binding does not necessarily lead to an enhancement of later metabolic steps. There have been several reports in the literature demonstrating a dissociation between effects on insulin binding and subsequent insulin action. Studies of the effects of starvation and re-feeding *in vitro* in rats (Kasuga *et al.*, 1977; Olefsky, 1976a) and *in vivo* in normal human subjects (De Fronzo *et al.*, 1978) have shown that, in general, changes in insulin binding do not reflect changes in insulin action. Although insulin binding increases on starvation, insulin-stimulated glucose oxidation shows a decrease in both sensitivity and maximum rate (Olefsky, 1976a; De Fronzo *et al.*, 1978). A similar result was obtained by Beck-Nielsen (1978) for diabetic obese human subjects. In streptozotocin-diabetic rats, Kobayashi & Olefsky (1979) found an increase in insulin binding and insulin sensitivity of glucose transport but a decrease in the maximum glucose transport rate and an overall decrease in glucose oxidation. However, Le Marchand *et al.* (1976) found that streptozotocin treatment of *ob/ob* mice had no effect on the insulin sensitivity of liver or adipose tissue even though insulin binding was increased. Overall in these studies changes in insulin responsiveness and sensitivity do not reflect the alterations in binding of insulin to receptors. In general it has been found that glucose transport and insulin binding are more closely linked and that a dissociation occurs between insulin binding and glu-

cose oxidation (Olefsky, 1976b). As shown in the present study, trypsin treatment causes an additional dissociation between insulin binding and insulin action on glucose transport.

In conclusion, it can be seen that trypsin can cause a stimulation in insulin binding to adipocytes by increasing insulin-receptor affinity. However, this leads not to a subsequent stimulation of glucose transport or oxidation but rather to a dissociation between binding and post-receptor events. These findings highlight the importance of post-receptor events in the biological action of insulin. Trypsin treatment may prove a useful tool for studying processes involved in the coupling of insulin-receptor binding to insulin action.

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