Receptor Binding and Biological Effect of Insulin in Human Adipocytes

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Summary. The binding of ^{125}I -labelled insulin to human adipocytes was studied at 37°C . The precipitability of the ^{125}I -labelled insulin preparation (0.03 nmol/l) in trichloroacetic acid and the concentration of biologically active insulin (7.5 nmol/l) remained constant in buffer incubated with human adipocytes (100 μl cells/ml suspension) for 30–60 minutes at 37°C , whereas more than half of the insulin was inactivated by rat fat cells under the same conditions. A constant level of binding of ^{125}I -labelled insulin (0.03 nmol/l) to human adipocytes was obtained after 45 minutes. The apparent dissociation constant of receptor binding was about 0.2 nmol/l as compared to about 2 nmol/l for rat adipocytes. Conversion of $[\text{U-}^{14}\text{C}]\text{glucose}$ to lipids was stimulated half-maximally by about 0.05 nmol/l of insulin (similar to rat adipocytes). Thus, half-maximal stimulation of human adipocytes was obtained with a receptor occupancy of about 20–30 per cent.

Key words: Insulin receptors, human adipocytes, insulin degradation, lipogenesis.

Insulin-receptor studies in man have in most cases been carried out using circulating mononuclear cells as the receptor source. However, Olefsky et al. [1] and Amatruda et al. [2] have studied the binding of insulin to one of its target cells, the adipocyte, using the oil separation method [3]. Furthermore, Olefsky [4] recently compared insulin binding to mononuclear cells and isolated adipocytes from the same individuals and found a significant, although weak, positive correlation ($r = 0.53$).

Receptor binding of insulin is often measured at a relatively low temperature in order to minimize

degradation of hormone. The studies cited above were carried out at 24°C where no effect of insulin on glucose metabolism has been demonstrated in human adipocytes. Insulin stimulates the conversion of ^{14}C -labelled glucose to lipids (glyceride-glycerol) in isolated adipocytes from normal adults at 37°C , although the effect is much smaller than that observed in cells from young rats [5]. We therefore decided to study the binding of insulin to human adipocytes at 37°C and compare the binding with the effect of insulin on the conversion of glucose to lipids.

Material and Methods

The patients were 17 non-diabetic, non-obese males (35–69 years, $101.8 \pm 3.9\%$ ideal body weight: mean $\pm$ SEM), who underwent surgery for gastric or duodenal ulcer. Adipose tissue was taken at the beginning of the operation from the greater omentum and within 10 minutes transported to the laboratory in Krebs-Ringer bicarbonate buffer (pH 7.4) containing albumin (10 mg/ml) and glucose (0.55 mmol/l) at 37°C . The same buffer was used throughout the experiment. Human adipocytes were prepared by incubation of carefully minced fat tissue with collagenase (0.5 mg/ml) for 90 min at 37°C . The isolated cells were washed five times. The diameters of 100 cells from each donor were measured after fixation with osmium tetroxide as described previously [6]. The means ranged from 59 to 118 μm , median 88 μm , $n = 12$.

The isolated adipocytes were incubated in 2 ml buffer (2% v/v) at 37°C . In the receptor studies the cells were incubated for 45 min with ^{125}I -labelled insulin and porcine insulin in the concentrations indicated in Figure 2. In the lipid synthesis studies the

cells were incubated for 120 minutes with [U - ^{14}C]-glucose or in a few experiments with [3 - 3H]glucose (both approximately 0.25 $\mu Ci/ml$) and porcine insulin as indicated in Figure 3. When receptor studies and lipid-synthesis studies were to be compared, the cells were taken from the same suspension of washed cells and the incubations were carried out in parallel. All incubations were carried out in duplicate and terminated by removing 3 aliquots of 300 μl from the suspension followed by centrifugation of the cells through silicone oil as described before [3, 7]. Lipids were extracted by the addition of scintillation fluid directly to the incubation vial [9].

The rats (male Wistar, specific pathogen-free) were fed ad libitum and weighed 100–150 g when used. The isolation of rat adipocytes from the epididymal fat pads using collagenase (0.5 mg/ml) and the bioassay of insulin using [3 - 3H]glucose have been published in detail before [8, 9].

Measurement of radioactivity followed previously published procedures [3, 7, 9]. ^{125}I -activity in the cell pellets and in aliquots of the medium was measured in a Selectronic gammacounter with an efficiency of 55% and a background of 2.5 counts per minute. At least 1000 counts were accumulated.

Porcine insulin (about 27 U/mg) and ^{125}I -labelled insulin (about 30 $\mu Ci/\mu g$) were obtained from NOVO. The properties of this ^{125}I -labelled insulin preparation have been described previously [7]. Crude collagenase (type II) and bovine serum (fraction V) were from Sigma. [U - ^{14}C]glucose and [3 - 3H]glucose were from the Radiochemical Centre, Amersham. Silicone oil was from Midland Silicones (MS 200, specific gravity 0.97, viscosity 500 centistokes).

Results

Binding of Insulin as a Function of Time

The precipitability of the ^{125}I -labelled insulin preparation (0.03–0.6 nmol/l) in trichloroacetic acid remained nearly constant in the experiments with human adipocytes described below (range 95–98%). Since insulin is rapidly degraded by incubation with rat adipocytes at 37°C [7], we compared the rate of degradation of insulin in a suspension of human adipocytes at 37°C with that in a suspension of rat adipocytes. Figure 1A shows that the concentration of biologically active insulin (7.5 nmol/l) remained constant for up to 60 minutes in the concentrated suspension of human adipocytes (10% v/v), whereas the concentration of biologically active insulin decreased to about 65% after 30 min of incubation with rat adipocytes. It should be noted that the ap-

parent K_m for insulin degradation by a suspension of rat adipocytes in Krebs-Ringer bicarbonate buffer with 10 mg of albumin per ml was about 300 nmol/l (7).

Figure 1B shows the binding of 0.03 nmol/l ^{125}I -labelled insulin as a function of time in the absence and the presence of 1 $\mu mol/l$ unlabelled insulin. The binding is expressed as ^{125}I -activity per 1 cells/ ^{125}I -activity per 1 medium (B/F). It appears that a steady state of binding was obtained after about 45 minutes and maintained until 120 min. Similar results were obtained at 22°C, although about 90 minutes were required to achieve steady state of binding (data not shown). No change was found in the amount of insulin bound after 45 minutes at 37°C when studies were performed with isolated cells from one donor after incubation with collagenase for 90, 120 and 150 min, respectively.

Binding at Steady State

Figure 2A shows the concentration dependence of binding after 45 min of incubation at 37°C. The curve is the best fit according to the hypothesis that the total binding is accounted for by a binding to one homogeneous group of receptors plus a non-specific binding (cf. text to Figure 2A). In 15 experiments with adipocytes from 15 persons the median apparent K_d for receptor binding was 0.16 nmol/l, as calculated from the computerized fit (lower and upper quartiles 0.10 and 0.23 nmol/l). The fraction of ^{125}I -labelled insulin bound to the cells was always less than 1% of the total amount present. The concentration of insulin receptors was: Median 3.8×10^{-2} nmol/l cells, lower and upper quartiles 2.7×10^{-2} and 5.2×10^{-2} ($n = 15$). The number of cells per incubation vial was calculated in 12 experiments and the amount of specific binding sites per cell was: Median: 7.4×10^3 , lower and upper quartiles: 5.0×10^3 and 11.7×10^3 .

Figure 2B shows that the maximal binding was increased at 22°C (120 min incubation). There was no significant difference between the apparent K_d calculated at the two temperatures.

Relationship between Binding and Action

No 3H -activity was incorporated into the lipids of human adipocytes when [3 - 3H]glucose was used as substrate (data not shown). Figure 3 shows the insulin dose-response relationship of receptor binding (A) and the conversion of [U - ^{14}C]glucose to lipids (B) in cells from the same person. In this experiment the apparent K_d was 0.21 nmol/l and the insulin concentration needed for halfmaximal stimulation of the incorporation of [^{14}C]glucose into lipids

Fig. 1. A Time course of degradation of insulin by human ($\blacktriangle$ — $\blacktriangle$) and rat ($\triangle$ — $\triangle$) adipocytes. The human cells and the rat cells were prepared in parallel and incubated in concentrations of 100 μ l cells per ml of suspension. The concentration of insulin was 7.5 nmol/l. The samples were incubated for 0, 30 and 60 min and medium was removed after centrifugation of the cells through oil. The transferred aliquots of medium were diluted to give the concentrations 0.4, 0.2, 0.1, 0.05, 0.025 nmol/l if no degradation had occurred. The concentration of biologically active insulin was assessed by incubation with a dilute suspension of rat adipocytes (5 μ l/ml) using the conversion of [3- 3 H]glucose to lipids as response variable [9]. The ordinate indicates the concentration of biologically active insulin in per cent of that at zero-time (mean $\pm$ SEM, $n = 12$). **B** Time course of binding of 0.03 nmol/l 125 I-labelled insulin to human adipocytes at 37°C in the absence (4—4) and the presence (○—○) of 1 μ mol/l native insulin. The cell concentration was 20 μ l per ml of suspension. Aliquots of suspension were removed at the indicated times and the cells were centrifuged through oil. The ordinate, B/F, denotes the ratio 125 I-activity per 1 cells/ 125 I-activity per 1 medium. Thus, the fraction of the total amount of 125 I-labelled insulin (0.03 nmol/l) bound to the cells at steady state was about 0.005. All values were corrected for trapped medium which was assessed with L-[14 C]glucose as marker and amounted to 1.1 per cent of the cell volume, i.e. $B/F + 0.0011$. The points indicate the mean of 6 replicates $\pm$ SEM.

Fig. 2. Concentration dependence of binding at steady state. **A** Cells (2 per cent v/v) from one person were incubated either with 125 I-labelled insulin of constant specific activity from 0.01 nmol/l to 0.9 nmol/l (○—○) or with progressively decreasing specific activity from 0.09 nmol/l (●—●). The incubation time was 45 min at 37°C. The results are corrected for trapped medium, cf. Fig. 1B. The points indicate the mean of 6 values $\pm$ SEM. The curve was computed according to the hypothesis, that two types of binding occurred, one proportional to the concentration of insulin in the range 0.01 to 1000 nmol/l (non-specific binding) and on to a single group of homogeneous saturable binding sites (receptor binding). The calculation was carried out as described previously [7]. The apparent K_d for receptor binding was 0.10 nmol/l with the 95 per cent confidence limits 0.09 nmol/l to 0.12 nmol/l. **B** Cells (2 per cent v/v) from one person were incubated either at 22°C for 120 min (○—○, $K_d = 0.17$ nmol/l, 95 per cent confidence limits 0.15 nmol/l to 0.19 nmol/l) or at 37°C for 45 min (●—●, $K_d = 0.20$ nmol/l, 95 per cent confidence limits 0.17 nmol/l to 0.23 nmol/l).

Fig. 3. Binding and action of insulin. **A** The cells (2 per cent v/v) were incubated at 37°C with ¹²⁵I-labelled insulin and calculations were carried out as explained in the text to Fig. 2 ($K_d = 0.21$ nmol/l, 95 per cent confidence limits 0.18 nmol/l to 0.24 nmol/l). **B** Cells (2 per cent v/v) from the same donor as in A were incubated with [U-¹⁴C]glucose and insulin as indicated for 120 min at 37°C. Aliquots were taken as explained in the text to Fig. 2. ED_{50} was determined graphically as 0.03 nmol/l. The points represent the mean of 6 values $\pm$ SEM

(ED_{50}) was 0.03 nmol/l, i. e. halfmaximal activation was obtained with a receptor occupancy of about 12 per cent. In 15 experiments the median ED_{50} was 0.05 nmol/l (lower and upper quartiles 0.03 and 0.09 nmol/l). In 13 experiments the apparent K_d and ED_{50} were estimated on cells from the same individuals and the median occupancy at ED_{50} was 29 per cent (lower and upper quartiles 11 and 40 per cent).

Discussion

Binding and Degradation as a Function of Time

We have found that a steady state of insulin binding was obtained after about 45 min of incubation at 37°C and maintained until 120 min. The insulin concentration in the medium appeared to remain nearly constant. Furthermore, prolonged incubation of the cells with collagenase (from 90 to 150 mins) did not change the binding of insulin during a following 45 min incubation. It is therefore unlikely that marked changes in the receptor concentration occur within a 2 hour incubation period. The results are quite different from those obtained by Olefsky et al. [1] who found that binding decreased markedly from 10 min to 60 min of incubation at 37°C. These authors found a constant binding from 30 min to 45 min at 24°C followed by a decrease, even though insulin degradation was virtually absent at this temperature. We think that disruption of the cells is the most likely explanation for the observed decrease in binding since insulin bound to membranes or vesicles containing little or no fat will not be recovered in cell pellets after centrifugation through oil.

Terris and Steiner [10] have observed that about 40 per cent of the insulin bound to rat hepatocytes

at steady state (30°C) is subsequently degraded. A receptor-linked degradation of insulin also occurs in rat adipocytes even though this mechanism accounts for only a small fraction of the total insulin degradation in that system [11]. It should be noted that the total turnover on the human insulin receptors is so low that the present study does not rule out a receptor-linked insulin degradation. About 0.5 per cent of the insulin was bound to the cells at steady state, cf. legend to Figure 1B. Assuming a dissociation rate constant of 0.07 min^{-1} , as has been found for rat adipocytes [7], the total turnover amounts to about 2 per cent per hour of the insulin present in the incubation vial, i. e. a receptor-linked degradation would hardly be measurable even if all of the label dissociated from the cells as degradation products.

Binding at Steady State

The curves fitted to the results are computed according to the assumption that only one group of saturable binding sites exists. It appears that such a curve can be fitted quite well to the data from a particular experiment. However, in all experiments less ¹²⁵I-labelled insulin than expected was bound in the presence of 1 $\mu\text{mol/l}$ unlabelled insulin, suggesting some heterogeneity of the binding sites or negative homotropic cooperativity (cf. Figures 2 and 3A). We have not performed Scatchard analysis due to the uncertainty about which value to subtract for non-specific binding. It should also be noted that the binding is not well-described at the very low insulin concentrations where receptor binding should be proportional to the insulin concentration. In any case, the concentration of insulin which gives half of the maximal B/F-ratio is very close to the calculated apparent K_d , i. e. about 0.2 nmol/l

both at 37°C and 24°C. This is one order of magnitude lower than the value reported by Olefsky et al. [1] and Olefsky [4]. The reason for this discrepancy is not clear, although it may be pointed out that incubation for 45 min at 24°C as employed by Olefsky et al. seems too short to ensure a steady state at the low insulin concentrations. This will tend to increase the apparent K_d . Recently, Harrison et al. [12] studied insulin binding to adipocytes from obese patients at 24°C. The binding curves obtained after incubation for 30 min were concave upwards when analysed according to Scatchard. The dissociation of ^{125}I -label from the cells was accelerated by native insulin (83 nmol/l) suggesting negative homotropic cooperativity. The insulin concentration causing 50 per cent inhibition of specific ^{125}I -labelled insulin binding was about 1.3 nmol/l. However, the halftime of dissociation of ^{125}I -label from the cells at 24°C in the absence of unlabelled insulin in the washout medium exceeded 60 min. Therefore, steady state of binding cannot have been achieved after incubation for 30 min.

Relationship between Binding and Action

The receptor binding of insulin in rat adipocytes exhibits an apparent K_d of about 3 nmol/l, whereas the conversion of $[3\text{-}^3\text{H}]\text{glucose}$ or $[\text{U-}^{14}\text{C}]\text{glucose}$ [7, 8] to lipids is stimulated half-maximally with about 0.06 nmol/l of insulin (ED_{50}). We observed that no ^3H -activity was incorporated into lipids of human adipocytes when $[3\text{-}^3\text{H}]\text{glucose}$ was used as substrate. In rat adipose tissue the ^3H -atoms are predominantly incorporated into fatty acids via labelled NADPH formed in the pentose cycle [13]. However, Goldrick [5] previously suggested, on the basis of studies on the metabolism of $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$, that the pentose cycle activity is virtually absent in human adipocytes and this is probably the reason for the lack of incorporation of ^3H -atoms from $[3\text{-}^3\text{H}]\text{glucose}$. $[\text{U-}^{14}\text{C}]\text{glucose}$ is incorporated in the glycerol moiety of triglycerides in human fat cells and this process is stimulated by insulin [5]. We do not know the mechanism of this biological effect of insulin and particularly whether transmembrane glucose transport is rate determining for the glucose metabolism in human adipocytes under the conditions employed. ED_{50} was 0.05 nmol/l for human adipocytes, which is very close to the value obtained for rat adipocytes. In the experiment shown on Figure 3 the vials containing ^{125}I -labelled insulin were incubated for 45 min, whereas those containing $[\text{U-}^{14}\text{C}]\text{glucose}$ were incubated for 120 min with insulin present from time zero. Otherwise, the vials were identical. In some previous experiments with rat adipocytes we have added the

labelled glucose when insulin binding was at steady state [7]. However, the addition of labelled glucose at time zero causes only a very small change in ED_{50} , when the total incubation time is 120 min. In rat adipocytes ED_{50} is achieved with a receptor occupancy of about 2 per cent [7], whereas the present data indicate that a considerably higher occupancy is required in human adipocytes to cause half of the maximal incorporation of glucose carbon into lipids. Thus, the phenomenon of "excess receptors" seems less marked in adipocytes from adult humans than in adipocytes from young growing rats.

Acknowledgements. This study was supported by grants from Nordic Insulin Foundation, The Danish Diabetes Association and Johann and Hanne Weimann's legat.

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Received: February 24, 1977, and in revised form June 10, 1977

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