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The Monocyte as a Model for the Study of Insulin Receptors in Man

H. Beck-Nielsen, O. Pedersen, K. Kragballe, and N. Schwartz Sørensen

Medical Department III and II and Department of Clinical Chemistry, County Hospital of Aarhus, Aarhus, Denmark

Summary. We have characterized the cellular composition of preparations isolated from peripheral blood by Ficoll-Isopaque gradient centrifugation. ^{125}I-insulin binding to every cell type was measured. A highly significantly positive correlation between specific cell binding fraction and the monocyte concentration of the heterogeneous cell suspension was demonstrated. Depletion of monocytes reduced the insulin binding approximately 80%, which confirms previous findings by other investigators. The granulocytes possessed the second highest binding ability, but only one fourteenth of that of monocytes. Compared to the lymphocyte the monocyte had about 25 times greater insulin binding. Also thrombocytes bound insulin and contamination with these meant that their contribution to the total specific cell binding was not negligible. A reduction in these contaminants is essential. We found that insulin binding to erythrocytes was insignificant. A method of calculating the specific insulin binding to monocytes alone is introduced. The monocyte-insulin-receptor possesses specificity. Only an insignificant degradation of receptor bound insulin could be shown. Evidence of negative cooperativity between receptors was found. Consequently monocytes are considered a useful model for insulin receptor studies in man.

Key words: Insulin receptor, monocytes, thrombocytes, negative cooperativity, insulin degradation.

In the last few years populations of mononuclear cells isolated from peripheral blood have been used extensively for the study of insulin binding to its receptor. In comparative studies of lymphocytes, granulocytes and erythrocytes isolated by Ficoll-Hypopaque gradient centrifugation, Gavin et al. [1] found the highest binding to lymphocytes. However, no significant insulin binding to lymphocytes purified by passage over nylon wool could be shown [2]. Recently Schwartz et al. [3] demonstrated that about 90% of the specific insulin binding of a preparation of mononuclear leucocytes could be accounted for by its content of monocytes. Therefore, an ideal cell preparation for the study of insulin receptor-binding would be a purified, highly concentrated suspension of monocytes. Because of technical difficulties in obtaining pure monocyte preparations, however, a composite population of monocytes and lymphocytes with a small admixture of granulocytes, erythrocytes and platelets is still used.

We have characterized the cellular composition of mononuclear cell preparations derived from Ficoll-Isopaque gradients and we have measured insulin binding to the different cell types using another approach than the one employed by Schwartz et al. [3]. The results of our studies have enabled us to elaborate a method of calculating the amount of insulin bound to the monocytes alone. The present study was designed to examine the specificity of the receptor, the possibility of site to site receptor interaction and insulin degradation in the medium and at the receptor site.

Materials and Methods

Insulin

In the comparative studies of insulin binding to different cell types, ^{125}I-insulin with a specific activity of about 70 μCi/ug (Nordisk Insulin Laboratorium, Copenhagen) was used. ^{125}I-insulin with a specific

activity of about 25 μCi/ug (Novo Research Institute) was employed to measure insulin binding to monocytes in a group of 25 normal subjects.

Preparation of Cells

Monocytes, lymphocytes, erythrocytes and granulocytes were isolated from peripheral blood drawn from a cubital vein after an overnight fast. The blood was collected in tubes containing EDTA (dipotassium salt) and the cells were fractionated on Ficoll-Isopaque (Nygård, Oslo) gradients according to Boyum [4]. With this technique the interface between the Ficoll and the plasma contains the lymphocytes and the monocytes while the erythrocytes and the granulocytes are spun to the bottom of the tubes. The isolated cells were resuspended in a 25 mmol/l Tris-HCl buffer, pH 8.0 at 15° C [5].

Monocytes and lymphocytes were isolated together and resuspended in the buffer to a final concentration of 70×10^6 per ml. The monocytes were identified by morphological and cytochemical criteria in cytocentrifuged smears using an alpha naphthyl acetate esterase method [6]. B lymphocytes carry receptors for the activated part of complement C_3. Binding of sheep erythrocytes sensitized with antisheep IgM and complement C_3 was used to identify these cells [7]. T lymphocytes were detected by sheep erythrocyte rosette formation [7].

Insulin binding to lymphocytes was studied after removal of monocytes. Freshly isolated mononuclear leucocytes were suspended in 10 ml Hanks balanced salt solution and 1 ml fetal calf serum; 0.2 g carbonyl iron powder (Grade SF, GAF comp., New York) was added, and the mixture was incubated at 37° C for 30 min. The iron particles and the phagocytic monocytes were removed with a magnetic stick [8].

Erythrocytes were isolated from the bottom of the tubes. Twenty μl cell suspension was diluted with 1 ml saline and washed twice. Erythrocytes were then resuspended to a final concentration of about 100×10^6 per ml. The granulocyte content was insignificant.

Granulocytes were likewise isolated from the bottom of the tubes. After mononuclear leucocytes had been harvested and the Ficoll layer had been removed, cells at the bottom of the tubes were washed 3 times with Hanks balanced salt solution [9]. Five ml plasma and 4 ml 6% isotonic dextran were added to 10 ml cell suspension. After incubation at 37° C for 45 min in a tube tilted 45°, the erythrocytes settled and the plasma layer containing the granulocytes was collected. The final concentration was 10×10^6 per ml.

Platelets were isolated from acid-citrate-dextrose blood on the Blood Bank and Blood Grouping Laboratorium, University of Aarhus [10]. The platelet suspension was adjusted to a concentration of about 100×10^6 per ml.

Binding Studies

All cell types were incubated in duplicate with ^{125}I-insulin at a concentration of 172 pmol/l in Tris-HCl buffer (pH = 8.0) at 15° C for 100 min [5]. Changes in pH in the medium during the 100 min incubation were less than 0.1 pH unit. The cell concentration for each cell type is given in legends to figures. Insulin binding to erythrocytes, lymphocytes, granulocytes and platelets was measured only at tracer concentration, 172 pmol/l, whereas a competition study was performed for ^{125}I-insulin binding to monocytes by adding increasing concentrations of native insulin to the incubation medium. At the end of the incubation period cell-bound and free insulin were separated by centrifugation through silicone oil (density = 1.04) [11], except for platelets which could not sediment through oil. These were isolated from the incubation medium by centrifugation through aqueous buffer following the method of Rodbell [12]. "Specific cell binding fraction" is defined as total binding fraction minus non-specific binding fraction. Radioactivity which remained bound in the presence of an excess of native insulin at 7000 nmol/l was considered "non-specific". The non-specific cell-binding fraction for each cell type is given in the legends.

Cell Binding Analysis

The results of binding studies are presented in four ways 1) The specific cell binding fraction (bound/total insulin) measured at tracer concentration (172 pmol/l). 2) The specific cell binding fraction plotted as a function of total insulin concentration (competition curve). 3) Bound/free (B/F) insulin plotted as a function of bound (B) insulin (Scatchard plot) [13]. The validity of this plot has previously been discussed [5]. From the intercept on the x-axis the total receptor concentration per cell is derived (R_o). 4) De Meyts et al. [14] have shown that the average affinity constant ($\overline{K}_i$), at any point, i, on the Scatchard curve can be calculated from the formula:

$\overline{K}_i = \frac{(B/F)_i}{R_o - B_i}$; $K_e = (\overline{K}_{empty})$ represents the affinity of the empty sites. In this study we have designated $\overline{K}_e$ as the value of K at the tracer concentration used, 172 pmol/l.

Studies of Receptor Cooperativity. Using the method described by de Meyts et al. [15] we have estimated

the possibility of site to site receptor interaction. After incubation cells were sedimented at 4° C and the supernatant was replaced by an equal aliquot of chilled buffer. The cells were resuspended and samples of 150 µl were transferred to tubes containing 10 ml Tris-HCl buffer, 1% bovine serum albumin. Native insulin at 1.7×10^{-7} mol/l was added to half of the tubes. Tubes were then incubated at 15° C. At the times indicated two tubes of each set were centrifuged, and the radioactivity of the cell pellet was determined. The radioactivity of the cells as a percentage of the value present at t = 0, was plotted as a function of time.

Degradation Studies. The integrity of ^{125}I-insulin recovered from the cells was estimated by 10% trichloroacetic acid (TCA) precipitation of the diluting medium in the dissociation experiments described above. ^{125}I-radioactivity was measured both in the TCA precipitate and in the supernatant. All studies were done in duplicate.

The degradation of ^{125}I-insulin in the incubation medium of receptor experiments was measured in the same manner.

Statistical Methods

For correlation studies, Spearman's coefficient of rank (R) was employed, except for the correlation between insulin binding and monocyte concentration, where a linear regression analysis was applied (r).

Results

Cell Composition

The concentration of mononuclear cells was 69.7 ± 7.2 × 10^6/ml (mean ± S.D.) in 25 young normal persons. Monocytes comprised 12.9 ± 4.5% of these. Lymphocytes were sub-divided into 21% B cells and 58% T cells. The mononuclear leucocytes were contaminated with 20 ± 15 × 10^6 erythrocytes, 1.2 ± 0.9 × 10^6 granulocytes and 200 ± 80 × 10^6 platelets, per ml.

Binding Studies

Monocytes and Lymphocytes. Insulin binding to mononuclear leucocytes (at a concentration of 62 ± 10 × 10^6/ml) was studied in 29 healthy young persons. The cellular insulin binding was positively correlated to the number of monocytes in the suspension ($r = 0.84$ and $p < 0.001$) (Fig. 1). The intercept on the ordinate was 0.7, which for the great-

Table 1. Insulin binding to purified preparations of erythrocytes, granulocytes and platelets from 6 normal persons. Erythrocytes, granulocytes and platelets were incubated with ^{125}I-insulin at 15° C for 100 min. After separation of cell-bound and free insulin, the specific cell binding fraction at tracer concentration was estimated. The non-specific cell binding fraction averaged for erythrocytes about 75%, for granulocytes about 45% and for platelets about 50% of the total binding fraction

	Cell number concentration $10^6 \times ml^{-1}$	Specific cell binding fraction
Erythrocytes	108 ± 30	0.1 ± 0.1 × 10^{-2}
Granulocytes	10.7 ± 3.4	0.2 ± 0.1 × 10^{-2}
Platelets	90 ± 10	0.3 ± 0.1 × 10^{-2}

Table 2. The relative insulin binding to blood cells

	Specific cell binding fraction per 10 × 10^6 cells
Monocytes	2.8 × 10^{-2}
Granulocytes	0.2 × –
Lymphocytes	0.1 × –
Platelets	0.05 × –
Erythrocytes	0.01 × –

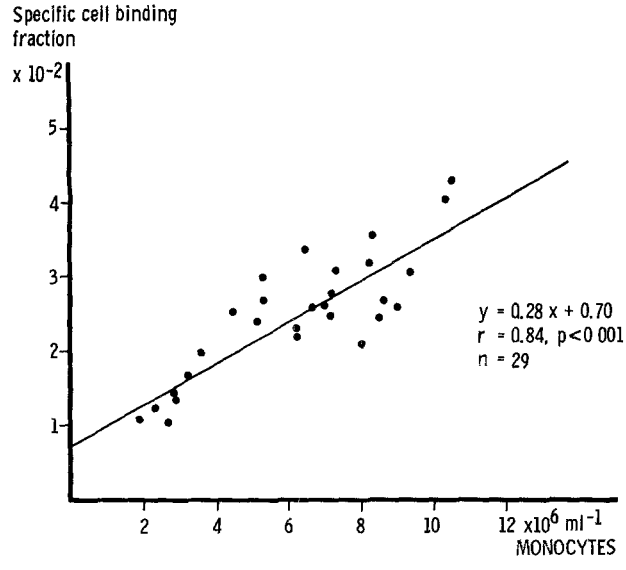

Fig. 1. Correlation between insulin binding to mononuclear leucocytes (62 ± 10 × 10^6 per ml) and the monocyte content in the isolated cell suspension from 29 normal persons. To obtain a desired range in the monocyte concentration of the cell suspension, mononuclear cells were isolated from both EDTA-blood and defibrinated blood. The tracer concentration of ^{125}I-insulin was 172 pmol/l

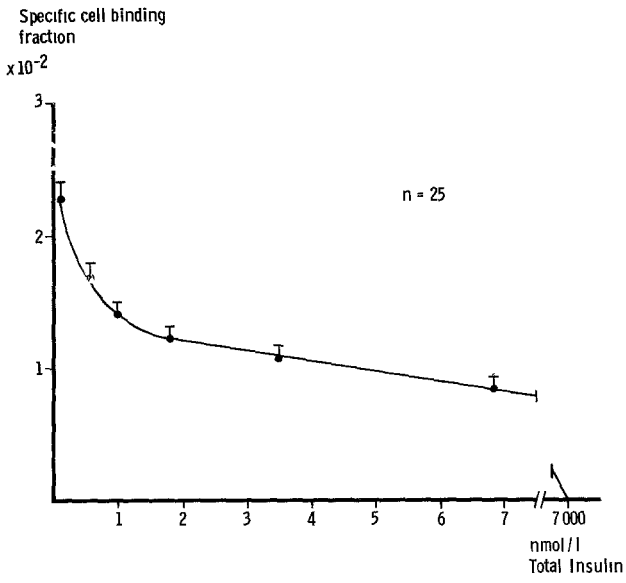

Fig. 2. The inhibiting effect of native insulin on ^{125}I-insulin binding to monocytes from 25 normal persons (mean ± SEM). The specific cell binding fraction is corrected to a monocyte concentration of 10^7 per ml, using the formula described above Non-specific binding averaged 25% of the total binding. The tracer concentration of ^{125}I-insulin was 172 pmol/l

est part represents binding to lymphocytes. There was no significant correlation between the total number of lymphocytes in the suspension and the insulin binding ($R = 0.28$ and $p > 0.1$).

No correlation between insulin binding and the number of B cells could be demonstrated ($R = 0.07$). An inverse, non-significant correlation was found between insulin binding and the number of T cells. ($R = -0.44$ and $p > 0.1$). Incubation of mononuclear leucocytes with carbonyl iron reduced the monocyte concentration from 8.7×10^6 to 1.7×10^6/ml. This decrease of monocyte concentration was followed by a reduction of specific insulin binding fraction from 2.8×10^{-2} to 1.0×10^{-2}. Insulin binding was expected to fall a further 0.5×10^{-2}, if the remaining 1.7×10^6 monocytes had also been removed. Lymphocytes therefore bound only about 0.5×10^{-2}, which is close to the binding calculated from Fig. 1. Monocytes probably account for about 80% of the insulin binding to mononuclear leucocytes.

Erythrocytes, Granulocytes and Platelets. The results of insulin binding to erythrocytes, granulocytes and platelets at different cell concentrations are given in Table 1. The relative insulin binding adjusted to the same cell concentration is shown in Table 2.

Correction of Binding Fraction

In the mononuclear cell preparation the lymphocytes account for about 20% of the cell-bound insulin and of course both the concentration and the binding ability of lymphocytes vary. A monocyte-receptor study based on simple normalization of the cell count to a mean value of the monocyte concentration probably results in biased insulin-binding values, i.e. unstable and too high specific cell binding fractions. Thrombocytes cause the same problem, which under our experimental conditions was solved by using silicone oil centrifugation.

We have elaborated a correction formula to correct for insulin binding to lymphocytes and to adjust the insulin binding to the mean monocyte concentration in the cell preparation (10^7 monocytes/ml). The correction formula yields a more precise estimate of the monocyte-bound insulin. Let us assume that the ratio between insulin binding to one monocyte (B_m) and one lymphocyte (B_l) is constant ($B_l/B_m = k$).

The specific insulin binding to 1 ml of an arbitrary suspension (x) of monocytes and lymphocytes is expressed:

$B_x = M_x \times B_m + L_x \times B_l$ or $B_x = M_x \times B_m + L_x \times k \times B_m$,

where M_x is the number of monocytes per ml of the suspension x and L_x is the number of lymphocytes per ml of the suspension x. From this equation we can derive B_m:

$$B_m = \frac{B_x}{M_x + L_x \times k}$$

Hence insulin binding to a suspension of 10^7 monocytes per ml ($B_{10}7$) is:

$$B_{10}7 = M_{10}7 \times B_m = \frac{M_{10}7 \times B_x}{M_x + L_x \times k}$$

($M_{10}7 = 10^7$ monocytes per ml).

The Monocyte Insulin Receptor

Insulin binding to monocytes from 25 healthy 20–30 years old persons was measured (Fig. 2). Scatchard plots (Fig. 3) of the binding data were curvilinear with an upward concavity, which can be explained by the presence of multiple classes of binding sites with different affinities or by the existence of site to site interaction, which has been referred to as "negative cooperativity" [15]. Kinetic studies on the dissociation of ^{125}I-insulin from monocytes (Fig. 4) indicated negative cooperativity, since the presence of native insulin accelerated the rate of dissociation of ^{125}I-insulin. Using the method described by De Meyts and Roth [14] the mean number of receptors per monocyte (R_o) was estimated to approximately 7.000, which is about 10 receptors per μm^2 surface

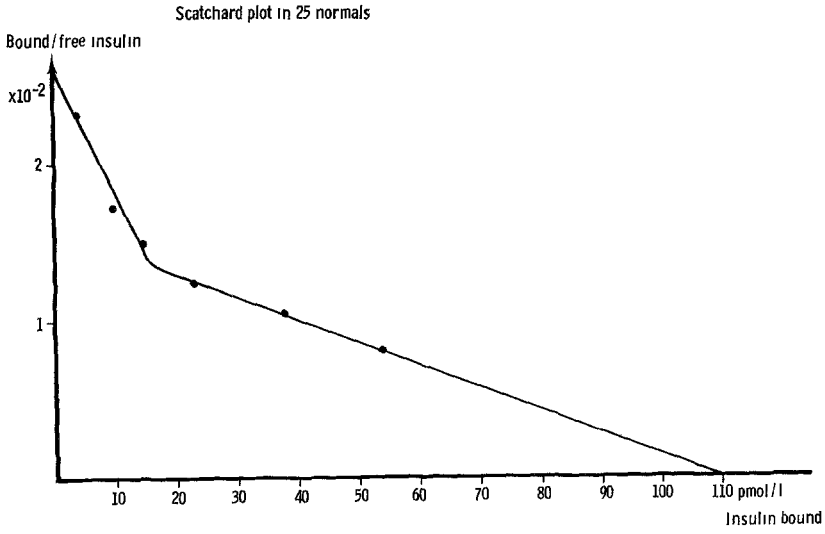

Fig. 3. Scatchard plot of the binding results in Figure 2

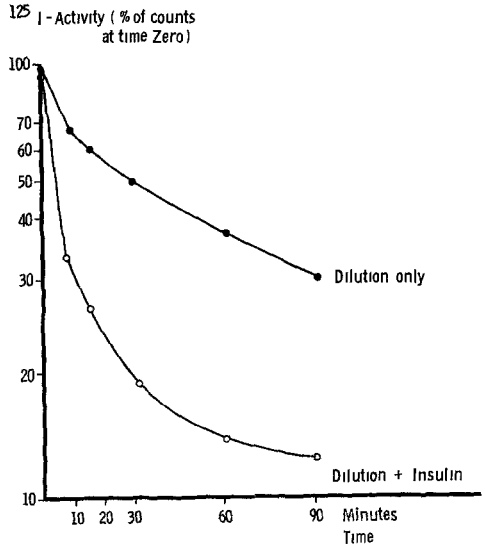

Fig. 4. Dissociation of ^{125}I-insulin from monocytes in buffer and in buffer plus 1.7 × 10^{-7} mol/l of native insulin (mean values of 5 replicates). In these experiments the concentration of mononuclear leucocytes was 100 × 10^6 per ml and the proportion of monocytes 16%. The ^{125}I-insulin concentration was 172 pmol/l

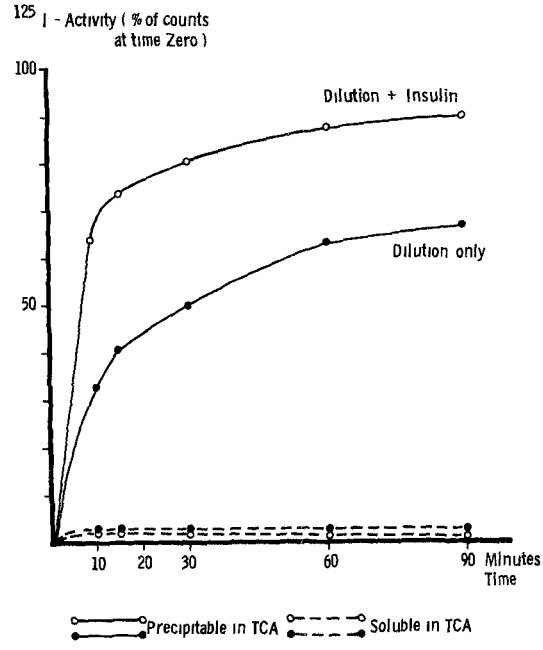

Fig. 5. Dissociation of insulin and degraded insulin into the washout medium. The counts appearing in the medium were separated into a fraction soluble in trichloroacetic acid and one precipitable by trichloroacetic acid. The counts which were present in the medium at zero time have been subtracted from all points (mean values of 5 replicates). Cell concentration and ^{125}I-insulin concentration are mentioned in legend to Figure 4

area. The apparent high affinity constant, ($\bar{K}_c$), was about 2.2 × 10^8 mol^{-1}.

Degradation. The ^{125}I-insulin degradation in the incubation medium after incubation of mononuclear cells for 100 min at 15° C was 5%. Degradation studies of ^{125}I-insulin which was dissociated from receptors into the dilution medium revealed that 5% of the dissociated insulin was soluble in TCA, whereas tracer insulin degradation was reduced to about 1% in the tubes containing native insulin at a concentration of 1.7 × 10^{-7} mol/l (Fig. 5).

Receptor Specificity. To test the specificity of the monocyte receptor we examined the inhibitory effect of porcine insulin, proinsulin and glucagon on the ^{125}I-insulin binding (Fig. 6). Glucagon inhibited insulin binding very slightly and compared to native

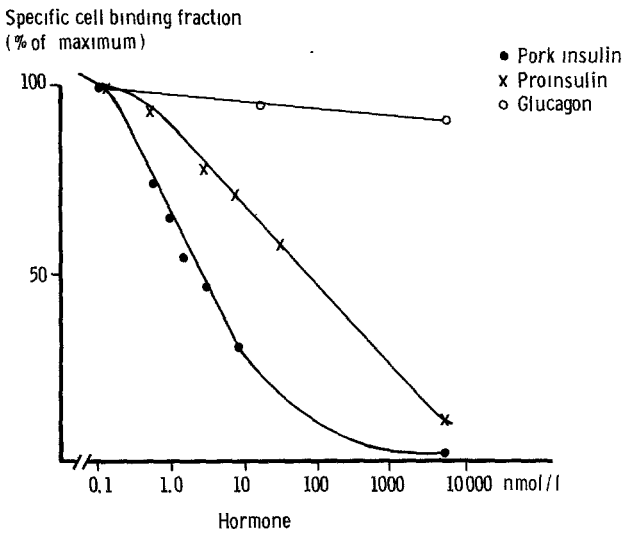

Fig. 6. The inhibitory effect of porcine insulin, proinsulin and glucagon on the ^{125}I-insulin binding (mean values of 5 replicates)

insulin proinsulin had a competitive effect of about 2%.

Precision of the Method. All insulin binding studies were done in duplicate. After gamma-counting the total cell binding fraction and the non-specific cell binding fraction were calculated. The standard deviation of the first parameter was in 50 determinations 0.19 and the corresponding standard deviation for the second parameter was 0.11. The standard deviation of the specific cell binding fraction (total ÷ nonspecific binding) is 0.22. The detection limit of the method is about 0.6 (3 × s.d.).

Discussion

Insulin Binding to Different Blood Cells

We have showed a highly significantly positive correlation between insulin binding and the monocyte concentration, but no significant correlation to the number of lymphocytes in the suspension, neither T nor B cells. Depletion of monocytes decreased the insulin binding by about 80%; therefore the monocyte is the major insulin binding cell in the preparation of mononuclear leucocytes. These findings corroborate the study of Schwartz et al. [3].

Granulocytes possess the second highest insulin binding, but of the total number of leucocytes in the preparation granulocytes comprise 1% and their contribution to the total insulin binding is consequently negligible. Fussganger et al. [16] found about 1000 insulin receptors per granulocyte, which supports our finding that the binding ability of granulocytes is much lower than that of monocytes. Insulin binding to erythrocytes was insignificant and the presence of a small amount of these cells in the preparation is unimportant. Thrombocytes, the insulin binding ability of which has not earlier been described, may comprise about 10–20% of the total insulin binding. This considerable share in insulin binding taken in consideration with the wide range of platelet numbers, makes it necessary to reduce these contaminates. This can be done in two ways: 1) by use of silicone oil centrifugation to separate cells from supernatant (most platelets are layered in the interface between oil and supernatant) and 2) by use of defibrinated blood [5]. Defibrination results in depletion of monocytes too. For this reason reduction of platelets by silicone oil centrifugation is preferable.

The Monocyte Insulin Receptor

Specificity of insulin binding to mononuclear leucocytes has been demonstrated [1, 17]. Our experiments were therefore confined to the inhibitory effect of proinsulin and glucagon on the ^{125}I-insulin binding. Glucagon inhibits insulin binding very slightly and compared to native insulin proinsulin has a competitive effect of about 2%. Like De Meyts et al. [15] we also found that native insulin accelerates the dissociation of ^{125}I-insulin from the receptors, which is interpreted as evidence of negative cooperativity. Steiner and Terris [18] have, in liver cells, found close on 40% degradation of receptor bound insulin. Granulocytes also degrade insulin, as shown by Fussganger et al. [16]. In a previous study [5] we measured insulin inactivation at 15° C by examining the ability of rebinding of ^{125}I-insulin to a fresh preparation of mononuclear cells after a normal incubation period. Only 5% of the binding ability of labelled insulin was lost after the first exposure to cells. In this study slight (5%) degradation of insulin both in the washout-medium and in the primary incubation medium was observed. However, addition of an excess of native insulin to the washout-medium reduced the degradation of tracer insulin from 5% to about 1%, which suggests that ^{125}I-insulin is degraded in the medium rather than at the receptor site.

Addendum. Since completing this study Bar et al. [19] have reported approximately twice the insulin binding to monocytes compared with our data. The higher insulin binding might be a result of 1) the use of a five times lower tracer concentration (which implies a lower degree of negative cooperativity), 2) no correction for insulin binding to lymphocytes or 3) no reduction of insulin binding thrombocytes.

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H. Beck- Nielsen
Medical Department III
County Hospital
Tage Hansensgade
DK-8000 Aarhus C
Denmark