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Control of erythrocyte membrane microviscosity by insulin

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The human erythrocyte membrane binds insulin through high-affinity, low-capacity binding sites (dissociation constant K_{d1} $2.45 \cdot 10^{-9}$ M; capacity n_1 207 fmol/mg protein) and low-affinity, high-capacity binding sites (K_{d2} $0.63 \cdot 10^{-6}$ M; n_2 37 pmol/mg protein). Treatment of the erythrocyte membrane or the intact cells with the physiological concentration of insulin, which is within the range of K_d value of the high-affinity sites, results in a significant reduction of the membrane microviscosity and the filtration time of the intact cells. Use of supraphysiological concentrations of the hormone reverses the effect of the lower concentration of insulin on the membrane microviscosity and the filtration time.

The biological effects of insulin in various tissues have been shown to be mediated through the interaction of the hormone with specific and high affinity receptors of plasma membranes [1,2]. Although human erythrocytes contain highly specific insulin receptors similar to the receptors of well known target tissues for the hormone action, no function of either the receptors or the ligand in the case of these cells is known [3–5]. However, studies with erythrocytes from diabetic patients have shown that these cells are less deformable and have higher membrane microviscosity than the red blood cells from normal individuals [6–9]. Since erythrocytes must often pass through capillaries considerably smaller than their own diameters, the deformability of these cells is critically important for rapid and homogeneous perfusion of oxygen in the microcirculation [10,11]. It has been proposed that these physical changes in the erythrocyte membrane may predispose the diabetic to tissue hypoxia and, in the long run, microangiopathy [6]. Since diabetes is a disease due either to absolute or relative insulin deficiencies, these studies might suggest a role of insulin in the control of erythrocyte deformability. In this communication, we report that the interaction of insulin with the high-affinity receptors of the human erythrocyte membrane results in a significant reduction of the membrane microviscosity, a phenomenon that in vivo may facilitate the movement of the red blood cells in the microcirculation.

The concentration dependent binding of ^{125}I-insulin to the erythrocyte membrane of normal individuals was analyzed by a Scatchard plot [12]. Erythrocyte membranes were prepared according to Hanahan and Ekholm [13]. The membrane preparation (80–100 μg protein) was incubated with 0.2 nM ^{125}I-insulin [3] in the presence of increasing concentrations of unlabeled human insulin (0–24 nM; Actrapid, Novo, Squibb) in a total volume of 200 μl binding mixture containing 50 mM Tris-HCl buffer (pH 7.4), and 5 mM $MgCl_2$ for 3 h at 22°C in siliconized glass tubes. After incubation, the assay mixture was layered over 30% sucrose (1 ml) solution in the above buffer in 1.5 ml plastic Eppendorf centrifuge tubes and centrifuged at $8000 \times g$ for 10 min at 8°C. The bottom part of the centrifuge tube, containing the

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membrane pellet, was cut off and the radioactivity of the pellet was determined in a gamma counter. The dissociation constants and the binding capacities of the insulin receptors of the membrane preparation were calculated by a non-weighted, iterative, least square algorithmic analysis in a microcomputer (Radio Shack, TRS 80, Model 4). The non-specific binding was determined in the presence of 1000-fold excess of the unlabeled hormone in the assay mixture. The specific binding was calculated by subtracting the non-specific binding from the total binding and used in the above calculation. The Scatchard plot was curvilinear in nature. Whether the curvilinearity of the plot was due to heterogeneity of binding sites or arose from negative cooperative interaction within a single class of binding site [14] is not known. Computer analysis of the binding data showed the presence of one high-affinity, low-capacity binding site population (dissociation constant K_{d1} $2.45 \cdot 10^{-9}$ M and capacity n_1 207 fmol/mg protein) and one low-affinity, high-capacity binding site population (K_{d2} $6.35 \cdot 10^{-7}$ M; n_2 37 pmol/mg protein). The degradation of ^{125}I-insulin by the membrane preparation was determined according to Dwenger and Zick [15] except the reaction was performed as described above. It was found that at 23°C, less than 2% of the total insulin was degraded in 3 h and as such the degradation would have little or no effect on the true concentration of the hormone in the incubation mixture. The above investigators reported similar results on the degradation of ^{125}I-insulin by the human erythrocyte membrane at 22°C [15]. Since the physiological concentration of insulin varies between 0.03 nM ($6 \cdot 10^{-6}$ international units/ml blood) and 1 nM ($150 \cdot 10^{-6}$ international units/ml blood [16], the importance of low-affinity, high-capacity binding sites of the red blood cell membrane is not obvious. However, in other tissues, both the high-affinity and low-affinity receptors have been shown to be physiologically important [17].

To determine the role of insulin on erythrocyte deformability, we next measured the change of the membrane microviscosity of these cells in the presence of physiological concentrations of the hormone by a fluorescence polarization method [18]. The human erythrocyte membranes were fluorescence-labeled by incubating the red cell ghosts with 1,6-diphenyl-1,3,5-hexatriene as the fluorescence probe. Incubation of the diphenylhexatriene-labeled membranes with insulin resulted in a significant decrease of the membrane microviscosity of these cells (Fig. 1). While the membrane preparation in the control experiments showed microviscosity of 5.47 ± 0.26 (mean $\pm$ S.D.) poise, the presence of 1.6 and 2.4 nM insulin in the incubation mixture showed microviscosities of 4.26 ± 0.21 poise ($p < 0.01$, paired 't' test) and 4.31 ± 0.23 poise ($p < 0.01$, paired 't' test), respectively. The kinetics of reduction of membrane microviscosity ran parallel to the kinetics of binding of the hormone to the membrane. Both the steady-state binding of ^{125}I-insulin to the erythrocyte membrane and the maximum reduction of the membrane microviscosity were obtained after incubating the assay mixture for 3 h at 22°C. The concentration-dependent decrease of microviscosity induced by insulin in the erythrocyte membrane showed an 'S' profile indicating that the effect of the hormone was cooperative in nature. The concentration of insulin (1.6–2.4 nM) needed to produce the maximum decrease of the membrane microviscosity is within the range of physiological concentrations in blood and compares with K_{d1} of the high-affinity, low-capacity receptors. The use of higher concentrations of insulin (3.2 nM) tended to reverse the effect of microviscosity induced by the lower concentrations of the hormone. Whether the increase of microviscosity in the presence of higher concentrations of insulin was due to the binding of the hormone to the low-affinity, high-capacity receptors or due to the formation of dimers as a result of insulin-insulin interaction on the cell membrane is not known. Nevertheless, the stability of the lowered state of microviscosity only in the physiological range of insulin may imply that both hypo- and hyperinsulinemia are detrimental to the membrane fluidity.

Since membrane microviscosity and deformability of the red blood cells are reciprocally related [19], we also studied the effect of insulin on the deformability of these cells by the filtration method of Teitel [20]. As expected, the incubation of the human erythrocytes with physiological concentrations of the hormone decreased the filtration time significantly when compared with the control (Ta-

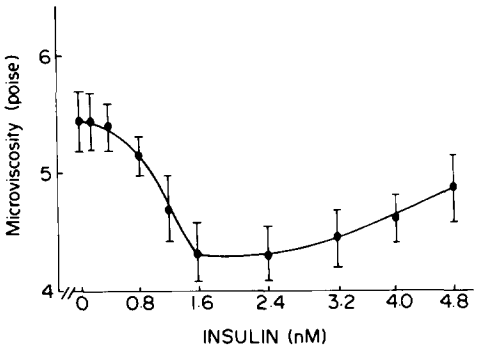

Fig. 1. The decrease of erythrocyte membrane microviscosity by insulin. The microviscosity ($\bar{\eta}$) of the erythrocyte membrane preparation was studied by fluorescence polarization using diphenylhexatriene as the fluorescence probe for the hydrocarbon core of the membrane bilayer [18]. Typically, $5.6 \cdot 10^6$ erythrocyte ghosts/ml in 50 mM Tris-HCl buffer (pH 7.4), containing 5 mM $MgCl_2$ were labeled with the fluorescence probe by incubating an equal volume of 2 μM diphenylhexatriene dispersion in the same buffer for 1 h at 22°C. The degree of fluorescence polarization (P) was measured in a Perkin-Elmer luminescence spectrometer (LS-5) fitted with a polarizer accessory.

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular, respectively, to the direction of the polarized excitation beam. The fluorescence anisotropy r was obtained from P by Eqn. 2.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} - 2I_{\perp}} = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 2} = \frac{2P}{3 - P} \quad (2)$$

Microviscosity $\bar{\eta}$ was determined from the Perrin equation:

$$\frac{r_o}{r} = 1 + C(r)\frac{T\tau}{\bar{\eta}} \quad (3)$$

where r_o is the limiting fluorescence anisotropy, τ is the life time of the excited state, T is the absolute temperature and $C(r)$ is a structural parameter of the probe. A calibration curve r_o/r versus $T\tau/\bar{\eta}$ was constructed from Eqn. 3 and used for the determination of $\bar{\eta}$. P was determined at 22°C. The effect of insulin on the erythrocyte membrane microviscosity was determined by adding different concentrations of the hormone to previously labelled membrane preparations and incubating the suspension for 3 h at 22°C. Each point is the mean ± S.E. of five separate experiments, each with triplciate determinations. Identical results were obtained by using human insulin from two different commercial sources (Actrapid, Squibb; Humulin, Eli Lilly).

ble I). The filtration time was maximally reduced when the cells were incubated with 0.8–1.6 nM insulin. As in the case of the membrane micro-

TABLE I

THE DECREASE OF FILTRATION TIME OF WASHED INTACT RED BLOOD CELLS BY INSULIN

Blood from normal volunteers who had fasted for at least 12 h was collected in 0.15 mM sodium citrate. The washed red blood cells were prepared and the filtration time was determined according to Teitel [19], except the cells were washed three times at 4°C at intervals of 1 h between each washing and suspended in 0.9 % NaCl containing 5 mM $MgCl_2$ ($(5–6) \cdot 10^6$ cells/ml). Different concentrations of insulin were added to the cell suspension and incubated at 22°C for 3 h. The results shown here are the half filtration time ($t_{1/2}$) ± S.E. of five different experiments, each in triplicate. The erythrocyte preparation had less than 1% lymphocytes, monocytes and granulocytes as contaminants [21].

Insulin (nM)	Half filtration time, $t_{1/2}$ (s)
0	131 ± 5
0.4	90 | + 9
0.8	69 ± 7
1.6	72 ± 6
2.4	95 ± 10
4.0	117 ± 11
8.0	121 ± 13

viscosity, the use of supraphysiological concentrations of the hormone increased the filtration time of these cells. The washing of erythrocyte preparation of 0.155 M sodium phosphate buffer (pH 7.1) containing 5 mM glucose did not influence the change of filtration time influenced by insulin on these cells.

The effect of insulin on the membrane microviscosity and erythrocyte deformability are apparently specific and independent of glucose concentration. Substitution of insulin (M_r 5734) by a peptide hormone of similar molecular weight, like porcine α-adrenocorticotropic hormone (M_r 4566), produced no effect on the membrane microviscosity when used in the concentration of 0.2 to 100 nM. Similarly, albumin (M_r 67000) either in the same range or at 0.5 mM concentration had no effect on the erythrocyte membrane microviscosity. Addition of glucose (4.5 mM) to the membrane suspension did not affect the reduction of the microviscosity induced by insulin.

The physiological implication of our observation is apparent. The lowering of the microviscosity and consequently the incresed deformability

may play an important role in red blood cell diffusion in the microcirculation. The insulin binding data as well as the observation that microviscosity and deformability effects occurred within the K_{d1} values of the insulin binding, indicate that the insulin receptors of these cells are not inactive structural components as commonly supposed but are functional elements of the membrane, modulating one of the most important properties of the red blood cells in the delivery of oxygen to the tissues through the capillary circulation. Our results on the direct effects of insulin on erythrocyte membrane microviscosity support the hypothesis of Vague and his co-workers who proposed earlier that insulin is capable of controlling erythrocyte deformability [9,22,23].

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