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Regulation of insulin receptor activity of human erythrocyte membrane by prostaglandin E_1

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Incubation of human erythrocyte membrane with low concentration of prostaglandin E_1 or prostacyclin increased the binding of ^{125}I-labeled insulin to the membrane. The binding of the radioiodinated hormone was maximally stimulated at 3 nM prostaglandin E_1 and the use of higher concentrations (above 8 nM) of the autacoid tended to reverse its own effect at lower concentrations. While prostaglandins A_1, A_2, B_1, B_2, D_2, $F_{1\alpha}$, $F_{2\alpha}$ or 6-keto-prostaglandin $F_{1\alpha}$ had no effect on the binding of insulin to the erythrocyte membrane, prostaglandin E_2 at similar concentrations decreased the binding of the hormone. The effect of prostaglandin E_1 on the increased binding of the insulin was found to be reversible and depended on the occupancy of the autacoid molecules on the membrane and showed positive cooperativity. Scatchard analysis of the binding of ^{125}I-labeled insulin to the erythrocyte ghosts indicated that in the presence of the autacoid, the binding capacity of the insulin receptor increased 2-fold (from 207 to 424 fmol/mg protein) without any change in the ghosts affinity for the ligand (K_d $2.4 \cdot 10^{-9}$ versus $2.49 \cdot 10^{-9}$ M). As a consequence of increased binding of insulin to the erythrocyte membrane in the presence of prostaglandin E_1 (3.0 nM), the optimal concentration of the peptide hormone for the maximal reduction of the membrane microviscosity decreased from approx. 1.6 to approx. 0.4 nM. Addition of prostaglandin E_1 alone at the above concentration to the assay mixture had no effect on the membrane microviscosity.

Introduction

Prostaglandins have been shown to produce a regulatory effect on the β-cell function through the inhibition of glucose-stimulated insulin secretion [1]. It has also been reported that inhibitors of prostaglandin synthesis, such as aspirin and ibuprofen [1–3], but not indomethacin [3,4], augment the secretion of insulin. Although these studies indicate that prostaglandins might influence the level of insulin in the system, no direct role of the autacoids in the biological effects of insulin is known.

In this paper, we report that when the human erythrocyte membranes, which contain a highly specific insulin receptor [5], are treated with prostaglandin E_1 or prostacyclin, the hormone receptor number is increased with no apparent change in the receptor affinity. It was found that the increase in the insulin receptor number of the human erythrocyte membrane was reversible in nature and depended on the occupancy of the prostaglandin molecules on the membrane surface.

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Materials and Methods

Materials. ^{125}I-labeled insulin (spec. act. 100 μCi/μg) and [5,6(n)-^{3}H]prostaglandin E_1 (spec. act. 50.3 Ci/mmol) were obtained from ICN Radiochemicals, Irvine, CA and New England Nuclear, Boston, MA, respectively. Human insulin was the product of Squibb (Actrapid). All other reagents used were of analytical grade.

Preparation of Human Erythrocyte Membrane. Human erythrocyte membranes were prepared as previously described [6]. Care was taken to assure that the volunteers had not taken any medication for at least 2 weeks prior to blood donation.

Insulin binding assay. The erythrocyte membrane preparation (80–100 μg protein) was incubated with 0.6 nM ^{125}I-labeled insulin in the presence of increasing concentrations of unlabeled insulin (0–24 nM) in a total volume of 200 μl binding mixture containing 50 mM Tris-HCl buffer (pH 7.4) and 5 mM $MgCl_2$ for up to 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 8000xg for 10 min at 8°C. The bottom part of the centrifuge tube containing the membrane pellet was cut off and the radioactivity of the pellet was determined in a gamma counter. Parallel experiments were run by adding 1000-fold excess unlabeled insulin (0.6 μM) to the above incubation mixture to determine nonspecific binding. This value was subtracted from the total ^{125}I-labeled insulin binding to the membrane preparation to calculate the specific binding.

Kinetic analysis of ^{125}I-labeled insulin binding to the erythrocyte membrane. The binding of ^{125}I-labeled insulin to the erythrocyte membrane preparation was analyzed by the method of Scatchard [7]. The dissociation constants (K_d) and the number of binding sites were obtained from non-linear regression analysis of equilibrium binding by a non-weighted, iterative, least-squares algorithm microcomputer analysis.

Prostaglandin binding assay. The binding of [^{3}H]prostaglandin to the erythrocyte membrane was studies by the method described previously [6]. Briefly, the membrane preparation (80 μg protein) was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and 3.0 nM [^{3}H]prostaglandin E_1 (25000–30000 dpm) in a total volume of 200 μl. After incubation at 22°C for 30 min, the mixture was vacuum-filtered through a Whatman glass microfibre filter (GF/C) which had been presoaked with the assay buffer. The filter paper was washed with 15 ml of the buffer at 0°C. The filtered papers were then dried and suspended in 10 ml of ACS-II scintillation solvent and counted in a Beckman scintillation spectrometer (LS-8000) with 45% efficiency for ^{3}H. Nonspecific binding was obtained by adding 1000-fold excess unlabeled prostaglandin E_1 to the above assay mixture. This values was subtracted from total binding to calculate the specific binding of [^{3}H]prostaglandin to the membrane preparation.

Degradation of ^{125}I-labeled insulin by the erythrocyte membrane. The degradation of ^{125}I-labeled insulin by the membrane preparation was determined according to Dwenger and Zick [8], except that the incubation was performed in the reaction mixture described above.

Protein estimation. Protein concentration of the membrane preparation was determined according to Lowry et al. [9].

Determination of erythrocyte membrane microviscosity. The microviscosity ($\bar{\eta}$) of the erythrocyte membrane preparation was studied by fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene as the fluorescence probe for the hydrocarbon core of the membrane bilayer as described previously [10]. 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.

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

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 the equation (2):

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

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

$$\frac{r_0}{r} = 1 + C(r)\frac{T_{\tau}}{\bar{\eta}} \quad (3)$$

where r_0 is the limiting fluorescence anisotropy, τ is the lifetime of the excited state, T is the absolute temperature and $C(r)$ is a structural parameter of the probe. A calibration curve r_0/r versus $T_{\tau}/\bar{\eta}$ was constructed from the Eqn. 3 and used for the determination of $\bar{\eta}$. P was determined at 22°C.

Results

Effect of prostaglandin E_1 on the binding of ^{125}I-labeled insulin to erythrocyte membrane

Incubation of human erythrocyte membrane with ^{125}I-labeled insulin resulted in a rapid and reversible binding of the hormone to the membrane preparation. Equilibrium was attained within 30 min of incubation at 22°C. Addition of prostaglandin E_1 to the reaction mixture progressively increased the binding of the radioligand with the increase of incubation time and at equilibrium (30 min), the specific binding of ^{125}I-labeled insulin was increased almost 2-fold in the presence of the autacoid when compared with the control (Fig. 1). The effect of prostaglandin E_1 on insulin binding to the membrane preparation was biphasic in nature. At 3 nM concentration, the autacoid showed maximal stimulation of the binding of the peptide hormone to the erythrocyte membrane. The use of higher concentrations of the prostaglandins (8 nM or more) in the assay mixture tended to decrease the binding of insulin (not shown). To determine the specificity of the effect of prostaglandin E_1, the autacoid was substituted by various prostaglandins in the reaction mixture. With the exception of prostacyclin, no other prostaglandin, including prostaglandins A_1, A_2, B_1, B_1, D_2, $F_{1\alpha}$, $F_{2\alpha}$ or 6-keto-prostaglandin $F_{1\alpha}$ at various concentrations (0.5–15 nM), had any effect on the binding of ^{125}I-labeled insulin to the erythrocyte membrane. Prostaglandin E_2, unlike the other prostaglandins at 3 nM or higher concentrations, decreased the binding of ^{125}I-labeled insulin to the receptor (Fig. 1).

Reversibility of the stimulatory effect of prostaglandin E_1 on the binding of ^{125}I-labeled insulin

Incubation of 3.0 nM [^{3}H]prostaglandin E_1 with the membrane preparation showed 160 fmol of the autacoid bound per mg of membrane protein at equilibrium (30 min). By repeated washings, the amount of prostaglandin bound to the membrane preparation was gradually decreased. Incubation of these washed membrane preparations containing decreasing amounts of the prostaglandin with ^{125}I-labeled insulin showed a gradual decrease of the binding of the radioiodinated hormone to the erythrocyte membrane (Fig. 2a). However, removal of about 50% prostaglandin E_1 from the membrane preparation decreased the binding of ^{125}I-labeled insulin to the basal level. When the amounts of ^{125}I-labeled insulin bound to the membrane after different stages of washings were plotted against the quantities of prostaglandin E_1 which remained bound to the preparation, an 'S'-shaped curve was obtained (Fig. 2b). These results indicate that the effect of prostaglandin E_1 on the

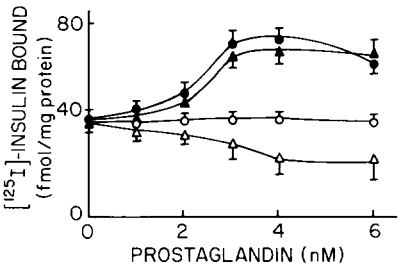

Fig. 1. Effect of prostaglandins on the specific binding of ^{125}I-labeled insulin to the human erythrocyte membrane. The membrane preparation was incubated with ^{125}I-labeled insulin (0.6 nM) in the presence of various prostaglandins as described in the Materials and Methods. The nonspecific binding of the radioiodinated hormone was determined by adding excess unlabelled insulin (0.6 μM) to the assay mixture and the specific binding was calculated by substracting the nonspecific binding from the total binding. Each point represents mean ± S.E. of three to six experiments. Prostaglandin E_1, ●; prostacyclin, ▲; prostaglandins A_1, A_2, B_1, B_2, D_2, $F_{1\alpha}$, $F_{2\alpha}$ and 6-ketoprostaglandin $F_{1\alpha}$, ○; prostaglandin E_2, △.

interaction of insulin with its receptors was cooperative in nature. Re-incubation of the washed membranes (after the final washing) with prostaglandin E_1 increased the binding of ^{125}I-labeled insulin to the membrane by 77% (64 ± 1.6 fmol/mg protein) when compared to the control in the absence of prostaglandin E_1 (36 ± 1.6 fmol/mg protein).

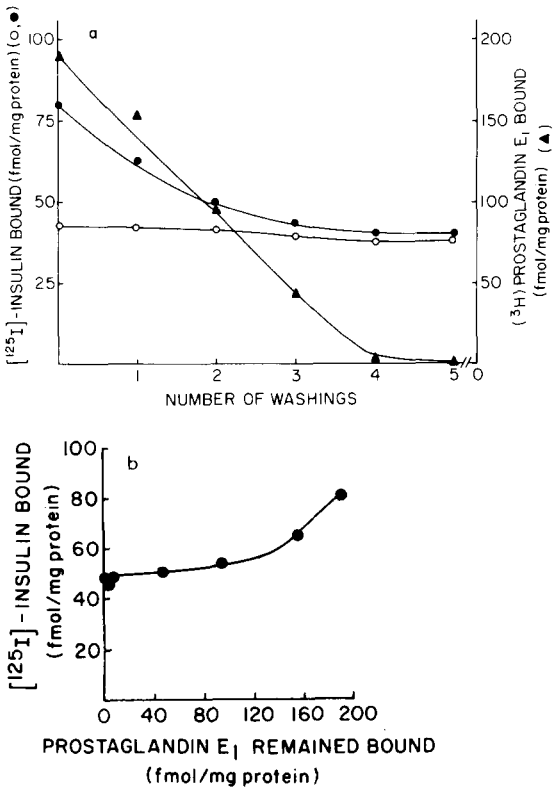

Fig. 2. Relation between the degree of occupancy of prostaglandin E_1 and the binding ^{125}I-labeled insulin to human erythrocyte membrane. The membrane preparation was incubated with prostaglandin E_1 (3.0 nM) as described. After equilibrium (30 min), the membranes were either incubated with ^{125}I-labeled insulin (0.6 nM) or washed five times with 1.0 ml of the assay buffer by centrifugation (12000 × *g*) for 30 min at 0°C. After each washing, the membranes were incubated with the radioiodinated hormone to determine the binding of insulin at various stages of washing procedures. Parallel experiments were run using [^{3}H]prostaglandin E_1 (3.0 nM) to determine the amounts of the autacoid remaining bound to the membrane after each washing (a). Control experiments were run by washing the red cell ghosts under identical conditions in the absence of prostaglandin E_1 in the assay mixture. Results shown here are specific bindings for both insulin and the prostaglandin. Each point represent the mean of three experiments. Binding of ^{125}I-labeled insulin in the control experiments (○) and in the presence of prostaglandin E_1 (●); binding of [^{3}H]prostaglandin E_1 alone (▲).

Characteristics of the binding of ^{125}I-labeled insulin to the erythrocyte membrane in the presence of prostaglandin E_1

The Scatchard plot of the binding of ^{125}I-labeled insulin to the erythrocyte membrane was curvilinear in nature (Fig. 3). 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 [11] is not known.

The Scatchard plot of analysis of total binding of ^{125}I-labeled insulin to the membrane preparation showed the presence of two classes of binding site corresponding to low-capacity specific sites and high-capacity non-specific sites. The specific binding sites displayed a high-affinity ($K_{d1} = 2.45 \cdot 10^{-9}$ M) and low-capacity ($n_1 = 207$ fmol insulin per mg protein) interaction with the ligand ($r^2 = 0.98665$). The nonspecific binding sites showed low-affinity ($K_{d2} = 0.6 \cdot 10^{-6}$ M), high-capacity ($n_2 = 38$ pmol insulin per mg protein) characteristics. In the presence of 3 nM prostaglandin E_1, the Scatchard plot analysis of ^{125}I-labeled binding to

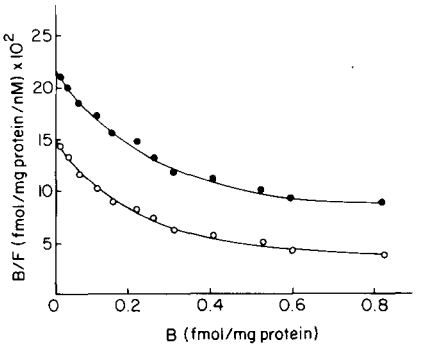

Fig. 3. Scatchard plots of total ^{125}I-labeled insulin binding to human erythrocyte membrane in the presence and absence of prostaglandin E_1. The erythrocyte membrane (80–100 μg protein) was incubated with 0.6 nM ^{125}I-labeled insulin plus 0–0.6 μM unlabeled hormone with or without 3 nM prostaglandin E_1 for 30 min at 23°C. Total binding (expressed as fmol/mg protein) for each point was determined by dividing cpm by the calculated specific activity (cpm/fmol) obtained by diluting 0.6 nM ^{125}I-labeled insulin with known concentrations of the unlabeled hormone. Each point represent the mean of four experiments. ^{125}I-labeled insulin, ○; ^{125}I-labeled insulin plus prostaglandin E_1, ●.

the erythrocyte membrane showed an increase in the binding capacity (n_1) from 207 fmol to 424 fmol insulin per mg protein ($P < 0.005$, $n = 6$) without affecting K_{d1} ($2.49 \cdot 10^{-9}$ M) compared to the control. However, binding of ^{125}I-labeled insulin in the presence of prostaglandin E_1 to the membrane preparation through the nonspecific sites was also significantly increased. The binding capacity (n_2) of the nonspecific sites increased from 38 pmol to 140 pmol insulin per mg protein ($P < 0.005$, $n = 6$) with simultaneous decrease of low affinity ($K_{d2} = 2.75 \cdot 10^{-6}$, $P < 0.001$, $n = 6$) when compared with the controls.

As described by Klotz and Hunston [12], total binding can be expressed as

$$r = \underbrace{\frac{n_1 k_1 [\text{insulin}]}{1 + k_1 [\text{insulin}]}}_{\text{(I)}} + \underbrace{\frac{n_2 k_2 [\text{insulin}]}{1 + k_2 [\text{insulin}]}}_{\text{(II)}}$$

where r is total insulin binding, n_1 is the capacity of the high-affinity binding sites, k_1 the association constant of the high-affinity binding sites, n_2 the capacity of the low-affinity binding sites and k_2 the association of low-affinity high-capacity binding sites. The components I and II of the equation indicate the 'true' specific and nonspecific contribution of the total binding, respectively. By substituting the values of n_1, $K_1 = 1/K_{d1}$, n_2 and $K_2 = 1/K_{d2}$ in the above equation, the specific and nonspecific binding of ^{125}I-labeled insulin to the erythrocyte membrane estimated by theoretical calculation was found to be within 10% of the observed values by the experimental procedure (subtracting the amount of ^{125}I-labeled insulin bound to the membrane preparation in the presence of 1000-fold excess of unlabeled insulin from the total binding (Fig. 1)).

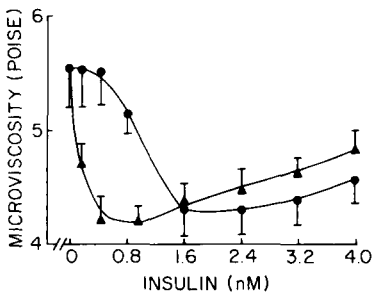

Fig. 4. Effect of prostaglandin E_1 on the insulin-induced reduction of erythrocyte membrane microviscosity ($\bar{\eta}$). The microviscosity of the membrane preparation was determined by fluorescence polarization method as described in Materials and Methods. Insulin alone, ●; insulin plus 3.0 nM prostaglandin E_1 ▲. Each point is the mean ± S.E. of five separate experiments.

Degradation of ^{125}I-labeled insulin by erythrocyte membrane preparation

Incubation of ^{125}I-labeled insulin with the membrane preparation at 22°C indicated that less than 2% of the total insulin was degraded in 30 min. Addition of prostaglandin (3 nM or 9 nM) to the assay mixture produced no effect on the degradation of the hormone.

Effect of prostaglandin E_1 on the reduction of erythrocyte membrane microviscosity induced by insulin

We have recently shown that insulin at physiological concentration reduces the microviscosity of the erythrocyte membrane [10]. Since prostaglandin E_1 increases the binding of ^{125}I-labeled insulin to the membrane preparation (Fig. 1), experiments were performed to determine the effects of prostaglandin E_1 on the insulin-induced reduction of membrane microviscosity of human erythrocyte. As reported before [10], the addition of increasing concentrations of insulin gradually decreased the microviscosity, and at 1.6 nM concentration of the hormone the membrane microviscosity was maximally reduced. On the other hand, the presence of prostaglandin E_1 (3 nM) in the assay mixture decreased from 1.6 nM to 0.4 nM the concentration of insulin needed for a similar reduction of membrane microviscosity (Fig. 4). Prostaglandin E_1 alone at this or higher concentration (up to 7 nM) had no effect on the membrane microviscosity (not shown).

Discussion

It is generally accepted that many of the biological effects of insulin are initiated by the interaction of the ligand with highly specific receptors on the membrane (see Ref. 13 for review). The availability and the affinity of the receptors which determine, at least in part, the physiological ef-

fects of insulin are regulated by various intrinsic and extrinsic factors including the status of growth of the cells [14,15]. For example, it has been reported that glucocorticoids increase the number of insulin receptors on the cell surface without affecting the affinity of the binding sites [16,17]. In contrast, insulin 'down regulates' its own effects by decreasing the number of receptors without changing their affinity [18]. In both cases, the regulation of receptor density depends on de nov protein synthesis. The plasma membrane of the human erythrocyte, which contains highly specific insulin receptors, does not have the ability to synthesize new receptor proteins. Nevertheless, our results showed that prostaglandins directly modify the insulin receptor interaction in human erythrocyte membrane by increasing the hormone receptor number. These results also showed that the insulin receptor number in erythrocyte is not necessarily correlated only to the protein synthetic ability or to the mean age of the erythrocytes [19]. On the other hand, the observed changes of insulin receptor numbers erythrocytes in pathological conditions [20,21], might be due to the consequence of changes of the prostaglandin level in the circulation.

The increase in insulin receptor number on the membrane preparation brought about by prostaglandin E_1 was very specific. With the exception of prostacyclin, no other prostaglandin produced a similar effect on the insulin receptor number (Fig. 2). We have previously reported that the human red-cell membrane contains highly specific prostaglandin E_1 binding sites [6]. It was also found that prostacyclin and its hydrolysis product, 6-ketoprostaglandin $F_{1\alpha}$, compete equally with prostaglandin E_1 for the ligand-binding sites. Although prostacyclin, like prostaglandin E_1, increased the insulin receptor number on erythrocyte membrane (Fig. 2), 6-ketoprostaglandin $F_{1\alpha}$ produced no effect on the hormone receptor number. These results indicate that the effect of prostaglandins on the increase in insulin receptor number not only depends on the specificity of the binding sites of the autacoids but also relates to the intrinsic properties of the prostanoids. The cooperative nature of the binding characteristics of ^{125}I-labeled insulin to its receptors in the presence of prostaglandin E_1 (Fig. 1, 2) indicate that the autacoid is probably acting as a positive effector in the interaction. However, the insulin receptors and prostaglandin E_1 binding sites in erythrocyte membrane are shown to be separate entities [5,6] and the autacoid increased the insulin receptor number without changing the affinity of the ligand for its receptors. It is possible that the binding of prostaglandin E_1 to its receptors on the membrane might induce a reversible exposure of cryptic insulin receptors due to conformational changes in membrane topography rather than a direct interaction of the autacoid with the hormone receptors. The effect of prostaglandin E_1 on the increased binding of insulin is not restricted to the human erythrocyte membrane alone. Preliminary results have indicated that the autacoid also increased the binding of the hormone in the case of human lymphocytes (unpublished data).

The erythrocyte deformability, which is reciprocally related to the membrane microviscosity, is critically important for rapid and homogeneous perfusion of oxygen in the microcirculation [22]. Although human erythrocytes contain highly specific insulin receptors, their functions in these cells are unknown. Recently, we [10] and other investigators [23] have reported that insulin at physiological concentration increases erythrocyte deformability and, thereby, the hormone might facilitate the movement of red blood cells in the microcirculation. The increase in insulin receptor number of human erythrocyte membrane by prostaglandin E_1 and consequently the reduction of optimum concentration of the peptide hormone for the maximal decrease of the membrane microviscosity (Fig. 3) indicate that the prostaglandin might favorably modify the effect of insulin on the red blood cells in the microcirculation in vivo.

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