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DISTRIBUTION OF INSULIN RECEPTORS IN HUMAN ERYTHROCYTE MEMBRANES

INSULIN BINDING TO SEALED RIGHT-SIDE-OUT AND INSIDE-OUT HUMAN ERYTHROCYTE VESICLES

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Analyses of insulin binding to human erythrocytes and to resealed right-side-out and inside-out erythrocyte membrane vesicles have revealed that high affinity insulin binding receptors are present on both sides of the erythrocyte membranes. Insulin binding to human erythrocytes was examined with the use of a binding assay designed to minimize the potential errors arising from the low binding capacity of this cell type and from non-specific binding in the assay. Scatchard analysis of equilibrium binding to the cells revealed a class of high affinity sites with a dissociation constant (K_d) of $(1.5 \pm 0.5) \cdot 10^{-8}$ M and a maximum binding capacity of 50 ± 5 sites per cell. Interestingly, both resealed right-side-out and inside-out membrane vesicles exhibited nearly identical specific sites for insulin binding. At the high affinity binding sites, for both right-side-out and inside-out vesicles, the dissociation constant (K_d) was $(1.5 \pm 0.5) \cdot 10^{-8}$ M, and the maximum binding capacity was 17 ± 3 sites per cell equivalent. These findings suggest that insulin receptors are present on both sides of the plasma membrane and are consistent with the participation of the erythrocyte insulin receptors in an endocytic/recycling pathway which mediates receptor-ligand internalization/externalization.

Although a well-defined physiological response by erythrocytes to insulin is lacking, their ready availability, their potential for the monitoring of hormone receptor status under various conditions of age and disease, and their well-characterized cell membranes have made erythrocyte insulin receptors the subject of intensive investigation [1–18]. We have recently isolated and partially characterized the insulin receptors of human erythrocytes [18] which appear to have a somewhat different subunit structure from insulin receptors present in the classic target cells. The aim of the present communication is to address the question of whether the erythrocyte insulin receptor is a fixed peripheral protein located exclusively on the external surface of the red cell membrane, or whether it is distributed on both sides of the plasma membrane. To accomplish this objective, experiments were carried out using sealed right side-out and inside-out vesicles derived from human erythrocyte membranes.

Because our preliminary experiments indicated a substantial amount of insulin binding to the walls of plastic centrifuge tubes [19], for these studies we adopted glass tubes which bind insulin

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to a much lesser extent. In addition, aliquots of the precipitated ^{125}I-labeled insulin bound cells were quantitatively removed from the glass centrifuge tubes for radioactivity determination. Fig. 1 shows a typical Scatchard plot of data obtained from insulin binding to human erythrocytes at 4°C. The binding profile is curvilinear and shows two distinct binding characteristics. One of these shows higher affinity, but lower capacity; and the other shows lower affinity, but greater capacity. At the high affinity binding region, the dissociation constant (K_d) and maximum insulin binding capacity were $(1.5 \pm 0.5) \cdot 10^{-8}$ M and 50 ± 5 sites per cell, respectively.

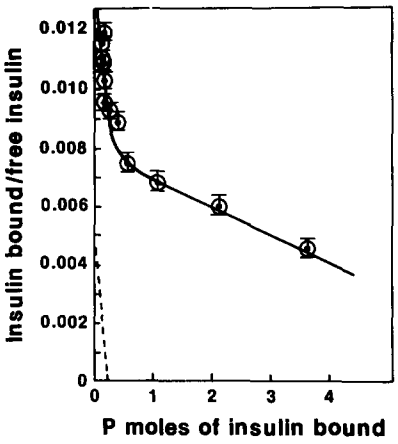

Fig. 1. Scatchard plot of insulin binding to human erythrocytes. Human erythrocytes were prepared according to the procedures of Kim and Luthra [24]. Packed erythrocytes (0.4 ml) were added to a series of 2 ml glass centrifuge tubes (Kimble, No. 45150) containing the same amount of ^{125}I-labeled insulin (New England Nuclear) but various amounts of cold insulin (porcine, Eli Lilly & Co). The range of concentrations of cold insulin used was from 0.333 nM to 500 nM in 0.6 ml of balanced salt solution (BSS). The final volume of each incubation mixture was 1 ml. The balanced salt solution (BSS) was composed of 137 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 10 mM sodium phosphate buffer (pH 7.6). The tubes were gently agitated overnight (16 h) at 4°C and total radioactivity was measured. The tubes were then centrifuged for 15 min at $1350 \times g$. The radioactivity of a 0.2 ml aliquot of each supernatant was measured. After removing the supernatant, 1 ml of dibutylphthalate was added and the tubes were recentrifuged. The remaining supernatant and dibutylphthalate was removed by aspiration. 0.1 ml of packed cells (approx. $9.8 \cdot 10^8$ cells) was quantitatively transferred with a Drummond pipet to a tube containing 2 ml of distilled water and the pipet was rinsed several times with the water. The radioactivity of the cells was measured. Corrections for non-specific binding were made by subtracting the binding in the presence of excess unlabeled insulin (16.7 μM). The average results from six separate binding experiments were analyzed according to the Scatchard plot [25].

To determine the sidedness of the insulin receptors, sealed right side-out and inside-out vesicles were prepared [20,21], and sidedness assays were carried out by the determination of acetylcholin-

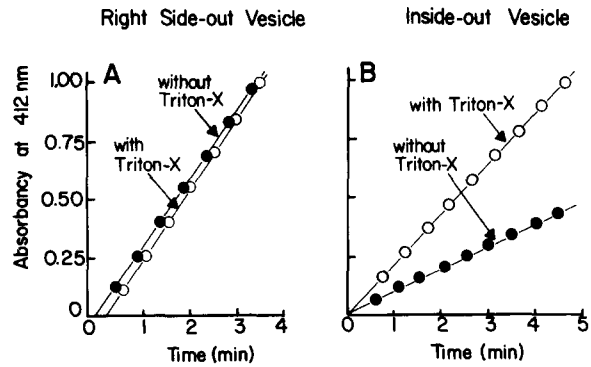

Fig. 2. Determination of the sealed right side-out and inside-out vesicles of human erythrocyte membranes by measurement of acetylcholinesterase activity as a membrane marker. Erythrocyte vesicles were prepared according to the procedure of Steck and Kant [20,21] from about 500 ml of human blood depleted of white cells by aspiration of the buffy coat after centrifugation. The blood was then washed three times with 5 mM sodium phosphate buffer (pH 8.0) containing 150 mM NaCl, and the remaining white cells were removed by aspiration. Ghosts were prepared by hypotonic lysis of the packed cells four times in 20 volumes of ice cold 5 mM sodium phosphate buffer (pH 8.0). After each centrifugation, the white button at the bottom of the centrifuge tube was removed by aspiration. Membrane vesiculation was initiated in 0.5 mM sodium phosphate buffer (pH 8.0) and the membranes were further treated with 0.1 mM $MgCl_2$ (for right side-out vesicles) or without addition of $MgCl_2$ (for inside-out vesicles). Immediately after salt addition, the vesicles were centrifuged at $40000 \times g$ for 30 min. to obtain a tightly packed pellet. The vesicle suspension was passed several times through a syringe attached to a No. 27 gauge needle. Protein yields were generally 40–70% that of ghost protein. The sidedness of the sealed right side-out and inside-out vesicles was assayed by the acetylcholinesterase accessibility technique. Aliquots of vesicle protein in 0.1 ml were briefly preincubated at 37°C in cuvettes with an equal volume of 5 mM sodium phosphate buffer (pH 8.0) either with or without 0.2% (v/v) Triton X-100. Then 0.5 ml of sodium phosphate buffer (100 mM, pH 7.5) was added to make the volume to 0.7 ml, followed by an addition of 0.05 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mM sodium phosphate (pH 7.0) containing 17.69 mM $NaHCO_3$. The reaction was initiated by adding 0.05 ml of 12.5 mM acetylthiocholine chloride and the change in absorbancy was followed by spectrophotometer at 412 nm at room temperature. The result shown is a typical result of four separate experiments.

esterase activity as a membrane marker [20,21]. The results of the sidedness assays (Fig. 2) show that the right side-out vesicle preparation consisted almost totally of right side-out vesicles in agreement with the findings reported by Crane et al. [22], whereas the inside-out vesicle preparation was composed of about 60% sealed inside-out vesicles. The sidedness of the vesicles during the insulin binding assays remained essentially unchanged.

The results of insulin binding to these vesicle preparations are shown in Fig. 3. The binding profiles are curvilinear as in the case of the intact cells. In Table I, the insulin binding parameters at the high specificity binding sites for the intact cells and for both types of the vesicles are summarized.

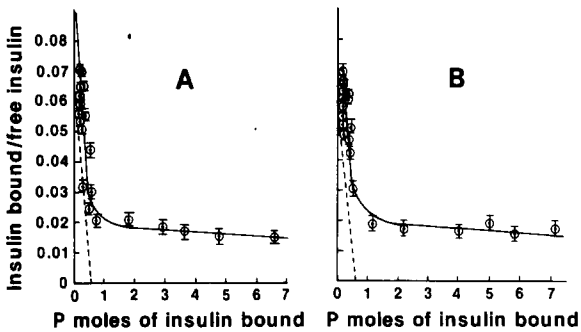

Fig. 3. Scatchard plot of insulin binding to right side-out (A) and inside-out vesicles (B). The sealed right side-out and inside-out vesicles were prepared as described in Fig. 2. The vesicles (0.4 ml) were added to a series of Beckman polyallomer tubes containing the same amount of ^{125}I-labeled insulin but varying amounts of unlabeled insulin (0.333 nM to 500 nM) in a final volume of 1 ml. Following gentle agitation of the tubes overnight at 4°C, the radioactivity of each tube was measured, and then the tubes were centrifuged at 195000 × g for 20 min at 4°C. The radioactivity of aliquots of the supernatant was measured. After removal of the supernatant, the wall of the tubes was wiped with moistened cotton swabs and then the sedimented vesicles were frozen by placing the tubes in holes made in a block of solid CO_2. The frozen pellet was transferred in one piece to a test tube with the use of a small spatula at the instant when the wall of the frozen tube began to thaw. The radioactivity of the transferred vesicles was measured and corrections for non-specific binding were made by subtracting the binding in the presence of excess unlabeled insulin (16.7 μM). The pellet was dissolved in 2 ml of 1 M NaOH by sonication. The protein content of each sample was determined according to the procedure of Lowry et al. [26]. The radioactivity bound by the vesicles was expressed per mg of protein. The results shown are the average of four separate experiments.

TABLE I

DISSOCIATION CONSTANTS (K_d) AND MAXIMUM NUMBER OF INSULIN BINDING SITES PER HUMAN RED BLOOD CELL AND VESICLES OF THE CELL AT HIGH SPECIFICITY BINDING SITES

The cell equivalent of vesicles was determined by comparing the amounts of proteins of the ghosts and the vesicles.

	Dissociation constants (K_d) (M)($\times 10^8$)	Maximum number of insulin binding sites per cell or cell equivalent of vesicles
Human erythrocytes	1.5 ± 0.5	50 ± 5
Right side-out vesicles	1.5 ± 0.5	17 ± 3
Inside-out vesicles	1.5 ± 0.5	17 ± 3

There is no significant difference in dissociation constant or maximum number of high affinity binding sites between the right side-out and inside-out vesicles. Data presented in Table I also show that although the dissociation constants for the intact cells and vesicles at the high-affinity binding site are practically the same, the maximum number of binding sites per cell equivalent vesicle is approximately one third of intact cells. These findings suggest that human erythrocytes have insulin receptors facing the cytoplasmic side as well as the outside face of the plasma membranes. If the observed insulin binding to the inside-out vesicle preparations were entirely due to contamination by right side-out vesicles, the insulin binding would have been less than 40% of the value we observed. Therefore, these data support the conclusion that insulin receptors are present on both sides of the erythrocyte membrane. It is of interest to note that in our previous studies [18], only about one-half of the labeled insulin-receptor complex was extracted from erythrocyte membranes with Triton X-102, perhaps indicating that there may be a difference in accessibility of the detergent to receptors on the outside and inside face of the cell membranes. Similar results have been reported for the asialoglycoprotein receptors of isolated hepatocytes [23].

The Scatchard plots (Figs. 1 and 3) of insulin binding data show curvilinearity. The curvilinearity has been attributed either to negative cooperativity between binding sites [27] or to two distinct

receptor populations of differing affinity for insulin [28]. Recently Donner [29] has reported that in hepatocytes, the observed curvilinearity of Scatchard plots was caused by the binding of insulin degradation products. However, this seems to be an unlikely possibility in our present studies, since Sephadex G-50 gel filtration chromatography of a Triton X-102 extract from a mixture of ^{125}I-labeled insulin and isolated erythrocyte ghosts which was maintained at 4°C overnight did not show any significant amount of ^{125}I-labeled insulin degradation products (Im, J.H., unpublished data).

The number of insulin binding receptors per human erythrocyte at the high affinity binding site reported in the literature varies greatly [1–17]. Quite discordant values have been published in different studies by the same investigators [7,8,15–17]. One of the reasons for the large variation in the insulin binding values may be due to the failure to control the extent of insulin binding to the plastic microfuge tubes used which is appreciable.

In the determination of insulin binding characteristics by equilibrium binding techniques, the following points should be noted. For any cell such as erythrocytes where insulin binding is exceedingly small, it is desirable to choose centrifuge tubes with a low insulin binding capacity, and to use a larger amount of both insulin and cells or membrane vesicles in order to obtain accurate insulin binding data. Furthermore, following the complete removal of the supernatant, withdrawal of an aliquot of sedimented cells for radioactivity determination will minimize errors due to ^{125}I-labeled insulin binding to the centrifuge tubes (see legend to Fig. 1).

Gorden et al. [30] and Goldfine et al. [31] reported that the initial binding of ^{125}I-labeled insulin to hepatocytes is restricted to the membrane, and that ^{125}I-labeled insulin molecules are symmetrically distributed on both sides of the plasma membrane. Further incubation resulted in systematic and progressive translocation of ^{125}I-labeled insulin to the inside of the cell (see also for review Ref. 32). Our findings that insulin binds equally well to right side-out and inside-out vesicles of human erythrocytes may provide additional supporting evidence for the internalization of the insulin-receptor in this cell type and raises the possibility that the human erythrocyte insulin receptor may participate in an endocytic/recycling pathway which mediates receptor-ligand internalization/externalization. Evidence supporting the downregulation (endocytotic internalization) of insulin receptors in human erythrocytes incubated with insulin has recently been presented [33], and also supports the recycling of insulin receptors. Since mature red cells lack intracellular organelles and the ability to synthesize proteins, receptor internalization/externalization within the domain of the plasma membrane would provide an effective mechanism by which this cell could bind, internalize, and degrade insulin while preserving receptor integrity. Extensive evidence for a reversible internalization/externalization pathway for other receptor-ligand systems has also been reported [23,34–39].

Recently, Baumann et al. [40] reported a high-affinity insulin binding to right side-out human erythrocyte membrane vesicles, but little or no binding to inside-out vesicles. These studies differed from ours in that binding experiments were carried out at 22°C for 3 h while we determined binding at 4°C for 16 h. Although both conditions for insulin binding were sufficient to achieve equilibrium, degradation of ^{125}I-labeled insulin, known to occur in red cells [41] and even with isolated membranes (Kim et al., unpublished observations), could be substantial at 22°C, while it is negligible at 4°C. If degradative activity is predominantly associated with the inside face of the erythrocyte membrane as appears probable, this might result in an apparent lack of ^{125}I-labeled insulin binding at this site. It is interesting that Peterson et al. [33] have reported that human erythrocytes whose receptors had been downregulated by exposure to insulin for 3 h at 37°C recover 90% of their specific binding of the hormone when incubated in insulin-free buffer an additional 16 h at 15°C. In contrast, incubating the cells for 16 h at 37°C instead of 15°C did not result in an increase in specific insulin binding.

In any event, our results are consistent with evidence that the erythrocyte insulin receptor participates in an endocytic/recycling pathway, while the results of Baumann et al. [40] argue against significant transmembrane movement of the insulin receptor. Further studies will be necessary to

resolve this discrepancy.

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References

1 Insel, J.R., Kolterman, O.G., Seakow, M. and Olefsky, J.M. (1980) Diabetes 29, 132–139
2 DePirro, R., Fusco, A., Lauro, R., Testa, I., Ferreti, F. and DeMartinis, C. (1980) Diabetes 29, 96–99
3 Pederson, O., Beck-Nielsen, H. and Heding, L. (1980) N. Engl. J. Med. 302, 886–892
4 Dons, R.F., Corash, L.M. and Gorden, P. (1981) J. Biol. Chem. 256, 2982–2987
5 Gambhir, K.K., Archer, J.A. and Carter, L. (1977) Clin. Chem. 23, 1590–1595
6 Eng, J., Lee, L. and Yalow, R. (1980) Diabetes 29, 164–166
7 Herzberg, V., Boughter, J.M., Carlisle, S. and Hill, D.E. (1980) Nature 286, 279–281
8 Herzberg, V.L., Boughter, J.M., Carlisle, S.K., Ahmad, F. and Hill, D.E. (1980) Pediatr. Res. 14, 4–7
9 Sinha, M.K., Ganguli, S. and Sperling, M.A. (1981) Diabetes 30, 411–415
10 Kappy, M.S. and Plotnick, L. (1979) Diabetes 28, 1001–1005
11 Kobayashi, M., Ohgaku, S., Iwasaki, M., Harano, Y., Maegawa, H. and Shigeta, Y. (1980) Endocrinol. Japan 27, 337–342
12 Tsibris, J.C.M., Raynor, L.O., Buhi, W.C., Buggie, J. and Spellacy, W.N. (1980) J. Clin. Endocrinol. Metab. 51, 711–717
13 Wachslicht-Rodbard, H., Gross, H.A., Rodbard, D., Ebert, M.H. and Roth, J. (1979) N. Engl. J. Med. 300, 882–887
14 Yasuda, K. and Kitabchi, A.E. (1980) Diabetes 29, 811–814
15 Robinson, T.J., Archer, J.A., Gambhir, K.K., Hollis, Jr., V.W., Carter, L. and Bradley, C. (1979) Science 205, 200–202
16 Gambhir, K.K., Nerurkar, S.G., Cruz, I.A. and Hosten, A.O. (1980) Horm. Metab. Res. 12, 414–415
17 Gambhir, K.K., Archer, J.A. and Bradley, C.J. (1978) Diabetes 27, 701–708
18 Im, J.H., Meezan, E., Rackley, C.E. and Kim, H.D. (1983) J. Biol. Chem. 258, 5021–5026
19 Im, J.H., Meezan, E. and Kim, H.D. (1980) Fed. Proc. 40, 441
20 Kant, J.A. and Steck, T.L. (1972) Nature New Biology 240, 26–28
21 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 30, 172–180
22 Crane, F.L., Crane, H.E., Sun, L., MacKellar, W.C., Greb-ing, C. and Low, H. (1982) J. Bioenerg. Biomembranes 14, 425–433
23 Weigel, P.H. and Oka, J.A. (1982) J. Biol. Chem. 257, 1201–1207
24 Kim, H.D. and Luthra, M.G. (1977) J. Gen. Physiol. 70, 171–185
25 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biol. Chem. 193, 265–275
27 DeMeyts, P., Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877–1888
28 Krupp, M.N. and Livingston, J.N. (1978) Proc. Natl. Acad. Sci. USA 75, 2593–2597
29 Donner, D.B. (1980) Proc. Natl. Acad. Sci. USA 77, 3176–3180
30 Gorden, P., Carpentier, J.L., Freychet, P. and LeCam, A. (1978) Science 200, 782–785
31 Goldfine, I.D., Jones, A.L., Hradek, G.T. and Wong, K.Y. (1981) Endocrinology 108, 1821–1828
32 Gorden, P., Carpentier, J.L., Freychet, P. and Orci, L. (1980) Diabetologia 18, 263–274
33 Peterson, S.W., Miller, A.L., Kelleher, R.S. and Murray, E.F. (1983) J. Biol. Chem. 258, 9605–9607
34 Weigel, P.H. and Oka, J.A. (1983) J. Biol. Chem. 258, 5095–5102
35 Weigel, P.H., Ray, D.A. and Oka, J.A. (1983) Anal. Biochem. 133, 437–449
36 Weigel, P.H. (1980) J. Biol. Chem. 255, 6111–6120
37 Tolleshaug, H., Chindemi, P.A. and Rogoeczi, E. (1981) J. Biol. Chem. 256, 6526–6528
38 Weigel, P.H. (1981) Biochem. Biophys. Res. Commun. 101, 1419–1425
39 Tolleshaug, H. and Berg, T. (1979) Biochem. Pharmacol. 28, 2919–2922
40 Baumann, G., Abramson, E.C. and Drobny, E.C. (1983) J. Cell. Biochem. 21, 233–237
41 Gambhir, K.K., Nerurkar, S.G., Das, P.D., Archer, J.A. and Henry, W.L. (1981) Endocrinology 109, 1787–1789