Erythrocyte Insulin Receptors in Non-insulin-dependent Diabetes Mellitus

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SUMMARY

Insulin binding was studied on circulating erythrocytes isolated from 12 normal and 12 untreated, adult, nonobese, nonketotic, non-insulin-dependent diabetic subjects. Insulin binding in normal subjects was higher than in diabetics ($P < 0.01$); binding variation was caused mainly by a reduction in insulin receptor concentration. Insulin binding was inversely correlated with fasting serum insulin levels ($R = 0.49$; $P < 0.01$). The close agreement between the present data and previous data on other cell populations suggests that isolated erythrocytes may be a useful tool in clinical investigations on the human insulin receptor.

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Now that insulin receptors have been identified on the monocyte cell membrane,[1,2] it is no longer necessary to take biopsy specimens when doing human studies.[3–11] A major drawback in the use of the monocyte method, however, is the large quantity of blood (100 ml or more) required for each receptor study, because of the low monocyte concentration in white blood cells.

It has recently been shown that insulin receptors are also present on circulating erythrocytes,[12,13] and their binding characteristics are almost identical to those of the monocyte system. Since a sufficient number of erythrocytes are easily isolated from 15 ml of blood, it was suggested that they may provide an ideal model for clinical evaluation of insulin receptors.[12] Nevertheless, before replacing the monocyte model, the insulin receptor present on the erythrocyte should be evaluated in some of the conditions previously studied on other cell membranes.

In the present investigation the insulin receptor on erythrocytes in normal subjects is compared with that in subjects with non-insulin-dependent diabetes mellitus.

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MATERIAL AND METHODS

Subjects. 12 male volunteers with normal glucose tolerance and 12 nonketotic, non-insulin-dependent diabetic male patients who had fasting blood glucose concentrations >140 mg $\cdot$ dl^{-1} on at least three occasions were studied. All subjects were of normal body weight (Geigy table, 7th edit.), and none presented with any disease other than diabetes. None was taking any drug known to affect carbohydrate or insulin metabolism. No females were included in the study, because there is a wide variation in insulin receptors during the menstrual cycle.[4,14]

Buffer used for binding studies consisted of Hepes (50 mM), Tris (50 mM), $MgCl_2$ (10 mM), EDTA (2 mM), dextrose (10 mM), $CaCl_2$ (10 mM), NaCl (50 mM), KCl (5 mM), and bovine serum albumin (0.1%; pH 8 at 15°C).[12,13]

Preparation of cells. Blood (15 ml) was collected from an antecubital vein in tubes containing 4 ml 3.8% sodium citrate. Each sample was diluted with isotonic saline to a final volume of 30 ml and then transferred on a Ficoll-Angiographin gradient for cell fractionation according to the method of Boyum.[15] Saline, lymphomonocytes, Ficoll-Angiographin, granulocytes, and the upper layer of the erythrocyte phase were aspirated after centrifugation ($600 \times g$ at 4°C for 30 min). The cell pellet was resuspended with 20 ml isotonic saline and the above procedure repeated. The new eythrocyte pellet was washed first with 20 ml isotonic saline and then with 20 ml buffer. The last pellet was suspended with buffer at the final cell concentration; contamination by mononuclear cells was minimal. Cell concentration was determined using an automatic Coulter counter (Royco 920-A, California) and by hematocrit.

Binding studies were performed as previously described.[12,13] Erythrocytes ($2.5 \times 10^9 \times$ ml^{-1}) were incubated at 15°C with ^{125}I-insulin (34 pmol/L, 110–130 μCi/μg, Sorin, Italy), with or without varying amounts of native insulin (Organon, Holland), in a total volume of 0.5 ml. After 210 min of incubation, duplicate 200-μl samples were placed in 1.5-ml

TABLE 1
Clinical and metabolic characteristics of the study group*

	No.	Age (yr)	Wt (kg)	Ht (cm)	Fasting blood glucose (mg · dl^{-1})	Fasting serum insulin (μU/ml)	Specific cell† binding fraction (2.5 × 10^9 eryth./ml)
Normals	12	48 ± 10	72 ± 11	169 ± 10	85 ± 5	10.4 ± 1.7	6.39 ± 0.50
Diabetics	12	49 ± 11	69 ± 10	165 ± 9	208 ± 30	14.5 ± 2.0	5.39 ± 0.56

* Mean ± SD.
† Insulin specifically bound/total insulin (× 10^{-2}) at the tracer concentration of 34 pmol/L.

tubes containing 0.8 ml ice-cold buffer and 200 μl di-n-butyl phthalate (density=1.044). Cell-bound and free insulin were separated by centrifugation in a Microfuge B (Beckman Instruments, Fullerton, California) for 2.5 min. TCA precipitation or rebinding of supernatants with freshly prepared cells showed that ^{125}I-insulin degradation was less than 3%. Nonspecific binding was defined as the amount of labeled insulin that remained bound in the presence of 17 μmol/L of native insulin. Nonspecific binding was about 16% of total binding (~1% of total radioactivity).

Analytic procedures. Plasma glucose was determined by the glucose-oxidase method using a Beckman glucose analyzer.[16] Serum immunoreactive insulin was measured with the method described by Herbert et al.;[17] the coefficient of interassay variation was 8.1% and that of intra-assay was 7.8%.

Statistical analysis. Analysis of variance was employed to compare results from normal and diabetic subjects. Spearman's coefficient of rank was applied in correlation studies.

RESULTS

Clinical characteristics and results are briefly reported in Table 1. The specific cell-binding fraction in normal subjects (6.39 ± 0.05) is higher than in diabetics (5.39 ± 0.56)($P < 0.01$;df = 1.22;F = 18.57). In the presence of a single class of binding sites exhibiting negative cooperativity, the decrease in binding is caused mainly by a decreased receptor concentration (R_0) rather than by a change in receptor affinity ($\bar{K}_e$), even if a change in receptor affinity cannot be excluded (Figure 1). On the other hand, if the so-called "apparently higher affinity binding sites" are taken into account, it can be seen that changes are due only to variations in receptor concentration (Figure 2).

A significant inverse relationship ($R = 0.49$, $P < 0.01$) was found between fasting serum insulin concentration and the insulin-specific cell-binding fraction (Figure 3).

DISCUSSION

Insulin binding on erythrocytes from non-insulin-dependent diabetics is significantly lower than it is in normal subjects. According to the negative cooperativity model,[18] the decrease in binding is caused mainly by a lower concentration of insulin receptors rather than by a change in receptor affinity; on the other hand, a change in receptor affinity cannot be excluded, since average affinity profiles do not entirely overlap (Figure 1,inset). In this regard, it is well known that the slope of the profiles is considerably influenced by the R_0

FIGURE 1. Scatchard plot[39] of mean values from competition-inhibition studies in 12 normal subjects (■——■) and 12 non-insulin-dependent diabetic subjects (●——●) in fasting conditions. Bound and free ratio of ^{125}I-insulin (B/F) is plotted as a function of the insulin bound. Extrapolation of the curve to the horizontal axis is used to obtain the total receptor concentration (R_0) (Inset[18]) Average affinity ($\bar{K}$) is plotted as a function of the occupancy of insulin receptors at that concentration of insulin (B/R_0). Theoretically, $\bar{K}_e$ represents the highest affinity reached at zero occupancy of the insulin receptor. In practice, $\bar{K}_e$ has been taken to be equal to $\bar{K}$ at an insulin concentration of 0.2 ng/ml (34 pmol/L).

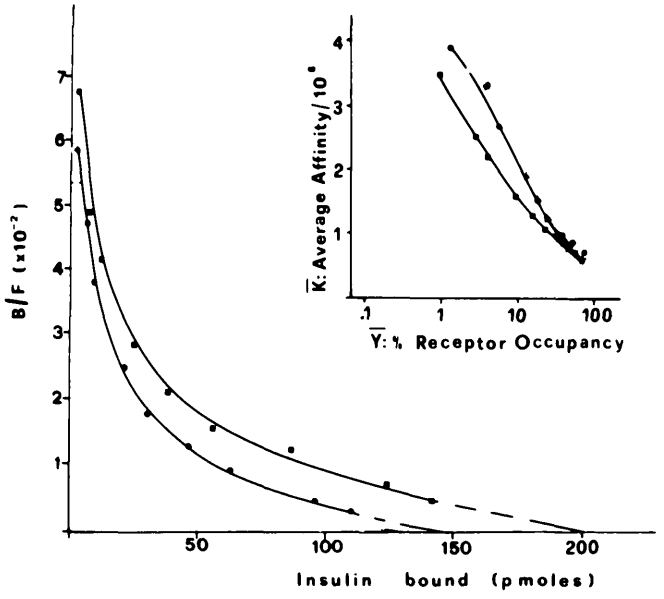
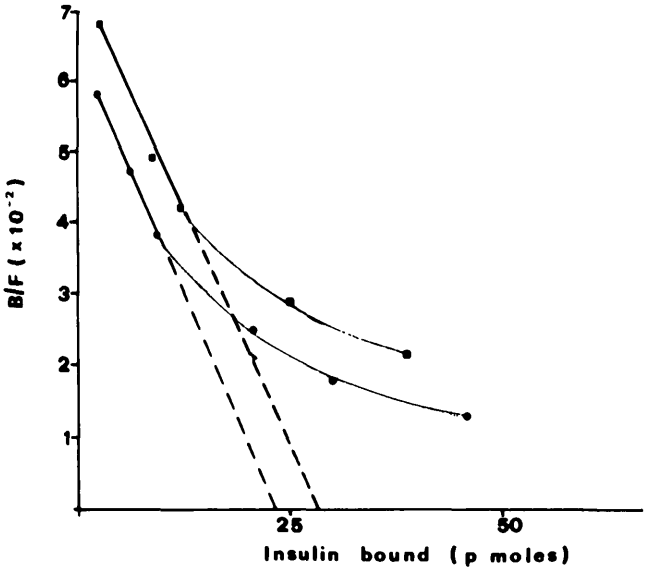


FIGURE 2. Scatchard plots, given in figure 1, on a larger scale. Straight lines represent so-called "apparently higher affinity binding sites".



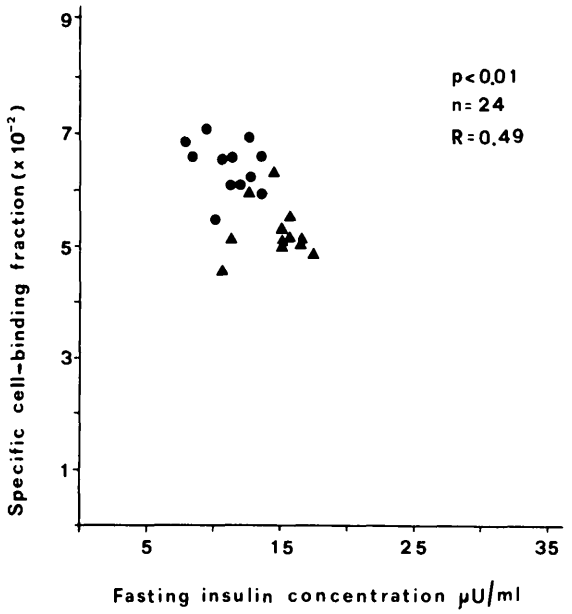


FIGURE 3. Relationship between specific cell-binding fraction (per 2.5 × 10^9 erythrocytes/ml) and fasting serum insulin level in 12 normal subjects (●) and 12 non-insulin-dependent diabetic subjects (▲).

value, which is an extrapolation and is grossly evaluated.[19,20]

The question of negative cooperativity[21] versus the presence of two or more classes of binding sites,[22] or a single class of sites not exhibiting negative cooperativity,[23] is still under debate; therefore, at present, the so-called "apparently higher affinity binding sites" should also be taken into consideration. According to this model the decrease in binding is caused only by a reduction of insulin receptors. The present data are in agreement with results obtained on other tissues.[8,10,11,24–30]

Subsequent to the hypothesis advanced by Gavin et al.[31] that the insulin receptor may be subject to negative feedback regulation by the height of ambient insulin concentration (downregulation), significant negative correlations between insulin binding and fasting insulin levels have been found in certain hyperinsulinemic states.[3,9,32–34] In the present study, a negative correlation was found between insulin binding and fasting serum insulin levels, suggesting that insulin might also regulate the erythrocyte insulin receptor. On the other hand, the ability of insulin to modulate its receptor is based primarily on studies in vitro, and situations in which insulin receptor variations do not appear to be related to insulinemia have been described.[4–7,35,36] The relationship found might, thus, be an epiphenomenon of a more important, controlling influence.

These data suggest that changes exist in the erythrocyte insulin receptor associated with or occurring with diabetes mellitus in a similar form to those described for other cell populations. Therefore, even if excessive amounts of cells have to be employed (because of the low number of receptor sites per cell), sufficient information about the insulin receptor may be obtained from erythrocytes.

The close parallelism between findings on monocytes[8,10,11] and those on erythrocytes seems to support the possibility that the erythrocyte model might replace the monocyte model in future clinical studies; this would undoubtedly have certain advantages. In fact, about 15 ml of blood is used for each erythrocyte receptor determination versus 80–100 ml for monocytes. Moreover, erythrocytes can easily be separated as a pure preparation, while monocyte samples are composed of a mixed cell population of lymphocytes, monocytes, and granulocytes.[1,2,5] The biologic role of insulin receptors present on monocytes and erythrocytes has not yet been elucidated, and, even if it has been suggested that monocyte insulin reflect a similar situation to that occurring on the cell membrane of other tissues,[33] it has not yet been shown that insulin binding on different cells is regulated by the same factors. In this respect, it must be borne in mind that the erythrocyte has certain peculiarities distinguishing it from other cells (lack of nucleus and lack of internalization of cell surface proteins). Thus, if it is assumed that the nucleus plays a role in insulin action and that cells internalize insulin as a consequence of insulin receptor interaction,[37,38] some differences might also exist between erythrocytes and other cells.

In conclusion, the present report demonstrates that, in agreement with previous studies on other cell populations, non-insulin-dependent diabetes mellitus is associated with a loss of insulin receptors on the erythrocyte membrane, thus suggesting that erythrocytes might be used in clinical studies instead of monocytes. In this respect, it is interesting to note that the monocyte and the erythrocyte insulin receptors from three obese subjects (~200% of ideal body weight) have shown the same behavior during a 5-day-fasting period (data not reported) and that patients with impaired glucose tolerance in basal conditions have a reduced number of erythrocyte insulin receptors in comparison with normals.[40] Nevertheless, before replacing the monocyte model, further comparative studies are required.

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REFERENCES

[1] Schwartz, R. H., Bianco, A. R., Handwerger, B. S., and Kahn, C. R.: Demonstration that monocytes rather than lymphocytes are the insulin-binding cells in preparation of human peripheral blood mononuclear leukocytes preparation: implication for studies of insulin-resistance states in man. Proc. Natl. Acad. Sci. USA. 72:474–78, 1975.

[2] Beck-Nielsen, H., Pedersen, O., Kragballe, K., and Sørensen N. S.: The monocyte as a model for the study of insulin receptors in man. Diabetologia *13*:563–69, 1977.

[3] Bar, R. S., Gordon, P., Roth, J., and Kahn, C. R.: Fluctuations in the affinity and concentration of the insulin receptors on circulating monocytes of obese patients. J. Clin. Invest. *58*:1123–35, 1976.

[4] De Pirro, R., Fusco, A., Bertoli, A., Greco, A. V., and Lauro, R.: Insulin receptors during the menstrual cycle in normal women. J. Clin. Endocrinol. Metab. *47*:1387–89, 1978.

[5] De Pirro, R., Bertoli, A., Greco, A. V., Gelli, A. S., and Lauro, R.: The effect of food intake on insulin receptor in man. Acta Endocrinol. (Copen) *90*:473–80, 1979.

[6] De Pirro, R., Tamburrano, G., Fusco, A., Natoli, C., and Lauro, R.: Insulin receptor during arginine infusion test in man. Diabetologia *15*:227, 1978. Abstract.

[7] Muggeo, M., Bar, R. S., and Roth, J.: Change in affinity of insulin receptors following oral glucose in normal adults. J. Clin. Endocrinol. Metab. *44*:1206–09, 1977.

[8] Beck-Nielsen, H.: The pathogenic role of an insulin-receptor defect in diabetes mellitus of the obese. Diabetes *27*:1175–81, 1978.

[9] Beck-Nielsen, H. Pedersen, O., Bagger, J. P., and Sorensen, N. S.: The insulin receptor in normal and obese persons. Acta Endocrinol. (Copen) *83*:565–75, 1976.

[10] Olefsky, J. M., and Reaven, G. M.: Insulin binding to monocytes and total mononuclear leukocytes from normal and diabetic patients. J. Clin. Endocrinol. Metab. *43*:232–34, 1976.

[11] Olefsky, J. M., and Reaven, G. M.: Insulin binding in diabetes. Diabetes *26*:680–88, 1977.

[12] Gambhir, K. K., Archer, J. A., and Bradley, C. J.: Characteristics of human erythrocyte insulin receptors. Diabetes *27*:701–08, 1978.

[13] Lauro, R., Greco, A. V., Bertoli, A., Fusco, A., Spallone, L., Magnatta, R., and De Pirro, R.: A study on insulin receptors of human erythrocytes. LAB, J. Res. Lab. Med. 6:297–308, 1979.

[14] De Pirro, R., Bertoli, A., Greco, A. V., Fusco, A., and Lauro, R.: Differences in erythrocyte insulin receptor between man and woman. Acta Endocrinol. (Copen) (Suppl.) *225*:310, 1979. Abstr.

[15] Boyum, A.: Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. *21*(Suppl. 97):77–89, 1968.

[16] Hugget, A. St. G., and Nixon, D. A.: Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. Lancet *2*:368–70, 1957.

[17] Herbert, V., Lan, K. S., Gottlieb, C. W., and Bleicher, S.: Coated charcoal immunoassay of insulin. J. Clin. Endocrinol. Metab. *25*:1375–84, 1965.

[18] De Meyts, P., and Roth, J.: Cooperativity in ligand binding: a new graphic analysis. Biochem. Biophys. Res. Commun. *66*:1118–26, 1975.

[19] Muggeo, M., Bar, R. S., Roth, J., Kahn, C. R., and Gorden, P.: The insulin resistance in acromegaly: evidence for two alterations in the insulin receptor on circulating monocytes. J. Clin. Endocrinol. Metab. *48*:17–25, 1979.

[20] Beck-Nielsen, H., and Pedersen, O.: Diurnal variation in insulin binding to human monocytes. J. Clin. Endocrinol. Metab. *47*:385–90, 1978.

[21] Pollet, R. J., Standaert, M. L., and Haase, B. A.: Insulin binding to the human lymphocyte receptor. Evaluation of the negative cooperativity model. J. Biol. Chem. *252*:5828–34, 1977.

[22] Krupp, M. N., and Livingstone, J. N.: Insulin binding to solubilized material from fat cell membranes: evidence for two binding species. Proc. Natl. Acad. Sci. USA *75*:2593–97, 1978.

[23] Maturo, J. M., III, and Hollemberg, M. D.: Insulin receptor: interaction with nonreceptor glycoprotein from liver cell membranes. Proc. Natl. Acad. Sci. USA *75*:3070–74, 1978.

[24] Kern, P., Picard, J., Caron, M., and Veissiere, D.: Decreased binding of insulin to liver plasma membrane receptors in hereditary diabetic mice. Biochim. Biophys. Acta *389*:281–89, 1975.

[25] Harrison, L. C., Billington, T., Clark, S., Nichols, R., East, I., and Martin, F.: Decreased binding of insulin by receptors on placenta membranes from diabetic mothers. J. Clin. Endocrinol. Metab. *44*:206–09, 1977.

[26] Freychet, P., and Forgue, E.: Insulin receptors in the heart muscle: impairment of insulin binding to the plasma membrane in the obese hyperglycemic mouse. Diabetes *23*(Suppl. 1):354, 1974. Abstr.

[27] Freychet, P., Laudat, M. H., Laudat, P., Rosselin, G., Kahn, C. R., Gorden, P., and Roth, J.: Impairment of the insulin binding to the fat cell membrane in the obese hyperglycemic mouse. FEBS Lett. *25*:339–42, 1972.

[28] Soll, A. H., Goldfine, I. D., Roth, J., and Kahn, C. R.: Thymic lymphocytes in obese (ob/ob) mice: a mirror of the insulin receptor defect in liver and fat. J. Biol. Chem. *249*:4127–31, 1974.

[29] Dahms, W. T., Atkinson, R. L., and Bray, G. A.: Insulin resistance and the binding of insulin to adipocytes. Clin. Res. *24*:116A, 1976. Abstract.

[30] Goldstein, S., Blecher, M., Binder, R., Perrino, P. V., and Recant, L.: Hormone receptors: binding of glucagon and insulin to human circulating mononuclear cells in diabetes mellitus. Endocr. Res. Commun. *2*:367–76, 1975.

[31] Gavin, J. R., III, Roth, J., Neville, D. M., Jr., De Meyts, P., and Buell, D. N.: Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture, Proc. Natl. Acad. Sci. USA *71*:84–88, 1974.

[32] Olefsky, J. M.: Decreased insulin binding to adipocytes and circulating monocytes from obese persons. J. Clin. Invest. *57*:1165–72, 1976.

[33] Olefsky, J. M.: The insulin receptor: its role in insulin resistance of obesity and diabetes. Diabetes *25*:1154–64, 1976.

[34] Harrison, L. C., Martin, F. I., and Melick, R.: Insulin receptor binding in isolated fat cells and insulin sensitivity in obesity. J. Clin. Invest. *58*:1435–41, 1976.

[35] Bennet, G. V., and Cuatrecasas, P.: Insulin receptor of fat cells in insulin-resistant metabolic states. Science *176*:805–06, 1972.

[36] Kahn, C. R., Goldfine, I. D., Neville, D. M., Jr., and De Meyts, P.: Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. Endocrinology *103*:1054–66, 1978.

[37] Goldfine, I. D., and Smith, G. J.: Binding of insulin to isolated nuclei. Proc. Natl. Acad. Sci. USA *73*:1427–31, 1976.

[38] Goldfine, I. D., Jones, A. L., Hradek, G. T., Wong, K. Y., and Mooney, J. S.: Entry of insulin into human cultured lymphocytes: electron microscope autoradiographic analysis. Science *202*:760–63, 1978.

[39] Scatchard, G.: The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. *51*:660–72, 1949.

[40] De Pirro, R., Fusco, A., Lauro, R., Testa I., Ferretti, F., and De Martinis, C.: Erythrocyte insulin receptor in patients with impaired glucose tolerance. Submitted for publication.