Measurement of Insulin Binding by Erythrocyte Ghosts

A Method That Allows Storage Without Loss of Binding Characteristics

PAUL F. WILLIAMS, SUE K. WATSON, DENNIS K. YUE, AND JOHN R. TURTLE

SUMMARY

The insulin binding of red cell ghosts was studied and found to be similar to insulin binding of the original erythrocyte. Red cell ghosts were stable on storage, and serial samples can be measured in one assay, thus eliminating the problem of interassay variation. Erythrocyte ghosts may be preferable to whole red cells for long-term insulin receptor studies. DIABETES 32:644–647, July 1983.

Binding of insulin to human erythrocytes has been used to examine insulin receptor changes in obesity,[1,2] chronic renal failure,[3] anorexia nervosa,[4] diabetes,[5,6] experimental hyperinsulinemia,[7] and during the menstrual cycle.[8] Changes in tracer insulin binding to erythrocyte receptors have correlated well with insulin sensitivity and insulin binding in other tissues.[9] In pregnancy this correlation does not occur.[10,11] Red cell insulin receptor concentrations and binding have been shown to change in relation to the stage of maturity of the erythrocyte.[12–15] Insulin binding decreases exponentially with cell age, but insulin binding in any sample was not wholly dependent on the reticulocyte concentration.[15]

Problems associated with erythrocyte insulin receptor assays include low binding,[16] high nonspecific binding,[16–19] and interassay variation.[20] These are difficulties encountered frequently in the study of membrane receptors. In monitoring the erythrocyte insulin receptor changes during treatment of diabetes, interassay variation may introduce significant errors. Storage of red blood cells at 4°C does not halt the loss of receptor in vitro and cryopreservation of erythrocytes does not preserve all membrane components.[21] In order to allow samples from different time periods to be assayed together, we have investigated insulin binding by human erythrocyte ghosts stored for up to 2 yr at −70°C and have shown that they retain the receptor binding characteristics of the original red cell. The use of erythrocyte ghosts in insulin binding studies allows detection of sequential changes in insulin receptor parameters with increased precision.

From the Department of Endocrinology, Royal Prince Alfred Hospital, and Department of Medicine, the University of Sydney, Australia.
Address reprint requests to Dr. Paul F. Williams, Department of Medicine, University of Sydney, Sydney, N.S.W., Australia 2006.
Received for publication 6 October 1982.

METHODS

Assay conditions. Blood samples (20 ml) from diabetic patients attending the Diabetic Clinic between 0900 and 1500 h were collected into ammonium heparin tubes. The specimens were centrifuged at room temperature and the plasma removed. Control samples were obtained from healthy individuals of comparable age, within 10% of ideal body weight, and with no history of carbohydrate intolerance. Reticulocyte counts were performed routinely and one sample with greater than 1% reticulocytes was discarded. Cells were washed twice with an equal volume of isotonic saline and stored at 4°C overnight. Tubes with visible hemolysis were discarded since it increased nonspecific binding and affected specific binding randomly. This did not occur commonly provided the samples were centrifuged and the cells stored at 4°C within a few hours of venepuncture. Red cells were prepared using a Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) modification of the Boyum gradient technique[22] and divided into two aliquots. From one, red cell ghosts were prepared by the Hanahan and Ekholm modification of the method of Dodge et al.,[23] and the other was used for the red cell insulin receptor assay. Both ghost and red cell insulin receptors were measured in a Hepes-Tris buffer (Hepes 50 mmol/L, Tris 50 mmol/L, glucose 10 mmol/L, NaCl 97.5 mmol/L, KCl 5 mmol/L, $CaCl_2$ 30 mmol/L, pH 8.0, bovine serum albumin 0.5% in the case of red cells and 1% in the case of ghosts). The total incubation volume of 300 μl was made up of red cell or ghost ($1–4 \times 10^9$/ml) in 200 μl of Hepes-Tris buffer, insulin ($5 \times 10^{-11}–10^{-6}$ mol/L) in 50 μl of Hepes buffer (50 mmol/L, pH 8.0, bovine serum albumin 2.5%), and ^{125}I-insulin in 50 μl of Hepes-Tris buffer. ^{125}I-Insulin was prepared to a specific activity of 160–220 mCi/mg,[24] stored at −70°C, and

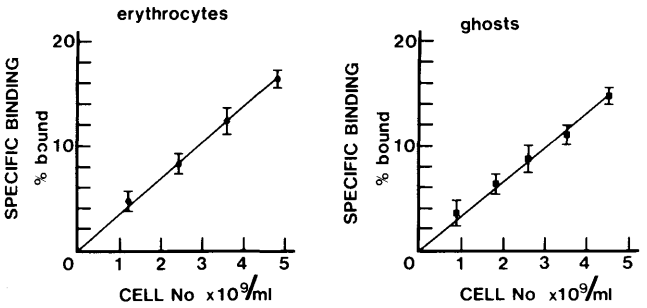

FIGURE 1. The relationship between insulin binding and erythrocyte or ghost numbers. The erythrocytes were prepared from a pooled sample of blood obtained from diabetic patients. The ghosts were prepared from the erythrocytes of a single healthy donor. Each point represents specific binding ± 1 SD of triplicate determinations.

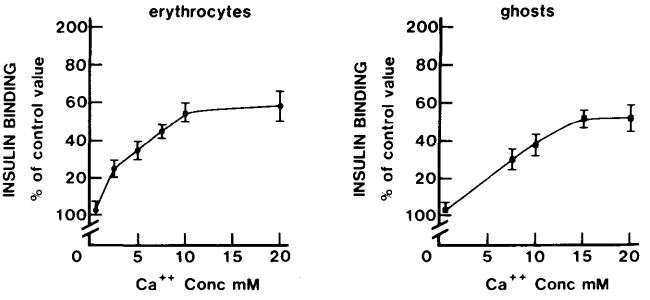

FIGURE 2. The effect of Ca^{2+} on insulin receptor of erythrocytes and erythrocyte ghosts. Erythrocytes (3.3 × 10^9/L) and their derived ghosts (2.0 × 10^9/L) were incubated at 4°C for 4 h in Hepes-Tris buffer containing varying Ca^{2+} concentration. Each point represents specific binding ± 1 SD of triplicate determinations.

used within 1 mo of iodination. The incubation mixture was shaken at 110 cycles/min at 4°C for 4 h. Afterward 1 ml of ice-cold Hepes-Tris buffer was added before the incubation mixture was centrifuged, decanted and washed again in 1 ml of Hepes-Tris buffer, and counted in a Wallac-LKB Gamma counter. Results were expressed as specific insulin binding per 4 × 10^9 red cells or ghosts. Red cell and ghost numbers were determined by counting on a Linson cello-scope (Linson Instruments AB, Sweden). Ghost numbers were also confirmed manually by counting on a Neubauer hemocytometer, and the two methods differed by less than 10%.

Effects of time, cell numbers, calcium concentration, and pH. To determine the time required to reach equilibrium, erythrocyte and ghost assays were incubated from 0 to 6 h. Total and nonspecific binding at tracer concentration were determined at hourly intervals. Incubations with increasing numbers of erythrocytes or ghosts in the assay medium were performed to study the relationship between insulin binding and red cell or ghost numbers up to a maximum of 5 × 10^9 cells/ml. Because of the effects of both Ca^{2+} and H^+ ions on erythrocyte insulin binding, a comparison of the effects of these ions on erythrocytes and ghosts was carried out. Buffers with various Ca^{2+} ion concentrations were kept isoosmotic by appropriate alterations to the Na^+ concentration. The pH of the incubation medium was varied between 6.0 and 8.2.

Effects of storage on red cell and ghost. To determine whether storage overnight affected the erythrocyte insulin binding, five samples were collected and each divided into two aliquots. One sample was analyzed immediately and the other was studied after storage overnight at 4°C in saline. The displacement curves generated were then tested for similarity using the "Allfit" program as described below.[25]

Batches of ghosts were prepared from three diabetic patients requiring venesection for hemochromatosis. Each was divided into three aliquots. The first was analyzed immediately and the other two were stored separately in Hepes-Tris buffer at −70°C and analyzed at monthly intervals. The three curves from each patient were again tested by the Allfit program.

Serial blood samples (N = 25) were obtained from eight diabetic patients during treatment of diabetes by diet therapy, oral hypoglycemic agents, or insulin. Red cell insulin receptors were measured within 24 h of obtaining the samples. Ghosts were prepared from the same samples but stored in Hepes-Tris buffer at −70°C and assayed in one batch. For this series of experiments only specific insulin binding at tracer concentration was examined.

Analysis of data. Dose-response curves for insulin displacement from the receptors of erythrocytes and erythrocyte ghosts were compared using the Allfit program.[25] This program tested statistically whether the erythrocyte or ghost insulin dose-response curves shared common binding parameters. The binding of insulin y (%) was described by the equation

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

where x = insulin concentration (mol/L); a = binding at zero dose (%); b = slope factor; c = 50% maximum effective dose (mol/L of insulin); and d = binding at infinite dose (%). Statistical evidence for the goodness of fit for any two curves was provided by a variance ratio F test; a nonsignificant F indicates that the curves share common binding parameters. A runs test was used to detect nonrandomness of residuals.[25]

TABLE 1
The binding parameters shared by freshly prepared red cell and ghost insulin receptors

Parameters	Normal	Diabetic 1	Diabetic 2
a	17.17 ± 0.92	12.60 ± 0.90	6.97 ± 0.15
b	0.23 ± 0.30	0.71 ± 0.12	1.25 ± 0.13
c	0.92 × 10^{-9} ± 0.18 × 10^{-9}	0.62 × 10^{-9} ± 0.17 × 10^{-9}	0.82 × 10^{-9} ± 0.07 × 10^{-9}
d	1.9 ± 0.9	0.21 ± 0.1	0.61 ± 0.13
	F = 1.8 (P = 0.18)	F = 1.46 (P = 0.3)	F = 0.47 (P = 0.8)

*a, b, c, and d are parameters obtained by Allfit that describe the insulin dose-response curves of both erythrocyte and ghost receptors. A nonsignificant value for F (P > 0.05) indicates that the curves share common binding parameters.

TABLE 2
The binding parameters shared by stored ghost insulin receptors

Parameters	Patient 1	Patient 2	Patient 3
a	3.39 ± 0.14	5.48 ± 0.83	4.0 ± 0.25
b	1.00 ± 0.18	0.28 ± 0.10	0.47 ± 0.12
c	$1.4 \times 10^{-9} \pm 0.28 \times 10^{-9}$	$3.9 \times 10^{-9} \pm 3.9 \times 10^{-9}$	$5.8 \times 10^{-9} \pm 3.8 \times 10^{-9}$
d	0.07 ± 0.18	−1.43 ± 1.54	−0.76 ± 0.75
	F = 1.5 (P = 0.23)	F = 0.89 (P = 0.54)	F = 1.23 (0.33)

*a, b, c, and d are parameters obtained by Allfit that describe the insulin dose-response curves of each sample of erythrocyte ghosts stored over a period of 2 mo and assayed on three occasions. A nonsignificant value for F (P > 0.05) indicates that the curves share common binding parameters.

Results were expressed as mean ± 1 SD. Linear correlation was calculated by the least-squares method.

RESULTS

The binding of insulin to ghosts and erythrocytes was related linearly to cell numbers up to 5×10^9/ml (Figure 1). Increased calcium concentration in the incubation medium was associated with increased insulin bindings to both erythrocytes and ghosts (Figure 2). For both erythrocytes and ghosts the maximal insulin binding was reached after 4 h of incubation at pH 8.0.

Insulin displacement curves for erythrocytes and their freshly prepared ghosts in 6 normal and 12 diabetic patients were not significantly different in any sample. Representative curves for one normal and two diabetic patients are illustrated in Figure 3 and the parameters best describing each pair of curves are given in Table 1.

Binding parameters of the ghost from three hemochromatotic patients did not change significantly during storage for 8 wk at −70°C. When analyzed with Allfit the series of curves from each patient was found to share the same parameters. Results are summarized in Table 2.

There was excellent correlation ($r = 0.92$, $P < 0.01$) between tracer insulin binding to erythrocytes (studied within 24 h of sample collection) and to ghosts (studied after a mean storage time of 13.5 ± 10.7 mo, range 1–27 mo). Results of four patients followed for longer than 3 mo are shown in Table 3.

Storage of erythrocytes (five specimens) for up to 30 h at 4°C had no effect on erythrocyte binding when the insulin displacement curves obtained before and after storage were analyzed by the Allfit program. Hemolysis and inaccuracy became serious problems for samples stored beyond this time.

The intraassay variation (N = 30) for the erythrocyte assay was 3.5% at the tracer binding point and 4% at the half-maximal displacement point. Due to the problems of storing intact red cells beyond 30 h it was not possible to estimate the long-term interassay variation of erythrocyte insulin receptors. Intraassay variation for erythrocyte ghosts (N = 27) was 3.5% for tracer binding and 4.2% for the half-maximal displacement point. Interassay variation of a ghost sample assayed over a 4-mo period on 13 occasions was 10.4% at tracer binding.

DISCUSSION

The erythrocyte insulin receptor offers several advantages as a model for studying the receptor changes seen in diabetes. It is readily available and does not require a large volume of blood samples. Insulin binding to erythrocytes decreases as an exponential function of the cell age and it is only in the younger cells that regulation of insulin receptor concentration may take place.[15] In diabetes a slight decrease in red cell survival has been recorded.[26] The shortened average cell age will tend to obscure the decrease of insulin receptor in diabetes. However, the difference in mean cell age cannot account for the difference between normal subjects and patients under treatment. Reticulocyte counts carried out on all samples allowed us to discard specimens that were obviously influenced by a decrease in average cell age.

TABLE 3
The binding of insulin to freshly prepared erythrocyte and stored ghost insulin receptors

	Insulin binding to receptors (%)					
	Initial*		1 mo*		3–6 mo*	
Patient	Erythrocyte†	Ghost†	Erythrocyte†	Ghost†	Erythrocyte†	Ghost†
V.V.	14.6	12.8 (12 mo)	15.8	15.1 (11 mo)	16.6	17.7 (9 mo)
R.C.	5.3	4.2 (27 mo)	7.0	5.5 (26 mo)	6.4	8.0 (21 mo)
E.D.	6.3	5.0 (4 mo)	10.2	9.2 (3 mo)	6.3	5.0 (1 mo)
M.M.	9.1	8.3 (6 mo)	—	—	15.1	17.1 (1 mo)

*Time from beginning of diet, oral hypoglycemic agents, or insulin treatment.
†Erythrocyte insulin receptors were measured within 24 h of obtaining the samples. Ghosts were stored and insulin receptors measured in one assay with the duration of storage shown in parentheses.

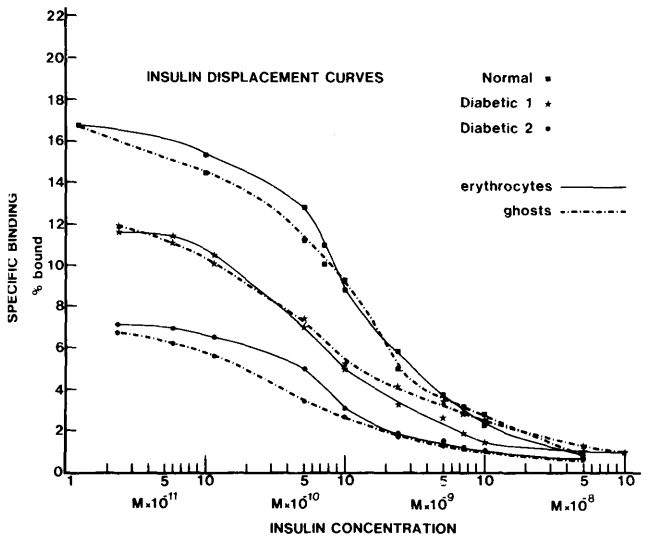

FIGURE 3. Competition curves for insulin binding to erythrocytes and ghosts from one normal and two diabetic patients. Each point is the mean of triplicate determinations for specific binding and normalized to 4 × 10^9 cells or ghosts/ml.

To monitor the changes of insulin receptor numbers in diabetic patients undergoing therapy, it is necessary to allow time for new erythrocytes to appear in the circulation. Therefore, interassay variation may be a complicating factor in the interpretation of results. Erythrocytes have a low number of receptors and insulin binding is sensitive to tracer damage. Receptor studies may be carried out on erythrocytes stored for 24–30 h at 4°C, but longer storage was not reported. Cryopreservation in glycerol has also been reported, but membrane protein changes with storage were not prevented.[21]

Erythrocyte ghost insulin receptors share many receptor properties of the original cells. The kinetics of insulin binding, the pH optima of the reaction, and the effect of Ca^{2+} are very similar for erythrocytes and their ghosts. The high Ca^{2+} concentration of the incubation medium used in this study was found to increase specific binding of insulin to both erythrocytes and ghosts. The inclusion of high Ca^{2+} concentration in the assay and storage buffer also served to prevent the formation of inside-out erythrocyte ghost vesicles.[23] The excellent agreement of insulin binding sites per cell between the red cell ghost and intact erythrocytes suggests that loss of insulin receptor due to the formation of inverted erythrocyte ghosts was not a serious problem. No difference can be found between the insulin displacement curves of erythrocytes and ghosts prepared at the same time. Even after storage for greater than 2 yr, binding to red cell ghosts reflects accurately the initial binding to the red cell of the same sample. Thus samples taken from patients over a considerable period may be compared in a single assay where interassay variation is eliminated completely. This will assist in quantitating the long-term changes in insulin receptors and evaluate the effects of various treatment regimens.

The red cell is a tissue readily obtainable with little discomfort to the patient. Good correlation between changes in monocyte and erythrocyte insulin receptor tracer binding had been demonstrated in studies of insulin sensitivity. However, the combinations of low bindings and high interassay variation limit the practical application of this model. The use of erythrocyte ghosts improves the flexibility and accuracy of the studies of red cell insulin receptors.

ACKNOWLEDGMENT

This work was supported by the John Claude Kellion Foundation.

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