Insulin causes insulin-receptor internalization in human erythrocyte ghosts

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The effect of incubation with insulin on insulin-receptor internalization by erythrocyte ghosts was investigated. The number of surface insulin receptors decreased by 30–40% after incubation of ghosts with insulin. Total insulin-receptor binding to solubilized ghosts was the same in insulin-incubated and control ghosts, whereas insulin binding to an internal vesicular fraction was substantially increased in insulin-incubated ghosts. Our findings suggest that erythrocyte-ghost insulin receptors are internalized to a vesicular compartment in response to incubation with insulin.

INTRODUCTION

Human erythrocytes, like a variety of eukaryotic cells, have high- and low-affinity surface insulin receptors. These erythrocyte insulin receptors have been shown to be down-regulated in response to cell-surface insulin binding (Peterson *et al.*, 1983; Scherthaner *et al.*, 1985) and are subsequently recycled to the cell surface (Peterson *et al.*, 1983). Although the number of surface insulin receptors on human erythrocytes is low (2000 receptors/cell) there is evidence that binding of insulin to these receptors stimulates glucose transport across the erythrocyte plasma membrane (Dustin *et al.*, 1984). The number of surface insulin receptors on erythrocytes decreases with age, and this decrease in surface receptors diminishes the capability of the remaining receptors to be down-regulated (Wilson & Peterson, 1986).

Because mature human erythrocytes lack intracellular organelles and protein-synthesizing components, insulin-receptor internalization in this system may require only the plasma membrane and underlying cytoskeletal components. For this reason, we conducted a study to determine whether insulin-receptor internalization in response to insulin occurs in haemoglobin-free resealed erythrocyte plasma membranes (ghosts). We report here that the number of insulin receptors on the surface of human erythrocyte ghosts decreases when ghosts have been exposed to insulin, whereas the total ghost content of receptors (soluble receptors) does not change. This decrease in surface receptors corresponds to an increase in specific insulin binding to an intracellular vesicular fraction. These results indicate that insulin receptors on the surface of erythrocyte ghosts are internalized in response to insulin in the medium.

EXPERIMENTAL

Materials

Fresh human erythrocytes, used to prepare erythrocyte ghosts, were obtained from the Center for Blood Research, Boston, MA, U.S.A. Mono[¹²⁵I]iodinated insulin (pig) was purchased from New England Nuclear. Insulin (pig), phenylmethanesulphonyl fluoride, bovine

serum albumin, bovine IgG and polyethylene glycol 8000 were purchased from Sigma.

Buffer G was used in all insulin binding assays. It consists of 50 mM-Tris, 50 mM-Hepes, 10 mM-MgCl₂, 2 mM-EDTA, 10 mM-glucose, 10 mM-CaCl₂, 50 mM-NaCl, 5 mM-KCl and 0.1% (w/v) bovine serum albumin.

Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared by the method of Ronner *et al.* (1977), by using cycles of erythrocyte haemolysis for 3 min at 4 °C in low-salt buffer, resealing of membranes in iso-osmotic buffer, and pelleting of resealed membranes. The low temperature and short interval of exposure of membranes to low-ionic-strength buffer is insufficient to cause removal of cytoskeletal components (Hargreaves *et al.*, 1980). After the final wash, pelleted ghost membranes were resealed with a final concentration of 2 mM-ATP inside, unless otherwise stated. Pelleted ghosts were suspended in iso-osmotic buffer G. Phenylmethanesulphonyl fluoride (final concn. 1 mM) was included in all washes. Ghosts, suspended in a 3% (w/v) dextran solution, appeared bright when viewed under phase light microscopy, indicating that they were impermeable to added dextran (Hargreaves *et al.*, 1980). Ghost protein was determined by the method of Lowry *et al.* (1951), and ghost phosphate content by the method of Lowry *et al.* (1954). Methods of biochemical characterization of ghosts are described at the end of this section.

Measurement of insulin binding

Specific insulin binding to the surface of ghosts was assayed by a method modified from that described by Gambhir *et al.* (1981), in which filtration, rather than centrifugation, was used to remove free from bound insulin. Quadruplicate samples of erythrocyte ghosts (3 mg of protein/ml) in a total volume of 0.25 ml were incubated for 90 min at 15 °C with ¹²⁵I-insulin, with or without an excess of unlabelled insulin. Samples were then diluted by addition of 0.75 ml of buffer G and filtered over 2.4 cm Whatman GF/A glass microfibre filters. Filters were rinsed with 3 × 3 ml of buffer G. The radioactivity retained on the filters was counted in a

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gamma counter. Specific binding was calculated as the difference between total (incubated without excess of unlabelled insulin) and non-specifically bound label (incubated with 0.1 mg of unlabelled insulin/ml). Non-specific binding was 10–20% of total binding. Statistical significance of differences in specific insulin binding was determined by using a non-directional two-tailed Student's *t* test.

For Scatchard-analysis experiments, erythrocyte ghosts were incubated with 0.5 ng of ^{125}I -insulin and various amounts of unlabelled insulin (0–0.001 mg) in buffer G (Gambhir *et al.*, 1977) in a total volume of 0.25 ml. The suspension was then incubated at 15 °C for 90 min. Samples, in quadruplicate, were filtered over Whatman GF/A glass microfibre filters as described above. The radioactivity retained on the filters was counted in a gamma counter. The resulting data were analysed by the method of Scatchard (1949) and fitted to a non-co-operative computer model. Binding constants were generated from Scatchard plots by using a best-fit computer program with an iterative approximation method. Both binding constants and receptor numbers for high-affinity receptors were adjusted to minimize deviation of the B/F (bound/free) value.

Insulin incubation conditions

In order to determine effects of insulin on insulin binding, ghosts were incubated with or without 1 μg of insulin/ml for 3 h at 37 °C, then washed with 3×5 volumes of buffer G (pH 7.5) over a 3 h period. Specific binding to the surface receptors was measured by the ^{125}I -insulin radio-receptor assay described above.

Low-pH washes to remove surface-bound insulin

To remove surface-bound insulin specifically, ghosts were treated with a low-pH solution, in accordance with a modification of the method of Haigler *et al.* (1980). Erythrocyte ghosts (3 mg/ml) were incubated with ^{125}I -insulin (0.5 ng) with and without 1 μg of insulin/ml at 4 °C and 37 °C for 3 h. After the incubation, ghosts were washed twice in 4 vol of 0.2 M-acetic acid in buffer G (pH 2.86) solution. Cell-associated radioactivity was then determined.

Assay for soluble insulin receptors

The soluble-receptor assay used was a modification of the procedure described previously for erythrocytes (Dustin *et al.*, 1984). Erythrocyte ghosts, at an original concentration of 3 mg of protein/ml, were solubilized for 60 min at 4 °C in buffer G containing Triton X-100 at a final concentration of 1%. Binding to solubilized receptors was determined by incubating the suspension at a final Triton X-100 concentration of 0.08% after dilution with buffer G (pH 7.9) with 25 ng of ^{125}I -insulin, with or without excess insulin, in a final volume of 0.25 ml. After 1 h at 24 °C, the receptor-hormone complexes were precipitated by polyethylene glycol with bovine IgG as a carrier (Cuatrecasas, 1972), collected, and washed on Whatman GF/F glass microfibre filters. Specific binding was calculated as the difference between total binding (without excess insulin) and non-specific binding (with excess insulin).

Isolation of internalized vesicular fraction

Isolation of an internalized vesicular fraction from erythrocyte ghosts was accomplished by differential

centrifugation by modification of the method described by Stadel *et al.* (1983). A 3 ml suspension of erythrocyte ghosts (3 mg/ml) was lysed with 10 mM-Tris/HCl/0.6 mM-phenylmethanesulphonyl fluoride and centrifuged at 40000 *g* for 10 min to pellet the plasma membrane. Supernatant was removed to a level far enough above the pellet to avoid contamination with plasma membrane. The supernatant was centrifuged at 158000 *g*_{av} for 1.5 h to pellet the light vesicular fraction.

Assays for plasma-membrane markers

Accessibility of externally oriented (sialic acid residues) and cytoplasm-facing (NADH-cytochrome *c* oxidoreductase and glyceraldehyde-3-phosphate dehydrogenase) plasma-membrane markers was determined as described by Steck & Kant (1974). Total marker content was determined by permeabilizing membrane vesicles with either Triton X-100 or saponin.

RESULTS AND DISCUSSION

Scatchard analysis of insulin binding to human erythrocyte ghosts

Erythrocyte ghosts prepared from intact human erythrocytes retain surface insulin receptors that have similar insulin-binding parameters to those of the original receptors. A Scatchard plot of insulin binding to human erythrocyte ghosts resulted in a biphasic curve (Fig. 1), which is characteristic of insulin binding to most cell types, including intact human erythrocytes (Peterson *et al.*, 1983). Scatchard analysis of insulin binding to erythrocyte ghosts (Table 1) yielded similar receptor affinities, and relative proportions of high- and low-affinity receptor sites, to those reported for intact erythrocytes (Peterson *et al.*, 1983). Estimation of average diameter of ghosts, by examination under phase light microscopy (see the Experimental section), indicated

Fig. 1. Scatchard plot of insulin binding to human erythrocyte ghosts

Erythrocyte ghosts, at 3 mg of protein/ml in buffer G, were incubated in quadruplicate for 90 min at 15 °C with ^{125}I -insulin, with or without various concentrations of unlabelled insulin as described in the Experimental section. The graph was plotted from data from a representative experiment. Two other experiments yielded similar results.

Table 1. Summary of data derived from Scatchard plots

Freshly thawed erythrocyte ghosts, sealed with ATP, were incubated at 37 °C with or without insulin (1 µg/ml) for 3 h, then washed. The ghosts were then incubated, in quadruplicate, with 0.5 ng of ¹²⁵I-insulin and various amounts of unlabelled insulin (0–0.1 mg), at 15 °C for 60 min. At the end of that time, samples were filtered to separate free insulin from that bound to the ghosts. The radioactivity retained on the filter was then counted in a gamma counter. The resulting data were analysed by the method of Scatchard (1949).

Incubation condition	High-affinity sites		Low-affinity sites	
	K_d (M)	No. of receptors/ghost	K_d (M)	No. of receptors/ghost
Without insulin	17×10^{-10}	7	3.7×10^{-7}	300
With insulin	13×10^{-10}	2	0.87×10^{-7}	173

that these ghosts were about one-third the size of the original erythrocytes. From calculations using this value, the receptor concentrations (per unit area of surface membrane) were found to be similar for both ghosts and intact erythrocytes, even though the numbers of surface receptors per ghost were less than for intact erythrocytes (Peterson *et al.*, 1983).

Effect of insulin on subsequent insulin binding to erythrocyte ghosts

Since intact erythrocyte insulin receptors have been shown to be down-regulated in response to insulin (Peterson *et al.*, 1983), we determined whether insulin-receptor binding on the erythrocyte ghost surface also decreased in response to insulin. Erythrocyte ghosts were incubated for 3 h at 37 °C with or without insulin (1 µg/ml). These incubation conditions were optimal for down-regulation of insulin receptors of intact erythrocytes (Peterson *et al.*, 1983). After this incubation, the ghosts were washed in Buffer G three times over a 3 h period, to remove free insulin. This washing procedure is sufficient to remove 92% of surface-bound insulin (results not shown). Ghosts were then assayed for surface specific insulin binding. In a representative experiment there was a 40% decrease in specific insulin binding to the surface of ghosts that had been incubated with insulin, as compared with control ghosts. Scatchard analysis of insulin binding to erythrocyte ghosts shows that the decrease in receptor number after exposure to insulin is primarily accounted for by a decrease in the number of low-affinity sites (Table 1).

ATP requirement for decreases in specific insulin binding

In order to determine whether ATP is required for the decrease in surface receptor binding after exposure to insulin, experiments were performed with erythrocyte ghosts which had been sealed with or without 2 mM-ATP. In a series of four experiments, ghosts, with and without ATP, were incubated with and without insulin (1 µg/ml), and then washed. Subsequent surface insulin-binding assays showed that, after exposure to insulin, there was a decrease of 39% in specific binding to surface insulin receptors of ghosts sealed with ATP, but no decrease in specific binding to ghosts sealed without ATP (Table 2). This finding is consistent with reports that ATP is required for drug-induced endocytosis in intact erythrocytes and resealed ghosts (Penniston & Green, 1968; Ginn *et al.*, 1969; Schrier *et al.*, 1975).

Low-pH wash of surface-bound insulin

Washing cells in low-pH buffers has been shown to dissociate peptide hormones, such as epidermal growth factor (Haigler *et al.*, 1980) and insulin (Waelbroek, 1982; Possner *et al.*, 1978) from their surface receptors. If insulin–insulin-receptor complexes have been internalized in response to insulin binding, then the insulin would be expected to remain associated with the ghosts after low-pH washes remove surface-bound insulin. In order to determine this, ghosts were incubated with radiolabelled insulin for 3 h at 37 °C and 4 °C, then subjected to low-pH washes to remove specifically surface-bound radioactivity (Table 3). Acetic acid-washed and pelleted ghosts, which had been incubated under conditions that caused decreases in surface receptor binding (37 °C), retained 28% original specifically bound radioactivity. Ghosts incubated at 4 °C had no decrease in surface receptor binding, and acetic acid washing removed 98% of specifically bound radioactivity. These results support the contention that the decrease in surface binding is due to internalization of insulin-receptor complexes.

Total insulin binding to erythrocyte ghosts incubated with and without insulin

If the decreased number of surface receptors, after incubation with insulin, is a result of receptor internalization, then the total number of insulin receptors should

Table 2. ATP-dependence of receptor internalization

Freshly thawed ghosts, sealed with or without 2 mM-ATP (final concn.), were incubated for 3 h at 37 °C with or without insulin (1 µg/ml). Insulin-incubated and control ghosts were washed and then assayed for specific surface binding of insulin as described in the Experimental section. Results are presented as means ± S.D. ($n = 4$). Similar results were obtained in three additional experiments. Significance testing was done between 'Without insulin' and 'With insulin' under each ATP condition.

Incubation	Ghosts sealed ...	Insulin binding (c.p.m.)	
		Without ATP	With ATP
Without insulin		508 ± 43	595 ± 86
With insulin		517 ± 45	364 ± 56
<i>P</i>		> 0.2	> 0.01

Table 3. Low-pH wash to remove surface-bound insulin

Freshly thawed ghosts, sealed with ATP, were incubated for 1 h at 15 °C, with radiolabelled insulin with and without an excess of unlabelled insulin, and then assayed to determine surface bound insulin as described in the Experimental section. The same ghosts were also incubated at 4 °C and at 37 °C for 3 h, then subjected to acetic acid washes as described in the Experimental section to remove surface bound insulin. Results are presented as means $\pm$ S.D. ($n = 4$) from one experiment. Similar results were obtained in one additional experiment.

Condition	Specifically bound insulin	
	(c.p.m.)	(% remaining)
Surface-bound: 1 h, 15 °C	3264 $\pm$ 552	
Acetic acid-washed: 3 h, 4 °C	64 $\pm$ 65 ($P < 0.001$)	2
Acetic acid-washed: 3 h, 37 °C	918 $\pm$ 163 ($P < 0.001$)	28

be the same in insulin-incubated and control ghosts. Total receptor binding was measured by the soluble-receptor assay (see the Experimental section) and was found to be the same in ghosts incubated with or without insulin (Table 4). Specific surface binding of insulin to insulin-incubated ghosts was 42% lower than insulin binding to control ghosts. Thus receptors that are no longer available for surface binding are still present in the ghost, indicating that receptors were internalized after incubation with insulin, rather than inactivated.

Insulin-receptor identification in an internalized vesicular fraction

If insulin receptors are internalized by an endocytotic mechanism, then they should be identifiable in an internal vesicular fraction. Stadel *et al.* (1983) have reported that in the frog erythrocyte there are endocytotic vesicles, containing insulin receptors, that can be separated from the plasma membrane by differential centrifugation. Using this differential-centrifugation tech-

nique, a light-density internal vesicular fraction was isolated from ghosts. Insulin binding to the internal vesicular fraction was determined by the soluble-receptor assay. It should be noted that insulin binding to an internal vesicular fraction isolated from untreated intact erythrocytes represented 46% of total insulin binding (results not shown), whereas that to the internal-vesicular fraction isolated from untreated ghosts represented only 8% of total insulin binding. We attribute this finding to the fact that the method for making ghosts is similar to that for separating out the internal vesicular fraction, and thus a portion of the internal vesicular fraction is lost in the ghost preparation procedure.

Insulin binding to the internal vesicular fraction of ghosts increased nearly 3-fold after incubation of ghosts with insulin (Table 4). There was a corresponding decrease in insulin binding to solubilized plasma-membrane receptors isolated from insulin-incubated erythrocyte ghosts. These results indicate that the receptors have been internalized in response to insulin. The ratio of protein to lipid was higher in the internal vesicular fraction (30 μ g of protein/ μ mol of phosphate) than in the plasma membrane (13 μ g of protein/ μ mol of phosphate), suggesting that internalization was by concentrative endocytosis.

The ghosts and the internal vesicular fraction were biochemically characterized by methods described by Steck & Kant (1974). The sidedness of the membrane vesicles was ascertained by measuring the extent to which intrinsic membrane markers were accessible to probes in the presence or absence of permeabilizing detergent. It was found that cytoplasm-facing enzymes (NADH-cytochrome *c* oxidoreductase and glyceraldehyde-3-phosphate dehydrogenase) were 90% inaccessible to exogenously added substrate in sealed ghosts, but were only 0–13% inaccessible in the internal vesicular fraction (Table 5). Sialic acid residues, which have external orientation in intact erythrocytes, were 71% inaccessible to sialidase added exogenously to internal-vesicular-fraction membranes but were completely accessible when sialidase was added to sealed ghosts. These results indicate that ghosts are sealed and right-side out, and that the internalized vesicular fraction consists mostly of inside-out vesicles.

Our data support the contention that exposure of

Table 4. Total insulin binding to insulin-incubated and control ghosts

Freshly thawed ghosts, sealed with ATP, were incubated for 3 h at 37 °C with or without insulin (1 μ g/ml), then washed. Plasma membranes and internal vesicular fractions were isolated as described in the Experimental section. Insulin-incubated and control ghosts were assayed for surface binding of insulin or subjected to the soluble-receptor assay described in the Experimental section. Results are presented as total specifically bound insulin per fraction. Results are expressed as means $\pm$ S.E.M. for 16 determinations from four experiments. Significance testing was done between values for 'Without insulin' and 'With insulin' conditions.

Incubation condition . . .	Total specifically bound insulin (fmol)		
	Without insulin	With insulin	
Surface binding			
Intact ghosts	60 $\pm$ 2.3	35 $\pm$ 6.7	($P < 0.001$)
Soluble-receptor binding			
Whole ghosts	134 $\pm$ 23	141 $\pm$ 13	($P > 0.2$)
Plasma membrane	108 $\pm$ 10	50 $\pm$ 7.1	($P < 0.001$)
Internal vesicular fraction	17 $\pm$ 3.0	47 $\pm$ 10	($P < 0.001$)

Table 5. Accessibility of plasma-membrane markers in erythrocyte ghosts and internal vesicular fraction

Ghosts and vesicles were assayed for the presence of plasma-membrane markers as described in the Experimental section. Sialic acid released is expressed as nmol of *N*-acetylneuraminic acid/mg of protein. NADH-cytochrome *c* oxidoreductase activity is expressed in nmol of product/min per mg of protein. Glyceraldehyde-3-phosphate dehydrogenase activity is expressed as μ mol of product/min per mg of protein. Membranes permeabilized with detergent were incubated at room temperature for 30 min with either 0.1% (v/v) Triton X-100 or 0.01% (w/v) saponin; '% Accessible' is (value without detergent/value with detergent) $\times$ 100.

Marker	Sealed ghosts	Vesicles
Sialic acid released		
No Triton X-100	26.8	18.6
+ Triton X-100	22.6	64.2
% Accessible	118	29
NADH-cytochrome <i>c</i> oxidoreductase		
No saponin	0.6	1.2
+ Saponin	5.8	1.1
% Accessible	10	109
Glyceraldehyde-3-phosphate dehydrogenase		
No Triton X-100	0.25	1.88
+ Triton X-100	2.86	2.17
% Accessible	9	87

erythrocyte ghosts to insulin results in internalization of insulin receptors to an intracellular vesicular compartment. Even though the amount of internalization in ghosts is not as great as that observed with intact erythrocytes (60–70%) (Peterson *et al.*, 1983), our findings suggest that receptor internalization may require only the plasma membrane, with its underlying cytoskeletal components, and ATP. The human erythrocyte ghost may thus provide an excellent model system for

investigation of the mechanism of this type of receptor internalization.

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