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Increased Turnover of Surface Insulin Receptors in Fibroblastic Cultures From Genetically Diabetic (DB/DB) Mice

Ranjana Kadle, Mohan K. Raizada, and Robert E. Fellows

Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242 (R.K., R.E.F.), and Department of Physiology, University of Florida, Gainesville, Florida 32610 (M.K.R.)

The turnover of surface insulin receptors in fibroblastic cultures from genetically diabetic (db/db) mice and nondiabetic (m/m) littermates has been determined by combining a heavy isotope density shift technique with cross-linking of insulin to surface receptors. Our results indicate that the surface insulin receptors turn over faster in diabetic cells than in nondiabetic cells. In addition, fewer receptors are incorporated into the plasma membrane per hour in diabetic cells than in nondiabetic cells. It is possible to propose a model to account for the altered expression of surface insulin receptors in diabetic cells on the basis of abnormalities of receptor incorporation and turnover.

Key words: insulin receptors, cell culture, db/db mouse, density shift technique

It has been suggested that the insulin resistance observed in non-insulin-dependent (type II) diabetes mellitus (NIDDM) is due to a decrease in insulin binding resulting primarily from a reduction in the number of insulin receptors [1,2]. Decreased binding has been observed both in freshly isolated cells, including adipocytes, lymphocytes, and monocytes from patients with NIDDM [3], and in fibroblasts cultured from the skin of patients with severe insulin resistance [4–6]. In the latter, the persistence of the abnormality for several generations in culture [4–6] points to an underlying genetic defect. However, the finding of decreased insulin binding in freshly isolated adipocytes, but not in cultured skin fibroblasts [7], suggests that increased insulin resistance of NIDDM may result from nongenetic factors as well.

In studies of fibroblasts cultured from the skin of diabetic C57BLKsJ db/db mice, we have found that the number of surface insulin receptors is decreased to 40–60% of that seen in paired cultures of fibroblasts from nondiabetic (m/m) littermates, while the receptor affinity is unchanged [8]. The decreased number of receptors on the cell surface is the result of a redistribution of receptors from the surface to the intracellular compartment without a decrease in the total number of receptors per cell [9]. This down-regulation in surface receptors in diabetic fibroblasts is clearly not

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attributable to circulating insulin, since it is observed up to 20 passages in culture [10]. Based on these data, we have used fibroblasts from the db/db mouse as a model to investigate cellular defects that may be analogous to those occurring in NIDDM.

Under equilibrium conditions, the number of receptors at the cell surface is a function of both the rate at which receptors are inserted into (and removed from) the plasma membrane and the half-life of surface receptors. The latter, a measure of receptor turnover, is based on intrinsic properties of the receptor and its interaction with the plasma membrane.

The heavy isotope density shift technique, adapted by Reed and Lane [11] from the method developed by Devreotes et al [12] for the study of the acetylcholine receptor, is a relatively new methodology for determining the kinetics of insulin receptor turnover. When this technique is used in conjunction with cross-linking of insulin to its surface receptor [13], the rate of receptor externalization and internalization is reflected in the rate of appearance of heavy and disappearance of light surface receptors, while receptor turnover can be determined from the $t_{1/2}$ of the disappearance of surface receptors.

The present investigation was carried out to determine if the observed redistribution of receptors in fibroblasts from db/db mice could be explained on the basis of alterations in membrane incorporation of receptors, receptor turnover, or both in these cells. Our results indicate that turnover of surface receptors is faster and the number of receptors incorporated into the plasma membrane is lower in fibroblasts from diabetic mice than in comparable cells from nondiabetic littermates. Thus it appears that the defects responsible for the altered distribution of receptors in diabetic cells involve both turnover and incorporation.

MATERIALS AND METHODS

Four-week-old nondiabetic (m/m) and diabetic (db/db) male mice of the C57BLKsJ strain were obtained from Jackson Laboratories (Bar Harbor, ME). Temin's modification of Eagle's medium (TMEM), fetal bovine serum (FBS), minimum essential medium (MEM)-vitamin solution, Earle's buffered salt solution (EBSS), and sodium bicarbonate were purchased from Grand Island Biological Co. (Grand Island, NY). Crystalline porcine insulin (lot no. 4BR88AM) was purchased from Elanco (Indianapolis, IN). Ethlene glycol bis(succinimidyl succinate) (EGS) was purchased from Pierce Chemical Co. (Rockford, IL). Penicillin and streptomycin were from Pfizer Inc. (New York, NY). A mixture of amino acids containing ^{2}H, ^{13}C, and ^{15}N was purchased from Merck, Sharpe and Dohme (Canada). Bovine serum albumin (BSA), heavy water (D_2O), Tris (hydroxymethyl)aminomethane hydrochloride, Triton X-100, N-2 hydroxyethyl piperazine N'-2 ethane sulfonic acid (HEPES), and cesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorinert was purchased from ISCO (Lincoln, NE). Porcine insulin iodinated with chloramine T to a specific activity of 150–250 μCi/μg was supplied by the University of Iowa Diabetes and Endocrinology Research Center.

Cell Culture

Fibroblastic cultures were prepared from the skin of mice as described previously [8,10]. Cells grown from skin explants were plated in 35-mm culture dishes at a density of 1×10^5 cells/dish or in 100-mm culture dishes at a density of 1×10^6

cells/dish and incubated in TMEM containing 10% FBS. The fibroblasts became confluent in 3–4 days and were maintained in culture up to 20 passages. Cultures were used for experiments after the sixth passage to remove them from the influence of in vivo environmental factors.

Heavy Isotope Labeling

For the preparation of "heavy" medium, an amino acid mixture containing 95% ^{15}N, ^{13}C, and ^{2}H was used. A liter of the heavy medium contained the following: amino acid mixture, 1.0 g; MEM vitamin mixture (100×), 20.0 ml; EBSS (10×), 100 ml; sodium bicarbonate, 4.2 g; glucose, 3.5 g; glutamine, 60.0 mg; cystine, 32.8 mg; tryptophan, 84.0 mg; penicillin, 1×10^5 units; and streptomycin, 100.0 mg. Confluent cultures in 100-mm culture dishes were incubated with 6.0 ml heavy medium containing 10% FBS. After incubation for various times, cultures were used for binding and cross-linking experiments.

Covalent Cross-linking of Insulin to Its Receptor

Cultures were washed twice with HEPES buffer (25 mM HEPES, 116 mM NaCl, 3.0 mM KCl, 1.8 mM CaCl, 0.8 mM $MgSO_2.7H_20$, 1.0 mM $NaH_2PO_4.H_20$, and 5.6 mM glucose), pH 8.0, and incubated with 3.5 ml HEPES buffer, pH 8.0, containing 0.22 nM ^{125}I-insulin and 1.6% BSA for 30 min at 24°C. Under these conditions less than 5% of bound insulin is internalized as measured by dissociation of labeled hormone in the presence of excess unlabeled insulin [8]. For determination of nonspecific binding, 16.6 μM unlabeled insulin was included in the reaction mixture. Following incubation, cultures were washed three times with ice-cold HEPES buffer, pH 8.0, containing 0.8% BSA and once with ice-cold HEPES buffer without BSA. The cultures were then incubated with 2 mM EGS in 3.5 ml PBS, pH 7.4, for 10 min at 24°C and washed three times with 2 ml PBS, pH 7.4. Cell monolayers were scraped from the plates with a rubber policeman and centrifuged at 300g. The pellet was suspended in .25 ml 50 mM Tris in D_2O, pH 7.4, containing 4% Triton X-100 and centrifuged at 44,000 rpm in a Beckman type 50 rotor at 4°C for 60 min. The supernatant was subjected to CsCl density gradient ultracentrifugation.

To measure the dissociation of ^{125}I-insulin after cross-linking, ^{125}I-insulin binding was carried out in 35-mm plates as described above. After removal of unbound radioactivity by washing, the bound ^{125}I-insulin was cross-linked to the receptor by incubation with 0.5 ml 2 mM EGS in 25 mM HEPES buffer, pH 7.4, for 15 min at 24°C. Cells were washed three times with 2 ml HEPES buffer, pH 7.4, and 2.0 ml HEPES buffer was added to each plate. Plates were incubated at 24°C for various time periods and then washed three times with 2 ml HEPES buffer to remove dissociated radioactivity. Cells were dissolved in 2.0 ml 2 N NaOH, and radioactivity was counted in a Beckman 8000 gamma counter.

Isopycnic Banding of Insulin-Receptor Complex

A solution of 42.7% CsCl was prepared in D_2O buffered with 50 mM Tris, pH 7.4. For preparation of CsCl gradients, 4 ml of the CsCl solution was added to cellulose nitrate ultracentrifugation tubes. To this, 0.2 ml of solublized receptor was added and mixed thoroughly. Tubes were filled to the top with paraffin oil by Pasteur pipette and centrifuged at 50,000 rpm for 20 h at 4°C in a Beckman SW 50.1 rotor. Paraffin oil was removed without disturbing the CsCl gradient, and 150-μl (12-drop)

fractions were collected from the top with an ISCO density gradient fraction collector, using Fluorinert as the chase solution. The fractions were counted in a Beckman 8000 gamma counter.

RESULTS

Cultures of nondiabetic fibroblasts incubated with ^{125}I-insulin for 30 min at 24°C bound .78 ± .03 fmol/mg protein (Fig. 1a). After further incubation of these cultures with 2 mM EGS for 15 min, the binding was 90% of the initial value. Following removal of EGS and incubation with fresh HEPES at 24°C for 60 min, 82% of the ^{125}I-insulin remained bound, while only 48% remained bound in cultures not cross-linked with EGS.

A similar effect of EGS was observed in diabetic cultures. Incubation of these cells with ^{125}I-insulin for 30 min at 24°C resulted in binding of .31 ± .008 fmol/mg protein (Fig. 1b). After incubation with EGS for 15 min, binding was 92% of the initial value. Sixty minutes after cross-linking of insulin to its receptor, 82% of hormone remained bound, compared to 46% in un-cross-linked cultures. In both nondiabetic and diabetic cultures, the cross-linked hormone did not dissociate further when the cultures were incubated up to 24 h at 4°C.

To confirm that ^{125}I-insulin was cross-linked specifically to its receptor, cultures were incubated with ^{125}I-insulin in the presence of excess unlabeled insulin, washed to remove unbound insulin, and incubated with EGS for 15 min. Cells were solublized in 4% Triton X-100, and the soluble fraction was subjected to density gradient centrifugation. As shown in Figure 2, the amount of ^{125}I-insulin cross-linked in the presence of 16.6 μM unlabeled insulin owing to nonspecific binding (open circles) was insignificant in both diabetic and nondiabetic cultures.

In order to determine the rate at which insulin receptors appear on the surface, confluent cultures in 100-mm dishes were incubated with medium containing heavy

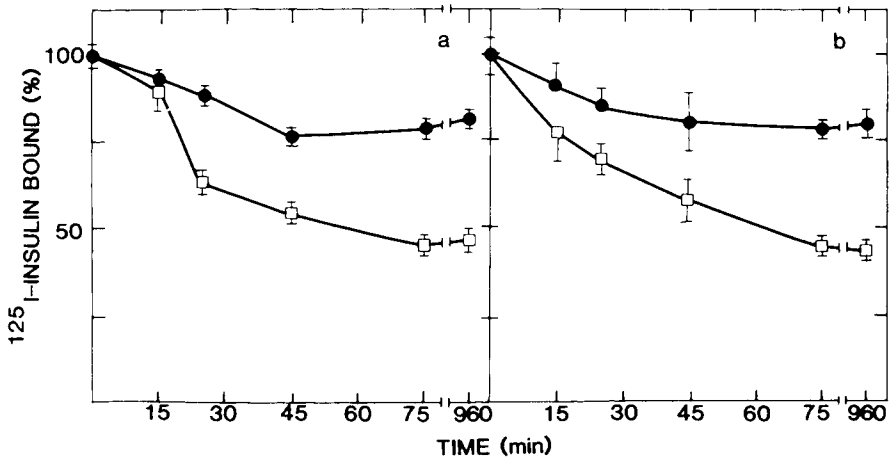

Fig. 1. Effect of cross-linking on dissociation of ^{125}I-insulin bound to confluent nondiabetic and diabetic cultures. Nondiabetic (a) and diabetic (b) cultures were incubated with 0.22 nM ^{125}I-insulin and 1.6% BSA in 0.5 ml HEPES buffer, pH 8.0, for 30 min at 24°C. Bound ^{125}I-insulin was cross-linked to its receptor by EGS as described in the Materials and Methods section. Each point is the mean ± SEM of triplicate determinations for cross-linked (●) and un-cross-linked (□) cultures.

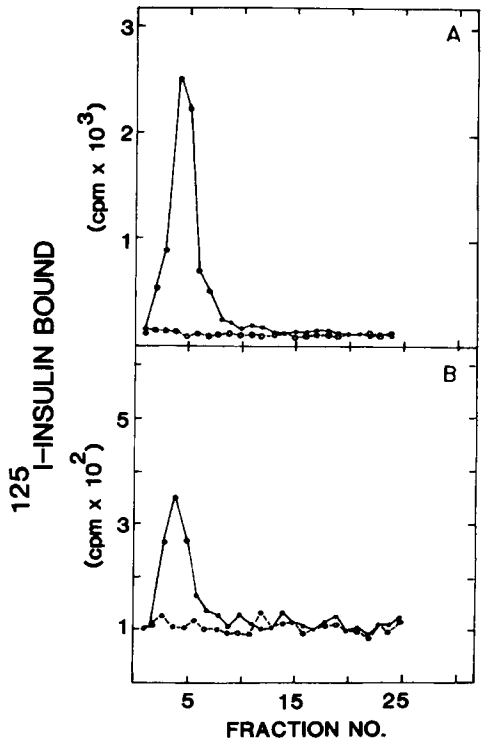

Fig. 2. Isopycnic banding of solublized ^{125}I-insulin-receptor complex from nondiabetic (A) and diabetic (B) cultures. ^{125}I-insulin was bound and cross-linked in the presence (○) or absence (●) of excess unlabeled insulin. The solublized cross-linked insulin-receptor complex was centrifuged on a 56.8% CsCl density gradient.

amino acids for various periods of time. The cross-linked soluble ^{125}I-insulin-receptor complex was centrifuged on a 42.7% CsCl density gradient for 20 hr. The profiles for various times of incubation in heavy medium are shown for nondiabetic (Fig. 3) and diabetic (Fig. 4) cultures. After 6 hr of incubation in heavy medium, newly synthesized heavy receptors could be detected in both nondiabetic and diabetic cultures. Table I shows the heights of the heavy and light peaks as a percentage of the total peak height (heavy + light). Corrections were made for peak overlaps to obtain true peak heights. As shown in Table I, after 6 hr of incubation, the heavy peak in nondiabetic cultures was 9.5% of the total, while in diabetic cultures it was 21.4% of the total. After 12 hr the heavy peak constituted 34.2% of the total in nondiabetic cultures and 45.7% of the total in diabetic cultures.

Since the peaks are symmetrical, peak heights were used to calculate the half-life of the receptor using a semi-log plot of the peak height of the light receptor as a function of time (Fig. 5). The plot is linear for both nondiabetic and diabetic cells, indicating that the decay of the light receptor follows first-order kinetics, described by the equation $R_t = k_s/k_d\ (1 - e^{-k_d t}) + R_o e^{-k_d t}$ where R_t is the receptor level at time t, k_s is the constant synthetic rate, k_d is the rate constant for disappearance, and R_o is the receptor level at time 0. By this method, the half-life of the insulin receptor at the cell surface is 11.0 hr for diabetic cells and 13.7 hr for nondiabetic cells. The

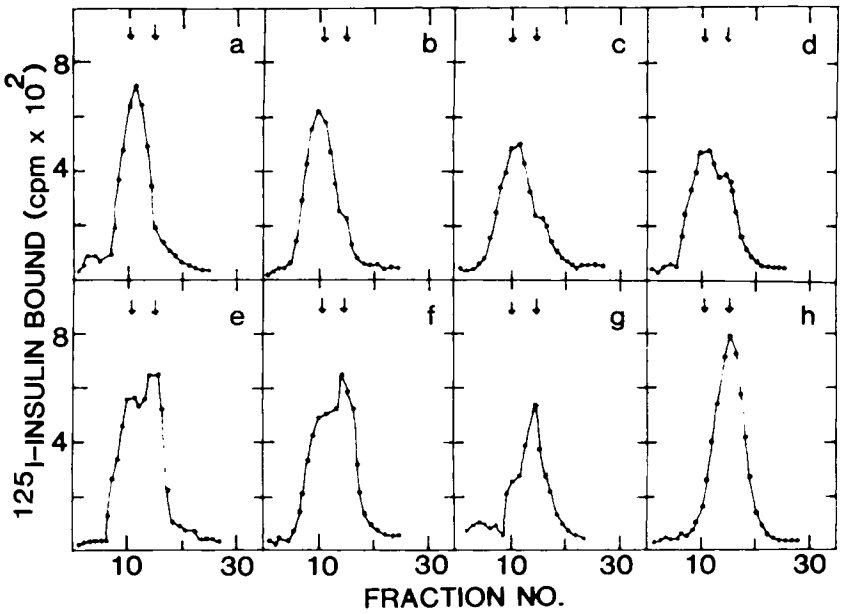

Fig. 3. Isopycnic banding of cross-linked, solublized ^{125}I-insulin-receptor complex from nondiabetic cultures shifted from normal to heavy medium. Confluent cultures were incubated with heavy medium for various time periods. ^{125}I-insulin binding and cross-linking of bound insulin to its receptor was carried out as described in the Materials and Methods section. The cross-linked insulin-receptor complex was solublized in Triton X-100 and centrifuged on 42.7% CsCl density gradient for 20 hr. Times of incubation in the heavy medium were as follows: a) 0 hr; b) 6 hr; c) 9 hr; d) 12 hr; e) 16 hr; f) 21 hr; g) 27 hr; and h) 36 hr. Pairs of arrows indicate the position of light (left) and heavy (right) peaks.

decay constant (k_d) for disappearance of the insulin receptor, calculated from the equation: $t_{1/2} = .693/k_d$, is estimated to be .05 hr^{-1} for nondiabetic cells and .063 hr^{-1} for diabetic cells.

The number of insulin receptors incorporated into the plasma membrane per hour can be estimated from the total number of surface receptors for nondiabetic and diabetic fibroblasts. Based on our findings of 77,000 receptors per cell in nondiabetic cultures and 29,000 receptors per cell in diabetic cultures [8], approximately 2,800 receptors are incorporated per hour in nondiabetic cells and 1,300 per hour in diabetic cells.

DISCUSSION

The heavy isotope density shift technique [12] permits both quantitative comparison of the kinetics of receptor synthesis, and when combined with irreversible binding of ligand to surface receptor, the turnover of surface receptors in cultured cells [14,15]. In the present investigation we have used these techniques to compare the turnover and rate of incorporation of surface insulin receptors in fibroblastic cultures from db/db mice and their nondiabetic (m/m) littermates. We have found that the half-life of the insulin receptor at the cell surface is 20% less (and therefore the turnover is 20% faster) in diabetic cells than in nondiabetic cells. The half-life of the

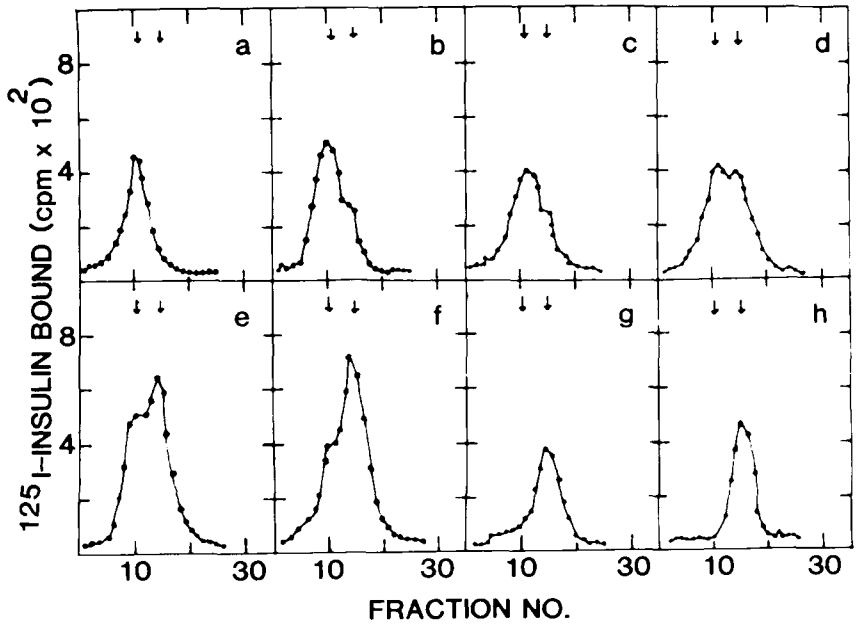

Fig. 4. Isopycnic banding of cross-linked, solublized ^{125}I-insulin-receptor complex from diabetic cultures shifted from normal to heavy medium. Confluent cultures were incubated with heavy medium for various time periods. ^{125}I-insulin binding and cross-linking of bound insulin to its receptor were carried out as described in the Materials and Methods section. The cross-linked insulin-receptor complex was solublized in Triton X-100 and centrifuged on 42.7% CsCl density gradient for 20 hr. Times of incubation in the heavy medium were as follows: a) 0 hr; b) 6 hr; c) 9 hr; d) 12 hr; e) 16 hr; f) 21 hr; g) 27 hr; and h) 36 hr. The arrows indicate the positions of heavy and light peaks.

TABLE I. Proportion of Heavy and Light Receptors During Incubation of Nondiabetic and Diabetic Fibroblasts in Heavy Medium (Heavy and Light Peak Heights are Given as the Percent of Total Peak Height [heavy + light])

Hours of incubation	Nondiabetic		Diabetic	
	Light (%)	Heavy (%)	Light (%)	Heavy (%)
0	100.0	0.0	100.0	0.0
6	90.5	9.5	78.6	21.4
12	63.8	34.2	54.3	45.7
16	47.0	53.0	38.3	61.7
21	39.3	60.7	26.9	73.1
27	23.7	77.3	8.1	91.9
36	0.0	100.0	0.0	100.0

surface insulin receptor of nondiabetic mouse fibroblasts is somewhat longer than that of 3T3-L1 adipocytes (10 hr) determined by ultracentrifugation [15] but approaches the range of IM-9 lymphocytes (9–12 hr) determined by surface iodination and immunoprecipitation [16]. This study also indicates that the number of receptors inserted into and removed from the plasma membrane per hour in diabetic cells is less than half that in nondiabetic cells.

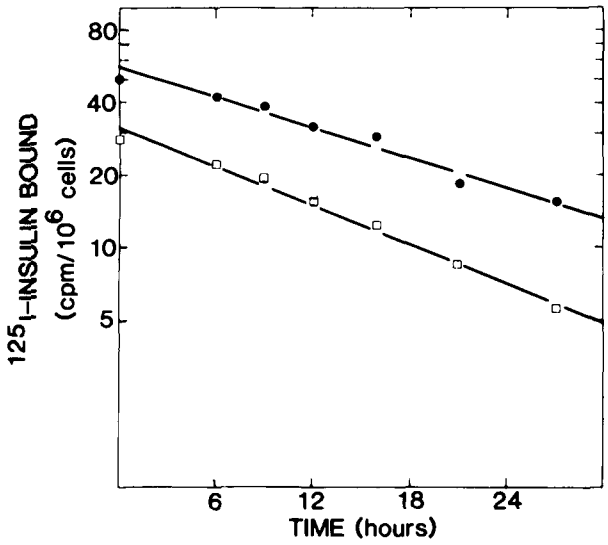

Fig. 5. Kinetics of disappearance of light receptor from the plasma membrane. Peak heights from Figures 3 and 4, expressed as cpm/ 10^6 cells, are plotted on a semi-log scale as a function of time of incubation of nondiabetic (●) and diabetic (□) cultures in heavy medium. The half-lives of cell surface receptors estimated from this graph are 11.0 hr and 13.7 hr for nondiabetic and diabetic cells, respectively.

As reported previously [9], although diabetic fibroblasts consistently show a 40–60% decrease in surface ^{125}I-insulin binding, the total cellular binding of diabetic and nondiabetic cells is not significantly different. As a result, diabetic fibroblasts have a greater number of receptors in the intracellular compartment. Since the total number of receptors are the same in both diabetic and nondiabetic cells, the defect(s) in diabetic cells leading to decreased surface binding apparently is not due to a major defect in the ability to synthesize functional receptors in sufficient numbers. Alternately, the receptors of diabetic cells may be defective in a way that modifies turnover but not binding capacity, or altered membrane properties may affect turnover of normal receptors.

The data obtained in this study suggest that the abnormal expression of surface insulin receptors in diabetic cells results from a defect in the process of insertion of intracellular receptors into the plasma membrane leading to a decrease in the number of receptors incorporated per unit time. A decrease in the rate of incorporation by itself will lead to a shift in the distribution of receptors toward the intracellular compartment. This effect will be enhanced if the same or a separate defect increases surface receptor turnover. The steady state that results should be characterized by a lower-than-normal rate of receptor incorporation and a greater proportion of receptors in the intracellular compartment, consistent with our findings in the diabetic db/db mouse fibroblast.

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