Insulin Elicits a Redistribution of Transferrin Receptors in 3T3-L1 Adipocytes through an Increase in the Rate Constant for Receptor Externalization*

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Incubation of 3T3-L1 adipocytes with insulin at 37°C resulted in a 2-fold increase in specific binding of transferrin to cell-surface receptors, as measured by a subsequent incubation of cells at 4°C with 125I-transferrin. The insulin concentration required for half-maximal effect was 10 nM, and the half-time for insulin action was 40 s. By comparison, insulin stimulated hexose transport in 3T3-L1 adipocytes with a half-maximal effect at 8 nM and a half-time of 105 s. Scatchard analysis of 125I-transferrin binding to cells at 4°C showed that the insulin-induced increase in transferrin receptor binding was due to an increase in the number of surface transferrin receptors. When cells were incubated for 2 h at 37°C with 125I-transferrin to achieve steady-state binding and then exposed to insulin, there was a 1.7-fold increase in surface-bound transferrin (acid-sensitive) and a corresponding decrease in intracellularly bound transferrin (acid-insensitive). Thus, insulin elicits translocation of intracellular transferrin receptors to the plasma membrane. Concomitant with the 2-fold increase in surface receptors in response to insulin, there was a 2-fold increase in the rate of 59Fe* uptake from 59Fe*+-loaded transferrin. The rate of externalization of the intracellular 125I-transferrin-receptor complex at 37°C was determined for basal and insulin-treated cells. Insulin increased the first-order rate constant for this process 1.7-fold. The effect of insulin on the rate of externalization is sufficient to account for the increase in surface transferrin receptors.

The steady-state level of plasma membrane transferrin receptors is subject to hormonal regulation. Insulin elicits a 3-fold increase in the level of surface transferrin receptors in rat adipocytes due to the redistribution of intracellular receptors to the plasma membrane (8). The simplest kinetic description of the steady-state condition for an unoccupied receptor that is recycling continuously between the plasma membrane and the intracellular membranes is (9): 

\[ k_e[\text{intracellular receptors}] = k_e[\text{plasma membrane receptors}] \]

where \( k_e \) and \( k_n \) are the first-order rate constants for externalization and endocytosis of the receptor, respectively. Thus, according to this analysis, the ratio of plasma membrane receptors to intracellular receptors is equal to \( k_e/k_n \), and the insulin-induced increase in this ratio found for adipocytes could be due to either an increase in \( k_e \) or a decrease in \( k_n \).

In the present study, we show that insulin also causes net translocation of transferrin receptors to the plasma membrane in 3T3-L1 adipocytes, and we have examined the basis of this effect. By direct measurement of the rate of release of intracellular transferrin, we have established that the locus of insulin action is on the rate constant for externalization of the transferrin-receptor complex. In addition, the time course and concentration dependence of the stimulatory effect of insulin on surface transferrin receptors and hexose transport have been compared, since the latter process involves the translocation of glucose transporters from an intracellular site to the plasma membrane (10, 11).

EXPERIMENTAL PROCEDURES

Materials—Human transferrin (Behring Diagnostics) was saturated with iron as described (6) and dialyzed overnight at 4°C against phosphate-buffered saline (150 mM NaCl, 10 mM NaH2PO4, pH 7.4). Iodination of ferrotransferrin with Na125I (Amersham Corp.) was performed using lactoperoxidase/glucose oxidase immobilized on agarose beads (Enzymobeads, Bio-Rad) (6). Diferric I25I-transferrin (specific activity, 9–10 Ci/g) was isolated by gel filtration in phosphate-buffered saline (Sephadex G-25, Pharmacia F-L Biocornerials), made 1 mg/ml in BSA, filtered (0.22 μm Millipore, Millipore), and stored at 4°C. [59Fe]Transferrin (specific activity, 20 mCi/g) was prepared by incubation of iron-free transferrin with NaFeCl2 (Amersham Corp.) as described (6). 2-Deoxy-d-[1,2-3H]glucose was obtained from ICN Pharmaceuticals. Insulin was a gift from Dr. Ronald Chance of Lilly. Tissue culture media and sera were obtained from GIBCO.

Cell Culture—3T3-L1 fibroblasts were cultured and differentiated in 6-well cluster dishes (6.5 cm2/well) as previously described (12). Mature 3T3-L1 adipocytes (2 × 106 cells/well, 2 mg of protein/well) were used between 8 and 12 days after initiation of differentiation, at which time greater than 95% of the cells exhibited the adipocyte phenotype (12). Cells were incubated for 2 h in serum-free Dulbecco’s modified Eagle’s medium at the beginning of each experiment.

Hexose Transport—Hexose transport was assayed by the uptake of 2-deoxyglucose, a process for which transport has previously been

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shown to be rate-limiting in 3T3-L1 adipocytes (12). Measurement of uptake at 4°C after exposure of cells to vehicle or insulin at 37°C (see Fig. 1) involved the following procedure. Cell monolayers were washed at 37°C with three 1-ml aliquots of KRP (128 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.26 mM CaCl2, 5 mM NaH2PO4, pH 7.4), and then KRP was added to 1 ml/well. The pH of KRP after this addition was 7.28. At the desired time, a 6-well dish was transferred to ice, and buffer was replaced with a ml/well KRP at 4°C. Each well was washed subsequently with two additional 1-ml aliquots of KRP at 4°C and then incubated for 2 h at 4°C with 0.95 ml of KRP containing 1 mg/ml BSA. Hexose uptake was initiated by addition of 50 μl of 2-deoxy[3H]glucose (final concentration, 50 μM; 0.3 μCi/well) and stopped 20 min later by aspiration of the medium, followed by three rinses with KRP at 4°C. The cells were solubilized with 1.0 ml of 1% Triton X-100, and radioactivity was measured by liquid scintillation spectrometry. The uptake of 2-deoxy[3H]glucose at 4°C in basal and insulin-stimulated cells is linear for at least 30 min. This protocol was adopted so that cells were treated in an identical manner in the comparison of the time courses of insulin action on surface transferrin receptors and hexose transport (see Fig. 1).

When hexose uptake at 37°C was assessed (see Fig. 2), cell monolayers were first washed with three 1-ml aliquots of KRP and then incubated for 20 min at 37°C with 0.95 ml of KRP and vehicle (0.01 ml of 10 mM HCl) or various concentrations of insulin. The pH of KRP after this additional incubation was 7.25. Measurement of uptake was initiated by addition of 50 μl of 2-deoxy[3H]glucose (final concentration, 50 μM; 0.3 μCi/well) and stopped 5 min later by three rinses with phosphate-buffered saline at 4°C. Cell-associated radioactivity was measured as described above. The uptake of 2-deoxyglucose at 37°C in basal and insulin-treated cells is linear for 20 min.

**Rate of Externalization of Intracellular [125I]Transferrin**—Cells were incubated with 3 nm [125I]transferrin at 37°C for 2 h, exposed to vehicle or insulin at 37°C for 5 min, and washed on ice as described above. Measurement of the uptake was initiated by addition of 50 μl of 2-deoxy[3H]glucose (final concentration, 50 μM; 0.3 μCi/well) and stopped 5 min later by three rinses with phosphate-buffered saline at 4°C. The uptake of 2-deoxy[3H]glucose at 4°C in basal and insulin-treated cells is linear for 20 min. The initial rate of uptake at 4°C was determined by a 2-h incubation at 37°C followed by addition of 2.0 ml of KRP and 50 μl of vehicle (10 mM HCl) or insulin (see the figure legends for insulin concentration and length of exposure). The final pH of KRP was 7.25. At the desired time, a 6-well dish was transferred to ice, and buffer was replaced with 1 ml of KRP at 4°C. The well was subsequently washed with three additional 1-ml aliquots of KRP at 4°C and incubated for 2 h with 1 ml of 3 nm [125I]transferrin (unless stated otherwise) in KRP containing 1 mg/ml BSA at 4°C (100,000–150,000 cpm/well). At the end of the 2-h incubation, unbound ligand was aspirated, and nonspecific binding was reduced by three 1-ml washes (1 min each) with cold KRP. Cells were solubilized with 1 N NaOH, and the radioactivity was determined in a Beckman γ-counter. A control experiment, in which the efficacy of three rapid washes was compared with three 1-min washes, indicated that none of the specifically bound [125I]transferrin was lost in the latter procedure. Control experiments established that nonspecific binding was reduced. Nonspecific binding was taken as the [125I]transferrin bound in the presence of an excess (1 μM) of unlabeled dextriferric transferrin; it ranged from 20 to 50% of the total binding. The value for nonspecific binding probably consists of both unbound transferrin trapped with the cells and transferrin bound to unknown sites of low affinity present in relatively large amounts. All data have been corrected for nonspecific binding.

In control experiments, the time course of binding of 3 and 10 nm [125I]transferrin to cells at 4°C was examined over a 5-h period. Specific binding of [125I]transferrin exhibited two phases: a rapid increase and then a slow linear rise. The initial phase was complete within the 30 min and 1 h for 10 and 3 nm transferrin, respectively, and constituted about 90% of the binding at 2 h. We have therefore defined the specific binding after 2 h at 4°C as equilibrium binding to surface receptors. We have been unable to determine the cause of the slow second phase of specific binding.

**Transferrin Binding at 37°C**—Cell monolayers were washed with three 1-ml aliquots of KRP at 37°C and then incubated with 3 nm [125I]transferrin in KRP containing 1 mg/ml BSA for 2 h at 37°C. Vehicle or insulin was then added as described for Table I. The cells were then incubated with three 1-ml washes of 1-min duration with cold KRP. Surface-bound ligand was discriminated from intracellular ligand by an acid-stripping procedure (13). Cells were incubated for 8 min at 4°C with 1 ml of 200 mM acetic acid, 500 mM NaCl, pH 2.5, and rinsed with another 1 ml of cold phosphate-buffered saline. Cells were then incubated with three additional 1-ml washes with 500 mM acetic acid, 500 mM NaCl, pH 2.5, abolished insulin responsiveness, as measured by stimulation of hexose transport. Therefore, an alternative method was developed based on the fact that apotransferrin binds with much lower affinity than dextriferric transferrin to transferrin receptor at pH 7.4 (4.5). Cells were incubated for 15 min at 4°C in 25 mM acetic acid, 150 mM NaCl, 2 mM CaCl2, pH 5.0, with 50 μM deferoxamine mesylate (Ciba Pharmaceuticals, Inc.) in order to release and chelate the iron from the surface-bound dextriferric transferrin (4). Cells were then washed twice with 1 ml of KRP and incubated for 30 min at 4°C in 1 ml of KRP with 50 μM deferoxamine and 125 nM unlabeled transferrin. A control experiment showed that 30 min is sufficient time for complete dissociation of surface-bound 125I-apotransferrin at 4°C. Moreover, basal cells that had been treated in this manner were fully as insulin-responsive as cells maintained in KRP at 37°C, as measured by stimulation of hexose transport. Subsequently, cells that had been stripped of surface-bound transferrin were washed once with KRP and externalization of intracellular [125I]transferrin was initiated by the addition of KRP containing 1 mg/ml BSA, 1 μM transferrin with or without 1 μM insulin at 37°C and immediate transfer of each to 4°C at the 30-min time point. This protocol was repeated at the desired time by transfer of the cells to ice, followed at once by one wash with KRP at 4°C. Cells were solubilized with 1 N NaOH in order to measure the remaining 125I-transferrin. Parallel measurements were made with cells exposed to 1 μM unlabeled transferrin during the initial 2-h incubation at 37°C, and all values have been corrected for nonspecific binding.

**Rate of Iron Uptake**—Cell monolayers were washed with three 1-ml aliquots of KRP at 37°C, and then 0.94 ml of KRP containing 1 mg/ml BSA at 37°C was added. Vehicle (0.01 ml of 10 mM HCl) or insulin (final concentration, 100 nM) was added for 5 min prior to addition of 50 μl of [55Fe]transferrin (final concentration, 17 nm). The pH of KRP after addition of vehicle was 7.35. Iron uptake was stopped at the desired time by transfer of the 6-well dishes to an ice bath, followed at once by three 1-ml washes with KRP at 4°C. The cells were solubilized with 1% Triton X-100, and the radioactivity was determined by scintillation spectrometry.

**RESULTS**

**Time Course and Concentration Dependence of Insulin Stimulation of Surface Transferrin Binding and Hexose Transport**—The time course of insulin stimulation of transferrin binding to surface receptors is shown in Fig. 1. Insulin (100 nM) elicited a 2-fold increase in binding to transferrin receptors with a half-time of 40 ± 6 s (n = 3, ± S.E.). At the later time points, 5 and 15 min, there was a consistent 30–40% reduction in the insulin-stimulated level of surface transferrin binding. For purposes of comparison, the time course
previously published studies (7 nM (12) and 6 nM (16)).

In experiments, with 100 nM insulin for various periods of time at 37 °C, followed by measurement of $^{125}$I-transferrin binding at 4 °C (9) or the uptake of 2-deoxy[3H]glucose at 4 °C (3) as described under "Experimental Procedures." Insulin increased surface-bound $^{125}$I-transferrin from 9.6 to 24 fmol/well after 3.7 min. Each point is the average value of triplicate determinations from a single experiment, and similar results were obtained in two other experiments. The uptake of 2-deoxyglucose was increased from 94 to 650 pmol/20 min/well after a 10-min exposure to insulin. Each point is the average of triplicate measurements. A second experiment gave virtually identical results, with a half-time of 108 s.

Receptors—The insulin-induced increase in binding of $^{125}$I-transferrin to surface receptors could be elicited by an increase in either the affinity or the number of surface transferrin receptors. Scatchard analysis of $^{125}$I-transferrin binding at 4 °C showed that insulin increased the number of surface transferrin receptors from 17 to 28 fmol/well (Fig. 3). The values for the dissociation constant in basal and insulin-treated cells were 2.3 and 1.5 nM, respectively. In a second separate experiment, the number of surface receptors for basal and insulin-treated cells were 9 and 22 fmol/well, respectively, and the dissociation constants were 1.1 and 1.0 nM. Thus, insulin caused a 2-fold increase in the number of surface transferrin receptors without a significant effect on receptor affinity.

The rapidity of the effect of insulin on the number of surface transferrin receptors on 3T3-L1 adipocytes indicates that protein synthesis is not involved. An alternative mechanism, expected on the basis of results obtained in rat adipocytes (8), is an insulin-induced redistribution of transferrin receptors from an intracellular pool to the plasma membrane. Incubation of cells with 3 nM $^{125}$I-transferrin for 2 h at 37 °C resulted in the maximal steady-state level of 68 fmol bound per well, of which 11 fmol were acid-sensitive and thus represented binding to surface transferrin receptors (Table I). When cells were exposed to 100 nM insulin for 5 or 8 min, there was a 1.7-fold increase in acid-sensitive binding (19 fmol/well) and a corresponding decrease in acid-insensitive binding (representing intracellular receptors) from 56 to 49 fmol/well. There was no change in the total $^{125}$I-transferrin bound (acid-sensitive + acid-insensitive) in response to insulin. Therefore, insulin

for the effect of insulin on hexose transport under the same conditions is also presented in Fig. 1. This process proceeded more slowly; the 7-fold increase in the uptake of 2-deoxyglucose in response to insulin occurred with a half-time of 105 s.

The concentration dependence of the effect of insulin on surface transferrin binding was also examined (Fig. 2). The increase was half-maximal at 10 nM insulin (average of two experiments, with individual values of 9 and 11 nM). Similarly, hexose uptake was stimulated half-maximally by 8 nM insulin (identical values obtained in two experiments). Thus, insulin regulates surface transferrin binding and hexose uptake with the same concentration dependence. The concentration of insulin that elicited half-maximal stimulation of these two responses was very similar to that required to elicit stimulation of 2-deoxyglucose uptake in 3T3-L1 adipocytes in two previously published studies (7 nM (13) and 6 nM (16)).

**Effect of Insulin on Surface and Intracellular Transferrin Receptors—**The insulin-induced increase in binding of 3 nM $^{125}$I-transferrin to surface receptors could be elicited by an increase in either the affinity or the number of surface transferrin receptors. Scatchard analysis of $^{125}$I-transferrin binding at 4 °C showed that insulin increased the number of surface transferrin receptors from 17 to 28 fmol/well (Fig. 3). The values for the dissociation constant in basal and insulin-treated cells were 2.3 and 1.5 nM, respectively. In a second separate experiment, the number of surface receptors for basal and insulin-treated cells were 9 and 22 fmol/well, respectively, and the dissociation constants were 1.1 and 1.0 nM. Thus, insulin caused a 2-fold increase in the number of surface transferrin receptors without a significant effect on receptor affinity.

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**Fig. 1.** Time course of insulin stimulation of surface transferrin binding and 2-deoxyglucose uptake. Cells were incubated with 100 nM insulin for various periods of time at 37 °C, followed by measurement of $^{125}$I-transferrin binding at 4 °C (9) or the uptake of 2-deoxy[3H]glucose at 4 °C (3) as described under "Experimental Procedures." Insulin increased surface-bound $^{125}$I-transferrin from 9.6 to 24 fmol/well after 3.7 min. Each point is the average value of triplicate determinations from a single experiment, and similar results were obtained in two other experiments. The uptake of 2-deoxyglucose was increased from 94 to 650 pmol/20 min/well after a 10-min exposure to insulin. Each point is the average of triplicate measurements. A second experiment gave virtually identical results, with a half-time of 108 s.

**Fig. 2.** Effect of insulin concentration on surface transferrin binding and 2-deoxyglucose uptake. 3T3-L1 adipocytes were incubated with various concentrations of insulin in KRP for 20 min at 37 °C. The binding of 3 nM $^{125}$I-transferrin at 4 °C (9) and the 5-min uptake of 2-deoxy[3H]glucose at 27 °C (9) were then measured as described under "Experimental Procedures." Insulin elicited an increase in $^{125}$I-transferrin binding from 11 to 26 fmol/well. The points are average values of duplicate determinations, and similar results were obtained in another experiment. The basal rate of 2-deoxyglucose uptake was 0.63 nmol/5 min/well, and the maximal effect of insulin was an increase to 6.4 nmol/5 min/well. The points are average values of duplicate determinations, and similar results were obtained in a second experiment.

| Insulin Concentration (nM) | Acid-sensitive Binding (fmol/well) | Acid-insensitive Binding (fmol/well) | Total Binding (fmol/well) |
|---------------------------|---------------------------------|-----------------------------------|-------------------------|
| Basal                     | 11.4 ± 1.4                      | 56.3 ± 1.2                        | 68 ± 1.8                |
| Insulin 8 min             | 19.0 ± 0.2                      | 49.6 ± 0.8                        | 69 ± 0.8                |
| 8 min                     | 19.2 ± 0.7                      | 47.8 ± 1.3                        | 67 ± 1.5                |

**Table I**

Effect of insulin on transferrin binding to surface receptors of control and insulin-treated cells. Cells were incubated with vehicle (9) or 100 nM insulin (9) for 10 min in KRP at 37 °C (9). Transferrin binding to control and insulin-treated cells at 4 °C was then measured as described under "Experimental Procedures" with concentrations of $^{125}$I-transferrin from 0.5 to 25 nM (250,000 cpm/ml). Each point is the average obtained from triplicate determinations.
Receptor—The insulin-induced change in the steady-state were obtained in two other experiments. Each point is the average of triplicate determinations. Similar results

uptake was measured as described under "Experimental Procedures." Incubation with vehicle ferrin receptors, transferrin on ice, and measurement of the rate of decrease to reach a steady state, removal of the surface-bound 'I-

stant for externalization of intracellular transferrin receptors, described above could be effected through a reduction in the rate constant for internalization of plasma membrane trans-

receptors that was due to the translocation of intracellular receptors to the surface. Our results on the effect of insulin on surface transferrin receptors of 3T3-L1 adipocytes are similar to results obtained in rat adipocytes (8). There are 8 and 30 fmol of surface transferrin receptors/2 × 10⁶ rat adipocyte cells in the basal and insulin-treated states, respectively, with a dissociation constant of 1–2 nM; similar values were obtained in the present study (Fig. 3). Subcellular fractionation and analysis of the receptor content in the plasma membrane and low density microsome fractions from basal and insulin-treated rat adipocytes showed that the increased number of surface transferrin receptors is due to translocation of receptors from an intracellular location to the plasma membrane (8). Insulin also elicits translocation of transferrin receptors in 3T3-L1 adipocytes since the insulin-induced increase in the surface transferrin-receptor complex was accompanied by a stoichio-

elicited an increase in the number of surface transferrin receptors that was due to the translocation of intracellular receptors to the surface.

Effect of Insulin on Rate of Externalization of Transferrin Receptor—The insulin-induced change in the steady-state distribution of surface and intracellular transferrin receptors described above could be effected through a reduction in the rate constant for internalization of plasma membrane transferrin receptors, $k_{in}$, or through an increase in the rate constant for externalization of intracellular transferrin receptors, $k_{ex}$ (see the Introduction). The rate of externalization of intracellular transferrin receptors was examined directly by incubation of cells with 3 nM [125I]-transferrin at 37°C for 2 h to reach a steady state, removal of the surface-bound [125I]-transferrin on ice, and measurement of the rate of decrease in intracellular [125I]-transferrin at 37°C. When the effect of insulin was examined, insulin was included in the last 5 min of incubation with the [125I]-transferrin at 37°C, as well as during the subsequent incubation at 37°C, so that the cells would be fully stimulated by insulin at the beginning of the second incubation at 37°C.

The results of a representative experiment are shown in Fig. 4A. About 90% of the intracellular [125I]-transferrin was rapidly released from the cells. When the data are plotted on a semilogarithmic scale, after correcting for the small percentage of [125I]-transferrin that is not released after 45–60 min, it is apparent that about 80% of the intracellular [125I]-transferrin was externalized by a first-order process (Fig. 4B). Insulin treatment of the cells increased the first-order rate constant for release of intracellular [125I]-transferrin by a factor of 1.7. The average values of the first-order rate constant for basal and insulin-treated cells were 0.111 ± 0.003 and 0.194 ± 0.006 min⁻¹, respectively (n = 3, ± S.E.). The results in Fig. 4A also show that insulin treatment reduced the amount of intracellular [125I]-transferrin from 59 to 50 fmol/well at time 0. This effect is due to the translocation of transferrin receptors to the surface during the initial 5-min exposure of cells to insulin (see above and Table I).

Effect of Insulin on Uptake of Iron—The functional consequence of an insulin-induced increase in the number of surface transferrin receptors was examined by comparison of the rate of $^{59}$Fe³⁺ accumulation in control and insulin-treated cells (Fig. 5). The rate of $^{59}$Fe³⁺ uptake was linear for at least 90 min and was increased from 12 to 21 fmol/min/well in the presence of 100 nM insulin. In a parallel experiment performed on the same day, insulin elevated surface transferrin binding from 7.5 to 14 fmol of $^{125}$I-transferrin bound per well (protocol is in legend of Table I). Thus, a 1.8-fold increase in the rate of $^{59}$Fe³⁺ accumulation was paralleled by a 1.9-fold increase in [125I]-transferrin bound to surface receptors.

**DISCUSSION**

Our results on the effect of insulin on surface transferrin receptors of 3T3-L1 adipocytes are similar to results obtained in rat adipocytes (8). There are 8 and 30 fmol of surface transferrin receptors/2 × 10⁶ rat adipocyte cells in the basal and insulin-treated states, respectively, with a dissociation constant of 1–2 nM; similar values were obtained in the present study (Fig. 3). Subcellular fractionation and analysis of the receptor content in the plasma membrane and low density microsome fractions from basal and insulin-treated rat adipocytes showed that the increased number of surface transferrin receptors is due to translocation of receptors from an intracellular location to the plasma membrane (8). Insulin also elicits translocation of transferrin receptors in 3T3-L1 adipocytes since the insulin-induced increase in the surface transferrin-receptor complex was accompanied by a stoichio-

**FIG. 4.** A, effect of insulin on rate of release of intracellular [125I]-transferrin. Cells were incubated at 37°C with [125I]-transferrin (3 nM) for 2 h, followed by addition of vehicle (■) or 1 μM insulin (■) for 5 min. Cells were then transferred to ice where surface-bound ligand was removed. Release of intracellular [125I]-transferrin was initiated by replacement of 4°C buffer with 37°C buffer without (■) or with (■) 1 μM insulin and transfer of cells to a 37°C bath. After various times at 37°C, the remaining cell-associated [125I]-transferrin was measured. See "Experimental Procedures" for details of the method. Each point is derived from the average of triplicate determinations in three separate experiments. B, first-order plots of release of intracellular transferrin. The average values (8.3 fmol, basal; 5.7 fmol, insulin) for unreleased [125I]-transferrin at 45 and 60 min were subtracted from those at the other time points shown in A, and the resulting values are presented as a semilogarithmic plot of cell-associated transferrin versus time.

**FIG. 5.** Effect of insulin on rate of iron uptake. Cells were incubated with vehicle (■) or 100 nM insulin (■) for 5 min in KRP at 37°C prior to addition of [59Fe]transferrin. The rate of [59Fe]³⁺ uptake was measured as described under "Experimental Procedures." Each point is the average of triplicate determinations. Similar results were obtained in two other experiments.
Insulin Regulation of Transferrin Receptors

**Scheme 1.** FeTf, ferrotransferrin; Rn, cell-surface receptor; FeTf-Rn, ferrotransferrin-surface receptor complex; Tf-Ri, intracellular apotransferrin-receptor complex.

metrical decrease in the intracellular transferrin-receptor complex (Table I).

Determination of the site at which insulin acts to alter the steady-state distribution of receptors between the plasma membrane and intracellular membranes would contribute to an understanding of the mechanism of action of insulin. The following analysis of the results from Table I and Fig. 4 demonstrates that insulin increased the rate constant for externalization of transferrin receptors and had no significant effect upon the rate constant for receptor internalization. A kinetic model for the recycling of transferrin and its receptor in the human hepatoma cell line, HepG2, has been described (14) (Scheme 1). According to this scheme, ferrotransferrin binds to its receptor on the cell surface. The ferrotransferrin-surface receptor complex undergoes endocytosis by a first-order process with the rate constant \( k_{\text{en}} \) and then releases its iron within an acidic compartment. The resulting apotransferrin-receptor complex returns to the surface (rate constant, \( k_{\text{en}}' \)), and apotransferrin dissociates into the extracellular medium at pH 7.4. It is assumed in Scheme 1 that the rate constants for release of iron from the internalized ferrotransferrin-receptor complex and for the release of apotransferrin from the surface receptor are relatively large so that these steps are not rate-limiting. The latter assumption has been validated for hepatoma cells (14) where dissociation of apotransferrin from the cell surface at pH 7.3 and at 37 °C occurs with a half-time of 16 s. In terms of Scheme 1, the observed first-order process for the release of intracellular transferrin (Fig. 4) corresponds to the externalization of the apotransferrin-receptor complex (\( k_{\text{en}}' \)). Thus, insulin increased the value of the rate constant for externalization by a factor of 1.7.

This kinetic model also allows analysis of the effect of insulin on the internalization rate of the ferrotransferrin-receptor complex. At steady state, the rate of internalization of the ferrotransferrin-receptor complex (FeTf-Rn) equals the rate of externalization of the apotransferrin-receptor complex (Tf-Ri).

\[
k_{\text{en}}[\text{FeTf-Rn}] = k_{\text{en}}'[\text{Tf-Ri}]
\]

The values for [FeTf-Rn] and [Tf-Ri] obtained from steady-state binding of 59Fe3' at 37 °C to basal and insulin-treated cells (Table I) and the values for \( k_{\text{en}}' \) (Fig. 4) can be substituted into Equation 1 to solve for \( k_{\text{en}}' \). The values for \( k_{\text{en}}' \) in basal and insulin-treated cells were 0.55 and 0.50 min⁻¹, respectively. It is unclear whether this 10% decrease in \( k_{\text{en}}' \) in response to insulin is significant; but, if so, it is a small effect compared to the 70% increase in \( k_{\text{en}} \). The 1.7-fold effect of insulin on \( k_{\text{en}} \) accounts almost entirely for the observed 1.7-fold increase in the level of the ferrotransferrin-receptor complex at the surface (Table I).

Although the rate constants for internalization and externalization of the unoccupied transferrin receptor (\( k_{\text{en}} \) and \( k_{\text{en}}' \) in the Introduction) have not been measured in this study, it seems likely that insulin regulates unoccupied transferrin receptors in the same manner. This conclusion is supported by the similar magnitudes of the effect of insulin on occupied (Table I) and unoccupied (Fig. 3) surface transferrin receptors. Insulin elicited a 2-fold increase in unoccupied surface transferrin receptors, the amounts of which were subsequently measured by Scatchard analysis of 125I-transferrin binding at 4 °C (Fig. 3).

The observation that the transferrin receptor-mediated uptake of 59Fe3' is stimulated 1.8-fold by insulin (Fig. 5) is consistent with the above analysis. At steady state, the rate of iron uptake is equal to \( k_{\text{en}}[\text{FeTf-Rn}] \); as shown in Table I, insulin increased the concentration of [FeTf-Rn] by a factor of 1.7.

Since insulin affects the rate of externalization of intracellular transferrin receptors, investigation of the biochemical basis for insulin regulation should be directed toward an understanding of the distribution and movement of the intracellular receptors. The actual process that determines the rate constant for externalization of the transferrin receptor and that is regulated by insulin is unknown. Internalized transferrin receptors are initially found in peripheral endosomes and then in juxtanuclear endosomes (15). The receptors probably return to the plasma membrane from both these sites by vesicular trafficking that has yet to be defined. Given the complexity of the process, it is remarkable that it follows first-order kinetics.

A previous study from this laboratory has shown that insulin elicits a 2-fold increase in the rates of both fluid-phase endocytosis and exocytosis in 3T3-L1 adipocytes (16); and thus, insulin stimulates overall membrane recycling. A detailed analysis of the kinetics of fluid-phase exocytosis indicates that insulin increases the size of the endosomal compartment by a factor of 1.6 and the rate constant for fluid-phase exocytosis from this compartment by a factor of only 1.25. Thus, the effect of insulin on the rate constants for externalization of the transferrin receptor and of endosomal fluid are different, and the relationship between the two effects remains to be elucidated.

The effect of insulin on the transferrin receptors of 3T3-L1 adipocytes is similar to the effect of epidermal growth factor on the transferrin receptors of human fibroblasts (17). Epidermal growth factor elicits a 2-fold increase in the number of surface transferrin receptors on fibroblasts, which is associated with a 2-fold increase in the rate constant for externalization of the intracellular transferrin-receptor complex.

Two other adipocyte membrane proteins, the receptor for insulin-like growth factor II (IGF-II) (18, 19) and the glucose transporter (10, 11), undergo redistribution from intracellular sites to the plasma membrane in response to insulin. The IGF-II receptor, like the transferrin receptor, continuously recycles (20, 21). Since insulin has no effect on the rate constant for internalization of photoaffinity-labeled IGF-II receptor (20), it is probable that the basis for the redistribution of this receptor is also an increase in \( k_{\text{en}} \). A proposed alternative explanation, that redistribution is due to an inhibition of the rate of internalization as the result of a change in the phosphorylation state of the plasma membrane IGF-II receptor (22), seems unlikely. It is not known whether the glucose transporter continuously recycles. The half-times for insulin activation of hexose transport at 37 °C in 3T3-L1 adipocytes (105 s, Fig. 1) and rat adipocytes (120 s, Ref. 23) are considerably longer than the half-times for redistribution of the transferrin receptor (40 s, Fig. 1) and the IGF-II receptor (45 s, Ref. 23). Thus, the translocation of the transporter may occur by a distinct pathway. Recent work from our laboratory has described the isolation of vesicles containing the insulin-responsive intracellular glucose transporters (24). It will be of interest to determine if the insulin-responsive intracellular transferrin receptors are also located in these vesicles.
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