Kinetics of Insulin Action on Protein Synthesis in Isolated Adipocytes

ABILITY OF GLUCOSE TO SELECTIVELY DESSENSITIZE THE GLUCOSE TRANSPORT SYSTEM WITHOUT ALTERING INSULIN STIMULATION OF PROTEIN SYNTHESIS*

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When adipocytes were exposed to [3H]leucine for times ranging from 5 to 180 s, leucine was found to enter cells rapidly and equilibrate with the cell interior within 5 s. After an additional 15–30 s [3H]leucine was incorporated into nascent protein, and the rate of incorporation was linear for up to 6 h in both control and insulin-treated cells. Since treatment of adipocytes with 10 ng/ml insulin enhanced the rate of leucine incorporation 2–3-fold with minimal or no effect on the rate of protein degradation or leucine uptake, we conclude that the predominant effect of insulin is on enhancement of protein synthesis. To assess the time required for insulin to stimulate protein synthesis, we preincubated cells with 10 ng/ml of insulin for various times from 2 to 30 min and then measured [3H]leucine incorporation into protein during a 4-min assay. These results revealed that the insulin stimulation of protein synthesis is rapid ($t_0$ of 4.4 min), but 9-fold slower than insulin activation of glucose transport ($t_0 < 0.5$ min under identical conditions). In contrast to the rapidity of insulin activation, we found that deactivation proceeded at much slower rates ($t_0$ of 32 and 21 min for protein synthesis and glucose transport, respectively).

Desensitization of the glucose transport system has previously been shown to occur after adipocytes are exposed to high glucose and insulin. To examine the specificity of desensitization, we treated cells for 6 h with 20 mM glucose and 25 ng/ml insulin and then examined insulin sensitivity and maximal insulin responsiveness of both the glucose transport and protein synthesis systems. After treatment, the glucose transport was markedly insulin-resistant (60% loss in maximal insulin responsiveness and a marked loss in insulin sensitivity), whereas the protein synthesis system exhibited neither diminished insulin responsiveness nor loss of insulin sensitivity. In fact, insulin sensitivity actually increased, as indicated by the finding that less insulin was required to stimulate protein synthesis (insulin $ED_{50}$ values of 0.25 and 18 ng/ml at 0 and 6 h of treatment). From these studies we conclude that desensitization of the glucose transport system by glucose and insulin treatment appears to be specific for this particular effector system and does not reflect a state of generalized cellular insulin resistance.

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Treatment of primary cultured adipocytes with insulin and glucose desensitizes the insulin-responsive glucose transport system by reducing both maximal insulin responsiveness and insulin sensitivity (1–3). To clarify the mechanisms underlying desensitization, we recently examined the role of glucose in the induction of cellular insulin resistance. Several salient findings emerged from these studies. First, we found that neither glucose nor insulin alone was capable of inducing desensitization of the glucose transport system; however, in the presence of insulin, physiological concentrations of glucose did effectively decrease maximal insulin responsiveness in a dose-dependent manner. Our interpretation of this observation is that the intracellular metabolism of glucose is an important regulator of the insulin-responsive glucose transport system and that glucose (or a glucose metabolite) forms part of a negative feedback loop that decreases both insulin responsiveness and sensitivity. In this scheme, the role of insulin is simply to enhance glucose uptake into the cell. A second and particularly important finding of this study is related to a novel mechanism involved in induction of desensitization. Specifically, we found that glucose and insulin treatment diminishes maximal insulin responsiveness, not by reducing the total number of intracellular glucose transporters, but rather by impairing the ability of glucose transporters to translocate from the cell interior to the cell surface (2).

To investigate the locus of glucose action, we have now focused on the question of specificity by testing the hypothesis that desensitization of glucose transport is not due to generalized desensitization of all insulin effector systems, but rather is due to a localized effect of glucose within the insulin action-glucose transport cascade. Our approach in testing this hypothesis was to treat cells with high glucose and insulin to induce insulin resistance of the glucose transport system and then examine the insulin sensitivity and maximal insulin responsiveness of another insulin-responsive effector system. The one we chose for study was the insulin-responsive protein synthesis system. Since relatively few studies have examined the kinetics of insulin action on protein synthesis in adipocytes, we examined in detail the rapidity of [3H]leucine uptake and incorporation into protein and the kinetics of insulin activation and deactivation on the protein synthesis system. These studies demonstrated that leucine uptake and incorporation into protein is remarkably rapid in adipocytes and that insulin enhances the rate of protein synthesis 2–3-fold within minutes. In the second part of this paper, we examined the specificity of glucose-induced desensitization, and these results revealed that glucose selectively desensitizes the glucose transport system without influencing insulin's action on protein synthesis.

EXPERIMENTAL PROCEDURES

Materials—Porcine monocomponent insulin was generously supplied to Dr. Ronald Chance of the Eli Lilly Company (Indianapolis,
Kinetics of Insulin Action on Protein Synthesis in Adipocytes

IN-[3H]deoxyglucose, l-[3,4,5-3H]leucine, and mono-[125I]-insulin were purchased from Du Pont-New England Nuclear; collagenase was obtained from Worthington; Dulbecco’s modified Eagle’s medium was from Gibco; d-2-deoxyglucose, Heps, 

phloretin, and cycloheximide were from Sigma; bovine serum albumin (tragent grade CFB), Fischer Scientific Company (Kankakee, IL); and octyl glucoside was from Pierce Chemical Co.

Preparation of Isolated Adipocytes—Male Sprague-Dawley rats weighing 160–225 g were killed by cervical dislocation, and the epicardial fat pads were removed. Isolated adipocytes were obtained using a modified method of Rodbell (4) by shaking finely minced tissue (1–3 g) in 4-ounce polypropylene containers at 37 °C for 1 h in 4 ml of Dulbecco’s modified Eagle’s medium (containing 25 mM HEPES and 1% bovine serum albumin), pH 7.4, containing 25 mM Hepes, collagenase (2 mg/ml), and albumin (40 mg/ml). Adipocytes were then filtered through nylon mesh (250 pm) and washed three times by centrifuging cells in 50-ml conical polypropylene tubes at 100 rpm for 1 min, aspirating the infranatant, and resuspending the cells in Heps-buffered saline, pH 7.4, containing 20 mM Heps, 120 mM NaCl, 1.25 mM MgSO4, 2.0 mM CaCl2, 2.5 mM KCl, 1.0 mM Na2HPO4, 1.0 mM pyruvate, and 1% bovine serum albumin. After the third aspiration, Heps-buffered saline was added to obtain a cell concentration of approximately 400,000 cells/ml (14 ml buffer/g wet weight of unminced epicardial fat pad).

Measurement of Protein Synthesis by [3H]Leucine Incorporation into Protein—Adipocytes (200 μl) were aliquoted into 12 × 75-mm polystyrene tubes containing 200 μl of medium consisting of Dulbecco’s modified Eagle’s medium (minus glucose), 1% bovine serum albumin, and 20 mM Heps, pH 7.4. Cells were preincubated for 30 min at 37 °C with or without 10 μg/ml cycloheximide and in the absence or presence of 10 ng/ml insulin. Precursor incorporation into protein was initiated by adding 20 μl of [3H]leucine (0.02 μCi in Heps-buffered saline, 140 Ci/mmol) and incubating cells at 37 °C for various times depending on the experimental protocol. To stop the reaction, polystyrene tubes containing cells were rapidly transferred from a 37 °C water bath to a 4 °C ice bath, which completely inhibited protein synthesis in <1 min (data not shown). A 350-μl cell suspension was withdrawn from each tube, transferred to a 400-μl aliquoting cell, and incubating cells at 37 °C for 30 min at 37 °C with or without 10 μg/ml cycloheximide.

RESULTS

Development of a Rapid Assay to Estimate [3H]Leucine Incorporation into Protein—The classical method for estimating net rates of protein synthesis is to measure [3H]leucine incorporation into trichloroacetic acid-precipitable protein. However, we found this method to be time-consuming, limited in the number of samples that could be processed, and environmentally undesirable because of the caustic HCl waste. Therefore, we developed and characterized an alternate assay that was free of the above disadvantages. The employed method is called the whole cell method and entails pretreating 400 μl of intact adipocytes (both control and insulin-stimulated) in the absence or presence of 10 μg/ml cycloheximide, a concentration which inhibits protein synthesis by 90–95%. After 30 min, 20 μl of [3H]leucine is added, and the cells are incubated for 1 h at 37 °C before the reaction is stopped by cooling adipocytes to 4 °C in an ice bath. Cells are then separated from the extracellular medium by centrifugation through silicone oil, and the excised cell plug is counted in a scintillation counter.

To validate the assumption that our whole cell method provides an accurate estimate of [3H]leucine incorporation into protein, we compared our whole cell method with the classical trichloroacetic acid method. Incorporation of [3H]leucine into protein was measured by the trichloroacetic acid method using a method modified from the procedure of Folks et al. (7). This entailed transferring 350 μl of cells to a 1.5-ml Microtuge, washing cells four times with Heps-buffered saline (to remove extracellular [3H]leucine), and then solubilizing cells in a mixture containing Heps-buffered saline, 1% octyl glucoside, 0.5 mg/ml bacitracin, and 0.2 mM phenylmethylsulfonyl fluoride. After trichloroacetic acid was added (final concentration 10%), the mixture was centrifuged, the supernatant was aspirated, and the protein pellet was reconstituted and counted.

As shown in Table I, both methods gave remarkably similar results. Basal levels of protein synthesis were comparable, and protein synthesis was enhanced by insulin to an equal extent (165% for the whole cell method versus 149% for the trichloroacetic acid method). Moreover, measurement of insulin sensitivity by construction of complete insulin dose-response curves yielded similar values (ED50 of 0.21 ng/ml for the whole cell method versus 0.23 ng/ml for the trichloroacetic acid method).

Fig. 1 reveals that cycloheximide is a potent inhibitor of

1 The abbreviation used is: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.


### Table I

**Comparison of protein synthesis assays**

Incorporation of [3H]leucine into protein was estimated using two methods, our newly developed whole cell method and the classical trichloroacetic acid method. The whole cell method is described under "Experimental Procedures," and the trichloroacetic acid method is outlined under "Results." Each value represents the mean ± S.E. of three experiments.

| Condition                  | Whole cell method | Trichloroacetic acid method |
|----------------------------|-------------------|----------------------------|
| Basal                      | 2798 ± 122 dpm    | 2758 ± 105 dpm             |
| Basal + cycloheximide      | 625 ± 38 dpm      | 245 ± 18 dpm               |
| Nonspecific trapping       | 269 ± 20 dpm      | 269 ± 20 dpm               |
| Inhibition by cycloheximide| 90.8%             | 91.2%                      |
| Insulin-treated            | 7428 ± 381 dpm    | 6893 ± 431 dpm             |
| Insulin + cycloheximide    | 762 ± 88 dpm      | 510 ± 25 dpm               |
| Nonspecific trapping       | 93.4%             | 92.7%                      |
| Inhibition by cycloheximide|                  |                            |
| Insulin stimulation        | 165%              | 149%                       |
| Insulin sensitivity        | 0.25 ng/ml        | 0.28 ng/ml                 |

(Insulin ED50)

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**Fig. 2. Time course of protein synthesis.** Adipocytes were pretreated at 37 °C for 30 min in the absence (control) or presence of either 10 ng/ml insulin or 10 μg/ml cycloheximide and then exposed to 0.02 μCi of [3H]leucine. After a period of hours (A) or minutes (B), cells were cooled to 4 °C, separated from the medium by centrifugation, and the amount of cell-associated [3H]leucine was determined. Each point represents the mean ± S.E. of three replicates.

**Fig. 3. Uptake of [3H]leucine and incorporation into protein.** A protocol identical to that described in Fig. 2 was used with the exception that the reaction was stopped at various times from 1 to 90 s.

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Protein synthesis in isolated adipocytes and inhibits protein synthesis equally well under basal and insulin-stimulated conditions. (ED50 of 80 ng/ml in insulin-treated cells and 85 ng/ml in control cells). In both Table I and Fig. 1, cycloheximide inhibited protein synthesis by 90–95% at the highest concentration (10 μg/ml), and similar results were obtained when puromycin was used to inhibit protein synthesis. It should be noted that the inhibitory effects of cycloheximide could be effectively reversed upon removal of cycloheximide. Thus, when cells were exposed to 10 μg/ml cycloheximide for 1 h at 37 °C and then washed three times in cycloheximide-free buffer, basal rates of protein synthesis returned to control values (Fig. 1, inset). Overall, these results are in excellent agreement with earlier studies demonstrating that insulin could stimulate the incorporation of labeled amino acids into protein (indicative of de novo protein synthesis) in adipose tissue and that puromycin could inhibit protein synthesis by >95% (8).

Kinetics of [3H]Leucine Uptake and Incorporation into Protein in Control and Insulin-treated Cells—As shown in Fig. 2A, the amount of cell-associated [3H]leucine in both control and insulin-treated cells was linearly related to time for up to 6 h. Thus, it appears that adequate amounts of metabolic energy were available to maintain protein synthesis at a constant rate. Moreover, linearity in insulin-treated cells for these prolonged periods of time suggests that the insulin-responsive protein synthesis system is not undergoing insulin-induced desensitization. If it were, we would expect to see linearity deviate at the later times concomitant with the development of insulin resistance.

As shown in Fig. 2B, when the amount of cell-associated [3H]leucine was plotted for control and insulin-treated cells over a 60-min period, very similar results were obtained. However, the observation that at the earliest time point (6 min) cell-associated radioactivity was noticeably higher in insulin-pretreated cells compared to cycloheximide controls suggests that [3H]leucine incorporation into protein occurs very rapidly.

To further investigate the rate at which [3H]leucine is incorporated into protein, we performed the experiment shown in Fig. 3. Adipocytes were pretreated for 30 min at 37 °C with either insulin or cycloheximide. Radiolabeled leucine was then added for various periods of time from 5 to 90
s, and the reaction was terminated by rapidly cooling cells to 4 °C in an ice bath. As can be seen, cycloheximide-pretreated cells exhibited a marked increase in cell-associated [{}^{3}H]leucine at 5 s (the earliest time point), and this value remained constant throughout the 90-s time course. We interpret this to mean that [{}^{3}H]leucine is internalized and rapidly reaches equilibrium within 5 s. Insulin-pretreated cells exhibited a similar “jump-up” in cell-associated radioactivity at 5 s; however, from 5 to 90 s the amount of cell-associated radioactivity progressively increased in contrast to cycloheximide-treated cells. We believe this reflects the incorporation of [{}^{3}H]leucine into protein. Since the amount of cell-associated radioactivity in insulin and cycloheximide-treated cells was significantly different by 30 s, we interpret this to mean that within 30 s [{}^{3}H]leucine is transported into adipocytes, binds to tRNA, and migrates to the rough endoplasmic reticulum, where it is incorporated into nascent protein, an extremely rapid chain of events.

Because [{}^{3}H]leucine uptake into adipocytes is rapid and equilibrates before our first 5-s time point, we employed a modified protocol in which reduced temperature was used to slow uptake in order to make quantitative measurements. Specifically, we pretreated cells for 30 min at 37 °C in the absence or presence of 10 ng/ml insulin to maximally stimulate protein synthesis. Adipocytes were then cooled to 4 °C, exposed to [{}^{3}H]leucine, rewarmed to 37 °C, and then rapidly transferred back to a 4 °C ice bath at various times. As shown in Fig. 4, the progressive increase in cell-associated radioactivity was nearly identical in control, insulin, and cycloheximide-treated cells during the first 60 s of the experiment. As expected, there was no further increase in cell-associated radioactivity in cycloheximide-treated cells, whereas a gradual increase was observed in control cells and a marked increase was seen in insulin-treated cells. From these data we draw two conclusions. First, it is apparent that the uptake rate of [{}^{3}H]leucine can be slowed significantly by reducing both temperature and time. Thus, the window of leucine uptake can be expanded from under 5 s (Fig. 3) to 1 min through the use of reduced temperature. More importantly, it is apparent that [{}^{3}H]leucine uptake is similar between control and insulin-treated cells, indicating that insulin’s ability to augment protein synthesis is not mediated by effects on the rate of leucine uptake into adipocytes.

To confirm that the rate of [{}^{3}H]leucine uptake is unaffected by insulin, as suggested by the data in Fig. 4, we repeated these experiments using a protocol in which cell-associated [{}^{3}H]leucine was assessed at three times, 0, 1, and 3 min. The results of these experiments, depicted in Fig. 4 and Table II, clearly show that at 1 min there are no differences among the three groups, whereas at 3 min the amount of radioactivity associated with control and insulin-treated cells is significantly greater than that of cycloheximide-treated cells. In this study the mean ± S.E. represents the average of three separate experiments sampled in triplicate.

**Effects of Insulin on Protein Degradation**—Shown in Fig. 5 is an experiment in which isolated adipocytes were incubated with [{}^{3}H]leucine for 1 h (37 °C) and then extensively washed at 4 °C to remove all extracellular radioactivity. After aliquotting cells to 12 × 75-mm tubes, we added 10 μg/ml cycloheximide to all samples, and 10 ng/ml insulin to half the tubes, and then warmed cells to 37 °C for the indicated times. As can be seen from these results, protein degradation proceeded relatively slowly under basal conditions (33% loss of cell-associated radioactivity after 4 h), and insulin had only a minimal effect on slowing the overall rate of degradation after 4 h and no discernible effect after 1 h. Since insulin markedly enhances incorporation of cell-associated [{}^{3}H]leucine (Fig. 2) without altering the uptake of [{}^{3}H]leucine (Fig. 4 and Table II) or protein degradation (Fig. 5), it appears likely that the site of insulin action is at the level of protein synthesis.

**Time Courses of Insulin Activation and Deactivation**—To determine the times required for insulin to stimulate protein synthesis and glucose transport, we performed the experiments depicted in Fig. 6. Cells were preincubated with 10 ng/ml insulin for various times from 1 to 30 min, after which
be spare receptors for stimulation of protein synthesis, just as there are for the glucose transport system. In other words, response curves were constructed (Fig. 7). As can be seen, the sensitivity between effector systems, complete insulin dose-synthesis. It should also be mentioned that there appears to be less than the effect of insulin on activation of glucose transport (37-4-37 °C) have no adverse cellular effects.

**Specificity of Glucose for the Glucose Transport System**—Desensitization of the glucose transport system has previously been shown to occur after adipocytes are exposed to high glucose and insulin (1-3). To examine the specificity of desensitization, we performed the experiment depicted in Fig. 9. Adipocytes were treated for 6 h (at 37 °C) with 20 mM glucose and 25 ng/ml insulin and then thoroughly washed to remove insulin and allow the stimulated insulin effector systems to deactivate down to basal levels. When we measured basal and maximally insulin-stimulated rates of glucose transport (upper panel) and [3H]leucine incorporation into protein (lower panel), we found that the glucose transport system was markedly insulin-resistant (60% loss in maximal responsiveness and reduced insulin sensitivity), whereas the insulin-responsive protein synthesis system exhibited neither decreased insulin responsiveness nor sensitivity. In fact, insulin sensitivity appeared to actually increase after 6 h of treatment. From these studies we conclude that desensitization of the concentration that occupies less than 10% of the total receptors (9).

Deactivation of the protein synthesis and glucose transport systems after insulin removal is shown in Fig. 8. When adipocytes were pretreated with 10 ng/ml insulin for 30 min (at 37 °C), cooled to 4 °C, washed to remove ligand, and then warmed to 37 °C to allow deactivation of insulin action, we found that both protein synthesis and glucose transport rates deactivated to basal values over a 60-min time period. Interestingly, rates of deactivation were similar for both effector systems with a t½ of 32 and 21 min for protein synthesis and glucose transport, respectively. To determine whether the rapid cooling and rewarming of cells had any deleterious effects on insulin action, we include a parallel control group that was allowed to deactivate for 60 min, after which time cells were re-exposed to insulin for an additional 30 min. These cells were fully responsive to a second insulin challenge demonstrated that temperature transition (37-4-37 °C) have no adverse cellular effects.

**Comparison of insulin dose-response curves for protein synthesis and glucose transport.** Cells were preincubated in the absence (basal) or presence of the indicated concentrations of insulin for 30 min at 37 °C, after which time dose-response curves were constructed based on the rates of [3H]leucine incorporation into protein and glucose transport rates.

Time rates of protein synthesis or glucose transport were assessed during a 4-min assay. It is apparent from these results that the stimulatory effect of insulin in protein synthesis is very rapid (t½ of 4.4 min), but still nine times slower than the effect of insulin on activation of glucose transport (t½ of <0.5 min when compared under identical experimental conditions).

To eliminate the possibility that the observed differences in insulin activation time were due to differences in insulin sensitivity between effector systems, complete insulin dose-response curves were constructed (Fig. 7). As can be seen, the sensitivity of adipocytes to insulin were similar with an ED₅₀ of 0.19 for glucose transport and 0.26 ng/ml for protein synthesis. It should also be mentioned that there appears to be spare receptors for stimulation of protein synthesis, just as there are for the glucose transport system. In other words, insulin exerts a maximal effect on protein synthesis at a
the incorporation of [3H]leucine into protein. This newer
alternative assay that was free of the above
disadvantages. Our method, termed the whole cell assay,
was validated and shown to yield results nearly iden-
tical to those obtained using the classical method of trichlo-
oroacetic acid precipitability. It is of interest to note that using
our whole cell method, the epididymal fat pads from one rat
provided sufficient cells to perform experiments which typi-
ically would include 30–200 individual determinations, 10–100
treatment groups each containing two to three replicates.

FIG. 9. Specificity of glucose for the glucose transport sys-
tem. Cells were cultured for 6 h at 37 °C in the presence of glucose
(20 mM) and/or insulin (25 ng/ml). After washing cells and allowing
the stimulatory effects of insulin to deactivate to basal levels, we
measured basal (open bars) and maximally insulin-stimulated (solid bars) rates of glucose transport (upper panel) and protein synthesis (lower panel). Complete insulin dose-response curves were performed to assess insulin activity, and the calculated insulin ED_{50} values are shown in white on the bars.

![Glucose Transport and Protein Synthesis](image)

**FIG. 10.** Schematic summary depicting [3H]leucine uptake and incorporation into protein and the effect of insulin on protein synthesis and glucose transport systems.

**glucose transport system by glucose and insulin treatment is**
specific for this particular effector system and does not reflect
a state of generalized cellular insulin resistance.

**DISCUSSION**

The three major objectives of the current study were to: 1) develop a method for rapidly estimating the incorporation of [3H]leucine into protein; 2) use this method to examine the kinetics of insulin action on protein metabolism; and 3) test the hypothesis that desensitization of the glucose transport system (by glucose and insulin treatment) is not a consequence of generalized cellular insulin resistance, but is rather a localized effect confined to the glucose transport system. Each of these objectives will be discussed in turn, but the salient findings are schematically presented in Fig. 10.

The classical method for estimating net rates of protein synthesis is to measure [3H]leucine incorporation into trichloroacetic acid-precipitable protein. However, we found this method to be time-consuming, limited in the number of samples that could be processed, and environmentally undesirable because of the caustic HCl waste. Therefore, we developed and characterized an alternate assay that was free of the above disadvantages. Our method, termed the whole cell assay, entailed preincubating cells in the absence (controls) or presence of cycloheximide (which inhibits protein synthesis by 90–95%), adding [3H]leucine, and then measuring the amount of radioactivity associated with intact cells. By subtracting counts in cycloheximide-treated cells (nonspecific component) from control cells and insulin-treated cells, we estimated the incorporation of [3H]leucine into protein. This newer method was validated and shown to yield results nearly identical to those obtained using the classical method of trichlo-

Among the prominent observations that emerged from our kinetic experiments was the finding that leucine uptake into adipocytes is extremely rapid (<5 s) and unaffected by insulin. Thus, the ability of insulin to enhance [3H]leucine incorporation into protein does not appear to be mediated by a direct effect of insulin on the rate of amino acid transport. This conclusion is in accordance with the earlier studies of Mune-mura et al. (10) using isolated adipocytes, Rodbell (11) using adipocyte ghosts, and Goodman (12) using adipose tissue. In keeping with the rapid uptake of [3H]leucine, we also found that as early as 15–30 s after the extracellular addition of radiolabeled leucine, incoming [3H]leucine is incorporated into nascent protein. This latter conclusion is based on the observation that a significant difference in cell-associated [3H]leucine was seen between cycloheximide and insulin-treated cells 30 s after the addition of leucine (Fig. 3). Thus, it appears likely that incoming [3H]leucine binds to tRNA, migrates to the endoplasmic reticulum, and undergoes insertion into nascent protein all within a time frame of less than 30 s, a remarkably rapid cascade of cellular events. However, it should be mentioned that the rapidity of this process may be unique to adipocytes, since this cell has only a thin cytoplasmic rim due to large glycogen droplets. Thus, diffusion of leucine into the cell interior, association with cytoplasmic enzymes, and transit to intracellular organelles may not be rate limiting. It should be emphasized that these rapid kinetic studies would not have been possible without our whole cell
Assay for rapidly estimating leucine incorporation into protein and protocols employing low temperatures to effectively slow or halt protein metabolism.

A widely held belief is that insulin enhances protein synthesis over a period of hours rather than minutes (13); however, this clearly is not case for insulin stimulation of protein synthesis in isolated adipocytes. Under our experimental conditions employing a 4-min assay, protein synthesis was stimulated 2-3-fold by insulin with a t½ of 4.4 min (maximal by 15-20 min). In related adipocyte studies using different techniques, Haasson and Ingelman-Sundberg (14) found an initial lag of about 4 min with full expression of insulin action after an additional 2-6 min. Interestingly, they also observed that the time course of insulin action on enhancement of $[^{3}H]$ leucine incorporation into protein was temporarily correlated with insulin-mediated incorporation of $^{32}$P into ribosomal protein S6, suggesting that these two phenomena are causally linked. Moreover, based on the finding that insulin influenced neither the rate of protein elongation nor the specific activity of leucine-tRNA, these authors also concluded that insulin increases the number of initiated, active ribosomes.

Many of the discrepancies regarding the kinetics of insulin action and the cellular mechanisms underlying insulin action on protein synthesis can be attributed to fundamental differences between various cell types. For example, although insulin has no acute effects on amino acid uptake in adipocytes (10-12, 14-15), it is well established that insulin rapidly enhances the rate of amino acid transport in hepatocytes (16), hepatoma cells (17), fibroblasts (18), and muscle (19, 20).

Similarly, insulin rapidly enhances the rate of protein synthesis over a period of minutes in adipocytes (Fig. 3), whereas insulin influences the rate of protein synthesis over a period of hours in other cell types through mechanisms involving de novo protein synthesis (18, 21). Thus, it is apparent that insulin can regulate protein metabolism through diverse mechanisms depending on the phenotypic characteristics of the cell; therefore, generalizations regarding the mechanisms and rapidity of insulin action on protein synthesis should be limited to the cell type(s) under study.

The predominant site of insulin action in adipocytes appears to be at the level of protein synthesis. We base this supposition on our finding that insulin rapidly ($t_{1/2}$ of 4.4 min) and markedly (2-3-fold increase) enhanced leucine incorporation into protein without influencing $[^{3}H]$ leucine uptake and that adipocytes degraded proteins relatively slowly with only minimal inhibitory effects by insulin on protein degradation. Particularly relevant to this aspect of our work are the early findings of Minemura et al. (10) who in 1970 systematically investigated whether the site of insulin action in isolated adipocytes was at the level of amino acid uptake, protein breakdown, or protein synthesis. Using $^{14}$C-labeled proteins and the method of acid precipitation of proteins on filter discs, these investigators concluded that the predominant effect of insulin was on some unidentified step in protein synthesis. Further evidence supporting an effect of insulin on protein synthesis in adipocytes was provided by the more recent studies of Lyons et al. (15). These investigators prepared polyribosomes from fat pads of control and insulin-injected rats and found a 2-fold increase in the protein synthetic activity of isolated ribosomes after insulin treatment, directly demonstrating a persistent and direct effect on ribosomal protein synthesis. This finding eliminated the possibility of major insulin effects on amino acid transport or protein degradation. Moreover, based on the observed shift in the ribosomal distribution pattern in sucrose gradients, it appeared likely that insulin increased the number of initiated ribosomes (15).

Several experimental approaches have been used to study the mechanism(s) of insulin action at the cellular level, one of which has been to elucidate the very early events in the insulin action cascade. This approach has led to a much clearer understanding of the itinerary and metabolic fate of insulin and insulin receptors after endocytotic uptake of insulin-receptor complexes (6, 22-24), greater insights into insulin action (13, 25), and a more detailed knowledge of the intimate relationship between insulin binding, receptor function, and insulin action. Particularly exciting have been the recent discoveries that insulin receptors are tyrosine-specific protein kinases that can undergo ligand-induced autophosphorylation (26-31) and that a novel inositol-containing glycolipid mediates many of insulin's biological actions after being released into the cell interior following insulin binding to cell surface receptors (25). Concomitant with these discoveries have come new theories relating to the causes of insulin resistance. For example, reports have appeared ascribing insulin resistance to both decreased kinase activity of insulin receptors (32-35) and changes in the ability of cells to liberate second messengers in response to insulin (36-38). In evaluating these reports, however, it must be realized that regulation of these events would most likely lead to a state of generalized insulin resistance due to the fact they occur very early in the cascade of cellular events. Therefore, it is apparent that other regulatory mechanisms must exist to exert more selective control over the pleiotropic effects of insulin.

To explore post-receptor regulation of insulin at distal sites along the insulin action pathway, we tested the hypothesis that desensitization of glucose transport is not due to generalized desensitization of all insulin effector systems, but rather is due to a localized action of glucose within the glucose transport cascade. Our approach in testing this hypothesis was to treat cells with high glucose and insulin (to induce insulin resistance of the glucose transport system) and then examine insulin sensitivity and responsiveness of the protein synthesis system. Results of these studies clearly revealed that glucose can selectively desensitize the glucose transport system without inducing desensitization of other insulin-responsive effector systems. Thus, we found that after a 6-h incubation with high glucose and insulin, the glucose transport system of isolated adipocytes was markedly insulin-resistant, maximal insulin responsiveness was reduced by about 60%, and insulin sensitivity was decreased 7-fold (from an ED₅₀ of 0.2 ng/ml to 0.78 ng/ml in control and insulin-treated cells, respectively). In contrast, no diminution of either maximal insulin responsiveness or sensitivity of the protein synthesis system was observed under identical treatment conditions. We postulate from these findings that many, if not all, insulin effector systems are independently regulated and that various metabolic substrates play important roles in regulating the pleiotropic effects of insulin.

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Kinetics of Insulin Action on Protein Synthesis in Adipocytes

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