Insulin-Stimulated Glucose Transport and Insulin Internalization Share a Common Postbinding Step in Adipocytes

ALBERT L. JOCHEN AND PAULOS BERHANU

SUMMARY

We recently demonstrated that chymotrypsin substrate analogues inhibit receptor-mediated insulin internalization in isolated rat adipocytes. In this study, the effect on glucose transport of inhibiting insulin internalization with these agents was examined. Glucose transport was assayed by measuring [^{3}H]-2-deoxyglucose uptake, and internalized insulin was measured after rapidly dissociating surface-bound insulin with an acidic buffer. The chymotrypsin substrate analogue *N*-acetyl-Tyr ethyl ester inhibited insulin internalization by 85% while increasing surface-bound insulin by 80–110%. Under these conditions, ATP levels were minimally altered, and basal glucose transport was unchanged; however, insulin-stimulated glucose transport was decreased by 86%. The inhibition of insulin-stimulated glucose transport was not overcome by supramaximal concentrations (400 ng/ml) of insulin. When insulin internalization and insulin-stimulated glucose transport were measured in the presence of increasing concentrations of *N*-acetyl-Tyr ethyl ester (0.1–1 mM), a strong and highly significant correlation ($r = .97$, $P < .001$) was found between inhibition of insulin internalization and inhibition of insulin-stimulated glucose uptake. Fragments of *N*-acetyl-Tyr ethyl ester that do not inhibit insulin internalization were also without effect on insulin-stimulated glucose transport. In addition to *N*-acetyl-Tyr ethyl ester, four other chymotrypsin substrate analogues that are effective inhibitors of insulin internalization also markedly inhibited insulin-stimulated glucose transport. These results indicate that insulin internalization and insulin-stimulated glucose transport share a common postbinding step in adipocytes and that this step is inhibitable by chymotrypsin substrate analogues. *Diabetes* 36:542–45, 1987

From the Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado.

Address correspondence and reprint requests to Paulos Berhanu, University of Colorado Health Sciences Center, Department of Medicine, B151, 4200 East Ninth Avenue, Denver, CO 80262.

Present address of A.L.J.: Endocrine-Metabolic Section, Froedtert Memorial Lutheran Hospital, Medical College of Wisconsin, 9200 West Wisconsin, Milwaukee, WI 53226.

Received for publication 1 December 1986 and accepted in revised form 26 January 1987.

After the binding of insulin to its specific cell surface receptors, the resulting insulin-receptor complexes are rapidly internalized by absorptive endocytosis (1–6). Although the functional role of this process is not completely understood, suggested potential functions include regulation of cell surface receptor number (7), termination of insulin action by delivery of insulin to intracellular sites of degradation (8), and mediation of certain insulin effects (9,10). It is also not known whether or how the relatively rapid postbinding processes of insulin internalization and insulin-stimulated glucose transport are related. One approach to clarifying such relationships would be to study insulin-mediated glucose transport under conditions where insulin internalization is blocked. We recently characterized a class of agents, chymotrypsin substrate analogues, that efficiently inhibit insulin internalization in isolated rat adipocytes (11). To determine the relationship between insulin internalization and insulin-stimulated glucose transport, we examined the effect of inhibiting insulin internalization with chymotrypsin substrate analogues on insulin-stimulated glucose uptake.

MATERIALS AND METHODS

Materials. Porcine monocomponent insulin was supplied by Dr. Ronald Chance of Eli Lilly (Indianapolis, IN). ^{125}I-labeled Na^+ was purchased from Amersham (Arlington Heights, IL), [^{3}H]-2-deoxyglucose and L-[^{3}H]glucose from New England Nuclear (Boston, MA), bovine serum albumin (BSA; Fraction V) from Armour Pharmaceutical (Chicago, IL), silicone oil from Thomas Scientific (Swedesboro, NJ), and chymotrypsin substrate analogues and other chemicals from Sigma (St. Louis, MO).

Preparation of isolated adipocytes. Isolated adipocytes were prepared by collagenase digestion of epididymal fat pads obtained from male Sprague-Dawley rats weighing

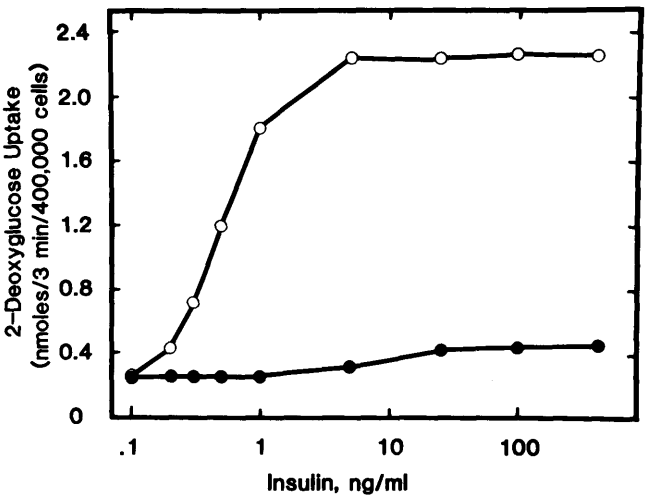

FIG. 1. Effect of insulin on 2-deoxyglucose uptake in control and *N*-acetyl-Tyr ethyl ester–treated cells. Adipocytes were incubated with indicated insulin concentrations for 30 min at 37°C in absence (○) or presence (●) of 1 mM *N*-acetyl-Tyr ethyl ester. [^{3}H]-2-deoxyglucose was then added, and uptake was measured after 3 min and corrected for L-[^{3}H]glucose uptake. Results shown represent means of 3 separate experiments.

180–220 g (12). The cells were filtered through a polystyrene mesh, washed three times, and suspended in Krebs-Ringer phosphate (pH 7.4) containing 1% BSA (KRP/BSA). Adipocyte counts were performed as previously described (11).

Measurement of insulin internalization. Insulin was iodinated as previously described (11) and chromatographed on a Sephadex G-50 column to remove free ^{125}I. Isolated rat adipocytes (400,000 cells/ml) were incubated with 5 ng/ml ^{125}I-labeled insulin in KRP/BSA at 37°C in the presence or absence of *N*-acetyl-Tyr ethyl ester. Nonspecific binding was determined by performing parallel incubations in the presence of 50 μg/ml unlabeled porcine insulin. At the indicated times, intracellular ^{125}I-insulin was measured with a modification (11) of an acid-dissociation technique (13) that rapidly and quantitatively removes surface-bound insulin, leaving behind intracellular insulin. Details of this procedure have been previously described (11).

Measurement of glucose transport. Isolated adipocytes (400,000 cells) were incubated in a final volume of 1 ml KRP/BSA at 37°C in the presence or absence of *N*-acetyl-Tyr ethyl ester with the indicated amounts of insulin. After 30 min of incubation, [^{3}H]-2-deoxyglucose uptake was measured as previously described (14). [^{3}H]-2-deoxyglucose was added at a concentration of 0.1 mM (0.2 μCi/ml), and the uptake was terminated after 3 min by centrifuging the cell aliquots (300 μl) through silicone oil. The uptake of L-[^{3}H]glucose was measured in parallel incubations to correct for nonspecific trapping of isotope (14).

Measurement of adipocyte ATP. Perchloric acid extracts of adipocytes neutralized with KOH were assayed for ATP with a luciferin-luciferase kit (ATP Bioluminescence CLS, Boehringer, Mannheim, FRG) as described previously (15). Bioluminescence was monitored in a liquid scintillation counter with the coincidence circuit turned off.

RESULTS

To assess the relationship between insulin internalization and insulin's ability to stimulate glucose transport, dose-response curves for 2-deoxyglucose uptake were determined in the absence and presence of 1 mM *N*-acetyl-Tyr ethyl ester (Fig. 1). We have previously shown that this chymotrypsin substrate analogue inhibits intracellular insulin accumulation by 85% without decreasing binding to surface receptors (11). Basal uptake was similar in control cells and *N*-acetyl-Tyr ethyl ester–treated cells (0.16 ± 0.02 vs. 0.20 ± 0.02 nmol · 3 min^{-1} · 400,000 cells^{-1}, mean ± SE). In contrast, insulin-stimulated 2-deoxyglucose uptake was markedly inhibited by *N*-acetyl-Tyr ethyl ester (2.24 ± 0.12 vs. 0.31 ± 0.03 nmol · 3 min^{-1} · 400,000 cells^{-1} at 5 ng/ml insulin). The

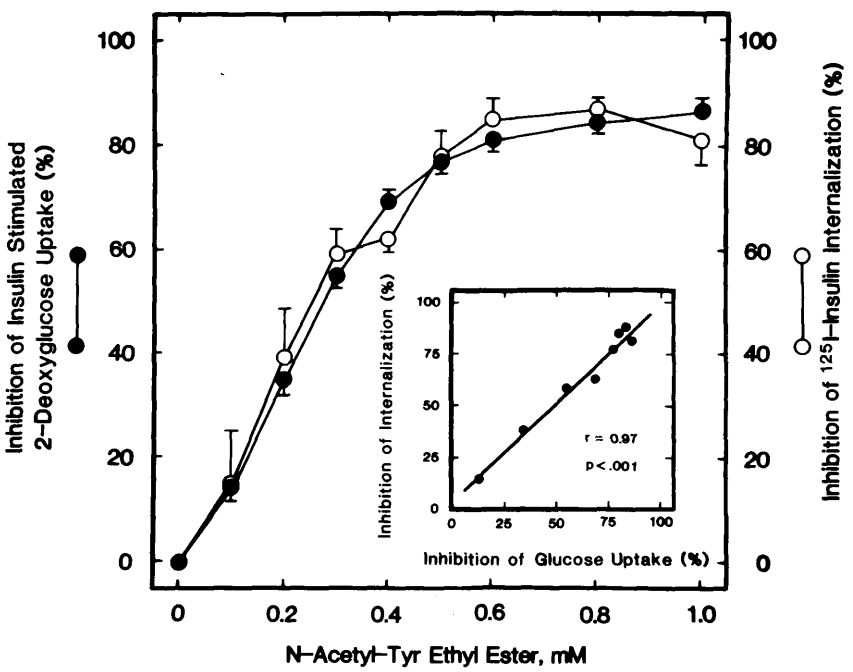

FIG. 2. Relationship between inhibition of insulin-stimulated glucose transport and inhibition of insulin internalization. Adipocytes were incubated for 30 min at 37°C with 5 ng/ml unlabeled insulin in presence of indicated *N*-acetyl-Tyr ethyl ester concentrations and [^{3}H]-2-deoxyglucose uptake then determined over 3-min period (●). In parallel, cells were incubated with indicated *N*-acetyl-Tyr ethyl ester concentrations and 5 ng/ml ^{125}I-labeled insulin. After 30 min, intracellular insulin was determined after dissociation of surface-bound insulin by acid wash (○). Results shown are corrected for nonspecific hexose uptake and nonspecific insulin binding and represent means ± SE of 3 separate experiments.

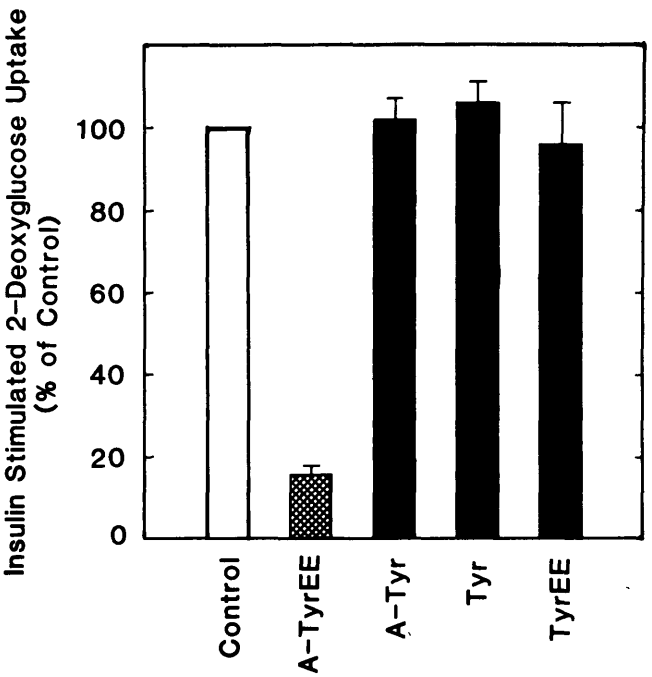

FIG. 3. Structural requirements for inhibition of insulin-stimulated 2-deoxyglucose uptake by *N*-acetyl-Tyr ethyl ester. Adipocytes were incubated for 30 min at 37°C with 5 ng/ml insulin in presence of: no further additions (control, *open bar*), 1 mM *N*-acetyl-Tyr ethyl ester (A-TyrEE), 1 mM *N*-acetyl-Tyr (A-Tyr), 1 mM Tyr (Tyr), or 1 mM Tyr ethyl ester (TyrEE). [^{3}H]-2-deoxyglucose uptake was then measured during 3 min incubation at 37°C. Results shown represent means ± SE of 3 separate experiments.

inhibition of insulin-stimulated 2-deoxyglucose uptake by *N*-acetyl-Tyr ethyl ester could not be overcome by supramaximal (400 ng/ml) concentrations of insulin (Fig. 1).

Figure 2 shows the effects of increasing concentrations of *N*-acetyl-Tyr ethyl ester on insulin internalization and insulin-stimulated 2-deoxyglucose uptake. Both insulin-stimulated glucose transport and insulin internalization were decreased to the same extent at each concentration of *N*-acetyl-Tyr ethyl ester. Thus, a strong and highly significant correlation (r = .97, $P < .001$) was found between the inhibition of the two processes by increasing *N*-acetyl-Tyr ethyl ester concentrations (Fig. 2). Concomitant with the inhibition of intracellular insulin accumulation by *N*-acetyl-Tyr ethyl ester, there was a proportionate increase (80–110% above control) in the amount of insulin remaining surface bound (data not shown).

Inhibition of insulin-stimulated glucose uptake required the intact *N*-acetyl-Tyr ethyl ester molecule, because its fragments (*N*-acetyl-Tyr, Tyr, and Tyr ethyl ester) were without effect (Fig. 3). Thus, similar to effects on insulin internalization (11), the inhibitory effect on glucose uptake depends on the full structural complement of a chymotrypsin substrate analogue (16). Furthermore, in addition to *N*-acetyl-Tyr ethyl ester, four other chymotrypsin substrate analogues (*N*-acetyl-Phe ethyl ester, *N*-acetyl-Trp ethyl ester, benzoyl-Tyr ethyl ester, and benzoyl-Tyr amide), which were previously shown to inhibit insulin internalization (11), also inhibited insulin-stimulated glucose transport (Fig. 4).

ATP levels were measured in control and *N*-acetyl-Tyr ethyl ester–treated cells to assess the effects of this agent on cellular metabolic energy. In the presence of 1 mM *N*-acetyl-Tyr ethyl ester at 37°C, ATP levels were 109 ± 5% (*P* NS) and 83 ± 9% (*P* NS) of those in control cells after 10 and 30 min of incubation, respectively (data not shown).

DISCUSSION

The results of this study demonstrate that chymotrypsin substrate analogues block insulin's ability to stimulate glucose transport in isolated rat adipocytes. This inhibition directly correlated with the ability of these agents to block receptor-mediated insulin internalization based on the dose dependency of inhibition (Fig. 2) and on the structural requirements for inhibition. Thus, fragments of *N*-acetyl-Tyr ethyl ester that were ineffective in inhibiting insulin internalization were also without effect on insulin-stimulated glucose transport (11; Fig. 3). Similarly, a series of established chymotrypsin substrate analogues that effectively inhibited insulin internalization (11) also inhibited insulin-stimulated glucose transport (16; Fig. 4).

The effects of these agents are unlikely to be a result of nonspecific cell toxicity, because *N*-acetyl-Tyr ethyl ester did not significantly alter adipocyte ATP levels or affect basal glucose uptake. Furthermore, our previous results showed that among a large group of structurally similar compounds tested, only chymotrypsin substrate analogues inhibited insulin internalization (11). The decreases in insulin internalization and insulin-stimulated glucose transport were not the result of an inability of insulin to bind to its receptor, because cell surface (receptor) binding actually increased in the presence of *N*-acetyl-Tyr ethyl ester.

The dual inhibition of insulin internalization and insulin-

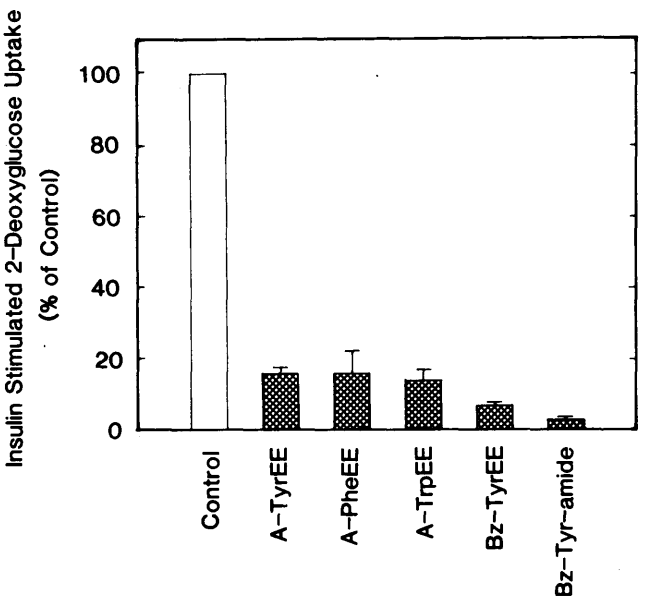

FIG. 4. Effects of various chymotrypsin substrate analogues on insulin-stimulated 2-deoxyglucose uptake. Adipocytes were incubated for 30 min at 37°C with 5 ng/ml insulin in presence of: no further additions (control, *open bar*), 1 mM *N*-acetyl-Tyr ethyl estr (A-TyrEE), 0.75 mM *N*-acetyl-Phe ethyl ester (A-PheEE), 0.5 mM *N*-acetyl-Trp ethyl ester (A-TrpEE), 0.2 mM benzoyl-Tyr ethyl ester (Bz-TyrEE), or 10 mM benzoyl-Tyr amide (Bz-Tyr-amide). [^{3}H]-2-deoxyglucose uptake was then measured during 3 min incubation at 37°C. Results shown represent means ± SE of 3 separate experiments.

stimulated glucose transport suggests that these two processes are related. This does not necessarily imply that glucose-transport activation requires the interaction of internalized insulin or its degradation products with intracellular sites of action, because compounds structurally unrelated to insulin, such as anti-receptor antibodies and lectins, can stimulate glucose transport via the insulin receptor (17–19). A more likely explanation is that insulin internalization and insulin-stimulated glucose transport share a common postbinding step or pathway that is inhibitable by chymotrypsin substrate analogues. This step could represent an early shared postbinding step involving the insulin receptor itself or could involve pathways distal to the insulin receptor. For example, because it is thought that activation of glucose transport by insulin involves the recruitment of intracellular glucose transporter units to the plasma membrane (19,20), it is possible that the chymotrypsin substrate analogues may be blocking a step in vesicular trafficking that is common to both the recruitment of glucose transporter units to the plasma membrane and the translocation of cell surface insulin-receptor complexes to the cell interior. Thus, the demonstration of chymotrypsin substrate analogues as novel inhibitors of insulin internalization and glucose-transport activation should prove useful in further studies in this area.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent secretarial assistance of Melissa Adams in the preparation of this manuscript.

This work was supported by Grant AM-32880 from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). A.L.J. was supported by Fellowship Grant IF32 AM-07383 from the NIDDK.

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