Kinetics of Biosynthetic Human Proinsulin Action in Isolated Rat Adipocytes

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SUMMARY

The binding and biologic potencies of human biosynthetic proinsulin (HPro) were determined in isolated rat adipocytes. At both 16°C and 37°C, proinsulin was found to have 3% (on a molar basis) the potency of porcine insulin for displacing bound [(^{125}I)Tyr^{A14}]-insulin from insulin receptors. Human biosynthetic proinsulin also had 3% of the molar potency of insulin for stimulation of deoxyglucose transport (EC_{50} = 8.8 ± 0.05 × 10^{-11} M for insulin and 2.9 ± 0.55 nM for HPro). However, both hormones produced the same maximal effect on glucose transport. In order to determine if the delay in onset and persistence of proinsulin action seen in vivo was due to any differences at the cellular level, the time course of HPro action on glucose transport was determined. Biologically equivalent submaximal concentrations of insulin (0.166 nM) and HPro (4.44 nM) gave identical time courses for stimulation of deoxyglucose transport at 37°C with half-maximal effects at 4 min and full effects by 30 min. Maximally stimulating concentrations of insulin (1.66 nM) and HPro (22.2 nM) also had superimposable time courses. Deactivation of stimulated glucose transport was determined by incubating equivalent concentrations of insulin (0.166 and 1.66 nM) and HPro (4.44 and 22.2 nM) until full stimulation was achieved, washing cells free of unbound hormone, and initiating dissociation and deactivation by resuspension in hormone-free buffer. Both the absolute activities of transport and rates of deactivation were the same for insulin and HPro. At the submaximal concentrations, 50% of the hormones' effects were lost by 20 min, while 50 min was required after maximal stimulation for 50% deactivation. Therefore, the activation and deactivation kinetics of HPro were no different at the cellular level than for insulin; the delayed onset and prolonged persistence of action in vivo may be due to differences in in vivo pharmacokinetics and/or metabolism. DIABETES 1986; 35:318–23.

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Proinsulin is the direct precursor molecule to insulin, and a small fraction (2–5%) of uncleaved proinsulin is secreted by the beta cells into the circulation.[1] This, coupled with the fact that the metabolic clearance rate for proinsulin is approximately one-fourth that of insulin,[2–4] means that serum proinsulin levels can be 10–30% as high as insulin levels under basal conditions. Although the biologic role of proinsulin is unclear, it does possess weak insulin activity with a molar potency ranging from 1% to 16% that of insulin, depending on the source and response studied.[2,5–12]

With the recent availability of purified human biosynthetic proinsulin (HPro) made by recombinant DNA techniques[13] it has been possible to perform more extensive studies into proinsulin action. In vitro studies using intact cells or purified receptors have found that HPro has a reduced affinity for insulin receptors.[14–18] Kinetic studies demonstrated that tracer concentrations of HPro take far longer to reach equilibrium compared with insulin while the two hormones dissociate at a similar rate.[16,17] This indicates that the reduced binding affinity of proinsulin is due to a decreased association rate constant with no change in the dissociation rate constant. However, once HPro binds to receptors, it is internalized at the same rate as insulin. Functional studies of HPro action have revealed a close correlation between binding and biologic potencies.[15–17]

In vivo studies of HPro action have established that the prohormone differs significantly from insulin. HPro has 7–12% the biologic potency of insulin to stimulate in vivo glucose disposal or inhibit hepatic glucose production.[2,5–7,19] However, insulin and HPro act through the same receptors, since submaximal concentrations are additive in their effects while maximal concentrations are not. The effects of proinsulin to increase glucose disposal rate and suppress hepatic glu-

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cose output develop more slowly compared with insulin.[3,19,20] Most strikingly, however, the biologic half-life of HPro is markedly prolonged relative to insulin.[20] Thus, after cessation of a constant infusion of HPro or insulin, 50% of the hormone's stimulatory effect on peripheral glucose disposal was still present at 35 and 71 min for insulin and HPro, respectively. Furthermore, 50% suppression of hepatic glucose output persisted for 55 and 188 min after cessation of the insulin or HPro infusion, respectively. The differences in biologic half-life are only partially explained by differences in circulating half-life of insulin (5 min) and HPro (42 min). These findings suggested that some process distal to serum HPro levels accounts for the markedly prolonged biologic half-life of this hormone.

Thus, the purpose of this study was to further elucidate the mechanisms underlying the observed in vivo differences in kinetics of insulin and proinsulin action. By using an isolated cell system one can study the kinetics of hormone action without the confounding variables of in vivo pharmacokinetics.

MATERIALS AND METHODS

Materials. Porcine insulin was a gift from Dr. R. E. Chance of Eli Lilly and Co. (Indianapolis, Indiana). HPro and [(^{125}I)Tyr^{A14}]-human proinsulin were gifts of Dr. Bruce Frank of Eli Lilly Laboratories, the preparation and characterization of which have been described elsewhere.[21] [(^{125}I)Tyr^{A14}]-insulin was also a gift of Dr. Frank. Bovine serum albumin (Fraction V) was purchased from Boehringer–Mannheim (Indianapolis, Indiana). Collagenase was purchased from Worthington Biochemicals (Freehold, New Jersey); deoxyglucose and L-glucose were from Sigma (St. Louis, Missouri), and phloretin from ICN Pharmaceuticals (Plainview, New York). The silicone oil for deoxyglucose transport and binding assays was from A. H. Thomas Co. (Philadelphia, Pennsylvania), while that for 3-O-methylglucose was from Union Carbide Corp. (Danbury, Connecticut). L-[1-^{3}H(N)]-glucose, 2-(1,2-^{3}H)-deoxy-D-glucose, and 3-O-[^{14}C(U)]-methyl-D-glucose were purchased from New England Nuclear (Boston, Massachusetts).

Preparation of isolated adipocytes. Fat cells were isolated from the epididymal fat pads of Sprague Dawley rats (180–220 g) by a modification of the method of Rodbell.[22] Minced tissue was incubated for 60 min at 37°C in Krebs-Ringer phosphate buffer (KRP) containing 3 mM glucose-4% BSA and collagenase (2 mg/ml), pH 7.4. The cell suspension was filtered through a 500-μm nylon mesh and washed twice in reaction buffer. The cells were then filtered a second time through 250-μm mesh and washed two additional times. Washes were performed with a buffer containing 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 10 mM Hepes, and 1% BSA (pH 7.4), and this buffer was used in all subsequent binding and transport studies.

Cell counting was performed using a modification[23] of Method III of Hirsch and Gallian[24] and a Model ZM Coulter Counter with 400-μm aperture tube.

Insulin binding. Measurement of insulin binding was performed as described previously using the buffer detailed above.[23] Adipocytes were incubated with ^{125}I-A^{14}-insulin (0.2 ng/ml) or ^{125}I-HPro (2 ng/ml) and various concentrations of unlabeled insulin and HPro. Triplicate 200-μl aliquots were removed, layered over silicone oil in 400-μl microfuge tubes, and then centrifuged at 11,000 × *g* in a Beckman microfuge (Beckman Instruments, Fullerton, California). The tubes were sliced through the oil layer and the cell plugs placed into tubes for direct measurement of radioactivity. Nonspecific binding of either hormone was determined in the presence of 200 μg/ml unlabeled insulin and all results are corrected for this value.

Glucose transport. Stimulation of glucose transport was used as the measure of biologic activity of insulin and HPro. Two different glucose analogues were used to measure transport activity and both gave comparable results. 3-O-methylglucose was used for the deactivation studies where brief reaction times were needed, and 2-deoxyglucose was employed for steady-state experiments and activation kinetics.

2-deoxyglucose transport was measured by a modification of a previously reported technique.[25] After preincubation for various times with insulin or HPro, the reaction was initiated by adding a small volume of mixed ^{3}H-deoxyglucose and unlabeled sugar (0.1 mM, 0.2 μCi/ml) to the cells. For steady-state studies substrate was added directly to the 1-ml preincubation. For activation studies, 1 ml of the cell suspension was removed from the preincubation flask and added to substrate. Triplicate 200-μl aliquots were layered over silicone oil as in the binding studies, and the reactions were terminated after 3 min by centrifugation. The contributions of trapping and diffusion were corrected for by parallel incubations with ^{3}H-L-glucose. This value was subtracted from total uptake to determine carrier-mediated transport.

The rapid assay of initial rates of 3-O-methylglucose transport was accomplished using the microfuge technique of Foley et al.[26] Reactions were performed in 3-ml cryotubes in which 20 μl of substrate (0.2 μCi) was placed. The reaction was started by adding 50 μl of cell suspension (10^6/ml) to substrate (final concentration 15 μM), and was terminated after 2–10 s by addition of 400 μl of 0.1 mM phloretin. A 400-μl aliquot was layered over 100 μl of silicone oil in 550-μl tubes and centrifuged in the microfuge for 30 s. Diffusion was insignificant during the brief assay period, and trapping was corrected for by subtracting cell-associated radioactivity after a time-zero assay.

Incubation conditions. For steady-state studies of binding and glucose transport stimulation, initial incubations were done in 1-ml volumes in 17 × 100-mm polypropylene tubes. Time courses of binding and transport activation were carried out in large volumes and initiated by adding labeled (binding) or unlabeled hormone (transport). Aliquots were removed at specified times for direct measurement of binding or subsequent deoxyglucose transport assay. Use of the same conditions for measurement of binding and glucose transport permits direct comparison of functional activity and receptor occupancy.

Deactivation studies employed the standard buffer with the addition of 1 mM pyruvate. The deactivation assay was performed as previously described.[27] Cells were incubated with insulin or HPro at 37°C, collected by centrifugation for 2 min at 50 × *g*, the buffer was removed, and dissociation and deactivation initiated (t = 0) by adding insulin-free buffer back to the original volume. At specified times, 5 ml of the suspension was removed, concentrated by centrifugation

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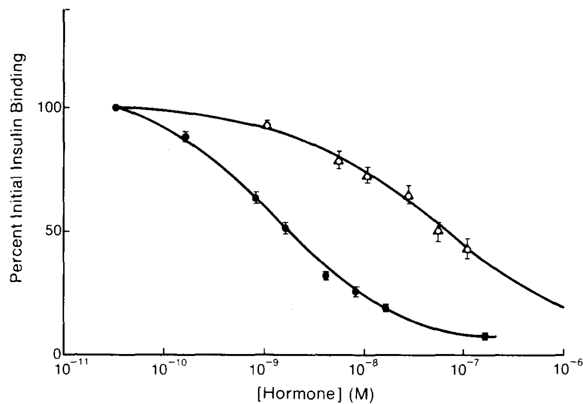

FIGURE 1. Ability of insulin and HPro to compete for ^{125}I-insulin binding to adipocytes. Adipocytes (2×10^5/ml) were incubated with ^{125}I-insulin (33.3 pM) and various concentrations of insulin (•) and HPro (△). Incubation was for 90 min at 37°C. Degradation did not exceed 15%. Results are the average ± SEM of five experiments for HPro and eight experiments for insulin.

and removal of 4.5 ml of buffer, and this cell suspension was used in the 3-O-methylglucose transport assay. Cells not previously exposed to insulin were concentrated in the same way to measure basal transport activity.

RESULTS

Comparison of biologic potencies of insulin and HPro. The ability of insulin and HPro to compete for insulin binding sites on adipocytes was measured at both 16°C and 37°C. Previous studies[16,17] showed that HPro displayed a slower association rate than insulin and our current studies have confirmed this finding (data not shown). At 16°C, only 2 h are needed for ^{125}I-insulin to reach a steady state of binding, while a tracer concentration of ^{125}I-HPro (2 ng/ml) requires 4 h to attain the steady state. At 37°C, steady-state binding at tracer hormone concentrations is reached at 15 and 60 min for insulin and HPro, respectively. All subsequent binding studies were carried out under steady-state conditions. At 16°C, 50% displacement of specifically bound ^{125}I-insulin (a measure of affinity) occurred at an insulin concentration of 0.82 ± 0.08 nM (average ± SEM) and an HPro concentration of 26.3 ± 5.9 nM. At 37°C, the ED_{50} values were 1.74 ± 0.22 nM for insulin and 61.1 ± 10.2 nM for HPro (Figure 1). Thus, at both temperatures, HPro had 3% the binding potency of insulin. The biologic potencies of HPro and insulin to stimulate 2-deoxyglucose transport are compared in Figure 2. A half-maximal effect was obtained at an insulin concentration of 0.08 nM, while concentrations above 4 nM were fully stimulating. The curve for HPro is parallel and right-shifted and the corresponding half-maximal value is 3.3 nM, while maximal stimulation occurs at concentrations >30 nM. Thus, HPro has 3% the biologic potency of insulin but, at maximal concentrations, gives the same absolute effect as insulin. The similarity between the binding and biologic potencies of HPro suggests that HPro-receptor complexes are coupled to action with the same efficiency as insulin-receptor complexes.

Kinetics of insulin and HPro action. We then studied the time courses of insulin and HPro action in vitro, and related the results to the known differences in the onset of action in vivo.[20,21] Since the initial step in action is formation of the hormone-receptor complex, the lower association rate for HPro[16,17] (also see below) could complicate interpretation. However, formation of hormone-receptor complexes is a function of hormone and receptor concentrations as well as the association rate; thus, it is possible to increase the HPro concentration to overcome the difference in association rates and insure that insulin- and HPro-receptor complexes are formed at similar rates. When this is done, one can study the onset of stimulation of glucose transport and ascribe any differences to the nature of the hormone-receptor complex, itself. Since the affinity of HPro is 1/30th that of insulin, using an HPro concentration 30 times that of insulin should negate the effect of the affinity difference. The concentrations of insulin and HPro compared in Figure 3 meet these criteria, achieve the same fractional receptor occupancies, and also give equivalent steady-state glucose transport stimulation (Figure 2). Figure 3 displays the time courses for stimulation of glucose transport by submaximal (0.16 and 4.44 nM) and maximal (1.66 and 22.2 nM) concentrations of insulin and HPro, respectively. Results are expressed as the absolute amount of 2-deoxyglucose transport with the 3-min assay started at the indicated time after insulin addition. These functionally equivalent concentrations increase glucose transport with similar time courses for insulin and HPro. Submaximal concentrations reached half of their full effect by 4 min and full effect within 30 min (Figure 3A). The higher hormone concentrations (Figure 3B) are half-maximal by 2–3 min and complete by 20 min. While the 3-min deoxyglucose transport assay limits the resolution at early time points, there were no significant differences between the onset of insulin and HPro action when equivalent doses were compared.

Deactivation rates. In order to determine whether the prolonged in vivo biologic half-life of HPro is due to persistent hormone action at the cellular level, deactivation of glucose transport after stimulation by insulin or HPro and subsequent removal of the hormone was compared. Hormone concentrations and preincubation periods were determined from the results in Figures 2 and 3 to give comparable biologic effects at both submaximal and maximal concentrations (see legend

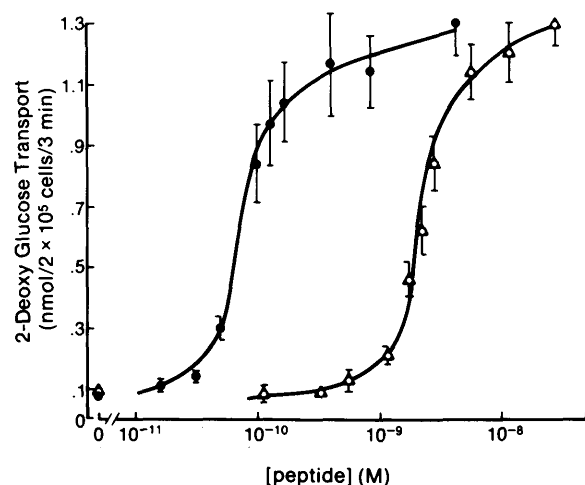

FIGURE 2. Ability of insulin and HPro to stimulate 2-deoxyglucose uptake. Adipocytes (2×10^5/ml) were preincubated for 60 min at 37°C with varying concentrations of insulin (•) and HPro (△); the uptake of 2-deoxyglucose over a 3-min period was then determined. Results are the average ± SEM of six experiments.

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to Figure 4 for absolute numbers). Since the absolute levels of transport were the same for each peptide, the results were expressed as the loss of maximal hormone effect (measured as the difference between basal and stimulated transport at t = 0) at each time point. At the lower concentrations, insulin and HPro showed similar deactivation rates with 50% deactivation at 20 min (Figure 4A). Increasing the hormone concentration slows the rate of deactivation,[28] but does not create any differences between insulin and HPro (Figure 4B). The time required for 50% deactivation was 50 min for both hormones. In neither case does HPro action persist beyond that of insulin.

DISCUSSION

Initial studies into the biologic action of proinsulin were complicated by large interspecies differences in amino acid sequence and possible contamination with insulin. Such variables could account in large part for the fact that reported potencies of proinsulin have ranged from 1% to 16% that of insulin.[5-15] The recent availability of human proinsulin (HPro) synthesized through recombinant DNA techniques[13] has made possible more detailed investigation into the nature of proinsulin action.

The genesis for the current studies was in the observation that during glucose clamp studies in normal subjects, the onset and deactivation of HPro biologic effects were far slower compared with insulin.[20] Thus, during primed continuous infusion, steady-state insulin levels were reached at 30 min; glucose disposal plateaued by 3 h with half-maximal stimulation at 40 min, and hepatic glucose output was suppressed by 50% at 30 min and 100% by 60 min. In contrast, serum HPro levels rose more gradually reaching steady state at 145 min. The biologic effects of HPro were also delayed, with plateau stimulation of glucose disposal at 5 h and half-maximal stimulation at 2.5 h; hepatic glucose output was suppressed 50% at 90 min and 100% by 180 min. From these in vivo results it was not clear if the delayed development of HPro effects was secondary to the slow rise in serum HPro levels or due to differences in HPro action at the cell level. The current studies, using an isolated cell system with rapid control of hormone concentrations, allows us to resolve this question.

To compare the rate at which insulin and HPro stimulate glucose transport distal to the hormone binding step, the hormone concentrations used must result in the same absolute number of occupied insulin receptors and the same final maximal response. Earlier work[15,18] supports the conclu-

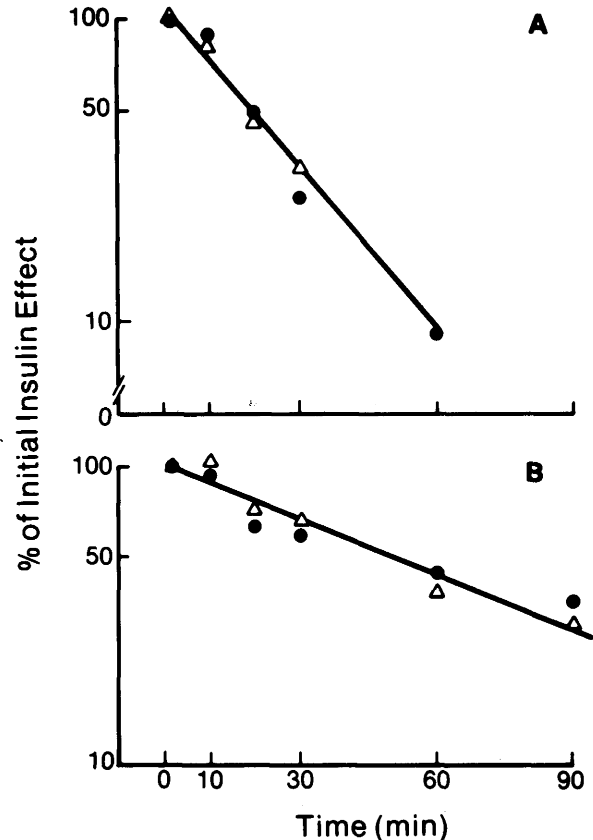

FIGURE 4. Deactivation of glucose transport after stimulation by insulin or HPro. Cells were incubated with various concentrations of the peptides for the times noted below, washed free of unbound hormone, and resuspended in insulin-free buffer. Results are expressed as a percentage of the difference between basal rates of 3-O-methylglucose transport and the stimulated rate at t = 0 (100% initial effect). The horizontal axis represents the time after resuspension at which the transport assay was performed. (A) Cells were incubated with submaximal insulin (•, 0.166 nM) and HPro (△, 4.44 nM) for 60 min. Basal transport was 0.027 ± 0.005 pmol/2 × 10^5 cells/s (N = 9), while stimulated transport was 0.374 ± 0.039 for insulin (N = 9) and 0.358 ± 0.123 for HPro (N = 4). (B) Maximal insulin (1.66 nM) and HPro (22.2 nM) concentrations were incubated with cells for 30 min. Basal transport was 0.018 ± 0.003 (N = 6), while stimulated transport was 0.415 ± 0.054 for insulin (N = 5) and 0.381 ± 0.066 for HPro (N = 4).

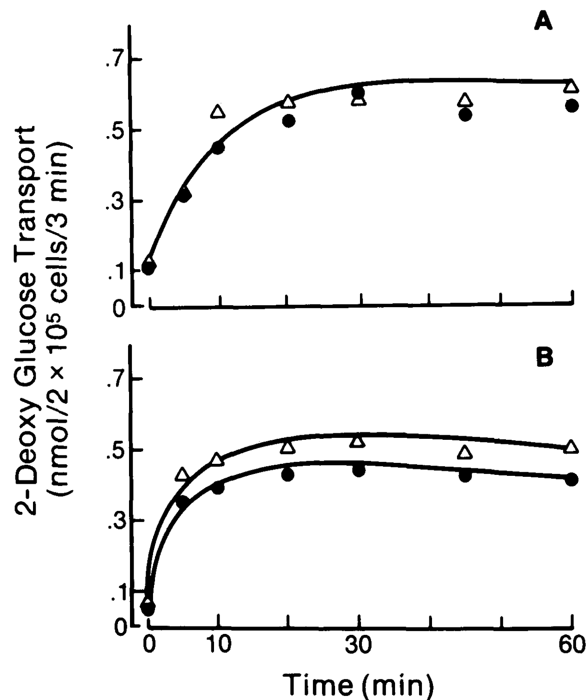

FIGURE 3. Time courses for stimulation of 2-deoxyglucose transport. Adipocytes were incubated without hormone for 30 min at 37°C. Basal transport was measured, and a small volume of insulin or HPro added. One-milliliter aliquots of the cell suspension were removed after the indicated times of preincubation, and the 3-min uptake of deoxyglucose was measured. (A) Submaximal concentrations of insulin (•, 0.166 nM) and HPro (△, 4.44 nM) were used. (B) Maximal concentrations of insulin (1.66 nM) and HPro (22.2 nM) were used. Results are the average of three experiments.

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sion that the lower binding affinity of HPro is due in major part to a reduced association rate constant. This difference in the rate of receptor complex formation can be overcome by increasing the HPro concentration so that by mass action hormone complexes are formed at the same rate as with insulin. The results in Figure 3 show that when concentrations of insulin and HPro are used that give equivalent formation of hormone receptor complexes, the two peptides stimulate glucose transport with similar time courses. At the highest concentrations (Figure 3B), half-maximal stimulation occurs within 3 min for both insulin and HPro while stimulation is complete by 20 min. Lowering the hormone concentration slows activation slightly but does not change the similarity of the time courses between insulin and HPro (Figure 3A). The time required for the 2-deoxyglucose transport assay (3 min) limits the resolution obtained at earlier time points, but no difference is seen in vitro that could account for the large in vivo differences. Thus, once an insulin receptor is occupied, the mechanism coupling occupancy to stimulation of glucose transport does not discriminate between insulin or proinsulin.

Even more striking than the slow in vivo development of HPro action is the marked prolongation of HPro action after the hormone infusion is stopped.[20] Thus, after cessation of the infusion, insulin was cleared from the circulation with a half-time of 5 min while HPro displayed a half-life of 42 min.[20] Deactivation of HPro biologic effects were even more delayed. The stimulated glucose disposal rate declined by 50% at 36 min after insulin and 72 min after HPro; suppression of hepatic glucose output deactivated by 50% at 55 and 188 min for insulin and HPro, respectively. Whether these differences in deactivation rates for insulin and HPro were due to pharmacokinetic factors* or differences in cellular actions could not be determined from the in vivo studies. However, in the current in vitro studies the cellular events in HPro action can be isolated and separately evaluated. Thus, when adipocyte glucose transport is stimulated to comparable levels with either insulin or HPro and followed by rapid removal of unbound hormone, the HPro- or insulin-stimulated glucose transport system deactivates at the same rate. Furthermore, since we have previously shown that insulin and HPro dissociate from insulin receptors at the same rate,[17] the current results indicate that the steps in deactivation of insulin and HPro action distal to dissociation from receptors are also comparable. These results suggest that the in vivo differences in deactivation of insulin and HPro effects on glucose disposal are not due to differences at the cell level. In a strict sense, the conclusions from the current studies can only be extended to HPro effects on glucose disposal. Suppression of hepatic glucose output is mediated through hormone effects on the liver, and although unlikely, it is still possible that differences exist in the kinetics of the cellular actions of insulin and HPro in liver cells.

The relative potency of HPro for receptor binding and biologic activity is the same, suggesting that HPro-receptor complexes and insulin-receptor complexes are coupled to biologic effector systems in an identical manner. Much of the reported range in proinsulin potencies can be accounted for by variations in experimental systems and the purity of the proinsulin preparations used. However, two recent papers[17,18] and the current report, all utilizing isolated rat adipocytes and HPro, differ somewhat in the reported values for HPro binding and biologic potency (1–10%). Peavy et al.[16] noted that while the ED_{50} for insulin action is nearly constant ($5.0–8.8 \times 10^{-11}$ M), there are large variations in the ED_{50} for HPro (0.5–8.5 nM). One possible explanation for the modest variability in the ED_{50} values reported for HPro (refs. 17, 18, and current results) could involve differences in degradation of HPro as the cell concentration varied l0-fold between studies. It has been found that some partial degradation products of HPro (which are still judged intact by the criteria of TCA precipitability) are biologically more active than proinsulin.[12] Increased production of more active intermediates under some conditions could result in a higher relative potency compared with proinsulin alone.

The major conclusions from this work are that once bound to receptors, HPro causes the same magnitude of biologic effect as insulin and does so with similar activation and deactivation time courses. Thus, the slower kinetics of HPro action and deactivation in vivo are not due to differences at the peripheral cell level, but must involve differences in pharmacokinetics, compartmentalization, or processing of the HPro molecule.

*This refers to all aspects of insulin and proinsulin transport, distribution, and metabolism before interaction with cells.

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