Insulin Binding, Biologic Activity, and Metabolism of Biosynthetic Human Insulin

JERROLD M. OLEFSKY

Insulin binding to receptors, insulin action, and cellular insulin metabolism were studied using biosynthetic human insulin (BHI). The results demonstrated that this insulin preparation binds to receptors with full binding potency and also exerts normal biologic activity to stimulate adipocyte glucose oxidation and glucose transport. Furthermore, following initial binding to adipocyte receptors, BHI is internalized and degraded in a manner indistinguishable from purified pork insulin (PPI). Thus, these results demonstrate that BHI has full biologic activity, is metabolized normally, and should produce the full insulin effect when administered to diabetic patients. DIABETES CARE 4: 244–247, MARCH–APRIL 1981.

As a result of recent advances in recombinant DNA technology, it has now become possible to clone certain human genes and translate the gene products in the laboratory. The technology to accomplish this feat has recently been developed for the human insulin gene and it is anticipated that biosynthetic human insulin (BHI) will become available for wide-scale clinical use in the near future.[1] In addition to potential future economic advantages, the use of BHI for the treatment of diabetes may also have clinical advantages. Thus, BHI will undoubtedly be much less antigenic than insulins derived from various animal species, and this should help ameliorate allergic responses to insulin and could help circumvent the complexities of diabetic management that result from the development of anti-insulin antibodies. However, before clinical studies can be adequately evaluated one must be certain that BHI has full biologic activity and is metabolized in a normal manner. On a theoretical basis, there is no reason to suspect that this will not be the case since pork insulin has full biologic potency and has been used for experimental and clinical purposes for many years. Pork insulin and human insulin differ by only one amino acid residue (B30), and position B30 is not in the active site of the insulin molecule.[2] To test this concept, we have studied insulin binding, insulin degradation, and insulin action using a purified preparation of BHI. The results demonstrate that BHI has full biologic activity and is metabolized in a normal manner.

MATERIALS AND METHODS

Materials. Pork monocomponent insulin, BHI, and insulin purified from human pancreases were generously supplied by Dr. Ronald Chance of the Eli Lilly Company (Indianapolis), Na ^{125}I and L-1-^{3}H-glucose were purchased from the New England Nuclear Company, 2-deoxy-D-1-^{3}H-glucose and ^{14}C-1-glucose from Amersham, bovine serum albumin (fraction V) from Armour and Company, and collagenase from Worthington Biochemicals.

Iodination of insulin. ^{125}I-Insulin was prepared to a specific activity of 100–150 μCi/μg according to the modification by Freychet et al.[3] of the method of Hunter and Greenwood,[4] as previously described.[5]

Preparation of isolated adipocytes. Male Sprague-Dawley rats weighing 150–210 g were used for all experiments. Rats were stunned by a blow to the head, decapitated, and their epididymal fat removed. Isolated fat cells were prepared by shaking at 37°C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (3 mg/ml) and albumin (40 mg/ml) according to the method of Rodbell.[6] Cells were then filtered through nylon mesh (250 μm), centrifuged at 400 rpm for 2 min, and washed twice in 35 mM TRIS buffer. Adipocyte counts were performed according to a modification of method III of Hirsch and Gallian,[7] in which the cells were fixed in 2% osmium tetroxide in 0.05 M collidine buffer (made isotonic with saline) for 24 h at 37°C and then taken

up in a known volume of 0.154 M NaCl for counting. Counting was performed with a model ZB Coulter Counter with a 400-μm aperture.

Binding studies. Isolated adipocytes (2–3 × 10^5 cells) were incubated in a total volume of 1 ml (Tris-BSA buffer, pH 7.6) with ^{125}I-insulin (0.2–0.3 ng/ml) in the absence and presence of unlabeled insulin. Incubations were performed in polypropylene tubes (17 × 100) in a shaking water bath at the indicated temperatures and times. The binding reactions were terminated, and free ^{125}I-insulin was separated from cell-bound radioactivity by removing aliquots (300 μl) from the cell suspension and rapidly centrifuging the cells in plastic microtubes to which 100 μl of silicone oil had been added.[8] Silicone oil has a specific gravity intermediate between buffer and cells; therefore, after centrifugation, three layers result: cells on top, oil in the middle, and buffer on the bottom. The cells were then removed and the radioactivity was determined. Adipocyte-associated radioactivity was determined in triplicate from each incubation tube, and each experiment is a representative example of at least three similar experiments unless otherwise indicated. Insulin degradation was monitored by assessing the ability of ^{125}I-insulin remaining in the incubation media to precipitate with 10% trichloroacetic acid (TCA). With this method,[9] TCA-precipitable radioactivity is considered to be intact insulin, and TCA-soluble material is taken to be degraded insulin.

Nonspecific binding. In these experiments, nonspecific binding is defined as the amount of ^{125}I-insulin remaining in the cell layer in the presence of a large excess (50 μg/ml) of unlabeled insulin. When cells equilibrate with a tracer concentration of ^{125}I-insulin (0.1–0.3 nl/ml), only 3% to 7% of the bound insulin represents nonspecific binding. For all samples, total and nonspecific binding was determined, and the total binding was corrected to reflect specific binding.

Glucose transport studies. Control and insulin-treated adipocytes (2–3 × 10^5 cells/ml) were incubated in Krebs-Ringer bicarbonate, 1% BSA, pH 7.4, in the absence and presence of various concentrations of insulin, as previously described in detail.[10] Incubations were performed in polypropylene tubes (total volume 1 ml) in a shaking water bath at 37°C for 1 h, unless otherwise indicated. Glucose uptake was then measured by adding 10 μl of 2-deoxyglucose (12.5 mM) containing 0.2 μCi 2-deoxy-1-^{3}H-glucose. The reaction was terminated at the end of 3 min by transferring 300-μl aliquots from the incubation mixture to plastic microfuge tubes containing 100 μl of silicone oil. The tubes were centrifuged for 30 s in a Beckman microfuge and the assay was considered terminated when centrifugation began. This assay measures the total uptake of radiolabeled 2-deoxyglucose and is based on the principle that while 2-deoxyglucose is transported and phosphorylated by the same process as D-glucose, it cannot be further metabolized. For all experiments, glucose uptake for each point represents the mean of triplicate samples, and each experiment is a representative example of at least three similar experiments unless otherwise indicated. The distribution space of radiolabeled L-glucose was determined in all experiments and used to correct the uptake data for nonspecific carry-over of radioactivity with the cells and the uptake of hexose by simple diffusion.

Glucose oxidation. Measurements of adipocyte ^{14}C-1-glucose oxidation were made according to the method of Rodbell[6] as previously described.[10]

RESULTS

As shown by the competition curves in Figure 1, the displacement of ^{125}I-insulin binding to insulin receptors of isolated rat adipocytes was studied over a range of concentrations of PPI, BHI, and purified human pancreatic insulin. As can be seen, the ability of these three insulin preparations to compete for binding to receptors is identical, and the same results were found when other insulin receptor systems were used, such as cultured IM-9 lymphocytes and human adipocytes. Thus, these data demonstrate that BHI has full binding potency and interacts with insulin receptors in a normal manner.

Direct binding studies using labeled BHI were also performed. These experiments are summarized in Figure 2. Monoiodinated BHI and PPI exclusively labeled in the A14-tyrosine position of the insulin A-chain were studied. In addition, the results were compared to insulin binding by ^{125}I-pork insulin comprised of a mixture of A14-labeled and A19-labeled species. The ability of unlabeled pork insulin to displace these three labeled insulin preparations from adipocyte insulin receptors is shown in Figure 2. As can be seen, the A14-labeled BHI and PPI yielded identical competition curves, demonstrating that both insulin preparations bind to the same population of insulin receptors. It is also apparent that at lower insulin concentrations, adipocyte insulin receptors bind the A14-insulin preparations 28% more, compared with the iodinated insulin preparation containing insulin-la beled at the A14-tyrosine as well as the A19-tyrosine. This

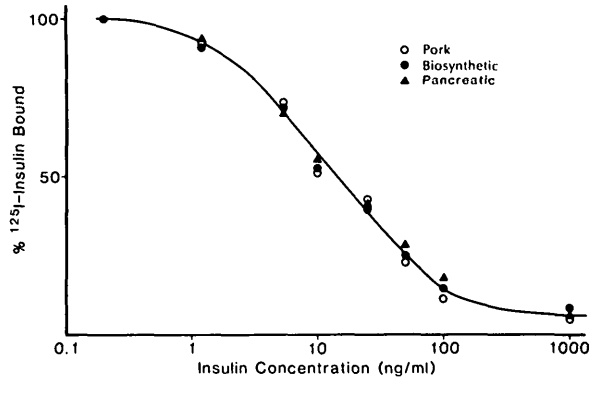

FIG. 1. The ability of pork insulin (○), BHI (●), and purified insulin from human pancreases (▲) to compete for ^{125}I-pork insulin binding to insulin receptors of isolated rat adipocytes. Cells were incubated with 0.2 ng/ml of ^{125}I-insulin plus the indicated concentrations of unlabeled insulin for 90 min at 24°C. All data are normalized to percent of maximal insulin binding (at 0.2 ng/ml ^{125}I-insulin) and are corrected for nonspecific binding.

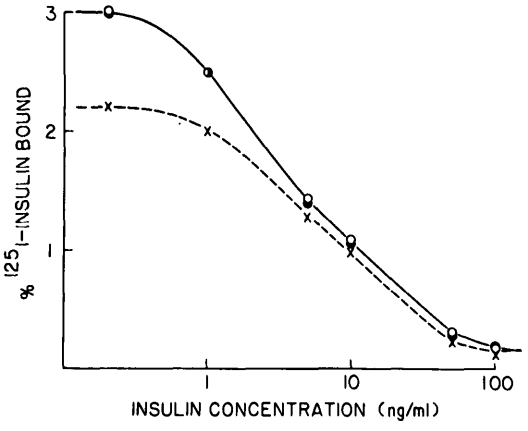

FIG. 2. Insulin binding to isolated adipocytes by monoiodinated A14 BHI (●), monoiodinated A14 pork insulin (○), and a ^{125}I-pork insulin preparation containing a mixture of A14 and A19-labeled species (X). The unlabeled insulin used in all these studies was PPI, and all the data are corrected for nonspecific binding.

latter finding confirms the previously reported observations that A14-labeled insulin has greater binding affinity for the insulin receptor as compared with the A19-iodinated insulin.[11]

The biologic activity of PPI, BHI, and purified human pancreatic insulin was also compared. These results are seen in Figure 3, and demonstrate that all three insulin species have identical stimulatory effects over the entire dose-response curve on adipocyte glucose oxidation (Figure 3A) and glucose transport (Figure 3B).

After the initial binding step, a number of metabolic events follow, including biologic action, hormone internalization, and hormone degradation. It has now been demonstrated that after the formation of the insulin-receptor complex on the cell surface, some portion of the bound insulin is internalized via endocytosis[12,13] and is then processed and eventually degraded intracellularly.[12–14] The precise biochemical mechanisms and intracellular sites of insulin degradation are not completely known, but it is apparent that lysosomes play an important role in this process.[12,13] Consequently, when cells are treated with chloroquine (a lysosomotropic agent that inhibits intralysosomal proteolysis), the intracellular processing and degradation of insulin are inhibited and intact insulin accumulates within adipocytes.[12–14] Thus, the rate of accumulation of insulin by chloroquine-treated adipocytes can be used as an index of the rate of internalization. Using this approach, the time course of binding, internalization, and hormone degradation was determined using A14-labeled pork insulin and BHI. As can be seen in Figure 4A, the time course of binding and rate of accumulation of these two insulin preparations by chloroquine-treated adipocytes is identical, indicating that both insulins are internalized in the same manner. Furthermore, the rate of degradation of these two insulins is also the same (Figure 4B), and chloroquine inhibits degradation of both preparations in a similar manner. These results provide evidence

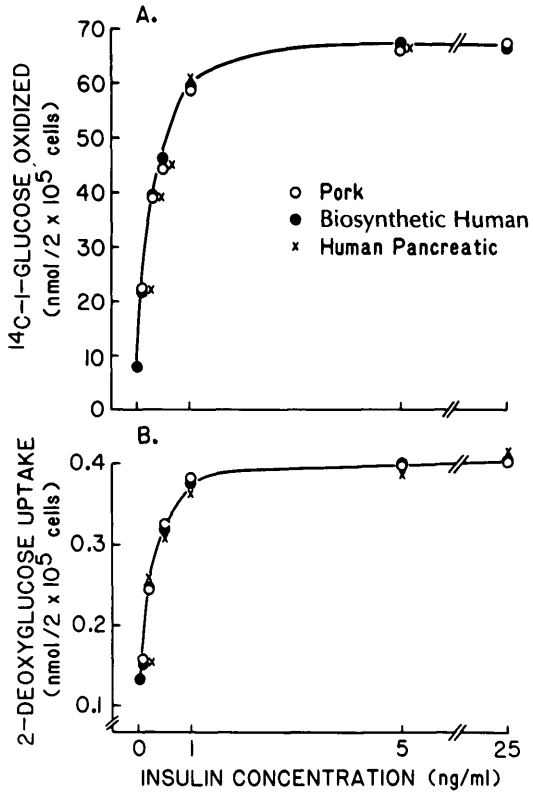

FIG. 3. Biologic activity of PPI (○), BHI (●), and purified insulin from human pancreases (X). (A) Dose-response curve for stimulation of ^{14}C-1-glucose oxidation by isolated rat adipocytes. (B) Dose-response curve for 2-deoxyglucose uptake by isolated rat adipocytes.

that the intracellular processing and degradation of BHI and pork insulin are comparable, indicating that the overall in vivo metabolism of these insulins will also be identical.

DISCUSSION

Methods are now available to produce BHI in vitro by recombinant DNA technology.[1] This advance should lead to the eventual use of BHI for clinical purposes on a widespread scale. Because of the potential clinical use of this new insulin preparation, it is important to ascertain its biologic and metabolic properties. In this study we have examined the ability of BHI to bind to insulin receptors and to promote biologic activity. In addition, we have also assessed the subsequent cellular metabolism of insulin following the initial binding event. The results have demonstrated that, compared with PPI or purified human insulin extracted from human pancreases, BHI has full binding and biologic potency. Thus, using either labeled or unlabeled insulin preparations, insulin-binding properties of these three insulin preparations were indistinguishable in several cell types. This demonstrates that BHI binds to the full population of insulin receptors at a normal rate and with the same affinity as pork insulin.

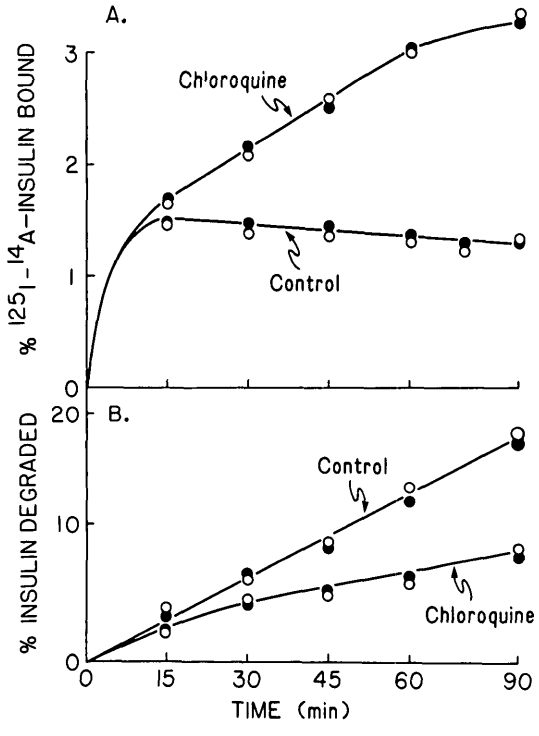

FIG. 4. (A) Time course of binding and accummulation of monoiodinated A14 BHI (●) and monoiodinated A14 PPI (○) by control and chloroquine-treated (0.2 mM) rat adipocytes. (B) Time course of degradation of the A14-labeled human and pork insulin preparations by control and chloroquine-treated adipocytes. In these experiments, control and chloroquine-treated cells were incubated with the different insulin preparations at 37°C and samples were obtained at the indicated time points. Cell-associated radioactivity was measured in the usual fashion (see METHODS), and insulin degradation was assessed by measuring the ability of the insulin remaining in the buffer to precipitate with 10% TCA.

Following the initial binding step, several physiologic events ensue.[15] These include initiation of biologic activity and internalization and degradation of the hormone. The results presented in the current studies demonstrate that all three insulin preparations have the same ability to stimulate glucose metabolism, as assessed by measurements of adipocyte glucose oxidation and transport. Furthermore, based on the studies using chloroquine-treated cells, it can be inferred that BHI is internalized and subsequently metabolized and degraded in a manner similar to that of pork insulin.

As a result of these findings it can be concluded that BHI will have full biologic potency in vivo when administered to patients. In addition, since cellular internalization and metabolism of this insulin preparation is normal, it can also be inferred that the pharmacokinetics of BHI will be normal. The biologic potency and subsequent metabolism of BHI are normal, and if the potential for markedly reduced antigenicity is born out in clinical studies,[1] the availability of BHI for the treatment of diabetic patients should provide a significant advance in the treatment of this complex disease.

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From the University of Colorado Health Sciences Center, Department of Medicine, B151, 4200 East Ninth Avenue, Denver, Colorado 80262.

Address reprint requests to J. M. Olefsky at the above address.

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