Potency of Biosynthetic Human Insulin Determined In Vitro

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Biosynthetic human insulin (BHI) obtained from separately synthesized A- and B-chains by recombinant DNA technology with *Escherichia coli* fermentation was compared with human and pork insulin of high purity in vitro. Applying four biologic tests (glucose oxidation and glucose incorporation into the lipids by rat epididymal fat pads, inhibition of lipolysis, and ATP depletion of isolated fat cells) and three receptor assays (binding competition with human fat cells, IM-9 lymphocytes, and rat liver cell plasma membranes), we could not discern significant differences of the half-maximum response by these seven methods. The only variance occurred with the ATP-depletion assay. This method disclosed 10% greater maximum reversion of isoproterenol-induced ATP depletion by BHI when compared with pork insulin. DIABETES CARE 4: 228–234, MARCH–APRIL 1981.

Natural human[1] and pork[2] insulin differ only by one amino acid of the C-terminal B-chain. B-30-Alanine of pork insulin substitutes for the more hydrophilic amino acid threonine found in human insulin. This amino acid, however, does not seem to be necessary for full biologic potency, as shown by Carpenter.[3] Thus it was of interest to determine whether BHI, shown by multiple criteria[4] to be of high purity, showed any differences in potency when compared to pork insulin, as well as to natural human insulin. It has been suggested from in vivo studies that totally synthesized human insulin (CGP 12-831)[5] and BHI (46 L 35)[6] might differ in their potency when compared with pork insulin. No in vitro data have been reported. This paper reports studies in vitro, comparing three highly purified insulin preparations: biosynthetic and natural human insulins and natural pork insulin. In addition, the very weak analogue des-octapeptide (B23–B30) insulin has been included in some experiments.

MATERIALS AND METHODS

Biosynthetic and pancreatic human insulin of very high purity were obtained from Dr. F. Enzmann, Lilly (Bad Homburg, Germany) (Batches 615-70 N-174-10 and 615-1054 B-214-1). Pork insulin (cryst., Batch 615 07-256) and des-octapeptide insulin (DOP) (Batch 615-108 2 B-246) were obtained from Dr. R. Chance, Lilly (Indianapolis).

Tyrosine A14-monoiodinated ^{125}I-biosynthetic human insulin (Lot J84-02 N-140, spec. act. 320 μCi/μg, labeled June 19, 1980) and pork insulin (Lot J84-020 N-140, spec. act. 367 μCi/μg, labeled May 29, 1980) were obtained from Dr. F. Enzmann, Lilly (Bad Homburg). For studies with IM-9 lymphocytes and rat liver cell plasma membranes, routinely labeled ^{125}I-pork insulin (according to Roth[7]) (spec. act. 180 μCi/μg) was used, from Dr. B. Hansen, Nordisk (Gentofte, Denmark) (Lot Pbl. 14/I Kry. P.: 449-55).

D-(U ^{14}C)-glucose (Lot 255042-A, spec. act. 1.38 mCi/mg) was obtained from Amersham-Buchler (Braunschweig, Germany). Luminescence reagents NRS (nucleotide releasing reagent) and Lumit HS (purified firefly luciferin–luciferase, specific to ATP), as well as standard ATP, were obtained from Abimed (Düsseldorf, Germany). All other reagents used were of analytic grade, obtained from Boehringer, Mannheim, or Serva (Heidelberg, Germany).

Adipose tissue and adipocytes from male CHbb rats, 180 ± 20 g, were obtained from Thomae Biberach (Germany). Human adipocytes were obtained from paraumbilical fat tissue during bile–bladder surgery. Collagenase (Serva, Heidelberg), 0.6 U/mg, was used to digest fat tissue according to Rodbell.[8] Fat cells were filtered prior to use through Japanese silk screen, Monyl 73 HD. Fat cell counts were performed according to Lorch and Rentsch,[9] after staining with acridine orange HCl in Hanks' solution, 1 mg/ml (Merck, Darmstadt, Germany, Cat. no. 1333). The IM-9 lympho-

cytes were obtained from and cultured according to De Meyts.[10]

The following methods were used to study insulin potencies:

1. Glucose oxidation according to Renold et al.[11] Rat epididymal fat pads, 100 mg/ml, obtained according to Faulhaber et al.,[12] were incubated in Krebs-Ringer's phosphate (KRP) buffer[12] supplemented with bovine serum albumin: 2 g/dl, D-glucose, 150 mg/dl, U-^{14}C-glucose, 0.08 μCi/ml, insulin with or without addition of a surplus of anti-insulin antiserum.

Incubation: 2 h at 37°C with gentle shaking; $^{14}CO_2$ released by 5 N H_2SO_4, 200 μl, was absorbed by hyamine hydroxide, 200 μl, during 40 min of further incubation. Studies were performed in triplicate.

2. Glucose incorporation into the lipid fraction. In another set of incubations performed as in (1) above, tissue slices were removed, dried on filter paper, and extracted with chloroform–methanol (2:1), 7 ml for 60 h, according to Dole and Meinertz.[14] After repeated addition of 2 ml of KCl, 0.37 g/dl, to the extracts and phase separation by decantation (three times in 12 h), extracts were evaporated to dryness and evaluated for ^{14}C-incorporation by addition of Bray's solution and counting.

3. Inhibition of lipolysis of isolated human fat cells. Fat cells were isolated by collagenase, 15 mg/2 ml, 37°C, 45–55 min, filtered through a silk screen, washed several times with incubation buffer (KRP + BSA, 2 g/dl; glucose, 150 mg/dl), and incubated in a concentration of approximately 5 × 10^5 cells (25–45 mg lipid/ml) in a total volume of 0.5 ml for 90 min at 37°C. Isoproterenol was added prior to the experiments in a concentration of 100 ng/ml. Insulin was added simultaneously at the concentrations indicated in RESULTS. Glycerol release was determined according to Chernik[15] with the fluorometric method of Wieland[16] using an Aminco fluorometer.

4. Inhibition of ATP depletion. Fat cells were isolated as indicated in (3) above and incubated in KRP buffer, pH 7.4 + BSA 2 g/dl + glucose 100 mg/dl, 5 × 10^5 cells/ml, in a total volume of 0.5 ml for 5 h at 37°C. Insulin was added as indicated in RESULTS, 15 min before the addition of isoproterenol, 1000 ng/ml. At regular intervals, 10 μl of the cell suspension was taken off during gentle shaking with a whirlmix and added to 30 μl of nucleotide releasing reagent (NRS) for somatic cells.

After 15 sec, the cuvette, in which extraction was performed, was placed directly into the bioluminescence detector, Lumac cell tester (M 1060) (Abimed, Düsseldorf). Relative light units (RLU) obtained during a 10-s integration time were corrected by internal standardization, adding 20 ng ATP/10 μl to a second probe of cells. Light emission (measured in relative light units) in this system proved to be linearly related to the ATP content of the cells over the entire range of 0.5 to 200 RLU studied; 1 RLU corresponded to 800 pg ATP.

5. Binding of ^{125}I-insulin to human fat cells. Fat cells were isolated according to (3) above. Binding studies were performed according to Gammeltoft and Gliemann;[17] 5 × 10^5 fat cells were incubated in 1 ml of KRP buffer (BSA 2 g/dl, pH 7.6) at 24°C for 30 min in the presence of tracer, 50 pg/ml, with or without the addition of various concentrations of cold pork insulin. Bound and free ^{125}I-insulin were separated according to Olefsky and co-workers[18] by centrifugation of 2 × 0.2 ml of suspended fat cells through 0.1 ml of dinonylphthalate in a Beckman microfuge. Specifically bound ^{125}I-insulin was calculated by the amount of totally bound ^{125}I-insulin minus nonspecifically bound tracer in the presence of an excess of cold hormone, 10^{-6} M.

6. Binding of ^{125}I-insulin to IM-9 lymphocytes. Lymphocytes were used in the late logarithmic phase of replication, 60 h after medium change; 2 × 10^6 cells/ml were incubated in 0.5 ml total volume of Hepes buffer, 0.12 M, pH 7.6, according to De Meyts.[10] ^{125}I-pork insulin, 120 pg/ml, was incubated with and without the addition of various concentrations of BHI, pancreatic human insulin, or pork insulin, respectively, for 120 min at 15°C. Two aliquots of 0.2 ml of suspended cells were centrifuged through 0.2 ml of ice-cold incubation buffer in a Beckman microfuge. ^{125}I-insulin in the sediment was counted after aspiration of the supernatant by cutting the tip of the microfuge tubes (0.55 ml total volume). Data are expressed as percentage of bound ^{125}I-insulin in relation to the total amount of ^{125}I-insulin added.

7. Binding of ^{125}I-insulin to rat liver plasma membranes. Liver cell plasma membranes were prepared from full-term pregnant rats following the procedure of Neville;[9] 200 μg membrane protein in 0.1 ml buffer (10 mM phosphate, pH 7.0, 150 mM NaCl, 2.5 mM KCl, 1 mM EDTA, 100 KIE/ml aprotinin, and 10 μl human plasma in 1 ml buffer) was incubated in a total volume of 0.3 ml with ^{125}I-pork insulin (Nordisk) (100 pg/ml), or with ^{125}I-pork insulin (Lilly) (33 pg/ml), or with ^{125}I-BHI (Lilly) (33 pg/ml), and with increasing amounts of unlabeled pork insulin (Nordisk), pancreatic human insulin (Lilly) or BHI (Lilly). The test tubes were incubated at 4°C for 18 h. Free tracer was separated from the ligand-bound tracer by centrifugation of the radioactivity found in the tube, containing 1000 ng/ml nonlabeled insulin.

RESULTS

The dose-response relationship of U-^{14}C-glucose oxidation to $^{14}CO_2$ in nmol glucose/100 mg fat tissue lipids is shown in Figure 1 (top). The amount of U-^{14}C-glucose incorporated into the lipid fraction during 120 min of incubation of rat epididymal fat pads in the presence of 3 × 10^{-10} to 3 × 10^{-9} M insulin is also shown in Figure 1 (bottom). Both biosynthetic and pancreatic human insulin were as potent as pork insulin. An excess of antipork insulin antibodies completely inhibited the effects of insulin, 3 × 10^{-9} M. Half-maximum effects were obtained with 8.3, 6.9, and 10.4 × 10^{-10} M insulin of biosynthetic, natural human, or pork origin, respectively, regarding glucose oxidation, and with 6.9, 5.4, and 5.6 × 10^{-10} M regarding glucose incorporation into lipids. Statisti-

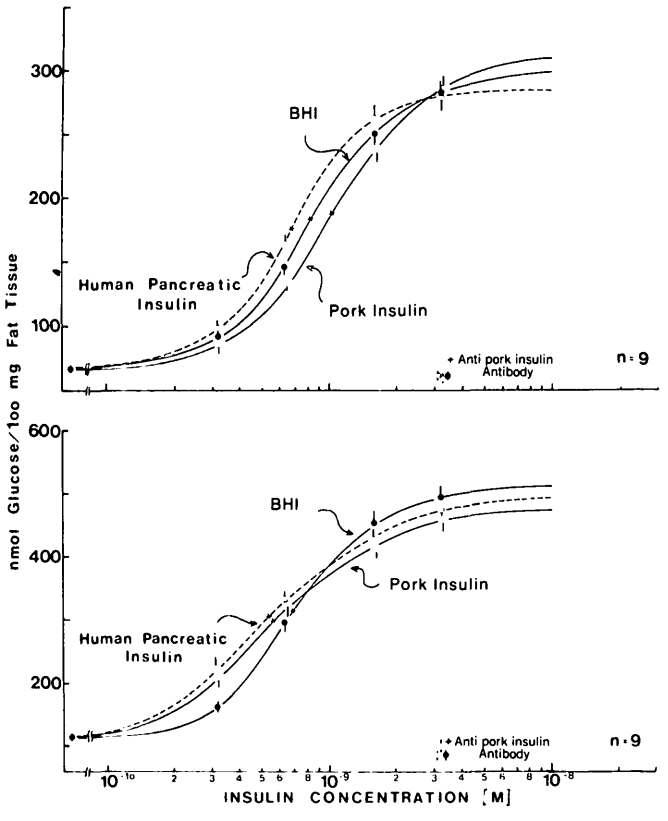

FIG. 1. Top: D-U^{14}C-glucose oxidation to $^{14}CO_2$ by rat epididymal fat tissue slices (100 mg/ml) in the presence of insulin, 3 × 10^{-10} – 3 × 10^{-9} M, without and with the addition of antipork insulin antiserum. Two hours of incubation, 37°C in KRP–BSA buffer, supplemented with glucose, 150 mg/dl. Data are expressed in nmol glucose/100 mg fat tissue lipids, extracted. Mean ± SEM of three experiments performed in triplicate. Bottom: D-U-^{14}C-glucose incorporation into the lipid fraction of rat epididymal fat tissue slices. Incubation conditions as indicated in the top half of figure.

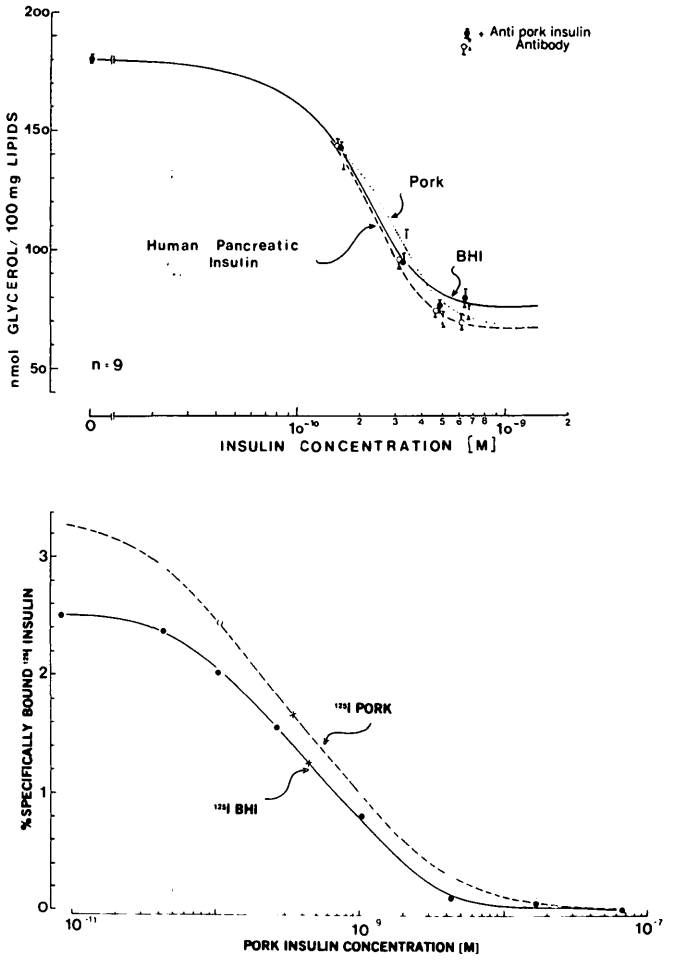

FIG. 2. Top: Lipolysis of human adipocytes (5 × 10^5 cells/ml) after isoproterenol (100 ng/ml) without and with the addition of insulin (1.6 to 6.4 × 10^{-10} M) in the absence or presence of antipork insulin antiserum. Data are expressed as nmol glycerol/100 mg lipid, released during 90 min, 37°C. Bottom: Specific binding of ^{125}I-insulin (50 pg/ml) to isolated human adipocytes (5 × 10^5 cells/ml) in the presence of various concentrations of unlabeled pork insulin, within 30 min, 24°C, pH 7.6. Non specific binding in the presence of an excess of cold insulin (10^{-6} M) has been subtracted. Mean of two experiments.

cally significant differences could not be discerned at the 5% level. The inhibition of isoproterenol-induced lipolysis of human fat cells by insulin is shown in Figure 2 (top). Half-maximum responses have been observed with 2 × 10^{-10} M insulin with all three analogues. Binding of ^{125}I-pork insulin and BHI (50 pg/ml) to isolated human adipocytes, in the absence or presence of various concentrations of cold pork insulin, is shown in Figure 2 (bottom). Nonspecific binding in the presence of an excess of cold insulin (10^{-6} M) has been subtracted. For half-maximum displacement of the tracers, 3.4 × 10^{-10} M pork insulin with respect to pork tracer, or 4.5 × 10^{-10} M with respect to the BHI tracer, was necessary.

The competition inhibition curve of insulin binding to IM-9 lymphocytes is illustrated in Figure 3. The standard error of the mean of five experiments with pork ^{125}I-insulin (Nordisk, 180 μCi/μg, 120 pg/ml) in the absence and the presence of various amounts of cold pork insulin (Lilly) is indicated by the shaded area. The competition with BHI shown by the closed line and that with pancreatic human insulin (Lilly) is shown by the dotted line. Half-maximum displacement was obtained with 7.9, 7.2, and 4.3 × 10^{-10} M biosynthetic or pancreatic human or pork insulin, respectively. Scatchard analysis of these data (Figure 4) revealed flattening of the slope of the curval linearity, observed with both human insulins as compared with pork insulin, suggesting the possibility of a slight decrease of the negative cooperative interaction of human insulin with its receptor sites.

The displacement of iodinated pork insulin (Nordisk), iodinated pork insulin (Lilly), and iodinated BHI (Lilly) by pork insulin (Nordisk), pancreatic human insulin (Lilly) and BHI (Lilly) is shown in Figure 5. Pork insulin and BHI are displaced from binding sites on rat liver plasma membranes by the three insulin preparations. Differences in activity between these three insulin preparations were not detected.

The time course of ATP depletion of rat adipocytes after

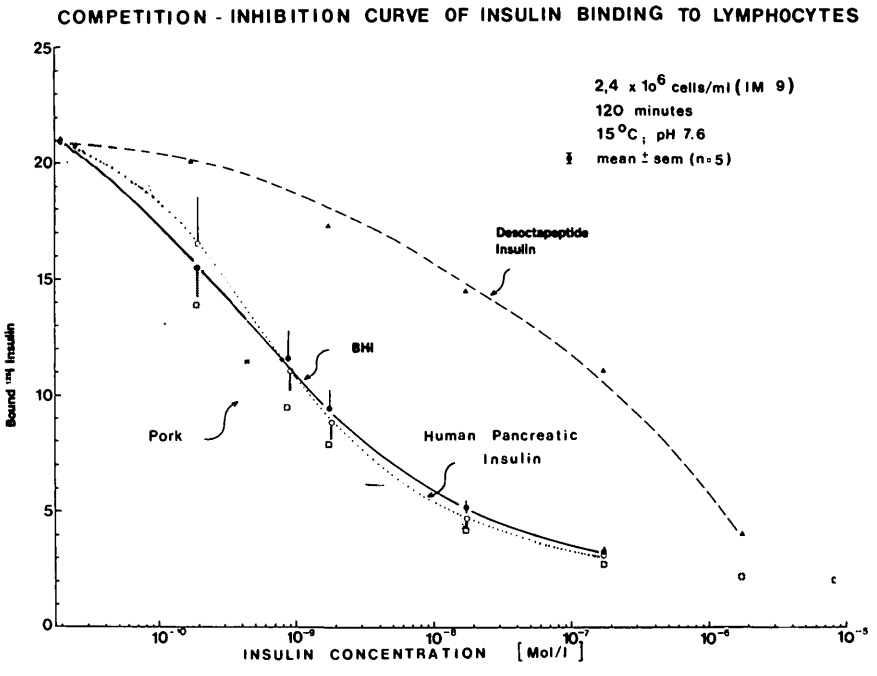

FIG. 3. Binding of ^{125}I-insulin to IM-9 lymphocytes (2.4 × 10^6 cells/ml) within 120 min, 15°C, pH 7.6, in the presence of various amounts of four insulin analogues. Mean ± SEM of five experiments.

addition of isoproterenol, 1000 ng/ml, is shown in Figure 6. The ATP content of fat cells throughout the 5-h incubation period was stable, as shown by the asterisks. ATP content was determined with 4 pg/fat cell. The open dots indicate the effect of isoproterenol. ATP is depleted almost completely within 4 h. Addition of small amounts of insulin 15 min before isoproterenol retard this effect of ATP depletion, and higher concentrations of insulin (1–4 ng/ml) gradually prevent depletion until a maximum is obtained that cannot be surpassed by twice as much insulin or more (8–80 ng/ml). The subsiding effects of small amounts of insulin are due to progressive degradation of insulin during the incubation period. A threshold level of 1 ng/ml, kept stable by a second addition 2 h after the first dose, is as efficient as 4 ng/ml insulin added once initially.

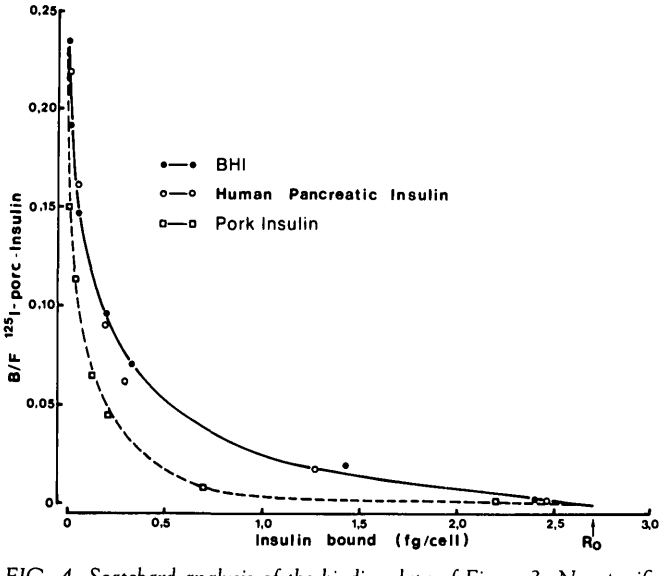

FIG. 4. Scatchard analysis of the binding data of Figure 3. Nonspecific binding in the presence of an excess of unlabeled pork insulin has been subtracted. Data of the experiments with human insulins are calculated on the assumption that nonspecific binding of the experiments with pork insulin is comparably high at 10^{-5} M.

The effects of various concentrations of BHI and pancreatic human insulin, compared with pork insulin and des-octapeptide insulin, analyzed within the fourth and fifth hour of incubation of rat fat cells in the presence of isoproterenol, are depicted in Figure 7. As can be seen, all three analogues were nearly as active to prevent depletion of half the amount, which could be restored. In contrast, the very weak analogue, des-octapeptide insulin, exhibited only 2.5% of this potency. BHI (1–8 ng/ml) could prevent significantly more ATP depletion (10%; $P < 0.01$) when compared with pork insulin. On the other hand, des-octapeptide insulin showed a decreased maximum effect.

DISCUSSION

We have analyzed the potency of three highly active and one weak insulin analogue. The classical in vitro bioassays could not discern any difference of these fully potent preparations with significance. This is in accordance with the data of Chance et al.,[4] and also with the data of Märki and Albrecht,[20] who compared totally synthesized and natural human and pork insulin in various in vitro bioassays. The observation with IM-9 lymphocytes disclosed the possibility of a small change of the negative cooperative region of human insulin; however kinetic experiments to prove this supposition have not been performed. As has been shown by

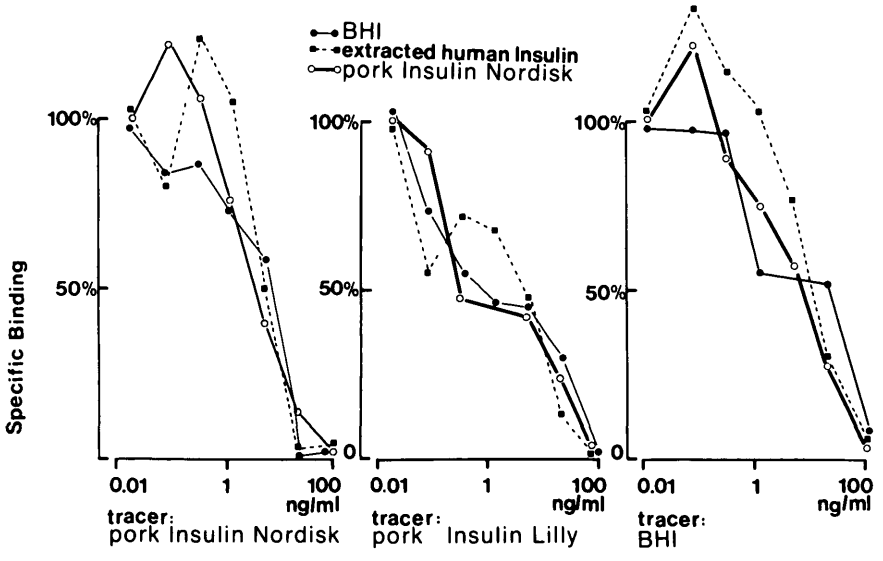

FIG. 5. Binding of iodinated insulin to rat liver cell plasma membranes. Specific binding is obtained by subtraction of the bound radioactivity in the presence of an excess of cold insulin. The x-axis indicates the amounts of added nonlabeled hormone.

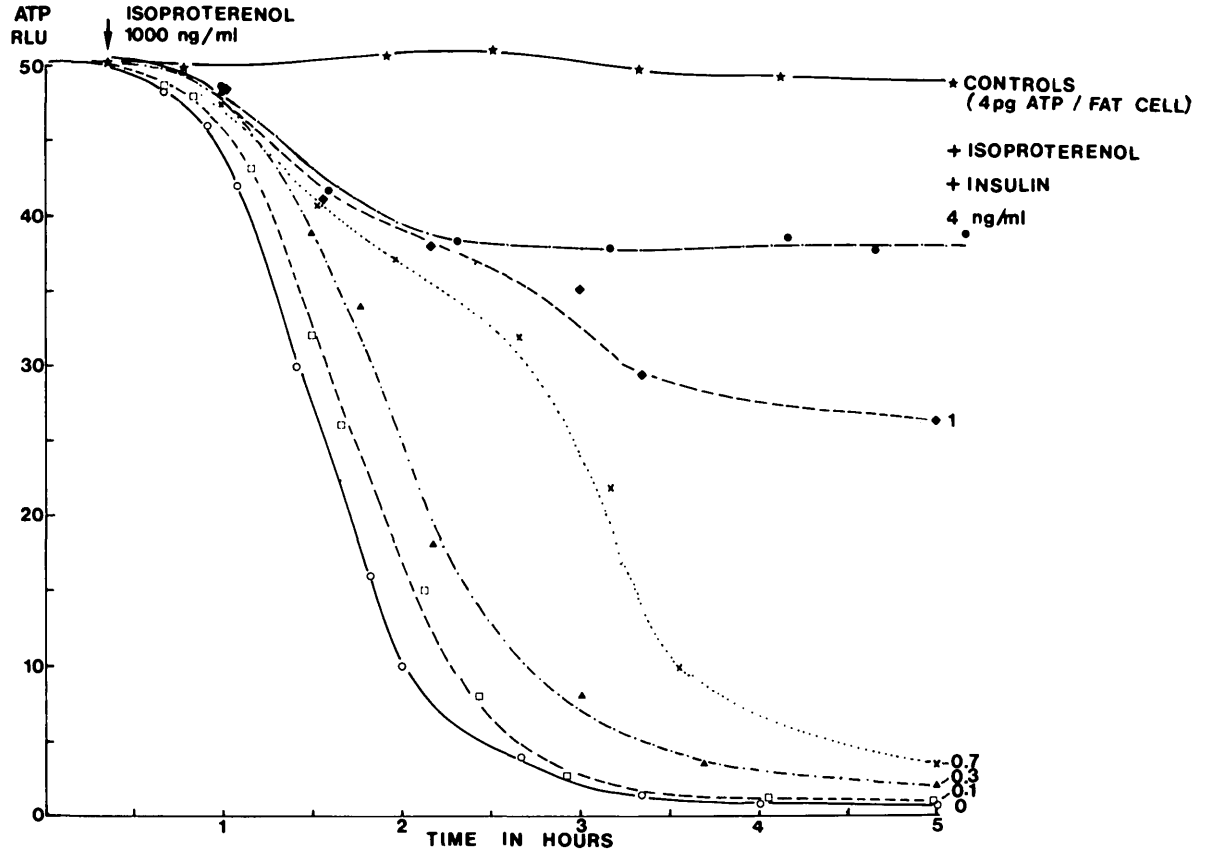

FIG. 6. ATP content of rat adipocytes (5 × 10^5 cells/ml) incubated for 5 h, 37°C, pH 7.4, in KRP–BSA (2 g/dl) + glucose (100 mg/dl); in the absence of insulin or isoproterenol, control level: 4 pg ATP/fat cell; in the presence of isoproterenol (1 μg/ml) and before (15 min) addition of various amounts of pork insulin (0–4 ng/ml). Data with 8 and 80 ng/ml were comparable to those with 4 ng/ml, but have been omitted for clarity.

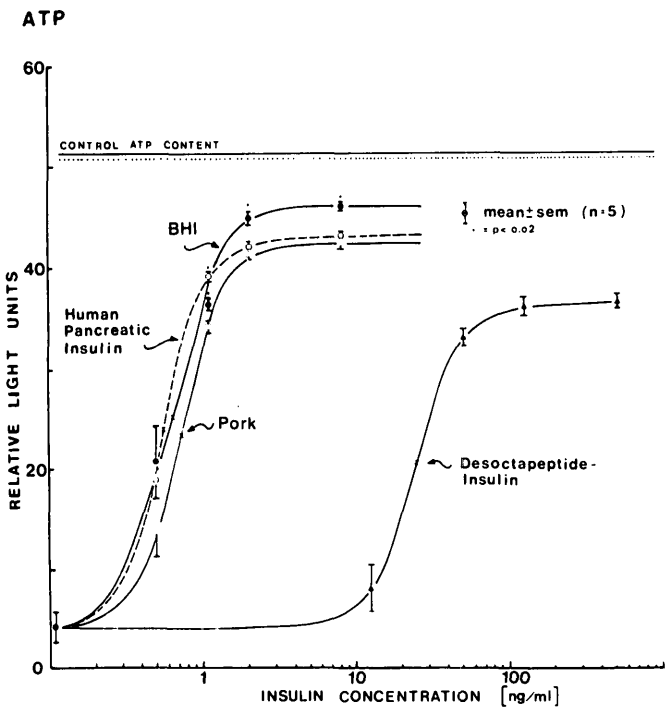

FIG. 7. Dose-related inhibition of isoproterenol (1 μg/ml) induced ATP depletion of rat adipocytes in the presence of various concentrations of four insulin analogues. Mean ± SEM of five experiments. Data are expressed in relative light units (RLU); 1 RLU corresponds to 0.8 ng ATP. Incubation conditions as indicated in Figure 6. Data are derived from measurements within the fifth hour of incubation.

De Meyts et al.[21] for the des-B-30-alanine analogue from natural pork insulin[3] and again with BHI (Keefer et al., this issue, pp. 209–14), B-30 seems without influence on negative cooperative interactions of the insulin molecule with its receptor site.

Alternatively, curvilinear Scatchard plots have been related to degradation.[22] Thus decreased degradation of BHI, compared with pork insulin, might have contributed to the effects observed. Under comparable conditions to the studies presented, as summarized by Ginsberg,[23] IM-9 lymphocytes degrade insulin at 15°C by 3–5%. On the other hand, recently, Sönne and Gliemann[24] reported lack of receptor-mediated insulin degradation with IM-9 cells. These studies were performed in the presence of the degradation inhibitor bacitracin. Thus, further studies need to be performed until definite conclusions can be drawn regarding this point of discussion. Nevertheless, the studies with rat adipocytes indicate that BHI is less prone to degradation, indirectly supporting the assumption noted above. Kinetic data of the ATP-depletion assay permitted us to calculate the degradation rate of insulin in the presence of low initial concentrations. We observed a degradation rate of 3 pg/min for BHI, but of 5 pg/min for pork insulin in the presence of 500 pg/ml insulin and 5 × 10^5 cells/ml. Moreover, degradation differences should be the basis of the minor variations observed until now between BHI and pork insulin in vivo, as will be shown by several reports in this issue. Keen et al.[6] showed that low doses of BHI, whether infused intravenously or injected subcutaneously, are more potent than pork insulin by either route.

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