Comparative Study of the Effects of Porcine Proinsulin and Insulin on Lipolysis and Glucose Oxidation in Rat Adipocytes

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SUMMARY

Proinsulin blocked lipolysis activation by epinephrine, glucagon or theophylline. The antilipolytic effect of proinsulin was not blocked by Kunitz pancreatic trypsin inhibitor (KPTI). The concentrations of proinsulin and insulin required to cause 50 per cent inhibition of epinephrine-stimulated lipolysis were estimated to be approximately 1×10^{-9} M and 3×10^{-11} M respectively. Proinsulin which had been reduced and allowed to reoxidize was found to have approximately the same antilipolytic activity as native proinsulin, indicating that the antilipolytic effect was due to proinsulin and not insulin contamination. The concentrations of proinsulin and insulin required to give 50 per cent of maximal stimulation of glucose oxidation were estimated to be approximately 5×10^{-9} M and 1×10^{-11} M respectively. The effect of proinsulin on glucose oxidation did not appear to be caused by any insulin contamination since reduced-reoxidized proinsulin had approximately the same activity as native proinsulin. KPTI had no effect on proinsulin-stimulated glucose oxidation in isolated fat cells, in contrast to the marked inhibitory effect observed using intact epididymal fat pad. This suggests that the KPTI-sensitive proteolytic activity of the epididymal fat pad is not located in the fat cell but in some other type cell or extracellular space. DIABETES *19*:209-16, April, 1970.

The existence of proinsulin, a precursor of insulin, was discovered by Steiner and Oyer in 1967.[1] While investigating the biosynthesis of insulin from radioactive amino acids in human islet cell adenoma slices, they observed a second labeled protein fraction which reacted with guinea pig anti-insulin serum but appeared to be a larger molecule than insulin. Treatment with trypsin converted the larger protein to a molecule which appeared to be insulin. Steiner et al.[2] then studied the time course of incorporation of radioactive amino acids into insulin and the second protein fraction and found that the label was initially incorporated into the larger protein and subsequently appeared in insulin. Steiner and his associates concluded that the larger protein was a precursor in the biosynthesis of insulin and named it "proinsulin."

Chance et al.[3,4] isolated porcine proinsulin from pancreatic extracts and determined the amino acid sequence. Porcine proinsulin was shown to contain eighty-four amino acids including the twenty-one amino acid-containing A chain, the thirty amino acid B chain and a thirty-three amino acid-connecting peptide which joins the terminal carboxyl group of the B chain with the terminal amino group of the A chain. The molecular weight of 9,082 calculated from amino acid composition agrees well with the molecular weight calculated from physical data by Frank and Veros.[5]

Shaw and Chance[6] have recently reported studies on the effect of porcine proinsulin on the rat epididymal fat pad in vitro. They found that proinsulin enhanced the oxidation of labeled glucose to CO_2 and was approximately one tenth as effective as single component insulin on a molar basis. They also found that Kunitz pancreatic trypsin inhibitor (KPTI) blocked this effect of proinsulin on the epididymal fat pad. The proinsulin-stimulated conversion of labeled glucose to fatty acids in rat epididymal fat pads was also blocked by KPTI. They concluded that proinsulin itself had little if any effect on glucose metabolism in rat adipose tissue and that the observed activity was due to proteolytic activity which catalyzed the conversion of proinsulin to insulin or insulin-like material which caused the metabolic effects.

In this paper we report our observations on the effects of proinsulin on lipolysis and glucose oxidation in isolated fat cells* and the effect of KPTI thereon.

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MATERIAL AND METHODS

Male albino rats (Charles River, CD) weighing 100 to 140 gm. were used in these studies. The rats were fed ad libitum with Purina rat chow.

Fat cells were prepared by the method of Rodbell.[7,8] All operations in the preparation and utilization of the fat cells were conducted in plastic containers. The cells were prepared by incubating approximately 1 gm. of adipose tissue (cut in small pieces) with 10 mg. of crude collagenase in 3 ml. of Krebs Ringer phosphate solution (pH 7.4) containing 4 per cent bovine serum albumin (KRP-BSA). This mixture was incubated for one hour at 37° C. with shaking. The mixture was then forced through Japanese silk screen, size 12xx, to disperse the fragments into free cells which passed through the silk screen and to remove some of the debris. A 20-ml. plastic syringe which had been cut off at the tip and covered with silk screen (fastened by means of a rubber band) was used to carry out this filtration step. The filtered suspension was then centrifuged very gently for one minute (ca. 400xg), causing the fat cells to collect at the top of the tube. The cells were then removed by means of a plastic syringe, with a short length of plastic tubing in place of a needle; the cells were resuspended in 10 ml. of room temperature KRP-BSA, and the centrifugation and washing procedure was repeated four times. The cells were finally suspended in KRP-BSA using 20 ml. of KRP-BSA per gram (wet weight) of original adipose tissue.

The lipolysis assays were conducted in plastic scintillation counting vials. The total reaction volume was 3 ml. of KRP-BSA, which included 1 ml. of fat cells (50 mg. of original tissue) and any lipolytic or antilipolytic agents as indicated. The reaction mixtures were incubated for two hours at 37° C. with shaking. At the end of two hours, a 0.50-ml. aliquot of the reaction mixture was added to 0.50 ml. of 10 per cent trichloroacetic acid to stop the reaction, and the sample was deproteinized by centrifugation. The extent of lipolysis was determined by measuring the amount of glycerol released by the method of Lambert and Neish[9] as modified by Korn.[10] A 0.50-ml. aliquot of the supernatant fluid was mixed with 0.10 ml. of 0.05 M sodium periodate and allowed to stand at room temperature for five minutes. Then 0.10 ml. of 0.50 M sodium arsenite was added and the mixture allowed to stand at room temperature for ten minutes. Five milliliters of chromotropic acid was then added (prepared by combining 100 ml. of 0.025 M chromotropic acid [4,5-dihydroxy-2,7-naphthalene-di-sulfonic acid] with 400 ml. of 24·N H_2SO_4). The mixture was then heated for thirty minutes at 100° C. and cooled; its absorbance was then measured at 570 mμ.

The results were expressed as micromoles of glycerol released per gram of original adipose tissue per hour. Protein was not measured because the cells were prepared in the presence of 4 per cent BSA. Fat cells can be prepared in the absence of BSA,[11] but we have found in several experiments that the lipolytic activity obtained per milliliter of cells prepared in the absence of BSA is only about 60 per cent that observed with cells prepared in the presence of BSA. The lower activity may be caused by increased lysis of cells in the absence of BSA and simply reflect a lower yield of cells. Because of the greater activity obtained in the presence of BSA, we think that this method is preferable except in experiments in which there is a definite need to determine protein levels.

The glucose oxidation studies were performed in plastic scintillation counting vials capped with rubber serum stoppers. One milliliter of the fat cell preparation was added to 1 ml. of Krebs-Ringer phosphate buffer (pH 7.4) containing in the final 2 ml. 0.1 mg. glucose-U-C-14 (ca. 200,000 cpm per micromole glucose C), 0.50 mg. human albumin, fraction V, and any other components as indicated. The reaction mixtures were incubated for two hours at 37° C. under air with shaking in a Dubnoff metabolic shaker. A plastic cup containing 0.30 ml. 4 N NaOH was suspended from the rubber stopper. At the end of the two-hour incubation period, 0.50 ml. 5 N H_2SO_4 was injected into the medium, and the bottle was shaken for another thirty minutes to allow the carbon dioxide formed to be absorbed by the alkali. The alkali was then transferred to a conical centrifuge tube, the volume was made up to 1 ml. (using CO_2-free glass distilled water), and a 0.10-ml. aliquot was plated for counting in a windowless flow counter. The results were expressed as micromoles of CO_2 formed per gram original tissue per two hours.

Epinephrine bitartrate and norepinephrine bitartrate were purchased from Nutritional Biochemical. Collagenase and KPTI were purchased from Worthington Biochemical. It is worth noting that lots of KPTI vary considerably in their ability to block proinsulin-stimulated glucose oxidation by intact epididymal fat pads. Therefore, the quantity of KPTI necessary to block proinsulin conversion must be determined by testing the

TABLE 1

Effects of insulin and proinsulin on lipolysis. The concentrations of single component insulin (SCI) and proinsulin (PI) were 4.3×10^{-9} M and 2.0×10^{-8} M respectively. KPTI where used was 50 μg./ml. which was sufficient, for that particular preparation, to block proinsulin-stimulated glucose oxidation in intact epididymal fat pads.

Lipolytic agent	Glycerol release: μmoles/gm./hr.			
	+ None†	+ SCI	+ PI	+ PI + KPTI
None*	0.32± .17			
Norepinephrine 0.05 μg./ml.	10.96±1.01	2.40±.12	2.00±.24	1.92‡
Norepinephrine 0.10 μg./ml.	27.16± .87	17.32±.58	10.28±.08	7.80‡
None	0.92± .03			
Theophylline 5×10^{-4} M	9.04± .17	3.08±.10	2.84±.03	3.16±.10
None	1.72± .40			
Glucagon 5×10^{-7} M	7.76± .99	3.36±.00	3.20±.52	2.96±.45

*The word none in this column indicates that no lipolytic agent was added and that fat cells alone were incubated in the standard incubation media.

†The word none indicates that no insulin, proinsulin, or KPTI was added to the reaction mixtures in this column.

‡These experiments were done in duplicate and the average is given; all others were done in triplicate and the mean ± the standard error of the mean is reported.

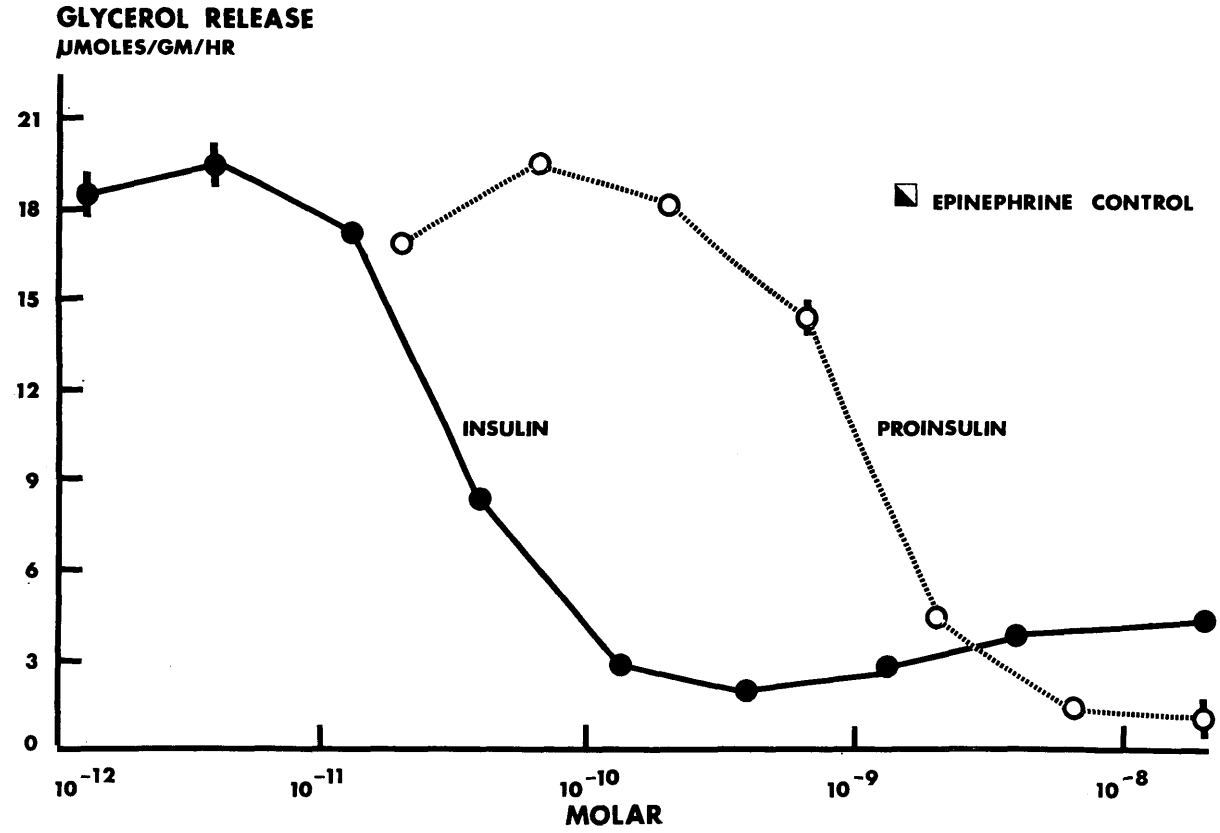

FIG. 1. Inhibition of epinephrine-stimulated lipolysis by insulin and proinsulin. Each assay vial contained 0.10 μg./ml. epinephrine bitartrate to activate lipolysis, and proinsulin or insulin was added as indicated. These assays were done in triplicate and the mean is represented by a circle and the standard error of the mean is represented by a bar. Some of the points had standard errors which were no larger than the circle used and in these cases bars were not added.

particular lot for its ability to block proinsulin-stimulated glucose oxidation by intact epididymal fat pads. Chromotropic acid was purchased from Matheson, Coleman, and Bell; bovine serum albumin (Fraction V), from Sigma; and glucose-U-C-14 (0.25 mC./9.2 mg.) from New England Nuclear. The human albumin preparation was obtained through the courtesy of Dr. H. N. Antoniades, Blood Research Institute, Inc., Boston, Mass. The porcine proinsulin and single component porcine insulin were obtained through the courtesy of Dr. R. E. Chance, Lilly Research Laboratories, Indianapolis, Ind. The glucagon (cysteine treated to inactivate any insulin present) and the reduced-reoxidized proinsulin[13,14] were obtained through the courtesy of Dr. W. W. Bromer, Lilly Research Labs, Indianapolis.

RESULTS

Lipolysis

Proinsulin blocked the lipolytic effect of norepinephrine, glucagon, and theophylline as shown in table 1. This spectrum of antilipolytic activity was similar to that observed with insulin. The effect of proinsulin on lipolysis in isolated fat cells was not blocked by the addition of KPTI at a concentration of 50 μg./ml. Control experiments indicated that KPTI by itself had no effect on norepinephrine-activated lipolysis.

The antilipolytic activity of proinsulin was not as great as that of insulin, as indicated by a comparison of their dose response curves shown in figure 1. An I_{50} value was calculated, this value being the molar concentration which produced 50 per cent of the maximal inhibition observed. The I_{50} of insulin was estimated to be 3.2×10^{-11} M, whereas the I_{50} of proinsulin was 1.1×10^{-9} M.

Figure 2 depicts the antilipolytic dose response curve of proinsulin in the presence and absence of KPTI. The presence of KPTI caused a slight shift to the right of the dose response curve, the I_{50} in the absence of KPTI being approximately 0.6×10^{-9} M and in the presence of KPTI, 1.3×10^{-9} M. This difference is relatively small and may be of no real significance since the I_{50} for proinsulin calculated from figure 1 was 1.1×10^{-9} M which is almost identical with the figure of 1.3×10^{-9} M obtained in the presence of KPTI in

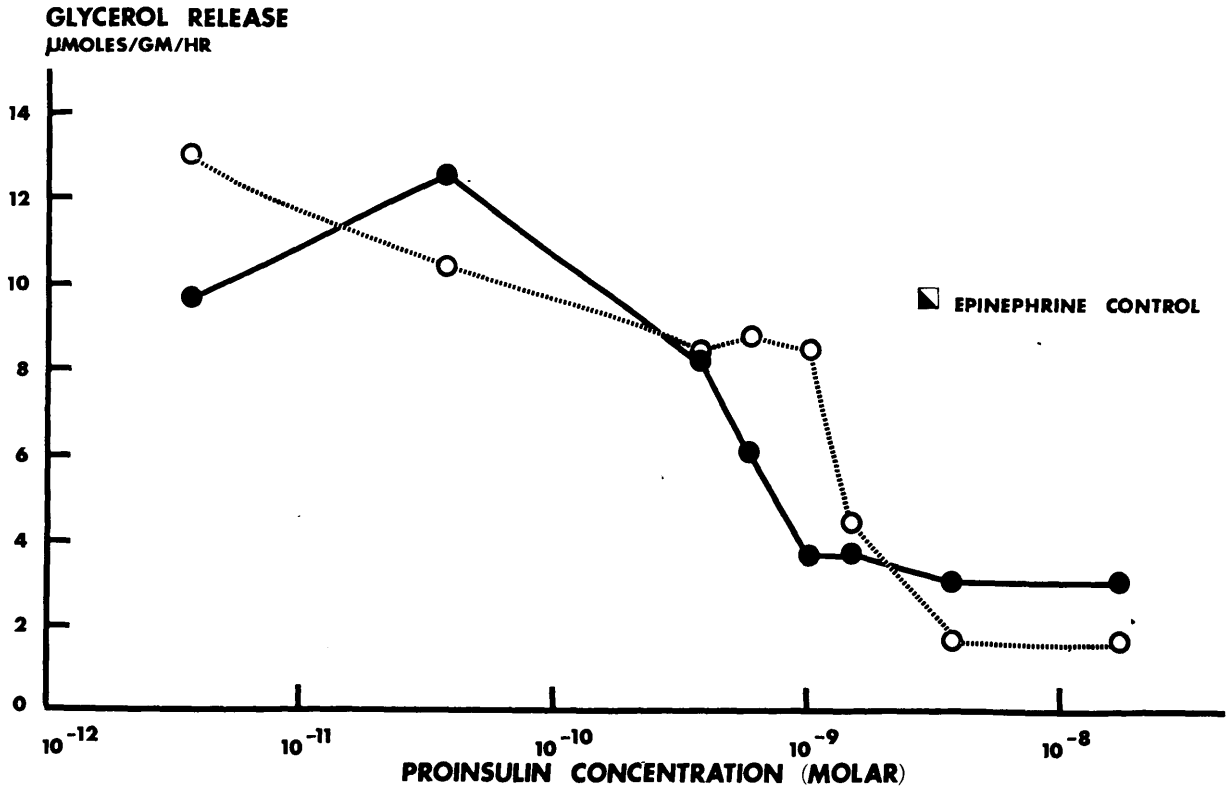

FIG. 2. Effect of KPTI on antilipolytic dose response curve of proinsulin. Each assay vial contained 0.10 μg./ml. epinephrine bitartrate to activate lipolysis, and proinsulin as indicated. KPTI was present at 100 μg./ml. in the assays represented by the dashed line. This assay was done in duplicate and the average is given.

this experiment. It would seem unlikely that the difference between the two curves in figure 2 is due to KPTI blocking the conversion of proinsulin to insulin-like activity since similar experiments on the dose response curve of proinsulin-stimulated glucose oxidation in the presence and absence of KPTI gave curves which were almost identical, as shown in figure 6.

A dose response curve of the antilipolytic activity of insulin and proinsulin when theophylline was used as the lipolytic agent is shown in figure 3. The I_{50} of insulin was approximately 2.7×10^{-11} M and the I_{50} of proinsulin was 0.5×10^{-9} M. These values are similar to those observed when epinephrine was used as the lipolytic agent.

A preparation of proinsulin that had been reduced with mercaptoethanol to disrupt the disulfide bonds and then allowed to reoxidize under air to reform proinsulin was tested for its antilipolytic activity against epinephrine, and the dose response curve obtained is shown in figure 4. Proinsulin which had been reoxidized for 2.5 hrs. had an I_{50} of 1.4×10^{-9} M. A similar experiment using the same proinsulin sample after twenty-four hours of reoxidation gave a dose response curve which was essentially identical except that the I_{50} was approximately 0.8×10^{-9} M. Native proinsulin had an I_{50} of 1.1×10^{-9} M which is quite close to the values obtained with the two recombined samples.

Glucose oxidation

Figure 5 shows a comparison of the effects of proinsulin, insulin, and reduced-reoxidized proinsulin on the oxidation of glucose-U-C-14 to C-14-O_2 in isolated fat cells. The data are expressed as the percentage increase of glucose oxidation above the basal rate observed in the absence of insulin or proinsulin. Each point in the figure represents the mean value from two separate experiments, each run in triplicate with the exception of the reduced-reoxidized proinsulin dose response curve which was determined once in triplicate. Insulin was more active than proinsulin, the A_{50} (molar concentration causing 50 per cent of the highest observed activation of glucose oxidation) for insulin being 1.2×10^{-11} M whereas the A_{50} for proinsulin was 5.5×10^{-9} M. The A_{50} for reduced-reoxidized proinsulin was 6.5×10^{-9} M, which is essentially identical with that observed for native proinsulin. The above data indicate that proinsulin has an intrinsic ability to stimulate glucose oxidation and that this effect is not caused by insulin contamination, since reduction and reoxidation should inactivate more than 95 per cent of any insulin present.[12,14]

Figure 6 depicts the effects of proinsulin on glucose oxidation in the presence or absence of KPTI at a

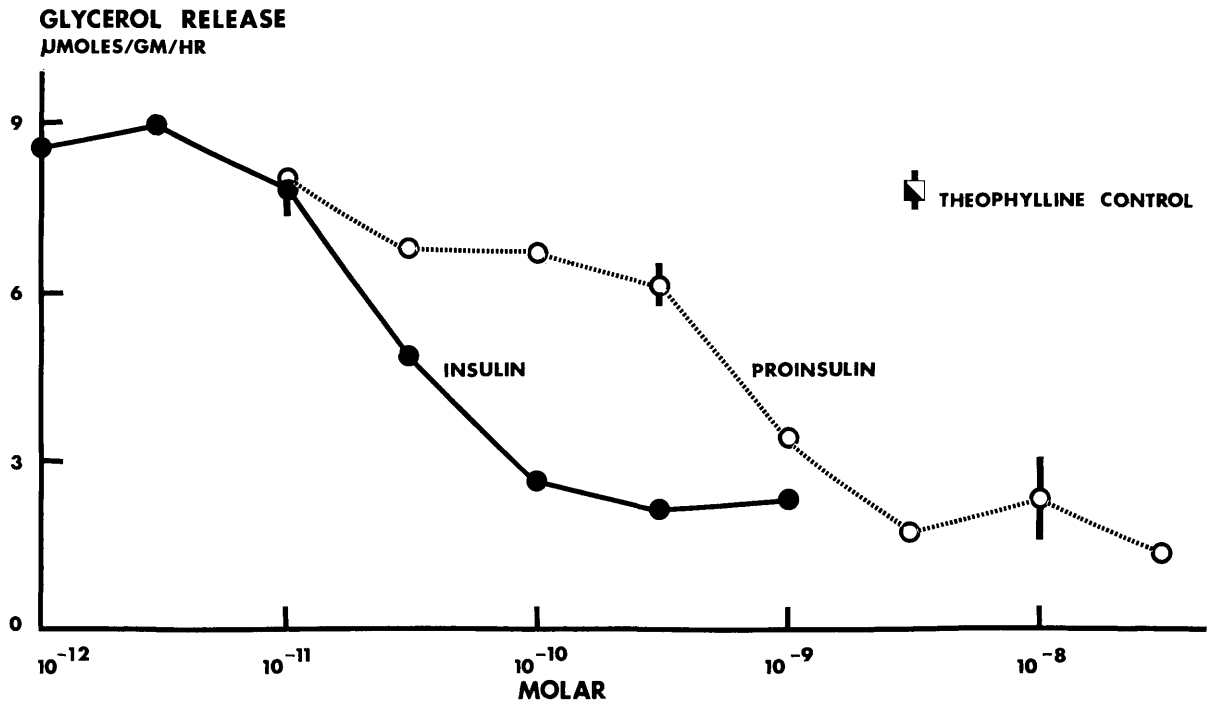

FIG. 3. Inhibition of theophylline-stimulated lipolysis by insulin and proinsulin. Each assay vial contained 5×10^{-4} M theophylline to activate lipolysis, and proinsulin or insulin was added as indicated. These assays were done in triplicate.

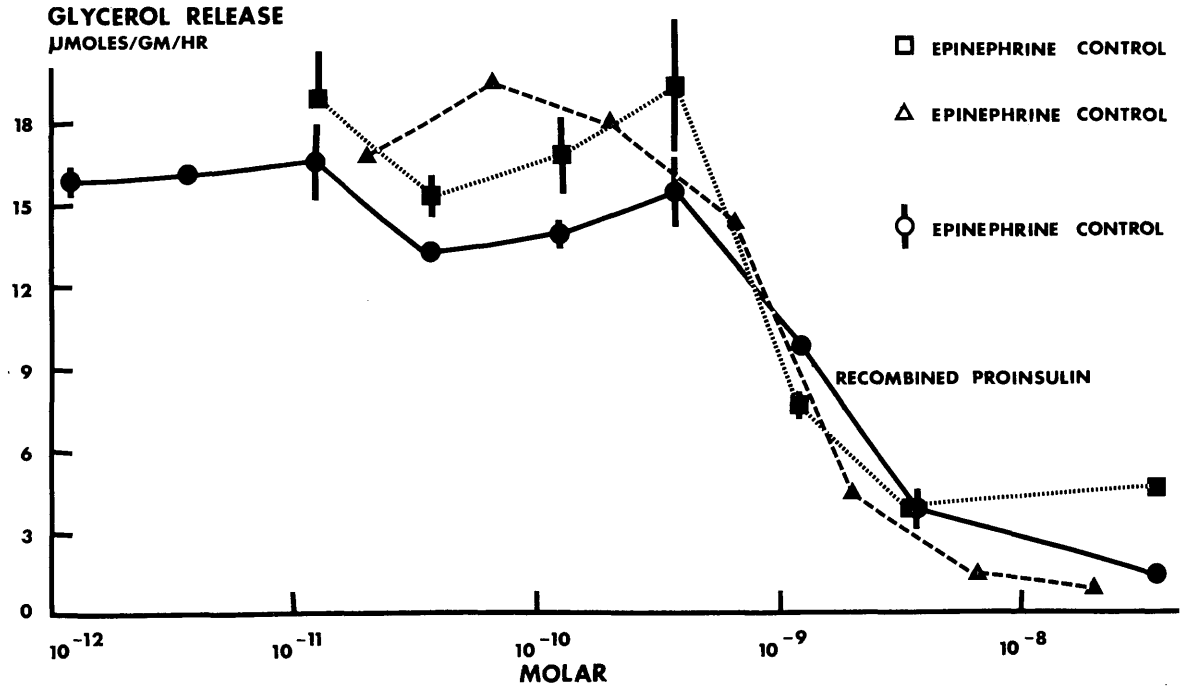

FIG. 4. Antilipolytic dose response curve of reduced-reoxidized proinsulin. Each assay vial contained 0.10 μg./ml. epinephrine bitartrate to activate lipolysis. These experiments were each done on different days. The solid triangles represent native proinsulin and the open triangle is the epinephrine control for that day. The solid circles represent 2.5-hr. reoxidized proinsulin and the open circle is the appropriate control. The solid squares represent 24-hr. reoxidized proinsulin and the open square is the epinephrine control for this experiment. The assays were done in triplicate.

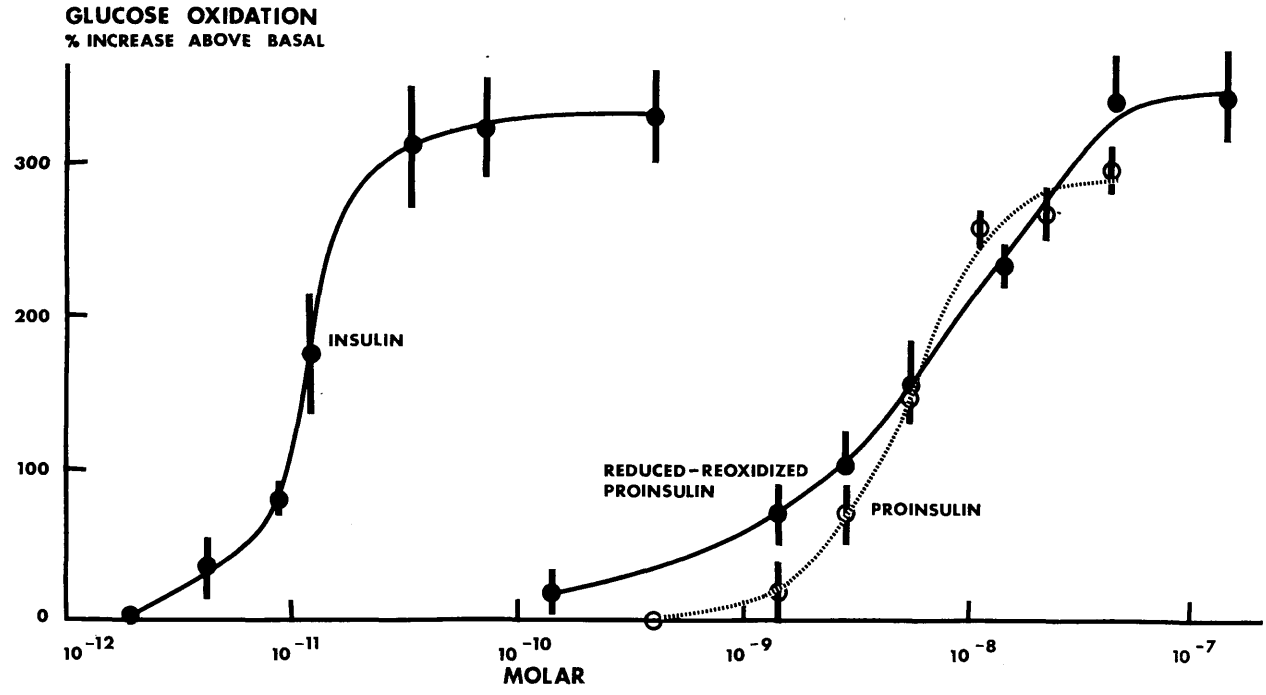

FIG. 5. Stimulation of glucose oxidation by insulin, proinsulin, and reduced-reoxidized proinsulin.

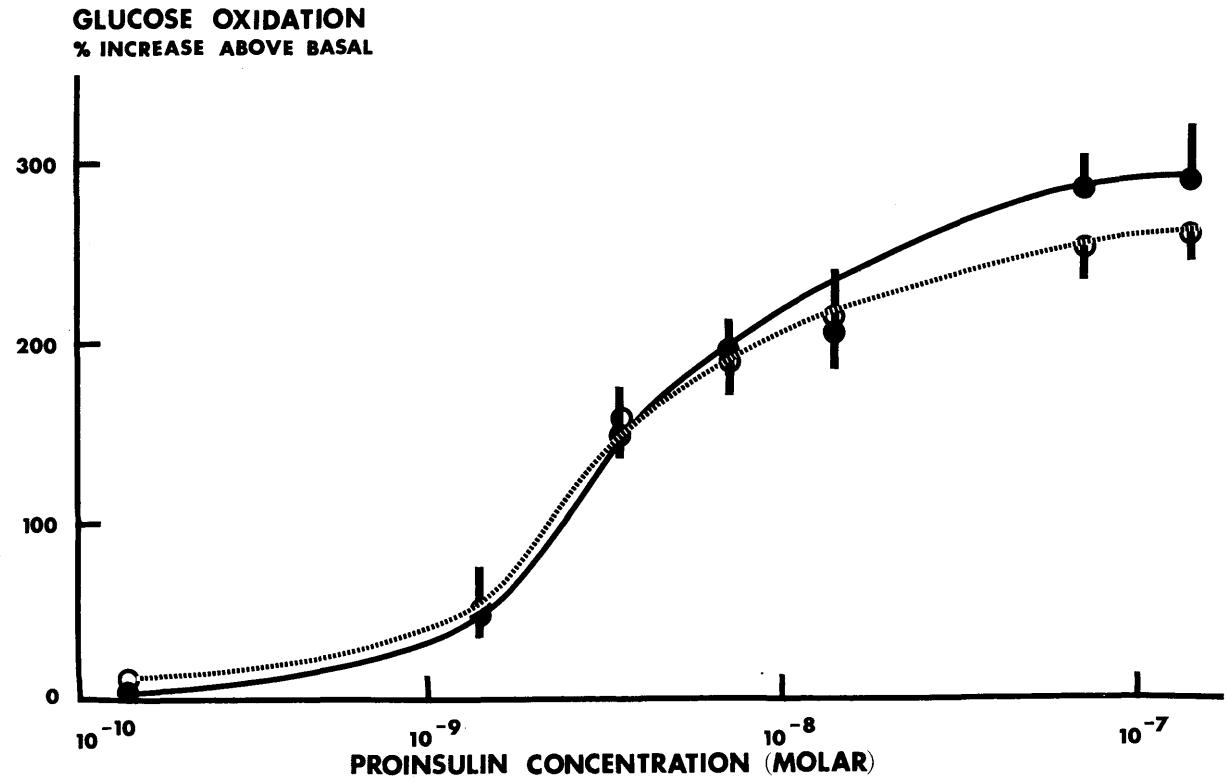

FIG. 6. Stimulation of glucose oxidation by proinsulin in the presence and absence of KPTI. KPTI was present at 100 μg./ml. in the assays represented by the dashed line.

concentration of 100 μg./ml. Each point in this figure represents the mean value from two separate experiments, each run in triplicate. KPTI was without effect in this experiment, the A_{50} being 3.2×10^{-9} M both in the presence and absence of KPTI.

DISCUSSION

The effects of proinsulin on inhibition of lipolysis and stimulation of glucose oxidation appeared to be quite similar to those of insulin except that much higher concentrations of proinsulin were required to produce an equivalent response. The maximal response obtained, however, was approximately the same regardless of whether insulin or proinsulin was used. Proinsulin, like insulin, blocked the lipolytic effect of epinephrine, glucagon, and theophylline. Insulin was about twenty to thirty times as potent as proinsulin in blocking lipolysis stimulated by either epinephrine or theophylline. The antilipolytic activity of proinsulin could not be accounted for by insulin contamination because at least 3 to 5 per cent insulin contamination would have been required. It is doubtful that there was this much insulin in the proinsulin preparation based on two lines of evidence. First, there was no detectable insulin band when proinsulin was examined electrophoretically by Dr. R. E. Chance using polyacrylamide disc gel electrophoresis. Secondly, proinsulin which had been reduced and reoxidized had approximately the same antilipolytic activity as native proinsulin.

The ability of insulin to stimulate glucose oxidation was much greater than that of proinsulin, as indicated by their A_{50} values of 1.2×10^{-11} M and 5.5×10^{-9} M, respectively. The A_{50} of proinsulin calculated from figure 6 was 3.2×10^{-9} M. Based on these data the ability of proinsulin to stimulate glucose oxidation could be explained by the presence of 0.2 to 0.4 per cent insulin as a contaminant. The fact that the A_{50} of reduced-reoxidized proinsulin was approximately the same as normal proinsulin indicated that there was no insulin contamination present in this proinsulin preparation. These data indicate that proinsulin has an intrinsic ability to stimulate glucose oxidation.

KPTI did not block the effect of proinsulin on either lipolysis or glucose oxidation in isolated fat cells. This is in contrast to the ability of KPTI to block the stimulation of glucose oxidation by proinsulin in pieces of

rat epididymal fat pad. This observation suggests that the KPTI-sensitive proteolytic activity present in intact tissue is lost during the preparation of fat cells. Kitabchi et al.[15] have recently reported that proinsulin stimulated glucose oxidation and lipogenesis in rat adipocytes and that these effects were not blocked by pancreatic trypsin inhibitor or trasylol, which is in agreement with our data.

Since the intrinsic activity of proinsulin on isolated fat cells is quite small, it is apparent that if proinsulin is to affect adipose tissue in vivo, then it must be converted to a more active form by some proteolytic activity such as the KPTI-sensitive protease described by Shaw and Chance,[6] or serum proteolytic activity. If proinsulin is actually secreted by the pancreas, then these extrapancreatic proteases would be an important means of converting circulating proinsulin to more active insulin-like material.

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