Differential Effect of Porcine Proinsulin on Rat Epididymal Fat Cells and Fat Pieces

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SUMMARY

Proinsulin is a biosynthetic precursor of insulin which reacts with insulin antibody and has been found in the peripheral circulation. The present studies were undertaken to determine the extent of its intrinsic biologic activity as compared with insulin and to provide evidence regarding the possibility and anatomic location of its conversion to insulin or biologically active related products in peripheral tissues.

The effects of single component porcine insulin and proinsulin on the conversion of glucose-1-C-14 to C-14-O_2 and inhibition of the theophylline-stimulated lipolysis were studied in rat adipose tissue cells and pieces. Kunitz pancreatic trypsin inhibitor (KPTI) was used to block tissue conversion of proinsulin to insulin. Proinsulin was twenty-three to twenty-seven times less effective than insulin when assayed on isolated fat cells. These results were not changed significantly by KPTI. Whereas insulin appeared less effective on fat pieces than on cells—presumably due to difficulty with diffusion into the more intact tissue—proinsulin, an even larger molecule, was as effective in fat pieces as fat cells. Thus proinsulin was only nine to eighteen times less effective than insulin when assayed on fat pieces. With KPTI, fat pieces became markedly insensitive but still responsive to proinsulin.

These results suggest that the proinsulin molecule has biologic activity not necessitating conversion to insulin for expression. Intact tissues contain trypsin-like activity capable of converting proinsulin to insulin. This activity must be located between the vascular endothelium and before access to, but not including, the cell membrane. DIABETES *19*:289-95, May, 1970.

Proinsulin has recently been demonstrated to be a synthetic precursor of insulin in the pancreatic beta cell.[1,2] Preliminary studies have suggested that proinsulin cross-reacts with most antibodies to insulin,[1] thus raising the possibility that some portion of serum "immunoreactive insulin" might be proinsulin. Subsequently, Rubenstein, Cho, and Steiner have presented evidence that proinsulin is present in both blood and urine[3] and have suggested that Roth's "big insulin"[4] might be further evidence for this phenomenon. This is of particular interest, since preliminary studies have suggested that the biologic activity of proinsulin is significantly less than insulin[5] and could account for the high but "ineffective" levels of immunoreactive insulin seen in early stages of diabetes and other syndromes of "insulin resistance."[3]

Thus further in vitro studies of the extent and mechanism of the biologic activity of proinsulin seem indicated. Since Shaw and Chance have reported that proinsulin is effective on fat pads[5] and Glieman (as quoted by Rubenstein et al.[3]) showed little activity on fat cells, it was felt these phenomena should be studied in detail under identical conditions in the same laboratory. If proinsulin is made more active after conversion to insulin, these studies might point to the anatomic site of the enzymatic machinery for this conversion.

METHODS

Male 140 to 160 gm. ad libitum-fed Wistar rats were killed by decapitation and the epididymal fat pads excised. Isolated fat cells were prepared by collagenase* digestion as described by Rodbell[6] and modified by Glieman[7] and, in separate experiments, fat pad pieces were prepared as described by Bray.[8]

Both tissue preparations were incubated identically in Krebs-ringer bicarbonate buffer containing 40 mg./ml. bovine serum albumin.† Dose response curves for insulin-like activity of single component porcine insulin‡ and proinsulin‡ were determined for the conver-

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*Crude Bacterial Collagenase, Worthington Biochemicals, Freehold, New Jersey.

†Lot E29907, Armour and Co., Chicago, Illinois.

‡Kindly provided by Dr. Walter Shaw, Eli Lilly & Co., Indianapolis, Indiana.

TABLE 1

Summary of half-maximal concentrations* (K) for the effects of insulin and proinsulin on adipose tissue

Effect studied	KPTI†	Isolated cells			Pieces		
		Insulin	Proinsulin	Ratio‡	Insulin	Proinsulin	Ratio‡
Antilipolysis	0	56(36-89)	1,530(534-4,370)	27	159(106-239)	1,510(683-3,340)	9
	+	41(9-188)	1,360(468-3,960)	33	86(61-122)	4,130(2,325-7,330)	48
C-14-O_2	0	93(37-230)	2,150(1,036-4,450)	23	197(161-241)	3,530(2,565-4,870)	18
	+	74(53-103)	5,860(4,130-8,320)	79	259(212-316)	11,500(6,980-19,000)	44

*X 10^{-12} M/L., parentheses contain the 95 per cent confidence limits.
†50 μg./ml. Kunitz pancreatic trypsin inhibitor.
‡K proinsulin/K insulin.

sion of glucose-I-C-14-O_2* and for antilipolysis[9] after stimulation with theophylline† as summarized below. Kunitz pancreatic trypsin inhibitor‡ was added as specified (50 μg./ml.).[5] Preliminary studies of C-14-O_2 production with proinsulin and fat pieces had confirmed that this dose of KPTI produced maximum inhibition. Concentrations of KPTI up to 500 μg./ml. had no effect on lipolysis or C-14-O_2 production. All data were referred to cell count[7] in the case of the isolated cells, and weight in the case of fat pieces.

Conversion of glucose-1-C-14 to C-14-O_2

Fat cells (approximately 200,000/ml.) or fat pieces were incubated for two hours at 37° C. in 2 ml. of buffer in equilibrium with 95 per cent O_2-5 per cent CO_2 as described by Glieman.[7] The buffer contained 0.1 mg./ml. glucose and 0.1 to 0.2 mc of glucose-1-C-14/ml. and varying quantities of insulin and proinsulin. C-14-O_2 was collected and counted in a Packard Tri Carb Scintillation Counter.§ After internal standardization, glucose conversion to C-14-O_2 was calculated from the initial specific activity of glucose and expressed as μmoles glucose converted per mg. (pieces) or per 200,000 fat cells.

Antilipolysis

Fat cells or pieces were incubated without glucose for one hour as 37° C. under 95 per cent O_2-5 per cent CO_2 in the presence of 1×10^{-4} M theophylline. Previous experiments had established this theophylline concentration as near maximum lipolysis. Glycerol was determined by the method of Chernick[10] and expressed as μmoles glycerol released per mg. (pieces) or per 200,000 cells. Insulin or proinsulin was added in increasing amounts until maximum inhibition of lipolysis was achieved.

Since there was some day-to-day variability in basal C-14-O_2 production and maximum lipolysis and only one full dose response condition could be studied at a time, all data were normalized to per cent increase in C-14-O_2 production over control which was caused by insulin, and per cent of the maximum lipolysis seen in the absence of insulin. All data are expressed as the mean ± S.E.M. The mean ± S.E.M. value of the actual basal C-14-O_2 production and maximum lipolysis are presented in the appropriate figure legends for reference.

Statistical consideration of the data was by computer with the determination of the best fit linear regression over the straightest portion of the dose response curve for each consideration.[11] A K, or half-maximal response, or midpoint of each dose response curve and its 95 per cent confidence limits was then determined for each condition and agent studied[11] (table 1). In addition, where the linear regressions were appropriately parallel (antilipolysis), the relative potencies with KPTI/without KPTI at similar levels of response and their 95 per cent confidence limits were calculated[12] (table 2).

RESULTS

Antilipolysis

Isolated rat fat cells and fat pieces were incubated in the presence of 10^{-4} M theophylline to stimulate lipolysis. Increasing amounts of single component porcine insulin and proinsulin were used to inhibit this lipolysis (figure 1). To be noted is the rather gentle inflection in the antilipolysis curves (figure 1) which make determination of the K or half-maximal values in these studies less precise than those from C-14-O_2 production to follow (figure 2).

In fat cells (figure 1A, table 1) the antilipolytic effect

*Lot CFA72, Batch 3, Nuclear Chicago, Chicago, Illinois.
†Mathieson, Coleman, Bell, Cincinnati, Ohio.
‡Worthington Biochemicals, Freehold, New Jersey.
§Model 314EX, Packard Instrument Company, La Grange, Illinois.

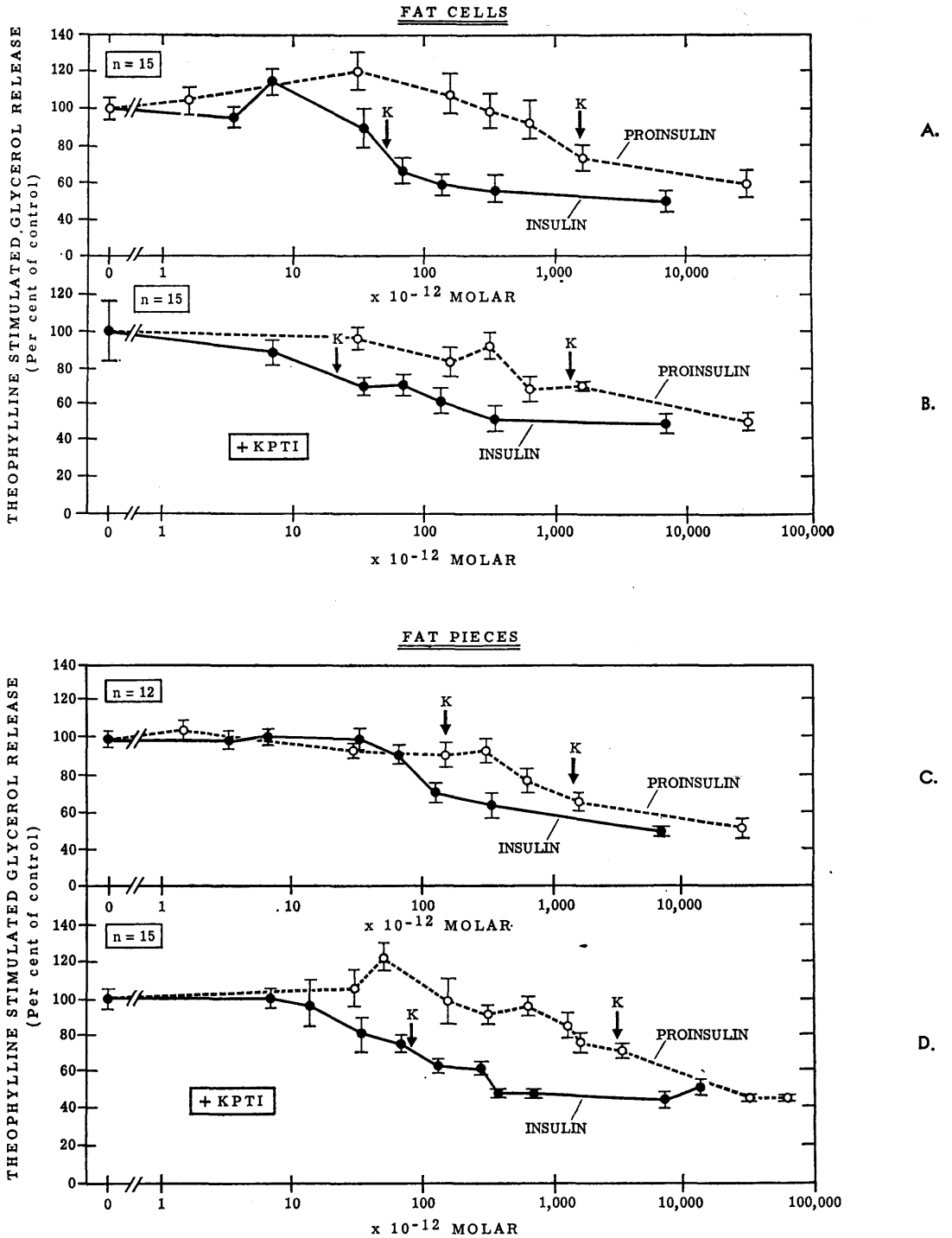

FIG. 1. Effect of single component porcine proinsulin and insulin on glycerol release in rat adipose pieces and cells. Fat cells or pieces were incubated for one hour at 37° C. in 2 ml. of Krebs bicarbonate buffer equilibrated with 95 per cent O_2-5 per cent CO_2 in the presence of 1×10^{-4} M theophylline. Concentrations of insulin and proinsulin were as indicated. Glycerol release was determined and expressed as μmoles glycerol/hr./200,000 cells or 100 mg. fat tissue. Results are expressed as per cent ± S.E.M. of control (theophylline-stimulated) glycerol release for each day's experiment. The actual control values were: A. Fat cells: 89.5 ± 10.0. B. Fat cells plus 50 μg./ml. KPTI: 108.1 ± 15.9. C. Fat pieces: 254.4 ± 4. D. Fat pieces plus 50 μg./ml. KPTI: 284.5 ± 11.0.

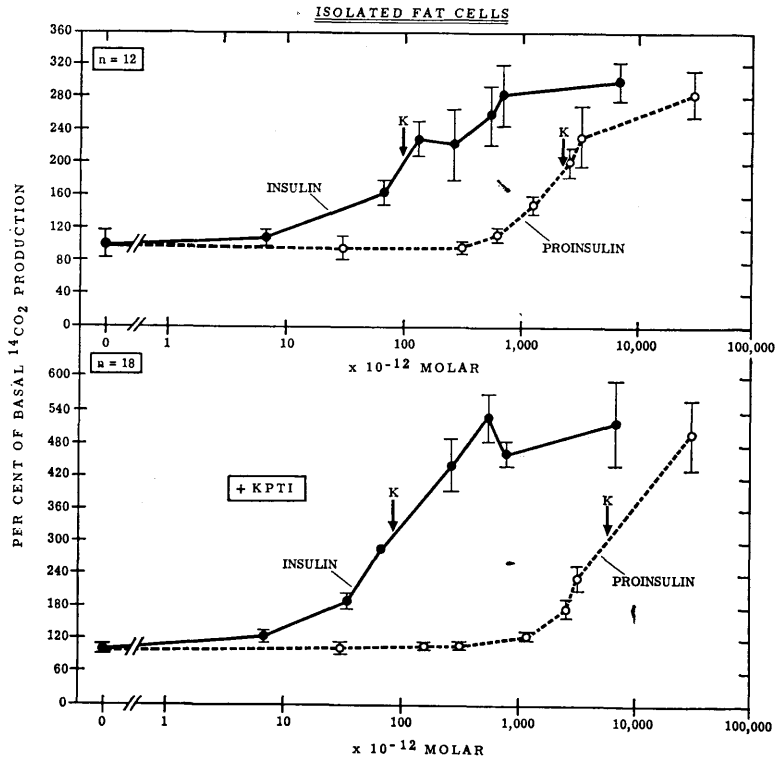

FIG. 2. Effect of single component porcine proinsulin and insulin on production of C-14-O_2 from glucose-1-C-14 in rat adipose pieces and cells. Fat cells or fat pieces were incubated for two hours at 37° C. and CO_2 trapped and counted as described in Methods. Concentrations of insulin and proinsulin were as indicated. Results were calculated as mμ moles glucose-1-C-14 oxidized to C-14-O_2/2 hr./200,000 cells or 100 mg. fat tissue and converted to per cent of control (no insulin) glucose oxidation for each day's experiment. The actual control values were: A. Fat cells: 12.7 ± 1.9. B. Fat cells plus 50 μg./ml. KPTI: 9.7 ± 0.9. C. Fat pieces: 138.2 ± 15.4. D. Fat pieces plus 50 μg./ml. KPTI: 118.9 ± 8.4.

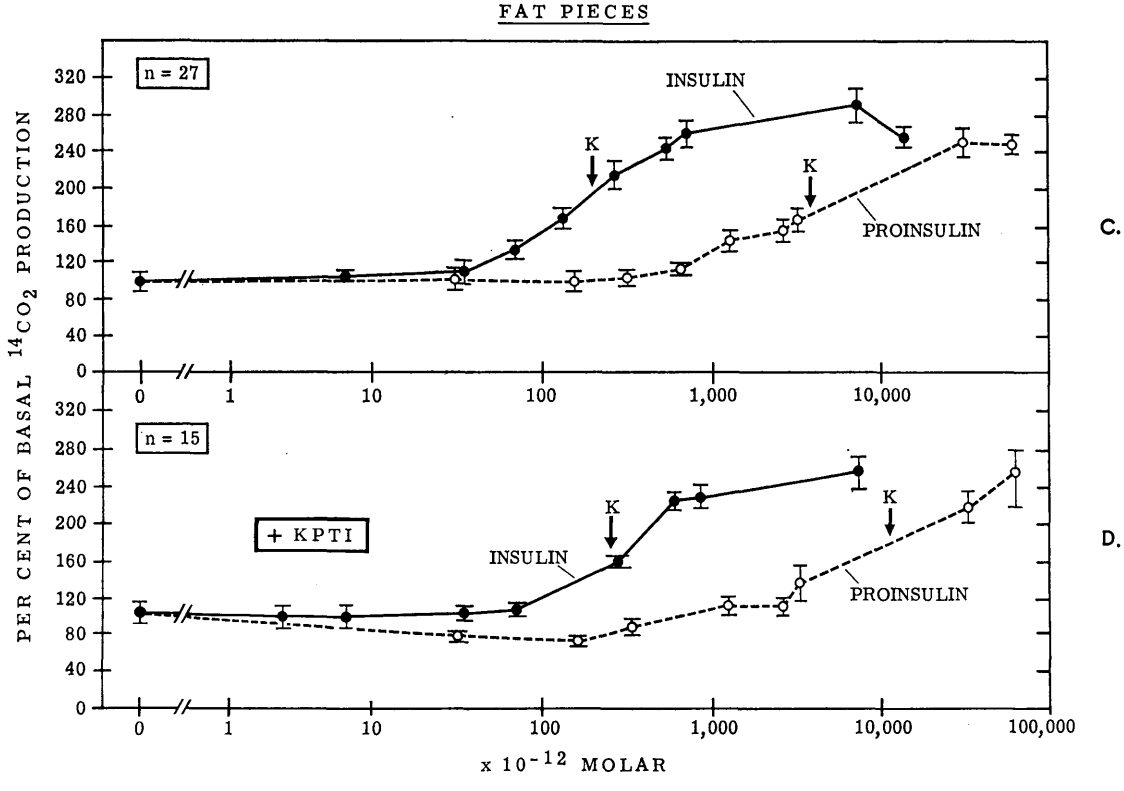

TABLE 2

Relative potencies (M')* at same responses

Effect studied	Condition	M'	95% confidence limits
Antilipolysis	Fat cell, insulin	0.83	0.31-2.26
	Fat cell, proinsulin	0.26	0.06-1.11
	Fat pieces, insulin	0.42	0.25-0.71
	Fat pieces, proinsulin	1.9	0.78-4.65

*With KPTI/without KPTI.

of insulin first appeared at a concentration of approximately 35 $\times$ 10^{-12} M with a K or half-maximal effect at 56 $\times$ 10^{-12} M insulin. The fat cells appeared to be much less sensitive to proinsulin with the earliest inhibition of lipolysis at 320 to 640 $\times$ 10^{-12} M proinsulin and a K of 1,530 $\times$ 10^{-12} M proinsulin. These same studies then were repeated in the presence of 50 mg./ml. of Kunitz pancreatic trypsin inhibitor (KPTI) (figure 1B, table 1). The effectiveness of neither insulin (K = 41 $\times$ 10^{-12} M) nor proinsulin (K = 1,360 $\times$ 10^{-12} M) was inhibited by KPTI.

The preparation of fat pieces appeared to be less sensitive than fat cells to insulin with the earliest effect becoming apparent at 70 $\times$ 10^{-12} M insulin and a K value of 159 $\times$ 10^{-12} M insulin (figure 1C, table 1). However, the K value for proinsulin with fat pieces was the same as that found with fat cells (1,510 $\times$ 10^{-12} M proinsulin). Though it is unclear precisely where the lowest dose effect of proinsulin becomes apparent, it is probably somewhere in the range of 50 to 200 $\times$ 10^{-12} M proinsulin. Thus both the K value and the earliest effect of proinsulin are unchanged and certainly not higher, indicating fat pieces are at least as sensitive to proinsulin as fat cells while, conversely, fat pieces appear *less* sensitive than fat cells to insulin. As with fat cells, the incubation of fat pieces with KPTI (figure 1D, table 1) had little effect on the insulin dose response curve, the K value being 86 $\times$ 10^{-12} M insulin. However, the effect of proinsulin on fat pieces appears to be inhibited by KPTI since the initial antilipolytic effect was found at 300 $\times$ 10^{-12} M proinsulin with a K value of 4,130 $\times$ 10^{-12} M proinsulin. This is also shown by the relative potencies (M') for antilipolysis from table 2. Here a value for M' greater than 1 indicates an inhibitory effect of KPTI. The only value for M' greater than 1 is for fat pieces-proinsulin, (M' = 1.9); however, it must be pointed out that the lower confidence limit is just below 1. Thus there is a suggestion that the presence of KPTI is inhibitory, if at all, only on the effect of proinsulin on antilipolysis in fat pieces.

C-14-O_2 production

An effect of insulin on C-14-O_2 production by rat fat cells was seen at the lowest dose tested, 7 $\times$ 10^{-12} M insulin, while half-maximal effect was at a K of 93 $\times$ 10^{-12} M insulin (figure 2A, table 1). Porcine proinsulin was significantly less effective with the initial inflection at 300 with a K of 2,150 $\times$ 10^{-12} M proinsulin in fat cells. When the cells were incubated with 50 mg./ml. of KPTI, the insulin curve was unchanged (K = 74 $\times$ 10^{-12} M insulin) (figure 2B, table 1). The effect of proinsulin on fat cells in this case appears to be inhibited by KPTI: initial inflection at 1,200 and K of 5,860 $\times$ 10^{-12} M proinsulin (figure 2B). This difference is not statistically significant since the lower confidence limit of this figure (4,130) overlaps with the upper limit in the absence of KPTI (4,450).

Similar to the lipolysis studies, the production of C-14-O_2 by fat pieces was found to be less sensitive to insulin than that of fat cells: initial inflection between 70 to 140 and K of 197 $\times$ 10^{-12} M insulin (figure 2C, table 1). As in the lipolysis studies, fat pieces (K = 3,530 $\times$ 10^{-12} M proinsulin) appeared almost as sensitive as fat cells (K = 2, 150 $\times$ 10^{-12} M proinsulin) to the stimulation of C-14-O_2 production by proinsulin and the small difference here is not significant due to the extensive overlap of the confidence limits.

As in the lipolysis studies, KPTI did not shift the insulin dose response curve on fat pieces significantly: initial inflection approximately 70 and K = 259 $\times$ 10^{-12} M insulin (figure 2D, table 1). However, the fat pieces were made markedly insensitive to proinsulin by KPTI: K = 11,500 $\times$ 10^{-12} M proinsulin (figure 2D).

These values for K are summarized in table 1. Thus fat cells were more sensitive than fat pieces to the effects of insulin. The insulin effects were not significantly changed by KPTI in either preparation. Conversely, in the absence of KPTI, fat pieces appeared to be as sensitive to proinsulin as fat cells. Proinsulin was found to be twenty-seven times less effective than insulin on cells but only nine times less effective on pieces (antilipolysis). In the case of C-14-O_2 production proinsulin was twenty-three times less effective on cells and eighteen times less effective on pieces. In the presence of KPTI, fat pieces were made insensitive to proinsulin while the response of fat cells to proinsulin was probably unchanged. The overlap in the confidence

limits of the K values compared above suggests these comparisons must conservatively be regarded as trends only, without statistical surety.

DISCUSSION

Previous reports have suggested that porcine proinsulin is approximately ten times less effective than insulin in the stimulation of glucose disposition to lipids and CO_2 by intact rat epididymal fat pads.[5] In addition Glieman is quoted in a personal communication as finding "extremely low biologic potency" on isolated fat cells.[3] Finally, Shaw and Chance[5] have speculated that proinsulin is biologically effective only after its tryptic-like conversion to insulin, an enzymatic activity which they blocked with Kunitz pancreatic trypsin inhibitor (KPTI). The present studies were undertaken in an attempt to determine if, under comparable conditions in a single laboratory, there might be a difference in the biological effectiveness of proinsulin on fats cells or fat pieces and to examine more closely the possibility of intrinsic biological activity in the proinsulin molecule. If fat pieces were found to be more sensitive to proinsulin than fat cells, this would tend to implicate the interstitial material which is digested away by collagenase and not the remaining fat cell plasma membrane as the site of the tryptic-like proinsulin-converting system. Another logical deduction from this hypothesis would be that KPTI should not significantly inhibit any proinsulin effect on isolated fat cells but should be inhibitory with fat pieces.

The present results demonstrate an effect of porcine proinsulin, like insulin, to inhibit lipolysis and stimulate the production of C-14-O_2 from glucose-1-C-14 in both isolated epididymal fat cells and epididymal fat pieces of rats. Proinsulin is significantly less effective than insulin in performing these biological functions, however. In fat cells on a molar basis insulin is approximately twenty-three to twenty-seven times more effective than proinsulin and in fat pieces some nine to eighteen times (table 1). In all cases the ratio of the K's (proinsulin:insulin) is decreased in fat pieces when compared with cells. This difference is accounted for by a decrease in the molar effectiveness of insulin on fat pieces, presumably due to greater difficulty in accession of insulin to the cellular membrane,[14] and little change in the effectiveness of proinsulin, in spite of the presumed *decrease* in permeability for this larger molecule.

The persistence of the effects, especially on fat cells in the presence of KPTI, suggests that the proinsulin molecule has intrinsic though lower biologic activity which does not need conversion to insulin for expression. This is contrary to the suggestion of Shaw and Chance[5] but they did not test the higher concentrations studied here. The results obtained in the presence of KPTI would indicate that, when the converting system is blocked, the remaining presumed intrinsic proinsulin activity was thirty-three to seventy-nine times less than insulin when assayed on fat cells and forty-four to forty-eight times less in fat pieces (table 1).

Two alternative explanations to intrinsic activity of proinsulin must be considered. First, conversion of proinsulin to insulin by other tryptic or chymotryptic systems not inhibited by these doses of KPTI cannot be ruled out. Preliminary studies for these experiments as well as the data of Shaw and Chance[5] had indicated 50 μg./ml. KPTI to be maximally effective on fat pieces. If such systems exist, they must be intimately associated with the cell membrane and not completely inhibited by the even more ready access of KPTI since proinsulin was effective on fat cells in the presence of KPTI. In addition they must be less active than that system presumably in the interstitium since fat pieces were as sensitive to proinsulin as fat cells. Secondly, minor contamination of the proinsulin by insulin is possible but is unlikely in view of the fact that the material used in this study can be easily separated from insulin on Sephadex, and by polyacrilamide disc gel electrophoresis, and does not produce "A" chains on sulfitolysis. Contamination at most must be significantly less than 1 per cent.[5,13] In addition, W. Bromer of Eli Lilly & Co. (personal communication) reports that the proinsulin used in these studies has been subjected to reduction and reoxidation with persistence of biologic activity on fat cells, fat pads, and the mouse convulsion assay. This rules out contamination with insulin.

As mentioned above, fat pieces were found to be equally as sensitive to proinsulin as fat cells. This was in spite of the observation that fat pieces were from two to three times *less* sensitive to insulin than fat cells. This latter observation has usually been explained by increased difficulty of accession of materials by diffusion to the cells in relatively intact tissues when compared with isolated cell preparations.[14] With a molecular weight of 9,000, proinsulin should, if anything, have greater difficulty than insulin (MW = 6,000) reaching the cell membrane in fat pieces. Since it was concluded above that proinsulin had some, but lesser, biologic activity than insulin, these results imply some conversion of proinsulin to insulin by fat pieces. By inference the converting systems would therefore be located be-

tween the vascular lumen and the cellular membrane, i.e., those materials digested away by collagenase. This is further supported by the marked inhibition by KPTI of proinsulin effect found in fat pieces (table 1) whereas the insulin effect was unchanged, and the lack of significant effect of KPTI on proinsulin or insulin action in fat cells (antilipolysis).

A word of caution is needed at this point. These studies involved the use of porcine proinsulin and insulin on rat tissue. Despite the minor amino acid differences in rat and porcine insulin, the latter is effective on rat tissues. However, we do not yet know the comparative amino acid sequences of the connecting peptide of rat and porcine proinsulin or whether this peptide might direct a greater amount of species specificity in the various activities studied here.

Rubenstein et al. have suggested that proinsulin is released from the beta cells of the pancreas and enters the bloodstream.[3] Upon reaching the periphery the present results suggest it could act directly on cells, though with low molar activity. Alternatively, an enzymatic system exists in the interstitial spaces of adipose tissue capable of converting proinsulin to a more active substance, presumably insulin.[15] The nature and activity of such a proteinase, now that it has tentatively been located, should be of great interest. A deficiency of such activity, either in the interstitial spaces of the pancreas or the peripheral tissues or both, could act to inhibit glucose disposition.

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