In Vitro Activity of Biosynthetic Human Proinsulin

Receptor Binding and Biologic Potency of Proinsulin and Insulin in Isolated Rat Adipocytes

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SUMMARY

The receptor binding characteristics and biologic potency of biosynthetic human proinsulin (rDNA) were determined in isolated rat adipocytes and compared with those of insulin. In competition with ^{125}I(A14)-iodoinsulin for binding to adipocyte receptors at 15°C, proinsulin showed a 100-fold lower affinity for binding than did insulin. A proinsulin concentration of $3.2 \pm 0.8 \times 10^{-7}$ M was required for 50% inhibition of tracer binding as compared with a concentration of $1.7 \pm 0.3 \times 10^{-9}$ M for insulin. These results were confirmed in direct competition studies using proinsulin and ^{125}I-iodoproinsulin. A similar 100-fold difference was also observed in competitive binding experiments conducted at 37°C. The biologic potency of human proinsulin was evaluated by its ability to stimulate glucose incorporation into total fat cell lipid and also by its antilipolytic activity. Glucose incorporation into lipid was half-maximal at a proinsulin concentration of $1.5 \pm 0.4 \times 10^{-8}$ M, whereas the same response was observed at an insulin concentration of $5.2 \pm 1 \times 10^{-11}$ M. Proinsulin also demonstrated an antilipolytic potency that was <1% that of insulin. The time course over which insulin and proinsulin stimulated glucose incorporation into lipid was the same, as was the time course over which the stimulation dissipated after removal of the hormones. No synergism of insulin and proinsulin stimulation of lipogenesis was observed when fat cells were incubated with submaximal concentrations of the two hormones. It is concluded that biosynthetic human proinsulin has approximately 1% the receptor binding affinity and biologic potency of insulin in the isolated rat adipocyte system. DIABETES **1984; 33:1062–67.**

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Whereas most proinsulin is converted to insulin or is degraded in the pancreas, small amounts of proinsulin are secreted from the pancreas and are found in the circulation in both normal and abnormal states.[1] The biologic role for this hormone precursor in peripheral tissue, however, is not known. From various in vitro studies, the biologic activity of proinsulin has been found to be qualitatively similar to insulin, although quantitatively the activity is much less on a mole-to-mole basis. The range of potency for proinsulin, as compared with insulin, has varied among different studies from <1% to as much as 16%.[2,3]

With the advent of recombinant DNA technology, it is now possible to obtain large amounts of purified human proinsulin and studies are underway exploring the potential clinical use of this material.[4] As a result, it has now become even more important to understand the action of this prohormone at the cellular level. To explore this in depth, we have examined the receptor binding and biologic activity of human proinsulin (rDNA) in a well-characterized rat adipocyte preparation.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from Harlan Industries, Indianapolis, Indiana. Animals were given free access to water and a commercial pellet diet and were housed in a controlled environment with a 12-h light:12-h dark cycle for at least 5 days before use. Only rats weighing 150–200 g were selected for use in these studies.

Fat cell isolation. Adipocytes were prepared from epididymal fat pads by a modification[5] of the collagenase digestion procedure of Rodbell.[6] Krebs-Ringer-HEPES (KRH) buffer,[5] pH 7.4, containing 4% bovine albumin and 0.55 mM glucose was used in all isolation and incubation steps.

Insulin and proinsulin iodination. Porcine single-site ^{125}I(A14)-iodoinsulin (sp act 300–340 μCi/μg) was prepared and isolated as described previously.[7] The preparation of human ^{125}I-monoiodoproinsulin (sp act 210–230 μCi/μg) in

general used the same methodology as for the preparation of the iodoinsulin.[8] The ^{125}I-iodoproinsulin used in these studies was specifically labeled on the tyrosine of proinsulin (Tyr-79) corresponding to the A14 tyrosine of insulin.

Quantitation of insulin and proinsulin binding and degradation. Unless indicated otherwise in the figure legend, approximately 2 × 10^5 adipocytes were incubated in 1 ml of buffer with ^{125}I-labeled pork insulin or human proinsulin and varying concentrations of unlabeled insulin or proinsulin. Incubations were conducted at either 15°C or 37°C and for the times indicated for the individual experiment. At the end of the incubation period, triplicate samples were withdrawn for determination of binding (cell-associated radioactivity) and degradation, as described previously.[7] Degradation was assessed by solubility in 5% trichloroacetic acid. Samples were counted in a Tracor Analytic Model 1285 gamma scintillation spectrometer with a counting efficiency of 85%.

Measurement of insulin and proinsulin biologic activity. The ability of the hormones to stimulate lipogenesis was assessed according to the method of Moody et al.[9] by monitoring the incorporation of 2-^{3}H-glucose into total fat cell lipid. Cells were incubated at 37°C for the indicated period of time and the reaction was then terminated by the addition of 10 ml of Liquifluor (New England Nuclear, Boston, Massachusetts). Radioactivity was determined in a Searle Isocap 300 liquid scintillation counter at an efficiency of approximately 30%. Blanks were prepared in which the scintillation fluid was added to the vials before the addition of cells. The average counts obtained from these vials were subtracted from those observed in all other samples.

The antilipolytic activity of insulin and proinsulin was determined in experiments in which cells were incubated at 37°C in buffer supplemented with 0.1 mg/ml ascorbic acid, which was included to limit oxidation of added epinephrine. Cells were incubated in buffer alone, with 1 μg/ml epinephrine, or with epinephrine and the indicated concentration of insulin or proinsulin. After 1 h of incubation, the reaction was terminated by adding 100 μl of 4.4 N perchloric acid. Samples were neutralized by addition of 100 μl of 2.4 M K_2CO_3 and an aliquot analyzed for glycerol content by a fluorometric method as described previously.[10] Addition of epinephrine typically resulted in a 20-fold increase in the rate of glycerol release as compared with the control condition, and a maximally effective concentration of insulin inhibited the epinephrine-stimulated increase in lipolysis by >85%.

Statistical analyses. Competitive binding curves and biologic activity dose-response curves were analyzed using the PREFIT and ALLFIT programs[11] based on a four-parameter logistic model. These analyses indicated the concentration of insulin or proinsulin necessary to produce a half-maximal response, as well as the maximal and minimal values. All values are presented as the mean ± SEM. Differences between insulin and proinsulin were compared by Student's *t*-test. When more than two sample means were compared, a one-way analysis of variance followed by the Student-Newman-Kuels multiple-range test was performed.

Materials. Collagenase (type I, lot 40P080 or 43D289) was purchased from Worthington Biochemicals, Freehold, New Jersey. Pentex bovine serum albumin (fraction V) was from Miles Biochemicals, Elkhart, Indiana, and was exhaustively dialyzed before use as described previously.[5] D-[2-^{3}H]-glucose was purchased from Amersham Corp., Arlington Heights, Illinois. Crystalline porcine insulin (lot 615-07J-256 or 615-2H2-300) was provided by Dr. R. E. Chance of the Eli Lilly Research Laboratories, Indianapolis, Indiana. Biosynthetic human proinsulin was prepared as previously described.[12] The studies described here were done with lot L31-1U2-165. Two additional lots (581-5H7-24A and 759-0B6-265) were also tested with identical results.

Proinsulin solutions were freshly prepared by dissolving the contents of vials that contained calibrated quantities of human proinsulin. The protein content of the vials had previously been determined by dissolving contents in 0.01 N HCl and then subjecting aliquots to amino acid analysis and to HPLC analysis. The protein content was calculated from the results of the amino acid analyses and agreed with results of a UV determination of protein content. Freshly prepared solutions of insulin were calibrated by UV determination.

RESULTS

The time course of insulin and proinsulin binding to isolated adipocytes at 15°C is shown in Figure 1. Whereas insulin binding reached apparent equilibrium by 60 min, maximal proinsulin binding was not attained at this temperature until approximately 4.5 h. Maximal specific proinsulin binding was only 1.8% of that observed with insulin. The much lower apparent affinity of proinsulin relative to insulin necessitated a higher cell concentration in these studies than we have used previously,[7] which accounts for the much higher percentage of insulin binding compared with what we[7] and others[13] have reported. To allow proinsulin binding to reach equilibrium, therefore, all competitive binding studies conducted at 15°C were carried out for 4.5 h.

In Figure 2 are shown the results of experiments in which

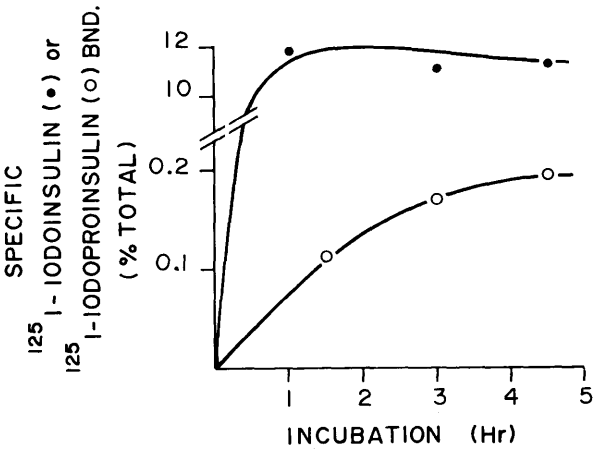

FIGURE 1. Time course of insulin and proinsulin binding to isolated adipocytes. Adipocytes (1 × 10^6/ml) were incubated with tracer concentrations of ^{125}I(A14)-iodoinsulin ([●] 1 × 10^{-11} M) or ^{125}I-iodoproinsulin ([○] 6 × 10^{-11} M) at 15°C. At the indicated times, samples were taken and the amount of cell-bound radioactivity was determined as described in MATERIALS AND METHODS. Nonspecific binding was determined by including 1 × 10^{-5} M insulin during the incubation. The resulting value was subtracted from total binding to give an estimate of specific binding. Nonspecific binding of ^{125}I(A14)-iodoinsulin was estimated to be 0.29% of the total radioactivity added, and that for ^{125}I-iodoproinsulin was 0.22%. Results shown are the mean of 2 experiments in which individual determinations were made in triplicate.

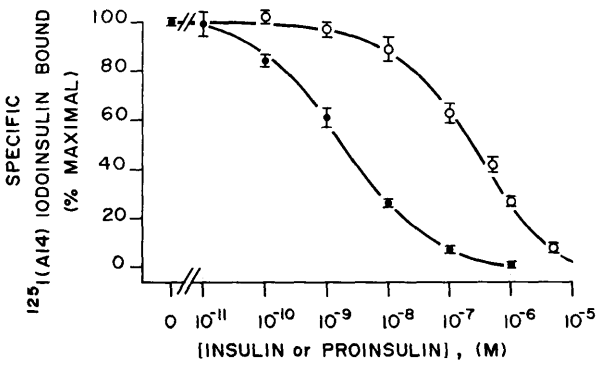

FIGURE 2. Competitive binding of insulin and proinsulin to isolated adipocytes at 15°C. A tracer concentration of ^{125}I(A14)-iodoinsulin (1×10^{-11} M) and the indicated concentrations of unlabeled insulin (●) or proinsulin (○) were incubated with fat cells for 4.5 h. The amount of cell-bound radioactivity was then determined. Results are expressed relative to the maximal specific ^{125}I-iodoinsulin binding that was observed and are the mean ± SEM of 3 experiments. Nonspecific binding of ^{125}I-iodoinsulin averaged 7% of total binding.

^{125}I(A14)-iodoinsulin competed for receptor binding with native pork insulin or human proinsulin at 15°C. The results of these experiments indicated a difference >100-fold in apparent affinity, as 50% inhibition of tracer insulin binding occurred at an insulin concentration of $1.7 \pm 0.3 \times 10^{-9}$ M as compared with a human proinsulin concentration of $3.2 \pm 0.8 \times 10^{-7}$ M.

Similar experiments were also conducted at 37°C. An approximate 100-fold difference in apparent affinity was also observed in these studies, as the concentration of native insulin necessary for 50% inhibition of tracer insulin binding was $4.5 \pm 0.9 \times 10^{-9}$ M as compared with the corresponding value of $4.7 \pm 0.7 \times 10^{-7}$ M for human proinsulin (Figure 3). Both insulin and proinsulin inhibited the degradation of ^{125}I-iodoinsulin in direct proportion to their ability to inhibit receptor binding of the tracer material (data not shown).

To further examine the apparent low affinity of proinsulin for binding to adipocytes, competitive binding studies were done using ^{125}I-iodoproinsulin and increasing amounts of unlabeled proinsulin (Figure 4). The results of these studies were very similar to those obtained when ^{125}I-iodoinsulin was used as the tracer. The concentration of proinsulin required for 50% displacement of the ^{125}I-iodoproinsulin was 2.0×10^{-7} M, as compared with the 3.2×10^{-7} M concentration of proinsulin required for 50% displacement of ^{125}I-iodoinsulin. These results support the concept that proinsulin and insulin compete for the same receptor.[14]

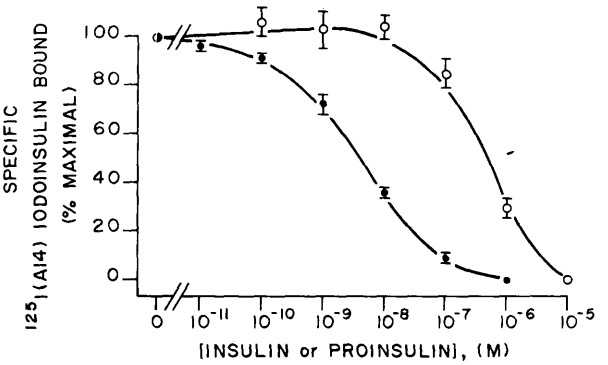

FIGURE 3. Competitive binding of insulin and proinsulin to isolated adipocytes at 37°C. Experiments similar to those described in Figure 2 were conducted in which fat cells were incubated with tracer insulin and either unlabeled insulin (●) or proinsulin (○) for 1 h at 37°C. Results shown are the mean ± SEM of 4 experiments. Average nonspecific binding of the ^{125}I(A14)-iodoinsulin was 20.3% of total binding.

The biologic potencies of insulin and human proinsulin were compared using the assay of Moody et al.[9] to monitor the incorporation of glucose into total fat cell lipid. The time course of insulin and proinsulin stimulated conversion of glucose into lipid was compared using hormone concentrations that, from preliminary experiments, were known to produce slightly greater than half-maximal responses. After a short lag period of 5 min or less, the time course of glucose incorporation into lipid was linear and was the same for both hormones (data not shown). The full dose-response curves for both hormones are shown in Figure 5. At high concentrations, both insulin and human proinsulin produced the same maximal response. Compared with porcine insulin, however, human proinsulin displayed <1% of the potency. Half-maximal response to insulin was observed at $5.2 \pm 1 \times 10^{-11}$ M, whereas $1.5 \pm 0.4 \times 10^{-8}$ M human proinsulin was required to produce the same half-maximal response.

For comparison, we also examined the relative potency of the hormones based on their ability to inhibit epinephrine-stimulated lipolysis. As shown in Figure 6, proinsulin also demonstrated <1% the potency of insulin in this assay. Note that the dose-response curve for insulin is biphasic, with higher concentrations being less effective.[15] Dose-response curves for proinsulin and insulin stimulation of 2-deoxyglucose uptake also showed a >100-fold difference between the dose required for 50% of maximal response (data not shown).

The duration of the biologic response to insulin or proinsulin after removal of the hormones is shown in Figure 7.

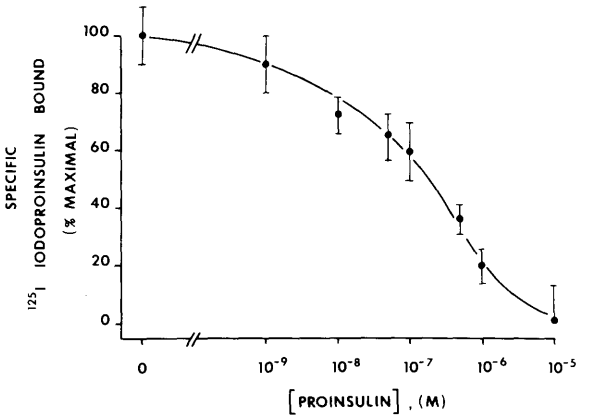

FIGURE 4. Competitive binding of ^{125}I-iodoproinsulin and unlabeled proinsulin to isolated adipocytes. Approximately 10^6 fat cells/ml were incubated with ^{125}I-iodoproinsulin (6×10^{-11} M) and the indicated concentration of unlabeled proinsulin. After incubation for 4.5 h at 15°C, the amount of cell-bound radioactivity was determined and is expressed relative to the binding observed with only tracer proinsulin. Binding in the presence of 10^{-5} M unlabeled proinsulin (nonspecific binding) was subtracted from total binding to provide an estimate of specific binding at each proinsulin concentration. Nonspecific binding averaged 0.22% of the total ^{125}I-iodoproinsulin tracer added. Results shown are the mean ± SEM of 3 experiments in which individual determinations were made in duplicate.

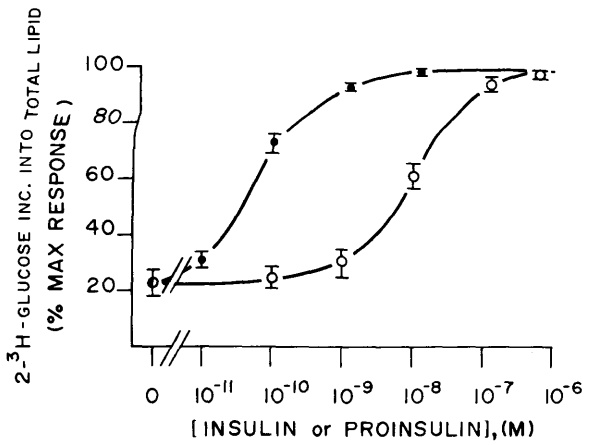

FIGURE 5. Dose-response curves of insulin- and proinsulin-stimulated incorporation of glucose into total fat cell lipid. Isolated adipocytes were incubated in buffer containing 0.55 mM 2-^{3}H-glucose for 1 h at 37°C either with no additions (◑) or with the indicated concentrations of insulin (●) or proinsulin (○). Incorporation of glucose into lipid was determined after the addition of toluene-based scintillation fluid to the vials. Results are expressed relative to the response observed with 1×10^{-8} M insulin. Values shown are the mean ± SEM of 7 experiments.

Cells were preincubated for 1 h with a concentration of either hormone that produced a maximal response. The cells were then rapidly washed (<5 min) and resuspended in buffer containing 2-^{3}H-glucose. The incorporation of glucose into lipid was then followed as a time course and compared with cells preincubated in the absence of either hormone. As shown (Figure 7), the biologic response to both hormones dissipated over the same time interval. For comparison, the response of cells preincubated and then incubated with a maximally effective concentration of insulin is shown.

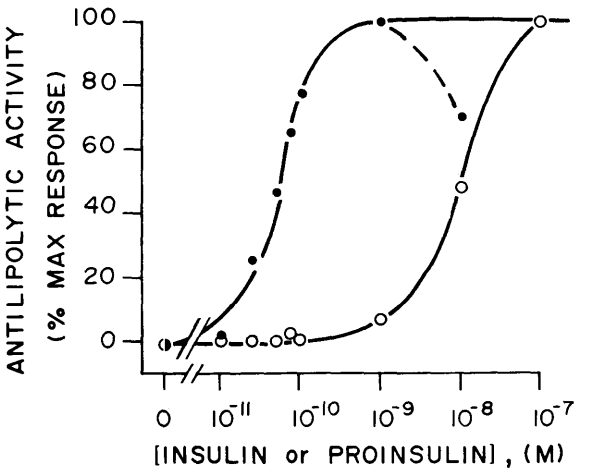

FIGURE 6. Dose-response curves of antilipolytic activity of insulin and proinsulin. Fat cells were incubated with either 1 μg/ml epinephrine alone (◑) or with epinephrine plus insulin (●) or proinsulin (○) at the indicated concentrations. After 1 h of incubation at 37°C, the reaction was terminated by acidification and the amount of glycerol released was determined by a fluorimetric assay as described in the MATERIALS AND METHODS section. All results are expressed relative to the antilipolytic response observed with 10^{-9} M insulin. A half-maximal response to insulin was observed at a concentration of 5.3×10^{-11} M, whereas a proinsulin concentration of 8.5×10^{-9} M was required to produce the same response. Results shown are the means of 2 experiments in which individual determinations were made in triplicate.

In a recently published abstract, Schlüter et al.[16] reported that preincubation of monocytes with human proinsulin results in accentuated insulin binding. In some of our receptor binding experiments (Figures 2 and 3) we noted a slight *trend toward greater ^{125}I-iodoinsulin binding in cells incubated* with low concentrations of proinsulin, although this did not reach statistical significance. We therefore examined the effect of preincubation with low concentrations of proinsulin on insulin binding at 37°C. Cells were preincubated for 2 h with 10^{-10}, 10^{-9}, or 10^{-8} M proinsulin. ^{125}I-iodoinsulin was added to the vials, and binding was determined after a 1-h incubation. Preincubation with proinsulin had no effect on insulin binding, as compared with cells preincubated with no additions (data not shown). To further test the possibility that proinsulin potentiates or augments the response to insulin, the experiments presented in Table 1 were conducted. As shown, simultaneous incubation of cells with submaximal concentrations of insulin and proinsulin produced a simple additive effect of the two hormones. Thus, in these experiments, proinsulin apparently did not alter the fat cells' responsiveness to insulin other than in producing its own insulin-like response.

DISCUSSION

Human proinsulin (rDNA), as is evident from the present results, has a much lower biologic activity in vitro in adipocytes than previously appreciated. Using glucose incorporation into lipids, antilipolysis, and glucose transport as measures of activity, the biologic potency of proinsulin rel-

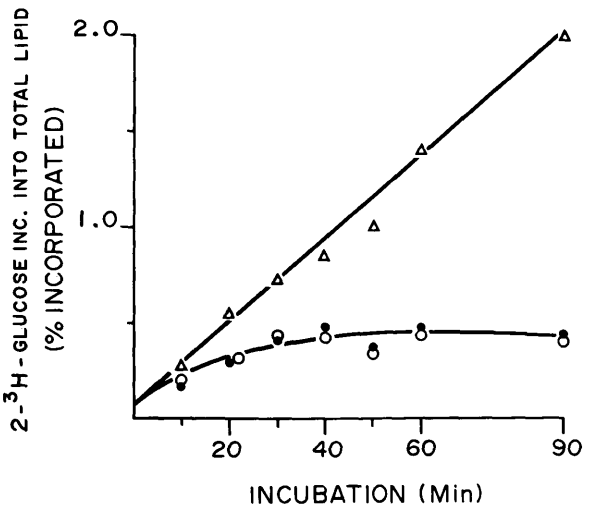

FIGURE 7. Termination of insulin- and proinsulin-stimulated biologic activity. Cells were incubated for 1 h at 37°C either in buffer alone or with 10^{-8} M insulin or 10^{-6} M proinsulin. The fat cells were then rapidly washed 3 times with approximately 10 vol of buffer per wash. After the final wash, cells were resuspended for the experimental period in buffer containing 0.55 mM 2-^{3}H-glucose. Samples were taken at the indicated times and the extent of incorporation of glucose into lipid was determined. The incorporation observed with control cells (preincubated and then incubated in buffer only) was subtracted from all other values, such that the results shown represent the net stimulation above basal. Results shown are from a single representative experiment in which individual determinations were made in duplicate. Two other experiments gave similar results. ●, Preincubated with 10^{-8} M insulin and then incubated with buffer alone; ○, preincubated with 10^{-6} M proinsulin and then incubated with buffer alone; and △, preincubated with 10^{-8} M insulin and then reincubated with 10^{-8} M insulin during the experimental period.

TABLE 1
Effects of insulin and proinsulin on ^{3}H-glucose incorporation into total fat cell lipid

Addition	Glucose converted to lipid (nmol/2 × 10^5 cells/h)
6×10^{-11} M Insulin	182 ± 6*
1×10^{-9} M Proinsulin	30 ± 11
6×10^{-11} M Insulin + 1×10^{-9} M proinsulin	216 ± 11
1×10^{-8} M Insulin	348 ± 22

*Results shown are the means ± SEM.

ative to insulin was 1% or less. Similar results were obtained in measurements of glucose oxidation (data not shown). Some previous reports have found activities in this general range. Gliemann and Sorenson[17] reported bovine proinsulin to have 2% of the activity of insulin, and Toomey et al.[3] found a range of 0.4%–3% for porcine proinsulin. Steele et al.[18] also found porcine proinsulin to have 1% of the antilipolytic activity of insulin. Solomon and Duckworth,[19] using the perifused adipocyte, also found porcine proinsulin to have <1% of the antilipolytic activity of insulin.

In contrast, many studies have found considerably higher activities for proinsulin. Kitabchi[20] and Yu and Kitabchi[21] obtained values for porcine and bovine proinsulin of 9–12% as compared with insulin for both antilipolysis and glucose oxidation. Lavis et al.[15,22] reported 7–9% and Solomon et al.[23] 6% using isolated adipocytes in an incubation system. In a recent report, Podlecki et al. found biosynthetic human proinsulin to have approximately 10% of the receptor binding and biologic potency of insulin in isolated rat adipocytes.[24]

The explanation for the divergent values in the literature is not totally clear. The differences among the various studies are primarily due to differences in potency of proinsulin rather than insulin. The concentration of insulin required to produce a 50% inhibition of lipolysis varied from 1.7 to 7.2 × 10^{-11} M in six separate studies, whereas the comparable concentration for proinsulin varied from 0.27 to 6 × 10^{-9} M in the same studies, a fourfold range for insulin and a 22-fold range for proinsulin. In the present study, the concentration of insulin producing a half-maximal antilipolytic response, 5.3 × 10^{-11} M, agrees well with the reported values, but the corresponding concentration for proinsulin of 8.5 × 10^{-9} M is higher than previously reported.

Most of the studies on the biologic activity of proinsulin were performed ten or more years ago using proinsulin extracted from either bovine or porcine pancreata. The possibility exists that a small amount of contamination with either insulin or partially cleaved proinsulin could have been present. Split proinsulin has significantly greater biologic activity than intact proinsulin.[21] Of interest, several studies that used reduced-reoxidized proinsulin, which would remove insulin or cleaved proinsulin, tended to find lower activities of proinsulin.[3,17] This possibility, however, would not explain all of the divergent results and, in particular, the explanation for the difference between the present study and that by Podlecki et al.[24] is not apparent.

Some early studies using intact fat pads suggested that the biologic activity of proinsulin might be due to conversion to insulin by proteolytic enzymes in the tissue, since KPTI, an inhibitor of proteolysis, decreased the biologic activity of proinsulin.[25] Subsequent studies using isolated fat cells, however, failed to find evidence of conversion of proinsulin to insulin.[20,26]

The insulin-like effect of proinsulin appears to be due to interaction with the insulin receptor,[14] and, thus, the relative binding potency of proinsulin should be comparable to its biologic activity. This result was found in the present study. Unlabeled proinsulin had <1% the potency of unlabeled insulin for competitive displacement of ^{125}I(A14)-iodoinsulin in isolated adipocytes. Relatively few studies exist in the literature concerning the binding potency of proinsulin in adipocytes. Gammeltoft and Gliemann,[27] however, found proinsulin to have approximately 2% of the activity of insulin for binding to rat adipocytes. In contrast, Etherton and Walker[28] found proinsulin to have 6% of the activity of insulin for binding to swine adipocytes.

In the present studies, we have shown similar apparent affinities of proinsulin for adipocyte receptors using either ^{125}I-iodoinsulin or ^{125}I-iodoproinsulin in competitive binding experiments. These results support the concept that proinsulin and insulin bind to the same receptor, but with proinsulin having a 100-fold lower affinity. The binding potency of proinsulin to other tissues besides adipocytes has varied widely in the literature. Early studies reported as much as 15–20% of the activity of insulin in liver,[2] but later this was reevaluated and reported as 3% in a careful study by Freychet,[14] suggesting that earlier preparations might have been less pure. In recent unpublished work, we have also found that human proinsulin has approximately 3% of the binding affinity of insulin in isolated rat hepatocytes.

Some of the reported differences could be due to species differences, since, unlike insulin, proinsulin structure varies considerably among different species. Prager and Schernthaner[29] reported that human proinsulin had approximately 1% of the binding potency of human insulin to freshly isolated human monocytes. Human proinsulin also has about 2% of the binding potency of human insulin in IM-9 lymphocytes, a human-derived cell line (C. S. Hooker, personal communication). These observations are, therefore, consistent with our findings that human proinsulin has approximately 1% of the activity of insulin in adipocytes in vitro. However, it was recently reported that in vivo, human proinsulin has approximately 7% of the activity of insulin.[4] In vivo data, obviously, reflect more than just interaction with a homogeneous cell type. Proinsulin has a prolonged half-life as compared with insulin, reflecting a slower clearance rate and decreased susceptibility to degradation by insulin-degrading enzymes.[1] In addition, proinsulin may have differential effects in different tissues. The suggestion has been made that proinsulin has a greater effect on liver than on peripheral tissue,[4] which is supported by our finding of threefold greater binding of proinsulin to hepatocytes as compared with adipocytes. In addition, very little is known about proinsulin interaction with muscle, the major nonhepatic insulin-sensitive tissue.

Interactions between insulin and proinsulin could also exist at the tissue level. Schlüter et al.[16] recently reported, in abstract form, that proinsulin potentiated the activity of insulin. In the present study, however, we could find no significant potentiation of insulin action by proinsulin and found that the two hormones produced additive effects at submaximal con-

centrations, in agreement with several previous studies. We also found no prolongation of the effect of proinsulin on the adipocyte as compared with insulin and no differences in the onset of action of the two hormones. Thus, once bound to the receptor, both proinsulin and insulin appear to have similar effects on adipocytes with the markedly reduced biologic activity of proinsulin, as compared with insulin, due to a decreased affinity for the insulin receptor.

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