Rapid Publications

Early Detection of Degraded A_{14}-^{125}I-Insulin in Human Fibroblasts by the Use of High Performance Liquid Chromatography

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SUMMARY

We studied the metabolism of A_{14}-^{125}I-insulin in intact human fibroblasts using high performance liquid chromatography (HPLC) to detect and separate its early degradation products. The high resolving power of HPLC enabled us to separate what has been considered "intact insulin" by Sephadex G-50 chromatography or TCA precipitability into two additional peaks that had decreased biochemical properties with respect to immunoprecipitability and receptor binding but not decreased TCA precipitability. We conclude that human fibroblast is capable of metabolizing insulin within 2 min at 37°C into intermediate molecules that can be detected by HPLC but not by TCA precipitability or molecular sieve chromatography. DIABETES 32:474–477, May 1983.

Biologically active ^{125}I-insulin is bound to receptors and internalized by endocytosis in insulin-sensitive tissues.[1–3] Subsequent to binding, cellular processing of the hormone occurs with the production of low-molecular-weight, TCA-soluble material.[4–6] The majority of studies have used molecular sieve chromatography to examine the products. Earlier studies from our laboratories have shown that with purified insulin protease from skeletal muscle of rat and man, incubation of ^{125}I-insulin with enzyme results in material that is indistinguishable from the "insulin peak" on molecular sieve chromatography.[7,8] However, these incubation products have decreased immunoprecipitability and binding to insulin receptors compared with the native insulin. Studies of insulin processing in intact human target tissues are limited and, when available, have used either TCA precipitability and/or molecular sieve chromatography to assess the intactness of insulin. Therefore, we used intact human fibroblasts to study cell-associated insulin degradation using not only TCA precipitability and molecular sieve chromatography but also a more sensitive method of separation by high performance liquid chromatography (HPLC) to isolate the intermediate products for studying the biochemical properties in regard to TCA precipitability, immunoprecipitability, and liver membrane binding. Our studies indicate that neither TCA precipitability nor molecular sieve chromatography is sensitive enough to detect early product formation of cell-associated insulin degradation. We found HPLC to be the most useful tool to separate the products of initial insulin degradation in intact human fibroblasts.

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MATERIALS

Fetal bovine serum, trypsin, and fungizone were purchased from Grand Island Biologicals (Grand Island, New York). Eagles Minimal Essential Medium (MEM) was obtained from Flow Laboratories (McLean, Virginia). Guinea pig antiserum to insulin and rabbit anti-guinea pig serum were purchased from Cappell (West Chester, Pennsylvania). Urea obtained from Malinkrodt (St. Louis, Missouri) was purified by passing a 9 M solution through a Rexyn 1-300 column (Fisher Scientific Products, Fairlawn, New Jersey) before being diluted to 6 M urea for use. All other reagents were obtained as described in our previous paper.[9]

METHODS

Insulin was iodinated by a chloramine T method as modified by the authors and purified to carrier-free monoiodinated A_{14} insulin with specific activity of 360 μCi/μg by purification on HPLC.[9]

Human diploid fibroblast cultures were established from human foreskins as described by Mott et al.[10] Stock cultures of human fibroblasts were maintained in MEM and supplemented with nonessential amino acids (1% vol/vol), bovine fetal serum (10% vol/vol) and 10 mM HEPES in 75-cm^2 cul-

ture flasks containing 15 ml of growth medium. Cells were used between the fifth and twelfth passages upon reaching confluent monolayers and 2 days after the last addition of fresh MEM.

The monolayers of 6.0×10^6 cells/flask were washed three times with 10 ml of Dulbecco's phosphate buffered saline, pH 7.6, at 4°C to remove the growth medium. For the binding and degradation studies, 5 ml of HEPES buffer (50 mM HEPES, 90 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, 10 mg/ml BSA, pH 7.6) containing 2×10^6 cpm A_{14}-^{125}I-insulin (10^{-10} M) was added to the flasks and incubated at 4°, 22°, and 37°C for various time periods. The incubation solution was poured off and saved to measure insulin degradation in the medium by TCA precipitability using 1-ml aliquots with 1 ml of 10% TCA. For study of cell-associated binding and degradation, the flasks were then rinsed three times with 10 ml HEPES buffer at 4°C and 3 ml of 5% TCA was added to each flask. The cells were scraped off the flask and pulled into a 5-ml syringe with a 20-gauge needle to break up the cells before placing the material in 12×75 tubes containing 0.1 ml of 9% dialyzed bovine albumin. The tubes were vortexed, then centrifuged at $400 \times g$ for 15 min. The supernatant was poured into another tube and the supernatant and precipitate were counted in a gamma counter. Total counts were taken as an indication of insulin binding to the cells and percent degradation was calculated by measurement of cpm in the TCA-soluble portion over the total count. Leakage of insulin-degrading enzyme in the fibroblasts was evaluated by incubating HEPES buffer without A_{14}-^{125}I-insulin with the fibroblasts for the various times at temperatures of 4°, 22°, and 37°C. The buffer was then removed from the cells and the A_{14}-^{125}I-insulin was added to this buffer and incubated again at the same corresponding time and temperature. The measurement of TCA-solubility was calculated as described above.

To obtain the highest amount of binding without degradation of A_{14}-^{125}I-insulin, preliminary experiments were performed at different times and temperatures. These experiments showed the best conditions for the most binding with minimal degradation were 1 h at 4°C in HEPES pH 7.6; therefore, all subsequent degradation studies were carried out after labeled insulin (2×10^6 cpm) was first incubated in the fibroblast culture dish for 1 h at 4°C. The buffer was poured off and the cells were rinsed three times with HEPES buffer at 4°C without any radioactivity. Buffer at 37°C was then added to the flasks and the flasks were incubated at 0, 2, 5, 10, 15, 30, and 60 min at 37°C. When TCA solubility was used as an index of degradation, at the end of each time period the buffer was removed, the cells were washed again three times with buffer at 4°C, 3 ml of 10% TCA was added, and degradation was calculated as before.

For determination of degradation products by chromatography, the A_{14}-^{125}I-insulin that was cell associated at the end of the various time and temperature incubations was extracted from the cells using either (1) 3 ml of 0.1% TFA (for HPLC study) or (2) 1 M acetic acid with 6 M urea and 0.1% Triton X-100 (for molecular sieve chromatography). Evaluation of incubation products by Sephadex G-50 at various times was performed by eluation of labeled molecules on superfine columns (0.9×60 cm) with 1 M acetic acid, 6 M urea, and 0.1% Triton X-100.

For HPLC the TFA-solubilized material was injected on an HPLC with two Waters μC_{18} Bondapak columns in line with a precolumn filter of the same packing material and eluted isocratically with 0.1% TFA:acetonitrile (66:34 vol/vol). One-milliliter fractions were collected and counted for radioactivity. Each fraction was then checked for TCA precipitability, immunoprecipitability using excess anti-insulin antibodies (1:1000 dilution first antibody), and binding to liver membranes.[11]

RESULTS

Figure 1A shows the percent binding of A_{14}-^{125}I-insulin to human fibroblasts with time at 4°, 22°, and 37°C without

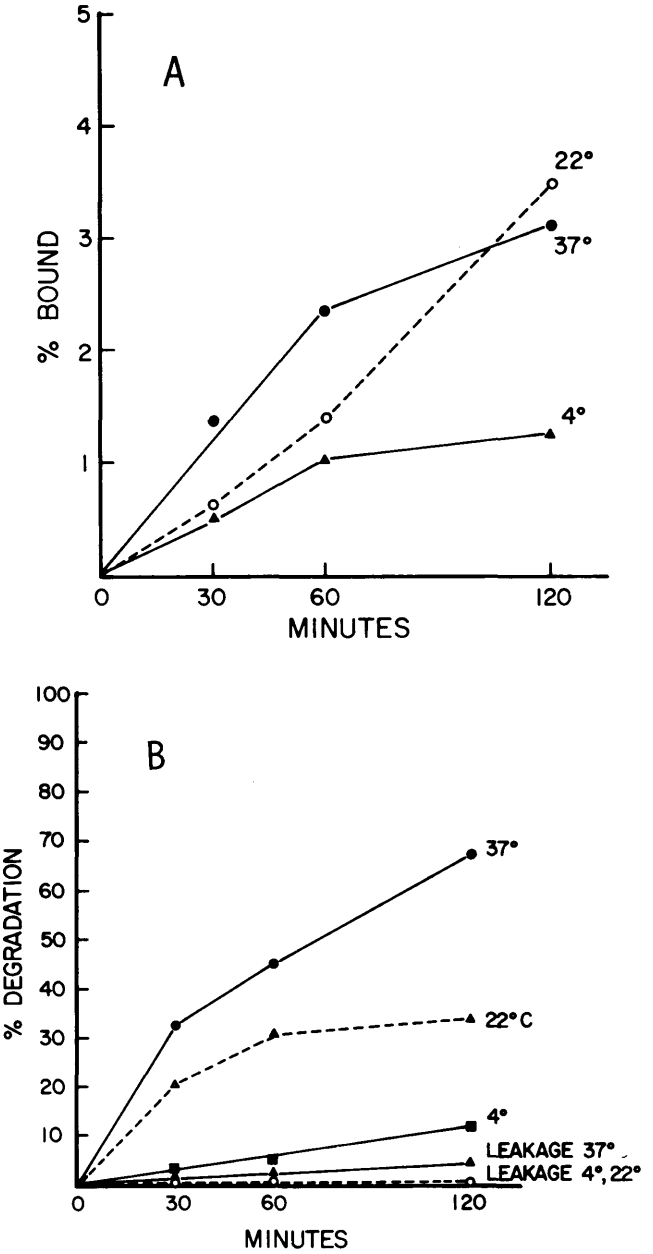

FIGURE 1. Effect of time and temperature on A_{14}-^{125}I-insulin binding (A) and degradation (B) to human fibroblasts. The data are representative values of five experiments each from duplicate flasks.

preincubation. Figure 1B shows the percent degradation as well as leakage of enzymes for the corresponding times. The binding at 37°C was approximately 2½ times that at 4°C; however, the degradation was almost 10 times more at 37°C at 1-h incubation. The binding at 22°C was only slightly more than at 4°C but the degradation was about six times greater. Although the binding at 4°C was less than at the other temperatures, the degradation was considerably less and, more importantly, there was no leakage of any degrading enzymes from the cells. As can be seen, binding was affected by temperature, but 4°C appeared to provide a more constant level of binding from 60–120 min. Hence, incubation at 4°C was taken as the optimal condition to preincubate labeled insulin with fibroblasts for study of cell-associated binding and degradation.

To follow the products of degradation at various times the solubilized incubations were chromatographed on a Sephadex G-50 column. Figure 2 shows the elution profile of the 0-min and 10-min incubations of the fibroblasts with A_{14}-^{125}I-insulin. One major peak (#2), which eluted at the A_{14}-^{125}I-insulin position, can be seen at the 0 time. At 10 min another peak (#3) appeared that eluted in the iodotyrosine range with a decrease in peak #2 and a slight skewing of the peak.

The biochemical properties of this "insulin peak" from the Sephadex G-50 column were measured with respect to TCA precipitability, immunoprecipitability, and liver membrane binding at 0, 2, 5, and 30 min as seen in Table 1. At no time was the "insulin peak" TCA soluble; however, immunoprecipitability of the "insulin peak" decreased from 93% maximum at 0 min to 66% at 30 min. The liver membrane binding results were even more dramatic, showing "insulin peaks" to bind to liver membrane from 14% at time 0 to 3% at 30 min.

TABLE 1
Biochemical properties of "insulin peak" from Sephadex G-50 chromatography

	0 min	2 min	5 min	30 min
TCA precipitability	97%	97%	97%	97%
Immunoprecipitability	93%	86%	78%	66%
Liver membrane binding	14%	11%	8%	3%

For methods see text. The values are averages of duplicate flasks and are representative of four separate experiments.

In an effort to further analyze these products of degradation, the solubilized media from various time incubations were injected on the HPLC and peaks were identified as described in METHODS. Figure 3 shows the elution profiles of the product peaks at various incubation times. At time 0 the only peak (D) seen was the A_{14}-^{125}I-insulin with a retention time of 26 min. After incubation for 2 min, however, three more peaks were evident. Peak A, ^{125}I-tyrosine, eluted at 9 min; peak B at 14 min; and peak C at 19 min. With increasing incubation time there was a decrease in the height of the A_{14}-^{125}I-insulin (peak D) and a corresponding increase in the height of the ^{125}I-tyrosine peak (peak A). A most significant finding was the order of appearance of the two intermediate peaks, B and C. Although peak C showed its greatest height at 2 min, by 30 min this had reduced to less than 50% of its height with concomitant appearance of increased peak B at 5 min. This suggests that the order of the degradation process involves the orderly disappearance of intact insulin (peak D) with progression to intermediate production of peak C followed by B, and then appearance of the final degradation product—^{125}I-tyrosine.

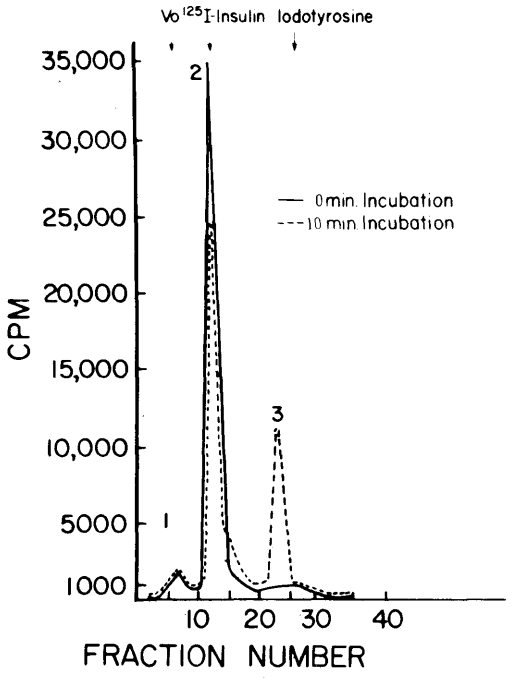

FIGURE 2. Sephadex G-50 eluation profile of cell-associated A_{14}-^{125}I-insulin with human fibroblasts at 0- and 10-min incubations at 37°C. See METHODS for details. The data are averages of four experiments from duplicate flasks.

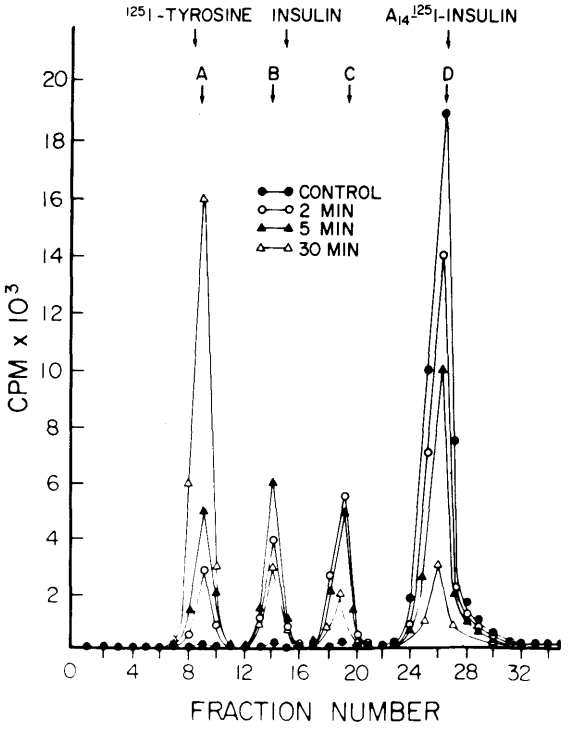

FIGURE 3. Chromatographic profile on HPLC of incubation products of cell-associated A_{14}-^{125}I-insulin with human fibroblasts at different incubation times. For details of studies, see METHODS. The data are representative of four experiments run in duplicate flasks.

TABLE 2
Biochemical properties of various HPLC fractions of insulin intermediates

Fraction	Control*					2 min*					5 min*					30 min*				
	A	B	C	D	Total	A	B	C	D	Total	A	B	C	D	Total	A	B	C	D	Total
TCA precipitability	—	—	—	97%	97%	3%	97%	97%	97%	87%	3%	97%	97%	97%	78%	3%	97%	97%	97%	36%
Immunoprecipitability	—	—	—	93%	93%	0%	63%	78%	93%	83%	0%	63%	78%	93%	71%	0%	63%	78%	93%	30%
Liver membrane binding	—	—	—	14%	14%	0%	3%	7%	14%	7%	0%	3%	7%	14%	4%	0%	3%	7%	14%	2%

*Results are expressed as percent TCA precipitability, immunoprecipitability, or binding according to the methods described in the text. The data are averages of duplicate flasks and representative of four experiments.

To ensure that HPLC-obtained chromatographic peaks were not artifacts of the methodology, peaks A, B, C, and D were combined and rechromatographed on the Sephadex G-50 column. Peaks B, C, and D chromatographed as the "insulin peak." Peak A eluted at fraction 24. Hence, the unresolved "insulin peak" on Sephadex was demonstrated to be actually three components.

The biochemical properties of these HPLC peaks were measured as to TCA precipitability, immunoprecipitability, and liver membrane binding as shown in Table 2. The total values refer to the incubation mixture before it was injected onto the HPLC. These values decreased in all three parameters with time from the initial 97% TCA precipitability, 93% immunoprecipitability, and 14% liver membrane binding, which was the same as peak D (A_{14}-^{125}I-insulin) at all times. Peaks B, C, and D, maintained TCA precipitability, but peak A (iodotyrosine) was not TCA precipitable. Peak C had decreased immunoprecipitability and liver membrane binding properties, with peak B showing even further decrease. Peak A was essentially devoid of all three physical and biochemical properties. Hence the "insulin peak" on Sephadex G-50 was actually composed of three components, two of which were altered molecules of A_{14}-^{125}I-insulin (peaks B and C).

DISCUSSION

We have shown that physiologic concentrations of biologically active insulin, when incubated with human fibroblasts, bind to cell membranes at 4°C without significant degradation. Furthermore, as there appears to be no leakage of insulin-degrading activity at 37°C, this constitutes cell-associated A_{14}-^{125}I-insulin interaction with membranes at physiologic temperature.

Our studies demonstrate that there is a stepwise alteration of the cell-associated insulin molecule as assessed by immunoprecipitability and receptor binding. That such alteration is the result of change in the physical properties of the molecule is further demonstrated by the elution pattern on HPLC. This suggests that interaction of A_{14}-^{125}I-insulin (peak D) with fibroblasts first results in production of peak C with properties intermediate between peak D and iodotyrosine with subsequent conversion of peak C to a less immunoprecipitable intermediate product (peak B) and final conversion to iodotyrosine. Neither of these two peaks (C or B) was detectable in the early periods of incubation if molecular sieve chromatography or TCA precipitability was chosen as the measure of insulin degradation.

In conclusion, we have shown that the human fibroblast is capable of cell-associated insulin degradation in a time- and temperature-dependent fashion with alteration of its molecular properties as early as 2 min. This can be detected by HPLC but not by the conventional methods of TCA precipitability or molecular sieve chromatography.

ACKNOWLEDGMENTS

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REFERENCES

[1] Carpentier, J. L., Gordon, P., Amherdt, M., VanObberghen, E., Kahn, R., and Orci, L.: ^{125}I-insulin binding to cultured human lymphocytes: initial localization and fate of hormone determined by quantitative electron microscopic autoradiography. J. Clin. Invest. 1978; 61:1057–70.

[2] Schlessinger, J., Shechter, Y., Willingham, M. C., and Pastan, I.: Direct visualization of the binding, aggregation, and internalization of insulin and epidermal growth factor on fibroblastic cells. Proc. Natl. Acad. Sci. USA 1978; 75:2659–68.

[3] Jarett, L., and Smith, R. M.: Ultrastructural localization of insulin receptors on adipocytes. Proc. Natl. Acad. Sci. USA 1975; 72:3526–31.

[4] Olefsky, J. M., Kobayashi, M., and Chang, H.: Interactions between insulin and its receptors after the initial binding event. Functional heterogeneity and relationship to insulin degradation. Diabetes 1979; 28:460–71.

[5] Terris, S., and Steiner, D. F.: Binding and degradation of [^{125}I]-insulin by rat hepatocytes. J. Biol. Chem. 1975; 250:8389–98.

[6] Duckworth, W. C., and Kitabchi, A. E.: Insulin metabolism and degradation. Endocr. Rev. 1981; 2:210–33.

[7] Duckworth, W. C., Stentz, F. B., Heinemann, M., and Kitabchi, A. E.: Initial site of cleavage by insulin protease. Proc. Natl. Acad. Sci. USA 1979; 76:635–39.

[8] Neal, G. W., and Kitabchi, A. E.: Insulin degradation by human skeletal muscle. Biochim. Biophys. Acta 1982; 719:259–66.

[9] Stentz, F. B., Wright, R. K., and Kitabchi, A. E.: A rapid means of separating A_{14}-^{125}I-insulin from heterogeneously labeled insulin molecules for biologic studies. Diabetes 1982; 31:1128–31.

[10] Mott, D. M., Howard, B. V., and Bennett, P. H.: Stoichiometric binding and regulation of insulin receptors on human fibroblasts using physiological insulin levels. J. Biol. Chem. 1979; 254:8762–67.

[11] Swislocki, N. J., Tierney, J., and Essner, E. S.: Isolation and characterization of a liver plasma membrane fraction enriched in glucagon-sensitive adenylate cyclase. Arch. Biochem. Biophys. 1976; 174:291–97.