Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 163-170

THE EFFECT OF INHIBITORS OF INSULIN PROCESSING ON GENERATION OF INSULIN INTERMEDIATE PRODUCTS FROM HUMAN FIBROBLAST AS DETECTED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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Received February 27, 1985

Summary: To assess the role of various modulators of insulin processing on cell-associated A_{14}-^{125}I-insulin intermediates in human fibroblasts, we have studied the effect of N-ethylmaleimide (NEM), chloroquine, bacitracin, dansylcadavarine, and phenylarsine oxide on generation of these intermediate products with the use of HPLC. NEM completely inhibited generation of intermediate peaks or iodotyrosine. Chloroquine inhibited conversion of A_{14}-^{125}I-insulin to iodotyrosine by about 75 percent and the remaining A_{14}-^{125}I-insulin was not susceptible to acid wash. Bacitracin, dansylcadavarine, and phenylarsine oxide, on the other hand, stimulated formation of intermediate products with concomitant inhibition of iodotyrosine formation. We conclude that there are at least three components of insulin degradation in human fibroblasts. These include the sulfhydryl group inhibitor-sensitive, the intracellular chloroquine-sensitive, and membrane site inhibitor-sensitive components.

INTRODUCTION: Insulin elicits its biological effect through a series of events involving binding, internalization (endocytosis), and degradation of the hormone followed by exocytosis of the smaller molecular weight products (1-4).

Cell-mediated insulin degradation has been demonstrated in all insulin-sensitive mammalian tissues (For review, see 5). Although processing of insulin, including internalization and degradation, has been proposed by some to be related to mechanism of action of the hormone, (6, 7), unanimous agreement as to the role of insulin degradation in its biological action is lacking. Although TCA solubility is the most often used method for assessment of insulin degradation, and can detect total degradation of insulin, it suffers from lack of sensitivity and specificity, especially when used to assess degradation of physiologic concentration of the hormone in the early events of insulin degradation.

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0006-291X/85 $1.50

We have recently shown that intermediate products of insulin degradation are formed from cell-associated A_{14}-^{125}I-insulin in intact human fibroblasts in the early events of insulin processing. These intermediates consist of at least two peaks which can be detected by high performance liquid chromatography (HPLC), but not by either TCA solubility or molecular sieve chromatography (Sephadex G-50) method (8). Since many studies dealing with the inhibitors of insulin processing and their action on isolated cells had previously used TCA solubility for degradation studies, we conducted a series of experiments to investigate the role of these inhibitors in generation of these newly-described intermediate products. Our studies described herein present hitherto unknown effects of major inhibitors of insulin processing on generation of these intermediates detected by HPLC. They provide some evidence for the existence of at least three components of cell-associated insulin degradation. They further suggest that the use of TCA solubility or molecular sieve chromatography for assessment of intracellular insulin degradation in the presence of inhibitors of insulin processing should be interpreted with caution.

MATERIALS AND METHODS

Materials

Purified pork insulin was kindly provided by Dr. Ronald Chance of Eli Lilly Company (Indianapolis, IN). Insulin was iodinated by a chloramine T method as described previously and purified to carrier-free monoiodinated A_{14}-^{125}I-insulin with specific activity of 360 μCi/μg by purification on HPLC (9). All materials for fibroblast tissue cultures and other reagents were obtained from commercial sources as described elsewhere (8-10).

Methods

Fibroblast tissue cultures were established from human foreskin as described by Howard et al (11), and were maintained in Eagle's MEM, supplemented with nonessential amino acids, 10% fetal calf serum (v/v) and HEPES buffer (10mM) in enclosed vessels 75 cm^2 (250 ml vol). Cells were obtained from cultures between the 8th and 12th passages upon reaching confluent monolayers and two days after the last addition of fresh MEM.

The monolayers of 6.0 x 10^6 cells/flask were washed three times with 10 ml of Dulbecco's phosphate buffered saline (PBS), pH 7.6, at 4°C to remove the growth medium. The procedures for study of binding and degradation of A_{14}-^{125}I-insulin were similar to those described previously (8-10).

For the study of the effect of inhibitors of insulin degrading activity (IDA) in intact fibroblasts, various inhibitors when added were first preincubated for one hour at 37°C, then the inhibitors were incorporated at the same time that A_{14}-^{125}I-insulin was incubated with the fibroblasts at 4° C for one hour. At the end of one hour, the buffer was poured off and the cells were washed three times in 10 ml HEPES buffer at 4° C. 5 ml of buffer plus inhibitors at 37° was added to the cells and incubated for the various times.

The methods for determination of degradation products of A_{14}-^{125}I-insulin by TCA solubility or HPLC were identical to those described previously (8-10).

RESULTS

A. Effect of Inhibitors on Generation of Insulin Intermediate Products

Figure 1 demonstrates the effect of various inhibitors on generation of two intermediate products (Peaks B & C), intact insulin (Peak D), and iodotyrosine

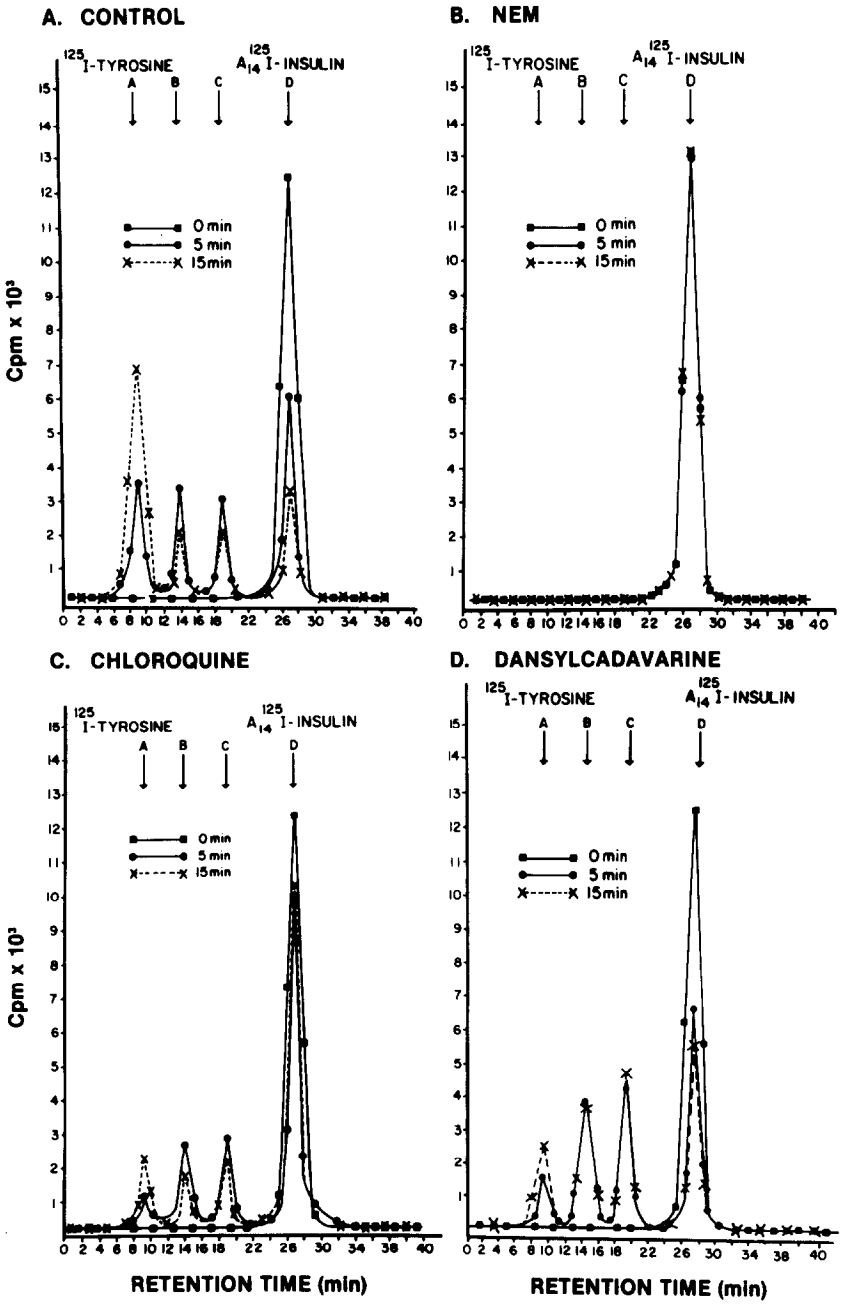

Figure 1. Effect of various inhibitors of insulin processing on formation of HPLC-detected peaks from incubation products of cultured human fibroblast and A_{14}-^{125}I-insulin.

(Peak A) at 5 and 15 minutes, as compared to the control without the presence of inhibitors. TCA solubility measurements (CPM) in all the above situations were similar to the iodotyrosine values detected by HPLC (See Below). Thus TCA solubility was not capable of detecting Peaks B & C. In the control experiment (Fig. 1A), there was a time-dependent conversion of A_{14}-^{125}I-insulin to intermediate, Peaks B & C, and generation of ^{125}-I tyrosine (Peak A). The latter value was 25% at 5 minutes and 53% at 15 minutes. TCA solubility as an indicator of insulin degradation at the corresponding times was 22% and 51% respectively.

Figure 1B demonstrates the profile of the products in the presence of 1mM NEM. As can be seen, NEM completely inhibited conversion of A_{14}-^{125}I-insulin to intermediates and iodotyrosine as formation of these compounds was undetectable on HPLC.

Figure 1C and Table 1 depict the HPLC profile of chloroquine-treated cells, and demonstrate two interesting phenomena: (1) formation of iodotyrosine was inhibited by a greater degree (74-78%) than formation of intermediate products (<10%); and (2) accummulated A_{14}-^{125}I-insulin was not susceptible to acid wash, indicating that intracellular transport of intact insulin preceded inhibition of its conversion to intermediate products and iodotyrosine.

Figure 1D demonstrates cell-associated insulin degradation in the presence of dansylcadavarine. Unlike chloroquine, dansylcadavarine did not inhibit intermediate product formation. In fact, it enhanced the accumulation of intermediates, but it inhibited formation of iodotyrosine. Similar results were also obtained with bacitracin and phenylarsine oxide (Table 1). Also, unlike chloroquine, with the use of these latter inhibitors, the unreactive intact insulin was about 78 percent dissociated by acid wash at five minutes and 51 percent at 15 minutes.

B. Measurement of Products of Incubation Medium in the Presence of Inhibitors

In order to evaluate the nature of the material exocytosed from intact cells, the extract of incubation medium in the presence and absence of various inhibitors was placed on HPLC. The only radioactive component detected on HPLC

TABLE 1

EFFECT OF VARIOUS INHIBITORS ON INSULIN INTERMEDIATES FORMED IN THE DEGRADATION PROCESS IN INTACT FIBROBLASTS*

	HPLC PEAKS				
	A_{14}-^{125}I-Insulin (D)	(C)	(B)	Iodotyrosine (A)	%AD†
5 Minutes					
Control	37.4 ± .62	19.3 ± .53	17.7 ± .60	25.6 ± .57	6
NEM	100 ± .01	0	0	0	
Bacitracin (1mg/ml)	44.1 ± .72	25.2 ± .63	25.1 ± .66	5.6 ± .51	78
Dansylcadavarine (.5mM)	41.3 ± .68	25.5 ± .71	25.0 ± .69	9.2 ± 2.47	76
Chloroquine (.5mM)	59.8 ± .73	17.3 ± .55	17.2 ± .61	5.7 ± .51	78
Phenylarsineoxide (1uM)	39.8 ± .94	26.2 ± .91	24.5 ± .96	9.5 ± .93	
Bacitracin (1mg/ml) + Dansylcadavarine (.5mM)	46.8 ± .78	23.9 ± .62	23.8 ± .64	5.5 ± .58	
Bacitracin (1mg/ml) + Chloroquine (.5mM)	61.5 ± .83	17.6 ± .75	15.1 ± .69	5.8 ± .61	
15 Minutes					
Control	21.1 ± .58	12.9 ± .53	12.8 ± .59	53.2 ± .85	1.8
NEM (1mM)	100.0 ± .03	0	0	0	
Bacitracin (1mg/ml)	38.0 ± .57	25.8 ± .61	22.1 ± .59	14.1 ± .51	55
Dansylcadavarine (.5mM)	32.3 ± .66	26.8 ± .69	23.9 ± .71	17.0 ± .63	51
Chloroquine (.5mM)	57.9 ± .86	14.1 ± .77	13.7 ± .72	14.3 ± .69	3
Phenylarsineoxide (1uM)	34.9 ± 1.07	26.7 ± .98	20.2 ± .96	18.2 ± .99	
Bacitracin (1mg/ml) + Dansylcadavarine (.5mM)	37.1 ± .72	24.9 ± .75	21.7 ± .77	16.3 ± .68	
Bacitracin (1mg/ml) + Chloroquine (.5mM)	58.7 ± .88	18.3 ± .86	9.8 ± .73	13.2 ± .81	

*Values are reported as percent of total radioactivity and are mean ± SEM of three separate experiments run in triplicate. For details of experiments, see Methods.

† Percent Acid Dissociable

was ^{125}I-tyrosine. No intermediate product B or C was detected from the incubation media of the control or inhibitor-treated cells at any time period.

C. The Effect of Combination of Inhibitors on Intermediate Product Formation

The effects of combination of chloroquine and bacitracin or bacitracin and dansylcadaverine were investigated at 5 minutes and 15 minutes. The results in Table 1 demonstrate that the effect of chloroquine plus bacitracin was the same as chloroquine alone. Furthermore, the effects of bacitracin and dansylcadavarine were not additive.

Although there were some quantitative changes between membrane-sensitive inhibitors, dansylcadavarine, bacitracin or phenylarsine oxide,

the qualitative changes were the same between all these three compounds and resulted in accumulation of intermediate products with significant inhibition in formation of iodotyrosine.

DISCUSSION

The present studies demonstrate that with the use of HPLC, the well-known inhibitors of insulin processing can serve as useful metabolic probes to dissect the cellular events underlying cell-associated insulin degradation product formation in intact human fibroblasts, which hitherto had not been possible with the use of TCA solubility or molecular sieve chromatography.

Our studies with 1mM NEM clearly indicate that this sulfhydryl group inhibitor totally prevents the conversion of radioactive insulin to intermediate products and iodotyrosine. The unreactive radioactive insulin has the same HPLC profile as A_{14}-^{125}I-insulin (Figure 1B).

The studies with chloroquine demonstrate that this agent inhibits conversion of A_{14}-^{125}I-insulin to iodotyrosine (but not to insulin intermediates) with concomitant accumulation of intact A_{14}-^{125}I-insulin, which is not susceptible to acid wash. This suggests intracellular localization of chloroquine for its inhibitory effect on the formation of iodotyrosine from intact insulin.

The studies with bacitracin, dansylcadavrine, and phenylarsine oxide are of interest as (a) all three compounds produced qualitatively similar HPLC profiles; (b) these compounds at the concentration used stimulated formation of intermediate products; and (c) all these compounds inhibited iodotyrosine formation. Furthermore, the remaining intact A_{14}-^{125}I-insulin was 78 percent acid washable at five minutes and 51 percent acid washable at 15 minutes, suggesting presence of intact insulin molecule at the cell membrane site. As bacitracin, dansylcadavarine, and phenylarsine oxide are known to act at cell membrane site (12), transglutamidase (13) or endocytosis (14, 15) respectively, this information taken together with the result of our study suggests that the major action of these compounds on cell-associated insulin degradation is at the cell membrane site. Increased formation of intermediate products by these

compounds could be the result of increased conversion of A_{14}-^{125}I-insulin to intermediate products or, more likely, inhibition of intermediate product conversion to iodotyrosine. Since recent work from this laboratory has shown that the major enzyme responsible for degradation of insulin in subcellular fractions of human fibroblast is similar to intact human fibroblast and consists of cytosolar neutral protease (10), it is plausible to assume that the above membrane-sensitive inhibitors may act at the cytoplasmic side of membrane where insulin protease is presumably located (5). Based on the above information and the work in the literature, we hypothesize that there are at least three components of insulin degradation in intact human fibroblast as follows:

1. A sulfhydryl group inhibitor-sensitive component where total inhibition of insulin degradation occurs with complete inhibition of the formation of insulin intermediates and iodotyrosine;

2. A chloroquine-sensitive component which is located intracellularly and results in inhibition of iodotyrosine but not intermediate products formation, and

3. A cell membrane inhibitor-sensitive component which brings about accummulation of intermediate products and inhibition of iodotyrosine.

Clearly, more detailed studies on dose response curves of these inhibitors in intact cells and purified insulin protease and on chemical identification of the products are needed before the mechanism of insulin degradation in these cells can be fully elucidated.

Preliminary work from this laboratory has suggested a possible role of insulin intermediate products on activation of pyruvate dehydrogenase complex (PDC) on rat heart mitochondria (16). It would, therefore, be of interest to study the effect of inhibitors of insulin processing on PDC activity of intact human fibroblasts in the presence and absence of insulin.

ACKNOWLEDGMENTS

The authors are grateful to Mary Ann Roberts for technical assistance and to Bettye J. Hammack for secretarial support. This work was supported by USPHS training grant AM07088 and Clinical Research Center grant RR00211.

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