Inhibition of Insulin Degradation by Nonsuppressible Insulin-Like Activity

GEORGE A. BURGHEN, WILLIAM C. DUCKWORTH, ABBAS E. KITABCHI, SOLOMON S. SOLOMON, and PHILLIP L. POFFENBARGER

From the Departments of Pediatrics, Medicine, and Biochemistry, University of Tennessee Center for the Health Sciences, and Laboratories of Endocrinology and Metabolism Research Service, Veterans' Administration Hospital, Memphis, Tennessee 38163, and Department of Medicine, University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT Nonsuppressible insulin-like activity, provided by three sources, was evaluated for its effect on the proteolytic degradation of insulin utilizing insulin protease obtained from rat liver homogenate as well as liver cell membranes. All three preparations of nonsuppressible insulin-like activity were found to be competitive inhibitors of insulin degradation.

In addition human plasma was fractionated yielding an acetone precipitate which was found to have nonsuppressible insulin-like activity and to be a competitive inhibitor of insulin protease.

INTRODUCTION

Nonsuppressible insulin-like activity (NSILA) obtained from pork plasma has been demonstrated to inhibit the proteolytic degradation of insulin by liver insulin protease (1–3). The mechanism of inhibition was found to be competitive in nature. It was further shown that human plasma contained a fraction which possessed NSILA and also inhibited insulin degradation in a competitive manner (1–3). We have now shown that two additional preparations of NSILA extracted from human serum or plasma competitively inhibit insulin degradation by a partially purified insulin degrading enzyme from liver. Furthermore, these preparations of NSILA also competitively inhibit insulin degradation by liver cell membranes.

METHODS

Chemicals. NSILA preparations were obtained from three sources. The first was extracted from porcine serum by Solomon et al. by a modification (4) of the method of Antoniades (5). The second was prepared from human serum by Burgi et al. (6) and has a mol wt of about 7,500. The third preparation was prepared from human plasma by the method of Poffenbarger (7) and has a mol wt of about 90,000.

Porcine insulin was the generous gift of Dr. Ronald Chance of Eli, Lilly and Co., Indianapolis, Ind. Radioiodinated ^{125}I-insulin containing less than 1 g atom of iodine/mol of insulin was obtained from Nuclear International Corp., Burlington, Mass. Bovine serum albumin, (fraction V) was obtained from Armour Pharmaceutical Co., Chicago, Ill. All other chemicals, analytical reagent grade, were obtained from commercial sources.

Preparation of plasma inhibitor. Pooled plasma from normal fasted subjects was dialyzed. After dialysis, sufficient acetone at 0°C was added to produce an 80% solution of acetone. The acetone precipitate formed was lyophilized, and the dry precipitate was dissolved in a volume of distilled deionized water equal to the original plasma volume. This acetone precipitate was dialyzed three times for a total of 20 h against 20 vol of distilled deionized water, then tested for its ability to inhibit insulin degradation by the $Ca_3(PO_4)_2$ gel purified insulin protease. The insulin-like biological activity was also assayed (4).

Enzymatic assay. $Ca_3(PO_4)_2$ gel purified rat liver insulin protease was prepared by a previously described method (8). The trichloracetic acid (TCA) method (8) of determining insulin protease activity was utilized. The incubation mixture, with a total vol of 0.5 ml, containing 0.1 M Tris-HCl buffer, pH 7.6, 0.005 M EDTA, 0.3 g/dl bovine serum albumin, 0.1 nM ^{125}I-insulin, and $Ca_3(PO_4)_2$ gel puri-

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TABLE I

Insulin-like Activity and Inhibitory Activity of Various Preparations of NSILA

	μU Insulin-like activity/mg protein	% Enzyme inhibition/mg protein	Ratio of insulin-like activity to inhibitory activity
Plasma	1.3	4.9	3.77
Plasma Extract*	10.7	30	2.80
NSILA—Ensinck‡	150	577	3.85
NSILA-s—Froesch*	1,000	2,229	2.23
NSILA, large—Poffenbarger*	4,300	1,900	0.44

* These preparations contained no detectable insulin or proinsulin by immunoassay.

‡ The insulin in this preparation was 11.0 μU per incubation mixture as determined by immunoassay.

fied insulin protease was incubated 15 min at 37°C. After incubation the reaction was terminated by adding 1.5 ml of 8% TCA. The percentage of insulin degraded was determined as the net amount of radioactive material produced which was not precipitable with 6% TCA.

NSILA. Pooled human plasma and an acetone precipitable extract from this plasma were evaluated for NSILA using the isolated fat cell system (4, 9). Activity is expressed as nanoatoms of [U^{14}C]glucose conversion to $^{14}CO_2$. Microunits of insulin activity were determined from insulin standards.

Membrane preparation and assay. Liver cell membranes were prepared by the method of Neville (10) through Step 11 and stored in liquid nitrogen. Insulin degradation by membranes was assayed by incubation of ^{125}I-insulin, membranes in the specified concentration, and other additions as indicated in 0.1 M Tris-Ringer bovine serum albumin, pH 7.6. After 30 min at 37°C the membranes were sedimented by centrifugation in a Beckman microfuge. Portions of the supernate were transferred to the tubes containing 0.05 M Tris-HCl, pH 7.0, with 0.5% bovine serum albumin, and the degradation determined by the use of TCA as described previously (8, 11).

RESULTS

A comparison of the insulin-like activity in microunits per milligram protein with the insulin degrading inhibitory activity expressed as percent inhibition per milligram protein of the five plasma preparations containing NSILA are shown in Table I. With increase in insulin-like activity there tends to be an increase in enzyme inhibitory activity, except for the large NSILA of Poffenbarger which demonstrates relatively greater biological activity than enzyme inhibitory activity. Very small amounts of insulin and proinsulin were detectable in these preparations (Table I). These amounts added to the incubation mixture did not produce detectable inhibition of ^{125}I-insulin degradation.

Lineweaver-Burk enzyme kinetic studies showed inhibition to be consistent with a competitive type of inhibition for all three NSILA preparations as seen in Fig. 1. The K_m for insulin degradation by the enzyme was found to be 0.11 μM. The apparent K_i's for the NSILA inhibitors are as follows: NSILA-Ensinck 76 μg/ml, NSILA-Froesch 6.0 μg/ml, and NSILA-Poffenbarger 9.2 μg/ml. Human plasma extracts (see Table I) were found to contain inhibitor(s) of insulin protease which were shown by kinetic studies to be competitive in type (data not shown).

Fig. 2 shows the inhibitory effects of NSILA-s and the large NSILA of Poffenbarger on insulin degradation by partially purified liver cell membrane preparation to be competitive in nature. The apparent K_m was 3×10^{-7} M, similar to previous studies (12).

DISCUSSION

The material in plasma which has insulin-like activity and is not suppressible by anti-insulin antibody is heterogeneous. Depending upon the conditions used to prepare and to assay the material, several different components have been described. Of these, the best characterized is the NSILA-s fraction studied extensively by Burgi et al. and Oelz et al. (6, 13). This is a peptide with a mol wt of approximately 7,500, which has been extensively purified and characterized (13). Another apparently different NSILA is a large (90,000) peptide isolated and characterized by Poffenbarger (7). This material has been purified to one band on polyacrylamide gel and does not appear to be an aggregate or a smaller molecule attached to a carrier.

Insulin protease which has been demonstrated in most tissues (14) has now been highly purified in muscle (11) and kidney (15), and appears to be proteolytic in nature (16). This enzyme is inhibited by plasma (17), and proinsulin intermediates (18) but not by a number of other peptides and proteins which have been examined (8, 15).

Previous work from this laboratory had shown that the inhibition of proteolytic degradation of insulin by

plasma could be attributed to a fraction which contained NSILA (1, 3). This prompted us to examine the effects of various preparations of NSILA on insulin degradation by the liver. A crude plasma NSILA extract and

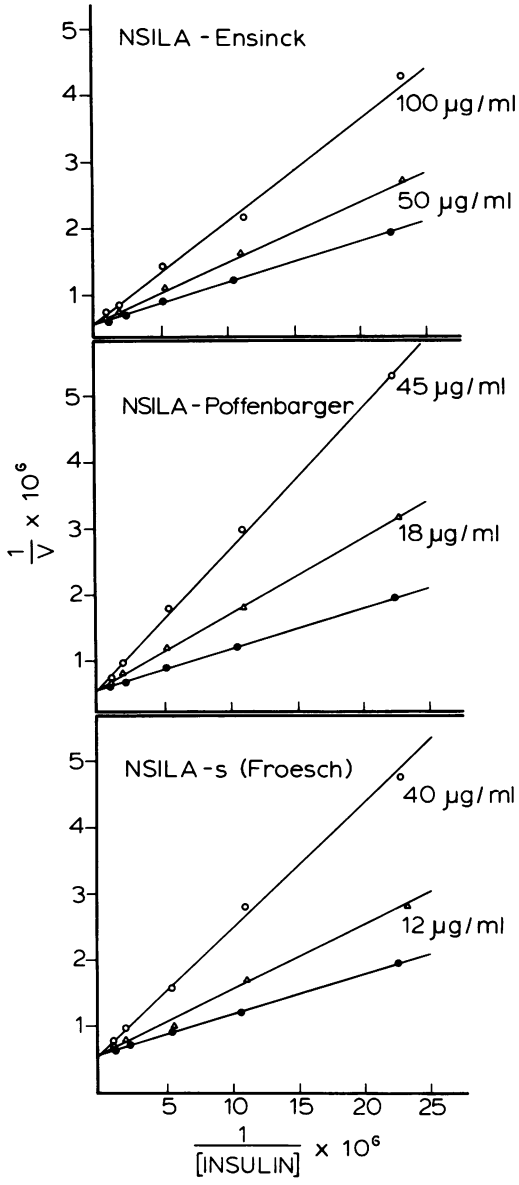

FIGURE 1 Reciprocal plot of insulin protease with insulin as substrate in the presence and absence of three NSILA preparations. The incubation system contained 0.1 M Tris-HCl buffer, pH 7.6, 0.005 M EDTA, 0.3% bovine serum albumin, 0.1 nM ^{125}I-insulin, 15 µg of $Ca_3(PO_4)_2$ gel purified insulin protease in each tube, and varying amount of unlabeled pork insulin in a total volume of 0.5 ml. The concentration of insulin ranged from 5×10^{-8} M to 1×10^{-6} M. Samples were incubated for 15 min at 37°C. The reaction was stopped with 1.5 ml of 8% TCA. The insulin concentration is expressed as moles and velocity as moles of insulin degraded per minute per milliliter enzyme.

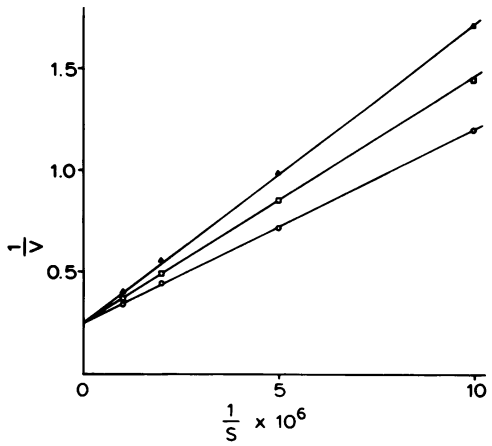

FIGURE 2 Reciprocal plot of insulin degradation by liver cell membranes in the presence and absence of two different preparations of NSILA. The procedure was as described in text. The values represented by circles were obtained in the absence of NSILA. The squares represent insulin degradation in the presence of NSILA-s (Froesch), 16 µg/ml. The triangles represent insulin degradation in the presence of the large molecular weight NSILA (Poffenbarger), 45 µg/ml. Velocity is expressed as picomoles insulin degraded/0.07 mg membrane protein/30 min.

partially purified preparations of both NSILA-s and the larger NSILA of Poffenbarger were tested. All preparations tested were competitive inhibitors of insulin degradation by the liver insulin protease. The inhibitory potency correlated well with the NSILA content except for Poffenbarger's NSILA which was shown to have relatively more insulin-like activity than enzyme inhibitory activity; this may be due to structural differences in the NSILA preparations. Since purification of these NSILA preparations are not comparable one cannot, with certainty, compare these preparations on a molar basis. It should be recognized, however, that the apparent molecular weight of NSILA-Poffenbarger is considerably greater than the other two preparations. The partially purified NSILA preparations were also competitive inhibitors of insulin degradation by liver cell membranes. Preliminary evidence suggests that a highly purified enzyme preparation does not degrade NSILA but further studies will be required to confirm this observation.

The concentration of NSILA in normal plasma is approximately 100-230 µU/ml (13, 19). This is equivalent to 100 µg/ml of our preparation of NSILA-s or to 25 µg/ml of the large NSILA. In either case, significant inhibition of insulin degradation by the liver could be produced by these concentrations of NSILA. Thus it is clear that the plasma concentration of NSILA could affect the rate of insulin degradation and that this could be a control mechanism for insulin metabolism. Additional studies are needed to clarify the relationship of

NSILA to insulin degradation under a variety of conditions when concentrations of NSILA are altered.

Determination of proinsulin using insulin protease (17) could conceivably be affected by plasma inhibition of this enzyme. Although this does not affect proinsulin determination in postprandial blood samples from normal and diabetic subjects (20, 21), fasting samples may show falsely elevated proinsulin values when insulin protease is used for the assay (21–23).

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