INSULIN AND NON-SUPPRESSIBLE INSULIN-LIKE ACTIVITY (NSILA-s): EVIDENCE FOR SEPARATE PLASMA MEMBRANE RECEPTOR SITES

K. Megyesi[+1], C. R. Kahn[+], J. Roth[+], E. R. Froesch[‡], R. E. Humbel[‡], J. Zapf[‡], and D. M. Neville, Jr.[o]

[+]Diabetes Section, Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health Bethesda, Maryland

[‡]Department of Medicine and Institute of Biochemistry, University of Zurich, Zurich, Switzerland

[o]Section of Biophysical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

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SUMMARY: The soluble fraction of the non-suppressible insulin-like activity of plasma (NSILA-s) is a purified serum peptide with insulin-like bioactivity but not immunoreactivity. A preparation of NSILA-s with 0.3 percent the bioactivity of insulin inhibited the binding of ^{125}I-insulin to lymphocytes, adipocytes and liver membranes and also inhibited ^{125}I-NSILA-s binding to lymphocytes in direct proportion to its bioactivity. In contrast, NSILA-s was 300,000 times more potent than insulin and 1,000 times more potent than proinsulin in inhibiting binding of ^{125}I-NSILA-s to liver membranes. Thus, it appears that, in liver, insulin and NSILA-s bind to two types of receptors with markedly different affinities.

Insulin at physiological concentrations regulates blood glucose and affects profoundly the fate of all energy-producing substrates. At high concentrations insulin also stimulates cell division and growth (1-5). In vitro, serum can mimic most, if not all, of the effects of insulin (3,5-10). Of the total insulin-like biological activity of fasting serum in vitro, only 5-10% can be suppressed by anti-insulin antibodies and is accounted for by insulin itself (8,9,11). The majority of the insulin-like activity persists in the presence of anti-insulin antibody and is referred to as "non-suppressible insulin-like activity" (NSILA)[2]. NSILA represents multiple substances (9,11,12). Most of the NSILA is not well defined chemically but is precipitated in acid-ethanol. A minority of NSILA is soluble in acid-ethanol and is referred to as NSILA-s. The latter

[1]Guest worker on leave from the First Department of Medicine, Semmelweis Medical University, Budapest, Hungary.

[2]The abbreviations used are: NSILA, non-suppressible insulin-like activity and NSILA-s, non-suppressible insulin-like activity soluble in acid-ethanol.

has been purified extensively (9,11,12), well characterized (9,11-17), and appears to be closely related to other insulin-like substances, the somatomedins (10-20) and multiplication-stimulation activity (21). NSILA-s lacks the immunological reactivity of insulin but has all of the metabolic and growth promoting properties of insulin. For the metabolic effects, NSILA-s is less potent than insulin, whereas for stimulation of cell growth NSILA-s is more active (4,5).

In our previous studies we have shown that purified plasma membranes of rat liver (22-24), isolated rat adipocytes (22) and cultured human lymphocytes (25,26) possess specific insulin binding sites. A wide variety of insulin analogues compete with labeled insulin for binding to these receptors in direct proportion to their bioactivities (23,25). In the present study, we have demonstrated that the plasma membrane of liver contains two sets of receptors both of which bind both insulin and NSILA-s. The "insulin receptor" has a greater affinity for insulin than NSILA-s, while with the "NSILA-s receptor" the reverse is true. We postulate that binding with either substance to the "insulin receptor" initiates the metabolic responses while binding of either to the "NSILA-s receptor" generates growth stimulating effects. This specific NSILA-s receptor assay will be of great help in the purification and characterization of NSILA and in the study of plasma NSILA-s in man.

MATERIALS AND METHODS. NSILA-s was extracted from pooled plasma into acid ethanol and purified by gel filtration (11). Unless otherwise noted, the NSILA-s used in these experiments had insulin-like activity *in vitro* with fat pads equivalent to 70 mU/mg. This preparation is about 15-20 percent as potent on a weight basis as the most highly purified NSILA-s and represents a 500,000 fold purification over plasma. Less than 0.00001 percent of the biologic activity of the NSILA-s could be accounted for by its content of insulin by radioimmunoassy (27). We do not know whether the immunoreactivity represented a trace contamination with insulin or a very slight intrinsic reactivity of NSILA-s with anti-insulin antibodies.

NSILA-s was iodinated at specific activities of 80-150 μCi/μg by a modi-

fication (28) of the chloramine T method (29). The iodination mixture contained 30 µl of 0.3 M phosphate buffer (pH 7.4), 1-2 mCi $Na^{125}I$, and 10 µl NSILA-s (1 mg/ml in 0.01 N HCl); 3-24 µl of chloramine T (60 µg/ml) were added stepwise. After the addition of 1 ml of 0.3 M phosphate buffer containing 1 mg of bovine serum albumin (BSA) the mixture was transferred to a column of Sephadex G-50 to separate ^{125}I-NSILA-s from free iodide and any damaged aggregates (Fig. 1). The ^{125}I-NSILA-s migrated as a single peak with an apparent molecular weight of 7100 (Fig. 1, insert), which was in agreement with the

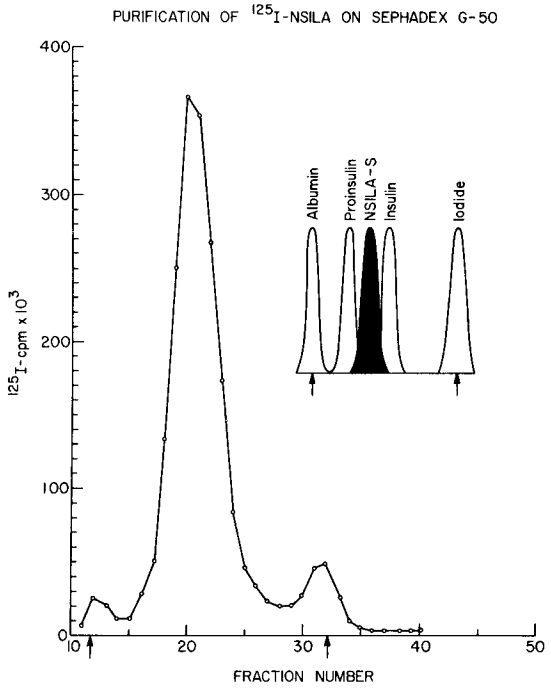

FIG. 1. Gel filtration of ^{125}I-NSILA-s. NSILA-s was labeled with ^{125}I (see Methods) and applied to a 0.9 x 53 cm column of Sephadex G-50 (fine). The column was equilibrated in and eluted with 0.03 M phosphate buffer, pH 7.4, containing 0.5 mg/ml BSA. Flow rate was 10 ml/hr. Fractions of 1 ml were collected and counted for radioactivity. The arrows mark the positions of albumin and iodide markers. The few fractions corresponding to the peak of the radioactivity were used as the ^{125}I-NSILA-s for binding studies. The inset is a schematic composite of several experiments performed with a column (1.5 x 90 cm) of Sephadex G-50 (fine) in 0.05 M $(NH_4)_2CO_3$, pH 8.6 calibrated with ^{125}I-labeled components.

molecular weight of 7400 estimated from the amino acid composition (11).

Porcine insulin (25 U/mg) and porcine proinsulin were gifts of Eli Lilly Co. Guinea pig insulin was kindly supplied by Dr. L. F. Smith and human growth hormone by Dr. A. E. Wilhelmi. The ^{125}I-insulin (30), cultured human lymphocytes (25,26), purified plasma membranes from rat liver (31), and methods for measuring the binding of iodinated hormones to their receptors (22-28) have been previously described. Specific experimental condition are in the legends to the figures.

RESULTS AND DISCUSSION. NSILA-s competed with ^{125}I-insulin for binding to the insulin receptor in direct proportion to its insulin-like bioactivity (Figs. 2A, 2B and 3A), a finding similar to that for all insulin analogues tested

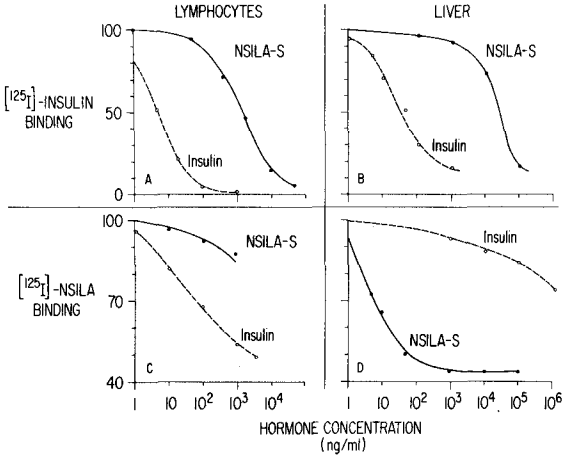

FIG. 2. Binding of ^{125}I-insulin and ^{125}I-NSILA-s to lymphocytes and liver. A. ^{125}I-insulin (1 x 10^{-11} M) was incubated in 0.5 ml with cultured human lymphocytes (IM-9), 20 x 10^6 cells/ml for 90 min at 15°. B. ^{125}I-insulin (3 x 10^{-11} M) was incubated in 0.15 ml with rat liver plasma membranes (0.2 mg/ml) for 60 min at 30°. C. ^{125}I-NSILA-s (0.5 ng/ml) was incubated in 0.5 ml with cultured human lymphocytes (IM-9), 40 x 10^6 cells/ml, for 90 min at 20°. D. ^{125}I-NSILA-s (0.5 ng/ml) was incubated in 0.15 ml with rat liver plasma membranes (1 mg/ml) for 90 min at 20°. In each experiment bound and free labeled peptides were separated by centrifugation in a Beckman microfuge and the radioactivity bound to the cells or membranes was counted. In the absence of unlabeled hormone, 30%, 12%, 5%, and 10% of the total radioactivity was bound in experiments A, B, C, and D, respectively. On the ordinate is the percent of maximum tracer binding, defined as 100% for each experiment; on the abscissa is the hormone concentration from 1 to 10^6 ng/ml. In each experiment maximum tracer binding (100%) occurred at hormone concentrations of less than 1 ng/ml. Each point is the mean of duplicate or triplicate samples. Non-specific binding has not been subtracted.

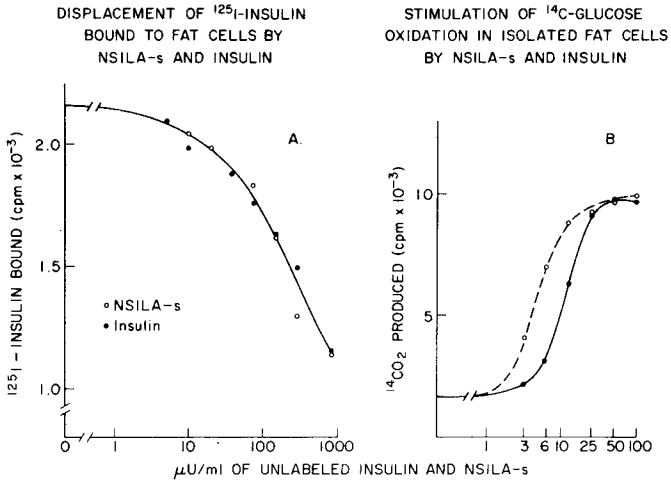

FIG. 3. NSILA-s (17 mU/mg) and insulin in isolated rat adipocytes. A. Competition for ^{125}I-insulin binding. Isolated fat cells (34) equivalent to 38 mg dry weight, were incubated in 0.6 ml of Dulbecco-buffer-1% albumin, pH 7.4, with unlabeled insulin or NSILA-s for 20 min at 30°. ^{125}I-insulin (1.1×10^{-10} M) was added for another 40 min. The ^{125}I-insulin bound to fat cells was separated from free insulin on cellulose acetate Millipore filters (EAWP, 1μ) and washed twice with 3 ml aliquots of buffer. The dried filters were counted in a liquid scintillation counter. The binding that occurred in the presence of 2.8×10^{-6} M unlabeled insulin (non-specific binding) has been subtracted from each value. B. Stimulation of glucose oxidation by NSILA-s and insulin. Glucose-1-^{14}C oxidation was measured (15) for 1 hr at 37° in the above buffer containing 10 mg% of glucose-1-^{14}C and insulin or NSILA-s (with insulin antiserum 2.5 U/ml). The slight difference in potency here between the NSILA-s and insulin reflects differences between the isolated fat cell assay and the fat pad assay.

(23,25,32). With purified rat liver plasma membranes or cultured human lymphocytes, NSILA-s, which had insulin-like bioactivity (70 mU/mg) equivalent to 0.3 percent that of porcine insulin (25 U/mg), displaced ^{125}I-insulin from its receptor with 0.2-0.4 percent of the potency of insulin (Figs. 2A and 2B). With isolated rat adipocytes a less pure preparation of NSILA-s was used (17 mU/mg); again, competition by NSILA-s was proportional to its insulin-like potency in the fat pad assay (Fig. 3A). As can be seen in Fig. 3B, this NSILA-s preparation was slightly more active in stimulating glucose oxidation in the isolated adipocyte than had been noted in the fat pad assay. Clearly neither the insulin-like bioactivity nor receptor binding activity of NSILA-s could be

accounted for by its content of immunoreactive insulin (see Materials and Methods).

To elucidate further the nature of the interaction of NSILA-s with receptors, binding of ^{125}I-NSILA-s was studied directly. As with labeled insulin, the binding of ^{125}I-NSILA-s to lymphocytes was inhibited by unlabeled insulin at concentrations as low as 10 ng/ml and unlabeled NSILA-s was only about 0.3% as effective as insulin in competing for binding to these cells (Fig. 2C). This suggested that most, if not all, of the ^{125}I-NSILA-s was, in fact, bound to the "insulin receptors".

In contrast to all of the previous experiments, when ^{125}I-NSILA-s was incubated with liver membranes, unlabeled insulin was very ineffective in competing for binding, whereas unlabeled NSILA-s was very potent (Fig. 2D). At insulin concentrations up to 1 mg/ml, the binding of ^{125}I-NSILA-s was reduced by only 25%. Unlabeled NSILA-s, on the other hand, reduced the binding of ^{125}I-NSILA-s by 35% at 10 ng/ml and reduced binding by 50-70% at 1 μg/ml. Further increases in the NSILA-s concentration failed to produce further displacement; the undisplaced portion of radioactivity corresponds to the "non-specific binding" observed in studies with other hormones (23). Porcine proinsulin, though much less potent than NSILA-s, was more than a hundred times as potent as insulin in inhibiting ^{125}I-NSILA-s binding. This is opposite to the findings with the insulin receptor, where proinsulin is only 5-20% as potent as insulin in displacing ^{125}I-insulin (23,25,32). At high concentrations proinsulin reduced the binding of ^{125}I-NSILA-s to the same level as unlabeled NSILA-s. NSILA-s that had been biologically inactivated by reduction and aminoethylation (11) as well as human growth hormone and guinea pig insulin (up to 1 μg/ml), had no effect on displacing the ^{125}I-NSILA-s from its receptor. NSILA-s preparations from different steps during the purification procedure, which differed by 70-fold in ther insulin-like biological potencies, inhibited the binding of ^{125}I-NSILA-s to liver in direct proportion to their bioactivities (manuscript in preparation). These data suggest that in liver there are two dis-

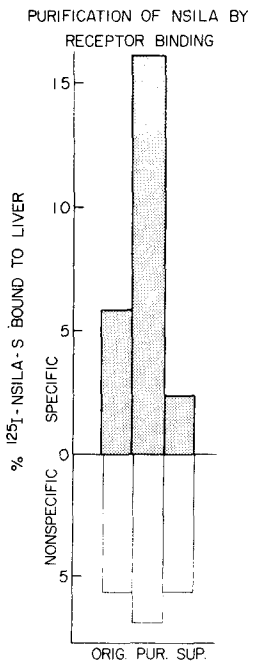

FIG. 4. Purification of ^{125}I-NSILA-s receptor binding. ^{125}I-NSILA-s (3 ng/ml) was bound to liver membranes (1 mg/ml) by incubation at 20° for 90 minutes. The membranes, separated from the supernatant ("SUP") by centrifugation, were resuspended in 0.01 M acetic acid pH 3. Ninety percent of the bound radioactivity was released into the medium. The membranes were separated from the medium by centrifugation, and the medium was restored to pH 7.4 ("PUR"). The original ^{125}I-NSILA-s that had not been exposed to liver membranes ("ORIG"), receptor purified ^{125}I-NSILA-s ("PUR"), and the radioactivity that had remained unbound during the first incubation ("SUP") were each incubated with fresh aliquots of liver membranes in the absence and presence of unlabeled NSILA-s, 1 μg/ml. The specifically bound ^{125}I-NSILA-s (shaded bars) is the difference between the total ^{125}I-NSILA-s bound (shaded and clear bars) and that bound in the presence of 1 μg/ml of unlabeled peptide (clear bars).

tinct but specific receptor sites - one for insulin and the other for NSILA-s. On a weight basis, NSILA-s (70 mU/mg) possesses about 3×10^{-4} of the potency of insulin in competing for the "insulin receptor" while insulin possesses about 3×10^{-6} of the activity of NSILA-s for the "NSILA receptor".

The NSILA-s used for iodination, although greatly enriched over plasma, was only about 15-20 percent as active biologically as the most purified preparations. The ^{125}I-NSILA-s was, therefore, presumably a mixture of ^{125}I-labeled components, only a portion of which interacted with the receptor. The

^{125}I-NSILA-s was purified by binding it to the liver membrane receptor followed by elution with acid (Fig. 4). The radioactivity recovered from the receptor showed a two and one-half fold enhancement in specific binding to both NSILA-s receptors in liver and insulin receptors in lymphocytes, whereas the ^{125}I-NSILA-s in the supernatant was depleted by 50% of material capable of binding to both of these receptors. This suggested that the same component of the ^{125}I-NSILA-s interacted with both the NSILA-s receptor in liver and the insulin receptor of the lymphocyte and that, in both cases, the amount of ^{125}I-NSILA-s capable of binding to receptors could be increased by purification on the receptor.

The somatomedins are a family of substances that appear in the plasma of hypophysectomized rats following the administration of growth hormone. One or more of these appear to be closely related to NSILA-s and like NSILA-s it displaces ^{125}I-insulin from its receptor in liver and fat in direct proportion to its insulin-like bioactivity (33). Somatomedin is more potent than insulin in displacing ^{125}I-insulin from chondrocytes (33) and ^{125}I-somatomedin from both chondrocytes and placenta (20) suggesting that separate receptors for these peptides may exist in these tissues. Whether the "NSILA-s receptor" and the "somatomedin receptor" are the same or share overlapping specificities remains to be determined.

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