

Inhibition of the Action of Nonsuppressible Insulin-Like Activity on Isolated Rat Fat Cells by Binding to its Carrier Protein

J. ZAPF, E. SCHOENLE, G. JAGARS, I. SAND, J. GRUNWALD, and E. R. FROESCH,
*Metabolic Unit, Department of Medicine, University Hospital,
CH-8091 Zürich, Switzerland*

ABSTRACT Nonsuppressible insulin-like activity extracted and purified from human serum (NSILA-S) mimics all insulin-like effects *in vitro* and, after injection, *in vivo* in the presence of excess insulin antibodies. However, there is no evidence that it exerts acute insulin-like effects in its native form in the circulation, where it is almost completely bound to a specific large molecular weight carrier protein. In this paper we show that partially purified NSILA-S-carrier protein, devoid of endogenous insulin-like activity, inhibits the stimulatory effect of NSILA-S, but not of insulin, on 3-O-methylglucose transport and on lipogenesis from [U-¹⁴C]glucose in isolated rat fat cells. Concomitantly, it prevents binding of ¹²⁵I-labeled NSILA-S to the insulin receptor and to the NSILA-S-binding site.

The following explanation is, therefore, offered for the absence of acute insulin-like effects of native NSILA-S *in vivo*: In native serum NSILA-S occurs almost exclusively as NSILA-S-carrier complex. According to recent findings the passage of this complex through blood capillaries is restricted. The present results indicate that, in addition, it is metabolically inactive, or, at least, possesses reduced metabolic activity. The well-known phenomenon that whole serum, nevertheless, exerts pronounced nonsuppressible insulin-like effects on adipose tissue *in vitro* seems, therefore, to be mainly caused by the presence of a large molecular weight insulin-like protein not identical to the NSILA-S-carrier complex.

INTRODUCTION

Nonsuppressible insulin-like activity extracted from human serum by acid-ethanol (NSILA-S)¹ (6, 7) mimics

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¹Nonsuppressible insulin-like activity extracted from

all acute metabolic effects of insulin on adipose tissue both *in vitro* (8) and, after intravenous or intraperitoneal injection, *in vivo* (8-10) even in the presence of an excess of insulin antibodies.

Human whole serum contains $\approx 200 \mu\text{U/ml}$ of insulin equivalents not suppressible by insulin antibodies, as measured by the rat fat pad assay (6, 10, 11). A similar amount of NSILA-S can be extracted from whole serum by Sephadex chromatography at acidic pH (10, 12). Despite this large amount of NSILA-S present in the circulation, adipose tissue and muscle are not permanently stimulated. Native NSILA-S, does, therefore, not seem to influence glucose homeostasis *in vivo*. It has been shown that in native serum NSILA-S is bound to a large molecular weight carrier protein (13-15), which displays a high degree of specificity and affinity for NSILA-S (12-14). Subsequently, the hypotheses have been advanced (*a*) that the blood capillary bed might constitute a diffusion barrier for the NSILA-S-carrier complex and that the "bioavailability" for NSILA-S at the cell membrane would thus be restricted (13-15) or (*b*) that the complex was metabolically inactive (15). Evidence in favor of the first concept has recently been presented by Meuli et al. (16). They demonstrated that partially purified NSILA-S-carrier protein, stripped of endogenous NSILA-S, abolished the action of NSILA-S on the perfused rat heart. It also prevented the binding of ¹²⁵I-labeled NSILA-S to the NSILA-S binding sites, which mediate the metabolic effects of NSILA-S on rat heart muscle (17, 18). However, the possibility could not be excluded

human serum by acid-ethanol and further purified. Partially purified NSILA-S preparations contain a mixture of two polypeptides with nonsuppressible insulin-like activity (1), which have recently been chemically (2-4) and biologically (5) characterized and termed insulin-like growth factors I and II (2). In this paper partially purified preparations will be referred to as NSILA-S.

that, in addition to its restricted capillary permeability, the NSILA-S-carrier complex might also be metabolically inactive. To test this, we measured the metabolic response of isolated rat fat cells to NSILA-S in the presence and absence of partially purified NSILA-S-carrier protein. Isolated rat fat cells are very sensitive to NSILA-S (19) and, in contrast to whole tissue, freely accessible to large molecules. We found that the action of NSILA-S on fat cells and the binding of ^{125}I -labeled NSILA-S to the insulin and the NSILA-S receptor were inhibited by NSILA-S-carrier protein.

METHODS

NSILA-S preparations

NSILA-S and unlabeled whale insulin (24 U/mg, identical amino acid sequence to porcine insulin) were kindly supplied by Dr. Rinderknecht and Dr. Humbel. NSILA-S preparations were standardized in the rat fat pad assay (11) or in the fat cell assay (19) with insulin as the reference. The NSILA-S preparation used for iodination (specific biological activity 200 mU/mg, $\approx 65\%$ pure) as well as the iodination procedure were the same as described earlier (20). It contains almost exclusively insulin-like growth factor (IGF)² I and $<2\%$ IGF II by radioimmunoassay. The binding characteristics of this preparation have been defined previously (12, 13, 20). For the metabolic studies and for determination of nonspecific binding, a NSILA-S preparation with a specific biological activity of 4.5 mU/mg was used to save pure material. It contained a $\approx 1:1$ mixture of IGF I and II as determined by radioimmunoassay.³ The specific biological activity of this preparation standardized in the fat cell assay was 6.8 mU/mg, i.e., somewhat higher than in the fat pad assay. The reason for this is a result of the fact that in fat cells IGF II is somewhat more potent than IGF I, whereas both are equipotent in the fat pad assay (5).

Partial purification of NSILA-S-carrier protein

The starting material was an acetone powder from a serum fraction (precipitate B) obtained by a modified Cohn fractionation and containing mostly α - and β -globulins (6). 2 g of this material was dissolved in 60 ml of 1 M acetic acid, stirred overnight at 4°C, and centrifuged. The supernate was chromatographed on a 3-liter Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (6 cm Diam) in 1 M acetic acid. Specific relative ^{125}I -labeled NSILA-S binding activities of the fractions were determined in aliquots, dialyzed against 0.1 M phosphate buffer pH 7.0, by the protein-binding assay (12) in the absence and presence of 250 $\mu\text{U}/\text{ml}$ of unlabeled NSILA-S. The peak of binding activity eluted between 60 and 80% bed volume. The fractions between 60 and 70% and between 70 and 80% were pooled, ultrafiltered, dialyzed extensively against Krebs-Ringer bicarbonate buffer, and tested in the fat cell assay for intrinsic insulin-like activity.

The 70–80% pool did not contain detectable amounts of intrinsic insulin-like activity and was, therefore, used exclusively in this study. Its protein content was 380 mg/100 ml. In the presence of 150 μU of NSILA-S, 1 mg of the partially

purified carrier protein bound $\approx 130 \mu\text{U}$ (Table I) compared to $\approx 5 \mu\text{U}$ bound/mg of protein in whole serum, which already contains $\approx 5 \mu\text{U}$ of endogenous NSILA-S/mg of protein (activities determined in the fat cell assay).

Preparation and incubation of fat cells

Lipid synthesis from $[\text{U-}^{14}\text{C}]$ glucose. Isolated fat cells from epididymal fat pads of normal male Zbz Cara (formerly Osborne-Mendel) rats, weighing between 100 and 120 g, were obtained by collagenase (Worthington Biochemical Corp., Freehold, N. J.) treatment according to the method of Rodbell (21). The cells ($1.5\text{--}2.0 \times 10^6$) were incubated for 1 h at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer containing 0.2 g/100 ml of human serum albumin (HSA) (Swiss Red Cross, Bern, Switzerland), 20 mg/100 ml of glucose, 0.2 μCi of $[\text{U-}^{14}\text{C}]$ glucose (Radiochemical Centre, Amersham, England), NSILA-S (as indicated in the figures) and 0.76 mg or no NSILA-S-carrier protein. The carrier protein or buffer (control) had been preequilibrated with NSILA-S for 2 h at room temperature in 0.4 ml of the same buffer before the cells were added. ^{14}C incorporation into total lipids was determined in 300- μl aliquots of the cell suspension centrifuged ($\approx 10,000\text{g}$, 20 s, Beckman microfuge B Beckman Instruments, Inc., Fullerton, Calif.) through a layer of dinonyl phthalate (50 μl) in 400- μl microtubes as described by Gliemann et al. (22). The tops of the microtubes that contained the cell layer were cut off and counted for radioactivity in 5 ml of Instagel (Packard Instrument Co., Downers Grove, Ill.) in a beta counter (Tricarb, Packard Instrument Co.).

3-O-methylglucose transport. 3-O-methylglucose transport was measured by the method of Vinten et al. (23) modified as described in detail earlier (19). Briefly, fat cells were preincubated under gentle stirring for 45 min at 37°C in 4 ml of Krebs-Ringer bicarbonate buffer that contained HSA, 1 g/100 ml, and 30 mM $[\text{U-}^{14}\text{C}]$ -3-O-methylglucose ($\approx 20 \mu\text{Ci}$). The cells were then centrifuged at 500 g through a layer of dinonyl phthalate and rapidly resuspended in 5 ml of the same buffer that contained 30 mM cold 3-O-methylglucose alone (control) and the additions as indicated in Fig. 2. After different time intervals, 300- μl aliquots were removed and the cells separated from the aqueous phase as described above. The radioactivity recovered from the packed cell layer after 10 min in the presence of 0.7 μM insulin corresponds to the extracellular water space determined with $[\text{3H}]$ inulin (24). It was subtracted from all values and the radioactivity extrapolated to zero time as described earlier (19).

Binding studies. The rationale underlying the binding studies described below was derived from earlier experiments, which had indicated that NSILA-S bound to two different sites on the fat cell, to the insulin receptor and to a specific NSILA-S acceptor site different from the insulin receptor (25). Fat cells preincubated with an excess of unlabeled NSILA-S and washed after preincubation had been shown to bind nearly as much ^{125}I -labeled insulin as cells that had not been preincubated with NSILA-S, whereas binding of labeled insulin in the presence of excess unlabeled NSILA-S decreased. In contrast, binding of ^{125}I -labeled NSILA-S after preincubation with an excess of unlabeled NSILA-S and subsequent washing of the cells had been found to be reduced as compared to cells not preincubated with NSILA-S, but it had been higher than nonspecific binding. From these and other experiments it had been concluded that NSILA-S bound to the insulin receptor with low affinity and that binding to the latter could, therefore, be easily reversed by washing, whereas binding of NSILA-S to its own receptor could not. The difference between (a) the binding of labeled NSILA-S

² Abbreviations used in this paper: HSA, human serum albumin, IGF, insulin-like growth factor(s).

³ Manuscript in preparation.

subsequent to washing the cells after preincubation with unlabeled NSILA-S and (b) the nonspecific binding had been attributed to reflect binding of the NSILA-S tracer to the insulin receptor, from which unlabeled NSILA-S had been removed by prior washing. The following binding studies were, therefore, designed according to the above procedure to examine whether the NSILA-S-carrier protein could block the binding of ^{125}I -labeled NSILA-S to the insulin receptor of the fat cell.

Fat cells were preincubated for 45 min at 37°C in Krebs-Ringer bicarbonate buffer that contained 1 g/100 ml of HSA together with or without $500\ \mu\text{U/ml}$ of NSILA-S. Binding of ^{125}I -labeled NSILA-S ($\approx 200,000\ \text{cpm}$, $\approx 0.7\ \mu\text{U}$) in the absence and presence of 0.38 mg/ml of partially purified NSILA-S-carrier protein was then determined before or after washing the cells three times with 5 ml of Krebs-Ringer bicarbonate-HSA buffer (centrifugation for 20 s at 500 g and room temperature). This second incubation in the presence of the tracer was carried out for 30 min at 37°C in 0.5 ml of the same buffer. Bound and free radioactivity were separated by centrifugation of 300- μl aliquots of the cell suspension through 50 μl of dinonyl phthalate in 400- μl microtubes (10,000 g, 20 s, Beckman microfuge B), and the cell layer on the top was cut off and counted in a gamma counter (Packard Instrument Co.).

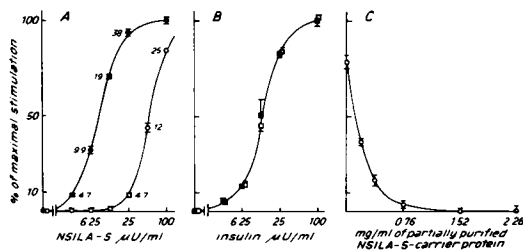


FIGURE 1 Stimulation of lipid synthesis from $[\text{U-}^{14}\text{C}]$ glucose in rat adipocytes by (A) increasing concentrations of NSILA-S, (B) by insulin in the absence (closed symbols) and presence (open symbols) of 0.76 mg/ml of partially purified NSILA-S-carrier protein, and (C) by 16.5 $\mu\text{U/ml}$ of NSILA-S in the presence of increasing concentrations of NSILA-S-carrier protein. 0.4 ml of Krebs-Ringer bicarbonate buffer that contained 1 mg of HSA, 0.1 mg of glucose, 0.2 μCi of $[\text{U-}^{14}\text{C}]$ -glucose, different amounts of NSILA-S (A) or insulin (B), and 0.76 mg or no (control) binding protein was incubated for 2 h at room temperature to allow for equilibration of NSILA-S binding to the carrier. To all samples that contained NSILA-S, 1 μl of guinea pig anti-insulin serum (neutralizing capacity 1 mU) was added. Then, 0.6 ml of a fat cell suspension (1.5×10^5 cells) in the same buffer (without labeled glucose, binding protein, and hormones) was added. Incubation was continued for 1 h at 37°C . 300- μl aliquots of the cell suspension were centrifuged through dinonyl phthalate and the packed cell layers counted in Instagel in a β -counter. All values are expressed as percent of maximal stimulation by 100 $\mu\text{U/ml}$ of insulin (same as for 100 $\mu\text{U/ml}$ of NSILA-S). (A and B) The abscissas give NSILA-S activities in insulin equivalents determined in the fat pad assay. The numbers on the curves of A indicate NSILA-S activities in insulin equivalents of the fat cell assay (see Methods). (C) The experimental procedure was the same as in A and B, except that increasing concentrations of the carrier protein were preincubated with a constant NSILA-S concentration (16.5 μU). All points are the means of triplicates from the same experiment, brackets give the SEM.

Determination of free and bound NSILA-S after equilibration with the carrier protein

To assess how much NSILA-S was bound when added at increasing concentrations to a constant amount of carrier protein, 19, 38, 76, and 152 μU of NSILA-S were equilibrated for 2 h at room temperature with 0.76 mg of carrier protein and a trace of ^{125}I -labeled NSILA-S (10,000 cpm, $\approx 0.04\ \mu\text{U}$) in 0.4 ml of Krebs-Ringer bicarbonate buffer that contained 0.2 g/100 ml of HSA. The mixture was then chromatographed on a Sephadex G-50 (medium) column (35 ml bed vol, 1 cm Diam) in 0.15 M ammonium acetate, pH 7.3. This procedure separates carrier-bound from free NSILA-S (Fig. 3). The corresponding fractions were counted for radioactivity, collected in 2 pools, and lyophilized.

The pool containing the free NSILA-S was dissolved in a small amount (3 ml) of 1 M acetic acid, transferred to a 5-ml plastic vial, relyophilized, taken up in 0.3–1.0 ml of Krebs-Ringer bicarbonate buffer-HSA (0.2 g/100 ml), and counted again for radioactivity. The final recovery was 80–84% of the radioactivity found in the “free NSILA-S pool” immediately after chromatography. NSILA-S was then determined by the competitive protein binding assay, described in detail elsewhere (12), and by the fat cell assay (19).

The lyophilized pool that contained the NSILA-S-carrier complex was dissolved in a small volume (3 ml) of 0.1 M ammonium bicarbonate, transferred to a 5-ml plastic vial, and relyophilized. It was then dissolved in 0.4 ml of 1 M acetic acid and chromatographed on a Sephadex G-50 (medium) column (35 ml bed vol, 1 cm Diam), with 1 M acetic acid. This chromatographic step dissociates the NSILA-S-carrier complex (10, 12) (Fig. 3). The fractions that contained the dissociated NSILA-S were pooled, lyophilized, and taken up in 0.5 ml of Krebs-Ringer bicarbonate-HSA buffer. The final recovery of radioactivity was between 65 and 70% of that eluted with the complex after the first neutral chromatographic step. NSILA-S was then determined in the protein binding (12) and the fat cell assay (19). Protein determinations were carried out by the method of Lowry et al. (26).

RESULTS

Fig. 1 shows the effect of partially purified NSILA-S-carrier protein on lipid synthesis from $[\text{U-}^{14}\text{C}]$ glucose in isolated rat fat cells. On the abscissa of Fig. 1A activities are expressed in insulin equivalents obtained in the fat pad assay; the numbers on the curves give the corresponding insulin equivalents for the fat cell assay as read from Fig. 1B (see Methods). In the presence of 0.76 mg/ml of the carrier protein the NSILA-S dose-response curve is shifted to the right. Whereas 12.5 $\mu\text{U/ml}$ of NSILA-S (equivalent to 18 $\mu\text{U/ml}$ in the fat cell assay) elicits a more than half-maximal response in the absence of the carrier, no detectable stimulation of lipid synthesis is seen in its presence. 25 $\mu\text{U/ml}$ (38 μU in the fat cell assay), which cause a near-maximal effect in the absence of the carrier result in only 9% stimulation when incubated together with the carrier protein. In sharp contrast, the stimulatory effect of insulin (Fig. 1B) is not affected by the NSILA-S binding protein, and the two dose-response curves are superimposable.

Increasing concentrations of the carrier protein (Fig.

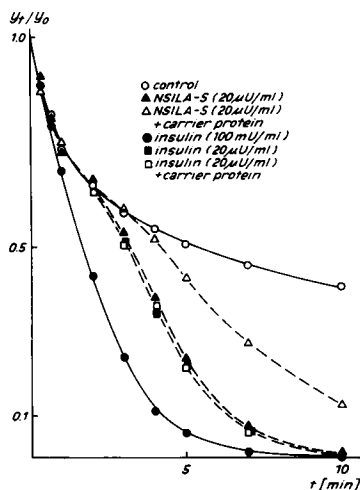


FIGURE 2 Inhibitory effect of 0.38 mg/ml of partially purified NSILA-S-carrier protein on the stimulation by NSILA-S (20 μ U/ml) of 3-O-methylglucose transport in rat adipocytes. Cells were preincubated for 45 min at 37°C in Krebs-Ringer bicarbonate buffer that contained 1 g/100 ml HSA and 30 mM of [14 C]3-O-methylglucose. After the cells were separated by centrifugation through dinonyl phthalate, they were resuspended in the same buffer that contained only unlabeled 3-O-methylglucose (30 mM) and one of the following additions; none (\circ), NSILA-S, 20 μ U/ml (\blacktriangle), NSILA-S 20 μ U/ml + 0.38 mg/ml carrier protein (\triangle), insulin, 20 μ U/ml (\blacksquare), insulin, 20 μ U/ml + 0.38 mg/ml carrier protein (\square), insulin, 100 mU/ml (\bullet). The efflux of the sugar was measured after different time intervals as described in Methods. All points are single determinations from one experiment with the same cell batch. Y_0 represents the radioactivity present at time zero in the packed cell layer after centrifugation of the cells through the oil phase. This value was extrapolated on a semilogarithmic plot of radioactivity associated with the cells against time, which gives a straight line (19). Y_t = radioactivity of the cells at time t .

1C) cause increasing inhibition of the stimulation of lipid synthesis by a constant concentration of NSILA-S (16.5 μ U/ml corresponding to 25 μ U/ml in the fat cell assay). At concentrations above 0.76 mg/ml of the carrier the stimulatory effect is completely abolished.

The inhibitory effect of the NSILA-S-carrier protein is also observed on NSILA-S-stimulated 3-O-methylglucose transport (Fig. 2). The time-course of 3-O-methylglucose efflux in the presence of 20 μ U/ml (fat pad activity) of NSILA-S and 0.38 mg/ml of the binding protein is comparable to that of \approx 5 μ U/ml of NSILA-S in the absence of binding protein (not shown). The binding protein does not inhibit the stimulatory effect of 20 μ U/ml of insulin.

The results of Table I demonstrate that inactivation of NSILA-S by the carrier protein is, indeed, a result of binding. The activities of NSILA-S determined in the experiment explained in Fig. 1A in the presence of the carrier protein are recovered as nonbound NSILA-S

after separation from carrier-bound NSILA-S by neutral Sephadex G-50 chromatography, although the amounts of nonbound NSILA-S measured after this procedure are not exactly the same as those calculated from the experiment in Fig. 1A. When the isolated NSILA-S-carrier complex is rechromatographed on Sephadex G-50 at acidic pH, NSILA-S is dissociated from the binding protein and recovered by two completely different assays, the competitive protein binding assay and the fat cell assay. Thus, after binding to and dissociation from the carrier protein NSILA-S still competes effectively with 125 I-labeled NSILA-S and it is also still biologically active.

In the experiments shown in Fig. 3 the chromatographic procedure applied above (Table I) was performed with 125 I-labeled instead of unlabeled NSILA-S. After equilibration of the binding protein with the tracer and gel filtration on Sephadex G-50 at neutral pH, most of the radioactivity eluted in the void volume (carrier complex). A small amount of radioactivity eluted as free [125 I]iodine or 125 I-labeled degradation products at around 100% bed volume. This latter peak was also present when 125 I-labeled NSILA-S was chromatographed without the binding protein, but the main radioactive peak now appeared at 61% bed volume, the elution volume of free NSILA-S. After isolation of the labeled NSILA-S-carrier complex from the experiment explained in Fig. 3A and rechromatography on Sephadex G-50 at acidic pH, the void volume peak had nearly disappeared, and the main radioactive peak now coincided with the one obtained on acidic chromatography of the free tracer. No radioactivity was detected at 100% bed volume on acidic chromatography of the labeled carrier complex indicating that no measurable degradation of 125 I-labeled NSILA-S had occurred during binding, isolation, and dissociation from the carrier protein.

Fig. 4 shows that the NSILA-S-carrier protein does not only prevent the binding of 125 I-labeled NSILA-S to the NSILA-S binding site (Fig. 4, column 6), but also to the insulin receptor (Fig. 4, columns 2 and 3). When fat cells are preincubated with an excess of unlabeled NSILA-S (500 μ U/ml), extensively washed, and reincubated with 125 I-labeled NSILA-S, binding of the tracer is \approx 40% (Fig. 4, column 2) of that observed in cells not preincubated with unlabeled NSILA-S (specific binding, Fig. 4, column 1). When the same experiment is carried out in the presence of NSILA-S-binding protein, binding falls from 40 to 0% (Fig. 4, column 3). This means that binding of the tracer now becomes the same as nonspecific binding measured in the presence of an excess of unlabeled NSILA-S together with (Fig. 4, column 5) or without the carrier protein. In the presence of carrier protein alone specific binding is also reduced to zero (Fig. 4, column 6).

TABLE I
Recovery of Increasing Concentrations of NSILA-S in the Presence of 0.76 mg of Partially Purified NSILA-S-Carrier Protein before and after Separation into the Free and Bound Form

NSILA-S added (standardized in fat cell assay)	Fat cell assay in the presence of carrier protein		NSILA-S recovered after neutral Sephadex chromatog- raphy and after acidic chro- matography of the NSILA-S- carrier complex			
	NSILA-S		Fat cell assay		Protein binding assay	
	Free	Apparently neutralized	Free	Bound	Free	Bound
μU		μU			μU	
19	<2	>17	*	17.4	1.4	12.0
38	4.7	33.3	*	35.7	2.6	24.9
76	11.9	64.1	19.8	80.2	16.1	49.7
152	25.0	127.0	52.0	106.0	41.8	87.2

Microunits in insulin equivalents of free and apparently neutralized NSILA-S in the presence of carrier protein have been read from Fig. 1B. The separation of free and bound NSILA-S and the dissociation of the NSILA-S-carrier complex by Sephadex chromatography was the same as described in Fig. 3 and in Methods. All values have been corrected for losses of activity as followed by the recovery of initially added tracer (see Methods).

* NSILA-S not detectable in the fat cell assay.

DISCUSSION

The early observation that native whole serum contains $\approx 200 \mu U/ml$ of nonsuppressible insulin-like activity as determined in the fat pad *in vitro* (10, 11) has always been puzzling because there is no evidence whatever that NSILA-S in its native form exerts acute metabolic effects *in vivo*. In contrast, pronounced insulin-like effects were seen *in vivo* when NSILA-S, extracted and partially purified from human serum, was injected into animals (8–10). The discovery of a specific carrier protein in serum to which NSILA-S is tightly bound (12–15) offered an explanation for these conflicting findings: In accord with the earlier suggestion of Rasio *et al.* (27), Bürgi *et al.* (6), and Froesch *et al.* (8) the hypothesis was again advanced that the vascular bed constituted a diffusion barrier for the NSILA-S-carrier complex ($\approx 200,000$ mol wt) (13, 28) and, therefore, restricted its bioavailability. Further support in favor of this hypothesis was raised by the work of Meuli *et al.* (16). They demonstrated that in the presence of partially purified NSILA-S-carrier protein, NSILA-S was metabolically inactive in the perfused rat heart. Furthermore, binding of ^{125}I -labeled NSILA-S to the NSILA-S binding sites, which mediate the effects of NSILA-S on heart (17, 18) and skeletal muscle,⁴ was reduced when NSILA-S-binding protein was added to the perfusion medium (16). The present work demonstrates that the

NSILA-S-carrier complex is metabolically inactive on isolated fat cells, *i.e.*, in the absence of diffusion barriers (Figs. 1 and 2). This concept is further corroborated by the results presented in Table I. They show (a) that the amount of NSILA-S that is biologically inactivated by the carrier protein, as calculated from Fig. 1A, corresponds reasonably well to the amount that is bound to and can again be dissociated from the carrier complex by acidic gel filtration, and (b) that after dissociation of the NSILA-S-carrier complex the NSILA-S is still biologically active. The validity and effectiveness of the chromatographic procedure that was applied to separate free from carrier-bound NSILA-S and to dissociate the NSILA-S-carrier complex is underlined by the experiment shown in Fig. 3. It further demonstrates, as is also apparent from the results of Table I, that degradation of NSILA-S during binding to its carrier is very unlikely to occur.

In adipocytes NSILA-S has been shown to act via the insulin receptor rather than via the NSILA-S binding site (5, 19, 25). Therefore, the question cannot be answered at the present time whether in rat heart the lack of biological activity of the NSILA-S-carrier complex is caused only by its restricted capillary permeability or to the carrier protein covering the biologically active site of NSILA-S or to both. With respect to the fat cell, however, binding of NSILA-S to its carrier protein apparently results in the loss of its ability to interact with the insulin receptor, which mediates the effects of NSILA-S on adipose tissue (5, 19, 25). This reasoning

⁴ Poggi, C., Y. LeMarchand-Brustel, J. Zapf, E. R. Froesch, and P. Freychet. Manuscript in preparation.

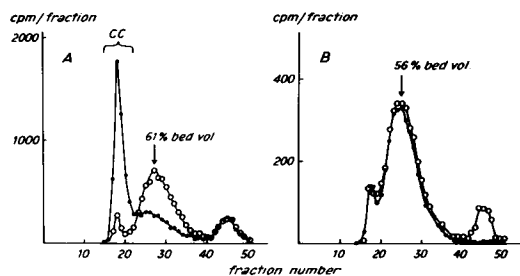


FIGURE 3 (A) Chromatography on Sephadex G-50 at neutral pH of ^{125}I -labeled NSILA-S preincubated with or without NSILA-S-carrier protein. Preincubation of ^{125}I -NSILA-S (9,500 cpm, $\approx 0.04 \mu\text{U}$) was carried out for 2 h at room temperature in 0.4 ml of Krebs-Ringer bicarbonate buffer that contained either 0.76 mg of partially purified NSILA-S-carrier protein (\bullet) or 0.8 mg of HSA (\circ). The mixture was chromatographed on a Sephadex G-50 (medium) column (35 ml bed vol, 1 cm Diam) with 0.15 M ammonium acetate, pH 7.3 and the fractions counted in a gamma counter. (B) Chromatography on Sephadex G-50 at acidic pH of the ^{125}I -labeled NSILA-S-carrier complex (CC) (\bullet) from the experiment shown (A) or of ^{125}I -labeled NSILA-S together with the carrier protein, but without prior preincubation (\circ). The fractions from the experiment shown (A) that contained the carrier complex ($\approx 3,500$ cpm) were pooled, lyophilized, taken up in 0.4 ml of 1 M acetic acid, and chromatographed with 1 M acetic acid on a Sephadex G-50 (medium) column (35 ml bed vol, 1 cm Diam). The same chromatographic procedure was applied for a mixture (0.4 ml) of ^{125}I -labeled NSILA-S (similar radioactivity as the NSILA-S-carrier complex) and 0.76 mg of the carrier protein without preincubation. All fractions were counted in a gamma counter. The radioactivity peak at 100% bed vol represents free ^{125}I -labeled iodine or small molecular weight degradation products. ^{125}I -labeled NSILA-S elutes at 56% bed vol on the acidic and at 61% on the neutral column. Neither neutral nor acidic Sephadex gel filtration alone resulted in significant losses of radioactivity.

is supported by the experiment of Fig. 4. Evidence has been obtained earlier (25) that after preincubation of fat cells with an excess of NSILA-S, subsequent washing results in rapid dissociation of NSILA-S from the insulin receptor, for which the binding affinity is low (5, 19, 25), but not in rapid dissociation from the NSILA-S binding site. After washing the cells and adding ^{125}I -NSILA-S, a certain amount of the tracer still binds specifically (Fig. 4, column 2). This amount probably reflects binding to the insulin receptor. It is no longer observed in the presence of the NSILA-S-carrier protein (Fig. 4, column 3). Therefore, the carrier protein appears to block the interaction of NSILA-S with the insulin receptor. In addition, it also blocks binding of NSILA-S to its own acceptor site (Fig. 4, column 6).

These results, however, leave another question to be answered. In native serum, NSILA-S is nearly completely bound to its carrier protein and $<2\%$ (2–4 $\mu\text{U/ml}$) is present in the free, dissociated form.⁵ Why is

⁵ Unpublished observations.

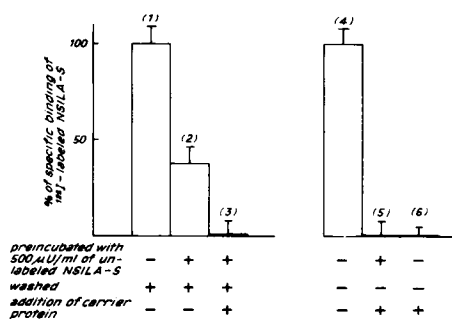


FIGURE 4 Binding of ^{125}I -labeled NSILA-S in the presence and absence of 100 $\mu\text{g/ml}$ of partially purified NSILA-S-carrier protein by rat adipocytes preincubated with or without unlabeled NSILA-S (500 $\mu\text{U/ml}$) and with or without washing after preincubation. ^{125}I -labeled NSILA-S ($\approx 200,000$ cpm, $\approx 0.7 \mu\text{U}$) was equilibrated for 2 h at room temperature in 0.1 ml of Krebs-Ringer bicarbonate buffer that contained 1 g/100 ml HSA and 100 μg or no NSILA-S-carrier protein. The cells ($\approx 2 \times 10^5$), pretreated as indicated below the figure, were then added and the incubation was continued for 30 min at 37°C. Bound and free radioactivity were then separated by centrifugation of the cells through dinonyl phthalate, and the cell layer on the top was counted in a gamma counter. Non-specific binding in the presence of 500 $\mu\text{U/ml}$ of unlabeled NSILA-S (32% of total binding) was subtracted from all values. They were expressed as percent of specific binding (0.4% of the added counts), which was set 100%. All determinations were performed in quadruplicate and the bars give the SEM.

it, then, that one detects $\approx 200 \mu\text{U/ml}$ of nonsuppressible insulin-like activity in unextracted whole serum in the fat pad assay? Recently, Poffenbarger (29) has purified from human serum a large molecular weight (90,000) protein with nonsuppressible insulin-like activity which could not be dissociated into small molecular weight NSILA-S and, therefore, seems to represent a different molecular species, similar to NSILA-P described by Jakob et al. (7). During the past years, we have failed to detect significant amounts of nonsuppressible insulin-like activity in the large molecular weight serum fractions obtained by Sephadex chromatography in 1 M acetic acid, a routine procedure to extract NSILA-S from serum (10, 12). However, when we reinvestigated this discrepancy, we found considerable amounts of nonsuppressible insulin-like activity in large molecular weight serum fractions after acidic Sephadex chromatography, provided that lyophilization of the fractions was replaced by ultrafiltration and extensive dialysis against Krebs-Ringer bicarbonate buffer (30). In accord with earlier observations (7) and with Poffenbarger's work (29) we were unable to dissociate large molecular weight nonsuppressible insulin-like activity into NSILA-S. Thus, at least two different molecular entities of nonsuppressible insulin-like activity are present in native serum: (a) NSILA-S that is almost completely bound to a specific carrier protein and that can be dissociated

under acidic conditions (10, 12, 15) (the NSILA-S-carrier complex is inactive on rat heart muscle [16] and adipocytes, this paper) and (b) large molecular weight nonsuppressible insulin-like activity that is active on adipose tissue *in vitro* (29, 30). The latter form seems to account mainly for the nonsuppressible insulin-like activity measured in whole serum by the fat pad assay. As for the NSILA-S-carrier complex, its passage through blood capillaries seems also to be restricted as indicated by the absence of nonsuppressible insulin-like effects of circulating plasma *in vivo*.

NSILA-S as well as either of its constituents, IGF I and IGF II, promote cell growth (1, 5, 20, 31, 32), stimulate sulfation of cartilage (5, 33, 34), and can be classified as somatomedins (35, 36). In this context, the question will have to be examined next whether or not these effects, which are mediated via specific high affinity cell membrane receptors different from the insulin receptor (5, 20, 34), persist in the presence of the carrier protein. One would expect this to be the case on the basis of the following observations: As first reported by Daughaday et al. (37), whole serum of acromegalic patients stimulates sulfate incorporation into rat cartilage to a significantly greater extent than whole serum of normal subjects, and the latter, in turn, is more active in this assay than whole serum of hypopituitary patients. In contrast, the fat pad assay carried out in these sera measures similar activities (10). After separation of small molecular weight NSILA-S from the binding protein by acidic Sephadex gel filtration, both the sulfation and the fat pad assay detect elevated activities in the small molecular weight extracts of acromegalic sera and decreased activities in those of hypopituitary sera (10). These findings make it appear likely that the sulfation assay does detect carrier-bound NSILA-S, but not large molecular weight nonsuppressible insulin-like activity, which, as reported by Poffenbarger (29), does not appear to be a sulfation factor. Thus, besides the much higher sensitivity towards NSILA-S of tissues concerned with growth as compared with typical insulin target tissues (5, 20, 31, 33), the former may dispose of mechanisms to extract NSILA-S from its binding protein. These two characteristics would also provide an explanation why, despite its limited capillary permeability, the NSILA-S-carrier complex is active on growing tissues *in vivo*.

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REFERENCES

1. Rinderknecht, E., and R. E. Humbel. 1976. Polypeptides with nonsuppressible insulin-like and cell-growth

- promoting activities in human serum: isolation, chemical characterization and some biological properties of forms I and II. *Proc. Natl. Acad. Sci. U. S. A.* 73: 2365-2369.
2. Rinderknecht, E., and R. E. Humbel. 1976. Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth promoting activities: Evidence for structural homology with insulin B chain. *Proc. Natl. Acad. Sci. U. S. A.* 73: 4379-4381.
3. Rinderknecht, E., and R. E. Humbel. 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J. Biol. Chem.* 253: 2769-2776.
4. Rinderknecht, E. and R. E. Humbel. 1978. Primary structure of human insulin-like growth factor II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 89: 283-286.
5. Zapf, J., E. Schoenle, and E. R. Froesch. 1978. Insulin-like growth factor I and II: some biological actions and receptor binding characteristics of two purified constituents of nonsuppressible insulin-like activity of human serum. *Eur. J. Biochem.* 87: 285-296.
6. Bürgi, H., W. A. Müller, R. E. Humbel, A. Labhart, and E. R. Froesch. 1966. Nonsuppressible insulin-like activity of human serum. I. Physicochemical properties, extraction and partial purification. *Biochim. Biophys. Acta.* 121: 349-359.
7. Jakob, A., C. Hauri, and E. R. Froesch. 1968. Nonsuppressible insulin-like activity of human serum. III. Differentiation of two distinct molecules with nonsuppressible ILA. *J. Clin. Invest.* 47: 2678-2688.
8. Froesch, E. R., W. A. Müller, H. Bürgi, M. Waldvogel, and A. Labhart. 1966. Nonsuppressible insulin-like activity of human serum. II. Biological properties of plasma extracts with nonsuppressible insulin-like activity. *Biochim. Biophys. Acta.* 121: 360-374.
9. Oelz, O., A. Jakob, and E. R. Froesch. 1970. Nonsuppressible insulin-like activity (NSILA) of human serum. V. Hypoglycemia and preferential metabolic stimulation of muscle by NSILA-S. *Eur. J. Clin. Invest.* 1: 48-53.
10. Schlumpf, U., R. Heimann, J. Zapf, and E. R. Froesch. 1976. Nonsuppressible insulin-like activity and sulfation activity in serum extracts of normal subjects, acromegalics and pituitary dwarfs. *Acta Endocr. (Kbh).* 81: 28-42.
11. Froesch, E. R., H. Bürgi, E. B. Ramseier, P. Bally, and A. Labhart. 1963. Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity. *J. Clin. Invest.* 42: 1816-1834.
12. Zapf, J., U. Kaufmann, E. Eigenmann, and E. R. Froesch. 1977. Determination of nonsuppressible insulin-like activity in human serum by a sensitive protein binding assay. *Clin. Chem.* 23: 677-683.
13. Zapf, J., M. Waldvogel, and E. R. Froesch. 1975. Binding of nonsuppressible insulin-like activity to human serum. Evidence for a carrier protein. *Arch. Biochem. Biophys.* 168: 638-645.
14. Kaufmann, U., J. Zapf, B. Torretti, and E. R. Froesch. 1977. Demonstration of a specific serum carrier-protein of nonsuppressible insulin-like activity *in vivo*. *J. Clin. Endocrinol. Metab.* 44: 160-166.
15. Megyesi, K., C. R. Kahn, J. Roth, and P. Gorden. 1975. Circulating NSILA-S in man. Preliminary studies of stimuli *in vivo* and of binding to plasma components. *J. Clin. Endocrinol. Metab.* 41: 475-484.
16. Meuli, C., J. Zapf, and E. R. Froesch. 1978. NSILA-carrier protein abolishes the action of nonsuppressible

- insulin-like activity (NSILA-S) on perfused rat heart. *Diabetologia*. 14: 255-259.
17. Meuli, C., and E. R. Froesch. 1976. Binding of insulin and nonsuppressible insulin-like activity to isolated perfused rat heart muscle. Evidence for two separate binding sites. *Arch. Biochem. Biophys.* 177: 31-38.
 18. Meuli, C., and E. R. Froesch. 1977. Insulin and nonsuppressible insulin-like activity stimulate the same glucose transport system via two separate receptors in rat heart. *Biochem. Biophys. Res. Commun.* 75: 689-695.
 19. Schoenle, E., J. Zapf, and E. R. Froesch. 1977. Effects of insulin and NSILA on adipocytes of normal and diabetic rats: receptor binding, glucose transport and glucose metabolism. *Diabetologia*. 13: 243-249.
 20. Zapf, J., M. Mäder, M. Waldvogel, D. S. Schalch, and E. R. Froesch. 1975. Specific binding of nonsuppressible insulin-like activity to chicken embryo fibroblasts and to a solubilized fibroblast receptor. *Arch. Biochem. Biophys.* 168: 630-637.
 21. Rodbell, M. 1964. Metabolism of isolated fat cells. 1. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* 239: 375-380.
 22. Gliemann, J., K. Osterlind, J. Vinten, and S. Gammeltoft. 1972. A procedure for measurement of distribution spaces in isolated fat cells. *Biochim. Biophys. Acta.* 286: 1-9.
 23. Vinten, J., J. Gliemann, and K. Osterlind. 1976. Exchange of 3-O-methylglucose in isolated fat cells. *J. Biol. Chem.* 251: 794-800.
 24. Schoenle, E., J. Zapf, and E. R. Froesch. 1979. Receptor binding and effects of insulin and NSILA-S on glucose transport and metabolism in adipocytes from hypophysectomized rats. *Diabetologia*. 16: 41-46.
 25. Schoenle, E., J. Zapf, and E. R. Froesch. 1976. Binding of nonsuppressible insulin-like activity (NSILA) to isolated fat cells: evidence for two separate membrane acceptor sites. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 67: 175-179.
 26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.
 27. Rasio, E. A., E. Mack, R. H. Egdahl, and M. G. Herrera. 1968. Passage of insulin and inulin across vascular membranes in the dog. *Diabetes*. 17: 668-672.
 28. Zapf, J., and E. R. Froesch. 1979. Binding protein(s) for NSILA in serum, protein binding assay and serum NSILA levels in health and disease. In Proceedings of the International Symposium on Somatomedins and Growth. Santa Margherita Ligure, Italy. 1-3 March.
 29. Poffenbarger, P. L. 1975. The purification and partial characterization of an insulin-like protein from human serum. *J. Clin. Invest.* 56: 1455-1463.
 30. Zapf, J., G. Jagars, I. Sand, and E. R. Froesch. 1978. Evidence for the existence in human serum of large molecular weight nonsuppressible insulin-like activity (NSILA) different from the small molecular weight forms. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 90: 135-140.
 31. Morell, B., and E. R. Froesch. 1973. Fibroblasts as an experimental tool in metabolic and hormone studies. II. Effects of insulin and nonsuppressible insulin-like activity (NSILA-S) on fibroblasts in culture. *Eur. J. Clin. Invest.* 3: 119-123.
 32. Rechler, M. M., J. M. Podskalny, I. D. Goldfine, and C. A. Wells. 1974. DNA synthesis in human fibroblasts. Stimulation by insulin and nonsuppressible insulin-like activity (NSILA-S). *J. Clin. Endocrinol. Metab.* 39: 512-521.
 33. Zingg, A. E., and E. R. Froesch. 1973. Effects of partially purified preparations with nonsuppressible insulin-like activity (NSILA-S) on sulfate incorporation into rat and chicken cartilage. *Diabetologia*. 9: 472-476.
 34. Froesch, E. R., J. Zapf, T. K. Audhya, E. Ben-Porath, B. J. Segen, and K. D. Gibson. 1976. Nonsuppressible insulin-like activity and thyroid hormones: Major pituitary dependent sulfation factors for chick embryo cartilage. *Proc. Natl. Acad. Sci. U. S. A.* 73: 2904-2908.
 35. Chochinov, R. H., and W. H. Daughaday. 1976. Current concepts of somatomedin and other biologically related growth factors. *Diabetes*. 25: 994-1007.
 36. Zapf, J., E. Rinderknecht, R. E. Humbel, and E. R. Froesch. 1978. Nonsuppressible insulin-like activity (NSILA) from human serum: recent accomplishments and their physiologic implications. *Metab. Clin. Exp.* 27: 1803-1828.
 37. Daughaday, W. H., W. D. Salmon, Jr., and F. Alexander. 1959. Sulfation factor activity of sera from patients with pituitary disorders. *J. Clin. Endocrinol. Metab.* 19: 743-758.