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# THE EFFECTS OF NONSUPPRESSIBLE INSULIN-LIKE PROTEIN (NSILP) ON CYCLIC NUCLEOTIDE METABOLISM IN RAT LIVER

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## SUMMARY

Nonsuppressible insulin-like protein (NSILP), 100 ng/ml, inhibited cyclic AMP accumulation in rat liver, as stimulated by glucagon, $10^{-7}$M, from 493 ± 12 to 183 ± 7 pmoles/gm tissue ($p<0.001$), but did not alter basal levels of cyclic AMP, 143 ± 2 pmoles/gm tissue. NSILP, 100 ng/ml, also inhibited cyclic AMP accumulation, stimulated by epinephrine, 5 X $10^{-4}$M, from 387 ± 12 to 233 ± 9 pmoles/gm tissue. With 1 µM as substrate, NSILP, 100 ng/ml, increased cAMP-dependent phosphodiesterase activity in liver slices from 19.08 ± 0.18 to 24.94 ± 0.38 pmoles cAMP hydrolyzed/mg protein/min ($p<0.001$), but did not alter this enzyme activity in broken cell preparations of rat liver. Cyclic GMP levels in liver slices, 22.5 ± 0.3 pmoles/gm tissue, were increased by NSILP to 36.3 ± 0.5 pmoles/gm tissue ($p<0.01$). NSILP had no effect on adenylate cyclase activity. These changes, caused by NSILP in cyclic nucleotide metabolism in liver, resemble those described for insulin, and suggest that alterations in cyclic nucleotide levels in liver may be relevant to other hepatic effects of NSILP.

## INTRODUCTION

Nonsuppressible insulin-like protein (NSILP) is one of the growth-promoting factors in human serum (1-3), and may have insulin-like effects on glucose and fat metabolism in various tissues (4). In part, insulin modifies glucose and fat metabolism by altering cyclic nucleotide metabolism. Insulin suppresses cyclic AMP accumulation in target tissues (5-7) via an indirect stimulation of low Km, cAMP-dependent phosphodiesterase activity (8-12), and may increase cyclic GMP levels in target tissues (13, 14). Whether NSILP affects cyclic nucleotide metabolism as insulin is not established. The purposes of the present study were to determine if NSILP has effects similar to insulin on cyclic AMP metabolism and cyclic GMP accumulation in preparations of rat liver.

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## MATERIALS AND METHODS

NSILP was prepared from out-dated Blood Bank plasma as previously described (15). Partially purified NSILP, representing the active fraction derived from the G-200 fractionation step, was employed because of the scarcity of completely purified product. This fraction exhibited a biological specific activity of 5.4 mU NSILA/mg protein assayed by the rat epididymal fat pad segment insulin bioassay (16). NSILP comprised 20% of the protein by weight, determined by NSILP specific immunoassay. Quantitative immunoelectrophoresis determined that the G-200 NSILP fraction contained albumin as the major contaminant (72%) plus haptoglobin (6%) and trace transferrin (<1%). These contaminants were not biologically active in the bioassay, and did not cross-react in either the NSILP immunoassay or liver membrane NSILP receptor assay. Nevertheless, bovine serum albumin (Fraction V), was added as a control to all experiments. No detectable insulin was found in the NSILP G-200 fraction by insulin radioimmunoassay (16).

All experiments were carried out on liver obtained immediately after the sacrifice of fed, Sprague-Dawley rats, 200-250 mg. Animals were sacrificed by cervical fracture. The livers were removed from the animals and placed in iced sucrose, 0.25 M, until further use.

For the generation of cyclic nucleotides, slices of rat liver, 40-70 mg, were made with a Stadie-Riggs microtome and preincubated in Krebs-Ringers-bicarbonate buffer (17), at 37°C., under a 95% $O_2$ - 5% $CO_2$ atmosphere for 30 minutes. Bovine serum albumin, (Fraction V, Sigma Chemical Co., St. Louis, Missouri), 250 mg/100 ml, and dextrose, 200 mg/100 ml, were added. For the generation of cyclic GMP only, theophylline, 180 mg/100 ml, was also added. After 30 minutes, the slices were removed from the preincubates and placed immediately in fresh incubates, containing the same substances plus the hormones under study or their diluent, bovine serum albumin. The incubations were terminated after 5 or 10 minutes by removing the slices from the incubates and quick-freezing them in liquid nitrogen. As described by Chase (18), the slices were boiled for 10 minutes in 0.05 acetate buffer, pH 6.25, and homogenized in the acetate buffer with Duall glass grinding tubes and a mechanical homogenizer. The homogenates were then centrifuged at 10,000 xg for 30 minutes, again quick-frozen in liquid nitrogen, thawed, and recentrifuged at 10,000 xg for 30 minutes, to remove substances, which may interfere with the radioimmunoassay for cyclic AMP or cyclic GMP, as described by Steiner, et al (19), and by Chase (18). The supernatant from the second 10,000 xg centrifugation was retained for radioimmunoassay of cyclic AMP and cyclic GMP. Radioimmunoassay of these cyclic nucleotides was carried out, according to the method of Steiner, et al (20), using a commercial kit (New England Nuclear, Boston, Massachusetts).

The reliability of methods for measuring cyclic nucleotides were confirmed, in addition to the validation claimed by Chase (18). [$^3$H]-cAMP and [$^3$H]-cGMP, repurified over neutral alumina (21), were added to homogenates of liver slices, prepared as described below, just prior to the quick-freezing step of the tissue fixation. After quick freezing and boiling these homogenates, as in the fixation of the liver slices, the conversion of [$^3$H]-cAMP or [$^3$H]-cGMP to [$^3$H]-AMP or [$^3$H]-GMP by endogenous phosphodiesterase activity was estimated, using our modification (22) of the method of Rutten et al (23) for cAMP-dependent phosphodiesterase activity and the method of Russell, et al (24) for cGMP-dependent phosphodiesterase activity. Less than 6% of the [$^3$H]-cAMP or [$^3$H]-cGMP was catabolized during tissue fixation. The reliability of the radioimmunoassay method was confirmed by documenting that there was no cross-reactivity between cyclic AMP and cyclic GMP, over a wide concentration range. In some experiments, the cyclic nucleotide content of samples were also assayed after acetylating the samples (25). The results using acetylated versus non-acetylated samples correlated very well (r=0.955); however, the cyclic nucleotide levels measured in these liver slices were within the range of

the assay using non-acetylated samples more than acetylated samples. Therefore, the experiments presented report values using non-acetylated samples.

Assays of phosphodiesterase or adenylate cyclase activity were carried out on homogenates or fractions of homogenates, prepared from fresh rat liver, or liver preincubated for 10 minutes in Krebs buffer with dextrose and bovine serum albumin added, as described above. Whole liver or liver slices were minced with surgical scissors and then homogenized over ice in 25 mM Tris HCI, pH 7.4, 800 µl/100 mg tissue weight, with a Ten Broeck glass homogenizer. Homogenates were filtered once over gauze before further use. In some experiments, partially purified plasma membranes were prepared from the homogenates by the method of Marx, et al (26). In others, a 50,000 xg supernatant was separated from the homogenates by centrifuging a partially purified, 2000 xg supernatant for 30 minutes.

Cyclic AMP-dependent phosphodiesterase activity was estimated by our modification (22) of the method of Rutten et al (23). In most experiments, cyclic AMP, 1 µM, was used as substrate, and assays were carried out on partially purified plasma membranes from liver homogenates in order to preferentially activate the low Km enzyme form (27). In other experiments, assays were carried out on whole homogenates; or the 50,000 xg supernatants of liver homogenates was used with cyclic AMP, 100 µM, in order to perferentially activate the high Km enzyme form (27). Adenylate cyclase activity in whole homogenates or partially purified plasma from homogenates of rat liver, was assayed in the presence of adenosine triphosphate (ATP), 1.6 mM, with an ATP-regenerating system by the method of Krishna, et al (28), with the modification of Ramachandran (29) to separate cyclic AMP from its precursor. Details of our method have been published previously (22, 30). Guanylate cyclase activity was assayed by the method of Helwig, et al (31) on whole homogenates, or 50,000 xg supernatants from liver homogenates. Protein concentrations in these assays were estimated by the method of Lowry, et al (32).

All statistics were analyzed on a TI-59 programmable calculator (Texas Instruments, Dallas, Texas), using programs and a program module supplied by the manufacturer. Statistical significance was evaluated by paired T-tests.

## RESULTS

A physiologic concentrations of NSILP, 100 ng/ml (16), had no significant effect on basal levels of cyclic AMP in rat liver slices, but did decrease cyclic AMP accumulation, as stimulated by glucagon, $10^{-7}$M, or epinephrine, 5 X $10^{-4}$M (Table 1). Subsequent experiments were carried out to determine if this inhibitory effect of NSILP on cyclic AMP accumulation may be related to changes in either cAMP-dependent phosphodiesterase activity or adenylate cyclase activity. In intact liver slices, with 1 µM cAMP as substrate, NSILP, 100 or 500 ng/ml significantly increased cAMP-dependent phosphodiesterase activity (Table 2). Lesser concentrations of NSILP were ineffective. In contrast, phosphodiesterase activity, which was 17.7 $\pm$ 0.2 pmoles cAMP hydrolyzed/mg protein/min when assayed in whole homogenates of rat liver, or 18.30 $\pm$ 0.12 when assayed in partially purified plasma membranes from liver homogenates with cyclic AMP, 1 µM as substrate, was not altered by the direct addition of NSILP, 50-500 ng/ml. No

Table 1

Effects of NSILP on Basal and Hormonally-Stimulated Cyclic AMP Accumulation in Slices of Rat Liver *

| Addition | Cyclic AMP Concentration ** (pmoles cAMP/gm wet tissue weight) | p< |
|---|---|---|
| bovine serum albumin | 143 ± 2 | - |
| NSILP, 100 ng/ml | 139 ± 4 | ns |
| glucagon, $10^{-7}$M | 439 ± 12 | 0.001 |
| glucagon, $10^{-7}$M, + NSILP, 100 ng/ml | 183 ± 7 *** | 0.05 |
| epinephrine, 5 X $10^{-4}$M | 387 ± 12 | 0.001 |
| epinephrine, 5 X $10^{-4}$M, + NSILP, 100 ng/ml | 233 ± 9 **** | 0.01 |

* Slices were preincubated for 30 minutes and then incubated in the additions noted for 10 minutes, as described in the text.

** Values represent the mean ± S.E.M. of 11 determinations

*** $p<0.001$ for difference between this value and value with glucagon, alone.

**** $p<0.01$ for difference between this value and value with epinephrine alone.

direct effect of NSILP could be demonstrated on adenylate cyclase activity in partially purified plasma membranes from liver homogenates. Basal adenylate cyclase activity, 81.6 ± 1.7 pmoles cAMP formed/mg protein/10 min, and adenylate cyclase activity stimulated by glucagon, $10^{-9}$M, 394.9 ± 11.1, or epinephrine, 5 X $10^{-4}$M, 355.4 ± 5.6 pmoles cAMP formed/mg protein/10 min, were unaffected by NSILP, 100 ng/ml or 500 ng/ml.

NSILP, 100 or 500 ng/ml, also increased the levels of cyclic GMP in liver slices (Table 3). Lesser concentrations of NSILP caused small, insignificant increases. However, these same concentrations of NSILP had no direct effect on guanylate cyclase activity. In whole homogenates guanylate cyclase activity was 15.5 ± 0.2 pmoles cGMP formed/gm protein/10 min in the absence of NSILP, and 15.0 ±

Table 2

Effects of NSILP on cAMP-Dependent Phosphodiesterase Activity in Liver Slices*

| Addition | Phosphodiesterase Activity** (pmoles cAMP hydrolyzed/mg protein/min) | p< |
|---|---|---|
| bovine serum albumin*** | 19.08 ± 0.18 | - |
| NSILP, 50 ng/ml | 17.96 ± 0.42 | ns |
| NSILP, 100 ng/ml | 24.94 ± 0.38 | 0.001 |
| NSILP, 500 ng/ml | 25.54 ± 1.69 | 0.005 |

* Slices were preincubated, as described in the text, in bovine serum albumin or NSILP for 10 minutes, before being homogenized and assayed for phosphodiesterase activity. cAMP, 1 μM, was added as substrate.

** Values represent the mean ± S.E.M. of 8 determinations.

*** Diluent of NSILP, added at the appropriate concentrations.

0.2, 15.9 ± 0.2 and 16.2 ± 0.5 in the presence of NSILP, 50, 100 and 500 ng/ml, respectively. Similar observations were made with 50,000 xg supernatants from liver homogenates (not shown).

## DISCUSSION

Serum contains several proteins capable of exerting insulin-like effects in various tissue in vitro, and following injection in vivo (1-3). Serum non-suppressible insulin-like activity (NSILA) is the term used to describe the total insulin-like bioactivity of serum not suppressed by insulin specific antiserum (4, 16, 33). Serum NSILA represents the composite effect of two related but distinct insulin-like proteins, one a large molecular weight (88,000 Daltons) protein or protein complex, the other a smaller molecular weight protein(s) (5,700 Daltons) which is carried in blood by a specific transport protein (15, 34). The larger species is

Table 3

Cyclic GMP Concentrations in Rat Liver Slices *

| Addition | Cyclic GMP Concentrations** (pmoles cGMP/gm wet weight) | p< |
|---|---|---|
| bovine serum albumin | 22.5 ± 0.3 | - |
| NSILP, 50 ng/ml | 25.7 ± 1.0 | ns |
| NSILP, 100 ng/ml | 36.3 ± 0.5 | 0.01 |
| NSILP, 500 ng/ml | 37.5 ± 0.6 | 0.01 |

* Slices, 40-70 mg, were preincubated for 30 minutes as described in the text, and then incubated for 10 minutes in the substances noted.

** Values represent the mean ± S.E.M. of 10 determinations.

called non-suppressible insulin-like protein (NSILP) and contributes 60 to 90% of the total NSILA of serum. The smaller species is called NSILA-s and contributes 10 to 30% of total serum NSILA (15, 35). The exact molecular relationship between NSILP and NSILA-s remains poorly defined but homology may exist in their respective structures, (16).

Both NSILP and NSILA-s have been found elevated in the plasma of patients experiencing profound hypoglycemia associated with extra-pancreatic tumors (36, 37). Recently NSILP production by one such tumor has been reported (36). NSILA-s appears to exert its insulin-like effect in insulin-like effect in insulin target tissues by cross-reaction with insulin receptors (38); however, NSILP shows no cross-reactivity in similar receptor systems (16, 39, 40). Therefore NSILP may act by enhancing effects of insulin on target cells, or by exerting insulin-like effects via different receptors specific for NSILP. That NSILP may augment effects of insulin on target cells was recently suggested by the demonstration that NSILP may enhance the binding of insulin to insulin-specific receptors (16, 39). This study focused on whether NSILP itself has insulin like effects on target cells.

We demonstrated that NSILP, as insulin (5-7), suppresses the stimulation of cyclic AMP accumulation by glucagon and epinephrine in liver. As insulin (8-12),

NSILP may inhibit cyclic AMP accumulation via an indirect stimulation of low Km, cAMP-dependent phosphodiesterase activity in liver. This effect is believed to be indirect, because NSILP, as insulin, increases phosphodiesterase activity in preparations of intact cells only, and has no effect on the enzyme in broken cell preparations. We are unable to show any effect of NSILP on adenylate cyclase activity in liver. Some investigators have reported that insulin may inhibit hormonally-stimulated adenylate cyclase activities (41-43); however this possible effect of insulin has not been confirmed in all studies (44-47). We also observed a stimulation of cyclic GMP accumulation in rat liver slices by NSILP. Insulin has been observed to have a similar effect (13, 14). As insulin (46), NSILP may not directly enhance cyclic GMP formation, because it does not stimulate guanylate cyclase activity. Thus, NSILP appears to cause changes in the hepatic metabolism of cyclic nucleotides, which are similar to those caused by insulin.

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