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A self-regulating insulin delivery system. II. In vivo characteristics of a synthetic glycosylated insulin

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Summary

These studies were designed to evaluate some of the in vivo characteristics of the insulin derivative, succinyl amido phenyl glucopyranoside insulin (SAPG-insulin). The in vitro bioeffectiveness studies in isolated rat fat cells demonstrated that the monosubstituted SAPG-insulin derivatives retained their full biological activity, while disubstituted SAPG-insulin had a reduced bioactivity. This is in good agreement with previous in vivo biological assay results. The antigenicity of both SAPG-insulin and native bovine insulin was examined in a rabbit model; no antibody formation was observed, indicating that the derivatization of insulin does not result in an immune response. In addition, plasma disappearance and liver uptake studies in dogs demonstrated that SAPG-insulin is handled in the same manner as native insulin in vivo.

Introduction

A glycosylated insulin, succinyl amido phenyl-α-D-glucopyranoside insulin (SAPG-insulin) was synthesized and characterized for use in the self-regulating insulin delivery system (Jeong et al., 1984; Seminoff et al., 1989). Clinical utility of this system requires demonstration that the biological properties of the glycosylated insulin are comparable to those of native insulin. In this paper the biological potency, immunological activity, and pharmacokinetic behavior of SAPG-insulin are assessed in several animal models.

The in vitro bioeffectiveness of the insulin derivative was evaluated by its ability to stimulate glucose oxidation and lipogenesis in isolated rat fat cells. These results were compared with previously obtained in vivo results.

An important facet of the in vivo characterization of SAPG-insulin is a demonstration that the glycosylated insulin does not cause an immunological effect in vivo. The immunological response to glycosylated insulin was assessed in rabbits by measuring the antibody response after immunization.

Pharmacokinetic studies included determination of plasma half-life following i.v. injection,

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and determination of first-pass hepatic uptake in a canine model. In order to estimate hepatic uptake of glycosylated insulin and commercial bovine insulin, the disappearance of measurable insulin following intraportal and vena caval injection were compared. In addition the effect of different heparin concentrations on hepatic uptake was shown.

Materials and Methods

Materials

[U-^{14}C]glucose was obtained from ICN Biomedical Inc. and [3-^{3}H]glucose was purchased from New England Nuclear. Crude collagenase was obtained from Worthington Biochemical Corp. Innovar Vet was purchased from Pitman-Moore, and sodium heparin from Invenex Labs. Bovine albumin and insulin were purchased from Sigma Chemical Co., and SAPG-insulin was synthesized and purified as described in previous papers (Jeong et al., 1984; Seminoff et al., 1989). The fractions of SAPG-insulin which were used in these studies were the B-1 phenylalanine monosubstituted, A-1 glycine monosubstituted and the A-1 glycine, B-1 phenylalanine disubstituted derivatives. Native bovine insulin was used as a control.

In vitro bioeffectiveness

The adipocyte preparation for these experiments was performed using methods outlined by Cosmatos et al. (1978) and Gliemann (1965). Briefly, the epididymal fat pads of male Wistar rats (150–200 g) were removed and dissected to eliminate blood vessels and connective tissue. The fat cells were then incubated for 45 min at 37 °C in a metabolic shaker (130 cycles/min), with collagenase (3 mg/ml) in Krebs–Ringer buffer (KRB) with 3% albumin. The digested contents were filtered through a nylon screen and the suspended fat cells were centrifuged in polyallomer tubes for 2 min at 1400 rpm. The sedimented stromal–vascular cells were removed by gentle aspiration. The fat cells floating on the surface were washed by suspension in 37 °C KRB–albumin buffer followed by centrifugation and decantation, this procedure was repeated 4 times.

The insulin stimulated $^{14}CO_2$ production from labelled glucose was measured by counting the amount of $^{14}CO_2$ produced by incubation of the fat cells and ^{14}C-labelled glucose in the presence of either native or fractions of SAPG-insulin (Gliemann and Gammeltoft, 1974). Fat cells were incubated at 37 °C for 2 h in a metabolic shaker (56 cycles per min) with 1 ml KRB-albumin buffer containing 0.5 mM ^{14}C-glucose and 0–133 nmol of the test insulin. After 2 h, the vials were fitted with CO_2 traps which were subsequently counted for DPM's of $^{14}CO_2$ retained by the trap (1500 TRI-CARB™ Liquid Scintillation Analyzer, Packard Instrument Co.).

Insulin stimulated lipogenesis was measured by determination of the amount of tritium from [^{3}H]glucose incorporated into glycerol (Moody et al., 1974). This was accomplished using the same methodology described for glucose oxidation, except [^{3}H]glucose was utilized instead of [^{14}C]glucose. The glycerol produced was extracted into a toluene-based scintillation fluid and counted.

Immunological studies

The immune system response to glycosylated insulin was tested in rabbits. New Zealand white female rabbits weighing less than 3 kg were used, and each sample was tested in two rabbits. The potential antigens tested were bovine insulin, unseparated SAPG-insulin, and SAPG-albumin (synthesized from bovine serum albumin in a manner similar to SAPG-insulin). The albumin conjugate served as a positive control, since bovine albumin is known to elicit an immune response in rabbits.

The rabbits were given a local intramuscular analgesic, Innovar Vet, which is also a potent vasodilator (Stickrod and Chadwick, 1982). Initially, 3 ml of blood was withdrawn from the ear vein to serve as a control for determination of antibody production. On day 1 of the experiment, an emulsion containing 1 mg of sample in 2 ml of isotonic buffer and 2 ml of Freund's complete adjuvant was injected i.m. into several sites. Two weeks later the same procedure was repeated, except the booster injection contained only 200 ng

of sample and was made with incomplete Freund's adjuvant. This step was repeated every two weeks until a total of 4 boosters had been given. Two weeks following the last booster, the final blood samples were taken.

The presence of antibody was detected using a radial immunodiffusion plate method (Jurd, 1981). A 1% agarose gel solution was cast on a thin plastic film. Several sets of center wells with 5 surrounding wells were punched into the gel. Then, 2–4 μl of 1 mg/ml sample (test antigen) was placed in the center of the well and dilute solutions of test rabbit serum were placed in the surrounding wells. The gels were kept in a moist environment for 1 week and then stained with Coomassie blue R250 to determine if an antibody–antigen complex had formed.

Plasma disappearance and liver uptake studies

Healthy mongrel dogs were used for all studies. Animals were fasted for 16–18 h and anesthetized with i.v. sodium pentobarbital (USP) before each study. Dextrose (5% in water) was infused as needed during the study to maintain blood glucose concentrations between 50 and 70 mg/dl. The SAPG-insulin fraction utilized in all dog studies was the B-1 phenylalanine monosubstituted component.

Three dogs were studied for the plasma disappearance experiments. Intravenous cannulae were placed in two limb veins and sodium heparin (300 U/kg) was administered. Basal insulin concentrations were determined. SAPG-insulin or native insulin (2 μg/kg in 5 ml isotonic saline) was injected as a bolus into one limb vein and blood samples (1 ml) were taken from another vein at 2 min intervals for 20 min following injection.

The dogs for the hepatic uptake studies were fasted and anesthetized as before, and a ventral midline incision was made. For the sampling, one femoral vein was cannulated with a Swan–Ganz catheter and the tip of the catheter was advanced to the inferior vena cava (IVC) 6 cm anterior to the diaphragm. This permitted sampling of blood from the IVC cephalad to the entry of the hepatic vein. To facilitate mixing of blood derived from the hepatic vein and distal IVC, the balloon was left partially inflated. An 18 gauge i.v. catheter was inserted into a mesenteric vein and advanced to the level of the main portal vein (3 cm caudal to the liver), for injection of insulin or SAPG-insulin. The other femoral vein was cannulated with a single lumen cannula which was advanced to the level of the terminal IVC. The dog was then i.v. injected with either 100 U/kg or 300 U/kg of sodium heparin.

At 10 s after beginning free flow collection of 1 ml (in 10 seconds) aliquots of blood from the IVC Swan–Ganz catheter, a 10 s bolus of test insulin (2 μg/kg) was given into the portal vein. The collection of aliquots every 10 s from the IVC was continued for 3 min. All catheters were flushed with heparinized saline after use. The procedure was repeated, except the insulin was infused into the terminal IVC. These experiments were performed on at least 3 dogs for each test insulin. Blood glucose levels were monitored with a dextrometer before and after each infusion and there was a 20 min break between injections. Plasma insulin levels were determined using a commercially available (Micromedic) radioimmunoassay kit as described by Seminoff et al. (1989).

Results and Discussion

In vitro bioeffectiveness

Glucose oxidation and lipogenesis were stimulated by both native insulin and various SAPG-insulin derivatives. Table 1 shows the maximal effects (±S.D.) for each test insulin in both experiments. B-1 monosubstituted SAPG-insulin re-

TABLE 1

Maximal effects of insulins (% conversion)

Insulin type	Oxidation	Lipogenesis
B-1 monosubstituted SAPG-insulin	99.5 ± 3.9	98.4 ± 3.0
A-1 monosubstituted SAPG-insulin	90.6 ± 8.6	86.1 ± 2.1
A-1, B-1 disubstituted SAPG-insulin	74.8 ± 1.5	69.9 ± 8.1
Bovine insulin	99.4 ± 4.8	97.3 ± 2.0

TABLE 2

Immunological study results

	Week 0	Week 2	Week 4	Week 6	Week 8	Week 10
Bovine insulin	–	–	–	–	–	–
SAPG-insulin	–	–	–	–	–	–
SAPG-albumin	–	–	+	+	+	+

+ Denotes presence of antibody.
– Denotes absence of antibody.

tained virtually 100% of the effect of bovine insulin while the A-1 monosubstituted SAPG-insulin showed a slight decrease in its maximal effect. The A1, B1 disubstituted conjugate showed a significantly reduced biological activity which is in agreement with previous findings using the in vivo mouse convulsion assay (Seminoff et al., 1989).

Immunological studies

The immunological study results, using bovine insulin, unseparated SAPG-insulin and SAPG-albumin are shown in Table 2. The presence of a precipitation band indicated a positive immune response. The heterogeneous (unseparated) mixture of SAPG-insulin components showed no immunogenicity, therefore the testing of the separated homogeneous fractions of the glycosylated insulins was not performed. The only compounds which elicited an immunogenic response were the derivatized albumins and this was expected due to the antigenic properties of bovine albumin in rabbits. This response was specific for albumin when rabbit serum was tested separately against SAPG and albumin.

Thus, it has been demonstrated that SAPG-insulin is not apt to be antigenic. It has also been reported by researchers (Geiger et al., 1979) that substitution of insulin at the B-1 phenylalanine position actually decreases the immunogenicity of insulin. It was hypothesized that the area surrounding the B-1 phenylalanine site participates in the formation of the antibody combining site, and therefore, alterations at this site may decrease the affinity for the antibody (Lindsay and Shall, 1971).

Plasma disappearance

The experimentally derived plasma concentration vs time curves were modelled using a two exponential term function (two-compartment model).

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

A and B refer to the y-axis intercept of each exponential section of the curve and α and β are rate constants for the distribution and elimination phases, respectively. Natural log plots of the plasma insulin level versus time are shown in Figs. 1 and 2 and demonstrate that the disappearance of both bovine and SAPG-insulin follows a two-compartment model. The values for A, B, α, β and the corresponding half-lives, and elimination rate constants are given in Table 3.

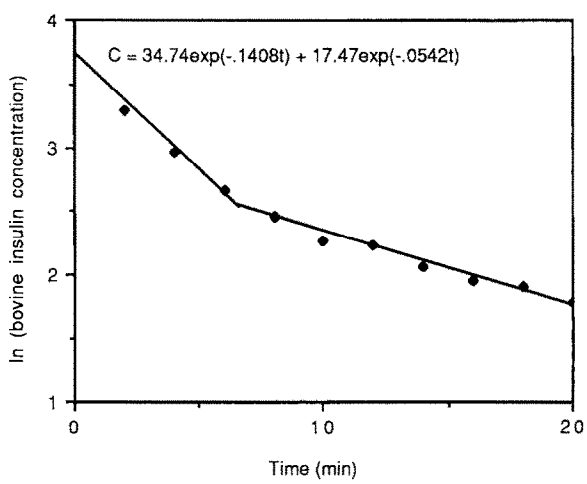

Fig. 1. Plasma concentration–time curve for bovine insulin following i.v. injection using a two-compartment model.

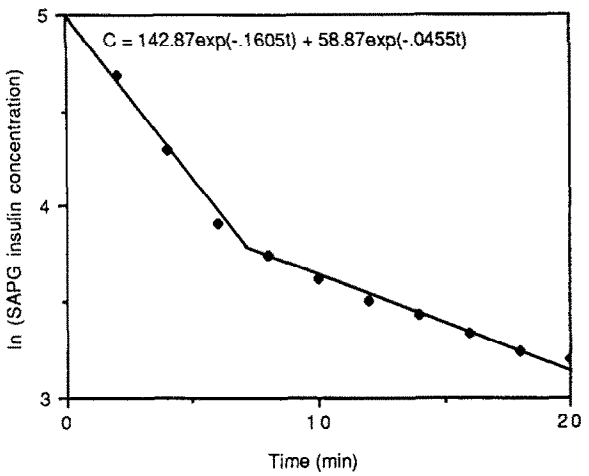

Fig. 2. Plasma concentration–time curve for SAPG-insulin following i.v. injection using a two-compartment model.

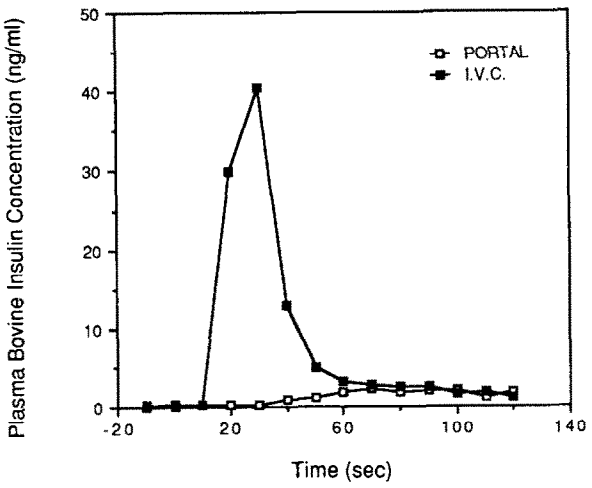

Fig. 3. Plasma concentration profiles of bovine insulin following injection into IVC and portal veins (100 U/kg heparin).

Although the rate constants α and β are very close for both insulins, the values of A and B are significantly larger for SAPG-insulin. This is believed to be due to the larger volume of distribution for bovine insulin ($V_d = (\text{dose}/A + B)$). Since bovine insulin is more hydrophobic than SAPG-insulin, it can partition to a greater extent into the second compartment, leading to reduced plasma concentrations, and lower values of A and B.

The elimination rate constants for both bovine and SAPG-insulin are quite similar (0.0917 min^{-1} and 0.0923 min^{-1}, respectively) and agree with data from other studies using porcine and human insulin (Brogard et al., 1987). In addition, the two-phase first order kinetics exhibited by the derivatives follows the predicted pattern of unmodified insulin plasma disappearance (Halban et al., 1981). This indicates that there are no significant differences between the elimination of SAPG-insulin vs native bovine, porcine or human insulin.

Hepatic uptake

Normally, 60–70% of insulin secreted into the portal system is bound by hepatic insulin receptors and removed from the circulation during the first pass. These studies were designed to determine if the same process occurred for SAPG-insulin.

The insulin levels in the vena cava cephalad to the liver following bolus insulin administration, either in the portal vein or IVC are shown in Figs. 3–6. The profiles shown in Figs. 3 and 4 were generated using heparin doses of 100 U/kg and the profiles in Figs. 5 and 6 were found using 300 U/kg heparin. The ratio of the areas under the curves represent the percent of the dose which was not absorbed by the liver. This allowed an esti-

TABLE 3

Pharmacokinetic parameters

Insulin	A (ng/ml)	B (ng/ml)	α (min^{-1})	β (min^{-1})	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	k_{el} (min^{-1})
Bovine	34.74	17.47	0.1408	0.0542	4.90	12.79	0.0917
B-1 mono-substituted SAPG	142.87	58.87	0.1605	0.0455	4.32	15.20	0.0923

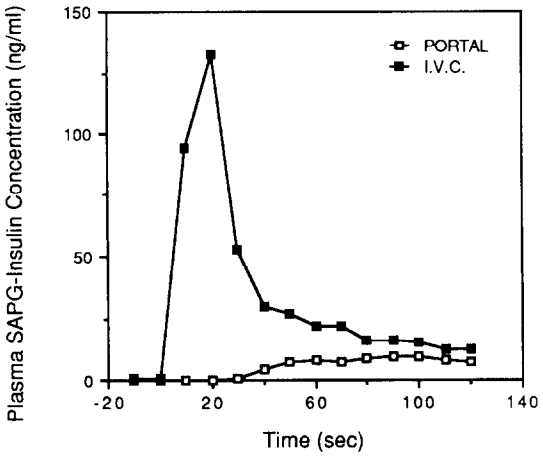

Fig. 4. Plasma concentration profiles of SAPG-insulin following injection into IVC and portal veins (100 U/kg heparin).

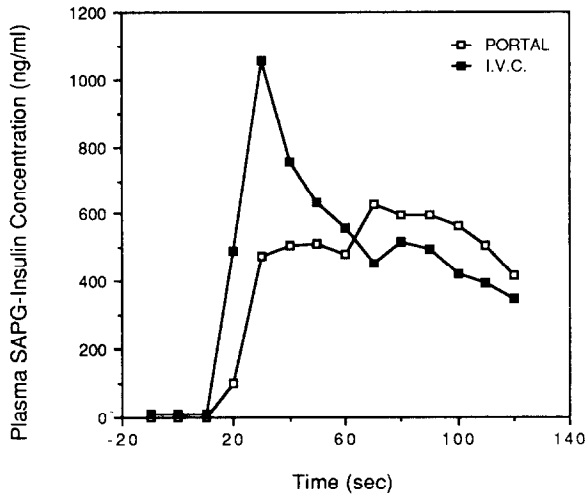

Fig. 6. Plasma concentration profiles of SAPG-insulin following injection into IVC and portal veins (300 U/kg heparin).

mate of hepatic uptake to be made without measuring actual blood flow rates. Although, the plasma concentrations of the insulins did not return to baseline levels within 2 min, this effect on the ratio of the areas under the curve should be minimal, and was neglected.

There were two important points elucidated by these experiments. First, native insulin and SAPG-insulin did not show significantly different liver uptake with either dose of heparin. Secondly, the dose of heparin markedly affected the liver uptake of native or glycosylated insulin. The low dose heparin experiment resulted in liver uptake of 86% and 89% for bovine and SAPG-insulin, respectively. The value for the high heparin dose was 13% for both insulins. These results suggest that heparin may alter insulin interaction with hepatic receptors.

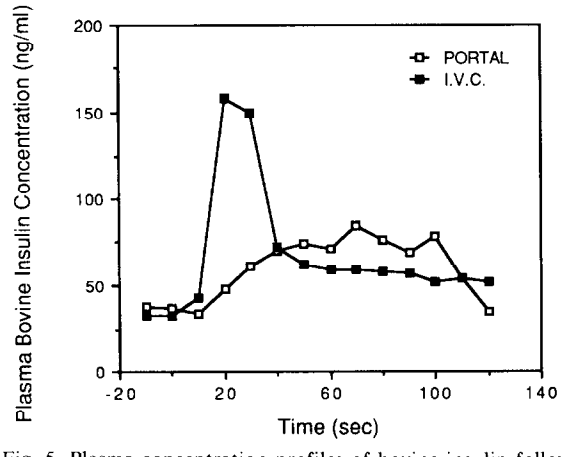

Fig. 5. Plasma concentration profiles of bovine insulin following injection into IVC and portal veins (300 U/kg heparin).

Heparin is a large, highly sulphated, negatively charged glycosaminoglycan which occurs naturally in many tissues. It is known to modify the activity of many proteins, and has been shown to interact with several receptors including fibroblast low-density lipoprotein receptors (Brown et al., 1978) and hepatic glucocorticoid receptors (Hubbard and Kalami, 1983). Kriauciunas et al. (1987) have shown in cultured human lymphocytes that heparin, in concentrations comparable to those used in these experiments, decreased both the number and affinity of insulin receptors in a dose-dependent manner. These results suggest that a similar heparin-induced deactivation of hepatic insulin receptors is occurring in vivo.

Conclusions

It is apparent from these studies that the in vivo behavior of SAPG-insulin and bovine insulin do not differ significantly. The biological activity of this insulin derivative is equivalent to that of bovine insulin as measured by both in vitro and in

vivo assays. In addition, no immunological response was measured in a rabbit model. Finally, neither the plasma half-life, nor the liver uptake of SAPG-insulin differed measurably from bovine insulin. Therefore, these studies have demonstrated that in vivo administration of this glycosylated insulin derivative should have the same effect as that of native bovine insulin.

Acknowledgements

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