Intravenous Infusions of Sulfated Insulin Normalize Plasma Glucose Levels in Pancreatectomized Dogs

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SUMMARY

Sulfated insulin (SI) differs radically from regular crystalline zinc insulin (CZI). To date, SI has been used mainly for the subcutaneous treatment of diabetics with resistance or local allergic reactions to CZI. In this regard, SI exists as a soluble monomer at pH 7.4 and is not inclined to self-association even when agitated and exposed for long periods to materials known to aggregate CZI. To compare its stability and biologic activity when used in conjunction with intravenous infusion pumps, diabetic dogs were infused portally for 140 days with SI and for 140 days with CZI. These studies demonstrated a significant improvement of glycemic control obtainable with SI compared with CZI. Mean ± SD fasting glycemias were normalized for the SI group (99 ± 19 mg/dl) and were significantly ($P < 0.001$) less than the mean of 148 ± 64 mg/dl for the CZI group. Mean ± SD coefficient of variation of the fasting plasma glucose concentrations was 18 ± 1% for the SI- versus 43 ± 3% for the CZI-infused dogs, both significantly greater than normal values of 4.5 ± 0.5%. Basal insulin requirements under these conditions also differed significantly ($P < 0.001$). The CZI group received 0.35 ± 0.07 mU/kg/min compared with 0.20 ± 0.05 mU/kg/min for the SI group,[1] the former resulted in mean ± SD plasma levels of 14 ± 7 μU/ml and the latter resulted in concentrations of 47 ± 12 μU/ml. Insulin clearance rates were 28 ± 11 ml/kg/min with CZI compared with 5 ± 3 ml/kg/min with SI ($P < 0.001$). CZI but not SI resulted in progressive obstruction of the delivery systems employed. In conclusion, porcine SI is more stable and remarkably more resistant to aggregation than regular CZI preparations. It maintains its bioactivity and affords improved long-term glycemic control when infused i.v. With SI insulin requirements were reduced 1.75-fold while insulin clearance was reduced 5.6-fold compared with CZI. These findings reflect the essentially monomeric nature of the hormone and emphasize its remarkable stability in the difficult environment presented by a portable pumping system. DIABETES 32:788–792, September 1983.

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Previous long-term studies with intravenous insulin infusion in diabetic dogs resulted in good glycemic control only when the reservoirs were changed or flushed every 3–4 days.[1,2] Purified porcine insulins readily self-associate when exposed to various materials[3] and such aggregation is accelerated by motion.[4] Such aggregates include high-molecular-weight polymers of insulin and have reduced biologic activity.[5] When infused at a constant basal rate, aggregating insulin results in poor control of fasting glycemia and severely limits the usefulness of insulin delivery devices in which the hormone has to be stored for prolonged periods.[6] Most subcutaneous delivery devices minimize this problem since the insulin-containing component is replaced every day or so.[7–9] Furthermore, there is some evidence from animal studies that the intravenous infusion of such aggregates of insulin may be toxic.[10,11]

To circumvent these problems, we sought to find a stable insulin that was in monomeric form and would not self-associate or lose its bioactivity when exposed for long periods to the continuous motion and the materials of our portable pumping systems and their delivery catheters. Sulfated insulin (SI) appears to have some promise in this regard. However, its use has been restricted previously to subcutaneous treatment of the occasional patient with insulin resistance or allergy.[12,13] There has been only one report on its use in pumps.[14] We report here the significant improvements obtained in glycemic control of pancreatectomized dogs in-

fused with SI as compared with normal controls and similar diabetic animals infused with crystalline zinc insulin (CZI).

MATERIALS AND METHODS

Eleven male beagle dogs were used in these studies. Their body weights were all normal, averaging 13.8 ± 0.5 kg in the nondiabetic control group (N = 5), 14.3 ± 1.2 kg in the diabetic group (N = 3) infused with regular CZI, and 13.8 ± 0.5 kg in the SI-infused group (N = 3). All were fed in the morning at 1000 h with a 620-g mixed meal consisting of 260 g dry food (Master Premium Dinner, Maple Leaf Mills, Toronto, Ontario, Canada) and 360 g soft meal (Meat Mix, Derby Pet Foods, Toronto, Ontario, Canada). The light/dark cycle was 14/10 h.

Six of the dogs were pancreatectomized as previously described,[1,2] at which time two silicone rubber catheters were placed as detailed previously.[15] Catheters were placed (1) into an external jugular vein and (2) into the portal vein either via the splenic or a mesenteric vein, the former for blood sampling and the latter for exogenous insulin infusion. Insulin solutions were delivered from a refillable silicone rubber reservoir using a miniature peristaltic pump[16] and battery-powered flow-rate controller.[17] In the postprandial period, insulin infusion was accelerated above basal rates and for time durations in keeping with reducing the postprandial glycemic excursion to approximate the normal response without hypoglycemia, similar to methods detailed previously.[1,2]

Insulin solutions were prepared as follows. Porcine SI 100 U/ml (Connaught Laboratories Ltd., Toronto, Ontario, Canada) was diluted in buffered bacteriostatic water containing 1% benzyl alcohol and 10 mM TRIS-HCl to a final concentration of 0.5 U/ml and pH adjusted to 7.6. Regular CZI solutions (1 U/ml) were similarly prepared by diluting U100 highly purified porcine insulin (Nordisk Insulinlaboratorium, Gentofte, Denmark) into bacteriostatic water containing 1% benzyl alcohol, using either 10 mM TRIS-HCl, 1.2 mM bicarbonate, or 1% autologous serum all at pH 7.4. These insulin solutions were introduced into the silastic reservoirs carried on the dogs and refilled periodically through integral bacterial filters (Millex, GS0.22 μm filter unit, Millipore Corporation, Bedford, Massachusetts). Results obtained with the bicarbonate and serum were entirely similar to those obtained with the TRIS-HCl buffer and, therefore, only the latter was used in the present study.

Contents of the reservoirs were checked for pH change. Aggregates were recovered from the reservoirs containing CZI insulin, centrifuged, and repeatedly washed with distilled water. Samples were lyophilized and aliquots were prepared for examination at 1200–24,000× magnification in a scanning electron microscope (Model #JSM35, Jeol, Japan). Aliquots of approximately 50 mg were added to solutions of 6 M HCl, 6 M urea, 6 M guanidine HCl at pH 10.5, 150 mM sodium bicarbonate, and fresh human or canine serum.

Blood samples. Fasting blood samples were taken at 0900 h through an indwelling jugular catheter consecutively for 20 wk (5 samples/wk) in the pancreatectomized dogs for a total of 140 days. In the normal dogs, fasting blood samples were also taken in the morning at 0900 h but only on six occasions each. Samples were immediately transferred into 1.5-ml microtubes containing 0.03 ml heparin (1000 U/ml Hepalean, Harris Laboratories, Toronto, Ontario, Canada), kept on ice, and then centrifuged. After measuring glucose, plasma samples were immediately frozen at −20°C until assay for insulin concentration as described below.

Analytic methods. Fasting plasma glucose concentration was measured using a glucose-oxidase method (Beckman Instruments, Fullerton, California). Fasting insulin levels were measured by radioimmunoassay. In the nondiabetic dogs, immunoreactive insulin was assayed using porcine insulin standard and ^{125}I porcine insulin tracer (Novo Research Institute, Gentofte, Denmark), antiserum kindly provided by Dr. Peter H. Wright, and a dextran-coated charcoal separation technique.

For diabetic animals treated with porcine SI (Connaught Laboratories Ltd., Toronto, Ontario, Canada), the above assay was performed as for porcine insulin, but using SI standards with a potency of 5.75 U/mg.

A comparison of the standard curves obtained with regular and sulfated porcine insulins is shown in Figure 1. These curves are consistent with the expected decreased antibody binding of the SI. Adequate sensitivity is maintained, particularly at the lower concentrations, for the accurate measurement of this modified insulin. Plasma samples, when diluted, gave values that fell along the SI standard curve.

The inter- and intraassay coefficients of variation were, respectively, ±11.3% and ±4.0% at a concentration of 1 ng/ml. Statistical analyses were done using the unpaired *t* test. All results are presented as mean ± SD.

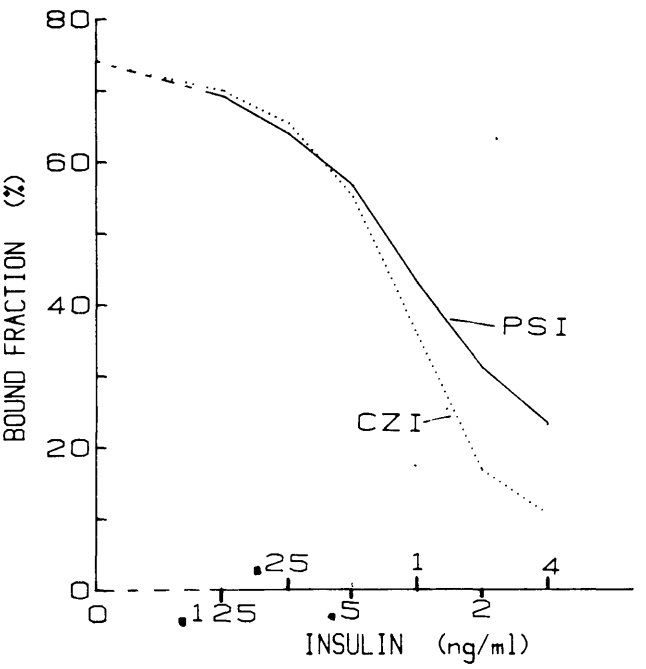

FIGURE 1. Typical binding curves for porcine sulfated (PSI) and porcine crystalline zinc (CZI) insulins in the radioimmunoassay described in METHODS. Potencies: 1 ng PSI = 5.75 μU; 1 ng CZI = 25.6 μU. The inter- and intraassay coefficients of variation of PSI were, respectively, ±11.3% and ±4.0% at a concentration of 1 ng/ml.

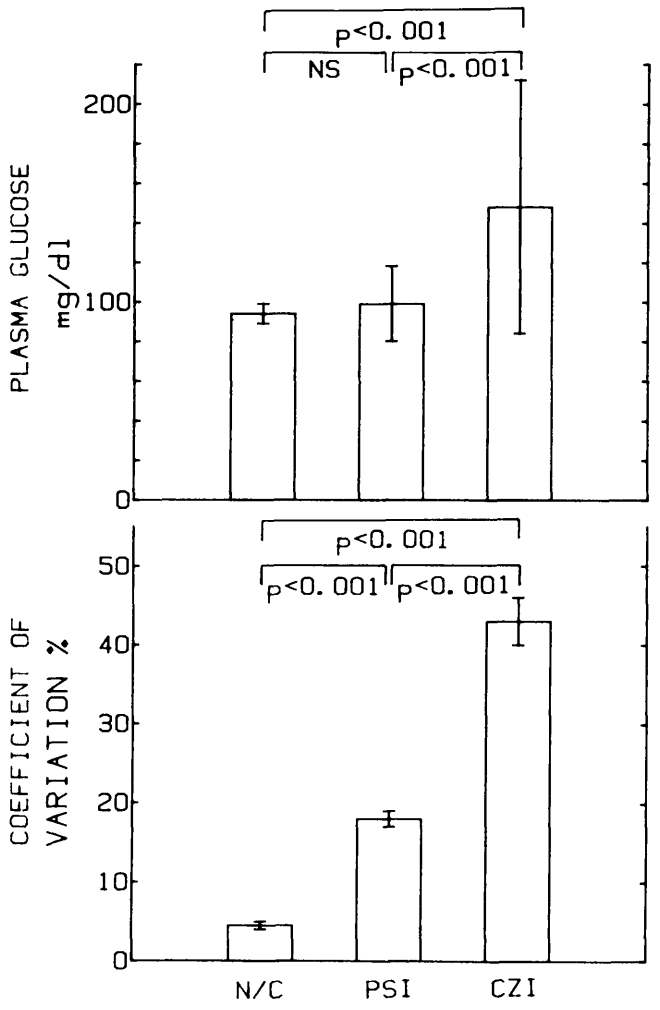

FIGURE 2. Mean fasting plasma glucose concentrations and their respective coefficients of variation of PSI in healthy controls (N/C, N = 5) and in diabetic dogs infused with porcine sulfated insulin (PSI, N = 3) and crystalline zinc insulin (CZI, N = 3) for 140 consecutive days. (Data shown as mean ± 1 SD.)

RESULTS

Mean ± SD fasting plasma glucose levels in the three groups are shown in Figure 2. Mean fasting glycemia in normal dogs was 94 ± 5 mg/dl. In diabetic dogs receiving SI, it was also normal (99 ± 19 mg/dl), but in those receiving CZI it was elevated (148 ± 64 mg/dl) significantly ($P < 0.001$). In these studies, obstructive occlusion occurred a total of six times in the group receiving CZI infusion. While in contrast, such episodes were never observed in the porcine SI-infused group. All solutions remained stable with respect to their initial pH. Aggregates recovered from the insulin reservoirs were generally of a heterogeneous nature but contained fibrillar structures. Aggregates were insoluble in 100% serum, 150 mM bicarbonate, 6 M HCl, or 6 M urea. However, dissolution could be achieved in 6 M guanidine HCl at pH 10.5.

Figure 2 also shows the coefficients of variation of the mean fasting plasma glucose concentrations in these three groups. In normal dogs it was 4.5 ± 0.5%, in the purified porcine insulin-infused group it was 43 ± 3%, while in the SI-infused group it was 18 ± 1%. Variation in fasting glycemia in the purified porcine insulin-infused group was significantly higher ($P < 0.001$) than either the normal or SI-infused groups. There was also a significant difference ($P < 0.001$) between the SI-infused group and the normals.

Figure 3 shows the mean ± SD fasting immunoreactive insulin (IRI) levels in the three groups. In normal animals, mean fasting IRI levels were 12 ± 6 μU/ml. In the purified porcine insulin-infused group, mean fasting IRI levels were 14 ± 7 μU/ml, similar to normal, while in the SI-infused group, mean fasting IRI levels (47 ± 12 μU/ml) were significantly elevated ($P < 0.001$). Mean ± SD basal portal infusion rates in the diabetic dogs were 0.20 ± 0.05 mU/kg/min for SI and 0.35 ± 0.07 mU/kg/min for CZI ($P < 0.001$). For reference, the generally accepted rate of endogenous insulin release in the basal fasting state in normal animals is considered to be 0.2 mU/kg/min.[18,19] Again for reference, the clearance rate of insulin in normal dogs is shown. Insulin clearance in the healthy animals fell between the calculated clearance rates for diabetic animals treated either with the SI or the CZI formulations. Insulin clearance in the fasting state in the SI group was 5.4 ± 3.7 ml/kg/min, significantly

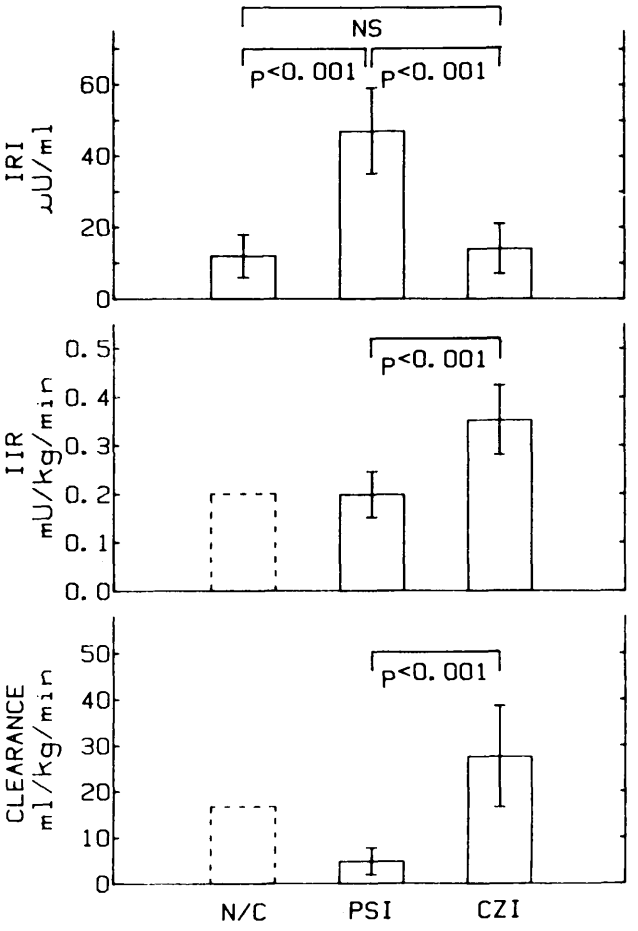

FIGURE 3. Fasting immunoreactive insulin levels (IRI), basal insulin infusion rates (IIR), and insulin clearances in healthy dogs (N/C, N = 5) and diabetic dogs infused with porcine sulfated insulin (PSI, N = 3) and crystalline zinc insulin (CZI, N = 3) for 140 consecutive days. Data outlined with broken lines are representative of normal controls.[18,19] (All data shown as mean ± SD.)

less than the 28 ± 11 ml/kg/min calculated for the CZI group ($P < 0.001$).

DISCUSSION

By the simple expedient of adding bicarbonate or autologous serum to the solution, we improved the stability of insulin in portable delivery devices, as previously reported.[20,21] However, in such long-term applications, insulin is continuously agitated, exposed to small air bubbles in the reservoir, and in repeated contact with the surfaces of the reservoir, pump tubes, and delivery catheters. In our experience, all regular CZI preparations gradually aggregated[3] or formed higher-molecular-weight polymers[4,5] under these conditions. Such aggregates may simply obstruct the system,[6] thereby gradually reducing the flow of insulin, which in turn leads to a loss of glycemic control. Alternatively, the higher-molecular-weight polymers of insulin may not so much obstruct the system as express a reduced biologic activity because they do not dissolve in the blood before being extracted or degraded. Either or both of these insulin by-products (which have a reduced bioactivity when infused) caused the significantly higher mean fasting plasma glucose levels in the purified porcine insulin-infused group and contributed to a significant deviation from the normal of the diurnal glycemic profiles (data not shown). Furthermore, the infusion rate on the average had to be increased about 1.8-fold above normal values to compensate for the inactive portion of the CZI infusate.

Until recently, SI has been used predominantly for insulin-resistant diabetic patients.[12,13] However, SI has only been administered by subcutaneous injection. Its use for intravenous infusion was stimulated initially because it was so highly soluble. Furthermore, in in vitro tests, it did not have a tendency to self-associate even when exposed to continuous motion at room temperature and in contact with the surfaces usually encountered in insulin delivery systems.[3,5] These surfaces to which insulin was exposed in our device were predominantly polymeric in nature; the reservoir, pump tubes, and delivery catheters were all made of silicone rubber.

By using this insulin, we succeeded in the long-term (140 consecutive days) to normalize fasting plasma glucose levels in pancreatectomized dogs. Interestingly, the coefficient of variation of the mean fasting plasma glucose concentration was not reduced to normal. Whether this remaining instability is due to the nature of the SI preparation, the portal route of delivery, iatrogenic, or essential to this type of experimental diabetes is not known. Further studies are underway to explore its causes and the degree to which this instability varies when the infusion is directed peripherally rather than portally.

Also of interest are the high fasting plasma IRI levels in the SI-treated group (about 5 times normal). The higher circulating levels of the modified hormone in the circulation even when infused directly into the portal circulation of the liver and the decreased clearance rates imply a longer half-life. In diabetic man, treated with SI and in whom high levels of anti-insulin antibodies were initially present, the half-life of ^{35}S-labeled SI in the circulation was 60 min.[13] Both these effects are probably a consequence of the reduced degradation, secondary to its reduced receptor binding, which in turn is a consequence of the significant numbers (2–8) of negative charges imparted to each molecule of insulin by the sulfation-sulfonation process.[22] However, binding of SI to various receptors has only recently been studied.[23]

Because SI is essentially monomeric in nature and remains this way,[5] it is not surprising that exogenous insulin requirements were similar to those said to occur endogenously in healthy animals[18] or in studies of an acute nature using crystalline zinc preparations not subjected to agitation and before self-association could otherwise occur.[19] Remarkably, in over 1½ yr of continuous exogenous insulin infusions in 16 animals, obstructive occlusion of the pumping system by aggregates simply never occurred when SI was used uniquely (data not shown). It is apparent that for prolonged insulin infusion and glycemic normalization with pumping systems currently used or under development presently available CZI will probably not be adequate and may well be contraindicated.[10,11] Sulfated or otherwise chemically modified insulins may prevent the symptoms, but will they solve the problem?

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