Rapid Publication
Prevention of Insulin Aggregation by Dicarboxylic Amino Acids During Prolonged Infusion

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SUMMARY

The dicarboxylic amino acids, aspartic acid and glutamic acid, at their isoelectric pH, reduced aggregation of insulin solutions in vitro for 16 days during continuous agitation at 37°C. Unprotected insulin solutions, when infused via a 14-day implantable infusion device in diabetic Chinese hamsters, controlled plasma glucose levels for only 2 days, followed by escape coincident with insulin aggregation. However, when insulin solutions were protected with glutamic acid, euglycemia was maintained for the 14-day life of the device. DIABETES *30*:83–85, January 1981.

Aggregation of insulin solutions in reservoirs and catheters of artificial pancreatic devices has posed an unexpected problem.[1–3] The duration of insulin infusion is presently limited to a few days without frequent replacement of the system or flushing of the insulin reservoir.[4–9] Incorporation of purified insulin preparations in diverse buffers at neutral or acid pH has not prevented aggregation,[2,3] and the addition of serum to insulin solutions, recently reported to promote solubilization,[2] is impractical in long-term devices.

Early studies suggested that certain amino acids may form chelates with insulin in the presence of heavy metals.[10] Therefore, we have studied the protective effect of naturally occurring amino acids, as well as some organic acids, during continuous agitation in vitro. Glutamic and aspartic acids, at their isoelectric pH, were found to be unique in reducing insulin aggregation. In vivo, glutamic acid also protected insulin solutions administered via 2-wk implantable minipumps in fully ambulatory diabetic Chinese hamsters.

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MATERIALS AND METHODS

Neutral insulin solutions were prepared by appropriate dilutions of highly concentrated (500 U/ml) single-component regular neutral pork zinc insulin (U-500 regular Iletin II; Eli Lilly and Company) in unbuffered fluid containing 16 mg of glycerin and 2 mg of phenol per milliliter of distilled water (Diluting Fluid, Eli Lilly and Company). For in vitro experiments, the insulin concentration was 50 U/ml; for in vivo infusions, concentrations ranged from 100 to 140 U/ml because, in preliminary studies, these were necessary to normalize plasma glucose levels by the second day in severely diabetic hamsters. (Small variations in concentration were used to compensate for minor differences in body weight or variation in insulin sensitivity in a specific, previously tested animal.) Amino or organic acids (Calbiochem/Behring Corp., La Jolla, California) were added directly to the diluted neutral insulin in a final concentration of 7 mg/ml, and the mixture was shaken gently at room temperature until solution was complete. The final pH of the aspartic and glutamic acid/insulin solutions was 3.5 and was not adjusted further. The pH of other amino or organic acid/insulin solutions was adjusted to 7.4 or 3.5 with NaOH or HCl. Saline/glutamic acid mixtures were prepared by addition of glutamic acid at the same concentration in saline.

For in vitro experiments, 5-ml plastic test tubes, 12 × 75 mm (Sarstedt) containing 2 ml of the solutions were sealed and continuously agitated in a Dubnoff shaking incubator at 60 to 80 rpm at 37°C for 16 days. Light diffraction of the final suspension was estimated as optical density (O.D.) at wavelength 600 nm.

For in vivo experiments, implantable pumps (Alzet osmotic minipump No. 2002; Alza Corp., Palo Alto, California), which release their contents (244.0 ± 4.7 μl) at a constant rate (0.50 ± 0.02 μl/h) were used during a 14-day period. Pumps were weighed before and after filling to determine the initial fluid volume. After the cap was removed, a 2-cm vinyl catheter [i.v. soft medical polyvinyl tubing (i.d. = 0.023 in) Bolab Inc., Derry, New Hampshire] was attached to the tube extension beyond its flange.

Fourteen diabetic Chinese hamsters of both sexes from

the Chinese Hamster Program Project (Kalamazoo, Michigan) were studied. Only those animals with plasma glucose values exceeding 220 mg/dl at three different samplings in the fed state were included. Three hamsters (control group) received minipumps filled with saline/glutamic acid solution; four received minipumps containing either neutral insulin solution alone or glutamic acid/insulin solution in alternating sequence; and seven other hamsters received only one implant each, with either neutral insulin alone (N = 4) or glutamic acid/insulin solution (N = 3).

Pumps were implanted s.c. on the backs of anesthetized animals (ketamine hydrochloride) through a 2-cm incision in the skin of the neck, as previously described.[11] Animals were fed ad libitum, and blood samples were drawn daily between 1000 and 1100 h from the orbital sinus beginning 2 days before implantation through day 14. Plasma glucose levels were determined with a Beckman glucose analyzer. On the fourteenth day, the pumps were removed and their residual contents centrifuged in microfuge tubes to evaluate the extent of insulin precipitation.

RESULTS

In vitro experiments. By the fourth day, shaking of the unprotected insulin solutions resulted in aggregation (light microscopy indicated that aggregates consisted of 10- to 20-μm crystals, aggregated crystals, and possibly some fibrils), which increased progressively through the 16 days (final O.D. = 0.60 ± 0.08 nm; N = 21). None of the amino acids was effective at pH 7.4: O.D. was >0.25 nm for aspartic and glutamic acids, glycine, lysine, alanine, cystine, valine, serine, threonine, methionine, arginine, phenylalanine, tryptophan, proline, leucine, histidine, asparagine, and glutamine. At their isoelectric pH (3.3–3.5), however, glutamic acid (O.D. = 0.12 ± 0.02; N = 17) and, particularly, aspartic acid (O.D. = 0.08 ± 0.01; N = 7) prevented aggregation of insulin solutions. Other amino acids, as well as citric, lactic, and acetic acids, were ineffective at pH 3.3 to 3.5.

In vivo experiments (Figure 1). Minipumps containing saline/glutamic acid solution did not significantly modify plasma glucose levels. In hamsters infused with insulin alone, glycemia was normalized on the second day after implantation (84 ± 6 mg/dl), but hyperglycemia recurred as early as the third day. By the sixth day, plasma glucose levels (246 ± 32 mg/dl) reached preimplantation (268 ± 22 mg/dl) or control animals' levels (335 ± 13 mg/dl).

Glutamic acid-protected insulin solutions induced a significant decrease in glycemia on the second and third days. Despite higher initial plasma glucose levels than those of unprotected controls, normalization (87 ± 18 mg/dl) occurred by the fourth day and was maintained throughout the subsequent 2-wk life of the minipumps. Two glutamic acid/insulin-treated animals showed persistent hypoglycemia (<50 mg/dl) during infusion, in spite of oral sucrose feedings. However, in two other similarly treated animals, plasma glucose was increased on the fourteenth day, indicating some impaired insulin efficiency.

Examination of residual solutions, removed from the pumps and centrifuged, revealed a major precipitate in each unprotected insulin sample, a minor precipitate in the glutamic acid-protected samples from the two animals in which a loss of plasma glucose control was noted, and no precipitate in the other glutamic acid/insulin or saline/glutamic acid samples. Corresponding aggregates were also seen in the catheters of all minipumps containing precipitates.

The volumes of final liquid in all minipumps were equally depleted at day 14, indicating that even those pumps containing precipitates had not become completely occluded.

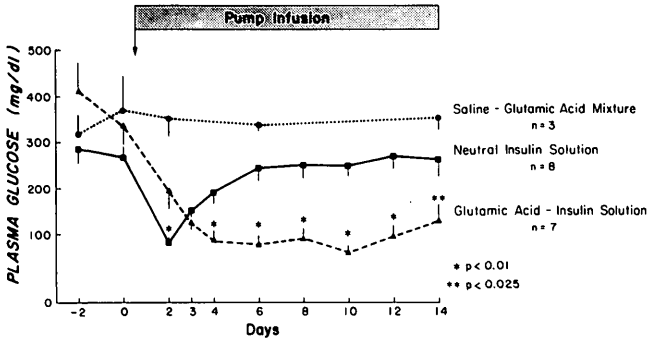

FIGURE 1. Effectiveness of insulin plus glutamic acid delivered subcutaneously via minipump in the diabetic Chinese hamster. Mean flow rates were 0.5 μl/h; daily insulin dose averaged 56–57 U/kg body wt/day. Data given as mean ± SEM; significance was determined by Student's *t* test on unpaired data.

DISCUSSION

These results support previous findings that insulin aggregation can occur in reservoirs and catheters of implantable or portable delivery systems[3] and that in vitro shaking of neutral purified insulin induces a gross aggregation in all vials within 3 to 8 days.[3]

Serum has been used successfully by Albisser et al.[2] as an insulin anti-aggregant in portable infusion devices filled with relatively dilute (5 U/ml) insulin solutions. However, the same group has found no such anti-aggregation characteristics, in vitro, with albumin, sodium bicarbonate, cystine, histidine, tryptophan, or glycine;[2,3] in our in vitro experiments, precipitation was not prevented by addition of albumin, lipoproteins, or bicarbonate (data not shown). Sixteen amino acids tested at neutral pH also did not give positive results. However, two amino acids, glutamic acid and, particularly, aspartic acid, at their isoelectric pH, reduced aggregation of concentrated insulin when agitated at 37°C for 16 days. Glutamic acid was preferred for the in vivo study because of its better solubility, although, in all in vitro experiments, aspartic acid was the more effective dicarboxylic amino acid and should be the amino acid of choice if reduced solubility is not a factor.

It has been suggested[3] that low flow rate, elevated temperature, and motion are important factors contributing to insulin aggregation. Our experimental system is particularly challenging because: (1) the infusion rate of the 2-wk minipump is very low (0.50 μl/h), (2) diameter of the catheter is small, (3) the insulin solutions are shaken by the frequent movement of the hamsters, (4) the implants are exposed to body temperature, and (5) the insulin solutions are highly concentrated. Each of these promotes insulin aggregation.

The fact that 1-wk minipumps have been used success-

fully in the Chinese hamster[11,12] is presumably due to their higher flow rate and larger apertures with no catheters. As anticipated, the 2-wk infusion devices were more sensitive to aggregation problems, as shown in the in vivo experiments using unprotected neutral insulin. A dramatic rise in plasma glucose occurred in the diabetic hamsters as early as the third day, reflecting the presence of insulin aggregates that were directly visible in the residual solution removed from the pump. Part of the loss of effectiveness was likely caused by retention of these insoluble aggregates in the pumps, but, since most of the contents had been released during the 14 days, a decline in biologic effectiveness of infused aggregates is also indicated.

In contrast to the results with unprotected insulin, the addition of glutamic acid resulted in normalization of blood glucose for the life of the minipump, and aggregation at 14 days was minimal.

The process responsible for the anti-aggregation effect of glutamic and aspartic acids is not known. Acid pH (3.5), obtained by simple addition of either amino acid to the neutral insulin solutions, seems to be necessary, since aggregation was not prevented when these mixtures were adjusted to neutral pH before shaking. However, previous studies have shown acid pH to be insufficient in itself,[2,3] and our tests with other amino acids at acid pH did not produce positive results. Similarly, lactate and the polycarboxylic organic acid, citrate, were ineffective at pH 3.5, even though they were at their isoelectric points at this pH.

Therefore, a specific action of the dicarboxylic amino acids, glutamic acid and aspartic acid, at acid pH (3.5)—corresponding approximately to their isoelectric point (at which partial protonation of their carboxyl groups occurs)—appears to be responsible for the anti-aggregation effect on highly concentrated insulin solutions. Because metals in insulin preparations have been involved in aggregation mechanisms, this effect could be due to the chelation of the carboxyl group(s) to the zinc in insulin, thereby producing more soluble metallobiomolecular complexes.[10,13–16] However, a direct reaction of these amino acids with the insulin polypeptide is not excluded. Further studies are needed to confirm the efficiency and tolerance of glutamic acid and aspartic acid as insulin anti-aggregation agents in infusion devices used in humans.

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