Neutralization of the Biologic Activity of Human Serum Bound Insulin by Potent Insulin Antisera

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SUMMARY

The in vivo biologic activity of human serum bound insulin is significantly neutralized in vivo, in intact mice and rats, by undiluted guinea-pig insulin antisera (GPAIS). Significant neutralization of biologic activity on both muscle and adipose tissue glycogen synthesis was observed with the addition of GPAIS.

The present studies demonstrate that the concentration of insulin antisera is critical for the neutralization of in vivo biologic activity of bound insulin. In addition, the results suggest that bound insulin injected intraperitoneally into mice or rats may be modified in such a way to allow reactivity with insulin antisera, as judged by loss of biologic activity. DIABETES *21*:930-34, September, 1972.

The presence of macromolecules in blood exerting insulin-like activity but differing from insulin in their physicochemical properties was described initially by Beigelman et al.[1] and Antoniades et al.[2] Partially purified preparations have been obtained from blood by resin adsorption and elution[2-6] or by acid-ethanol extraction of plasma or plasma fractions.[5-9] These partially purified preparations exert in vivo and in vitro biologic activities similar to those of crystalline insulin. However, they exhibit a higher molecular weight and a slower electrophoretic mobility than crystalline insulin and are unreactive with insulin antisera as judged by radioimmunoassay. Furthermore, unlike insulin or proinsulin, no tissue or gland has been identified as the sole source of these substances. Their only source is the blood, of which they seem to constitute only a trace amount.

Antoniades and associates[4] have called these substances "bound insulin" or "insulin complexes" and have suggested the possibility that they represent insulin metabolites formed in vivo by metabolic transformation in extrapancreatic sites. This suggestion is consistent with the fact that bound insulin exerts in vivo and in vitro biologic activities similar to those of crystalline insulin. Differences in the physicochemical properties and immunologic reactivity of bound insulin and insulin could result from the modification of the free-insulin molecule. However, if the biologic activity of bound insulin is due to the release of insulin from its bound form, one would expect neutralization of the biologic activity by insulin antisera.

The present report provides evidence that the in vivo biologic activity of human serum bound insulin is significantly neutralized in vivo, in intact mice and rats, by undiluted insulin antisera.

MATERIALS AND METHODS

Preparation of bound insulin from human sera. One thousand milliliters of pooled human sera is passed at room temperature through a glass column (8 x 97 cm.) containing 2.5 L. wet volume Dowex-50w-x8 resin (Na^+ cycle) at a rate of 30 ml. per minute. A description of the technic for sodium cycling the resin has been reported elsewhere.[10] The serum is allowed to equilibrate with the resin for about thirty minutes and is subsequently washed with 5 L. of 0.15 M NaCl at a flow rate of about 30 ml. per minute. Following the saline wash the resin is eluted with 5 L. of dilute ammonium hydroxide (0.02 N) at a rate of about 150 ml. per minute. The pH of the alkaline eluate is maintained between 5 and 9 during elution with 0.36 N sulfuric acid and is adjusted to a final pH 7.2 $\pm$ 0.2.

The eluate is subjected to ultrafiltration at 2° C. (50 psi nitrogen pressure) in a 2 L. capacity stirred cell utilizing Amicon Diaflow membranes (UM-10) which allow the passage of molecules with a molecular weight of less than 10,000. The concentrated solution (retentate) inside the ultrafiltration cell is lyophilized. With the 2 L. stirred cell fitted with a 150 mm. diameter UM-10 membrane and connected to a stainless steel reservoir, about 6 L. of alkaline eluate containing the bound insulin can be ultrafiltered in twenty-four hours at an operating pressure of 50 psi.

The lyophilized material is stored in a desiccator at 5° C. This preparation of bound insulin contains, per milligram protein, less than 10 μU. of immunoassayable

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insulin, as measured by the Morgan and Lazarow technic.[11]

The partially purified serum bound insulin obtained with the above technic contains about 100 mg. protein per liter of original serum and its biologic activity corresponds to about 400 to 600 μU. insulin activity per milligram protein as judged by the rat intraperitoneal assay.

Protein determination. Protein was determined by the method of Lowry et al.[12] using crystalline bovine albumin as the standard.

Insulin antisera. Undiluted lyophilized guinea-pig insulin antisera (GPAIS) were obtained through the courtesy of Dr. Peter H. Wright, Department of Pharmacology, School of Medicine, Indiana University, and Dr. Edward L. Grinnan, Eli Lilly and Company, Indianapolis. The following lot of GPAIS from Dr. Wright was used in these studies: Lot 550 with a neutralizing potency of 1.36 U./ml. Lot GPIN20925GP14, with a neutralizing potency of 3.8 U./ml., was obtained from Dr. Grinnan. All lyophilized GPAIS powders were reconstituted to original volumes with distilled water.

Intraperitoneal assay for insulin activity in intact mice and rats. Insulin bioassays were carried out in vivo in intact mice and rats by the intraperitoneal assay described by Rafaelsen et al.,[13] as modified by Cahill et al.[14] Samples were prepared by dissolving either crystalline bovine insulin (Eli Lilly and Company) or bound insulin in 5 per cent human serum albumin (HSA) dialyzed beforehand for twenty-four hours at 2° C. against 100 volumes Gey and Gey bicarbonate buffer equilibrated with 95 per cent O_2:5 per cent CO_2. Final volume of solutions for injection was achieved by the addition of either 5 per cent HSA or GPAIS.

Each rat received intraperitoneally 5 ml. of sample containing 1 mg. glucose and 2 μC. of glucose-U-C-14 (New England Nuclear Corporation; specific activity about 4.3 mc. per millimole). Each mouse received intraperitoneally 1 ml. of sample containing 1 mg. glucose and 2 μC. of glucose-U-C-14. The food was removed from the cages of the animals two hours before the assay.

Before injection the rats were narcotized lightly with 50 per cent CO_2:50 per cent O_2, the testes displaced into the abdominal cavity, and the communication between peritoneal and scrotal spaces closed by a transcutaneous suture. The mice were sacrificed one hour, and the rats two hours after injection, and both hemidiaphragms and epididymal pads removed rapidly. The epididymal pads were weighed immediately, placed in separate tubes each containing 20 ml. chloroform:methanol (2:1) and shaken for four hours. The hemidiaphragms were weighed rapidly, placed in tubes containing 30 per cent KOH at 100° C., hydrolyzed, and the glycogen precipitated[13] and transferred to planchettes for counting. The extracted epididymal pads were similarly hydrolyzed, and then glycogen was isolated and counted for radioactivity.

The values are expressed as the mean ± SEM of the average counts per minute incorporated into the glycogen of two hemidiaphragms or two epididymal pads per gram tissue. Five animals were used per group.

The male CD (cesarean delivered) mice and rats used in these studies were obtained from the Charles River Breeding Laboratories and were maintained on Purina Chow fed ad libitum. The body weight of the rats was between 110 and 120 gm. and that of the mice between 30 and 35 gm.

RESULTS

Figure 1 presents data on the effect of undiluted GPAIS on the biologic activity of bound insulin and crystalline insulin, as measured by the rat intraperitoneal assay. Significant neutralization of biologic activity on both muscle and adipose tissue glycogen synthesis was observed with the addition of 0.6 ml. GPAIS.

Figure 2 demonstrates the effect of various concentrations of undiluted GPAIS on the biologic activity of bound insulin as measured by the rat intraperitoneal assay. Significant neutralization of biologic activity on both muscle and adipose tissue glycogen was seen with 0.1 ml. GPAIS with little or no further neutralization observed with higher concentrations of GPAIS. The results become even more significant from the fact that in figures 1 and 2 different preparations of bound insulin and GPAIS were used.

As noted in previous publications,[15,16] bound insulin appears to be more potent on muscle than on adipose tissue glycogen synthesis compared to crystalline insulin as judged by the intraperitoneal assay.

Figure 3 shows the effect of GPAIS on the biologic activity of bound insulin on the muscle and adipose tissue of intact mice. Some neutralization of the biologic activity of bound insulin on muscle was apparent with the addition of 0.2 ml. GPAIS per animal with a significant effect occurring with the addition of 0.6 ml. GPAIS per mouse. A significant neutralization of biologic activity on adipose tissue glycogen was observed with both 0.2 and 0.6 ml. GPAIS.

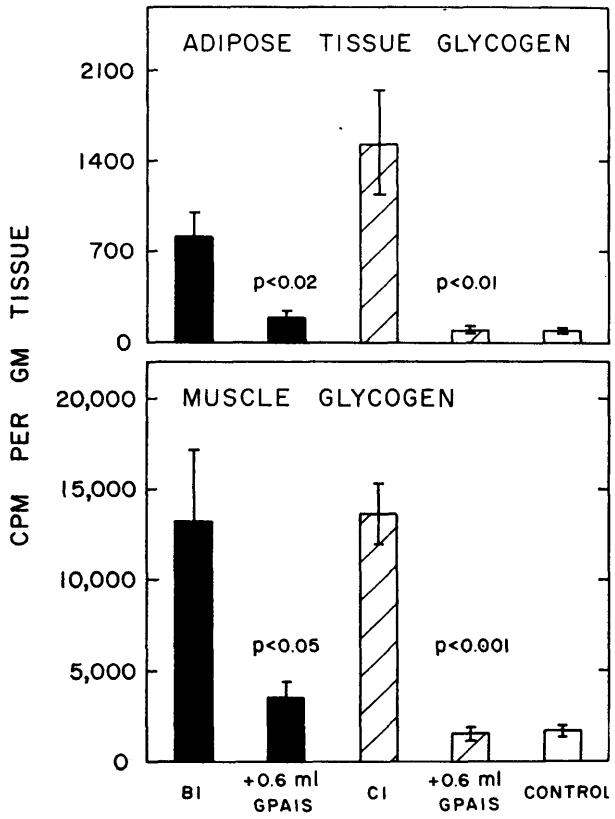

FIG. 1. Effect of guinea pig anti-insulin antisera (GPAIS) on the in vivo activity of partially purified human bound insulin (BI) and crystalline bovine insulin (CI) in intact rats. Neutralizing potency of GPAIS is 1.8 U. porcine crystalline insulin/ml. Control consists of 5 per cent HSA in Gey and Gey buffer with 0.6 ml. GPAIS. Each rat received bound insulin (1.6 mg. protein) or 1,000 μU. crystalline insulin. Each value represents the mean ± SEM of five rats.

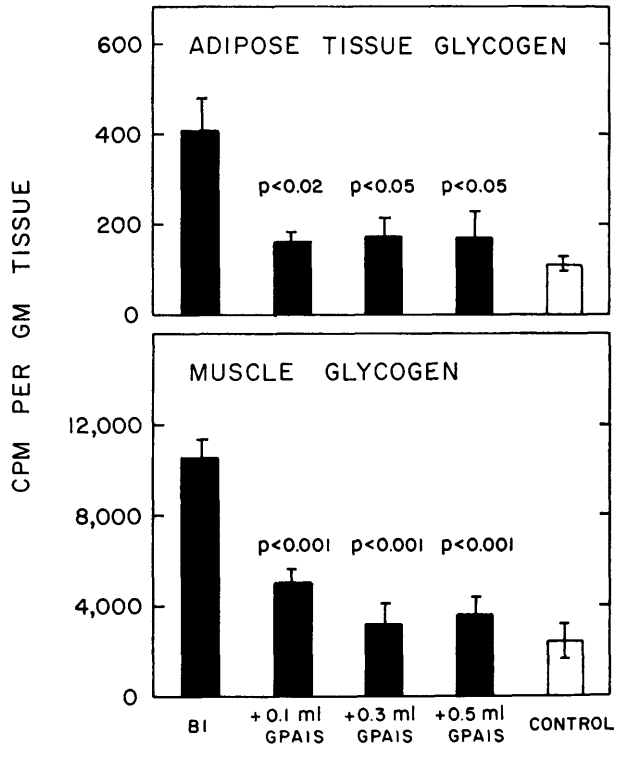

FIG. 2. Effect of various concentrations of guinea pig anti-insulin antisera (GPAIS) on the in vivo activity of partially purified human bound insulin (BI) in intact rats. Neutralizing potency of GPAIS is 1.36 U. porcine crystalline insulin/ml. Control consists of 5 per cent HSA in Gey and Gey buffer with 0.5 ml. GPAIS. Each rat was injected with bound insulin (1.5 mg. protein). The p-values are compared to the bound insulin (BI). Each value represents the mean ± SEM of five rats.

Table 1 presents control studies in intact rats and mice injected with and without GPAIS. In these studies groups of rats and mice were injected, as described above, with 5 per cent HSA in Gey and Gey bicarbonate buffer containing 1 mg. glucose and 2 μC. glucose-U-C-14, without and with various amounts of GPAIS. The results presented in table 1 indicate that the addition of GPAIS did not affect significantly the baseline values.

DISCUSSION

The present studies demonstrate that the biologic activity of human bound insulin is significantly neutralized in intact mice and rats by undiluted insulin antisera. Bound insulin is not reactive with the antisera as judged by in vitro radioimmunoassay technics. This has been considered by some as evidence that bound insulin does not contain insulin. However, as discussed previously by Antoniades et al.[4] and more recently by Yalow and Berson,[17] one should distinguish between reactivity with insulin antisera as demonstrated by detectability in radioimmunoassay and by neutralization of biologic activity. It is now generally accepted that it is possible to dissociate biologic activity from immunologic reactivity. Modification of the insulin molecule may prevent its reactivity with insulin antisera rendering the modified insulin molecule undetectable by radioimmunoassay. On the other hand, as mentioned above, if the biologic activity of modified insulin is due to release of insulin, one would expect neutralization of its biologic activity by insulin antisera.

The biologic effect of insulin, measured by the intraperitoneal assay, is due to a localized effect and is not affected by the endogenous circulating insulin. Rafaelsen et al.[13] reported that intravenous injection of up to 10,000 μU. of crystalline insulin per rat did not produce any significant effect on the incorporation of glucose carbon into the hemidiaphragm and epididymal

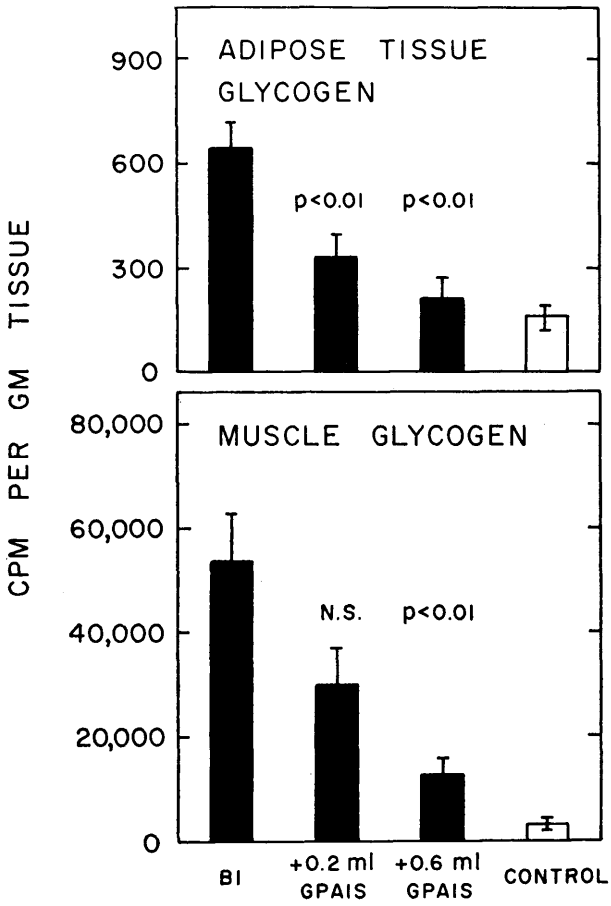

FIG. 3. Effect of guinea pig anti-insulin antisera (GPAIS) on the in vivo activity of partially purified human bound insulin (BI) in intact mice. Neutralizing potency of GPAIS is 1.36 U. porcine crystalline insulin/ml. Control consists of 5 per cent HSA in Gey and Gey buffer with 0.6 ml. GPAIS. Each mouse was injected with bound insulin (1.5 mg. protein). The p-values are compared to the bound insulin (BI). Each value represents the mean ± SEM of five mice.

adipose tissue glycogen of the animals, while intraperitoneal injection of only 100 μU. per animal produced significant biologic activity. Similar results were reported by Antoniades et al.[16] These data make most unlikely the possibility that the reduction of the biologic activity of bound insulin by the GPAIS is due to the inactivation of the endogenous insulin by the antisera. Consistent with this is the lack of any significant change in the control baseline values by the presence of GPAIS as shown in table 1.

Previous studies by Stern et al.[18] have demonstrated the neutralization of the biologic activity of goat serum bound insulin in intact rats by undiluted antisera (0.2 ml. per rat). Significant neutralization of the biologic activity of human bound insulin on isolated rat hemidiaphragm in the presence of adipose tissue extracts (ATE) has been reported by Shaw and Shuey.[19] Partial inactivation of human bound insulin in isolated rat epididymal adipose tissue was reported from our laboratory during prolonged incubation with the antisera.[4] A number of other investigators have reported partial inactivation by insulin antisera of the insulin-like activity of whole sera or of serum fractions obtained by acid-ethanol extraction[20,21] or by electrophoretic procedures.[22] However, other investigators reported that insulin antisera did not affect the biologic activity of serum bound insulin or serum fractions with insulin-like activity in isolated rat epididymal adipose tissue. On the basis of this observation they have used the term "nonsuppressible insulin-like activity" in describing bound insulin or preparations obtained by acid-ethanol extraction of serum or plasma fractions.[5-8]

Some of these differences between investigators may be due to the potency of the antisera used. For example, in our earlier studies[4] with isolated rat adipose tissue,

TABLE 1

Effect of GPAIS on control baseline values in intact rats and mice

I. Intact Fed Rats	Muscle glycogen cpm/gm. tissue Mean + SEM	p	Adipose tissue glycogen cpm/gm. tissue Mean + SEM	p
Control buffer (5% HSA) (6)	1,795 ± 414		127 ± 31	
+0.3 ml. GPAIS (6)	1,402 ± 277	N.S.	93 ± 11	N.S.
+0.6 ml. GPAIS (6)	1,720 ± 472	N.S.	139 ± 17	N.S.
II. Intact Fed Mice				
Control buffer (5% HSA) (6)	1,878 ± 861		77 ± 9.3	
+0.2 ml. GPAIS (6)	2,028 ± 400	N.S.	87 ± 9.3	N.S.
+0.6 ml. GPAIS (6)	2,187 ± 145	N.S.	76 ± 12.9	N.S.

Neutralizing potency of GPAIS is 1.36 U. porcine crystalline insulin/ml.
Each value represents the mean ± SEM of six animals.
p compared with control buffer (5 per cent HSA).

diluted antisera were used, capable of neutralizing the biologic activity of 1,000 μU. crystalline insulin. Ensinck et al.[23] have used antisera which were diluted 100 to 500-fold before use. These diluted antisera were capable of inactivating crystalline insulin. However, interaction of crystalline insulin with antisera occurs by mixing the insulin with the antisera in a test tube even before incubation with the tissue. This is not the case with bound insulin which is not reactive with the antisera. The degree of inactivation of bound insulin by antisera apparently depends upon the concentration of the antisera since neutralization may represent the net effect of the rate of bound insulin activation and utilization by the tissue and the rate of combination of activated bound insulin with the antisera. It is possible that antisera compete with the tissue for the binding of insulin released from its bound form. The present studies indicate that the concentration of insulin antisera is critical in the demonstration of neutralization of biologic activity of bound insulin in the muscle and adipose tissue of intact mice and rats assayed by the intraperitoneal technic. Furthermore, they suggest that bound insulin injected in these animals may be modified in such a way to allow reactivity with the antisera as judged by loss of biologic activity.

ACKNOWLEDGMENT

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