Serum Insulin-like Activity in Hypophysectomized and Depancreatized (Houssay) Dogs

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SUMMARY

Serum insulin-like activity was measured serially by the epididymal fat pad method in twelve dogs over several weeks. The animals were first hypophysectomized and subsequently pancreatectomized; no insulin was administered at any stage of the experiment. Hypophysectomy resulted in a reduction of serum ILA of some 50 per cent from an average normal fasting value of 420 μU. per ml.

Subsequent pancreatectomy caused no further appreciable reduction in serum ILA over the entire observation period of five weeks. In a selected number of serum samples residual ILA was detectable also by the rat hemidiaphragm procedure. None of the Houssay dogs had measurable amounts of insulin in blood by immunoassay. It was considered unlikely that activities measured by the two bioassays represented insulin. DIABETES *14*:658-62, October 1965.

The insulin-like activity (ILA) in sera of completely depancreatized animals has been studied by a number of investigators. No insulin was found by immunoassay,[1,2] a substantial reduction or no ILA was found by the rat diaphragm technic,[3-5] but results obtained with the epididymal fat pad method indicated that considerable insulin-like activity persisted after removal of the pancreas.[1,6-12]

Steinke and associates[7] measured ILA in sera of dogs of the Toronto group and reported a sharp decline in ILA within twenty-four hours following pancreatectomy. The preoperative ILA of some 400 μU. per ml. fell to 200 μU. per ml., and was maintained at that to the time of death which occurred within five to eight days. In view of the concepts of "atypical," "bound" and "nonsuppressible insulin,[11,13-18] the possibility had to be considered that the epididymal fat pad method measured some form of insulin in blood which had a low turnover and was biologically inactive while circulating in vivo.

It was, therefore, of considerable interest to know how long this type of ILA could persist in animals deprived both of endogenous and exogenous insulin supply. The depancreatized dog, because of its short life span, was not considered suitable for this type of experiment and, therefore, in the present study ILA was measured by the epididymal fat pad assay in Houssay dogs which survived for several weeks. A sharp decline was noted after hypophysectomy, but subsequent removal of the pancreas caused little alteration in serum ILA. In a selected number of serum samples residual ILA was detectable also by the rat diaphragm technic, while none of the Houssay dogs had measurable amounts of insulin in blood by immunoassay.

MATERIAL AND METHODS

The hypophysis was removed from twelve mongrel dogs (average weight 12 kg.) under aseptic conditions during sodium pentobarbital anesthesia. The transbuccal approach of Essex and Astarabadi[19] was chosen, because it does not require exposure of the brain and the operation takes no more than about thirty minutes. Two weeks later a total pancreatectomy was performed in eleven

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animals. In one dog both hypophysectomy and pancreatectomy were performed in one session. Blood loss was kept to a minimum by atraumatic ligation of all minute blood vessels by an instrument devised by one of us (A.S.[20]). The integrity of larger pancreatico-duodenal vessels was meticulously preserved to avoid ischemia and necrosis of the duodenal loop. At autopsy no pancreatic remnants were found in the operating field, and no aberrant pancreatic tissue was seen along the small intestine. The sella turcica was empty.

In addition to antibiotics, the hypophysectomized animals received, twenty-four hours before pancreatectomy, 25 mg. of cortisone and 5 mg. of desoxycorticosterone intramuscularly, and the dose was gradually reduced over the next seven days. No exogenous insulin was administered at any time of the experiment.

Blood samples were obtained at weekly intervals (nonfasting) and the glucose concentration in whole blood was determined by the micromethod of King.[21] Serum was stored in the frozen state and aliquots were packed in dry ice and shipped to Germany by air. Animal experiments were carried out in the Toronto laboratories and so was the immunoassay for insulin in blood. All other laboratory procedures were carried out in Frankfurt.

Serum ILA on epididymal fat pad was measured essentially according to the original method of Martin, Renold and Dagenais,[22] with modifications introduced by Ditschuneit and co-workers.[23] Each assay was carried out in duplicate using fat pad segments from six different rats in two different incubation flasks and serum in a 1:2 dilution. Radioactivity of the CO_2 formed from glucose-1-C-14 was measured in a liquid scintillation counter using hyamine-hydroxide as adsorbant. Most of the reported figures are the mean of 2 to 6 separate duplicate assays. The bioassay with rat hemidiaphragm was performed according to Vallance-Owen and Hurlock.[24] Glucose in the incubation medium was measured by the method of Huggett and Nixon,[25] and the activity of the 1:4 diluted serum was calculated as net glucose uptake (i.e., subtracting basal uptake) per 10 mg. of dry diaphragm. One mU. of crystalline pork insulin served as a standard.

For the immunological assay of insulin the double antibody technic of Morgan and Lazarow[26] was chosen. Pork insulin labeled with I-131 (20-30 mC./mg.) was purchased from Abbott Laboratories. No suitable dog insulin standard was available to us, and therefore pork insulin was used instead; the sequence of amino acids for both insulins appears to be the same.[27] A pure pork insulin standard was the gift of the Connaught Medical Research Laboratories of the University of Toronto, by courtesy of Dr. A. M. Fisher. Anti-beef insulin serum from guinea pig was purchased from Immunology, Inc., (Lombard, Illinois), and the anti-guinea pig serum from rabbit was purchased from Hyland Laboratories (Los Angeles, California). All determinations were carried out at least in duplicate and multiple assays were performed on a number of samples always using 1 ml. of serum and 10 μU. of radioactive insulin per tube. In the absence of unlabeled insulin about 40 per cent of the total radioactivity was precipitated upon incubation with 0.1 ml. of anti-insulin serum in a 1:5000 dilution for twenty-four hours, and subsequent incubation with 0.2 ml. of rabbit anti-guinea pig serum for another twenty-four hours. The latter amounts of antiserum were sufficient in antibody content to precipitate the insulin-anti-insulin complex in a reproducible fashion when undiluted, 1:1 and 1:2 diluted sera from normal fasting dogs containing around 10 μU. insulin I-131 per ml. were assayed. Since the recoveries from a diluted sample usually checked within 5 per cent, the use of EDTA was omitted.[28]

For further studies of the ILA on fat pad, acid alcohol extracts were prepared from sera of Houssay dogs according to the method of Scott and Fisher.[29] Anti-insulin sera were prepared from guinea pigs which were immunized with pure beef insulin according to Robinson and Wright.[30] In a 1:10 dilution, 0.1 ml. of antiserum was potent enough to neutralize the effect of 1 mU. of insulin in the fat pad assay.

RESULTS

Serum insulin-like activity was measured serially in all twelve animals by the epididymal fat pad method and the results are reproduced in figure 1. The variable number of animals included in each period of study was either due to breakage of tubes during shipment or to death of the animals at different stages of the experiment. Following hypophysectomy the mean value for ILA fell by some 50 per cent from the mean value obtained in normal dogs, and the difference was statistically highly significant. This is in keeping with results obtained in hypophysectomized monkeys by the epididymal fat pad assay[31] and hypophysectomized rats by the diaphragm method.[32] It is remarkable, however, that subsequent pancreatectomy failed to lower the serum ILA of hypophysectomized animals. The serum ILA fluctuated closely to the level of 200 μU. per ml. over the entire observation period of five weeks. There

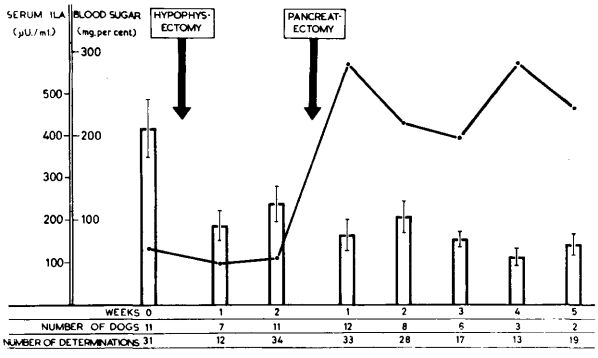

FIG. 1. Serum ILA measured by the epididymal fat pad assay. Mean ± S.E. is based on average values obtained for each serum sample. Note the marked fall of ILA following hypophysectomy and persistence of ILA after pancreatectomy.

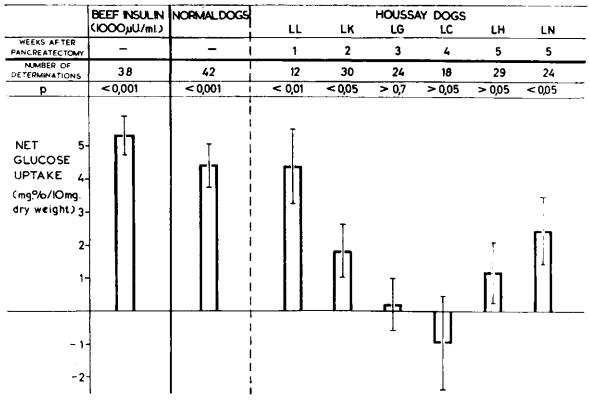

FIG. 2. Serum ILA measured by the rat diaphragm assay. Mean ± S.E. Note that sera of dogs LL, LK and LN stimulated glucose uptake.

was no statistically significant difference between ILA values of hypophysectomized and Houssay dogs at any stage of the experiment.

Serum ILA was also determined by the rat hemidiaphragm technic in six Houssay dogs. The results obtained with six representative serum specimens, each from one dog at different time intervals, are given in figure 2. In one animal (LL) the glucose uptake was virtually identical with that of normal dogs, and in two other animals (LK and LN), of which one was five weeks after pancreatectomy, the glucose uptake was still significantly different from zero (see p values).

The effect of acid alcohol extraction and of anti-insulin serum on ILA as measured by the epididymal fat pad assay was studied, and preliminary results are presented in table 1. Three serum samples from normal dogs were separately treated with acid alcohol and multiple ILA measurements in the extracts gave a mean value of 174 μU. per ml. This value represented some 70 per cent of the figures obtained for native undiluted serum. The acid alcohol extractable ILA was suppressible by adding 0.01 ml. of anti-insulin serum to the incubation medium prior to the addition of the extract. The residual ILA of 11 μU. per ml. was in the range of basal fat pad activity measured with or without the addition of anti-insulin serum. When serum samples of three Houssay dogs were tested, the ILA of undiluted specimens could be effectively suppressed by addition of anti-insulin serum without the necessity for acid alcohol extraction, leaving some residual activity of 27 per cent of the original value. Finally, representative serum samples of three other Houssay dogs were extracted with acid alcohol which procedure had decreased the ILA to some 33 per cent of that found in the non-extracted sera; extractable ILA could be suppressed by anti-insulin serum.

At least one serum sample from each of our twelve Houssay dogs was assayed for insulin by the double antibody technic described above. The samples were chosen to cover the entire observation period from one to five weeks following pancreatectomy. There was no trend in the values obtained from individual serum samples regardless of the time of pancreatectomy. Therefore, the results were pooled and the mean ± S.E. for

TABLE 1

Insulin-like activity by epididymal fat pad assay of undiluted serum and of acid alcohol extracts with and without addition of anti-insulin serum

Group	Number of dogs	Number of determinations	Serum μU./ml.	Serum + immune serum μU./ml.	Serum extract μU./ml.	Serum extract + immune serum μU./ml.
Normal	3	16	250 ± 51*	—	174 ± 47	11 ± 5
Houssay	3	20	168 ± 41	46 ± 2	—	—
	3	22	148 ± 21	—	49 ± 11	11 ± 3

*Mean ± S.E. of all determinations.

twelve dogs was 1.3 ± 0.1 μU. per ml. This value is at the limit of the sensitivity of the method as set up in our laboratory and, therefore, cannot be considered as a measurable amount of insulin.

DISCUSSION

The primary purpose of this investigation was to study the persistence of serum ILA in depancreatized animals by the epididymal fat pad assay. The persistence of considerable ILA over a period of five weeks without any tendency to diminish in concentration even at the time of death of the animals does not speak for "bound," "atypical" or "nonsuppressible" ILA as easily acceptable concepts of biological significance.[11,13-18] Serum containing only "bound" insulin is not supposed to be reactive by the diaphragm method of assay and this was by no means uniformly the case in our Houssay dogs. It is difficult to accept that insulin, in whatever form it may circulate in blood, would not gradually disappear after removal of the organ of origin.

The over-all experimental evidence in our study tends to point out that the ILA in sera of Houssay dogs was of nonpancreatic origin. The material apparently had some properties of insulin for which there is only incomplete evidence in that the fat pad ILA was suppressed by immune serum under certain conditions, while under other conditions, suitable for the double antibody assay, no immunological reaction for serum insulin took place. This apparent discrepancy is currently being studied in greater detail (Frankfurt). Finally, there was no evidence at any time that the ILA demonstrated by the bioassays in vitro corresponded to the clinical picture in vivo. On the other hand, gradual wasting and death of our Houssay dogs was in keeping with lack of insulin in blood as demonstrated by the immunoassay.

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normal level of 1.23. Administration of actinomycin D to the insulin treated diabetic rats resulted in a rate of acetate incorporation of 0.23, thus the restoration by insulin of fatty acid synthesis in the epididymal fat of diabetic rats was completely blocked by actinomycin. It is likely that this effect is due to a block in the DNA dependent synthesis of messenger RNA.

These workers also studied the rate of olefin synthesis in the different groups of rats. Liver microsomes were incubated with stearic acid-1-C-14 in the presence of appropriate cofactors. From the reaction mixture the fatty acids were separated as their methyl esters by means of gas-liquid chromatography, and the per cent conversion of stearic acid to oleic acid was determined by liquid scintillation spectrometry. In agreement with earlier experiments by the same investigators, they found a conversion of 31 per cent of the stearic acid to oleic acid with a microsome preparation from normal rat liver during the incubation. Liver microsomes from untreated diabetic rats showed less than 3 per cent conversion.

Insulin treatment of diabetic rats for twenty-six hours restored the microsomal synthesis of oleic acid from stearic acid to normal (33 per cent). Treatment with 10 μg. per 100 gm. body weight of actinomycin D prior to each insulin treatment prevented insulin repair of the enzyme deficiency, resulting in a rate of conversion of stearic to oleic acids of 12.5 per cent. Raising the actinomycin D level to 25 μg. per 100 gm. further reduced the per cent conversion to a level of 4. Suitable controls showed that actinomycin D did not have a direct toxic effect on the microsomal enzyme activity.

These results, showing the correction by insulin of acetylcarboxylase activity and microsomal monoene synthesis in diabetic rats, plus the blocking of the insulin repair by actinomycin D, are strongly in support of the hypothesis that an important role of insulin is in the induction of specific enzyme synthesis. The induction is brought about by a stimulation of messenger RNA production. Wool and Munro suggested that insulin combines with a repressor molecule whose function is to prevent the transcription of specific messages from DNA to messenger RNA. The nature of this or any other repressor molecule is still unknown, although all investigators agree that some type of chemical compound functioning in this way very likely exists. These studies with insulin will be of particular value if they offer some clue to the type of molecule which could act as a repressor.

This study also points out the clear separation of the hypoglycemic effect of insulin from the induction of enzymes. It would seem likely that the transport of glucose mediated by insulin is not dependent on protein synthesis.

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