Conditioned Insulin Secretion in the Albino Rat (34605)

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(Introduced by Daniel Porte, Jr.)
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Several studies have shown that various effects of repeated injections of insulin can be conditioned. In particular, Reiss (1) reported a conditioned comatose-like state in rats; Balagura (2) reported a conditioned insulin-like hyperphagia in rats; and several studies have reported a conditioned hypoglycemia in dogs (3–6) and in rats (7–9). Although all of these findings could be explained by a conditioned release of insulin, there has been no direct evidence for such a mechanism. Therefore, the present study was designed to ascertain whether or not a conditioned insulin release can account for the conditioned hypoglycemia.

Since repeated insulin injections are given throughout the conditioning period in the conditioning paradigm, a direct assay for insulin via the immunoreactive assay method would be complicated by the presence of insulin antibodies built up in the animals during conditioning. Therefore, a less direct approach was taken. This report gives the results of two experiments designed to determine if insulin release is, in fact, conditioned in this paradigm.

General Method. The basic conditioning procedure has been described in detail elsewhere (7). Briefly, it involves milking a blood sample from a lesion in the skin in the tip of the tail of a rat. The rat then gets a subcutaneous injection of either 1 unit/20 g of body weight of insulin (Iletin, regular injection insulin, U80 Lilly), or the equivalent volume of physiological saline, and is placed in an individual conditioning chamber for a period of 20 min. The chamber is permeated with the odor of Mentholatum, which is applied to a gauze pad that is taped to the inside of the closed lid of the chamber. After this period, a second blood sample is taken from its tail, and the rat is returned to its home cage. Blood glucose determinations by a glucose oxidase method (10) are subsequently done on the blood samples. Six such conditioning trials are given at the same time of the day on alternate days. Double-blind procedures are used to prevent any possible influence on either the rats' behavior or the glucose determinations.

Expt. 1. This study was undertaken to determine whether or not the destruction of the beta cells of the islets of Langerhans of the pancreas has an effect on the conditioned hypoglycemia.

Method. Subjects were 24 male albino rats derived of Wistar origin, obtained from the Simonsen Laboratories, Inc., Gilroy, California. They were between 90 and 100 days old at the beginning of the experiment. The study consisted of two identical replications of 12 rats each, run 2 months apart.

In both replications, each of the 12 rats was given 6 trials of the conditioning procedure, but the 12 rats were randomly divided into two groups: one group received injections of insulin on each conditioning trial, and the other received injections of saline. The seventh trial was the test trial.

Fourteen hr before the test trial, half the subjects in each group were given 85 mg/kg of the diabetogenic drug, streptozotocin. The other rats received the equivalent volume of the vehicle for the streptozotocin, a 21% solution of citric acid in physiological saline. The injections were given intravenously while the rats were under halothane anesthesia. The choice of a time for this injection was made on the basis of the findings of Junod and collaborators (11). They found that af-

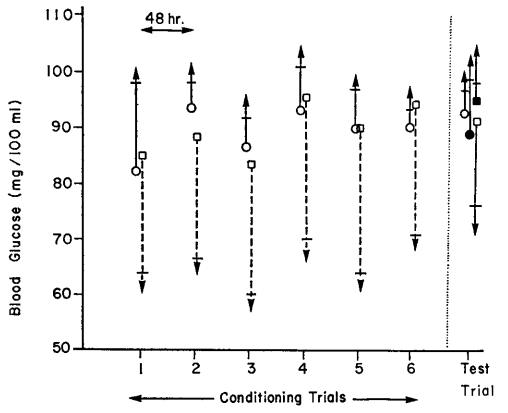

FIG. 1. Blood glucose levels before and after the 20-min retention in the conditioning chamber: (○, ●), animals conditioned with saline injections; (□, ■), animals conditioned with insulin injections. On the test trial: (solid symbols), animals getting streptozotocin; (open symbols), animals getting its vehicle. (Dotted vertical line), the point in time at which the streptozotocin or its vehicle was given.

ter the administration of streptozotocin to rats, the rats' blood glucose levels fall to near zero and then rise, passing through the normal range at about 14 hr. Use of this time interval has the advantage that all animals began the test trial with approximately equal blood glucose levels.

At the time at which the next conditioning trial would normally have occurred (*i.e.*, 14 hr later), the usual conditioning procedure was followed except that all animals got an injection of saline. After this trial, the pancreas of each animal was removed and subsequently prepared for histological examination.

Results. As there were no detectable differences between the results of the two replications, the results have been pooled. They are shown in Fig. 1. The mean blood glucose levels before the 20-min period in the conditioning chambers are represented by squares and circles for the two groups. Arrows have been drawn from these points to the mean level of blood glucose at the end of the 20-min period. These means are indicated by the tips of the arrows. The horizontal lines intersect the arrows at a distance from the tip that corresponds to one standard error of the mean. One rat that had gotten streptozotocin had a basal glucose level that exceeded the limits of the measuring instrument; its data, therefore, were omitted from the analysis.

During conditioning, the subjects getting the insulin showed a decrease in blood glucose, whereas those getting saline showed a slight rise in blood glucose. On the test day, when all subjects received only saline, only one group showed a decrease in blood glucose: those were the rats that had been conditioned with insulin, and that had been given only the vehicle for the streptozotocin 14 hr before testing. The rats that had been conditioned with insulin and which had received the streptozotocin showed a rise in blood glucose level on the test trial of approximately the same magnitude as that shown by the group that had received saline during the conditioning trials. There was no reliable difference between the rats trained with saline that had received streptozotocin and those that had not. The change in blood glucose shown by the rats conditioned with insulin and not given streptozotocin was significantly different from both the conditioned rats given the vehicle for streptozotocin ($t = 4.07$, 9 df, $p < 0.01$) and from the control rats (put through a conditioning procedure but given saline instead of insulin) given the vehicle for the streptozotocin ($t = 6.08$, 10 df, $p < 0.01$). Histological examination of the pancreases of the animals revealed that the beta cells had been extensively destroyed in the rats given the streptozotocin.

Even though these data imply that the conditioned hypoglycemia is due to a release of insulin, it is still possible that the hypoglycemia is due to some other mechanism, such as a decrease in some antagonist to insulin. The blood glucose level cannot drop appreciably over a 20-min period unless some insulin is present in the blood. Thus, it is not possible to exclude completely the possibility that in Expt. 1 the rats given streptozotocin did in fact experience a decrease in insulin antagonists. Such a phenomenon might have no effect on blood glucose levels because insufficient insulin would remain in the blood at the time of the test trial, 14 hr after the

administration of the streptozotocin. Therefore, a second experiment was undertaken in order to determine whether or not the conditioned hypoglycemia was associated with increased insulin-like activity (ILA).

Expt. 2. Method. Subjects were 12 male rats of the same type as in Expt. 1. They were randomly divided into two groups of 6 subjects each. One group had 6 conditioning trials with injections of insulin, and the other group had 6 conditioning trials with injections of saline.

On the test trial, all subjects (a) had a blood sample removed from the tail, (b) received a saline injection, and (c) were placed in the conditioning chamber for either 5 or 10 min. At the end of this time, enough blood (0.5 ml) for an ILA assay was milked from the tail.

The ILA assay used was a modification of that of Randle (12). A weighed amount of fresh hemidiaphragm from 1 of 6 additional, starved rats was incubated in 0.8 ml of buffer (13) containing 2.5 mg/ml of glucose and 0.2 ml of plasma from 1 of the 12 experimental subjects. The mixtures were incubated in Warburg manometer flasks in a Gilson Differential Respirometer (model GR 20/5) for 3 hr at 37°. The gas phase during the incubation was maintained at 93.14% oxygen and 6.86% carbon dioxide. The shake rate was 160 cpm.

The glucose level of each flask was determined before and after the incubation, the difference representing the glucose uptake of the isolated rat diaphragm. The procedure is based upon the assumption that the glucose uptake by the muscle tissue increases with increased concentration of insulin in the blood sample. As a control for any differences between diaphragms, each was cut in half, and the plasma from a control subject was incubated with a piece from one half, the plasma from a subject conditioned with insulin was incubated with the corresponding piece from the other half.

Results. The responses of the two groups during the 6 conditioning trials were the same as for the analogous groups in expt. 1. On the test day, the pieces of diaphragm incubated with the plasma from rats trained on insulin took up significantly more glucose (mg of glucose/g of wet wt diaphragm/hr) than those incubated with plasma from the controls ($F = 44.64$; 1 and 7 df; $p < 0.01$). The data are given in Table I.

TABLE I. Means and Standard Errors of the Mean for the Various Groups in Exp. 2.

Time (min)	Glucose uptake on test trial (mg of glucose/g of wet wt diaphragm/hr)	
	Mean	SEM
Conditioned		
5	4.47	0.638
10	4.16	0.045
Controls		
5	2.21	0.236
10	1.14	0.214

One rat conditioned with insulin injections was not given an injection on the test day. This rat had the same ILA level as the control subjects, whereas the other 5 rats conditioned with insulin all showed an increased ILA relative to the controls. This rat has been omitted from the analysis because of the procedural difference.

Samples taken at 5 min showed significantly greater ILA levels than those taken 10 min after the onset of the conditioning situation ($F = 6.15$; 1 and 7 df; $p < 0.01$).

Discussion. Expt. 1. The relatively new drug, streptozotocin, was used in preference to the more well-known drug, alloxan, because it is a superior diabetogenic agent and has fewer side effects (14). In fact, there is not the shift from carbohydrate to lipid metabolism that occurs following alloxan administration (14). The dosage and the time the streptozotocin was injected were chosen, on the basis of the data of Junod and collaborators (11), so that at the beginning of the test trial the blood glucose of the rats given streptozotocin would be as close as possible to that of the rats given its vehicle. This choice was satisfactory, for the mean basal glucose level of the rats given streptozotocin was 91.4 mg/100 ml of blood, which is to be compared with 92.0 for the controls.

Although the effect of the streptozotocin could conceivably have been caused by some hypothetical, nonspecific or general debilitat-

ing action rather than its specific action on the beta cells, the rats given the streptozotocin behaved no differently from the controls, nor were their glucose levels high enough to indicate undue stress. Therefore, we attribute the absence of a conditioned response in the rats given streptozotocin to its specific action on the beta cells.

Expt. 2. The fact that the ILA activity was greater 5 min after the beginning of the test trial than it was 5 min later suggests that the conditioned release of insulin occurs shortly after the onset of the conditioned stimulus. The half-life of insulin, which may be as short as 3.3 min (15), is consistent with the decrease observed over the 5 min. The delayed hypoglycemic response to the release of insulin, which peaks about 20 min after the onset of the conditioned stimulus (7), is also in line with expectations.

The decrease of ILA in the rats given the vehicle may have been caused by an increase in stress-related hormones, such as epinephrine, which inhibit release of insulin (16) and cause a slight rise in glucose level that peaks about 1 hr after the injection (7). The gradual accumulation of these hormones and the delay in their effect on the release of insulin may account for the decrease in ILA in these rats between 5 and 10 min after the injection.

It might be argued that the difference between insulin-conditioned and saline-conditioned rats is due to some cumulative effect of the insulin given during the conditioning trials instead of being due to a conditioned release of endogenous insulin. There are, however, three lines of evidence against this: (a) the basal glucose levels of rats trained on insulin are the same as those of rats trained on saline, (cf. Fig. 1); (b) the 48 hr intervening between the last injection of insulin and the test trial is between 96 [see Ref. (17)] and 850 half-lives (15) of insulin; and (c) one rat that had been conditioned with insulin, but which received no injection at all on the test trial, showed no increase in ILA relative to the controls. The fact that this rat, without an injection, did not show the increased ILA indicates that the ILA increase depends upon the injection procedure on the test trial. This is inconsistent with an explanation based upon an hypothesized accumulation effect of repeated injections of insulin, but it follows directly from the observation that the injection procedure is an essential component of the conditioned stimulus (9).

Significance. The results of these two experiments, taken together, indicate that this conditioning paradigm causes a conditioned increase in some hypoglycemic agent detected by the ILA procedure, that is eliminated by destroying the beta cells. That hypoglycemic agent almost certainly is insulin.

This conclusion is difficult to reconcile with a report of conditioned hypoglycemia in pancreatectomized dogs (3). However, that report contains no evidence that all of the pancreas had been removed, a difficult feat at best. Further, the data reported were based on the only three dogs that survived, out of 23 dogs operated upon. Perhaps the reason they survived was incomplete removal of their pancreases. This experiment also lacked an appropriate control group against which the conditioned response could be compared. Thus, this report fails to cast serious doubt on the conclusion that the hypoglycemia observed here depends upon a conditioned release of insulin.

To condition an effect is to bring it under a particular kind of neural control, whether directly or indirectly. Thus, these results demonstrate that release of insulin can be elicited by a neural mechanism that can be conditioned. There have been several recent reports (18–20) that electrical stimulation of the vagus nerve can cause release of insulin, but this matter has long been controversial (21). The advantage of a conditioning technique over gross electrical stimulation of the vagus nerve trunk is that the experimenter can rely upon the functional organization of the nervous system to activate specifically those fibers of a functionally distinct system, whereas electrical stimulation unselectively activates populations of fibers that may have antagonistic and interacting effects.

The mechanism or mechanisms whereby nervous activity increases the titer of insulin in the blood is unknown. At least three possi-

bilities can be advanced at present: (a) a direct release of insulin by means of activity in neurons making direct contact with the beta cells (22); (b) modulation of insulin release via local vasodilation in the proximity of the beta cells (23); and (c) secondary release of insulin caused by a release of some gut factor such as pancreozymin (24), secretin (25), or gastrin (26).

These results indicate that in normal rats blood glucose can be lowered directly by injection of insulin, or indirectly by means of a neural mechanism that can be conditioned. When the mechanism for this conditioned release of insulin becomes known, perhaps it will clarify the functional utility of a release of insulin under conditions in which, in the past, the animal's blood glucose level had been physiologically low at the outset. Not only does this appear to be maladaptive, but it conflicts with current views of the processes of conditioning.

Summary. Although the phenomenon of conditioned hypoglycemia is now well established, little is known about its mechanism. The present experiments lead to the conclusion that the mechanism involves a release of insulin: Experiment 1 showed that rats given an injection of streptozotocin, a drug which destroys the beta cells of the islets of Langerhans, did not show a conditioned hypoglycemia, whereas rats given only the vehicle for the streptozotocin did; and in experiment 2, blood drawn from conditioned rats just before the conditioned hypoglycemia would normally occur showed greater insulin-like activity than blood drawn from control rats. These results demonstrate a conditioned release of some hypoglycemic agent that depends upon the integrity of the beta cells. A neural control over release of insulin must be inferred to explain them.

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