A Decreased Response of Cyclic Adenosine Monophosphate Concentrations to Glucagon in Liver Slices from Streptozotocin-induced Diabetic Rats

KAMEJIRO YAMASHITA, SOMO YAMASHITA, HIROKO YASUDA, YUJI OKA, AND ETSURO OGATA

SUMMARY

Responses to glucagon from the adenylate cyclase–cyclic adenosine monophosphate (cAMP) system in liver slices from control and streptozotocin-induced diabetic rats were compared. Tissue cAMP levels were similar in the basal state but responded poorly to glucagon (20 pg/ml–2 μg/ml) in diabetic rats. Insulin treatment of diabetic rats in vivo led to a reversal of the glucagon stimulation towards the values in the control rats. The basal and glucagon-stimulated activities of adenylate cyclase in crude membrane fractions were similar in both groups.

Plasma immunoreactive glucagon levels in diabetic rats were approximately three times higher than those in normal rats.

Liver slices obtained from normal rats, which were injected with glucagon (0.2 mg, i.m.) 45 min previously, also showed an impaired responsiveness to glucagon of tissue cAMP levels, while no significant difference in adenylate cyclase activity was observed between the normal and glucagon-treated rats.

These results suggest that the responsiveness of liver slices from the streptozotocin-induced diabetic rat has been modified by the preceding hyperglucagonemia. The reason for the observed differences between slices and crude membranes is not known.

DIABETES 29:188–192, March 1980.

Diabetes has been described as a bihormonal disease caused not only by insulin deficiency but also by glucagon excess.[1] From this point of view, it is conceivable that deteriorations in hepatic metabolism in diabetes are attributable to abnormal plasma levels of either hormone or both.

Recently it has been demonstrated that several hormones, including insulin and catecholamines, regulate the concentration and function of their own receptors in target cells.[2,3] For example, hyperinsulinemia due to a variety of causes is accompanied by a decrease in the specific insulin-binding capacity of target cells. Regarding glucagon, Soman and Felig reported that the hyperglucagonemia, lasting 5 h, in rats resulted in a 45% decrease in glucagon binding and a comparable decrease in the glucagon-stimulated adenylate cyclase activity of a membrane fraction from the liver.[4] However, preceding and subsequent reports of theirs and of others were or were not in harmony with this conclusion.[5–7] It is possible that the disparity in conclusions may come from a difference in the tissue preparations employed in the experiments.

The present study was undertaken to determine if responses to glucagon of the hepatic adenylate cyclase–cyclic AMP (cAMP) system were altered in streptozotocin-induced–diabetic rats and whether the alteration resulted from the preceding hyperglucagonemia. For this purpose the responses of liver slices and those of liver membrane preparations were compared.

Evidence is presented in this communication that the preceding hyperglucagonemia may cause a diminished response of the adenylate cyclase–cAMP system in the slices but not in the membrane fractions.

MATERIALS AND METHODS

Wistar rats, weighing between 200 and 250 g, were used. Diabetes was induced by a single injection of streptozotocin (SZ) (60 mg/kg of body weight) into the tail vein. Control rats received the vehicle. SZ was dissolved immediately before use in a citric acid buffer (50 mM) adjusted to pH 4.5, and 0.3 ml of solution was injected. On days 7–14 after SZ injection, plasma glucose was measured, and only those rats with fasting plasma glucose of 200 mg/dl or higher were used in the study. Several rats in the diabetic group were injected subcutaneously with 0.5 U of Lente insulin per day for four days, and those rats with fasting plasma glucose of 120 mg/dl or lower were used as insulin-treated diabetic rats. In another set of experiments the normal

From the Department of Medicine, University of Tsukuba, the Department of Medicine, University of Tokyo, and the Division of Endocrinology, National Cancer Institute, Japan.
Address reprint requests to Kamejiro Yamashita, M.D., Department of Medicine, University of Tsukuba, Niihari-gun, Ibaraki-ken, 300-31, Japan.
Received for publication 8 January and in revised form 22 October 1979.

animals received 200 μg of glucagon i.m. 45 min before sacrifice, with the control receiving the appropriate vehicle (saline). The rats had free access to standard rat chow and tap water. The experiments were started at between 10 and 11 a.m. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.), and the abdominal wall was opened. Two milliliters of blood was withdrawn from the abdominal aorta with a heparinized syringe; 20 μl of the blood sample was used for glucose determination. An aliquot of the plasma was stored for immunoreactive insulin (IRI) assay. For glucagon assay, 50 μl of Trasylol (250 U) was added immediately to 1 ml of the blood (before separation of the plasma).

After being perfused with 20–40 ml of cold normal saline from the portal vein, the liver was removed and sliced. Thirty to 40 mg of the slices was incubated at 37°C in test tubes containing 1 ml of Krebs-Ringer bicarbonate buffer, glucose (1 mg/ml), theophylline (10^{-2}M), bovine albumin (1 mg/ml), and appropriate substances to be tested. In all incubations the gas phase was 95% O_2 and 5% CO_2. The slices were preincubated for 10 min without test substances, and then the incubation was performed as indicated in the legends of the figures. After incubation, the tissue was extracted with 0.5 ml of hot 50 mM sodium acetate. After centrifugation, the cAMP content in the supernate was determined by radioimmunoassay[8] using the Yamasa kit.

Liver homogenates were prepared in 0.25 M sucrose and filtered through four layers of gauze. The 8000 *g* pellet of the filtrate was used as a crude membrane fraction.

Adenylate cyclase activity in the liver crude membrane fraction was assayed according to the procedure described previously.[9] In short, incubation of 10–30 μg of membrane protein was performed at 37°C in 200 μl of a medium consisting of 2.5 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 30 mM Tris-HCl buffer (pH 7.6), and an ATP-regenerating system (10 mM phosphenolpyruvate and 5 μg of pyruvate kinase). The reaction was terminated by boiling for 3 min. After centrifugation, the supernate was assayed for cAMP by the above-mentioned method. Glucagon was added in the concentrations indicated in the figures. The rate of cAMP production was used as an index of the enzyme activity.

The specific activity of a plasma membrane marker enzyme, 5′-nucleotidase, and a microsomal marker enzyme, glucose-6-phosphatase, was measured by the method described by Aronson and Touster.[10] Protein content was estimated by the method of Lowry et al.[11]

Plasma glucose was measured by a glucose oxidase method.[12] IRI was assayed with the two antibody method by use of a kit provided by Daiichi Isotope Co., and immunoreactive glucagon was assayed with the two antibody method employing antiserum 30 K.[13] Glucagon was a generous gift from Kodama Co. SZ was purchased from Upjohn Co., Kalamazoo, Michigan.

RESULTS

As summarized in Table 1, plasma glucose and glucagon levels were higher and insulin levels were much lower in the diabetic than in the control rats.

Figure 1 indicates that glucagon (200 pg/ml to 2 μg/ml) significantly stimulated cAMP levels in a dose-dependent fashion during 3 min of incubation in slices from control rats. The stimulation by glucagon of cAMP levels in liver slices was much lower than that obtained in perfusion experiments,[14] but it was consistent with the results obtained by others in slice experiments.[15] In contrast, no significant stimulation was observed in slices from diabetic rats by glucagon at concentrations lower than 200 ng/ml. The stimulation of cAMP levels by 2 μg/ml glucagon in liver slices from the diabetic rats was markedly lower than that from the normal rats. Basal levels (obtained without glucagon) were the same between the two groups.

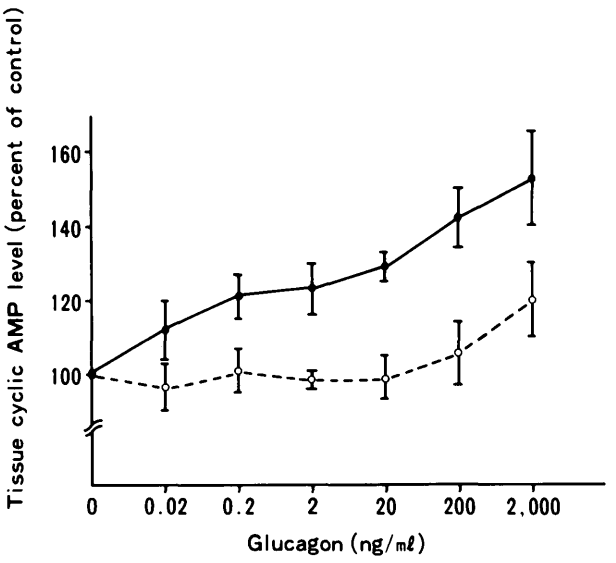
In liver slices from the control rats, the peak tissue cAMP response to glucagon was obtained at 3 min of incubation (Figure 2). Thereafter, the cAMP levels declined, reaching the basal by 10 min. The response was significantly lower in slices from diabetic than in the control rats at 3 and 5 min of incubation. Insulin treatment (0.5 U of Lente insulin subcutaneously for 4 days) led to a reversal of the glucagon stimulation towards the values in the control group.

TABLE 1
Plasma glucagon, insulin, and glucose levels in normal and streptozotocin-induced diabetic rats

	Glucagon (pg/ml)	Insulin (μU/ml)	Glucose (mg/100 ml)
Normal rats (N = 10)	115 ± 8.6	13.7 ± 2.1	104 ± 5.0
Diabetic rats (N = 8)	296 ± 38.3	2.3 ± 1.2	381 ± 44.7

Data are presented as means ± SE.

FIGURE 1. Effect of various concentrations of glucagon on cAMP levels in liver slices from control and SZ-induced diabetic rats.
After preincubation for 10 min, the slices were incubated in triplicate for 3 min in the medium containing various concentrations of glucagon, as described under MATERIALS AND METHODS. The basal levels (obtained in the absence of glucagon) of tissue cAMP in the control and the diabetic rats were 0.22 ± 0.03 and 0.20 ± 0.04 (mean ± SE of five experiments in each group) nmol/g tissue, respectively, and the difference between them was not significant statistically. In each experiment the basal levels were compared, the difference being not significant statistically. The values (mean ± SE, N = 5) in the figure are expressed as percentage of the basal of the respective group. The differences of values between the respective counterparts are significant ($P < 0.05$ by Student's *t* test) from 200 pg/ml to 2μg/ml of glucagon concentrations. (●——● Normal, N = 5; ○------○ diabetic, N = 5.)



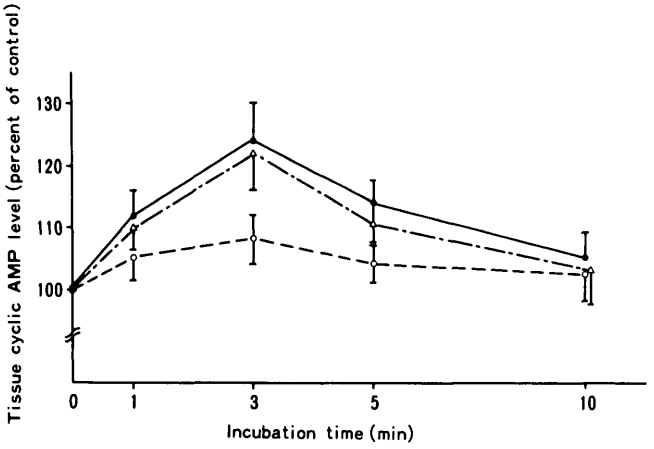


FIGURE 2. Time course of glucagon stimulation of cAMP levels in liver slices from control, SZ-induced diabetic, and insulin-treated diabetic rats.

Slices were incubated for various times in the medium containing glucagon (200 pg/ml). The values in the figure are expressed as percent of the basal. The other details are as in Figure 1. The cAMP responses of the diabetic rats at 3 and 5 min are significantly lower than the respective counterparts of control rats and insulin-treated diabetic rats ($P < 0.05$). (●——● Normal, N = 10; ○-------○ diabetic, N = 8; △— - — - —△ insulin-treated diabetic, N = 8.)

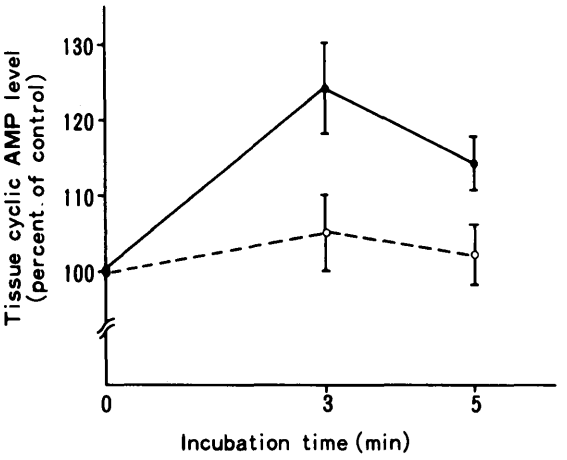


FIGURE 3. Time course of glucagon stimulation of cAMP levels in liver slices from control and glucagon-pretreated rats.

Normal rats were injected with 200 μg of glucagon or the vehicle (saline) i.m. 45 min before sacrifice. The other procedures were as described under MATERIALS AND METHODS. The slices were incubated in the medium containing 200 pg/ml glucagon.

The basal levels of tissue cAMP in the control and the glucagon-pretreated rats were 0.24 ± 0.04 and 0.25 ± 0.06 nmol/g tissue, respectively (N = 4 in each group), and the difference was not significant statistically. The values in the figure are expressed as in Figure 1. The values of the glucagon-pretreated rats are significantly lower than those of the respective control rats at 3 and 5 min of incubation ($P < 0.05$). (●——● Control, ○------○ glucagon-pretreated.)

Although the cAMP levels stimulated by norepinephrine (100 μM) were much lower than those by glucagon, the hormone-induced change was statistically significant (data not shown). Similar small but significant stimulation by norepinephrine was reported in liver perfusion experiments.[14] The rate of stimulation was identical in both the normal and the diabetic rats.

To examine adenylate cyclase activity in the membrane fraction, we prepared the crude membrane fraction as described in METHODS. The purity of the preparation was checked by assaying 5′-nucleotidase and glucose-6-phosphatase activities. As previously reported,[16] liver homogenates and membrane fractions from diabetic rats had significantly decreased 5′-nucleotidase activity when compared with controls (normal liver: homogenates 0.34 ± 0.05 μmol/mg protein/10 min, crude membranes 0.67 ± 0.06; diabetic liver: homogenates 0.20 ± 0.03, crude membranes 0.41 ± 0.06; values are mean ± SEM of four different experiments in each group), whereas glucose-6-phosphatase activity was increased in diabetic rats (normal liver: homogenates 0.71 ± 0.06 μmol/mg protein/10 min, crude membranes 1.24 ± 0.15; diabetic liver: homogenates 1.16 ± 0.21, crude membranes 2.32 ± 0.35). Comparison of these data indicates that the membrane fraction was concentrated by approximately twofold in both the control and the diabetic sample and that both preparations were accompanied by similar contamination with microsomal fractions. The adenylate cyclase activity in the crude membrane fraction prepared from either the normal or the diabetic livers was increased in a similar fashion by increasing concentrations of glucagon (20 pg/ml to 2 μg/ml) (data not shown). These results contrast sharply with those obtained in the slice experiments (Figure 1).

The effect of glucagon administration in vivo on the hepatic cAMP response to glucagon in vitro was next examined in normal rats (Figure 3). The basal tissue cAMP levels were the same for control and glucagon-pretreated animals. However, the response to glucagon in the tissue from the glucagon-pretreated animals was significantly lower than that in the control animals. The cAMP response to norepinephrine in slices and the adenylate cyclase activity in the crude membrane fractions were similar between the two groups of animals (data not shown).

Glucose (1.5 g/kg body weight) was injected i.p. 45 min before sacrifice to induce similar hyperglycemia (higher than 200 mg glucose/100 ml) to that produced by glucagon or SZ treatment, and the liver tissue cAMP response to glucagon was examined. The responsiveness was not altered by this maneuver (data not shown).

DISCUSSION

The present study demonstrates that SZ-diabetic rats exhibit hyperglycemia, hypoinsulinemia, and hyperglucagonemia. These results are consistent with the previous report.[16] Furthermore, we have demonstrated that responses to glucagon of the adenylate cyclase–cAMP system in liver slices from these diabetic rats are significantly lower than those in liver slices from normal rats and that insulin treatment of diabetic rats leads to a reversal of the responsiveness towards the values of the control rats. These phenomena were observed when physiologic or supraphysiologic quantities of glucagon were employed in the stimulation of the cAMP system.

Several, conflicting observations have been reported regarding the binding and biologic activity of glucagon in liver preparations from SZ-diabetic rats.[7,16] Srikant et al. reported that the concentration of specific glucagon-binding sites was significantly reduced in SZ-diabetic rats.[7] In isolated hepatocytes, Bhathena et al. demonstrated that specific glucagon binding was decreased in diabetic rats compared with controls and that hepatocyte cAMP response to

glucagon was almost abolished in diabetic rats.[17] When adenylate cyclase activity was examined in membrane fractions, Pilkis et al.[18] and Srikant et al.[7] observed that glucagon-stimulated adenylate cyclase activities were not different in SZ-diabetic rats and control rats.

In contrast, Soman and Felig reported that the specific binding of ^{125}I-glucagon to liver plasma membranes was approximately twofold higher in the diabetic as compared with the control rats.[16] They suggested that it was due to an increase in the number of binding sites rather than to a change in the binding affinity. Simultaneously, they reported that both the basal and glucagon-stimulated adenylate cyclase activities in the SZ-treated rats were twofold higher than in controls. Similar results were reported in membrane fractions by Hepp.[19] It is difficult to reconcile these diverse conclusions, but one likely explanation is that they are due to the difference in the experimental settings, particularly to that in liver preparations. To minimize inclusion of possible artifacts in the present study, we relied on a simple tissue slice system, and the results indicate, as in the case of those in the isolated cell experiments,[17] that tissue cAMP response to glucagon was diminished in diabetic rats. In our hands, also, the crude liver membrane preparation (intentionally prepared without drastic manipulations) did not exhibit the differences in the responsiveness to glucagon between diabetic, glucagon-treated, and control rats. Therefore, the results of membrane experiments including ours were much different from those obtained in intact cells. Similar dissociation between the glucagon-responsive hepatic adenylate cyclase activity and the hepatic cAMP response to glucagon was reported in hyperglucagonemic rats previously.[19] It seems likely that such factors as phosphodiesterase activity contribute to regulation of the tissue cAMP levels in diabetic livers, since phosphodiesterase activity in homogenates is still present, even after the addition of 10 mM theophylline. The reduced responsiveness to glucagon in vitro in liver slices from glucagon-injected rats has been shown, although the level and time course of hyperglucagonemia may be different from those in diabetic states. The present results are consistent with the report of DeRubertis and Craven.[21] Similar line of results was also reported by Soman and Felig in the rats pre-infused with glucagon for 5 h.[4] In addition, our results indicate that hyperglycemia per se did not reproduce the state of diabetic rats. These data support the thesis that impairment of the liver cAMP response to glucagon in diabetic rats is caused by preceding hyperglucagonemia. Similar reduction in hepatic cAMP responses to glucagon has been described in cultured fetal hepatocytes after preceding exposure to glucagon.[20]

Since Exton et al.[14] and Hepp[19] reported the activation of adenylate cyclase by norepinephrine, we also examined the effect of norepinephrine on tissue cAMP levels. In contrast with the glucagon stimulation, no difference in the norepinephrine stimulation of tissue cAMP levels was observed between diabetic and normal livers and between glucagon-pretreated and control rats (data not shown). The above-mentioned phenomenon (the reduced responsiveness to glucagon in vitro in liver slices from the diabetic and glucagon-injected rats) may be explainable as a hormone-specific desensitization or refractoriness induced by endogenous overproduction or an exogenous administration of the α-cell hormone. Similar phenomena have been reported in terms of other peptide hormones and catecholamines.[22–24]

The pathophysiologic significance of hyperglucagonemia and of altered glucagon responses in diabetic states has not been elucidated. However, it seems that the hyperglycemia maintained in the diabetic rats is an expression of a persistent, though less efficient, action of glucagon on hepatic glucose production. In conclusion, the present data support the concept that regulation by glucagon of hepatic metabolism is impaired in diabetic livers.

ACKNOWLEDGMENTS

The authors thank Dr. James B. Field for his help in preparing the manuscript.

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