Time Course of the Defective α-Cell Response to Hypoglycemia in Diabetic BB Rats

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Although it is understood that patients with insulin-dependent diabetes mellitus (IDDM) lose the ability to release glucagon during a hypoglycemic challenge, the relationship of this defect to the disease onset and loss of β-cell function is not well defined. To address this issue, we measured the counterregulatory response in three groups of BB/wor rats during sequential 90-minute euglycemic (7 mmol/L) and hypoglycemic (3 mmol/L) insulin clamps (180 pmol/kg · min). Group 1 (n = 8) consisted of nondiabetic BB rats (aged 84 ± 3 days), and groups 2 and 3 were rats studied 1 day (n = 7) or 7 days (n = 6) after diabetes onset. Plasma glucagon concentrations were similar in all groups during euglycemia (244 ± 47 ng/L for nondiabetic, 308 ± 38 for 1 day of diabetes, and 277 ± 30 for 7 days of diabetes). Moreover, after 1 day of diabetes, the increase in plasma glucagon during hypoglycemia was similar to that seen in controls (to 581 ± 94 and 650 ± 118 ng/L, respectively) even though insulin production by the pancreas was virtually absent. However, after 7 days of diabetes, plasma glucagon only increased to 339 ± 59 ng/L during hypoglycemia (*P* = nonsignificant *v* basal), despite normal pancreatic glucagon content (11.5 ± 1.2 *v* 10.8 ± 0.6 μg/g in nondiabetic controls). In conclusion, the hypoglycemia-associated defect in glucagon release occurs early in the course of diabetes in BB rats and is not associated with decreased baseline plasma or pancreatic glucagon levels. This impairment, although not immediately linked to the decrease in pancreatic insulin content, occurs soon afterward, implying that the two events are related.

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THE PROFOUND BENEFITS of intensive therapy, such as a decreased occurrence of neuropathy and microvascular complications reported by the Diabetes Control and Complications Trial,[1] demonstrate a clear need to improve long-term glycemic control in insulin-dependent diabetes mellitus (IDDM). Unfortunately, this form of therapy results in more frequent episodes of severe hypoglycemia, which can then lead to altered counterregulatory glycemic thresholds and hypoglycemia unawareness,[2] provoking still more hypoglycemia. In addition, as the duration of IDDM increases, defects in the counterregulatory pathways themselves emerge, making hypoglycemia even more likely. In particular, the release of glucagon, which normally functions as a primary defense against hypoglycemia,[3-5] is lost in diabetic patients.[6-9] This plus an impairment in epinephrine release that develops later[7,10,11] act in concert to limit the diabetic patient's ability to prevent serious hypoglycemic events that may adversely affect brain function.[12-14]

The factors that contribute to the α cell's failure to respond to a hypoglycemic challenge and its relationship to the onset of IDDM are not well defined. Paradoxically, circulating levels of glucagon are normal or elevated in the postabsorptive state in patients with IDDM,[15-17] despite the presence of hyperglycemia. In addition, glucagon release in response to other stimuli such as arginine and alanine remains intact,[16,17] suggesting that it is not simply a defect in the glucagon synthesis and secretion pathway.

Defective glucagon responses to hypoglycemia generally appear within the first 2 to 3 years of disease,[15] and as early as several weeks in some patients. Inasmuch as this time course is closely related to the disappearance of endogenous insulin secretion in IDDM, it has been suggested that the absence of insulin within the islet might desensitize the α cell to changes in glucose concentrations.[18] It is noteworthy that restoring circulating insulin levels and normalizing glucose concentrations are not sufficient to restore the glucagon response to hypoglycemia.[15,19] Nonetheless, it could still be argued that the insulin concentration in the local environment of the α cell is much higher in a healthy pancreas than the amount that reaches the peripheral circulation. Alternatively, it may be that the normal decrease in endogenous insulin secretion during hypoglycemia is necessary to stimulate the α cell to secrete glucagon.

The current study was therefore undertaken to define more precisely the relationship between disease onset, loss of pancreatic insulin secretory capacity, and appearance of the α-cell defect in response to a hypoglycemic challenge. The BB rat was chosen as a model of IDDM for several reasons. Like its human counterpart, it develops a severe impairment in glucagon release during hypoglycemia but retains the capacity to secrete glucagon in response to amino acids or physical stress.[20] The onset of IDDM in BB rats is extremely abrupt (ie, within 1 to 2 days) and is not followed by a "honeymoon" phase, thus allowing more precise timing of the loss in β-cell function than in the human disease. Finally, the animal model offers the opportunity to obtain pancreatic tissue for direct measurement of islet hormone levels.

MATERIALS AND METHODS

Animal Model

Diabetes-prone BB/wor male rats were obtained from the University of Massachusetts breeding facility. All animals were housed in the Yale Animal Care Facility, kept on a 12-hour light/dark cycle, and fed a standard ad libitum rat chow diet consisting of 51% carbohydrate, 5% fat, and 22% protein, with the

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remaining weight accounted for by moisture and nonmetabolizable solids such as ash (Agway Prolab 3000, Syracuse, NY). The animals were divided into three experimental groups based on the appearance of disease. The first two groups of animals underwent surgery using pentobarbital anesthesia (40 mg/kg intraperitoneally) 2 to 3 weeks before the predicted date of disease onset (based on breeding records provided by the University of Massachusetts) to implant long-term catheters in a carotid artery and jugular vein for subsequent use in hypoglycemic clamp experiments, as described previously.[21] This protocol was based on preliminary studies showing that if surgery (and the stress accompanying it) were performed during the week preceding the predicted date of diabetes onset, hyperglycemia was either delayed or never occurred in these rats. Following surgery, the animal's body weight and AM fed plasma glucose levels were monitored daily to determine the precise time of onset of diabetes. Group 1, the nondiabetic control group, consisted of eight BB rats that had not developed diabetes within the time frame of the study. Group 2 (n = 7) were BB rats studied within 24 hours after tail vein plasma glucose values exceeded 13.5 mmol/L in the fed state.

In addition, a third group of rats (n = 6) were studied after they had developed diabetes for 7 days. Because of the difficulty in maintaining vascular access, as well as a delay of disease onset caused by the problems associated with surgery catheters were implanted in this group immediately after hyperglycemia was observed. Thereafter, they remained hyperglycemic for 7 days while being treated with single daily injections of PZI insulin (8 U kg; Eli Lilly & Co, Indianapolis, IN) before the study. Only animals in which the treatment was sufficient to permit weight gain and that exhibited no other signs of illness were subsequently studied using an infusion protocol approved by the Animal Care and Use Committee of Yale University School of Medicine.

Infusion Protocol

All animals were allowed ad libitum access to food and water except on the morning of study, when food was removed. Arterial (infusion) and central venous (sampling) catheters were then opened, flushed with normal saline, and kept patent with a 20-μL/min infusion of saline containing a small amount of heparin (1 to 2 U/mL). The animals were studied 1 hour later while awake and freely moving around in the cages.

Diabetic and nondiabetic rats then underwent identical infusion studies according to the following protocol. Before initiating the infusion protocol, two samples were obtained from each animal for measurement of plasma glucose, epinephrine, and norepinephrine levels over a 30-minute time interval under basal conditions. During initial studies, glucagon samples were also obtained before the insulin clamp. Results yielded levels similar to those seen during insulin infusion; since each sample required 800 μL blood, basal glucagon samples were not collected in subsequent studies. At 0 minutes, an infusion of regular porcine insulin (180 pmol/kg · min; Eli Lilly & Co) was begun, and continued for 180 minutes. Preliminary experiments demonstrated the need for high-dose insulin to consistently decrease plasma glucose in rats after the onset of diabetes. In the first 90 minutes of the study, plasma glucose was maintained at euglycemic levels (7 mmol/L) using a variable-rate dextrose infusion based on 5-minute plasma glucose determinations. Thereafter (90 to 180 minutes), plasma glucose was allowed to decrease to hypoglycemic levels (3 mmol/L) and was clamped there for 30 minutes. Blood samples for measurement of catecholamines, glucagon, and insulin levels were obtained at 15-minute intervals over the last 30 minutes of the euglycemic and hypoglycemic phases. The total volume of blood withdrawn was approximately 5 mL and was replaced with heparinized donor blood obtained by cardiac puncture of a nondiabetic BB rat.

Pancreatic Hormone Content

In a separate group of animals, the insulin and glucagon content of pancreatic tissue was measured in the three treatment groups already mentioned, nondiabetic controls (n = 8), diabetes of 1 day's duration (n = 8), and diabetes of 7 days' duration (n = 8). Tissue content was measured in this additional series of rats (rather than in the infusion series) to eliminate potential effects of the 3-hour hyperinsulinemic-hypoglycemic clamp protocol on pancreatic glucagon and insulin content. All animals were killed using an overdose of pentobarbital, and the entire pancreas was quickly removed, weighed, and homogenized in acid/ethanol to extract islet hormones. Tissue extracts were stored at −20°C until analyzed by radioimmunoassay.

Analytical Measurements and Calculations

Plasma glucose level was measured in duplicate using a Beckman glucose analyzer (Fullerton, CA), and plasma epinephrine and norepinephrine were analyzed using a radioenzymatic method (Amersham, Arlington Heights, IL). Glucagon concentrations were determined in plasma and in pancreatic tissue homogenates (after acid/ethanol extraction) by a double-antibody radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). Insulin measurements in plasma and pancreatic tissue (acid-extracted) used rat insulin standards in a double-antibody assay (Binax, South Portland, ME). Pancreatic insulin and glucagon content are expressed as picomoles per gram and nanograms per gram tissue, respectively. All data expressed as the mean ± SE were analyzed using ANOVA (CRUNCH software; San Francisco, CA).

RESULTS

All three groups were closely matched in terms of age (Table 1). Body weight at the time of study was slightly but not significantly reduced in 1-day diabetic rats. As expected, there were striking differences in plasma glucose levels between diabetic and nondiabetic groups at the outset of the study. Animals in whom diabetes occurred only 24 hours before the study reached this level of hyperglycemia spontaneously, whereas those with diabetes for 7 days were kept at hyperglycemic levels by adjusting the single daily insulin dose. Circulating basal glucagon levels in hyperglycemic diabetic animals (252 ± 24 ng/L, n = 8) were similar to those observed in euglycemic nondiabetic controls (223 ± 16 ng/L) before starting the insulin infusion.

During the high-dose insulin clamp, plasma insulin concentrations increased rapidly to 17.1 ± 1.8 nmol/L. Plasma glucose in both diabetic groups decreased to euglycemic levels within 30 minutes (time course not shown); the glucose plateau (coefficient of variation <10%) during the last 30 minutes of euglycemia was comparable in each group (Table 1). When glucose was decreased further to hypoglycemic levels, all groups reached the target of 3 mmol/L within 30 to 40 minutes and remained constant at

Table 1. Comparison of Age, Body Weight, and Plasma Glucose Plateau in Three Groups of Rats

	Age (d)	Body Weight (g)	Plasma Glucose (mmol/L)		
			Basal	Euglycemia	Hypoglycemia
Nondiabetic	84 ± 3	286 ± 10	8.2 ± 0.2	6.9 ± 0.1	2.9 ± 0.1
1-day diabetic	78 ± 6	232 ± 26	20.0 ± 1.2	7.4 ± 0.3	3.1 ± 0.1
7-day	82 ± 3	272 ± 11	23.9 ± 1.0	7.3 ± 0.3	3.1 ± 0.1

that level for an additional 30 minutes (coefficient of variation <8%; Table 1). Plasma epinephrine and norepinephrine concentrations during the euglycemic and hypoglycemic phases of the study are shown in Fig 1. During the euglycemic basal period, circulating epinephrine levels were not significantly different in the nondiabetic group and both diabetic groups (1.03 ± 0.35, 2.99 ± 1.19, and 1.48 ± 0.33 nmol/L for nondiabetic and 1-day and 7-day diabetic, respectively). Norepinephrine values were also comparable among the three groups, although euglycemic levels after 1 day of diabetes (3.65 ± 0.78 nmol/L), like epinephrine levels, tended to be slightly but not significantly higher compared with levels observed after 7 days of diabetes (2.31 ± 0.35) or in nondiabetic controls (2.28 ± 0.45). Moreover, there was a marked stimulation ofepinephrine release at 3 mmol/L glucose that was comparable in all groups (to 16.37 ± 1.68, 17.09 ± 4.95, and 19.12 ± 4.40 nmol/L in nondiabetic and 1-day and 7-day diabetic, respectively). Likewise, the norepinephrine response to hypoglycemia was at least as great in 1-day and 7-day diabetic rats (to 8.09 ± 2.15 and 5.57 ± 1.32 nmol/L, respectively) compared with nondiabetic rats (to 4.16 ± 0.71). If variations in baseline values are taken into account, increments of norepinephrine above basal were similar in all three groups (1.8-fold in nondiabetic, 2.2-fold in 1-day diabetic, and 2.4-fold in 7-day diabetic).

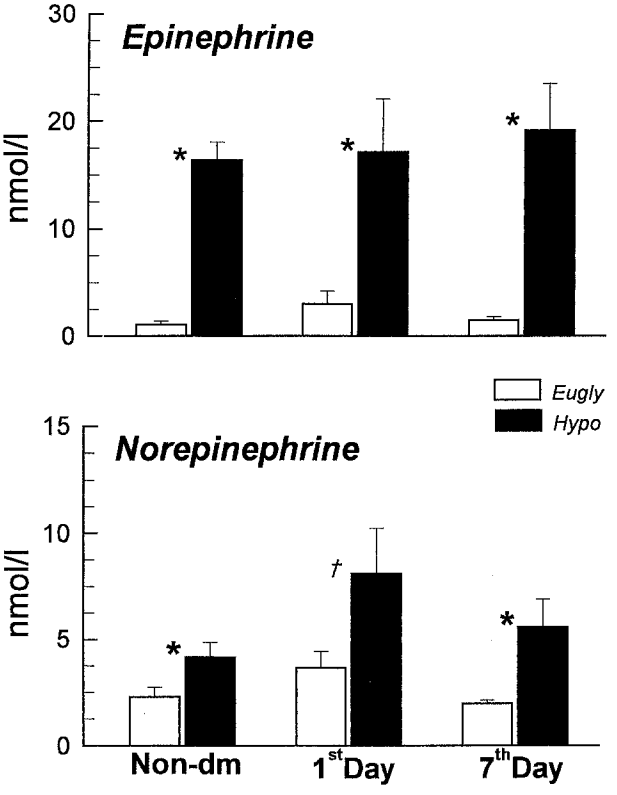

Fig 1. Effects of hypoglycemia on plasma epinephrine and norepinephrine in 1-day and 7-day diabetic and nondiabetic (non-dm) rats. The mean levels of 2 samples obtained during the final 30 minutes of the euglycemic (□) and hypoglycemic (■) phases of the insulin infusion protocols are shown. Data are expressed as the mean ± SE. *P < .05, †P < .06: v euglycemia.

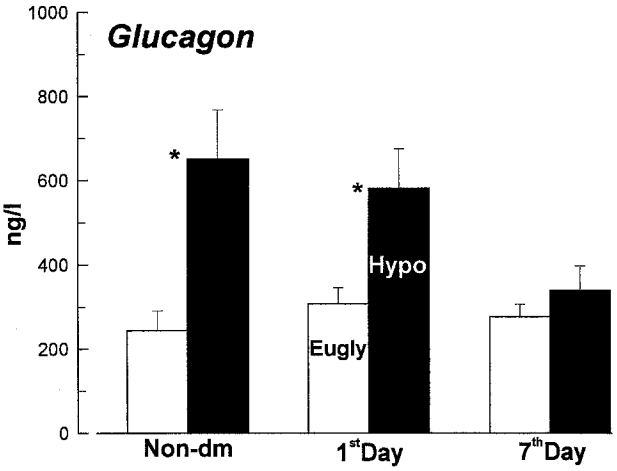

Fig 2. Circulating glucagon levels during hyperinsulinemic euglycemia (□) and hypoglycemia (■) in control and diabetic rats. Results are the mean of 3 measurements obtained during the final 30 minutes of both intervals in nondiabetic controls and diabetic rats 1 day or 7 days after disease onset. *P < .05 v euglycemia.

Plasma glucagon concentrations during euglycemia (7 mmol/L) and hypoglycemia (3 mmol/L) are shown in Fig 2. Circulating glucagon levels were similar during the euglycemic phase of the study in nondiabetic (244 ± 47 ng/L), 1-day diabetic (308 ± 38), and 7-day diabetic (291 ± 34) rats. Moreover, these values were similar to those obtained before insulin infusion in all groups. However, systemic glucagon levels following the decrease in glucose to 3 mmol/L were strikingly diminished in BB rats after 7 days of diabetes (Fig 2). Stimulated glucagon values in 1-day diabetic animals increased to 581 ± 94 ng/L, a value indistinguishable from that in nondiabetic rats, which increased to 658 ± 130 ng/L (P = NS v 1-day diabetic). In contrast, after 7 days of diabetes, only a small increase above basal levels was observed (to 350 ± 55), which was not statistically significant. Furthermore, the glucagon response of the 7-day diabetic group throughout the hypoglycemic period was significantly diminished compared with that of the nondiabetic controls and 1-day diabetic rats (both $P < .05$).

Pancreatic glucagon and insulin content are depicted in Fig 3. Despite differences in glucagon responses to hypoglycemia, glucagon content was not significantly different in the three groups (10.8 ± 0.6, 8.6 ± 1.0, and 11.5 ± 1.2 μg/g pancreas in nondiabetic and 1-day and 7-day diabetic rats). In contrast, pancreatic insulin content was markedly reduced in diabetic rats. After only 1 day of diabetes, pancreatic insulin content was decreased by 98%, and within 7 days it was decreased by 99% (to 0.3 ± 0.1 and 0.07 ± 0.05 nmol/g pancreas, respectively) compared with values in nondiabetic controls (14.2 ± 2.5 nmol/g).

DISCUSSION

These data demonstrate that a defect limiting the glucagon response to hypoglycemia develops rapidly and is clearly present within 7 days after disease onset in diabetic BB rats, even though basal circulating and tissue levels of

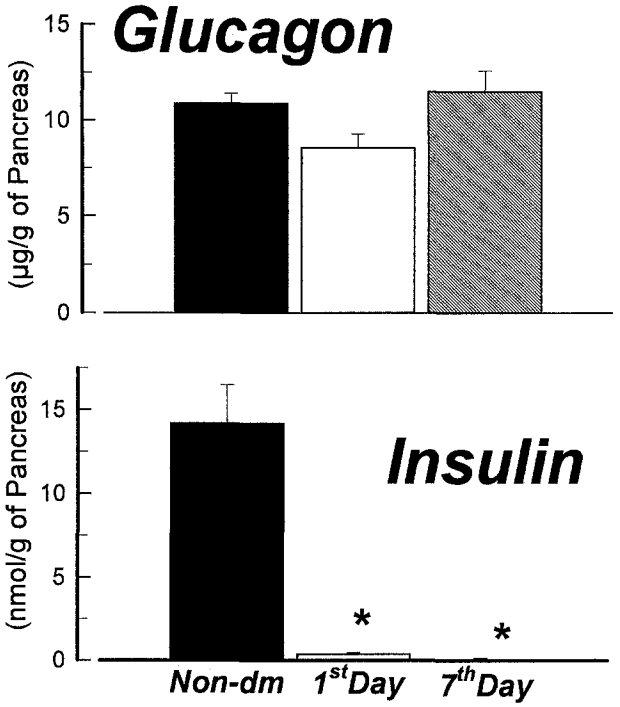

Fig 3. Effects of diabetes on glucagon and insulin content of the pancreas. *$P < .001$ *v* nondiabetic.

glucagon are not diminished. In addition, although this defect in glucagon release did not appear simultaneously with the abrupt loss of pancreatic insulin content, it occurred soon afterward. In contrast, the epinephrine response to hypoglycemia (~3 mmol/L) was robust in these newly diabetic animals. This finding contrasts with data obtained in BB rats with diabetes of longer duration (~3 months),[20] in whom, like some humans with long-standing IDDM,[7,10] the epinephrine response to hypoglycemia is blunted.

It has been suggested that the appearance of the defective glucagon response to hypoglycemia in IDDM is related to the disappearance of insulin secretory capacity.[18] However, it has been difficult to define the relationship of the α-cell defect to the loss of β cells as achieved in the present report, due to a variety of factors inherent in the human model—factors this animal model did not have to contend with. To begin with, in human diabetes the progression from insulitis to complete loss of β-cell function is prolonged, typically taking anywhere from many months to years to occur. Furthermore, identifying and sequentially analyzing patients before and after disease onset to determine the time sequence of events leading to impaired α-cell response would be problematic and labor-intensive. In contrast, the BB rat provides a model in which diabetes occurs in over 50% of the animals at a predictable age of onset. Furthermore, the accelerated development of hyperglycemia, β-cell loss, and defective α-cell function during hypoglycemia facilitates the dissection of cause-and-effect relationships.

With regard to underlying mechanisms, it is clear that altered synthesis and storage of glucagon do not account for the glucagon defect observed after 7 days of IDDM. Circulating glucagon levels and the total amount of glucagon recovered from pancreatic tissue in both diabetic groups were comparable to those in nondiabetic animals. Insulin content, on the other hand, was almost completely lost 24 hours after the appearance of hyperglycemia. Thus, glucagon release during hypoglycemia was preserved for at least 1 day (but < 7 days) after endogenous insulin production was lost. Acute generalized insulin deficiency did not appear to be immediately responsible for the hypoglycemia-specific glucagon defect. The data suggest a cause-effect relationship between a lack of glucagon response to hypoglycemia after 7 days of disease and the decline of insulin due to β-cell loss. However, until the effects of other factors associated with β-cell loss (such as decreased amylin, GABA, and C-peptide) have been examined, the role of insulin deficiency cannot be established with certainty.

It is also noteworthy that short-term administration of large doses of insulin did not restore α-cells responsiveness to hypoglycemia. This insulin dose resulted in a rapid elevation of plasma insulin to levels that were approximately 100-fold above those routinely observed in the peripheral circulation under basal conditions. These elevations were initiated 90 minutes before and continued throughout the 90-minute hypoglycemic interval. Despite this, the glucagon response to hypoglycemia after 7 days of diabetes was virtually absent in these animals. Conversely, it is also known that elevations in circulating insulin per se may attenuate the release of glucagon,[22,23] and this also may have occurred in the present studies to some extent. Regardless, this potential effect was readily overcome during moderate hypoglycemia in 1-day diabetic rats, as well as in the nondiabetic control group.

Part of the difficulty in defining the mechanism of the observed α-cell defect lies in the fact that it is not at all clear whether hypoglycemia is detected in the α cell or elsewhere. In this regard, we have recently reported that the ventromedial hypothalamus (VMH) region of the brain is capable of detecting hypoglycemia and initiating a systemic glucagon response.[24] It seems unlikely that damage to the VMH glucose sensor explains the observed defect, since it was restricted to the α cell and did not involve the sympathoadrenal system, which is also activated by the VMH during hypoglycemia.

It has been suggested that the β cell is the glucose sensor for the entire islet. Alpha cells only respond to changes in the concentration of insulin or some other locally released factor in a paracrine fashion. This may play a role in the observed impairment in IDDM, although it should be pointed out that in 1-day diabetic rats in whom functional β-cell mass was small, the glucagon response to hypoglycemia is preserved. Although it is conceivable that this small residual β-cell mass might have preserved α-cell function, it is also possible the impact of β-cell loss on α-cell physiology is not immediate—perhaps trophic effects on an α-cell glucose-sensing mechanism requiring several days to become manifest.

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