Impaired Hormonal Responses to Hypoglycemia in Spontaneously Diabetic and Recurrently Hypoglycemic Rats

Reversibility and Stimulus Specificity of the Deficits

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Abstract

To evaluate the roles of iatrogenic hypoglycemia and diabetes per se in the pathogenesis of defective hormonal counterregulation against hypoglycemia in insulin-dependent diabetes mellitus (IDDM), nondiabetic, and spontaneously diabetic BB/Wor rats were studied using a euglycemic/hypoglycemic clamp. In nondiabetic rats, recurrent (4 wk) insulin-induced hypoglycemia (mean daily glucose, MDG, 59 mg/dl) dramatically reduced glucagon and epinephrine responses by 84 and 94%, respectively, to a standardized glucose fall from 110 to 50 mg/dl. These deficits persisted for > 4 d after restoring normoglycemia, and were specific for hypoglycemia, with normal glucagon and epinephrine responses to arginine and hypovolemia, respectively. After 4 wk of normoglycemia, hormonal counterregulation increased, with the epinephrine, but not the glucagon response reaching control values. In diabetic BB rats (MDG 245 mg/dl with intermittent hypoglycemia), glucagon and epinephrine counterregulation were reduced by 86 and 90%, respectively. Chronic iatrogenic hypoglycemia (MDG 52 mg/dl) further suppressed counterregulation. Prospective elimination of hypoglycemia (MDG 432 mg/dl) improved, but did not normalize hormonal counterregulation. In diabetic rats, the glucagon defect appeared to be specific for hypoglycemia, whereas deficient epinephrine secretion also occurred during hypovolemia. We concluded that both recurrent hypoglycemia and the diabetic state independently lead to defective hormonal counterregulation. These data suggest that in IDDM iatrogenic hypoglycemia magnifies preexisting counterregulatory defects, thereby increasing the risk of severe hypoglycemia. (*J. Clin. Invest.* 1993. 92:2667–2674.) Key words: glucagon • epinephrine • insulin • BB rats • counterregulation

Introduction

Symptomatic hypoglycemia is a common complication in the treatment of insulin-dependent diabetes mellitus (IDDM).[1] The incidence of severe episodes of hypoglycemia resulting in seizure or coma, or requiring another individual to administer therapy, is increased two- to threefold in patients practicing intensive insulin therapy (1, 2). This phenomenon has become the principal factor limiting the attainment of normoglycemia in IDDM, thereby frustrating attempts to diminish the complications associated with long-standing diabetes (3–7).

Although hypoglycemia in IDDM is mediated in part by overinsulinization, it is now appreciated that a deficient hormonal counterregulatory response to hypoglycemia is a key pathogenic factor (8–12). IDDM patients lose their capacity to release glucagon during hypoglycemia relatively early in the course of the disease (13), and as a result, are dependent on epinephrine to reverse hypoglycemia (14–17). Unfortunately, many IDDM patients lose their epinephrine counterregulation as well, and therefore are particularly vulnerable to severe hypoglycemia (5, 13). Intensive insulin treatment regimens designed to achieve near normoglycemia in IDDM also suppress the epinephrine response to hypoglycemia, thereby compounding the problem of deficient counterregulation and increasing the risk of hypoglycemia (3, 18, 19). Recent studies suggest that this effect of intensive insulin treatment may be due, at least in part, to hypoglycemia itself (3, 20). Heller et al. (21) have reported that one episode of antecedent hypoglycemia may blunt glucagon and epinephrine secretion in humans. The impact of recurrent hypoglycemia remains uncertain. Furthermore, it is not known whether the suppression of counterregulatory hormone release persists after return to normoglycemia, and whether such effects are stimulus specific for hypoglycemia.

The aims of this study were to determine (*a*) the influence of recurrent insulin-induced hypoglycemia, independent of diabetes, on hormonal counterregulation in nondiabetic BB rats; (*b*) the time course for recovery of defective counterregulation after restoration of normoglycemia; (*c*) the stimulus specificity of the hormonal defect induced by recurrent hypoglycemia; and (*d*) the contribution of recurrent hypoglycemia to, and the stimulus specificity of deficient hormonal counterregulation observed in IDDM.

Methods

Animals.

Male nondiabetic and autoimmune, spontaneously diabetic BB rats were supplied by the BB/Wor lab (University of Massachusetts, Worcester, MA) (22–25). Nondiabetic BB rats were members of the "diabetes resistant" strain of BB rats, of which less than 1% develop diabetes (26). 10 groups of rats were studied; their treatment data are summarized in Table I. For each group, the mean glucose during insulin treatment was determined in the fed state by measuring plasma glucose from the tail vein at least four times during a 24-h cycle on at least three different days, and often more frequently, to adequately determine the extent of the diurnal glycemic response to each insulin treatment, including the times of the greatest probability of hypoglycemia, normoglycemia, or hyperglycemia.

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1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; PZI, protamine zinc iletin.

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Table I. Treatment Protocols

Group	Rat	*n*	Age	Wt	Initial insulin treatment	Subsequent treatment	Experiment
				g			
I	Nondiabetic	12	4 mo	329±9	None	None	Hypoglycemia
II	Nondiabetic	9	4–5 mo	440±8	3–4 wk recurrent hypoglycemia	None	Hypoglycemia
III	Nondiabetic	7	4–5 mo	418±10	3–4 wk recurrent hypoglycemia	3–6 d recovery	Hypoglycemia
IV	Nondiabetic	6	5 mo	413±15	3–4 wk recurrent hypoglycemia	3–4 wk recovery	Hypoglycemia
V	Nondiabetic	9	4–5 mo	363±19	None	None	Arginine/ hypovolemia
VI	Nondiabetic	5	4–5 mo	426±12	3–4 wk recurrent hypoglycemia	4 d recovery	Arginine/ hypovolemia
VII	Diabetic	7	4 mo	339±6	6 wk intermediate control	None	Hypoglycemia
VIII	Diabetic	6	5 mo	349±9	6 wk intermediate control	3–4 wk chronic hypoglycemia	Hypoglycemia
IX	Diabetic	8	5 mo	341±11	6 wk intermediate control	3–4 wk poor control	Hypoglycemia
X	Diabetic	7	5 mo	315±13	6 wk intermediate control	3–4 wk poor control	Arginine/ hypovolemia

Aim 1. The influence of recurrent insulin-induced hypoglycemia, independent of diabetes, on hormonal counterregulation was examined in two groups of nondiabetic rats using the hyperinsulinemic euglycemic/hypoglycemic clamp. Group I consisted of nondiabetic rats aged 4 mo that had a mean glucose of 120±10 mg/dl (mean±SD), and served as controls. Group II were nondiabetic rats that at 3–4 mo of age were made recurrently hypoglycemic using a gradually increasing protamine zinc iletin (PZI; Eli Lilly & Co., Indianapolis, IN) insulin dose regimen over 5 d, followed by 3–4 wk of treatment with twice-daily injections of ~ 8–9 U/kg PZI insulin (mean glucose 59±21 mg/dl).

Aim 2. The time course for recovery of deficient hormonal counterregulation after recurrent hypoglycemia was assessed in two groups of recurrently hypoglycemic nondiabetic rats that were treated similarly to group II. These animals underwent euglycemic/hypoglycemic clamp studies at 3–6 d (group III) or 3–4 wk (group IV) after discontinuation of insulin treatment and restoration of normoglycemia.

Aim 3. The stimulus specificity of the hormonal defect induced by recurrent hypoglycemia was evaluated by quantitating glucagon and epinephrine responses to arginine infusion and hypovolemic stress, respectively, in two groups of nondiabetic rats during a euglycemic clamp. Group V were nondiabetic rats similar to group I that served as controls. Group VI were similar to group III, nondiabetic rats made chronically hypoglycemic for 3–4 wk and then allowed to recover to normoglycemia for 4 d before the experiment.

Aim 4. The contribution of recurrent hypoglycemia to deficient counterregulation observed in IDDM was examined using the euglycemic/hypoglycemic clamp in three groups of animals from a rat model of IDDM (diabetic BB rats), in whom the insulin treatment regimen was varied to produce differing degrees of glycemic control and antecedent hypoglycemia. Group VII were diabetic rats treated from the onset of diabetes (age of onset ~ 2.5 mo) for 6–8 wk with a standard daily injection regimen of ~ 8 U/kg PZI insulin. This treatment produced intermediate control as reflected by a mean glucose of 245±180 mg/dl. Glucose monitoring revealed that this group had intermittent episodes of hypoglycemia lasting several hours per day. Groups VIII and IX were initially treated in the same manner as group VII, and then were prospectively switched to insulin regimens designed to achieve chronic hypoglycemia (group VIII), or to eliminate antecedent hypoglycemia (group IX), for 3–4 wk. Group VIII received two daily injections of 7 U/kg PZI for 3–4 wk (mean glucose 52±19 mg/dl). Group IX received two daily injections of 1.6–2 U/kg PZI that resulted in a mean glucose of 432±106 mg/dl without episodes of hypoglycemia detected by frequent monitoring throughout the 24-h diurnal cycle during the 3–4 wk treatment (dubbed "poor control"). The stimulus specificity of the counterregulatory defects in diabetes was studied in a similar group of poorly controlled diabetic rats (group X) using the arginine infusion and hypovolemic stress protocol. The use of poorly controlled diabetic rats avoided the potentially confounding effect of antecedent hypoglycemia on hormonal responses.

Experimental procedures

Euglycemic/hypoglycemic clamp. The hyperinsulinemic glucose-clamp technique, as adapted for the rat (27), was used to provide a fixed hypoglycemic stimulus to rats in groups I to IV and VII to IX. At least 3 d before the experiment, under pentobarbital anesthesia, catheters were inserted into a carotid artery for blood sampling and one or two jugular veins for infusion of test substances, and filled with a heparin/polyvinylpyrrolidone solution, as previously described (27). The catheters were fastened to skin on the rat's back with tape and surgical staples. The rats were fasted for ~ 16 h before the study. On the day of the study, the heparin/polyvinylpyrrolidone solution was withdrawn and the catheters were infused with heparinized saline (~ 25 μl/min) for at least 30 min before the experiment. The rats were conscious, unstressed, and allowed to roam freely in their cage during the experiment. At the outset, a primed (360 mU over 1.5 min)-constant insulin infusion [20 mU/(kg·min)] was begun and a variable infusion of exogenous glucose was adjusted based on plasma glucose measurements obtained at 5-min intervals to achieve the desired glucose level. During the first 90 min of the experiment, the rats were brought to euglycemia (mean plasma glucose ~ 110 mg/dl). Thereafter, plasma glucose was allowed to fall to hypoglycemic levels (~ 50 mg/dl) and was maintained for 90 min. Experiments were terminated if the plasma glucose fell below 80 mg/dl during the last 45 min of the euglycemic phase, or rose above 70 mg/dl (secondary to glucose infusion), or inadvertently fell below 35 mg/dl during the hypoglycemic phase. Because a supraphysiologic insulin infusion rate of 20 mU/(kg·min) was

necessary to induce a hypoglycemic stimulus of 50 mg/dl in the nondiabetic controls, this insulin infusion was used in all groups (except group IX). The chronically hyperglycemic rats (group IX) were more insulin resistant, and thus this group received a higher insulin dose [50 mU/(kg · min)] to achieve the appropriate level of hypoglycemia.

Blood samples for measurement of glucagon and epinephrine were taken during the baseline period and during the euglycemic (75 and 90 min) and hypoglycemic (120, 135, 150, 165, and 180 min) portions of the experiments. Blood obtained from nondiabetic BB rats at least 30 min before the experiment was transfused during the experiment to quantitatively replace blood withdrawn during the study.

Arginine infusion/hypovolemic stress study. To assess glucagon and epinephrine secretion to a nonhypoglycemic stimulus (groups V, VI, and X), arginine infusion and hypovolemic stress were sequentially administered while maintaining a euglycemic (110 mg/dl), hyperinsulinemic [20m U/(kg · min)] clamp throughout the study using a variable glucose infusion. The first 90 min of the experiment were identical to the euglycemic portion of the euglycemic/hypoglycemic clamp described above. However, at 90 min, an arginine infusion [30 mg/(kg min), pH · 7.4] was superimposed for 60 min (28). At 150 min, arginine infusion and blood replacement were discontinued and 10 mg/kg of blood was withdrawn to initiate the hypovolemic stress phase of the study. Subsequently, 5 ml/kg of blood was withdrawn (and not replaced) every 15 min for ~ 75 min.

Blood samples for measurement of glucagon and epinephrine were taken at the baseline and at the end of the euglycemic phase of the experiment. During the arginine infusion, glucagon was determined at 15-min intervals. Epinephrine measurements were obtained before the hypovolemic stress (at 150 min), and every 15 min thereafter during the hypovolemic phase of the study.

Analytic procedures. Plasma glucose was measured by the glucose oxidase method (Beckman Instrs., Inc., Fullerton, CA). Glucagon (ICN Biomedicals, Inc., Costa Mesa, CA) and insulin (Binax, South Portland, ME) levels were measured by a double antibody RIA, while catecholamines were assayed using a radioenzymatic assay (Amersham Corp., Arlington Heights, IL).

Calculations. Mean plasma glucose levels during the euglycemic phase were calculated from the interval 45 to 90 min, during the hypoglycemic phase from 105 to 180 min, during the arginine infusion from 95 to 150 min, and during the hypoglycemic stress from 155 min to the end of the study. Peak glucagon and epinephrine responses to acute hypoglycemia represent the difference between maximal hypoglycemic values (the mean of the two greatest values during hypoglycemia) and mean euglycemic values. Glucagon and epinephrine responses during the sequential arginine infusion/hypovolemic stress study were calculated by subtracting the prestimulus values from the mean of the two greatest values during arginine infusion or hypovolemic stress.

Data are reported as the mean or as the mean±SE, except for data for mean treatment glucose, which are reported as mean±SD. Comparisons between two groups were performed using the two-tailed Student's *t* test, or between three or more groups using ANOVA.

Results

The effect of, and recovery from, recurrent hypoglycemia in nondiabetic rats (groups I to IV). Mean glucose levels for the control and each recurrent hypoglycemia group were not significantly different during the euglycemic (111±2, 112±2, 110±1, and 113±2 mg/dl) and hypoglycemic (52±1, 50±1, 52±2, and 55±2 mg/dl, respectively for groups I, II, III, and IV) phases of the experiment. The effects of recurrent hypoglycemia on hormonal counterregulation in nondiabetic rats studied with the hyperinsulinemic euglycemic/hypoglycemic clamp are depicted in Fig. 1, and peak hormonal responses are summarized in Table II. In nondiabetic rats studied immediately after 3–4

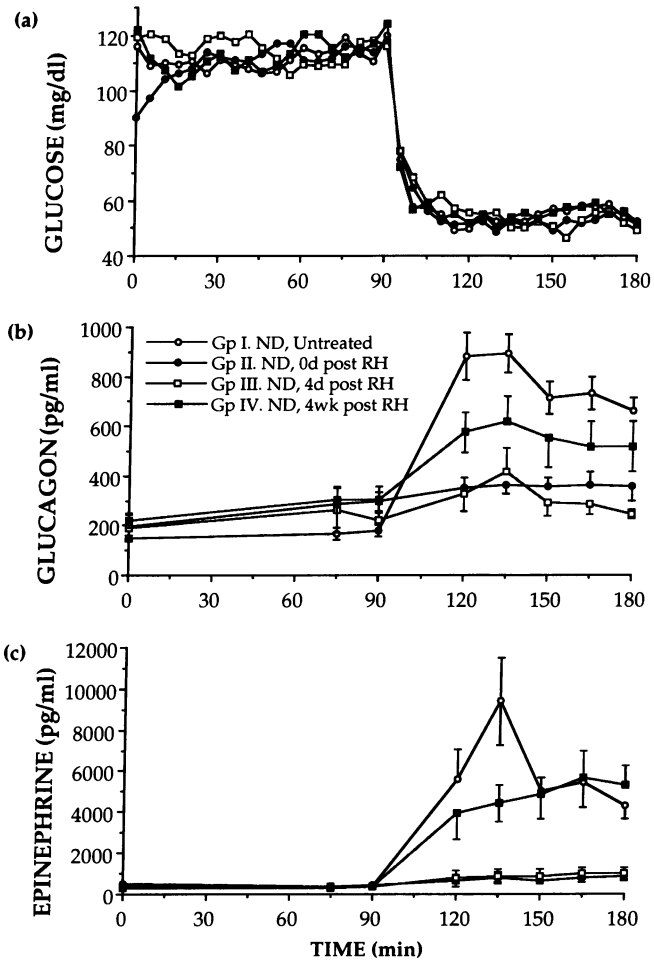

Figure 1. The effect of recurrent hypoglycemia (RH) on hormonal counterregulation in nondiabetic (ND) rats. Study glucose (*a*), glucagon (*b*), and epinephrine (*c*) counterregulation. Results are mean±SE.

wk of recurrent hypoglycemia (group II), peak glucagon and epinephrine responses during the hypoglycemic phase of the clamp were reduced by 84 ($P < 0.0001$) and 94% ($P < 0.001$), respectively, when compared to the untreated, nondiabetic controls (group I). 3–6 d after restoration of normoglycemia (group III), suppression of hormonal counterregulation persisted, i.e., the responses remained indistinguishable from those immediately after the hypoglycemic treatment (P = NS vs group II). However, 3–4 wk after restoration of normoglycemia (group IV), the epinephrine and glucagon responses increased significantly, rising 11-fold ($P < 0.01$, Fig. 1 *c*) and 3-fold ($P < 0.05$, Fig. 1 *b*), respectively, above the responses immediately after the hypoglycemic treatment (group II). 3–4 wk after restoration of normoglycemia (group IV), the epinephrine response increased to a value not statistically different from the control's response ($P = 0.11$ vs group I). Although the early epinephrine response of group IV was marginally blunted ($P = 0.054$ vs group I, at t = 135 min, Fig. 1 *c*), values at other time points were virtually identical to the control's response. The glucagon response of group IV, while rising above that in group II, remained 59% below that seen in the controls ($P < 0.002$). Plasma insulin levels (715±110, 831±212, 1293±171, and 1061±72 μU/ml, respectively, for

Table II. Peak Hormonal Responses to Hypoglycemic, Arginine, and Hypovolemic Stimuli

Group	Rat*	Experiment	Glucagon	Epinephrine
			pg/ml	*pg/ml*
I	ND, untreated	Hypoglycemia	748±91	8660±1560
II	ND, 0 d post RH	Hypoglycemia	117±33‡	480±170‡
III	ND, 3–6 d post RH	Hypoglycemia	184±63‡	700±280‡
IV	ND, 3–4 wk post RH	Hypoglycemia	328±69‡,§	5330±1140§,‖
V	ND, untreated	Arg/hypovolemia	493±139	21140±2740
VI	ND, 4 d post RH	Arg/hypovolemia	404±60	21180±3280
VII	DM, intermediate control	Hypoglycemia	105±21‡	900±370‡
VIII	DM, chronic hypoglycemia	Hypoglycemia	45±10‡,¶	260±90‡
IX	DM, poor control	Hypoglycemia	188±36‡,**	4030±880‡,¶,**
X	DM, poor control	Arg/hypovolemia	282±22‡‡	4840±1250‡

* ND: Nondiabetic; RH: recurrent hypoglycemia; DM: diabetic. ‡ $P < 0.05$ vs group I (ND, untreated). § $P < 0.05$ vs group II (ND, 0 d post RH). ‖ P = NS vs group I (ND, untreated). ¶ $P < 0.05$ vs group VII (DM, intermediate control). ** $P < 0.05$ vs group VIII (DM, chronic hypoglycemia). ‡‡ P = NS vs group V (ND, untreated).

groups I to IV) were higher in both groups III and IV than in group I ($P < 0.05$).

Stimulus specificity of hormonal defects in nondiabetic rats (groups V, VI). Mean glucose levels for the nondiabetic control (V) and 4-d post-hypoglycemia (VI) groups were maintained at euglycemia before (111±3 and 109±1 mg/dl) and during the arginine infusion (107±3 and 114±4 mg/dl, respectively). Plasma glucose rose slightly during the hypovolemic stress phase of the experiment (116±2 and 126±5 mg/dl, respectively), however, these changes did not reach statistical significance. Plasma insulin concentrations during the clamps were not significantly different in groups V (751±279 μU/ml) and VI (1100±64 μU/ml). The glucagon responses to arginine infusion (during which the epinephrine values remained at baseline) and the epinephrine responses to hypovolemic stress in the nondiabetic controls (group V) and in the nondiabetic rats recovering from recurrent hypoglycemia (group VI) are depicted in Fig. 2. The hormonal responses to hypoglycemia of similar treatment groups are given for comparison in Fig. 2 (groups I and III). The glucagon and epinephrine responses to acute hypoglycemia (Fig. 2, *b* and *d*) in rats studied after 3–6 d of recovery from recurrent hypoglycemia (group III) were suppressed. In marked contrast to the deficient responses to hypoglycemia in group III, both the glucagon response to arginine (Fig. 2 *a*), and the epinephrine response to hypovolemia (Fig. 2 *c*) displayed by group VI were similar to those seen in untreated nondiabetic controls (P = NS vs group V).

The effects of glycemic control on hormonal counterregulation in diabetic rats (groups VII to IX). The effects of glycemic control on hormonal counterregulation in the diabetic BB rats during the euglycemic/hypoglycemic clamp experiments are shown in Fig. 3 and peak hormonal responses are summarized in Table II. Diabetic rats under intermediate control (group VII) had peak glucagon and epinephrine responses to hypoglycemia that were markedly reduced, by 86 ($P < 0.0001$, Fig. 3 *b*) and 90% ($P < 0.001$, Fig. 3 *c*), respectively, compared to the nondiabetic controls (group I). This hormonal suppression occurred despite a slightly greater hypoglycemic stimulus in the diabetic group (46±1 mg/dl) than the nondiabetic controls (52±1 mg/dl, $P < 0.01$). Plasma insulin levels during the clamp experiments were comparable (715±110 and 657±62 μU/ml, respectively for groups I and VII). Plasma glucose levels during the euglycemic phase in group VII (111±5 mg/dl), as well as in the other diabetic groups (VIII and IX), during both the euglycemic (111±3 and 113±1 mg/dl) and hypoglyce-

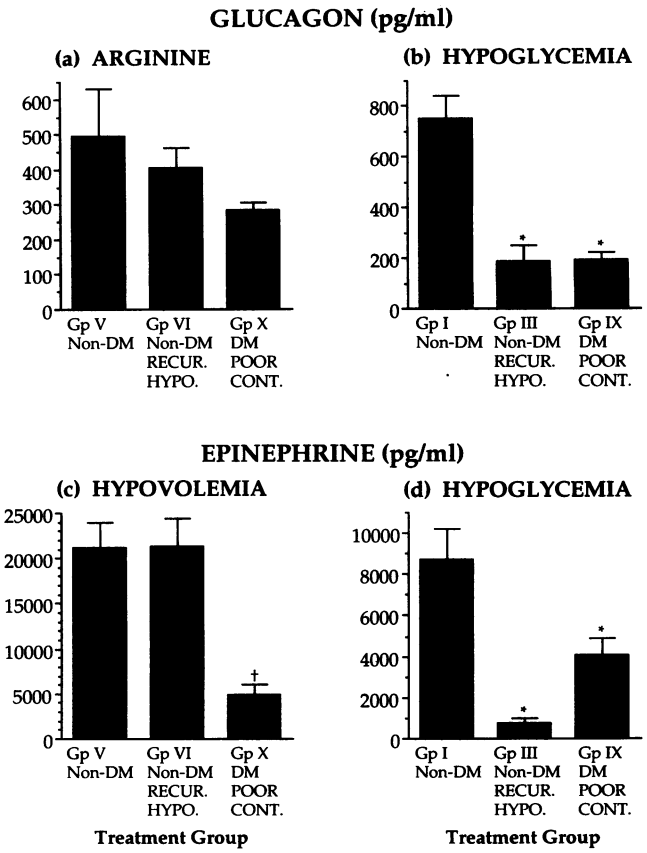

Figure 2. Stimulus specificity of counterregulatory defects caused by recurrent hypoglycemia and diabetes. The glucagon response to (*a*) arginine infusion, and (*b*) hypoglycemia for comparison. The epinephrine response to (*c*) hypovolemic stress, and (*d*) hypoglycemia for comparison. Results are expressed as mean response±SE. * $P < 0.05$ vs group I, + $P < 0.05$ vs group V.

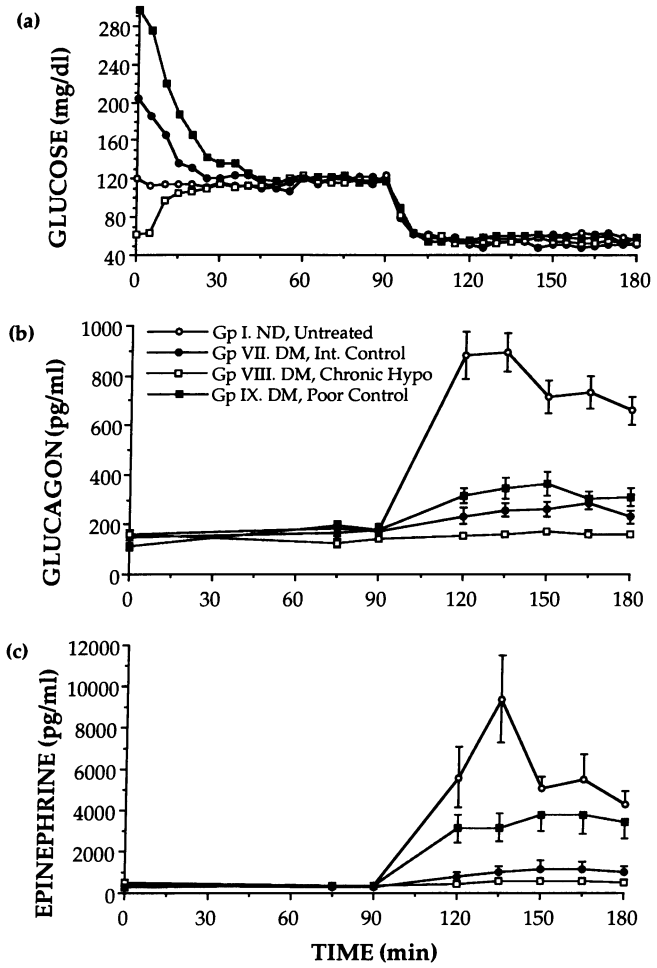

Figure 3. The effect of antecedent hypoglycemia on hormonal counterregulation in diabetes (DM). Study glucose (*a*), glucagon (*b*), and epinephrine (*c*) counterregulation. Results are mean±SE.

mic phases (48±1 and 52±4 mg/dl, respectively) were similar to the nondiabetic controls. Therapy provoking chronic hypoglycemia (group VIII) further suppressed the glucagon counterregulatory response by 57% ($P < 0.05$ vs group VII). Although chronic hypoglycemia also decreased the epinephrine response by 70%, this change did not reach statistical significance when compared with the intermediately controlled diabetic group ($P = 0.10$). Glucagon responses in the poorly controlled diabetic rats (group IX) were significantly greater than those of rats experiencing chronic hypoglycemia ($P < 0.01$ vs group VIII). Elimination of episodes of antecedent hypoglycemia, on the other hand, did not significantly augment the peak glucagon response compared to rats experiencing intermittent hypoglycemia while receiving intermediate control ($P = 0.08$ vs group VII). Moreover, the peak glucagon response in the poorly controlled rats remained markedly blunted at 75% less than the nondiabetics' response ($P < 0.0001$ vs group I). In contrast, elimination of antecedent hypoglycemia increased the epinephrine response to acute hypoglycemia fourfold above diabetic rats in the intermediate control group ($P < 0.02$ vs group VII) and ~16-fold above diabetic rats experiencing chronic hypoglycemia ($P < 0.01$ vs group VIII). The early peak epinephrine response to hypoglycemia in the poorly controlled group (IX) remained significantly below that seen in the nondiabetic control group ($P < 0.05$ vs group I). However, comparison of time points beyond 1 h revealed no significant difference between the two groups (Fig. 3 *c*). Plasma insulin levels during the study were not significantly higher in the chronically hypoglycemic diabetic group (1004±63 μU/ml, P = NS), but in the poorly controlled diabetic group that received a higher insulin infusion rate, insulin levels were significantly elevated (3600±220 μU/ml, $P < 0.001$), compared to the nondiabetic controls.

Stimulus specificity of hormonal defects in diabetic rats (groups V, X). Mean glucose levels during the euglycemic (109±3 mg/dl) and arginine infusion (107±1 mg/dl) phases, as well as a slight rise in glucose during the hypovolemic stress phase of the experiment (123±4 mg/dl) in the diabetics (group X) were similar to those in the nondiabetic controls (group V). The plasma insulin level in the diabetic group (609±123 μU/ml) was also similar to the control group. The glucagon response to arginine infusion and the epinephrine response to hypovolemic stress in the nondiabetic controls (group V) and in the poorly controlled diabetic rats (group X) are depicted in Fig. 2. The hormonal responses to hypoglycemia of similar treatment groups are given for comparison in Fig. 2 (groups I and IX). The glucagon response to arginine in the diabetic group (Fig. 2 *a*) tended to be slightly less, but not significantly different than that in the nondiabetic controls ($P > 0.20$ vs group V), in contrast to the markedly blunted response to hypoglycemia (Fig. 2 *b*). On the other hand, much like the blunted epinephrine response to hypoglycemia in this group (Fig. 2 *d*), the epinephrine response to hypovolemia (Fig. 2 *c*) was significantly diminished in the diabetic group, 78% less than the control group's response ($P < 0.001$ vs group V).

Discussion

Deficient secretion of glucagon and epinephrine plays a major etiologic role in the high morbidity of iatrogenic hypoglycemia in IDDM (4, 5, 8–12). Glucagon counterregulation is diminished within a few years after disease onset, while epinephrine counterregulation decreases years later (13). Although the pathogenesis of the adrenergic deficit is poorly understood, and in some cases may be the result of autonomic neuropathy (29), some patients display the defect without detectable autonomic dysfunction (3, 30, 31). The demonstration that short-term intensive insulin therapy in patients with IDDM commonly results in a sharp diminution in the adrenergic response to acute hypoglycemia suggests that iatrogenic hypoglycemia during the course of treatment is an important contributory factor to defective counterregulation (3, 19). This possibility is supported by recent experimental data in nondiabetic humans suggesting that a brief period of antecedent hypoglycemia may blunt hormonal counterregulation (20, 21). However, those studies did not establish whether the effect is (*a*) persistent or counterbalanced by adaptation chronically, and (*b*) specific for hypoglycemic stimuli.

In the current studies we examined the effect of more prolonged periods of recurrent hypoglycemia on hormonal counterregulation. Rats were chosen as an experimental model because of (*a*) the high morbidity of chronic recurrent hypoglycemia; (*b*) the ability to monitor, manipulate, and ensure compliance with different treatment regimens; (*c*) the ability to provide a significant and standardized hypovolemic stress stimulus for assessing the specificity of defective epinephrine secretion; and (*d*) the potential for future histologic and whole-or-

gan physiologic experimentation after the characterization of the models herein. The diabetic BB rat was used because this animal model develops spontaneous, autoimmune, insulin-dependent diabetes that bears a striking resemblance to type I diabetes in humans, though more rapid in onset (22–25). Moreover, the diabetic BB rat provided us with an opportunity to characterize the in vivo counterregulatory response of an animal model of type I diabetes free of the potentially multiple confounding toxic effects, and often incomplete diabetes, inherent in the use of alloxan or streptozotocin (32).

The hyperinsulinemic, euglycemic/hypoglycemic clamp technique was used to control glucose levels and provide a standardized, fixed hypoglycemic stimulus. During the arginine infusion/hypovolemic stress experiments, the clamp technique was used to reproduce the supraphysiologic hyperinsulinemia accompanying the hypoglycemia study while fixing glucose levels at baseline values. This approach diminished the confounding effect of varying hyperglycemia on hormone secretion in response to arginine infusion and hypovolemic stress, thus providing a more accurate comparison of the effects of each treatment on responses to the different stimuli.

The current studies demonstrate a profound defect in hormonal counterregulation following a 3–4-wk period of recurrent hypoglycemia in nondiabetic rats (Fig. 1). Immediately after treatment, the glucagon and epinephrine response to hypoglycemia decreased 84 and 94%, respectively, relative to control values (Table II). This defect persisted, without significant change, even after 3–6 d of recovery. The epinephrine, and to a lesser extent the glucagon, response to hypoglycemia began to increase 1–2 wk after restoration of normoglycemia (results not shown). By 3–4 wk after treatment, the epinephrine response had fully recovered, and the glucagon response had increased significantly, but still remained significantly less than the untreated nondiabetic control (Fig. 1). These findings imply that the hormonal defects produced by recurrent hypoglycemia are persistent, but reverse over time. It is possible that a more prolonged recovery from antecedent hypoglycemia would have further restored glucagon counterregulation.

To determine whether the deficient hormonal counterregulation subsequent to recurrent hypoglycemia was stimulus specific, nonhypoglycemic stimuli for glucagon (arginine infusion) and epinephrine secretion (hypovolemic stress) were administered to nondiabetic rats 4 d after restoration of normoglycemia. Rats 4 d after hypoglycemic treatment were used because of the stability of their metabolic state (i.e., absence of recent hypoglycemia), while their hormonal counterregulatory response to acute hypoglycemia remained maximally suppressed. Interestingly, these rats exhibited completely normal glucagon and epinephrine secretory responses to nonhypoglycemic stimuli (Fig. 2), indicating that the secretory reserves of the α-cells and adrenal medulla were intact and that the persistent hormonal defects induced by recurrent hypoglycemia were not due to glandular hormonal depletion. Instead, our data suggest that recurrent hypoglycemia itself, independent of diabetes, blunts hormonal responses specifically to hypoglycemic stimuli.

The role of recurrent hypoglycemia in the deficient counterregulation observed in IDDM was also investigated using BB rats 6–8 wk after diabetes onset that were receiving a standard insulin regimen (Fig. 3). These rats displayed a striking (85–90%) diminution of their glucagon and epinephrine responses to hypoglycemia (Table II). It is noteworthy that although these diabetic rats had a mean glucose of 245 mg/dl, they experienced intermittent episodes of hypoglycemia averaging several hours in duration daily. To examine the contribution of recurrent hypoglycemia to their deficient counterregulation, we studied diabetic rats treated initially by the standard insulin regimen and then prospectively switched to either chronic hypoglycemia (mean glucose = 52±19 mg/dl) or elimination of antecedent hypoglycemia (mean glucose = 432±106 mg/dl). As in the nondiabetic rats, chronic hypoglycemia further decreased the glucagon and epinephrine responses to acute hypoglycemia. In contrast, elimination of the hypoglycemic episodes seen in the intermediate control group for 3–4 wk caused a fourfold increase in the epinephrine response to near normal levels, and a more modest increase in the glucagon response to acute hypoglycemia, demonstrating the malleability of the hormonal counterregulation in diabetes and its dependence on the level of antecedent glycemia. However, even with elimination of antecedent hypoglycemia, the peak glucagon and epinephrine responses by the diabetic rats remained significantly reduced by 75 and 53%, respectively, relative to the nondiabetic controls. It is possible that a more prolonged period free from hypoglycemia would have further increased hormonal counterregulation. It should be noted that insulin levels, by necessity, were significantly higher in the poorly controlled diabetic group (IX). Thus, it is conceivable that more pronounced hyperinsulinemia during the experiments in the poorly controlled diabetic rats contributed in part to the suppression of glucagon and epinephrine release observed in these studies. Smaller, though significant, increases in insulin levels were also observed in groups III and IV despite identical infusion rates, so that the hormonal responses of these nondiabetic rats after 4 d and 4 wk of recovery from recurrent hypoglycemia may have been closer to the controls if we had achieved lower insulin levels. However, previous studies have demonstrated that a two- to fourfold variation in insulin concentration has little or no effect on hormonal counterregulation (33–36). The influence of more pronounced insulin differences, on the other hand, remains unclear, with both suppression and augmentation of hormone secretion reported with 8–10-fold variations in insulin levels (37, 38). These reports (37, 38) compared supraphysiologic with low-to-mid-physiologic hyperinsulinemia, a range in which the greatest effect is likely to be observed. In our study, insulin levels were by necessity supraphysiologic in all groups (> 600 μU/ml), and the relative differences in insulin levels between groups were small, making it unlikely that differences in insulin concentrations between the groups contributed to any differences seen in hormonal counterregulation. On the other hand, the large doses of insulin used in these studies potentially limit the clinical application of our data.

We next investigated whether the blunted hormonal counterregulation that persisted despite elimination of antecedent hypoglycemia in the diabetic rats was specific for hypoglycemic stimuli. Previous studies in human IDDM have found an increased glucagon response to arginine (39–42), which decreases to normal values if insulin is administered concomitantly (41, 42). However, those studies did not control the plasma glucose or insulin concentrations during the experiment, and did not treat nondiabetic controls with similar insulin infusions. Histologic studies in treated or stable diabetic BB rats have found normal islet glucagon content (22, 43, 44), but the in vivo glucagon response to intravenous arginine infusion

in the diabetic BB rat has not been adequately studied. In the current study, diabetic rats after 3–4 wk without hypoglycemia were used to eliminate the effect of antecedent hypoglycemia on the results. While the glucagon response to arginine infusion in the diabetic group tended to be less, it was not significantly different from that in the nondiabetic controls. However, it is possible that the lack of a significant difference represents a type II error due to the large variation in the response of the nondiabetic controls. The diabetic's response suggests a minimal reduction in α-cell secretory reserve or function. This small change could reflect a relatively greater inhibition of glucagon secretion in the diabetic rat by supraphysiologic hyperinsulinemia. The similarity of the experimental conditions during administration of arginine or hypoglycemic stimuli allows a more valid comparison of glucagon responses to different stimuli. In this case, defective glucagon secretion appears to be restricted to hypoglycemic stimuli in the diabetic rat.

Previous studies examining epinephrine secretion in IDDM have been limited by the difficulty of providing a standardized epinephrine stimulus. It has been reported that humans with diabetes have epinephrine responses to exercise that are similar or greater than those in nondiabetics, even when the diabetics exhibit a deficient epinephrine response to acute hypoglycemia (45). However, the exercise protocol (45) produced only a 20–50% increase in epinephrine for nondiabetics, compared to a 600% increase in response to hypoglycemia. Thus, the exercise provided a relatively mild stimulus for epinephrine secretion, inadequate to assess the specificity of a hormonal deficit. We used gradual blood volume depletion on a volume per unit weight basis to provide quantitatively similar stimuli within and between groups of rats. Glucose was fixed at euglycemic levels using a hyperinsulinemic clamp to eliminate the confounding effect of different degrees of ensuing hyperglycemia on epinephrine release. In contrast to the stimulus specificity of the glucagon deficit, the epinephrine response to hypovolemic stress in the diabetic BB rat was markedly reduced by 78% relative to the nondiabetic controls' response. This observation suggests that the disturbance in adrenomedullary function seen in the diabetic BB rat is nonspecific in nature. Interestingly, the defect develops over a relatively short period; rats had been diabetic for a total of only 9–10 wk. Whether this nonspecific dysfunction is a consequence of the neuropathic effects of poorly controlled diabetes or due to autoimmune adrenalitis is uncertain. Although the poorly controlled rats had free access to water and maintained their weight, it is possible that a subtle degree of chronic hypovolemia decreased their response to acute hypovolemia.

To the extent that our findings are relevant in the clinical setting, they have important implications for the treatment of type I diabetics. In as much as patients with type I diabetes have almost completely absent glucagon response to hypoglycemia, epinephrine must partially counterbalance the glucagon deficit and become the primary counterregulatory hormone. As recurrent hypoglycemia profoundly diminishes epinephrine counterregulation, those diabetics experiencing recurrent hypoglycemia will have lost their last remaining acute counterregulatory mechanism. In an attempt at maintaining tighter glycemic control with the aim of reducing the long-term vascular and neural complications of diabetes, the morbidity from hypoglycemia may be increased greatly. Extrapolating the results in our rat studies to humans, antecedent hypoglycemia diminishes future glucagon and epinephrine responses to hypoglycemia, so that hypoglycemia begets hypoglycemia. Furthermore, decreased hormonal counterregulation makes future episodes of hypoglycemia not only more likely, but also more severe, especially for those individuals with hypoglycemia unawareness (46). A similar scenario has been suggested by Cryer et al. based on studies after one episode of hypoglycemia in humans (21, 46). The effect of antecedent hypoglycemia to diminish the sympathetic response to acute hypoglycemia may also make hypoglycemia more difficult to detect by the patient, contributing to the syndrome of hypoglycemia unawareness (47).

The stimulus specificity of the deficient glucagon and epinephrine responses of nondiabetic rats to acute hypoglycemia has implications for the nature of the defect(s) caused by antecedent hypoglycemia. In analysis of his results from nondiabetic humans, Cryer suggested that antecedent hypoglycemia causes a hypoglycemia-associated autonomic failure, and in turn induces the counterregulatory defects seen after recurrent hypoglycemia (47). However, our results do not support this view. We observed a completely normal epinephrine response to hypovolemia, despite the near absence of a response to hypoglycemia in nondiabetic rats studied 4 d after recurrent hypoglycemia, suggesting that the adrenal medulla and the sympathetic pathways involved in autonomic activation of the adrenal medulla remain intact.

In summary, we examined the effect of recurrent hypoglycemia on glucagon and epinephrine counterregulation, and the persistence and stimulus specificity of deficient hormonal counterregulation after recurrent hypoglycemia in nondiabetic and diabetic BB rats. We found that (*A*) recurrent hypoglycemia results in profound defects in hormonal counterregulation independent of diabetes. (*B*) These defects are persistent for at least 4 d after return to normoglycemia. (*C*) Both glucagon and epinephrine counterregulation increase significantly after 4 wk of recovery from hypoglycemia, with the epinephrine, but not the glucagon, response returning to normal levels. (*D*) The effect of recurrent hypoglycemia to blunt hormone secretion is stimulus specific for hypoglycemia. (*E*) The effect of recurrent hypoglycemia to profoundly diminish hormonal counterregulation extends to IDDM. However, diabetes, independent of antecedent hypoglycemia, leads to blunted glucagon and epinephrine counterregulation. (*F*) In the diabetic rat, glucagon secretion is diminished specifically to glycemic stimuli, but epinephrine secretion is nonspecifically diminished. These data indicate that both the diabetic state and recurrent hypoglycemia independently impair hormonal counterregulation in the BB rat. Our data suggest that in IDDM, iatrogenic hypoglycemia magnifies preexisting counterregulatory defects, thereby increasing the risk of severe hypoglycemia.

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References

1. The DCCT Research Group. 1987. Diabetes Control and Complications Trial (DCCT): results of feasibility study. *Diabetes Care.* 10:1–19.

2. The DCCT Research Group. 1991. Epidemiology of severe hypoglycemia in the Diabetes Control and Complications Trial. *Am. J. Med.* 90:450–459.

3. Simonson, D. C., W. V. Tamborlane, R. A. DeFronzo, and R. S. Sherwin. 1985. Intensive insulin therapy reduces counterregulatory hormone responses to hypoglycemia in patients with type I diabetes. *Ann. Intern. Med.* 103:184–190.

4. Santiago, J. V., N. H. White, D. A. Skor, L. A. Levandoski, D. M. Bier, and P. E. Cryer. 1984. Defective glucose counterregulation limits intensive therapy of diabetes mellitus. *Am. J. Physiol.* 247:E215–E220.

5. White, N. H., D. A. Skor, P. E. Cryer, L. A. Levandoski, D. M. Bier, and J. V. Santiago. 1983. Identification of Type I diabetic patients at increased risk for hypoglycemia during intensive therapy. *N. Engl. J. Med.* 308:485–491.

6. Unger, R. H. 1982. Meticulous control of diabetes: benefits, risks, and precautions. *Diabetes.* 31:479–483.

7. Flier, J. S., and L. H. Underhill. 1988. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318:1315–1321.

8. Amiel, S. A., W. V. Tamborlane, L. Sacca, and R. S. Sherwin. 1988. Hypoglycemia and glucose counterregulation in normal and insulin-dependent diabetic subjects. *Diabetes Metab. Rev.* 4:71–89.

9. Cryer, P. E., C. Binder, G. B. Bolli, A. D. Cherrington, E. A. M. Gale, J. E. Gerich, and R. S. Sherwin. 1989. Hypoglycemia in IDDM. *Diabetes.* 38:1193–1199.

10. Amiel, S. A. 1991. Glucose counter-regulation in health and disease: current concepts in hypoglycaemia recognition and response. *Q. J. Med.* 80:707–727.

11. Cryer, P. E. 1988. Hypoglycemia and insulin-dependent diabetes mellitus. *Diabetes Annu.* 4:272–310.

12. Gerich, J. E., and P. J. Campbell. 1988. Overview of counterregulation and its abnormalities in diabetes mellitus and other conditions. *Diabetes Metab. Rev.* 4:93–111.

13. Bolli, G., P. DeFeo, R. Compagnucci, M. G. Cartechini, G. Angeletti, F. Santeusanio, P. Brunetti, and J. E. Gerich. 1983. Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. *Diabetes.* 32:134–141.

14. Popp, D. A., S. D. Shah, and P. E. Cryer. 1982. Role of epinephrine-mediated β-adrenergic mechanisms in hypoglycemic glucose counterregulation and posthypoglycemic hyperglycemia in insulin-dependent diabetes mellitus. *J. Clin. Invest.* 69:315–326.

15. Bolli, G., P. DeFeo, P. Compagnucci, M. G. Cartechini, G. Angeletti, F. Santeusanio, and P. Brunetti. 1982. Important role of adrenergic mechanisms in acute glucose counterregulation following insulin-induced hypoglycemia in type I diabetes. Evidence for an effect mediated by beta-adrenoreceptors. *Diabetes.* 31:641–647.

16. DeFeo, P., G. Bolli, G. Perriello, S. DeCosmo, P. Compagnucci, G. Angeletti, F. Santeusanio, J. E. Gerich, M. Motolese, and P. Brunetti. 1983. The adrenergic contribution to glucose counterregulation in type I diabetes mellitus. Dependency on A-cell function and mediation through $beta_2$-adrenergic receptors. *Diabetes.* 32:887–893.

17. Cryer, P. E., T. F. Tse, W. E. Clutter, and S. D. Shaw. 1984. Roles of glucagon and epinephrine in hypoglycemic and non-hypoglycemic glucose counterregulation in humans. *Am. J. Physiol.* 247:E198–E205.

18. Amiel, S. A., W. V. Tamborlane, D. C. Simonson, and R. S. Sherwin. 1987. Defective glucose counterregulation after strict glycemic control of insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 316:1376–1383.

19. Amiel, S. A., R. S. Sherwin, D. C. Simonson, and W. V. Tamborlane. 1988. Effect of intensive insulin therapy on glycemic thresholds for counterregulatory hormone release. *Diabetes.* 37:901–907.

20. Davis, M. R., and H. Shamoon. 1991. Counterregulatory adaptation to recurrent hypoglycemia in normal humans. *J. Clin. Endocrinol. & Metab.* 73:995–1001.

21. Heller, S. R., and P. E. Cryer. 1991. Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans. *Diabetes.* 40:223–226.

22. Nakhooda, A. F., A. A. Like, C. I. Chappel, F. T. Murray, and E. B. Marliss. 1977. The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. *Diabetes.* 26:100–112.

23. Nakhooda, A. F., A. A. Like, C. I. Chappel, C.-N. Wei, and E. B. Marliss. 1978. The spontaneously diabetic Wistar rat (the "BB" rat). Studies prior to and during development of the overt syndrome. *Diabetologia.* 14:199–207.

24. Marliss, E. B., A. F. Nakhooda, P. Poussier, and A. A. Like. 1982. The diabetic syndrome of the 'BB' Wistar rat: possible relevance to type I (insulin-dependent) diabetes in man. *Diabetologia.* 22:225–232.

25. Logothetopoulos, J., N. Valiquette, E. Madura, and D. Cvet. 1984. The onset and progression of pancreatic insulitis in the overt, spontaneously diabetic, young adult BB rat studied by pancreatic biopsy. *Diabetes.* 33:33–36.

26. Walker, R., A. J. Bone, A. Cooke, and J. D. Baird. 1988. Distinct macrophage subpopulations in pancreas of prediabetic BB/E rats. Possible role for macrophages in pathogenesis of IDDM. *Diabetes.* 37:1301–1304.

27. Rossetti, L., D. Smith, G. I. Shulman, D. Papachristou, and R. A. DeFronzo. 1987. Correction of hyperglycemia with phlorizin normalizes tissue sensitivity to insulin in diabetic rats. *J. Clin. Invest.* 79:1510–1515.

28. Kuku, S. F., J. B. Jaspan, D. S. Emmanouel, A. I. Katz, and A. H. Rubenstein. 1978. Plasma glucagon, insulin and glucose responses to intravenous arginine infusion in the rat. *Horm. Metab. Res.* 10:99–100.

29. Polonsky, K., R. Bergenstal, G. Pons, M. Schneider, J. Jaspan, and A. Rubenstein. 1982. Relation of counterregulatory responses to hypoglycemia in type I diabetics. *N. Engl. J. Med.* 307:1106–1112.

30. Windom, B., and D. C. Simonson. 1990. Glycemic control and neuropsychologic function during hypoglycemia in patients with insulin-dependent diabetes mellitus. *Ann. Intern. Med.* 112:904–912.

31. Ryder, R. J., D. R. Owens, T. M. Hayes, M. A. Chatei, and S. R. Bloom. 1990. Unawareness of hypoglycemia and inadequate hypoglycemic counterregulation: no causal relationship with diabetic autonomic neuropathy. *Br. Med. J.* 301:783–787.

32. Junod, A., A. E. Lambert, W. Stauffacher, and A. E. Renold. 1969. Diabetogenic action of streptozotocin. Relationship of dose to metabolic response. *J. Clin. Invest.* 48:2129–2139.

33. Bolli, G., P. DeFeo, G. Perriello, S. De Cosmo, P. Compagnucci, F. Santeusanio, P. Brunetti, and R. H. Unger. 1984. Mechanisms of glucagon secretion during insulin-induced hypoglycemia in man. Role of the beta cell and arterial hyperinsulinemia. *J. Clin. Invest.* 73:917–922.

34. Kerr, D., M. Reza, N. Smith, and B. A. Leatherdale. 1991. Importance of insulin in subjective, cognitive, and hormonal responses to hypoglycemia in patients with IDDM. *Diabetes.* 40:1057–1062.

35. Davis, M., M. Mellman, and H. Shamoon. 1991. Effect of physiologic hyperinsulinemia on counterregulatory hormone (CRH) responses during hypoglycemia in humans (HYPO). *Diabetes.* 40:72*a*. (Abstr.)

36. Liu, D., E. Moberg, M. Kollind, P.-E. Lins, and U. Adamson. 1991. A high concentration of circulating insulin suppresses the glucagon response to hypoglycemia in normal man. *J. Clin. Endocrinol. & Metab.* 73:1123–1128.

37. Davis, S. N., R. Dobbins, C. Tarumi, C. Colburn, D. Neal, and A. D. Cherrington. 1992. Effects of differing insulin levels on response to equivalent hypoglycemia in conscious dogs. *Am. J. Physiol.* 263:E688–E695.

38. Diamond, M. P., L. Hallarman, K. Starick-Sych, T. W. Jones, M. Connolly-Howard, W. V. Tamborlane, and R. S. Sherwin. 1991. Suppression of counterregulatory hormone response to hypoglycemia by insulin per se. *J. Clin. Endocrinol. & Metab.* 72:1388–1390.

39. Unger, R. H., E. Aguilar-Parada, W. A. Müller, and A. M. Eisentraut. 1970. Studies of pancreatic alpha cell function in normal and diabetic subjects. *J. Clin. Invest.* 49:837–848.

40. Gerich, J. E., M. Langlois, C. Noacco, J. H. Karam, and P. H. Forsham. 1973. Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science (Wash. DC)* 182:171–173.

41. Gerich, J. E., M. Lorenzi, E. Tsalikian, N. V. Bohannon, V. Schneider, J. H. Karam, and P. H. Forsham. 1976. Effects of acute insulin withdrawal and administration on plasma glucagon responses to intravenous arginine in insulin-dependent diabetic subjects. *Diabetes.* 25:955–960.

42. Kawamori, R., M. Shichiri, M. Kikuchi, Y. Yamasaki, and H. Abe. 1980. Perfect normalization of excessive glucagon responses to intravenous arginine in human diabetes mellitus with the artificial beta-cell. *Diabetes.* 29:762–765.

43. Patel, Y. C., T. Wheatley, F. Malaisse-Lagae, and L. Orci. 1980. Elevated portal and peripheral blood concentration of immunoreactive somatostatin in spontaneously diabetic (BBL) Wistar rats. Suppression with insulin. *Diabetes.* 29:757–761.

44. Tannenbaum, G. S., E. Colle, L. Wanamaker, W. Gurd, H. Goldman, and T. A. Seemayer. 1981. Dynamic time-course studies of the spontaneously diabetic BB Wistar rat. II. Insulin-, glucagon-, and somatostatin-reactive cells in the pancreas. *Endocrinology.* 109:1880–1887.

45. Hirsch, B. R., and H. Shamoon. 1987. Defective epinephrine and growth hormone responses in type I diabetes are stimulus specific. *Diabetes.* 36:20–26.

46. Cryer, P. E. 1992. Iatrogenic hypoglycemia as a cause of hypoglycemia-associated autonomic failure in IDDM. A vicious cycle. *Diabetes.* 41:255–260.

47. Gerich, J. E., M. Mokan, T. Veneman, M. Kortykowski, and A. Mitrakou. 1991. Hypoglycemia unawareness. *Endocr. Rev.* 12:356–371.