

**Antecedent hypoglycemia impairs autonomic cardiovascular function -
implications for rigorous glycemic control**

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ABSTRACT

Objective: Glycemic control decreases the incidence and progression of diabetic complications, but increases the incidence of hypoglycemia. Hypoglycemia can impair hormonal and autonomic responses to subsequent hypoglycemia. Intensive glycemic control may increase mortality in individuals with type 2 diabetes at high risk for cardiovascular complications. We tested the hypothesis that prior exposure to hypoglycemia leads to impaired cardiovascular autonomic function.

Research Design and Methods: Twenty healthy subjects (age 28 ± 2 years; 10 males) participated in two 3-day in-patient visits, separated by 1-3 months. Autonomic testing was performed on days 1 and 3 to measure sympathetic, parasympathetic and baroreflex function. A 2 hour hyperinsulinemic [hypoglycemic (2.8 mmol/l) or euglycemic (5.0 mmol/l)] clamp was performed in both the morning and afternoon of day 2.

Results: Comparison of the day 3 autonomic measurements demonstrated that antecedent hypoglycemia leads to: (1) reduced baroreflex sensitivity (16.7 ± 1.8 vs. 13.8 ± 1.4 ms/mmHg, $P=0.03$); (2) decreased muscle sympathetic nerve activity response to transient nitroprusside-induced hypotension (53.3 ± 3.7 vs. 40.1 ± 2.7 bursts/minute, $P<0.01$); and (3) reduced ($P<0.001$) plasma norepinephrine response to lower body negative pressure (3.0 ± 0.3 vs. 2.0 ± 0.2 nmol/l at -40 mmHg).

Conclusions: Baroreflex sensitivity and the sympathetic response to hypotensive stress are attenuated following antecedent hypoglycemia. Because impaired autonomic function, including decreased cardiac vagal baroreflex sensitivity, may contribute directly to mortality in diabetes and cardiovascular disease, our findings raise new concerns regarding the consequences of hypoglycemia.

Control of blood glucose is the cornerstone of diabetes management as glycemic control decreases the incidence and progression of diabetic microvascular (1-4) and, in some studies, macrovascular complications (2;5). However, rigorous glycemic control leads to an increased incidence of hypoglycemia (1;6). Even a single episode of hypoglycemia may impair the counterregulatory metabolic and autonomic responses to subsequent hypoglycemia (7). Recently, evidence has emerged suggesting an association between hypoglycemia and increased mortality in critically ill patients receiving insulin therapy (8). An increase in mortality was also observed in the highly intensive treated limb (targeting HbA1c values of <6%) of a multi-center clinical trial of individuals with type 2 diabetes at high risk for cardiovascular disease events (9). The cause of the mortality in these studies could not be directly attributed to hypoglycemia.

Cardiovascular autonomic impairment is associated with and may cause increased mortality (10-14). Autonomic neuropathy is a predictor of increased mortality in many diabetic cohort studies (10;11). In addition, impaired heart rate variability is associated with increased risk of mortality in patients after a myocardial infarct (12). More recent studies in the post-myocardial infarction population have shown that impaired baroreflex sensitivity is an independent predictor of cardiac mortality (13;14).

In an effort to extend our understanding of the effect of hypoglycemia on the autonomic nervous system, we tested the hypothesis that prior exposure to hypoglycemia would lead to impaired control of cardiovascular autonomic function. We therefore examined cardiovascular autonomic function using standardized tests measuring sympathetic, parasympathetic and baroreflex function before and after euglycemic hyperinsulinemic

and hypoglycemic hyperinsulinemic clamp studies.

METHODS

We recruited healthy men and women, aged 18 to 50 years. A complete medical history, physical examination, screening autonomic testing were performed, and blood for electrolytes, liver function tests and complete blood count were obtained to determine subject eligibility. Each subject participated in two 3-day inpatient study visits, separated in time by one to three months (**Figure 1**). On days 1 and 3 of each study visit, subjects underwent autonomic nervous system testing. On day 2, either two (morning and afternoon) hypoglycemic hyperinsulinemic clamp studies or two euglycemic hyperinsulinemic clamp studies were performed. Those subjects randomized to hypoglycemia on the first inpatient study visit were exposed to euglycemia on the second study visit, and *vice versa*. All autonomic testing was performed at Beth Israel Deaconess Medical Center by investigational staff unaware of subject condition, and subjects were transferred by wheelchair and taxi (0.4 Km) to and from Brigham and Women's Hospital General Clinical Research Center (GCRC) for overnight stays and day 2 study procedures. The institutional review boards at both institutions approved all study procedures and all subjects gave written informed consent.

Diet and activity. Subjects refrained from vigorous exercise for 7 days prior to each admission. Subjects consumed an isocaloric diet provided by the GCRC containing 125 ± 10 mmol/day sodium, 100 ± 10 mmol/day potassium and 200 ± 13 mmol/day calcium with water ad lib. for four days prior to study procedures and continuing through to end of day 3. Subjects received a minimum of 2000 ml fluid orally on day 2.

Hyperinsulinemic clamp protocols. After completing day 1 autonomic testing, subjects were admitted to the Brigham and Women's Hospital GCRC. After fasting and remaining supine overnight, subjects received a primed, constant infusion of insulin (160 and 120 mU/m² body surface area (BSA)/minute sequentially for 5 minutes each, followed by 80 mU/m² BSA/minute for 125 minutes of Novolin R, Novo Nordisk, Princeton, NJ). At T=-15, 0, 45, 75, 105 and 135 minutes of the insulin infusion, blood was withdrawn for determination of hormone levels via an indwelling intravenous catheter that was placed in a retrograde fashion near the wrist of a hand resting in a warm box (66°C). Blood was withdrawn for glucose determination every five minutes and the rate of a 20% dextrose infusion was adjusted to achieve target blood glucose levels of 5.0 mmol/l for the euglycemic clamps and 2.8 mmol/l for the hypoglycemic clamps. At the end of the 135-minute insulin infusion, subjects received a small snack and the dextrose infusion rate was adjusted to achieve blood glucose levels of at least 5.0 mmol/l. After a 90-minute break, the hyperinsulinemic clamp procedure was repeated. Afternoon insulin infusions during hypoglycemic clamp studies were extended as necessary to achieve 90 minutes of hypoglycemia. In four subjects, the insulin infusion was increased by 50% to achieve the target glucose level of 2.8 mmol/l. Twenty-four-hour urine collections were obtained on day 2. The next morning subjects returned to Beth Israel Deaconess Medical Center for the day 3 autonomic testing.

Autonomic Testing. Testing was performed at 9 A.M. in the autonomic laboratory of the Beth Israel Deaconess Medical Center on days 1 and 3 of each study visit. All autonomic investigators were unaware as to whether subjects had been exposed to hypoglycemia or euglycemia on day 2. Subjects received a light breakfast 2 hours prior to testing. Subjects were allowed at least

a 20-minute rest in the supine position in a quiet, dim lit environment to attain psychological and physiological equilibration prior to testing. RR interval, beat-to-beat blood pressure (Finometer, FMS, Amsterdam, the Netherlands), and oscillometric blood pressure (Dinamap, Critikon Company, Tampa, FL) were measured. Muscle sympathetic nerve activity (MSNA) was recorded from the peroneal nerve with microneurography. Neural signal was filtered (bandwidth 0.7-2.0 kHz), rectified and integrated (time constant, 0.1 second) (Nerve Traffic Analyzer, Model 662c-3, University of Iowa Bioengineering, Iowa City, IA). Sympathetic bursts were identified by their characteristic morphology and relationship to R waves on the ECG using an automated sympathetic neurogram analysis program (15). The number of sympathetic bursts was expressed as burst/minute. The following tests were performed in sequential order:

Paced breathing heart rate variability. The high frequency region of the RR interval power spectrum during supine paced breathing was used to assess baseline cardiac vagal function. Subjects were instructed to initiate a breath with each tone of a series of computer generated auditory cues at a preset, evenly spaced rate of 12 breaths/minute. The autospectra of the RR interval were estimated for the 7-minute segment using the Blackman Tukey method. Power spectral estimates of the RR interval heart rate were quantified using the area (power) of the spectrum in the high frequency region (HF, 0.15-0.50 Hz) (16).

Baroreflex assessment. The modified Oxford technique was used to assess cardiac vagal and sympathetic baroreflex function (17). After a resting period of 30 minutes, a 5-minute baseline recording was made followed by the baroreflex test: sequential administration of bolus injections of 100 µg sodium nitroprusside and of 150 µg phenylephrine hydrochloride produced a drop

in systolic blood pressure of ~15 mmHg below baseline followed by a rise of ~15 mmHg above baseline. The cardiac vagal baroreflex was assessed by the relation between RR interval and systolic blood pressure (**Figure 2B**). The muscle sympathetic nerve response to transient hypotension was determined (17).

Simulated orthostatic stress with lower body negative pressure (LBNP). Graded lower body negative pressure was used to simulate orthostatic stress without the confounding effects of muscle contraction. Supine subjects were sealed at the waist in a metal tank. Following a 5 minute baseline data collection period, pressures of -10, -20, -30, and -40 mmHg were generated sequentially. Four minutes were spent at each negative pressure level. Blood was drawn for hormone measurements via an indwelling venous catheter at baseline and after 3 minutes of each negative pressure level. Subjects rated their symptoms of lightheadedness, dizziness, weakness, nausea, sweating and feelings of impending blackout or fainting on a scale of 0 to 10 (with 10 indicating the most severe symptom level) using a standardized questionnaire at minute 3 of each level of LBNP. A score greater than or equal to 3 was considered a clinically significant symptom.

Laboratory tests. Serum insulin levels were measured using the Insulin-Coat-A Count Kit from Diagnostic Products Corporation (Los Angeles, CA), with a lower limit of detection of 18 pmol/l. Serum glucose levels were measured using the Beckman Glucose Analyzer 2 (Beckman Coulter, Chaska, MN). Plasma norepinephrine, plasma epinephrine and 24 hour urinary epinephrine levels were assayed using the 2 CAT RIA kit (Immuno Biological Laboratories, Inc, Minneapolis, MN). 24 hour urinary collections were assayed for free cortisol levels using the Cortisol Diagnostic Products Coat-A-Count RIA kit and creatinine using the Roche

reagent (COBAS Integra 400, Roche Diagnostics, Indianapolis, IN).

Statistical analysis. Data were analyzed using repeated-measures analysis of variance with main effects of treatment (antecedent hypoglycemia and antecedent euglycemia) and either characteristic points on the cardiac baroreflex relation curve or lower body negative pressure level. Non-repeated measures assessed in two conditions were analyzed using Student's two-tailed paired t-test. Categorical variables were compared using Fisher's exact test. Data are expressed as means \pm standard error of the mean (SEM).

RESULTS

Demographics, screening and baseline (day 1) assessments. 20 healthy subjects (age 28 ± 2 years; 10 males) were studied. Subjects had an average body mass index of 24.1 ± 0.6 kg/m², resting heart rate 64 ± 2 bpm, systolic blood pressure 107 ± 2 mmHg, and diastolic blood pressure 69 ± 2 mmHg at screening. Autonomic testing performed at screening was within normal limits in all subjects. Baseline assessments of all autonomic measures were similar on both of the day 1 studies.

Insulin clamp. Morning baseline levels of glucose (5.3 ± 0.2 mmol/l and 5.1 ± 0.1 mmol/l) and insulin (3.9 ± 0.3 μ U/ml and 3.8 ± 0.4 μ U/ml) were similar on euglycemic and hypoglycemic clamp study days, respectively. Serum insulin levels averaged 123 ± 3 μ U/ml during the clamp studies, with no significant differences between any of the four clamp studies. Blood glucose averaged 2.8 ± 0.0 mmol/l for the final 105 minutes of both the morning and afternoon hypoglycemic clamp procedures, and 5.0 ± 0.0 mmol/l for both corresponding periods of the euglycemic clamps (**Figure 3**).

Cortisol and epinephrine were elevated in 24-hour urine collections obtained on the day of the hypoglycemic clamps compared with levels in urine collections on the day of the

euglycemic clamps, whereas urinary creatinine levels were similar (**Figure 4**). Urine sodium levels were similar on the euglycemic (119 ± 8 mmol/TV) and hypoglycemic (102 ± 7 mmol/TV) clamp study days.

Cardiac vagal and sympathetic baroreflex assessment. Cardiac vagal baroreflex sensitivity, as determined by the slope of the relation between RR interval and blood pressure during the modified Oxford test performed on Day 3, was significantly reduced after antecedent hypoglycemia as compared to antecedent euglycemia (**Figure 5A and B**).

Further, to take into account any potential influence of the variability in baroreflex sensitivity between admissions, we determined the baroreflex sensitivity difference Δ BRS (baroreflex sensitivity on Day 3 minus baroreflex sensitivity on Day 1 of the same admission). Δ BRS with antecedent hypoglycemia on Day 2 (-1.6 ± 4.3 ms/mmHg) was significantly less than Δ BRS with antecedent euglycemia on Day 2 (2.3 ± 6.3), $P<0.01$.

Characteristics of the cardiac vagal baroreflex function curve (threshold, mid and saturation points) are shown in the **Table** and demonstrate a shift towards the right after antecedent hypoglycemia, as compared to antecedent euglycemia. Sympathetic nerve activity during the hypotensive period of the modified Oxford test was significantly lower after antecedent hypoglycemia compared to antecedent euglycemia (**Figure 5C**).

There was no association between BMI, gender, clamp insulin levels, HOMA index and the effect of antecedent hypoglycemia on baroreflex sensitivity.

Cardiac vagal function during paced breathing. The RR interval high frequency spectral power determined during paced breathing tended to be lower after the hypoglycemic clamp compared to the euglycemic clamp (609 ± 103 vs. 804 ± 206

ms^2 , $P=0.13$). There was a positive correlation between the power spectral density and cardiac vagal baroreflex sensitivity determined after antecedent hypoglycemia ($r=0.67$, $P<0.01$) and after antecedent euglycemia ($r=0.57$, $P<0.05$).

Simulated orthostatic stress with lower body negative pressure (LBNP). The RR interval and systolic blood pressure at baseline and in response to increasing levels of lower body negative pressure were similar, irrespective of whether the study was performed after antecedent hypoglycemia or antecedent euglycemia (**Figure 6A and B**). Plasma norepinephrine levels rose in response to increasing levels of negative pressure ($p<0.0001$), however this response was significantly blunted after antecedent hypoglycemia as compared to antecedent euglycemia (**Figure 6C**). Plasma epinephrine levels also increased during LBNP ($p<0.05$), but this response was not significantly affected by the antecedent glucose level (**Figure 6D**).

In the post-euglycemic condition a total of 5 of 20 (25%) subjects experienced clinically significant symptoms or signs of impending syncope, compared to 12 of 20 (60%) subjects in the post-hypoglycemic condition ($P=0.05$, Fisher's exact test).

DISCUSSION

Our data demonstrate that antecedent hypoglycemia results in a significant decrease in: (i) cardiac vagal baroreflex sensitivity, and (ii) the sympathetic response to both a transient pharmacologically induced hypotensive stress and a graded simulated orthostatic stress using lower body negative pressure. It is well established that prior exposure to hypoglycemia attenuates the autonomic nervous system response to subsequent hypoglycemia (7;18). The present data, which show that prior hypoglycemia attenuates the autonomic response to specific cardiovascular stresses, extend those findings.

Furthermore, the evidence that antecedent hypoglycemia attenuates cardiovascular autonomic control may have significant clinical implications; impaired autonomic function is associated with and may be a contributor to mortality in diabetes and cardiovascular disease (10;12-14).

It is well established from studies in healthy and diabetic subjects that exposure to recent hypoglycemia reduces the counterregulatory hormone (e.g., epinephrine, glucagon, adrenocorticotrophic hormone) and autonomic nervous system responses to subsequent hypoglycemia. This hypoglycemia associated autonomic failure/dysfunction leads to decreased ability to sense hypoglycemia and restore euglycemia (7). However, there are conflicting reports as to whether antecedent hypoglycemia impairs the autonomic response to non-hypoglycemic stimuli as well hypoglycemic stimuli. In one study of type 1 diabetic subjects, the epinephrine and norepinephrine responses to exercise and upright posture were intact following antecedent hypoglycemia (19). Other studies of type 1 diabetic subjects suggested that the deficit is more generalized (20;21); the epinephrine and norepinephrine responses to a cold pressor test were reduced in well controlled type 1 diabetic subjects (20) and antecedent hypoglycemia reduced the normal exercise-induced rise in epinephrine, norepinephrine, glucagon, growth hormone, pancreatic polypeptide and cortisol in healthy individuals (21). Our data are consistent with these latter studies and strongly support the view that the effects of antecedent hypoglycemia on the autonomic nervous system are more generalized and not specific to subsequent hypoglycemic stimuli.

Prior studies have shown that *during* hypoglycemia there is acquired prolongation of the rate corrected QT interval.(22) Our findings show that antecedent hypoglycemia leads to impaired control of cardiovascular

function, even after euglycemia is restored, and that the effects of antecedent hypoglycemia on autonomic function can last for at least 16 hours. We demonstrated that antecedent hypoglycemia attenuated the sympathoadrenal and muscle sympathetic nerve activity outflow responses to simulated orthostatic stress and transient hypotension. Thus, in the clinical setting, it is possible that recent exposure to hypoglycemia could impair sympathetic nervous system responses to cardiovascular stresses.

Antecedent hypoglycemia also decreased baroreflex sensitivity, which indicates impairment in vagal cardiac modulation. Decreases in vagal autonomic function may lead to attenuation of vagal protection against sudden arrhythmic death. Impaired cardiac vagal control, as determined by reduced heart rate variability, is associated with increased risk of post-myocardial infarction mortality, even after adjusting for clinical, demographic, other Holter features, and ejection fraction (12;23-25). In addition, impairment of baroreflex sensitivity has been associated with adverse outcomes in clinical (13;14) and preclinical (26) studies.

Baroreflex sensitivity, calculated from the measurement of the rate-pressure relation following an intravenous bolus of phenylephrine, was a significant independent risk predictor of cardiac mortality in the Autonomic Tone and Reflexes After Myocardial Infarction (ATRAMI) study, an international multicenter prospective study of 1,284 patients with a recent myocardial infarction (14). The 2-year mortality for individuals with baroreflex sensitivity <3.1 ms per mmHg was 9%, versus 2% for those with preserved baroreflex sensitivity. In a 5-year follow-up study of a subset of 244 patients with ST-segment elevation myocardial infarction and normal ejection fraction, impaired baroreflex sensitivity (<3.1 ms per mmHg) 4 to 6 weeks after the myocardial infarction identified patients with

a relative risk of cardiovascular mortality of 11.4, compared with those without impaired baroreflex sensitivity (13).

In the current study baroreflex sensitivity in normal subjects after antecedent hypoglycemia did not reach the level of 3.1 ms per mmHg, however, given the observed reduction that was evident in our studies (6 of 20 subjects had a decrease in baroreflex sensitivity of >5 ms/mmHg and 3 subjects had a decrease in baroreflex sensitivity of ≥ 10 ms/mmHg), it is possible that similar changes after hypoglycemia in individuals impaired baroreflex sensitivity at baseline would lead to baroreflex sensitivity below 3.1 ms per mmHg. Thus, hypoglycemia-induced impairment in baroreflex sensitivity and/or sympathetic nervous system cardiovascular control (as noted above) could have clinical implications, especially in critically ill patients or in patients at high risk for cardiovascular events.

The high frequency power of the RR interval in response to paced breathing, also an index of cardiac vagal control, correlated positively with cardiac vagal baroreflex sensitivity in our study. There was a strong tendency toward a reduction in the high frequency RR interval power in the post-hypoglycemic state but this did not reach statistical significance. Several possible explanations for this finding exist. These include: (1) the provocation for the cardiac vagal baroreflex assessment (transient hypotension) provided a more consistent and potent stimulus than the provocation for respiratory mediated RR interval variability (we did not monitor or control the depth of respiration); (2) there is a differential effect of hypoglycemia on the autonomic pathways involved in the two reflexes. In future studies, both frequency and amplitude of respiration should be monitored.

The present data may have implications for diabetic subjects particularly those with

autonomic neuropathy who may have an increased mortality risk (10).

Furthermore, it is possible that the consequences of antecedent hypoglycemia on autonomic function may be amplified in diabetic subjects, particularly those with autonomic dysfunction. For example, while the reduced counterregulatory and hormonal response to antecedent hypoglycemia occurs in the absence of diabetic autonomic neuropathy, as measured by standard tests of autonomic function (18;27), several (28-30), although not all (22), studies suggest that the presence of autonomic dysfunction further attenuates the autonomic response to hypoglycemia.

Other populations in which the consequences of antecedent hypoglycemia on autonomic function may be more apparent are critically ill patients and individuals with diabetes at risk for cardiovascular events. There is a significant increase in mortality in critically ill patients who experience hypoglycemia. The risk is similar irrespective of whether the critically ill patients received intensive or conventional insulin therapy and is not directly caused by hypoglycemia (8). Similarly, in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, which enrolled individuals with diabetes at increased risk of cardiovascular events, intensively lowering blood glucose to a goal glycated hemoglobin (HbA1c) level of less than 6.0% increases the risk of death compared with a less intensive treatment goal of 7.0-7.9% (9). The difference does not appear to be directly due to hypoglycemia or the use of a specific drug. Further, and perhaps of mechanistic importance, while the relative risk of nonfatal myocardial infarction is lower in the intensively treated group, all cause mortality and mortality from cardiovascular causes is greater. The effect of antecedent hypoglycemia on autonomic cardiovascular function in individuals with diabetes and in vulnerable diabetic

subpopulations, such as critically ill patients or patients with cardiovascular disease, is an important area for additional study.

The randomized cross-over design of this study provides the advantage of each subject serving as his/her own control. Additionally, subjects were exposed to similar insulin levels during the hypoglycemic and euglycemic clamp procedures. However, this trial design also imposed a potential limitation of residual effects from exposure to hypoglycemia/euglycemia during the first study visit. Therefore, we reevaluated subjects at least 1 and up to 3 months after the initial study visit. To ensure no interval change in autonomic function between clamps, baseline autonomic testing was performed prior to each clamp. Further, physical activity and dietary intake (including electrolytes) were rigorously controlled.

In summary, these data suggest that cardiovascular autonomic function, specifically, baroreflex sensitivity and the sympathetic response to a hypotensive stress, is attenuated following antecedent hypoglycemia. Attenuation of cardiac vagal

baroreflex sensitivity is an independent predictor of mortality in post-myocardial infarction patients. As our findings have potential implications for rigorous glycemic control in diabetes, studies are needed to determine the effects of antecedent hypoglycemia on autonomic cardiovascular function in individuals with type 1 and type 2 diabetes.

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Table 1: Baroreflex function characteristics

	Post-Euglycemia	Post-Hypoglycemia
Threshold		
SBP (mmHg)	120±4	123±4
RR interval (ms)	790±37	813±33
Midpoint		
SBP (mmHg)	127±4	132±3*
RR interval (ms)	966±39	977±39
Saturation		
SBP (mmHg)	134±4	138±3
RR interval (ms)	1142±50	1140±47
Baroreflex Slope		
(mmHg/ms)	16.7±1.8	13.8±1.4*

* denotes $P < 0.05$

FIGURE LEGENDS

Figure 1. Study design. Subjects participated in two, 3 day/2 night protocols separated in time by 1 to 3 months. Subjects were randomized by admission to participate in either euglycemic (target blood glucose 5.0 mmol/l) hyperinsulinemic clamp studies or hypoglycemic (target blood glucose 2.8 mmol/l) hyperinsulinemic clamp studies on day 2. Autonomic testing was conducted on days 1 and 3 by investigators unaware of whether a subject was participating in the euglycemia or hypoglycemia protocol.



