The Effects of Intensive Therapy and Antecedent Hypoglycemia on Counterregulatory Responses to Hypoglycemia in Type 2 Diabetes

Stephen N. Davis, MD\textsuperscript{1, 2}, Stephnie Mann, BSN\textsuperscript{1}, Vanessa J. Briscoe, PhD\textsuperscript{1}, Andrew C. Ertl, PhD\textsuperscript{1} and Donna B. Tate, MS\textsuperscript{1}

Departments of Medicine, Vanderbilt University\textsuperscript{1} and Veterans Affairs\textsuperscript{2}, Nashville, TN.

Please address all correspondence to:
Stephen N. Davis, MD.
Division of Diabetes, Endocrinology and Metabolism
Vanderbilt University
7465 MRB IV
2213 Garland Avenue
Nashville, TN  37232-0475.

Submitted 4 September 2008 and accepted 26 November 2008.
ABSTRACT

Objective: The physiology of counterregulatory responses during hypoglycemia in intensively treated Type 2 DM subjects is largely unknown. Therefore, the specific aims of the study tested the hypothesis that 1) 6 months of intensive therapy to lower HBA1C below 7.0% would blunt autonomic nervous system (ANS) responses to hypoglycemia and 2) antecedent hypoglycemia will result in counterregulatory failure during subsequent hypoglycemia in patients with suboptimal and good glycemic control.

Research Design and Methods: Fifteen Type 2 DM (8M/7F) underwent 6 month combination therapy of metformin, glipizide XL and acarbose to lower HBA1C to 6.7% and 2-day repeated hypoglycemic clamp studies before and after intensive therapy. A control group of eight non-diabetic subjects participated in a single 2-day repeated hypoglycemic clamp study.

Results: Six months therapy reduced HBA1C from 10.2±0.5 to 6.7± 0.3%. Rates of hypoglycemia increased to 3.2 episodes per patient/month by study end. Hypoglycemia (3.3±0.1 mmol/L) and insulinemia (1722±198 pmol/L) were similar during all clamp studies. Intensive therapy reduced (p<0.05) ANS and metabolic counterregulatory responses during hypoglycemia. Antecedent hypoglycemia produced widespread blunting (p<0.05) of neuroendocrine, ANS and metabolic counterregulatory responses during subsequent hypoglycemia before and after intensive therapy in Type 2 DM patients and in non-diabetic controls.

Conclusion: Intensive oral combination therapy and antecedent hypoglycemia both blunt physiologic defenses against subsequent hypoglycemia in Type 2 DM. Prior hypoglycemia of only 3.3±0.1 mmol/L can result in counterregulatory failure in Type 2 DM patients with suboptimal control and can further impair physiologic defenses against hypoglycemia in intensively treated Type 2 DM.
Large randomized controlled multi-center clinical trials have demonstrated the benefit of improved glycemic control on microvascular complications in both Type 1 and Type 2 diabetes (1, 2). These compelling data have produced a paradigm shift in the treatment of diabetes (particularly Type 2 DM) striving for HBA1C values below 7.0% (3). The major drawbacks of tight metabolic control in patients with Type 1 DM are well documented and include increased hypoglycemia and weight gain (4-8).

Recently three large studies have investigated the effects of rigorous metabolic control (HBA1C < 7.0%) on the prevalence of macrovascular disease in Type 2 DM (9-11). The overall conclusion of these studies was that HBA1C values below 7.0% did not produce a statistically significant reduction in macrovascular events, but did produce a marked increase in hypoglycemia in Type 2 DM. The effects of intensive therapy on physiologic counterregulatory responses during hypoglycemia in Type 2 DM have not been thoroughly investigated. Burge, et al. (12) demonstrated that improving glycemic control during an eight day in-patient admission could lower symptom responses and plasma glucose levels for activation of epinephrine during hypoglycemia. Levy, et al. (13) using a cross-sectional study design also concluded that improved glycemic control in Type 2 DM shifts the thresholds for counterregulatory hormone release to lower plasma glucose concentrations during hypoglycemia. Kurzon-Burakowska, et al. (14) improved HBA1C from 11.3±1.1 to 8.1±0.9% during a 4-month period. Thresholds (i.e. plasma glucose values) for counterregulatory hormone release, as well as epinephrine and cortisol responses were lowered by improved glycemic control. Spyer, et al. (15), investigating a group of 7 Type 2 DM with an HBA1C of 7.4%, also found that the glycemic thresholds for counterregulatory hormone release were reduced from elevated to normal physiologic glucose levels. However, similar to some (13, 16) but not all studies (17), there was no difference in values of the key counterregulatory hormones, epinephrine and glucagon, during hypoglycemia when compared to non-diabetic controls. Studies investigating the mechanisms regulating counterregulatory responses during hypoglycemia in Type 2 DM are even fewer. Segal, et al. (16) determined that antecedent hypoglycemia in a group of Type 2 DM with an HBA1C of 8.1% resulted in hypoglycemia associated autonomic failure similar to patients with Type 1 DM. Despite the above data, two questions remain unanswered, 1) What are the effects of a period of rigorous glycemic control to reduce HBA1C below 7.0% on counterregulatory responses in Type 2 DM, and 2) what are the effects of antecedent hypoglycemia on autonomic nervous system, neuroendocrine and metabolic counterregulatory mechanisms before and after a period of rigorous metabolic control in Type 2 DM. In the present study, we tested the hypothesis that 6 months intensive therapy to lower HBA1C below 7.0% would impair counterregulatory response to hypoglycemia and that antecedent hypoglycemia would further impair key homeostatic counterregulatory mechanisms during subsequent hypoglycemia in Type 2 DM.

**RESEARCH DESIGN AND METHODS**

**Subjects.** Fifteen (8M/7F) patients with Type 2 DM (age 47±2), body mass index of 33±2 kg/m², glycosylated hemoglobin of 10.2±0.5% (normal range 4-6.5%), anti-GAD and anti-islet cell antibody negative and disease duration of 6±2 years were studied. Patients were receiving diet and exercise (n=2) or oral agent monotherapy (metformin...
(mean 750 mg/day, n=10) or sulfonylurea (glyburide or glipizide mean 5 mg/day, n=3)). Eight non-diabetic control subjects (4M/4/F), aged 48±3, BMI 30±2 kg/m², and HBA1C 5.1±0.2% were also studied. Each subject had a normal blood count, plasma electrolytes, liver and renal function. All gave written informed consent. Studies were approved by the Vanderbilt University Human Subjects Institutional Review Board.

**Experimental Design.**

**Type 2 DM Subjects.** Fifteen patients participated in two 2-day hypoglycemia experiments separated by 6 months. Subjects were asked to avoid any exercise and consume their usual weight maintaining diet for 3 days before each study. Two days prior to a study, sulfonylurea and metformin tablets were omitted. After six months when the patients were in good metabolic control, these medications were replaced with injections of regular insulin before each meal. The dose of regular insulin was carefully adjusted so that hypoglycemia (<3.9 mmol/L) and hyperglycemia (>11.1 mmol/L) were avoided in the two days prior to a study. Each subject was admitted to the Vanderbilt General Clinical Research Center (CRC) at 5:00 PM on the evening before an experiment. At this time, two intravenous cannulae were inserted under 1% lidocaine local anesthesia. One cannula was placed in a retrograde fashion into a vein on the back of the hand. This hand would be placed in a heated box (55-60°C) during the study so that arterialized blood could be obtained (18). The other cannula was placed in the contralateral arm for infusions. Patients then received a standardized evening meal and a continuous low-dose infusion of insulin was started to normalize plasma glucose. The insulin infusion was adjusted overnight to maintain blood glucose between 4.4 and 7.2 mmol·L⁻¹.

**Hypoglycemia Experiments.** After an overnight 10 hr fast at 0 min, a primed (18μCi) continuous infusion (0.18 μCi·min⁻¹) of HPLC purified [3⁻³H] glucose (Perkin Elmer Life Sciences, Boston, MA; 11.5 mCi·mmol⁻¹·L⁻¹) was administered via a precalibrated infusion pump (Harvard Apparatus, South Natick, MA). A period of 90 min was allowed to elapse followed by a 30 min basal control period. Plasma glucose was maintained at euglycemia during this period by continuing the overnight basal insulin. At time 120 min, a primed constant (15.0 pmol·kg⁻¹·min⁻¹) infusion of insulin (Human Regular Insulin, Eli Lilly, Indianapolis, IN) was started and continued until 240 min. The rate of fall of glucose was controlled (0.07 mmol·min⁻¹) and the hypoglycemic nadir (3.3 mmol·L⁻¹) was achieved using a modification of the glucose clamp technique (19). During the clamp period, plasma glucose was measured every five min and a 20% dextrose infusion was adjusted so that plasma glucose levels were held constant at 3.3±0.1mmol·L⁻¹ (20). Potassium chloride (20mmol·L⁻¹) was infused during the clamp to reduce insulin-induced hypokalemia. After completion of the 2 hr test period, the insulin infusion was turned down to the basal rate and the plasma glucose was rapidly restored to euglycemia with 20% dextrose. In the afternoon following a 2 hr period of euglycemia, a second 2 hr hypoglycemic clamp of 3.3 mmol/L was performed similar to the morning study. (No tritiated glucose was infused during the afternoon studies). After completion of the afternoon hypoglycemic clamp, a basal insulin infusion was restarted and a standardized evening meal and snack was given. Similar to the previous night, a variable low-dose infusion was used to maintain glucose levels (4.4 to 7.2 mmol/L) overnight. The next morning after a 10 hr overnight fast, an identical (including a tritiated glucose infusion) 2 hr hypoglycemic clamp at 3.3±0.1 mmol/L was performed as described for day 1.
Study Medication. Following completion of the first 2-day clamp studies, patients were started on triple oral combination therapy. Metformin and acarbose were increased to 1 gm twice a day and 50 mg three times a day over a 4-week period, respectively. Glipizide XL was increased to 10 mg once or twice a day over a period of 3 weeks. All patients performed intensive self blood glucose monitoring before main meals, snacks and at bedtime or any time that they felt low glucose or a second person thought they had a low glucose. Thus, study subjects tested their glucose levels between 4-7 times per day. Patients were contacted by study nurses twice a week and by a dietician weekly to adjust diet, exercise, and medication and discuss any treatment side effects. Patients were seen by either SND, study nurses and/or a dietician monthly. Patient adherence to protocol was assessed at monthly visits and found to be excellent. One patient moved out of state during the 6 month treatment period and was lost to follow up. After completion of 6 months intensive treatment period, patients were readmitted to the Vanderbilt GCRC for an identical 2-day hypoglycemic clamp protocol as described above. The non-diabetic control subjects underwent a single 2-day in-patient hypoglycemic clamp protocol similar (without overnight glucose control) to the type 2 DM patients.

Direct Measurement of Muscle sympathetic Nerve activity (MSNA). MSNA was recorded from the peroneal nerve at the level of the fibular head and popliteal fossa (21, 22). The approximate location of this nerve was determined by transdermal electrical stimulation (10-60 V, 0.01 msec duration). This stimulation produced painless muscle contraction of the foot. Following this, a reference stainless steel microelectrode with a shaft diameter of 200 µm was placed subcutaneously. A similar tungsten electrode, with an uninsulated tip (1-5 µm) was inserted into the nerve and used for recording of muscle sympathetic nerve activity (MSNA).

Criteria for acceptable MSNA recordings were: 1) electrical stimulation produced muscle twitches but not paresthesia, 2) nerve activity increased during phase II of the Valsalva maneuver (hypotensive phase) and was suppressed during phase IV (blood pressure overshoot), and 3) nerve activity increased in response to held expiration.

Tracer Calculations. Rates of glucose appearance (Ra), endogenous glucose production (EGP), and glucose utilization were calculated according to the methods of Wall et al. (23). EGP was calculated by determining the total Ra (this comprises both EGP and any exogenous glucose infused to maintain the desired hypoglycemia) and subtracting it from the amount of exogenous glucose infused. It is now recognized that this approach is not fully quantitative, since underestimates of total Ra and rate of glucose disposal (Rd) can be obtained. The use of a highly purified tracer and taking measurements under steady-state conditions (i.e., constant specific activity) in the presence of low glucose flux eliminates most, if not all, of the problems. In addition, in order to maintain a constant specific activity, isotope delivery was increased commensurate with increases in exogenous glucose infusion. During these studies, only glucose flux results from the steady state basal and the final 30 min periods of the hypoglycemic clamps are reported.

Analytical Methods. Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Glucagon was measured according to a modification of the method of Aguilar-Parada et al. with an interassay coefficient of variation (CV) of 12% (24). Insulin was measured as previously described (25) with an interassay CV of 9%. Catecholamines were determined by HPLC (26) with an
interassay CV of 12% for epinephrine and 8% for norepinephrine. Two modifications to the procedure for catecholamine determination were made: 1) a five-point rather than a one-point standard calibration curve was used; and 2) the initial and final samples of plasma with known amounts of epinephrine and norepinephrine were spiked so accurate identification of the relevant respective catecholamine peaks could be made. Cortisol was assayed using the Clinical Assays Gamma Coat Radioimmunoassay (RIA) kit with an interassay CV of 6%. Growth hormone was determined by RIA (27) with a CV of 8.6%. Pancreatic polypeptide was measured by RIA using the method of Hagiopian et al. (28) with an interassay CV of 8%. Lactate, glycerol, alanine and β-hydroxybutyrate were measured in deproteinized whole blood using the method of Lloyd et al. (29). Non-esterified fatty acids (NEFA) were measured using the WAKO kit adopted for use on a centrifugal analyzer (30).

Blood for hormones and intermediary metabolites were drawn twice during the control period and every 15 min during the experimental period. Cardiovascular parameters (pulse, systolic, diastolic, and mean arterial pressure) were measured non-invasively by a Dinamap (Critikon, Tampa, FL) every 10 min throughout each study starting at 80 min.

Hypoglycemic symptoms were quantified using a previously validated semiquantitative questionnaire (31). Each individual was asked to rate his/her experience of the symptoms twice during the control period and every 15 min during experimental periods. Symptoms measured included sweaty, tremor/shaky, hot, thirsty/dry mouth, agitation/irritability, palpitations, tired/fatigued, confusion, dizzy, difficulty thinking, blurriness of vision, and sleepy. The ratings of the first six symptoms were summed to get the autonomic score while the ratings from the last six symptoms provide a neuroglycopenic symptom score.

**Statistical Analysis.** Data are expressed as mean ± SE and were analyzed using standard, parametric, one- and two-way analysis of variance (ANOVA) and with repeated measures where appropriate (SigmaStat; SPSS Science, Chicago, IL). Tukey’s post hoc analysis was used to delineate statistical significance across time within each group, and for each group compared to day 1 of the PRE intensive therapy group. A P value of <0.05 was accepted as statistically significant. The baseline and final 30 min of hypoglycemia was compared for most parameters, as steady state glucose levels, insulin levels, and glucose infusion rates were achieved by this time.

**RESULTS**

**Effects of 6 Months Intensive Therapy on Counterregulatory Responses to Hypoglycemia, Insulin, Glucose and HBA_{1C} Levels.** HBA_{1C} levels were reduced from 10.2±0.5 to 6.7±0.3% during the 6 months of intensive therapy. Body weight remained stable throughout 6 months of intensive therapy (97.6±7 to 96.1±6 kg). Plasma glucose levels were controlled overnight and were similar at the start of the pre-intensive therapy (PRE) and post-intensive therapy (POST) (5.6±0.3 and 5.3±0.2 mmol/L, respectively). Basal plasma glucose levels in the control (CONTROL) group were also equivalent at 5.3±0.2 mmol/L. Plasma glucose levels (3.3±0.1 mmol/L) were equivalent during PRE, POST and CONTROL hypoglycemic clamps (Figure 1).

Basal insulin levels were 198±36, 210±30 and 66±18 pmol/L in the PRE, POST and CONTROL groups, respectively. Insulin levels during the clamp studies were similar among groups (1722±198 pmol/L). C-peptide levels decreased from 0.75±0.12 to 0.17±0.02 ng/L during hypoglycemia in PRE. Following intensive therapy, basal C-peptide
levels were increased (1.2±0.2 ng/ml; p<0.05) and were higher during POST clamps (0.3±0.03 ng/L; p<0.05) as compared to PRE. **Neuroendocrine Responses.** Following 6 months intensive therapy, epinephrine responses were significantly blunted in POST as compared to PRE and CONTROL values (2033±343 vs. 3788±414 and 3837±441 pmol/L, respectively; p<0.05) (Figure 2). Intensive therapy had no effect on other neuroendocrine (glucagon, growth hormone, cortisol, norepinephrine or pancreatic polypeptide) responses to hypoglycemia (Figure 3). Cortisol levels were higher (p<0.05) in PRE as compared to CONTROLS (801±77 vs. 635±80 nmol/L).

**Glucose Kinetics and Intermediary Metabolism.** Basal endogenous glucose production (EGP) was similar in all three groups (PRE 9.9±1.1, POST 9.5±1.1, CONTROL 9.4±1.1 µmol/kg/min). EGP declined significantly in all three groups (p<0.01). However, EGP was higher (p<0.01) during hypoglycemia in PRE (6.6±1.1 µmol/kg/min) as compared to POST and CONTROL values where there was no measurable EGP. Rates of glucose infusion were significantly greater (p<0.01) in POST (10.5±3.3 µmol/kg/min) and CONTROL (16.5±3.9 µmol/kg/min) as compared to PRE (2.2 µmol/kg/min).

**Intermediary Metabolism.** NEFA (193±22 vs. 303±38 µmol/L), lactate (1.52±0.17 vs. 1.96±0.18 mmol/L) and glycerol (49±7 vs. 69±9 µmol/L) were significantly blunted (p<0.05) during POST compared to PRE intensive therapy, respectively (Figure 4). Plasma lactate, glycerol and blood NEFA levels were higher (p<0.05) during POST intensive therapy vs. CONTROL.

**Muscle Sympathetic Nerve Activity.** Basal rates of MSNA were similar in PRE (34±3 burst/min), POST (31±4 burst/min) and CONTROL (38±6 bursts/min). MSNA increased by 17±3 burst/min during PRE which was increased (p<0.05) compared to the responses during POST (5±3 bursts/min) and CONTROL clamps (8±2 burst/min) (Figure 5).

**Hypoglycemic Symptom Scores.** Total hypoglycemic scores increased by 33±6 during hypoglycemia in PRE which was increased (p<0.01) compared to the response during POST (19±4). Symptom responses were lower (p<0.01) in CONTROL (12±2) as compared to both PRE and POST intensive therapy studies (Figure 5). Intensive treatment blunted autonomic symptom responses (PRE 18±3 vs. POST 10±4; p<0.05) and neuroglycopenic symptom responses to hypoglycemia (15±3 vs. 9±3; p<0.05).

**Cardiovascular Responses.** Intensive therapy significantly blunted cardiovascular responses to hypoglycemia. Systolic blood pressure increased from 120±6 to 129±6 mmHg (p<0.05) during hypoglycemia in PRE but remained similar to baseline (129±6 to 130±7 mmHg) in POST. Heart rate also increased during hypoglycemia in PRE (74±5 to 85±6 beats/min; p<0.05) but remained similar to baseline (70±4 to 73±4 beats/min) in POST. Systolic blood pressure increased by a non-significant amount in controls (119±5 to 125±4 mmHg), but heart rate increased significantly (62±4 to 72±4 beats/min; p<0.05).

**Hypoglycemic Events during Intensive Treatment.** Self-reported blood glucose readings below 3.9 mmol/L are shown in Table 1. No patient experienced any hypoglycemia in the month prior to the start of the study. During the study, all patients experienced hypoglycemic readings between 3.3 and 3.9 mmol/L. Thirteen patients had readings between 2.8 and 3.3 mmol/L, and frequency of hypoglycemia increased to 3.2 episodes per patient per month by the end of the 6 month study. No major episodes of hypoglycemia occurred during the study. Four patients documented hypoglycemic readings below 2.8 mmol/L.
Effects of Antecedent Hypoglycemia on Counterregulatory Responses to Hypoglycemia Before and After 6 Months Intensive Therapy.

**Insulin, C-peptide and Glucose Levels.** Insulin levels were similar during the morning of day 1 and day 2 clamp studies in all three groups (1534±174 to 1890±222 pmol/L). Plasma glucose levels were equivalent during morning day 1 and day 2 studies (3.3±0.1 mmol/L) in all groups. Antecedent hypoglycemia attenuated the fall in C-peptide during day 2 in both PRE (day 1, -0.58±0.08 vs. day 2, -0.39±0.11 ng/L) and POST studies (day 1, -0.98±0.15 vs. day 2, -0.61±0.11 ng/L; p<0.05).

**Neuroendocrine Levels.** Baseline neuroendocrine hormone levels were similar at the start of PRE, POST and CONTROL studies (Table 2). Despite equivalent glucose and insulin levels, antecedent hypoglycemia significantly blunted day 2 epinephrine levels in PRE (day 1, 3788±414 vs. day 2, 2191±332 pmol/L; p<0.01) POST (day 1, 2033±343 vs. day 2, 1281±316 pmol/L; p<0.05) and in CONTROL groups (day 1, 3837±441 vs. day 2, 1880±611 pmol/L; p<0.05). Plasma glucagon levels were also significantly blunted by antecedent hypoglycemia in PRE (day 1, 81±7 vs. day 2, 49±5 ng/L; p<0.05), POST (day 1, 74±11 vs. day 2, 50±5.2 ng/L; p<0.05) and CONTROL groups (day 1, 83±12 vs. 63±7 ng/L; p<0.05). Similarly cortisol levels were blunted by antecedent hypoglycemia in PRE (day 1, 801±77 vs. 635±75 nmol/L; p<0.05), POST (day 1, 718±72 vs. day 2, 582±88 nmol/L; p<0.05) and CONTROL groups (day 1, 635±80 vs. day 2, 492±86 nmol/L; p<0.05). Antecedent hypoglycemia did not significantly blunt day 2 growth hormone, pancreatic polypeptide or norepinephrine responses in the pre or post patient studies. However, growth hormone (day 1, 7.8±0.6 vs. day 2, 3.9±0.4 ng/L; p<0.05) and pancreatic polypeptide (day 1, 128±26 vs. day 2, 83±28 ng/L; p<0.05) levels were significantly blunted by antecedent hypoglycemia in the CONTROL group.

**Glucose Kinetics.** Glucose specific activity was in a steady state at the start and final 30 min of each hypoglycemic clamp (Table 3). Antecedent hypoglycemia significantly reduced endogenous glucose production during PRE (day 1, 6.6±0.6 vs. day 2, 0.6±1.1 µmol/kg/min; p<0.01). Endogenous glucose production was not measurable during both days of POST and CONTROL studies. Antecedent hypoglycemia resulted in greater glucose infusion rates to maintain target glucose in PRE (day 1, 2.2±1.1 vs. day 2, 8.8±1.6 µmol/kg/min; p<0.05) POST (day 1, 10.5±3.3 vs. day 2, 15.4±2.7 µmol/kg/min; p<0.05)) and CONTROL (day 1, 16.5±3.3 vs. day 2, 23.1±3.9 µmol/kg/min; p<0.05).

**Intermediary Metabolism.** Plasma glycerol responses were blunted (p<0.05) by antecedent hypoglycemia in PRE (day 1, 13±3 vs. day 2, 3±1 µmol/L), POST (day 1, -2±1 vs. day 2, -18±3 µmol/L) and CONTROL studies (day 1, -22±3 vs. day 2, -41±4 µmol/L). Similarly, plasma lactate levels were also blunted (p<0.05) by day 1 hypoglycemia in PRE (day 1, 1.95±0.2 vs. day 2, 1.31±0.2 mmol/L), POST (day 1, 1.5±0.22 vs. day 2, 1.2±0.1 mmol/L) and CONTROL (day 1, 1.6±0.1 vs. day 2, 1.4±0.1 mmol/L).

**Muscle Sympathetic Nerve Activity (MSNA).** Day 1 hypoglycemia reduced MSNA responses during day 2 hypoglycemia in PRE (17±3 vs. 4±2 bursts/min; p<0.05) and in CONTROL (8±2 vs. 3±2 burst/min; p<0.05, respectively). MSNA responses during day 1 hypoglycemia in POST were significantly blunted by intensive treatment and did not decline further during day 2 hypoglycemia (5±2 vs. 6±1 bursts/min).

**Hypoglycemia Symptoms.** Day 1 hypoglycemia reduced symptom responses (p<0.05) during day 2 hypoglycemia in PRE (17±3 vs. 4±2 bursts/min; p<0.05) and in CONTROL (8±2 vs. 3±2 burst/min; p<0.05, respectively). MSNA responses during day 1 hypoglycemia in POST were significantly blunted by intensive treatment and did not decline further during day 2 hypoglycemia (5±2 vs. 6±1 bursts/min).
Day 1 hypoglycemia blunted (p<0.05) both autonomic (18±3 vs. 9±4) and neuroglycopenic (15±4 vs. 7±2) scores during day 2 hypoglycemia in PRE. In POST, day 1 hypoglycemia predominantly blunted day 2 neuroglycopenic symptoms (7±2 vs. 3±2).

**Cardiovascular Responses.** Blood pressure and heart rate responses were similar during day 1 and day 2 hypoglycemia in all groups.

**DISCUSSION**

This study has prospectively determined the effects of 6 months intensive triple combination oral therapy with improvement of HBA$_{1C}$ to 6.7% on integrated counterregulatory responses during repeated hypoglycemia in a group of patients with Type 2 DM. Our results demonstrate that intensive treatment with oral combination therapy substantially reduces autonomic nervous system (ANS), metabolic (endogenous glucose production, lipolysis), symptoms and cardiovascular responses to hypoglycemia. Additionally, antecedent hypoglycemia can produce further widespread (i.e. neuroendocrine) reductions in the above physiologic counterregulatory mechanisms in Type 2 DM patients both before and after intensive therapy.

Three recent large multi-centered trials have examined the effects of intensive glucose control with an average HBA$_{1C}$ below 7.0% on macro and microvascular complications in Type 2 DM (9-11). All three studies identified an increased prevalence of hypoglycemia with lowering of HBA$_{1C}$ in Type 2 DM (9-11). To date, there are no published prospective studies examining the effects of intensive glucose control (i.e. HBA$_{1C}$ below 7.0%) on physiologic responses to hypoglycemia in Type 2 DM. Our study demonstrates that achieving a HBA$_{1C}$ of 6.7% with triple oral therapy (glipizide XL, metformin, acarbose) for six months in the absence of exogenous insulin therapy can significantly reduce neuroendocrine, metabolic and ANS responses to hypoglycemia. Furthermore, repeated episodes of relatively mild hypoglycemia (3.3 mmol/L) can further significantly reduce counterregulatory responses in Type 2 DM patients, thus creating a syndrome of hypoglycemia associated autonomic failure which occurs irrespective of moderate or tight glycemic control.

The patients in the present study were diagnosed with Type 2 DM for a mean of 6±2 years, which was similar to Spyer, et al. and Israelian, et al. (15, 17), but of a shorter duration than other recent studies investigating counterregulatory responses to hypoglycemia in Type 2 DM (12-14, 16). Thus, the present results provides data in a group of more recently diagnosed individuals whom may be predicted to benefit most from rigorous metabolic control. Additionally, it should be noted that the results in this present study are applicable to a group of patients whom underwent a period of significant glycemic control with a specific triple oral therapy and lifestyle change regimen. Thus, we cannot determine if other therapeutic regimens (i.e. with insulin, thiazolidinediones and/or agents that increase GLP-1 axis activity) would have produced similar results. Intensive therapy in this study had marked effects on blunting ANS responses to hypoglycemia. The key counterregulatory hormone epinephrine was reduced by almost 50%. Similarly MSNA was reduced by ~66% and hypoglycemic symptom scores were blunted by ~40%. Additionally, blood pressure and heart rate responses were also significantly blunted by intensive therapy. The above results demonstrate that central sensing of hypoglycemia combined with end-organ responses were both reduced by intensive therapy. The reduced sympathetic nervous system activity also impacted important metabolic counterregulatory mechanisms during hypoglycemia following intensive therapy. Endogenous glucose
production was not measurable and lipolysis (NEFA, glycerol) together with glycogenolysis (lactate) were reduced by \( \geq 30\% \) following intensive therapy. However, it should be noted that with the exception of epinephrine, (which was lower during POST as compared to CONTROL), that intensive therapy reduced the exaggerated counterregulatory responses observed in the PRE patients to the usual physiologic responses observed in the CONTROLS. Additionally, we would like to point out that the above metabolic findings may have been caused by a combination of reduced ANS input and improvement in glucotoxicity/insulin sensitivity (32, 33). We did not identify any effects of intensive therapy to significantly blunt other neuroendocrine responses (glucagon, cortisol, growth hormone, norepinephrine, pancreatic polypeptide). With the exception of cortisol, levels of the above neuroendocrine hormones were not different from age and BMI matched non-diabetic individuals. Spyer, et al. also reported similar findings in a group of Type 2 DM with an HBA1C of 7.4\% (15). However, Israelian, et al. reported that both glucagon and growth hormone responses were blunted during similar hypoglycemic conditions to this study in Type 2 DM subjects. The fact that the blunting effects of intensive therapy on counterregulatory responses in this study were confined almost exclusively to the sympathetic nervous system is different to the situation in Type 1 DM where intensive therapy has been reported to result in a more widespread blunting of neuroendocrine responses during hypoglycemia (34). The reasons for the targeted effects on the sympathetic nervous system are not apparent from the present study. Previous work (13, 14) has determined that the threshold for counterregulatory hormone release in Type 2 DM remain elevated even during relatively good glycemic control (HBA1C 7.4\%). Thus we believe that it is unlikely that the depth of hypoglycemia in our study (3.3 mmol/L) could have mitigated against finding a difference in neuroendocrine responses after intensive therapy. In addition, the magnitude of the neuroendocrine responses was sufficiently robust to have been able to determine a difference if one had been present. However, we cannot exclude the possibility that deeper hypoglycemia may have provoked a more widespread difference in maximal neuroendocrine responses between the pre and post treatment groups (13, 14).

The second part of our study addressed a possible mechanism for our findings of reduced counterregulatory mechanisms during hypoglycemia following intensive therapy. We tested the hypothesis that antecedent hypoglycemia was a mechanism responsible for acquired counterregulatory failure during hypoglycemia in Type 2 DM. Self blood glucose monitoring of 4-6 times per day had demonstrated an aggregate frequency of hypoglycemia of 36–48 episodes per month or up to 3.2 events per patient a month. Over 95\% of these episodes were in the range of 2.8 to 3.9 mmol/L. Although this level of hypoglycemia is often considered “mild” in clinical practice, these present results clearly demonstrate the profound blunting effects of a plasma glucose of 3.3 mmol/L on subsequent physiologic responses to hypoglycemia. Epinephrine and symptom responses were blunt by antecedent hypoglycemia in both pre and post intensive therapy studies. Of note, despite equivalent insulinemia and glycemia, antecedent hypoglycemia blunted the fall of C-peptide during day 2 hypoglycemia in both PRE and POST intensive therapy studies. This obviously has some concern as suppression of endogenous insulin during hypoglycemia is a primary physiologic counterregulatory defense. MSNA was substantially reduced during day 2 hypoglycemia in PRE. However due to the inherent blunting effects of intensive therapy,
MSNA was substantially suppressed during day 1 hypoglycemia in POST and was not further reduced during day 2 hypoglycemia. Cortisol and glucagon responses were significantly reduced by antecedent hypoglycemia in both PRE and POST intensive therapy studies. In addition to the above, growth hormone and pancreatic polypeptide (a marker of parasympathetic activity) were both blunted by antecedent hypoglycemia in non-diabetic control studies. Our present findings extend the elegant studies of Segel, et al. (16) whom also described blunting of ANS and neuroendocrine responses following antecedent hypoglycemia in longer duration, moderately controlled (HBA$_{1C}$ 8.6±1.1%) Type 2 DM patients. The widespread reduction of ANS and neuroendocrine responses following day 1 hypoglycemia also led to significant blunting of metabolic counterregulatory mechanisms (glucose kinetics, lipolysis and glycogenolysis) in both PRE and POST studies. As discussed, Type 2 DM patients typically have higher thresholds (i.e. increased plasma glucose values) for counterregulatory defenses against hypoglycemia (7). This is an important protective mechanism which serves to reduce hypoglycemia in Type 2 DM. The findings of increased MSNA, cortisol and symptom scores coupled with elevated EGP, lipolysis and glycogenolysis in PRE as compared to the non-diabetic controls are consistent with higher plasma glucose thresholds and/or glucotoxicity providing added protection against hypoglycemia in these individuals. Thus, the finding that antecedent hypoglycemia and intensive glucose control reduced counterregulatory defenses (e.g. symptoms, EGP, MSNA) back down to normal control values indicates that the risk for iatrogenic hypoglycemia had been increased as important physiologic protective mechanisms against hypoglycemia had been removed in these Type 2 DM patients.

A limitation of this study was that cognitive function was not formally assessed during our repeat hypoglycemia studies before and after intensive therapy. We did not identify any gross changes in cognitive function within our T2DM or healthy control subjects during any of our hypoglycemia studies. Whether this indicates that cognitive function is relatively preserved during repeated hypoglycemia and intensive therapy or that more sophisticated methodology is needed to detect differences in cognitive function will require further study.

In summary, this present study has demonstrated that 6 months intensive glycemic control with triple oral combination therapy to near normalize HBA$_{1C}$ levels can result in substantial reductions of epinephrine responses during hypoglycemia in individuals with Type 2 DM. In addition, the exaggerated neuroendocrine, ANS and metabolic counterregulatory responses which were present in the PRE intensive therapy group (and thus acting as increased defenses against hypoglycemia) were reduced to levels similar to the non-diabetic CONTROL group. Near normalization of HBA$_{1C}$ resulted in an increased frequency of $\geq 3.0$ hypoglycemic episodes per patient month by study end. Our results clearly demonstrate that antecedent hypoglycemia can also induce autonomic nervous system, neuroendocrine and metabolic counterregulatory failure in Type 2 DM patients with either elevated or near normal glycemic control. Furthermore, the combination of repeated hypoglycemia and intensive glycemic control produces additive effects to further reduce physiologic defenses against subsequent hypoglycemia. Therefore, we would conclude that antecedent hypoglycemia appears to be the likely cause of the blunted sympathoadrenal counterregulatory responses occurring during hypoglycemia following intensive treatment in our Type 2 DM patients. Additionally, even relatively mild hypoglycemia (3.3±0.1
mmol/L) can produce significant blunting of subsequent counterregulatory mechanisms in Type 2 DM and increase the risk for future hypoglycemia. This further reinforces the therapeutic goal of achieving good glycemic control while minimizing the occurrence of any hypoglycemia.

ACKNOWLEDGEMENTS
The authors would like to thank the expert technical assistance of Eric Allen, Pam Venson and Wanda Snead. We also thank the nursing staff of the Vanderbilt CRC, Ms. Caroll Moffat, Ms. Linda Balch and Jerri Brown for superb dietary and diabetes management of our patients. This work was supported in part by NIH grants, R01-DK-069803, MO1-RR-000095, P01-HL-056693 and P60-DK-020593 and an award from the JDRFI/VA.
REFERENCES

1. The Diabetes Control and Complication Trial Research Group (1993). The effect of intensive treatment of diabetes on the development and progression of long term complication in insulin-dependent diabetes mellitus. N. Engl. J. Med. 329, 977-986.
2. United Kingdom Prospective Study Group (1998). Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complication in patients with type 2 diabetes (UKPDS). Lancet 352(9131), 837-853.
3. American Diabetes Association (2008) Standards of medical care in diabetes. Diabetes Care 31: S3-S78.
4. The Diabetes Control and Complications Trial Research Group (1991). Epidemiology of severe hypoglycemia in the diabetes control and complication trial. American Journal of Medicine, 90(40), 450-459.
5. Diabetes Control and Complications Trial (DCCT) Research Group (1997). Hypoglycemia in the diabetes control and complications trial. Diabetes, 46(2), 271-286.
6. Gabriely, I., and Shamoon, H. (2004). Hypoglycemia in diabetes: common, often unrecognized. Cleveland Clinic Journal of Medicine, 71(4), 335-342.
7. Briscoe, V. J., & Davis, S. N. (2006). Hypoglycemia in type 1 and type 2 diabetes: physiology, pathophysiology and management. Clinical diabetes, 24:115-121.
8. Briscoe, V. J., & Davis, S. N. (2008). Hypoglycemia, In Type 1 Diabetes in Adults: Principles and Practice. Edited by Drs. S. Jabbour, E. Stephens, I. Hirsch, B. Goldstein, S. Garg and M. Riddle. Taylor 7 Francis Informa, New York, NY.
9. Gerstein, H. C., Pogue, J., Mann, J. F., Lonn, E., Dagenais, G. R., McQueen, M., Yusuf, S; HOPE investigators. (2005). The relationship between dysglycaemia and cardiovascular and renal risk in diabetic and non-diabetic participants in the HOPE study: a prospective epidemiological analysis. Diabetologia, 48(9): 1749-55.
10. The Action to Control Cardiovascular Risk in Diabetes Study Group. (2008) Effects of intensive glucose lowering in type 2 diabetes. New England Journal of Medicine, 358: 2545-2559.
11. The ADVANCE Collaborative Group. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. New England Journal of Medicine, 358: 2560-2572.
12. Burge, M. R., Sobhy, T. A., Qualls, C. R., and Schade, D. S. (2001). Journal of Clinical Endocrinology & Metabolism, 86(11): 5471-5478.
13. Levy, C. J., Kinsley, B. T., Bajaj, A., & Simonson, D. C. (1998). Effect of glycemic control on glucose counterregulation during hypoglycemia in NIDDM. Diabetes Care, 21(8): 1330-1338.
14. Korzon-Burakowska, A., Hopkins, D., Matyka, K., Lomas, J., Pernet, A., Macdonald, I., and Amiel, S. (1998). Effects of glycemic control on protective responses against hypoglycemia in type 2 diabetes. Diabetes Care, 21(2): 283-290.
15. Spyer, G., Hattersley, a. T., MacDonald, I. A., Amiel, S., and MacLeod, K. M. (2000). Hypoglycaemic counter-regulation at normal blood glucose concentrations in patients with well controlled type-2 diabetes. Lancet, 365(9246): 1970-1974.
16. Segel, S. A., Peramore, D. S., and Cryer, P. E. (2002). Hypoglycemia-associated autonomic failure in advanced type 2 diabetes. Diabetes, 51: 724-733.
17. Israeli, Z., Szoke, E., Woeber, J., Bokhari, S., Shorr, M., Schwenke, D., Cryer, P.E., Gerich, J., Meyer, C. (2006). Multiple defects in counterregulation of hypoglycemia in modestly advanced type 2 diabetes mellitus. *Metabolism, 55: 593-598.*

18. Abumrad, N. N., Rabin, D., Diamond, M. C., and lacy, W. W. ((1981). Use of a heated superficial hand vein as an alternative site for measurement of amino acid concentration and for the study of glucose and alanine kinetics in man. *Metabolism, 30, 936-940.*

19. DeFronzo, R. a., Tobin, K. and Andres, R. (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol 237: E216-E223.*

20. Amiel, S. A., Tambolane, W. V., Simonson, D. C., Sherwin, R. (1987). Defective glucose counterregulation after strict control of insulin-dependent diabetes mellitus. *N Engl J Med, 316: 1376-1383.*

21. Wallin, B. G., Sundlof, G., Eriksson, B. M., Dominiaik, P., Grobecker, H., Lindblad, L. E. (1981). Plasma noradrenaline correlates to sympathetic muscle nerve activity in normotensive man. *Acta Physiol Scand, 111(1): 69-73.*

22. Sandoval, D. A., Ertl, A. C., Richardson, M. A., Tate, D. B., Davis, S. N. (2003). Estrogen blunts neuroendocrine and metabolic responses to hypoglycemia. *Diabetes, 52(7), 1749-1755.*

23. Wall, J. S., Steele, R., Debodo, R. D., and Altszuler, N. (1957). Effect of insulin on utilization and production of circulating glucose. *Am J Physiol, 189: 43-50.*

24. Aguilar-Parada, E., Eisentraut, A. M., and Unger, R. H. (1969). Pancreatic glucagon secretion in normal and diabetic subjects. *Am J Med Sci, 257: 415-419.*

25. Wide, L., and Porath, J. (1966). Radioimmunoassay of proteins with the uses of sephadex-coupled antibodies. *Biochem Biophys Acta, 130: 257-260.*

26. Causon, R., Caruthers, M., Rodnight, R. (1982). Assay of plasma catecholamines by liquid chromatography with electrochemical detection. *Anal Biochem, 116: 223-226.*

27. Hunter, W., and Greenwood, F. (1962). Preparation of [\(^{131}\)I]-labeled human growth hormone of high specific activity. *Nature, 194: 495-496.*

28. Hagopian, W., Lever, E., Cen, D., Emmonoud, D., Polonsky, K., Pugh, W., Moosa, A., Jaspan, J. (1983). Predominance of renal and absence of hepatic metabolism of pancreatic polypeptide in the dog. *Am J Physiol, 245: 171-177.*

29. Lloyd, B., Burrin, J., Smythe, P., Alberti, KGMM. (1978). Enzymatic fluorometric continuous flow assays for blood glucose, lactate, pyruvate, alanine, glycerol and 2-hydroxybutyrate. *Clin Chem 24: 1724-1729.*

30. Ho, R. J. (1970). Radiochemical assay of long chain fatty acids using \(^{63}\)NI as tracer. *Anal Biochem, 26: 105-113.*

31. Deary, L., Hepburn, D., Macleod, K. and Frier, B.M. (1993). Partitioning the symptoms of hypoglycemia using multi-sample confirmatory factor analysis. *Diabetologia, 36: 771-770.*

32. Hawkins, M., Gabriely, L., Wozniak, R., Reddy, K., Rossetti, L., Shamon, H. (2002). Glycemic control determines hepatic and peripheral glucose effectiveness in type 2 diabetic subjects. *Diabetes, 51: 2179-2189.*

33. Donath, M. Y., Schumann, D. M., Faulenback, M., Ellingsgaard, H., Perren, A., Ehsses, J. A. (2008). Islet inflammation in type 2 diabetes: from metabolic stress to therapy. *Diabetes Care, 31(S2), S161-S164.*

34. Davis, M.R., Mellman, M., Shamon, H. Further defects in counterregulatory response induced by recurrent hypoglycemia in IDDM. (1992). *Diabetes, 41: 1335-1340.*
Figure 1
Plasma glucose and insulin levels during repeated 2 day hyperinsulinemic hypoglycemic clamps in overnight fasted individuals with Type 2 DM and healthy controls (CONTROL). Type 2 DM patients are studied before (PRE) and after (POST) six months of intensive triple oral combination anti-diabetes therapy.
Figure 2
Plasma epinephrine, glucagon and cortisol levels during repeated 2 day hyperinsulinemic hypoglycemic clamps in overnight fasted individuals with Type 2 DM and healthy controls. * Plasma epinephrine, glucagon and cortisol levels are significantly reduced (p<0.05) following day 1 hypoglycemia in healthy controls and patients with Type 2 DM both before (PRE) and after (POST) six months intensive triple oral combination anti-diabetes therapy. † Plasma epinephrine levels are significantly reduced (p<0.05) following six months intensive triple oral combination anti-diabetes therapy. ‡ Plasma cortisol levels are significantly reduced (p<0.05) in healthy controls compared to Type 2 DM patients before intensive therapy.

Final 30 minutes of hypoglycemia
Figure 3
Plasma growth hormone and pancreatic polypeptide levels during repeated 2 day hyperinsulinemic hypoglycemic clamps in overnight fasted individuals with Type 2 DM and healthy controls. * Plasma growth hormone and pancreatic polypeptide levels are significantly reduced (p<0.05) following day 1 hypoglycemia in healthy controls.

Final 30 minutes of hypoglycemia
Figure 4
Blood glycerol, lactate and plasma NEFA levels during repeated 2 day hyper insulimetic hypoglycemic clamps in overnight fasted individuals with Type 2 DM and healthy controls. * Blood glycerol, lactate and plasma NEFA levels are significantly reduced (p<0.05) in healthy controls and patients with Type 2 DM both before (PRE) and after (POST) six months intensive triple oral combination anti-diabetes therapy. † Blood glycerol, lactate and plasma NEFA levels are reduced (p<0.05) following six months intensive therapy in Type 2 DM. ‡ Blood glycerol, lactate and plasma NEFA are significantly reduced (p<0.05) in healthy controls compared to Type 2 DM before intensive therapy.

Final 30 minutes of hypoglycemia
Figure 5
Delta responses from baseline in muscle sympathetic nerve Activity (MSNA) and hypoglycemic symptom scores during repeated 2 day hyperinsulinemic hypoglycemic clamps in overnight fasted individuals with Type 2 DM and healthy controls. * Δ MSNA and symptom responses are significantly reduced (p<0.05) in Type 2 DM and healthy controls. † Δ MSNA and symptom responses are significantly reduced (p<0.05) in Type 2 DM following six months intensive triple oral combination anti-diabetic therapy. ‡ Δ MSNA and symptom responses are significantly reduced (p<0.05) in healthy controls as compared to Type 2 DM before intensive therapy (PRE).

Final 30 minutes of hypoglycemia
Table 1. Self-reported blood glucose readings (<3.9 mmol/L) by patients during 6 months intensive treatment period.

| Level of hypoglycemia | Month 1 | Month 2 | Month 3 | Month 4 | Month 5 | Month 6 |
|-----------------------|---------|---------|---------|---------|---------|---------|
| <3.3-3.9 mmol/L       | 12      | 26      | 27      | 33      | 26      | 34      |
| <2.9-3.3 mmol/L       | 5       | 8       | 6       | 6       | 12      | 13      |
| <2.9 mmol/L           | 7       | 3       | 2       | 2       | 1       |         |
| Total episodes recorded| 17      | 41      | 36      | 41      | 40      | 48      |
| Frequency per Patient/month | 1.1 | 2.7 | 2.4 | 2.7 | 2.7 | 3.2 |

All 15 patients recorded episodes of hypoglycemia (<3.9 mmol/L) during the 6 months intensive treatment period.
Table 2. Baseline neuroendocrine hormone levels at the start of day 1 and day 2 hyperinsulinemic hypoglycemic clamps in healthy individuals (CONTROL) and patients with T2DM pre intensive therapy (PRE) and post intensive therapy (POST).

|                        | T2DM PRE-Intensive Therapy | T2DM POST-Intensive Therapy | Healthy Individuals |
|------------------------|-----------------------------|-----------------------------|---------------------|
|                        | Day 1 | Day 2 | Day 1 | Day 2 | Day 1 | Day 2 |
| Epinephrine (pg/ml)    | 278±82 | 180±38 | 202±44 | 218±33 | 223±48 | 184±48 |
| Norepinephrine (pg/ml) | 1.07±0.15 | 1.0±0.14 | 0.9±0.2 | 0.9±0.18 | 0.7±0.2 | 1.17±0.26 |
| Glucagon (ng/L)        | 54±5   | 45±5  | 48±3   | 46±4  | 48±8   | 47±6  |
| Growth Hormone (µg/L)  | 2±1    | 2±0.5 | 1±0.5  | 1.5±0.5 | 1±0.3  | 1±0.2 |
| Cortisol (pmol/L)      | 345±61 | 248±69 | 248±55 | 237±44 | 254±43 | 235±58 |
| Pancreatic Polypeptide (pmol/L) | 48±19 | 31±9  | 30±9  | 29±8  | 28±8  | 25±7  |

Data are mean ± SEM.
**Table 3.** Glucose specific activity (dpm/mmol) during the basal period and the final 30 min of day 1 and day 2 hyperinsulinemic-hypoglycemic clamps in healthy individuals (CONTROL) and patients with T2DM pre intensive therapy (PRE) and post intensive therapy (POST).

|                  | 100 mins | 110 mins | 120 mins | 210 mins | 225 mins | 240 mins |
|------------------|----------|----------|----------|----------|----------|----------|
| Day 1-PRE T2DM   | 286±33   | 283±31   | 279±27   | 310±27   | 314±26   | 306±25   |
| Day 2-PRE T2DM   | 299±40   | 300±35   | 305±36   | 303±21   | 295±21   | 304±22   |
| Day 1-POST T2DM  | 257±51   | 266±51   | 265±50   | 290±41   | 288±43   | 290±42   |
| Day 2-POST T2DM  | 330±27   | 329±26   | 325±26   | 332±20   | 336±20   | 326±20   |
| Day 1-CONTROL    | 373±30   | 374±30   | 374±29   | 306±26   | 320±29   | 312±33   |
| Day 2-CONTROL    | 370±30   | 375±36   | 363±33   | 308±34   | 295±39   | 294±38   |

Data are mean ± SEM.