

# Effects of Differing Durations of Antecedent Hypoglycemia on Counterregulatory Responses to Subsequent Hypoglycemia in Normal Humans

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The aim of this study was to determine whether the duration of antecedent hypoglycemia regulates the magnitude of subsequent counterregulatory failure. A total of 31 lean healthy overnight-fasted individuals (16 men/15 women) were studied. There were 15 subjects (8 men/7 women) who underwent two separate 2-day randomized experiments separated by at least 2 months. On day 1, 2-h hyperinsulinemic ( $9 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) euglycemic ( $5.2 \pm 0.1 \text{ mmol/l}$ ) or hypoglycemic ( $2.9 \pm 0.1 \text{ mmol/l}$ ) glucose clamps (prolonged hypoglycemia) were carried out in the morning and afternoon. Of the other subjects, 16 participated in a 2-day study in which day 1 consisted of morning and afternoon short-duration hypoglycemia experiments (hypoglycemic nadir of  $2.9 \pm 0.1 \text{ mmol}$  for 5 min), and 10 of these individuals underwent an additional 2-day study in which day 1 consisted of morning and afternoon intermediate-duration hypoglycemia (hypoglycemic nadir of  $2.9 \pm 0.1 \text{ mmol}$  for 30 min). The next morning (day 2) all subjects underwent an additional 2-h hyperinsulinemic-hypoglycemic clamp ( $2.9 \pm 0.1 \text{ mmol/l}$ ). The rate of fall of glucose ( $0.07 \text{ mmol/min}$ ) was carefully controlled during all hypoglycemic studies so that the glucose nadir was reached at 30 min. Despite equivalent day 2 plasma glucose and insulin levels, there were significant differences in counterregulatory physiological responses. Steady-state epinephrine, glucagon, growth hormone, cortisol, and pancreatic polypeptide levels were similarly significantly blunted ( $P < 0.01$ ) by the differing duration day 1 hypoglycemia compared with day 1 euglycemia. Muscle sympathetic nerve activity and endogenous glucose production were also similarly blunted ( $P < 0.01$ ) by day 1 hypoglycemia (relative to day 1 euglycemia). Day 2 hypoglycemic symptoms were significantly reduced ( $P < 0.01$ ) after day 1 prolonged intermediate- but not short-duration hypoglycemia. In summary, two episodes of short-duration moderate hypoglycemia can produce significant blunting of key neuroendocrine and metabolic counterregulatory responses.

Hypoglycemic symptom scores are reduced by prolonged but not short-duration prior hypoglycemia. We conclude that in healthy overnight fasted humans, 1) neuroendocrine, autonomic nervous system, and metabolic counterregulatory responses are sensitive to the blunting effects of even short-duration prior hypoglycemia, and 2) the duration of antecedent hypoglycemia results in a hierarchy of blunted physiological responses with hypoglycemic symptom awareness less vulnerable than neuroendocrine responses. *Diabetes* 49:1897–1903, 2000

Numerous studies have demonstrated that antecedent hypoglycemia can blunt subsequent counterregulatory responses to hypoglycemia (1–7). Recent work has focused on determining the in vivo factors and mechanisms responsible for this finding. Depth of antecedent hypoglycemia (8), number of prior episodes of hypoglycemia (2,4,8), and sex (9) can all independently regulate the magnitude of subsequent counterregulatory failure.

To date, the effects of duration of prior hypoglycemia on subsequent counterregulatory failure have not been defined. We have previously demonstrated that 2-h episodes of hypoglycemia in the morning and afternoon can result in substantial blunting of counterregulatory responses during next-day hypoglycemia (8). Heller and Cryer (4) have demonstrated a similar magnitude of counterregulatory failure after hypoglycemia of 30 min in the morning and 2 h in the afternoon. On the other hand, Widom and Simonson (2) have demonstrated that four episodes of only 60 min of moderate hypoglycemia on consecutive days could diminish neuroendocrine responses to subsequent hypoglycemia. Thus, previous studies have used experimental designs incorporating differing durations of antecedent hypoglycemia ranging from 30 to 120 min. A relevant clinical question, therefore, arises as to how short a duration of antecedent hypoglycemia is necessary to blunt subsequent neuroendocrine responses. Many patients with diabetes have early cues that they are hypoglycemic but often choose to delay immediate remedial action. This hesitancy to self-treat prolongs duration and possibly depth of hypoglycemia. However, it is unknown whether duration of prior hypoglycemia can independently regulate the magnitude of subsequent counterregulatory failure. Therefore, the specific aim of the present study was to test the hypothesis that two episodes of short-duration moderate hypoglycemia can result in blunted counterregulatory responses to subsequent hypoglycemia in

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CRC, Clinical Research Center; CV, coefficient of variation; EGP, endogenous glucose production; HPLC, high-pressure liquid chromatography; MSNA, muscle sympathetic nerve activity; NEFA, nonesterified fatty acid;  $R_a$ , glucose appearance;  $R_d$ , glucose utilization; RIA, radioimmunoassay; SNS, sympathetic nervous system.

normal humans. The glucose clamp technique was used to control glucose and insulin levels. Neuroendocrine, metabolic, hypoglycemic symptom, autonomic nervous system, and cardiovascular responses were measured to provide an integrated evaluation of in vivo counterregulatory physiology. This experimental design also permits identification of the elements of the counterregulatory hierarchy most vulnerable to the effects of prior short-duration hypoglycemia.

## RESEARCH DESIGN AND METHODS

**Subjects.** We studied 31 healthy subjects (16 men/15 women), age  $27 \pm 2$  years, BMI  $22.7 \pm 0.5$  kg/m<sup>2</sup>, and HbA<sub>1c</sub>  $5.1 \pm 0.1\%$  (normal range 4–6.5%). None were taking medication or had a family history of diabetes. Each subject had normal blood count, plasma electrolytes, and liver and renal function. All gave written informed consent. Studies were approved by the Vanderbilt University Human Subjects Institutional Review Board. The subjects were asked to follow their usual weight-maintaining diet for 3 days before each study. Each subject was admitted to the Vanderbilt Clinical Research Center (CRC) at 5:00 P.M. on the evening before an experiment. All subjects were studied after an overnight 10-h fast. Fifteen of the subjects in the present study also participated in related experiments investigating the effects of prolonged (2-h) hypoglycemia on counterregulatory responses to subsequent hypoglycemia (9,10).

### Experimental design

**Glucose clamp studies.** Fifteen subjects had previously participated in two 2-day randomized experiments (antecedent euglycemia and antecedent prolonged hypoglycemia [9,10]). Sixteen other subjects participated in the 2-day studies investigating the effects of day 1 short-duration hypoglycemia. Of these additional individuals, 10 of 16 also participated in a 2-day study investigating the effects of day 1 intermediate-duration hypoglycemia. Subjects were not informed of the protocol in which they participated. (Single blinding of the experiments was not possible because all subjects would experience hypoglycemic symptoms and could therefore deduce the duration of the hypoglycemia experiment in which they were participating.) Studies in women were performed in the follicular phase of a menstrual cycle. On the morning of each study after an overnight fast, 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 was placed in a heated box (55–60%) so that arterialized blood could be obtained (11). The other cannula was placed in the contralateral arm so that 20% glucose could be infused via a variable rate volumetric infusion pump (Imed, San Diego, CA).

**Antecedent euglycemia experiments.** On the morning of day 1 after the insertion of venous cannulae, a period of 90 min was allowed to elapse followed by a 30-min basal period and a 120-min hyperinsulinemic-euglycemic experimental period. At time zero, a primed continuous infusion of insulin (12) was administered at a rate of  $9 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 120 min. Plasma glucose levels were measured every 5 min, and a variable infusion of 20% dextrose was adjusted so that plasma glucose levels were held constant (13). Potassium chloride (20 mmol/l) was added to the glucose infusate in each study. After completion of the initial 2-h test period, the insulin infusion was discontinued and plasma glucose was maintained at euglycemia for 2 h. At that point, insulin was restarted, and a second 2-h hyperinsulinemic-euglycemic clamp identical to the morning's study was performed. At completion of the second glucose clamp, subjects consumed a large meal, consumed a bedtime snack, and remained in the CRC (Fig. 1).

**Antecedent hypoglycemia experiments.** These experiments followed a similar format to previously described euglycemia experiments. Short-duration, intermediate-duration, or prolonged hypoglycemia was induced during identical 2-h morning and afternoon hyperinsulinemic glucose clamp experiments. During short-duration hypoglycemia, plasma glucose was allowed to fall from baseline to  $\sim 2.9$  mmol/l over a period of 30 min. Plasma glucose was maintained at  $\sim 2.9$  mmol/l for 5 min and then was rapidly restored to and maintained at euglycemia for the remainder of the 2-h experiment by the use of an exogenous 20% glucose infusion. In the intermediate-duration hypoglycemia studies, plasma glucose was allowed to fall from baseline to 2.9 mmol/l over a period of 30 min, maintained at this level for a further 30 min, and then rapidly restored to euglycemia for the remainder of the 2-h study. During the prolonged hypoglycemia studies, plasma glucose fell to 2.9 mmol/l over a period of 30 min and was maintained at this level for 90 min. Plasma glucose was maintained at euglycemia for the 2 h between all morning and afternoon hypoglycemic clamp experiments (Fig. 1).

**Day 2 experiments.** Day 2 experiments involved a single 2-h hypoglycemic clamp at  $2.9 \pm 0.1$  mmol/l to assess the effects of differing day 1 glycemia on subsequent hypoglycemic counterregulatory responses. Studies were performed after a 10-h overnight fast. To measure glucose kinetics, a primed (18  $\mu\text{Ci}$ ) constant infusion (0.18  $\mu\text{Ci}/\text{min}$ ) of [<sup>3</sup>H]glucose was started at  $-120$  min and continued throughout the 240-min experiment. At time zero, a primed con-

stant infusion of insulin at a rate of  $9 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  was started. The rate of fall of plasma glucose was equated in all studies by adjusting an exogenous infusion of glucose via a feedback algorithm based on 5-min bedside glucose values. The exogenous glucose infusion was adjusted to ensure that plasma glucose fell at a rate of  $0.35 \pm 0.01$  mmol/l every 5 min. The hypoglycemic plateau ( $\sim 2.9$  mmol/l) was controlled by a modification of the glucose clamp technique (14).

Rates of glucose appearance ( $R_a$ ), endogenous glucose production (EGP), and glucose utilization ( $R_d$ ) were calculated during day 2 studies according to the methods of Wall et al. (15). EGP was calculated by determining the total rate of  $R_a$  (which comprises both endogenous glucose production and exogenous glucose infused to maintain the desired hypoglycemia) and subtracting from it the amount of glucose infused. It is now recognized that this approach is not fully quantitative because underestimates of total  $R_a$  and  $R_d$  can be obtained. The use of a highly purified tracer and the taking of measurements under steady-state conditions (i.e., constant specific activity) in the presence of a low glucose flux minimizes the major problems.

**Direct measurement of muscle sympathetic nerve activity.** Microneurographic activity was recorded from the peroneal nerve at the level of the fibular head (16). The approximate location of this nerve was determined by transdermal electrical stimulation (10–60 V, 0.01-ms duration). This stimulation produced painless muscle contraction of the foot. Next, a reference tungsten electrode with a shaft diameter of 200  $\mu\text{m}$  was placed subcutaneously. A similar electrode, with an insulated tip (1–5  $\mu\text{m}$ ), was inserted into the nerve and used for recording muscle sympathetic nerve activity (MSNA). A recording of MSNA was considered adequate when 1) electrical stimulation produced muscle twitches but not paresthesia; 2) stretching of the tendons in the foot evoked proprioceptive afferent signals, whereas cutaneous stimulation by slight stroking of the skin did not; 3) nerve activity increased during phase II of the Valsalva maneuver (hypotensive phase) and was suppressed during phase IV (blood pressure overshoot); and 4) nerve activity increased in response to held expiration.

Two types of sympathetic fibers (skin and muscle) can be identified from recordings of peripheral nerves. MSNA was recorded in the present studies because this has been demonstrated to reflect increased sympathetic activity during insulin-induced hypoglycemia (17), 2-deoxyglucose-induced neuroglycopenia (18), and hyperinsulinemic euglycemia in normal humans (19).

Sympathetic nerve activity is expressed as bursts per minute. Measurements of MSNA were made from the original tracings using a digitizer tablet (HIPAD; Houston Instruments, Austin, TX) coupled with Sigma Scan Software (Jandel Scientific, Coite Modena, CA) in a microcomputer.

**Analytical methods.** The collection and processing of blood samples have been described elsewhere (20). Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Glucagon was measured according to the method of Aguilar-Parada et al. (21) with an interassay coefficient of variation (CV) of 15%. Insulin was measured as described previously (22) with an interassay CV of 11%. Catecholamines were determined by high-pressure liquid chromatography (HPLC) (23) with an interassay CV of 12% for epinephrine and 12% for norepinephrine. We made two modifications to the procedure for catecholamine determination: we used a five-point rather than one-point standard calibration curve, and we spiked the initial and final samples of plasma with known amounts of epinephrine and norepinephrine so that accurate identification of the relevant respective catecholamine peaks could be made. Cortisol was assayed by using the Clinical Assays Gamma Coat radioimmunoassay (RIA) kit with an interassay CV of 6%. Growth hormone was determined by RIA (24) with a CV of 8%. Pancreatic polypeptide was measured by RIA using the method of Hagopian et al. (25) with an interassay CV of 8%. Lactate, glycerol, alanine, and 3-hydroxybutyrate were measured on deproteinized whole blood using the method of Lloyd et al. (26). Nonesterified fatty acids (NEFAs) were measured using the WAKO kit adopted for use on a centrifugal analyzer (27).

Blood samples for glucose flux were taken every 10 min throughout the control period and every 15 min during the experimental period. 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 noninvasively by a Dinamap (Critikon, Tampa, FL) every 10 min throughout each 300-min study. MSNA was measured continuously throughout each 300-min study. Hypoglycemic symptoms were quantified using a previously validated semiquantitative questionnaire (28). Each patient and healthy control were asked to rate his or her experience of the symptoms twice during the control period and every 15 min during the experimental period. Symptoms measured included fatigue, confusion, hunger, dizziness, difficulty thinking, blurred vision, sweating, tremors, agitation, heat, thirst, and pounding heart. The ratings of the first six symptoms were summed to get a neuro-

glycopenic score, whereas the ratings from the last five symptoms provided an autonomic symptom score.

**Materials.** HPLC-purified [ $^3\text{H}$ ]glucose (New England Nuclear, Boston, MA) was used as the glucose tracer ( $11.5 \text{ mCi} \cdot \text{mmol}^{-1} \cdot \text{l}^{-1}$ ). Human regular insulin was purchased from Eli Lilly (Indianapolis, IN). The insulin infusion solution was prepared with normal saline and contained 3% (vol/vol) of the subjects' own plasma.

**Statistical analysis.** Data are expressed as means  $\pm$  SE unless otherwise stated. Between-group analysis was analyzed using standard parametric 2-way analysis of variance with a repeated-measures design. This was coupled with Duncan's post hoc test to delineate at which time statistical significance was reached. A value of  $P < 0.05$  indicated significant difference.

## RESULTS

**Insulin and glucose levels from day 1 studies.** Steady-state insulin levels were similar during morning and afternoon protocols ( $558 \pm 54$ ,  $546 \pm 48$ ,  $552 \pm 56$ , and  $534 \pm 54 \text{ pmol/l}$ ) for antecedent euglycemia, prolonged prior hypoglycemia, intermediate-duration hypoglycemia, and short-duration prior hypoglycemia, respectively (Fig. 1). Plasma glucose levels were maintained at  $5.2 \pm 0.1 \text{ mmol/l}$  during day 1 euglycemia. The rate of fall of plasma glucose was equated during all hypoglycemia protocols. During morning and afternoon prolonged day 1 hypoglycemia, the hypoglycemic plateau of  $2.9 \text{ mmol/l}$  was reached after 30 min and maintained at this level for the remainder of the 120-min experiments. During intermediate-duration prior hypoglycemia, the hypoglycemic plateau of  $2.9 \text{ mmol/l}$  was reached after 30 min and maintained at this level for a further 30 min, and then plasma glucose was rapidly restored to euglycemia for the remainder of the 120-min experiment. During short-duration prior hypoglycemia, the fall in plasma glucose levels were  $3.9 \pm 0.1 \text{ mmol/l}$  at 20 min,  $2.9 \text{ mmol/l}$  for 5 min at 30–35 min, and then rapidly restored to euglycemia. Therefore, plasma glucose levels were  $<3.9 \text{ mmol/l}$  for 25 min in the morning and an identical duration in the afternoon.

**Insulin, glucose, and neuroendocrine values from day 2 morning studies.** Insulin infusions resulted in equivalent steady-state levels by 30 min in all groups ( $528$ – $546 \pm 54 \text{ pmol/l}$ ).

Plasma glucose fell at an equivalent rate ( $0.07 \text{ mol/min}$ ) and reached a similar steady-state hypoglycemic plateau of  $2.9 \pm 0.1 \text{ mmol/l}$  (Fig. 1). Despite equivalent hypoglycemia and insulinemia, there were large group differences in neuroendocrine responses. After day 1 euglycemia, arterial plasma epinephrine (Fig. 2) increased from  $0.2 \pm 0.01$  to  $4.0 \pm 0.5 \text{ nmol/l}$  during the final 30 min of hypoglycemia. Epinephrine levels were similarly significantly blunted ( $P < 0.01$ ) after short-duration, intermediate-duration, and prolonged day 1 hypoglycemia ( $2.6 \pm 0.4$ ,  $2.7 \pm 0.4$ , and  $2.4 \pm 0.3 \text{ nmol/l}$ ) compared with day 1 euglycemia, respectively. Norepinephrine levels increased to similar values in all groups during day 2 hypoglycemia. However, because of baseline differences, the incremental responses were similarly significantly blunted ( $P < 0.05$ ) after both short-duration, intermediate-duration, or prolonged hypoglycemia compared with day 1 euglycemia (Fig. 2). Glucagon responses were also significantly blunted ( $P < 0.01$ ) by all differing-duration day 1 hypoglycemia (Fig. 3). Glucagon levels after short-duration, intermediate-duration, and prolonged hypoglycemia increased to  $138 \pm 8$ ,  $140 \pm 12$ , and  $141 \pm 18 \text{ ng/l}$ , respectively. These values were considerably reduced compared with responses after euglycemia ( $262 \pm 34 \text{ ng/l}$ ). Plasma cortisol responses were reduced in a stepwise fashion by differing-duration day 1 hypoglycemia (Fig. 3). Cortisol levels increased to  $745 \pm 55 \text{ nmol/l}$  after euglycemia but only  $580 \pm 55$ ,  $611 \pm 75$ , and  $497 \pm 55 \text{ nmol/l}$  after antecedent short-duration, intermediate-duration, or prolonged hypoglycemia, respectively. Growth hormone levels (Fig. 3) increased to  $47 \pm 9 \text{ } \mu\text{g/ml}$  after day 1 euglycemia and were significantly increased ( $P < 0.01$ ) compared with values after short-duration ( $27 \pm 3 \text{ } \mu\text{g/ml}$ ), intermediate-duration ( $24 \pm 3$ ), and prolonged day 1 hypoglycemia ( $29 \pm 4 \text{ } \mu\text{g/ml}$ ). Pancreatic polypeptide responses (Fig. 2) followed a similar pattern to the above counterregulatory hormones. Day 1 short-duration ( $193 \pm 22 \text{ pmol/l}$ ), intermediate-duration ( $172 \pm 26 \text{ pmol/l}$ ), and prolonged hypoglycemia ( $203 \pm 28 \text{ pmol/l}$ ) produced

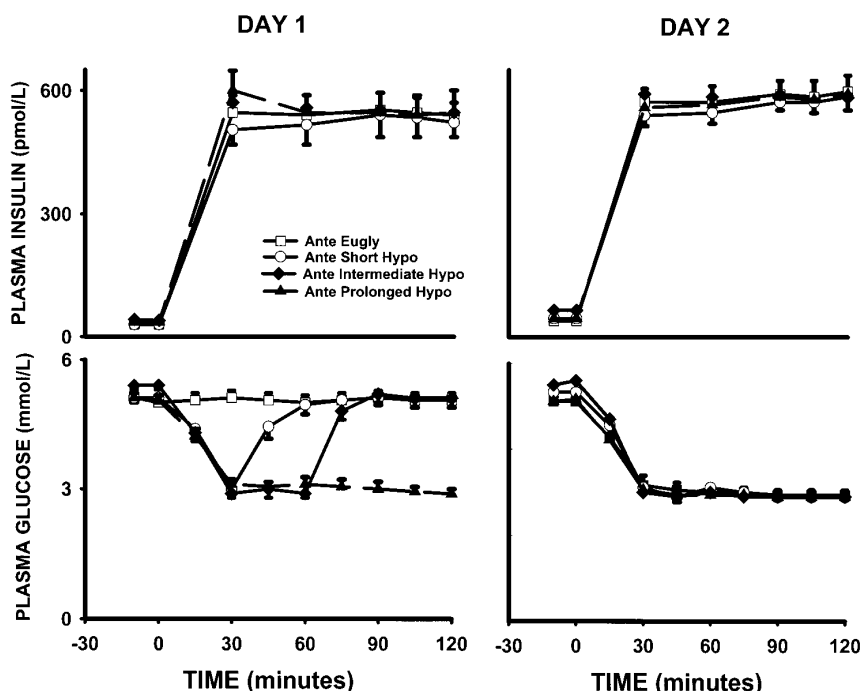


FIG. 1. Plasma insulin and glucose levels during day 1 and day 2 morning euglycemic and hypoglycemic clamp studies. Ante, antecedent; Eugly, euglycemia; Hypo, hypoglycemia.

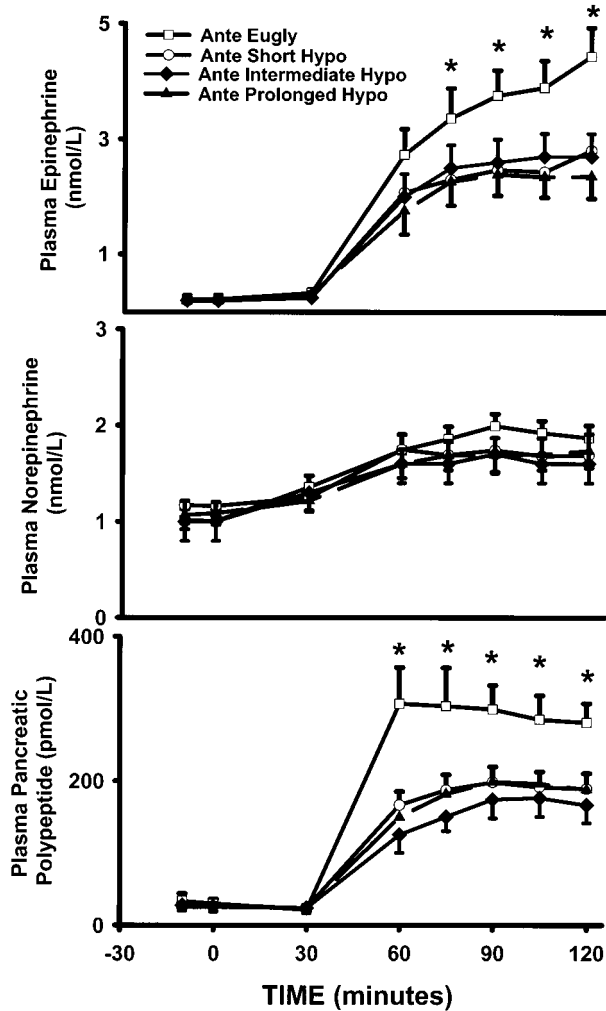


FIG. 2. Effects of day 1 hyperinsulinemic euglycemia and differing durations of hyperinsulinemic hypoglycemia on plasma epinephrine, norepinephrine, and pancreatic polypeptide responses during day 2 hyperinsulinemic hypoglycemia ( $2.9 \pm 0.1$  mmol/l) in overnight fasted healthy individuals. \*Plasma epinephrine and pancreatic polypeptide responses are significantly ( $P < 0.01$ ) reduced after day 1 short-duration, intermediate-duration, and prolonged hypoglycemia compared with day 1 euglycemia. Incremental increases in plasma norepinephrine after day 1 euglycemia are increased ( $P < 0.05$ ) compared with day 1 short-duration, intermediate-duration, and prolonged hypoglycemia. Ante, antecedent; Eugly, euglycemia; Hypo, hypoglycemia.

significant blunting ( $P < 0.01$ ) in pancreatic polypeptide levels compared with antecedent euglycemia ( $288 \pm 29$  pmol/l). **MSNA.** Incremental responses in MSNA were significantly blunted ( $P < 0.01$ ) by all day 1 hypoglycemia (Fig. 4). After day 1 euglycemia, MSNA increased by  $12 \pm 2$  bursts/min ( $27 \pm 4$  to  $39 \pm 3$  bursts/min). After day 1 short-duration, intermediate-duration, or prolonged hypoglycemia, MSNA responses were similarly attenuated at  $5 \pm 2$  bursts/min ( $33 \pm 4$  to  $38 \pm 3$ ),  $6 \pm 2$  bursts/min ( $26 \pm 3$  to  $32 \pm 3$ ), and  $5 \pm 1$  bursts/min ( $26 \pm 6$  to  $31 \pm 4$ ), respectively.

**Glucose kinetics.** Glucose specific activity (disintegrations per minute per millimole) was in a steady state during the control period and final 30 min of each day 2 experimental group (CV all  $\leq 2.3\%$ ). EGP remained similar to baseline after day 1 euglycemia ( $11.0 \pm 0.6$  to  $9.4 \pm 1.1$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). EGP was similarly significantly blunted ( $P < 0.01$ ) after short-duration

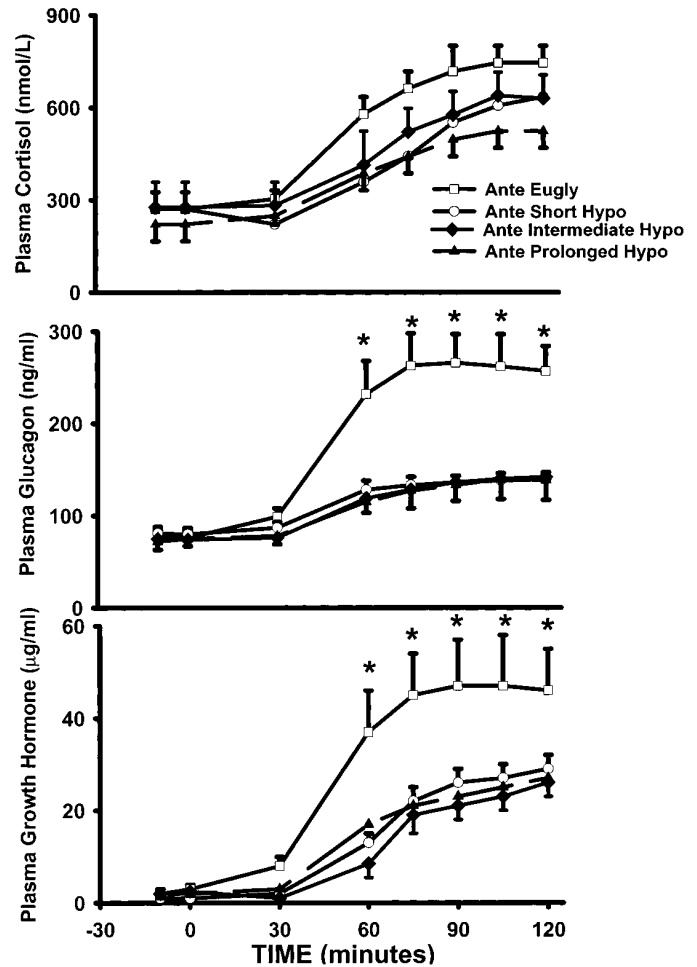
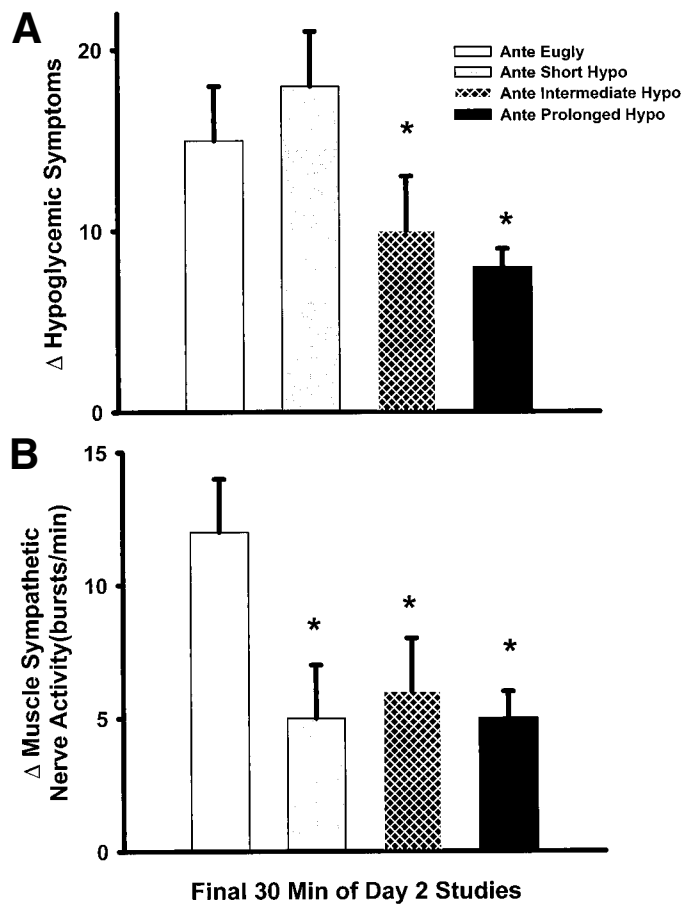


FIG. 3. Effects of day 1 hyperinsulinemic euglycemia and differing durations of hyperinsulinemic hypoglycemia on plasma cortisol, glucagon, and growth hormone responses during day 2 hyperinsulinemic hypoglycemia ( $2.9 \pm 0.1$  mmol/l) in overnight fasted healthy individuals. \*Plasma glucagon and growth hormone responses are significantly ( $P < 0.05$  to  $P < 0.01$ ) reduced after day 1 short-duration, intermediate-duration, and prolonged hypoglycemia compared with day 1 euglycemia. Plasma cortisol is significantly reduced by day 1 prolonged hypoglycemia compared with day 1 euglycemia. Ante, antecedent; Eugly, euglycemia; Hypo, hypoglycemia.

( $11.0 \pm 0.6$  to  $3.9 \pm 1.6$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), intermediate-duration ( $10.5 \pm 1.1$  to  $4.4 \pm 2.2$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), and prolonged day 1 hypoglycemia ( $11.6 \pm 0.6$  to  $5.5 \pm 1.1$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) compared with antecedent euglycemia. Glucose infusion rates used to maintain day 2 hypoglycemia were significantly increased ( $P < 0.01$ ) after short-duration ( $9.7 \pm 2.3$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), intermediate-duration ( $6.6 \pm 2.2$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), or prolonged antecedent hypoglycemia ( $7.0 \pm 2.5$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) compared with antecedent euglycemia ( $2.3 \pm 0.7$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). Rates of glucose utilization were similar in each group during the final 30 min of day 2 hypoglycemia ( $11.6 \pm 1.1$  to  $13.6 \pm 1.6$   $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ).

**Intermediary metabolism during day 2 hypoglycemia.** Blood glycerol levels remained similar to baseline after day 1 euglycemia ( $45 \pm 6$  to  $45 \pm 9$   $\mu\text{mol/l}$ ). Glycerol levels were significantly and similarly blunted ( $P < 0.01$ ) after short-duration ( $54 \pm 4$  to  $37 \pm 4$   $\mu\text{mol/l}$ ), intermediate-duration ( $50 \pm 5$  to  $38 \pm 4$   $\mu\text{mol/l}$ ), or prolonged hypoglycemia ( $41 \pm 3$  to  $29 \pm 3$   $\mu\text{mol/l}$ ).



**FIG. 4.** Effects of day 1 hyperinsulinemic euglycemia and differing durations of hyperinsulinemic hypoglycemia on incremental responses of hypoglycemic symptoms (A) and muscle sympathetic nerve activity (B). \*Day 2 increment in hypoglycemic symptoms are significantly reduced ( $P < 0.01$ ) after day 1 intermediate-duration and prolonged hypoglycemia compared with day 1 euglycemia or short-duration hypoglycemia. Day 2 incremental responses in muscle sympathetic nerve activity are significantly reduced ( $P < 0.01$ ) after day 1 short-duration, intermediate-duration, and prolonged hypoglycemia compared with day 1 euglycemia. Ante, antecedent; Eugly, euglycemia; Hypo, hypoglycemia.

Blood lactate levels increased similarly after day 1 euglycemia ( $561 \pm 85 \mu\text{mol/l}$ ) and short-duration ( $651 \pm 92 \mu\text{mol/l}$ ), intermediate-duration ( $705 \pm 110 \mu\text{mol/l}$ ), or prolonged hypoglycemia ( $519 \pm 85 \mu\text{mol/l}$ ). Blood  $\beta$ -hydroxybutyrate levels were suppressed similarly after day 1 euglycemia ( $32 \pm 8$  to  $10 \pm 3 \mu\text{mol/l}$ ), short-duration ( $37 \pm 6$  to  $8 \pm 2 \mu\text{mol/l}$ ), intermediate-duration ( $47 \pm 18$  to  $11 \pm 4 \mu\text{mol/l}$ ), or prolonged hypoglycemia ( $35 \pm 6$  to  $8 \pm 2 \mu\text{mol/l}$ ). Day 2 suppression of NEFA levels from baseline was also similar after day 1 euglycemia or differing duration hypoglycemia.

**Day 2 hypoglycemic symptoms.** Total hypoglycemic symptom scores increased  $15 \pm 3$  (autonomic  $9 \pm 2$ , neuroglycopenic  $6 \pm 1$ ) after day 1 euglycemia. Increases in hypoglycemic symptoms were unaffected by day 1 short-duration hypoglycemia ( $18 \pm 3$ ; autonomic  $10 \pm 2$ , neuroglycopenic  $8 \pm 1$ ). Hypoglycemic symptom scores, particularly autonomic symptoms, were significantly suppressed ( $P < 0.05$  to  $P < 0.01$ ) by intermediate-duration ( $10 \pm 3$ ; autonomic  $5 \pm 2$ , neuroglycopenic  $5 \pm 1$ ) and prolonged ( $8 \pm 1$ ; neuroglycopenic  $4 \pm 1$ , autonomic symptoms  $4 \pm 1$ ) hypoglycemia.

**Day 2 cardiovascular parameters.** Cardiovascular responses during day 2 were unaffected by day 1 euglycemia or differing-duration hypoglycemia. Heart rate increased  $10 \pm 1$ ,  $9 \pm 1$ ,  $9 \pm 1$ , and  $10 \pm 1$  beats/min after day 1 euglycemia and long-duration, intermediate-duration, and short-duration hypoglycemia, respectively. Mean arterial pressure changed minimally during all day 2 studies (day 1 euglycemia  $-1 \pm 1$  mmHg, prolonged hypoglycemia  $-2 \pm 1$  mmHg, intermediate-duration hypoglycemia  $-1 \pm 1$  mmHg, and short-duration hypoglycemia  $-3 \pm 2$  mmHg).

## DISCUSSION

This present study has examined the effects of 2 episodes of differing-duration hypoglycemia on counterregulatory responses to subsequent hypoglycemia. Our results demonstrate that short durations of hypoglycemia (5 min at a plasma glucose of  $2.9 \pm 0.1$  mmol and 20 min of lowering and raising glucose levels between 3.9 and 2.9 mmol/l) in the morning and afternoon produce a similar magnitude of blunted neuroendocrine, glucose kinetic, and muscle sympathetic nerve activity responses compared with two episodes of 30 or 90 min of moderate hypoglycemia ( $2.9 \pm 0.1$  mmol/l). Hypoglycemic symptoms (principally autonomic symptoms) were significantly blunted by prolonged and intermediate-duration hypoglycemia but were unaffected by prior short-duration hypoglycemia.

Antecedent hypoglycemia can produce blunted counterregulatory responses to subsequent hypoglycemia (1–8). Recent studies have focussed on elucidating the in vivo mechanisms responsible for this finding (8,9,29–31). To date, depth of prior hypoglycemia (8), sex (9), increased plasma cortisol (29), lactate (30), or ketone body levels (30) and elevated cerebral glucose extraction (31) have all been identified as independent factors regulating the magnitude of hypoglycemia-associated counterregulatory failure. The role played by duration of prior hypoglycemia in causing subsequent counterregulatory failure is unknown. In the present study, we have addressed this question by determining if two episodes of very brief (5-min) moderate hypoglycemia ( $2.9 \pm 0.1$  mmol) could blunt subsequent counterregulatory responses. The target glycemia level of 2.9 mmol/l was chosen because, in previous studies, we have demonstrated that prolonged (90-min) antecedent hypoglycemia of 3.9 or 3.3 mmol/l in women had virtually no effect on blunting counterregulatory responses (9). In fact, in healthy women, antecedent hypoglycemia of 2.9 mmol/l was required to produce significant blunting of subsequent counterregulatory responses. Antecedent hypoglycemic periods of 5 min were chosen because this was the shortest period of time that we could reliably ascertain (i.e., triplicate glucose readings) that the glycemic nadir had been obtained. Although the desired target of 5 min at 2.9 mmol/l was obtained in all day 1 morning and afternoon studies, it should be noted that the duration of hypoglycemia (defined as glycemia  $< 3.9$  mmol/l) was  $\sim 25$  min during each glucose clamp. This duration of hypoglycemia consisted of three elements. The first element consisted of the time taken for plasma glucose to drop from 3.9 to 2.9 mmol/l ( $\sim 10$  min). The second element consisted of 5 min at 2.9 mmol/l. The third element consisted of the time taken to elevate plasma glucose from 2.9 to  $> 3.9$  mmol/l ( $\sim 10$  min). It is relevant to account for all hypoglycemia below a plasma glucose value of 3.9 mmol/l because we have previously

demonstrated that prolonged antecedent hypoglycemia of 3.9 and 3.3 mmol/l in healthy men can significantly blunt subsequent counterregulatory responses.

The number of episodes of prior hypoglycemia required to produce significant blunting of next-day counterregulatory responses is controversial. Heller and Cryer (4) demonstrated that one episode of prior hypoglycemia produces no blunting of counterregulatory responses to next-day hypoglycemia. On the other hand, Veneman et al. (5) reported that one episode of prolonged nocturnal hypoglycemia can produce significant blunting of next-day counterregulatory responses. We should point out that the experimental approach in this study uses a model of two episodes of prior hypoglycemia on day 1. Therefore, it should be emphasized that the results of this study relate to two rather than one episode of prior hypoglycemia.

Our present results demonstrate that two episodes of prolonged, intermediate-duration, or short-duration antecedent hypoglycemia produce similar blunting of day 2 neuroendocrine responses. The reduction in day 2 neuroendocrine responses was homogeneous and existed across the spectrum of all major counterregulatory hormones. This appears to rule out the possibility that short-duration antecedent hypoglycemia could selectively blunt some but not all neuroendocrine responses. MSNA was also equivalently blunted by antecedent short-duration or prolonged hypoglycemia. The MSNA responses underscore the sensitivity of the sympathetic nervous system (SNS) to the deleterious effects of even minimal prior hypoglycemia. Key metabolic counterregulatory responses (EGP and lipolysis) were also reduced commensurately with diminished neuroendocrine and SNS activation and are also therefore significantly blunted by short-duration antecedent hypoglycemia. Interestingly, glucose utilization, another important metabolic counterregulatory mechanism, was unaffected by prior hypoglycemia of any duration. We believe the explanation for this finding is that epinephrine's maximal effect to antagonize insulin-stimulated glucose uptake occurs at relatively modest concentrations of the catecholamine (~1.5 nmol/l [32]). In the present study, even the day 2 blunted levels of epinephrine after the differing prior hypoglycemia exceeded this ceiling, and thus the major mechanism for limiting glucose utilization was able to function at full capacity.

The second significant finding from this study is the determination that hypoglycemic symptoms were blunted by prior prolonged and intermediate-duration but not short-duration hypoglycemia. This finding is discordant with neuroendocrine and SNS responses and appears to indicate that the duration of antecedent hypoglycemia can produce a hierarchy of blunting effects on physiological defenses against subsequent hypoglycemia. Neuroendocrine and SNS responses (in men) are exquisitely sensitive to even modest prior episodes of hypoglycemia (8). Hypoglycemic symptoms, however fundamental in the defense against hypoglycemia, were preserved after short-duration hypoglycemia. These present findings may have mechanistic implications in the development of hypoglycemia-associated autonomic failure (33). Consistent with previous work (34), our present results confirm the multifactorial nature of the pathogenesis of the syndromes of counterregulatory failure of which hypoglycemic symptom unawareness is an important component. Although there is a consensus that neuroendocrine responses are blunted by

prior hypoglycemia (1–8), there is considerable debate whether cognitive function is also similarly affected (5,6,35–37). Several studies have reported discordant effects of blunted neuroendocrine responses but preserved cognitive testing during subsequent hypoglycemia (6,37). Furthermore, data concerning the effects of prior hypoglycemia on hypoglycemic symptoms in type 1 patients are also conflicting (33,38,39). Studies have reported that prior hypoglycemia had either no effect on thresholds for hypoglycemic symptoms (34,38) or significantly reduced the glucose level necessary to provoke symptoms (33,38). In addition, Dagogo-Jack et al. (40) have demonstrated that a period of scrupulous avoidance of hypoglycemia in type 1 patients results in a return of hypoglycemic symptoms but not neuroendocrine responses to subsequent hypoglycemia. Taken together, the above studies indicate that prior hypoglycemia can produce differential (hierarchical) responses in physiological defenses against subsequent hypoglycemia. This present study extends the concept further by presenting evidence of compartmentalization of ANS responses after antecedent hypoglycemia. ANS neuroendocrine markers (epinephrine, norepinephrine, and pancreatic polypeptide) and MSNA were concordant and exhibited significant blunting after short-duration or prolonged hypoglycemia. Cardiovascular responses, on the other hand, were unaffected by any prior hypoglycemia, whereas hypoglycemic symptoms were blunted by intermediate-duration or prolonged but not short-duration hypoglycemia. The finding that prior hypoglycemia can blunt some but not all ANS responses to subsequent hypoglycemia has also recently been observed by Paramore et al. (41).

To date, prior elevations of cortisol (29) increased cerebral blood glucose uptake (31), and increases in alternative cerebral metabolic fuels (lactate and ketone bodies [30]) have been identified as potential mechanisms responsible for hypoglycemia-associated counterregulatory failure. It would appear that any mechanism(s) responsible for the syndrome of hypoglycemia-associated autonomic failure must act at either multiple sites or one dominant coordinating center within the brain. Borg et al. (42) demonstrated that destruction of, or local neuroglycopenia in, the ventral medial nucleus of the hypothalamus can significantly regulate neuroendocrine responses to hypoglycemia in rats. Other studies have demonstrated the importance of hindbrain catecholaminergic cells in the ANS responses to stress (43). Thus, if there is one unifying mechanism responsible for the syndromes of hypoglycemia-associated autonomic failure (e.g., cortisol), then the present results imply that the duration of, and perhaps levels obtained, of the putative agent is important to the qualitative as well as quantitative blunting of physiological defenses against subsequent hypoglycemia.

Caution always needs to be applied when extrapolating results from normal humans to patients with diabetes. However, these present findings appear to suggest that advocating clinical paradigms limiting the duration of hypoglycemia in diabetic patients may have therapeutic usefulness and could help preserve symptomatic defenses against hypoglycemia even if neuroendocrine responses are blunted.

We conclude that in healthy overnight fasted humans, 1) neuroendocrine, autonomic nervous system, and metabolic counterregulatory responses are sensitive to the blunting effects of even short-duration prior hypoglycemia, and 2) the duration of antecedent hypoglycemia results in

a hierarchy of blunted physiological responses, with hypoglycemic symptom awareness less vulnerable than neuroendocrine responses.

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