Effects of Antecedent $GABA_A$ Activation With Alprazolam on Counterregulatory Responses to Hypoglycemia in Healthy Humans

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OBJECTIVE—To date, there are no data investigating the effects of $GABA_A$ activation on counterregulatory responses during repeated hypoglycemia in humans. The aim of this study was to determine the effects of prior $GABA_A$ activation using the benzodiazepine alprazolam on the neuroendocrine and autonomic nervous system (ANS) and metabolic counterregulatory responses during next-day hypoglycemia in healthy humans.

RESEARCH DESIGN AND METHODS—Twenty-eight healthy individuals (14 male and 14 female, age 27 ± 6 years, BMI 24 ± 3 kg/m^2, and A1C 5.2 ± 0.1%) participated in four randomized, double-blind, 2-day studies. Day 1 consisted of either morning and afternoon 2-h hyperinsulinemic euglycemia or 2-h hyperinsulinemic hypoglycemia (2.9 mmol/l) with either 1 mg alprazolam or placebo administered 30 min before the start of each clamp. Day 2 consisted of a single-step hyperinsulinemic-hypoglycemic clamp of 2.9 mmol/l.

RESULTS—Despite similar hypoglycemia (2.9 ± 1 mmol/l) and insulinemia (672 ± 108 pmol/l) during day 2 studies, $GABA_A$ activation with alprazolam during day 1 euglycemia resulted in significant blunting ($P < 0.05$) of ANS (epinephrine, norepinephrine, muscle sympathetic nerve activity, and pancreatic polypeptide), neuroendocrine (glucagon and growth hormone), and metabolic (glucose kinetics, lipolysis, and glycogenolysis) counterregulatory responses. $GABA_A$ activation with alprazolam during prior hypoglycemia caused further significant ($P < 0.05$) decrements in subsequent glucagon, growth hormone, pancreatic polypeptide, and muscle sympathetic nerve activity counterregulatory responses.

CONCLUSIONS—Alprazolam activation of $GABA_A$ pathways during day 1 hypoglycemia can play an important role in regulating a spectrum of key physiologic responses during subsequent (day 2) hypoglycemia in healthy man. ***Diabetes*** **59: 1074–1081, 2010**

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Received 13 October 2009 and accepted 6 January 2010. Published ahead of print at http://diabetes.diabetesjournals.org on 19 January 2010. DOI: 10.2337/db09-1520. Clinical trial reg. no. NCT00592332, clinicaltrials.gov.

Hypoglycemia continues to be the major limiting factor to good glycemic control in patients with diabetes. During the last two decades, there have been many studies demonstrating that antecedent hypoglycemia can blunt counterregulatory responses to subsequent hypoglycemia in healthy and type 1 and type 2 diabetic individuals (1). Despite the clinical importance and many elegant studies addressing this topic, there remain gaps in our knowledge regarding the mechanisms regulating neuroendocrine and autonomic nervous system (ANS) responses during episodes of repeated hypoglycemia in man.

The three major acute neuroendocrine/ANS counterregulatory defenses against a falling plasma glucose include release of glucagon and epinephrine combined with inhibition of endogenous insulin release. All of these mechanisms either fail (i.e., insulin modulation and glucagon release within ~5 years of type 1 diabetes duration) or become substantially reduced with disease duration (type 2 diabetes). Furthermore, repeated hypoglycemia has been demonstrated to reduce epinephrine and glucagon responses, which are important defenses against subsequent falling blood glucose levels in both type 1 (epinephrine) and type 2 (epinephrine and glucagon) diabetes (2).

For many years, the problem of severe or frequent hypoglycemia was thought to be confined almost exclusively to type 1 diabetes. Recent multicenter trials aimed at improving glycemic control both within hospitals and in the community have identified excess adverse events and death plausibly related to hypoglycemia in type 2 diabetes (3,4). The glucagon response to hypoglycemia is initially relatively preserved in type 2 diabetes (although there is decrease with disease duration) (5). However, as the prevalence of hypoglycemia is increasing in type 2 diabetes, it continues to be of importance to understand the mechanisms regulating release of both glucagon and epinephrine during repeated episodes of hypoglycemia.

γ-Aminobytyric acid (GABA) is a major inhibitory neurotransmitter. Previous studies have demonstrated increases in GABAergic tone within the ventromedial hypothalamus in rats with repeated hypoglycemia, which is associated with blunted glucagon and epinephrine responses (6). Chan et al. (7) have also demonstrated that blockade of $GABA_A$ receptors within the ventromedial hypothalamus in rats results in increased glucagon and epinephrine responses during hypoglycemia. Studies investigating the effects of $GABA_A$ modulation on counterregulatory responses during hypoglycemia in humans are scarce. In fact, previous studies have used activation of $GABA_A$ receptors rather than changes in GABA concen-

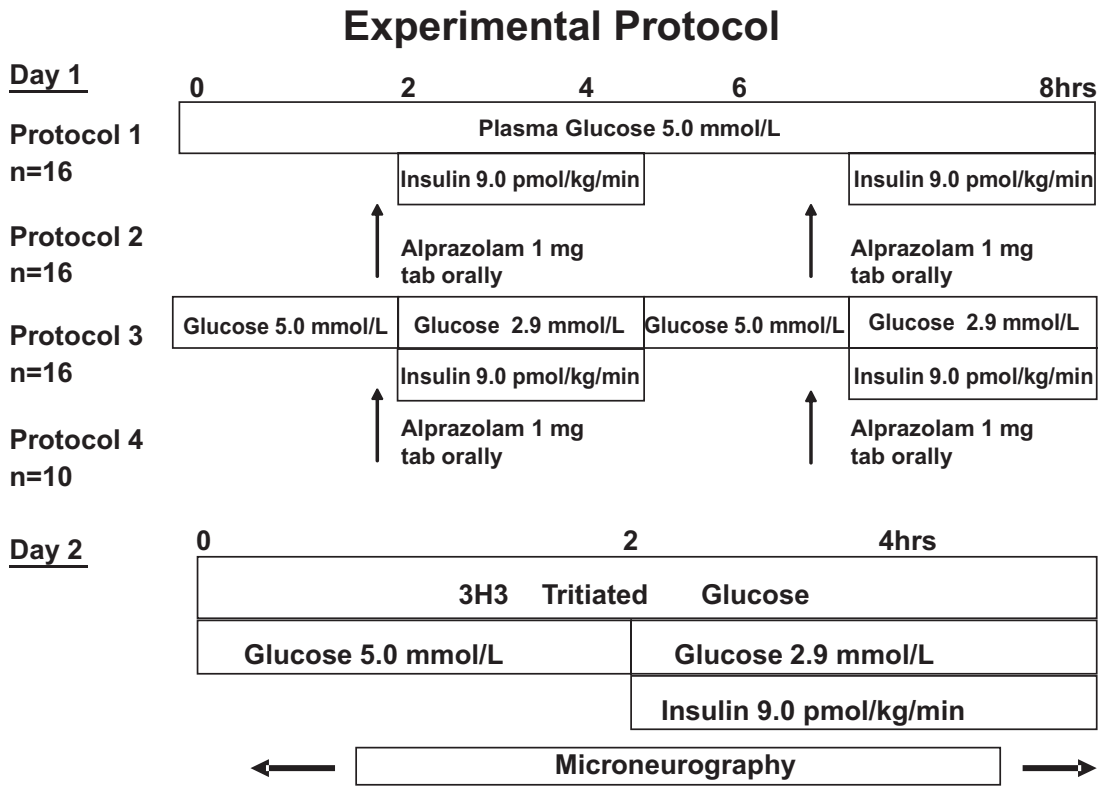

FIG. 1. Study procedures.

trations to investigate the role of GABAergic pathways in ANS and neuroendocrine counterregulatory responses during hypoglycemia in humans and primates. van Vugt et al. (8) demonstrated that alprazolam (a potent pharmacologic activator of the benzodiazepine-$GABA_A$ receptor) can inhibit anterior pituitary neuroendocrine responses during acute hypoglycemia in rhesus monkeys. Giordano et al. (9) reported that alprazolam also reduced neuroendocrine and epinephrine responses to acute intravenous insulin bolus–induced hypoglycemia in healthy humans. Breier et al. (10), using a model of 2-deoxyglycose–induced glucoprivic stress in humans, also demonstrated that alprazolam blunted ACTH and epinephrine responses during neuroglycopenia. Lastly, Smith et al. (11), using modafinil to acutely lower GABA levels during clamped hypoglycemia in healthy humans, reported increased heart rate and improved cognitive function with the drug. Thus, available data would indicate that $GABA_A$ activation can acutely reduce, whereas $GABA_A$ blockade can increase, neuroendocrine and sympathoadrenal responses to hypoglycemia. However, it is unknown whether $GABA_A$ activation can play a mechanistic role in causing neuroendocrine and ANS failure during repeated hypoglycemia in healthy humans. Therefore, in the present study, we have tested the hypothesis that antecedent pharmacologic activation of benzodiazepine-$GABA_A$ receptors with alprazolam can result in counterregulatory failure during next-day hypoglycemia in healthy humans.

RESEARCH DESIGN AND METHODS

Twenty-eight healthy individuals (14 male and 14 female, aged 27 ± 6 years, BMI 24 ± 3 kg/m^2, and A1C 5.2 ± 0.1%) were studied. Subjects were nonsmokers, had no family history of diabetes, and were not taking any medications. All subjects had normal liver, renal, and hematological parameters. Studies were approved by the Vanderbilt University Human Subjects Institutional Review Board, and all subjects gave informed written and verbal consent.

Experimental design. The volunteers participated in four separate, randomized, double-blind 2-day experiments, with differing day 1 protocols, separated by at least 2 months (Fig. 1). Women were studied at the same point in their menstrual cycle for each arm of the study so as to reduce variability associated with phase of menstrual cycle. All subjects were instructed to avoid intense exercise and alcohol and to consume their usual weight-maintaining diet for 3 days before each study. Each subject was admitted to the Vanderbilt University Clinical Research Center the evening before an experiment. The next morning, after an overnight 10-h fast, subjects had intravenous cannulae placed into each arm under local 1% lidocaine anesthesia. One cannula was placed in a retrograde fashion into a vein in the back of the hand. This hand was placed in a heated box (55–60°C) so that arterialized blood could be obtained (12). The other cannula was placed in the contralateral arm for infusions of dextrose, insulin, potassium chloride, and labeled glucose.

Day 1 consisted of different antecedent challenges (morning and afternoon hypoglycemia or euglycemia with or without prior [30 min before each clamp] administration of 1 mg alprazolam or placebo in a randomized double-blind manner) (Fig. 1). Day 1 studies consisted of a baseline period (0–120 min) and a 2-h hyperinsulinemic experimental clamp period (120–240 min). An insulin-infusion solution was prepared with normal saline containing 3% (vol/vol) of the subject's own plasma. At the onset of the experimental period, a primed continuous infusion of insulin (Eli Lilly, Indianapolis, IN) was administered at a rate of 9 pmol · kg^{-1} · min^{-1} for 120 min (Medfusion 3010; Medex-A Furon Healthcare Company, Deluth, GA). Potassium chloride (5 mmol/h; Imed pump) was also infused during the clamp period to reduce insulin-induced hypokalemia. 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) in the prior euglycemia studies. During hypoglycemia, the rate of fall of glucose was controlled (0.08 mmol/min) and the hypoglycemic nadir (3.0 mmol/l) was achieved and held constant using a modification of the glucose clamp technique (14). After completion of the initial 2-h test period, plasma glucose was maintained at euglycemia for 2 h. At that point, insulin was restarted, and a second hyperinsulinemic-euglycemic clamp, or hyperinsulinemic-hypoglycemic clamp, identical to that of the morning's study was performed (i.e., 1 mg alprazolam or placebo administered 30 min before the start of glucose clamp). At completion of the second glucose clamp,

subjects consumed a standardized meal and a bedtime snack prior to 10 P.M. and remained in the Clinical Research Center.

Day 2 hypoglycemia. Day 2 was identical for all four protocols and was started after an overnight 10-h fast. Each study consisted of a tracer equilibration period (0–90 min), a basal period (90–120 min), and a 2-h experimental period (120–240 min). A primed (18 μCi) continuous infusion (0.18 μCi/min) of high-pressure liquid chromatography–purified [3-3H] glucose (11.5 mCi/mmol/l; Perkin Elmer Life Sciences, Boston, MA) was administered starting at 0 min and continued throughout the study for measurement of glucose kinetics. Also during the equilibration period, isolation of the peroneal nerve for microneurography (technique described below) was started. At the onset of the experimental period, a primed constant (9 pmol · kg^{-1} · min^{-1}) infusion of insulin was started and continued for the next 2 h. The rate of fall of glucose was controlled (~0.08 mmol/min), and the hypoglycemic nadir (2.9–3.0 mmol/l) was achieved and then held constant for the remainder of the study.

Tracer calculations. Glucose R_a, endogenous glucose production (EGP), and glucose utilization (R_d) were calculated according to the methodology of Wall et al. (15). EGP was calculated by determining the total R_a (which comprises both EGP and any exogenous glucose infused to maintain the desired hypoglycemia) and subtracting from it the amount of exogenous glucose infused. It is now recognized that this approach is not fully quantitative because it underestimates the total R_a and R_d that 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. To minimize changes in specific activity, isotope delivery was increased commensurate with increases in exogenous glucose infusion. For this study, only glucose flux results from the basal and the final 30-min periods of the hypoglycemic clamps are reported.

Direct measurement of muscle sympathetic nerve activity via microneurography. Muscle sympathetic nerve activity (MSNA) was recorded because it provides a measurement of direct sympathetic nervous system activity during insulin-induced hypoglycemia (16). MSNA was measured in the peroneal nerve at the level of the fibular head or popliteal fossa. A recording of MSNA was considered adequate when there was *1*) spontaneous appearance of pulse-linked bursts, *2*) increased nerve activity during phase II (hypotensive phase) and suppressed activity during phase IV (blood pressure overshoot) of the Valsalva maneuver, *3*) increased nerve activity in response to held expiration (apnea), or *4*) proprioceptive afferent signals in response to stretching the tendons in the foot or tapping the muscle belly but not cutaneous stimulation by stroking the skin.

Sympathetic nerve activity was expressed as bursts per minute. Measurements of MSNA were made from original tracings or online recordings (DI-220; Dataq Instruments, Akron, OH) by an operator blinded to the sequence of experiments. Bursts were selected if the signal:noise ratio was >2:1.

Analytical methods. The collection and processing of blood samples have previously been described (17). Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Blood for hormones and intermediary metabolites were drawn twice during the basal period and every 15 min during the experimental period. Glucagon was measured according to the method of Aguilar-Parada et al. (18), with an interassay coefficient of variation (CV) of 15%. Insulin was measured as previously described (19), with an interassay CV of 11%. Catecholamines were determined by high-pressure liquid chromatography (20), with an interassay CV of 12% for both epinephrine and norepinephrine. We made two modifications to the procedure for catecholamine determination: *1*) we used a five-point rather than one-point standard calibration curve, and *2*) we spiked the initial and final samples of plasma with known amounts of epinephrine and norepinephrine so that accurate identification of the relevant catecholamine peaks could be made. Growth hormone (21) (interassay CV 8%), cortisol (Clinical Assays Gamma Coat Radioimmunoassay kit) (interassay CV 6%), pancreatic polypeptide (interassay CV 8%) (22), and glucagon (Linco Research, St. Louis, MO) (interassay CV 15%) were measured using radioimmunoassay techniques. Lactate and β-hydroxybutyrate were measured on deproteinized whole blood using the methodology of Lloyd et al. (23). Nonesterified fatty acids (NEFAs) were measured using a WAKO kit (24).

Cardiovascular parameters. Heart rate and systolic, diastolic, and mean arterial blood pressure were measured noninvasively by a Dinamap (Critikon, Tampa, FL) every 10 min throughout each 2-h insulin clamp. Hypoglycemic symptoms were quantified using a previously validated semiquantitative questionnaire (25). Each individual was asked to rate his or her experience of the symptoms twice during the control period and every 15 min during experimental periods. Symptoms measured included the following: 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 means ± SE and were analyzed using standard parametric one- and two-way ANOVA 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 with the prior euglycemia control group. A P value of <0.05 was accepted as statistically significant. The baseline and final 30 min of hypoglycemia on day 2 were compared for most parameters because steady-state glucose levels, insulin levels, and glucose infusion rates were achieved by this time. Baseline period data represent an average of two time points (110 and 120 min), and final 30-min data represent an average of three measurements taken during this time (210, 225, and 240 min).

RESULTS

Day 1 glucose and insulin levels. Plasma glucose levels were similar in the morning and afternoon during the prior euglycemic studies with and without alprazolam (5.2 ± 0.1 mmol/l). Plasma glucose during the day 1 morning and afternoon hypoglycemia studies were also similar with and without alprazolam (2.9 ± 0.1 mmol/l). Plasma insulin levels were similar among all groups during the morning and afternoon hyperinsulinemic-euglycemic and -hypoglycemic clamps (672 ± 108 pmol/l).

Day 2 glucose, insulin, and neuroendocrine counterregulatory hormones. Plasma glucose was equivalent (2.9 ± 0.1 mmol/l) during all of day 2 hypoglycemia. Plasma insulin was also similar (612 ± 58 pmol/l) during all of day 2 hypoglycemia studies (Fig. 2).

Plasma glucagon levels (Fig. 3) were significantly reduced ($P < 0.01$) during the final 30 min of day 2 hypoglycemia following day 1 euglycemia and alprazolam (131 ± 21 ng/l) and day 1 hypoglycemia (132 ± 18 ng/l) compared with day 1 euglycemia (241 ± 34 ng/l). Day 1 hypoglycemia and alprazolam resulted in a greater reduction ($P < 0.05$) in day 2 glucagon (76 ± 8 ng/l) than that in the other groups during day 2 hypoglycemia.

Day 2 growth hormone responses were also lower ($P < 0.05$) following day 1 euglycemia and alprazolam (20 ± 4 μg/l) than those of day 1 euglycemia (31 ± 5 μg/l) or day 1 hypoglycemia (28 ± 4 μg/l). Growth hormone responses were further reduced ($P < 0.05$) following day 1 hypoglycemia and alprazolam (13 ± 3 μg/l) compared with those of day 1 hypoglycemia and day 1 hypoglycemia and alprazolam (Fig. 3). Day 2 plasma cortisol responses were similar in all groups following the differing day 1 interventions.

ANS responses during day 2 hypoglycemia. Day 2 plasma epinephrine levels (Fig. 4) were significantly lower ($P < 0.05$) during the final 30 min of hypoglycemia following day 1 euglycemia and alprazolam (3,397 ± 339 pmol/l), day 1 hypoglycemia (2,230 ± 290 pmol/l), and day 1 hypoglycemia and alprazolam (2,943 ± 515 pmol/l) than those of day 1 euglycemia (4,209 ± 389 pmol/l).

Day 2 baseline and final 30 min of hypoglycemia norepinephrine values (Fig. 4) were also significantly lower ($P < 0.05$) following day 1 euglycemia and alprazolam (0.7 ± 0.1 and 1.2 ± 0.16 nmol/l, respectively) and day 1 hypoglycemia and alprazolam (0.6 ± 0.1 and 1.5 ± 0.15 nmol/l) than those of day 1 euglycemia (1.1 ± 0.1 and 1.9 ± 0.17 nmol/l). Pancreatic polypeptide levels during the final 30 min of day 2 hypoglycemia were also significantly lower ($P < 0.05$) after day 1 euglycemia and alprazolam (163 ± 27 pmol/l), day 1 hypoglycemia (197 ± 28 pmol/l), and ($P < 0.01$) day 1 hypoglycemia and alprazolam (128 ± 32 pmol/l) than those of day 1 euglycemia (263 ± 33 pmol/l).

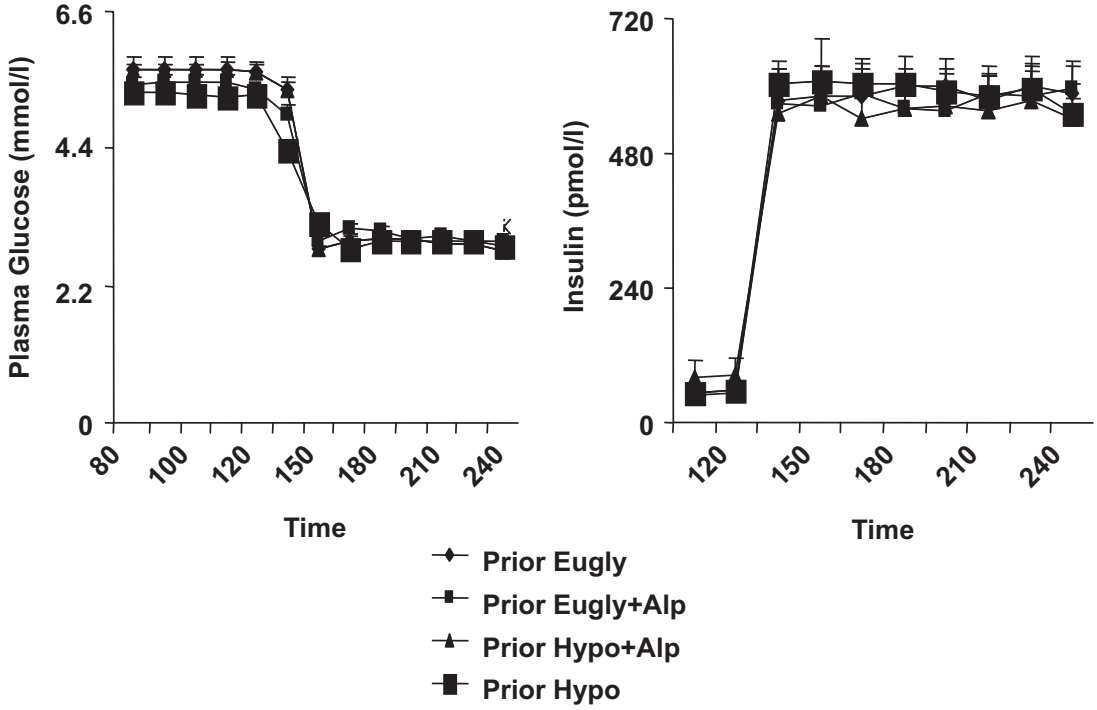

FIG. 2. Plasma glucose and insulin levels during day 2 hypoglycemia in healthy individuals fasted overnight following either day 1 euglycemia (Eugly), day 1 euglycemia and alprazolam (Eugly+Alp), day 1 hypoglycemia (Hypo), or day 1 hypoglycemia and alprazolam (Hypo+Alp).

Day 2 pancreatic polypeptide responses were blunted by a greater extent ($P < 0.05$) following day 1 alprazolam and hypoglycemia compared with day 1 hypoglycemia and day 1 euglycemia.

Basal MSNA (Fig. 5) was significantly reduced ($P < 0.05$) following day 1 alprazolam administration. MSNA responses during the final 30 min of day 2 hypoglycemia were also reduced following day 1 euglycemia and alprazolam ($\Delta 3 \pm 1$ bursts/min) and day 1 hypoglycemia ($\Delta 7 \pm 2$ bursts/min) compared with those ($\Delta 12 \pm 2$) following day 1 euglycemia. MSNA responses were blunted by a greater extent ($P < 0.05$) following day 1 hypoglycemia and alprazolam ($\Delta -2 \pm 1$ bursts/min) than those of day 1 euglycemia and alprazolam, day 1 hypoglycemia, or day 1 euglycemia.

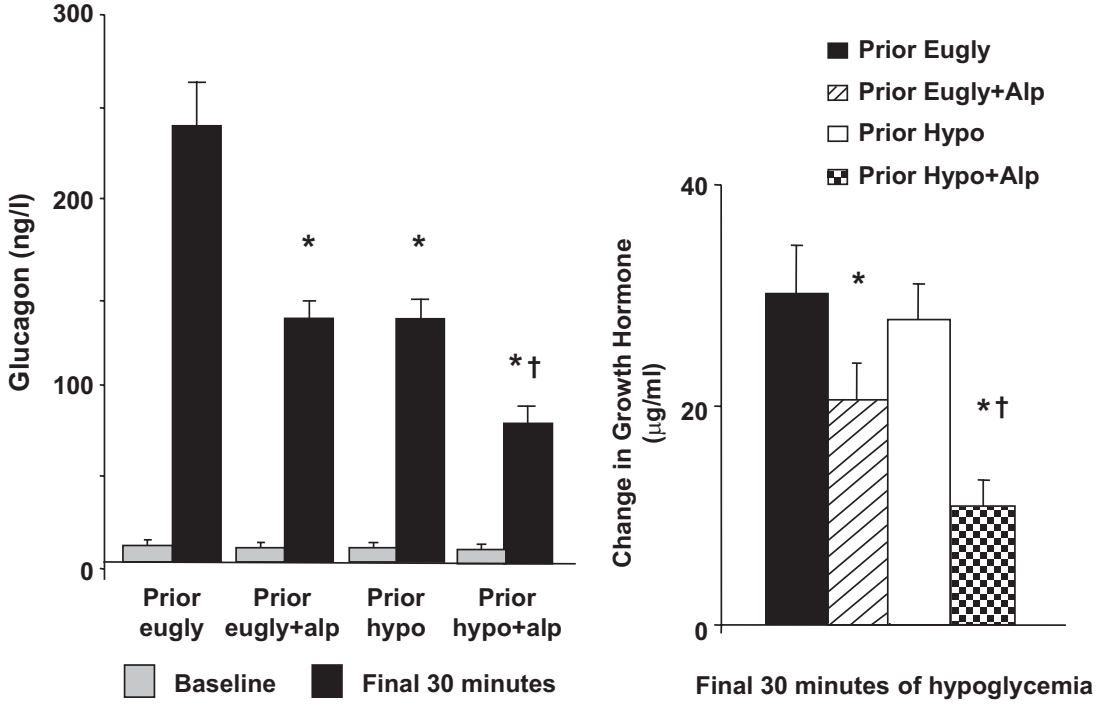

FIG. 3. Day 2 glucagon and growth hormone responses (baseline and final 30 min of hypoglycemia) in healthy individuals fasted overnight following either day 1 euglycemia (Eugly), day 1 euglycemia and alprazolam (Eugly+Alp), day 1 hypoglycemia (Hypo), or day 1 hypoglycemia and alprazolam (Hypo+Alp). *Final 30-min levels are significantly reduced ($P < 0.05$) compared with those of day 1 euglycemia. †Final 30-min levels are significantly reduced ($P < 0.05$) compared with day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 euglycemia.

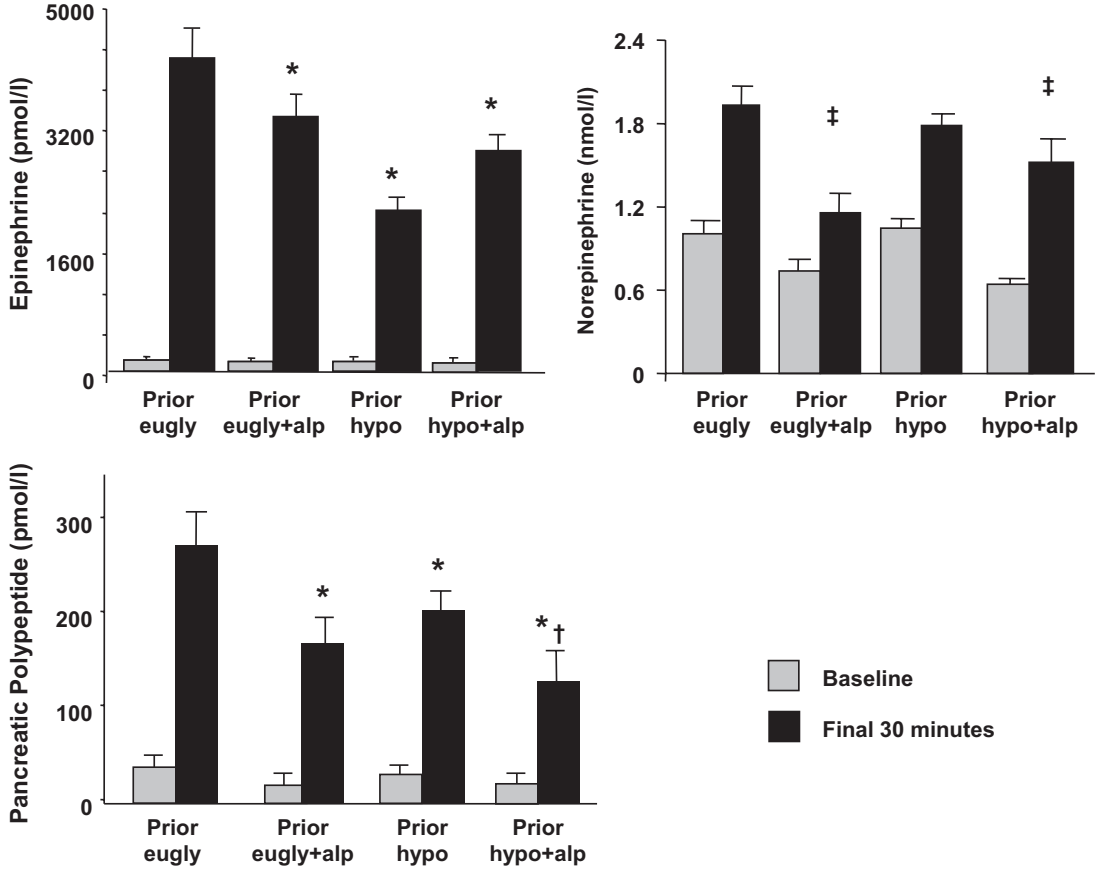

FIG. 4. Day 2 epinephrine, norepinephrine, and pancreatic polypeptide (baseline and final 30 min of hypoglycemia) in healthy individuals fasted overnight following either day 1 euglycemia (eugly), day 1 euglycemia and alprazolam (eugly+alp), day 1 hypoglycemia (hypo), or day 1 hypoglycemia and alprazolam (hypo+alp). *Final 30-min levels are significantly reduced ($P < 0.05$) compared with those of day 1 euglycemia. ‡Basal and final 30-min norepinephrine values are significantly reduced ($P < 0.05$) compared with those of day 1 euglycemia.

Day 2 glucose kinetics. Rates of EGP were significantly reduced ($P < 0.05$) during the final 30 min of day 2 hypoglycemia following day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 hypoglycemia and alprazolam (7.2 ± 1.7, 6.1 ± 1.6, and 8.8 ± 1.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively) with those of day 1 euglycemia (12.2 ± 1.7 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Table 1). Glucose infusion rates were significantly increased ($P < 0.05$) during the final 30 min of day 2 hypoglycemia following day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 hypoglycemia and alprazolam (7.5 ± 1.1, 8.8 ± 3.3, and 8.3 ± 2.2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively) compared with 2.5 ± 1.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ following day 1 euglycemia.

Day 2 intermediary metabolism. Blood lactate levels were significantly reduced ($P < 0.05$) basally and during the final 30 min of day 2 hypoglycemia following day 1 euglycemia and alprazolam and day 1 hypoglycemia and alprazolam compared with day 1 euglycemia (Table 2). Plasma NEFA levels were also significantly reduced ($P < 0.05$) at baseline and during the final 30 min of hypoglycemia following both day 1 alprazolam groups. NEFA levels were also reduced ($P < 0.05$) during the final 30 min of day 2 hypoglycemia following day 1 hypoglycemia (Table 2).

Day 2 cardiovascular responses and symptom responses. There were similar changes in blood pressure (systolic, diastolic, and mean arterial pressure) and heart rate during the final 30 min of hypoglycemia in all groups (Table 3). Hypoglycemic symptoms were reduced in all groups during the final 30 min of day 2 hypoglycemia. Following day 1 euglycemia and alprazolam, symptoms were reduced by ~25%, which did not reach statistical significance. Following day 1 hypoglycemia and alprazolam and day 1 hypoglycemia, there were significant reductions ($P < 0.05$) of 30 and 38%, respectively. Day 2 autonomic and neuroglycopenic symptom scores were similarly reduced following day 1 hypoglycemia or day 1 hypoglycemia and alprazolam.

DISCUSSION

This study tested the hypothesis that day 1 pharmacologic activation of GABA$_A$ receptors in healthy man with alprazolam can blunt neuroendocrine and ANS responses to day 2 hypoglycemia. Our results demonstrate that prior GABA$_A$ receptor activation with alprazolam has widespread effects to blunt anterior pituitary, sympathoadrenal, parasympathetic, and sympathetic neural counterregulatory responses to next-day hypoglycemia. GABA$_A$ activation resulted in significant blunting of a spectrum of key neuroendocrine, ANS, and metabolic counterregulatory responses/mechanisms (glucagon, epinephrine, endogenous glucose production, and lipolysis) during next-day hypoglycemia.

Numerous studies have investigated the mechanisms responsible for the acquired ANS and neuroendocrine counterregulatory failure occurring following hypoglycemia (1). To date, a unifying mechanism responsible for the syndrome of acquired hypoglycemia-associated counterregulatory failure

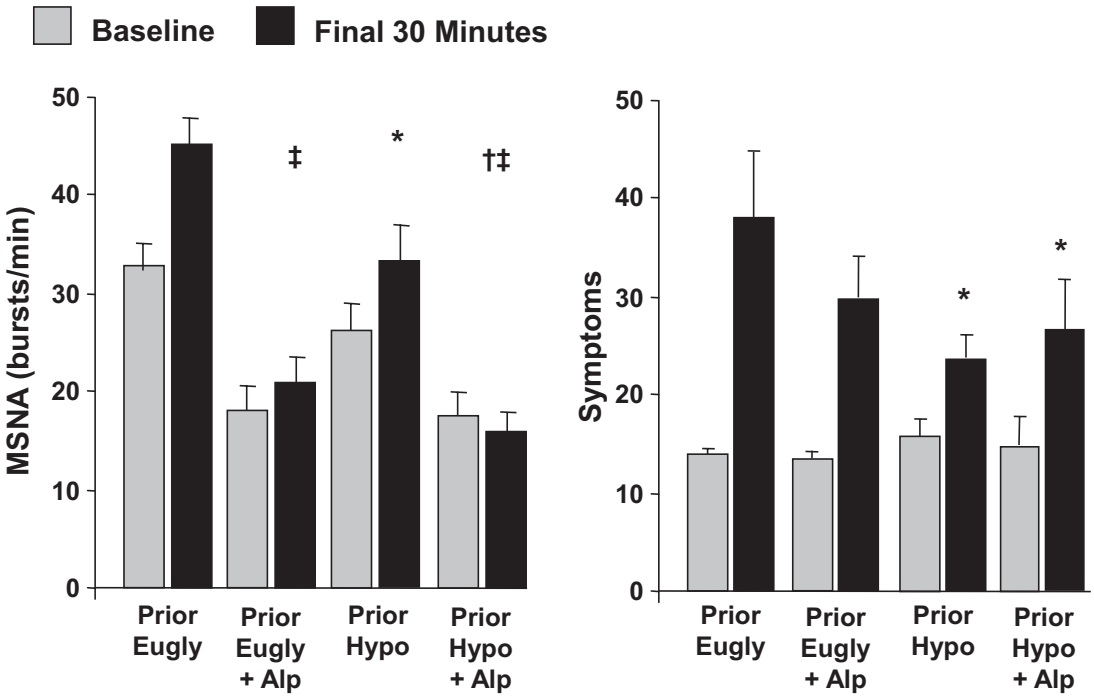

FIG. 5. MSNA and hypoglycemia symptoms at baseline (gray boxes) and during final 30 min (black boxes) of day 2 hypoglycemia in healthy individuals fasted overnight following either day 1 euglycemia (Eugly), day 1 euglycemia and alprazolam (Eugly+Alp), day 2 hypoglycemia (Hypo), or day 2 hypoglycemia and alprazolam (Hypo+Alp). *Final 30-min levels are significantly reduced ($P < 0.05$) compared with those of day 1 euglycemia. †Final 30-min responses are significantly reduced ($P < 0.05$) compared with those of day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 euglycemia. ‡Basal and final 30-min levels are significantly reduced ($P < 0.05$) compared with those of prior euglycemia.

has not been determined (26). GABA is a major inhibitory neurotransmitter and is known to regulate many physiologic responses (27–29). Previous work has demonstrated that increases of gabergic tone in the ventromedial nucleus in rats can downregulate counterregulatory responses to hypoglycemia and indeed subsequent hypoglycemia (6). Additionally, blockade of $GABA_A$ in the ventromedial nucleus of rats increases neuroendocrine and sympathetic nervous system responses to hypoglycemia (7). Determination of the effects of activation of specific gabergic neurons in discrete areas of the human brain is not possible at this time. To overcome this limitation, previous studies have used alprazolam, a commonly used anxiolytic to specifically activate brain benzodiazepine-$GABA_A$ receptors (8,9). Two previous studies (one in primates and the other in healthy humans) have demon-

TABLE 1
Rates of endogenous glucose production, glucose disappearance, and glucose infusion during baseline and final 30 min of hypoglycemia in men fasted overnight following day 1 euglycemia, day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 hypoglycemia and alprazolam

	Baseline period	Final 30 min hypoglycemia
Endogenous glucose production ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)		
Euglycemia	9.9 ± 0.6	13.2 ± 1.7*
Euglycemia and alprazolam	9.9 ± 1.1	7.2 ± 1.7*†
Hypoglycemia	11.6 ± 0.6	6.1 ± 1.7*†
Hypoglycemia and alprazolam	11.0 ± 1.1	8.8 ± 1.1*†
R_d ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)		
Euglycemia	10.5 ± 2.2	15.6 ± 1.7*
Euglycemia and alprazolam	9.9 ± 0.6	14.8 ± 1.7*
Hypoglycemia	11.6 ± 1.7	14.9 ± 1.1*
Hypoglycemia and alprazolam	10.5 ± 1.1	17.1 ± 1.7*
Glucose infusion rate ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)		
Euglycemia	0	2.5 ± 1.1
Euglycemia and alprazolam	0	7.5 ± 1.1†
Hypoglycemia	0	8.8 ± 3.3†
Hypoglycemia and alprazolam	0	8.3 ± 2.2†

*$P < 0.05$: significantly different from baseline. †$P < 0.05$: significantly different from euglycemic controls.

TABLE 2
Intermediary metabolite levels during baseline and final 30 min of hyperinsulinemic hypoglycemia in healthy individuals fasted overnight following day 1 euglycemia, day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 hypoglycemia and alprazolam

	Baseline period	Final 30 min hypoglycemia
Blood lactate (mmol/l)		
Euglycemia	0.9 ± 0.1	1.4 ± 0.1*
Euglycemia and alprazolam	0.6 ± 0.1†	1.1 ± 0.1*‡
Hypoglycemia	0.9 ± 0.1	1.4 ± 0.1*
Hypoglycemia and alprazolam	0.6 ± 0.1†	0.9 ± 0.1*‡
Plasma NEFA ($\mu mol/l$)		
Euglycemia	329 ± 43	147 ± 22*
Euglycemia and alprazolam	174 ± 27†	80 ± 12*‡
Hypoglycemia	346 ± 31	98 ± 23*†
Hypoglycemia and alprazolam	186 ± 40†	73 ± 21*‡
Blood β-hydroxybutyrate ($\mu mol/l$)		
Euglycemia	40 ± 20	10 ± 4*
Euglycemia and alprazolam	70 ± 10	33 ± 20*
Hypoglycemia	30 ± 8	9 ± 2*
Hypoglycemia and alprazolam	20 ± 10	10 ± 5*

*$P < 0.05$ significantly different from baseline. †$P < 0.05$ significantly different from euglycemia and hypoglycemia. ‡$P < 0.05$ significantly different from euglycemia.

TABLE 3
Cardiovascular responses during baseline and final 30 min of hyperinsulinemic hypoglycemia in men fasted overnight following day 1 euglycemia, day 1 euglycemia and alprazolam, day 1 hypoglycemia, and day 1 hypoglycemia and alprazolam

	Baseline period	Final 30 min hypoglycemia
Monthly systolic blood pressure (mmHg)		
Euglycemia	111 ± 3	119 ± 4*
Euglycemia and alprazolam	116 ± 4	126 ± 5*
Hypoglycemia	114 ± 3	123 ± 4*
Hypoglycemia and alprazolam	120 ± 6	131 ± 9*
Monthly diastolic blood pressure (mmHg)		
Euglycemia	69 ± 3	62 ± 2*
Euglycemia and alprazolam	73 ± 3	68 ± 3*
Hypoglycemia	67 ± 3	68 ± 4*
Hypoglycemia and alprazolam	73 ± 3	66 ± 4*
Monthly mean arterial pressure (mmHg)		
Euglycemia	82 ± 4	78 ± 4
Euglycemia and alprazolam	82 ± 3	79 ± 4
Hypoglycemia	81 ± 2	79 ± 2
Hypoglycemia and alprazolam	85 ± 4	83 ± 5
Heart rate (bpm)		
Euglycemia	63 ± 3	76 ± 5*
Euglycemia and alprazolam	62 ± 3	72 ± 4*
Hypoglycemia	65 ± 4	75 ± 4*
Hypoglycemia and alprazolam	65 ± 3	74 ± 3*

*$P < 0.05$ significantly different from baseline.

strated that acute alprazolam administration blunts neuroendocrine and sympathetic nervous system responses during intravenouos insulin bolus–induced hypoglycemia (8,9). In the present study, prior GABA$_A$ activation with alprazolam during day 1 euglycemia resulted in significant reductions in ANS and neuroendocrine responses during next-day hypoglycemia. Multiple limbs of the ANS response to hypoglycemia were blunted by prior GABA$_A$ activation. Epinephrine, norepinephrine, MSNA, and hypoglycemic symptoms were reduced, indicating a diffuse blunting effect upon the sympathetic nervous system. Furthermore, basal sympathetic neural outflow was also reduced by prior GABA$_A$ activation. Additionally, pancreatic polypeptide, a marker of parasympathetic nervous system activity, was also significantly reduced. The site of GABA$_A$ sensing to downregulate the ANS responses cannot be precisely determined in this study. The down regulation of MSNA responses points to central nervous system sensing. However, there are GABA$_A$ receptors in islet cells that may be regulating the pancreatic polypeptide and glucagon responses. Additionally, previous work has demonstrated that GABA$_A$ receptors may also modulate catecholamine secretion directly from the adrenal medulla (30). Day 1 GABA$_A$ activation with alprazolam also reduced the response of growth hormone and glucagon during day 2 hypoglycemia. The regulation of glucagon release during hypoglycemia is still under study. Previous work has provided evidence for local control (i.e., within the pancreatic islets) of glucagon release secondary to inhibition of β-cell insulin release (31). Other studies have pointed to regulation via the ANS (32). In the present study, we cannot definitively state whether prior activation of GABA$_A$ inhibited glucagon release via a direct effect on pancreatic islets or via inhibition of neural (presumably ANS) pathways.

Similar to the findings of Giodano et al. (7) in humans and Chan et al. (8) in rats, we did not find significant reductions of cortisol during hypoglycemia following alprazolam. However, both Breir et al. (10), using 2-deoxyglycose glucose to create a glucoprivic state in humans, and Giodano et al. (9) reported that ACTH levels were blunted during hypoglycemia following acute GABA$_A$ activation with alprazolam. We did not measure ACTH in the current study, but our finding that growth hormone responses were blunted following alprazolam supports previous findings that GABA$_A$ activation can blunt hypothalamo-anterior pituitary responses during hypoglycemia in humans.

Important metabolic counterregulatory responses/mechanisms were also blunted by GABA$_A$ activation. Endogenous glucose production, lipolysis (as reflected by NEFA levels), and glycogenolysis (as reflected by lactate levels) were blunted during day 2 hypoglycemia following alprazolam. These reduced metabolic counterregulatory responses during day 2 hypoglycemia can be explained by the blunted ANS and neuroendocrine drive caused by the GABA$_A$ activation. The reduced day 2 basal NEFA and lactate levels following day 1 alprazolam may also be explained by the observed reduced sympathetic neural activity (i.e., reduced lipolysis and glycogenolysis) (27). Blood pressure and heart rate responses were not different during day 2 hypoglycemia in any of the groups despite the differences in ANS activity. The mechanism for this finding is not known but may be explained by offsetting effects of GABA$_A$ activation on the sympathetic and parasympathetic nervous system.

This present study also studied whether activation of GABA$_A$ receptors during prior hypoglycemia had any additional effects on subsequent counterregulatory responses. Our results do demonstrate that pharmacologic activation of GABA$_A$ receptors during prior hypoglycemia with alprazolam results in additional blunting of some counterregulatory responses. Epinephrine and norepinephrine responses were not further blunted during day 2 hypoglycemia by the addition of alprazolam during day 1 hypoglycemia. Additionally, important metabolic counterregulatory mechanisms such as glucose kinetics (EGP, glucose disappearance, and lipolysis) were not farther decreased by the combination of hypoglycemia and GABA$_A$ activation). However, MSNA, glucagon, pancreatic polypeptide, and growth hormone responses were further reduced following day 1 hypoglycemia and alprazolam. We believe that there may be two possible explanations for this finding: *1*) the combination of hypoglycemia and alprazolam resulted in greater activation of GABA$_A$ receptors or *2*) prior alprazolam and hypoglycemia operate through different mechanisms for which the combined effects are additive. Our results also suggest that blunted counterregulatory responses are not due to exhaustion of individual neuroendocrine hormones and that the ANS response to hypoglycemia is heterogeneous, with some elements more susceptible to downregulation following certain stimuli (i.e., GABA$_A$ activation) than others (in this study, MSNA and parasympathetic nervous system were more susceptible than adrenomedullary and symptom responses). Lastly, although this is a study investigating physiologic responses to hypoglycemia in healthy subjects, it may be useful to discuss the possible clinical implications of our study. Benzodiazepines are commonly used in the clinical management of patients with diabetes. The findings that alprazolam can significantly reduce key neuroendocrine and ANS counterregulatory responses during next-day hypoglycemia and that the combination of prior hypoglycemia with

alprazolam can further blunt certain counterregulatory responses raise concerns about the possible effects of benzodiazepines on the prevalence of hypoglycemia in clinical practice.

The dose of alprazolam (1 mg before each of two glucose clamps) used in the present study was relatively modest. In the U.S., the drug is approved to be used up to a dose of 10 mg daily. The present study dose of alprazolam was typical of usual starting doses, which range from 0.5 to 1 mg three times a day. However, we do want to point out that the present study was not a clinical outcomes study. We were using alprazolam as a specific pharmacologic probe for $GABA_A$ activation. As a result of to the present study design, we cannot comment whether higher (or lower) doses of day 1 alprazolam would have had greater or lesser effects on day 2 counterregulatory responses. It should also be noted that although alprazolam has a quick onset of action reaching maximum levels within 1–2 h, the plasma half-life is longer with a typical duration of 9–11 h. Thus, as day 2 hypoglycemia was induced ~21 h after the last administration of alprazolam, it is possible that the day 1 administration of the drug still resulted in some acute effects during subsequent (day 2) hypoglycemia. However, what is clear from the present study is that submaximal activation of $GABA_A$ receptors can result in rapid and widespread downregulation of subsequent homeostatic responses to hypoglycemia in healthy man.

In summary, this study has demonstrated that prior activation of $GABA_A$ receptors by alprazolam can produce a spectrum of reduced ANS (adrenomedullary, direct sympathetic nerve activity, and parasympathetic nervous system), neuroendocrine (growth hormone and glucagon), and metabolic (endogenous glucose production, lipolysis, and glycogenolysis) counterregulatory responses during next-day hypoglycemia. We conclude that prior activation of $GABA_A$ pathways can play an important role in regulating a number of key physiologic responses to subsequent hypoglycemia in healthy man. Further studies are required to determine whether $GABA_A$ receptors exert similar effects in individuals with diabetes.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01 DK-069803, M01 RR-000095, P01 HL-056693, and P60 DK-020593.

No potential conflicts of interest relevant to this article were reported.

We are thankful for the expert technical assistance of Eric Allen and Wanda Snead. We also thank the nursing staff of the Vanderbilt General Clinical Research Center and Jan Botts Hicks for her expert secretarial skills.

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