Effect of Oral Amino Acids on Counterregulatory Responses and Cognitive Function During Insulin-Induced Hypoglycemia in Nondiabetic and Type 1 Diabetic People

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OBJECTIVE—Amino acids stimulate glucagon responses to hypoglycemia and may be utilized by the brain. The aim of this study was to assess the responses to hypoglycemia in nondiabetic and type 1 diabetic subjects after ingestion of an amino acid mixture.

RESEARCH DESIGN AND METHODS—Ten nondiabetic and 10 diabetic type 1 subjects were studied on three different occasions during intravenous insulin (2 mU · kg^{-1} · min^{-1}) plus variable glucose for 160 min. In two studies, clamped hypoglycemia (47 mg/dl plasma glucose for 40 min) was induced and either oral placebo or an amino acid mixture (42 g) was given at 30 min. In the third study, amino acids were given, but euglycemia was maintained.

RESULTS—Plasma glucose and insulin were no different in the hypoglycemia studies with both placebo and amino acids ($P >$ 0.2). After the amino acid mixture, plasma amino acid concentrations increased to levels observed after a mixed meal (2.4 ± 0.13 vs. placebo study 1.7 ± 0.1 mmol/l, $P = 0.02$). During clamped euglycemia, ingestion of amino acids resulted in transient increases in glucagon concentrations, which returned to basal by the end of the study. During clamped hypoglycemia, glucagon response was sustained and increased more in amino acid studies versus placebo in nondiabetic and diabetic subjects ($P < 0.05$), but other counter-regulatory hormones and total symptom score were not different. β-OH-butyrate was less suppressed after amino acids (200 ± 15 vs. 93 ± 9 μmol/l, $P = 0.01$). Among the cognitive tests administered, the following indicated less deterioration after amino acids than placebo: Trail-Making part B, PASAT (Paced Auditory Serial Addition Test) (2 s), digit span forward, Stroop colored words, and verbal memory tests for nondiabetic subjects; and Trail-Making part B, digit span backward, and Stroop color tests for diabetic subjects.

CONCLUSIONS—Oral amino acids improve cognitive function in response to hypoglycemia and enhance the response of glucagon in nondiabetic and diabetic subjects. ***Diabetes*** **57: 1905–1917, 2008**

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In type 1 diabetes, there is not only failure of the pancreatic β-cell but also severe impairment of the α-cell in terms of response to hypoglycemia (1,2). Adequate glucagon secretory response to falling plasma glucose concentration, which, along with adrenaline response, is normally the primary line of defense against hypoglycemia (3), is lost within a few months of diabetes onset (2). This process appears unavoidable, irreversible, and unrecoverable in the natural history of the disease (4), contributing to impaired glucose counterregulation and risk of severe hypoglycemia (5). As a result, in type 1 diabetes, the therapeutic goal of near-normoglycemia with intensive insulin treatment may carry the burden of greater frequency of hypoglycemia, as indicated by the Diabetes Control and Complications Trial (6).

Several attempts have been made to reduce the risk of hypoglycemia in intensively treated type 1 diabetes. The efforts have been substantially focused in two directions. The first has been to define more rational and physiological strategies of insulin replacement (7–9). The second has been to explore the possibility of inducing a recovery of glucagon response to hypoglycemia. The finding that a number of amino acids may stimulate glucagon release from pancreatic α-cells (10) has prompted a series of studies to test the hypothesis that amino acid stimulation may sustain glucagon secretion in response to hypoglycemia in type 1 diabetes (11–13). The majority of studies have been conducted by giving amino acids intravenously as a mixture (11), as dipeptide (alanine and glutamine) (12), or as a single amino acid (alanine) (13), with conflicting results. Our group has recently shown that intravenous alanine is able to partially restore glucagon response to insulin-induced hypoglycemia (13). Surprisingly, to the best of our knowledge, no study so far has examined whether amino acids given orally may stimulate glucagon response during insulin-induced hypoglycemia in type 1 diabetes. This hypothesis might be interesting, because previous studies have shown that the failure of glucagon response to hypoglycemia in type 1 diabetes recovers after food intake (14,15). This has led to the hypothesis that it is the protein component of the meal that induces such an effect (15). In fact, oral alanine may improve glucose recovery from hypoglycemia mediated by a sustained increase in plasma glucagon in type 1 diabetes (16,17). In addition to the potential effect on recovery of glucagon responses, amino acids might also serve as a substrate alternative to glucose for the brain and therefore limit cognitive dysfunction during hypoglycemia (18), as do lactate (19) and β-hydroxybutyrate (20).

The aim of the present study was to examine the effects

of oral administration of a mixture of amino acids on hormonal counterregulatory responses, particularly glucagon, and responses of symptoms and cognitive function during insulin-induced hypoglycemia in people with type 1 diabetes compared with nondiabetic subjects.

RESEARCH DESIGN AND METHODS

Institutional review board approval was obtained for these studies. Ten healthy nondiabetic volunteers (5 men, age 32 ± 7 years, BMI 23 ± 2 kg/m^2, C-peptide 1.2 ± 0.2 nmol/l, A1C 4.6 ± 0.3% [means ± SD]) were studied along with 10 subjects with type 1 diabetes on long-term intensive insulin treatment (6 men, age 30 ± 8 years, diabetes duration 17 ± 7.8 years, BMI 22.7 ± 1.8 kg/m^2, A1C 7.4 ± 1.0% [means ± SD]). At the time of the study, all type 1 diabetic subjects were free of any detectable microangiopathic complication and were negative at the screening for autonomic neuropathy, as judged on the basis of a standard battery of cardiovascular tests (21).

The study was carried out according to the Declaration of Helsinki after obtaining written informed consent from all subjects. All nondiabetic and diabetic volunteers were studied on three different occasions in a random, computer-generated sequence at 2- to 3-week intervals with the modified hyperinsulinemic glucose clamp technique used to either maintain euglycemia or induce hypoglycemia. In diabetic subjects, care was taken to avoid preprandial, postprandial, and nocturnal blood glucose <72 mg/dl over the week before studies as previously reported (22). Briefly, the glycemic targets were blood glucose 110–130 mg/dl in the fasting state, before meals, and at bedtime, and blood glucose 140–180 mg/dl 2 h after meals. Blood glucose was measured in the fasting state, before meals, and 2 h after meals and three times a week at 0300 h. In addition, blood glucose was measured whenever subjects believed their sugar was low. Patients were advised to decrease or increase the dose of basal insulin if their fasting blood glucose was repeatedly <110 mg/dl or >130 mg/dl, and to decrease or increase the dose of rapid-acting insulin at meals if their 2-h postprandial blood glucose was repeatedly <140 mg/dl or >180 mg/dl. Adjustments of rapid-acting insulin dose were made according to the carbohydrate content of meals. Four subjects had six values of blood glucose between 55 and 72 mg/dl in the week before the study, which caused the postponing of the study to the next week. Finally, to avoid hypoglycemia the day before the experiment, the total daily insulin dosage was cut by 20%, and patients were asked to contact one investigator by phone to receive advice on insulin doses.

On the morning of the studies, all nondiabetic and diabetic subjects were admitted to the General Clinical Research Center of the Department of Internal Medicine at ~0700 h. A hand vein of the nondominant arm was cannulated retrogradely and maintained in a hot box (~60°C) for sampling of arterialized-venous blood (23). A superficial vein of the ipsilateral arm was also cannulated for infusion of insulin and glucose (see below). The two veins were maintained patent by means of 0.9% NaCl infusion (0.5 ml/min). In the diabetic subjects, an intravenous infusion of human regular insulin (diluted to 1 unit/ml in 2 ml of the subject's blood and 0.9% NaCl to a final volume of 100 ml) was begun at ~0730 h in a feedback fashion, using a syringe pump (Harvard Apparatus, Ealing, South Natick, MA), according to an algorithm described previously (24), to reach the target plasma glucose of 100 mg/dl by 0830 h.

In all studies at time 0900 h (time 0 min), in both nondiabetic and diabetic subjects, intravenous insulin at the rate of 2 mU · kg^{-1} · min^{-1} was started and continued until the end of the study (i.e., 160 min). Intravenous glucose at variable rate was also infused to maintain euglycemia (plasma glucose at 90 mg/dl) throughout the study on one occasion (clamped euglycemic study), whereas on the other two occasions, the rate of glucose infusion was decreased after 30 min to allow plasma glucose to fall and reach the target plasma glucose of 47 mg/dl at 120 min. This hypoglycemic plateau was maintained for the next 40 min, that is, until the end of the study (clamped hypoglycemic studies).

On each occasion, at 30 min, subjects ingested over a 5-min period a 200-ml drink containing either a mixture of amino acids (3.1 g lysine, 1.7 g histidine, 3.1 g arginine, 2.8 g aspartate, 2.3 g threonine, 2 g serine, 1 g glutamate, 3.3 g proline, 2.7 g glycine, 1.7 g alanine, 1.1 g cysteine, 3 g valine, 0.7 g methionine, 2.7 g isoleucine, 4.6 g leucine, 4.1 g tyrosine, 0.5 g phenylalanine, 0.9 g tryptophan, and 2.1 g glutamine) or a seemingly identical placebo rendered palatable by flavoring it with artificial fruit flavor and adding 2 g sucrose and 1 g aspartame. All drinks were prepared and administered by a research nurse not involved in the further execution of the study. The mixture of amino acids was given both in the clamped euglycemia study (euglycemia–amino acid study) and in one of the two clamped hypoglycemia studies (hypoglycemia–amino acid study), whereas in the other clamped hypoglycemia study subjects ingested placebo (hypoglycemia-placebo study).

In all studies, blood samples were drawn at 5- to 10-min intervals for bedside plasma glucose measurement and at 30-min intervals for measurement of plasma insulin, C-peptide, pancreatic polypeptide, amino acid, counterregulatory hormone, and nonglucose substrate concentration (see below).

A semiquantitative symptom questionnaire (25) was administered every 30 min. Subjects were asked to score from 0 (none) to 5 (severe) on each of the following symptoms: seven autonomic/neurogenic (adrenergic: heart pounding, tremor, anxiety, and irritability; cholinergic: sweating, hunger, and tingling); five neuroglycopenic (difficulty in thinking, weakness, dizziness, blurred vision, and drowsiness); and three nonspecific (thirst, nausea, and headache) (26). The sum of each of these constituted the total symptom score.

In addition, at baseline, before inducing hypoglycemia, and at the hypoglycemic plateau (indicated as "time −30," "time 0," and "time 120," respectively) and at similar times during the euglycemic studies, cognitive function was assessed by applying a battery of hypoglycemia-sensitive tests: Trail-Making parts A and B (27); verbal fluency (28); verbal memory (28); digit vigilance (28); forward and backward digit span (29); Stroop word, color, and color-word (interference) subtests (30); and the Paced Auditory Serial Addition Test (PASAT; 2 and 3 s) (31). Tests were always performed in this order.

Analytical methods. Plasma glucose was measured by means of a Beckman glucose analyzer (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Plasma insulin, C-peptide, pancreatic polypeptide, counterregulatory hormone, glucagon, adrenaline, norepinephrine, glycerol, β-OH-butyrate, lactate, and alanine were measured by previously described assays (32). Plasma concentrations of amino acids were measured by high-performance liquid chromatography with postcolumn o-phalaldehyde derivatization (33). To remove antibody-bound insulin, plasma was mixed with an equal volume of 30% polyethylene glycol immediately after blood collection in both type 1 diabetic and nondiabetic subjects (34). A1C was determined by high-performance liquid chromatography using a Hi-AUTO A1C, TM HA 8121 apparatus (DIC; Kyoto Daiichi, Kogaku, Japan). Plasma free fatty acid (FFA) concentrations were measured using a commercial kit (Wako NEFA C test kit; Wako Chemicals, Neuss, Germany).

Statistical analysis. All data were subjected to repeated-measures ANOVA with Huynh-Feldt adjustment for nonsphericity (35). The ANOVA model included the sequence of studies as the between-subjects factor, whereas test condition (hypoglycemia-placebo/hypoglycemia–amino acid/euglycemia–amino acid) and time were the within-subjects factors. Subjects were entered into the model as random factors. The factor group (nondiabetic/diabetic subjects) was entered into the model as a between-subjects factor. If there were significant differences between baseline values, these were used as covariates. In this way, the data over the serial time points could be adjusted for any differences in baseline values (35). Post hoc comparisons (Newman-Keuls test) were carried out to pinpoint specific differences on significant interaction terms.

The area under the curves (AUCs) for counterregulatory hormones and substrates at the clamped hypoglycemia period (120–160 min) were calculated according to the trapezoidal rule. The incremental AUC (iAUC) for glucagon response was calculated by subtracting plasma glucagon values before amino acid ingestion from the total AUC.

A modified Bonferroni procedure (36) for multiple cognitive test adjustments was used to maintain an overall type 1 error rate of 5% ($\alpha = 0.05$).

Data are given as means ± SE, except where SD is specified. We considered differences to be statistically significant if the P value was 0.05 or less. We conducted the statistical analyses by using NCSS 2007 software (Kaysville, UT) and Statistica software, version 6.0 (StatSoft, Tulsa, OK).

RESULTS

Plasma glucose and insulin concentrations and rates of glucose infusion (Fig. 1). In all study conditions, plasma glucose was maintained at the preselected plateaus, with no differences between groups and study conditions ($P > 0.2$).

Plasma insulin concentrations achieved during the study did not differ between nondiabetic and diabetic subjects or in relation to treatment conditions. On average, baseline plasma insulin concentrations were lower in nondiabetic compared with diabetic subjects ($P < 0.001$) in all study conditions.

The rates of glucose infusion were lower during the hypoglycemia plateau in the hypoglycemia–amino acid

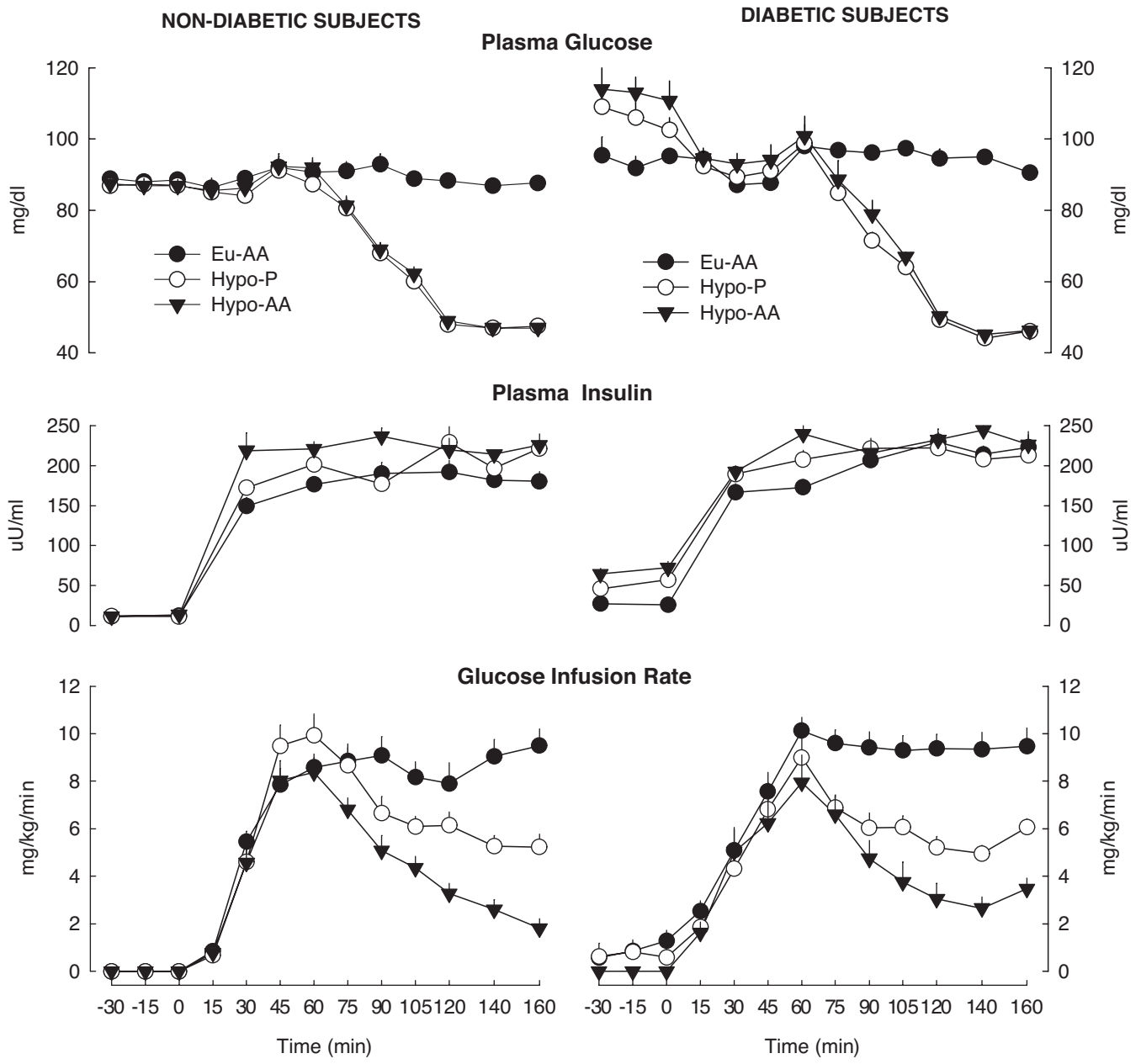

FIG. 1. Plasma glucose and free insulin concentrations and rates of glucose infusion during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

study compared with hypoglycemia-placebo and euglycemia–amino acid studies in nondiabetic subjects (2.6 ± 0.4 vs. 5.5 ± 0.6 and 8.8 ± 0.9 mg · kg^{-1} · min^{-1}, respectively, $P < 0.001$) and in diabetic subjects (3.1 ± 0.7 vs. 5.4 ± 0.5 and 9.4 ± 0.9 mg · kg^{-1} · min^{-1}, respectively, $P < 0.001$). Glucose infusion rates were not different between nondiabetic and diabetic subjects ($P = 0.661$).

Plasma glucagon, C-peptide, and pancreatic polypeptide concentrations. Plasma glucagon concentrations were similar at baseline in nondiabetic and diabetic subjects in all study conditions. In the hypoglycemia-placebo study, plasma glucagon concentrations after an initial decrease at 60 min increased ($P = 0.001$) in nondiabetic subjects, whereas it did not increase in diabetic subjects ($P = 0.867$). The ingestion of amino acids in the hypoglycemia–amino acid study increased significantly the response of glucagon to hypoglycemia in nondiabetic and, to a lesser extent, in diabetic subjects compared with the hypoglycemia-placebo study (Table 1). Amino acids stimulated glucagon response also in the euglycemia–amino acid study, which was maximal between 75–120 min and then tended to decrease to baseline values at 120–160 min. In diabetic subjects, the response of glucagon to hypoglycemia stimulated by amino acids was lower than that of nondiabetic subjects. However, it was superimposable on that of nondiabetic subjects in the hypoglycemia-placebo study ($P = 0.581$; Table 1). Interestingly, although the iAUC of glucagon response in the hypoglycemia–amino acid study was 4.7 times greater in nondiabetic compared with diabetic subjects (7,468 ± 835 and 1,599 ± 142 ng/l, respectively, $P < 0.001$), the estimated contribution of amino acids per se in directly stimulating glucagon secretion, as derived from the

TABLE 1
Basal plasma levels, maximal concentrations, and AUCs for glucagon, adrenaline, noradrenaline, cortisol, growth hormone, and pancreatic polypeptide in 10 nondiabetic and 10 type 1 diabetic subjects studied during clamped hypoglycemia (120–160 min) with amino acid and placebo ingestion and during clamped euglycemia (120–160 min) with amino acid ingestion

	Nondiabetic subjects				Type 1 diabetic subjects			
	Hypoglycemia-placebo	Hypoglycemia–amino acids	Euglycemia–amino acids	*P* value	Hypoglycemia-placebo	Hypoglycemia–amino acids	Euglycemia–amino acids	*P* value
Glucagon								
Basal levels (ng/l)	67 ± 4	69 ± 5	77 ± 4	0.373	49 ± 7	56 ± 4	51 ± 3	0.843
C_{max} (ng/l)	142 ± 20	394 ± 36§	137 ± 18	0.001	49 ± 9	124 ± 25§	64 ± 9	0.016
AUC ($ng \cdot l^{-1} \cdot min^{-1}$)	116 ± 16	318 ± 30§	111 ± 13	0.001	44 ± 7	101 ± 18§	57 ± 7	0.028
Adrenaline								
Basal levels (nmol/l)	0.2 ± 0.1	0.1 ± 0.1†	0.4 ± 0.1§	0.034	0.5 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.756
C_{max} (nmol/l)	2.8 ± 0.4	2.7 ± 0.5	0.7 ± 0.1§	0.001	1.6 ± 0.4	1.3 ± 0.1	0.8 ± 0.3	0.165
AUC ($nmol \cdot l^{-1} \cdot min^{-1}$)	2.1 ± 0.4	1.6 ± 0.3	0.4 ± 0.1§	0.003	1.0 ± 0.2	0.8 ± 0.1	0.5 ± 0.1§	0.048
Noradrenaline								
Basal levels (nmol/l)	0.8 ± 0.2	0.6 ± 0.2	1.1 ± 0.2	0.124	1.3 ± 0.3	1.0 ± 0.2	1.3 ± 0.2	0.411
C_{max} (nmol/l)	1.5 ± 0.2	1.3 ± 0.2	1.9 ± 0.2	0.132	2.0 ± 0.5	1.7 ± 0.2	1.9 ± 0.3	0.796
AUC ($nmol \cdot l^{-1} \cdot min^{-1}$)	1.2 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	0.060	1.3 ± 0.3	1.3 ± 0.2	1.2 ± 0.1	0.963
Cortisol								
Basal levels (μg/l)	9.1 ± 1.0	9.8 ± 0.9	10 ± 1.2	0.794	11.3 ± 1.3	12 ± 1.7	11.8 ± 1.1	0.846
C_{max} (μg/l)	22 ± 4.5	21 ± 4.5	11 ± 1.2§	0.013	20 ± 2.1§	17 ± 1.2	14 ± 0.4	0.014
AUC ($\mu g \cdot dl^{-1} \cdot min^{-1}$)	15 ± 3.4	13 ± 2.8	6.6 ± 0.6§	0.011	16 ± 2.1	15 ± 1.2	9.0 ± 0.5§	0.004
Growth hormone								
Basal levels (μg/l)	0.6 ± 0.2	0.9 ± 0.3	0.9 ± 0.3	0.451	2.0 ± 1.6	2.0 ± 1.9	1.6 ± 1.2	0.772
C_{max} (μg/l)	17 ± 3.7	16 ± 2.0	3.2 ± 1.7§	0.002	19 ± 3.1	20 ± 3.9	3.1 ± 0.9§	0.001
AUC ($\mu g \cdot l^{-1} \cdot min^{-1}$)	9.5 ± 2.3	7.5 ± 1.0	0.5 ± 0.2§	0.001	8.7 ± 1.9	8.4 ± 1.8	0.9 ± 0.6§	0.003
Pancreatic polypeptide								
Basal levels (pmol/l)	22 ± 1	23 ± 1.3	24 ± 1.2	0.799	18 ± 1.1	21 ± 1.1	20 ± 1.2	0.684
C_{max} (pmol/l)	168 ± 19†	194 ± 11§	30 ± 4.0	0.001	127 ± 40	141 ± 34	23 ± 3.0§	0.012
AUC ($pmol \cdot l^{-1} \cdot min^{-1}$)	131 ± 21	143 ± 11	26 ± 2.0§	0.001	70 ± 23†	80 ± 21§	20 ± 3.0	0.047

Data are means ± SE. Basal levels are the average of −30- and 0-min values. *P* values calculated from repeated-measures ANOVA. §/†Significant within-group differences.

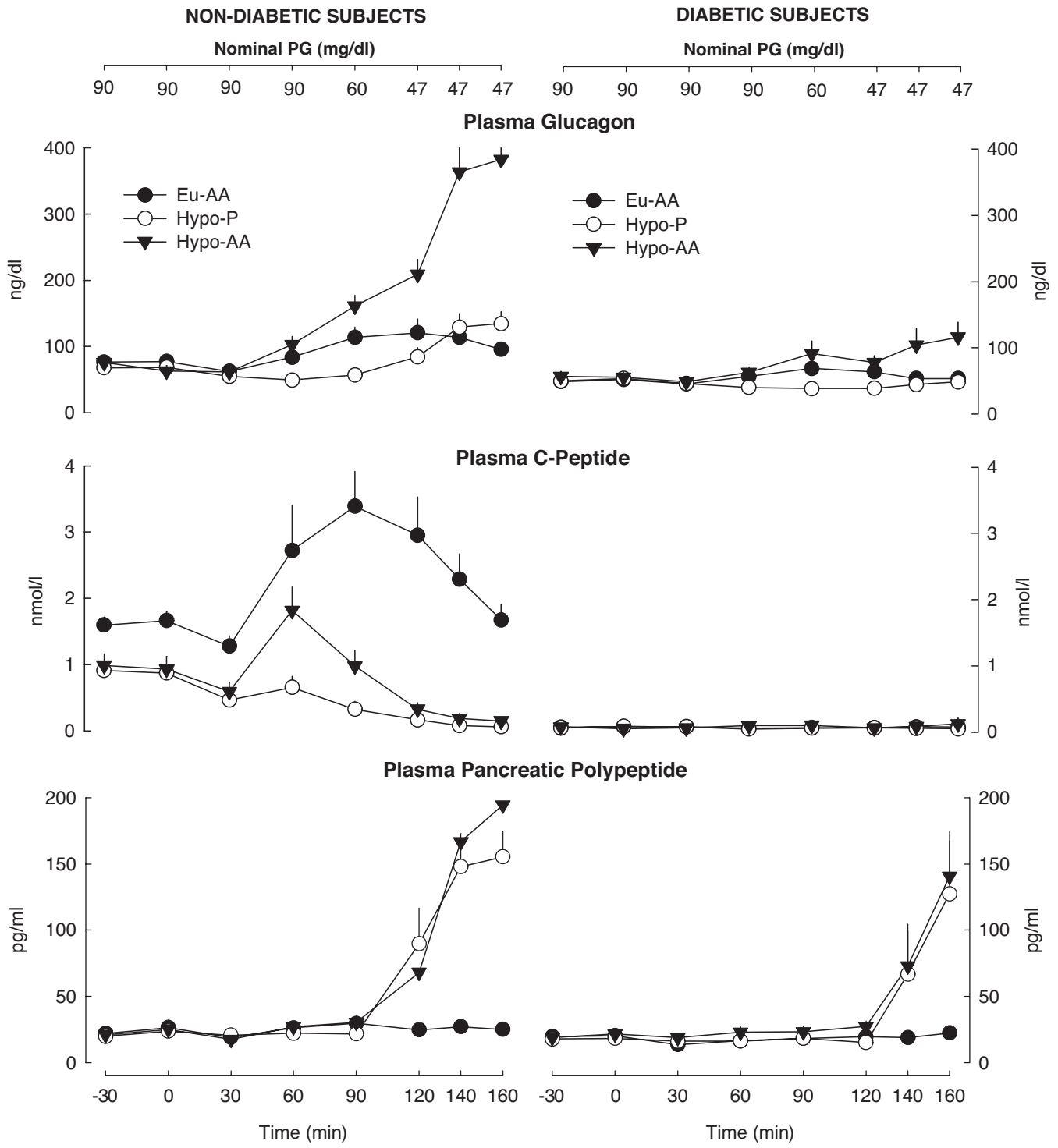

FIG. 2. Plasma glucagon, C-peptide, and pancreatic polypeptide concentrations during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

euglycemia–amino acid study, was not statistically different in both nondiabetic and diabetic subjects (19 ± 4 and 35 ± 7%, respectively, $P = 0.061$).

Plasma C-peptide concentrations in nondiabetic subjects decreased at 30 min in all studies and then remained suppressed in the hypoglycemia-placebo study, whereas they increased after the ingestion of amino acids more in the euglycemia–amino acid than in the hypoglycemia–amino acid study ($P < 0.05$, Fig. 2). By the end of the study, however, C-peptide concentrations returned to basal values and were suppressed in the euglycemia–amino acid and hypoglycemia–amino acid studies, respectively. Plasma C-peptide concentrations were undetectable in diabetic subjects in all studies.

Plasma pancreatic polypeptide concentration increased during hypoglycemia-placebo and hypoglycemia–amino acid studies in both nondiabetic and diabetic subjects, although the peak response was higher in the hypoglycemia–amino acid study in nondiabetic subjects but not in diabetic subjects (Table 1). Pancreatic polypep-

tide levels did not change in euglycemia–amino acid studies.

Plasma adrenaline, norepinephrine, cortisol, and growth hormone concentrations. Plasma adrenaline levels increased in the hypoglycemia-placebo and hypoglycemia–amino acid studies in both nondiabetic and diabetic subjects. However, responses were lower in diabetic compared with nondiabetic subjects (Table 1; $P < 0.05$). In the euglycemia–amino acid study, adrenaline levels did not change compared with basal values in both groups (Table 1).

Plasma norepinephrine concentrations were not different in all study conditions in both nondiabetic and diabetic subjects (Table 1; $P > 0.2$).

Responses of plasma cortisol and growth hormone increased similarly in response to hypoglycemia-placebo and hypoglycemia–amino acid compared with euglycemia–amino acid in both nondiabetic and diabetic subjects (Fig. 3; Table 1).

Plasma nonglucose substrate (Fig. 4). Plasma FFA levels decreased similarly in all studies in both nondiabetic and diabetic subjects. However, FFA concentrations at 120–160 min were higher in the hypoglycemia-placebo and hypoglycemia–amino acid compared with euglycemia–amino acid studies ($P = 0.018$) in nondiabetic subjects. They were not different in diabetic subjects ($P = 0.712$).

Plasma glycerol concentrations decreased from basal values in all studies in both nondiabetic and diabetic subjects. However, similar to FFA, glycerol levels were higher in the hypoglycemia-placebo and hypoglycemia–amino acid compared with euglycemia–amino acid studies ($P = 0.018$) only in nondiabetic subjects.

Plasma β-OH-butyrate concentrations, after an initial decrease to nadir values at 90 and 60 min in nondiabetic and diabetic subjects, respectively, increased in the hypoglycemia–amino acid and euglycemia–amino acid but not in the hypoglycemia-placebo studies in both nondiabetic ($P < 0.001$) and diabetic subjects ($P < 001$).

Plasma lactate concentrations increased in all studies in both nondiabetic and diabetic subjects. In nondiabetic subjects, plasma lactate increased more in the hypoglycemia-placebo compared with both the hypoglycemia–amino acid ($P = 0.025$) and the euglycemia–amino acid ($P = 0.008$) studies. In diabetic subjects, lactate was similar in the hypoglycemia-placebo, hypoglycemia–amino acid, and euglycemia–amino acid studies ($P > 0.2$). Overall, plasma lactate response in nondiabetic was greater than that of diabetic subjects (nondiabetic vs. diabetic subjects: hypoglycemia-placebo, $P < 0.001$; hypoglycemia–amino acid, $P = 0.005$; and euglycemia–amino acid, $P = 0.046$).

Plasma branched and nonbranched chain amino acid concentrations. Branched and nonbranched chain amino acid (BCAA and N-BCAA, respectively) concentrations were similar at baseline in nondiabetic and diabetic subjects in all studies (Fig. 5). After the ingestion of amino acids, both BCAA and N-BCAA levels increased similarly in the hypoglycemia–amino acid and euglycemia–amino acid studies. In the hypoglycemia-placebo studies, both BCCA and N-BCCA concentrations tended to decrease from basal values by the end of study with no difference between the two groups (Fig. 5).

Symptoms. The score of autonomic and neuroglycopenic symptoms increased in the hypoglycemia-placebo and hypoglycemia–amino acid studies with no difference ($P > 0.2$) between studies in both nondiabetic and diabetic subjects, whereas it did not change in the euglycemia–amino acid studies in nondiabetic and diabetic subjects (Fig. 6).

Cognitive function. With the exception of Trail-Making part A, digit vigilance, and Stroop words and colors tests in nondiabetic subjects and of Trail-Making part A and digit vigilance tests in diabetic subjects, all cognitive tests deteriorated significantly during hypoglycemia compared with euglycemia–amino acid, in both hypoglycemia-placebo and hypoglycemia–amino acid (Table 2). However, the degree of deterioration was lower in hypoglycemia–amino acid than in hypoglycemia-placebo in the following tests: Trail-Making part B, PASAT (2 s), digit span forward, and Stroop colored words and verbal memory tests in nondiabetic subjects and Trail-Making part B, digit span backwards, and Stroop color tests in diabetic subjects.

DISCUSSION

The present study was undertaken to examine the effects of the ingestion of a mixture of amino acids on the counterregulatory, symptomatic, and cognitive responses to hypoglycemia. Both nondiabetic and type 1 diabetic subjects were studied. First, the results indicate that oral amino acids enhance glucagon response to hypoglycemia in both nondiabetic and diabetic subjects, although to a lesser extent in the latter. Second, oral amino acids affect the responses of β-cells of pancreatic islets, as shown by the lower suppression of C-peptide. Third, amino acids preserve several aspects of cognitive responses to hypoglycemia, which, to the best of our knowledge, is a novel finding.

In type 1 diabetic subjects, the glucagon response to hypoglycemia with amino acid administration was nearly similar to the glucagon response to hypoglycemia alone (without amino acid administration) in the nondiabetic subjects. Such a result is in line with recent evidence indicating that the sensitivity of pancreatic α-cells to amino acids is markedly reduced in diabetic subjects compared with nondiabetic subjects and that a near-normal glucagon response is seen when the amino acid alanine is infused in diabetic subjects in hypoglycemia (13).

Interestingly, the near-normal response of glucagon to hypoglycemia after oral amino acids observed in the present study in diabetic subjects is also similar to that described after ingestion of a mixed meal (15). It is currently believed that the protein component of the meal is responsible for such an effect. As reported previously, the mechanisms of amino acid–induced amplification of the glucagon response to hypoglycemia likely involve both direct and indirect effects (13). In this study, a direct stimulatory effect of oral amino acids on glucagon response was evident at 60–90 min in both the euglycemic clamp and the hypoglycemic clamp before hypoglycemia was established. However, the stimulatory effect of oral amino acids on glucagon release was modulated by plasma glucose concentration. It was amplified by the induction of hypoglycemia in both nondiabetic and diabetic subjects but suppressed by euglycemia in both groups of subjects. This indicates that amino acids directly stimulate glucagon secretion from the α-cell (37) and that hypoglycemia amplifies the direct stimulatory effect of amino acids on glucagon release. Furthermore, oral amino acids may also act through different indirect mechanisms that involve gastroenteric peptides such as glucagon-like peptide 1 (38) and a decrease of tonic intra-islet α-cell inhibition by

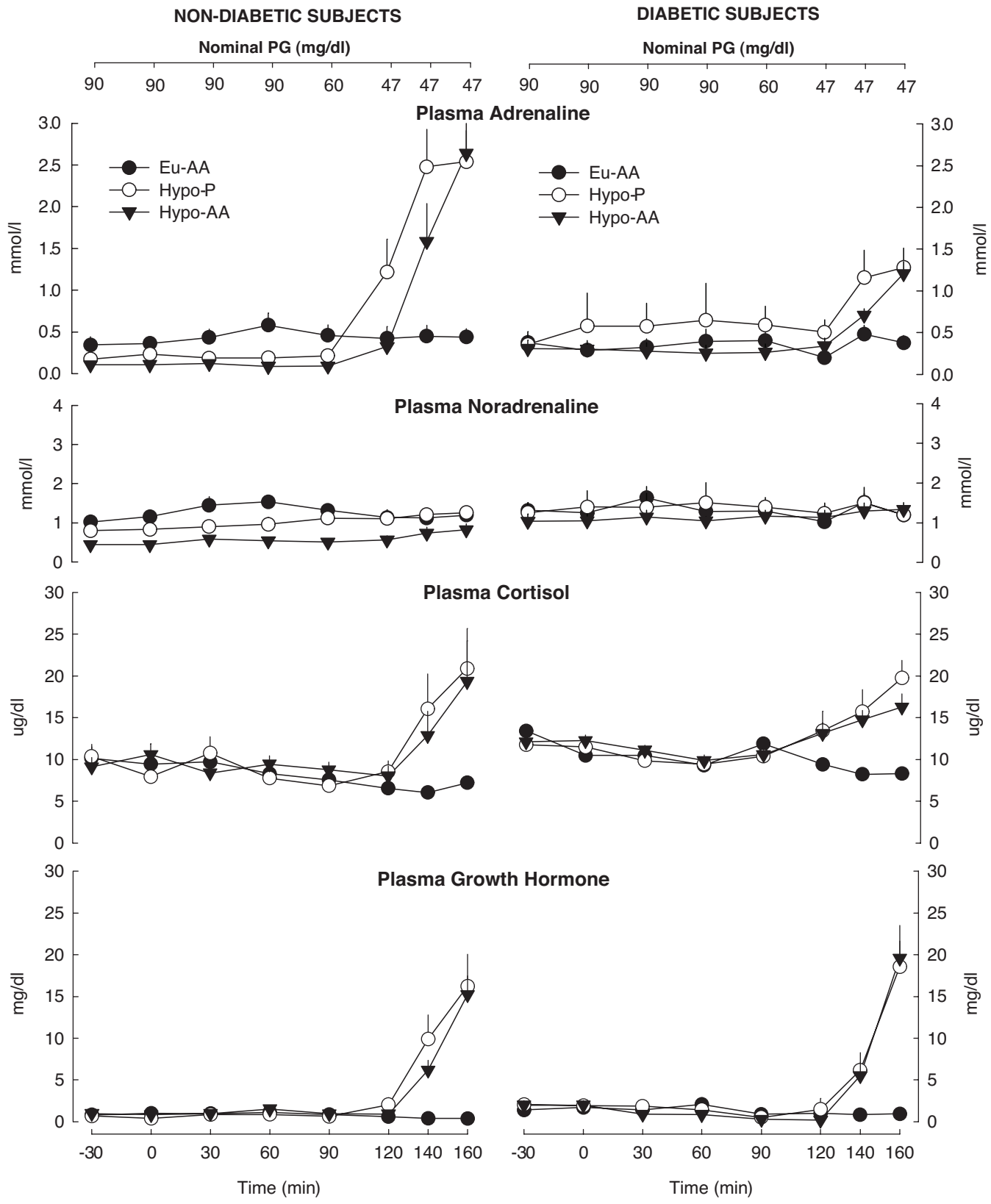

FIG. 3. Plasma adrenaline, norepinephrine, cortisol, and growth hormone concentrations during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

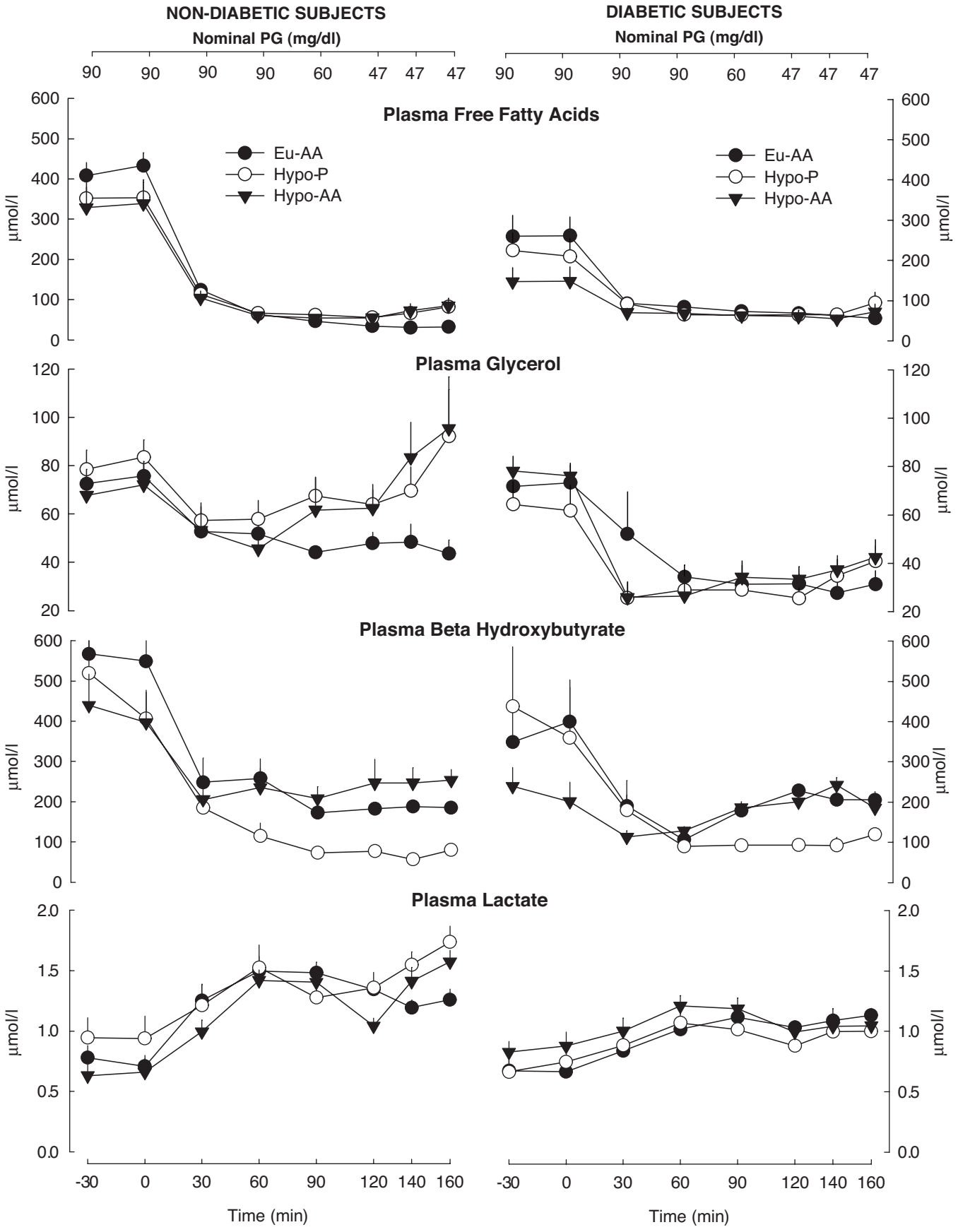

FIG. 4. Plasma concentrations of FFAs, glycerol, β-hydroxybutyrate, lactate, and alanine during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

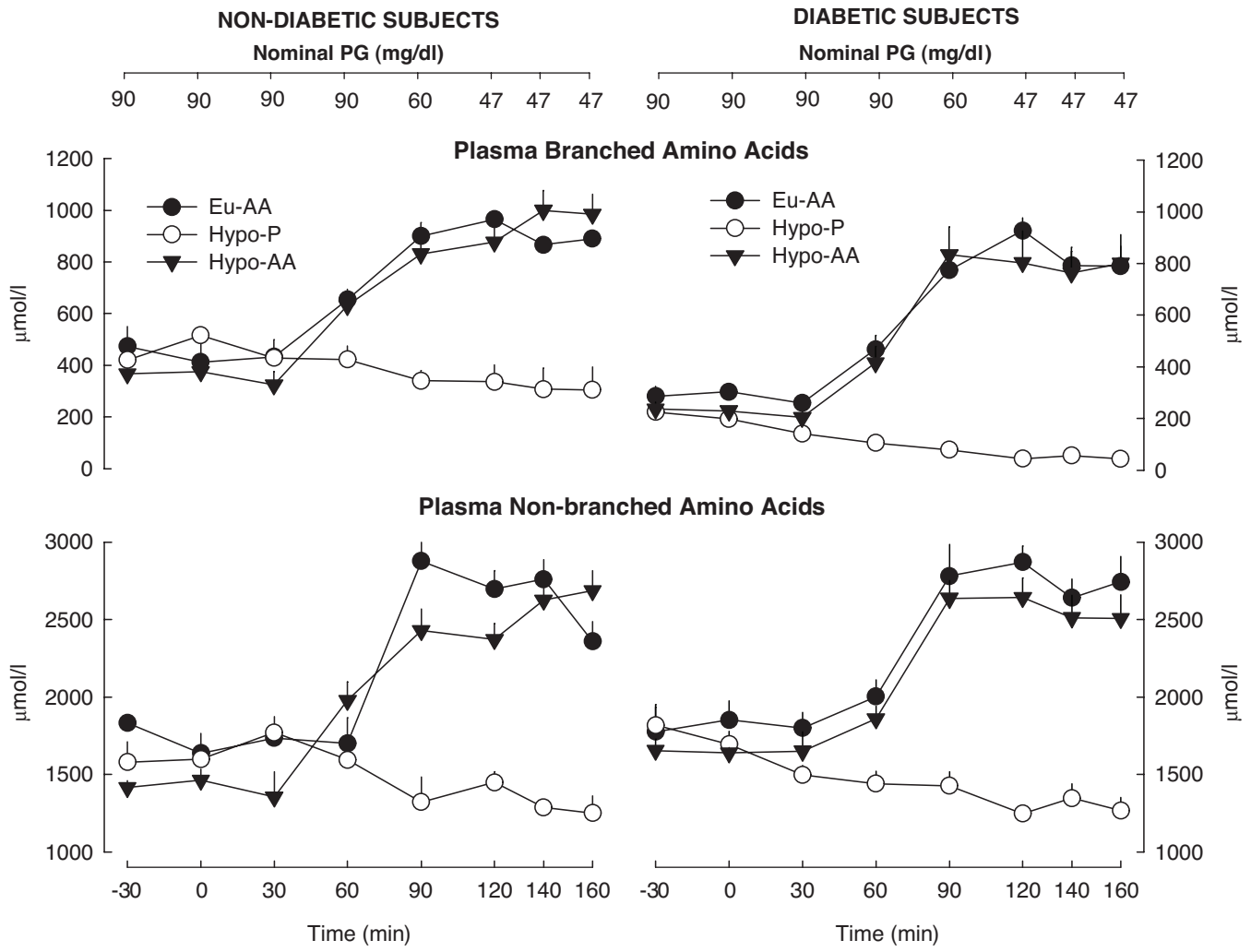

FIG. 5. Plasma concentrations of BCAAs and N-BCAAs during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

insulin and hypoglycemia (39). With regard to the latter, it is possible that the initially greater stimulation of β-cell function by amino acids was followed by a greater decrement in intra-islet insulin concentration during hypoglycemia, resulting in a greater decrease in tonic intra-islet α-cell inhibition by insulin and signaling for increased glucagon secretion during hypoglycemia in normal nondiabetic subjects (39). Most likely, the decrement in intra-islet insulin may be an important factor for the increase in glucagon secretion during hypoglycemia (40).

The ingestion of amino acids induced a greater peak response of pancreatic polypeptide compared with placebo in the hypoglycemic clamp studies in nondiabetic but not in diabetic subjects. It is well known that pancreatic polypeptide secretion after ingestion of a mixed meal is mostly mediated by the vagus nerve or by the extravagal cholinergic system (41). In addition, hypoglycemia per se represents a strong stimulus to pancreatic polypeptide secretion (41). Furthermore, autonomic vagal neuropathy blunts pancreatic polypeptide response to hypoglycemia (41). Although the diabetic subjects we studied had no evident signs of autonomic neuropathy, we cannot exclude the possibility that the lower response of pancreatic polypeptide concentrations to hypoglycemia in these subjects compared with nondiabetic subjects might be related to subclinical autonomic neuropathy or to other diabetes-related causes, including diabetes duration (41).

Elevation of plasma amino acid concentration after ingestion of amino acids did not affect counterregulatory hormones, with the exception of glucagon, and symptomatic responses to hypoglycemia, whereas the elevation clearly resulted in preservation of some aspects of cognitive function during hypoglycemia. This is a new finding that points toward a net effect of amino acids in supporting cognition while having no relevant effects on hormonal and symptomatic responses to hypoglycemia. It should be noted that previous studies indicate only a partial or no role of amino acids in sustaining cognition during hypoglycemia. In fact, Evans et al. (18) have shown that alanine infusion can sustain performance of Stroop word and color tests during hypoglycemia, although a contribution of elevated lactate levels to performance in those tests cannot be completely excluded (18). In contrast, M'Bemba et al. (12) found no effect of infusion of a dipepetide made of alanine and glutamine on a four-choice reaction time test. One possible explanation for a specific effect of amino acids on cognitive function is that elevated plasma amino acids do not affect metabolism and neurotransmission of brain centers that physiologically direct counterregulation and symptoms, whereas they may sustain brain

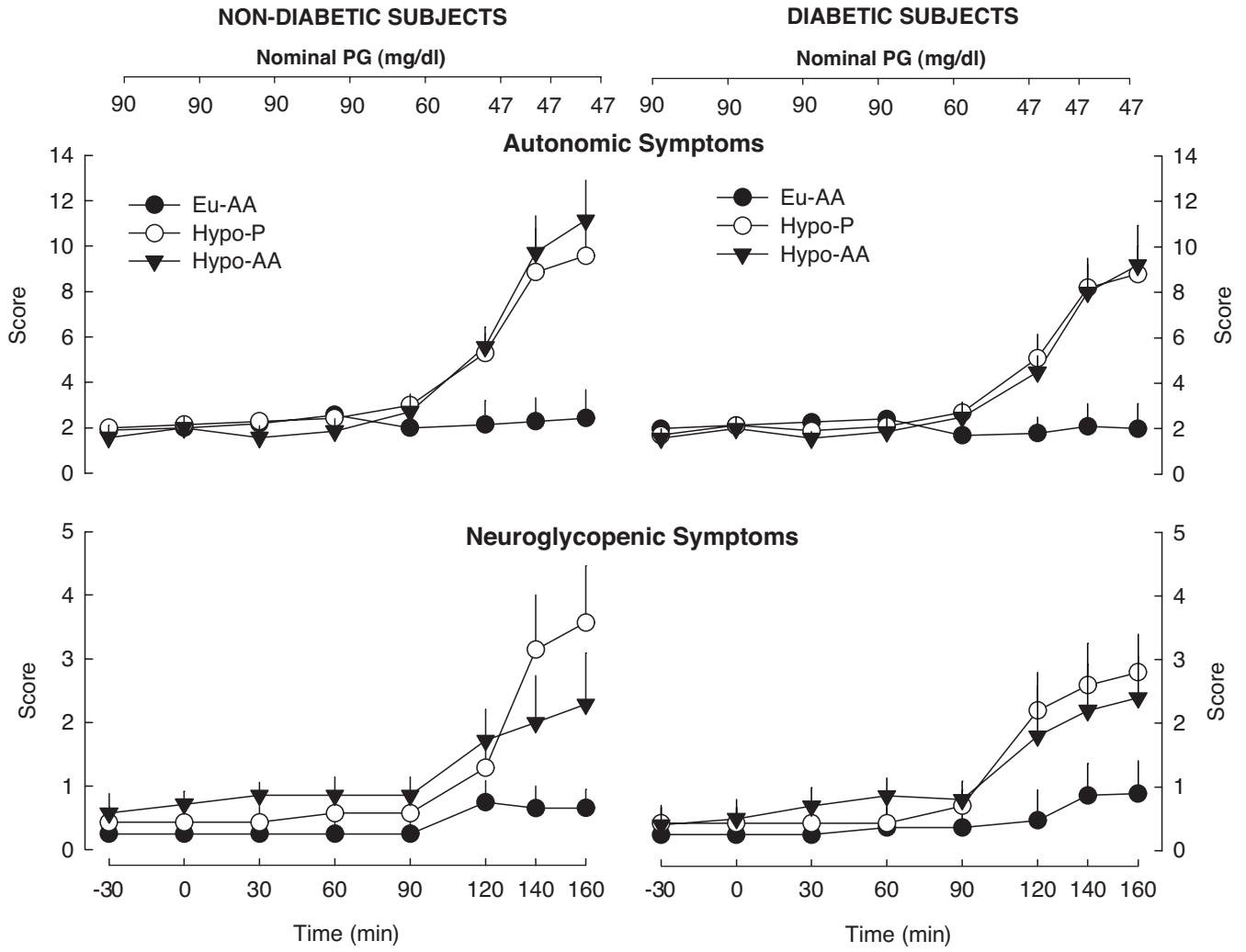

FIG. 6. Autonomic and neuroglycopenic symptom scores during clamped euglycemia with amino acid (●) ingestion and clamped hypoglycemia both with placebo (○) and amino acid (▲) ingestion in 10 nondiabetic (*left*) and 10 type 1 diabetic (*right*) subjects.

areas capable of controlling hypoglycemia-induced deterioration of cognitive function. Therefore, in contrast to the role of other nonglucose substrates such as lactate (19) and ketones (20), amino acids limit their effect only to cognitive function. That would seem advantageous because the reduced counterregulatory hormone release and lower symptoms associated with lactate (19) or ketone (20) use by the brain might weaken defensive responses to hypoglycemia and generate hypoglycemia unawareness, a well-known risk factor for severe hypoglycemia (42), even in the face of preserved cognitive function.

Earlier evidence indicates that amino acids can serve as energy sources in the brain during insulin-induced hypoglycemia (43). Therefore, under such circumstances, they can be used by neurons and glia cells as an alternative substrate to glucose to limit the detrimental effect of glucopenia on cerebral metabolism and function. However, it has been recently shown that brain amino acid (alanine and leucine) uptake is not sufficient to offset energy deficit due to reduced glucose uptake during hypoglycemia (44) and that increased availability of amino acids does not result in net brain uptake of amino acids during hypoglycemia (45). Additionally, several amino acids such as tryptophan, tyrosine, phenylalanine, histidine, and arginine are used by the brain for the synthesis of various neurotransmitters and neuromodulators (46) that play a variety of functions in the brain, including a role in the regulation of mood state, fatigue, attention, and memory (47). Therefore, on theoretical grounds, our study result of preservation of some aspects of cognitive function during hypoglycemia supplemented with oral amino acids might be due either to a direct use of amino acids by neuronal cells to derive energy and maintain cognitive function or to their conversion to neurotransmitters and/or neuromodulators able to affect cognition. However, whichever the mechanism, levels of different classes of amino acids in the brain depend largely on their availability in blood, and when individuals are exposed to stressful conditions, such as hypoglycemia, brain requirements for specific amino acids may be particularly critical. In the present study, supplementation of oral amino acids not only prevented the insulin-induced fall in plasma amino acids observed in the hypoglycemia and placebo studies, it actually increased the overall concentrations of amino acids. Likely, these increased concentrations favored amino acid use by the brain.

The battery of tests that we used assessed a broad range of cognitve aspects. Overall, amino acid ingestion sustained cognitive domains pertaining to memory, attention, psychomotor efficiency, information processing, and im-

TABLE 2
Cognitive test scores in 10 nondiabetic and 10 type 1 diabetic subjects studied during clamped hypoglycemia with amino acid and placebo ingestion and during clamped euglycemia with amino acid ingestion

	Nondiabetic subjects				Type 1 diabetic subjects			
	−30 min	0 min	160 min	*P* value	−30 min	0 min	160 min	*P* value
Nominal								
PG (Eu/Hypo) (mg/dl)	90/90	90/90	90/47		90/90	90/90	90/47	
Trail-Making part A*								
Hypoglycemia-placebo	39 ± 2.6	34 ± 5.0	70 ± 13	0.234	42 ± 5.2	60 ± 9	109 ± 27	0.072
Hypoglycemia–amino acids	36 ± 4.6	36 ± 5.7	65 ± 8.0		32 ± 4.9	68 ± 7.2	63 ± 9.1	
Euglycemia–amino acids	30 ± 3.8	67 ± 4.7	61 ± 3.6		39 ± 6.2	65 ± 9.6	78 ± 5.4	
Trail-Making part B*								
Hypoglycemia-placebo	53 ± 4.9	71 ± 9.4	89 ± 10	0.002	49 ± 2.2	64 ± 9.3	115 ± 26	0.014
Hypoglycemia–amino acids	54 ± 4.1	56 ± 7.6	74 ± 6.6†‡		52 ± 5.3	63 ± 7.8	69 ± 9.9†	
Euglycemia–amino acids	62 ± 6.4	57 ± 6.4	50 ± 2.5†		64 ± 5.6	62 ± 3.5	64 ± 6.7†	
PASAT (3 s)§								
Hypoglycemia-placebo	59 ± 0.6	58 ± 0.6	41 ± 7.5‡	0.044	55 ± 0.5	55 ± 0.2	51 ± 1.7‡	0.031
Hypoglycemia–amino acids	58 ± 0.5	58 ± 0.7	49 ± 3.2‡		59 ± 1.2	55 ± 0.4	54 ± 1.0‡	
Euglycemia–amino acids	52 ± 2.3	54 ± 1.9	57 ± 0.8		53 ± 1.7	54 ± 1.3	58 ± 1.0	
PASAT (2 s)§								
Hypoglycemia-placebo	47 ± 2.8	52 ± 2.5	33 ± 5.9	0.042	36 ± 1.2	38 ± 3.5	35 ± 3.2‡	0.038
Hypoglycemia–amino acids	50 ± 2.3	51 ± 3.0	46 ± 2.4†		40 ± 2.8	47 ± 1.7	36 ± 1.6‡	
Euglycemia–amino acids	40 ± 3.5	48 ± 3.8	45 ± 2.4†		41 ± 1.5	48 ± 1.2	45 ± 0.8	
Digit span forward¶								
Hypoglycemia-placebo	4.0 ± 0.3	4.6 ± 0.3	3.3 ± 0.5	0.034	4.2 ± 0.2	5.0 ± 0.3	3.3 ± 0.3‡	0.028
Hypoglycemia–amino acids	4.3 ± 0.1	4.5 ± 0.2	4.4 ± 0.2†		4.0 ± 0.3	3.8 ± 0.3	3.6 ± 0.2‡	
Euglycemia–amino acids	4.3 ± 0.1	4.5 ± 0.1	4.3 ± 0.2†		4.1 ± 0.1	4.2 ± 0.1	4.0 ± 0.1	
Digit span reverse¶								
Hypoglycemia-placebo	5.0 ± 0.4	4.6 ± 0.4	3.8 ± 0.6‡	0.038	4.8 ± 0.3	4.7 ± 0.1	4.0 ± 0.3	0.011
Hypoglycemia–amino acids	5.1 ± 0.4	4.4 ± 0.4	4.3 ± 0.5‡		4.9 ± 0.3	4.5 ± 0.3	4.8 ± 0.2†	
Euglycemia–amino acids	4.3 ± 0.6	4.5 ± 0.4	4.8 ± 0.3		4.8 ± 0.2	4.8 ± 0.3	4.5 ± 0.3†	
Digit vigilance‖								
Hypoglycemia-placebo	33 ± 2.4	33 ± 2.1	28 ± 3.6	0.149	31 ± 1.2	36 ± 1.2	33 ± 1.1	0.065
Hypoglycemia–amino acids	33 ± 1.4	33 ± 1.9	30 ± 2.5		33 ± 1.4	38 ± 1.4	32 ± 1.0	
Euglycemia–amino acids	32 ± 2.1	31 ± 2.7	33 ± 1.5		36 ± 1.4	32 ± 1.3	37 ± 1.5	
Verbal fluency**								
Hypoglycemia-placebo	9.5 ± 0.5	11.3 ± 0.9	8.2 ± 1.3‡	0.001	13.5 ± 0.1	12.4 ± 1.0	10.8 ± 0.9‡	0.002
Hypoglycemia–amino acids	13 ± 0.9	11.4 ± 1.0	9.8 ± 1.1‡		12.5 ± 0.3	10.8 ± 0.7	9.0 ± 0.6‡	
Euglycemia–amino acids	12.3 ± 0.3	11.8 ± 0.6	13.8 ± 0.7		11.0 ± 1.0	12.0 ± 1.4	14.0 ± 1.0	
Stroop word††								
Hypoglycemia-placebo	94 ± 5.8	104 ± 8.0	80 ± 15.8	0.458	104 ± 4.0	98 ± 3.8	96 ± 1.9‡	0.014
Hypoglycemia–amino acids	107 ± 7.8	104 ± 7.3	94 ± 8.0		103 ± 1.4	108 ± 1.7	102 ± 1.3‡	
Euglycemia–amino acids	98 ± 4.3	103 ± 5.0	104 ± 5.5		106 ± 3.3	109 ± 4.1	114 ± 2.8	
Stroop color††								
Hypoglycemia-placebo	73 ± 3.2	82 ± 3.4	55 ± 10	0.066	78 ± 2.5	79 ± 2.9	59 ± 0.8	0.002
Hypoglycemia–amino acids	78 ± 4.1	79 ± 2.7	69 ± 3.8		76 ± 0.9	77 ± 1.7	75 ± 2.3†‡	
Euglycemia–amino acids	74 ± 1.2	79 ± 3.1	79 ± 1.1		81 ± 2.5	83 ± 2.5	87 ± 1.9	
Stroop color-words††								
Hypoglycemia-placebo	51 ± 2.3	55 ± 1.3	38 ± 6.8	0.025	48 ± 1.7	52 ± 0.9	50 ± 0.4‡	0.042
Hypoglycemia–amino acids	62 ± 3.2	59 ± 2.0	51 ± 2.0†‡		49 ± 0.5	50 ± 1.7	49 ± 1.6‡	
Euglycemia–amino acids	56 ± 2.6	55 ± 2.2	60 ± 3.2		46 ± 2.1	53 ± 1.5	60 ± 1.7	
Verbal memory test‡‡								
Hypoglycemia-placebo	4.8 ± 0.1	3.6 ± 0.6	2.2 ± 0.7	0.042	5.0 ± 0.0	5.0 ± 0.0	3.5 ± 0.4‡	0.015
Hypoglycemia–amino acids	4.3 ± 0.2	4.2 ± 0.3	3.4 ± 0.5†		5.0 ± 0.0	4.8 ± 0.1	4.0 ± 0.3‡	
Euglycemia–amino acids	4.2 ± 0.2	4.0 ± 0.4	3.2 ± 0.5†		4.8 ± 0.1	4.8 ± 0.1	4.5 ± 0.2	

Data are means ± SE. *P* values are calculated from repeated-measures ANOVA. †$P < 0.05$ vs. hypoglycemia-placebo. ‡$P < 0.05$ vs. euglycemia–amino acids. *Time(s) required to complete the task. §Number of correct responses. ¶Number of digit sequences correctly repeated. ‖Number of correct targets crossed out in 90 s. **Number of words named in 60 s. ††Number of correct responses in 45 s. ‡‡Number of words recalled.

mediate memory in nondiabetic subjects and all of these except immediate memory in diabetic subjects. This intergroup difference can be most likely attributed either to diabetes or to a general verbal ability and/or general intelligence condition. Notably, these results have been obtained with plasma amino acid concentrations in the physiological range of the postprandial condition in humans (~2–3 mmol/l) (15).

Plasma β-OH-butyrate concentrations were suppressed in all studies. However, they were less suppressed after oral amino acid ingestion in both euglycemia and hypoglycemia. Their use by the brain as an alternative substrate to

sustain brain metabolism and function during hypoglycemia cannot be completely excluded. However, this is unlikely because, first, the levels of β-OH-butyrate in our study were much lower than those that have been shown to be effective in maintaining cognitive function during hypoglycemia in previous studies (20), and second, β-OH-butyrate diminished counterregulatory and symptomatic responses to hypoglycemia in those studies (20) but not in our present study.

In conclusion, the present study indicates that oral amino acids improve some aspects of cognitive function in response to hypoglycemia and potentiate (in nondiabetic subjects) and recover (in subjects with type 1 diabetes) responses of glucagon to hypoglycemia. Additional studies are required to prove that these beneficial effects of oral amino acids during hypoglycemia translate into clinical advantages for people with type 1 diabetes and to possibly demonstrate that they contribute to reduction of the risk of severe hypoglycemia in type 1 diabetes.

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REFERENCES

1. Gerich J, Langlois M, Noacco C, Karam J, Forsham P: Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha-cell defect. *Science* 182:171, 1973
2. Bolli G, de Feo P, Compagnucci P, Cartechini MG, Angeletti G, Santeusanio F, Brunetti P, Gerich JE: Abnormal glucose counterregulation in insulin-dependent diabetes mellitus: interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. *Diabetes* 32:134–141, 1983
3. Bolli GB, Fanelli CG: Physiology of glucose counterregulation to hypoglycemia. In *Hypoglycemic Disorders*: *Endocrinology and Metabolism Clinics of North America*. Vol. 28. Service FJ, Ed. W.B. Saunders, Philadelphia, 1999, p. 467–493
4. Cryer PE: Hypoglycemia: the limiting factor in the management of IDDM. *Diabetes* 43:1378–1389, 1994
5. White NH, Skor DA, Cryer PE, Levandoski LA, Bier DM, Santiago JV: Identification of type 1 diabetic patients at increased risk for hypoglycemia during intensive therapy. *N Engl J Med* 308:485–491, 1983
6. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
7. Fanelli C, Pampanelli S, Epifano L, Rambotti AM, Di Vincenzo A, Modarelli F, Ciofetta M, Lepore M, Annibale B, Torlone E, Perriello G, De Feo P, Santeusanio F, Brunetti P, Bolli GB: Long-term recovery from unawareness, deficient counterregulation and lack of cognitive dysfunction during hypoglycaemia, following institution of rational, intensive insulin therapy in IDDM. *Diabetologia* 37:1265–1276, 1994
8. Bolli GB: How to ameliorate the problem of hypoglycemia in intensive as well as non-intensive treatment of type 1 diabetes mellitus. *Diabetes Care* 22 (Suppl. 2):B43–B52, 1999
9. Rossetti P, Porcellati F, Bolli GB, Fanelli CG: Prevention of hypoglycemia while achievinggood glycemic control in type 1 diabetes mellitus: the role of insulin analogs. *Diabetes Care* 31 (Suppl. 2):S113–S120, 2008
10. Kuhara T, Ikeda S, Ohneda A, Sasaki Y: Effects of intravenous infusion of 17 amino acids on the secretion of Gh, glucagon and insulin in sheep. *Am J Physiol* 260:E21–E26, 1991
11. Caprio S, Tamborlane WV, Zych K, Gerow K, Sherwin RS: Loss of potentiating effect of hypoglycemia on the glucagon response to hyperaminoacidemia in IDDM. *Diabetes* 42:550–555, 1993
12. M'bemba J, Cynober L, de Bandt P, Taverna M, Chevalier A, Bardin C, Slama G, Selam JL: Effects of dipeptide administration on hypoglycaemic counterregulation in type 1 diabetes. *Diabete Metab* 29:412–417, 2003
13. Porcellati F, Pampanelli S, Rossetti P, Busciantella Ricci N, Marzotti S, Lucidi P, Santeusanio F, Bolli GB, Fanelli CG: Effect of the amino acid alanine on glucagon secretion in non-diabetic and type 1 diabetic subjects during hyperinsulinaemic euglycaemia, hypoglycaemia and post-hypoglycaemic hyperglycaemia. *Diabetologia* 50:422–430, 2007
14. Fanelli CG, Pampanelli S, Porcellati F, Bartocci L, Scionti L, Rossetti P, Bolli GB: Rate of fall of blood glucose and physiological responses of counterregulatory hormones, clinical symptoms and cognitive function to hypoglycaemia in type I diabetes mellitus in the postprandial state. *Diabetologia* 46:53–64, 2003
15. Porcellati F, Pampanelli S, Rossetti P, Cordoni C, Marzotti S, Scionti L, Bolli GB, Fanelli CG: Counterregulatory hormone and symptom responses to insulin-induced hypoglycemia in the postprandial state in humans. *Diabetes* 52:2774–2783, 2003
16. Wiethop BV, Cryer PE: Alanine and terbutaline in treatment of hypoglycemia in IDDM. *Diabetes Care* 16:1131–1136, 1993
17. Wiethop BV, Cryer PE: Glycemic actions of alanine and terbutaline in IDDM. *Diabetes Care* 16:1124–1130, 1993
18. Evans ML, Hopkins D, Macdonald IA, Amiel SA: Alanine infusion during hypoglycaemia partly supports cognitive performance in healthy human subjects. *Diabet Med* 21:440–446, 2004
19. Maran A, Cranston I, Lomas J, Macdonald I, Amiel SA: Protection by lactate of cerebral function during hypoglycaemia. *Lancet* 343:16–20, 1994
20. Veneman T, Mitrakou A, Mokan M, Cryer P, Gerich J: Effect of hyperketonemia and hyperlacticacidemia on symptoms, cognitive dysfunction, and counterregulatory hormone responses during hypoglycemia in normal humans. *Diabetes* 43:1311–1317, 1994
21. Ewing DJ, Clarke BF: Autonomic neuropathy: its diagnosis and prognosis. *Clin Endocrinol Metab* 15:855–888, 1986
22. Fanelli CG, Epifano L, Rambotti AM, Pampanelli S, Di Vincenzo A, Modarelli F, Lepore M, Annibale B, Ciofetta M, Bottini P, Porcellati F, Scionti L, Santeusanio F, Brunetti P, Bolli GB: Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM. *Diabetes* 42:1683–1689, 1993
23. McGuire E, Helderman J, Tobin J, Andres R, Berman M: Effects of arterial venous sampling on analysis of glucose kinetics in man. *J Appl Physiol* 41:565–573, 1976
24. De Feo P, Perriello G, Ventura MM, Calcinaro F, Basta G, Lolli C, Cruciani C, Dell'Olio A, Santeusanio F, Brunetti P, Bolli GB: Studies on overnight insulin requirements and metabolic clearance rate of insulin in normal and diabetic man: relevance to the pathogenesis of the dawn phenomenon. *Diabetologia* 29:475–480, 1986
25. Mitrakou A, Ryan C, Veneman T, Mokan M, Jenssen T, Kiss I, Durrant J, Cryer P, Gerich J: Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *Am J Physiol* 260:E67–E74, 1991
26. Cryer PE: Symptoms of hypoglycemia, thresholds for their occurrence, and hypoglycemia unawareness. *Endocrinol Metab Clin North Am* 28:495–500, 1999
27. Boll T, Barth J: Neuropsychology of Brain Damage. In *Handbook of Clinical Neuropsychology*. Filskov S, Boll T, Eds. New York, Wiley, 1981, p. 418–452
28. Lezak MD, Howieson DB, Loring DW, Hannay HJ, Fischer JS: *Neuropsychological Assessment*. 4th ed. New York, Oxford University Press, 2004, p. 429–550
29. Wechsler D: *Manual of the Wechsler Adult Intelligence Scale-Revised*. New York, The Psychological Corporation Limited, 1981
30. Golden C: *Stroop Color and Word Test*. Chicago, Stoelting, 1978
31. Gronwall DMA: Paced auditory serial-addition task: a measure of recovery from concussion. *Percept Mot Skills* 44:367–373, 1977
32. Fanelli CG, De Feo P, Porcellati F, Perriello G, Torlone E, Santeusanio F, Brunetti P, Bolli GB: Adreneric mechanisms contribute to the late phase of hypoglycemic glucose counterregulation in humans by stimulating lipolysis. *J Clin Invest* 89:2005–2013, 1992
33. Nair KS, Welle SL, Tito J: Effect of plasma amino acid replacement on glucagon and substrate responses to insulin-induced hypoglycemia in humans. *Diabetes* 39:376–382, 1990
34. Kuzuya H, Blix PN, Horwitz DL, Steiner DF, Rubenstein AH: Determination of free and total insulin and C-peptide in insulin treated diabetics. *Diabetes* 26:22–29, 1977
35. Winer BJ, Brown DR, Michels KM: *Statistical Principles in Experimental Design*. 3rd ed. New York, McGraw-Hill, 1991, p. 497–582
36. Holland BS, Copenhaver M: Improved Bonferroni type multiple testing procedures. *Psychol Bull* 104:145–148, 1988
37. Iversen J: Secretion of glucagon from the isolated, perfused canine pancreas. *J Clin Invest* 50:2123–2136, 1971
38. Dunning BE, Foley JE, Ahrén B: Alpha cell function in health and disease:

influence of glucagon-like peptide-1. *Diabetologia* 48:1700–1713, 2005
39. Raju B, Cryer PE: Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin deficient diabetes. *Diabetes* 54:757–764, 2005
40. Banarer S, McGregor VP, Cryer PE: Intraislet hyperinsulinemia prevents the glucagon response to hypoglycemia despite an intact autonomic response. *Diabetes* 51:958–965, 2002
41. Krarup T, Schwartz TW, Hilsted J, Madsbad S, Overlaege O, Sestoft L: Impaired response of pancreatic polypeptide to hypoglycaemia: an early sign of autonomic neuropathy in diabetics. *Br Med J* 2:1544–1546, 1976
42. Gold AE, MacLeod KM, Frier BM: Frequency of severe hypoglycemia in patients with type 1 diabetes with impaired awareness of hypoglycemia. *Diabetes Care* 17:697–703, 1994
43. Wong KL, Tyce GM: Glucose and amino acid metabolism in rat brain during sustained hypoglycemia. *Neurochem Res* 8:401–415, 1983
44. Lubow JM, Piñón IG, Avogaro A, Cobelli C, Treeson DM, Mandeville KA, Toffolo G, Boyle PJ: Brain oxygen utilization is unchanged by hypoglycemia in normal humans: lactate, alanine, and leucine uptake are not sufficient to offset energy deficit. *Am J Physiol Endocrinol Metab* 290: E149–E153, 2006
45. Wahren J, Ekberg K, Fernqvist-Forbes E, Nair S: Brain substrate utilisation during acute hypoglycaemia. *Diabetologia* 42:812–818, 1999
46. Betz AL, Goldstein GW, Katzman R: Blood-brain-cerebrospinal fluid barriers. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*. 5th ed, Siegel GJ, Ed. New York, Raven Press, 1994, p. 681–698
47. Lieberman HR: Nutrition, brain function and cognitive performance. *Appetite* 40:245–254, 2003