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Vascular endothelial growth factor during hypoglycemia in patients with type 1 diabetes mellitus: relation to cognitive function and renin-angiotensin system activity

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Abstract

In healthy adults, levels of vascular endothelial growth factor (VEGF) increase in response to mild hypoglycemia. VEGF is implicated in glucose transport over the blood-brain barrier, and the increase during hypoglycemia has been positively correlated with preservation of cognitive function during hypoglycemia. High activity in the renin-angiotensin system (RAS) is associated with an increased risk of severe hypoglycemia in patients with type 1 diabetes mellitus. Renin-angiotensin system possibly exerts its mechanism in hypoglycemia via VEGF. We studied the impact of mild hypoglycemia on plasma VEGF in patients with type 1 diabetes mellitus and high or low RAS activity and analyzed associations between VEGF levels and cognitive function during hypoglycemia. Eighteen patients with type 1 diabetes mellitus—9 with high and 9 with low RAS activity—underwent a single-blinded, placebo-controlled, crossover study with either mild hypoglycemia or stable glycemia. Cognitive function was assessed by the California Cognitive Assessment Package and the Alzheimer Quick Test. Nadir plasma glucose was 2.2 (0.3) mmol/L. During the control study, plasma VEGF did not change. During hypoglycemia, plasma VEGF increased from 39 to 58 pg/L in the high-RAS group ($P = .004$) and from 76 to 109 pg/L in the low-RAS group ($P = .01$), with no difference between RAS groups ($P = .9$). A weak association between reduced preservation of cognitive function during hypoglycemia and low VEGF response was observed. Plasma VEGF levels increase during mild, short-term hypoglycemia in patients with type 1 diabetes mellitus. The VEGF response is not dependent on RAS activity and only weakly associated with preservation of cognitive function during hypoglycemia. Thus, the previously described association between low RAS activity and better cognitive performance during hypoglycemia does not seem to be mediated by VEGF.

1. Introduction

During hypoglycemia, the supply of glucose to the brain depends on plasma glucose, cerebral blood flow, and transport of glucose across blood-brain barrier (BBB). The plasma glucose concentration depends on several factors, for example, the ability to mobilize stored glucose in the liver and the concentration of insulin. Although the effect of cerebral blood flow changes during hypoglycemia varies throughout the brain [1], the transport of glucose across BBB depends on the insulin-independent glucose transporter 1 (GLUT 1), which is located at very high density in the endothelial cells [2]. Under normal conditions, GLUT 1–mediated BBB glucose transport is not rate limiting for glucose metabolism in the brain. However, when blood glucose levels drop to 2 to 3 mmol/L, the transport of glucose through GLUT 1 becomes rate-limiting for glucose metabolism in the brain [3].

Vascular endothelial growth factor (VEGF) is a potent regulator of normal and pathologic angiogenesis [4]. Recent research indicates that VEGF is also implicated in glucose metabolism because VEGF enhances glucose transport across the blood-retina barrier, most likely because of increased translocation of GLUT 1 to the plasma membrane [5]. Glucose transporter 1 messenger RNA is also up-regulated by VEGF in an endothelial cellular model [6].

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Plasma VEGF has been shown to increase during hypoglycemia in a human monocytic cell line [7] and in healthy adults [8-10]. Moreover, the increase in VEGF during hypoglycemia is positively correlated with the preservation of cognitive function during hypoglycemia in healthy adults [8]. It has been proposed that VEGF is a part of the counterregulatory response to hypoglycemia because hypothalamic cells with connections to plasma glucose responsive cells can synthesize VEGF (reviewed by Peters et al [11]).

High activity of the angiotensin-converting enzyme (ACE) is associated with poorer cognitive function during hypoglycemia in healthy subjects [12]. In accordance with this, several independent studies have reported a positive correlation between serum ACE activity and risk of severe hypoglycemia in type 1 diabetes mellitus [13-16]. Other components of the renin-angiotensin system (RAS), that is, high circulating angiotensinogen concentration and the angiotensin II receptor subtype 2 genotype 1675 polymorphism, are also associated with increased risk of severe hypoglycemia [17]. The physiologic pathways involved are not known. Angiotensin II, the main effector hormone in RAS, stimulates VEGF production in smooth muscle cells [18]. However, the effect of basal RAS activity on human in vivo VEGF production during hypoglycemia is unknown.

The aims of this study were to assess plasma VEGF levels during hypoglycemia in patients with type 1 diabetes mellitus, to explore the possible influence of basal RAS activity, and to evaluate if any association exists between VEGF concentrations and cognitive function during insulin-induced mild hypoglycemia in patients with type 1 diabetes mellitus.

2. Material and methods

2.1. Design

The study was a single-blinded, controlled, crossover study. Hypoglycemia was induced with a standardized intravenous insulin infusion. Each subject was studied during hypoglycemia and at maintained baseline glycemia (designated *control*), separated by at least 4 weeks. Primary end point was plasma VEGF changes during hypoglycemia.

2.2. Subjects

Nine patients with high RAS activity and 9 patients with low RAS activity were selected from a cohort of 171 individuals with type 1 diabetes mellitus from our outpatient clinic. No subjects were treated with ACE inhibitors or angiotensin II receptor antagonists (or other antihypertensive agents) because these drugs interfere with natural-occurring ACE activity and therefore potentially alter the hypoglycemic response and levels of circulating VEGF. Selection according to RAS group was based on 3 factors, all independently contributing to risk of hypoglycemia: ACE activity [13-16], concentration of angiotensinogen [17], and the angiotensin II receptor subtype 2 genotype 1675 polymorphism [17]. Angiotensin-converting enzyme activity was scored from 1 to 4 points (lowest activity = 1 point); plasma concentration of angiotensinogen was scored from 1 to 4 points (lowest concentration = 1 point); and angiotensin II receptor subtype 2 genotype GG, AG, and AA were scored as 1, 2, and 3 points, respectively.

The frequency of diabetic complications was low: 6 had untreated background retinopathy (3 in each RAS group), 1 had microalbuminuria (in the high-RAS group), 2 had asymptomatic peripheral neuropathy (1 in each RAS group), and 1 had symptomatic autonomic neuropathy (in the high-RAS group). None of the subjects had overt kidney disease, hypertension, macrovascular disease, or heart failure; and RAS groups did not differ in baseline characteristics (Table 1). None of the subjects were treated with psychopharmacologic drugs, strong analgesics, or β-blockers, which could interfere with hypoglycemic symptoms, or had known malignant disease, which could alter VEGF concentrations. The study was approved by the Regional Ethics Committee, and written informed consent was obtained from all subjects.

2.3. Experimental protocol

2.3.1. Week before experiment

Subjects were instructed to live and eat as usual and to avoid any rigorous exercise as well as the use of alcohol or psychoactive drugs in the week preceding the experiment.

Table 1
Demographic, diabetes-related, and RAS characteristics of the 18 subjects at baseline

	RAS activity		*P*
	Low (n = 9)	High (n = 9)	
Demographic and diabetes-related characteristics			
Age (y)	41 (30-57)	39 (26-61)	.71
Sex, F/M (n)	3/6	2/7	.60
Body mass index (kg/m^2)	25.4 (19.7-29.6)	25.6 (23.0-28.7)	.86
Duration of diabetes (y)	17 (7-27)	20 (8-40)	.50
Detectable C-peptide, +/− (n)[a]	5/4	4/5	.64
Daily insulin dose (IU)	62 (42-92)	57 (42-74)	.52
Hemoglobin A_{1c} (%)	8.4 (7.3-9.2)	8.2 (6.2-9.7)	.59
Severe hypoglycemia (episodes/patient-y)	0.3 (0-2)	2.9 (0-10)	<.05
RAS characteristics at baseline			
Serum ACE activity (U/L)	28 (22-45)	59 (45-82)	–
Angiotensin II receptor subtype 2 genotype GG, AG, AA (n)	9, 0, 0	0, 0, 9	–
Plasma angiotensinogen (nmol/L)	760 (554-926)	1099 (573-3014)	–
Plasma prorenin (ng/[mL/h])	25 (12-47)	22 (3.6-47)	.57
Plasma renin (ng/[mL/h])	3.5 (1.8-5.6)	3.6 (0.8-13)	.91
Plasma angiotensin II (pmol/L)	4.7 (2.3-6.8)	3.6 (1.8-4.7)	.07
Total RAS activity (points[b])	4.4 (3-5)	9.6 (9-11)	–

Numbers are mean (range) unless otherwise stated. *P* values refer to comparisons between RAS groups.

[a] Detection limit: 10 pmol/L. [b] Possible range: 3 to 11 points.

2.3.2. Day before experiment

To avoid any possible hypoglycemia-induced prestudy changes of plasma VEGF, a continuous glucose monitoring system device (CGMS) (Medtronic MiniMed; Medtronic Diabetes, Northridge, CA) was mounted to monitor the glycemic level the night before the experiment.

2.3.3. Day of experiment

Subjects arrived at 7:30 AM in the laboratory after an overnight fast. Data from the CGMS were downloaded. If hypoglycemia had been present during the preceding night, as indicated by 2 consecutive CGMS values less than 3.5 mmol/L or 1 self-monitored blood glucose concentration less than 3.5 mmol/L, the experiment was postponed for at least 14 days. This happened at 8 occasions. Two experiments were postponed because of hyperglycemia (blood glucose >20 mmol/L). If hypoglycemia had not been present, the subject was introduced to the experimental setting, and an intravenous cannula was inserted in an antecubital vein in both forearms. The experiment was carried out in a cyclic manner with a total of 6 cycles: 2 baseline cycles, 2 cycles with hypoglycemia or maintained glycemia (control), and 2 postexperiment cycles (recovery). Each cycle began with bedside blood glucose measurement. Subsequently, venous blood was drawn for measurement of plasma glucose, plasma VEGF, and other hormones. Afterwards, bedside blood glucose was measured again. In the end of the cycles, cognitive function was assessed. In hypoglycemic cycles, bedside blood glucose was measured again at the end of the cycle.

2.4. Intravenous insulin infusions

2.4.1. Hypoglycemia

(Waters Corp, Milford, MA)After the second cycle, 500% of the subject's insulin morning dose (human insulin, Actrapid; Novo Nordisk, Bagsværd, Denmark) mixed with 500 mL of isotonic saline was infused intravenously (100 mL/h) until the bedside blood glucose was less than 5 mmol/L, after which the infusion was stopped. If the desired blood glucose level was not achieved, the insulin infusion was restarted with a lower, customized rate. In pilot studies, this dosage scheme resulted in stable hypoglycemia with nadir in the range between 2.0 and 2.5 mmol/L, which was the hypoglycemic target in this study. Bedside monitoring of blood glucose level during experiments was done every 10 minutes. After the fourth cycle, the subject drank 0.5 L of apple juice to reestablish a normal glucose level.

2.4.2. Control (maintained glycemia)

To stabilize the glucose level, human insulin (Actrapid, Novo Nordisk) and glucose were infused intravenously throughout the study. After the second cycle, an infusion of 500 mL of 10% glucose (50 mL/h) was started; and if the subject's blood glucose concentration at the end of the second cycle was between 3.5 and 4.9 mmol/L, 6 IU of insulin was added to the glucose infusion. If the blood glucose was 5 to 10 mmol/L, 10 IU of insulin was added; and if the blood glucose was greater than 10 mmol/L, 18 IU of human insulin was added.

2.5. Biochemical analyses

Bedside capillary blood glucose measurements used for monitoring during the experiments were done by a HemoCue Analyzer (HemoCue, Ängelholm, Sweden). Plasma glucose concentrations used for evaluation of the hypoglycemic challenge were measured enzymatically (COBAS INTEGRA; Roche, Basel, Switzerland). Plasma VEGF (isoform VEGF-165) was measured using enzyme-linked immunosorbent assay technique (Quantiglo Human VEGF Immunoassay; R&D Systems, Europe, Abingdon, United Kingdom; reference range, 13-182 pg/mL; mean, 61). Plasma glucagon concentrations were measured with a radioimmunoassay directed against the C-terminus of the glucagon molecule (antibody code no. 4305), which mainly measures glucagon of pancreatic origin [19]. Catecholamines were determined by high-performance liquid chromatography with fluorometric detection [20]. Serum growth hormone concentration was determined with a commercial fluorometric method (Delfia; Wallac, Turku, Finland) using 2 monoclonal antibodies against 2 separate antigenic determinants on the growth hormone molecule. (Waters Corp, Milford, MA) Concentrations of cortisol were determined by routine method. Plasma renin and prorenin were determined by radioimmunoassays of generated angiotensin I [21]. Plasma angiotensinogen was determined as the maximum quantity of angiotensin I generated during incubation of plasma in the presence of excess recombinant human renin [21]. Serum ACE activity was determined by a commercial kinetics-based assay (Sigma Diagnostics, St Louis, MO) [22]. Plasma angiotensin II was determined after Sep-Pak (Waters Corp, Milford, MA) extraction from plasma with an in-house radioimmunoassay [23]. The angiotensin II receptor subtype 2 1675G→A polymorphism was determined by polymerase chain reaction as previously described [24].

2.6. Hypoglycemia symptom scoring

Hypoglycemic symptoms were assessed with the Edinburgh Hypoglycemia Scale, which is a questionnaire in which the subjects must indicate the severity of predefined hypoglycemic symptoms on a scale from 1 to 7 [25].

2.7. Assessment of cognitive function

2.7.1 California Cognitive Assessment Package

The Danish version of the abbreviated California Cognitive Assessment Package (CalCAP) test (EN Miller, *California Cognitive Assessment Package*, Norland Software, Los Angeles, CA, 1990) runs on a computer screen and consists of 4 different reaction time tasks with increasing complexity: (1) a simple reaction time task where subjects must strike a key whenever a number is presented, (2) a choice reaction task with reaction to a

specific number, (3) a choice reaction task with reaction to 2 identical numbers in a sequence, and (4) a choice reaction test with reaction to 2 numbers in a sequence (increasing order). The 2 latter tests require use of working memory. We calculated the individual mean reaction times (MRTs) for each test (MRT 1, MRT 2, MRT 3, and MRT 4) in milliseconds and counted the error rates (ERs) for the 3 choice reaction tests (ER 2, ER 3, and ER 4). Participants performed the test once before the study to get familiar with the test and thereby reduce any practice effect.

2.7.2. Alzheimer Quick Test

The Alzheimer Quick Test (AQT) measures cognitive and perceptual speed and has been shown to assess function of the parietal lobe [26,27]. In this study, the last section of part A was used. Subjects must name 40 objects (circle, line, square, and triangle) in different colors (black, blue, red, and yellow), for example, red square, blue triangle etc. Because of its design, the test can be used across linguistic codes and cultural domains. The time to perform the task was measured in seconds.

2.8. Statistic analysis

Calculations were performed using statistical software (SPSS version 13.0 for Windows; SPSS, Chicago, IL). Analysis of plasma VEGF, adrenaline, glucagon, cortisol, and growth hormone concentrations is based on mean values in cycles 1 + 2, 3 + 4, and 5 + 6. Shapiro-Wilk *W* test of normality showed that plasma VEGF was not normally distributed in most cycles (positively skewed). The VEGF values were logarithmic (log10) transformed before statistical testing. To assess the impact of RAS activity (high-RAS group vs low-RAS group) and intervention (hypoglycemia vs control) on changes in plasma VEGF, a mixed linear model (analysis of covariance [ANCOVA]) was used on logarithmic VEGF values (SPSS: analyze > mixed models > linear > continue). Because different baseline values in the 2 RAS groups could potentially affect any hypoglycemia-induced changes in VEGF levels, baseline values were considered as a covariate (and a fixed factor). Renin-angiotensin system group and intervention were fixed factors, and patient number was a random factor. Back-transformation to percentage was done on parameter estimates and confidence intervals. Before running the ANCOVA, paired *t* tests were done on the logarithmic VEGF values. Because a few *ΔVEGF values* (defined as mean plasma VEGF in cycle 3 + 4 minus mean plasma VEGF in cycle 1 + 2) were negative, transformation of this variable was not possible; and therefore, Wilcoxon signed ranked test was used to compare ΔVEGF in the 2 RAS groups.

A post hoc analysis of the impact of baseline VEGF and ΔVEGF (plasma VEGF during hypoglycemia minus plasma VEGF at baseline) concentrations on cognitive function (MRT and AQT) and hypoglycemic symptoms during hypoglycemia was done using ANCOVA. VEGF or ΔVEGF tertiles, RAS group and baseline MRT/AQT values, and hypoglycemia symptom score were fixed factors (the latter also covariates); and subject number was a random factor. When multiple comparisons were done, the Sidak correction method was applied. Dividing of VEGF values into tertiles was arbitrary and based on the number of subjects. Regression analyses were done on all 18 subjects and in both RAS groups to determine if VEGF changes were associated with changes in counterregulatory hormones (adrenaline, glucagon, growth hormone, and cortisol). Demographic and diabetes-related characteristics, nadir plasma glucose, insulin doses, and insulin concentrations in the 2 RAS groups were compared using an independent *t* test or a nonparametric test if needed. Changes in concentrations of counterregulatory hormones during the experiments (baseline vs hypoglycemia) were analyzed using a paired *t* test. Comparisons of Δ values of each hormone between RAS groups were done by independent *t* tests. Level of statistical significance was less than .05 (2-sided).

3. Results

3.1. Plasma glucose, insulin dose, and serum insulin

3.1.1. Day of hypoglycemia

Nadir plasma glucose was 2.2 (0.3) mmol/L, and mean plasma glucose in the stimulus period was 2.5 (0.32) mmol/L (mean [SD], n = 18). The hypoglycemic stimulus was similar in both groups: In the high-RAS group, nadir plasma glucose was 2.2 (0.4) mmol/L; and in the low-RAS group, nadir plasma glucose was 2.3 (0.3) mmol/L (mean [SD], $P = .41$). The insulin doses used to induce hypoglycemia did not differ between RAS groups: high-RAS group, 0.29 (0.13) IU/kg vs low-RAS group, 0.23 (0.09) IU/kg (mean [SD], $P = .28$). Neither did serum insulin concentrations differ: Baseline serum insulin concentration was 30 pmol/L in the high-RAS group and 40 pmol/L (median) in the low-RAS group ($P = .32$). The serum insulin concentration during hypoglycemia was 108 pmol/L in the high-RAS group and 95 pmol/L (median) in the low-RAS group ($P = .92$).

3.1.2. Day of maintained blood glucose level

Plasma glucose, insulin doses, and insulin concentrations did not differ significantly between RAS groups (data not shown).

3.2. VEGF during hypoglycemia

Overall, plasma VEGF increased during hypoglycemia from 61 (20-162) to 76 (23-300) pg/mL (median [range], $P < .0001$ by paired *t* test with logarithmic values, n = 18). In the recovery cycles, VEGF decreased compared with the hypoglycemic period ($P = .02$) (Fig. 1).

In the high-RAS group, VEGF increased from 39 (20-131) to 58 (23-300) pg/mL during hypoglycemia (median [range], $P = .004$). In the recovery cycles, VEGF levels

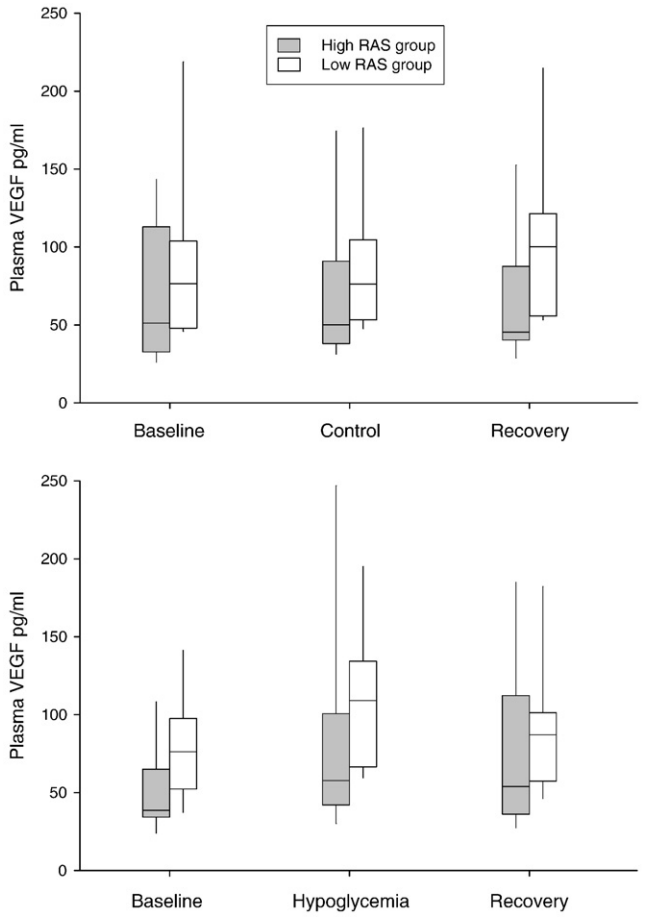

Fig. 1. Box plot of plasma concentrations of VEGF in 9 subjects with high and 9 subjects with low basal activity in the RAS and type 1 diabetes mellitus during the experiments. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Lines above and below the box indicate the 90th and 10th percentiles. Upper section: day of control. Lower section: day of hypoglycemia. Subjects in the high-RAS group: gray shade. Subjects in the low-RAS group: no shade.

decreased insignificantly (P = .43, hypoglycemia vs recovery). In the low-RAS group, VEGF increased from 76 (28-162) to 109 (56-214) pg/mL (median [range], P = .01). In the recovery cycles, VEGF decreased (P = .01, hypoglycemia vs recovery). When comparing ΔVEGF in the 2 RAS groups, no difference was found (P = .9). During the control study, VEGF levels did not change in the high-RAS group (P = .72, baseline vs hypoglycemia) or the low-RAS group (P = .98, baseline vs hypoglycemia).

Sex affected neither the level of baseline plasma VEGF nor the ΔVEGF. Baseline VEGF levels were 78 (51-131) pg/mL in women and 53 (20-162) pg/mL in men at the day of hypoglycemia (P = .16). At the day of control, VEGF levels were 99 (46-149) pg/mL in women and 55 (22-282) pg/mL in men (P = .46). The ΔVEGF levels were 16 (15-169) pg/mL in women and 16 (−1 to 104) pg/mL in men at the day of hypoglycemia (P = .44).

By ANCOVA, including all VEGF values at baseline and during hypoglycemia from all 18 subjects during both the hypoglycemia and the control study, the results were confirmed: The parameter estimate of the percentage increase of hypoglycemia-related (compared with control) plasma VEGF concentration was 39% (14%-70%, 95% confidence interval [CI]; P = .002). There was no effect of RAS group because plasma VEGF levels among subjects in the high-RAS group was only 0.06% (−19% to 23%, 95% CI) higher than those in the low-RAS group (P = 1.0).

3.3. Cognition during hypoglycemia

The hypoglycemic stimulus resulted in significant prolongation of MRTs in reaction time tasks 2 to 4 (MRT 2-4) and in prolongation of the completion time in AQT (Table 2).

3.4. VEGF and cognition during hypoglycemia

There was no effect of baseline VEGF on MRT 1 to 4 or ER. However, there was a trend toward slower reaction time in the upper baseline VEGF tertile in MRT 3 and 4. In MRT 3, the estimated MRT in the upper tertile was 673 (597-749) milliseconds (mean [95% CI]) vs 565 (511-619) milliseconds (mean [95% CI]) in the middle tertile (P = .07) and 570 (503-636) milliseconds (mean [95% CI]) in the lower tertile (P = .15). In MRT 4, the estimated MRT in the upper tertile was 684 (609-761) milliseconds (mean [95% CI]) vs 569 (516-623) milliseconds (mean [95% CI]) in the middle tertile (P = .05) and 598 (533-664) milliseconds (mean [95% CI]) in the lower tertile (P = .27). There was no effect of baseline VEGF on completion time in AQT (Table 3).

There was no statistical association between ΔVEGF tertile and MRT during hypoglycemia, except in MRT 1. Belonging to the lowest ΔVEGF tertile was associated with

Table 2
Cognitive function at baseline and during hypoglycemia in 18 subjects with type 1 diabetes mellitus

Test	Reaction time (ms)	P	Errors (n)	P
MRT 1 baseline	362 (53)	.12	–	
MRT 1 hypoglycemia	379 (72)		–	
MRT 2 baseline	451 (50)	.003	1.8 (2.2)	.001
MRT 2 hypoglycemia	479 (72)		5.4 (5.0)	
MRT 3 baseline	515 (83)	.001	4.6 (2.6)	.002
MRT 3 hypoglycemia	591 (123)		7.9 (4.7)	
MRT 4 baseline	518 (93)	<.0005	0.1 (0.3)	.12
MRT 4 hypoglycemia	605 (134)		0.9 (2.0)	
	Completion time (s)			
AQT baseline	50.1 (12.8)	<.0005		
AQT hypoglycemia	62.9 (16.4)			

Reaction time and errors in the 4 CalCAP tests and completion time in the AQT before induction of hypoglycemia (baseline) and during hypoglycemia. Numbers are mean (SD). Paired t tests have been used to compare baseline with hypoglycemia.

Table 3
Estimated reaction times (in milliseconds) in the CALCAP test and completion times (in seconds) in the AQT during hypoglycemia in 18 patients with type 1 diabetes mellitus belonging to different VEGF tertiles or ΔVEGF tertiles

		Reaction time (ms) (95% CI)	*P* vs middle	*P* vs high	Reaction time (ms) (95% CI)	*P* vs middle	*P* vs high
		Baseline VEGF tertile			ΔVEGF tertile		
MRT 1	Low	393 (345-441)	.92	.80	444 (400-488)	.029	.013
	Middle	376 (339-413)	–	.96	368 (337-399)	–	1.0
	High	363 (309-417)	–	–	364 (339-389)	–	–
MRT 2	Low	477 (441-513)	.99	1.0	519 (481-558)	.13	.12
	Middle	483 (453-512)	–	1.0	471 (444-498)	–	1.0
	High	479 (438-520)	–	–	473 (451-495)	–	–
MRT 3	Low	570 (503-636)	1.0	.15	618 (518-719)	.69	1.0
	Middle	565 (511-619)	–	.07	558 (485-631)	–	.68
	High	673 (597-749)	–	–	603 (546-661)	–	–
MRT 4	Low	598 (533-664)	.87	.27	638 (534-742)	.69	.96
	Middle	569 (516-623)	–	.05	576 (502-650)	–	.81
	High	684 (609-761)	–	–	613 (554-672)	–	–
		Completion time (s) (95% CI)			Completion time (s) (95% CI)		
		Baseline VEGF tertile			ΔVEGF tertile		
AQT	Low	70 (58-81)	.6	.38	73 (59-88)	.53	.29
	Middle	61 (52-71)	–	.9	62 (52-73)	–	.97
	High	56 (41-70)	–	–	60 (51-68)	–	–

P values are corrected for multiple comparisons (Sidak method). Statistics: ANCOVA.

a prolonged reaction time: Estimated MRT in the lowest ΔVEGF tertile in MRT 1 was 444 (400-488) milliseconds (mean [95% CI]) vs 368 (337-399) milliseconds (mean [95% CI]) in the middle tertile ($P = .029$) and 364 (339-389) milliseconds (mean [95% CI]) in the upper tertile ($P = .013$). There was no effect of VEGF response on completion time in AQT.

3.5. VEGF and hypoglycemic symptoms during hypoglycemia

There was no association between baseline and ΔVEGF and hypoglycemic symptoms during hypoglycemia (Table 4).

3.6. Counterregulatory hormones during hypoglycemia

Concentrations of adrenaline, glucagon, cortisol, and growth hormone increased significantly in the low-RAS group during hypoglycemia (compared with baseline). In the high-RAS group, only adrenaline and growth hormone levels increased during hypoglycemia. When comparing Δ values in the 2 RAS groups, we found a significantly higher glucagon response in the low-RAS group than in the high-RAS group (Table 5).

3.7. VEGF and counterregulatory hormones during hypoglycemia

In univariate regression analysis, ΔVEGF was not associated with changes in any counterregulatory hormone. The regression coefficient of Δglucagon in a multivariate regression analysis (with adrenaline, glucagon, cortisol, and growth hormone as explanatory variables) was 19.7 (0.76-38.7 CI, $P = .04$). When repeating the multiple regression analysis in both RAS groups, changes in any of the counterregulatory hormone were not associated with ΔVEGF.

4. Discussion

This study shows that the plasma concentration of VEGF increases during hypoglycemia among individuals with type

Table 4
Estimated mean hypoglycemia symptom score during hypoglycemia in 18 patients with type 1 diabetes mellitus belonging to different VEGF tertiles and ΔVEGF tertiles

	Symptom score (95% CI)	*P* vs middle	*P* vs high	Symptom score (95% CI)	*P* vs middle	*P* vs high
Baseline VEGF tertile				ΔVEGF tertile		
Low	23 (14-32)	.68	.84	23 (11-35)	1.0	.97
Middle	28 (22-35)	–	.17	23 (15-32)	–	.96
High	18 (8-28)	–	–	25 (19-32)	–	–

P values are corrected for multiple comparisons (Sidak method). Statistics: ANCOVA.

Table 5
Counterregulatory hormone levels (mean [SD]) at baseline and during hypoglycemia in 18 patients with type 1 diabetes mellitus and high or low activity in RAS

	Low-RAS group (n = 9)		High-RAS group (n = 9)		P^a
	Baseline	Hypoglycemia	Baseline	Hypoglycemia	
Adrenaline (pg/mL)	36.2 (15.3)	339 (302)*	26.3 (12.8)	150.8 (37.1)*	.12
Glucagon (pmol/mL)	7.7 (1.7)	7.8 (2.0)*	6.9 (2.5)	5.4 (1.4)†	.04
Cortisol (nmol/L)	354 (88.3)	526 (108)†	412 (145)	526 (212)	.54
Growth hormone (mU/L)	1.7 (2.0)	17.3 (15.3)*	4.14 (7.79)	32.2 (17.9)†	.19

Baseline values are average of hormone levels in cycles 1 and 2. Levels during hypoglycemia are average of hormone levels in cycles 3 and 4.
[a] P values when comparing Δ values of each hormone between RAS groups (independent t test).
*$P < .05$ and †$P < .01$ compared with baseline (paired t test).

1 diabetes mellitus. This finding is in accordance with previous studies in healthy adults [8-10] and in a human cell line [7]. It is not known whether differences in VEGF levels during hypoglycemia exist between individuals with type 1 diabetes mellitus and matched controls or between individuals with or without hypoglycemia unawareness.

There were no differences in VEGF response between the 2 RAS groups, which indicate that the beneficial effects of low RAS activity on cognitive function during mild hypoglycemia [12] are not mediated by differences in VEGF concentrations. It is possible that basal RAS activity influences the VEGF response during more severe hypoglycemia. However, nadir blood glucose in this study was 2.2 (0.3 SD) mmol/L, which is low compared with other studies in which experimental hypoglycemia is induced. In addition, a blood glucose of about 2 to 2.5 mmol/L as in this study is a clinically relevant hypoglycemic stimulus. As in other studies [8,9], the increase in VEGF during hypoglycemia was independent of the increase in adrenaline concentration. The lack of correlation between adrenaline and VEGF is underlined by the fact that the counterregulatory response during hypoglycemia was different in the 2 RAS groups, whereas this was not the case for the VEGF response.

The origin of the plasma VEGF rise is unclear. A study by Merl et al [9] indicates that the pituitary gland, in which VEGF production has been demonstrated [28], may secrete VEGF during hypoglycemia because antecedent hypoglycemia attenuates VEGF response to subsequent hypoglycemia. This phenomenon is typical of the classic hypoglycemic counterregulatory response and is assumed to result from a central nervous adaptive process [29]. Furthermore, activated platelets and neutrophils can release VEGF [30,31]; and activation of both cell types has been demonstrated during hypoglycemia [32,33]. Parallel measurements of VEGF concentrations in plasma (in which platelet activation is inhibited) and serum were not performed in our study. Insulin is another candidate trigger because insulin has been shown to be involved in the stabilization of the hypoxia inducible factor 1, which is a transcription factor for the VEGF gene. However, in a hyperinsulinemic euglycemic clamped study by Dantz et al [8], VEGF did not rise; and in a study by Dullaart et al [34], VEGF levels were unaffected by acute hyperglycemia, hyperinsulinemia, and the combination of both. Because the insulin concentrations in our study are substantially lower (≈110 pmol/L) than those in typical hypoglycemic clamp studies, the hypoglycemic stimulus per se—and not the infused insulin—probably is the main stimulator of VEGF secretion.

In conflict with the findings of Dantz et al [8], we found no positive association between reduced preservation of cognition during hypoglycemia and belonging to the lowest ΔVEGF tertile, except in MRT 1. However, MRT 1 was the least complex reaction time task, in which hypoglycemia had the least impact on cognitive performance (Table 2). Lack of association in the other tasks may indicate that the finding is a result of coincidence or that a possible positive effect of the VEGF rise on cognitive function during short-duration hypoglycemia is only exerted in simple tasks. Furthermore, we found no association between low ΔVEGF and prolonged completion time in AQT. In the study by Merl et al [9], it was not possible to demonstrate any correlation between VEGF response and preservation of cognitive function during hypoglycemia in healthy adults.

The reason for the conflicting results in this study, the study by Merl et al [9], and the study by Dantz et al [8] may be explained by differences in the hypoglycemic stimulus and different cognitive tasks in the 3 studies. The duration of the hypoglycemic stimulus in our study was about 1 hour. In the study by Merl et al [9], it was 2.5 hours; and in the study by Dantz et al [8], it was 6 hours. Dantz et al [8] used the Stroop test that measures executive function and attention [35] and a memory test. Merl et al [9] used a short-term memory task and a classic auditory attention task. We used a reaction time task in which the use of working memory is required and the AQT, which is derived from the Stroop test. The depth of hypoglycemia in the 3 studies was comparable: In our study, nadir blood glucose was 2.2 (0.3 SD) mmol/L. In the study by Dantz et al, it was 2.3 mmol/L (0.4 SD, n = 16); and in the study by Merl et al, it was 2.5 mmol/L (SD unknown, n = 15).

The weak association between cognition and VEGF in this study does not rule out the possibility that VEGF has an important (paracrine) function in cerebral glucose

metabolism during prolonged hypoglycemia, for example, during nocturnal hypoglycemia. However, the true clinical usefulness of VEGF measurements in diabetes treatment has yet to be proven. Furthermore, our results regarding cognition and plasma VEGF concentrations, which are based on post hoc analysis, should be interpreted with caution and be confirmed in other studies.

5. Conclusions

We have demonstrated that mild insulin-induced hypoglycemia for approximately 1 hour triggers an increase in plasma VEGF concentrations in patients with type 1 diabetes mellitus. Basal activity in RAS influenced neither basal nor hypoglycemia-induced VEGF concentrations. A questionable association between low VEGF response and reduced cognitive preservation during hypoglycemia was observed. Whether measurement of VEGF concentrations is useful as a marker of future risk of hypoglycemia among patients with type 1 diabetes mellitus remains to be studied. Further studies are needed to elucidate the origin of VEGF during hypoglycemia and to detect if any differences in the hypoglycemic VEGF response between individuals with or without diabetes exist. In addition, studies of VEGF in the human BBB are warranted.

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