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Vascular Endothelial Growth Factor: A Novel Endocrine Defensive Response to Hypoglycemia

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Glucose, the most important fuel for the brain, is supplied by the actions of counterregulatory hormones and the sympathetic nervous system. Yet to obtain access to the brain, glucose must pass the blood-brain barrier. Here we show that vascular endothelial growth factor (VEGF), a potent regulator of blood vessel function, is a candidate hormone for facilitating glucose passage across the blood-brain barrier under critical conditions. In 16 healthy men, VEGF serum concentrations increased under 6 h of insulin-induced hypoglycemic conditions from 86.1 ± 13.4 to 211.6 ± 40.8 pg/ml (P = 0.002), whereas in the hyperinsulinemic euglycemic control condition, no change was observed. During hypoglycemia serum VEGF, but no other counterregulatory hormone, was associated with preserved neurocognitive function, as measured with a memory test (r = 0.539; P = 0.031) and the Stroop interference task (r = 0.569; P = 0.021). Findings show that acute hypoglycemia is accompanied by a brisk increase in circulating VEGF concentration and that VEGF could mediate rapid adaptation of the brain to neuroglycopenia. (*J Clin Endocrinol Metab* 87: 835–840, 2002)

CONTROL OF BRAIN glucose concentration is of utmost importance for higher organisms. Lack of brain glucose causes cognitive dysfunction, and lowest glucose concentrations can induce seizures, loss of consciousness, and death. Therefore, brain glucose must persistently be maintained above a critical threshold. Because the brain is isolated from the general circulation by the blood-brain barrier (BBB), glucose is transported by carrier mechanisms through microvessel endothelial cells and the plasma membranes of neurons and glia. The carrier protein specific for glucose transport across the BBB is GLUT-1 (1). Mutations of the glucose transporter GLUT-1 are associated with intractable seizures (2). Reduced availability of blood glucose (*i.e.* during hypoglycemia) enhances uptake of glucose into the brain (3). Thus, glucose transport across the BBB is a critical mechanism to compensate for hypoglycemic conditions.

No hormonal mediator has yet been identified that directly enhances brain glucose uptake. However, during acute hypoglycemia, classical glucose counterregulation is known to enhance brain glucose uptake indirectly by reducing uptake of glucose into peripheral tissues. This response is mediated mainly via secretion of counterregulatory hormones such as epinephrine into the circulation. Release of these hormones and sympathetic activation induce adrenergic symptoms, such as trembling, and neuroglycopenic symptoms, such as sweating, blurred vision, and sleepiness, giving the individual awareness of being hypoglycemic (4). Repeated episodes of hypoglycemia lead to defective brain glucose sensing. Even a single bout of hypoglycemia reduces both awareness and counterregulatory response to subsequent hypoglycemia (5). Prolonged mild hypoglycemia also results in reduced awareness but does not impair the counterregulatory response (6, 7). The absence of symptoms in systemic hypoglycemia suggests that the brain can adapt to low levels of blood glucose by increasing cerebral blood flow and glucose transport across the BBB (8, 9).

Here we propose that vascular endothelial growth factor (VEGF) (10), also called vascular permeability factor (11), is a candidate for directly regulating brain glucose uptake under critical conditions. The brain exhibits highest densities of VEGF-binding sites, which are high-affinity cell surface receptors. These receptors are located on the brain microvessel endothelium forming the BBB (12). VEGF is a potent polypeptide regulator of blood vessel function (13) and has been demonstrated to enhance GLUT-1 gene expression and glucose transport in vascular endothelial cells (14). Particularly, in endothelial cells forming the blood retina barrier (which resembles the BBB), VEGF has been demonstrated to mediate enhancement of glucose transport by increasing translocation of cytosolic GLUT1 to the plasma membrane surface (15). Furthermore, VEGF mediates induction of endothelial fenestrations (16, 17), thereby increasing the transport of small molecules such as sucrose and fluorescein across the BBB (18, 19). Local administration of VEGF into the cerebral microcirculation in rats did not only increase BBB permeability to 10-kDa dextran but also dilated cerebral arterioles (20). Importantly, this response was rapid, occurring within 10 min after the administration of VEGF. Thus, VEGF can increase brain glucose uptake by dilating cerebral arterioles, enhancing the transport of glucose via GLUT-1, or inducing endothelial fenestrae in conditions of brain glucose deprivation. Here we show that in humans the systemic VEGF concentration is tightly associated with the level of hypoglycemia. Furthermore, data indicate that VEGF release during hypoglycemia is positively correlated to the maintenance of neurocognitive function.

Abbreviations: BBB, Blood-brain barrier; CV, coefficient of variation; PVN, paraventricular nucleus; VEGF, vascular endothelial growth factor.

Materials and Methods

Subjects

Sixteen young healthy men participated in hypoglycemic clamp experiments (age 25 ± 1 yr.; body mass index 22.5 ± 0.5 kg/m^2), another eight in control euglycemic clamp experiments (age 27 ± 2 yr.; body mass index 21.7 ± 0.6 kg/m^2). Exclusion criteria for both groups were chronic or acute illness, current medication of any kind, smoking, alcohol or drug abuse, obesity, and diabetes in first-degree relatives. The study was approved by the local ethics committee, and each volunteer gave written informed consent.

Study design and clamp session

Clamp experiments lasting 6 h were performed in all 24 men. On the day of the clamp session, subjects reported to the medical research unit by 0800 h. Ten hours before testing until completion of the clamp session, they had to fast and to abstain from coffee, tea, and alcoholic beverages. The experiments were carried out in a sound-attenuated room with the subject resting in a supine position with his trunk in an almost upright position (about 60 degrees). To obtain arterialized venous blood, a cannula was inserted into a dorsal vein of the subject's hand, which was placed in a heated box (50–55 C). A second cannula was inserted into an antecubital vein of the contralateral arm. Both cannulas were connected to long thin tubes, which enabled blood sampling and adjustment of the rate of dextrose infusions from an adjacent room without being noticed by the subject. After a 1-h baseline period, a bolus of insulin (30 mU/kg H-Insulin; Hoechst) was injected followed by a continuous infusion at a rate of 1.5 mU min^{-1}kg^{-1} for the next 6 h. Simultaneously, a 20% dextrose solution was infused at variable rates to achieve predefined target plasma glucose levels. Arterialized blood was drawn at 5-min intervals to measure plasma glucose. During the euglycemic control clamps (eight subjects), plasma glucose concentration was held between 5.0 and 5.5 mmol/liter. In the stepwise hypoglycemic clamp session (16 subjects), we reduced plasma glucose to achieve successive plateaus of 4.2, 3.6, 2.9, and 2.3 mmol/liter. Each plateau was maintained for a period of 45 min, and the next lower plateau was induced gradually within the following 45 min. Standard cognitive tests (a memory test and the Stroop test) were performed at each of the hypoglycemic plateaus. To determine VEGF, insulin, epinephrine, and cortisol blood was collected every 90 min. Blood was centrifuged within 1 min after withdrawal and serum was kept at −72 C until assay.

Memory test

Memory testing was comprised of the consecutive presentation of two different word lists each containing 15 words. To enable repeated testing, 10 different lists were formed from a pool of words. The 15 words were presented orally at a rate of one word per 3 sec. After each list and a subsequent break of 1 min, the subject was asked to recall verbally all words he remembered from the preceding list. The number of words correctly recalled at each testing was summed across the two lists.

Stroop test

On the Stroop interference test, the subject was presented with a series of color names (green, red, blue, yellow) printed in inks of different colors. The task was to selectively attend to and to name as quickly as possible the print color of each word. The total number of correct responses within 45 sec was determined. Before the proper experiment, the subject was allowed to practice. For the clamp session, five alternate forms of the test were prepared.

Laboratory methods

Concentration of $VEGF_{165}$ in serum samples was measured in duplicate by a sandwich-type enzyme-linked immunoassay (Quantikine; R&D Systems, Minneapolis, MN). Antibodies were directed against human recombinant $VEGF_{165}$. The immobilized antibody was monoclonal, and the second horseradish peroxidase-coupled antibody was polyclonal. The intra- and interassay coefficients of variation (CV) were <5% and <7.5%, respectively; the lower detection limit was 9 pg/ml (21).

Plasma glucose was measured in duplicate using the glucose oxidase method (intra- and interassay CV <1.8% and <2.6%, respectively; glucose analyzer, Beckman Coulter, Inc. Instruments GmbH, Munich, Germany). The serum concentration of insulin was measured by RIA (Insulin RIA 100, Pharmacia AB, Uppsala, Sweden) with an intraassay CV of 5.8% and an interassay CV of 6.5%. High-pressure liquid chromatography was used to measure serum epinephrine (intraassay CV <2.9%; interassay CV <4.2%; Chromsystems, Munich, Germany) and cortisol was determined by ELISA (intraassay CV <2.0%; interassay CV <3.9%; Roche Molecular Biochemicals Diagnostica, Mannheim, Germany).

Statistical methods

Data are reported as means ± SEM. A P value less than 0.05 was considered significant. For comparisons between the effects of hypoglycemic and euglycemic clamps and between values at baseline and the last plateau of the clamp, respectively, unpaired and paired t tests were applied. Because of considerable interindividual variability at baseline, changes in serum VEGF at the end of the clamp (at 360 min) were expressed as ΔVEGF (percent), with the level at baseline set to 100%. Also, Δvalues (percent) were calculated for memory performance, performance of the Stroop interference task, and the counterregulatory hormones. These variables (memory recall test, Stroop test, VEGF, epinephrine, and the other counterregulatory hormones) were normally distributed (Kolmogorov-Smirnov, P values all > 0.05).

Glycemic thresholds for hormonal responses have been calculated according to a standard method described by Mitrakou *et al.* (22). The glycemic threshold is defined as the plasma glucose concentration at which mean response of subjects first differed significantly from corresponding response in control (euglycemic) experiments.

Multiple linear regression analysis was used to determine dependence of cognitive function (memory test and Stroop interference test) on VEGF, cortisol, epinephrine, norepinephrine, GH, ACTH, and glucagon responses to hypoglycemia. The variables VEGF, cortisol, epinephrine, and the other counterregulatory hormones were conditionally selected in a forward stepwise procedure (inclusion criteria, $P < 0.05$). All calculations were performed using the SPSS statistical program, version 9.0 (SPSS, Inc., Chicago, IL).

Results

During the hypoglycemic clamp, serum VEGF concentrations strongly increased reaching values 2.5 times higher than baseline values at the end of the clamp (86.1 ± 13.4 to 211.6 ± 40.8 pg/ml; $P = 0.002$). During the euglycemic control clamp, serum VEGF concentrations did not change (Fig. 1). The increase in VEGF reached significance at the first plateau already ($P < 0.05$) (*i.e.* at a blood glucose concentration of 4.2 ± 0.1 mmol/liter). A similar increase during hypoglycemia was observed in serum epinephrine concentrations (Fig. 1). The glycemic thresholds for activation of counterregulatory responses are summarized in Table 1.

In the memory test, the number of words correctly recalled decreased from 7.8 ± 0.3 at baseline testing to 5.0 ± 0.5 at testing during the lowest hypoglycemic plateau ($P = 0.001$; Fig. 1). In the Stroop interference test, the total number of correct responses decreased from 59.5 ± 2.9 to 47.9 ± 3.2 ($P = 0.001$; Fig. 1). The deterioration of cognitive function measured with the memory test and the Stroop interference task correlated with the increase in serum VEGF (Fig. 2): The stronger the increase in VEGF, the more words were correctly recalled in the memory test ($r = 0.539$; $P = 0.031$) and the greater the number of correct responses on the Stroop task ($r = 0.569$; $P = 0.021$). No similar correlations were found for the increase in epinephrine (memory test: $r = 0.227$; $P = 0.324$; Stroop task: $r = 0.206$, $P = 0.387$; Fig. 2) during hypoglycemia. Also, after application of multiple linear regres-

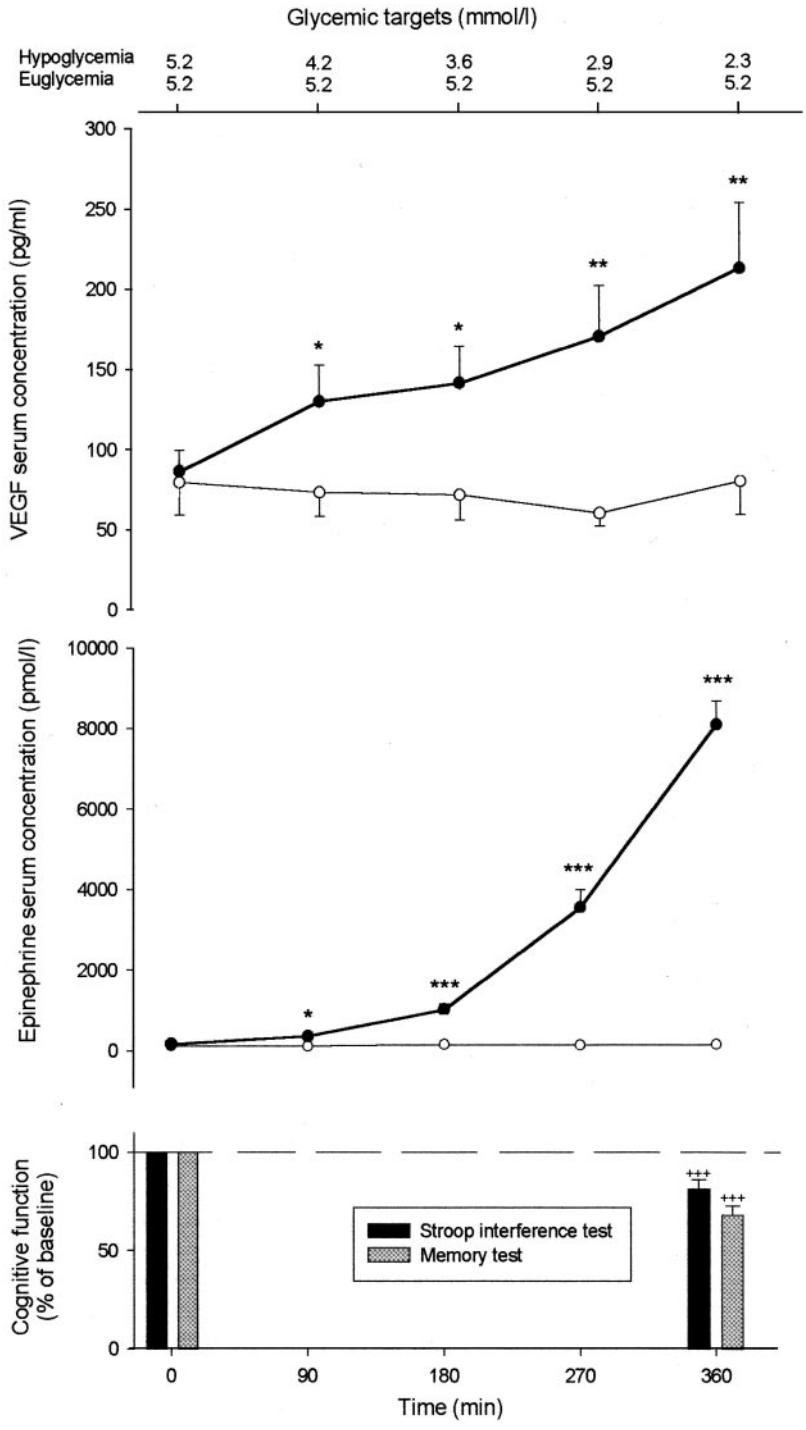

FIG. 1. Effects of hypoglycemia on VEGF, epinephrine serum concentration, and cognitive function (*bar graph*). Values are means ± SEM calculated for the stepwise hypoglycemic (●) and euglycemic (○) conditions. The bottom panel shows ratios for performance on the Stroop interference test and the memory test, under baseline conditions (glycemic target 5.2 mmol/liter) and under last plateau conditions (glycemic target 2.3 mmol/liter). The number of correct responses and the number of words correctly recalled, respectively, at baseline were set to 100%. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* euglycemic control; +++, $P < 0.001$ *vs.* baseline.

sion analysis, both cognitive function tests were associated with VEGF but not with cortisol, epinephrine, norepinephrine, GH, ACTH, or glucagon.

Hypoglycemic clamps were performed in a stepwise procedure. Plasma glucose concentrations at baseline and 90, 180, 270, and 360 min after the beginning of insulin infusion were 5.2 ± 0.1, 4.6 ± 0.1, 3.8 ± 0.1, 3.1 ± 0.0, and 2.3 ± 0.1 mmol/liter, respectively. During euglycemia respective glucose concentrations were 5.3 ± 0.1, 5.1 ± 0.1, 5.1 ± 0.1, 5.3 ± 0.2, and 5.3 ± 0.2 mmol/liter. Corresponding serum insulin concentrations were closely comparable during hypoglycemic (7 ± 1, 104 ± 4, 95 ± 3, 92 ± 5, and 99 ± 5 pmol/liter) and euglycemic clamp sessions (7 ± 2, 101 ± 4, 89 ± 5, 98 ± 5, and 102 ± 4 pmol/liter). Serum cortisol concentrations were 260 ± 20, 250 ± 20, 280 ± 20, 500 ± 30, and 670 ± 30 nmol/liter during hypoglycemic clamps and 260 ± 20, 240 ± 20, 240 ± 20, 210 ± 30, and 160 ± 40 nmol/liter during euglycemic clamps.

TABLE 1. Glycemic thresholds for activation of counterregulation

Hormone	Glycemic threshold (mmol/liter)
VEGF	4.2 ± 0.1
Epinephrine	4.2 ± 0.1
Norepinephrine	3.8 ± 0.2
C-peptide	4.2 ± 0.1
GH	3.8 ± 0.2
Glucagon	3.7 ± 0.1
ACTH	3.7 ± 0.1
Cortisol	3.6 ± 0.2

Values are mean ± SEM. A glycemic threshold was defined as the plasma glucose concentration at which the mean response of subjects first differed significantly from corresponding response in control (euglycemic) experiment.

Discussion

This study showed that acute hypoglycemia is associated with an increase in serum VEGF in humans. Of note, enhanced VEGF secretion during hypoglycemic conditions correlated positively with the maintenance of cognitive performance during hypoglycemia, but no such relation was found for epinephrine, cortisol, or other counterregulatory hormones.

The origin of the observed VEGF response is uncertain. VEGF mRNA is expressed in high concentrations throughout the brain, with particularly high concentrations in the hypothalamus and pituitary (10). In the hypothalamus, many early responses to hypoglycemia including that of epinephrine release are triggered by specific glucose-responsive neurons that are mainly located in the ventromedial hypothalamus (23–26). These neurons respond directly to glucose that has entered the brain via the BBB by glucose transporters. The ventromedial hypothalamus glucose-responsive neurons have intrahypothalamic projections to the paraventricular nucleus (PVN), which is linked to both neuroendocrine and autonomic efferent pathways (23). A putative mechanism of VEGF release in response to hypoglycemia relies on a stimulatory effect of the specific glucose-responsive neurons on the release of neuropeptides from neurons of the PVN. Cells of the PVN synthesize VEGF, which can be excreted together with other neuropeptides such as CRH, vasopressin, and the pituitary adenylate cyclase-activating polypeptide (27, 28). In the pituitary, VEGF has been detected in folliculostellate cells located in the anterior lobe (29, 30). The release of VEGF from folliculostellate cells is stimulated by pituitary adenylate cyclase-activating polypeptide and inhibited by glucocorticoids (31). VEGF from hypothalamic nuclei and the pituitary may have direct access to the systemic blood circulation.

Brain cells develop neuroglycopenia at plasma glucose concentrations below about 3.0 mmol/liter. *In vitro*, VEGF mRNA expression and VEGF synthesis are up-regulated in response to glucose deprivation in glioma cell cultures (32, 33), retinal cells (34), embryonic stem cells (35), and the human monocytic cell line U-937 (36). These findings add VEGF to a growing list of glucose-regulated proteins that also includes GLUT-1 (37). Because VEGF mRNA is diffusely expressed throughout the brain, VEGF production could be considered a general stress response of neurons to glucose deprivation. Along this line, decreasing brain glucose concentrations stimulate VEGF release both within the central nervous system and from the hypothalamus-pituitary system into the peripheral compartment. VEGF's early response to hypoglycemia and its presumed direct access to the systemic circulation suggest the hypothalamus-pituitary system is the major source of the observed increase in serum VEGF.

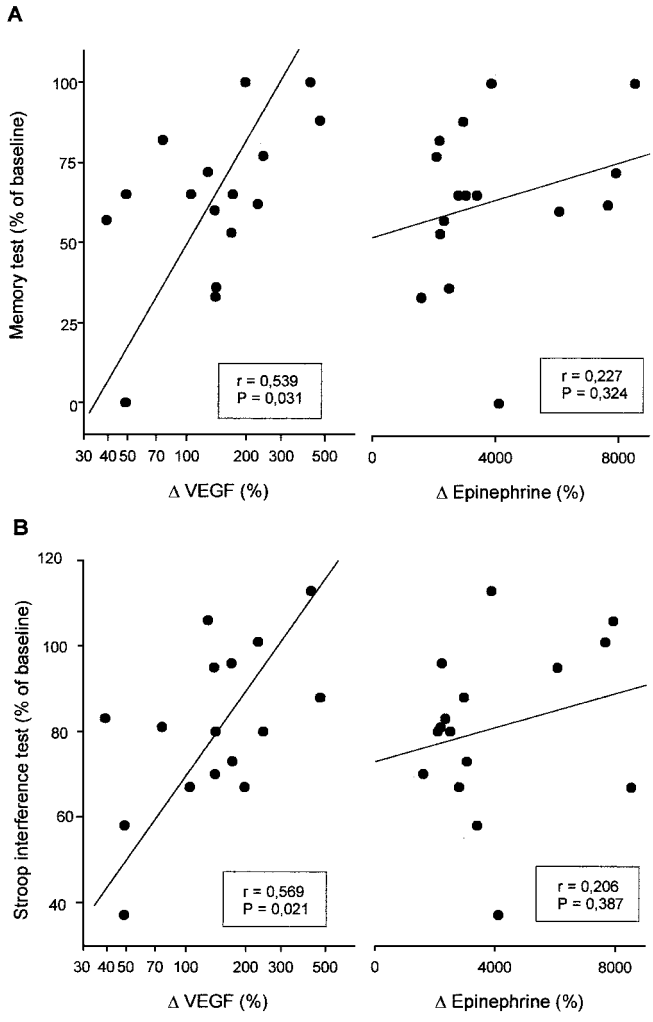

FIG. 2. Linear correlation between decrease in (A) short-term memory (number of words correctly recalled in the memory test) and (B) attention performance (number of correct responses on the Stroop interference test), on the one hand, and the increases in VEGF and epinephrine to hypoglycemia, on the other hand. Δ values refer to the difference between serum concentrations at the end of the clamp (at 360 min) and at baseline (at 0 min). Changes in VEGF, epinephrine, recalled words, and correct responses are expressed in percent, with the level at baseline set to 100%. Note significant correlation between VEGF response and preserved cognitive performance during hypoglycemia, whereas no such correlation occurred for epinephrine.

Peripheral tissues (*e.g.* muscle and fat) can also produce VEGF. Activation of the sympathoadrenal system has been shown to exert a stimulatory effect on brown fat VEGF production and release (38–40). Because the glycemic threshold for serum VEGF release was 4.2 mmol/liter (Table 1) and a similar threshold was observed for serum epinephrine increase, it can be speculated that adrenergic activation at the peripheral tissue level might also have contributed to the increase in serum VEGF.

In contrast to the brain, peripheral insulin-sensitive organs (*e.g.* muscle and fat) were not glucose deprived during the hyperinsulinemic conditions established in the present experiments. During the hyperinsulinemic clamps, peripheral glucose uptake is expected to be distinctly enhanced under hypoglycemic (2.0 g/kg dextrose infused) and even more enhanced under euglycemic conditions (3.8 g/kg dextrose infused) (41). Extremely high glucose concentrations may increase VEGF mRNA expression and peptide production in vascular smooth muscle cells (42, 43) Also, insulin has been shown to increase the level of the regulatory α subunit of hypoxia-inducible factor-1, a factor inducing VEGF mRNA, in peripheral tissues such as human and murine hepatoma cells, rat skeletal muscle myoblasts, and murine embryonic fibroblasts (44, 45). In the present study, however, serum VEGF failed to increase during the hyperinsulinemic euglycemic clamps. Thus, a relevant contribution of peripheral VEGF secretion because of insulin or changes in peripheral glucose uptake appears unlikely.

A direct central nervous effect of VEGF during hypoglycemia was not demonstrated here. However, our data provide evidence indicating a potential influence of VEGF on cognitive brain function. We found a relation between the amplitude of the VEGF-secreting response and cognitive performance at the hypoglycemic level of 2.4 mmol/liter (Fig. 2, A and B). Under hypoglycemic conditions subjects with strong VEGF responses showed superior performance on a short-term memory task as well as on a selective attention task (Stroop interference task). Both measures indicate an improved cognitive processing of stimuli in association with stronger VEGF responses to hypoglycemia. A weakening of hypoglycemia induced cognitive impairments has been shown also to occur already during an ongoing hypoglycemic state after a period of about 90 min in healthy (6) and type 1 diabetic subjects (7). This observation indicates a fast adaptive response of the brain to neuroglycopenia, which we propose be established via release of VEGF.

In conclusion, we demonstrated that acute hypoglycemia was accompanied by a brisk increase in circulating VEGF in humans. The temporal response pattern of VEGF corresponds to that of a counterregulatory hormone. Thus, VEGF should be included in the list of hormones (epinephrine, ACTH, cortisol, and GH) that are released during hypoglycemia.

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