Local Ventromedial Hypothalamus Glucopenia Triggers Counterregulatory Hormone Release

Walter P. Borg, Robert S. Sherwin, Matthew J. During, Monica A. Borg, and Gerald I. Shulman

To test the hypothesis that nuclei of the ventromedial hypothalamus (VMH) play a key role in the detection of counterregulatory responses to hypoglycemia, we delivered the glucopenic agent 2-deoxyglucose via bilaterally placed microdialysis probes into the VMH of conscious, chronically catheterized rats. The goal was to produce cellular glucopenia localized to the VMH. The volume of brain tissue exposed to 2-deoxyglucose was determined by adding [^{3}H]2-deoxyglucose to the dialysate; its distribution in cerebral tissue was almost exclusively limited to the VMH. Rats with microdialysis probes placed into the frontal lobes served as a control group. Local perfusion of 2-deoxyglucose (but not glucose) into the VMH caused a prompt twofold increase in plasma glucose in association with a striking elevation of plasma glucagon (3.5-fold), epinephrine (30-fold), and norepinephrine (3.5-fold). No effect was seen when 2-deoxyglucose was delivered into the frontal lobes. We conclude that glucopenia localized to the VMH triggers the release of counterregulatory hormones that defend against hypoglycemia. Thus, the neurons that sense glucopenia may be situated in the VMH. *Diabetes* 44:180–184, 1995

While the role of the individual counterregulatory hormones in hypoglycemia correction has been studied extensively (1), the mechanisms that link glucopenia with activation of the counterregulatory system are poorly understood. Specifically, there has been controversy concerning the tissues that sense glucopenia and coordinate the counterregulatory response. Both the central nervous system (CNS) and extracerebral glucose sensors have been implicated in the activation of counterregulatory hormone release during hypoglycemia (2–7). Recent studies from our laboratory, based on chemical lesioning of various brain regions, have suggested that the neurons located in the ventromedial hypothalamus (VMH) are essential for the integrated hormonal response to glucose deprivation (8). To further clarify the role of the VMH in hypoglycemia detection and counterregulation, we delivered 2-deoxyglucose, a metabolic inhibitor commonly used to generate cellular glucopenia, via microdialysis probes directly into the VMH of awake rats. It was reasoned that, if glucosensors are located in the VMH, this manipulation should cause a counterregulatory response despite systemic normoglycemia. The microdialysis technique allowed us to deliver 2-deoxyglucose directly into a localized brain region of awake, unrestrained animals, eliminating such confounding factors as anesthesia and hypoglycemia in other areas of the body.

From the Departments of Internal Medicine and Surgery (M.J.D.), Yale University School of Medicine, New Haven, Connecticut.

Address correspondence and reprint requests to Dr. Gerald I. Shulman, Yale University School of Medicine, Department of Internal Medicine/Endocrinology, 333 Cedar St., Fitkin 1, New Haven, CT 06520-8020.

Received for publication 28 September 1994 and accepted in revised form 6 October 1994.

CNS, central nervous system; VMH, ventromedial hypothalamus; FL, frontal lobe; LHA, lateral hypothalamic area.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats were purchased from Charles River Laboratories. Animals were housed in an environmentally controlled room with a 12-h light/dark cycle, and were maintained on standard ad libitum rat diet (Prolab 3000, AGWAY, Waverly, NY) comprised of 22% protein, 5% fat, and 51% carbohydrate (the remaining 22% consists of ash, crude fiber, and moisture). Rats (body wt 270–310 g) were anesthetized by intraperitoneal injection (1 ml/kg) of a mixture of xylazine (20 mg/ml) and ketamine (100 mg/ml) in a ratio of 1:2 (vol:vol) and placed on the stereotaxic frame. The skull was exposed, and holes were drilled bilaterally in chosen coordinates, through which the guide cannulas were lowered slowly into the brain. All stereotaxic coordinates were determined from the atlas of Paxinos and Watson (9). Two groups of animals were prepared as follows. For group 1, VMH cannulas were placed by using the coordinates 2.6 mm posterior and 3.8 mm lateral in relation to bregma, and at the angle of 20° in relation to the horizontal plane passing through bregma and lambda. For group 2, frontal lobe (FL) cannulas were placed by using the coordinates 2.0 mm anterior and 2.0 mm lateral in relation to bregma at the angle of 90° in relation to the vertical bregma-lambda plane. The cannulas were then secured to the skull with stainless steel screws and dental acrylic. Animals were then allowed to recover from the stereotaxic procedure for 12–18 days before study. One day before each experiment, standard microdialysis probes of side-by-side design (10) were inserted into guide cannulas. The lengths of the VMH probes and FL probes were 10.5 and 2.0 mm, respectively (as measured from bregma-lambda plane). On the morning of the experiment, perfusion medium was loaded into 1-ml syringes and delivered at a flow rate of 2.5 μl/min by using a Harvard perfusion pump (model 22, Harvard Bioscience). Three kinds of perfusates were used: 5 mmol/l glucose, 100 mmol/l glucose, and 100 mmol/l 2-deoxyglucose. A sterile, pyrogen-free, artificial, extracellular, fluid solution (135 mmol/l NaCl, 3 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.2 mmol/l $CaCl_2$, 200 mmol/l ascorbate, and 2 mmol/l sodium phosphate buffer to pH 7.4) served as a solvent for these solutions. At the end of each experiment, probe placement was verified histologically by cresyl violet staining. Only animals that showed bilateral probe placement into the desired brain regions were included. Of rats with VMH, 5 of 18 (28%) and none of the rats with FL probes failed the histological criteria. Histological results were the only criteria for excluding or including the studies for data analysis.

At 6–10 days before study (i.e., 6–8 days after stereotaxic surgery), animals underwent an additional aseptic surgical procedure for placement of internal jugular vein and carotid artery catheters under intraperitoneal pentobarbital anesthesia (Nembutal, 35 mg/kg body wt). The polyethylene carotid artery catheter was extended to the level of the aortic arch, and the silicone internal jugular vein catheter was advanced to the level of the right atrium. At the end of the procedure, both catheters were flushed and filled with heparin (42 U/ml) and polyvinylpyrrolidone (1.7 g/ml) solution, plugged, tunneled subcutaneously around the side of the neck, and externalized behind the head through a skin incision. Catheters remained sealed until the day of the study.

TABLE 1
Basal characteristics of the animals

	Perfusion through		
	VMH	FL	VMH (reversed order)
n	7	6	6
Insulin (pmol/l)	246 ± 60	384 ± 66	264 ± 42
Glucagon (ng/l)	130 ± 8	152 ± 17	148 ± 23
Epinephrine (nmol/l)	0.8 ± 0.2	0.8 ± 0.3	0.7 ± 0.2
Norepinephrine (nmol/l)	2.0 ± 0.5	1.2 ± 0	1.7 ± 0.3

Data are means ± SE.

Only those animals that had completely recovered and showed no signs of infection within 36 h after surgery were used.

The rats were food-deprived for ~2 h before the start of each experiment. The catheters were flushed with saline and maintained patent by a slow infusion of saline (20 μl/min) that contained a small amount of heparin (1–2 U/ml). Animals were fully awake and freely moving about in their cages. After a 60-min rest period, arterial blood samples were withdrawn from carotid artery catheter for assay of baseline plasma glucose, insulin, glucagon, epinephrine, and norepinephrine concentrations. At the same time, perfusions through the microdialysis probes were started. Three groups of experiments were performed. In the first group (n = 7), VMH probes were perfused sequentially with a solution that contained 5 mmol/l glucose for the first 30 min, followed by 100 mmol/l glucose for 60 min, 5 mmol/l glucose again for 30 min, and finally 100 mmol/l 2-deoxyglucose for the last 60 min of the study. In the second group (n = 6), perfusates were delivered in the same order as in the first group, but the FLs were perfused instead of the VMH. In the third group (n = 6), 100 mmol/l 2-deoxyglucose was introduced after the initial 5 mmol/l glucose perfusion. Arterial blood samples were withdrawn during the perfusion to measure glucose every 10 min and glucagon, catecholamines, and insulin every 30 min. During blood withdrawal, sample dilution by fluid in the dead space of the catheter was avoided by withdrawal of ~0.5 ml of blood before collection of each blood sample (~0.1 ml) with a second syringe. Subsequently, the contents of the initial syringe were reinfused to minimize blood loss. In addition, blood obtained from littermates was transfused during the study via the jugular vein catheter to quantitatively replace blood withdrawn during the experiment.

The volume of brain exposed to 2-deoxyglucose was estimated by including the [^{3}H]2-deoxyglucose in the dialysate and measuring its distribution in the cerebral tissue in four rats. In these experiments, VMH probes were perfused with a 100 mmol/l 2-deoxyglucose solution containing 0.06 μCi/ml of [^{3}H]2-deoxyglucose for 60 min. At the end of the experiment, the rats were anesthetized with pentobarbital (Nembutal, 35 mg/kg body wt) and decapitated, and the brains were removed and put into a rodent brain slicer. Frozen 1-mm coronal sections of brain containing probes were made and placed on an ice-cold flat surface.

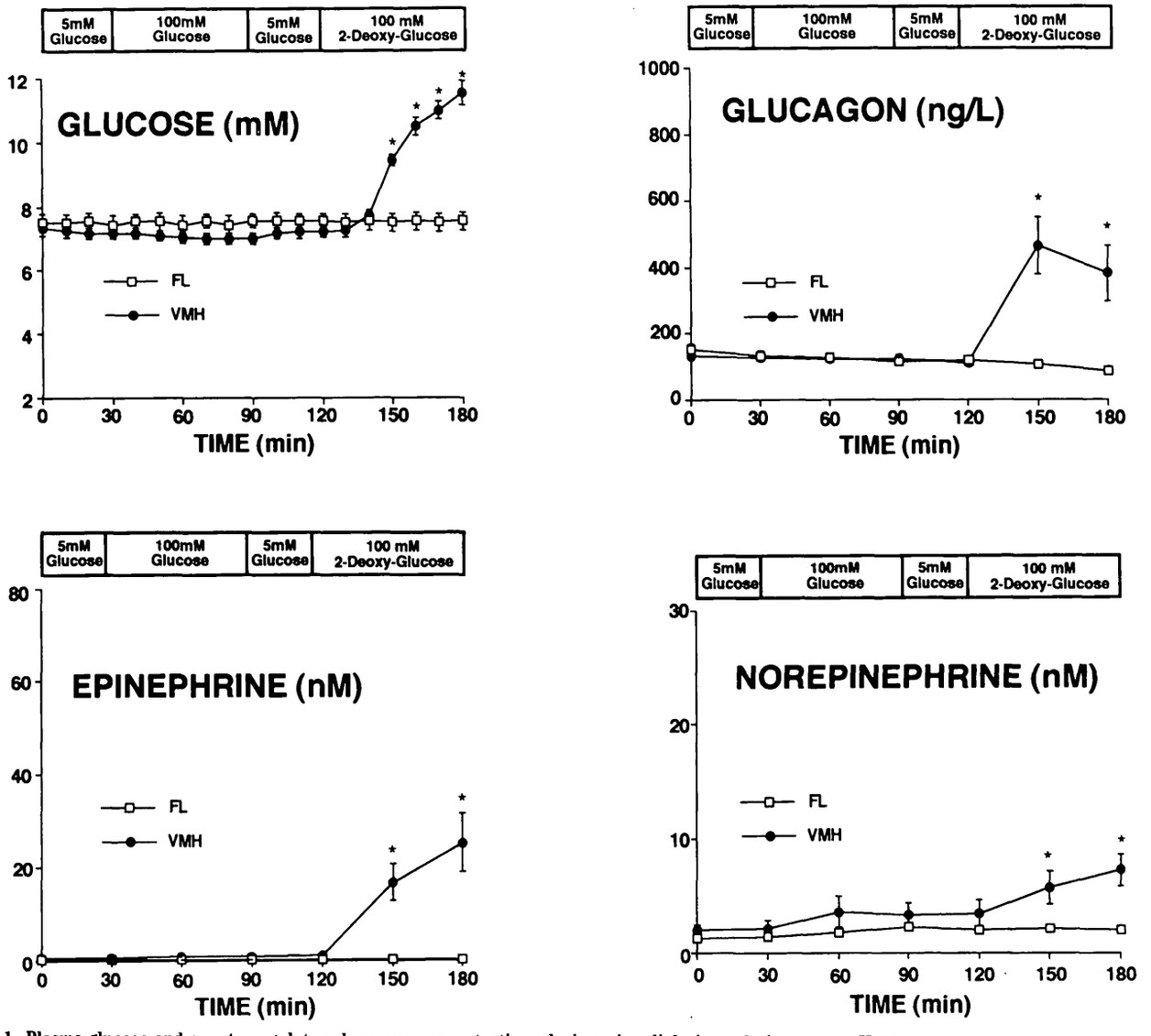

FIG. 1. Plasma glucose and counterregulatory hormone concentrations during microdialysis perfusions of the VMH and FLs. *Significant statistical difference as compared with FL perfusion ($P < 0.05$).

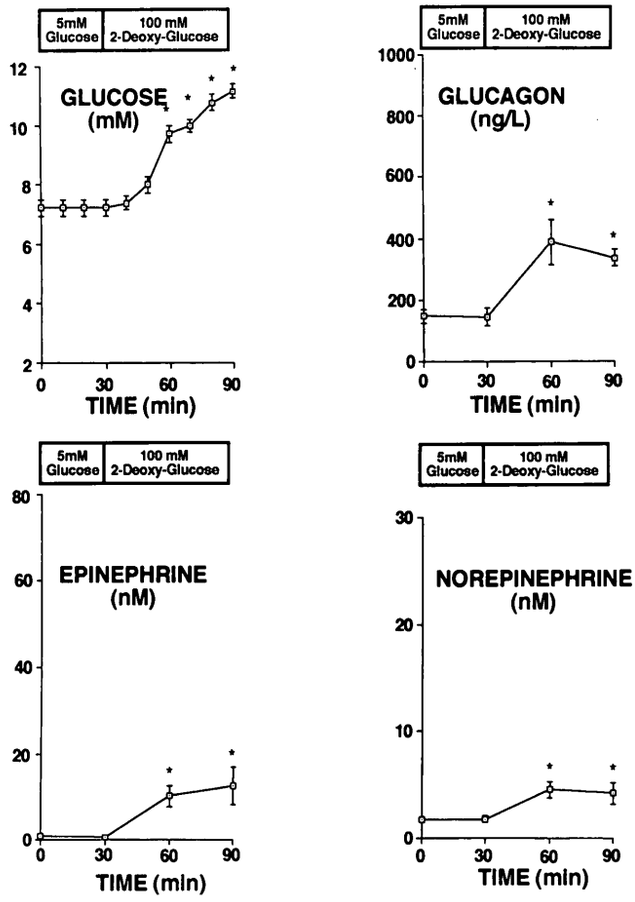

FIG. 2. Effect of VMH perfusion (with 2-deoxyglucose introduced at the beginning of the study, i.e., after the initial 5 mmol/l glucose perfusion) on plasma glucose and counterregulatory hormone concentrations. *Significant statistical difference between samples taken during and after 2-deoxyglucose perfusion, as compared with the baseline and the initial 5 mmol/l glucose perfusion period.

Thereafter, tissue samples were collected using an 18-gauge biopsy needle from the VMH and surrounding cerebral tissue, including the inferior part of the arcuate nucleus, dorsomedial nucleus, perifornical nucleus, medial amygdaloid nucleus, lateral hypothalamic area (LHA), and third verticle. Tissue samples were placed in scintillation liquid (Ultima-Gold, Packard), sonicated, and counted in a liquid scintillation counter (Tri-carb 1900; Packard). The protocol was reviewed and approved by the Yale Animal Care and Use Committee.

Plasma glucose was measured in duplicate by using a Beckman glucose analyzer II (Beckman). Plasma glucagon (ICN) and plasma insulin (Binax) concentrations were determined by a double-antibody radioimmunoassay with a porcine standard and a rat standard, respectively. Plasma concentrations of epinephrine and norepinephrine were measured by a radioenzymatic method (Amersham).

Data are expressed as means ± SE. Comparison between the study groups was made by analysis of variance with a repeated measure design, followed by Student's *t* test to localize effects.

RESULTS

As summarized in Table 1, basal plasma glucose, insulin, glucagon, epinephrine, and norepinephrine concentrations were not significantly different between any of the study groups. The average weights of the animals on the day of the study were also not significantly different between the groups (340 ± 20 g [VMH perfusion], 320 ± 30 g [FL perfusion], 340 ± 30 g [VMH perfusion with reversed order of the perfusates]).

The effects of VMH and FL perfusions on plasma glucose and counterregulatory hormone concentrations are depicted in Fig. 1. Both VMH and FL perfusions with 5 and 100 mmol/l

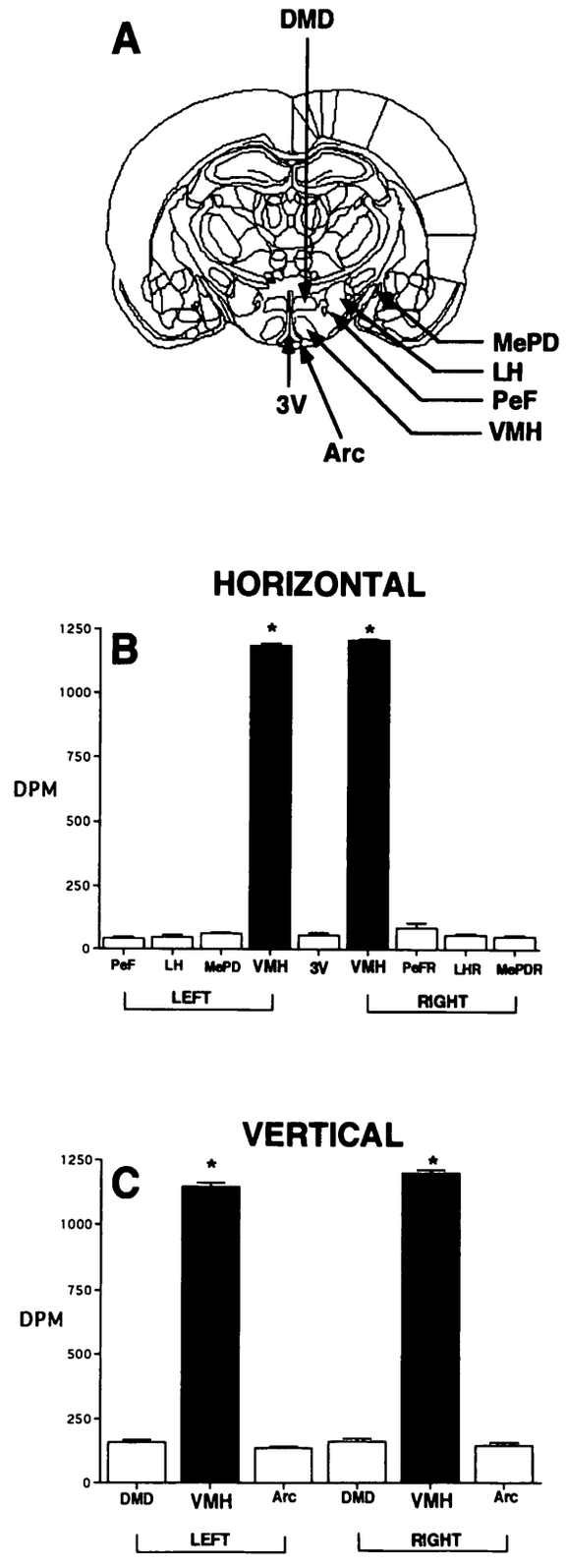

FIG. 3. The results of the measurements of distributions of the radiolabeled 2-deoxyglucose, perfused through VMH probe in four rats. *A* shows the location of the samples: *VMH*; *Arc*, inferior part of arcuate nucleus; *DMD*, dorsomedial nucleus; *PeF*, perifornical nucleus; *LH*, lateral hypothalamic area; *MePD*, medial amygdaloid nuclei; *3V*, third verticle. *B* and *C* show the radioactivity (expressed as dpm) of the samples from cerebral tissue. *Significant statistical difference between VMH and other samples ($P < 0.001$).

glucose had no effect on plasma glucose concentration. However, perfusion of 100 mmol/l 2-deoxyglucose into the VMH caused a significant increase in plasma glucose concentration as compared with that during 100 mmol/l glucose perfusion ($P < 0.05$ vs. 100 mmol/l glucose perfusion). Moreover, 2-deoxyglucose had no such effect when delivered to the FLs. As presented in Fig. 1, 2-deoxyglucose perfusion into VMH caused a marked increase in circulating glucagon, epinephrine, and norepinephrine concentrations as compared with FL perfusion ($P < 0.05$). No such elevation was observed during the earlier phase of the study when 5 or 100 mmol/l glucose was delivered to the VMH. In addition, the concentrations of the counterregulatory hormones during FL perfusion with 2-deoxyglucose were virtually identical to that observed during 100 mmol/l glucose perfusion.

When 2-deoxyglucose was introduced at the beginning of the study, perfusion of 2-deoxyglucose through VMH also had a hyperglycemic effect (Fig. 2). Furthermore, under these conditions, elevation of the counterregulatory hormone has also been noted (Fig. 2).

During the FL perfusions, plasma insulin concentrations were not significantly different during both 100 mmol/l 2-deoxyglucose and 100 mmol/l glucose perfusion (50 ± 156 vs. 276 ± 54 pmol/l; NS). Also, 2-deoxyglucose perfusion into VMH produced no significant change in circulating insulin concentration as compared with that seen during the 100 mmol/l glucose perfusion (114 ± 30 vs. 150 ± 18 pmol/l, NS).

Figure 3 shows the distribution of radiolabeled 2-deoxyglucose in rats perfused through the VMH probe. The radioactivity of samples obtained from the VMH was markedly higher than that of the surrounding tissues (i.e., inferior part of arcuate nucleus; dorsomedial, perifornical, and medial amygdaloid nuclei; LHA; and third verticle; $P < 0.001$). Furthermore, the radioactivity found in each of the samples of tissue outside of the VMH was not significantly different from the background level (NS).

DISCUSSION

This study demonstrates that localized glucopenia produced by delivery of 2-deoxyglucose directly into the VMH of awake rats triggers the release of counterregulatory hormones in the absence of systemic hypoglycemia. These findings provide strong evidence for the existence of hypothalamic glucoreceptors.

The role of the CNS in the regulation of counterregulatory responses to hypoglycemia is controversial. Although several lines of evidence strongly implicated the CNS in hypoglycemia detection and counterregulation (2,4,8,11), the precise brain regions involved were not established. Previous studies have demonstrated that injections of 2-deoxyglucose into the third ventricle of anesthetized animals causes hyperglycemia (12,13), suggesting that glucosensitive tissue may be situated in close proximity to the third ventricle. However, the specific nuclei involved are difficult to determine by using this approach because many important nuclei (arcuate, periventricular, perifornical, dorsomedial, VMH nuclei, etc.) are situated near the third ventricle. Furthermore, the widespread circulation of cerebrospinal fluid throughout the cerebral ventricles makes any conclusion regarding the location of the potential glucoreceptors even more uncertain. For these reasons, such experiments do not provide precise information about the location of the brain glucosensors.

Among the structures located near the brain ventricles, the nucleus of VMH, known as a regulator of food intake or the satiety center (12,14), has been proposed to contain glucosensitive neurons that could mediate the counterregulatory responses to hypoglycemia (15,16). This possibility mainly derives from the observation that mechanical or electrical stimulation of the VMH produces hyperglycemia in anesthetized rats (14,17). However, several studies have not supported this concept. DiRocco and Grill (18) reported that the sympathetic response to systemic infusion of 2-deoxyglucose was not abolished by complete decerebration of the rat. Cane et al. (19) observed that counterregulatory responses were not inhibited when glucose was infused in the carotid arteries to abolish forebrain hypoglycemia during systemic hypoglycemia. In addition, Ritter et al. (20) showed that obstruction of the cerebral aqueduct (which connects the fourth ventricle located in the hindbrain with the third and lateral ventricles situated in the forebrain) suppressed the hyperglycemic response to the metabolic inhibitor 5-thioglucose injected into the lateral ventricle, whereas the hyperglycemic response to 5-thioglucose injected into the fourth ventricle was attenuated, but not abolished. These data suggested that glucoreceptors might be located in the hindbrain (which consists of the pons and medulla oblongata) and not in the hypothalamus. However, this view is not supported by data of Frizzell et al. (4), who showed that bilateral glucose infusion into the vertebral circulation did not attenuate the counterregulatory response to hypoglycemia. They did, however, show that the release of counterregulatory hormones in response to hypoglycemia could be inhibited when glucose was infused into the carotid and vertebral arteries simultaneously, suggesting that the glucose sensor for hypoglycemia was indeed located within the CNS.

Although it has long been recognized that efferents from the ventral region of the hypothalamus impinge on the endocrine pancreas (21), the VMH was initially considered as a sympathetic center, controlling mainly catecholamine responses (22–25). Thus, when it was shown that electrical stimulation of the VMH in rats promoted glucagon secretion (17), this was thought to reflect activation of the autonomic nervous system, a potent stimulator of glucagon release (11,26). However, more recent studies have suggested that the VMH may play a key role in the detection and coordination of the counterregulatory responses to hypoglycemia. In these experiments, bilateral VMH lesions were produced in Sprague-Dawley rats by local ibotenic acid injection (8). Rats with lesions in the LHA and FL, sham-operated (stereotaxic needle placement into hypothalamus without injection) animals, and naive animals served as control groups. Glucagon, epinephrine, and norepinephrine responses to insulin-induced hypoglycemia were markedly inhibited in the VMH-lesioned rats as compared with the other control groups (8).

This study was intended to further clarify the contribution of the VMH to hypoglycemic counterregulation. We reasoned that, if glucosensors are located in the VMH, glucopenia limited to this neural center should cause a counterregulatory response despite peripheral normoglycemia. The microdialysis technique (10) provided a means of delivering 2-deoxyglucose directly into a specifically defined region of awake, unrestrained animals. Delivery of 2-deoxyglucose into the VMH caused a rapid and marked increase in circulating counterregulatory hormones accompanied by hyperglycemia. These responses were not caused by a nonspecific

hyperosmolar effect of the 100 mmol/l 2-deoxyglucose solution, because the same concentration of glucose perfused as a control had no such effect. Potential nonspecific effects of the microdialysis technique per se were excluded by applying the identical microdialysis procedure to a localized volume of FLs. Finally, the possibility that these changes were simply due to a time-dependent phenomenon was excluded by showing the same effect when 2-deoxyglucose was introduced at the beginning of the study, i.e., after the initial 5 mmol/l glucose perfusion. Note that the degree of glucopenia induced with 2-deoxyglucose is usually difficult to control and, therefore, difficult to relate to physiological hypoglycemia. Nevertheless, utilization of radiolabeled 2-deoxyglucose perfused through a microdialysis probe clearly showed that the local glucopenia produced in our experiments was limited to the VMH.

In summary, our studies demonstrated that glucopenia localized to the VMH triggers the release of glucagon and catecholamines. These findings support the hypothesis that the VMH contains neurons that sense glucopenia and that this brain region is important for mediating a counterregulatory response to hypoglycemia.

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service Grants DK-20495, DK-40936, DK-45735, and NS-28227 and by a fellowship grant from the Juvenile Diabetes Foundations International (W.P.B., M.A.B.).

We are indebted to Drs. William Tamborlane and Michael McCaleb for comments and suggestions. We appreciate the assistance of Aida Groszmann and Andrea Belous for their help in measuring plasma hormones. We also gratefully acknowledge Karen Davis for expert technical assistance.

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