Influence of Insulin in the Ventromedial Hypothalamus on Pancreatic Glucagon Secretion In Vivo

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OBJECTIVE—Insulin released by the β-cell is thought to act locally to regulate glucagon secretion. The possibility that insulin might also act centrally to modulate islet glucagon secretion has received little attention.

RESEARCH DESIGN AND METHODS—Initially the counterregulatory response to identical hypoglycemia was compared during intravenous insulin and phloridzin infusion in awake chronically catheterized nondiabetic rats. To explore whether the disparate glucagon responses seen were in part due to changes in ventromedial hypothalamic (VMH) exposure to insulin, bilateral guide canulas were inserted to the level of the VMH and 8 days later rats received a VMH microinjection of either 1) anti-insulin affibody, 2) control affibody, 3) artificial extracellular fluid, 4) insulin (50 μU), 5) insulin receptor antagonist (S961), or 6) anti-insulin affibody plus a γ-aminobutyric acid A (GABA_A) receptor agonist muscimol, prior to a hypoglycemic clamp or under baseline conditions.

RESULTS—As expected, insulin-induced hypoglycemia produced a threefold increase in plasma glucagon. However, the glucagon response was fourfold to fivefold greater when circulating insulin did not increase, despite equivalent hypoglycemia and C-peptide suppression. In contrast, epinephrine responses were not altered. The phloridzin-hypoglycemia induced glucagon increase was attenuated (40%) by VMH insulin microinjection. Conversely, local VMH blockade of insulin amplified glucagon twofold to threefold during insulin-induced hypoglycemia. Furthermore, local blockade of basal insulin levels or insulin receptors within the VMH caused an immediate twofold increase in fasting glucagon levels that was prevented by coinjection to the VMH of a GABA_A receptor agonist.

CONCLUSIONS—Together, these data suggest that insulin’s inhibitory effect on α-cell glucagon release is in part mediated at the level of the VMH under both normoglycemic and hypoglycemic conditions. Diabetes 59:1521–1527, 2010

The pancreatic β-cell is the primary regulator of glucose homeostasis and glucagon secretion (1,2). In the presence of changes in blood glucose concentration, local changes in insulin together with zinc and/or γ-aminobutyric acid (GABA) release from the β-cell are thought to act in concert to regulate α-cell glucagon secretion (3,4). However, during hypoglycemia the central nervous system (5,6), and the VMH in particular, also plays a critical role in stimulating the release of glucagon and other counterregulatory hormones, via changes in the activity of specialized glucose-sensing neurons (7–10). Moreover, mice with selective loss of glutamate release in steroidogenic factor 1 neurons, which are expressed exclusively in the VMH, show a markedly impaired glucagon secretory response to acute hypoglycemia (11). These and other studies suggest that central regulation of pancreatic α-cell glucagon secretion may play a greater role than previously anticipated, at least during insulin-induced hypoglycemia (2,6–8).

The fact that the VMH may directly influence α-cell glucagon release, and that glucose-sensing neurons in the VMH appear to use similar mechanisms for detecting fluctuations in glucose to the pancreatic α- and β-cells (7,8), suggested that insulin might play a similar regulatory role through a central mechanism. The possibility that insulin might exert a central effect is consistent with evidence that brain insulin levels mirror those in the circulation (12), that glucose-sensing neurons express insulin receptors, and that insulin alters the firing rate of these neurons (8,13–15). Moreover, insulin has been reported to act on the hypothalamus to regulate hepatic glucose production (16). However, studies in humans evaluating whether the level of circulating insulin during exogenous insulin administration influences counterregulatory hormone responses have yielded conflicting results. It has been reported that higher levels of plasma insulin may amplify (17,18) or inhibit (19–21) epinephrine responses during hypoglycemia. One study noted a significant effect of circulating insulin on accompanying glucagon responses, specifically an inhibitory action (20). Whether insulin acts centrally to influence glucose counterregulation is also uncertain. In animal studies, insulin delivered directly into the brain in very large doses has been reported to amplify counterregulatory hormone secretion (22–24) or to have no direct effect (25).

This study was undertaken to determine whether insulin acts within the ventromedial hypothalamus (VMH) to modulate the secretion of glucoregulatory hormones. For this purpose, we either blocked insulin action or administered insulin to the VMH, during hypoglycemia produced in the presence or absence of elevated circulating insulin levels, respectively. In addition, we examined whether
insulin acts locally in the VMH under basal conditions to regulate glucoregulatory hormone secretion in the absence of elevated levels of insulin. Our findings provide in vivo evidence for the first time that insulin acts within the VMH to influence the release of glucagon from α-cells both during hypoglycemia and under basal conditions.

RESEARCH DESIGN AND METHODS

Animal preparation for in vivo experiments. Adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were studied. Animals were housed in individual cages in the Yale Animal Resource Center, fed a standard pellet diet (Agway Probal 3000, Syracuse, NY), and maintained on a 12-h day/night cycle. At 7–10 days before each study, rats were anesthetized with an intraperitoneal injection (1 ml/kg) of a mixture of xylazine (AnaSed 20 mg/ml; Lloyd Laboratories, Shenandoah, IA) and ketamine (Ketaset 100 mg/ml; Aveco Co., Fort Dodge, IA) in a ratio of L2:vol:vol). The rats initially underwent cardiovascular surgery for the implantation of chronic vascular catheters, in the right internal jugular vein and the left carotid artery as described. The catheters (PE50 tubing with a tip made from silastic laboratory tubing [0.51 mm intradurally]) are inserted via a neck incision into the internal jugular vein and carotid artery and extended to the level of the right atrium and aortic arch, respectively. They are then tunneled subcutaneously and externalized at the nape of the neck where the catheter ends are left free. The patency of catheters is maintained by filling them with a heparin/polyvinylpyrrolidone solution; the venous catheter was used for infusions and the artery, for blood sampling. In addition, bilateral microinjection guide cannulas were stereotaxically inserted 1 mm above the VMH for delivery of test substances as described previously (26). Experimental protocols are in accordance with laboratory animal care guidelines and were approved by the Yale Animal Care and Use Committee.

In vivo experiments. Metabolic experiments were conducted ~8 days after complete recovery from surgery. Insulin was diluted and phloridzin dissolved in saline. All microinjected solutions were diluted in artificial extracellular fluid (aECF). Prior to the start of experiments, animals were fasted overnight and in the morning they were studied while awake and able to move about their cages. Initially, blood was withdrawn to assess baseline hormone levels. Thereafter, animals received bilateral microinjections via the guide cannulas of either aECF (n = 6), insulin (50 μU) (n = 5), control affibody (1 μg) (n = 8), anti-insulin affibody (AB21006, 1 μg; Abcam, Cambridge MA) (n = 8), anti-insulin affibody + muscimol (1 nmol/l) (n = 6), or high-affinity insulin receptor antagonist SR961 (1 μg) (Novo Nordisk, Bagsvaerd, Denmark) (n = 6). Affibody molecules mimic antibodies in function but have advantages of a smaller size (~6 kDa) and stability. After microinjection, hypoglycemic clamps were performed for 90 min using either an insulin (6 mU/kg/min) or phloridzin (100 μg/kg/min) infusion and fixing blood glucose at ~70 mg/dl using a variable rate glucose infusion. Blood samples were collected at 30, 60, and 90 min for measurement of plasma glucagon, C-peptide, and insulin by radioimmunooassay (Linco Research) and catecholamines using high-performance liquid chromatography. Peak glucagon responses occurred at 60 min. After euthanasia, the erythrocytes were resuspended in an equivalent volume of artificial plasma and reinfused back into the animal to prevent volume depletion and anemia. Animals were killed using sodium pentobarbital and the brains removed and frozen in dry ice. Subsequently, the accuracy of probe placements was determined by histological inspection of coronal brain sections. Only data obtained from animals in which the probes were positioned beside the VMH were used (~15% of the animals were excluded).

In vitro and in vivo validation of the anti-insulin affibody. 3T3L1 cells were cultured to confluence in high-glucose Dulbecco’s modified Eagle’s medium containing 10% bovine growth serum (Hyclone) and 1% penicillin-streptomycin. Confluent cells were then differentiated using high-glucose Dulbecco’s modified Eagle’s medium containing 10% FBS, isobutylmethylxanthine, insulin, and dexamethasone, as described (27). Cells were considered fully mature 8 days after the induction of differentiation. After complete differentiation, cells were serum starved overnight and treated with either PBS, 3 mM insulin alone, or 3 mM insulin preincubated with 10 μg anti-insulin affibody. After stimulation, cells were washed with ice-cold PBS and total protein was isolated. Total cell lysate (15 μg) was subjected to Western blotting and membranes were probed using antibodies against p-Akt (no. 9271; Cell Signaling) or glyceraldehyde-3-phosphate dehydrogenase (Sigma) for 1 h. Two validation studies, hypoglycemic clamps were conducted with VMH anti-insulin or control affibody microinjection, as described above, and brains from animals were collected at 30 min. The brains were sectioned and total protein from VMH micropunches was obtained. Akt phosphorylation in the VMH in response to insulin treatment was analyzed using Western blotting using antibodies against p-Akt and normalized against β-actin as a loading control. These studies confirmed the insulin-blocking action of the affibody in vitro and in vivo. Addition of the anti-insulin affibody in vitro prevented the phosphorylation of Akt in 3T3L1 cells (Fig. 1A), and VMH microinjection of anti-insulin affibodies suppressed the 2.5-fold local increase in Akt phosphorylation produced during insulin-induced hypoglycemia (Fig. 1B).

Additional methods. All values are mean ± SEM. Treatment effects were analyzed using repeated-measures two-way ANOVA followed by post hoc analysis to localize effects or two-tailed Student t tests as appropriate using the Prism analytical software. P < 0.05 was set as the criterion for statistical significance.

RESULTS

Glucagon responses to insulin— versus phloridzin— induced hypoglycemia. To test the effects of circulating insulin on glucagon responses to hypoglycemia, we intravenously infused rats with either insulin or phloridzin and allowed plasma glucose to fall to ~70 mg/dl using the glucose clamp technique. Plasma insulin declined with phloridzin (7 ± 1 to 4 ± 1 μU/ml) and rose with insulin (8 ± 1 to 151 ± 21 μU/ml) infusion, but plasma glucagon was identically reduced in both groups (Fig. 1). The exogenous glucose infusion rate (GIR) to maintain identical hypoglycemia was, however, markedly higher with insulin hypoglycemia compared with phloridzin (8.7 ± 0.3 vs. 0.5 ± 0.1 mg/kg/min; insulin vs. phloridzin P < 0.01). Glucagon levels increased during both phloridzin-induced (33 ± 12 baseline to 777 ± 122 ng/l) and insulin-induced (55 ± 10 to 178 ± 45 ng/l) hypoglycemia (P < 0.01), however, the magnitude of the rise was fourfold to fivefold greater in the absence of systemic hyperinsulinemia. This difference occurred despite identical 70–90% decreases in
C-peptide levels and comparable increases in plasma catecholamines (Fig. 2). To evaluate whether the phloridzin infusion might act directly to stimulate glucagon secretion, we administered phloridzin while maintaining euglycemia using a variable glucose infusion. Under these conditions, plasma glucagon remained unchanged (38 ± 12 baseline vs. peak levels of 55 ± 11 ng/l during clamp, n = 6; P = not significant [NS]), suggesting the lack of a direct effect on α-cell secretion. Because these studies do not distinguish between the relative contribution of intraislet and hypothalamic insulin to the disparate glucagon response, additional studies were conducted to manipulate VMH insulin action under hyperinsulinemic and hypoglycemia.

**VMH insulin action suppresses glucagon response to insulin-induced hypoglycemia.** To investigate whether the observed disparate glucagon responses to hypoglycemic stimuli could at least in part be attributed to differences in the exposure of the VMH to insulin, we microinjected specific anti-insulin or control (non-insulin binding) affibodies bilaterally into the VMH to locally bind and reduce local insulin levels during a 6 mU/kg/min hyperinsulinemic hypoglycemic clamp. Blockade of insulin action within the VMH reduced by 50% the GIR required to maintain the same hypoglycemic plateau compared with microinjection of a control affibody (4.4 ± 0.4 anti-insulin affibody vs. 8.7 ± 0.3 mg/kg/min control, P < 0.05). This effect was associated with a 2.5-fold greater glucagon increase in rats that received the VMH anti-insulin affibody (430 ± 95 anti-insulin affibody vs. 178 ± 45 ng/l control affibody; P < 0.05). In contrast, blockade of insulin within the VMH had no effect on the suppression of either C-peptide or catecholamine levels during hypoglycemia, suggesting that insulin acts directly on the VMH to selectively suppress glucagon secretion independent of intraislet insulin or input from circulating catecholamines (Fig. 3).

**VMH insulin microinjection suppresses glucagon response to phloridzin hypoglycemia.** Conversely, when insulin (50 μU) or aECF (control) was microinjected bilaterally into the VMH during a phloridzin hypoglycemic clamp study, local insulin delivery reduced glucagon-mediated counterregulation. Local VMH insulin delivery caused an increase in the glucose infusion rate required to maintain the same hypoglycemic plateau (1.7 ± 0.2 VMH insulin vs. 0.4 ± 0.1 mg/kg/min control, P < 0.05) and suppressed glucagon levels by 40% (777 ± 122 control vs. 498 ± 60 ng/l insulin, P < 0.05; Fig. 3). No significant changes were observed in plasma insulin, C-peptide, or catecholamines levels. The inability of VMH insulin delivery to completely suppress the glucagon response to phloridzin hypoglycemia to levels seen with insulin-induced hypoglycemia may be due to insulin actions within other target areas, such as the islet.

**Local blockade of VMH insulin increases basal glucagon levels.** To examine whether the β-cell might regulate basal α-cell glucagon secretion via insulin delivery to the VMH in the absence of exogenous insulin, we microinjected either 1) an anti-insulin affibody (or a control affibody), 2) a potent insulin receptor antagonist (28) (S961; Novo Nordisk), or 3) aECF bilaterally into the VMH of awake overnight-fasted rats. As shown in Fig. 4, blockade of endogenous VMH insulin action (anti-insulin affibody) caused an immediate twofold increase in plasma glucagon within 5 min. This resulted in a rise in plasma glucose (15 ± 5 mg/dl), limited by a small but insignificant
Clamp

Insulin affibody

In part, by an enhancement of local GABA tone. Thus, insulin's effect on the VMH may be mediated, at least has no effect on either glucagon or glucose levels (29). In contrast, we have previously shown that in over-abolished by the simultaneous injection of muscimol (Fig. 4). The rapid increase in glucagon release caused by local blockade of VMH insulin was completely because of rapid disinhibition of glucagon secretion. No such changes in either study were observed in control animals (Fig. 4). The rapid increase in glucagon release caused by local blockade of VMH insulin was completely abolished by the simultaneous injection of muscimol (Fig. 4). In contrast, we have previously shown that in overnight-fasted rats VMH microinjection of muscimol alone has no effect on either glucagon or glucose levels (29). Thus, insulin’s effect on the VMH may be mediated, at least in part, by an enhancement of local GABA tone.

DISCUSSION

Insulin regulates glucose homeostasis through its actions on multiple sites, including the liver, muscle, and adipose tissue (30). Insulin also exerts important effects on the central nervous system, including neural development, satiety/feeding, cognitive function, and aging (31,32). Glucagon release from the neighboring a-cell, a major determinant of hepatic glucose production, has also been shown to be under the control of insulin levels within the islet (2,33). The current study extends these observations by providing evidence that in addition to its local paracrine action, insulin acts in the VMH to influence and possibly fine-tune the release of pancreatic glucagon under both hypoglycemic and fasting conditions. Previous studies from our laboratory have demonstrated that a variety of factors involved in glucose sensing by VMH neurons and the neurotransmitter input to these neurons, such as GABA, generally alter both glucagon and sympathoadrenal counterregulatory responses (26). Thus, the failure of VMH insulin delivery to act similarly and simultaneously alter the sympathoadrenal response to hypoglycemia implies that separate neural pathways might activate glucagon and sympathoadrenal responses to glucose deprivation, or that those other systems contribute to a final common pathway, thereby altering both responses. It is noteworthy in this regard that mice lacking VGLUT2 specifically in steroidogenic factor 1 neurons (a major subset of VMH neurons), and thus unable to release glutamate from these neurons, show a much more profound effect on glucagon than catecholamine release in response to glucopenia (11). The possibility that the brain and pancreas operate in concert to control glucagon secretion is consistent with the reported similarities found between the mechanisms used by glucose-sensing β-cells and their neuronal counterparts in the VMH (8).

In contrast to the current findings, previous studies using neuronal insulin receptor knockout mice suggested that brain insulin amplifies catecholamine but not glucagon responses to hypoglycemia (24). The differences between our findings and those studies may be explained by developmental adaptations within the central nervous system and/or the global neuronal insulin receptor knock-down produced in the neuronal insulin receptor knockout mouse. Moreover, a recent study failed to detect an effect of brain insulin infusion on catecholamine or glucagon responses to hypoglycemia or glucoprivation (25). However, those observations may be complicated by the very large pharmacological doses of exogenous insulin delivered to the brain. In the current study, the role of insulin in glucose counterregulation was assessed by two complementary approaches. Phloridzin or insulin was infused peripherally to compare the hormone response with a comparable fall in plasma glucose in the presence or absence of insulin. These studies demonstrated marked differences in glucagon secretory responses, however, the tissues responsible for the disparate response to the two hypoglycemias could not be established with this approach. To explore this question, we locally increased or

| GIR (mg/kg/min) | Plasma glucagon (pg/ml) | Plasma C-peptide (pM) | Plasma epinephrine (pg/ml) |
|----------------|------------------------|-----------------------|---------------------------|
| Control        | Affibody               | Insulin               | VMH aECF VMH Insulin (50uU) |
| 0.0            | 200                    | 100                   | 1000                      |
| 0.5            | 300                    | 150                   | 2000                      |
| 1.0            | 400                    | 200                   | 3000                      |
| 1.5            | 500                    | 250                   | 4000                      |
| 2.0            | 600                    | 300                   | 5000                      |

FIG. 3. Effect of local blockade of VMH insulin during insulin-induced hypoglycemia (above) and VMH delivery of insulin during phloridzin-induced hypoglycemia (below) on GIR, peak glucagon, C-peptide, and epinephrine levels. Blockade of VMH insulin action reduced GIRs and amplified glucagon responses during systemic insulin infusion, whereas VMH insulin microinjection during phloridzin infusion increased GIR and suppressed glucagon responses. (All values ± SEM, *P < 0.05.)
blocked insulin within the VMH, a key brain glucose-sensing region, during infusion of phloridzin and insulin, respectively. We used a mild hypoglycemic stimulus (70 mg/dl) that minimized the confounding effects on pancreatic glucagon secretion of simultaneous marked increases in sympathoadrenal activity caused by more severe hypoglycemia. Such increases independently stimulate α-cell glucagon secretion.

It should be noted that although our microinjection probes were directed predominately at the ventromedial nucleus of the VMH (which includes both the ventromedial and arcuate nucleus), very small volumes were used, and because this approach largely restricts delivery of agents to the VMH, it is difficult to completely limit spread to neighboring areas (34). Thus, we cannot exclude the possibility that spillover effects to surrounding regions might also have contributed to the observed changes in glucagon secretion. It is noteworthy in this regard that genetically engineered mice with predominant absence of insulin receptors in hypothalamic neurons display hepatic insulin resistance (30). Furthermore, conditional knock-in of insulin receptors in Agouti related peptide (Agrp) neurons of these mice reduced hepatic glucose production (glucagon was not measured) (35). Whether the effects seen during VMH microinjection in the current study reflect spread to Agrp neurons in the arcuate nucleus remain uncertain. In addition, previous studies have demonstrated that delivery of insulin into the hypothalamus directly suppresses hepatic glucose production (16). Thus, we cannot exclude the possibility that direct stimulation of glucose release from the liver as well as stimulation of glucagon secretion contributed to the lower glucose infusion rates observed after blockade of VMH insulin during the clamp study. Finally, our observation that systemic insulin delivery increases Akt phosphorylation within the VMH is consistent with previous studies showing that insulin exerts its effects on the hypothalamus via activation of the phosphatidylinositol 3 kinase signaling pathway (36).

Of particular interest, insulin within the VMH also appears to regulate glucagon secretion under basal conditions, lending even greater significance to the role of hypothalamic insulin action in glycemic regulation. Blockade of insulin action in the VMH by two separate mechanisms caused an immediate increase in plasma glucagon in the absence of a decline in insulin secretion. These findings support the view that the hyperglucagonemia was induced at the level of the VMH rather than the islet and raise the possibility that impaired VMH insulin action may also contribute to disordered systemic glucose regulation. This view is consistent with reports of the development of a diabetes phenotype in Drosophila after ablation of brain
insulin (37) and of insulin resistance in mice with the knockdown of brain insulin receptors (38).

With respect to the possible mechanism for insulin’s effect within the VMH, studies conducted in islets have shown that GABA is released from the β-cells together with insulin and that insulin acts on the α-cell to increase GABAₐ receptor trafficking to the cell surface, thereby enhancing GABA’s inhibitory effect on glucagon secretion (39,40). Moreover, we have reported that VMH GABAergic tone exerts a powerful effect on the counterregulatory response to hypoglycemia (26,29). GABA levels in VMH interstitial fluid decrease in response to hypoglycemia, promoting hormone release (26), whereas inappropriately elevated GABA levels in the VMH appear to contribute to the absent glucagon response to insulin-induced hypoglycemia observed in rodent models of type 1 diabetes (O.C., unpublished observations). It has also been reported that insulin exerts direct electrophysiological effects on neurons in vivo, which may be influenced by GABAergic inputs (14). The observation that GABAₐ receptor antagonist reverses insulin’s effect on the VMH implies that insulin may act by enhancing GABAergic tone in the VMH, as is seen in the islet (38,39). In addition to potential effects of insulin on GABAergic tone within the VMH, studies have demonstrated that VMH ATP-sensitive K⁺ channel activity influences both glucose counterregulation and hepatic gluconeogenesis (41). These studies coupled with data showing insulin’s effects on the opening of ATP-sensitive K⁺ channels (41,42) make this a plausible downstream mechanism, which might explain the effects observed here. The striking similarities between the functioning of the pancreatic β-cells and glucose-sensing neurons, as well as evidence that insulin functions as a neuronal peptide in more primitive organisms, are in keeping with the emerging view that the brain and the pancreatic islet share common evolutionarily conserved mechanisms (7,43,44).

The current study does not, however, suggest that insulin within the VMH has the primary role in regulation of glucagon secretion. Intraislet insulin undoubtedly plays a critical role in glucagon secretion. This view is supported by our failure to see complete suppression of the glucagon response during local VMH insulin delivery during phlorizin-induced hypoglycemia. The suppressive effect of local insulin was less than 50%. Moreover, a recent study reported a very similar effect of insulin signaling in the islet to regulate glucagon release (33). On the other hand, given that the rise in basal glucagon levels observed after VMH insulin blockade was not accompanied by a fall in endogenous insulin secretion and that C-peptide was not accompanied by a fall in basal glucagon levels observed after VMH insulin delivery in the absence of VMH insulin blockade, it is unlikely that the marked differences in glucagon responses can be attributed solely to differences in intraislet insulin levels.

The primary metabolic evolutionary challenge faced by humans has been starvation, during which insulin and glucose levels decrease in unison, thereby releasing the restraining effect of insulin on the α-cell at the level of both the islet (45,46), and the VMH. This results in an inappropriate disinhibition of glucagon secretion, allowing the release of stored glucose from the liver. The current data suggest that the decrease in glucose provoked by systemic insulin administration may be not only perceived as a signal of metabolic deprivation but also seen as a signal of concomitant food availability, thereby resulting in a muted or maladaptive glucagon response. It is noteworthy that a similar enhancement of glucagon secretion has been reported with drug-induced insulin-independent decrements in blood glucose in the dog, which was attributed to suppression of intraislet insulin, but that is equally consistent with the loss of the central inhibitory actions of insulin (47). One might speculate that the loss of glucagon response to hypoglycemia in type 1 diabetic patients lacking endogenous insulin might result in part from the simultaneous increase in insulin levels both in the VMH and locally in the islet caused by exogenous insulin administration. This possibility is consistent with our previous report showing marked stimulation of glucagon and epinephrine release in diabetic BB rats when hypoglycemia was produced by systemic infusion of 5’-aminoisooxazol-4-carboxyamide-1-β-D-ribofuranoside (a chemical activator of AMP-kinase) and phlorizin rather than insulin (48).

We conclude that β-cell insulin secretion acts to inhibit α-cell glucagon secretion both at the level of the islet and the VMH. Insulin’s central effect appears to occur under hypoglycemia as well as basal conditions and thus might contribute to disorders of glucose homeostasis.

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