 Activation of ATP-Sensitive K+ Channels in the Ventromedial Hypothalamus Amplifies Counterregulatory Hormone Responses to Hypoglycemia in Normal and Recurrently Hypoglycemic Rats

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The mechanism(s) by which glucosensing neurons detect fluctuations in glucose remains largely unknown. In the pancreatic β-cell, ATP-sensitive K+ channels (KATP channels) play a key role in glucosensing by providing a link between neuronal metabolism and membrane potential. The present study was designed to determine in vivo whether the pharmacological opening of ventromedial hypothalamic KATP channels during systemic hypoglycemia would amplify hormonal counterregulatory responses in normal rats and those with defective counterregulation arising from prior recurrent hypoglycemia. Controlled hypoglycemia (~2.8 mmol/l) was induced in vivo using a hyperinsulinemic (20 mU · kg⁻¹ · min⁻¹) glucose clamp technique in unrestrained, overnight-fasted, chronically catheterized Sprague-Dawley rats. Immediately before the induction of hypoglycemia, the rats received bilateral ventromedial hypothalamic microinjections of either the potassium channel opener (KCOs) diazoxide and NN414 or their respective controls. In normal rats, both KCOs amplified epinephrine and glucagon counterregulatory responses to hypoglycemia. Moreover, diazoxide also amplified the counterregulatory responses in a rat model of defective counterregulation. Taken together, our data suggest that the KATP channel plays a key role in vivo within glucosensing neurons in the ventromedial hypothalamus in the detection of incipient hypoglycemia and the initiation of protective counterregulatory responses. We also conclude that KCOs may offer a future potential therapeutic option for individuals with insulin-treated diabetes who develop defective counterregulation. Diabetes 54:3169–3174, 2005

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recently shown in vivo that pharmacological closure of the impaired glucose counterregulation (38), and we have excited neurons and can alter the response of glucose-excited neurons to changes in ambient glucose levels. In animal models, transgenic Kir6.2 knockout mice show impaired glucose counterregulation (38), and we have recently shown in vivo that pharmacological closure of the \( K_{\text{ATP}} \) channel in the VMH via direct microinjection of glibenclamide suppressed hormonal counterregulatory responses to systemic insulin-induced hypoglycemia (39).

The present study was designed to answer the perhaps more clinically relevant question; namely, would pharmacological opening of ventromedial hypothalamic \( K_{\text{ATP}} \) channels during systemic hypoglycemia amplify the hormonal counterregulatory response? Furthermore, we also sought to determine whether we could reverse the counterregulatory hormone defect that ensues from recurrent antecedent hypoglycemia through pharmacological opening of ventromedial hypothalamic \( K_{\text{ATP}} \) channels during a subsequent episode of systemic hypoglycemia.

**RESEARCH DESIGN AND METHODS**

Three separate studies were performed: 1) an examination of the effect of acute microinjection of the potassium channel opener (KCO), diazoxide, to the VMH on counterregulatory responses to hypoglycemia; 2) an examination of the effect of acute microinjection of the SUR-1 selective KCO, NN414 (40), to the VMH on counterregulatory responses to hypoglycemia; and 3) an examination of the effect of acute microinjection of the KCO, diazoxide, to the VMH on counterregulatory responses to hypoglycemia in rats that had experienced recurrent acute microinjection of the potassium channel opener (KCO), diazoxide, to the VMH on counterregulatory responses to hypoglycemia in rats that had experienced recurrent

**RESULTS**

**Effect of ventromedial hypothalamic microinjection of the KCO, diazoxide, on counterregulatory responses to acute hypoglycemia.** In the first study, the effect of bilateral microinjection of the KCO, diazoxide (\( n = 11 \)), in comparison with vehicle-injected rats (CON-1; \( n = 11 \)), on counterregulatory responses to acute hypoglycemia was examined. Plasma glucose profiles under the two study conditions did not significantly differ (mean glucose 60–90 min: 2.9 ± 0.1 vs. 2.8 ± 0.1 mmol/l, diazoxide vs. CON-1, respectively). In contrast, the glucose infusion rates (GIRs) from 60 to 90 min required to maintain hypoglycemia were reduced by ~45% following VMH diazoxide (9.9 ± 1.9 vs. 17.6 ± 2.6 mg · kg\(^{-1} \) · min\(^{-1} \) infusion of human regular insulin (Eli Lilly) was begun. The plasma glucose was allowed to fall to ~2.8 mmol/l (50 mg/dl) and was then maintained at this level for 90 min using a variable-rate 20% dextrose infusion based on frequent plasma glucose determinations. Samples for measurement of the hormones epinephrine, norepinephrine, glucagon, and insulin were taken at ~10, 45, 60, 75, and 90 min.

**Analytical procedures.** Plasma levels of glucose were measured by the glucose oxidase method (Beckman, Fullerton, CA). Catecholamine analysis was performed by high-performance liquid chromatography using electrochemical detection (ESA, Acton, MA); plasma insulin and glucagon were measured by radioimmunoassay (Linco, St. Charles, MO). All data are expressed as means ± SE. Area under the curve (AUC) for each hormone was calculated for each study and then divided by time of study (90 min). Means from each group were then compared using a t test (SPSS 11.0 for Windows; SPSS).

**Effect of ventromedial hypothalamic microinjection of the SUR-1 selective KCO, NN414, on counterregulatory responses to acute hypoglycemia.** NN414 is a novel KCO that selectively activates Kir6.2/SUR-1 (40). We also compared the effect of bilateral ventromedial hypothalamic microinjection of NN414 (\( n = 7 \)) with control rats (CON-2; \( n = 6 \)) on counterregulatory responses to hyperinsulinemic hypoglycemia. Mean plasma glucose during each hypoglycemic plateau did not significantly differ...
(mean plasma glucose 60–90 min: 2.7 ± 0.1 vs. 2.7 ± 0.1 mmol/l for NN414 vs. CON-2, respectively). As with the
diazoxide study, we found that NN414 ventromedial hypo-
thalamic–microinjected rats required significantly less ex-
ogenous glucose (~65%) to maintain equivalent
hypoglycemia (5.2 ± 2.0 vs. 14.7 ± 2.3 mg · kg⁻¹ · min⁻¹;
P < 0.05; Fig. 1A). NN414-injected rats also demonstrated
significant increases in plasma epinephrine (AUC/time
9.1 ± 2.0 vs. 3.5 ± 0.7 nmol/l; P < 0.05; Fig. 1B) and
glucagon (AUC/time 186.5 ± 32.9 vs. 100.0 [22.1] ng/l; P <
0.05; Fig. 1C) but not norepinephrine (1.8 ± 0.4 vs. 1.6 ±
0.3 nmol/l; P = NS) responses to hypoglycemia in compar-
ison with control rats. Plasma insulin did not differ be-
tween groups during the clamp procedure.

**Effect of ventromedial hypothalamic microinjection**
of the KCO, diazoxide, on counterregulatory re-
sponses to acute hypoglycemia in rats who had
experienced recurrent episodes of insulin-induced
hypoglycemia. Plasma glucose profiles during the hyper-
insulinemic-hypoglycemic clamp studies in recurrently
hypoglycemic Sprague-Dawley rats did not differ between
the diazoxide (n = 10) or control (n = 14) rats (mean
glucose 60–90 min: 2.9 ± 0.1 vs. 2.9 ± 0.1 mmol/l,
respectively; P = NS). However, once again, ventromedial
hypothalamic microinjection of diazoxide resulted in a
significant reduction in the GIR required to maintain the
hypoglycemic plateau (11.1 ± 2.2 vs. 21.0 ± 2.1 mg · kg⁻¹
· min⁻¹ in controls; P < 0.05; Fig. 2A). The reduction in
GIR following diazoxide was of a similar magnitude to that
seen in the normal rats (~45%). This was accompanied by
significant increases in epinephrine (AUC/time: 4.4 ± 0.7
vs. 1.6 ± 0.3 nmol/l; P < 0.05; Fig. 2B) and glucagon
(173.2 ± 28.6 vs. 77.3 ± 15.2 ng/l; P < 0.05; Fig. 2C) but not
norepinephrine (2.3 ± 0.4 vs. 1.9 ± 0.3 nmol/l; P = NS)
secretory responses during subsequent hypoglycemia.
Plasma insulin levels did not differ between groups during
the clamp procedure in this experiment.

Comparison of our two control groups in the diazoxide
studies showed that the recurrent hypoglycemia protocol
had resulted in a significant impairment of the epinephrine
(3.4 ± 0.6 vs. 1.6 ± 0.3 nmol/l; normal control vs. recur-
rently hypoglycemic control; P < 0.05) but not the gluca-
gon (88.1 ± 17.9 vs. 77.3 ± 15.2 ng/l; P = NS) response to
the study hypoglycemia (Table 1). VMH diazoxide in
recurrently hypoglycemic rats restored the counterregula-

![FIG. 1. GIRs (A), plasma epinephrine (B), and plasma glucagon (C) following ventromedial hypothalamic microinjection in normal Sprague-Dawley rats under each study condition. ■, diazoxide group; □, NN414; and controls (CON-1 [■] and CON-2 [□]). Values are shown as means ± SE and represent AUC/time. *P < 0.05 vs. control study.](image1)

![FIG. 2. Epinephrine and glucagon levels during hypoglycemia in recurrently hypoglycemic Sprague-Dawley rats following ventromedial hypothalamic microinjection of diazoxide or control. Results shown as means ± SE plasma values at each sampling time point (A and B: ■, diazoxide; □, control) and as AUC/time. C and D: ■, diazoxide; □, control. *P < 0.05 vs. control.](image2)
VMH DIAZOXIDE AND GLUCOSE COUNTERREGULATION

DISCUSSION

There is substantial evidence in vitro (10,14,21,33,35,42-44) and in vivo (38,39) indicating a key role for the K<sub>ATP</sub> channel in glucosensing in the hypothalamus and, in particular, the VMH. This evidence includes 1) demonstration of K<sub>ATP</sub> channels in brain, including the VMH (33,43); 2) RT-PCR amplification of cytoplasm harvested at the end of fura-2 Ca<sup>2+</sup> imaging studies identifying SUR-1 and Kir6.2 in ventromedial hypothalamic neurons (23); 3) electrophysiological studies in brain-slice preparations showing ventromedial hypothalamic K<sub>ATP</sub> channel activity that is responsive to both changes in extracellular substrate and SUR-1 ligands and moreover that K<sub>ATP</sub> responses to substrate can be modified by SUR-1 ligands (14,17,21,24,38); and 4) the in vitro demonstration that ventromedial hypothalamic neurons in Kir6.2<sup>−/−</sup> mice are unresponsive to changes in extracellular glucose and SUR-1 modulation (38). In keeping with these observations, the current study together with our previous report provide data demonstrating that in vivo delivery of agents that either open or close the K<sub>ATP</sub> channel within the VMH of the rat have converse effects on the normal hormonal counterregulatory response to acute hypoglycemia. These in vivo studies extend earlier work by providing the specificity that limits interpretation of data from the study of transgenic mice where there is a more generalized defect in the target gene and from the in vitro study of brain-slice preparations or cells in culture where normal interneuronal connectivity is disrupted.

K<sub>ATP</sub> channels consist of pore-forming Kir6.x subunits that associate with different kinds of regulatory sulfonylurea receptor subunits: SUR-1, SUR-2A, and SUR-2B. Diazoxide acts predominantly through Kir6.2/SUR-1; however, it can also act on SUR-2B regulatory subunits found on vascular smooth muscle fibers, which suggests that under certain conditions it will have a vasodilatory action. To investigate whether the action of diazoxide in the VMH to amplify counterregulatory responses to acute hypoglycemia might have resulted from an alteration in local cerebral blood flow through activation of Kir6.2/SUR-2B, we chose to perform a further series of in vivo studies using a second potassium channel activator, NN414. NN414 has been shown to selectively activate KATP channels of the Kir6.2/SUR-1 type (40). Dabrowski et al. (40) compared the effects of NN414 and diazoxide on whole-cell K<sup>+</sup> currents in an HEK293 cell line stably expressing the pancreatic β-cell-type K<sub>ATP</sub> channel Kir6.2/SUR-1 and reported an EC<sub>50</sub> for NN414 of 0.45 ± 0.1 µmol/l and for diazoxide 31 ± 5 µmol/l. In contrast, NN414 had no activating effect on oocytes expressing either Kir6.2/SUR-2A or Kir6.2/SUR-2B channels. Interestingly, when the investigators examined Kir6.2/SUR-2A and Kir6.2/SUR-2B channels in inside-out membrane patches, they found no significant effect of NN414 when the channels were preblocked with 100 µmol/l MgATP or preactivated with 100 µmol/l MgADP, but, in the absence of nucleotide, NN414 actually had an inhibitory effect on these channels with an IC50 for SUR-2A and SUR-2B of 10 ± 2 and 7.1 ± 0.8 µmol/l, respectively. We found that microinjection of NN414 bilaterally to the VMH also amplified counterregulatory responses to acute hypoglycemia, an effect that was greater in magnitude to that seen following diazoxide microinjection. Taken together, these studies provide compelling evidence that the Kir6.2/SUR-1 form of K<sub>ATP</sub> channel is involved in the glucosensing mechanism used by neurons in the VMH.

While in vivo microinjection certainly provides a greater specificity by targeting specific brain regions, it is not possible to completely exclude effects outside a region of interest. The small volume of injection (0.2 µl) and rapid fall in drug concentration from the injection site suggest that a primary action in other central glucosensing regions (e.g., hindbrain) is unlikely. We also considered the possibility of nonspecific effects resulting from microinjection of diazoxide. We think this is unlikely because we were able to replicate the diazoxide study with an alternate KCO (NN414) and because our previous study showed that microinjection of a KCC had the opposite effect on hormonal counterregulation. DMSO, used as a vehicle to dissolve diazoxide, could potentially have independent effects on neuronal activity. However, no significant differences were apparent when we compared counterregulatory responses between the controls in the acute diazoxide study (CON-1) with those of the controls in the acute NN414 study (CON-2), where DMSO was not present in the solution. This suggests that any potential independent effect of the DMSO is unlikely to have had a significant impact on our findings.

Taken together, the acute studies support the view that modulation of the K<sub>ATP</sub> channels in the VMH has a direct effect on neuronal responses to changing extracellular glucose. Recent studies (35,45,46) implicating glucokinase in hypoglycemia sensing provide support for the hypothesis that the mechanism by which specialized glucosensing neurons within the VMH detect a change in extracellular glucose is similar to that used by the pancreatic β-cell. It is unlikely, however, that this is the sole mechanism used, given that not all glucosensing neurons express glucokinase or Kir6.2 (23), and there may be other potential sensing mechanisms, e.g., AMP-activated protein kinase (47). However, overall the data to date indicate the presence of at least one signaling system in the VMH for detecting a falling glucose that uses glucokinase and the K<sub>ATP</sub> channel as key regulatory steps.

Recent severe hypoglycemia is a risk associated with, and a primary limitation to, intensive insulin therapy (48). Single (49) or multiple (50) episodes of acute hypoglycemia induce defective counterregulation in individuals with (51) and without (50) type 1 diabetes. The mechanism(s) by which this defect is induced is not yet known, although current data suggest that the defect may, directly or indirectly, arise as a consequence of hypothalamic-pituitary-adrenal axis activation during acute hypoglycemia (41,52). Given that we had demonstrated an acute effect of KCO to amplify counterregulatory responses, we...
sought to determine whether we could also restore counterregulatory responses in an animal model of defective hormonal counterregulation through the direct application of a KCO to the VMH. Normal male Sprague-Dawley rats were subjected to three consecutive daily episodes of acute hypoglycemia before undergoing a hyperinsulinemic-hypoglycemic clamp study. Consistent with a previous report (41), this model induced a defective epinephrine counterregulatory response as assessed by the hyperinsulinemic-hypoglycemic clamp (Table 1). Ventromedial hypothalamic microinjection of diazoxide produced an amplification of hormonal counterregulatory responses, and a reduction in the amount of exogenous glucose required to maintain the hyperinsulinemic-hypoglycemic clamp, in rats with defective counterregulation secondary to recurrent hypoglycemia. The responses generated were in fact greater than those seen in the control rats that had not undergone the recurrent hypoglycemia protocol. It is of note that recurrent hypoglycemia had only a small effect on glucagon secretion in the control rats (comparison of the two control groups). This may be a reflection of the model we chose; it is likely that factors such as depth of hypoglycemia, its duration, and the frequency of induced episodes all have an impact on hormone counterregulatory responses. In our experience, it takes a more chronic exposure to recurrent once-daily hypoglycemia to induce a glucagon secretory defect in normal rats (41,42). This may reflect the evidence now accruing that abnormalities in glucagon secretion during hypoglycemia primarily result from the failure of intraislet insulin levels to fall in type 1 diabetes (53,54). Despite this, the fact that we saw an amplification of the glucagon secretory response to hypoglycemia in both normal and recurrently hypoglycemic rats underscores the importance of the autonomic nervous system in determining the magnitude of the glucagon secretory response to acute hypoglycemia. Our data indicate that providing an additional pharmacological stimulus to open KATP channels in the VMH of rats who have experienced recurrent hypoglycemia markedly enhances both epinephrine and glucagon responses to a subsequent episode of hypoglycemia and that the defect induced by recurrent episodes of hypoglycemia may operate in a different way on those circuits effecting epinephrine and glucagon secretion.

The clinical applications of diazoxide, the only commercially available KCO in clinical use, are limited because it lacks sufficient specificity, strongly activating β-cell and smooth muscle KATP channels but additionally having a weak stimulatory effect on cardiac and vascular KATP channels (40). Because of this, diazoxide has many undesired side effects (e.g., vasodilation and hirsutism). Moreover, although research in this area is scarce, very little diazoxide is thought to cross the blood-brain barrier (55), and hence effects on central glucosensing systems may be limited. However, the different composition, tissue expression patterns, and functional roles of the KATP channel subtypes offer a potential means of developing novel therapies for specific conditions. Our data would suggest that a SUR-1–specific KCO that is able to cross the blood-brain barrier would amplify counterregulatory responses to insulin-induced hypoglycemia. As such, as proof of concept, our study offers the first in vivo demonstration of the potential use of KCOS in the treatment of individuals with type 1 diabetes who develop the complication of defective hypoglycemia counterregulation.

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