Inactivation of the DMH selectively inhibits the ACTH and corticosterone responses to hypoglycemia

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Evans, Scott B., Charles W. Wilkinson, Pam Gronbeck, Jennifer L. Bennett, Aryana Zavosh, Gerald J. Taborsky, Jr, and Dianne P. Figlewicz. Inactivation of the DMH selectively inhibits the ACTH and corticosterone responses to hypoglycemia. Am J Physiol Regul Integr Comp Physiol 286: R123–R128, 2004; 10.1152/ajpregu.00328.2003.—We have previously reported that repeated bouts of insulin-induced hypoglycemia (IIH) in the rat result in blunted activation of the paraventricular, arcuate, and dorsomedial hypothalamic (DMH) nuclei. Because DMH activation has been implicated in the sympathoadrenal and hypothalamic-pituitary-adrenal (HPA) responses to stressors, we hypothesized that its blunted activation may play a role in the impaired counterregulatory response that is also observed with repeated bouts of IIH. In the present study, we evaluated the role of normal DMH activation in the counterregulatory response to a single bout of IIH. Local infusion of lidocaine (n = 8) to inactivate the DMH during a 2-h bout of IIH resulted in a significant overall decrease of the ACTH response and a delay of onset of the corticosterone response compared with vehicle-infused controls (n = 9). We observed suppression of the ACTH response at time (t) = 90 and 120 min (50 ± 12 and 63 ± 6%, respectively, of control levels) and early suppression of the corticosterone response at t = 30 min (59 ± 13% of the control level). The epinephrine, norepinephrine, and glucagon responses were not altered by DMH inactivation. Our finding suggests that DMH inactivation may play a specific role in decreasing the HPA axis response after repeated bouts of IIH.

METHODS

Subjects. Male Wistar rats (350–400 g; Animal Technologies Limited, Kent, WA) were maintained on a 12:12-h light-dark schedule (lights on at 6:00 A.M., off at 6:00 P.M.), with ad libitum access to food and water and were studied during the lights-on portion of the light cycle. All procedures were approved by the Animal Studies Subcommittee of the Veterans Affairs Puget Sound Health Care System Research and Development Committee and adhere to the guiding principles for research set forth by the American Physiological Society (3).

Surgery. All animals underwent bilateral implantation of intraventricular Silastic catheters under ketamine-xylazine anesthesia [60 mg/kg ketamine (Ketalar, Abbott Laboratories, Chicago, IL), 7.8 mg/kg xylazine (Xyla-Ject; Phoenix Pharmaceutical, St. Joseph, MO)] as described by Evans et al. (16). Catheters were tunneled subcutaneously and exteriorized through a midline incision in the scalp. Each rat also received bilateral 26-gauge stainless steel guide cannulas (Plastics One, Roanoke, VA) aimed at the DMH using the stereotaxic coordinates [−2.2 anterior-posterior, ± 0.5 medial-lateral, −7.0 dorsal-ventral from bregma according to the atlas of Paxinos and Watson (28)]. The intracranial cannula and intravenous catheters were held in place with acrylic cement (Lang Dental, Wheeling, IL) and four skull screws (Small Parts, Miami Lakes, FL). Animals received subcutaneous 3 ml lactated Ringer solution (Baxter Pharmaceutical Products, New Providence, NJ) and intramuscular 0.2 ml Gentamicin antibiotic (Bayer, Leverkusen, Germany) and were maintained on a circulating water heating pad until recovery from anesthesia. Catheter lines were filled with 25–60% polyvinylpyrrolidone (PVP10; Sigma, St. Louis, MO) heparin (1,000 U/ml; Elkins-Sinn, Cherry Hill, NJ) and kept patent by a heparin (100 U/ml) flush every 3 days. All animals regained weight to at least the presurgical level and were on a positive weight gain trajectory before study. Figure 1A shows examples of DMH placements to illustrate the range of typical DMH cannula placements and a plotting of the actual placements. Animals that were brain regions over the control of components of the counterregulatory response. In this study, to further probe central nervous system (CNS) mechanisms of the counterregulatory response and HAAF, IIH was induced in animals using the same parameters as in our HAAF model, whereas the DMH was inactivated with lidocaine. The DMH has been demonstrated to be involved in the autonomic and HPA response to stressors such as air stress but not in others such as hemorrhagic stress (13, 21, 30, 42, 43). This rather specific role of the DMH, along with the fact that the DMH is activated by IIH and its activation is blunted in our HAAF model (15), led us to ask the question of how DMH inhibition might contribute to a blunted counterregulatory response to IIH.
found to have placements outside of the DMH were included in an anatomical control group. Additionally, this anatomical control group consisted of animals receiving cannulas aimed 1 mm dorsal to the DMH coordinates listed above (final n = 10; 5 vehicle infused, 5 lidocaine infused). Figure 1B shows the placements for the anatomical control group.

**Experimental procedures.** Before performing experiments, animals were familiarized with square acrylic test chambers (~30 cm × 30 cm × 30 cm) and the microinjection procedure, as described by Evans et al. (16). Subsequent to this, the animals were placed in the test chambers with ad libitum access to food and water for at least 2 h before experimental procedures began. Food was removed 1 h before the experiment, injection and withdrawal cannulas were connected, and the experiment began once the animals were observed to be calm (~10–15 min after connecting cannulas; 10:00 A.M.). Animals received insulin (Novolin R, regular human insulin, recombinant DNA origin; Novo Nordisk, Princeton, NJ; 0.5 U·100 g body wt⁻¹·h⁻¹) intravenously over 120 min along with DMH infusions of lidocaine (2%; n = 8; Sigma) or vehicle (0.1 M PBS, pH = 7.4; n = 9; Oxoid, Basingstoke, UK). Lidocaine is a local anesthetic that blocks sodium channels and some calcium channels in neurons, thus blocking conduction (7, 29, 41). It has been widely used in a variety of studies to inactivate specific CNS targets (for example, Refs. 2, 17, 32, 35). Microinjections and intravenous infusions were carried out by programmable syringe pumps (SP101i; World Precision Instruments, Sarasota, FL), as described in Evans et al. (16). A 10-min microinjection was made every 25 min. Lidocaine or vehicle was infused at a rate of 0.1 μl/min, a rate that causes no discernible tissue damage upon histological evaluation (unpublished observations). The dose, infusion rate, and timing were chosen based on studies showing inactivation for ~15–20 min and diffusion of ≤1.0 mm² for similar rates and volumes (1, 2, 18, 26, 35). Blood samples (1.5 ml) were drawn every 30 min and immediately replaced with donor blood drawn from unstressed rats immediately before the procedure.

**Histology.** After the termination of infusions, each animal was overdosed with pentobarbital sodium (Nembutal; Abbott Laboratories). Fast green dye (0.3 μl; VWR, West Chester, PA) was infused in the DMH to mark the site of injection, and then animals were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde at 4°C for 3 days. Brains were submersed in 30% sucrose followed by freezing at ~−80°C in embedding media (Fisher, Pittsburgh, PA), until sectioning at 40 μm. Tissue sections were mounted on slides to verify cannula placement. Before performing experiments, animals were familiarized with square acrylic test chambers (~30 cm × 30 cm × 30 cm) and the microinjection procedure, as described by Evans et al. (16). Subsequent to this, the animals were placed in the test chambers with ad libitum access to food and water for at least 2 h before experimental procedures began. Food was removed 1 h before the experiment, injection and withdrawal cannulas were connected, and the experiment began once the animals were observed to be calm (~10–15 min after connecting cannulas; 10:00 A.M.). Animals received insulin (Novolin R, regular human insulin, recombinant DNA origin; Novo Nordisk, Princeton, NJ; 0.5 U·100 g body wt⁻¹·h⁻¹) intravenously over 120 min along with DMH infusions of lidocaine (2%; n = 8; Sigma) or vehicle (0.1 M PBS, pH = 7.4; n = 9; Oxoid, Basingstoke, UK). Lidocaine is a local anesthetic that blocks sodium channels and some calcium channels in neurons, thus blocking conduction (7, 29, 41). It has been widely used in a variety of studies to inactivate specific CNS targets (for example, Refs. 2, 17, 32, 35). Microinjections and intravenous infusions were carried out by programmable syringe pumps (SP101i; World Precision Instruments, Sarasota, FL), as described in Evans et al. (16). A 10-min microinjection was made every 25 min. Lidocaine or vehicle was infused at a rate of 0.1 μl/min, a rate that causes no discernible tissue damage upon histological evaluation (unpublished observations). The dose, infusion rate, and timing were chosen based on studies showing inactivation for ~15–20 min and diffusion of ≤1.0 mm² for similar rates and volumes (1, 2, 18, 26, 35). Blood samples (1.5 ml) were drawn every 30 min and immediately replaced with donor blood drawn from unstressed rats immediately before the procedure.

**Statistical analysis.** Data from the plasma assays were analyzed using repeated-measures ANOVA with time as the repeated measure and treatment (lidocaine or vehicle) as the between-groups factor. In the event of significant main effects or interactions, Fisher’s protected least-significant difference post hoc tests were done to determine significant differences, and t-tests were done where appropriate. Significance for all tests was taken as P ≤ 0.05.

**RESULTS**

**DMH lidocaine blunts the HPA response.** Table 1 presents the neuroendocrine data for the experimental DMH groups.

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**Fig. 1.** A: left, 3 representative photomicrographs showing the extent of typical cannula placements in the dorosomedial hypothalamus (DMH). *Sites of injection. The dark color is a dye deposited at the time of perfusion. Right, plot of all the placements. B: plot of cannula placements for experimental anatomic controls.
DMH lidocaine did not affect baseline (t₀) values of any of the neuroendocrine parameters (Table 1). Both DMH vehicle (n = 9) and DMH lidocaine (n = 8) rats demonstrated significant decreases in plasma glucose during intravenous insulin infusion [Table 1; main effect of time: F(4,15) = 406, P < 0.0001]. The declines in plasma glucose levels did not differ between the two groups [no interaction between time and treatment: F(4,60) = 0.88, P = 0.48].

Table 1 and Fig. 2B show that intra-DMH lidocaine infusion blunted the ACTH response to IIH [treatment effect: F(1,15) = 8.1, P = 0.01; time × treatment interaction: F(4,60) = 3.65, P = 0.0099]. Lidocaine rats demonstrated a significantly decreased ACTH response to IIH at both 90 and 120 min into the insulin infusion (Fig. 2B; P = 0.02 and P = 0.007, respectively). The release of corticosterone was also decreased by intra-DMH lidocaine [Table 1 and Fig. 2A; time × treatment interaction: F(4,60) = 2.48, P = 0.05]. Corticosterone remained at baseline at 30 min in the lidocaine group (P = 0.89, t₀ vs. t₃₀) and then proceeded to steadily increase over 120 min (Table 1 and Fig. 2A). In contrast, the corticosterone in the vehicle group had increased already at 30 min (P < 0.0001, t₀ vs. t₃₀) and was significantly higher than the lidocaine group (P = 0.03, vehicle vs. lidocaine at t₃₀).

The plasma catecholamine response to IIH was not suppressed by DMH inactivation. Plasma epinephrine levels increased in both groups across the 120-min session (Table 1), with no effect of intra-DMH lidocaine [no treatment effect: F(1,15) = 0.255, P = 0.62; no time × treatment interaction: F(4,60) = 0.224, P = 0.92]. The increase of plasma norepinephrine was also not affected by DMH lidocaine [Table 1; no treatment effect: F(1,15) = 0.406, P = 0.53; no time × treatment interaction: F(4,60) = 0.228, P = 0.92].

**Table 1. Plasma glucose and hormone levels during hypoglycemia after lidocaine or vehicle infusion in the DMH**

<table>
<thead>
<tr>
<th>Time Point, min</th>
<th>Glucose, mg/dl</th>
<th>ACTH, pg/ml</th>
<th>Corticosterone, µg/dl</th>
<th>Epinephrine, pg/ml</th>
<th>Norepinephrine, pg/ml</th>
<th>Glucagon, pmol/ml</th>
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<tr>
<td></td>
<td>DMH Vehicle (n = 9)</td>
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<td></td>
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<tr>
<td>0</td>
<td>104±2</td>
<td>21±4</td>
<td>7.8±1.5</td>
<td>95±25</td>
<td>371±57</td>
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<td>243±24</td>
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Values are means ± SE; n, no. of rats. DMH, dorsomedial hypothalamus. *P < 0.05 vs. DMH vehicle.
lidocaine had no effect on pancreatic glucagon release in response to IIH [Table 1; no treatment effect: $F(1,15) = 0.218$, $P = 0.65$; no interaction between time and treatment: $F(4,60) = 0.068$, $P = 0.99$].

Control injections do not blunt the response. Data for the anatomic control group are presented in Table 2. Figure 2, C and D, shows the corticosterone and ACTH data graphically for comparison with the DMH group data in Fig. 2, A and B. As is evident, lidocaine injected 1 mm dorsal to the DMH did not blunt the indexes of the counterregulatory response measured in the current study [time × dose: glucagon, $F(4,32) = 0.27$, $P = 0.89$; epinephrine, $F(4,32) = 0.46$, $P = 0.77$; norepinephrine, $F(4,32) = 0.45$, $P = 0.77$; ACTH, $F(4,32) = 0.53$, $P = 0.72$; corticosterone, $F(4,32) = 0.49$, $P = 0.74$].

DISCUSSION

Inactivation of the DMH (and not regions dorsal to it; see Fig. 1B and Table 2) delayed and reduced the increase of ACTH resulting from IIH (Fig. 2B). Despite having a drop in blood glucose that was closely matched to the vehicle group (Table 1), the lidocaine group did not experience a rise in ACTH until 90 min into the IIH ($t_0$ vs. $t_{60}$: $P = 0.34$; $t_0$ vs. $t_{90}$: $P = 0.0008$). This is delayed by 30 min compared with the vehicle group, which demonstrated an earlier rise of ACTH ($t_0$ vs. $t_{30}$: $P = 0.07$; $t_0$ vs. $t_{60}$: $P = 0.005$). Both groups reached a plateau of ACTH levels at 90 min into the IIH (for vehicle $t_0$ vs. $t_{120}$: $P = 0.69$; for lidocaine $t_0$ vs. $t_{120}$: $P = 0.34$), but the peak ACTH levels were lower in the lidocaine group (Fig. 1A; $t_{120}$ vehicle vs. $t_{120}$ lidocaine: $P = 0.007$). This result is consistent with studies in which microinjection of the GABA agonist muscimol into the DMH decreased the ACTH response to air stress (33). Conversely, stimulation of the DMH with the glutamatergic agonist kainate or the GABA antagonist bicuculline methiodide increased plasma ACTH (4, 22). Thus DMH inhibition, whether by an inhibitory neurotransmitter analog or by inactivation, blunts the activation of the HPA axis. The influence of DMH activity on the HPA response to a stressor may be mediated by direct projections from the DMH to the parvocellular PVN (6, 9, 36–38). In fact, Ter Horst and Luiten (37) indicate that the PVN is the primary target for DMH projections. Much evidence indicates that the corticotropin-releasing hormone (CRH) neurons in the parvocellular PVN are principally responsible for ACTH release by the anterior pituitary (see Ref. 4 for a summary). Evidence also indicates that the projection from the DMH to the parvocellular PVN is excitatory (5, 20). Infusion of muscimol, a GABA$_A$ receptor agonist, in the DMH blunts the ACTH response to air stress and the associated rise in the number of activated neurons in the PVN, as assessed by c-Fos expression in the PVN (13). The parsimonious explanation for this would be that the DMH projection neurons to the PVN were inhibited by muscimol, removing an excitatory input to CRH-producing neurons in the PVN.

Because ACTH stimulates corticosterone release from the adrenal cortex, one would predict a decrease in the corticosterone response to IIH with the blunted ACTH response. Although the corticosterone response was initially delayed, it was progressively less reduced thereafter. The terminal $t_{120}$ corticosterone levels were identical for both groups (vehicle vs. lidocaine for $t_{120}$: $P = 0.40$). We have observed in past studies that ACTH must be blunted substantially before it is associated with a decreased corticosterone response at this level of IIH (Refs. 15 and 16 and unpublished observations). Additionally, a blunted ACTH response in the lidocaine group might primarily decrease the duration of the corticosterone response (23, 25, 40). It would only be possible to confirm this if we extended our observations beyond the plateau, i.e., beyond 120 min. A blunted corticosterone response after DMH inhibition is consistent with the findings of Keim and Shekh (22), in which chemical stimulation of the DMH increased corticosterone levels.

The effect of DMH inactivation was very specific to the HPA response to IIH. The plasma catecholamine profile, reflecting the sympathoadrenal component of the counterregulatory response, was not altered by DMH lidocaine. The present finding coupled with our previous observations (15, 16) suggests a fundamental difference in the roles of different regions of the hypothalamus in the counterregulatory response to IIH. Our previous study, in which we inactivated the PVN during IIH, demonstrated that the PVN, unlike the DMH, is important for the sympathoadrenal response to IIH (16), particularly the last 30 to 60 min of the response. Inactivation of the PVN did not delay the ACTH response (or for that matter the corticosterone or catecholamine responses); it decreased the magni-

Table 2. Plasma glucose and hormone levels during hypoglycemia after lidocaine or vehicle infusion

<table>
<thead>
<tr>
<th>Time Point, min</th>
<th>Glucose, mg/dl</th>
<th>ACTH, pg/ml</th>
<th>Corticosterone, μg/dl</th>
<th>Epinephrine, pg/ml</th>
<th>Norepinephrine, pg/ml</th>
<th>Glucagon, pmol/ml</th>
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<td>464±151</td>
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Values are means ± SE; $n$ = 5 rats in each group.
ROLE OF THE DMH IN THE COUNTERREGULATORY RESPONSE TO HYPOGLYCEMIA

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tude of the peak response at 90 and 120 min (16). This profile was similar to the blunting of the epinephrine and corticosterone responses in our model of HAAF (15) and suggests that PVN inhibition may serve a more prominent role in HAAF because inactivating the PVN alone produces more of a HAAF-like neuroendocrine profile. We suggest that the degree of DMH inhibition is important in shaping the altered counterregulatory neuroendocrine profile of HAAF. Thus neither inhibition of the PVN alone, nor inhibition of the DMH alone, during IIH fully replicates the neuroendocrine profile of HAAF. Therefore, future studies must examine the role of other structures that we have observed to have altered activation with repeated IIH, i.e., the hypothalamic Arc and the PVN of the thalamus (15). These studies may bring us closer to defining HAAF in terms of altered activity in multiple brain regions.

A caveat for the above interpretation that DMH inactivation blunts the HPA response to IIH is that we did not measure plasma glucose at a higher temporal resolution, e.g., every 10 min. It is possible, for example, that glucose levels may have decreased at a slower rate in the lidocaine-infused rats over the first 30 min of insulin infusion, thus providing a lesser stimulus to the HPA axis. However, given that the results are consistent with the literature demonstrating a role for the DMH in the HPA response to other stressors (see above), and the fact that the ACTH response is inhibited even after 2 h with similar plasma glucose values, it seems unlikely that this would be a satisfactory explanation for the results.

Although we found no change in the catecholamine response to IIH with DMH inactivation, other studies have shown that stimulation and inactivation of the DMH alter indexes of autonomic activity at baseline and in response to a particular stressor. For example, stimulation or disinhibition of the DMH has been shown to increase heart rate, blood pressure, and renal sympathetic nerve activity (4, 19, 30). However, there are conflicting reports. For instance, one study has reported that injection of bicuculline methiodide, a GABA\(\_\alpha\) receptor antagonist, in the DMH increased the temperature of intercapsular brown adipose tissue (iBAT; see Ref. 44), whereas another study showed that injection of \(\gamma\)-glutamate in the DMH decreased the firing rate of sympathetic nerves innervating iBAT (42). Although there are some discrepancies, taken together, this literature nevertheless argues for the potential stimulatory effects of the DMH on the sympathetic nervous system. Importantly, as DiMicco et al. (13) point out in a recent review, the role of the DMH is stressor specific; i.e., although muscimol injection in the DMH inhibits the increases of blood pressure, heart rate, and Fos-positive neurons in the PVN accompanying air stress (27, 33, 34), the same treatment does not do this in the context of hemorrhagic stress. It appears that, in the context of IIH, the DMH may be more important for the HPA components of the response rather than the sympathoadrenal components.

Perspectives

Our previous studies have shown that PVN activation plays a critical role in the sympathoadrenal response to IIH but that it is not responsible for the complete counterregulatory response to a single bout of IIH, and its inactivation cannot be solely responsible for the impaired counterregulatory response, which occurs with multiple bouts of IIH. Another hypothalamic region that is both activated by IIH and inhibited by repeated IIH in our HAAF model is the DMH. In the current study, we found the DMH to be important for the HPA component of the counterregulatory response to IIH, particularly during the initial phase of this response. The DMH was not important for the sympathoadrenal component of the response, or for the glucagon component. Thus DMH inhibition in the context of HAAF would likely contribute to the inhibition of the HPA response. The complete blunted neuroendocrine profile of HAAF is the result of varying levels of PVN, DMH, and Arc inhibition (with the lack of inhibition of other structures, such as the thalamic PVN), and perhaps inhibition of other CNS sites in the brain stem (31) and peripheral end organs, which we have not yet investigated.

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