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Brain insulin infusion does not augment the counterregulatory response to hypoglycemia or glucoprivation

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Abstract

Although high dosages of insulin can cause hypoglycemia, several studies suggest that increased insulin action in the head may paradoxically protect against severe hypoglycemia by augmenting the sympathoadrenal response to hypoglycemia. We hypothesized that a direct infusion of insulin into the third ventricle and/or the mediobasal hypothalamus (MBH) would amplify the sympathoadrenal response to hypoglycemia. Nine-week–old male rats had insulin (15 mU) or artificial cerebrospinal fluid (aCSF, control) infused bilaterally into the MBH or directly into the third ventricle. During the final 2 hours of the brain insulin or aCSF infusions, the counterregulatory response to either a hyperinsulinemic hypoglycemic (~50 mg/dL) clamp or a 600-mg/kg intravenous bolus of 2-deoxyglucose (2DG) was measured. 2-Deoxyglucose was used to induce a glucoprivic response without peripheral insulin infusion. In response to insulin-induced hypoglycemia, epinephrine rose more than 60-fold, norepinephrine rose more than 4-fold, glucagon rose 8-fold, and corticosterone rose almost 2-fold; but these increments were not different in aCSF vs insulin treatment groups with either intracerebroventricular or bilateral MBH insulin protocols. Intracerebroventricular insulin infusion stimulated insulin signaling as noted by a 5-fold increase in AKT phosphorylation. In the absence of systemic insulin infusion, 2DG-induced glucopenia resulted in an equal counterregulatory response with brain aCSF and insulin infusions. Under the conditions studied, although insulin infusion acted to stimulate hypothalamic insulin signaling, neither intrahypothalamic nor intracerebroventricular insulin infusion augmented the counterregulatory response to hypoglycemia or to 2DG-induced glucoprivation. Therefore, it is proposed that the previously noted acute actions of insulin to augment the sympathoadrenal response to hypoglycemia are likely mediated via mechanisms exterior to the central nervous system.

1. Introduction

The greatest clinical challenge associated with the use of insulin in the management of diabetes is to fully correct hyperglycemia without causing hypoglycemia. Counterregulation in response to hypoglycemia is impaired in insulin-treated diabetic patients who lack a glucagon response [1]. Diabetic patients are usually solely dependent on their sympathoadrenal response to prevent severe hypoglycemia. Unfortunately, even a single episode of hypoglycemia has been shown to reduce the sympathoadrenal response to future episodes of hypoglycemia [2,3]. Diabetic patients who develop this impaired sympathoadrenal response are at markedly increased risk for severe, life-threatening hypoglycemia [1,4]. Thus, extensive research efforts have focused on elucidating the mechanisms that underlie this impaired sympathoadrenal response with the hope of finding novel approaches to preventing and/or restoring the impaired counterregulatory response to hypoglycemia.

There has been much debate about whether insulin per se may increase the sympathoadrenal response to hypoglycemia [5]. At equivalent levels of hypoglycemia, some studies have demonstrated that increased insulin levels augment the sympathoadrenal response in nondiabetic [6,7] and diabetic humans [8,9], whereas other studies failed to demonstrate an effect of increased insulin levels to alter the sympathoadrenal response to hypoglycemia in nondiabetic [10,11] and diabetic humans [10,12,13]. Paradoxically, one study has

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demonstrated that the increased insulin levels significantly diminished the sympathoadrenal response [14]. The site of insulin action could not be determined from the above reports. Hypoglycemia experiments in dogs demonstrated that a selective increase in carotid and vertebral artery insulin levels profoundly augmented the epinephrine response, suggesting that insulin acts in the head to augment the sympathoadrenal response to hypoglycemia [15,16]. Supporting the notion that insulin may be acting in the central nervous system (CNS) to augment the sympathoadrenal response to hypoglycemia, brain/neuron-specific insulin receptor knockout (NIRKO) mice have a blunted sympathoadrenal response to insulin-induced hypoglycemia [17]. Consistent with reports that the mediobasal hypothalamus (MBH) (composed of the ventromedial hypothalamus and the arcuate nucleus) is a primary site of glucose sensing [18-20] and insulin action [21,22], NIRKO mice have been shown to have changes in glucose transporter expression in the MBH [23]. Taken together, these studies suggest that the cerebral circulation or the CNS, and perhaps the MBH, may be the sites where increased insulin levels act to augment counterregulation. Therefore, it was hypothesized that a direct infusion of insulin into brain (ie, into the cerebrospinal fluid [CSF] within the third ventricle or directly into the parenchyma of the MBH) would amplify the sympathoadrenal response to hypoglycemia.

2. Research design and methods

2.1. Animals and procedures

Nine-week–old male Sprague-Dawley rats were fed a standard rat chow and housed on a 12-hour/12-hour day/night cycle. Two weeks before each study, the animals were anesthetized with isoflurane; and microinjection cannulae (Plastics One, Roanoke, VA) were inserted into the MBH or the third ventricle. At the border of the ventromedial hypothalamus and the arcuate nucleus, the MBH was targeted by bilateral cannulae (coordinates from bregma: posterior, 2.8 mm; targeted depth, 10.1 mm; lateral, ±0.6 mm). For intracerebroventricular (ICV) injections, a solitary ICV cannula was inserted 2.8 mm posterior to bregma, on the suture line, to a targeted depth of 10.1 mm. After a 1-week recovery period, the animals were anesthetized with ketamine/xylazine (87/13.4 mg/kg, intraperitoneal); and MicroRenathane catheters (Braintree Scientific, Braintree, MA) were implanted into the left carotid artery and right jugular vein. The animals were then allowed approximately 1 week to recover before the experiment, and a recovery of presurgery body weight was used as an index of the animals' health. Animals were excluded from analysis if the intrahypothalamic (IH) or ICV cannulae were not positioned properly as determined on posthumous examination with Evans blue dye (0.0075%). All animal procedures were approved by the Animal Studies Committee of Washington University.

2.2. Hyperinsulinemic hypoglycemic clamp protocol

After a 15-hour fast, each animal was briefly anesthetized with isoflurane (<5 minutes) to insert and secure the internal brain cannulae to the designated depth as well as to attach the vascular catheters. Insulin (total dose of 15 mU in artificial CSF [aCSF]) or vehicle (aCSF) was infused into the brain for 5 hours via the bilaterally implanted IH cannulae at 0.025 mU/min (0.05 μL/min bilaterally), whereas insulin was infused into the ICV cannula at 0.05 mU/min (0.05 μL/min). Data from the literature indicated that such a pharmacologic dose of insulin would be optimal to observe an augmentation of the counterregulatory response to hypoglycemia because pharmacologic insulin doses are necessary to elicit insulin-signaling events in the hypothalamus [24] and an insulin-induced augmentation of the counterregulatory response is best observed when insulin levels are raised 100- to 600-fold [6,25-27]. After a 3-hour basal rest period in which the animals were not handled, baseline arterial blood samples were obtained in awake, unrestrained rats. During the final 2 hours of the brain insulin or aCSF infusions, hypoglycemia was induced with an intravenous infusion of regular human insulin (Lilly, Indianapolis, IN) diluted in phosphate-buffered saline with 1% bovine serum albumin. Systemic insulin was administered at a low dose in the IH experiments (50-mU/kg bolus and 5 mU/[kg min]) and at a high dose in the ICV experiments (150-mU/kg bolus and 15 mU/[kg min]). Arterial blood samples (0.3 μL) were drawn at 10-minute intervals to measure blood glucose. A 50% dextrose infusion was adjusted to achieve blood glucose levels of approximately 50 mg/dL within 30 minutes. When the blood glucose reached approximately 50 mg/dL, the clock was reset to 0; and blood samples were collected at 0, 30, 60, 90, and 120 minutes. Red blood cells were resuspended in heparinized (20 U/mL) saline and reinfused after each blood sample to avoid anemia and volume depletion. The dextrose infusion was titrated to maintain blood glucose at 40 to 50 mg/dL throughout the clamp. At the end of the experiment, some rats were briefly anesthetized with isoflurane to obtain samples of CSF by cervical spine needle insertion.

2.3. 2-Deoxyglucose–induced glucoprivation protocol

A similar protocol as described above for the hyperinsulinemic hypoglycemic clamp protocol was used except that, in lieu of systemic insulin and glucose administration, a bolus of 2-deoxyglucose (2DG) (600 mg/kg, intravenous) was given 3 hours after insulin or aCSF was infused into the 2 IH cannulae. A basal blood sample was taken immediately before the 2DG bolus and then at 30, 60, 90, and 120 minutes after the 2DG bolus.

2.4. Insulin signaling

To determine whether the dose of insulin was effective in eliciting insulin-signaling events in the hypothalamus,

Western blot analysis was performed to quantify AKT phosphorylation given its relatively robust response to insulin when using low specific protein content harvested from the hypothalamus [24]. Previously ICV-cannulated, fasted rats were administered either insulin (10 mU in 5 μL delivered over 5 minutes, n = 3) or an equal volume of aCSF (n = 3). Thirty minutes later, brains were rapidly frozen in liquid nitrogen (<30 seconds after decapitation of anesthetized rats). The *dissected hypothalamus* was defined anatomically as posterior to the optic chiasm, anterior to the mammillary body, inferior to the thalamus, and ±1 mm lateral to the midline. Hypothalami were homogenized in radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium dodecyl sulfate, 0.1 mmol/L phenylmethylsulfonyl fluoride), complete protease inhibitors cocktail (Roche, Indianapolis, IN), and phosphatase inhibitors: 1 mmol/L active sodium orthovanadate and 1 mmol/L NaF. Protein concentration was measured with the bicinchoninic acid protein quantification kit (Pierce, Rockford, IL). Protein extracts (100 μg) were fractionated by electrophoresis on a 10% Bis-Tris Criterion XT (Biorad, Hercules, CA) gel and transferred to nitrocellulose membranes. Membranes were probed with the antibody against the phospho-AKT and total AKT (Cell Signaling Technology, Boston, MA). Primary antibody binding was detected by enhanced chemiluminescence reagents (Perkin Elmer, Wellesley, MA) on ISO-MAX films and quantified by ImageQuant software analysis (Amersham Pharmacia, Piscataway, NJ).

2.5. Analytical procedures

Plasma levels of glucose were measured by the glucose oxidase method (BD Logic Glucometer, Franklin Lakes, NJ). Epinephrine and norepinephrine analysis was measured with a single isotope derivative (radioenzymatic) method [28]. Plasma corticosterone (MP Biomedicals, Orangeburg, NY) and glucagon (Linco Research, St Charles, MO) were measured by radioimmunoassay. Plasma insulin was measured by ultrasensitive rat insulin enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove, IL). In a few instances, values that were beyond the detectable range of an assay and could not be repeated were recorded, for statistical purposes, as the minimum or maximum detectable limit of that assay.

2.6. Statistical analysis

Results are presented as the mean ± SEM. Statistical analysis was by 2-way analysis of variance using SigmaStat 3.1 (Systat Software, San Jose, CA) or by an unpaired Student *t* test. Unless otherwise stated, statistical significance was set at *P* less than .05.

3. Results

The counterregulatory response to insulin-induced hypoglycemia was measured in rats receiving an IH or ICV infusion of either vehicle aCSF or insulin. In the basal state, weight, glucose, insulin, glucagon, corticosterone, epinephrine, and norepinephrine levels were not significantly different between the groups. In response to the peripheral insulin infusion, hypoglycemia (40-50 mg/dL) was maintained with a coinfusion of glucose, although blood glucose levels trended down to a similar extent with both treatment groups (Figs. 1A, 2A). During the hyperinsulinemic hypoglycemic clamp, insulin levels rose significantly with systemic insulin infusion and were not different between treatment groups (Figs. 1B, 2B). With systemic insulin infusion, insulin levels rose 5-fold with low insulin doses (Fig. 1B) and 50-fold with high insulin doses (Fig. 2B). During the clamp, there were no significant differences in the glucose infusion rates between the aCSF and insulin infusion protocols; but as expected, the glucose infusion rates were significantly lower in response to low insulin doses (5 mU/[kg min]) (Fig. 1C) than with high insulin doses (15 mU/[kg min]) (Fig. 2C). In response to hypoglycemia, the rate of glucose infusion was similarly matched with brain insulin or aCSF treatments. Epinephrine levels rose more than 60-fold (Figs. 1D, 2D) and norepinephrine levels rose more than 4-fold (Figs. 1E, 2E) without any significant differences between the aCSF- and insulin-treated groups. Likewise, glucagon increased more than 8-fold (Figs. 1F, 2F) and corticosterone levels nearly doubled (Figs. 1G, 2G) without any significant differences between the groups.

The counterregulatory response to a 2DG-induced glucoprivic signal was measured in rats receiving an IH infusion of either vehicle (aCSF) or insulin (15 mU over 5 hours). In the basal state, weight, glucose, insulin, glucagon, corticosterone, NE, and epinephrine were not significantly different between the 2 groups (IH aCSF vs IH insulin). In response to 2DG, plasma glucose levels rose dramatically in both groups (Fig. 3A); but there were no significant differences between the 2 groups. In response to 2DG and the subsequent rise in glycemia, insulin levels rose approximately 2-fold (Fig. 3B) without any significant differences between the 2 groups. However, the increase in insulin levels achieved after 2DG was significantly less than the 5-fold increase in insulin levels that was achieved with the 5-mU/(kg min) insulin infusion during the hyperinsulinemic clamp protocol (Fig. 1B). Quantitatively similar counterregulatory responses were noted with 2DG administration as compared with insulin-induced hypoglycemia protocols. Specifically, epinephrine levels rose more than 60-fold (Fig. 3C), norepinephrine rose approximately 5-fold (Fig. 3D), glucagon rose approximately 8-fold (Fig. 3E), and corticosterone levels more than doubled (Fig. 3F) without any significant differences between the IH insulin vs aCSF treatment groups.

In the absence of brain insulin infusion, CSF insulin concentrations were consistently 2.1% ± 0.2% of plasma insulin levels and were strongly correlated with plasma insulin levels (R^2 = 0.82, Fig. 4A). In a separate group of rats, ICV administration of 10 mU of insulin increased AKT phospho-

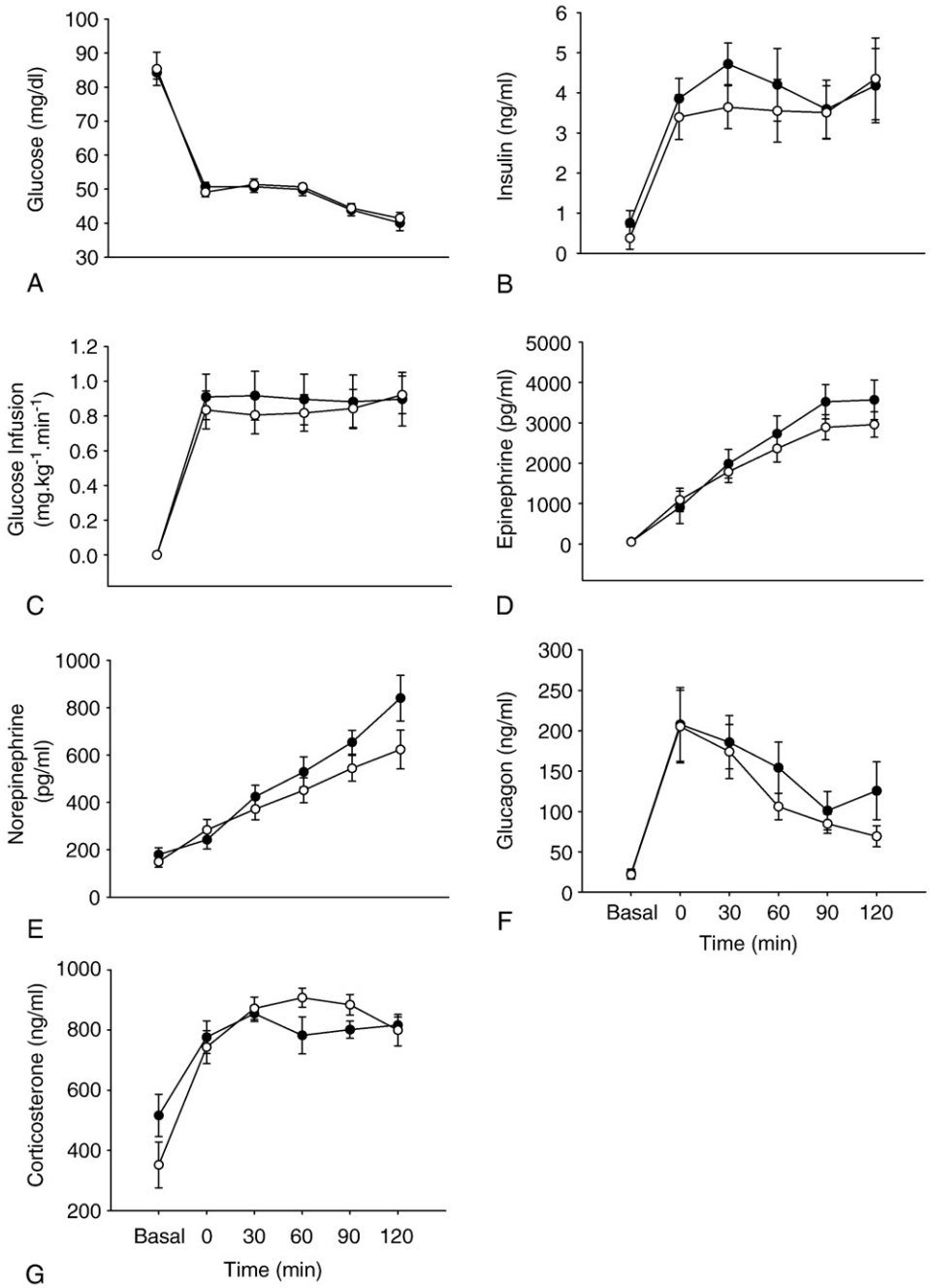

Fig. 1. A-G, The counterregulatory response to a 2-hour, low-dose (5 mU/[kg min]) hyperinsulinemic hypoglycemic clamp (~40-50 mg/dL) was measured in IH vehicle (aCSF)–treated (solid circles) or IH insulin (15 mU)–treated (open circles) rats. Systemic metabolite and hormone measurements are shown at baseline and at 0, 30, 60, 90, and 120 minutes of hypoglycemia for (A) glucose, (B) insulin, (C) glucose infusion rates, (D) epinephrine, (E) norepinephrine, (F) glucagon, and (G) corticosterone.

rylation 5-fold ($P < .05$), but not total AKT (not shown), as compared with aCSF-treated control rats (Fig. 4B).

4. Discussion

Studies have shown that the sympathoadrenal response to hypoglycemia may be enhanced [6-9], not altered [10-13], or inhibited [14] by insulin. Animal studies have suggested that insulin might act in the cerebral circulation or in the brain to augment the sympathoadrenal response to hypoglycemia [15-17]. In this study, the acute infusion of insulin directly into either the third ventricle or the MBH did not augment the sympathoadrenal response to insulin-induced hypoglycemia. These doses of insulin did reach high levels in the CSF, and these doses were effective in eliciting insulin-

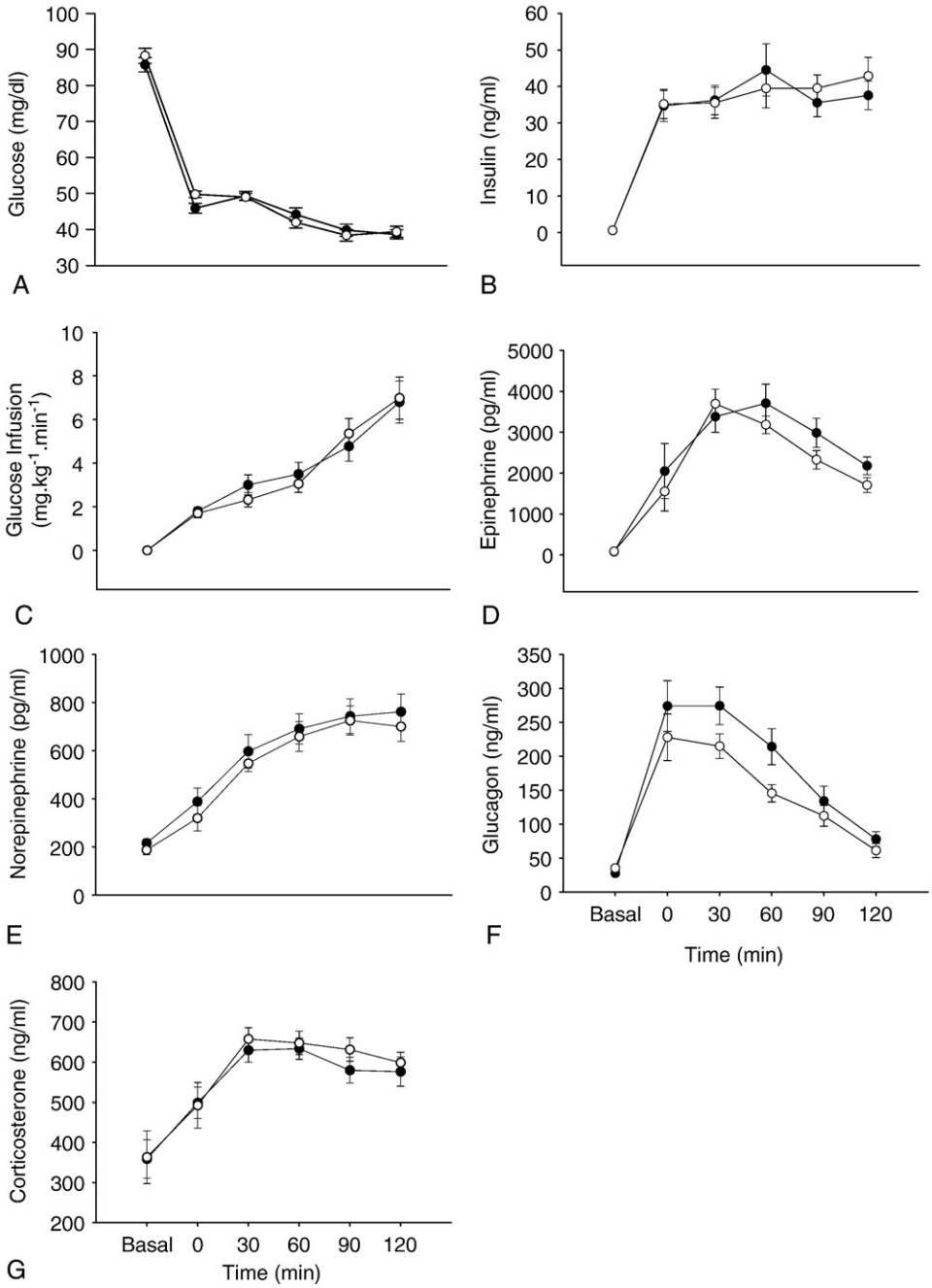

Fig. 2. A-G, The counterregulatory response to a 2-hour, high-dose (15 mU/[kg min]) hyperinsulinemic hypoglycemic clamp (~40-50 mg/dL) was measured in ICV vehicle (aCSF)–treated (solid circles) or ICV insulin (15 mU)–treated (open circles) rats. Systemic metabolite and hormone measurements are shown at baseline and at 0, 30, 60, 90, and 120 minutes of hypoglycemia for (A) glucose, (B) insulin, (C) glucose infusion rates, (D) epinephrine, (E) norepinephrine, (F) glucagon, and (G) corticosterone.

signaling events. Similarly, in the absence of systemic insulin infusion, insulin infusion directly into the MBH does not augment the counterregulatory response to 2DG-induced glucopenia. Thus, under the conditions studied, acute brain insulin infusion did not augment the sympathoadrenal response to hypoglycemia or to glucoprivation.

These studies were designed to optimize our ability to observe an effect of brain insulin infusion. Specifically, (1) the rats were fasted overnight to ensure low basal levels of insulinemia; (2) a relatively low dose of systemic insulin was administered to achieve hypoglycemia during the clamp to maximize our ability to detect a specific effect of IH insulin; (3) unlike milder degrees of hypoglycemia, a moderate depth of hypoglycemia (ie, 40-50 mg/dL) was chosen because this depth of hypoglycemia was shown to more highly correlate epinephrine response to insulin levels [29]; (4) to avoid the

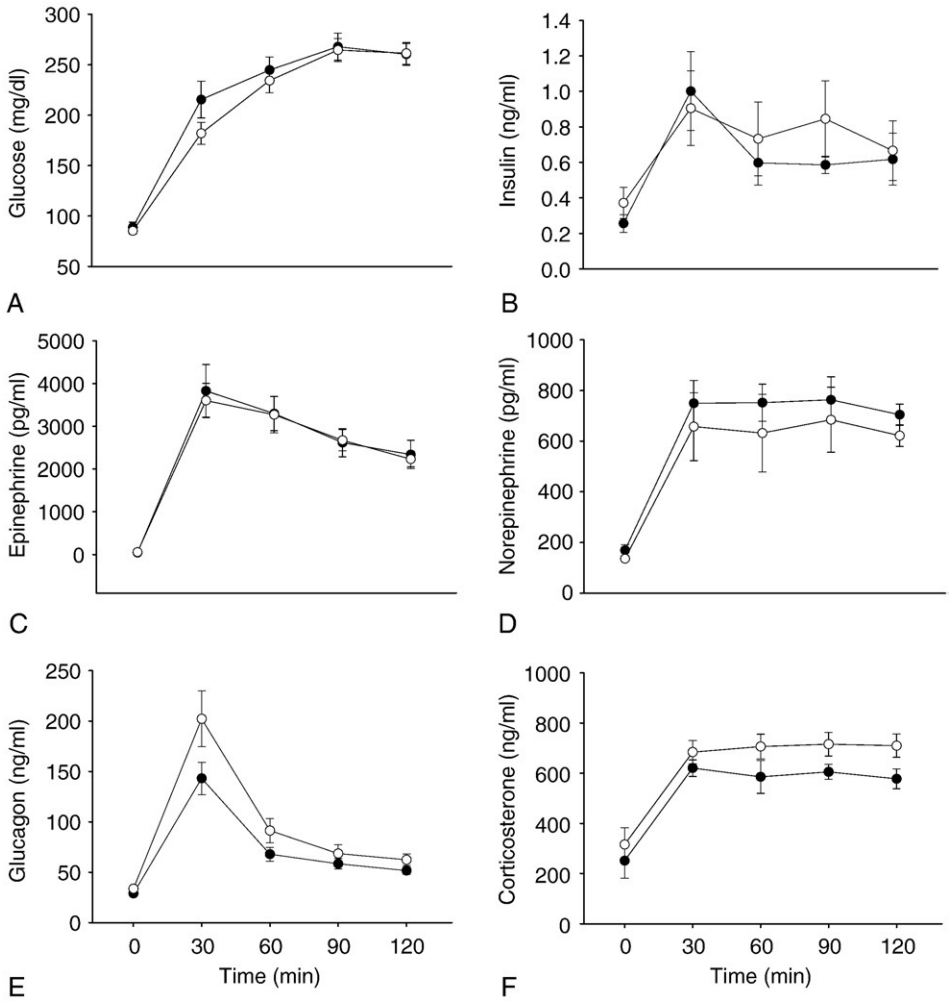

Fig. 3. A-F, The glucoprivic response to 2DG (600-mg/kg intravenous bolus) was measured in IH vehicle (aCSF)–treated (solid circles) or IH insulin (15 mU)–treated (open circles) rats. Systemic metabolite and hormone measurements are shown at baseline (time 0) and at 30, 60, 90, and 120 minutes after administration of 2DG for (A) glucose, (B) insulin, (C) epinephrine, (D) norepinephrine, (E) glucagon, and (F) corticosterone.

confounding effects associated with systemic insulin infusion, 2DG experiments were performed to elicit a glucoprivic counterregulatory response; (5) because the passage of insulin across the blood-brain barrier may be limited by a specific insulin transporter [30,31], the ICV and IH insulin administration experimental approach was designed in order that insulin could bypass the blood-brain barrier; (6) high pharmacologic doses of IH and ICV insulin were chosen to maximally elicit effects in the brain [32,33], as they more closely correlate with augmentation of the sympathoadrenal response to hypoglycemia [5]; and (7) the MBH was specifically targeted for insulin infusion because nuclei within this region have been shown to be involved in both mediating brain insulin action [34,35] and containing glucose-sensing neurons that mediate the sympathoadrenal response to hypoglycemia [18,19,21].

The current set of experiments allowed for a comparison of the sympathoadrenal responses to high- and low-dose systemic insulin infusion clamp protocols by comparing the responses in the aCSF-infused control rats. In the absence of brain insulin infusion and at matched degrees of hypoglycemia, low-dose insulin infusion (5 mU/[kg min]) increased systemic insulin levels 5-fold and resulted in a net epinephrine response (area under the curve) of $3.3 \pm 0.5 \times 10^5$ pg/(min mL). Higher-dose insulin infusion (15 mU/[kg min]) increased systemic and CSF insulin levels 10-fold above low-dose insulin infusion; yet at matched degrees of hypoglycemia, higher insulin doses yielded a net epinephrine response (area under the curve) similar to low-dose insulin infusion ($3.9 \pm 0.4 \times 10^5$ pg/[min mL], P = not significant). These findings indicate that, even in the absence of brain insulin infusion, marked increases in systemic insulin levels fail to increase the sympathoadrenal response to hypoglycemia in rats.

Davis et al [15,16] demonstrated that infusion of insulin into the carotid and vertebral circulation of dogs augmented the sympathoadrenal response to hypoglycemia. There are many experimental variables that could have accounted for

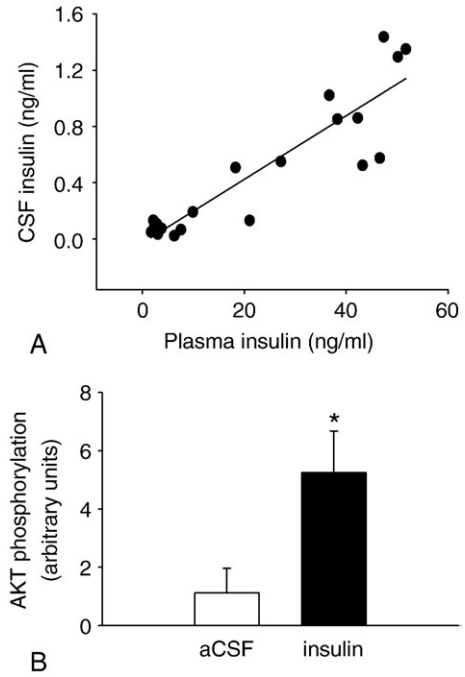

Fig. 4. A, In the absence of CNS insulin infusion, CSF insulin levels were highly correlated (R^2 = 0.82) with plasma insulin levels during the hyperinsulinemic hypoglycemic clamps. B, In a separate group of rats, ICV administration of 10 mU of insulin increased AKT phosphorylation 5-fold (*P < .05) as compared with aCSF-treated control rats.

the notable differences between these previous results and our current study. Firstly, it is possible that a difference in species may account for the apparent discrepancy between the studies, although brain insulin actions seem to be more prominent in murine models than in the dog [36]. Secondly, it is conceivable that insulin-induced changes in cerebral blood flow [37], rather than the direct actions of insulin in brain parenchyma, were responsible for the previously observed enhanced sympathoadrenal response to hypoglycemia with vertebral and carotid artery insulin infusion [15,16]. Thirdly, insulin actions in the brain are not restricted to the hypothalamus. Areas in the hindbrain, particularly the nucleus of the tractus solitarius, are involved in the sensing of hypoglycemia and have been shown to be insulin sensitive [38,39]. Given the free distribution of insulin in the CSF with ICV insulin administration, the absence of an effect on the counterregulatory response to hypoglycemia with ICV insulin experiments argues against any significant effect of insulin action in other brain areas, including the nucleus of the tractus solitarius. However, when considering possible brain regions affected, carotid and vertebral artery infusion would more likely engage a wider neuronal territory than the more focal delivery of insulin in the current studies, possibly accounting for the observed differences between studies. Finally, the effect of systemic hyperinsulinemia to augment the adrenomedullary response may be mediated by direct actions on the adrenal medulla [40]. However, because systemic (and presumably adrenal) insulin levels are matched during hyperinsulinemic hypoglycemic clamp experiments, adrenal insulin actions do not explain why head insulin infusion [15,16] but not brain insulin infusion should alter the adrenomedullary response to hypoglycemia.

Because previous reports have shown that insulin entry into the CNS may be saturable at low levels of insulin [30,31], it could be argued that the peripheral insulin levels achieved during the hyperinsulinemic clamp could have elicited a maximal effect on brain insulin action and theoretically could also have masked any additional effect of increased brain insulin infusion. There are several reasons to suggest that insulin's effect in the brain was not saturated. Firstly, our data did not show evidence for saturation, but rather a linear increase of CSF insulin concentration with increases in systemic insulinemia (Fig. 4A). Secondly, many other studies indicate that the effect of insulin in augmenting the counterregulatory response to hypoglycemia is not saturated at low insulin levels but rather is enhanced with pharmacologic insulin infusions [6,25-27]. Thirdly, a highly significant linear relationship between high plasma insulin levels and augmented epinephrine and norepinephrine responses to hypoglycemia [5] supports the supposition that the effect of insulin in the brain was not already maximal with the doses of insulin infusions used in these experiments. Finally, in the absence of a systemic insulin infusion, experiments performed using 2DG also demonstrated that brain insulin infusion did not augment counterregulation, which supports the contention that insulin effects in the brain were not saturated by systemic insulin infusion during the hyperinsulinemic clamp experiments.

Although increased brain insulin did not increase the sympathoadrenal response, these experiments do not refute the notion that a small amount of insulin action in the brain may be critical for normal brain glucose sensing and the generation of a full sympathoadrenal response to hypoglycemia. Indeed, one of us (SJF) has previously demonstrated that the absence of insulin signaling in NIRKO mice causes a blunted sympathoadrenal response to insulin-induced hypoglycemia [17]. The defective sympathoadrenal response in the NIRKO mice appears to be specific to glucose sensing and may be related to changes in glucose transporter expression in the MBH [23]. Consistent with the notion that a basal amount of insulin is critical for normal brain glucose sensing, lowering basal insulin levels has been shown to decrease brain glucose uptake [41]. Furthermore, consistent with a role for basal hypothalamic insulin in modulating the counterregulatory response to hypoglycemia, acute blockade of insulin signaling in the ventromedial hypothalamus has recently been shown to augment the glucagon response to hypoglycemia [42].

Unlike the putative effect on the sympathoadrenal medullary response, the central effect of insulin in regulating the glucagon secretion is less well established. This study demonstrated that CNS insulin infusion did not affect the glucagon response (Figs. 1F, 2F, 3E), although

other studies report that insulin may act in the brain to increase [16], decrease [42], or not effect [17] the glucagon response to hypoglycemia.

As a marker of the hypothalamic-pituitary-adrenocortical response to hypoglycemia, the increased corticosterone levels were not significantly altered by brain insulin infusion in these experiments (Figs. 1G, 2G, 3F). Insulin's effect on this hypothalamic-pituitary-adrenocortical response to hypoglycemia has also been equivocal, with some studies indicating a significant stimulatory [6,8,25,26] or inhibitory [13] effect, or no significant effect [7,9,11,12].

In summary, under the conditions studied, it was found that neither IH nor ICV insulin augmented the sympathoadrenal response or the response of other counterregulatory hormones to insulin-induced hypoglycemia or to 2DG-induced glucoprivation in male Sprague-Dawley rats. Therefore, it is proposed that the previously noted acute actions of insulin to augment the sympathoadrenal response to hypoglycemia are likely mediated via insulin actions outside the CNS.

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