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RECURRENT INSULIN-INDUCED HYPOGLYCEMIA CAUSES SITE-SPECIFIC PATTERNS OF HABITUATION OR AMPLIFICATION OF CNS NEURONAL GENOMIC ACTIVATION

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Abstract—Antecedent hypoglycemia is a primary factor in hypoglycemia-associated autonomic failure, a pathophysiological condition characterized by impaired glucose counterregulatory function. Conventional therapeutic strategies involving administration of intermediate dosage-release formulations of insulin in the management of insulin-dependent diabetes mellitus result in frequent iatrogenic hypoglycemia. This study investigated the neuroanatomical location, direction, and magnitude of CNS neuronal genomic activation by singular versus repeated induction of hypoglycemic bouts of greater than 6 h duration achieved by administration of the intermediate-acting insulin, humulin neutral protamine Hagedorn (NPH). Adult male rats injected subcutaneously with Humulin NPH exhibited robust immunolabeling for the nuclear transcription factor, Fos, in discrete telencephalic, diencephalic, midbrain, and caudal hindbrain loci in a pattern that was not identical to that described for regular insulin. Administration of four doses of insulin on as many days significantly diminished or extinguished Fos immunostaining within the parvocellular hypothalamic paraventricular nucleus, lateral hypothalamic area, dorsomedial hypothalamic nucleus, thalamic paraventricular nucleus, nucleus tractus solitarius, and area postrema, but did not modify labeling of other metabolic loci. However, numbers of Fos-immunoreactivity-positive magnocellular neurons in the hypothalamic paraventricular and supraoptic nuclei were significantly increased after the second and fourth insulin doses, relative to the single-dose group. Concurrent observations of exacerbated hypoglycemia and modified patterns of glucoregulatory hormone secretion after serial injections of intermediate-acting insulin suggest that central mechanisms governing compensatory endocrine responses, specifically glucagon, become habituated to repetitive hypoglycemia of extended duration. Resultant alterations in CNS-islet and -adrenomedullary output and hypothalamic–pituitary–adrenal activity may reflect diminished neuronal activation within one or more of the brain loci characterized here by nonuniform transcriptional activation. The current studies provide a neuroanatomical foundation for further investigation of the neurochemical phenotypes and interconnectivity of functionally adaptive neurons, underlying cellular and molecular mechanisms of diminished or enhanced activation, as well as the impact of these modified cellular responses on glucose counterregulation during administration of intermediate-acting insulin.

Key words: Humulin NPH, recurrent insulin-induced hypoglycemia, Fos immunocytochemistry, glucagon, insulin, hypothalamus.

The metabolic stressor, hypoglycemia, is a potent threat to neurological function since the brain requires high substrate fuel input, yet maintains only limited energy reserves. Conventional therapeutic management of insulin-dependent diabetes mellitus, defined as a regimen based upon one or two daily injections of an intermediate-acting insulin formulation, is highly correlated with iatrogenic hypoglycemia (Cryer and Polonsky, 1998). Diabetic patients utilizing such treatment regimens suffer on average one or two episodes of symptomatic hypoglycemia weekly. Antecedent hypoglycemia is a primary factor in the development of hypoglycemia-associated autonomic failure, a pathophysiological condition in diabetic patients that is characterized by diminished hypoglycemic awareness and impaired glucose counterregulation. By attenuating compensatory mechanisms for relief of neuroglucopenia, recurring induction of insulin-induced hypoglycemia thus exacerbates the potential in these individuals for neural dysfunction and injury.

It is well established that the CNS regulates counterregulatory glucagon and adrenomedullary catecholamine secretion via visceral efferent outflow (Helman et al., 1982; Rohner-Jeanrenaud et al., 1983; LeMagnen, 1984; Niijima, 1988), and that several central autonomic structures, including the hypothalamic paraventricular (PVH), dorsomedial (DMH), and ventromedial (VMH) nuclei, and lateral hypothalamic area (LHA) participate in this function (Frohman and Bernardis, 1971; Berthoud and Jeanrenaud, 1979; Sawchenko et al., 1981; Yoshimatsu et al., 1984). Recent transneuronal tracing studies have verified the neuroanatomical connectivity of these and other brain loci with sympathetic and parasympathetic preganglionic neurons that project to the pancreas and adrenal medulla. Retrograde pseudorabies viral labeling approaches demonstrate the polysynaptic linkage of these autonomic motor neurons with upstream cells in the medulla [nucleus tractus solitarius (NTS), area postrema (AP), gigantocellular/

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Abbreviations: AP, area postrema; DMH, dorsomedial paraventricular nucleus; -ir, immunoreactivity; LHA, lateral hypothalamic area; NPH, humulin neutral protamine Hagedorn; NTS, nucleus tractus solitarius; PAG, periaqueductal gray; pNTS, precomissural nucleus tractus solitarius; PVH, hypothalamic paraventricular nucleus; PVHm, magnocellular division of the hypothalamic paraventricular nucleus; PVHpm, posterior magnocellular part of the hypothalamic paraventricular nucleus; PVT, paraventricular thalamic nucleus; RIIH, recurrent insulin-induced hypoglycemia; TBS, Tris-buffered saline; t_o, time zero; VMH, ventromedial paraventricular nucleus.

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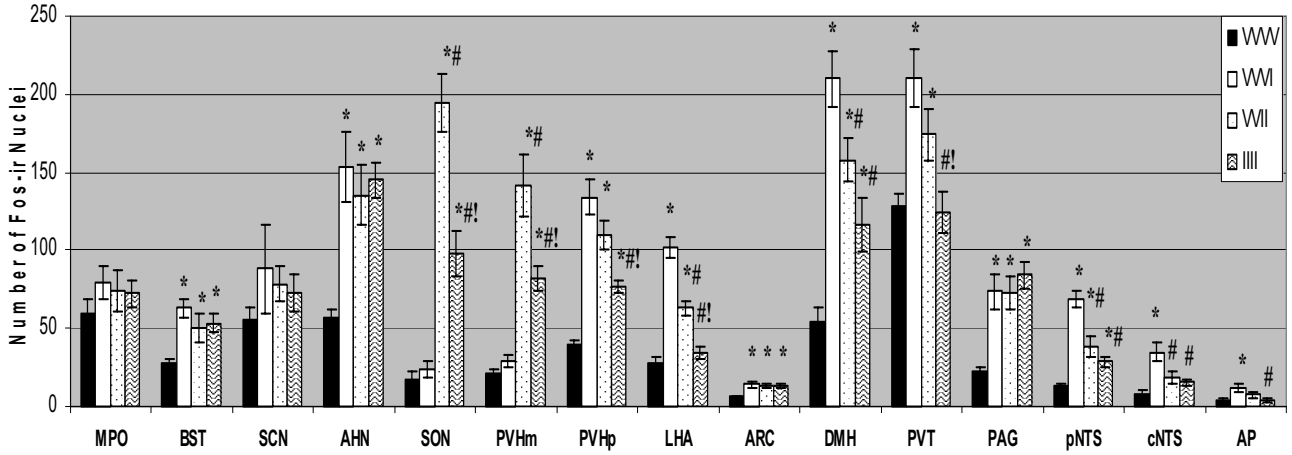

Fig. 1. Effects of single versus repetitive insulin administration on Fos immunolabeling in CNS autonomic metabolic loci. Anteroposterior coordinates relative to bregma (Swanson, 1998) are provided for representative sampled levels through each neural structure. The bars depict mean bilateral counts±S.E.M. of Fos-ir-positive neurons in the median preoptic nucleus (MEPO; −0.11 mm), bed nucleus of the stria terminalis (BST; −0.11 mm), suprachiasmatic nucleus (SCN; −1.08 mm), anterior hypothalamic nucleus (AHN; −1.53 mm), supraoptic nucleus (SON; −1.33 mm), PVH, PVHm (−1.78 mm); PVH, medial (dorsal zone) and lateral parvicellular divisions (PVHp; −2.00 mm), LHA (−1.78 mm), arcuate nucleus (ARC; −2.45 mm), DMH (−2.45 mm), PVT (−2.00 mm), PAG (−4.45 mm), pNTS (−13.44 mm), commissural NTS, medial and lateral parts (cNTSm; −14.16 mm), and AP (−14.16 mm). * $P<0.05$ versus VVVV; # $P<0.05$ versus VVVI; ! $P<0.05$ versus VVII. Fos labeling was evaluated 2 h after treatments on day 4 of the experiment.

lateral paragigantocellular reticular nuclei, lateral reticular nucleus, and raphe pallidus/obscurus]; pons (A5 cell group, lateral parabrachial nucleus, locus coeruleus, Barrington's nucleus); midbrain [periaqueductal gray (PAG)]; diencephalon (PVH, LHA, perifornical area, DMH, and arcuate nucleus); and telencephalon (central amygdaloid nucleus, medial preoptic area, and bed nucleus of the stria terminalis; Loewy and Haxhiu, 1993; Loewy et al., 1994; Jansen et al., 1997). Fos immunocytochemistry is an effective functional mapping tool for identification of individual neurons that are transcriptionally activated during glucopenia, and labeling for this protein has been reported in different subsets of the neural loci described above in response to regular (short-acting) insulin or glucose antimetabolite injection (Pacak and Palkovits, 2001, review; Briski, 1999; Briski and Brandt, 2000). Despite the widespread clinical utilization of intermediate-release forms of insulin characterized by a 6- to 12-h span of activity (Unger and Foster, 1996), neither the impact of a single dose, nor more importantly, repetitive administration of these longer-acting pharmaceutical formulations on the functional status of central neurons has been investigated. Preliminary studies in our laboratory reveal that CNS patterns of Fos immunostaining induced by a single injection of the intermediate-acting insulin, humulin neutral protamine Hagedorn (NPH), are not identical to that previously described for regular insulin or glucose antimetabolites. We thus sought to establish an experimental model for recurrent insulin-induced hypoglycemia (RIIH) based on an intermediate-acting insulin formulation in order to investigate the impact of antecedent hypoglycemic episodes of prolonged duration on central neuronal Fos immunoexpression and counterregulatory hormone secretion. The objective of the present experiments was to examine whether recurrent induction of prolonged hypoglycemia results in similarly unique modifications in direction and magnitude of Fos labeling in brain loci that regulate autonomic and neuroendocrine responses to hypoglycemia, and to determine if local habituation of neuronal genomic responsiveness is correlated with modified patterns of glucoregulatory hormone secretion.

EXPERIMENTAL PROCEDURES

Experimental design

All animal protocols were conducted in accordance with NIH guidelines for care and use of laboratory animals and approved by the ULM Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Adult male Sprague–Dawley rats (300–400 g bw) were housed in groups under a 14-h/10-h light/dark lighting schedule, and permitted free access to standard rat chow and water. The animals were divided into four treatment groups ($n=6$/group), and injected s.c. at 11:00 h on 4 consecutive days with either 12.5U/kg bw NPH (Humulin NPH; Henry Schein, Inc., Melville, NY, USA) or diluent as follows: group 1: days 1–4, diluent; group 2: days 1–3, diluent; day 4, insulin; group 3: days 1 and 2, diluent; days 3 and 4, insulin; group 4: days 1–4, insulin. The final working dilution of Humulin NPH used was 12.5 U/ml. This particular dose was selected on the basis of preliminary dose-response analyses demonstrating an approximate reduction in circulating glucose of greater than 50% of baseline for more than 4–5 h. Two hours after the fourth and final injection, each animal was killed by transcardial perfusion with 0.9% saline containing 2% sodium nitrite, followed by 0.1 M potassium phosphate buffer, pH 7.6, containing 4.0% paraformaldehyde and 0.2% picric acid. The brains were postfixed in fresh fixative, sunk in 25% sucrose, and cut into 25 μm serial sections on a Reichert/Jung freezing microtome. Tissues were stored at −20 °C in cryoprotectant containing 30% sucrose. In order to obtain a sufficient volume of blood to permit all analytes of interest to be measured in individual animals, additional rats were injected with either one, two, or four doses of