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HINDBRAIN MEDULLA CATECHOLAMINE CELL GROUP INVOLVEMENT IN LACTATE-SENSITIVE HYPOGLYCEMIA-ASSOCIATED PATTERNS OF HYPOTHALAMIC NOREPINEPHRINE AND EPINEPHRINE ACTIVITY

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Abstract—Cell-type compartmentation of glucose metabolism in the brain involves trafficking of the oxidizable glycolytic end product, L-lactate, by astrocytes to fuel neuronal mitochondrial aerobic respiration. Lactate availability within the hindbrain medulla is a monitored function that regulates systemic glucostasis as insulin-induced hypoglycemia (IIH) is exacerbated by lactate repletion of that brain region. A2 noradrenergic neurons are a plausible source of lactoprivic input to the neural gluco-regulatory circuit as caudal fourth ventricular (CV4) lactate infusion normalizes IIH-associated activation, e.g. phosphorylation of the high-sensitivity energy sensor, adenosine 5′-monophosphate-activated protein kinase (AMPK), in these cells. Here, we investigated the hypothesis that A2 neurons are unique among medullary catecholamine cells in directly screening lactate-derived energy. Adult male rats were injected with insulin or vehicle following initiation of continuous L-lactate infusion into the CV4. Two hours after injections, A1, C1, A2, and C2 neurons were collected by laser-microdissection for Western blot analysis of AMPK$_{\alpha 1/2}$ and phosphoAMPK$_{\alpha 1/2}$ proteins. Results show that AMPK is expressed in each cell group, but only a subset, e.g. A1, C1, and A2 neurons, exhibit increased sensor activity in response to IIH. Moreover, hindbrain lactate repletion reversed hypoglycemic augmentation of pAMPK$_{\alpha 1/2}$ content in A2 and C1 but not A1 cells, and normalized hypothalamic norepinephrine and epinephrine content in a site-specific manner. The present evidence for discriminative reactivity of AMPK-expressing medullary catecholamine neurons to the screened energy substrate lactate implies that that lactoprivation is selectively signaled to the hypothalamus by A2 noradrenergic and C1 adrenergic cells.

Key words: A2 noradrenergic neurons, C1 adrenergic neurons, insulin-induced hypoglycemia, laser-catapult microdissection, Western blotting, pAMPK.

INTRODUCTION

The neural network that controls glucostasis is extensive, linking multiple populations of metabolic-sensory cells situated within and outside the brain to a hierarchy of integrative, premotor, and motor components situated at various levels of the central neuroaxis (Watts and Donovan, 2010). The need to characterize the neurochemical elements of this system and their connectivity is undisputed. Hindbrain catecholamine signaling is evidently requisite for optimal function of this circuitry as collective destruction of noradrenergic (A1, A2) and adrenergic (C1, C2) cells by toxin uptake at a common hypothalamic projection field prevents feeding and autonomic responses to systemic pharmacological glucoprivation (Ritter et al., 2011). There remains the need to distinguish the respective roles (or lack thereof) of these individual cell groups in brain reactivity to insulin-induced hypoglycemia (IIH), a recurring complication of insulin-dependent diabetes mellitus management (Cryer, 2008). In particular, there is a keenness to know if one or more of these populations function to detect hypoglycemia-associated reductions in cellular energy. Signals of nerve cell metabolic instability derive from a small number of brain sites where specialized neurons adjust synaptic firing in response to diminished substrate fuel supply, including the dorsal vagal complex (DVC) of the dorsomedial medulla, where A2 and C2 catecholamine cells reside (Mizuno and Oomura, 1984; Adachi et al., 1995; Balfour et al., 2006). The efficacy of anti-glycolytic drugs to induce hyperphagia and hyperglycemia upon delivery into the DVC or ventrolateral medulla (the location of A1 and C1 cells) demonstrates connectivity of metabolic sensors in these sites with the central glucoregulatory circuitry (Ritter et al., 2000).

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Abbreviations: aCSF, artificial cerebrospinal fluid; AMPK, adenosine 5′-monophosphate-activated protein kinase; ARH, arcuate hypothalamic nucleus; AVPV, anteroventral periventricular nucleus; BSA, bovine serum albumin; CORT, corticosterone; CV4, caudal fourth ventricular; DMH, dorsomedial hypothalamic nucleus; DVC, dorsal vagal complex; E, epinephrine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; I, insulin; IgG, immunoglobulin G; IIH, insulin-induced hypoglycemia; LHA, lateral hypothalamic area; MPN, medial preoptic nucleus; NE, norepinephrine; O.D., optical densities; pAMPK, phosphoAMPK; PVH, paraventricular hypothalamic nucleus; TBS, tris-buffer saline; TH, tyrosine hydroxylase; TH-ir, TH-immunoreactive; V, vehicle; VMH, ventromedial hypothalamic nucleus.

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The ultrasensitive energy gauge adenosine 5′-monophosphate-activated protein kinase (AMPK) is phosphorylated by upstream kinases in response to metabolic stressors that deplete ATP (Hardie, 2011), and acts in the brain to regulate cellular and systemic energy homeostasis (Ronnett et al., 2009). In the hindbrain, AMPK is implicated in mechanisms controlling leptin- or glucose anti-metabolite-associated feeding patterns (Hayes et al., 2009; Li et al., 2011) and glucostasis (Ibrahim et al., 2013). Our work was the first to localize AMPK to a specific neurochemical phenotype in the DVC, e.g. A2 noradrenergic neurons, where it is co-expressed with other metabolo-sensory biomarkers and is activated during hypoglycemia (Cherian and Briski, 2011); yet, it is unclear if other hindbrain catecholamine cells feature this sensor or if AMPK is hypoglycemia-sensitive in those populations.

Metabolism of glucose, the primary energy source to the brain, is compartmentalized by cell-type and involves exchange of metabolites between astrocytes and neurons (Laming et al., 2000). The astrocyte-neuron lactate shuttle hypothesis (ANLSH) postulates that glucose is acquired from the circulation by astrocytes and either stored as glycogen, a complex branched polymer, or catabolized to the oxidizable fuel L-lactate for trafficking to neurons (Pellerin and Magistretti, 1994; Pellerin et al., 1998). Lactate is released into the extracellular space as a vital energy substrate for nerve cell aerobic respiration. Despite high energy needs, neurons are ironically devoid of energy stores and exhibit a truncated glycolytic pathway that favors pentose phosphate metabolism and antioxidative protection over energy production (Barros, 2013). Nerve cell reliance upon astrocyte-derived lactate is indicated by its preferred use over glucose as an *in vivo* energy substrate when both substrates are available (Wyss et al., 2011). The astrocyte glycogen reservoir is the principal alternative to blood-derived glucose as a source of energy. This energy reserve is dynamic during normal brain activity and metabolic stasis, and is an important reserve of lactate equivalents during states of heightened activity or glucose deficiency (Stobart and Anderson, 2013). Unlike neurons, astrocytes maintain a high rate of glycolysis; internal glycogen stores thus favor glial and neuronal energetic stability as stored glucose can be rapidly converted to energy and exportable fuel for respective needs of each cell type (Obel et al., 2012).

Lactate utilization by neurons is an integral component of hindbrain monitoring of neuro-metabolic stability. Utilization of hindbrain signals of lactoprivation by the energy balance network is proved by induction of hyperglycemia and Fos expression in hypothalamic metabolic loci by pharmacological restraint of hindbrain monocarboxylate transporter function and exacerbation of glucose decrements by hindbrain lactate infusion during hypoglycemia (Briski and Patil, 2005). Hindbrain lactoprivic control of blood glucose, feeding, counterregulatory hormone secretion, and hypothalamic AMPK activation is impeded by neurotoxic lesions of medullary catecholamine hindbrain neurons (Gujar et al., 2014). A2 neurons are one presumptive source of this regulatory signaling as AMPK activity in these cells during hypoglycemia is lactate-dependent. The DVC is typical of brain nuclei in its heterogeneous neurotransmitter cell composition. This cellular diversity complicates efforts to determine molecular mechanisms of metabolo-sensory function in this site, necessitating the selective analysis of homogeneous cell samples. Laser microdissection is a powerful technology that permits acquisition of single CNS neurons of interest, based upon morphological or neurochemical criteria, for downstream molecular, e.g. DNA, RNA, and protein analyses. The present studies utilized a combinatory approach, involving *in situ* immunocytochemistry, single-neuron laser-catapult microdissection, and high-sensitivity Western blotting (Cherian and Briski, 2011, 2012) to determine if (1) medullary noradrenergic and adrenergic cell groups express AMPK; (2) this sensor is activated during hypoglycemia in all or some catecholamine populations; and (3) lactate regulation of AMPK is common to all catecholamine cell groups in the hindbrain medulla. The current project also evaluated effects of hindbrain lactate repletion on hypoglycemia-associated changes in norepinephrine (NE) and epinephrine (E) content of individual micropunch-dissected hypothalamic metabolic loci to identify sites where catecholamine activity communicates hindbrain lactate dyshomeostasis.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (300–400 g *bw*) were maintained under a 14-h-light:10-h-dark lighting schedule (light on at 05.00 h), and allowed to feed (Harlan Teklad LM-485; Harlan Industries, Madison, WI, USA) and water *ad libitum*. 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. Animals were divided into three treatment groups ($n = 6$/group). On day 1 of the study, animals were implanted with a 26-gauge stainless-steel cannula guide (prod no. C315G/SPC; Plastic One, Inc., Roanoke, VA, USA) into the caudal fourth ventricle (CV4) [coordinates: 0 mm lateral to midline; 13.3 mm posterior to *bregma*; 6.1 mm ventral to skull surface] under ketamine/xylazine anesthesia (0.1 mL/100 g *bw i.p.*, 90 mg ketamine:10 mg xylazine/mL; Putney, Inc., Portland, ME; LLOYD laboratories Inc., Shenandoah, IO, USA), and transferred to individual cages. On day 10, CV4 infusion of L-lactate (25 μM/2.0 μL/h (Briski and Patil, 2005) or the vehicle (V), artificial cerebrospinal fluid (aCSF), was initiated 10 min before time zero (t_0; 11.00 h), and continued until +120 min. Infusions were performed using 33-gauge internal injection cannulas (prod. no. C315I/SPC; Plastics One, Inc.) projecting 0.5 mm beyond the cannula guide into the CV4. At t_0, groups of aCSF-infused rats were injected *s.c.* with sterile vehicle (V; Eli Lilly & Co., Indianapolis, IN, USA; $n = 6$) or neutral protamine Hagedorn insulin (I; 12.5 U/kg *bw s.c.* (Paranjape and Briski, 2005); Butler Schein, Melville, NY, USA; $n = 6$), while lactate-infused rats received an I injection ($n = 6$). Animals were sacrificed by decapitation at +120 min for