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DORSOMEDIAL HINDBRAIN CATECHOLAMINE REGULATION OF HYPOTHALAMIC ASTROCYTE GLYCOGEN METABOLIC ENZYME PROTEIN EXPRESSION: IMPACT OF ESTRADIOL

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Abstract—The brain astrocyte glycogen reservoir is a vital energy reserve and, in the cerebral cortex, subject among other factors to noradrenergic control. The ovarian steroid estradiol potently stimulates nerve cell aerobic respiration, but its role in glial glycogen metabolism during energy homeostasis or mismatched substrate supply/demand is unclear. This study examined the premise that estradiol regulates hypothalamic astrocyte glycogen metabolic enzyme protein expression during normo- and hypoglycemia *in vivo* through dorsomedial hindbrain catecholamine (CA)-dependent mechanisms. Individual astrocytes identified *in situ* by glial fibrillary acidic protein immunolabeling were laser-microdissected from the ventromedial hypothalamic (VMH), arcuate hypothalamic (ARH), and paraventricular hypothalamic (PVH) nuclei and the lateral hypothalamic area (LHA) of estradiol (E)- or oil (O)-implanted ovariectomized (OVX) rats after insulin or vehicle injection, and pooled within each site. Stimulation [VMH, LHA] or suppression [PVH, ARH] of basal glycogen synthase (GS) protein expression by E was reversed in the former three sites by caudal fourth ventricular pretreatment with the CA neurotoxin 6-hydroxydopamine (6-OHDA). E diminished glycogen phosphorylase (GP) protein profiles by CA-dependent [VMH, PVH] or -independent mechanisms [LHA]. Insulin-induced hypoglycemia (IIH) increased GS expression in the PVH in OVX + E, but reduced this protein in the PVH, ARH, and LHA in OVX + O. Moreover, IIH augmented GP expression in the VMH, LHA, and ARH in OVX + E and in the ARH in OVX + O, responses that normalized by 6-OHDA. Results demonstrate site-specific effects of E on astrocyte glycogen metabolic enzyme expression in the female rat hypothalamus, and identify locations where dorsomedial hindbrain CA input is required for such action. Evidence that E correspondingly increases and reduces basal GS and GP in the VMH and LHA, but augments the latter protein during IIH suggests that E regulates glycogen content and turnover in these structures during glucose sufficiency and shortage.

Key words: ventromedial hypothalamic nucleus, estradiol, glycogen synthase, glycogen phosphorylase, insulin-induced hypoglycemia, laser-catapult microdissection.

INTRODUCTION

Brain astrocytes engage in numerous activities that benefit nerve cell function and survival, including release of regulatory glio-transmitters, control of local blood supply, and provision of oxidative phosphorylation substrates (Stobart and Anderson, 2013). Glucose, the primary energy source to the brain, is taken up from the circulation for use by neurons and astrocytes, and stored as glycogen within the latter cell compartment (Nehlig et al., 2004). In the brain and elsewhere, glycogen metabolism is controlled by antagonistic actions of glycogen synthase (GS) and glycogen phosphorylase (GP), which respectively catalyze glycogen synthesis and depletion. These enzymes are regulated in turn by glycogen synthase kinase-3-β (GSK3β), which inactivates GS, and phosphoprotein phosphatase-1 (PP1), which stimulates GS and deactivates GP. CNS glycogenolysis is increased when energy provision falls short of demand circumstances, e.g. during seizure, sleep deprivation, and hypoglycemia (Gruetter, 2003; Brown, 2004). While physiological hypoglycemia is a rare event, insulin-induced hypoglycemia (IIH) is a recurring complication of obligatory meticulous control of insulin-dependent diabetes mellitus in the clinical setting (Cryer, 2005, 2008, 2011). As vital nerve cell functions are maintained at high energy expense, hypoglycemia-associated reductions in glucose supply to the brain pose a serious risk of neuronal injury. There is thus keen interest in understanding the capability of the brain glycogen reserve to fend off against this threat (Gruetter, 2003; Suh et al., 2007; Herzog et al., 2008; Oz et al., 2009) and exploration of possible ways to maximize such protection.

There is growing recognition of neural and endocrine influence on astrocyte glycogen metabolism. The catecholamine (CA) neurotransmitter, norepinephrine,

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Abbreviations: 6-OHDA, 6-hydroxydopamine; ARH, arcuate hypothalamic nucleus; CA, catecholamine; CV4, caudal fourth ventricle; GP, glycogen phosphorylase; GS, glycogen synthase; GSK3β1, glycogen synthase kinase-3-β; IIH, insulin-induced hypoglycemia; LHA, lateral hypothalamic area; O, oil; OVX, ovariectomized; PP1, phosphoprotein phosphatase-1; PVH, paraventricular hypothalamic nucleus; TBS, tris-buffered saline; VMH, ventromedial hypothalamic nucleus.

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stimulates glycogen breakdown in cerebral cortex *in vivo* (Harik et al., 1982) and cultured cortical astrocytes *in vitro* (Pellerin et al., 1997), but its role in astrocyte glycogen metabolism during hypoglycemia is unclear. The hypothalamus maintains glucostasis through its ability to monitor cellular metabolic stability and process this and other sensory information to regulate behavioral and autonomic and neuroendocrine motor functions. Substrate fuel-sensing neurons in characterized hypothalamic structures increase synaptic firing during hypoglycemia (Ashford et al., 1990; Silver and Erecińska, 1998) to likely control local and body-wide compensatory responses. It would be informative to learn if and how hypoglycemia may influence astrocyte glycogen metabolism in those sites. Astrocytes express estrogen receptor-alpha and -beta (Azcoitia et al., 1999; Hösli et al., 2000) and are implicated in estrogenic neuroprotective and anti-inflammatory actions on the female brain (Spence et al., 2011, 2013). Estradiol benefits neuronal energetic stability by stimulating oxidative phosphorylation (Nilsen et al., 2007), but its impact on astrocyte glycogen metabolism during glucostasis or dyshomeostasis is not known. Estradiol controls noradrenergic input to the preoptic area-hypothalamus through regulation of norepinephrine neurotransmission volume (Wise et al., 1981; Adler et al., 1983) and direction of norepinephrine effects on substrates (Herbison and Dyer, 1991; Herbison, 1998). The present studies utilized a characterized ovariectomized (OVX) female rat animal model involving reinstatement of plasma estradiol levels within the physiological range to investigate the hypothesis that this hormone regulates astrocyte glycogen metabolic enzyme protein expression in hypothalamic metabolo-sensory loci during normo- and hypoglycemia. Subsets of estradiol- and non-estradiol-treated animals were pretreated by caudal fourth ventricular administration of the selective CA neurotoxin 6-hydroxydopamine (6-OHDA) to assess whether dorsomedial CA input is required for such hormonal control. Astrocytes identified *in situ* by glial fibrillary acid protein immunolabeling were harvested individually by laser-catapult microdissection from ventromedial, arcuate, and paraventricular nuclei and the lateral hypothalamic area (LHA), and pooled for each structure prior to high-sensitivity Western blot analysis of GS, PP1, GSK3 β, and GP protein expression.

EXPERIMENTAL PROCEDURES

Animals

Female Sprague Dawley rats (270–350 g bw) were housed in individual cages under a 14 h light: 10 h dark cycle (lights on at 05.00 h) and fed standard laboratory chow diet and tap water *ad libitum*. All surgical and experimental 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. On day 1, animals were implanted with a 26-gauge stainless-steel cannula guide (prod. No. C315G/SP; 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; 90 mg ketamine:10 mg xylazine/mL; Henry Schein Inc., Melville, NY, USA). On day 7, each rat was re-anesthetized for bilateral ovariectomy and *sc* silastic capsule (i.d. 0.062 in., o.d. 0.125 in.; 10 mm/100 g bw) implantation; capsules contained either 17 β estradiol-3-benzoate (E; 30 μg/mL safflower oil; $n = 20$) or oil alone (O; $n = 20$).

Experiment design

On days 14 and 16, animals were injected into the CV4 with the CA neurotoxin 6-OHDA (N; 75 μg/1.0 μL/day; $n = 10$ E; $n = 10$ O) (Selvage et al., 2004) or vehicle (V; sterile apyrogenic water containing 0.2% ascorbic acid; $n = 10$ E; $n = 10$ O), as described (Gujar et al., 2014). This treatment paradigm results in significant loss of dorsal vagal complex CA nerve cells without change in numbers of local neuropeptidergic neurons (Gujar et al., 2014). On day 18, one half of 6-OHDA- or vehicle-pretreated rats were injected *sc* with neutral protamine Hagedorn insulin (I; 12.5 U/kg bw), while the remainder were given sterile diluent (V) alone (Eli Lilly Co., Indianapolis, IN, USA). Rats were sacrificed by decapitation at +120 min for trunk blood and brain collection as this time point constitutes the hypoglycemic nadir resulting from this insulin formulation (Paranpape and Briski, 2005; Nedungadi et al., 2006). Each brain was snap-frozen in liquid nitrogen-cooled isopentane and stored at −80° C.

Blood glucose measurement

Whole blood glucose levels were measured with an Accuchek Advantage glucometer (prod. No. 860; Roche Diagnostic Corporation, Indianapolis, IN, USA).

Immunocytochemical assessment of neurotoxin destruction of dorsal vagal complex neurons

Hindbrains from CV4 N- or V-pretreated animals injected *sc* with V were immersion-fixed (12 h) in 0.1 M potassium phosphate buffer, pH 7.6, containing 4.0% paraformaldehyde and 0.2% picric acid, sunk in 25% sucrose, and cut into 25 μm serial sections. For each animal, three tissue sections were processed, per level, from rostral (−13.28 to −13.60 mm relative to bregma), commissural (−13.76 to −14.16 mm), and caudal (−14.36 to −14.86 mm) levels of the dorsal vagal complex for tyrosine hydroxylase immunoreactivity (-ir). Sections were washed with 0.05 M Tris-buffered saline (TBS, pH 7.6), pre-incubated (1 h) with 4.0% normal donkey serum (prod. No. S30; Millipore, Billerica, MA, USA), and incubated (48 h) at 4 °C with mouse monoclonal antibodies against tyrosine hydroxylase (prod. No. 22941, 1:10,000, Immunostar, Hudson, WI, USA) diluted in TBS containing 0.05% Triton X-100 (TBS-Tx). Sections were then incubated (2 h) with an AlexaFluor-488 donkey anti-mouse antiserum (prod. No. A-21202, 1:400; Molecular Probes, Eugene, OR, USA) diluted in TBS containing 2.0% normal donkey serum,