Neuropeptides 47 (2013) 139–147

Contents lists available at SciVerse ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

Hypoglycemia differentially regulates hypothalamic glucoregulatory neurotransmitter gene and protein expression: Role of caudal dorsomedial hindbrain catecholaminergic input

Amit D. Gujar, Baher A. Ibrahim, Pratistha Tamrakar, Karen P. Briski *

Department of Basic Pharmaceutical Sciences, College of Pharmacy, The University of Louisiana at Monroe, Monroe, LA 71201, USA

ARTICLE INFO

Article history:
Received 20 September 2012
Accepted 28 January 2013
Available online 13 March 2013

Keywords:
6-Hydroxydopamine
A2 noradrenergic neurons
Insulin
Neuropeptide Y
Orexin-A
Oxytocin
RT-PCR
Western blotting

ABSTRACT

The hypothalamic neurochemicals neuropeptide Y (NPY), orexin-A (ORX), and oxytocin (OXY) exert glucoregulatory effects upon intracerebral administration, findings that support their potential function within neural pathways that maintain glucostasis. Current understanding of how these neurotransmitter systems respond to the diabetes mellitus complication, insulin-induced hypoglycemia, is limited to knowledge of neuropeptide gene transcriptional reactivity. We investigated the hypothesis that hypoglycemia elicits hypothalamic site-specific alterations in levels of these neurochemicals, and that adjustments in local neurotransmitter availability may be regulated by catecholaminergic (CA) input from the caudal dorsomedial hindbrain. The arcuate (ARH) and paraventricular (PVH) hypothalamic nuclei and lateral hypothalamic area (LHA) were each microdissected from adult male rats pretreated by caudal fourth ventricular administration of the selective CA neurotoxin, 6-hydroxydopamine (6-OHDA), or vehicle prior to insulin (INS)-induced hypoglycemia. Hypoglycemia stimulated ARH NPY gene expression and NPY accumulation in the ARH and LHA, but not PVH. 6-OHDA pretreatment did not modify the positive NPY mRNA response to INS, but blunted hypoglycemic augmentation of ARH and LHA NPY content while increasing PVH NPY levels in response to hypoglycemia. INS-treated rats exhibited diminished LHA ORX gene expression and increased [ARH; LHA] or decreased [PVH] tissue ORX protein levels. 6-OHDA + INS animals showed a comparable decline in ORX transcripts, but attenuated augmentation of ARH and LHA ORX content and elevated PVH ORX levels. OT mRNA and protein were respectively decreased or unchanged during hypoglycemia, responses that were uninfluenced by hindbrain CA nerve cell destruction. These results illustrate divergent adjustments in glucoregulatory neurotransmitter gene expression and site-specific protein accumulation in the hypothalamus during hypoglycemia. Evidence that 6-OHDA pretreatment does not modify NPY or ORX transcriptional reactivity to hypoglycemia, but alters hypoglycemic patterns of NPY and ORX accretion implicates dorsomedial hindbrain CA neurons in regulation of translation/post-translational processing and site-specific availability of these neurotransmitters in the hypothalamus during hypoglycemia.

1. Introduction

Hypoglycemia poses a significant risk of neurological dysfunction and injury as disruption of a continuous glucose supply threatens nerve cell transmembrane ionic equilibrium. The brain counteracts hypoglycemia by activating autonomic, neuroendocrine and behavioral outflow that ultimately increases glucose availability. Current understanding of this critical brain function is impeded by limited knowledge of the unified function of diverse, wide-ranging components of the central gluco-regulatory network. The hypothalamus, brainstem, and spinal cord contain elements of this network (Watts and Donovan, 2010), but the neurochemical identity and functional connectivity of neuron populations that comprise this extensive polysynaptic circuitry remain unclear. The hypothalamus plays a pivotal role in gluco-regulation as it is the final common source of motor commands to autonomic motor neurons and the pituitary-adrenal endocrine axis, and is a principal source of signals that regulate feeding behavior. Several hypothalamic neuropeptides, e.g. neuropeptide Y (NPY), orexin-A (ORX), and oxytocin (OT) exert effects on blood glucose levels upon intracerebral administration (Bjorkstrand et al., 1996; Marks and Waite, 1997; Parikh and Marks, 1997; Sakurai et al., 1998; Yi et al., 2009), findings that support their potential function *in vivo* in neural pathways that regulate glucostasis. This notion is bolstered by

* Corresponding author. Address: Department of Basic Pharmaceutical Sciences, College of Pharmacy, The University of Louisiana at Monroe, 356 Bienville Building, 1800 Bienville Drive, Monroe, LA 71201, USA. Tel.: +1 318 342 3283; fax: +1 318 342 1737.
E-mail address: briski@ulm.edu (K.P. Briski).

0143-4179/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.npep.2013.01.004

evidence that NPY or ORX receptor antagonism alters temporal patterns of insulin-induced hypoglycemia (Paranjape et al., 2007; Briski and Nedungadi, 2010). Current understanding of how these neurotransmitter systems react to hypoglycemia is constrained by reliance on customary indirect approaches to investigation of neuropeptide production involving measurement of gene transcripts by real-time RT-PCR, northern blotting, *in situ* hybridization, etc. There is mounting awareness that gene transcription rates cannot always be reliably extrapolated to predict protein yield; indeed, functional genomics analyses disclose divergence of gene transcript and protein product profiles in various models (Gygi et al., 1999; Chen et al., 2002; Guo et al., 2008). Needed information regarding hypoglycemic regulation of hypothalamic gluco-regulatory neuropeptides includes knowledge of projection sites wherein neurotransmitter levels are changed. For example, intra-hypothalamic NPYergic projections are functionally diverse, as delivery of exogenous NPY to various structures elicits location-specific actions (Nguyen et al., 2011). Thus, hypoglycemia may exert disparate effects on NPY activity within different terminal fields. Insight gained from neuroanatomical mapping of locations of hypoglycemia-associated adjustments in neuropeptide content would be a logical foundation for efforts focused on cellular substrates and molecular mechanisms of hypoglycemia-driven neurotransmitter regulation of glucose counter-regulatory motor outflow. Here, we investigated the hypothesis that hypoglycemia exerts differential effects on hypothalamic NPY, ORX, and OT mRNA versus neuropeptide levels, and furthermore, that hypoglycemia-associated adjustments in these neurotransmitters may vary among intra-hypothalamic projection sites.

Brainstem catecholaminergic (CA) neurons are a likely upstream source of input to glucostatic hypothalamic elements during hypoglycemia, since these myriad adrenergic and noradrenergic cell populations project extensively to the vicinity of those effectors (Sawchenko, 1983; Rinaman, 2010) and collective depletion of these brainstem catecholamine cell groups impairs hyperphagic (Ritter et al., 2001) and hypercorticosteronemic (Ritter et al., 2003) responses to glucose antimetabolite-induced glucoprivation. Hindbrain dorsal vagal complex (DVC) CA neurons are a plausible derivation of such signaling, as these cells are selectively activated in response to direct glucose anti-metabolite delivery to the hindbrain (Briski and Marshall, 2000), and are transcriptionally reactive to hypoglycemia-associated decrements in the glucose metabolite, lactate, in this part of the brain (Patil and Briski, 2005a,b). A2 noradrenergic neurons, located in the caudal DVC, express hypoglycemia-sensitive metabolo-sensory biomarkers, e.g. the low-affinity, high K_m hexokinase, glucokinase (GCK); the inwardly-rectifying, ATP-dependent potassium channel, K_{ATP}; and the cellular energy gauge, adenosine 5′-monophosphate-activated protein kinase (AMPK) (Briski et al., 2009b; Koshy Cherian and Briski, 2011, 2012). The present studies evaluated the premise that DVC CA signaling is critical for gluco-regulatory gene and/or neuropeptide responses to hypoglycemia and mediates hypoglycemic effects on site-specific availability of these neurotransmitters within the hypothalamus.

2. Methods and materials

2.1. Animals

Adult male Sprague Dawley rats (350–400 g *bw*) were maintained under a 14 h light:10 h dark lighting schedule (light on at 05.00 h), and allowed free access to standard laboratory rat chow (Harlan Teklad LM-485; Harlan Industries, Madison, WI) and tap water. 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. On day 1 of the study, animals were implanted with a PE-20 cannula into the caudal fourth ventricle (CV4) [coordinates: 0 mm lateral to midline, 13.3 mm posterior to bregma, and 6.6 mm ventral to the skull surface] under ketamine/xylazine anesthesia (0.1 ml/100 g *bw ip*, 90 mg ketamine/10 mg xylazine/mL; Henry Schein, Inc., Melville, NY, USA), and transferred to individual cages. All animals utilized in the present studies exhibited cerebrospinal fluid reflux from the tip of the cannula on day 1 of the study; post-mortem histological examination of each brain confirmed cannula placements within the caudal fourth ventricle and lack of structural damage to underlying hindbrain structures, including the DVC.

2.2. Experimental design

On days 10 and 12, groups of rats were injected into the CV4 with 6-OHDA (75 μg/day (Selvage et al., 2004) (group 1; $n = 10$) or vehicle, sterile apyrogenic water containing 0.2% ascorbic acid (VEH) (group 2; $n = 10$), in a total volume of 1.0 μL. The additive, ascorbic acid, attenuates immediate oxidation of 6-OHDA. This 6-OHDA treatment paradigm significantly reduces brain tissue catecholamine levels, but has no effect on serotonin content (Selvage et al., 2004). On day 14, subsets of each group were injected *sc* at 11.00 h with either neutral protamine Hagedorn insulin (INS; 12.5 U/kg *bw*; $n = 5$) or saline (SAL; $n = 5$). The animals were sacrificed 2 h after treatments, e.g. 13.00 h, for brain tissue and trunk blood collection, a time point that coincides with the hypoglycemic nadir elicited by this intermediate-release formulation in this animal model (Paranjape and Briski, 2005). Separate groups of 6-OHDA- ($n = 8$) and vehicle ($n = 8$) – pretreated rats were evaluated for feeding responses to *sc* injection of INS ($n = 4$) or SAL ($n = 4$) on an hourly basis between +1 and +6 h on day 14, as described (Briski and Nedungadi, 2009, 2010).

2.3. Blood analyte measurements

Blood glucose levels were measured with an Accu-Check Advantage glucometer (Roche diagnostics, Indianapolis, IN), as described (Nedungadi et al., 2006).

2.4. Immunocytochemical assessment of neurotoxin destruction of DVC catecholaminergic neurons

Dissected hindbrains were immersed for 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 collected, per level, from the rostral (−13.28 to −13.60 mm posterior to bregma), commissural (−13.76 to −14.16 mm posterior to bregma), and caudal (−14.36 to −14.86 mm posterior to bregma) DVC for processing for tyrosine hydroxylase (TH) immunoreactivity. Sections were preincubated for 60 min. with 4.0% normal donkey serum (prod. No. S30, Millipore, Billerica, MA), then incubated for 48 h at 4 °C with mouse monoclonal antibodies against tyrosine hydroxylase (prod. No. 22941, 1:10,000, Immunostar, Hudson, WI) diluted in 0.05 M Tris-buffered saline (TBS, pH 7.6) containing 0.05% Triton X-100, as described (Briski et al., 2001). Tissues were incubated for 2 h with AlexaFluor-488 donkey anti-mouse antibody (prod. No. A-21202, 1:400, Molecular Probes, Eugene, OR) in TBS containing 2.0% normal donkey serum, mounted on glass slides, and coverslipped. Images were captured with an LSM 5 PASCAL confocal scanning laser microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY), and bilateral counts of TH-ir-positive neurons were obtained for each DVC level.