Brain Research 1667 (2017) 19–27

Contents lists available at ScienceDirect

Brain Research

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

Research report

Nitric oxide in the nucleus of the tractus solitarius is involved in hypoglycemic conditioned response

Beatriz A. Alvarado [a,1], Mónica Lemus [a,1], Sergio Montero [a,b], Valery Melnikov [b], Sonia Luquín [c], Joaquín García-Estrada [c], Elena Roces de Álvarez-Buylla [a,*]

[a] Department of Neuroendocrinology, University Center of Biomedical Research, Colima University, Colima, Mexico
[b] Faculty of Medicine, Colima University, Colima, Mexico
[c] Department of Neurosciences, University Center of Health Sciences, Guadalajara University, Guadalajara, Mexico

ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form 31 March 2017
Accepted 26 April 2017
Available online 5 May 2017

Keywords:
Pavlovian conditioning
Glucose homeostasis
Guanylate cyclase
YC1
L-NAME
Nitric oxide
Nucleus tractus solitarius

ABSTRACT

The repeated injection of insulin (unconditioned stimulus, UCS) immediately followed by exposure to sensory stimulation (e.g. sound or odor; conditioned stimulus, CS) results in a learned conditioned reflex in which the exposure to the CS alone lowers blood glucose. The brain regions participating in this hypoglycemic Pavlovian response remain unknown. Here we investigate if nitric oxide (NO) in the nucleus tractus solitarius (NTS), a nucleus known to be involved in glucose homeostasis, participates in this hypoglycemic reflex. Insulin injections (UCS) were paired with exposure to menthol odor (CS). After 8–10 reinforcements (4–5 days training), rats acquire the learned hypoglycemic response. An increase in c-Fos expression was observed in the NTS, the ventrolateral hypothalamic nucleus (VLH) and other brain regions of conditioned rats. Microinjections of 3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole (YC-1) a stimulator of soluble guanylate cyclase (sGC) into NTS before the UCS accelerated the acquisition of the learned hypoglycemic response; 5–6 reinforcement produced pronounced glucose drop when exposed to the CS. In contrast, an inhibitor of NO synthase (NOS) N^{ω}-Nitro-L-arginine methyl ester (L-NAME) in the NTS prolonged the required training period (11–15 reinforcements) to obtain the hypoglycemic reflex, and reduced the glycemic response. The number of c-Fos expressing cells in the NTS and VLH in rats receiving YC-1was significantly higher than that observed in rats receiving L-NAME. These findings suggest that NO-cGMP-PKG signaling in the NTS can modify the acquisition of conditioned hypoglycemia, and suggests that this nucleus directly participates in this reflex.

1. Introduction

Hypoglycemia is a major complication of insulin therapy for diabetes. The loss of counterregulatory responses (CRR) in glucose homeostasis significantly increases the risk of severe hypoglycemic episodes (Hurst et al., 2012). Glucose homeostasis requires humoral and neural signaling for an integrated control of glucose release and its utilization by the different organs (Levin et al., 2011; Verberne et al., 2014; Wasserman, 2009). The brain's participation in glucose homeostasis is a subject of intense research (Ritter et al., 2011; Göbel et al., 2013). Hypothalamus and brainstem areas play an important role in mediating the CRR to hypoglycemia, controlling metabolic fuels intake and hepatic glycogenolysis (Cota et al., 2007; Levin et al., 2008; Coll and Yeo, 2013; Seoane-Collazo et al., 2015). The paraventricular (PVH) and ventrolateral hypothalamic (VLH) nuclei extend projections to the nucleus of the tractus solitarius (NTS) and the lateral amygdala (LA) (Petrovich et al., 2005; Bailey et al., 2006; Lindberg et al., 2013). The NTS situated proximal and just lateral to the dorsal motor nucleus of the vagus, receives afferents from multiple classes of glucose sensors (Davis et al., 2004; Marty et al., 2007). Among these sensors that project through the petrosal ganglion to NTS, are the carotid body chemoreceptors which have been shown to respond to hypoglycemia and to participate in systemic glucose homeostasis (Álvarez-Buylla and de Álvarez-Buylla, 1988; Pardal and López-Barneo, 2002; Kumar, 2007). The integration of signals from the periphery by the central nervous system (CNS)

Abbreviations: CCR, counterregulatory responses; CNS, central nervous system; L-NAME, N^{ω}-Nitro-L-arginine methyl ester; LS, lateral amygdala; LTP, long term potentiation; NO, nitric oxide; NOS, NO synthase; NTS, nucleus tractus solitarius; PVH, paraventricular hypothalamic; SGC, soluble guanylyl cyclase; VLH, ventrolateral hypothalamus; YC-1, [3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole.

* Corresponding author at: CUIB, Universidad de Colima, Ave. 25 de Julio 965, Villas San Sebastián, Colima 28045, Mexico.

E-mail addresses: nuskaroces@gmail.com, rab@ucol.mx (E. Roces de Álvarez-Buylla).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.brainres.2017.04.016
0006-8993/

induces peripheral changes to maintain glucose homeostasis (DeFalco et al., 2001).

The in vivo study of integral glucose control, and specifically the participation of the brain, requires an awake physiologically stable animal model system (Wasserman, 2009). Early work using awake dogs or rats showed that the hypoglycemic response induced by insulin can be conditioned. Pavlovian conditioning is acquired when a previously neutral stimulus is paired with the stimulus to be conditioned (Pavlov, 1927; Johnson et al., 2000). In this study insulin injection (unconditioned stimulus) (US) was paired with the conditioned stimulus (CS) (menthol odor exposure) (Álvarez-Buylla and Carrasco-Zanini, 1960; Woods et al., 1969; Flaherty et al., 1987). Following repeated conditioned plus unconditioned presentations, the CS alone elicits a hypoglycemic response. This conditioned response has also been observed in humans (Stockhorst et al., 2011). Since only small amounts of insulin are required as unconditioned stimulus (0.05 U/100 g in rats and 2 U/kg in dogs) (Álvarez-Buylla and Roces de Álvarez-Buylla, 1975; Álvarez-Buylla and Carrasco-Zanini, 1960), it is likely that this reflex engages normal endogenous blood sugar regulatory mechanisms. Interestingly, Pavlovian glucose conditioning can be established in pancreatectomized dogs (Álvarez-Buylla and Carrasco-Zanini, 1960), but requires an intact vagus nerve and is abolished by atropine (Álvarez-Buylla et al., 1961; Woods and Shogren, 1972). The above suggests that β-cells insulin secretion is not required for conditioned hypoglycemia and that neural (vagally-mediated) signaling is involved (Stockhorst et al., 2011).

Nitric oxide (NO), a highly diffusible gas produced from l-arginine (l-arg) by NO synthase (NOS), activates the soluble guanylyl cyclase (sGC) (Marsault and Frelin, 1992 Komsuoglu Celikyurt et al., 2014) and participates in synaptic plasticity, including memory consolidation in classical fear conditioning (Schafe et al., 2005). A major target of NO, sGC generates the intracellular second messenger cGMP (Boulton et al., 1995) that stimulates protein kinase G (Zhuo et al., 1994). The NO-independent activator of sGC, YC-1[3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazole] (Friebe et al., 1996) can enhance rodent learned behavior (Thielen et al., 2004). YC-1 intensifies long-term potentiation (LTP) (Chien, 2003; Boulton et al., 1995) indicating that the NO-cGMP-PKG signaling pathway participates in synaptic-plasticity and fear memory formation (Ota et al., 2008). Therefore, NO plays a key role in synaptic plasticity and the establishment of a conditioned reflex (Antonov et al., 2007; Kelley et al., 2011), but its role in the conditioned hypoglycemia has not been investigated.

In this study in rats we identified brain regions that become activated, increasing c-Fos expression, after conditioned association of insulin with menthol odor. Many c-Fos expressing cells were detected in NTS. We show that enhanced NO signaling in NTS reduced the required period to establish the conditioned hypoglycemia and resulted in lower glucose levels. In contrast, decreasing NOS signaling in NTS, prolonged the acquisition latency required to establish the hypoglycemic reflex and the observed hypoglycemia reduced. We conclude that the NTS and NO are involved in the learned association between insulin effects and an environmental stimulus.

2. Results

2.1. Saline control did not evoke conditioned hypoglycemia (Control group)

In order to test whether experimental manipulations (placing the rat in the conditioning chamber, blood sampling and injections through the i.p. catheter) affected basal blood glucose levels, 5 rats were subjected to experimental conditions equivalent to those to be used during conditioning in Experimental group 1 (see below), but the exposure to menthol odor was paired with saline (0.3 mL) injections instead of insulin (Control group (pseudoconditioned)). In these animals a small, but significant, increase in glucose concentration in blood samples (B1 *vs.* B2) was observed ($p = 0.0023$, $F = 31.294$) (Fig 1/**CS**−). This is likely due to the stress associated with the manipulation or the exposure to the menthol odor alone (Woods and Shogren, 1972).

2.2. Conditioned hypoglycemia (Experimental group 1)

Five rats subjected to conditioning (Experimental group 1) received 8–10 reinforcements (4–5 days training) (Álvarez-Buylla and Roces de Álvarez-Buylla, 1975), pairing the unconditioning stimulus (insulin injection) and the conditioning stimulus (menthol odor) (**CS+**). Mean glucose levels in plasma 20 min after both stimuli presentation at the 9th reinforcement, decreased from 106.2 ± 1.2 mg/dL in blood sample (B1) to 51.6 ± 4.3 mg/dL in B2 (Fig. 1), 51.4 % from basal level ($p = 0.005$, $F = 31.294$). Presentation of the conditioning stimulus alone (test of conditioning) (test) resulted in plasma glucose levels dropping from 103.6 ± 7.3 mg/dL to 64.8 ± 3.2 mg/dL. Examples of the UCS and CS glycemic response of individual animals is shown in Fig. S2. The first basal blood sample (B1) (25 μL), was obtained simultaneously with the beginning of insulin i.p. injection (unconditioning stimulus). At the end of this period the CS was turned off, the rat was placed again in the elevated plastic box and a second blood sample (B2) (25 μL) was taken (t = 20 min) (Fig. 1, panels "a" and "b") ($p = 0.04$) (Fig. 1/**CS+**) (37.7 % from the basal); differences between conditioned hypoglycemic effects during conditioning *a*nd those during tests were not significant ($p = 0.21$, $F = 31.294$). We conclude that in trained rats, menthol alone activates endogenous mechanisms to induce hypoglycemia.

2.3. Conditioned stimulus increases c-Fos expression in the NTS and VLH (Experimental group 1)

In order to determine which regions of the brain are activated by the conditioned stimulus, we compared the expression of immediate early gene c-Fos (Ao et al., 2005) between conditioned (Experimental group 1) (n = 5) and Control group (n = 5). Immediately following the conditioning test (exposure to menthol alone), rats were perfused and the brains processed for Fos-ir staining. An increase in the number of Fos-ir cells was observed in arcuate nucleus, dorsal hypothalamic nucleus, LA, cerebellum, NTS and VLH. A marked increase was noted in the NTS and the VLH from Experimental group 1, areas known to be involved in glucose regulation (Levin et al., 2011; Wasserman, 2009). In NTS the mean number of Fos-ir cells increased from 13.83 ± 0.8 (Control group) to 33.8 ± 2.1 (Experimental group 1) ($p < 0.01$, $F = 23–322$) and in VHL from 18.2 ± 2.7 to 45 ± 2.3 mg/dL ($p < 0.00$, $F = 23.322$); following conditioning, the number of Fos-ir cells increased 2.4 and 2.5 times, respectively in NTS and VHL (Fig. 2, panels "a" and "b"). For the present study we focused on the NTS and investigated if changes in NO signaling within this nucleus modified the learning of the hypoglycemic conditioned reflex.

2.4. YC-1 in NTS increased the acquisition latency of conditioned hypoglycemia (Experimental group 2)

NO has presynaptic and postsynaptic effects, and is involved in synaptic plasticity and memory formation (Schafe et al., 2005; Nazeri et al., 2014). We investigated the effects of local application of YC-1 into NTS on the establishment of the hypoglycemic conditioned reflex. YC-1 stimulates sGC in an NO-independent way and sensitizes it to NO (Friebe et al., 1996). In Experimental group 2