Nitric Oxide in the Solitary Tract Nucleus (STn) Modulates Glucose Homeostasis and FOS-ir Expression After Carotid Chemoreceptor Stimulation

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Abstract We evaluate in rats the role of NO in the solitary tract nucleus (STn) after an anoxic stimulus to carotid body chemoreceptor cells (CChrc) with cyanide (NaCN), on the hyperglycemic reflex with glucose retention by the brain (BGR) and FOS expression (FOS-ir) in the STn. The results suggest that nitroxidergic pathways in the STn may play an important role in glucose homeostasis. A NO donor such as sodium nitroprusside (NPS) in the STn before CChrc stimulation increased arterial glucose level and significantly decreased BGR. NPS also induced a higher FOS-ir expression in STn neurons when compared to neurons in control rats that only received artificial cerebrospinal fluid (aCSF) before CChrc stimulation. In contrast, a selective NOS inhibitor such as Nω-nitro-L-arginine methyl ester (L-NAME) in the STn before CChrc stimulation resulted in an increase of both, systemic glucose and BGR above control values. In this case, the number of FOS-ir positive neurons in the STn decreased when compared to control or to NPS experiments. FOS-ir expression in brainstem cells suggests that CChrc stimulation activates nitroxidergic pathways in the STn to regulate peripheral and central glucose homeostasis. The study of these functionally defined cells will be important to understand brain glucose homeostasis.

Keywords Nitric oxide solitary tract nucleus · Glucose · Sodium nitroprusside · Fos · Cerebrospinal fluid · Carotid chemoreceptor

1 Introduction

The mammalian brain depends on an efficient supply of oxygen and glucose. Carotid chemoreceptor cells that are strategically located at the initiation of brain circulatory system are of unique importance for CNS glucose homeostasis. CChrc function as peripheral glucose sensors, which in response to hyperglycemia or

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glucopenia release neurotransmitters to excite the adjacent nerve terminals of the carotid sinus nerves (CSN) (Álvarez-Buylla and Roces de Álvarez-Buylla, 1975; López-Barneo, 2003, Zhang et al., 2007). Afferent pathways reach the STn through CSN to participate in respiratory and cardiovascular functions, as well as in the CNS energy metabolism (Mifflin, 1996). But the precise mechanism of the glucose sensor activity and central projections to achieve central glucose regulation remains unclear.

Nitric oxide (NO) has been identified in several brain areas implicated in a diversity of biological functions (Moncada et al., 1991), such as blood pressure regulation and neuroendocrine responses (Housley and Sinclair, 1988; Sunico et al., 2005). NO synthases and their mRNA are present in the hypothalamic magnocellular neurons in the supraoptic and paraventricular nuclei (Kadowaki et al., 1994) where vasopressin and oxytocin are synthesized. NO induces ATP depletion in the CNS and modulates metabolic pathways during glycolysis (Almeida et al., 2005). Furthermore, in vitro experiments showed that NO functions as a neuromodulator by several mechanisms, which include reduction of the excitability of the CChrc in the hypoxic chemoreception (Prabhakar, 1994). In addition to the well known activity of CChrc in oxygen sensing, we found that NO likely participates in brain glucose homeostasis (Montero et al., 2006). The findings that mitochondria contain an isoform of NO synthase, which produces significant amounts of NO for regulating their own oxygen demands (Brodsky et al., 2002), indicates that NO may be important for glucose metabolism.

Our laboratory has previously observed that NO donors centrally applied into the cisterna magna, increase hyperglycemic response and BGR during normoxia in anesthetized rats. By contrast, during the hypoxic state, as after CChrc stimulation with NaCN, central NO administration inhibits the glucose parameters studied. In the same way, carotid baroreceptor activation induces NO production in the STn and c-fos proto-oncogen expression after CREB phosphorylation through a soluble guanylate cyclase/GMPc/proteinkinase G1 (Chan et al., 2004). In a further attempt to define the significance of centrally produced NO after CChrc stimulation on glucose homeostasis we have manipulated the hyperglycemic reflex with BGR after applying an anoxic stimulus to the CChrc, infusing a NO donor or a NOS inhibitor into the STn. Selective concentrations of the nitroxidergic drugs, capable of altering nitric oxide levels in blood, were subsequently used to study their effects on the activity of STn neurons measured as FOS protein expression (Fos-ir).

2 Methods

2.1 Animals, Surgical Procedures and CChrc Stimulation

Male Wistar rats (280–300 g) were used. They were housed at 22–24°C on a 12:12 h light:dark cycle. Al procedures were in accord to the Guide for the Care and Use of Laboratory Animals from the National Research Council. Rats were anesthetized with a bolus injection of sodium pentobarbital (3 mg/100 g i.p.) and a continuous

i.p. infusion of the same anesthetic (0.063 mg/min). The rats were maintained with artificial respiration controlling body temperature.

CChrc stimulation was performed by slowly injecting NaCN (5 μg/100 g in 0.25 ml saline) into the left carotid sinus. To ensure that responses were due to NaCN reaching only the rat's left carotid sinus, it was temporarily isolated from the cephalic circulation, while the right carotid sinus was denervated (Álvarez-Buylla and Roces de Álvarez-Buylla, 1975). Both, the left external carotid artery (beyond the lingual branch) and the internal carotid near the jugular foramen were temporarily occluded (15–20 s); the perfusion liquid that bathed the left carotid body was withdrawn by means of a catheter introduced into the lingual artery (Álvarez-Buylla and Álvarez-Buylla, 1988).

2.2 STn Microinjections

Glass cannulas (Microcaps, Drummond) were placed in the STn according Paxinos and Watson (1986) coordinates: P=12.7 mm, L=1.5 mm, V=7.7 mm At the end of the experiment the whole brain was rapidly extracted and stored at −70°C until cut on a cryostat (Leica VT 1000E) at 40 μm, to verify the injection place.

2.3 Drugs

Sodium cyanide (NaCN); artificial cerebrospinal fluid [aCSF (NaCl, KCl, $MgCl_2$, $CaCl_2$, ascorbate)]; saline (100 nl); sodium nitroprusside (NPS, 5 nmol); N-nitro-L-arginine methyl ester (L-NAME, 10 nmol).

2.4 Blood Collection and Measurements

Blood samples were withdrawn from catheters inserted into the femoral artery and jugular sinus without interrupting the circulation in these vessels (0.12 ml of arterial blood and 0.12 ml of venous blood proceeding from the brain). Blood samples were drawn at t= −10 min and t= −5 min (before nitroxidergic drugs infusion into the STn was made), and t=5 min, t=10 min, t=20 and t=30 min (after CChrc stimulation). In a complete sampling period, 1.2 ml of blood was taken (less than 8% of total blood volume). Fluid loss was replaced by injecting 0.3 ml saline after each pair of samples was taken. Plasma glucose concentration was measured by the glucose-oxidase method in mg/dl, in a Beckman Autoanalyzer. Brain glucose uptake was calculated in mg/dl, between glucose concentration in the abdominal aorta and glucose concentration in the jugular sinus.

The data are expressed in absolute values as mean ± SEM. The statistical comparisons were performed using Student's paired *t*-test, and ANOVA for repeated measurements when comparisons were made between groups. $^{*}P<0.05$, statistical significant when the result was compared with its own basal; $^{+}P<0.05$, statistical

significant when comparison was made between control group and experimental groups.

2.5 Immunohistochemistry

After the last sample was taken, animals were perfused intracardially (t=40 min after CChrc stimulation) with warm isotonic saline solution followed by ice-cold 4% paraformaldehyde in 0.1 M phophate-buffered saline (PBS). The brain was removed, post-fixed by submersion in PBS overnight. Serial transverse sections of the medulla oblongata were cut on a cryostat (50 μm) and collected in PBS. Free-floating sections that contained the STn were processed for immunohistochemistry using a primary sheep polyclonal antiserum (Jackson Merk) generated against the N-terminal of rat FOS protein to detect FOS-like immunoreactivity (FOS-ir) (Kobelt et al., 2004). As a secondary antiserum, biotinilated BA-1000 antirabbit IgG made in goat (Vector lab) was used. Visualization of immunoreactive product was enhanced with diaminobenzidine (DAB). Positive FOS-ir cells were counted manually.

2.6 Experimental Protocol

Experiment 1: infusion of aCSF (control) in the STn 4 min before CChrc stimulation with NaCN; arterial glucose, BGR and FOS-ir expression in STn neurons in rats were determined. Experiment 2: same as in "experiment 1", after an infusion of a NO donor (sodium nitroprusside- SNP) in the STn 4 min before CChrc stimulation with NaCN. Experiment 3: same as in "experiment 1" after an infusion of a NOS inhibitor (N-nitro-L-arginine methyl ester- L-NAME) in the STn 4 min before CChrc stimulation with NaCN.

3 Results

In the rats in which aCSF (100 nl) was injected into the STn 4 min before CChrc stimulation with NaCN (5 μg/100 g) an increase in arterial glucose levels accompanied by an increase in BGR was observed (Fig. 1). When a SNP (5 nmol/100 nl) injection was made in the STn 4 min before CChrc stimulation, arterial glucose levels also increased, while the increase in BGR was inhibited. In this case BGR decreased down to 13.6±3.8 at t=10 min, and to 13.3±2.4 mg/dl at t=20 min. Comparing these values with those obtained after an aCSF infusion, significant differences were obtained (P=0.02 and P=0.03 respectively, ANOVA for repeated measures) (Fig. 1). When a NOS inhibitor (L-NAME) was injected in the STn 4 min before CChrc stimulation, the hyperglycemic reflex did not result in significant changes, while BGR increased significantly in comparison with control experiments (P=0.04, ANOVA for repeated measures) (Fig. 1).

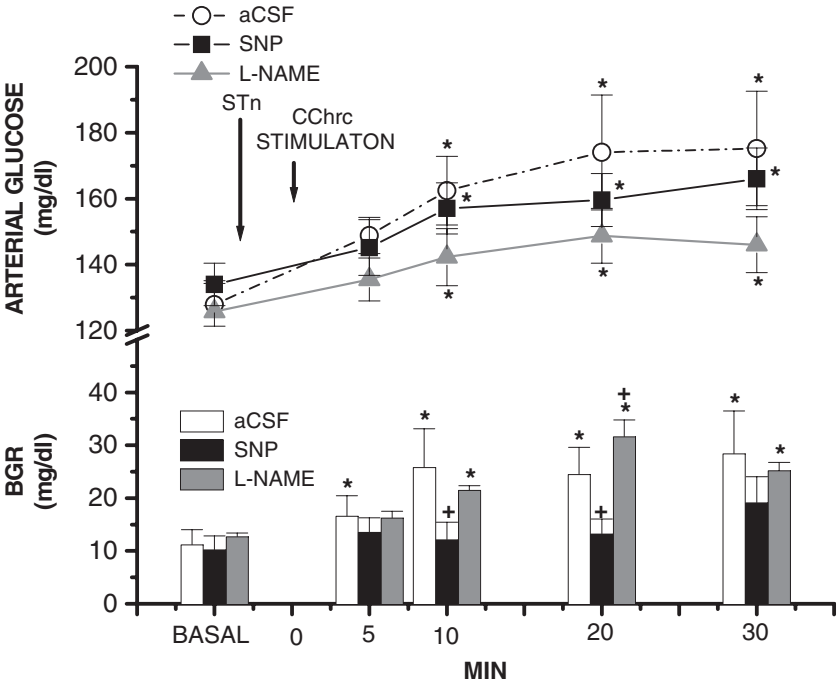

Fig. 1 CChrc stimulation with NaCN (5 μg/100 g 4 min after an aCSF injection (n=5) or a SNP infusion (5 nmol/100 nl) (n=6) or a L-NAME infusion (10 nmol/100 nl) (n = 4) in the STn in anesthetized rats (n=6). aCSF, artificial cerebrospinal fluid; L-NAME, Nω-nitro-L-arginine methyl ester; NaCN, sodium cyanide; SNP, sodium nitroprusside; STn solitary tract nucleus. The values are means ± SE, $^{*}P<0.05$, compared with their own basal, Student *t*-test; $^{+}P<0.05$ comparing the control group (aCSF), ANOVA for repeated measures

To verify whether the STn participates in the hyperglycemic reflex with BGR, the number of FOS-ir positive neurons in this nucleus was determined 40 min after CChrc stimulation and unilateral injections of aCSF (control), SNP or L-NAME in the STn 4 min before the stimulation. Figures 2 and 3 show the results obtained in FOS-ir expression distribution in these groups. In control rats, the number of positive-marked cells 40 min after CCrhc stimulation was 56±4; while when SNP was injected, the number of positive STn neurons increased up to 71±7 after CChrc stimulation ($P=0.05$, ANOVA one way, SNP vs aCSF); the injection of L-NAME in the STn reduced the number of FOS-ir cells down to 30±2 in the STn neurons after CChrc stimulation ($P=0.001$, ANOVA one way, L-NAME vs aCSF).

4 Discussion

Our findings suggest that nitroxidergic drugs have a unique relationship with CChrc stimulation effects on glucose variables. When aCSF injections were made into the STn 4 min before CChrc local stimulation with NaCN in control rats, a significant

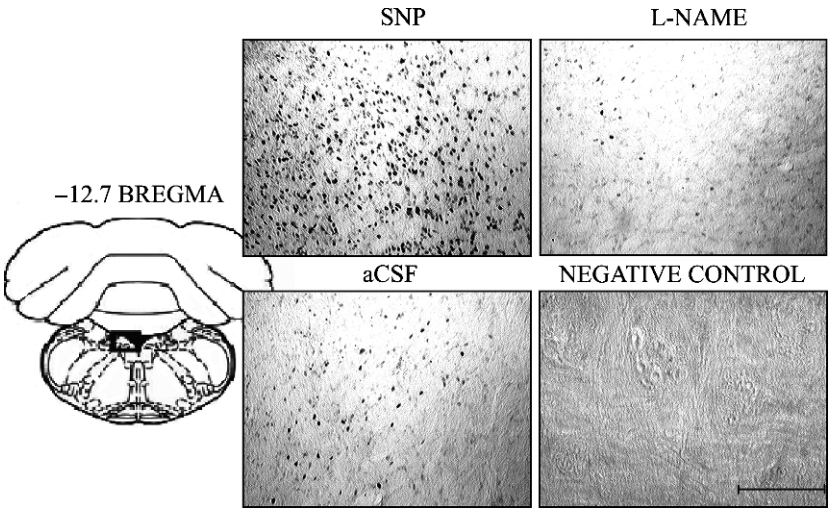

Fig. 2 Photomicrographs of FOS-ir neurons in the STn after: aCSF (100 nl), SNP (5 nmol/100 nl) or L-NAME (10 nmol/100 nl) infusions in the STn rats 4 min before CChrc stimulation of normal anesthetized rats, 100 X. Black dots represent individual nuclei with FOS-ir expression. Large numbers of FOS-ir cells were detected after SNP infusion. The enclosure shows the STn in a brain stem section. Scale bar: 1 mm

increase in arterial glucose concentration and BGR levels were obtained. An infusion of SNP into the STn 4 min before CChrc stimulation elicited an increase in arterial glucose levels with a significant reduction on BGR (when compared with the results obtained in control rats). Chemoreceptor activation also induced a transcriptional activation of c-fos gene in STn of rats that received a NO donor, indicating a

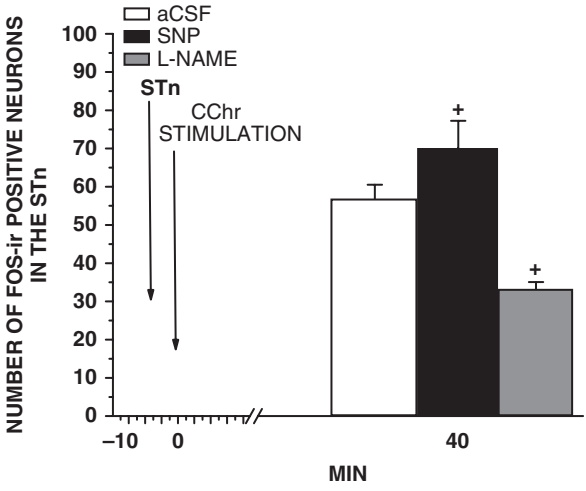

Fig. 3 Number of Fos-ir neurons in the STn 40 min after CChrc stimulation with NaCN and a previous infusion of aCSF (100 nl), or SNP (5 nmol/100 nl), or L-NAME (10 nmol/100 nl) in the STn in normal anesthetized rats. The values are means ± SE, $^{*}P < 0.05$ with aCSF, $^{+}P < 0.05$, comparing aCSF group with SNP or L-NAME groups, ANOVA one way

robust Fos-ir neuron expression. We conclude that nitroxidergic neurons in the STn, participate in the glycemic responses obtained here after CChrc stimulation, as was previously observed for baroreceptor stimulation (Talman et al., 2001). Moreover, L- NAME infusion into the STn 4 min before CChrc stimulation induced a significant increase in BGR, decreasing FOS-ir neurons in STn sections. This discrepancy could be due to differential effect of L-NAME in the STn depressing glutamate and adenosine as well as the hyperglycemia induced by 2-deoxyglucose (Sugimoto et al., 1997). NOS inhibitors also reduce baro-cardiopulmonary reflexes (Dias et al., 2005).

As was previously observed in in vitro experiments, NO induces a dual effect on carotid chemosensory discharges depending on the PO_2 level. Low levels of PO_2 inhibit the increase in chemosensory discharges, whereas during normoxia, NO increases the discharges (Iturriaga et al., 2000). SNP also reduces the increase in sensitivity and amplitude in the CSN discharges induced by acetylcholine, but L-NAME enhances it (Alcayaga et al., 1999). We cannot dismiss a possible inhibitory effect of NO on STn blood vessels. The increase in Fos-ir positive cells in the STn after SNP is probably due to NO responding neurons activation after CChrc stimulation with an anoxic stimulus, inhibiting the BGR. By the contrary, L-NAME effects were the opposite. It is also possible that GABAergic activity to inhibit the anoxic stimulus on BGR has been reflected in STn neurons increasing FOS-ir expression.

In conclusion, unilateral microinjections of a NO donor as SNP into the STn before CChrc stimulation with NaCN induced significant changes in BGR, when the results were compared with those obtained in control rats that received aCSF in the STn. In these experiments, FOS-ir expression in STn neurons also changed, indicating a significant increased when compared with STn cells obtained in control experiments. These local changes could be able of modulating the CChrc function by means of nitroxidergic pathways to participate in glucose homeostasis.

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