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INTERACTION BETWEEN THE CARBON MONOXIDE AND NITRIC OXIDE PATHWAYS IN THE LOCUS COERULEUS DURING FEVER

R. N. SORIANO,[a]* M. KWIATKOSKI,[b] M. E. BATALHAO,[a] L. G. S. BRANCO[c] AND E. C. CARNIO[a]

[a]*Nursing School of Ribeirão Preto, 14040-902, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

[b]*Medical School of Ribeirão Preto, 14049-900, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

[c]*Dental School of Ribeirão Preto, 14040-904, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

Abstract—We have documented that the locus coeruleus (LC), the main noradrenergic nucleus in the brain, is part of a thermoeffector neuronal pathway in fever induced by lipopolysaccharide (LPS). Following this pioneering study, we have investigated the role of the LC carbon monoxide (CO) and nitric oxide (NO) pathways in fever. Interestingly, despite both CO and NO are capable of activating the same intracellular target, soluble guanylate cyclase (sGC), our data have shown that LC CO is an antipyretic molecule, whereas LC NO is propyretic. Thus, aiming at further exploring the mechanisms underlying their anti- and propyretic properties, we investigated the putative interplay between the LC CO and NO pathways. Male Wistar rats were implanted with a guide cannula in the fourth ventricle (4V) and a temperature datalogger capsule in the peritoneal cavity. The animals were microinjected into the 4V with an inhibitor of heme oxygenase (HO) (ZnDPBG [zinc(II)deuteroporphyrin IX 2,4 bis ethylene glycol]), or a CO donor (CORM-2 [tricarbonyldichlororuthenium-(II)-dimer]), or an inhibitor of nitric oxide synthase (NOS) (l-NMMA [N^G-monomethyl-L-arginine acetate]), or an NO donor (NOC12 [3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene]), and injected with LPS (100 μg/kg i.p.). Two hours later, the rats were decapitated, and the brains were frozen and cut in a cryostat. LC punches were processed to assess LC bilirubin and nitrite/nitrate (NOx) levels. Microinjection of ZnDPBG reduced LC bilirubin and increased LC NOx, whereas l-NMMA diminished LC NOx and reduced LC bilirubin. Furthermore, NOC12 caused an increase in LC bilirubin, whereas CORM-2 caused a reduction in LC NOx. These findings are consistent with the notion that in the LC during LPS fever the CO pathway downmodulates NOS activity and the NO pathway upmodulates HO activity, and, together with previous data, allow us to conjecture that LC CO blunts fever by downmodulating NOS (antipyretic property), LC NO upmodulates HO and sGC activities favoring the development of LPS fever (propyretic effect).

*Corresponding author. Tel: +55-16-3602-4051; fax: +55-16-3633-0999.
E-mail address: rensoriano@gmail.com (R. N. Soriano).

Abbreviations: cGMP, cyclic guanosine 3′,5′-monophosphate; CO, carbon monoxide; COX, cyclooxygenase; CSF, cerebrospinal fluid; HO, heme oxygenase; LC, locus coeruleus; l-NMMA, N^G-monomethyl-L-arginine acetate; LPS, lipopolysaccharide; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrite/nitrate; SE, standard error of the mean; Tb, deep body temperature; 3V, third ventricle; 4V, fourth ventricle.

Key words: bilirubin, heme oxygenase, nitrite/nitrate, nitric oxide synthase, thermoregulation, lipopolysaccharide.

About two decades ago, it was first documented that nitric oxide (NO), a gaseous short-lived free radical, acts as a messenger in the CNS (Garthwaite et al., 1988) modulating the release of neurotransmitters (Prast and Philippu, 1992). Endogenous NO is produced by nitric oxide synthases (NOS) from oxidation of the guanidino-nitrogen atom of L-arginine. Similarly, carbon monoxide (CO), another diatomic gaseous messenger, has also been shown to modulate neuronal activity (Brune and Ullrich, 1987; Marks et al., 1991; Stevens and Wang, 1993; Verma et al., 1993; Zhuo et al., 1993; Wilkinson et al., 2009). Endogenous synthesis of CO, yielded by heme oxygenases (HO), occurs from oxidative cleavage of heme molecules by oxidation of the α-meso carbon of porphyrin ring. Actually, besides CO, the reaction generates equimolar amounts of free iron (which leads to induction of ferritin, an iron-binding protein) and biliverdin. Biliverdin, in turn, is rapidly reduced to bilirubin by biliverdin reductase, an enzyme that exerts its activity under both acidic and alkaline conditions, being nicotinamide adenine dinucleotide (reduced form; NADH) the cofactor required under acidic condition, whereas nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) is the cofactor at basic pH (Tenhunen et al., 1969; Vítek and Ostrow, 2009).

The roles of CO and NO have been widely studied for the past decades in biological systems (Arnold et al., 1977; Verma et al., 1993) under physiological and pathophysiological conditions, including fever (Steiner et al., 1999, 2002, 2004; Steiner and Branco, 2002; Feleder et al., 2007; Ravanelli et al., 2007; Soszynski and Chelminiak, 2007), an adaptive, regulated increase in deep body temperature (Tb) initiated by a concatenation of events triggered by pyrogens and modulated by pro- and anti-inflammatory mediators (for review see Blatteis, 2006). It is well established that the CNS plays a crucial role in integrating peripheral and central thermoafferent signals, driving efferent pathways (controlling the thermoeffectors) to adjust body's thermal balance to face environmental (e.g. shifts in ambient temperature) and immunoinflammatory challenges such as that one employed in experimental model of systemic inflammation, in which bacterial endotoxin (lipopolysaccharide, LPS) is peripherally administered (Steiner et al., 2002; Almeida et al., 2004; Ravanelli et al., 2007; Soszynski and Chelminiak, 2007; Soriano et al., 2010). Studying such a model, we documented that the locus coeruleus (LC)—the main noradrenergic nucleus in the brain (group A6), located in the vicinity of the external

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laterodorsal walls of the fourth ventricle (4V) in the pons—is part of a thermoeffector neuronal pathway (Almeida et al., 2004) in fever induced by LPS (Hare et al., 1995; Xu et al., 2003). Because of its importance as the principal source of noradrenaline in the brain (Dahlstroem and Fuxe, 1964), bearing diffusely branched axons and dendrites (Xu et al., 1994), the LC has been considerably explored, specially its connections (functional neuroanatomy) (Xu et al., 1994; Aston-Jones et al., 1995; Modirrousta et al., 2004; Kodama and Koyama, 2006) and biochemistry (Xu et al., 1994; Castellani et al., 1996; Hundahl et al., 2008).

Following studies in the literature, in particular those showing that LC cells express both the CO-synthesizing enzyme HO (Castellani et al., 1996; Hundahl et al., 2008) and the NO-synthesizing enzyme NOS (Xu et al., 1994; Ye et al., 1997; Cuellar et al., 2000) as well as the electrophysiological studies by Pineda et al., (1996) reporting that CO and NO can activate the firing rate of most noradrenergic neurons of the LC, we have investigated in the LC of rats the role of CO and NO in modulating fever induced by LPS. Interestingly, despite both CO and NO are known to activate the same intracellular target, that is, the cyclic guanosine 3′,5′-monophosphate (cGMP)-synthesizing enzyme, the soluble form of guanylate cyclase (sGC) (Arnold et al., 1977; Verma et al., 1993), our previous studies have shown that LC CO and NO evoke opposite effects, that is, LC CO is antipyretic (Ravanelli et al., 2007), whereas LC NO is propyretic (Soriano et al., 2010).

CO effects in comparison with NO have been suggested to be additive, overlapping, or opposite in different nuclei or biological systems, effects which have been considered to be most likely dependent upon the tissue microenvironment (Verma et al., 1993; Zhuo et al., 1993; Henningsson et al., 1999; Artinian et al., 2001; Yang and Qin, 2004; Motterlini et al., 2000; Gomes et al., 2004; Grion et al., 2007; Kitamura et al., 1998; Kurauchi et al., 2009; Ding et al., 1999; Ingi et al., 1996; Ryter et al., 2004; Botros and Navar, 2006; Mancuso et al., 1998; Polte et al., 2000; Foresti et al., 1997; Bouton et al., 2000; Thom et al., 1997; Thorup et al., 1999). Thus, in the present work, we investigated the putative interplay between the CO and NO pathways in the LC aiming at further exploring the mechanisms underlying their anti- and propyretic properties, respectively. To attain this goal we performed microinjections of inhibitor of HO or NOS, or of donor of CO or NO, into the 4V and measured the LC levels of stable products of the activity of both HO and NOS.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats, obtained from institutional vivarium sources, were group housed (4–5 animals per cage) and acclimated in a temperature-controlled chamber at 25 °C (model: ALE 9902001; Alesco, Monte Mor, São Paulo, Brazil), with a 12-h/12-h light/dark cycle (lights on at 6.00 AM) for 1 week before experimental use. All experiments were performed on rats weighing 300–340 g at the time of the experiments, hydrated and fed *ad libitum*, and singly housed in cages with a metallic grid lid and the floor covered with wood chip bedding material. To obviate effects of circadian variation, experiments started between 8.00 and 10.00 AM. Animal care was carried out in compliance with the guidelines set by the Brazilian College of Animal Experimentation (COBEA), an affiliate of the International Council for Laboratory Animal Science (ICLAS), which included minimizing the number of animals used and their suffering, and had the approval of the Animal Care and Use Ethics Committee of the University of São Paulo (CEUA, protocol 10.1.305.53.9).

Surgeries

Surgical procedures were performed under ketamine-xylazine general anesthesia (100 and 10 mg/kg, respectively; 1 ml/kg, i.p.). Antibiotics (160,000 U/kg benzylpenicillin, 33.3 mg/kg streptomycin, and 33.3 mg/kg dihydrostreptomycin; 1 ml/kg i.m.; prophylactically) and analgesic medication (flunixin; 2.5 mg/kg, 1 ml/kg s.c.) were provided immediately after the end of the surgeries. The animals were fixed (prostrate) on a stereotaxic frame to be implanted in the 4V with a stainless-steel guide cannula (16-mm long, 22 ga outer diameter) at the following coordinates: 8.9 mm caudal to bregma; at the mid-line (the sagittal suture); 5.5–6.5 mm ventral from the skull surface; incisive bar: −5.0 mm; inclination of the vertical stereotaxic bar at 10° The guide cannula was fixed to the skull with stainless-steel screws and acrylic cement. A tightly fitting stylet was inserted into the guide cannula to maintain patency and prevent infection. Afterward, the rat was turned over, and a median laparotomy was performed to insert a temperature datalogger capsule (SubCue, Calgary, AB, Canada) into the peritoneal cavity. All animals were kept under deep general anesthesia throughout the surgical procedures, receiving a supplementary dose of anesthetic whenever necessary. After the surgical procedures, the animals were housed singly in cages with a metallic grid lid and the floor covered with wood chip bedding material. Before the experimental procedures, the animals were allowed to recover from the surgical interventions for at least 5 days.

Drugs

The non-selective NOS inhibitor (N^G-monomethyl-L-arginine acetate, l-NMMA) was purchased from Tocris (Ellisville, MO, USA); the non-selective inhibitor of HO (zinc(II)deuteroporphyrin IX 2,4 bis ethylene glycol, ZnDPBG) from Porphyrin Products (Logan, UT, USA); the NO donor (3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene, NOC12) from Calbiochem (La Jolla, CA, USA); the CO donor (tricarbonyldichlororuthenium-(II)-dimer, CORM-2) and endotoxin (bacterial lipopolysaccharide, LPS, serotype 0111: b4) from Sigma (St. Louis, MO, USA). The l-NMMA and NOC12 were dissolved (the latter protected from light) in pyrogen-free saline; the CORM-2 was dissolved in 0.5% dimethylsulfoxide (DMSO); and the ZnDPBG was dissolved in Na_2CO_3 (50 mM). The l-NMMA and ZnDPBG were dissolved and stored at −20 °C; the NOC12 and CORM-2 were freshly dissolved in their respective vehicles.

Microinjection

A 10-μl syringe (Hamilton, Reno, NV, USA), connected to a microinjection needle (30 ga outer diameter) with a polyethylene tube (PE 10; protected from light), and a microinjection device (model 310, Stoelting, Wood Dale, IL, USA) were used to perform the microinjection within the 4V at a flow rate of 50 nl/min. The microinjection needle (0.1 mm longer than the guide cannula) was inserted into the guide cannula solely at the moment of the microinjection.

Tb recordings

Deep (abdominal) body temperature (Tb) was recorded throughout the experiments with the temperature datalogger capsule