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Involvement of neuronal nitric oxide synthase (nNOS) in the regulation of migrating motor complex (MMC) in sheep

M. Castro [a], J.M. Muñoz [a], M.P. Arruebo [a], M.D. Murillo [a], C. Arnal [b,c], J.I. Bonafonte [b], M.A. Plaza [a,*]

[a] Departamento de Farmacología y Fisiología, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, 50013 Zaragoza, Spain
[b] Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, 50013 Zaragoza, Spain
[c] CIBER de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Spain

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ABSTRACT

The objectives of this study were to evaluate the role of nitric oxide (NO) synthase isoforms (nNOS, eNOS, and iNOS) in the regulation of the migrating motor complex (MMC) in sheep using electromyography and their expression in the gastrointestinal (GI) tract by Western blot (WB) and immunohistochemistry. Intravenous administration of L-NAME or the nNOS inhibitor 7-nitroindazole (7-NI) decreased the MMC interval. Myoelectric activity of intestinal phase II was increased, whereas antral activity was reduced. These effects were blocked by L-arginine. Inhibitors of either iNOS (aminoguanidine and S-methylisothiourea) or eNOS (L-NIO) were ineffective. The NO donor sodium nitroprusside decreased GI myoelectric activity, inhibited the MMC pattern, and prevented the effects induced by L-NAME and 7-NI in the intestine. Intracerebroventricular administration of these agents did not modify GI motility.

In the rumen, abomasal antrum, duodenum, and jejunum, WB showed three bands at about 155, 145, and 135 kDa corresponding to nNOS, and a 140-kDa band (eNOS); however iNOS was not detected. Positive nNOS immunostaining was observed in neurons of the myenteric and submucous plexus of all GI tissues, while eNOS was found in the endothelial cells, ruminal and intestinal epithelium, as well as in some enteric neurons and in endocrine-like cells of the duodenal Brunner's glands. In contrast, only weak iNOS immunoreactivity was found in ruminal epithelium. Taken together, our results suggest that NO, synthesized at a peripheral level by nNOS, is tonically inhibiting the MMC pattern and intestinal motility in sheep.

Introduction

Nitric oxide (NO) is synthesized from L-arginine in a reaction catalysed by nitric oxide synthase (NOS). In the gastrointestinal (GI) tract, NO is one of the main inhibitory non-adrenergic non-cholinergic neurotransmitters (NANC) (Boeckxstaens et al., 1990). Thus, NO inhibits oesophageal, gastric, and intestinal motility, promotes gastric accommodation, and relaxes gut sphincters and, hence, participates in gastric emptying and intestinal transit.

Altered NO generation has been linked to several GI disorders such as achalasia, functional dyspepsia, diabetic gastroenteropathy, postoperative ileus, and slow-transit constipation (Shah et al., 2004). In ruminants, abomasal displacement has been related to increased NOS activity (Doll et al., 2009). In addition, NO regulates the migrating motor complex (MMC) in rats, chickens, dogs, humans, and sheep (Martinez et al., 1993; Sarna et al., 1993; Rodriguez-Membrilla et al., 1995; Russo et al., 1999; Onaga et al., 2000), but not in pigs (Lefebvre et al., 2005). Besides, the intestine is under tonic inhibition of the nitrergic system (Delbro, 1996). The role of nNOS, eNOS and iNOS has been studied in the colonic MMC (Tameyasu et al., 2004). Nevertheless, the specific NOS isoforms that are involved in the regulation of MMC in the stomach and small intestine are not known.

There are three isoforms of NOS, called neuronal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS III), and inducible NOS (iNOS or NOS II). Both nNOS and eNOS are constitutive and are present in normal conditions (Palmer and Moncada, 1989). In contrast, iNOS expression in physiological situations is minimal and increases in the presence of endotoxins or cytokines, among other agents. In the GI tract, nNOS-derived NO released from the enteric nervous system regulates the peristalsis and the sphincter function of the intestine. Furthermore, eNOS, expressed in endothelial and smooth muscle cells, mediates vasodilation, prevents leukocyte and platelet adhesion, and is essential for the maintenance of mucosal blood flow and for the protection of mucosal barrier function. In the gut, inflammatory, epithelial, endothelial, and neuronal cells can express iNOS. Large quantities of NO produced by iNOS may increase gut permeability and stimulate intestinal secretion (Dijkstra et al., 2004).

No studies to date have evaluated the specific NOS isoforms involved in the control of the MMC pattern in the stomach and small

* Corresponding author. Tel.: +34 976 761648.
E-mail address: aplaza@unizar.es (M.A. Plaza).

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intestine. Moreover, only few studies have evaluated the expression of NOS in the ovine digestive tract and those were mainly focused on nNOS. Thus, our aim was to elucidate the role of NO in the control of the MMC in sheep and to determine the NOS isoforms involved in it. Furthermore, we wanted to study the expression and localization of nNOS, eNOS, and iNOS in the rumen, abomasal antrum, and small intestine of sheep.

Materials and methods

Animal preparation

All procedures were carried out under Project Licence PI40/05 (February 8th, 2006) approved by the in-house Ethics Committee for Animal Experiments at the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Ewes, 4–5 year old and weighing 50–60 kg, were used. Under general anaesthesia with intravenous (IV) sodium thiopental (20 mg/kg; Pentothal, Abbott), eight triplets of 120 μm nickel/chrome electrodes (Microfil Industries) were implanted in the muscular wall of the abomasal antrum (5 cm from the pylorus), duodenum (40 cm from the pylorus), and proximal jejunum (0, 1, 2, 3, 4, and 5 m from the ligament of Treitz). A stainless-steel cannula was also inserted into the right lateral ventricle of the brain to perform intracerebroventricular (ICV) administrations, as previously described (Bueno et al., 1983). Analgesic and anti-inflammatory agents, buprenorphine (20 μg/kg, intramuscular injection; Buprex, Schering-Plough) and meloxicam (0.2 mg/kg, subcutaneous injection; Metacam), were administered the day of surgery and the following 3 days. For Western blot and immunohistochemistry, ewes were euthanased with an IV injection of sodium pentobarbital (Dolethal, Vétoquinol). Samples of the rumen, abomasal antrum, duodenum, and jejunum were collected and washed in cool phosphate-buffered saline (PBS).

Myoelectric recordings

The myoelectric activity was amplified (MT 8P, Lectromed) and filtered by low-pass (50 Hz) and high-pass filters (15 Hz). A computer-based method (Datasystem EMG v4.0, Panlab) converted the analogue signal into digital values with a sampling frequency of 100 samples/s. The myoelectric activity was integrated as the sum of the absolute values of the signal amplitude over 1-min intervals. Integrated myoelectric activity (IMA) of the phase II of the MMC cycles obtained after saline or nitrergic agents is expressed as a percentage relative to the mean value of IMA in the phases II recorded in the control period (3 h before drug administration) as previously described (Plaza et al., 1996). The jejunal data showed in this work correspond to those obtained at 2 m from the ligament of Treitz.

In the first series of experiments, one of the following drugs was administered: N (G)-nitro-L-arginine methyl ester (L-NAME, 1, 2, 5, and 10 mg/kg/h, IV or 0.5 mg/kg/h, ICV; 1 h), D-NAME (5 mg/kg/h, IV, 1 h), 7-nitroindazole (7-NI, 1, 5, 10, and 25 mg/kg/h, IV or 2.5 mg/kg/h, ICV; 1 h), N^5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO, 1 and 3 mg/kg/h, IV or 0.3 mg/kg/h ICV; 1 h), aminoguanidine (AG, 5, 10, and 25 mg/kg/h, IV or 0.25 mg/kg/h, ICV; 1 h), S-methyl-isothiourea (SMT, 0.5 and 1 mg/kg/h, IV or 0.1 mg/kg/h, ICV; 1 h), L-arginine (L-Arg, 25 and 125 mg/kg/h, IV, 4.5 h), D-Arg (25 mg/kg/h, IV, 4.5 h), sodium nitroprusside (SNP, 0.025, 0.1, 0.5, and 1 mg/kg/h, IV or 0.025 mg/kg/h, ICV; 3.5 h) or angiotensin II (50 ng/kg/min, IV, 1 h).

During the second series of experiments, L-NAME (5 mg/kg/h, IV, 1 h) was administered concomitantly with L-Arg or D-Arg (25 mg/kg/h, IV, 4.5 h) or SNP (0.025, 0.5, and 1 mg/kg/h, IV or 0.025 mg/kg/h, ICV; 3.5 h). Following the same protocol, 7-NI (25 mg/kg/h, IV, 1 h) was infused with L-Arg (125 mg/kg/h, IV, 4.5 h) or SNP (0.5 mg/kg/h, IV, 3.5 h). The administration of L-NAME or 7-NI started 30 min after the beginning of the infusion of L-Arg, D-Arg, or SNP. IV infusions of drugs were carried out by means of a cannula placed in the jugular vein. Each treatment was executed in a randomised order in six sheep. Experiments were performed in each sheep at 4-day intervals.

Western blot (WB)

The tissues were homogenized in cold radio-immunoprecipitation assay buffer (RIPA), containing PBS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate (SDS), supplemented with a complete EDTA-free protease inhibitor cocktail (Roche), phenylmethylsulfonyl fluoride (PMSF, 0.1 mM), pepstatin A (5 μg/mL), and bestatin (5 μg/mL). To exclude the possibility that the three band pattern observed for nNOS in the RIPA homogenates was due to a protease-mediated cleavage of the 155 kDa band, tissues for nNOS studies were also homogenized in cold N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer (20 mM HEPES, 1 mM EDTA, and 0.2 M sucrose, pH 7.2), that did not include detergents. The homogenates were centrifuged at 15,000 *g* at 4 °C for 20 min, and the protein concentration of the supernatants was assessed using the Lowry (Lowry et al., 1951) or the Bradford (Bio-Rad) methods (Bradford, 1976) depending on the buffer used in the homogenization process (RIPA or HEPES, respectively).

The proteins were separated on 6.5% or 7.5% SDS–PAGE gels (Bio-Rad) and transferred to a PVDF membrane (Immobilon-P; Millipore). The total protein loaded per lane was 50, 25 and 60 μg for nNOS, eNOS, and iNOS, respectively, except for nNOS in rumen (60 μg). The blots were blocked with 5% non-fat dry milk in PBS plus 0.1% Tween 20 (PBST) at room temperature (RT) for 1 h (nNOS, eNOS) or with 4% non-fat dry milk and 1% bovine serum albumin (BSA) in PBST at 4 °C overnight (iNOS).

C-terminal mouse monoclonal antibodies against human nNOS (1:16000), human eNOS (1:1000), and mouse iNOS (1:200) were applied at 4 °C overnight (nNOS, eNOS) or at RT for 2 h (iNOS). After washing, the membranes were incubated with a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:1000, BD Biosciences) at RT for 1 h. The immunocomplexes were detected by chemiluminescent reaction (ECL Plus, Amersham Biosciences). The nNOS and eNOS monoclonal antibodies have previously been validated for sheep in brainstem tissues (Wood et al., 2005). Rat brain and heart homogenates were used as nNOS and eNOS positive controls respectively, according to the antibody manufacturer's information about its reactivity. Commercial lysates of mouse macrophages, stimulated with LPS and IFNγ or unstimulated (BD Biosciences), were used as positive or negative iNOS expression controls, respectively.

Immunohistochemistry (IHC)

GI samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4 μm-thick sections. After deparaffination and rehydration, the slices were blocked for endogenous peroxidase in 0.3% H_2O_2/methanol. Non-specific binding was blocked with 15% of normal goat serum (nNOS) or following the instructions of the Vectastain ABC Kit (Vector Laboratories) (eNOS and iNOS). Slices were then incubated at 4 °C overnight with anti-nNOS (1:1000), -eNOS (1:200) or -iNOS (1:10) antibodies. Antibodies against eNOS and iNOS were the same as those used in the WB studies, whereas for nNOS a C-terminal rabbit anti-human nNOS polyclonal antibody was used.

After washing, sections were incubated at RT for 30 min with a biotin-conjugated goat anti-rabbit antibody (1:200, Vector Laboratories) (nNOS) or following the instructions of the Vectastain ABC Kit for mouse IgGs (eNOS and iNOS). Then, the tissues were treated with a horseradish peroxidase-labelled avidin–biotin complex (Vectastain Elite ABC kit for nNOS or Vectastain ABC Kit for eNOS or iNOS) and with diaminobenzidine (Vector Laboratories). The sections were counterstained with Carazzi's haematoxylin. Negative controls were performed without primary antibodies following the same protocol described above.

Reagents

Primary antibodies were obtained from BD Biosciences. L-NAME, D-NAME, 7-NI, AG, SMT, L-Arg, D-Arg, SNP, angiotensin II, HEPES, EDTA, sucrose, IGEPAL CA-630, SDS, sodium deoxycholate, PMSF, bestatin, pepstatin A, and BSA were purchased from Sigma–Aldrich. L-NIO and Tween 20 were obtained from Tocris and Acros Organics respectively. All the other reagents were purchased from Panreac. For IV or ICV administrations, agents were dissolved in sterile saline (2 mL) or water (200 μL), respectively, except 7-NI, which was dissolved in DMSO (up to 245 mg/mL). Equivalent volumes of these vehicles did not modify GI myoelectric activity.

Statistical analysis

Results are expressed as the mean ± SEM from six animals. The WB and IHC results are representative experiments of six different animals carried out in triplicate. Data were analysed by one-way ANOVA followed by Scheffé's *F* test. $P < 0.05$ was considered statistically significant.

Results

Effects of NOS inhibitors on GI motility

The GI myoelectric activity in sheep is organized into MMC cycles that recurred every 120.5 ± 5.9 min in our study. This motor pattern was characterized by a duodenal activity front with spike bursts at their maximal frequency (phase III) that lasted 2.8 ± 0.1 min and migrated to the jejunum. Phase III was followed by a period of quiescence (phase I) lasting 9.3 ± 0.9 min. The subsequent phase II consisted of a phase of irregular spiking activity which duration varied depending on the MMC cycle length. Coinciding with the duodenal phases III and I, antral activity decreased to 45.1 ± 3.2% from phase II values for 12.8 ± 0.8 min.