Influence between NO and CO in guinea pig stomach fundus

Boris Kadinov¹, Dimitar Itzev¹

¹ Institute of Neurobiology, Sofia, Bulgaria

Corresponding author: Boris Kadinov (kadinovb@gmail.com)

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Abstract

The interaction between carbon monoxide and nitric oxide and their role in modulation of stomach fundus excitability was studied. The presence and colocalization of heme oxygenase 1 (HO-1) and nitric oxide synthase (NOS) was verified in myenteric ganglia by immunohistochemistry. The role of inducible heme oxygenase isoenzyme was investigated after in vivo treatment of animals with CoCl₂ (80 mg kg⁻¹ b.w.) injected subcutaneously 24 hours before euthanasia. This treatment resulted in positive staining for the inducible isoform in stomach smooth muscle.

Keywords

Contraction; guinea-pig; hemin; heme oxygenase; histochemistry; smooth muscle

Introduction

The heme oxygenase (HO) pathway is primarily responsible for the endogenous production of carbon monoxide (CO). It has been postulated to be a second gaseous neurotransmitter (Costa et al. 1996; Farrugia and Szurszewski 1996). CO is produced together with ferrous iron and bilirubin by the action of heme oxygenase, in collaboration with cytochrome P450 reductase and biliverdin reductase (Xue et al. 2000). To date, two isoforms of HO have been characterized, HO-1 and HO-2. These isoforms are distinct in their regulation and expression. HO-1 is a stress protein and is induced by a wide variety of stimuli such as metal ions, hypoxia, and bacterial and viral toxins (McCoubrey et al. 1997). HO-2 is the constitutive isoform and is more prevalent in peripheral neural tissues, the brain, testes, and endothelial cells of the arteries (McCoubrey et al. 1997). Another isoform, called HO-3, has recently been found in brain, thymus, liver, prostate, kidney, spleen and testes (Xue et al. 2000).

Only a few studies have examined the presence of HO, HO activity, and CO effects in the gastrointestinal tract. HO-2 has been localized primarily in the enteric neurons of different regions of the gastrointestinal tract, the parietal and gastrin-containing cells of the gastric mucosa, and the interstitial cells of Cajal (ICC) networks of the mouse small intestine (Motterlini et al. 1996; Fan et al. 1998). The expression of HO is best visualized by immunohistochemical reaction with antibodies to the respective isoforms of the enzyme. The advantage of this method is its specificity. It allows the monitoring of the intracellular distribution of HO in thin tissue sections.

Nitric oxide (NO), produced by neuronal nitric oxide synthase (nNOS), is an established neural messenger in the central, peripheral, and enteric nervous systems (ENS), (Jaffrey and Snyder 1995; Stark and Szurszewski 1992; Goyal and Hirano 1996). In the ENS it acts as an intercellular inhibitory neurotransmitter by diffusing from neurons to adjacent smooth muscle cells where it activates soluble guanylate cyclase, resulting in smooth muscle relaxation.
NOS and HO have been colocalized in the murine ileum (Zakhary et al. 1997) and opossum anorectum (Battish et al. 2000). In addition, mice with gene deletions respectively targeted for HO-2 and nNOS exhibited profound changes in the gastrointestinal motility (Xue et al. 2000). Specifically, nonadrenergic noncholinergic (NANC) relaxation was found to be reduced dramatically in HO-2 and nNOS knockout mice compared with normal wild-type mice (Zakhary et al. 1997).

NANC nerves intrinsic to the enteric nervous system mediate relaxation of the stomach gastric fundus smooth muscle. Because HO2 and nNOS are colocalized in some enteric neurons, CO and NO may function as co-neurotransmitters (Maines 1997; Ny et al. 1997).

Recent studies by Ny et al. (Ny et al. 1997) have shown the presence of HO-2 and its colocalization with nNOS in different regions of the gastrointestinal tract. Heme and NO may be important regulators of HO-1 and heme turnover in skeletal muscle tissue in vivo (Vesely et al. 1998). However, the specific details of the presence of HO-1 and its colocalization with NOS in the myenteric plexuses and their distribution in the gastric fundus have not been examined.

Therefore, the aim of the present study was to examine the localization of HO-2 and its colocalization with nNOS in the gastric fundus. To date, the bulk of the physiological data regarding the neural regulation in the stomach, especially the NOS pathway, have been obtained in an experimental animal model, guinea pig. The presence of HO and its copresence with NOS, however, have not been examined in this animal model.

For immunohistochemical investigation 16 guinea-pigs (8 control and 8 treated with CoCl2) were killed as described above. The stomach’s fundus were cut in several slabs and were immediately fixed by immersion with 4% paraformaldehyde and 0.15% picric acid in 0.1 mol L⁻¹ phosphate buffer (PB), pH 7.3 for 24 h at 4°C. Some of the pieces were processed for paraffin embedding. Tissue sections (5 µm thick) were mounted on chrom alum-coated slides, deparaffinized in xilol, rehydrated in a graded series of ethanol and washed in 0.05 mol L⁻¹ phosphate buffered saline (PBS). Other slabs were washed over night in 20% sucrose solution in 0.1 mol L⁻¹ PBS. Consecutive 30 µm thick sections from muscle coat of the fundus were prepared on “Reichert Yung” freezing microtome and collected in solution of sucrose. They were washed overnight in PBS after several changes.

The color pictures of the sections obtained were taken via digital camera, and the intensity of the staining into the smooth muscle layer was analyzed using Corel Draw software. The data obtained from HO-1 stained sections were compared between treated and non-treated animals as follows. The intensity of the staining of at least 20 different sections prepared from each animal was taken as an average after subtracting the background staining separately for each section. Background staining was defined as an immunonegative staining that could be observed in the areas of the vascular lumen. After performing such a normalizing procedure for the sections taken from each animal, the differences in the staining intensity between treated and non-treated animals were statistically evaluated. The intensity of HO-2 staining was assessed by comparing the average intensity of the non-specific staining obtained as a background taken from selected immunonegative areas of each section with the staining of the smooth muscle layer. Comparison for the staining intensity between different sections was not performed. For the purpose of such analysis only 30 µm thick sections were used.

Contraction experiments. The preparations for contractile studies were done according to the previously described procedure. After excision fundic strips (10 mm long) were mounted vertically in 10 ml organ baths and stretched under tension of 10 mN. The organ baths were filled with Ca-free Krebs’ solution (see below) and strips were left for 30 min to relax. The bath solution was then changed to 2.5 mmol L⁻¹ Ca-containing Krebs to initiate contractions, and the strips were left to equilibrate for 45–60 min until a stable spontaneous contractile activity developed. The bath solutions were kept in dark, thermostatically controlled at 37 °C and continuously bubbled with a gas mixture (95% O₂ and 5% CO₂). During the equilibrium period the bath solution was changed every 20 min. The spontaneous isometric contractions of preparations were registered using Grass Instruments force-dis-
placement transducers (capacitance ± 10 g) and recorded on paper line recorder (type MTA 175, Kutesz, Hungary). The neurally independent tone of the tissue was evaluated in the presence of 1 μmol L⁻¹ tetrodotoxin to block the release of neurotransmitters. The responses of the strips to field electrical stimulation (FES) were registered in

Figure 1. Immunohistochemistry of NADPH-d.
Figure 2. Immunohistochemistry of HO-1 and HO-2. Immunohistochemically stained 30 µm thick sections of the guinea pig stomach fundus for non-treated and treated animals.
the presence of atropine (1 µmol L\(^{-1}\)) and L-ω-Nitro-L-arginine and evoked on the plateau of the \(\text{PgF}_2\alpha\) induced tone. Parameters of stimulation were as follows: 2 Hz and 5 Hz, at 50 V, for 30 sec, applied via platinum electrodes placed into the bath chamber at 2.5 mm distance from both sides of the strips. The relaxatory responses of the strips to electrical stimulation were estimated as a percentage of the maximal tone developed before addition of CaCl\(_2\) as compared to the basal level of the tone as measured in Ca\(^{2+}\)-free Krebs following the stretching. The data were assessed for statistical significance using paired Tukey-Kramer Multiple Comparison Test at p < 0.05.

**Solutions**

Krebs’ solution, used in contraction experiments, consisted of (in mmol.L\(^{-1}\)): 110 NaCl, 6 KCl, 1.2 MgCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 11 glucose, 25 NaHCO\(_3\), 2.5 CaCl\(_2\), which was bubbled with 95% O\(_2\) and 5% CO\(_2\) gas mixture to achieve a pH of 7.4 at 37 °C.

**Drugs**

All substances used for solutions contraction experiments were obtained from ICN Biomedicals, Irvine, CA, USA, except NaOH (for pH adjustment), vitamin E and atropine sulphate, which were SIGMA products. Hemin and Sn-protoporphyrin IX were purchased from ALEXIS Biochemicals, Switzerland.

**Results**

**Immunohistochemistry**

In the untreated animals, immunohistochemical studies revealed the presence of NADPH-d-positive neurons (Figure 1). The staining is in single neurons from the ganglia of the myenteric plexus, with weak staining in the smooth muscle tissue. In the treated guinea-pigs, the majority of neurons in the myenteric ganglia of the gastric fundus are NADPH-positive, with the staining mainly on the bodies of the neurons and the neural pathways not stained. In addition, smooth muscle tissues are also intensely stained, indicating a large amount of NADH-d in these tissues.

Immunohistochemically stained 30 µm thick sections of the guinea pig stomach fundus for non-treated and treated animals. Circles (A and B) shows myenteric ganglia. White arrows (C and D) point to immunopositive nerve cell bodies in the myenteric ganglia. Black arrows (E and F) point to immunonegative cell bodies.

Immunohistochemical studies for HO-1 revealed the presence of immunopositive neurons mainly in the treated animals (Figure 2), which is indicative of the induction of the enzyme in the tissues examined.

**Figure 3.** (a) Original trace representing the effect of hemin (0.01 µmol.L\(^{-1}\)) on the FES-evoked relaxation in non-treated guinea-pig gastric fundus strips. (b) Original trace representing the effect of hemin (0.01 µmol.L\(^{-1}\)) on the FES-evoked relaxation in treated guinea-pig gastric fundus strips. \(\uparrow\) – adding \(\text{PgF}_2\alpha\) (0.5 nmoL.L\(^{-1}\)) to evoke contractile response. Incubation with hemin is for 20 min., with Vitamin E – 10 min. before hemin application. The responses of the strips to EFS were registered in the presence of atropine (1 µmol.L\(^{-1}\)).
Contraction experiments – nontreated animals

Pretreating the strips of untreated animals with PGF\_2\_\alpha in the presence of atropine (1 µmol L\(^{-1}\)) and L-NNA (0.1 mmol L\(^{-1}\)) resulted in increased muscle tone. After its stabilization, a 2Hz and 5Hz FES, each of 30 sec duration, was applied. After the first stimulation, the re-stabilization of muscle tone (Figure 3a) was awaited. Blocking NOS resulted in a 76% increase in the effect of Hemin on the FES-evoked response at a stimulation frequency of 2 Hz (Figure 4). The presence of VitE in the solution reduces the effect of Hemin by up to 49%. At 5Hz, the increase in Hemin’s effect is 51%, and the presence of VitE reduces it to 41%. On the other hand, blocking NOS results in a 23% ±7) effect of SnPPIX (Table 1). The presence of Hemin results in a 34% reduction in the effect of SnPPIX. At high stimulation frequency (5Hz), blocking has no significant effect on the effect of SnPPIX.

Contraction experiments – treated animals

Treatment of experimental animals with CoCl\_2 did not affect the spontaneous contractile activity and tone of the preparations. The increase in the tone of the preparations in the presence of atropine (1 µmol L\(^{-1}\)) and L-NNA (0.1 mmol L\(^{-1}\)) before electrical stimulation takes into account the longer time required to stabilize the tone after PGF\_2\_\alpha, instability, as well as changing the configuration of the relaxer response (Figure 3b).

After relative stabilization of muscle tone, a 2Hz and 5Hz FES, each lasting 30 s, was administered. After the end of the first stimulation, a re-stabilization of muscle tone was awaited.

Hemin has an effect on the relaxation response only at low stimulation frequency – 2Hz, and blocking NOS increases its effect by 18% (Figure 5). However, the pres-

![Figure 4. FES-evoked relaxations in non-treated and treated guinea pigs. (a) Effect of Hemin (10 µmol.L\(^{-1}\)) on 2 Hz (n = 5) evoked relaxations. (b) Effect of Hemin (10 µmol.L\(^{-1}\)) on 5 Hz (n = 5) induced relaxatory responses of the strips. Data are the means ±SEM of experiments (* p < 0.5).](image1)

![Figure 5. FES-evoked relaxations in non-treated and treated guinea pigs in presence of VitE (100 µmol L\(^{-1}\)). (a) Effect of Hemin (10 µmol L\(^{-1}\)) on 2 Hz (n = 5) evoked relaxations. (b) Effect of Hemin (10 µmol L\(^{-1}\)) on 5 Hz (n = 5) induced relaxatory responses of the strips. Data are the means ±SEM of experiments (* p < 0.5).](image2)
Table 1. Effect of SnPPPIX (1 µmol.L⁻¹) alone and in the presence of hemin (10 µmol.L⁻¹) on EFS-evoked relaxations in non-treated (n = 5) and treated (n = 5) guinea-pigs; ( * p < 0.05 vs control).

|                  | Nontreated animals | Treated animals |
|------------------|--------------------|-----------------|
|                  | 2Hz                | 5Hz             | 2Hz              | 5Hz              |
| SnPPPIX          | *85.36 (±9.42)      | 95.52 (±5.15)   | 99.61 (±16.23)   | 105.08 (±10.41)  |
| Hemin + SnPPPIX  | 91.19 (±11.9)      | *110.25 (±2.73) | *134.63 (±20.05) | 107.68 (±16.16)  |

ence of VitE in the solution increases the Hemin effect by up to 61%. Blocking NOS eliminates the effect of SnPPPIX (Table 1). Treatment with Hemin before SnPPPIX increases the amplitude of the relaxation response. At 5Hz, blocking the NOS has no significant effect on the effect of SnPPPIX.

Discussion

The present immunohistochemical data demonstrate the presence of HO-1 and iNOS in myenteric plexus of the guinea-pig gastric fundus and show that the treatment with heavy metals (CoCl₂) affects NOS induction as the induction of HO. The presence of both enzymes in myenteric neurons in the guinea-pig gastric fundus points to the possible existence of a mutual relationship between the two systems.

As we have shown previously (Kadinov et al. 2002), the neurally derived CO is the actual modulator of the smooth muscle contractility in the stomach fundus. The above presented results suggest, that the release of CO from myenteric synapses can be modulated by the neuronal NOS by released NO. The increased effect of hemin when blocking NOS in both treated and non-treated animals suggests the suppressive role of NOS/NO system on the heme oxygenase activity. This supports the scheme of interaction between the two enzyme systems proposed by Foresti and Motterlini (1999) in its part to regulate the induction and activity of HO-1 by NOS and NO. In our studies, the effect of NO stimulation on electrical stimulation cannot be explained by the effect on HO-1 expression. In the other side, this effect is more distinct in non-treated animals, the constitutive isoform of the enzyme HO-2 is also affected. Also, blocking NOS in treated animals did not significantly differentiate the effect of Hemin, respectively on endogenous CO, on relaxation. This indirectly indicates a reduced level of NOS in the treated animals, suggesting that the influence of heavy metals may adversely affect the expression of NOS and, accordingly, NO-mediated relaxation upon electrical stimulation.

The presence of an antioxidant increases the effect of the endogenous CO. With blocked NOS the effect of hemin on the relaxatory response on EFS is more distinct, but in low frequency of stimulation.

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