Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: Evidence from genomic deletion of biosynthetic enzymes


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In the present study, we employ mice with targeted genomic deletions of HO2 (HO2Δ/Δ), nNOS (nNOSΔ/Δ), or both (HO2Δ/Δ nNOSΔ/Δ) to evaluate the physiologic roles of CO and NO in gastrointestinal neuromuscular transmission. We report that the resting membrane potential (RMP) of gastrointestinal smooth muscle cells is significantly depolarized in HO2Δ/Δ, nNOSΔ/Δ, and HO2Δ/Δ nNOSΔ/Δ mice and that mechanical and electrical responses to nerve stimulation in these mutant mice are substantially attenuated. Our results show that CO is an inhibitory neurotransmitter and indicate that CO and NO collaborate as coneurotransmitters to mediate intestinal inhibitory neurotransmission.

Materials and Methods

Solutions and Drugs. Krebs’ solution was freshly prepared daily and had the following ionic composition (in mM): Na+ 127.4; K+ 5.9; Ca2+ 2.5; Mg2+ 1.2; Cl− 134; HCO3− 15.5; H2PO4− 1.2; and glucose 11.5. The solution was continuously bubbled with 97% oxygen, 3% CO2 and maintained at pH 7.4. Atropine (1 mM), propranolol (1 mM), and phentolamine (1 mM) were present in all solutions used in this study to block adrenergic and cholinergic neurotransmission. Unless otherwise indicated, all chemicals were obtained from Sigma. CO was obtained from Scott Specialty Gasses (Troy, MI).

Animals and Initial Dissection of Jejunal Smooth Muscle Specimens. Adult male 129SVEV (wild-type) mice from The Jackson Laboratory and HO2Δ/Δ, nNOSΔ/Δ, and HO2Δ/Δ nNOSΔ/Δ mice were killed at 6–8 weeks of age with CO2 gas with the prior approval of the Mayo Animal Care and Use Committee. After the abdomen was opened, a segment of jejunum approximately 10 cm distal to the pylorus was removed and placed in preoxygenated Krebs’ solution. The jejunal segments were opened along their anti-mesenteric borders and transferred to fresh oxygenated Krebs’ solution. The mucosal layer was removed under direct vision by using a binocular microscope, and full thickness muscle strips (0.5 mm × 0.2 mm) were prepared, with the long axis cut parallel to the circular muscle layer for subsequent use in immunostaining, histochemical staining, or physiologic recordings (described below). For immunohistochemical or histochemical staining and for electrophysiologic studies, the mucosa was peeled away from the jejunal smooth muscle.

Abbreviations: ENS, enteric nervous system; nNOS, neuronal nitric oxide synthase; HO1 and HO2, heme oxygenase isoforms; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; HO1, heme oxygenase-1; HO2, heme oxygenase-2; ICC, interstitial cells of Cajal; RMP, resting membrane potential; EFS, electrical field stimulation; NANC, nonadrenergic noncholinergic; IP, inhibitory junctional potential.

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layers of all specimens before further preparation as described below.

**Immunostaining.** The mucosa-free dissected muscle layers were fixed in 4% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by incubation at 4°C for 12 h. Next, tissues were rinsed with PBS before immunostaining by using antibodies to c-kit, PGP 9.5, or HO2 or histochemical staining for NADPH-diaphorase. The antibodies, amounts or titers, and their sources were as follows: rat monoclonal anti-c-kit (GIBCO; 5 μg/ml), rabbit polyclonal anti-PGP 9.5 (Biogenes, Bournemouth, U.K.; 1/400), rabbit polyclonal anti-HO2 (StressGen Biotechnologies, Victoria, Canada; 1/1,000), and rabbit polyclonal anti-GST-HO2 (1/1,200). Secondary antibodies (donkey anti-rabbit IgG; Chemicon; 1/100) were conjugated to Cy2, rhodamine, and Cy5. NADPH-diaphorase histochemistry was performed either directly after fixation or after immunohistochemistry for HO2 by incubating tissues for 15–20 min in 0.1 M Tris (pH 7.4, 24°C) containing 0.3% Triton X-100, 2 mg/ml nitroblue tetrazolium, and 1 mg/ml β-NADPH. Staining was analyzed by examining the tissues with an epifluorescent (Zeiss Axiophot) light and laser scanning confocal microscopy (Zeiss LSM510).

**Electrophysiological Measurements.** After initial dissection as described above, muscle strips were placed in a recording chamber with the circular muscle facing upward. One end of the muscle strip was pinned down to a sylgard-coated (Dow-Corning) chamber to record intracellular electrical activity, and the other end was attached to an isometric force transducer to record contractile activity of the entire muscle strip. The chamber (3-ml total volume) was perfused with warmed (37°C) and oxygenated Krebs’ solution at a constant flow rate of 3 ml/min. After an equilibration period of at least 2 h, the muscle strips were stretched to an initial tension of 50–100 mg above the baseline tension. Recordings of intracellular electrical activity from smooth muscle cells were obtained by using glass capillary microelectrodes filled with 3 M KCl and with resistances ranging from 30 to 80 MΩ. Intracellularly recorded potentials were amplified by using a WPI M-707 amplifier (WPI Instruments, New Haven, CT) and displayed on an oscilloscope (Tektronix 5113). Force was measured isometrically and amplified with a bridge circuit amplifier. Both electrical signals and mechanical activity were recorded on chart paper (Gould 220, Gould, Cleveland) and also on an FM tape recorder (3964A, Hewlett-Packard). For electrical field stimulation (EFS) of nerves in the muscle strips, two platinum wires were placed parallel to the long axis of the preparation and were connected through a square wave stimulator (Grass 588, Grass Instruments, Quincy, MA) and a stimulus isolation unit (Grass SIU 5A). In all cases, EFS was applied as a train of six pulses of 0.35-ms duration, 100 V, and 30 Hz, for a total duration of 200 ms.

**Statistical Analysis.** All observed values are expressed as means ± SEM. The number of cells recorded is designated (n) in the results or figure legends. Statistical significance was determined by using paired and unpaired Student’s t tests. A P value of less than 0.05 was considered significant.

**Results**

***Myenteric Neurons and ICC Appear Normal in HO2−/−, nNOS−/−, and HO2−/− nNOS−/− Mice.*** Because loss of certain genes, such as GDNF, c-kit, and ICC, to migrate and/or develop (23–26), we explored the morphology and function of the ENS and ICC in mutant mice. We monitored PGP 9.5 and c-kit expression by immunostaining as markers for ENS and ICC, respectively. HO2−/−, nNOS−/−, and HO2−/− nNOS−/− intestinal preparations stain normally for PGP 9.5 and c-kit, indicating normal development of enteric neurons and intact ICC network (data not shown). NADPH-diaphorase histochemistry, reflecting nNOS expression, appears normal in HO2−/− mice but is absent in nNOS−/− and HO2−/− nNOS−/− intestines. HO2 immunoreactivity is present in a subset of enteric neurons, with 25–50% of these also staining for NADPH-diaphorase histochemistry in wild-type mice. We also observe HO2 but not NADPH-diaphorase staining in ICC of wild-type mice, consistent with our previous findings (12, 27). HO2 staining is absent in the HO2−/− and the HO2−/− nNOS−/− specimens.

Functional ICC are required for normal electrical slow waves and smooth muscle contraction (28–30). We recorded phasic contractions and electrical slow waves in jejenum and observed no significant abnormalities in HO2−/−, nNOS−/−, or HO2−/− nNOS−/− mice, consistent with functionally normal ICC in these mice.

***Jejunal Smooth Muscle Cells Are Depolarized in HO2−/−, nNOS−/−, and HO2−/− nNOS−/− Mice.*** We measured the RMP in smooth muscle cells of the jejenum from wild-type and mutant mice (Fig. 1). In comparison to wild-type cells, the RMP is depolarized about 8 and 5 mV in HO2−/− and nNOS−/− smooth muscle cells, respectively. Compared with the RMP of wild-type mice, the RMP of HO2−/−/nNOS−/− mice is depolarized by about 13 mV, reflecting an apparent additive effect of the two genomic deletions. Thus, HO2 and nNOS seem to be crucial for maintenance of a normal RMP, and NO and CO formed in ICC or enteric neurons act together to determine the RMP.

We wondered whether the NO and CO that contribute to establishing the RMP of the muscle cells are derived from ICC or enteric neurons. Action potentials of enteric neurons can be abolished by treatment with tetrodotoxin, which should not affect ICC. Tetrodotoxin treatment has no influence on jejunal muscle cell RMP (Fig. 1). This lack of influence suggests that action potentials of the enteric neurons are not required for regulation of RMP; thus, CO or NO regulating RMP comes either from the basal activity of these neurons or from ICC.
Mechanical and electrical responses to EFS. Mechanical (Upper) and electrical (Lower) response to EFS (*) recorded from jejunal circular smooth muscle cells from wild-type (WT), nNOS \(^{\Delta\Delta} \), and HO2 \(^{\Delta\Delta} \)/nNOS \(^{\Delta\Delta} \) mice. EFS evokes an UP of only \(-0.75 \pm 0.8 \text{ mV} \) in nNOS \(^{\Delta\Delta} \)-derived specimens compared with \(-7.6 \pm 0.6 \text{ mV} \) in wild-type muscles (\(n = 4\); \(P < 0.05\)). However, the decrease in slow wave amplitude was not different between nNOS \(^{\Delta\Delta} \) and wild-type specimens, with a decreased amplitude of 45 \% and 47 \% (respectively) (\(n = 12\); \(P > 0.05\)). Both mechanical and electrical inhibitory responses to EFS were nearly abolished in the HO2 \(^{\Delta\Delta} \)/nNOS \(^{\Delta\Delta} \)-derived samples, and an excitatory electrical response was unmasked.

![Diagram](https://example.com/diagram.png)
HO2-derived CO for normal function. This notion is supported by the partial restoration of NANC inhibitory transmission by CO gas (Fig. 4B), which may allow endogenously produced NO to mediate inhibitory transmission comparable to wild-type preparations. To examine the interaction between NO and CO further, we treated the HO2knockout strips with l-nitroarginine for 20 min, followed by exposure to 10% CO for 10 min. The l-nitroarginine treatment blocks the effects of CO such that less inhibitory transmission is evident than in the HO2knockout preparations treated with CO alone (Fig. 4C). We also examined intestinal preparations of HO2knockout/nNOSknockout mice in the presence of exogenous CO. The behavior of these muscle strips is essentially the same as that of the HO2knockout preparations treated with l-nitroarginine in addition to CO (Fig. 4D).

**Discussion**

One of the most striking findings of the present study is that the RMP of jejunal circular smooth muscle cells is determined by HO2 and nNOS. Smooth muscle cells are depolarized in HO2knockout and nNOSknockout, and the HO2knockout/nNOSknockout manifest additional depolarization, reflecting additive effects of the two enzymes. Do the NO and CO responsible for the RMP derive from enteric neurons or ICC? Mice with genomic deletion of c-ret lack an ENS (26, 31), but the RMP of their small intestinal smooth muscle cells is the same as that of wild-type mice (S. Ward, personal communication). On the other hand, mice with a mutation in c-kit (W/W) lack ICC, and their intestinal smooth muscle cells are depolarized by about 8 mV (23). This level of depolarization is essentially identical to what we observe in HO2knockout specimens, suggesting that HO2-derived CO from the ICC plays a major role in establishing the RMP. How does NO affect the RMP? Immunohistochemical studies suggest that, although nNOS is abundant in enteric neurons, it may not be present in ICC (12, 27). Conceivably, ICC express low levels of nNOS that were not detected in these studies. Despite the observations that the loss of the entire ENS does not affect the smooth muscle RMP, nNOS in enteric neurons may influence RMP; because loss of the whole ENS results in the loss of many excitatory and inhibitory enteric neurotransmitters that may contribute directly or indirectly to the RMP, the effect of the singular loss of NO may be obscured.

Previously, we found that intestinal relaxation is partially reduced in HO2knockout and in nNOSknockout mouse ileum (8). We also found that cGMP levels of ileal muscle strips were reduced in HO2knockout and nNOSknockout specimens, and depolarization-induced augmentation of cGMP was also reduced in HO2knockout and nNOSknockout mouse ileum (8). The relevance of these findings to intestinal physiology in the intact organism was established by our observation that gastrointestinal transit time was prolonged in HO2knockout and nNOSknockout animals (8). These findings suggest that both CO and NO are neurotransmitters in the ENS regulating intestinal motility in mice. Because our earlier study did not monitor the electrical responses of intestinal smooth muscle cells to neural input and did not evaluate the smooth muscle RMP, it was not possible to provide definitive evidence of a neurotransmitter role for CO. In the present study, we have directly demonstrated that, after EFS, IJPs are reduced in HO2knockout and

**Table 1. Targeted genomic deletion of HO2 attenuates the electrical response to EFS**

<table>
<thead>
<tr>
<th>Wild type (n = 12)</th>
<th>HO2knockout (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperpolarization, mV</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>Reduction in amplitude of first slow wave, %</td>
<td>47 ± 3.3</td>
</tr>
<tr>
<td>Reduction in amplitude of second slow wave, %</td>
<td>22 ± 4.6</td>
</tr>
<tr>
<td>Reduction in amplitude of third slow wave, %</td>
<td>9.7 ± 2</td>
</tr>
</tbody>
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Targeted genomic deletion of HO2 results in attenuation of electrical response to EFS in jejunal smooth muscle preparations. The initial hyperpolarization or IJP in response to EFS is only 2.6 mV in HO2knockout-derived specimens compared to 7.6 mV in wild-type samples. In addition, the reduction in the amplitude of the first three electrical slow waves after EFS is significantly attenuated in the HO2knockout-derived specimens compared to wild-type samples. The number of independent measurements is indicated by (n), and statistical significance is indicated (*, P < 0.05).
HO2 and nNOS have overlapping expression in 25–50% of myenteric neurons, implying that CO and NO can be produced by the same neurons (8, 13–15). Our present study provides insight into the role of CO and NO as coregulators. CO might sensitize intestinal smooth muscle cells to the effects of NO. Alternatively, CO may enhance nNOS catalytic activity or facilitate NO release from enteric neurons.

ICC, enteric neurons, and intestinal smooth muscle cells interact to mediate physiologic inhibitory transmission and smooth muscle relaxation. Our studies of CO and NO interactions may clarify such relationships. nNOS has not been reliably demonstrable in ICC, suggesting that the dominant role of ICC in regulating muscle RMP involves influences of CO on neuronal NO as well as potential sensitization of muscle cells to NO. It is also possible that CO and NO from enteric neurons affect ICC, perhaps regulating CO production in ICC.

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