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HEME-OXYGENASE-2 IMMUNOLABELLING IN PIG JEJUNUM

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SUMMARY

Heme-oxygenase-2 generates carbon monoxide in the enteric nervous system and in interstitial cells of Cajal in the canine, mouse and human jejunum. Carbon monoxide is considered a non-adrenergic and non-cholinergic inhibitory neurotransmitter and it establishes and maintains the resting membrane potential in the stomach and small intestine. The aim of this study was to determine the distribution of heme-oxygenase-2 in the enteric nervous system of the pig jejunum. Heme-oxygenase-2 immunoreactivity was found in neurons of myenteric ganglia and in nerve fibers in the circular and longitudinal muscle layers. These results suggest that carbon monoxide is produced in the enteric nervous system of the pig jejunum and might mediate inhibitory neural activity in myenteric ganglia and inhibitory neural input to smooth muscle cells in the circular and longitudinal muscle layers.

Keywords

enteric nervous system; heme-oxygenase-2; smooth muscle; non-adrenergic and non-cholinergic; pig jejunum

INTRODUCTION

Heme oxygenase (HO) generates carbon monoxide (CO), biliverdin and iron (Baranano and Snyder, 2001; Baranano et al., 2001). Three isoforms of HO - HO-1, HO-2 and HO-3 - have been identified. The constitutive isoform HO-2 appears to be responsible for CO synthesis in the gastrointestinal tract (Baranano and Snyder, 2001; Baranano et al., 2001) and its expression has been detected in enteric nerves in several species (reviewed in Gibbons and Szurszewski, 2004).

CO has been reported to hyperpolarize and relax circular smooth muscle of the small intestine in dog, human and mouse, internal anal sphincter circular smooth muscle in the opossum and circular smooth muscle of the lower esophageal sphincter and jejunum in pig by modulating a cGMP-dependent delayed rectifier K^+ current (Colpaert et al., 2002; Farrugia et al., 1993; Farrugia et al., 1998; Farrugia et al., 2003; Rattan and Chakder, 2000; Zakhary et al., 1997).

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CO has also been described in the mouse and opossum gastrointestinal tract as a non-adrenergic and non-cholinergic (NANC) inhibitory neurotransmitter (Farrugia et al., 1993; Rattan and Chakder, 1993) and in mouse, dog and human intestine as a hyperpolarizing agent that establishes and maintains the resting membrane potential (Farrugia et al., 2003; Sha et al., 2007).

The objective of the present study was to demonstrate by immunohistochemistry whether HO-2 is present in enteric nerves in pig jejunum using protein gene product (PGP) 9.5 as a marker of enteric neuronal network (Miller et al., 2001) and thus to determine whether CO might be a candidate gastrointestinal NANC transmitter in this species. Jejunum was chosen because it is often used to study the action of neurotransmitters in the gastrointestinal tract.

MATERIAL AND METHOD

Sample tissues were obtained from four adult pigs of either sex anesthetized with thiopental sodium following procedures approved by the Animal Care and Use Committee of the Mayo Clinic. After the abdomen was opened, a segment of 10 cm of jejunum ~20 cm distal to the ligament of Treitz was collected and placed in oxygenated Krebs solution at room temperature. A piece of jejunum measuring ~1 × 1 cm, containing the entire thickness of the intestinal wall was cut and immersed in freshly prepared 4% paraformaldehyde fixative overnight at 4 °C. The tissues were rinsed thoroughly in phosphate-buffered saline (PBS; 0.1 mol/L; pH 7.4), immersed overnight at 4 °C in PBS with 30% su crose, and frozen in isopentane at −40 to −50 °C. Cryostat sections, 12– 20 μm thick, were cut, thaw-mounted on gelatin–chrome alum-coated glass slides and air-dried. Approximately 20 sections were prepared from each tissue sample. Alternate sections were selected for immunolabelling. Methods and antibodies used for HO-2 and PGP 9.5 immunohistochemistry were as previously described by Farrugia et al., (1998) and Miller et al., (2001). Briefly, tissue sections were incubated in PBS containing 0.3% Triton X-100 and 10% normal donkey serum (NDS; Jackson Immuno Research Lab, Inc., West Grove, PA) in a humid chamber at room temperature for 60 min and then in rabbit polyclonal antiserum raised against HO-2 (StressGen Biochemicals, Victoria, Canada) or PGP 9.5 (Biogenesis, Kingston, NH) diluted 1:1000 in 5% NDS overnight at 4 °C. Sections were then incubated for 60-90 min at room temperature with CY3-labelled donkey anti-rabbit IgG (Jackson Immuno Research Lab, Inc.) diluted 1:100 in 2.5% NDS to visualize sites of HO-2 or PGP 9.5 immunoreactivity. Sections were washed thoroughly between each step using PBS. Sections were coverslipped in antifade mounting medium (InVitrogen Molecular Probes, Eugene, OR) and examined by epifluorescence (Zeiss Axiophot) or laser scanning confocal microscopy (Zeiss LSM 510; Zeiss, Thornwood, NY). In control experiments, no immunoreactivity was detected in sections incubated with only secondary antibody or when the primary antibodies were replaced with normal serum at the same dilution. The specificity of the HO-2 antibody has been previously demonstrated (Farrugia et al., 1998; Porcher et al., 1999).

For double HO-2 and PGP 9.5 immunolabeling experiments, tissue sections were first incubated for 1 h in PBS containing 10% normal goat serum (NGS; Jackson Immuno Research Lab, Inc.) and 0.3% Triton X-100 and then for 12 h at 4 °C in a mixture of HO-2 and PGP 9.5 antisera (guinea pig anti-PGP 9.5, Novus Biological Inc., Littleton, CO and rabbit anti-human HO-2, kind gift from Dr. Snyder, Johns Hopkins University, Baltimore, MA) diluted 1:500 and 1:1000, respectively in PBS containing 0.3% Triton X-100 and 5% NGS. After several rinses with PBS for 1 hour, the sections were incubated for 3 h at room temperature in a mixture of secondary antibodies (CY3 conjugated goat anti-guinea pig and FITC conjugated goat anti-rabbit, Jackson Immuno Research Lab, Inc.), both diluted 1:800 with PBS containing 2.5% NGS and 0.3% Triton X-100. Tissue sections were finally coverslipped in antifade mounting medium and examined by epifluorescence microscopy.

RESULTS

HO-2 immunoreactivity was localized in neuronal bodies in myenteric ganglia between the longitudinal and circular smooth muscle layers, in nerve-like fibers in the circular and longitudinal smooth muscle layers of pig jejunum (Figure 1). Figure 2 illustrates the distribution of PGP 9.5 immunopositivity in myenteric ganglia, long nerve fibers in the circular muscle layer and cross sections of nerve fibers in the longitudinal muscle layer similar to the distribution of HO-2 immunolabelling seen in Figure 1. Figure 3 illustrates that HO-2 and PGP 9.5 immunolabelling were co-localized in myenteric ganglion neurons.

DISCUSSION

In the gastrointestinal tract of several species including, mouse (Zakhary et al., 1996, 1997; Miller et al. 1998; Xue et al., 2000), rat (Donat et al., 1999), cat (Ny et al. 1996), dog (Farrugia et al., 1998), opossum (Battish et al., 2000), pig (Werkstrom et al., 1997; Colpaert et al. 2002) and humans (Porcher et al., 1999; Miller et al. 2001; Piotrowska et al., 2003) the CO producing enzyme, HO-2 is found in myenteric neurons and interstitial cells of Cajal. CO relaxes and hyperpolarizes gastrointestinal smooth muscle in different regions of the gastrointestinal tract, such as the lower esophageal sphincter, gastric fundus, jejunum and internal anal sphincter (Zahkary et al., 1997; Farrugia et al., 1998; Rattan and Chakder, 2000; Xue et al., 2000; Colpaert et al., 2002; Matsuda et al., 2004). CO plays a role in neurally-mediated nonadrenergic noncholinergic (NANC) relaxation of mouse ileum (Zakhary et al., 1997) and opossum internal anal sphincter (Rattan and Chakder, 2000) and in NANC hyperpolarization and relaxation of mouse jejunum (Xue et al., 2000). However, a previous study showed that CO was not a likely inhibitory mediator of neurotransmission in the pig jejunum (Matsuda et al., 2004).

Recently it has been suggested that endogenously generated CO, through the action of HO-2 in interstitial cells of Cajal, establishes and maintains the resting membrane potential gradient across the circular muscle layer in mouse, dog and human jejunum (Farrugia et al., 2003; Sha et al., 2007). Support for this concept has come from experiments using the HO-2 knockout mouse which showed that the gradient in resting membrane potential is abolished and that gastrointestinal motor activity is disordered (Xue et al., 2000; Zakhary et al., 1997) In humans, loss or damage to enteric neurons or interstitial cells of Cajal or both has been demonstrated in patients with diabetic gastrointestinal disorder, intestinal constipation and in Chagas' disease of the colon (Adachi et al., 2008; Hagger et al., 2000; Lyford et al., 2002; Miller et al., 2008; Nakahara et al., 2002; Yu et al., 2002).

In summary, the present study shows that HO-2 is present in the enteric nervous system in the pig jejunum. Since CO does not appear to function as an inhibitory mediator of neural transmission in pig jejunum (Matsuda et al., 2004), we suggest that endogenously generated CO released from enteric neurons may help to establish and maintain a resting membrane potential gradient across the circular muscle layer as it does when it is released from the interstitial cells of Cajal in the dog stomach and in the dog, mouse and human jejunum. Further studies including direct measurement by intracellular recording of the resting membrane potential gradient across the circular muscle layer is necessary to test this hypothesis.

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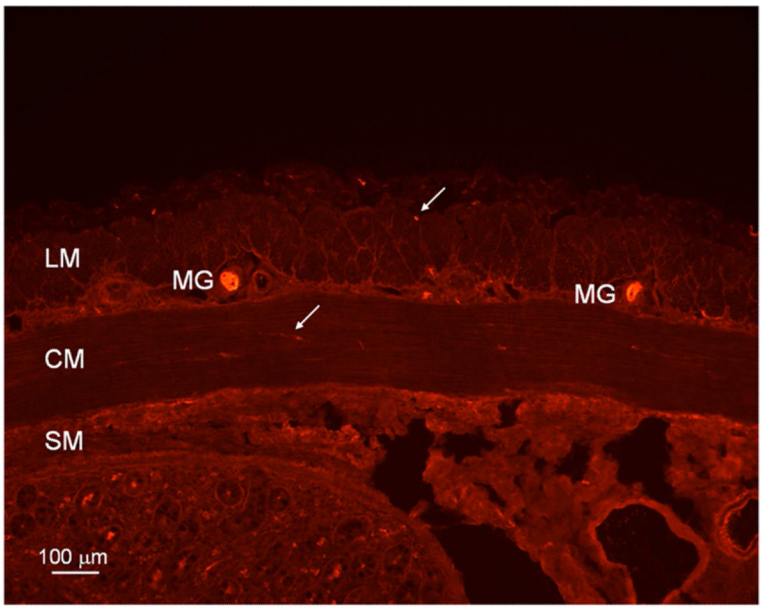

Figure 1.
Heme-oxygenase-2 (HO-2) imunoreactivity in pig jejunum. HO-2 imunoreactivity is localized in neuronal bodies in myenteric ganglia (MG), in varicose nerves fibers (arrow) in circular muscle layer (CM) and cross section of nerve fibers (arrow) in longitudinal muscle layer (LM). Bar: 100 μm; SM: submucosa.

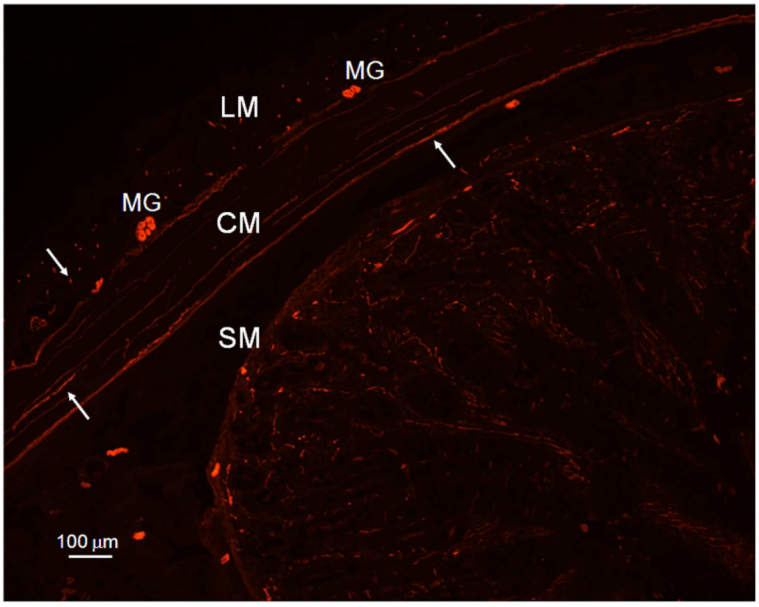

Figure 2.
Immunolocalization of PGP 9.5, a neuronal marker, reveals myenteric ganglia (MG), long nerve fibers (arrow) in the circular muscle layer (CM) and cross sections of nerve fibers (arrow) in the longitudinal muscle layer (LM) in the pig jejunum. Bar: 100 μm; SM: submucosa.

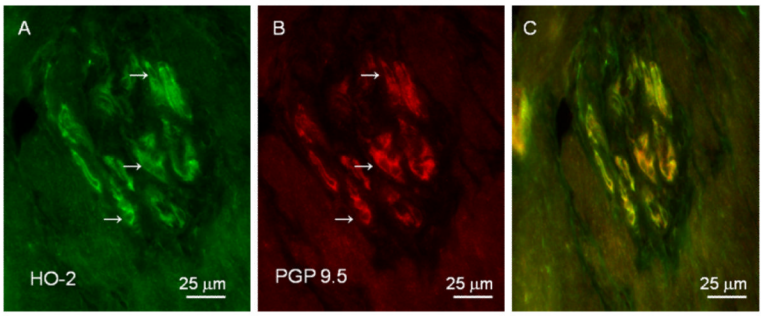

Figure 3.
Double immunolabeling for HO-2 and PGP 9.5 in pig jejunum. HO-2 (A) and PGP 9.5 (B) are co-localized (C) in myenteric ganglion neurons (arrows). Bar: 25 μm.