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Investigation of the potential modulatory effect of biliverdin, carbon monoxide and bilirubin on nitrergic neurotransmission in the pig gastric fundus

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Abstract

In porcine gastric fundus, we have investigated the colocalization of the bile pigment biosynthetic enzymes heme oxygenase-2 and biliverdin reductase with neuronal nitric oxide synthase (nNOS), the effect of carbon monoxide (CO) on fundic circular smooth muscle and the possible modulatory effect of the bile pigments biliverdin and bilirubin on CO-mediated relaxations and on nitrergic relaxation. Heme oxygenase-2 and biliverdin reductase immunoreactivity was present in all nNOS containing myenteric neurons. CO induced a concentration-dependent relaxation of fundic circular smooth muscle strips, which was completely blocked by the specific guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ). 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), biliverdin and bilirubin strongly enhanced the amplitude of the CO-induced relaxation. Tin protoporphyrin had no effect on electrically induced nitrergic relaxation, but spectrophotometric analysis learned that incubation of porcine gastric fundus circular muscle strips with tin protoporphyrin did not influence heme oxygenase activity. In conclusion, our data suggest that nitrergic neurons in the pig gastric fundus are able to produce biliverdin and bilirubin, and that these agents potentiate the relaxant effect of CO, which is formed concomitantly with biliverdin by heme oxygenase-2.

Keywords: Heme oxygenase-2; Biliverdin reductase; Bile pigment; Carbon monoxide; Nitrergic relaxation; Gastric fundus, pig

1. Introduction

It is now well known that the main endogenous source for production of carbon monoxide (CO) is through the oxidative cleavage of heme by the enzyme heme oxygenase (E.C.1.14.99.3) (Maines, 1988, 1993). Similar to nitric oxide synthase (NOS), the heme oxygenase family consists of three distinct isozymes: a stress-inducible heme oxygenase-1, which is upregulated by a variety of different stimuli (Otterbein and Choi, 2000); the constitutive heme oxygenase-2, which is expressed under normal physiologic conditions (Maines, 1997); and a newly identified heme oxygenase-3, which displays poor heme catalytic activity but may have a regulatory role in heme-dependent cellular processes (McCoubrey et al., 1997). Evidence has been gathered that CO might function as a messenger molecule in signal transduction in both the central and peripheral nervous systems (Maines, 1993; Rattan and Chakder, 1993; Dawson and Snyder, 1994).

Although the exact role of CO in the gastrointestinal smooth muscle control remains elusive, it is well established that CO causes direct smooth muscle relaxation in a number of preparations including the internal anal sphincter (Rattan and Chakder, 1993), the lower oesophageal sphincter (Ny et al., 1996) and the jejunum (Farrugia et al., 1998). The involvement of the heme oxygenase/CO pathway in gastrointestinal motility control is further corroborated by a number of experimental findings: myenteric neurons immunoreactive for heme oxygenase-2 are present in several gastrointestinal tissues (Ny et al., 1996; Farrugia et al., 1998; Porcher et al., 1999), while in heme oxygenase-2 knockout mice a strong reduction in the non-adrenergic non-cholinergic (NANC) relaxation and in the hyperpolarization

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of gastrointestinal muscle was observed (Zakhary et al., 1997; Xue et al., 2000).

Metabolism of heme by heme oxygenase leads, in addition to CO, to the concomitant generation of free iron and biliverdin. The bile pigment biliverdin can subsequently be reduced by the enzyme biliverdin reductase (E.C.1.3.1.24) to another bile pigment, i.e. bilirubin (Maines, 1997). Whereas bilirubin was usually thought of as a waste product of heme metabolism, recent evidence highlights the various antioxidant and modulatory properties it possesses. Indeed, bilirubin is a gifted player in the field of radical biology: prevention of the oxidation of polyunsaturated fatty acids (Stocker et al., 1987), inhibition of α-tocopherol consumption by reducing its chromanoxyl radical (Stocker and Peterhans, 1989), prevention of peroxynitrite-mediated protein oxidation (Minetti et al., 1998) and scavenging of superoxide anions (Robertson and Fridovich, 1982) are amongst the antioxidant effects which have been attributed to it. Administration of bilirubin to rats modulates lipopolysaccharide-induced E- and P-selectin expression in the vascular system, providing support that bilirubin is able to modulate this inflammatory response (Vachharajani et al., 2000). Cytoprotective properties in a model of ischemic heart injury (Clark et al., 2000) and in a model of hydrogen peroxide-induced oxidative injury to neurons (Doré et al., 1999) also suggest that bilirubin is more than an end product of heme oxygenase activity that needs to be eliminated.

In the pig gastric fundus, nitric oxide (NO) is the main mediator of NANC inhibitory neurotransmission (Lefebvre et al., 1995) which enables relaxation of this part of the stomach during food intake. Superoxide anions, produced by all aerobic cells during mitochondrial respiration and other normal metabolic cell processes, can chemically react with NO and reduce its biological activity. In a previous study on the effect of antioxidant depletion on nitrergic neurotransmission in the pig gastric fundus, inhibition of the endogenous antioxidant enzyme Cu^{2+}/Zn^{2+} superoxide dismutase rendered the relaxation of circular smooth muscle strips elicited by electrical stimulation of nitrergic nerves sensitive to superoxide anions generated by 6-anilino-5,8-quinolinedione (LY83583) (Colpaert et al., 2002b). Besides Cu^{2+}/Zn^{2+} superoxide dismutase other tissue antioxidants might also be involved in the protection of free radical NO as nitrergic neurotransmitter in the pig gastric fundus (Colpaert and Lefebvre, 2002): of these bilirubin seems to be an eligible candidate since it can interact with various NO-inactivating species and, moreover, was shown to partially protect the relaxation induced by exogenously administered NO against superoxide attack by LY83583 (Colpaert and Lefebvre, 2000). Finally, colocalization of heme oxygenase-2 and neuronal nitric oxide synthase (nNOS) is demonstrated in different gastrointestinal regions (Ny et al., 1997; Donat et al., 1999; Battish et al., 2000).

One might hypothesize from these data that bilirubin can modulate the NO-signaling pathway in pig gastric fundus provided that biliverdin reductase is also present and can reduce heme oxygenase-2 formed biliverdin into bilirubin. The incomplete inhibition exerted by nNOS-inhibitors on electrically induced relaxations in NANC conditions in porcine gastric fundus circular smooth muscle strips (Dick and Lefebvre, 1997), suggests that other neurotransmitters such as CO might have a role in the inhibitory control of mechanical activity in this tissue. The aim of the present study is to investigate the possible contribution of CO and/or bilirubin to NANC neurotransmission in the pig gastric fundus by examining the influence of the heme oxygenase inhibitor tin protoporphyrin. The colocalization of heme oxygenase-2 and biliverdin reductase with nNOS was investigated as well as the relaxant effect of exogenous CO.

2. Material and methods

2.1. Preparation of smooth muscle strips

Experiments were carried out on isolated circular smooth muscle strips of the porcine gastric fundus. The stomach was removed from healthy 6-month-old male castrated pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution. After the mucosa was removed, strips (15 × 3 mm) were cut from the fundus in the direction of the circular muscle layer. All tissues were used immediately. Strips were mounted vertically between two platinum plate electrodes under a load of 2 g in organ baths, containing physiological salt solution at 37 °C and gassed with 95% O_2/5% CO_2. The composition of the physiological salt solution was (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 124.1, HCO_3^- 25, $H_2PO_4^-$ 1.2 and glucose 11.5 (Mandrek and Milenov, 1991). The Krebs solution always contained 10^{-6} M atropine and 4×10^{-6} M guanethidine to inhibit cholinergic and noradrenergic responses. Changes in length were recorded via isotonic transducers (T3, Palmer Bioscience) on a recorder (FWR3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Graphtec). Electrical field stimulation (40 V, 0.1 ms, 0.5–16 Hz, 10 s) was applied by means of a stimulator (S88, Grass). The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment.

2.2. Protocols for functional experiments

After the equilibration period, all strips were first contracted with 3×10^{-7} M 5-hydroxytryptamine (5-HT) and subsequently relaxed by 10^{-5} M sodium nitroprusside; tissues were then washed at 15 min interval for 1 h before continuing the experiment.

A first set of experiments was performed to study the effect of the heme oxygenase inhibitor tin protoporphyrin on relaxations induced by electrical field stimulation (40 V, 0.1

ms, 0.5–16 Hz, 10 s with an interval of 5 min between each 10 s train), boli of exogenous NO (2×10^{-6}, 10^{-5} and 10^{-4} M with an interval of 5 min in between), vasoactive intestinal polypeptide (VIP, 10^{-7} M) or isoprenaline (10^{-5} M) using the following protocol. Tone was raised again with 3×10^{-7} M 5-HT and when a stable plateau contraction was obtained, the different types of relaxant stimuli were applied (only one type of stimulus was tested per strip). After repetitive rinsing for 30 min, tin protoporphyrin (10^{-6}–10^{-4} M to study the effect on electrical field stimulation; 10^{-6}–5×10^{-5} M to investigate the effect on NO and VIP; 5×10^{-5} M for isoprenaline) was added into the organ bath and left in contact with the experimental tissue for another 30 min. The preparations were again contracted with 5-HT (3×10^{-7} M) and when the contraction amplitude was stable, the relaxant stimuli were studied for the second time. In parallel time control strips, the solvent of tin protoporphyrin {dimethylsulphoxide (DMSO)} was administered. The influence of tin protoporphyrin (5×10^{-5} M) on the effect of VIP (10^{-7} M) was also studied in the presence of 3×10^{-4} M L-N^G-nitroarginine methyl ester (L-NAME).

In a second series of investigations the influence of exogenously applied carbon monoxide (CO) on the porcine gastric fundus circular smooth muscle strips was studied. Tone was raised by administration of 3×10^{-7} M 5-HT. Once a stable contraction was obtained, exogenous CO was administered in a cumulative way (10^{-5}–10^{-3} M). To study the influence of 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ, 10^{-5} M), 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1, 3×10^{-5} M), biliverdin (2×10^{-4} M) and bilirubin (2×10^{-4} M) upon these relaxations, a protocol similar to the one used in the tin protoporphyrin series was followed. CO (10^{-5}–10^{-3} M) was given cumulatively on top of a second 5-HT-induced contraction and, after washout, administered once again 1 h later on top of a third 5-HT-induced plateau; ODQ (10^{-5} M) was added 15 min before this third 5-HT-induced contraction, while YC-1 (3×10^{-5} M), biliverdin (2×10^{-4} M) or bilirubin (2×10^{-4} M) were given on top of this third 5-HT-induced contraction 5 min before the addition of CO. Each preparation was exposed to only one treatment, and parallel experiments with the solvents were run as controls.

In all protocols studying the influence of tin protoporphyrin, biliverdin or bilirubin, the organ baths were covered with aluminium foil to avoid light exposure.

2.3. Immunohistochemistry

For immunohistochemistry, tissue of the gastric fundus was obtained from 6-week-old domestic pigs that were euthanised by an overdose of pentobarbital (50 mg kg^{-1}) intravenously. A fundic area (± 4 cm^2) was dissected out along the major curvature and fixed for 2 h at room temperature in freshly prepared paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The tissues were then rinsed thoroughly in PBS, immersed overnight at 4 °C in 30% sucrose in PBS, embedded in Tissue-Tek OCT compound (Miles) and cut into 12 μm thick cryostat sections which were mounted on chrome alum-gelatine-coated glass slides. For double immunostaining for heme oxygenase-2 or biliverdin reductase and neuronal nitric oxide synthase (nNOS), the cryosections were preincubated with a blocking mixture containing 0.25% Triton X-100, 1% bovine serum albumin and 10% normal goat serum in PBS (30 min, room temperature). They were then simultaneously incubated in a rabbit polyclonal heme oxygenase-2 antiserum (OSA-200; diluted 1:400; Stressgen Biotechnologies) or a rabbit polyclonal biliverdin reductase antiserum (OSA-400; diluted 1:100; Stressgen) and a mouse monoclonal nNOS antiserum (diluted 1:2000; Sigma) overnight at room temperature in a humid chamber. Visualization of the primary antisera was achieved by immersion in biotinylated goat anti-rabbit antibody (diluted 1:500; Sigma) and CY3-labelled goat anti-mouse antibody (diluted 1:1000; Jackson Immunoresearch Laboratories) for 2 h followed by a 2-h incubation period with fluorescein isothiocyanate (FITC)-conjugated streptavidin (diluted 1:500; Jackson Immunoresearch Laboratories). Preparations were coverslipped with Vectashield (Vector Labs) and examined under a Zeiss Axiophot fluorescence microscope equipped with the appropriate filter set.

2.4. Heme oxygenase activity assay

Porcine gastric fundus strips from 6-month-old male castrated pigs were incubated in physiological salt solution with tin protoporphyrin (10^{-4} M) for 45 min; control strips coming from the same experimental animal were incubated in parallel in the absence of tin protoporphyrin. The strips were subsequently blot-dried on filter paper, weighed, frozen in liquid nitrogen and homogenized with a micro-dismembrator (B. Braun) for 60 s. The pulverized material was dissolved in five volumes of ice-cold 0.02 M Tris–HCl/0.25 M sucrose buffer (pH 7.4) containing protease inhibitors and centrifuged at $19\,000 \times g$ for 10 min at 4 °C; the supernatant was removed and centrifuged again at $105\,000 \times g$ for 60 min at 4 °C. Finally, the microsomal pellet was resuspended in 0.5 ml of 0.02 M Tris–HCl/0.25 M sucrose buffer (pH 7.4). Heme oxygenase activity was determined by a spectrophotometric assay that measures the formation of bilirubin as a difference in absorbance between 464 and 530 nm (extinction coefficient for bilirubin 40 mM^{-1} cm^{-1}) (Motterlini et al., 1996). Briefly, the heme oxygenase reaction mixture is composed of the following final concentrations: 0.4 mg/ml fresh microsomal protein, 1 mM β-NADPH, 2 mM glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 1 mM $MgCl_2$, 400 ng/ml purified rat liver biliverdin reductase (a kind gift of Dr. Mahin Maines),

25 μM hemin, 0.25 M sucrose and 20 mM Tris–HCl in a final volume of 1 ml (pH 7.4). The reaction mixture was incubated at 37 °C in the dark for 60 min. Heme oxygenase activity was also determined when tin protoporphyrin (10^{-4} M) was directly added to the heme oxygenase reaction mixture. The reaction mixture without the NADPH-generating system served as a blank. Heme oxygenase activity was expressed as amount of bilirubin formed per mg protein per hour. The protein concentration was determined by the method of Bradford (1976).

2.5. Data analysis

Relaxations are expressed as percentage of the relaxation induced by 10^{-5} M sodium nitroprusside at the beginning of the experiment. Responses in the presence of interfering drugs are related to those obtained before administration of these drugs. Experimental data are expressed as means ± S.E.M. and *n* refers to the number of strips from different animals. Results within tissues are compared by a paired *t*-test and results between tissues with an unpaired *t*-test; a difference is considered statistically significant at $P < 0.05$.

2.6. Chemicals

The following drugs were used: bilirubin ditaurate from Calbiochem; vasoactive intestinal polypeptide (VIP) from Bachem; isoprenaline from Abbott; 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) and tin protoporhyrin IX dichloride from Tocris Cookson; biliverdin hydrochloride from ICN biomedicals; 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) from Alexis Biochemicals; hemin from Affiniti Research Products; protease inhibitor cocktail tablets from Boehringer Mannheim; 5-hydroxytryptamine creatinine monosulphate from Janssen Chimica; bovine serum albumin, dimethylsulphoxide (DMSO), glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, β-NADPH, L-N^G-nitroarginine methyl ester (L-NAME), normal goat serum, paraformaldehyde, pentobarbital, sodium nitroprusside, sucrose and Triton X-100 from Sigma. The purified biliverdin reductase enzyme was kindly donated by Dr. Mahin D. Maines (Department of Biophysics and Environmental Medicine, University of Rochester School of Medicine, NY, USA). Drugs were dissolved in deionized water except ODQ which was dissolved in 100% ethanol, biliverdin that was dissolved in 0.2% NaOH, and YC-1 and

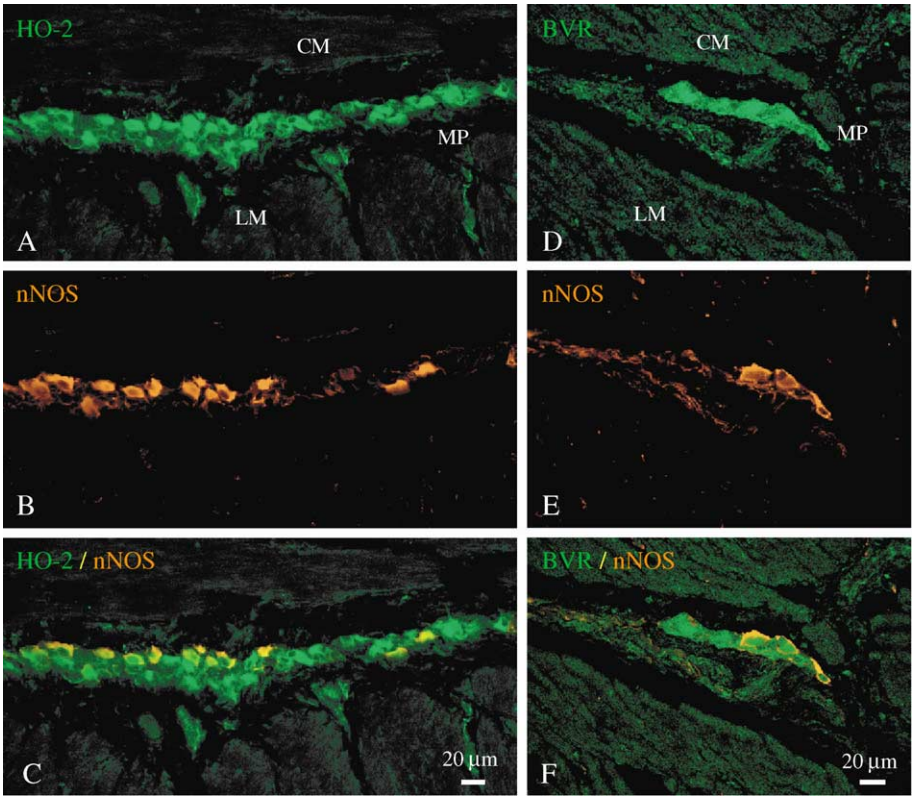

Fig. 1. Heme oxygenase-2 (A) and biliverdin reductase (D) immunoreactivity (green FITC-fluorescence) found in neurons of the myenteric plexus (MP) lying between the circular muscle (CM) and longitudinal muscle (LM) layers of porcine gastric fundus. In (B) and (E), the red CY3-fluorescence indicates the staining for neuronal nitric oxide synthase (nNOS) in the same cryostat sections as in (A) and (D). The double exposure photomicrographs (=combined fluorescence; C and F) clearly demonstrate that all nitrergic neurons colocalize for both heme oxygenase-2 and biliverdin reductase.

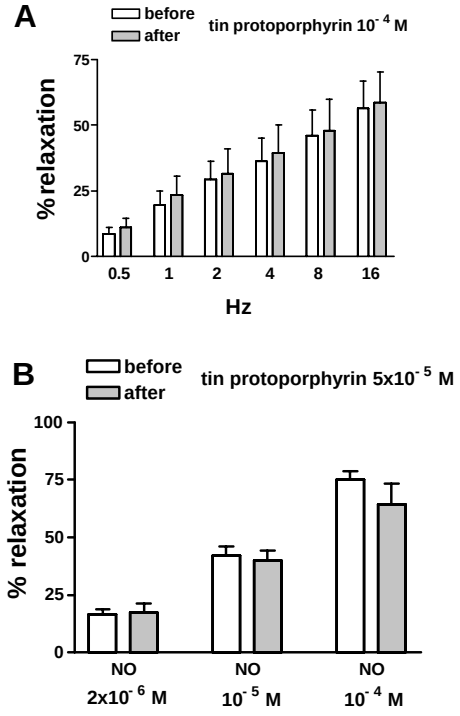

Fig. 2. Relaxant responses of circular smooth muscle strips of porcine gastric fundus induced by electrical field stimulation (0.5–16 Hz) (A) or exogenous nitric oxide (NO; 2×10^{-6}–10^{-4} M) (B) before and after addition of 10^{-4} M (A) or 5×10^{-5} M (B) tin protoporphyrin. Relaxations are expressed as percentage of the relaxation induced by sodium nitroprusside at the beginning of the experiment. Values are means ± S.E.M. of $n = 6$–7.

tin protoporphyrin which were dissolved in dimethylsulphoxide (DMSO). All solutions were prepared on the day of the experiment and solvents themselves were without significant effect at the concentrations used in the experiments.

Saturated CO and NO solutions were prepared from gas (Air Liquide, Belgium), as described by Rattan and Chakder (1993) and Kelm and Schrader (1990), respectively, yielding a vial containing CO or NO in a concentration taken to be 2×10^{-3} M.

3. Results

3.1. Double immunolabelling for nNOS and heme oxygenase-2 or biliverdin reductase

We have described before the distribution of heme oxygenase-2 and biliverdin reductase in porcine gastric fundus (Colpaert et al., 2002a). Heme oxygenase-2 protein is present in mucosal epithelial cells, in the endothelium of intramural blood vessels, and in a subpopulation of interstitial cells of Cajal. Heme oxygenase-2 immunoreactivity is further confined to intrinsic neurons of both the submucous and myenteric plexus, while in nerve fibers running through the circular and longitudinal muscle layers, heme oxygenase-2 positive staining is rare. Biliverdin reductase immunostaining showed an identical distribution pattern as heme oxygenase-2.

We now double labelled porcine gastric fundus cryosections with an antibody specific for heme oxygenase-2 and another specific for nNOS; this revealed that all myenteric neurons which expressed the nNOS protein also showed immunofluorescence for heme oxygenase-2 (Fig. 1A–C). Although nNOS positive nerve fibers were frequently observed in the circular and longitudinal smooth muscle layers, no co-staining for heme oxygenase-2 was found. Very similar results were obtained when double labelling with the biliverdin reductase and nNOS antibody; again, all

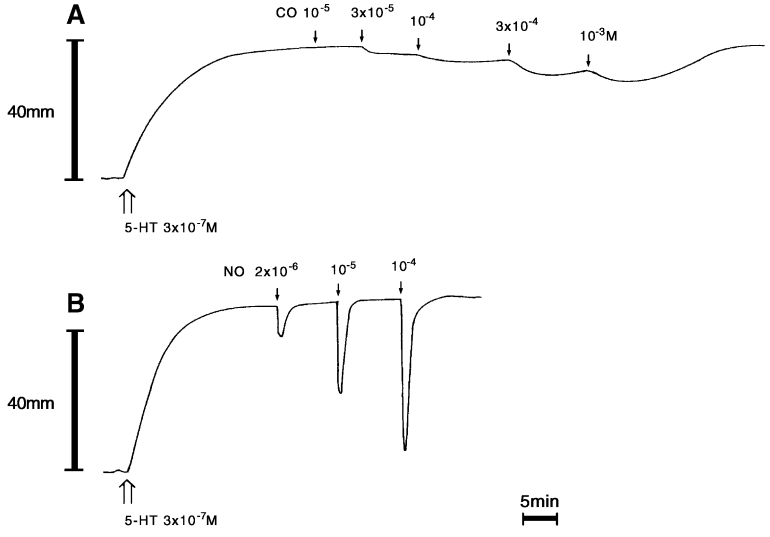

Fig. 3. In (A) a representative tracing is shown demonstrating the effect of exogenous carbon monoxide (CO) administered in a cumulative way (10^{-5}–10^{-3} M) on circular smooth muscle strips of porcine gastric fundus precontracted with 3×10^{-7} M 5-hydroxytryptamine (5-HT). Similarly, the bottom tracing (B) shows the effect of 2×10^{-6}, 10^{-5} and 10^{-4} M of exogenous nitric oxide (NO) administered at 5 min intervals.

nNOS immunoreactive neurons contained the biliverdin reductase protein (Fig. 1D–F). In contrast to the findings with heme oxygenase-2, some nerve fibers were detected expressing both nNOS and biliverdin reductase.

3.2. Influence of tin protoporphyrin on relaxations induced by electrical field stimulation, NO, VIP and isoprenaline

Train stimulation with 10 s trains at 5 min intervals in the presence of atropine and guanethidine induced short-lasting frequency-dependent relaxations of the porcine gastric fundus circular smooth muscle strips. At a frequency of 0.5 Hz, the amplitude of the relaxation was 8.8 ± 2.4% ($n = 6$) of the relaxation induced by sodium nitroprusside; at 16 Hz, the amplitude was 56.5 ± 10.3% ($n = 6$). These relaxations were reproducible in the presence of DMSO, the solvent of tin protoporphyrin. Tin protoporphyrin, 10^{-6}, 10^{-5}, 5×10^{-5} and 10^{-4} M ($n = 6$ for each concentration) did not influence electrically induced relaxations as illustrated for 10^{-4} M in Fig. 2A.

Exogenous NO (2×10^{-6}–10^{-4} M) induced concentration-dependent short-lasting relaxations (Fig. 3B). At 10^{-4} M, the amplitude was 75.0 ± 3.7% of the relaxation induced by sodium nitroprusside ($n = 6$). These relaxations were reproducible and were not influenced by tin protoporphyrin (10^{-6}–5×10^{-5} M) (data shown for tin protoporphyrin 5×10^{-5} M in Fig. 2B). VIP (10^{-7} M) induced a relaxation, that developed at a slow rate and was sustained (maximal amplitude = 105.0 ± 3.5% of the relaxation induced by sodium nitroprusside, $n = 7$). In the presence of 5×10^{-5} M tin protoporphyrin, the response to VIP was significantly decreased to 78.9 ± 7.4% of that before tin

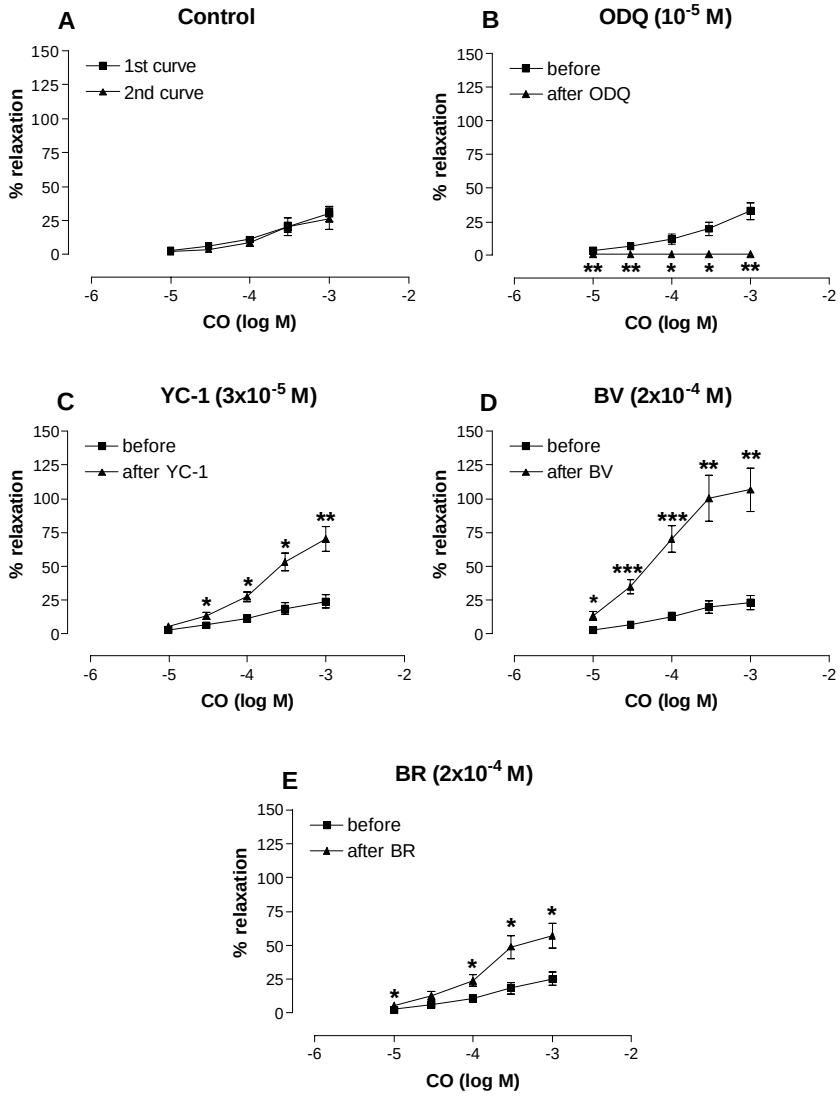

Fig. 4. Relaxant responses of circular smooth muscle strips of porcine gastric fundus induced by CO before and after addition of ODQ (10^{-5} M, B), YC-1 (3×10^{-5} M, C), biliverdin (BV; 2×10^{-4} M, D) and bilirubin (BR; 2×10^{-4} M, E). In (A), the reproducibility of the response to CO is shown. Relaxations are expressed as percentage of the relaxation induced by sodium nitroprusside at the beginning of the experiment. Values are means ± S.E.M. of $n = 6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: significantly different from the response before addition (paired t-test).

protoporphyrin ($n=7$; $P<0.05$). Co-incubation of tin protoporphyrin with L-N^G-nitroarginine methyl ester (L-NAME, 3×10^{-4} M) did not modify this inhibitory effect of tin protoporphyrin upon the VIP-induced relaxation (data not shown). The β-adrenoceptor agonist isoprenaline (10^{-5} M) induced a slowly developing relaxation which was not sustained (the amplitude of relaxation was $42.7\pm6.6\%$ of the relaxation induced by sodium nitroprusside); this was not influenced by 5×10^{-5} M tin protoporphyrin ($n=4$).

3.3. Heme oxygenase activity assay

The heme oxygenase activity, indirectly measured as biliverdin derived bilirubin formation by biliverdin reductase, in tissue homogenates from porcine gastric fundus strips which were incubated for 45 min in physiological salt solution was 1453 ± 392 pmol bilirubin mg^{-1} protein h^{-1} ($n=4$). Incubation of the smooth muscle strips with tin protoporphyrin (10^{-4} M) did not result in a decrease of heme oxygenase activity: in homogenates of these tin protoporphyrin-pretreated strips, the rate of bilirubin production was 1588 ± 315 pmol bilirubin mg^{-1} protein h^{-1} ($n=4$). However, the rate of bilirubin production in tissue homogenates from porcine gastric fundus strips decreased from 1355 ± 139 pmol bilirubin mg^{-1} protein h^{-1} ($n=6$) to an undetectable level, when tin protoporphyrin (10^{-4} M) was directly added to the heme oxygenase reaction mixture.

3.4. Porcine gastric fundus smooth muscle relaxation by CO

Addition of exogenous CO caused a concentration-dependent long-lasting relaxation of gastric fundus circular smooth muscle strips which became evident starting from a concentration of 3×10^{-5} M CO (Fig. 3A).

The characteristics of the CO-induced relaxation differed from those of the NO-induced relaxation (Fig. 3A and B): (1) the rate of development of relaxation was markedly slower for CO than for NO; (2) the potency of CO to relax the porcine gastric fundus smooth muscle was far less than that of NO since 2×10^{-6} M NO already induced a clearcut relaxation, while CO only induced relaxation from 3×10^{-5} M onwards; (3) the duration of the CO-mediated relaxant responses was more prolonged than those obtained with NO.

3.5. Effect of ODQ, YC-1, biliverdin and bilirubin on CO-mediated relaxation

Tissues, which had been regularly rinsed after a first application of CO (10^{-5}–10^{-3} M), responded to 5-HT (3×10^{-7} M) in a reproducible manner. The amplitude of relaxation to exogenous CO (10^{-5}–10^{-3} M) was well maintained in the time control strips (Fig. 4A) and in strips receiving the solvents of interfering agents. When the strips were incubated with the selective guanylate cyclase inhibitor ODQ (10^{-5} M), the relaxant effect of CO (10^{-5}–10^{-3} M) was completely antagonized (Fig. 4B).

YC-1 (3×10^{-5} M) induced a small fall in tone when given on top of the 5-HT-induced contraction; in its presence, the relaxation to exogenous CO (10^{-5}–10^{-3} M) was significantly enhanced (Fig. 4C), e.g. the amplitude of the relaxation to 10^{-3} M exogenous CO increased from $23.7\pm4.9\%$ before to $70.2\pm9.0\%$ after addition of YC-1 ($P<0.01$; Fig. 4C). Similarly, we observed a significant increase in the magnitude of the relaxant effect of CO (10^{-5}–10^{-3} M) in the presence of either biliverdin (2×10^{-4} M) or bilirubin (2×10^{-4} M). For biliverdin the response to 10^{-3} M exogenous CO was increased from $23.0\pm5.1\%$ to $106.6\pm16.2\%$ ($P<0.01$; Fig. 4D) and for bilirubin from $25.4\pm4.9\%$ to $56.9\pm9.1\%$ ($P<0.05$; Fig. 4E) after their application.

4. Discussion

The present study reports for the first time on the possible colocalization of both heme oxygenase-2 and biliverdin reductase with neuronal nitric oxide synthase (nNOS) in myenteric neurons of a gastrointestinal tissue, more specifically the gastric fundus of pig. Our results obtained via indirect immunohistochemistry demonstrate that all intrinsic nitrergic (i.e. nNOS immunoreactive) neurons of the myenteric plexus are also labelled for heme oxygenase-2 and biliverdin reductase. Since heme oxygenase-2 and biliverdin reductase are the biosynthetic enzymes of respectively biliverdin and bilirubin we can thus conclude that in porcine gastric fundus all nitrergic myenteric neurons possess the biochemical pathway for production of these antioxidant bile pigments, and for the production of CO. The complete colocalization of heme oxygenase-2 with nNOS in the fundic part of the pig stomach differs from the degree of colocalization found by Miller et al. (2001) in the antrum of human stomach, namely 70–80%. A nearly 100% colocalization of heme oxygenase-2 with nNOS has, however, also been detected in the internal anal sphincter region of the opossum (Battish et al., 2000).

To further investigate whether CO and/or bile pigments might have a physiological role in non-adrenergic non-cholinergic (NANC) neurotransmission in porcine gastric fundus, we pretreated our experimental preparations with a competitive inhibitor for the heme oxygenase enzyme, i.e. tin protoporphyrin. Recently, Rattan and Chakder (2000) propagated tin protoporphyrin as the most selective metalloporphyrin inhibiting heme oxygenase activity in the gastrointestinal smooth muscle. However, tin protoporphyrin (in a concentration up to 10^{-4} M) did not alter relaxations of circular smooth muscle strips induced by electrical field stimulation at different frequencies in the presence of atropine and guanethidine. At first sight, these data preclude a role for CO in NANC neurotransmission in the porcine gastric fundus. Moreover, the hypothesis that biliverdin and

bilirubin might protect endogenous free radical NO against superoxide anions by virtue of their antioxidant properties and thus enable free radical NO (released by electrical field stimulation) to function as endogenous nitrergic neurotransmitter, seems to be ruled out by these findings. However, quantitative spectrophotometric analysis learned that tin protoporphyrin is not able to inhibit heme oxygenase activity in the pig gastric fundus. In homogenates of circular smooth muscle strips which were incubated with tin protoporphyrin approximately the same amount of bilirubin (1500 pmol mg^{-1} protein h^{-1}) was formed as in homogenates of untreated control preparations; this value corresponds with previous results obtained in the untreated feline lower oesophageal sphincter (Ny et al., 1996). The non-effect of pretreating strips with tin protoporphyrin on heme oxygenase activity contrasts to the complete inhibition of heme oxygenase activity that was observed when adding tin protoporphyrin directly to the heme oxygenase reaction micture, and points to a problem of transport of the heme oxygenase inhibitor tin protoporphyrin towards its microsomal target protein in the experimental tissues. Moreover, when examining the effect of tin protoporphyrin on circular smooth muscle relaxation elicited by vasoactive intestinal polypeptide (VIP), we observed that tin protoporphyrin significantly attenuated the VIP-induced relaxation of our experimental strips. In contrast, the relaxation induced by isoprenaline, a β-adrenoceptor agonist which also induces smooth muscle relaxation by stimulation of adenylate cyclase, was not influenced by tin protoporphyrin. The non-selective action of tin protoporphyrin on the VIP response seems thus to be situated at the receptor level. This inhibition of VIP-mediated relaxation is a well-recognized side effect of many metalloporphyrins; in smooth muscle strips from the opossum lower oesophageal sphincter, Rattan et al. (1999) concluded that Zn protoporphyrin attenuates VIP-induced relaxation by inhibition of VIP binding to G protein-coupled receptors linked to adenylate cyclase at a point proximal to G protein activation, which thus not requires membrane penetration. Because of this issue of non-selectivity and problematic transmembrane transport of tin protoporphyrin in the porcine gastric fundus, the exact role of CO and of the bile pigments biliverdin and bilirubin in electrically induced nitrergic relaxation can thus not be determined at the present time.

In various species, exogenously applied CO induces smooth muscle relaxation at different parts of the gastrointestinal tract: opossum internal anal sphincter, feline lower oesophageal sphincter and canine jejunum (Rattan and Chakder, 1993; Ny et al., 1996; Farrugia et al., 1998). In porcine stomach, we demonstrate relaxant effects of exogenous CO on fundic circular smooth muscle preparations. This CO-induced relaxation differed from the relaxation obtained by exogenous NO in that the development of relaxation was slower and the relaxation was more prolonged when compared to the NO-induced relaxation; also CO was clearly less potent than NO. The more persistent response to CO can be explained by the fact that CO has a greater stability than NO in solution and therefore its actions on the tissue may persist for a longer period of time.

CO is thought to induce relaxation of smooth muscle by activation of soluble guanylate cyclase and formation of intracellular cGMP (Ramos et al., 1989; Furchgott and Jothianandan, 1991). In addition to this cGMP-dependent mechanism, another proposed mechanism for the action of CO involves the activation of K^+-channels leading to hyperpolarization of cell membranes as was demonstrated in human and canine jejunal smooth muscle (Farrugia et al., 1993, 1998). In our experimental tissues, the relaxant action of CO was completely counteracted by the specific soluble guanylate cyclase inhibitor ODQ. This proves that the soluble guanylate cyclase/cGMP system is the principal if not the only pathway through which exogenous CO evokes relaxations in porcine gastric fundus. As CO is generally believed to be a less potent stimulator of soluble guanylate cyclase than NO (Stone and Marletta, 1994), this corresponds with the lower potency of CO compared to NO in our preparation. In porcine oesophagogastric junction, the CO-induced relaxation was partly mediated by a cGMP-dependent mechanism and partly by activation of high and low conductance Ca^{2+}-activated K^+-channels (Werkström et al., 1997). This might explain the higher potency of CO in this preparation versus the pig gastric fundus; also in feline oesophageal sphincter (Ny et al., 1996) and in opossum internal anal sphincter (Rattan and Chakder, 1993), CO was much more potent in inducing smooth muscle relaxation than in porcine gastric fundus.

Friebe et al. (1996) showed that the benzylindazole derivative YC-1 turns CO into a potent activator of soluble guanylate cyclase: in the presence of YC-1, CO led to a 100-fold increase in enzyme activity which is comparable to the stimulatory effect induced by NO. Results illustrating that YC-1 is an allosteric activator of soluble guanylate cyclase and sensitizes the enzyme towards its activators NO or CO, have been gathered in vascular smooth muscle, platelets and endothelial cells (see Wu et al., 1995; Mulsch et al., 1997; Schmidt et al., 2001). Our study shows that YC-1 enhances the CO-mediated relaxation also in gastrointestinal smooth muscle. The small decrease in tone by YC-1, when given on top of a 5-HT-induced contraction, might be explained by a potentiation of basal soluble guanylate cyclase activation via tonically released NO. NO synthase inhibition indeed consistently induces contraction in pig gastric fundus circular smooth muscle strips (Lefebvre et al., 1995). The relaxant effect of YC-1 might also be due to NO-independent direct soluble guanylate cyclase activation as demonstrated in vascular smooth muscle cells (Mulsch et al., 1997). Also biliverdin and bilirubin enhanced the CO-induced relaxation through a yet unknown mechanism. This is an interesting finding: indeed, biliverdin is generated concomitantly with CO during heme breakdown, while the co-expression of heme oxygenase-2 and biliverdin reductase in porcine gastric fundus enables biliverdin-derived bilirubin forma-

tion. The potentiating effect of the bile pigments biliverdin and bilirubin on the relaxant action of the messenger molecule CO gives further credence to the hypothesis that by-products of heme metabolism might be of significance in signal transduction. The enhancement by bilirubin of relaxations induced by exogenous NO, observed in previous work in porcine gastric fundus (Colpaert and Lefebvre, 2000), further supports this notion.

In conclusion, our results in porcine gastric fundus show that the nitrergic myenteric neurons are endowed with heme oxygenase-2 and biliverdin reductase, the biosynthetic enzymes leading to generation of CO, biliverdin and bilirubin. While the role of CO and the bile pigments biliverdin and bilirubin in NANC neurotransmission cannot be further substantiated due to a lack of specific heme oxygenase inhibitors, exogenous CO can induce smooth muscle relaxation through the soluble guanylate cyclase/cGMP pathway, a response which is amplificated by the synthetic compound YC-1 and by biliverdin and bilirubin. This suggests that bile pigments might have a modulatory influence on responses to signaling molecules in porcine gastric fundus.

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References

Battish, R., Cao, G.-Y., Lynn, R.B., Chakder, S., Rattan, S., 2000. Heme oxygenase-2 distribution in anorectum: colocalization with neuronal nitric oxide synthase. Am. J. Physiol. 278, G148–G155.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72, 248–252.

Clark, J.E., Foresti, R., Sarathchandra, P., Kaur, H., Green, C.J., Motterlini, R., 2000. Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. Am. J. Physiol. 278, H643–H651.

Colpaert, E.E., Lefebvre, R.A., 2000. Influence of bilirubin and other antioxidants on nitrergic relaxation in the pig gastric fundus. Br. J. Pharmacol. 129, 1201–1211.

Colpaert, E.E., Lefebvre, R.A., 2002. Role of antioxidants in the protection of the nitrergic neurotransmitter. Acta Neurol. Belg. 102, 68–72.

Colpaert, E.E., Timmermans, J.-P., Lefebvre, R.A., 2002a. Immunohistochemical localization of the antioxidant enzymes biliverdin reductase and heme oxygenase-2 in human and pig gastric fundus. Free Radic. Biol. Med. 32, 630–637.

Colpaert, E.E., Timmermans, J.-P., Lefebvre, R.A., 2002b. Influence of antioxidant depletion on nitrergic relaxation in the pig gastric fundus. Br. J. Pharmacol. 135, 917–926.

Dawson, T.M., Snyder, S.H., 1994. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J. Neurosci. 14, 5147–5159.

Dick, J.M.C., Lefebvre, R.A., 1997. Influence of different classes of NO synthase inhibitors in the pig gastric fundus. Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 488–494.

Donat, M.E., Wong, K., Staines, W.A., Krantis, A., 1999. Heme oxygenase immunoreactive neurons in the rat intestine and their relationship to nitrergic neurons. J. Auton. Nerv. Syst. 77, 4–12.

Doré, S., Takahashi, M., Ferris, C.D., Hester, L.D., Guastella, D., Snyder, S.H., 1999. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc. Natl. Acad. Sci. U. S. A. 6, 2445–2450.

Farrugia, G., Irons, W.A., Rae, J.L., Sarr, M.G., Szurszewski, J.H., 1993. Activation of whole cell currents in isolated human jejunal circular smooth muscle cells by carbon monoxide. Am. J. Physiol. 264, G1184–G1189.

Farrugia, G., Miller, S.M., Rich, A., Liu, X., Maines, M.D., Rae, J.L., Szurszewski, J.H., 1998. Distribution of heme oxygenase and effects of exogenous carbon monoxide in canine jejunum. Am. J. Physiol. 274, G350–G358.

Friebe, A., Schultz, G., Koesling, D., 1996. Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. EMBO J. 15, 6863–6868.

Furchgott, R.F., Jothianandan, D., 1991. Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 28, 52–61.

Kelm, M., Schrader, J., 1990. Control of coronary vascular tone by nitric oxide. Circ. Res. 66, 1561–1575.

Lefebvre, R.A., Smits, G.J.M., Timmermans, J.-P., 1995. Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. Br. J. Pharmacol. 116, 2017–2026.

Maines, M.D., 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. 2, 2557–2568.

Maines, M.D., 1993. Carbon monoxide: an emerging regulator of cGMP in the brain. Mol. Cell. Neurosci. 4, 389–397.

Maines, M.D., 1997. The heme oxygenase system: a regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. 37, 517–554.

Mandrek, K., Milenov, K., 1991. Responses of porcine gastric and duodenal smooth muscle to VIP. J. Autonom. Pharmacol. 11, 353–364.

McCoubrey, W.K., Huang, T.J., Maines, M.D., 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur. J. Biochem. 247, 725–732.

Miller, S.M., Reed, D., Sarr, M.G., Farrugia, G., Szurszewski, J.H., 2001. Haem oxygenase in enteric nervous system of human stomach and jejunum and co-localization with nitric oxide synthase. Neurogastroenterol. Motil. 13, 121–131.

Minetti, M., Mallozzi, C., DiStasi, A.M.M., Pietraforte, D., 1998. Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. Arch. Biochem. Biophys. 352, 165–174.

Motterlini, R., Foresti, R., Intaglietta, M., Winslow, R.M., 1996. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. Am. J. Physiol. 270, H107–H114.

Mulsch, A., Bauersachs, J., Schafer, A., Stasch, J.-P., Kast, R., Busse, R., 1997. Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br. J. Pharmacol. 120, 681–689.

Ny, L., Alm, P., Ekström, P., Larsson, B., Grundemar, L., Andersson, K.-E., 1996. Localization and activity of haem oxygenase and functional effects of carbon monoxide in the feline lower oesophageal sphincter. Br. J. Pharmacol. 118, 392–399.

Ny, L., Alm, P., Larsson, B., Andersson, K.-E., 1997. Morphological relations between heme oxygenases, NO-synthase and VIP in the canine and feline gastrointestinal tracts. J. Auton. Nerv. Syst. 65, 49–56.

Otterbein, L.E., Choi, A.M.K., 2000. Heme oxygenase: colors of defense against cellular stress. Am. J. Physiol. 279, L1029–L1037.

Porcher, C., Orsoni, P., Berdah, S., Monges, G., Mazet, B., 1999. Distribution of heme oxygenase 2 in nerves and c-Kit+ interstitial cells in human stomach. Histochem. Cell. Biol. 112, 317–322.

Ramos, K.S., Lin, H., McGrath, J.J., 1989. Modulation of cyclic guanosine

monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide. Biochem. Pharmacol. 38, 1368–1370.

Rattan, S., Chakder, S., 1993. Inhibitory effect of CO on internal anal sphincter: heme oxygenase inhibitor inhibits NANC relaxation. Am. J. Physiol. 265, G799–G804.

Rattan, S., Chakder, S., 2000. Influence of heme oxygenase inhibitors on the basal tissue enzymatic activity and smooth muscle relaxation of internal anal sphincter. J. Pharmacol. Exp. Ther. 294, 1009–1016.

Rattan, S., Fan, Y.-P., Chakder, S., 1999. Mechanism of inhibition of VIP-induced LES relaxation by heme oxygenase inhibitor zinc protoporphyrin IX. Am. J. Physiol. 276, G138–G145.

Robertson Jr., P., Fridovich, I., 1982. A reaction of the superoxide radical with tetrapyrroles. Arch. Biochem. Biophys. 213, 353–357.

Schmidt, K., Schrammel, A., Koesling, D., Mayer, B., 2001. Molecular mechanisms involved in the synergistic activation of soluble guanylyl cyclase by YC-1 and nitric oxide in endothelial cells. Mol. Pharmacol. 59, 220–224.

Stocker, R., Peterhans, E., 1989. Synergistic interaction between vitamin E and the bile pigments bilirubin and biliverdin. Biochim. Biophys. Acta 1002, 238–244.

Stocker, R., Yamamoto, Y., McDonagh, A., Glazer, A., Ames, B.N., 1987. Bilirubin is an antioxidant of possible physiological importance. Science 235, 1043–1045.

Stone, J.R., Marletta, M.A., 1994. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry 33, 5636–5640.

Vachharajani, T.J., Work, J., Sekutz, A.C., Granger, D.N., 2000. Heme oxygenase modulates selectin expression in different regional vascular beds. Am. J. Physiol. 278, H1613–H1617.

Werkström, V., Ny, L., Persson, K., Andersson, K.-E., 1997. Carbon monoxide-induced relaxation and distribution of haem oxygenase isoenzymes in the pig urethra and lower oesophagogastric junction. Br. J. Pharmacol. 120, 312–318.

Wu, C.C., Ko, F.N., Kuo, S.C., Lee, F.Y., Teng, C.M., 1995. YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. Br. J. Pharmacol. 116, 1973–1978.

Xue, L., Farrugia, G., Miller, S.M., Ferris, C.D., Snyder, S.H., Szurszewski, J.H., 2000. Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: evidence from genomic deletion of biosynthetic enzymes. Proc. Natl. Acad. Sci. U. S. A. 97, 1851–1855.

Zakhary, R., Poss, K.D., Jaffrey, S.R., Ferris, C.D., Tonegawa, S., Snyder, S.H., 1997. Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide. Proc. Natl. Acad. Sci. U. S. A. 94, 14848–14853.