TOPICAL REVIEW

The role of carbon monoxide in the gastrointestinal tract

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Carbon monoxide (CO) is a biologically active product of haem metabolism that contributes to the normal physiology of the gastrointestinal tract. In this article, we review recent data showing that CO is an integral regulator of gastrointestinal motility and an important factor in the response to gastrointestinal injury. CO is generated by haem oxygenase-2 (HO-2), which is constitutively expressed in many inhibitory neurones of the vertebrate enteric nervous system. The membrane potential gradients along and across the muscle layers of the gastrointestinal tract require the generation of CO by haem oxygenase-2. The presence of CO is also necessary for normal inhibitory neurotransmission in circular smooth muscle and appears to permit nitric oxide-mediated inhibitory neurotransmission. Genetic deletion of the haem oxygenase-2 gene in mice slows gut transit. The other major CO synthetic enzyme, haem oxygenase-1 (HO-1) is induced under conditions of stress or injury. Recent studies have demonstrated that up-regulation of haem oxygenase-1 protects the gut from several types of gastrointestinal injury, suggesting that CO or induction of HO-1 may find therapeutic use in gastrointestinal diseases and injuries. Furthermore, it is anticipated that the understanding of CO-mediated signalling in the gastrointestinal tract will inform studies in other tissues that express haem oxygenases.

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General introduction

The physiological function of carbon monoxide (CO) has become the subject of intensive research in recent years and studies on the gastrointestinal tract have been at the forefront of these investigations. It is now understood that CO is an important chemical signal that regulates neurotransmission, smooth muscle tone and the response to cellular injury (Fig. 1). In addition, other products of haem degradation, biliverdin and Fe^{2+}, are attracting attention for their physiological effects. There are several comprehensive reviews on the physiology and pathophysiology of CO in general including the proceedings of a recent symposium devoted to the subject (Choi *et al.* 2002, see also Maines, 1997). However, the role of CO in the gut has not been reviewed since 1999 (Farrugia & Szurszewski, 1999) and the field has advanced significantly since then, with clear demonstrations of CO as a nerve-derived signalling molecule and as a suppressor of cellular injury. There are several products of haem degradation but CO has received most attention in the gut. Therefore, it is the focus of this review. The significance of biliverdin and Fe^{2+} to gut physiology is still unclear and will not be discussed in detail.

Synthesis of carbon monoxide

Two haem oxygenase enzymes, haem oxygenase-1 (HO-1) and haem oxygenase-2 (HO-2) are located in the endoplasmic reticulum and catalyse the synthesis of carbon monoxide from Fe protoporphyrin IX (haem) (Table 1, reviewed by Maines, 1997). A third isoform, HO-3, has also been described but it does not generate CO from haem and the functional role of this protein is unclear (McCoubrey *et al.* 1997). These proteins are structurally quite different but the reaction chemistry is the same, relying on oxidation of NADPH and using molecular oxygen in the cleavage of haem. Carbon monoxide (CO), biliverdin, Fe^{2+} and H_2O_2 are generated by this reaction. Until the late 1980s haem oxygenases were considered simply responsible for removal of excess haem. It is now evident that generation of CO, biliverdin, Fe^{2+} and H_2O_2 are also important functions of haem oxygenase

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and that all of the products of haem degradation have biological effects. The anatomical distribution of the synthetic enzymes and the regulation of the enzymes largely determine the contribution of CO to intercellular signalling.

Distribution of haem oxygenases in the gastrointestinal tract. Haem can be synthesized *de novo* in all mammalian cells tested to date (Maines, 1980). Under normal, non-diseased conditions, the rate-limiting step to CO production appears to be the activity of the haem oxygenases. HO-1 and HO-2 are regulated by strikingly different mechanisms, which may reflect different physiological and pathological roles.

HO-1 is expressed at very low levels in the gut unless induced by disease, injury and/or inflammation. On the other hand, HO-2 expression has been reported from studies throughout the healthy gut in a number of species. Predictably, there are species differences but the overall pattern of HO-2-like immunoreactivity is similar in the animal models commonly used in studies of motility and enteric neuroscience. A proportion of enteric neuronal cell bodies and fibres in the myenteric plexus and nerve fibres in the deep muscular plexuses express HO-2-like immunoreactivity in human (Miller *et al.* 2001), mouse (Zakhary *et al.* 1997; Miller *et al.* 1998), dog (Farrugia *et al.* 1998), cat (Ny *et al.* 1996, 1997), opossum (Battish *et al.* 2000; Chakder *et al.* 2000), guinea-pig (Vollerthun *et al.* 1996) and pig (van Ginneken *et al.* 2001; Colpaert *et al.* 2002*a*). Neurones in the pyloric and ileocaecal sphincters are reported to have particularly high levels of HO-2 (Ny *et al.* 1997), although other regions of the gut, particularly the stomach and small intestine, also contain an abundance of immunoreactive neurones (e.g. Miller *et al.* 2001). HO-2 immunoreactivity is typically clearest in cell bodies but is also detected in nerve fibres.

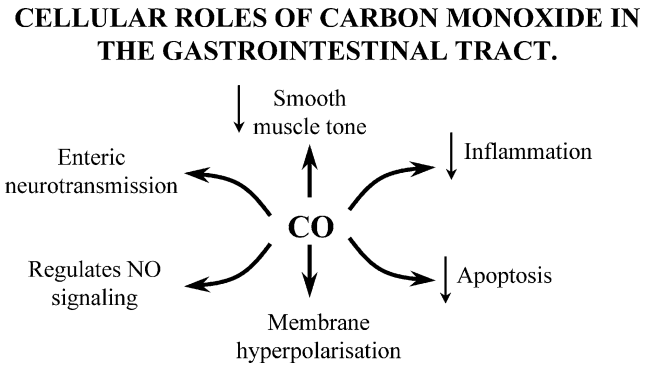

Figure 1.
Cellular roles of carbon monoxide in the gastrointestinal tract.

Therefore the cell can potentially release CO and bilirubin either at the neurites or from the cell body. Often, HO-2-immunoreactive neurones also express nitric oxide synthase (NOS) and vasoactive intestinal peptide, with the degree of colocalization between HO-2 and NOS varying between 10% for human submucous ganglia and 100% for pig fundus (Colpaert *et al.* 2002*a*). Non-neuronal cells in the gastrointestinal tract also express HO-2, including a population of cells in the mucosal epithelium, the smooth muscle of blood vessels, the endothelium of blood vessels and interstitial cells of Cajal (Grozdanovic & Gossrau, 1996; Ny *et al.* 1996, 1997; Zakhary *et al.* 1997; Farrugia *et al.* 1998; Hu *et al.* 1998; Miller *et al.* 1998, 2001; Donat *et al.* 1999; Porcher *et al.* 1999; Piotrowska *et al.* 2003). Interstitial cells of Cajal are essential for normal gastrointestinal motility (Ward *et al.* 1994; Huizinga *et al.* 1995) and in mouse, interstitial cells of Cajal from the colon and small intestine express HO-2. However, HO-2 is absent from interstitial cells of Cajal in the mouse gastric fundus and fundic smooth muscle is depolarized compared to the rest of the gastrointestinal tract due to lack of CO production. Also the membrane potential gradient observed across the circular smooth muscle of colon and small intestine is not seen in the gastric fundus (Farrugia *et al.* 2003). HO-2 is expressed in interstitial cells of Cajal identified in rat ileum by morphological criteria (Donat *et al.* 1999), but the data on human gut are contradictory. Studies identifying HO-2-immunoreactive interstitial cells of Cajal in human gastric antrum (Porcher *et al.* 1999) and human colon (Piotrowska *et al.* 2003) have been published, but in another report HO-2 was not detected in interstitial cells of Cajal from either gastric antrum or jejunum (Miller *et al.* 2001). HO-2 immunoreactivity was absent from the few interstitial cells of Cajal observed in the colon of individuals with Hirschsprung's disease (Miller *et al.* 2001). The expression of HO-2 in intrinsic blood vessels in the gut and in mesenteric arteries (Naik *et al.* 2003) indicates that haem catalysis affects vascular tone in these vessels as in other blood vessels (Koehler & Traystman, 2002; Kourembanas, 2002). A role for HO-2 in enteric blood flow has not been reported, but chronic hypoxia does up-regulate HO-1 and cause smooth muscle hyperpolarization in mesenteric arteries from chronically hypoxic rats (Naik *et al.* 2003). There are no published data on the role of HO-2 in the physiology of epithelial cells from intestinal mucosa.

Regulation of haem oxygenase activity

Regulation of HO-1. An abundance of data on the regulation of HO-1 throughout the body has been

Table 1. Haem oxygenase isoforms

	HO-1	HO-2	HO-3
Isoform	Widely inducible	Constitutive, inducible by adrenal glucocorticoids	Unknown
Regulation	Regulation of transcription, haem availability	Phosphorylation by ser/thr/tyr kinases, haem availability	Unknown
Transcripts	Single 1.8 Kb transcript	1 widely expressed transcript, 4 add'l transcripts in testis 1.3-2.1 Kb	2.1-5 Kb
Homology	43% with HO-2 50% with HO-3	43% with HO-1 90% with HO-3	50% with HO-1 90% with HO-2
Proposed role	Antioxidant and inflammation	Generates signalling molecules	Regulation of haem-dependent genes

obtained using many different models and many of the preliminary observations were made in tissue and cells from outside the gut. However, studies led by Bauer and colleagues in the gut have identified a number of novel mechanisms for regulation of HO-1 and demonstrated a very important role for regulation of HO-1 in the health of the gastrointestinal tract. HO-1 is predominantly expressed in the spleen, where haemoglobin from senescent erythrocytes is broken down. However, transcription of the gene is readily induced throughout the body by a number of factors associated with cell injury or inflammation. A frequently cited and vivid example is the response to a haematoma in which HO-1 is induced and breaks down the red–purple haemoglobin to green biliverdin and yellow bilirubin (Foresti & Motterlini, 1999). In that situation, HO-1 has a clear role in removal of haem from the site of injury. HO-1 is also induced by hypoxia, UV irradiation, oxidative stress, reactive oxygen species, heavy metals, cytokines, lipopolysaccharide, shear stress and heat shock (Choi & Alam, 1996; Foresti & Motterlini, 1999). In fact, HO-1 is the heat shock protein HSP32 (Keyse & Tyrrell, 1989) and appears to be a generic part of the response to cellular stress regardless of the involvement of haem. In the absence of HO-1, mutant mice (Poss & Tonegawa, 1997) exhibit a greatly reduced resistance to cellular stress, the animals die young and suffer from chronic inflammation and growth retardation. In humans, loss of HO-1 produced similar symptoms (Yachie *et al.* 1999), and in another study, impaired HO-1 transcription, due to a mutation in the promotor region of the gene, seems to predispose individuals to pulmonary emphysema (Yamada *et al.* 2000), and coronary artery disease of type 2 diabetics (Chen *et al.* 2002). The converse is also true; it has been demonstrated that increased HO-1 expression protects many tissues from injury including muscle (Nath *et al.* 1992), endothelium (Balla *et al.* 1992) and lung (Choi & Alam, 1996).

The mechanism and effects of inducing HO-1 expression have been studied in many tissues. Hypoxia, CO in low concentrations, haem lysinate, NO in the presence of reactive oxygen species and a number of cytokines, including interleukin-1β and tumour necrosis factor-α, rapidly activate a short-term increase in HO-1 transcription and stabilize existing HO-1 mRNA (Kourembanas, 2002). For example, HO-1 transcription is induced in pulmonary vascular smooth muscle within 6 h of exposure and returns to baseline after 48 h, probably due to feedback inhibition by high concentrations of CO on HO-1 transcription (Morita *et al.* 1995). During the period of elevated HO-1 activity, a number of inflammatory mediators including NO appear to be suppressed (McQuillan *et al.* 1994), and this has prompted several studies on the effects of prolonged HO-1 induction. In the lung sustained expression of HO-1, either by repeated haem (McQuillan *et al.* 1994) administration (Christou *et al.* 2000) or viral infection with HO-1 cDNA (Minamino *et al.* 2001) prevented the deleterious pulmonary responses to hypoxic injury. HO-1 and CO increase levels of the anti-inflammatory cytokine IL-10 (Otterbein *et al.* 2000; Moore *et al.* 2003), and IL-10 can induce HO-1 (Visner *et al.* 2003). Other studies have shown that induction of HO-1 suppresses hyper-acute rejection during organ transplantation and that hearts from HO-1 knockout mice are more quickly rejected than organs from wild-type animals (Soares *et al.* 2001).

The significance of HO-1 induction to gastrointestinal health and disease is only just becoming appreciated. In common with other tissues outside the spleen, the healthy gut does not appear to express significant amounts of HO-1 (Miller *et al.* 2001), although there are reports of HO-1 expression in nerves and unidentified cells of the smooth muscle layers in the cat gut (Ny *et al.* 1996, 1997). At the cellular level, HO-1 can be induced in cultured human intestinal epithelial cells and, consistent with a

potential anti-inflammatory effect, this inhibits cytokine-mediated inducible NOS (iNOS) expression (Cavicchi *et al.* 2000). HO-1 activity in the gut is up-regulated by heavy metal administration. Cadmium causes a 300% increase in HO-1 activity in epithelial cells of rat small intestine (Rosenberg & Kappas, 1991), and in guinea-pig stomach, HO-1 is induced in the smooth muscle layers by cobalt administration, resulting in decreased excitability of smooth muscle strips and single cells (Kadinov *et al.* 2002). For the injured gut, several papers have recently reported that HO-1 can be induced with beneficial effects (Wang *et al.* 2001; Murthy *et al.* 2002; Fujii *et al.* 2003; Moore *et al.* 2003; Nakao *et al.* 2003). In animal models of colitis, the condition is associated with increased HO-1 levels (Wang *et al.* 2001) and further induction of HO-1 results in less inflammation and evidence of more rapid repair (Murthy *et al.* 2002). In mice with postoperative ileus following mild surgical manipulation of the small intestine, transient expression of HO-1 is detected in macrophages, leucocytes and other cells activated by inflammation. Administration of low concentrations of CO further induces HO-1 expression and improved gut motility and circular muscle contractility in these animals (Moore *et al.* 2003). In rats injected with lipopolysaccharide, as a model of sepsis, HO-1 is induced in intestinal epithelium and inhibition of HO-1 activity produces much greater mucosal injury in response to the insult (Fujii *et al.* 2003). CO administration is also effective at reducing inflammatory injury and reducing ileus in transplanted small intestine in rats. Inhalation of CO suppressed production of several inflammatory mediators and lowered blood nitrite levels consistent with reduced induction of iNOS (Nakao *et al.* 2003).

Clearly, induction of HO-1 represents a surprising and potent anti-inflammatory pathway that acts in many ways to ameliorate tissue injury. Although the toxic effects of chronic CO production or possibly haem depletion are not known, the effects of over-expression of HO-1 in mice are subtle (Maines, 1997). Therefore, it appears that regulation of HO-1 is a promising target for treatment of gastrointestinal diseases that involve oxidative stress and/or inflammatory injury such as diabetic gastroenteropathy, suppression of rejection in organ transplants and inflammatory bowel disease.

Regulation of HO-2. HO-2 is known as the constitutive form of HO, but HO-2 activity can be increased in response to an acute stimulus such as non-adrenergic non-cholinergic nerve stimulation in the internal anal sphincter of the opossum by vasoactive intestinal peptide (VIP) (Chakder *et al.* 2000). Studies on the mechanisms of HO-2 induction have demonstrated that Ca^{2+} influx, activation of protein kinase C (Dore *et al.* 1999) and activation of tyrosine kinases (Leffler *et al.* 2003) are all likely mediators of HO-2 activation. These messenger systems increase CO and/or bilirubin production through HO-2 in several tissues, indicating that intracellular second messengers regulate HO-2 activity. The response to glutamate of vascular smooth muscle from pig cerebral microvessels is a particularly robust, receptor-mediated effect that is rapid and involves activation of tyrosine kinases (Leffler *et al.* 2003). This is one of the clearest demonstrations of regulated CO release, a prerequisite for a genuine intercellular signalling molecule. Recently it has been shown that HO-2 is regulated by the protein kinase CK2. HO-2 is phosphorylated and activated by CK2 resulting in CO-mediated neurotransmission (Boehning *et al.* 2003). Regulation of HO-2 by CK2 has also been shown in the gut, with the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) inhibiting murine internal anal sphincter relaxation (Boehning *et al.* 2003). Regulation of HO-2 by CK2 (also known as casein kinase 2) provides a mechanism for the rapid production of HO-2 in response to an activating stimulus such as Ca^{2+}. In addition, as discussed below, the likely regulation of HO-2 by nitrosylation of cysteines (Ding *et al.* 1999; Hartsfield, 2002) may have physiological relevance, given the frequent colocalization of NOS and HO-2 in enteric nerves.

Transcriptional regulation of HO-2 expression has been reported for glucocorticoids (Raju *et al.* 1997), which implies that the level of HO-2 will be increased in times of stress. The connection to stress has not been directly demonstrated, but HO-2 expression does increase in pig duodenum during the first few days after birth when the adrenal cortex matures and glucocorticoids are first synthesized (van Ginneken *et al.* 2001).

Measurement of haem oxygenase activity

There are a number of methods for the measurement of haem oxygenase activity, including detection of CO in radioactive pulse chase experiments, gas chromatography, UV spectrophotometry (Marks *et al.* 2002) and spectrophotometric assays for biliverdin-IXα production (Nath *et al.* 1992; Liang *et al.* 2000). These assays are not especially sensitive and are off-line, so the data are obtained with some delay. However, it has proven possible to reproducibly measure HO activity in smooth muscle from dog stomach and small intestine using these techniques (Farrugia *et al.* 2003). One technique for

real time measurements of CO production uses laser absorption spectroscopy and holds the promise of rapid and sensitive measurements of haem oxygenase activity from much smaller samples of tissue or from cultured cells (Morimoto *et al.* 2001). This new technique has not to our knowledge been tested on gut tissue, but may prove very useful in determining the real time changes in haem oxygenase activity and CO production in response to potential regulators of the enzymes.

Physiological effects of CO in the gastrointestinal tract

There is a temptation to regard CO as 'NO-light' because of the molecular similarities between CO and NO, common downstream targets and the frequent overlap in the expression of the synthetic enzymes. However, CO has effects that distinguish it from NO, particularly the high affinity of CO for ferrous (but not ferric) haem molecules, the ability of CO to modify histidine residues and the greater stability of CO. CO does not react freely with oxygen or thiols and therefore it has a physiological half-life of minutes compared to seconds for NO. It appears that CO is indeed 'a paradigm unto itself', as suggested in a previously published review on the physiological effects of CO (Cary & Marletta, 2001).

CO is required for the membrane potential gradients along and across the gastrointestinal muscle layers. CO appears to be a hyperpolarizing factor in the gastrointestinal muscle layers. In the mouse and dog, CO production and haem oxygenase activity mirror the smooth muscle membrane potential (Farrugia *et al.* 2003). In the gastrointestinal tract, there is a large gradient in membrane potential along the long axis of the stomach from the fundus to the pylorus. There is also a membrane potential gradient across the thickness of circular muscle layer (Bauer *et al.* 1985; Bauer & Sanders, 1985, 1986). In the distal stomach and small intestine, the membrane potential in the circular smooth muscle region next to the longitudinal muscle is approximately 10 mV hyperpolarized compared to circular smooth muscle cells at the inner circular smooth muscle region, next to the submucosa (Bauer *et al.* 1985; Bauer & Sanders, 1985, 1986). In the large intestine, the gradient is present but it is reversed. CO production and haem oxygenase activity are higher in the hyperpolarized regions of the stomach, small intestine and colon and lower in the more depolarized regions (Farrugia *et al.* 2003). In regions of the gut with the same smooth muscle mechanical threshold the smooth muscle gradient may allow a graded contractile response to a stimulus, with a weak stimulus recruiting only the more depolarized smooth muscle and a stronger stimulus recruiting more hyperpolarized smooth muscle, suggesting a role for CO in controlling intestinal contractility.

Nitric oxide and carbon monoxide. Nitric oxide (NO) was described in 1987 as an intercellular messenger (Ignarro *et al.* 1987; Palmer *et al.* 1987). The discovery that a gas, NO, can function as a regulator of physiological function led several investigators to look for other gases that may also function as intercellular messengers. Carbon monoxide (CO) was suggested as a second gaseous messenger based on its marked similarities to NO (Marks *et al.* 1991; Schmidt, 1992). Like NO, CO activates guanylyl cyclase by binding to the haem at the active site of guanylyl cyclase, resulting in increased cyclic GMP (cGMP) levels (Rich *et al.* 1994). In addition, the frequent colocalization of nitric oxide synthase and haem oxygenase (see Xue *et al.* 2000; Miller *et al.* 2001 for examples in the gut) led investigators to study not only CO as a signalling molecule in its own right, but also how CO and NO interact.

Multiple levels of interaction have now been identified. NO appears to increase HO-1 expression by inducing transcription and stabilizing HO mRNA (Hartsfield *et al.* 1997), although the mechanism of action is still not well understood. Induction of HO-1 by NO may be dependent on their relative concentrations (Hartsfield, 2002). CO regulates NOS activity in a concentration-dependent manner, with high CO levels inhibiting NOS activity and low CO levels stimulating NO production (Ingi *et al.* 1996; Thorup *et al.* 1999). In intestinal smooth muscle cells, CO appears to induce NO formation via neuronal and endothelial NOS (nNOS and eNOS) activation (I. Lim, S. J. Gibbons, G. Farrugia *et al.*, unpublished observations). Increased NO levels were not due solely to release of preformed NO by CO, as previously reported (Thorup *et al.* 1999), as the effects of CO were blocked by inhibitors of NOS.

In the enteric nervous system, CO appears to play a crucial role with NO in inhibitory neurotransmission and setting the resting membrane potential in enteric smooth muscle cells (Xue *et al.* 2000; Farrugia *et al.* 2003). The resting membrane potential is depolarized in both HO-2 and nNOS knockout mice (Xue *et al.* 2000). Double knockout mice have even greater depolarization. CO and NO also interact to mediate inhibitory neurotransmission. NO generated from nNOS mediates a significant portion of gastrointestinal inhibitory neurotransmission in several

species (Xue *et al.* 2000). Non-adrenergic non-cholinergic neurotransmission is nearly abolished in HO-2 knockout mice and can be restored by addition of exogenous CO (Fig. 2). L-NNA (N^G-nitro-L-arginine), an inhibitor of nNOS, blocks the effect of CO, suggesting that CO is an obligate co-messenger for NO-mediated neurotransmission. Functional gastrointestinal studies reveal different patterns of disturbed transit, with delayed gastric emptying, but apparently normal small and large bowel transit times in nNOS knockouts, while HO-2 knockout animals exhibit overall slowed transit times (Zakhary *et al.* 1997).

The apparent marked overlap between the properties of CO and NO raises the question of why there are two molecules with the same function. An accumulation of data suggests that together with their overlapping functions CO and NO also function as distinct signalling molecules. NO is a free radical with an unpaired electron. Loss of the electron results in the nitrosonium ion, which can participate in oxidative and reductive reactions. CO is a stable compound without an unpaired electron. A major difference between CO and NO is the anti-inflammatory and antiapoptotic effect of CO. CO activates the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is involved in proliferation, apoptosis and cytokine release (Morse *et al.* 2002). CO generated from HO-1 inhibits endothelial cell tumour necrosis factor α-induced apoptosis via a mechanism that includes activation of the p38 mitogen-activated protein kinase signal pathway and nuclear factor-κ((Brouard *et al.* 2002; Soares *et al.* 2002). Both CO and NO directly modify the function of other proteins, but even when acting on the same protein they may have different sites of action. This has been best worked out for calcium-activated large conductance potassium channels. Nitric oxide increases the activity of calcium-dependent potassium channels via modifications to sulfhydryl groups. Carbon monoxide modulates the activity of the same channel by modification of histidine residues. The effects of NO are mediated on the β subunit of the potassium channel while those of CO are on the α subunit, again indicating the divergent mechanisms of action of NO and CO (Wu *et al.* 2002).

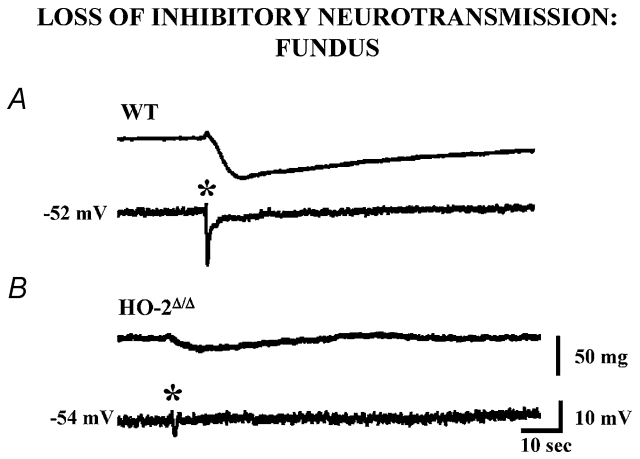

Figure 2. Loss of inhibitory neurotransmission in the gastric fundus of the HO-2 knockout mouse
A, electrical stimulation of enteric nerves (*) in mouse gastric fundus under non-adrenergic, non-cholinergic conditions evokes a transient hyperpolarization and relaxation of the circular smooth muscle. *B*, the response to electrical stimulation is significantly reduced in the HO-2 knockout mouse (HO-$2^{\Delta(\Delta}$) and is restored by application of CO (see Xue *et al.* 2000).

CO as a neurotransmitter. Gases, unlike conventional neurotransmitters, are not stored in vesicles and therefore neuronal stimulation requires the rapid induction of the biosynthetic enzyme and production of the gas on demand for it to act as a neurotransmitter. For production of NO, neuronal activation results in activation of nNOS by calcium–calmodulin (Bredt & Snyder, 1990). The mechanisms by which CO can be produced on demand have only recently begun to be elucidated. The question of whether CO production can be regulated on demand was addressed in studies of pig cerebellar microvessels (Leffler *et al.* 2003) and on hippocampal neuronal cultures and the murine internal anal sphincter (Boehning *et al.* 2003). Boehning and colleagues demonstrated a regulatory pathway following neuronal stimulation in which Ca^{2+} entry activates protein kinase C (PKC), which in turn phosphorylates CK2, resulting in phosphorylation and activation of HO-2 (Boehning *et al.* 2003) and providing a mechanism for the regulated release of CO.

CO is the second gas to fulfil many of the criteria for a neurotransmitter. CO is an effective hyperpolarizing factor of enteric smooth muscle and acts on the same molecular targets as the endogenous neurotransmitter. CO is not stored but can be generated by haem oxygenase in enteric neurones. Genetic deletion or pharmacological inhibition of HO-2 reduces the size of the inhibitory junction potential and this effect can be restored by application of exogenous CO. CO is water-soluble, quickly diffuses away from the site of release and is inactivated by binding to haemoglobin. To date the principal problems with calling CO a neurotransmitter have been the issues of regulated release and the overlap between the molecular targets of CO and NO. While the issue of regulated release is now the focus of intense study, it is not possible using the current technology to measure real-time CO release and separate the effects from the effects of NO. The issue of CO as a

neurotransmitter is further confounded by the expression of HO-2 in interstitial cells of Cajal, because these cells play a major role in neurotransmission between enteric nerves and smooth muscle. Does the knockout of the HO-2 gene impair neurotransmission because CO generation is lost from nerves or from interstitial cells of Cajal? If the answer is that CO from interstitial cells of Cajal is more significant, then our definition of a non-adrenergic non-cholinergic neurotransmitter in the gut should either exclude CO or also cover signalling molecules generated by non-neuronal cells such as interstitial cells of Cajal, fibroblasts or glia, as has been previously suggested (Snyder & Ferris, 2000).

Mechanism of action of CO

Activation of guanylate cyclase. CO is a weak activator of soluble guanylate cyclase *in vitro* with much lower potency and efficacy than NO. However, application of CO to a number of different tissues results in increased cGMP production, activation of type I cGMP-dependent protein kinase and smooth muscle relaxation (Maines, 1997; Denninger & Marletta, 1999), suggesting that *in vivo* CO does modulate cGMP levels. In the ileum of HO-2 knockout mice, basal levels of cGMP are significantly lower compared to wild type and nNOS knockout animals. In addition, activation of cGMP production by the nicotinic receptor agonist DMPP (1,1-dimethyl-4-phenylpiperazinium) is reduced in HO-2 knockout mice, and levels of cGMP are restored by application of CO or NO (Zakhary *et al.* 1997). Thus CO does contribute to cGMP production in the gut and several mechanisms have been proposed to explain the enhanced *in vivo* effects of CO on soluble guanylate cyclase activity. One proposal suggests that CO is particularly potent in some cell types because the conformation of soluble guanylate cyclase is altered by the presence of a hitherto unidentified cofactor similar to the synthetic molecule YC-1 (Stone & Marletta, 1998). YC-1 acts in synergism with CO as activators of soluble guanylate cyclase (Friebe *et al.* 1996). Another possibility is that CO elevates NO levels, possibly by activation of one of the NOS isozymes or displacement of NO from intracellular stores (see above, Thorup *et al.* 1999) and that the two molecules act together to increase cGMP production. However, in the mouse ileum CO activates cGMP production in nNOS knockout mice and therefore it appears this isoform of NOS does not mediate the effect. In addition, inhibitors of NOS do not affect CO-stimulated cGMP production in other tissues including vascular smooth muscle (Sammut *et al.* 1998).

Many cellular responses to CO appear to depend on cGMP production. cGMP-dependent protein kinase I is one target that is an important mediator of smooth muscle relaxation by direct effects on the contractile machinery as well as by altering Ca^{2+} homeostasis and voltage-gated ion channel activity (Carvajal *et al.* 2000). In some cells, cGMP also activates cyclic nucleotide-gated ion channels and can regulate the levels of cAMP by inhibition or activation of certain isoforms of phosphodiesterases (Denninger & Marletta, 1999). In pig fundus and guinea-pig ileum, cGMP appears to be required for relaxation of smooth muscle strips (Utz & Ullrich, 1991; Colpaert *et al.* 2002*b*). At the cellular level, cGMP-dependent protein kinase mediates activation by CO of voltage-dependent K^+ currents in intestinal smooth muscle cells (below).

Activation of potassium channels. CO has been shown to activate K^+ channels in a variety of tissues, including the gastrointestinal tract. In human and canine intestinal smooth muscle, CO activated a delayed rectifier-like K^+ current resulting in membrane hyperpolarization (Farrugia *et al.* 1993, 1998). The current was blocked by quinidine and was also activated by exogenous cGMP, suggesting the cGMP pathway as the mechanism of action of CO. Similar results were seen in rabbit corneal epithelial cells. Exogenous CO activated a non-Ca^{2+}-activated large conductance K^+ channel. CO increased intracellular cGMP levels and did not activate the large conductance K^+ channel in excised patches, again suggesting that the effects of CO on the K^+ channel were through cGMP. CO activates a 70 pS channel in rat thick ascending limb cells (Liu *et al.* 1999). A direct activation of K^+ channels is reported for large conductance Ca^{2+}-activated K^+ channels through an interaction with histidine residues on the α subunit. As outlined above this is distinct from the mechanism of action of NO which appears to be through the β subunit.

Binding to other ferrous haem molecules. The ability of CO to bind ferrous haem is familiar from the effects of this molecule on haemoglobin and the toxic consequences on the oxygen-carrying capacity of erythrocytes. In addition, CO binds to other haem-containing proteins including cytochrome *c* oxidase, cytochrome P450, NOS and haem-containing transcription factors. Some of these proteins appear to be mediators of the cellular effects of CO. CO regulates circadian gene transcription by binding to the haem-containing transcription factor NPAS2 (Dioum *et al.* 2002). Cytochrome P450 converts arachidonic acid into a number of eicosanoids that alter vascular smooth muscle tone (Edwards & Weston, 1998) and inhibition of cytochrome P450 appears to be the intermediary for CO-induced vasodilatation in lamb ductus arteriosus (Coceani

et al. 1996). CO inhibition of cytochrome *c* has minor effects in healthy tissue, but under hypoxic conditions CO does cause cellular injury due to oxidative stress in tissues with high O_2 requirements (Piantadosi, 2002). The significance of these haem-containing proteins for the effects of CO on the gastrointestinal tract is not clear and requires further investigation.

Mitogen-activated protein kinase (MAPK). Lipopolysaccharide (LPS) is a constituent of the bacterial cell wall and plays an important role in mediating the effects of sepsis. LPS activates pro-inflammatory mediators and activates MAPKs including ERK1/2, JNK and p38 (Otterbein *et al.* 2000). HO-1 through CO inhibits the effects of LPS through the MKK–p38 MAPK pathway and IL-10 (Otterbein *et al.* 2000). A similar mechanism of action may underlie the effects of CO in preventing postoperative ileus.

Effects of other products of haem metabolism

Biliverdin/bilirubin. The biliverdin produced by haem oxygenases is converted to bilirubin by biliverdin reductase, an enzyme that is often colocalized with haem oxygenases. (e.g. pig fundus enteric neurones (Colpaert *et al.* 2002*a*). Bilirubin is generally considered to be a toxic waste product, particularly in the case of neonatal kernicterus (McDonagh, 1990), but it is also a potent antioxidant that scavenges peroxide radicals and inhibits lipid peroxidation (Stocker *et al.* 1987; Dore *et al.* 1999). Bilirubin decreases the generation of peroxynitrates and injury of arterial endothelial cells (Foresti & Motterlini, 1999) reduces the contractility of tracheal smooth muscle (Samb *et al.* 2002) and inhibits migration and adhesion of leucocytes (Ishikawa *et al.* 1997; Hayashi *et al.* 1999). There are few published studies on the effects of bilirubin in the gastrointestinal tract, but in the pig fundus bilirubin potentiates inhibitory neurotransmission (Colpaert & Lefebvre, 2000), possibly by preventing the oxidation of NO. These observations indicate that HO-2 activity contributes both a hyperpolarizing factor in CO and a regulator of nitrergic neurotransmission in bilirubin and CO. In addition, bilirubin could be an effective contributor to the anti-inflammatory effects of HO-1 induction. The apparent redundancy of this 'belts and braces' mechanism with overlapping roles for CO and bilirubin probably reflects the significant biological hazards due to inflammation and oxidative stress.

Fe^{2+}. Iron is toxic to cells, it is an effective catalyst for generation of reactive oxygen species and free radicals (Paller, 1988; McCord, 1998). Intracellular iron levels are therefore tightly controlled. Induction of HO-1 occurs in parallel with increased ferritin levels and increased transferrin levels. The results are cells generating large amounts of Fe^{2+} from haem catalysis that are able to quickly clear this potentially toxic metabolite (Balla *et al.* 1992; Nath *et al.* 1992). A physiological function for iron as a messenger has not been described.

Summary

The emerging roles for CO in the gastrointestinal tract now cover an extraordinary range of physiological functions both in health and in disease. CO is now being seriously considered as a therapeutic anti-inflammatory agent. These are indeed exciting times for a molecule once relegated to a useless byproduct of haem metabolism and a much-feared toxic byproduct of incomplete combustion.

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