

Themed Section: Pharmacology of the Gasotransmitters

REVIEW

CO and CO-releasing molecules (CO-RMs) in acute gastrointestinal inflammation

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Carbon monoxide (CO) is enzymatically generated in mammalian cells alongside the liberation of iron and the production of biliverdin and bilirubin. This occurs during the degradation of haem by haem oxygenase (HO) enzymes, a class of ubiquitous proteins consisting of constitutive and inducible isoforms. The constitutive HO2 is present in the gastrointestinal tract in neurons and interstitial cells of Cajal and CO released from these cells might contribute to intestinal inhibitory neurotransmission and/or to the control of intestinal smooth muscle cell membrane potential. On the other hand, increased expression of the inducible HO1 is now recognized as a beneficial response to oxidative stress and inflammation. Among the products of haem metabolism, CO appears to contribute primarily to the antioxidant and anti-inflammatory effects of the HO1 pathway explaining the studies conducted to exploit CO as a possible therapeutic agent. This article reviews the effects and, as far as known today, the mechanism(s) of action of CO administered either as CO gas or via CO-releasing molecules in acute gastrointestinal inflammation. We provide here a comprehensive overview on the effect of CO in experimental *in vivo* models of post-operative ileus, intestinal injury during sepsis and necrotizing enterocolitis. In addition, we will analyse the *in vitro* data obtained so far on the effect of CO on intestinal epithelial cell lines exposed to cytokines, considering the important role of the intestinal mucosa in the pathology of gastrointestinal inflammation.

LINKED ARTICLES

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Abbreviations

CO, carbon monoxide; COHb, carbon monoxide-haemoglobin; CO-RM, CO-releasing molecule; ER, endoplasmic reticulum; FGF, fibroblast growth factor; GI, gastrointestinal; HO, haem oxygenase; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible NOS; I/R, ischaemia/reperfusion; KC, keratinocyte chemoattractant; NEC, necrotizing enterocolitis; NOX, NADPH oxidase; Nrf2, nuclear factor erythroid 2-related factor; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PERK, protein kinase R-like endoplasmic reticulum kinase; POI, post-operative ileus; ROS, reactive oxygen species; sGC, soluble guanylate cyclase

The haem oxygenase (HO) pathway in the gastrointestinal (GI) tract

Physiological role of HO enzymes

Carbon monoxide (CO) is produced during the metabolism of haem by HO; this reaction also leads to the generation of ferrous iron (Fe²⁺) and biliverdin, the latter being subsequently reduced to bilirubin by biliverdin reductase. Originally

thought of as a mechanism that only serves to dispose of haem, it is now clear that the products of haem metabolism have important functions under physiological and pathophysiological conditions. Three isoforms of HO have been identified (for nomenclature see Alexander *et al.*, 2013). HO1 is the inducible isoform while HO2 is constitutive. In the GI tract, HO2 is present not only in enteric neurons (Miller *et al.*, 2001; Colpaert *et al.*, 2002), but also in non-neuronal cells, in particular interstitial cells of Cajal

(Grozdanovic and Gossrau, 1996; Miller *et al.*, 1998). A third isoform (HO3) has been reported but there are doubts whether HO3 has a functional role as it was never convincingly reported at the protein level (Hayashi *et al.*, 2004; Wu and Wang, 2005). CO generated by HO enzymes binds preferentially and with high affinity to transition metals, in particular to the reduced form of haem iron (Fe^{2+}) present in haemoproteins; it can thus be expected to bind to the iron in the haem group of soluble guanylate cyclase (sGC), the primary target of NO for GI smooth muscle relaxation. Still, CO is a weak activator of purified sGC in comparison to NO (Stone and Marletta, 1994; Friebe *et al.*, 1996), but the short-lasting relaxations induced by bolus administration of CO in murine fundus and colon strips were abolished in strips of apo-sGC mice expressing haem-free sGC, suggesting that sGC is indeed essential for CO to cause relaxation in the GI tract (Cosyns *et al.*, 2013). However, both sGC-dependent and sGC-independent mechanisms are involved in the effects induced by prolonged exposure of GI smooth muscle to CO (De Backer *et al.*, 2008). CO has been proposed as another gaseous GI neurotransmitter but the evidence in favour of this is weaker than for NO. The possible role of CO as an inhibitory neurotransmitter in the GI tract or as an hyperpolarizing factor regulating GI smooth muscle membrane potential was very recently reviewed by Gibbons *et al.* (2013).

Protective effects of HO1 induction

HO1 is induced by a variety of physical and chemical stressors including haem, endotoxin, UV irradiation, heat shock and haemorrhagic shock, all sharing the ability to cause oxidative stress (Maines, 1997). Since the first report showing that HO1 induction crucially controls inflammation in a model of carrageenan-induced pleurisy in rats (Willis *et al.*, 1996), evidence has accumulated that induction of HO1 is a fundamental adaptive response to oxidative stress and acute inflammation (Bauer *et al.*, 2008), providing antioxidant, anti-inflammatory and cytoprotective effects. The homeostatic role of HO1 induction in mitigating the inflammatory response makes sense because reactive oxygen species (ROS) are indeed involved in the initiation and progression of inflammation (Mittal *et al.*, 2014). Possible mechanisms include activation of toll-like receptors and the Nalp-3 inflammasome (Cannizzo *et al.*, 2011) and formation of peroxynitrite by rapid combination of superoxide with NO (Mittal *et al.*, 2014). In the GI tract, expression of HO1 is increased in many experimental models such as i.p. treatment with LPS (Otani *et al.*, 2000), intracolonic administration of trinitrobenzene sulfonic acid (Wang *et al.*, 2001) and surgical intestinal manipulation (De Backer *et al.*, 2009). In patients with inflammatory bowel disease, it was found that macrophages and epithelial cells of colonic mucosa exhibit an increased expression of HO1 although levels of this protein decreased in the case of high inflammatory activity (Paul *et al.*, 2005). Induction of HO1 by exogenously administered haemin reduces GI inflammation and tissue injury (Attuwaybi *et al.*, 2004; Zhong *et al.*, 2010; Yoriki *et al.*, 2013). HO1 induction was also shown to mediate the protective effects of the amino acid glutamine in the GI tract (Uehara *et al.*, 2005; Giriş *et al.*, 2006; 2007) and to be a mechanism of action of 5-aminosalicylic acid, a common agent used for the treatment of inflammatory bowel disease (Whittle and Varga,

2010). This opens the possibility of exploiting the HO1 pathway as a therapeutic target for GI inflammation in a similar way to what has been proposed for the nervous and cardiovascular systems. A pilot clinical study using haemin to treat diabetic gastroparesis, which has been shown experimentally to be associated with insufficient HO1 expression in a particular set of macrophages (Choi *et al.*, 2008; 2010), is ongoing (NCT01206582; <http://clinicaltrials.gov>).

Role of the by-products of HO1

The antioxidant and anti-inflammatory effects of HO1 induction are related to the breakdown of haem, which is known for its pro-oxidant and cytotoxic properties, and to a concerted action of the three products of haem metabolism, namely Fe^{2+} , biliverdin and CO (Ryter and Choi, 2009). These emerging findings led gradually to the investigation of the biological effects and possible pharmacological application of each single by-product of HO1. First of all, free Fe^{2+} could be expected to act as a pro-oxidant through the generation of hydroxyl radicals in the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot$). However, induction of HO1 is accompanied by a concomitant up-regulation of ferritin (Nath *et al.*, 1992; Ren *et al.*, 2007), which sequesters Fe^{2+} and has antioxidant properties (Arosio *et al.*, 2009). Additionally, an anti-apoptotic effect of heavy chain ferritin was shown in an ischaemia/reperfusion (I/R) model of the liver (Berberat *et al.*, 2003). Biliverdin is quickly reduced by biliverdin reductase to bilirubin, which has been reported to act as potent intracellular antioxidant against lipid peroxidation (Stocker *et al.*, 1987). Exogenous administration of nanomolar concentrations of bilirubin has been reported to restore post-ischaemic myocardial function and minimize infarct size similarly to induction of HO1 by haemin, suggesting a primary role of bilirubin in HO1-mediated tissue protection against reperfusion injury (Clark *et al.*, 2000). Bilirubin also protects against I/R injury in the intestine, an effect that is associated with a decrease in intestinal lipid peroxidation products (Ceran *et al.*, 2001; Hammerman *et al.*, 2002). In a Dutch study, Caucasian patients with Crohn's disease are more likely to develop this pathology when not expressing the UDP-glucuronyltransferase 1A1*28 homozygous genotype, which is associated with Gilbert's syndrome and increased bilirubin levels (de Vries *et al.*, 2012). Correspondingly, patients with Crohn's disease showed significantly lower levels of serum bilirubin compared with controls in a study in the Czech Republic (Leniček *et al.*, 2014). Exogenous applications of bilirubin might thus be considered for the treatment of GI conditions where oxidative stress is involved. As biliverdin is rapidly reduced to bilirubin, exogenous administration of biliverdin was also attempted with success. In the GI tract, biliverdin attenuates transplantation-induced injury of the small bowel (Nakao *et al.*, 2004) and ameliorates dextran sodium sulfate-induced colitis (Berberat *et al.*, 2005). When oxidized by ROS, bilirubin is converted back to biliverdin, and a recycling loop between bilirubin and biliverdin via biliverdin reductase has been proposed (Wegiel and Otterbein, 2012) although there are also arguments against this antioxidant redox cycle (Jansen and Daiber, 2012). CO seems to contribute most significantly to the antioxidant, anti-inflammatory and cytoprotective effects of the HO1 pathway (Mottlerlini and Otterbein, 2010) in line with early

reports that exogenous CO mimicked the protective effects of HO1 induction even under inhibition of HO1 activity (Sato *et al.*, 2001). This explains the recent efforts to exploit the use of CO as a therapeutic agent to mimic the inherent beneficial actions of the HO1 pathway.

Mechanisms of action of CO

Although the exact molecular target of CO remains elusive, several mechanisms of action for this gaseous molecule have been proposed, mainly on the basis of data in cell lines and non-GI tissue. The protective effect of CO in acute GI inflammation during post-operative ileus (POI), sepsis and necrotizing enterocolitis (NEC) will be discussed in the respective sections.

Activation of sGC/p38 MAPK by CO

In the innate immune system, macrophages are one of the main players involved in the acute inflammatory response in reaction to pathogens, which is achieved by secretion of pro-/anti-inflammatory cytokines and pro-oxidant molecules. Interestingly, in a seminal paper by Otterbein and co-workers, it was shown that CO reduces the production of TNF- α and IL-1 β and increases the synthesis of the anti-inflammatory IL-10 in LPS-stimulated macrophages; these effects do not involve activation of the sGC-cGMP pathway (Otterbein *et al.*, 2000). In contrast, the inhibitory effect of CO on leukocyte adhesion in mesenteric venules and on neutrophil migration into the peritoneal cavity induced by carrageenan is counteracted by the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Freitas *et al.*, 2006). Similarly, the inhibitory effect of CO on apoptosis of neuroblastoma cells, induced by the inhibitor of mitochondrial complex I rotenone to mimic ischaemic respiratory arrest, is reversed by inhibition of sGC (Schallner *et al.*, 2013). Notably, Otterbein and co-workers found that the inhibitory effect of CO on LPS-stimulated macrophages was related to further enhancement of p38 MAPK expression (Otterbein *et al.*, 2000) and the involvement of p38 MAPK activation in the beneficial effects of CO has since been reported in many models *in vivo* (Otterbein *et al.*, 2003a; Zhang *et al.*, 2003; Kohmoto *et al.*, 2007). However, CO is unlikely to activate p38 MAPK directly as it lacks a transition metal centre in the protein structure that would function as a binding site for the gaseous molecule. Thus, the p38 MAPK activation by CO might be the result of another upstream target. Interestingly, the inhibitory effect of CO on smooth muscle cell proliferation is linked to sGC-dependent activation of p38 MAPK (Otterbein *et al.*, 2003b).

Interaction of the HO1/CO pathway with inducible NOS (iNOS)

An essential enzyme up-regulated in immune cells involved in the acute inflammatory response such as macrophages is iNOS. In LPS-stimulated macrophages, CO decreased nitrite (marker of NO production) levels without changing iNOS protein expression (Sawle *et al.*, 2005), suggesting that CO might inhibit iNOS activity. However, in the same model, Tsoyi *et al.* (2009) reported that CO is able to reduce iNOS

expression, and this was also observed in LPS-treated human umbilical vein endothelial cells (Sun *et al.*, 2008).

Anti-/pro-oxidant effect of CO

In view of the contribution of ROS to the initiation and propagation of inflammation, inhibition of ROS production by CO might contribute to its anti-inflammatory action. NADPH oxidases (NOX) are a family of multicomponent enzymes solely devoted to the production of superoxide and macrophages contain the patriarch enzyme of the family, NOX2. Inhibition of NOX by CO, probably by binding to the haem-containing gp91^{phox}/NOX2 unit, was shown to be involved in the anti-inflammatory actions of CO in LPS-stimulated macrophages (Nakahira *et al.*, 2006) and its anti-proliferative actions in human airway smooth muscle cells (Taillé *et al.*, 2005). The inhibitory effect of CO on vascular smooth muscle cell migration has been related to inhibition of NOX1 (Rodriguez *et al.*, 2010). In contrast, binding of CO to cytochrome c oxidase (complex IV of the mitochondrial respiratory chain) was reported to result in a significant burst of mitochondrial ROS production and a concomitant conditioning of the cell with up-regulation of antioxidant and cytoprotective genes, which protects it from subsequent stress stimulation (Bilban *et al.*, 2008). Moreover, in a caecal ligation and puncture model, CO induced a mild mitochondrial oxidative stress response in the heart which stimulated mitochondrial biogenesis and improved bioenergetics in association with a reduced inflammatory response (Lancel *et al.*, 2009). This was confirmed in a recent study performed in rat isolated heart mitochondria (Lo Iacono *et al.*, 2011) showing that low micromolar concentrations of CO, probably by partially binding to cytochrome c oxidase and/or other unidentified targets in the respiratory chain, increase oxygen consumption and mitochondrial hydrogen peroxide production when physiologically stimulating the electron transfer chain with pyruvate/malate, but significantly decrease the burst of ROS induced by stimulating the reverse electron flow in mitochondria, a phenomenon that appears to typify oxidative and inflammatory disease states (Chen *et al.*, 2007).

Induction of HO1 by CO

The beneficial effects of CO might also be related, at least in some circumstances, to induction of HO1 providing a positive feedback loop (CO \rightarrow induction of HO1 \rightarrow more CO \rightarrow further induction of HO1) and allowing the ROS scavenging properties of biliverdin/bilirubin to come into play (Rodella *et al.*, 2006). In human endothelial cells, CO induces HO1 expression through a mechanism involving Nrf2 (nuclear factor erythroid 2-related factor) activation. This has been shown to be associated with upstream activation of PERK (protein kinase R-like endoplasmic reticulum kinase), one of the three endoplasmic reticulum stress sensors; this correlated with protection against endoplasmic reticulum stress-induced endothelial cell apoptosis (Kim *et al.*, 2007).

Administration of CO as a therapeutic agent

CO gas

The recognition that HO1-derived CO serves as a signalling and cytoprotective intracellular mediator prompted scientists

to devise strategies to deliver CO safely for therapeutic purposes. CO administered as gas at doses that do not compromise the oxygen carrying capacity of haemoglobin was firstly used *in vivo* to show that it protects against hyperoxic injury in rat lung (Otterbein *et al.*, 1999). Since then, inhalation of CO gas at doses of 250 p.p.m. for a short period of time has shown efficacy in many animal models of disease, and devices for applying CO gas by inhalation in man have been developed (Motterlini and Otterbein, 2010). Using this protocol, Vanova *et al.* (2014) showed that the distribution of CO after 1 h CO inhalation is tissue specific and results in increased CO levels ranging from 32-fold in spleen to 2-fold in intestine.

CO in preservation solution

In transplantation surgery, CO has also been proposed to be used *ex vivo* as an adjuvant to preservation solution in which organs are normally stored prior to grafting. Indeed, cold storage of rat intestinal grafts in University of Wisconsin solution vigorously bubbled with 5% CO for 5 min before transplantation in recipient animals has been reported to markedly reduce the up-regulation of inflammatory mediators and improve graft blood flow and mucosal barrier function (Nakao *et al.*, 2006b). Similar data have been obtained for rat kidney and lung grafts (Kohmoto *et al.*, 2008; Nakao *et al.*, 2008) and for renal transplantation in pigs (Yoshida *et al.*, 2010). In contact with air, the CO-bubbled storage solution quickly loses CO by release into air, so that the CO-bubbled solution must be kept in a tightly sealed container without an air layer to keep the CO concentration constant (Kohmoto *et al.*, 2008).

CO-releasing molecules (CO-RMs)

For this and other reasons, CO-RMs were developed to create the opportunity to deliver CO in a more practical, controllable and accurate way to the target site in comparison to CO gas inhalation. The most extensively studied CO-RMs today consist of two classes: (i) metal carbonyl complexes containing ruthenium, manganese or molybdenum which carry CO bound to metals and (ii) boranocarbonates that do not contain transition metals and release CO spontaneously based on pH changes. While the original CO-RMs had to be dissolved in organic solvents (Motterlini *et al.*, 2002), water-soluble CO-RMs such as CORM-A1 and CORM-3 have been subsequently developed (Clark *et al.*, 2003; Motterlini *et al.*, 2005). In many animal models of inflammation, I/R injury and oxidative stress, these and other similar CO-RMs have been shown to have beneficial effects similar to CO gas (Motterlini, 2007; Motterlini and Otterbein, 2010). In the context of organ preservation, and similar to what has been described with CO gas above, addition of CORM-A1 and CORM-3 during kidney cold storage markedly increased glomerular filtration rate, vascular activity and energy metabolism at reperfusion (Sandouka *et al.*, 2006). Despite the fact that these agents need to be optimized, and one has to keep in mind the possible toxicity originating from the molecule after release of CO especially when used chronically, CO-RMs offer a great opportunity in drug discovery as they differ from classical organic drugs and represent one of the few examples where transition metals are used as scaffolds to deliver the

active principle. For instance, a metal within the CO-RMs scaffold can be viewed as the 'Achilles heel' in the development of these molecules to drugs due to the common perception that transition metals *per se* can catalyse cytotoxic reactions. However, a great portion of structural and enzymatic proteins in cells contain transition metals, and several metal-handling proteins that protect cells against potentially toxic metal-based reactions are known, suggesting that biological tissues may be able to tolerate low levels of metal carbonyls (Foresti and Motterlini, 2013). Interestingly, we are gradually learning that the transition metal in CO-RMs appears to be influential in transferring CO into the cells efficiently and limiting the potential toxic effects of free CO gas (Foresti and Motterlini, 2010; Michel *et al.*, 2012).

Toxicity of exogenous CO

The interaction of CO with transition metals is central to the dichotomous nature (toxicity vs. beneficial effects) of this gaseous molecule. CO binds to haemoglobin with a 200–250 times higher affinity than oxygen, forming carbon monoxide-haemoglobin (COHb) that decreases the capacity of blood to deliver oxygen to the tissues. The basal COHb level in man is 0.1–1% in the absence of environmental contamination or smoking (Ryter and Choi, 2013). Toxicity symptoms can occur with COHb levels from 10% and above (Von Burg, 1999), although COHb may not be the only reliable biomarker for determining the toxicity of this gas (Foresti *et al.*, 2008). In experimental studies with CO or CO-RMs, one thus usually attempts to keep COHb levels below 10%, levels that are also occasionally encountered in heavy smokers. Still, one can be reluctant to apply chronically CO or CO-RMs, for example, for treatment of inflammatory bowel disease, in doses inducing COHb levels of 5–10%. Deleterious effects to the heart were indeed shown for chronic ambient CO levels as low as 30 p.p.m. with peaks of 100 p.p.m. (Andre *et al.*, 2010; Reboul *et al.*, 2012). Short-term CO therapy should be devoid of such effects and must be expected to be easily acceptable for application in man. This review therefore considers the findings with CO gas and CO-RMs for conditions involving acute GI inflammation, that is, POI, septic ileus and NEC with particular attention on its role in the GI mucosa. Acute I/R injury of the gut will not be addressed here as this topic was very recently reviewed elsewhere (Liao *et al.*, 2013).

CO in POI

Pathogenesis of POI

POI refers to the transient impairment of bowel motility after major abdominal surgery; it lasts 2–4 days until the first passage of flatus or stool combined with adequate oral intake for 24 h. Patients suffer from abdominal discomfort, nausea and vomiting. POI can delay post-operative recovery, induce morbidity and increase the length of hospital stay; the economic impact of POI has been estimated at \$750 million per year (Doorly and Senagore, 2012). In the first hours after surgery, inhibition of GI motility is mainly due to

activation of reflex inhibitory nervous pathways (Bauer and Boeckxstaens, 2004; Boeckxstaens and de Jonge, 2009). In a rat model of this early phase, it was reported that endogenous CO contributes to the retarded GI transit at 1.5 h after surgery as HO inhibitors reversed surgery-induced inhibition of transit (Korolkiewicz *et al.*, 2004). The prolongation of GI motility suppression during POI is due to a complex immune-regulated inflammatory process in the intestinal muscularis, with a critical role for activation of the resident muscularis macrophage network (Kalff *et al.*, 1999). The kinetically active substances NO (via iNOS) and prostaglandins (via COX-2) are released, as well as pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α , chemokines (MCP-1) and adhesion molecules (intercellular adhesion molecule-1; ICAM-1). This leads to additional recruitment of circulating leukocytes and further release of inflammatory mediators (Boeckxstaens and de Jonge, 2009).

Effect of CO on POI

The preclinical studies investigating the influence of CO on the inflammatory phase of POI by manipulating the small intestine after laparotomy and measuring transit 24 h after surgery are summarized in Table 1.

CO gas. Moore *et al.* (2003) reported that inhaled CO (250 p.p.m. for 1 h before and for 24 h after surgery) in mice significantly improved the prolongation of transit induced by surgery, corresponding to improvement of the surgery-induced suppression of spontaneous and betanechol-induced activity in small intestinal circular muscle strips obtained 24 h after surgery. No negative effect of inhaled CO, induced by its possible GI relaxant effect, was observed, as CO inhalation in control non-operated animals did not significantly influence transit. Surgery increased iNOS mRNA expression in the small intestinal muscular layer at 3 and 6 h, and nitrite release in the culture medium of muscular material obtained 24 h after surgery; these effects were suppressed by CO inhalation, suggesting that CO might act by inhibiting iNOS gene expression as well as iNOS activity. Surgery also increased the mRNA expression of pro-inflammatory IL-1 β , IL-6 and COX-2; CO reduced IL-1 β expression and the anti-inflammatory IL-10 and HO1 further enhanced IL-10 and HO1 expression. This supports the possibility that the beneficial effects of CO are at least partially due to increased HO1 expression. Interestingly, the increase in muscular leukocyte infiltration induced by surgery was not suppressed by CO.

The same group (Moore *et al.*, 2005) showed that inhalation of 250 p.p.m. CO for 3 h before surgery in rats was sufficient to fully reverse surgery-induced retardation in transit, indicating a more feasible protocol for application in humans than prolonged inhalation. They also showed that 250 p.p.m. for 3 h before surgery is effective in pigs receiving repetitive opioid analgesia to mimic the human condition, whereby the GI inhibitory effects of opioids also contribute to ileus. Blood COHb levels reached 6% just before surgery and progressively declined thereafter. Although CO inhalation suppressed the increase in total blood white cell count at 4 h after surgery, it did not suppress muscular leukocyte infiltration at 24 h, once again suggesting that CO by inhalation is not able to inhibit additional leukocyte recruitment during the pathogenesis of POI.

CO in i.p. solution. Following the report that the intestinal grafts performance is enhanced by their preservation in solutions saturated with CO (Nakao *et al.*, 2006b), Nakao *et al.* (2006a) showed that an i.p. injection of Ringer's lactate solution bubbled with 100% CO for 15 min just before closure of the abdomen prevents postsurgical ileus in mice. COHb levels reached almost 8% at 5 min after administration of CO but decreased to less than 4% within 30 min. This study showed that treatment with solutions containing CO affects surgery-induced mRNA expression of pro- and anti-inflammatory parameters as reported by Moore *et al.* (2003) for inhaled CO. Importantly, i.p. injection of CO suppressed muscular leukocyte infiltration. The study further showed that surgery induced activation of MAPK p38, ERK and JNK, and the transcription factor NF- κ B, and that CO suppressed the activation of all these signalling proteins except p38. The majority of the effects of CO were diminished by pretreatment with the sGC inhibitor ODQ, suggesting activation of sGC by CO early in its protective pathway.

CO-RMs. An i.p. injection of the 'fast' CO-releaser CORM-3 at 3 and 1 h before surgery in mice (40 mg·kg⁻¹) was found to increase blood COHb levels to 2.3% after 10 min from injection; this was associated with partial protection against retarded transit, while the inactive compound i-CORM-3 was not effective (De Backer *et al.*, 2009). A similar effect was obtained with equimolecular amounts of the 'slow' CO-releaser CORM-A1 but COHb levels in this case increased to 8.4% at 20 min after injection. Similar to data obtained with CO in i.p. solution, the i.p. administration of CORM-3 suppressed muscular leukocyte infiltration at 6 and 24 h after surgery, decreased ERK activation but increased MAPK p38 activation. Surgery induced a progressive increase in protein expression of HO1 and in total HO activity; this was further enhanced by CORM-3 at 1–6 h after surgery in a MAPK p38-dependent manner. Finally, the study showed that CORM-3 suppresses the progressive increase in muscular oxidative stress levels induced by surgery, illustrating that the anti-oxidative properties of CO might also contribute to its beneficial effect in POI. In contrast to previous studies, De Backer *et al.* (2009) also investigated the role of the mucosal layer. Surgery did not induce pronounced expression of pro-inflammatory cytokines in the mucosa, but led to an early mucosal oxidative stress burst 1 h after surgery, which might contribute to intestinal epithelial barrier dysfunction during POI (Snoek *et al.*, 2012). CORM-3 reduced this early mucosal oxidative burst in a fully HO1-dependent way, as shown with the HO inhibitor chromium mesoporphyrin, while the anti-inflammatory/anti-oxidative effects of CORM-3 in the muscularis were only partially HO1-dependent.

Cells involved in the protective effect of CO in POI. None of the studies described investigated the type of cells that are targeted by CO in promoting its beneficial effects in POI. In view of the crucial role of resident macrophages in the muscle layer in the pathogenesis of POI, the mechanisms and effects of CO reported in LPS-stimulated macrophages are rather instructive (see Mechanisms of Action: Otterbein *et al.*, 2000; Sawle *et al.*, 2005; Nakahira *et al.*, 2006; Tsoyi *et al.*, 2009). Although very recently contested (Gomez-Pinilla *et al.*, 2014), activation of peritoneal mast cells is also considered to play

Table 1

Effects of CO on GI motility and on inflammatory parameters in the muscle layer in preclinical models of post-operative ileus

Reference	1	2	3	4
Species	Mouse	Rat	Mouse	Mouse
CO source	Inhalation 250 p.p.m. 1 h before to 24 h after surgery	Inhalation 250 p.p.m. 3 h before surgery	i.p. Ringer's lactate, 100% CO bubbled at the end of surgery	i.p. CORM-3 40 mg·kg ⁻¹ 3 and 1 h before surgery
Blood COHb	-	-	8%	2.3%
GI transit (24 h)	POI ↓	POI ↓	POI ↓	POI ↓
<i>In vitro</i> muscle activity	+CO ↑	+CO ↑	+CO ↑	+CO ↑
<i>In vitro</i> betanechol	↑	↑	-	↑
sGC involvement in CO effect	↑	↑	-	-
p38 MAPK phosphorylation	-	-	Yes	↑
ERK MAPK phosphorylation	-	-	↑	↑
JNK MAPK phosphorylation	-	-	↑	↑
iNOS mRNA	↑	↓	↑	-
Activity	-	-	-	↑
ROS	-	-	-	↑
HO1 mRNA	↑	-	-	↑
Protein	-	-	-	↑
HO total activity	-	-	-	↑
MPO (infiltrating leukocytes)	↑	↑	↑	↑
IL-1β mRNA	↑	↑	↑	↑
IL-6 mRNA (protein in 4)	↑	↑	↑	↑
IL-10 mRNA (protein in 4)	↑	↑	↑	↑
COX-2 mRNA	↑	↑	↑	↑
ICAM-1 mRNA (protein in 4)	-	-	↑	↑
MCP-1 mRNA (protein in 4)	-	-	↑	↑
NF-κB DNA binding activity	-	-	↑	-

1: Moore et al. (2003); 2: Moore et al. (2005); 3: Nakao et al. (2006a); 4: De Backer et al. (2009). -, not determined.

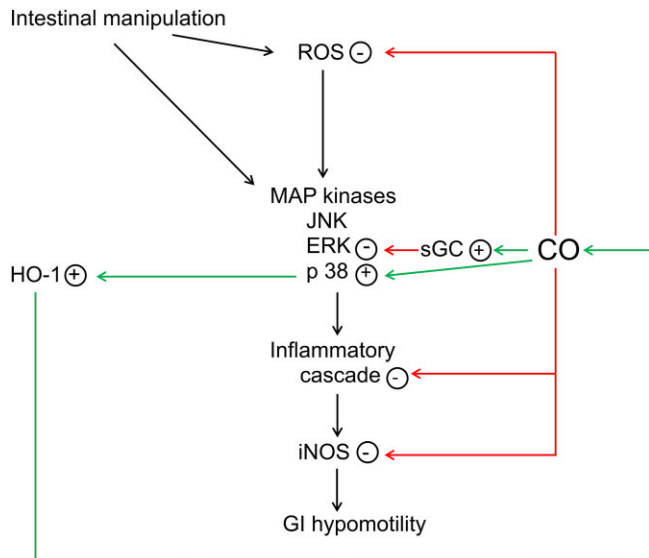


Figure 1

Graphical representation of the possible mechanisms of action of CO in POI. Intestinal manipulation induces oxidative stress and an inflammatory cascade, leading to increased expression/activity of iNOS and GI hypomotility via iNOS-derived NO. CO reduces the inflammatory cascade and iNOS expression/activity (Moore *et al.*, 2003; Nakao *et al.*, 2006a; De Backer *et al.*, 2009). Contributing mechanisms can be inhibition of ROS production (De Backer *et al.*, 2009), suppression of ERK MAPK phosphorylation (De Backer *et al.*, 2009) in a sGC-dependent way (Nakao *et al.*, 2006a) and induction of HO1 (Moore *et al.*, 2003) via enhanced p38 MAPK phosphorylation (De Backer *et al.*, 2009).

an important role in the inflammatory cascade during POI (de Jonge *et al.*, 2004; The *et al.*, 2008; Boeckxstaens and de Jonge, 2009). The compound 48/80 or antigen-induced release of histamine from rat and guinea pig mast cells is inhibited by an incubation medium saturated with CO or by addition of a CO-RM; this is associated with calcium sequestration through a cGMP-dependent mechanism (Di Bello *et al.*, 1998; Ndisang *et al.*, 1999; Vannacci *et al.*, 2004). Whether this mechanism plays any role in the protective effect of CO in POI needs further investigation.

Perspectives

Thus, all studies so far show that CO is able to protect against POI but i.p. administration of CO seems to have a more pronounced anti-inflammatory effect than inhalation. The results obtained with one specific CO-RM cannot be automatically extrapolated to all CO-RMs available especially with regard to safety. A scheme of the possible mechanisms of action of CO in POI is shown in Figure 1. The first clinical trial in patients requiring colonic resection is ongoing with a protocol consisting of CO inhalation for 1 h before and 1 h after resection (NCT01050712; <http://clinicaltrials.gov>).

CO and the intestine in sepsis

Sepsis is a frequent cause of mortality in intensive care. It is defined as a systemic inflammatory response to infection,

which can progress to severe sepsis with multiple organ failure and further to septic shock with acute circulatory failure and refractory hypotension. Sepsis is driven by a complex cascade of events, initiated by bacteria-derived molecules such as LPS (endotoxin) with subsequent formation of inflammatory cytokines, ROS and nitrogen species, I/R and mitochondrial dysfunction (Cinel and Opal, 2009). Animal models of sepsis consist of administration of LPS or bacteria, or caecal ligation and puncture (De Winter and De Man, 2010). In these models, CO either inhaled as a gas or supplied as CO-RMs has been shown to increase survival and to decrease inflammation and tissue damage in critical organs involved in septic multiple organ failure including the lung, liver, heart and kidney (Sarady *et al.*, 2004; Hoetzel *et al.*, 2007; Cepinskas *et al.*, 2008; Lancel *et al.*, 2009; Mizuguchi *et al.*, 2009; Lee *et al.*, 2014). In most studies, CO is applied before or just after administering the septic stimulus, which is clinically irrelevant in patients with established sepsis, but a recent study showed that inhalation of CO in a therapeutic mode (i.e. 2.5 h after starting the LPS infusion) still provides some degree of protection (Koulouras *et al.*, 2011).

The role of the intestine in the pathogenesis of sepsis

While the beneficial effects of CO in the critical organs during sepsis can certainly be attributed to a local anti-inflammatory and antioxidant action, the therapeutic action of CO might also affect the intestine. It is surprising that experimental studies on CO in sepsis rarely include intestinal readouts as the intestine is very vulnerable during systemic inflammation and can trigger and perpetuate sepsis (Clark and Coopersmith, 2007). Conditions such as major trauma, extensive burns and hypovolemia lead to I/R of the intestine, triggering an intestinal inflammatory reaction and intestinal epithelial barrier dysfunction (Magnotti *et al.*, 1998; de Haan *et al.*, 2009; Flessas *et al.*, 2011). Pro-inflammatory mediators produced in the intestine also reach the systemic circulation, promoting systemic inflammation. Dysfunction of the epithelial barrier allows translocation of live bacteria and/or their components such as LPS through the intestinal mucosa, further reaching the circulation via the intestinal lymph nodes. The intestinal events are accompanied by severe impairment of intestinal motility (septic ileus), promoting bacterial colonization of the normally sterile small intestine and stomach; this will facilitate bacterial translocation and predisposes to pneumonia by aspiration of gastric content (Hassoun *et al.*, 2001; Deitch, 2002; Balzan *et al.*, 2007). The mechanism of sepsis-induced ileus, as assessed by injecting LPS, shows similarity with that of the inflammatory phase of POI (Eskandari *et al.*, 1997), although differences in the degree of particular responses were observed when comparing animals with a similar degree of transit retardation, induced either by surgical intestinal manipulation or by i.p. injection of LPS. The POI model induced a greater inflammatory response and a greater degree of leukocyte infiltration (Schmidt *et al.*, 2012). In experimental sepsis, HO1 is induced counteracting intestinal tissue injury (Fujii *et al.*, 2003) and additional HO1 induction by pretreatment of animals with haemin 24 h before administration of LPS protected against the retardation of transit and the intestinal circular muscle dysmotility (Bortscher *et al.*, 2012).

Table 2

Effects of CO on intestinal tissue integrity and on inflammatory parameters in preclinical models of sepsis

Reference	1		2		3		4	
Species	Rat		Rat		Rat		Mouse	
Model	LPS injection i.v.		LPS injection i.v.		LPS injection i.v.		CLP	
CO source	Inhalation 250 p.p.m. from 1 to 4 h after LPS challenge		i.p. perfusion 2 L·min ⁻¹ 250 p.p.m. CO from 1 to 7 h after LPS challenge		i.p. 2 mL·kg ⁻¹ 250 p.p.m. CO 1 h after LPS challenge		i.v. CORM-2 8 mg·kg ⁻¹ immediately after CLP	
Blood COHb	6.9%		7.0%		2.6%		–	
	LPS	+CO	LPS	+CO	LPS	+CO	CLP	+CO
Intestinal tissue damage	↑	↓	↑	↓	↑	↓	↑	=
Intestinal cell apoptosis	↑	↓	↑	↓	↑	↓	–	–
p38 MAPK phosphorylation	–	–	↑	↑	↑	↑	–	–
iNOS protein	–	–	–	–	–	–	↑	↓
NO production	–	–	–	–	–	–	↑	↓
ROS	↑	↓	↑	↓	↑	↓	↑	↓
SOD activity	–	–	–	–	↓	↑	–	–
HO1 mRNA	↑	↑	–	–	–	–	–	–
MPO (infiltrating leukocytes)	↑	↓	↑	↓	↑	↓	↑	↓
TNF- α protein	–	–	–	–	–	–	↑	↓
IL-1 β protein	–	–	–	–	–	–	↑	↓
IL-10 protein	–	–	–	–	↓	↑	–	–
ICAM-1 protein	↑	↓	↑	↓	↑	↓	↑	↓
PAF-1 protein	↑	↓	↑	↓	↑	↓	–	–

1: Liu *et al.* (2007); 2: Liu *et al.* (2010a); 3: Liu *et al.* (2010b); 4: Wang *et al.* (2012). CLP, caecal ligation and puncture; –, not determined.

Effect of CO on the intestine in sepsis

Studies concentrating on the effect of CO on the intestine during experimental sepsis are summarized in Table 2. The intestinal readouts involve whole tissue homogenates of small intestinal fragments without separation of mucosal or muscular layers. Liu *et al.* (2007) reported that CO inhalation at 250 p.p.m. from 1 to 4 h after injection of LPS in rats ameliorates intestinal injury. This effect was associated with significant suppression of the LPS-induced intestinal cell apoptosis. Although inhalation of CO gas induced an increase in arterial COHb levels to 6.9%, no significant differences in the partial arterial oxygen pressure and arterial oxygen saturation were observed. Injection of LPS increased intestinal HO1 mRNA expression, which was further augmented following CO inhalation. CO inhalation reduced the LPS-induced increase in intestinal ICAM-1 and platelet activator factor expression as well as leukocyte infiltration and lipid peroxidation, so CO might exert its protective effect via anti-inflammatory, anti-oxidative and anti-apoptotic actions. Similar protective effects were obtained when continuously perfusing 2 L·min⁻¹ of 250 p.p.m. CO (from CO gas compressed at 250 p.p.m. with balanced air in a cylinder) into the peritoneal cavity (through an inlet in the hypogastric right region with an outlet in the epigastric left section) from 1 to 7 h after i.v. administration of LPS (Liu *et al.*, 2010a). Parameters were

measured after 1, 3 and 6 h of CO treatment and illustrated that the beneficial effect of CO was maintained for the whole perfusion period. As measured at 3 h after LPS challenge, p38 MAPK phosphorylation was increased by LPS and further enhanced by CO treatment. The group of Liu *et al.* (2010b) then also investigated the effect of a single i.p. injection of 2 mL·kg⁻¹ of 250 p.p.m. CO 1 h after LPS treatment. At 1 and 3 h after injection of CO, the LPS-modified parameters were beneficially influenced by CO but no longer at 6 h. In addition to their previous studies, intestinal levels of superoxide dismutase activity and IL-10 protein were measured; both markers were suppressed by LPS but partially reversed by CO, with IL-10 being still elevated 6 h after CO treatment. Dal-Secco *et al.* (2010) confirmed in mice the importance of ICAM-1 for LPS-induced neutrophil migration. The increased expression of ICAM-1 on mesenteric venular endothelium observed 2 h after an i.p. injection of LPS was significantly reduced by treating the animals with the CO donor dimanganese decacarbonyl (3 mg·kg⁻¹ s.c.) 30 min before LPS challenge. The effect of the CO donor was prevented by ODQ, illustrating that its effect depends on sGC activation. Recently, Wang *et al.* (2012) studied the mouse small intestine 24 h after caecal ligation and puncture. The intestine showed increased lipid peroxidation, leukocyte infiltration (myeloperoxidase activity), and TNF- α , IL-1 β , ICAM-1 and iNOS protein levels, the latter corresponding to increased NO

Table 3

Effects of CO on intestinal tissue integrity and on inflammatory parameters in preclinical models of conditions predisposing to sepsis

Reference	1		2		3		4	
Species	Mouse		Mouse		Mouse		Mouse	
Model	Haemorrhagia		Burn injury		Burn injury		Hindlimb I/R	
CO source	Inhalation 250 p.p.m. from onset of shock		i.v. CORM-2 8 mg·kg ⁻¹ immediately after burn injury		i.v. CORM-2 8 mg·kg ⁻¹ immediately after burn injury		Inhalation 250 p.p.m. concomitant with reperfusion	
Blood COHb	-		-		-		8.4%	
	Haemorrhage	+ CO	Burn	+CO	Burn	+CO	I/R	+ CO
Intestinal tissue damage	↑	↓	↑	=	-	-	-	-
iNOS protein	-	-	-	-	↑	↓	-	-
NO production	-	-	-	-	↑	↓	-	-
ROS	-	-	-	-	↑	↓	↑	=
HO1 protein	-	-	↑	↑	-	-	-	-
MPO (infiltrating leukocytes)	-	-	↑	↓	-	-	-	-
TNF- α protein	-	-	-	-	↑	↓	↑	↓
IL-1 β protein	-	-	-	-	↑	↓	=	=
ICAM-1 protein	-	-	↑	↓	-	-	↑	↓
NF- κ B DNA binding activity	-	-	↑	↓	-	-	-	-

1: Zuckerbraun *et al.* (2005a); 2: Sun *et al.* (2007); 3: Liu *et al.* (2008); 4: Scott *et al.* (2009). -, not determined.

production. All these markers of inflammation were attenuated by CORM-2 (8 mg·kg⁻¹) injected i.v. immediately after the caecal ligation procedure. Histological analysis revealed hydropic degeneration and granulocyte infiltration by the septic procedure; while CORM-2 decreased granulocyte infiltration, it did not improve the hydropic degeneration.

Effect of CO on the intestine in conditions predisposing to sepsis

Experimental studies also confirmed that conditions clinically predisposing to the development of sepsis such as haemorrhagic shock and burn injury induce intestinal damage and inflammation. The effects of CO gas inhalation or CO-RMs on the intestine in these conditions are summarized in Table 3, and show similar results to the sepsis models *per se*. In a mouse model of haemorrhagic shock (90–150 min at an arterial pressure of 25 mmHg by blood withdrawal followed by resuscitation with the shed blood), inhalation of 250 p.p.m. CO from the onset of shock until intestinal harvest 4 h after resuscitation prevented the shock-induced intestinal damage (oedema, loss of epithelial and mucosal architecture with areas of focal inflammatory cell influx; Zuckerbraun *et al.*, 2005a). Two studies investigated the intestinal consequences 24 h after a 15% total body surface full-thickness thermal injury (Sun *et al.*, 2007; Liu *et al.*, 2008). An i.v. treatment with CORM-2 immediately after burn injury had the same beneficial effect on pro- and anti-inflammatory mediators as observed in sepsis models. Only one of the two studies included histological analysis. Although CORM-2 treatment decreased burn-induced granulocyte infiltration, it did not

improve hydropic degeneration (oedema and sloughing of the villous tips; Sun *et al.*, 2007). Finally, Scott *et al.* (2009) reported that even remote I/R from the intestine (hindlimb) increases leukocyte recruitment, as measured by intravital microscopy of the submucosal postcapillary venules, and intestinal TNF- α , ICAM-1 and ROS levels. Inhalation of 250 p.p.m. CO for 3 h from the start of hindlimb reperfusion completely prevented the increase in intestinal leukocyte recruitment and TNF- α and ICAM-1 expression, but not the increase in ROS levels; this suggests that CO is mainly active through its anti-inflammatory properties in this model.

Perspectives

The studies summarized in Tables 2 and 3 support the idea that the beneficial effect of CO in sepsis might be due, at least in part, to prevention or recovery of intestinal inflammation and tissue damage, therefore, preventing the triggering role of the intestine in sepsis. Although none of the studies in Tables 2 and 3 measured gut barrier function, it is expected that the anti-inflammatory and antioxidant effects of CO on the intestine will also decrease the intestinal barrier dysfunction in sepsis. Additionally, the enhancement of host bacterial clearance (Chung *et al.*, 2008; Onyiah *et al.*, 2013) and direct bacteriostatic/bactericidal effects by CO and CO-RMs (Desmard *et al.*, 2012) might contribute to their beneficial effect in sepsis. Still, when healthy volunteers were subjected to CO gas inhalation (500 p.p.m. CO for 1 h) just before i.v. injection of LPS (2 ng·kg⁻¹), increasing COHb levels to 7%, no changes in plasma neutrophil counts and cytokine levels were observed compared with LPS alone (Mayr *et al.*, 2005).

However, Takaki *et al.* (2010) reported that arterial CO levels and monocyte HO1 protein expression are increased in patients in intensive care, with a significantly more pronounced increase in patients with severe sepsis/septic shock than in non-septic patients. In the septic patients, there was a positive correlation between survival and CO/HO1 levels. These findings further support the importance of trials with CO treatment in septic patients, especially if the beneficial effects of acute CO treatment is confirmed in humans in the ongoing clinical study in colonic surgery patients mentioned above. However, this will first require additional preclinical work to select the optimal protocol and procedures of CO delivery (concentration of CO, selection of CO-RM).

CO in NEC

Pathogenesis of NEC

NEC is an important cause of death in premature infants. It is estimated that up to 12% infants with a birth weight of less than 1500 g will develop the disease, and 30% of them will not survive as overwhelming sepsis can occur when the intestinal mucosal barrier breaks down (Gephart *et al.*, 2012). The clinical signs are often non-specific but when developing, diffuse areas of bowel necrosis can occur with the possibility of perforation, necessitating emergency surgery. The pathogenesis of NEC is not fully understood but NEC does not occur until at least 8–10 days postpartum when bacteria have colonized the gut. The intestinal immaturity with inadequate intestinal barrier function and an excessive inflammatory response towards the post-natal microbial colonization of the intestine predisposes to NEC (Neu and Walker, 2011). As tissues resected from infants with NEC are obtained at later severe stages of the disease, they cannot reveal early pathogenesis. Animal models have therefore been developed, usually combining enteral feeding and hypothermic or hypoxic stress in the preterm or early postterm period, to identify the mediators of GI inflammation in NEC (Sodhi *et al.*, 2008). A unifying pathogenetic hypothesis was recently put forward (De Plaen, 2013). Following the introduction of food, there is a proliferation of the intestinal bacteria. These bacteria and their products adhere to the epithelium, breach the immature intestinal mucosal barrier and activate lamina propria immunocytes. These produce inflammatory agents that attract further inflammatory cells, in particular neutrophils, and inflict further damage to the intestinal barrier with bacterial translocation and mucosal necrosis. The way to sepsis and shock is then open.

Role of HO1/effect of CO in NEC

In line with observations that intestinal specimens of children with NEC show increased expression of HO1 protein (Zuckerbraun *et al.*, 2005b), two recent experimental studies suggest that the impaired ability of the intestine to react with HO1 expressed in the immature intestine contributes to the development of NEC. Exposure of newborn rats (day 1) to hypercapnia followed by re-oxygenation induced gut barrier failure (increased peroral FITC-dextran uptake) and increased intestinal iNOS expression and caspase-3 activity, but not so in weanling rats (15 days). In the latter case, the procedure

induced the expression of HO1 protein in the GI tract within 24 h, while HO1 protein was only increased at 72 h in newborn rats. Pretreatment of newborn rats with haemin induced earlier expression of HO1 and attenuated gut barrier failure and apoptosis (Pietzcker *et al.*, 2012). Using 7-day-old heterozygous HO1 (^{+/}) mice, Schulz *et al.* (2013) showed that a partial lack of HO1 promotes the development of NEC induced by combining oral gavage of formula food and exposure to hypoxia; the enterocolitis incidence was higher and the survival rate lower than in wild-type mice. Whether either one or both of the HO1 products, CO and biliverdin-bilirubin, prevent enterocolitis was not investigated in the studies of Pietzcker *et al.* (2012) and Schulz *et al.* (2013). Zuckerbraun *et al.* (2005b) showed that exposure to 250 p.p.m. CO for 1 h per day in neonatal rats exposed to hypoxia and formula feeding from day 1 to 3 decreased terminal ileal inflammation and reduced mortality on day 4. The beneficial effects of CO were associated with attenuation of the hypoxia/formula-induced iNOS expression and protein nitration in terminal ileum mucosa. CO also prevented the increase in serum nitrite, TNF- α and IL-1 β levels, suggesting that CO also has a systemic effect on inflammatory cells.

Perspectives

CO might thus be considered as a therapeutic agent in human NEC. As systematic exposure of premature infants to CO will not be acceptable, additional experimental studies are required to investigate whether CO can still be effective when the signs and symptoms of suspected NEC occur, and whether the protective effects can also be obtained with CO-RMs.

CO in intestinal epithelial cells

From the above sections, it is clear that the intestinal epithelial cell layer plays a crucial role in acute GI inflammation. This underlines the importance of studies with intestinal epithelial cell lines; publications on the protective effect of CO in intestinal epithelial cell lines are summarized in Table 4. The intestinal epithelial cell lines studied are from murine, rat and human origin; the rodent cell lines (MODE-K, YAMC and IEC-6) are derived from non-cancerous GI tissues while the human cell lines (Caco-2, HT-29 and DLD-1) are from tumorous tissue sources. The pro-inflammatory cytokine TNF- α is an important inflammatory mediator in GI inflammation (Holtmann *et al.*, 2002) explaining why studies so far investigating the effect of CO or CO-RMs involved exposing monolayers of enterocytes to either TNF- α alone or in combination with other pro-inflammatory cytokines such as IL-1 β and IFN- γ (cytokine mix). All these studies involve acute measurements where the cells were exposed to TNF- α or cytokine mix from 30 min to maximally 24 h followed by cellular and transcriptional readouts.

CO treatment protects against intestinal epithelial cell death induced by inflammatory cytokines, as shown with CO gas in rat and CORM-A1 in mouse small intestinal epithelial cells (Zuckerbraun *et al.*, 2005b; Babu *et al.*, 2012). With regard to the mechanism of its protective effect, none of the

Table 4

Effects of CO on intestinal epithelial cell lines

Cell type	Inflammatory trigger	CO source	Effect of inflammatory markers/overall outcome	Reference
Human colonic epithelial cell line, DLD-1	CM	250–400 p.p.m. CO	↓ iNOS mRNA expression	1
Rat small intestinal epithelial cell line, IEC-6	CM	250 p.p.m. CO	↓ iNOS protein expression ↓ Nitrite production	2
	TNF- α plus actinomycin D	250 p.p.m. CO	↓ Cell death (apoptosis)	
	LPS/hypoxia	250 p.p.m. CO	↓ Cell death ↓ iNOS protein expression ↓ Transcriptional activation of iNOS promoter	
Human colonic epithelial cell line, HT-29	TNF- α	CORM-2 (5–20 μ M)	↓ IL-8 mRNA expression ↓ ICAM-1 and COX-2 protein expression	3
Human colonic epithelial cell line, Caco-2	CM	CORM-2 (50–150 μ M)	↓ Nitrite production ↓ iNOS mRNA expression ↓ IL-8 protein expression ↓ Transcriptional activation of NF- κ B ↓ I κ B α phosphorylation ↓ MAPK (p38, ERK 1/2 and JNK 1/2) phosphorylation	4
Young adult mouse colonic epithelial cell line, YAMC	No trigger*	CORM-2 (1–10 μ M)	↑ Wound repair of cell monolayer	5
Young adult mouse colonic epithelial cell line, YAMC	TNF- α	CORM-2 (10 μ M)	↓ KC mRNA expression ↓ KC production ↓ NF- κ B activity	6
Mouse duodenal epithelial cell line, MODE-K	TNF- α plus cycloheximide	CORM-A1 (100 μ M)	↓ Cell death (apoptosis) ↑ GSH levels ↓ ROS production ↓ Caspase-3/7 activity	7

1: Dijkstra *et al.* (2004); 2: Zuckerbraun *et al.* (2005b); 3: Lee *et al.* (2007); 4: Megias *et al.* (2007); 5: Uchiyama *et al.* (2010); 6: Takagi *et al.* (2011); 7: Babu *et al.* (2012). CM, cytokine mixture.

*No inflammatory trigger is used; central manual scraping of monolayer creating a wound.

studies have investigated the involvement of sGC and only the study by Megias *et al.* (2007) examined a possible contribution of p38 MAPK. In fact, CORM-2 treatment reduced the cytokine-induced increase in phosphorylation of MAPK p38, similar to its effect on ERK 1/2 and JNK 1/2 phosphorylation. This is in contrast with many *in vivo* studies on acute GI inflammation reporting that CO further enhances inflammation-induced p38 MAPK phosphorylation (see Tables 1 and 2). An up-regulation of iNOS and sustained overproduction of NO have been associated with intestinal mucosal damage and barrier dysfunction of the gut in acute GI inflammation. Hence, several studies investigated the effect of CO on iNOS expression/NO production in intestinal epithelial cell lines. Consistently, CO treatment reduced the cytokine-induced expression of iNOS at the mRNA or protein level, correlating with suppression of nitrite production (Dijkstra *et al.*, 2004; Zuckerbraun *et al.*, 2005b; Megias *et al.*, 2007). No evidence for a selective effect of CO on iNOS activity has been obtained (Sawle *et al.*, 2005). In a study with the rat small intestinal epithelial cell line IEC-6 (Zuckerbraun

et al., 2005b), CO also inhibited LPS- and/or hypoxia (1% oxygen)-induced increase in iNOS protein expression, which was corroborated by the decrease in the transcriptional activation of the iNOS promoter. The effect of CO on iNOS in intestinal epithelial cells might be related to its upstream influence on the transcription factors involved in inflammation-induced iNOS expression. In the elaborate study of Megias *et al.* (2007) in human Caco-2 cells, CORM-2 indeed reduced the transcriptional activation of NF- κ B triggered by a cytokine mixture. Moreover, I κ B α phosphorylation after cytokine challenge was also prevented by CORM-2, thus implying that CO might directly influence NF- κ B activation. Interestingly, this study also reported that CORM-2 reduced the increased expression of IL-8. IL-8 is the best characterized member of the neutrophil chemoattractant family (Rot and von Andrian, 2004). It is rapidly up-regulated in human colonic epithelial cell lines in response to stimulation with bacteria, TNF- α or IL-1 β and mimics acute mucosal inflammatory conditions (Eckmann *et al.*, 1993; Jung *et al.*, 1995; Gewirtz *et al.*, 2001). Also increased IL-8 mRNA

expression has been observed in the intestinal epithelial layer from infants with NEC (Nadler *et al.*, 2001). Moreover, IL-8 expression is transcriptionally regulated by MAPK phosphorylation and NF- κ B activation in intestinal epithelial cells (Berin *et al.*, 2002). Rodents lack IL-8, but express keratinocyte chemoattractant (KC), a functionally related homologue of IL-8 in humans. This KC derived from epithelial cells has been reported to be involved in the pathophysiology of neutrophil-dependent animal models of intestinal inflammation (Li *et al.*, 2004; Liu *et al.*, 2010c). CORM-2 inhibited KC gene expression and protein production along with inhibition of NF- κ B transcriptional activation in TNF- α -stimulated young adult mouse colonic epithelial cells (Takagi *et al.*, 2011).

The anti-inflammatory effects of CO/CO-RMs required HO1 induction in other isolated cell systems such as hepatocytes (Zuckerbraun *et al.*, 2003), macrophages (Sawle *et al.*, 2005), microglia (Min *et al.*, 2006) and endothelial cells (Kim *et al.*, 2007); however, treatment with CO-RMs does not influence HO1 protein expression in human colonic Caco-2 cells (Megias *et al.*, 2007) and murine small intestinal epithelial MODE-K cells (Babu *et al.*, 2012), and thus no evidence for a positive feedback loop CO/HO1/CO was obtained. In another human colonic epithelial cell line, HT-29, Lee *et al.* (2007) reported that bilirubin *per se* had a similar inhibitory effect as CORM-2 on TNF- α -induced IL-8, ICAM-1 and COX-2 expression, and the effect was additive when CORM and bilirubin were incubated together, suggesting that pharmacological induction of HO1 might lead to more pronounced beneficial effects than exogenous CO. However, the HO1 inducer cobalt protoporphyrin decreased TNF- α plus actinomycin D-induced apoptotic cell death of IEC-6 cells only to the same extent as exogenous CO gas (Zuckerbraun *et al.*, 2005b). Only one study investigated the influence of CO on ROS production in intestinal epithelial cells (Babu *et al.*, 2012). The increased ROS production and the decreased levels of cellular reduced GSH induced by TNF- α /cycloheximide in MODE-K cells were partially prevented by treatment with CORM-A1. This correlated with diminished caspase-3/7 activity, apoptosis and cell death, suggesting that modulation of ROS/oxidative stress might be involved in the anti-apoptotic and cytoprotective effects of CORM-A1.

During acute GI inflammation associated with oxidative stress, impairment of the epithelial surface barrier with accompanying erosion demands rapid resealing of this barrier to maintain homeostasis. The whole process of rapid resealing of superficial wounds involving migration of the epithelial cells adjacent to the injured surface to the site of mucosal wound defect, differentiation and reorganization of the cytoskeleton with ultimate closure of the denuded area to establish the continuity of surface epithelium is termed 'epithelial restitution'. Epithelial restitution is a critical process which does not involve epithelial cell proliferation, but contraction of the cytoplasmic processes of underlying myofibroblasts (Moore *et al.*, 1989). Uchiyama *et al.* (2010) provided evidence in YAMC cells that CO can promote colonic mucosal restitution. Myofibroblast-conditioned medium enhanced the wound repair of a manually denuded monolayer of YAMC cells to a more pronounced extent when the myofibroblasts were treated with CORM-2. This beneficial effect of CORM-2 on epithelial cell restitution was abolished

by treating the myofibroblasts with siRNA for fibroblast growth factor (FGF)15. CORM-2 increased FGF15 mRNA and protein expression probably by down-regulating microRNA miR-710.

All studies in intestinal epithelial cell lines thus support the hypothesis that CO can reduce acute mucosal inflammation and damage, and even stimulate intestinal epithelial cell restitution. The beneficial effect of CO in *in vivo* models of sepsis and NEC might thus at least be partially due to its effects at the mucosal level. It is thus advisable that intestinal inflammation and the effect of CO in these models are studied separately in the mucosal versus the muscular layer of the GI tract to further elucidate the mechanism of action in these conditions.

Conclusion

In preclinical *in vivo* models of acute GI inflammation during POI after abdominal surgery, sepsis and NEC, treatment with CO gas or CO-RMs suppresses the intestinal infiltration of leukocytes and the expression of inflammatory mediators, leading to improved GI transit (POI) and decreased intestinal damage (sepsis, NEC). This beneficial effect is confirmed in intestinal epithelial cell lines exposed to cytokines. The mechanism of action of CO in acute GI inflammation has not been profoundly examined, but enhanced p38 MAPK phosphorylation, suppressed iNOS expression and decreased production of ROS are most consistently reported. However, the beneficial effects of CO in humans affected by acute GI inflammation still need to be confirmed. The most straightforward indication to attempt CO for acute GI inflammation in humans, as administration can be started in a preventive way before the planned operation starts, is abdominal surgery, and that is exactly where a clinical trial with inhaled CO is now ongoing. Even though this trial will lead to positive results with CO, the application of CO in humans with sepsis or NEC will require more extensive preclinical work to determine the optimal route of CO administration. For instance, what is the maximal tolerable and beneficial dose of CO gas inhalation? Which CO-RM should be used and what is the dose and the best route of administration? When is the best time to start CO treatment once sepsis or NEC has developed? How long should the CO gas inhalation be applied for? Are single or repetitive administrations of CO-RM or CO gas needed? Is the residual molecule formed after CO release from CO-RMs safe or toxic? All these and similar important issues are required to be properly addressed in animal studies before moving on to humans.

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Conflict of interest

No conflict of interest.

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