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Review

Carbon monoxide: the bad and the good side of the coin, from neuronal death to anti-inflammatory activity

P. F. Mannaioni, A. Vannacci, E. Masini

Department of Preclinical and Clinical Pharmacology, University of Florence, 50139 Florence, Italy, Fax: ++39 (0)554271280, e-mail: pierfrancesco.mannaioni@unifi.it

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Abstract. The double origin of carbon monoxide (CO) as an atmospheric pollutant or as an endogenous gaseous modulator of many pathophysiological processes prompted us to review some aspects of the bad side and of the good side of coin among the pleiotropic effects of CO.
On the bad side of the coin, we focus on the interval form in acute CO poisoning, discussing experimental evidence suggesting that the delayed neuropathology after CO poisoning is a free radical-driven event. In this context, we challenge the mandatory place of hyperbaric oxygen therapy (HBO) in CO poisoning as a possible summation of oxy-radicals generated by HBO and the free radical cascade set in motion during the reoxygenation phase of acute CO-poisoning. We also discuss an opposing view, which provides evidence suggesting that HBO therapy actually decreases the load of free radicals in acute CO-poisoning and may be beneficial in preventing delayed neuropsychiatric sequelae.
On the good side of the coin, we briefly outline the endogenous generation of CO and the leading role of heme–oxygenases (HO) in relation to the place of CO in biology and medicine.
The main focus of this section is on the growing literature on CO and inflammation. Here we report on in-vitro and in-vivo studies on the modulation afforded by exogenously administered/endogenously produced CO in a variety of experimental and clinical settings of inflammation.
Our recent studies on experimental models of allergic inflammation are also discussed, and the CO-releasing molecules envisaged as potential anti-inflammatory drugs suitable for clinical use.

Key words: Carbon monoxide – Heme oxygenase – CO poisoning – inflammation, CO-releasing molecules

Correspondence to: P. F. Mannaioni

The bad side of the coin

Acute CO poisoning

The figures for the morbidity and the mortality of acute CO poisoning fluctuate widely, according to the country and the methods of epidemiological surveillance. In the United States CO poisoning results in approximately 40,000 visits in the Emergency Department annually [1], with a mortality toll of 600 deaths per year [2]. In the UK, Blumenthal [3] reports a mortality toll of 1000 deaths per year; in France, CO poisoning accounts for 5000–8000 hospital admissions yearly [4]; in Italy, in the years 1993–1994, 12,000 cases have been admitted to hospital, averaging about 6000 cases every year, with more than 300 deaths per year [5]. However, the true incidence of CO poisoning is not known, since many non-lethal exposures go undetected [6]. Mortality rate as high as 31 % has been reported in large series, although in other surveys it has been only 1–2 % [7]. Notwithstanding the variability in the epidemiological data, CO poisoning is a common condition with a significant risk of mortality, and in those surviving the intoxication there is a substantial incidence of long term brain dysfunction [8–10].

An updated sample of the epidemiology of CO poisoning is reported in Table 1. The Greater Florence area, from which patients are conveyed to Florence General Hospital (A.U.O.C. Careggi, Florence, Italy) encompasses about one million inhabitants. Our retrospective epidemiological survey of CO poisoning, relative to the years 1999–2003, revealed 125 hospital admissions for acute CO poisoning. The demography of our population shows a prevalence of middle-aged patients almost equally represented as male and female; 32 % of the patients experienced loss of consciousness with average COHb levels of 23 %. The vast majority of intoxications were due to faulty indoor heaters, only 3 % being attempted suicide. Interestingly, there were no casualties in our sample, while a consistent incidence of late neuropathological sequelae (6.4 %) was observed at 12 month follow up.

Age, Yr	Sex	Loss of consciousness %	COHb %	Outcome R	D	N	Total
	M44.8 %						
42.9 ± 20.1		32	23.0 ± 10.2	93.6	0	6.4	125
	F 55.2 %						

Table 1. Retrospective epidemiology of carbon monoxide poisoning in the city of Florence, Italy. The data show the absence of mortality and the presence of late neurological sequelae.

R: Recovered on release from the hospital
D: Died
N: Late neurological sequelae

The interval form of CO poisinong

The pathophysiology and clinical findings of acute CO poisoning have been extensively reviewed [11]. The course of CO poisoning may be roughly divided into interval and non-interval forms, the former referring to neuropsychiatric symptoms occurring within several days or weeks of an asymptomatic period which follows recovery from unconsciousness in an acute state of poisoning. The cause is thought to be progressive, diffuse demyelinization of cerebral white matter in rather specific brain areas. The most common neuropsychiatric manifestations of the interval form are lethargia, behaviour changes, forgetfulness, memory loss and Parkinsonian features [12]. Three cases of interval form of CO poisoning have been reported [13], showing akinetic mutism related to white matter hyperintensities in the bilateral globus pallidus. Brain injury associated with cognitive sequelae and white matter hyperintensities have been reported in seventy three consecutive CO poisoned patients [14], with extended localization in the brain (see [14], for a review). Magnetic resonance (MR) images revealed multiple lesions in the subcortical white matter and basal ganglia in 12 patients who had delayed encephalopathy, out of 89 patients with CO intoxication, showing that the neurological manifestations correlated roughly with neuroimaging changes [15]. A lack of correlation was found between some biochemical markers for brain damage and CO poisoning: neuron-specific enolase and S-100 beta protein, the structural protein of astroglia, were not increased after CO poisoning, failing to be predictive of the delayed neuropathological sequelae [16]. However, no direct information on S-100 beta protein in the interval form of CO poisoning is available.

Is the delayed neuropathology after CO poisoning a free radical-driven event?

Some pathophysiological aspects of CO poisoning are consistent with post-ischemic reperfusion injury, in that in CO poisoning there is an hypoxic phase usually followed by reoxygenation. In fact, the severity of CO poisoning does not correlate with COHb levels [17]. It is worth noting that both the hypoxic stress and the variability in COHb levels are a function of the variable degree of underlying pathologies in the patient population. In keeping with the idea that CO poisoning may precipitate an oxidative stress, many reports have described the generation of free radicals in CO poisoning. In the brain of CO poisoned rats, xanthine dehydrogenases are converted to xanthine oxidases in the late phase of intoxication, parallel to the increase in conjugated dienes, suggesting that xanthine oxidase-derived reactive oxygen species are responsible for the lipid peroxidation of neuronal membranes [18]. In the brain of rats subjected to CO-induced hypoxia and then reoxygenated, hydroxyl radicals were detected, both in the hypoxic and in the reoxygenation phase [19]. Therefore, cellular hypoxia does not account for some aspects of CO poisoning, as shown by the poor correlation between clinical status and COHb levels, and by the remnant effects of CO after COHb has been cleared from circulation [20]. Studies conducted with rats made leukopenic, or treated with inhibitors of leukocyte adhesion to the vasculature, show that these groups of animals did not exhibit the biochemical changes observed in the brain of sham operated rats, such as the conversion of xanthine dehydrogenase to xanthine oxidase, and lipid peroxidation of neuronal membranes, occurring at 90 min following CO poisoning [21]. These results suggest a key role for leukocytes in the late brain damage of CO poisoning, to be regarded as a non-bacterial inflammation [21]. A 10 fold increase in nitrotyrosine production was found in brain of CO poisoned rats [22]. The data further show that peroxynitrite may be generated during CO poisoning, due to the enhanced rate of production of both nitric oxide (NO) and superoxide [22]. Taken together, these observations suggest that one of the possible mechanisms of brain damage in CO poisoning is the stimulation of free radical formation, producing neuronal death, responsible for the late neuropathological sequelae.

Among the free radical species produced in the late phase of neuropathology, the role of reactive oxygen species (ROS) is pre-eminent since the hydroxyl radical has been detected in the late re-oxygenation phase [18] and superoxide anion as a cause of nitrosative stress [22]. The involvement of other ROS, such as hypochlorite anion, may be inferred from experiments showing a lack of brain damage in the absence of granulocytes and of myeloperoxidase generating hypochlorite anions [21]. Thus CO-mediated brain lipid peroxidation is the result of a free radical cascade, which degrades membrane lipids according to the classical mechanism of a redox reaction, strongly dependent on the balance between oxidative stress and the antioxidant system. However, information on the limitation of antioxidant defence in CO poisoning is lacking.

Hyperbaric oxygen therapy in CO poisoning

Hyperbaric oxygen therapy (HBO) is considered to be the treatment of choice for CO poisoning, since it shortens the

duration of coma and decreases the early mortality [23]. As far as the neuronal death in the late neuropathological sequelae, HBO is more controversial, since hyperoxia induces the formation of free radicals, which could be additive to the free radicals actually generated in CO poisoning. In fact, increased formation of free radicals in hyperoxia has been extensively reported [24]. Hyperoxia induces oxygen radical production in rat lung mitochondria [25]; lung injury by hyperoxia in the newborn lambs is linked to the increase in membrane lipid peroxidation, and to the decrease in lung reduced glutathione (GSH) levels [26]. In man, HBO treatment of healthy volunteers induced DNA damage in the alkaline Comet assay with leukocytes [27]; in twelve patients treated with HBO for pathological conditions related to hypoxia, HBO led to a significant accumulation of plasma reactive oxygen metabolites [28].

On the other hand, HBO was found to prevent brain lipid peroxidation when administered to rats for 45 min beginning and 45 min subsequent to CO poisoning, suggesting that HBO could prevent CO-mediated brain lipid peroxidation. The authors conclude that there is a tissue level effect of HBO, unrelated to the diminution of COHb, which antagonizes CO-mediated brain lipid peroxidation.

Moreover, recent evidence suggests that HBO could be considered as an anti-inflammatory tool, tentatively offerring a rationale for its use in acute CO poisoning, besides the increased clearance of COHb. In considering acute CO poisoning as an hypoxia/reoxygenation disease, in which free radicals are produced in an inflammatory-like cascade, recent data on the anti-inflammatory effects of HBO on experimental paradigms have to be considered. Using a lipopolysaccaride (LPS)-stimulated rodent model, it has been shown that HBO attenuates LPS-induced lung injury [29], and this attenuation involves inducible nitric oxide synthase (iNOS) [30]. Using a cultured lens epithelium model, it was also shown that HBO induces HO-1 expression [31], and increases the level of HO-1 in human lymphocytes [32]. More recently, in an LPS-induced acute lung injury in the rat, HBO attenuates the morphometric and biochemical parameters of inflammation. The beneficial effect of HBO involves both the inhibition of iNOS and the subsequent decrease in NO synthesis, as well as the induction of HO-1 and the subsequent increase in CO generation [33]. Interestingly, hemin (a HO-1 inducer) significantly mitigates the inflammatory parameters, either alone or in combination with HBO, while tin protoporphyrin (a HO-1 inhibitor) reinstates the inflammatory injury [33]. Therefore, if acute CO poisoning, either in the interval or non-interval form, is regarded, at least in part, as an inflammatory reaction, HBO may be an effective therapeutic modality to reduce the free radical-driven injury, by downregulating iNOS and upregulating HO-1.

The interaction between the two gaseous modulators, CO and NO has been repeatedly addressed (see [34], for a review), showing cross-talk between the iNOS/NO and HO-1/CO pathways (Fig. 4). That NO and NO releasing agents stimulate HO-1 expression thus increasing CO production, has established over many years in a variety of in-vitro/in-vivo settings in different tissues and experimental models [34]. Conversely, inhibition of HO-1 greatly enhances NO production [34], suggesting that endogenous CO down-regulates the generation of NO. In fact, spinal motor neurons from HO-1-null mice are strikingly more sensitive to NO cytotoxicity than cells expressing HO-1 [35], suggesting a feed-back mechanism excited by CO towards NO-induced cytotoxicity. Thus, in neuronal cells, HO-1 may be viewed as a front-line defence against NO toxicity [35]. The complexity of the relationship between NO and HO-1 is highlighted in the commentary by Zuckerbraun et al. [36]: in a model of liver injury, not only does NO up-regulate NO-1 in the liver, but HO-1 and CO also increase iNOS expression. The current literature is almost unanimous in considering NO as an inducer of HO-1 [36]. Less clear is the interplay between CO and NO, since CO has been shown to down-regulate or up-regulate iNOS depending on the organ (brain, lung, intestines, skeleton, muscle) and the experimental settings [37]. However, the data derived from the above mentioned studies cannot be directly applied to humans.

Surveys of the outcome of CO poisoning in patients treated with HBO in comparison with normobaric oxygen therapy (NBO) have been carried out repeatedly. A 9 month analysis of the outcome of CO poisoning was carried out in 1985 [38] in 230 consecutive hospital admissions. Out of 230 subjects, 4 patients (1.7 %) died in hospital, 3 of them having received HBO therapy. Out of 203 subjects treated with HBO therapy, 9 (4 %) showed delayed neuropathological sequelae, within a free interval of 2 weeks after hospital discharge. A further survey was carried out 10 years later [39] on 900 patients subdivided into three groups of 300, according to three different protocols, using HBO at 2.45, 2.80, and 3.00 Atm. Seizure activity occurred during HBO therapy in 16 out of the 900 patients (1.8 %). Seizures developed in 1 out of 300 patients treated at 2.45 Atm (0.3 %), in 9 out of 300 patients treated at 2.80 Atm (3.00 %), and in 6 out of 300 patients treated with 3.00 Atm (2 %). An overview of the differential outcome in terms of delayed neuropathological sequelae in CO poisoning has been given recently by Gorman [40], showing a small, but significant trend towards a protective effect of HBO therapy. Furthermore, a recent study showed that repetitive HBO treatments may prevent the delayed neuropsychiatric sequelae of CO poisoning when applied individually, by monitoring the peak alpha frequency as an indicator of efficacy [41].

Our recent observations on the incidence of late neurological sequelae, comparing HBO with NBO therapy, show that out of 26 patients treated with HBO, 3 (11.5 %) developed neuropathological sequelae, in comparison to 14 out of 82 patients (17.1 %) treated with NBO therapy, at 1 year follow up from acute CO intoxication, although the difference did not reach statistical significance.

In conclusion, no controversy exists about the effectiveness of HBO in abating the mortality and the length of stay in hospital of patients with acute CO poisoning, while the protective role of HBO in preventing the neuropathological sequelae is still an issue. Several unblinded, non-randomized trials have suggested that the use of HBO prevents the development of neuropathology [42]. A recent analysis was carried out to examine the effectiveness of HBO compared with NBO for the prevention of neurological sequelae in patients with acute CO poisoning. On the basis of Medline (1966 to present) and Embase (1980 to present), six randomized

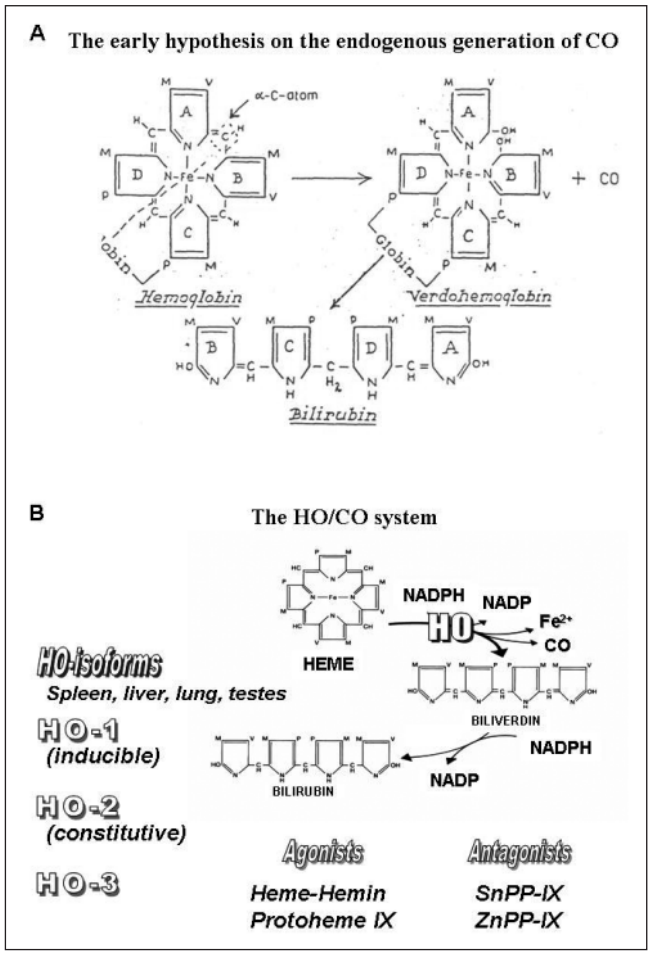

Fig. 1. (A) The early hypothesis on the endogenous generation of CO. Modified from Engstedt et al. [47]. (B) Schematic representation of the HO/CO pathway.

controlled trials were identified. Out of these six trials, four revealed no benefit of HBO for the reduction of neurological sequelae, while two other trials did [43]. Therefore, existing randomized trials have not established a preventive effect of HBO on the occurrence of delayed neurological sequelae, and call for further research involving multi-center randomized controlled trials [42].

The good side of the coin

The endogenous generation of CO

As early as 1894, the physiological presence of a combustible gas in the blood of the dog was detected by Grehant, and subsequently confirmed as CO by De Saint-Martin [43, 44]. The following observations by Sjostrand [45] were relevant to the endogenous generation of CO: in bedridden patients it was found that the mean concentration of CO in the expired air was significantly higher than in the inspired air [45] and that the amount of exhaled CO was considerably increased in patients with hemolytic anemia and other diseases with increased hemoglobin breakdown [43–45]. Therefore, it was suggested, as early as 1957, that CO would be produced by opening at the alpha-C atom of the tetrapyrrol ring of heme into an open pigment chain [42] (Fig. 1A). The question whether the physiological levels of COHb could be accounted for by breathing CO-polluted air, or endogenously produced CO, has been repeatedly addressed. The observation that the seals of the Weddell Sea in the Antarctic have a CO content in the blood six times higher than the mean values for human non-smokers, despite breathing non-polluted air, lent further support to the endogenous generation of CO, produced by the breakdown of hemoglobin, which is 40–50 % higher in the seals than the average values in human adults [47]. A clear cut estimation of CO production in man was given by measuring the COHb levels in normal subjects rebreathing in a closed system. Under these circumstances, a steady increase in the blood COHb levels was observed [48] allowing the evaluation of CO production in normal man [49].

The breakthrough in the pathophysiology of CO has been the discovery that the enzymatic degradation of heme, arising from the catabolism of hemoglobin and of the prosthetic moieties of hemoproteins, is carried out by the family of heme oxygenases (HO), resulting in the generation of Fe^{2+}, CO and biliverdin, to be further oxidized to bilirubin. The HO enzyme system was first described by Tenhunen [50], and its activity repeatedly reviewed [51, 52]. In most mammalian cells there are at least three forms of HO: the oxidative stress and heme inducible HO-1, and the constitutively expressed HO-2 and HO-3 [53, 54] (Fig. 1B). Thus, the HO/CO system, which shares some of biochemical and biological properties with the nitric oxide (NO)/nitric oxide synthase (NOS) system, may play a role as a widespread signal transduction mechanism in the regulation of cell function and communication [55].

The role of CO in biology and medicine

The role of CO in biology and medicine has been recently reviewed [56]. It appears that CO has a variety of pathophysiological roles, as represented in Figure 2. To briefly summarize the most relevant observations, in endothelium-denuded rings of mesenteric arteries of piglets, precontracted with the thromboxane A_2 mimetic U46619, CO produces a concentration dependent relaxation, completely abolished by the soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) [57]. CO inhibits human airway smooth cell proliferation, in a way that is not blocked by ODQ, but involves activation of the mitogen-activated protein kinase (MAPK) pathway [58]. CO generated by HO-1 suppresses apoptosis in endothelial cells [59], while overexpression of HO-1 increases caspase-3 activation in vascular smooth muscle cells [60]. Inhaled CO shows cytoprotective efficacy during intestinal ischemia/reperfusion injury associated with small intestine transplantation in Lewis rats [61], and in rat orthotopic lung transplantation [62]. CO inhibits human platelet aggregation triggered by threshold levels of agonists, probably acting by increasing cGMP levels [63]. Only circumstantial evidence is available on the role of CO in the central nervous system. In cultures of primary olfactory neurons, odor-

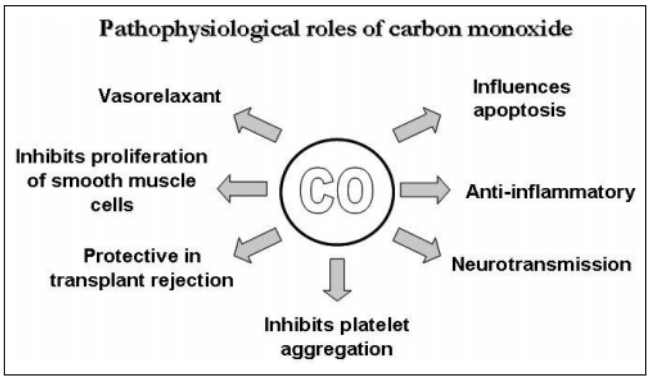

Fig. 2. A scheme representing the most relevant pathophysiological roles of carbon monoxide.

ants markedly enhance cGMP concentrations, which were restored to basal levels by hemoglobin, a CO scavenger. In the same experiments, CO directly raised cGMP levels, suggesting its role as a neural messenger associated with the maintenance of endogenous cGMP concentrations in the brain [64]. Indirect evidence, based on the use of HO-1 inhibitors also suggests a role for endogenous CO in the modulation of nociceptive transmission [65] and of neuronal activation during seizures [66].

The role of CO in inflammation

In vitro studies

The group of Otterbein has provided solid evidence for the anti-inflammatory effects of CO in vitro. Exposure of wild type RAW-264 macrophages to low concentrations of CO selectively inhibited the expression of LPS induced pro-inflammatory cytokines, while increasing the LPS-induced expression of anti-inflammatory cytokines [67]. Interestingly, the anti-inflammatory effects of CO were not mediated through a guanylyl-cyclase/cGMP pathway, but through a pathway involving MAPKs. Other direct proof of the anti-inflammatory effect of CO was given subsequently [68]. In purified CD4+T cells from the Jurkat clone EG-1, exposure to CO produces a suppressive effect on tritiated thymidine uptake in response to stimulation with anti-CD3 plus anti-CD28 antibodies. In the same experimental setting, the other two products of HO-1, Fe^{2+} and bilirubin, failed to show any antiproliferative effect. The inhibitory effect was independent of the guanylyl-cyclase/cGMP pathway, and possibly linked to inhibition of extracellular signal-regulated kinase activation [68].

In-vitro experiments performed with fresh biopsy specimens of colonic mucosa from healthy humans and with human colon carcinoma cell and DLD-1 have shown that incubation with CO 250–400 ppm, prior to exposure to a cytokine mix to mimic inflammation, directly inhibits iNOS mRNA induction, as well as serving a protective function in epithelial cells [69].

In immortalized human hepatocytes, the induction of HO-1 has anti-apoptotic effects through an increase in Bcl-XL expression. The HO-1 mediated Bcl-xL expression depends on the activation of the p38 MAPK pathway, and is potentiated by exogenous CO which increased cellular ATP levels, suggesting that CO can directly stimulate the generation of ATP in human hepatocytes [70]. Accordingly, the emerging role of CO in the gastrointestinal tract now encompasses an extraordinary range of physiological functions in health and disease, such as enteric neurotrasmission, the regulation of NO signalling, smooth muscle tone, membrane hyperpolarization, and antiapoptotic and anti-inflammatory effects (see [71] for a review).

The emerging data suggest that CO could be seriously considered as a therapeutic anti-inflammatory agent in liver and intestinal diseases. However, a negative profile for the HO/CO system is likely both in clinical and experimental cirrhosis. In the isolated perfused mesenteric artery of rats, made cirrhotic by combined treatment with CCl_4 and phenobarbital, the constriction responses to KCl, phenylephrine and endothelin-I were decreased. The vasoconstriction responses were augmented by stepwise inhibition of CO production. These findings suggest that CO exerts a vasorelaxant effect on the mesenteric circulation, thus contributing to portal hypertension, the major complication of cirrhosis.

Surprisingly, the expression of the constitutive isoform of HO (HO-2) was increased in the mesenteric region of cirrhotic rats. It is suggested that inhibition of HO may serve as a therapeutic option in cirrhotic patients, possibly improving portal hypertension [72]. However, outstanding evidence in favour of a pleiotropic protection afforded by the HO/CO system, with special emphasis on hepatobiliary dysfunction, have been reported continuously [73].

Indirect evidence for the anti-inflammatory effect of CO was provided in experiments carried out in cells overexpressing HO-1. Overexpression of HO-1 resulted in protection against *Pseudomonas aeruginosa*-induced injury in a cystic fibrosis airway epithelial cell line [74]. Dendritic cells play a central role in the induction of immunity and tolerance, by undergoing maturation in response to danger signals, resulting in the secretion of pro-inflammatory cytokines. Induction of HO-1 expression in human and rat dendritic cells inhibits LPS-induced phenotypic maturation and secretion of pro-inflammatory cytokines, resulting in a decrease in alloreactive T-cell proliferation. This novel immune function of HO-1, presumably shared by the products of its activity, CO among them, may be of interest for the inhibition of the immune response in autoimmune diseases [75]. However, the concomitant effects of ferritin and bilirubin cannot be ruled out in the interpretation of the anti-inflammatory effect due to the overexpression of HO-1.

In vivo studies

In-vivo studies on the anti-inflammatory effect of CO can be subdivided into experiments dealing with direct exposure to CO and experimental settings using HO-1 transfection and HO-1 induction. Ventilator-induced lung injury is a major cause of morbidity and mortality in intensive care units, through increased alveolar-capillary permeability, recruitment of neutrophil leukocytes and alveolar macrophages,

causing inflammation in the lung. The demonstration that CO provides protection against hyperoxic lung injury [76] prompted the testing of the hypothesis that inhaled CO could exert anti-inflammatory effects on an experimental model of ventilation-induced lung injury. In the broncho-alveolar lavage fluid of rat, ventilation (15 min to 3 h) or ventilation plus LPS treatment significantly raises the number of cells and the amount of tumor necrosis factor-alpha (TNF-alpha). Low concentrations of inhaled CO significantly reduced TNF-alpha levels and total cell count in lavage fluid of rats under ventilation alone and plus LPS. The mechanisms of the CO-mediated anti-inflammatory effect are suggested to involve the p38 MAPK pathway [77]. CO is increased in the exhaled air of asthmatic patients [78] and in newborn infants at term with sepsis [79]. However, it is not known whether the increased levels of CO in the exhaled air [78] is a causative link in the biology of asthma or whether the increased plasma levels of CO contribute to the vasodilatatory characteristic of sepsis [79]. Recent studies have consistently demonstrated that the level of exhaled CO is associated with the clinical severity of asthma [80].

In humans, the anti-inflammatory effects of CO have been thoroughly analyzed. In a randomized, double-blind, placebo-controlled trial, 9 healthy volunteers inhaled synthetic air (as placebo), and 500 ppm CO for 1 h in a random order and received a 2 ng/Kg intravenous bolus of LPS after inhalation. LPS infusion transiently increased plasma concentrations of a host of pro-inflammatory mediators (TNF-alpha, IL-6, IL-8, IL-10, IL-1alpha and IL-11beta mRNAs). Interestingly, the LPS induced increase in pro-inflammatory mediators was not influenced by CO inhalation [81]. Therefore, inhalation of 500 ppm CO for 1 h had no anti-inflammatory effect in a systemic inflammation model in humans. These data are in sharp contrast to similar experiments carried out in rodent models. In particular, inhalation of 250 ppm CO for 1 h significantly reduced TNF-alpha and IL-1 beta in the mouse experimental endotoxemia model [67]. However, it is worth noting that the human model of experimental endotoxemia [81], which is not protected by CO inhalation, is not comparable to the rodent model of sepsis, in which CO inhalation is protective [67]. In fact, LPS was given at a sublethal dose (1 mg/kg) in the rodent model, causing a striking increase in serum levels of pro-inflammatory cytokines, while in the human models LPS was given in minute amounts (2 ng/kg) causing a less sustained increase in plasma concentration of pro-inflammatory cytokines. Moreover, the results also differ in that, in the rodent model, the serum anti-inflammatory cytokine, IL-10, increased in response to LPS in animals pretreated with 260 ppm CO, while in the human model, CO had no significant influence on the LPS-induced production of IL-10.

Indirect information on the anti-inflammatory role of CO is available from in-vivo studies using experimental models of HO-1 induction or transfection. Focussing on HO-1, the role of CO as a potential anti-inflammatory agent and the clinical implication of the HO system in inflammation have been extensively reviewed [51, 54] and HO-1 as a target for the modulation of the inflammatory response has been proposed from many years. In a model of carrageenan pleurisy in the rat, the exudate volume and the number of inflammatory cells reached a maximum 24 h after carrageenan injection. At 48 h, the pleural inflammation was completely resolved. HO-1 activity in blood inflammatory cells proceeded inversely with the inflammatory process, the highest activity being present at the time of recovery from inflammation. Moreover, both the exudate volume and the number of inflammatory cells were increased by treatment with an HO inhibitor (tin protoporphyrin, SnPP) and increased by an HO inducer (iron protoporphyrin, FePP). The authors suggest that up-regulation of HO-1 may be of benefit in the treatment of inflammatory diseases [82]. In a model of systemic inflammation in mice, ICAM-1 expression was increased in liver cells. Induction of HO-1 via hemin resulted in a significant reduction of the ICAM overexpression, whereas HO-1 inhibition via chromium mesoporphyrin did not significantly alter ICAM-1 expression [83]. HO-1 modulates the early inflammatory response. In HO-1 knockout mice, splenocytes secrete disproportionately high amounts of Th1 cytokines, as compared with the wild type. These data confirm and extend the previous observation that HO-1 null mice and a human patient with HO-1 deficiency developed unusual and abnormal inflammatory diseases [84, 85]. The ETS proteins are a family of transcriptional factors that are known to be involved in inflammatory responses. In murine macrophages (RAW 264.7), LPS induced ETS-2, which in turn up-regulated HO-1, once again suggesting a modulatory role for HO-1 as a negative feed-back factor in inflammation [83].

Ischemia/reperfusion (I/R) injury may be considered, at least in part, as an inflammatory process, due to neutrophil accumulation at the site of tissue injury, and to the release of inflammatory mediators, such as oxygen free radicals and pro-inflammatory cytokines. In a model of renal I/R in the rat, inhalation of CO nullified the inflammatory parameters and significantly reduced I/R injury [86].

Hind limb I/R in mice is associated with the systemic inflammatory response syndrome, leading to liver injury during the multi-organ dysfunction syndrome. Inhalation of CO (250 ppm) significantly reduced hepatocellular injury, while HO inhibition increased inflammation and hepatocyte injury [87]. The place of CO as an anti-inflammatory agent in the gastrointestinal tract has been repeatedly shown, in addition to liver protection. Thus, inhaled CO significantly improved post-transplant motility in transplanted grafts of a small intestine transplant in the rat [88]; CO administration is effective as a therapeutic modality in mice with established chronic colitis [89], and protects against the development of experimental necrotising enterocolitis in neonatal rats [90].

In the above quoted papers, although the induction of HO-1 is indeed associated with anti-inflammatory effects, the sole participation of CO in the defence against inflammation is uncertain, since ferritin and bilirubin, the other end-products of HO activity, may be involved as anti-inflammatory mediators. Moreover, some studies indicate that HO-1 enzymatic activity is not always associated with beneficial effects; thus, iron released by HO-1 activity may contribute to membrane lipid peroxidation, whereas HO-1 inhibition, and not overexpression, is endowed with anti-inflammatory actions. This is the case with the therapeutic administration of the HO-1 inhibitor, SnPP IX, which was able to control the symptoms of adjuvant arthritis in the rat [91].

Studies in experimental models of allergic inflammation

Studies on mast cells

The initial observation that exposure to CO (100 %; 30 min) significantly reduced the release of histamine and the sequential exocytosis induced by compound 48/80 in isolated purified rat serosal mast cells [92], prompted an extension of the experiments on the modulation afforded by the HO/CO system on the allergic response of immunological cells and of sensitized tissue in vitro.

Isolated purified guinea pig serosal mast cells generate a small, but detectable, amount of bilirubin, showing the presence of the constitutive isoform, HO-2. Exposure of guinea pig mast cells to hemin, an HO-1 inducer, increased several times the generation of bilirubin, showing the presence of the inducible isoform, HO-1 [93]. Preincubation with hemin nullified the release of histamine by exposure to antigen of guinea pig mast cells from actively sensitized animals [93]. The immunological release of histamine was reinstated by HbO_2, a scavenger of CO, and by the inhibitor of guanylyl-cyclase, ODQ [93]. The anti-allergic effect of hemin was mainly due to the generation of CO. In fact, exposure to CO (100 % for 30 min) reproduced entirely the inhibitory effect of hemin on the immunological release of histamine. Also in the case of CO, the release of histamine was reinstated by HbO_2 and by ODQ, and left unchanged by exposing mast cells to nitrogen [93]. Both preincubation with hemin and direct exposure to CO produced a net increase in cGMP levels, and a diminution of intracellular Ca^{2+} [93]. The results of these experiments suggest that the HO/CO system downregulates the immunological response of guinea pig mast cells, a primary cell lineage in mounting and expanding the IgE-mediated allergic response.

Studies on basophils

Mast cells are involved mainly in the early phase of allergic inflammation [94]. Among the inflammatory cells, circulating basophils are of paramount relevance in the late-phase reaction of IgE-mediated immune response [95]. In experiments using semi-purified human basophils, hemin increased concentration-dependently the generation of bilirubin, restored to control values by an HO-1 blocker, Zn-protoporphyrin IX [96]. Exposure of human basophils to anti-IgE produced the expected release of histamine and increased the membrane protein CD63, expressed only by activated basophils. Preincubation with hemin, at concentrations suitable to produce a 4-fold increase in HO-1 activity, significantly reduced both the anti-IgE-induced release of histamine and the expression of CD63 [96], blocked by the HO-1 antagonist Zn-protoporphyrin IX, and restored by scavenging CO (HbO_2) and by blocking guanylyl-cyclases [96]. In a fashion similar to the results obtained in guinea pig mast cells, the direct exposure to CO inhibited the immunological release of histamine and the CD63 expression in human basophils challenged with anti-IgE. HbO_2 and ODQ reset the immunological activation of human basophils, which was unaffected by exposure to nitrogen. Induction of HO-1 and exposure to CO both increased cGMP levels and decreased intracellular Ca^{2+}, in the same way as in guinea pig mast cells [96]. These results suggest that the HO/CO system downregulates, not only the mast cell-driven early phase of the allergic response, but also the late phase in which basophils are mainly involved.

Studies on cardiac anaphylaxis

The HO/CO system modulates the allergic response not only in isolated immunological cells such as mast cells and basophils, but also in isolated tissues from actively sensitized animals. Cardiac anaphylaxis has been described as the increase in rate and strength of contraction, decrease in the coronary outflow and severe arrhythmias, in isolated hearts of actively sensitized animals, challenged in vitro with the specific antigen. Cardiac anaphylaxis is recognized as a type-I hypersensitivity reaction, in which the release of histamine participates in the myocardial injury, the cardiac damage being reduced by the hormone relaxin, by the agonists of histamine H_2 receptors, and by a variety of NO donors and generators [97]. In a series of experiments, isolated hearts from actively sensitized guinea pigs reacted to the administration of the antigen through the aortic cannula with a net increase in rate and strength of contraction, accompanied by the appearance of histamine in the perfusates [98–100] and by the onset of severe arrhythmias. Pre-treatment with hemin provided protection against cardiac anaphylaxis, in that the response to antigen was fully inhibited, and the release of histamine significantly reduced. Similarly in isolated immunological cells, the inhibitory effect on some parameters of allergic inflammation was associated with an increase in cardiac cGMP levels and with a decrease in tissue Ca^{2+} overload. The inhibitory effect was fully mimicked by exogenous CO, and completely antagonized by ZnPP-IX, an HO-1 inhibitor. These experiments show that pretreatment with hemin increases the HO-1 activity and expression in a way that is temporally related to the suppression of the immune response [98–100].

CO and inflammation: possible mode of action

Ample evidence has been, therefore, provided that both endogenously generated and exogenously administered CO downregulate the inflammatory process and especially the allergic response. The anti-inflammatory effect of CO has been accounted for by a variety of possible events. CO has been shown to affect several intracellular signalling pathways, including guanylyl-cyclase which generates cGMP, and the MAPK system [56]. Other possible signalling mechanisms would entail the interaction with Ca^{2+} modulated K^+ channels [101, 102], and cross-talk with the other gaseous mediator, NO [103] (Fig. 3). The issue of the interplay among the two physiological gaseous mediators is relevant. Motterlini et al. [103] found that incubation of endothelial cells with various NO-releasing agents resulted in a marked increase in the HO-1 activity, associated with cytoprotection. Beside the NO-donating drugs, also endogenous NO-generators (LPS) were found to regulate HO-1 gene expression in Kupfer cells [104]. Therefore, NO, either released by pharmacological interventions or endogenously produced within the inflammatory process or in I/R, results in the increased

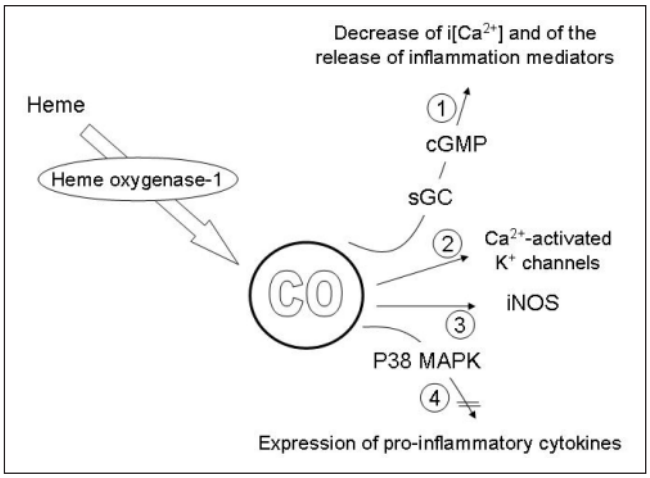

Fig. 3. The putative mode of action of HO-1-generated CO as a potential anti-inflammatory agent. (1) HO-1-generated CO binds to soluble guanylate cyclase (sGC), increases cyclic guanosine monophosphate (cGMP) levels, and decreases i[Ca^{2+}]. (2) Co modulates Ca^{2+} activated K^+ channels resulting in the decrease of i[Ca^{2+}]. (3) Cross talk with NO. (4) CO blocks the p38 mitogen activated expression of pro-inflammatory cytokines.

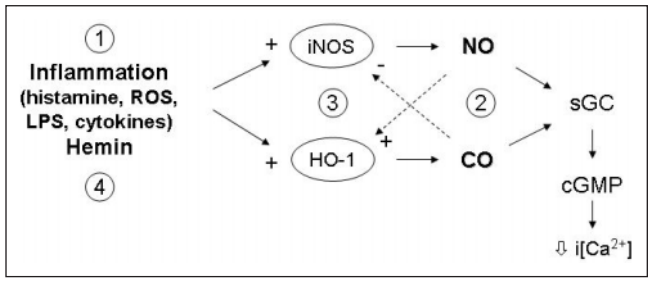

Fig. 4. Cross talk between NO and CO in inflammation. Inflammatory mediators (1) increase the generation or both NO (2) and CO (2) by overexpression of iNOS (3) and HO-1 (3). NO upregulates HO-1, while CO downregulates iNOS (3). Hemin (4) produced in inflammation upregulates both iNOS and HO-1, the two gaseous mediators co-operate in binding to sGC raising cGMP levels, decreasing i[$Ca]^{2+}$ and resulting in a downregulation of inflammation.

generation of CO. The NO-driven increase in CO production may indicate that CO exerts an anti-inflammatory action by decreasing NO synthesis, under conditions in which high concentrations of NO exert pro-inflammatory effects [105]. However, in a rat model of cardiac focal I/R (FIR), treatment of the animals with hemin (4 mg/kg i.p., 18 h before FIR) increased both iNOS protein expression and NOS activity [106]. These data show that the end products of HO-1, CO among them, actually increase the generation of NO, thereby offerring an explanation for the anti-inflammatory action of CO under conditions when low concentrations of NO exert a cytoprotective/anti-inflammatory effect. Taken together, these findings suggest that the HO/CO and NOS/NO systems co-operate to modulate allergic inflammation depending on the local concentration of their end products (only NO in the case of NOS induction; CO, ferritin and bilirubin in the case of HO-1 induction) (Fig. 4).

The use of hemin to induce HO-1 expression and activity, thus increasing the generation of CO, is widespread [96, 97, 105]. Hemin is a protoporphyrin which is generated during the breakdown of hemoproteins, whenever cells die of apoptotic or necrotic death, as in inflammation. Protoporphyrins at high concentration are known to be photosensitive pigments, capable of causing local cell injury through the generation of free radicals [107]. However, at a dose of 4mg/kg, hemin in the cardiac model of FIR may represent a rather specific defensive mechanism against I/R injury.

Carbon monoxide – releasing molecules

As previously reported, induction of HO-1 by means of chemicals and/or endogenous substrates, is associated with significant anti-inflammatory effects. That HO-1-derived CO is mainly responsible for downregulating inflammation is substantiated by the pharmacological responses observed when CO is applied exogenously to models of inflammation in vivo and in vitro, mimicking the potent anti-inflammatory actions of HO-1 overexpression. Therefore, it is conceivable that CO could be a therapeutic tool for the treatment of immune conditions, such as allergic inflammation. The direct administration of CO as a gas has been proposed [108], but it proved difficult to establish precise dose-related effects, due to the uncertain titration of the gaseous moiety. The use of prodrugs, such as methylene chloride, which are bioactivated to form CO, has been tried and found to be relatively unpredictable, due to the variable extent of metabolism [109]. A third possibility involves the synthesis of specific CO carriers, i.e. molecules containing CO moieties and capable of releasing CO, in strict analogy to the pharmacology of NO donors. In the past few years, the laboratory of Motterlini has succeeded in synthesising and characterizing transition metal carbonyls as potential CO-releasing molecules (CO-RMs) [110].There are three generations of CO-RMs. The first generation was based on transition metal carbonyls. These compounds contain a heavy metal (nickel, cobalt, iron, manganese, ruthenium) [111] surrounded by carbonyl groups as co-ordinate ligands. In order to release CO, many of these compounds need to be activated by light [111]. Interestingly, a metal carbonyl complex, tricarbonyl-dichlororuthenium dimer [Ru $(CO)_3$-$Cl_2]_2$ spontaneously elicited the release of CO, depending on the initial concentration, in a myoglobin detection assay [112]. This compound was not cytotoxic, except at very high concentrations and after prolonged exposure. It relaxed isolated rat aorta, precontracted with phenyephrine, in a manner that was antagonized by myoglobin and by ODQ [112]. In isolated perfused rat hearts, the coronary constriction induced by N^G-monomethyl-L-arginine methylester (L-NAME) was mitigated by [Ru $(CO)_3$-$Cl_2]_2$. As a potential anti-inflammatory agent, [Ru $(CO)_3$-$Cl_2]_2$ has been evaluated on immunological cells, such as guinea pig mast cells and human basophils. In isolated purified guinea pig mast cells from actively sensitized animals, [Ru $(CO)_3$-$Cl_2]_2$ significantly decreased both the antigen-induced release of histamine and the expression of the activation marker CD203c [113]; in human basophils, it similarly blocked the anti-IgE-induced release of histamine, and the expression of the activation marker CD203c [113]. The effects were parallel to the decrease in antigen-induced Ca^{2+} overload,

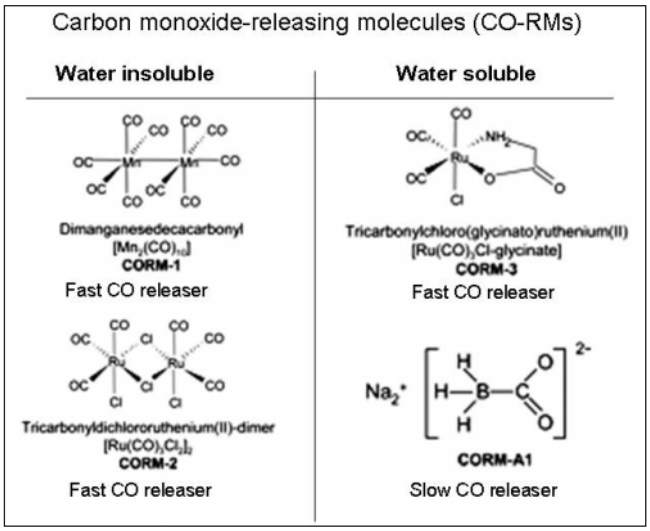

Fig. 5. Classes of CO-RMs and their chemical properties. From Mottelini et al. [100].

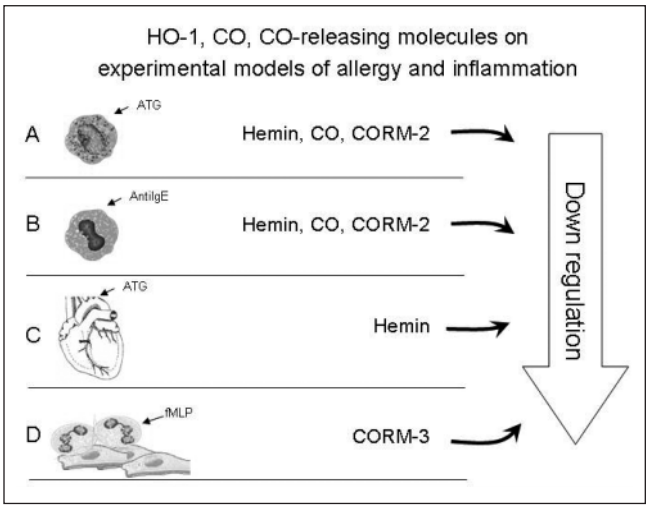

Fig. 6. Hemin, CO and CORM-2 down regulate the immunological activation of guinea-pig mast cells (A) and of human basophils (B). Hemin (C) suppresses cardiac anaphylaxis. CORM-3 (D) down regulates the inflammatory response of endothelial cells.

tentatively explaining the anti-allergic effect. At the concentrations used, mast cell viability was unaffected, cytotoxic effects occurring only at higher concentrations [113].

The shortcoming of $[Ru(CO)_3\text{-}Cl_2]_2$ is that it is soluble in organic solvents only, which renders the compound poorly compatible with biological systems. Therefore, a second generation of water soluble metal carbonyls was synthesized, carrying a glycine molecule in the structural backbone (tricarbonyl-chloro-glycinated ruthenium, CORM-3 [114]. In a careful study, CORM-3 liberated CO under physiological conditions and protected myocardial cells and tissues against I/R injury, as well as cardiac allograft rejection [114]. A possible involvement of K-ATP channel activation has been proposed, as a tentative mode of action [114]. In addition, CORM-3 reduced infarct size in vivo, when given in mice at the time of reperfusion, after a 30 min coronary artery occlusion [115]. In a model of vascular inflammation, consisting of rat coronary endothelial cells co-incubated with human polymorphonuclear granulocytes (PMN), the addition of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) resulted in a significant increase in CD54 (ICAM) expressed by the endothelial cells. The increase in the ICAM expression was inhibited by CORM-3 in a concentration-dependent way, and was left unchanged by the inactive form (iCORM-3) that is incapable of liberating CO. The expression of the surface integrin CD11b by fMLP-activated human PMN was reduced consistently by CORM-3 and unmodified by iCORM-3 [116]. Conceivably, the overexpression of adhesion molecules by endothelial cells in the presence of fMLP-stimulated PMN could be due to the production of superoxide anion by PMN, a process which is also inhibited by CORM-3 [unpublished observation].

A third generation of CO releasers has been synthesized recently. CORM-A1 does not contain a transition metal and liberates CO at a slower rate under physiological conditions. The chemical backbone is sodium borane-carbonate, and the absence of transition metals sets it free from the risk of metal-related toxicity. The characteristics of the three molecules are reported in Figure 5. Like the other compounds, in rat aortic strings precontracted with phenylephrine, CORM-A1 caused relaxation with a time-course more sustained than that of CORM-3 [117] and decreased the arterial pressure in the anesthetized rat. The effects were not reproduced by the inactivated form of CORM-A1, and were potentiated by the indazole compound 3-(5'-hydroxymethyl-2'-furyl-1-benzylindazole (YC-1), which is known to sensitize guanylyl cyclase to activation by CO [118], but is blocked by the guanylyl cyclase inhibitor, ODQ. Therefore, the discovery of compounds capable of carrying CO to and releasing CO in tissues, calls for the development of novel pharmacological agents suitable for therapeutic application.

Conclusions

On the bad side of the coin, CO poisoning is a paradigm of hypoxia/reoxygenation, in which free radicals represent a source of major injury in the development of the interval form of CO poisoning. We have already hypothesized that HBO could increase free-radical induced damage by generating further ROS in addition to the free radicals generated during the hypoxia/reoxygenation. However, there is some evidence that HBO conversely slows CO-mediated lipid peroxidation, although how this occurs is not known. It has been suggested [119] that, if oxy radicals derived from a CO-associated disturbance in mitochondrial electron transport initiate lipid peroxidation, the HBO effect may be due to a dissociation of the cytochrome–CO complex, as reported by Brown et al. [120].

In conclusion, the undisputed therapeutic value of HBO in acute CO poisoning may rest mainly on the detachment of CO from the tissue heme moiety, rather than on the accelerated clearance of COHb. The addition of free radical scavengers, such as GSH, acetylcysteine, tempol, to the standardized international protocols for the treatment of acute CO poisoning may be advisable.

On the good side of the coin, among the pleiotropic effects of CO in isolated cells, in experimental animals and in humans, we have focussed on the anti-inflammatory role of CO, mainly in allergic inflammation. Immunological cells are fully responsive to CO. The immunological and non-immunological activation of rat and guinea pig mast cells is inhibited by CO and restored by blocking guanylyl cyclases (ODQ) and by scavenging CO (HbO_2). The IgE-dependent stimulation of human basophils is greatly decreased by CO, as is the expression of the CD11b integrin and the generation of superoxide anions by human PMN. Results obtained on the activation of immunological cells using HO-1 induction and exogenous CO and CO-releasing molecules are reported in Figure 6 (see text for details). An overwhelming literature is available on the antioxidant and anti-inflammatory effects of the induction and overexpression of HO-1 in wild type and/or genetically manipulated animals (see [54] for a review), which is supportive of an identical anti-inflammatory effect of exogenous CO and the resulting enhanced HO-1 activity. Whether the anti-inflammatory action of endogenous CO is exerted through the stimulation of guanylyl cyclases, via activation of Ca^{2+}- dependent K^+ channels, or via interaction with the MAPK pathway remains to be elucidated. However, all these mechanisms lead to a net decrease in intracellular Ca^{2+}, which fully explains the inhibition of the release by inflammatory cells of preformed or newly synthesized mediators of inflammation, a process which is highly dependent on intracellular Ca^{2+} levels.

Thus, induction of HO-1 is a potent endogenous negative feed-back mechanism in inflammation, in which CO co-operates with other endogenous anti-inflammatory agents (NO, cannabinoids) in mitigating inflammation [121]. Within this framework, the new class of CO-releasing drugs is worth investigating. The attachment of NO to aspirin is under investigation and a carbonyl aspirin is conceivable.

The concept of carbonyl-aspirin has become more attractive since the demonstration of a novel mechanism of action of aspirin linked to HO-1. It was recently shown that aspirin is able to increase HO-1 protein levels in human endothelial cells in cultures [122]. Moreover, it was shown that aspirin, by acetylating COX-2, switches the conversion of arachidonic acid to 15-R-HETE, which is released by endothelial and epithelial cells and transformed via 5-lipo-oxygenase (5-LO) to 15-epimer lipoxin (aspirin-triggered lipoxin, ATL). Low doses of aspirin actually trigger the formation of inflammation-resolving 15-epi lipoxin A-4 in healthy individuals, which may account for the anti-inflammatory action of aspirin in vivo [123]. Interestingly, the group of Nascimento-Silva has provided evidence that an aspirin-triggered lipoxin A-4 stable analogue is able to induce HO-1 expression in cultured human endothelial cells [124]. Thus, aspirin increases HO-1 activity either *per se*, or via the generation of ATL and the aspirin-induced generation of CO, Fe^{2+} and bilirubin could pinpoint the HO/CO system as a major modulator in the resolution phase of the acute inflammatory response.

Finally, we fully agree with the statement of Barinaga [125], who wrote that "CO was likely to provide fuel for plenty of labs". The prediction, dating from 1993, has turned out to be true.

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