The Role of Carbon Monoxide as a Gasotransmitter in Cardiovascular and Metabolic Regulation

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The role of CO as a gasotransmitter in cardiovascular and metabolic regulation

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Abstract Carbon monoxide (CO) is produced endogenously through the oxidative catabolism of heme by heme oxygenase (HO). Firstly described as a putative neuronal signalling messenger, CO is now also regarded having been involved in a variety of physiological and pathophysiological processes in the cardiovascular system, including regulating blood pressure, smooth muscle cell proliferation, anti-inflammatory, anti-apoptotic and anti-coagulation effects. CO contributes substantially to the protective effects of HO enzymes as a mediator of cell and tissue protection. The diverse actions of this diatomic gas mainly depend on the stimulation of soluble guanylate cyclase, opening of BK_{Ca} channels as well as activation of mitogen-activated protein kinases and/or Akt signalling pathways. The cellular and molecular consequences of CO signalling are only partially characterized and appear to differ depending on cell types and circumstances. This chapter provides an overview of the many roles CO plays as a gasotransmitter in the cardiovascular system.

Keywords Carbon monoxide · Heme oxygenase · Cardiovascular · Gasotransmitter · Signal transduction systems

Abbreviations

AMPK	AMP-activated protein kinase
BK_{Ca}	Big-conductance calcium-activated potassium channels
C/EBP	CCAATT-enhancer-binding protein
cGMP	cyclic guanosine 3′, 5′-monophosphate
CO	Carbon monoxide
CoPP	Cobalt protoporphyrin
CORM	Carbon monoxide releasing molecule
EDRF	Endothelium-derived relaxing factor
ENaC	Epithelial Na^+ channel
eNOS	endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinases
ETC	Electron transport chain
GC	Guanylate cyclase
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HEK293	Human embryonic kidney
HIF-1α	Hypoxia-inducible factor-1α
HO	Heme oxygenase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
iNOS	inducible nitric oxide synthase
JNK	Jun-activated kinases

LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	Nicotinamide-adenine dinucleotide phosphate oxidase
O_2^-	Superoxide anion
PAH	Pulmonary arterial hypertension
PI3K	Phosphatidylinositol 3-kinase
PPARγ	Peroxisome proliferator-activated receptor-γ
ROS	Reactive oxygen species
sGC	soluble guanylate cyclase
SHRs	Spontaneously hypertensive rats
STATs	Signal transducers and activators of transcription
STZ	Streptozotocin
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
ZDF	Zucker diabetic fatty

1 Introduction

For almost a century, carbon monoxide (CO) has been commonly stigmatized as the "silent killer" due to its strong affinity to haemoglobin [1,2], 240 times that of oxygen, resulting in tissue hypoxia and death [3-5]. Because of its association as a toxic gas, it won't be until 1968 when it was proposed that CO is endogenously produced from heme catalysis [6]. Not understanding the significance of this discovery at the time, the endogenous synthesis of CO was seen as a metabolic waste product of heme oxygenase (HO)-catalyzed heme degradation [7]. However, the breakthrough discovery detailing the possible physiological role CO may play in the mammalian system came in 1987 when Brune and Ullrich (1987) [8] showed that, like nitric oxide (NO), CO can also activate soluble guanylate cyclase (sGC). This laid the foundation for the next discovery in 1993 where Synder and associates (1993) [9] showed that CO, again like NO, might also have a physiological role to play. Since then, there have been numerous findings of CO having anti-inflammatory, anti-apoptotic and anti-proliferative properties (for review see [10]). This Chapter will focus on the signalling targets of CO contributing to its physiological and pathophysiological role in the cardiovascular system.

2 How the concept of “gasotransmitter” evolved

The scientific community was thrilled when the final verdict of the mysterious endothelium-derived relaxing factor (EDRF) [11], a vasorelaxant substance synthesized and secreted from endothelial cells, was a gaseous molecule. The conclusion that NO is an endogenous gaseous molecule, termed gasotransmitter, triggered the exploration of other possible gasotransmitters, including CO and H_2S [12]. Unlike neurotransmitters, gasotransmitters are not stored in vesicles, thus they must be rapidly synthesized in response to stimulation. There is no exocytosis upon the release of gasotransmitters, and in fact, gases are not contained by membranes at all; they can freely enter a cell with no need for receptors or active endocytosis to influence a cell.

The discovery of NO, CO, and H_2S as small signalling gasotransmitters has spawned a new type of science: that endogenously derived gases could elicit crucial biological functions, as well as contribute to the pathogenesis of human diseases. Several other gases are currently under investigation to determine if they too act as endogenous mediators, including acetaldehyde (CH_3CHO), sulfur dioxide (SO_2), dinitrogen oxide (N_2O), and ammonia (NH_3). Overall, these new insights have improved our understanding of physiological importance of gasotransmitters not only in physiological functions, but also in the pathogenesis of human diseases.

3 Classification of a gasotransmitter

Before the discovery of NO in 1980 [11] the idea that a gaseous substance can induce an array of cell signalling processes, such as the regulation of cardiovascular, immune and nervous system function, was unfathomable. The concept and terminology of 'gasotransmitter' was firstly proposed by Rui Wang in 2002 [12], which implies a class of endogenous gaseous substances that can induce an array of signalling responses in a cell. Now with this new science, standards must be laid out to distinguish a gasotransmitter so it may not be confused with other classifications of signalling molecules. The criteria for establishing a signalling molecule as a gasotransmitter are described below in Table 1 [10,12].

Table 1 Classification of the gasotransmitter

1. They are to be small molecules of gas, such as NO, CO and H_2S.[1]
2. The gasotransmitter must be membrane permeable and not have to rely on cell membrane cognate receptors, nor vesicle-releasing machinery to elicit their cell signalling response.
3. The gaseous substance must be endogenously and enzymatically synthesized and its formation must be regulated by a physiological stimuli.
4. The biological effects of gasotransmitters can be mimicked by the same molecules applied exogenously.
5. They must have specific cellular and molecular targets. To illustrate this, both NO and CO can activate GC thus increasing the intracellular concentrations of the secondary messenger, cGMP and H_2S can induce the opening probability of K_{ATP} channels in vascular tissues.
6. Finally, the cellular effects of gasotransmitters may or may not be mediated by second messengers.

[1]The molar mass of NO, CO and H_2S are 30.01, 28.01 and 34.08 g mol^{-1}, respectively.

One of the most intriguing features of gasotransmitters corresponds to their unique chemical signalling mechanism. Unlike classic signalling molecules that interact through the stimulation of G protein coupled receptors or tyrosine kinase receptors to elicit their distinct signalling cascade, gasotransmitters chemically modify their intracellular targets. For instance, NO and H_2S induce *S*-nitrosylation or *S*-sulfhydration of the targeted protein, respectively. Through post-translational modification, gasotransmitters are able to induce immediate effects in the targeted cell. This Chapter will solely look at the signalling process of CO. If the reader wishes to learn about the signalling process of NO and H_2S, they are invited to read other excellent reviews [13,14].

3.1 CO as a gasotransmitter

As a small molecule of gas, CO can freely pass through cell membranes without having to rely on the assistance of the targeted cell's transportation machinery. This allows CO to rapidly influence cellular behaviour and function. CO is endogenously synthesized by HO. There are currently three known isoforms of HO, including HO-1, HO-2 and HO-3. These isoforms are discussed in detail in the following section.

Unlike NO and H_2S, gaseous CO is not metabolized in mammals [15,16]. In fact, elimination of CO is strictly through exhalation in the lungs without biochemical modification [17]. Under normoxic conditions, this diatomic gas has a half life of 3-7 hours [15]. Indeed, out of all three gases CO is the most biologically stable gasotransmitter. CO has weak chemical reactivity, mainly because it does not have unpaired electrons, and does not chemically dissociate in an aqueous solution to form different chemical species. Thus, CO might be capable of exerting its effects during longer time periods and distances compared to NO or H_2S.

4 Production of CO in the cardiovascular system

Long before the concept of gasotransmitters was even established [12], the scientific community was aware that living organisms can endogenously synthesize CO [18-20]. In the presence of O_2 and NADPH, the endogenous production of CO raises principally from the catabolism of heme by HO (~86%) to subsequently give equimolar amounts of biliverdin, Fe^{II} and CO [21] (Fig. 1). All of these three products of HO are biologically active. Biliverdin formed in this reaction is reduced to bilirubin by biliverdin reductase [22]. HO catalyzes the rate-limiting step in the degradation of heme to CO and biliverdin [21]. Although most of the heme oxidation occurs in the liver and spleen, HO is ubiquitously expressed in mammalian cells [6]. This gives HO the potential to continuously produce CO [6].

As mentioned in the previous section, there are three mammalian paralogues of HO. These isoforms of HO are sub-classified into either inducible (HO-1) by inflammation or oxidative stress, or constitutive (HO-2), meaning they are constantly active [22]. The inducible HO-1 is a redox-sensitive response protein whose activity is up-regulated by enhanced oxidative stress [23], either by inflammatory or disease-like conditions. Furthermore, HO-1 is a heat-shock protein [24,25] and is considered to be an essential anti-oxidant enzyme up-regulated in response to cellular stress [22]. On the other hand, HO-2 is constitutive expressed and is mainly responsible for basal HO activity [22,26], and thus CO production. HO-2 is activated by calcium-calmodulin [27], as well as casein kinase 2 in neurons [28]. Similar to eNOS, HO-2 is also localized to the endothelial layer of

blood vessels [29]; where like NO, CO too plays an important physiological role in the regulation of vascular tone [30].

Although ubiquitously expressed in all tissues examined so far, there is particularly a high density of these enzymes in the brain (HO-2), liver (HO-2 and HO-1), spleen (HO-1), vascular endothelial cells and smooth muscles tissues (HO-1 and HO-2) [22]. Likewise, these tissues are also central to erythrocyte turnover and contain large heme pools, further contributing to the enhanced carboxyhaemoglobin levels detected in these tissue beds. Finally, HO-3 appears to be an inactive paralogue and is so far thought to represent a pseudogene [31]. HO-3 was cloned from rat brain, but other than that, not much is known about the HO-3 isoform [32].

The remaining 14% of CO production lies within lipid peroxidation, bacteria, photo-oxidation and xenobiotic (foreign compound) metabolism [33]. Ideally, healthy humans produce about 20 $\mu M\ h^{-1}$ of CO [34-36]; however, elevated levels of exhaled CO from critically ill patients have been observed in conditions of asthma [37,38], bronchiectasis [39], cystic fibrosis [40,41], upper [42] and lower [43] respiratory tract viral infections, diabetes [44], rhinitis [45] and metabolic syndrome [46]. Major emphasis will be focused on cardiovascular-related diseases in this Chapter.

5 Physiological functions of CO in the cardiovasculature

The diverse actions of CO have been mainly attributed to its regulation of common signalling pathways, such as the stimulation of sGC, opening of BK_{Ca} channels, activation of mitogen-activated protein kinases (MAPK) and Akt. The signalling outcomes of this activation largely depend on cell types and circumstances.

5.1 CO-mediated vasorelaxation

CO stimulates vasorelaxation mainly through three major cellular mechanisms. These include the activation of soluble guanylate cyclase (sGC) [47-49], stimulation of big-conductance calcium-activated potassium channels (BK_{Ca}) [50,51], as well as NOS induction [52,53]. The interaction between CO/HO and NO/NOS are discussed in further detail in Section 8.

CO releasing molecules (CORMs) were shown to relax precontracted aortic rings [52], dilate renal afferent arterioles [53], decrease intrahepatic vascular resistance and increase perfusion flow in cirrhotic livers [54]. CO can also indirectly reduce vascular resistance by blocking the synthesis of the potent vasoconstrictors, including endothelin-1 [55] and the cytochrome P450-mediated generation of vasoconstrictors [56,57].

Under oxidative stress, CO may act as a vasoconstrictor [58]. Both bubbled CO and CORM-3 constricted rat renal arteries, whereby anti-oxidants (e.g. tempol and ebselen) inhibited CO-induced reactive oxygen species (ROS) production and converted CO from constrictor to dilator [58]. Furthermore, inhibition of the pro-oxidant enzymes, such as NOS, NADPH oxidase (Nox), xanthine oxidase and complex IV of the mitochondrial electron chain, converted CO from constrictor to dilator [58]. Thus, the redox state of the cell may play a key role in determining whether CO induces vasodilation or vasoconstriction; thereby, allowing CO to contribute to the fine tuning of vascular tone.

5.2 CO effects on cell proliferation and apoptosis

5.2.1 Anti-apoptotic effects of CO

The effects of CO signalling on cellular apoptosis are tissue and cell specific. For instance, CO acts as an anti-apoptotic agent in endothelial cells [59,60], hepatocytes [61,62] and cardiomyocytes [63], thus preventing cell and tissue injury. The anti-apoptotic effects of CO appear to be dependent on the activation of the p38 MAPK signalling transduction pathway [59,60], phosphorylation of protein kinase R-like endoplasmic reticulum kinase and/or through Akt activation [61]. CO was shown to prevent tumor necrosis factor-(TNF)-α-induced cellular apoptosis [59] and endoplasmic reticulum (ER) stress-induced apoptosis [60] *via* a p38 MAPK-dependent mechanism. Additionally, CO protected hepatocyte against apoptosis and fulminant hepatitis through the activation of nuclear factor (NF)-κB *via* ROS generation and Akt pathways in primary rat or mouse hepatocytes and Hep3B cells (a human hepatoma cell line) [61]. Interestingly, low CO concentrations (10-100 μM) can prevent mitochondrial membrane permeabilization in isolated mouse hepatocytes, thus blocking the release of pro-apoptotic factors [64]; however, high CO concentrations (250 and 500 μM) triggered mitochondrial swelling [64]. It remains to be seen if therapeutic application of CO also elicits these pathways observed under *in vitro* analysis.

5.2.2 Pro-apoptotic effects of CO

The pro-apoptotic effect of CO was seen in hyperproliferative smooth muscle cells [65,66] and fibroblasts [67]. CO also inhibits the proliferation of human airway smooth muscle cells [68] and rat vascular smooth muscle cells under both hypoxic [55,69] and normoxic [69] conditions. Morse and associates (2005) [67] demon-

strated that both chronic (14 days) and transient exposure (3 hours) of inhaled CO (250 ppm) to mice inhibited fibroblast proliferation, thus reducing the degree of fibrosis. The authors showed that CO arrested fibroblast proliferation at the G0/G1 phase of the cell cycle through a cGMP-dependent mechanism that involved changes in the expression of cell-cycle regulatory proteins (increased cellular levels of the cyclin-dependent kinase inhibitor, p21Cip1 and decreased levels of cyclins A and D) [67]. Peyton et al. (2002) [66] reported that 100-200 ppm CO arrested serum-stimulated vascular smooth muscle cell (VSMC) proliferation at the G(1)/S transition phase and blocked the expression of cyclin A, as well as the activation of cyclin A-associated kinase activity and cyclin-dependent kinase 2 activity. Likewise, 250 ppm CO inhibited α–smooth muscle actin proliferation through enhanced expression of small proline-rich protein-1A expression *via* stimulation of the extracellular regulated kinases (ERK) pathway [65]. It is becoming increasingly clear that the HO/CO signalling system is an important mediator in regulating cell survival.

5.3 CO-mediated anti-aggregatory effects

In addition to regulating cell growth and relaxing blood vessels, CO may also preserve blood flow at sites of vascular damage by blocking platelet aggregation. Monolayers of cultured rat aortic VSMCs subjected to shear stress stimulated time-dependent increases in the inducible HO-1, the production and release of CO, as well as increases in intracellular cGMP levels in co-incubated platelets [70]. The authors showed that treatment with the HO-1 inhibitor (30 μM SnPP-IX) blocked the stimulatory effect on platelet cGMP concentration induced by sheared VSMCs [70]. Likewise, CO was shown to inhibit the production of platelet-derived growth factor-B in endothelial cells under hypoxic conditions [71].

These results suggest that CO is an endogenous VSMC-derived messenger that may be selectively induced by hemodynamic forces to block platelet aggregation and preserve blood flow at sites of vascular damage. Further investigation is needed to elucidate the actual mechanism by which shear stress up-regulates HO-1 and if CORMs can inhibit platelet aggregation *in vivo*.

5.4 CO-related angiogenesis

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis [72]. Exposure to CO gas or CORMs increased the expression of VEGF in VSMCs [73], human microvascular endothelial cells [74,75], human umbilical vein endothelial cells (HUVECs) [76], rat primary cardiomyocytes and H9C2 myocytes [77]. This mechanism of action was shown to occur through a p38 kinase-

dependent pathway [77] or phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin and MEK/ERK-dependent pathways [76]. As such CORM-2 [76], -1 and -3 [75] were shown to be potent stimulators of angiogenesis. Additionally, methylene chloride (CO-donor; single dose 500 mg/kg) promoted angiogenesis in the infarct heart through the induction of VEGF-B and hypoxia-inducible factor (HIF)-1α, thus speeding up cardiac repair [78].

5.5 Anti-inflammatory effects of CO

The anti-inflammatory properties of CO and CORMs are partially due to the activation of the signal transduction pathways such as p38 MAPK [79-81], HIF-1α [82,83], peroxisome proliferator-activated receptor-γ (PPARγ) [84,85], GC signalling [86] and CCAATT-enhancer-binding protein (C/EBP)-β/δ [87]. Due to its ability to regulate these important pathways or through direct interaction, CO was shown to suppress the activation of pro-inflammatory enzymes, such as inducible NO synthase (iNOS) [84,88,89] and cyclo-oxygenase-2 [90,87], as well as inflammatory transcription factor NF-κB [90,88,89,91,92]. As such, CO was shown to inhibit the production of pro-inflammatory cytokines, including macrophage inflammatory protein-1, prostaglandin-2, IL-1β, -2, -6, -10 and intercellular adhesion molecule (ICAM)-1 [80,85,87,89,90-93], as well as and the expression of toll-like receptor (TLR)-3, -4 [81,93] in macrophages [80,81,87,88], T cells [94], colonic epithelial cells [89], alveolar epithelial cells [91], dendritic cells [93], HUVECs [85,91] or in joint tissues of a collagen-induced arthritis model [90]. Needless to say, CO or CORMs administration was shown to decrease leukocyte rolling, adhesion and neutrophil migration into the inflammatory sites [86,95] and lipopolysaccharide (LPS)-stimulated activation of macrophages [96,97] and HUVECs [98].

While a large body of evidence suggests CO to be an anti-inflammatory agent in experimental models, it has yet to be shown if the same can be said in humans. In a randomized, double-blinded, placebo-controlled, two-way cross-over clinical trial, inhalation of CO (500 ppm for 1 hr) had no anti-inflammatory effect, as measured by cytokine production (TNF-α, IL-1α/β, -6, -8), against an LPS infusion [99].

5.6 Cardiac protection from tissue reperfusion injury

Numerous studies have demonstrated the cytoprotection of CO following vascular injury [63,83,92,96-98,100]. After an ischemic injury, 10 to 50 μM CORM-3 produced a significant recovery in myocardial performance and a marked reduction in cardiac muscle damage and infarct size [63]. The cardioprotective effects mediated

by CORM-3 in cardiac cells and isolated hearts were abolished by 5-hydroxydecanoic acid, an inhibitor of mitochondrial ATP-dependent potassium channels [63]. These authors also found that using a model of cardiac allograft rejection in mice, CORM-3 considerably prolonged the survival rate of transplanted hearts. Recently, it was shown that CORM-2 (8 mg/kg) protected the liver from ischemia-reperfusion injury by up-regulating the expression of the anti-apoptotic protein Bcl-2, down-regulating caspase-3 activation, as well as reducing cellular apoptosis after ischemia-reperfusion injury [92]. Lastly, CO protected against ischemia-reperfusion injury by inhibiting L-type Ca^{2+} channel mediated influx of Ca^{2+}, which prevented Ca^{2+} overloading and calpain activation; thereby, reducing cellular energy demand required for contraction and preventing ischemic death in H9c2 cells (rat heart cell line) [101].

6 Pathophysiological changes of CO functions and metabolism

The lungs are the only site of CO elimination. Exhaled CO is reflective on total blood CO (*via* carboxyhemoglobin content), due to the equilibrium of CO between the alveolar-capillary barrier in the lungs and blood [102,103]. In fact, the amount of CO exhaled in the breath may correlate to the severity of diabetes [44] and asthma [104]. Recently, it was reported that individuals who exhaled high levels of CO were more likely to develop metabolic syndrome and cardiovascular disease events; thus, underscoring the importance of CO in the pathogenesis of metabolic and vascular risk [46].

6.1 Diabetes

Diabetes is a disease characterized by chronic high blood sugar levels, where type 1 diabetes is classified as insulin-dependent and type 2 diabetes as non-insulin-dependent diabetes. Interestingly, exhaled CO levels are significantly increased in diabetic patients [44,105]. Peter Barnes and associates (1999) [44] found that exhaled CO levels are elevated in patients with type 1 and type 2 diabetes by 4.0 and 5.0 ppm, respectively, compared to healthy subjects (2.9 ppm). Indeed, the authors observed a positive correlation between exhaled CO levels and the incidence of glycemia in all subjects and the duration of the disease [44]. An oral glucose tolerance test performed in healthy non-smoking volunteers demonstrated a correlation in plasma glucose levels (3.9 to 5.5 mM) and exhaled CO (3.0 to 6.3 ppm), which returned to normal after 40 min of glucose administration [44].

To possibly explain the correlation between CO and glucose levels, Lundquist and associates [106] showed that glucose stimulates HO activity in intact mouse islets. Interestingly, HO-2 is strongly expressed in both insulin and glucagon secreting cells in the mouse

[106] and in the rat endocrine pancreas [106]. In fact, Goto-Kakizaki rats (a model with defective pancreatic β-cell HO-2) exhibited reduced CO and insulin insufficiency, suggesting that HO-2 plays a critical role in insulin release and glucose metabolism [107]. Studies have shown both hemin and exogenous CO enhanced insulin secretion from glucose-stimulated islets through a GC-dependent pathway [106,108]. Exogenous CO also induced the release of glucagon, which was abrogated by GC inhibition [106]. These studies show that HO/CO pathway constitutes a novel regulatory system in the stimulation of insulin and glucagon release, and that acute raises in CO may act as a counter-regulatory mechanism in response to increased plasma glucose levels.

On the contrary, studies have shown that HO-1 activity is reduced in aorta and kidney tissues in the Zucker diabetic fatty (ZDF) rats [109,110] and in aorta extracts from streptozotocin (STZ)-induced rats [111]. In fact, CO release from aorta extracts was significantly lower in ZDF rats compared to Zucker lean rats, suggesting that HO-1 activity is inhibited under obese/hyperglycemic conditions [109]. Administration of cobalt protoporphyrin (CoPP), an inducer of HO-1 activity, increased CO production in ZDF rats and STZ-induced rats. CoPP-treated ZDF rats showed improved insulin sensitivity, hyperinsulinemia, remodelled adipose tissue and reduced inflammatory cytokines [109]. These metabolic improvements are likely due to increased phosphorylation of Akt and AMP-activated protein kinase (AMPK) in the aorta and kidney, as well as serum adiponectin [109]. Studies have shown that activation of AMPK and p-Akt increases glucose transport, fatty acid oxidation [112,113] and phosphorylated endothelial nitric oxide synthase (eNOS) [114]. However, it is not clear whether enhanced CO, not biliverdin or released Fe^{II}, is the main contributor to the significant metabolic improvements observed in CoPP-treated ZDF rats. Further research is needed to identify the source response for the increased exhaled CO levels in diabetic patients and if exhaled CO can be used as a prognostic factor in the development of and monitoring diabetes.

Interestingly, STZ-treated rats exhibited reduced sensitivity to CO-induced vasorelaxation compared to normal rats [115]. The decreased vasorelaxant effect of CO was related to diminished sensitivity of BK_{Ca} channels in VSMCs due to hyperglycaemia-induced glycation [115]. This constitutes a novel mechanism for the diabetic vascular complications.

6.2 Vascular proliferative diseases

The hallmark of vascular proliferative diseases includes VSMC hypertrophy and hyperplasia due to severe imbalance in the redox homeostatic signalling of cellular proliferation and apoptosis. CO was shown to prevent intimal hyperplasia by arresting hyperproliferative VSMCs [82,116-118], increase mobilization and recruitment of

bone-marrow-derived progenitor cells to denuded vessels, as well as enhance re-endothelialization due to its pro-proliferative actions on endothelial cells [82,119]. The later is said to be dependent upon NOS and NO, involving the modulation of RhoA and Akt [119] or HIF-1α/VEGF [82] signalling pathways and may help prevent restenosis.

6.2.1 Hypertension

Chronic hypertension is characterized by vascular structural changes, such as vessel wall hypertrophy and hyperplasia, which contributes to elevated resistance and high blood pressure [120]. HO-1 is pathophysiologically activated by hemodynamic stress in response to elevated blood pressure, where the expression and activity of HO-1, sGC and cGMP in VSMCs are associated with different stages of hypertension development [120]. Kobayashi et al. (2007) [121] demonstrated that inhaled CO (60 ppm) significantly decreased left ventricular hypertrophy and aortic hypertrophy, which attenuated the development of angiotension II-dependent hypertension in mice. These cytoprotective mechanisms of CO were due to reduced ROS production *via* the reduction of Nox and Akt phosphorylation [121]. Furthermore, both CoPP and CORM-3 (2 mg/100 g) increased the dilatory response to acetylcholine (ACh) in Sprague-Dawley rats, which was likely due to a CO-induced decrease in iNOS expression leading to improved vascular reactivity [111].

Pulmonary arterial hypertension (PAH) is characterized by vascular proliferation and remodelling in the small pulmonary arteries, which leads to a progressive increase in pulmonary vascular resistance, eventually resulting in right heart failure. Exposure to inhaled CO (250 ppm, 1 hour/day for 2 or 3 weeks) reversed established PAH and right ventricular hypertrophy, restored right ventricular and pulmonary arterial pressures, as well as pulmonary vascular architecture in mice [122]. The mechanism of action was dependent upon CO-induced up-regulation of eNOS and NO generation, as well as stimulated apoptosis and inhibition of cell proliferation in VSMCs [122]. In fact, the HO/CO system seems to attenuate hypertension only when the NOS pathway is fully operative [123]. Additionally, Dubuis et al. (2005) [51] demonstrated that chronic CO inhalation (50 ppm for 21 days) attenuated hypoxic PAH development, likely through BK_{Ca} channel activation.

Hemin supplementation has been used extensively to up-regulate the HO-1/CO system and has shown success in the treatment of hypertension [124-128]. Interestingly, hemin therapy was shown to lower blood pressure in young spontaneously hypertensive rats (SHRs) (8 weeks), but not in adult SHRs (20 weeks). Indeed, a desensitized HO/CO-sGC/cGMP system may take the blame in mesenteric artery [124] and aortic tissues [125] of adult SHRs;

leading to treatment failure. Young SHRs have a defective, yet responsive, HO/CO-sGC/cGMP system, where hemin supplementation was shown to normalize high blood pressure [124,125]. Intriguingly, chronic hemin treatment (15 mg/kg per day for 21 days) established long-lasting anti-hypertension protection for 9 months after the removal of implanted hemin osmotic minipumps in 12-week old SHRs [127]. Additionally, this hemin protocol reversed SHR-featured arterial eutrophic inward remodelling and decreased expression levels of vascular endothelial growth factor in SHRs [127]. The sustained up-regulation of HO-1 expression in vascular tissues and normalization of blood pressure of SHRs may have been due to residual hemin accumulation within VSMCs. As such, Chang et al. (2008) showed that hemin treatment (5 μM for 21 days) contributes to vascular remodelling by inhibiting proliferation of cultured rat aortic VSMCs by arresting cells at G0/G1 phases [128]. Hemin treatment also decreased the expression of p21 protein (an important negative regulator for cell proliferation) and the level of ROS [128]. Overall, application of this hemin technology at physiologically or therapeutically relevant concentrations will pave the way for novel and long-lasting treatment regimens for hypertension.

The transient up-regulation of HO-1 may represent the first line of defence in the pathophysiological development of hypertension. However, the protective effects of HO/CO system in hypertension are not without controversy. For instance, in Dahl salt-sensitive rats, vascular reactivity to ACh was restored immediately upon inhibition of HO [129]. In agreement, HO inhibition improved ACh-induced vasodilation and lowered systolic blood pressure in obese Zucker rats [130]. This suggests that CO overproduction in the vasculature induces endothelial dysfunction, leading to hypertension.

6.2.2 Atherosclerosis

Atherosclerosis is a multifacet disease involving endothelial dysfunction, inflammation and vascular proliferation. Considerable evidence suggests that HO-1 is up-regulated and plays a beneficial role in against atherosclerosis development (for review, see [131]); however, less is known about the direct effects of CO signalling in atherosclerosis.

CO may serve as a strong anti-atherosclerotic agent due to its VSMCs apoptotic properties, induction on endothelial cell proliferation and anti-inflammatory effects. Indeed, exposure to CO (250 ppm; 1 hour before vascular injury) blocked the development of arteriosclerotic lesions associated with chronic graft rejection and balloon-angioplasty–induced vessel injury in rats [116]. The protective effect of CO is mainly attributed on its ability to suppressing intimal hyperplasia arising from balloon injury, as well as blocking leukocyte infiltration/activation and VSMC proliferation [116]. Recently, it was showed that the reciprocal relationship between the HO-1/CO and NOS/NO systems plays an inhibitory role in atherosclerotic

plaque formation [132]. It has also been reported that CO-induced VSMC proliferation inhibition is dependent on the GC/cGMP and p38 MAPK signalling pathways [116] and the down-regulation of endothlin-1 (a mediator known to induce VSMC proliferation) [132].

6.3 Myocardial infarction

CO can also contribute protective actions in the heart. CORM-3 treatment was shown to reduce cardiac muscle damage and infarct size, as well as preserve cell viability and myocardial performance against hyperoxia-reoxygenation damage in isolated cardiac cells or hearts [63,133], mimicking the late phase of ischemic preconditioning [134]. Pre-exposure to inhaled CO gas (1000 ppm) prior to myocardial ischemia-reperfusion injury reduced infarct area and suppressed the migration of macrophages and monocytes into the infarcted zone in rats [135]. The cardioprotective effects of CO on cardiac ischemia–reperfusion injury seemed to be mediated by the p38 MAPK and Akt–eNOS pathways, including cGMP production [135]. Likewise, another study demonstrated that CO (250 ppm) inhalation improved cardiac energetics and protected the heart during reperfusion after cardiopulmonary bypass in pigs [136] and attenuated ischemia-reperfusion injury in mice due to its anti-apoptotic action [137]. Additionally, Lakkisto et al. (2010) [78] demonstrated that methylene chloride (500 mg/kg) promoted cardiac repair in the infarct heart by inducing angiogenesis and cardiomyocyte proliferation in the infarct border area.

7 The cellular and molecular mechanisms of CO effects

CO has a strong affinity for metal atoms; specifically iron in the reduced state, thereby allowing CO to exclusively react with a number of metalloproteins, such as hemoglobin, sGC, COX, cytochrome p450, cytochrome *c* oxidase, NOS and Nox [57,138-142]. To a weaker extent, CO can interact with non-metal-containing molecules, such as amino acids and lipids [142]. Although weak, these interactions can trigger a biochemical change in the structure of these proteins, thus altering their function and likely signalling cascades.

7.1 CO-induced ROS production

Low concentrations of ROS act as important second messengers to modulate intracellular signalling pathways [143] by affecting the activity, as well as protein-protein and protein-DNA interaction of en-

zymes and transcription factors [144]. CO can modulate cellular ROS generation, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) from mitochondria-dependent and cytosolic sources. Regulation of ROS production is the main mechanism by which CO alters major cell signalling pathways. This includes the phosphorylation of Akt [61,145], activation of NF-κB [61], p38 MAPK [122], Bax and Bid [146], up-regulation of PPARγ [147], HIF-1α and TGF-β [83], secretion of TNF-α, as well as inhibition of ERK-1/2 and cyclin D1 expression [148], mitochondrial cytochrome *c* release [146] and TLR-2, -4, -5 and -9 signalling [149].

The electron transport chain (ETC) is the major site of ATP production in the mitochondria, which heavily relies on oxygen to power the electron cycle [143]. The ETC is also the major for ROS production, including O_2^- and H_2O_2 [143]. CO interferes with the ETC by binding to cytochrome *c* oxidase for oxygen, thus modulating the enzyme's catalytic activity [150-152]. Cytochrome *c* oxidase is located within Complex IV and is the terminal enzyme of the ETC. CO also binds to cytochrome P450 thus directly inhibiting Complex IV [153,154]. As a result, CO promotes electron leakage from the ETC leading to an induction in ROS production. Interestingly, CO was also shown to inhibit Nox, thus leading to the subsequent down-regulation of O_2^- production [148,146,149]. However, the affinity of CO to Nox is weak, and thus the reaction is slow [155,156]. Nonetheless, ROS production and signalling by Nox is decreased, but there is a simultaneous increase in ROS production from the mitochondria [148].

The transient increase in CO-induced ROS production is likely responsible for the protective induction of anti-oxidant enzymes and protective genes, because it forces the cell to undergo an oxidative conditioning to attenuate further ROS production [145,157]. CO was shown to have anti-oxidant properties, because it reduced hepatic lipid peroxidation, re-established total hepatic glutathione and glutathione disulfide levels and protected HUVECs against TNF-α-induced oxidative stress [157]. Yet, chronic exposure to CO (30 ppm/day plus five 1-hour peaks at 100 ppm, 4 weeks) promotes oxidative stress in rats [158]. Thus, it seems the cytoprotective effects of CO signalling are dependent on exposure duration and CO concentration.

7.2 Ion channel signalling

Crucial to the role of CO in physiology and disease is its ability to regulate several classes of ion channels. These include BK_{Ca}, Ca^{2+} channel (L-type) families, ligand-gated P2X receptors (P2X2 and P2X4) and the epithelial Na^+ channel (ENaC). These important ion channel targets of CO are summarized in Fig. 2.

7.2.1 BK_{Ca}

Opening of BK_{Ca} channels leads to hyperpolarization, which closes the voltage-dependent Ca^{2+} channels, reduces intracellular Ca^{2+} concentrations, leading to vascular relaxation. BK_{Ca} are by far the most studied ion channel target of CO. CO signalling was shown to mediate the activation of BK_{Ca} in systemic arterial smooth muscle [159-162], oxygen-sensitive glomus cells of the carotid body [163,164], pulmonary artery myocytes [51] and in venous endothelial cells [165].

The conductance and opening probability of BK_{Ca} channels are determined by specific amino acid residues that compose the channel protein. Wang et al. (1997) [160,161] showed that CO increases the opening probability, but not the conductance, of BK_{Ca} channels, suggesting that the gating mechanism is likely modified by CO. Administration of diethyl pyrocarbonate, a chemical that reacts only with the unprotonated imidazole ring of histidine, abolished the effect of CO on BK_{Ca} channels, which was stored upon removal of diethyl pyrocarbonate [160]. Additionally, photo-oxidation with rose bengal, which modifies only histidine residues located on the external surface of the cell membrane, also blocked the stimulatory effect of CO on BK_{Ca} channels [160]. These observations suggest that an interaction between CO and the imidazole group of an extracellular histidine residue could be largely responsible for CO-induced BK_{Ca} channel activation in rat VSMCs [160].

A site-directed mutagenesis of a cysteine residue at position 911 in the intracellular C-terminal domain significantly reduced, but not completely, the ability of CO to activate human BK_{Ca} channel subunit [166]. Another study showed that mutations of histidine 365, histidine 394 and aspartate 367 (amino acid residues located in the cytoplasmic high-affinity divalent cation sensor in the RCK1 domain) appear to alter CO affinity to BK_{Ca} channels [167]. Therefore, it is possible that these residues [167], the extracellular histidine residue [160] and the C911 residue [166] all contribute to the site of action of CO. Furthermore, different subtypes of BK_{Ca} channels are expressed in different types of cells from different species. This would also explain the aforementioned reports.

Intriguingly, the stimulatory effects of CO and NO on BK_{Ca} activation may have different molecular basis in regards to their unique molecular and functional interactions with the channel subunits. Wu et al. (2002) [159] demonstrated that NO activates BK_{Ca} channels by interacting with the BK_{Ca},β subunit, whereas CO stimulates channel opening *via* the BK_{Ca},α subunit in rat VSMCs. Moreover, NO has a stronger potency for BK_{Ca} activation compared to CO, and pretreatment of NO abolished CO-induced activation of the BK_{Ca} channels [159]. Indeed, NO binding to the BK_{Ca},β subunit may exert an allosteric effect on the neighbouring BK_{Ca},α subunit, thus suggesting a feedback mechanism between NO and CO signalling in order to regulate vascular contractility [159].

7.2.2 Ca^{2+} channel (L-type) receptors

CO regulation of L-type Ca^{2+} channels is controversial. CORM-2 or CO inhibits cardiac L-type channel ($\alpha 1_C$ or CACNA1C) in native rat cardiomyocytes [168], HEK293 cells [168,169] or in H9c2 cells (rat heart cell line) [101]. CO inhibits channel activation *via* the transient increase in mitochondrial ROS formation, leading to the modulation of three specific cysteine residues (at positions 1789,1790 and 1810) in the C-terminal tail of L-type Ca^{2+} channel [168]. In stark contrast, CO was shown to activate L-type Ca^{2+} channels in human recombinant intestinal smooth muscle cells [170]. In fact, CO induces channel activation through a NO-dependent mechanism [170]. These contradicting observations may be due to differences in the cellular redox state or tissue-specific splice variation, which was also observed in oxygen regulation of L-type channels [171].

7.2.3 Ligand-gated P2X receptors

P2X receptors are ligand-gated ion channels that are opened by the binding of extracellular ATP [172]. P2X receptors initiate contraction in cardiomyocytes and in various smooth muscle cells, and are the only ligand-gated ion channel to be modulated by CO [172,173]. CORM-2 enhance ATP-evoked P2X2 currents in rat pheochromocytoma cells, but inhibits currents evoked by P2X2/3 and P2X4 receptors in the presence of high ATP in HEK293 cells [174]. Recently, it was shown that CORM-2 inhibits human recombinant P2X4 channels in HEK293 cells [175]. CO likely regulates P2X channels through a cGMP-independent pathway [175,174] and mitochondrial-ROS generation [175].

7.2.4 ENaC

ENaC plays an important role in reabsorption of sodium primarily in the kidney, colon and lung [172]. CO regulation of ENaC is also controversial. For instance, CORM-2 activates ENaC in inside-out membrane patches in a kidney cortical collecting duct cell line [176]; yet, CORM-3 inhibits ENaC in rat cultured alveolar type II cells and in a human airway cell line [177]. The latter occurs through a diethyl pyrocarbonate-dependent mechanism, which is dependent upon important histidine residues [177]. These divergent results may reflect tissue-specific differences in ENaC subunit composition, species variation in amino acid sequences and experimental methodology.

7.3 CO-induced activation of major signalling pathways

7.3.1 MAPK

Numerous studies have linked the p38 MAPK pathway to the anti-inflammatory, anti-apoptotic and anti-proliferative effects of CO [59,80,98,157,178]. MAPK is a non-metal protein that plays a critical role in cellular signal transduction, where all three MAPK members (Jun-activated kinases; JNK, p38 and ERK) may be involved in proliferation and cell-cycle progression [179]. In fact, CO signalling was shown to inhibit the induction of ERK1/2 in human airway smooth muscle cells [68,148], in the rat kidney [180] and rat lung [181], as well as induce JNK-1 and -2 activation in rat aorta tissues [116].

The p38 isoforms (α, β, γ and δ) are broadly sensitive to oxidative, environmental and inflammatory stress and are thus up-regulated in response to these stressors [179]. Therefore, CO likely up-regulates p38 MAPK primarily through the transient burst of ROS [148]. CO was also shown to up-regulate p38 MAPK through the cGMP/GC pathway [116]. Interestingly, CO (250 ppm) up-regulated p38, specifically p38β and down-regulated p38α expression, which mediated CO-induced cytoprotection against hyperoxic stress in human alveolar epithelial cells [98]. However, in the same cell line, CORM-3 (1 mM) inhibited the expression of p38 [178]. This controversy may be due to the physical form of CO (CO gas *vs.* CORM-3), the concentration (250 ppm *vs.* 1 mM) and the experimental conditions (hyperoxic *vs.* TNF-α-stimulation). Also, it is not clear which p38 isoform CO regulated in the later study [178].

7.3.2 PI3K-Akt

PI3K/Akt signalling is involved in pro-survival signalling, glucose metabolism and cell proliferation [182]. CO signalling was shown to induce Akt expression in rat endothelial cells [183], isolated mouse cardiomyocytes [145], rat hepatocytes [61] and rat hearts [135]. The mechanism of action by which CO actives Akt is through CO-induced mitochondrial H_2O_2 production. Excess H_2O_2 oxidizes the functional thiol groups on counter-regulatory phosphatase like phosphatase and tensin homolog and protein-tyrosine phosphatase-1B, leading to their inactivation [184,185]. The inactivation of these phosphatase permits unopposed activity of Akt. Induction of Akt, observed after CO treatment, leads to oxidative phosphorylation, heme synthesis and mitochondrial biogenesis [145], angiogenesis by stabilization HIF-1α [76], as well as cell survival [61,135,183].

7.3.3 STAT

The signal transducers and activators of transcription (STAT) family of transcription factors mediate cytokine- and growth factor-induced signals that regulates cellular proliferation and differentiation [186]. STAT3 was shown to up-regulate manganese superoxide dismutase [187] and molecules essential for cell growth and survival, such as heat shock proteins and growth factors [188,189]. CO can differentially modulate STAT1 and STAT3 activation, where STAT3 activation by CO is responsible for the anti-apoptotic effect in endothelial cells during anoxia-reoxygenation injury [183]. Additionally, it was shown that endothelial STAT3 is essential for the protective effects of CO in oxidant-induced lung injury and cell death [190]. Likewise, exogenous CO was unable to fully block hyperoxia-induced apoptosis in STAT3-deficient mice or in lung endothelial cells transfected with STAT3 siRNA [190].

7.3.4 PPAR

PPARs play essential roles in adipogenesis and glucose homeostasis, and negatively regulate inflammatory responses [191]. So far, three PPAR isoforms have been identified, PPARα, PPARβ/δ and PPARγ. Numerous studies report the induction of PPARγ with CO treatment [84,85,147,192-194]. Recently, it was demonstrated that exogenous CO enhances SUMOylation of PPARγ in LPS-treated macrophages *via* mitochondrial ROS production [192]. SUMOylation is a protein modification that has significant barring on inflammatory responses [192]. Through the induction of PPARγ, CORM-2 was shown to down-regulate iNOS in LPS-activated macrophages [84] and inhibit high-glucose-induced ICAM expression in HUVECs [85], thus reducing the inflammatory state. Inhaled CO was also shown to protect against ventilator-induced lung injury *via* PPARγ activation [193].

7.3.5 HIF-1α

HIF-1α is an oxygen sensor and a transcriptional complex involved in the regulation of cell survival, angiogenesis, glucose metabolism and inflammation [195]. CO was reported to increase the stability of HIF-1α in macrophages [83], endothelial cells [76], vein grafts from the inferior vena cava [82] and in cardiomyocytes [78]. CO-induced HIF-1α expression is necessary to prevent anoxia/reoxygenation-induced apoptosis, preserve cellular homeostasis at the site of injury [83] and promote cardiac regeneration in a myocardial infarct model [78]. It was demonstrated that CORM-2 increases the stability of the HIF-1α protein by suppressing its ubiquitination and increasing HIF-1α/HSP90α interaction, which is responsible for HIF-1α stabilization [76]. Lastly, *ex vivo* CO treatment of vein grafts resulted in

increased HIF-1α activation in the vein grafts, thus preventing intimal hyperplasia [82].

On the contrary, one study showed that CO (10 and 80%) suppressed the activation of HIF-1α by triggering destabilization of HIF-1α in Hep3B cells under hypoxia conditions [196]. However, the authors of this study used high concentrations of CO since CO has a low affinity for direct HIF-1α binding [196], whereas others have shown that low concentrations of CO induced HIF-1α activation, indirectly, likely through the P13K/Akt and MAPK pathways [76] or mitochondrial-ROS production [83].

7.3.6 sGC

Similar to NO, CO can stimulate sGC leading to an elevation of intracellular cGMP levels. This effect was shown to occur in VSMCs [47], human internal thoracic and radial artery rings [48] and in the liver [49]. CO binds to the heme moiety of sGC, thus stimulating sGC and elevating intracellular levels of cGMP leading to vasorelaxation [47-49], anti-inflammation [86], inhibition of anti-platelet activation [8] and regulation of glucose metabolism [106,108]. Interestingly, studies have shown that NO is about 30-100-fold [197] more potent than CO in stimulating sGC *in vitro* [198,199] and NO-induced vasorelaxation is about 1000-fold greater than CO-mediated vasodilation [197]. However, Zakhary et al. (1997) [200] demonstrated that intestinal cGMP levels were depleted in HO-2 knockout mice to the same extent as nNOS knockout mice.

8 The interaction of CO with NO and H_2S

It is abundantly clear that CO, NO and H_2S interact amongst themselves (Fig. 3). NO up-regulates HO-1 expression thus enhances the generation of endogenous CO [201-203]. In fact, NO is the one of the most potent inducers of HO-1 [204,205]. In turn, CO attenuates iNOS activity by binding to the heme moiety of the enzyme [206-208] and mediates iNOS protein expression levels through p38 [203] and protein turnover [206,209,210]. Taking this interrelationship into account, it has been suggested that CO regulates iNOS expression and activity in order to modulate the tissue stress response [203,209-211]. In other words, CO could act as a feedback inhibitor of iNOS when NO concentrations exceed the homeostatic threshold [212]. Indeed, NO derived from iNOS induces HO-1 expression [213] and potentiates HO-1 induction [214], whereby HO-1 activity inhibits iNOS expression [212]. On the contrary, CO was shown to increase iNOS protein expression following TNF-α/D-galactosamine treatment in mice, which resulted in liver protection [215]. How CO differently regulates iNOS expression in different organs or different stimuli is not clear.

Interaction of CO with H_2S is another interesting signalling event. One report showed that H_2S administered to rats with hypoxia pulmonary hypertension increased plasma CO concentration and HO-1 expression in the pulmonary artery [216]. In fact, when rats were given DL-propargylglycine (an inhibitor of cystathionine γ-lyase) to inhibit endogenous H_2S production, HO-1 gene and protein expression were significantly reduced, and consequently, plasma CO concentration [216]. The mechanism by which H_2S up-regulates the HO-1/CO system is not fully understood; however, hemoglobin, the common "sink" may have been involved in the displacement of CO in the presence of high H_2S, thereby increasing the release of CO. In agreement, another group showed that H_2S up-regulates HO-1 expression, *via* ERK activation, in LPS-stimulated macrophages [88]. H_2S-induced up-regulation of HO-1 resulted in the down-regulation of iNOS/NO system and NF-κB activation [88]. Other indirect evidence shows that H_2S inhibits all three NOS isoforms (eNOS, iNOS and neural NOS) [217], thus suggesting an interference in the NOS/NO-induced up-regulation of the HO-1/CO system. We have only begun to scratch the surface of the complex interrelationship of CO with NO and H_2S. This field of research holds exciting prospects related to gasotransmitter interactions.

9 Perspectives

CO has come a long way from solely being considered an environmental pollutant to an important gasotransmitter involved in a number of physiological processes in both physiological and pathophysiological systems. Toxicology studies are needed to establish the safety profile of prolonged exposure to various CO concentrations in pre-clinical studies. The routes for CO administration as a therapeutic agent will require better controlled and designed examination to optimize the absorption, distribution and the systemic impacts of CO. The next important objective is to validate the therapeutic benefits of CO observed in various animal models in humans. Moreover, the ability to use exhaled CO as a reliable, non-invasive biomarker in diabetes and metabolic syndrome would hold promise for gauging disease severity and therapeutic efficacy. Furthering our understanding of the metabolism and function of CO will lead to rational drug design for novel therapeutic gain in preventing and treating different diseases.

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Fig. 1. Endogenous production of CO from heme oxygenase

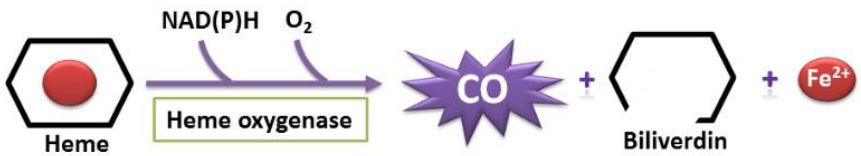

Fig. 2. CO regulation of important ion channels in the cardiovascular system

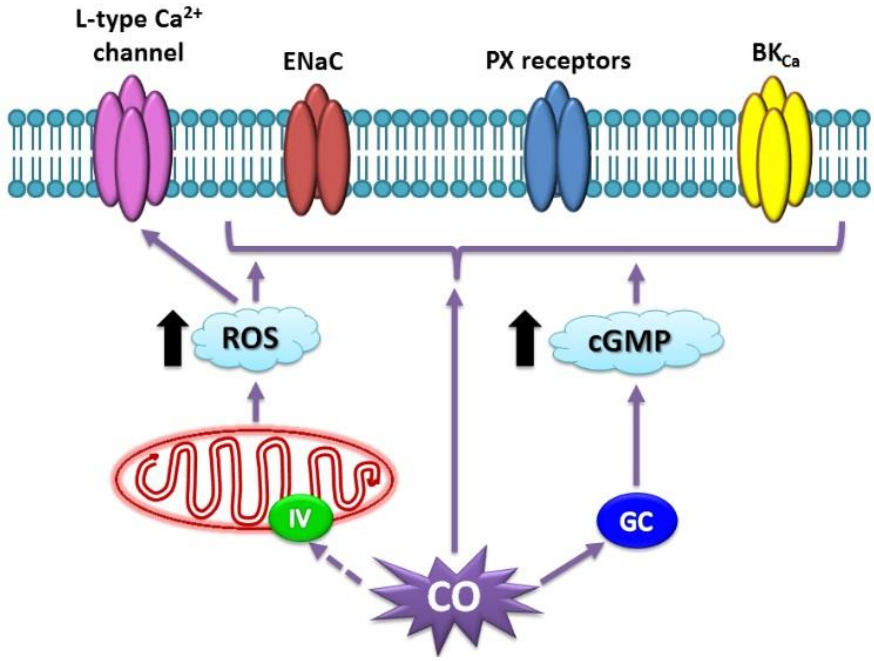

Fig. 3. Interrelationship between CO, NO and H_2S