Acta Biochimica Polonica

Vol. 50 No. 1/2003

31–47

www.actabp.pl

Review

Carbon monoxide — a "new" gaseous modulator of gene expression★✪

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Received: 02 January, 2003; revised 13 February, 2003; accepted: 04 March, 2003

Key words: nitric oxide, oxidative stress, vascular endothelial growth factor, angiogenesis, atherosclerosis

Carbon monoxide (CO) is an odorless, tasteless and colorless gas which is generated by heme oxygenase enzymes (HOs). HOs degrade heme releasing equimolar amounts of CO, iron and biliverdin, which is subsequently reduced to bilirubin. CO shares many properties with nitric oxide (NO), an established cellular messenger. Both CO and NO are involved in neural transmission and modulation of blood vessel function, including their relaxation and inhibition of platelet aggregation. CO, like NO, binds to heme proteins, although CO binds only ferrous (FeII) heme, whereas NO binds both ferrous and ferric (FeIII). CO enhances the activity of guanylate cyclase although it is less potent than NO. In contrast, CO inhibits other heme proteins, such as catalase or cytochrome P450. The effects of CO on gene expression can be thus varied, depending on the cellular microenvironment and the metabolic pathway being influenced. In this review the regulation of gene expression by HO/CO in the cardiovascular system is discussed. Recent data, derived also from our studies, indicate that HO/CO are signifi-

✪This work was partially supported by grants from the State Committee for Scientific Research (KBN, Poland), No. 3 P04A 049 22 and 6 P04B 013 21.

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Abbreviations: cGMP, 3′,5′-cyclic guanosine monophosphate; HIF, hypoxia inducible factor; ET-1, endothelin-1; HMEC-1, human microvascular endothelial cells; HOs, heme oxygenases; HRE, hypoxia response element; iNOS, inducible nitric oxide synthase; IL-10, interleukin-10; MIF, macrophage inflammatory protein 1β; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor α; VSMC, vascular smooth muscle cells.

cant modulators of inflammatory reactions, influencing the underlying processes such as cell proliferation and production of cytokines and growth factors.

Carbon monoxide has been known since 17th century as a poisonous gas ("the silent killer"). In 1857 Claude Bernard determined that the gas produces asphyxia by reversibly combining with hemoglobin (Piantadosi, 2002). Innumerable deaths have resulted from CO created by incomplete combustion of organic materials. Recently its concentration in the atmosphere, being a result of technological processes and fumes produced by our cars, is monitored and alerts our consciousness when reported to be too high in the smoggy air of our cities. Additionally, CO is a component of cigarette smoke. Due to such a bad reputation not many people, including scientists, are willing to consider its significance as a player of physiological, not only pathological processes in our organism.

Formation of CO in the body was demonstrated in 1952 by Sjöstrand, who reported that decomposition of hemoglobin *in vivo* led to CO production (Sjöstrand, 1952). In 1968 heme oxygenase, the enzymatic source of CO was identified by Tenhunen *et al.* (1968; 1969; 1970), and in the mid 80's two isoforms of the HO enzyme were discovered and cloned (Rotenberg & Maines, 1990; Shibahara *et al.,* 1985; Yoshida *et al.,* 1988). Heme oxygenase-1 (HO-1), an inducible isoform and HO-2, a constitutive form cleave and oxidize the α-methene bridge of the heme molecule yielding equimolar amounts of biliverdin, CO and iron (for a review see: Maines, 1997) (Fig. 1). The catalytic activity of HO requires a concerted action of microsomal NADPH-cytochrome P450 reductase to transfer electrons to the HO-heme complex.

In the last few years evidence has accumulated showing that the HO enzymes and their by-products are important players in the cellular metabolism. This review is intended to discuss some of those data demonstrating the significance of CO in modulation of cellular inflammatory reaction.

HEME OXYGENASES AND THEIR ENZYMATIC ACTIVITY

Currently three isoforms of HO are known: HO-1, HO-2 and HO-3. HO-1 (termed also hsp32) is a stress inducible enzyme which can be expressed most probably in every cell facing contact with noxious stimuli (for a review see: Maines, 1997). Hence, HO-1 induction can be regarded as a general response to oxidant stress (Applegate *et al.,* 1991). HO-1 can represent a secondary protective system, while the primary defense mechanism against oxidative stress is glutathione (Meister, 1994). Interestingly, glutathione depletion results in a strong induction of HO-1 (Applegate *et al.,* 1991; Lautier *et al.,* 1992; Ewing & Maines, 1993) supporting the importance of HO-1 in cellular protective mechanisms.

HO-2 is a constitutive gene, expressed in neurons, endothelium and many other cell types. The only known inducers of HO-2 activity are adrenal glucocorticoids (Weber *et al.,* 1994) but it is also possible that activation of protein kinase C can result in an increased degradation of heme by the HO-2 isoform (Baranano & Snyder, 2001). HO-2 is involved in the regulation of neural system functioning, modulating the neural transmission in central nervous system, digestive system and in male copulatory organ (for a review see: Maines, 1997; Baranano & Snyder, 2001; Snyder & Baranano, 2001).

HO-3 is a newly identified isoform (McCoubrey *et al.,* 1997) and has been found only in rats (Scapagnini *et al.,* 2002). It is constitutively expressed in the liver, spleen, brain and kidney, but its ability to degrade heme is much limited in comparison with HO-1 and HO-2 (McCoubrey *et al.,* 1997). The *HO-3* gene does not contain introns which suggests that *HO-3* could have arisen by retrotransposition of the *HO-2* gene (Scapagnini *et al.,* 2002). HO-3 is believed to function as a heme sensing

or heme binding protein rather than a heme-degrading enzyme (McCoubrey *et al.,* 1997), but its biological role requires further elucidation.

The activity of HO-1 generates colour effects. When large amounts of heme are released from destroyed erythrocytes due to a physical insult on blood vessels in the skin and muscles, HO-1, induced by heme, starts its degradation. The black heme is transformed to green biliverdin, and when the bruise is dissapearing, the yellowish bilirubin appears.

Basing on the biochemical activities of HO, its products have for a long time been recognized only as the waste of heme cleavage. Additionally, when heme degradation occurs under pathological conditions, significant health disturbances may arise. Hence, it has been customary to regard HO as an enzyme, the activity of which results in the clinical problem of hyperbilirubinemia leading to jaundice. However, ample evidence has recently accumulated indicating that HO activity and all its by-products play important physiological roles (for reviews see: Choi & Otterbein, 2002; Foresti & Motterlini, 1999; Hill-Kapturczak *et al.,* 2002; Maines, 1997; Otterbein & Choi, 2000) (Fig. 1).

First, HO activity removes the prooxidant heme (Balla *et al.,* 1991; Jeney *et al.,* 2002; Quan *et al.,* 2002; Vercellotti *et al.,* 1994). Second, both biliverdin and bilirubin have antioxidant properties, efficiently scavenging reactive oxygen species and inhibiting lipid peroxidation (Baranano *et al.,* 2002; Stocker *et al.,* 1987a; 1987b). Third, iron released from heme enhances the synthesis of ferritin, which additionally has antioxidant capabilities (Balla *et al.,* 1992). Fourth, HOs, or at least HO-1, prevent free iron accumulation in the cells not only indirectly by stimulating ferritin production, by also directly by extruding iron oustide the cell (Ferris *et al.,* 1999). In this activity HO-1 cooperates with a recently identified Fe-ATP pump (Baranano *et al.,* 2000). An indication for a role of HO-1 in iron extrusion is the accumulation of iron in the cells of *HO-1* knockout mice (Poss & Tonegawa, 1997b; 1997c) or in the liver and kidney of a boy who lacked a functional *HO-1* gene (Yachie *et al.,* 1999). Accordingly, gene transfer of *HO-1* to cells derived from *HO-1* knockout animals restored the cells' capability to control the cellular iron level (Ferris *et al.,* 1999).

Finally, CO, the "notoriously infamous" by-product of HO activity enters recently the

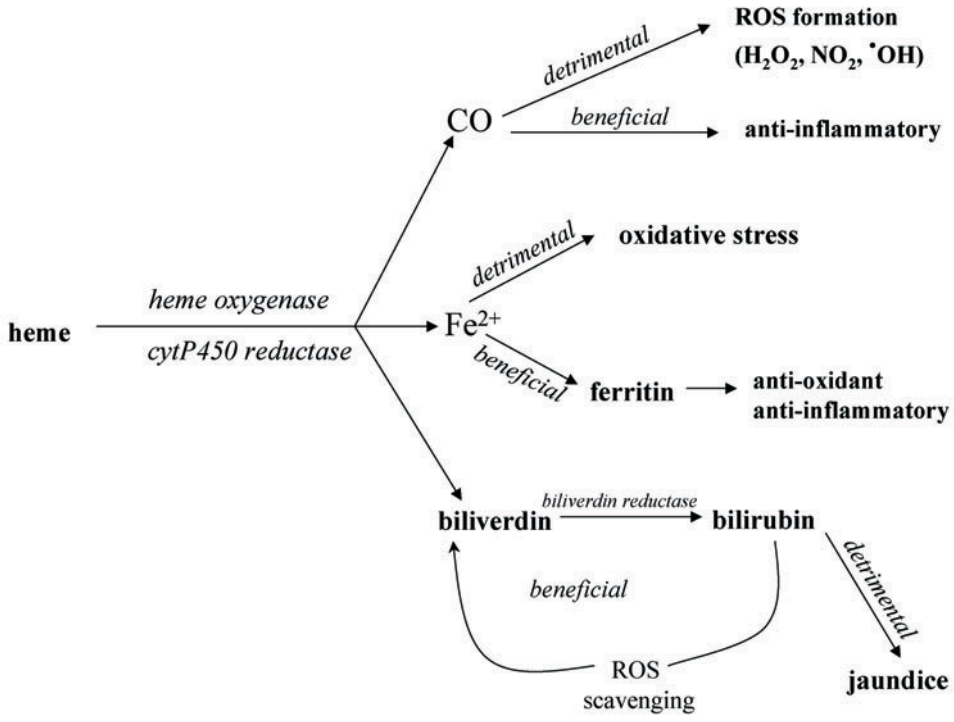

Figure 1. Mechanisms of heme oxygenase activity

scene as an important modulator of many physiological processes. Particularly, it plays a role in neural transmission, is necessary for ejaculation, involved in the homeostatic control of cardiovascular function and appears to modulate gene expression in many cell types (for reviews see: Foresti & Motterlini, 1999; Snyder & Baranano, 2001). However, due to space limitations we will discuss here only the recently investigated activities of CO in the pathways governing the synthesis of inflammatory cytokines and growth factors.

CO PRODUCTION AND MECHANISMS OF CO EFFECTS ON CELLULAR FUNCTION

HOs are the main producers of CO in the human body. Much smaller amounts of CO can also derive from other sources, like lipid peroxidation (Fig. 2) (for a review see: Piantadosi, 2002). CO is formed at the rate of 16.4 μmol/h in the human body and the daily production of CO is substantial, reaching more than 12 ml (500 μmole). However, average physiological concentrations of CO in tissues are rather in the low nanomolar range (for a review see: Piantadosi, 2002). On the other hand, increased production of CO has been demonstrated in chronic inflammatory lung diseases, such as obstructive pulmonary disease, cystic fibrosis, and asthma as well as in infectious pulmonary diseases (Kharitonov & Barnes, 2002).

The action of CO depends primarily on its ability to bind heme proteins and to inhibit or alter their biochemical functions (for a review see: Piantadosi, 2002). By interacting with heme proteins CO differentially influences electron-transport reactions producing either prooxidant or antioxidant effects. The action of CO is dependent on its concentration, the concentration of O_2 as well as on the availability of reduced transition metals, such as iron or copper (cited after: Piantadosi, 2002).

Some activities of CO resembles those of NO (for reviews see: Baranano & Snyder, 2001; Snyder *et al.,* 1998). CO, like NO, avidly binds heme proteins with iron in the reduced ferrous state ([Fe(II)], but unlike NO, does not bind ferric iron [Fe(III)]. Like NO, CO activates soluble guanylate cyclase (Brune *et al.,* 1990; Furchgott & Jothianandan, 1991; Karlsson *et al.,* 1985; Utz & Ullrich, 1991), leading to a several-fold increase in the production of cGMP, although its potency in stimulation of this effect is about 30–100 times lower than that of NO (Kharitonov *et al.,* 1995). Therefore, due to such a weak influence of CO on guanylate cyclase, the physiological significance of this activation is in doubt. It is, however, suggested that the effect of CO on guanylate cyclase can be potentiated

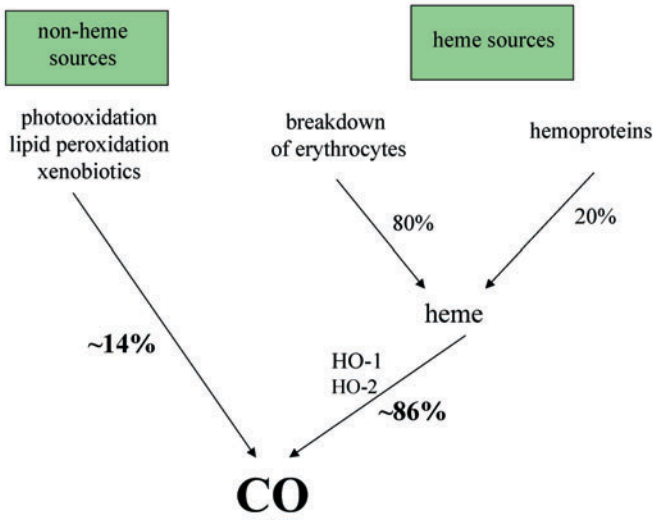

Figure 2. The sources of carbon monoxide (based on data from Vreman *et al.*, 2002)

by some as yet unknown co-stimulators. The rationale for such a hypothesis was a discovery of YC-1, a benzyloid derivative, which augments CO-mediated induction of cGMP production to a level attained by NO (Friebe & Koesling, 1998; Friebe *et al.,* 1996).

Additionally, it is possible that CO may amplify the NO-mediated activation of guanylate cyclase (Ingi *et al.,* 1996; Cao *et al.,* 2000). CO may also exert its activity through a direct influence on NO. Indeed, low concentrations of CO stimulate NO release and augment the production of the strong oxidant peroxynitite in vascular cells (Thom *et al.,* 1999; 2000).

Other heme proteins, such as myoglobin, cytochrome *c* oxidase, cytochrome P450, catalase and tryptophan dioxygenase can be also influenced by CO (for a review see: Piantadosi, 2002). In contrast to guanylate cyclase, however, CO inhibits their catalytic activities. As a result, a significant cellular oxidative stress can be produced by CO in vascular endothelium (Thom *et al.,* 1999; 2000) and in other cell types *in vivo* (Piantadosi, 2002). This occurs already after low-level CO exposure (100 p.p.m.) and can generate a significant lipid peroxidation. Such an effect can be blocked by superoxide dismutase and iron chelators (Piantadosi, 2002).

CO can influence gene expression in several ways. First, an increase in CO concentration *in vivo* will result in carboxyhemoglobin formation and decreased oxygenation and hypoxia. Hypoxia is a physiological regulator of important biological processes, including erythropoiesis, angiogenesis, glycolysis and tissue remodeling (for a review see: Kourembanas, 2002). It may originate from a decrease in O_2 concentration, but can also occur when blood hemoglobin is blocked by CO.

Second, local effects of CO may derive from its interaction with NO (for a review see: Hartsfield, 2002). CO can cause a release of NO from its heme-bound intracellular pool, which may result not only in activation of guanylate cyclase, but also in nitrosylation of protein thiol groups (Thorup *et al.,* 1999; Foresti *et al.,* 1999; Marshall *et al.,* 2000). Through those ways NO can influence gene expression (for reviews see: Dulak & Józkowicz, 2003; Marshall *et al.,* 2000). Such effects can occur either in cells constitutively generating NO, like neurons and endothelial cells, or in cells producing significant amounts of NO by the action of inducible nitric oxide synthase (iNOS). A combination of high concentrations of NO and CO can influence mitochondrial cytochromes, causing their inhibition and formation of reactive oxygen species (ROS) leading to lipid peroxidation (Agarwal *et al.,* 1995; Koehler & Traystman, 2002). ROS induced by CO can influence the activity of several transcription factors and kinases, as has been demonstrated so far for NF-κB and p38 kinase (Brouard *et al.,* 2002) (see below).

Third, the cellular effect of CO can be independent of hypoxia and interaction with NO. As mentioned, CO can induce the generation of hydrogen peroxide, maybe through induction of the expression of manganese superoxide dismutase (MnSOD) (Frankel *et al.,* 2000) or by inhibition of catalase activity (Zhang & Piantadosi, 1992; Piantadosi, 2002) (Fig. 3). H_2O_2 is not only a toxic oxidant, but it is also an important cellular messenger, regulating the expression of numerous genes.

Besides the similarities striking differences between CO and NO exist. NO is a free radical and it is the most reactive of physiological gases (Piantadosi, 2002). The reaction of NO with ROS can result in formation of numerous potent intermediates. It is likely that NO toxicity, which is higher than that of CO and occurs above 100 p.p.m., derives from its reaction with superoxide to form peroxynitrite (Piantadosi, 2002). Accordingly, the potential therapeutic doses of inhaled NO are probably lower (65 p.p.m.) than of carbon monoxide (500–1000 p.p.m.) (Thiemermann, 2001).

In vivo CO is almost immediately toxic at the concentration of 0.4% (4000 p.p.m.) or more, but the concentration of 0.01% (100 p.p.m.) is tolerable and allowable for an exposure of several hours (Otterbein & Choi, 2000). CO at low

concentrations (10–500 p.p.m.) is well tolerated by cells, and the rodents can be exposed to 500 p.p.m. continuously for up to 2 yr without deleterious effects (Stupfel & Bouley, 1970; Otterbein & Choi, 2000).

The cellular effects of CO was studied basing on the activity of HO-1 and the use of HO inhibitors, but data on cellular CO concentrations are usually lacking. In contrast, exogenous CO was applied at very different amounts, sometimes very high. Such varied modes of treatment may result in discrepant results as will be discussed further.

REGULATION OF GENE EXPRESSION IN HYPOXIA BY CO

In vascular smooth muscle cells (VSMC) cultured under hypoxic conditions CO is produced as a result of induction of HO-1, and an increase in cGMP content is observed (Morita *et al.,* 1995). The response is transient, with cGMP peaking at 15 h and returning to baseline by 48 h. It has been reported that very high exogenous CO concentrations (5% or more, even up to 80%) inhibited hypoxic induction of erythropoietin (Huang *et al.,* 1999), vascular endothelial growth factor (VEGF) (Goldberg & Schneider, 1994), endothelin-1 (ET-1) and platelet-derived growth factor (PDGF) genes (Morita & Kourembanas, 1995). Endogenous CO, derived from HO activity in VSMC growing in co-culture with endothelial cells, inhibited the hypoxic induction of PDGF-B and ET-1 in endothelium (Morita & Kourembanas, 1995). In a feedback response, the decreased production of those mitogens by endothelial cells resulted in slowing down the proliferation of the co-cultured VSMC (Morita *et al.,* 1997).

The underlying mechanism is not known. It was suggested that it can be due to the inhibition of cytochrome P450 (Wang, 1998). Indeed, cytochrome P450-linked monooxygenase is responsible for the generation of vasoconstricting substances, such as certain arachidonic acid metabolites or ET-1. CO is an inhibitor of cytochrome P450 and the level of cytochrome P450 is controlled by the availability of cellular heme, which can be degraded to CO and biliverdin (Wang, 1998). A decreased formation of vasconstrictors in response to CO would lead to vascular relaxation.

The complex effect of CO on gene expression in hypoxia requires elucidation. It has been hypothesized that CO inhibits the hypoxic induction of genes encoding vasoconstrictors in smooth muscle cells in the early hypoxic phase (Kourembanas, 2002). During chronic hypoxia, however, low CO may tilt the balance toward increased production of growth factors and vasoconstrictors that promote vessel-wall remodeling (Kourembanas, 2002).

The main molecular sensor of the level of O_2 in the cell is the transcription factor HIF-1 (for a review see: Semenza, 2002). In the functional state it is a heterodimer consisting of two subunits, HIF-1α and HIF-1β. This heterodimer binds to a recognition site (HRE – hypoxia response element) present in the promoter of many genes regulated by hypoxia, such as erythropoietin, VEGF, HO-1 or inducible nitric oxide synthase (iNOS) (for a review see: Semenza, 2002).

HIF-1 subunits are constitutively produced in the majority of cells, but dimer formation is prevented by oxygen-dependent degradation of HIF-1α. Degradation occurs after hydroxylation of specific proline residues (P402 and P564) in the HIF-1α protein (for a review see: Maxwell & Ratcliffe, 2002). At normal oxygen tension the hydroxylation is performed by a prolyl hydroxylase, which requires iron, O_2 and 2-oxoglutarate as cofactors.

The data on the effect of CO on HIF-1 production and activity are scarce and inconclusive. It has been suggested that the cellular hypoxia sensor is a heme-containing protein (Goldberg & Schneider, 1994; Huang *et al.,* 1999) because cobalt chloride or iron chelators can mimic the effects of hypoxia. It has been hypothesized that in the presence of

O_2 that putative protein can bind O_2 at a heme site attaining a "relaxed" configuration, whereas the absence of O_2 confers a "tense" conformation (cited after Kourembanas, 2002). It was claimed that CO, a molecule known to interact with heme groups, can inhibit the hypoxic induction of genes by behaving similarly to O_2 and shifting the heme-protein to the relaxed configuration (Kourembanas, 2002). However, the recent discovery of the prolyl hydroxylase which requires iron but is not a heme protein sheds some doubts on this attractive hypothesis.

Recently, a first eukaryotic transcription factor selectively affected by CO was discovered. Dioum *et al.* (2002) have demonstrated that binding of NPAS2, a member of the same family of proteins to which HIF-1α belongs, is inhibited by CO, but is not influenced by NO nor O_2. NPAS2 is a homologue of CLOCK, a transcription factor involved in modulation of circadian activity in the suprachiasmatic nucleus (Rutter *et al.,* 2002). NPAS2 is also present in cells outside the central nervous system (Dioum *et al.,* 2002; Rutter *et al.,* 2002), and, interestingly, binds to a very similar DNA sequence (CACGTG) as HIF-1 (TACGTG). The relationship and potential reciprocal influence of NPAS2 and HIF-1 on their activity is not known and opens a new fascinating area for further investigations.

The binding of the HIF-1 protein to HRE, as determined by gel shift assay, was attenuated in cells treated with exogenous CO in hypoxia (Liu *et al.,* 1998). However, the amount of HIF-1α protein seemed not to be influenced at 5% CO concentration (Liu *et al.,* 1998), while it was decreased at high, 80% exposure (Huang *et al.,* 1999). How this relates to the physiological situation is not known as the concentrations of CO used in those experiments were extremely high.

Interestingly, and in contrast to the studies discussed above, our recent data indicate that CO can be a positive regulator of VEGF synthesis. We observed a significant induction of VEGF in vascular smooth muscle cells cultured in the presence of 1% CO (10000 p.p.m.) in otherwise normoxic conditions (Dulak *et al.,* 2002; Dulak & Józkowicz, 2003). It appears that CO can modulate VEGF synthesis also in other cell types. Accordingly, we observed an increase in VEGF synthesis in microvascular endothelial cells (HMEC-1) treated with ruthenium carbonyl compound, a representative of a new class of substances named carbon monoxide releasing molecules (CO-RM) (Jozkowicz *et al.,* in press). Those chemicals can release CO, and thus are equivalent to the widely used NO donors (Motterlini *et al.,* 2002). Additionally, in HMEC-1 the expression of VEGF was more potently increased by 15d-PGJ_2, a strong activator of HO-1, which induces the generation of higher amount of CO than that attained after CO-RM treatment (Jozkowicz *et al.,* in press).

THE EFFECT OF CO ON INFLAMMATORY REACTION

Great attention has been recently paid to the presumed anti-inflammatory functions of CO. It has been demonstrated that CO at a physiological concentration (100–500 p.p.m.) inhibits the production of pro-inflammatory cytokines (Otterbein *et al.,* 2000). In macrophages treated with LPS the synthesis of TNFα, MIF and IL-1 is a marker of the inflammatory processes. When such cells were kept in the presence of CO, the production of those pro-inflammatory molecules decreased. Interestingly, CO upregulated the synthesis of the anti-inflammatory cytokine IL-10 (Otterbein *et al.,* 2000). Looking further for the mechanisms governing this potentially preventive CO activity Otterbein *et al.* (2000) found that CO action is independent of cGMP, but rather p38 kinase is necessary (Fig. 3). Accordingly, CO failed to inhibit cytokine production in cells derived from animals with targeted mutation in the

MKK3 gene encoding a kinase activating p38 (Otterbein *et al.,* 2000).

Interestingly, a positive loop may operate in the anti-inflammatory functions of HO-1. As mentioned, CO induces IL-10 synthesis in monocytes, indicating for a role of HO-1. Recently Lee & Chau (2002) have demonstrated that IL-10 induces HO-1 expression, utilizing CO as a mediator of anti-inflammatory activities.

The anti-inflammatory activity of CO may underlie the protective effect of HO in such processes like graft rejection (Ke *et al.,* 2001; 2002; Sato *et al.,* 2001) or development of atherosclerosis (Ishikawa & Maruyama, 2001; Ishikawa *et al.,* 2001). It has been elegantly demonstrated that HO activity is necessary for inhibition of xenograft or allograft rejection. When hearts are transplanted from mice to rats, the acute rejection can be prevented by ablation of the complement system and suppression immune response in the recipients by cobra venom factor treatment and cyclosporine delivery (Soares *et al.,* 1998). However, when HO activity was additionally blocked by HO inhibitors the grafts were rejected (Soares *et al.,* 1998).

Through the influence on inflammatory reactions HO-1 overexpression may also prevent the development of atherosclerosis (Shi *et al.,* 2000). The beneficial effect of HO-1 can be exerted by CO-dependent inhibition of vessel constriction (Suematsu *et al.,* 1995), while bilirubin can attenuate the adhesiveness of leukocytes to the vessel wall (Duckers *et al.,* 2001; Hayashi *et al.,* 1999; Kozma *et al.,* 1997; 1999; Zhang *et al.,* 2001). Additionally, HO-1 activity may modulate the inflammatory processes by augmenting iron extrusion from the cells of the blood vessel wall (Juan *et al.*, 2001).

Disruption of the HO-1 gene provides very interesting data supporting the anti-inflammatory role of HO products. The life of HO-1 knockout mice is strongly affected by progressive chronic inflammation characterized by hepatosplenomegaly, lymphadenopathy and leukocytosis. Animals are debilitated and die at a young age from massive iron overload in the liver and kidneys (Poss & Tonegawa, 1997a; 1997b). In response to chronic hypoxia, they exhibit enhanced lipid peroxidation, accentuated oxidative damages, and increased right ventricular infarcts with organized mural thrombi. Exposure to endotoxin results in strong hepatocellular necrosis and high mortality from endotoxic shock. The cells derived from HO-1 targeted mice are highly susceptible to heme- and hydrogen peroxide-mediated toxicity. HO-1 knockout mice are

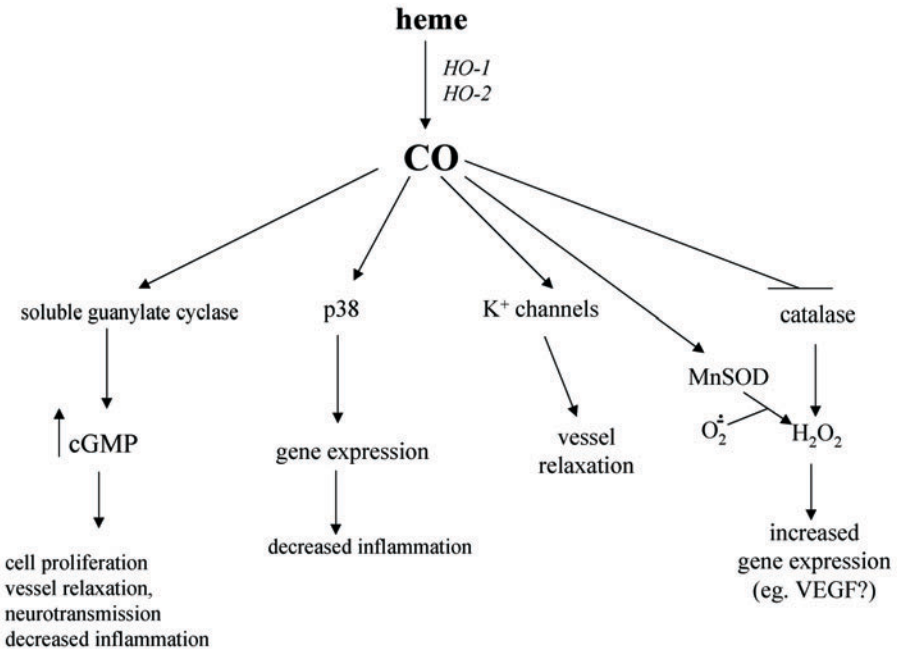

Figure 3. The effect of CO on gene expression and physiological functions in the cardiovascular system.

In this figure only the potential beneficial effects of CO are mentioned. However, it remains to be established whether the detrimental effects, which can occur when HO-1 activity is very high (Suttner & Dennery, 1999), are solely due to the increased release of free iron or are also dependent on CO.

also more sensitive to pulmonary ischemia compared to wild type counterparts (Fujita *et al.,* 2001). This could be prevented by inhalation of CO (0.1%), which inhibited the hypoxic induction of plasminogen activator inhibitor (PAI-1), resulting in higher activity of tissue plasminogen activator (tPA) and decreased fibrin deposition and lung inflammation (Fujita *et al.,* 2001).

Interestingly, the first case of human HO-1 deficiency was described after initial studies on HO-1 targeted mice. The human case of HO-1 deficiency exhibited similar features, including iron deposition in renal and hepatic tissues (Kawashima *et al.*, 2002; Ohta *et al.*, 2000; Yachie *et al.*, 1999). Lack of HO-1 activity resulted in extreme vulnerability of vessels to common stressful stimuli, including infections and environmental toxic substances. A cascade of inflammatory reactions and sustained oxidative stress led to severe and persistent vascular endothelial damage and detachment. Cell lines derived from this patient were strongly sensitive to hemin-induced injury (Jeney *et al.*, 2002). Importantly, HO-2, which was expressed normally, did not provide efficient defense. These clinical symptoms demonstrate the critical importance of HO-1 rather than HO-2 in iron metabolism and in protection of vessels against oxidative stress.

THE INHIBITORY EFFECT OF CO ON CELL PROLIFERATION AND APOPTOSIS

Growth factors, such as PDGF-BB (Durante *et al.,* 1999) or transforming growth factor β (TGF-β) (Kutty *et al.,* 1994; Hill-Kapturczak *et al.,* 2000) which induce VSMC proliferation, enhance also HO-1 expression. It has been hypothesized that induction of HO-1 expression represents a counterbalanced autocrine mechanisms which limits the SMC proliferation (Kourembanas, 2002). Indeed, gene transfer of HO-1 (Duckers *et al.,* 2001; Liu *et al.,* 2002; Peyton *et al.,* 2002; Zhang *et al.,* 2002) or delivery of CO (Morita *et al.,* 1997) blocked SMC proliferation. Accordingly, inhibition of HO-1 activity potentiates SMC growth (Peyton *et al.,* 2002; Togane *et al.,* 2000). The same effect was obtained by treatment of growing SMC with hemoglobin, a CO scavenger (Peyton *et al.,* 2002).

The effect of CO on the proliferation of VSMC can be mediated by influence on transcription factors. Among them E2F is a family of cell-cycle specific transcription factors, which regulate the expression of many genes involved in cell proliferation, and govern the transition of cells from the G_1 to S phase. It has been demonstrated that E2F is affected by CO in a cGMP-dependent manner (Durante, 2002). The involvement of cGMP in the inhibition of VSMC proliferation was also corroborated by experiments with YC-1, which augments CO-dependent activation of guanylate cyclase (Friebe & Koesling, 1998). Accordingly, YC-1 attenuated the growth of smooth muscle cells (Durante, 2002). Moreover, inhibition of guanylate cyclase with methylene blue or ODQ ([1,2,4]-oxadiazolo-[4,3-α] quinoxaline-1-one) prevented CO effect on SMC cell cycle progression (Morita *et al.,* 1997).

The inhibition of the growth of VSMC can have important consequences for prevention of the vessel narrowing after balloon angioplasty, a therapeutic treatment applied to patients with atherosclerosis developing in their coronary peripheral vessels. It is presumed that quick regeneration of endothelial cells after angioplasty can inhibit restenosis due to the restoration of endogenous modulatory mechanisms governing vessel functions (for review see: Dulak & Józkowicz, 2002). Interestingly, it has been recently demonstrated that CO protects endothelial cells from apoptosis induced by various stimuli (Brouard *et al.,* 2000). This effect requires the activation of the NF-κB transcription factor and is dependent on p38 kinase activity (Brouard *et al.,* 2002) indicating for a common mechanism of

the protective action of CO in endotoxin- or cytokine-induced inflammation and in regenerative processes after mechanical injury.

Of particular interest are observations demonstrating that CO (or HO-1 transfection) enhanced proliferation of endothelial cells (Li Volti *et al.,* 2002; Malaguarnera *et al.,* 2002; Deramaudt *et al.,* 1999). Thus, CO may behave similarly like NO, which prevents the apoptosis of endothelial cells and stimulates their proliferation, but which inhibits the growth of SMC (Kibbe *et al.,* 2000; Tzeng *et al.,* 1997). The mechanisms of such effects of those gases on endothelial cells are not known. It can be speculated that both CO and NO enhance the expression of VEGF, a mitogen and anti-apoptotic factor for endothelial cells (Dulak, 2001; Dulak *et al.*, 2002; Jozkowicz *et al.,* in press). However, as endothelial cells do not always produce detectable quantities of VEGF, other mechanisms may lay behind the effect of CO in endothelial cells.

Again, contradictory data have been also obtained concerning the effect of CO on viability of endothelial and vascular smooth muscle cells. Thom *et al.* (2000) showed that maintaining of bovine pulmonary artery endothelial cells in the presence of 100 p.p.m. CO for more than 1 h caused cell death, which could be prevented by a caspase-1 inhibitor. The effect of CO was mediated by NO, as it was blocked by an NO synthase inhibitor, S-isopropylisothiourea, and the peroxynitrite scavenger selenomethionine. Interestingly, prior exposure of endothelial cells to a lower concentration of CO, 10 p.p.m., conferred resistance against the lethal effects of 100 p.p.m. CO, suggesting that the anti- and pro-apoptoic effect of CO is concentration dependent (Thom *et al.,* 2000).

CONCLUSIONS

There is no doubt that CO exerts significant effects on many pathways of the cellular metabolism. In cells of the cardiovascular system CO inhibits inflammatory response, influencing synthesis of cytokines, cell proliferation and preventing cell apoptosis (Fig. 3). Those effects are mediated through both cGMP-dependent and cGMP-independent ways. The physiological activity of CO may result in inhibition of inflammatory reactions in hyperoxia, ischemia/reperfusion injury, atherosclerosis and graft rejection.

It cannot be longer taken as a dogma that CO is only a deadly substance with no physiological functions. Rather, CO can be regarded as a signaling molecule involved in, and maybe even critical for many aspects of cellular metabolism. Apparently, like in the case of the majority of substances, its potentially harmful or beneficial effects are dependent on the concentration. Regarding the physiological role, low concentration of CO, equivalent to the amount released by local activity of heme oxygenase, can influence underlying processes, inhibiting the inflammatory reactions. However, the range of HO activity is much broader, as it concomitantly releases biliverdin and iron. The final outcome of HO activity is thus probably different than the effect expected from the action of separately delivered CO. The beneficial, and possibly therapeutic window of HO-1 appears to be quite narrow, with protective effects exerted at moderately increased activity, and harmful influences prevailing when iron release from degraded heme is very high (Dennery *et al.,* 2003; Suttner & Dennery, 1999). Therefore, the potential beneficial and even therapeutic effects ascribed to increased HO-1 expression or CO supplementation have to be carefully reconsidered regarding the hypothetical risk of aggravation of the oxidative stress due to the increased CO and free iron release.

Although the complexity of the mechanisms underlying CO actions on gene expression is not well known, the results obtained in the last few years have demonstrated its importance in modulation of inflammatory reaction and cellular growth. They shed light on many unknown aspects of CO functions and suggest

new avenues for further investigations and presumably therapeutic applications.

We are grateful to Professor Aleksander Koj for his comments.

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