

CO AS A CELLULAR SIGNALING MOLECULE

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■ **Abstract** Many biological functions of heme oxygenase (HO), such as cytoprotection against oxidative stress, vasodilation, neurotransmission in the central or peripheral nervous systems, and anti-inflammatory, anti-apoptotic, or anti-proliferative potential, have been attributed to its enzymatic byproduct carbon monoxide (CO), although roles for biliverdin/bilirubin and iron have also been proposed. In addition to these well-characterized effects, recent findings reveal that HO-derived CO may act as an oxygen sensor and circadian modulator of heme biosynthesis. In lymphocytes, CO may participate in regulatory T cell function. A number of the known signaling effects of CO depend on stimulation of soluble guanylate cyclase and/or activation of mitogen-activated protein kinases (MAPK). Furthermore, modulation of caveolin-1 status may serve as an essential component of certain aspects of CO action, such as growth control. In this review, we summarize recent findings of the beneficial or detrimental effects of endogenous CO with an emphasis on the signaling pathways and downstream targets that trigger the action of this gas.

INTRODUCTION

Living organisms have developed highly conserved enzymatic systems that generate small gaseous molecules, such as nitric oxide (NO),* carbon monoxide (CO), and hydrogen sulfide (H₂S) (1–4). Coinciding with the development of industry,

*Abbreviations: bHLH: basic helix loop helix; cGMP: guanosine 3',5'-monophosphate; CNS: central nervous system; CO: carbon monoxide; EC: endothelial cells; HBP1: high mobility group-box protein-1; HO: heme oxygenase; HO-1: heme oxygenase-1; HO-2: heme oxygenase-2; Hsp70: 70-kDa heat shock protein; I/R: ischemia/reperfusion; LDL: low-density lipoprotein; MAPK: mitogen activated protein kinase; NO: nitric oxide; NPAS-2: neuronal PAS domain protein-2; p38 MAPK: p38 mitogen activated protein kinase; PDGF: platelet-derived growth factor; PKG: protein kinase G; pRB: retinoblastoma protein; sGC: soluble guanylate cyclase; SnPP-IX: tin-protoporphyrin IX; SMC: (vascular) smooth muscle cells; TGF- β 1: transforming growth factor- β 1; TNF- α : tumor necrosis factor-alpha; UVA: ultraviolet-A; ZnPP-IX: zinc protoporphyrin-IX.

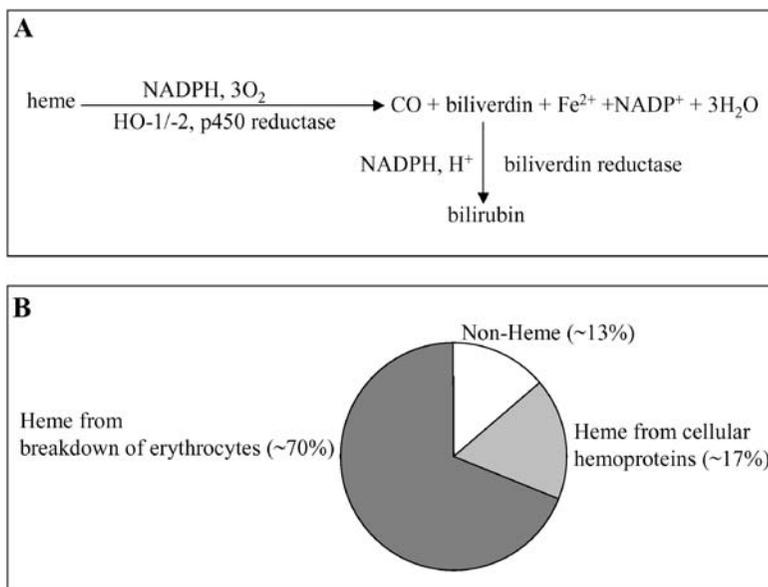


Figure 1 Catabolism of heme by heme oxygenase (HO). Equimolar CO, iron, and biliverdin are produced by HO enzymatic activity. Biliverdin is further metabolized by biliverdin reductase to bilirubin (A). A major source of endogenous CO arises from the degradation of erythrocytes and hemoproteins (B).

these molecules are also widely known as toxic air pollutants. Regardless of their notoriety, recent findings reveal that trace amounts of these gaseous molecules are indispensable for maintaining the biological system and defending the host from hostile environments (5). Physiological NO, as implicated in vasoregulation and immune function, arises from the conversion of L-arginine to L-citrulline by constitutive and inducible nitric oxide synthase (NOS) enzymes. On the other hand, endogenous CO evolves primarily from the enzymatic degradation of heme by the heme oxygenase enzyme system (HO; E.C. 1:14:99:3) (6–8) (Figure 1A and B). HO provides the first and rate-limiting step in the oxidation of heme, leading to the formation of biliverdin-IX α , with the concomitant liberation of CO and reduced heme iron. Heme metabolism concludes with the enzymatic reduction of biliverdin-IX α by NAD(P)H: biliverdin reductase (6, 9). At least two distinct isoforms of HO exist, a constitutive form (HO-2) and an inducible form (HO-1) responsive to transcriptional activation by a broad array of chemical and physical stress (8, 10).

Over the past decade, HO-1 has attracted intensive interest as a mediator of tissue protection and host defense. Efforts to dissect the underlying mechanisms of cell and tissue protection have revealed possible contributory mechanisms based on the evolution of all the major HO end-products, including CO (reviewed in 11). Because this represents primarily a review on the role of CO in intracellular

signaling, we refer the reader to other recent reviews for discussion of the possible roles of biliverdin/bilirubin and iron metabolism in HO-mediated protection (11–13).

HO-derived CO was formerly considered as a catabolic elimination product with no physiological significance (14). Indeed, elevated CO concentrations cause tissue hypoxia by virtue of competitive binding with the oxygen binding sites of hemoglobin (15, 16). A signaling role for CO first emerged from observations that CO can weakly stimulate soluble guanylate cyclase (sGC) *in vitro* by heme binding to produce guanosine 3',5'-monophosphate (cGMP), as well as stimulate the production of cGMP when directly applied to vascular smooth muscle cells (SMC) (17–19). HO-derived CO and downstream effects on cGMP formation have been implicated in a number of neuronal signaling processes, including olfactory neurotransmission (5, 20, 21–24). Furthermore, CO has been implicated in vascular processes such as regulation of vessel tone (25–30), SMC proliferation (31–34), and platelet aggregation (34–36). An exclusive or unequivocal role for sGC in all signaling processes triggered by CO has not been established, leaving the question open for the resolution of other possible molecular targets. Indeed, CO has been shown to modulate the activation state of mitogen-activated protein kinases (MAPK) critical for cellular signal transduction in response to stress and inflammation. CO exerts novel anti-inflammatory, anti-apoptotic, and anti-proliferative effects dependent on the modulation of the p38 MAPK signaling pathway (Figure 2). Over the past decade, CO applied at low concentration has been shown to confer cyto- and tissue-protective effects akin to HO-1 elevation in numerous models of tissue injury and disease, including oxidative lung injury, inflammation, and organ transplantation (37–39, reviewed in 40). Recent observations from this laboratory

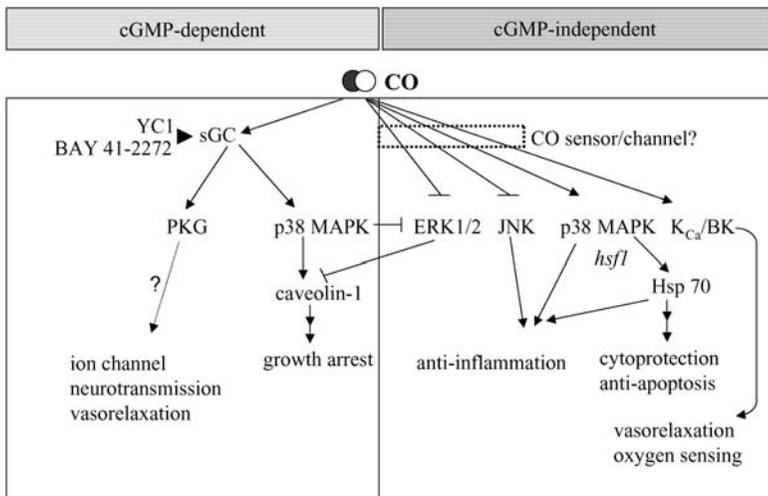


Figure 2 c-GMP-dependent or -independent CO signaling.

reveal additional candidate molecules that function as downstream targets of CO signaling, including the 70-kDa heat shock protein (Hsp-70) and caveolin-1 (31, 41). This review describes the major signaling pathways by which CO exerts potent and diverse actions on cellular homeostasis.

TRANSCRIPTIONAL REGULATION OF HO-1

HO-1 is transcriptionally upregulated as a sensitive cytoprotective protein by various types of oxidative stress, such as oxidized low-density lipoprotein (LDL), ultraviolet-A (UVA) radiation, thiol-reactive substances and by growth factors, hormones, disease states, and dietary antioxidants including various classes of natural products. There is no commonality among HO-1 inducers, which have diverse chemical structures ranging from proteins to small organic compounds to a gas molecule (NO) (reviewed in 42). HO-1 is widely accepted as a cellular defense mechanism against harmful or noxious stimuli, such as sodium arsenite or metal ions (43, 44, reviewed in 45). Although clearly depictive, we further categorize the HO-1 induction depending on the modes of action mechanism.

Synergistic Induction

If the induction and function of HO-1 parallels with the physiological activity of a given stimulus, it may be grouped in the category of a synergistic response. In such examples, the presence of HO-1 is required for the therapeutic effect of the primary stimuli, which is mediated presumably by the reaction products of HO activity. In this way, HO-1 can amplify the therapeutic effects of certain stimuli, such as the anti-inflammatory cytokine IL-10 (46), the anti-proliferative rapamycin (47), 15-PGJ₂ (48), and aspirin/salicylic acid (49). For instance, IL-10 stimulates the upregulation of HO-1, and subsequently a product(s) of the degradation of heme by HO-1 mediates the therapeutic effect of IL-10. Accumulating data with clinical therapeutics and Oriental medicines also support that HO activity may amplify therapeutic potential. Cholesterol-independent, pleiotropic actions of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exert anti-inflammatory and antioxidant action (50). Grosser et al. show that HO-1 is a target site of statins in endothelial cells. In cultured human umbilical vein endothelial cells, simvastatin and lovastatin increase HO-1 mRNA levels in a concentration- and time-dependent, but NO-independent fashion. The increased transcriptional expression of HO-1 was associated with elevated HO-1 protein and reduction of free radical formation (50). Recently, antioxidants (vitamin E, polyphenols) have received much attention as potential anti-atherosclerotic agents (51, 52). Among the polyphenols that induce HO-1, resveratrol, a phytoalexin of grape, may protect the vascular wall from oxidation, inflammation, platelet aggregation, and thrombus formation (52). Both caffeic acid and curcumin are plant extracts that exert potent anti-inflammatory effects, which have also been shown to function through upregulation of HO-1 (53) (see Table 1).

TABLE 1 Various stimuli induce HO-1 induction

Growth factor	Oxidative stress
FGF (178)	Endoplasmic reticulum stress (205)
HGF (179)	Heavy metals (206)
VEGF (180)	Oxidized LDL (207, 208)
NGF (181, 182)	Hydroperoxide (209)
TGF- β (183)	Diphenyl- and dithiol-compounds (210)
KGF (184)	HNE (208, 211)
BMP-7 (185)	Dieldrin (212)
PDGF (186)	Cigarette smoke extract (213)
Disease state (reviewed in 187)	Alcohol (214)
Ischemia reperfusion	Dietary antioxidant/natural product
Inflammation	Synthetic terpenoid (215)
Immune dysfunction	Quercetin/flavonoid (216)
Transplantation	Mycotoxin (food contaminant; 217)
Myocardial infarction	Ferulic acid/ethyl ferulic acid (218)
Atherosclerosis	Zerumbone (sesquiterpene; 219)
Hypertension	Selegiline (220)
Vascular restenosis	Turpentine oil (221)
Acute renal failure	3-O-caffeoyl-methyl quinic acid (222)
Glomerulonephritis	Diallyl sulfide (garlic; 223)
Diabetic kidney disease	Chalcone/rosolic acid (224)
Hypoxia/hyperoxia induced lung injury	Isoflurane (225)
Emphysema	Taurine (226)
Asthma	Curcumin (53)
Spinal cord injury	Cafestol/kahweol (227)
Cerebrovascular injury	Avicins (triterpenoid; 228)
Alzheimer's disease	Resveratrol (52, 229)
Hormone	Physiological changes
Estrogen (188)	Hypertonic saline (osmotic pressure; 230)
Prolactin (189)	Transient glucose deprivation (231)
Adrenocorticotrophin (190, 191)	Acidosis (232)
Atrial natriuretic peptide (192)	Nitric oxide (233–242)
Melatonin (193)	Cytokine (LPS, IFN- γ , TNF- α , IL-1 β ; 243)
Angiotensin II (55, 194)	Mechanical shear stress (244, 245)
Progesterone (195)	
Glucocorticoid (regulation of HO-2; 196)	
Chorionic gonadotropin (testis) (197)	
Medicines/chemicals	Miscellaneous
<i>Immunosuppressant/anti-inflammatory</i>	Dopamine (246)
IL-10 (46)	Nicotine (247)
Aspirin/salicylic acid (49)	β -amyloid (248)
Cyclopentenone PG (15d PGJ2, PGA2; 48, 198)	Cysteamine (249)

(Continued)

TABLE 1 (Continued)

Rapamycin (47)	UV irradiation (250)
Statins (50, 199)	Co-protoporphyrin (251)
<i>Antioxidant</i>	Proteasome inhibitor (252)
Vit E (51)	
Selenium (ebselen; 200, 201)	
Probucol (202)	
Paclitaxol (<i>antitumor</i> ; 203)	
Hydralazine (<i>vasodilator</i> ; 204)	

Abbreviations: FGF, fibroblast growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial cell growth factor; NGF, nerve growth factor; TGF, transforming growth factor; KGF, keratinocyte growth factor; BMP, bone morphogenic protein; PDGF, platelet derived growth factor; oxLDL, oxidized low density lipoprotein; HNE, hydroxynonenal; LPS, lipopolysaccharide; IFN, interferon; IL, interleukin; UV, ultraviolet.

Antagonistic Induction

In response to certain stimuli, the organism may induce HO-1 to counteract the action of the stimuli in a feedback mechanism (54, 55). For example, serum stimulates HO-1 gene expression and subsequent CO synthesis in SMC (54). CO acts in a negative feedback fashion to inhibit vascular SMC growth by regulating specific components of the cell cycle machinery, including retinoblastoma protein (pRb) phosphorylation or the expressions of p21^{Waf1/Cip1} and cyclin A. The ability of serum components to stimulate HO-1 gene expression is also observed after the administration of platelet-derived growth factor (PDGF), transforming growth factor- β 1 (TGF- β 1), or angiotensin II, and it may represent a counterregulatory mechanism against mitogen-stimulated SMC growth (55). In some cases, the inhibitor of HO-1 or scavenger of CO tends to exaggerate the effect of a given stimulus. For example, serum-stimulated DNA synthesis is augmented in the presence of HO inhibitors zinc- or tin-protoporphyrin IX (SnPP-IX, ZnPP-IX), indicating a growth inhibitory role for HO-1. In another example, angiotensin infusion causes hypertensive effects involving increased blood pressure and also induces HO-1 in the vasculature. Inhibition of HO-1 with metalloporphyrins aggravates the hypertensive effects of angiotensin, thus highlighting HO-1 as an antagonistic response to the stimulus (55).

HEMOPROTEINS AS CO RECEPTORS

The affinity of CO for metal atoms allows it to interact with a number of metalloproteins (56). This property implicates all heme-containing proteins as possible candidate biological CO sensors even though they are known to have other physiological ligands. These include proteins in which heme functions as a prosthetic group, for example, hemoglobin, myoglobin, sGC, cyclooxygenase, cytochrome p450, cytochrome *c* oxidase, inducible NOS, NADPH: oxidase, and the transcription factors Bach-1 and -2 (57, reviewed in 58). Furthermore, proteins that contain PAS (Per-Arnt-Sim) motifs, such as neuronal PAS domain protein-2 (NPAS-2),

are known to be gas responsive. CO can modulate the biological activity of these hemoproteins by virtue of its ability to bind the central iron group contained within their heme prosthetic groups (59, 60). When this occurs, CO induces conformational changes in these proteins that can regulate their biological activity (56). CO also potentially binds to proteins that contain other metal ions, such as zinc, copper, and manganese (58).

Soluble Guanylyl Cyclase

Vertebrate sGC is a heterodimeric (α/β) heme protein that senses NO (61, 62). sGC is expressed in the cytoplasm of almost all mammalian cells and mediates a wide range of important physiological functions (61). Based on the lipophilic property of NO and its site of generation, the plasma membrane localization of sGC was examined in rat heart and human platelets (63). Zabel et al. proposed that sGC dynamically translocates to the plasma membrane, where it is sensitized to NO. When NO binds to the heme motif of sGC, its activity in converting GTP to cGMP is enhanced by several hundredfold. cGMP acts as a second messenger that plays a pivotal role in a variety of physiological processes, including vasodilation and neuronal signal transduction (61).

The overall structure of sGC is unknown, but its catalytic domain is composed of the C-terminal regions of the both subunits, which have sequence homology to regions in the particulate sGC and adenylate cyclase. Although both subunits are essential for catalytic activity, the N-terminal region of the β -subunit alone binds the heme prosthetic group (64). The presence of the heme prosthetic group is required for activation of sGC by NO (17). NO has been shown to trigger a conformational change in sGC through the cleavage of the proximal histidine ligand, His105, resulting in a five-coordinate high-spin NO adduct (65). The reconstitution of sGC with a series of metalloporphyrins supports the hypothesis that the cleavage of the His-Fe bond is critical for activation (66, 67). For example, Mn(II)-protoporphyrin IX-reconstituted sGC binds NO but is not active, consistent with the role of the retention of the His-Fe bond. Conversely, Ni(II)- or Cu(II)-protoporphyrin IX-reconstituted sGC are active without NO. Ni(II) or Cu(II) protoporphyrin IX favors four-coordinate geometry, and therefore these reconstituted sGC variants lack the His-Fe bond. sGC with a free-base protoporphyrin IX, lacking an iron and therefore incapable of forming the His-Fe bond, is also active. However, the observation that porphyrin-free sGC is not active indicates that cleavage of the His-Fe bond is not sufficient and that the heme-vicinity also plays a crucial role in the activation mechanism of sGC.

Recently, CO has been shown to inhibit platelet aggregation and to relax vascular smooth muscle (35, 68), which are activities also attributed to the action of NO on sGC. These CO-dependent activities have been shown to be cGMP-dependent (35, 68–70). CO can enhance sGC activity *in vitro*, although its fourfold increase is much lower than that of NO and does not seem to explain the degree of the visible physiological responses. Indeed, because CO binding to sGC leads to a six-coordinate low-spin heme adduct, with an intact His-Fe bond, the

activation mechanism of CO must differ from that of NO. Importantly, however, the presence of YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole], or BAY-41-2272, small synthetic molecules that have been found to enhance the CO effect *in vitro*, allows activation by CO to reach the same level as that of NO-stimulated sGC (71–73). The molecular basis for the stimulation mechanism of YC-1 to the CO-bound sGC is unclear, but YC-1 apparently does not force the cleavage of the Fe-His bond (73), as might be expected if it also led to a five-coordinate adduct. However, the production of substantial amounts of the five-coordinate CO adduct upon addition of the BAY 41-2272 was demonstrated (72). Thus, several out-of-plane heme deformation modes are simultaneously activated, an observation similar to that realized upon NO activation. The question remains whether CO is a physiological ligand of sGC and whether there exists a physiological modulator *in vivo* that mimics the synergism triggered by YC-1 or BAY 41-2722. Although there is no evidence available for the latter question, there are strongly suggestive data in support of the former. First, CO-induced vasorelaxation can be blocked by inhibiting sGC activity (36), consistent with a direct role *in vivo* for CO and sGC. Second, there is the striking evidence that HO, the source of CO in nerve cells, colocalizes with sGC in cells with little or no NOS expression, supporting a link between CO and sGC (74, 75). Although the possible role of sGC in mammalian CO sensing is tantalizing but firmly unproven, the participation of other hemoprotein species in CO-sensing remains likely.

GC-INDEPENDENT EFFECTS OF CO CO has potential actions distinct from modulating cGMP levels. Wang et al. noted that CO-induced relaxation of rat-tail arteries was not completely blocked by inhibitors of the cGMP pathway, and that the residual relaxation could be blocked by inhibitors of large conductance calcium-activated K^+ -channels (K_{Ca}) (76). When activated, these channels relax smooth muscle by hyperpolarizing the membrane. CO (1 μ M) activates these channels to relax smooth muscle by membrane hyperpolarization (28, 76). CO increases the single channel open probability of K_{Ca} channels by a direct action on the channel. Chemical modification of a single histidine on the extracellular surface of the channel blocks the activation by CO (76). The effects of CO on K_{Ca} channels in SMCs have been confirmed by multiple laboratories (29, 30, 76). CO can also increase the activity of other families of K^+ channels (77). These findings establish an sGC-independent mechanism for the relaxant actions of CO on smooth muscle. Hemin and NADPH, HO substrates, mimic the effects of exogenously added CO when applied to excised membrane patches containing K^+ channels, and these effects are reversed by HO inhibitors (77). This suggests that HO is tightly associated with the K^+ channels on the plasma membrane.

NPAS2, Circadian Clock, and CO

The circadian clock is the central timing system that controls numerous physiological processes (78, 79). In peripheral tissues, the circadian clock generates

transcriptional rhythms (80, 81). The gases NO and CO are increasingly appreciated as major neurotransmitters (5). One of the principal means by which NO and CO transmit signals between neurons is through binding to a heme moiety at the active site of sGC and generation of the intracellular second messenger molecule cGMP. Recently, HO-derived CO has also been implicated in the regulation of circadian rhythms through transcriptional mechanisms. Dioum et al. have characterized a mammalian transcription factor NPAS2 that regulates circadian rhythm and that is regulated by CO binding (60, 82). NPAS2 modulates circadian activity in the suprachiasmatic nucleus but is also present in cells outside the central nervous system (CNS). NPAS2 was first identified as a member of the basic helix-loop-helix (bHLH) family of transcription factors (83). NPAS2 together with its binding partner BMAL1, another bHLH transcription factor, binds DNA as an obligatory heterodimer (60). The NPAS2 monomer contains two heme moieties that can act as CO-binding sites, which are both six-coordinate in their resting states. The binding of CO to NPAS2 inhibits the DNA binding of active NPAS2-BMAL1 complexes, which confer negative modulation of circadian clock machinery. CO binding to NPAS2 results in the accumulation of the BMAL1 homodimers at the expense of NPAS2-BMAL1 heterodimers. When Takahashi et al. (84, 85) identified Clock as a crucial regulator of circadian rhythms, they noted its close sequence similarity to NPAS2. Clock and NPAS2 regulate the activating portion of the circadian transcriptional feedback cycle by heterodimerizing with BMAL1. NPAS2-BMAL1 and Clock-BMAL1 heterodimers direct the transcription of period (Per) and cryptochrome (Cry) proteins, which are the negative regulatory components of the circadian clock. Per and Cry inactivate the Clock-BMAL1 and NPAS-BMAL1 heterodimers, thus completing the transcriptional loop. Considering that Clock displays significant sequence homology to NPAS2 in both PAS domains, it therefore might also bind to CO (85, 86).

One of the features of circadian clocks is their entrainment by environmental stimuli such as light, temperature, activity, and food intake (60). The molecular mechanisms that enable environmental stimuli to abruptly alter circadian rhythms remain obscure. However, modulation of Clock and NPAS2 activity according to the redox state of the cell may provide a clue (86). The reduced cofactors NADH and NADPH greatly enhance binding of Clock-BMAL1 and NPAS2-BMAL1 heterodimers to DNA, whereas the oxidized forms of the same molecules, NAD⁺ and NADP⁺, inhibit the DNA binding of these dimers (86). Alterations in DNA-binding activity occur with modest changes in the ratio of oxidized to reduced cofactors. This implicates the cofactors as molecular switches that direct NPAS2-BMAL1 and Clock-BMAL1 dimers to bind to DNA in response to changes in cellular redox state. PAS domains with modules of 130 amino acids, previously characterized in organisms ranging from bacteria to mammals, respond to variations in stimuli, including oxygen, voltage, light, and redox potential. The PAS domains of some bacterial proteins operate as oxygen sensors via a heme prosthetic group. Because both PAS domains of the NPAS2 monomer contain a heme molecule, the absorption spectrum of heme-bound NPAS2 resembles that of gas-sensing proteins from

bacteria, including the CO-sensor protein *CooA* from *Rhodospirillum rubrum* (56). Furthermore, CO bound to heme-containing NPAS2 with a dissociation constant of about 1 μM but failed to bind to NPAS2 lacking heme. In addition, CO inhibited the DNA-binding capacity of NPAS2-BMAL1 heterodimers with a similar molar potency. By contrast, NO did not bind to NPAS2 at physiologic concentrations, and O_2 bound irreversibly (60). In contrast, sGC is activated by nanomolar concentrations of NO but requires high micromolar concentrations of CO for activation. Because the characterization of CO regulation of NPAS2 results from *in vitro* experiments, it remains necessary to determine whether CO regulates NPAS2 in intact cells and organisms. If CO regulates NPAS2 *in vivo*, it would represent the first example of a mammalian protein selectively regulated by CO.

CO is formed in neurons exclusively by HO-2 (87, 88). The enzyme occurs in neuronal populations in numerous parts of the brain and partially overlaps with the distribution of NPAS2 (23, 42). HO-2 activity is dynamically regulated by neuronal impulses through a kinase cascade in which protein kinase C activates casein kinase-2, which in turn phosphorylates and activates HO-2 (23). HO-2 activity generates low micromolar concentrations of CO in the intact brain, which is sufficient to regulate the DNA-binding activity of NPAS2 (60). Dioum et al. suggest that the production of CO fluctuates according to the circadian rhythm of heme metabolism. Recent findings propose that the heme biosynthesis pathway, rate-limited by δ -aminolevulinic synthase-1 (ALAS1), falls under the control of circadian rhythm (78, 79). Indeed, heme controls the activity of the BMAL1-NPAS2 transcription complex *in vitro* by inhibiting DNA binding in response to CO. Heme differentially modulates expression of the mammalian *Period* genes *mPer1* and *mPer2* *in vivo* by a mechanism involving NPAS2 and *mPER2*. *mPER2* positively stimulates the activity of the BMAL1-NPAS2 transcription complex and, in turn, NPAS2 transcriptionally regulates ALAS1. Vitamin B_{12} and heme compete for binding to NPAS2 and *mPER2*, but they exert opposite effects on *mPer2* and *mPer1* expression *in vivo* (78). These data show that the circadian clock and heme biosynthesis are reciprocally regulated and suggest that porphyrin-containing molecules as potential candidates for therapy of circadian disorders (Figure 3). Elevated heme levels are likely to inhibit heme synthesis by modulating circadian transcription factors through *mPer2* or through HO-derived CO, which thus may constitute a cellular feedback loop for heme homeostasis.

SIGNALING GAS: p38 MAPK AND CO

Of the signaling pathways influenced by CO, we will emphasize the role of MAPK, especially p38 MAPK (Figure 4). Accumulating evidence suggests that p38 MAPK activation is essentially involved in the cytoprotective, anti-inflammatory, anti-apoptotic, and anti-proliferative effects of CO (31, 38, 41, reviewed in 89). Recently, Zhang et al. reported that CO inhibits apoptosis via p38 MAPK-STAT

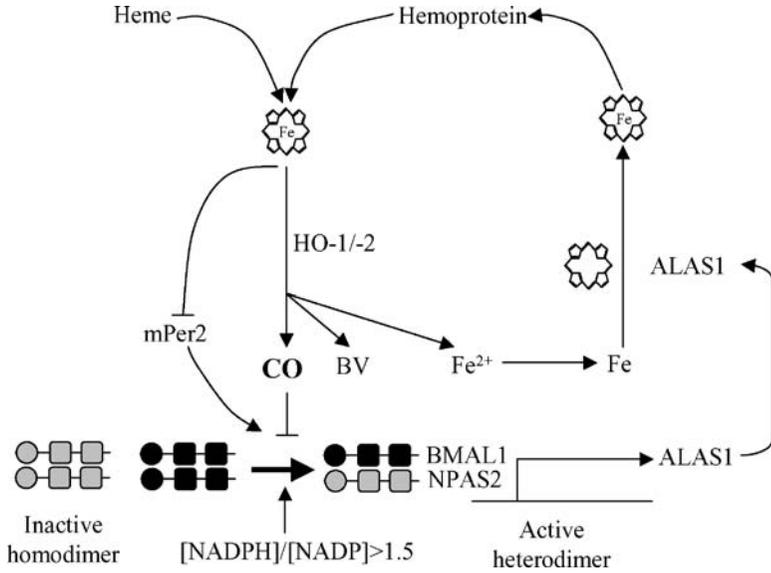


Figure 3 Reciprocal modulation of heme biosynthesis by NPAS-2 and possible involvement of CO in the processes.

or Akt/PKB-STAT pathways during ischemia reperfusion (I/R) injury (90–92). Hence, we discuss the specific roles of p38 MAPK in the following sections.

Properties of p38 MAPK Members

p38 α (p38) was first isolated as a 38-kDa protein rapidly tyrosine phosphorylated in response to lipopolysaccharide (LPS) stimulation (93, 94). p38 MAPK was cloned as a molecule that binds pyridinyl imidazole derivatives, which are known to inhibit biosynthesis of inflammatory cytokines such as interleukin-1 (IL-1) and tumor-necrosis factor- α (TNF α) in LPS-stimulated monocytes (95). To date, four splice variants of the p38 MAPK family have been identified: p38 α , p38 β (96), p38 γ (ERK6, SAPK3) (97, 98), and p38 δ (SAPK4) (99, 100). Of these, p38 α and p38 β are ubiquitously expressed, whereas p38 γ and p38 δ are differentially expressed depending on tissue type. All p38 MAPKs can be categorized by a Thr-Gly-Tyr (TGY) dual phosphorylation motif (101). Sequence comparisons have revealed that each p38 MAPK isoform shares ~60% identity within the p38 MAPK group but only 40%–45% to the other three MAPK family members. Mammalian p38 MAPKs show similar roles and activation has been shown to occur in response to extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines (TNF- α and IL-1), and growth factors (94, 102). This plethora of activators conveys the complexity of the p38 MAPK pathway. Activation of p38 α is not only dependent on stimulus but also on cell type (103). For example, insulin can stimulate p38 MAPK in 3T3-L1 adipocytes (104), but downregulates

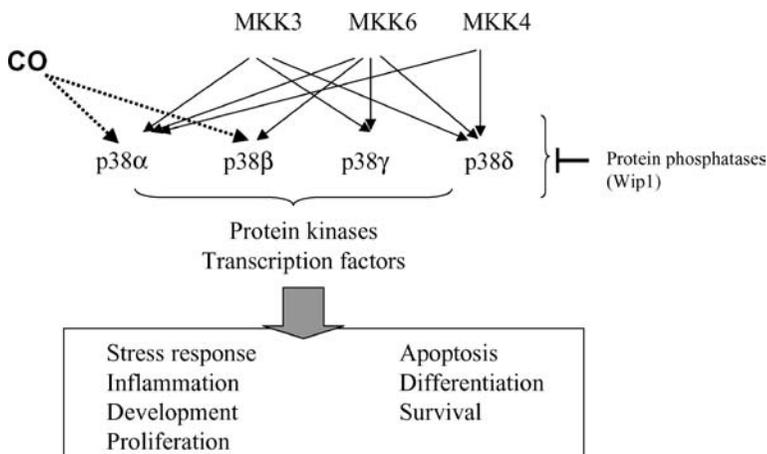


Figure 4 Effects of CO on diverse biological processes through p38 MAPK. The α - and β -isoforms of p38 MAPK actively participate in the functions of CO including anti-apoptotic, anti-proliferative, and anti-inflammatory activity.

p38 MAPK activity in chick forebrain neuron cells (105). Although the other three p38 MAPK isoforms have been cloned, little is known regarding their activation. Despite all four p38 MAPK group members displaying similar activation profiles (103, 106), differences have been observed in the kinetics and level of activation of these isoforms. Furthermore, the activation of p38 MAPK isoforms can be specifically controlled through different regulators and coactivated by various combinations of upstream regulators.

CO, p38 MAPK, and Apoptosis

We and others showed that expression of HO-1 or exposure of endothelial cells (ECs) to exogenous CO enhances p38 MAPK activation by TNF- α (41, reviewed in 89). Specific inhibition of p38 MAPK activation by SB 203580 or through overexpression of a p38 MAPK-dominant negative mutant abrogates the anti-apoptotic effect of HO-1. To the contrary, evidence exists for the concomitant activation of p38 MAPK and apoptosis induced by a variety of agents, such as nerve growth factor withdrawal and Fas ligation (107). In Jurkat T cells, Fas/CD95-induced cell death is augmented by exposure to CO, and this occurs in part via inhibition in the activity of ERK MAPK (108, 109). Thus, the role of p38 MAPK in apoptosis is cell type- and stimulus-dependent. Although p38 MAPK signaling can promote cell death in some cell lines, it may enhance survival, cell growth, and differentiation in others (reviewed in 106).

More recently, we demonstrated that Hsp70 mediates the cytoprotective activity of CO in ECs. The expression of Hsp70 appears as a downstream target of p38 β MAPK (41). Several studies demonstrate the protective capacity of Hsp70

against heat shock and other toxic stimuli. In rats, ischemic preconditioning of the liver strongly induced Hsp70, resulting in resistance to subsequent hepatic I/R injury (110). The downregulation of Hsp70 can facilitate the induction of apoptosis, whereas the upregulation of Hsp70 inhibits TNF- α -induced apoptosis (111, 112). The expression of Hsp70 conferred resistance in models of cardiac I/R injury or acute respiratory distress syndrome (113). Accumulating evidence demonstrates that the heat shock response can confer anti-inflammatory effects involving the inhibition of pro-inflammatory gene expression (114, 115). Hyperthermia pretreatment protected against the lethal effect of LPS or TNF- α treatment in mice, which correlated with systemic Hsp70 induction (111). Similarly, Zhang et al. have reported that p38 α MAPK activation is important for modulation of apoptosis during lung I/R injury (90–92). In a model of hepatic I/R injury, p38 MAPK activation was essential to exert the effect of CO (116).

We also suggested a critical role for the β -isoform of p38 MAPK in mediating the effects of CO on cytoprotection and Hsp70 expression because these effects were abrogated in endothelial cells by SB 203580, a selective inhibitor of α and β isoforms, and in p38 β -null fibroblasts. The observed inhibition of Hsp70 expression by SB 203580 results from inhibition of HSF-1 activation potentially involving phosphorylation and nuclear localization. The augmentation of p38 MAPK by CO over the TNF- α /Act D-induced background led to the significant increase in HSF nuclear translocation. Activation of HSF-1 by CO was also expressed as increased HSF DNA-binding activity. CO, however, did not further modulate the phosphorylation of HSF, which was also determined to require p38 MAPK activity (41).

CO, p38 MAPK, and the Cell Cycle

The participation of p38 α in cell growth has been observed in yeast and mammals (117). Overexpression of p38 α in yeast led to significant inhibition of proliferation, whereas treatment in mammalian cells with p38 α / β inhibitor SB 203580 increased proliferation. Likewise, inactivation of protein phosphatase 2C (Wip1) inhibited cellular growth and tumorigenesis by activating p38 MAPK (118, 119). p38 MAPK has been implicated in G1 and G2/M phases of the cell cycle (120–122). G1 arrest of NIH 3T3 cells caused by microinjection of Cdc42 was found to be p38 α -dependent. Also, a link between p38 MAPK and G1 cell cycle control has been proposed through the regulation of the p38 MAPK substrates high mobility group-box protein 1 (HBP1) and p21 (117). HBP1 is thought to have a role in regulating G1 cell cycle progression through the repression of cell cycle regulatory genes, similar in function to pRB, whereas the p21 CDK inhibitor is established as a crucial factor in preventing G1 progression through blockage of CDK activity. Several examples implicate p38 MAPK involvement in G2/M phase as well. p38 α is activated in mammalian cells upon M phase arrest by disruption of the spindle with nocodazole (120). Furthermore, p38 α and p38 γ are required for UV-induced G2 cell cycle arrest (122). Interestingly, p38 α activity increases in confluent cultures of human

fibroblasts compared with proliferating cultures, implying that α -isoform of p38 MAPK is required for contact inhibition (123). Lee et al. have demonstrated that overexpression of the *ho-1* gene delays the growth of A549 lung epithelial cells (124). This slowing of cell growth was associated with an increased number of cells in G0/G1 phase during the exponential growth phase and decreased entry into the S phase. Furthermore, the transgenic cells accumulated in the G2/M phase and failed to progress through the cell cycle when stimulated with serum, whereas the control cells exhibited normal cell cycle progression. Similarly, exogenous administration of CO recapitulated the same phenomena, resulting in the accumulation of SMC in G0/G1 phase (34).

Anti-Proliferative Effect of p38 MAPK

A common feature of tumor cells is a loss of senescence. p38 MAPK appears to play major roles in tumorigenesis and senescence (125–127). The activation of MKK6 and MKK3 led to a senescent phenotype dependent on p38 MAPK activity (126, 127). Also, p38 MAPK activity was shown to be responsible for senescence in response to telomere shortening, H₂O₂ exposure, and chronic *ras* oncogene signaling (106). Furthermore, p38 MAPK activation may be reduced in tumors. The loss of components of the p38 MAPK pathway, such as MKK3 and MKK6, resulted in increased proliferation and tumorigenic conversion regardless of the cell line or the tumor-inducing agent used in these studies (128). Bulavin et al. describe the critical role of p38 MAPK in inhibiting tumorigenesis or cell proliferation (118, 119). Wip1 is a serine-threonine phosphatase encoded by *Ppm1d*. It was first identified as a gene that is induced by the p53 tumor-suppressor protein in response to DNA damage. The importance of the activation of this phosphatase by p53 became clear when it was found that Wip1 specifically inactivates the protein kinase p38 MAPK, which can activate p53 to cause cell cycle arrest and apoptosis in response to certain environmental stress. In addition, p38 MAPK can inhibit cell cycle stimulatory proteins, such as cyclin D1 and the Cdc25 phosphatases. Thus, *Ppm1d* activation by p53 constitutes a negative feedback loop responsible for downregulation of genotoxic stress-induced signaling, which leads to suppression of the cellular stress response. Consistent with a role for Wip1 in cell proliferation is the finding that *Ppm1d* can cooperate with an activated *Hras* oncogene in transformation of primary mouse embryo fibroblasts. Conversely, Wip1-null cells have a reduced proliferative capacity in vitro and suffer from premature senescence. Suppression of *Ppm1d* expression in neuroblastoma cell lines suppressed growth and induced apoptosis. Together, these data establish Wip1 as a central component of the cellular stress response, whose inhibition enhances anti-proliferative effects of environmental stress signals by modulating p38 MAPK activity. Based on these findings, it is also important to note that upregulation of caveolin-1 expression is found in senescent cells, mice, rat, and human subjects (129). Collectively, the interaction between caveolin-1 expression and p38 MAPK activity may control a variety of key biological programs, such as cell proliferation, differentiation, and

senescence (31, 127, 129). Because trace amounts of exogenous CO treatment can cause upregulation of caveolin-1 expression and p38 MAPK activation, we also speculate that CO modulates cell growth or differentiation. We showed that CO inhibits the vascular SMC proliferation and neointima formation during balloon injury in rats by modulating the GC-cGMP-p38 MAPK-caveolin-1 signaling pathways (31).

EFFECTS OF CO ON PROLIFERATION AND NEOINTIMA FORMATION: INVOLVEMENT OF CAVEOLAE The importance of HO-1 as a protective gene in humans is supported by two population studies. First, individuals vary in terms of the strength of their HO-1 response to a given stimulus based on a GT-length polymorphism in the promoter of the *ho-1* gene. Individuals with a robust HO-1 response (short GT repeats) are less likely to have restenosis after balloon angioplasty, as well as less likely to have chronic rejection of an allograft and to undergo other pathological processes (reviewed in 130). Individuals with longer GT repeats, who respond more weakly in terms of upregulating HO-1 activity to a particular stress stimulus, are more likely to experience restenosis or chronic rejection. Thus, the ability to upregulate HO-1 more strongly to a specific stress stimulus appears to be physiologically important. Second, multiple studies show that individuals with high normal or just above normal levels of bilirubin, which is produced as a consequence of HO-1 action, have less atherosclerosis-related diseases than individuals with low normal levels of bilirubin (131). The combination of these association studies with experimental findings that administration of either CO or biliverdin suppresses atherosclerosis-related pathologies in rodents (31, 34, 132, 133) strongly supports the importance of HO-1, bilirubin/biliverdin, and CO as protective products in humans. Intimal hyperplasia is a major morphological feature of various arterial pathologies, such as atherosclerosis, postangioplasty restenosis, and transplantation arteriopathy (33).

Substantial evidence indicates that HO-1 is a negative regulator of growth in vascular SMCs, and the inhibition of SMC proliferation by HO-1 appears to be mediated via release of CO (31, 32, 34). The exogenous administration of CO at concentrations of 100 to 250 ppm can substitute for HO-1 in blocking SMC proliferation (31, 34). The CO scavenger, hemoglobin, enhances the proliferative response of vascular SMC to several growth factors. CO appears to block SMC growth by increasing the intracellular levels of cGMP. CO activates sGC and elevates cGMP levels in SMC. In fact, inhibition of sGC blocks the rise in cGMP and proliferative action of HO-1 (134). Recent studies clarified that inhibition of HO-1 activity by ZnPP-IX potentiates the mitogenic action of serum, angiotensin II, PDGF, endothelin-1, and hypoxia in SMCs (31, 55, 89). Thus, SMC-derived CO is an important regulator of SMC proliferation. Moreover, vascular SMCs obtained from HO-1 knockout mice exhibit enhanced proliferation and DNA synthesis compared with those from wild-type animals (32, 34). HO-1 may also indirectly modulate cell growth by affecting the release of growth factors. Alternatively, the induction of HO-1 by gene transfer

or hemin administration blocks SMC proliferation and neointimal formation (32, 34).

We have further investigated the signaling pathways that mediate the anti-proliferative effects of CO. We showed that the activation of the p38 β MAPK signaling pathway by CO led to an enhanced expression of caveolin-1 in fibroblasts and SMC, which in turn mediated the increased expression of p21^{Waf1/Cip1} and the down-regulation of cyclin A, leading to growth arrest. The inhibition of intimal hyperplasia by CO treatment in a vascular injury model involved the enhanced expression of caveolin-1 in vascular SMC (31).

Besides the anti-proliferative effect of CO in SMCs, CO may act as a multifunctional level against every stage of neointimal development owing to its potential anti-inflammatory and anti-apoptotic activity. In line with this notion, treatment with anti-inflammatory cytokines (i.e., IL-10), rapamycin (135, 136), cell migration inhibitors (Rho kinase inhibitor and endothelin B null) (137, 138), receptor signaling modulators (PDGFR) (139), and regulators of extracellular matrix formation (MMP, TIMP) (140, 141) inhibit neointimal formation in various models. The inhibitory role of IL-10 or rapamycin against neointimal development may depend on the induction of HO-1. Moreover, the latter three events, namely growth factor receptor signaling, cell migration, or extracellular matrix metabolism, occur at caveolae or lipid rafts directly or indirectly (see Table 2). Recent studies in caveolae-deficient animals show that caveolae sequester and regulate a variety of signaling intermediaries (142). Both G protein-coupled receptors and tyrosine kinase receptors are believed to cluster in caveolae, and the possibility that caveolae provide a platform for interactions between the sarcoplasmic reticulum and plasmalemmal ion channels is emerging (143, 144). Moreover, messengers involved in Ca²⁺ sensitization of myosin phosphorylation and contraction may depend on caveolae or caveolins. Caveolae, indeed, appear to constitute an important signaling domain that plays a role not only in regulation of smooth muscle tone but also in proliferation, such as seen in neointima formation and atherosclerosis.

CO in Innate Immunity: Effect on Macrophages

A strong link has been established between the p38 MAPK pathway and inflammation. Rheumatoid arthritis, Alzheimer's disease, and inflammatory bowel disease may be influenced in part by the p38 MAPK pathway (106). p38 MAPK plays essential roles in the production of pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-6) (93–95) and in the induction of enzymes such as COX-2 and iNOS and in the induction of VCAM-1 and other adherent proteins and inflammatory-related molecules. In addition, a regulatory role for p38 MAPK in the proliferation and differentiation of immune system cells by factors such as GM-CSF, EPO, CSF, and CD-40 has been established (107). If, however, macrophages overexpress HO-1 or are exposed to CO *in vitro* before stimulation with LPS, the pro-inflammatory (i.e., TNF- α and IL-6 production) response

TABLE 2 Caveolae-residing proteins. Some are localized to caveolae endogenously, whereas others localize transiently by stimuli

Growth factor receptor	Calcium and eNOS signaling
EGF receptor (253)	IP3 receptor like protein (282, 283)
VEGF receptor-2 (Flk-1/KDR; 254)	PLA2 (284)
TGF- β receptor (255)	Estrogen receptor (285)
Insulin receptor, Insulin-like growth factor receptor (256)	Cationic amino acid transporter (CAT) 1 (286)
PDGF receptor (257)	eNOS (287, 288)
NGF receptor (258)	Na/Ca Exchanger (NCX-2; 289)
Angiotensin II type I receptor (259)	Bradykinin receptor (290)
TNF receptor 1 (260)	
Cell migration/contraction	Heme oxygenase system (291, 292)
Integrin (261)	Heme oxygenase 1
RhoA/Rac (262, 263)	NADPH:cytochrome P450 reductase (NPR)
	Biliverdin reductase (BVR)
Endothelin receptor A (264)	Heme oxygenase 2
MT-MMP1 (265)	
MMP-2 (266)	
uPAR (267, 268)	
Protein kinase	Chemokine/Toll-like receptor signaling
PKA (269)	CCR5 (HIV-1 co-receptor; 293)
PKC (270)	TLR2 (294)
JAK/STAT (271)	MyD88 (295)
	IRAK-1 (296)
Structural protein	Ion channel
Caveolin (272)	Maxi-K channel (297)
Flotillin (273, 274)	BK channel (298)
Actin (275–278)	TRPC1 (calcium channel; 299)
Dynamin (279–281)	Ca ²⁺ -ATPase (300)
Vimentin (278)	
Miscellaneous	
Adenylyl cyclase/guanylyl cyclase (301, 302)	Na/K ATPase (303)
Glucose transporter GLUT4 (304)	Nogo-B (305)
Prostacyclin synthase (306)	src family kinases (272)
Heterotrimeric G proteins (307)	H-ras (308)
CD36 (309)	

Abbreviations: EGF, epidermal growth factor; TNF, tumor necrosis factor; MT-MMP, membrane type matrix metalloproteinase; MMP, matrix metalloproteinase; PKA, protein kinase A; PKC, protein kinase C; JAK/STAT, janus kinase-signal transducers and activators of transcription; PLA2, phospholipase A2.

is markedly inhibited, whereas the anti-inflammatory (i.e., IL-10 production) response is enhanced (38, 145). Three other modes of CO action contribute to its anti-inflammatory effects. First, CO prevents platelet activation and aggregation, thereby suppressing thrombosis and the pro-inflammatory response stimulated by activated platelets (34–36). Second, CO downregulates the expression in macrophages of plasminogen activator inhibitor type 1; this action appears to be crucial for the ability of CO to exert a protective effect in a model of lung I/R injury (36, 146). Third, CO prevents apoptosis in several cell types, including endothelial cells (41, 147, 148), fibroblasts (149), hepatocytes (150), and pancreatic β -cells (151). The anti-thrombotic effect of CO in platelets requires GC-cGMP and p38 MAPK. CO suppresses production of TNF- α and induces IL-10 in macrophages through activation of p38 MAPK but not by activation of the sGC/cGMP pathway. However, the action mechanisms underlying the anti-inflammatory effect of CO via p38 MAPK are quite different from the main paradigm where activation of p38 MAPK triggers pro-inflammatory reactions (152). It remains necessary to clarify the isotype-specific contributions of p38 MAPK to its anti-inflammatory action.

Regulation of the p38 MAPK pathway is not an isolated cascade and many different upstream signals can lead to p38 MAPK activation. These signals may be p38 MAPK specific (MKK3/6), or involve general MAPKKs (MKK4) or MAPKK-independent signals (TAB1 and ASK1) (153, 154). Downstream signaling pathways of p38 MAPK are quite divergent, and each component may interact with other cellular components, both upstream and downstream, to coordinate cellular processes such as feedback mechanisms. Furthermore, in vivo p38 MAPK is a complex event and exists in the presence of other MAPK and a plethora of other signaling pathways. The subcellular localization of p38 MAPK activation may also determine the end-point biological effect and may add yet another order of complexity. Future work would benefit from attention to the interaction between different pathways, the balance/regulation among signaling events, and the subcellular compartmentalization of p38 MAPK activation.

CO in Adaptive Immunity: Effect on Regulatory T Cells

A decade ago, Sakaguchi et al. suggested that a relatively small population of CD4⁺ T cells coexpressing CD25 (the α -chain of the IL-2R) was responsible for the prevention of autoimmunity (155). Since then, a plethora of studies have supported the notion that CD4⁺CD25⁺ T cells, often referred to as regulatory T cells (Treg), play a major role in suppressing immune reactivity (156, 157). The forkhead box P3 transcription factor (Foxp3) was identified as required for the development and maintenance of the CD25⁺ Treg cell compartment (158–161). The disruption in this gene leads to multi-organ-specific autoimmunity in both mice (scurfy mouse phenotype) and in humans [immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome] (162). We and others have demonstrated that CO/HO-1 is a potent modulator of innate immunity including

monocyte/macrophage cells (38, 41, 145, 163). Recent reports implicate that CO/HO-1 might be involved in cell-mediated, adaptive immunity, including transplant rejection (39, 164–165). Pae et al. have demonstrated that HO-1 is differentially expressed between CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell populations in a manner analogous to the aforementioned findings for human FOXP3 expression (166). Upon anti-CD3/anti-CD28 costimulation, human CD4⁺CD25⁻ T cells could also be induced to express HO-1; a finding that once again paralleled the expression pattern of human FOXP3. Stable transfection of HO-1 into Jurkat T cells inhibited cellular proliferation (166). This latter observation, when applied to natural Treg, could explain the anergic and refractory phenotype of this cell population. Interestingly, transfection of Foxp3 into Jurkat T cells reveals that Foxp3 subsequently induced expression of HO-1 (159). HO-1 gene expression in these regulatory cells suppressed proliferation and cytokine production of nontransfected cells in a cell-cell contact-dependent manner. Furthermore, the suppressive capacity of Foxp3-transfected Jurkat T cells and primary human CD4⁺CD25⁺ T cells was blocked in the presence of an HO-1 inhibitor, SnPP-IX. CO produced by HO-1, but not the other reaction products (iron or bilirubin), is capable of mediating the anti-proliferative effects of HO-1 in human CD4⁺ T cells (108, 109). Specifically, CO was shown to block production of IL-2, a principal cytokine responsible for T cell proliferation (159, 167). The anti-proliferative effect of CO in T cells is independent of the sGC/cGMP pathway and p38 MAPK and may involve caspase activation (108).

sGC and p38 MAPK Activation by CO

It is well established that p38 MAPK can be activated by a large number of environmental factors, and all of them do not activate the same signaling mechanism. For example, NO activates p38 MAPK via the sGC-protein kinase-G (PKG) pathway, whereas TNF- α uses ASK1 (153, 168, 169). Owing to the complexity of upstream signaling pathways of p38 MAPK in response to stimuli, it is possible that the intensity or duration of the kinase activation could be dependent on the specific signaling pathways that they utilize. Now, we have evidence that CO activates p38 MAPK by sGC-dependent or -independent pathways dependent on the experimental model (Figure 2). For example, the anti-inflammatory effects of CO in macrophages were determined to depend on p38 MAPK but not sGC. The induction of Hsp70 by CO is also not likely to use sGC as an intermediate signaling protein. However, caveolin-1 expression by the gas adopts sGC-p38 MAPK cascades, as assessed by chemical inhibitors and genetic deletion experiments. The role of sGC in caveolin-1 expression through p38 MAPK is also supported by the recent observation that reduced cGMP signaling is associated with neointimal proliferation and vascular dysfunction in hypercholesterolemic rabbits. Whether CO also uses PKG for the upregulation of caveolin-1 and growth inhibition in response to PDGF treatment or balloon-injured neointimal hyperplasia remains under investigation.

OXYGEN SENSING

Animals require oxygen to survive, and have evolved different mechanisms to sense and respond to low-oxygen tensions. When faced with low blood oxygen levels (hypoxia), humans and other mammals reflexively increase the lung ventilation rate to restore normal oxygen tensions to vital organs (170). This compensatory mechanism relies on oxygen-sensing glomus cells in the carotid body located in the carotid artery. Glomus cells sense hypoxia and respond by rapid membrane depolarization. This results in production of action potentials, influx of calcium ions (Ca^{2+}) into the glomus cells through voltage-gated Ca^{2+} channels, and release of the neurotransmitter dopamine. Dopamine then activates postsynaptic sensory neurons, and this afferent discharge initiates a variety of responses by the CNS that ensures appropriate oxygenation of different organs.

A recent study showed that the sensitivity of BK channels to hypoxia may be mediated by CO and that the underlying mechanism may involve an increasingly common principle in cellular signal transduction: a spatially tuned local signaling pathway mediated by a large protein complex of NPR, HO-2, and BK channel formed in the CNS (170).

On the vascular bed, CO can dilate blood vessels by directly acting calcium-dependent potassium channel (K_{Ca}). Kaide et al. demonstrated that decreased CO production was accompanied by a decreased number of open potassium channels in SMCs and increased vascular contractility; these effects were reversed with exogenous CO (30). In a study investigating the mechanisms of CO-mediated dilation of porcine cerebral arterioles, CO was found to dilate arterioles by increasing the effective coupling of calcium sparks to channels. The mechanism by which CO mediates these effects is still unclear, but the authors suggest the involvement of cGMP, another membrane-associated heme protein, or even direct action of CO on the receptors.

Moreover, chronic inhalation of CO (50 ppm) attenuates hypoxic pulmonary artery hypertension development presumably through activation of BK_{Ca} channels because CO inhalation slightly increases single-channel conductance of BK_{Ca} channels and induces a large increase in the sensitivity of BK_{Ca} channels to Ca^{2+} of pulmonary artery myocytes from normoxic and chronic hypoxic rats (171, 172). Consequently, BK_{Ca} currents are increased and play a more prominent role in controlling resting membrane potential of myocytes. From these observations, it is questioned whether the proposed mechanism for CO-dependent oxygen sensing in the CNS through BK channel is effective in peripheral vascular SMCs.

CO AND CAVEOLAE/CAVEOLIN-1: A NEW HYPOTHESIS

The membrane systems in any type of cell exhibit substantial and specific differences in their lipid compositions. In addition, specific differences exist between the two leaflets of most bilayers, and lipids in individual leaflets are not distributed

homogeneously in the plane of the membrane. Mainly because of their distinct biophysical properties, sphingolipids and cholesterol play a predominant part in generating microdomains in biological membranes. These sphingolipid- and cholesterol-dependent microdomains are designated as lipid rafts or caveolae (173). Lipid rafts are first assembled at the Golgi, and take major roles in specific trafficking of proteins and lipids to and from cellular compartments. Different types of cells can differ substantially in their raft contents, and association with lipid rafts influences cell signaling. Therefore, it is plausible that if a molecule can affect the composition of lipid rafts or caveolae, or the status of the structural protein caveolin, the molecule may play a role in cell signaling or trafficking. Li et al. proposed that endothelial nitric oxide synthase (eNOS)-dependent signaling could be terminated by a NO/cGMP-dependent process that leads to the disruption of caveolin-1 oligomers (174). Furthermore, they provided evidence that NO interferes with the integrity of caveolin-1 scaffolding function. Intracellular trafficking of caveolin and lipid microdomains may correspond to plasmalemmal caveolae after budding from the plasmalemmal membrane. Indeed, dynamin, which is known to play an essential regulatory role in the process of endocytosis through clathrin-coated pits, was recently found to promote cholera toxin B chain internalization within caveolae after budding from the plasmalemmal membrane. Therefore, both caveolar fission and apparent caveolin deoligomerization could represent the same phenomenon. Collectively, these data show that the integrity of the scaffolding function of caveolin-1 is potentially important for the spatial organization of signaling systems localized to caveolae and that NO modulates signal transduction by dissociating caveolin-1 oligomers (175). On the other hand, CO increased the oligomerization of caveolin-1 in the rat pulmonary artery endothelial cells (H.P. Kim & A.M.K. Choi, unpublished observation).

Caveolae have been proposed to play an important role in cell signaling and trafficking. Epidermal growth factor and platelet-derived growth factor receptors, endothelin-A and bradykinin B2 receptor, MAPKs, src-family nonreceptor tyrosine kinase, G proteins, protein kinase C, eNOS, and HO-1, among others, are concentrated in these signalosomes (Table 2). Caveolar-coat protein caveolin forms high-molecular-weight Triton-X-100 insoluble complexes through oligomerization mediated by N-terminal residues 61–101. NO can modulate signaling initiated via receptors (i.e., M2 muscarinic acetylcholine receptor) residing in caveolae. On the contrary, the involvement of CO in receptor-mediated signaling remains unclear. Hyperactivation of p38 MAPK by CO in response to PDGF-BB treatment provides a clue. Because the balance of upstream kinases and phosphatases may determine the phosphorylation strength by growth factor receptor signaling, CO may play a role in the signaling of growth factor receptor-mediated events by altering the activities of kinases, phosphatases, or both. Interestingly, some kinases and phosphatases are localized to caveolae to act properly in response to stimuli (176, 177). Because the effects of the two gases, NO and CO, can yield similar or different outcomes depending on the cells or stimuli, we need to investigate their roles in terms of caveolin-1 oligomerization or trafficking case by case.

It would be reasonable to speculate that, however, especially in acute phase, CO may modulate the signaling in a fashion opposite to NO owing to the apparent effects of the gases on caveolin-1 oligomerization. The questions arising from this paradox are (a) Can oligomerization of caveolin-1 inhibit signaling generally? (b) Can caveolin-1 oligomeric status modulate signaling depending on stimuli or cell type? (c) Can pretreatment of CO modulate activation of eNOS by agonists such as bradykinin? (d) Can oligomerization of caveolin-1 affect the activation of sGC, which is a well-known protein activated by both gases? Answering the above questions may not only shed light on the biology of caveolae, a signalosome, but also on the understanding of the crosstalk between the two gases.

CONCLUSION

In this review we have summarized the major known mechanisms by which CO, an endogenously produced gas, can affect cellular signaling pathways. Activation of sGC appears to be a functional intermediate in some of the biological effects of CO, including neurotransmission and vasodilation. The lack of involvement of sGC in some processes triggered by CO suggests that additional proximal targets of CO action may exist. Of these, modulation of K⁺-channel activity by CO has recently been described. MAPK signaling pathways, including p38 MAPK appear to represent major mediators of CO action, with respect to anti-inflammatory, anti-proliferative, and anti-apoptotic effects. An understanding of the molecular mechanisms of CO action at the cellular level may be useful in the rational design of therapeutic strategies for the treatment of inflammatory or proliferative disorders.

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CONTENTS

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS: HOW THEIR EFFECTS ON MACROPHAGES CAN LEAD TO THE DEVELOPMENT OF A NEW DRUG THERAPY AGAINST ATHEROSCLEROSIS, <i>Andrew C. Li and Wulf Palinski</i>	1
CYTOCHROME P450 AND XENOBIOTIC RECEPTOR HUMANIZED MICE, <i>Frank J. Gonzalez and Ai-Ming Yu</i>	41
HUMAN FLAVIN-CONTAINING MONOOXYGENASES, <i>John R. Cashman and Jun Zhang</i>	65
CANNABINOID RECEPTORS AS THERAPEUTIC TARGETS, <i>Ken Mackie</i>	101
REGULATION OF DRUG-METABOLIZING ENZYMES AND TRANSPORTERS IN INFLAMMATION, <i>Alison E. Aitken, Terrilyn A. Richardson, and Edward T. Morgan</i>	123
ACCESSORY PROTEINS FOR G PROTEINS: PARTNERS IN SIGNALING, <i>Motohiko Sato, Joe B. Blumer, Violaine Simon, and Stephen M. Lanier</i>	151
THE PROTEASOME AND PROTEASOME INHIBITORS IN CANCER THERAPY, <i>Peter M. Voorhees and Robert Z. Orłowski</i>	189
NUCLEAR AND MITOCHONDRIAL COMPARTMENTATION OF OXIDATIVE STRESS AND REDOX SIGNALING, <i>Jason M. Hansen, Young-Mi Go, and Dean P. Jones</i>	215
THE REGULATION AND PHARMACOLOGY OF ENDOTHELIAL NITRIC OXIDE SYNTHASE, <i>David M. Dudzinski, Junsuke Igarashi, Daniel Greif, and Thomas Michel</i>	235
REGULATION OF PLATELET FUNCTIONS BY P2 RECEPTORS, <i>Christian Gachet</i>	277
FUNCTIONAL IMAGING OF TUMOR PROTEOLYSIS, <i>Bonnie F. Sloane, Mansoureh Sameni, Izabela Podgorski, Dora Cavallo-Medved, and Kamiar Moin</i>	301
PHARMACOGENOMICS OF ACUTE LEUKEMIA, <i>Meyling H. Cheok, Sanne Lugthart, and William E. Evans</i>	317
REGULATION OF PHOSPHOLIPASE C ISOZYMES BY RAS SUPERFAMILY GTPASES, <i>T. Kendall Harden and John Sondek</i>	355

ROLE OF ABCG2/BCRP IN BIOLOGY AND MEDICINE, <i>P. Krishnamurthy and J.D. Schuetz</i>	381
CO AS A CELLULAR SIGNALING MOLECULE, <i>Hong Pyo Kim, Stefan W. Ryter, and Augustine M.K. Choi</i>	411
FUNCTION OF RETINOIC ACID NUCLEAR RECEPTORS: LESSONS FROM GENETIC AND PHARMACOLOGICAL DISSECTIONS OF THE RETINOIC ACID SIGNALING PATHWAY DURING MOUSE EMBRYOGENESIS, <i>Manuel Mark, Norbert B. Ghyselinck, and Pierre Chambon</i>	451
MOLECULAR MECHANISM OF 7TM RECEPTOR ACTIVATION—A GLOBAL TOGGLE SWITCH MODEL, <i>Thue W. Schwartz, Thomas M. Frimurer, Birgitte Holst, Mette M. Rosenkilde, and Christian E. Elling</i>	481
INDEXES	
Subject Index	521
Cumulative Index of Contributing Authors, Volumes 42–46	535
Cumulative Index of Chapter Titles, Volumes 42–46	538

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