A fact is a simple statement that everyone believes. It is innocent, unless found guilty. A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective.

- Edward Teller

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Carbon monoxide (CO) is well known for its toxic effects and has strong affinity for hemoglobin (Hb), which is nearly 245 times that of oxygen (Smith RP, 1986; Chance B et al., 1970). In addition, partial occupation of CO at the heme binding sites inhibits the release of O\textsubscript{2} from the remaining heme groups, shifting the \textit{O}_{2} dissociation curve to the left. These actions of CO reduce the \textit{O}_{2} carrying capacity and delivery potential leading to tissue hypoxia. Higher concentrations of CO also bind to cytochromes P450, cytochrome c, and myoglobin further amplifying the detrimental actions of CO (Piantadosi CA, 2002). Paradoxically, studies in the mid 20th century reported that CO is also generated endogenously in humans, and that under specific pathophysiological conditions CO production is greatly increased (Sjorstrand T, 1949; Vreman HJ et al., 2000). The daily production of CO in the human body is quite substantial; approaching nearly 20-\textmu\text{M}/hour (Coburn RF et al., 1965). The predominant biological source of CO (> 86\%) is from the degradation of heme by the enzyme heme oxygenase (HO) with minor amounts formed by photo-oxidation, lipid peroxidation, and xenobiotic metabolism (Sjorstrand T, 1949). HO cleaves \textalpha- meso carbon bridge of heme yielding equimolar amounts of biliverdin, iron, and CO. This oxidative reaction serves as the first and rate-limiting step in heme catabolism and is catalyzed by two distinct isoforms of HO: HO-1 is a ubiquitously distributed isoform that is strongly induced by biochemical and biophysical stress while HO-2 is constitutively expressed and concentrated in specific organs such as the brain and testes (Maines MD, 1989). HO-3, recently identified, is similar to HO-2 but less efficient heme catalyst (Perrella MA and Yet SF, 2003). HO-1 and HO-2 are expressed in the heart and blood vessels, and both proteins are found in vascular endothelium and smooth muscle. Moreover, HO is catalytically active in cardiovascular tissue as reflected by the HO-1-mediated production of bilirubin and CO (Durante W, 2002). Although long considered an obscure byproduct of heme metabolism with potential toxicological implications, the finding that another structurally similar poisonous gas, nitric oxide (NO) plays a significant role in human health, raised the possibility that CO may also serve an important physiological function (Marks GS et al., 1991). Studies in the past decade have clearly established the biological significance of CO in numerous organ systems. Although long considered an insignificant and potentially toxic waste product of heme catabolism, CO is now recognized as a key-signaling molecule that regulates numerous cardiovascular and other functions. In fact, many of the cytoprotective actions resulting from the induction of HO-1 are attributable to the generation
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of CO. Heme breakdown by HO-1 produces CO, bilirubin and iron (Fe⁺), in which a byproduct CO is reported as cytoprotective in diseased conditions mimicking the role of HO-1 (Otterbein LE et al., 2003). The direct evidence of the clinical significance of HO-1 in coronary artery disease (CAD) comes from a study demonstrating that HO-1 expression and activity are associated with atherosclerosis. Human arterial samples were obtained from normal subjects during surgery for vascular trauma or from patients with atherosclerotic diseases. Interestingly, vascular endothelial growth factor (VEGF) protein and HO-1 activity, as measured by bilirubin release per mg of aorta, were only present in the advanced atherosclerotic lesions (Morsi WG et al., 2006). Furthermore, leukocytes from patients with CAD also express HO-1 and the level of HO-1 expression was correlated with the severity of their disease, with patients suffering from acute myocardial infarction (AMI) being highest, followed by patients with unstable angina (Chen SM et al., 2005). It has also be shown that among patients with documented coronary artery disease, HO-1 level is correlated with plaque burdens (Li YG et al., 2006). Mice with a HO-1 null mutation have been shown to develop anemia associated with hepatic and renal iron overload and this contributes to the oxidative tissue injury and chronic inflammation (Poss KD and Tonegawa S, 1997). Moreover, HO-1 deficient mice develop right ventricular infarction after chronic hypoxia exposure and are more susceptible to ischemic and reperfusion (I/R) injury (Yet SF et al., 1999; Yoshida T et al., 2001; Liu X et al., 2005). The first human case of HO-1 deficiency has also been reported (Yachie A et al., 1999). This patient suffered persistent hemolytic anemia and an abnormal coagulation/fibrinolysis system, which were associated with elevated thrombomodulin and von Willebrand factor, indicating persistent endothelial damage. Likewise, studies on HO-2 deficient mice revealed that these animals exhibit hypoxemia and hypertrophy of the pulmonary venous myocardium and are more susceptible to hyperoxic lung damage, which is associated with increased expression of HO-1 (Dennery PA et al., 1998; Adachi T et al., 2004). These findings provide strong evidence to support that HO has important functions in normal physiology and pathophysiology, especially with regard to the cardiovascular system. HO-1 overexpression protects the myocardium from ischemic and reperfusion injury (Yet SF et al., 2001). Several lines of evidence suggest that the anti-inflammatory properties of CO and the anti-oxidant effects of bilirubin mediate the myocardial protection induced by HO-1. In a rat model, CO or biliverdin alone did not alter the survival of heart grafts, while a combined therapy was able to increase the survival from 0% to 80% (Nakao A et al., 2005). An earlier study from Hu CM (2004) and his group demonstrated that HO-1 has a role in the myocardial remodeling response (Hu CM et al.,
Cardiac hypertrophy induced by angiotensin II can be significantly suppressed by HO-1 overexpression either by cobalt protoporphyrin or HO-1 adenovirus. Results from Hu CM (2004) and his group support the hypothesis that bilirubin suppresses angiotensin II-induced cardiac hypertrophy via a reduction in reactive oxygen species (ROS) production. Others have also shown that antioxidants are effective at preventing cardiomyocyte hypertrophy and HO-1 induction attenuates cardiac hypertrophy in stroke-prone SHR rats (Seki T et al., 1999). ROS are implicated in various pathological myocardial dysfunctions and, therefore, the attenuation of ROS by HO-1 is considered to be a potential therapeutic target in myocardial diseases.

Our evolution in the understanding of CO has grown from studies throughout the 1900's investigating cellular respiration. CO is now known to influence both intra- and extracellular signaling (Otterbein LE et al., 2003). The findings that CO can confer protective (i.e., anti-inflammatory, anti-apoptotic, anti-proliferative) effects at low concentration in cells led to much intensive investigation as to the underlying molecular mechanisms involved. The primary known molecular target of CO is the heme iron center of hemoproteins. By this mechanism, high CO concentrations cause hypoxemia by competitive binding to the oxygen-binding sites of hemoglobin to form COHb, with an affinity approximately 245 times that of oxygen (Von Burg R, 1999). In humans, prolonged or elevated CO exposures can cause a number of acute clinical effects, including nausea, dizziness, and loss of consciousness. Symptoms of CO poisoning begin to appear at 20% COHb, while death occurs between 50 and 80% COHb (Von Burg R, 1999). In contrast, CO exposures associated with cytoprotective effects in rodents (i.e., against the injurious effects of mechanical ventilation) resulted in attained COHb levels typically within the 15 to 20% range (Hoetzel A et al., 2008, Dolinay T et al., 2004). CO, when applied at low concentrations, can influence a number of signaling pathways in cultured cells, including those regulated by soluble guanylate cyclase (sGC) and/or mitogen-activated protein kinase (MAPK) (Kim HP et al., 2006; Maines MD, 1997; Ryter SW et al., 2004; Morita T et al., 1995; Otterbein LE et al., 2003). At a cellular level, CO can stimulate sGC to increase the production of cyclic guanosine 3', 5'-monophosphate (cGMP), which has been demonstrated in vascular smooth muscle cells (VSMC) (Morita T et al., 1995). HO-derived CO and its effects on cGMP formation have been implicated in several neuronal signaling processes, as well as in the regulation of vascular functions (i.e., vessel tone, smooth muscle proliferation and platelet aggregation) (Ryter SW et al., 2006; Ryter SW et al., 2004). CO treatment can modulate the activation of MAPK pathways that are critical for cellular signal transduction in response
to stress and inflammation. In particular, the p38 MAPK signaling pathway has been implicated in the anti-inflammatory, anti-apoptotic, and anti-proliferative effects of CO (Otterbein LE et al., 2000; Otterbein LE et al., 2003; Brouard S et al., 2000; Otterbein LE et al., 2003). The integration of these pathways appears to vary in a cell type-specific fashion (Ryter SW et al., 2006). In one example, CO inhibits smooth muscle cell proliferation by activation of sGC, downstream activation of p38 beta MAPK, leading to increased expression of the cyclin-dependent kinase inhibitor p21Waf1/Cip1 (Otterbein LE et al., 2003). On the other hand, the activation of p38 MAPK by CO has been reported to be independent of sGC with respect to macrophages, indicating that the proximal target, presumably a heme protein, remains incompletely understood (Otterbein LE et al., 2000). The modulation of cellular ROS production from membrane or mitochondria-dependent sources has been implicated in CO-dependent signaling. Inhibition of membrane NADPH: oxidase by CO and subsequent down-regulation of O$_2$ production has been implicated in the antiproliferative and anti-inflammatory effects of CO (Nakahira K et al., 2006; Taillefer C et al., 2005). Recent observations from many laboratories reveal additional candidate molecules that function as downstream effector molecules of CO signaling, including the 70-kD heat shock protein (Hsp-70), peroxysome proliferator-activated receptor-γ (PPAR-γ), an anti-inflammatory nuclear regulator, Egr-1, and caveolin-1 (Kim HP et al., 2005; Bilban M et al., 2006; Kim HP et al., 2005; Wang XM et al., 2009). Recent studies have also identified novel mechanisms by which CO may exert anti-inflammatory effects involving the down-regulation of Toll-like receptor (TLR) trafficking and activation (Nakahira K et al., 2006; Wang XM et al., 2009).

In addition, studies employing the exogenous application of CO have confirmed the protective properties of this gas in several pathological conditions. Interestingly, alterations in CO synthesis are associated with many cardiovascular disorders, including atherosclerosis (Otterbein LE et al., 2003), hypertension (Minamino T et al., 2001), metabolic syndrome (Johnson FK et al., 2006), and ischemia-reperfusion injury (Stein AB et al., 2005). Significantly, restoration of physiologic CO levels exerts a beneficial effect in many of these settings, suggesting a crucial role for CO in maintaining cardiovascular homeostasis. CO has recently emerged as a potential therapeutic modality for the treatment of cardiovascular disease. Several strategies have been proposed for targeting HO-1 or CO to cardiovascular tissue such as (i) enhancing endogenous CO synthesis and/or activity by pharmacological induction of HO-1 or gene delivery of HO-1 or increasing substrate availability or application of CO-sensitizing compounds and (ii) exogenous delivery of CO by inhalation of
CO or CO-containing solutions or use of prodrug to generate CO or use of CO-releasing molecules (CORMs). The use of pharmacological inducers of HO-1 offers a promising approach. Many potent inducers of HO-1 have been identified and shown to exert beneficial effects in the circulation. Heme and its synthetic analogues are strong inducers of HO-1 and have been demonstrated to protect against cardiovascular disease in numerous animal models. In addition to inducing HO-1, heme may also promote CO synthesis by providing additional substrate for the enzyme. Morimoto Y et al. (2001) demonstrated that CO synthesis is likely substrate-limited in vascular cells, and that endogenous application of heme results in an immediate increase in the rate of CO synthesis (Morimoto Y et al., 2001). However, heme and its derivatives possess pro-oxidant properties and will require caution in their use. Natural antioxidants and dietary supplements offer an alternative approach to stimulate HO-1 expression that may not provoke tissue damage (Ogborne RM et al., 2004). Interestingly, there is increasing recognition that many of the vanguard drugs used to treat cardiovascular disease, including aspirin, statins, nitrovasodilators, rapamycin, and paclitaxol, are effective inducers of HO-1 and exert their clinical benefits, at least in part, through the release of CO (Durante W et al., 2005). A possible concern with the use of pharmacological inducers of HO-1 relates to the GT length polymorphism in the HO-1 promoter that may make such an approach difficult in patients with the long GT repeats that are more resistant to HO-1 induction. Increasing HO-1 expression via viral-mediated delivery of HO-1 circumvents this problem and provides for a more selective approach in targeting this gene to specific tissues (Juan SH et al., 2001; Duckers HJ et al., 2001; Tulis DA et al., 2001, Melo LG et al., 2002; Liu X et al., 2006; Sabaawy HE et al., 2001). Gene therapy approaches with HO-1 have proven highly effective in animal studies, and the recent development of inducible and/or tissue specific vectors will allow for a more refined pattern of HO-1 expression. However, current limitations in human gene therapy are well known and will require further improvements in vector design and certification of clinical safety and efficacy. The administration of CO provides a direct approach in delivering the gas. Inhalation of CO has been demonstrated to be effective in several animal models of cardiovascular disease (Otterbein LE et al., 2003; Fujimoto H et al., 2004; Lavitrano M et al., 2004; Akamatsu Y et al., 2004; Mazzola S et al., 2005). However, reports on tolerance to CO inhalation are contradictory and require further investigation (Stupfel M and Bouley G, 1970; Loennechen JP et al., 1999; Penney DG and Formolo JM, 1993). The use of prodrugs to generate CO provides another route for the systemic administration of CO. In particular, dichloromethane is readily metabolized by cytochrome P450 isozymes to CO
and CO₂. Interestingly, the production of CO following the oral ingestion of dichloromethane markedly attenuates intimal thickening in a model of chronic allogeneic aorta rejection in rats, demonstrating the feasibility of this technique to convey biologically relevant concentrations of CO (Chauveau C et al., 2002). More recently, the generation of novel CORMs provides another alternative for the delivery of CO. Several CORMs with various solubility and release kinetics have been synthesized and their biological activity validated in both vascular and cardiac tissue (Foresti R et al., 2005). These compounds may allow for a more controlled delivery of CO and could easily be impregnated onto various medical devices, including coronary stents. Furthermore, the combined use of CO with CO-sensitizing agents, such as 3-(5'-hydroxymethyl-2-furyl)-1-benzyl indazole (YC-1), may circumnavigate the possible development of tissue hypoxia by decreasing the amount of CO required to exert its therapeutic effect (Tulis DA, 2004). Since emerging studies suggest that CO may in certain instances promote cardiovascular dysfunction by stimulating the production of ROS and/or by inhibiting heme-containing proteins (e.g. endothelial NO synthase-eNOS), approaches that prevent the formation of CO may also be of therapeutic relevance. Metalloporphyrins are well-recognized and potent inhibitors of HO that have been widely employed to block the endogenous formation of CO. These pharmacological inhibitors resemble heme in their porphyrin structure, but the iron core is substituted by a heavy metal such as zinc, tin, or cobalt. These substituted porphyrins compete with heme for binding to HO and can be used to block HO activity both acutely and chronically (Tulis DA et al., 2001; Johnson RA et al., 1996). However, at high doses these metalloporphyrins are not selective for HO and can paradoxically induce the expression of HO-1 (Durante W, 2002). In addition, some metalloporphyrins are photosensitive and can undergo non-enzymatic degradation to release CO. Interestingly; recent work has identified novel non-porphyrin inhibitors of HO. Several imidazole-dioxolane derivatives have been demonstrated to be potent inhibitors of HO (Vreman HJ et al., 2002, Kinobe RT et al., 2006). Significantly, these compounds have no effect on NO synthase (NOS) or sGC activity, and a subset of these derivatives exhibit high selectively for HO-1 relative to HO-2. These later compounds may serve as important pharmacological tools to further define the role of HO-1 and CO in the cardiovascular system. Inhibiting the expression of HO-1 may block CO production. Molecular approaches targeting HO-1 mRNA using both antisense and small interference RNA technology have been successfully employed and may provide a more specific approach in downregulating CO synthesis (Quan S et al., 2001; Deng YM et al., 2004). Delivery of CO in gas form has no control on release pattern and concentration, which
may lead to undesired effects. Emerging evidence suggests that exogenously applied CO could have beneficial and therapeutic effects (Kim HP et al., 2006). This is conceivably true if delivery of CO can be probably controlled to convert toxic effects into beneficial signaling activities. Tricarbonyldichlororuthenium (II) dimmer known as CO-releasing molecule-2 (CORM-2), a lipid soluble molecule that was able to deliver CO in a controlled manner and simulate the cytoprotective action of HO-1 derived CO in biological systems (Józkwicz A et al., 2003; Choi BM et al., 2003). Subsequently tricarbonyldichloro (glycinato) ruthenium (II) (CORM-3), a water-soluble form also demonstrated protection against cardiac I/R injury (Motterlini R et al., 2002; Clark JE et al., 2003; Guo Y et al., 2004; Stein AB et al., 2005; Fujimoto H et al., 2004). A recent report by Bak I et al. (2005) showed that low concentration of CO in perfusion buffer reduces the infarct size, improves hemodynamic parameters and reduces ventricular fibrillation whereas higher concentration led to severe ventricular fibrillation in isolated perfused rat hearts. CO has been shown to inhibit NOS and it has also been reported that NO can inhibit human HO-1, leading to the suggestion that inhibition of human HO-1 by NO can contribute to the signaling interplay between NO and CO (Wang J et al., 2003). In a comparative study of the vasoactive effects of NO and CO in isolated rabbit aorta, exogenous CO produced an endothelial-independent vasorelaxant response, albeit with a 1000-fold less potency than NO under the same conditions (Furchgott RF and Jothianandan D, 1991). In contrast, the vasodilation elicited by CORM-3 required intact endothelium and an accessory role for endogenous NO production (Foresti R et al., 2004). CORM-2 mediated cardioprotection in intact and dysfunctional coronary endothelium has not been studied in isolated heart. Many published literature emphasizes the role of p38MAPK (Kim HP et al., 2005; Otterbein LE et al., 2000), relatively limited studies suggested the role of protein kinase (PK) A/G/C (Immenschuh S et al., 1998) and few studies have demonstrated the role of phosphatidylinositols 3-kinase (PI3K) pathway (Zhang X et al., 2005) in CO-mediated cytoprotection. Recent studies demonstrate that increased ROS generation, especially superoxide and hydrogen peroxide, is implicated in the mechanisms of doxorubicin (DXR)-induced cardiotoxicity (Wang S et al., 2004). The high level of DXR could damage membranes, proteins (e.g., enzymes, structural, and receptors), and DNA that may lead to cardiac dysfunction and apoptosis (Green PS and Leeuwenburgh C, 2002). H9c2 is a clonal cell line derived from embryonic rat heart tissue (Kimes BW and Brandt BL, 1976) and exhibits many of the properties of cardiac muscle, including electrophysiology, ion channels and receptors (Fujita T et al., 2006). Despite not being fully differentiated, H9c2 cells have been used frequently as a model of cardiac cells in...
several studies (Kim MS et al., 2006). CO can confer protection against apoptosis in several cell culture models, including endothelial cells. Exogenous CO has been shown to inhibit tumor necrosis factor (TNF)-alpha-initiated apoptosis in mouse fibroblasts (Petrache I et al., 2000) and endothelial cells (Brouard S et al., 2000). A similar in vitro antiapoptotic effect was observed with HO-1 overexpression (Petrache I et al., 2000). Recent evidence indicate that CO may be a strong contributor to the defensive action attributed to HO-1 and participate more directly in protecting cells against oxidative and nitrosative stress. The use of CORM has been instrumental to show this new role of CO. CORM-2 has been shown to reduce the production of ROS and NO derived from up-regulation of inducible-nitric oxide synthase (iNOS) in RAW 264.7 macrophages stimulated with lipopolisaccharide (Srisook K et al., 2006). Both CORM-2 and CORM-3 are able to reduce NO production in different cellular systems such as microglial cells (Bani-Hani MG et al., 2006) and murine macrophages (Sawle P et al., 2005) without affecting iNOS expression. Recently, Suliman HB et al. (2007) suggested that DXR disrupts cardiac mitochondrial biogenesis, which promotes intrinsic apoptosis, while CO/HO promotes mitochondrial biogenesis and opposes apoptosis, forestalling fibrosis and cardiomyopathy in DXR-induced cardiotoxicity using mice model. The progressive mitochondrial damage and mitochondrial initiation of apoptosis in DXR-induced cardiomyopathy (Childs AC et al., 2002), along with the antiapoptotic mitochondrial profile that accompanies stimulation of the CO/HO system (Plantadosi CA, 2006), support the idea that CO would oppose intrinsic apoptosis after DXR administration. CO modulates intracellular signaling pathways involving hemoproteins and downstream effectors. An organism is known to experience accelerated erythropoiesis when subjected to higher altitudes, to atmospheres containing CO, and when injected with androgens or cobalt (Erslev AJ, 1975). One way of assaying the degree of this response is by measurement of hematological profile and reticulocyte count. An increase in erythropoiesis under hypoxic conditions is essentially a response to a feedback system of the kidney that monitors tissue pO2. The response is an increase in red blood cell (RBC) production in the bone marrow (Fisher JW, 1975).

Emerging studies indicate that CO may also exert important protection against thrombosis. Both endogenously derived and exogenously applied CO inhibits platelet aggregation by stimulating the activation of sGC (Brune B and Ullrich V, 1987). In addition, CO mitigates platelet adhesion to venular endothelium in response to inflammation (Morisaki H et al., 2001). Furthermore, CO inhibits platelet aggregation and thrombosis following organ transplantation, and may contribute to the inhibition of platelet-dependent
thrombosis following the induction of HO-1 in a rodent artery injury model (Sato K et al., 2001; Peng L et al., 2004). Significantly, inhalation of CO rescues mice from lethal ischemic injury by preventing microvascular thrombosis and the accumulation of fibrin (Fujita T et al., 2001). There are only a limited number of studies on the mechanisms by which CO inhibits platelet aggregation. Interestingly, most of them use gaseous CO and reveal a similarity with NO in the ability of both gases to inhibit platelet function via a common target, soluble guanylate cyclase (sGC) (Brune B and Ullrich V, 1987). Recent study by Chlopicki S et al. (2006) used CORM-3, a water soluble CO-releaser and shown to inhibit human platelets by a mechanism independent of sGC. They also proposed that CORM-3 inhibits platelet aggregation to almost similar degree when 10 fold higher concentration of thrombin used. They also suggested that intracellular target (like $K_{\text{ATP}}$ channel, NO, mitogen-activated protein kinase -MAPK etc.) for antiplatelet activity of CO released by CORM-3 has to be established.

Although numerous biological effects associated with endogenous CO generation or pharmacological applications are now reported, the mechanism by which CO acts or the nature of its biological targets remain unestablished. The beneficial effect of CO and CORM, especially on cardiovascular system has inspired us to work on the mechanistic aspect of CORMs. We outlined following objectives based on published literature evidences:

**Objective 1:** To observe the role of CO-releasing molecule-2 (CORM-2) in isolated rat heart: Concentration-dependent effect of CORM-2 for cardioprotection and to elucidate the involvement of NO, coronary endothelium, $K_{\text{ATP}}$ channel and various protein kinases (MAPK, PKC, PI3K etc.) involved in I/R injury.

**Objective 2:** To elucidate the role of CORM-2 in doxorubicin (DXR)-induced cytotoxicity and apoptosis using H9c2 rat cardiomyocyte cells *in vitro*.

**Objective 3:** To study the role of CORM-2 in DXR-mediated cardiotoxicity and oxidative stress in mice model *in vivo*.

**Objective 4:** To find out the role of CORM-3 (water soluble CO-releaser) in platelet aggregation, thrombosis and mechanism(s) involved in it using in *vitro*, *ex vivo* and *in vivo* approaches.