The Evolution of Carbon Monoxide Into Medicine

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The discovery that carbon monoxide (CO)—a highly publicized toxic gas molecule—can have powerful benefits and curative effects not only changed how we view CO, but has, with tremendous contradiction, resulted in clinical trials of CO for the treatment of various pathologies. There is sound preclinical evidence that, at a low concentration, CO has benefits in numerous and diverse diseases in rodents, large animals, and humans. CO especially has potential benefits in inflammatory disorders. As CO moves ahead in the clinic, we continue to advance our understanding of how it functions, especially as the number of potential clinical applications expands. CO's mechanisms of action at the cellular level depend on the disease and the experimental focus, but the one constant is that CO reestablishes homeostasis. *Key words: carbon monoxide, inflammation, homeostasis*. [Respir Care 2009;54(7):925–932. © 2009 Daedalus Enterprises]

Introduction

When most hear the concept that carbon monoxide (CO) can be used to treat disease, the question usually arises as to how that can possibly make sense, given the intense media coverage of the danger of inhaling this poison gas. From advertisements that cigarettes contain CO to numerous newspaper reports on the perils of CO from leaky furnaces and car exhaust, one would think there is no question that avoidance of CO is the best option. Interestingly, the fact that those sources of CO also emit numerous known carcinogens and substances that are poisonous even at very low concentration goes largely ignored, and those substances might explain the morbidity and mortality that is attributed to CO.

For well over a century the deadly effects of high-concentration CO have been studied, confirmed, and reconfirmed. Warnings on cigarette packages alert the smoker to the fact that cigarette smoke contains CO. Homeowners equip their houses with CO detectors to protect their families from its silent, invisible lethality. The Environmental Protection Agency warns of CO in automobile exhaust, and radio and television stations in urban areas broadcast "bad air" alerts. Edgar Allen Poe was a victim of long-

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term CO exposure, from living in a house with leaky gas lighting. His story "The Fall of the House of Usher" details the symptoms (experienced by the main character) of chronic CO poisoning, which came to be termed *neurasthenia*.

Nearly every organism on the planet generates CO as a normal cellular function,[1,2] and, perhaps counter-intuitively, cellular CO output increases during stress and disease.[3-5] The fundamental metabolic catabolism of heme by the heme oxygenases has probably been occurring since the beginning of life on earth. In fact, the origin of life on earth may have required CO as a building block of amino acids and proteins.[6] Heme is an essential moiety for the functioning of numerous proteins and enzymes, so the ability to turn heme over necessitates the presence of heme oxygenases and, therefore, CO. But is CO simply a waste product (as most concluded), or could it serve an important physiologic purpose?

Heme Oxygenase: The Endogenous Carbon Monoxide Generator

Heme oxygenase is a ubiquitous enzyme that has been identified in nearly all species.[7] Heme oxygenase-2 is constitutively expressed, whereas heme oxygenase-1 is strongly inducible.[8] The original description of its function was heme catalysis. Heme oxygenase was simply a metabolic enzyme system that developed to handle the large number of hemoproteins and the turnover of heme molecules. Since the original discovery and characterization, the number of agents that induce heme oxygenase-1 expression has exploded.[9,10] Additionally, numerous agents, including statins, acetaminophen, and prostaglandins, require the induction and activity of heme oxygenase-1 to impart their benefits,[11-13] and blockade of heme oxygenase-1 results in a loss of effects. Heme oxygenase-1 catabolizes heme, including the heme in hemoproteins, which results in the generation of 2 additional products: biliverdin and Fe^{++}.[7] In addition to the benefits of removing excess heme, the 3 products generated and their biological actions provide protection and reestablish homeostasis.[9,14,15] Of the three, when administered exogenously, CO is the most well studied and most closely mimics heme oxygenase-1. If induced prior to stress, heme oxygenase-1 and its products provide remarkable protection against cell and tissue damage.[16-21] Biliverdin, Fe^{++}, and ferritin have not been tested sufficiently to make such a conclusive statement, but bile pigments and ferritin expression can also be protective.[22] Numerous studies with dozens of models have shown that heme oxygenase-1 has cytoprotective and restorative properties—a finding supported by the facts that: heme-oxygenase-1-deficient mice have chronic inflammatory sequelae that progress with age; and a human who lacked heme-oxygenase-1 enzymatic activity died of an inflammatory syndrome.[23,24] Though the protection afforded by heme oxygenase-1 is no longer a subject of debate, the mechanisms of that protection are still under investigation. I and others have posited that the protection is largely mediated by endogenously generated CO.

The effect of CO on the inflammatory response is best illustrated by studies of endotoxic shock, in vitro and in vivo.[25-28] Macrophages or mice pre-treated with CO prior to endotoxin had lower production of tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1) and IL-6, among other pro-inflammatory cytokines, and higher production of anti-inflammatory IL-10.[29] CO's actions are also largely anti-apoptotic. In endothelial cells, hepatocytes, T cells, macrophages, and fibroblasts, CO prevents cell death[30-34] induced by administration of TNF-α. The one exception is that CO augments cell death in Fas and tumor-necrosis-factor-related-apoptosis-including-ligand (TRAIL) induced cell death in Jurkat T cells[35] and prostate cancer cells treated with chemotherapeutics, which speaks to the specificity of CO and the pathways involved (unpublished data and Reference 36). More striking are the data from a model of pulmonary hypertension, in which CO-exposed pulmonary-artery endothelial cells induced death of pulmonary-artery smooth-muscle cells, requiring physical contact of the endothelial cells and pulmonary-artery smooth-muscle cells.[37] In that setting CO increases the pulmonary-artery endothelial cells' generation of nitric oxide (NO), which induces death of the pulmonary-artery smooth-muscle cells. Exposure of either cell type alone to CO has no effect.

The diversity of CO's effects was further elucidated in investigations of signal transduction. CO clearly modulates soluble guanylate cyclase, peroxisome proliferator-activated receptor (PPARγ), heat-shock protein 70, hypoxia-inducible factor 1 (HIF1α), mitogen-activated protein (MAP) kinases, signal transducers and activators of transcription (STAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor (IRF), phosphatidylinositol-3-kinase (PI3K/AKT), and NO synthase (NOS)/NO, which have all been implicated in cell-specific protection and homeostasis actions.[15] Though many of these signaling pathways are interlinked, CO seems to modulate their activation and function differently, depending on the cell type. For example, CO requires cyclic guanosine monophosphate (cGMP), generated via soluble-guanylate-cyclase activation, to inhibit smooth-muscle-cell proliferation, and this is independent of NOS.[38,39]

In contrast, in endothelial cells, cGMP is not involved in proliferation, but rather requires AKT and NOS (unpublished data). CO's effect on vascular smooth-muscle cells and endothelial cells is the opposite of its effect on cell proliferation. CO blocks vascular smooth-muscle cells, but enhances endothelial cell proliferation. The selective acti-

vation of one or more of these signaling pathways probably contributes to CO's benefit in models of colitis, malaria, autoimmune encephalitis, chronic rejection, delayed graft function following transplantation, and pulmonary hypertension.[37,40-43] However, CO was not delivered in the same manner in all of those models, which complicates interpretation of the mechanism of action. What is needed is a comprehensive understanding of the dosage, administration period, and the ideal measure of CO delivery and exposure.

Currently, carboxyhemoglobin (COHb) measurement is the standard method to assess the presence of CO in the body, but the evidence is somewhat weak that the COHb level corresponds with efficacy, particularly since COHb reflects only the CO in the blood, not the CO in the tissues. We will focus on COHb until a better CO marker is identified, and it remains under strong debate as to what the allowed COHb level should be in humans. Based on preclinical data, short CO exposure is as efficacious as longer exposure, and a range of 12–20% for an hour may be the ideal range with which to obtain the benefits of CO. Taking into account all the models and data on CO efficacy, the tolerable and allowable COHb level (15–18%) occurs with a CO concentration of 250 ppm for 1 hour. Further rigorous testing is necessary to determine if COHb is the ideal measure of CO exposure and whether or not additional soluble CO-response factors can be assessed.

Vreman et al found reliable gas-chromatography evidence of CO in tissues following CO inhalation, which supports CO tissue access and distribution.[44] Research is underway on identifying CO-releasing molecules, which principally function as a pro-drug: CO is released or transferred via a chemical scaffold to its target after systemic administration. Different CO-releasing molecules release CO at different rates, and in some instances no measureable COHb is created yet potent protective effects are observed (eg, in models of stroke and autoimmune encephalitis).[45] CO delivery via CO-releasing molecules also adds to the complexity of CO administration and our ability to correlate the exposure to a physiologic benefit, particularly if COHb is the only measure of CO exposure. CO-releasing molecules are under intense preclinical evaluation and hold great promise for alternative CO-delivery routes, including oral and intravenous administration.

CO is now recognized and accepted as a potential therapy, as evidenced by approval of clinical trials. At 250 ppm for a relatively short period, CO has been remarkably salutary in several disorders/diseases in rodent and pig models, when administered prophylactically or therapeutically.[27,29] Such a CO dose is about 5–10% of a lethal exposure, and lower doses and regimens have not yet been evaluated. I propose that CO's therapeutic effects, which appear to be largely based on its modulation of cellular inflammation, apoptosis, and proliferative behavior, is dictated by the cell's situation and milieu. Of course, the discovery of CO's therapeutic values does not diminish the fact of its toxicity at high doses. Table 1 summarizes the effects of CO at various concentrations and exposure periods.

Table 1. Biological Effects of Carbon Monoxide

Concentration ppm (%)	Medical Relevance
10,000 (1)	Lethal in minutes
3,000 (0.3)	D_{LCO} in PFT laboratory (10 s)
10–500 (0.001–0.05)	Preclinical efficacy (1 h)
100 (0.01)	Cigarette (3–5 min)
10 (0.001)	Ambient air
35 (0.035)	EPA limit for 8-h workday

D_{LCO} = diffusing capacity of the lung for carbon monoxide
PFT = pulmonary function test
EPA = Environmental Protection Agency

Carbon Monoxide in Transplantation

Perhaps the greatest volume of data on CO effectiveness is in the field of organ transplantation. CO's protective effects were initially demonstrated in a model of lung injury, and subsequently in a mouse heart to rat xenotransplantation system.[17,42] Mouse hearts transplanted to immunosuppressed rats survive indefinitely. However, if the mouse heart cannot express heme-oxygenase-1 activity (either because it lacks the heme-oxygenase-1 gene or because heme-oxygenase-1 activity is inhibited), the heart is rejected rapidly. This was the first demonstration that an organ can contribute to its own survival by expressing a gene. Heme-oxygenase-1 expression in the transplanted heart is essential to prevent rejection. Surprisingly, if donor and recipient are both treated with CO, the heart survives indefinitely, even when the heart cannot express heme oxygenase-1. That is, CO can fully substitute for heme oxygenase-1 and suppress the inflammatory response that would cause rejection.

To date CO has been tested in rodent models of heart, lung, kidney, small bowel, and islet cell transplantation, which included models of ischemia reperfusion injury and acute and chronic allograft rejection (review in Reference 46 and unpublished data). More recently, CO was found to reduce the delay to functioning of a post-transplant kidney in swine (unpublished data). Saturating the organ-preservation solution with CO reduces organ injury and improves post-transplantation functioning,[47] without needing to administer CO to the donor or recipient, and thus may permit longer preservation time. What remains unclear is the optimal amount, duration, and frequency of CO exposure, and the role of treating the donor, the organ, and/or the recipient.

Cellular Basis of the Therapeutic Effects of Carbon Monoxide

The cellular basis of CO's action remains under intense investigation. Our studies of the response of monocytes/macrophages to pro-inflammatory stimuli provide models. Monocytes/macrophages stimulated with bacterial lipopolysaccharide normally produce several pro-inflammatory cytokines, including TNF-α. The anti-inflammatory cytokine IL-10 is also synthesized. If monocytes/macrophages over-express heme oxygenase-1 or are exposed to CO before stimulation with lipopolysaccharide, the pro-inflammatory response (eg, TNF-α) is markedly inhibited, and the anti-inflammatory response (IL-10 production) is enhanced.[25] Similar results are seen in vivo. Thus, CO suppresses the pro-inflammatory response and boosts the anti-inflammatory response of monocytes/macrophages, which probably control the balance of inflammation in many conditions.

Two other known actions of CO contribute to its anti-inflammatory effects. First, CO prevents platelet activation/aggregation, thereby suppressing thrombosis,[48] and down-regulates monocyte/macrophage expression of the pro-thrombotic plasminogen activator inhibitor type-1 (PAI-1)—an action that appeared to be critical in CO's protection against ischemia reperfusion injury in a lung model.[49,50] Second, CO prevents apoptosis of several cell types, including endothelial cells, fibroblasts, and β cells of the pancreas.[32,51] Given that apoptosis of certain cell types can exacerbate the deleterious effects of inflammatory reactions, CO's anti-apoptotic effect may contribute to the overall protective effect. In a recent expansion of the endotoxin model into a live bacterial sepsis infection model, the expectation was that reducing the inflammatory response with CO (as was observed with lipopolysaccharide during a bacterial infection) would permit rapid bacterial proliferation and hasten the onset of septic shock and end-organ failure, but the exact opposite occurred. Chung et al found that exposing infected animals to CO enhanced bacterial clearance.[52] Desmard et al recently found that a CO-releasing molecule was bactericidal; it acted directly on the bacteria, unlike CO gas.[53]

Published[54] and unpublished data from our laboratory show that CO augments macrophages' ability to kill bacteria by enhancing radical-generation and phagocytosis (Fig. 1). CO increases reactive oxygen species and NO generation, probably via mitochondrial and NO synthases, respectively, that can participate in the killing.[34] We posit that amplification of killing, via increased formation of the phagolysosome, where radical-generation is concentrated, is an important mechanism by which CO protects from overwhelming infection. Gaseous CO does not seem to directly effect bacterial killing (unpublished data), which potentially speaks to the CO delivery mode's effects on immune response and bacteria. A conclusion of one CO-releasing-molecule study was that CO is delivered directly to the mitochondria of the bacteria and interferes with respiration by blocking the electron-transport chain.[53] Perhaps local concentrations of CO explains the different effects on the bacterial response.

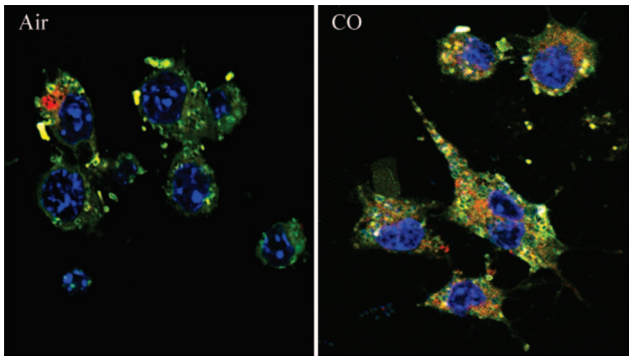

Fig. 1. Gaseous carbon monoxide (CO) at 250 ppm increases phagocytosis and bacteria clearance by macrophages. Compared to the preparation that was exposed to only air, the CO-exposed preparation has more enterococci inside the macrophages. The bacteria were fluorescently labeled and are red, the lysosomes are green, and the nuclei are blue. These images span 4–6 fields of view.

CO affects other cells that participate in inflammation. Both in vitro and in vivo, CO suppressed the proliferative response of smooth-muscle cells that contribute to neointimal proliferation (which characterizes atherosclerosis) in several models of vascular injury in vivo.[16] CO treatment of a rat recipient of an allogeneic aortic graft suppressed the post-transplant arteriosclerosis and transplant vascular stenosis that occurred without CO treatment.[16] Likewise, a one-hour CO pretreatment of a rat or mouse, in a vascular injury model, very significantly reduced (virtually ablated) the neointimal proliferation seen at 14 days post-angioplasty without CO treatment (unpublished data and Reference 16). Recent unpublished data from our laboratory shows that CO also affects endothelial cells.[37] At days 3–5 the animals exposed to CO had greater re-endothelialization of the denuded vessel than did the controls.

CO also increases the motility of endothelial cells. In vitro in the scratch assay, quantitation of endothelial-cell motility via time-lapse video showed that CO doubled the migration speed of endothelial cells across a field. We hypothesize that enhanced endothelial repair in vivo involves contributions of the cells neighboring the injury in the vessel and enhanced recruitment of endothelial progenitor cells. Collectively, the ability of the vessel to replace the denuded area probably contributes to the decreased stenosis observed at 2 weeks. The post-trauma effects of CO on luminal stenosis and the development of intimal hyperplasia have also been observed in pig models.[55]

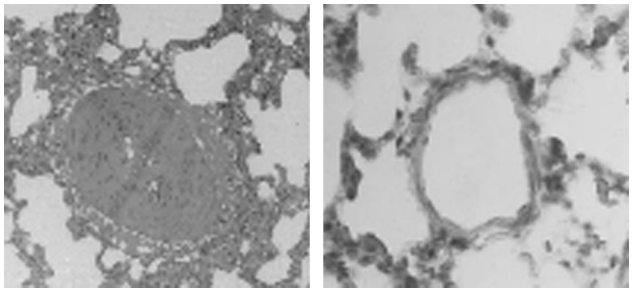

Fig. 2. Pulmonary arteriole from rats not treated (left) and treated (right) with carbon monoxide (CO), 6 weeks after administration of monocrotaline to induce pulmonary hypertension. At the peak of hypertension (day 29) CO exposure was initiated at 250 ppm for 1 h/d, and continued to day 42. CO reversed pulmonary hypertension to near normal. These images are representative of 6–8 sections per lung from 4–6 rats per treatment group. (From Reference 37, with permission.)

Heme Oxygenase-1/Carbon Monoxide and Nitric Oxide Synthase/Nitric Oxide

One of CO's mechanisms involves NO generation.[28,37] NO has been extensively studied. It is used to treat neonatal pulmonary hypertension, and is in clinical trials for other indications. Recent data indicates that NO up-regulates expression of heme oxygenase-1 and, thus, CO production. This raises the question of whether NO's therapeutic effects are mediated by or at least involve CO. Alternatively, CO increases the expression and activation of the inducible NOS and endothelial NOS isoforms and NO generation, but in a tissue-specific fashion. CO increased inducible NOS, but not endothelial NOS, in a model of acute hepatitis, whereas CO increased endothelial NOS, but not inducible NOS, in a model of pulmonary hypertension.[28,37] Brief CO exposure retro-remodeled thickened pulmonary arterioles to normal architecture (Fig. 2) and restored right-heart size to near normal.[37] The enhanced proliferation of endothelial cells (described above) requires activation and phosphorylation of endothelial NOS. Additionally, CO's ability to enhance bacterial killing is dependent on inducible NOS (unpublished data) and not endothelial NOS. Clearly these enzyme/gas-generating systems are interrelated and function as a metabolic gas cycle (Fig. 3). The enhancement or inhibition of enzyme activity by NO and CO probably depends on the needs of the cell. CO's high diffusivity and lack of reactivity in cells and tissues permits it to access more cellular targets than NO, which is highly reactive and therefore has a short half life and cannot have effects far from its generation source. Table 2 compares the physical properties and some of the preclinical efficacy of NO and CO that has been observed.[56-59]

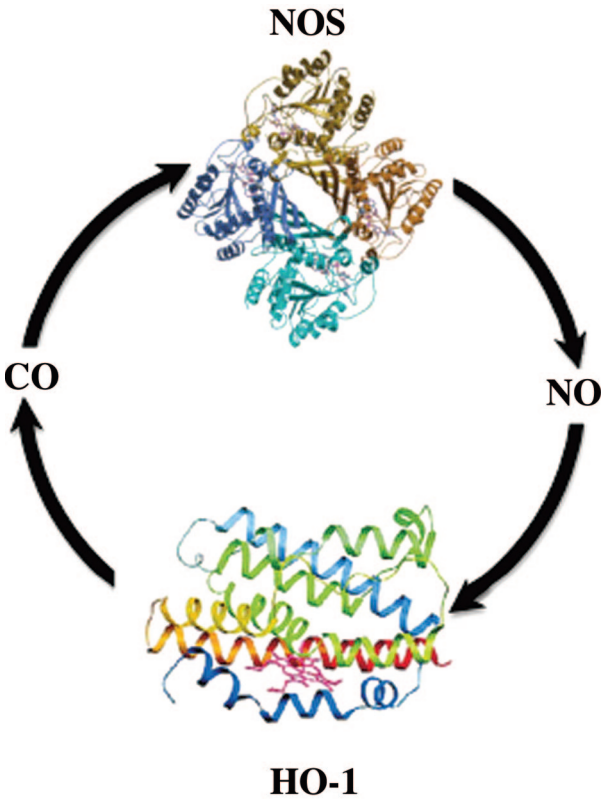

Fig. 3. Hypothetical carbon monoxide (CO) and nitric oxide (NO) gas cycle. The enzymes nitric oxide synthase (NOS) and heme oxygenase 1 (HO-1) produce NO and CO, respectively, and NO and CO may can enhance or inhibit enzyme activity, as dictated by cellular need.

Is Carbon Monoxide Ready for the Clinic?

Ikaria (Clinton, New Jersey) has developed an inhaled-CO-delivery device for clinical studies. Rather than parts per million, the device meters the gaseous CO in mg/kg units, so the CO dose is based on patient weight. A phase-2 trial is underway of CO for kidney-transplant recipients, and the study is designed to determine if inhaled CO affects post-transplant renal function. The vast evidence of CO's toxicity in higher concentrations commands the greatest of care in the clinical testing of CO, but we believe CO may have a niche before, during, and after some procedures, and probably in inflammatory syndromes, including pulmonary hypertension, cerebral malaria, sepsis, and colitis.

CO may not have to be administered to the transplant recipient. CO treatment of the donor and the ex vivo organ before implantation in the recipient, without CO treatment of the recipient, appears to impart a similar benefit of improved survival of the transplanted organ. A swine kidney model of delayed graft function found that intraoperative CO treatment of the recipient was sufficient to re-

Table 2. Inhaled Carbon Monoxide Versus Nitric Oxide

Physical Property	Carbon Monoxide	Nitric Oxide
Molecular weight	28.01	30.06
Boiling point (°C)	–191.5	–151.8
Melting point (°C)	–205	–163.6
Solubility in water (mg/L)	30	67
Density (kg/m^3 vapor)	788.6	3.027
Specific gravity (g/L)	1.250	1.037
Reactivity	Inert, except binds to hemoproteins	Highly reactive, very short half life
Metabolism	None	Rapid conversion to nitrite/nitrate
Preclinical efficacy		
Pulmonary hypertension	250 ppm for 1 h/d has long-term efficacy.[37]	20–80 ppm has rapid efficacy.[59]
Sepsis/acute respiratory distress syndrome	250 ppm for 4 h promotes bacteria clearance and decreases inflammation.[52]	0.2–20 ppm for 4 h decreases pulmonary hypertension but has no effect on inflammation.[58]
Myocardial ischemia	250–1,000 ppm for 24 h prevents ischemia-reperfusion injury.[56]	80 ppm for 60 min prevents ischemia-reperfusion injury.[57]

duce delayed graft function (unpublished data). With most of the preclinical research completed or ongoing, including large-animal testing, the clinical use of CO is now justified and has begun in transplant centers across the United States. In patients with chronic obstructive pulmonary disease, inhaled CO reduced lung inflammation.[60]

On the negative side is the vast literature, spanning decades, on the toxic effects of CO—almost all related to formation of COHb and interference with oxidative phosphorylation. However, those effects were from CO concentrations well above those used for testing and treatment. On the positive side, CO appears to be relatively non-toxic to cells: cells maintained in vitro in an atmosphere of 100,000 ppm CO for several days showed no evidence of damage or loss of function. Further, it may be that we can obtain CO's benefits with a lower CO concentration and a shorter CO administration time than has been used in most studies to date. Intermittent dosing is also effective and reduces the COHb level. The CO toxicity reported in many studies was related to long-term, high-concentration CO exposure. To date, in vivo CO therapy has been in the range 250–400 ppm in most studies, and has shown no adverse events. Significant, although not maximum, anti-inflammatory effects can be obtained in vivo with 10 ppm,[25] so a lower CO concentration may suffice and needs to be explored. The duration of CO administration that achieved a benefit has differed substantially (range in animal studies of 2 hours to several weeks). In an animal model of angioplasty balloon injury, CO at 250 ppm for one hour suppressed the development of re-stenosis. Shorter durations and lower concentrations have not been evaluated. Such CO treatment results in a COHb level of about 12–15%, which would be regarded as safe by most, particularly for a short duration. For comparison, smokers achieve COHb within that range. In vitro CO treatment of pancreatic islets for 30 min had a profound anti-apoptotic effect, and CO exposure for 2 hours (shorter times were not tested) very significantly improved islet function after transplantation. Further studies are needed to ascertain the best CO concentrations, durations, and treatment frequencies for various conditions.

Summary

CO administered to rodents and large animals at concentrations 10–20 fold below the lethal concentration have had remarkable therapeutic value. In almost all cases the animal was treated before challenge (eg, lipopolysaccharide, transplantation of a heart or islets of the pancreas, angioplasty), yet important and powerful therapeutic effects also occurred in the liver and lung.[18,37] In vitro and in vivo studies suggest that CO acts primarily to suppress inflammation and apoptosis. The early observations of CO's effects on monocytes were particularly instructive because they showed CO's ability to regulate the cellular response to stress. CO pretreatment of the cells suppresses the pro-inflammatory response and increases the anti-inflammatory cytokine IL-10. CO blocks vascular smooth-muscle cells, enhances endothelial-cell proliferation, and helps restore vascular homeostasis. CO increases T regulatory cells that protect transplanted organs and decreases T effector cells that drive graft rejection. CO markedly impacts the response to stress and trauma stimulation in various conditions.

We predict CO will have a benefit in numerous, if not all, situations in which inflammation plays a damaging role. Pretreatment and therapeutic treatment with CO may become a part of our therapeutic armamentarium. Whether the CO dose and duration of administration will be acceptable for patients remains a matter of speculation. CO's physiologic role as an endogenous defense mechanism au-

gurs well for its exogenous administration in appropriate circumstances.

Did organisms evolve the heme oxygenase-1 system simply to deal with heme? Or is it that organisms evolved when the atmosphere had more NO and CO and thus adapted to them? A tenet of evolutionary biology is that a trait remains in a population if it provides a survival advantage. The organisms in this case not only survived environments that contained CO, they developed an elegant system by which to produce it continuously within their cells and tissues, particularly during periods of stress. Perhaps as the atmosphere was depleted of CO in the early years of life on this planet, organisms that required CO for survival developed mechanisms to meet their CO needs. CO, by the mechanisms and arguments detailed above, was and continues to be simply a homeostatic mediator required for survival. A high concentration of inhaled CO is certainly toxic, as Edgar Allen Poe, who succumbed to "CO poisoning," attested, but a low concentration can be beneficial in various pathologies.

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