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Forum Review

Carbon Monoxide: Innovative Anti-inflammatory Properties of an Age-Old Gas Molecule

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ABSTRACT

Observations of the effects of carbon monoxide (CO) on mammalian systems have been known for thousands of years. To be sure, CO is deadly under certain conditions and concentrations, but perhaps as the data presented here will make clear, it also possesses other diverse functional and immunomodulatory properties. This review, together with the other reviews in this issue, will detail that over the past three decades, fundamental functional role(s) for this gas molecule are beginning to emerge. This review outlines that at low concentrations, exogenously administered CO is a molecule involved in the regulation of the inflammatory response in a variety of disease models. CO has been shown to modulate such cellular functions as cytokine production, cell proliferation and apoptosis, protecting the lungs and hearts of rodents from such stressors as endotoxin, ischemia/reperfusion injury, cardiac xenograft rejection, and asthma. Although the mechanism by which this simple diatomic gas provides this protection remains obscure, the conclusions are the same: CO at low concentrations, concentrations that are well below those that would otherwise create toxic effects, is proving beneficial in models of acute injury. CO, akin to nitric oxide, is proving to be an extraordinary signaling molecule generated by the cell that is vital in the regulation of cellular homeostasis. Antioxid. Redox Signal. 4, 309–319.

INTRODUCTION

THE FIRST DETECTION OF A COMBUSTIBLE GAS in the blood occurred in 1894 by Grehant (22). This gas was supposed by de Saint Martin and Nicloux to be carbon monoxide (CO) (14, 55). Nicloux and others attempted to show that CO was formed in the body and first asserted that the origins of CO in the body arose via carbohydrate metabolism (56). Other investigators are finding levels of this gaseous molecule present in the central nervous system. The proof should have been on the determination of the CO in inspired and expired air simultaneously combined with the measurement of carboxyhemoglobin (COHb) in the blood. Unfortunately, these measurements were not possible with the methods available before 1940. With the onset of the industrial revolution and the invention of the combustion engine, it became urgent to work out methods to measure and determine COHb because it was rapidly discerned that CO levels in the atmosphere were dangerous. It was discovered very early on that the COHb levels in the blood reflected the CO concentration in the alveolar air determined using rebreathing techniques. It was not until 1949 that Sjorstrand, and later Coburn, discovered that endogenously produced CO arose from the degradation of hemoglobin released from senescing erythrocytes (12, 13, 73). CO measurements via COHb or by rebreathing techniques in the 1970s, were used by clinicians to determine the life span of erythrocytes and the rate of heme turnover in their patients. Greater than 75% of CO produced in humans arises from erythrocyte turnover generated as a by-product of heme metabolism.

In 1969, the source of endogenous CO was discovered. Tenhunen *et al.* described and characterized heme oxygenase (HO) as the enzyme responsible for breaking down heme in the body, demonstrating that heme catalysis resulted in the subsequent release of CO and free iron as by-products (79). This enzymatic cleavage also resulted in the production of

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biliverdin, which was subsequently found to be rapidly converted to bilirubin via biliverdin reductase. Certainly the products of HO activity have been observed for decades, if not centuries, because unlike most biochemical functions, heme catalysis is color-coded and readily observable. For instance, a hematoma arising from a blow to the body is initially black, the color of heme. Over a number of days, the color changes to green, the color of biliverdin, and finally to yellow, the color of bilirubin. Use of these visual observations can be dated back to the time of Hippocrates, when patients with liver disease presented as hyperbilirubinemic, and were recognized because their skin was yellow in color. The generation of CO would also generate a pink skin hue as it bound tightly to hemoglobin.

Similarly, the prsence of CO has also been observable well before there were scientific "instruments" by which to test the atmosphere. With the advent of fire, it is not hard to imagine that primitive man, taking refuge in caves, brought fire inside and learned rapidly that when some of his co-dwellers did not survive the night, they should next time be sure to extinguish the flames lest they not awaken to greet the following day. Perhaps they recognized that if they began to turn bright red (from CO's binding to hemoglobin), it was time to get outside for some fresh air. And thus the first CO monitor and/or spectrophotometric assay was created [adapted from Penney (65)].

This review will address many aspects regarding recent findings demonstrating antiinflammatory aspects of CO in a number of inflammatory models in rodents that implicate CO as a functional biological mediator in numerous organ and tissue systems. Clinical investigators have also begun to use CO analysis of the exhaled breath as a biomarker related to increase in levels of oxidative stress. It has clearly been demonstrated that CO increases in the breath in correlation with the severity of an inflammatory state, such as asthma and diabetes, and in critically ill intensive care patients. In an effort to determine if this increase in exhaled CO is indicative of an attempt by the cells of a given tissue and organ system to reinstate homeostasis, or simply a waste product to be eliminated, a series of investigations have been completed to examine a possible role for CO in modulating the inflammatory response. By using both cellular and animal models of inflammation, including sepsis, hyperoxia, asthma, ischemia/reperfusion, and xenotransplantation, exogenous CO was administered at low concentrations that were well tolerated. Markers of injury and inflammation were measured to assess a functional role for CO in the cellular and molecular responses and found to be markedly attenuated when the animals were present in an atmosphere of CO (7, 8, 60, 61, 69).

It has been hypothesized that CO generated from the increased activity of HO-1 is important in the modulation of vascular smooth muscle tone (19, 50). In fact, it has been suggested that the HO/CO pathway is critical in the regulation of blood pressure under stress conditions (51, 67). The functional role of the HO-1/CO axis has also been evidenced in other tissue and organ systems, including the liver where it is involved in the regulation of bile canalicular contractility in hepatocytes, uterine contractions in the pregnant rat, and leukocyte adhesion in venular endothelial cells. Other systems where the HO/CO pathway constitutes a regulatory role is in the pancreatic islets of Langerhans where CO has been shown to stimulate glucagon and insulin release (30, 31, 71). The interest in the functional role of this metabolite of heme catalysis is continuing to expand with ever-increasing data suggesting that, like nitric oxide (NO), CO possesses a cellular and molecular function and should not be simply classified as a toxic waste product.

HO has been well established as a stress response gene induced by a diverse array of non–heme-induced cellular stresses, including lipopolysaccharide (LPS), heat shock, hyperoxia, heavy metals, and UV irradiation. It was hypothesized, based on the premise that HO-1 was induced by such stress responses, that CO as a by-product would simultaneously be generated and could be quantitated. Because CO does not undergo further metabolism in the body, its only source of excretion is via exhalation as a gas. The Sasaki and Barnes groups have plainly shown that not only is CO present and measurable in the exhaled air of a normal subject, levels of this gas increase correlatively with the severity of numerous inflammatory disease states (1, 35, 49, 70). In inflammatory conditions such as asthma, medical intervention such as the use of inhaled glucocorticoids was shown to reduce bronchoconstriction with subsequent correlative decreases in the amount of exhaled CO measured in the breath (86). Exhaled CO may also provide complementary data for assessment of asthma control in children, where exhaled CO has also been shown to increase during an episode of acute asthma (81). Patients with upper respiratory infections have increased levels of CO in the exhaled breath, suggesting that perhaps HO-1 possesses an antiviral effect in the airway (87). Because of the noninvasiveness of exhaled breath collection, it is being proposed that increased CO levels may be an early and noninvasive marker of airway and systemic inflammation and disease (34, 35, 64). It has been established that CO modulates histamine release from mast cells in response to allergen, as well as the immunological activation of human basophils (47, 48, 53). In cases of cystic fibrosis, CO measurements may be clinically useful in the management and monitoring of oxidation and inflammatory mediated lung injury (63). It has been demonstrated that the generation of CO directly affects cardiac output, vascular resistance, and subsequently blood pressure (37). It has also become evident that CO is involved in pathological conditions, including ischemia, endotoxic shock/sepsis, and excitotoxicity, acting as a cytoprotective agent (15, 46).

In light of the ever-increasing reports showing that CO is responsible for numerous cellular and organ functions, there are certainly more delineating the toxicity and lethality of CO exposure (33, 40, 52, 80). Over the past 100 years, CO has demonstrated its lethality again and again. In rigorous examinations in scientific laboratories over the span of a century, its deadly effects have been repeatedly and compellingly demonstrated (12). Yet there are also numerous inconsistencies throughout these research studies. In an extensive review of the literature regarding CO effects on behavior and brain injury, Benignus concludes:

> Barring the advent of a very well documented and replicable demonstration of COHb with behavioral effects, a physiological understanding of the mechanism of CO effects appears to be the only hope of resolution of the uncertainty of the effects of CO exposure. Too little is known about the effects on average, normal,

young, healthy, at rest subject and much less about cases that do not fit into categories of toxicity and yet are exposed to CO in their normal environment (3).

He goes on to state:

There is reason to believe that research done in the climate of environmental activism during which much of the CO research was performed, an experiment that produced no significant effect when others had already reported such would not have been published . . . if this were the case and many findings were never published because of no statistical effects, a review of the literature could easily take on a different appearance (3).

From these conclusions, it could be possible that perhaps CO has been labeled as toxic regardless of concentration and exposure time. Ironically, although CO has been extensively studied for over a century, the literature citings are a mere fraction of what they might otherwise be, and it is clearly possible that critical data supporting other "nontoxic" effects of CO may never be known.

And so, despite its demonstrable life-ending effects, CO in more recent studies is emerging as a functional molecule in an innumerable number of life-sustaining organ and cellular activities as described above, dependable, ubiquitous, and inexplicable, a role that has both bemused and dismayed scientists. In the late 19th century, it was Haldane who used CO to discover that hemoglobin was the blood protein that carried and delivered oxygen to the tissues (24). Perhaps it is this enigmatic nature of CO that has prompted some investigators, in recent research efforts, to begin to pay more attention to CO, if for no other reason than to explain the mechanisms of its toxicity and perhaps to turn its aberrant behavior into something comprehensible. CO insists on being attended to—and understood and appreciated. But that explication requires a context, one that provides the conceptual framework within which the mechanisms of CO's beneficial effects may be most precisely described. Since the late 19th century, scientists have known that CO was present in the blood of normal man and animals. Claude Bernard described CO's affinity for hemoglobin; this initial research became grounding for an essentially universal scientific tenet assigning CO to the category of poisons and toxins, which later included arsenic, nicotine, and opium (4, 5).

With the discovery of the enzyme HO, however, the sole origin of endogenously generated CO was identified. Although these revelations have indeed provoked much research, the mechanism by which HO-1 provides protection against injury remains unanswered. In an effort to address this question, it was hypothesized that CO was subserving a unique functional role in the mechanisms that generate the effects seen with induction of HO-1 itself. The investigation of CO's functions in acute lung pathology, including both endogenous and exogenous determinants, is currently being investigated in models of sepsis, asthma, xenotransplantation, ischemia/reperfusion, and hyperoxia, all well established models of acute inflammation and injury.

Additionally, studies have demonstrated that, far from being "waste" by-products of heme metabolism, bilirubin, ferritin, and CO are perhaps products serving a function. In fact, bilirubin, ferritin, and CO are all now accepted as being able to exercise other critical physiologic functions.

There is strong evidence in the literature to support the emerging paradigm that HO-1 plays a vital role in maintaining cellular and tissue homeostasis in various *in vitro* and *in vivo* models of oxidant-induced injury. Despite these convincing data indicating the protective role of HO-1 in oxidant stress, the precise mechanism(s) by which HO-1 serves as a potent cytoprotectant remains elusive. Although many investigators have speculated as described above that the catalytic by-products of heme catabolism, including bilirubin, and ferritin, generated by released iron, may mediate the protective function of HO-1, the evidence remains unresolved (2, 43, 76). CO, the remaining major end product of heme catalysis by HO, has been implicated as a chemical messenger in neuronal transmission and as a potent vasodilator. However, little is known regarding its functional role in potential cytoprotection against oxidative stress.

Recent reports by a number of investigators are beginning to show a functional role for CO in models of inflammation. Ndisang *et al.* showed that CO can modulate the guinea pig mast cell release of histamine, supporting the idea that increases in exhaled CO, such as during an asthmatic response, are perhaps an effort by the lung to immunomodulate histamine release and subsequent bronchoconstriction (53). In rodent models of hemorrhagic shock and sepsis, CO, albeit via indirect measurements, has been shown to provide protection against hepatic dysfunction (62).

To address the hypothesis that the by-product CO was potentially the mechanism of cytoprotection observed with HO-1, *in vivo* and *in vitro* exposure systems were developed to administer directly low concentrations of CO exogenously. Although it is difficult to correlate exogenously administered concentrations with that of endogenously generated CO from HO-1, the correlation between cytoprotection with HO-1 versus CO is striking. In other words, in models where HO-1 has been shown to be protective, low concentrations of CO administered exogenously show similar, if not identical, protection.

Subsequent studies unraveled an important function of exogenously administered CO in mammalian systems. This review will serve to provide evidence that CO, when administered exogenously at low concentrations, can exert potent antiinflammatory effects in both *in vivo* and *in vitro* models of LPS-induced inflammation, hyperoxic lung injury in rodents, allergen-induced inflammation, apoptosis, ischemia/reperfusion injury, and cardiac xenotransplantation that otherwise mimic effects seen with HO-1. The concentration of CO (<0.03%) used in these studies is one twentieth of the lethal dose of CO and significantly less than the concentration administered to humans (0.3%), albeit the exposures are continuous and ranged from 1 h to 4 weeks of exposure. A novel and intriguing aspect of these observations is that CO, even at low concentrations, selectively modulated the proinflammatory/anti-inflammatory cascade of cytokines and mediators.

ENDOTOXIC SHOCK

CO exposure selectively inhibited in a concentration-dependent fashion LPS-induced tumor necrosis factor-α (TNF-α) production and augmented interleukin (IL)-10 production measured in the same cells and mice. Surprisingly,

these biological effects of CO did not involve the guanylate cyclase/cyclic GMP (cGMP) pathway, which has been implicated in the biological effects of CO in the neuronal and vascular systems akin to NO (50, 81). Rather, the anti-inflammatory effects of CO appear to involve the mitogen-activated protein (MAP) kinase signaling pathway, in particular the MKK3/p38 MAP kinase pathway (see Fig. 1).

The precise biochemical mechanism by which CO can modulate the MAP kinases is not clear at this time. Based on current knowledge that none of the upstream kinases in the MAP kinase pathway contain a heme moiety (a common target of CO), and the current observation that CO mediates the antiinflammatory effects via a cGMP-independent pathway, it is plausible that CO could be modulating the upstream kinases through an unknown or unidentified intermediate molecule. This speculation is supported by observations that the protective effects of CO require new protein synthesis in TNF-α-induced apoptosis (unpublished observations). Our findings demonstrating the CO-induced augmentation of IL-10 production and induction of the p38 MAP kinase are consistent with previous reports that the p38 MAP kinase is critical in regulating IL-10 production (17).

There have been a number of reports showing that inhibition of TNF-α independent of augmented IL-10 release can occur following increases in cyclic AMP, decreased intracellular Ca^{2+}, or β-adrenoceptor stimulation (29, 54, 78). These studies suggest that CO inhibits LPS-induced TNF-α production via an IL-10 independent pathway, by affecting posttranscriptional synthesis of TNF-α. The data raised the immediate question as to how there could be augmentation in the activation of p38—a potent intermediate signal transducer involved in the production of TNF-α following LPS administration (27)—while simultaneously showing decreased production of TNF-α by enzyme-linked immunosorbent assay. To address this fundamental question, we looked at TNF-α mRNA and protein levels. Results found that CO was regulating LPS-induced TNF-α production posttranscriptionally. Western blot analysis of both cellular protein and secreted protein in the media indicated decreased amounts of TNF-α protein in the presence of normal transcription of the TNF-α gene as seen by Northern blot analysis. Conclusions from these studies are that CO is acting directly on posttranscriptional processes, or a more intriguing possibility is that CO is somehow acting indirectly via the increased activation of p38 in the posttranscriptional regulation of this cytokine. A third possibility is that there exists a delicate balance in the actions of p38; subtle cellular activation (as with LPS alone) stimulates TNF-α synthesis, whereas a hyperstimulation (as seen in the CO/LPS treated-cells) somehow becomes inhibitory, resulting in posttranscriptional regulation of the TNF-α protein. Taken together, it appears that CO is exerting posttranscriptional or secretional regulation of LPS-induced TNF-α expression. The regulation of LPS-induced TNF-α expression by CO exemplifies the accumulating evidence in the literature highlighting the complexity of the molecular regulation of TNF-α (25). Complex transcriptional and translational control of TNF-α expression has also been reported in similar studies (6, 20, 26). Studies of mechanism and localization are included as studies to investigate in the future. This study revealed a novel physiological function of the gaseous molecule CO in a model of LPS-induced inflammation.

CARDIAC XENOTRANSPLANTATION IN RODENTS

The ability of HO-1 and CO to suppress proinflammatory responses in macrophage activation may also contribute to the suppression of xenograft rejection, as demonstrated in a

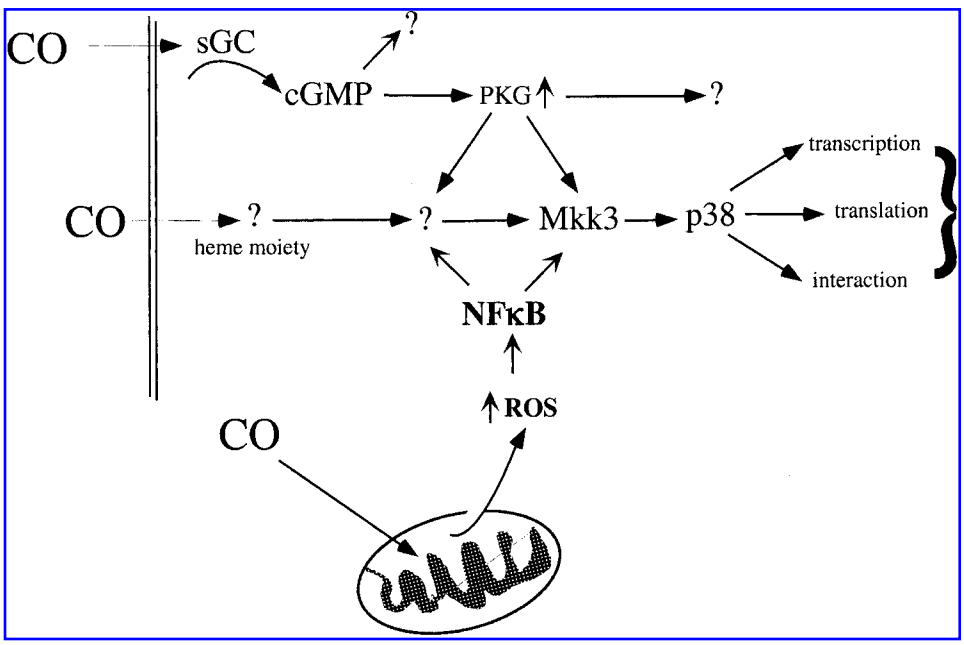

FIG. 1. Potential pathways for anti-inflammatory effects of CO. cGMP, cyclic GMP; MKK3, MAP kinase kinase 3; NFκB, nuclear factor-κB; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase.

recent study by Sato *et al.* (69). In this study, the authors demonstrate the beneficial effects of CO in avoiding rejection of a cardiac xenograft, which was similar to their previously published work showing that HO-1 can provide protection against acute rejection (74). Exposure to 400 ppm for only 2 days following transplant prevented rejection for up to 50 days, whereas air-treated control hearts were rejected in 5–7 days. CO likely mediates these protective effects, in part by modulating platelet function, by causing vasodilatation, and/or by inhibiting the proinflammatory responses of monocytes as described above. Activated monocytes are present in rejecting xenografts, and there are data suggesting that they may well play a role in rejection (41, 42). In unpublished observations by Sato *et al.,* the time of exposure required to observe protective effects of CO was only 2 days. In other words, 2 days of exposure was sufficient to protect the graft over the following weeks as the animals subsisted in room air. These data begin to address the functional mechanisms by which CO exerts its effects and are leading us toward the identification of potential cellular and molecular targets. Moreover, the time requirements for exposure to CO appear to be shrinking and thus minimalizing the toxicity issues associated with this gas molecule. Additionally, the anti-inflammatory effects of CO explain how exogenous administration of HO-1 by gene transfer or CO inhalation protected against hyperoxia-induced lung injury and inflammation as described below (59, 60). Despite the long-standing accepted paradigm that CO in our environment is toxic or even lethal, the data presented here, as well as the Sato data, make clear that CO can serve as a potent anti-inflammatory molecule. It is tempting to speculate that CO might be used therapeutically by (over)expressing HO-1, by inducing HO-1 by genetic engineering, or by local CO administration in areas of inflammation. CO might therefore be used therapeutically not only in sepsis and xenotransplantation, but in other inflammatory conditions.

ALLERGEN-INDUCED INFLAMMATION IN MICE

CO concentrations are increased in the exhaled breath of asthmatic patients (49). These levels subside following administration of anti-inflammatory nonsteroidal agents. Barnes and colleagues clearly demonstrate this phenomenon (34). The fundamental question asked by investigators was this: why would this "toxic" gas molecule be increased in the inflammatory state? Perhaps it serves as a defense mechanism in the lungs to counteract the bronchoconstriction, mediator release, and mucous production illicited by the allergen. To address this possibility, Chapman *et al.* examined the role of CO administrated to allergen-challenged mice (8). They found that CO at similar concentrations (0.025%; 250 ppm) used in the above models was able to block eosinophil influx and reduce the production of IL-5. Furthermore, the effects of CO in this model were selective, having no effect on other allergen-induced cytokine production such as eotaxin and IL-12. CO was also able to reduce increases in airway resistance in response to methacholine challenge in allergen-sensitized mice (unpublished observations). These data provide further credence to the notion that CO has a functional role in models of inflammation.

HYPEROXIA-INDUCED LUNG INJURY IN RATS

In these studies, it was hypothesized that a possible mechanism subserving HO-1-induced protection is through one of its by-products, CO. By using low concentrations of CO previously shown to be nontoxic (77), rats were exposed to lethal hyperoxia in the presence of low amounts of CO (similar to that used in the endotoxin and transplantation studies) to determine if protection was similar to that observed with HO-1 when administered intratracheally expressed in an adenovirus (59). Exogenous administration of low CO concentrations did indeed provide protection against oxidative stress in models of inflammation. It should be noted that the concentration of CO used in these studies, in the order of 50–500 ppm, corresponds to 0.005–0.05% CO. As differences in arterial pO_2 levels have been implicated in other models of tolerance to hyperoxia (9), the pO_2 content was measured in the experimental groups. No difference was observed between rats exposed to hyperoxia and rats exposed to hyperoxia in the presence of a low concentration of CO.

The precise mechanism(s) by which CO mediates protection in this model is not clear. The observation that CO attenuated hyperoxia-induced influx of neutrophils into the airways is interesting in that it is well established that neutrophil influx in the bronchoalveolar lavage is of paramount importance in the development of hyperoxia-induced lung injury in *in vivo* models and in human patients with adult respiratory distress syndrome (ARDS) (9, 11, 75). Moreover, identical experiments were performed using a second model of oxidant-induced lung injury and inflammation. LPS administered to rats induces profound neutrophil influx into the airways; however, this neutrophil influx was significantly inhibited in the lungs of rats given LPS and exposed to CO. Willis *et al.* recently reported that HO-1 modulates the inflammatory response *in vivo,* and a recent report by Soares *et al.* also showed that HO-1 modulates the inflammatory response and apoptosis *in vivo* in a model of cardiac xenotransplantation (74, 84). These findings support a mechanism to explain the anti-inflammatory properties observed with HO-1, which have been demonstrated in our laboratories and others (57, 59, 74, 84). However, in this study the use of exogenous CO did not directly prove that it is mimicking endogenous CO, and therefore cannot be compared meaningfully with CO produced during heme metabolism by endogenous HO-1. De-signing experiment(s) to show that endogenous CO from HO-1 actually mediates the protective effect of HO-1 *in vivo* is technically very difficult, perhaps not feasible because current available technology to measure CO *in vivo* (COHb) is not sensitive enough to detect increased CO levels after HO-1 induction. However, observations that exogenous CO could completely ablate or reverse the increased pleural effusion in rats treated concomitantly with a selective inhibitor of HO-1, tin protoporphyrin, suggested that exogenous CO could provide cytoprotective effects even in conditions where endogenous HO activity is completely inhibited. Nevertheless, the marked protection against hyperoxia-induced lung injury by exogenous CO at low concentrations observed here provides another suitable *in vivo* model to investigate further the functional physiologic role of CO in oxidant-induced lung injury.

Furthermore, oxygen exposure results in an increase in programmed cell death or apoptosis in the lung as demonstrated by Mantell (45). Exposure to hyperoxia in the presence of CO showed an inhibition in apoptosis, which may represent an additional mechanism by which CO provides protection against oxidant-induced injury and inflammation. Although the precise physiological function of apoptosis in the lung has yet to be established, emerging data strongly suggest that the total lung apoptotic index can serve as a useful marker of lung injury in response to oxidative stress such as hyperoxia (38, 58). Studies by Soares and Petrache also showed that HO-1 and CO may function as an antiapoptotic molecule in *in vitro* models of TNF-α-induced apoptosis (7).

The known observations that CO can avidly bind to heme moieties such as guanylyl cyclase and thereby increase cGMP, similar to the action of NO, may provide clues for future studies (39) (see Fig. 1). However, CO could also act via a pathway not involving cGMP, as has recently been described in other *in vitro* models examining HO-1 regulation by NO (25).

Presented here is evidence demonstrating that exogenous CO at low concentrations provides protection against hyperoxia-induced lung injury. The concentrations of CO needed to achieve these dramatic protective effects are far less than the known toxic concentrations, and even lower than the concentrations used in pulmonary function tests in humans. Although the precise mechanism by which CO exerts its protective effects has yet to be established, the inhibition of neutrophil inflammation and attenuation of total lung apoptotic index represent potential avenues to investigate in the future. This work continues to raise the intriguing possibility for the potential therapeutic use of low concentrations of CO in clinical settings not only in lung disorders such as ARDS and sepsis but also in a variety of other inflammatory disease states.

In a second model of acute lung injury and inflammation in mice, a low concentration of CO when mixed with >98% O_2 protects the lungs of mice when compared with those exposed to O_2 alone. Of obvious concern is the toxicity associated with this gas molecule. The concentration (<0.03%) of CO used, however, is one twentieth the lethal dose, and when administered at this concentration mixed with air produced no untoward effects on the mice. Stupfel and Bouley have shown that mice exposed to 500 ppm of CO for up to 2 years showed no untoward effects on multiple physiological or biochemical parameters (77). This concentration is 10-fold lower than that used in the measurement of lung diffusing capacity (D_LCO), a standard pulmonary function test used in humans. Here, in a clinically relevant model of ARDS, we show that not only was CO cytoprotective as it reduced markers of lung injury, but also and more importantly at this low concentration selective inhibition of hyperoxia-induced TNF-α, IL-1β, and IL-6 occurred at the tissue level. At a more functional level, survival was extended in those animals exposed to O_2 and CO.

There is increasing evidence that the MAP kinases are strong regulators of proinflammatory cytokines, in particular the p38 MAP kinase pathway (68). In the endotoxin model, the MKK3/p38 pathway proved to be an important variable in enabling CO to modulate cytokine production (see Fig. 1). The data collected in this study showed that CO prevented the increased transcription of TNF-α, IL-1β, and IL-6 in response to hyperoxia exposure requiring the MKK3/p38 pathway. It is therefore a formal possibility that modulation of these cytokines is perhaps the mechanism subserving the ability of CO to extend the survival of mice exposed to hyperoxia. Numerous studies have shown that if TNF-α and IL-1β are inhibited, the inflammatory response is reduced (23, 66). In fact, clinical trials are under way to treat sepsis and rheumatoid arthritis by interfering with TNF-α production (44). We took this approach a step further and showed that the ability of CO to modulate these cytokines involves the MAP kinase signaling cascades, in particular the MKK3/p38 MAP kinase pathway.

The precise molecular target(s) for CO is not clear at this juncture, but based on our current knowledge that it preferentially binds heme moieties present in numerous proteins we gain some insight into potential cellular target(s). In this model, CO modulates proinflammatory cytokine production through the MAP kinase MKK3/p38, so a plausible cellular target is an upstream intermediate involved in the modulation of these pathways. Obvious targets in the lung would include guanylyl cyclase or NADPH oxidase, both heme-containing enzymes that lie upstream of the p38 MAP kinase. Inhibition of NADPH oxidase has been shown to protect against oxidant-induced injury (36). In unpublished observations, CO inhibits the production of free radicals generated by NADPH oxidase. CO in this instance is most likely interfering with NADPH oxidase-induced superoxide production by binding to the heme moiety contained in the p47 subunit of the complex and thereby preventing the increased reactive oxygen species (ROS) generated in the above models, ROS that have been shown to initiate cytokine expression. Furthermore, the specificity of CO fails to modulate significantly a number of other hyperoxia-induced cytokines such as the transforming growth factor-β family members. Although some of these are also regulated by the MAP kinases, these cytokines are also dependent on other transcriptional activators, including the Stat and Smad family members (10, 32, 83).

These studies reveal a novel physiologic function of the gaseous molecule CO in a murine model of acute respiratory distress. Despite the long-standing and widely accepted paradigm that CO is toxic and even lethal, these data suggest otherwise. First, we demonstrate here that in a model of acute lung injury, CO at low concentrations is anti-inflammatory. Second, we show that it does so in part through inhibition of proinflammatory cytokines. Third, we demonstrate that it somehow modulates these cytokines via the MKK3/p38 MAP kinase axis.

It is tempting to speculate that CO might be used therapeutically by overexpressing HO-1, by inducing it via genetic engineering, or by local administration of a low concentration of CO in areas of inflammation. CO might prove therapeutic in not only acute respiratory distress syndrome, but also other inflammatory conditions. CO at low concentrations provides cytoprotection and acts as an anti-inflammatory agent. The evidence in support of CO as an anti-inflammatory agent continues in the mouse hyperoxia model with functional potential. TNF-α and IL-1β expression is once again inhibited, and suggests that the improved survival with decreased lung in-

jury is reflective of this phenomenon. Furthermore, survival in the Mkk3 null mice could not be extended with CO, nor did CO have any effect on the same injury markers that were otherwise inhibited in the wild-type mice exposed to O_2 in the presence of CO. Interestingly, in whole tissue, message levels of the proinflammatory cytokines were decreased by CO, unlike that seen in the RAW 264.7 macrophages, where inhibition occurred posttranscriptionally. This might point to cell-type specificity or cell versus tissue effects or even model differences where the milieu is different. Future studies will examine these phenomena.

SUMMARY

Although heme catalysis liberates three by-products, the only gaseous by-product, CO, was examined as a possible mechanism by which HO-1 provides beneficial cytoprotective properties. In identical models in which HO-1 showed protective effects, CO produced the same effects. What has been observed and described herein is that CO was able not only to mimic these protective effects, but also to do so at very low concentrations. At the same time, intracellular targets (MAP kinase) were identified that could be responsible for the cytoprotective response.

These studies also investigated derivative issues related to possible mechanisms by which CO was able to exercise its cytoprotective effects. Obvious research choices included downstream gene products including cytokines, which play important roles in the sequelae of tissue, and organ injury that occurred in the models of shock and hyperoxic lung injury used in these studies. On the other hand, upstream molecular targets involved in signal transduction and gene regulation, and responsible for the observed downstream effects, were examined to understand better the potential mechanism(s) by which CO behaved. To be sure, there are numerous pathways to consider. Certainly, the effects of CO in any given system could also vary. Any heme-containing protein of the hundreds that exist could potentially be a target or at least participate indirectly in the cellular response to any given stress.

Fortunately, the models of inflammation described in this review represent clinically relevant research models. Each provides pertinent insights that advance our understanding of clinical disease because although they diverge in terms of their relative cytotoxic etiologies and tissue injury dynamics, they are closely intertwined at the intracellular level. All generate enormous amounts of ROS that represent the fundamental underlying mechanisms by which they generate pathology. Ironically, a large percentage of patients with sepsis end up with ARDS, and the only common treatment for these patients is high levels of inspired oxygen. Despite the necessity of such a therapy to maintain normal arterial oxygen tensions for delivery to distal tissues, these high levels of inspired oxygen exacerbate the oxidative burden on lung tissue. Understanding the mechanisms of injury and the role of the stress response, including that of HO-1, will better enable the design of treatment regimens by which to treat both sepsis and ARDS, without resorting to the counterproductive oxygenation therapies. In animal studies, it is apparent that HO-1 is involved in the defense against these syndromes and is therefore a potential therapeutic target. The unanswered question is how and why HO-1, an enzyme simply designed to break down heme, and CO, an inert gas, are involved in these complex disease states.

Although the gaseous molecule CO has been studied for >100 years, those investigations have almost universally focused on its toxic features and effects, and CO remains, quite understandably, a molecule to be avoided. Despite those repeated indictments of CO as an exceptionlessly lethal agent, studies are under way perhaps suggesting otherwise. Put simply, CO possesses potent beneficial properties based on the findings presented here, properties that offer potentially enormous benefits in the treatments of sepsis, ARDS, asthma, and transplantation, disease states for which no substantial improvements in treatment have occurred over the past 30 years. The mechanisms of CO's toxicity are in theory well understood—it binds to hemoglobin and causes life-threatening hypoxia, resulting in brain damage and acute cardiovascular collapse. It also binds to cytochrome oxidase and prevents life-sustaining generation of ATP. Among these almost monolithic truths about CO, however, dwell uncertainties. Why is it that when blood is saturated with CO and administered to animals intravenously, they suffer no adverse effects despite the fact that COHb levels rise to magnitudes that would otherwise create serious, if not lethal, consequences (16, 21)? Why is it that there are reports of CO regulating carotid body signaling and neurotransmission? Why is it that HO-2 exists, a constitutively expressed isoform present in the body relentlessly producing CO, releasing it into the bloodstream to regulate vasomotor tone and/or into the brain to support long-term potentiation or memory (89)? Certainly, these would seem important functions for survival. But in the end, there is the most fundamental question: if CO is so toxic, why is it constantly generated in the body and, most paradoxical of all, increased in inflammatory states as measured in exhaled breath, creating a hypoxic environment limiting O_2 delivery to tissues and interfering with ATP synthesis?

Of course, CO may simply be a by-product that, like others, is waste that must be eliminated. But it may indeed possess a functional role in the maintenance of normal cellular homeostasis. It does seem coincidental that in mice and humans deficient in HO-1, there is profound inflammation that supercedes all other abnormalities (85). This inflammation does not appear to occur as a result of infection or otherwise exogenously determined sources; rather the inflammation exists from birth ultimately leading to death (of those who are born). In light of these observations, CO via HO-1 based on data presented here would appear to be the antidote, as it were, for this dysfunctional inflammatory, response, as the data in this dissertation strongly suggest. When HO-1 and therefore CO are present, perhaps these molecules lead to homeostatic regulation of the inflammatory response. HO-1 is induced via a variety of inflammatory stimuli, some no more stressful than the normal oxidative stress present in all O_2-breathing organisms. It is believed that oxidative stress caused by reactive radicals is an essential, if not exclusive, determinant of the aging process, and that it is involved in most, if not all, disease states (16a).

Perhaps CO acts as an antioxidant. Weinstock first hypothesized that CO could react with the hydroxyl radical to form

CO_2 and H^+ and thereby eliminate a potent free radical. Similarly, many of the ROS originate via the mitochondrial cytochrome oxidases, NADPH oxidases, xanthine oxidases, and cyclooxygenases, to name a few, present in most cells. Each of these enzymes contains a heme moiety, reactive with both O_2 and certainly CO. Hence, it is certainly a possibility that CO is acting as a potent antioxidant interfering with the generation of ROS. The potent antioxidant enzyme catalase also contains a heme moiety, and perhaps the binding of CO results in an increase in its activity level.

Future studies will examine this issue and, if confirmed, help explain most, if not all, of the cellular and tissue responses. Blockade of radicals has been shown to prevent cytokine release, inhibit apoptosis, and certainly prevent oxygen-mediated cellular injury like lipid peroxidation that leads, of course, to changes in permeability and increases in leukocyte trafficking.

Other formal possibilities would include indirect actions of CO on other non–heme-containing proteins or its possible gene-regulating function acting as a regulator of gene expression, a transcription factor generated endogenously via HO-1 during a stress response that modulates gene expression responsible for regulation of the downstream cellular responses. One obvious cellular target is the MAP kinase pathway. It appears that CO, by augmenting p38, could be acting via signaling of downstream events. Perhaps CO via p38 is increasing the expression of other cytoprotective genes. Augmented release of IL-10 would suggest as much.

CO has been studied for >100 years, and until the last few years has been touted as a molecule to avoid. During the industrial revolution, miners used canaries to detect CO levels, assuming that their higher metabolic rates would predispose them to CO poisoning well before a worker would succumb to such. Shortly thereafter, the Japanese waltzing mouse was used in mines for much the same purpose and reason. It was argued that by the time the bird died or the mouse began to convulse (hence the waltzing), a miner had been exposed only to sublethal CO concentrations and had time therefore to escape. It is ironic that the mouse once used to predict toxicity is now, 100 years later, acting in much the same capacity. Now, however, it offers itself the same, in experimental tests designed to determine whether and by what means CO exposure might be beneficial to man. Presented here, and confirmed by other vascular and neurobiologists, is a compelling reason for a renewed effort to understand this most extraordinary molecule.

ABBREVIATIONS

ARDS, adult respiratory distress syndrome; cGMP, cyclic GMP; CO, carbon monoxide; COHb, carboxyhemoglobin; HO, heme oxygenase; IL, interleukin; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NO, nitric oxide; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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