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Carbon Monoxide and the Brain: Time to Rethink the Dogma

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Abstract

Carbon Monoxide (CO), long thought to be a simple environmental pollutant is now known to have a critical role in cellular functions ranging from vasodilation to circadian rhythms. In this review, we will begin with a discussion of the enzyme responsible for CO production: heme oxygenase. Because this review will focus on the effects of CO in the brain, we will transition to CO toxicology and determine if this simple diatomic gas has really earned its nefarious reputation. An in depth analysis of the roles for CO in circadian rhythms and as a gasotransmitter will be provided in the neurological functional role section, followed by its vascular effects derived mainly from interactions with soluble guanylyl cyclase. We will then describe the evidence for CO's protective roles through the MAPK pathway, and finally touch upon the potential therapeutic roles for CO in neurological diseases including ischemic stroke, multiple sclerosis, and neuropathic pain.

Keywords

Carbon monoxide; heme oxygenase; stroke; brain; intracerebral hemorrhage; protective

INTRODUCTION

Carbon monoxide (CO), a simple diatomic gas, plays anything but a simple role in signal transduction. Long thought to be an environmental pollutant and a neurotoxin, CO has emerged to have many of the functions of nitric oxide (NO) including vasodilation, neurotransmission, inhibition of platelet aggregation, anti-proliferative effects on smooth muscle, as well as being an anti-inflammatory agent under certain conditions and concentrations. CO and NO, as well as hydrogen sulfide (H_2S), have the distinction of being potent gaseous mediators in the body and therefore their signal transduction is unique from all other canonical pathways. All three gases are polar, however, each can diffuse into any cell along a concentration gradient, explaining why none of the molecules has a plasma membrane receptor [1]. In this review, we will examine the role of carbon monoxide in the brain along the lines of neuro-immunology and cerebrovascular regulation, as well as

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CONFLICT OF INTEREST

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potential therapeutic roles for neurological disorders. We will also address the pervasive notion that CO is a neurotoxin and its vilification as the cause of hypoxic ischemic encephalopathy after suicide attempts with vehicle exhaust and burn injury, even though many other combustible gases are involved in both these cases.

HEME OXYGENASES

As further evidence of the benefits of CO, this gas is produced in every cell in the body by enzymes known as the heme oxygenases. The heme oxygneases, oringinlly. Described in 1968 were classified as simply metabolic enzymes until the early 1990's when reports speaking to their cytoprotective properties began to emerge. There are 2 isoforms of heme oxygenase (HO). HO-1 is the inducible form of the enzyme whereas HO-2 is constitutively expressed (2). Both HOs convert the heme substrate to CO, ferrous iron, and biliverdin, while oxidizing 1 mole of NADPH. HO-1 is constitutively expressed in liver and spleen where its main function is breakdown of heme arising from senescent erythrocytes, however under inflammatory conditions its' expression can be induced almost ubiquitously, but especially in the brain [2]. HO-2, on the other hand, is expressed in neurons, glial cells, and cerebral vasculature (2). HO-1 activity is rapidly upregulated by a variety of pathophysiological stimuli such as glutamate, seizures, hypoxia, and hypotension (2). HO-2, being a constitutively expressed enzyme, can be further upregulated with functional increases in enzyme activity without altering HO-2 expression. There have been many mechanisms that have been discussed through which HO-2 activity is altered [2,4]. In the vasculature of the brain, HO-2 activation has been shown to involve a Ca^{2+}/calmodulin-dependent mechanism by glutamate and iGLuR agonists [5,6]. NO also plays a role in modulating HO-2 activity, by binding a regulatory site on HO-2 or increasing HO-2 activity via a cGMP-dependent pathway and has also been shown to affect vasomotor function of piglet arterioles [5,7]. HO-2 has also been classified as a hemoprotein that can bind heme groups to the heme regulatory motif [8].

CO - TOXICOLOGY

The primary tenet of CO toxicity is due to its high affinity for hemoglobin, 230 times that of oxygen, resulting in a relative ischemia. CO is produced by incomplete combustion of organic matter such as gasoline or wood. It is a common byproduct present in the exhaust of motor vehicles, heaters, and cooking equipment, along with many other substances generated during combustion. When CO binds to hemoglobin, it is called carboxyhemoglobin, as opposed to oxyhemoglobin when oxygen is bound. Chronic smokers have been known to have carboxyhemoglobin levels of up to 9–12%, with normal being less than 1%. When patients succumb to the toxic effects of inhalation of combustion products, one of the first symptoms reported is headache, followed by more severe headache, vertigo, and nausea at higher concentrations and longer exposure times. Convulsions, respiratory arrest, and death result at the highest concentrations of CO. Importantly, many of the substances found in exhaust are potently toxic including carbon particulate, benzo-a-pyrene and even NO. Patients are generally symptomatic at carboxyhemoglobin levels of between 20–30% with convulsions and respiratory arrest ensuing with presentation at levels higher then 30%. The effects of combustion poisoning on the brain are pathognomonic: bilateral necrosis of the globus pallidi. The question then becomes, what is the evidence that CO is the underlying and sole molecule responsible for these problems when many of the combustion products are also inhaled and not being measured or evaluated for toxicity? Perhaps, CO is just a marker for combustion poisoning, but not actually the cause of any of these untoward neurological deficits? In fact, there is a significant amount of evidence pointing to the contrary; CO is not neurotoxic, but neuroprotective.

This evidence comes from animal data where CO has been used to ameliorate the effects of ischemic stroke in a rat middle cerebral artery occlusion model, a pig model of hypovolemic circulatory arrest, and improved outcomes in a mouse model of intraparenchymal hemorrhage [9,10]. Finally, in a model of multiple sclerosis there is evidence that CO treatment can reduce the cerebral inflammatory burden [11]. Furthermore, there are 4 clinical trials in various phases using doses of CO up to 250ppm for diseases ranging from idiopathic pulmonary fibrosis and acute respiratory distress syndrome to treatment with CO after kidney transplant and after colon resection to prevent paralytic ileus all of which are based on promising preclinical evidence. CO clearly cannot be marginalized solely as a toxin.

CO - NEUROLOGICAL FUNCTIONAL ROLES

HO-1 and HO-2 have been well-studied in the brain for its potential to generate CO, where it has been shown to serve a variety of roles as a neurotransmitter [12]. HO-2 has been found to be localized to certain cells in the olfactory bulb, cortex, hippocampus, hypothalamus, cerebellum, and caudal brain stem [12,13]. Given these known aspects of CO function as critical in neurocircuitry, we pose the question as to how CO can be neurotoxic?

Much attention has been garnered by the fact that CO may be involved in circadian rhythm regulation via a transcription factor known as neuronal PAS domain protein 2 (NPAS2). NPAS2 bears significant sequence homology to other transcription factors involved in circadian regulation such as Clock [14]. NPAS2 and Clock form heterodimers with BMAL1 before binding to response elements in the nucleus resulting in circadian regulation. Curiously, NPAS2 was found to have 2 heme domains. In a study by Dioum *et al*, the ability of NPAS2 to bind to DNA was studied in the presence of NO, CO, and O_2. NO only bound to the heme group at supraphysiological concentrations and O_2 bound irreversibly, however CO bound with a dissociation constant of 1 μm, implying relatively specific binding [15]. Furthermore, CO binding resulted in the destabilization of the heterodimeric transcription factor and dissociation from the DNA response element. Inhibition of NPAS2 would be the first protein that is directly regulated by CO [15].

The HO/CO system has also been studied in the hypothalamic-pituitary-adrenal axis and has been shown to inhibit the release of endocrine factors such as oxytocin and arginine vasopressin (AVP), in an *in vitro* culture of rat hypothalamic neurons [12,16,31]

Parkes *et al* did similar experiments on rat hypothalamic neurons and studied corticotropin-releasing factor (CRF) levels [17]. In this case, CO increased basal secretion of CRF. The cells were treated with CO, hematin, or ZnPP, an HO inhibitor. Treatment with both CO and hematin resulted in an increase in CRF levels, while the opposite was true for cells treated with ZnPP [17].

Besides the potential role CO may have in modulating other neurotransmitters and hormones, there is evidence that it alone can act as neurotransmitter and is involved in long-term potentiation (LTP). LTP is defined as a ganglion that remains persistently depolarized after a tetanic stimulation, also known as the induction phase. When sympathetic ganglia are pretreated with ZnPP (an HO inhibitor), LTP was completely inhibited. And when both ZnPP and CO were added to the ganglia during the induction phase, LTP was restored. However, when ZnPP was added during the maintenance phase of the LTP, it had no effect. This implies that CO is critical to initiate the LTP, but is not required for its maintenance [18]. Additionally, CO has an established role in LTP based on the fact that it is a weak agonist of soluble guanylyl cyclase (sGC) and therefore protein kinase G (PKG). NO also has a defined role in LTP via this signal transduction pathway [1,19,20]. Whether an

interaction exists between CO and NO in the brain is unknown, but given the relationship in other organs makes a neuronal interrelationship a distinct possibility.

CO - VASCULAR EFFECTS

CO, like NO, has been established as influencing vasomotor tone. Many studies have shown that CO can cause vasodilation and serve to regulate basal vascular tone. [1,20]. CO is thought to act through soluble guanylyl cyclase (sGC) and cyclic GMP (cGMP) and also activates BK_{Ca} channels in the vascular smooth muscle cells leading to vasodilation [21–23,32,33]. Unlike NO, CO is only a weak modulator of vasomotor tone in the large vessels. Unpublished data in small capillary beds suggests differential effects in the microvasculature.

The effects of CO on vasomotor tone have however, been well studied. In smooth muscle cells, CO acts through sGC to induce vasodilation [27,28]. Decaluwé et. al. looked at the involvement of soluble guanylyl cyclase (sGC) in CO mediated vasodilation of rat aortic segments [26,35,42]. CO saturated solution was used to bathe the segments that had been treated with either 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or YC-1, an sGC inhibitor and sensitizer, respectively. The results showed that in aortic slices treated with ODQ, CO did not cause vasodilation whereas aortic slices treated with YC-1 augmented CO's vasodilatory effects [26]. They also found that the extent of vasodilation in rat aortic segments was dose-dependent and interestingly, mouse aortic segments failed to show significant dilation after the application of CO [26].

For the most part, most groups have noted that CO is not as effective in increasing the activation of sGC and that studies demonstrating otherwise are effectively showing that CO influences the generation of NO by NOS that leads to elevations in cGMP necessary for vasodilation [24,27,28]. One in-vivo study on platelets showed that YC-1 augmented the effects of CO [29,30] on coagulation. Piglet pial arterioles were treated with dimanganese decacarbonyl (DMDC), a CO donor, and vasodilation was observed, but it did not show a significant increase in the concentration of cGMP in the cerebrospinal fluid (3). The use of ODQ, in addition to DMDC, led to constriction of the pial arterioles. However, the application of ODQ plus DMDC in addition to 8-bromo cGMP resulted in dilation, indicating that DMDC is likely dependent on sGC for its activity (3).

In contrast to the systemic circulatory effects, in the cerebral arterioles, CO also thought to modulate vasodilation through its interactions with the BK_{Ca} channels in the smooth muscle cells. The BK_{Ca} or big potassium channel is located on cell membranes and is activated by changes in intracellular Ca^{2+} levels called "calcium sparks" [12,13,18]. Activation allows the flow of K^+ ions down an electrochemical gradient effectively causing hyperpolarization of the vascular smooth muscle cells. CO is thought to activate BK_{Ca} channels through its coordination to an attached heme group on one of the channel's subunits [13,18]. Increases in intracellular Ca^{2+} normally results in smooth muscle contraction through binding of Ca^{2+} to calmodulin and myosin light chain kinase. These calcium sparks or increases in intracellular Ca^{2+} are however transient and BK_{Ca} channels are sensitive to Ca^{2+} in the micromolar range [36]. This mechanism has been most considered because of CO's established ability to bind iron in hemoglobin and other transition metals such as nickel and copper [22,35].

Astrocytes have also been implicated in cerebrovascular regulation through their glutamate receptors. The activation of these channels in astrocytes has been shown to increase intracellular Ca^{2+} in astrocytes themselves. This in turn is thought to bring about a decrease in the intracellular Ca^{2+} of neighboring vascular smooth muscle cells lending itself to CO mediated vasodilation [41]. Glutamate is an important neurotransmitter in the brain and is

known to cause vasodilation [23]. In recent studies, glutamate has also been shown to induce vasodilation through activation of HO expressed in astrocytes and the subsequent endogenous production of CO [41]. Astrocytes express ionotropic glutamate receptors (iGluR) and Parefenova *et al.* hypothesized that these receptors mediated the HO/CO pathway of cerebral vasodilation [41]. Piglet pial arteriole responses to iGluR agonists were studied and it was shown that glutamate and other iGluR agonists caused dilation of pial arterioles [42]. The use of a glia-specific toxin called L-2-α-amino-adipic acid (L-AAA) in addition to iGluR agonists showed that vasodilation was effectively blocked. Pure astrocyte cultures were taken and exposed to iGluR agonists and sharp increases in CO were observed supporting belief that glutamate-induced vasodilation was mediated through actions of astrocytes [25,26]. Interestingly, the group also looked at the difference between the two isoforms of HO expressed on the astrocytes concluding that HO-2 was the major isoform found on astrocytes and that HO-2 was more sensitive to iGluR agonist stimulation.

Interestingly, HO-2 knockout mice showed a relatively smaller extent of vasodilation than WT mice in response to hypoxia [43]. It was noted that because hypoxia-induced vasodilation was mediated by relieving the constrictor tone of the HO/CO system that the lack of such a system would result in less vasodilation. Finally, it is also worthy to note that different cerebral vascular beds respond differently to hypoxia depending on HO-2 expression. Arterioles that penetrate into the parenchymal of the brain continue to have normal hypoxic-vasodilation despite the lack of HO-2 expression. On the other hand, surface arterioles lacking HO-2 lose this response completely (43).

Morikawa et. al. hypothesized that CO arising from HO in the cerebral vasculature can exhibit properties of vasoconstriction rather than vasodilation. Under conditions of hypoxia, H_2S was the primary mediator of vasodilation in the cerebral microvasculature. H_2S is formed by cystathionine β-synthase (CBS), and shown to be localized to Bergmann glia and astrocytes. CO seems to exert a regulatory effect on CBS in the brain inhibiting the production of H_2S. However, in the presence of hypoxia, the level of CO production is reduced allowing the formation of H_2S to proceed causing vasodilation. A similar response to hypoxia was seen in CBS null mice also where surface arterioles were unreactive and penetrating arterioles dilated [43].

CO - SIGNALING MOLECULE

CO coordinates with certain transition metals and is thought to target molecules such as heme containing proteins such as mito-chondrial complexes of the electron transport chain (ETC) including cytochrome oxidase, and nitric oxide synthase (NOS). The fact that CO can bind such proteins, many of which produce damaging reactive oxygen species (ROS), suggests that CO can exert neuro-protective effects through its direct interaction with these proteins. A study has shown that ROS production by endothelial cells in the cerebral vasculature increases with stimulation with pro-inflammatory cytokines such as TNF-α and the neuromediator, glutamate. The use of a CO releasing molecule (CORM), CORM-A1 reduced the amount of oxidative stress caused by these same stimuli and also resulted in increased release of anti-inflammatory cytokines such IL-10. HO-1 knockout mice present with lymphadenopathy and splenomegaly with increased CD4 to CD8 ratios indicating a chronic inflammatory state, thereby implying that HO-1 has a role in decreasing inflammation [43]. In addition, an HO-1 deficient patient presented with many of the characteristics of the HO-1 knockout mouse dying at age 6, with complications of severe mental retardation further exemplifying the homeostatic role of HO-1/CO in the brain [44].

Many reports over the past ten years have elucidated the fact that in addition to the hemoprotein targets for CO, there are also numerous reports that CO influences a number of

intracellular pathways through interaction with proteins that do not contain transition metals. These intracellular pathways modulated by CO are involved in a wide variety of physiological functions including vascular homeostasis, inflammation, apoptosis, and cell proliferation. Many of the anti-inflammatory effects of CO are mediated through p38 MAP kinase activation and inhibition of ERK 1-2 [40,44,45,46]. There are other reports that CO influences PPARγ, HIF1α, NADPH oxidase, and STAT3 among others [39]. How many of these pathways are active and modulated by CO in the brain and peripheral nervous system remains an active area of study. Nitric oxide and ion channel activity have been described in the brain and enteric nervous system [39].

CO -- ROLE IN NEUROLOGICAL DISEASE

Given that HO-2 is highly and constitutively expressed in the brain and HO-1 can be induced in conditions of stress; it should come as no surprise that CO is a major regulator of not only cerebrovascular hemodynamics, but also the general inflammatory milieu of the brain [38]. The two areas of neurological disease that have been well studied with respect to the heme oxygenase system are multiple sclerosis and stroke, both ischemic and hemorrhagic. Multiple sclerosis is an autoimmune disease characterized by inflammatory lesions, demyelination, and axon loss.

Stroke, as was alluded to earlier, can be separated into 2 types. Ischemic stroke is defined by thrombotic occlusion of one or multiple cerebral arteries resulting in infarction. The vast majority of hemorrhagic stroke is caused by hypertension and results in an intraparenchymal hemorrhage [30]. In this case, damage is done by compression of cerebral structures that surround the hematoma. In all these cases, whether the problem is inflammatory, ischemia-reperfusion, or hemorrhagic compression; a role for HO and CO is likely.

Neuronal apoptosis is found in all types of neurological disease. Changes in intracellular K^+ levels play a key role in the apoptotic cascade and it has been hypothesized that CO can block neuronal damage and cell death by blocking the action of $K_V2.1$ channels [37]. These channels are inserted into the plasma membrane when apoptosis is initiated resulting in an efflux of K^+ ions. This is a key step in the apoptosis cascade. Using primary hippocampal neurons cultures, Dallas *et al.* showed the $K_V2.1$ channels were the principal channels through which this the efflux of K^+ occurred and that CO was highly selective in inhibiting $K_V2.1$ channel [39,37]. The inhibitory action of CO seems to involve ROS formation in the mito-chondria and that PKG and ERK1/2 may play a role in mediating these action [34].

The experimental autoimmune encephalitis (EAE) mouse is used as a well-characterized and accepted model for multiple sclerosis. The EAE mouse is created by injecting myelin oligodendrocyte glycoprotein, which leads to disease symptoms. When the EAE model is performed in HO-1 knockout mice, the knockout animals exhibit exaggerated paralysis, demyelination, and mortality compared to wild type EAE mice. Furthermore, wild type mice when treated with cobalt protoporphyrin IX, a HO-1 inducer, mice exhibit improved outcomes compared to untreated EAE mice. Immunohistochemistry shows decreased MHC II antigen presenting cells, decreased T lymphocytes, and less inflammatory infiltrate [37]. However, given the extensive research into multiple sclerosis, there is also conflicting evidence as to the role of CO/HO Chakrabarty *et al.* found that, treatment with tin protoporphyrin, a selective inhibitor of HO-1, yielded improved outcomes. The likely resolution between these conflicting results lies in the nonspecific nature of treatment with heavy metals along with different treatment times during the disease course [41].

In ischemic stroke, the protective role of CO is mediated by the upregulation of Nuclear factor-erythroid 2-related factor 2 (Nrf2), a transcriptional factor that binds to response elements on the promoter region of the HO-1 gene. Nrf2 is constitutively bound and

regulated by Keap1 and during oxidative stress Nrf2 dissociates from Keap1 and translocates to the nucleus. CO exposure in mice with middle cerebral artery occlusion show smaller infarct sizes in the brain versus mice without CO treatments indicating CO's protective role (8). CO exposed mice had significantly lower Nrf2 bound to keap1 than those exposed to air, and Nrf2 was found to be increasingly localized to the nucleus after CO treatment. Essentially, CO was unable to rescue mice deficient in Nrf2. These data suggest that CO is perhaps dependent on HO-1 expression for salutary effects in stroke. CO treatment exerted the most protective effect after a 1-hour delay in CO treatment versus a 3-hour delay in treatment after an ischemic episode (8). It is of interest to note here that like other pharmacologics, a therapeutic window of time in which CO is most effective in its protective role needs to be elucidated in different model systems.

Furthermore, just as in ischemic stroke where there may be a therapeutic window for the use of CO, which also holds true for hemorrhagic stroke, specifically intraparenchymal hemorrhage [10]. The model for intraparenchymal hemorrhage was created by intraparenchymal injection of collagenase. In this case, rats were treated with a CORM or a CO releasing molecule, before injury, 3 hours after injury, or 3 days later. Pretreatment improved outcome and decreased measures of inflammation, however treatment 3 hours after injury exacerbated both inflammation and worsened outcome. Treatment 3 days after injury resulted in improved outcome and decreased inflammatory mediators, although less so than pretreatment.

Lastly, recent evidence has shown that CO may also have a role in modulating neuropathic pain [47]. Following sciatic nerve injury in wild type mice, there was increased expression of HO and NOS2 along with an increased number of microglia, known to contribute to neuropathic pain. When mice were treated with CORM-2 or an HO-1 inducer the expression of NOS-1 and 2 decreased, along with the microglial number and the neuropathic pain, strongly implicating CO as an important mediator in modulating neuropathic pain. As observed with other models, there seems to be a strong interrelationship between CO and NO generation. In this nerve injury model, CO limited the pain response by inhibiting NOS1 and NOS2 expression and the generation of NO [47]. Seminal reports such as those described above continue to expand the breadth of impact CO seems to have in the central and peripheral nervous systems and underscore how much is still yet to understand about the intricate roles played by HO and CO in the brain and the regulation of neurologic function.

CONCLUSION

The media and physiologic dogma remains keen to continue to portray CO as the silent killer, as if it's sole purpose is to eliminate life. Much of the toxicity described is focused on neurocognitive effects such as memory, nausea and headache much of which is based on combustion where hundreds of toxic molecules are present. What we have attempted to detail here is the appreciation that CO is neuroprotective, critical in memory as a neurotransmitter and even in basic physiological processes such as circadium rhythm and vasomotor tone. As with all molecules both endogenous to the body and exogenous through the environment, the amounts and exposures are critical to the response of the organism. Low amounts of CO are indeed therapeutic which have resulted in clinical application. Numerous clinical trials are ongoing with exciting potential. A therapeutic role for CO and HO is expanding as research into this topic progresses. CO has evolved from a simple environmental pollutant to a critical gaseous mediator that has roles in decreasing inflammation, neurotransmission, and modulating cerebral and systemic hemodynamics. It is not surprising that such a multifunctional gas would be studied in numerous neurological diseases yielding a variety of effects depending on when CO is given and if it is given directly as pure gas or in the form of a CORM. Early results in stroke seem to show that

there may be a critical therapeutic window to give CO to optimize the chance to improve outcomes.

In addition to the direct binding of CO to transition metals, CO also seems to effect different functions through unknown mediators that result in numerous molecular targets that influence cell and tissue function. One cannot overlook that there may be an analogous enzyme similar to soluble guanylyl cyclase (sGC) that selectively binds and is modulated by CO that has yet to be discovered and open the floodgates for a new class of pharmacology, similar to the discovery of the NO/sGC pathway. The arrival of NPAS2 as the “CO transcription factor” [15] suggests that this ancient, yet simple molecule provides critical survival benefits to the organism and that we are just on the cusp of understanding its diverse functions.

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