# The case of CO signaling: why the jury is still out

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**Commentary**

Opening statements If you pose the question “Is carbon monoxide a signaling molecule in mammals?” to a group of informed scientists, you will hear a spectrum of answers that, in simple form, range from yes to no. Why is there still no consensus on this potentially important question? Those convinced that CO is a signaling agent cite circumstantial evidence: CO is synthesized in vivo by heme oxygenase (HO) (1), HO colocalizes with soluble guanylate cyclase (sGC) in numerous brain regions (2), and HO knockout mice demonstrate enteric dysfunction (3, 4). Also, assays of brain and smooth muscle, in which HO activity is disrupted or CO is added exogenously, document CO-modulated physiological responses, some involving the nitric oxide–cGMP (NO-cGMP) pathway (4, 5), others independent of cGMP. Indeed, the arguments favoring CO as a signaling agent usually draw analogies to NO signaling, now an established signaling system that uses a diatomic diffusible gas as the messenger. How valid is this analogy? Is CO a paradigm unto itself?NO and CO compared The elements of NO signaling can be summarized as follows. (a) NO is synthesized from L-arginine, a readily available substrate, by NO synthase (NOS). Cosubstrates for the reaction are NADPH and O2 (reviewed in ref. 6). (b) Constitutive isoforms of NOS are tightly regulated by physiological stimuli; activation of NOS is transient […]



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Commentary

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## Opening statements

If you pose the question "Is carbon monoxide a signaling molecule in mammals?" to a group of informed scientists, you will hear a spectrum of answers that, in simple form, range from yes to no. Why is there still no consensus on this potentially important question? Those convinced that CO is a signaling agent cite circumstantial evidence: CO is synthesized in vivo by heme oxygenase (HO) (1), HO colocalizes with soluble guanylate cyclase (sGC) in numerous brain regions (2), and HO knockout mice demonstrate enteric dysfunction (3, 4). Also, assays of brain and smooth muscle, in which HO activity is disrupted or CO is added exogenously, document CO-modulated physiological responses, some involving the nitric oxide–cGMP (NO-cGMP) pathway (4, 5), others independent of cGMP. Indeed, the arguments favoring CO as a signaling agent usually draw analogies to NO signaling, now an established signaling system that uses a diatomic diffusible gas as the messenger. How valid is this analogy? Is CO a paradigm unto itself?

## NO and CO compared

The elements of NO signaling can be summarized as follows. (a) NO is synthesized from L-arginine, a readily available substrate, by NO synthase (NOS). Cosubstrates for the reaction are NADPH and $O_2$ (reviewed in ref. 6). (b) Constitutive isoforms of NOS are tightly regulated by physiological stimuli; activation of NOS is transient (coupled to $Ca^{2+}$ release), leading to a burst in NO synthesis (7, 8). (c) NO is freely diffusible but has a limited lifetime principally because it reacts with $O_2$ and metals. (d) The NO receptor, sGC, is a highly efficient and sensitive trap for NO (9) and is activated up to 400-fold by NO. (e) Physiological responses to NO are clear and occur at low concentrations (reviewed in ref. 10).

The profile of CO differs substantially from NO. (a) CO is a stable product of the reaction catalyzed by the microsomal enzyme HO. The substrate is ferric protoporphyrin IX and the other products of the reaction are ferrous iron (which will lead to an equivalent of ferric iron and superoxide) and biliverdin (11, 12). NADPH, $O_2$, and flavoprotein reductase (cytochrome P450 reductase) are also required for turnover. (b) Two isoforms of HO have been characterized: an inducible form (HO-1) that is upregulated, especially in the spleen and liver, in response to various types of stress, and a constitutive form (HO-2) that is expressed throughout the brain, in nerves innervating smooth muscle, and, likely, in all other tissues at low levels (13). HO-2 does not appear to be induced, and free heme is the limiting factor of CO production. (c) CO is a very weak activator of sGC (fivefold) (14, 15) and does not appear to induce any major cellular responses.

## The NO response

Once NO binds to and activates sGC, cGMP levels rise rapidly. The main function of cGMP appears to be to reduce cytoplasmic calcium levels ($[Ca^{2+}]_I$). Primarily, cGMP activates cGMP-dependent protein kinase (PKG), which phosphorylates several important regulators of $[Ca^{2+}]_I$, inhibiting $Ca^{2+}$ gates and activating $Ca^{2+}$ pumps on the endoplasmic reticulum (ER) and activating $K_{Ca}$ channels on the plasma membrane. cGMP also regulates several classes of phosphodiesterases and is itself rapidly degraded by phosphodiesterases, whereupon $[Ca^{2+}]_I$ rises again (Figure 1). The physiological outcome of this transient drop in $[Ca^{2+}]_I$ varies depending on cell type, but in smooth muscle, a drop in $[Ca^{2+}]_I$ leads to relaxation, and a rise causes contraction (reviewed in ref. 16).

## The CO story

Over the last decade, numerous reports have documented possible roles for CO as a gaseous second messenger in neuronal signaling and smooth muscle regulation. One major hypothesis posits that CO is a modulator of NO signaling. Indeed, some compelling evidence has emerged from studies of the enteric nervous system and enteric smooth muscle in *HO-2*$^{-/-}$ mice and *HO-2*$^{-/-}$/*nNOS*$^{-/-}$ mice. For example, intestinal transit and smooth muscle relaxation are altered in *HO-2*$^{-/-}$ mice (5). CO appears to be required for proper maintenance of enteric smooth muscle resting membrane potential, and exogenous CO restores inhibitory transmission in contracted muscle of *HO-2*$^{-/-}$ mice. Surprisingly, these effects appear to depend on the synthesis of NO. Indeed, CO may be epistatic to NO in this context, since *HO-2*$^{-/-}$/*nNOS*$^{-/-}$ mice demonstrate a similar phenotype to that of *HO-2*$^{-/-}$ mice, but one that cannot be rescued by providing CO (4). Detailed studies of the ability of CO to induce NO synthesis are still needed. Indeed, if CO modulates cGMP levels by affecting NO synthesis, one might postulate the existence of novel CO receptors. However, as discussed below, the unregulated nature of CO synthesis confounds this hypothesis. Although these knockout studies imply that CO acts through cGMP, there is scarce evidence that physiological concentrations of CO directly activate sGC to synthesize relevant levels of cGMP.

Other emerging hypotheses suggest that CO effects are cGMP-independent and that CO targets novel proteins. Patch-clamp studies of rat tail artery

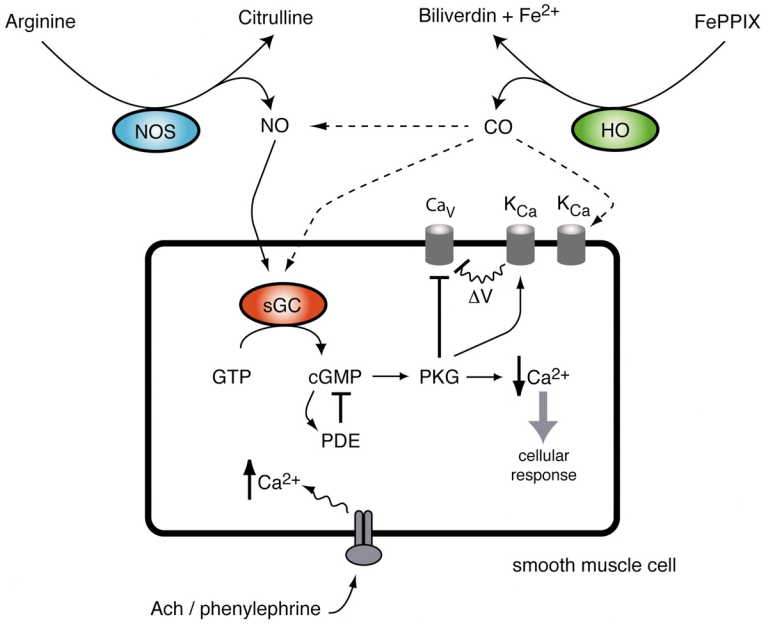


**Figure 1**

The potential interplay of NO and CO. Established interactions that lead to physiological responses are shown in solid lines, and dashed lines indicate interactions where some experimental support exists. NOS catalyzes the formation of NO and citrulline from arginine. NO directly activates the soluble isoform of guanylate cyclase (sGC), leading to 400-fold increased activity. cGMP then activates a PKG cascade and cellular $Ca^{2+}$ levels are lowered. In part, PKG potentiates calcium-activated potassium channels ($K_{Ca}$), and the resulting hyperpolarization inhibits voltage-gated $Ca^{2+}$ channels ($Ca_V$). PKG also appears to directly inhibit $Ca_V$. HO catalyzes the formation of CO from iron protoporphyrin IX. Results from $HO^{-/-}$ mice suggest that CO action depends on NO. While CO has been shown to activate the α1β1 isoform of sGC, the activation is very weak compared with that of NO (14). The action of CO on $K_{Ca}$ would also lead to a hyperpolarization and inhibition of $Ca_V$. Agonists such as acetylcholine (Ach) and phenylephrine act by increasing cellular $Ca^{2+}$.

smooth muscle cells revealed that exogenous CO activates a 238 pS $K_{Ca}$ channel independently of cGMP and without altering $[Ca^{2+}]_I$ (17). Kaide et al. suggest in this issue of the *JCI* that endogenously produced CO directly or indirectly activates a 105pS $K_{Ca}$ channel in renal artery smooth muscle, again apparently without inducing sGC (18). The resulting hyperpolarization renders the cell less sensitive to contractile agonists. This report opens up numerous questions that may help narrow the gap between CO and its apparent biological effects: Does endogenous CO affect cGMP or $[Ca^{2+}]_I$? What are the effects of the contractile agonists on these levels in the cell? Is this $K_{Ca}$ channel also regulated by PKG, as are other $K_{Ca}$ channels in smooth muscle cells? Does NO cause a similar activation of these $K_{Ca}$ channels?

## Reasonable doubts

Several significant concerns still envelop the issue of HO-2/CO signaling. Perhaps most troublesome is the apparent lack of regulation of HO-2, since the existence of a switch to turn a response on or off is usually taken as a sine qua non of signaling. Is the physiological action of CO mediated in some tonic fashion such that no switch is required? Perhaps even more problematic is the availability of the heme substrate. There is little free heme in the cell (no doubt owing to the toxicity of heme). How, then, is sufficient CO generated?

There are two sources of heme to serve as a substrate: biosynthesis and heme salvage. The eight-step biosynthetic pathway begins in the mitochondrion, continues in the cytosol, and concludes in the mitochondrion with the insertion of iron by ferrochelatase generating iron protoporphyrin IX (19). Details of how heme is chaperoned around the cell are lacking. For CO production, the substrate must be delivered to the ER, where both HO-1 and HO-2 reside. Studies on the various types of porphyrias emphasize the complex aspects of heme biosynthetic regulation. The other avenue for substrate would be that derived from the degradation of hemoproteins. The use of recycled heme as a substrate would certainly be an energy-efficient way to produce CO but would still require transport of heme from the proteosome to the ER. Perhaps diffusion and membrane solubility from both avenues would provide the requisite amounts of heme. While the reported $K_m$ for HO-2 of 0.4 μM (20) indicates an efficient enzyme, if the concentration of free heme is below that value, CO production will be low. Some of these enzymatic issues have been addressed in the context of CO signaling in olfactory receptor neurons (21), but rigorous determinations of free heme concentrations in brain and smooth muscle are needed.

How much CO is actually made? Because scaling reported values to actual tissue concentrations is difficult, studies in which micromolar concentrations of CO are added exogenously are subject to artifacts. On the other hand, since CO can ligate many hemoproteins, it might merely be an endogenous nuisance, but a modulator all the same. If CO signaling directly upregulates cGMP, as some evidence suggests, then a novel sGC must exist since the well-characterized α1β1 heterodimeric isoform is only weakly activated, even at high concentrations of CO. Given the toxicity of heme and the stringent regulation over its biosynthesis, it seems possible that at least some of the intriguing observations made in HO-2 knockout mice simply reflect the stress that is placed on the cell because of inefficient heme removal in the absence of this key enzyme. Unfortunately, the currently available HO inhibitors are not specific (22), so measurements of CO biosynthesis that rely on these agents are difficult to interpret.

## Closing arguments

CO as a signaling agent was and remains an intriguing possibility. Critical questions still need to be answered before a consensus can be reached about the existence and significance of the process. What does colocalization of HO-2 and sGC mean functionally? All nucleated cells synthesize and turn over hemoproteins. Does HO-2 serve a simple "housekeeping" function to eliminate low concentrations of toxic free heme? If cells have an additional capacity to respond to pathologically high heme concentrations, does HO-1

mediate this function? As always in science, experimental work will continue and answers will ultimately be found. The creed of the antitrust attorney David Boies seems appropriate: "You want to have a consistent, coherent set of themes that you establish and stick to, and that's particularly important the more complicated the case is. The more complicated it is, the more important it is to define what your simple truths are." (23)


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