ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 2, 2002

Forum Review

Biological Chemistry of Carbon Monoxide

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ABSTRACT

Carbon monoxide (CO) has many effects in biology due to its complex biochemical activities. These actions of CO depend primarily on its ability to bind heme proteins (Hp) and to inhibit or alter their biochemical functions. Whether CO is derived from exogenous or endogenous sources, its cellular activity is related to its concentration and the concentration of molecular O_2, as well as to the availability of reduced transition metals such as Fe(II). In this respect, the CO/O_2 ratio and O_2-dependent changes in local oxidation–reduction state assume critical importance in determining the physiological effects of CO by affecting the functions of specific Hp. By interacting with Hp, CO influences electron-transport reactions in a variety of ways, which can produce either prooxidant or antioxidant effects. Similarly, Hp relationships also govern how changes in CO concentration influence the physiological and pathological effects of nitric oxide and the relationships of the two biologically active gases to metal-catalyzed oxidations. This article provides a brief update on the biochemistry of CO as it relates to Hp binding, chemical oxidative processes, and cellular function. Antioxid. Redox Signal. 4, 259–270.

INTRODUCTION

CARBON MONOXIDE (CO) holds a prominent place in the history of the contemporary biological sciences. Since its recognition as a potent chemical asphyxiate in the middle of the 19th century, CO has been widely studied in physiology, biochemistry, pharmacology, toxicology, and medicine (5, 34, 44, 108). It has also served as a valuable tool for studying the biochemical characteristics and reactions of a variety of heme proteins (Hp), most notably hemoglobin, myoglobin, and the cytochromes (16, 44, 45). The primary value of CO for study of Hp comes from its ability to interact with iron, which is coordinated at the center of all heme-containing proteins. CO binds iron in its reduced state [Fe(II)] resulting in characteristic shifts in the optical spectrum of the Hp that can be used for its identification. CO interferes with Hp function by inhibiting oxygen (O_2) binding (*e.g.,* hemoglobin) or oxidation–reduction (redox) reactions requiring the transfer of electrons through the heme moiety, usually to molecular O_2 (*e.g.,* cytochromes). CO also binds to proteins that contain other transition metals at the active site, *e.g.,* copper, and will interfere with their functions. This review provides a brief update on the biochemistry of CO as it relates to Hp and cellular function. The effects of CO on Hp function account for most of its known and likely many of its unknown mechanisms of actions in biology.

A BRIEF HISTORY OF CO IN BIOLOGY

CO came to biological attention in 1857 when the French physiologist Claude Bernard determined that the gas produces asphyxia by reversibly combining with hemoglobin (5). In 1895, J.S. Haldane demonstrated that CO binding to hemoglobin could be antagonized by high partial pressures of O_2 (PO_2) and that mammals would survive lethal CO poisoning if a large amount of O_2 was dissolved in blood plasma (34). During World War II, Roughton and Darling reported that carboxyhemoglobin (COHb) shifted the oxyhemoglobin dissociation curve to the left because the unoccupied hemes of the hemoglobin tetramer bound O_2 with greater affinity after addition of CO to one site (80). This effect of CO made it more difficult for the hemoglobin molecule to unload oxygen in the tissues.

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These pioneering studies gave rise to the common concept of the pathophysiology of CO poisoning based on tissue hypoxia. Accordingly, the decreased arterial O_2 content (CaO_2) in combination with increased affinity of COHb for O_2 led to a decrease in tissue PO_2, which produced manifestations of hypoxia in the tissues (91). Although these principles have formed a sound scientific basis for understanding many biological effects of CO and the treatment of CO poisoning, they do not tell the whole story. They fail to explain the classical differences between the cellular effects of simple hypoxia and those of CO hypoxia, to be discussed later.

In addition to early work on its physiological effects, CO was used as a tool to study tissue respiration. In groundbreaking studies to discover the source of cellular respiration, Otto Warburg, David Keilin, and others found CO invaluable as a tool to study the biochemical behavior of intrinsic tissue pigments, identified subsequently as myoglobin and cytochrome c oxidase (cytochrome a,a_3) (44, 45, 108). In the 1920s, Warburg, engaged in the study of the role of iron in respiration, found that CO could inhibit respiration in yeast in a light-sensitive manner, extending 1896 observations of Haldane and Smith, who had discovered that COHb could be dissociated by exposure to light of appropriate wavelengths (35). Even now, photodissociation of CO from Hp at picosecond and nanosecond resolution is a powerful approach for studying molecular ligand binding (62). In the 1930s, Fenn and Cobb reported that living tissues burned CO by oxidizing it to CO_2 (27); however, the mitochondrion was not shown to be the source of this oxidizing power for almost 50 years.

The many fascinating and curious aspects of CO biology stimulated efforts in the latter half of the 20th century to understand the biological chemistry of CO. In 1952, Sjostrand reported that decomposition of hemoglobin *in vivo* led to CO formation (87). Increases in COHb were attributable to metabolic CO production, particularly after heme degradation had been stimulated by hemolysis (18, 19). Specific drugs and chemicals were found to accelerate endogenous CO production, including progesterone (15, 23). At about the same time, biochemical studies indicated endogenous CO was produced primarily from heme catabolism by heme oxygenase (HO) (95). Furthermore, in the microsomal fractions of cells, CO was found to bind to unique cytochromes, now called mixed-function oxidases, and the appearance of the broad CO band of the reduced enzyme in the Soret (UV) region of the spectrum led to the name cytochrome P_{450} (26).

At the University of Pennsylvania in the 1960s and 1970s, largely through physiological studies of Coburn, the CO store in the body was measured, and uptake of CO by tissues, particularly skeletal and cardiac muscle, was shown during ambient exposures. Coburn recognized that the amount of CO in tissues increased substantially during hypoxia due to transfer of the gas from blood to tissue and binding of CO to Hp such as myoglobin (17). Similarly, it was demonstrated that CO diffused out of the maternal circulation across the placenta and bound to fetal hemoglobin *in vivo* (38).

Within the last 25 years, it has been documented that CO is oxidized in the body to CO_2 by mitochondria (115–117). CO alters mitochondrial redox state and energy provision in the brain; these effects persist after COHb has been cleared from the blood (7). By activating guanylate cyclase, CO inhibits platelet aggregation (8) and acts as a direct vasodilator (75). Toxic and presumed subtoxic CO exposures are associated with significant oxidative (97, 99, 118) and nitrosative stress (98). Finally, over the past decade, endogenous CO production has been associated with changes in intra- and intercellular signaling processes, *e.g.,* neurotransmission and vasodilation by activating guanylate cyclase (55, 102). Some of these effects of CO appear to be closely related to those of nitric oxide (NO), whereas others may be direct influences of CO on the behavior of NO through interactions with iron or other metals. As discussed below and in the following articles, effects of CO are proving to be important in regulating inflammation, cell death, and cell proliferation *in vivo*, although the links to specific biochemical reactions in the cell still await elucidation.

CHEMICAL PROPERTIES OF CO

CO, the diatomic oxide of carbon, is a colorless, ubiquitous gas at temperatures above −190°C. It has a specific gravity of 0.967 relative to air and a density of 1.25 g/L at standard temperature and pressure (STP). Its water solubility is low, ~354 ml/dl (44.3 ppm by mass) at STP (3). CO is the anhydride of formic acid; however, it does not react with water without substantial energy input. Although CO is combustible, its formal triple bond makes it chemically quite stable under physiological conditions. Its reaction with molecular O_2 is slow and has a high energy of activation (213 kJ/mol); however, CO is involved in redox reactions (3).

In its free state, CO resists attack by most common reducing agents, including hydrogen. The reactivity of coordinated CO is much greater than the free gas, and transition metals are particularly effective in promoting reduction of CO (85). CO readily forms metal carbonyls, which are susceptible to the attack of the CO oxygen atom by electrophiles. Chemical reduction of CO, however, requires temperatures well above the normal physiological range (>100°C). Once formed in the body, metal carbonyls are relatively stable until CO is displaced, *e.g.,* by molecular O_2. The oxidation or oxygenation of CO to CO_2 is also well appreciated in chemistry, *e.g.,* the metal-catalyzed water gas shift reaction generates H_2 and CO_2 from CO and H_2O (85). Under known conditions, CO oxidation also requires temperatures beyond the physiological tolerance of most organisms.

Chemical reactions of CO with reactive oxygen species are widely known, particularly to atmosphere and soil chemists, who have traced the fate of atmospheric CO produced by photochemical oxidation of methane and other small organic molecules (4, 78). The primary route of elimination of CO from the atmosphere is by reaction with the hydroxyl radical (·OH) (4). This reaction involves two pathways: the bimolecular reaction yields atomic H and CO_2, whereas the addition reaction produces a carboxyl radical (HOCO). In the presence of molecular O_2, the carboxyl is rapidly converted to CO_2 + HO_2 (hydroperoxyl radical). Therefore, the reaction of CO with ·OH produces primarily HO_2 and CO_2. In the Earth's atmosphere, CO oxidation by ·OH can either produce or deplete ozone depending on the amount of HO_2 and NO in the air. For example, CO may be involved in the production of NO_2 in the atmosphere by the overall reaction:

$$CO + NO + O_2 \rightarrow CO_2 + NO_2$$

The extent to which this or related reactions reflect CO participation in cellular reactions with NO is unknown, however, any production of NO_2 would be toxic. If CO reacts with ·OH in biological systems, the reactions are almost certainly variable and site-specific due to the extremely short lifetime of the hydroxyl radical.

Some comments about the chemical interactions between CO and NO should be made for systems where iron and other redox-active transition metals are available. CO, like NO, avidly binds Fe(II)Hp, but unlike NO does not bind Fe(III)Hp (44). Experimentally, low concentrations of CO stimulate NO release and production of the strong oxidant peroxynitrite, *e.g.,* in blood platelets and vascular cells (98). A chemical mechanism for CO-mediated NO release has been proposed based on redistribution of NO in the cell, a hypothesis consistent with different equilibrium constants for metal binding of the two gases. NO is the most reactive of the physiological gases, having the same effective size and polarity as the O_2 molecule. The rate-limiting step for the overall NO binding reaction to Hp such as myoglobin is actually NO entry into the binding pocket (83). Thus, the overall association constants for NO with Fe(II)Hp are much faster than the association constants for CO. For hemoglobin, the affinity of NO for Fe(II) is 1,500 times greater than that of CO (44). For sperm whale myoglobin, the overall association rate constants for CO, O_2, and NO are 0.5, 17, and 22 $\mu M\ s^{-1}$, respectively (62). However, the dissociation constants for CO are considerably longer than for NO or O_2 (32). Therefore, NO is displaced gradually from Hp Fe(II) in the presence of CO, *e.g.,* nitrosyl-myoglobin or nitrosyl-hemoglobin (58). For Hp in aqueous solution, NO displacement requires minutes to hours even at excess CO concentration, and pH dependence of the displacement reaction for some Hp indicates conformational dependence of the effect. Displacement of NO from Fe(II) by CO may be enhanced if reduced thiols are present to serve as a sink for NO. Whether such *in vitro* measurements are relevant to living systems is not yet clear because the CO concentrations that have been used are orders of magnitude above those that occur in tissues. Hence, the extent to which CO displaces NO from Hp at physiological concentrations of CO, NO, and O_2 is unknown. It seems more likely that preformed CO bound to Fe(II), *e.g.,* of cellular Hp, would influence (increase or decrease) the bioactivity of newly synthesized NO in response to physiological stimulation or pathological events. This effect would produce differences in the apparent effects of CO as a function of the concentrations and metabolic fates of all three gases. Direct CO–NO interactions will not occur in met-Hp because CO does not bind to Fe(III). Finally, the interactions among CO, NO, and O_2 will be influenced by generation of reactive oxygen species that depend on the presence of reduced transition metals.

THE CO STORES OF THE BODY

The presence of CO in the tissues and cells of the body occurs by two routes: exogenous uptake from ambient by the lungs and endogenous production by the cells of the body

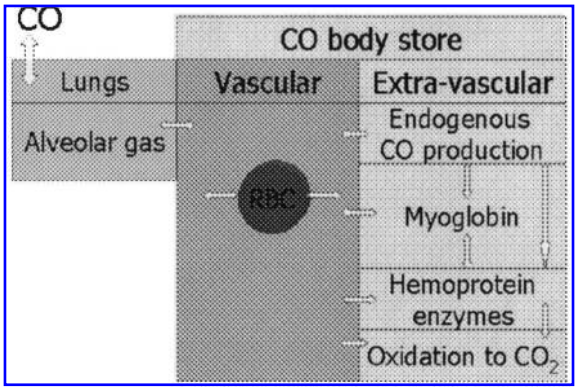

FIG. 1. Block diagram of the CO body stores. Under normal conditions, most of the CO in the body (80%) is bound to hemoglobin in the red blood cells (RBC). The rest is distributed among various tissues, primarily bound to myoglobin and other intracellular heme proteins. Small amounts of CO are produced by the HOs during heme degradation (<0.5 ml/h) and perhaps by lipid peroxidation. A small amount of CO is also oxygenated to CO_2 by cytochrome *c* oxidase in mitochondria.

(Fig. 1). CO in the lungs diffuses rapidly across the alveolar–capillary membrane at a rate that depends on several physiological variables, including alveolar gas volume, ventilation, and the concentration of hemoglobin in the pulmonary capillaries (16). It is also influenced by the rates of endogenous CO production and metabolism. Most of the CO in the body (~80%) is bound to hemoglobin as COHb (14). The remainder of the CO is distributed in the tissues. The cellular concentrations of CO depend upon the local partial pressures of both CO and O_2 because the two gases compete for the same iron or copper binding sites. This relationship to PO_2 has major implications for the actions of CO in the cell that will be discussed in more detail later. Among cellular Hp, myoglobin, cytochrome *c* oxidase, cytochrome P_{450}, catalase, guanylate cyclase, and tryptophan dioxygenase bind sufficient CO to alter function *in vitro*. After binding to Hp enzymes, CO usually inhibits electron transfer and/or catalytic activity. However, CO binding activates guanylate cyclase similarly to NO (55). Although the effects of CO on myoglobin and certain cytochromes are well understood, less well defined CO binding effects on other Hp and copper proteins may also be functionally important under some circumstances.

Endogenous CO production

CO is produced normally by enzymatic heme degradation by HO. Minute amounts of CO also can be produced as a by-product of iron-catalyzed lipid peroxidation (61, 104). HO enzymes are widely distributed in mammalian tissues; however, the bulk of heme catabolism, and hence endogenous CO production, occurs in the reticuloendothelial system of the spleen and liver (54). The enzyme forms a complex with NADPH-dependent cytochrome P_{450} reductase and biliverdin reductase on the endoplasmic reticulum and provides the normal mechanism and rate-limiting step for heme catabolism (54). HO breaks the porphyrin ring at the α-methene carbon,

which stoichiometrically releases biliverdin, molecular iron, and CO (95). Because balance is maintained between synthesis and catabolism of heme, HO influences cellular processes both by removing heme and by generating active metabolites. Free heme is cytotoxic, and heme-catalyzed oxidative reactions can initiate or exacerbate pathological conditions (24, 81, 82). Hp-derived oxidants include radical and nonradical forms of Hp-Fe^{4+} that oxidize a variety of substrates, including lipids, thiols, proteins, carbohydrates, and nucleic acids (81, 82).

Of the known HO isoforms (HO-1, 2, and 3), only HO-1 is a stress protein induced by heme, heat shock, oxidants, metals, lipopolysaccharide, hypoxia, and hyperoxia (13, 50, 81). Thus, endogenous CO production is stimulated by the stress response. HO-1 induction can attenuate oxidant injury *in vitro* and *in vivo*, and protection is blocked by enzyme inhibition (13, 64, 103). In cell-transfection studies, moderate HO-1 overexpression is cytoprotective, whereas massive overexpression worsens oxidative damage in part by increasing intracellular free iron (24, 93). Other functions remain to be elucidated; biliverdin (and bilirubin) is an antioxidant (82), whereas CO and iron provide cell signals (24, 81, 82). Free iron creates oxidative stress that is dealt with in part by a rapid increase in ferritin translation through its regulatory protein binding and activation of iron response elements (37, 68).

Due to the potential multiplicity of effect of HO-1, determining precise roles for endogenous CO production in cell signaling is not straightforward. The concept of CO signaling is perhaps best worked out for guanylate cyclase. Endogenous CO has been proposed as a diffusible second messenger or transmitter in the brain by activating guanylate cyclase (59, 60, 102). Although the ability of CO to activate guanylate cyclase *in vitro* may be 30–100 times less than that of NO, HO and NO synthase (NOS) are regulated very differently. Possible CO signaling effects also may derive through interactions with other Hp either by inhibiting function, altering their ability to generate oxidants (25), or by influencing the effects of NO as discussed earlier. In this respect, endogenous CO may be either antioxidant or prooxidant depending upon the redox environment in which it is generated and the activities of the metal centers to which it binds.

Daily endogenous CO production is substantial; the human body generates more than 12 ml of CO (STP) per day from heme catabolism (18). However, based on normal levels of COHb of 1–2%, average physiological concentrations of CO in living tissues would be expected to be in the low nanomolar range and vary inversely with local PO_2.

CO produced by xenobiotic metabolism

The production of CO by xenobiotic metabolism was discovered in 1972, when Stewart reported that methylene chloride (dichloromethane), a component of paint remover, increased the COHb level after inhalation of the vapor (90). Subsequently, it was found that methylene chloride and other dihalomethanes, most notably dibromomethane, are metabolized to CO enzymatically by cytochrome P_{450} in the liver *in vivo* and *in vitro* (47–49). Humans exposed to these chemicals in poorly ventilated spaces for up to 6 h have been found with COHb levels of 20–50% (66).

Dihalomethane-derived CO is produced primarily by oxidative dechlorination of the compounds by the CYP2E1 isoform of hepatic cytochrome P_{450} on the endoplasmic reticulum (30). This reaction has a high substrate affinity, but low capacity, and competes with a high-capacity cytosolic metabolic pathway involving the glutathione redox cycle (30, 66, 67, 76). The amount of CO produced by CYP2E1 metabolism of inhaled dihalomethane compounds is sufficient to inhibit cytochrome *c* oxidase activity in the brain, lungs, and muscle by as much as 50% (51, 66). This effect on cellular respiration is reversible and can be prevented by inhibition of CYP2E1, which implicates CO in the toxicity of these compounds (51). When CYP2E1 is induced before exposure to dihalomethanes, the rate of production of CO is accelerated.

CO AND HEMOGLOBIN

Because iron in hemoglobin is normally in the Fe(II) state, CO binds avidly to it. Fe(II) is present in both the deoxyhemoglobin (T) and oxyhemoglobin (R) states, and the metal is oxidized to Fe(III) in methemoglobin, which carries neither O_2 nor CO. CO binding of heme competes with O_2 binding, and the affinity of hemoglobin for CO is ~220 times greater than for O_2 (4). The equilibrium constant is known as the Haldane constant or M value. The steady-state relationship between the constant M and the partial pressures of CO and O_2 is described by the Haldane expression:

$$COHb/HbO_2 = M\ (PCO/PO_2)$$

where COHb is carboxyhemoglobin, HbO_2 is oxyhemoglobin, and PCO and PO_2 are the partial pressures of CO and O_2, respectively. Because M is large, CO at very low partial pressures will occupy most of the O_2 binding sites on hemoglobin. For example, at an inspired PCO of only 50 ppm, the COHb level at steady state will be ~5%.

Once CO is bound to hemoglobin, its rate of release is slow. The measured COHb half-time in the adult human body varies, but the values are usually between 200 and 360 min of breathing air (91). Because the COHb molecule carries no O_2, CO decreases the O_2 content of arterial blood (CaO_2) in proportion to the amount of COHb present. The CaO_2, neglecting dissolved oxygen, is the product of hemoglobin concentration [Hb], O_2 saturation (SaO_2), and the O_2 carrying capacity of hemoglobin (1.36 ml of O_2/g). In the presence of CO, [Hb] and SaO_2 are constant, and CaO_2 decreases in direct proportion to the decrease in the O_2 carrying capacity of hemoglobin as COHb is formed.

When CO binds to hemoglobin, the oxygen dissociation curve (ODC) of hemoglobin changes shape from its normal sigmoid to hyperbolic. This change in shape is due to an increase in cooperative O_2 binding produced by CO, which shifts the ODC to the left. In other words, the SaO_2 of hemoglobin at a given PO_2 in the presence of COHb is higher than in its absence. The COHb-mediated decrease in CaO_2 and left shift of the ODC lower the venous PO_2 and hence the tissue PO_2 for any amount of oxygen extraction by the tissues (29).

HEMOGLOBIN AND THE CELLULAR HEME PROTEIN EFFECTS OF CO

In the past it has been argued that the high affinity and rapid equilibration of CO with hemoglobin meant that uptake of CO by tissues was relatively unimportant to CO pathophysiology. According to this argument, hemoglobin acts as a buffer for CO in the circulation to prevent it from being taken up by the tissues. This line of reasoning, however, fails in the face of kinetic arguments, and experimental data demonstrate that CO is taken up by tissue, particularly when the PO_2 falls. Therefore, substantial cellular effects of CO are to be expected, although most of the evidence for intracellular effects is indirect. Cellular effects of CO have long been suggested by the poor correlation of COHb level with signs and symptoms of poisoning, physiological effects at low COHb (91), and remnant effects after COHb elimination (36).

In cells and tissues, the competitive relationship between CO and O_2 is described classically by the Warburg partition coefficient:

$$K = (n/1 - n)(CO/O_2)$$

where n, the fraction of a compound bound to CO, is equal to 0.5. Thus, K is the ratio of CO to O_2 needed to half-saturate the binding site with CO. Warburg coefficients are 0.4 for myoglobin, 1.0 for cytochrome P_{450}, and 5–15 for cytochrome *c* oxidase measured *in vitro* (16). The Warburg coefficient computed from *in vitro* measurements is difficult to translate to intracellular conditions because the CO/O_2 ratio in tissues is difficult to determine. The difficulty arises from technical and biological limitations for measuring PCO and PO_2. PCO and CO content are largely unknown due to lack of methods to enable precise, local measurements. As a result, tissue PCO estimates vary (17, 33). Tissue and intracellular PO_2 measurements are made with microelectrodes, or by spectroscopy of intrinsic O_2-labile chromophores, vital dyes, or fluorescent compounds. Values for tissue PO_2 also vary considerably and depend on location, *e.g.,* proximity to vessels. They also depend on technique, *e.g.,* O_2 consumption and tissue compression by electrodes (2). Nonetheless, tissue PO_2 is equal to or less than venous PO_2 (96), and may be quite low in organs such as brain, heart, and liver (5–30 torr) (2). Furthermore, as PO_2 falls, less CO is needed to produce an effect, and when O_2 is absent, CO binds to cellular Hp noncompetitively. Sufficient cellular uptake of CO has been demonstrated for several Hp to affect their functions (16, 20).

CO and myoglobin

In cardiac and red skeletal muscle, the globular Hp myoglobin participates in O_2 transfer to mitochondria. As noted above, ferrous myoglobin binds CO with a low K (~0.4) and has been demonstrated in heart at COHb levels below 2% and in skeletal muscle at 1% (17, 20, 110–112). The ratio of carboxymyoglobin (COMb) to COHb in the heart of the dog is close to one for a wide range of COHb values owing in large part to the lower PO_2 in the sarcoplasm of the myocyte than in blood. During muscle hypoperfusion or hypoxemia, CO uptake by myoglobin increases. Similarly, CO shifts from blood to muscle in humans at near maximal exercise as tissue PO_2 falls (20).

CO uptake by myoglobin is potentially quite important in working muscle because myoglobin facilitates O_2 diffusion from sarcoplasm to mitochondria to help satisfy the continuous demand for O_2 (110–112). In the beating heart, myoglobin is not fully oxygenated, indicating PO_2 is considerably lower in the myocyte than in the blood (41). This assumption is reasonable because P_{50} (partial pressure at 50% saturation) for O_2 of myoglobin *in vitro* is 3–4 mm Hg compared to 20–30 mm Hg for most mammalian hemoglobins. Although myoglobin provides a small reserve of O_2 to muscle, its main role in respiration is probably as an O_2 buffer to maintain an adequate PO_2 for mitochondria when the O_2 supply changes. Myoglobin helps maintain maximum O_2 uptake and contractile force in exercising skeletal muscle (22), where it facilitates oxidative phosphorylation (110–112).

The possibility that CO interferes with myoglobin-facilitated O_2 diffusion has been explored both experimentally (111) and in simulations (1, 39). In isolated cardiac myocytes at physiological PO_2 (~5 mm Hg), CO decreases the ratio of phosphocreatine to ATP when the COMb/myoglobin ratio approaches 0.6. The investigators could find no evidence that this effect on high-energy phosphate production was mediated by inhibition of cytochrome *c* oxidase by CO. They also predicted that COMb formation would impair myoglobin-dependent oxidative phosphorylation *in vivo* only when COHb levels exceeded 20%.

A three-compartment mathematical model (arterial and venous blood and myoglobin) predicts that COMb in low PO_2 regions of the heart will impair O_2 transport to mitochondria at COHb levels of 5–10% (1). The model also predicts increases in COMb concentration during hypoxia, decreases in blood flow, and periods of increased O_2 utilization. Whether increased COMb at COHb levels of 4–5% actually account for decreases in maximal O_2 uptake remains to be determined for exercising humans.

CO AND MIXED-FUNCTION OXIDASES (CYTOCHROME P_{450})

Mixed-function oxidases or oxygenases, *e.g.,* cytochrome P_{450}, named by the 450-nm absorption peak of the CO minus reduced difference spectrum, are distributed widely in mammalian tissues. These Hp isozymes are involved in steroid and xenobiotic metabolism, particularly in the liver, kidney, adrenal gland, and lungs (26). They catalyze hydroxylation reactions involving the uptake of a pair of electrons from NADPH with reduction of one atom of O_2 to H_2O and incorporation of the other into the substrate (109). The *in vitro* Warburg coefficients for CO of these Hp range from 0.1 to 12 (16). The sensitivity of cytochrome P_{450} to CO increases during rapid electron transport (26); however, tissue PCO *in vivo* is normally too low to inhibit these enzymes significantly. As COHb increases to nonphysiological and toxic levels, *e.g.,* 15–20%, significant CO binding to these enzymes does occur, and has been proposed to cause biological effects, such as changes in drug metabolism and vascular tone.

Values of K for CO binding to mixed-function oxidases have been estimated in several intact organs. In rabbit lung, the effects of CO suggest apparent average Warburg coefficients of ~0.5 for cytochromes P_{450} (28). In the liver, CO exposure decreases the metabolism of barbiturates and other drugs (57, 79). These effects of CO on drug metabolism are often attributed to cellular hypoxia unless they are greater than "equivalent" levels of hypoxic hypoxia. This line of reasoning is not necessarily valid unless drug metabolism is restored promptly on reintroduction of O_2. In studies of hemoglobin-free perfused rat liver, optical measurements have demonstrated uptake of CO by cytochrome P_{450} systems at CO/O_2 ratios of 0.03–0.10 (40, 86, 94). These CO/O_2 ratios, if translated directly to COHb, are incompatible with life (~85–95% COHb). However, such calculations are inherently limited by lack of an accurate basis for determining local PO_2 and Hp redox state in tissues, as mentioned earlier.

The possibility has been proposed that CO mediates vascular relaxation by inhibiting mixed-function oxidases. The hypothesis is based on CO interference with cytochrome P_{450}-dependent synthesis of endogenous vasoconstrictor substances (21, 105–107). Alternatively, there is just as good a reason to hypothesize that CO interferes with normal metabolism of vasodilator substances. Such mechanisms are alternative explanations for vasorelaxation by CO-mediated activation of guanylate cyclase, via either direct activation or activation by NO redistribution. However, the extent to which any of these mechanisms occurs under physiological conditions is not known, *e.g.,* for endogenous CO concentrations in the nanomolar range. Further studies are needed in this area.

CO and cytochrome c *oxidase*

Cytochrome *c* oxidase, also known as cytochrome a,a_3, the terminal enzyme in the respiratory chain of mitochondria, reduces molecular O_2 to water in a four-electron reaction. This reaction accounts for ~90% of the oxygen utilized by tissues, and mitochondrial electron transport produces most of the ATP in the cell. Cytochrome oxidase has a Michaelis–Menten constant (K_m) for O_2 *in vitro* of <1 mm Hg (11). As intracellular PO_2 is normally >1 mm Hg, cytochrome oxidase should remain oxidized until significant hypoxia is present. Indeed, in isolated mitochondria under low ADP conditions (State 4), the enzyme remains oxidized until the concentration of O_2 falls to 10^{-6} M and its reduction level increases to 4% after ADP addition (State 3) (11). At very high turnover rates, however, the reduction level of the oxidase may approach 20% *in vitro* (63).

The Warburg coefficient of cytochrome *c* oxidase is greater than that of either myoglobin or cytochrome P_{450}, and only reduced cytochrome a_3 binds CO (45, 113). Nonetheless, significant effects of CO on mitochondria have been observed *in vitro* and *in vivo*. Although the apparent K_m for O_2 of cytochrome *c* oxidase is low, cellular O_2 gradients are steep, mitochondrial PO_2 is low (29), and some of this enzyme is reduced *in vivo* (41, 42, 46, 63). This reduced enzyme (a_3 heme and Cu reduced) binds CO (113). In uncoupled heart mitochondria, Chance *et al.* found that CO/O_2 ratios as low as 0.2 markedly delayed recovery of cytochrome redox state from anoxia and normoxia (12). This suggests mitochondrial PO_2 would have to be ~0.1 torr for half of cytochrome *c* oxidase to be saturated with CO at COHb encountered in heavy smokers (10%).

Other studies of respiring tissues found that CO/O_2 ratios five to 10 times higher were needed to inhibit O_2 uptake by 50% (summarized in 16). It is important to recognize, however, that CO must inhibit more than half of the oxidase to inhibit O_2 consumption by 50%. A reserve exists because unblocked cytochrome oxidase may accept electrons from more than one cytochrome *c* molecule. Thus, oxidase inhibition increases mitochondrial reduction state, but O_2 consumption falls more slowly than expected for a strictly linear respiratory chain (cushioning). The cellular implications of partial electron transport inhibition by CO, which increases reduction state without energy failure, have not been investigated to any significant extent.

Despite the high value of its *in vitro* CO binding constant, CO binding to cytochrome *c* oxidase has been observed *in vivo*. Tissues with high O_2 requirements tend to have steep intracellular PO_2 gradients and maintain a small fraction of the cytochrome *c* oxidase in the reduced state. The amount of reduced enzyme increases as hypoxia develops (46), which allows any CO present to then bind in the absence of O_2 (infinite CO/O_2). The presence of the cytochrome a_3–CO complex is readily detected in the brain of bloodless animals (72) and in hemoglobin-circulated animals exposed to CO (6). Mitochondrial redox responses are measurable *in vivo* at arterial CO/O_2 ratios of <0.01. Cytochrome *c* oxidase–CO ligand formation increases the reduction level in the cytochrome $b–c_1$ region of the respiratory chain *in vivo* (71) and generates oxidative stress (74). As PO_2 declines, CO-mediated increases in mitochondrial reduction state can be observed by differential spectroscopy *in vivo* (see Fig. 2).

It has been known for years that oxidation of CO in the body to CO_2 proceeds more slowly than the rate of endogenous CO production. The rate of CO oxidation, however, increases in proportion to the tissue CO store (53). Oxidation of CO to CO_2 by reduced cytochrome *c* oxidase was reported in 1965 (101). Ten years later, Young *et al.* showed that oxidized cytochrome oxidase promotes CO oxygenation (117). Later it was found that cytochrome oxidase catalyzed the reaction at a CO/O_2 ratio of four in heart and brain mitochondria (115). Furthermore, the CO/O_2 ratio determines the molecular configuration of the oxidase (116). High CO/O_2 ratios favor ferrous carbonyl formation, whereas intermediate CO/O_2 ratios favor the CO-oxygenating species, and low CO/O_2 ratios favor the oxidized form. In any case, after CO binds to an oxidase molecule, that enzyme cannot transfer electrons to O_2 until after oxygenation of CO is complete (116). The ease with which CO is oxygenated to CO_2 by cytochrome *c* oxidase raises the possibility that this metabolic pathway plays an important role in regulating the effects of endogenous CO in tissues.

CO AND CELLULAR HYPOXIA

The sections above emphasize the importance of O_2 in the effects of CO on Hp binding. CO-Hp effects are augmented at low PO_2 because CO binds only ferrous Hp, which are predominant in reductive environments. Our knowledge of the behavior of CO in hypoxia also has assisted our understand-

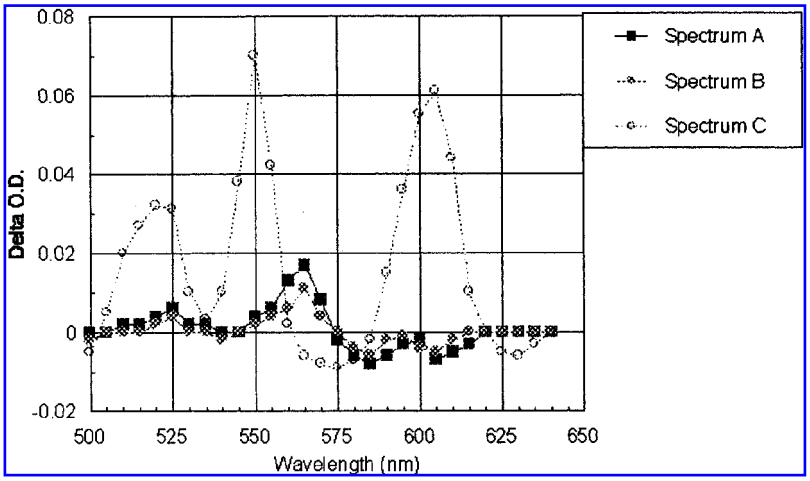

FIG. 2. Mitochondrial effect of CO after decreasing cellular PO_2 *in vivo*. A rat (postsplenectomy) was exchange-transfused with 10 blood volumes of fluorocarbon emulsion while breathing 100% O_2. Spectrum of the brain cortex was obtained by scanning reflectance spectroscopy in the visible region. Spectrum A shows the steady-state difference between breathing 99% O_2 + 1% CO minus 100% O_2. The loss of absorption at 605 nm and small increase at 590 nm indicate CO binding to the reduced a_3 component of cytochrome *c* oxidase. The increase in absorption at 564 nm is consistent with reduction of mitochondrial *b*-type cytochrome. Spectrum B is the steady-state difference between breathing 90% O_2 + 1% CO minus 99% O_2 + 1% CO. The decrease in inspired O_2 concentration decreased arterial PO_2 by ~10%. The resulting decrease in cellular PO_2 increased the amount of CO binding to cytochrome a_3 and further increased the reduction level of cytochrome *b* (seen at 564 nm). Spectrum C shows the difference spectrum for the fully reduced state (100% N_2) minus 90% O_2 + 1% CO. The absorption peaks of cytochromes *a* (605 nm) and *c* (551 nm) are apparent, however, the cytochrome *b* peak is missing due to earlier reduction by the effects of CO on cytochrome a_3. All difference spectra were normalized to 620 nm.

ing of how CO affects rapidly metabolizing tissues such as the brain and heart, where PO_2 tends to be low. For example, in the brain, CO hypoxia increases the reduction level of cytochrome *c* oxidase and favors binding of CO to the enzyme. This cellular CO uptake occurs at high COHb (6) and is slowly reversed after CO hypoxia (7). It is not clear where the lower limit lies for this effect on mitochondria. In addition, this phenomenon has been demonstrated *in vivo* in the beating heart (89). The point to be emphasized is that tissue O_2 depletion allows CO to bind cellular Hp noncompetitively.

The recovery of Hp function after CO binding also depends on the PO_2 in the tissue. In the brain, where recovery of cytochrome a,a_3 redox state after hypoxia depends on tissue PO_2 during reoxygenation, O_2 and CO compete for cytochrome a,a_3. In rats exposed to sufficient CO to interfere with cerebral energy metabolism, depletion of high-energy stores (phosphocreatine) and cellular acidosis can worsen after CO exposure despite removal of COHb (7). Deteriorating energy metabolism during reoxygenation correlates with cytochrome a,a_3 inhibition and can be prevented by hyperoxygenation, again indicating the critical competition between CO and O_2 at Hp binding sites.

Prolonged alveolar hypoxia induces responses in the pulmonary system, including right ventricular hypertrophy and pulmonary hypertension (88, 100). Similar changes are brought about by CO and have been attributed to hypoxia of vascular cells and ventricular myocytes (69). Acute hypoxia reversibly constricts the pulmonary arteries (hypoxic pulmonary vasoconstriction; HPV) whereas chronic hypoxia structurally modifies pulmonary vascular smooth muscle by stimulating cell proliferation and hypertrophy, elevating vascular pressures.

Exogenous CO activates guanylate cyclase, thereby enhancing cyclic GMP production and smooth muscle relaxation, but the importance of endogenous CO in the hierarchy of regulators of vascular tone has not yet been established (9, 10, 59, 92). This problem is due to the potential interactions of CO and NO with Fe(II) and lack of direct evidence for *in situ* CO binding to cellular constituents, including guanylate cyclase, integrally involved in the signaling pathways. Although some evidence implicates endogenous CO as a vasodilator in systemic blood vessels, little is known about its role in pulmonary vessels, which constrict instead of dilating during hypoxia. In dogs, ventilation with CO prevents HPV, and the evidence suggests a cytochrome P_{450} mechanism (56). In contrast, low concentrations of CO do not prevent HPV in isolated rat lungs (9). Although the importance of CO in modulating pulmonary vascular changes in hypoxia is unclear, one study showed a delay in the appearance of hypoxic pulmonary hypertension in neonatal rats exposed to CO (70).

During hypoxia, endogenous CO production continues even though HO requires O_2 to degrade heme and release CO. An important determinant of the enzyme's function in hypoxia is its K_m for O_2. The apparent K_m for O_2 of HO in liver microsomes is 12 μM (~8 torr); thus, the enzyme remains active during moderate to severe hypoxia (see Fig. 3). In comparison, NOSs, which also require molecular O_2, may have K_m for O_2 of two to four times greater than HO (77). Therefore, NO production may be more susceptible to limitation by hypoxia than the rate of endogenous CO production. Furthermore, CO produced by HO can bind to and inactivate NOS and decrease enzyme synthesis (25).

Hypoxia increases HO-1 protein, activity, and mRNA, and cyclic GMP levels in cultured vascular smooth muscle cells (59), and increases HO-1 mRNA in rat lungs after 2 h (50). By continuing to produce CO, HO-1 may potentially oppose

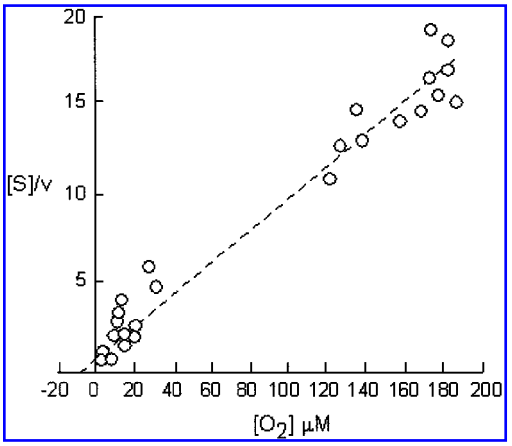

FIG. 3. Apparent K_m for O_2 of HO isolated from rat liver microsomes. The activity of the enzyme was measured as a function of PO_2. Data have been graphed on a plot of [S]/V versus [S], where S is oxygen concentration and V is the reaction velocity. The apparent K_m is 12 μM (courtesy of M.S. Carraway).

hypoxic vasoconstriction. Not unexpectedly, CO at supraphysiological concentrations (5%) inhibits proliferation of cultured smooth muscle cells (60); whether this means HO-1 activity actually delays pulmonary hypertension in chronic hypoxia is unknown. HO-1 induction is regulated in part by hypoxia inducible factor-1 (50), which activates hypoxia responsive genes such as erythropoietin, NOS, vascular endothelial growth factor, and glycolytic enzymes (65, 84). Regulation of HO-1 gene expression by hypoxia inducible factor-1 suggests unique functions for the enzyme in hypoxia beyond a response to increased heme turnover. In any event, the stage is set for further exploration of the physiological role of endogenous CO production in hypoxia.

CO AND OXIDATIVE STRESS

Significant cellular oxidative stress is produced by exogenous CO exposure in vascular endothelium (98, 99) and mitochondria *in vivo* (74, 118). The extent to which these effects are due to hypoxia–reoxygenation or direct effects of CO on cellular Hp function has not always been clear. Vascular oxidative stress occurs after low-level CO exposure (100 ppm) and may persist for many hours (98). CO has been associated in rats with conversion of xanthine dehydrogenase to xanthine oxidase accompanied by increases in brain lipid peroxidation measured by formation of conjugated dienes; however, the mechanism is unknown (97). Inhibition of xanthine oxidase, which has been linked to superoxide production and oxidative organ damage, prevented the rise in conjugated dienes. In addition, superoxide dismutase and iron chelators decreased CO-dependent lipid peroxidation.

High concentrations of CO increase intracellular H_2O_2 production in the brain accompanied by increases in hydroxyl radical production and decreases in the reduced to oxidized glutathione (GSH/GSSG) ratio in mitochondria (73). Generation of oxidative stress by CO, at least in the brain, differs from that of simple hypoxia (74). The latter finding suggests CO brings into play unique cellular mechanisms of oxidant generation *in vivo*, which would be expected to produce distinct effects on cell injury, repair, and proliferation.

Another intriguing possibility is that small amounts of CO are produced by lipid peroxidation in the body during periods of oxidative stress (61, 104, 114). CO formation by NADPH-dependent peroxidation of microsomal lipids was reported first in 1968 (61), and subsequent studies confirmed production of CO during iron-ascorbate-catalyzed peroxidation of membrane phospholipids (114) and tissue extracts (104). Increased CO excretion after carbon tetrachloride exposure *in vivo* also has been attributed in part to this mechanism (52). The physiological significance of this source of endogenous CO production is currently unknown and will require investigation in the future.

SUMMARY AND CONCLUSIONS

The biochemical effects of CO are complex and depend largely on the ability of the molecule to bind various Hp and inhibit or otherwise alter their functions. Thus, a multiplicity of actions of CO of both exogenous and endogenous derivation is expected to depend significantly on the concentrations of both CO and reduced transition metals, *e.g.,* Fe(II), in relation to the availability of molecular O_2. In this respect, the CO/O_2 ratio and O_2-dependent changes in the redox state of the cell or different compartments within a cell assume critical importance in the effects of CO on specific protein functions. Because CO may influence the reactions involving Hp in different ways, it can be expected to have both prooxidant and antioxidant effects in the cell. These Hp relationships also govern how changes in cellular CO concentration influence the complex physiological and pathological effects of NO and the relationships of these two gases to metal-catalyzed oxidative reactions in cells.

ABBREVIATIONS

CaO_2, arterial oxygen content; CO, carbon monoxide; COHb, carboxyhemoglobin; COMb, carboxymyoglobin; HO, heme oxygenase; Hp, heme protein; HPV, hypoxic pulmonary vasoconstriction; K_m, Michaelis–Menten constant; NO, nitric oxide; NOS, nitric oxide synthase; ODC, oxygen dissociation curve; PCO and PO_2, partial pressure of CO and O_2, respectively; redox, oxidation–reduction; SaO_2, arterial oxygen saturation; STP, standard temperature and pressure.

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Received for publication June 15, 2001; accepted August 15, 2001.

This article has been cited by:

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