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Forum Review

Measurement of Endogenous Carbon Monoxide Formation in Biological Systems

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ABSTRACT

Endogenous carbon monoxide (CO) formation has been measured in different biological systems using a variety of analytical procedures. The methods include gas chromatography–reduction gas detection, gas chromatography–mass spectroscopic detection, laser sensor–infrared absorption, UV-visible spectrophotometric measurement of CO–hemoglobin or CO–myoglobin complex, and formation of ^{14}CO from ^{14}C-heme formed following [2-^{14}C]glycine administration. CO formation ranged from a low of 0.029 nmol/mg of protein/h in chorionic villi of term human placenta to a high of 0.28 nmol/mg of protein/h in rat olfactory receptor neurons in culture and rat liver perfusate. Antioxid. Redox Signal. 4, 271–277.

INTRODUCTION

Approximately a decade ago, we observed that carbon monoxide (CO) relaxed smooth muscle when bubbled into a tissue bath containing Krebs' solution in which rabbit aortic smooth muscle, precontracted with phenylephrine, was mounted (13, 14). An examination of the literature revealed that CO had previously been reported to relax the lamb ductus venosus sphincter (2), several dog arterial preparations (8, 21), and several rat arterial preparations (27). Moreover, CO had been reported to share the biochemical property of nitric oxide (NO) to activate soluble guanylyl cyclase (4, 6). With this background we published an article in 1991 entitled "Does carbon monoxide have a physiological function?" (18). We suggested that CO, which is formed endogenously from heme catabolism by heme oxygenase (HO) and which shares some of the chemical and biological properties of NO, may play a similar role. We hypothesized that the heme-CO pathway would play a similar role to the L-arginine-NO pathway, which is accepted as a widespread signal transduction mechanism for the regulation of cell function and communication (21). We proposed that CO would bind to the iron atom of the heme moiety of soluble guanylyl cyclase and result in activation of this enzyme, followed by elevation of cyclic GMP (cGMP) content, which would lead through a biological cascade to the physiological effect (see Fig. 1). In our original article, we outlined a series of experimental investigations that needed to be conducted in order to test the hypothesis that CO has a physiological function. Among these were the following: (a) measurement of CO production by various tissues; (b) quantification of the amount of CO formed in tissues, to determine if it was sufficient to produce physiological effects; and (c) determination of the physiological effects of inhibitors of HO.

The objective of the present article is to review the analytical procedures for the measurement of endogenous formation of CO in a variety of tissues that have been developed. The term "endogenous formation of CO," as used in this article, refers to formation of CO in tissue in the absence of additional heme substrate or the cofactor NADPH. Where possible, we will describe the correlation of endogenous CO formation with a physiological parameter. The source of endogenous CO could be either HO-1 (inducible form), HO-2 (constitutive form) (17), or a combination of both isozymes. The role of a newly identified isozyme, HO-3 (19), remains to be clarified.

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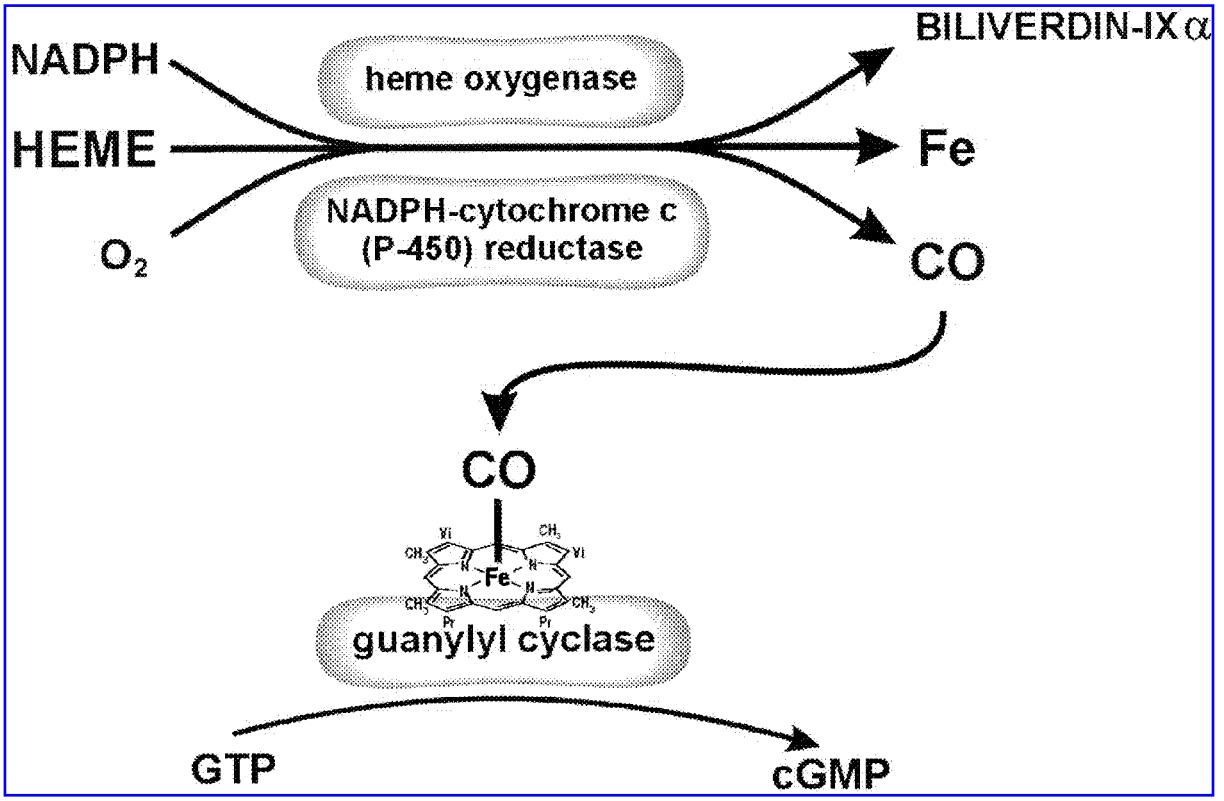

FIG. 1. Schematic illustration of the formation of biliverdin-IXα, iron (Fe) and carbon monoxide (CO) following heme metabolism, catalyzed by heme oxygenase acting in concert with NADPH-cytochrome c (P-450) reductase. It is proposed that CO binds to the iron atom of the heme moiety of soluble guanylyl cyclase in the cell in which CO is produced or in an adjacent cell. Activation of guanylyl cyclase results in elevated cGMP content, which leads via a series of enzymatic reactions to the physiological effect, e.g., smooth muscle relaxation.

MEASUREMENT OF CO BY GAS CHROMATOGRAPHY–REDUCTION GAS DETECTION (GC-RGD)

In hippocampus of guinea pig

Gas chromatography coupled to a suitable detector is considered to be an ideal method for measurement of low CO concentration (32, 33). Advantages of this method include precision, accurate calibration with standard gas, and low susceptibility to interfering substances. Further selectivity of the method is achieved by a chemical reduction involving CO and HgO at 210°C, resulting in the release of elemental Hg, which is detected spectrophotometrically in the gas phase at 254 nm (32, 33). We utilized this method (Fig. 2) to measure endogenous CO formation in hippocampal slices of the guinea pig (5). Transverse hippocampal slices of fetal and adult guinea pig were placed in artificial cerebrospinal fluid in sealed vials and incubated for 30 min at 37°C to allow newly formed CO to collect in the headspace gas. The amount of CO was quantitated by the GC-RGD procedure described above (32, 33). A higher rate of hippocampal CO formation was found in the fetus at gestational day (GD) 50 and GD62 (term, about GD68) compared with the adult. The CO formation rate at GD62 was 0.034 nmol/mg dry weight/h (0.094 nmol/mg of protein/h; Table 1). The data are consistent with our hypothesis of a physiological role for CO in cell function and communication.

In chorionic villi of term human placenta

It has been suggested that CO plays a complementary role with NO in the regulation of placental hemodynamics (15, 20, 23, 24, 26). We and others have demonstrated the presence of HO in term human placenta by measurement of enzymatic activity (20) and by means of immunohistochemistry (15). We have initiated experiments to determine whether endogenous CO formation occurs in intact human placenta. Samples of freshly isolated chorionic villi of term human placenta from cesarean-section delivery were incubated in vials containing Krebs' solution (pH 7.4) at 37°C during a 60-min time course. CO formation in the headspace gas was determined by the gas chromatographic procedure described previously. In an initial experiment, time-dependent formation of endogenous CO was measured at a rate of 0.029 nmol/mg of protein/h (Table 1). This finding is consistent with the hypothesis that CO plays a role in the regulation of placental hemodynamics.

In rat and mouse tissues

It has been pointed out that *in vitro* measurement of HO enzymatic activity indicates only the potential for *in vivo* CO formation. However, to determine the amounts of CO that could be formed under various *in vivo* conditions, it may be useful to measure tissue CO concentration. A method has been

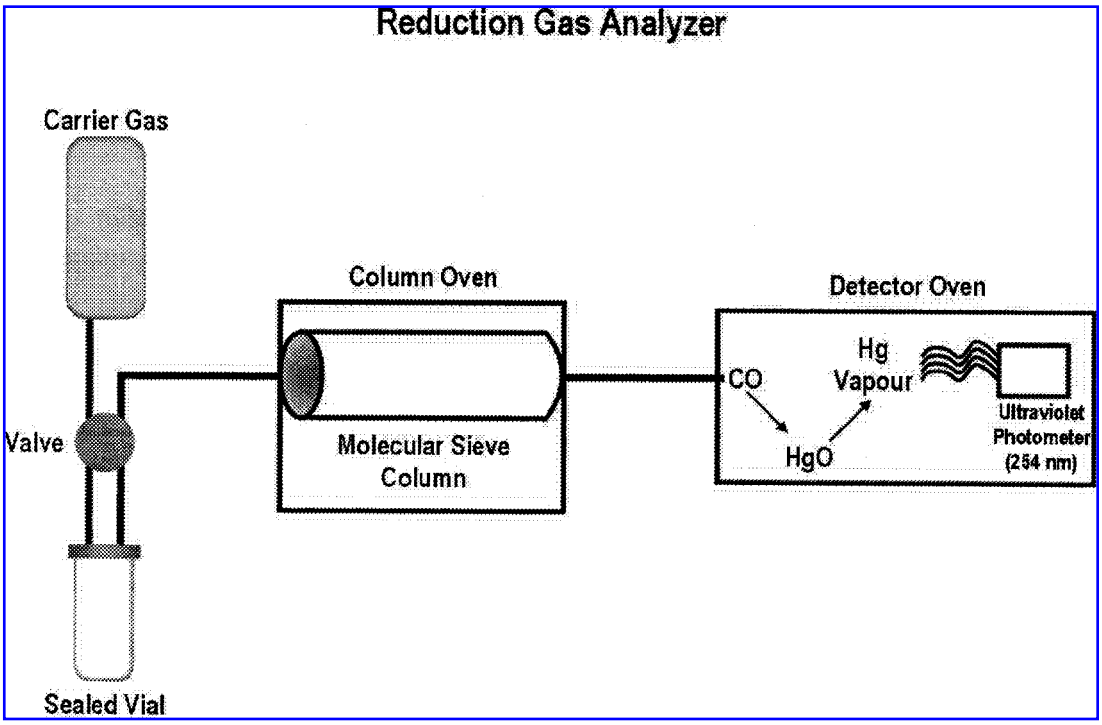

FIG. 2. Schematic illustration of the gas chromatographic–reduction gas detection method for separation and measurement of carbon monoxide in headspace gas.

developed for measuring CO concentration in tissues obtained from adult rats and mice (11, 34). The tissues are blanched by perfusion or devascularized and then sonicated in cold phosphate buffer, and aliquots of sonicate are incubated in vials at 0°C for 30 min with 50% (wt/vol) sulfosalicylic acid. The CO released in the vial headspace is measured by the GC-RGD method described previously (32, 33). In preliminary experiments, it was shown that blood had the highest CO concentration. Tissues such as spleen, heart, kidney, skeletal muscle, and liver, which contain substantial levels of hemoproteins, had higher concentrations of CO than brain, lung, intestine, and testes. The highest CO concentration values, other than in blood, were found in rat spleen (11 pmol of CO/mg fresh weight) and mouse muscle (10 pmol of CO/mg fresh weight).

MEASUREMENT OF CO BY QUANTITATION OF HEMOPROTEIN-BOUND CO

In human myometrium

CO formation by tissue explants of human nonlaboring pregnant myometrium at term was quantitated by measuring the percentage of carboxyhemoglobin relative to hemoglobin in the conditioned medium by UV-visible spectrophotometry (1). When the tissue was incubated with progesterone, an approximately sixfold increase in CO production was observed after 2 h. Concentration of CO decreased thereafter and returned to control value after 12 h. Progesterone was found to induce HO-1 protein synthesis and to induce the expression of HO-1 and HO-2 mRNAs. Activation of the HO-CO pathway in myometrium by hemin, a HO inducer, inhibited spontaneous contractility of human nonlaboring pregnant myometrial strips. The authors suggest that the induction of HO in the human myometrium leads to CO production that limits uterine contractility in pregnant myometrium.

In rat liver

Suematsu *et al.* (29, 30) have studied the possible role of CO as an endogenous modulator of sinusoidal tone in perfused rat liver. The livers were perfused with Krebs'–Henseleit bicarbonate-buffered solution (pH 7.4, 37°C) gassed with a mixture of 95% O_2 and 5% CO_2. Bile output was monitored at 5-min intervals via a cannula placed in the common bile duct. Effluent buffer was collected from the hepatic venous outlet, and the concentration of CO was measured spectrophotometrically by chemical reaction of ferrous myoglobin with CO to form the ferrous-CO complex of myoglobin. The CO concentration was found to be 0.7 nmol/min/g of liver (0.28 nmol/mg of protein/h; Table 1). To determine whether endogenous CO served as a vasorelaxant in the hepatic microcirculation, dual-color microfluorography was used to visualize the microvasculature by alternately monitoring sinusoidal lining and fat-storing Ito cells. When zinc protoporphyrin (ZnPP), an inhibitor of HO, was present in the perfusate, vascular resistance was elevated by 30%, baseline CO generation could not be measured, and constriction was found to occur predominantly in local sinusoidal segments colocalized with Ito cells. On the basis of these findings, it was proposed that CO functions as an endogenous modulator of hepatic sinusoidal perfusion and that Ito cells

TABLE 1. ENDOGENOUS CO FORMATION IN BIOLOGICAL SYSTEMS

Tissue preparation	*Analytical method*	*Rate of release of CO (nmol/mg of protein/h)*
Rat olfactory receptor neurons in culture	Radioactive [2-^{14}C]glycine	0.28
Rat cerebellar granule cells in culture	Radioactive [2-^{14}C]glycine	0.052
Rat interlobar artery	GC-MS*	0.095
Rat aortic vascular smooth muscle cells in culture	Laser sensor-infrared absorption	0.072
Chorionic villi of term human placenta	GC-RGD†	0.029
Fetal guinea pig hippocampus	GC-RGD	0.094‡
Rat liver perfusate	CO-myoglobin	0.28§
Rat liver hepatocytes in culture	CO-myoglobin	0.18§

*GC-MS, gas chromatography–mass spectrometry.

†GC-RGD, gas chromatography–reduction gas detection.

‡This value has been derived from the original data, expressed per milligram dry weight, on the basis of data showing that the protein content of mammalian brain is ~36% of dry weight (36).

§This value has been derived from the original data, expressed per gram weight of tissue, on the basis of data that the protein content of mammalian liver is ~15% of wet weight (36).

are involved in the relaxing mechanism. As the CO concentration that was required to increase cGMP in cultured Ito cells was ~100 times higher than that found in the perfused liver, it was suggested that CO elicited vasorelaxation by a cGMP-independent pathway. Several other investigators have provided evidence for the involvement of a cGMP-independent pathway (25, 35) in CO-induced vasorelaxation, and for CO-induced vasorelaxation in small-diameter blood vessels via the opening of high-conductance K_{Ca} channels. In a subsequent study, spectrophotometric measurement of CO-myoglobin was used to determine CO formation in the culture medium of isolated rat hepatocytes (7). The rate of CO formation was 0.45 nmol/min/g of liver (0.18 nmol/mg of protein/h; Table 1), which is comparable to the CO formation rate measured in the venous perfusate of rat liver (0.7 nmol/min/g of liver). On the basis of these results, it was suggested that hepatocytes are a major source of CO generation in the perfused rat liver.

MEASUREMENT OF CO BY QUANTITATION OF ^{14}C-LABELED COMPOUND

In rat olfactory bulb and cerebellar granule cells in culture

Ingi and Ronnett (9) selected cultured rat olfactory receptor neurons to measure endogenous CO production and its relationship to cGMP levels. The decision to select this system for the study was based on the fact that NO formation is not measurable in this system, and therefore changes in cGMP content would be dependent on CO production. To measure CO production, primary cultures of olfactory receptor neurons were prepared from neonatal rats and incubated on each day of culture with [2-^{14}C]glycine for 5 h. As a result, the carbon atoms that link the four pyrrole rings of heme become labeled with ^{14}C by the [2-^{14}C]glycine molecule. The actions of HO on [^{14}C]heme result in the formation of the open-chain tetrapyrrole, biliverdin, and ^{14}CO from the α-carbon atom linking the vinyl-containing rings, A and B, of [^{14}C]heme. The rate of CO formation was 0.28 nmol/mg of protein/h (Table 1). Tissue cGMP concentration was found to change in parallel with CO concentration. Moreover, CO formation and cGMP concentration were inhibited by the HO inhibitors, ZnPP and zinc deuteroporphyrin IX 2,4-bisethylene glycol. The investigators interpret their data as consistent with the concept that CO serves as a neuronal messenger linked to cGMP formation.

Ingi *et al.* (10) measured endogenous CO formation in primary cultures of cerebellar granule cells that were prepared from neonatal rats. On each day of culture, the cultures were incubated with [2-^{14}C]glycine for 5 h and ^{14}CO formation was measured. In addition to ^{14}CO formation, cellular uptake of [^{14}C]glycine (Table 1), total cellular heme, and [^{14}C]heme and biosynthesis of [^{14}C]δ-aminolevulinic acid (δ-ALA) were measured. Through these measurements, it was determined that the rate of endogenous CO formation was 0.052 nmol/mg of protein/h, which was considerably less than that measured in olfactory receptor neurons (0.28 nmol/mg of protein/h). The HO inhibitor, ZnPP, inhibited ^{14}CO formation demonstrating that the major amount of endogenous CO was derived from the enzymatic biotransformation of heme by HO. An important criticism of the hypothesis that the HO/CO system has a physiological role is that there is insufficient heme available as a substrate for HO. The above investigators were able to demonstrate via their measurements of glycine, δ-ALA, and heme that rapid turnover of heme occurs in these cultured neuronal cells, thereby producing appreciable amounts of CO. The investigators demonstrated that endogenous CO and exogenous CO at similar concentrations inhibited the NO-induced increase in cGMP content in these cells. They conclude that the major function of endogenous CO is to modulate the NO-cGMP signaling system in rat cerebellar granule cell cultures.

In rat aorta

To measure CO formation in rat aortic rings, Sammut *et al.* (28) constructed special equipment containing two separate upper and lower chambers separated by a 0.4-μm filter. Rat aortic tissue was incubated for 6 h in the lower chamber, which contained [2-^{14}C]glycine in oxygenated Krebs'–Henseleit buffer at 37°C. The [2-^{14}C]glycine was biotransformed into [^{14}C]heme, which was metabolized by HO in the rat aortic tissue to biliverdin and ^{14}CO. The ^{14}CO diffused into the upper chamber that contained a solution of deoxyhemoglobin and formed a ^{14}CO-deoxyhemoglobin complex, which was quantitated by liquid scintillation spectrometry. cGMP content was measured in the freeze-clamped aortic tissue. When the rat aortic rings were treated for 1 hour with the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), HO-1 was induced and ^{14}CO formation and cGMP content were markedly increased relative to control. The investigators conclude that regulation of vessel tone in untreated vessels is due primarily to the NO synthase/NO pathway. However, when blood vessel HO-1 is induced, then the HO/CO pathway plays an important role in CO production and regulation of blood vessel tone.

MEASUREMENT OF CO BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC-MS)

In rat interlobar artery

Balazy and Jiang (3) separated CO from nitrogen and other gases on a GS-Molsieve capillary column, 30 m in length and with an internal diameter of 0.53 mm. Previously, quantitation of CO by mass spectrometry was hindered by lack of an appropriate standard. These investigators have overcome this problem by using stable isotope-labeled CO, *e.g.,* ^{13}C^{18}O, as an internal standard. A series of CO concentrations in water was prepared, and a known amount of ^{13}C^{18}O dissolved in water was added to each of the CO solutions. The headspace gas from these solutions was analyzed by means of GC-MS, and there was a linear relationship between CO concentration and the ratio of intensity of ions at *m/z* 28 and 31. The investigators conclude that this technique provides a sensitive assay for CO in biological fluids. This analytical procedure was used to measure CO formation from rat interlobar artery during incubation with Krebs' buffer (12). CO was found to be formed at a rate of 0.095 nmol/mg of protein/h (Table 1), and its formation was markedly inhibited by chromium mesoporphyrin, a HO inhibitor.

MEASUREMENT OF CO BY LASER SENSOR-INFRARED ABSORPTION

In rat aortic vascular smooth muscle cells

Morimoto *et al.* (22) carried out real-time measurement of endogenous CO production in rat aortic vascular smooth muscle cells in culture using an ultrasensitive laser sensor and measurement of infrared absorption at 4.67 μm for CO quantitation without interference from other gases. The investigators have compared the sensitivity of their method with that of the GC-RGD method of Vreman and Stevenson (32, 33). In contrast to the GC-RGD instrument with a lower limit of sensitivity of 12 ppb of CO, the ultrasensitive laser sensor instrument had a sensitivity limit of 4.5 ppb of CO. The basal rate of CO production was 0.072 nmol/mg of protein/h (Table 1). Increased CO production was found after treatment of cells with hemin for 24 h. The investigators attribute the increase in CO production after hemin addition to induction of the HO-1 enzyme. Decreased CO production in the hemin-treated cells occurred after addition of the HO inhibitor, tin protoporphyrin. The investigators have provided a useful new analytical procedure for noninvasive and real-time quantitation of low CO concentrations, which should be valuable in studies of the physiological role of CO.

SUMMARY

Endogenous CO formation has been measured in different biological systems using a variety of analytical methods, and it is of interest to compare the basal rates of CO formation that were determined experimentally. For this comparison, all rates of CO formation have been converted to nmol of CO/mg of protein/h, and the values are presented in Table 1. Despite the fact that a variety of methods have been used for measurement of CO formation by different tissues, the rate of CO formation varies over a relatively narrow range from 0.029 nmol/mg of protein/h in chorionic villi of term human placenta to 0.28 nmol/mg of protein/h in cultures of rat olfactory receptor neurons and in rat liver perfusate. Certain brain regions and liver have high rates of CO formation, which may be due, at least in part, to the fact that liver and brain have higher levels of HO activity than most other tissues (16).

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ABBREVIATIONS

δ-ALA, δ-aminolevulinic acid; cGMP, cyclic GMP; CO, carbon monoxide; GC-MS, gas chromatography–mass spectrometry; GC-RGD, gas chromatography–reduction gas detection; GD, gestational day; HO, heme oxygenase; NO, nitric oxide; ZnPP, zinc protoporphyrin.

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