HEI HEALTH EFFECTS INSTITUTE

Effects of Carbon Monoxide on Isolated Heart Muscle Cells

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 62

Effects of Carbon Monoxide on Heart Muscle Cells

BACKGROUND

Human exposure to carbon monoxide can occur from automobile emissions, industrial processes, sidestream or mainstream cigarette smoke, and poorly ventilated appliances such as space heaters and gas stoves. The U.S. Environmental Protection Agency has set National Ambient Air Quality Standards for carbon monoxide of 9 ppm averaged over eight hours, and 35 ppm averaged over 1 hour. Despite reductions in ambient carbon monoxide concentrations over the past decade, approximately 20 million people live in areas of the United States that exceed the carbon monoxide standard.

Exposure to high concentrations of carbon monoxide is clearly dangerous and can be lethal. Inhaling low levels of carbon monoxide has the potential to produce adverse health effects, especially in people with coronary artery disease. Most researchers consider the major mechanism for the toxicity of carbon monoxide to be its ability to compete with oxygen for binding to hemoglobin, the protein that transports oxygen through the bloodstream and releases it to cells and tissues. Cells need oxygen to produce adenosine triphosphate (ATP), the energy source for muscle contraction and other body functions. The binding of carbon monoxide to hemoglobin, forming carboxyhemoglobin, reduces the amount of oxygen available to the heart muscle and other tissues for synthesizing ATP.

Some researchers speculate that an additional mechanism for carbon monoxide toxicity may involve carbon monoxide binding to myoglobin in the heart muscle cells called myocytes. They propose that under normal conditions myoglobin binds the oxygen released by hemoglobin and transports it to the site of ATP synthesis. Carbon monoxide, after being released from hemoglobin, could also bind to myoglobin, forming carboxymyoglobin, thereby reducing the amount of oxygen transported by myoglobin, diminishing the amount of ATP synthesized, and decreasing the ability of muscle cells to contract. Drs. Beatrice and Jonathan Wittenberg sought evidence for this additional mechanism of the cardiotoxicity of carbon monoxide.

APPROACH

The investigators isolated myocytes from rat hearts and exposed them in a chamber for one hour to gas mixtures containing a wide range of both oxygen and carbon monoxide concentrations. In this way they produced carboxymyoglobin levels ranging from 0% to 100% of the total myoglobin. One strength of the experimental design was the absence of hemoglobin, which also binds avidly to carbon monoxide. The investigators made simultaneous measurements of oxygen uptake and carboxymyoglobin content, and also determined the effect of increasing carboxymyoglobin levels on ATP synthesis. Thus, they correlated carboxymyoglobin levels in the exposure chamber with oxygen uptake and energy production by the myocytes.

RESULTS

The investigators reported that when they grouped their data into three ranges of carboxymyoglobin, the uptake of oxygen by cardiac myocytes was suppressed when the carboxymyoglobin levels exceeded 40%. (These levels are higher than those that would be expected from normal ambient exposure to carbon monoxide.) However, an independent statistical analysis showed only a weak relationship between increased carboxymyoglobin levels and decreased oxygen uptake. The investigators also obtained suggestive evidence that ATP synthesis decreased when carboxymyoglobin levels were elevated. However, this is a complex area and, in the absence of additional research, factors other than carboxymyoglobin cannot be excluded as being responsible for the observed effects.

The extent to which these findings with isolated rat cells apply to the intact rat heart, to lower carbon monoxide levels, or to the human heart is still unknown. The investigators' hypothesis, that increased carboxymyoglobin reduces ATP synthesis, which in turn decreases the work output of the heart, may be correct. However, future research is needed to test this hypothesis in more relevant experimental systems, such as intact tissues and animals, before we can conclude that carbon monoxide binding to myoglobin is a mechanism of toxicity that is relevant to low-level environmental exposures.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1989 to 1992. This study was conducted by Drs. Beatrice and Jonathan Wittenberg at Albert Einstein College of Medicine. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

By sequestering intracellular myoglobin of cardiac muscle cells in the nonfunctioning carboxymyoglobin form, carbon monoxide blocks myoglobin-facilitated diffusion of oxygen, as well as myoglobin-mediated oxidative phosphorylation. Here, we explore the hypothesis that the carbon monoxide blockade of myoglobin function may be responsible at the cellular level for a component of the cardiotoxicity of carbon monoxide observed during exercise. Suspensions of isolated rat cardiac myocytes were held in near steady states of oxygen pressure near the intracellular partial pressure of oxygen of the working heart (2 to 5 torr) and near the end-venous partial pressure of oxygen (20 torr). These suspensions were exposed to CO at low pressure (0.07 to 70 torr; 90 to 90,000 parts per million). The fraction of intracellular carboxymyoglobin, determined spectrophotometrically, was in good agreement with the fraction predicted from the ratio of carbon monoxide partial pressure to oxygen partial pressure. The effects observed were related to the fraction of intracellular myoglobin bound to CO. At physiological oxygen pressures no greater than 5 torr, after sequestration of approximately 50% of the myoglobin, steady-state oxygen uptake decreased significantly and was significantly less than the respiration of cell groups for which the fraction of carboxymyoglobin was 0% to 40%. When respiration is diminished, the rate of aerobic adenosine triphosphate synthesis (oxidative phosphorylation) also decreases. As in the whole heart, cytoplasmic adenosine triphosphate concentration in isolated heart cells is controlled at a constant level by the creatine phosphokinase equilibrium. When adenosine triphosphate utilization is unchanged, a sensitive monitor of the decreased adenosine triphosphate synthesis is the ratio of phophocreatine to adenosine triphosphate. When carboxymyoglobin was at least 40% of the total intracellular myoglobin, we found that the ratio of phosphocreatine to adenosine triphosphate in carbon monoxide–treated heart cells was significantly lower than that in control cells from the same preparation.

Thus, we concluded that sequestering intracellular myoglobin as carboxymyoglobin significantly decreased the rate of oxidative phosphorylation of isolated cardiac myocytes. We estimate that intracellular myoglobin-dependent oxidative phosphorylation will be inhibited when approximately 20% to 40% of the arterial hemoglobin in the whole animal is carboxyhemoglobin.

INTRODUCTION

At sublethal levels, carbon monoxide (CO)* binds to blood hemoglobin, thereby diminishing oxygen (O_2) delivery to the tissues and exerting toxic effects on the brain and heart. Specific inactivation of intracellular myoglobin decreases the work output of red skeletal muscle or cardiac muscle. This project addresses the cellular effects of low levels of CO on aerobic adenosine triphosphate (ATP) synthesis of heart muscle cells, which is directly related to the heart's ability to perform work.

Myoglobin present in cells of the heart and aerobic skeletal muscle binds CO with an affinity that is much less than that of blood hemoglobin and much greater than that of cellular cytochrome oxidase. When CO levels are increased in the presence of O_2, myoglobin becomes saturated long before cytochrome oxidase is affected (Wittenberg and Wittenberg 1987). Therefore, CO in the bloodstream will bind primarily to hemoglobin and then to myoglobin, before binding lethally to cytochrome oxidase (Agostoni et al. 1988; Sangalli and Bidanset 1990). Clinical trials have shown that with increasing levels of CO, the exercise-induced maximum respiratory rate decreases (Horvath et al. 1988). This decrease was independent of altitude; at maximum work loads, CO shifted reversibly into extravascular spaces. These conditions would be expected to load intracellular cardiac and skeletal muscle myoglobin with CO. In addition, it has been shown in dog gastrocnemius muscle that the O_2-dif-

This Investigators' Report is one part of Health Effects Institute Research Report Number 62, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Beatrice A. Wittenberg, Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461.

Although this document was produced with partial funding by the United States Environmental Protection Agency under assistance agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

* A list of abbreviations appears at the end of the Investigators' Report.

fusive conductance from blood to mitochondria was reduced by carboxyhemoglobin (COHb) in the blood (Hogan et al. 1990). This could be the result of inactivation of functional myoglobin in the muscle by CO.

Thus, intracellular myoglobin is a probable site for the specific cardiotoxicity of low concentrations of CO. The functions of intracellular myoglobin in the heart are only now being understood (Wittenberg and Wittenberg 1989). The current view is that specific inactivation of intracellular myoglobin does not kill the heart but diminishes the rate of respiratory O_2 uptake and, thus, the rate of oxidative phosphorylation as well. One-third of the resting O_2 consumption of the cardiac muscle cell is mediated by a CO-sensitive pathway, myoglobin-mediated oxidative phosphorylation, demonstrated at superabundant O_2 pressures (Wittenberg and Wittenberg 1987). Evidence suggests that the intracellular O_2 pressure in the working heart is near 2 to 5 torr (Gayeski and Honig 1991). At these O_2 pressures, myoglobin is partially deoxygenated, and functional myoglobin facilitates O_2 diffusion within the sarcoplasm (Wittenberg 1970; Wittenberg and Wittenberg 1989). This pathway is also CO-sensitive. In the absence of functional myoglobin, O_2 arrives at intracellular mitochondria by the diffusive flow of dissolved diatomic O_2 to cytochrome oxidase. This pathway is inhibited only at very high, nearly lethal levels of CO (ratio of partial pressure of CO [P_{CO}] to partial pressure of O_2 [P_{O_2}] is at least 6) (Wittenberg and Wittenberg 1987).

This study was designed to explore the hypothesis that CO binding to the intracellular myoglobin of heart cells may be responsible for the decreased work output and decreased tolerance to limitations of O_2 supply observed in people exposed to low levels of CO (Allred et al. 1989; Sheps et al. 1990). Epidemiological studies are beset by problems of sample homogeneity, statistical analysis, diversity of populations, and the necessity to avoid lethal consequences. Understanding the effects of CO on the heart depends on analyzing the mechanism of its effects on heart cells. We have tried to define the lower limits of CO levels required to bind intracellular myoglobin and to diminish oxidative phosphorylation in resting heart cells. We established that by inhibiting the function of intracellular myoglobin in resting rat heart cells at physiological O_2 pressures, CO decreases the rate of oxidative phosphorylation of those cells.

METHODS

PREPARATION OF HEART CELLS

Isolated cells were prepared from the hearts of adult rats by a method described previously (Wittenberg et al. 1981, 1988). Briefly, hearts were perfused initially with low-calcium minimal essential medium (J.R.H. Biosciences, Denver, PA) containing 117 mM sodium chloride; 5.7 mM potassium chloride; 4.4 mM sodium bicarbonate; 1.5 mM sodium phosphate; 1.7 mM magnesium chloride; 21.1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 11.7 mM glucose, amino acids, and vitamins; 2 mM L-glutamine; 10 mM taurine; and 0.01 mM calcium chloride; pH was adjusted to 7.2 with sodium hydroxide. This solution was 285 mosm. The free calcium activity was 5 μM, as measured with a calcium ion-selective electrode (Möller, Glasblaserei, Zurich, Switzerland). After blood washout, collagenase (0.1%) (Worthington Type II, Worthington Biochemical Corp., Freehold, NJ) was added to the perfusion medium, and perfusion continued for 25 minutes. The tissue was minced, and cells were collected in incubation medium (minimum essential medium with 1 mM calcium chloride and 0.5% dialyzed bovine serum albumin, Fraction V). Intact rectangular cells were separated from damaged rounded cells and broken cell fragments by centrifugation through isotonic Percoll (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden). The final yield was 52 ± 16 mg protein of heart cells, of which 76% ± 5% were rectangular cells. Washed heart cells were resuspended in incubation medium to a concentration of 0.7×10^6 cells/mL to measure respiratory O_2 uptake, optical spectra, and energy metabolites of cell suspensions.

The suspensions contained rectangular heart cells, with clearly defined striations, with the sarcomere length nearly 1.9 μm. The cells were quiescent in 1 mM calcium chloride, but shortened and relengthened specifically in response to electrical stimulation, indicating functional integrity of the sarcolemma and functionally intact excitation-contraction coupling mechanisms. The ATP and phosphocreatine (PCr) levels were comparable to those in the whole heart; intracellular calcium levels and excitation-contraction coupling mechanisms were intact; intracellular myoglobin and intracellular mitochondrial cytochromes were maintained in different cellular compartments in concentrations comparable to those in the whole heart. The sarcolemma of these cells was functionally intact, as indicated by permeability criteria and electron microscopy. All rectangular, functionally intact cells excluded trypan blue, but this criterion is considered less sensitive than the other criteria (Wittenberg and Robinson 1981). Although visibly clean and free of noncardiac cells (including red blood cells), the suspension also contained rounded heart cells. These were hypercontracted, energy-depleted, or calcium-overloaded heart cells, which did not fulfill our criteria for functional integrity (see above). In our preparations, round heart cells had no ATP or PCr, some still excluded trypan blue, and the respiratory O_2 uptake was low and variable. Cells were counted before and after experimental manipulations to de-

termine the proportion of rectangular cells in suspension. The initial count before incubation showed that approximately 75% to 80% of the cells were rectangular. If the final count was less than 60% rectangular cells, the experiment was rejected. After the experiments, the suspensions usually contained 65% to 70% rectangular cells. The loss of rectangular cells during the experiment was of the same order of magnitude in different preparations. Respiratory rates were normalized to the initial aerobic respiratory rate of the same cell suspension. The ambient medium was maintained at pH of 7.2 throughout the experiments.

MEASUREMENT OF RESPIRATION AND OPTICAL SPECTRA

Respiration measurements were made under steady states of O_2 pressure and uptake (Wittenberg and Wittenberg 1985). A suspension of myocytes was stirred in a chamber containing both liquid (3.7 mL) and gas (5 mL) phases. Composition of the gas phase was set by a mass flow controller (Tylan, Torrance, CA). Solution O_2 pressure, which is the difference betweeen O_2 entering and O_2 consumed by the myocytes, was monitored by a sensitive polarographic O_2 electrode (model 2110, Orbisphere Laboratories, Geneva, Switzerland). At constant temperature and stirring rate, the rate of O_2 uptake is derived from the difference between the solution's P_{O_2} in the absence of cells and that in the presence of cells using a mass transfer coefficient determined daily. The chamber, a cylindrical quartz cuvette with a 1.5-cm light path, was held in a block with a thermostat, which was placed in the sample light beam of a spectrophotometer (model 14, Cary) equipped with a scattered transmission accessory. The temperature was 30°C.

Optical spectra of heart cell suspensions or myoglobin solutions were acquired digitally from 650 to 340 nm, and difference spectra were constructed using a data acquisition system (Aviv Associates, Lakewood, NJ). During the course of the experiment, voltage readings from the O_2 electrode and absorbance readings at 425 nm were recorded continuously.

MEASUREMENT OF INTRACELLULAR CARBOXYMYOGLOBIN

The extent of the intracellular myoglobin in combination with CO was monitored spectrophotometrically. Because intracellular myoglobin is half saturated with O_2 at a P_{O_2} of 1.3 torr at 30°C (Wittenberg and Wittenberg 1985), intracellular myoglobin was largely in the oxygenated form, with only a minor contribution from deoxymyoglobin when the ambient P_{O_2} was greater than 10 torr. Under these conditions (initial P_{O_2} greater than 10 torr), the fraction of myoglobin combined with CO was measured by comparing the fractional absorbance change at 422 nm minus 408 nm in the difference spectrum of a suspension of heart cells with and without added CO, with the change observed in the transition from 0% to 100% carboxymyoglobin (COMb) (Wittenberg and Wittenberg 1987) (see Figure 1b). At a P_{O_2} of 0.5 to 10 torr, there was considerable spectral contribution from deoxymyoglobin. Under these conditions, the extent of myoglobin ligation with CO was determined from the fractional increase at 425 nm in the direct spectrum (425 nm is near the absorption maximum of COMb [422 nm] and is isosbestic [optical densities of deoxymyoglobin and oxymyoglobin (O_2Mb) are the same at the isosbestic point] for the transition from O_2Mb to deoxymyoglobin [see Figure 1a]). In most cases, the fractional COMb determined spectrophotometri-

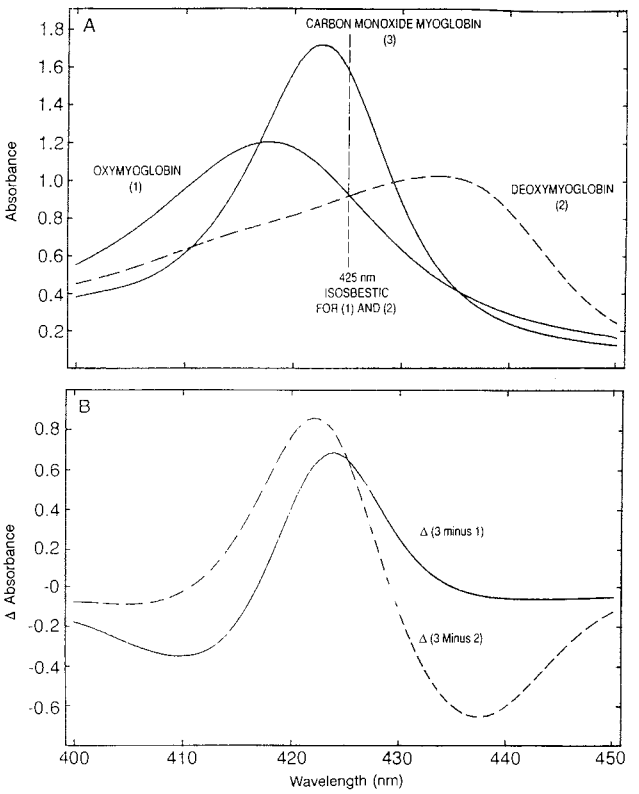

Figure 1. A: Direct spectra of purified rat (1) O_2Mb, (2) deoxymyoglobin, and (3) COMb in solution. At 425 nm, the optical densities of O_2Mb and deoxymyoglobin are the same, whereas the optical density of COMb is near its peak value. Consequently, in the presence of mixtures of O_2Mb and deoxymyoglobin, COMb is measured by the optical density change at 425 nm in direct spectra, without interference from changes in O_2Mb and deoxymyoglobin. **B: Difference spectra of purified rat COMb minus O_2Mb** (solid line) **and COMb minus deoxymyoglobin** (dashed line). The value of Δ absorption at 422 nm minus Δ absorption at 408 nm in the difference spectra for COMb minus O_2Mb (3 minus 1) is a sensitive index of the extent of conversion of O_2Mb to COMb in the absence of deoxymyoglobin. However, at physiological P_{O_2}, with O_2Mb, deoxymyoglobin, and COMb present simultaneously, these difference spectra cannot be used, so measurements are made at the isosbestic point (425 nm) (see Figure 2a) for O_2Mb and deoxymyoglobin.

cally was within the range of COMb calculated from the ambient P_{O_2} and P_{CO} during the course of the experiment, using a value for the partition coefficient $M = P_{O_2}/P_{CO} \times COMb/O_2Mb = 20$ (Wittenberg and Wittenberg 1987).

HEART CELL ENERGY METABOLITES

Samples of heart cell suspensions were deproteinized with ice-cold perchloric acid, and the supernatants were neutralized with cold potassium carbonate. After centrifugation, the supernatants were stored at −10°C. The PCr and ATP levels were determined by high-pressure liquid chromatography (Doeller and Wittenberg 1990). Samples of thawed, filtered extract (0.1 mL) were chromatographed by high-pressure liquid chromatography (series 400 pump, LC Autocontrol, and LC1–100 Integrator, Perkin-Elmer Corp., Norwalk, CT). The ATP and PCr were separated on a 15-cm anion exchange column (Partisil SAX 10, Whatman Inc., Clifton, NJ) by modifying the method of Harmsen and colleagues (1982). We subjected the samples to 5 mM ammonium phosphate, pH 2.85, for 10 minutes at 1 mL/min, followed by a linear gradient to 750 mM ammonium phosphate, pH 4.4, for 25 minutes at 2 mL/min. The column eluant's optical density was recorded at 210 nm.

DESIGN OF EXPERIMENTS

A sample of 2.6×10^6 heart cells was added to the chamber and equilibrated at 30°C for 20 minutes, with a flowing gas phase consisting of nitrogen and 5% to 9% O_2. With glucose in the medium, there was enough exogenous substrate for nine hours of incubation. During the equilibration period, the O_2 pressure dropped from an initial value to the steady-state O_2 pressure determined by the balance between O_2 inflow from the gas phase and O_2 uptake by cells in the fluid phase. A spectrum of aerobic heart cells was obtained. The fraction of O_2 in the inflowing gas was changed to achieve the initial steady-state P_{O_2} desired for the experiment. A new spectrum was obtained, and the steady-state rate of O_2 consumption was calculated under these initial conditions. The initial respiratory rate was always determined at P_{O_2} greater than 1 torr, because in this system, respiration becomes limited at lower O_2 pressures. After the initial equilibration and measurements were complete, CO was introduced into the inflow gas mixture. All subsequent measurements of respiration were normalized to the initial measurement and are reported as fractions of initial respiration. The total gas flow was maintained at 100 mL/min, and the percentage of O_2 was held constant. The kinetic traces of voltage (O_2 electrode) and absorption at 425 nm (near the COMb maximum) were initiated and maintained for one hour. The time course of a typical experiment is presented in Figure 2a. At the end of an hour, the kinetic traces were terminated, the spectrum of the heart cell suspension was recorded (Figure 2b), and the respiratory rate was calculated. To determine the fraction of intracellular myoglobin converted to COMb during the experiment, the CO concentration was increased to 5% for 10 minutes, and nitrogen was decreased so that the O_2 flow remained the same. The fully ligated intracellular COMb spectrum was recorded (Figure 2b), and the respiratory rate with myoglobin fully inhibited was calculated. The percentage of COMb was calculated from the difference at 422 nm minus 408 nm in the difference spectrum of aerated cells minus the cells treated with low CO, and divided by this difference between aerated cells and cells totally in the COMb form. Alternatively, in the presence of deoxymyo-

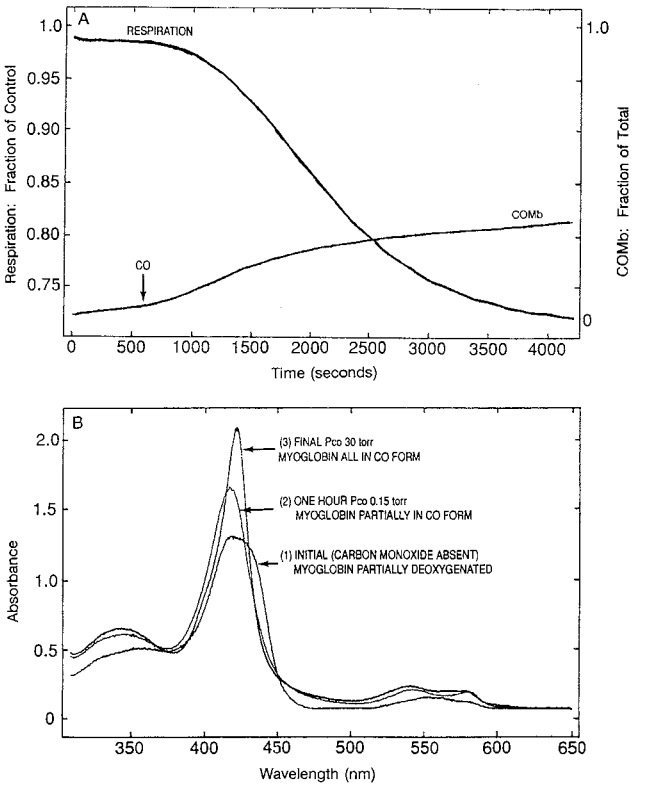

Figure 2. A: Time course of a representative experiment using a suspension of isolated heart cells. Carbon monoxide (180 ppm, 0.15 torr) was introduced after 600 seconds. The upper trace indicates respiration; the lower trace shows intracellular COMb. Under these conditions, medium P_{O_2} was 1.3 torr initially and increased to 12 torr after 4,200 seconds; 34% of intracellular myoglobin was converted to COMb. Combination was largely complete 15 minutes after CO was introduced; there was no detectable deoxymyoglobin after one hour. Respiration dropped to about 70% of the initial value one hour after CO was introduced. The substrate was 5.4 mM glucose. **B: Direct spectra of the heart cell suspension described in Figure 1a.** (1) Initially, CO was absent, myoglobin was partially deoxygenated, and the medium P_{O_2} was 1.3 torr. (2) After a one-hour exposure to CO, P_{CO} was 0.15 torr, the medium P_{O_2} was 12 torr, and 34% of intracellular myoglobin was converted to COMb. (3) This trace shows the final spectrum at saturating CO pressure. Myoglobin was completely converted to the COMb form with P_{CO} of 30 torr.

globin, the percentage of COMb was calculated by comparing the change in absorbance at 425 nm in the presence of low CO with the change in absorbance at 425 nm with maximal COMb.

The cells were removed rapidly from the chamber, deproteinized with perchloric acid, and frozen for high-performance liquid chromatography assay of PCr and ATP. Separate portions of the same cells at the same concentration had been incubated aerobically at 30°C in Erlenmeyer flasks swirled by a wrist-action shaker for equal periods of time. These cells were deproteinized separately to determine the maintenance of energy reserves in air in the absence of CO and to determine the paired control values. The ATP and PCr measurements were normalized for the fraction of rectangular cells at the end of the experiment. If the energy reserves of the control cells were low, the experiment was rejected. Preliminary experiments showed that under control conditions, ratios of PCr to ATP did not decrease significantly between 0 and 120 minutes of incubation, either in the observation chamber or in the Erlenmeyer flasks.

DATA HANDLING

Results are presented as mean ± standard error. The statistical significance of differences between experimental groups was determined using the unpaired *t* test, and analysis of variance (ANOVA) was determined using the Scheffe *F* test. The statistical significance of differences in a given preparation with and without CO was determined using the paired *t* test. Statistics were calculated with the Statview Graphics software package (Abacus Concepts, Berkeley, CA). Data are considered significantly different when $p \leq 0.05$.

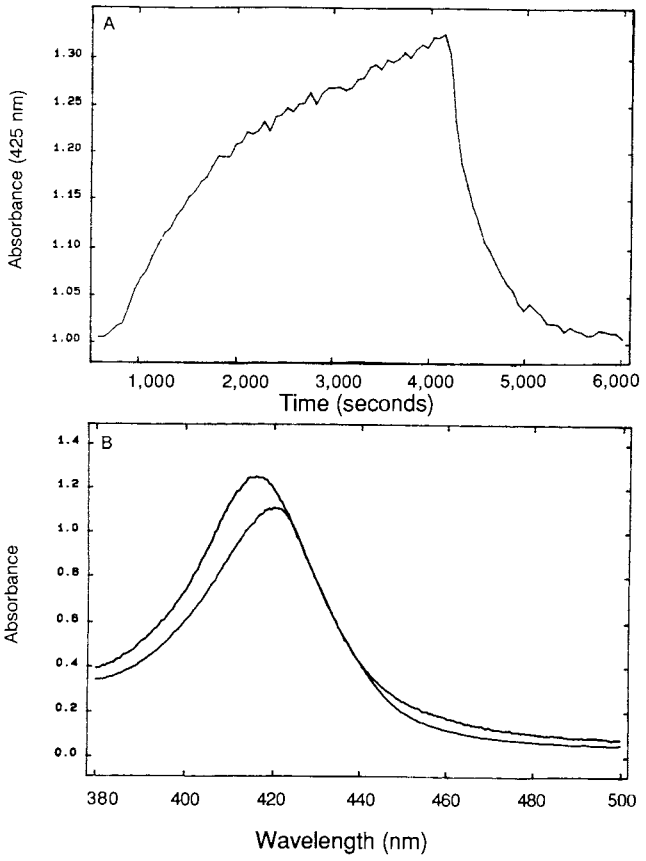

Figure 3. The reversibility of binding of carbon monoxide to intracellular myoglobin of isolated heart cells in stirred suspension. A: Time course of changes in absorbance at 425 nm. Increased absorbance indicates increase in COMb. CO at P_{CO} of 0.22 torr was introduced after 600 seconds and removed after 3,800 seconds. There was a rapid decrease in intracellular COMb after carbon monoxide was removed from the ambient solution, with P_{CO} at 0 torr and P_{O_2} at 56 torr. **B: Upper trace: direct spectrum of the heart cell suspension monitored at a single wavelength in Figure 3a**, after CO removal at the end of the kinetic trace. **Lower trace: direct spectrum of heart cells in a comparable experiment at the end of exposure to P_{CO} of 0.18 torr** for the entire experimental period.

RESULTS

EFFECTS OF CARBON MONOXIDE ON STEADY-STATE RESPIRATION

Typical effects of CO on steady-state respiration and the spectral absorption at 425 nm of a single suspension of heart cells are shown in Figure 2a. After CO was added, there was a relatively rapid increase in optical absorption, signaling partial conversion of intracellular myoglobin to the COMb form. Respiration, which had been maintained at a steady level, decreased to a new steady state. In the absence of CO, there was no change in optical density. However, the respiratory rate occasionally decreased. We cannot at this time account for the downward drift in respiration in the absence of CO, which may have been due to the loss of endogenous substrates, unidentified intracellular regulation in these resting cells, or, less probably, the loss of cell viability during the experiment.

Our study comprised 41 experiments, with varying partial pressures of extracellular CO (P_{CO}): 0 torr (n = 4); 0.074 torr (n = 4); 0.15 torr (n = 7); 0.2 torr (n = 4); 0.5 torr (n = 1); 0.74 torr (n = 3); 3.0 torr (n = 3); 7.4 torr (n = 2); 22 torr (n = 5); 30 to 37 torr (n = 6); and 74 torr (n = 2). These values correspond to 0, 90, 180, 270, 600, 900, 4,000, 9,000, 30,000, 40,000 and 90,000 ppm CO, respectively. At the onset of CO exposure, the P_{O_2} values were 0.2 to 5.0 torr (n = 20); 5 to 10 torr (n = 7); 10 to 20 torr (n = 10); and 20 to 35 torr (n = 4). Sequestering of myoglobin as COMb depended on the ratio of P_{CO} to P_{O_2} during each experiment,

as predicted from the experimentally determined partition coefficient $M = 20$ (Wittenberg and Wittenberg 1987).

In three experiments, the measured COMb was significantly lower than the calculated value based on the measured P_{O_2} and P_{CO} values. We could not distinguish whether experimental error or physiological response caused this discrepancy. In one experiment, CO was removed from the inlet gases after an initial exposure to 0.22 torr CO (Figure 3). Figure 3a shows a kinetic trace of the optical density at 425 nm recorded over 6,000 seconds. Carbon monoxide was introduced after 600 seconds. The optical density increased and approached a plateau, indicating the conversion of about 30% of the intracellular myoglobin to COMb. After 3,800 seconds, CO was removed from the inlet stream, and the optical density rapidly returned to a lower value. After the kinetic trace was terminated, we recorded an optical spectrum of the heart cell suspension. This is shown in the top trace of Figure 3b, which is the typical

Table 1. Effects of Increasing Intracellular Carboxymyoglobin on Respiration and High-Energy Phosphates of Heart Cells

Experiment Number	P_{CO} (torr)	P_{O_2} (torr)	COMb (% total myoglobin)	Respiration with CO (fraction of initial respiration)	PCr:ATP with CO (fraction of control value)
1	0	3.0–21.0	0	0.72	0.86
2	0	0.5–3.9	0	0.93	ND[a]
3	0	0.15–1.5	0	0.97	0.82
4	0	8.8–17.7	0	0.78	0.90
5	0.07	8.6–25	14	0.72	0.81
6	0.30	6.8–20.0	17	0.62	1.20
7	0.07	1.56–2.1	19	1.00	ND
8	0.15	6.0–9.1	19	0.67	0.82
9	0.07	0.5–9.7	21	0.84	0.90
10	0.11	1.3–16.7	21	0.85	0.92
11	0.30	26.4–32.0	22	0.63	ND
12	0.15	1.1–2.7	27	0.97	1.03
13	0.15	3.0–5.9	27	0.73	0.79
14	0.22	1.1–22.8	28	0.76	1.15
15	0.18	1.1–4.0	32	0.94	1.15
16	0.07	0.47–0.53	33	0.98	0.90
17	0.15	2.7–5.9	36	0.84	1.17
18	0.74	8.3–20.4	42	0.60	0.81
19	0.45	1.7–21.7	43	0.67	0.88
20	0.74	10.9–24.3	44	0.51	ND
21	0.15	1.3–14.0	45	0.71	0.86
22	0.15	4.6–5.0	46	0.54	0.81
23	0.74	2.1–11.4	50	0.74	0.97
24	2.96	17.3–24.7	76	0.65	ND
25	2.96	13.2–24.9	79	0.54	ND
26	2.96	13.9–19.4	79	0.76	ND
27	7.40	14.5–20.8	91	0.69	0.81
28	7.40	10.7–17.5	94	0.72	ND
29	37.10	14.2–17.1	100	0.80	ND
30	22.20	0.56–10.4	100	0.56	0.71
31	37.10	6.9–14.9	100	0.74	1.14
32	29.70	13.2–17.7	100	0.78	0.85
33	22.30	15.5–18.3	100	0.56	0.76
34	22.30	5.2–21.7	100	0.64	0.86
35	44.50	31.5–37.7	100	0.61	ND
36	29.70	17.1–27.0	100	0.55	0.73
37	29.70	22.3–24.7	100	0.57	0.76
38	22.00	14.3–17.0	100	0.76	0.80
39	74.20	20.0–25.0	100	0.60	0.84
40	20.78	3.0–13.6	100	0.81	1.19
41	22.30	1.3–30.2	100	0.44	0.74

[a] ND = not determined.

spectrum of intracellular O_2Mb with no detectable indication of COMb. The bottom trace of Figure 3b, from a comparable experiment, shows the optical spectrum with 30% COMb. Thus, the intracellular myoglobin spectrum reverted to that of O_2Mb, with no detectable bound CO after 30 minutes at a minimum P_{O_2} of 25 torr. The binding of intracellular myoglobin to CO was governed by the ambient P_{CO}. In many experiments, the P_{O_2} value at the end of the one-hour incubation was higher than the initial P_{O_2} value, as a consequence of declining respiration. The measured COMb at the end of the experiments was generally much lower than what would be expected from the initial ambient P_{O_2} and somewhat higher than the level expected from the P_{O_2} measured during the spectral analysis. This finding suggests that under the conditions of these experiments, CO is not irreversibly bound to intracellular myoglobin but, rather, is released from the myoglobin molecule when ambient P_{O_2} is increased.

The measured values for P_{O_2}, P_{CO}, COMb, respiration, and PCr and ATP levels in 41 experiments are presented in Table 1 in order of increasing COMb. Within any one experiment, an increase in intracellular COMb was always followed by a decrease in respiration to a new steady state (Figure 2a). To quantitate this effect and compare different experiments, we divided the results of all the experiments into groups based on the measured intracellular fraction of COMb in each experiment. The effects of increasing COMb on the steady-state rate of respiration (41 experiments) are shown in Figure 4. The correlation coefficient, $r^2 = 0.23$, was very low. The ANOVA was determined with the Scheffe F test, examining significant differences in the respiration of populations of heart cells with different fractions of intracellular COMb. We found that the steady-state respiration of suspensions of resting isolated heart cells was significantly lower when intracellular COMb comprised 80% to 100% of the total intracellular myoglobin ($n = 15$) than when intracellular COMb was 0% to 40% ($n = 17$) (one- factor ANOVA with the Scheffe F test). Similarly, the respiration of cells with 40% to 80% COMb ($n = 9$) was significantly lower than the respiration of cells with 0% to 40% intracellular COMb, and was not significantly different from the respiration of cells with 80% to 100% COMb (one-factor ANOVA with the Scheffe F test).

These results show that sequestering more than 40% of intracellular myoglobin by CO significantly decreased the mean steady-state respiration of heart cell populations. We used resting heart cells in these experiments; a larger effect would be expected in the working heart respiring at a higher rate.

Respiration was also significantly inhibited by CO (unpaired t test and ANOVA with the Scheffe F test) in cells

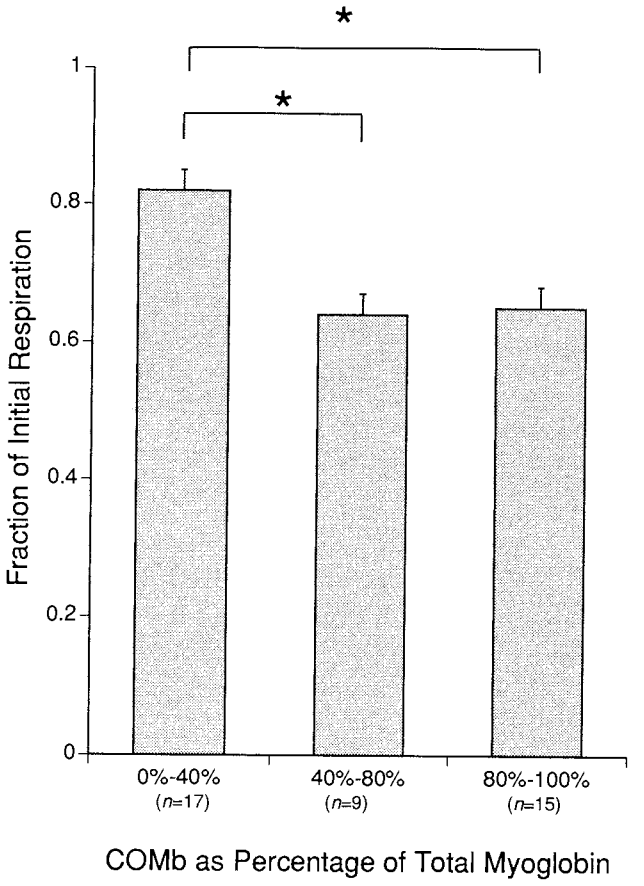

Figure 4. Effect of increasing conversion of intracellular myoglobin to the COMb form on the steady-state respiration of isolated heart cells. Control respiration was measured before CO treatment. An asterisk (*) indicates that steady-state respiration in these groups was significantly different from that of cells maintained in the chamber for one to two hours at low P_{CO} with 0% to 40% COMb, as determined by ANOVA with the Scheffe F test. COMb of 0% to 40% was recorded with P_{CO} of 0.07, 0.11, 0.15, 0.2, and 0.3 torr, and P_{O_2} of 0.15 to 30 torr. COMb of 40% to 80% was recorded with P_{CO} of 0.15, 0.45, 0.74, and 3 torr and P_{O_2} of 1.1 to 25 torr. COMb of 80% to 100% was recorded with P_{CO} of 7.4 to 74 torr and P_{O_2} of 0.6 to 38 torr. For all data, initial P_{O_2} was no greater than 35 torr.

held at sufficiently low levels of initial O_2 pressure (P_{O_2} no greater than 10 torr) to deoxygenate intracellular myoglobin partially. At these low physiological O_2 pressures, the respiration of cells with intracellular COMb ranging from 0% to 40% was 0.83 ± 0.03 of the initial value ($n = 16$). Respiration of cells with COMb ranging from 80% to 100% was 0.64 ± 0.07 of the initial value ($n = 5$), which is significantly different from values obtained with COMb ranging from 0% to 40%, using ANOVA with the Scheffe F test. The respiration of cells with COMb ranging from 40% to 80% was 0. 65 ± 0.04 ($n = 5$) which is significantly different from that of cells with 0% to 40% COMb, as judged by rigorous ANOVA with the Scheffe F test.

With P_{O_2} less than or equal to 5 torr, the respiration was

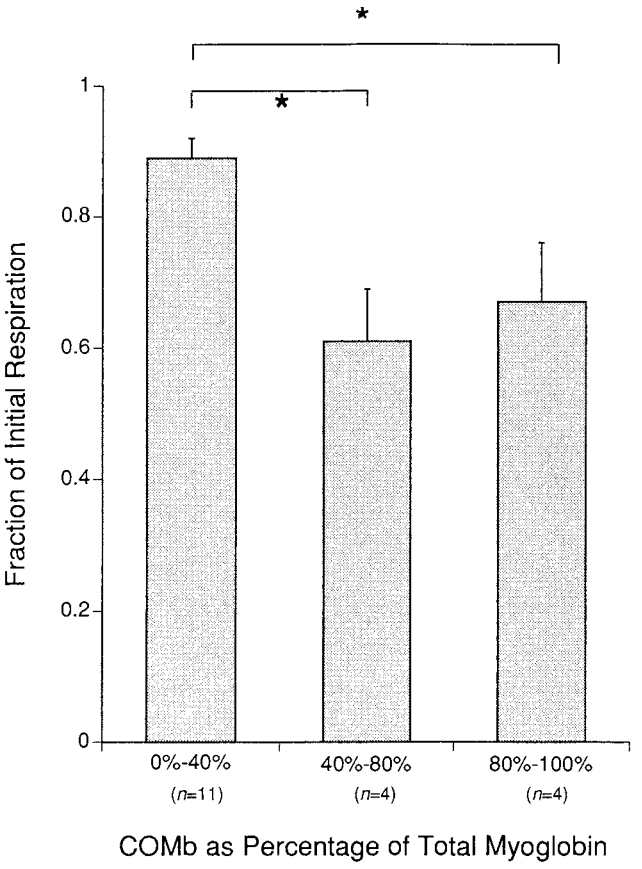

Figure 5. Effect of increasing conversion of intracellular myoglobin to the carbon monoxide form on the steady-state respiration of isolated heart cells with initial P_{O_2} no greater than 5 torr. Control respiration was measured before CO treatment. An asterisk (*) indicates that steady-state respiration in these groups was significantly different from that of cells maintained in the chamber for one to two hours at low P_{CO} with 0% to 40% COMb as determined by ANOVA with the Scheffe F test.

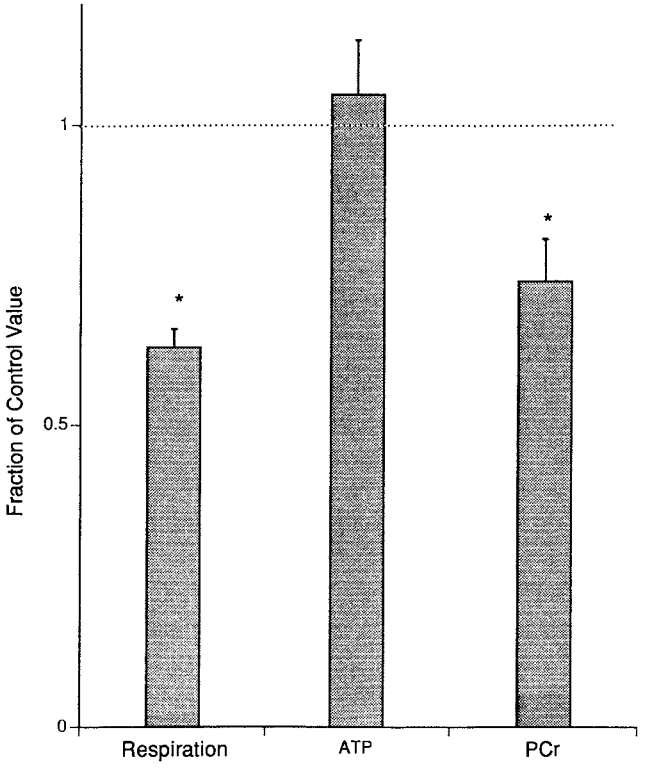

Figure 6. Effects of myoglobin inactivation by CO on isolated cardiac myocytes (P_{CO} of 37 to 74 torr, P_{O_2} of 15 to 38 torr). The broken line shows control values. Sequestering myoglobin with CO significantly decreased the rate of respiratory O_2 uptake and the level of PCr, but ATP levels remained unchanged. An asterisk (*) indicates a significant difference, $p \leq 0.05$, compared with controls using a one-paired t test.

significantly lower when COMb ranged from 80% to 100% (0.61 ± 0.08 [n = 4]) or 40% to 80% (0.67 ± 0.04 [n = 4]) of initial respiration, than when COMb ranged from 0% to 40% (0.88 ± 0.03 [n = 12]) of initial respiration (ANOVA with the Scheffe F test) (Figure 5). Although the number of experiments was small, the correlation coefficient, r^2, increased to 0.4 in this subset.

We make the conservative conclusion that at physiological P_{O_2}, sequestering 40% or more of intracellular myoglobin as COMb significantly decreases the rate of O_2 uptake of resting isolated heart cells at steady-state O_2 pressure.

EFFECTS OF CARBON MONOXIDE ON HIGH-ENERGY PHOSPHATE LEVELS

We monitored the levels of PCr and ATP in isolated heart cells exposed to CO for about two hours. As we have shown previously (Doeller and Wittenberg 1991; Gupta and Wittenberg 1991), ATP concentration was conserved, but an imbalance of energy synthesis and energy utilization in heart cells resulted in a drop in the PCr level (Figure 6). When ATP utilization is unchanged, a sensitive monitor of decreased ATP synthesis is the ratio of PCr to ATP. High energy stores, monitored by intracellular ratios of PCr to ATP, decreased as COMb increased (see Table 1). Because there was a large day-to-day variability in the ratio of PCr to ATP, we could not distinguish changes in this ratio without analyzing paired data from the same preparation of heart cells. We found that the ratio of PCr to ATP of CO-treated heart cells was significantly lower than that of control cells from the same preparation (n = 17, paired t test, p = 0.0004) when COMb constituted more than 40% of the total intracellular myoglobin. There was no significant difference in the ratios of PCr to ATP for heart cells with intracellular COMb of 0% to 40% and their respective controls (n = 14, p = 0.20).

We conclude that sequestering more than 40% of the intracellular myoglobin as COMb decreased the rate of oxidative ATP synthesis of resting heart cells at steady states of O_2 pressure.

Table 2. Effects of Substrate on Myoglobin-Dependent Oxidative Phosphorylation

Substrate Addition[a]	Number of Experiments	P_{CO} (torr)	P_{O_2} (torr)	COMb (% of total)	Respiration with CO (fraction of initial respiration)	PCr:ATP with CO (fraction of control value)
None	2	26	4–20	100	0.69	0.81
Glucose	8	0.74–37	0.6–21	97 ± 1.5	0.68 ± 0.04	0.87 ± 0.07
Glucose + octanoate	6	15–74	8–37	100	0.78 ± 0.08	0.89 ± 0.07
Glucose + palmitate	1	22	14–17	100	0.76	0.80
Palmitate	3	30–45	17–38	100	0.58 ± 0.022	0.75
Lactate	2	22–45	18–30	100	0.62	0.89
Glucose + lactate	3	22–30	5–33	100	0.70 ± 0.10	1.01 ± 0.28
Glucose + pyruvate	1	3	2–7	91	0.88	0.99

[a] Concentrations of substrates: 5.4 mM glucose throughout, 2 mM octanoate, 0.13 mM palmitate throughout, 2 mM lactate throughout, and 2 mM pyruvate.

EFFECTS OF SUBSTRATE ON CARBON MONOXIDE INHIBITION OF MYOGLOBIN-DEPENDENT OXIDATIVE PHOSPHORYLATION

The effects of different substrates on respiration and on the maintenance of PCr levels in the presence of 100% COMb are presented in Table 2. There was a well-defined drop in respiration and in the ratio of the PCr to ATP in myoglobin-inactivated heart cells when physiological substrates, including glucose and fatty acids, were added to the medium.

EFFECTS OF OLIGOMYCIN ON MYOGLOBIN-DEPENDENT OXIDATIVE PHOSPHORYLATION

We previously reported that when mitochondrial ATP synthase is inhibited with oligomycin, myoglobin inhibition no longer decreases the rate of respiration or the PCr levels of heart cells (Wittenberg and Wittenberg 1989; Gupta and Wittenberg 1991). Results from 10 experiments with 1 to 100 μM oligomycin are shown in Table 3. Although the respiration of resting heart cells was not affected by oligomycin, inactivating 100% of intracellular myoglobin as COMb led to no further reduction in respiratory rate. On the other hand, the PCr levels of heart cells were massively reduced in oligomycin-treated heart cells. Because oligomycin inhibits oxidative phosphorylation at the ATP synthesis step, all ATP production in these cells must have arisen from glycolysis. Thus, the inactivation of intracellular myoglobin with CO did not affect the rate of ATP synthesis, as reflected by changes in the PCr levels. This strongly suggests that the respiratory inhibition and decreased levels of PCr observed with 100% intracellular COMb are mediated by mitochondrial oxidative phosphorylation. These effects depend on a functional mitochondrial ATP synthase.

DISCUSSION AND CONCLUSIONS

This is the first report of the effects of prolonged CO exposure on heart cell suspensions at physiological ambient O_2 pressure. The entire range of physiological O_2 pressures has been examined, from P_{O_2} near 10 to 20 torr, the end-venous O_2 pressure, to P_{O_2} near 2 to 5 torr, the intracellular O_2 pressure of cells of the working heart (Gayeski and Honig 1991). We found that heart cell myoglobin was increasingly bound to CO as the ambient CO increased (90 to 90,000 ppm) at a given O_2 pressure, until all intracellular myoglobin was ligated. The steady-state rate of respiratory O_2 uptake and the intracellular high-energy phosphate levels (pooled results at all O_2 pressures) were significantly lower when more than 50% of the intracellular myoglobin was sequestered as COMb. This suggests that CO exerts its full effect on the respiration of resting heart cells when

Table 3. Effect of Oligomycin on Myoglobin-Mediated Oxidative Phosphorylation[a]

Addition	Respiration (fraction of initial respiration)	Respiration with CO (fraction of initial respiration)	PCr (fraction of control value)	PCr with CO (fraction of control value)
Control (glucose alone)	1.0	0.63 ± 0.03	1.0	0.74 ± 0.07
Glucose + oligomycin	0.96 ± 0.01	1.04 ± 0.04	0.41 ± 0.04	0.39 ± 0.07

[a] n = 10.

most of the myoglobin is preempted by CO. When P_{O_2} was at least 10 torr, we saw no deoxymyoglobin in the heart cell spectra. Facilitated diffusion did not make any further contribution to O_2 flux when myoglobin was fully saturated everywhere. Therefore, at these high O_2 pressures, the greater O_2 flux with functional myoglobin was due to myoglobin-mediated oxidative phosphorylation. When P_{O_2} was no greater than 10 torr, myoglobin was partially deoxygenated, and facilitated diffusion may have played a significant role in myoglobin function. Under these conditions, respiration was also significantly lower in groups of cells with COMb ranging from 40% to 100% than in groups of cells with COMb ranging from 0% to 40%. If it is assumed that ATP utilization is the same in the presence and absence of CO, the observed decrease in the ratio of PCr to ATP, with ATP levels constant, suggests that the rate of ATP synthesis decreased and the PCr pool was partially depleted to reach a new steady state and to maintain a constant ATP concentration. The ATP utilization was required to maintain ionic gradients and preserve cell viability, and there is no a priori reason why these requirements should change when O_2 uptake is decreased. Previously, we reported that CO toxicity (as monitored by respiratory O_2 uptake and PCr levels) is not observed when electron transport through the mitochondrial chain is inhibited by myxothiazol, antimycin A, or cyanide (Wittenberg and Wittenberg 1987). Carbon monoxide causes no further decrease in the rate of oxidative phosphorylation when mitochondrial ATP synthase has been previously inhibited by oligomycin. Thus, the data show that ambient CO enters the cells and, at physiological O_2 pressures, binds to intracellular myoglobin to an extent that is determined by the relative P_{O_2} and P_{CO} values. Sequestering 40% to 100% of the intracellular myoglobin caused a decrease in the rate of oxidative phosphorylation by intracellular mitochondria. The outstanding question is how myoglobin in the sarcoplasm affects oxidative phosphorylation at the inner mitochondrial membrane. To enhance mitochondrial oxidative phosphorylation, some carrier of reducing or oxidizing equivalents must transduce the effect of O_2Mb across the mitochondrial membranes.

The effect of CO on respiration was observed clearly in each experiment. The day-to-day variability makes it difficult to describe the relation between the fraction of intracellular myoglobin bound to CO and steady-state respiration in different experiments. This relation is the most accurate approach to a dose-response curve because the O_2 pressure changes within each experiment as a consequence of changing respiration. To analyze the scattered data we used an ANOVA with the rigorous Scheffe F test to find significant differences. The data are arbitrarily grouped in blocks of comparable size. These blocks are too broad to define the shape of the relation. The apparent step-function (Figures 4 and 5) may or may not be illusory. Although the shape of the relation is indeterminate, the two extremes are well defined.

Respiratory inhibition with CO may be the consequence of binding to cytochrome oxidase, the only mitochondrial component known to react with CO. However, the affinity of myoglobin for CO is much greater than the affinity of cytochrome oxidase for CO. We previously showed that although CO binding to myoglobin of heart cells is half the maximal level when P_{CO}/P_{O_2} is 0.05 and is complete when P_{CO}/P_{O_2} is 1.0, there is no detectable effect of CO on the spectrum of intracellular cytochrome oxidase until P_{CO}/P_{O_2} is 6 (Wittenberg and Wittenberg 1985, 1987). In these experiments, P_{CO}/P_{O_2} was kept below 2. Spectrophotometry showed no evidence of CO binding to cytochrome oxidase. The effect of CO on respiration in these experiments was not a consequence of CO binding to cytochrome oxidase.

A preparation of freshly isolated, functionally intact heart cells was used in this study to allow rigorous control of O_2 pressure, CO pressure, and ambient substrate (Wittenberg et al. 1988). The experiments excluded modulation by neural or circulatory feedback regulation and changes in O_2 delivery due to the presence of COHb in the blood. Cellular architecture and biochemical interactions were maintained in this preparation (see Methods section), so that the interaction of sarcoplasmic myoglobin and intracellular mitochondrial oxidative phosphorylation could be studied. We defined the conditions under which CO specifically inactivates intracellular myoglobin function and decreases the rate of oxidative phosphorylation of heart cells. Clearly, to the extent that this phenomenon occurs in the intact heart, it will limit the rate of oxidative phosphorylation, thereby limiting the rate of ATP production and the maximal rate at which the heart can pump blood. The outstanding question is whether we can speculate on the extent to which these conditions apply to whole-animal CO cardiotoxicity.

Gayeski and Honig (1991) have shown that intracellular myoglobin is approximately half-saturated with O_2 (corresponding to intracellular P_{O_2} of 1.3 torr at 30°C) in the working rat heart. We found that maximum inhibition of myoglobin-dependent respiration and ATP synthesis occurs at about 40% sarcoplasmic COMb. The intracellular P_{CO} needed to achieve 40% COMb at a P_{O_2} of 1.3 torr may be calculated from the partition coefficient M = 20: P_{CO} = COMb/O_2Mb × P_{O_2}/M = 0.4/0.6 × 1.3/20 = 0.04 torr or 50 ppm free CO. In turn, to achieve 50 ppm free CO in equilibrium with blood hemoglobin in the lung, we calculate, taking M of 240 for hemoglobin (the value for human blood at 37°C) and arterial P_{O_2} of 100 torr: COHb/O_2Hb = P_{CO}/P_{O_2} × M = 0.04/100 × 240 = 0.092 torr, equivalent to 12% COHb

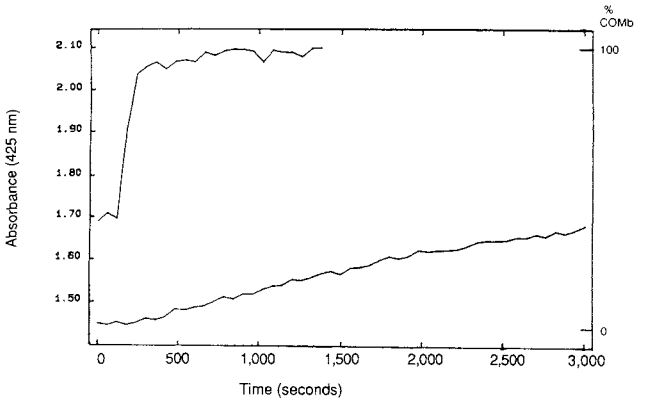

Figure 7. Kinetic traces of a suspension of isolated heart cells showing the relatively slow rate of combination of intracellular myoglobin with carbon monoxide at low concentrations of carbon monoxide. The lower trace shows values for P_{CO} of 0.22 torr and P_{O_2} of 1.1 to 4.0 torr. The upper trace shows results for the same preparation of cells in the chamber after a full spectrum was recorded (COMb = 28%). The P_{CO} was increased from 0.22 to 29.9 torr two minutes after the initiation of the trace. The P_{O_2} was 4 to 23 torr, with a final COMb of 100%.

in the arterial blood. To attain 80% COMb requires 38% COHb in the arterial blood. In clinical trials, Allred and associates (1989) observed "significant effects on cardiac function during exercise" with 4% venous COHb in subjects with coronary artery disease. Exacerbating preexisting cardiovascular disease by exposing subjects to low levels of CO is discussed elsewhere in detail (Chaitman et al. 1992). The present study provides an almost unique opportunity to distinguish tissue effects from effects of diminished blood O_2 delivery.

The data suggest that when the intracellular myoglobin of heart cells is exposed to sufficient CO to bind 50% of the total myoglobin, the maximum respiratory O_2 uptake and aerobic ATP synthesis by the heart become limited, especially in states of hypoxia. A heart with limited energy can pump only up to the limits of its energy supply. This would be expected to show up as a limitation in the maximum rate of work output. It is gratifying that, despite the uncertainties inherent in these highly speculative calculations, our estimate that approximately 20% arterial COHb is necessary to inhibit myoglobin-dependent oxidative phosphorylation in isolated rat cardiac myocytes agrees reasonably well with findings that less than 30% venous COHb can result in mild poisoning in patients, causing headaches and shortness of breath (Chaitman et al. 1992).

A study of dog gastrocnemius muscle, perfused in situ with 30% COHb, P_{CO} near 0.075 torr, and arterial P_{O_2} at 30 torr, demonstrated that the maximum rates of O_2 consumption and sustainable work output during exercise were 26% lower during CO treatment, although O_2 delivery was the same for exposures with and without CO (Hogan et al. 1990). With CO treatment, the intracellular COMb was calculated to be 40%. Our data show that at this level of intracellular COMb, the rate of aerobic ATP synthesis of resting heart muscle cells may well be decreased.

We observed with spectrophotometry that, at low levels of CO, the rate of conversion of intracellular myoglobin to COMb was considerably slower than at higher CO concentrations (Figure 7). This means that at low levels of CO, the onset of cardiotoxicity might be delayed by 30 to 40 minutes. On the other hand, Figure 3 shows that when CO is removed from the ambient solution, the removal of CO from the intracellular myoglobin is quite rapid.

We conclude that sequestering 50% or more of the intracellular myoglobin as COMb significantly decreases the rate of respiration and oxidative phosphorylation of cardiac myocytes. Although the extrapolation to whole-animal or human conditions is still highly speculative, the data suggest that, to the extent that it binds intracellular myoglobin of the heart, CO may affect the maximum work output of the heart, particularly under hypoxic conditions. Because the precision of the assay is low, we have not been able to define a lower limit for the CO dose required to produce this effect.

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ABOUT THE AUTHORS

Beatrice A. Wittenberg received her Ph.D. degree in pharmacology from Western Reserve University in 1954. After two postdoctoral years with Dr. R. Berne of Western Reserve University and with Dr. A. Goldfeder at New York University, she came to Albert Einstein College of Medicine where she has been Professor in Physiology and Biophysics since 1985. She is a member of the American Society of Biological Chemistry, International Society of Heart Research, American Physiological Society, and the Biophysical Society. Her research interests are cardiac bioenergetics, and myoglobin function in isolated heart cells.

Jonathan B. Wittenberg received his Ph.D. degree in biochemistry from Columbia University in 1950. After postdoctoral years at Harvard Medical School and the National Institutes of Health, he became Instructor and subsequently Assistant Professor of Biochemistry in Medicine at Case Western Reserve University School of Medicine. In 1955, he moved to Albert Einstein College of Medicine, first in the Department of Biochemistry and then in the Department of Physiology as Associate Professor. Since 1981 he has been Professor of Physiology and Biophysics at the Albert Einstein College of Medicine. He is a member of the American Society of Biochemistry, the Biochemical Society, Society of General Physiologists, American Physiological Society, Biophysical Society, Red Cell Club, and the Harvey Society. His research interests include the chemistry and kinetics of the reactions of intracellular hemoglobins, and the intracellular transport of oxygen by facilitated diffusion and other pathways.

ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine triphosphate
CO	carbon monoxide
COHb	carboxyhemoglobin
COMb	carboxymyoglobin
O_2	oxygen
O_2Mb	oxymyoglobin
M	partition coefficient
PCr	phosphocreatine
P_{CO}	partial pressure of carbon monoxide
P_{O_2}	partial pressure of oxygen
ppm	parts per million
r^2	square of the correlation coefficient

INTRODUCTION

The Health Effects Institute (HEI) occasionally issues a Request for Preliminary Applications (RFPA), soliciting proposals for studies of the "Health Effects of Automotive Emissions." The purpose of preliminary applications is to invite investigators to submit short descriptions of research proposals on topics outside of those defined by the more targeted Requests for Applications. In response to an RFPA issued in 1987, Dr. Beatrice Wittenberg, of the Albert Einstein College of Medicine of Yeshiva University, submitted a preliminary application entitled "Effects of Carbon Monoxide, Hydrogen Cyanide, and Hydrogen Sulfide on the Heart Muscle Cell." The Health Research Committee requested a full application for a pilot project focused on testing the effect of low concentrations of carbon monoxide on the respiration of cardiac muscle cells.

After the full proposal, "Effects of Carbon Monoxide on Isolated Heart Muscle Cells," was reviewed, the Research Committee approved the study, which began on March 1, 1989. The study lasted two and one-half years, and cost $124,332. Dr. Wittenberg's final report was received at the Health Effects Institute for review on February 7, 1992. A revised report, received on October 22, 1992, was accepted for publication by the Health Review Committee in November 1992. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of the Health Effects Institute and to the public.

REGULATORY BACKGROUND

Section 109 of the Clean Air Act, as amended in 1990, mandates that the U.S. Environmental Protection Agency (EPA)* establish primary and secondary National Ambient Air Quality Standards (NAAQS) for air pollutants, based on their health effects, at levels "requisite to protect the public health . . . allowing an adequate margin of safety." The Senate report on the 1970 Clean Air Act Amendments states that "[a]n ambient air quality standard . . . should be the maximum permissible air level of an air pollution agent or class of such agents (related to a period of time) which will protect the health of any group of the population" (U.S. Senate 1970).

Although this reference to "the health of any group of the population" is not clearly defined in the Clean Air Act itself or in the Senate Report, the Senate Report does specify that "included among those persons whose health should be protected by the ambient standard are particularly sensitive citizens (such as bronchial asthmatics and emphysematics) who in the normal course of daily activity are exposed to the ambient environment."

The Senate Report also states that "in establishing an ambient standard necessary to protect the health of these persons, reference should be made to a representative sample of persons comprising the sensitive group rather than to a single person in such a group."

The current primary NAAQS for carbon monoxide is 9 parts per million (ppm), averaged over eight hours, and 35 ppm, averaged over one hour, both not to be exceeded more than once a year (U.S. Environmental Protection Agency 1985). As part of the periodic reevaluation of the criteria pollutant standards mandated by Section 109(d)(1), the EPA reviewed several recent studies of the effects of exposure to carbon monoxide on the human cardiovascular system. Current evidence suggests that the health threat from carbon monoxide is serious for those who suffer from cardiovascular disease, particularly those with angina or peripheral vascular disease (U.S. Environmental Protection Agency 1991).

In March 1990, the Agency released for public comment an external review copy of its draft document "Air Quality Criteria for Carbon Monoxide." A draft Staff Paper of the Office of Air Quality Planning and Standards, which recommended no changes in the carbon monoxide standards, was circulated for technical review and comment in April of 1992, and accepted by the Clean Air Scientific Advisory Committee in August of 1992.

SCIENTIFIC BACKGROUND

Carbon monoxide is a ubiquitous air pollutant. Although exposure to high concentrations of carbon monoxide is known to be lethal, the effects of exposure to low levels of carbon monoxide are also of public health and regulatory concern because low level exposures can adversely affect cardiovascular function in patients with cardiovascular disease (Torino 1981; Allred et al. 1989a,b).

The source of most human exposures to carbon monoxide is the incomplete combustion of fossil fuels. Transportation

* A list of abbreviations appears at the end of the Investigators' Report.

sources account for approximately two-thirds of the nation's carbon monoxide emissions, with the largest contribution coming from motor vehicles (U.S. Environmental Protection Agency 1991). Urban carbon monoxide levels are about 3 ppm near roadways (Akland et al. 1985), 5 ppm to 30 ppm near heavily traveled roads during commuter hours (Akland et al. 1985; Bevan et al. 1991; Chan et al. 1991), and can exceed 40 ppm in highway tunnels (Stern et al. 1988). Other combustion sources, industrial processes, and solid waste disposal also contribute to the rest of the carbon monoxide emissions (U.S. Environmental Protection Agency 1991).

Indoor concentrations of carbon monoxide are usually lower than 2 ppm, but can reach dangerous levels, as high as 100 to 400 ppm, when combustion appliances malfunction. The U.S. Consumer Product Safety Commission directly attributed seventy deaths during 1980 to carbon monoxide exposures resulting from the use of unvented gas space heaters in confined spaces (cited in Coultas and Lambert 1991). Cigarette smoke, either mainstream or sidestream, is another frequent source of inhaled carbon monoxide (Turino 1981). The possible link between carbon monoxide exposure and adverse effects on patients with cardiovascular disease is an important public health issue because of the widespread exposure to this pollutant and the size of the population at risk. Approximately 22 million people live in areas of the United States where carbon monoxide levels exceed the NAAQS (U.S. Environmental Protection Agency 1991).

Inhaling carbon monoxide is dangerous because it interferes with the normal oxygen-carrying capacity of the blood. A decreased level of oxygen in tissues (hypoxia) can cause transient or permanent damage, especially in organs that demand high oxygen delivery to perform their normal functions, such as the brain and heart. Experimental studies using model systems suggest that, besides interfering with the oxygen-carrying capacity of the blood, additional mechanisms of carbon monoxide's toxicity are possible; these involve reactions occurring within heart muscle cells. The study by Drs. Beatrice and Jonathan Wittenberg addresses one of these possible mechanisms: the inhibition of oxygen transport within heart muscle cells caused by carbon monoxide, and the possible consequences of this inhibition on the cells' ability to produce the energy for contraction. The remainder of this section discusses the importance of oxygen for cellular energy production and carbon monoxide's known role in decreasing oxygen transport to cells. It concludes with a description of proposed mechanisms of how carbon monoxide could interfere with intracellular reactions that play a role in providing the energy required for heart muscle contraction.

OXYGEN TRANSPORT AND ENERGY PRODUCTION

All the processes involved in the growth and metabolism of cells require an input of energy. Muscle cells have an additional energy requirement because their specialized function, contraction, requires a high energy output. During contraction, chemical energy is released as a result of the breakdown of an important intracellular compound, adenosine triphosphate (ATP), and muscle cells convert this energy into mechanical work (Alberts et al. 1983; Darnell et al. 1986).

The continued production of ATP to provide the energy required for muscle contraction depends on the availability of oxygen; however, oxygen has only a low solubility in the body's aqueous fluids, such as blood. To resolve this problem, vertebrates have developed specialized proteins that bind oxygen and increase the amount of oxygen that can be transported within the body (Stryer 1981). Oxygen binds reversibly to one such specialized protein in red blood cells, the iron-containing protein hemoglobin. When oxygen binds to hemoglobin it forms oxyhemoglobin (O_2Hb); it is in this form that oxygen is transported through the blood until it is taken up by cells and used in the metabolic pathway that produces the majority of a cell's store of ATP (Alberts et al. 1983; Wittenberg and Wittenberg 1989). The process of ATP synthesis, which takes place inside cells within the mitochondria, is termed "oxidative phosphorylation." The chemical reactions that carry out the process of oxidative phosphorylation are driven by a series of enzyme complexes that are closely coupled with protein and nonprotein cofactors. Among these enzyme complexes is cytochrome oxidase, an enzyme that metabolizes oxygen and plays a critical role in ATP synthesis.

In order to reach the mitochondria, oxygen first detaches from hemoglobin, then diffuses out of red blood cells, through the capillary wall and the interstitial space, and finally through the external membranes of cells, such as muscle cells, and into the aqueous intracellular space therein (Haab 1990). A portion of the dissolved oxygen moves directly through the intracellular space to the mitochondria, where it is used for ATP synthesis. It has been proposed that myoglobin, another specialized iron-containing protein that reversibly binds oxygen, enhances intracellular oxygen transport in skeletal, smooth, and cardiac muscle cells (called myocytes) (Wittenberg and Wittenberg 1989).

One proposed mechanism for enhancing transport of oxygen within myocytes is as follows. As oxygen diffuses into the cells, it binds to deoxymyoglobin to form oxymyoglobin (O_2Mb). Oxymyoglobin diffuses through the soluble intracellular space and releases its oxygen in the vicinity of mitochondrial cytochrome oxidase, thereby facilitating ATP

synthesis. This process of oxygen diffusion to cytochrome oxidase is termed "myoglobin-facilitated oxygen diffusion" (Wittenberg et al. 1975; Wittenberg and Wittenberg 1989). Braunlin and coworkers (1986) later demonstrated the presence of this oxygen transport pathway in strips of cardiac muscle.

INTERFERENCE WITH OXYGEN TRANSPORT BY CARBON MONOXIDE

Carbon monoxide presents problems for biological systems because it interferes with the ability of cells to generate energy. Inhaled carbon monoxide binds reversibly to hemoglobin in red blood cells, forming carboxyhemoglobin (COHb). Carboxyhemoglobin impairs oxygen transport to cells by two mechanisms. First, carbon monoxide binding to hemoglobin reduces the amount of functional hemoglobin available to bind oxygen. Second, the presence of carbon monoxide on the hemoglobin molecule increases the affinity of the remaining functional hemoglobin for oxygen; thus, the oxygen bound as O_2Hb is less readily released to cells (Haab 1990).

The principal mechanism of toxicity associated with inhaling carbon monoxide is the tissue hypoxia induced by elevated levels of COHb. Increasingly, however, researchers have speculated that carbon monoxide's interaction with myoglobin and cytochrome oxidase may represent additional mechanisms of carbon monoxide toxicity. It has long been recognized that, in addition to binding to hemoglobin, carbon monoxide also binds to tissue myoglobin, forming carboxymyoglobin (COMb) (Coburn et al. 1971; 1973). In an in vitro model system, carbon monoxide was transferred from COHb to myoglobin, forming COMb (Agostini et al. 1988). If such a reaction occurred in vivo, it would reduce the deoxymyoglobin levels available for oxygen diffusion facilitated by myoglobin.

Until recently, myoglobin-facilitated oxygen diffusion to the vicinity of mitochondrial cytochrome oxidase was believed to be the only pathway by which myoglobin enhanced oxygen transport, and the only point at which carbon monoxide might inhibit oxygen transport in myocytes. In 1987, Wittenberg and Wittenberg proposed that there is another pathway by which myoglobin transports oxygen within the myocyte, stimulating ATP synthesis, and that this pathway is also inhibited by carbon monoxide. The existence of this alternate pathway was suggested by a series of in vitro experiments in which the contribution of the diffusion pathway to oxygen transport was eliminated (Wittenberg and Wittenberg 1987).

Wittenberg and Wittenberg (1987) reasoned that because myoglobin-facilitated oxygen diffusion requires deoxymyoglobin molecules to accept new molecules of oxygen as they arrive from the bloodstream, if all the myoglobin were in the oxygenated form, added carbon monoxide could not affect ATP synthesis stimulated by this pathway. To fully oxygenate deoxymyoglobin, the investigators exposed suspensions of cardiac myocytes to oxygen levels that produced concentrations of dissolved intracellular oxygen that were one hundred-fold higher than the level needed for optimum mitochondrial ATP synthesis. When the investigators added carbon monoxide to the cell suspensions that had been exposed to these high oxygen levels, they noted that COMb formed as oxygen was partially displaced from O_2Mb by carbon monoxide. In these experiments, one-third of the cells' oxygen uptake was abolished by the formation of COMb, and oxygen uptake decreased linearly with the decrease in O_2Mb. Other experiments indicated that the formation of COMb caused a decreased rate of ATP synthesis by mitochondrial oxidative phosphorylation. Because the decreased rate of ATP synthesis could not be attributed to the inhibition of myoglobin-facilitated oxygen diffusion by carbon monoxide, there being no deoxymyoglobin to bind carbon monoxide, Wittenberg and Wittenberg (1987) postulated that there is an additional pathway by which myoglobin transports oxygen within the myocyte. They termed this pathway "myoglobin-mediated oxygen delivery to mitochondria." In contrast to myoglobin-facilitated oxygen diffusion, which is believed to deliver oxygen to a site near cytochrome oxidase, the site at which oxygen may be delivered to mitochondria by the postulated myoglobin-mediated oxygen delivery pathway is not known (Wittenberg and Wittenberg 1987, 1989; B. Wittenberg, personal communication). The investigators have since renamed this newly-discovered pathway "myoglobin-mediated oxidative phosphorylation" (see the Investigators' Report).

The evidence for these alternate pathways of carbon monoxide-mediated toxicity in in vitro systems has led to the hypothesis that the toxicity of carbon monoxide toward cardiac tissue might result from its interaction with several proteins (hemoglobin, myoglobin, and cytochrome oxidase). In their Health Effects Institute-supported study, the Wittenbergs sought additional evidence for an alternate pathway of carbon monoxide's cardiotoxicity by attempting to correlate COMb levels with decreased oxygen uptake and ATP synthesis by cardiac myocytes. Myoglobin's binding of carbon monoxide could add to the reduction in oxygen's availability to mitochondria caused by COHb formation. A decreased oxygen level would reduce ATP synthesis in heart muscle and reduce the energy available for contraction. Reducing the heart's pumping capacity presents a risk to people with cardiovascular disease, whose ability to deliver oxygen to the heart is already diminished.

JUSTIFICATION FOR THE STUDY

Automotive emissions, particularly carbon monoxide, are thought to be risk factors in the development of heart disease (Clarkson 1988). In addition, the myocardial hypoxia caused by exposure to carbon monoxide is a risk factor for patients with preexisting coronary artery disease.

Investigators in the Health Effects Institute's program of carbon monoxide research have studied the effects of exposure to low levels of carbon monoxide in people with angina and with elevated baseline levels of cardiac arrhythmias (see the listing of Related Health Effects Institute Publications at the back of this volume). Dr. Wittenberg's proposal complemented HEI's ongoing research program on the effects of carbon monoxide on heart function. Dr. Wittenberg proposed to study whether there were additional mechanisms by which carbon monoxide caused cardiac toxicity, other than by COHb formation. At the time of her application, she and Dr. Jonathan Wittenberg had implicated a new pathway in the cardiac myocyte that was inhibited by carbon monoxide: myoglobin-mediated oxygen delivery to mitochondria. The Wittenbergs proposed that this pathway accounted for one-third of the resting oxygen consumption of the cardiac muscle cell, and that this pathway might represent the most sensitive point of attack by carbon monoxide on the energetics of heart muscle contraction (Wittenberg and Wittenberg 1987). Because these results were obtained at extremely high partial pressures of oxygen, ranging from 40 to 340 torr (Wittenberg and Wittenberg 1987), and the oxygen requirements of isolated cardiac myocytes are fully met at less than one torr (Wittenberg and Wittenberg 1989), the significance of this pathway at physiological levels of oxygen remained to be established.

In support of her application, Dr. Wittenberg noted that she and Dr. Jonathan Wittenberg were fully conversant with this area of research, having studied the chemistry and physiological role of cytoplasmic oxygen-binding proteins for almost twenty years. The Wittenbergs had also developed methods to prepare homogeneous suspensions of physiologically and morphologically intact cardiac myocytes, and had improved the procedures over time. The Health Effects Institute's Research Committee believed that these researchers were well qualified to investigate the effects of a broad range of oxygen and carbon monoxide levels on respiration and energy production in cardiac myocytes, and to help resolve the issue of whether carbon monoxide exerted its cardiotoxicity by mechanisms other than reducing the oxygen-carrying capacity of hemoglobin and decreasing its release of oxygen.

OBJECTIVES AND STUDY DESIGN

The long-term goal of the investigators was to explore the hypothesis that carbon monoxide binding to myocyte myoglobin is responsible, in part, for the decreased cardiac work output and lowered tolerance to diminished oxygen supply in humans exposed to low levels of carbon monoxide (Sheps et al. 1990; Allred et al. 1989b). The overall objectives of the investigators' study were to test the effect of low concentrations of carbon monoxide on respiratory oxygen uptake and the rate of ATP synthesis in isolated myocytes. The specific aims were to determine:

1. the level of steady-state oxygen consumption at a range of oxygen and carbon monoxide levels;
2. the partition of myoglobin between deoxymyoglobin, O_2Mb, and COMb at the various carbon monoxide and oxygen levels; and
3. the effect of increasing COMb levels on the intracellular ATP and phosphocreatine (PCr) levels.

In muscle cells, PCr is the energy source that rapidly regenerates ATP from adenosine diphosphate, which is formed when muscles contract and ATP is broken down to liberate chemical energy. Phosphocreatine replenishes the myocyte's ATP content by transferring its phosphate group to adenosine diphosphate, thus reforming ATP when mitochondrial ATP synthesis is inhibited. This reaction can occur until the cell's store of PCr is depleted. Because of ATP's replenishment via the PCr pathway, measuring ATP levels alone could be misleading, and might not accurately reflect the effect of COMb formation on ATP synthesis. However, measuring both ATP and PCr would allow the investigators to determine whether myocytes maintained a normal level of ATP synthesis in the presence of increasing COMb. A drop in PCr would indicate that a normal level of mitochondrial ATP synthesis was not maintained, and that the ATP level reflected replenishment by PCr.

All experiments used homogeneous suspensions of myocytes freshly isolated from the hearts of adult rats. Because the myocytes were not working against an external load, their oxygen uptake closely resembled that of the resting heart. The investigators stated that their experimental system offered the advantages of cell homogeneity and accurate control of extracellular oxygen pressure. Furthermore, they indicated that using an experimental system that did not depend on blood supply to muscle cells removed the binding of carbon monoxide to hemoglobin as a potential source of interference.

The cells were exposed to the gas mixtures in a tempera-

ture-controlled chamber placed in the light path of a recording spectrophotometer equipped with a data recording system. The rate of oxygen uptake was determined from the difference between the partial pressure of oxygen in solution in the absence and presence of cells. (The partial pressure of a gas is the pressure that would be exerted by one component of a mixture of gases if that component were present alone.) Determining the oxygen uptake depended on the use of a mass transfer coefficient that was determined for each day's cell preparation. The oxygen pressure of the solution was monitored by a polarographic oxygen electrode.

The extent to which myoglobin combined with oxygen or with carbon monoxide (that is, the deoxymyoglobin, O_2Mb, and COMb levels) was monitored using the spectrophotometric procedures described above. The levels of each of these compounds were determined by sophisticated spectral recordings that are clearly described in the Investigators' Report. The presence of an oxygen electrode in the chamber used for spectrophotometric analyses allowed the investigators to measure simultaneously the effect of carbon monoxide on oxygen uptake and the extent of carbon monoxide binding to myoglobin. The investigators used these data to determine the relationship between increasing levels of COMb and myocyte oxygen uptake.

The levels of ATP and PCr present at various oxygen and carbon monoxide levels were determined by high-pressure liquid chromatography.

Experiments were performed using the following partial pressures of carbon monoxide, expressed as torr (one torr is equal to 1/760 of normal atmospheric pressure); the corresponding ppm concentrations are given in parentheses: 0, 0.074 (90), 0.15 (180), 0.20 (270), 0.5 (600), 0.74 (900), 3.0 (4,000), 7.4 (9,000), 22 (18,000), 30–37 (30,000–40,000), and 74 (90,000). As a reference point for these values, an inspired concentration of 100 ppm carbon monoxide produces 14% blood COHb, a level that can cause headaches in healthy humans, and angina in patients with coronary artery disease; an inspired concentration of 500 ppm carbon monoxide can cause blood COHb levels to rise to 45%, a level just below that which causes respiratory failure (Turino 1981; Ilano and Raffin 1990).

The partial pressure of oxygen at the onset of carbon monoxide exposure was also varied. Experiments were performed over several ranges of oxygen partial pressure: 0.2 to 5 torr, 5 to 10 torr, 10 to 20 torr, and 20 to 35 torr. For reference, a study by Gayeski and Honig (1991) suggests that the intracellular oxygen pressure in the working heart of several animal species is near 3 to 5 torr. The range of 10 to 20 torr represents the venous oxygen pressure (see Investigators' Report). This level of oxygen pressure is typically found where blood enters the veins from capillaries in active peripheral tissues. Thus, the investigators performed their experiments over several ranges of oxygen pressures that are relevant to peripheral tissues.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators successfully completed most of the aims of their study. They used a novel and highly sophisticated experimental system, with which they have considerable expertise, to study the oxygen uptake of isolated myocytes in the presence of increasing levels of COMb. They correlated these data with measurements of the energy state of myocytes by determining the levels of ATP and PCr. However, because the precision of their assay was low, they could not determine the lower limit of carbon monoxide that affected respiration and oxidative phosphorylation. In addition, the investigators did not determine whether the myoglobin-mediated oxidative phosphorylation pathway was active at low oxygen pressures.

ASSESSMENT OF METHODS AND STUDY DESIGN

In a series of 54 experiments, the investigators exposed isolated cardiac myocytes to a wide range of gas mixtures containing varying levels of oxygen and carbon monoxide. These combinations produced COMb concentrations ranging from 0% to 100% of the total intracellular myoglobin. In several experiments, both cellular respiration and PCr:ATP ratios were determined as a function of the various gas mixtures. Using isolated cardiac myocytes as an experimental system allowed more definitive spectral and chemical analyses to be performed than would have been possible in intact tissues or animals. This approach also permitted the investigators to interpret their data at a more mechanistic level.

The investigators' employed a technically demanding method of continuous spectrophotometric analysis of living cells exposed to varying gas mixtures under controlled conditions. Using this technique permitted them to study the cells over relatively long periods of time (one hour), and to take continuous measurements during this period. They assessed the effects of sustained changes in COMb on the energy state of the cells by chemically extracting the cells

and quantifying the ATP and PCr levels. This analytical technique has obvious advantages over many other experimental systems. First, the relevant COMb measurements were obtained directly and rapidly after input of carbon monoxide; second, the target cells were exposed directly to the gas mixtures; and third, only the cell type of interest (cardiac myocytes) was analyzed for PCr and ATP content.

A major advantage of the investigators' in vitro experimental system was that it eliminated the presence of hemoglobin, which could bind carbon monoxide and confound the measurements. In addition, the isolated cells were not exposed to the influences of the nervous system or changes in blood flow, both of which have marked effects on cardiac function. This allowed detailed studies of the direct molecular responses of the cardiac cells and, potentially, the mechanisms that regulate these responses.

Thus, this experimental system is an excellent one for present and future studies. It should be noted, nonetheless, that using isolated cells does not reflect the complex environment found in intact animals and humans, which includes hemoglobin, other carbon monoxide binding proteins, and factors that influence the behavior of cardiac cells. Therefore, it will be important to verify the basic findings of the present study by analyzing other experimental systems that contain more of the complex characteristics of intact animals. These systems might include isolated tissue slices, isolated perfused hearts, and whole animals.

While the use of cardiac cells is clearly a strength, the characteristics of the cells were not fully defined in the report, and the investigators suggest that there was variability in the cell preparations used for different studies. The investigators noted that, on average, 25% of the cells used for the studies were not intact and, therefore, nonfunctional; this group was composed of damaged cardiac cells. The investigators believe that these cells did not contribute to their measurements in a meaningful way; however, the study would have been strengthened by including biochemical and spectrophotometric data supporting this conclusion.

To determine whether incubation in the spectrophotometric chamber itself had a significant adverse effect on various cellular functions, the investigators measured the viability of their cells at the end of the one-hour experimental period. They determined the number of cells that retained the characteristically rectangular shape and the capacity of the cells to contract. They observed a 10% to 15% decrease in the viable myocyte population over the exposure period. Decreased cell viability may have resulted from many factors, including incubating the cells at 30°C rather than at 37°C, and providing glucose as a metabolic substrate rather than the preferred substrate of fatty acids. Decreased cell viability suggests that a progressive loss of cellular function over the time the cells were incubated probably preceded the loss of normal cellular architecture. Whether this loss, or the variability in the cell preparations, or both, contributed to the considerable variability in the key data was incompletely addressed by the investigators.

By obtaining baseline data (myoglobin concentration, oxygen consumption, etc.) before carbon monoxide was added to the incubation chamber, each sample of cells could serve as its own control. This compensated for the variability among different experiments that might have been expected from small differences in procedures and cells. However, because determining PCr:ATP ratios required chemical extraction of the cells, this experimental design could not provide information on the initial cellular ratios of these compounds. Control values for PCr and ATP levels at the end of the incubation period were obtained from another aliquot of cells incubated in air at 30°C outside of the incubation chamber for the same duration as the experimental cells. To substantiate their claim that the PCr:ATP ratios measured in control cells after the incubation period were representative of the initial levels, the investigators performed an experiment in which they noted that there were no significant differences in PCr:ATP ratios during 120 minutes of incubation, either in the experimental cells maintained in the spectrophotometric chamber in the absence of carbon monoxide, or in the control cells incubated outside the experimental chamber.

STATISTICAL METHODS

The statistical methods employed were properly chosen for the analyses performed. The investigators used analysis of variance with the Scheffe F test to test for significant differences in the respiration of populations of heart cells with different fractions of COMb. The Scheffe F test is a multiple range test for unplanned comparisons, and is a more rigorous test for defining statistical differences than the t test. Data were considered to be significantly different when $p \leq 0.05$.

The investigators did not use regression analysis to assess the relationship between increasing COMb and decreasing respiration. A technical reviewer's regression analysis of the data presented in Table 1 of the Investigators' Report showed only a weak relationship between the two events, and is discussed below.

RESULTS AND INTERPRETATION

The investigators provided new data supporting the hypothesis that carbon monoxide binding converts myoglobin

to an inactive form and suppresses oxygen uptake and oxidative phosphorylation in isolated cardiac myocytes. However, not all of the inhibition is necessarily due to carbon monoxide because, as the report indicates, the respiratory rate of control cells not exposed to carbon monoxide also decreased during incubation. Thus, it is likely that the myocytes may be responding to factors other than carbon monoxide. The nature of these factors and how they affect the cells is currently unknown.

By subdividing different experiments into groups based on differing percentages of COMb, the investigators examined the effects of increasing concentrations of COMb on oxygen uptake by myocytes. Figures 4 and 5 of the Investigators' Report illustrate the relationship between COMb and respiration at high oxygen levels ($\leq$ 35 torr), and at low oxygen levels ($\leq$ 5 torr). The data presented in Figure 4 suggest that, at high oxygen levels, myocyte respiration was not inhibited by carbon monoxide until the COMb concentration reached 40% to 80% of the total myoglobin. Within this range the effect became maximal; there was no additional inhibition of myocyte respiration when the level of COMb reached 80% to 100% of total myoglobin. This is an unusual type of dose-response effect for a biological system; however, the investigators point out that this apparent "all or none" response may be a function of their arbitrary grouping of data into their chosen subsets.

Comparing the data from each individual experiment (presented in Table 1 of the Investigators' Report) suggests a greater variability than that seen in the grouped data, presented in Figures 4 and 5, which are taken from Table 1. Regression analysis of the data in Table 1 of the Investigators' Report, performed by an outside technical reviewer (Figure 1 in this Commentary), showed that the myocytes' responses to increasing carbon monoxide were highly variable, and were consistent with a linear relationship over the entire range of COMb concentrations. Thus, regression analysis does not provide clear evidence for a threshold COMb concentration, above which myocyte respiration decreases. This suggests that other variables may affect oxygen uptake directly or indirectly under these experimental conditions.

Lowering the oxygen pressure to less than 10 torr partially deoxygenates O_2Mb. Under these conditions, the investigators observed a decreased oxygen uptake by their myocyte preparations at COMb concentrations from 40% to 80% and from 80% to 100% of total myoglobin. Decreasing the partial pressure of oxygen to less than 5 torr (a level approaching the intracellular partial pressure of oxygen in cardiac cells in a working rat heart) resulted in significant decreases in oxygen uptake at 40% to 80% and 80% to 100% COMb concentrations. Because oxygen uptake at physiological oxygen partial pressures (less than 10 torr) was suppressed consistently at the higher COMb levels, the investigators concluded that a conservative estimate of the COMb levels required to suppress oxygen uptake most probably ranged from 50% to 100% of the total myoglobin.

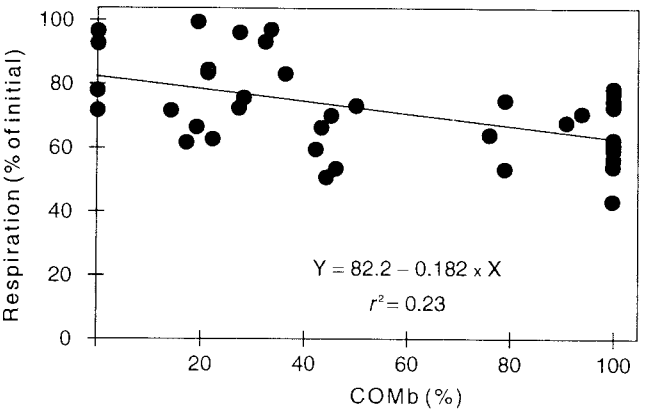

Figure 1. Regression analysis of data in Table 1 of the Investigator's Report.

The investigators calculated the concentration of blood COHb that could release sufficient carbon monoxide to produce the levels of COMb that decreased myocyte oxygen uptake in their experimental system (40% to 80% COMb). They proposed that a concentration of 40% COMb within a myocyte required 12% COHb in arterial blood, while 80% COMb could arise when approximately 38% of arterial blood hemoglobin existed as COHb. There are usually no adverse symptoms in healthy subjects at a COHb level of 10%; however, patients with coronary artery disease may experience a decreased threshold for angina at this level of COHb. Blood COHb levels between 10% to 20% can cause healthy subjects to experience headache, and patients with coronary artery disease may have an even greater risk for angina. Blood COHb levels between 30% and 40% cause moderate carbon monoxide poisoning, in the form of severe headaches, nausea, and other neurological symptoms, including a sudden fall in blood pressure on exertion (Ilano and Raffin 1990).

The investigators ruled out the possibility that the decline in respiration was due to a direct effect of carbon monoxide on cytochrome oxidase. They observed no spectral evidence for carbon monoxide to bind to cytochrome oxidase, and all experiments were conducted at ratios of carbon monoxide and oxygen partial pressures below that which allows carbon monoxide to bind to the enzyme. Because the myocytes required no blood supply, the investigators also ruled out COHb formation as having any effect on the myocyte's decline in respiration.

The investigators reported decreased PCr:ATP ratios

when more than 40% of the total myoglobin was in the nonfunctional COMb form. Because the PCr level dropped while the ATP level remained constant, they proposed that carbon monoxide-induced inhibition of mitochondrial ATP required the utilization of PCr to be increased to maintain a constant level of ATP. This is valid only if ATP utilization was unchanged, an assumption stated in the text. Whether this assumption is valid is unknown. Direct measurements of ATP utilization under the experimental conditions used for the spectral analyses should help to resolve this issue.

IMPLICATIONS FOR FUTURE RESEARCH

The investigators' findings are interesting, and their experimental system and analyses are excellent for exploring the role of carbon monoxide on cardiac myocyte function. Because their findings were obtained with "resting" (noncontracting) cells, the investigators suggested that the effects might be greater on cells undergoing active contraction, as in an intact heart. This hypothesis warrants future testing.

It will be important to test the hypothesis that carbon monoxide alters the key function of heart cells, which is, contraction, and to determine the nature of other functional changes related to carbon monoxide exposure. At some point there is always the necessity of testing observations on cells in a more relevant environment, such as intact tissues and animals. Results from these types of studies may one day allow researchers to extrapolate the concepts proposed by the present investigators to humans with cardiovascular disease.

CONCLUSIONS

In this study, the Wittenbergs evaluated the effects of increasing intracellular concentrations of COMb on oxygen uptake and ATP synthesis by myocytes. Using isolated cardiac muscle cells allowed more definitive chemical analyses than would be possible in intact tissues or animals, and allowed the investigators to interpret their data at a more mechanistic level. Their observations led them to conclude that myocyte oxygen uptake and ATP synthesis were suppressed when the COMb level increased.

The investigators grouped their data on myocyte oxygen uptake into subsets of 0% to 40%, 40% to 80%, and 80% to 100% COMb. At physiological oxygen pressures, COMb levels between 40% and 100% of the total myoglobin consistently reduced oxygen uptake. However, in some experiments performed at physiological oxygen pressures, the investigators reported statistically significant reductions in oxygen uptake at COMb levels between 40% and 80% that did not increase as COMb levels rose to between 80% to 100% of the total myoglobin. It is possible that this "all or nothing" response resulted from the investigators' arbitrary choice of the three subsets.

An independent examination of all of the oxygen uptake data (rather than the grouped data) by regression analysis did not strongly support the investigators' conclusions of a clear correlation between COMb content and suppression of oxygen uptake. Analyzing the data by this technique showed only a weak relationship between increasing COMb and decreasing oxygen uptake, and did not support the concept of a threshold COMb concentration above which respiration decreased. Therefore, other components of the experimental system, including variability in the cell preparations used in different experiments, may have affected myocyte respiration.

The extent to which these findings with isolated myocytes apply to the intact heart is unknown. In the long run, the hypothesis proposed in this report, that is, that increased COMb compromises ATP production, which in turn alters the maximum work output of the heart, may be correct. However, the lack of data from experiments using intact tissues or animals, does not allow extrapolation of these results to humans, and the investigators appropriately conclude that their hypothesis is speculative at this time.

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