EFFECT OF CO ON HUMAN CHEMOREFLEXES

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science,
Graduate Department of Physiology,
in the University of Toronto

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Despite decades of study, the precise mechanism by which the carotid body transduces the hypoxic stimulus remains controversial. CO inhibits carotid body output; Prabhakar has recently hypothesized that heme oxygenase 2 (HO-2) located in the glomus cells is the oxygen sensor, and that during hypoxia, endogenous CO production by HO-2 decreases, which disinhibits the carotid body. It was hypothesized that human subjects with mild carboxyhemoglobinemia would exhibit a reduced ventilatory response to hypoxia. Ten healthy male volunteers performed iso-oxic rebreathing tests at PO₂ of 50 and 150 mmHg before and after inhalation of ~1200 ppm CO in air, resulting in COHb values of 10.2 ± 0.2 % (mean ± SEM). No statistically significant differences in threshold or sensitivity were observed due to CO. Mild increases in COHb in healthy humans do not affect chemoreflex sensitivity, suggesting that endogenous CO production may not be important in transducing the hypoxic stimulus.
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<td>PO₂</td>
<td>partial pressure of oxygen (mmHg)</td>
</tr>
<tr>
<td>PCO₂</td>
<td>partial pressure of carbon dioxide (mmHg)</td>
</tr>
<tr>
<td>PCO</td>
<td>partial pressure of carbon monoxide (mmHg)</td>
</tr>
<tr>
<td>COHb</td>
<td>carboxyhemoglobin</td>
</tr>
<tr>
<td>O₂Hb</td>
<td>oxyhemoglobin</td>
</tr>
<tr>
<td>M</td>
<td>affinity constant of hemoglobin for CO relative to O₂</td>
</tr>
<tr>
<td>HO-2</td>
<td>heme oxygenase 2</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>ZnPP-9</td>
<td>zinc protoporphyrin 9</td>
</tr>
<tr>
<td>SnPP-9</td>
<td>tin protoporphyrin 9</td>
</tr>
<tr>
<td>CuPP-9</td>
<td>copper protoporphyrin 9</td>
</tr>
<tr>
<td>rPCO₂</td>
<td>rate of rise of PCO₂ during rebreathing</td>
</tr>
<tr>
<td>VєB</td>
<td>basal minute ventilation (L/min)</td>
</tr>
<tr>
<td>VтB</td>
<td>basal tidal volume (L)</td>
</tr>
<tr>
<td>fB</td>
<td>basal breathing frequency (min⁻¹)</td>
</tr>
<tr>
<td>VєS</td>
<td>slope of ventilation response (L·min⁻¹·mmHg⁻¹)</td>
</tr>
<tr>
<td>VтS</td>
<td>slope of tidal volume response (mL/mmHg)</td>
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<td>fS</td>
<td>slope of frequency response (min⁻¹·mmHg⁻¹)</td>
</tr>
<tr>
<td>VєT</td>
<td>threshold PCO₂ for ventilation response (mmHg)</td>
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<td>VтT</td>
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INTRODUCTION

Chemoreflexes

The chemoreflexes: role in maintenance of CO\textsubscript{2} and O\textsubscript{2} homeostasis

Breathing is an automatic motor act that originates in the brainstem, which is generated by neural mechanisms and is regulated by a neural control system (Nattie, 1999). A respiratory rhythm generator is responsible for automatically producing cyclic contractions of respiratory muscles, the function of which is to refresh the air contained within the lungs in order to provide oxygen to and remove carbon dioxide from actively metabolizing tissues via the blood (Feldman and McCrimmon, 1999). As such, a feedback control system is required in order to regulate the amount of ventilation according to the needs of the organism. This feedback control system includes mechanical sensory receptors that respond, for example, to lung volume or rate of change of lung volume, and chemical sensory receptors that respond to the local partial pressures of carbon dioxide (PCO\textsubscript{2}) and oxygen (PO\textsubscript{2}) and to pH. These chemical sensory receptors are termed "chemoreceptors", and these can be classified into central and peripheral types. Central and peripheral chemoreceptors contribute to ventilation in different ways by their different responses to PO\textsubscript{2} and PCO\textsubscript{2}, but collectively, the chemoreceptors are designed to promote homeostasis with respect to CO\textsubscript{2} and O\textsubscript{2} by causing ventilation to increase with increased PCO\textsubscript{2}, and/or low PO\textsubscript{2}. Changes in ventilation induced by changes in these variables are termed ventilatory chemoreflexes, or from here on, simply chemoreflexes.

Central chemoreceptors and the chemoreflex response to hypercapnia

The central chemoreceptors appear to consist of multiple chemoreceptive sites in the brainstem (Nattie, 1999). The ventral medulla has been a much-studied area and is a well accepted chemosensitive location that can affect ventilation; for example, changes in pH at the surface of
the ventral medulla affect breathing in anesthetized animals (Mitchell et al., 1963a; Mitchell et al., 1963b). Studies utilizing focal injections of acetazolamide, which acidifies the tissue, have identified other sites including: the NTS, locus coeruleus, medullary Raphe, and VRG (Nattie, 1994). However, the cellular and synaptic mechanisms of CO₂ chemoreception remain unknown (Feldman and McCrimmon, 1999). Generally, pH is assumed to be the final common pathway in the sensing of PCO₂ (Nattie, 1999): carbonic anhydrase catalyses the reaction of CO₂ and water, which combine to form carbonic acid, which rapidly dissociates to form primarily bicarbonate, but also carbonate anions and one or two protons:

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \rightleftharpoons 2\text{H}^+ + \text{CO}_3^-.
\]

CO₂ freely crosses the blood brain barrier, and the above reaction happens inside the neurons; thus the intracellular hydrogen ion concentration is greatly influenced by blood PCO₂.

The central chemoreceptors respond to increasing PCO₂ past a certain threshold value (see Figure 1). Indeed, one of the most fundamental and powerful responses of the body is that of increasing ventilation in response to increased PCO₂. This is a reflex that persists, albeit with somewhat less sensitivity, during sleep and even during anesthesia. Peripheral chemoreceptors also respond to increased PCO₂ (see below), however at normal PO₂, the central chemoreceptor drive is quantitatively much more important (Feldman and McCrimmon, 1999).
Peripheral chemoreceptors and the chemoreflex response to hypoxia

Peripheral chemoreceptors include the aortic and carotid bodies. In humans, the carotid bodies are more important (West, 1985), and henceforth, the aortic body chemoreceptors will be ignored. The carotid body is a small, very vascular organ located bilaterally at the bifurcation of the carotid artery (see Figure 2).

It is innervated by the petrosal ganglion via the carotid sinus nerve. It contains two main cell types: Type I (glomus) and Type II (sustentacular) cells; most evidence suggests that the glomus cells are the primary oxygen sensing cells (Prabhakar, 2000).

The function of the carotid bodies is to adjust our ventilation in response to oxygen level (for a review of oxygen sensing in the carotid body, see Gonzalez et al., 1995). If the carotid bodies are removed or denervated, the ventilatory response to low oxygen partial pressures is lost. The carotid bodies are quieted by high oxygen, and therefore even when PCO₂ is high, when PO₂ is 100 mmHg or more, carotid body discharge remains fairly flat (Feldman and McCrimmon, 1999). However, at low PO₂s, carotid body discharge increases dramatically with PCO₂ (Gonzalez et al., 1995).
A common view of the carotid bodies in the past has been as primarily oxygen sensors, for example, "the main stimulus of the type I cells is the low blood PO$_2$" (sic) (Gonzalez et al., 1995), with secondary effects by hydrogen ion concentration (see Figure 3). But for reasons discussed in Duffin (1990), it may be preferable to view peripheral chemoreceptors as primarily hydrogen ion sensors that are modified by oxygen (see Figure 4). There are two key benefits of this alternative viewpoint. First, it clarifies the form of the interaction between hydrogen ion and hypoxic stimuli: hypoxia sensitizes the response to hypercapnia, and this can be observed as an increase in slope and/or decrease in threshold of the ventilatory response to CO$_2$ with low O$_2$. Second, it makes it easy to consider the peripheral response to CO$_2$/O$_2$ as additive to the central response, and to other behavioural drives to ventilation to obtain the overall integrated ventilatory response.

Figure 3. Peripheral chemoreflex response to PO$_2$. Sensitivity is influenced by arterial PCO$_2$. (Reprinted from Duffin, 1990 with permission from the Canadian Journal of Anesthesia.)

Figure 4. Peripheral chemoreflex response to PCO$_2$. Sensitivity increases as PO$_2$ decreases (Reprinted from Duffin, 1990 with permission from the Canadian Journal of Anesthesia.).
Summary: model of chemoreflex control of breathing

The ventilatory response to CO₂ and O₂ can be considered as the sum of three drives: wakefulness drive, central chemoreflex drive, and peripheral chemoreflex drive (Duffin et al., 2000) (see Figure 5). Wakefulness drive, as reviewed by Shea (1996), describes cerebral activity associated with wakefulness that is part of the normal drive to breathe; this drive remains constant for all values of PCO₂. At low PCO₂, since there is no chemoreflex drive to ventilation, ventilation stays constant and is determined by wakefulness drive. Past a certain threshold PCO₂, peripheral and central chemoreflex drives increase. Central chemoreflex drive does not change between hyperoxia and hypoxia, but peripheral chemoreflex drive increases in slope (chemosensitivity). In our current understanding, the peripheral response may pivot around a value located below the x-axis. Resting ventilation is determined by the point of intersection of total ventilatory drive with the metabolic hyperbola. Therefore, at PCO₂ values above threshold, ventilation rises linearly with increasing PCO₂, the sensitivity being increased by low oxygen. Thus, measurement of the response to CO₂ and O₂ can be accomplished by measuring ventilation at different PCO₂ levels at various constant levels of PO₂.

Figure 5. Model of chemoreflex control of breathing. Total drive to ventilation (solid line), is a sum of wakefulness drive (dot-dashed line), central chemoreflex drive (dotted line), and peripheral chemoreflex drive (dashed lines).
**Hypoxia transduction in the carotid body:**

**downstream events**

The final step in the chemotransduction of hypoxia by the carotid body is an increase in firing of the carotid sinus nerve. The steps immediately preceding this increase are now fairly well agreed upon (Obeso et al., 2000). Figure 6 shows a "minimal schema" for chemoreception at low PO₂.

**Mechanism of O₂ chemoreception: hypotheses**

Despite decades of study, however, the precise mechanism by which the carotid body transduces the hypoxic stimulus ("Sensing", see Figure 6) is not well understood and remains controversial (Buckler, 1999; Donnelly, 1999; Prabhakar, 2000; Roy et al., 2000; Wilson et al., 2000). Understanding this mechanism is not only of scientific interest, but may also have clinical implications, as abnormalities of carotid body function have been implicated in Sudden Infant Death Syndrome (Donnelly, 2000).
Numerous hypotheses have been advanced as to the identity of the oxygen sensor (see Prabhakar and Overholt, 2000 for a recent review), including oxygen sensitive potassium and/or calcium channels (Montoro et al., 1996; Peers, 1990), membrane-bound hemoproteins, cytochrome c oxidase (Rozanov et al., 2000; Wilson et al., 2000), NADPH oxidase (Cross et al., 1990), nitric oxide synthase (Abu-Soud et al., 1996; Buerk and Lahiri, 2000; Iturriaga et al., 2000; Kline and Prabhakar, 2000), and heme oxygenase (Prabhakar et al., 1995) (see Figure 7). The issue is very complex because for every one of these theories there are many studies providing both supporting and contradictory evidence. Further explanation of these theories can be found in the Discussion chapter of this document. However, the next section will focus on the theory most relevant to the present study, namely that heme oxygenase is the oxygen sensor.
**Heme oxygenase as the primary oxygen sensor**

One of the more recent hypotheses, that heme oxygenase 2 (HO-2) located in the glomus cells is the oxygen sensor, has been proposed by Prabhakar (Prabhakar, 2000; Prabhakar, 1998; Prabhakar, 1999; Prabhakar et al., 1995; Prabhakar and Overholt, 2000).

Heme oxygenase is the major enzyme responsible for CO production. Two forms of heme oxygenase have been characterized: HO-1, which is inducible by a number of stimuli, including hypoxia, and HO-2, which is constitutively expressed in certain cell types. HO produces CO by catalysing the breakdown of heme (iron protoporphyrin 9); this process also requires O₂ (see Figure 8).

![Diagram of heme oxygenase and cytochrome P-450 reductase](image)

**Figure 8. Production of CO by heme-oxygenase. (Modified and reprinted from Prabhakar, 1998 with permission from Elsevier Science.)**

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The primary observations that have led to the formation of this hypothesis have been:

1. HO-2 has been shown to be present in Type I cells (glomus cells) of the carotid body (Prabhakar et al., 1995) of cats and rats.

2. CO production by HO-2 requires O₂, and hence in hypoxia, oxygen could theoretically become limiting and CO production could be reduced.

3. Tin protoporphyrin 9 (SnPP-9), a "false substrate" for HO-2 and potent HO-2 inhibitor, potentiates the respiratory response to hypoxia in rats, while elevating carboxyhemoglobin (COHb) from 2-24% reversed this potentiation (Yang et al., 1998). Similarly, another HO-2 inhibitor, zinc protoporphyrin 9 (ZnPp-9), increases carotid body output, measured directly; this effect is reversed by administration of exogenous CO (Prabhakar et al., 1995). Copper protoporphyrin 9 (CuPP-9), which does not significantly inhibit HO-2, showed no stimulatory response.

Therefore, Prabhakar has suggested that CO produced endogenously in glomus cells acts in an autocrine or paracrine manner to provide tonic inhibition of carotid body output. According to this theory, during hypoxia, decreased O₂ supply decreases the rate of synthesis of CO by HO-2, which results in disinhibition, and increased output (Prabhakar, 1998; Prabhakar, 1999; Prabhakar and Overholt, 2000).

Supporting evidence includes work by Lahiri et al, in which the output of in vitro cat carotid bodies perfused with hypoxic medium decreased when a PCO of 60-70 Torr was added to the perfusion medium (Lahiri et al., 1993) (see Figure 9. CO depresses hypoxic stimulation of in vitro perfused cat carotid body preparation. (Reprinted from Lahiri et al., 1993 with permission from Elsevier Science.)
9). Further evidence can be found in 1966 work by Cunningham and colleagues (Lloyd et al., 1966, Cunningham et al., 1967), who showed that human subjects breathing hypoxic hypercapnic gas decreased ventilation immediately after a bolus of CO was inhaled.

Other work by Prabhakar and colleagues suggests the mechanism by which CO might act in the carotid body. Overholt et al. (1996) found that ZnPP-9 increased intracellular Ca\(^{2+}\) if and only if extracellular calcium was present, whereas CuPP-9 did not. They also found that calcium currents were augmented and potassium currents were decreased with the HO-2 inhibitor. CO might also act to decrease carotid body discharge by causing vasodilatation, thus increasing blood flow and possibly improving oxygenation (although it should be noted that this is not a sensing mechanism per se).

**Hypothesis**

Inhalation of exogenous CO to cause elevated blood COHb levels will maintain inhibition to the carotid body during hypoxia, and cause the ventilatory response to hypoxia to be diminished or altogether abolished.

**Testing the hypothesis**

To test the hypothesis presented above, the ventilatory responses of healthy human volunteers to iso-oxic progressive hypercapnia (iso-oxic rebreathing) was compared at low PO\(_2\), before and after exposure to carbon monoxide (mild carboxyhemoglobinemia). To accept the hypothesis, one would expect to see a reduced ventilatory response to hypoxic rebreathing with increased COHb. Hyperoxic rebreathing tests performed before and after CO exposure served as a control.
Chemoreflex measurement

Previous modifications of Read's original rebreathing test (Read, 1967), first described by Casey et al., (1987) and Duffin and McAvoy (1988), have been recently updated (Mohan and Duffin, 1997), as well as the interpretation of its results (Duffin et al., 2000). In its hyperoxic form, the modified rebreathing test measures central chemoreflex sensitivity equivalent to those measured using Read's technique and the end-tidal forcing technique (Mohan et al., 1999).

The modifications made to Read's rebreathing technique (Read, 1967) are as follows. First, rebreathing is preceded by a 5-minute hyperventilation to lower stores of carbon dioxide, thereby allowing the chemoreflex threshold to be discerned as carbon dioxide rises through it. This modification also permits measurement of the sub-threshold ventilation independent of the chemoreflexes; this basal ventilation estimates the “wakefulness drive” (Fink, 1961).

For the second modification, iso-oxia is maintained during rebreathing by supplying oxygen to the rebreathing bag, so that both hyperoxic (150 mmHg) and hypoxic (50 mmHg) iso-oxic ventilatory responses to carbon dioxide can be examined. Comparison of hypoxic and hyperoxic measurements can be made to determine if changes occurred in peripheral or central chemoreflexes or both, making assumptions discussed in (Duffin et al., 2000) and listed in Appendix A.

Uptake, distribution and elimination of CO

CO is produced from the incomplete combustion of carbon-containing compounds. CO enters the body via inhalation and diffusion across the alveolar capillary membrane. It dissolves in the blood, where it binds reversibly, but with high affinity (approximately 200 times that of oxygen) to the oxygen binding site on hemoglobin, forming carboxyhemoglobin (COHb). The Haldane
equation (Douglas et al., 1912) describes the relationship between PCO in the blood and COHb concentration: 

\[
\frac{\text{PCO}}{\text{PO}_2} M = \frac{[\text{COHb}]}{[\text{O}_2\text{Hb}]},
\]

where \( M \) is a dimensionless affinity constant of hemoglobin for CO relative to \( O_2 \) which equals approximately 200 (Turino, 1981). CO is also capable of diffusing freely into tissues and binding to myoglobin, cytochromes, and other heme containing molecules, although the relative affinity of these molecules for CO compared to \( O_2 \) is not as high as hemoglobin (Coburn, 1979).

CO is metabolized by the body in skeletal muscle, probably in the mitochondria. Bovine cytochrome c oxidase slowly oxidizes CO to \( CO_2 \) using \( O_2 \) (Coburn and Forman, 1987), but the rate of metabolic consumption of CO is insignificant in resting human subjects. Even at a COHb of 20%, the rate of metabolism is small compared to the rate of endogenous production of CO by the breakdown of heme in the spleen and other tissues. Essentially, CO is eliminated only through the lung, and then only when the inspired PCO is less than that of the blood. The rate of elimination of CO from the lung is a function of the partial pressure gradient between mixed venous blood and alveolar gas. Therefore, the rate of elimination is increased by raising the \( \text{PO}_2 \), which increases the PCO at a given COHb/\( O_2\text{Hb} \) ratio (see the Haldane equation above), and by increasing ventilation, which decreases the steady-state alveolar PCO. Both of these act to maximize the gradient for diffusion of CO out of the blood and into the lung.

**Synopsis**

The carotid body is known to be the sensory organ responsible for detecting low oxygen in the blood and causing a compensatory increase in ventilation. Exactly how the stimulus of low oxygen is transduced by the carotid body into a change in firing rate is controversial. One recent hypothesis suggests that heme-oxygenase 2, a CO-producing enzyme located in the glomus cells.
of the carotid body, is the oxygen sensor that CO provides tonic inhibition to the carotid body, and that a decrease in CO production during hypoxia is the first event in the transduction of a hypoxic stimulus. The hypothesis tested here was that during hypoxia, provision of exogenous CO would maintain the inhibition to the carotid body and depress the ventilatory response to hypoxia.
METHODS

Subjects, consent, setting

After the Human Ethics Review Committee of the University of Toronto approved the study, a group of 10 healthy male volunteers between the ages of 22 and 52 gave their informed consent, subsequent to an explanation of the experimental protocols and possible risks. Volunteers were non-smokers without medication, and with no history of cardio-respiratory diseases. Their age, height and body mass were 28 ± 9 y, 180 ± 6 cm and 76 ± 8 kg, respectively (mean ± SD).

Protocol

Subjects were studied in the sitting position. A mask was fitted to the subject's face and the subject was then connected to a circle breathing circuit and was allowed to breathe normally for a few minutes. The subject then performed "practice" hyperoxic and hypoxic rebreathing tests in no particular order in order to check equipment function and to ensure that the subject was comfortable with each of the rebreathing tests. Following the practice rebreathing tests, the subject was disconnected from the circuit while an intravenous sampling catheter was inserted. The subject was once again connected to the circuit, and performed control hypoxic and hyperoxic rebreathing tests, in random order. Next, the subject was exposed to CO at approximately 1000-1200 ppm until COHb reached approximately 10%. Finally, post-exposure hypoxic and hyperoxic rebreathing tests were performed in the same order as the control rebreathing tests. Following the experiment, the circuit was modified to allow isocapnic hyperpnea, and the subject breathed at 20-40 L/min in order to facilitate the washout of the CO, while the PETCO₂ was maintained constant, until COHb fell below 6-7%.
Breathing circuits

Figure 10 illustrates the experimental apparatus. The subject breathed through a facemask (Hans Rudolph) connected via a MQ303 bacterial filter (Vacumed, Ventura, CA) and a VMM-400 turbine (Interface Associates) to one side of a Series 2870 three-way sliding valve (Hans Rudolph Inc.) that allowed switching between the circle circuit (A) and the rebreathing bag (B). A sticky silicone seal ensured a leak-proof seal with the subject’s face. A sampling port at the mouth allowed measurement of inspired and expired CO₂ and O₂ partial pressures. All circuit components, including valves, tubing, and connectors were of large internal diameter, in order to provide minimal resistance to breathing during hyperventilation; dead space of the mask and connectors was approximately 150 mL.

![Figure 10. Experimental apparatus.](image)

Circle circuit

The subject was connected to the circle circuit during the period of hyperventilation prior to each modified rebreathing test and during CO exposure. The circuit consisted of a non-rebreathing
valve (the point of connection to the three-way valve), a specially made CO₂ absorber filled with soda lime, and a reservoir bag of approximately 10 L capacity. An inlet located in the CO₂ absorber allowed gases entering the circuit to be well mixed before being inhaled by the subject, and oxygen, carbon monoxide and returned gas from CO₂ and O₂ analysers entered the circuit through this inlet.

A sampling port located at the reservoir bag allowed inspired air to be analysed for CO concentration using a CO monitor (PK Morgan, Chatham, Kent, England) whose analog output signal was digitised on a personal computer at 60 Hz using a DI-200 analog to digital convertor (DATAQ Instruments, Inc., Akron OH) and recorded using Windaq Pro (DATAQ Instruments, Inc., Akron OH). A three-lead ECG monitor (Hewlett Packard 78342A) was used to monitor the ECG continuously for the duration of the experiment to ensure that ST segment depression did not occur as a possible consequence of CO inhalation and hypoxia.

Rebreathing circuit

The rebreathing bag was of approximately 7 L capacity and contained an inlet for oxygen. Ventilation and end-tidal PCO₂ and PO₂ were monitored breath-by-breath throughout. Subjects’ O₂Hb was monitored by pulse oximetry. Ventilation was measured using a turbine, and PCO₂ and PO₂ were measured using gas analysers (Oxigraf, Fast Oxygen Analyzer and BCI International, Capnocheck Plus, respectively) which drew a continuous flow (150 ml min⁻¹) from the mouthpiece connection. Heart rate was calculated from the pulse oximeter tracing (BCI International, Capnocheck Plus).

During rebreathing, analog signals were digitised using a 16-bit analog to digital convertor (National Instruments, AT-MIO-16XE-50) for on-line computer analysis using specially written
software (National Instruments, LabVIEW, source code available on request). The software calculated tidal volumes, inspiratory and expiratory times, ventilation, end-tidal PCO₂ and PO₂, arterial oxygen saturation and heart rate on a breath-by-breath basis. The initial rebreathing equilibration was monitored, and unless a plateau in carbon dioxide was observed during equilibration, the rebreathing test was aborted.

CO exposure

During exposure, subjects were distracted by reading, sitting quietly, or listening to music. A flow of oxygen was supplied to the CO circuit at 200-400 mL/min (adjusted manually) in order to keep the FiO₂ at approximately 21%. A bolus of approximately 20 mL of pure CO was added to the circuit, and an infusion pump (Harvard Apparatus) was then used to add CO to the circuit at a rate of approximately 200 mL/hour. This flow rate was adjusted as necessary to keep the circuit CO concentration at approximately 1200 ppm. Every 5 minutes, approximately 2 mL of venous blood was withdrawn from the sampling catheter and analysed for COHb concentration. When the subject had achieved between 9 and 10% COHb (approximately 30-45 minutes), the infusion pump was turned off to allow the subject to equilibrate with the CO concentration of the circuit. Venous blood samples were taken as before; when two consecutive samples showed COHb values within 0.2%, the subject was deemed to be fully equilibrated, and the modified rebreathing tests were conducted (see below).

Rebreathing tests

Each rebreathing test consisted of 5 minutes of hyperventilation, during which volunteers remained connected to the circle circuit and were coached to maintain an end-tidal partial pressure of carbon dioxide between 19 and 25 mmHg. The rebreathing bag initially contained oxygen at a P₀₂ of 170 mmHg for the hyperoxic and 30 mmHg for the hypoxic test, and a PCO₂
~ 42 mmHg. Rebreathing began at the end of expiration and was followed by three deep breaths producing rapid equilibration of carbon dioxide partial pressures in the bag, lungs, and arterial blood to that of mixed venous blood. Equilibration was verified by observation of a plateau in the end-tidal carbon dioxide partial pressure, and was a prerequisite for continuing the test. During rebreathing, iso-oxia at 150 mmHg (hyperoxic) or 50 mmHg (hypoxic) levels was maintained by the addition of oxygen to the rebreathing bag under computer control. The experimenter terminated rebreathing either when ventilation exceeded 100 L/min, or end-tidal PCO$_2$ exceeded 60 mmHg, or if the subject experienced respiratory discomfort or was unable to tolerate the hypercapnia or hyperpnea. During chemoreflex testing, distracting sounds and motions in the room were minimized in order to minimize and keep constant any behavioural stimuli that might affect the subject’s breathing.

**COHb measurement**

Venous blood was obtained from a 20-gauge catheter inserted into a peripheral arm vein. A normal saline drip facilitated keeping the vein open for the duration of the experiment. Blood samples were obtained approximately every 5 minutes during carbon monoxide exposure. An OSM3 CO-Oximeter (Radiometer, Copenhagen, Denmark) was used to analyse the venous blood samples for COHb content.

**Data Analysis**

The data accumulated from the rebreathing tests were analysed using a specially designed spreadsheet (Microsoft Excel). First, breaths from the initial 3-breath equilibration, as well as sighs, swallows and breaths incorrectly detected by the software, were excluded from further analysis. Next, the breath-by-breath end-tidal partial pressures of carbon dioxide were plotted against time and fitted with a least-squares regression line. The equation for this line provided a
predicted value of end-tidal partial pressure of carbon dioxide as a function of time, thereby
minimising inter-breath variability. Subsequently, tidal volume (ml BTPS), respiratory rate
(breaths/min) and ventilation (L/min BTPS) were plotted against the predicted end-tidal partial
pressure of carbon dioxide (mmHg).

Each of these plots was fitted with a model made up of the sum of two or three segments
separated by one or two breakpoints, respectively (Duffin et al., 2000). Model fitting was based
on minimising the sum of least squares for non-linear regressions using commercial software
(Sigma Plot 5.0, SPSS). The first fitted segment was an exponential decline to a final value,
which was taken as a measure of the basal ventilation, basal tidal volume and basal breathing
frequency (VEB, VTB and fB characteristics, respectively). The exponential decline was chosen
to fit any waning of short-term potentiation of ventilation produced by hyperventilation that
might have occurred. In those volunteers without such a trend (most), the decay constant of the
exponential decline reverted to a value less than 1, so that the basal values were equivalent to the
mean values below the first breakpoint.

The second and possible third segments were fitted as straight lines from the first to the second
breakpoint, and above the second breakpoint, respectively. The first breakpoint was taken as a
measure of the chemoreflex threshold for the ventilation, tidal volume and breathing frequency
responses to carbon dioxide (VEF, VTF and fF characteristics, respectively). The second break
point was taken as the point at which the ventilatory response pattern changed, in terms of tidal
volume and frequency, but was observed in so few tests that it is not reported here. The slopes of
the first straight-line portions were taken as the chemoreflex sensitivity for the ventilation, tidal
volume, and breathing frequency responses (VES, VTS and fS characteristics, respectively).
**Statistical analysis**

The rate of rise of the PCO$_2$ during rebreathing (\(\text{r}_\text{PCO}_2\)), basal levels VEB, VTB and fB, chemoreflex sensitivities VES, VTS and fS and chemoreflex thresholds VET, VTT and fT were analysed using a two-way repeated measures analysis of variance (RM-ANOVA) with \(p<0.05\) judged significant. The two factors were CO (present vs. absent) and iso-oxic level (hypoxic vs. hyperoxic). Results are reported as mean ± SEM.

**CO Elimination**

Following the completion of data collection, the subjects voluntarily hyperventilated O$_2$ at 20-40 L/min to facilitate the washout of the CO from the body (Takeuchi et al., 2000), while isocapnia was maintained by the addition of CO$_2$ in proportion to the ventilation (Sommer et al., 1998). In brief, the subjects breathed through a circuit which provided 100% O$_2$ (fresh gas) at a flow rate approximately equal to the resting minute ventilation, and the balance of all ventilation was provided by a flow of 6% CO$_2$, balance O$_2$ (reserve gas). The fresh gas allows the usual amount of CO$_2$ elimination as would occur at rest, while the reserve gas prevents any additional CO$_2$ elimination, because its PCO$_2$ is approximately equal to that of the arterial blood, and therefore it provides no gradient for CO$_2$ elimination. However, both the fresh gas and reserve gas allow CO elimination. The total gas flow was set to a level that was sustainable for the subject, who continued to breathe on the circuit in this way until the COHb fell below 6%.

**Calibration**

Instruments were calibrated before each experiment as follows:
Ventilation

A 3 L calibration syringe (Model R5530B, Vacumed, Ventura, CA) was used to pump 3 L tidal volumes through the circuit, and the gain was adjusted until the calculated tidal volume reading was 3 L.

$CO_2$

Compressed air and 7% CO$_2$, balance oxygen were used to set the zero and gain for the digital CO$_2$ signal. Accuracy of reading was verified on most occasions using a third gas mixture (2.5% CO$_2$, balance oxygen).

$O_2$

A two point calibration of the digital O$_2$ signal was performed using 21% O$_2$ and 50% O$_2$, balance N$_2$. Accuracy of reading was verified on most occasions using a third gas mixture.

$CO$

A two point calibration of the CO analyser was performed using compressed air and a standard lung diffusion mixture, consisting of 0.298% CO, 9.9% He, 21% O$_2$, balance N$_2$.

$COHb$

Accuracy of reading was verified according to Radiometer's instructions using the calibrating solutions Hemoximetry Qualicheck S2140 and Qualicheck-3 S3060, with COHb of 36.3-39.9 and 24.4-29.4%, respectively. Although both of these concentrations exceed the measurement range of 1-10% for this study, these solutions have the lowest concentrations of COHb in commercially available calibrating solutions for our device. The machine read normal saline as
having a COHb value of 0. The COHb readings of blood samples were in the predicted range and were reproducible.
RESULTS

The age, height and weight for all subjects are listed in Table 1.

Table 1. Anthropometric data for all subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>170</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>183</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>190</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>180</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>176</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>176</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>178</td>
<td>86</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>181</td>
<td>78</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>183</td>
<td>84</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>185</td>
<td>74</td>
</tr>
<tr>
<td>Mean</td>
<td>28.3</td>
<td>180.2</td>
<td>75.8</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>8.8</td>
<td>5.6</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Four subjects performed the hypoxic rebreathing tests first, while six subjects performed the hyperoxic rebreathing tests first. Figure 11 shows the end-tidal PCO₂, end-tidal PO₂ and minute ventilation versus time relationships for a typical rebreathing test. The subjects’ initial resting COHb levels averaged 1.0 ± 0.04 %, and after a mean CO exposure time of 39 ± 2 minutes, COHb levels averaged 10.2 ± 0.2 % just before the first post-exposure rebreathing test. Figure 12 illustrates carboxyhemoglobin values versus time over the course of the experiment for a representative subject.
Figure 11. End tidal PCO₂, end tidal PO₂ and minute ventilation versus time for a typical rebreathing test. PO₂ squares, PCO₂ circles, ventilation triangles.

Figure 12. COHb versus time for a single representative subject.
The mean end-tidal PCO2 at the start of the hyperoxic rebreathing tests with and without CO were 35.2 ± 0.6 and 33.6 ± 0.7 mmHg, respectively; those for the hypoxic tests were 34.1 ± 0.7 and 34.1 ± 0.6 mmHg, respectively. None of these values was significantly different from the others. The mean iso-oxic end-tidal PO2 for the hypoxic tests with and without CO was 52.2 ± 0.3 and 52.1 ± 0.4 mmHg, respectively, and for the hyperoxic tests was 153.0 ± 0.1 and 153.0 ± 0.7 mmHg, respectively. The iso-oxic levels for the tests with CO did not differ significantly from the tests without CO. The rates of rise of end-tidal PCO2 (rrPCO2) during the hyperoxic rebreathing tests with and without CO were 3.48 ± 0.12 and 3.36 ± 0.18 mmHg/s, respectively, and during the hypoxic tests, were 3.84 ± 0.18 and 3.60 ± 0.24 mmHg/s, respectively. None of these values was significantly different from the others. Heart rate (HR) increased significantly with hypoxia from 69 ± 2 to 79 ± 2 beats per min, but CO had no effect.

Figure 13. Ventilation versus PETCO2 response curves for all four conditions for a single representative subject. Triangles are PO2 = 150 mmHg, circles are PO2 = 50 mmHg, filled symbols are control, open symbols are with COHb = 10%.
Figure 13 shows the ventilation response to carbon dioxide of a typical subject under all four conditions (iso-oxic hypoxic and hyperoxic, with and without CO); Figure 14 shows the average response. Table 2 shows pooled chemoreflex characteristics for all conditions (individual data are presented in Appendix B). The 2-way RM-ANOVA tests of the chemoreflex characteristics showed that the presence or absence of CO was not a significant factor for any of them. While the iso-oxic level was a significant factor for the response thresholds, there was not a significant interaction between CO and iso-oxic level. Post-hoc tests showed that \( V_{ET} \), \( V_{IT} \) and \( f_T \) were significantly decreased by hypoxia (p<0.001).

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**Figure 14.** Average ventilation versus PETCO\(_2\) response curves, calculated using the model. Solid lines are control, dotted lines are with COHb = 10\%. 95% Confidence intervals are presented for control data.
Table 2. Pooled results showing mean ± SEM (n=10) for the basal levels, threshold values, and sensitivities for all conditions. *indicates a significant effect of O₂ level within the same CO condition.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hypoxia</th>
<th>Hypoxia</th>
<th>Hyperoxia + CO</th>
<th>Hypoxia + CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>RrPCO₂ (mmHg/min)</td>
<td>3.48 ± 0.12</td>
<td>3.84 ± 0.18</td>
<td>3.36 ± 0.18</td>
<td>3.60 ± 0.24</td>
</tr>
<tr>
<td>V&lt;sub&gt;EB&lt;/sub&gt; (L/min)</td>
<td>6.9 ± 0.7</td>
<td>7.4 ± 1.3</td>
<td>6.3 ± 1.3</td>
<td>10.4 ± 1.3</td>
</tr>
<tr>
<td>V&lt;sub&gt;ET&lt;/sub&gt; (mmHg)</td>
<td>43.6 ± 0.6</td>
<td>38.4 ± 0.7 *</td>
<td>43.2 ± 0.8</td>
<td>39.0 ± 0.9 *</td>
</tr>
<tr>
<td>V&lt;sub&gt;ES&lt;/sub&gt; (L.min⁻¹.mmHg⁻¹)</td>
<td>4.0 ± 0.6</td>
<td>6.5 ± 1.9</td>
<td>4.8 ± 0.9</td>
<td>7.7 ± 1.8</td>
</tr>
<tr>
<td>V&lt;sub&gt;T&lt;/sub&gt; (mL)</td>
<td>580 ± 90</td>
<td>530 ± 100</td>
<td>530 ± 80</td>
<td>810 ± 130</td>
</tr>
<tr>
<td>V&lt;sub&gt;TT&lt;/sub&gt; (mmHg)</td>
<td>41.9 ± 0.8</td>
<td>37.3 ± 0.6 *</td>
<td>41.9 ± 0.8</td>
<td>37.7 ± 0.7 *</td>
</tr>
<tr>
<td>V&lt;sub&gt;TS&lt;/sub&gt; (mL/mmHg)</td>
<td>181 ± 45</td>
<td>248 ± 65</td>
<td>208 ± 43</td>
<td>273 ± 49</td>
</tr>
<tr>
<td>fB (min⁻¹)</td>
<td>11.8 ± 0.9</td>
<td>11.4 ± 1.1</td>
<td>10.9 ± 0.8</td>
<td>10.2 ± 0.9</td>
</tr>
<tr>
<td>fT (mmHg)</td>
<td>44.6 ± 1.4</td>
<td>39.9 ± 1.2 *</td>
<td>45.5 ± 1.2</td>
<td>39.6 ± 1.4 *</td>
</tr>
<tr>
<td>fS (min⁻¹.mmHg⁻¹)</td>
<td>1.4 ± 0.5</td>
<td>2.7 ± 1.0</td>
<td>1.9 ± 0.6</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>69 ± 2</td>
<td>79 ± 2 *</td>
<td>69 ± 2</td>
<td>79 ± 2 *</td>
</tr>
</tbody>
</table>
DISCUSSION

The ventilatory responses of human subjects to CO₂ were compared in a random cross-over design under hypoxic and hyperoxic conditions before and after CO inhalation. It was found that hypoxia caused a statistically significant decrease in the threshold of the ventilatory response, and a trend to an increasing slope, although statistical significance was not attained in the latter. However, none of the parameters of the hypoxic, nor hyperoxic ventilatory response to CO₂ were different following CO exposure. In the following sections, possible errors in the study will be discussed, the results will be compared to those of other investigators, and finally, the results will be integrated with respect to current theories of oxygen sensing by the carotid body.

Limitations

Level of exposure

It is possible that even 10% COHb might be too high a concentration, and that the response, which might have been visible at lower concentrations, was offset by another effect at 10% COHb. The findings of Lahiri et al. (1993) suggest that a change in the net effect of CO from depression to stimulation might occur at higher (10⁴ times higher) PCO values. This possibility was tested in one subject by pausing the CO exposure at approximately 5% COHb, and performing both the hypoxic and hyperoxic CO₂ rebreathing tests. For both hyperoxic and hypoxic tests, the subject’s CO₂ responses were almost identical to those observed during the control rebreathing tests (see Figure 15).
Placebo and time effects

The subjects were not blinded as to which tests were control and which were post-exposure. In addition, the post-exposure chemoreflex testing occurred an average of 74 minutes after control tests. Therefore, it is possible that a placebo effect and/or general discomfort could have played a role in the outcome of the experiment. However, there was no significant difference between control and post-exposure hypoxic rebreathing tests, so it seems unlikely that placebo or time effects played a role because they would have had to exactly offset the effect of the CO on the hypoxic ventilatory response curves. The prediction was that the CO would cause changes in the hypoxic responses, but not the hyperoxic responses, therefore the hyperoxic rebreathing tests served as a control. The absence of any difference between control and post-exposure hyperoxic rebreathing tests provides evidence that time and placebo effects did not affect the responses. Sham control experiments, in which the CO is replaced with air without the subjects' knowledge,
might have provided further evidence to discount these effects, but would have doubled the time commitment required from each subject.

Chemoreflex measurement

It is possible that the method of measuring chemoreflexes was not optimal to detect differences caused by the CO. However, this method of iso-oxic rebreathing is an accepted way of assessing chemoreflex responses, for example, see (Mahamed et al., 2001), and one which allows measurement of both the threshold and sensitivity of the response, while avoiding the confounding effects of falling PO2. As such, the method should have been capable of detecting any differences caused by the CO.

Venous blood

In this study, the goal was to manipulate the PCO in the carotid body, but instead of assessing carotid body PCO, COHb concentration in the blood was measured. Blood COHb concentration should be a good reflection of carotid body PCO. When CO is inhaled, it diffuses across the alveolar-capillary membrane and into the blood. It is then carried throughout the body freely dissolved in the plasma and bound reversibly to hemoglobin at a concentration that is in equilibrium with the dissolved fraction. At tissue capillary beds, CO diffuses freely across the cell membrane, following its partial pressure gradient, and enters the tissues. When blood PCO rises, tissue PCO may theoretically lag behind. The time constant of equilibration between particular tissues and the blood is not well known, but in very well perfused tissues, such as the carotid body, the time constant should be negligible. And indeed, in other studies which have used CO in vitro (Lahiri et al., 1993), step changes in the PCO of the perfusing solution resulted in changes in the output which were complete within seconds.
Another interesting possibility is that the PCO at the oxygen sensor under control conditions could be higher than one might expect based on the blood PCO. This would occur if the sensor were located in close association with the heme oxygenase molecules, the site of CO production. In this case, it is possible that 10% COHb would not provide a high enough tissue PCO to maintain carotid body inhibition during hypoxia. Unfortunately, it is difficult to guess the PCO at the oxygen sensing molecule since the identity (and hence location) of the molecule itself is controversial.

Low power

The possibility that a type II error might have been made in the statistical analysis was considered, because the power of the tests using an alpha of 0.05 for CO was low (0.05 for both VET and VES). The expected outcome was a large change, and the low statistical power would indicate an inability to detect a small difference; nevertheless, the experimental findings are sufficient to show that the hypothesized change did not occur. Interestingly, other recent studies of the chemoreflexes have also used small numbers of subjects and yet have been able to demonstrate significant changes; for example Mahamed and Duffin (2001) showed that repeated hypoxic exposures increased chemoreflex response thresholds, using similar numbers of subjects with similar standard errors.

Significant methodological differences from other studies

Other researchers have reported that CO inhibits (Lloyd et al., 1966; Prabhakar et al., 1995, Lahiri et al., 1993), stimulates (Lahiri et al., 1993) or does not affect (Duke et al., 1952) carotid body output and/or integrated ventilatory responses under various circumstances. The null finding presented here does not necessarily contradict those studies showing significant effects of
CO because there are significant differences between the methodology used in the present study and that of previous studies in terms of CO level and experimental species.

**CO level**

Some previous investigators have used very high levels of CO, for example PCO values of 500 mmHg (Joels and Neil, 1962; Lahiri et al., 1993) or COHb concentrations *in vivo* of as much as 70% (Lahiri et al., 1981). At these concentrations, significant binding might occur to a heme containing oxygen sensor, even if its affinity for CO is low (e.g., cytochrome oxidase (Coburn, 1979)). Indeed, this was part of the rationale for Joels and Neil (1962) to use a high level of PCO. At high concentrations of COHb *in vivo*, oxygen carrying capacity of the blood is significantly reduced, possibly lowering tissue PO₂. Under no circumstances could these CO levels be considered “physiological”. By contrast, in the present study, the subjects inhaled a partial pressure of approximately 0.7 mmHg, and peak blood PCO values can be calculated to be approximately 0.055 mmHg using the Haldane equation (Douglas et al., 1912). If endogenously produced CO truly inhibits the carotid body, then this partial pressure should have been sufficiently high to maintain inhibition. At the same time, this PCO was low enough so that minimal binding of CO would have occurred for cytochrome oxidase (whose relative affinity for CO/O₂ is approximately 1) (Coburn, 1979) or other heme-containing molecules, whose affinities for CO are similarly low (Coburn and Forman, 1987).

**Experimental species**

While some studies have been conducted in humans (Chiodi et al. (1941), much of the work on oxygen sensing in the carotid body, and on the role of CO in the carotid body, has been done using various neurorespiratory animal models, namely, the rat (Lahiri et al., 1999; Prabhakar et al., 1995), the cat (Duke et al., 1952; Gautier and Bonora, 1983; Joels and Neil, 1962; Lahiri et
al., 1993; Lahiri et al., 1981), and the rabbit (Lopez-Lopez and Gonzalez, 1992). However there are interspecies variations in some important features of the carotid body, for example, in the vascular anatomy (Seidl, 1973), ion channels (Prabhakar and Overholt, 2000), and neurotransmitters (Prabhakar, 2000) of the carotid body, as well as functional differences in the ventilatory response to hypoxia (Cunningham, 1974; Iscoe et al., 1998). Given these diverse and substantial differences between the usual animal models employed in the study of chemoreception and humans, it is not clear to what extent results obtained in these species help us to understand human chemoreception. Therefore, the present study is important because it describes the chemoreflex response to hypoxia/hypercapnia in the presence and absence of CO in humans.

Previous research

CO has a long history of use in experiments in respiratory physiology. The effect of CO on the respiratory system was first studied in the context of the pathophysiology of CO poisoning around the turn of the century by Haldane (1895), who found that, in himself, COHb values greater than 35% caused hyperventilation. Haggard and Henderson (1921) found that dogs with COHb values of 50% or more hyperventilated enough to cause alkalosis. Therefore, Chiodi et. al. (1941) studied the effect of COHb on ventilation and chemosensitivity to determine if this was the source of the stimulation. They performed a total of 10 experiments on 2 human volunteers exposed to COHb values of 11-41%, and measured the steady state ventilation response to CO2 concentrations of 1.8-5.2% in air. They found a small decrease in ventilation of 0.3-1.3 L/min. However, interpretation of the results is somewhat difficult due to the small observed effect, coupled with the small number of subjects, variation in the inspired CO2 percentage used, and variability in the observed COHb. Furthermore, the use of air instead of a hypoxic mixture means that carotid body output would have been fairly low. Duke et al. (1952) studied
spontaneously breathing anesthetized (and therefore hypercapnic) cats, and concluded that COHb, even as high as 76%, did not increase carotid sinus nerve discharge under hyperoxia or hypoxia. Although they observed some increase in firing under hypoxia, they attributed this to a fall in mean arterial blood pressure from 175 mmHg to 80 mmHg. The results of the present study are therefore consistent with those of Duke et al. (1952).

A change in paradigm occurred when physiologists became interested in the physiology of chemoreception itself. Joels and Neil (1962) used a high PCO (approximately 500 mmHg) as a tool to specifically inhibit cytochrome oxidase. They found that CO stimulated carotid body firing in hypoxia. As discussed above, this data does not address the putative role of endogenous CO production in oxygen sensing due to the very high PCO used.

Lloyd and co-workers (Cunningham et al., 1967; Lloyd et al., 1966) suggested that the carotid body might contain a heme protein that was involved in oxygen sensing, and that CO might successfully compete with oxygen for that protein and thus affect ventilation. These authors had human subjects inhale a bolus of 260 mL of pure CO in hypoxic/hypercapnic gas mixture, and found a slight depression of ventilation following the bolus. They interpreted this as support for the heme-pigment theory of chemoreception. The volume of CO administered was calculated by the authors to be sufficient to fully saturate the hemoglobin in the pulmonary capillaries on a single respiratory cycle (however, the PCO would still have been orders of magnitude lower than that of Joels and Neil, 1962). Although the authors made no measurements of the changes in arterial COHb that resulted from this maneuver, the dose of CO to which the carotid body was transiently subjected must have been very high. As such, the responses observed might reasonably be expected to be different from those in the present experiment, and may once again be too high to draw conclusions about the role of endogenous CO production on chemoreception.
Lahiri and colleagues (Lahiri et al., 1981) studied the effect of CO on carotid body output in anesthetized ventilated cats. They found little or no stimulation of carotid bodies by COHb up to 50%. Although this agrees with the present results, measurements were made at a PCO$_2$ of less than 20 mmHg and a PaO$_2$ of approximately 90 mmHg. Under these conditions, the level of stimulation of the carotid bodies is low, and changes in output might not occur even if CO is involved. Consequently, data from this study do not help to address the hypothesis under study in the present investigation.

Lahiri et. al. (1993) used a perfused superfused cat carotid body preparation to again study the effects of CO. Their findings were complicated: a high concentration of CO (PCO = 500 mmHg) stimulated carotid body output in hyperoxia, an effect which was reversed by light. However, in hypoxia, PCO's of 550 and 140 mmHg depressed output, with light resulting in even more depression. At a PCO of 60 mmHg, CO still caused depression, but light had no additional effect. These observations can be explained by two distinct effects, one that causes stimulation, and is light sensitive, and simultaneously, a second effect that causes depression, and is not light sensitive. In hyperoxia, stimulation exceeds depression, in hypoxia the reverse is true; stimulation occurs when PCO exceeds some value between 60-140 mmHg. Therefore, these data are consistent with Prabhakar's hypothesis, but also indicate that at a high PCO level, a second effect comes into play. Lahiri et. al. have since confirmed and extended these observations (Lahiri and Acker, 1999; Lahiri et al., 1999) using a superfused rat carotid body preparation. This group also studied the photochemical action spectra for the reversal of CO inhibition of the carotid body (Wilson et al., 1994), and have since concluded that “the primary oxygen sensor of the carotid body can be identified unambiguously... as cytochrome a$_3$ of the mitochondrial respiratory chain” (Wilson et al., 2000), and that “The photo-reversible inhibition of oxygen
sensing activity by CO accounts for at least 80% of the oxygen chemosensory activity of the carotid body” (Wilson et al., 1994).

A third change in paradigm occurred relatively recently as physiologists have begun to understand the important roles of nitric oxide and carbon monoxide as signaling molecules in the brain and elsewhere in the body. Hence, CO has been recast once again as a potential endogenous modulator of carotid body activity, in particular by Prabhakar (see Introduction).

**Interpretation of results in the context of transduction by heme oxygenase**

The amount of CO produced endogenously is very small: total body endogenous CO production normally accounts for COHb levels of less than 1% (Turino, 1981), and presumably only a small fraction of this comes from the carotid body. The subjects in the present study inhaled CO which resulted in COHb concentrations (and therefore tissue PCO values) of approximately 10 times the control value. If endogenously produced CO provides tonic inhibition of the carotid body and hypoxia normally decreases its production, then this exogenous CO should have been sufficient to maintain the inhibition during hypoxia, and reduce or eliminate the ventilatory response of our subjects to hypoxia. In fact, the results showed that the degree of stimulation of ventilation by hypoxia was not affected by the presence of CO, which can be interpreted as evidence against the endogenous CO hypothesis. This finding supports the claim of Bunn and Poyton (1996) that the amount of CO produced endogenously in the carotid body is too small to affect the oxygen sensor. The results of the present study are consistent with the idea that at physiological levels, CO does not have important interactions with oxygen sensing molecules in the carotid body, but simply binds to Hb and reduces the oxygen-carrying capacity of the blood. At a COHb level of only 10%, one would not expect this small decrease in oxygen carrying capacity to have any
impact on intracellular PO₂ (Chiodi et al., 1941), and therefore, no increase in output should be observed.

**Interpretation of results in the context of transduction by another mechanism**

While the lack of a significant effect of COHb of 10% on hypoxic response argues against Prabhakar's hypothesis, this result is consistent with several of the other current hypotheses for chemotransduction of hypoxia, but cannot differentiate between them.

**Mitochondrial cytochromes**

The mitochondrion is the primary site of consumption of O₂, and therefore, it has been suggested as the location where changes in oxygen tension are detected (Mulligan et al., 1981); this is otherwise known as "the metabolic hypothesis". A cytochrome with an unusually low affinity for oxygen could serve as the oxygen sensor (Gonzalez et al., 1995). Hypoxia depolarizes the mitochondria in the glomus cells (Biscoe et al., 1989), and substances which interfere with mitochondrial respiration, e.g., cyanide, affect sensory discharge in the same way as hypoxia (Mulligan and Lahiri, 1982). Lahiri and colleagues have administered high levels of CO (several hundred mmHg) and found that carotid body activity is stimulated, as in hypoxia (Lahiri et al., 1994), while light reverses this effect by photo-dissociation (Lahiri et al., 1999; Lahiri et al., 1993). As mentioned above, Wilson and colleagues have recently asserted that the oxygen sensor of the carotid body is cytochrome a₃ (Wilson et al., 2000). The authors worded this claim very strongly; and indeed, the data seems compelling. Under this theory, CO simulates hypoxia by binding to the cytochrome and stopping ATP production. Because the affinity of the cytochrome for CO is low, one would predict that in the present experiment, the low PCO used would have no effect; therefore, the results presented here are consistent with this hypothesis.
Membrane-bound hemoproteins

Another popular idea has been that the oxygen sensor is a membrane-bound hemoprotein, whose saturation reflects PO₂ at physiological levels, and which interacts with K⁺ or Ca²⁺ channels, for example, to increase their probability of being open (Gonzalez et al., 1995). Proponents of this hypothesis have suggested that CO would act as an agonist in binding to this hemoprotein, thereby simulating oxygen (Lloyd et al., 1966). Although the importance of oxygen sensitive ion channels has been questioned recently (Donnelly, 1997; Lahiri et al., 1998), supporting evidence includes work by Lopez-Lopez and Gonzalez, who showed in rabbit chemoreceptor cells that a PCO of 70 mmHg inhibited the hypoxia-induced inhibition of K⁺ currents (Lopez-Lopez and Gonzalez, 1992). Depending on the relative affinity of the purported hemoprotein for CO relative to O₂, high concentrations of CO might be necessary to elicit any response; consequently, the results presented here are consistent with this hypothesis as well.

NADPH oxidase

Acker and co-workers have proposed that NADPH oxidase, a non-mitochondrial heme containing enzyme, serves as the oxygen sensor (Acker, 1994; Cross et al., 1990). Synthesis of H₂O₂ by this enzyme is linked to oxygen supply, and a decrease in O₂ causes a decrease in H₂O₂; this results in the closing of the K⁺ channels, and subsequent depolarization and transmitter release. This hypothesis seems to have been discredited by Gonzalez and coworkers (Obeso et al., 1999; Obeso et al., 2000), who showed that NADPH oxidase inhibitors had no effect on the response of cat and rat carotid bodies to hypoxia. Nevertheless, according to this hypothesis, CO might bind to NADPH oxidase, simulating hypoxia as in the previous hypothesis above. If so, the results presented here could be consistent with this idea, provided that NADPH oxidase has a sufficiently low affinity for CO.
Nitric oxide synthase

NO is now understood to play important roles in the body, the best known of which may be as a vasodilator in smooth muscle. There is increasing evidence that NO plays a role as an O₂ sensor (Buerk and Lahiri, 2000). For example, NO interacts with the O₂-binding site of cytochrome c oxidase, reversibly inhibiting respiration (Cleeter et al., 1994), NO-synthase couples its rate of NO synthesis to the O₂ concentration in the physiologic range (Abu-Soud et al., 1996), NO-synthase knockout mice have an increased sensitivity to hypoxia (Kline and Prabhakar, 2000), and NO inhibits a Ca²⁺ current in rabbit glomus cells (Summers et al., 1999). For a brief review of this topic, see Iturriaga et al. (2000). CO could potentially act in several ways with the NO generating system. NOS is a hemoprotein (Maines, 1997); if CO can bind to NOS, it could inhibit production of NO, thus simulating hypoxia, and causing an increased sensory output. On the other hand, CO might substitute for NO on the target molecule on which NO is supposed to act, thus simulating hyperoxia and causing no change in the sensory output during hypoxia. In other words, it is difficult to predict what effect 10% COHb would have on a carotid body in which nitric oxide is the oxygen sensor; therefore, the results presented here provide neither supporting nor contradictory evidence for this theory.

Therefore, the results of the present study may also be consistent with the hypotheses that the oxygen sensor is NO-synthase, a membrane heme protein, cytochrome oxidase, or a mitochondrial cytochrome. However, the affinity of the oxygen sensing molecule for CO would have to be low relative to the PO₂. For example, the relative affinity of cytochrome oxidase for CO/O₂ is such that at PCO values as low as 0.06 mmHg, mitochondrial PO₂ would have to be approximately 0.1 mmHg or lower for significant binding of CO to occur (Coburn, 1979). It is also entirely possible that more than one system is active in oxygen sensing in the carotid body.
Conclusion

The hypothesis presented at the outset of this report was that the inhalation of exogenous CO to elevate blood COHb levels would result in the maintenance of inhibition to the carotid body during hypoxia, and cause the ventilatory response to hypoxia to be diminished or abolished altogether. In my healthy human subjects, hypoxia (PO₂ = 50 mmHg) caused a significant increase in the ventilatory response to CO₂, as measured by iso-oxic rebreathing. By contrast, inhalation of exogenous CO, resulting in carboxyhemoglobin levels approximately 10 times greater than control values did not affect the ventilatory response to CO₂ under either hypoxia (PO₂ = 50 mmHg) or hyperoxia (PO₂ = 150 mmHg); therefore, the experimental hypothesis was rejected.

Implications

The results of this study suggest that in the human, carbon monoxide at physiological levels does not act as an important regulator of the carotid body’s response to oxygen. The use of low concentrations of COHb to reduce oxygen content without reducing PO₂ in studies of control of breathing, for example (Koike et al., 1990), is supported. To the extent that the results conflict with previous work done in animals, the conclusion also suggests that, in certain cases, these species may not be suitable models for chemoreception in humans.

Proposals for Future Work

While the present study provides evidence against heme oxygenase being the oxygen sensor, further experiments are needed because the PCO at the actual oxygen sensor is unknown (see Limitations). One possible strategy would be to model PCO gradients in the glomus cell, based on the rate of production, rate of diffusion and distance from the site of synthesis, in order to predict the PCO at locations of proposed receptors. It might also be possible to measure
intracellular PCO gradients directly using radioactive or fluorescent markers, electrodes or some other technique. These data might support the use of 10% COHb in the present study. Perhaps the most important experiment in assessing the heme oxygenase theory has yet to be reported, namely, measuring the rate of CO production (or PCO) in the carotid body as a function of PO₂.
REFERENCES


APPENDIX A

*Model assumptions:*

1. Three independent factors, a peripheral chemoreflex drive, a central chemoreflex drive, and a basal drive determine respiration.
2. The peripheral chemoreflex drive is linearly related to PCO₂ at the peripheral chemoreceptors, with a slope (SDₚ) that varies rectangular hyperbolically with PO₂.
3. An area constant, a PO₂ asymptote and a drive asymptote describe the rectangular hyperbolic variation of slope SDₚ.
4. The central chemoreflex drive is linearly related to PCO₂ at the central chemoreceptors with a slope independent of PO₂.
5. The basal drive to respiration is determined by factors independent of both PCO₂ and PO₂.
6. The central and peripheral chemoreflex drives share a common chemoreceptor threshold and add to produce a total chemoreflex drive.
7. The total chemoreflex drive does not affect breathing until a chemoreflex drive threshold is exceeded.
8. The total chemoreflex drive produces changes in tidal volume, breathing frequency and ventilation that are scaled versions of the drive, with scaling factors.
9. The control of the pattern of breathing changes when tidal volume exceeds a threshold.
APPENDIX B

Individual data: chemoreflex responses

For each individual subject, VE, VT, and f versus PETCO₂ are presented for all four conditions. Triangles indicate PO₂ = 150 mmHg, circles indicate PO₂ = 50 mmHg, filled symbols indicate control, open symbols indicate COHb = 10%.
Subject 10

- Ventilation (L/min) vs. PCO₂ (mmHg)
- Tidal volume (mL) vs. PCO₂ (mmHg)
- Frequency (respirations/min) vs. PCO₂ (mmHg)