RAT CAROTID BODY CHEMOSENSORY DISCHARGE AND GLOMUS CELL HIF-1α EXPRESSION IN VITRO: REGULATION BY A COMMON OXYGEN SENSOR.

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Running Head:
Oxygen sensing in the carotid body

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ABSTRACT

Addition of a partial pressure of carbon monoxide (PCO \approx 350 \text{Torr}) to a normoxic medium (PO₂ \approx 130 \text{Torr}), was used to investigate the relationship between carotid body (CB) sensory discharge and expression of hypoxia-inducible factor-1α (HIF-1α) in glomus cells. Afferent electrical activity measured for \textit{in vitro} perfused rat CB increased rapidly (1-2 seconds) with addition of high CO (PCO \approx 350 \text{Torr}; PO₂ \approx 130 \text{Torr}) and this increase was fully reversed by white light. At submaximal light intensities, the extent of reversal was much greater for monochromatic light at 430 nm and 590 nm than for light at 450 nm, 550 nm and 610 nm. This wavelength dependence is consistent with the action spectrum of the CO compound of mitochondrial cytochrome a₃. Interestingly, when isolated glomus cells cultured for 45 min in the presence of high CO (PCO \approx 350 \text{Torr}; PO₂ \approx 130 \text{Torr}) in the dark, the levels of HIF-1α, which turns over slowly (many minutes), increased. This increase was not observed if the cells were illuminated with white light during the incubation. Monochromatic light at 430 nm and 590 nm light was much more effective than that at 450, 550, and 610 nm in blocking the CO induced increase in HIF-1α, as was the case for chemoreceptor discharge. Although the changes in HIF-1α take min while those for CB neural activity occur in 1-2 sec, the similar responses to CO and light suggest the oxygen sensor is the same (mitochondrial cytochrome a₃).

\textbf{Keywords:} carbon monoxide; oxygen, carotid body; cytochrome a₃; hypoxia-inducible factor-1α; mitochondria; sensory discharge.
INTRODUCTION

The carotid body consists primarily of glomus cell units comprising of 5-10 cells innervated by afferent fibers that have cell bodies in the petrosal ganglia. These are on the path to brain stem neurons responsible for the respiratory and cardiovascular reflexes (23). Glomus cells are known to possess oxygen sensors (10), and within a breath of hypoxic mixture and subsequent fall of arterial oxygen pressure, the afferent nerves begin to show an increase in discharge rate. CO is competitive with O2 in the CB, and the afferent electrical activity of the carotid sinus nerve (CSN) increases with increasing CO if the oxygen pressure is kept constant (18,44). The onset of this CO effect has been shown to be rapidly and fully reversed by bright white light (44). This pattern is a very distinctive characteristic of CO compounds of reduced iron-porphyrin. The CO-iron porphyrin compounds were first shown to be dissociated by light by Haldane and Smith (13), and this property became an important tool for identifying and characterizing the role of heme proteins (e.g., biological oxidases) in oxygen metabolism. Warburg (41), for example, first demonstrated that inhibition of cellular oxygen consumption by CO was reversed by light. Warburg and Negelein (42) then measured the wavelength dependence of this reversal and obtained the spectrum of the CO-compound of cytochrome a3, showing this heme protein is responsible for most of the oxygen consumption by yeast and mammalian cells.

More recently, the light induced reversal of CO induced increase in oxygen sensory activity in an isolated perfused-superfused carotid body preparation (CB) (16) was used to identify the oxygen sensor of the carotid body (43,44). When using monochromatic light of different wavelengths but at equal intensities, the extent of the reversal is dependent on wavelength of light, with greatest efficacy at 432 ± 2 nm and 590 ± 2 nm. Light at 432 nm was
6-7 times more effective than was 590 nm light. These spectral characteristics show the inhibitory CO complex is that of mitochondrial cytochrome a₃ as first described by Warburg and Negelein (42). Lahiri et al. (20) reported that, in the dark, high CO diminished O₂ consumption (VO₂) in the CB, and this correlated with the increase in chemosensory activity. Both of these effects were reversed by bright (white) light, consistent with their being mediated by cytochrome a₃. Mulligan and Lahiri (29) also provided evidence for a role of mitochondrial respiratory chain in CB chemosensory activity by showing that there was transient stimulation of CSN activity when inhibitors of mitochondrial electron transport chain were added (also see Wyatt and Buckler 46).

CO, in addition to its effect on mitochondria, has been reported to hyperpolarize (increase K⁺ current) the glomus cell membrane in normoxia (5,24). The implication is that CO, under normoxia, binds to a heme protein that is attached to an ion channel and controls its conductance. The changes in conductance could control the membrane potential of the nerve endings (21). CO is, however, reported to block the hypoxia induced inhibition of whole cell K⁺ current and depolarization on glomus cell membrane, i.e., it increases whole cell K⁺ current with a left-ward shift of the reversal potential (25,34). As such, the reported CO mediated changes in cell membrane conductance cannot be responsible for the excitation of the chemosensory discharge.

Hypoxia Inducible Factor-1α (HIF-1α) is present in the glomus cells (3,4). Recently, CB chemosensory response has been linked to the glomus cell transcription factor HIF-1α and the level of HIF-1α shown to increase following in vitro exposure of the CB to hypoxia (3). The increased in HIF-1α during hypoxia is regulated at the HIF-1α mRNA level as determined by O₂ concentration in the 0-95% range (36). Kline et al. (17) reported that mice partly deficient in HIF-1α showed a significantly diminished chemosensory response to hypoxia. Both the CB
CSN response and the increase in HIF-1α with hypoxia are reported to be suppressed by inhibitors of mitochondrial respiration (2,4,12,27). As a result, it appears there could be a link between HIF-1α and chemosensory response in CB, but the nature of this link was not established.

In the present study, CO has been used as an experimental tool to examine the relationship of the hypoxia induced increase in HIF-1α and the CSN activity of the carotid body. It is shown that CO induces a hypoxia like response for both and that the wavelength dependence of the light induced reversal of CO effects are the same. It is concluded that the oxygen sensor for hypoxia induced increases in glomus cell HIF-1α expression and CB chemosensory response is the same. There are, however, substantial temporal differences between the two responses: the rate of change in the CSN response is maximal in less than 2 seconds while HIF-1α induction in the carotid body by either hypoxia or Fe²⁺ chelation becomes significant in 2 minutes and maximal in about 10 min (37). In cells, Huang et al (14) reported that when HIF-1α was induced in cells by hypoxia and then the cells given a step increase to 21% oxygen, the a half-time for the decrease of <5 min but the time to maximal change was > 5 min. Thus, although cytochrome a₃ of mitochondrial respiratory chain is the oxygen sensor for both responses, they have different physiological roles. The CSN activity provides the very rapid response necessary for controlling breathing on a breath to breath basis whereas HIF-1α induction contributes to the intermediate and long term adaptation of cells and tissue to chronic changes in oxygen pressures (for reviews see Semenza (38,39) and Guzy and Schumaker (11)).

**Materials and Methods**
Carotid body perfusion and superfusion

The carotid bifurcation was prepared from anesthetized male rats (200-225 g) for in vitro perfusion essentially as described previously (35). Single pass perfusion and superfusion were established using hydrostatic pressures of 90-100 Torr. The perfusate-superfusate was modified Tyrode solution containing 112 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.1 mM MgCl₂, 20.5 mM NaHCO₃, 5.0 mM HEPES, 5.5 mM glucose, 20.8 mM sodium glutamate, 4g/L dextran (mol. wt. 74,2000) and 0.25 mM L-ascorbic acid at pH 7.4. The perfusate was equilibrated with either 21% oxygen:5% CO₂ remainder nitrogen or a mixture of 21% oxygen:5% CO₂:remainder CO. The temperature in the perfusion chamber was maintained at 36.5 ± 0.5°C. Paraffin oil was layered over the superfusate and the whole de-sheathed sinus nerve was placed on a platinum electrode for recording chemosensory discharge.

Measurement of the light induced reversal of the CO effect (photochemical action spectrum)

The wavelength dependence (spectral response) of the light induced reversal of the CO-induced increase in CSN activity of the CB is that of the CO complex of reduced cytochrome a₃ (43,44). The spectrum was measured as the light-induced changes in CSN for the isolated CB perfused with buffer equilibrated with a mixture of CO (PCO ≈ 350 Torr) and oxygen (Po₂ ≈ 130 Torr). Details of the experimental procedure were the same as described earlier (42). The monochromatic illuminating light was obtained by passing the light of a tungsten-I₂ light through a monochromator into a light guide. The light from the light guide was focused on an approximately 1 mm area of the CB preparation and centered as best as possible. The required intensities of monochromatic light were relatively high and this requirement was further increased depending on the preparation geometry, light scattering and other factors. Five
different wavelengths monochromatic light, ranging from 430 nm to 610 nm, were used in order to determine the wavelength dependence of the CO reversal effect.

**Glomus cell preparation**

The glomus cells were separated enzymatically as described previously (3). Briefly, carotid bodies (N=20) were surgically removed from anesthetized rats, cleared of connective tissue, and digested with 0.2% collagenase P (Roche Diagnostic Corp., IN) and 0.1% trypsin (Sigma Chemicals) in a Ca$^{2+}$ and Mg$^{2+}$ free phosphate buffer for 30 min at 37°C. The digested tissue was transferred to a solution of growth medium, tritiated with a fire polished Pasteur pipette, washed and plated on 18-mm cover slips in a Petri dish. The growth medium contained fetal bovine serum plus streptomycin, penicillin G and insulin. The Petri dishes containing the separated cells were left undisturbed for a period of 24-30 h in a humidified incubator (37°C, circulated with 5% CO$_2$ and air) for cells to adhere on to the cover slip. In all cell measurements, tyrosine hydroxylase was measured to verify the cells were glomus cells. Glomus cells are the only cells in the carotid body that contain TH.

**Superfusion of the isolated glomus cells**

Cover slips containing adherent cells were in a closed chamber and superfused with HEPES buffered medium (pH ~ 7.3): 139 mM NaCl, 4.9 mM KCl, 1mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM HEPES, 10 mM glucose, 10 mM; 250 µM L-ascorbic acid. Glomus cells were superfused with either normoxic (Nx; PO$_2$ ~ 130 Torr) and normoxic with CO (PCO$_2$ ~ 350 Torr; PO$_2$ ~ 130 Torr) media for 45-50 min at 37°C. The high oxygen pressure in the medium and the continuous refreshing of the medium around the cells assured both that: 1. when the superfusion medium was changed to one with CO, the oxygen pressure in the medium and at the cell surface
was unchanged and near 130 Torr; and 2. when superfusing with CO, the CO:O₂ ratio was approximately 3, low enough to cause only partial decrease in mitochondrial respiratory capacity. At the end of the superfusion period, the glomus cells were fixed in 4% ice cold paraformaldehyde. They were later used for immunofluorescence measurements of HIF-1α and tyrosine hydroxylase (TH).

**HIF-1α and TH immunofluorescence**

Adherent cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.3; 0.01 M) for 10 min, washed three times with PBS, permeabilized with 0.05% Triton X-100 in PBS for 5 min and rinsed again in three times with PBS. Nonspecific binding was blocked by incubating with 10% fetal bovine serum in PBS for 1 h at room temperature. The cells were then incubated with anti-HIF-1α at 1:200 (rabbit IgG, epitope mapping with amino acids 329-530 of HIF-1α of human origin, Santa Cruz Biotechnology) or anti-TH antibody at 1:500 (mouse IgG, Sigma Chemicals, St Louis, USA) at 4°C overnight in a humidified chamber. After incubation with primary antibodies, cells were washed in PBS for 10 min and incubated in secondary antibodies for 1 h at room temperature. These consist of goat anti-mouse IgG conjugated to Texas red (1:200; Chemicon International, CA, USA) and goat anti-rabbit IgG conjugated to FITC (1:200; Jackson Immunoresearch Lab, West Grove, PA, USA). After a thorough rinse with PBS, cells were covered with a photobleaching reagent (Mowiol, Calbiochem, La Jolla, CA) containing DABCO before imaging with a fluorescence microscope (see below). Controls were achieved either by omitting primary antibodies, or incubating cells with goat serum instead of primary antibodies. No non-specific immunofluorescence was observed in the controls.
Quantification of HIF-1α and tyrosine hydroxylase by immunofluorescence

Quantification of the changes in immunolabelling of HIF-1α was by a modification of the original method described previously (3). Briefly, isolated, cultured glomus cells were viewed with a Nikon Eclipse TE300 fluorescence microscope (x 60, x100 oil-immersions) and equipped with an optical filter changer (Lambda DG-4, Sutter instruments, Novato, CA). Fluorescence excitation was accomplished using a mercury lamp (150 W) fiber-optic light source, and appropriate filter sets. To prevent photo bleaching of the fluorescent preparation, a neutral density filter (ND 0.3, Chroma Technology) was used to attenuate 50% of the light intensity. The HIF–1α and TH immunofluorescence intensity images were acquired with 12-bit digital cooled charged-coupled device camera (ORCA 100, Hamamatsu), using graphics control software (Metamorph Imaging System, Universal Imaging). Images of glomus cells were digitally marked, and the pixel intensities were calculated. The background regions outside the cells were also digitally marked and the average pixel intensity was subtracted from each cell image. For each cell, the average intensity was determined for the entire cell area and this used as a measure of the level of HIF-1α for that cell.

Statistical analysis

The summary data in graphs are for the averages over entire cells and are presented as means ± SE and number of experiments for each condition (normoxia, hypoxia and hypoxia with mitochondrial inhibitors). Results were analyzed by one-way ANOVA (SigmaStat, Jandel Scientific). Differences were considered statistically significant when $P< 0.05$. 
RESULTS

The effect of CO administration on the afferent electrical activity of the in vitro rat CB both in the dark and when illuminated with monochromatic light of different wavelengths.

When the perfusate was switched from normoxic medium without CO to one containing CO (PCO$_2$ ~ 350 Torr; PO$_2$ ~ 130 Torr) in the dark, CSN discharge (imp/s) increased very rapidly (1-2 seconds) from a basal average value of 60 imp/s to 420 imp/s. This increase in neural activity was completely reversed by illuminating the CB with bright white light (Fig. 1). The above procedure was repeated with experimentally indistinguishable results. The response to turning the light on or off was rapid and reproducible. On the other hand, as long as CO containing buffer was used and the light was off, the neural activity remained essentially constant and at a high level.

In a separate experimental preparation, CB perfused with CO (in normoxia) was exposed to monochromatic light for which the wavelength could be switched among the wavelengths 430, 450, 550, 590 and 610 nm. After each light exposure there was a longer dark period (light-off) to allow the preparation to return to the dark steady state. The dependence of the response to light, measured by reversal of the CO induced increase in CSN activity of the rat CB, is demonstrated in Fig. 2A. The CSN activity increased markedly, from a basal average of 42 imp/s to 325 imp/s, when perfusion with the CO containing buffer was initiated (light–off). Essentially complete suppression of the CO induced increase was observed with high intensity white light (Fig 2B). Similarly, nearly complete suppression was observed for illumination with monochromic light of wavelengths 430 nm (to 74 imp/s) and 590 nm (to 68 imp/s). The suppression of activity was much less when the illumination was with monochromatic light of equal intensity but at wavelengths of 450, 550, and 610 nm. Previously, Wilson and coworkers...
Roy et al. (1994) showed, in cat CB, that the wavelength dependence of the reversal of the CO effect on afferent neural activity had well defined maxima at near 430 nm and 590 nm. The present data for the rat carotid body are, therefore, in good agreement with the earlier data for the cat CB (44).

**Effect of CO administration on expression of HIF-1α in rat CB glomus cells in the dark and when illuminated with monochromatic light of different wavelengths.**

Figure 3A shows images of the immunofluorescence of glomus cells stained for HIF-1α and tyrosine hydroxylase (TH), along with an overlay of the two images to show co-localization. In order to compare the changes in HIF-1α induced by adding CO with those induced by hypoxia, glomus cells were superfused for 45 min with either normoxic medium (PO$_2$ ~ 130 Torr) (first row) or a medium with low oxygen (1% O$_2$) (second row). The low oxygen medium resulted in the well established hypoxia induced increase in HIF-1α, due to stabilization of HIF-1α, and an increase in TH fluorescence. Superfusing the glomus cells for 45 min in the normoxic medium plus CO (PCO$_2$ ~ 350 Torr; PO$_2$ ~ 130 Torr) while continuously illuminating with white light resulted in little or no increase in HIF-1α. (third row) compared to normoxic medium without CO (first row). When the superfusion with CO containing medium was carried out in the dark, however, there was a marked increase in HIF-1α (row 4). This increase was similar to that for low oxygen medium (row 2). The ability of light to block the CO induced increase in HIF-1α in glomus cells is summarized in Fig 3B. There was significant accumulation of HIF-1α in cells superfused with CO in the dark but not when they were illuminated with white light during the superfusion period.

In a separate set of experiments, glomus cells were superfused with CO (PCO$_2$ ~ 350 Torr) containing normoxic (PO$_2$ ~ 130 Torr) medium for 45 min while illuminating the cells with
monochromatic light having wavelengths of 430, 500, 590 and 610 nm (Fig 4A). When glomus cells were incubated in normoxic medium (no CO), illumination during incubation had no effect on the levels of HIF-1α or TH (first row). In contrast, if the glomus cells were kept in the dark while superfusing with normoxic media containing CO, the level of HIF-1α immunofluorescence greatly increased compared to normoxic controls. This CO induced increase was not obviously affected by illumination when the wavelength of the monochromatic light was 500 nm (third row) or 610 nm (fourth row). In contrast, when illumination was with light of 430 nm (second row) or 590 nm (fourth row), the HIF-1α immunofluorescence was markedly lowered, to only slightly above the normoxic controls. Although the increases in HIF-1α during illumination at 430 and 590 nm light were small, they are statistically significant when compared to cells superfused with media without CO (Fig. 4B) or with CO but illuminated with bright white light.

DISCUSSION

General agreement has not been reached regarding the mechanism by which the CB senses the oxygen pressure and uses this information to control its afferent CSN activity, although several hypotheses have been put forward (1,23,33,40,44). The CB has several other biochemical and physiological responses to altered oxygen pressure in the cellular environment, and one of these is to increase the levels of hypoxia inducible factor HIF-1α (3). The present study compared the responses of rat CB CSN activity and glomus cell HIF-1α to inclusion of CO in the medium. Both the CB sensory activity and glomus cell HIF-1α increased when the incubation medium contained CO, despite the fact that the oxygen pressure in the medium was
not changed. This observation is consistent with the addition of CO to the medium being functionally equivalent to decreasing the oxygen pressure.

Since light has been shown (19,44) to rapidly and reversibly block the increase in afferent neural activity induced by addition of CO, experiments were designed to determine the efficacy of light on the CO induced increase in HIF-1α. The data show that CO induced marked increases both in the afferent CSN activity and in HIF-1α and bright white light was fully effective in reversing both responses. Moreover, when monochromatic light was used, both responses showed the same dependence on the wavelength of light used for illumination. This is strong evidence that the CO induced increase in afferent neural activity of the CB and in HIF-1α levels in the glomus cells are mediated by the same oxygen sensing mechanism. The only known pigment with the measured properties (CO competitive with O2, CO effect reversed by moderate light intensities, CO complex having absorption maxima at 430 and 590 nm) is the CO complex of reduced cytochrome a3 of the mitochondrial respiratory chain.

Light has also been reported to increase the excitatory activity of the carotid body when that activity has been suppressed by “severe hypoxia” in the presence of CO (see Lahiri et al. (21)). As the CO/O2 ratio is increased, the CSN activity increases to the maximal value for very low oxygen and then, as synthesis of ATP by the mitochondria fails to meet the ATP requirement for neural discharge, decreases again. At these high CO/O2 ratios, where the CSN activity is suppressed due to a lack of ATP, illumination with white light can cause an increase in afferent CSN activity. The light, by relieving the inhibition of cytochrome c oxidase by CO, increases ATP synthesis by the mitochondria. With increased ATP availability, the neural activity increases, but only for as long as the ATP is increased (as long as the light is on).
A reasonable hypothesis for the mechanism of oxygen sensing and activation of the sensory responses and the mechanism of CO interaction with the oxygen sensor is as follows: Mitochondrial oxidative phosphorylation maintains the cellular energy state ([ATP]/[ADP][Pi]) at a nearly constant (steady state) value under normoxic conditions. When the oxygen pressure decreases, or CO increases (see below), there is a decrease in energy state and this decrease (possibly through increased AMP levels) results in increasing [Ca^{2+}]_{i} through release from internal calcium stores and by influx of extracellular calcium (20,28). Increased intracellular calcium causes increased release of neurotransmitters such as dopamine (6) and ATP (30), increasing afferent neural activity. The increase in HIF-1α could be mediated by the same sensor system because a decrease in cellular energy state would activate the cellular stress response system. In the case of HIF-1α, the response is slower due to the time required for complex regulatory functions at the level of alterations in protein stability and gene expression.

The effects of CO can be understood from its known reaction with the reduced form of mitochondrial cytochrome a_{3} where it is competitive with respect to oxygen. In the dark, increasing the pressure of CO at constant oxygen pressures is the metabolic equivalent of decreasing the pressure of oxygen in the absence of CO. Increasing CO would, like decreasing O_{2}, cause a decrease in oxygen consumption (19,20) and ATP production by the mitochondrial oxidative phosphorylation. The resulting decrease in cellular energy state would signal for induction of HIF-1α and other stress response elements.

There have been other effects of CO reported for the CB and glomus cells. Glomus cell membranes have been reported to hyperpolarize with high CO due to reversal of hypoxia induced suppression of the background leak K^{+} current (5) and Ca^{2+} activated K^{+} current (34). These CO effects were reported for CO together with hypoxia and were not light sensitive,
indicating they are not involved in the CO induced increase in chemosensory discharge or induction of HIF-1α described for this study. Huang and coworkers (14) reported that in Hep3B cells incubated under a low oxygen gas phase, CO decreased the level of HIF-1α protein. However, these authors did not determine the oxygen pressure in the cellular environment, making this effect hard to interpret (see later discussion).

The HIF-1α response when glomus cells are incubated in normoxic media with CO is much slower than the neural response (~ 45 min vs < 2 sec). In the present paper, we have demonstrated that despite the difference in response rate, the CO induced increase in glomus cell HIF-1α, and, like the CO induced increase in the CSN activity of the isolated carotid body, can be suppressed by light. Further, using monochromatic light, we have shown that induction of HIF-1α by CO was more strongly suppressed by monochromatic light at wavelengths 430 nm and 590 nm than at 550 nm and 610 nm (Fig. 4A,B). This spectral result indicates that mitochondrial cytochrome a₃ was the oxygen sensor that mediated the observed HIF-1α response.

In the present experiments, illumination with monochromatic light with wavelengths of 430 nm or 590 nm did not entirely abolish the increase in HIF-1α whereas illumination with white light did. This is not surprising because the white light was obtained by direct illumination by the light source, removing only the infrared (heat) component. To obtain monochromatic light, this white light beam was passed through a monochromator and separated into its component colors (wavelengths). The intensity of the monochromatic light was necessarily of lower efficacy than the white light from which it was isolated.

In attached cell cultures incubated in media covered with a low oxygen pressure gas phase, it has been reported that adding high concentrations of mitochondrial inhibitors, such as
CO and NO, results in suppression of the hypoxia induced HIF-1α increase (7,9,12,15). Hagen and coworkers (12) suggested the mitochondrial inhibitors block the hypoxia induced stabilized HIF-1α by redistribution of the oxygen within the cell whereas Huang and coworkers (15) proposed it was due to displacement of O₂ from oxygen–dependent degradation domain of HIF-1α. Calapina and coworkers (7) suggested it resulted from increased production of oxygen radicals. Doege and coworkers (9), however, measured the oxygen pressures in the cellular environment and showed that if the oxygen diffusion gradient generated by cellular oxygen consumption was eliminated, the mitochondrial inhibitors no longer induced a decrease in HIF-1α. They concluded “that reduction of oxygen consumption reduces the O₂ gradient in conventional cell cultures, causing elevation of the cellular O₂ concentration, which leads to degradation of HIF-α.”

In the present study, the cells were superfused with medium equilibrated with 130 Torr oxygen pressure, continuously replenishing the medium in contact with the cells. As a result, when the superfusion medium was changed to one with CO, the oxygen pressure in the medium and at the cell surface did not change. Moreover, the CO:O₂ ratio was low enough to cause only partial decrease in mitochondrial respiratory capacity. Under these conditions, addition of CO would induce only a transient decrease (a few seconds) in the rate of oxygen consumption that lasted only until the energy state decreased enough to restore the respiratory rate (rate of ATP synthesis). Thus CO, by competing with oxygen for the active site of cytochrome c oxidase and lowering the oxygen pressure “seen” by the oxidase, lowers the cellular energy state. Stabilization of HIF-1α is likely linked to the decrease in energy state.

The carotid body has a distinctive sensitivity to oxygen pressure, but oxygen sensing occurs in essentially all cells and tissues. The carotid, and aortic, bodies are notable not as much
because they sense oxygen as because they are small organs specifically designed to translate the sensed oxygen pressure into afferent neural activity. This neural activity then influences the function of other tissues in remote locations. In most cells and tissues, oxygen sensing is used to control local functions, of which a large number are oxygen dependent. Well known examples include oxygen delivery to the tissue, including autoregulation in the brain and heart, modulation of local blood vessel density (angiogenesis), and apoptotic cell death. The oxygen pressure dependence of these tissue responses can be altered. It is known, for example, that the carotid body of adult animals is much more sensitive to oxygen than is that of the newborn and this sensitivity changes continuously during the maturation process (see 8,26). In adult animals, the oxygen sensitivity of the carotid body is much greater than that of the aortic body (22). These differences are consistent with the oxygen sensitivity of the tissue response not being determined just by the oxygen sensor. Rather, it indicates the sensor and sensory signal are being modulated by the metabolic environment and transmission mechanisms. The oxygen sensitivity can be, and is, modulated at several levels, including oxygen delivery to the tissue (vascular density, blood flow), the “set point” for cellular energy metabolism, and sensitivity of the neurons to the “message”. It is the “secondary” signal modulation mechanisms that determine the differences in oxygen sensitivity observed, for example, in the carotid body during maturation and between the aortic and carotid bodies. There is good evidence that mitochondrial oxidative phosphorylation, through cytochrome c oxidase, is an important oxygen sensor for regulation of not only the carotid and aortic body activity, but also of cardiac blood flow (see Nuutinen and coworkers (31,32)) and many other functions. It should be noted, however, that mitochondrial cytochrome c oxidase is not the only oxygen sensitive component in the cells, and not all oxygen sensing can be attributed to a single “universal” sensor. The data presented in this paper focuses
only on carotid body neural activity and HIF-1α induction in the glomus cells, showing they share a common oxygen sensor, and this is mitochondrial cytochrome c oxidase.

In summary: carbon monoxide (PCO 350 Torr) added to normoxic medium (PO2 130 Torr), induced increases in both sensory discharge in the isolated perfused rat CB and expression of both HIF-1α and tyrosine hydroxylase in glomus cells. The changes in afferent electrical activity were rapid (2-3 seconds) and fully reversed by light, the extent of reversal was much greater for monochromatic light at 430 nm and 590 nm than for light at 450 nm, 550 nm and 610 nm. Isolated glomus cells cultured for 45 min in the presence of CO (PCO 350 Torr; Po2 130 Torr) in the dark showed marked increase in HIF-1α. This increase was blocked by continuous illumination with white light. Monochromatic light at 430 nm and 590 nm also effectively blocked the increase while that at 450, 550, and 610 nm did not. The changes in HIF-1α were much slower than those for the afferent electrical activity, but the similar dependencies on CO and light are consistent with their responding to the same oxygen sensor (mitochondrial cytochrome a3).

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Figure Legends

Figure 1. Neural discharge recorded from the carotid sinus nerve (CSN) of an isolated perfused rat carotid body. The CB was perfused with a normoxic medium (Po2 \( \approx 130 \) Torr) and then switched to a medium containing the same oxygen pressure and CO (PCO \( \approx 350 \) Torr). Introduction of the CO caused immediate excitation of the CB in the dark, as indicated by the increase in CSN discharge. Illumination with a bright white light while perfusing with CO containing medium returned the CSN activity to the pre-CO baseline. The light induced suppression of CSN activity could be repeated several times with reproducible results.

Figure 2 A,B. The photosensitivity of the CSN discharge of the rat CB, perfused with normoxic medium containing CO (PCO \( \approx 350 \) Torr). 2A. After an initial period of perfusion in normoxic medium without CO, the perfusion medium was changed to one with CO, the oxygen pressure remaining the same (PCO \( \approx 350 \) Torr; PO2 \( \approx 130 \) Torr). The afferent CSN activity increased markedly (light off) and after it stabilized the carotid body was illuminated with monochromatic light with wavelengths of 430 nm, 450 nm, 550 nm, 590 nm or 610 nm for brief periods. The periods of illumination were separated by periods of dark (light off) sufficient for the CSN activity to return to the steady state. The periods with the light on were all of equal length, as were the intervening periods with the light off. Much greater light induced suppression of the CSN activity occurred for wavelengths of 430 nm and 590 nm than for the other wavelengths. 2B. A bar graph showing the relative efficacy of light of the different wavelengths in suppressing the CO induced increase in CSN activity. CSN activities at 450, 550 and 610 nm compared to 430 and 590 (* \( P < 0.001 \)).
**Figure 3 A,B.** Fluorescence microscope images showing the immunofluorescence intensities of HIF-1α (left), tyrosine hydroxylase (TH; middle) in glomus cells and the extent of their co-localization (right). **3A.** First row: Low fluorescence intensities show the low levels of HIF-1α and TH in cells incubated in normoxic media. Second row: Glomus cell incubated in media containing 1% O2 for 45 min show a marked increase in the immunofluorescence due to HIF-1α, consistent with hypoxia induced increase in HIF-1α. Third row: Incubation of the cells in a medium containing CO (PCO ≈ 350 Torr) and oxygen (PO2 ≈ 130 Torr) for 45 min while illuminating with bright white light resulted in levels of HIF-1α similar to those for normoxic media without CO. Fourth row: Incubation in CO containing normoxic medium, but in the dark, resulted in substantial increase in HIF-1α. Mag. = X600: Bar = 15 μM.

**3B.** A bar graph showing the levels of HIF-1α in the glomus cells (immunofluorescence intensity in arbitrary units). There was significantly higher (P<0.001 compared to normoxia) HIF-1α when the glomus cells were incubated with CO in the dark than when they were incubated in either normoxic media without CO or CO containing media while illuminating with bright white light. The increase during incubation with CO in the dark was equivalent to that during incubation in a medium equilibrated with 1% oxygen.

**Figure 4 A,B.** Fluorescence images showing the wavelength dependence of the effect of light on CO induced accumulation of glomus cell HIF-1α (left) and tyrosine hydroxylase (TH; middle). **4A.** The co-localization of HIF-1α and TH. immunofluorescence is shown on the right. First row: When the cells were incubated in normoxic medium without CO and illuminated with bright white light the level of HIF-1α immunofluorescence was minimal. Second row: Glomus cells incubated while illuminated with monochromatic light at 430 nm in a
medium with CO show only slightly increased HIF-1α compared to control.  

Third row: Glomus cells incubated in CO containing medium and illuminated with 550 nm light show markedly increased HIF-1α.  

Fourth row: Glomus cells incubated in CO containing medium and illuminated with 590 nm light showed only slight increase in HIF-1α fluorescence, much like illumination with 430 nm light (above).  

Fifth row: Glomus cells illuminated incubated in CO containing medium and illuminated with 610 nm light have markedly increased the HIF-1α fluorescence.  

**4B.** The wavelength dependence of the effect of light on the CO induced increase in HIF-1α in glomus cells. The level of HIF-1α immunofluorescence in glomus cells was significantly higher (***P<0.001) when they were illuminated with 500 nm or 610nm light than when they were illuminated with 430 nm or 590 nm light. The latter values were still significantly higher (*P<0.05) than for normoxic controls illuminated with white light.
350 Torr CO

CSN discharge (imp / 0.5s)

Normoxia Light-on Light-off

R-03-00 60 s
mean ± sem  
$n = 20$ cells
CO (P_{CO} \sim 350 \text{ Torr})

<table>
<thead>
<tr>
<th>Wavelengths (nm) of monochromatic light</th>
<th>HIF-1α immunofluorescence intensity (arbitrary units)</th>
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<tbody>
<tr>
<td>Normoxia</td>
<td>mean ± sem  \ n = 20 cells</td>
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<tr>
<td>White Light</td>
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<td>430</td>
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<td>590</td>
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* indicates a significant difference from Normoxia (p < 0.05).
** indicates a significant difference from Normoxia (p < 0.01).