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The primary oxygen sensor of the cat carotid body is cytochrome a_3 of the mitochondrial respiratory chain

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Abstract Carbon monoxide was shown to be competitive with O_2 in oxygen sensing by perfused carotid bodies isolated from cats, afferent electrical activity increasing with either decreasing O_2 or increasing CO. The CO-induced increase in afferent activity was fully reversed by bright light. At submaximal light intensities the extent of reversal, after correcting to equal light intensity of light quanta at each wavelength, was maximal for light of 432 ± 2 and 590 ± 2 nm, with a ratio (432/590) of approximately 6. This spectrum is characteristic of the CO compound of mitochondrial cytochrome a_3. The photo-reversible inhibition of oxygen sensing activity by CO accounts for at least 80% of the oxygen chemosensory activity of the carotid body.

Key words: Carotid body; Oxygen; Chemosensor; Oxygen sensor; Cat; Carbon monoxide

1. Introduction

The carotid body is an important chemosensory organ which responds to alterations in oxygen pressure in the blood by changing the activity in the afferent nerves. The electrical activity is small for normoxic and hyperoxic conditions but increases continuously as the arterial oxygen pressure falls below about 100 Torr (see for example [1]). Thus the carotid body has an internal mechanism for sensing oxygen which operates over a wide range of oxygen pressures, accurately transducing this information into electrical impulses passed through the afferent nerves. Other tissues are also capable of quantitatively detecting and responding to changes in oxygen pressure, prominent among them the heart, with its highly regulated blood flow [2]. The biochemical basis of oxygen sensing has been extensively studied but remains not well understood. There have been many suggestions as to the identity of the primary oxygen sensor (for review see [3,4]), one of which is the mitochondrial respiratory chain (see [2,5,6]). This suggestion has been criticized by workers holding the view that mitochondrial oxidative phosphorylation is saturated with oxygen at pressures above about 1 Torr (see for example [7]) but more recent measurements have indicated that the oxygen dependence of oxidative phosphorylation extends to 15 Torr or higher [8,9]. In the carotid body, oxygen measurements have yielded conflicting results. The reported oxygen pressures in the carotid body of normoxic animals (PaO_2 near 100 Torr) measured by oxygen electrodes range from less than 10 Torr [10] to as high as 70 Torr [11]. Non-invasive measurements using oxygen dependent quenching of phosphorescence have shown that in vivo the oxygen pressure in the vasculature of the carotid body closely correlates with the afferent electrical activity, and that in normoxia the oxygen pressure in the microvasculature has values near 40 Torr [12,13].

In the present study, we have made use of the light-induced reversal of the effect of CO on oxygen sensing by the carotid body to identify the primary sensory element. The CO compounds of reduced iron-porphyrin compounds was first shown to be readily photodissociated by Haldane and Smith [14] and this property has proven an invaluable tool in determining the role of heme proteins in oxygen metabolism. Warburg [15] demonstrated in 1926 that light reversed the inhibition of respiration by CO and Warburg and Negelein [16] measured the wavelength dependence of the light effect, providing the first spectrum of the CO-compound of the hemeprotein responsible for most of the respiration by cells, now known as cytochrome a_3. This technique became the standard by which biological oxidases could be identified and has also been used to identify bacterial oxidases (see for example [17]) as well as to show cytochrome P_{450} is oxygenase responsible for metabolism of many drugs [18]. The use of this technique to identify the oxygen sensor of the carotid body has become possible due to the development of an isolated perfused preparation with long-term (hours) stability while retaining excellent coupling of afferent activity and oxygen pressure [13,18].

2. Materials and methods

2.1. Carotid body perfusion and superfusion

The carotid bifurcation was prepared from anesthetized female cats (2–5 kg) for in vitro perfusion essentially as described previously [19]. Single pass perfusion and superfusion were established using hydrostatic pressures of 80 Torr. The perfusate and superfusates were modified Tyrode-solution containing (in mM) 154 Na^+, 123 Cl^-, 4.7 K^+, 2.2 Ca^{2+}, 1.1 Mg^{2+}, 22 glutamate, 5 glucose, and 5 HEPES at pH 7.4. The perfusate was gassed with either 21% oxygen 5% CO_2 or a mixture of oxygen and CO with 5% CO_2. The temperature in the perfusion chamber was maintained at 36.5 ± 0.5°C. Paraffin oil was layered over the superfusate to a depth of less than 3 mm. The whole sinus nerve was placed on two platinum wire electrodes for recording the chemosensory discharge as used by Eyzaguirre and Koyano [20]. In these experiments, the electroneurogram consisted of almost entirely chemoreceptor impulses as characterized by their random pattern of discharge and the responses to nicotine, CO_2, and oxygen.

2.2. Measurements of the photochemical action spectrum

The photochemical action spectrum was measured as the light-in-

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duced response in carotid body perfused with a medium containing a mixture of CO and oxygen according to the method described by Warburg and Negelein [16] and by Melnick [21]. The illuminating light was obtained by passing the light of a tungsten-I_2 light through a monochromator into a light guide. The light from the light guide was focused on an approximately 1 mm area of the carotid body preparation centered, as best possible, on the carotid body. The required intensities of monochromatic light are relatively high and this requirement is further increased depending on the preparation geometry, light scattering and other factors. In the course of this study, two different illuminating systems were used, one based on a Bausch and Lomb monochromator and the other on a high intensity Oriel interference filter monchromater. The latter can provide up to about 5- to 10-fold more intense monochromatic light, providing the higher intensity necessary for some carotid body preparations, but has lower resolution and reproducibility of wavelength selection. In each case, the intensity of the light as a function of wavelength was determined using a photodiode of known spectral response, allowing correction of the measured response to equal light intensities at each wavelength.

The light-induced reversal of the CO effect is due to absorption of photons of light by the CO compounds, resulting in dissociation of the CO with generation of a form which can react with oxygen. The effect of light is, therefore, proportional to the number of quanta of light absorbed by the CO compound, i.e. the number of photons/s times the absorption coefficient. Thus the measured light response can be converted to the absorption spectrum of the CO compound by calculating the response which would have occurred for light of the same number of quanta per second at each wavelength.

3. Results

3.1. *Effect of illumination on the afferent electrical activity of the carotid body*

Experimental tracings demonstrating the behavior of the carotid body are presented in Fig. 1. The afferent activity of the carotid body increased markedly when the perfusate was changed to one containing CO and this was completely reversed by bright white light. The response to light is rapid, and through leaving the light on for different times it was established that the maximal response occurred with 6–7 s illumination. Thus, after a steady state was achieved the carotid body was exposed to monochromatic light of different wavelengths for 6-s periods separated by dark periods sufficient to allow the chemosensory activity to return to the steady state. The perfusate flow was then stopped to show the full hypoxic response followed by perfusion with a medium without CO to show recovery to the normoxic level. The lower trace shows the photoresponses to light at different wavelengths from 630 nm to 410 nm. The best conditions for obtaining the photochemical action spectrum are when the CO-induced increase in afferent activity is 50 to 70% of the stopped flow value. In Fig. 1, for example, the afferent activity in the steady state with CO containing perfusate is approximately 50% of the stopped flow value. The range of activity modulated by light (minimum to maximum) is approximately 70% of the difference between the values for the absence of CO and for stopped flow. This CO/O_2 ratio provides relatively large changes in afferent neural activity and yet there is still sufficient remaining respiration to provide the energy levels (ATP) required to maintain stable cellular and chemosensory function. Maximal afferent activities, whether induced by near zero oxygen in the absence of CO or by high CO/O_2 ratios, cannot be sustained by the cartid body and there is progressive loss of function. It should be noted that the effect of light was completely dependent on the presence of CO. In the absence of CO, illumination even with light intensities above those necessary for complete reversal of the CO effect had no detectable effect on activity under either normoxic or at various levels of hypoxia.

Addition of CO to the perfusate resulted in a large increase in afferent activity over a similar perfusate with the same oxygen pressure but with the CO replaced with nitrogen. The

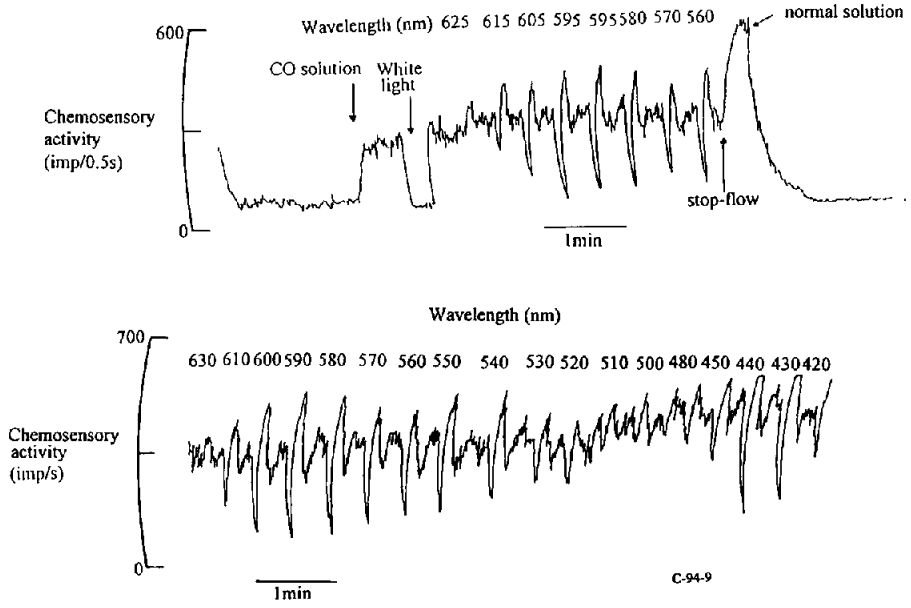

Fig. 1. The photosensitivity of the chemosensory discharge of carotid body being perfused with a medium containing CO. The carotid body was prepared as described in section 2. Upper trace: after the initial period of perfusion with no CO, the perfusion was changed to one with oxygen and carbon monoxide (130 Torr and 560 Torr respectively). The afferent activity increased markedly with the CO containing medium but this increase was completely reversed by exposure to bright white light. The carotid body was then exposed to monochromatic light (6.7 nm width at half height) of the indicated wavelengths for 6-s periods to measure the photoresponse of the afferent activity. The flow was then stopped to determine maximal oxygen chemosensory activity. Perfusion was restarted with a medium equilibrated with 21% oxygen and no CO. Lower trace: after a steady state activity was attained, the carotid body was given 6-s exposures to monochromatic light of the indicated wavelengths from 630 nm to 410 nm separated by periods of dark sufficient for the afferent activity to return to the steady state value.

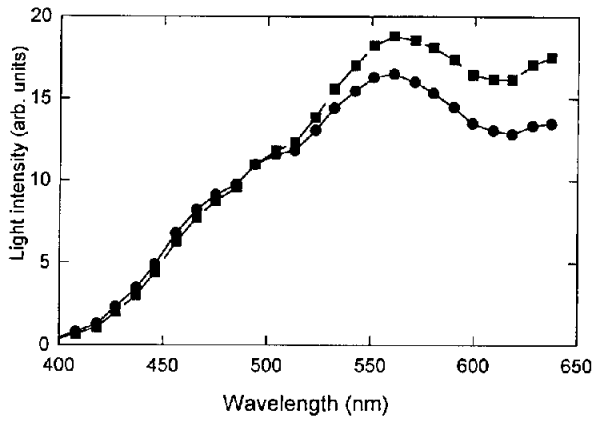

Fig. 2. The wavelength dependence of the illuminating light used for determining the photochemical action spectrum. The intensity of the light provided by the Oriel monochromatic light system was measured for a bandwidth at half height of 10 nm. The light energy was measured using a photodiode of known spectral response and plotted as the relative light energy (●) and after correction to the relative quantum intensity (■).

afferent activity increased with increasing $CO:O_2$ ratio, and the maximal CO-induced increase in activity was similar to the maximal increase observed with stopped flow hypoxia. At each level of increase in afferent activity induced by CO, illuminating with a sufficiently bright white light resulted in decrease in afferent activity to the steady state level for that oxygen pressure in the absence of CO or below. When the activity decreased to below the steady state level this was transient and if the light remained on it rose again to near the level for the absence of CO. A similar undershoot in activity was observed when hypoxic carotid bodies were reperfused with oxygenated medium, where the activity also temporarily fell below the steady state value.

When the moderate light intensities were used for brief periods of time, and then turned off, the activity transiently increased to above the steady state level. This is consistent with increased mitochondrial respiration depleting the oxygen pressure in the carotid body during illumination. When the light was then turned off, the activity rose well above the steady state, often approaching the value obtained by stopping the flow of perfusate, due to the lower oxygen pressure. In the dark, CO again inhibited respiration and oxygen diffusion returned the oxygen pressure, and therefore the activity, to the steady state levels. The CO-induced effects involve the full oxygen sensing activity of the carotid body i.e. there was no activity which needed to be attributed to a CO insensitive oxygen sensor or to an oxygen sensor which is sensitive to CO but with the CO effect insensitive to light.

3.2. *Wavelength dependence of the intensity of the illuminating light*

The light output of the tungsten-I_2 lamp was passed through an optical system which included a monochromator, with each part contributing to the final intensity of the emitted light. Fig. 2 shows the intensity of the illuminating light for the Oriel monochromater based illumination system. That for the Bausch and Lomb monochromator illumination system was similar in shape but lower in intensity at each wavelength. The light intensity is presented both in light energy, the conventional parameter, and in quantum intensity. Both increase markedly with increasing wavelength from 400 nm to 570 nm, reflecting in part the increase in energy emitted by the tungsten-I_2 lamp at longer wavelengths, and above 570 nm it is relatively wavelength independent. The profile shown in Fig. 2 can therefore be used to determine the relative response expected if the illuminating light were at constant quantum intensity. In the lower levels of response (<50% of maximum) the size of the response is approximately proportional to the intensity of the illumination light. This corrected response spectrum (action spectrum) is an accurate measure of the absorption spectrum of the CO compound which, when photodissociated, yields an unliganded heme capable of reacting with oxygen.

3.3. *The action spectrum of the photochemical response of oxygen sensor of the carotid body*

The light-induced response of the afferent neural activity of a typical carotid body preparation is shown in Fig. 3, upper panel. The response to illuminating light of each wavelength was corrected to equal quantum intensity (light quanta per unit time) for each wavelength according to Fig. 2 and shown in Fig. 3, middle panel. The Oriel illumination system has a reproducibility of approximately 2 nm and in this experiment the illuminating light had a width at half height of 10 nm in order to obtain the required light intensity (Fig. 3, middle panel). A given response at 430 nm required 6–7 times less light than that at 590 nm, indicating the absorption of the CO-compound was 6–7 times greater at 430 than at 590 nm. There is also a small maximum near 550 nm, although it is too small for clear resolution. The lower panel of Fig. 3 shows a photochemical action spectrum determined for a different carotid body preparation using the Bausch and Lomb illumination system with a bandwidth at half height of 6.7 nm and a wavelength reproducibility of ± 1 nm. The spectra in the middle and bottom panels are the same within experimental error. In separate experiments the positions of the maxima were more accurately determined using small (2 nm) changes in wavelength of illumination in the region of each maximum as 432 ± 2 nm and 590 ± 2 nm.

In any given carotid body preparation, it was possible to measure the spectrum 2 to 3 times over a period of 1–2 h. Measurements have been made for 6 independent experiments and the results are highly reproducible. Photochemical action spectra from two different carotid bodies, using the two different illumination systems, are presented for comparison. It should be noted that the sensitivity of the carotid bodies to illumination light intensity are often quite different since they have variable shapes, sizes, and positions with respect to the carotid bifurcation. This makes it impossible to provide the same illumination efficacy for different carotid body preparations. In addition, the variation in light response among carotid body preparations is consistent with the fact that, due to light scattering, the illumination light intensity rapidly decreases as it penetrates into the tissue. Nerve fibers coupled to sensory cells deep in the carotid body require higher illuminating light intensities for the photo-effect than do fibers coupled to sensory cells near the surface. Thus, each carotid body preparation has a different response to each given intensity of illumination light. This difference affects the absolute sensitivity to light but not the spectrum i.e. the same response in different carotid bodies may require several fold different incident light intensities but

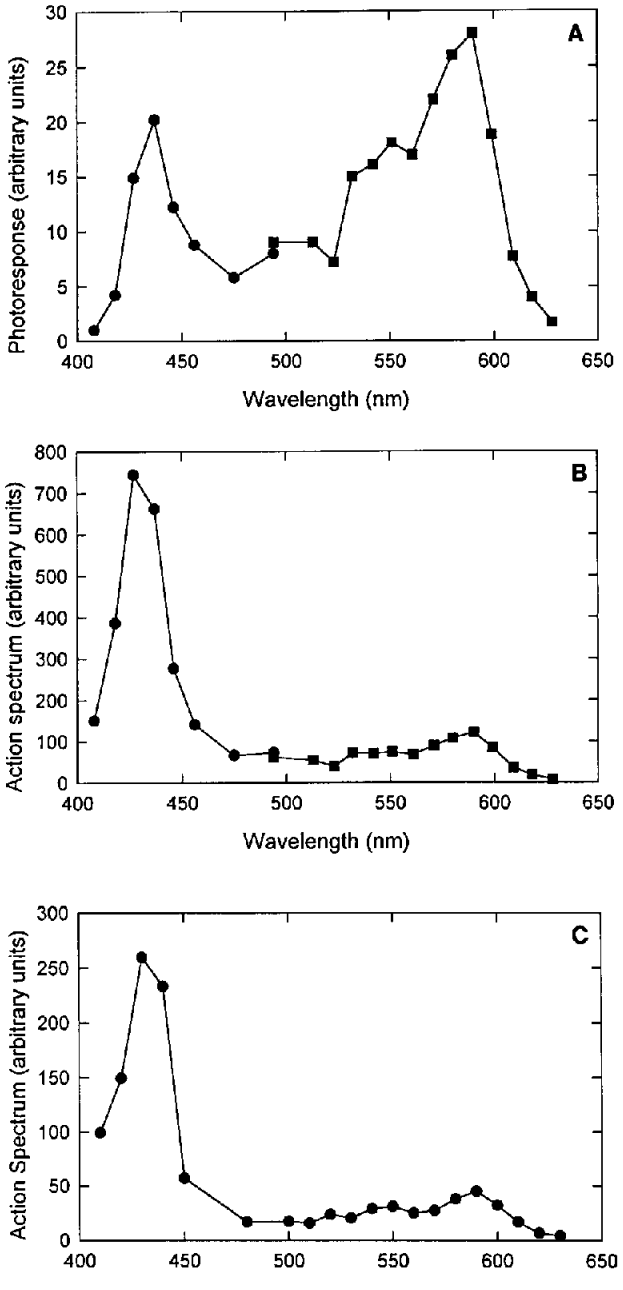

Fig. 3. The photochemical action spectrum of oxygen sensing by the cat carotid body. Isolated carotid body preparations were perfused with physiological saline containing 5 mM glucose and 22 mM glutamate and equilibrated with a gas containing 21% oxygen and 5% CO_2 . After the afferent neural activity had stabilized at the low value characteristic of the well oxygenated carotid body, the perfusion medium was then changed to a medium equilibrated with a gas containing $CO:O_2:CO_2$ at 23%, 67% and 5%, respectively. The afferent activity of the carotid body increased approximately 20-fold. The carotid body was then subjected to 6-s periods of illumination using monochromatic light of the indicated wavelengths, separated by brief recovery periods in the dark (Fig. 1). The uncorrected photo-response spectrum is shown in the upper panel. The extent of the decrease in afferent electrical activity of the carotid body induced by illumination was then corrected to the same quantum intensity of illuminating light (Fig. 2) and plotted against the wavelength of the light (middle panel). The top and middle panels are data taken using the Oriel monochromater system (10 nm bandwidth at half height) while the bottom panel is the spectrum obtained using the Bausch and Lomb monochromater system (6.7 nm bandwidth at half height).

when the response is measured as a function of wavelength, the same spectrum is obtained.

4. Discussion

Carbon monoxide is a very specific metabolic inhibitor. It is an structural analog of oxygen and thus binds at the oxygen reaction site of enzymes which react with oxygen, particularly hemoproteins. Thus CO acts as an inhibitor of the metabolic functions in which the hemes serve to either catalyze the reaction of oxygen (such as cytochrome a_3 or cytochrome P_{450}) or to transport oxygen to its reactive site (such as hemoglobin). This property has been an invaluable asset in the studies of cellular respiration, since it has proven an unambiguous method for determining the nature of the oxygen reactive component in living cells. Cytochrome a_3, for example, was identified as the primary respiratory oxidase in cells and it's spectrum was determined many years before this spectrum could be confirmed by other methods. Although the techniques for measuring absorption spectra in biological samples has improved enormously over the years, it is still impossible to accurately measure the spectrum of the pigments of the carotid body due to a combination of its small size, light scattering by the tissue, and the presence of several pigments with overlapping absorption spectra. Even if accurate absorption spectra could be measured, this would not identify the particular component responsible for oxygen sensing. Measuring the photochemical action spectrum is, in contrast, highly specific for the oxygen sensor. The light-induced activity changes are only affected by the presence of other tissue pigments (the light paths are so short that even in the Soret region of the spectrum, near 430 nm, the total absorbance by the tissue pigments is small) or by light scattering by the tissue. The carotid body response used to measure the photochemical action spectrum, i.e. its afferent electrical activity, is due only to the oxygen chemosensory activity.

The action spectrum for the reversal of CO effect on oxygen sensing by the carotid body has well defined maxima at near 432 and 590 nm as well as a small maximum near 550 nm. The ratio of the peak at 432 nm to that at 590 is between 6 and 7. This photochemical action spectrum is clearly that of cytochrome c oxidase of the mitochondrial respiratory chain as originally determined [16,17,22]. Values for the 432 nm to 590 nm ratio in the action spectrum of cytochrome a_3 have been reported to be 6.5 [22] or 6.7 [23] for yeast, and 6.0 for submitochondrial particles from heart muscle [21]. There is no evidence for contribution by other heme CO compounds, such as CO compound of cytochrome P_{450} or of a *b*-type cytochrome (see [24]). The former would show a distinctive maximum at 450 nm and the latter would both show other maxima in the region of 550 to 580 nm and alter the ratio of the absorption at 432 nm relative to that at 590 nm. Neither effect is observed.

Lopez-Barnes and coworkers [25,26] showed that the forward K^+-current in the glomus cells was lowered by lowering the PO_2 in the region from 150 to 80 Torr and suggested this was the mechanism of oxygen sensing. The decrease in K^+ current was reversed by CO [25], i.e. CO inhibited the effect of lowering oxygen pressure, but this effect occurred at oxygen pressures above the normal sensory range. Lahiri et al. [27], however, showed that CO caused a photolabile increase in chemosensory discharge but attenuated the hypoxic response.

The latter effect would be consistent with such a mechanism, but for a lower oxygen pressure range.

The maximal increase in afferent electrical activity induced by CO at moderate oxygen concentrations is similar to that induced by stopped flow hypoxia, indicating CO inhibits most of the oxygen sensing by the carotid body. This suggests that if there are oxygen sensors in the carotid body which are not affected by CO, they cannot account for very much of the sensory activity for oxygen. The CO induced increase in chemosensory activity is fully reversed by light and this can be achieved with either white light or higher intensity light at the wavelengths of the maximal effect. With the existing data, we can conclude that the photo-reversible oxygen sensing activity observed in the action spectrum accounts for at least 80% of the total chemosensory activity of the carotid body. Thus, mitochondrial cytochrome a_3 (and thereby mitochondrial oxidative phosphorylation) is responsible for most of the oxygen chemosensory activity of the carotid body. The contribution, if any, of other oxygen sensors to the remaining 20% of the chemosensory activity remains to be determined. It is reasonable to suggest, however, that mitochondrial cytochrome a_3 is also an important oxygen sensor in many other tissues.

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