Volume 256, number 1,2, 75–78 FEB 07666 October 1989

Indications to an NADPH oxidase as a possible pO_2 sensor in the rat carotid body

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Received 14 July 1989

The rat carotid body superfused with low pO_2 exhibited an optical absorbance spectrum which resembles the reduced spectrum of the NADPH oxidase in neutrophils. Diphenylene iodonium (DPI) as a specific inhibitor of the oxidase attenuated the reduced absorbance spectrum in the carotid body. Also absorbance bleaching by low doses of cyanide (50 and 100 μM) was inhibited by DPI, whereas higher doses of cyanide (300 μM) caused an absorbance spectrum typical for reduced cytochromes. It is concluded that an NADPH oxidase acts as a pO_2 sensor in the carotid body with low affinity for oxygen and high affinity for cyanide.

Carotid body; pO_2 sensor; NADPH oxidase; Diphenylene iodonium; Light absorbance spectrum; Hypoxia; Cyanide

1. INTRODUCTION

The carotid body located at the carotid sinus is able to transduce changes of oxygen pressure in the arterial blood into nervous signals regulating respiration and circulation in order to avoid hypoxic situations in the body. The mechanism of the transducing process is still a matter of discussion. A generally accepted concept defines this process as a pO_2-dependent transmitter release from carotid body type-I cells, which generates in postsynaptic afferent nerve endings action potentials via spontaneously depolarizing potentials (for review see [1]). In a metabolic hypothesis the respiratory chain of the carotid body mitochondria has often been considered to be the most probable candidate for a pO_2 sensor triggering the pO_2-dependent transmitter release. Anichkow and Belen'kii [2] as well as Joels and Neil [3] assumed that chemosensory excitation, especially under hypoxia, is caused by a decrease in ATP levels in the carotid body tissue. Biscoe [4] proposed that energy depletion under hypoxia triggers the nerve discharge by producing membrane instability in the sensory nerve endings. Using several inhibitors and uncouplers of the respiratory chain, Mulligan et al. [5] gave further support to the idea that oxidative phosphorylation is involved in the carotid body chemoreception. However, Obeso et al. [6] could show that carotid body excitation by cyanide mimicking the hypoxic chemoreceptor response does not alter the ATP content of the organ and, therefore, contradicting the metabolic hypothesis. This contradiction was supported by Acker and Starlinger [7] showing that the ATP content of the carotid body does not change under hypoxia.

To explain the responsiveness of the carotid body chemoreceptor under hypoxia, Mills and Jöbsis [8] discovered from their photometric studies on the respiratory chain of the carotid body with the dual wavelength method an unusually low affinity component for oxygen of cytochrome aa_3, which might act as a pO_2 sensor. Acker and Eyzaguirre [9] repeated these studies with a whole spectral analysis of the mouse carotid body showing unknown hypoxia-induced light transmission changes, which might have contaminated the dual wavelength measurements. The present paper

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

describes the same photometric measurements on the superfused rat carotid body with the indication that these hypoxia-induced light transmission changes might be caused by an oxidase similar to the respiratory burst oxidase, as described in neutrophils [10,11].

2. MATERIALS AND METHODS

2.1. *Carotid body superfusion*

After prolonged flushing of the common carotid arteries with Macrodex 6% (Knoll, FRG) to eliminate red cells from the tissue, carotid bodies and their immediate vessels were excised from rats anesthetized with pentobarbitone (3–4 mg/rat) and heparinized with 1300 USPE/animal. The carotid bodies were denuded of all other structures and placed in a small lucite chamber mounted on the stage of an upright photomicroscope (Zeiss, FRG). The cleaned organs (length, 500 μm; width, 300 μm; thickness, 150 μm) were then superfused with modified Locke's solution, as described by Delpiano and Acker [12], equilibrated either with different O_2 mixtures or containing KCN (50–300 μM) and viewed with a 40 × water immersion objective and 10 × oculars. Oxygenation of the superfusion medium was controlled close to the tissue with a needle pO_2 electrode [9] whose tip was located near the objective. Temperature was routinely maintained at 34–35°C and pH was kept at 7.36–7.37.

2.2. *Materials*

DPI, which was solved in DMSO (1%), was kindly supplied by Dr A.R. Cross, Department of Biochemistry, Bristol, England.

2.3. *Photometry*

For light absorption measurements, light from a halogen lamp (12 V, 100 W) filtered by a motor-driven monochromator (M20, Zeiss, FRG) trans-illuminated the organ continuously between wavelengths 410 and 620 nm with a halfbandwidth of 5–8 nm. Light signals crossing the preparation were recorded by a photomultiplier (Knott, FRG) placed on the third ocular tube of the microscope trinocular head. The photomultiplier signals together with the wavelength information of the monochromator were stored in a computer (Perkin Elmer 7/32). Light intensity changes of $(0.12 \pm 0.011) \times 10^{-3}$ OD can be detected with this device. This value can also be taken as an assessment of the baseline reproducibility in the selected wavelength range. To demonstrate the spectral characteristics of the carotid body, the organ under well oxygenated conditions served as a baseline, from which spectra under stimulatory conditions could be automatically subtracted by the computer. Only 1 min was required to record a whole spectrum. For each single experimental situation in one carotid body 5 spectra were recorded and averaged, counting as one experiment.

3. RESULTS AND DISCUSSION

3.1. *Hypoxia*

Fig.1 shows a hypoxic difference spectrum

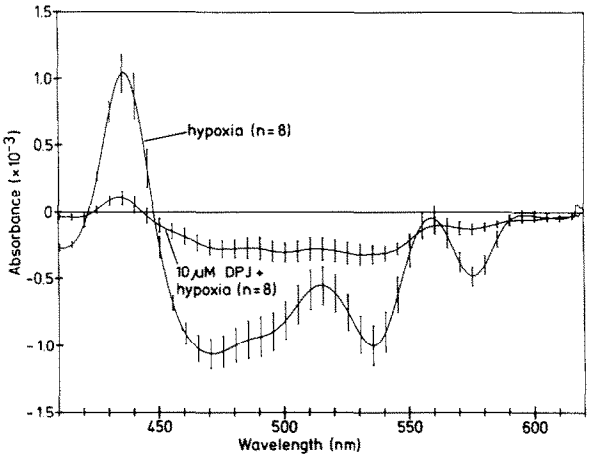

Fig.1. Effect of DPI on hypoxia-induced absorbance changes in the superfused rat carotid body. Spectra were recorded as described in section 2. Carotid body tissue superfused with a pO_2 of 340 Torr served as a baseline for the hypoxia-induced absorbance curve. Carotid body tissue superfused with a pO_2 of 340 Torr under addition of 10 μM DPI served as a baseline for the second spectrum. The full lines give the mean values. ± SE values are given every 5 nm. *n*, number of experiments.

(hypoxia) as a mean ± SE (standard error of the mean) of 8 carotid bodies. The absorbance spectrum during superfusion with a pO_2 of 340 Torr served as a baseline in these experiments from which spectra with hypoxic superfusion (pO_2 20 Torr) were automatically subtracted. The spectrum showed a typical absorbance maximum at 436 nm and a shoulder at 559 nm with a bleaching of the absorbance peaking at 413, 470, 535, and 575 nm. Since the form of the spectrum drew attention to the described hypoxic difference absorbance spectrum of the NADPH oxidase in neutrophils [10,11], carotid bodies of the same experiments were superfused with Locke's solution for 1 h containing 10 μM DPI, known to be a special inhibitor of a 45 kDa protein, which regulates the activity of the NADPH oxidase [10,11]. Fig.1 shows the mean ± SE of 8 hypoxic difference spectra under DPI conditions. The absorbance spectrum under superfusion with a pO_2 of 340 Torr and 10 μM DPI solved in 1% DMSO served as a baseline. It is to be seen that the spectrum is attenuated and that all maxima as seen in hypoxia, nearly disappeared. This reaction was taken as additional evidence for the involvement of an NADPH oxidase, keeping in mind that also other unidentified proteins might

contribute to the hypoxic spectrum. Assuming that the protein complex of the NADPH oxidase mainly determines the spectrum at 436 nm as characteristic for cytochrome *b*-559 and at 470 nm as characteristic for FAD [10,11], the pO_2 sensitivity of the light absorbance at these wavelengths was tested in another series of experiments (n = 8). Fig.2 shows that varying the pO_2 in the superfusion medium between 20 and 340 Torr revealed different sensitivities in the reduction of both components with a k_{mpO_2} of about 46 Torr for 436 nm and a k_{mpO_2} of about 71 Torr for 470 nm, if one takes the absorbance values at a pO_2 of 20 Torr as maximum.

3.2. *Cyanide*

Superfusing carotid bodies (n = 5) with different KCN concentrations resulted in light absorbance changes, as depicted in fig.3. The carotid body absorbance spectrum with a pO_2 of 340 Torr in the superfusion medium was taken as a baseline. As an example, the means ± SE (n = 5) of the difference spectra with 100 μM KCN and 300 μM KCN are shown. The lower dose resulted in a bleaching of the absorbance peaking at about 500 nm, whereas the higher doses induced an increase of the absorbance with characteristic maxima at 446 nm which might be related to reduced cytochrome aa_3, and at 520 as well as 550 nm which might be caused by the reduced form of cytochrome *c* [13]. To establish a dose response curve the absorbance changes at 550 nm were recorded and in fig.4 drawn in relation to different KCN doses. A decrease of the absorbance could be observed in five experiments at doses between 50 and 100 μM, which was followed by an increase of the absorbance at doses between 200 and 300 μM. Taking superfusion of the carotid body with 10 μM DPI and a pO_2 of 340 Torr as a baseline the different doses of KCN always resulted in an increase in absorbance (n = 5).

3.3. *Conclusions*

The light absorbance changes at 436 and 470 nm under different degrees of hypoxia (fig.2), presumably related to the reduction kinetics of cytochrome *b*-559 and FAD, resemble the hypoxic chemoreceptor nervous response curve [12] perhaps indicating a close relationship to the process of pO_2 chemoreception. Low doses of cyanide which excite the chemoreceptor like hypoxia in-

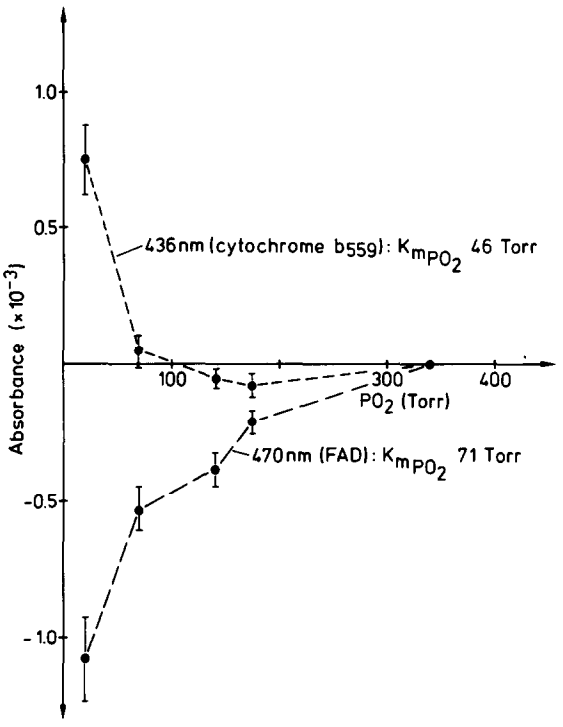

Fig.2. pO_2 response curve of absorbance changes of the carotid body tissue at 436 nm and 470 nm with the carotid body absorbance spectrum at a pO_2 of 340 Torr as a baseline. Means ± SE of eight experiments are shown.

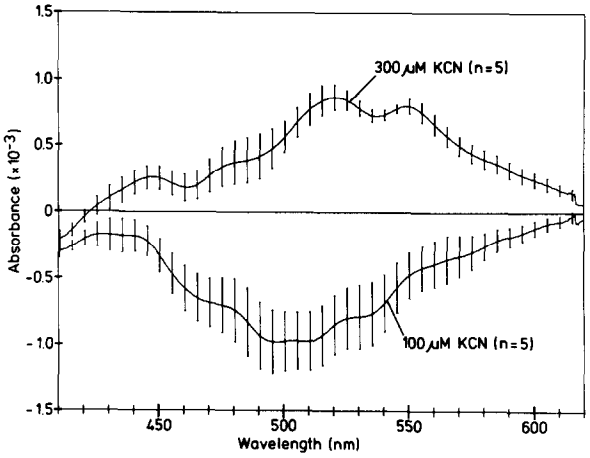

Fig.3. Effect of different doses of KCN on light absorbance in the rat carotid body. Carotid body tissue superfused with a pO_2 of 340 Torr served as a baseline. Means ± SE are shown. n, number of experiments.

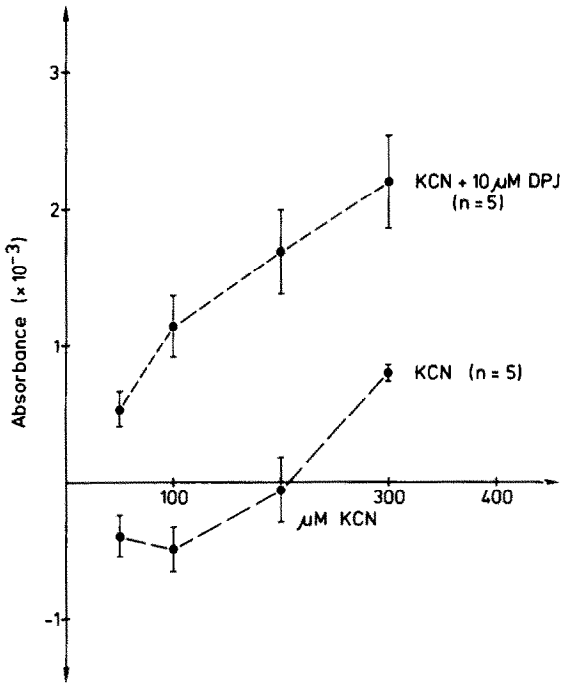

Fig.4. KCN response curves of absorbance changes in the carotid body tissue at 550 nm. Carotid bodies superfused with and without DPI at a pO_2 of 340 Torr served as baseline. Means ± SE are shown. *n*, number of experiments.

duce a bleaching of absorbance (fig.4). Since DPI attenuated or reversed these responses it might be speculated that low doses of cyanide as well as hypoxia act on a protein, which is described in the case of the NADPH oxidase as a 45 kDa protein [10,11]. Doses of cyanide as high as 300 μM are followed by an initial increase in chemoreceptor activity with a subsequent impairment [6]. Under these conditions the light absorbance in the carotid body reveals peaks typical for reduced cytochromes [13] which cannot be observed under hypoxic conditions. This underlines the importance of a functioning respiratory chain for a sustained chemoreceptor nervous activity under hypoxia of different degrees. Therefore, one might hypothesize that hypoxia, low doses of cyanide, and DPI are acting on the same protein which might represent the pO_2 sensor characterized by a low affinity for oxygen and a high affinity for cyanide. A pO_2 sensor located in the cell membrane of the carotid body like the NADPH oxidase in neutrophils [10,11] would be suitable to regulate the activity of different ion channels dependent on pO_2 to facilitate the transmitter release, irrespective of the respiratory chain. Further experiments have to prove this assumption and to clarify the involvement of this protein in the transducing process.

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