Type I cell ROS kinetics under hypoxia in the intact mouse carotid body ex vivo: a FRET-based study

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Bernardini A, Brockmeier U, Metzen E, Berchner-Pfannschmidt U, Harde E, Acker-Palmer A, Papkovsky D, Acker H, Fandrey J. Type I cell ROS kinetics under hypoxia in the intact mouse carotid body ex vivo: a FRET-based study. Am J Physiol Cell Physiol 308: C61–C67, 2015. First published October 15, 2014; doi:10.1152/ajpcell.00370.2013.—Reactive oxygen species (ROS) mainly originating from NADPH oxidases have been shown to be involved in the carotid body (CB) oxygen-sensing cascade. For measuring ROS kinetics, type I cells of the mouse CB in an ex vivo preparation were transfected with the ROS sensor construct FRET-HSP33. After 2 days of tissue culture, type I cells expressed FRET-HSP33 as shown by immunohistochemistry. In one population of CBs, 5 min of hypoxia induced a significant and reversible decrease of type I cell ROS levels (n = 9 CBs; P < 0.015), which could be inhibited by 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), a highly specific inhibitor of the NADPH oxidase subunits p47phox and p67phox. In another population of CBs, however, 5 min of hypoxia induced a significant and reversible increase of ROS levels in type I cells (n = 8 CBs; P < 0.05), which was slightly enhanced by administration of 3 mM AEBSF. These different ROS kinetics seemed to coincide with different mice breeding conditions. Type I cells of both populations showed a typical hypoxia-induced membrane potential (MP) depolarization, which could be inhibited by 3 mM AEBSF. These different ROS kinetics seemed to coincide with different mice breeding conditions. Type I cells of both populations showed a typical hypoxia-induced membrane potential (MP) depolarization, which could be inhibited by 3 mM AEBSF.

ROS; carotid body; FRET-HSP33; tissue oxygen; membrane potential; hypoxia; NADPH oxidase

CB type I cells located at the common carotid artery bifurcation transform changes in blood P\textsubscript{O}2 into corresponding electric activity conducted along sinus and glossopharyngeal nerves into the brainstem for regulating ventilation and blood circulation. The underlying mechanisms enabling this response are still unclear. Previously, we proposed an isoform of the neutrophil NADPH oxidase as a type I cell oxygen sensor (1, 8), which would be coupled to maxiK or TASK-1/TASK-3 (22) heterodimer channels to transfer P\textsubscript{O}2 sensitivity to these channels. It was proposed that hypoxia leads to a decrease of ROS production with subsequent inhibition of the potassium channels. Hypoxic inhibition of potassium channels was measured by patch-clamp technique in early studies (13) and leads to membrane depolarization and calcium influx. This results in transmitter release and ultimately induces action potentials in sinus nerve endings synthaptically connected to type I cells. Different subtypes of the NADPH oxidase as NOX2 with the corresponding subunits p22phox, gp91phox, p47phox, and p67phox and NOX4 with the subunit p22phox (5) have been detected in type I cells of human, guinea pig, rat, and mouse carotid bodies (CBs) (11, 15, 18). Lack of p47phox but not of gp91phox significantly potentiates CB response to hypoxia (21), likely due to low reactive oxygen species (ROS) levels and enhanced depression of potassium current and increased intracellular calcium levels (11). After 7 days of chronic hypoxia, the NADPH oxidase isoforms NOX2 and NOX4 together with p47phox were increased in rat CB type I cells. Thus increased ROS production might be responsible for dampening hyper-sensitivity in CB chemoreceptors during chronic hypoxia.

Specific inhibition of the overexpressed NADPH oxidase subunit p47phox by 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) (10) and herewith ROS production enhanced nervous chemoreceptor hypoxic response (12). This is in line with our hypothesis that low ROS levels cause high CB activity. However, measurements with the intracellular dye dihydroethidium (DHE) have shown enhanced ROS production by stimulation of the rat CB NADPH oxidase in acute hypoxia (11).

These contradicting findings highlight the main obstacle for further investigations on the significance of the NADPH oxidase as an oxygen sensor and leave the question unanswered whether hypoxia leads to ROS increase or decrease in CB tissue. The problem appears to lie in the lack of reliable techniques for measuring cellular ROS kinetics. The most commonly used intracellular dyes like 2’,7’-dichlorofluorescein (DCF) or DHE are chemically irreversibly oxidized by ROS (15a). Cellular ROS measurements in type I cells using these dyes, however, showed reversible reactions following changes in P\textsubscript{O}2 or drug application (11, 12). These dynamics in ROS levels make dyes with irreversible chemical changes unsuitable for continuous measurement of cellular ROS kinetics. In addition, the intracellular amount of the dyes may be influenced by P\textsubscript{O}2-dependent transporters also affecting the reliability of data obtained with these single wavelength-emitting dyes (3, 24). These problems can be solved by the very promising and state-of-the-art Förster resonance energy transfer (FRET)-based sensor for measuring ROS kinetics (24). The ROS sensor consists of enhanced cyan (ECFP) and yellow (EYFP) fluorescent protein motifs linked by the redox-dependent regulatory domain from the bacterial heat shock protein HSP33. ROS-induced oxidation of the HPS33 domain causes a conformational change upon which the FRET signal is decreased. CBs...
were transfected with plasmid DNA encoding for FRET-HSP33, which was detectable within 2 days of incubation. We detected a decrease in ROS as well as an increase under hypoxia, which seemed to depend on mice breeding conditions. AEBSF inhibited the hypoxia-induced decrease of ROS as well as the characteristic hypoxia-induced type I cell membrane potential (MP) depolarization indicating a pivotal role of the NADPH oxidase subunit p47phox for triggering CB response in dependence on PO2.

MATERIALS AND METHODS

Ethical approval. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the guidelines of the National Institutes of Health regarding the care and use of animals for experimental procedures. The study protocol was approved by the Institutional Animal Ethics Committee of the Universitätshospital Essen, University of Duisburg-Essen.

Procedure. Female C57BL/6J mice, 8–12 mo old, were used for the experiments as described (27). Mice were either bred in ambient environmental cage (AEC) or individual ventilated cages (IVC; Tecniplast, Hohenpein; Germany). IVC conditions have been described to produce chronic low grade hypoxic mice in contrast to truly normoxic AEC conditions (29).

Animals were euthanized by inhalation of isoflurane, and immediately thereafter, the bifurcations of both common carotid arteries together with the superior cervical ganglion were dissected and stored in ice-cold phosphate-buffered saline (PBS) solution until fine preparation. For transfection with the FRET-HSP33 ROS sensor, the tissue preparation was stored in a 0.2-cm electroporation cuvette (Bio-Rad, Munich, Germany) containing 1.6–1.9 µg/µl of the plasmid pFRET-HSP33 (24), kindly provided by Paul Schumacker (Chicago, IL), in ice-cold H2O. For electroporation, the Gene Pulser II (Bio-Rad) was applied using one single pulse of 200 Ω, 25 µF, and 0.2 kV.

Thereafter, the whole tissue preparation was kept under tissue culture conditions using DMEM (4.5 g glucose, 4 mM l-glutamine, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin; GibCO, Carlsbad, CA) with 5% CO2 in air and 37°C for 2 days. For measurements, CBS were dissected from the bifurcation using a stereomicroscope (American Optical, Southbridge, MA). For this purpose, the carotid bifurcation was carefully freed from connective tissue with tweezers until the nerve bundle between the CB and the superior cervical ganglion became visible. Transillumination of the bundle visualizes the CB as an opaque oval organ that can be easily separated from the arterial wall by blunt preparation.

The time of 2 days in tissue culture was sufficient for transfected CB type I cells to synthesize the FRET-HSP33 construct. For type I cell MP measurements, CBS were stained as described previously (27) with amino-naphthylethenylpyridinium dye with a propylsulfonate head group (di-4-ANEPPDHQ: 15 µM; Invitrogen) for 40 min in ice-cold PBS. For cellular oxygen measurements, CBS were incubated for 24 h with the intracellular localizing oxygen-sensitive dye Mitro-Image NanO2 (Luxtcel Biosciences) at a concentration of 100 µM/ml PBS (25). After being washed in 0.1% Triton X-100-PBS for 30 min and blocking in 5% bovine serum albumin-PBS for 1 h, CBS were stained with the primary antibodies sheep anti-tyrosine hydroxylase (anti-TH, diluted 1:25; Abcam, Cambridge, UK) and rabbit anti-GFP (diluted 1:20; BD Pharmingen, Heidelberg, Germany) for 1 h each. CBS were then washed in PBS and incubated with the secondary antibodies Alexa 568-conjugated goat anti-sheep immunoglobulin G (IgG, diluted 1:100; DiaNova, Hamburg, Germany) and Alexa 546-conjugated goat anti-rabbit IgG (diluted 1:400; Invitrogen) for 1 h. The stained CBS were washed in PBS and mounted on slides in Mowiol (Calbiochem, EMD Chemicals, Gibbstown, NY). For visualizing Alexa 514, an excitation wavelength of 440 nm and a 565/40 nm emission filter were used, whereas an excitation wavelength of 532 nm and a 660/50 nm emission filter were applied for Alexa 568.

Analysis. Relative changes of hypoxia-induced ECFP/EYFP ratio and oxygen and MP changes are graphically presented using TechPlot as described previously (27). Student’s paired two-sided t-test with unequal variance was used to calculate significant differences of hypoxia-induced ECFP/EYFP ratio changes whereas the unpaired t-test was used for AEBSF-induced MP changes. Pearson’s correlation coefficient r was used to show the strength of the correlation among oxygen, MP, and ROS and the degree of overlapping of colocalization in image analysis.
RESULTS

Localization of the FRET-HSP33 construct. Figure 1A shows the ratio of ECFP/EYFP fluorescence signals in a typical CB type I cell cluster (27) under experimental conditions. To prove the specificity of the localization, the identical CB was immunohistochemically stained. Figure 1B shows in red the same tissue spot with tyrosine hydroxylase (TH) staining typical for type I cells; Fig. 1C reveals the intracellular staining of the ECFP/EYFP in green with a similar topological pattern. Both ECFP and EYFP proteins are nearly identical mutants of GFP and thus were detected with the anti-GFP antibody. Figure 1D merges Fig. 1B and C, visualizing type I cells that express the FRET-HSP33 construct as yellow hot spots. Pearson’s correlation coefficient between both channels was 0.65.

Hypoxia-induced decrease of ECFP/EYFP (ROS) ratio in type I cells. After challenging two transfected CBs with one single hypoxic period resulting in a decrease in ROS as detected by a lowered ECFP/EYFP ratio (data not shown), we continued with exposing seven transfected CBs each to three hypoxic periods to document the reproducibility of our results.

Fig. 1. Immunohistochemistry. A: ratio of enhanced cyan (ECFP) and yellow (EYFP) fluorescent protein fluorescence of a typical carotid body (CB) type I cell cluster 2 days after transfection with pFRET-HSP33. B: identical CB type I cell cluster stained in red with anti-TH antibody. C: same CB type I cell cluster stained in green with anti-GFP antibody to detect ECFP/EYFP. D: merging B and C shows in yellow type I cells expressing the FRET-HSP33 construct. Bar = 10 μm.
Figure 2A demonstrates the highly reproducible and reversible hypoxia-induced decrease in ROS (indicated by the lower ECFP/EYFP ratio) of the same type I cell cluster (solid line with closed symbols) as seen in Fig. 1A. Three periods of 5 min of normoxia are each followed by hypoxic phases that last 5 min each. The animation (see Supplemental Data File; Supplemental Material for this article is available online at the Journal website) shows the time course of the changes in ROS of this type I cell cluster in relation to the analog data from Fig. 2A. Figure 2B shows as a solid line with closed symbols the mean ROS kinetic calculated from all seven CBs that showed hypoxia-dependent decreases in ROS. The three hypoxic minima of the mean ROS values show statistically significant differences from corresponding normoxic controls ($P < 0.015$). All mice of these experiments were bred under AEC conditions. The duration of the hypoxic periods can be estimated from oxygen kinetics in CB tissue (broken line) measured in a separate CB exposed to the same gas mixing conditions. The separate measurements in both cases are necessary due to spectral cross talk of the dyes disturbing the FRET analysis.

**Hypoxia-induced increase of ECFP/EYFP (ROS) ratio in type I cells.** Figure 3A demonstrates the highly reproducible and reversible hypoxia-induced increase of the ECFP/EYFP ratio (and thus ROS) of one mouse CB (solid line with closed symbols). Three periods of 5 min of normoxia are each followed by hypoxic phases that last 5 min each. Figure 3B shows as solid line with closed symbols the mean ROS values calculated from three CBs that showed a hypoxia-induced ROS increase and the corresponding standard deviation. The three hypoxic peaks of the mean ROS kinetics statistically significantly differ from corresponding normoxic controls (first peak $P < 0.016$; second peak $P < 0.012$; and third peak $P < 0.04$). All mice of these experiments were bred under IVC conditions. After exposure to 3 mM AEBSF for 30 min, this ROS decrease is inhibited (average of ~50 cells: solid line with open symbols). Figure 4B shows the mean hypoxia-induced ROS increase with standard deviation (solid line with closed symbols) of nine IVC mouse CBs due to a single hypoxic period in CB tissue (broken line). After exposure of five of the nine mouse CBs to 3 mM AEBSF for half an hour, the ROS increase is even slightly elevated (not statistically significant). Shown are the mean with standard deviation (solid line with open symbols). Each measurement is normalized to the mean of the first five data points (normoxic baseline). This finding suggests that there exist at least two simultaneously operating ROS-generating systems that are sensitive to changes in PO$_2$: one sensitive to AEBSF like the NADPH oxidase and the other of unknown origin insensitive to AEBSF.

**Kinetics of MP under different oxygen conditions and AEBSF inhibition.** Figure 5A shows the mean type I cell MP depolarization with standard deviation (solid line with closed symbols) of four AEC mouse CBs in the course of three subsequent hypoxic periods of CB tissue. Two of these four CBs were transfected with the FRET-HSP33 construct indicating the transfection procedure as not impairing the type I cell oxygen-sensing function. Figure 5B shows the mean...
type I cell MP depolarization with standard deviation (solid line with closed symbols) of seven IVC mouse CBs in the course of a single hypoxic period in CB tissue (broken line). After exposure of three of the seven mouse CBs to 3 mM AEBSF for 30 min, the hypoxic depolarization is significantly ($P < 0.005$) inhibited (solid line with open symbols). Each measurement is normalized to the mean of the first five data points (normoxic baseline).

Fig. 3. ROS increase kinetics. A: CB tissue exposed to 3 subsequent hypoxic periods as exemplified by the oxygen curve recordings in a separate CB (right $y$-axis: broken line) shows an increase of type I cell ROS levels measured by the ECFP/EYFP ratio (solid line, measured data as closed symbols; left $y$-axis). B: mean values and standard deviation ($n = 4$) of increases in ROS level induced by hypoxia are shown as a solid line with measured data as closed symbols (ECFP/EYFP ratio on the left $y$-axis). Mice of these experiments were bred under individual ventilated cages (IVC) conditions. The duration of hypoxic periods can be estimated by the separately measured oxygen kinetics in CB tissue (broken line). All values are shown as arbitrary units.

Fig. 4. ROS and 4-[(2-aminoethyl)benzenesulfonyl]fluorid (AEBSF) inhibition. A: hypoxic ROS decrease as measured by the ECFP/EYFP ratio in one AEC mouse CB (solid line with measured data as closed symbols) during a single hypoxic period in CB tissue (broken line). AEBSF inhibits this ROS decrease (solid line with measured data as open symbols). B: mean hypoxic ROS increase with standard deviation (solid line with measured data as closed symbols) of 9 IVC mouse CBs caused by a single hypoxic period in CB tissue (broken line). AEBSF even enlarges the amplitude of the ROS increase slightly. Shown is the mean with standard deviation of 5 CBs (solid line with measured data as open symbols). All values are shown as normalized arbitrary units.
Our experiments show that murine CB type I cells are capable of expressing heterologous proteins like the FRET-HSP33 and simultaneously preserve their electrical properties for sensing oxygen. It is generally accepted that ROS either from mitochondrial (24) or NADPH oxidase origin are the main determinants of the cellular redox state. Previous work has indicated that the NADPH oxidase seems to be the principal source for ROS in CB tissue (11). Herein, we measured the cellular redox state using the FRET-HSP33 ROS sensor. Specificity of this construct was tested using a control FRET construct with an ECFP-EYFP tandem construct linked by 12 amino acids instead of the redox-sensitive HSP33 hinge (26). When we used this control plasmid to transfect CBs, no hypoxia-induced changes in ECFP/EYFP fluorescence ratio were measured (data not shown).

Our experiments have shown that CB type I cells have several systems generating ROS. One is the AEBSF-sensitive NADPH oxidase, which decreases ROS production under hypoxia. The other probably mitochondria or endoplasmic reticulum oxidoreductin-1 (Ero1), both described to be involved in oxygen sensing (17, 24), responds to hypoxia with an increase of ROS production. The balance between the systems can apparently be shifted between individual CBs due to breeding conditions (AEC vs. IVC). The factors that influence the balance between these systems, however, remain unclear and are subject to further studies. However, only the AEBSF-sensitive hypoxia-induced ROS decrease seems to trigger MP depolarization even if the hypoxic ROS increase is predominant (see Figs. 4B and 5B). This might hint to a tightly localized cooperation between potassium channels and the NADPH oxidase.

ROS and MP kinetics followed closely the tissue oxygen levels supporting the hypothesis of a leading role of ROS in transferring oxygen sensitivity to potassium channels. However, other factors might also be involved in the potassium channel gating with CO, H2S, NO, or AMP as candidates that are currently discussed (18, 19). Measurements of their kinetics with similar FRET sensors under varying tissue oxygen conditions could very well be the method of choice to investigate their hierarchy in the oxygen-sensing process.

The same FRET-HSP33 sensor we used has previously been applied in pulmonary smooth muscle cells (PSMCs) to study their hypoxic response (25). In that study, a delayed beginning and long-lasting ROS increase under hypoxia were observed, reaching a maximum after ~20 min. A reversal of the signal and return to basement levels under reoxygenation could not be shown (25). Comparably slow ROS kinetics could also be measured with DHE in PSMCs where acute hypoxia led to a decrease of ROS as in the AEC CBs whereas chronic hypoxia resulted in increased ROS generation as in the IVC CBs. (28). From these data one can conclude that chronic hypoxia seems to influence the balance between the ROS-generating systems.

It is of interest to note in accordance with our experiments (Figs. 4B and 5B) that the NAD(P)H oxidases NOX2 or NOX4 are tightly colocalized with potassium channels potentially transferring oxygen sensitivity to these channels. Likewise, HEK293 cells transfected with NOX4 and TASK1 potassium channels show colocalization of both proteins in the plasma membrane of these cells. Moreover, TASK1 oxygen sensitivity was enhanced by overexpression of NOX4 and inhibited by silencing NOX4 (16). Similarly, the predominant oxygen sensors in H146 cells and neuroepithelial bodies are complexes of NOX2 and Kv+ channel proteins (7).
Our data show that the AEBSF-sensitive system, most probably the NADPH oxidase, is the key system for triggering hypoxia-induced MP depolarization as it was already shown on the p47phox knockout mouse CBs (11). AEBSF seems to mimic hypoxia by inhibiting the complex formation between p47phox and NADPH oxidase and consequently blocking ROS generation; this, however, also prevents any hypoxia-induced ROS decrease and depolarization of the MP.

In all, previous work and our findings strengthen the hypothesis of a substantial role of the NADPH oxidase (2, 4) in the oxygen-sensing process. Whether the NADPH oxidase, i.e., NOX2 or NOX4, transfers the oxygen sensitivity to potassium channels by means of ROS kinetics or reversible binding with the subunit p47phox is subject to further investigations.

The work herein may be a first step using a sensitive FRET sensor technique in viable and functional ex vivo CB preparations to get reliable data on P2o-dependent ROS kinetics as a solid base for a better understanding of oxygen sensing in CBs.

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REFERENCES

AUTHOR CONTRIBUTIONS


