Chapter 3

Measurement of Superoxide Production in Acute Hypoxia by Fixed-Cell Microscopy

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Abstract

Redox signaling implication in cell adaptation to hypoxia has been studied for a long time, both in long-term and acute responses. However, measurement of superoxide and other reactive oxygen species (ROS) in acute hypoxia is technically challenging, for example, because of the need to overcome the effect of cell reoxygenation before measurement.

Here we describe a method we have developed for measuring superoxide production in acute hypoxia using the fluorescent probe dihydroethidine in fixed-cell microscopy. The method allows measuring the kinetics of superoxide production (or other ROS with the appropriate probes) by incubating the probe in different time windows during hypoxia incubation.

Key words Acute hypoxia, Superoxide burst, Fluorescence microscopy, Redox signaling

1 Introduction

Animal cells respond to reduced oxygen availability (hypoxia) triggering different responses that rewire their metabolism and initiate systemic responses in the organism, such as hyperventilation or increased erythropoiesis. There has been an extensive search for the molecular mechanisms sensing the decrease in oxygen. The sensors to medium- and long-term hypoxia are well-known oxygen-dependent prolyl hydroxylases that modify the hypoxia-inducible factors (HIF), whose stability and transactivation is increased, modifying the expression of hundreds of genes [1]. However, the search for the mechanisms sensing acute reductions in oxygen availability is not finished, even though responses to acute hypoxia, such as hyperventilation induced by the carotid body or pulmonary vasoconstriction, have been known for a long time [2].

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Superoxide (O_2^-) is produced through the addition of one electron to a dioxygen molecule, O_2, and leads to generation of other reactive oxygen species (ROS), which collectively are known to be involved in redox cell signaling. Thus, when oxygen availability is reduced, one would expect a reduced production of superoxide. However, a paradoxical increase in superoxide and/or other ROS formation was described in some reports for hypoxic conditions [3, 4], while other reports did not find such increase [5]. More recently, superoxide and ROS production has been involved in acute oxygen sensing in the carotid body, providing a physiological role for this redox signaling effect [6, 7].

Some years ago, we had observed an increase in reversible cysteine oxidation in a number of proteins in endothelial cells subjected to relatively short times of hypoxia [8], and we aimed to characterize if superoxide production was increased in cells subjected to hypoxia. We developed a method to study superoxide production during acute hypoxia using dihydroethidine (or its mitochondria-directed version, mito-HE or mitosox) during 10-min time windows at different times of incubation in a hypoxia chamber, and we validated the microscopy method by comparing it with HPLC separation of the DHE products, coupled with fluorescent detection. Using this method, together with live microscopy imaging protocols in acute hypoxia, we were able to show that mitochondrial superoxide production was increased in the first minutes of hypoxia exposure, decreasing afterward; we called it a "superoxide burst" in acute hypoxia, which explained why some reports measuring ROS production at longer times of hypoxia did not see the increase in ROS production [9]. We have also used this method and complementary techniques to characterize the mechanism triggering this superoxide production in acute hypoxia, first showing that mitochondrial complex I deactivation is involved [10] and more recently that the mitochondrial Na^+/Ca^{2+} exchanger is a key player in this mechanism, through its activation and subsequent Na^+ import into the mitochondrial matrix [11].

The method uses different cell samples that are incubated in a hypoxia chamber (with media and solutions that are pre-equilibrated in the hypoxia chamber), and DHE is added for 10 min after different times of incubation in the chamber. Thus, each sample represents a different time window of hypoxic incubation, with a fixed window width (10-min windows). Cell fixation is performed at 4 °C, and the samples are covered just after fixation, thus minimizing the contact of the living cells to the oxygen-rich atmosphere outside the chamber. Controls are performed by treating the cells in the same way at room air atmosphere (what we call normoxia) and by adding antimycin A, an inhibitor of mitochondrial complex III that is well known to increase mitochondrial superoxide production.

2 Materials

1. Hypoxia chamber. We use the Invivo$_2$ 200 and Invivo$_2$ 400 workstations (Ruskinn-Baker).
2. Hanks' balanced salt solution with Ca^{2+}/Mg^{2+} (HBSS + Ca/Mg).
3. 5 mM dihydroethidium (DHE) stock in DMSO; store at −20 °C (*see* **Note 1**).
4. 4% paraformaldehyde (4% PFA) in phosphate-buffered saline; store at 4 °C (*see* **Note 2**).
5. 10 mM antimycin A stock in absolute ethanol; store at −20 °C.

3 Methods

For superoxide detection in fixed cells, firstly seed cells a day before experimentation on glass coverslips. Cells should not reach confluence since it leads to unspecific DHE signal. Typically, 60–70% confluence is best working for this protocol.

3.1 Superoxide Detection in Hypoxia

1. For treatments in hypoxia (*see* **Note 3**), pre-equilibrate all the solutions to hypoxic conditions before use and make a 5 μM DHE working solution (*see* **Notes 4** and **5**).
2. Introduce plated cells in the hypoxic chamber set at 1% O_2, 5% CO_2, and 37 °C, and incubate for the indicated times (0, 15, 30, 45, and 60 min) in pre-equilibrated hypoxic medium (*see* **Notes 6** and **7**). *See* Fig. 1 for a scheme of the time course for the different treatments.
3. After every time, wash cells three times with pre-equilibrated hypoxic HBSS + Ca/Mg and incubate with 5 μM DHE for 10 min, all in darkness and inside the hypoxia chamber.
4. After incubation, wash away excess of probe three times with pre-equilibrated hypoxic HBSS + Ca/Mg, and fix cells by adding 4% PFA. Immediately, remove cells from hypoxia chamber and incubate in darkness at 4 °C in a fridge for 15 min just after adding 4% PFA onto cells.
5. After fixation, wash wells three times with normoxic HBSS + Ca/Mg, gently remove excess of liquid from the coverslips, and mount them on slides (*see* **Note 8**).
6. Keep slides in darkness at 4 °C until quantification (*see* **Note 9**).

3.2 Superoxide Detection in Normoxia

1. For treatments in normoxia, change medium for new normoxic medium and treat cells as hypoxic cells, but in a standard cell incubator.

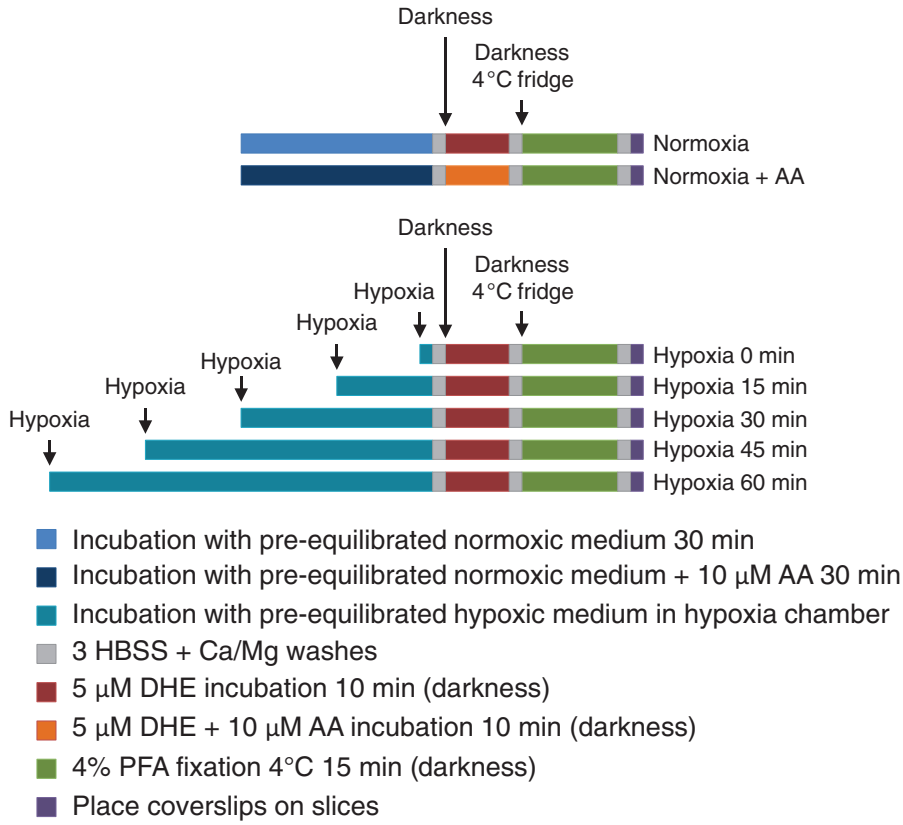

Fig. 1 Schematic timeline of the methodology described. For treatments in hypoxia, cells are introduced in hypoxia chamber where medium is changed by previously pre-equilibrated hypoxic medium and cells are incubated during different times (0, 15, 30, 45, and 60 min). These cells of hypoxic conditions remain in hypoxia chamber until fixation. For treatments in normoxia, the medium is changed by previously pre-equilibrated normoxic medium, and incubation happens in standard cell incubator. Normoxia + AA condition is the positive control; cells are incubated with 10 μM AA (diluted in pre-equilibrated normoxic medium) for 30 min. After these incubations, cells are washed three times with pre-equilibrated hypoxic or normoxic HBSS + Ca/Mg. From this point forward, cells are kept in darkness. Cells are incubated with 5 μM DHE (diluted in pre-equilibrated hypoxic or normoxic HBSS + Ca/Mg) for 10 min in hypoxia chamber or in standard cell incubator. In addition to 5 μM DHE, 10 μM AA is added onto cells in normoxia + AA condition. After that, cells are washed again three times with pre-equilibrated normoxic or hypoxic HBSS + Ca/Mg. Next, cells are fixed adding 4% PFA at 4 °C in a fridge for 15 min; thus, cells are removed from hypoxia chamber just after adding 4% PFA on cells. Then, cells are washed three times with pre-equilibrated normoxic HBSS + Ca/Mg. Finally, coverslips, where cells are seeded, are placed on slices, which are kept in darkness at 4 °C in fridge until quantification

2. The positive control for ROS production is performed with antimycin A. Add it to a final concentration of 10 μM onto normoxic cells 30 min before and during incubation with the probe.

3.3 Microscopy Imaging and Quantification

1. Take at least three images per coverslip in a fluorescence microscope, using the following excitation/emission filter pairs: 546-12/560 (*see* **Note 10**).

2. Quantify images using ImageJ software. The same threshold is set for all the images, and the mean value from the histogram is averaged for the three images of each coverslip.

Figure 2 shows a typical result of the superoxide burst in endothelial cells. Tiron treatment eliminates superoxide production, which is a control of the specificity of the method.

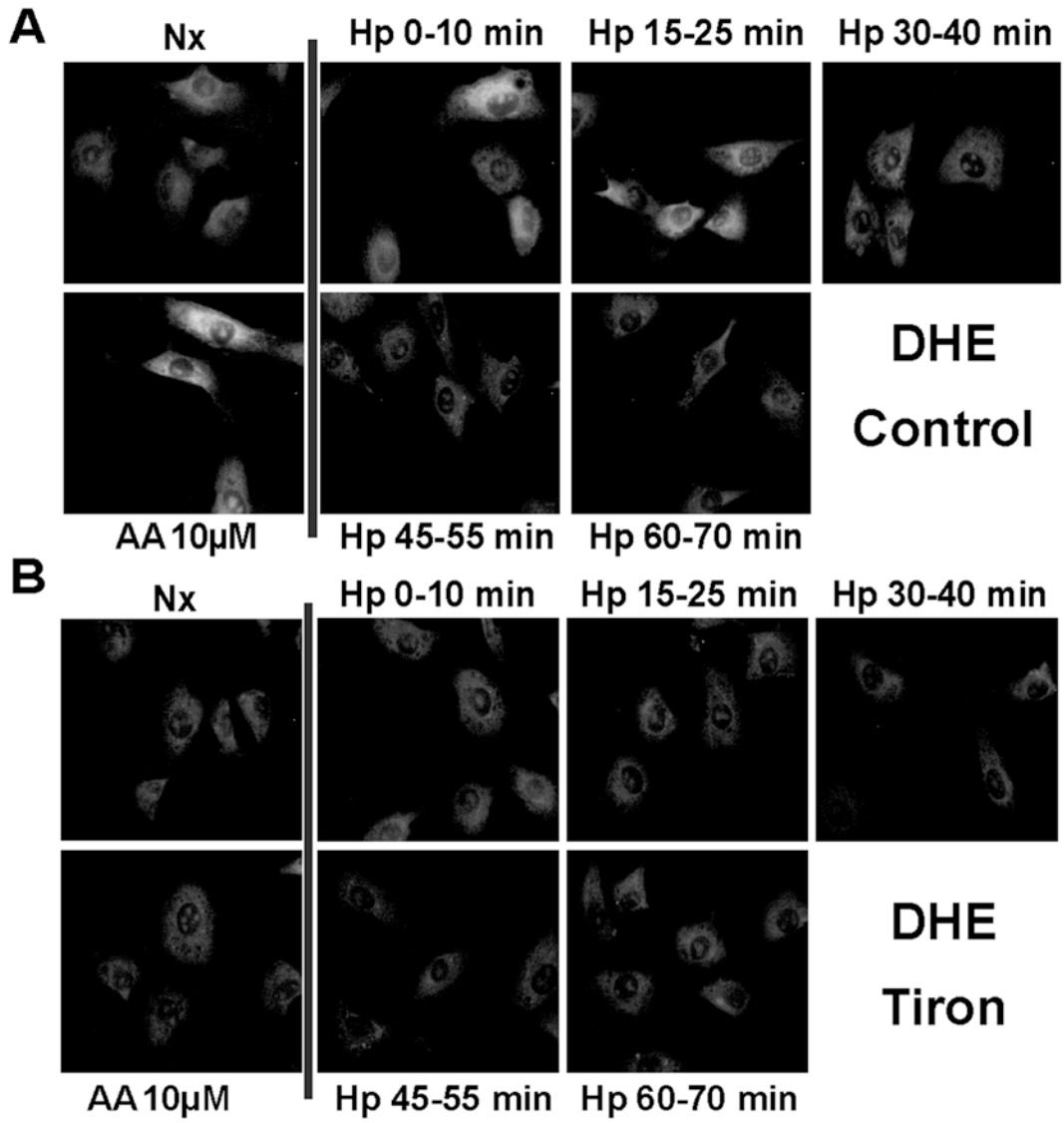

Fig. 2 Superoxide detection by DHE and fluorescence microscopy in fixed endothelial cells treated with 1 mM Tiron. Control bovine aortic endothelial cells (BAECs; **a**) and BAECs incubated with 1 mM Tiron (**b**) were treated as in Fig. 1. Tiron dismutates superoxide showing the specificity of the method. (**a**, **b**) Representative images showing DHE fluorescence. (**c**) Quantification of images from three independent experiments. Data are presented as mean ± s.e.m. $^{**}p < 0.01$ versus Nx. (Reprinted from [9] with permission from Elsevier)

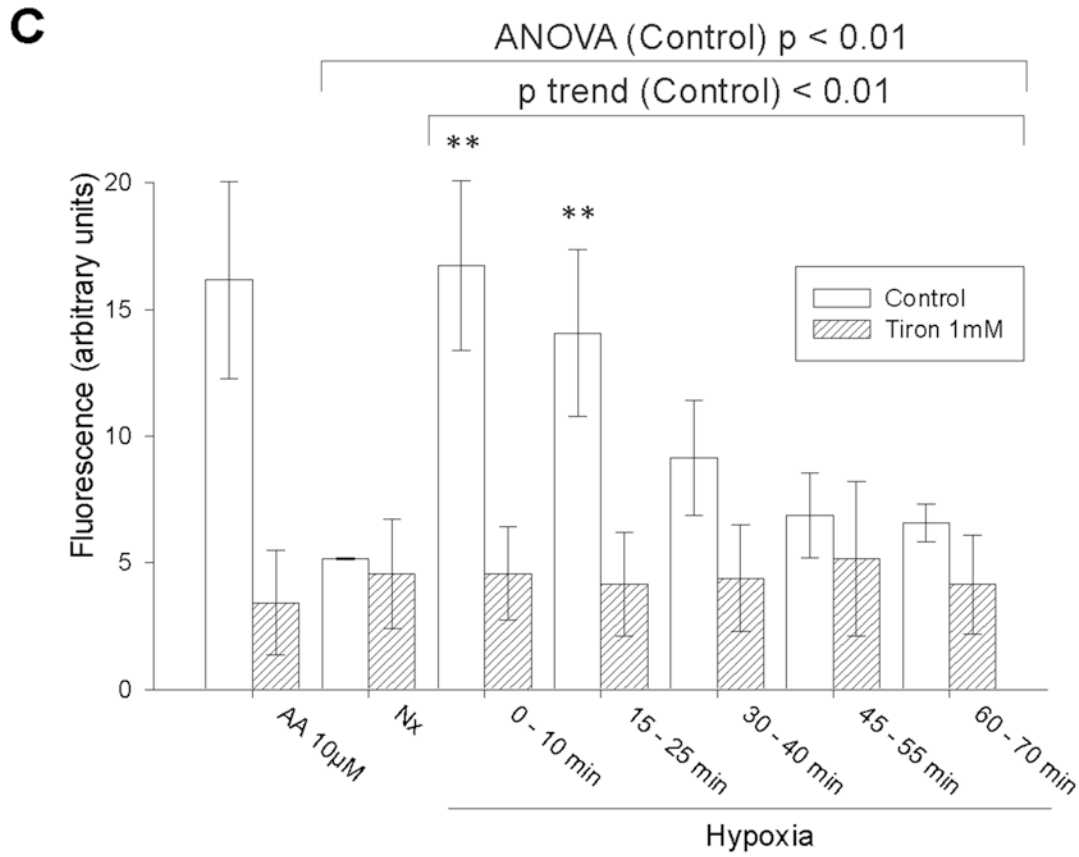

Fig. 2 (continued)

4 Notes

1. We have also used other probes to measure ROS production, such as dichlorofluorescein (DCF), adapting the concentration of the probe and the time of incubation [9].
2. Paraformaldehyde (PFA) solution should be suitable for microscopy.
3. Hypoxic chamber should be routinely calibrated every 2 months. O_2 sensors typically last no more than 1 or 2 years, depending on the use (at least for Ruskinn Invivo$_2$ 200 and Invivo$_2$ 400 chambers). Since expired O_2 sensors cannot detect the appropriate level of oxygen, uncalibrated hypoxic chambers normally insufflate gas in a very frequent fashion. This feature can be used as a clue to identify an uncalibrated hypoxic chamber containing an expired O_2 sensor.
4. Pre-equilibrate media and buffers from overnight to 2 h prior to the experiments in cell culture plates with the top on since

evaporation could occur if the plate remains open (the diameter of the plate depends on the volume to use in the experiment).

5. Prepare a 5 μM DHE working solution in HBSS + Ca/Mg from a 5 mM stock solution. Hypoxic DHE solution should be prepared from hypoxic pre-equilibrated HBSS + Ca/Mg solution.
6. Time 0 means that normal culture medium is changed for hypoxic pre-equilibrated medium and the rest of the protocol is continued immediately.
7. We usually incubate cells in hypoxia at 1% O_2, but different O_2 concentrations can be used. In our seminal study, we observed a higher superoxide burst changing cells from normoxia to 2% O_2, and we also saw that changing cells from 7% O_2 to 1% O_2 (using two hypoxia chambers) produced a similar burst than the change from normoxia to 1% O_2 [9].
8. It is advisable to remove excess of liquid and mount one coverslip at a time; otherwise, cells may dry out and fluorescence intensity may be disturbed due to changes in cell shape. Mounting media may contain DAPI or other markers which do not overlap with DHE.
9. Slides should be imaged before 1 week after the experiment.
10. We use a Leica DMR fluorescence microscope with a 63× objective. All images are collected with fixed exposure times and gain value. These values are set on the negative control (normoxia) which is in turn captured before the positive control (normoxia + antimycin A). In this way, minimum and maximum fluorescence values can be figured out prior to imaging the hypoxia treatments.

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