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Original Contribution

Acute hypoxia produces a superoxide burst in cells

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ABSTRACT

Oxygen is a key molecule for cell metabolism. Eukaryotic cells sense the reduction in oxygen availability (hypoxia) and trigger a series of cellular and systemic responses to adapt to hypoxia, including the optimization of oxygen consumption. Many of these responses are mediated by a genetic program induced by the hypoxia-inducible transcription factors (HIFs), regulated by a family of prolyl hydroxylases (PHD or EGLN) that use oxygen as a substrate producing HIF hydroxylation. In parallel to these oxygen sensors modulating gene expression within hours, acute modulation of protein function in response to hypoxia is known to occur within minutes. Free radicals acting as second messengers, and oxidative posttranslational modifications, have been implied in both groups of responses. Localization and speciation of the paradoxical increase in reactive oxygen species production in hypoxia remain debatable. We have observed that several cell types respond to acute hypoxia with a transient increase in superoxide production for about 10 min, probably originating in the mitochondria. This may explain in part the apparently divergent results found by various groups that have not taken into account the time frame of hypoxic ROS production. We propose that this acute and transient hypoxia-induced superoxide burst may be translated into oxidative signals contributing to hypoxic adaptation and preconditioning.

In most metazoans, oxygen has to be distributed through the organism to be used by cells within various organs. In several physiological and pathophysiological scenarios, cells undergo a decrease in the amount of available oxygen, known as hypoxia, which induces acute and long-term cellular, local, and systemic responses [1]. Most of the long-term responses are mediated by the induction of over 100 genes by the hypoxia-inducible factors (HIFs)[2], heterodimeric transcription factors composed of a constitutively expressed subunit (HIF-β) and an O_2-dependent HIF-α subunit. In normoxia the latter subunit is continuously degraded through a mechanism mediated by a family of HIF prolyl hydroxylases (EGLNs or PHDs), which carry out an O_2-dependent hydroxylation that is suppressed when oxygen concentration decreases, allowing HIF-α stabilization [2]. Some studies revealed that HIF-α stabilization was associated with an acute increase in reactive oxygen species (ROS) production in hypoxia [3,4].

It has been shown that acute responses to hypoxia involve local temporal changes in redox state due to the alterations in production of short-lived reactive oxygen species [5–8]. However, the nature of oxygen-sensing free radical generators, the targets of these radicals, and the resulting changes in enzyme activity and, finally, whether hypoxia causes a decrease or an increase in ROS production remain a matter of debate. Hypoxia-induced ROS production has been attributed mainly to the mitochondrial oxidative phosphorylation system (OXPHOS) and has been proposed to contribute to PHD inhibition and HIF-α stabilization [9–12] (reviewed in [13,14]).

Abbreviations: 2-OH-E, 2-hydroxyethidium; BAEC, bovine aortic endothelial cell; CDCFDA, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; DHE, dihydroethidium; HIF, hypoxia-inducible factor; Mito-HE , mito-hydroethidine; OXPHOS, mitochondrial oxidative phosphorylation system; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester

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This posed a paradox, as superoxide is directly produced from oxygen so its production rate should decrease with a decrease in oxygen availability [13,15]. Indeed, some of these findings and methodological approaches have been questioned [16,17] (reviewed in [18]). However, detailed investigations confirmed that increased ROS production occurred under conditions of mild hypoxia (1–3% O_2), but not during severe hypoxia or anoxia [13,19,20].

ROS production in hypoxia has often been measured with fluorescent probes that are oxidized by different species. The main limitation of some (if not most) of these studies is that the fluorescence measurements were performed outside the hypoxia chamber with reoxygenation occurring during the measurements. Several recent reports have confirmed hypoxic ROS production in cells exposed to hypoxia while recording the fluorescent signal from HSP-FRET or roGFP [5,9,21–23] (reviewed in [13,24]). Both thiol-based protein sensors are oxidized by H_2O_2, although oxidation by other intracellular oxidants cannot be ruled out. Superoxide anion is the primary ROS produced by the OXPHOS. However, there are few reports using a specific probe for direct measurement of this species in hypoxic cells [17,20,25] and we are not aware of any study showing the dynamics of superoxide production upon deoxygenation. Understanding the time course of superoxide production will help us approach the mechanisms underlying that response.

By using a novel proteomic method for detecting reversible thiol oxidation (redox fluorescence switch), we have recently shown a specific pattern of proteins in which cysteine thiol residues are reversibly oxidized when endothelial cells are subjected to acute hypoxia for 2 h [8]. This suggests a role for hypoxia-induced ROS production in cell signaling: the increased ROS levels can produce specific oxidative posttranslational modifications that may regulate the HIF pathway or promote other acute responses [8]. We herein present the data on superoxide production directly assessed in the cytosol and in the mitochondria of cells exposed to acute hypoxic challenge, combining different techniques to avoid potential artifacts.

Materials and methods

Cell culture

Bovine aortic endothelial cells (BAECs) were obtained from aortas donated by a local slaughterhouse and isolated as previously described [26]. BAECs were cultured at 37 °C in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. They were used between passages 3 and 9; endothelial morphology was assessed by visual inspection and by Western blot for endothelial nitric oxide synthase.

EA.hy926 cells (kindly provided by Dr. Cora-Jean S. Edgell, University of North Carolina, NC, USA) were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with HAT, 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin

HeLa cells were cultured at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

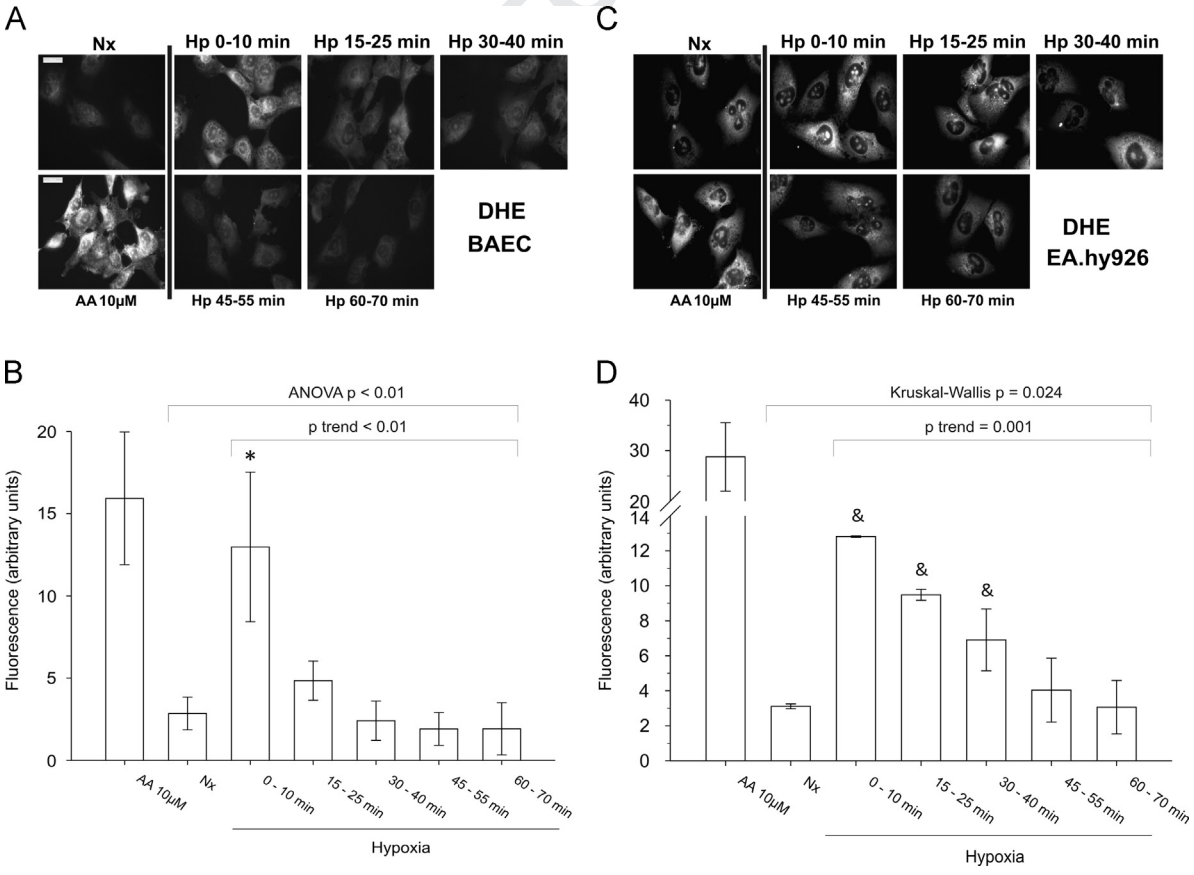

Fig. 1. Superoxide detection by DHE and fluorescence microscopy in fixed endothelial cells. (A, B) BAECs and (C, D) EA.hy926 cells were incubated for 60 min in normoxia (Nx) or in normoxia with antimycin A (AA, 10 μM for 30 min) or incubated in a hypoxia chamber at 1% O_2 with medium preequilibrated in the hypoxic condition (Hp) for 0, 15, 30, 45, or 60 min. 5 μM DHE was added for 10 min more, and the cells were fixed in the hypoxia chamber. (A, C) Representative images showing DHE fluorescence. (B, D) Quantification of images from four (B) or three (D) independent experiments. Data are presented as the mean ± SEM. $^*p < 0.05$, &$p = 0.0495$ versus Nx.

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