Redox Biology 12 (2017) 1040–1051

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Mitochondrial complex I deactivation is related to superoxide production in acute hypoxia

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ARTICLE INFO

Keywords:
Hypoxia
Oxygen sensing
Superoxide
Mitochondrial complex I
Redox signalling

ABSTRACT

Mitochondria use oxygen as the final acceptor of the respiratory chain, but its incomplete reduction can also produce reactive oxygen species (ROS), especially superoxide. Acute hypoxia produces a superoxide burst in different cell types, but the triggering mechanism is still unknown. Herein, we show that complex I is involved in this superoxide burst under acute hypoxia in endothelial cells. We have also studied the possible mechanisms by which complex I could be involved in this burst, discarding reverse electron transport in complex I and the implication of PTEN-induced putative kinase 1 (PINK1). We show that complex I transition from the active to 'deactive' form is enhanced by acute hypoxia in endothelial cells and brain tissue, and we suggest that it can trigger ROS production through its Na^+/H^+ antiporter activity. These results highlight the role of complex I as a key actor in redox signalling in acute hypoxia.

1. Introduction

Eukaryotic organisms use oxygen (O_2) as the final electron acceptor in the mitochondrial electron transport chain, producing water (H_2O) and driving the production of the high-energy molecule ATP through oxidative phosphorylation (OXPHOS). The OXPHOS system is located in the mitochondrial inner membrane and is composed of five complexes which couple the pumping of H^+ to the transfer of electrons from different substrates, such as NADH (oxidised by complex I) and succinate (oxidised by complex II). The difference in charges and pH generated across the mitochondrial inner membrane establish the mitochondrial membrane potential (ΔΨmt) and the pH gradient (ΔpH), respectively. Both parameters determine the protonmotive force (Δμmt) essential to drive OXPHOS. A series of reactive oxygen species (ROS) is also formed from the incomplete reduction of O_2 during respiration [1,2]. ROS can oxidise the majority of cellular components including nucleic acids, lipids and proteins, and are known to be associated with cell damage, particularly in conditions of oxidative stress [3]. Mitochondrial ROS are involved in many pathological scenarios [4] such as stroke [5], cancer [6], Parkinson's [7], Alzheimer's [8] or cardiovascular diseases, where its overproduction may contribute to disease progression. However, it is acknowledged that mitochondrial ROS also act as second messengers in cell signalling processes in a variety of physiological conditions [9–15].

Among the five complexes comprising OXPHOS, complex I is the largest and performs a reversible NADH-ubiquinone oxidoreductase reaction coupled to pumping four H^+ across the mitochondrial inner membrane. Complex I is formed by a hydrophilic arm which incorporates one flavin mononucleotide (FMN) and eight iron-sulfur clusters involved in electron transfer across this structure. The hydrophilic domain is attached to a hydrophobic arm involved in the H^+-pumping function of the complex. Energy transfer to the hydrophobic domain

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http://dx.doi.org/10.1016/j.redox.2017.04.025
Received 16 February 2017; Received in revised form 28 March 2017; Accepted 18 April 2017
Available online 21 April 2017
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occurs through the stabilization of the oxidised quinone in the ubiquinone-binding site which allows a series of conformational rearrangements necessary for H^+ pumping [16]. Complex I can also undergo a deactivation process named active/'deactive' transition (A/D transition) which implies a switch from a NADH-ubiquinone oxidoreductase activity to a Na^+/H^+ antiporter through its hydrophobic arm [17,18]. Importantly, deactivation includes a series of conformational changes in which the Cys39 of the complex I subunit ND3 becomes exposed. This exposure has been used as a marker of deactivation [19,20]. In addition, complex I can be modulated by proteins and lipids [21] whose deregulation can lead to pathophysiological scenarios. Among them, a genetic variant of Parkinson's disease involves the mutation of the PTEN-induced putative kinase (PINK1) gene which has been associated with lower complex I activity and increased ROS production [22,23].

Mitochondrial complex I is also a major site of superoxide anion production in the mitochondria [1,24] through both forward and reverse reactions (electron transfer from NADH to ubiquinone, or from reduced ubiquinone to NAD^+, respectively). The reverse reaction or reverse electron transfer (RET) needs a large pool of reduced ubiquinone which is normally generated from succinate oxidation through mitochondrial complex II, can be inhibited by rotenone and is dependent on high $\Delta\Psi mt$ [25,26]. RET has been implicated in exacerbated ROS production in reperfusion after ischemia [27,28].

Cells are frequently subjected to changes in oxygen availability and must adapt in order to survive. A decrease in oxygenation (hypoxia) induces a series of acute and long-term cellular, tissue-specific and systemic adaptive responses [29]. Both types of responses have been linked to the production of ROS. Whether ROS generation increased or decreased in hypoxia was strongly debated for years [30,31]. We have recently described that superoxide anion is produced in the first minutes of hypoxia by the mitochondria in different cell types, and correlates in endothelial cells with the oxidation of protein thiols [32,33].

More recently, it has been described that complex I is involved in the specialized acute response to hypoxia that takes place in the carotid body [34], where it triggers a ROS signal that activates ion channels provoking the release of neurotransmitters and hyperventilation [35]. Herein, we describe that complex I is involved in the ROS burst produced in acute hypoxia, in endothelial cells but also in brain tissue, and the mechanism by which complex I may be involved in triggering this response.

2. Materials and methods

2.1. Animals, cell culture and transfection

All animal experiments were performed following the Guide for the Care and Use of Laboratory Animals and were previously approved by the institutional ethics committee of the Universidad Autónoma de Madrid, Spain, according to the European guidelines for the use and care of animals for research in accordance with the European Union Directive of 22 September 2010 (2010/63/UE) and with the Spanish Royal Decree of 1 February 2013 (53/2013). All efforts were made to minimize the number of animals used and their suffering.

Cells were routinely maintained in cell culture incubators (95% air, 5% CO_2 in gas phase, 37 °C). Bovine aortic endothelial cells (BAECs) were isolated as previously described [36] and cultured in RPMI 1640 supplemented with 15% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. BAECs were used between passages 3–9. Endothelial morphology was assessed by visual inspection.

Transfection of 30 nM siRNA or 0.25 µg pHyPer-Cyto (CytoHyPer), C199S pHyPer-Mito (mitosypHer) or C199S pHyPer-Cyto (cytosypHer) vector DNA per 0.8 cm^2 well was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Experiments were carried out 48 h after transfection.

2.2. siRNA preparation

Doubled-stranded siRNAs against bovine NDUFS4, NDUFS2 and PINK1 were designed and purchased from Integrated DNA Technologies (NDUFS4 sense sequence GCUGCCGUUUCCGUUUCCAAGGUUUTT; NDUFS2 sense sequence TCGGACAGTCGACATTGGGATT; PINK1 sense sequence GGCUGCUAAUGUGCUUCAUUU). siSCR was purchased from Santa Cruz Biotechnology.

2.3. Detection of superoxide by fluorescence microscopy in fixed cells

BAECs were seeded on glass coverslips one day before experimentation. In some experiments, 1 µM rotenone or 1 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was added 30 min before experimentation and maintained during the experiment. For treatments in hypoxia, all the solutions were pre-equilibrated in hypoxic conditions before use; plated cells were introduced in an Invivo2 400 workstation (Ruskinn) set at 1% O_2, 5% CO_2, 37 °C, and incubated for the indicated times (0, 15, 30, 45 and 60 min) in fresh medium, washed three times with Hank's Balanced Salt Solution with Ca^{2+}/Mg^{2+} (HBSS + Ca/Mg) and incubated with 5 µM dihydroethidium (DHE) in HBSS + Ca/Mg for 10 min in the dark. Excess probe was removed by three washes with HBSS + Ca/Mg, the cells were fixed with 4% paraformaldehyde (PFA), and incubated in the dark at 4 °C for 15 min. After fixation, the cells were again washed three times with HBSS + Ca/Mg and coverslips were placed on slides. For normoxic treatments, the medium was changed for fresh normoxic medium, and treated as hypoxic cells, but in a standard cell incubator. Images (three images per each coverslip; the number of independent experiments is described in the figure legends) were taken with a Leica DMR fluorescence microscope with a 63x objective, using the 546-12/560 excitation/emission filter pairs and quantified using ImageJ software (NIH). The same threshold was set for all the images and the mean value from histograms was averaged for the three images of each coverslip.

2.4. Detection of intracellular ROS by live imaging fluorescence microscopy

BAECs were seeded in 6-well plates one day before experimentation. Plated cells were washed three times with HBSS + Ca/Mg and incubated with DHE for 20 min at 37 °C in the dark. 1 µM FCCP was also added at this time and maintained during the experiment. DHE was then washed out and new HBSS + Ca/Mg was added. After this time, the plate was placed into a Leica DM 16000B fluorescence microscope equipped with a Leica DFC360FXcamera, an automated stage for live imaging and a thermostated hypoxic cabinet. The planes were focused for image capture, and images were taken with a 20x objective every 2 min during 40 min, providing a total of 21 cycles. Normoxia experiments started and ended at 20% O_2 and 5% CO_2, whereas hypoxia experiments started at 20% O_2 and 5% CO_2 and then were switched to 2% O_2 and 5% CO_2 in cycle 2 (due to technical limitations of the hypoxia cabinet, it was not possible to set O_2 concentration below 2%). The excitation/emission filter pair used was 546-12/560. Images were quantified with Image J software. Three independent experiments were performed for each condition. For each experiment and condition, four regions of interest (ROIs) were created, each ROI surrounding an individual cell, and the mean fluorescence of each ROI for each time cycle was collected. In some analyses, for each experiment and condition, four identical linear ROIs were created and the maximum peak value of cycles 0, 5, 10, 15 and 20 were collected for each ROI.

2.5. Detection of intracellular ROS and intramitochondrial pH by live imaging confocal microscopy

To detect intramitochondrial pH, BAECs were transfected with the

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