Analytical Biochemistry 395 (2009) 134–143

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Flow cytometry-based assay for the activity of NAD(P)H oxidoreductases of the outer mitochondrial membrane

Alexey G. Kruglov *, Marina E. Solov'eva, Vera V. Teplova

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia

ARTICLE INFO

Article history:
Received 10 April 2009
Available online 11 August 2009

Keywords:
Method
Mitochondria
Outer membrane
NAD(P)H oxidoreductase
Lucigenin
Dimethylbiacridene
Intact cells
Permeabilized cells

ABSTRACT

NAD(P)H oxidoreductases of the outer mitochondrial membrane (OMM) are able to activate various xenobiotics and stimulate the production of reactive oxygen species and the opening of the mitochondrial permeability transition pore. However, the role of these systems in the cell damage by xenobiotics and chemotherapeutic drugs is poorly understood because the methods for the selective assessment of their activity have not been elaborated and specific inhibitors are unknown. Here we propose a method for the semiquantitative assessment of the activity of NAD(P)H oxidoreductases of the OMM in intact and permeabilized cells that is based on the flow cytometry detection of dimethylbiacridene, a fluorescent product of two-electron reduction of lucigenin. The method uses the structural feature of mitochondrial organization: the proximity of the sites of one-electron reduction of lucigenin to cation radical (NAD(P)H oxidoreductases of the OMM) to the sites of its subsequent oxidation (cytochrome *c* oxidase). The inhibition of cytochrome *c* oxidase by cyanide selectively activates the dimethylbiacridene formation by oxidoreductases of the OMM but not by other cellular oxidoreductases. The proposed protocol allows one to assess the lucigenin reductase (two-electron) activity of NAD(P)H oxidoreductases of the OMM and to compare it with the activity of other cellular systems that can be used for the analysis of the role of these systems in the cell damage by xenobiotics and antitumor drugs.

NADH- and NADPH-dependent oxidoreductases of the outer mitochondrial membrane (OMM)[1] reduce a number of natural and artificial autoxidizable substances, including quinones, chemotherapeutic drugs, and xenobiotics [1–7]. In isolated mitochondria, this can cause a rapid production of reactive oxygen species (ROS) and the opening of the mitochondrial permeability transition pore (mPTP), a key event in programmed cell death [1–6]. However, the role of NAD(P)H oxidoreductases of the OMM in the cell damage by chemotherapeutic drugs and xenobiotics is poorly understood. There are several reasons for this. First, there is no method for assessing the specific activity of these systems in the whole cell. Cellular organelles and compartments (mitochondria, microsomes, nuclear membrane, and cytosol) contain numerous NAD(P)H-dependent enzymes that are also capable of reducing and activating xenobiotics: NADH cytochrome *b*5 reductase, NADPH cytochrome P450 reductase, NAD(P)H:quinone acceptor oxidoreductases, nitric oxide synthase, carbonyl reductases, aldo–keto reductases, and respiratory chain complexes [2,4,8–14]. Because the nature of NAD(P)H oxidoreductases of the OMM has not yet been completely established [3,7,15], no methods for their quantification have been elaborated due to the absence of specific antibodies, short interfering RNA (siRNA), and selective inhibitors neutral toward other NAD(P)H-dependent enzymes. Second, the isolation of mitochondria from cell cultures and tissues usually presents a challenge owing to the shortage of material, cross-contamination of cellular fractions, and loss of integrity of mitochondrial membranes during isolation. The problem of the loss of integrity of mitochondrial membranes does not allow one to make a definite conclusion about the existence/nature of the tissue-specific rotenone-sensitive NADH oxidoreductase and its role in the cardiotoxicity of antitumor anthracyclines [7,15,16].

Recently, we showed that NAD(P)H oxidoreductases of the OMM reduce the redox cycling dication lucigenin (Luc^{2+}), a known chemiluminescent probe for superoxide, apparently via the one-electron (1ē) mechanism [5]. The resulting cation radical ($Luc^{+\bullet}$) readily transfers the electron to various acceptors: ferricytochrome

* Corresponding author. Fax: +7 4967 33 05 53.
E-mail address: krugalex@rambler.ru (A.G. Kruglov).

[1] *Abbreviations used:* OMM, outer mitochondrial membrane; ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore; siRNA, short interfering RNA; Luc^{2+}, lucigenin (*N*,*N*-dimethyl-9-9-biacridinium dinitrate); 1ē, one-electron; $Luc^{+\bullet}$, lucigenin cation radical; 2ē, two-electron; DBA, dimethylbiacridene; Cyt *c*, cytochrome *c*; COX, cytochrome *c* oxidase; IMM, inner mitochondrial membrane; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MTR, MitoTracker Red; RLM, rat liver mitochondria; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamide; FCCP, *p*-trifluoromethoxyphenylhydrazone; $\Delta\Psi_m$, inner membrane transmembrane potential; TPP^+, tetraphenylphosphonium; MCLA, 3,7-dihydro-2-methyl-6-(4-methoxyphenyl)imidazol[1,2-a]pyrazine-3-one; AU, arbitrary units; Ant A, antimycin A; EDTA, ethylenediaminetetraacetic acid; SEM, standard errors of the mean; MPP+, 1-methyl-4-phenyl-pyridinium.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.08.009

c (free or bound) [17], molecular oxygen [18], and (presumably) the thiols of mPTP [6], causing a fast ROS production and mPTP opening [5,6]. A repeated 1ē reduction of $Luc^{+\cdot}$ or the 2ē reduction of Luc^{2+} gives rise to stable and water-insoluble dimethylbiacridene (DBA) [19,20]. The spectral properties of DBA and Luc^{2+} are different, and these compounds can be distinguished both photometrically and fluorimetrically [20,21]. In complex biological systems, Luc^{2+} can be reduced by various reductases [22–25]. The exact mechanism of reduction of the majority of known reductases has not been studied. However, one may suggest that the 1ē reduction is a more widely distributed mechanism given that Luc^{2+} activates ROS production in many cellular, subcellular, and enzymatic systems [18,22–25]. Of interest is the fact that the 1ē reduction in several enzymatic systems can be switched to the 2ē reduction under anaerobic conditions [18]. This means that Luc^{2+} can undergo two consecutive steps of 1ē reduction: to $Luc^{+\cdot}$ and to DBA [18,26].

Here we propose a rapid and relatively selective method for assessing the activity of NAD(P)H oxidoreductases of the OMM in intact and permeabilized cells. The method is based on the selective activation of DBA formation by NAD(P)H oxidoreductases of the OMM under conditions of chemical anoxia. We show that the 1ē reduction of Luc^{2+} by NAD(P)H oxidoreductases of the OMM can be switched to DBA formation through the inhibition of oxidation of $Luc^{+\cdot}$ by cytochrome *c* (Cyt *c*) and cytochrome *c* oxidase (COX) in the inner mitochondrial membrane (IMM). The method does not require any specific antibodies or inhibitors of NAD(P)H oxidoreductases or isolation/purification of mitochondria.

Materials and methods

Materials

Cell lines (HEp-2, HL-60, COLO 320 HSR, and U251MG) were purchased from the Russian Cell Culture Collection (Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, Russia). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and phosphate-buffered saline (PBS) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). MitoTracker Red CMXRos (MTR) was purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA). The other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Isolation and purification of mitochondria

All manipulations with animals before the beginning of isolation of livers were performed in accordance with the Helsinki Declaration of 1975 (revised in 1983) and national requirements for the care and use of laboratory animals. Adult male Wistar rats were killed by cutting the neck after anesthesia with CO_2. Rat liver mitochondria (RLM) were isolated according to the standard differential centrifugation procedure [27]. The homogenization medium contained 220 mM mannitol, 70 mM sucrose, 10 mM Hepes (pH adjusted to 7.4 with Trizma base), 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 0.15% bovine serum albumin (BSA). Liver homogenate (Homo) was centrifuged at 600*g* for 8 min. Pellets containing cell debris were discarded, and the supernatant with the crude cytosolic fraction ($Super_1$) was centrifuged at 9000*g* for 10 min. The resulting crude microsomal fraction ($Super_2$) was kept for studies, and the crude RLM fraction (RLM_0) was washed one time (RLM_1), two times (RLM_2), or three times (RLM_3) with medium devoid of EGTA and BSA. At each stage, aliquots for the experiments and protein determination were taken. The final pellets were resuspended in the same medium to yield 70 to 90 mg protein/ml. Then RLM fractions were analyzed for the presence of glucose-6-phosphatase activity [28]. In a preliminary study, a comparison of preparations washed one, two, three, four, and six times revealed that after three washings the influence of contaminating microsomes on NAD(P)H oxidation and ROS production became negligible. (The medium for the two last washings was devoid of EGTA and BSA.) The traces of glucose-6-phosphatase activity were hardly detectable starting after four washings. In separate experiments, mitochondria were purified using Percoll gradient as described by Hovius and coworkers [29]. Briefly, a homogenized liver was centrifuged two times at 600*g* for 5 min. RLM were sedimented at 10,300*g* for 10 min. Pellets were layered over Percoll-containing (30%, v/v) mannitol medium and centrifuged at 95,000*g* for 30 min. The resulting RLM were washed two times with mannitol-based medium (8000*g* for 10 min). Final RLM possessed only traces (if any) of the glucose-6-phosphatase activity and were considered as pure (RLM_{pure}). Mitochondrial protein was assayed by the biuret method using BSA as a standard [30]. All measurements were performed at 30 °C in standard KCl-based medium (125 mM KCl, 20 mM mannitol, 10 mM Hepes, and 2 mM KH_2PO_4) supplemented with 5 mM glutamate and 5 mM malate unless otherwise indicated. Other experimental details are given in the figures and figure legends.

Determination of the content of mitochondrial protein in total protein of preparation

The content of the mitochondrial protein in 1 mg of total protein in RLM preparation was determined as a ratio of the maximal rates of cyanide-sensitive respiration in mitochondrial preparation and pure RLM (1 mg protein/ml) in the presence of 250 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamide (TMPD), 2 mM ascorbate, and 500 nM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). RLM purified with a Percoll gradient were considered as pure (RLM_{pure}, 1 mg mitochondrial protein/mg total protein). The mitochondrial respiration was detected by polarography using an oxygen Clark-type electrode and a computerized recording system Record 4 (Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Russia).

Recording of the permeabilization of mitochondrial membranes

The permeabilization of the IMM of RLM due to mPTP opening or treatment with the pore-forming peptide alamethicin causes the swelling of mitochondria, the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), and the release of Cyt *c*. Swelling was recorded as a decrease in absorbance at 540 nm (A_{540}) in 2-ml thermostated cuvettes using a Uvikon 923 spectrophotometer (Kontron Instruments, Milan, Italy). Changes in $\Delta\Psi_m$ were traced by the distribution of tetraphenylphosphonium (TPP^+) with the use of a thermostated electrode chamber and a TPP^+-selective electrode [31]. The electrode was connected to a computerized recording system Record 4. The release of Cyt *c* from RLM was measured as follows. RLM (2 mg/ml in a 6-ml chamber) were incubated in standard medium supplemented with respiratory substrates with continuous control of respiration and $\Delta\Psi_m$ and parallel control of swelling. Swelling and a fall in $\Delta\Psi_m$ were initiated by the addition of Ca^{2+}, alamethicin, or Luc^{2+} plus NAD(P)H. After a 10-min incubation with stirring, RLM were sedimented by high-speed centrifugation and the differential absorption spectra of the supernatant (reduced with dithionite/oxidized) were recorded in broad cuvettes (length = 3 cm). The extinction coefficient for Cyt *c* absorption (A_{550}–A_{540} nm) was taken to be 19.1 $mM^{-1}\,cm^{-1}$.

Measurement of the superoxide anion level

The superoxide anion level (an indirect measure of the steady-state concentration of $Luc^{+\cdot}$) was measured using the highly