Redox Biology 15 (2018) 109–114

Contents lists available at ScienceDirect

Redox Biology

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

Short communication

# Cytochrome $b_5$ reductase is the component from neuronal synaptic plasma membrane vesicles that generates superoxide anion upon stimulation by cytochrome *c*

Alejandro K. Samhan-Arias[a,*], Sofia Fortalezas[b], Cristina M. Cordas[a], Isabel Moura[a], José J.G. Moura[a], Carlos Gutierrez-Merino[b,*]

[a] UCIBIO, REQUIMTE, Departamento de Quimica, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[b] Department of Biochemistry and Molecular Biology, Faculty of Sciences, and Institute of Molecular Pathology Biomarkers, University of Extremadura, 06006 Badajoz, Spain

## ARTICLE INFO

*Abbreviations:*
$Cb_5R$, Cytochrome $b_5$ reductase
DTPA, Diethylenetriaminepentaacetic acid
DHE, Dihydroethidium
$E^+$, Ethidium
FAD, Flavin adenine dinucleotide
NADH, Reduced nicotinamide adenine dinucleotide
NBT, Nitroblue tetrazolium nitroblue tetrazolium
SPMV, Synaptic plasma membrane vesicles
TB, Terrific Broth terrific Broth
SOD, Superoxide dismutase
XA, Xanthine xanthine
XO, Xanthine oxidase

*Keywords:*
Cytochrome *c*
Superoxide anion
NADH oxidase
Cytochrome $b_5$ reductase
Neurons

## ABSTRACT

In this work, we measured the effect of cytochrome *c* on the NADH-dependent superoxide anion production by synaptic plasma membrane vesicles from rat brain. In these membranes, the cytochrome *c* stimulated NADH-dependent superoxide anion production was inhibited by antibodies against cytochrome $b_5$ reductase linking the production to this enzyme. Measurement of the superoxide anion radical generated by purified recombinant soluble and membrane cytochrome $b_5$ reductase corroborates the production of the radical by different enzyme isoforms. In the presence of cytochrome *c*, a burst of superoxide anion as well as the reduction of cytochrome *c* by cytochrome $b_5$ reductase was measured. Complex formation between both proteins suggests that cytochrome $b_5$ reductase is one of the major partners of cytochrome *c* upon its release from mitochondria to the cytosol during apoptosis. Superoxide anion production and cytochrome *c* reduction are the consequences of the stimulated NADH consumption by cytochrome $b_5$ reductase upon complex formation with cytochrome *c* and suggest a major role of this enzyme as an anti-apoptotic protein during cell death.

## 1. Introduction

The plasma membrane NADH oxidase activity of cerebellar granule neurons represents a disguisable activity producing superoxide anion ($O_2^{\cdot-}$) as a collateral product of NADH consumption [1–4]. The plasma membrane constituents associated to this activity are not well defined although it is known that cytochrome $b_5$ reductase ($Cb_5R$) is one of its major components present at the plasma membrane of rat cerebellar granule neurons in culture and of synaptic plasma membrane vesicles (SPMV) from rat brain [1]. This protein increases its association to lipids rafts in apoptosis [2]. In addition, 1–3 h after apoptosis induction an increment of $O_2^{\cdot-}$ has been detected at the peripheral neuronal plasma membrane [2]. This event correlates with the observed times for cytochrome *c* (Cyt *c*) release from mitochondria to the cytosol, as soon as 1 h after apoptosis induction, although the maximum peak for its release was found at 3 h [2].

In this work, we described the function of Cyt *c* as activator of the $O_2^{\cdot-}$ production by $Cb_5R$, as a component of SPMV, and results were experimentally confirmed with two isoforms of human $Cb_5R$. Due to the important role of Cyt *c* redox state in apoptosis and its reduction by $Cb_5R$, we propose a function of $Cb_5R$, as one the main defensive components during apoptosis after Cyt *c* release from mitochondria to the cytosol.

* Corresponding authors.
*E-mail addresses:* alejandro.samhan@fct.unl.pt (A.K. Samhan-Arias), carlosgm@unex.es (C. Gutierrez-Merino).

https://doi.org/10.1016/j.redox.2017.11.021
Received 19 October 2017; Received in revised form 23 November 2017; Accepted 24 November 2017
Available online 27 November 2017

## 2. Materials and methods

### 2.1. SPMV preparation

Rat brain SPMV were prepared using a standard procedure as described in [1,3].

### 2.2. Human $Cb_5R$ isoforms cloning

Cloning of $Cb_5R$ isoforms was performed as indicated in [5] using commercially available construct for soluble and primers described in Supplementary material.

### 2.3. Purification of recombinant human $Cb_5R$ isoforms

Clones of $Cb_5R$ isoforms were overexpressed in DE3 competent cells (Rosetta Gammi 2, Novagen) and the recombinant protein purified as indicated in [5].

### 2.4. NADH oxidase activity

NADH oxidase was measured at 37 °C as in [1,3,4,6,7].

### 2.5. $O_2$ consumption

$O_2$ consumption was measured using an Oxygraph Plus DW1 (Hansatech instruments) electrode in the same buffer described above, in presence of NADH (50 μM) and purified human recombinant $Cb_5R$ isoforms at 37 °C.

### 2.6. $O_2^{\cdot-}$ measurement with NBT

$O_2^{\cdot-}$ production by $Cb_5R$ was calculated measuring the reduction of NBT in the same buffer described above at pH 7.0, with NBT 200 μM and SOD 1 U/mL at 560 nm at 37 °C using a ε of 27.8 $mM^{-1}$ $cm^{-1}$ [8,9].

### 2.7. Cyclic voltammetry

Qualitative measurement of the $O_2^{\cdot-}$ generated by $Cb_5R$ was performed by cyclic voltammetry with a pyrolytic graphite electrode using the thin layer technique (membrane cut off 3.5 kDa) [5]. $Cb_5R$ (0.6 mM) or albumins (0.6 mM) as a control were loaded onto the electrode. The set up was completed with a silver/silver chloride (Ag/AgCl) reference electrode and a platinum counter electrode to complete the three electrodes cell configuration.

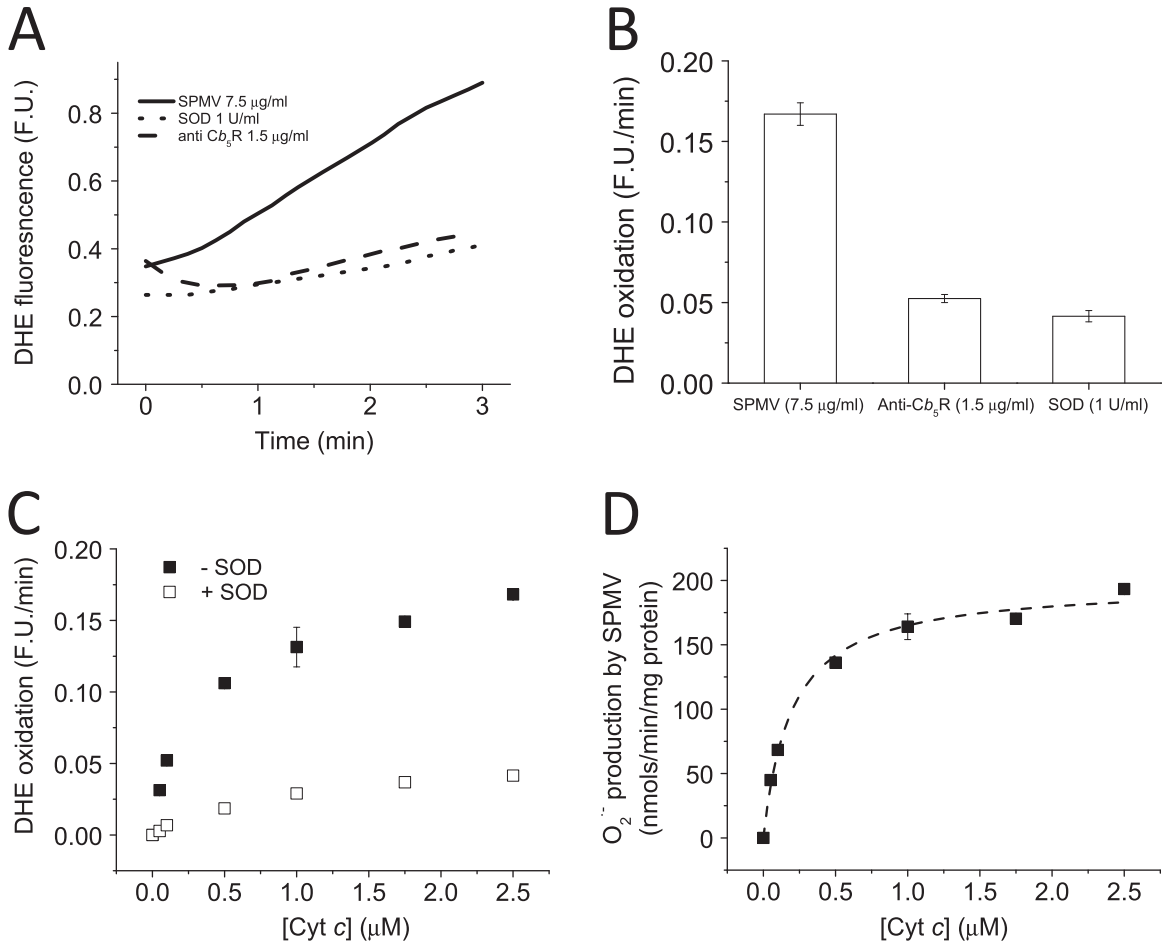


**Fig. 1. Cyt *c* stimulated NADH-dependent $O_2^{\cdot-}$ production by SPMV. Panel A** : Kinetics of the NADH dependent DHE oxidation by SPMV measured by fluorescence in the absence and presence of SOD (1 U/mL) and anti- $Cb_5R$ antibody (1.5 μg/mL). DHE oxidation was measured at 37 °C in potassium phosphate 20 mM plus DTPA 0.1 mM (pH 7.0), using a Perkin Elmer spectrofluorimeter with 470 nm and 605 nm excitation and emission wavelengths, respectively, and 10 nm excitation and emission slits. Representative traces of DHE oxidation by SPMV (7.5 μg/mL) in the presence of NADH (50 μM), oxidized Cyt *c* ($Fe^{3+}$) (2.5 μM) and DHE (2 μM),in the presence of 1.5 μg/mL anti-$Cb_5R$ (dashed line) or 1 U/mL SOD (dotted line) are shown. **Panel B**: Quantification of the inhibition induced by anti-$Cb_5R$ (1.5 μg/mL) and SOD (1 U/mL) on the DHE oxidation rate by SPMV (7.5 μg/mL) in the presence of NADH (50 μM) and oxidized Cyt *c* ($Fe^{3+}$) (2.5 μM). **Panel C**: Dependence of the NADH-dependent DHE oxidation rate by SPMV (7.5 μg/mL) upon Cyt *c* concentration in the absence (filled squares) or in the presence of SOD (1 U/mL) (open squares). **Panel D**: NADH dependent $O_2^{\cdot-}$ production by SPMV (7.5 μg/mL) dependence upon Cyt *c* concentration, measured with DHE. All the results shown in this Figure are the average ( ± standard errors) of experiments done by triplicate.