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:**
- Cb5R, Cytochrome \(b_5\) reductase
- DTPA, Diethylenetriaminepentaacetic acid
- DHE, Dihydroethidium
- E\(^+\), Ethidium
- FAD, Flavin adenine dinucleotide
- NADH, Reduced nicotinamide adenine dinucleotide
- NBT, Nitroblue tetrazolium
- SPMV, Synaptic plasma membrane vesicles
- TB, Terrific Broth
- SOD, Superoxide dismutase
- XA, Xanthine xanthine oxidase
- XO, Xanthine oxidase

**Keywords:**
- Cytochrome \(c\)
- Superoxide anion
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- 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 disguised activity producing superoxide anion (O\(_2^-\)) 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 (Cb5R) 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\(_2^-\) 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\(_2^-\) production by Cb5R, as a component of SPMV, and results were experimentally confirmed with two isoforms of human Cb5R. Due to the important role of Cyt \(c\) redox state in apoptosis and its reduction by Cb5R, we propose a function of Cb5R, as one the main defensive components during apoptosis after Cyt \(c\) release from mitochondria to the cytosol.

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

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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 Cb5R isoforms cloning

Cloning of Cb5R 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 Cb5R isoforms

Clones of Cb5R 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. O2 consumption

O2 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 Cb5R isoforms at 37 °C.

2.6. O2- measurement with NBT

O2- production by Cb5R 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 O2- generated by Cb5R was performed by cyclic voltammetry with a pyrolytic graphite electrode using the thin layer technique (membrane cut off 3.5 kDa) [5]. Cb5R (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 O2- 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-Cb5R 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) and oxidized Cyt c (Fe3+) (2.5 μM) and DHE (2 μM), in the presence of 1.5 μg/mL against Cb5R (dashed line) or 1 U/mL SOD (dotted line) are shown.

Panel B: Quantification of the inhibition induced by anti-Cb5R (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 (Fe3+) (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 O2- 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.
2.8. $O_2^-$ measurement with DHE

$O_2^-$ formation was measured by fluorescence using dihydroethidium (DHE) \[10\]. Measurements were performed at 37 °C in buffer (pH 7.0) potassium phosphate 20 mM, DTPA 0.1 mM, and DHE 2 μM and Cyt c at the concentration indicated in each experiment, using a quartz cuvette. Fluorescence of DHE was measured with 470 nm and 605 nm excitation and emission wavelengths, respectively, and slits of 10 nm. Xanthine/Xanthine oxidase (XA/XO) was used to calibrate the signal.

2.9. Cb5R:Cyt c complex formation

Complex formation was measured at 37 °C as indicated in \[5\].

3. Results

3.1. $O_2^-$ production by SPMV NADH oxidase activity is stimulated by Cyt c

We measured the effect of oxidized Cyt c (Fe$^{3+}$) on the NADH-dependent $O_2^-$ production by SPMV with DHE. Addition of Cyt c (2.5 μM) to the assay produced more than 3-fold increase in the oxidation of DHE, in the presence of SPMV (7.5 μg/mL) and NADH (50 μM) (Fig. 1A, continuous line and B). In addition, SOD added to the assay blocked the Cyt c stimulated DHE oxidation rate by SPMV (Fig. 1A, dotted line and B), pointing out that the increased DHE oxidation rate was due to production of $O_2^-$, as expected for a $O_2^-$ responsive dye \[11\]. The effect of a specific antibody against CbR (ProteinTech, Cat #4668234) in this assay was also tested (Fig. 1A, dashed line and B). The $O_2^-$ production by SPMV was almost completely inhibited, i.e. ≥ 90 % inhibition, in the presence of the specific antibody against CbR. We measured the DHE oxidation rate dependence upon Cyt c (Fe$^{3+}$) concentration, in the absence (filled squares) and presence of SOD (1 U/mL) (open squares) (Fig. 1C). Addition of increasing concentrations of Cyt c to the assay produced a Cyt c dependent increase of the DHE oxidation rate. Calibration curves for $O_2^-$ production vs. DHE oxidation were generated using increasing XO concentrations (Supplementary Fig. S1). Thereafter, we calculated that Cyt c was stimulating the NADH-dependent $O_2^-$ production by SPMV almost 20-fold, reaching a maximum value of 192 ± 41 nmoles/min/mg protein, in comparison to the activity measured in absence of Cyt c (10 nmoles/min/mg protein) (Fig. 1D). The NADH dependent $O_2^-$ production dependence upon Cyt c concentration yielded a $K_m$ for Cyt c stimulation of 0.2 ± 0.03 μM.

3.2. Measurement of the $O_2^-$ production by recombinant CbR isoforms

3.2.1. $O_2^-$ production by CbR

The oxidation of NADH by soluble and membrane purified CbR isoforms (Fig. 2 and Supp. Fig. S2A, respectively) was linearly dependent upon protein concentration (Fig. 2D). The calculated NADH oxidase activity of soluble and membrane CbR was 0.27 ± 0.02 and 0.15 ± 0.02 μmoles/min/mg of protein, respectively. Under the same experimental conditions the kinetics of $O_2$ consumption, in the presence of

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Fig. 2. Correlation between NADH oxidation, $O_2$ consumption and superoxide anion production by soluble CbR. Panel A: Representative traces of the NADH oxidation by soluble CbR (2.5 and 5 μg/mL) are shown. NADH oxidase activity was measured from absorbance decay at 340 nm at 37 °C, in the following assay medium (pH 7.0): potassium phosphate 20 mM, DTPA 0.1 mM, NADH 100 μM in presence of soluble CbR 2.5 (black line) and 5 μg/mL (grey line). Dotted lines indicate the slopes used to calculate the activity. Panel B: Oxygen consumption kinetics for soluble CbR 1 μg/mL (black line) 5 μg/mL (grey line). The reaction was started by addition of CbR at the time marked by a large drop of trace signals. $O_2$ consumption was measured in presence of soluble CbR using a Oxygraph Plus DW1 (Hansatech Instruments) electrode, filled with 2 mL of the assay medium indicated in the Panel A. Dotted lines indicate the slopes used to calculate the activity. Panel C: $O_2^-$ production by soluble CbR was measured with NBT. Representative traces of the kinetics of NBT reduction by soluble CbR isoform, and sensitivity to SOD is shown. NBT reduction was measured at 37 °C at 560 nm with soluble CbR 2.5 μg/mL in absence (black line) or presence of SOD 1 U/mL (dotted line) in the assay medium indicated in the Panel A, supplemented with NBT 200 μM. The dotted line indicates the slope used to calculate the activity. Panels D, E and F: Dependence upon CbR concentration for soluble (filled squares) and membrane (open squares) CbR, respectively, of NADH oxidation, oxygen consumption and superoxide production. All the results shown in this Figure are the average (± standard errors) of triplicate experiments.
of NADH, by the soluble and membrane isomorph of CbR (Fig. 2B and Supp. Fig. S2B, respectively) yielded an O₂ consumption rate of 0.54 ± 0.02 and 0.33 ± 0.04 μmoles/min /mg protein for soluble and membrane CbR respectively, calculated from the linear regression plot obtained with increasing enzyme concentrations (Fig. 2E). O₂ − was measured from the SOD-inhibited NBT reduction (Fig. 2C and Supp. Fig. S2C), yielding a rate of O₂ − production by soluble and membrane CbR of 0.49 ± 0.02 and 0.26 ± 0.02 μmoles/min /mg of protein, respectively, from the slope of the linear dependence with CbR concentration (Fig. 2F). Thus, these results yielded a stoichiometry of 2 molecules of O₂ − generated per molecule of oxidized NADH and a good coherence for the results obtained with these three methods.

Cyclic voltammetry can be used for experimental assessment of O₂ − production [12–16]. On these grounds, we have used this technique to further confirm O₂ − production by CbR. The measurement of this radical was first calibrated using K₂O₂ as a model compound. Two peaks appeared dependent on two generated components over the control: one at 1.25 V and another one at 0.8 V that were assigned to O₂ − and O₂, respectively (Fig. 3A). In presence of CbR (panel B), a signal similar to the observed for O₂ − (using K₂O₂) appears, at the same potential, and the O₂ signal decreased correlating with O₂ consumption by the enzyme to generate O₂ −. In presence of SOD, the O₂ − measured signal generated by CbR was equal to control (panel 3C).

3.2.2. Cyt c stimulated O₂ − production by CbR

The NADH-dependent DHE oxidation rate by purified CbR was almost completely inhibited by the presence of SOD in the assay medium (Fig. 4A). The Cyt c stimulated O₂ − production by CbR (1 mg/mL) was also reliably monitored with DHE (Fig. 4B) by the dependence upon Cyt c (Fe³⁺) of the initial DHE oxidation rate. As Cyt c reduction has also been used as an indicator to monitor O₂ − production [17,18], we have experimentally assessed whether the SOD inhibited reduction of Cyt c can reliably monitor the NADH-dependent O₂ − production by purified CbR. The kinetics of Cyt c reduction by CbR in absence (continuous line) and presence of SOD (dashed line) is shown in Fig. 4C. These results showed that SOD (1 U/mL) inhibits by 40–45 % the reduction of Cyt c upon incubation in the assay for 45 min, and about the same reduction of the initial rate of reduction up to 5–10 min. This result is in contrast with the almost complete inhibition by SOD (1 U/mL) of O₂ − production by CbR which can use Cyt c as a final electron acceptor, and (2) reduction of Cyt c by the O₂ − released by CbR.

Therefore, for a proper kinetic analysis of O₂ − production we measured the dependence of the DHE oxidation rate upon Cyt c and DHE concentration, using a fixed CbR concentration and a fixed concentration of one of the two substrates for O₂ − detection, as indicated in the Supp. material. The data were fit to a two substrate Michaelis-Menten kinetic model (Fig. 4 D and E). To calculate the O₂ − production, we calibrated the oxidation of DHE by XA/XO (Supp. Fig. 1B and C). From titration results with different Cyt c concentrations and fixed DHE (2 μM) and CbR (1 μg/mL), we calculated a kcat for O₂ − production by soluble and membrane CbR of 1.37 ± 0.02 and 0.49 ± 0.02 s⁻¹, with a Km for Cyt c of 0.29 ± 0.01 and 0.42 ± 0.02 μM, respectively (Fig. 4D). From titration with different DHE concentrations and fixed Cyt c (2.5 μM) and CbR (1 μg/mL)
Fig. 4. DHE oxidation by Chb5R is almost completely inhibited by SOD and Cyt c stimulated O$_2^-$ production by Chb5R. Panel A: Kinetics of DHE oxidation by soluble Chb5R (continuous line) vs. blank (dashed line) in absence (black line) and presence of 1 U/mL SOD (grey line). DHE oxidation was measured at 37 °C in the following assay medium (pH 7.0): potassium phosphate 20 mM, DTPA 0.1 mM, NADH 50 μM and 2 μM DHE, with a fixed reductase concentration (1 μg/mL or 27.6 nM). Excitation and emission wavelengths 470 nm and 520 nm, respectively, and 10 nm excitation and emission slits. Panel B: Kinetics of DHE oxidation by soluble Chb5R sensitive to SOD (1 U/mL), in the presence of increasing Cyt c concentrations. DHE oxidation was measured as indicated above, in the presence of the Cyt c concentrations listed in the figure. The traces shown are averages of experimental triplicates. Panel C: Representative traces for the kinetics of Cyt c reduction by soluble (black line) and membrane Chb5R (grey line) with Cyt c (3.75 μM), in the absence (continuous lines) and presence (dashed lines) of SOD 1 U/mL. Cyt c reduction was measured at 550 nm at 37 °C in the following assay medium (pH 7.0): potassium phosphate 20 mM, DTPA 0.1 mM, Cyt c 3.75 μM and NADH 100 μM, with soluble or membrane Chb5R (0.1 μg/mL). Panel D: The O$_2^-$ production rate by soluble (filled squares) and membrane (open squares) Chb5R isoforms dependence upon Cyt c is shown. The DHE oxidation rate was measured as indicated in the Panel A, with a fixed Chb5R (1 μg/mL or 27.6 nM) and DHE concentration (2 μM) in the assay medium. Panel E: The O$_2^-$ production by soluble (filled squares) and membrane (open squares) Chb5R isoforms depends upon Cyt c concentration. The DHE oxidation rate was measured as indicated in the Panel A, with a fixed Chb5R concentration (1 μg/mL or 27.6 nM) and Cyt c (2.5 μM). Panel F: Soluble (filled squares) and membrane Chb5R (open squares) flavin autofluorescence dependence upon Cyt c concentration. Cyt c elicits a large increase of Chb5R-flavin autofluorescence that allows measuring Chb5R-Cyt c complex formation by fluorescence. Y-axis: molar fraction of Chb5R saturated with Cyt c, which has been calculated as follows: SF/$S_{max}$ = ($F_{c+}/F_{c}$)/($F_{m}−F_{c}$), where F is the fluorescence intensity at each Cyt c concentration, and $F_{0}$ and $F_{m}$ are the fluorescence intensity in the absence and saturating concentrations of Cyt c, respectively. Fluorescence measurements were performed at 37 °C in the following buffer medium (pH 7.0): 20 mM potassium phosphate, 1 mM EDTA, and 2 μM Chb5R. Excitation and emission wavelengths 470 nm and 520 nm, respectively, with excitation and emission slits of 10 nm. The averages (± standard errors) of triplicate experiments are shown. Solid and dashed lines are the best non-linear squares fit to the one-binding site equation ($F_{c+}/F_{c}$)/($F_{m}−F_{c}$) = [Cyt c]/($K_d$+[Cyt c]), and yielded values of $R^2$ = 0.99 and 0.98, respectively, for soluble (black line) and membrane (grey line) Chb5R isoforms.

Table 1: O$_2^-$ production by human Chb5R.

|              | Soluble Chb5R (μmoles/min /mg protein) | Membrane Chb5R (μmoles/min /mg protein) |
|--------------|--------------------------------------|-----------------------------------------|
| NADH oxidase| 0.27 ± 0.02                          | 0.15 ± 0.02                              |
| O$_2^-$ consumption | 0.54 ± 0.02                           | 0.33 ± 0.04                              |
| O$_2^-$ production (SOD-inhibited NBT reductase) | 0.49 ± 0.02                           | 0.26 ± 0.02                              |
| Cyt c stimulated O$_2^-$ production (DHE) | 4.1 ± 0.2                        | 3.5 ± 0.3                                |
| Cyt c stimulated O$_2^-$ production (DHE) | 4.4 ± 0.1                        | 3.8 ± 0.4                                |

Values calculated by fitting the data obtained with DHE to one substrate Michaelis-Menten kinetics.

Values calculated by fitting the data obtained with DHE to two substrates Michaelis-Menten kinetics.

3.3. Measurement of Chb5R and Cyt c dissociation constant

The results shown above pointed out that Cyt c behaves as a redox partner of Chb5R, opening the possibility to use flavin autofluorescence of Chb5R to measure the interaction between these two proteins, see e.g. [5]. Fig. 4F shows Chb5R flavin autofluorescence intensity dependence upon Cyt c concentration, yielding a large increase of the fluorescence intensity, i.e. between 60 % and 300 % for soluble Chb5R and for membrane Chb5R at saturating concentration of Cyt c (5 μM). These results revealed that Cyt c interaction with Chb5R can be appropriately monitored by Chb5R flavin autofluorescence. The data can be fit to a hyperbolic curve as indicated in the Material and Methods section, yielding a dissociation constant of the Cyt c/Chb5R complex of 0.40 ± 0.05 and 0.38 ± 0.02 μM for soluble and membrane Chb5R, respectively. Scatchard plot analysis (Supp. Fig. S3) is consistent with the binding of one Cyt c molecule per Chb5R molecule for soluble and membrane isoforms.
4. Discussion

A scheme or the reactions described in this manuscript is shown in Supp. Fig. S4. Our data demonstrate that ChbR can use O$_2$ as an electron acceptor using NADH as substrate. Although the use of DHE for measurement of O$_2^-$ production by purified ChbR with DHE, using proper controls (i.e. calibrating the signal with XA/ XO in the presence of a large amount of catalase that avoid E$^{3+}$ formation when H$_2$O$_2$ is also produced) as shown in other reports [10,11,20]. Stoichiometric ratios between NADH and O$_2$ consumption indicated that ChbR uses one NADH molecule to reduce two O$_2$ molecules. Moreover, the values obtained for O$_2^-$ production correlated with O$_2$ consumption, indicating that the O$_2$ consumption is mainly due to O$_2^-$ production (Table 1). With the use of an anti-ChbR antibody, we confirmed that ChbR was responsible of the Cyt c (Fe$^{3+}$) stimulated production by SPMV, since 90% of the O$_2$ production was blocked by addition of specific antibodies against ChbR, added to the assay. As cytochrome P450s also display NAD(P)H-dependent production of O$_2^-$ [21] and some cytochrome P450 isoforms are associated the plasma membrane [22], it is likely that cytochrome P450s account for most of the ChbR-independent O$_2^-$ production [22,23], although we cannot discard other O$_2^-$ sources. Our results also show that Cyt c binds to purified ChbR isoforms with dissociation constants similar to the $K_m$ values for the Cyt c stimulated O$_2^-$ production by ChbR isoforms and close to the $K_m$ value obtained from the NADH-dependent production of O$_2^-$ by SPMV.

In the context of apoptosis, the function of Cyt c reduction by ChbR can be seen as part of the cellular defense system, because this protein can be seen as part of the cellular defense system, because this protein shows a widespread subcellular membrane localization, namely, endoplasmic reticulum, outer mitochondrial membrane and plasma membrane [24]. Cyt c reduction blocks apoptosis since its role in this type of cell death has been mainly attributed to the oxidized form [25-27]. For this reason, systems with ability to reduce Cyt c have an intrinsic anti-apoptotic function. Noteworthy, the payback for this reduction exerted by ChbR is the formation of O$_2^-$, a radical also described to be formed in mitochondria upon Cyt c release [28].

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.redox.2017.11.021](http://dx.doi.org/10.1016/j.redox.2017.11.021).

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