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Research paper

Functional expression of electron transport chain complexes in mouse rod outer segments

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ABSTRACT

Rod photoreceptors efficiently carry out phototransduction cascade, an energetically costly process. Our recent data in bovine rod outer segment (OS) demonstrated that ATP for phototransduction is produced by an extramitochondrial oxidative phosphorylation, thanks to the expression of the Electron Transport Chain (ETC) complexes and of F_1F_o ATP synthase in disks. Here we have focused on mouse retinas, reporting the activity of ETC complexes I, II, IV assayed directly on unfixed mouse eye sections, as well as immunogold TEM analysis of fixed mouse eye sections to verify the presence of ND4L subunit of ETC complex I and subunit IV of ETC complex IV in rod OS. Data suggest the presence of functional ETC in mouse rod OS, like their bovine counterpart. The protocol here developed for *in situ* assay of the ETC complexes activity represents a reliable method for the detection of ETC dysfunction in mice models of retinal pathologies. In fact, the ETC is a major source of reactive oxygen intermediates, and oxidative stress, especially when ectopically expressed in the OS. In turn, oxidative stress contributes to many retinal pathologies, such as diabetic retinopathy, age related macular degeneration, photoreceptor death after retinal detachment and some forms of retinitis pigmentosa.

1. Introduction

Vertebrate photoreceptors carry out light capture thanks to the presence of the visual pigment Rhodopsin (Rh) [1]. Rods, associated with achromatic vision, are composed of an inner part, the Inner Segment (IS), containing mitochondria, and an Outer Segment (OS). The OS is an elongated stack of membrane disks [2,1] surrounded by the plasma membrane, connected to IS by a cilium.

The proposed mechanism of energy supply for phototransduction of glycolytic ATP and phosphocreatine diffusion from the IS to the OS [3–5] has been challenged [6,7]. New findings point out to the existence of an extramitochondrial aerobic metabolism in bovine OS disks, which would better account for the ATP need of the light stimulated photoreceptor [8–10]. Our previous proteomic and functional studies showed the presence and activity in purified bovine rod OS disks, of the ETC complexes I to IV, F_1-F_o ATP synthase, and of the enzymes of the Tricarboxylic Acid Cycle [8–10]. Disks consume oxygen in the presence of various respiring substrates [9]. The main function of the ETC complexes I–V is to produce ATP through oxidative phosphorylation (OXPHOS), but it has been recognized as one of the major cellular generators of reactive oxygen intermediates (ROI) [11–13]. Respiratory chain defects can affect any organ, at any age [14] but usually, neurological manifestations are prevalent [15]. Oxidative stress is a risk factor for age related macular degeneration (AMD) [16,17]. Any impairment in the OXPHOS can increase ROI production and therefore oxidative stress [6].

In the present study we report for the first time the *in situ* activity of ETC complexes I, II, and IV in rod OS on unfixed mouse retinal sections by histochemical assay. The presence of ND4L subunit of ETC I and IV subunit of ETC IV in mouse OS was also confirmed by Transmission Electron Microscopy (TEM) analysis on mouse retinal sections. Besides confirming that the four ETC are expressed and catalytically active in mouse rod OS, like bovine ones, data may represent a reliable method to detect ETC dysfunction in oxidative stress related retinal pathologies in mouse models of disease.

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2. Materials and methods

2.1. Materials

Tissue-Tek OCT compound was purchased from Bio-Optica. All other chemicals and enzymes were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Eyes were excised from adult male Swiss mice (weighing 25 g; Charles River, Calco, Italy) housed at constant temperature (22 °C) and relative humidity (50%) under a regular light–dark schedule. Water and standard mouse fodder were freely available. For immunogold assays, eyes were filled with fixative (4% paraformaldehyde and 0.1% glutaraldehyde in PBS buffer solution) for 1.5 h and then washed overnight with 50 mM NH_4Cl, dehydrated and embedded in LR White Resin followed by polymerization at 58 °C. Ultrathin sections (80 nm) were placed on Formvar-coated nickel grids and used the next day for immunogold labelling. For histochemical reactions unfixed eyes were directly cryoprotected o.n. in 30% sucrose in PBS and in Tissue-Tek OCT (Electron Microscopy Sciences, Fort Washington, PA) and then cut transversally by cryostat Frigocut 2800E (Reichert-Jung, Germany) at 12 μm thickness. Animal manipulations were conducted in conformity with institutional guidelines, in accordance with the European legislation (European Communities Directive of 24 November 1986,86/609/EEC) and with the NIH Guide for the Care and Use of Laboratory Animals.

2.3. TEM immunogold microscopy of mouse retinas

For immunostaining of sections, the postembedding immunogold method was applied. Sections were first treated with blocking solution (10% goat serum, 0.1% Tween 20, PBS 1×), then incubated with mouse monoclonal anti-rhodopsin (diluted 1:200) (Sigma Aldrich, St. Louis, MO, USA) or rabbit polyclonal anti ND4L subunit of ETC complex I (diluted 1:25) (Abcam Cambridge, UK) or rabbit polyclonal anti subunit IV of ETC complex IV (diluted 1:25) (Abcam Cambridge, UK) overnight at 4 °C. Ab binding was detected using a secondary Ab (goat anti-rabbit IgG (Sigma Aldrich, St. Louis, MO, USA) (diluted 1:100), or goat anti-mouse IgG (British BioCell International) (diluted 1:100)) coupled to gold particles (10 nm diameter for anti-rabbit, and 5 nm diameter for anti-mouse). Sections were analyzed at an FEI Tecnai G^2 transmission electron microscope operating at 100 kV. In negative controls, instead of the specific primary Ab, the preimmune serum was applied to the sections. The images were acquired with TIA Fei software Cam, collected and typeset in Corel Draw X3. Controls were performed by omitting primary Ab, which resulted in absence of cross-reactivity (data not shown).

2.4. Histochemical reactions for ETC activity

Transversal sections were incubated in: 0.8 mM NADH, 1.3 mM nitro blue tetrazolium (NBT) in PBS for Complex I assay; 200 mM of succinic acid, 0.2 mM phenazine methosulfate (PMS), 1.5 mM NBT, in PBS for Complex II assay; 50 mM phosphate (pH 7.4), 0.75 mg/ml DAB (3,3′diaminobenzidine), 0.75 mg/ml cytochrome *c* for Complex IV assay. Histochemical reactions were performed at 37 °C in the dark for 1–2 h and checked every 30 min. Incubation was stopped in 0.1 M phosphate buffer once clear differentiation between highly reactive and nonreactive portions could be discerned. Control sections were incubated with PBS only in absence of substrate.

3. Results

3.1. Immunogold on mouse retinal sections

Our previous results on bovine rod OS prompted us to verify whether proteins of the mitochondrial OXPHOS are true components of the OS also in mouse retinas. To this end, immunogold TEM imaging was performed on mouse retinal sections. Panel A reports labelling with an Ab against Rhodopsin (Rh), (5 nm diameter gold particles). Rh signal is intense in OS disk while it is absent on resin, as reported in Inset (representing the enlarged vision of squared area in Panel A). Panels B–F show retinal sections labelled with an Ab against the ND4L subunit of ETC complex I (10 nm diameter gold particles). ND4L signal is present in rod OS (see Panel D and its enlarged OS detail in panel E and F). Labelling appears specific because it is absent in nuclei and cytoplasm (panel B) but present in the IS mitochondria (Panel C). In Panels G–K retinal sections labelled with Ab anti subunit IV of ETC complex IV (COX IV) (10 nm diameter gold particles) are reported. COX IV signal is present in the OS, as shown in Panel I and its magnification (Panels J and K), in the IS mitochondria (Panel H), but not in nuclei and cytoplasm (Panel G).

3.2. Activity of ETC I, II and IV on mouse retinal sections

Activity of the Electron Transport Chain (ETC) complexes I, II, IV was detected in unfixed transversal sections by histochemical reactions. Sections were transversally cut following the section plane showed in Fig. 2, Panel F, including the area containing only sclera, pigment epithelium (RPE) and rod outer segment (OS) layers, as confirmed by haematoxylin and eosin staining of these sections (Fig. 2 Panel D). Activity of ETC I, II and IV could be clearly detected in both pigmented epithelium mitochondria and rod OS layers (Fig. 2, Panels A, B, C) with respect to negative controls (i.e: transversal retinal section incubated in PBS (Fig. 1 Panel E)). Violet and brown colour in Fig. 2 panel A and B represent the product of the reduction reaction of NBT to NBT-formazan with the parallel oxidation of NADH and succinate by ETC complex I and ETC complex II, respectively. Cytochrome *c* oxidase activity oxidises DAB, producing a brown precipitate, shown in Fig. 2 Panel C.

4. Discussion

New findings on rod OS metabolism have been recently reported [8–10]. The present and previous data suggest that the mammalian OS, theta is devoid of mitochondria, is a site of extramitochondrial aerobic metabolism. Histochemical assays of ETC complexes I, II and IV on transversal not-fixed mouse retinal sections (Fig. 2 Panels A, B and C) show that ETC are active in both OS and RPE (retinal pigmented epithelium) layers, in comparison to control sections incubated in the absence of substrates (Fig. 2 Panel E). Activities were observed *in situ* on retinal sections, ruling out the possibility of a contamination. Previously activity of ETC IV was reported in mitochondria of RPE, photoreceptor IS, outer plexiform layer, and inner plexiform layer, but not in rod OS on rat [18] and human [19] longitudinal retinal sections. The ETC I and IV were found in different regions of mouse retina but not in rod OS by using histochemical reactions on fixed sections [20–22].

Immunogold shows the presence of Rh with the ETC proteins ND4L (a subunit of ETC I) and COX subunit IV in the rod OS on mouse retinal sections (Fig. 1). The ETC I, II and IV appear true components of the mouse OS, as its bovine counterpart.

The present histochemical protocol was developed for the visualization of the ETC complex activity *in situ* in the rod OS. It differs from previous ones as far as the treatment of samples

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