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Research paper

Impairment of extramitochondrial oxidative phosphorylation in mouse rod outer segments by blue light irradiation

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ABSTRACT

Exposure to short wavelength light causes increased reactive oxygen intermediates production in the outer retina, particularly in the rod Outer Segments (OS). Consistently, the OS were shown to conduct aerobic ATP production through the ectopic expression of the electron transfer chain complexes I–IV and F_1F_o-ATP synthase. These facts prompted us to verify if the oxidative phosphorylation in the OS is implied in the oxidative damage of the blue-light (BL) treated OS, in an organotypic model of mouse retina.

Whole mouse eyeball cultures were treated with short wavelength BL (peak at 405 nm, output power 1 mW/cm^2) for 6 h. Immunogold transmission electron microscopy confirmed the expression of Complex I and F_1F_o-ATP synthase in the OS. *In situ* histochemical assays on unfixed sections showed impairment of respiratory Complexes I and II after BL exposure, both in the OS and IS, utilized as a control. Basal O_2 consumption and ATP synthesis were impaired in the OS purified from blue-light irradiated eyeball cultures. Electron transfer capacity between Complex I and II as well as activity of Complexes I and II was decreased in blue-light irradiated purified OS.

The severe malfunctioning of the OS aerobic respiratory capacity after 6 h BL treatment may be the consequence of a self-induced damage. BL exposure would cause an initial over-functioning of both the phototransduction and respiratory chain, with reactive oxygen species production. In a self-renewal vicious cycle, membrane and protein oxidative damage, proton leakage and uncoupling, would impair redox chains, perpetuating the damage and causing hypo-metabolism with eventual apoptosis of the rod. Data may shed new light on the rod-driven retinopathies such as Age Related Macular Degeneration, of which blue-light irradiated retina represents a model.

1. Introduction

Due to its high energy demand, the retina is one of the most oxygen-consuming tissues in the body [1]. Visible light, acting as photosensitizer, supports a highly oxidative retinal milieu. In fact, a well-established cause of retinal damage is exposure to bright light, such as direct sunlight, or continuous ambient light [2]. This promotes the generation of Reactive Oxygen Intermediates (ROI) and oxidative stress [3]. Consistently, some of us have recently demonstrated that exposure of organotypic mouse retinal cultures to short wavelength blue light (BL) causes oxidative damage not only in the inner segment (IS) where mitochondria are present, but especially in the OS, a compartment devoid of mitochondria [4]. Moreover the enzyme SOD-1 as well as molecules indicators of protein oxidation (N(6)-Carboxymethyllysine, advanced glycation endproduct) and lipid oxidation (malondialdehyde and 4-hydroxy-nonenal) were also increased in the OS [4]. Oxidative stress

Abbreviations: AMD, age-related macular degeneration; ATP synthase, F_1F_o-ATP synthase; BL, blue light; ETC, electron transport chain; COX, Cytochrome c oxydase; OS, rod outer segment; OXPHOS, oxidative phosphorylation; PUFA, polyunsaturated fatty acids; ROI, reactive oxygen intermediates; ROP, retinopathy of prematurity; SD, standard deviation.

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eventually leads to photoreceptor cell death [5]. The OS, where phototransduction takes place, contains high levels of poly-unsaturated fatty acids, a typical ROI target [6]. For example, DHA, a very oxidizable fatty acid, is enriched in the rod outer limb membrane [7]. Nonetheless, the source of ROI under BL stress in the OS, traditionally considered exclusively glycolyzing, seems difficult to envisage. Indeed, the oxidative stress induced by BL-exposure inside the OS [4] [5], can be better explained assuming that the ROI production is inextricably linked to the impairment of the OXPHOS functioning in the rod OS. In fact, the functional presence of the Electron Transport Chain (ETC) complexes I–IV and F_1F_o-ATP synthase (ATP synthase) was demonstrated in the rod OS [8], unveiling an unsuspected site of oxidative stress generation in the outer retina [9]. Any impairment in the OXPHOS can increase ROI production and therefore oxidative stress [10]. In particular, Complex I is a main ROI producer [11] [17].

Aiming at verifying whether the origin of the oxidative stress, consequent to BL exposure, is the redox chain in the OS, we studied the expression and activity of the ETC complexes I − III and ATP synthase in mouse organotypic retinal eyeball cultures exposed to BL (peak at 405 nm) for 6 h. By immunogold Transmission Electron Microscopy (TEM) we demonstrate that the ETC Complex I and ATP synthase are expressed in the rod OS of both exposed cultures and controls. Biochemical analyses conducted on OS purified from BL-irradiated cultures, showed impairment of ATP synthesis, ETC complexes activity and O_2 consumption. Data were confirmed by *in situ* histochemical assays of rod OS Complex I and II on unfixed mouse retinal sections.

2. Materials and methods

2.1. Ethics statement

The ethics committee of the TU Dresden approved all animal experiments and the license for removal of organs was provided by the Landesdirektion Dresden (Az.: 24D-9168.24-1/2007-27).

2.2. Organotypic cultures

The organotypic model of photoreceptors is well established and has already been characterized in detail [4]. On postnatal day 24 ± 4 days (shortly after weaning), C57BL/6 mice of either sex were sacrificed by cervical dislocation. Their eyes were immediately enucleated and transferred into phosphate buffered saline (PBS). The eyeballs were punctured with a needle (BD Microlance 3, 27G60.5 Inch) at the ora serrata to create a small hole which enabled fluid exchange and were transferred into an optimized cell culture medium. The eyeballs were cultivated in almost their original form in medium (DMEM/F12 GIBCO (cell culture medium)+10% fetal calf serum (FCS)+2% B-27 supplement+1% penicillin-streptomycin+2 mM glutamine) in a 6-well culture plate at 37 °C with a CO_2 level of about 5% in a cell culture incubator for different lengths of time.

2.3. Irradiation with blue light

Illumination of the cultivated eyeballs was produced by a LED-based system (# LZ1- 00UA05 BIN U8; LedEngin, Santa Clara, USA) that was constructed in our lab [4]. It generated short wavelength BL (peak at 405 nm) with an output power of 1 mW/cm^2. The eyeballs were positioned in cut cell culture inserts (transparent; BD, Heidelberg, Germany) so that their corneas faced the BL diodes (1 per well) for 6 h. Non-irradiated eyes were used as controls.

2.4. Purified rod OS preparation

Rod outer segments were prepared according to previous protocols [12,13]. To better protect the tissue, protease inhibitor (Complete; Roche Diagnostics) was added to the solution. Six retinas were pooled per sample in 120 μl of 8% iodixanol (prepared from a 60% w/v OptiPrep solution) in Ringer's solution (130 mM NaCl; 3.6 mM KCl; 1.2 mM $MgCl_2$; 10 mM HEPES; 0.02 mM EDTA; in distilled H_2O, at pH 7.4). Samples were vortexed at maximum speed for 2 min and centrifuged at 200 g for 1 min. The upper 100 μl of the supernatant containing the outer segments was transferred to a new tube kept in ice. A total of 100 μl of 8% iodixanol solution was added to the original sample. The vortexing and sedimentation sequence was repeated four times, for a collection of 500 μl of supernatant. The collected supernatant was loaded on top of a discontinuous OptiPrep step gradient in Ringer's solution (0.7 ml, 10% to 0.7 ml, 18%) in a 2 ml tube. The tube was centrifuged at 3500 g for 30 min at 4 °C. The band between 10% and 18% OptiPrep, corresponding to outer segments was collected and diluted three times in Ringer's solution. The outer segments were pelleted by centrifugation at 30000*g* for 30 min at 4 °C. Then they were washed, resuspended in 80 μl Ringer solution and immediately frozen at −80 °C.

2.5. Immunogold transmission electron microscopy (TEM)

After BL exposure for 6 h at 37 °C in a cell culture incubator, each eyeball was cut in half through the equator to permit removal of the anterior segment and vitreous body. Next, the eyecup (retina still attached to retinal pigment epithelium) was fixed with 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. Ulthrathin section (80 nm) were used for postembedding immunogold method. Sections were treated with blocking solution, incubated with rabbit polyclonal anti ND4L subunit of ETC Complex I (diluted 1:25) (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) or rabbit anti-ATP synthase β-subunit (diluted 1:50) (Sigma Aldrich, St. Louis, MO, USA) antibody (Ab) overnight at 4 °C. The primary Ab was recognized by 10 nm gold particle-coupled goat anti-rabbit secondary Ab (BDI, Dublin, Ireland; diluted 1:30). Sections were analyzed using an EM 906 electron microscope (Carl Zeiss, Oberkochen, Germany). Controls were treated with the same protocol, except for light exposure. Controls with secondary Abs only showed absence of cross-reactivity (data not shown).

2.6. Histochemical reactions for ETC I & II activity

Cultivated, unfixed eyes were cryo-protected in 30% sucrose before embedding in OCT compound. Transversal sections were cut at 14-μm thickness on a cryostat and mounted onto Superfrost glass slides (Menzel, Braunschweig, Germany). To analyze NADH-Coenzyme Q oxidoreductase (Complex I) activity sections were incubated at 37 °C for 60–90 min with the following incubation medium: 2 mM NADH, 0.6 mM nitroblue tetrazolium chloride (NBT) in 0.1 M phosphate buffer, pH 7.4. For Succinic dehydrogenase (Complex II) histochemical assay, sections were incubated at 37 °C for 40–60 min with the following incubation medium: 50 mM succinic acid, 1.5 mM NBT; 5 mM EDTA, 1 mM sodium azide, 1 mM 1-Methoxy-5-methylphenazinium methyl sulfate (mPMS) in 0.1 M phosphate buffer, pH 7.6. Control sections were incubated with PBS only in absence of substrate. Sections were monitored by an Olympus IX81 inverted microscope (Olympus, Jena, Germany) equipped with vital microscopy chamber (37 °C). Reaction was checked every 10 min using automated time lapse imaging until clear differentiation between highly reactive and nonreactive parts