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Original article

Modulation of the rod outer segment aerobic metabolism diminishes the production of radicals due to light absorption

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ABSTRACT

Oxidative stress is a primary risk factor for both inflammatory and degenerative retinopathies. Our previous data on blue light-irradiated retinas demonstrated an oxidative stress higher in the rod outer segment (OS) than in the inner limb, leading to impairment of the rod OS extra-mitochondrial aerobic metabolism.

Here the oxidative metabolism and Reactive Oxygen Intermediates (ROI) production was evaluated in purified bovine rod OS in function of exposure to different illumination conditions. A dose response was observed to varying light intensities and duration in terms of both ROI production and ATP synthesis. Pretreatment with resveratrol, inhibitor of F_1F_o-ATP synthase, or metformin, inhibitor of the respiratory complex I, significantly diminished the ROI production. Metformin also diminished the rod OS Complex I activity and reduced the maximal OS response to light in ATP production.

Data show for the first time the relationship existing in the rod OS between its -aerobic- metabolism, light absorption, and ROI production. A beneficial effect was exerted by metformin and resveratrol, in modulating the ROI production in the illuminated rod OS, suggestive of their beneficial action also in vivo. Data shed new light on preventative interventions for cone loss secondary to rod damage due to oxidative stress.

1. Introduction

The Electron Transfer Chain (ETC) is a major producer of Reactive Oxygen Intermediates (ROI) [1,2], in turn responsible for the oxidative stress production, a set of alterations occurring in tissues, cells and macromolecules upon alteration of the physiological equilibrium between production and elimination of ROI. These, typically, trigger a vicious circle, since oxidative stress aggravates ROI production, causing hypo-metabolism, in turn lowering the ability of the cell to scavenge the damage. Markers of oxidative damage include malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) [3]. The body's antioxidant defense capacity relies on different Vitamins and antioxidants of both exogenous and endogenous origin among which polyphenols [4,5].

The retina is the central nervous system (CNS) tissue displaying the highest oxygen (O_2) consumption per gram of tissue [6], especially the outer retina adapted to darkness, particularly sensitive to damage by ROI. In fact, the rod OS membranes display an elevated content of polyunsaturated fatty acids (PUFA) [2,7]. In particular, photoreceptors consume about 4 times more O_2 than the other neurons of the CNS, thereby comprising the retina [8]. Consistently, rod OS may be responsible for the high O_2 consumption of the outer retina [6], as suggested by experimental evidence from biochemical proteomic and imaging analyses [9–12]. The ectopic expression of active of F_1F_o-ATP synthase (ATP synthase), as well as electron transfer chain (ETC) in the rod disks points to the occurrence of an aerobic metabolism, devoted to providing ATP for photo-transduction. Notably, irradiation of organotypic eye cultures (explants) with short wavelength blue light (BL) elicited a larger ROI production in the rod OS, than in the IS [7].

Abbreviations: AMD, age-related macular degeneration; Ap5A, di(adenosine) − 5-penta-phosphate; ATP synthase, FoF1-ATP synthase; DR, diabetic retinopathy; ETC, electron transport chain; MTF, Metformin: OS, outer segment; PUFA, polyunsaturated fatty acids; ROI, reactive oxygen intermediates; RP, retinitis pigmentosa; RV, resveratrol; TEM, transmission electron microscopy

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Exposure to BL for 6 h impaired the ETC and uncoupled ATP synthase, likely due to OXPHOS overwork caused by prolonged irradiation, with membrane damage. In fact, a dysfunctional oxidative phosphorylation increases the level of ROI [1].

ROI are believed to play a role in the aging process, both in acute and chronic conditions [13–15,16,4–6]. Furthermore, oxidative stress plays a central role in the retinal degenerations [17,18] Retinal degenerative diseases include Age-related macular degeneration (AMD) [17] and diabetic retinopathy (DR) [19]. DR is a progressive complication during the course of type 1 or 2 diabetes and the leading cause of blindness in developed countries [6,20]. About 60% of individuals with type 2 diabetes have some degree of retinopathy and develop initial DR within 20 years of diagnosis. DR is a microvascular disease, characterized by progressive changes in the retinal microvasculature with accompanying damage of neurons and Muller glial cells, that are eventually lost by apoptosis [6]. The ischemic retina releases growth factors that lead to pathological angiogenesis, neovascularization resulting in edema and intraretinal hemorrhages. A number of interrelated pathways that are the target of hyperglycemia are involved in the pathobiology of diabetic complications; among the most actively studied you include oxidative stress, the polyol pathway and the advanced glycation products (AGE) [21,22].

The American diabetes Association (ADA) and the European Association for the Study of diabetes (EASD) recommend metformin (MTF) now used for more than 60 years, as a first-line therapy of type 2 diabetes mellitus in non-pregnant adults [23]. Its benefits include low-cost, minimum hypoglycemic risk, and a decrease in low-density lipoproteins (LDL) and glycated hemoglobin (HbA1c) levels. MTF improves insulin sensitivity in muscle and adipose tissue and suppresses hepatic gluconeogenesis mainly through activation of adenosine monophosphate-activated protein kinase (AMPK) [24]. AMPK regulates metabolism and lipid biosynthesis, inactivates acetyl-CoA carboxylase and induces the expression of muscle hexokinase [25]. However, the metformin mechanism of action involved in insulin-sensitizing effects and bioactive lipids is not completely understood. By AMPK, MTF regulates metabolism and lipid biosynthesis, inactivating acetyl-CoA carboxylase and inducing the expression of muscle hexokinase [24]. MTF treatment improves insulin sensitivity and dyslipidemia, regulating the expression of the genes associated with lipid metabolism [26] (also promoting hepatocyte and pancreatic β cell regeneration [27]. However, MTF suppresses liver glucose-6-phosphatase expression by AMPK-independent mechanism involving complex I inhibition [28]. In fact, another MTF mechanism of action is ETC Complex I activity inhibition [29], recently suggested to be its main action also in vivo [30–32]. Resveratrol, a stilbene natural compound, was shown to act as an inhibitor of ATP synthase, and was shown to be able to inhibit the ectopic ATP synthase of the rod OS [33].

Here we present in vitro data on bovine rod OS homogenates showing, for the first time, a dependence of the rod OS aerobic metabolism and ROI production on absorbed light at varying intensities. We also report an increase in surface protein oxidation in the rod OS under saturating ambient light and show that both metformin and resveratrol can diminish the ROI production and the oxidative phosphorylation rates.

2. Material and methods

2.1. Sample preparations

2.1.1. Extraction of retinas

Retinas were extracted as previously described [34]. Briefly, the eye semicup, including the retina, from freshly enucleated bovine eyes (from a local certified slaughterhouse) after vitreous and lens removal, were incubated for 10 min with Mammalian Ringer (MR, 0.157 M NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 8 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ pH 6.9 plus protease inhibitor cocktail (Sigma-Aldrich, S. Louis, MO, USA) and 50 μg/ml Ampicillin). Each retina was then cut free of the optic nerve with scissors and collected.

2.1.2. Purified bovine rod OS preparations

Purified bovine rod OS were prepared under dim red light at 4 °C from 14 retinas, by sucrose/Ficoll continuous gradient centrifugation [12,35] in the presence of protease inhibitor cocktail (Sigma–Aldrich, S. Louis, MO) and ampicillin (100 μg/ml). Rod OS preparation were characterized for integrity of plasma membrane as reported [35]. Rod OS homogenates were obtained by Potter-Elveheim homogenization on ice in 1:1 (w/v) hypotonic medium (5 mM Tris-HCl, pH 7.4 plus protease inhibitor cocktail and 100 μg/ml ampicillin).

2.1.3. Osmotically intact rod OS disk preparations

Osmotically intact disks were prepared by Ficoll flotation [36] from purified rod OS. After letting the rod OS burst for 3 h in 5% Ficoll solution with 70 μg/ml leupeptin, and 100 μg/ml ampicillin, at 4 °C 2 ml of distilled water were layered onto Ficoll and sample centrifuged for 2 h at 25,000 rpm in a Beckman FW-27 rotor (100.000 × *g*). Disks were collected in the distilled water phase, under sterile conditions. Both purification procedures were carried out in the absence of Cyclosporin A and 2-Aminoethoxydiphenyl borate. These are inhibitors of the mitochondrial permeability transition pore (MTP) [37,38]. Such conditions therefore would promote the MTP formation in contaminant mitochondria, if any, so that these would not be functional.

2.2. Transmission electron microscopy

The front half of bovine eyes were excised and the vitreous humor and lens removed. The eye cups were then filled with fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS buffer solution. After fixation (1,5 h), retinas were removed from the eye capsule, cut into small pieces, washed overnight with 50 mM NH4Cl, dehydrated and embedded in LR White Resin [39] and polymerized at 58 °C. Ultrathin sections were placed on Formvar-coated nickel grids and used the next day for immunogold labeling.

For immunostaining of sections, the postembedding immunogold method was applied. Sections were treated with blocking solution (1% BSA, 0.1% Tween 20, PBS 1 ×), then incubated with mouse monoclonal anti-rhodopsin (1:100) (Sigma Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti-ND1 subunit of ETC I Antibody (Ab) (diluted 1:50) (Abcam, Cambridge, UK) overnight at 4 °C. Ab binding was detected using secondary anti-rabbit IgG (British BioCell International) (diluted 1:100) coupled to gold particles (25) nm, and goat anti-mouse IgG (British BioCell International) (diluted 1:100) coupled to gold particles (5 nm). Sections were analyzed at a FEI Tecnai G12 transmission electron microscope operating at 100 kV. In negative controls the pre-immune serum was applied to the sections instead of the specific primary Ab. Images were acquired with OSIS Veleta cameras, collected and typeset in Corel Draw X4. Controls were performed by omitting primary Ab, which resulted in absence of crossreactivity (data not shown).

2.3. Light exposure conditions for cytofluorimetric and ATP synthesis assay

For cytofluorimetric measurements and ATP production assays, rod OS were subjected to the following illumination conditions: i) dim light; ii) saturating ambient light exposure for 5 min; iii) saturating ambient light exposure for 1 h under a lens filter (lens filters, belonging to category 3 defined by NBR15111 Maximum value of spectral transmittance is 1 tV Maximum value of solar UVA transmittance (315–380 nm) is 0,5 tV Visible Light Transmission (VLT) is from 8% to 18%.) (according to: ABNT [40]), to mimic human sunglass wearing conditions; iv) saturating ambient light exposure for 1 h. For oxygen consumption assay rod OS were treated with ambient-sunlight exposure for 1 h or to dim light.

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