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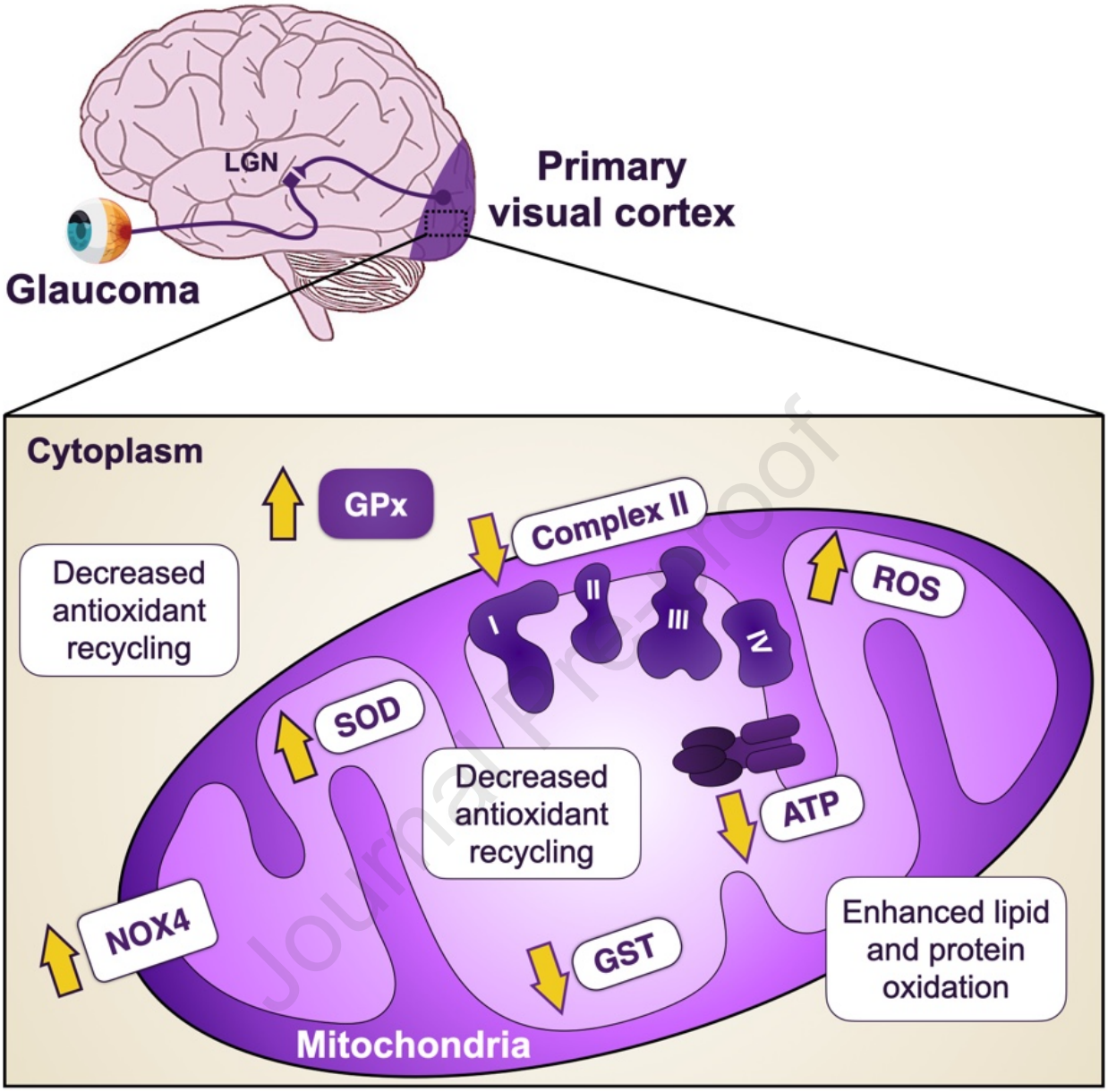

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Abstract

Glaucoma is a neurodegenerative disease that affects eye structures and brain areas related to the visual system. Oxidative stress plays a key role in the development and progression of the disease. The aims of the present study were to evaluate the mitochondrial function and its participation in the brain redox metabolism in an experimental glaucoma model.

3-month-old female Wistar rats were subjected to cauterization of two episcleral veins of the left eye to elevate the intraocular pressure. Seven days after surgery, animals were sacrificed, the brain was carefully removed and the primary visual cortex was dissected. Mitochondrial bioenergetics and ROS production, and the antioxidant enzyme defenses from both mitochondrial and cytosolic fractions were evaluated.

When compared to control, glaucoma decreased mitochondrial ATP production (23%, $p<0.05$), with an increase in superoxide and hydrogen peroxide production (30%, $p<0.01$ and 28%, $p<0.05$, respectively), whereas no changes were observed in membrane potential and oxygen consumption rate. In addition, the glaucoma group displayed a decrease in complex II activity (34%, $p<0.01$). Moreover, NOX4 expression was increased in glaucoma compared to the control group (27%, $p<0.05$). Regarding the activity of enzymes associated with the regulation of the redox status, glaucoma showed an increase in mitochondrial SOD activity (34%, $p<0.05$), mostly due to an increase in Mn-SOD (50%, $p<0.05$). A decrease in mitochondrial GST activity was observed (11%, $p<0.05$). GR and TrxR activity were decreased in both mitochondrial (16%, $p<0.05$ and 20%, $p<0.05$ respectively) and cytosolic (21%, $p<0.01$ and 50%, $p<0.01$ respectively) fractions in the glaucoma

group. Additionally, glaucoma showed an increase in cytoplasmatic GPx (50%, *p<0.01*). In this scenario, redox imbalance took place resulting in damage to mitochondrial lipids (39%, *p<0.01*) and proteins (70%, *p<0.05*).

These results suggest that glaucoma leads to mitochondrial function impairment in brain visual targets, that is accompanied by an alteration in both mitochondrial and cytoplasmatic enzymatic defenses. As a consequence of redox imbalance, oxidative damage to macromolecules takes place and can further affect vital cellular functions. Understanding the role of the mitochondria in the development and progression of the disease could bring up new neuroprotective therapies.

Keywords: mitochondria, glaucoma, primary visual cortex, oxidative stress, neurodegeneration

1. Introduction

Glaucoma is a disease characterized by an optic nerve specific pattern of damage and visual field loss, which if not controlled, may lead to blindness [1]. Glaucoma is not just an eye disease. In recent years, many studies have shown that glaucoma also damages brain structures, and that intraocular pressure (IOP) may be the most important risk factor for the progressive degeneration from the retina to the brain [2–4]. The loss of optic nerve fibers, formed by the axons of retinal ganglion cells (RGCs), leads to neuronal degeneration all along the entire optic projection into central visual targets [5]. Different mechanisms have been proposed to contribute to optic neuropathy. Among them, oxidative stress has been suggested to play a central role in the development of the disease [6,7]. Oxidative damage has been demonstrated in the aqueous humor of patients presenting different types of glaucoma [8,9]. In addition, using animal models of chronic IOP elevation, redox imbalance was shown, not only in eye structures such as retina [10] but also in optic nerve head, vitreous, and aqueous humor [11].

The occurrence of oxidative stress in the brain has been shown for the first time by our group [12]. Moreover, we demonstrated that glaucoma increases the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) from different sources, such as NOX family and iNOS in the primary visual cortex, in an context where the antioxidants defenses were overwhelmed as a consequence of impaired Nrf2 signaling [13].

Mitochondria are important organelles that participate in vital cellular processes such as energy production in the form of ATP via oxidative phosphorylation, regulation of ROS production, and cell apoptosis, among others [14]. In physiological conditions,

mitochondrial ROS production is extremely regulated, but when the mitochondrial electron transport chain fails an electron could be donated to O_2, generating superoxide anion ($O_2^{\bullet-}$) [15]. The $O_2^{\bullet-}$ is then converted into hydrogen peroxide (H_2O_2) by the mitochondrial matrix superoxide dismutase (Mn-SOD). H_2O_2 could be metabolized by mitochondrial glutathione peroxidase (GPx) or by cytosolic GPx or peroxisomal catalase, as it is capable of diffusion through the membrane. Therefore, mitochondria are considered the main source of $O_2^{\bullet-}$ and H_2O_2 in mammals [16,17]. In order to protect mitochondria against oxidative damage, these organelles also contain non-enzymatic antioxidants, including the glutathione and thioredoxin systems, with their corresponding recycling enzymes, glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively [18].

The central nervous system (CNS), and in particular RGCs highly relies on mitochondrial aerobic production of ATP that makes them more susceptible to mitochondrial alterations [19,20]. Moreover, the axon regions are rich in mitochondria, reflecting the high ATP demands that a neuron has in order to achieve synaptic transmission [21]. In this sense, an impairment in mitochondrial function accompanied by a decrease in respiratory complexes activity is known to contribute to the pathogenesis of several neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease [22].

Regarding the role of mitochondria in glaucoma pathogenesis, it has been described that elevated IOP alters mitochondrial structure and function with impaired ATP production in RGCs *in vitro* [23]. In addition, elevated IOP animal models showed mitochondrial alterations in the retina and in the optic nerve [24]. Furthermore, altered mitochondrial functions has been observed in both trabecular meshwork and inflammatory cells of patients with primary open angle glaucoma [25–27].

Even though the role of mitochondria has been evaluated in different eye structures, to our knowledge, the mitochondrial function and its role in glaucoma disease in brain visual targets, such as the primary visual cortex, remain to be revealed. Therefore, the aims of this study were to evaluate the mitochondrial function and its participation in the brain redox metabolism in the primary visual cortex in an experimental glaucoma model. In order to assess these purposes, mitochondrial bioenergetics and ROS production, and the antioxidant enzyme defenses from both mitochondrial and cytosolic fractions were evaluated. The elucidation of the mitochondrial function may explain the mechanism by which glaucoma damage extends to central visual targets, contributing to neurodegeneration.

2. Materials & Methods

2.1. Experimental model of glaucoma

3-month-old female Wistar strain rats (250 g of weight) were subjected to the cauterization of two of the four episcleral veins present on the left eye to induce glaucoma [28]. Before the surgical procedure, rats were anesthetized with a mixture of xylazine (0.5 mg/kg) and ketamine (50 mg/kg) that was administered intraperitoneally. A coaxial light microscope was used to operate the animals and the eyelids were retracted using a specially designed speculum. The conjunctiva was opened using atraumatic forceps and Vannas scissors and the veins of the limbus were exposed. Each vein was softly lifted from the sclera using a cyclodialysis spatula and cauterized with an ophthalmic cautery. The animals from the glaucoma group (total n=10) underwent this procedure, whereas the control group (total n=10) received the same surgical technique, but without performing the cauterization. The animals were housed in standard animal rooms in a 12 hours light/dark, under controlled conditions of temperature (21 ± 2 ºC) and humidity, and were fed with food and water *ad libitum*. In order to confirm the elevation of IOP, a Tonopen XL tonometer (Menor, Norwell, MA, USA) was used to measure the IOP as previously described [11].

Seven days after surgery rats were anesthetized as described above and were sacrificed with pneumothorax. The Committee for Care and Use of Laboratory Animals (CICUAL) of the School of Pharmacy and Biochemistry University of Buenos Aires approved every experimental procedure (CICUAL FFyB #3314).

2.2. Mitochondrial isolation and preparation of mitochondrial membranes

The brain was carefully removed from the skulls, the primary visual cortex was dissected and then homogenized in 5 mL of buffer containing 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA (pH=7.40). Homogenates were centrifuged at 600 g for 10 min at 4 °C and the resulting supernatant was then centrifuged at 8000 g for 10 min at the same temperature. The pellet, containing freshly isolated mitochondria, was resuspended in 500 µL of buffer containing 230 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH=7.40). The last supernatant obtained was called cytosolic fraction [29].

Submitochondrial membranes were obtained by freeze–thaw cycles of the mitochondrial preparation, followed by a passage through a tuberculin syringe with a needle [30]. Protein content was determined by Lowry's method using bovine serum albumin as standard [31].

2.3. Mitochondrial respiration

Mitochondrial O_2 consumption was assessed in freshly isolated mitochondria (0.5 mg protein) with a Clark-type O_2 electrode (Hansatech Oxygraph, Hansatech Instruments Ltd, Norfolk, England) for high-resolution respirometry. Mitochondrial respiration was measured in a reaction medium containing 120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES, 1 mg/mL BSA, 2 mM malate and 5 mM glutamate (pH=7.20) at 30 °C. In this condition, a state 4 respiration rate was measured (rest state respiration) and when 125 µM ADP was added, state 3 respiration rate was assessed (active state respiration) [32]. The ratio between state 3 and state 4 respiration rates was used to calculate the respiratory control ratio (RCR). Results were expressed as ng-at O/ min.mg protein (n=10 each group).

2.4. Mitochondrial ATP production rate

ATP production rate was determined in freshly isolated mitochondria (0.15 mg protein) using the luciferin-luciferase system in a microplate reader (Varioskan® LUX, Thermo Scientific, MA, USA). The reaction medium contained 150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 0.1% (W/V) BSA, 10mM K_2HPO_4/KH_2PO_4, 0.1 mM $MgCl_2$ (pH=7.40), supplemented with 0.8 mM luciferin, 20 µg/ml luciferase, at 30 °C. The reaction was triggered when 3 mM malate, 1.25 mM glutamate and 1 mM ADP were added. In order to establish the basal signal, a negative control with 2 µM oligomycin was performed. A calibration curve was performed using ATP as standard [33]. Results were expressed as nmol ATP/ min.mg protein (n=6 each group).

2.5. Mitochondrial membrane potential (ΔΨm)

The potentiometric cationic probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) was used for the evaluation of inner mitochondrial membrane potential by flow cytometry. A sample of freshly isolated mitochondria (15 µg protein) was incubated with $DiOC_6$ (30 nM) in reaction buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH=7.40), supplemented with 0.2 mM malate and 0.5 mM glutamate as mitochondrial substrates. The procedure was performed in the dark at 37 °C for 20 min. Data was acquired in a FACSCaliburg equipment (BD Biosciences). To exclude debris, mitochondria were gated based on light-scattering properties and 30,000 events per sample were collected. 10-N-nonyl acridine orange (NAO) (100 nM) was used to selectively stain mitochondria and to evaluate the purity of the mitochondrial preparations, due to its ability to selectively bind to cardiolipin at the inner mitochondrial membrane. Mitochondrial selected population was 95% NAO positive [34]. $DiOC_6$ signal was analyzed in the FL-1 channel with FlowJo software (TreeStar,

Ashland, OR, USA), and quantified as median fluorescence intensity (MFI) (n=6 each group) [35].

2.6. Mitochondrial superoxide anion ($O_2^{\bullet-}$) production

The MitoSOX probe was used for the determination of mitochondrial $O_2^{\bullet-}$ production by flow cytometry. The procedure was performed as previously described in section 2.5. A sample of freshly isolated mitochondria (15 µg protein) was incubated in the dark at 37 °C for 20 min with 5 µM MitoSOX in the reaction buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH=7.40), supplemented with 0.2 mM malate and 0.5 mM glutamate as mitochondrial substrates. MitoSOX signal was analyzed in the FL-2 channel with FlowJo software (TreeStar, Ashland, OR, USA) [36]. Results were expressed as the percentage of positive events for MitoSOX ($MitoSOX^+$) (n=4 each group).

2.7. Mitochondrial hydrogen peroxide (H_2O_2) production rate

The production of H_2O_2 was determined in freshly isolated mitochondria by the Amplex red-horseradish peroxidase (HRP) system, following the fluorescence intensity in a microplate reader (Varioskan® LUX, Thermo Scientific, MA, USA) at 587 nm (λem) and 563 nm (λexc) at 30 °C. The reaction medium contained 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 0.01% (W/V) BSA (pH=7.20), supplemented with 2 mM malate and 5 mM glutamate, as substrates for mitochondrial respiration. Mitochondria (0.15 mg protein) were added to the reaction medium with 25 mM Amplex red and 0.5 U/mL HRP. A calibration curve was performed using H_2O_2 as standard. Controls were performed in the presence of catalase and in the absence of isolated mitochondria or HRP, in order to evaluate

that non-specific probe oxidation was minimal (<1%) [37]. Results were expressed as nmol H_2O_2/ min.mg protein (n=6 each group).

2.8. Mitochondrial respiratory chain complexes activities

2.8.1. NADH-cytochrome c reductase (complex I–III)

Complex I-III activity was evaluated by a colorimetric assay in a microplate reader (Varioskan® LUX) following cytochrome c^{3+} reduction at 550 nm (ε = 19.6 $mM^{-1}cm^{-1}$). The mitochondrial membranes (2 mg protein/mL) were added to 100 mM phosphate buffer (pH = 7.40), 4 mM NADH, 0.5 mM cytochrome c and 10 mM KCN, at 30 °C [38]. Results were expressed as nmol reduced cytochrome c^{3+}/ min.mg protein (n=8 each group).

2.8.2. Succinate cytochrome c reductase (complex II–III)

Complex II-III activity was determined as described in section 2.8.1, except that instead of using NADPH, it was substituted by 0.1 M succinate [38]. Results were expressed as nmol reduced cytochrome c^{3+}/ min.mg protein (n=8 each group).

2.8.3. Cytochrome oxidase activity (complex IV)

Complex IV activity was determined spectrophotometrically at 550 nm by following the oxidation rate of 50 mM cytochrome c^{2+} in a Jasco V-730 Spectrophotometer (Jasco Analitica Spain, Madrid, Spain). Cytochrome c^{2+} was freshly prepared by reduction of cytochrome c^{3+} with dithionite, followed by Sephadex- G25 exclusion chromatography. Mitochondrial membranes (1 mg protein/mL) were added to the buffer mixture described in section 2.8.1. [39]. Cytochrome c^{2+} oxidation rate was

242 calculated from the pseudo-first reaction constant (k′) and results are expressed as
243 k′/ mg protein (n=8 each group).
244
245 *2.9. NADPH oxidase-4 (NOX4) expression*
246 In order to assess NOX4 expression, 10% SDS-page was performed to resolve
247 mitochondrial samples of 60 µg protein. Then, proteins were transferred to
248 membranes of nitrocellulose and blocked with 7.5% non-fat dry milk in PBS-Tween
249 for 1 h. Overnight incubation was done with anti-NOX4 (1:250; sc-30141, Santa Cruz
250 Biotechnology) or anti-VDAC (1:500; sc-390996, Santa Cruz Biotechnology). PBS-
251 Tween was used to wash the membranes for 10 min three times, and HRP-
252 conjugated anti-rabbit antibody (1:12500, 170-6515, BIO-RAD) or anti-mouse
253 antibody (1:5000, 170-6516, BIO-RAD) were employed for secondary detection.
254 Membranes were washed as previously described and chemiluminescent detection
255 was done using Clarity Max Western ECL substrate-Luminol Solution (BIO-RAD).
256 ImageJ program (1.50i, Wayne Rasband, National Institutes of Health, USA) was
257 used to quantify bands (n=5 each group).
258
259 *2.10. Markers of oxidative damage to macromolecules*
260 *2.10.1. Protein oxidation*
261 Protein oxidation was assessed by measuring the carbonyl groups content from
262 oxidatively modified proteins in mitochondrial fraction (0.75 mg protein) following the
263 method previously described by Levine and coworkers (1990) [40] using a Hitachi U-
264 2000 Spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). Results were
265 expressed as nmol/ mg protein (n=6 each group).
266

2.10.2. Lipid oxidation

Lipid damage was evaluated as thiobarbituric acid reactive substances following the fluorometric method previously described by Yagi (1976) [41] using a mitochondrial sample of 0.75 mg protein. The measure was performed using a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA, USA) at 515 nm (λexc) and 553 nm (λem). 1,1,3,3-tetramethoxypropane (MDA) was used to perform a calibration curve. Results were expressed as nmol MDA/ mg protein (n=6 each group).

2.11. Activity of enzymes associated with the regulation of the redox status

The following enzymatic activities were evaluated in both mitochondrial and cytoplasmatic cellular fractions: Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione S-transferase (GST), Glutathione reductase (GR), and Thioredoxin reductase (TrxR). The enzymatic activities were assessed following the adapted methods previously described by our group [12,13,42]. To evaluate SOD activity increasing amounts of mitochondrial or cytoplasmatic sample (5, 7, 10 µL) were used. In order to specifically evaluate the activity of the isoform Mn-SOD in mitochondrial fraction, 1mM KCN was added to inhibit Cu,Zn-SOD [43]. A sample of 10 µL of mitochondrial fraction and 30 µL of cytoplasmatic fraction was used to study the other enzyme activities (n=6, each group). All enzymatic measurements were performed by spectrophotometric assays using a Jasco V-730 Spectrophotometer (Jasco Analitica Spain, Madrid, Spain).

2.12. Chemicals

Antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and Bio-Rad Laboratories (Hercules, CA, USA). All the other chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA).

2.13. Statistical analysis

Statistical calculations were performed using the statistical software GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± standard error of the mean (SEM). The statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's *t*-test. A probability value of less than 0.05 indicates a statistically significant difference. Pearson's correlation coefficient (r) was employed to evaluate the association between complex II activity and superoxide anion production and ATP production rate.

3. Results

3.1. Mitochondrial respiration is preserved in glaucoma

In order to evaluate brain mitochondrial function in glaucoma, different parameters were assessed. The first step was to study mitochondrial respiration as O_2 consumption by isolated mitochondria in resting (state 4) and active (state 3) states. State 3 is the condition of highest physiological rate of ATP synthesis and O_2 consumption. As it is shown in Table 1, the glaucoma group showed no changes in O_2 consumption in state 4 and state 3, as well as in RCR, when compared to the control group.

3.2. Glaucoma alters mitochondrial ATP production rate

Another parameter of mitochondrial function is the ATP production rate as it is the main activity of mitochondria during oxidative phosphorylation. When mitochondrial function is impaired, the ATP synthesis could be compromised [17]. As it is shown in Figure 1A, when compared to controls, the ATP production rate decreased 23% in the glaucoma group ($p<0.05$), leading to a deficiency in the oxidative phosphorylation.

3.3. Mitochondrial membrane potential (ΔΨm) is conserved in glaucoma

Considering the decrease in ATP production rate, ΔΨm was evaluated, since the proton gradient across the inner membrane is the driving force for ATP synthesis. In order to assess ΔΨm, mitochondria were selected based on light-scattering properties (SSC vs FSC) and the fluorescence of NAO, that selectively binds to cardiolipin (Figure 1B). As shown in Figure 1B, more than 95% of events were

positive for NAO, indicating that contamination with debris through the isolation process was minimal. When $DIOC_6$ fluorescence was quantified, (Figure 1C) there were no significant changes between glaucoma and control groups, suggesting that in glaucoma the mitochondrial membrane potential is not altered.

3.4. Mitochondria is an important source of ROS in glaucoma

Mitochondrion is one of the main sources of $O_2^{\bullet-}$. In order to determine its production, the fluorogenic probe MitoSOX was employed. An increased production of mitochondrial $O_2^{\bullet-}$ was observed in the glaucoma group (28%, *p<0.01*), as it is shown in the overlaid histograms and quantification of $MitoSOX^+$ mitochondria (Figure 1D).

Mitochondrial H_2O_2 production rate was evaluated in freshly isolated mitochondria in state 4 using malate-glutamate as substrates to assess its highest production [44]. Glaucoma group displayed an increase in the H_2O_2 production rate when compared to the control (28%, *p<0.05*) (Figure 1E).

Altogether, these results suggest that in glaucoma the mitochondria are a source of ROS in the primary visual cortex.

3.5. Complex II activity is impaired in glaucoma, with no changes in the other mitochondrial respiratory chain complex activities

Due to the decrease in ATP production rate and the increase in ROS production, we further investigated the possible causes, analyzing mitochondrial respiratory chain complexes activities. When compared to the control group, complex II-III activity was 34% lower in glaucoma (*p<0.01*), while complexes I-III and IV activities remained unchanged (Table 2). In this context, we found a negative correlation between

355 complex II activity and mitochondrial superoxide anion production (r=-0.80; *p<0.05*),
356 suggesting that the decrease in complex II activity could promote ROS production
357 (Figure 2A). In addition, a positive correlation was found when comparing the
358 complex II activity and the ATP production rate (r=0.73; *p<0.05*), suggesting that the
359 impairment in complex II activity could be a possible cause for the decrease in
360 mitochondrial ATP production (Figure 2B).
361
362 *3.6. NADPH oxidase-4 (NOX4) contributes to mitochondrial ROS production in*
363 *glaucoma*
364 In order to assess the role of NADPH family in this pathology, the expression of
365 NOX4 was assessed by western blot, as this is the isoform present in the
366 mitochondria [45,46]. As it is shown in Figure 3, there was an increase in NOX4
367 expression in the glaucoma group compared to controls (27%, *p<0.05*), suggesting
368 that mitochondrial NOX4 is also a source of ROS in the primary visual cortex in
369 glaucoma.
370
371 *3.7. Glaucoma induces oxidative damage to mitochondrial macromolecules in the*
372 *brain*
373 To elucidate the consequence of a shift-toward a more oxidative environment
374 oxidative damage to macromolecules was evaluated in isolated mitochondria. The
375 content of carbonyl groups from oxidatively modified proteins was 70% higher in
376 glaucoma compared to the control group (*p<0.05)* (Figure 4A). In addition, oxidative
377 damage to lipids was evaluated showing an increase in the glaucoma group
378 compared to controls (39%, *p<0.01)* (Figure 4B). Based on these results,
379 mitochondrial macromolecules are a target of oxidative damage in glaucoma.

3.8. Glaucoma modifies the activity of mitochondrial and cytosolic antioxidant enzymes and enzymes associated with the regulation of the redox status

As it is shown in Table 3, SOD levels increased 34% in glaucoma compared to the control group (*p<0.05*). There are two main isoforms, the manganese superoxide dismutase (Mn-SOD) and the copper, zinc superoxide dismutase (Cu, Zn-SOD). While the Mn-SOD is localized in the mitochondrial matrix, the second one is localized in the mitochondrial intermembrane space and in the cytoplasm [47]. We found an increase of 50% in Mn-SOD activity in the glaucoma group (*p<0.05*), but no significant changes in the activity of Cu,Zn-SOD, suggesting that the increased activity of total SOD is a result of an enhancement in mitochondrial Mn-SOD activity. The activity of Mn-SOD is critical to metabolize the $O_2^{\bullet -}$ that arises from the electron leak, generating H_2O_2. This product is metabolized in the mitochondria by GPx or it can diffuse through the mitochondrial membrane into the cytoplasm. As it is shown in Table 3, there were no changes in GPx activity in the mitochondrial fraction, whereas its activity in the cytosol was increased by 50% (*p<0.01*). GST is an enzyme that uses GSH as a cofactor in order to protect the brain against oxidative stress [48]. This enzyme activity was decreased in the mitochondrial fraction in glaucoma compared to the control group (11%, *p<0.05*), while no significant changes were found in the cytosolic fraction. In addition, the activity of GR, the enzyme that is in charge of GSH recycling showed a decrease of 16% and 21% in both mitochondrial and cytosolic fraction, respectively (*p<0.05, p<0.01*). In order to study the thioredoxin system status, TrxR activity was evaluated in both mitochondria and cytosolic fraction. As it is shown in Table 3, the activity of this enzyme was decreased in both

405 fractions (28%, *p<0.01* and 50%, *p<0.01,* respectively) suggesting that the
406 thioredoxin system is also compromised in glaucoma.

4. Discussion

Glaucoma is a neurodegenerative disease that alters not only eye structures, but also brain areas related to the visual system such as the lateral geniculate nucleus and the primary visual cortex [5,12,13]. Oxidative stress, described as an imbalance between the generation of ROS and the antioxidant defenses in favor of the first ones that can lead to redox signaling disruption and macromolecules damage, has been proven to be a mechanism of glaucoma progression in patients and in animal models affecting the aqueous humor, optic nerve head, and visual brain structures [8,11–13,49,50].

Mitochondrial dysfunction plays a critical role in the pathogenesis of glaucoma since it was implicated in different cellular processes such as aging, death, excitotoxicity, and oxidative damage in the RGCs [51–55]. Considering this overwhelming evidence, it is tempting to hypothesize that the mitochondria from primary visual cortex could also be involved in the development of glaucoma disease. In addition, the visual system, including the CNS, has one of the highest energy demands and relative rates of oxygen consumption, and because of that is more susceptible to mitochondrial alterations [19]. ATP production by oxidative phosphorylation is the main mitochondrial function, providing the necessary energy for the cellular processes. A failure of this organelle correlates with impaired electron transfer chain and less ATP production [56,57]. Synaptic degeneration as well as neuronal loss and death could be a consequence of decreased ATP levels at synapses. Indeed, a reduction in mitochondrial ATP production has been described in several neurodegenerative diseases, such as Alzheimer [58], Parkinson’s disease [59], Huntington disease [60], and Amyotrophic lateral sclerosis [61]. Interestingly, when

ATP production was evaluated in our glaucoma model, a significant decrease was observed, suggesting that even though the respiratory control rate has not changed, the mitochondrial function is altered. In this case, the decrease in ATP production was not due to an alteration of mitochondrial membrane potential, since it was unaltered when compared to the control group.

The oxidative phosphorylation process involves the electrons transfer between the respiratory chain complexes, with releasing of H^+ into the intermembrane space. This situation generates the electrochemical H^+ gradient across the inner mitochondrial membrane necessary to produce ATP [62]. Several neurodegenerative diseases have been associated with impairment of mitochondrial complexes, for example, complex IV activity is impaired in Alzheimer's disease [63], complex I activity is compromised in Parkinson's disease [64], and complex II, III and IV activity are decreased in Huntington's disease [65]. In this sense, when we evaluated the activity of mitochondrial complexes, only complex II activity was found decreased in glaucoma, with no changes in complex I, III or IV activities. Among all mitochondrial complexes, complex II is unique: it is encoded by nuclear DNA, it has an important role in the tricarboxylic acid cycle and does not form supercomplexes as complex I, III, and IV do [66,67]. Moreover, it is not directly involved in the proton motive force generation, although complex II impairment has been associated with inefficient ATP synthesis [68]. Interestingly, we found a positive correlation between complex II activity and ATP production, indicating that an impairment in this complex could lead to a decrease in ATP production rate in glaucoma. In addition, in the last years, the role of complex II in mitochondrial ROS production has been widely discussed [69–71]. Recently, it has been shown that complex II is also a relevant source of ROS under both physiological and pathological conditions [72]. Moreover, the increased

457 production of $O_2^{\bullet-}$ has been reported in both forward and reverse reactions [73–76],
458 with rates comparable to the ones reached by complex I and III [77]. In addition,
459 oxidative modifications to complex II have been shown to impair its activity leading to
460 increased $O_2^{\bullet-}$ production [78]. In line with these findings, we showed a negative
461 correlation between complex II activity and mitochondrial $O_2^{\bullet-}$ production, suggesting
462 that complex II impairment may contribute to the increased mitochondrial ROS.
463 Altogether, we showed for the first time that glaucoma induces the impairment of
464 complex II in primary visual cortex mitochondria, suggesting that it could not only be
465 a relevant source of ROS in this pathology, but also that impacts on the
466 mitochondrial capacity to produce ATP.
467 A link between mitochondrial dysfunction and ROS production has been shown in
468 several neurodegenerative diseases [79]. Accordingly, we found that mitochondria
469 are important sources of ROS in the primary visual cortex in glaucoma, since not
470 only the $O_2^{\bullet-}$ production but also H_2O_2 levels were increased in the isolated
471 mitochondria. There are two main sources of H_2O_2 in the mitochondria. The first one
472 results from mitochondrial SOD activity which metabolizes the $O_2^{\bullet-}$ from the electron
473 transport chain into H_2O_2, and the second one is the activity of NOX4, which is the
474 only isoform of the NOX family that produces H_2O_2 and it is located in the
475 mitochondrial membrane [80,81]. In our model, we found an increase in NOX4
476 expression in isolated mitochondria, suggesting that indeed it could be one of the
477 causes of the H_2O_2 increased levels. On the other hand, there are two SOD isoforms
478 in mitochondria capable of metabolizing the $O_2^{\bullet-}$ into H_2O_2, the Cu,Zn-SOD, in the
479 intermembrane space, and Mn-SOD, in the mitochondrial matrix [82]. When the total
480 SOD activity was evaluated in mitochondria, we found an increase in glaucoma, that
481 was mainly at the expense of the Mn-SOD increased activity since there were no

changes in cytosolic Cu,Zn-SOD activity. The H_2O_2 can diffuse to the cytoplasm, triggering different signaling pathways, such as the one modulated by NF-kB [83]. It has been shown that this nuclear factor induces the expression of different genes, including the expression of Mn-SOD [84]. Hence, it is reasonable to hypothesize that in glaucoma, the observed increase in Mn-SOD activity could be a consequence of NF-κB activation, as previously described by our group [13].

As mitochondria is susceptible to oxidative stress, mainly due to an inefficient oxidative phosphorylation, a well-consolidated antioxidant system has evolved to protect them from oxidative damage [85]. In this sense, an important enzyme that detoxifies H_2O_2 is the GPx, using GSH as a cofactor and producing GSSG in the detoxifying process. In this model, mitochondrial GPx activity remained unchanged, whereas it was significantly increased in the cytoplasm. In this context, the GST activity becomes relevant, since mitochondrial GST has a glutathione peroxidase role in order to protect membrane against lipid peroxidation by scavenging peroxides and their end products [86]. Interestingly, mitochondrial GST activity is decreased in glaucoma, probably due to a decrease in its cofactor, GSH, resulting in oxidative damage to lipids. As mitochondria do not possess the enzymatic machinery necessary for the GSH *de novo* synthesis and rely on cytosolic synthesis and subsequent transport [87], the GSSG recycling by mitochondrial GR becomes crucial to maintain mitochondrial GSH levels. Previous work of our group showed a compromised GSH metabolism in the primary visual cortex in glaucoma due to a significant decrease in the cytosolic *de novo* synthesis [13]. In this context, we evaluated mitochondrial and cytosolic GR activity in order to assess the GSH recycling. We found a significant decrease not only in the mitochondrial but also in the cytosolic fractions, suggesting that the GSH metabolism alteration may also

affect the mitochondria redox status. Non-enzymatic antioxidants such as GSH and thioredoxin (Trx) systems are vital for redox homeostasis [88]. It has been found that dysregulation of these systems contribute to neurodegenerative progression in diseases such as Alzheimer, Parkinson and Huntington [89–91]. In particular, Trx directly reduces oxidized modified proteins [92] and in also the cofactor of peroxiredoxin, a relevant enzyme that detoxifies H_2O_2 in both cytoplasmatic and mitochondrial [93]. In this context, the activity of TrxR becomes vital since it is the enzyme that maintains the Trx pool by recycling the oxidized form [94]. In our glaucoma model, we found that TrxR was significantly decreased, leading to a disruption in Trx recycling in glaucoma. Taking together, glaucoma induces not only an increase in mitochondrial ROS production, but also affects the cell antioxidant capacity that fails to counteract the rise in oxidant species. In this scenario, redox imbalance takes place.

As a consequence, a significant damage to both proteins and lipids was found in isolated mitochondria, probably due to an increase in ROS production and a decrease in antioxidant defenses. The macromolecule damage could further contribute to the mitochondrial dysfunction, since it has been shown that protein and lipid oxidation could alter mitochondrial complexes enzymatic activity, ultimately impairing mitochondrial function [95,96].

Taken altogether, glaucoma induces alterations in mitochondrial bioenergetics leading to mitochondrial dysfunction in brain visual targets, specifically, in the primary visual cortex in an elevated IOP animal model. The mitochondrial dysfunction is evidenced by an impairment in complex II activity that may result in an inefficient oxidative phosphorylation, a deficient ATP generation, and increased production of ROS. In this scenario, where the mitochondrial antioxidant defenses

532 are overwhelmed, oxidative damage to both mitochondrial lipids and proteins takes
533 place, contributing to the cell redox imbalance (Figure 5). Maintaining mitochondrial
534 function is crucial in the CNS since it is the source of energy for neuronal function
535 and excitability. Hence, mitochondrial dysfunction could lead to neuronal loss and
536 has been associated with several neurodegenerative diseases [97]. The
537 mitochondrial role has been widely studied in glaucoma disease in the retina,
538 trabecular meshwork, and patients' blood samples [98], but to our knowledge this is
539 the first time that mitochondrial function is evaluated in the primary visual cortex.
540 Unraveling the role of mitochondria in glaucoma contributes to the better
541 understanding of the pathology progression in brain structures and opens the
542 possibility of finding a novel and effective neuroprotective treatment that could delay
543 the progression of the disease.
544

545 **5. Conclusions**

546

547 Mitochondrial function is crucial for neuronal survival in the CNS as a source of
548 energy. In this study we demonstrated, in a rat model, that glaucoma leads to
549 mitochondrial dysfunction in visual brain targets such as primary visual cortex. The
550 mitochondrial alteration is evidenced by an impairment in complex II activity that may
551 result in a deficient ATP generation, and increased production of ROS. In this
552 scenario, an overwhelmed enzymatic antioxidant system, not only in the
553 mitochondria, but also in the cytoplasm resulted in oxidative damage to
554 macromolecules. Understanding the role of the mitochondria in the progression of
555 glaucoma disease could bring up the possibility of novel neuroprotective therapeutic
556 approaches.

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure captions

Figure 1. Mitochondrial bioenergetics in control and glaucoma groups. (A) Mitochondrial ATP production was determined in freshly isolated mitochondria using the luciferin-luciferase system and shows a decrease in glaucoma vs control group (n=6 each group). (B) Flow cytometry was used to evaluate the inner mitochondrial membrane potential and superoxide anion production. Mitochondria were selected based on light scattering properties (SSC vs FSC), 30,000 events were collected, and purity of mitochondrial preparations was evaluated by selective staining for cardiolipin with NAO. (C) $DIOC_6$ was used for the evaluation of inner mitochondrial membrane potential. Representative overlaid histograms of $DIOC_6$ fluorescence in mitochondria and $DIOC_6$ fluorescence quantification of gated mitochondria in glaucoma and control groups shows no differences between them (n=6 each group). (D) MitoSOX probe was used for the determination of mitochondrial superoxide anion production. Representative overlaid histograms and fluorescence quantification of the experimental group displayed an increase in $MitoSOX^+$ mitochondria in glaucoma vs control group (n=4 each group). (E) Mitochondrial H_2O_2 production rate was performed by the amplex red-HRP system showing an increase in glaucoma group vs control (n=6 each group). Results are expressed as mean ± SEM. *$p<0.05$* and *$p<0.01$*. Statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's *t*-test.

Figure 2. Correlations associated with the impairment in complex II activity. (A) Pearson's correlation coefficient (r) shows a negative correlation between complex II activity and superoxide anion production (n=4 each group). (B) Pearson's correlation

coefficient (r) shows a positive correlation between complex II activity and the ATP production rate (n=5 each group).

Figure 3. NOX4 expression in control and glaucoma group was evaluated by western blot. (A) A representative blot is shown. Bands of approximately 70 kDa and 34 kDa correspond to NOX4 and VDAC, respectively. (B) Bars represent the relative protein level calculated by the ratio of NOX4/ VDAC displaying a higher expression of this protein in glaucoma vs control group. Results are expressed as mean ± SEM (n=5). **p<0.05.* Statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's *t*-test.

Figure 4. Oxidative damage to mitochondrial macromolecules. (A) Protein oxidation was assessed by measuring the carbonyl content in mitochondrial fraction and an increase in glaucoma group was displayed. (B) Lipid peroxidation was evaluated as thiobarbituric acid reactive substances in isolated mitochondria showing an increase in glaucoma vs control group. Results are expressed as mean ± SEM (n=6). **p<0.05* and ***p<0.01*. Statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's *t*-test.

Figure 5. Glaucoma induces redox imbalance in the primary visual cortex by altering the mitochondrial function and the cellular antioxidant capacity. Mitochondrial dysfunction is evidenced by an impaired complex II activity that results in a deficient ATP generation and increased production of superoxide anion ($O_2^{\bullet-}$). The $O_2^{\bullet-}$ is metabolized into H_2O_2 by SOD_2 (Mn-superoxide dismutase) in the mitochondrial matrix. In turn, H_2O_2 diffuses to the cytoplasm where it is metabolized by glutathione peroxidase (GPx) since mitochondrial GPx remains unchanged. GPx uses GSH as a cofactor, which is recycled by glutathione reductase (GR) using NADPH. Thioredoxin

reductase (TrxR) also uses NADPH to recycle oxidized thioredoxin ($TrxS_2$) into reduced thioredoxin ($Trx(SH)_2$). These recycling enzymes are decreased in both mitochondrial and cytoplasmic fractions, affecting the cell antioxidant capacity that fails to counteract the rise in oxidant species. In this scenario, where the mitochondrial antioxidant defenses are overwhelmed, redox imbalance takes place. Abbreviations: IM (inter membrane), OM (outer membrane), intermembrane space (IMS), SOD_1 (Cu,Zn-superoxide dismutase), NOX4 (NADPH oxidase-4), Glutathione S-transferase (GST), reduced glutathione (GSH), oxidized glutathione (GSSG).

964 **Tables**

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Table 1: Oxygen consumption rates and respiratory control ratio of isolated brain mitochondria from control and glaucoma group

Mitochondrial metabolic states	O_2 consumption (ng-at O/ min.mg prot)	
	Control	Glaucoma
State 4	21.6 ± 1.4	20.3 ± 0.9
State 3	71.7 ± 5.1	69.9 ± 4.6
RCR	3.3	3.4

Results are expressed as mean ± SEM for 10 animals in each group. Statistical analysis by unpaired Student's t-test.

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Table 2: Mitochondrial respiratory complexes I, II, IV activities from brain cortex of control and glaucoma group

	Control	Glaucoma
Complex I (nmol/ min.mg prot)	25.7 ± 1.8	27.7 ± 1.9
Complex II (nmol/ min.mg prot)	9.5 ± 0.7	6.1 ± 0.9**
Complex IV (*k'*/ mg prot)	10.2 ± 0.3	10.3 ± 0.5

Results are expressed as mean ± SEM for 8 animals in each group (***p<0.01*). Statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's t-test.

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Table 3: Brain subcellular fraction activity of antioxidant enzymes defenses and enzymes related to the regulation of the redox status control and glaucoma groups

Enzyme	Mitochondria	Cytosol

	Control	Glaucoma	Control	Glaucoma
SOD (U/ mg prot)	8.2 ± 0.4	10.9 ± 0.9*	12.7 ± 1.9	13.5 ± 1.5
Mn-SOD (U/ mg prot)	3.9 ± 0.6	6.3 ± 0.6*	-	-
GPx (nmol/ min.mg prot)	10.9 ± 0.6	11.8 ± 1.0	8.9 ± 0.6	13.5 ± 0.8**
GST (mU/ mg prot)	18.6 ± 0.6	16.1 ± 0.7*	196.2 ± 7.8	190.2 ± 8.2
GR (nmol/ min.mg prot)	6.0 ± 0.3	5.0 ± 0.3*	5.2 ± 0.1	4.1 ± 0.2**
TrxR (nmol/ min.mg prot)	7.4 ± 0.6	5.3 ± 0.3**	24.7 ± 4.1	14.6 ± 1.1*

Results are expressed as mean ± SEM for 6 animals in each group (*$p<0.05$ **$p<0.01$). Statistical significance of the differences between the glaucoma and control group was calculated by unpaired Student's t-test.

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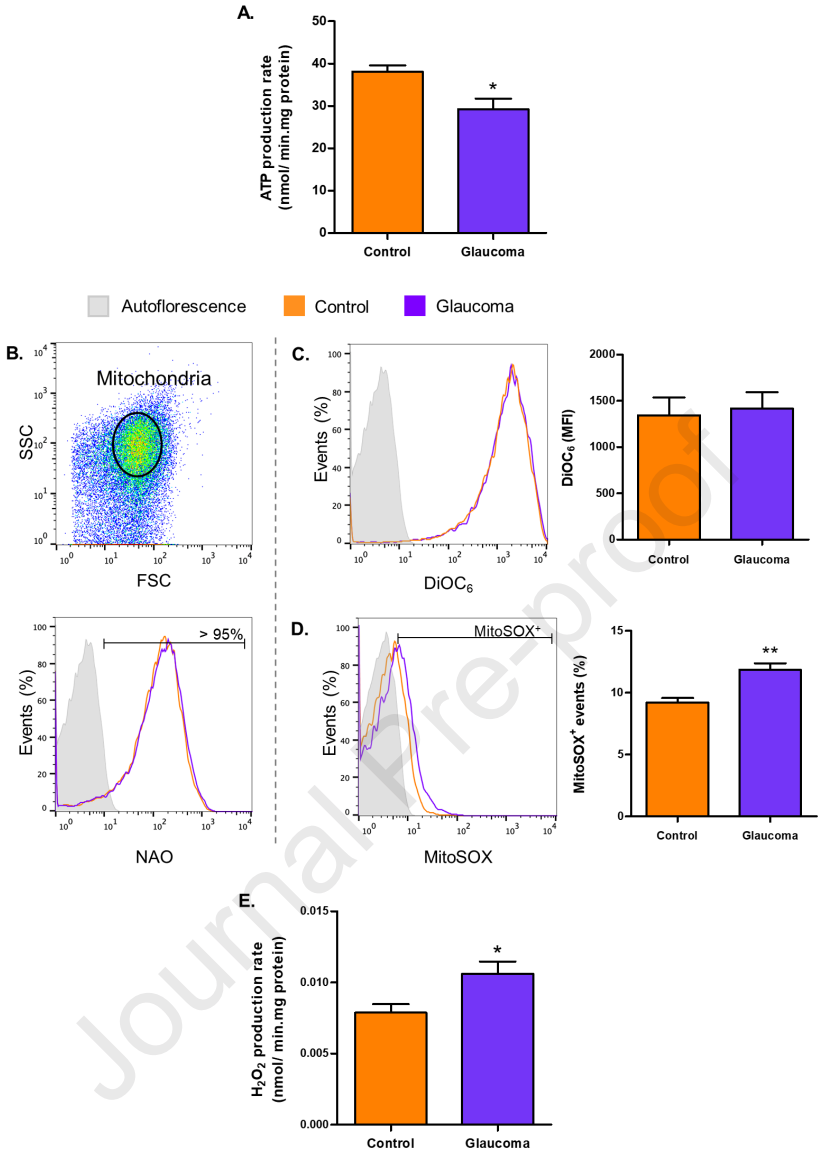

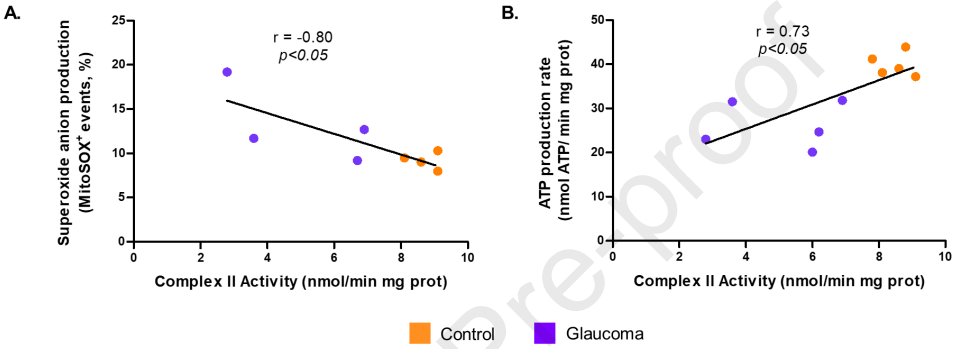

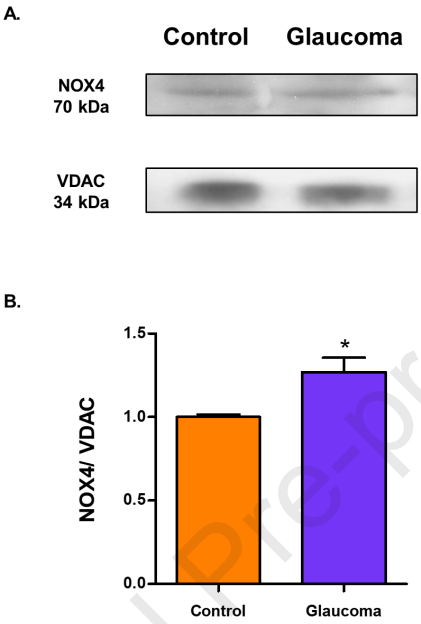

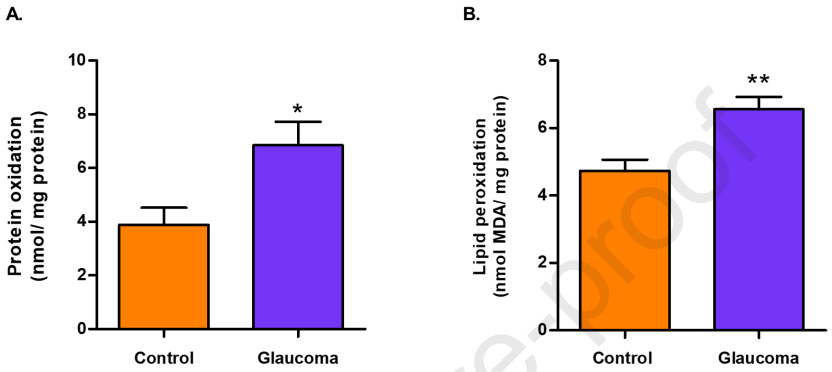

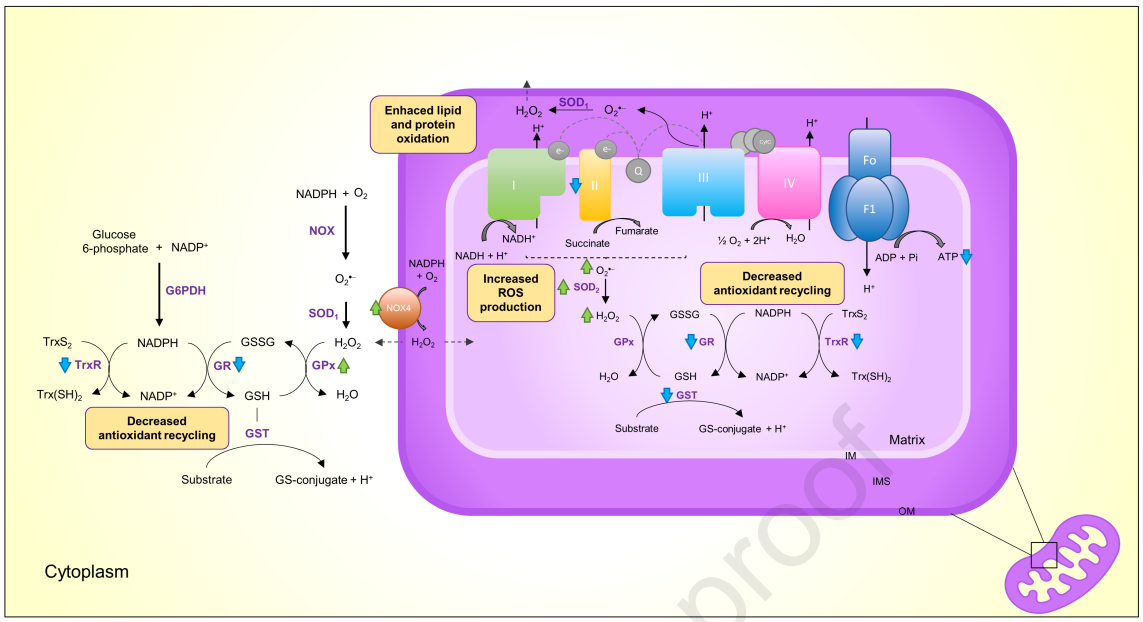

Highlights:

- Glaucoma alters mitochondrial ATP production rate in the primary visual cortex.
- Mitochondrial superoxide anion and hydrogen peroxide production increases in glaucoma.
- Complex II activity is impaired in glaucoma.
- Mitochondrial NOX4 is an additional source of ROS in the mitochondria in glaucoma.
- Glaucoma modifies mitochondrial and cytosolic enzyme defenses leading to redox imbalance.

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: