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Anthocyanins protect from complex I inhibition and APPswe mutation through modulation of the mitochondrial fission/fusion pathways

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ABSTRACT

Anthocyanins are a distinguished class of flavonoids with powerful free radical-scavenging activity that have been suggested as chemotherapeutic agents for the prevention of Alzheimer disease (AD). In this study, we examined the ability of nutraceutical Medox rich in purified cyanidin 3-O-glucoside (C3G), 3-O-b-glucosides and delphinidin 3-O-glucoside (D3G) to counteract mitochondrial deficiency induced by complex I inhibition and/or amyloid-β peptide (Aβ) induced toxicity. SH-SY5Y neuroblastoma cells were stably transfected with APP Swedish K670N/M671L double mutation (APPswe) or with the empty vector and treated with rotenone. We report that Medox treatment improves the metabolic activity and maintains cell integrity in both cell lines. At the mitochondrial level, APPswe and rotenone induced mitochondrial fragmentation, an effect that was counteracted by Medox through the modulation of fission and fusion proteins, resulting in a reshaped mitochondrial network. Although Medox was unable to fully neutralise the effects of rotenone on ATP levels and mitochondrial membrane potential, it was able to prevent rotenone-induced cytotoxicity.

Our findings suggest that Medox anthocyanins, on top of their antioxidant capacity, ameliorate mitochondrial dysfunction generated by Aβ overproduction or by chemical inhibition of mitochondrial complex I via stabilization of the fusion/fission processes. Modulation of the mitochondrial network has been suggested as a novel therapeutic approach in diseases involving mitochondrial dysfunction and oxidative stress. Hence, increasing the understanding of how anthocyanins influence mitochondrial dynamics in a neurodegenerative context, could be of future therapeutic value.

1. Introduction

Alzheimer disease (AD) is a devastating disorder of multifactorial etiology that leads patients into memory loss and cognitive decline. By 2035, the nearly 32 million patients suffering from this dementia worldwide is estimated to be doubled [1]. At the molecular level, AD is characterized by accumulation of amyloid-β peptide (Aβ) in extracellular plaques and hyperphosphorylated tau protein in intra-neuronal tangles in brain Today, no treatment can reverse the progression of AD, and available drugs can only ameliorate some symptoms once the pathology is diagnosed [2,3]. The scientific community is exerting a tremendous effort on conducting new disease-modifying strategies to delay its onset and maintain the independency of the diagnosed patients.

Anthocyanins are flavonoids belonging to a large natural group of hydrophilic pigments, known as polyphenols, that are ubiquitously found in the plant kingdom [4]. Recent epidemiological and pre-clinical research have suggested the health benefits of dietary anthocyanin-rich extracts in reducing incidence of AD [5,6,7,8]. It has been suggested that, by crossing the brain blood barrier (BBB) [9], anthocyanins may accomplish their ROS scavenging ability and modulate signaling pathways in the central nervous system [10,11]. Furthermore, the presence of intact glycosylated anthocyanins such as cyanidin-3-O-beta-glucoside (C3G) and delphinidin-3-O-beta-galactoside (D3G) in the hippocampus of adult rats leads to enhanced cognitive performance [12] and improved spatial memory as well as reduced lipid peroxidation, higher levels of reduced glutathione, and the induction of antioxidant enzymes [13].

Medox capsules, a nutraceutical compound that consists of natural anthocyanins purified from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) have been examined in clinical studies where it has diverse health-promoting effects by modulating pro-inflammatory chemokines [14] and HDL-cholesterol/LDL-cholesterol concentrations in plasma [15]. The characterization of the different anthocyanins in cellular models of Parkinson's disease (PD) and AD, revealed a differential

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impact on protective responses due to a biochemical structure-activity relationship [16]. Synergistic interactions involving multiple anthocyanins have been proposed for maximal neuroprotection [17]. In PD cellular models, D3G (but not C3G) was shown to protect primary neurons from the toxic effects of rotenone [16] whereas in SH-SY5Y cellular models of AD, C3G was reported to inhibit $A\beta_{25-35}$-induced intracellular ROS formation [18] as well as the toxicity of oligomeric and fibrillar $A\beta_{1-42}$ [19].

Mitochondrial deficiency is a prevailing feature of AD, wherein Aβ contributes by significantly reducing brain metabolism, increasing oxidative stress [20] and an imbalanced mitochondrial fission and fusion that results in abnormal mitodynamics and increased sensitivity to cell death [21]. In addition to the above mentioned study [16], recent evidence suggests that flavonoids can prevent mitochondrial dysfunction-associated pathologies by inhibiting H_2O_2 overproduction in damaged brains [22].

In the present study, we investigated whether Medox anthocyanins could mitigate the toxicity of mitochondrial complex I inhibition by rotenone in a cellular AD model. Rotenone has been shown to reduce cell viability and mitochondrial membrane potential ($\Delta\psi_m$), and induce mitochondrial fragmentation and autophagy in previous studies [23,24]. To explore Medox effects, we performed experiments using SH-SY5Y cells stably transfected with the familial AD APP Swedish KM670/671NL double mutation (APPswe). Our results demonstrate that the protective mechanisms of Medox involve an improvement of $\Delta\psi_m$, cell membrane integrity and mitochondrial dynamics.

2. Material and methods

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM; 11966025), fetal bovine serum (FBS), TrypLE™ Express, Geneticin® Selective Antibiotic (G418 Sulfate), and bicinchoninic protein assay (Pierce™ BCA) kit were obtained from Life Technologies, (CA, USA). Resazurin sodium salts suitable for cell culture, Rotenone ≥95% and dimethyl sulfoxide for molecular biology and D-(+)-Galactose (≥99%) were obtained from Sigma-Aldrich (MO, USA). The mitochondrial ToxGlo™ assay kit was purchased from Promega Corporation (Madison, WI). Complex IV Human Specific Activity Microplate Assay Kit was obtained from Abcam (Cambridge, UK).

2.2. Primary antibodies

The following antibodies were used in this study: mouse anti-Opa1 (1:1000; BD Biosciences, San Jose, CA); mouse anti-Mfn2 (1:1000; Abcam, Cambridge, UK); mouse anti-Drp1 (1:500; BD Biosciences, San Jose, CA); rabbit anti-β-actin (1:5000; Sigma, MO, USA).

2.3. Medox anthocyanins

The nutraceutical, Medox®, has been developed by Biolink Group (Sandnes, Norway). Medox capsules consist of natural purified anthocyanins isolated from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*). The concentration assessed for the study is expressed in μg/ml. The relative content of each anthocyanin in the capsules was 33.0% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of cyanidin; 58.0% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of delphinidin; 2.5% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of petunidin; 2.5% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of peonidin; 3.0% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of malvidin; and 1.0% of 3-*O*-rutinoside of cyanidin and delphinidin. The capsules also contained pullulan, maltodextrin, and citric acid (4%) to maintain the stability of anthocyanins.

2.4. Cellular culture and treatments

Human SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, USA). Stable transfection of APP with the Swedish KM670/671NL double mutation or empty pcDNA 3.1 vector used as control were performed as formerly described [25]. SH-SY5Y transfected cells were routinely grown at 37 °C in a humidified incubator with 5% CO_2 in DMEM + Glucose (10 mM) or +Galactose (10 mM), supplemented with 10% fetal bovine serum and geneticin. All experiments were performed after 24 h of incubation. Cells were treated with Medox anthocyanins (0.05 μg/ml; containing 20 nM C3G and 30 nM D3G) for 18 h. To study Medox effect against rotenone-induced toxicity, cells were incubated for 18 h with rotenone (100 nM) in presence or absence of Medox anthocyanins. To explore whether Medox was able to improve cell recovery after a short exposure to rotenone, cells were exposed to rotenone (100 nM) for 30 min and then incubated in the presence or absence of Medox anthocyanins for 18 h.

2.5. Resazurin assay

The Resazurin reagent was prepared in DMEM + glucose (10 mM) or galactose (10 mM) at a final concentration of 20 μg/ml. SH-SY5Y transfected cells were seeded in 24-well plates at 10×10^4 cells/well. After experimental treatments in glucose or galactose containing media, plates were washed once in 1× PBS and incubated in 400 μl of Resazurin working solution for 2 h at 37 °C. Fluorometric Resazurin reduction was measured in Tecan plate readers and the values were calculated by Magellan™ Data Analysis Software. Results were expressed as percentages of the values obtained from the appropriate controls.

2.6. Measurement of complex IV activity

The complex IV specific activity microplate assay kit (ab 1099910) was used to determine the activity and quantity of the enzyme in SH-SY5Y transfected cells. Cells were seeded in 6-well plates at 15×10^4 cells/well in glucose-containing media and treated in the absence or presence of Medox (0.05 μg/ml) for 18 h as previously described. The assay was performed following the manufacturer's instructions. Complex IV activity and protein quantity were determined colorimetrically at 550 nm and 405 nm respectively. The activity values were calculated by Magellan™ Data Analysis Software. Results were expressed by OD/min in relation to protein levels.

2.7. Measurement of cell membrane permeability and ATP levels

The mitochondrial ToxGlo™ assay kit (G80000) was used to assess mitochondrial deficits. Two different passages of control and APPswe transfected cells were seeded by duplicate in three independent 96-well plates at 5×10^4 cells/well in galactose-containing media. Cell membrane permeability was first assessed by the presence of bis-AAF-R110, a fluorogenic peptide substrate associated with protease activity, that gives significant signal with non-viable cells, in relation to viable cells. Results are expressed as fluorescence intensity. Next, ATP was measured over the same cell cultures by adding the ATP Detection Reagent, resulting in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The concentration of ATP was calculated from a calibration curve described in the kit and the data was expressed by nmole of ATP.

2.8. Analysis of mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry

Cells were seeded in 12-well plates at 10×10^4 cells/well in galactose containing medium and treated with rotenone and Medox as previously described. After treatment, cells were washed with PBS and stained with 100 nM tetramethyl rhodamine methyl ester (TMRM, Life Science Technologies) for 15 min at 37 °C, then washed with PBS. The cells were