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Resveratrol affects differently rat liver and brain mitochondrial bioenergetics and oxidative stress *in vitro*: Investigation of the role of gender

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ABSTRACT

Resveratrol (3,5,4′-trihydroxy-trans stilbene) is commonly recognized by its antioxidant properties. Despite its beneficial qualities, the toxic effects of this natural compound are still unknown. Since mitochondria are essential to support the energy-dependent regulation of several cell functions, the objective of this study was to evaluate resveratrol effects on rat brain and liver mitochondrial fractions from male and females regarding oxidative stress and bioenergetics. No basal differences were observed between mitochondrial fractions from males and females, except in liver mitochondria, the generation of H_2O_2 by the respiratory chain is lower for female preparations. Resveratrol inhibited lipid peroxidation in preparations from both genders and organs. Furthermore, brain mitochondria in both gender groups appeared susceptible to resveratrol as seen by a decrease in state 3 respiration and alterations in mitochondrial membrane potential fluctuations during ADP phosphorylation. As opposed, liver mitochondria were less affected by resveratrol. Our data also demonstrates that resveratrol inhibits complex I activity in all mitochondrial preparations. The results suggest that brain mitochondria appear to be more susceptible to resveratrol effects, and gender appears to play a minor role. It remains to be determined if resveratrol effects on brain mitochondria contribute to deterioration of mitochondrial function or instead to mediate hormesis-mediated events.

1. Introduction

The redox active polyphenol compound resveratrol (3,5,4′-trihydroxy-trans stilbene) was firstly identified in roots from white hellebore (*Veratrum album*) and later in roots from Japanese knotweed (*Polygonum cuspidatum*) (Nonomura et al., 1963). Resveratrol is also found in grapes (*Vitis vinifera*), grape juice, wine berries (*Vaccinium macropon*) and peanuts (*Arachis hypogaea*) (Fremont, 2000; Pervaiz, 2003). Resveratrol has been shown to trigger several physiological effects in laboratory animals, resulting in cancer prevention, microvascular and neuroprotection as well as antidiabetic effects (Baur and Sinclair, 2006). Resveratrol is one of the main components of red wine, the consumption of which is associated with a lower incidence of heart failure in France (Richard, 1987). The protective effects are associated with antioxidant proprieties that were confirmed in the heart in different models (Gresele et al., 2011), including lipopolysaccharide (LPS)-induced oxidative stress (Sebai et al., 2011) and doxorubicin (DOX)-induced cardiotoxicity (Xu et al., 2012).

Resveratrol crosses the blood–brain barrier (BBB) (Wang et al., 2002), demonstrating neuroprotective effects in several disorders such as cerebral ischemia and Alzheimer's disease (Baur and Sinclair, 2006). Resveratrol also increases spatial memory performances in the circular platform tasks in primates (Dal-Pan et al., 2011), thus demonstrating cognitive and neuroprotective effects (Agarwal and Baur, 2011; Huber and Superti-Furga, 2011). Fukiu et al. showed that resveratrol induces the expression of mitochondrial superoxide dismutase (SOD2) and as a consequence reduces mitochondrial oxidative stress and damage in neurons (Fukui et al., 2010).

Specifically in the liver, resveratrol up-regulates the expression of glucogenic genes by attenuating insulin signaling and by deacetylating FOXO1 (Park et al., 2010). Resveratrol also decreases

Abbreviations: ADP, adenosine diphosphate; ANT, adenosine nucleotide translocator; ATP, adenosine triphosphate; BBB, blood–brain barrier; DCPIP, 2,6-dichorophenolindophenol; DMSO, dimethyl sulfoxide; DOX, doxorubicin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; FOXO1, forkhead box protein 1; LDL, low density protein; LPS, lipopolysaccharide; NADH, nicotinamide adenine dinucleotide; Ox, oxidized; RCR, respiratory control ratio; ROT, rotenone; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species; TPP^+, tetraphenylphosphonium.

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fibrosis and promotes hepatocyte regeneration, which increased the survival of mice during cholestatic liver injury (Chan et al., 2011). Although the protective effects of resveratrol on heart mitochondria have been described (Gutierrez-Perez et al., 2011), mitochondrial-specific studies are lacking in liver and brain mitochondria.

Mitochondria are the cell powerhouses due to the synthesis of ATP by oxidative phosphorylation (Pereira et al., 2009a). Mitochondria are also a major endogenous source of reactive oxygen species, either under normal (Rigoulet et al., 2011) or pathological (Puente-Maestu et al., 2012; Reale et al., 2012) conditions. Macromolecular oxidative damage in mitochondria induces a decline in the efficiency of oxidative phosphorylation, and result in the induction of the mitochondrial permeability transition and in the release of pro-apototic factors that trigger apoptosis (Pereira et al., 2009a). With this in mind, several effects of resveratrol on cells may be derived from direct or indirect mitochondrial effects. To support the evidence that resveratrol presents direct effects on mitochondria, we have isolated fractions from rat liver and brain and investigated whether resveratrol alters mitochondrial bioenergetics and prevents induced oxidative damage. A second important question was whether resveratrol-induced mitochondrial effects are gender-dependent. To answer this latter question, mitochondrial fractions were isolated from female and male rats. Isolated mitochondrial fractions are a recognized model to measure compound toxicity (Pereira et al., 2009b). Nevertheless, the large majority of experiments is performed with mitochondrial fractions from male animals. Differences between mitochondrial fractions from male and female animal models may influence the final outcome of chemical-biological interactions at the mitochondrial level. The concentrations used in this study are within the concentration range used by others (Annabi et al., 2012; He et al., 2012; Price et al., 2012; Valdecantos et al., 2010).

2. Material and methods

2.1. Chemicals

All chemicals used in this work were purchased from Sigma Aldrich Co (St. Louis, MO), unless specified. Resveratrol was prepared in DMSO, the final volume used was lower than 0.1% (v/v); aqueous solutions were prepared in ultrapure water (Milli-Q Biocel A10 with pre-treatment via Elix 5, Millipore, Billerica, MA, USA). Non-aqueous solutions were prepared in ethanol. In this case, the final volume used was always lower than 0.1% (v/v).

2.2. Animals

Male and female Wistar rats (8–12 weeks) from our animal colony (Center for Neuroscience and Cell Biology, University of Coimbra) were housed in type III-H cages (Tecniplast, Italy) with irradiated corn cob grit bedding (Scobis Due, Mucedola, Italy), following environmental requirements with *ad libitum* acess to food (4RF21, Mucedola, Italy) and water and maintained at constant temperature (22 °C) and humidity with a 12 h light/dark cycle. Animal handling and sacrifice followed the procedures approved by the Federation of European Laboratory Animal Science Associations (FELASA). Animal handlers and the authors ACM and VAS are credited by FELASA (category C) for animal experimentation.

2.3. Mitochondria preparation

The livers were quickly removed and mitochondria were isolated by conventional methods (Moreno et al., 2007). Briefly, liver mitochondria were isolated using an homogenization media composed of 250 mM sucrose, 5 mM Hepes, 0.5 mM EGTA and 0.1% defatted bovine serum albumin containing medium (pH = 7.2). The mitochondrial pellet was washed twice and suspended in washing buffer (250 mM sucrose, 10 mM HEPES, pH = 7.4). Brain mitochondria were isolated by a previously published method (Rosenthal et al., 1987), using 0.02% digitonin to release mitochondria from the synaptosomal fraction. The whole brain except for the cerebellum was immediately removed, washed and homogenized at 4 °C in 10 mL of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml defatted BSA, pH 7.4) containing 5 mg of the bacterial protease (Subtilisin A, type VIII from *Bacillus licheniformis*, Sigma). Single brain homogenates were brought to 30 ml and then centrifuged at 746*g* (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 11,950*g* for 10 min. The pellet was then resuspended in 10 ml of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 11,950 xg for 5 min. Finally, the mitochondrial pellet was ressuspended in about 200 μl of resuspension medium. Mitochondrial protein was determined by the Biuret method calibrated with bovine serum albumin (Gornall et al., 1949).

2.4. Lipid peroxidation

Lipid peroxidation was evaluated following oxygen consumption using a Clark-type electrode in a glass chamber with magnetic stirring, at 30°. Mitochondria (1 mg and 0.8 mg for liver and brain, respectively) were pre-incubated for 3 min with resveratrol in 1 ml of medium containing 175 mM KCl and 10 mM Tris–Cl (pH = 7.4), supplemented with 2 μM rotenone (in the presence or absence of resveratrol) to inhibit mitochondrial respiration induced by endogenous substrates. Membrane lipid peroxidation was initiated by adding 1 mM adenosine diphosphate (ADP)/0.1 mM Fe^{2+} as oxidizing pair. Controls (basal levels) in absence of ADP/Fe^{2+}, were performed under the same conditions. Lipid peroxidation was also evaluated by thiobarbituric acid reactive species (TBARS) generation according to a modified procedure (Santos et al., 2001). Briefly, aliquots of mitochondrial suspension were obtained 10 min after the addition of ADP/Fe^{2+} and added to 0.5 ml of ice-cold 40% trichloroacetic acid. Then, 2 ml of aqueous thiobarbituric acid (0.67%) containing 0.001% of 2.6-di-*tert*-butyl-*p*-cresol was added to the samples. The mixtures were heated at 90° for 10 min and the supernatant fractions were collected and the absorbance read at 530 nm in a Spectronic 21 spectrophotometer (Bausch & Lomb, NY, USA). The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^{-5} mol^{-1} cm^{-1} and expressed as nmol TBARS/mg protein (Moreira et al., 2011; Santos et al., 2001).

2.5. Hydrogen peroxide generation

Hydrogen peroxide (H_2O_2) generation was measured fluorimetrically using a modification of a previously described method (Barja, 2002). Briefly, mitochondria were incubated with 1.5 mM of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 145 mM KCl, 30 mM Hepes, and 0.1 mM homovalinic acid and 6U/ml horseradish peroxidase. Resveratrol was incubated for 3 mins with mitochondrial fractions. The reactions were initiated by adding 5 mM/2.5 mM glutamate/malate. The fluorescence was measured with 312 nm as excitation wavelength and 420 nm as emission wavelength in Victor X3 Multilabel reader (Perkin Elmer, Waltham, USA). Hydrogen peroxide generation was calculated using a standard curve of H_2O_2, freshly prepared. The standards and the samples were incubated under the same conditions.

2.6. Mitochondrial respiration

Oxygen consumption was measured polarographically with a Clark-type Oxygen electrode connected to a recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were performed at 30 °C in 1 ml of standard respiratory medium with 1 mg of liver mitochondria or 0.5 mg of brain mitochondria. For liver mitochondria, the reaction medium used was composed by 130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4, 5 mM Hepes and 2 mM $MgCl_2$; for brain mitochondria, the reaction medium was composed of 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 0.01 mM EGTA (pH 7.4). Respiration was initiated with 5 mM glutamate/2.5 mM malate and state 3 started by adding ADP (150 nmol/mg protein for brain mitochondria and 125 nmol/mg protein for liver mitochondria). Respiration rates were obtained assuming an oxygen concentration of 236 nmol O_2/ml in the experimental medium at 30 °C (Rasmussen and Rasmussen, 2003). The respiratory state 2 (oxygen consumption before ADP addition – v2), state 3 (oxygen consumption in the presence of ADP – v3), state 4 (oxygen consumption after ADP phosphorylation – v4) and respiratory control ratio (RCR = state 3/state 4) were obtained according to Chance and Williams (1956). The ADP/O ratio is expressed as the ratio between the amount of ADP added and the oxygen consumed during v3. Resveratrol was pre-incubated with mitochondrial suspension for 3 min before ADP addition.

In order to determine the possible site of resveratrol interaction, mitochondrial fractions were frozen/thawed three times and oxygen consumption was assessed by using a Clark oxygen type electrode in 1 ml of reaction medium composed by 130 mM sucrose, 50 mM KCl, 2.5 mM KH_2PO_4, 5 mM Hepes and 2 mM $MgCl_2$ (liver mitochondria) or 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 0.01 mM EGTA (brain mitochondria). One or 0.8 mg mitochondrial protein was used for liver and brain preparations, respectively. Mitochondrial lysates were incubated with resveratrol for three minutes. The direct effects on complex I-sustained respiration were measured in the presence of 1 mM of NADH, while direct effects on complex II-sustained respiration were assessed in the presence of 5 mM of succinate and 2 μM rotenone.