



## 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<sub>2</sub>O<sub>2</sub> 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.

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### 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 macrocarpon*) 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

**Abbreviations:** ADP, adenosine diphosphate; ANT, adenosine nucleotide translocator; ATP, adenosine triphosphate; BBB, blood–brain barrier; DCPIP, 2,6-dichlorophenolindophenol; 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<sup>+</sup>, tetraphenylphosphonium.

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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 properties 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). Fukui 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

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-apoptotic 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* access 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 746g (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 11,950g 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 resuspended in about 200  $\mu$ l 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  $\mu$ M 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  $\text{Fe}^{2+}$  as oxidizing pair. Controls (basal levels) in absence of ADP/ $\text{Fe}^{2+}$ , 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/ $\text{Fe}^{2+}$  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  $1.56 \times 10^{-5} \text{ mol}^{-1} \text{ cm}^{-1}$  and expressed as nmol TBARS/mg protein (Moreira et al., 2011; Santos et al., 2001).

### 2.5. Hydrogen peroxide generation

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) 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  $\text{H}_2\text{O}_2$ , 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  $\text{KH}_2\text{PO}_4$ , 5 mM Hepes and 2 mM  $\text{MgCl}_2$ ; for brain mitochondria, the reaction medium was composed of 100 mM sucrose, 100 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$ , 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  $\text{O}_2$ /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  $\text{KH}_2\text{PO}_4$ , 5 mM Hepes and 2 mM  $\text{MgCl}_2$  (liver mitochondria) or 100 mM sucrose, 100 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$ , 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  $\mu$ M rotenone.

### 2.7. Mitochondrial transmembrane electric potential

The mitochondrial transmembrane electric potential ( $\Delta\psi_m$ ) was estimated by the transmembrane distribution of the lipophilic cation tetraphenylphosphonium ( $\text{TPP}^+$ ) by using a selective electrode prepared as previously described following the Kamo instructions (Kamo et al., 1979), using an  $\text{Ag}/\text{AgCl}_2$ -saturated electrode as reference.  $\text{TPP}^+$  uptake was measured from the decrease in  $\text{TPP}^+$  concentration in the medium as sensed by the electrode (Oliveira et al., 2004). Mitochondria (1 or 0.5 mg/ml) were incubated in the standard reaction medium supplemented with  $3 \mu\text{M}$   $\text{TPP}^+$  and energized by the addition of 5 mM glutamate/2.5 mM malate. After a steady-state distribution of  $\text{TPP}^+$  was reached (after about 1 min of recording), ADP was added and  $\Delta\psi_m$  fluctuations recorded. Resveratrol was pre-incubated with mitochondrial suspension for 3 min before ADP addition.

### 2.8. Complex I activity

The maximal activity of mitochondrial complex I was assessed in disrupted mitochondrial preparations after three cycles of freezing/thawing. One hundred and ninety  $\mu\text{l}$  of reaction medium (25 mM  $\text{KH}_2\text{PO}_4$ , pH = 7.5, 5 mM  $\text{MgCl}_2$ , 300  $\mu\text{M}$  KCN), supplemented with 4  $\mu\text{M}$  antimycin A, 3 mg/ml BSA, 60  $\mu\text{M}$  coenzyme  $\text{Q}_1$ , 160  $\mu\text{M}$  DCPIP and 10  $\mu\text{g}/\text{ml}$  of brain or liver mitochondria were transferred to a 96-well plate. One micromolar of complex I inhibitor rotenone, 25  $\mu\text{M}$  resveratrol or 5  $\mu\text{l}$  EtOH (vehicle) were added to the respective wells. Enzymatic activity of brain and liver preparations was measured through a decrease in absorbance of DCPIP after the addition of 100  $\mu\text{M}$  fresh-prepared NADH in a Victor X3 plate reader (Perkin Elmer, Waltham, USA) at 600 nm. Enzyme activity was calcu-

lated by using the slope achieved during the linear phase (15 cycles). Specific complex I activity was calculated through the difference with the basal activity in the presence of rotenone.

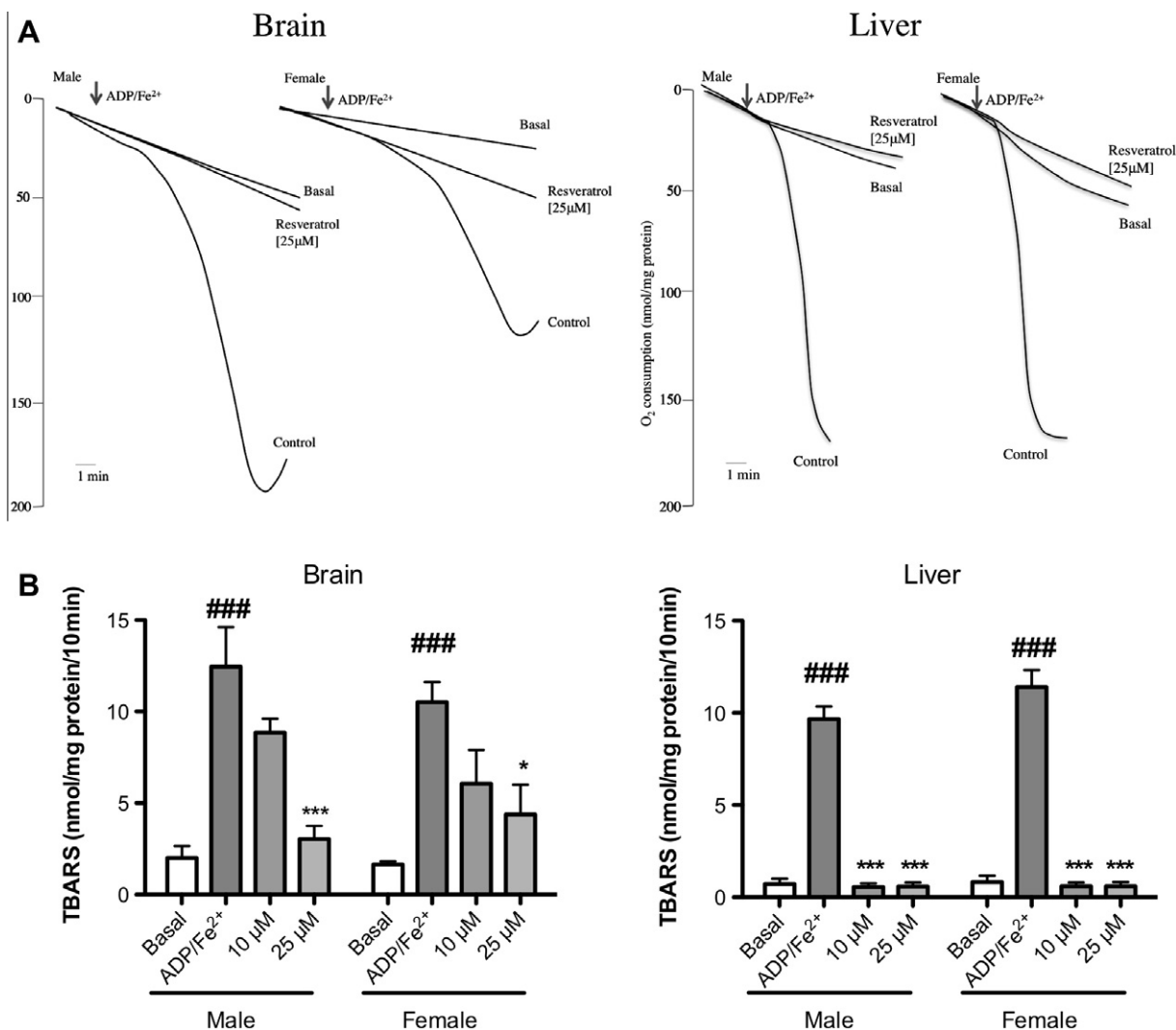
### 2.9. Statistical analysis

The results are representative of at least three different preparations from individual animals as described in the legends of the figures. Values are presented as mean  $\pm$  SEM and were compared by two-way ANOVA followed by Bonferroni post-test or t-student test. A value of  $p < 0.05$  was considered significant.

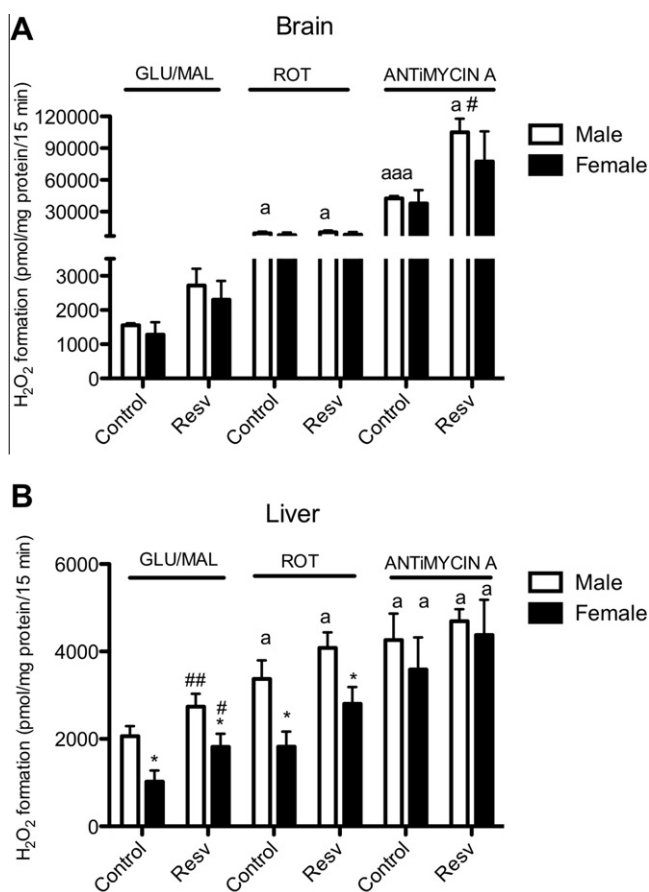
## 3. Results

### 3.1. Resveratrol decreases lipid peroxidation in brain and liver mitochondria

The effects of resveratrol on oxidative damage were assessed by mitochondria membrane peroxidation induced by the pro-oxidant pair  $\text{ADP}/\text{Fe}^{2+}$  (Fig. 1). This effect was evaluated by measuring oxygen consumption (Fig. 1A) and TBARS formation (Fig. 1B). In the absence of resveratrol and after the addition of  $\text{ADP}/\text{Fe}^{2+}$ , it is possible to observe a two-phase kinetic in oxygen consumption: an initial phase with a slower oxygen consumption that is followed



**Fig. 1.** Effects of resveratrol on membrane lipid peroxidation of rat and liver mitochondria induced by the pro-oxidant pair  $\text{ADP}/\text{Fe}^{2+}$ .  $\text{ADP}/\text{Fe}^{2+}$ -induced lipid peroxidation was evaluated following oxygen consumption resulting from membrane peroxidation (A) and TBARS colorimetric assay (B). Panel A represents a typical recording of mitochondrial preparations from brain and liver of male and female rats. The data in panel B represent the mean  $\pm$  SEM of four to six independent experiments. Statistical significance: ###  $p < 0.001$  compared with the respective basal levels, \*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared with the mitochondria in the presence of the pair  $\text{ADP}/\text{Fe}^{2+}$ .



**Fig. 2.** Effect of resveratrol on mitochondrial hydrogen peroxide ( $H_2O_2$ ) production. The production of hydrogen peroxide by the mitochondrial respiratory chain was evaluated as described in the materials and methods section. Mitochondrial fractions from brain (0.5 mg) and liver (1 mg) of male and female Wistar–Han rats were incubated with standard respiratory medium in the presence or absence of resveratrol. Basal levels of  $H_2O_2$  production were determined in the absence of mitochondrial substrates. Glutamate/malate (5 mM/2.5 mM) was used as substrate. Rotenone (1.0  $\mu$ M) and antimycin A (0.5  $\mu$ M) were used to increase  $H_2O_2$  production. Data represent mean  $\pm$  SEM from independent experiments. Statistical significance: \* $p < 0.05$ , when compared with respective gender preparation, # $p < 0.05$ , ## $p < 0.01$  when compared with respective control, <sup>a</sup> $p < 0.05$ , <sup>aaa</sup> $p < 0.001$  when compared with the absence of inhibitors.

by a rapid oxygen consumption phase (Fernandes et al., 2006). The initial phase is likely due to the time needed to generate the perferyl ion complex that has been suggested to be the responsible for the initiation of lipid peroxidation. The quickly oxygen consumption is due to the oxidation of the polyunsaturated fatty acid acyl chain of the phospholipidic membrane by ROS, leading to the propagation of lipid peroxidation (Fernandes et al., 2006). As observed in Fig. 1A, in both genders and both organs, the pro-oxidant pair  $ADP/Fe^{2+}$  induced an increase in oxygen consumption that is decreased in the presence of resveratrol to nearly no-oxidant basal levels. The data are in accordance with the results obtained by measuring TBARS formation. Pre-incubation with the highest concentration of resveratrol 25  $\mu$ M lead to a decrease in TBARS production in both organs and genders (brain mitochondria, males:  $12.5 \pm 2.5$  vs.  $3.0 \pm 0.7$ , brain mitochondria females:  $10.5 \pm 1.1$  vs.  $4.4 \pm 1.6$ ; male mitochondria, liver:  $9.7 \pm 0.7$  vs.  $0.6 \pm 0.2$ , female mitochondria, liver:  $11.4 \pm 0.9$  to  $0.6 \pm 0.2$  nmol TBARS/mg protein, without vs. with resveratrol) (Fig. 1B). For the lowest concentration used (10  $\mu$ M), resveratrol had no effect on brain mitochondrial fractions, as opposed to liver mitochondria, where protection against lipid peroxidation-induced TBARS generation was observed for both genders.

### 3.2. Resveratrol increases the mitochondrial $H_2O_2$ production in liver

Liver and brain mitochondria from male and female rats were incubated with resveratrol in order to investigate the effects of the compound on hydrogen peroxide generation by the respiratory chain.

In both genders, treatment of mitochondria with rotenone (a complex I inhibitor) or antimycin A (a complex III inhibitor) maximally induces  $H_2O_2$  generation, as expected (Chen et al., 2003; Leloup et al., 2006) (Fig. 2). When analyzing  $H_2O_2$  production in untreated mitochondria (Fig. 2), liver mitochondria from female rats have generally a lower basal  $H_2O_2$  generation when compared with preparations from male rats. The same effect was not observed in brain mitochondrial fractions. When resveratrol was added to the different experimental groups, some interesting effects were observed. When added to liver mitochondria, resveratrol increased  $H_2O_2$  generation when glutamate-malate was used alone (Fig. 2B), in both preparations from male and female rats. In brain mitochondrial fractions (male only), resveratrol amplified the effect of antimycin A in increased  $H_2O_2$  generation (Fig. 2A).

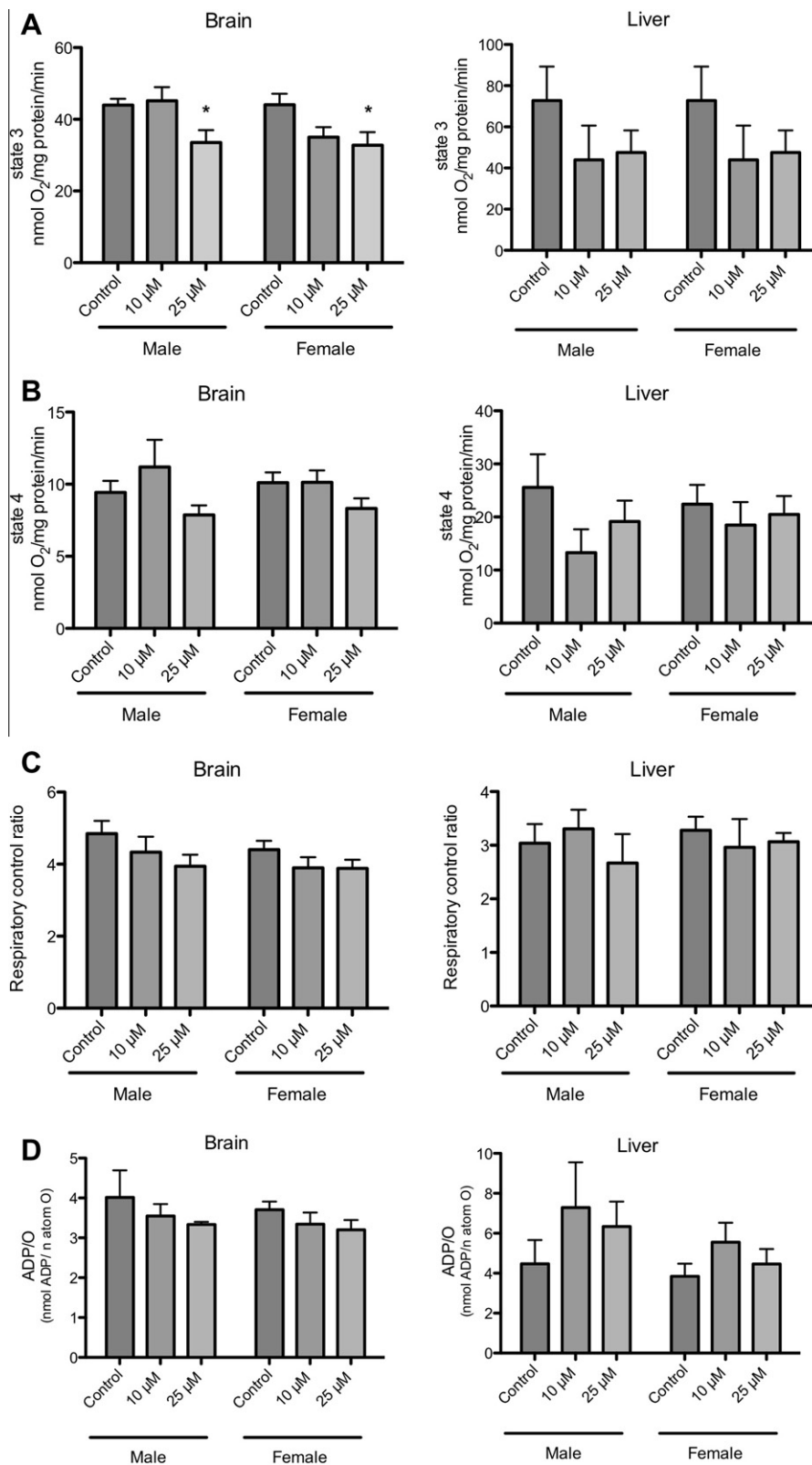
We also compared directly liver and brain mitochondrial fractions from both genders in terms of basal activity of glutathione peroxidase (GPx) and glutathione content. We have measured that in general, liver mitochondrial have increased GPx, with fractions from females having the highest activity ( $345.80 \pm 46.01$  to  $216.40 \pm 17.71$  U/mg protein of male fractions, Fig. S1). No differences were observed in terms of GSH, GSSG or GSH/GSSG ratio (Table S1).

### 3.3. Resveratrol decreases state 3 respiration in brain mitochondria

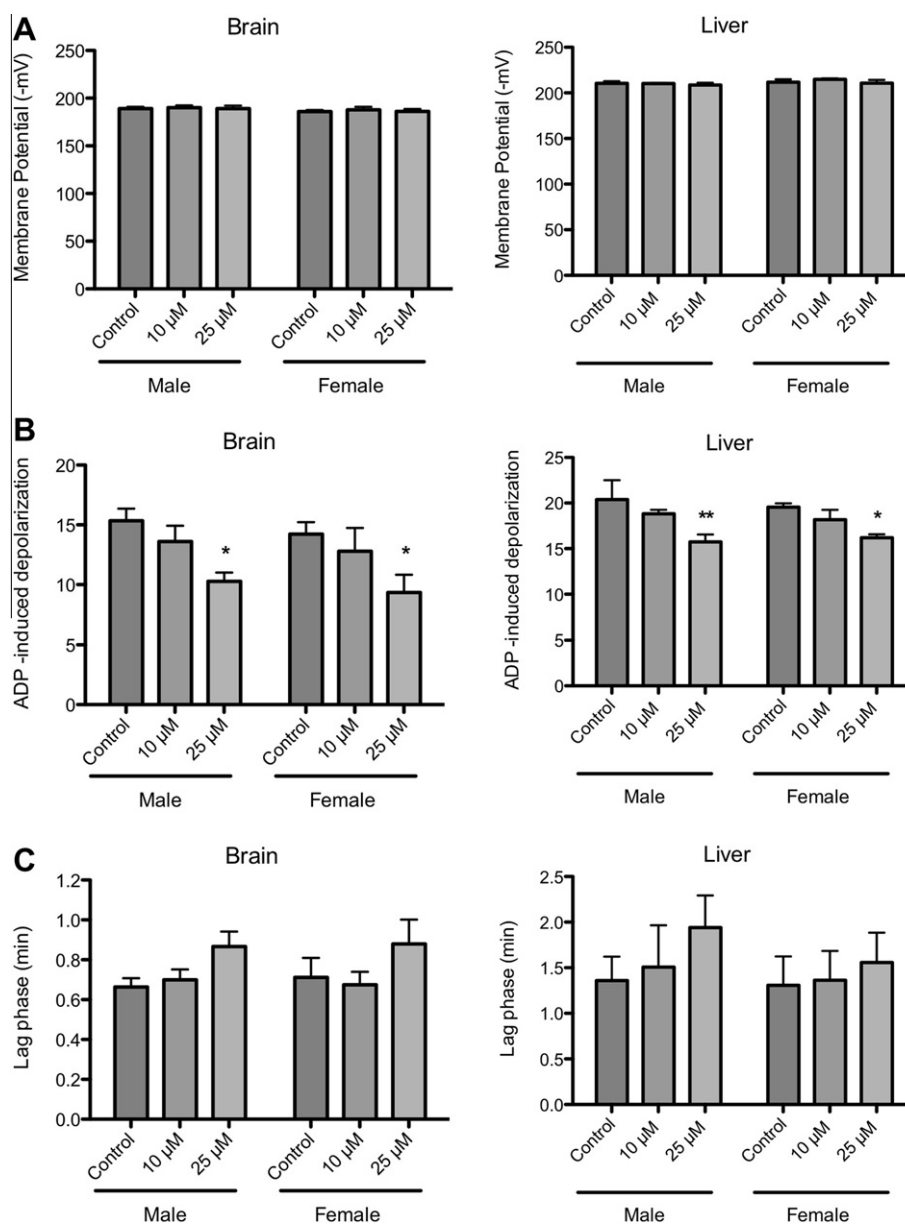
The next question in this work led us to investigate direct effects of resveratrol on liver and brain mitochondrial respiration for both genders. In the absence of resveratrol, no differences between genders were observed. The addition of resveratrol (10, 25  $\mu$ M) to freshly isolated brain mitochondria induced consumption decrease of state 3 respiration from  $44.0 \pm 1.8$  to  $33.5 \pm 3.5$  nmol  $O_2$ /mg protein for males and from  $44.1 \pm 3.0$  to  $32.8 \pm 3.7$  nmol  $O_2$ /mg protein for preparations from female animals (Fig. 3A). The alterations in respiration state 3 in brain mitochondria were not reflected in the RCR (Fig. 3C), may be due to the fact that the oxygen consumption has also has a tendency to be decreased by resveratrol during state 4 ( $p = 0.0591$ , Fig. 3B). No alterations were observed for liver mitochondria (Fig. 3). The ADP/O, a measure of the efficiency of oxidative phosphorylation, was not altered after resveratrol incubation for any of the groups studied (Fig. 3D).

### 3.4. Mitochondrial membrane potential during ADP phosphorylation is affected by resveratrol

Gender did not influence the different end-points regarding mitochondrial transmembrane electric potential (Fig. 4). Resveratrol did not affect the maximum transmembrane electric potential developed by mitochondria for both genders and organs (Fig. 4A). However, the depolarization induced by ADP is decreased by resveratrol at 25  $\mu$ M in brain and liver mitochondria (Fig. 4B). In brain mitochondria, the decrease was from  $15.3 \pm 1.0$  to  $10.3 \pm 2.7$  (preparations from males) and  $14.2 \pm 1.0$  to  $9.3 \pm 1.5$  (preparations from females), numbers in (–mV). When using liver mitochondria, the decrease was from  $20.4 \pm 2.1$  to  $15.7 \pm 0.8$  (preparations from male) and from  $19.5 \pm 0.4$  to  $16.2 \pm 0.4$  (preparations from females), results in (–mV). Although there is not a statistical difference, the lag phase showed a tendency to be increased in brain mitochondria from male rats when incubated with 25  $\mu$ M resveratrol ( $p = 0.0591$ ).



**Fig. 3.** Resveratrol effects on mitochondrial respiration: (A) mitochondrial state 3 respiration; (B) mitochondrial state 4 respiration; (C) respiratory control ratio (RCR); (D) ADP/O. Brain (0.5 mg) and liver (1 mg) mitochondria were incubated with 10 or 25  $\mu\text{M}$  of resveratrol for 3 min in 1 mL of respiration media supplemented with 5 mM glutamate and 2.5 ml malate. ADP (75 nmol for brain mitochondria and 125 nmol for liver mitochondria) was added to induce state 3 respiration. The RCR was calculated as the ratio between state 3 and state 4 respiration. The ADP/O was calculated as the number of nmol ADP phosphorylated per natom oxygen consumed during state 3. Data represent mean  $\pm$  SEM from four to six independent experiments. Statistical significance: \* $p < 0.05$ , when compared with the respective control.



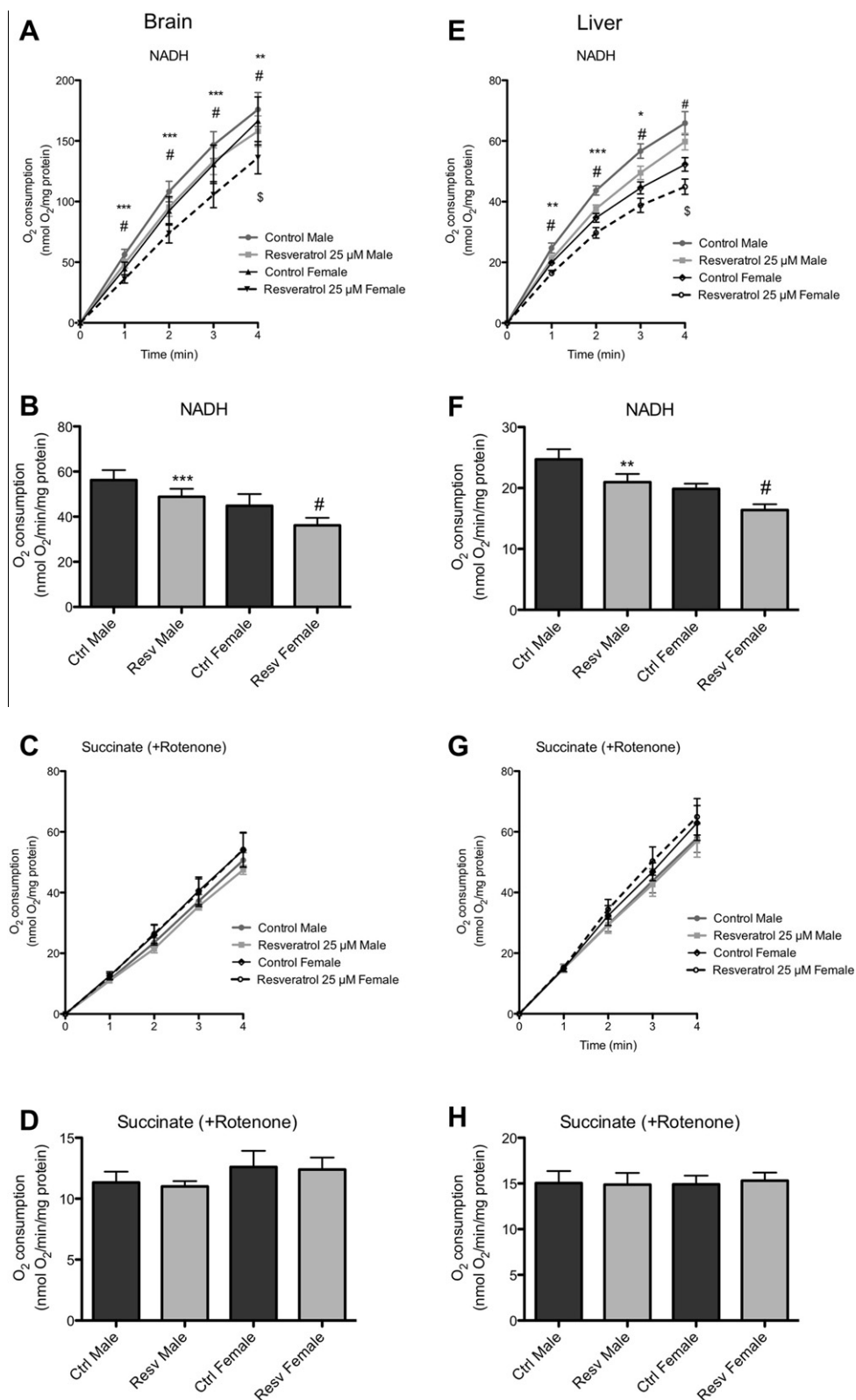
**Fig. 4.** Resveratrol effects on mitochondrial  $\Delta\Psi$  fluctuations: (A) Maximum mitochondrial membrane potential developed ( $\Delta\Psi$ ) after mitochondrial energization; (B) Depolarization induced by ADP addition; (C) Time elapsed during complete ADP phosphorylation (lag phase). Brain (0.5 mg) and liver (1 mg) mitochondria were incubated with 10 or 25  $\mu\text{M}$  of resveratrol for 3 min in 1 ml of standard respiratory medium. Mitochondria were energized with 5 mM glutamate and 2.5 ml malate and the phosphorylation cycle was initiated with ADP (75 nmol for brain mitochondria and 125 nmol for liver mitochondria). Data represent mean  $\pm$  SEM from four to six independent experiments. Statistical significance \* $p < 0.05$  and \*\* $p < 0.01$  when compared with the respective control.

### 3.5. Resveratrol demonstrates a direct effect on mitochondrial complex I

By using disrupted mitochondrial membranes and specific substrates, one can evaluate distinct sites of drug-induced toxicity. Resveratrol decreases oxygen consumption in frozen/thawed mitochondrial preparations from liver and brain when using NADH as substrate (Fig 5A and E). Preparations from brains of male rats showed a resveratrol-induced decrease in oxygen consumption from  $56.3 \pm 4.4$  to  $48.81 \pm 3.6$  nmol  $\text{O}_2$ / mg protein/ min; while a decrease from  $44.8 \pm 5.2$  to  $36.2 \pm 3.4$  nmol  $\text{O}_2$ /mg protein/ min was observed in brain preparations from female rats. When investigating liver mitochondrial preparations, resveratrol also inhibited complex I-sustained respiration in male ( $24.7 \pm 1.7$  to  $21.0 \pm 1.4$  nmol  $\text{O}_2$  consumed/mg/min) and female rats ( $19.9 \pm 0.9$  to  $16.4 \pm 0.9$  nmol  $\text{O}_2$  consumed/mg/min). Interestingly, NADH-

sustained oxygen consumption in both tissues was higher in preparations from males when compared with preparations from female animals. The same experiments were performed by using the complex II substrate succinate (in the presence of complex I inhibitor rotenone). In this case, resveratrol had no effect on succinate-induced mitochondrial respiration.

In parallel, the maximum activity of complex I was investigated using a colorimetric method. For both organs and genders, resveratrol decreased complex I specific activity. In brain mitochondria, a decrease of 22% and 11% was observed in preparations from male and female preparations, respectively. A higher magnitude effect was observed in liver mitochondria, where the activity in the presence of resveratrol decreased 73% for male preparations and practically 100% in the case of preparations from female rats. The maximal activity of complex I did not differ between genders.



**Fig. 5.** Resveratrol inhibition of mitochondrial complex I. Resveratrol effects on mitochondrial oxygen consumption in disrupted mitochondrial membranes was measured in a Clark-type electrode during 4 min after NADH (complex I, A, B, E, F) or succinate plus rotenone addition (complex II, C, D, G, H) in preparations from male and female Wistar rats. Data represent mean  $\pm$  SEM from five to eight independent experiments. Statistical significance: for male populations \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 when compared with the respective control and for female preparations # $p$  < 0.05 when compared with respective control, § $p$  < 0.05, compared with male.

#### 4. Discussion

There is a growing amount of studies focusing on natural compounds and their possible benefits in terms of human health. Although the antioxidant, anti-inflammatory and anti-tumoral effects of resveratrol (Delmas et al., 2006; Pervaiz and Holme, 2009) have been widely described, the cellular toxicity was not yet completely evaluated, especially regarding mitochondrial bioenergetics.

Following previous works showing protective effects of resveratrol on brain and liver (Morin et al., 2003; Park et al., 2010; Pervaiz and Holme, 2009), the present work investigates its toxicity on isolated brain and liver mitochondria at concentrations that act to decrease oxidative stress. The use of brain and liver mitochondria was due to the fact that resveratrol is able to cross the BBB (Wang et al., 2002) and it is highly metabolized in the liver (Walle, 2011), respectively. The present work highlights the relevance of using mitochondrial-isolated fractions from male and female rats, which is very rare in the literature. Although we did not observe basal differences in mitochondrial preparations from both genders in most of the parameters investigated, except when measuring maximal respiration due to freezing/thawing cycles, it is known that estrogens have protective effects, enhancing antioxidant defenses and decreasing mitochondrial dysfunction (Borras et al., 2007; Eskes and Haanen, 2007; Vina et al., 2011). For instance, in liver mitochondrial the  $H_2O_2$  levels are lower in females than in males (Fig. 2). This can be due to the activity of GPx, which is augmented in liver preparations from females (Fig. S1), although no differences were measured in brain or in reduced and oxidized GSH (Table S1). Female rats have better mitochondrial capacity showing less oxidative stress generation based in estrogen levels (Borras et al., 2003; Guevara et al., 2011). Sex hormones have neuroprotective effects, which have also been shown to increase mitochondrial efficiency (Nilsen and Brinton, 2004). Besides, the differences in gender, we aimed to discuss the different effects of resveratrol and to observe its effects on mitochondrial function for concentrations known to act as antioxidant. Our results confirm previous studies (Aftab et al., 2010; Ghanim et al., 2011; Kelsey et al., 2010; Toklu et al., 2010) regarding the antioxidant properties of resveratrol (Fig. 1). Our data shows that resveratrol indeed acts as an antioxidant by the measurement of TBARS and by following oxygen consumption during lipid peroxidation. In both cases (Fig. 1), resveratrol was able to decrease lipid peroxidation, disregarding the gender. Resveratrol increases  $H_2O_2$  generation in both male and female liver mitochondria in the presence of the substrate (glutamate-malate) alone. The effect is not observed with brain mitochondria (Fig. 2), except when mitochondria are incubated with complex III inhibitor, antimycin A (Fig. 2A). The resveratrol effects on liver mitochondria in terms of  $H_2O_2$  generation can be explained by a direct action on complex I, or instead, as previously reported, by modulation of mitochondrial Manganese-superoxide dismutase (Robb et al., 2008), increasing the flux of hydrogen peroxide production.

Concerning effects of resveratrol on mitochondrial bioenergetics, Zini et al., showed that resveratrol inhibits brain mitochondrial respiratory chain at the complexes I–III span (Zini et al., 1999). In this work, the enzymatic activity of ubiquinol cytochrome c reductase in the presence of resveratrol was decreased by 20% (Zini et al., 1999). The authors suggest that resveratrol can preserve mitochondrial functions by three different mechanisms: antioxidant-mediated effect, complex III direct effect and membrane stabilizing effects (Zini et al., 2002). A decrease of state 3 in brain mitochondria of female and male rats was observed in the presence of resveratrol (Fig. 3). Since the maximal transmembrane electric potential developed was not affected by resveratrol (Fig. 4), this

**Table 1**

Effect of resveratrol on brain and liver mitochondrial respiratory complex I maximum activity. Activity expressed as nmol DCPIP/min/mg protein.

	Male		Female	
	Ctrl	Resv	Ctrl	Resv
Brain	135.7 ± 16.82	106.6 ± 13.05*	112.4 ± 4.724	99.61 ± 4.3*
Liver	49.74 ± 12.14	13.65 ± 13.27*	52.12 ± 4.97	0.00 ± 12.47*

Data are the mean ± SEM of four independent experiment. Statistical significance: \* $p < 0.05$  when compared with the respective control.

suggests that the respiratory chain was not largely inhibited, although we cannot exclude that increased  $H_2O_2$  generation results from inhibition of complex I and/or III. Instead, the results from both respiration (Fig. 3) and transmembrane electric potential (Fig. 4) suggest an effect on the phosphorylative system, including in the adenine nucleotide transporter, the phosphate transporter, or even with the ATP synthase (Gledhill and Walker, 2005). Interestingly, inhibition of state 3 respiration was not observed in liver mitochondria. Although the reason for this difference is still unclear, it may have to do with differential accumulation of resveratrol in mitochondrial membranes from both gender groups or with the different number of target complex subunits present in both preparations.

We further described that resveratrol inhibits mitochondrial respiration induced by NADH, but not succinate, in freeze/thawed preparations, which indirectly allows to study the maximal respiration rate (Fig. 5). In accordance, resveratrol also reduced complex I specific activity as followed by the reduction of DCPIP (Table 1). This data demonstrates that resveratrol acts as a complex I inhibitor in mitochondrial preparations from both organs and genders. Interestingly, although resveratrol induced a larger inhibition of complex I activity in liver freeze/thawed preparations, the same type of effect was not visible when using intact mitochondria (Fig. 3). This may imply that the site of resveratrol inhibition on complex I faces the matrix side, being more accessible when mitochondrial membranes are disrupted to perform specific assays with NADH. The data also supplies evidence that resveratrol may also target complex I besides the already described complex III (Zini et al., 1999), thus contributing to increase the generation of hydrogen peroxide by the respiratory chain (Fig. 2).

The results may also suggest that increased generation of  $H_2O_2$  in liver and, under some conditions, in brain mitochondria by resveratrol can act to stimulate several signaling pathways, including those related with antioxidant defenses. The effects of resveratrol in the metabolism of different organs and gender-mediated effects should be explored in further detail to determine potential toxic effects and mechanisms by which resveratrol is described to cell fitness.

#### Conflict of Interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2012.11.031>.

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