J Bioenerg Biomembr (2011) 43:101–107
DOI 10.1007/s10863-011-9349-4

Protective effects of resveratrol on calcium-induced oxidative stress in rat heart mitochondria

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Received: 1 November 2010 / Accepted: 24 December 2010 / Published online: 30 March 2011

Abstract Trans-resveratrol is a nutraceutical with known antioxidant, anti-inflammatory, cardioprotective, and anti-apoptotic properties. The aim of this study was to evaluate the effects of resveratrol on heart mitochondria. Resveratrol significantly decreased Fe^{2+} + ascorbate oxidant system-induced lipid peroxide levels, preserved physiological levels of glutathione, and increased nitric oxide (NO) levels in mitochondria. Under calcium-mediated stress, there was a 2.7-fold increase in the NO levels, and a mild decoupling in the mitochondrial respiratory chain. These results provide a mechanism for and support the beneficial effects of resveratrol under pathological conditions induced by oxidative stress and calcium overload. In addition, these findings underscore the usefulness of resveratrol in the prevention of cardiovascular diseases.

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Keywords Cardioprotection · Resveratrol · Mitochondria · Oxidative stress · Calcium-overload

Introduction

Resveratrol is a polyphenolic phytoalexin (trans-3,4′,5-trihydroxystilbene) found in red wine, peanuts, grapes, berries (especially mulberries), various herbs, and propolis (bee products). It mediates diverse biochemical and physiological actions that include the ability to protect the brain, kidneys, and heart from ischemic injury (Cannon et al. 2004). It has estrogenic, antiplatelet, and anti-inflammatory properties (Blake and Ridker 2001). The cardioprotective effects of resveratrol have been attributed to its antioxidant and anti-inflammatory properties (Clarke et al. 1991). Resveratrol's antioxidant properties are due to its ability to scavenge superoxide radical ($O_2^{\cdot -}$) and its enhancing effect on expression/activity of oxidative enzymes (Jia et al. 2008; Fan and Mattheis 2001). In the ischemic-reperfused heart, resveratrol has been found to induce NO synthesis and to lower oxidative stress (Mukamal et al. 2003). The ability of resveratrol to stimulate NO production during ischemia-reperfusion is thought to play a crucial role in its heart protective activity (Clarke et al. 1991). Such remarkable properties have elicited a vast interest in the identification of resveratrol-inhibited enzymes and others whose activation is enhanced (Pirola and Fröjdo 2008).

Mitochondrial dysfunction and subsequent production of reactive oxygen species (ROS) are contributors to cardiac failure. In fact, the mitochondrial electron transport chain (ETC) is a source of ROS during heart ischemia reperfusion due to electron leakage at complexes I and III for producing $O_2^{\cdot -}$ and H_2O_2 (Paradies et al. 2004; Petrosillo et al. 2003). Additionally, there are quantitative and qualitative changes

in mitochondria during hypertension and cardiac hypertrophy (Leary et al. 2002). Resveratrol administration protects the heart by inducing pharmacological preconditioning and prevents myocardial reperfusion injury, presumably by targeting the mitochondrial permeability transition (MPT) pore. Xi et al. (2009) suggested that pharmacological preconditioning is due to the translocation of glycogen synthase kinase 3β from cytosol to mitochondria. Additionally, another explanation is that resveratrol may induce MPT closure and reduce ROS production in mice that overexpress human aldose reductase (Ananthakrishnan et al. 2009).

Calcium overload is an important factor in heart mitochondrial dysfunction and could result in pathological conditions such as ischemia-reperfusion (Gunter et al. 1994). The increase in mitochondrial calcium concentration stimulates ROS production, leading to MPT pore opening, cytochrome *c* release, and apoptosis (Piper et al. 1985; Borutaite et al. 2003).

Because of the participation of calcium overload and oxidative stress in heart mitochondria dysfunction and the cardioprotective effect of resveratrol under pathological conditions in which mitochondrial dysfunction is involved, the goal of the work reported in the present paper was to determine whether a physiologically achievable concentration of resveratrol can decrease mitochondrial dysfunction upon calcium overload or Fe^{2+} + ascorbate exposure.

Materials and methods

Reagents

All chemicals were of the highest purity available commercially and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation and purification of rat heart mitochondria

Adult, male Wistar rats (weighing 350–450 g) were sacrificed by decapitation according to our Institutional Animal Care and Use Committee guidelines and Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). Heart mitochondria were isolated according to the protocol described by Moreno-Sánchez and Hansford (1988). Rat hearts were finely minced and placed in an ice-cold Sucrose 250 mM, HEPES 10 mM, EGTA 1 mM, pH 7.4 (SHE) isolation medium. Nagarase (0.4 mg/heart) was added to the homogenate, which was then incubated on ice for 9 min. To remove the protease, the homogenate was centrifuged for 5 min at 750×g. The pellet was resuspended in SHE buffer and homogenized with Teflon potter. The homogenate was centrifuged at 314×g at 4 °C for 10 min to remove debris. The supernatant was decanted and centrifuged at 6,350×g for 10 min. The mitochondrial pellet was resuspended in isolation buffer and placed on ice during the experiments. Protein concentration was determined by the Biuret method (Gornall et al. 1949) by using bovine serum albumin as a standard.

Induction of oxidative stress

Oxidative stress was induced by incubating mitochondria for 5 min with 5.0 μM of free calcium, which was added from a stock solution of $CaCl_2$. Calculations for determination of the calcium required to achieve the free concentration desired for this cation were performed with WinMax software, which considers ionic strength in the reaction mixture and chelant concentration among other parameters (Patton 1999). Lipoperoxidation was stimulated by treatment with 0.25 mM $FeCl_2$ and 0.5 mM ascorbic acid for 15 min.

Measurement of mitochondrial oxygen consumption and swelling

Mitochondrial oxygen consumption (1 mg protein/ml) was measured polarographically by employing a Clark-type electrode (YSI, model 5,300) in KME buffer (120 mM KCl, 0.5 mM EGTA 20 mM MOPS: pH 7.4). Oxygen uptake at a resting state (state 4) was determined in the presence of succinate (10 mM) plus 2 μM rotenone to inhibit reverse electron transfer from complex II to complex I. Oxygen consumption in phosphorylating state (state 3) was stimulated by the addition of 300 μM ADP. Respiratory control ratios (RCRs) were calculated from the rate of oxygen consumption in state 3 divided by the rate of respiration in state 4. Oxygen uptake was expressed in nanoatoms of oxygen/min/mg of protein. The mitochondrial permeability transition pore (PTP) was measured in intact mitochondria (0.3 mg/ml) isolated from cardiac samples and was suspended in a buffer containing 120 mM KCl, 10 mM Tris–HCl, 5 mM KH_2PO_4, and 20 mM MOPS. Mitochondrial swelling was assessed spectrophotometrically as a decrease in absorbance at 520 nm (A_{520}) (Wang et al. 2005).

Determination of total glutathione (GSH)

Assessment of glutathione levels was conducted by spectrophotometric analysis of Dystrobrevin beta (DTNB) reduction, as described previously by Åkerboom and Siess (1981), following the manufacturer's recommendations (Sigma-Aldrich). Additionally, we used Ellman's reagent DTNB (5′,5-dithiobis-2-nitrobenzoic acid), which reacts with GSH to form a product with an absorption maximum at 412 nm. A calibration curve with GSH was performed for each experiment. Measurements were conducted on an

enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio Rad, Benchmark model). In addition, GSH levels were assessed after pre-treatments with Fe^{2+} + ascorbate and/or resveratrol for 5 min.

Determination of mitochondrial lipid peroxidation (LPO)

Changes in LPO levels were determined by thiobarbituric acid assay as described by Buege and Aust (1978). A 1-mg aliquot of mitochondrial protein suspension was mixed with 2 ml of acid solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl. The mixture was heated in boiling water for 15 min, ice-cooled, and centrifuged at 4,410×g for 5 min. Absorbance of the supernatant was measured at 532 nm with a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. Data were expressed as nanomoles of thiobarbituric acid reaction substances (TBARS)/mg protein and calculated by utilizing the molar extinction coefficient for malondialdehyde of $1.56\times10^5\ M^{-1}cm^{-1}$.

Measurement of NO

NO levels were estimated by the Griess reaction that measures nitrite quantities, as described by Green et al. (1982). Griess reagent containing 0.1% N-(1-naphthyl) ethylendiamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid was added to the mitochondrial suspension. The red-violet diazo dye formed was measured spectrophotometrically at 546 nm. The competitive inhibitor of the NOS, N-monomethyl-L-arginine (L-NMMA) (100 μM) and the NO donor, S-nitroso-N-acetylpenicilamine (SNAP) (50 μM) were used as negative and positive controls, respectively.

Statistical analysis

Data are presented as mean±SEM. To assess statistical differences, data were analyzed by means of the Student *t* test employing Sigma Plot v.9.0 software. Values of $p<0.05$ were considered statistically significant.

Results

Effect of resveratrol on mitochondrial respiration

To test the effect of resveratrol, we measured the oxygen (O_2) consumption and respiratory control ratios (RCR) of rat heart mitochondria. In the absence of calcium (Ca^{2+}) or Fe^{2+} + ascorbate, resveratrol (100 μM) increased mitochondrial respiration in both 3 and 4 states (Fig. 1, panels B and A, respectively). During state 4 (Fig. 1, panel A), resveratrol increased O_2 consumption by 29% compared to

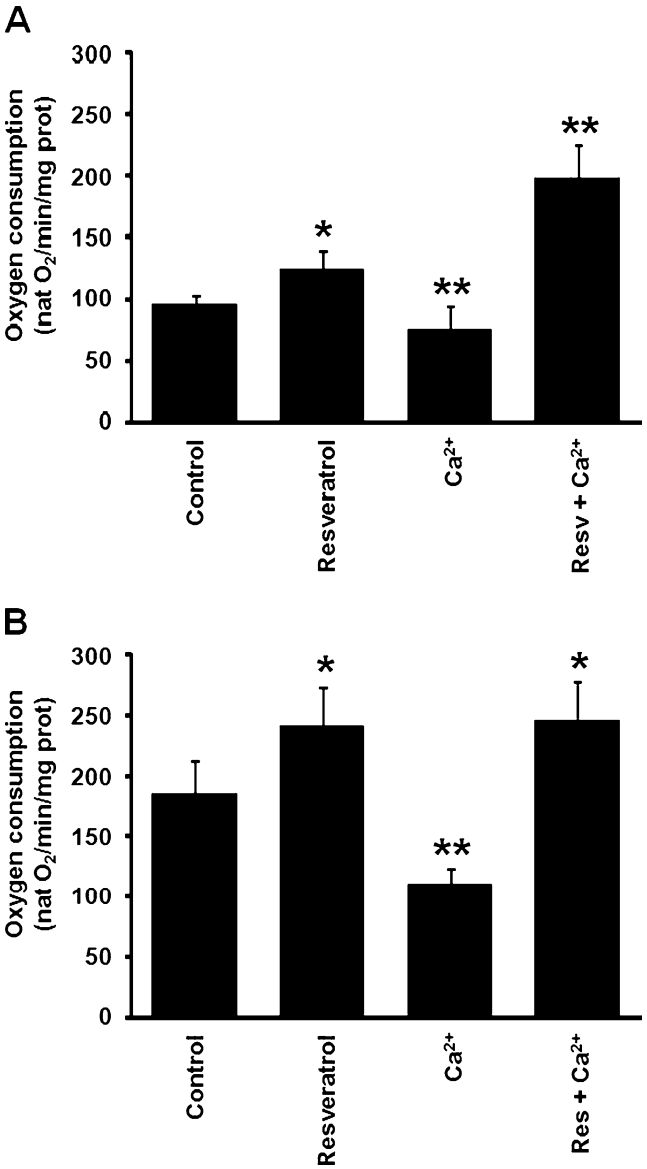

Fig. 1 Effect of resveratrol and Ca^{2+}-mediated changes in O_2 utilization. Heart mitochondria (1 mg/ml) were incubated in medium (KME), and succinate (10 mM) and rotenone (2 μM) were added. **a** state 4 oxygen consumption (in the absence of ADP); **b** state 3 oxygen utilization (mitochondria were energized by ADP (300 μM) (Materials and Methods). Concentration of resveratrol (Resv) was 100 μM; Ca^{2+}= 5 μM. Data represent the mean ± SEM. *$p<0.05$; **$p<0.01$ ($n=4$)

that in control mitochondria (123.2 vs. 95.3 nat O_2/min/mg protein). The addition of Ca^{2+} (5 μM) decreased O_2 consumption by 24% compared to the level in controls, while pre-incubation with resveratrol for 5 min prior to addition of Ca^{2+} prevented the decrease in O_2 utilization more than in those observed with resveratrol alone. In state 3 respiration (Fig. 1, panel B), resveratrol increased O_2 consumption by 30% compared with control mitochondria (241.25 vs. 185.26 natO_2/min/mg protein). Addition of Ca^{2+} inhibited state 3 respiration by 40%, while preincu-

bation with resveratrol prevented the decrease in O_2 consumption, and now it was nearly identical to those observed in control and resveratrol-treated mitochondria. These data indicate that resveratrol protects mitochondria from the effects of Ca^{2+} overload. Table 1 shows RCR, which is a measure of mitochondrial integrity and function. Control values in the absence of Ca^{2+} were 1.93 ± 0.23, and no significant change was observed when resveratrol was present due to a parallel increase in respiration in both state 3 and state 4. In the presence of Ca^{2+}, RCR decreased by 21%, which can be attributed to a decrease of higher magnitude in state 3 respiration than that observed in state 4 (Fig. 1, panels B and A, respectively). Although the decrease in RCR was even more pronounced in the presence of Ca^{2+} plus resveratrol (35%) (Table 1), this result can be attributed to mitochondrial uncoupling because we observed an increase in state 4 respiration under these conditions (Fig. 1, panel A). We also investigated mitochondrial swelling (Table 1) because a direct contribution of the mitochondrial permeability transition pore (PTP) under ischemia-reperfusion injury was shown (Xi et al. 2009). The addition of Ca^{2+} induced swelling by approximately 12%. Moderate, but consistent protection (6%), was achieved when resveratrol plus Ca^{2+} was present in the buffer containing mitochondria. No significant effect on swelling was observed by adding resveratrol alone (Table 1).

Effect of resveratrol on mitochondrial glutathione levels

To assess the influence of resveratrol on the redox state of mitochondria, we examined mitochondrial GSH levels (Fig. 2). Basal levels of total GSH were 4.6 nmoles/mg of protein in control mitochondria. No significant changes in GSH were observed in the presence of resveratrol. Under conditions of Ca^{2+} overload, total GSH levels decreased by 42% when compared with the control. Resveratrol prevented Ca^{2+}-mediated decrease in GSH levels, a finding nearly the same as that in control mitochondria (Fig. 2). These data suggested to us that resveratrol confers

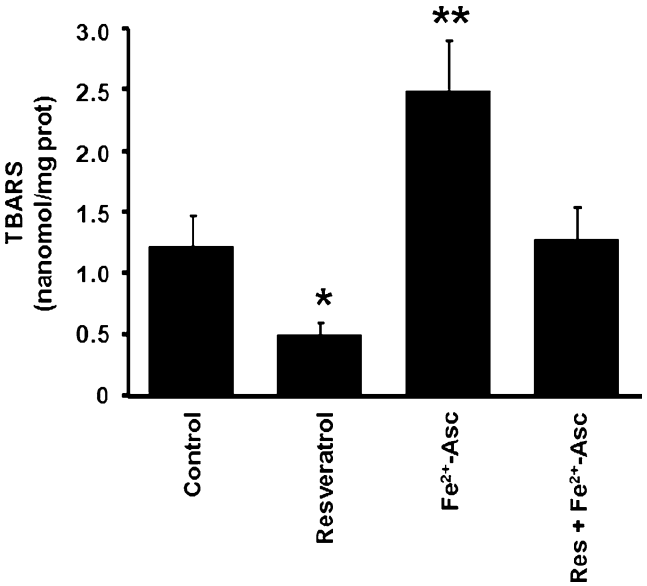

Fig. 2 Resveratrol inhibits Ca^{2+}-mediated GSH loss. One mg of the mitochondria was incubated with each treatment as described in the text. Ca^{2+}=5 μM; resveratrol=100 μM. Data represent the mean± SEM. *p<0.05 (n=4)

protection against Ca^{2+}-induced GSH loss, which could result in an oxidative environment in mitochondria.

Effect of resveratrol on the mitochondrial LPO

Changes in the levels of oxidized lipids are a measure of mitochondrial oxidative stress (Cortés-Rojo et al. 2009). To test the antioxidant properties of resveratrol, we examined lipid peroxide (LPO) levels (assessed via thiobarbituric acid reaction substances; TBARS) after treatment of mitochondria with the Fe^{2+} + ascorbate system (Fig. 3). Resveratrol decreased basal mitochondrial LPO levels by 40%. When Fe^{2+} + ascorbate were added to mitochondria, TBARS levels increased two-fold compared to the levels in mock-treated controls. Following treatment of mitochondrial suspensions with resveratrol prior to Fe^{2+} + ascorbate addition, LPO levels were similar to those of the controls (Fig. 3). These results suggested to us that resveratrol either

Table 1 Respiratory control ratio and swelling of heart mitochondria

Respiratory control ratio in rat heart mitochondria			
Control	Resveratrol (100 μM)	Ca^{2+} (5 μM)	Resveratrol/Ca^{2+} (100 μM/5 μM)
1.93±0.23 (100%)	1.97±0.19 (102%)	1.52±0.33 (79%)	1.26±0.27 (65%)
Swelling in rat heart mitochondria (% of inhibition)			
Control	Resveratrol (100 μM)	Ca^{2+} (5 μM)	Resveratrol/Ca^{2+} (100 μM/5 μM)
0	2%	12%	6%

Respiratory control ratios were obtained as described in Materials and Methods. Positive controls of swelling were assayed in the presence of 10 μM $CaCl_2$; negative controls were assayed in the presence of 1 μM cyclosporin A (data not show). Data of swelling results are representative of one experiment (n=10). RCR results represent the mean±SEM, n=4

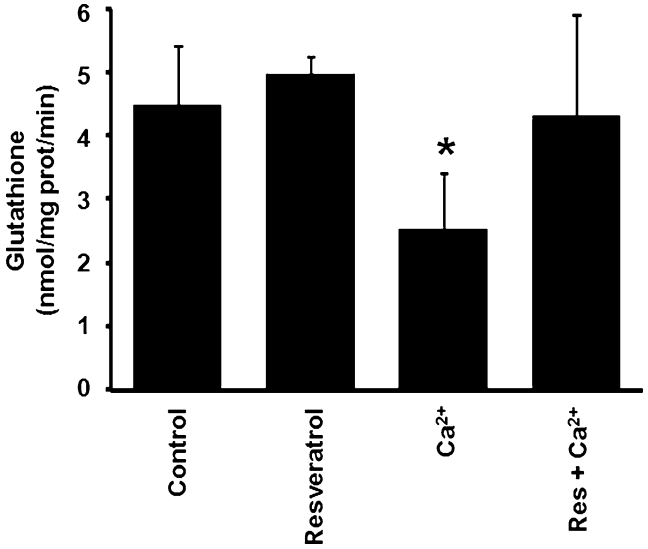

Fig. 3 Quantification of mitochondrial lipoperoxidation. Lipoperoxidation was induced with $FeCl_2$ (0.25 mM) and ascorbic acid (Asc) (0.5 mM). Treatments were carried out for 15 min in phosphate buffer containing mitochondria (1 mg/ml of protein) with gentle shaking. Resveratrol=100 μM. Data represent the mean±SEM. *$p<0.01$; **$p<0.001$ ($n=5$)

directly protect lipids from oxidation or inhibits mitochondrial ROS generation, thereby protecting the integrity of lipids in mitochondrial membranes.

Effect of resveratrol on mitochondrial NO production

NO in the mitochondria is generated by the mitochondrial nitric oxide synthase (mtNOS) isoform, which can regulate mitochondrial respiration and energy production (Ghafourifar and Saavedra-Molina 2006). To test the effect of resveratrol on NO production, mitochondria were incubated in the presence or absence of resveratrol. Preincubation with resveratrol increased (~75%) NO production compared to that in control mitochondria [1.25±0.35 vs. 0.71±0.13 μmol/mg protein, respectively (Fig. 4)]. In controls, L-NMMA (100 μM) decreased NO synthesis [55% and, as expected, NO donor SNAP (50 μM)] increased NO levels by three-fold. To examine whether resveratrol exerted some effect on NOS activity, we incubated the mitochondria suspensions with resveratrol plus L-NMMA. Data show a significant decrease in a change of diminution of 50% (0.63±0.13 vs. 1.25±0.35 μmoles/mg protein) in NO levels compared to the levels in mitochondria incubated with resveratrol.

Next, we investigated whether Ca^{2+} has an effect on mitochondrial NO production. Results show that Ca^{2+} increased the level of mitochondrial NO by nearly 2.3-fold (1.63±0.38 vs. 0.71±0.13 μmol/mg protein, respectively) (Fig. 4). In the presence of Ca^{2+} + resveratrol, mitochondrial NO production increased significantly by 2.7-fold. Treatment with Ca^{2+} + L-NMMA+resveratrol decreased NO levels compared to those in resveratrol-treated mitochondria. These data may indicate that resveratrol plays an important role in NOS activity, and, therefore, in mitochondrial NO production.

Discussion

Biologically active trans-resveratrol has unidentified properties by which it protects a variety of tissues, in brain, kidneys and heart from ischemia-reperfusion injury (Raval et al. 2008). Resveratrol also confers neuroprotection via the Sirtuin 1-Uncoupler 2 protein pathway (Della-Morte et al. 2009), as well as decreases the toxic effects of chemotherapeutic drugs, such as doxorubicin, by decreasing mitochondrial ROS production (Danz et al. 2009). Furthermore, resveratrol was demonstrated to modulate respiration and to decrease the respiratory control ratio of mitochondria isolated from rat brain and liver (Zini et al. 1999; Zhang et al. 2005). Here we examined the effects of resveratrol on the consequences of Ca^{2+} overload by using heart mitochondria. To our knowledge; there are no published studies as yet on this topic.

Our results demonstrate a protective effect of resveratrol on heart mitochondria upon Ca^{2+} overload (Table 1). We observed an inhibitory effect of Ca^{2+} overload on state 4 respiration (Fig. 1), which suggested to us that, through

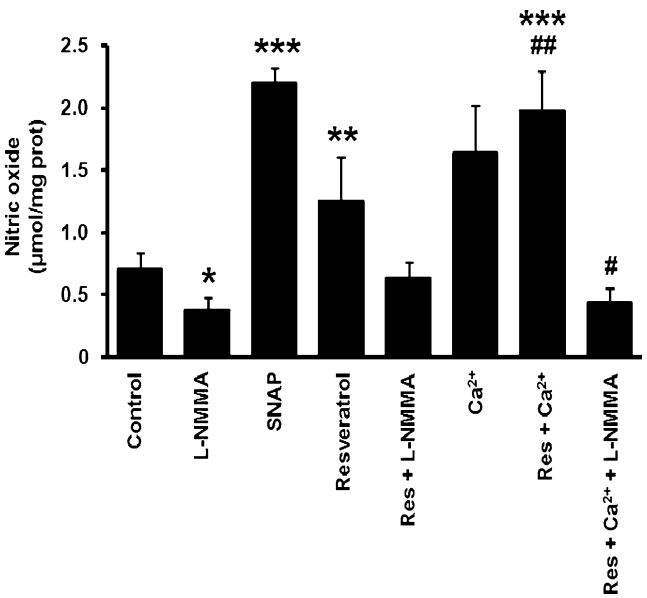

Fig. 4 Effects of resveratrol on nitric oxide levels. Mitochondria (1 mg/ml) were used as in Materials and Methods. Reactions were allowed to proceed for 10 min at room temperature. L-NMMA=100 μM; SNAP=50 μM. Ca^{2+}=5 μM. Resv, resveratrol (100 μM). Data represent the mean±SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. resveratrol. $n=6$

respiratory complexes, high concentrations of Ca^{2+} affect electron transfer. We also observed an inhibition of respiration in state 3 (Fig. 1) which provides evidence that some components, such as the F_1F_0-ATP synthase, phosphate carrier and/or adenine nucleotide translocator activities, are also modulated by Ca^{2+}. Most importantly, resveratrol ameliorated the observed effect of Ca^{2+} on heart mitochondria. This effect could be related to a slight uncoupling of the respiratory chain that decreases mitochondrial ROS production due to a drop in transmembrane potential and decreased concentration of semiquinone species at quinone redox sites of ETC. Another possibility is that resveratrol modulates the permeability transition pore opening in response to Ca^{2+} overload. The latter is supported by Ananthakrishnan et al. (2009) who reported that an inhibitor of aldose reductase, a known pro-oxidant enzyme, decreased the mitochondrial permeability transition pore opening in response to high concentrations of Ca^{2+}. It is also possible that resveratrol modulated NADH production via Krebs cycle dehydrogenases, which have been shown to be activated by increased intra mitochondrial Ca^{2+} levels, as suggested by Griffiths and Rutter (2009).

Alterations in GSH concentration is a well-established marker of oxidative stress (Lu and Armstrong 2007). Our results show that resveratrol was able to protect mitochondrial GSH levels even in the presence of Ca^{2+} overload (Fig. 2). At this time, based on these and studies by others, we are unable to discern whether the resveratrol itself functions as a mitochondrial antioxidant, or directly regulates the activity of respiratory complexes implicated in ROS generation. It is also possible that resveratrol creates an intra-mitochondrial environment that increases the activity of the major antioxidant enzymes, including Mn superoxide dismutase and/or glutathione peroxidases and reductases, and thereby decreases ROS levels and reestablishes physiological GSH levels.

Protection against lipoperoxidation is crucial for ETC function, as reported recently (Cortés-Rojo et al. 2007, 2009). Our present results showed that resveratrol decreased lipid peroxide levels. We observed a decrease in state 3 respirations by resveratrol in the presence of Ca^{2+} overload, which suggested to us that the anti-lipoperoxidative effect of resveratrol could be related to decreased oxidative stress. These findings become important because mitochondria contain the machinery necessary to carry out β-oxidation, especially in the heart, in which lipids are the most important energy source. Our data are in line with early reports, showing that resveratrol decreases lipid oxidation in various tissues, e.g., the liver, heart, brain, and testes (Kim et al. 2002; Rifici et al. 1999; Karlsson et al. 2000), and that in ischemia-reperfusion, heart injury-associated ROS production and lipoperoxidation are linked to mitochondrial respiratory complexes I and III (Paradies et al. 2004; Petrosillo et al. 2003).

NO was shown to regulate mitochondrial respiration and energy production (Ghafourifar and Richter 1997; Ghafourifar and Saavedra-Molina 2006; Calderón-Cortés et al. 2008). Resveratrol increases in mitochondrial NO (mtNO) synthesis itself and also in the presence of Ca^{2+} overload. These results are not fully understood, but are in agreement with data published by Dedkova et al. (2004). The significance of these observations is not fully understood. Increased NO production could be part of resveratrol's antioxidant properties, in that NO serves as a natural $O_2^{\cdot-}$ scavenger via reacting with $O_2^{\cdot-}$ and thus inhibiting its further reduction to the $^{\bullet}OH$ radical. Interactions between $^{\bullet}OH$ radicals and lipids result in lipid peroxides, and, therefore, scavenging $O_2^{\cdot-}$ by NO may decrease the levels of lipid oxidation products. It is also possible that resveratrol decreases state 3 respirations and also increases both catalase and glutathione peroxidase activity (Labinskyy et al. 2006); accordingly, together with NO, it may mount an effective antioxidant defense. It has been shown that resveratrol is involved in eNOS and iNOS expression and increase in its activity (Arunachalam et al. 2010); therefore NO is considered beneficial during heart infarct recovery and in ischemia-reperfusion episodes (Hattori et al. 2002).

Resveratrol was found to significantly decrease levels of TBARS, possibly due to its ability to scavenge free radicals (Hung et al. 2002; Kim et al. 2002). The mild, uncoupling effect of resveratrol could be attributed to its chemical structure, which is similar to that of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that induces an increase in O_2 consumption and decay in the transmembrane potential (Terada 1990). In addition, the uncoupling of the respiratory chain mediated by resveratrol observed in the present study could be important in mobilization of lipid reserves toward mitochondrial β-oxidation. These effects of resveratrol could be employed in the prevention or treatment of diabetes, obesity, and metabolic syndrome (Zaninovich 2005; Schrauwen et al. 1999).

Together these data show a mechanism by which resveratrol exerts its protective effects on mitochondria, including by the direct modulation of mitochondrial transmembrane potential, inhibition of state 3 respiration, increase in NO levels and protection of mitochondrial GSH loss. These properties and those published by others support the utility of resveratrol in treatment and prevention of diseases associated with mitochondrial dysfunction.

Acknowledgments The authors appreciate the financial support from CIC-UMSNH 2.16 (ASM); CONACYT (144250 to ASM during his sabbatical). CONACYT 130638 (CCR); PROMEP PTC-266 (CCR). We greatly appreciate editorial revision of Mardelle Susman (Dept. of Microbiology and Immunology, UTMB).

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