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Exploiting the Role of Resveratrol in Rat Mitochondrial Permeability Transition

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Abstract Resveratrol (RSV), a natural polyphenolic antioxidant, has been considered an anticarcinogenic agent as it triggers tumor cell apoptosis through activation of the mitochondrial pathway. In our study, the effects of RSV on mitochondria, especially on the mitochondrial permeability transition (MPT) process, were investigated by multiple methods. We found that RSV induced a collapse of membrane potential and matrix swelling related to MPT. We further demonstrated that Ca^{2+} was necessary for this RSV-induced MPT opening. In addition, RSV induced the inner membrane permeabilization to H^+ and K^+, the depression of respiration and changes in membrane fluidity. The results suggested that RSV-induced MPT was accompanied by mitochondrial dysfunction. But the prohibition on lipid peroxidation and different effects of low- and high-dose RSV on membrane fluidity and respiration showed that the interaction of RSV and the mitochondria could not be the result of a single simple mechanism.

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Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RSV) is a phytoalexin present in the skin of grapes and in a narrow range of other spermatophytes. RSV is well known for its biological activities, including antioxidant, anti-inflammatory, antiplatelet aggregation, cell growth modulation, anticarcinogenic, antiatherogenic and phytoestrogen-like effects as well as immunomodulation and chemoprevention (Baur and Sinclair 2006; Baur et al. 2006). The present study indicates that RSV is a promising natural compound for cancer prevention and for treatment of a variety of human cancers (Pervaiz 2001). Findings on RSV have been extensively reviewed and have initiated numerous studies on its molecular mechanisms (Gusman et al. 2001; Savouret and Quesne 2002; Aziz et al. 2003). Jang et al. (1997) reported that the cancer-preventive activity of RSV was linked to its ability to eliminate free radicals and to reduce oxidative and mutagenic stress and the inhibition of DNA polymerase (see also Fontecave et al. 1998). Further studies pointed to a general mechanism of RSV-induced anticancer activity that was dependent on mitochondria-mediated apoptosis (Yousuf et al. 2009). Effects of RSV and its derivatives on mitochondrial function and metabolic homeostasis have been studied in various in vitro and in vivo models (Gosslau et al. 2005; Lagouge et al. 2006; Biasutto et al. 2008), but the molecular mechanism responsible for the antineoplastic effects of RSV has not been fully elucidated. Although caspase activation,

alteration of expression of Bcl-2 family proteins and conformational changes of Bax and Bak are understood to participate in RSV-induced apoptotic death (Dorrie et al. 2001; Tinhofer et al. 2001; Delmas et al. 2003), many other studies have shown that RSV could also impact mitochondrial membrane potential, the respiration chain and ATP synthesis (Mahyar-Roemer et al. 2001; Dave et al. 2008). Furthermore, it is suggested that RSV inhibits tumor growth and prevents myocardial reperfusion injury by targeting the mitochondrial permeability transition (MPT) pore (Ma et al. 2007; van Ginkel et al. 2008; Xi et al. 2009). MPT is now considered to be a critical event in the induction of the apoptotic process, but unfortunately, many questions regarding the mitochondria-mediated cell-death pathways remain unanswered (Crompton 1999). The investigations described here are aimed at getting further information on the effects of RSV on mitochondria, especially on the MPT process. Absorption and fluorescence methods were used to measure the mitochondrial swelling, membrane potential and membrane fluidity of mitochondria. We wished not only to shed new light on the mechanism of RSV-induced apoptosis but also to provide a direction for mitochondrial event monitoring by spectroscopic methods.

Materials and Methods

Chemicals

Resveratrol, cyclosporin A (CsA), EGTA, adenosine diphosphate (ADP), oligomycin, rotenone, rhodamine 123 (Rh123), hematoporphyrin (HP), ruthenium red (RR) and $CaCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical reagent grade, and all solutions were prepared with asepsis double-distilled water. All the water-insoluble compounds were dissolved in ethanol as stock solutions that were diluted with double-distilled water before use.

Isolation of Mitochondria

Liver mitochondria from Wistar rats (200–250 g) were isolated according to standard differential centrifugation procedures. The liver tissue was briefly homogenized in medium A containing 250 mM sucrose, 0.5 mM EGTA and 3 mM Tris (pH 7.2) (Xia et al. 2002; Zhang et al. 2011). The protein concentration was determined by the Biuret method. The respiratory control ratio (RCR) was measured by a Clark electrode (Oxygraph; Hansatech, King's Lynn, UK). Only mitochondrial suspensions that demonstrated an RCR above 2.5 were used.

Determination of Mitochondrial Swelling

Mitochondrial swelling was measured spectrophotometrically by monitoring the absorbance at 540 nm over 7 min at 25 °C. Mitochondria (0.25 mg/ml) were suspended in 2 ml respiration buffer B (200 mM sucrose, 10 mM Tris–Mops, 20 μM EGTA–Tris, 5 mM succinate, 2 μM rotenone and 3 μg/ml oligomycin, pH 7.4), and 2.5 μM Ca^{2+} was added before injecting RSV and/or other reagents. Spectra were recorded at room temperature on a 4802 double beam spectrophotometer (UNICO, Dayton, NJ) equipped with 1.0-cm quartz cells.

H^+ and K^+ Mitochondrial Inner Membrane Permeabilization

H^+ and K^+ mitochondrial inner membrane permeabilization was also detected using the swelling method. Mitochondrial inner membrane permeabilization to H^+ was detected in medium C: 135 mM K-acetate, 5 mM HEPES, 0.1 mM EGTA, 2 μM rotenone and 0.2 mM EDTA, pH 7.4. Before measuring, 1 μg/ml valinomycin was added to permeabilize to K^+. Some assays were performed in the presence of 1 μM FCCP for total permeabilization to H^+. Mitochondrial inner membrane permeabilization to K^+ was detected in medium D: 135 mM KNO_3, 5 mM HEPES (pH 7.1), 0.1 mM EGTA, 2 μM rotenone and 0.2 mM EDTA, pH 7.4. Some assays were performed in the presence of 1 μg/ml valinomycin for total permeabilization to K^+.

Measurement of Membrane Potential

Changes in mitochondrial membrane potential ($\Delta\psi_m$) were indicated by the accumulation of Rh123 (250 μM) as monitored by the changes in fluorescence emission intensity (Zamzami et al. 1995). The $\Delta\psi_m$ was assessed by an LS-55 fluorophotometer (Perkin-Elmer, Norwalk, CT) at 25 °C equipped with a quartz cell of 1.0 cm pathlength ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm). Mitochondria (0.5 mg/ml) were suspended in buffer B (2 ml).

Assessment of Membrane Fluidity

Dynamic changes in mitochondrial membranes were measured by the fluorescence anisotropic changes of HP-labeled mitochondria. Fluorescence anisotropic (r) values were collected by measurement of $I_{\parallel}$ and $I_{\perp}$, i.e., the fluorescence intensities polarized parallel and perpendicular, respectively, to the vertical plane of polarization of the excitation beam. Anisotropy (r) is defined by the equation

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (1)$$

where $G = I_{\perp}/I_{\parallel}$ is the correction factor for instrumental artifacts (Ricchelli et al. 1999a; Lakowicz 1999). Free probes in the bulk medium do not contribute to the fluorescence anisotropy since they are almost fluorimetrically silent in aqueous media.

The HP stock solution was prepared in absolute ethanol. HP (final concentration of 5 μM) was added to the mitochondrial suspensions (0.5 mg/ml, buffer B). Anisotropic changes were recorded by an LS-55 fluorophotometer (Perkin-Elmer) at $\lambda_{ex} = 520$ nm, $\lambda_{em} = 626$ nm.

Measurement of Oxygen Consumption

All oxygen consumption measurements were made using a Clark-type oxygen electrode (Hansatech) in a 1-ml thermostatted, water-jacketed glass chamber maintained at 25 °C (Monteiro et al. 2008).

For mitochondrial respiration measurement, mitochondria (1 mg/ml) were suspended in respiration buffer E (100 mM sucrose, 10 mM Tris–MOPS, 1 mM EDTA, 50 mM KCl, 2 mM $MgCl_2$, 10 mM KH_2PO_4 and 2 μM rotenone, pH 7.4) in a sealed chamber equipped with a magnetic stirrer. State 4 respiration was considered upon addition of 5 mM succinate as the energizing substrate. To induce state 3 respiration, 0.3 mM ADP was added subsequently. Finally, 30 μM DNP was added to induce uncoupled respiration.

For lipid peroxidation measurement, mitochondria (1 mg/ml) were suspended in 1 ml buffer F (175 mM KCl, 10 mM Tris–Cl and 2 μM rotenone, pH 7.4) (Fernandes et al. 2006). Membrane lipid peroxidation was initiated by adding ADP/Fe^{2+} (1 mM/1 mM) as oxidizing agents.

Transmission Electron Microscopy of Mitochondria

Mitochondria in different experimental conditions were fixed for 30 min at 4 °C using glutaraldehyde at a final concentration of 2.5 % in 0.1 M cacodylate buffer, then postfixed with 1 % osmium tetroxide and dehydrated. Observations were made using a JEM-100CX II transmission electron microscope (JEOL, Tokyo, Japan).

Results and Discussion

RSV Induces Ca^{2+}-Mediated Mitochondrial Swelling and Decreases the Membrane Potential

Mitochondrial swelling and collapse of the transmembrane potential are the direct results of PT pore opening. The effects of different concentrations of RSV on mitochondrial swelling were evaluated by measuring the absorbance at 540 nm (A540) over 10 min. Before measuring, mitochondrial suspensions were incubated with Ca^{2+} (2.5 μM), at which concentration it may not induce MPT directly (Fig. 1a, curve a). As shown in Fig. 1a, RSV induced mitochondrial swelling as attested by the decrease in absorbance in a concentration-dependent manner, and a maximal effect was obtained at 200 μM. The results suggested that Ca^{2+} was involved in mitochondrial swelling induced by RSV.

The effect of RSV on the $\Delta\psi_m$ was also measured by the fluorescence probe Rh123. Rh123 can accumulate in the mitochondrial matrix with its fluorescence quenched. Upon collapse of $\Delta\psi_m$, Rh123 is released into the medium,

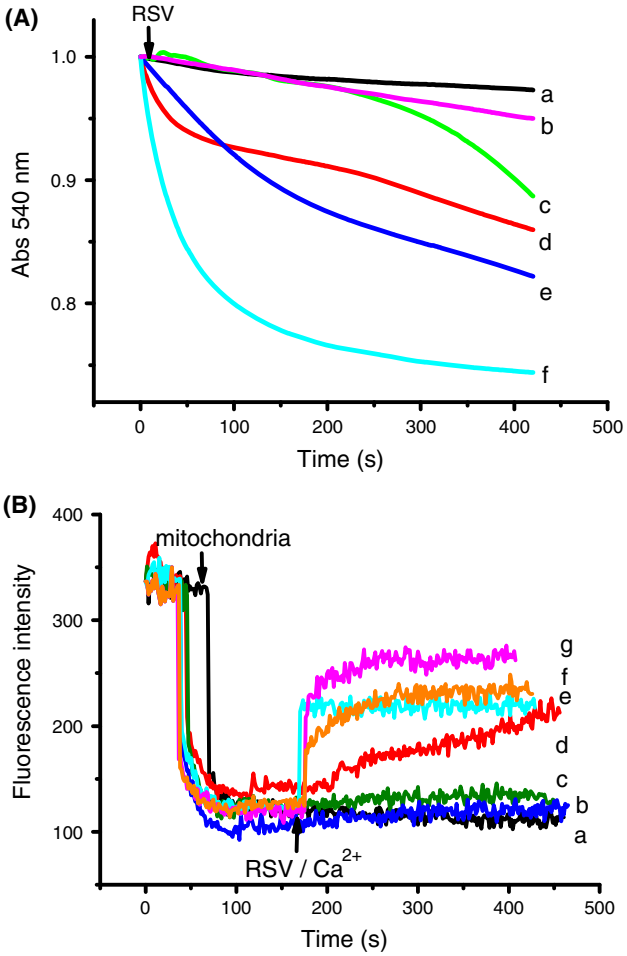

Fig. 1 RSV induced isolated mitochondrial swelling (**a**) and decreased the $\Delta\psi_m$ (**b**) in the presence of 2.5 μM Ca^{2+}. **a** Mitochondrial swelling was monitored at 540 nm as described (see "Determination of Mitochondrial Swelling" section). RSV was added to mitochondria (0.25 mg/ml) at concentrations (in μM) of *a* 0, *b* 20, *c* 50, *d* 100, *e* 150 and *f* 200. **b** Membrane potential was measured by the fluorescence of Rh123 as described (see "Measurement of Membrane Potential" section). Where indicated (*arrow*), mitochondria, RSV (traces *a–f*) or Ca^{2+} (trace *g*, 25 μM) was added. RSV was added at concentrations (in μM) of *a* 0, *b* 20, *c* 50, *d* 100, *e* 150 and *f* 200

causing an increase in fluorescence intensity. As shown in Fig. 1b, the decrease of $\Delta\psi_m$, indicated by the increase of fluorescence intensity of Rh123, was observed with the addition of RSV (Zhu et al. 2002). The decreased value of $\Delta\psi_m$ with increasing concentrations of RSV shows that the mitochondrial swelling was accompanied by changes in membrane potential. These results demonstrated that RSV induced Ca^{2+}-mediated MPT in mitochondria of normal rat liver.

Effects of RR, CsA and ADP on RSV-Induced MPT

Although the structure of the MPT pore, a protein channel, has not yet been identified, CsA is considered to be a well-established inhibitor of MPT by preventing the connection with cyclophilin D and adenine nucleotide translocator (Yarana et al. 2012). ADP acts as the next most effective protective agent and can be combined with CsA to prevent MPT synergistically (Brustovetsky and Dubinsky 2000). As shown in Fig. 2, addition of CsA or ADP can completely prevent the swelling induced by RSV and partially alleviate $\Delta\psi_m$ dissipation. The prevention of CsA and ADP confirms our conclusion that RSV-induced MPT should be related to the Ca^{2+} threshold determining the closed or unclosed state of the permeability transition pore in rat liver mitochondria (Petrosillo et al. 2004).

RR, a noncompetitive inhibitor of the Ca^{2+} uniporter in mitochondria, has been demonstrated to inhibit several mechanisms involved in intracellular Ca^{2+} regulation (Dikalov et al. 2012). As shown in Fig. 2a, b, pretreatment with RR totally abolished the swelling and collapse of $\Delta\psi_m$, indicating that Ca^{2+} accumulation inside mitochondria is needed for this process. In order to confirm that Ca^{2+} is needed for RSV-induced MPT opening, the swelling measurement was performed with addition of EGTA (Fig. 2a). Since Ca^{2+} was chelated by EGTA, no changes in the absorbance at 540 nm also indicated that Ca^{2+} was needed for RSV-induced MPT.

Effects of RSV on Mitochondrial Ultrastructure

The effects of RSV on mitochondrial ultrastructure were investigated by transmission electron microscopy (TEM). Mitochondria extracted from rat liver maintained their integrity with dense matrix (Fig. 3a). With addition of RSV in Fig. 3b, mitochondria were obviously swollen, with decreased matrix electron density and enlarged volume, which is a typical MPT configuration. Treatment with CsA, ADP and RR can inhibit the RSV-induced MPT efficiently (Fig. 3c). The TEM observation is in accordance with spectroscopic measurement, which also confirmed that the spectroscopic method can be considered as a reliable, cheap and convenient tool in detecting mitochondrial function.

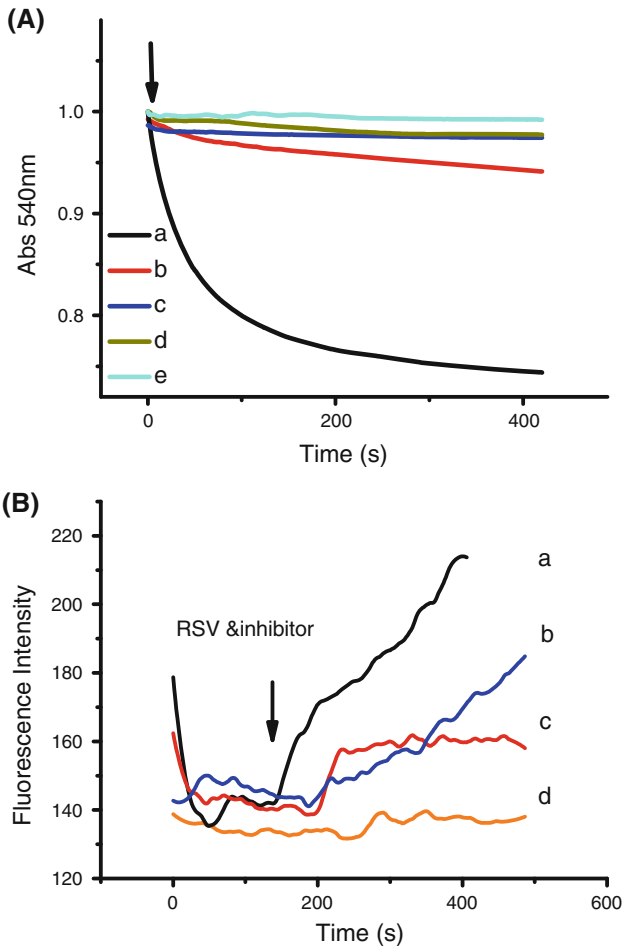

Fig. 2 **a** RSV induced isolated mitochondrial swelling in the presence of 2.5 μM Ca^{2+} inhibited by different types of inhibitor. Where indicated (*arrow*), 200 μM RSV (*a*) was added to 4 mM ADP (*b*), 1 μM CsA (*c*), 10 μM ruthenium red (*d*) or 4 mM EDTA (*f*). **b** RSV induced a decrease of $\Delta\psi_m$ inhibited by some inhibitors. Where indicated (*arrow*), 200 μM RSV (*a*) was added to 4 mM ADP (*b*), 1 μM CsA (*c*) or 10 μM ruthenium red (*d*)

Effects of RSV on Mitochondrial Inner Membrane Permeabilization to H^+ and K^+

As shown in Fig. 4, the effects of RSV on H^+ or K^+ mitochondrial inner membrane permeabilization were evaluated by swelling of nonrespiring mitochondria. The acetate can be transported across the inner membrane as the protonated species HOAc and then, in the mitochondrial matrix, dissociate to the acetate anion and H^+, leading to a proton gradient (Fernandes et al. 2008; Gamble and Lehninge 1973). A valinomycin-dependent swelling occurs only if the proton gradient is dissipated, as shown in Fig. 4a, curve a. The decrease of Abs in the presence of

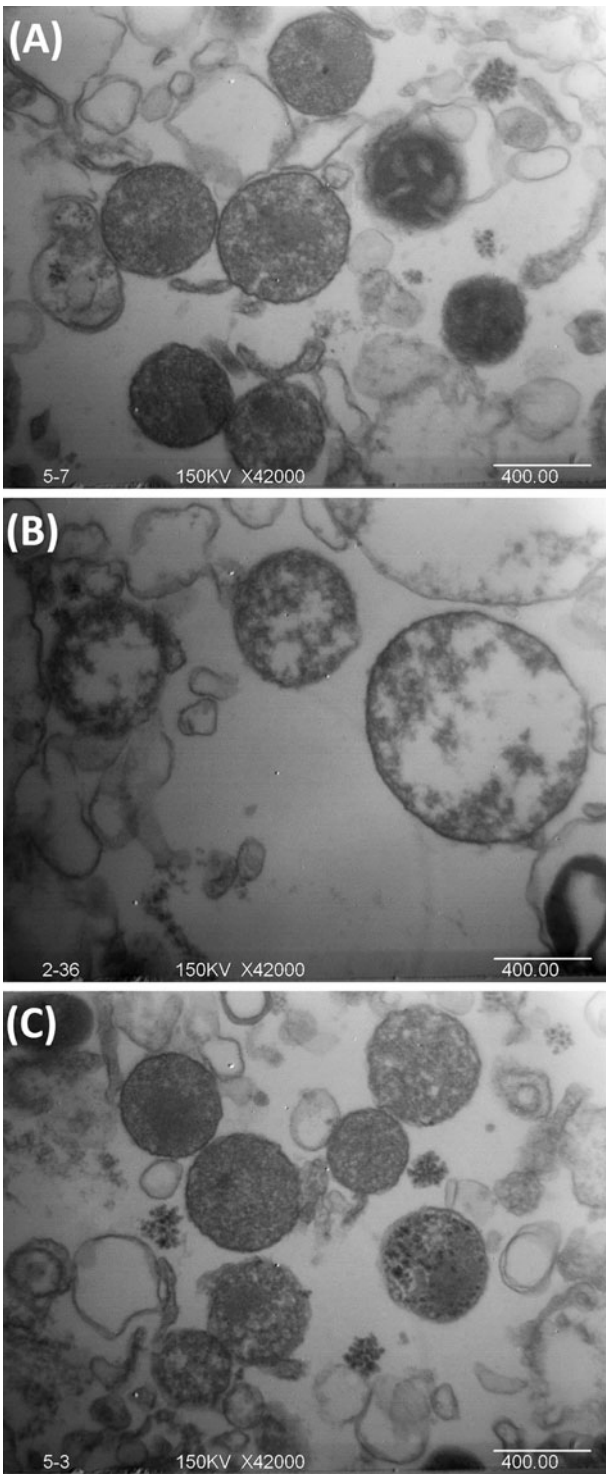

Fig. 3 Effects of RSV on mitochondrial ultrastructure. Mitochondria (0.5 mg/ml) were incubated for 2 min at 25 °C in standard medium without (**a**) or with some additions (**b** 200 μM RSV, **c** 200 μM RSV, 1 μM CsA, 4 mM ADP, 10 μM RR)

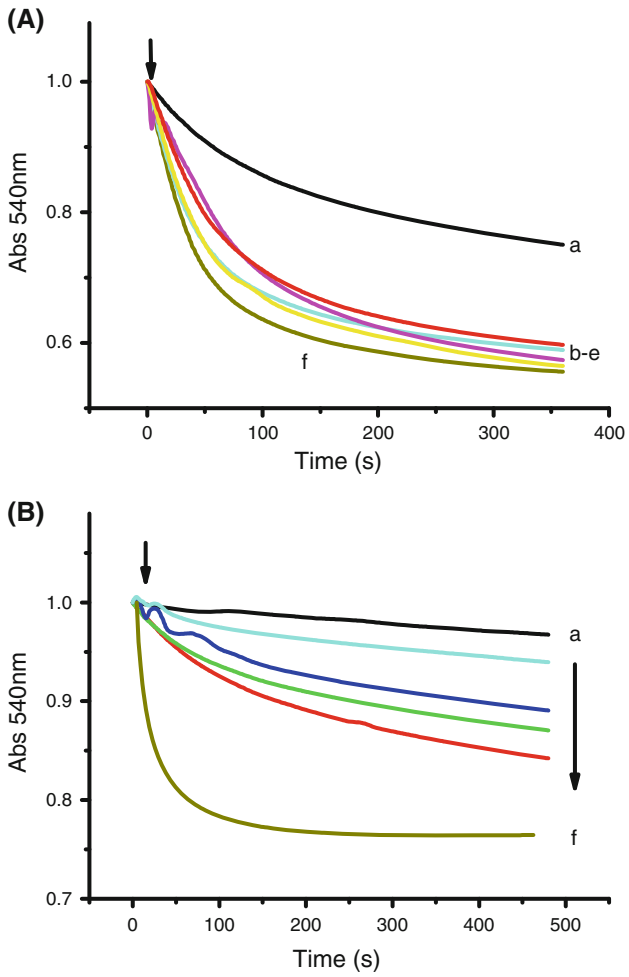

Fig. 4 Effect of RSV on mitochondrial inner membrane permeabilization to H^+ (**a**) and K^+ (**b**) as described (see "H^+ and K^+ Mitochondrial Inner Membrane Permeabilization" section). **a** Where indicated (*arrow*), mitochondria, 1 μg/ml valinomycin (*a–f*) and RSV (*b–e*)/FCCP (*f*, 1 μM) were added. RSV was added at the following concentrations (in μM): *a* 0, *b* 20, *c* 100, *d* 150, *e* 200. **b** Where indicated (*arrow*), mitochondria and RSV (*b–e*) or valinomycin (*f*, 1 μg/ml) were added. RSV was added at the following concentrations (in μM): *a* 0, *b* 20, *c* 100, *d* 150, *e* 200

RSV indicated that RSV led to an intensified valinomycin-dependent mitochondrial swelling, which is close to the value of maximum swelling estimated with FCCP (Fig. 4a). The results suggested a direct action of RSV on proton conductance through the mitochondrial inner membrane (Fernandes et al. 2008). Since the mitochondrial inner membrane is permeable to NO_3^-, there is a K^+ gradient between the two sides of the inner membrane when mitochondria are suspended in medium D. The optimal swelling method can also be used to observe mitochondria under the condition of K^+ permeabilization. The maximal rate of swelling is observed by adding valinomycin to provide K^+ entry into the intramitochondrial space and the mitochondrial matrix (Fig. 4b, curve f). As shown in Fig. 4b, addition of RSV induced swelling in a concentration-dependent manner, indicating that the K^+

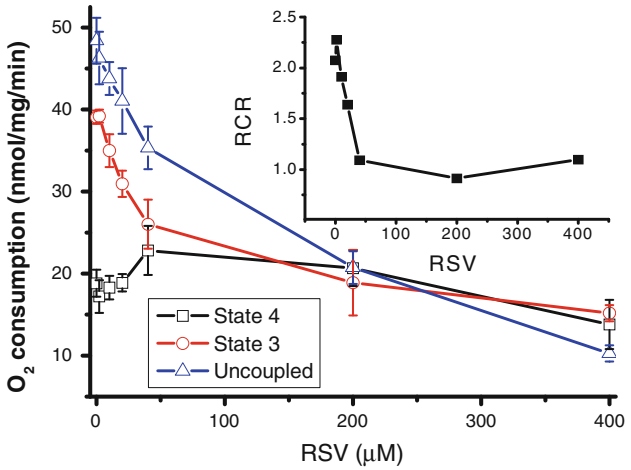

Fig. 5 Effects of RSV on the respiration of isolated mitochondria (1 mg/ml) in rat liver as described (see "Measurement of Oxygen Consumption" secation). O_2 consumption recordings allowed calculation of the rate of state 3 (ADP-stimulated) respiration, the rate of state 4 (non-ADP-stimulated) respiration and uncoupled respiration (DNP-stimulated state 4/uncoupled respiration). *Inset* the respiratory control ratio (RCR = state 3/state 4)

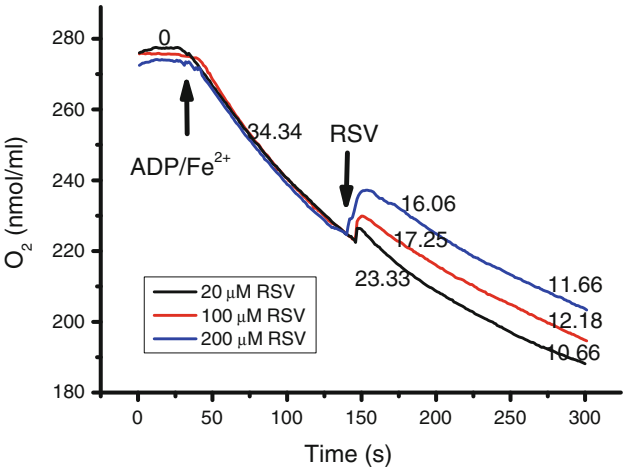

Fig. 6 Effect of RSV on membrane lipid peroxidation of mitochondria (1 mg/ml) induced by the pro-oxidant pair ADP/Fe^{2+} as described (see "Measurement of Oxygen Consumption" section). Where indicated (*arrow*), RSV at different concentrations was added. *Numbers* indicate rates of oxygen consumption as nanomoles of O_2 per minute per milliliter

conductance through the inner mitochondrial membrane was also affected by RSV.

Effects of RSV on Mitochondrial Respiration

Figure 5 shows a polarographic determination of oxygen consumption in mitochondria energized with the complex II substrate succinate. Under this condition, state 3 and uncoupled respiration were decreased with the increase of RSV. Meanwhile, state 4 respiration was stimulated by a lower concentration of RSV, reaching a maximum rate when RSV was 40 μM. But above 40 μM, although overall stimulation was still observed, the degree of stimulation decreased in a concentration-dependent manner, showing that RSV acts as an uncoupler below 40 μM and at higher concentrations it progressively inhibits mitochondrial respiration. A high state 3 rate indicates an intact respiratory chain and ATP synthesis, while a low state 4 rate indicates an intact mitochondrial inner membrane (Adlam et al. 2005). The decrease in RCR suggests that RSV causes extensive mitochondrial damage. RSV decreased both ADP-stimulated state 3 respiration and uncoupled respiration, suggesting that it is a respiratory inhibitor (Lim et al. 2009). Moreover, in isolated mitochondria, the major control over state 4 respiration is the proton leak through the mitochondrial inner membrane; the entrance of H^+ into the inner membrane and the decrease of $\Delta\psi_m$ will increase oxygen consumption, which is consistent with the data in Fig. 4a. This indicates that the RSV effect was not only due to a direct action on respiratory chain but also related to the damage of the inner membrane. This was confirmed by the experiments depicted in Fig. 1, showing that RSV is able to induce MPT.

Effects of RSV on Lipid Peroxidation

The effect of RSV on lipid peroxidation was evaluated by measuring oxygen consumption (Fig. 6). Membrane peroxidation was induced by the pro-oxidant pair ADP/Fe^{2+}. After addition of ADP/Fe^{2+} into the nonrespiring mitochondria, oxygen consumption increased due to oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids by ROS and, consequently, to the propagation phase of lipid peroxidation (Fernandes et al. 2006). RSV decreased the rate of oxygen consumption, and the effect was initially concentration-dependent. After a few seconds, the rates of oxygen consumption of the suspensions treated with different concentrations of RSV became similar. The present result shows that RSV can partly inhibit lipid peroxidation in the mitochondrial membrane.

Effects of RSV on the Fluidity of Mitochondrial Membranes

Recent studies have reported that induction of MPT in rat liver mitochondria was accompanied by fluidity changes of mitochondrial membranes (Ricchelli et al. 1999a). The changes of fluorescence excitation anisotropy (r) of mitochondria-bound dyes can reflect the membrane fluidity changes. The probe HP was used to monitor the changes in membrane fluidity induced by RSV, and the concentration dependence of RSV on the anisotropic changes of HP-labeled mitochondria were studied (Fig. 7). Interestingly,

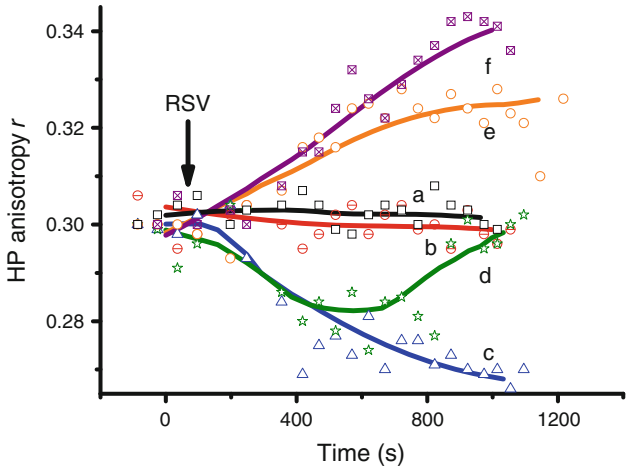

Fig. 7 Effects of different concentrations of RSV on the anisotropic changes of HP-labeled mitochondria (0.5 mg/ml) as described (see "Assessment of Membrane Fluidity" section). Where indicated (*arrow*), RSV was added at the following concentrations (in μM): *a* 0, *b* 20, *c* 50, *d* 100, *e* 150, *f* 200

we observed that the effects of RSV on mitochondrial membrane fluidity at high and low concentrations are different. The decrease of HP anisotropy, which corresponds to the increase in membrane fluidity, has been detected at low concentrations of RSV (20, 50 μM). The membrane fluidity first increased and then decreased, while the concentration of RSV reached about 100 μM. It seems that 100 μM is a critical concentration, and when the concentration was higher, the membrane fluidity decreased with the increase of RSV concentration. Since HP mainly accumulates in polar, solvent-accessible regions of the lipid bilayer and protein regions of the inner membrane (Ricchelli et al. 1999b), the results show that RSV-evoked mitochondrial dysfunction is accompanied by a remarkable change in membrane fluidity in the polar protein regions. The increase in membrane fluidity indicates that low concentrations of RSV may lead to the assembly of MPT pore and the intrinsic proton permeability of the lipid bilayer, the so-called proton leak (Garcia et al. 1998). The decrease of membrane fluidity at higher RSV concentrations indicates that the membrane becomes rigid and HP is anchored into the membrane, which shows a serious structural alteration in the polar protein region.

Conclusion

MPT is a Ca^{2+}-gated channel, and most MPT stimulators are Ca^{2+}-dependent. In this study, RSV induced MPT (Fig. 1) with a small amount of Ca^{2+}, which could be abolished by EGTA (Fig. 2a), suggesting that Ca^{2+} is involved in RSV-induced MPT opening. The prevention of RSV-induced MPT by inhibitors such as CsA, ADP and RR partly or totally (Figs. 2, 3) further demonstrates that Ca^{2+} is essential for RSV-induced MPT opening. Regarding the exact Ca^{2+} function in RSV-induced MPT, Ma et al. (2007) suggested that Ca^{2+}-induced Ca^{2+} release may play an important role. In spite of Ca^{2+}, our results show that RSV can disturb the balance of H^+ between the two sides of the mitochondrial matrix and the space between the inner and outer membrane (Fig. 4a). Since intracellular K^+←acts as a repressor of apoptotic effectors, the loss of K^+ may serve as a disaster signal to allow apoptosis (Yu 2003); the partial permeability of K^+ to the inner membrane (Fig. 4b) induced by RSV suggested that the mitochondrial K^+ channel may also take part in RSV-triggered apoptosis. The data also confirm that the permeabilization of K^+ is in accordance with collapse of the mitochondrial membrane (Dębska et al. 2001). In isolated mitochondria, the major control over respiratory state 4 is the proton leak, while most of the control is taken by electronic transport chain (ETC) and substrate transport in state 3 (Li et al. 2011). RSV causes the increase in state 4 and the decrease in state 3 (Fig. 5), suggesting that it affects both ETC and transport of the mitochondrial inner membrane. The ability of RSV to inhibit uncoupled respiration strongly suggested that the site of RSV interaction should be located in the ETC (Li et al. 2011). And this is consistent with the data that a high dose of RSV inhibits respiration of state 4. In addition, the effects of RSV on the fluidity of mitochondrial membranes (Fig. 7) show that a low dose of RSV induces assembly of the MPT pore while a high dose induces a serious structural alteration in the polar protein region. The result is in accordance with the respiration data. Although the effect of RSV on Ca^{2+}-triggered MPT has been demonstrated and studied by scientists in different fields (Ma et al. 2007; Zini et al. 2002), the current work presents new information about the effects of RSV on mitochondrial respiration, membrane fluidity and membrane pro-oxidation; and there are some interesting findings which have not been reported before.

In conclusion, this study demonstrates that RSV can induce MPT and that Ca^{2+} is essential during this process. The impairment by RSV of mitochondrial functions can be considered the trigger of its anticarcinogenic effects. The interaction site of RSV on the mitochondrial membrane has been proposed, which needs further investigation. The low doses of the agent have different effects on membrane fluidity and respiratory state 4 compared to higher doses of RSV, which should be taken into consideration during future clinical treatment. The prohibition by RSV of lipid peroxidation also suggested that the function of RSV on mitochondria is multiple. Further research is necessary to elucidate the details of the mechanism.

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