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Resveratrol-induced mitochondrial dysfunction and apoptosis are associated with Ca^{2+} and mCICR-mediated MPT activation in HepG2 cells

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Abstract Resveratrol, a natural polyphenolic antioxidant, has been reported to possess the cancer chemopreventive potential in wide range by means of triggering tumor cells apoptosis through various pathways. It induced apoptosis through the activation of the mitochondrial pathway in some kinds of cells. In the present reports, we showed that resveratrol-induced HepG2 cell apoptosis and mitochondrial dysfunction was dependent on the induction of the mitochondrial permeability transition (MPT), because resveratrol caused the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) with the concomitant release of cytochrome c (Cyt.c). In addition, resveratrol induced a rapid and sustained elevation of intracellular [Ca^{2+}], which compromised the mitochondrial $\Delta\Psi_m$ and triggered the process of HepG2 cell apoptosis. In permeabilized HepG2 cells, we further demonstrated that the effect of the resveratrol was indeed synergistic with that of Ca^{2+} and Ca^{2+} is necessary for resveratrol-induced MPT opening. Calcium-induced calcium release from mitochondria (mCICR) played a key role in mitochondrial dysfunction and cell apoptosis: (1) mCICR inhibitor, ruthenium red (RR), prevent MPT opening and Cyt.c release; and (2) RR attenuated resveratrol-induced HepG2 cell apoptotic death. Furthermore, resveratrol promotes MPT opening by lowering Ca^{2+}-threshold. These data suggest modifying mCICR and Ca^{2+} threshold to modulate MPT opening may be a potential target to control cell apoptosis induced by resveratrol.

Keywords Resveratrol · Apoptosis · Ca^{2+} · Cytochrome c · Mitochondrial permeability transition · mCICR

Introduction

The cancer chemopreventive properties of resveratrol were first appreciated when Jang et al. demonstrated that resveratrol possesses cancer chemopreventive activity against all the three major stages of carcinogenesis, initiation, promotion, and progression [1]. Following the observation by Jang et al., many studies in cell culture system as well as in animal models have shown the cancer chemopreventive as well as cancer therapeutic effects of resveratrol. It also has been found that the cancer chemopreventive activity of resveratrol was related to its ability to trigger tumor cell death by apoptosis [2, 3]. But how resveratrol exerts its anti-carcinogenic effects via trigger apoptosis is not very understood. Although alteration of expression of Bcl-2 family of proteins, caspase activation, changes of Bax and Bak conformation, enhancement of FasL expression have been accessed to participate in resveratrol-induced apoptotic death [4–6]. The findings that resveratrol-induced apoptosis was preceded by mitochondria transmembrane potential ($\Delta\Psi_m$) collapse suggested mitochondria may be the target, which resveratrol act on. Really, the succeeded studies demonstrated that resveratrol-induced apoptosis of tumor cells through a mitochondrial-dependent pathway [7, 8]. Dorrie et al. reported the resveratrol-induced extensive apoptosis by depolarizing

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mitochondrial $\Delta\Psi_m$ and activating caspase-9 in leukemia cells [7]. Resveratrol also increases expression of proapoptotic Bax and its translocation to the mitochondria [8]. Although it is clear that mitochondrial pathway is involved in apoptosis of cancer cells induced by resveratrol, but the exact mechanism of how resveratrol induced mitochondrial $\Delta\Psi_m$ collapse has not been clearly known.

It is known that at least three mitochondrial-specific events have been well defined in cells undergoing apoptosis, namely, loss of mitochondrial $\Delta\Psi_m$, induction of mitochondrial permeability transition (MPT), and cytosolic translocation of apoptogenic factors, such as cytochrome c (Cyt.c), and apoptosis-inducing factor [9, 10]. The reduction in mitochondrial $\Delta\Psi_m$ is thought to be mediated by the opening of the MPT pore, a dynamic multiprotein complex located at the contact site between the inner and the outer mitochondrial membranes [11, 12]. In some forms of apoptosis processes the release of Cyt.c from the mitochondria via MPT. MPT is a Ca^{2+}-, voltage-, and pH-gated channel with a molecular cutoff of approximately 1500 Da [13, 14]. Mitochondrial Ca^{2+} uptake, which is believed to be responsible for triggering of MPT opening during apoptosis [15, 16]. Ca^{2+}-induced calcium release from mitochondria (mCICR) is a special kind of mitochondria Ca^{2+} transport which presents in the mitochondria [17, 18]. It is also found out that mCICR can trigger MPT opening [19]. Prompted by these reports, we decided to evaluate the effect of MPT opening and mCICR in the process of HepG2 cell apoptosis induced by resveratrol.

In the current study, we investigated resveratrol-induced apoptosis in human hepatoblastoma-derived HepG2 cell line and the potential mechanisms involving mitochondria of resveratrol-induced HepG2 cell apoptosis. In particular, the relationship among MPT opening, Cyt. c release, and mCICR in resveratrol-induced apoptosis was analyzed. We confirmed that resveratrol induced HepG2 cell apoptosis. We showed that mitochondrial membrane depolarization, MPT opening, mCICR and Cyt. c release are associated with resveratrol-induced apoptosis. Our results indicate that resveratrol induces apoptosis via triggering Cyt. c release by MPT opening, and Ca^{2+} is necessary for resveratrol-induced MPT opening. In addition, resveratrol-induced MPT opening and cytochrome c release depends on mCICR.

Materials and methods

Reagents

Resveratrol, Cyclosporin A (CsA), ruthenium red (RR), trifluoperazine, digitonin, BAPTA/AM were purchased from Sigma. Resveratrol was dissolved in DMSO to make a stock solution of 100 mM sotred at –20°C before use. Calcein/AM, tetramethylrhodamine ethyl ester (TMRE), rhod-2/AM, fluo-3/AM, hoechst33342, and Alexa-Fluor 488 monkey anti-mouse secondary antibody were obtained from Molecular Probes. Annexin V-FITC Apoptosis Detection Kit and protease inhibitors mixture were obtained from Calbiochem. Mouse anti-Cytochrome c monoclonal antibody (mAb) was purchased from Promega Corporation.

Cell culture and treatment

Human HepG2 hepatoma cells (purchased from the Cell Bank of Shanghai Institute of Cell Biology, ShangHai, China) were cultured at 37°C, 5% CO_2 in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin. For each experiment, resveratrol was added to the cells to obtain the indicated final concentrations. As a control for the dissolving medium used for each chemical, an equivalent amount of the specific diluent was added to a control cell population in every experiment. When specified, CsA and other inhibitors were added to the cells before or after resveratrol treatment.

In the experiments, all fluorescent images of cells were collected using confocal microscope (TCS SP2, Leica, Germany). Flow cytometric analyses were performed by using flow cytometer (EPICS Elite ESP, Coulter, USA). At least 10,000 cells were analyzed from each group and the data were processed using Elite system workstation.

Apoptosis assay

To measure apoptotic cell death, an Annexin V-FITC apoptosis detection kit was used according to the manufacturer's protocol to analyze the extracellular facing plasma membrane phosphatidylserine residues with the confocal microscope and the flow cytometer. Briefly, cells were grown in 6-well microtiter plate at concentration of approximate 1×10^5 cells/ml within 24 h. After treated with resveratrol for 24 h, cells were harvested and suspended in the provided media binding reagent with the following centrifugation at 800 g for 2 min. Cells were resuspended in binding buffer and 10 μl media binding reagent and 1.25 μl Annexin V-FITC were added into the cell suspension. Then the mixture was incubated 15 min at room temperature in the dark and gently resuspened in 0.5 ml cold binding buffer including 10 μl PI. At last the cells were immediately analyzed with the flow cytometer and the confocal microscope.

Morphological changes of apoptotic cells were examined under the confocal microscope after stained with

hoechst33342 at a final concentration of 1 μg/ml. For electron microscopy, cells were fixed in 2.5% glutaraldehyde for 2 h at 4°C, and conventional transmission electron microscopy was performed following standard protocols [20]. The cell ultrastructure was collected using HITACHI H-7500 transmission electron microscope.

Assessment of mitochondrial $\Delta\Psi_m$

Mitochondrial $\Delta\Psi_m$ in whole cells was measured using TMRE, as described in Ref. [21]. Briefly, 1×10^5 cells/ml were plated in 6-well microtiter plate. After treated with resveratrol, cells were harvested and incubated 30 min with DMEM containing 250 nM TMRE in the cell incubator. Finally, cells were analyzed by flow cytometer.

Determinations of MPT in intact cells

MPT opening in whole cells was monitored using two fluorimetric probes, TMRE and calcein/AM, as previously described [22, 23]. In brief, 1×10^5 cells/ml were plated on 35-mm-diameter Petri dishes and treated with resveratrol. After 8 h treatment with resveratrol, cells were loaded with 250 nM TMRE and 0.5 μM calcein/AM in resveratrol-free DMEM for 20 min. After loading, cells were washed and the resveratrol-containing DMEM was added back. Subsequently, images of green calcein fluorescence and red TMRE fluorescence were collected from 8 to 12 h using confocal microscope. TMRE fluorescence was excited at 568 nm and emission was imaged at >590 nm using long pass filter. Calcein fluorescence was excited at 488 nm and emission was imaged at 515–560 nm using band pass filter.

Measurement of $[Ca^{2+}]_c$ in intact cells

Changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) were measured with the fluorescent probe fluo-3/AM as described previously [24]. Briefly, cells were plated in 35-mm-diameter Petri dishes at concentration of approximate 1×10^5 cells/ml within 24 h. After treatments, cells were loaded for 30 min with 8 μM fluo-3/AM in a dark room. The cells were washed with DMEM to remove nonhydrolyzed fluo-3/AM before test. Fluorescence measurements were performed at an excitation of 488 nm and an emission of 525 nm.

Measurement of mitochondrial $\Delta\Psi_m$, $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in permeabilized cells

Selective digitonin permeabilization of the plasma membrane was adopted from the ''Method'' described by Ref. [25, 26], which permeabilizes the plasma membrane to allow entry of mitochondrial substrates and permit the examination of mitochondrial $\Delta\Psi_m$, $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. Briefly, cells were cultured in 35-mm-diameter Petri dishes at concentration of approximate 1×10^5 cells/ml. After washed, cells were permeabilized with 30 μg/ml digitonin in 1.8 ml of an intracellular medium composed of 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES-Tris pH 7.2 supplemented with a mixture of protease inhibitors. All the measurements in permeabilized cells were carried out in ATP generating system containing 2 mM Mg^{2+}-ATP, 5 mM phosphocreatine, 5 U/ml creatine kinase. Mitochondria were energized with 2 mM succinate (complex II substrate). The final $[Ca^{2+}]$ of the intracellular medium was 350 nM.

In order to carry out simultaneous measurements of $[Ca^{2+}]_c$ and mitochondrial $\Delta\Psi_m$, the permeabilized cells were supplemented with 8 μM fluo-3/AM and 250 nM TMRE. Fluorescence was continuously monitored using confocal microscope at an excitation of 488 nm and an emission of 525 nm for fluo-3/AM at an excitation of 568 nm and an emission of >590 nm for TMRE. The relatively low affinity of rhod-2/AM for Ca^{2+} (K_d~570 nM for rhod-2/AM) was selected to measure mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) [27]. Rhod-2/AM fluorescence was excited at 549 nm and emission was imaged at 581 nm using band pass filter.

Immunofluorescence detection of Cyt.c

Cyt.c release was measured using flow cytometry as described previously with minor modifications as outlined below [28]. For immunofluorescence, cells were collected at different time points. Cell suspensions were washed twice in ice cold PBS, pH 7.4, and resuspended in 1 ml of mitochondrial medium (250 mM sucrose, 10 mM KCl, 20 mM HEPES-KOH, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$) supplemented with 1% protease inhibitors mixture. Cells were permeabilized for 30 s under vortexing with 0.001% digitonin and centrifuged for 3 min at 1,000*g*. Pellet was resuspended in 4% paraformaldehyde and incubated for 20 min at room temperature. After two washes with PBS, the fixed cells were incubate in labeling medium (2% FBS, 0.2% sodium azide, and 0.5% Triton X-100 in PBS) for 15 min, centrifuged at 3,000*g* for 5 min, and then incubated with 1 μg/ml of anti-cytochrome c antibody in a total of 200 μl of labeling medium at 4°C for 1 h. After incubation, cells were washed twice in the labeling medium and then incubated in 200 μl of the labeling medium containing 5 μg/ml of antimouse AlexaFluor 488-conjugated secondary antibody at 4°C for additional 1 h. Cells were washed twice under the same conditions, resuspended in 200 μl PBS, and immediately analyzed in a flow cytometer.

Statistical analysis

All assays were performed in triplicates and data were expressed as means ± SEM. One-way ANOVA test was performed and statistical significance was set at $P < 0.05$.

Results

Resveratrol induced HepG2 cells apoptosis

We analyzed the population of Annexin-V binding with flow cytometry. As shown in Fig. 1A, resveratrol caused a significant concentration-dependent increase in the Annexin-V binding apoptotic cell populations after 24 h compared to untreated control cells, and a concentration of 100 μM was chosen for subsequent experiments. The morphological changes were shown in Fig. 1B and C. There is also an increase in the number of apoptotic cells, which were suggested by exhibiting cell shrinkage, chromatin condensation, nuclear fragmentation and shedding, after treatment of cells with 100 μM resveratrol for 24 h.

Resveratrol induced HepG2 cell apoptosis via activating MPT and release of Cyt.c

Since it is known that resveratrol can induce cancer cells apoptosis, it was hypothesized that mechanism may be operative at the level of the MPT and Cyt.c release. As a consequence of MPT opening, solutes with molecular mass of up to 1,500 Da nonselectively diffuse across the mitochondrial inner membrane, leading to mitochondrial depolarization [11, 12]. As shown by Fig. 2A, after 8 h exposure of cells to 100 μM resveratrol, mitochondria began to depolarize and release TMRE but do not become permeable to calcein. After 10 h of resveratrol treatment, mitochondrial $\Delta\Psi_m$ began to depolarize concomitant with the redistribution of calcein into the mitochondria matrix, causing the voids and honeycomb appearance of calcein images to disappear. After 12 h, virtually all mitochondria were depolarized and permeable to calcein.

Our results showed that Cyt.c release was found to occur after resveratrol treated HepG2 cells for 12 h (Fig. 2C). This was in agreement with that the release of apoptogenic proteins including Cyt.c from mitochondria is mechanistically linked to MPT opening [9, 10]. As shown in Fig. 2B

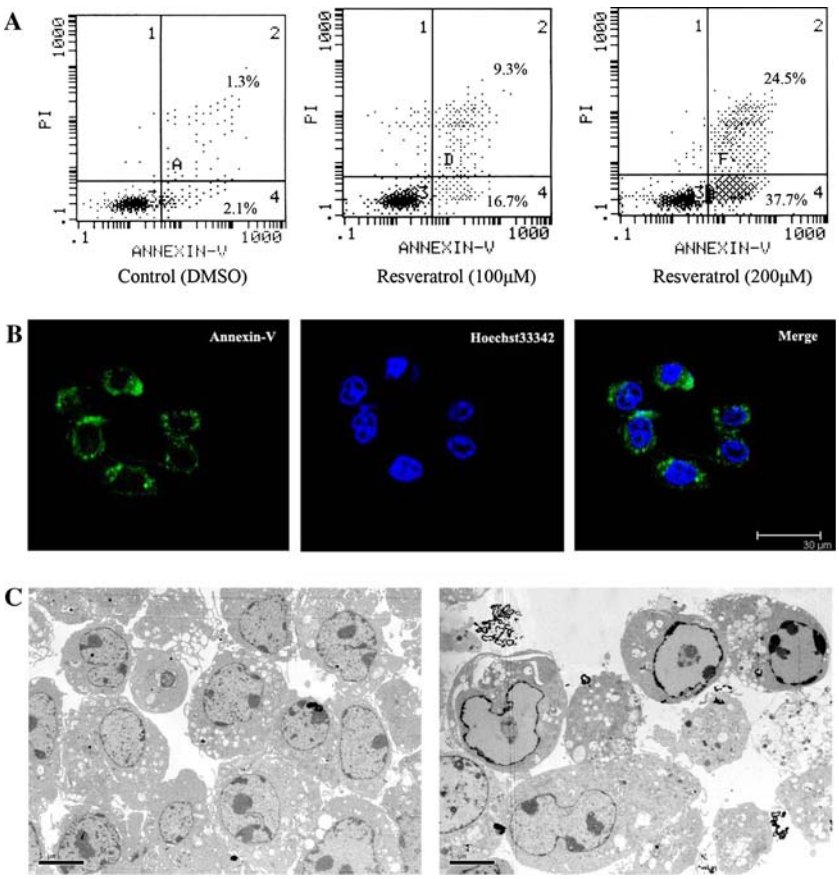

Fig. 1 Resveratrol induced HepG2 cell death via apoptosis. (**A**) HepG2 cells were treated with DMSO (control) and resveratrol (100 μM and 200 μM). After 24 h, cells were harvested for Annexin V-FITC and PI analysis. (**B**) HepG2 cells were treated with resveratrol (100 μM) for 24 h and live cells analyzed for phosphatidylserine externalization using a commercial Annexin V kit and hoechst33342 staining as described under ''Materials and methods'' (scale bars, 30 μm). (**C**) For ultrastructure, the control cells (*left*) and resveratrol-treated cells (100 μM, *right*) were fixed in 2.5% glutaraldehyde for 2 h at 4°C, and conventional transmission electron microscopy was performed. Finally, cells were observed under transmission electron microscope. Each experiment was performed in triplicate (scale bars, 5 μm)

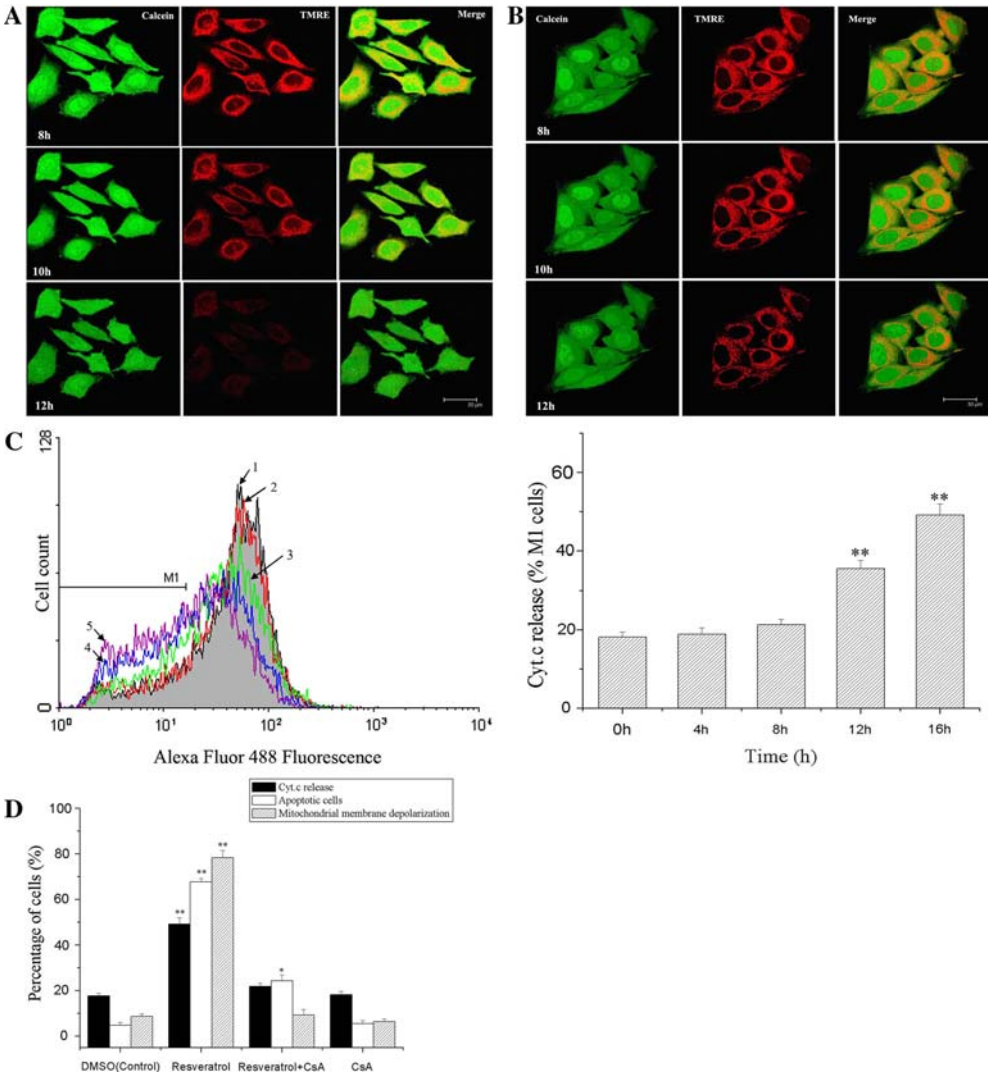

Fig. 2 Mitochondrial permeability transition (MPT) opening as well as Cyt.c release in resveratrol-treated HepG2 cells was blocked by CsA. The time-dependent effects of resveratrol on MPT opening (**A** and **B**). Cells were co-loaded with tetramethylrhodamine ethyl ester (TMRE) and calcein after their exposure to resveratrol (100 μM) as described in ''Materials and methods''. (**A**) The opening of MPT began to occur within 10 h of treatment, as indicated by decrease of red TMRE from mitochondria and permeabilization of green calcein from the cytosol into mitochondria. (**B**) Blockage of resveratrol-induced mitochondrial depolarization and MPT opening by CsA. Cells were pre-treated with 2 μM CsA for 30 min prior to the resveratrol (100 μM). One experiment was repeated three times (scale bars, 30 μm). (**C**) Time course of Cyt.c release in resveratrol-treated cells. The values of the percentage of cells in the Gate M1 regions of the fluorescence histograms were plotted for each time point. Cells were treated with resveratrol for 0 h (*1*), 4 h (*2*), 8 h (*3*), 12 h (*4*), 16 h (*5*), respectively, fluorescence of cells permeabilized with digitonin, and incubated with both antibodies. (**D**) Effects of CsA on the mitochondrial $\Delta\Psi_m$, Cyt.c release and apoptosis of cells after resveratrol treatment. The mitochondrial $\Delta\Psi_m$ and Cyt.c release after resveratrol (100 μM) treatment for 16 h was measured by flow cytometry as described in ''Materials and methods''. Cell apoptosis after resveratrol treatment for 48 h was determined by Annexin-V/PI staining. Data are expressed as means ± SEM of three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ as compared to medium alone control (DMSO)

and D, resveratrol-induced MPT opening was assessed in the presence of CsA. At a concentration of 2 μM CsA, inner membrane permeabilization and mitochondrial depolarization did not occur after resveratrol treatment. In addition, CsA almost completely inhibited the translocation of Cyt.c from mitochondrial to cytosol (Fig. 2D), indicating that the Cyt.c release was tightly controlled by CsA. These findings also implied that the onset of MPT induced by resveratrol preceded Cyt.c release. Figure 2D also showed CsA could obviously reduce this resveratrol-induced apoptosis, but not completely inhibit it. These demonstrated that resveratrol could directly target the MPT pore and induce mitochondrial MPT opening in a CsA-inhibitable manner.

Cytosolic Ca^{2+} was involved in resveratrol-induced MPT and apoptosis in HepG2 cells

Given the available literature showing a close relationship between Ca^{2+} and apoptosis [29], we investigated whether Ca^{2+} changes occurred in response to resveratrol. Our data demonstrated that resveratrol (100 μM) caused a sustained elevation of intracellular [Ca^{2+}] when compared to control (Fig. 3A). BAPTA/AM (50 μM) and EDTA (0.5 mM) were used to identify the origin of the increased intracellular free Ca^{2+} concentration. As shown in Fig. 3B, BAPTA/AM completely suppressed the cell apoptotic death induced by resveratrol. In contrast, EDTA alone revealed a weaker effect comparing to BAPTA/AM, suggesting that the increased intracellular free Ca^{2+} concentration in HepG2 cells was largely contributed by the redistribution of intracellular Ca^{2+}. Furthermore, BAPTA/AM was found to completely blocked the mitochondrial membrane depolarization in HepG2 cells treated with resveratrol (Fig. 3C), indicating that the elevation of intracellular free Ca^{2+} concentration was responsible for the collapse of mitochondrial $\Delta\Psi_m$ in resveratrol treated HepG2 cells and Ca^{2+} may be necessary in resveratrol induced mitochondrial membrane depolarization and MPT opening.

Ca^{2+}-mediated activation of MPT in permeabilized cells exposed to resveratrol

In order to investigate whether resveratrol affect $[Ca^{2+}]_c$ and mitochondrial $\Delta\Psi_m$ responses given to Ca^{2+} addition, we established simultaneous fluorometric measurements of mitochondrial $\Delta\Psi_m$ and $[Ca^{2+}]_c$ in permeabilized HepG2 cells. As shown in Fig. 4A and B, the addition of resveratrol and Ca^{2+} to the permeabilized cells yielded increases

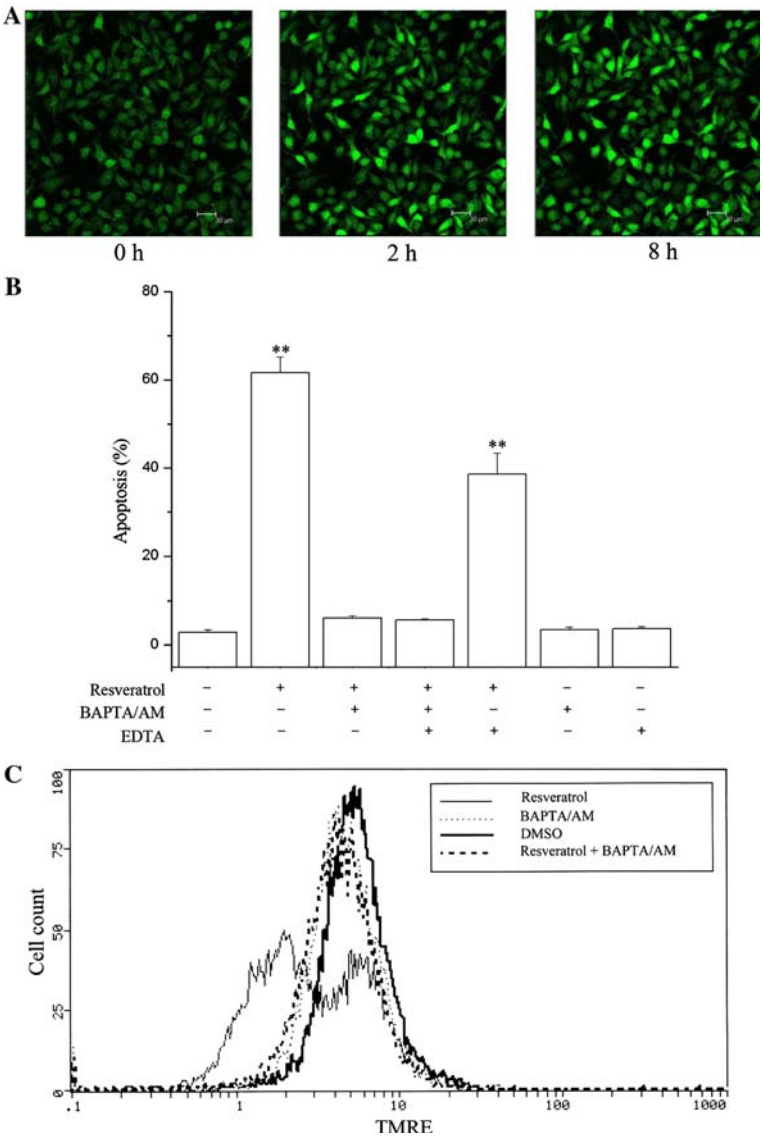

Fig. 3 Ca^{2+} was involved in HepG2 cell apoptosis induced by resveratrol. (**A**) Time-course effect of resveratrol (100 μM) on the intracellular [Ca^{2+}] in HepG2 cells after treatment for 0 h, 2 h, or 8 h. Cells loaded with Fluo-3/AM (8 μM) were observed under confocal microscope immediately (scale bars, 30 μm). (**B**) Effects of BAPTA/AM (50 μM) and EDTA (0.5 mM) on the cell apoptosis after treatment with resveratrol. (**C**) Effect of BAPTA/AM on the mitochondrial membrane depolarization of HepG2 cells after treatment with resveratrol. Cells after treatment were stained with tetramethylrhodamine ethyl ester (TMRE) (250 nM) for 30 min, and the change of mitochondrial $\Delta\Psi_m$ was determined by flow cytometry: control, BAPTA/AM alone, resveratrol + BAPTA/AM, resveratrol alone, respectively. Cells were preincubated with BAPTA/AM (50 μM) for 30 min (**B** and **C**) and further incubated with resveratrol (100 μM) for 12 h (**C**) or 48 h (**B**). Each experiment was performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$ as compared to control

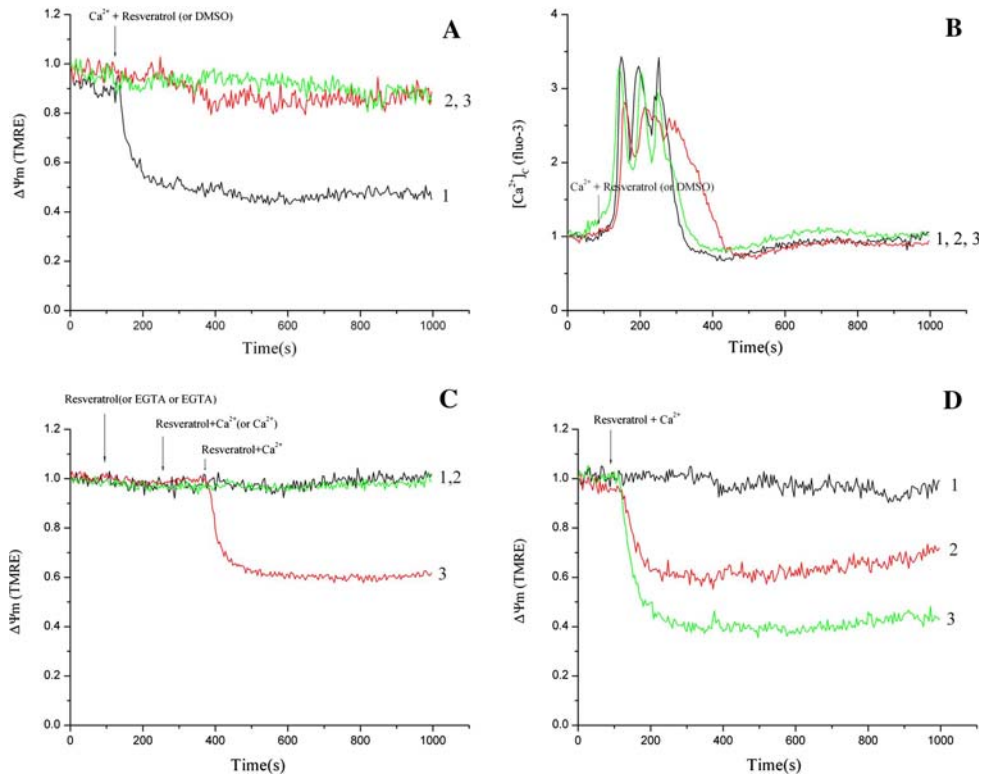

Fig. 4 Ca^{2+}-mediated mitochondrial permeability transition (MPT) opening and $[Ca^{2+}]_c$ responses in permeabilized cells exposed to resveratrol. Measurements of $\Delta\Psi_m$ and $[Ca^{2+}]_c$ were carried out in permeabilized HepG2 cells in the presence of 2 mM succinate using a mitochondrial membrane potential probe tetramethylrhodamine ethyl ester (TMRE) and a cytosolic Ca^{2+} indicator fluo-3 respectively. Effect of resveratrol on MPT (**A**) and cytosolic $[Ca^{2+}]$ spike (**B**). 1 (black trace), permeabilized cells were treated with 100 μM resveratrol plus 10 μM $CaCl_2$; 2 (red trace), permeabilized cells were treated with DMSO plus 10 μM $CaCl_2$; 3 (green trace), permeabilized cells were pre-treated with 2 μM CsA for 4 min prior to the 100 μM resveratrol plus 10 μM $CaCl_2$. (**C**) Ca^{2+} is necessary for resveratrol-induced MPT opening. 1 (black trace), permeabilized cells were treated with 100 μM resveratrol only; 2 (green trace), permeabilized cells were pre-treated with 0.5 mM EGTA for 4 min prior to the 100 μM resveratrol plus 10 μM $CaCl_2$; 3 (red trace), permeabilized cells were treated with 100 μM resveratrol plus 10 μM $CaCl_2$, 0.5 mM EGTA was added 4 min before and 1.0 mM $CaCl_2$ was added 100 s before treatment. (**D**) Ca^{2+} concentrations affected resveratrol-induced MPT opening. Permeabilized cells were treated with, 1 (black trace), 100 μM resveratrol plus 1 μM $CaCl_2$; 2 (red trace), 100 μM resveratrol plus 10 μM $CaCl_2$; 3 (green trace), 100 μM resveratrol plus 20 μM $CaCl_2$, respectively

of $[Ca^{2+}]_c$, which were transient (Fig. 4B, black trace) and the decrease of mitochondrial $\Delta\Psi_m$ which means the activation of MPT (Fig. 4A, black trace). With 1 μM CsA treatment, resveratrol plus Ca^{2+}-induced mitochondrial depolarization was inhibited, but had no effect on the transient increases of $[Ca^{2+}]_c$ except the third $[Ca^{2+}]_c$ pulses (Fig. 4B, green trace). In order to determine Ca^{2+} is needed for resveratrol-induced MPT opening, the experiments were performed as described in Fig. 4C and D. There occurred no decrease of mitochondrial $\Delta\Psi_m$ when cells were stimulated with 100 μM resveratrol only, or treated with 100 μM resveratrol plus 10 μM Ca^{2+} after pretreated with 0.5 mM EGTA to chelate the intracellular Ca^{2+}. However, if precultured with 1.0 mM Ca^{2+} after 0.5 mM EGTA, the resveratrol-induced mitochondrial $\Delta\Psi_m$ decrease occurred (Fig. 4C). Further experiments demonstrated that Ca^{2+} concentrations affected resveratrol-induced MPT opening. As Fig. 4D shown, 100 μM resveratrol plus 1 μM Ca^{2+} induced no changes in mitochondrial $\Delta\Psi_m$, while 10~20 μM Ca^{2+} plus 100 μM resveratrol could potentiate the fall of mitochondrial $\Delta\Psi_m$. These results demonstrated that Ca^{2+} was needed for resveratrol-induce MPT opening.

mCICR dependence of resveratrol-induced MPT opening

Mitochondrial have the ability to rapidly accumulate and then release large quantities of Ca^{2+} [29]. Ca^{2+} can induce mCICR during Ca^{2+}-induced MPT opening [19]. As shown in Fig. 5A, 100 μM resveratrol plus 10 μM Ca^{2+} triggered mitochondrial Ca^{2+} overloaded rapidly then declined over the starting baseline gradually. Ascend and descent of mitochondrial Ca^{2+} in the permeabilized cells were the

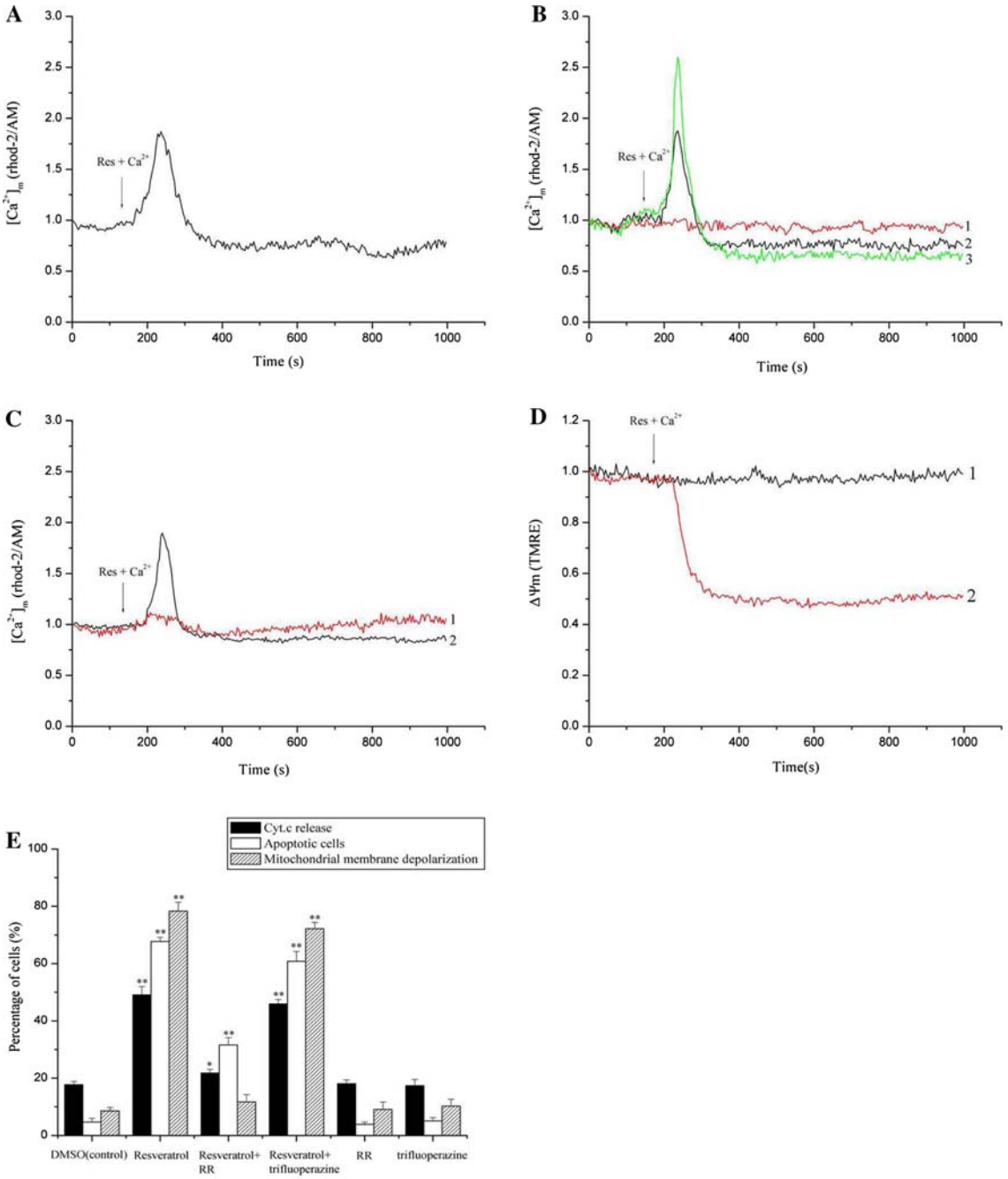

Fig. 5 Resveratrol-induced mitochondrial permeability transition (MPT) is dependent on mCICR. Measurements of mitochondrial [Ca^{2+}] were carried out in permeabilized HepG2 cells using the relatively low affinity of rhod-2/AM for Ca^{2+}. (**A**) mCICR was induced with 100 μM resveratrol plus 10 μM $CaCl_2$. (**B**) Ca^{2+} concentrations affected resveratrol-induced mCICR. Permeabilized cells were treated with, 1 (red), 100 μM resveratrol plus 1 μM $CaCl_2$; 2 (black), 100 μM resveratrol plus 10 μM $CaCl_2$; 3 (green), 100 μM resveratrol plus 20 μM $CaCl_2$, respectively. (**C**) The effects of trifluoperazine and RR on mitochondrial [Ca^{2+}] changes. Permeabilized cells were treated with 100 μM resveratrol plus 10 μM $CaCl_2$ and 4 min before detection, there were added by, 1 (red), 1 μM RR and 2 (black), 1 μM trifluoperazine, respectively. (**D**) The effects of trifluoperazine and RR on mitochondrial $\Delta\Psi_m$ changes. Permeabilized cells were treated with 100 μM resveratrol plus 10 μM $CaCl_2$ and 4 min before detection, there were added by, 1 (red), 1 μM trifluoperazine and 2 (black), 1 μM RR, respectively. (**E**) Effects of RR and trifluoperazine on the mitochondrial $\Delta\Psi_m$, Cyt.c release and apoptosis of HepG2 cells after resveratrol treatment. The mitochondrial $\Delta\Psi_m$ and Cyt.c release after resveratrol (100 μM) treatment for 16 h was measured by flow cytometry, as described in ''Materials and methods''. Cell apoptosis after resveratrol treatment for 48 h was determined by Annexin-V/PI staining. Data are expressed as means ± SEM of three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ as compared to medium alone control (DMSO)

proof of the free Ca^{2+} influx into mitochondria first and then efflux from mitochondria, indicating there occurred mCICR during resveratrol-induced MPT opening.

We carried out further experiments to determine the effects of mCICR on resveratrol-induced MPT opening. The results showed, 100 μM resveratrol plus 1 μM Ca^{2+} can not induce changes of mitochondrial Ca^{2+} and $\Delta\Psi_m$ in the permeabilized cells. Similarly, as shown in Fig. 5B, 10 μM or 20 μM Ca^{2+} plus 100 μM resveratrol, which can induce ascend and descent of mitochondrial Ca^{2+} in permeabilized cells, induced mitochondrial $\Delta\Psi_m$ decrease as well (Fig. 4D). These results suggested mCICR was necessary for resveratrol-induced MPT opening.

In attempt to demonstrate this suggestion, we studied the relationship between resveratrol-induced mCICR and MPT opening. As shown in Fig. 5C and D, resveratrol-induced mCICR was inhibited or weakened in the presence of 1 μM RR, an inhibitor of mitochondrial Ca^{2+} uniporter, and mitochondrial $\Delta\Psi_m$ decrease was inhibited too. These results indicated that part or full inhibition of mCICR could result in part or full inhibition of MPT. Moreover, with 1 μM trifluoperazine, a inhibitor of Ca^{2+}/Na^{+} exchanger that is one of the mitochondria Ca^{2+} extrusion pathways [30], resveratrol-induced mCICR and the mitochondrial $\Delta\Psi_m$ decline were unchangeable. These suggested that mCICR caused MPT alteration. In line with these observations, we found that the mitochondrial depolarization, Cyt.c release and cell apoptosis induced by resveratrol were inhibited by RR in intact cells (Fig. 5E), which further confirmed that resveratrol-induced MPT was determined by mCICR.

Resveratrol lowered the Ca^{2+} threshold for MPT opening

As shown in Fig. 6A and B, permeabilized cells were pretreated with resveratrol at the concentration of 100, 120, and 150 μM for 4 min prior to the addition of 10 μM Ca^{2+} which induce mitochondrial $\Delta\Psi_m$ decrease, respectively, and the decline accelerated along with the resveratrol concentrations, indicating that the effect of resveratrol on MPT is dose-dependent. On the other hand, pretreating permeabilized cells with resveratrol for 0, 4, 8, 12 min, 10 μM Ca^{2+} induce decrease of mitochondrial $\Delta\Psi_m$ at different level, indicating the effects of resveratrol on MPT is time-dependent. These results proved that resveratrol could facilitate MPT opening with time and dose-dependent manner in the presence of Ca^{2+}.

We also found that 10 μM Ca^{2+} could not induce the decrease of mitochondrial $\Delta\Psi_m$ in the absence of resveratrol, while being pretreated permeabilized cells with 100 μM resveratrol and 10 μM Ca^{2+} could induce the decrease of mitochondrial $\Delta\Psi_m$. Further experiments showed that, being pretreated permeabilized cells with 0, 25, 50, and 100 μM resveratrol respectively, the decrease of mitochondrial $\Delta\Psi_m$ could be triggered by 60, 40, 20, and 10 μM Ca^{2+} accordingly (data not shown). These data indicated that resveratrol could facilitate MPT opening by lowering the Ca^{2+} threshold for MPT opening.

Discussion

Several experimental studies suggest that resveratrol can induce the apoptotic cell death in numerous cancer cell lines [2–6]. However, few studies have evaluated the chemopreventive effects of resveratrol against liver cancer and the underlying mechanisms are not well known. Our study intended to investigate the mechanisms of apoptosis induced by resveratrol in human hepatoma cells line HepG2 cell and focus on the mitochondrial pathways in resveratrol-induced HepG2 cell apoptotic death. Our results showed that resveratrol could induce HepG2 cells apoptosis in dose-dependent manner and depolarize mitochondrial $\Delta\Psi_m$ in cells. We also demonstrated that resveratrol induced CsA-sensitive MPT opening and following Cyt.c release. Furthermore, we showed that mitochondrial membrane depolarization, MPT opening and Cyt.c release are involved in resveratrol-induced apoptosis, which are consistent with mitochondrial-specific events in cells undergoing apoptosis, namely, loss of mitochondrial $\Delta\Psi_m$, induction of MPT opening, and cytosolic translocation of apoptogenic factors, such as Cyt.c [9]. These results above also agree with reports in other studies that mitochondrial membrane depolarization, MPT opening and Cyt.c release are involved in drug-induced apoptosis [31, 32]. In addition, we found that MPT opening progresses gradually during resveratrol-mediated apoptosis, which began in a small quantity of mitochondria and required several hours to affect all mitochondria. It is suggested that relatively long lasting pore openings eventually caused Cyt.c release and then triggered cell apoptotic death. Thus, the cytotoxic action of resveratrol is most likely to be mediated by onset of the MPT and this process is irreversible.

MPT is a Ca^{2+}-gated channel and most MPT stimulators are Ca^{2+}-dependent implied that Ca^{2+} might play dominant role in MPT opening [25]. In the present study, we observed that resveratrol induced cytosolic Ca^{2+} increased and cytosolic Ca^{2+} was involved in resveratrol-induced MPT opening and apoptosis. In the experiments, we used BAPTA/AM and EDTA to identify the origin of the Ca^{2+}. BAPTA/AM could block the cell apoptotic death induced by resveratrol and suppress the mitochondrial $\Delta\Psi_m$ decrease, which implied that the intracellular $[Ca^{2+}]$ increase predominantly controlled the HepG2 cell apoptosis. In

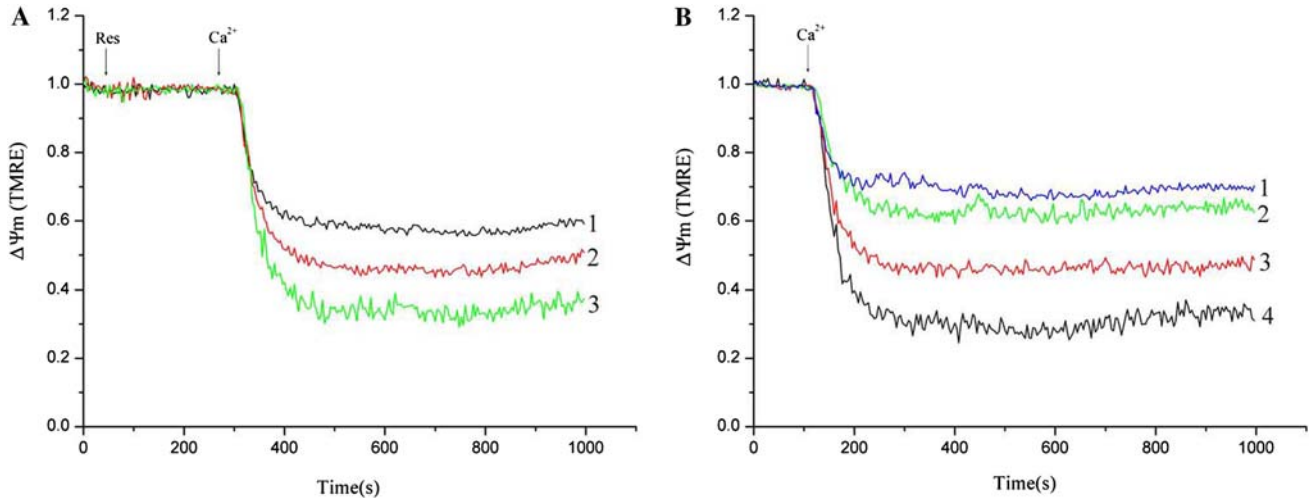

Fig. 6 The dose and time-dependent effects of resveratrol on mitochondrial permeability transition (MPT). (**A**) The dose-dependent effects of resveratrol on mitochondrial $\Delta\Psi_m$ changes 4 min after treated with, *1*, 100 μM; *2*, 120 μM; *3*, 150 μM resveratrol, respectively, the permeabilized cells were stimulated with 10 μM Ca^{2+}. (**B**) The time-dependent effects of resveratrol on mitochondrial $\Delta\Psi_m$ changes. The permeabilized cells were stimulated with 10 μM Ca^{2+} and treated with 100 μM resveratrol before detection, *1*, 0 min; *2*, 4 min; *3*, 8 min; *4*, 12 min, respectively

contrast to BAPTA/AM, EDTA revealed a minor effect on resveratrol-induce cell apoptosis, implying that extracellular Ca^{2+} influx made a small contribution to the increase of intracellular [Ca^{2+}]. We also found resveratrol induced MPT opening in the presence of Ca^{2+}, while resveratrol failed to induce MPT opening after chelating intracellular Ca^{2+} with BAPTA/AM. This demonstrated that Ca^{2+} is necessary for resveratrol-induced MPT opening.

Mitochondria play significant role in shaping cellular Ca^{2+} signals in all cell types that any disturbance either in the organelle distribution and morphology or in the driving force for Ca^{2+} accumulation may result in impaired cell function [24, 25]. Our results suggested that Ca^{2+} is essential for resveratrol-induced MPT opening. Then, what is the exact way for Ca^{2+} function in resveratrol-induced MPT? It is known that Ca^{2+} uptake by mitochondria is responsible for triggering of mCICR and mCICR can trigger MPT opening [19, 33]. Our experiments demonstrated that resveratrol could induce cytosolic Ca^{2+} increase and then induce mCICR. From our data it is presented that RR blocked the depolarization of mitochondrial $\Delta\Psi_m$, Cyt.c release and cell apoptosis, which indicated that Cyt.c release and the following cell apoptosis depended on level of the intracellular Ca^{2+} and mCICR. It suggested that mCICR may play important roles in apoptosis by inducing MPT opening. Meanwhile, these results suggest that pharmacological alteration of mCICR to alter MPT opening and Cyt.c release is possible, raising one of the possibilities of regulating apoptosis.

Though it is unknown how Ca^{2+} functions during MPT opening, it has been proposed that the MPT opening appears to be strictly dependent of the saturation of the internal Ca^{2+}-binding sites of the MPT [34]. Therefore, it is reasonable that there exists a Ca^{2+} threshold for MPT opening. Our experiments found that resveratrol could improve MPT opening in the presence of Ca^{2+}. Resveratrol also could facilitate MPT opening by lowering Ca^{2+} threshold. These results suggested resveratrol might function in apoptosis induction in this way. Meanwhile, these results suggest pharmacological alteration of Ca^{2+} threshold to alter MPT opening is possible.

In conclusion, our data provided functional evidence that resveratrol triggered HepG2 cells apoptosis via inducing MPT opening and Cyt.c release. In addition, the effect of the resveratrol in HepG2 cells was synergistic with that of Ca^{2+} and mCICR, which could be important in the control of mitochondrial functions, involving the depolarization of mitochondrial $\Delta\Psi_m$, MPT opening, Cyt.c release. Therefore, these findings suggested that it is possible to regulate reaveratrol-induced apoptosis in HepG2 cells by altering mCICR and Ca^{2+} threshold to alter MPT opening and Cyt.c release.

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