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Inhibition of ursolic acid on calcium-induced mitochondrial permeability transition and release of two proapoptotic proteins ☆

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Abstract

The possible inhibition of ursolic acid (UA) on mitochondrial permeability transition (MPT) in mouse liver was investigated to identify the mechanisms underlying the hepatoprotective effect of UA. The effect of UA on liver MPT induced by Ca^{2+} was assessed by measuring changes in mitochondrial volume, mitochondrial membrane potential (MMP), release of matrix Ca^{2+}, and transfer of cytochrome *c* (Cyt *c*) and apoptosis-inducing factor (AIF) from the intermembrane space to the cytoplasm. The results showed that obvious mitochondrial swelling, loss of MMP, and release of matrix Ca^{2+} occurred after the addition of 50 μM Ca^{2+}. However, preincubation with 20, 50 or 100 μg ml^{-1} UA significantly blocked the above changes. Addition of 100 μg ml^{-1} UA inhibited on mitochondrial swelling by 73.2% after 5 min, while the MMP dissipating and Ca^{2+} releasing were, respectively, suppressed by 59.3% and 54.1% after 3 min. In addition, Western blot analysis showed Cyt *c* and AIF transferred from mitochondrial pellet to the supernatant after the addition of 50 μM Ca^{2+}, but the process was significantly inhibited by various concentrations of UA. The results suggest that the mechanisms underlying the hepatoprotection of UA may be related to its direct inhibitory action on MPT.

Keywords: Ursolic acid; Mitochondrial permeability transition; Mitochondrial swelling; Mitochondrial membrane potential; Cytochrome *c*; Apoptosis-inducing factor

Ursolic acid (UA) is a triterpenoid compound that exists widely in food, medicinal herbs, and other plants. Previous studies show that UA has numerous pharmacological activities including antioxidant activity, as well as anti-inflammatory, anti-cancer, and hepatoprotective effects [1,2]. It can protect the liver against a number of hepatotoxicants such as carbon tetrachloride (CCl_4), acetaminophen, D-galactosamine (D-GalN), and ethanol, and has been used as a traditional medicine in China to treat human liver disorders [1,3]. However, the possible hepatoprotective mechanisms of UA remain unknown.

The accumulated evidence suggests that mitochondria play an important role in controlling cell survival and death. The function of mitochondria is not only to provide ATP by oxidative phosphorylation but includes other roles such as the modulation of intracellular Ca^{2+} homeostasis, pH control, and induction of apoptotic and excitotoxic cell

☆ *Abbreviations:* AIF, apoptosis-inducing factor; Apaf-1, apoptosis protease-activating factor-1; CCl_4, carbon tetrachloride; CsA, cyclosporine A; Cyt *c*, cytochrome *c*; D-GalN, D-galactosamine; *F*, fluorescent intensity; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; PAGE, polyacrylamide gel electrophoresis; PTP, permeability transition pore; Rh123, rhodamine 123; ROS, reactive oxygen species; UA, ursolic acid.

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death [4,5]. It is generally accepted that cell death is the most crucial step in the development of all kinds of liver injury, fibrosis, alcoholic liver disease, and hepatitis [6,7].

Recently, the mitochondrial permeability transition pore (PTP) was identified as a major player in the mitochondrial pathways leading to cell death. As a channel at the contact sites between the inner and outer mitochondrial membrane, PTP modulates apoptotic and excitotoxic cell death, and is involved in most kinds of liver injury and liver disease [8] by being switched between its two conductance states. At low conductance, PTP opening is reversible and does not entail large amplitude swelling of the mitochondrial matrix. It is pH operated and allows the diffusion of ions and can be blocked spontaneously during the normal life of the cell. At high conductance, PTP opening is irreversible and involved in the mediation of cell death. It causes mitochondrial permeability transition (MPT), which allows solutes of molecular weights greater than 1.5 kDa to pass between the mitochondrial matrix and the cytoplasm. This causes equilibration of ions within the matrix and the cytosol, dissipating the membrane potential and uncoupling the respiratory chain. The volume disregulation following the opening of the PTP results in the swelling of the matrix, leading to outer membrane disruption and the release of proapoptotic proteins such as cytochrome *c* (Cyt *c*) and apoptosis-inducing factor (AIF) into the cytosol, ultimately contributing to cell death [9]. The process of switching from low- to high-conductance state of PTP can be induced by high intracellular inorganic phosphate, Ca^{2+}, reactive oxygen species (ROS) or proapoptotic proteins such as Bax. It is inhibited by cyclosporine A (CsA), Bcl-2 or Bcl-$_{XL}$ and is strictly dependent on the saturation of the internal Ca^{2+}-binding sites of the PTP [9]. In fact, it is widely accepted that mitochondria finally induce apoptotic and necrotic cell death by inducing MPT and the release of Cyt *c* and AIF from the mitochondrial intermembrane space.

Previous reports have shown that MPT occurs in livers of ischemia–reperfusion, D-GalN and CCl_4 damaged animals. Indeed, it was suggested that inhibition of mitochondrial PTP opening might constitute a relevant pharmacological approach to protect cell from death, and the search for novel PTP inhibitors should be an important strategy for the treatment of liver diseases [10,11]. In this paper, we address the possible inhibition of UA on liver mitochondrial PTP opening, Ca^{2+} release, and both Cyt *c* and AIF transfer from mitochondria to cytosol in the search to identify the mechanisms underlying the hepatoprotective effect of UA.

Materials and methods

Chemicals. Fura-2/AM, rhodamine 123 (Rh123), succinate, rotenone, and cyclosporine A (CsA) were purchased from Sigma Chemical (St. Louis, MO, USA). Ursolic acid (UA, 95% purity) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Anti-Cyt *c* and AIF antibodies were purchased from Trevigen (Gaithersburg, MD, USA) and R&D Systems (Minneapolis, MN, USA), respectively. All other chemicals were of high purity from commercial sources.

Animals. Adult male ICR mice weighing 18–22 g (Experimental Animal Center of Southeastern University, Grade II, Certificate No. 97003) were housed at a temperature of 20–25 °C under a 12-h light/dark cycle with 50% of relative humidity and kept in filtered and pathogen-free air. This study complied with current ethical regulations on animal research in our university.

Isolation of liver mitochondria. Mitochondria were prepared from mice livers according to the method of Apprille et al. [12]. In brief, mice livers were excised and homogenized in isolation buffer containing 225 mM mannitol, 75 mM sucrose, 0.05 mM EDTA, and 10 mM Tris–HCl (pH 7.4) at 4 °C. The homogenates were centrifuged at 600*g* for 5 min to remove cell debris and the nuclear fraction. The resultant supernatants were centrifuged at 8800*g* for 10 min. The pellet was washed twice with the same medium. Protein concentration was determined using Coomassie brilliant blue.

Determination of mitochondrial swelling. Mitochondrial swelling was assessed by measuring the absorbance of their suspension at 540 nm. Liver mitochondria of normal mice were prepared in the assay buffer (1.0 mg protein ml^{-1}) containing 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 μM rotenone, 10 mM Hepes, and 5 mM succinate. For dose-dependent test, 20, 50 or 100 μg ml^{-1} UA was added 3 min prior to the addition of 50 μM Ca^{2+}. For time-dependent test, UA (50 μg ml^{-1}) was added to the same medium 1, 2 or 3 min following the treatment of 50 μM Ca^{2+}. CsA (5 μM) was used as a positive reference [13]. The extent of mitochondrial swelling was assayed by measuring the decrease in absorbance (A_{540}) 1, 2, 3, 4, and 5 min after the addition of 50 μM Ca^{2+} at 30 °C and the inhibitory rate of mitochondrial swelling was calculated as follows: $(\Delta A_{\text{Control}} - \Delta A_{\text{drug}})/\Delta A_{\text{Control}} \times 100\%$, $\Delta A = A_{0\ \text{min}} - A$ [14].

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (MMP) was evaluated according to Emaus et al. [15] from the uptake of the fluorescent dye rhodamine 123 (Rh123) which accumulates electrophoretically into energized mitochondria in response to their negative inside membrane potential. Liver mitochondria isolated from normal mice were incubated at 25 °C in the assay buffer (0.5 mg protein ml^{-1}) containing 225 mM D-mannitol, 70 mM sucrose, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 5 mM succinate, and 5 μM rotenone, pH 7.2. UA of 20, 50 or 100 μg ml^{-1} was added, and the discharge of Rh123 was induced by 50 μM $CaCl_2$. MMP was assessed spectrophotometrically (Hitachi 850) with excitation at 505 nm and emission at 534 nm after addition of 0.3 μM Rh123 at 25 °C [16]. CsA at 5 μM was used as a positive reference [13].

Measurement of mitochondrial-free calcium. The intramitochondrial Ca^{2+} level was assayed by Ca^{2+} indicator dye fura-2/AM. Liver mitochondria (0.5 mg protein ml^{-1}) of normal animals were incubated with the fluorescence fura-2/AM for 30 min at 30 °C in suspension medium containing 125 mM sucrose, 65 mM KCl, 5 mM succinate, 5 mM Hepes, and 1 μM fura-2/AM, pH 7.4, and then washed twice with the medium without the dye to eliminate free fura-2/AM. The final mitochondria pellet was diluted in the suspension medium to obtain a protein concentration of 0.5 mg ml^{-1}. Various concentrations of UA were added to the medium and incubated for 3 min. Ca^{2+} release was induced when challenged with 50 μM $CaCl_2$. Fluorescent intensity (*F*) of fura-2 loaded mitochondria was recorded on a Hitachi 850 fluorescence spectrometer at an excitation 340 nm and an emission 510 nm [14]. CsA at 5 μM was used as a positive reference [13].

Immunoblot analysis of Cyt c and AIF release. Both the amounts of Cyt *c* and AIF in the supernatant and in the mitochondrial pellet were determined by Western blot analysis [17,18]. Liver mitochondria were prepared in the same assay buffer as in the method of measurement on mitochondrial swelling and incubated at 30 °C with different concentrations of drugs for 3 min. Mitochondria pellets from all groups were obtained by centrifugation at 8800*g* for 10 min after the addition of 50 μM Ca^{2+}. Protein was extracted from the pellets with lysis buffer (50 mM Tris–HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0). The supernatant and protein from the mitochondrial pellets were separated by 10% or 15% SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred to

a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The blot was probed with a primary antibody against Cyt *c* at a dilution of 1:1000 or against AIF diluted at 1:2000. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies and the blots were visualized by ECL-based detection (ECL kit, Amersham).

Statistical analysis. Differences among all groups were analyzed by one-way analysis of variance (ANOVA), followed by SNK-q-test and P value <0.05 was accepted as statistical significance.

Results

Effect of UA on Ca^{2+}-induced mitochondrial swelling

As shown in Fig. 1A, we found obvious swelling of liver mitochondria after the addition of 50 μM Ca^{2+}. UA at various concentrations showed a dose-dependent attenuation against the swelling. After 5 min, 20, 50, and 100 μg ml^{-1} UA inhibited swelling by 27.0%, 58.8%, and 73.2%, respectively. Inhibition of Ca^{2+}-induced mitochondrial swelling by UA was also a function of elapsed time, the longer the delay in adding UA, the lower the inhibitory rate of swelling (Fig. 1B).

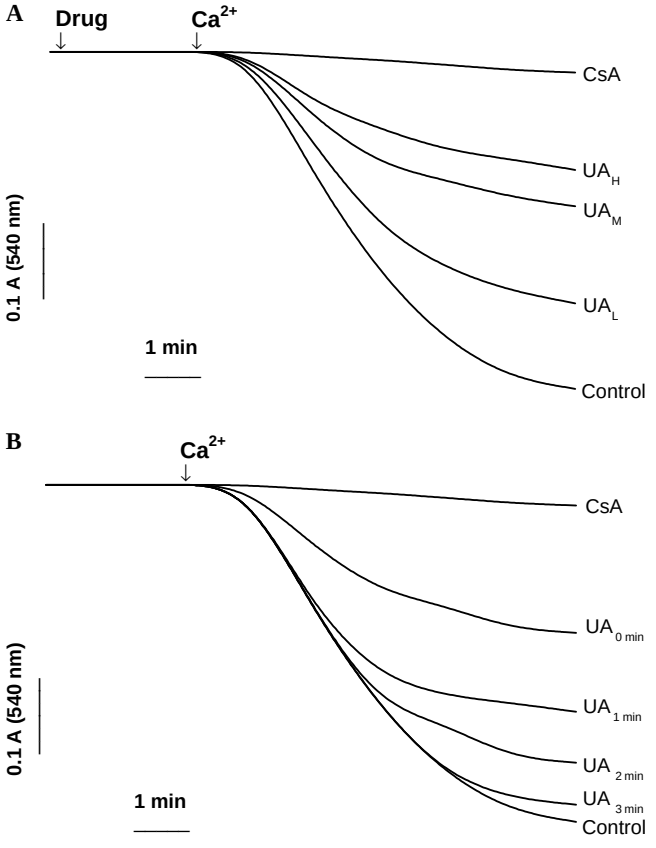

Fig. 1. Effect of UA on Ca^{2+}-induced mitochondrial swelling. (A) Dose-dependent inhibitory effect on Ca^{2+}-induced mitochondrial swelling by UA. UA_L, UA_M, and UA_H represented 20, 50 or 100 μg ml^{-1} UA, respectively. (B) Time-dependent inhibition of mitochondrial swelling by UA. UA (50 μg ml^{-1}) was added 3 min before or 1, 2, and 3 min after ($UA_{0\ min}$, $UA_{1\ min}$, $UA_{2\ min}$, and $UA_{3\ min}$) adding Ca^{2+} to the succinate-energized mitochondria. The control trace referred to Ca^{2+} alone, and CsA (5 μM) was used as a positive reference. The curves represent typical recordings from experiments of at least three different mitochondrial preparations.

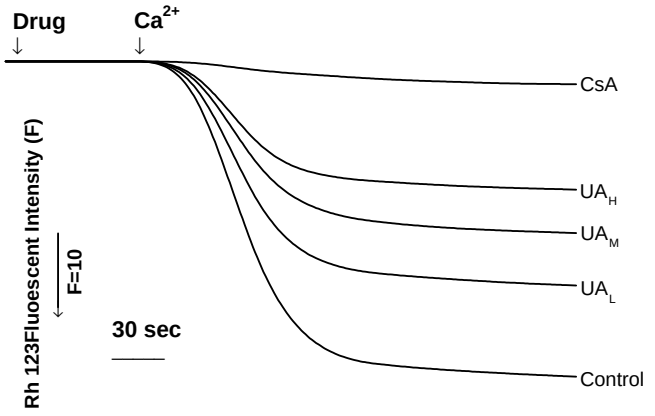

Fig. 2. Effect of UA on Ca^{2+}-induced dissipation of mitochondrial membrane potential. UA_L, UA_M, and UA_H represented 20, 50, and 100 μg ml^{-1} UA, respectively. CsA (5 μM) was used as a positive reference. The results represent one typical recording from three experiments.

Effect of UA on Ca^{2+}-induced dissipation of mitochondrial membrane potential

The addition of 50 μM Ca^{2+} to the isolated mitochondria induced a progressive increase in the discharge of Rh123 from the mitochondria, which indicated a loss of MMP. The fluorescent intensity (F) of the mitochondrial suspension increased significantly (45.9%) 3 min after the addition of Ca^{2+}. A dose-dependent inhibition of MMP loss was observed (Fig. 2) when UA was added to the isolated mitochondria 3 min before the addition of Ca^{2+}.

Effect of UA on Ca^{2+}-induced intramitochondrial-free Ca^{2+} release

Fig. 3 shows that 50 μM Ca^{2+} caused an obvious decrease (37.8%) in the fluorescent intensity of the mitochondrial suspension, which indicated intramitochondrial-free Ca^{2+} release. Against the control, the pretreatment of UA blocked the mitochondrial Ca^{2+} release in a dose-dependent manner. Treatment with 20, 50, and 100 μg ml^{-1} UA, respectively, inhibited Ca^{2+} release by 30.0%, 46.2%, and 54.1%.

Effect of UA on Cyt c and AIF release from liver mitochondria

Fig. 4 shows the addition of 50 μM Ca^{2+} induced the release of large amounts of both Cyt *c* and AIF from the mitochondria pellet to the supernatant. However, pretreatment with various concentrations of UA significantly blocked the mitochondrial Cyt *c* and AIF release.

Discussion

Previous studies have shown that UA can effectively block cell damage and death in liver from CCl_4, acetaminophen, D-GalN, and ethanol, and can also significantly

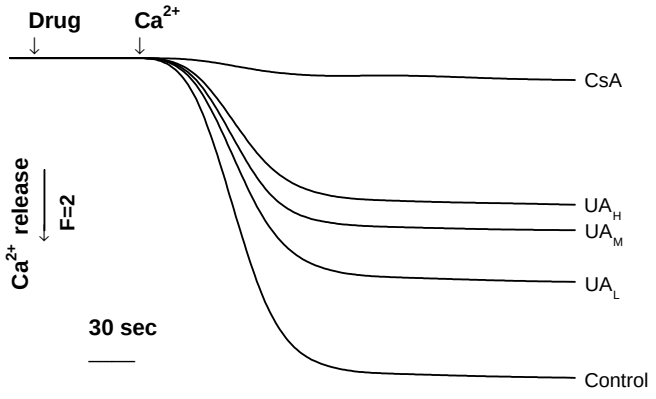

Fig. 3. Effect of UA on intramitochondrial-free Ca^{2+} release induced by Ca^{2+}. UA_L, UA_M, and UA_H represented 20, 50, and 100 μg ml^{-1} UA, respectively. CsA (5 μM) was used as a positive reference. The results are one representation of the experiments of three different mitochondrial preparations.

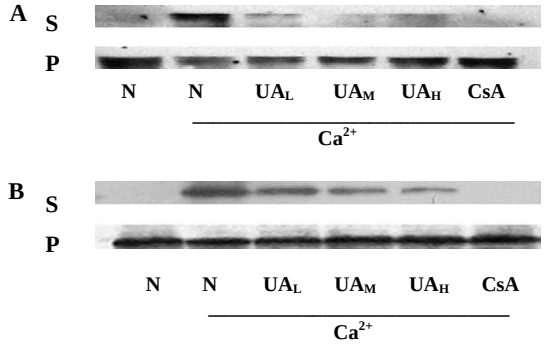

Fig. 4. Effect of UA on Ca^{2+}-induced intramitochondrial Cyt *c* and AIF release. Western immunoblots of Cyt *c* (A) and AIF (B) in suspensions of mitochondria exposed to Ca^{2+}. Liver mitochondria isolated from normal mice were incubated with drugs before the addition of 50 μM Ca^{2+}, and then mitochondrial suspensions were centrifuged and the supernatant (S, concentrated 5-fold) and pellet (P) were used for immunoblotting. UA_L, UA_M, and UA_H represented 20, 50, and 100 μg ml^{-1} UA, respectively. CsA (5 μM) was used as a positive reference. The data shown are typical of three experiments.

decrease the elevation in both serum AST and serum ALT in animals damaged by those hepatotoxicants [1,3]. The results suggest that the hepatoprotection of UA might be related to its improvement of function of liver mitochondria because it has been accepted that ALT enzyme is an indicator of the degree of the cell membrane damage while AST is for mitochondrial damage since mitochondria contain 80% of the enzyme [19]. However, the mechanisms underlying the protection of UA on the liver mitochondria were not determined. The present study demonstrated the direct protection of UA on liver mitochondria considering that MPT plays a key role in mitochondrial control of cell death.

There is growing evidence that it is crucial for the cell to maintain cytosolic Ca^{2+} at very low level (0.1–0.2 μM). However, all kinds of liver injury and liver disease can result in hepatocellular Ca^{2+} overload [20], which can activate the mitochondrial Ca^{2+} uniporter in the mitochondrial inner membrane and induce a mitochondrial Ca^{2+} influx. However, the excessive intramitochondrial Ca^{2+} can lead to the opening of mitochondrial PTP, and finally damage mitochondria and induce apoptotic or necrotic cell death. Ca^{2+}-induced liver MPT has therefore become a widely used model for evaluating the effects of drugs or other substances on MPT [11]. In this paper, MPT was induced by adding 50 μM Ca^{2+} to liver mitochondria isolated from mice and assessed by measuring release of matrix Ca^{2+} as well as mitochondrial swelling and MMP. The results showed that UA could block the overload of intramitochondrial Ca^{2+} in a dose-dependent manner, which might be one of the mechanisms underlying its protection on liver mitochondria.

The extent of mitochondrial swelling is believed to be an important indicator of mitochondria function. It has been found that significant liver mitochondrial swelling occurs in liver injury such as CCl_4-induced and ischemia–reperfusion liver injuries [13,21]. Our observations of liver mitochondria in vitro show that UA can prevent Ca^{2+}-induced mitochondrial swelling in a dose- as well as time-dependent manner in mice livers, indicating it strongly inhibits the triggering of MPT.

It is well documented that maintenance of the MMP is necessary for the mitochondria to carry out its functions. Disruption of the MMP is known as to be an early event in the process of MPT in the liver, which can be induced by the treatment of mice with CCl_4 and D-GalN, and by ischemia–reperfusion damage [21–23]. Data presented in our study demonstrate that 20, 50, and 100 μg ml^{-1} UA significantly prevented the collapse of MMP induced by the challenge of Ca^{2+}, which confirmed the suggestion that UA inhibits the opening of mitochondrial PTP.

PTP opening results in the release of Cyt *c* and another important mitochondrial intermembrane protein, AIF, from mitochondrial intermembrane space to the cytoplasm. Cyt *c* is thought to interact with the protein apoptosis protease-activating factor-1 (Apaf-1) to activate caspase-9, which in turn activates other caspases such as caspase-3, 6, 7, and induces nuclear apoptosis [24]. AIF can act as a direct activator of nuclear DNases in a caspase-independent way, thereby causing the characteristic oligonucleosomal DNA fragmentation pattern observed during apoptosis [24]. Various concentrations of UA were found significantly to block both the release of mitochondrial Cyt *c* and AIF from mitochondrial intermembrane space induced by Ca^{2+}, suggesting that UA protects hepatocytes against cell apoptosis in both caspase-dependent and -independent ways.

This study demonstrates that UA can protect liver mitochondria against Ca^{2+}-induced mitochondrial swelling, MMP dissipation, Ca^{2+} release from the mitochondrial matrix, as well as Cyt *c* and AIF transfer from the mitochondrial intermembrane space. These findings indicate that the mechanisms underlying the hepatoprotection of UA against hepatotoxing might be related to the direct inhibitory effect of UA on MPT, which interferes in cell

death in a caspase-dependent as well as -independent manner. Mitochondrial PTP should therefore be an important pharmacological target in screening for hepatoprotective agents with potential in clinical application.

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