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ORIGINAL ARTICLE

Tanshinone IIA protects rat primary hepatocytes against carbon tetrachloride toxicity via inhibiting mitochondria permeability transition

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Abstract
Tanshinone IIA (Tan IIA), one of the key components of *Salvia milthorrhiza* Bunge (Lamiaceae), is used to treat liver disease. The present study was carried out to investigate the possible mechanisms involved in the hepatoprotective effects of Tan IIA on carbon tetrachloride (CCl_4)-induced hepatocyte toxicity. In cultures treated with 1 or 2 µM CCl_4, Tan IIA (10–75 µM) significantly increased hepatocyte survival rates. However, only at a concentration of 75 µM could Tan IIA partially reverse the CCl_4 (3 µM)-induced decrease of survival rate (34 ± 3% vs. 18 ± 3%, $n = 8$, $p < 0.01$). In isolated mitochondria energized with succinate, Tan IIA could inhibit the large swelling effect induced by CCl_4 (1 and 2 µM). Base on these results, Tan IIA could protect rat primary cultured hepatocytes from CCl_4-induced toxicity partially by the inhibitory effect on the opening of mitochondrial permeability transition (MPT).

Keywords: *Carbon tetrachloride; hepatocyte toxicity; mitochondrial permeability transition; Tanshinone IIA*

Introduction

Tanshinone IIA (Tan IIA), a derivative of phenanthrene-quinone isolated from Danshen (*Salvia milthorrhiza* Bunge (Lamiaceae)), a widely used Chinese herbal medicine, has antioxidant properties. Tan IIA inhibits alcoholic liver disease (Yin et al., 2008), inhibits the inflammatory response of colitis (Bai et al., 2008), and suppresses the inflammation in atherosclerotic lesions (Fang et al., 2008) by means of antioxidant activity. Meanwhile, the effects of Tan IIA have been elucidated against carbon tetrachloride (CCl_4)-induced hepatotoxicity (Liu et al., 2001, 2002, 2003). However, the mechanisms underlining the hepatoprotective effects of Tan IIA have not been reported.

CCl_4 was found to induce severe oxidative stress in the liver more than 40 years ago (Friede, 1960). Numerous studies have show evidence that antioxidative supplements could rescue CCl_4-induced hepatocyte toxicity (Qiusheng et al., 2004; Wang et al., 2008), which triggers apoptosis via a mitochondria-initiated pathway (Cai et al., 2005). Moreover, an increase in the resistance of hepatic mitochondria to Ca^{2+}-stimulated permeability transition (mitochondrial permeability transition, MPT) protected mouse livers from CCl_4 toxicity (Chiu et al., 2007).

All of these data suggest that Tan IIA might have a protective role against CCl_4-induced hepatocyte toxicity by inhibition of MPT. In the present study, we investigated the involvement of MPT in CCl_4-induced hepatocyte toxicity.

Materials and methods

Chemicals

All chemicals and solvents were from Sigma (St. Louis, MO, USA) and of analytical grade unless otherwise stated. Tanshinone IIA (Tan IIA) was dissolved in dimethylsulfoxide (DMSO) to obtain the stock solution, which

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was further diluted to achieve the final concentration. DMSO (≤0.1%) was used as a control.

Animals and isolation of rat primary hepatocytes

Sprague-Dawley male rats with an average weight of 200 ± 30 g were supplied by Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). The rats were housed in cages under controlled conditions at 20 ± 3°C and 45–65% humidity with a 12 h light–dark cycle (lights on 06:00 h). Drinking water and food were provided *ad libitum* throughout the study. All procedures were performed in accordance with guidelines on the care and use of experimental animals set by Fudan University Cancer Hospital.

Hepatocytes were isolated from Sprague-Dawley male rats by two-step collagenase perfusion as described previously (Cai et al., 2005; Zhai et al., 2008) with collagenase IV. Monolayer hepatocytes were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) (1:1) supplemented with 15% fetal bovine serum (PAA Laboratories GmbH, Linz, Austria), 100 U/mL penicillin, and 70 μg/mL streptomycin. Hepatocytes provided with fresh medium throughout each test were referred to as the control cultures. In the present tests, Tan IIA was added to the culture medium 1 h prior to CCl_4 and cultures treated with Tan IIA alone were referred to as the vehicle control.

Hepatocyte survival rates

Rat primary hepatocytes were treated with Tan IIA in the presence or absence of CCl_4. The survival rates were estimated by the standard trypan blue exclusion assay. Briefly, rat primary hepatocytes were trypsinized and dyed with 0.4% Trypan blue after being collected by centrifugation. Viabilities were determined by counting an average of 100 cells per field in four different fields per culture.

Preparation of rat liver mitochondria and induction of MPT

Hepatic mitochondria were isolated from rats in a sucrose–HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer by differential centrifugation (Cai et al., 2005). Mitochondria (75 μg mitochondrial protein per mL) were suspended in 1 mL buffer (2 mM HEPES, pH 7.5, 0.25 M sucrose, 10 mM succinate, 1 mM potassium phosphate). MPT was initiated by the addition of CCl_4 with calcium (20 μM final concentration) as indicated in the figures. The progression of MPT was monitored by the change in absorbance at 540 nm at room temperature.

Measurement of hepatocyte mitochondrial membrane potential

Mitochondrial membrane potential was evaluated as the accumulation of TMRE (tetramethylrhodamine, ethyl ester, perchlorate; final concentration of 500 nM) according to the method described by Wu et al. (1990). CsA (1 μM) was used in this procedure as an inhibitor of mitochondrial permeability transition (MPT). Fluorescence readings were taken on a fluorimeter (NOVOstar; BMG LABTECH, Offenburg, Germany) with the excitation wavelength at 485 nm and the emission wavelength at 520 nm.

Statistical analysis

Group mean values and standard deviations were calculated. Data are expressed as the mean ± SD of three independent experiments unless otherwise stated. Statistically significant differences were determined by Student's *t*-test. Differences were considered statistically significant if $p < 0.05$.

Results

Tanshinone IIA reduces the hepatocytotoxic effect of CCl_4

CCl_4 (1, 2, and 3 μM) remarkably decreased the survival rates of rat primary hepatocytes to 30.2 ± 6.5%, 20.8 ± 4.3%, and 18.9 ± 3.1%, respectively ($n = 8$). In cultures treated with 1 or 2 μM CCl_4, Tan IIA (10–75 μM) significantly increased the survival rates (Figure 1). Moreover, Tan IIA (75 μM) successfully protected rat primary hepatocytes

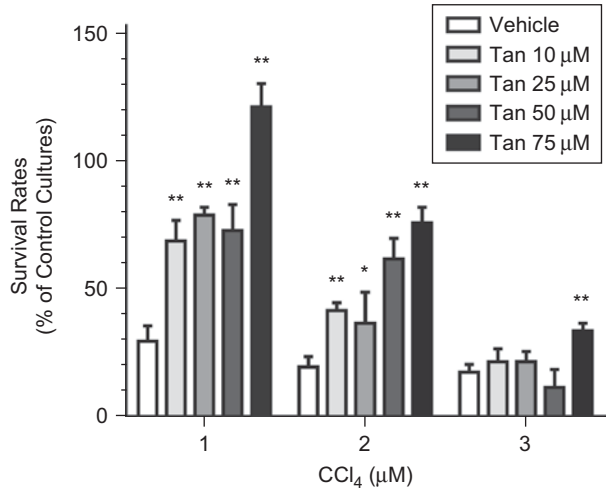

Figure 1. CCl_4-induced decrease of rat primary hepatocyte viability. Rat primary hepatocytes cultured with CCl_4 (1, 2, and 3 μM) for 24 h caused significant decrease of viability ($n = 8$). Significant difference from (vehicle) control cultures, $^*p < 0.05$; $^{**}p < 0.01$.

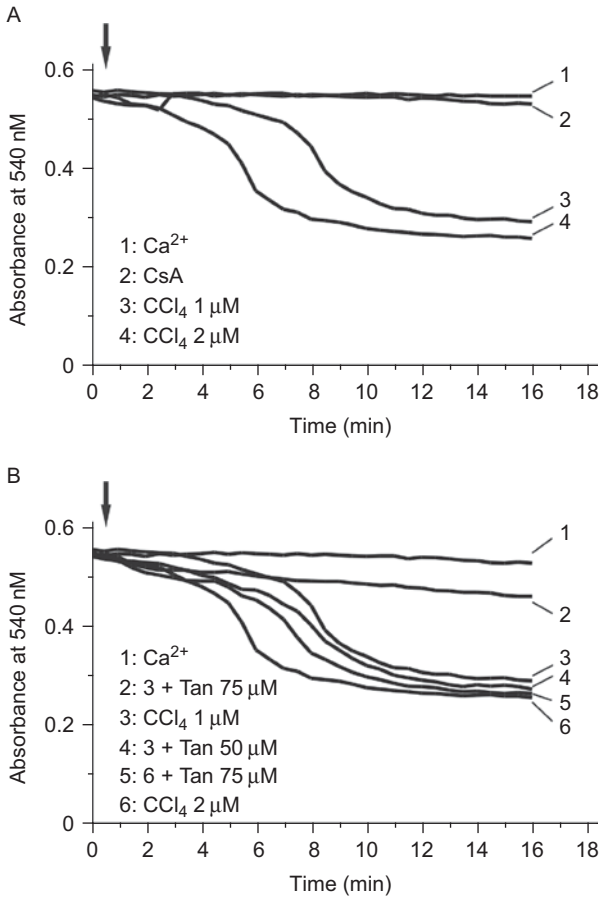

Figure 2. Induction of mitochondrial swelling by CCl_4 in isolated hepatic mitochondria. (A) Swelling effect monitored as the decrease of absorbance at 540 nm in energized mitochondria, CCl_4 was added where indicated. Note that 20 μM Ca^{2+} alone could not induce mitochondrial permeability transition (MPT). Mitochondrial swelling was inhibited by adding 1 μM CsA. (B) Swelling effect monitored at A540 as in (A). Ca^{2+} (20 μM final concentration) was used as the control. 75 μM Tan IIA could inhibit 1 μM CCl_4-induced mitochondrial swelling. The data represent a typical experiment conducted at least three times with similar results.

against CCl_4 (3 μM)-induced hepatocyte death. However, at lower concentrations (10–50 μM), Tan IIA failed to block the toxic effects of 3 μM CCl_4.

Tanshinone IIA inhibits CCl_4-induced mitochondrial permeability transition opening

To further investigate the role of mitochondrial permeability transition (MPT) in the protective effects of Tan IIA against CCl_4 hepatocyte toxicity, we monitored the apparent decrease in the absorbance of the mitochondria suspension at 540 nm. CCl_4 could induce mitochondrial swelling with a quick onset and large magnitude (Figure 2A). Mitochondrial swelling induced by CCl_4 was totally blocked when CsA was present (Figure 2A). In the presence of 75 μM Tan IIA, both the rate of onset

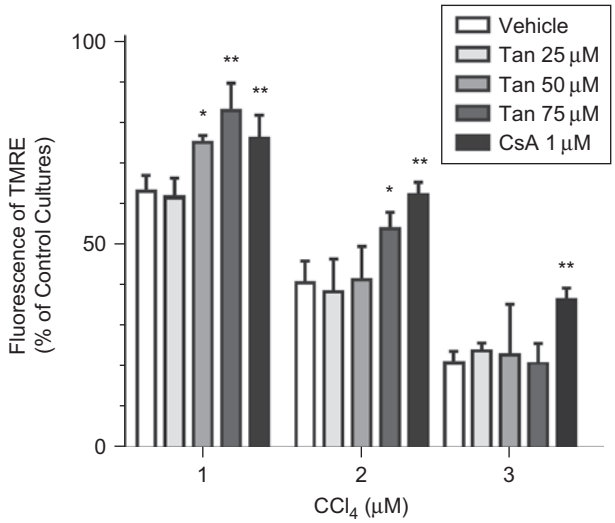

Figure 3. The effects of Tan IIA on CCl_4-induced decrease of the mitochondrial transmembrane potential (MMP) of rat primary hepatocytes ($n = 8$). CsA was referred to as the positive control. Significant difference from (vehicle) control cultures, $^*p < 0.05$; $^{**}p < 0.01$.

and the magnitude of 1 μM CCl_4-induced mitochondrial swelling were inhibited (line 2 vs. line 3, Figure 2B).

Tanshinone IIA reverses CCl_4-induced decrease of mitochondria membrane potential

The mitochondria membrane potential (MMP) was evaluated in hepatocytes treated with increased concentrations of CCl_4 in the absence or presence of cyclosporine A (CsA), a selective mitochondrial permeability transition (MPT) inhibitor.

As shown in Figure 3, MMP dropped to 63.72 ± 3.07%, 41.14 ± 8.02%, and 22.77 ± 1.97% of the control cultures after 24 h exposure to 1, 2, and 3 μM CCl_4, respectively. This effect also was reversed by the addition of Tan IIA or CsA.

Discussion

At least two different apoptosis pathways are involved in CCl_4-induced apoptosis, the mitochondrial pathway and the death-receptor pathway (Araragi et al., 2003). MPT, a typical character of mitochondrial dysfunction, is a major controlling mechanism in certain apoptotic systems that also contributes to the release of cytochrome c (Cai & Jones, 1998), which was detected in CCl_4-treated rat primary hepatocytes. At the same time, caspase 3 activation as well as a decrease in cellular glutathione (GSH) content and an increase in malondialdehyde level was observed (Cai et al., 2005). However, its pathologic role in CCl_4-induced liver injury needs to be further clarified.

MPT could be induced by CCl_4 in isolated rat hepatic mitochondria, and the addition of cyclosporine A (CsA), a selective MPT inhibitor, could block this phenomenon (Figure 2A). Tan IIA was able to decrease the mitochondrial sensitivity to Ca^{2+}-induced MPT (Figure 2B). A similar effect led to the protective influence of Schisandrin B against CCl_4 toxicity in mouse livers (Chiu et al., 2007). Our results as shown in Figure 1 also confirmed the hypothesis that the inhibitory effect of Tan IIA on MPT opening might result in increased viability.

Tan IIA at concentrations lower than 75 μM failed to block CCl_4 (1 and 2 μM)-increased mitochondria sensibility to Ca^{2+}-induced MPT (Figure 2B) while successfully inhibiting CCl_4 (1 and 2 μM)-induced hepatocyte toxicity and reversing the decrease of mitochondria membrane potential (Figures 1 and 3). Moreover, 75 μM Tan IIA failed to block 3 μM CCl_4-induced MPT or the decrease of MMP while showing an ability to inhibit 3 μM CCl_4-induced hepatocyte toxicity (Figure 1). These inconsistencies might result from the activities of Tan IIA on other cellular pathways, such as inhibition of JNK activation (Park et al., 2007; Yang et al., 2008) or p-ERK1/2 expression (Li et al., 2008), which were absent in cell-free conditions.

The most important findings of the present study were that Tan IIA could increase rat primary hepatocyte viabilities in the presence of CCl_4 and that the inhibitory effect of Tan IIA on CCl_4-induced mitochondria dysfunction might be involved in the protective effect against CCl_4 toxicity.

Declaration of interest

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