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Tetrandrine-induced apoptosis is mediated by activation of caspases and PKC-δ in U937 cells

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Abstract

Tetrandrine, which is isolated from Chinese herb *Stephania tetrandrae*, possesses anti-inflammatory, immunosuppressive, and cytoprotective properties. Though it was previously shown that tetrandrine causes a G1 blockade and apoptosis in various cell types, however, the mechanism by which tetrandrine initiates apoptosis remains poorly understood. In present study, we investigated the mechanisms of apoptosis induced by tetrandrine in U937 leukemia cells. Tetrandrine inhibited U937 cell growth by inducing apoptosis. After treatment of U937 cells with tetrandrine (10 μM) for 24 h, alteration of cell morphology, chromatin fragmentation, cytochrome *c* release, and caspase activation were observed. Tetrandrine also induced early oxidative stress, which resulted in activation of JNK, but not ERK and p38 MAPK. A broad-spectrum caspase inhibitor and antioxidants significantly blocked tetrandrine-induced caspase-3 activation. However, inhibition of the JNK activity with SP600125 did not block tetrandrine-induced apoptosis. Tetrandrine-induced apoptosis of U937 cells also required activity of PKC-δ, because pretreatment with a specific PKC-δ inhibitor greatly blocked tetrandrine-induced caspase-3 activation. In addition, the apoptotic response to tetrandrine was significantly attenuated in dominant-negative PKC-δ transfected MCF-7 cells, suggesting that PKC-δ plays an important role in tetrandrine-induced apoptosis and can induce caspase activation. These results suggest that tetrandrine induces oxidative stress, JNK activation, and caspase activation. However, JNK activation by ROS is not involved in the tetrandrine-induced apoptosis. In addition, tetrandrine induces caspase-dependent generation of a catalytically active fragment of PKC-δ, and this fragment also appears to play a role in the activation of caspases.

Keywords: Tetrandrine; Apoptosis; Caspase; ROS; JNK; PKC-δ

1. Introduction

Tetrandrine, a bis-benzylisoquinoline alkaloid isolated from the roots of *Radix stephania tetrandrae* (S. Moore), has been used in China for several decades to treat patients with arthritis, arrhythmia, hypertension, inflammation, and silicosis [1–3]. Tetrandrine has been known to possess immunosuppressive [4], non-selective Ca^{2+} channel blocking [5], anti-proliferative [6,7], and free radical scavenging effects [8]. In addition, tetrandrine has been considered to be a promising chemopreventive agent, because tetrandrine causes a G1 blockade and apoptosis in various cell types such as U937, HL60, A549, HepG2, and T-cell [7,9–13]. Many therapeutic and chemopreventive agents eliminate tumor cells by inducing apoptotic cell death. However, the mechanism by which tetrandrine

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly[ADP-ribose] polymerase; PKC, protein kinase C; ROS, reactive oxygen species; z-VAD-fmk, N-benzyloxcarbonyl-Val-Ala-Asp-fluoromethyl ketone; Ac-DEVD-*p*Na, Asp-Glu-Val-Asp-chromophore *p*-nitroanilide

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induces apoptosis of cancerous cells is not clearly understood.

Apoptosis is important for destruction of undesired cells during development and homeostasis of multi-cellular organisms, and is characterized by distinct morphological changes such as plasma membrane blebbing, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation [14]. Many proteins are known to involve in the process of apoptosis. Caspases are essential for the execution of cell death by various apoptotic stimuli [15]. Caspase activation is often regulated by various proteins, including the member of inhibitors of apoptosis (IAP) and Bcl-2 family [16,17]. In addition to caspases, it has been shown that specific protein kinases including MAPK and PKC family are involved in mediating apoptosis [18–21]. The family of MAPK is known to regulate cellular proliferation, survival, and differentiation [22]. Among the family of MAPK, JNK and p38 MAPK have been implicated in the regulation of the apoptotic cell death induced by diverse stimuli, including DNA damage, heat shock, oxidative stress, or ultraviolet light [19,20,22,23]. The PKC family of serine–threonine kinases is activated by diverse apoptotic stimuli and participates in various cellular functions [24]. Recently, many studies have suggested that specific isoforms of PKC play important roles in cell apoptosis [18,21,25].

In present study, the molecular events that mediate tetrandrine-induced apoptosis in U937 cells were examined. Our results demonstrate that tetrandrine induces oxidative stress, JNK activation, and caspase activation. But JNK activation by ROS is not involved in the tetrandrine-induced apoptosis. In addition, tetrandrine also induces generation of a catalytically active fragment of PKC-δ and this fragment presumably plays a role in the activation of caspases.

2. Materials and methods

2.1. Materials

Tetrandrine, DCFH-DA, Vitamin E, *N*-acetylcysteine, and glutathione were purchased from Sigma–Aldrich. Antibodies for caspase-3, -7, -9, cytochrome *c*, XIAP, HIAP-1, HIAP-2, and Bax were bought from PharMingen. Antibodies for Bcl-2 and PARP were purchased from Boehringer Mannheim. Anti-rabbit or mouse secondary horseradish peroxidase antibodies and ECL Western detection reagents were bought from Amersham Biosciences. SP600125 was purchased from Biomol. Ac-DEVD-*p*Na, z-VAD-fmk, and rottlerin were obtained from Calbiochem. Bradford reagent was from Bio-Rad. Antibodies for PKC-δ, phospho-PKC-δ (Thr505; p-PKC-δ), ERK, phospho-ERK, JNK, phospho-JNK, p38 MAPK, and phospho-p38 MAPK were obtained from Cell Signaling.

2.2. Cell culture

U937 cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Typically, 3×10^5 cells/ml of U937 cells were seeded in T-25 flasks as 4 ml cultures and maintained in the tissue culture incubator for 12–16 h before the addition of tetrandrine. At the indicated times, cells were harvested and washed twice with phosphate-buffered saline (PBS). Cell viability was determined by trypan blue exclusion. Tetrandrine was stored in powder and dissolved immediately in dimethyl sulfoxide before experiments.

2.3. Assessment of apoptotic nuclei and DNA fragmentation assay

For propidium iodide staining, cells were harvested and washed twice with ice-cold PBS and fixed with 4% paraformaldehyde in 0.1% phosphate buffer (pH 7.4) for 10 min at room temperature, followed by ethanol containing 1% HCl for 10 min at −20 °C. Fixed cells were placed on slides and stained with 1 μg/ml propidium iodide solution containing 100 μg/ml DNase-free RNase A for 30 min at 37 °C. Nuclear morphology of cells was examined by a fluorescence microscopy. For DNA fragmentation assay, cells were harvested, washed in PBS and then lysed in a digestion buffer containing 50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/ml proteinase K, and 1 mM EDTA at 55 °C for 3 h. RNase A (0.5 μg/ml) was added and further incubated at 55 °C for 18 h. The genomic DNA was extracted by phenol–chloroform–isoamylalcohol and analyzed by 1.7% agarose gel electrophoresis. The DNA was visualized and photographed under ultra-violet illumination after staining with ethidium bromide.

2.4. Measurement of caspase-3-like activity

Cells were collected and washed with PBS and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of each of the following: aprotinin, leupeptin, and pepstatin. Cell lysates were clarified by centrifugation at 12,000 rpm for 10 min, and 50 μg of protein were incubated with 50 μM Ac-DEVD-*p*Na colorimetric substrate at 37 °C for 1 h. Optical density was then measured at 405 nm.

2.5. Western blot analysis

Whole cell extracts were prepared in lysis buffer as described previously [26], and mitochondria-free cell extracts were prepared from cells as described previously [27]. For phospho-protein detection, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and 1 mM NaF, and

lysed in a buffer (20 mM Tris–Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine–HCl). The protein concentration of extracts was estimated with Bradford reagent using bovine serum albumin as the standard. Approximately, 50–100 μg of protein from each sample was separated on 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon P membranes (Millipore). Immunoblotting was performed by standard procedures, and proteins were detected using alkaline phosphatase-conjugated secondary antibody. Equal protein loading was confirmed by ponceau S staining of membrane as well as β-actin immunoblotting.

2.6. Measurement of ROS generation

DCFH-DA was used to detect intracellular generation of ROS by tetrandrine. DCFH-DA is a stable, nonfluorescent, and nonpolar compound that can diffuse through cell membranes. Once inside the cell, the acetyl groups are cleaved by cytosolic enzymes to form the polar nonfluorescent dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. Cells were harvested, washed twice with PBS, and suspended in PBS (1×10^6 cells/ml). Cell suspension (500 μl) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM, and incubated at 37 °C for 15 min. After the addition of tetrandrine, cells were incubated at 37 °C for various times (15–120 min). Then ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells with a FACS Calibur flow cytometer (Becton Dickinson). Mean fluorescence intensity was obtained by histogram statistics using the CellQuest program.

2.7. Transient transfection

MCF-7 cells were seeded into 100-mm plates for 1 day before transfection in density of 5×10^5 cells/ml. Cells were transiently transfected with 1 μg of empty vector (pCDNA3.1), pCDNA3.1-PKC-δ (wild type PKC-δ), or pCDNA3.1-PKC-δ (K378R) (dominant-negative PKC-δ) using FuGENE^{TM} 6 (Roche Diagnostics), according to the protocol provided by the company. Following transfection, cells were cultured for 19 h and exposed to 10 μM tetrandrine for various times in the presence of fetal bovine serum, and cell viability was determined using the trypan blue exclusion assay. Cells were co-transfected with 0.2 μg of pRL-TK (Promega) as a control for transfection efficiency.

2.8. Flow cytometry analysis

Cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol, and stored at 4 °C. Prior to analysis, cells were washed again with PBS, suspended in 1 ml of cold propidium iodide solution (100 μg/ml RNase A, 50 μg/ml propidium iodide, 0.1% (w/v) sodium citrate, 0.1% (v/v) NP-40), and further incubated on ice for 30 min in the darkness. Cytometric analyses were performed using a flow cytometer (FACS Calibur, Becton Dickinson) and CellQuest software. Approximately 15,000 cells were counted for each determination.

2.9. Statistical analysis

Statistical analysis was performed using paired or unpaired Student's *t*-test. The accepted level of significance was P-value <0.05.

3. Results

3.1. Cytotoxic effect of tetrandrine on U937 cells

To verify tetrandrine-induced cytotoxicity, we examined the effects of different concentrations of tetrandrine on viability of U937 cells. While treatment with tetrandrine at 1 μM had little effect, treatments with tetrandrine at 5 or 10 μM caused a time-dependent reduction of cell survival. After 24 h treatment, significant loss of viability was detected at 5 or 10 μM tetrandrine. Only 35 and 10% cells survived after treatment with 5 and 10 μM tetrandrine for 24 h, respectively (Fig. 1A). These results indicate that tetrandrine dose- and time-dependently decreases viability of U937 cells. To evaluate the nature of tetrandrine-induced apoptosis, cells were treated with 10 μM tetrandrine for 24 h, and examined by propidium iodide staining. Fluorescent propidium iodide staining of chromatin revealed the presence of apoptotic bodies in non-viable cells treated with tetrandrine in contrast to control cells (Fig. 1B). Tetrandrine also induced progressive accumulation of extra-nuclear fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis (Fig. 1C). To quantify the degree of apoptosis, we analyzed the amount of sub-G1 DNA by flow cytometry. U937 cells were treated with 10 μM tetrandrine for the indicated times. As shown in Fig. 1D, the addition of tetrandrine to U937 cells resulted in markedly increased accumulation of the sub-G1 phase in a time-dependent manner. Significant cell cycle arrest was not detected before the onset of apoptosis (Fig. 1D). Together, these results suggest that the mechanism of cytotoxic effects induced by tetrandrine on U937 cells is related to its apoptosis-inducing activity.

3.2. Caspases mediate tetrandrine-induced apoptosis

Caspases are essential for execution of cell death by various apoptotic stimuli [15]. To determine whether tetrandrine-induced apoptosis is associated with the activation of

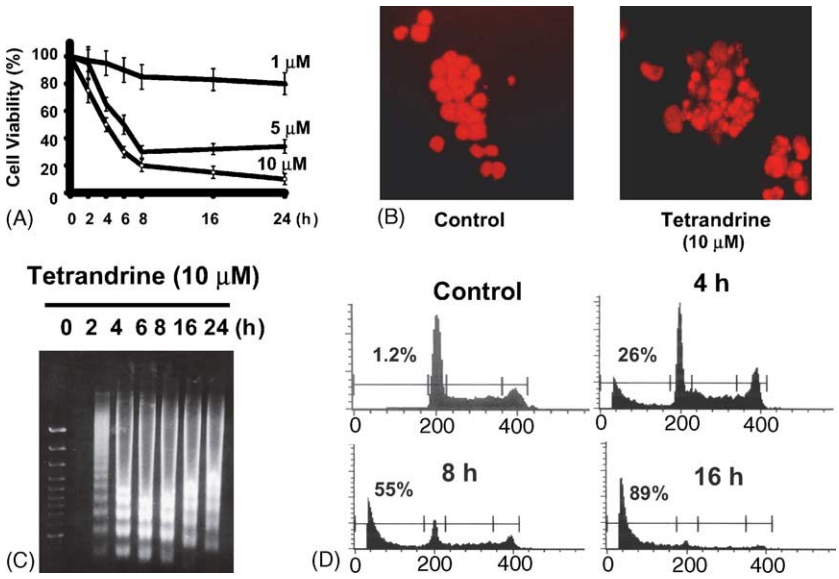

Fig. 1. Effects of tetrandrine on U937 cell survival and DNA integrity. (A) U937 cells were treated with the indicated concentrations of tetrandrine for different time periods. Cell viability was determined using the trypan blue exclusion assay. The results are expressed as mean ± S.D. ($N = 3$). (B) Phenotypic changes in U937 cells induced by 10 μM tetrandrine for 24 h after chromatin staining with propidium iodide. (C) Time course of internucleosomal DNA fragmentation induced by 10 μM tetrandrine, analyzed by agarose gel electrophoresis. (D) Flow cytometry analysis of apoptotic cells. Cells were treated with 10 μM tetrandrine for the indicated times, and at each time point, DNA content was evaluated by propidium iodide staining.

caspases, we measured the activities of caspases in U937 cells treated with tetrandrine. Activation of caspase-9, -3, or -7 during the apoptotic induction by tetrandrine was assessed by the decrease in levels of each inactive proform, which gets proteolytically cleaved upon activation. In U937 cells, treatment with tetrandrine resulted in a decrease of the inactive proform of both the upstream caspase-9 and the downstream effector caspase-3 and -7 in a time-dependent manner (Fig. 2A). In addition, there was a time-dependent activation of caspase-8 after treatment with tetrandrine in U937 cells. Activation of caspase leads to the cleavage of several target proteins such as PARP, PKC-δ, DNA-dependent protein kinase, or Bax [28–31]. Subsequent Western blot analysis disclosed progressive proteolytic cleavage of PARP and PKC-δ in U937 cells after treatment with tetrandrine. To quantify the proteolytic activity of caspase-3, we performed an in vitro assay, based on the cleavage of Ac-DEVD-*p*Na by caspase-3 into chromophore *p*-nitroanilide. As shown in Fig. 2B, treatment with tetrandrine induced a time-dependent increase of caspase-3 activity. There was about 10-fold increase of caspase-3 activity by 24 h treatment with 10 μM tetrandrine. To prove caspase activation is an essential step in the apoptotic pathway induced by tetrandrine, cells were pretreated with a cell-permeable broad spectrum caspase inhibitor z-VAD-fmk (100 μM) for 1 h, and then treated with 10 μM tetrandrine for 4 h. Tetrandrine strongly stimulated caspase-3 protease activity, which was abolished in cells pretreated with z-VAD-fmk (Fig. 2C). Furthermore, tetrandrine treatment of U937 cells resulted in the generation of a 85-kDa cleavage product of PARP and a 40-kDa cleavage product of PKC-δ. Pretreatment with z-VAD-fmk significantly decreased PARP and PKC-δ cleavage (Fig. 2C). Blockage of caspase activity by pretreatment of U937 cells with z-VAD-fmk markedly inhibited cell death induced by tetrandrine (data not shown). These results clearly indicate that tetrandrine-induced apoptosis is associated with caspase activation. The major mechanism of processing and activation of caspases is mediated by Apaf-1 [32]. Apaf-1 is activated by binding to cytochrome *c* from the mitochondria in early stage of apoptosis. Therefore, we next assessed the extent of cytochrome *c* translocation into cytoplasm in U937 cells in response to tetrandrine. A dramatic increase in cytoplasmic cytochrome *c* was detected following 1–2 h treatment with tetrandrine (Fig. 2D). These findings are consistent with the proteolytic activation of caspase-3 and activity determinations for Ac-DEVD-*p*Na cleavage. Together, these results suggest that tetrandrine-induced apoptosis in U937 cells is mediated through cytochrome *c*-dependent activation of caspases.

3.3. Modulation of Bcl-2 and IAP family in tetrandrine-induced apoptosis

Caspase activation is regulated by various proteins, including the IAP or Bcl-2 family [16,17]. To analyze whether tetrandrine induces U937 cell death by modulating the expression of IAP or Bcl-2 family members, we examined the expression of IAP or Bcl-2 family members in tetrandrine-treated U937 cells for the different times. As shown in Fig. 2E, treatment of U937 cells with tetrandrine for 24 h did not significantly alter the expression level of Bcl-2 or HIAP-1, but the expression level of Mcl-1 was greatly down-regulated. Though the expression level of

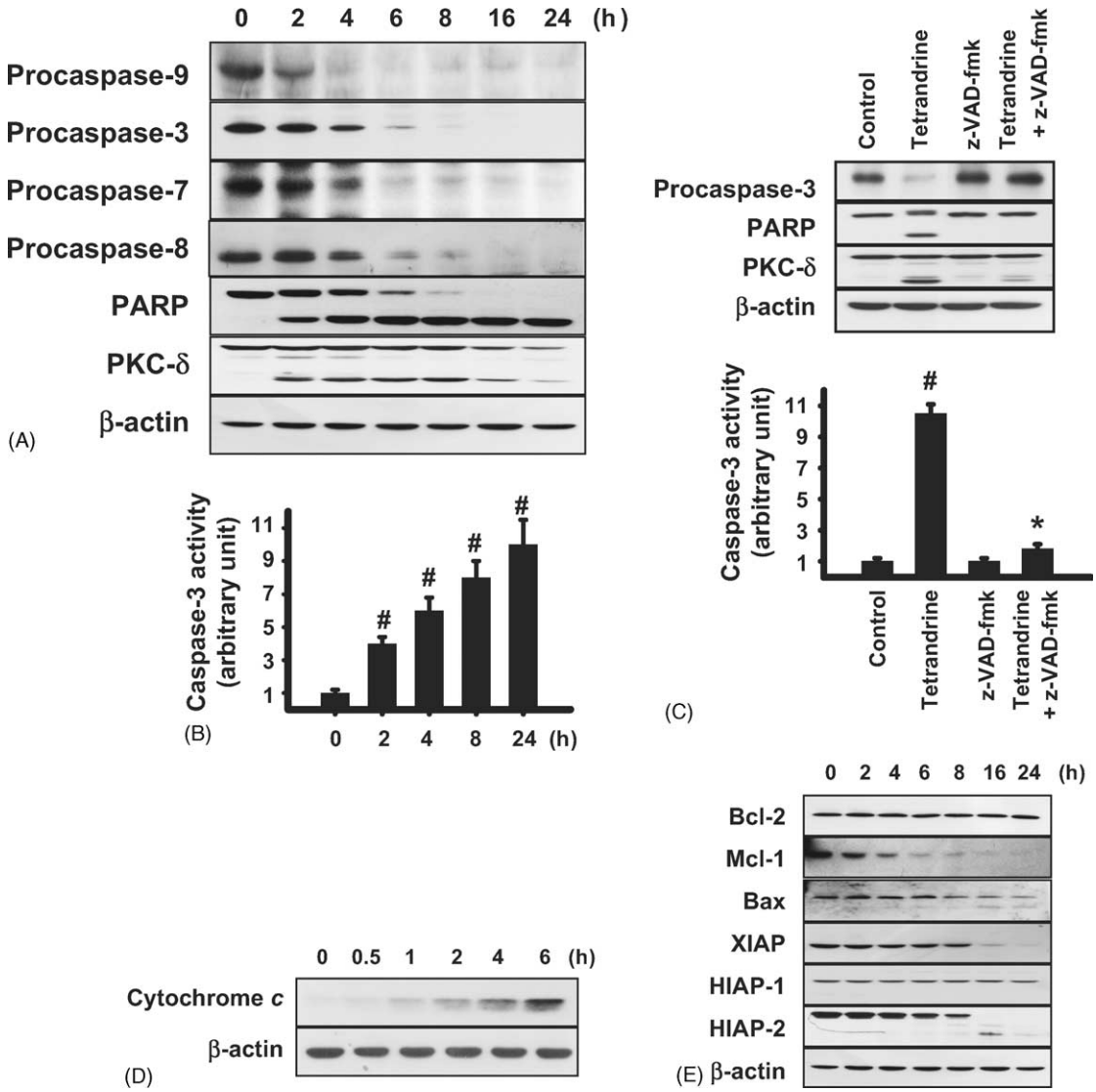

Fig. 2. Effects of tetrandrine on activation of caspases and expression levels of apoptosis related proteins in U937 cells. (A) Cells were treated with 10 μM tetrandrine for the indicated times, and equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of procaspase-9, -7, -3, PARP, and PKC-δ. To confirm equal loading, the same blot was stripped and reprobed with anti-β-actin. (B) U937 cells were treated with 10 μM tetrandrine for the indicated times. Enzymatic activities of caspase-3 were determined by incubation of 50 μg of total protein with 50 μM chromogenic substrate (Ac-DEVD-*p*Na) for 1 h at 37 °C. The release of chromophore *p*-nitroanilide was monitored at 405 nm. Data represent the mean ± S.D. ($N = 3$). $^{\#}P < 0.01$ vs. control (0 h). (C) Effects of z-VAD-fmk on tetrandrine-induced caspase-3 activation. U937 cells were incubated with z-VAD-fmk or vehicle for 1 h before treatment with 10 μM tetrandrine for 4 h. Equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of procaspase-3, PARP, and PKC-δ. Caspase activity was similarly determined as described above. Data represent the mean ± S.D. ($N = 3$). $^{\#}P < 0.01$ vs. control, $^{*}P < 0.01$ vs. tetrandrine. To confirm equal loading, the same blot was stripped and reprobed with anti-β-actin. (D) Cells were treated with 10 μM tetrandrine for the indicated times. The cytosolic fraction was prepared, and analyzed for measurement of cytosolic cytochrome *c* amount by Western blotting as described in Section 2. (E) Cells were treated for the indicated times with 10 μM tetrandrine and equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of Bcl-2, Mcl-1, Bax, XIAP, HIAP-1, and HIAP-2. To confirm equal loading, the same blot was stripped and reprobed with anti-β-actin.

Bax, XIAP, or HIAP-2 was not changed in early time of apoptosis induced by tetrandrine, however, after 8–16 h treatment, the expression level of Bax, XIAP, or HIAP-2 was dramatically decreased. The cleavage of Bax and HIAP-2 occurred several hours after cleavage of PARP or PKC-δ and DNA fragmentation. These results suggest that Bcl-2 and IAP family members may have no significant effects on activation of caspases in early apoptotic progress, but they may enhance caspases activity at late stage in tetrandrine-induced apoptosis.

3.4. Oxidative stress on tetrandrine-mediated apoptosis

Recent studies have demonstrated that some anti-tumor or chemopreventive drugs induce tumor cell death via ROS generation [33–35]. This led us to investigate whether the tetrandrine-induced apoptosis in U937 cells is through ROS generation. As shown in Fig. 3A, treatment with tetrandrine greatly increased the population of U937 cells with high DCF fluorescence, which indicated the increase of intracellular ROS. A significant increase of ROS was

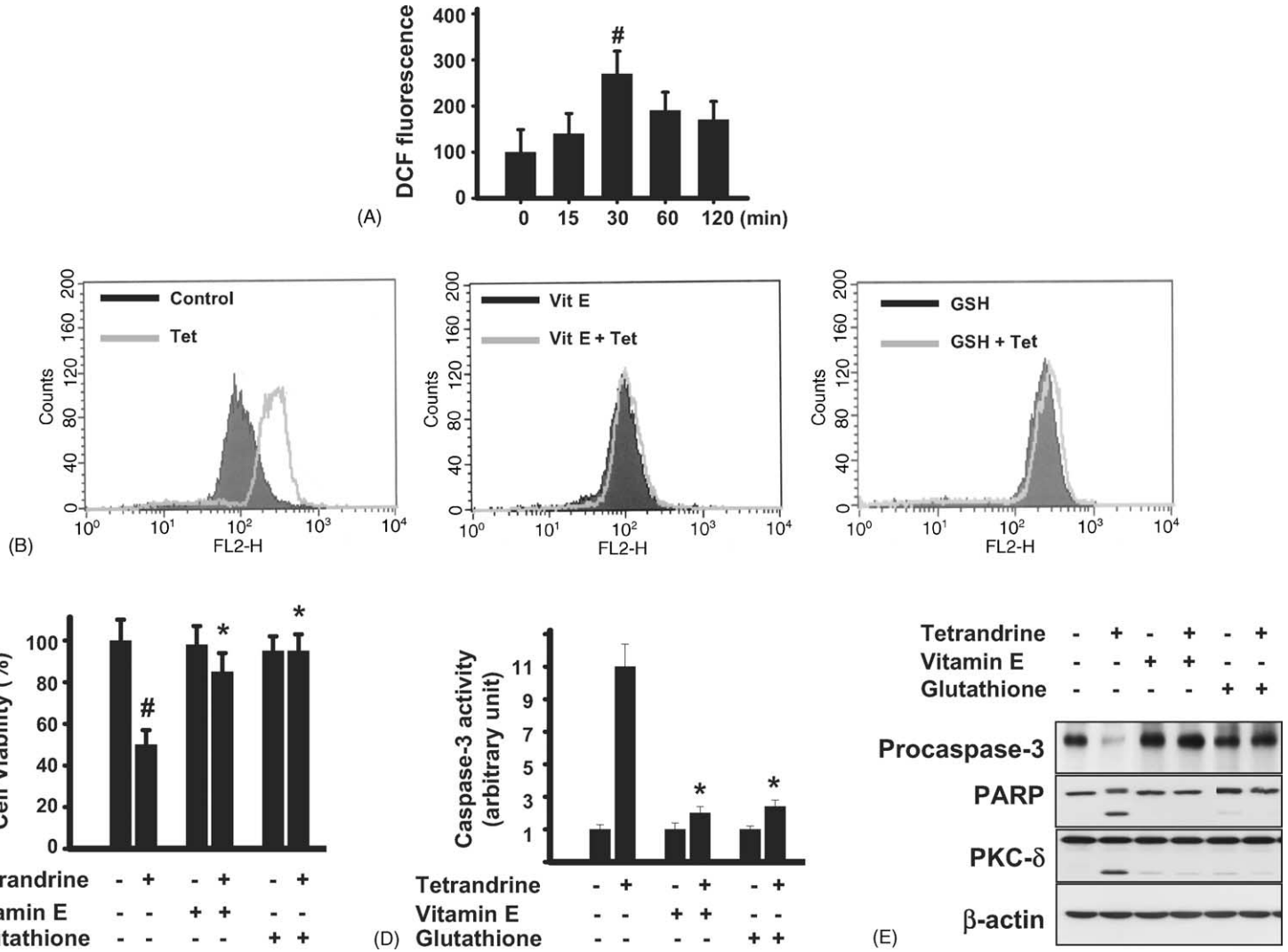

Fig. 3. Generation of ROS by tetrandrine in U937 cells, and protective effect of antioxidants against tetrandrine-induced apoptosis in U937 cells. (A) U937 cells were preloaded with 20 μM of DCFH-DA for 15 min at 37 °C. Tetrandrine (10 μM) was then added into the cells and incubated for the indicated times at 37 °C. DCF fluorescence was measured with a flow cytometer as described in Section 2. DCF fluorescence is presented as mean ± S.D. ($N = 3$) from the histogram statistics. $^{\#}P < 0.05$ vs. control (0 min). (B) Cells were pretreated with 50 μM Vitamin E (Vit E) or 20 mM glutathione (GSH) for 1 h, then harvested, and washed twice with PBS. Cells were then preloaded with DCFH-DA, and treated with tetrandrine (Tet) for 30 min. DCF fluorescence was analyzed with a flow cytometer, and expressed as histograms. (C) Protective effects of Vitamin E (50 μM) or glutathione (20 mM) against tetrandrine-induced cytotoxicity in U937 cells. Cells were preincubated with Vitamin E or glutathione for 1 h before treatment with 10 μM tetrandrine for 4 h. Cell viability was determined using the trypan blue exclusion assay. The results are expressed as mean ± S.D. ($N = 3$). $^{\#}P < 0.01$ vs. control, $^{*}P < 0.01$ vs. tetrandrine. Effects of Vitamin E or glutathione on the tetrandrine-induced caspase-3 activity (D) and cleavage of PARP and PKC-δ (E). U937 cells were preincubated with Vitamin E or glutathione for 1 h before treatment with 10 μM tetrandrine for 4 h. Caspase-3 activity was determined as described above. Data represent the mean ± S.D. ($N = 3$). $^{*}P < 0.01$ vs. tetrandrine. Equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of procaspase-3, PARP, and PKC-δ. To confirm equal loading, the same blot was stripped and reprobed with anti-β-actin.

observed at 30 min treatment with tetrandrine, and the level of ROS was decreased with time thereafter (Fig. 3A). To assess the role of ROS in the tetrandrine-induced apoptosis in U937 cells, we determined the viability in U937 cells that were pretreated for 1 h with Vitamin E (50 μM) or glutathione (20 mM) and then treated with tetrandrine (10 μM) for additional 4 h. Pretreatment with Vitamin E or glutathione effectively inhibited the increase of DCF fluorescence by tetrandrine (Fig. 3B), suppressed tetrandrine-induced cytotoxic effect in U937 cells (Fig. 3C), abolished caspase-3 activity by tetrandrine (Fig. 3D), and decreased PARP and PKC-δ cleavage by tetrandrine (Fig. 3E). These results indicate that tetrandrine-induced apoptosis in U937 cells is mediated by intracellular ROS generation.

3.5. JNK activation does not mediate tetrandrine-induced apoptosis

The family of MAPK has been shown to be activated in response to certain cellular stresses [22,23]. Therefore, we determined whether tetrandrine, which increases oxidative stress, induces the activation of MAPK pathway. As shown in Fig. 4A, treatment with tetrandrine resulted in phosphorylation (activation) of JNK, but not ERK and p38 MAPK, suggesting that JNK is specifically activated in tetrandrine-induced apoptotic pathway. Activation of JNK became apparent at 1 h following treatment with tetrandrine and maintained by 2 h. Striping and reprobing the same blot with an antibody against total JNK revealed no change in total JNK proteins after treatment with tetrandrine, indicating that tetrandrine-induced activation of pre-existing JNK. ROS are known to activate JNK or p38 MAPK, and activities of these kinases are linked to apoptosis [36–38]. To examine whether JNK activation is caused by tetrandrine-induced ROS, we tested the effect of Vitamin E. Pretreatment with Vitamin E effectively prevented JNK phosphorylation induced by tetrandrine, suggesting that JNK activation is mediated by ROS (Fig. 4B). To examine the role of JNK in tetrandrine-induced apoptosis, we tested the effect of SP600125, a JNK inhibitor. Inactivation of JNK with SP600125 that blocked JNK phosphorylation by tetrandrine did not block tetrandrine-induced PARP cleavage (Fig. 4C) and U937 cell death (data not shown). These results suggest that though JNK is activated by tetrandrine-induced ROS, JNK activation is not involved in the tetrandrine-induced apoptosis.

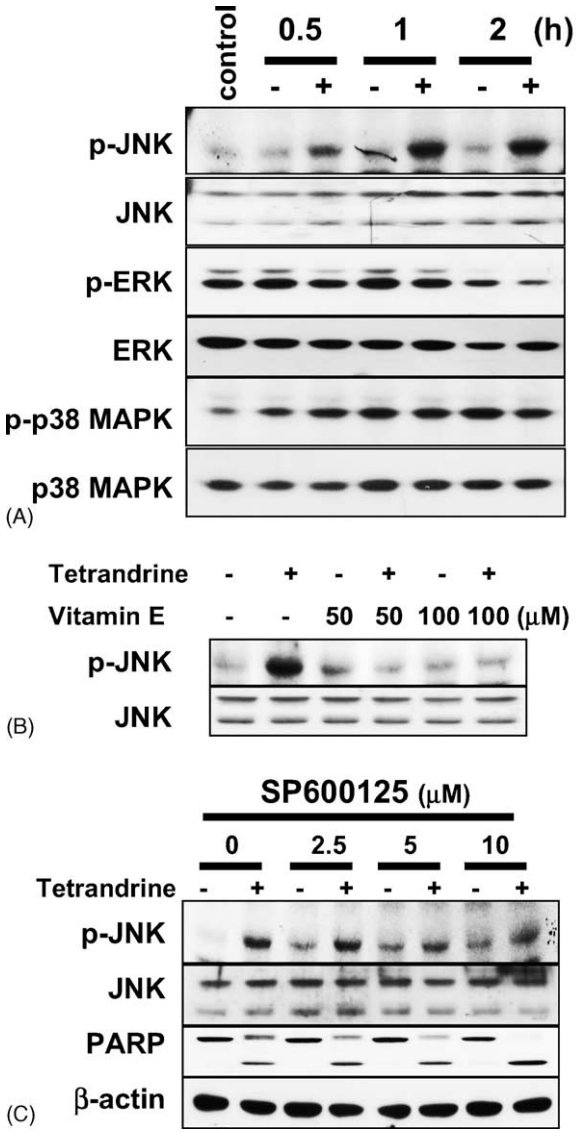

Fig. 4. Activation of JNK, but not p38 and ERK, by tetrandrine in U937 cells. (A) Cells were treated with 10 μM tetrandrine for the indicated times. At each time, cell lysates were prepared and used for Western blotting with antibodies against phospho-JNK (p-JNK), phospho-ERK (p-ERK), or phospho-p38 (p-p38) MAPK to detect phosphorylated forms of JNK, ERK, or p38 MAPK. The same membrane was striped and reprobed with antibodies against total JNK, ERK, or p38 MAPK. (B) Tetrandrine-induced JNK activation is attenuated by Vitamin E in U937 cells. Cells were preincubated with 50 or 100 μM Vitamin E for 1 h before treatment with 10 μM tetrandrine for 1 h. p-JNK and total JNK were analyzed by Western blotting as described above. (C) Effects of a JNK inhibitor, SP600125, on tetrandrine-induced apoptosis in U937 cells. Cells were pretreated with the indicated concentrations of SP600125 for 30 min before treatment with 10 μM tetrandrine for 4 h. p-JNK or PARP cleavage was analyzed by Western blotting. To confirm equal loading, the same blot was stripped and reprobed with antibodies against total JNK or β-actin.

3.6. Activation of PKC-δ is critical for the tetrandrine-induced apoptosis in U937 cells

It has been shown that PKC-δ participates in the process of apoptosis in cells upon treatment with many pro-apoptotic stimuli [18,39,40]. In this study, treatment with tetrandrine resulted in concomitant proteolytic cleavage and activation of PKC-δ as detected by Western blot analysis using antibodies against total and phosphorylated (Thr505) PKC-δ (Fig. 5A). The cleaved form of PKC-δ was detected at 120 min after tetrandrine treatment. Coincidently, phosphorylation of the cleaved PKC-δ was also detected at 120 min after tetrandrine treatment. Whether PKC-δ plays a critical role in tetrandrine-induced

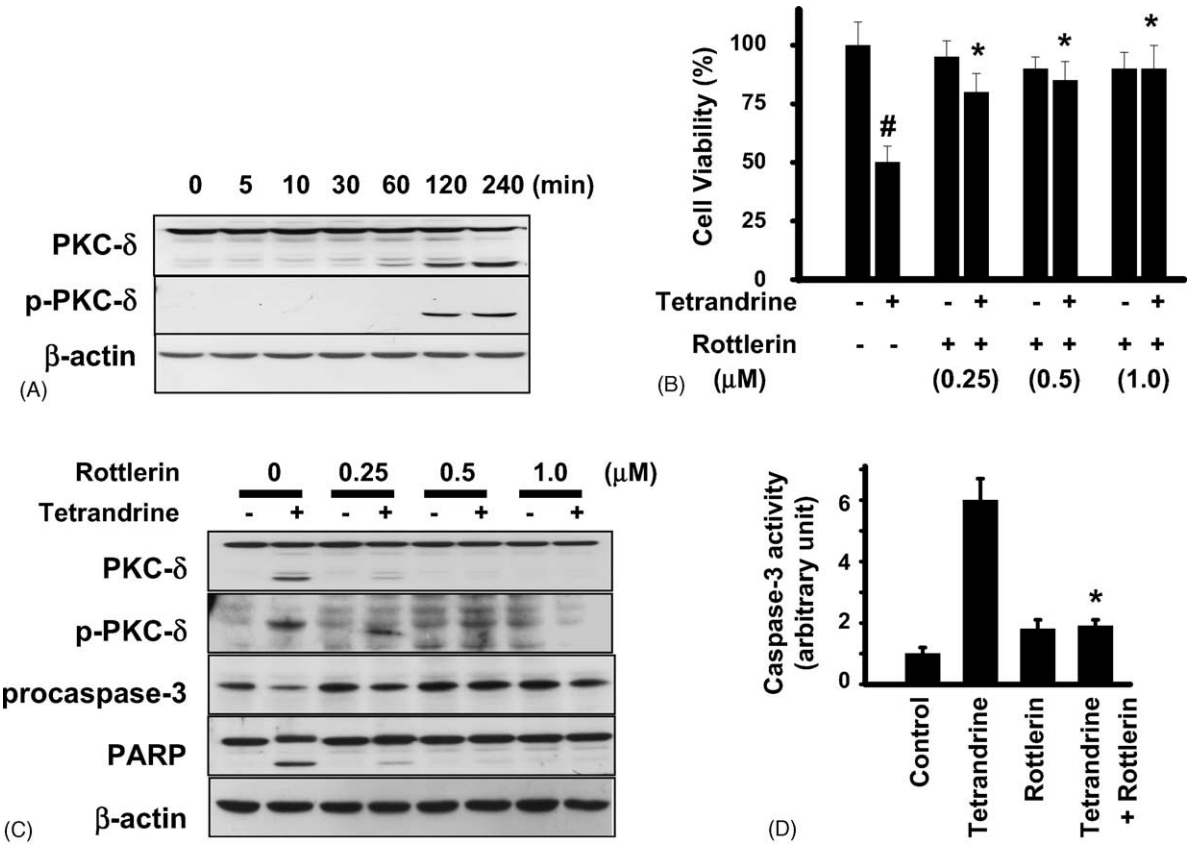

Fig. 5. Role of PKC-δ on the tetrandrine-induced apoptosis in U937 cells. (A) Cells were treated with 10 μM tetrandrine for the indicated times. Equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of total PKC-δ and p-PKC-δ. (B) Cells were preincubated with the indicated concentrations of rottlerin, an inhibitor of PKC-δ, for 1 h before treatment with 10 μM tetrandrine for 4 h, and cell viability was determined using the trypan blue exclusion assay. The results are expressed as mean ± S.D. ($N = 3$). $^{\#}P < 0.01$ vs. control, $^{*}P < 0.01$ vs. tetrandrine. (C) Effects of rottlerin on tetrandrine-induced PKC-δ cleavage and phosphorylation, caspase-3 activation, and PARP cleavage. Cells were preincubated with 0.5 μM rottlerin for 1 h before treatment with 10 μM tetrandrine for 4 h, and equal amounts of cell lysates (50 μg) were subjected to electrophoresis and analyzed by Western blotting to measure levels of total PKC-δ, p-PKC-δ, procaspase-3, or PARP. (D) Effects of rottlerin on tetrandrine-induced caspase-3 activity. Cells were preincubated with 0.5 μM rottlerin for 1 h before treatment with 10 μM tetrandrine for 4 h, and caspase-3 activity was determined as described in Section 2.

apoptosis of U937 cells was next examined using rottlerin, a specific pharmacological inhibitor of PKC-δ. Though rottlerin alone had a little cytotoxic effect on U937 cells, inactivation of PKC-δ with rottlerin greatly promoted the survival of tetrandrine-treated cells in a dose-dependent manner (Fig. 5B), significantly blocked proteolytic cleavage and phosphorylation of PKC-δ by tetrandrine (Fig. 5C), and efficiently blocked PARP cleavage (Fig. 5C) and caspase-3 activation (Fig. 5D) by tetrandrine. To further investigate the role of PKC-δ in tetrandrine-induced apoptosis, we transiently transfected MCF-7 cells with a vector expressing wild type or dominant-negative PKC-δ. The apoptotic response to tetrandrine was significantly attenuated in dominant-negative PKC-δ transfected MCF-7 cells (Fig. 6). These results suggest that PKC-δ plays an important role in tetrandrine-induced apoptosis and can induce caspase activation.

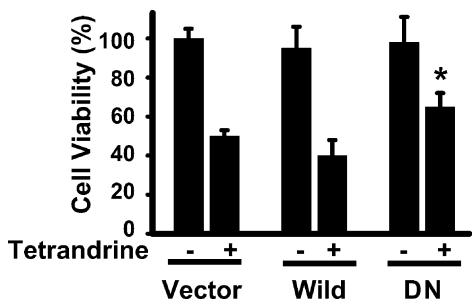

Fig. 6. Regulation of tetrandrine-induced apoptosis by PKC-δ. MCF-7 cells were transiently transfected with an empty vector (Vector), a vector containing wild type PKC-δ (Wild), or a vector containing dominant negative PKC-δ (K378R) (DN). The transfected cells were then treated with 10 μM tetrandrine for 24 h. Cell viability was determined using the trypan blue exclusion assay. The results are expressed as mean ± S.D. ($N = 3$). $^{*}P < 0.05$ vs. Vector and 10 μM tetrandrine.

4. Discussion

In the present study, we have demonstrated that tetrandrine induces apoptosis in association with the activation of caspases and that ROS play a crucial role in tetrandrine-induced apoptosis in U937 cells. Though activation of JNK

is mediated through tetrandrine-induced ROS, this kinase does not involve in the tetrandrine-induced apoptosis. Furthermore, we have shown that caspase-mediated PKC-δ activation promotes apoptotic process, and PKC-δ appears to further exert its activity as an upstream of a caspase cascade in tetrandrine-induced apoptosis in U937 cells.

It has been well understood that activation of caspases is critical in apoptosis by various apoptotic stimuli because active caspases proteolytically cleave many intracellular proteins such as PARP and PKC-δ [15]. Accordingly, several recent studies have suggested that activation of caspases is important for tetrandrine-induced apoptosis in various tumor cells [9,10,41]. Consistent with these previous reports, our data have indicated that activation of caspases seems to be associated with the induction of apoptosis in U937 cells by tetrandrine. In general, release of Apaf-1 and cytochrome *c* from mitochondria into cytosol implicates the activation of caspase-9, which triggers activation of downstream effector caspases such as caspase-7/-3 [32]. In tetrandrine-treated U937 cells, we have observed accumulation of cytosolic cytochrome *c* and a time-dependent decrease of each proform of caspase-9, -7, and -3, suggesting activation of these caspases by tetrandrine. There are some recent publications displaying either caspase-8-dependent or caspase-8-independent apoptosis by tetrandrine [13,41]. At the present study, we have found that tetrandrine also induces activation of caspase-8, which may be attributable to the tetrandrine-induced apoptosis in U937 cells. Subsequently, tetrandrine-induced strong proteolytic cleavage of PARP and PKC-δ, which is an indicator of apoptosis. In addition, the pan-caspase inhibitor, z-VAD-fmk, effectively prevented caspase-3 activation, blocked cleavage of PARP and PKC-δ, and promoted cell viability. Therefore, our results suggest that a cascade of caspase activation triggers the apoptosis in tetrandrine-treated cells. The release of cytochrome *c* from mitochondria induces the activation of caspases. Furthermore, caspase activation is regulated by various proteins, including the IAP family and the Bcl-2 family [16,17]. In tetrandrine-treated cells, the expression levels of IAP and Bcl-2 family proteins except Mcl-1 were not significantly changed, suggesting that these proteins do not link to activation of caspases. The IAP family of anti-apoptotic proteins inhibits caspase activity [17], and the release of cytochrome *c* from the intermitochondrial space is thought to be regulated by the Bcl-2 family members [16]. The cleavage form of Bax protein was reported to induce apoptosis [31]. In present study, we have found that proteolytic cleavage of Bax, XIAP, and HIAP-2 occurs in late stage of tetrandrine-induced apoptosis in U937 cells, suggesting that caspase activity may be further enhanced by activation of Bax and inactivation of these IAP proteins during late stage of the apoptotic process induced by tetrandrine.

ROS also play an important role in apoptosis [35,42]. It is reported that tetrandrine induces apoptosis through oxidative stress and glutathione depletion in Neuro 2a mouse neuroblastoma [43]. In our experiment, ROS were detected in cells treated with tetrandrine, and pretreatment with either Vitamin E or glutathione effectively suppressed ROS production, promoted cell viability, and prevented caspase activation and cleavage of PARP and PKC-δ, suggesting that the cytotoxic effect of tetrandrine is mediated by early oxidative stress. ROS are known to activate stress-activated protein kinases, such as p38 MAPK or JNK, and activation of these kinases is linked to apoptosis [36,37]. We have shown that tetrandrine leads to activation of JNK, but not p38 MAPK. Phosphorylation of JNK by tetrandrine is prevented by Vitamin E, suggesting a link between oxidative stress and JNK activation. However, SP600125, a JNK inhibitor, which effectively blocks JNK phosphorylation, does not exert a protective effect against tetrandrine-induced apoptotic cell death. Therefore, we demonstrate that ROS play a critical role in apoptosis by tetrandrine, but JNK activation by ROS is not involved in the tetrandrine-induced apoptosis.

PKC isozymes are known to play important roles in apoptosis [25]. Twelve members of PKCs have been identified so far, based on cofactors required for their maximal activation, and these PKC isozymes are known to be activated by proteolytic separation of the regulatory domain from the catalytic domain [21,24,25,39]. While the classical and atypical PKC isozymes are associated with cell survival, the novel PKC isozymes, notably PKC-δ, are proapoptotic in function [18,20,34,40]. We show here that inhibition of PKC-δ activity blocks tetrandrine-induced apoptosis, demonstrating that PKC-δ exerts as a pro-apoptotic signal. Proteolytic cleavage and activation of PKC-δ by caspases are shown to be an important step in apoptosis in response to many apoptotic stimuli [21,29,39]. In contrast, it has been reported that inhibition of PKC-δ by rottlerin, a specific inhibitor of PKC-δ, blocks drug-induced apoptosis as well as proteolytic activation of caspase-3 [18,44], suggesting that PKC-δ may lie upstream of caspases. In present study, we demonstrate that pretreatment with z-VAD-fmk, a pan-caspase inhibitor, could inhibit both PKC-δ cleavage and caspase-9/-3 activation. Interestingly, we observe that inhibition of PKC-δ by rottlerin could prevent caspase-3 activation and promote cell survival in tetrandrine-treated U937 cells. Furthermore, our data disclose that overexpression of a dominant-negative mutant of PKC-δ in MCF-7 cells attenuates tetrandrine-induced apoptosis. Therefore, PKC-δ appears to have some role in mediating the apoptotic pathway upstream of caspase cascade in tetrandrine-treated cells. These results suggest that the activation of caspases and PKC-δ are mutually regulated by each other and caspase-mediated activation of PKC-δ appears to enhance the

amplification of the caspase cascade and subsequently potentiates apoptotic process in cells treated with tetrandrine.

In conclusion, findings of the present study demonstrate that tetrandrine induces apoptosis in U937 cells through oxidative stress as well as activation of caspases and PKC-δ. PKC-δ appears to exert its activity as an upstream of a caspase cascade and caspase-mediated PKC-δ activation seems to further promote apoptotic process by tetrandrine, suggesting that there may be a mutual regulation between PKC-δ and caspases for their activation. From growing evidence of the tetrandrine-mediated cytotoxicity against various tumor cells, tetrandrine may be a potential candidate as an anti-cancer or chemopreventive agent. Further efforts to develop potentially therapeutic strategies with tetrandrine are warranted.

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