Original Article
MPTP related mitochondrial pathway in oroxylin A induced-apoptosis in HepG2 cancer cells

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Abstract: Oroxylin A is a flavonoid extracted from the root of Scutellaria baicalensis Georgi. Our previous work demonstrated that oroxylin A induced cell apoptosis via the mitochondrial pathway in cancer cells. The present study explored the mechanism underlying oroxylin A-induced apoptosis. We firstly showed that oroxylin A caused apoptosis through the mitochondrial pathway in human hepatoma HepG2 cells. We next demonstrated that mitochondrial permeability transition pore (MPTP) was activated, suggesting the elevated permeabilization of the mitochondrial outer membrane after oroxylin A treatment. These data indicated that Oroxylin A-induced apoptosis via a MPTP-dependent manner. The production of reactive oxygen species (ROS) is essential for the activation of MPTP, and we confirmed that Oroxylin A increased MPTP activation in a ROS-dependent manner. We finally showed that Oroxylin A inhibited the proliferation of HepG2 xerograph in vivo. Collectively, our results demonstrated that oroxylin A induced apoptosis by activating MPTP in human hepatoma cells.

Keywords: MPTP, oroxylin A, ROS, apoptosis

Introduction

Mitochondrial permeability transition (MPT) is an instant change of membrane permeability when cells are treated by noxious stimulation like oxidative stress and cytotoxic drugs [1]. This transformation is thought to be triggered by the opening of the mitochondrial permeability transition pore [2], which leads to nonspecific disintegration of the outer mitochondrial membrane. MPTP is a important multi-component protein which locates in the inner mitochondrial membrane that can connect the inner and the outer sites of the mitochondrial membrane [3, 4]. It has been demonstrated that the normal physiological function of cell is regulated by MPTP in mitochondrial [2]. The opening of this transition pore leads to permeability transition and the release of apoptotic proteins like cytochrome c and AIF, and finally activates the following apoptotic cascades to the final death of the cell [5, 6]. Mitochondria in cancer cells maintain in hyper-metabolic status which means their mitochondria are more vulnerable to various factors like ROS. Therefore, potential drug candidates that target mitochon-dria are supposed to have a tumor selectivity [7]. Regulation of cell apoptosis through MPTP is the balance between pro- and anti-apoptotic Bcl-2 family proteins [8]. Cancers with high levels of Bcl-2 protein exhibit a resistance to mitochondrial cell apoptosis, which indicates that Bcl-2 mitochondrial enrichment may de-activate MPTP [9]. Although the mechanism underlying MPTP activation remains to be known, it appears to be clear that MPTP might be a valuable target for cancer therapy.

Oroxylin A ($C_{16}H_{12}O_5$, **Figure 1**) is a flavonoid isolated from the root of Scutellaria baicalensis Georgi, a traditional herbal medicine generally regarded as an analgesic, antipyretic, anti-tumor, and anti-inflammatory agent. Oroxylin A has been demonstrated to inhibit cancer proliferation and metastasis both in vitro and in vivo [10, 11]. Recently, it was reported that oroxylin A stimulated the accumulation of intracellular ROS, which might trigger the activation of MPTP. Therefore, we hypothesized that oroxylin A might promote the activation of MPTP in HepG2 cells, and induce the release of apoptotic proteins from mitochondria to cytoplasm.
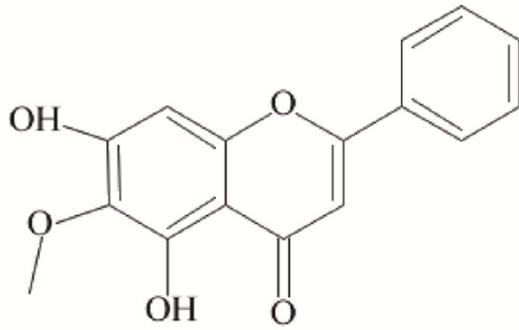
Figure 1. Molecular structure of oroxylin A (C16H-12O5, MW 284).

Materials and methods

Materials

Oroxylin A (C16H12O5) was isolated from the root of Scutellaria baicalensis according to a previously reported method and dissolved in DMSO [12]. Samples containing 99% or higher of oroxylin A were used. Oroxylin A was dissolved in DMSO to 200 mM and stored at -20°C. Before every experiment, the stock solution of oroxylin A was diluted with basal medium to various working concentrations. Antibodies to caspase-3 (sc-56052), caspase-9 (sc-56073), caspase-8 (sc-56070), Bcl-2 (sc-7382) were obtained from Santa Cruz (Santa Cruz, CA); antibody to cytochrome c was from Calbiochem (Merck, Darmstadt, Germany); antibodies to AIF (catalog number 4642) was purchased from Cell Signaling (Danvers, MA); antibody to Cox IV (ab14744) was from Abcam (UK); and antibody to β-actin (BM0627) was from Boster (Wuhan, China).

Cell culture

Human hepatoma HepG2 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in 90% DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China). Cells were cultivated at 37°C in a water-jacketed CO_2 incubator (Thermo Forma, Waltham, Massachusetts) in a humidified atmosphere with 5% CO_2.

Animal model

Male athymic BALB/c nude mice (35-40 days old) with body weight ranging from 18 to 22 g

were supplied by the Academy of Military Medical Sciences of the Chinese People's Liberation Army (Certificate No. SCXK-(Army) 2007-004). The animals were maintained at 22 ± 2°C with 55-65% humidity in stainless steel cages under controlled light (12 h light/day) and were fed with standard laboratory food and water. Animal care was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, USA.

Twenty nude mice were inoculated subcutaneously with 1×10^7 HepG2 cells into the right axilla. After 12 days of growth, tumor sizes were determined using micrometer calipers. Mice-inoculated HepG2 cells with similar tumor volumes were randomly divided into the following two groups (five mice/group): saline control, and oroxylin A (100 mg/kg, i.v., every 2 days). Tumor sizes were measured every 3 days using micrometer calipers, and tumor volume was calculated using the following formula: TV (mm^3) = d2 × D/2, where d and D were the shortest and the longest diameters, respectively. Mice were sacrificed on day 21, and tumor tissues were used for immunohistochemistry assay.

Cell metabolic activity assay

HepG2 cells were seeded in 96-well plates, incubated overnight, and treated with oroxylin A (100 µM). Then Alamar blue assay (Invitrogen, Carlsbad, CA) was performed according to the manufacturer's instructions. The fluorescence was tested once an hour for 9 h, at 530-560 nm excitation wavelength and 590 nm emission wavelength using a fluorospectro photometer. The Alamar blue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth.

SOD2 activity assay

SOD2 activity was assayed with the Cu/Zn-SOD and Mn-SOD Assay Kit (KeyGen, Nanjing, China) following the manufacturer's instructions. Briefly, cells were collected and lysed. The addition of 3 mM potassium cyanide to the

cell lysate inhibited both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity. Samples were assayed in the absence of xanthine oxidase to generate a sample background. After sample and SOD standard were prepared and added into a 96-well plate, we initiated the reaction by adding 20 µl of diluted xanthine oxidase to all the wells. The plate was incubated on a shaker for 30 min at room temperature. The OD values were detected using a spectrophotometer (Thermo) at 450 nm.

The SOD activity of the sample was calculated using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR, LR = (Ablank1-Ablank2-Asample)/(Ablank1-Ablank2) × 100%) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Thus, SOD activity sample (U) = LR sample/(1-LR sample) units.

Annexin V/PI staining

Apoptosis cells were identified by the Annexin V-FITC Apoptosis Detection Kit (KeyGen, Naijing, China) according to the manufacturer's instructions. In brief, 1×10^6 cells were harvested, and washed, and suspended with PBS. Cells were re-suspended in 500 µl binding buffer and then added 5 µl AnnexinV-FITC and 5 µl PI. Apoptotic cell death was examined by FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA) immediately after double supravital staining.

Measurement of mitochondrial permeability transition pore (MPTP)

The opening of MPTP was monitored by fluorospectro photometer using bis (bis-carboxymethyl amino methyl fluorescein)-acetoxymethyl ester, also called Calcien AM. 1×10^6 cells were collected and washed with cold PBS, then re-suspended in PBS and incubated for 30 min at 37°C with Calcien AM. The fluorescence intensity was measured at 488 nm excitation and 505 nm emission using a fluorospectro photometer.

Immunocytochemical study

Tissue sections and cells were rinsed with PBS and fixed with 4% paraformaldehyde in phos-

phate buffer (pH 7.4) for 20 min at room temperature. After washing with PBST and blocking with 3% bovine serum albumin (BSA) in PBST for 1 h, the cells were incubated with anti-Bcl-2 antibody (Santa Cruz, CA) overnight at 4°C and then washed with PBST. Tetramethylrhodamine labeled anti-mouse IgG antibody (Rockland) was added to the cells and incubated for 1 h. For identifying of the opening of MPTP, cells were rinsed with PBST and exposed to Calcien AM at 37°C for 30 min. For staining of nuclei, cells were rinsed with PBST and exposed to DAPI for 15 min. After washing with PBS, cells were examined under a laser scanning confocal microscope.

Measurement of intracellular ROS

Intracellular ROS was detected by DCFH-DA (Beyotime Institute of BioTechnology, Haimen, China). Cells were harvested from 6-well plates and washed with PBS, then re-suspended in serum-free medium and incubated with 10 µM DCFH-DA added for 30 min at 37°C in the dark. The fluorescence intensity was measured by FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA) immediately at 488 nm excitation and 525 nm emissions.

Cytochrome c and AIF release assay

The fractionation of the mitochondrial protein and cytosolic protein was extracted according to cytochrome c release apoptosis assay kit (Calbiochem, Germany) instruction. Briefly, 5×10^7 cells were collected by centrifugation at 600 g for 5 min at 4°C and washed with ice-cold PBS. Cells were re-suspended with 1 ml of 1 × Cytosol Extraction Buffer Mix containing dithiothreitol and Protease Inhibitors and incubated on ice for 10 min. Then cells were homogenized in an ice-cold grinder and the homogenate was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 700 g for 10 min at 4°C. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was collected as cytosolic fraction and the pellet was re-suspended in 0.1 ml Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors and then vortexed for 10 s and saved as mitochondrial fraction. Western blotting was used to detect cytochrome c and AIF of cytosolic fraction and mitochondrial fraction with cytochrome c anti-body
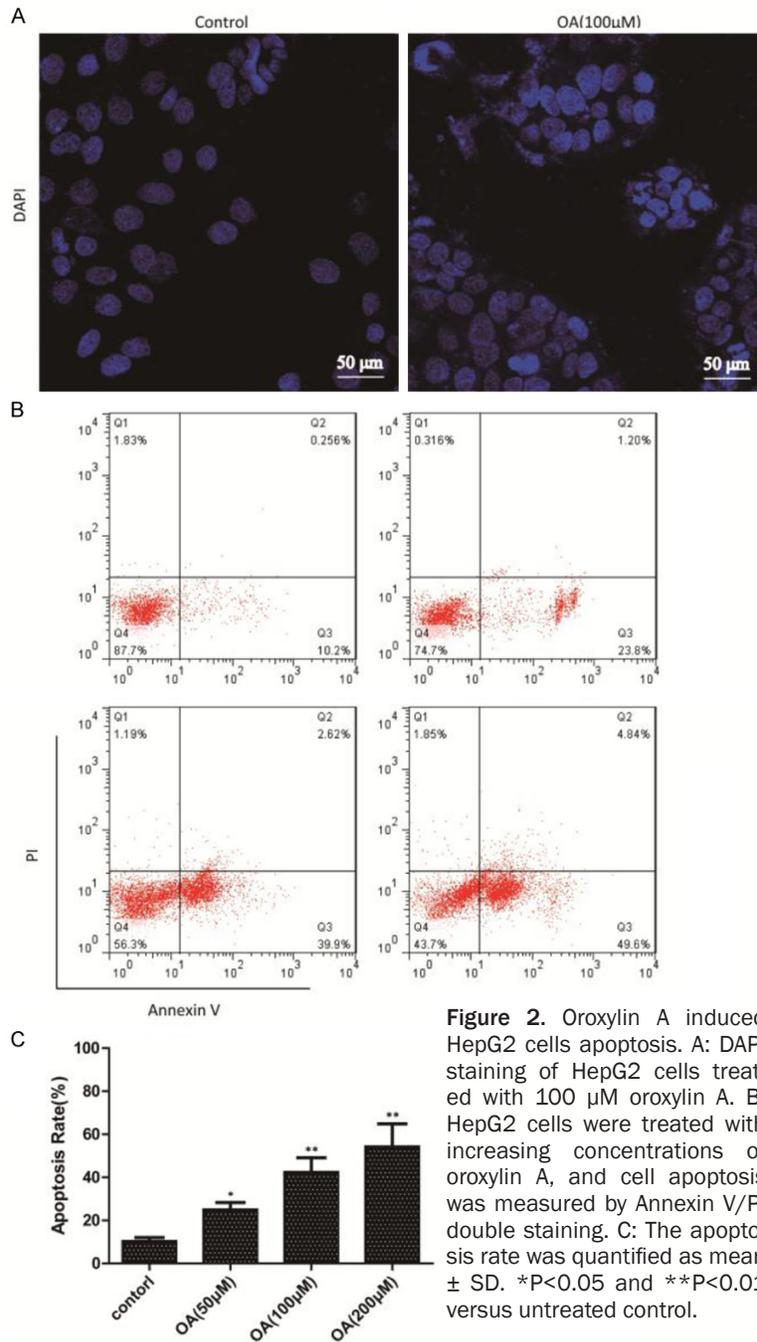
Figure 2. Oroxylin A induced HepG2 cells apoptosis. A: DAPI staining of HepG2 cells treated with 100 μM oroxylin A. B: HepG2 cells were treated with increasing concentrations of oroxylin A, and cell apoptosis was measured by Annexin V/PI double staining. C: The apoptosis rate was quantified as mean ± SD. *P<0.05 and **P<0.01 versus untreated control.

siRNA-Bcl-2 (30 pM) were introduced into the cells using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's recommendations.

Statistical analysis

Results were expressed as x̄ ± s. Data shown were representatives of at least three independent experiments. Statistical analysis of the data was performed using the unpaired Student's t test. *P* values were two-sided at which a value of 0.05 was considered statistically significant.

Results

Oroxylin A induced HepG2 cell apoptosis

To identify apoptosis induced by oroxylin A in HepG2 cells, we first performed DAPI staining and Annexin V/PI staining assay. Untreated HepG2 cells showed the steady chromatic distribution in nucleolus. Oroxylin A-treated cells emitted bright fluorescence, presenting the early phenomena of apoptosis, due to the chromatin agglutination and the nucleus pyknosis under the fluorescent microscope (**Figure 2A**). Annexin V/PI double staining assay was further employed to measure cell apoptosis. As shown in **Figure 2B** and **2C**, oroxylin A induced HegG2 cell apoptosis in a dose-dependent manner.

Oroxylin A induced the opening of MPTP in HepG2 cells

To detect whether ROS was involved in apoptosis, we evaluated the intracellular ROS levels in HepG2 cells after oroxylin A treatment. We showed that ROS production was enhanced by oroxylin A in a dose dependent manner (**Figure**

(Calbiochem, Germany) and AIF antibody (Cell Signaling, MA).

Plasmid and siRNA transient transfection

The pcDNA-Bcl-2 was obtained from addgene (Addgene 16461, Addgene Sidney St, Cambridge, MA). Small interfering RNAs of Bcl-2 were purchased from Santa Cruz. For transfection, HepG2 cells were seeded in 6-well plates at 70% confluency, then pcDNA-Bcl-2 (1.0 μg)/
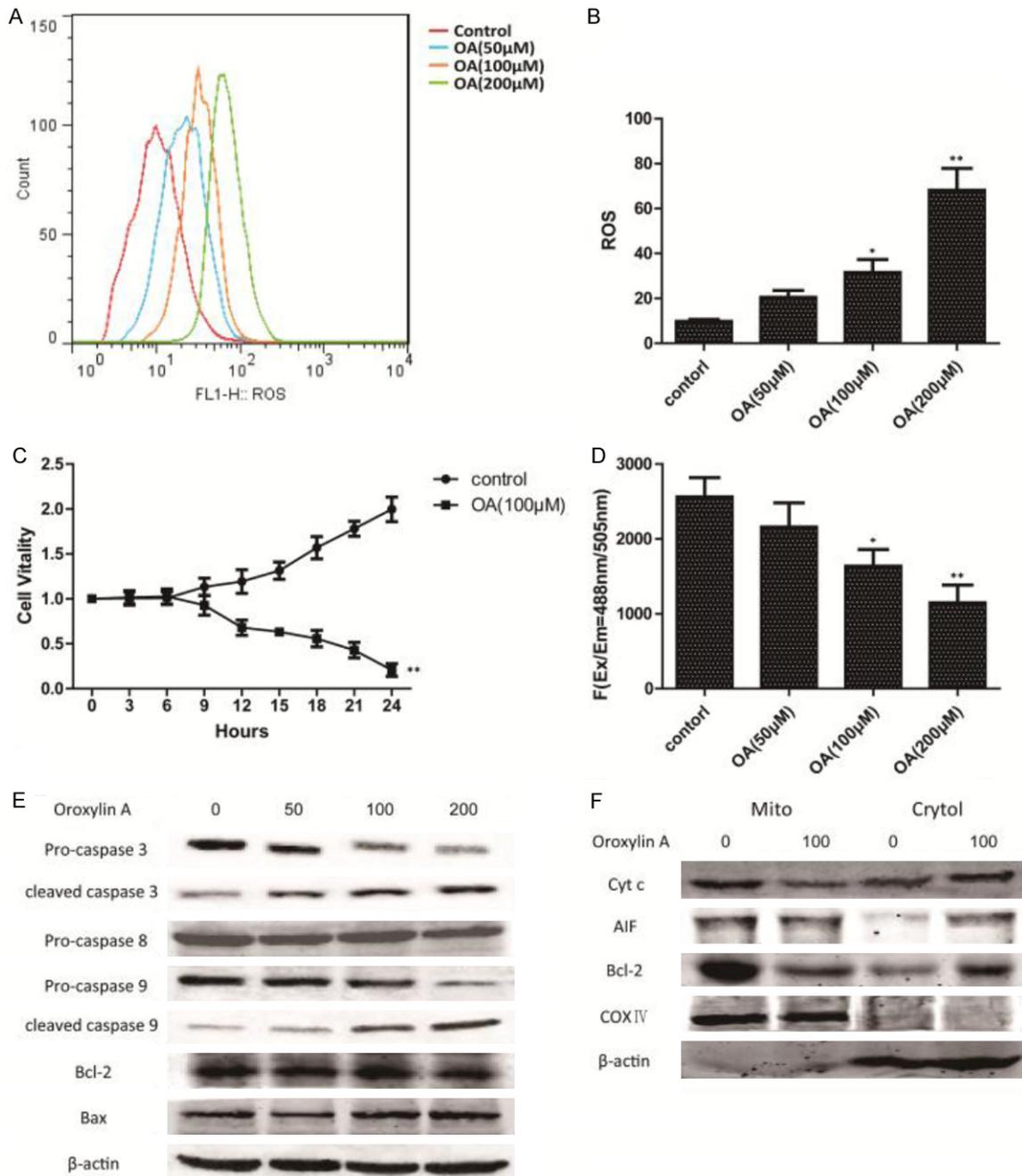
Figure 3. Oroxylin A induced the opening of MPTP in HepG2 cells. A: Cells were treated with different concentrations of Oroxylin A, and the production of ROS was monitored by FCM, using 10 μM DCFH-DA. B: The ROS levels were quantified and shown as means ± SD. C: Cell metabolic activity was investigated after treatment of 100 μM oroxylin A for 24 h. D: Calcien-AM staining of HepG2 cells after 24 h treatment of different concentrations of oroxylin A. The fluorescence intensity was measured at 488 nm excitation and 505 nm emission using a fluorospectro photometer and the results from the experiments are shown as means ± SD. E: HepG2 cells were treated with indicated concentration of oroxylin A for 24 h, then the expression of caspase-3, caspase-8, caspase-9, Bcl-2 and Bax were detected by western blot. F: Western blotting analysis of cytochrome c and AIF in the cytosolic fraction. The fractionation of the mitochondrial protein and cytosolic protein was performed according to the instruction of cytochrome c release apoptosis assay kit. *P<0.05; **P<0.01 versus untreated control.

3A, **3B**). Meanwhile, the growth of HepG2 cells was inhibited by oroxylin A (**Figure 3C**). To inves-

tigate whether the opening of MPTP was involved in cell apoptosis induced by oroxylin A,
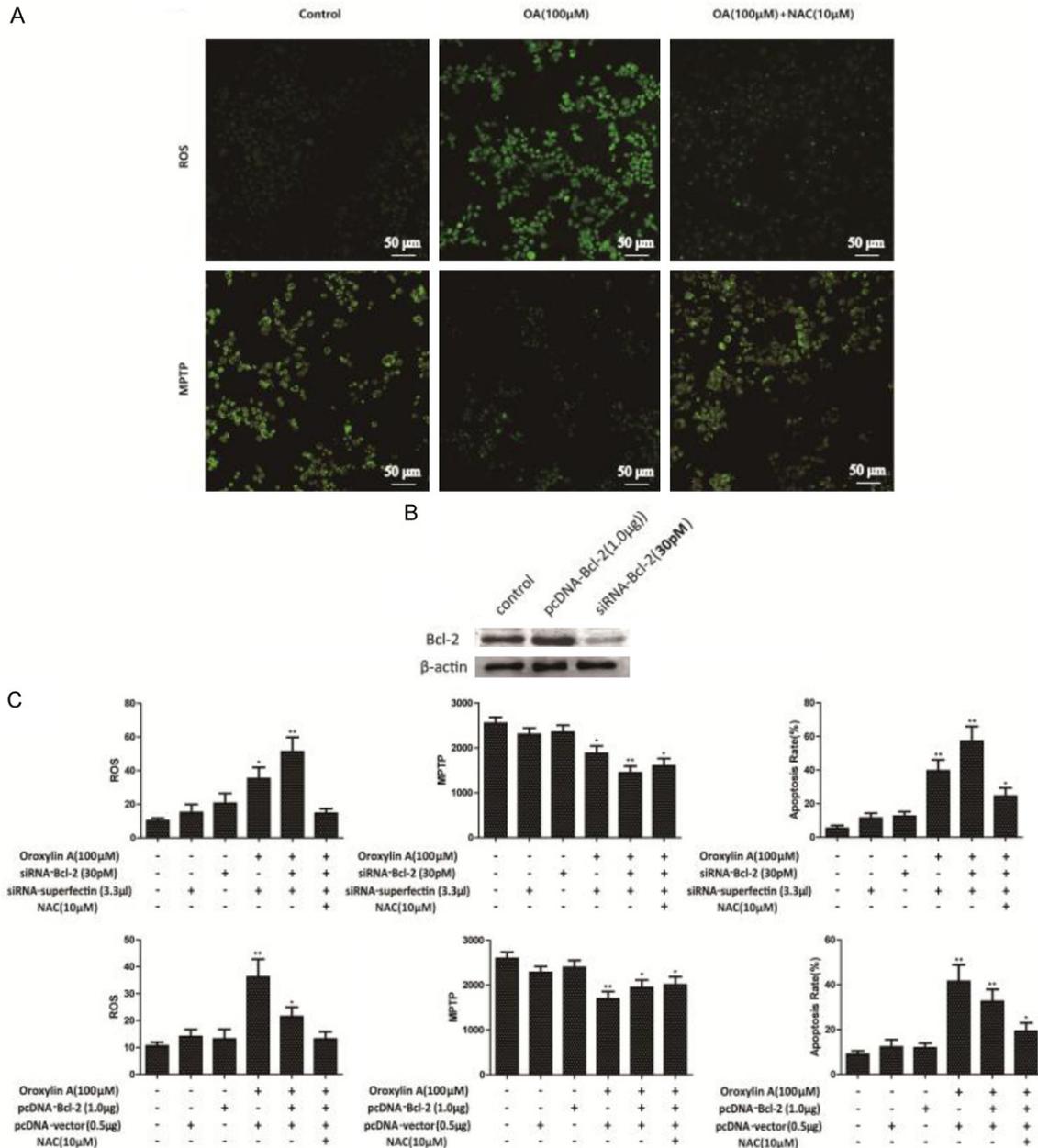
Figure 4. Oroxylin A-induced MPTP activation was ROS dependent. A: Confocal images of HepG2 cells after 100 µM Oroxylin A treatment. B: The over-expression or silencing of Bcl-2 in HepG2 cells was confirmed by western blot. C: ROS production, MPTP activation and apoptosis rates were monitored as above and the results were shown as means ± SD.

we evaluated the activation of MPTP by staining the mitochondria with calcein which can be captured by mitochondria. Calcein will be quenched by cobalt ions when MPTP is activated during wash buffer washing. The change of green fluorescence intensity was determined by flow cytometer FACS Calibur assay. After the cells were treated with oroxylin A for 24 h, MPTP was activated in HepG2 cells (**Figure 3D**).

We further examined the involvement of caspases in oroxylin A-induced apoptosis. After treatment with oroxylin A for 24 h, caspase-3, caspase-9 were markedly activated, while caspase-8 still remained unchanged (**Figure 3E**). These results indicated that oroxylin A-induced apoptosis was regulated through the mitochondria-mediated pathway rather than the death receptor-mediated pathway. To investigate wh-
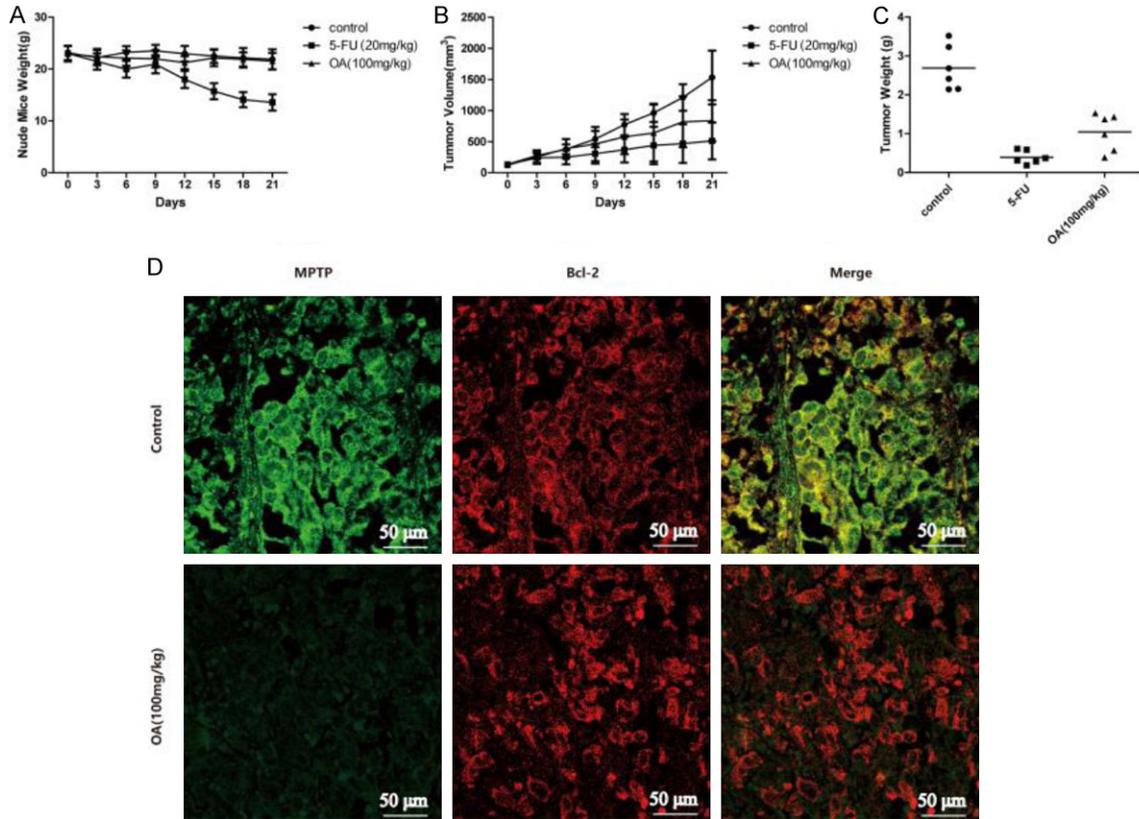
Figure 5. Oroxylin A induced Bcl-2 mitochondrial translocation in vivo. A-C: HepG2 cells were transplanted in BALB/C nude mice and 100 mg/kg of oroxylin A was administrated by intraperitoneal injection. The tumor volume, body weight and tumor weight were shown. D: Confocal images of tumors showing fluorescence of MPTP in green and Bcl-2 in red.

ether the opening of MPTP was involved in oroxylin A-induced apoptosis, we evaluated the the release of pro-apoptotic proteins such as Cyt-c and AIF from mitochondria. After HepG2 cells were treated with oroxylin A for 24 h, the expressions of Cyt-c, AIF and Bcl-2 were significantly decreased in mitochondria but increased in cytoplasm (**Figure 3F**).

Oroxylin A induced MPTP activation was ROS-dependent

As shown in **Figure 4A**, treatment with oroxylin A for 24 h significantly activated MPTP; however, pre-treatment with NAC in HepG2 cells, an antioxidant that could decrease intracellular ROS levels, totally blocked the activation of MPTP. These results strongly suggested that the oroxylin A-induced MPTP activation might be ROS-dependent. Another essential regulator of MPTP is Bcl-2; the enrichment of Bcl-2 deactivates MPTP in mitochondria. To probe whether the observed MPTP activation required

Bcl-2, we inhibited Bcl-2 expression in HepG2 cells through RNA interference (**Figure 4B**). As shown in **Figure 4C**, knockdown of Bcl-2 significantly increased the level of ROS and the activation of MPTP. The enhanced apoptosis rates were observed accordingly. Overexpression of Bcl-2 (**Figure 4B**), on the contrary, reduced the ROS levels, deactivated MPTP and inhibited cell apoptosis (**Figure 4C**). NAC greatly attenuated the changes induced by Bcl-2 (**Figure 4C**). These results demonstrated that oroxylin A could promote the opening of MPTP and triggered the mitochondria-mediated apoptotic pathway by increasing ROS levels.

Oroxylin A induced Bcl-2 mitochondrial translocation in vivo

Immunofluorescence analysis was used to detect the subcellular localization of Bcl-2 and mitochondria in vivo. HepG2 cells were transplanted in BALB/C nude mice and 100 mg/kg of oroxylin A was administrated by intraperito-

neal injection. 20 mg/kg 5-Fu was used as the positive control. After 21 days, tumor samples were collected and studied by immunohisto-chemical staining. The results clearly showed that oroxylin A significantly inhibited the growth of HepG2 tumors while did not cause obvious weight loss (**Figure 5A-C**). Compared with orox-ylin A, 5-Fu showed much greater cytotocity as evidenced by declined body weight (**Figure 5A**). The photographs of confocal microscopy dem-onstrated that Bcl-2 in cytoplasm distributed more widely than that in the control group (**Figure 5D**).

Discussion

Oroxylin A, a naturally occurring monoflavonoid extracted from S. baicalensis Georgi, has been shown to be a promising candidate for selec-tive and effective management of inflammation [13]. Oroxylin A can also induce cancer cell apoptosis and death via the mitochondrial pathway [10, 14]. Here, we examined the effect of oroxylin A on human hepatoma HepG2 cells, and demonstrated that oroxylin A induced HepG2 cell apoptosis by activating of MPTP. We also showed that oroxylin A increased the ROS levels and caused oxidative stresses, which in turn led to the opening of MPTP [15].

It is long been known that mitochondria can become leaky, uncoupled and massively swol-len if cells are exposed to high calcium concen-trations or under oxidative stress. Measurement of the permeability properties of mitochondria demonstrated that the MPTP is a non-specific pore with a diameter of about 2.3 nm [16]. Although the composition of MPTP is not fully understood yet, accumulating evidences con-vincingly suggest that MPTP is formed by TSPO (translocator protein, previously known as the peripheral benzodiazepine receptor) located in the mitochondrial outer membrane and cyclophilin-D in the mitochondrial matrix [3, 4, 17]. Opening of the MPTP makes the mem-brane of mitochondria freely permeable to pro-tons, small molecular weight metabolites, cofactors and ions. The activation of MPTP cause the decrease of mitochondrial mem-brane potentials and the swelling of mitochon-dria, which will break the outer membrane of mitochondria, and spill pro-apoptotic proteins, such as cytochrome c, AIF and pro-caspases, into cell cytoplasm and finally trigger cell apop-tosis [18, 19]. Therefore, the activation of MPTP

is detrimental not only on cell mitochondria, but also on the fate of the entire cell. We showed that oroxylin A treatment induced MPTP open-ing and significant cell apoptosis, suggesting that MPTP might be one of targets of oroxylin A. MPTP represents significant therapeutic tar-gets that should be considered in the future treatment of human cancer [20, 21].

It is well established that ROS plays an essen-tial role in many types of cancer. The increase of ROS is often observed in cell death, and the generation or up-regulation of ROS can induce cell apoptosis [22]. Mitochondrial respiration is the major source of ROS. Unless adequately detoxified, ROS causes mitochondrial oxidative stress, leads to the decline of mitochondrial functions, permeabilises the mitochondria and finally triggers the intrinsic pathway leading to apoptosis [23]. Many anticancer drugs, such as curcumin, have been known to increase the level of ROS as they induce the apoptosis in cancer cells. However, ROS has a dual role in carcinogenesis. It is demonstrated that high levels of ROS are involved in cancer metastasis [24]. In pancreatic cancer, ROS may activate pancreatic stellate cells by stabilization of HIF-1α and up-regulation of Gli1 expression, thus promoting pancreatic stellate cells to secret soluble factors such as IL-6, SDF-1, and VEGF-A to favor pancreatic cancer invasion [25]. ROS may act as an adaptive strategy to inhibit autophagic cell death by up-regulation of the AKT/mTOR pathway [26]. Indeed, some natural compounds, especially antioxidants (e.g., Metformin), can decrease ROS levels as they induce apoptosis in cancer cells. It is conclud-ed that although excessive ROS can lead can-cer cells to death, a relatively high levels of ROS is benefit for cancer initiation and progression. Our data showed that high concentrations of oroxylin A induced cell apoptosis via up-regula-tion of ROS levels; whether oroxylin A affects cancer cell ROS without cytotocity, remains to be investigated.

The Bcl-2 family is a well-studied group of pro-teins involving in multiple cell progresses like apoptosis, necrosis, and autophagy [27, 28]. Within this family, members such as Bcl-xl, Bcl-2 are overexpressed in cancer, indicating they may exert anti-apoptotic functions. Bcl-2 has also been shown to maintain the mitochon-drial membrane potential, and inhibit the open-

ing of MPTP in parallel [29]. It is reported that Bax positively mediated the opening of MPTP [30], and the increase of Bcl-2 protein and aberrant Bcl-2/Bax ratio could lead to the deactivation of MPTP [31]. We therefore hypothesized that Bcl-2 might exert its anti-apoptotic effects through mitochondrial translocation, and prevented the opening of MPTP induced by other cellular stresses like accumulation of ROS. Our results demonstrated that overexpression of Bcl-2 reduced the opening of MPTP, and declined the cell apoptosis rate. Immunofluorescence assay showed that Bcl-2's mitochondrial translocation was prevented, inducing MPTP opening.

In summary, we clearly demonstrated that oroxylin A induced activation of mitochondrial apoptotic pathway by inducing the opening of MPTP and the accumulation of ROS, and MPTP was involved in the pro-apoptotic effect of Bcl-2 in HepG2 cells.

Disclosure of conflict of interest

None.

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