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UCP2-Related Mitochondrial Pathway Participates in Oroxylin A-Induced Apoptosis in Human Colon Cancer Cells

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Oroxylin A is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi. Our previous research demonstrated that oroxylin A have various anti-tumor effects including apoptosis, cell cycle arrest, drug-resistant reversion, and others. This paper explores the mechanism how oroxylin A induce apoptosis by regulating uncoupling protein 2 (UCP2) in human colon cancer cells. We found that the inhibition of UCP2 by UCP2 siRNA significantly increased the sensitivity of cells to drugs, reactive oxygen species (ROS) generation and the opening of mitochondrial permeability transition pore (MPTP) of CaCo-2 cells. We also found that UCP2 inhibition could lead to ROS-mediated MPTP activation. Furthermore, we demonstrated that oroxylin A triggered MPTP-dependent pro-apoptotic protein release from mitochondria to matrix and then induced apoptotic cascade by inhibiting UCP2. Intriguingly, the inhibition of UCP2 by oroxylin A was able to block Bcl-2 translocation to the mitochondria, keeping MPTP at open-state. In conclusion, we have demonstrated that UCP2 plays a key role in mitochondrial apoptotic pathway; UCP2s inhibition by oroxylin A triggers the MPTP opening, and promotes the apoptosis in CaCo-2 cells.

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The studies on the role of UCP2 have been increasing. UCP2 is a protein belongs to the mitochondrial anion transporter superfamily (Mailloux and Harper, 2011). UCP2 can regulate the proton pump, allowing H^+ weaving through the inner mitochondrial membrane to be more efficient (Brand and Esteves, 2005; Derdak et al., 2008). UCP2 can render the electron flow via the respiratory complexes that give rise to part of the mitochondrial proton gradient leaking back into the matrix according to the "uncoupling to survive" hypothesis (Brand, 2000; Rose et al., 2011). Several researches have demonstrated that UCP2 is broadly over-expressed in cancer cells, especially human colon cancer (Derdak et al., 2006). This feature supports an adaptive mechanism to reduce reactive oxygen species (ROS) generation which means UCP2 can decrease the mitochondrial oxidative stress (Derdak et al., 2009). UCP2 is also active or over-expressed in several stages of tumor development, protecting cancer cells from apoptosis via negative regulation of mitochondrial ROS generation during ATP synthesis (Fulop et al., 2006). This evidence was later supported by the demonstration that UCP2 over-expressed by pcDNA decreases mitochondrial super-oxide ion generation in human colon cancer cells (Lee et al., 2005; Dalla Pozza et al., 2012). Kuai et al. (2010) found that UCP2 is highly expressed in human colon cancer, having an influence on tumor aggressiveness and cancer metastasis. UCP2 over-expression in tumors may be a general

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phenomenon which can serve as a potential anti-tumor therapeutic target.

There is also another important protein in the inner mitochondrial membrane called MPTP (Kinnally and Antonsson, 2007). It is a multi-component protein that can connect the inner sites with the outer sites of the mitochondrial membrane (Mathupala et al., 2006b; Gogvadze et al., 2009a). It has been demonstrated that this multi-component can regulate normal physiological functions of cells via mitochondria (Kinnally and Antonsson, 2007). The mitochondria mediated apoptosis pathway is initiated by various factors like high levels of cytoplasmic Ca^{2+}, ROS, the activation of pro-apoptotic Bcl-2 family proteins, releasing apoptogenic proteins like cytochrome *c*, AIF, which are apparently important for the propagation in the following apoptotic cascades to the final death of the cells (Liu et al., 1996; Antonsson, 2004). Apoptosis caused by release of apoptogenic protein is a common step of apoptosis through mitochondrial pathway, which seems to largely depend on MPTP (Gogvadze et al., 2009a). The mitochondria of cancer cells, as apoptosis machinery, are different from that of normal cells in structure and function, so the drugs aiming at mitochondria are supposed to have tumor selectivity (Fulda et al., 2010). Mitochondrial permeability transition (MPT) is an instant permeabilization when cells are exposed to noxious stimulus (McCommis and Baines, 2012). This transformation is thought to begin after the opening of the MPTP, which leads to nonspecific disintegration of the outer mitochondrial membrane. The critical regulation of the cellular apoptosis though mitochondria pathway via the MPTP is the balance between pro- and anti-apoptotic Bcl-2 family proteins (Gogvadze et al., 2009a; Kroemer and Reed, 2000). It has been demonstrated that there could be pore structures forming on the outer mitochondrial membrane (OMM) to release cytochrome when free Bax interacts with Bak and/or Bax (Willis et al., 2005). This phenomenon can be reversed if Bax is replaced by Bcl-2. Although the mechanisms underlying MPTP remain to be unknown, it appears to be clear that the MPTP is considered to be the most viable target for novel anti-cancer agents (Marzo et al., 1998). Bcl-2 mitochondrial translocation can block the MPTP from opening, preventing the apoptogenic proteins to leak from mitochondria. In addition, over-expressed UCP2 helped Bcl-2 enrichment on mitochondria.

Oroxylin A ($C_{16}H_{12}O_5$, Fig. 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, which has been proved to have multiple pharmacological effects on several types of cancer both in vitro and in vivo including apoptosis and metastasis inhibition (Liu et al., 2009; Wei et al., 2013). Results of this study have indicated that the UCP2 acts as an anti-apoptosis protein over-expressed in the membrane of mitochondria in Caco-2 cells in vitro. UCP2 can reduce ROS production, which is regarded as a critical factor for the opening of MPTP, reducing the apoptogenic proteins released from mitochondria to matrix. Moreover, we found, for the first time, that oroxylin A induced apoptosis via inhibiting UCP2 expression, motivating opening of MPTP in colon cancer cells.

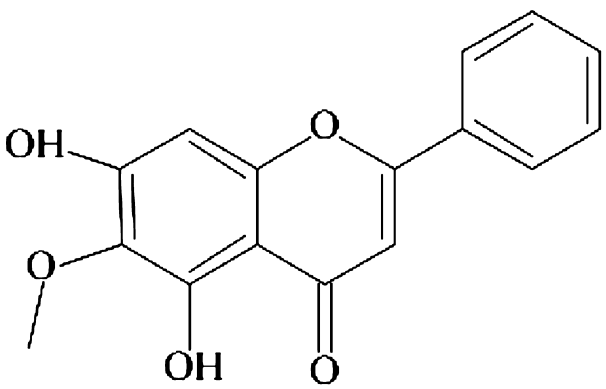

Fig. 1. Molecular structure of oroxylin A (C16H12O5, MW:284).

Materials and Methods

Materials

Oroxylin A ($C_{16}H_{12}O_5$) was isolated from the root of *Scutellaria baicalensis* according to a previously reported method and dissolved in DMSO (Li and Chen, 2005). Samples containing 99% or higher of oroxylin A were used in all experiments. 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. Adriamycin ($C_{27}H_{29}O_{11}N \cdot HCl$) was purchased from Pfizer Italia S.r.l and dissolved in normal saline. Caspase-8 inhibitor II was purchased from Calbiochem (EMD Chemicals, Inc. San Diego, CA) and dissolved in DMSO.

Antibody to UCP2(catalog number PL031926R) was obtained from PLlabs (Columbia, UK); 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 PARP (catalog number 9542) and AIF (catalog number 4642) were from Cell Signaling (Danvers, MA); antibody to Cox IV (ab14744) was from Abcam Ltd (HK, China); and antibody to β-actin (BM0627) was from Boster (Wuhan, China).

Cell culture

Human colorectal cancer CaCo-2 cells and HCT-116 cells were purchased from Cell Bank of Shanghai Institute of Bio-chemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). HCT-116 cells were maintained in 90% McCoy's 5A medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Invitrogen Corporation, Carlsbad, CA), 100 U/ml penicillin G, and 100 μg/ml streptomycin. Respectively CaCo-2 cells were maintained in 90% DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China). Both kinds of cells were cultivated at 37 °C in a water-jacketed CO_2 incubator (Thermo Forma, Waltham, MA) in a humidified atmosphere with 5% CO_2.

Annexin V/PI staining

Apoptosis cells were identified by Annexin V-FITC Apoptosis Detection Kits (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 resuspended in 500 μl binding buffer and then added 5 μl Annexin V-FITC and 5 μl PI. Apoptotic cell death was examined by FACSCalibur 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 Calcein-AM. 1×10^6 cells were collected and washed with cold PBS, then resuspended in PBS and incubated for 30 min at 37 °C with Calcein-AM. The fluorescence intensity was measured at 488 nm excitation and 505 nm emission using a fluorospectrophotometer.

Plasmid and siRNA transient transfection

The pcDNA3-UCP2 plasmid was obtained from GeneChem (GeneChem plasmid 7351). Small interfering RNAs of UCP2 were purchased from Santa Cruz. For transfection, HCT-116 cells were seeded in 6-well plates at 70% (UCP2 palsmid) confluency, then pcDNA-UCP2 (1.0 μg) were introduced into the cells using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's recommendations, respectively, CaCo-2 cells were incubated with 30% (UCP2 siRNA) then siRNA duplexes were introduced in the same method. Later the cells were exposed to oroxylin A, Adriamycin, and vehicle then harvested for further experiments.

Immunocytochemical study

Cells were rinsed with PBS and fixed with 4% paraformaldehyde in phosphate 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 Calcein-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 FV10-ASW [Ver 2.1] (Olympus Corp, MPE FV1000) for co-localization analysis.

Measurement of intracellular ROS

Intracellular ROS was detected by DCFH-DA (Beyotime Institute of BioTechnology, Haimen, China) sensitively. Cells were harvested from 6-well plates and washed with PBS, then resuspend 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 FACSCalibur 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 resuspended 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 resuspended in 0.1 ml Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors and then vortexed for 10 sec 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 (CALBIOCHEM, Germany) and AIF antibody (Cell Signaling, MA).

Results

UCP2 inhibition increase sensitivity of human colon cancer cells to Adriamycin

To identify the effect of UCP2 on apoptosis in human colon cancer cell, we first performed Annexin V/PI staining assay and DAPI staining on CaCo-2 cell and HCT-116 cell after Adriamycin treatment for 24 h. Figure 2A showed that the expression of UCP2 in CaCo-2 cell and HCT-116 cell were different and the higher level expression of UCP2 was observed in CaCo-2 cell. After cells were treated with Adriamycin 2 μM for 24 h, the apoptotic rate of CaCo-2 cell were significantly increased by UCP2 silencing (Fig. 2B, left), while the apoptosis rate of HCT-116 cell were decreased with UCP2 over-expressing (Fig. 2B, right). Under the fluorescent microscope, untreated CaCo-2 and HCT-116 cells were stained equally with blue fluorescence, showing the steady chromatic distribution in nucleolus. Both Adriamycin-treated CaCo-2 cells and HCT-116 cells showed chromatin agglutination and the nucleus pyknosis, which were the early phenomenon of apoptosis. After UCP2 was knocked down, the nucleus of CaCo-2 cells were injured heavily and there were more apoptotic bodies; instead the apoptotic phenomenon induced by Adriamycin in HCT-116 cells, which was abated after UCP2 over-expressed (Fig. 2C).

UCP2 inhibits ROS-mediated MPTP opening

Since UCP2 is a multifunction protein in inner membrane of mitochondria, it can affect mitochondria at the integral level (Derdak et al., 2009). We believed that there may be some connection between its anti-apoptosis ability and its ROS suppression ability. We evaluated the effect of UCP2 on intracellular ROS production through ROS assay on CaCo-2 cells and HCT-116 cells treated with H_2O_2 10 μM. As shown in Figures 3A–C, ROS accumulation was enhanced when UCP2 was knocked down in CaCo-2 cells and was reduced when UCP2 was over-expressed in HCT-116 cells. These results indicated that UCP2 played a key role in ROS accumulation. ROS is an important factor which affects MPTP opening, so UCP2 inhibition may change the status of MPTP indirectly. We evaluated the activation of MPTP by staining the mitochondria with Calcein-AM which can be captured by mitochondria. Calcein-AM will be quenched by cobalt ions if the MPTP is activated. The fluorescence intensity was measured at 488 nm excitation and 505 nm emission using a fluorospectro photometer. After H_2O_2 treatment for 24 h, the MPTP was activated in both cells with dosage dependent manner. After UCP2 silencing in CaCo-2 cells, the opening of MPTP was enhanced while this phenomenon was reversed when UCP2 over-expressed in HCT-116 cells (Fig. 3D).

Taken together, these results demonstrated that UCP2 might be a key role in ROS-mediated MPTP activation.

Oroxylin A sensitizes apoptosis in mitochondrial pathway by UCP2 inhibition

Our previous research has proved that oroxylin A has therapeutic potential in acute myelogenous leukemia by targeting peroxisome proliferator-activated receptor gamma (PPARγ), which is an intronic enhancer in UCP2 expression (Hui et al., 2014). Therefore, oroxylin A may affect UCP2 expression. We detected the changes of the UCP2 when CaCo-2 cells were incubated with 50, 100, and 200 μM by Western blot. As shown in Figure 4A, oroxylin A suppressed UCP2 expression with a dose-dependent manner. Subsequently, we evaluated intracellular ROS and

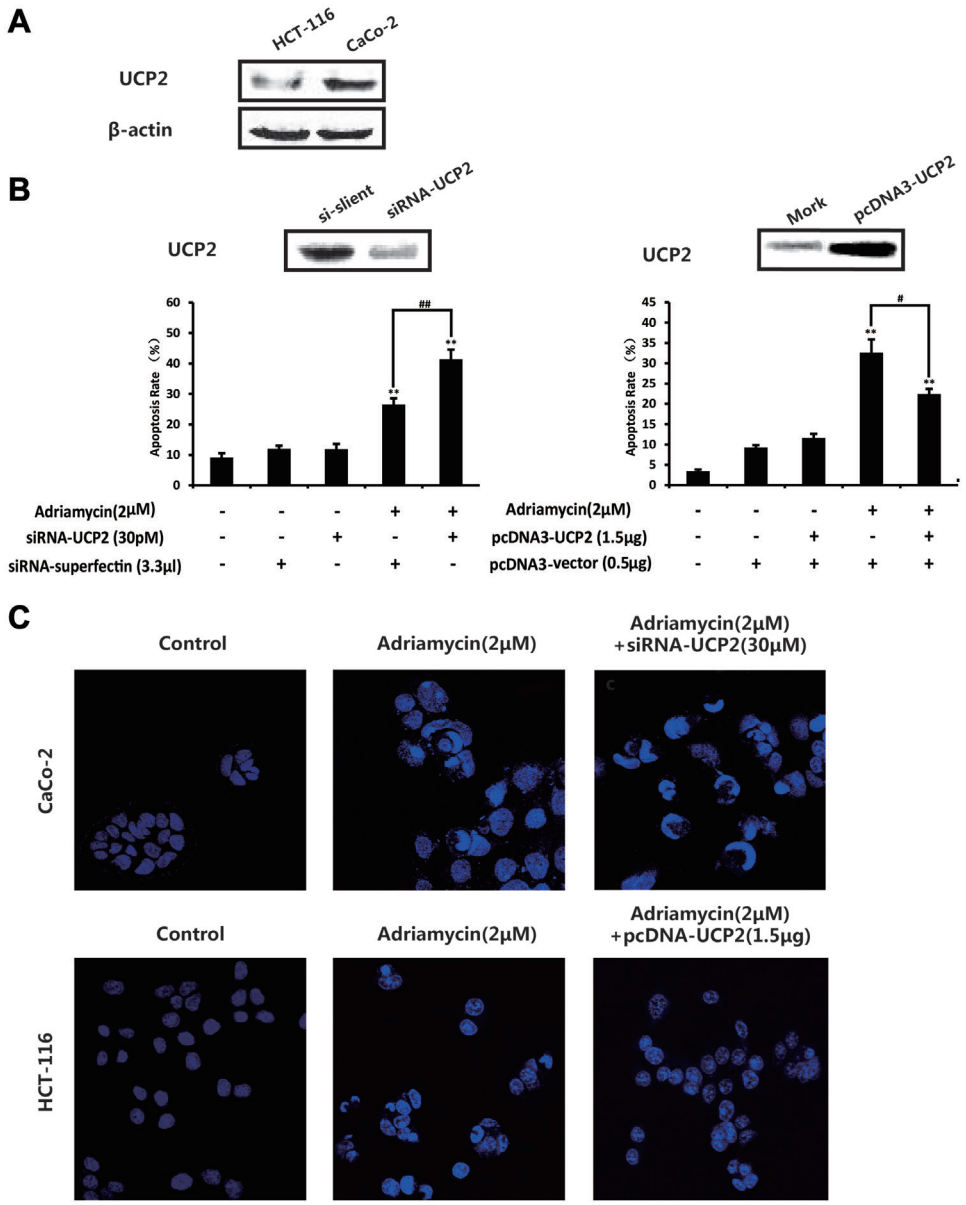

Fig. 2. UCP2 inhibition increased colon cancer cells sensitivity to Adriamycin. (A) UCP2 expression in CaCo-2 cell and HCT-116 cell was detected by Western blot. (B) CaCo-2 cells were seeded in 6-well plates, incubated overnight, and transfected with UCP2 siRNA (30 pM). Respectively, HCT-116 cells were transfected with UCP2 plasmid (1.5 μg). After Adriamycin (2 μM) treatment for 24 h, both CaCo-2 cells and HCT-116 cells were stained with Annexin V and PI and apoptosis cells were quantitated by flow cytometer. Results from the experiments are shown as means ± SEM. (C) DAPI staining of CaCo-2 cells were treated in the DMSO, Adriamycin (2 μM), Adriamycin (2 μM) + siRNA-UCP2 (30 pM), and HCT-116 cells were treated in the DMSO, Adriamycin (2 μM), Adriamycin (2 μM) + pcDNA-UCP2 (1.5 μg).

apoptotic rates of CaCo-2 cells and HCT-116 cells with different UCP2 levels, incubating with 100 μM oroxylin A for 24 h. The results showed that increased ROS was little affected even UCP2 was over-expressed in HCT-116 cells (Fig. 4B). The apoptotic rate was increased when low-level expression of UCP2 both in CaCo-2 cells and HCT-116 cells. Additionally, the apoptotic rate was little changed by caspase-8 inhibitor II, indicating that oroxylin A-mediated apoptosis was not mainly regulated through death-receptor pathway (Fig. 4C). These results demonstrated that oroxylin A eliminated antioxidant capacity of UCP2, and induced apoptosis in CaCo-2 cells and HCT-116 mainly though non-death-receptor pathway.

Oroxylin A induces apoptosis by MPTP activation in CaCo-2 cells

To prove oroxylin A-induced apoptosis is really via mitochondrial pathway, we further examined the involvement of caspases by Western blot. As shown in Figure 5A, PARP cleavage and caspase-9 activation were detected in CaCo-2 cells after oroxylin A 100 μM treatment for 24 h.

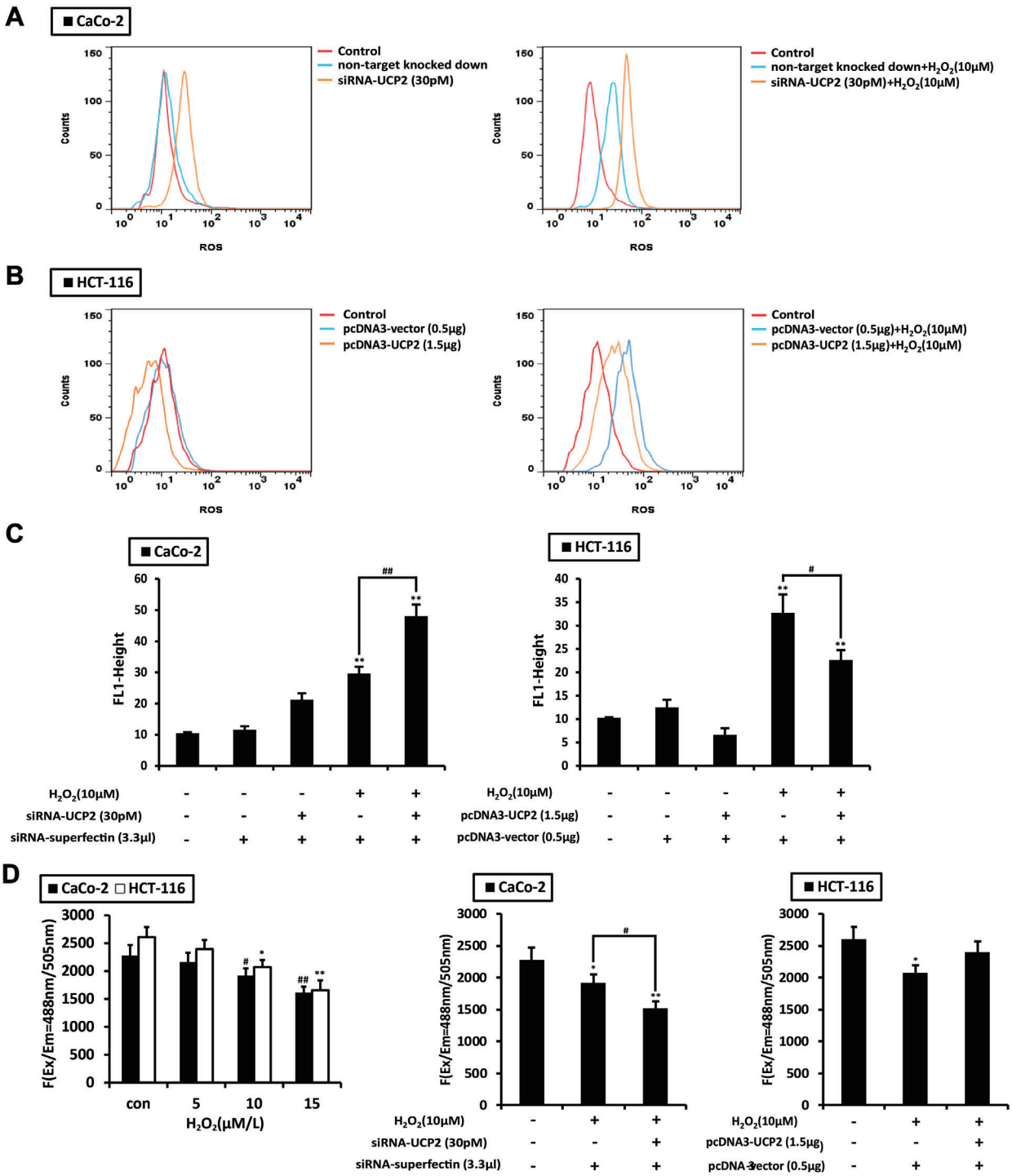

Fig. 3. UCP2 suppressed ROS accumulation and MPTP activation in CaCo-2 cells and HCT-116 cells. (A) CaCo-2 cells were transfected with UCP2 siRNA as above and ROS production was monitored by flow cytometry using 10 μM DCFH-DA before and after CaCo-2 cells were treated with H_2O_2 (10 μM) for 24 h. (B) HCT-116 cells were transfected with UCP2 pcDNA (1.5 μg) as above and ROS production was monitored before and after cells were treated with H_2O_2 for 24 h. (C) Quantitative evaluations of ROS production of CaCo-2 cells and HCT-116 cells treated with H_2O_2 (10 μM). (D) The opening of MPTP was detected by Calcien-AM staining after CaCo-2 cells and HCT-116. The fluorescence intensity was declined after cells were treated by H_2O_2 indicated the opening of MPTP. 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 ± SEM.

Consequently, leak of Cyt-c and AIF from mitochondrial were also observed (Fig. 5B). These results indicated that MPTP might be activated in CaCo-2 cells after oroxylin A treatment. Then, we evaluated the status of MPTP. As shown in Figure 5C, under the fluorescent microscope, untreated CaCo-2 cells were stained with green fluorescence. After cells were treated with oroxylin A for 24 h, the fluorescence intensity was declined in dosage dependent manner. These results suggested

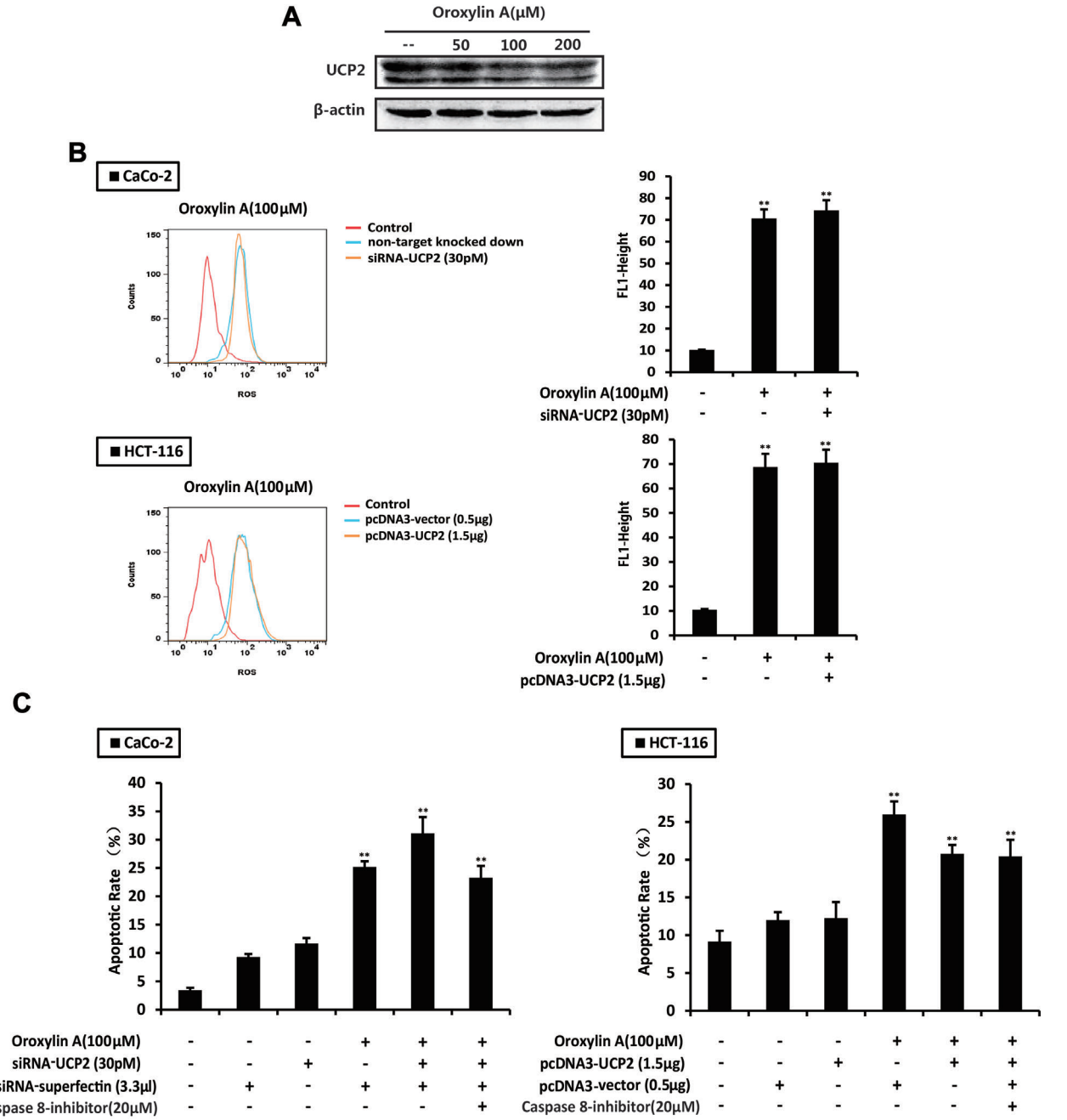

Fig. 4. Oroxylin A induced apoptosis of CaCo-2 cells and HCT-116. (A) UCP2 inhibition by oroxylin A was detected by Western blot. CaCo-2 cells were treated with indicated concentration of oroxylin A for 24 h. (B) ROS production was monitored by flow cytometry using 10 μM DCFH-DA after CaCo-2 cells were treated with oroxylin A and oroxylin A + siRNA-UCP2 (30 pM) for 24 h. Respectively HCT-116 cells were treated with oroxylin A and oroxylin A + pcDNA-UCP2 (1.5 μg). (C) The CaCo-2 cells was treated with indicated concentration of oroxylin A, siRNA-UCP2 (30 pM), and caspase-8 inhibitor II. Respectively, HCT-116 cells was treated with indicated concentration of oroxylin A, pcDNA-UCP2 (1.5 μg), and caspase-8 inhibitor II. After 24 h, cells were stained with Annexin V and PI and apoptosis cells were quantitated by flow cytometer. Results from the experiments are shown as means ± SEM.

that MPTP activation played a key role in oroxylin A-induced mitochondrial apoptosis.

Oroxylin A inhibits UCP2-dependent Bcl-2 mitochondrial translocation

Bcl-2 is an important influence factor of MPTP, its enrichment on mitochondria de-activates MPTP. Immunofluorescence analysis was used to detect the subcellular localization of Bcl-2 and mitochondria in HCT-116 cells (Fig. 6). Confocal images showed that Bcl-2 was mainly present in the cytosol of control cells. After UCP2 over-expression, Bcl-2's mitochondrial translocation was observed in response to H_2O_2 10 μM treatment for 24 h and the opening of MPTP was reduced. These results demonstrated that ROS-mediated MPTP activation can be reduced by enrichment of Bcl-2 on

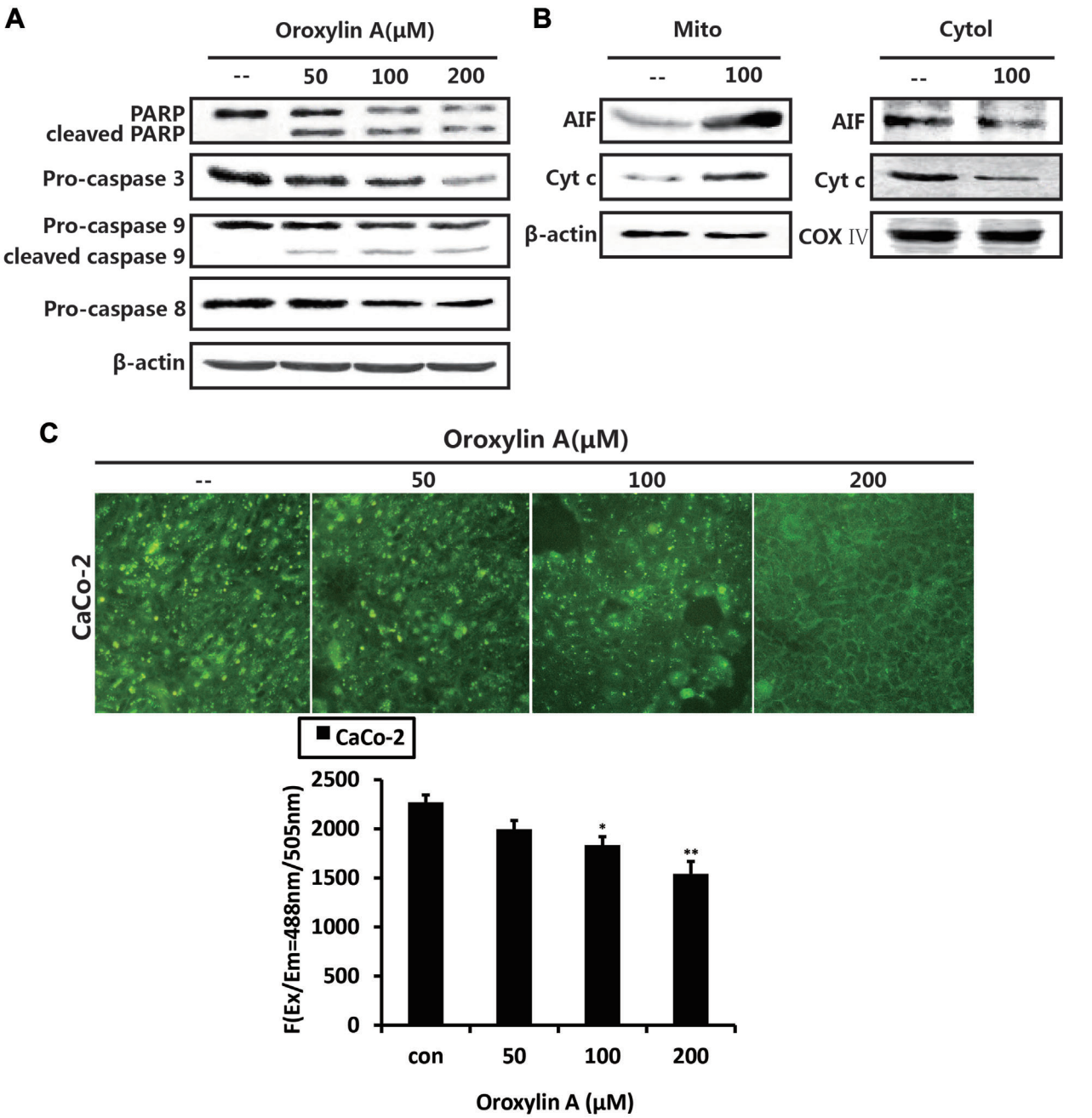

Fig. 5. Oroxylin A induces MPTP activation in CaCo-2 cells. (A) The CaCo-2 cells were treated with indicated concentration of oroxylin A. After 24 h, the effect of oroxylin A on PARP cleavage, caspase-3, caspase-8, caspase-9 were detected by Western blot. (B) Western blotting analysis of cytochrome c and AIF in the cytosolic fraction. The fractionation of the mitochondrial protein and cytosolic protein was performed according to the cytochrome c release apoptosis assay kit instruction. Cytochrome c and AIF in the cytosolic fraction was analyzed by Western blotting analysis with cytochrome c and AIF antibody. (C) Calcien-AM staining of the CaCo-2 cells treated with indicated concentration of DMSO, oroxylin A (50 μM), oroxylin A (100 μM), and oroxylin A (200 μM) for 24 h. 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 ± SEM.

mitochondria via UCP2. After treatment with oroxylin A 100 μM for 24 h, the phenomenon of translocation was eliminated and MPTP was activated.

Taken together, our findings demonstrated that oroxylin A induced MPTP activation by suppressing UCP2, causing ROS accumulation, and apoptosis in mitochondrial pathway.

Discussion

In the present study, we clearly elucidated for the first time the role of UCP2-mediated mitochondrial uncoupling on apoptosis in colon tumor cells. It is well known that UCP2 is an antioxidant mitochondrial protein whose inhibition induces oxidative stress (Dando et al., 2013). In several stages of tumor

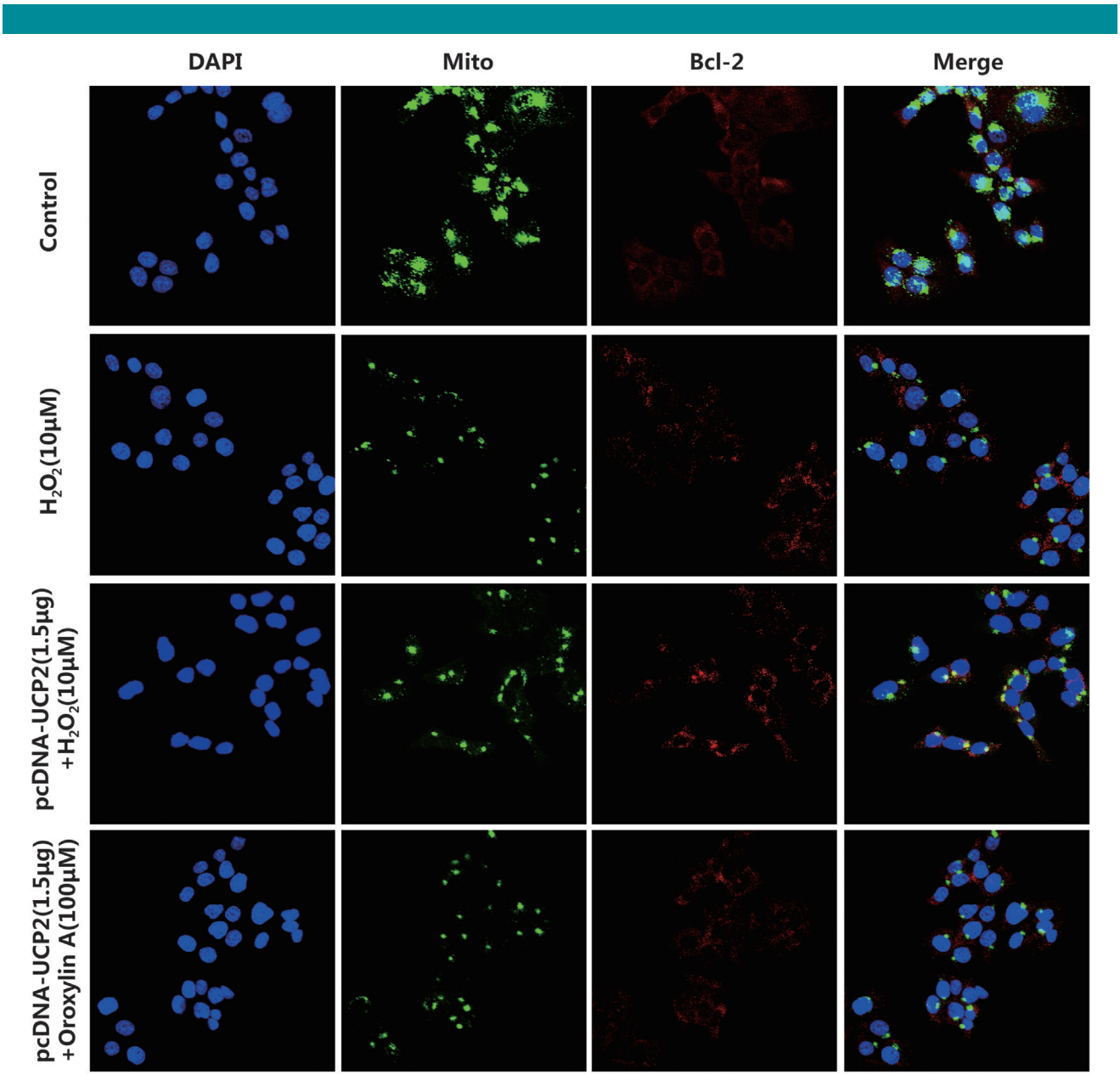

Fig. 6. UCP2 inhibition affects the enrichment of Bcl-2 on mitochondria. (A) HCT-116 cells were incubated with indicated concentration of DMSO, H_2O_2 (10 μM), pcDNA-UCP2 (30 μM) + H_2O_2 (10 μM), and pcDNA-UCP2 (30 μM) + oroxylin A (100 μM) for 24 h. Confocal images of cells show fluorescence of DAPI in blue, Mito in green, Bcl-2 in red, and are merged in Lane 4.

development, UCP2 is broadly over-expressed protecting cancer cells from apoptosis via negative regulation of mitochondrial ROS generation during ATP synthesis, especially in human colon cancer (Derdak et al., 2006). We demonstrated that oroxylin A triggered MPTP activation in human colon cancer cell by inhibiting UCP2, resulting in mitochondrial mediated apoptosis. Our data in agreement with the observations of Sanming Deng and colleagues who demonstrated that UCP2 inhibited ROS-mediated apoptosis in A549 under hypoxic conditions (Deng et al., 2012). The primary function of UCP2 is allowing the reentry of protons to the mitochondrial matrix by dissipating the proton gradient and subsequently decreasing ROS production. This function of UCP2 was previously indicated as ROS production regulator in many tissue types (Lee et al., 2005; Mailloux and Harper, 2011; Dando et al., 2013). In cancer cells, UCP2 may play an indispensable role in the resistance response during chemotherapeutics. The increased levels of UCP2 and diminished sensibility to cytotoxic treatment were observed in a drug-resistant subset of cancer cells derived from melanoma, leukemia, and human colon cancer cells (Pfefferle et al., 2013). We found that intracellular ROS was lower in cells with over-expressed UCP2 compared to control cells, while higher with UCP2 suppressed under the same treatment with 10 μM H_2O_2 (Figs. 3A and B). Integrating our results, regulation of ROS production may be the main function of UCP2 in cancer cells.

It has been demonstrated that MPTP is a potential selective target for anti-cancer therapy (Fulda, 2010; Suh et al., 2013).

Although the concept of the MPTP is still evolving (Nakagawa et al., 2005), plenty of evidence has demonstrated that the MPTP was directly responsible for cytochrome *c* release which was critical in mitochondrial pathway apoptosis (Kinnally and Antonsson, 2007). The opening of MPTP can be activated by oxidative stress, Ca^{2+} overload and cytotoxic drugs (Liu et al., 2009). We have acknowledged that over-expressed UCP2 can suppress ROS production, so was there any relationship between UCP2 and MPTP? In order to investigate our hypothesis, we evaluated the opening of MPTP at different level of UCP2. The results showed that the opening of MPTP was reduced when UCP2 expressed at a higher level (Fig. 3D). These results indicated that UCP2 inhibition might indirectly promote MPTP activation.

Oroxylin A has been shown to be a promising candidate for selective and effective management of inflammation with effective anticancer activities and low toxicities both in vivo and in vitro (Zhao et al., 2010). Our laboratory has proved that oroxylin A reversed MDR with more than fourfold in vitro, increased accumulation of Rhodamine 123 in MCF7/ADR cells notably by a concentration-dependent manner, decreased the MDR1 expression both in protein and mRNA level (Zhu et al., 2013). Our research suggested that oroxylin A might be a moderate complementary medicine in Adriamycin treatment. In order to confirm whether UCP2 was involved in oroxylin A-induced apoptosis, we examined the expression of UCP2, ROS, and the apoptotic rates under different UCP2 expression (Fig. 4). Our data showed that UCP2 was downregulated after treatment with oroxyin A for 24 h in dosage-dependent manner, and the level of ROS in both cell were maintained in a high level. Results of Annexin V/PI staining assay also indicated that oroxylin A could promote the apoptosis effectively when UCP2 was overexpressed. Western blot results indicated that oroxylin A induced apoptosis though induction of the caspase cascade and mitochondrial permeability transition (Fig. 5). We observed the release of cytochrome *c* and AIF from mitochondria to cytosol. These results indicated that MPTP activation might be a key role in oroxylin A-induced mitochondrial pathway apoptosis.

The composition of MPTP is not clearly understood yet, there is evidence accumulating for the molecular constituents of the MPTP (Kokoszka et al., 2004; Mathupala et al., 2006a; Gogvadze et al., 2009a). A critical regulation of the cellular apoptosis through the MPTP is the balance between pro- and anti-apoptotic Bcl-2 family proteins (Gogvadze et al., 2009b), the increase of Bax expression and aberrant Bcl-2/Bax ratio lead to the opening of MPTP (Kang et al., 2013). Therefore, we hypothesized that Bcl-2 might exert its anti-apoptotic effect through mitochondrial translocation, preventing the opening of MPTP induced by other cellular stresses like accumulation of ROS. We examined the subcellular localization of Bcl-2 after H_2O_2 treatment and our results demonstrated that the opening of MPTP was reduced due to the enrichment of Bcl-2 on mitochondria. When cells were incubated with oroxylin A 100 μM the translocation was prevented and MPTP was activated.

In the present study we have clearly demonstrated for the first time that the involvement of UCP2 in the ROS-dependent opening of MPTP and this anti-apoptosis effect of UCP2 could be reversed by oroxylin A. In summary, we have demonstrated that UCP2 inhibition played a key role in oroxylin A-induced activation of MPTP related mitochondrial apoptotic pathway in colon tumor cells.

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