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Different Apoptotic Effects of Wogonin Via Induction of H_2O_2 Generation and Ca^{2+} Overload in Malignant Hepatoma and Normal Hepatic Cells

Libin Wei,[1] Na Lu,[1] Qinsheng Dai,[1] Jingjing Rong,[1] Yan Chen,[1] Zhiyu Li,[2] Qidong You,[1] and Qinglong Guo[1*]

[1] *Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, The People's Republic of China*

[2] *Department of Medicinal Chemistry, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, The People's Republic of China*

ABSTRACT

Wogonin, a major active constituent of *Scutellaria baicalensis*, possesses potent anticancer activities both in vivo and in vitro. This paper describes the different apoptotic effects of wogonin in HepG2 and L02 cells and the possible mechanism for the differences. Through DAPI staining, Annexin-V/PI double-staining assay, JC-1 detection and the expressions of the key apoptotic proteins, we find that wogonin prefers to induce apoptosis in HepG2 cells through the mitochondrial pathway, while has much less effects on L02 cells. Moreover, overexpression of Bcl-2 can block wogonin-induced apoptosis in HepG2 cells. To illustrate the specific selective mechanism of wogonin in apoptosis induction, H_2O_2, $^{\cdot}O_2^-$ and Ca^{2+} are measured by 2′,7′-dichlorfluorescein-diacetate, dihydroethidium and Flou-3 AM assay, respectively. The results show that the different apoptotic effects of wogonin in HepG2 and L02 cells are due to the different regulations to the redox balance of reactive oxygen species and the Ca^{2+} release from endoplasmic reticulum. IP_3R-sensitive Ca^{2+} channels are the key targets of the wogonin-increased H_2O_2. Besides, the activation of PLCγ1 plays as a bridge between H_2O_2 signal molecules and Ca^{2+} release. Taken together, wogonin preferentially kills hepatoma cells by H_2O_2-dependent apoptosis triggered by Ca^{2+} overload. The results reveal that wogonin is a competitive anticancer drug candidate for the malignant hepatoma therapy. J. Cell. Biochem. 111: 1629–1641, 2010.

KEY WORDS: WOGONIN; DIFFERENT APOPTOSIS EFFECTS; H_2O_2; $^{\cdot}O_2^-$; CA^{2+}; IP_3R; PLCγ1

Hepatocellular carcinoma (HCC), a primary hepatic tumour with aggressive malignance and high prevalence, is the third leading cause of cancer death worldwide [Yamashita and Kaneko, 2010]. So far, the overall survival of patients with HCC is not optimistic because of the extreme chemoresistance of HCC and the low selectivity of chemotherapy drugs [Jia et al., 2007]. These drugs kill the tumour cells as well as the normal cells, leading to significant adverse effects hard to tolerance for patients. All these disadvantages result in a failure of chemotherapy. Therefore, much more efforts should be contributed to the development of the selective anticancer drugs for HCC therapy.

Mitochondria play significant roles during the cell apoptosis process. Loss of mitochondrial membrane potential (MMP) is a crucial step involved with apoptosis, triggering the activation

Abbreviations: 2-APB, 2,2-diphenyl-1,3,2-oxaza-borolidine internal salt; ADM, adriamycin; AIF, apoptosis inducing factor; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DHE, dihydroethidium; HCC, hepatocellular carcinoma; IP_3R, inositol-1,4,5-triphosphate receptors; MMP, mitochondrial membrane potentials; PARP, poly-ADP-ribose polymerase; PIAF, cisplatin/interferon α-2b/ADM/fluorouracil; PLCγ1, phosphoinositide-specific phospholopase gamma 1; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, Ca^{2+} re-uptake by the sarco/endoplasmic reticulum Ca^{2+} ATPase; SOD, superoxide oxido-reductase dismutase.

Libin Wei and Na Lu contributed equally to this work.

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*Correspondence to: Professor Qinglong Guo, PhD, Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, The People's Republic of China.
E-mail: anticancer_drug@yahoo.com.cn

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of apoptotic cascade and the execution of cell death [Kroemer et al., 2007]. In addition, mitochondria are the most important sources of cellular reactive oxygen species (ROS). The moderate increase of ROS in cancer cells has significant effects, such as stimulating cellular growth, promoting mutations and inducing resistance to anticancer agents [Zubovych et al., 2010]. However, ROS are chemically active and can cause severe cellular damage. Furthermore, the very fact that cancer cells are under increased intrinsic ROS stress may accomplish the purpose to kill the malignant cells preferentially because the further ROS insults are beyond their endurance [Pelicano et al., 2004].

H_2O_2 is a main member of ROS, which affects the functions of various ion channels and other proteins involved in signal transduction [Akaishi et al., 2004; Varela et al., 2004]. Therefore, H_2O_2 is competent to be an intracellular messenger. Ca^{2+} plays a significant role in the regulation of cell survival [Berridge et al., 2000]. Recently, more and more attention has focused on the mechanisms of Ca^{2+} mobilization in response to changes of cellular redox state. It has been shown that H_2O_2 can stimulate the mobilization of Ca^{2+} in many cell types by modifying Ca^{2+} channels/pumps and the activity of enzymes involved in Ca^{2+} signalling pathways.

Wogonin, a naturally monoflavonoid extracted from *Scutellaria baicalensis* radix [Chi et al., 2001], has been acknowledged as an anticancer drug candidate due to its high performance and low toxicity [Li-Weber, 2009]. It has been confirmed that wogonin induces apoptosis in some cancer cell lines such as human promyeloleukemic cells HL-60 [Lee et al., 2009b; Huang et al., 2010], human prostate cancer cells LNCaP [Lee et al., 2009a] and human hepatoma cells SMMC-7721 [Wang et al., 2006]. Moreover, it has been reported that wogonin selectively activates $PLC_\gamma 1$ via triggering Ca^{2+} release to cytoplasm and induces apoptosis in malignant T cells, but not in normal peripheral blood cells [Baumann et al., 2008]. However, data for the mechanisms, by which wogonin causes different apoptotic effects in cancer and normal cells, are still largely scarce. More investigations are needed aiming to show the distinct selection of wogonin to cancer cells and establish guided rationales for its clinical application.

In this study, we compared the different apoptotic effects of wogonin in HepG2 and L02 cells and investigated the reasonable mechanism. The results reveal that wogonin is a potent anticancer drug for HCC due to its preferential apoptosis induction in hepatoma cells.

MATERIALS AND METHODS

REAGENTS

Wogonin (purity ≥95%) was extracted from *S. baicalensis* radix according to the protocols reported previously with slight modifications [Hui et al., 2002]. In the experiments, the stock solution of wogonin was prepared in dimethyl sulphoxide (DMSO), stored at −20°C, and diluted with medium until needed. The final DMSO concentration did not exceed 0.1% throughout the study. The control groups were exposed to 0.1% DMSO in vitro experiment. N-acetyl-cysteine (NAC; Sigma–Aldrich, St. Louis, MO) was dissolved in the sterile double-distilled water to the concentration of 500 mM and stored at 4°C. 2,2-Diphenyl-1,3,2-oxaza-borolidine internal salt (2-APB, Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO to the concentration of 10^{-1} M and stored at −20°C. Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IRDyeTM800 conjugated second antibodies were obtained from Rockland Inc. (Bedford, PA).

CELLS CULTURE

Human hepatoma cell line HepG2 and human normal embryon hepatic cell line L02 were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Both the cells were cultured in RPMI-1640 medium (GIBCO, Invitrogen, Inc., Carlsbad, CA) supplemented with 10% heat-inactivated foetal bovine serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G and 100 μg/ml streptomycin at 37°C with 5% CO_2.

COLORIMETRIC MTT ASSAY

The logarithmic cells were detached to prepare 1.0×10^4/ml cell suspension, and partitioned into 96-well plates at 100 μl/well for 24 h at 37°C. Then cells were treated with wogonin at different concentrations (30–300 μM) at 100 μl/well. After 24 and 48 h incubation, 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide; Sigma–Aldrich) solution (20 μl/well) was added and cultured for 4 h. Then the supernatant was discarded and DMSO was added (100 μl/well). The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (EL800, BioTek Instruments Inc., Winooski, VT). The inhibitory ratio was calculated by the following formula:

$$\text{Inhibitory ratio } \% = \left(\frac{A_{control} - A_{treated}}{A_{control}}\right) \times 100\%$$

$A_{treated}$ and $A_{control}$ were the average absorbance of three parallel experiments from the treated and blank control groups, respectively. The results were presented as mean ± SD.

CELL MORPHOLOGICAL ASSESSMENT

Cells were seeded in 6-well tissue culture plates at a concentration of 1×10^5 cells/well and treated with wogonin (100, 150 and 200 μM) for 24 h. At the end of incubation, cells were fixed with ice-cold 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 25 min and washed with ice-cold PBS (2,000 rpm × 5 min). At last the cells were stained with fluorochrome dye DAPI (1 μg/ml) (4′,6-diamidino-2-phenylindole; Santa Cruz Biotechnology, Inc.) for 25 min and observed under a fluorescence microscope (Olympus IX51, Olympus Corporation, Tokyo, Japan) with a peak excitation wave length of 340 nm.

ANNEXIN-V/PI DOUBLE-STAINING ASSAY

Cells were incubated with wogonin (100, 150 and 200 μM) for 24 h, then harvested and resuspended with PBS. Apoptotic cells were identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated Annexin-V and PI, according to the manufacturer's instructions of the Annexin-V-FITC Apoptosis Detection kit (KeyGen, Nanjing, China). Apoptotic cell

death was examined by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA).

WESTERN-BLOT ANALYSIS FOR KEY APOPTOTIC PROTEINS

After cells were incubated with wogonin (100, 150 and 200 μM) for 24 h, the total proteins in cells were lysed and isolated by lysis buffer [100 mM Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethylsul-fonyl fluoride and 1 g/ml aprotinin]. The concentration of proteins was measured using the BCA assay method with Varioskan spectrofluorometer and spectrophotometer (Thermo, Waltham, MA) at 562 nm.

The Western-blot analysis for proteins was conducted according to our previous methods [Zhao et al., 2004]. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., Lincoln, NE).

MITOCHONDRIAL MEMBRANE POTENTIAL DETERMINATION

Quantitative changes of MMP at the early stage of the cell apoptosis were measured by the Mitochondrial Membrane Potential Detection kit (KeyGen). After being incubated with woginin (100, 150 and 200 μM) for 24 h, all floating and attached cells were harvested and resuspended with ice-cold PBS (2000 rpm × 5 min). Then the cell suspensions were incubated in JC-1 prepared with 1× Incubation Buffer for 20 min at 37°C, and detected by FACSCalibur flow cytometry (Becton Dickinson).

PLASMID TRANSIENT TRANSFECTION

The pcDNA3-Bcl-2 plasmid was obtained from Addgene (Addgene plasmid 8768). For transfection, HepG2 cells were seeded in 6-well plates at 65% confluency at first. Then pcDNA3-Bcl-2 (0.5 μg) was introduced into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After that, cells were exposed to wogonin or the vehicle and harvested for further experiments.

CYTOCHROME C AND AIF RELEASE ASSAY

After cells were incubated with wogonin (100, 150 and 200 μM) for 24 h, 1×10^7 cells were collected by centrifugation at 800*g* for 5 min at 4°C and washed with ice-cold PBS. The fractionation of the mitochondrial protein and cytosolic protein were extracted according to the instruction of Mitochondrial Protein Extraction kit (KeyGen), respectively. Cell nuclear and cytoplasmic fractions were prepared using a nuclear/cytosol fractionation kit of Biovision Inc. (Moutain View, CA) according to the manufacture's direction. Western blotting was used to detect cytochrome c (Cyt-c) and apoptosis inducing factor (AIF) of cytosolic fraction, mitochondrial fraction and nuclear fraction with Cyt-c antibody (Santa Cruz Biotechnology, Inc.) and AIF antibody (Cell Signaling, Beverlyis, MA).

MEASUREMENT OF H_2O_2 LEVEL

According to the method described previously [Lluis et al., 2007], the level of intracellular H_2O_2 was detected using fluorescent dye 2′,7′-dichlorfluorescein-diacetate (DCFH-DA, Beyotime Institute of BioTechnology, Haimen, China) sensitively. After wogonin-treatment (100, 150 and 200 μM) for 24 h, cells were collected and incubated with 100 μM DCFH-DA attenuated with serum-free medium for 30 min at 37°C in the dark. The fluorescence intensity was measured by FACSCalibur flow cytometry (Becton Dickinson) at Ex./Em. = 488/525 nm.

MEASUREMENT OF $^{\cdot}O_2^-$ LEVEL

Wogonin-treated (100, 150 and 200 μM for 24 h) cells were harvested and stained with 5 μM $^{\cdot}O_2^-$-sensitive dye dihydroethidium (DHE, Beyotime Institute of BioTechnology) for 60 min at 37°C in the dark. Subsequently, cells were washed three times with PBS (2000 rpm × 5 min), and the fluorescence intensity was assayed by FACSCalibur flow cytometry (Becton Dickinson) at Ex./Em. = 300/610 nm.

DETECTION OF INTRACELLULAR CALCIUM LEVEL

Wogonin-treated (100, 150 and 200 μM for 24 h) cells were loaded with 4 μM Fluo-3 AM (Beyotime Institute of BioTechnology) for 60 min at 37°C in the dark, then resuspended with PBS. The fluorescence intensity of loaded cells were measured by FACSCalibur flow cytometry (Becton Dickinson) at Ex./Em. = 488/525 nm.

STATISTICAL EVALUATION

Data are shown as mean ± SD from triplicate parallel experiments unless otherwise indicated. Statistical analyses are performed using an unpaired, two-tailed Student's *t*-test or one-way ANOVA. Significance of difference is indicated as $^{*}P < 0.05$ and $^{**}P < 0.01$.

RESULTS

WOGONIN CAUSES DIFFERENT GROWTH INHIBITION OF HEPG2 AND L02 CELLS

After cells were treated with wogonin for 24 and 48 h, respectively, the growth inhibitory rates of HepG2 cell were nearly twice as high as that of L02 cells. Even at high concentration (≥200 μM), the inhibitory rate of L02 cells was <20% (Fig. 1A).

WOGONIN INDUCES APOPTOSIS IN HEPG2 CELLS PREFERENTIALLY

After the cells were treated for 24 h by wogonin, the shape of HepG2 cells was distorted severely, but nearly no morphological change was observed in the L02 cells (data not shown). Under the fluorescent microscope, untreated HepG2 and L02 cells were stained equably with blue fluorescence, demonstrating the steady chromatinic distribution in nucleolus. Wogonin-treated HepG2 cells emitted bright fluorescence, the early phenomena of apoptosis, due to the chromatin agglutination and the nucleolus pyknosis. At the concentration of 150 and 200 μM, cellular nucleus of HepG2 cells disintegrated and formed many nuclear fragments. However, in L02 cells, the bright fluorescence could be observed just a little at the concentration of 200 μM (Fig. 1C).

To verify the apoptotic cell death induced by wogonin, the apoptosis ratios were detected by Annexin-V/PI double-staining assay. HepG2 cells treated with 100 μM wogonin presented much higher apoptosis ratios compared with the control group. At the concentration of 150 and 200 μM, the apoptosis ratios of HepG2 cells were 29 and 73%, respectively. L02 cells, which were treated with 100 and 150 μM wogonin, exhibited no significant changes compared with control group. Although the apoptosis ratio of L02

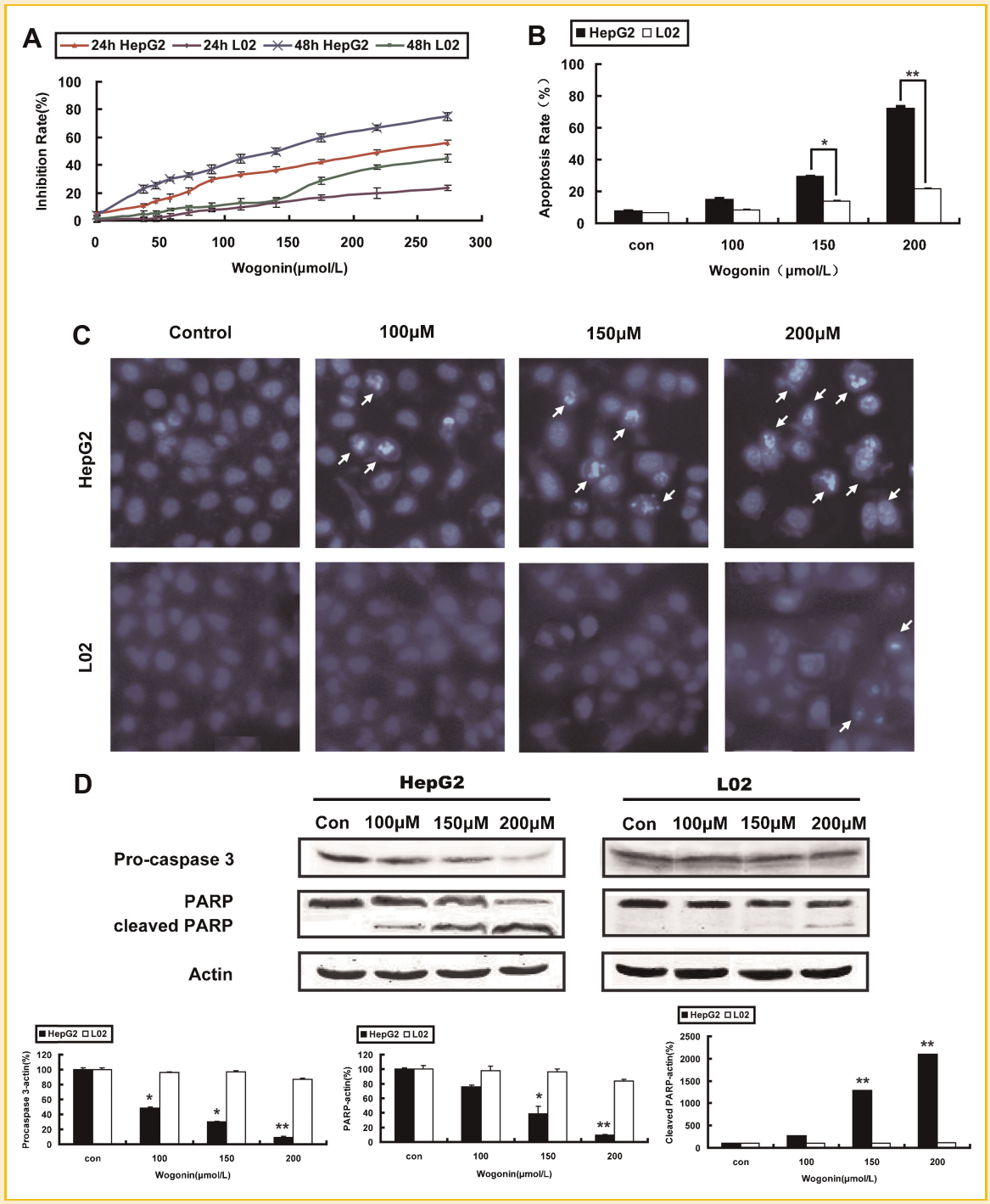

Fig. 1. Wogonin preferentially induces apoptosis in HepG2 cells but not in L02 cells. (A) Different growth inhibition of HepG2 and L02 cells induced by wogonin. Cells were treated with wogonin at the concentration ranged from 30 to 300 μM for 24 and 48 h, respectively. Cell viability was determined by MTT assay. Error bars represent SD(±). (B) Annexin-V/PI double-staining assay were measured after treatment with wogonin for 24 h. Histograms of apoptosis rates were quantitated, containing the early and late apoptosis. Error bars represent SD. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked. (C) Nucleolus morphologic changes by wogonin were observed under fluorescent microscope (400×). The white arrows indicate the apoptotic cells with nuclear fragments. (D) Western blot assays were used to examine expressions of (A) PARP, caspase 3, cleaved caspase 3. Error bars represent SD. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared control. [Colour figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

cells increased to 20% at the concentration of 200 μM, it was less than one-third of the apoptosis ratio in HepG2 cells (Fig. 1B).

These results revealed that wogonin preferentially induced apoptosis in HepG2 cells.

WOGONIN INDUCES APOPTOSIS THROUGH THE INTRINSIC DEATH PATHWAY

As shown in Figure 1D, the procaspase 3 cleaved to active form in HepG2 cells after treatment with 100 μM wogonin for 24 h. The

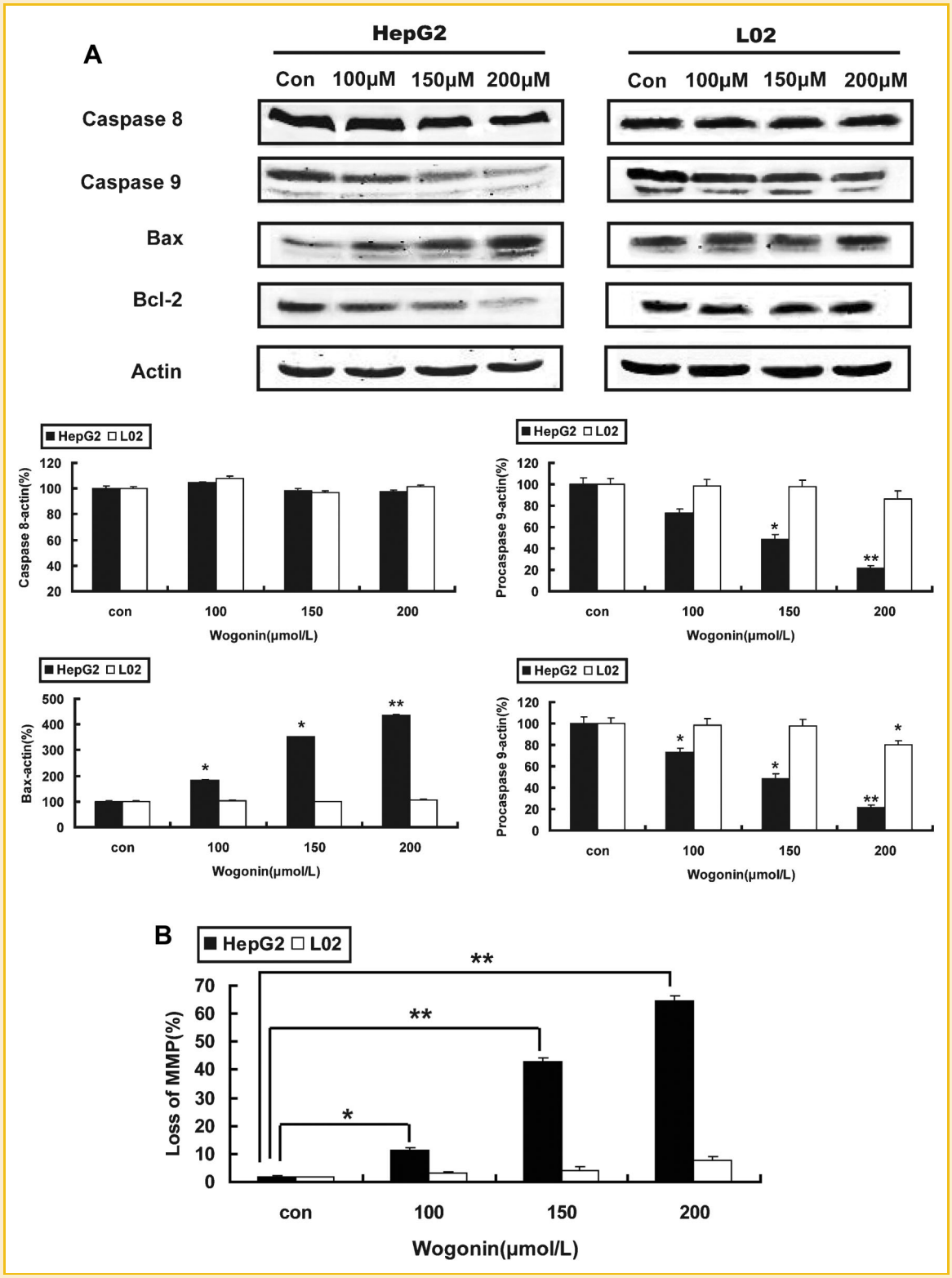

Fig. 2. Wogonin-induced apoptosis through the intrinsic death pathway. (A) Western blot assays were used to examine expressions of caspase 8, caspase 9, bax and bcl-2. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared control. (B) Wogonin decreases the MMP. Cells treated with wogonin for 24 h. The uptake of JC-1 analysed by flow cytometry. The per cent of the loss of MMP were represented by histograms. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked. Error bars represent SD.

expression of its downstream substrate PARP (poly-ADP-ribose polymerase) protein decreased, and the expression of cleaved PARP protein increased correspondingly in HepG2 cells in a concentration-dependent manner after wogonin treatment. However, in L02 cells the expression of the three proteins was not changed, except slight activation of cleaved PARP was observed at the concentration of 200 μM.

To investigate whether a mitochondria-mediated pathway or a receptor-mediated pathway were involved in wogonin-induced apoptosis in HepG2 and L02 cells, we measured the expression of caspase 8, caspase 9, bax and bcl-2 proteins (Fig. 2A). In HepG2 cells, caspase 8 was not activated after wogonin treatment (200 μM) for 24 h, while caspase 9 was activated after wogonin treatment (100 μM). These results indicated that wogonin-induced apoptosis was unlikely to be regulated through the receptor-mediated pathway. The expression of bax increased and the expression of bcl-2 decreased concentration dependently after wogonin treatment in HepG2 cells. Compared with the changes of key apoptosis proteins in HepG2 cells, caspase 9 was only activated in L02 cells after cells were treated with 200 μM wogonin for 24 h, and the expression of other proteins did not change after wogonin treatment.

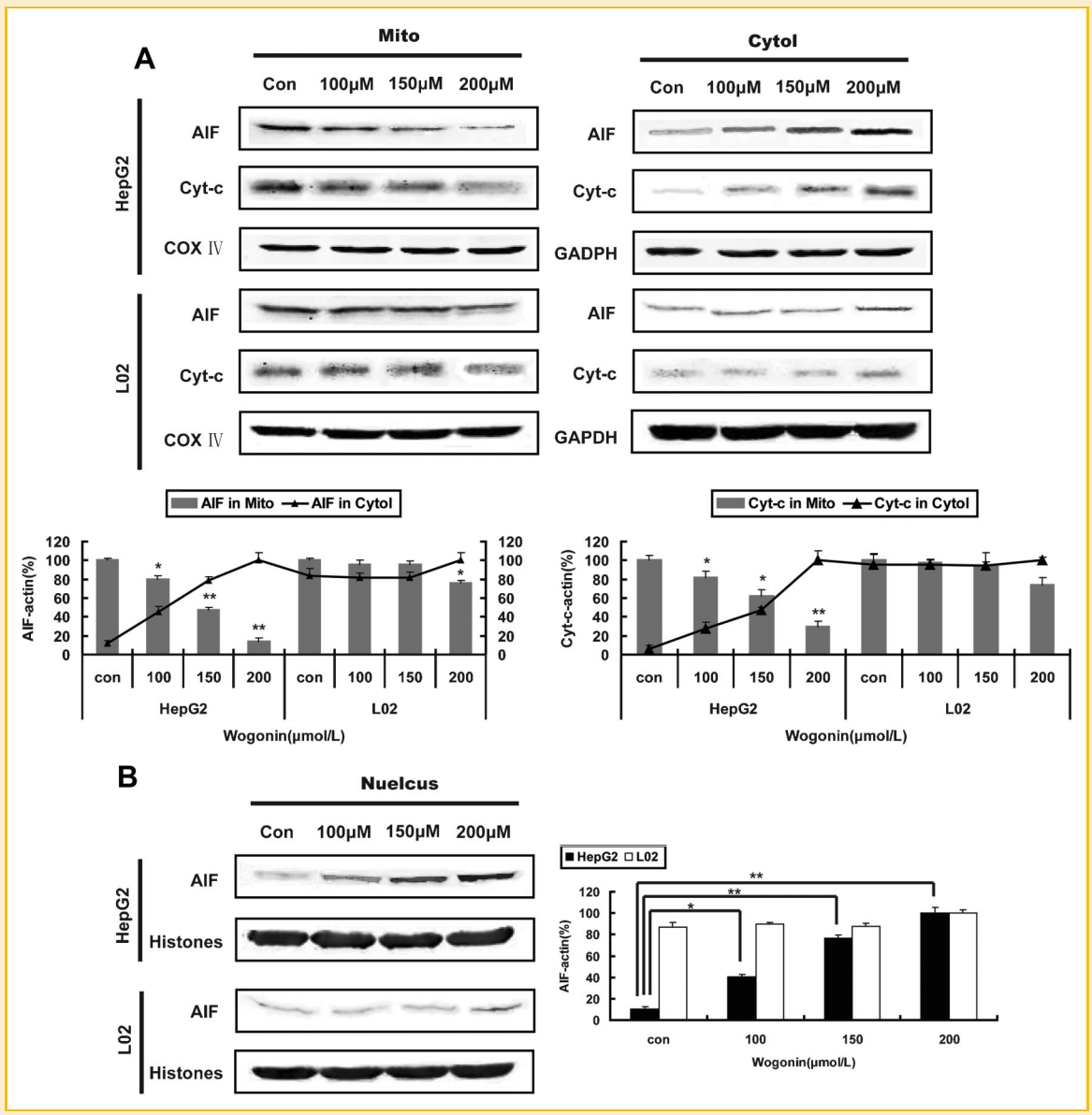

Fig. 3. Effects of wogonin on apoptosis-related proteins Cyt-c and AIF. (A) Wogonin influences the subcellular redistributions of Cyt-c and AIF in HepG2 and L02 cells. Mitochondrial and cytosolic fractions were isolated after 24 h wogonin-treatment, and subjected to Western blot analysis. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared control (represent AIF and Cyt-c at the same time). (B) Effect of wogonin on AIF translocation in HepG2 and L02 cells. Nuclei fractions were isolated as described after 24 h wogonin-treatment, and subjected to Western blot analysis. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked. Error bars represent SD.

The fate of cells succumbing to the intrinsic pathway is triggered by the loss of MMP [Galluzzi and Kroemer, 2007]. To verify the intrinsic pathway-mediated apoptosis induced by wogonin further, we measured the loss of MMP by JC-1 detection (Fig. 2B). After incubated with wogonin for 24 h, HepG2 cells exhibited increasing per cent of fluoresced green concentration dependently, standing for a continuous dissipation of MMP. However, no significant changes of MMP were observed in L02 cells after wogonin treatment.

The loss of MMP leads to opening of the permeability transition pore (PTP) and the release of proapoptotic proteins such as Cyt-c and AIF from the intermembrane space to the cytosol [Loeffler and Kroemer, 2000]. Cyt-c release is required for caspase activation that initiates the apoptotic program [Liu et al., 1996]. After HepG2 cells were treated with wogonin for 24 h, the amount of Cyt-c and AIF significantly decreased in mitochondria while increased in cytosol in a concentration-dependent manner. In response to the wogonin treatment of 100 and 150 μM in L02 cells, the amount of Cyt-c and AIF in mitochondria or cytosol changed neither. And upon 200 μM wogonin treatment, the increase of Cyt-c and AIF in cytosol and the decrease of them in mitochondria were observed in L02 cells (Fig. 3A). AIF can translocate from cytosol to nucleus, where it activates cyclophilin A and participate in chromatinolysis. Thus, we detected whether wogonin could induce relocation of AIF to be a nuclear hallmark. As shown in Figure 3B, a portion of AIF was found in the nucleus in wogonin-treated HepG2 cells while little AIF was detected in untreated HepG2 cells, untreated L02 and treated L02 cells.

These data strongly suggested that the apoptosis caused by wogonin was mainly mediated through the mitochondrial pathway and preferentially induced in HepG2 cells.

OVEREXPRESSION OF BCL-2 INHIBITS WOGONIN-INDUCED APOPTOSIS IN HEPG2 CELLS

Since the apoptotic effect of wogonin was never observed in Bcl-2-overexpressing cells, and Bcl-2 protein family participates in the regulation of intrinsic pathway [Autret and Martin, 2010]. To determine whether the events are required for wogonin-induced apoptosis, HepG2 cells were transiently transfected with a plasmid encoding Bcl-2, and apoptotic cells were detected by Annexin-V/PI double staining. As shown in Figure 4A, wogonin (150 μM) treatment-induced apoptosis in vector control cells, whereas Bcl-2-overexpressing cells exhibited a much lower apoptosis rate, with 50% inhibition under the same conditions. And overexpression of Bcl-2 also blocked the wogonin-caused downregulation on the expression of Bcl-2 protein in HepG2 cells (Fig. 4B). This result indicated that wogonin-induced apoptosis was dependent on the mitochondrial death pathway.

WOGONIN CHANGES THE REDOX BALANCE OF ROS IN HEPG2 CELLS BUT NOT IN L02 CELLS

Accumulation of ROS can lead to mitochondrial dysfunction and initiate apoptosis [Yu et al., 2007]. ROS are divided into two subgroups; nonradical such as hydrogen peroxide (H_2O_2) and free radicals such as superoxide radicals ($^{\cdot}O_2^-$).

In HepG2 cells, the intracellular H_2O_2 level exhibited a significant increase after cells were treated with 150 and 200 μM wogonin for 24 h. However, the generation of H_2O_2 did not change in L02 cells (Fig. 5Aa).

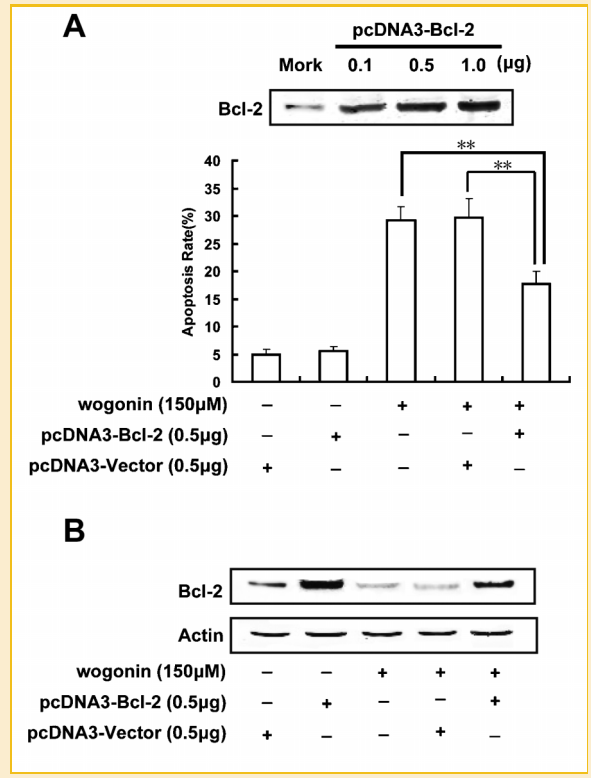

Fig. 4. Overexpression of Bcl-2 inhibits wogonin-induced apoptosis in HepG2 cells. HepG2 cells transfected with vector only, or with Bcl-2 plasmid were treated with 150 μM wogonin for 24 h. (A) Cells were stained with Annexin-V and PI, and apoptotic cells were quantitated by flow cytometer. (B) Western blot assays were used to examine the effects of wogonin in the expressions of bcl-2 after overexpression of Bcl-2. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked. Error bars represent SD.

Upon treatment of 150 μM wogonin for 24 h, the level of $^{\cdot}O_2^-$ decreased notably in HepG2 cells. But in L02 cells no changes of $^{\cdot}O_2^-$ level were observed after wogonin treatment (Fig. 5Ab).

The results revealed that wogonin did not act as a ROS generator, but a ROS scavenger, deoxidizing $^{\cdot}O_2^-$ to H_2O_2 by the antiradical enzyme superoxide oxido-reductase dismutase (SOD). Thus, wogonin shifted the redox balance of ROS to a more reductive state and heightened the total ROS level further in HepG2 cells, but no effects were shown in L02 cells (Fig. 5C).

DIFFERENT APOPTOTIC EFFECTS OF WOGONIN ARE NOT CAUSED BY THE INCREASED GENERATION OF ROS DIRECTLY

H_2O_2, is considered as a key component to ROS damages because it can accumulate in the cell at relatively high concentrations, occupy a stable state, and may convert to radical ROS ($^{\cdot}OH$) [Sasaki et al., 1998]. Therefore, increased H_2O_2 by wogonin might lead to cell apoptosis.

To investigate whether the increased generation of H_2O_2 was involved in wogonin-induced apoptosis, we measured the apoptosis rates in the presence or absence of antioxidant NAC. Interestingly, though NAC essentially abrogated the wogonin-mediated generations of H_2O_2 and $^{\cdot}O_2^-$ (Fig. 5B), NAC did not inhibit cell apoptosis induced by wogonin (Fig. 6A). But when cells were treated with 5 μM

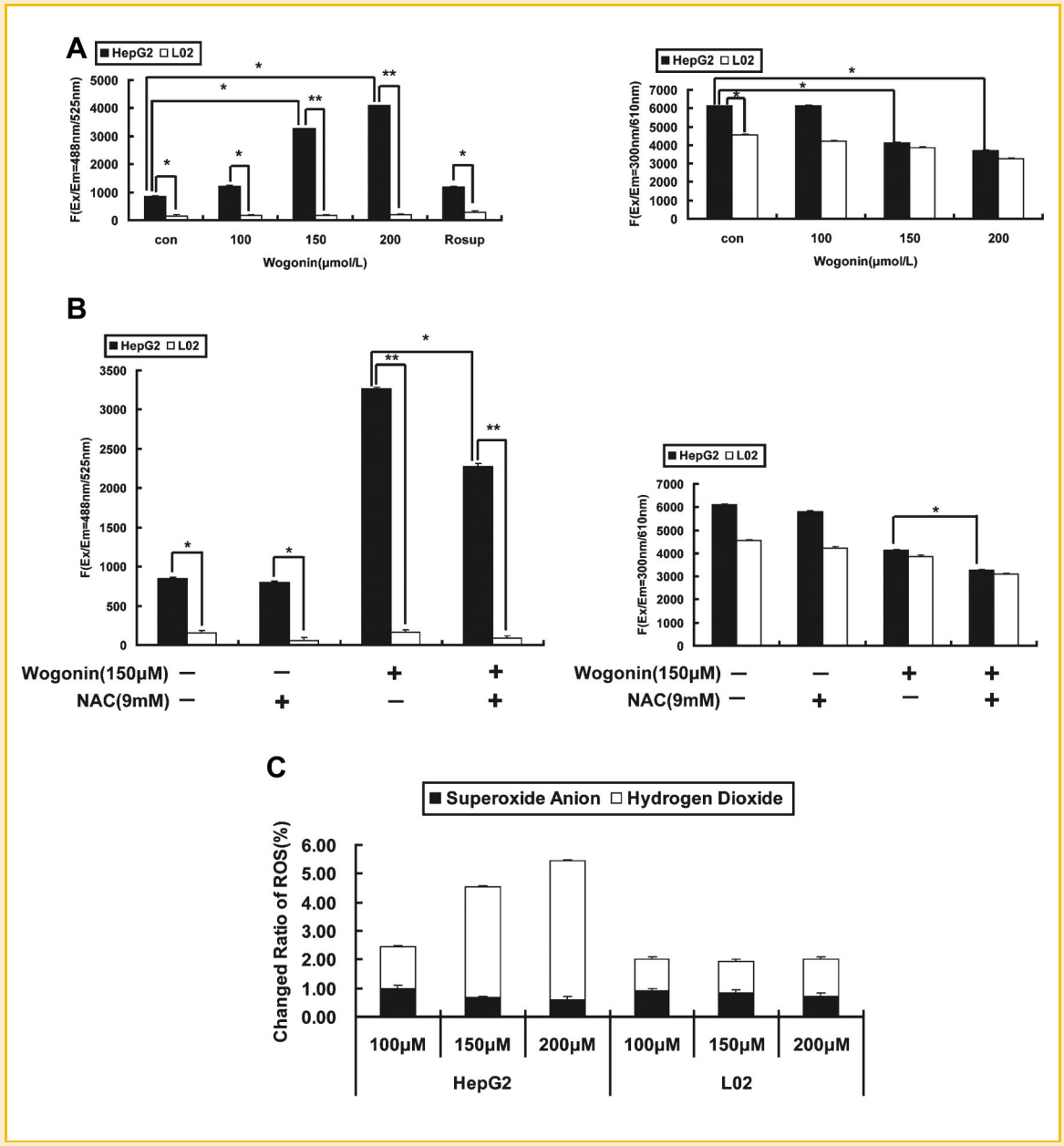

Fig. 5. Wogonin influences the intracellular ROS generation and ROS redox balance. (A) The effects of wogonin on the generation of intracellular H_2O_2 and $^{\cdot}O_2^-$ after treatment with wogonin for 24 h. (a) The level of H_2O_2 was detected by flow cytometry (Ex./Em. = 488/525 nm). Rosup (50 mg/ml) is a positive control of H_2O_2 generation. (b) The level of $^{\cdot}O_2^-$ was detected by flow cytometry (Ex./Em. = 300/610 nm). (B) The changes of intracellular ROS caused by wogonin in the presence of NAC. Pre-treated with 9 mM NAC for 1 h, cells were treated with 150 μM wogonin for 24 h. (a, b) The measurement of H_2O_2 and $^{\cdot}O_2^-$ were the same as that of Figure 5A. (C) Wogonin changed the ROS redox balance in HepG2 cells but not in L02 cells. The levels H_2O_2 and $^{\cdot}O_2^-$ in wogonin-treatment groups were compared with the control group of each cells, respectively. Then the changed ratios were added up. Error bars represent SD. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked.

rotenone, a compound inducing apoptosis via generation of ROS directly [Radad et al., 2006], NAC blocked the apoptosis induction as well as the generation of ROS (Fig. 6A). Interestingly, the apoptotic rates increased in HepG2 cells, but decreased in L02 cells when wogonin combined with rotenone for the treatment. The effects of these two drugs combination were also blocked by NAC (Fig. 6A).

The results demonstrated that wogonin-induced apoptosis did not involve the increased generation of H_2O_2 directly.

DIFFERENT APOPTOTIC EFFECTS OF WOGONIN ARE INITIATED BY THE OVERLOAD OF CYTOSOLIC CA^{2+}

The level of cytosolic Ca^{2+} increased significantly after treatment with 150 μM wogonin in HepG2 cells, but just slightly in L02 cells (200 μM wogonin) (Fig. 6B). In the presence of 2-APB (50 μM), a specific blocker of inositol-1,4,5-triphosphate receptors (IP_3R) and inhibited calcium release form the endoplasmic reticulum (ER) [Ma et al., 2000], the level of cytosolic Ca^{2+} decreased in response to 150 μM wogonin

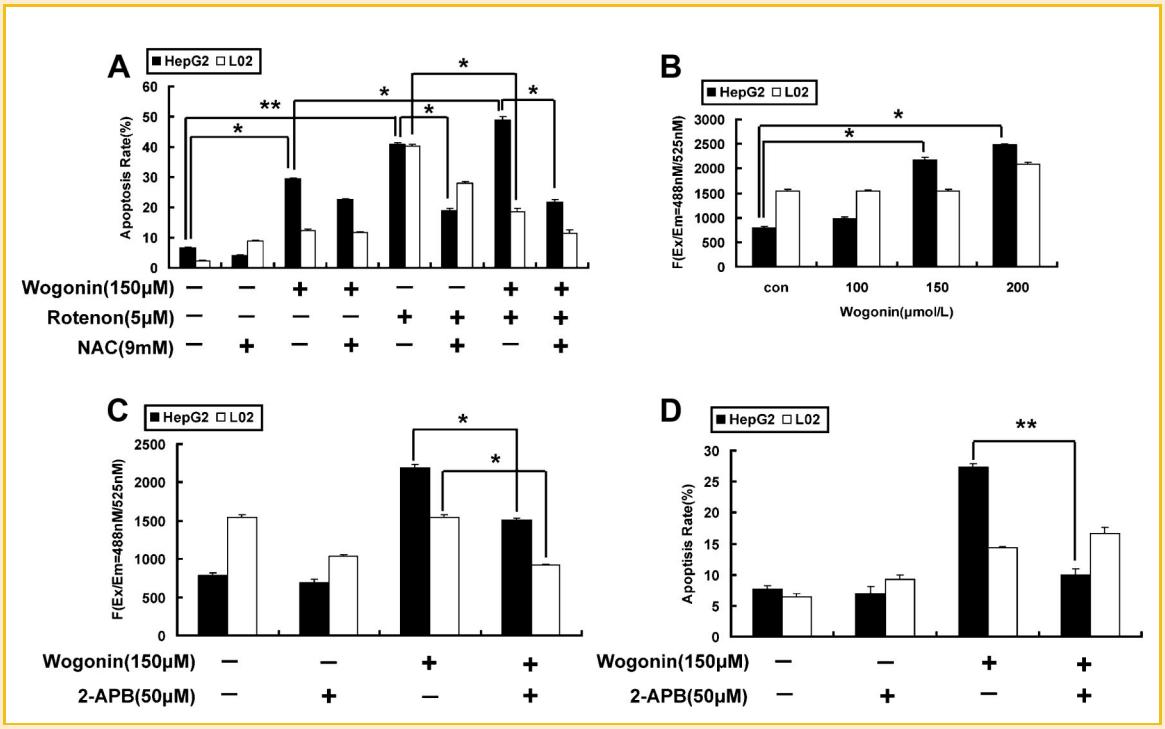

Fig. 6. Wogonin-induced apoptosis is not involved with the generation of ROS directly, but the Ca^{2+} overload. (A) Wogonin-induced apoptosis in HepG2 and L02 cells could not be reversed by NAC. Pre-treated with 9 mM NAC for 1 h, cells were treated with 150 μM wogonin or 5 μM rotenone for 24 h. Then cells were incubated with Annexin-V and PI and subjected to flow cytometry analysis. (B) Wogonin changed the intracellular cytoplasm Ca^{2+} concentration. Cells were treated with wogonin for 24 h and the level of cytoplasm Ca^{2+} was detected by flow cytometry (Ex./Em. = 488/525 nm). (C) Antagonism of 2-ABP on Ca^{2+} release caused by wogonin in HepG2 and L02 cells. Cells were pre-treated with 2-ABP (50 μM) without extracellular Ca^{2+} (in PBS) for 15 or 30 min, respectively, then treated with wogonin (150 μM) for 24 h and detected by flow cytometry (Ex./Em. = 488/525 nm). (D) Wogonin-induced apoptosis was partially resisted by 2-ABP only in HepG2 cells. Cells were treated as the manner of Figure 6C. Then the apoptosis rates were measured using Annexin-V and PI analysis by flow cytometry. Error bars represent SD. Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked.

treatment both in HepG2 and L02 cells (Fig. 6C). The results revealed that wogonin influenced the release of Ca^{2+} from ER by targeting IP_3R.

To study whether the breakdown of intracellular Ca^{2+} homeostasis played as a key in the apoptosis induced by wogonin, apoptosis rate was measured in the presence of 2-APB. However, reduced apoptosis was only observed in HepG2 cells treated with wogonin in the presence of 2-APB (Fig. 6D).

These results showed that wogonin-induced apoptosis was involved with a Ca^{2+}-mediated mechanism.

WOGONIN-INDUCED ACTIVATION OF PLCγ1 WAS ESSENTIAL FOR THE RELEASE OF CA^{2+}

Phosphoinositide-specific phospholipase gamma 1(PLCγ1) is known as an important enzyme in Ca^{2+} signalling pathways. The activation of PLCγ1 was investigated after wogonin treatment in HepG2 and L02 cells. Among the three tyrosine residues of PLCγ1 (Tyr-771, -783 and -1254), Tyr-783 is an essential tyrosine residue for IP_3 formation [Kim et al., 1991]. Therefore, a phosphospecific Tyr-783 antibody was used to detect the phosphorylation of PLCγ1 induced by wogonin.

As shown in Figure 7A, treatment of the wogonin for 24 h induced PLCγ1 phosphorylation on Tyr-783 in a concentration-dependent manner in HepG2 cells. Furthermore, the phosphorylation of PLCγ1 induced by wogonin in HepG2 cells was inhibited by 9 mM NAC. In the experiment, 1 mM H_2O_2, which could activate the PLCγ1 phosphorylation on Tyr-783 significantly [Wang et al., 2001], was used as a positive control. In L02 cells, wogonin treatment led to a very low generation of H_2O_2 (Fig. 4); therefore, no or very little PLCγ1 activation was observed (Fig. 7C).

These data suggested that wogonin-induced activation of PLCγ1 by inducing the generation of H_2O_2 in HepG2 cells, leading to the release of Ca^{2+} from IP_3-dependent Ca^{2+} channels and induction of apoptosis.

DISCUSSION

Currently, increasing efficacy and decreasing side effects have become a capital and urgent task for the development of anticancer drugs. *Scutellaria baicalensis*, which is a popular and multi-purpose herb used in China traditionally, has been convinced of possessing anticancer activities [Lu et al., 2008; Zhang et al., 2008a, 2008b]. It has been reported that wogonin and its homologous compounds inhibited the viabilities of human hepatoma cells accompanying the loss of MMP and the exhaustion of glutathione content [Chang et al., 2002; Liu et al., 2009]. In this paper, the data prove that wogonin

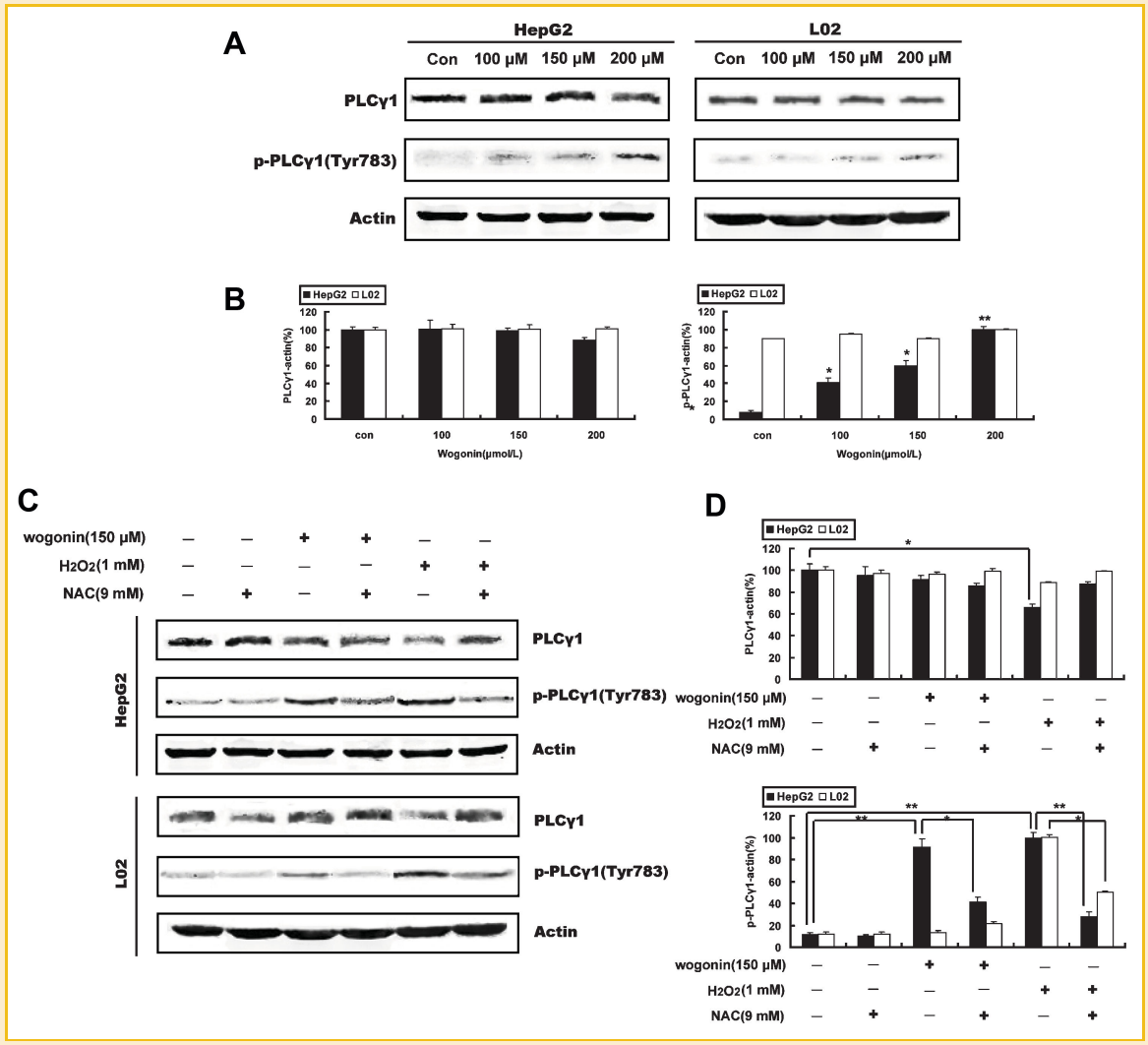

Fig. 7. Activation of PLCγ1 plays an essential role in wogonin-induced Ca^{2+} release. (A, B) After cells were treated with wogonin for 24 h, Western blot assays were used to examine expressions of PLCγ1 and its phosphotyrosine (Tyr-783). Quantitation of results in (B). Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared control. (C, D) Pre-treated with 9 mM NAC for 1 h, cells were treated with 150 μM wogonin or 1 mM H_2O_2 for 24 h, Western blot assays were used to examine expressions of PLCγ1 and phosphotyrosine (Tyr-783). Quantitation of results in (D). Asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$) indicate significant difference compared between the two items linked. Error bars represent SD.

preferentially induces apoptosis through the intrinsic pathway in human hepatoma HepG2 cells by the measurement of the typical indexes of apoptosis and the influence of over expression of Bcl-2. However, wogonin does not affect the growth of embryon hepatic L02 cells at lower concentrations except induces slight apoptosis at high concentration (200 μM). It seems that wogonin preferentially kills tumour cells.

It has been convinced that ROS play a key role in the intrinsic pathway-mediated apoptosis [Archer et al., 2008]. The damage of ROS to cells is because of the high oxidative capacity of oxidizing radicals. ROS react with DNA and lipid, leading to DNA (nuclear and mitochondrial) single strand breaking and the loss of MMP [Trachootham et al., 2009]. The results show that wogonin shifts the redox balance of ROS exclusively in HepG2 cells, trending to deoxidize $^{\cdot}O_2^-$ to H_2O_2 by SOD. To our surprise, wogonin-induced apoptosis is not caused by direct effects of increased H_2O_2 because the apoptosis is not inhibited by neutralizing H_2O_2 with NAC.

Then what is the bridge between the increased H_2O_2 and the wogonin-induced apoptosis? ROS detoxification pathway can minimize oxidative damage, but excessive H_2O_2 production can cause changes in Ca^{2+} homeostasis and further alterations of ROS production [Hool and Corry, 2007; Feissner et al., 2009]. For example, H_2O_2 treatment resulted in Ca^{2+} release from ER in Hela cells [Bootman et al., 1992]. Mitochondrial Ca^{2+} is an essential effector of ATP synthesis, yet Ca^{2+} overload can also lead to mitochondrial dysfunction and cell death. Through the detection of Ca^{2+}, the results reveal that wogonin induces cell apoptosis by the overload of cytosolic Ca^{2+}.

2-ABP has been extensively used as a membrane permeable modulator of IP_3-sensitive Ca^{2+} channels and plays a critical role

in Ca^{2+} fluxion in non-excitable cell. The results suggest that 2-ABP can inhibit the Ca^{2+} release in the presence of wogonin both in HepG2 cells and L02 cells. IP_3R channels are the primary Ca^{2+} release channels in the ER in non-excitable cells, which is initiated by binding of the signalling molecule IP_3 [Berridge, 1993]. Redox modification is thought to directly stimulate IP_3R-mediated Ca^{2+} release from the ER [Wesson and Elliott, 1995]. So the results reveal that wogonin targets IP_3R channels via increasing H_2O_2 and induces apoptosis.

In the further studies, we found PLCγ1 was activated by wogonin and responsible for the release of Ca^{2+} via IP_3-dependent Ca^{2+} channels from ER. It has been shown that H_2O_2 could activate PLCγ1 in some cell types [Hong et al., 2006]. The activated PLCγ1 produces diacylglycerol (DAG) and IP_3 from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the activation of protein kinase C and increases Ca^{2+} release [Berridge et al., 2003].

Therefore, we infer that the increased H_2O_2 acts as a signalling molecular to phosphorylate PLCγ1, which initiates the opening of IP_3R Ca^{2+} channels and accommodates Ca^{2+} transport. The over-uptake of Ca^{2+} to mitochondria stimulates the opening of PTP, dissipates MMP and initiates the apoptotic program finally (Fig. 8). Based on this apoptosis inducing pathway, what is the key factor underlying the high antitumour effects and less side effect of wogonin?

It has been well documented that cancer cells are under higher oxidative stress level than normal cells [Fruehauf and Meyskens, 2007; Fang et al., 2009]. The direct proof of oxidative stress in the cancer cells is shown through the analysis of significantly elevated levels of ROS such as $\cdot O_2^-$ [Gupte and Mumper, 2009]. Therefore, the effects of wogonin are like 'adding fuel to the flame'. By the exclusive accommodation of wogonin on ROS redox balance in HepG2 cells, the increased H_2O_2 becomes a spanking apoptosis inducing signal. Finally, wogonin exclusively induces apoptosis in HepG2 cells.

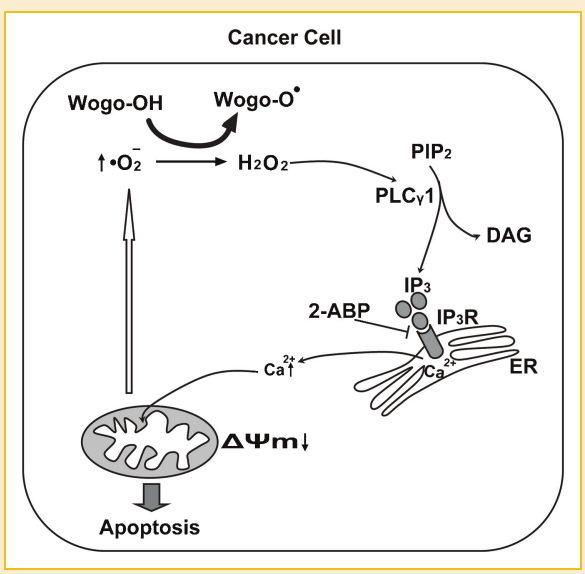

Fig. 8. Hypothetical diagram of mechanism for wogonin-induced apoptosis in malignant hepatoma cells. In the condition that malignant hepatoma cells went through an elevated ROS stress, wogonin deoxidized $\cdot O_2^-$ to H_2O_2. H_2O_2 acted as a signalling molecule to phosphorylate PLCγ1, which catalysed PIP_2 to generate DAG and IP_3. Then IP_3 binded with IP_3R in the ER, inducing Ca^{2+} release from endocytoplasmic reticulum stores. 2-ABP could inhibit IP_3R selectively. Cytosolic Ca^{2+} overload leaded to collapse of the mitochondrial membrane.

Mitochondria, ROS and Ca^{2+}, which form a positive feedback loop and influence each other, are the core in this study. Mitochondria, as a central control in energy metabolism and apoptosis, can be disrupted by ROS and Ca^{2+} [Valko et al., 2006]. Conversely, mitochondrion is a significant source of cellular ROS and crucial to proper cellular Ca^{2+} signalling [O'Rourke, 2007]. Furthermore, mitochondrial Ca^{2+} uptake can lead to free radical production. Cellular redox state can still significantly modulate Ca^{2+} signalling [Csordas and Hajnoczky, 2009]. Though continuously debating around their cross-relationships is advancing, there are clearly more questions than answers in this field.

Sarco/ER Ca^{2+}-ATPase (SERCA) is another important Ca^{2+} pumps, which is a Ca^{2+} ATPase which transfers Ca^{2+} from the cytosol of the cell to ER at the expense of ATP hydrolysis. It has been reported that H_2O_2 can inhibit Ca^{2+} re-uptake by SERCA [Redondo et al., 2004; Voss et al., 2008; Strosova et al., 2009]. Withstanding the experiment limitations, our data do not imply SERCA. Further study should be done latter, but this paper clearly indicated the selectively apoptotic effects of wogonin to the malignant hepatoma cells, and provided reasonable mechanism for the differences.

Collectively, the different apoptotic effects are due to three points: (i) wogonin induces apoptosis by a specific pathway, involving deoxidizing $\cdot O_2^-$ to H_2O_2, activating PLCγ1, promoting Ca^{2+} release from ER and dissipating MMP, (ii) wogonin generates many apoptosis inducing signals H_2O_2 in hepatoma cells based on the fact that cancer cells are under higher ROS stress level than normal cells and (iii) wogonin makes cytosolic Ca^{2+} overload via IP_3-sensitive Ca^{2+} channels, which is activated by the over increased H_2O_2 and phosphorylated PLCγ1.

The prognosis of HCC is poor, as systemic chemotherapy is of low efficacy, even with the combined treatment of curative resection and adjuvant chemoradiotherapy [Simonetti et al., 1997]. The most widely used treatment for HCC is adriamycin (ADM), either alone or in combination with other drugs. The response rate of ADM in a phase III clinical trials was only 10.5% when used alone and 20.9% for PIAF (cisplatin/interferon α-2b/ADM/fluorouracil) combination therapy [Yeo et al., 2005], but chemotherapy usually causes cytotoxicity with poor selection. Thus, the Chinese herbal compound wogonin may be extremely competitive as anticancer drugs against malignant hepatoma due to its high anticancer effects and low toxicity to normal tissues. Moreover, a synergistic treatment combining wogonin and ADM may provide a new approach to improve the efficacy of chemotherapy for HCC.

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