Daphnoretin-induced apoptosis in HeLa cells: a possible mitochondria-dependent pathway

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Abstract Daphnoretin is a bicoumarin compound isolated from a natural product, *Wikstroemia indica*, which has been used to treat many diseases. It has strong antiviral and anti-tumor activities. Taking the anti-tumor activity of daphnoretin as a starting point, the present study aimed to test the pro-apoptotic effect of daphnoretin and its underlying mechanism in HeLa cells. The inhibitory effects of daphnoretin on viability and proliferation of HeLa cells were determined by the MTT assay. Daphnoretin-induced apoptotic morphological changes were analyzed by mitochondrial membrane potential and Hoechst staining. The number and stage of apoptotic HeLa cells were determined by flow cytometry. Gene expression was determined by reverse-transcription polymerase chain reaction.

Protein expression was determined by western blot. The caspase activity of HeLa cells was detected by a caspase-3 and caspase-9 colorimetric assay kit. We found that daphnoretin significantly inhibited HeLa cells' viability by the MTT assay and flow cytometry. The nuclei of the apoptotic cells exhibited strong, blue fluorescence in Hoechst staining. *Bax* mRNA and protein levels were increased while *bcl-2* mRNA levels were decreased after daphnoretin treatment. Daphnoretin also activated both caspase-3 and caspase-9. These findings suggest that daphnoretin promotes apoptosis of HeLa cells in a mitochondria-mediated way. Daphnoretin therefore has potential to be a promising drug to treat uterine cervix cancer.

Keywords Daphnoretin · Anti-tumor · Apoptosis · Mitochondria · HeLa cells

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Introduction

Daphnoretin (7-hydroxy-6-methoxy-3, 7'-dicoumaryl ether) is a bicoumarin isolated from *Wikstroemia indica* C.A., a root traditionally used in the treatment of arthritis, tuberculosis, syphilis, and pertussis (Ko et al. 1993). Since it was first isolated in 1979 (Kato and Hashimoto 1979), more and more papers have been published on how to more effectively extract and isolate the compound. Meanwhile, many pharmacological effects of daphnoretin have been demonstrated, including anti-tumor (Hall et al. 1982; Zhang et al.

2007; Yang et al. 2008; Diogo et al. 2009), antiviral (Hu et al. 2000; Ho et al. 2010), antioxidant (Deiana et al. 2003), anti-complement (Park et al. 2006), activation of PKC (Wang et al. 1995; Ko et al. 1993; Chen et al. 1996), and induction of respiratory burst effects (Wang et al. 1995). Some mechanisms of its anti-tumor activity have been investigated (Yang et al. 2008; Diogo et al. 2009). Here, we focus on the mechanism of daphnoretin-induced apoptosis, especially via the mitochondrial pathway.

Apoptosis, also called programmed cell death, is a natural style of death in organisms (Benedict et al. 2002; Fadeel and Orrenius 2005). It involves a succession of biochemical events that eventually lead to characteristic morphological changes of the cells, including blebbing, cell shrinkage, chromatin condensation, and nuclear fragmentation. Mechanisms of apoptosis are sorted into the extrinsic pathway, also called the death receptor-mediated pathway, and the intrinsic pathway, also called the mitochondria-dependent pathway. In the latter pathway, the proteins Bax and Bcl-2, which both belong to the Bcl-2 family, play adverse roles in apoptosis, with their ratio being an important determinant of the susceptibility to apoptosis (Cory and Adams 2002). In addition, caspase-9, a member of the cysteine aspartic acid-specific protease family, is uniquely involved in the mitochondria-dependent pathway (Taylor et al. 2008). Based on our previous work (Yang et al. 2008) and the results of our investigation presented here, we propose a possible mitochondria-regulated pathway in daphnoretin-induced apoptosis.

Methods and materials

Chemicals and drug treatment

Dried roots of *W. indica* C.A. (600.4 g) were twice extracted via reflux 75 % EtOH (750 ml $\times$ 3 h) and the solvent was then removed under reduced pressure under vacuum. The residue (25.97 g) was dissolved in water and extracted with EtOAc (150 ml $\times$ 3, 23.67 g). Then part of the extract (10.1 g) was subjected to column chromatography on a silica gel and eluted with CHCl₃/MeOH (49:1). After recrystallization, the extraction amount of daphnoretin was 260 mg.

A stock solution of daphnoretin was made at 50 μ g/ml in minimum essential medium (MEM), and our positive

control carboplatin (1120092ER, Qilu Pharmaceutical CO., LTD, Shandong, China) was dispensed at 5 mg/ml. Both drugs were diluted with Eagle's MEM (Gibco) for all cell experiments.

3D cell culture

The human cervix adenocarcinoma cell line HeLa (TCHu187) was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, and cultured in fresh complete MEM containing 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco), and 10 % newborn calf serum (Gibco) at 37 °C in 5 % CO₂.

To develop disposable microcarrier cell culture, a BioLevigator 3D Cell Culture System (Hamilton, Shanghai, China) was pre-warmed and the CO₂ adjusted to the appropriate levels (Justice et al. 2009). 0.5 ml of GEM-3030 (Hamilton) was added to a tube containing 8.5 ml of complete culture medium (MEM) further added during inoculation for adjusting to a cell concentration of 10⁶ c/ml. The total volume in the tube is 10 ml. The vial of washed GEM was inverted as needed to re-suspend the GEM substrate. 0.5 ml of re-suspended GEM slurry was quickly drawn up in a pipette and dispensed into the BioLevigator tube. The HeLa cell suspension, containing approximately 2 million cells, was added to the tube, making the total volume in the tube approximately 10 ml. This was capped and placed in the BioLevigator for 24 h.

Cell viability inhibition assay

The inhibitory effect of daphnoretin on the proliferation of HeLa cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, OH, USA) assay, with carboplatin as a positive control. The cells were seeded in 96-well plates with 5 $\times$ 10⁴ cells/ml, and cultured for 24 h. Then, after cultured for 24 h the medium without serum was used and cells were exposed to different concentrations of daphnoretin and 500 μ g/ml carboplatin. After incubation for 12 or 24 h, MTT (5 mg/ml) was added for further incubation for 4 h. Then the medium was removed, and Dimethyl sulfoxide (DMSO) was added to dissolve the reduced formazan product. The absorbance was read at 570 nm with a reference filter of 660 nm.

Analysis of mitochondrial membrane potential

The changes in the mitochondrial membrane potential ($\Delta\psi$ M) were determined using a fluorescent dye 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl-carbocyanineiodide, JC-1 (Beyotime, Jiangsu, China), which accumulates in the mitochondrial membrane as a monomer (fluoresced green) or dimer (fluoresced red–orange) depending on the mitochondrial membrane potential in living cells (Wu et al. 2012; Shi et al. 2011). Briefly, cells were plated at a seeding density of 4×10^5 cells/well in a 6-well plate. After 12 or 24 h of treatment with different concentrations of daphnoretin (0.1, 1, 10, 50 μ g/ml) and 500 μ g/ml carboplatin, cells were incubated with 5 μ M JC-1 for 20 min at 37 °C, then washed, and placed on a thermostat at 37 °C in the dark. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 μ M, Beyotime) was used as a positive control (fluoresced green) which evokes total mitochondrial membrane depolarization. Fluorescent images were visualized by a Zeiss inverted fluorescence microscope.

Apoptotic morphology analysis

Daphnoretin-induced apoptotic morphological changes were analyzed by Hoechst staining (Hoechst 33258 kit; Beyotime). After incubation with fixative solution for 10 min and staining solution for 5 min, fluorescence of stained cells was captured under a fluorescent microscope using a charge-coupled device camera.

Flow cytometry

The number and stage of apoptotic HeLa cells were determined by flow cytometry with an Annexin V-FITC apoptosis detection kit (Beyotime). After collection by centrifugation, the apoptotic cells were resuspended in Annexin V-FITC binding buffer. Annexin V-FITC and PI were successively added to incubate with cells. The quantity of stained cells was determined by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA, USA).

Quantitative real-time RT-PCR analysis

Gene expression was determined by Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) (Wong and Medrano 2005). Trizol

Fig. 1 Daphnoretin reduces viability of HeLa cells. After treatment with daphnoretin (0.1, 1, 10, 50 μ g/ml) or carboplatin (500 μ g/ml) for 12 or 24 h, the MTT assay was performed. The absorption values positively correlated with cell viability. (a) In the 12-h group. The viability of HeLa cells was significantly inhibited following administration of daphnoretin at 10 and 50 μ g/ml (* P < 0.05 vs. control group) (n = 6). (b) In the 24-h group, the viability of HeLa cells was also inhibited following administration of daphnoretin at 1 μ g/ml (* P < 0.05 vs. control group) and 10 and 50 μ g/ml (# P < 0.01 vs. control group) (n = 6). Carb represents carboplatin (500 μ g/ml)

Reagent (Takara, Dalian, China) was used for isolating total RNA. Cells were directly lysed in a 3.5-cm diameter dish by adding 1 ml of Trizol reagent. Then 0.2 ml chloroform was added, and the homogenized sample was incubated for 15 min at room temperature. Subsequently, RNA was precipitated by mixing with isopropyl alcohol. Total RNA yield was quantified by UV spectrophotometry measured at 260 nm. Then mRNA was isolated from total RNA by using Oligo (dT) cellulose. The extracted mRNA of each sample was reverse transcribed into first-strand complement DNA (cDNA) and amplified using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China).

Fig. 2 Daphnoretin induced inhibition of mitochondrial membrane potential in HeLa cells. After treatment with daphnoretin (1 μ g/ml, 10 μ g/ml, 50 μ g/ml) or carboplatin (500 μ g/ml) for 12 h, apoptotic HeLa cells stained with JC-1 fluorescence exhibited *green* fluorescence, especially in the carboplatin and 50 μ g/ml daphnoretin groups (a) CCCP was used as a positive control. The same results were also seen at 24 h (b)

A total volume of 25 μ L reaction mixture containing 2 μ L cDNA, 12.5 μ L $2 \times$ SYBR Green 1 Master Mix (Takara, Dalian, China), and 1 μ L of each primer. The cycling conditions were as follows: pre-incubation at 95 $^{\circ}$ C for 30 s, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 5 s, annealing at 60 $^{\circ}$ C for 30 s and extension at 72 $^{\circ}$ C for 30 s using iQ5 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA). The relative quantitative value was expressed by the $\Delta\Delta C_t$ method. GAPDH was used as an internal control to compare the amount of total mRNA of each sample. Each experiment was performed in duplicate and repeated 3 times. The following primers were used: GAPDH: sense 5'-GTGAAGGTCGGAGTCAACG-3', anti-sense 5'-GGTGAAGACGCCAGTGGACTC-3'; human Bax: forward, 5'-ATGCGTCCACCAAGAA

GC-3' and reverse, 5'-GTCCACGGCGGCAATCA-3'; human bcl2: forward, 5'-AACTGGGGGAGGATTG TG-3' and reverse, 5'-AGGTGCCGGTTCAGGTAC-3' (Kaium et al. 2011; Liu et al. 2012).

Western blotting assay

The drug-treated cells were washed twice with ice-cold PBS and lysed in cell and tissue protein extraction reagent (Kangchen Inc., Shanghai, China) for 30 min on ice. Cell debris was then removed by centrifugation at 12,000 rpm for 10 min at 4 $^{\circ}$ C. The protein content in the samples was determined using the BCA Protein Quantitative Analysis Kit (Beyotime). 50 μ g of protein samples was boiled at 95 $^{\circ}$ C for 5 min prior to loading and separating in 12 % polyacrylamide gels

Fig. 3 Daphnoretin induced apoptotic morphological changes in HeLa cells. After treatment with daphnoretin (0.1 µg/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml) or carboplatin (500 µg/ml) for 24 h, HeLa cells were stained with Hoechst 33258 to detect characteristic apoptotic nuclei. Photofluorographic images ($\times 200$) show both

carboplatin (a) and 50 µg/ml daphnoretin (a) groups exhibiting strong, nuclear blue fluorescence (arrow), while the control group (a) shows little. (b) Daphnoretin at 50 µg/ml and 10 µg/ml significantly induced apoptosis in HeLa cells (# $P < 0.01$ and * $P < 0.05$ vs. control group, respectively) ($n = 4$)

before being transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking with 5 % nonfat dry milk in Tris-buffered saline containing 0.1 % Tween-20, membranes were incubated overnight at 4 °C on a rocking platform with rabbit polyclonal anti-Bax (Proteintech Group, Chicago, IL, USA), anti-Bcl-2 (Abcam, Cambridge, UK), anti-caspase-3 (Cell Signaling, Boston, MA), and anti-caspase-9 (Cell Signaling, Boston, MA) antibodies in 5 % bovine serum albumin (Amresco) at a dilution of 1:1000, 1:100, 1:1000, and 1:1000, respectively. Then membranes were incubated for 1 h with the secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (ICL Lab, Newberg, OR, USA) at a dilution of 1:8000. GAPDH (1:8000 dilution) was used as a loading control for protein quantity on immunoblots. Semiquantitative analysis was performed using a laser densitometer with an imaging system (Alpha Innotech, San Leandro, CA, USA).

Results were analyzed with AlphaEaseFC Software and reflected as the fold difference in protein expression of different treatment groups to that of the loading control group.

Caspase-3/9 activity detection

The caspase activity of HeLa cells was detected by a caspase-3 and caspase-9 colorimetric assay kit (Biovision, Milpitas, CA, USA). After collection by centrifugation, the apoptotic cells were resuspended in 50 µl chilled cell lysis buffer and incubated on ice for 10 min. The lysed cells were centrifuged for 1 min (10,000g), then the supernatant was transferred to a fresh tube and the protein concentration determined. Reaction buffer (50 µl; containing 10 mM DL-dithiothreitol) and 5 µl of 4 mM LEHD-pNA (caspase-9 substrate) substrate were added to each sample and incubated at 37 °C for 1–2 h. The absorbance was read

Fig. 4 Daphnoretin induced cell apoptosis at different stages. After treatment with daphnoretin (0.1 µg/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml) or carboplatin (500 µg/ml) for 24 h, HeLa cells were stained with Annexin V-FITC and PI. Compared with control (a),

50 µg/ml daphnoretin (b) and 500 µg/ml carboplatin (c) induced apoptosis in HeLa cells. (d) Daphnoretin (50 µg/ml) mainly induced cell apoptosis at an early stage, while carboplatin induced cell apoptosis at both stages (early and late)

at 405 nm in a microtiter plate reader (TECAN Systems Inc., Männedorf, Switzerland).

Statistical analysis

SPSS 15.0 and OriginPro 8 were used to analyze descriptive statistics. Statistical comparisons among groups were analyzed using One-Way ANOVA. Differences between two groups were determined using Student's *t* test.

Results

Daphnoretin HeLa cells viability

After treatment with daphnoretin (0.1, 1, 10, 50 µg/ml) or carboplatin (500 µg/ml) for 12 or 24 h, the MTT assay was performed. Carboplatin was used as a positive control. In the 12-h group (Fig. 1a), the viability of HeLa cells was significantly inhibited following administration of daphnoretin at 50 and 10 µg/ml ($P < 0.05$ vs. control group). In the 24-h group (Fig. 1b), the viability of HeLa cells was also

inhibited following administration of daphnoretin at 1 µg/ml ($P < 0.05$ vs. control group) and 50 and 10 µg/ml ($P < 0.01$ vs. control group). The IC_{50} values of daphnoretin to HeLa cells at 12 and 24 h were 5.82 and 5.52 µg/ml, respectively. The results demonstrate that daphnoretin could effectively reduce the viability of HeLa cells.

Daphnoretin induced apoptosis in HeLa cells

To further assess the effects of daphnoretin on the mitochondrial apoptotic pathway, the $\Delta\psi_m$ of HeLa cells was measured using JC-1 fluorescence. At a highly polarized $\Delta\psi_m$, JC-1 aggregates and emits red fluorescence, but forms monomers and emits green fluorescence when $\Delta\psi_m$ is depolarized occurring in the early stage of apoptosis. After treatment with daphnoretin (0.1, 1, 10, 50 µg/ml) or carboplatin (500 µg/ml) for 12 h, apoptotic HeLa cells exhibited green fluorescence, especially in the carboplatin and 50 µg/ml daphnoretin groups (Fig. 2a). Similar results were obtained at 24 h (Fig. 2b). Daphnoretin at 50 and 10 µg/ml significantly induced early stage of apoptosis in HeLa cells in 12 and 24 h.

Fig. 5 Daphnoretin increases *bax* mRNA level while decreasing *bcl-2* mRNA level. After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, *bax* and *bcl-2* mRNA levels of HeLa cells were determined by qRT-PCR. As shown in (a) Daphnoretin at 10 and 50 $\mu\text{g/ml}$ led to a significant increase in *bax* mRNA levels (the values have been normalized via *GAPDH* mRNA levels); (b) Daphnoretin at 50 $\mu\text{g/ml}$ led to a significant decrease in *bcl-2* mRNA levels relative to *GAPDH*. (* $P < 0.05$ vs. control) (n = 3)

After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, HeLa cells were stained with Hoechst 33258 to detect characteristic apoptotic nuclei. Apoptotic HeLa cells exhibited strong, nuclear blue fluorescence, especially in the carboplatin and 50- $\mu\text{g/ml}$ daphnoretin groups (Fig. 3a, b). Daphnoretin at 50 and 10 $\mu\text{g/ml}$ significantly induced apoptosis in HeLa cells ($P < 0.01$ and $P < 0.05$ vs. control group).

We further investigated by flow cytometry the exact number of apoptotic cells at different stages. Apoptotic cells of a late stage could be stained by both Annexin V-FITC and PI. Compared with control (Fig. 4a), 500 $\mu\text{g/ml}$ carboplatin (Fig. 4b) and 50 $\mu\text{g/ml}$ daphnoretin (Fig. 4c) induced apoptosis in HeLa cells. However, daphnoretin mainly induced cell apoptosis at an early stage, while carboplatin induced cell apoptosis nearly equally at early and late stages (Fig. 4d).

Daphnoretin increased *bax* mRNA levels while it decreased *bcl-2* mRNA levels

After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, total mRNA of HeLa cells was extracted and reverse transcribed into cDNA, then specifically amplified by PCR. The level of the pro-apoptotic *bax* mRNA was increased in the 50- $\mu\text{g/ml}$ daphnoretin group, while the level of the anti-apoptotic *bcl-2* mRNA was decreased (Fig. 5a). Meanwhile, daphnoretin at 50 $\mu\text{g/ml}$ led to a significant decrease in *bcl-2* mRNA levels compared to control (Fig. 5b).

Daphnoretin promoted Bax, caspase-3, and caspase-9 protein expression with decreased Bcl-2 protein expression

After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, proteins of HeLa cells were extracted and separated, then blotted on nitrocellulose membrane and incubated with antibodies. Relative to the control group, the expression of the pro-apoptotic mitochondrial protein Bax was clearly increased in both the 50- $\mu\text{g/ml}$ daphnoretin and carboplatin groups (Fig. 6a). However, expression of the anti-apoptotic mitochondrial protein Bcl-2 appeared to be decreased (Fig. 6b). The elevated expressions of *bax*, caspase-3 and caspase-9 suggest possible mitochondria-mediated apoptosis (Fig. 6b, c).

Daphnoretin induced both caspase-3 and caspase-9 activity

After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, the caspase-3 and caspase-9 activities of HeLa cells were detected by a colorimetric assay kit. The activities of both caspase-3

Fig. 6 Daphnoretin promotes Bax protein expression with little influence on Bcl-2 protein expression. After treatment with daphnoretin (0.1, 1, 10, 50 μg/ml) or carboplatin (500 μg/ml) for 24 h, Bax, Bcl-2, caspase-3 and caspase-9 protein expression was investigated by western blot (a) Clear elevated expression

(Fig. 7a) and caspase-9 (Fig. 7b) were elevated. Daphnoretin at 50 μg/ml could induce the activities of caspase-3 ($P < 0.01$ vs. control) and caspase-9 ($P < 0.05$ vs. control). The elevated activity of caspase-3 and caspase-9 thus suggests possible mitochondria-mediated apoptosis.

Discussion

Based on previous research on the anti-tumor activity of daphnoretin (Yang et al. 2008), we designed a series of experiments to investigate how this compound affects apoptosis. To confirm the negative influence on cancer cells, we first performed a cell viability inhibition assay. As expected, daphnoretin (10 and

of Bax protein relative to control following 50 μg/ml daphnoretin and carboplatin can be observed (b) Daphnoretin at 50 μg/ml significantly increase caspase-3 protein and caspase-9 protein relative to control (c) ($*P < 0.05$ vs. control group) (n = 3)

50 μg/ml) significantly inhibited the viability of HeLa cells after both 12 and 24 h of incubation. This result corresponds with those on K1735-M2 mouse melanoma cells and MDA-MB-231 human breast cancer cells (Diogo et al. 2009). Apoptosis of cells could be detected by the reduction of the mitochondrial membrane potential, Hoechst staining or flow cytometry; the former is used to observe the morphology of apoptotic cells, while the latter is widely performed to precisely determine the number and stage of apoptotic cells (Wu et al. 2010; Mahfouz et al. 2009). With Hoechst staining, more HeLa cells exhibited strong, nuclear blue fluorescence in the daphnoretin-treated group compared with the control group. This suggests that the inhibitory effect of daphnoretin on HeLa cells may involve apoptosis. Furthermore, the positive shift

Fig. 7 Daphnoretin induces both caspase-3 and caspase-9 activity. After treatment with daphnoretin (0.1, 1, 10, 50 $\mu\text{g/ml}$) or carboplatin (500 $\mu\text{g/ml}$) for 24 h, caspase activity of HeLa cells was detected by a colorimetric assay kit. The absorbance of cleaved substrate represents the caspase activity. (a) Daphnoretin at 50 $\mu\text{g/ml}$ significantly induces caspase-3 activity ($\#P < 0.01$ vs. control) ($n = 3$). (b) Daphnoretin at 50 $\mu\text{g/ml}$ significantly induces caspase-9 activity ($*P < 0.05$ vs. control) ($n = 3$)

of Annexin V-FITC-stained cells in the daphnoretin-treated group as detected by flow cytometry confirmed the pro-apoptotic effect of daphnoretin. In addition, unlike carboplatin, daphnoretin (10 and 50 $\mu\text{g/ml}$) promoted apoptotic cells more in an early stage of apoptosis, which suggests that the pro-apoptotic effect of daphnoretin may be regulated through mitochondrial pathway.

We further investigated the mechanism involved in this daphnoretin-induced apoptosis. Mechanisms of apoptosis include the extrinsic pathway and the intrinsic pathway. In the intrinsic pathway, which is

also known as the mitochondria-dependent pathway, several well-known molecules are involved, such as the Bcl-2 family, cytochrome c, and the caspase family (Kroemer et al. 2008). Following the main line from upstream Bcl-2/Bax to downstream caspase-3/9, we explored the possible mechanism of daphnoretin-induced apoptosis. We found that at the protein level, daphnoretin could promote the expression of the pro-apoptotic protein Bax. At the mRNA level, daphnoretin also decreased the amount of anti-apoptotic *bcl-2* while it increased the amount of pro-apoptotic *bax*. In addition to the elevated activity of caspase-3, daphnoretin could raise the activity of caspase-9, which is specifically involved in the mitochondria-dependent pathway of apoptosis. All results provide evidence that daphnoretin-induced apoptosis in HeLa cells involves the mitochondria-dependent pathway of apoptosis. In previous research of our group, we discovered via increased fluorescence intensity detected by laser scanning confocal microscopy that daphnoretin led to elevated calcium concentration in the cytoplasm (Yang et al. 2008). In fact, calcium plays an important role in many paradigms of cell apoptosis. Moreover calcium regulates the whole event by translocation among the cytoplasm, endoplasmic reticulum (ER), and mitochondria (Berridge et al. 2000; Ott et al. 2007). Calcium release from the ER calcium pool raises the calcium concentration in the cytoplasm, followed by elevated calcium concentration in the mitochondria. The overloaded calcium in mitochondria could lead to inositol 1,4,5-trisphosphate (IP_3)-induced opening of the mitochondrial permeability transition pore and the following cytochrome c release, which could activate caspases (Pinton et al. 2008). In addition, calcium also has a relationship with the Bcl-2 family. For example, enforced expression of Bax has been shown to result in the accumulation of itself in the ER and mitochondria, promoting calcium translocation from the ER to mitochondria and following cytochrome c release (Tabas and Ron 2011). In another experiment, Bax and Bak double knock-out cells appeared to have a decreased uptake of calcium by mitochondria (Teles et al. 2008). These results both coincide with the changes of calcium concentration and Bcl-2/Bax expression found in our research. Many researchers have reported that daphnoretin can act as a protein kinase C (PKC) activator. In fact, according to previous research, there is also a relationship between PKC and apoptosis (Griner and Kazanietz 2007). For

example, activation of PKC could lead to apoptosis of LNCaP cells (Xiao et al. 2009), prostate cancer cells, and neutrophils (Nagarsekar et al. 2008), while activation of PKC α is related to the suppression of apoptosis in human pre-B REH cells, in which Bcl-2 phosphorylation could be induced by PKC α activation (Reyland and Bradford 2010). It should be noted that different PKC subtypes might play different roles in cell apoptosis. Therefore, it is worthy to investigate through which subtype of PKC daphnoretin activates pro-apoptotic factors.

Conclusions

We demonstrated the pro-apoptotic effect of daphnoretin and investigated the possible involvement of the mitochondria-mediated pathway in daphnoretin-induced apoptosis in HeLa cells. All results suggest that daphnoretin might be a potential alternative agent for anti-cancer therapy. Future work will explore the regulatory mechanism involved in this pathway to better understand the pro-apoptotic effect of daphnoretin.

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