Fas/FasL Signaling Allows Extracellular-Signal Regulated Kinase to Regulate Cytochrome c Release in Oridonin-Induced Apoptotic U937 Cells

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Previously, we found that human histocytic lymphoma U937 cells possessed high susceptibility to oridonin-induced cell death, but the molecular mechanisms in response to oridonin remain unclear. In this study, U937 cells showed susceptible to apoptosis induced by 27 μM oridonin and an agonistic anti-Fas IgM mAb (CH-11) (500 ng/ml) as a Fas-sensitized positive control. Caspase 8 inhibitor z-IETD, but neither caspase 1 inhibitor Ac-YVAD nor caspase 10 inhibitor z-AEVD, effectively blocked oridonin-induced cell death as well as DNA fragmentation. Western blot analysis showed the up-regulated expression of Fas, FasL, and FADD, and down-regulated expression of procaspase 8, suggesting that Fas/FasL pathway was activated in oridonin-induced cell apoptosis. Further, stimulation of U937 cells with oridonin and CH11 resulted in significant ERK MAPK activation. However, inhibition of ERK by PD98059 reversed oridonin-induced cell death as well as the activation of caspase 8, indicating that ERK-mediated control occurred upstream of caspase 8. Simultaneously, ERK activation accounted for the release of cytochrome c, but failed to influence decreased Bel-2 expression induced by oridonin. Taken together, these results suggest that Fas/FasL signaling pathway-mediated ERK activation sensitized U937 cells to mitochondrial pathway-mediated apoptosis induced by oridonin.

Key words oridonin; apoptosis; Fas; extracellular-signal regulated kinase (ERK); cytochrome c

Apoptosis is a mechanism of cell death and is an important process for normal development and suppression of oncogenesis. It is believed that apoptosis is largely initiated either from mitochondria (the intrinsic pathway) or through cell death receptors (the extrinsic pathway), followed by recruitment of cascade responses of caspases, a group of aspartate-specific cysteine proteases, ultimately resulting in apoptosis. Among cell surface receptors, Fas receptor (CD95), a number of tumor necrosis factor (TNF) receptor family, exerts a critical role in triggering the apoptotic signaling and effector machinery. Upon stimulation of Fas with an agonistic anti-Fas IgM mAb or its natural ligand, FasL, several proteins with distinct functions, such as a cytosolic adaptor protein, FADD, are recruited to the cytoplasmic domain of the Fas and in turn bind to the initiator caspase, caspase 8. Subsequently, the cleaved and activated caspase 8 causes a strictly regulated process involving activation of downstream effector caspases including caspase 3, 6 and 7, eventually leading to apoptosis. However, stimulation of Fas also results in activation of multiple kinases such as phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) other than those of effector caspases, indicating that phosphorylation-mediated signaling is likely to be involved in some aspects of Fas activation and regulation. In addition, MAPK proteins seem to be responsible for mitochondrial pathway-mediated apoptosis. In some cases, the intrinsic pathway is thought to be a signal amplifier of the extrinsic pathway, due to the presence of intermediators such as Bid and phosphorylation-mediated kinases.

Oridonin (Fig. 1), a major component isolated from the aerial of Rabdosia rubescens, has been used for treatment of pharyngitis, has anti-bacterial, anti-mutagenesis, scavenging active oxygen free radicals and anti-tumor effects. In the process of investigating the cytotoxic effects of oridonin on tumor cell lines, we found that human histocytic lymphoma U937 cells possessed much susceptibility to apoptosis induced by oridonin relative to other solid tumor cells such as human cervical cancer HeLa, and murine fibrosarcoma L929. Therefore, the focus of our current study is to investigate the distinct mechanism of oridonin-induced U937 cell death and find out the novel pathway responsible for the high sensitivity of U937 to oridonin-induced apoptosis.

In this study, we for the first time demonstrated that oridonin induced cell death through Fas/FasL signaling, that is, up-regulation of Fas, FasL, and FADD expression; activation of caspase 8. Importantly, ERK functioned upstream of caspase 8, at the same time, regulated cytochrome c release from mitochondria, indicating induction of cytochrome c release amplified Fas/FasL signaling in response to oridonin through ERK as an intermediary.
MATERIALS AND METHODS

Chemical Material Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The purity of oridonin was measured by HPLC and determined to be about 99%. Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death.

Biological Materials Agonistic anti-Fas IgM mAb (clone CH-11) and anti-Fas IgG (clone UB2) were purchased from Medical & Biological Laboratories (Nagoya, Japan). Caspase 1 inhibitor, Ac-Val-Ala-Asp-CMK (Ac-YVAD-CMK) was from Bachem (Bubendorf, Switzerland); caspase 8 inhibitor, z-Ile-Glu-Thr-Asp-FMK (z-IETD-FMK) and caspase 10 inhibitor, z-Ala-Glu-Val-Asp-FMK (z-VEID-FMK) were from Enzyme Systems (Livermore, CA, U.S.A.). Genistein, RNase A, proteinase K, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). MEK inhibitor U0126 was briefly dissolved in 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

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Culture of U937 Cells Human histiocytic lymphoma U937 (&#8801;CRL-1593.2) cells were obtained from American Type Culture Collection, (Manassas, VA, U.S.A.). The cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% FCS, 0.03% L-glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml), and maintained at 37 °C with 5% CO2 in a humidified atmosphere.

Growth Inhibitory Test U937 cells (2×10^5/well) were seeded into 96-well culture plates (Nunc, Roskilde, Denmark). After overnight incubation with 10% FCS, various concentrations of oridonin, CH11 or UB2 were added to the plates. Following incubation, cell growth was measured at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described with a plate reader (Bio-Rad, Hercules, CA, U.S.A.)

Observation of Morphologic Changes U937 cells in RPMI-1640 medium containing 10% FCS were seeded into 96-well culture plates. After overnight culture, oridonin was added to the cell culture and the cellular morphology was observed using phase-contrast microscopy (Olympus, Tokyo).

Flowcytometric Analysis U937 cells were harvested and washed with PBS. The cell pellets were fixed in 75% ethanol at 4 °C overnight. After washing twice with PBS, the cells were stained with 1.0 ml of propidium iodide (PI) solution containing PI 50 µg/ml, RNase A 1 g/l and 0.1% Triton X-100 in sodium citrate 3.8 mmol/l, followed by incubation on ice in the dark condition for 30 min. Samples were analyzed by a FACSscan flowcytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

DNA Extraction and Detection of DNA Fragmentation U937 cells (1×10^6 cells) were collected by centrifugation at 1500 g for 5 min and washed once with Ca2+ - and Mg2+-free phosphate buffered-saline (PBS). The cell pellet was suspended in 100 µl cell lysis buffer (Tris–HCl 10 mmol/l pH 7.4, EDTA 10 mmol/l pH 8.0, Triton X-100 0.5%) and kept at 4 °C for 30 min. The lysate was centrifuged at 7200×g for 20 min. The supernatants were incubated with 20 µg/ml RNase A (Sigma) at 37 °C for 60 min, then incubated with 20 µg/ml proteinase K (Merck, Rahway, NJ, U.S.A.) at 37 °C for 60 min. The supernatants were again mixed with NaCl 0.5 mol/l and 50% isopropyl alcohol overnight at -20 °C, followed by centrifugation at 7200×g for 15 min. After drying, DNA sample was dissolved in TE buffer pH 7.8 (Tris–HCl 10 mmol/l pH 7.4 and EDTA 1 mmol/l pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 mg/ml ethidium bromide.

PKC Activity Assay PKC activity assay was carried out according to the instructions of PepTag® Non-Radioactive Protein Kinase C Assay Kit (Promega, WI, U.S.A.). Briefly, the cells (1×10^6 cells) were washed once with PBS and lysed in lysis buffer, containing 20 mM Tris–HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mg/l leupeptin (pH 7.5). Assays were performed at 30 °C in a total volume of 25 µl containing 5 µl PKC reaction 5×buffer, 5 µl PLSR7SVAK peptide, 5 µl PKC activator, 1 µl peptide protection solution and 9 µl sample. Reactions were initiated by the addition of 9 µl sample and terminated after 30 min by incubation of the reaction mixture at 95 °C for 10 min. After adding 1 µl of 80% glycerol, each sample was separated by 0.8% agarose gel electrophoresis at 100 V for 15 min.

Western Blot Analysis Western blot analysis was performed as previously described. Briefly, both adherent and non-adherent cells were harvested; washed twice with PBS; and then lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml aprotonin and 10 µg/ml leupeptin) at 4 °C for 60 min. The lysate was centrifuged at 15000×g for 10 min and the supernatants were used for Western blotting analysis. Protein concentration was determined by the Folin assay. Equal amounts of total protein were separated by 12% gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Proteins were detected with the primary antibodies against Fas, FasL, FADD, caspase 8, ERK, phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized by using 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

Statistical Analysis All data represent at least three independent experiments and are expressed as mean±S.D., unless otherwise indicated. Statistical comparisons were made using Student’s t-test. p-Values of less than 0.05 were considered to represent a statistically significant difference.
RESULTS

Differential Sensitivity of U937 Cells to Apoptosis Induced by Oridonin, Agonistic Anti-Fas IgM mAb CH-11, and Anti-Fas IgG mAb UB2

To examine whether U937 cells were sensitive to Fas pathway-mediated apoptosis, agonistic anti-Fas IgM mAb CH-11 and anti-Fas IgG mAb UB2 were applied on the cells. As shown in Fig. 2A, treatment with CH11 200–500 ng/ml showed 25–30% reduction in U937 cell growth, relative to UB2 treatment group. More significant results were obtained when the cells were incubated with 27 μM oridonin for 12 h, which was consistent with our previous study. Flowcytometric analysis further confirmed that CH11 and oridonin, but not UB2, had the ability to induce U937 cell apoptosis due to observation of sub-G1 peaks (Fig. 2B). These results suggest that U937 cells might express Fas in certain degree, resulting in the sensitivation to agonistic anti-Fas IgM mAb CH-11.

Oridonin-Induced Apoptosis in U937 Cells Is Caspase 8 Dependent, but Caspase 1 and Caspase 10 Independent

The death receptor pathway-mediated apoptosis is known to assemble receptor complexes that recruit initiator caspases such as caspase 1, 2, 8 and/or 10. These activated initiator caspases then activate other downstream effector caspases, leading to apoptosis. In our previous study, oridonin-induced apoptosis was insensitive to inhibitors of caspase 8 and caspase 1 in several cell types such as L929 cells. To determine whether these initiator caspases are involved in oridonin-induced U937 cell apoptosis, relevant caspase inhibitors, caspase 1 inhibitor Ac-YVAD, caspase 8 inhibitor z-IETD and caspase 10 inhibitor z-AEVD were applied. Among these, z-IETD effectively inhibited oridonin-induced apoptosis in U937 cells, whereas Ac-YVAD and z-AEVD had no effect, suggesting that caspase 8 was likely to be involved (Fig. 3). This conclusion gained further support from the morphologic observation that application of caspase 8 inhibitor z-IETD in combination with oridonin decreased the number of apoptotic bodies, in contrast with oridonin alone cases (Fig. 4A); and DNA fragmentation analysis revealed that oridonin-induced DNA ladder was significantly attenuated by addition of z-IETD, but addition of Ac-YVAD and z-AEVD only slightly blocked DNA fragmentation (Fig. 4B).

Up-Regulation of Death-Inducing Signaling Complex

![Fig. 2. Effect of Oridonin, Agonistic Anti-Fas mAb CH11, and UB2 on U937 Cell Apoptosis](image)

The cells were incubated with oridonin, CH11, and UB2 at various doses for 12 h. The ratio of cell death was examined by MTT assay (A); and DNA content after PI staining was analyzed by flowcytometry (B). Values represent the mean±S.D. (n=3).

![Fig. 3. Impact of Inhibitors of Caspase 1, Caspase 10, and Caspase 8 on Oridonin-Induced Cell Death](image)

The cells were cultured in the absence or presence of the caspase inhibitors, Ac-YVAD-CMK (A), z-AEVD-FMK (B), or z-IETD-FMK (C) for 1 h prior to addition of oridonin, and then incubated for 12 h. Black, striated and white bars represent oridonin, oridonin plus caspase inhibitors, and caspase inhibitors, respectively. ∗∗p<0.05 vs. oridonin-treated group.
DISC Protein Expression, Including Fas, FADD and Caspase 8, by Oridonin

To further confirm the involvement of Fas/FasL signaling in oridonin-induced apoptosis, the DISC protein expression was examined by Western blot analysis. As shown in Fig. 5, the expression levels of Fas and FasL significantly and persistently increased after administration of oridonin accompanied by a relative increase in the expression of cytosolic adaptor protein, FADD. In line with the results in Fig. 3, the result of Western blot analysis also revealed that the protein level of procaspase 8 was reduced rapidly upon 3 h, and then remained at a relatively stable level for the rest of the induction time, indicating that procaspase 8 was cleaved in the early phase to initiate capase cascade, ultimately resulting in apoptosis.

Taken together, these results suggest that Fas/FasL signaling was activated in response to oridonin, which partially or totally accounted for U937 cell apoptosis induced by oridonin.

Oridonin-Induced Cell Death Was Promoted by ERK, but not by PTK and PKC

MAPK signaling pathways have been known to play important roles in cell growth and cell death. To evaluate the involvement of MAPK cascade in oridonin-induced cell death, specific inhibitors of ERK (PD98059), p38 (SB203580), JNK (SP600125) were employed. After 12 h incubation with oridonin, 10 μM PD98059 effectively decreased oridonin-induced cell death, while SB203580 and SP600125 failed (Fig. 6). There is evidence that protein tyrosine kinase (PTK) and protein kinase C (PKC) regulate ERK activation and participate in Fas/FasL pathway-mediated apoptosis.17) To assess whether such a mechanism is involved in oridonin-induced U937 cell death, PTK inhibitor genistein was applied to examine the function of PTK in oridonin-treated U937 cell death, and simultaneously, PKC activity assay was carried out. The results showed that treatment with 50 μM genistein in combination with oridonin did not cause the decrease in cell death as observed in the presence of PD98059, on the contrary, genistein increased cell death (data not shown). Also, the failure of oridonin to induce PKC activation (data not shown) indicates neither PTK nor PKC promoted oridonin-induced cell death.

ERK Was Activated by Fas/FasL Signaling in Oridonin-Treated Cells

Several recent studies have shown
that activation of mitogen-activated protein kinase (MAPK) proteins may result from Fas/FasL signaling pathway.\(^{18}\) Expression of phosphorylated ERK began to increase from 3 h, and then was sustained until 24 h, consistent with our previous study.\(^{14}\) In contrast, stimulation of U937 with CH11 led to a transient ERK phosphorylation (Fig. 7). Both oridonin and CH11 had no impact on the ERK protein expression. Since oridonin induced U937 cell apoptosis through Fas/FasL pathway, in combination with evidence that agonistic anti-Fas CH11 induced ERK phosphorylation, these results indicate that ERK activation in response to oridonin might result from Fas/FasL signaling.

To analyse the role of ERK in Fas/FasL signaling pathway in more detail, we examined the impact of ERK inhibitor PD98059 on the decreased expression of procaspase 8 and increased expression of FADD. Exposure of PD98059 (10 \(\mu\)M) to U937 cell culture prior to oridonin treatment, then the cells were treated with oridonin for 12 h. FADD and procaspase 8 were analyzed by Western blot analysis.

Modulation of Bcl-2 Family Proteins, Cytochrome c Release, and Caspase-3 Activation in Oridonin-Induced U937 Cell Apoptosis Accumulating evidence suggests that the mitochondria play a critical role in apoptosis by modulating Bcl-2 family members, such as Bcl-2, Bcl-X\(_L\), and Bax, and releasing apoptogenic factors, such as cytochrome c and apoptosis-inducing factor (AIF).\(^{19}\) After treatment with oridonin for 12 h, the expression of Bcl-2 began to decrease, simultaneously, cytochrome c levels in the cytoplasm increased (Fig. 9). Caspase-3 was activated by proteolytic processing of the 32-kDa procaspase into two smaller subunits of 17 and 20 kD, after treatment with 27 \(\mu\)M oridonin for 12 h. These results indicated that oridonin-induced apoptosis was involved in the mitochondria-mediated pathway: the initial phase mediated by Bcl-2 down-regulation, resulting in cytochrome c release from the mitochondria; and the executive phase mediated by the caspase-3 activation.

**Contribution of ERK Activation to Cytochrome c Release in Oridonin-Induced Apoptotic U937 Cells** To examine whether Bcl-2 downregulation and cytochrome c upregulation in response to oridonin treatment is dependent on ERK activation, MEK inhibitor PD98059 or U0126 are administered. Treatment with 10 \(\mu\)M PD98059 significantly reversed the increased expression of cytochrome c induced by oridonin and CH11, but had no effect on the downregulation of Bcl-2 protein (Fig. 10). Similar effects were observed with another ERK inhibitor U0126 treatment. These data suggest activation of ERK by oridonin was required for the release of cytochrome c in the apoptotic U937 cells.

**DISCUSSION**

Fas/FasL pathway has been implicated as an important cel-
lular pathway regulating the induction of apoptosis in diverse cell types and tissues. However, some Fas-expressing cancer cells seem to be resistant to apoptotic signaling through this receptor on the basis of a strategy to escape apoptosis mediated by FasL-expressing cytotoxic T cells. For U937 cells tested in this study, engagement of Fas antigen with its agonistic antibody CH11 resulted in apoptosis as monitored by MTT assay and flowcytometric analysis, suggesting U937 cells with the characteristics of Fas sensitivity. This result was in line with the previous report that incubation of U937 cells with an anti-Fas antibody for 4h induced apoptosis. Our previous studies showed that oridonin inhibited L929 and HeLa cells growth at the doses ranging from 41.2 to 68.7 μM. In contrast, 27 μM oridonin was able to decrease U937 cell growth by 50%, indicating a high sensitivity of U937 cells to oridonin-induced cell death. Caspase 8 has been considered to be the first caspase activated following Fas stimulation with the subsequent assembly of the death-inducing signaling complex (DISC). Our results showed that caspase 8 inhibitor z-IETD reduced oridonin-induced cell death and procaspase 8 expression decreased after treatment with oridonin. This contrasts with our previous study in L929 and HeLa cells lacking caspase 8 activation in the presence of oridonin, indicating that oridonin-induced apoptosis in L929 and HeLa cells is caspase 8 independent. The reason for this discrepancy is not clear, but might be related to the different sensitivity of tumor cells to Fas. In addition, expression of Fas, FasL, and FADD was up-regulated by oridonin treatment. These results suggest that oridonin induced U937 cell death through Fas/FasL signaling.

MAPK members including ERK, JNK, and p38, are reported to play important roles in cell growth and apoptosis. In this study, ERK inhibitor PD98059, but not JNK inhibitor SP600125 and p38 inhibitor SB203580, effectively suppressed oridonin-induced cell death and DNA fragmentation, indicating that ERK MAPK was required for oridonin-induced cell apoptosis. Several previous reports have demonstrated that signal pathways of apoptosis in endothelial cells involve activation of PTK and PKC. A study by Karsan and his colleagues showed that endothelial cell death induced by TNFα was mediated via a PKC pathway. PTK and PKC were thought to transmit signaling events through ERK MAPK. This is in contrast with the present study, in which oridonin fails to induce PKC activation. Moreover, PTK inhibitor genistein did not decrease oridonin-induced cell death, on the contrary, increased cell death. These results suggest that unlike the effect of ERK, PTK and PKC did not contribute to oridonin-induced cell death and ERK was likely to be activated by other signal molecules.

There is evidence that Fas/FasL signaling has the ability to induce JNK and MAPK activation. The present results indeed show that oridonin activated Fas/FasL signaling, in turn, upregulated ERK phosphorylation, as agonistic anti-Fas antibody CH11 did. In addition, PD980598 reversed decreased expression of procaspase 8, but failed to abrogate increased expression of FADD, implying that ERK functions upstream of caspase 8, but FADD independent. Previous studies showed that MAPK was activated in a variety of cell types upon TNFR1 stimulation. This kind of activation also applies for Fas signaling through another adaptor protein, Fas-interacting serine/threonine kinase receptor-interacting protein (RIP). Combination of the previous results with our present observation indicates that ERK activation might be mediated by RIP but not by FADD in response to Fas/FasL signaling. Therefore, further research will be required to elucidate the regulatory mechanisms of ERK activation.

Accumulating evidence shows that apoptosis induction through mitochondrial pathway is related to ERK activation. A study in U937 cells in response to Epigallocatechin-3-gallate (EGCG) plus vanadate indicated that ERK phosphorylation was required for cytochrome c release and caspase-3 activation. Thus, ERK inhibitor PD98059 is used in the present study to investigate the role of ERK activation in changed expression of Bcl-2 protein and cytochrome c release. Pretreatment with PD98059 blocked the increased expression of cytochrome c, but had no influence in down-regulated Bcl-2 protein expression induced by oridonin, suggesting that ERK allows cytochrome c release in the cytoplasm to mediate the mitochondria-dependent apoptotic pathway in response to oridonin. In addition, we have found that Bcl-XL downregulation and Bax upregulation in response to oridonin treatment is dependent on ERK activation, implying that ERK might influence the release of cytochrome c through alteration of ratio of Bcl-XL/Bax, independent of Bcl-2. This is in contrast with the previous study, in which inhibition of ERK activation reversed Bcl-2 downregulation and cytochrome c release in gemetabine-induced human nonsmall cell lung cancer H1299 cell apoptosis.

In summary, we demonstrated that sensitivity of U937 cells to oridonin-induced apoptosis was associated with Fas/FasL signaling events including increased expression of Fas, FasL, and FADD; and caspase 8 activation. Importantly, this signaling was amplified by ERK pathway, in turn, promoted cytochrome c release from mitochondria, eventually resulting in caspase 3 activation.

REFERENCES