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Original Article

Oridonin induces apoptosis and autophagy in murine fibrosarcoma L929 cells partly via NO-ERK-p53 positive-feedback loop signaling pathway

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Aim: To investigate the role of nitric oxide (NO) in oridonin-induced apoptosis and autophagy in murine fibrosarcoma L929 cells and the underlying molecular mechanisms.
Methods: Cell viability was measured using MTT assay. Intracellular NO level, $SubG_1$ cell ratio and autophagy cell ratios were analyzed with flow cytometry after diaminofluorescein-2 diacetate (DAF-2DA), propidium iodide (PI) and monodansylcadaverine (MDC) staining, respectively. Protein expression was examined using Western blot analysis.
Results: Exposure of L929 cells to oridonin (50 μmol/L) for 24 h led to intracellular NO production. Pretreatment with NOS inhibitor 1400w or *L*-NAME inhibited oridonin-induced apoptosis and autophagy in L929 cells. The pretreatment decreased the apoptosis-related protein Bax translocation and cytochrome *c* release, increased Bcl-2 level, reversed the autophagy-associated protein Beclin 1 increase and conversion of LC3 I to LC3 II. Furthermore, pretreatment with NO scavenger DTT completely inhibited oridonin-induced apoptosis and autophagy in L929 cells. In addition, oridonin (50 μmol/L) activated ERK and p53 in L929 cells, and the interruption of ERK and p53 activation by PD 98059, pifithrin-α, or ERK siRNA decreased oridonin-induced apoptosis and autophagy. The inhibition of NO production reduced oridonin-induced ERK and p53 activation, and NO production was down-regulated by blocking ERK and p53 activation.
Conclusion: NO played a pivotal role in oridonin-induced apoptosis and autophagy in L929 cells. Taken together with our previous finding that ERK contributes to p53 activation, it appears that NO, ERK, and p53 form a positive feedback loop. Consequently, we suggest that oridonin-induced apoptosis and autophagy are modulated by the NO-ERK-p53 molecular signaling mechanism in L929 cells.

Keywords: oridonin; murine fibrosarcoma L929 cells; apoptosis; autophagy; NO; ERK; p53

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Introduction

NO, which was identified as an endothelial-derived relaxing factor (EDRF) in 1987[1], is a ubiquitous cellular messenger molecule in the cardiovascular, nervous and immune systems, where it has been shown to participate in activities involving cardiovascular function, neurodegenerative diseases and cancer[2–4].

Oridonin (Figure 1A), an active diterpenoid that was isolated from *Rabdosia rubescens*, has various pharmacological and physiological functions, particularly for the treatment of cancers[5].

Apoptosis, also known as type I programmed cell death, is characterized by an ensemble of morphological features, including cellular shrinkage, plasma membrane blebbing, chromatin condensation, nuclear fragmentation and apoptotic body formation[6]. Apoptosis can be triggered by intrinsic and extrinsic pathways that involve either mitochondria or death receptors[7]. Macroautophagy (hereafter referred to as autophagy) is the most active form of autophagy and affects various physiological and pathological processes, including immunity, cancer and neurodegenerative diseases[8, 9]. Autophagy is the process by which organelles and cytosolic macromolecules are sequestered into double-membrane structures known as autophagosomes that are subsequently delivered to the lysosome for degradation[10]. Autophagy is regulated by several autophagy-related ATG genes, many of which have mammalian orthologs. Two well-known ATG genes in mammals are Beclin 1 and microtubule-associated

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protein light chain 3 (LC3). Beclin 1, which is the mammalian homolog of Atg6 and functions in either the autophagic pathway or the early formation of autophagosomes, plays a central role in the regulation of autophagy[11]. LC3, which is the mammalian homolog of Atg8, is associated with the autophagosomal membranes after autophagocytic processing. LC3 I normally exists in the cytosol; when autophagy is induced, LC3 I conjugates with phosphatidylethanolamine to form the autophagosome-associated LC3 II. The accumulation of LC3 II is correlated with the number of autophagosomes present[12].

The L929 murine fibrosarcoma cell line was derived in March 1948. Strain L was one of the first cell strains to be established in continuous culture, and clone 929 was the first cloned strain developed. The parent L strain was derived from normal subcutaneous areolar and adipose tissues of a 100-d-old male C3H/An mouse. Clone 929 was established (by the capillary technique for signal cell isolation) from the 95th subculture generation of the parent strain. It is widely believed that L929 is sensitive to tumor necrosis factor alpha (TNFα) and that TNFα-induced cell death is caspase-independent[13]. Our previous study demonstrated that oridonin could induce L929 cell caspase-independent apoptosis by the activation of the ERK-p53 signaling pathway[14, 15]. Interestingly, NO is known to activate both ERK and p53 via phosphorylation[16], and it appears to be involved in the regulation of apoptosis[17]. In this study, oridonin induced NO production, which mediated L929 cell apoptosis and autophagy and led to the activation of ERK and p53.

Materials and methods

Reagents

Oridonin was obtained from the Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China), and its purity was determined to be 99.4% by HPLC measurements. Fetal bovine serum was purchased from TBD Biotechnology Development (Tianjin, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), monodansylcadaverine (MDC), RNase A, diaminofluorescein-2 diacetate (DAF-2DA), 1400w, *L*-NAME, sodium nitroprusside (SNP), DTT, PD98059 (PD), and pifithrin-α (PFT-α) were purchased from Sigma Chemical (St Louis, MO, USA). ERK small interfering RNA (siRNA), control siRNA and Lipofectamine 2000 were from Invitrogen (Invitrogen, Carlsbad, CA, USA). Rabbit polyclonal antibodies against Bax, Beclin 1, LC3, ERK, p-ERK, p53, and p-p53, mouse polyclonal antibodies against Bcl-2, cytochrome *c*, and β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

L929 cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Beijing Yuanheng Shenyang Research Institution of Biotechnology, Beijing, China), 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.03% *L*-glutamine and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. All of the experiments were performed on logarithmically growing cells.

Cell viability assay

Cells were seeded in 96-well plates at a concentration of 5×10^4 cells/mL. After 48 h of incubation, they were treated with oridonin in the presence or absence of the indicated inhibitors for 24 h. Cell viabilities were measured by MTT staining.

$$\text{Cell viability (\%)}=100-\frac{A_{492,\ \text{control}}-A_{492,\ \text{experimental}}}{A_{492,\ \text{control}}-A_{492,\ \text{blank}}}\times100$$

Flow cytometric analysis of NO levels

The intracellular NO was detected using the NO fluorescent probe diaminofluorescein-2 diacetate (DAF-2DA). Once diacetate groups of the DAF-2DA reagent are hydrolyzed by cytosolic esterases, DAF-2 is released and sequesters the reagent inside of the cell. NO production converts the non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2 triazole (DAF-2T)[18]. The L929 cells were treated with oridonin for the indicated periods or co-incubated with the given inhibitors for 24 h. They were then incubated with 10 μmol/L DAF-2DA at 37 °C for 30 min to measure NO production. Next, the cells were harvested, and the pellets were suspended in 0.5 mL PBS. Finally, the samples were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of $SubG_1$ cells

The L929 cells were treated with oridonin for the indicated periods or co-incubated with the given inhibitors for 24 h. The collected cells were fixed in 70% ethanol, stained for DNA content with PI at 4 °C in the dark for 30 min, and measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of autophagy

After incubation with oridonin for the indicated periods, the cells were cultured with 0.05 mmol/L MDC at 37 °C for 1 h and then analyzed by flow cytometry.

siRNA transfection

The cells were transfected with siRNA by using Lipofectamine 2000 according to the manufacturer's instructions. The transfected cells were used for subsequent experiments 24 h later.

Western blot analysis

The L929 cells were treated with oridonin for the indicated periods or co-incubated with the given inhibitors for 24 h. Equivalent amounts (30 μg) of total protein were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed as described previously[19].

Preparation of mitochondrial and cytosolic extracts

Cells were collected by centrifugation at 200×*g* at 4 °C for 5 min and washed twice with ice-cold PBS. The cell pellets were resuspended in ice-cold homogenizing buffer (250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L

EDTA, 1.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin). The cells were homogenized with 20 strokes of a Dounce homogenizer at 4 °C. Nuclei and intact cells were removed by centrifugation at 500×*g* at 4 °C for 12 min. The supernatants were subjected to centrifugation for 30 min (12 000×*g*, 4 °C) to precipitate the mitochondria. The resultant supernatants were used as the cytosolic fraction, and the pellets were lysed in lysis buffer (50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 2 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L PMSF) on ice for 1 h. The lysates were subjected to centrifugation at 12 000×*g*, 4 °C for 30 min, and the supernatants were used as the mitochondrial fractions.

Statistical analysis

All of the presented data and results were confirmed in at least three independent experiments. The data are expressed as the mean±standard error (SEM). The data were analyzed with a one-way ANOVA using the Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), and the statistical comparisons were performed using least significant difference (LSD) *post-hoc* tests. $P<0.05$ was considered to be statistically significant.

Results

Oridonin induces NO production

The production of NO was evaluated by a flow cytometric analysis with the NO fluorescent probe DAF-2DA. In oridonin-treated L929 cells, the DAF-2T-positive cell ratio increased over time (Figure 1B). These data suggest that oridonin induces NO production.

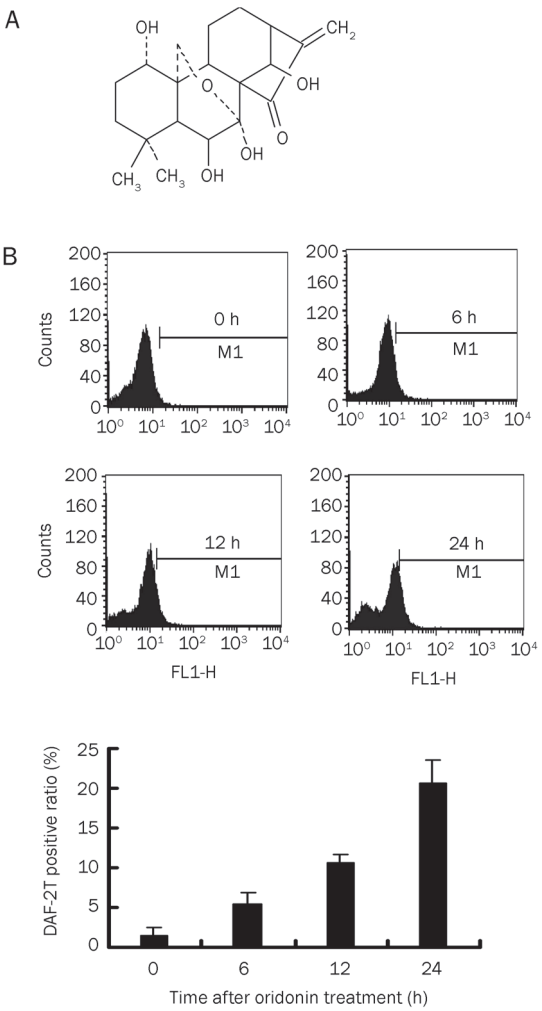

Figure 1. Oridonin induced the generation of nitric oxide. (A) Chemical structure of oridonin. (B) Cells were incubated with 50 μmol/L oridonin for 0, 6, 12, and 24 h followed by flow cytometric analyses of NO level changes after DAF-2DA staining. Mean±SEM. *n*=3.

NO mediates apoptosis and promotes cytoprotective autophagy in oridonin-treated L929 cells

Cellular NO is primarily synthesized from L-arginine by a family of NOSs[20]. Thus, we introduced the NOS inhibitors 1400w[21] and *L*-NAME[22] to investigate the role of NO in oridonin-induced cell growth inhibition. As shown in Figure 2A, the increase in NO was suppressed by 1400w or *L*-NAME. Additionally, the 1400w or *L*-NAME pretreatment reversed the oridonin-induced cell growth inhibition (Figure 2B). These results suggested that oridonin-induced NO production caused cell death in L929 cells.

Our previous study demonstrated that oridonin induced apoptotic cell death and protective autophagy[23]. To investigate the role of NO in oridonin-induced L929 cell apoptosis and autophagy, the cells were pretreated with 1400w and *L*-NAME. Pretreatment with 1400w or *L*-NAME decreased the number of $SubG_1$ cells compared to the oridonin alone treatment group (Figure 2C). These findings indicated that NO induced apoptosis in the oridonin-treated L929 cells. Our previous study also showed that oridonin induced apoptosis mainly through the mitochondrial pathway[15]. Hence, the expression of the mitochondria-related proteins Bax, cytochrome *c*, and Bcl-2 were examined using Western blot analyses (Figure 1S). Pretreatment with 1400w or L-NAME sequestered the mitochondrial Bax and cytosolic cytochrome c and increased Bcl-2 levels (Figure 2E). The inhibition of NO by 1400w or L-NAME also attenuated the induction of autophagy (Figure 2D). Furthermore, we obtained similar western blot results from the assessment of Beclin 1 levels and the conversion of LC3 I to LC3 II (Figure 2E). Collectively, these results indicated that NO was involved in oridonin-induced apoptosis and autophagy in L929 cells. To further confirm the role of NO in oridonin-induced apoptosis and autophagy, we introduced DTT to eliminate NO production[18] (Figure 2F). DTT pretreatment reversed oridonin-induced apoptosis (Figure 2G) and autophagy (Figure 2H). Further studies verified that pretreatment with DTT decreased Bax translocation and cytochrome *c* release and increased Bcl-2 levels, whereas this pretreatment reversed the Beclin 1 increase and conversion of LC3 I to LC3 II (Figure 2I). On the basis of the above results, we suggest that the oridonin-induced production of NO is

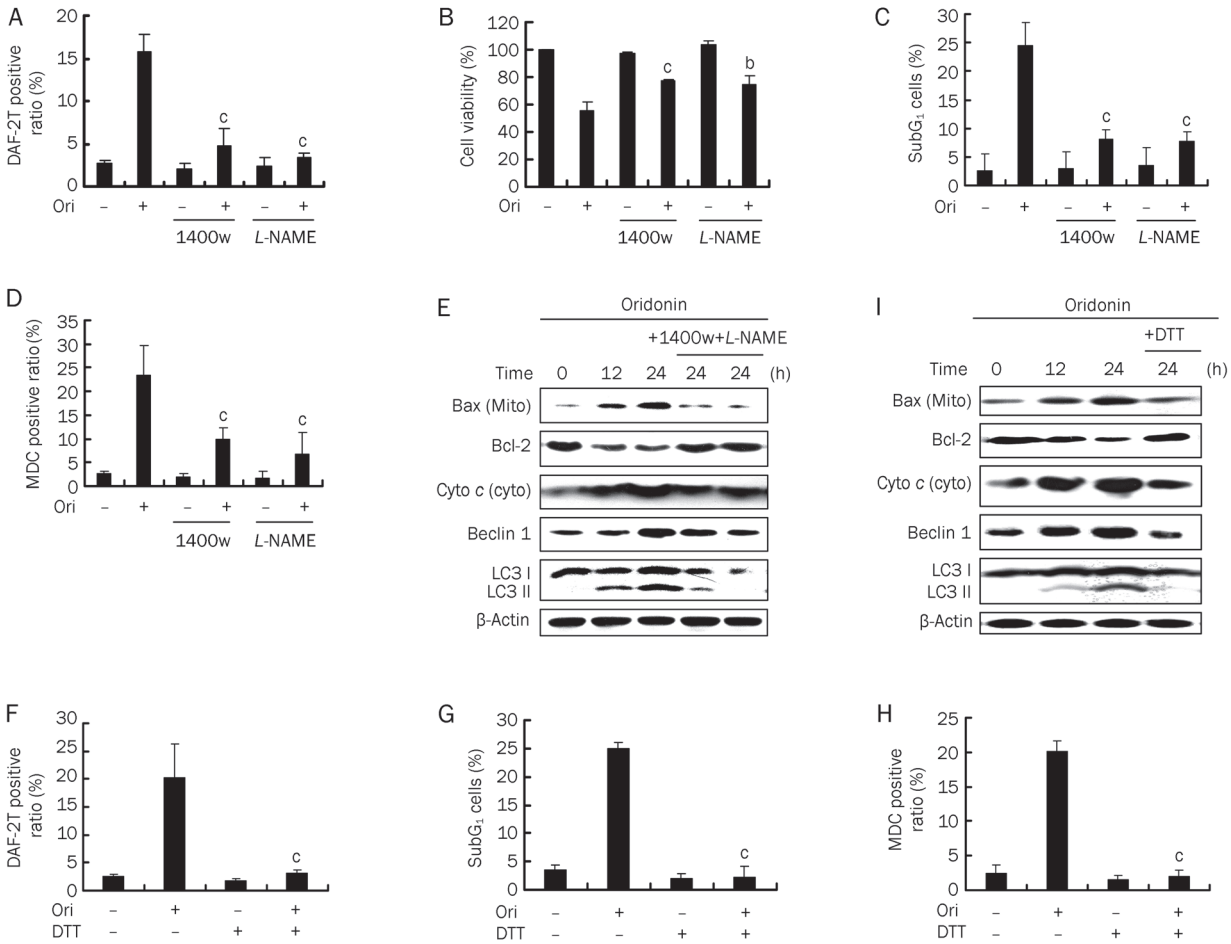

Figure 2. Effect of nitric oxide on oridonin-induced apoptosis and autophagy. (A) Cells were incubated with 50 μmoL/L oridonin for 24 h in the presence or absence of 1400w (10 μmoL/L) or *L*-NAME (20 μmoL/L) for 3 h and then analyzed by flow cytometry for NO level changes after DAF-2DA staining. (B) Cell viability was measured by MTT assay. (C–E) Cells were incubated with 50 μmoL/L oridonin for 24 h in the presence or absence of 1400w (10 μmoL/L) or L-NAME (20 μmoL/L) for 3 h. (C) Flow cytometric analyses of apoptotic cell ratios after PI staining ($SubG_1$ cells). (D) Flow cytometric analyses of autophagic cell ratios after MDC staining. (E) Western blot analyses for the detection of mitochondrial Bax, Bcl-2, cytosolic cytochrome c, Beclin 1, and LC3 levels. β-Actin was used as an equal loading control. (F–H) Cells were pretreated with 0.5 mmoL/L DTT and then cultured with 50 μmoL/L oridonin for 24 h. (F) Flow cytometric analyses of NO level changes after DAF-2DA staining. (G) Apoptotic cell ratios after PI staining ($SubG_1$ cells). (H) Autophagic cell ratios after MDC staining. (I) Cells were incubated with 50 μmoL/L oridonin for 24 h in the presence or absence of 0.5 mmoL/L DTT and then analyzed by Western blotting to detect mitochondrial Bax, Bcl-2, cytosolic cytochrome c, Beclin 1, and LC3 levels. β-Actin was used as a loading control. Mean±SEM. n=3. $^bP<0.05$, $^cP<0.01$ *vs* oridonin-only treatment group.

bifunctional in oridonin-induced cell death; it induces mitochondrial malfunction to promote the mitochondrial apoptotic pathway and simultaneously induces autophagy to protect the cells from dying.

ERK-p53 is involved in oridonin-induced apoptosis and autophagy

Extracellular signal-regulated kinase (ERK), which is a member of the mitogen-activated protein kinase family, and tumor suppressor protein p53 have been reported to be invovled in apoptosis and autophagy[24–26]. It is not known whether ERK and p53 participate in oridonin-induced cell apoptosis and autophagy. To address this question, the cells were pretreated with 5 μmol/L MEK inhibitor PD98059 (PD) and 5 μmol/L p53 inhibitor pifithrin-α (PFT-α) to inhibit ERK and p53 activation, respectively. As shown in Figure 3A, compared with the oridonin alone group, the PD pretreated group and the PFT-α pretreated group had significantly increased cell viabilities. The Western blot analyses demonstrated that p-ERK and p-p53 levels rose over time after oridonin administration, despite the lack of obvious changes in ERK and p53 levels (Figure 3B). Furthermore, the inhibition of ERK or p53 activation decreased apoptotic cell death (Figure 3C) and MDC positive ratios (Figure 3D). The Western blot analyses also showed that PD or PFT-α pretreatment reversed the oridonin-induced Bax translocation, cytochrome *c* release and declining Bcl-2 levels along with Beclin 1 activation and the conversion of LC3 I to LC3 II (Figure 3E). These results indicate that ERK and p53 are involved in oridonin-induced apoptosis and autophagy. In our previous work[15] oridonin-induced ERK contributed to p53 activation; thus, we introduced ERK-directed siRNA

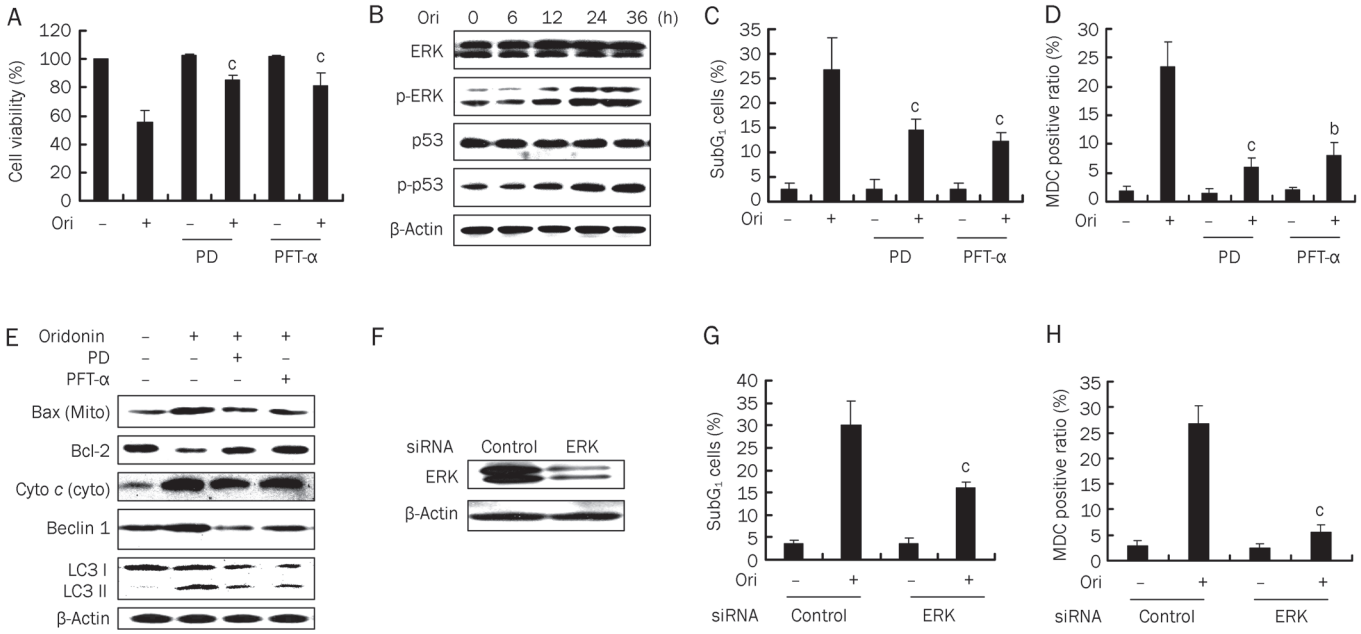

Figure 3. Effects of ERK and p53 on oridonin-induced apoptosis and autophagy. Cells were incubated with 50 μmol/L oridonin for 24 h in the presence or absence of PD 98059 (PD) (5 μmol/L) or pifithrin-α (PFT-α) (5 μmol/L) for 3 h. (A) Cell viabilities were measured by MTT assay. (B) Western blot analyses for the detection of ERK, p-ERK, p53, and p-p53 levels after oridonin treatment for the indicated time periods. β-Actin was used as a loading control. (C) Flow cytometric analyses of apoptotic cell ratios after PI staining ($SubG_1$ cells). (D) Autophagic cell ratios after MDC staining. (E) Western blot analyses for the detection of mitochondrial Bax, cytosolic cytochrome c, Beclin 1 and LC3 levels. β-actin was used as a loading control. (F) The cells were transfected with control or ERK-directed siRNA for 24 h, and then ERK levels were examined by Western blot analyses. β-Actin was used as a loading control. (G–H) After transfection, flow cytometric analyses of apoptotic (G) and autophagic (H) cell ratios were conducted after oridonin treatment. Mean±SEM. n=3. ^bP<0.05, ^cP<0.01 *vs* oridonin-only treatment group.

to confirm the above results (Figure 3F). As shown in Figure 3G and H, the oridonin-induced apoptotic and autophagic ratios decreased after transfection with ERK-directed siRNA. Collectively, these results provided strong evidence that the ERK-p53 pathway is involved in the apoptotic and autophagic pathways simultaneously.

NO contributes to ERK-p53 activation, which provokes NO production in oridonin-treated L929 cells

It should be noted that both NO, ERK and p53 participate in oridonin-induced apoptosis and autophagy. Hence, exploring the correlation between NO and ERK-p53 was the logical next step. Compared with the oridonin alone group, 1400w or *L*-NAME pretreated cells showed decreased ERK and p53 activities as shown by p-ERK and p-p53 protein levels, suggesting that NO contributes to ERK and p53 activation (Figure 4A). Next, to investigate whether ERK and p53 affected NO production, the cells were pretreated with PD, PFT-α or ERK-directed siRNA. As shown in Figure 4B and 4C, compared with the oridonin alone group, the PD, PFT-α, and ERK-

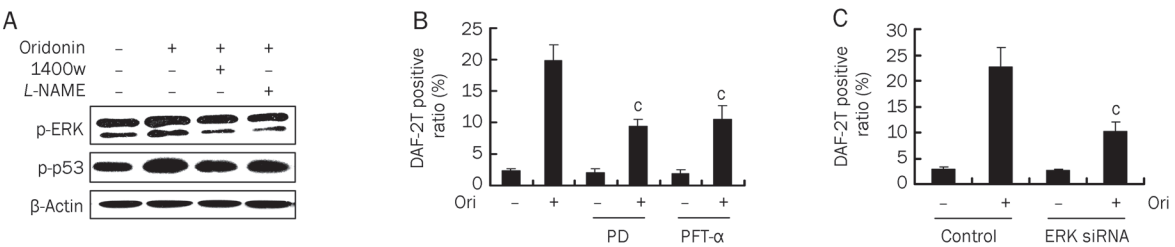

Figure 4. Relationship between nitric oxide and ERK-p53. (A) Cells were incubated with 50 μmol/L oridonin for 24 h in the presence or absence of 1400w (10 μmol/L) or *L*-NAME (20 μmol/L) for 3 h and then analyzed by Western blotting to assess the p-ERK and p-p53 levels. β-Actin was used as a loading control. (B) Cells were incubated with 50 μmol/L oridonin for 24 h in the presence or absence of PD98059 (PD) (5 μmol/L) and pifithrin-α (PFT-α) (5 μmol/L) for 3 h and then analyzed by flow cytometry to measure NO level changes after DAF-2DA staining. (C) Cells were transfected with control or ERK-directed siRNA for 24 h, and then flow cytometric analyses were carried out to evaluate NO levels after oridonin treatment. Mean±SEM. n=3. ^cP<0.01 *vs* oridonin-only treatment group.

directed siRNA pretreated groups showed markedly reduced DAF-2T ratios, indicating that ERK and p53 are positive regulators in oridonin-induced NO production.

Discussion

NO is a simple diatomic gas and free radical, and its biochemical features not only enable it to be an ideal signaling molecule but also exert a wide range of biological effects that can be summarized into three main categories: regulatory, deleterious and protective[27]. NO production during pre-apoptosis has been shown to be associated with mitochondrial dysfunction[28]. The mitochondrion works as a pivotal executioner in apoptotic signaling pathways. Here, we showed that the mitochondria-associated pro-apoptotic protein Bax translocated to the mitochondria, and cytochrome *c* was released from the mitochondria into the cytosol after oridonin treatment. In contrast, expression of the anti-apoptotic protein Bcl-2 was down-regulated. Moreover, the inhibition of NO production by 1400w or *L*-NAME reduced Bax translocation and cytochrome *c* release and increased Bcl-2 levels. These results indicate that oridonin-induced NO production results in mitochondrial dysfunction leading to apoptosis. As an inducer of apoptosis, little is known regarding the role of NO in the modulation of autophagy. In this study, we found that oridonin-induced NO production provoked autophagy as demonstrated by Beclin 1 activation and the conversion of LC3 I to LC3 II.

NO has been reported to induce the activation of ERK and p53 in various cell types, suggesting the involvement of ERK and p53 in oridonin-induced cell death[17, 29, 30]. Consistent with these observations, our results demonstrated that NO production regulated ERK and p53 activation, resulting in apoptotic cell death and protective autophagy in oridonin-treated L929 cells. Thomas *et al* have shown that low levels of NO induce ERK phosphorylation, whereas p53 phosphorylation occurs at considerably higher levels[16]. In our study, the activation of ERK and p53 via phosphorylation was accompanied by the sustained production of NO after oridonin administration. It should be noted that pretreatment with DTT but not 1400w or *L*-NAME was able to completely eliminate NO production and thoroughly inhibit apoptosis and autophagy. In addition to NO clearance, DTT is also a scavenger of reactive oxygen species (ROS) and peroxynitrite ($ONOO^-$)[31], and NO can react with superoxide radicals, leading to the formation of $ONOO^-$ [32]. Thus, we speculate that (in addition to NO) ROS and reactive nitrogen species (RNS), such as $ONOO^-$, may participate in ERK and p53 activation and mediate apoptosis and autophagy in oridonin-treated L929 cells.

Paradoxically, the NO-ERK-p53 pathway activated apoptotic cell death and autophagy, although autophagy has previously been demonstrated to antagonize apoptosis and thereby protect cells from dying[23]. This phenomenon might occur because of the crosstalk between apoptosis and autophagy, which was first recognized when Beclin 1 was initially identified as a Bcl-2-interacting protein[33]. The binding of the anti-apoptotic proteins Bcl-2 and Bcl-xL to Beclin 1 inhibits autophagosome formation to decrease Beclin 1-dependent autophagy[34]. Bcl-2, the first protein that has been found to interact with Beclin 1, has been shown to inhibit autophagy, in addition to its well-established role in apoptotic inhibition[9]. The oridonin treatment down-regulated Bcl-2 levels, whereas the 1400w or L-NAME pretreatment reversed this decline. The inhibition of ERK or p53 activation also reversed this decrease in Bcl-2 levels. These results support the idea that the inhibition of Bcl-2 levels by the NO-ERK-p53 pathway leads to Beclin 1 activation and thereby induces autophagy. By contrast, a series of studies have reported that cytoplasmic p53 suppresses autophagy and that nuclear p53 triggers it[7, 33, 34]. PFT-α has been shown to specifically block the transcriptional activity of p53[35]. In our study, the PFT-α pretreatment resulted in decreased apoptosis and autophagy, accompanied by reductions in Bax and cytochrome *c*, and increased Bcl-2 levels with the simultaneous suppression of expression of the autophagic protein Beclin 1 and the conversion of LC3 I to LC3 II. Although the total p53 levels did not significantly change over time, the p-p53 levels markedly increased, and p53 translocated from the cytoplasm to the nucleus (Figure 2S). It is conceivable that nuclear p53 both triggered autophagy and activated Bax-dependent apoptotic signaling pathways.

In conclusion, oridonin induced NO production, which mediated apoptosis and autophagy. NO production activated the ERK-p53 signaling pathway; in turn, ERK-p53 activation provided positive feedback for NO production, which stimulated apoptosis and autophagy. This dual action of NO as both an important player in apoptotic cell death and protective autophagy might aid in the development of an effective treatment to target NO toxicity.

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Author contribution

Yuan-chao YE, Wei-wei LIU, Bin-bin LIU, and Takashi IKEJIMA designed the research; Yuan-chao YE, Hong-ju WANG and Lei XU performed the research; Shin-Ichi TASHIRO and Satoshi ONODERA contributed some reagents; Yuan-chao YE analyzed the data and wrote the paper; Takashi IKEJIMA revised the paper.

Supplementary information

Supplementary figures are available at the Acta Pharmacologica Sinica website.

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