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Activation of Phosphoinositide 3-Kinase, Protein Kinase C, and Extracellular Signal-Regulated Kinase Is Required for Oridonin-Enhanced Phagocytosis of Apoptotic Bodies in Human Macrophage-Like U937 Cells

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Abstract. Our previous study showed that oridonin isolated from *Rabdosia rubescens* enhanced phagocytosis of apoptotic cells by macrophage-like U937 cells through tumor necrosis factor (TNF) α and interleukin (IL)-1β release. In this study, we further investigated signaling events involved in oridonin-augmented phagocytosis. Phagocytic stimulation was significantly suppressed by inhibitors, including a phosphoinositide 3-kinases (PI3K) inhibitor (wortmannin), a protein kinase C (PKC) inhibitor (stauroporine), and a phospholipase C (PLC) inhibitor (U73122). Exposure of U937 cells to oridonin caused an increase in PKC activity time-dependently, which was prevented by pretreatment with inhibitors of PI3K and PLC. Simultaneously, the activation of protein kinase B (PKB/Akt) and the increased expression of PLCγ2 were also blocked by wortmannin. In addition, an extracellular signal-regulated kinase (ERK) MAPK inhibitor, PD98059, suppressed oridonin-augmented phagocytosis, whereas the p38 MAPK inhibitor (SB203580) and c-Jun N-terminal kinase (JNK) MAPK inhibitor (SP98059) had no inhibitory effect. Furthermore, pretreatment of U937 cells with anti-TNFα and anti-IL-1β antibodies blocked oridonin-induced phagocytic stimulation as well as phosphorylation of ERK, but did not block the activation of PKC, indicating that these signaling events are triggered by oridonin, whereas secreted TNFα or IL-1β only activate the ERK-dependent pathway. Taken together, oridonin is suggested to enhance phagocytosis of apoptotic bodies by activating PI3K, PKC, and ERK-dependent pathways.

Keywords: oridonin, phagocytosis, phosphoinositide 3-kinase, protein kinase C, extracellular signal-regulated kinase

Introduction

Oridonin, a diterpenoid isolated from the plant *Rabdosia rubescens*, which has been used in traditional Chinese medicine for treatment of human cancers, has various pharmacological and physiological effects such as anti-bacteria, anti-tumor, scavenging active oxygen free radicals, and antimutagenetic effects (1 – 4). Previous reports have demonstrated that oridonin exerted inhibitory effects on various cancers such as human prostate carcinoma, non-small cell lung cancer, acute promyelocytic leukemia, and glioblastoma multiforme (5). In our previous study, we found that oridonin induced A375-S2 cell apoptosis via the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathway (6). In addition, we have shown that oridonin enhances 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 cells to phagocytose UV-irradiated apoptotic U937 cells by a mechanism involving tumor necrosis factor (TNF) α and interleukin (IL)-1β release (7). However, little is known about intermediates required for oridonin-induced TNFα and IL-1β

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secretion.

PI3K enzymes are divided in three classes. Class IA has been reported to regulate diverse cellular processes including cell growth, apoptosis, endocytosis, phagocytosis, and chemotaxis (8 – 10). Class IA mammalian enzymes are made of a catalytic subunit, p110, and a regulatory subunit, p85. PI3K inhibition reduced phagocytosis and neutrophil migration by polysaccharide purified from *Ganoderma lucidum* (11). Neutrophils isolated from mice lacking PI3K have been reported to be defective in phagocytosis-related chemotaxis (12). *Pi3k 1/2* null *Dictyostelium* cells are defective in chemoattractant-mediated activation of protein kinase B (PKB/Akt), which is important in modulating chemotaxis in *Dictyostelium* (13). Accumulated evidences have linked the roles of phospholipase Cγ (PLCγ) in phagocytosis (14). PI3K induced activation of PLCγ1, but not that of PLCγ2 in mast cells (15). Activated PLCγ translocated to the inside surface of the cell membrane and activated protein kinase C (PKC) and the calcium pathway in fibroblast Swiss 3T3 cells (16). Moreover, it has been shown that PKC inhibition prevents phagocytosis in human monocytes and polymorphonuclear cells (PMN) (17).

Several studies suggest that enzymes of the MAPK family have a critical role in modulating phaogocytosis and chemotaxis (18 – 20). This family includes extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK). The role of MAPK members in phagocytosis appears to be diverse, depending on cell types and stimulus. Polysaccharide from *Ganoderma lucidum* has been reported to enhance neutrophil engulfment by activating p38 MAPK but not ERK (11). However, phagocytosis of *Salmonella typhimuriumo* in murine BAC-1.2F5 cells triggered both ERK and JNK phosphorylation (21). In addition, it has been demonstrated that the mechanism involved in the phagocytosis of apoptotic cells differ from those involved in the phagocytosis of bacteria. P38 inhibition impaired phogocytosis of *E. coli* but not that of apoptotic cells by bone marrow derived macrophages (22).

In addition to regulation of endocytosis, ERK MAPK has been reported to modulate TNFα production in lipopolysaccharide-treated human monocytes (23). On the other hand, TNFα and IL-1β function to activate the ERK pathway. In human articular chondrocytes, ERK phosphorylation is significant after stimulation with TNFα and IL-1β (24).

U937 cells are induced to differentiate into macrophagic-phenotype cells that possess the ability to phagocytose apoptotic cells and microspheres. Differentiated U937 cells have been reported to provide an excellent tool for determining the function of macrophages (25).

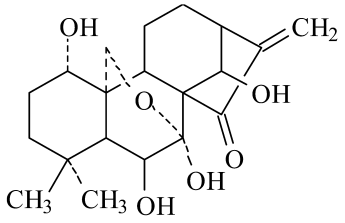
In the present study, we demonstrate that the PI3K pathway participates in oridonin-enhanced phaogocytosis. Among the PI3K downstream targets are PKB/Akt and PLCγ. Inhibitors of PI3K and PLC prevent PKC activation. In particular, ERK, but neither p38 nor JNK, is activated by stimulation of U937 cells with oridonin. Oridonin, therefore, recruits interacting moleucules and induces the following signaling events required for the enhanced phagocytosis: PI3K, PKB/Akt phosphorylation, PLCγ2 overexpression, PKC activation, and an increase in ERK activity.

Materials and Methods

Reagents

Oridonin was obtained from the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). The chemical structure of oridonin is shown in Fig. 1A. The purity of oridonin was measured by HPLC

A



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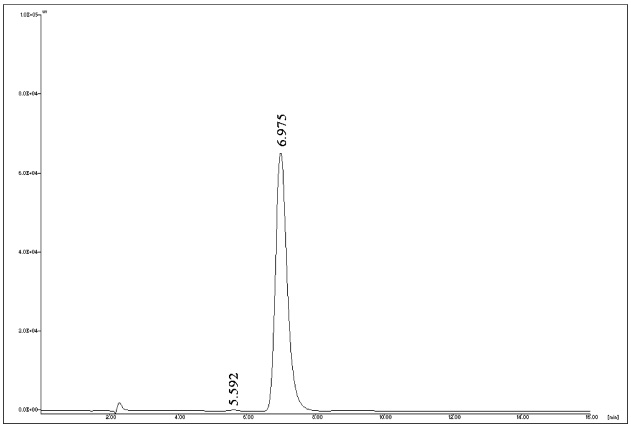


# Name	RT	Area [uv.Sec]	%Area
1	5.592	4650	0.313
2	6.975	1479713	99.687

Total Area of Peak = 1484363 [uv.Sec]

Fig. 1. Chemical structure (A) and HPLC profile (B) of oridonin. Determination and quantitation of oridonin were performed by HPLC (JASCO system, Tokyo) using a column (Dikma, Diamonsil TMC18, 200 × 4.6 mm, 5 μm) at 30°C with MeOH-H_2O (65:35, v/v) as the mobile phase at 0.8 ml/min and detection of the elute at 238 nm. The retention time (RT) for oridonin was 6.975 min.

and determined to be about 99% (Fig. 1B). Oridonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The 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.

PI3-K family inhibitor wortmannin, TPA, Hoechst 33258, and acridine orange were purchased from Sigma Chemical (St. Louis, MO, USA). ERK inhibitor PD98059, P38 MAPK inhibitor SB203580, and JNK inhibitor SP600125 were from Calbiochem (La Jolla, CA, USA). The PKC activity assay kit was obtained from Promega Corporation (Madison, WI, USA). Fetal calf serum (FCS) was purchased from the Dalian Biological Reagent Factory (Dalian, China). Polyclonal rabbit anti-human TNFα antiserum and anti-human IL-1β antiserum were prepared in our laboratory.

Culture of U937 cells

Human histocytic lymphoma (U937 #CRL-1593.2) cells were obtained from American Type Culture Collection, (Manasas, VA, USA). The cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) 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% CO_2 in a humidified atmosphere.

Induction of differentiation of U937 cells into macrophages

U937 cells were placed into the individual wells of the 6-well plates at a seeding density of 1×10^5 cells/ml. TPA was added into each well from a DMSO stock solution to reach the final concentration of 50 ng/ml (final DMSO concentration was 0.02%, which had no detectable effect on cell differentiation). The cells were incubated at 37°C for 15 h before being used in the experiments. Seventy percent of the cells adhered to the plate's bottom by 15 h.

Induction and measurement of apoptosis

Apoptosis of U937 cells was induced by UV irradiation and analyzed by observation of chromatin condensation and DNA fragmentation. U937 cells (1×10^6 cells/ml) were seeded into a culture plate and cultured overnight. Then, the cells were treated with UV irradiation (52.1 J/m^2) followed by incubation for the indicated time. UV-irradiated U937 cells were fixed with 3.7% paraformaldehyde at 37°C for 30 min, and then they were washed twice with PBS and stained with Hoechst 33258 at 167 μM at 37°C for 15 min. Nuclear morphology of the cells was observed under a fluorescence microscope (Olympus, Tokyo).

For measurement of DNA fragmentation, U937 cells treated with UV irradiation (52.1 J/m^2) (1×10^6 cells) were collected by centrifugation at $150 \times g$ for 5 min and washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The cell pellet was suspended in 100 μl cell lysis buffer pH 8.4 (10 mM Tris-HCl, pH 7.4; 10 mM EDTA, pH 8.0; 0.5% Triton X-100) and kept at 4°C for 30 min. The lysate was centrifuged at $7,200 \times g$ for 20 min. The supernatant was incubated with 20 μg/μl RNase A (Sigma) at 37°C for 60 min and then incubated with 20 μg/μl proteinase K (Merck, Rahway, NJ) at 37°C for 60 min. The supernatant was again mixed with 0.5 M NaCl and 50% isopropyl alcohol overnight at −20°C, followed by centrifugation at $7,200 \times g$ for 15 min. After drying, DNA was dissolved in TE buffer, pH 7.8 (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 mg/l ethidium bromide.

Phagocytosis assays

U937 cells in RPMI 1640 supplemented with 10% FCS were seeded into 6-well plates (2×10^5 cells/well), in which coverslips (20×20 mm) were placed, and cultured with 80 nM TPA at 37°C with 5% CO_2 atmosphere for 15 h. The monolayers of U937 cells on coverslips were washed three times with RPMI 1640 medium. The cells were treated with inhibitors for 1 h prior to the administration of oridonin. Then the cells were challenged with UV-irradiated U937 cells after being labeled with acridine orange (10 mg/l) and stood at 37°C for 1.5 h. Floating cells were washed three times with PBS, and the adhering U937 cells and macrophage-like cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h and then stained with 167 μM Hoechst 33258 at 37°C for 10 min. The coverlips were examined by fluorescence microscopy, and the number of macrophage-like cells that bound and/or engulfed U937 cells was scored. A mininum of 200 cells were counted in random fields in each experimental condition. Results are expressed as the percentage of macrophage-like cells that had taken up U937 cells.

Flow cytometric analysis

In some experiments, phagocytosis was further measured by flow cytometry. UV-irradiated U937 cells were labeled with acridine orange (10 mg/l) at room temperature for 5 min. After washing twice with PBS, the labeled cells were co-incubated with macrophages similarly to the assay described above. After washing, the cells on the plates were scraped and suspended in PBS, and then 10,000 cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

PKC activity assay

PKC activity assay was carried out according to the instructions of the PepTag® Non-Radioactive Protein Kinase C Assay Kit (Promega). Briefly, the cells were washed once with PBS and lysed in lysis buffer, including 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mg/l leupeptin (pH 7.5). Assays were then performed at 30°C in a total volume of 25 μl containing 5 μl PKC reaction 5 × buffer, 5 μl PLSRTLSVAAK 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 blotting analysis

Western blotting analysis was performed as previously described (26). Briefly, both adherent and non-adherent cells were harvested; washed twice with ice-cold 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 aprotinin and 10 μg/ml leupeptin) at 4°C for 60 min. The lysate was centrifuged at 15,000 × *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 electrophoretially transferred to nitrocellulose membranes. Proteins were detected with the primary antibodies against AKT/PKB, phospho-AKT/PKB, p38, phospho-p38, ERK, phospho-ERK, JNK, and phospho-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish perosidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized by using 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

Statistical analyses of data

All results were confirmed in at least three separate experiments. The data are expressed as means ± S.D. One way ANOVA followed by Tukey's *post-hoc* test was used for multiple comparisons. $P<0.05$ was considered significant.

Results

Effects of inhibitors of PI3K, PKC, and PLCγ on phagocytic stimulation

U937 cells were induced to undergo apoptosis by treatment with UV irradiation (52.1 J/m^2). Apoptosis was confirmed by examining the nuclear morphology after Hoechst 33258 staining and DNA fragmentation. In the control group, U937 cell nuclei were stained homogeneously with Hoechst 33258 (Fig. 1Aa), whereas after treatment with UV irradiation followed by 12-h culture, the cells showed marked blebbing of nuclei and apoptotic bodies (Fig. 2Ab). Furthermore, UV-irradiated U937 cells exhibited a typical DNA fragmentation that is a hallmark of apoptosis (Fig. 2B). The phagocytosis assay was performed using UV-irradiated apoptotic bodies as phagocytic targets. Treatment with 200 nM PI3K inhibitor (wortmannin), 100 nM PKC inhibitor (stauroporine), and 10 μM PLCγ inhibitor (U73122) significantly reversed the effect of oridonin (2.7 μM) (Fig. 2C). It was suggested that oridonin enhanced phagocytic activity of macrophage-like U937 cells through activating the PI3K-dependent signaling pathways.

PKC activity increased time dependently during oridonin-induced phagocytic stimulation

Since a PKC inhibitor, stauroporine, effectively inhibited the effect of oridonin on phagocytosis, PKC activity was assayed under the influence of these inhibitors. The specific fluorescent substrate phosphorylation was observed by treatment with 2.7 μM oridonin. The extent of phosphorylation continued to increase and reached the maximal upon 24 h of treatment with 2.7 μM oridonin (Fig. 3). Oridonin time-dependently increased PKC activity. However, PKC activation was attenuated by pretreatment of U937 cells with the PI3K inhibitor wortmannin, PLC inhibitor U73122, and PKC inhibitor stauroporine, suggesting PKC activation in response to oridonin required the PI3K pathway.

Expression of PLCγ2 and phosphorylation of PI3K downstream, PKB/Akt, were involved in oridonin-enhanced phagocytosis

It has been reported that PKB/Akt works downstream of PI3K in the signal transducing pathway to regulate endocytosis (17). To ascertain whether PKB/Akt activation was involved in the phagocytic stimulation, Western blot analysis was performed to examine phospho-PKB/Akt and PKB/Akt expression. The expression level of phospho-PKB/Akt began to increase from 3 to 12 h, peaked at 12 h, and then slightly declined, whereas the expression of PKB/Akt protein in

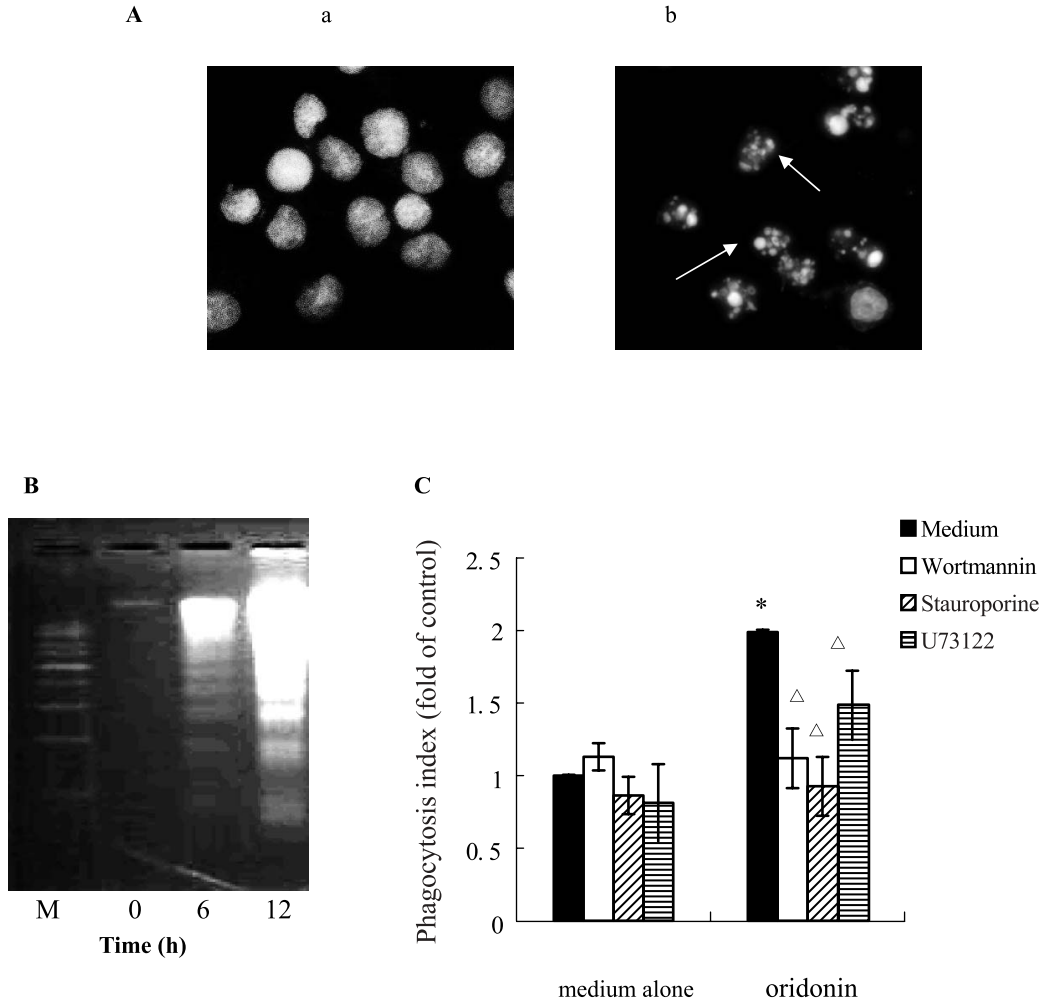


Fig. 2. Effects of inhibitors on enhanced phaogocytic activity of oridonin. A: UV-irradiation induced apoptosis in U937 cells. Morphological changes of U937 cell nuclei were observed at 0 h (a) and 12 h (b) after UV irradiation (52.1 J/m^2) followed by treatment with Hoechst 33258 staining at ×200 magnification. Arrows indicate fragmented nuclei. B: DNA fragmentation induced by UV irradiation. U937 Cells were incubated for 0, 6, and 12 h after UV irradiation (52.1 J/m^2). C: Macrophage-like U937 cells were preincubated with 200 nM wortmannin, 100 nM stauroporine, or 10 μM U73122 for 1 h and then incubated with 2.7 μM oridonin for 12 h. Following incubation, the cells were co-cultured with apoptotic bodies at 37°C for another 90 min. Phagocytosis was then determined by light microscopy. n = 3. Mean ± S.D. Asterisk indicates significant difference between cells treated with vehicle and oridonin-treated cells; Triangles indicate significant difference between the cells treated with inhibitors and the oridonin-treated cells.

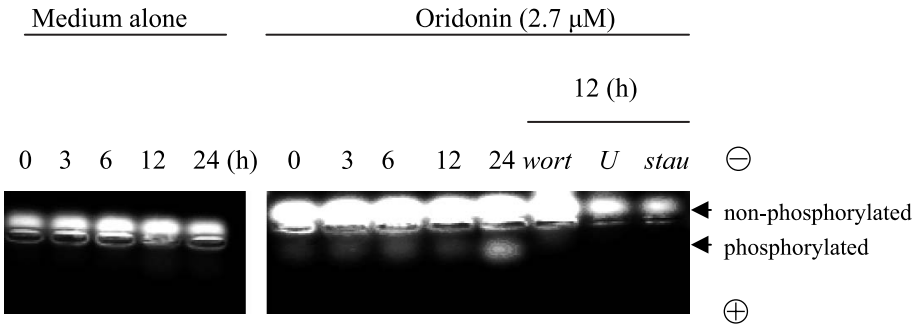


Fig. 3. Effects of oridonin on PKC activity in macrophage-like U937 cells. U937 cells were pretreated with wortmannin (wort), U73122 (U), and stauroporin (stau) for 60 min, followed by the addition of oridonin. After incubation for different time periods as indicated, PKC activity was determined using a PKC activity assay kit, according to the manufacturer's instructions. Triplicate experiments gave similar results.

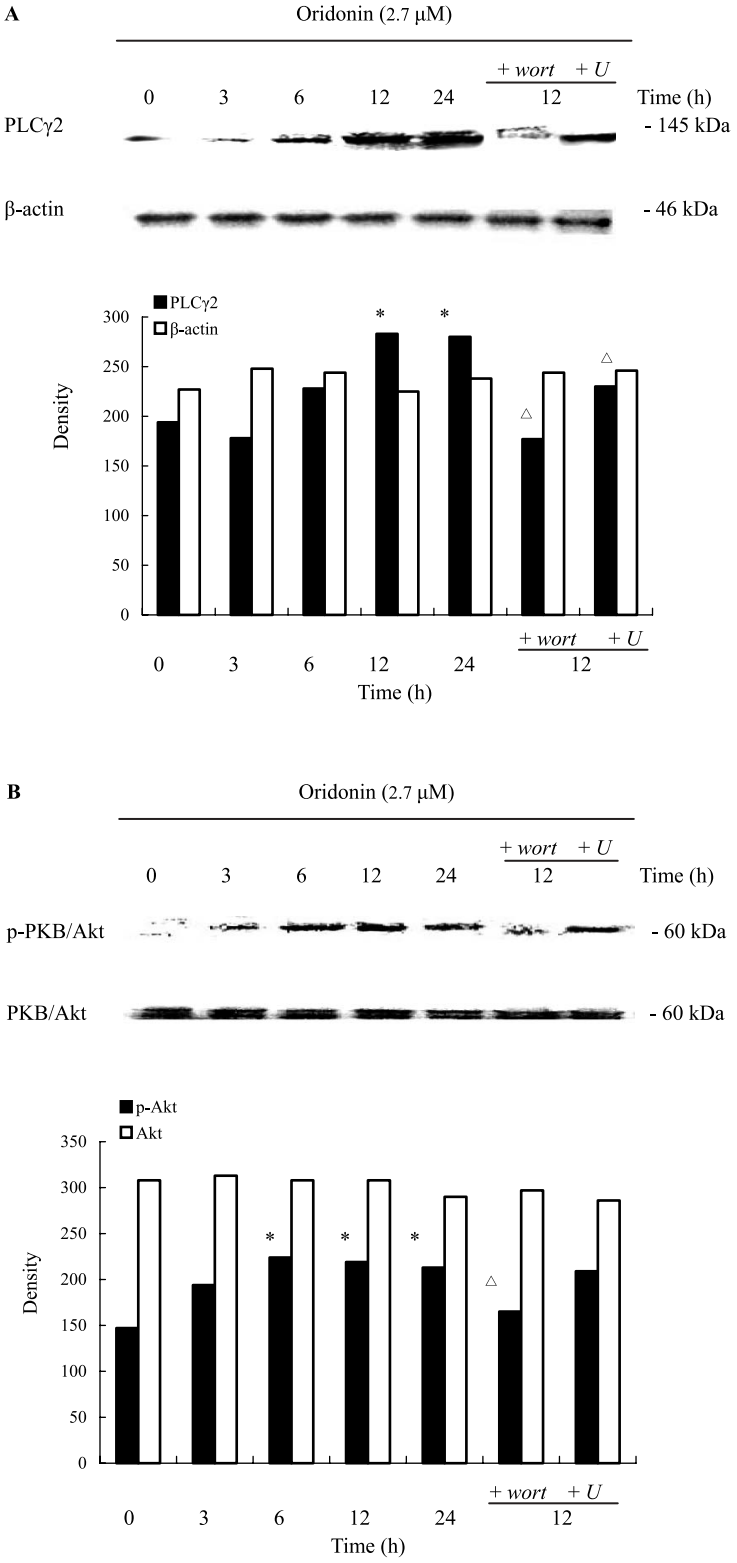


Fig. 4. Oridonin-induced up-regulated PLCγ2 expression and PKB/Akt phosphorylation. Cells were pretreated with 200 nM wortmannin (wort) or 10 μM U73122 (U) for 60 min and then incubated with 2.7 μM oridonin for the indicated time period. Cell lysates were separated by 12% SDS-PAGE; and PLCγ2 (A), phospho-PKB/Akt, and PKB/Akt proteins (B) bands were detected by Western blot analysis. Triplicate experiments gave similar results. Asterisks indicate significant difference between cells treated with vehicle and oridonin-treated cells; Triangles indicate significant difference between the cells treated with inhibitors and the oridonin-treated cells at 12 h.

a whole cell lysate did not change (Fig. 4B). In contrast, the expression of PLCγ2 protein continued to increase in that time period (Fig. 4A). Pretreatment with wortmannin, followed by 12 h incubation, reduced both the phosporylation of PKB/Akt and the expression of PLCγ2 protein, compared with those at 12 h in the oridonin-treated cells without inhibitors. The PLCγ inhibitor U73122 blocked PKC activation (Fig. 3), but had no effect on PKB/Akt phosphorylation (Fig. 4B). These results indicated that two PI3K downstream parallel targets, PKB/Akt and PLCγ2, were involved in response to oridonin, and PLCγ2 expression was required for PKC activation.

Effects of inhibitors of ERK, p38, and JNK on oridonin-enhanced phagocytosis

To determine whether MAPK family was involved in oridonin-enhanced phagocytosis, 10 μM ERK inhibitor PD98059, 10 μM p38 MAPK inhibitor SB203580, and 10 μM JNK inhibitor SP600125 were administrated. The percentage of phagocytosis was determined by light microscopy or by flow cytometery. Macrophage-like U937 cells were pretreated with 10 μM PD98059, SB203580, and SP600125 for 60 min, and then cultured with 2.7 μM oridonin for 12 h. The results showed that the stimulated effect of oridonin was unaffected in the presence of SB203580 or SP600125, while the ERK inhibitor PD98059 significantly reduced the enhancement (Fig. 5A). Simultaneously, fluorescence intensity of apoptotic bodies labeled with acridine orange was measured by flow cytometery. Apoptotic bodies engulfed by U937 cells corresponded to the peak. Pretreatment with PD98059 resulted in a decrease in the number of apoptotic bodies engulfed by U937 cells, but SB203580 and SP600125 had no effect (Fig. 5B). In addition, phagocytosis was further observed by incubating UV-irradiated apoptotic bodies that had been labeled with acridine orange with macrophage-like U937 cells tagged with Hoechst 33258 staining (Fig. 6A). As expected, addition of PD98059 decreased the number of macrophages that have engulfed apoptotic bodies (Fig. 6B). These observations indicated that oridonin-stimulated phagocytosis did not require p38 MAPK and JNK activation; however, it was dependent on ERK activity.

ERK phosphorylation was activated in oridonin-induced phagocytic stimulation

After U937 cells were exposed to 2.7 μM oridonin for 12 h, expression of phospho-ERK increased in a time-dependent manner, whereas the expression of phospho-p38 and phospho-JNK did not change in that time period (Fig. 7). Although PD98059, SB203580, and SP600125 were able to inhibit phosphorylation of their specific proteins, the expressions of ERK and JNK as well as p38 MAPK protein in a whole cell lysate did not change.

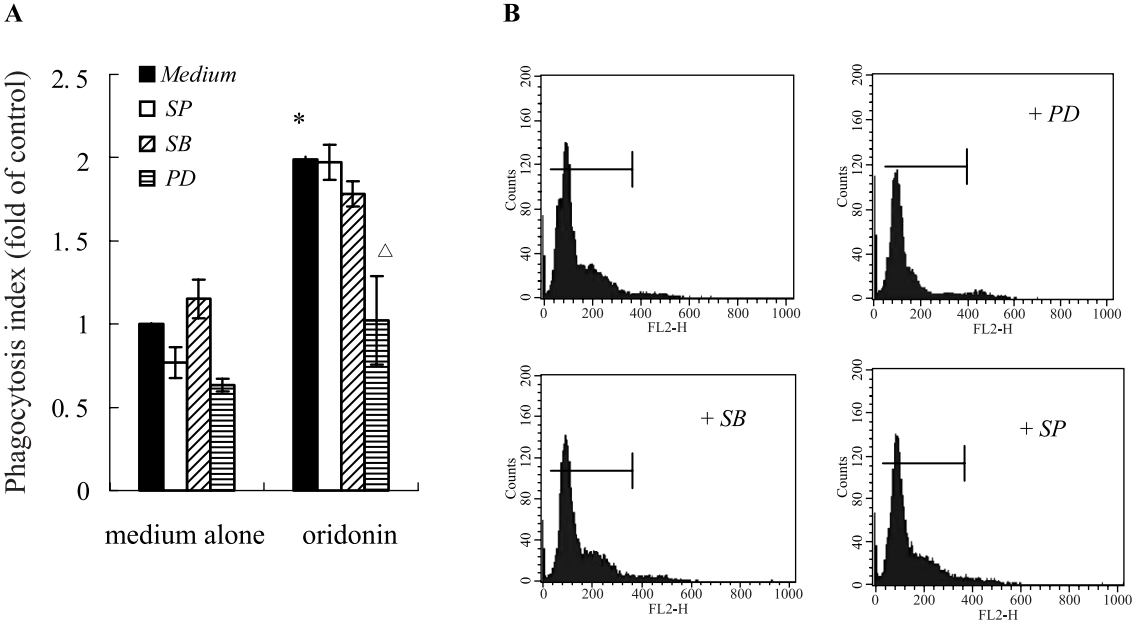


Fig. 5. Effect of MAPK inhibitors on oridonin-induced phagocytic stimulation. U937 cells were pretreated with 10 μM PD98059 (PD), SB203580 (SB), and SP600125 (SP) for 60 min, followed by the addition of oridonin. After 12 h, phagocytosis was determined by both light microscopy (A) and flow cytometry (B). n = 3. Mean ± S.D. Asterisk indicates significant difference between cells treated with vehicle and oridonin-treated cells; Triangle indicates significant difference between the cells treated with inhibitors and the oridonin-treated cells.

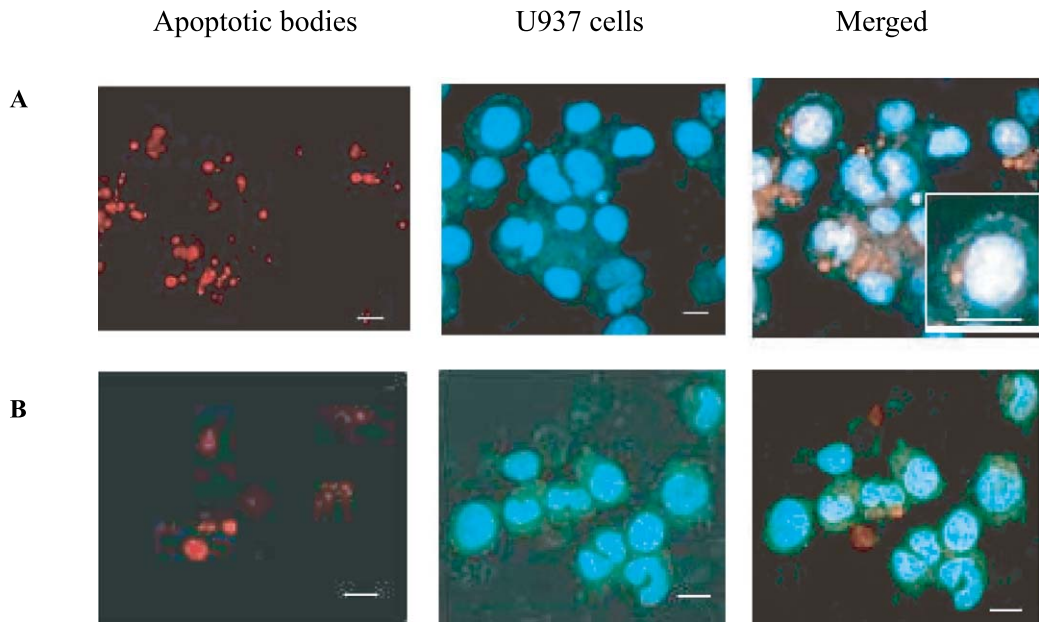


Fig. 6. Morphological observation of phagocytosis of apoptotic bodies by macrophage-like U937 cells. U937 cells were pretreated in the absence (A) and presence (B) of 10 μM PD98059 for 60 min, followed by the addition of oridonin. After 12 h, cells were co-cultured with acridine orange-labeled apoptotic bodies for 90 min, stained with Hoechst 33258, and observed by fluorescence microscopy at ×200 magnification. Insertion shows apoptotic bodies engulfed by the macrophage-like U937 cell at ×400 magnification. Bar represents 15 μm. The results are representative of those of three independent experiments.

Effects of anti-TNFα and anti-IL-1β antibodies on PKC activity and ERK phosphorylation

Our previous reports have demonstrated that oridonin exhibited remarkable stimulatory effect on phagocytosis through TNFα and IL-1β release (7). To confirm the involvement of moleucular events by oridonin, but not by secreted TNFα and IL-1β, U937 cells were incubated with anti-TNFα and anti-IL-1β antibodies for 12 h. As shown in Fig. 8A, although anti-TNFα and anti-IL-1β antibodies exert significant inhibitory effect on oridonin-enhanced phagocytosis, activity of the key phagocytic regulator PKC was not inhibited in the presence of these two antibodies (Fig. 8B). Both antibodies and the PKC inhibitor stauroporine caused decrease in ERK phosphorylation (Fig. 8C). These results suggest that the signal events involved were triggered by oridonin, and TNFα and IL-1β acted on the downstream ERK pathway to regulate phagocytosis.

Discussion

In this study, we have demonstrated that wortmannin, stauroporine, and U73122 significantly reduced phagocytic stimulation by oridonin. In addition, wortmannin and U73122 markedly reduced PLCγ2 expression and PKC activation, but U73122 had no effect on PKB/Akt phosphorylation. The MAPK family member, ERK was phosphorylated in oridonin-induced stimulation. These observations indicate that the following signal molecules are involved in the action of oridonin: PI3K, PLCγ2, PKB/Akt, PKC, and ERK.

PI3K is an important regulator of cellular processes including phagocytosis (27). Downstream of PI3K lies PKB/Akt and PLCγ2, a second messenger in phagocytosis, which in turn stimulate the process of endocytosis by up-regulation of PKC activation (23). Our results show that at 12 h after administration of wortmannin, stauroporine, and U73122, oridonin-augmented phagocytosis was suppressed markedly, indicating that the PI3K signal cascade including PI3K, PLCγ, and PKC was involved in the enhancement. It was further confirmed by up-regulated PLCγ2 expression, which was reversed by the PI3K inhibitor wortmannin or PLC inhibitor U73122. In addition, PKB/Akt has been reported to modulate Fcγ receptor-mediated endocytosis (17). At 12 h after oridonin treatment, PKB/Akt phosphorylation was significant, indicating that PKB/Akt was activated in this process. However, further study will be required to elucidate the relationship of PKB/Akt phosphorylation and the phagocytic action of oridonin. Accumulated evidence has linked the crucial roles of PKC in macrophagic phagocytosis (28 – 30). The results in this study show that PKC activity in response to oridonin increased in a time-dependent manner. However, by inhibiting PI3K activity, wortmannin prevented the expression of PLCγ2 and, in turn,

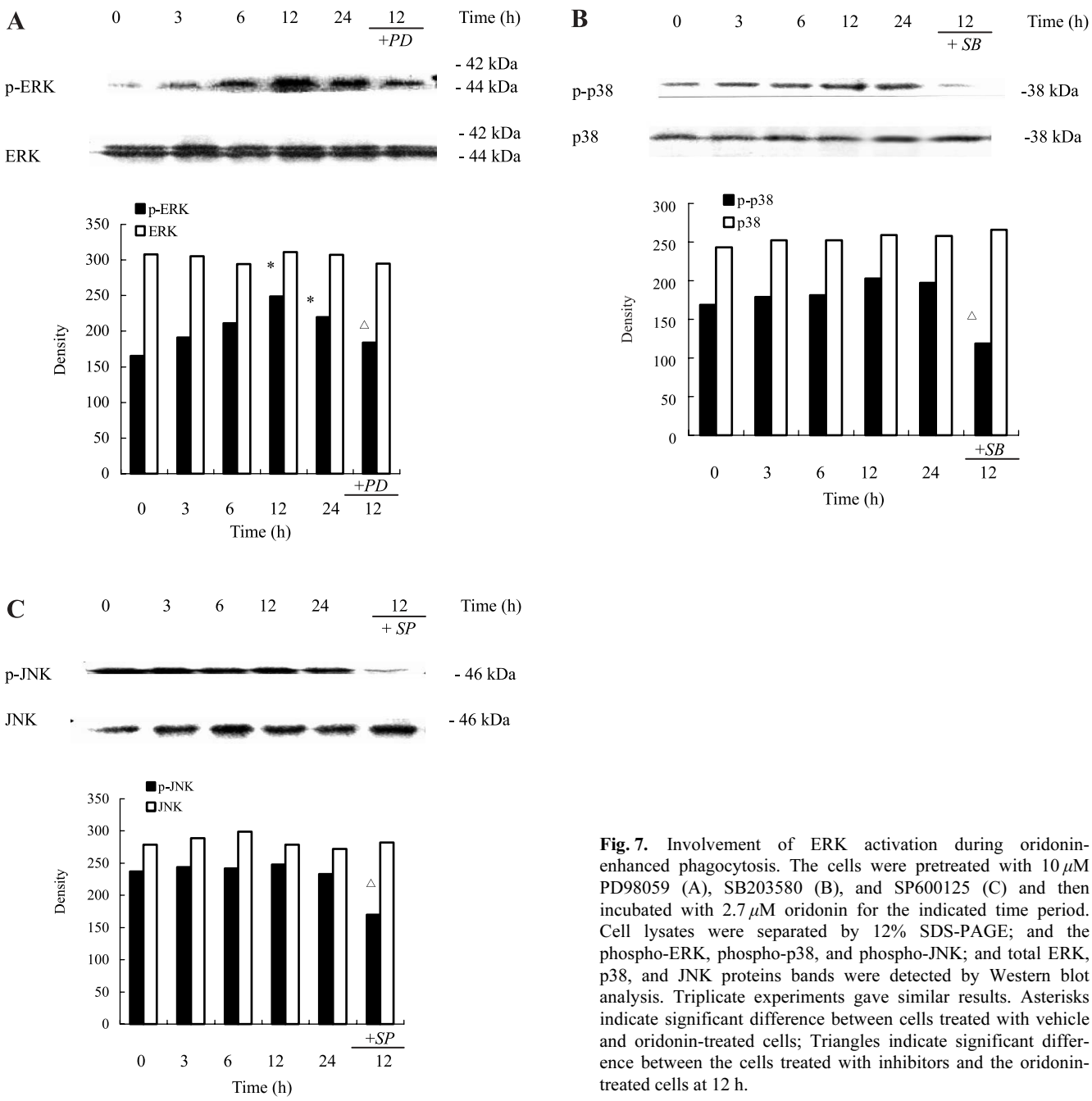


Fig. 7. Involvement of ERK activation during oridonin-enhanced phagocytosis. The cells were pretreated with 10 μM PD98059 (A), SB203580 (B), and SP600125 (C) and then incubated with 2.7 μM oridonin for the indicated time period. Cell lysates were separated by 12% SDS-PAGE; and the phospho-ERK, phospho-p38, and phospho-JNK; and total ERK, p38, and JNK proteins bands were detected by Western blot analysis. Triplicate experiments gave similar results. Asterisks indicate significant difference between cells treated with vehicle and oridonin-treated cells; Triangles indicate significant difference between the cells treated with inhibitors and the oridonin-treated cells at 12 h.

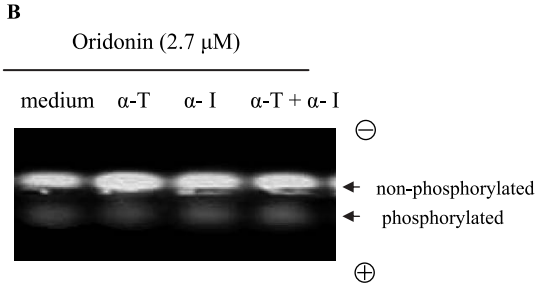
inhibited the subsequent activation of PKC, resulting in inhibition of phagocytosis. In addition, our previous study showed that PI3K and the downstream p53 were involved in oridonin-induced A375-S2 cell apoptosis (6). These results indicated that oridonin-mediated phagocytosis and apoptosis share a common PI3K pathway and then diverge downstream of PI3K.

On the other hand, it is reported that downregulation of MEK/ERK activation blocked BAC-1.2F5 cell phagocytosis of *Salmonella typhimurium* (21). Also, studies in neutrophil in response to a variety of stimuli have indicated that phagocytosis is regulated by p38 MAPK (31, 32). Therefore, the MAPK family is investigated in the present study. Neither the p38 inhibitor SB203580 nor the JNK inhibitor SP600125 blocked oridonin-stimulated phagocytosis of apoptotic bodies, whereas the MEK/ERK inhibitor PD98059 did. Western blot analysis further confirmed that phosphorylation of ERK increased, but those of JNK and p38 did not change. These results indicated that phagocytic stimulation required activation of ERK but not that of p38 MAPK, which might be dependent on the type of

A

	Medium alone	Oridonin (2.7 μM)			
		control	α-T	α- I	α-T + α- I
Phagocytosis (%)	13.3 ± 0.8	27.0 ± 1.1*	16.0 ± 2.1△	10.2 ± 1.4△	15.3 ± 0.2△

B



C

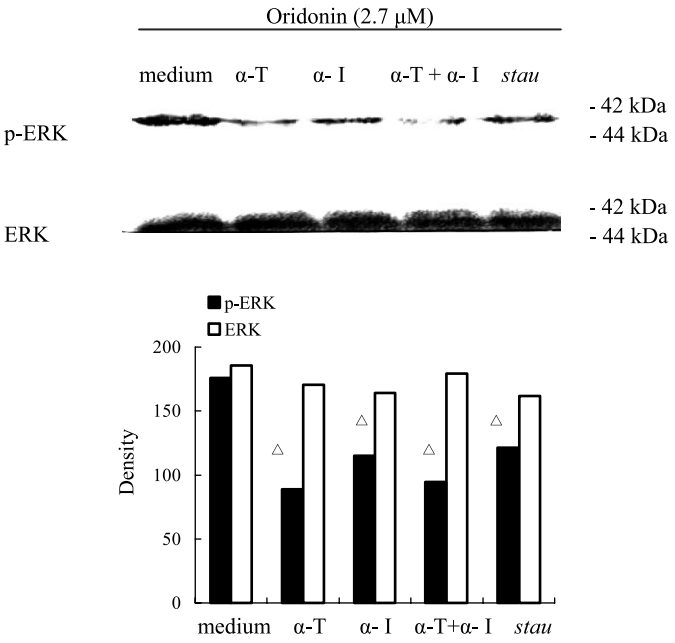


Fig. 8. Effects of anti-TNFα and anti-IL-1β antibodies on PKC or ERK activation. A: U937 cells were cultured for 12 h in the presence of oridonin with anti-TNFα antiserum (α-T) (1:10000 dilution), anti-IL-1β antiserum (α-I) (1:1000 dilution), or anti-TNFα plus anti-IL-1β antiserum. n = 3, Mean ± S.D. B: PKC activity was assayed by using the PKC activity assay kit. C: phospho-ERK and ERK were analyzed by Western blotting. Triplicate experiments gave similar results. Asterisk indicates significant difference between cells treated with vehicle and oridonin-treated cells; Triangles indicate significant difference between the cells treated with antibodies or inhibitors and the oridonin-treated cells.

macrophages and phagocytic stimuli.

Our previous study demonstrated that oridonin stimulated the secretion of TNFα and IL-1β in macrophage-like U937 cells (7). In this study, anti-TNFα and anti-IL-1β antibodies neutralized TNFα and IL-1β activities resulting in inhibition of phagocytosis, whereas they did not attenuate PKC activation, indicating that participating signal cascades including PI3K, PLCγ2, PKB/Akt, and PKC are independent of secreted TNFα and IL-1β. However, the downstream target of ERK involves pathways influencing TNFα or IL-1β production, and TNFα and IL-1β conversely phosphorylate ERK MAPK (24, 33). In this study, decreased ERK phosphorylation in the presence of anti-TNFα and anti-IL-1β antibodies suggests that TNFα and IL-1β function downstream of the ERK pathway to regulate phagocytosis. Furthermore, it is possible that oridonin stimulates TNFα and IL-1β release through activating ERK MAPK. Further study remains required.

The human macrophage cell line U937 has been used extensively as an in vitro model for human macrophage differentiation and effector functions including phagocytosis (34). U937 cells have been shown to have a relatively efficient phagocytosis pathway for processing pathogen and particles (35). In this study, we used U937 as macrophage-like cells to investigate oridonin-enhanced phagocytosis of apoptotic bodies through the PI3K-dependent pathway. Thus, we hope the present results may provide a basic view for a further study on the phagocytic effect of oridonin.

Taken together, the present study shows that oridonin enhances phagocytosis of apoptotic bodies by macrophage-like U937 cells through activation of the PI3K pathway involving PKB/Akt phosphorylation and PLCγ2 overexpression, leading to the activation of downstream PKC. Simultaneously, ERK activation is involved in the process.

References

1 Zhang Y, Wang J, Lou LG, Zhang TM, Hou JW, Xin WJ. Scavenging effect of oridonin on active oxygen free radicals. Henan Medical Research. 1999;8:100–104.

2 Fuji K, Node M, Sai M, Fujita E, Takeda S, Unemi N. Terpenoids. LIII. Antitumor activity of trichorabdals and related compounds. Chem Pharm Bull. 1989;37:1472–1476.

3 Fujita T, Takeda Y, Sun HD, Minami Y, Marunaka T, Takeda S, et al. Cytotoxic and antitumor activities of *Rabdosia* diterpenoids. Planta Med. 1988;54:414–417.

4 Osawa K, Yasuda H, Maruyama T, Morita H, Takeya K, Itokawa H. Antibacterial trichorabdal diterpenes from *Rabdosia Trichocarpa*. Phytochemistry. 1994;36:1287–1291.

5 Ikezoe T, Chen SS, Tong XJ, Heber D, Taguchi H, Koeffler HP. Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. Int J Oncol. 2003;23:1187–1193.

6 Zhang CL, Wu LJ, Zuo HJ, Tashiro S, Onodera S, Ikejima T. Cytochrome *c* release from oridonin-treated apoptotic A375-S2 cells is dependent on p53 and extralellular signal-regulated kinase activation. J Pharmacol Sci. 2004;96:155–163.

7 Liu YQ, You S, Zhang CL, Tashiro S, Onodera S, Ikejima T. Oridonin enhances phagocytosis of UV-irradiated apoptotic U937 cells. Biol Pharm Bull. 2005;28:461–467.

8 Buczynski G, Grove B, Nomura A, Kleve M, Bush J, Firtel RA, et al. Inactivation of two *Dictyosteliumdiscoideum* genes, *DdPIK1* and *DdPIK2*, ncodingproteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosometransport, and actin cytoskeleton organization. J Cell Biol. 1997;136:1271–1286.

9 Vanhaesebroeck B, Waterfield MD. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res. 1999;253:239–254.

10 Leevers SJ, Vanhaesebroeck B, Waterfield MD. Signaling through phosphoinositide 3-kinases: The lipidstake centre stage. Curr Opin Cell Biol. 1999;11:219–225.

11 Hsu MJ, Lee S, Lee ST, Lin W. Signaling mechanism of enhanced neutrophil phagocytosis and chemotaxis by the polysaccharide purified from *Ganoderma lucidum*. Brit J Pharmacol. 2003;139:289–298.

12 Tohru Y, Osamu I, Hajime NYDC, Mikinori K. Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. FEBS Lett. 2000;467:253–258.

13 Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, et al. Central role for G protein-coupled phosphoinositide 3-kinase in inflammation. Science. 2000;287:1049–1053.

14 Perskvist N, Zheng LM, Stendahl O. Activation of human neutrophils by *Mycobacterium tuberculosis* H37Ra involves phospholipase Cg2, Shc adapter Protein, and p38 mitogen-activated protein kinase. J Immunol. 2000;164:959–965.

15 Barker SA, Caldwell KK, Pfeiffer JR, Wilson BS. Wortmannin-sensitive phosphorylation, translocation, and activation of PLCγ1, but not PLCγ2, in antigen-stimulated RBL-2H3 mast cells. Mol Biol Cell. 1998;9:483–496.

16 Kanazawa H, Ohsawa K, Sasaki Y, Kohsaka S, Imai Y. Macrophage/microglia-specific protein Iba1 enhances membrane ruffling and rac activation via phospholipase C-γ-dependent pathway. J Biol Chem. 2002;277:20026–20032.

17 Ganesan LP, Wei G, Ruma A, Pengal RA, Leni Moldovan L, Moldovan N, et al. The serine/threonine kinase Akt promotes Fcγ receptor-mediated phagocytosis in murine macrophages through the activation of p70S6 kinase. J Biol Chem. 2004; 279:54416–54425.

18 Nick JA, Avdi NJ, Gerwins P, Johnson GL, Worthen GS. Activation of a p38 mitogen activated protein kinase in human neutrophils by lipopolysaccharide. J Immunol. 1996;156:4867–4875.

19 Schnyder B, Meunier C, Car BD. Inhibition of kinases impairs neutrophil activation and killing of *Staphylococcus aureus*. Biochem J. 1998;331:489–495.

20 Downey GP, Butler JR, Tapper H, Fialkow L, Saltiel AR, Rubin BB, et al. Importance of MEK in neutrophil microbicidal responsiveness. J Immunol. 1998;160:434–443.

21 Procyk KJ, Kovarik P, Gabain A, Baccarini M. *Salmonella typhimurium* and lipopolysaccharide stimulate extracellularly regulated kinase activation in macrophages by a mechanism involving phosphatidylinositol 3-kinase and phospholipase D as novel intermediates. Infect Immun. 1999;67:1011–1017.

22 Blander J, Medzhitov R. Regulation of phagosome maturation by signals from Toll-like receptors. Science. 2004;304:1014–1018.

23 Bruggen T, Nijenhuis S, Raaij E, Verhoef J, Asbeck BS. Lipopolysaccharide-induced tumor necrosis alpha production by human monocyte involves the Raf-1/MEK1-MEK2/ERK1-ERK2 pathway. Infect Immun. 1999;67:3824–3829.

24 Geng Y, Valbracht J, Lota M. Selective activation of the mitogen-activated protein kinase subgroups c-Jun NH2 terminal kinase and p38 by IL-1 and TNF in human articular chondrocytes. J Clin Invest. 1996;98:2425–2430.

25 Passmore JS, Lukey PT, Ress SR. The human macrophage cell line U937 as an in vitro model for selective evaluation of mycobacterial antigen-specific cytotoxic T-cell function. Immunology. 2001;102:146–156.

26 Suzuki K, Hino M, Kutsuna H, Hato F, Sakamoto C, Takahashi T, et al. Selective activation of p38 mitogen-activated protein kinase cascade in human neutrophils stimulated by IL-1beta. J Immunol. 2001;167:5940–5947.

27 Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem. 1999;274:8347–8350.

28 Liu MK, Herrera-Velit P, Brownsey RW, Reiner NE. CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. J Immunol. 1994;153:2642–2652.

29 Greenberg S, Chang P, Silverstein SC. Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. J Exp Med. 1993;177:529–534.

30 Zhou MJ, Brown EJ. CR3 (Mac-1, alpha M beta 2, CD11b/CD18) and Fc gamma RIII cooperate in generation of a neutrophil respiratory burst: requirement for Fc gamma RIII and tyrosine phosphorylation. J Cell Biol. 1994;125:1407–1416.

31 Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, et al. P38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. J Immunol. 1998;160:1982–1989.

32 McLeish KR, Knall C, Ward RA, Gerwins P, Coxon PY, Klein JB, et al. Activation of mitogen-activated protein kinase cascades during riming of human neutrophils by TNF-α and GM-CSF. J Leukocyte Biol. 1998;64:537–545.

33 Carter AB, Monick MM, Hunninghake GW. Both Erk and p38 kinases are necessary for cytokine gene transcription. Am J Respir Cell Mol Biol. 1999;20:751–758.

34 Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer. 1976;17:565–577.

35 Harris PE, Colovai AI, Maffei A, Liu Z, Suciu Foca N. MHC class I presentation of exogenous and endogenous protein-derived peptides by a transfected human monocyte cell line. Immunology. 1995;86:606–611.