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Ursolic acid promotes the release of macrophage migration inhibitory factor via ERK2 activation in resting mouse macrophages

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Abstract

Macrophage migration inhibitory factor (MIF) plays some pivotal roles in innate immunity and inflammation. Ursolic acid (UA), an anti-inflammatory triterpene carboxylic acid, was recently reported to induce the release of pro-inflammatory mediators in resting macrophages (Mϕ). We investigated the effects of UA on MIF protein release in resting RAW264.7 mouse Mϕ, and found that it decreased intracellular MIF protein levels and promoted the release of MIF into the culture media in dose- and time-dependent manners, without affecting mRNA levels. Further, the triterpene strikingly induced activation of mitogen-activated protein kinase kinase 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) within 30 min, whereas no phosphorylation of p38 MAPK or JNK protein was observed. In addition, UA-promoted MIF release was significantly inhibited by PD98059, a MEK1/2 inhibitor, while siRNA for ERK2, but not ERK1, significantly decreased the amount of MIF protein released. These results suggest that UA triggers the release of intracellular MIF protein through the ERK2 activation.

Keywords: Macrophage; Macrophage migration inhibitory factor; Ursolic acid; Extracellular signal-regulated kinase 2; Dietary factor

1. Introduction

Macrophage migration inhibitory factor (MIF), initially identified as a soluble factor derived from activated T lymphocytes, is a cytokine that plays critical roles in several inflammatory conditions by regulating the activation of macrophages (Mϕ) and T cells [1–4]. MIF shows a variety of biological functions, including tumoricidal and parasiticidal activities [5–7], as well as induction of tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and toll-like receptor 4 (TLR4), a signal-transducing molecule of the lipopolysaccharide receptor complex [8–10], though most of its molecular mechanisms remain unclear [11]. This pro-inflammatory cytokine is physiologically unique because of its ability to functionally inactivate the tumor suppressor protein p53 [12,13]. These findings suggest that MIF is a key molecule in the convergence of inflammatory processes with those of carcinogenesis. Although the production of MIF has been reported to be induced by oxidative stress, endotoxins, and pro-inflammatory cytokines [14–16], much remains unknown regarding the underlying molecular mechanism. Further, there have been no reports of the small molecules that regulate MIF production.

Some triterpenoids, which are ubiquitously distributed throughout nature, have been long considered to have anti-inflammatory activities [17] and are often utilized in folk medicine in many Asian countries, though their molecular

Abbreviations: COX, cyclooxygenase; DMEM, dulbecco's modified eagle medium; DMSO, dimethylsulfoxide; ELISA, Enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; ICE, IL-1β-converting enzyme; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MIF, macrophage migration inhibitory factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PMA, phorbol 12-myristate-13-acetate; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; siRNA, short small interfering RNA; TACE, TNF-α-converting enzyme; TLR, toll-like receptor; TNF, tumor necrosis factor; UA, ursolic acid

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mechanisms remain to be fully elucidated. Ursolic acid (UA; 3β-hydroxy-12-urs-12-en-28-oic acid), a pentacyclic triterpene carboxylic acid found in various plants in the form of an aglycone or as glycosides [18–25], is well known to possess many important biological functions such as anti-cancer, anti-inflammatory, hepato-protective, anti-ulcer, hypolipidemic, and anti-atherosclerotic activities [17,26–28]. Further, it has been reported that UA attenuates the expression of inducible nitric oxide synthase (iNOS) and COX-2 expression through nuclear factor-kappa B (NF-κB) repression in lipopolysaccharide (LPS) or interferon-γ (IFN-γ) activated mouse Mϕ [29,30]. Intriguingly, You et al. recently reported that UA induced nitric oxide (NO) and TNF-α production via NF-κB activation in resting RAW264.7 mouse Mϕ [31], implying that the effects of UA on NF-κB activities are dependent on the biological status of Mϕ.

In the present study, we investigated the effects of UA on the induction and production of MIF in resting RAW264.7 Mϕ, in an attempt to determine the action mechanism by which UA promotes MIF protein release.

2. Materials and methods

2.1. Reagents

Dulbecco's modified eagle medium (DMEM), Opti-MEM®, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). LPS was from Sigma–Aldrich (St. Louis, MO). UA, actinomycin D, and cycloheximide were obtained from Funakoshi (Tokyo, Japan). PD98059, SB203580, and SP600125 came from Calbiochem (La Jolla, CA). Non-specific control, extracellular signal-regulated kinase 1 (ERK1), and ERK2 small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Lipofectamine™ 2000 was obtained from Invitrogen (Carlsbad, CA). Antibodies were purchased from the following sources: rabbit anti-MIF was from Cosmo Bio (Tokyo, Japan); rabbit anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38 MAPK, rabbit anti-p38 MAPK, rabbit anti-active c-Jun NH_2-terminal kinase 1/2 (JNK1/2), rabbit anti-JNK1/2, rabbit anti-phospho-mitogen-activated protein kinase kinase 1/2 (MEK1/2), rabbit anti-MEK1/2, rabbit anti-phospho-c-Jun (Ser^{73}), rabbit anti-phospho-mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), and anti-rabbit antibody horseradish peroxidase-linked IgG antibodies came from Cell Signaling Technology Inc. (Beverly, MA); goat anti-β-actin antibody was from Santa Cruz Biotechnology Inc.; anti-goat IgG was obtained from Dako (Glostrup, Denmark). Oligonucleotide primers were synthesized by Proligo (Kyoto, Japan). A Qiashredder™ and RNeasy Mini Kit® were from Qiagen (Hilden, Germany). An RNA polymerase chain reaction (PCR) Kit (AMV) Ver. 2.1 and a Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit® came from TaKaRa Bio (Shiga, Japan), while a rat/mouse MIF immunoassay kit was from Sapporo Immunodiagnostic Laboratory (Sapporo, Japan). Mouse interleukin (IL)-1β, mouse IL-6, and mouse TNF-α enzyme-linked immunosorbent assay kits were purchased from Endgen Inc. (Wobum, MA). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) unless specified otherwise.

2.2. Cell culture

RAW264.7 Mϕ were obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% FBS, L-glutamine (330 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

2.3. Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 Mϕ were seeded onto a 96-well plate at a density of 2×10^5 cells/ml in 200 μl of DMEM including 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. After 12 h, the cells were washed twice with phosphate buffered saline (PBS) and cultured in serum-free medium. The cells were then treated with UA (0.16, 0.8, or 4 μM) that was dissolved in dimethylsulfoxide (DMSO) (0.1%, v/v, as a final concentration). Control cells were treated only with 0.1% (v/v) DMSO, which showed no effect on the assay systems (data not shown). After incubation for 12 h, the supernatant (5 μl) was subjected to ELISA and the amounts of MIF were measured according to the protocol of the kit. Similarly, the concentrations of TNF-α, IL-1β, and IL-6 were measured using the supernatant (50 μl) according to the protocol of the kit. For time-course experiments, the cells were treated with UA (0.8 or 4 μM) for 0, 3, 6, 12, or 24 h.

2.4. Cell viability

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue-exclusion, and LDH release tests. For the MTT assay, after the cells were washed twice with PBS, 50 μl of serum-free medium and 5 μl of MTT stock solution (5 mg/ml) were added to each culture, and the cells were further incubated at 37 °C for 1 h. After incubation, 100 μl of DMSO was added and they were sonicated for 5 min. Fifty microliters of HCl/2-propanol (3.4 μl/ml) was then added to each well. Visible absorbance at 600 and 650 nm was measured using an MPR-A4*i* microplate reader (TOSHO, Japan). For the trypan blue-exclusion assays, the harvested cell suspensions

(50 μl) were mixed with 50 μl of trypan blue (0.2%) and the cells were observed under a light microscope. LDH release was measured according to the protocol of the kit. Briefly, to detect the membrane-toxic effect of UA, RAW264.7 Mϕ were cultured with UA at a density of 1×10^6 cells/ml for 12 h. Maximal LDH release from the cells was determined following the addition of a 1% Triton lysis solution to the non-treated cells. Supernatants were transferred to another 96-well plate and a reconstituted substrate mixture was added. After incubation at room temperature for 30 min, visible absorption at 492 nm was measured using an MPR-A4*i* microplate reader (TOSHO).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 Mϕ were cultured with or without UA at a density of 1×10^6 cells/ml for 12 and 24 h. Total cellular RNA was extracted from the cells using a Qiashredder™ and RNeasy Mini Kit®. *Glyceraldehyde phosphate dehydrogenase* (*GAPDH*) transcript served as the internal control. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was then performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA). PCR was conducted with 0.25 μM of MIF sense (5′-CACCATGCCTATGTTCATCGTGAACA-3′) and 0.25 μM of MIF anti-sense (5′-GGGCTCAAGGCGAAGGTGGAACCGTT-3′) primers, 381 bp, and 0.1 μM of GAPDH sense (5′-TCTTTGGCCTACCTATAACTGG-3′) and 0.1 μM of GAPDH anti-sense (5′-CTAGACTGCTACCATCCGTC-3′) primers, 496 bp. PCR conditions consisted of 14 or 17 cycles, with 1 min of denaturation at 94 °C, 2 min of annealing at 53 °C, and 1 min of primer extension at 72 °C. Amplified cDNA was electrophoresed on 2% agarose gels. Image analysis was performed using NIH Image.

2.6. Western blotting

For Western blot analysis, 1.0×10^6 cells were lysed in lysis buffer [protease inhibitor, phosphatase inhibitor (TaKaRa Bio, Shiga, Japan), 10 mM Tris (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1 mM sodium vanadate (V)] and the lysates were boiled for 5 min. Protein concentration was determined using a DC protein assay (Bio-Rad Laboratories Ltd., Kyoto, Japan), with γ-globulin used as the standard. Denatured proteins (10 or 40 μg) were separated using SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, MA). After blocking overnight at 4 °C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were first incubated with each antibody [rabbit anti-MIF, goat anti-β-actin, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-active JNK1/2, anti-JNK1/2, anti-phospho-MAPKAPK-2, and anti-phospho-c-Jun (Ser^{73}) antibodies] at dilutions of 1:1000. The second incubation was performed with horseradish peroxidase-conjugated secondary anti-rabbit IgG or anti-goat IgG antibody (1:1000 dilution each). The blots were developed using an ECL Advance Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was analyzed using NIH Image.

2.7. RNA interference (RNAi) of protein kinases

Transfection of siRNA was conducted with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's specifications. Briefly, 24 h before transfection, the cells were seeded onto a 96- or 24-well plate at a density of 2.0×10^5 cells/ml or 1.0×10^5 cells/200 μl in DMEM that included 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2, in order to reach 40–60% confluence on the day of transfection. An siRNA solution (non-specific control, ERK1, ERK2, or combined ERK1/2) was added to 50 μl of serum-free Opti-MEM I® (1 μM final concentration). In another microtube, 2 μl of Lipofectamine™ 2000 was diluted in 50 μl of serum-free Opti-MEM I®. After adding the siRNA solution to the Lipofectamine™ 2000 solution, the transfection mixture was incubated for 25 min at room temperature. This transfection mixture was diluted in 500 μl of serum-free Opti-MEM I®, after which 50 or 500 μl of the mixture was added to the 96- and 24-well plates, and the cells were incubated for 6 h. After the media were replaced by 200 μl or 1 ml of DMEM, respectively, the cells were incubated for another 36 h. After being washed twice with PBS, the cells were treated with serum-free DMEM containing DMSO (0.1%, v/v) or UA (4 μM). After 12 h, the concentrations of MIF in the media were quantified by ELISA. Alternatively, the cells were lysed at 30 min after DMSO- or UA-exposure for Western blotting, as described above.

2.8. Statistical analysis

Each experiment was performed at least three times and the data are shown as the mean ± standard deviation (S.D.), where appropriate. Statistically significant differences between groups in each assay were determined using Student's *t*-test (two-sided).

3. Results

3.1. UA dose-dependently promotes MIF protein release without cellular membrane damage

To investigate the effects of UA on the release of MIF protein into the media, RAW264.7 Mϕ were treated with

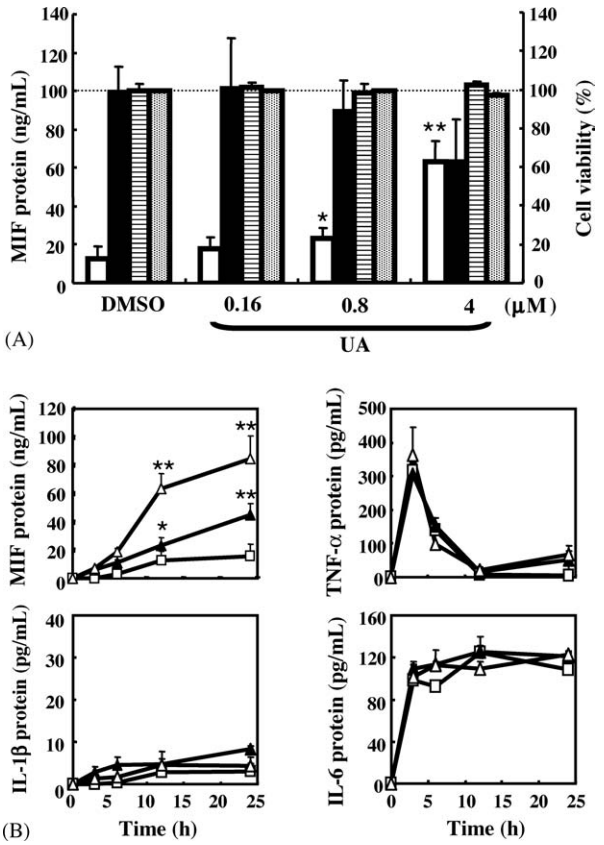

Fig. 1. UA promoted MIF protein release in a dose- and time-dependent manner without cellular membrane damage in RAW264.7 Mϕ. (A) RAW264.7 Mϕ (2.0×10^5 cells/200 μl) were treated with 0–4 μM of UA and incubated for 12 h. Supernatants were collected and the amounts of MIF were determined by ELISA, then cell viability was determined using MTT, trypan blue-exclusion, and LDH release tests, as described in Section 2. (□) media MIF protein; (■) cell viability in MTT test; (▤) trypan blue-exclusion test; (▨) LDH release test. (B) RAW264.7 Mϕ (2.0×10^5 cells/200 μl) were incubated with DMSO (0.1%, v/v) or UA (0.8 or 4 μM) for the indicated times. Supernatants were collected and the amounts of MIF, TNF-α, IL-1β, and IL-6 were determined by ELISA, as described in Section 2. (□) DMSO; (▲) UA (0.8 μM); (△) UA (4 μM). $^{*}P < 0.01$; $^{**}P < 0.001$ vs. DMSO by Student's *t*-test. Data are shown as the mean ± S.D. from three independent experiments.

various concentrations of UA for 12 h. Fig. 1A shows the levels of MIF protein in the media and cell viability, the latter of which was measured using MTT, trypan blue-exclusion, and LDH release assays. Resting RAW264.7 Mϕ spontaneously released MIF protein after 12 h of incubation (12.5 ± 8.6 ng/ml), whereas UA stimulation (0.16–4 μM) increased it in a dose-dependent manner (1.4-, 1.9-, and 5.5-fold at 0.16, 0.8, and 4 μM, respectively) as compared to the DMSO-treated cells. Although cell viability, determined by MTT tests, tended to decrease with UA treatment, it was not statistically significant. In addition, both trypan blue-exclusion and LDH release tests, which were used to reflect cell membrane damage, suggested that treatment with UA for 12 h, even at a concentration of 4 μM, did not cause detectable membrane damage.

3.2. UA time-dependently releases MIF protein

We next investigated the time course of MIF, TNF-α, IL-1β, and IL-6 release from Mϕ treated with UA for 3, 6, 12, and 24 h by ELISA. The release levels of those cytokines in 0.1% DMSO-treated cells were identical to those in non-treated cells (data not shown). As shown in Fig. 1B, UA treatment increased MIF release in concentration- and time-dependent manners (45 ± 7.9 and 85 ± 15.8 ng/ml with 0.8 and 4 μM UA, respectively, at 24 h), though the DMSO-treated control cells also released MIF (16 ± 8.6 ng/ml at 24 h). In contrast, the concentrations of TNF-α, IL-1β, and IL-6 in the media were not increased by UA treatment for 0–24 h as compared with the DMSO-treated cells (Fig. 1B). Cytotoxicity was not seen in any of the experiments (data not shown).

3.3. UA reduces intracellular MIF protein amounts without affecting mRNA expression levels

The effects of UA on the levels of *MIF* mRNA and its intracellular protein were examined by RT-PCR and Western blotting, respectively. As shown in Fig. 2A, constitutive *MIF* mRNA expression was observed in non-treated RAW264.7 Mϕ and the level was not changed by treatment with 0.8 or 4 μM of UA for 12 and 24 h. In parallel, MIF protein was also expressed in a constitutive manner (Fig. 2B). UA stimulation (4 μM) for 12 h decreased intracellular MIF protein levels by 26% as compared with the DMSO-treated cells. A distinct reduction was observed (by 81%) when the cells were treated with UA (4 μM) for 24 h.

3.4. UA triggers MEK1/2 and ERK1/2 phosphorylation

Since the induction and production of pro-inflammatory cytokines were partially regulated by MAPKs, we next examined which MAPK pathway is activated by UA to induce MIF release. RAW264.7 Mϕ were treated with UA (4 μM) and the phosphorylation of MEK1/2, ERK1/2, p38 MAPK, and JNK 1/2 was determined by Western blotting. UA strikingly induced both MEK1/2 and ERK1/2 activation within 10 and 30 min, respectively, as compared with the DMSO-treated cells (Fig. 3), whereas p38 MAPK and JNK protein were not phosphorylated under any of the experimental conditions. Reactivity of the antibodies for these kinases was confirmed using cell lysates from LPS-treated cells. Notably, the UA-increased expression level of ERK2 was higher than that of ERK1.

3.5. Effects of specific inhibitors and MAPK siRNA on UA-induced MIF protein production

To demonstrate the involvement of MEK/ERK activation in UA-induced MIF release, several kinase specific

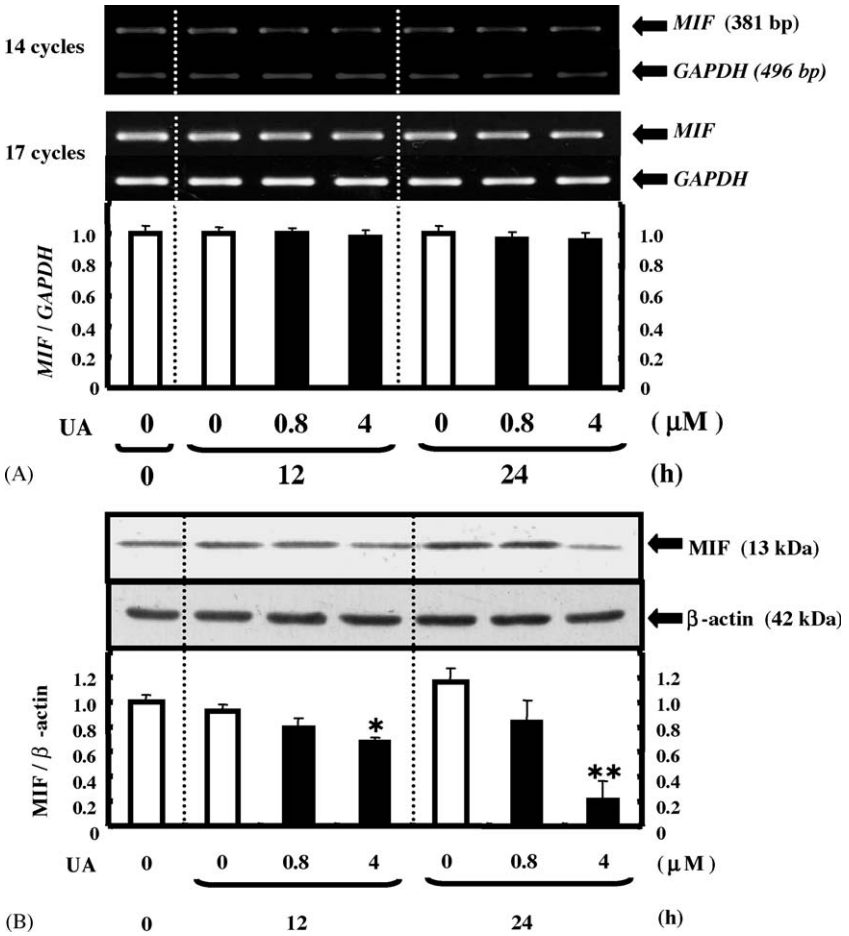

Fig. 2. Levels of *MIF* mRNA expression and its protein in UA-treated RAW264.7 Mϕ. RAW264.7 Mϕ (5.0×10^6 cells/5 ml) were treated with 0.8 or 4 μM of UA dissolved in DMSO (0.1%, v/v), and then incubated for 12 or 24 h before being subjected to RT-PCR (A) and Western blotting (B) analyses, as described in Section 2. The levels of *MIF* mRNA and protein were corrected by *GAPDH* and β-actin, respectively. $^{*}P < 0.01$ vs. DMSO at 12 h; $^{**}P < 0.001$ vs. DMSO at 24 h by Student's *t*-test. Three independent experiments were performed with representative images shown.

inhibitors and siRNA for the target kinases were employed. Each kinase inhibitor was added to the cells when cell viability was maintained at 90% or more (data not shown). PD98059 (an inhibitor of MEK1/2), SP600125 (JNK1/2), and SB203580 (p38 MAPK), suppressed LPS-induced phosphorylation of the respective substrates of those kinases, i.e., ERK1/2, c-Jun (Ser^{73}), and MAPKAPK-2 (Fig. 4A), confirming the selective inhibition of those kinases, except for PD98059, which also inhibited the JNK1/2 activity. PD98059, but not SP600125, SB203580, actinomycin D, or cycloheximide, markedly reduced UA-induced MIF secretion by 50% (Fig. 4C). Next, we examined the effects of siRNAs for ERK1/2. Transfection with ERK1/2 siRNA for 6 h resulted in a significant knockdown of the corresponding active form of each kinase (Fig. 4B). Of importance, UA-induced MIF protein release was significantly reduced by 73% and 88% by ERK2 and ERK1/2 siRNA, respectively, whereas ERK1 siRNA did not show any notable suppression (Fig. 4C).

4. Discussion

Evidence supporting a central role for MIF in host responses has emerged rapidly over the past few years. In the context of immune and inflammatory responses, MIF is released systemically by the anterior pituitary gland [14] and locally by cells such as Mϕ [8], T cells [4], and eosinophils [32]. MIF may be one of the most abundant secreted factors initially detected as a consequence of immune cell activation, which was partially supported by our results of comparisons with IL-1β, TNF-α, and IL-6 levels (Fig. 1B). This cytokine is released in response to extremely low levels of bacterial toxins, thereby promoting the expression of subsequent pro-inflammatory mediators [3,4,8,15]. Although MIF production has been reported to be induced by oxidative stress, endotoxins, and pro-inflammatory cytokines [14–16], knowledge of the underlying molecular mechanism of production remains sparse. Thus, identification of a molecular probe that regulates MIF production may provide a clue to address

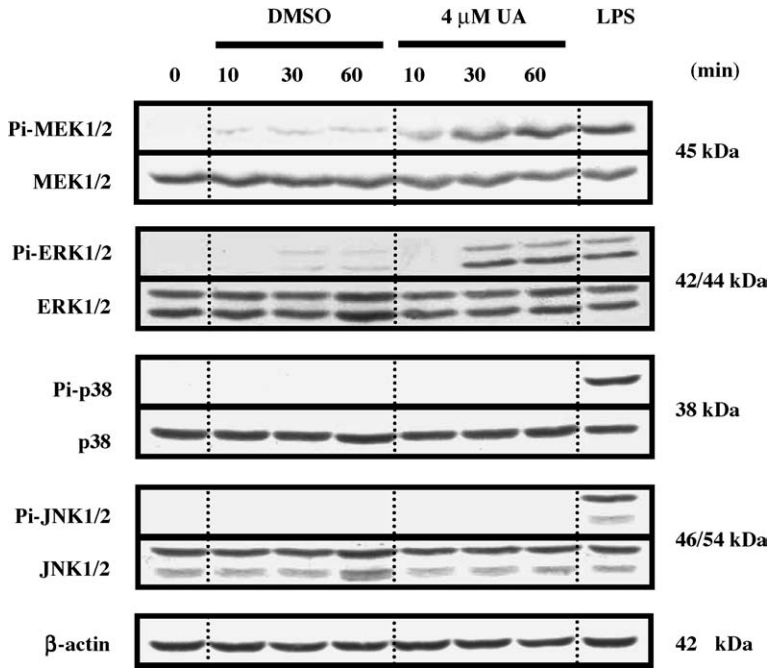

Fig. 3. UA phosphorylated MEK1/2 and ERK1/2, but not p38 MAPK or JNK1/2, in RAW264.7 Mϕ. RAW264.7 Mϕ (1.0×10^6 cells/ml) were treated with 0.1% DMSO or 4 μM UA for 0, 10, 30, and 60 min, or LPS for 10 min (p38) and 30 min (MEK1/2, ERK1/2, and JNK1/2), after which Western blotting was performed as described in Section 2. The experiments were repeated three times independently, with one representative result shown for each.

those issues. To the best of our knowledge, this is the first report of a small molecule that promotes MIF production.

MIF mRNA was found to be expressed in a constitutive manner in non-stimulated RAW264.7 Mϕ (Fig. 2A), which is consistent with other previous reports [8,33,34]. While LPS has been reported to up-regulate *MIF* mRNA expression [8], UA did not show such an effect under the present experimental conditions (Fig. 2A), which is consistent with our data showing that actinomycin D did not suppress UA-induced MIF secretion (Fig. 4C). Further, it is of paramount importance to note that, while UA reduced intracellular MIF protein (Fig. 2B), cycloheximide did not suppress MIF production (Fig. 4C), indicating that UA secretes MIF protein via post-translational mechanisms. Therefore, the transportation system of intracellularly stored MIF protein may be activated by UA leading to its secretion. This notion is supported by the results of a previous study that found that a large amount of MIF protein is stored in the cytosol of a variety of cells, in contrast to other cytokines [35], and the total MIF content within Mϕ and in culture media did not change when stimulated with LPS and calcium ionophore A23187 [36]. UA does not appear to passively diffuse MIF protein due to cellular membrane damage, because it did not exhibit any cytotoxicity in either trypan blue-exclusion or LDH release tests (Fig. 1A). Of note, the MIF secretion mechanism proposed in the present study is not similar to others described for other pro-inflammatory cytokines in several aspects. For example, in addition to transcriptional regulation, pro-IL-1β is processed by an IL-1β-converting enzyme (ICE, caspase-1) to generate biologically active IL-1β [37]. In addition, physiological TNF-α levels are regulated by at least three levels of transcription, including post-transcription, which is related to mRNA stability [38], and post-translation, in which it is shed from the cellular membrane by the TNF-α-converting enzyme (TACE) [39]. On the other hand, UA-induced MIF production, as mentioned above, is probably regulated only at post-translational levels, while the precursor of MIF protein is still unknown.

MAPKs control many cellular events, including differentiation, proliferation, and death, as well as short-term changes required for homeostasis and acute hormonal responses [40]. To date, at least three major MAPK cascades have been described that involve the activation of ERK1/2, JNK1/2, and p38 MAPKα/β/γ. The ERK cascade is mostly responsive to mitogenic and differentiation stimuli, whereas the JNK1/2 and p38 MAPK pathways are preferentially activated by pro-inflammatory cytokines and extracellular stress such as UV radiation and osmotic shock [41,42]. It has also been reported that the LPS-triggered p38 MAPK pathway plays an important role in *IL-1β* mRNA expression and ICE activity in mouse Mϕ [43]. Similarly, LPS- and phorbol 12-myristate-13-acetate (PMA)-induced ERK1/2 activation has been shown to up-regulate *TNF-α* mRNA expression [44,45] and induce TACE activity [45,46].

The molecular mechanisms by which UA activates MIF release are not fully understood. In the present study, we used specific inhibitors and siRNAs to identity which MAPKs are involved in MIF release. Our results showed that UA activated the MEK/ERK pathway (Fig. 3), while

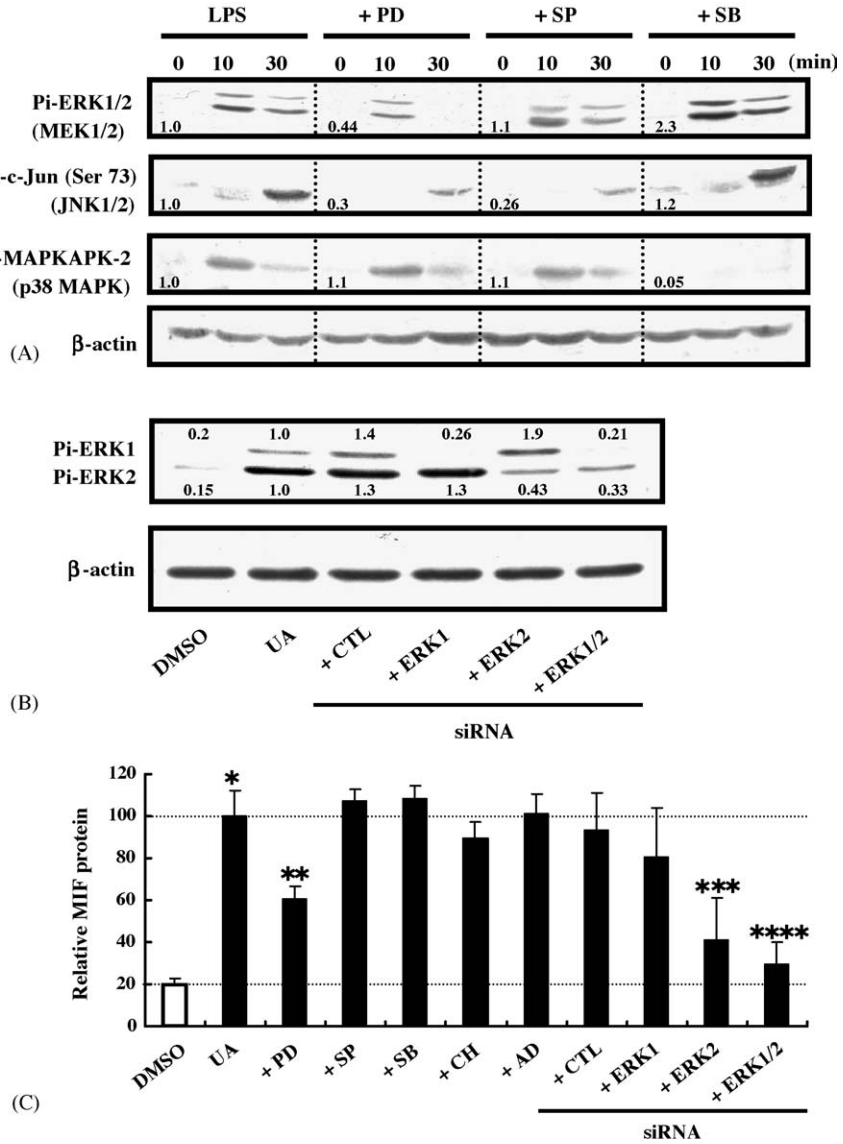

Fig. 4. Effects of specific inhibitors and siRNAs for protein kinases toward UA-induced MIF secretion in RAW264.7 Mϕ. (A) RAW264.7 Mϕ (1.0×10^6 cells/ml) were seeded onto a 6-well plate and cultured for 12 h. Following pre-incubation of the cells with 0.1% DMSO, 50 μM of PD98059 (PD), 50 μM of SB203580 (SB), or 10 μM of SP600125 (SP) for 30 min, they were treated with LPS for 0, 10, and 30 min, after which the cells were subjected to Western blotting as described in Section 2. Values shown in each blot panel indicate the relative kinase activity from 0 to 30 min after LPS exposure, with β-actin employed as the internal standard. (B) RAW264.7 Mϕ (2.0×10^5 cells/well) were seeded onto a 24-well plate and cultured for 12 h. After the cells were treated with siRNA solution (control, ERK1, ERK2, or combined ERK1/2, final concentration 1 μM each) for 6 h, the medium was replaced with 1 ml of DMEM. After 36 h, the cells were treated with DMSO (0.1%, v/v) or UA (4 μM) for 30 min. Total cell lysates were subjected to Western blotting as described in Section 2. Each band intensity was corrected using β-actin as an internal standard. (C) RAW264.7 Mϕ (2.0×10^5 cells/200 μl) were seeded onto a 96-well plate and cultured for 12 h. After being treated with DMSO (0.1%, v/v), 50 μM of PD, 50 μM of SB, 10 μM of SP, 0.16 μM of actinomycin D (AD), and 0.12 μM of cycloheximide (CH) for 30 min, the cells were treated with 4 μM of UA for 12 h. For the RNAi experiments, RAW264.7 Mϕ (2.0×10^5 cells/well) were seeded onto a 24-well plate and cultured for 12 h. After the cells treated with siRNA (control, ERK1, ERK2, or combined ERK1/2) for 6 h, the medium was replaced with 1 ml of DMEM. After 36 h, the cells were treated with DMSO (0.1%, v/v) or UA (4 μM) for 12 h. Each supernatant was collected and the amounts of MIF were determined by ELISA, as described in Section 2. $^{*}P < 0.0001$ vs. DMSO; $^{**}P < 0.005$; $^{***}P < 0.0005$; $^{****}P < 0.0001$ vs. UA, in Student's *t*-test. Data are shown as the mean ± S.D. from triplicate cultures.

PD98059 and siRNA for ERK2, but not that for ERK1, markedly suppressed UA-induced MIF release (Fig. 4C), indicating a critical role for ERK2. However, the molecular mechanisms which UA activates ERK2 are not fully understood. It is possible that ERK2 activation is induced not only by MEK1/2, but also by unknown kinases, because maximal ERK1/2 inhibition rate by PD98059 was 50% (Fig. 4A), which was related to MIF inhibition (Fig. 4C). Our results are not consistent with a previous finding by Fukuzawa et al. that MIF secretion is not regulated by ERK1/2, but rather by protein kinase C in myocardial cells stimulated with H_2O_2 [16], though the discrepancies may have been derived from the different cell types and stimuli used.

An increasing amount of evidence suggests that certain phytochemicals, particularly those in food, have marked anti-inflammatory cancer chemopreventive properties [47,48]. UA is one such dietary compound that has attracted considerable interest because of its remarkable biological functions [17,26–28]. For example, we and others previously showed that UA has inhibitory effects toward PMA-induced skin tumor promotion in mice and Epstein-Barr virus activation in Raji cells [49–51]. Further, it attenuated LPS- and IFN-γ-induced *iNOS* and *COX-2* expression through NF-κB abrogation in mouse Mϕ [29,30]. In addition, You et al. recently presented intriguing data showing that UA induced *iNOS* and *TNF-α* expression via NF-κB activation in resting Mϕ [31], and proposed a hypothesis that the effects of UA on NF-κB activity are dependent on the biological status of Mϕ. In contrast, UA enhanced IFN-γ-induced MIF production in RAW264.7 Mϕ (Ikeda et al., unpublished data).

In conclusion, we found that UA enhances MIF secretion via ERK2 activation at a post-translational level in resting Mϕ. Previous findings have shown that the production of MIF protein is linked to pro-inflammatory events, which may lead to carcinogenesis and rheumatoid arthritis, though UA has also been shown to be an effective and promising anti-inflammatory and anti-carcinogenic agent in vivo by counteracting endogenous and exogenous stimuli. Additional extensive investigations on the effect of UA toward MIF release in vivo are required to determine the efficacy and toxicity of this triterpenoid.

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