

A novel synthetic chalcone derivative promotes caspase-dependent apoptosis through ROS generation and activation of the UPR in MH7A cells

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Received: 12 October 2015 / Accepted: 14 October 2015 / Published online: 22 October 2015
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Abstract Chalcones, one class of polyphenolic compounds synthesized as secondary metabolites during flavonoid biosynthesis in plants, have anti-inflammatory and antitumorogenic effects. In this study, we investigated the roles of a novel chalcone derivative, (E)-3-(3,5-dimethoxyphenyl)-1-(1-hydroxynaphthalen-2-yl)prop-2-en-1-one (DK-59), on rheumatoid arthritis (RA) fibroblast-like synoviocyte MH7A cells. DK-59 treatment reduced cell viability and induced caspase-mediated apoptosis in MH7A cells; activation of caspase-7 and caspase-3, as well as fragmentation of poly (ADP-ribose) polymerase (PARP-1), occurred in a time-dependent manner, and treatment with a pan-caspase inhibitor, Z-VAD, significantly reduced DK-59-mediated PARP-1 cleavage. DK-59-mediated apoptosis was caused by the production of intracellular reactive oxygen species (ROS); DK-59 treatment induced ROS production in a time-dependent manner, and treatment with the antioxidant *N*-acetyl-L-cysteine significantly inhibited DK-59-mediated fragmentation of PARP-1 and recovered cell viability. Many genes that function in the unfolded protein response (UPR), including inositol-requiring protein 1, X-box binding protein 1, and

C/EBP homologous protein (CHOP), were upregulated by DK-59 treatment. CHOP knockdown by lentivirus infection reduced DK-59-mediated PARP-1 cleavage, suggesting that CHOP plays a proapoptotic role in DK-59-mediated apoptosis in MH7A cells. Taken together, the results suggest that DK-59-mediated induction of ROS and activation of the UPR can be used as a target for the therapeutic treatment of RA.

Keywords Chalcones · Unfolded protein response · RA-FLSs · Rheumatoid arthritis · ROS · CHOP

Introduction

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease characterized by hyperplasia of synovial tissue, inflammation, and progressive destruction of joint cartilage and bone (Bartok and Firestein 2010; Choy and Panayi 2001). The surface of synovial tissue is composed mainly of fibroblast-like synoviocytes (FLSs), combined with an infiltration of lymphocytes and macrophages. The FLSs in patients with RA (RA-FLSs) produce inflammatory cytokines, and increased proliferation and insufficient apoptosis of RA-FLSs contribute to the progression of RA (Baier et al. 2003; Bottini and Firestein 2013). RA-FLSs also show resistance to immunosuppressive therapies because of their cancer-like characteristics, such as somatic mutations and epigenetic alterations (Bottini and Firestein 2013; Ishihara and Igarashi 2012). Non-immune therapies, such as inhibiting abnormal proliferation and/or inducing apoptosis of RA-FLSs, have therefore become an important approach to reducing disease severity.

Numerous studies have reported diverse pharmaceutical efficacies of polyphenol compounds for various diseases.

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Polyphenol-based products possess many advantages, including low toxicity to normal cells. Flavonoids, one of the polyphenolic compounds that are abundant in edible plants, have a general C6–C3–C6 structure. Among the flavonoids, chalcone has a C3 bridge (1,3-diaryl-2-propen-1-ones) composed of a linear chain of carbon atoms (an α,β -unsaturated carbonyl group). Both hydroxylated and methoxylated chalcones are frequently produced in plants, and chalcones have been reported to possess antitumor, anti-inflammatory, antioxidative, and antiproliferative properties (Echeverria et al. 2009; Nowakowska 2007).

During the course of investigating the effects of novel synthetic chalcone derivatives on apoptosis in RA-FLSs, we found that a cellular response to endoplasmic reticulum (ER) stress [i.e., the unfolded protein response (UPR)] is involved in this process. The UPR is activated under diverse stress conditions that perturb ER homeostasis, including calcium depletion and perturbation of the redox environment in the ER, which cause an accumulation of unfolded proteins. Three ER membrane sensors, inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK), and activating transcription factor 6 (ATF6), play critical roles in initiation of the UPR. These three UPR components are coordinated to exert an adaptive response by attenuating overall protein synthesis and inducing expression of folding enzymes and chaperones. However, under severe or chronic ER stress conditions, the apoptotic pathway is initiated (Kaufman et al. 2002; Malhotra and Kaufman 2007; Ron and Walter 2007).

Activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP), which function as transcription factors downstream of PERK, are involved in redox control through interaction with nuclear factor E2-related factor 2 (Nrf2), in the initiation of the ER stress-mediated apoptotic pathway through alterations in the B cell lymphoma 2 (BCL-2) family and death receptor 5 (DR5), as well as in selective protein synthesis (Afonyushkin et al. 2010; Cullinan and Diehl 2006; Szegezdi et al. 2006; Tabas and Ron 2011). Because RA-FLSs have cancer-like characteristics and because selective killing of cancer cells targeting the reactive oxygen species (ROS)-mediated mechanisms have been reported, targeting the ROS system to control abnormal proliferation of RA-FLSs may be an effective approach toward treating RA-FLSs (Raj et al. 2011; Trachootham et al. 2009).

In the present study, we investigated the effects of a synthetic chalcone-derived flavonoid, (E)-3-(3,5-dimethoxyphenyl)-1-(1-hydroxynaphthalen-2-yl)prop-2-en-1-one, on abnormal proliferation of RA-FLSs. We examined its effects on cell viability, apoptosis, and ROS production, and characterized its underlying mechanisms of action using MH7A human RA-FLS cells.

Materials and methods

Reagents, chemicals, cells, and cell culture

The chalcones used in this study were synthesized as described previously (Hwang et al. 2011). The crystal structure of (E)-3-(3,5-dimethoxyphenyl)-1-(1-hydroxynaphthalen-2-yl)prop-2-en-1-one has been previously described (Lee et al. 2012). Antibodies against cleaved caspase-3, cleaved caspase-7 (Asp198), IRE1 α , CHOP, and glucose-regulated protein 78 (GRP78) were purchased from Cell Signaling Technology (Beverly, MA). Antibody against the phosphorylated form of IRE1 α (P-IRE1, Ser724) was purchased from Novus Biologicals (Littleton, CO). Antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Alexa Fluor[®] 488-conjugated- and Alexa-Fluor[®] 555-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). The pan-caspase inhibitor Z-VAD-FMK was purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). MH7A cells and immortalized human RA-FLSs (Miyazawa et al. 1998) were cultured as previously described (Jeong et al. 2014).

Cell viability assay and fluorescence-activated cell sorting (FACS) analysis

MH7A cells were seeded onto 96-well plates and treated with 0–50 μ M DK-59 for 24 h. When needed, cells were treated with 10 mM *N*-acetyl-L-cysteine (NAC) for 1 h before DK-59 treatment. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) or the MTT assay (Sigma-Aldrich, St. Louis, MO), as previously described (Shin et al. 2009). FACS analysis was performed using a Becton–Dickinson Flow Cytometer (Becton–Dickinson Bioscience, San Jose, CA), as previously described (Jeong et al. 2014).

TUNEL assay

Apoptosis was determined by a TUNEL assay using an ApoBrdU in situ DNA Fragmentation Assay kit (BioVision, Milpitas, CA), as previously described (Shin et al. 2014).

Immunofluorescence assay, immunoblot analysis, and RT-PCR

The immunofluorescence assay was performed using primary antibodies specific to cleaved caspase-7 (Asp198) and α -tubulin, with secondary antibodies conjugated with

Alexa Fluor 488[®] (for α -tubulin) and Alexa-Fluor[®] 555 (for caspase-7), as previously described (Shin et al. 2014). Nuclear DNA was stained with 1 μ g/mL Hoechst 33258 (Sigma-Aldrich) for 10 min. Labeled cells were examined using an EVOSfl[®] fluorescence microscope (Advanced Microscopy Group, Bothell, WA). Immunoblot analysis was performed as previously described (Jeong et al. 2014). RT-PCR was performed using Improme II (Promega, Madison, WI) and EX Taq (Takara, Otsu, Japan) following the manufacturer's instructions. X-box binding protein 1 (XBPI) fragments were amplified using a forward primer (5'-ACA GTA GCA GCT CAG ACT GC-3') and a reverse primer (5'-CAT TAA TGG CTT CCA GCT TG-3'). PCR products were digested with *Pst*I to separate the unspliced form of XBPI from the spliced form.

Detection of intracellular ROS levels

ROS levels in the DK-59-treated cells were determined by fluorescence microscopy (Eclipse TS-200; Nikon) or flow cytometry (Becton–Dickinson Bioscience, San Jose, CA), as previously described (Jeong et al. 2014; Shin et al. 2009). When needed, cells were treated with 10 mM NAC for 1 h before DK-59 treatment.

Small hairpin RNA (shRNA)

MH7A cells were infected with DNA damage inducible transcript 3 (DDIT3) MISSION[®] shRNA lentiviral transduction particles expressing scrambled or CHOP shRNAs (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Two combined lentiviral plasmid clones were used to target CHOP nucleotides 149–169 and 483–503.

Results

DK-59 treatment reduced cell viability and induced apoptosis in MH7A cells

To investigate the effects of chalcone derivatives on RA-FLSs, we synthesized novel chalcone derivatives and tested their effects on MH7A cell proliferation. Among the 10 tested chalcone derivatives, (E)-3-(3,5-dimethoxyphenyl)-1-(1-hydroxynaphthalen-2-yl)prop-2-en-1-one (code name: DK-59) markedly reduced MH7A cell viability (Fig. 1a). Previously, we reported the synthetic procedures, complete assignment of NMR data, and crystal structure of DK-59 (Hwang et al. 2011; Lee et al. 2012). DK-59 treatment reduced cell viability in a dose-dependent manner, reaching the lowest viability at 30 μ M (Fig. 1b). We

subsequently used 10–20 μ M DK-59 for further studies. This concentration was approximately 3–4-fold lower compared to previously reported flavonoids that induced similar degrees of viability in MH7A cells. To determine whether DK-59 induced cell death through apoptosis, we examined the populations of sub-G1 cells in propidium iodide (PI)-stained cells using flow cytometry. The proportion of cells in the sub-G1 phase increased \sim 33 and \sim 46 % upon treatment with 20 μ M DK-59 for 24 h and 48 h, respectively, compared to DMSO-treated control cells (Fig. 1c). In addition, in situ DNA fragmentation was observed using a TUNEL assay, indicating that DK-59 induced apoptosis in MH7A cells (Fig. 1d).

DK-59 induced cell death via the caspase-mediated pathway in MH7A cells

Next, we investigated whether the apoptosis observed in DK-59-treated MH7A cells was caspase-dependent. We examined the activation of the effector caspases (caspase-7 and caspase-3) and fragmentation of poly (ADP-ribose) polymerase 1 (PARP-1) via immunoblot analysis and immunofluorescence microscopy. Treatment of MH7A cells with 10 μ M DK-59 for 12 or 24 h significantly induced activation of caspase-7 and caspase-3, as well as PARP-1 fragmentation (Fig. 2a). To directly test caspase dependency, cell viability was examined in the presence of DK-59 with or without the pan-caspase inhibitor, Z-VAD-FMK. Pretreatment of cells with Z-VAD-FMK for 1 h significantly reduced DK-59-mediated PARP-1 cleavage (Fig. 2b). When the MH7A cells cultured on coverslips were treated with 10 μ M DK-59 and analyzed by immunofluorescence microscopy, the cleaved form of caspase-7 was observed in the nuclei of DK-59-treated cells (Fig. 2c). Taken together, these results show that DK-59-induced apoptosis is mediated through a caspase-dependent pathway in MH7A cells.

DK-59 induced oxidative stress-mediated apoptosis in MH7A cells

Because chalcone derivatives function as pro-oxidants or anti-oxidants, we tested whether DK-59-mediated apoptosis was associated with the generation of oxidative stress. First, we measured the induction of intracellular ROS using a fluorescent ROS probe [dichloro-dihydro-fluorescein diacetate (DCFH-DA)] and flow cytometry. The ROS signal increased in a time-dependent manner in DK-59-treated MH7A cells (Fig. 3a). Similarly, fluorescence microscopy showed that the ROS signal was increased in DK-59-treated cells, while the ROS signal was reduced in cells co-treated with the antioxidants NAC and DK-59

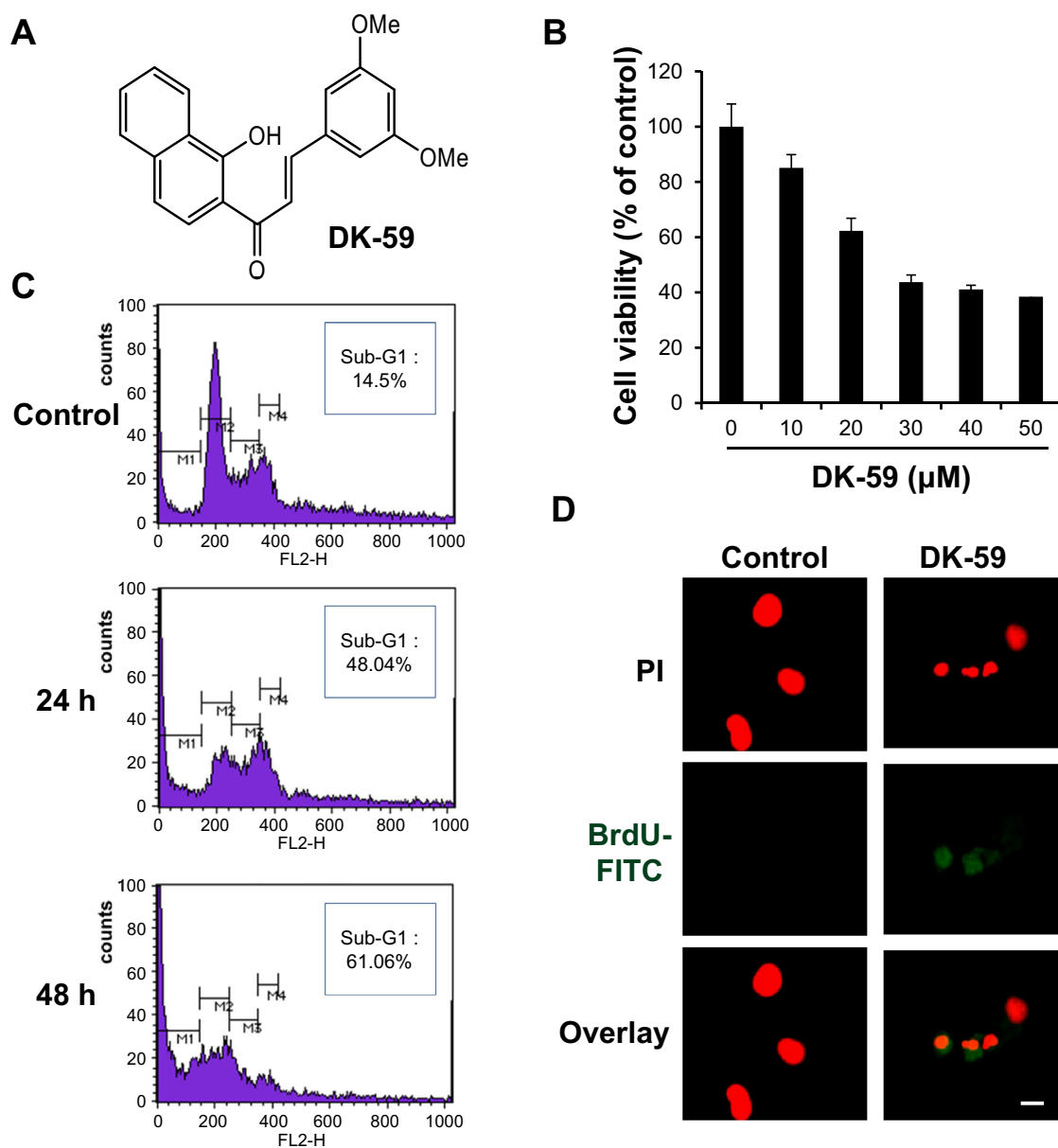


Fig. 1 DK-59 treatment reduced cell viability and induced apoptosis in MH7A cells. **a** Chemical formula of (E)-3-(3,5-dimethoxyphenyl)-1-(1-hydroxynaphthalen-2-yl)prop-2-en-1-one (DK-59). **b** MH7A cells were treated with various concentrations of DK-59 for 24 h as indicated, and cell viability was measured using a CCK-8 assay. **c** MH7A cells were treated with 20 μM DK-59 for 24 h or 48 h, and

cellular DNA content was characterized by flow cytometry. **d** MH7A cells treated with 10 μM DK-59 for 24 h were stained with 5-bromo-2'-deoxyuridine 5'-triphosphate (Br-dUTP) and a fluorescein isothiocyanate (FITC)-labeled anti-BrdU antibody. Nuclear DNA was stained with propidium iodide (PI). Fluorescence was observed using an EVOSfl[®] fluorescence microscope. *Scale bar* indicates 10 μM

(Fig. 3b). When cell viability was measured with or without NAC, NAC treatment increased the viability of DK-59-treated cells (Fig. 3c). Using immunoblot analysis, the cleaved form of PARP-1 was clearly observed in DK-59-treated cells, while it was barely observed in NAC-treated cells before DK-59 treatment (Fig. 3d). Together, these results show that DK-59-mediated accumulation of oxidative stress causes apoptosis in MH7A cells.

During DK-59-mediated apoptosis, the UPR is activated, and CHOP acts as a proapoptotic factor

Some chalcone derivatives have been reported to induce ER stress (Kuo et al. 2010; Shin et al. 2014; Yang et al. 2013) and ROS-mediated apoptosis (Singh et al. 2014; Wu et al. 2013). We therefore investigated the relationships between DK-59, ER stress, the UPR, and ER stress-induced

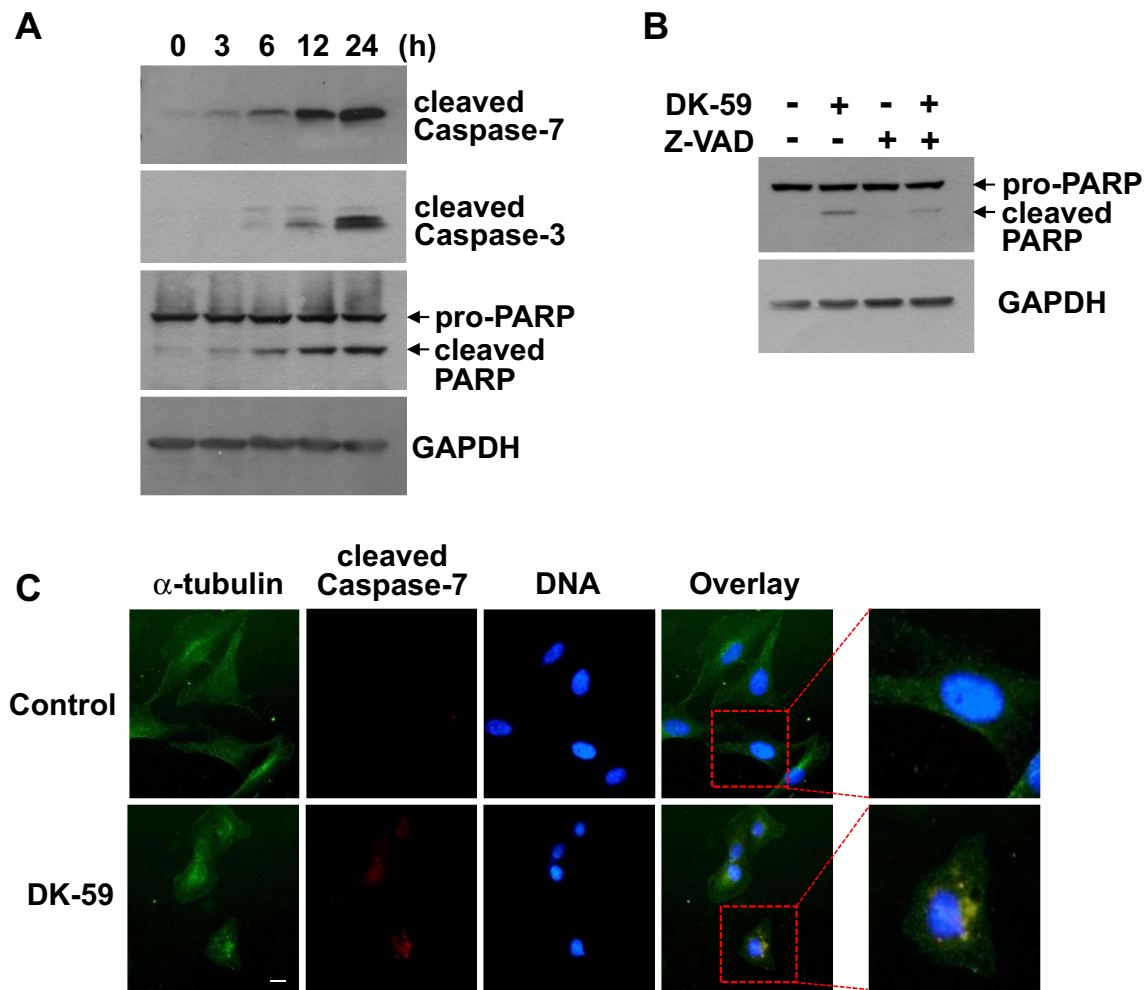


Fig. 2 DK-59 treatment induced caspase-mediated apoptosis in MH7A cells. **a** MH7A cells were treated with 10 μ M DK-59 for various periods and cell lysates were subjected to immunoblot analysis using antibodies specific to the cleaved form of caspase-7, the cleaved form of caspase-3, and PARP-1, or GAPDH. **b** Cells pretreated with or without 40 μ M Z-VAD for 1 h were treated with 10 μ M DK-59 for 12 h. Cell lysates were subjected to immunoblot analysis using antibodies specific to PARP-1 or GAPDH. **c** MH7A

cells cultured on coverslips were treated with 10 μ M DK-59 for 24 h and stained with antibodies specific to α -tubulin or the cleaved form of caspase-7, followed by treatment with secondary antibodies (*green* and *red* signals). Nuclear DNA was stained with Hoechst 33258 (*blue* signal). Fluorescent cells were examined using an EVOSf1[®] fluorescence microscope. Overlay images are shown on the *right*. Scale bar indicates 20 μ M. (Color figure online)

apoptosis. Immunoblot analyses using antibodies specific to IRE1 α , the phosphorylated form of IRE1 α , GRP78, and CHOP showed activation of the UPR by DK-59 treatment (Fig. 4a). Splicing of XBPI mRNA, which is initiated by ribonuclease activity of the phosphorylated form of IRE1 α , was observed following DK-59 treatment (Fig. 4b). When we performed knockdown experiments using lentivirus-expressing shRNAs, immunoblot results showed that PARP-1 fragmentation was reduced by knocking down CHOP in DK-59-treated cells, suggesting that CHOP acts as a proapoptotic factor and mediates DK-59-induced apoptosis (Fig. 4c). Taken together, these results show that DK-59 induces the UPR and that, in this pathway, CHOP functions as a death factor in DK-59-mediated apoptosis in MH7A cells.

Discussion

Inhibiting proliferation or inducing apoptosis of RA-FLSs is important in reducing the disease severity of RA (Baier et al. 2003; Bottini and Firestein 2013). In the present study, we investigated the effects of a chalcone derivative, DK-59, on MH7A RA-FLSs, and found that DK-59-induced ROS and oxidative stress-activated UPR caused caspase-mediated apoptosis.

Blocking ROS production rescued cell viability and fragmentation of PARP-1, suggesting that DK-59-mediated apoptosis was caused by ROS accumulation (Fig. 3c, d). One possible molecular mechanism of ROS induction in DK-59-treated cells is depletion of reduced glutathione

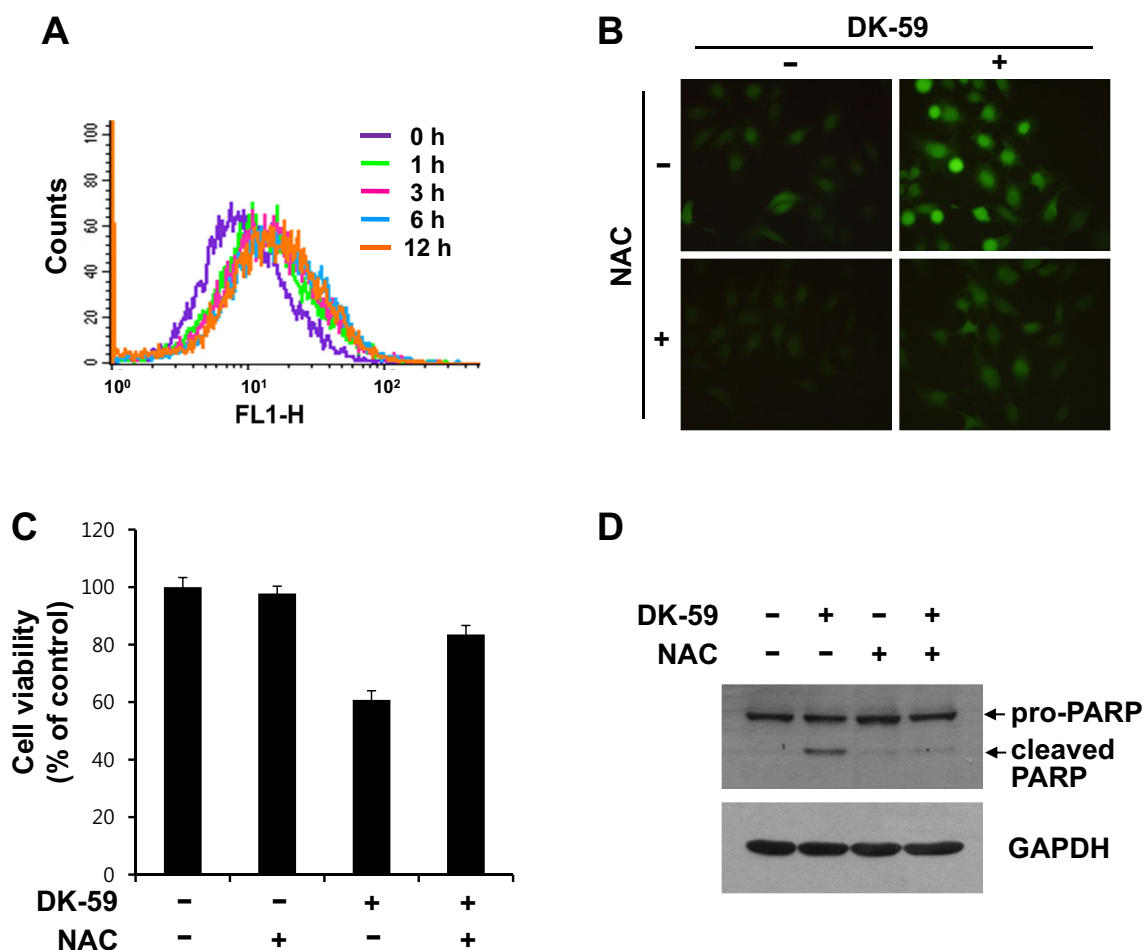


Fig. 3 DK-59 treatment induced ROS-mediated apoptosis in MH7A cells. **a** Cells treated with 10 μ M DK-59 for various periods were treated with 5 μ M DCFH-DA and analyzed by flow cytometry. **b** Cells pretreated with or without 10 mM NAC for 1 h were treated with 20 μ M DK-59 for 12 h, followed by DCFH-DA treatment. Fluorescence intensity was detected by microscopy. **c** Cells pretreated

with or without 10 mM NAC for 1 h were treated with 20 μ M DK-59 for 24 h and cell viability was determined using the MTT assay. **d** Cells pretreated with or without 4 mM NAC for 1 h were treated with 10 μ M DK-59 for 12 h and cell lysates were subjected to immunoblot analysis using antibodies specific to PARP-1 or GAPDH

(GSH) via direct interaction with DK-59. Because the α,β -unsaturated carbonyl groups of the chalcone (Michael acceptor) are known to interact with a thiol (Dimmock et al. 1999; Go et al. 2005; Guzy et al. 2010), it is likely that the interaction between the unsaturated α - β double bond and/or the carbonyl groups of DK-59 and the thiol of GSH reduced the amount of GSH, resulting in a decrease in the ratio of GSH/GSSG. A decreased GSH/GSSG ratio will reduce the ROS scavenging capacity of cells. As a result, ROS will accumulate in the ER lumen and cytosol, in addition to a decline in GSH-mediated reduction of misfolded proteins in the ER. Consistent with these possibilities, our group recently reported that a synthesized methoxychalcone reduced GSH levels and caused ROS accumulation in cancer cells (Shin et al. 2014). However, when compared with other synthetic chalcones tested, we did not know what structural feature(s) of DK-59

facilitated its increased pro-oxidant activity. Due to the methoxy substituents, one possible reason could be the increased activity of the Michael acceptor group. DK-59 contains two methoxy substituents at positions 3 and 5 of an aromatic ring. Methoxy substituents at these positions have been shown to increase the activity of a Michael acceptor group by withdrawing electrons. This activity was reduced by making the Michael acceptor group less electron-deficient with methoxy substituents at positions 4' or 2, 4, and 6 of the aromatic rings (Sawle et al. 2008). It is probable that the increased activity of a Michael acceptor group influences the redox status by directly interacting with thiol-containing antioxidants or by affecting the expression of ROS-forming enzymes such as NADPH oxidases and NO synthases. Methoxychalcone-mediated expression of a gene was abolished in macrophages when the α - β double bond and/or the carbonyl groups of the

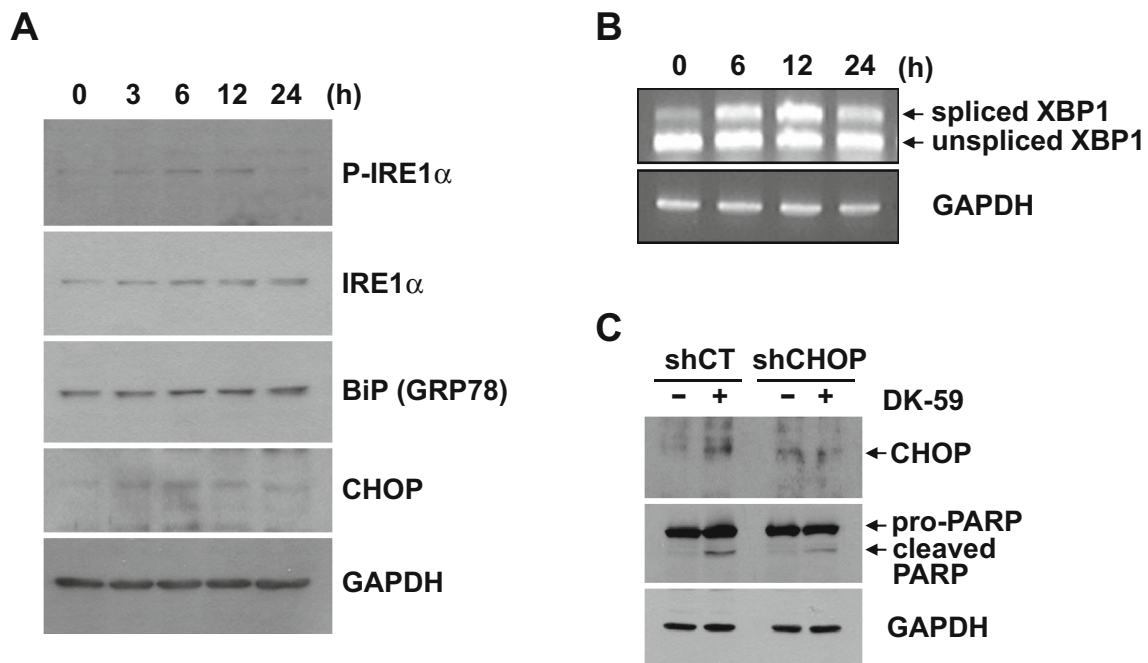


Fig. 4 The unfolded protein response is activated by DK-59 treatment, and CHOP is involved in DK-59-mediated apoptosis in MH7A cells. **a** MH7A cells were treated with 20 μ M DK-59 for various periods and cell lysates were subjected to immunoblot analysis using antibodies specific to P-IRE1 α , IRE1 α , BiP/GRP78, CHOP, or GAPDH. **b** MH7A cells were treated with 20 μ M DK-59 for various periods as indicated. Expression of XBP1 mRNA was determined by

RT-PCR. XBP1 PCR bands were treated with *Pst*I. **c** MH7A cells were infected with lentivirus particles expressing control (shCT) or CHOP (shCHOP) shRNAs. At 48 h postinfection, cells were treated with 10 μ M DK-59 for 8 h and cell lysates were subjected to immunoblot analysis using antibodies specific to CHOP, PARP-1, or GAPDH

Michael acceptor group were reduced (Sawle et al. 2008). These possibilities are consistent with the results that treatment with NAC, a thiol-containing antioxidant, abolished the apoptotic effects of DK-59 (Fig. 3c, d), while treatment with Tiron, an antioxidant lacking thiol groups, did not (data not shown).

Because RA-FLSs show some cancer-like characteristics, apoptosis in DK-59-treated MH7A cells could be related to the reliance of the cells on the ROS control system. Despite high ROS production due to rapid proliferation and metabolism, ROS levels are maintained within a tolerable range in cancer cells because they have developed an efficient ROS scavenging system, making cancer cells more dependent upon ROS scavenging systems than normal cells (Trachootham et al. 2009). It is therefore possible that DK-59 treatment accumulates ROS levels above the toxic ROS threshold in MH7A cells by affecting ROS scavenging components, in addition to its direct role as a pro-oxidant. Consistent with this possibility, we previously reported that apigenin-mediated induction of ROS caused apoptosis in MH7A cells, supporting the idea that MH7A cells are vulnerable to ROS generation under pro-oxidant conditions (Shin et al. 2009).

The UPR in the ER can be initiated under various stress conditions, including accumulation of ROS and

disturbances in redox status. If the GSSG/GSH balance is altered in the ER lumen, abnormal redox homeostasis reduces the protein folding capacity of the ER and results in an accumulation of unfolded proteins in the ER lumen, leading to activation of the UPR (Bhandary et al. 2012; Malhotra and Kaufman 2007). ATF4 and CHOP, transcription factors activated by the UPR downstream of PERK, alter the redox system by changing the expression of antioxidant genes and affect oxidative stress-mediated cell death (Han et al. 2013; Lange et al. 2008; Wang et al. 2014). In addition, oxidation/reduction processes mediated by protein disulfide isomerase (PDI) and ER oxidoreductin 1 (Ero1) that are involved in the protein folding process can change the ratio of GSH/GSSG (Bhandary et al. 2012). Taken together with our results showing increased expression of ATF4 and CHOP (protein and mRNA) in DK-59-treated MH7A cells (Fig. 4 and data not shown), it is reasonable to speculate that expression of ATF4 and CHOP is essential for adjusting the redox homeostasis-mediated UPR and cell death under pro-oxidant conditions.

In addition to its role in the redox system, CHOP functions as a death factor by downregulating Bcl-2, inducing translocation of Bcl-2-associated X protein (Bax) to the mitochondria, inducing the expression of Bcl-2 homology domain 3 (BH3) only proteins, and mediating

calcium-dependent apoptosis through inositol trisphosphate receptor 1 and Ero1 (Kim et al. 2006; Li et al. 2009; McCullough et al. 2001; Tabas and Ron 2011). The role of CHOP in DK-59-mediated apoptosis in RA-FLSs is complex due to its diverse activities and the specific characteristics of RA-FLSs. Because ROS were involved in DK-59-mediated cell death that was suppressed by NAC, and knockdown of CHOP suppressed caspase-mediated apoptosis, it is likely that components connecting oxidative stress to CHOP-mediated cell death are downstream effectors of CHOP in MH7A cells. Proteins that contain redox-regulated cysteine residues at their active sites would be good candidates. In this system, CHOP and ATF4 that are induced by ROS will further produce ROS and induce apoptosis (Kim et al. 2013; Wang et al. 2014). However, the production of inflammatory cytokines and the differential expression of CHOP in the inflammatory synoviocytes may make the mechanism more complicated (Feng et al. 2014). Nonetheless, because synovial cells are prone to ER stress due to uncontrolled proliferation, characterizing synovial cell-specific roles of CHOP and synovial cell-specific effectors that function downstream of CHOP will significantly increase the possibility of using chalcone derivatives for the prevention or treatment of RA.

Acknowledgments This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2010-0024997). This work was supported by the Konkuk University.

Conflict of Interest The authors declare that there are no conflicts of interest.

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