Hempseed oil induces reactive oxygen species- and C/EBP homologous protein-mediated apoptosis in MH7A human rheumatoid arthritis fibroblast-like synovial cells

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Ethnopharmacological relevance: The medicinal efficacy of hempseed (Cannabis sativa L.), which is rich in polyunsaturated fatty acids, in atopic dermatitis, inflammation, and rheumatoid arthritis (RA) has been suggested for centuries. Hempseed has been used as a treatment for these diseases in Korean and Chinese folk medicine. The aim of the study is to investigate the effects of hempseed oil (HO) on MH7A human RA fibroblast-like synovial cells.

Materials and methods: MH7A cells were used to study the anti-rheumatoid effects of hempseed (Cannabis sativa L., cv. Cheungsam/Cannabaceae) oil by investigating cell viability, apoptosis, lipid accumulation, oxidative stress, and endoplasmic reticulum (ER) stress-induced apoptosis.

Results: HO treatment reduced the survival rate of MH7A cells and promoted apoptotic cell death in a time- and dose-dependent manner. Both lipid accumulation and the level of intracellular reactive oxygen species (ROS) increased in HO-treated MH7A cells. Co-treatment with the antioxidant Tiron effectively abrogated the cytotoxic effects of HO: the ROS level was reduced, cell viability was recovered, and apoptotic cell death was significantly diminished. Moreover, HO-treated cells exhibited increased expression of the major ER stress markers, glucose-regulated protein 78 and C/EBP homologous protein (CHOP). The siRNA-mediated knockdown of CHOP prevented HO-induced apoptosis.

Conclusions: Our results suggest that HO treatment induced lipid accumulation, ROS production, CHOP expression, and apoptosis in MH7A cells, and that CHOP functions as an anti-rheumatoid factor downstream of HO in MH7A cells.

1. Introduction

Hempseed (Cannabis sativa L.) has been used as a health food and medicinal plant resource for centuries (Al-Khalifa et al., 2007; Callaway, 2004; Prociuk et al., 2008; Zias et al., 1993; Zuardi, 2006). For example, hempseed has been used as a treatment for glaucoma, menstrual cramps, asthma, atopic dermatitis, inflammatory diseases, and arthritis (Benhaim, 2000; Callaway et al., 2005; Zuardi, 2006). In Korean and Chinese folk medicine, brewed hemp has traditionally been used to reduce chronic knee pain in patients with arthritis. According to the Donguibogam, one of the major resources for Oriental medicine, the hempseed known as “Mazain” can improve blood circulation, relieve constipation, and build strength in combination with other oriental medicinal herbs (Cho, 2005). These medicinal effects are closely related to the abundant essential fatty acids and nutrients in hempseed.

Hempseed contains about 30% oils and 25% proteins in addition to dietary fiber, vitamins, and minerals. Hempseed consists of many fatty acids including palmitic acid, stearic acid, oleic acid, linoleic acid (LA, 18:2 omega-6), α-linolenic acid (AL, 18:3 omega-3), arachidic acid, gamma-linolenic acid (GLA), and stearidonic acid; the fatty acid patterns vary slightly depending on the hempseed genotype (Callaway, 2004; Carvalho et al., 2006; Kriese et al., 2004). The protein content and amino acid values of hempseed were previously reported (Callaway, 2004) and a proteomic profiling of hempseed proteins from Cheungsam, a nondrug-type hemp, was also recently described (Park et al., 2012).
Hempseed oil (HO) is composed of > 80% polyunsaturated fatty acids (PUFAs) and is a good source of the two essential fatty acids, LA and AL (Callaway, 2004). Omega-3 fatty acids, which are essential for development and growth, have curative effects on inflammation, cardiovascular disease, autoimmune diseases, and cancer (Berquin et al., 2008; Leaf and Weber, 1988; Simopoulos, 1991, 2002b). A lower ratio of omega-6:omega-3 fatty acids has important beneficial effects on disease (Simopoulos, 2002a). Since HO contains > 80% PUFAs and its ratio of omega-6 to omega-3 is about 2.5:1, which is quite low, there have been many attempts to use HO as a drug for rheumatoid arthritis (RA), cancer, and other inflammatory diseases. Additionally, hempseed meal affects body weight, cell growth, and neurodegenerative disease in Drosophila melanogaster (Lee et al., 2010, 2011).

RA is an autoimmune inflammatory disease characterized by chronic synovial tissue inflammation, synovial tissue hyperplasia, and progressive articular cartilage destruction (Choy, 2012; Liu and Pope, 2003; Malaviya and Ostor, 2012; Scott and Steer, 2007). In the joints of patients with RA, the aggressive front of synovial tissue, called pannus, invades and destroys the articular tissue. Pannus is composed mainly of fibroblast-like synoviocytes (FLSs) combined with an infiltrate of lymphocytes and macrophages. Both increased proliferation and insufficient apoptosis of FLSs contributes to synovial tissue hyperplasia (Baier et al., 2003; Choy, 2012; Kawakami and Eguchi, 2002). Since synovial hyperplasia is primarily caused by inflammation, immunosuppressive agents have been suggested as antiarthritic treatments to inhibit the production of inflammatory cytokines and abnormal FLS proliferation (Hui et al., 1997; Jeong et al., 2009; Libby, 2008; Lorenz and Kalden, 1999; Ma et al., 2002). Recent studies have demonstrated the efficacy of PUFAs in decreasing the disease severity of RA in animal studies and beneficial effects on some clinical outcomes of RA patients (Ruggiero et al., 2009).

Recently, we showed that immunoglobulin binding protein (BiP)/glucose-regulating protein 78 (GRP78), a representative endoplasmic reticulum (ER) chaperone, plays a critical role in synoviocyte proliferation and angiogenesis (Yoo et al., 2012). BiP works as a regulator of three ER stress sensor proteins and has been recovered as an autoantigen in RA (Panayi and Corrigall, 2006). The unfolded protein response (UPR) is induced by the activation of three mammalian UPR sensor proteins, inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK) (Kaufman, 1999; Kaufman et al., 2002). The UPR is caused by the accumulation of unfolded proteins in the ER lumen under conditions of insufficient protein folding. IRE1α induces the transcription of ER chaperones and folding enzymes, including BiP/GRP78, through the bZIP transcription factor XBP1 (Lee et al., 2002). C/EBP homologous protein (CHOP)/growth arrest and DNA damage 153 (GADD153), a UPR-regulated protein, is a transcription factor that forms heterodimers with C/EBP family members through its bZIP domain and controls the expression of genes that function in cell survival or cell death (Oyadomari and Mori, 2004; Yamaguchi and Wang, 2004). CHOP functions as a proapoptotic factor by downregulating Bcl-2 expression and by disrupting the cellular redox state via the depletion of cellular glutathione (Kim et al., 2006; McCullough et al., 2001). CHOP also acts as a regulator of inflammation, sometimes without inducing cell death, in several cell types (Endo et al., 2006; Jeong et al., 2009). Despite several pieces of evidence indicating that CHOP works as a proapoptotic factor, CHOP-mediated apoptosis is controversial.

In this study, we investigated the anti-rheumatoid effect of HO and the UPR using MH7A human FLSs from patients with RA (RA-FLS). Since hempseed has been used to prevent RA or to improve the symptoms of RA for years via an unknown mechanism, understanding the molecular mechanism of HO in RA-FLS would improve the application of hempseed to RA treatment. In our attempt to identify the factors involved in HO-mediated cell death, we found that the induction of reactive oxygen species (ROS) and CHOP play critical roles in the apoptotic death of HO-treated RA-FLS.

2. Materials and methods

2.1. Chemicals, reagents, cells, and cell culture

2',7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). DAPI, 4,5-di-hydroxy-1,3-benzenedisulfonic acid (Tiron), and Oil Red O were purchased from Sigma (St. Louis, MO). Anti-poly(ADP-ribose) polymerase (PARP) and –GADD153 (CHOP) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-KDEL antibodies, which recognize both GRP78 and GRP94, were obtained from StressGen Biotechnologies Co. (Victoria, BC, Canada). HO was extracted from Cheungsam (Cannabis sativa L., cv. Cheungsam/ Cannabaceae), a nondrug-type hemp variant. Cheungsam was developed by crossing the IH3 Netherland variety with a local Korean variety (Moon et al., 2002). Hempseeds were provided by Dangjin Agricultural Technology Center (DATC), a branch office of the local government in Korea. We obtained government permission to use hempseeds from the Ministry of Food and Drug Safety (permission # 2007-230). Hexane-extracted HO was prepared as previously described by Jeun et al. (2011). Briefly, hempseed was washed, dried, and finely ground with a high-speed mixer. Hempseed powder was mixed with three volumes of hexane for 24 h with mild stirring. The extract was serially filtered using a No. 2 filter (Whatman International Ltd., Maidstone, Kent, UK) and a nylon filter (0.45 μm, Whatman International Ltd.). The extraction procedure was repeated twice with the residue. The solvent was evaporated using a rotary vacuum evaporator (Eyla, Tokyo, Japan) at 35 °C. Residual solvent was re-evaporated twice at 70 °C for 10 min. To increase the solubility of HO extract in culture media, two volumes of ethanol were added to the hexane-extracted HO and mixed by vortexing for 5 min at room temperature. The upper ethanol phase was used in this work. The final concentration of HO-containing ethanol in most cases was 2% in culture. All of the chemicals used for the Griess assay were purchased from Sigma. The MH7A cells, immortalized human RA fibroblast-like synoviocytes (human RA-FLS) with SV40 T antigen (Miyazawa et al., 1998), were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco) under a humidified atmosphere in 5% CO₂ at 37 °C.

2.2. Measurement of cell viability

EZ-cytox (Daeiltech Co., Seoul, Korea) was used to determine the relative number of viable cells. MH7A cells were seeded in 48-well cell culture plates and incubated for 16 h. The cells were treated with 0–2.5% HO for 24 h or 0–20 μM methotrexate (MTX) for 24 as a positive control. When needed, the cells were pretreated with the antioxidant Tiron for 1 h, and then transfected with siRNA oligos or plasmids for 18 h before HO treatment. After washing the cells with phosphate-buffered saline (PBS), 300 μl of fresh medium was added to each well together with 15 μl of EZ-cytox and incubated for 30 min at 37 °C. The absorbance of the soluble substrate was measured at 450/690 nm using an ELISA Reader (UYM 340; ASYS Hitech, Salzburg, Austria).
2.3 Plasmids, siRNA oligos, and transfection

CHOP cDNA was obtained from MH7A cells by reverse transcription (RT)–PCR. A 0.5-kb BamHI–HindIII fragment was subcloned into the same sites of the pcDNA 3.1 plasmid. siRNA oligonucleotides specific for CHOP (siC1 and siC4) were purchased from Sigma-Proligo (The Woodlands, TX) and scrambled oligonucleotides (nonspecific control, NC) were purchased from Bioneer (Daejeon, Korea). Transfection of the CHOP plasmid (pcDNA-CHOP) was carried out using Lipofectamine 2000 (Life Technologies Co., Carlsbad, CA) according to the manufacturer’s protocols.

2.4 Identification of apoptotic cells by microscopy and fluorescence-activated cell sorting (FACS) analysis

To monitor apoptotic cell death by fluorescence microscopy, control or HO-treated cells were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature. The cells were then stained with 1 μg/ml DAPI in PBS for 10 min at room temperature. After washing three times with PBS, nuclear morphology was observed under a fluorescence microscope (ECLIPSE TS-200; Nikon, Tokyo, Japan) with an inverted fluorescence microscopy (400 × ). Cells showing fragmented or condensed nuclei were considered apoptotic cells. For the evaluation of apoptotic cell death by FACS, attached and floating cells grown in 60-mm culture dishes were pooled in conical tubes, pelleted by centrifugation, washed with PBS, and fixed with cold 70% ethanol at 4 °C overnight. Cells were washed, re-suspended in 1 ml of a propidium iodide (PI) solution containing 20 μg/ml RNase A and 100 μg/ml PI, incubated for 30 min at 37 °C, and assayed using a Becton Dickinson Flow Cytometer (Becton–Dickinson Biosciences, San Jose, CA) at 488 nm. The data were analyzed with WinMDI version 2.9 software (Joe Trotter, Scripps Research Institute, La Jolla, CA). Sub-G1 cells were considered to be apoptotic. The percentage of apoptotic cells was calculated as the ratio of sub-G1 cells to that of the entire cell population.

2.5 Oil Red O staining and Griess assay

Cells cultured in 12-well plates were treated with HO for 24 h, fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature, and washed with 60% isopropanol three times. A working solution of Oil Red O was incubated with the completely dried samples for 10 min. After removal of the Oil Red O solution, water was added immediately and the cells were washed four times. Stained lipid droplets in the cells were observed using a fluorescence microscope (ECLIPSE TS-200; Nikon) using 488 nm for excitation. For the Griess assay, Oil Red O was eluted from the cells with 100% isopropanol for 10 min. The absorbance of Oil Red O was measured at 500 nm (corrected for the background absorbance at 690 nm) using a microplate reader (UYM 340; ASYS Hitech).

2.6 Immunoblot analysis

Cells were harvested using RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, and 2 mM EDTA) with 1% phosphatase inhibitors and protease inhibitors. The protein concentration was quantified using the Bradford method (Bio-Rad, Hercules, CA). Proteins boiled in 1 × sample buffer [500 mM Tris–HCl (pH 6.8), 10% SDS, 20% glycerol, 0.05% bromophenol blue, and 1% β-mercaptoethanol] for 5 min at 100 °C were separated on SDS-polyacrylamide gels. The proteins were then electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and blotted with the indicated antibodies at 4 °C overnight in Tris-buffered saline containing 0.08% Tween 20 (TBST) and 1% nonfat milk. The membranes were then incubated with horseradish peroxidase-conjugated antibodies at room temperature for 2 h, and the band signal was detected using an LAS-3000 Luminescent Image Analyzer (Fujiﬁlm, Tokyo, Japan). To ensure equal loading of the samples, the blots were stripped in stripping buffer [100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.8)] at 50 °C for 30 min, washed twice with TBST buffer for 15 min each, and re-probed with anti-β-actin antibodies.

2.7 Measurement of intracellular ROS levels

Intracellular ROS production was detected using DCFH–DA as an intracellular probe. Briefly, cells were treated with 5 μM DCFH–DA for 1 h at 37 °C. After washing twice with PBS, the cells were treated with HO for 3 h at 37 °C. When needed, Tiron was added 1 h before HO treatment. The fluorescence intensity of dichloroﬂuorescein was observed under a fluorescence microscope (ECLIPSE TS-200; Nikon) using 488 nm for excitation.

2.8 Observation of endocytosis

Endocytosis was observed using dextran staining. Cells were pre-stained with 5 μM dextran for 1 h, washed with 1 × PBS twice, and treated with 1% and 2% HO or ethanol (as the control) for 24 h. The cells were then observed under a fluorescence microscope at 560 nm.

2.9 Statistical analysis

A one-way analysis of variance was used for all statistical analyses with independent experiments. Following this, post hoc analysis by Student’s or Duncan’s t-test was conducted using SPSS 12.0K0 for Windows (SAS Institute Inc., Cary, NC). P-values < 0.05 were considered to be signiﬁcant, unless otherwise speciﬁed. All data are expressed as the mean ± the standard error of the mean (SEM) of three independent experiments performed in triplicate.

3. Results

HO containing high levels of unsaturated fatty acids has been used as a source of food and medicine for centuries without knowledge of its mechanism of action. In this study, we examined the effect of HO on MH7A human RA-FLS cells. We ﬁrst investigated the effect of HO on cell viability and apoptosis using RA-FLS cells. To increase the solubility of the HO extract in culture media, HO extracted with hexane was further extracted with ethanol (see Section 2). MH7A cells treated with increasing concentrations of HO for 24 h showed decreased viability (Fig. 1A, right). When the cells were treated with MTX, a drug used for treatment of RA, for 24 h as a positive control, cell viability was reduced in a dose-dependent manner (Fig. 1A, left). When the cells treated with 2% HO were observed for apoptotic markers, fragmentation of PARP and nuclear condensation were observed (Fig. 1B and C), suggesting that HO inhibited the proliferation of MH7A cells by inducing apoptosis.

To verify whether apoptotic cell death was induced by HO, we measured lipid accumulation in MH7A cells using Oil Red O, which stains oxidized lipid droplets in cells (Laughton, 1986). Microscopic observation of the HO-treated MH7A cells revealed that the number of lipid droplets was increased in a dose-dependent manner (Fig. 2A). The same pattern was observed when the amount of the Oil Red O extracted from the stained cells was measured quantitatively using a microplate reader (Fig. 2B). It seems that the uptake of HO by the cells occurred through...
endocytosis, as shown by dextran staining of HO-treated cells (Fig. 2C). These results suggest that HO-mediated lipid accumulation in the cells caused apoptotic cell death by increasing oxidative stress through lipid peroxidation.

We tested whether oxidative stress was increased in the HO-treated MH7A cells. First, we investigated the induction of intracellular ROS in HO-treated MH7A cells using DCFH-DA, a fluorescent ROS probe. By fluorescence microscopy, the ROS signal was clearly increased in the HO-treated cells (Fig. 3A). To determine whether HO-induced cell death was relevant to the generation of ROS, we measured the viability of cells co-treated with HO and the antioxidant Tiron. While the HO-treated cells showed reduced viability, consistent with our previous results (Fig. 1), the viability of the cells pretreated with Tiron just before HO treatment was similar to that
of the control cells. This demonstrates that treatment with Tiron inhibited HO-induced cell death in MH7A cells (Fig. 3B). Lastly, we examined the sub-G1 population, a hallmark of apoptosis, using flow cytometry to confirm whether HO treatment induced ROS-mediated apoptosis. The proportion of HO-treated cells in sub-G1 was ~21%, while that of cells co-treated with Tiron and HO was ~14%, indicating recovery by treatment with the antioxidant (Fig. 3C). These results indicate that HO induces ROS-mediated apoptotic cell death in MH7A cells.

Since HO is rich in PUFAs, and PUFAs were reported to induce ER stress in some cancer cells (Jakobsen et al., 2008; Pan et al., 2004), we investigated the relationship among HO, ER stress, and ER stress-induced apoptosis. Western blot analyses using antibodies specific for KDEL, which recognizes both GRP78 and GRP94, and CHOP, which is involved in the ER stress pathway, revealed the induction of GRP94 and CHOP by HO treatment in a dose-dependent manner (Fig. 4A). Expression of CHOP was increased by HO treatment in a time-dependent manner. CHOP is a proapoptotic factor that downregulates Bcl-2 or upregulates other proapoptotic factors, although there are some discrepancies depending on the cell type. Therefore, we investigated the role of CHOP in cell viability and apoptosis in HO-treated MH7A cells by knocking down CHOP expression using siRNAs specific for CHOP. Our cell viability assay results showed that the siRNA-mediated knockdown of CHOP inhibited HO-induced death in MH7A cells (Fig. 4B and C), and FACS analysis showed that knocking down CHOP clearly reduced the sub-G1 population in HO-treated cells from 25.9% to ~11%, suggesting that CHOP acts as a proapoptotic factor (Fig. 4D). In contrast, overexpression of CHOP slightly increased the percentage of sub-G1 cells. Taken together, these results suggest that CHOP functions as a proapoptotic factor in HO-treated MH7A cells.

4. Discussion

Hempseed is rich in PUFAs and essential amino acids and has long been used as food and a medicinal resource. In this study, we investigated the mechanism of action for HO in MH7A synovial cells and found that HO reduced cell viability, increased lipid accumulation and oxidative stress, and induced CHOP expression and apoptosis. These results support the idea that hempseed is a good resource for the prevention or treatment of RA, consistent with traditional beliefs from Korean and Chinese medicine. We believe that our results are closely tied to the effects of the omega-3 fatty acids contained in hempseed because HO contains > 80% PUFAs, including the omega-3 fatty acids, which have anti-inflammatory properties and beneficial effects on some clinical outcomes of RA patients (Ruggiero et al., 2009; Simopoulos, 2002b). Indeed, AL is converted to docosahexaenoic acid (DHA, C22:6n3) and eicosapentaenoic acid (EPA, C20:5n3) in some eukaryotic cells. DHA and EPA are also known to have anti-inflammatory effects and to suppress the proliferation of synovocytes in patients with RA (Hamaguchi et al., 2008). Kim (2011), and Kim and Lee (2011) have shown that Cheungsam hempseed contains ~32.5% oil and that the major constituents of HO, such as palmitic acid, stearic acid, oleic acid, and LA, are detected in Cheungsam HO, similar to previous reports on different hemp genotypes (Borhade, 2013; Callaway, 2004; Carvalho et al., 2006; Kim, 2011; Kim and Lee, 2011; Kriese et al., 2004; Park et al., 2012). In addition, the total percentages of PUFAs and the two essential fatty acids, LA and AL, are similar to those previously reported (Callaway, 2004; Karimi and Hayatghaibi, 2006; Kriese et al., 2004). For these reasons, along with the reasons discussed above, we believe that our results are tightly correlated with the effects of the omega-3 fatty acids. At this point, whether any single
Fig. 4. CHOP is involved in hempseed oil (HO)-induced cell death in MH7A cells. (A) Cells were treated with various concentrations of HO for 24 h (top) or with 2% HO for various periods (bottom) and cell lysates were subjected to immunoblot analysis. Thapsigargin (Tg, 1 μM) was used as a positive control. (B) Cells were transiently transfected with siRNA oligonucleotides specific for CHOP (siC1 or siC4) or scrambled oligonucleotides (nonspecific control, NC). At 18 h post-transfection, the cells were treated with 2% HO for 24 h and cell viability was determined. (C) and (D) Cells were transiently transfected with a pDNA-CHOP plasmid or siRNA oligonucleotides specific for CHOP (siC1 and siC4). (C) Cell lysates were subjected to immunoblot analysis using antibodies specific for CHOP (indicated by the arrowhead) or β-actin. (D) Cells stained with PI were subjected to FACS analysis. Apoptosis is expressed as the percentage of cells in sub-G1 phase as indicated by the bar in the histogram.
compound or combination of compounds derived from PUFA's caused the effects observed in our study is unclear. One of the possible candidates that exerted anti-proliferative effects is GLA because hempseed oil contains about 4% GLA and it can be converted to dihomo-γ-linolenic acid (DGLA), and eventually to prostaglandin E1 (PGE1), which is known to have anti-proliferative properties (Callaway, 2004; Fan and Chapkin, 1998; Wang et al., 2012). Currently, we are assessing the effects of several pure compounds found in HO on synovial cells. 

One possible explanation for HO-mediated cell death is lipid peroxidation and ROS accumulation. We observed lipid accumulation (Fig. 2A and B), ROS production (Fig. 3A), and apoptosis (Fig. 1B and C) in HO-treated synovial cells. An analogous interpretation of these results is that significant portions of the accumulated lipids were converted to highly reactive and oxidative molecules by lipid peroxidation, and that the resultant aldehydes (e.g., 4-hydroxyhexenal) served as ROS donors. These products, which are relatively stable in the cell, would serve as alkyl donors, causing ROS-mediated cell death (Lee et al., 2006). The roles of ROS are complicated because ROS can be induced by many different sources and exert diverse effects depending on the cell type. Nevertheless, the results of this study show that ROS are mediators of apoptosis in HO-treated, lipid-accumulated synovial cells. These results are consistent with our previous report regarding apigenin-mediated synovial cell death in the induction of ROS and its apoptotic role in synovial cells (Shin et al., 2009).

An interesting feature of HO-mediated apoptotic cell death is the involvement of CHOP. CHOP is a transcription factor that functions in the cell death pathway under ER stress conditions. CHOP is known to induce apoptosis by downregulating transcription of the anti-apoptotic factor Bcl-2, by disrupting the cellular redox state, and by upregulating proapoptotic factors such as Bax, as well as the calcium-mediated activation of calcium–calmodulin kinase II (CaMKII) (Kim et al., 2006; McCullough et al., 2001). The primary regulators of CHOP expression in HO-treated synovial cells are not known, but if the UPR in synovial cells follows the typical pattern in eukaryotic cells, CHOP expression is regulated by the PERK-eIF2α-ATF4 pathway rather than IRE1α-XBP1 or ATF6, two other UPR pathways with relatively weak activation. Due to their cancer and inflammatory cell-like features, however, it is unlikely that the synovial cell UPR is similar to that of normal eukaryotic cells. Downstream effectors of CHOP-mediated apoptosis are complicated and the main effectors in synovial cells are unknown. Possible targets are ERO1α-mediated hyperoxidation of the ER lumen, inositol trisphosphate receptor 1 (IP3R)-mediated calcium release from the ER, and the resultant activation of CaMKII and Bax–Bak-mediated mitochondrial permeabilization (Hetz, 2012; Marciniak et al., 2004; Tabas and Ron, 2011). Since our results showed that ROS are involved in HO-mediated cell death, which is suppressed by Tiron, it is obvious that at least CHOP and ERO1α contribute to the induction of oxidative stress markers and apoptosis. However, we cannot rule out the possibility that there are synovial cell-specific roles for CHOP because, as mentioned above, synovial cells have both cancer cell-like and inflammatory cell-like features where the intercellular and intracellular environments are prone to ER stress due to the uncontrolled cell cycle. It will be necessary to elucidate which pathway is involved in our system by specifically blocking downstream targets or using mutants for ERO1α, IP3R, and CaMKII. By defining the roles of effectors other than CHOP (e.g., GADD34, DR5, JNK, TRB3, and RIDD) in synovial cells and by identifying the primary component of HO that exerted the effects shown in this study, our understanding of synoviocyte survival and death will be significantly improved and the opportunity to use HO-derived drugs for RA treatment will be increased.

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References


