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백굴채 추출물의 항산화 활성과 기전

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Antioxidant Activity and Its Mechanism of *Chelidonium majus* Extract

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ABSTRACT : *Chelidonium majus* (CM) contains several isoquinoline alkaloids that have been reported to have various biological activities such as anti-inflammatory, antimicrobial, antioxidant, immune-modulatory, and antitumoral. It has been reported that the extract of CM had an antioxidant potential, however the mechanism has not been verified. In this study, we found that CM extract activated FOXO3a. FOXO3a is a transcription factor that involved in various biological processes such as cell cycle arrest, apoptosis, DNA repair, and ROS detoxification. Transcriptional activities of FOXO3a were regulated by post-translational modifications including phosphorylation, acetylation, and ubiquitination. Protein level of FOXO3a was increased by CM extract. Promoter activities of FOXO-transcriptional target genes such as MnSOD, p27 and GADD45 were activated by CM extract in a dose dependent manner. In addition, protein level of MnSOD, major antioxidant enzyme, was increased by CM extract. Thereby ROS level was decreased by CM in old HEF cells. These results suggest that CM extract has an antioxidant activity through FOXO activation.

Key Words **:** *Chelidonium majus*, FOXO3a, Antioxidant

INTRODUCTION

Chelidonium majus (CM) is known as greater celandine. It contains isoquiniline alkaloids such as sanguinarine, chelidonine, chelerythrine, berberine, propopine, and coptisine (Gilca *et al.*, 2010). CM has a various biological activities such as anti-inflammatory (Park *et al.*, 2011), antimicrobial (Meng *et al.*, 2009), antioxidant (Nadova *et al.*, 2008), anticancer (Habermehl *et al.*, 2006; Noureini and Wink, 2009; Paul *et al.*, 2012), immunomodulatory (Chung *et al.*, 2004), radioprotective (Song *et al.*, 2003), and nephron-protective activities (Koriem *et al.*, 2013).

FOXO3a is a subgroup of the forkhead family that has a conserved DNA binding domain. FOXO3a is a transcription factor that binds to the consensus FOXO binding site of its target genes. FOXO3a involved in various biological functions such as cell cycle control, apoptosis, DNA repair, and ROS detoxification through regulation of its target genes (Salih and Brunet, 2008; van der Horst and Burgering, 2007). FOXO3a is regulated by posttranslational modifications including phosphorylation, acetylation, methylation, and ubiquitination (Zhao *et al.*, 2011). These post translational

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modifications affect stability of protein, DNA binding activity, and cellular localization. Akt phosphorylates FOXO3a, leading to nuclear exclusion of FOXO3a and inhibits FOXO3a activity (Brunet *et al.*, 1999). IkB kinase (IKK) phosphorylates and inhibits FOXO3a activity (Hu *et al.*, 2004). ERK phosphorylates FOXO3a at Ser 294, Ser344, and Ser 425 and degrades via an MDM2-mediated ubiquitination pathway (Yang *et al.*, 2008). MST1 phosphorylates FOXO3a at Ser 207 that disrupts binding with 14-3-3 proteins and promotes nuclear translocation (Lehtinen *et al.*, 2006). SIRT1 interacts with FOXO3a in response to oxidative stress and deacetylates FOXO3a. Deacetylated FOXO3a induces cell cycle arrest and resistance to oxidative stress (Brunet *et al.*, 2004).

It has been reported that the extract of CM had an antioxidant potential (Nadova *et al.*, 2008), however the mechanism has not been verified. Therefore, we investigate the mechanism of antioxidant activity of CM extract. Here we show that FOXO activity was upregulated by CM extract. The level of FOXO3a protein was increased by CM extract. Target genes of FOXO3a such as MnSOD, p27, and GADD45 were activated and thereby CM extract resulted in removal of ROS in old HEF cells.

MATERIALS AND METHODS

1. Cell culture

A primary human embryonic fibroblast (HEF) cells were established from an abortus at Hallym University Hospital (Kim *et al.*, 2005). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Bio-Whitaker, Basel, Switzerland) at 37℃ in a humidified atmosphere containing 5% CO_2.

2. Preparation of CM Extract

CM was purchased from Dae Kwang Herb Medicine Co., Ltd., Chuncheon, Korea and the voucher specimen (No. RIC-1283) was deposited at the center for efficacy assessment and development of functional foods and drugs (Regional Innovation Center), Hallym University, Republic of Korea. Aerial part of CM (500 g) mixed with 3 liter of 70% ethanol‐water solution in a 5000 ml round bottom flask fitted with a cooling condenser which was used to perform the extraction. The extraction temperature was controlled at 70℃ with a water bath to allow ethanol boiled continuously. Extraction was carried out for 6 h. The extracts were combined and concentrated under reduced pressure with a Model EYELA N-1000 rotary evaporator (Tokyo Rikakikai, Tokyo, Japan), which yielded 86.2 g (17.2%) of the crude extract for biological test.

3. Production of U2OS-FHRE Stable Cell Lines

To produce the FHRE-GFP vector, CMV promoter of pEGFP-N2 vector (Clontech, Mountain View, CA, USA) was removed by restriction enzyme and FHRE (three copies of forkhead responsive element) was inserted. U2OS cells were transfected with FHRE-GFP vector using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). After 24 h, cells were splited and maintained with DMEM media containing G418 (0.8 ㎎/㎖). After 2 weeks, stable transfectants were obtained and analyzed.

4. High Content Screening

U2OS-FHRE stable cells were seeded in 96-well plate. After 24 h, cells were treated with CM extract for 24 h. Cells were fixed using 3.7% formaldehyde for 10 min and washing with 1x PBS, 3 times. GFP intensity was measured by high content screening (ArrayScan V, Cellomics, Pittsburgh, PA, USA).

5. Transfection and Luciferase Assay

The day before transfection, the HEF cells were seeded in 24 well plates. Cells were transfected with plasmid DNA using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were treated with CM for 24 h. The luciferase activity in the extract of the transfected cells was measured using a commercial kit, Luciferase Assay Reagent (Promega, Fitcgburg, WI, USA), and the β-galactosidase activity was measured. For each sample, the luciferase activity was divided by the β-galactosidase activity to correct for the transfection efficiency.

6. Western Blot Analysis

Cells were harvested and lysed in lysis buffer (10 mM Tris‐HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% NP-40 containing a mixture of protease inhibitors). 40 ug of protein was separated on SDS-polyacrylamide gel and then transferred to immune-blot PVDF membrane. The membranes were incubated with primary antibodies. The membranes were then incubated with the secondary antibody conjugated with anti-mouse or anti-rabbit IgG-horseradish peroxidases. Protein detection was

performed using the ECL system (Amersharm Biosciences, Piscataway, NJ, USA).

The anti-FOXO3a and the anti-phospho-FOXO3a (T32) antibody were purchased from Cell Signaling Technology (Essex, MA, USA). The anti-MnSOD antibody was purchased from BD PharMingen (Franklin lakes, NJ, USA). The anti-actin antibody was purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase-coupled anti-rabbit and anti-mouse were from Pierce Technology Corporate (Meridian RD, Rockford, IL, USA).

7. ROS Measurement

Cells were incubated with 30 μM DCDHF-DA for 30 min at 37℃. Cells were washed twice with PBS, and detached with trypsin. Cells were sonicated and centrifuged (12,000 g, 20 min, 4℃). Fluorescence intensity was measured in the supernatants using a fluorescence spectrometer (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) (λ_{ex}: 485 nm; λ_{em}: 530 nm). Fluorescence intensities were normalized by total amount of proteins (Alaimo *et al.,* 2011).

8. Statistical Analysis

Data were analyzed using GraphPad Prism 4 (Ver. 4.03, GraphPad Software, San Diego, CA, USA). Standard deviation and comparison of data have been performed by paired student's t-test.

RESULTS

1. FOXO3a Activation by CM Extract

FHRE-GFP vector has a promoter containing three FOXO3a response elements. Expression of GFP protein was increased by FOXO3a activation (Fig. 1A). U2OS cells were stably transfected with FHRE-GFP vector. To investigate if the CM extract activates FOXO3a, U2OS-FHRE-GFP stable cells were seeded in 96-well plate. After 24 h, CM extract was treated into U2OS-FHRE-GFP stable cells for 24 h. GFP intensities were measured by high content screening as described in materials and methods. CM extract activates FOXO3a (Fig. 1B). To confirm this, HEF cells were transiently transfected with plasmid DNA containing 3X FHRE-luciferase. After 24 h, cells were treated with CM extract for 24 h, and luciferase activity was measured. As seen in Fig. 1C, 3X FHRE-luciferase activities were increased by CM extract. These results show that CM extract activates FOXO3a.

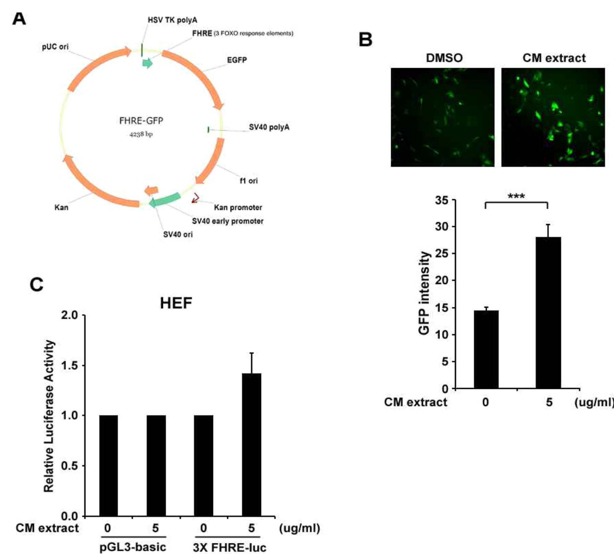

Fig. 1. Activation of FOXO3a in response to CM extract. (A); Vector map of FHRE-GFP, (B); U2OS-FHRE-GFP stable cells were treated with CM extract for 24 h. GFP intensity was analyzed by high content screening, (C); Promoter activities of pGL3-basic and 3X FHRE-luc were measured, and the *β*-galactosidase activity was measured. To correct for the transfection efficiency, the luciferase activity was divided by the *β*-galactosidase activity. The error bars indicate standard deviations of results obtained from three independent experiments and the statistical significance is indicated as ***p< 0.001 by Student's t-test.

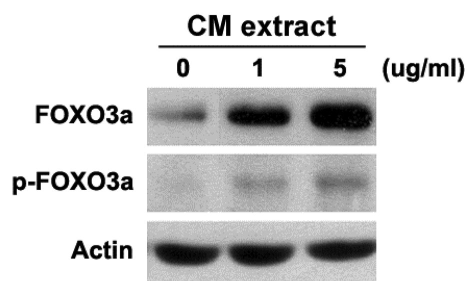

Fig. 2. Increase of FOXO3a protein level by CM extract. HEF cells were treated with CM extract for 24 h. Protein levels FOXO3a, p-FOXO3a, and actin were measured by western blot analysis.

2. Protein Level of FOXO3a Increase in Response to CM Extract

Because CM activates FOXO3a, the protein level of FOXO3a was measured by western blot analysis. HEF cells were treated with CM extract for 24 h. FOXO3a protein was increased by CM extract in dose-dependent manner (Fig. 2). Phosphorylation of FOXO3a (T32) was also increased slightly.

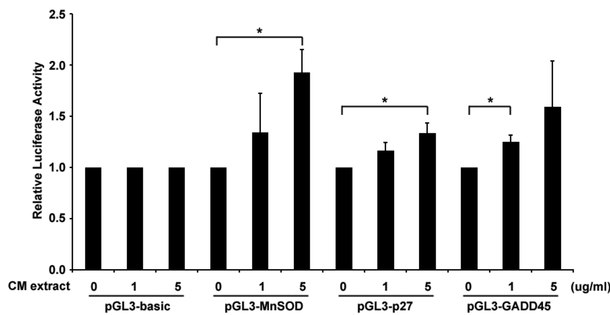

Fig. 3. Target genes of FOXO3a were activated by CM extract. HEF cells were transfected with pGL3-basic, pGL3-MnSOD, pGL3-p27, and pGL3-GADD45, respectively. After 24 h, cells were treated with CM extract for 24 h. Promoter activities were measured using a commercial kit, and the β-galactosidase activity was measured. To correct for the transfection efficiency, the luciferase activity was divided by the β-galactosidase activity. The error bars indicate standard deviations of results obtained from three independent experiments and the statistical significance is indicated as *p < 0.05 by Student's t-test.

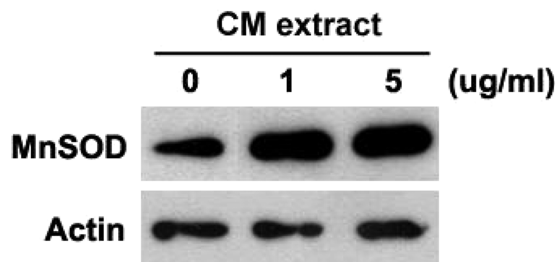

Fig. 4. Increase of MnSOD protein level by CM extract. HEF cells were treated with CM extract for 24 h. Protein levels of MnSOD and actin were measured by western blot analysis.

3. Promoter Activities of FOXO3a Target Genes Increase by CM Extract

MnSOD, p27, and GADD45 are transcriptional target genes of FOXO3a (Dijkers *et al.*, 2000; Kops *et al.*, 2002; Tran *et al.*, 2002). HEF cells were transfected with pGL3-MnSOD, pGL3-p27, and pGL3-GADD45, respectively. After 24 h, transfected cells were treated with CM extract for 24 h. These promoter activities were increased by CM extract (Fig. 3). This result showed that FOXO3a target genes were activated by CM extract through activation of FOXO3a.

4. MnSOD Protein Level Increase by CM Extract

MnSOD is a major antioxidant enzyme that modulates the cellular redox environment (Dhar and St Clair, 2012). MnSOD is reported to be a transcriptional target gene of FOXO3a (Kops *et al.*, 2002). Human MnSOD gene promoter has FOXO binding elements at position -1249 and -997. To investigate whether CM extract regulates MnSOD protein level, HEF cells were treated with CM extract for 24 h and protein level of MnSOD was measured by western blot analysis. Protein level of MnSOD was increased by CM (Fig. 4).

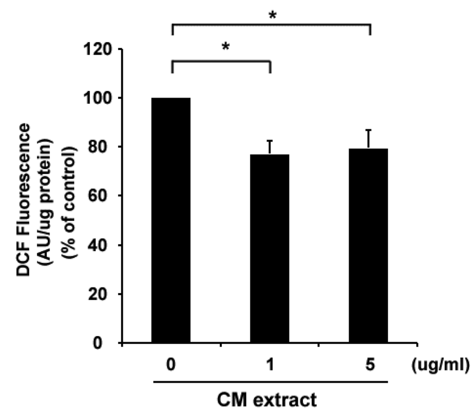

Fig. 5. Decrease of ROS level by CM extract. HEF cells were treated with CM extract for 24 h. Cells were incubated with 30 μM DCDHF-DA for 30 min at 37℃ ROS levels were measured by fluorescence spectrometer as described in materials and methods. The error bars indicate standard deviations of results obtained from five independent experiments and the statistical significance is indicated as *p < 0.05 by Student's t-test.

5. ROS Level Decrease by CM Extract

ROS level is known to be increased with age in HEF cells. Old HEF cells were treated with CM extract for 24 h. ROS level was measured by staining with DCDHF-DA. ROS level was decreased up to 20% by treatment of CM extract (Fig. 5).

DISCUSSION

Reactive oxygen species (ROS) is a natural byproduct of the metabolism and have crucial role in various biological processes. When ROS level increases gradually in cells, oxidative stress to cells begins to show up. Oxidative stress causes damage of nucleic acids, proteins, and lipids, and eventually results in various disease such as cancer, neurodegeneration, atherosclerosis, diabetes, and aging (Ray *et al.*, 2012). It has been reported that the extract of CM had an antioxidant potential and antiproliferative activity through apoptosis in leukemia cells, although the mechanism has not been verified (Nadova *et al.*, 2008). In this study

we showed that CM extract regulates FOXO3a to promote the removal of ROS in old HEF cells.

FOXO3a is involved in various biological functions. FOXO3a protects cells from oxidative stress through directly increases of manganese superoxide dismutase (MnSOD) RNA and protein levels (Kops *et al.*, 2002). FOXO3a also contributes to repair the damaged DNA by regulation of direct target gene, GADD45 (Tran *et al.*, 2002). FOXO3a controls apoptosis through transcriptional regulation of its target gene, Bim (Sunters *et al.*, 2003). FOXO3a regulates cell cycle progression by transcriptional regulation of p27, inhibitor of cell cycle progression (Dijkers *et al.*, 2000). FOXO3a was activated by CM extract (Fig. 1B) and target genes of FOXO3a such as MnSOD, p27, and GADD45 were activated (Fig. 3). However, how FOXO3a activity is increased by CM extract is still unknown. It may phosphorylate or acetylate FOXO3a to activate. Or it may upregulate transcriptional activity of FOXO3a by other mechanisms.

CM extract contains isoquinoline alkyloids such as sanguinarine, chelidonine, chelerythrine, berberine, protopine, and coptisine (Gilca *et al.*, 2010). Expression of pgp (p-glycoprotein) was increased by chelidonine which is the component of CM extract. Chelidonine caused apoptosis and decreased expression level of Bcl-2. Telomerase activity was reduced by low doses of chelidonine and expression level of hTERT RNA was decreased. Growth rate of HepG2 cells was declined by very low doses of chelidonine (Noureini and Wink, 2009). It was also reported that chelidonine and hydroxydihydrosanguinarine inhibit LPS-induced NO production in macrophage RAW264.7 cells. Cox-2 and iNOS mRNA level were decreased by chelidonine and hydroxydihydrosanguinarine in LPS treated RAW264.7 cells (Park *et al.*, 2011). Chelidonine and sanguinarine caused apoptosis in human acute T-lymphoblastic leukaemia MT-4 cells (Philchenkov *et al.*, 2008). Recently, it has been reported that chelidonine promotes apoptosis through upregulation of p38, p53, and other pro-apoptotic genes. And protein kinase B (AKT), PI3K, JAK3, STAT3, E7, E7, and other antiapoptotic genes were downregulated by chelidonine (Paul *et al.*, 2012). However, which component activates FOXO3a has not been verified. Further detailed characterization of component of CM that activates FOXO3a will elucidate the mechanism.

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