Korean J. Plant Res. 29(3):281-288(2016)
http://dx.doi.org/10.7732/kjpr.2016.29.3.281

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Activating Transcription Factor 3 is a Molecular Target for Apoptotic Effect of Silymarin in Human Colorectal Cancer Cells

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Abstract - Apoptosis has been regarded as a therapeutic target because apoptosis is typically disturbed in human cancer. Silymarin found in the seeds of the milk thistle (*Silybum marianum*) has been reported to exert anti-cancer properties through apoptosis. This study was performed to investigate the molecular target for silymarin-mediated apoptosis in human colorectal cancer cells. Silymarin reduced the cell viability and induced an apoptosis in human colorectal cancer cells. ATF3 overexpression increased PARP cleavage by silymarin. Increased ATF3 expression in both protein and mRNA was observed in silymarin-treated cells. In addition, silymarin increased the luciferase activity of ATF3 promoter. Inhibition of JNK and IκK-α blocked silymarin-mediated ATF3 expression. The results suggest that silymarin induces apoptosis through JNK and IκKα-dependent ATF3 expression in human colorectal cancer cells.

Key words - Silymarin, Activating transcription factor 3, Apoptosis, Human colorectal cancer, Cancer chemoprevention

Introduction

As a member of ATF/CREB family of bZIP transcription factors, activating transcription factor 3 (ATF3) has been reported to be stress-responsive gene product (Liang *et al*., 1996). In astrocytes, ATF3 activation exerts the protective activity against oxidative insults (Das *et al*., 2011). However, there are other observations indicating that ATF3 expression plays an important role in cancer chemopreventive properties by various anti-cancer agents such as conjugated linoleic acid, LY294002, curcumin and 3,3'-diindolymethane (Lee *et al*., 2005; Lee *et al*., 2006; Yamaguchi *et al*., 2006; Lee *et al*., 2013), which ATF3 could function as a pro-apoptotic protein. Furthermore, ATF3 activates p53 (Yan *et al*., 2005) and attenuates cyclin D1 (Yan *et al*., 2005) and MMP-2 expression (Chen and Wang, 2004).

Silymarin is a complex of three flavonolignans (silybin, silydianin and silychristin) and two flavonoids (tamoxifen and quercetin) found in the seeds of the milk thistle (*Silybum marianum*) (Abenavoli *et al*., 2010). Although silymarin has been long used for treatment of liver diseases (Mereish *et al*., 1991), silymarin has been reported to exert anti-cancer properties. Silymarin inhibits the growth of human breast cancer cells through increasing p21 expression and decreasing CDKs (Zi *et al*., 1998). In previous study, we have reported that silymarin induces cyclin D1 proteasomal degradation, which results in the growth arrest of human colorectal cancer cells (Eo *et al*., 2015). Furthermore, silymarin inhibits angiogenesis, invasion and metastasis (Ramasamy and Agarwal, 2008). In apoptotic activity, silymarin induces apoptotic cell death in human malignant melanoma cells through increasing the expression of Fas-associated proteins with death domain (FADD) (Ramasamy and Agarwal, 2008). In addition, silymarin causes apoptosis in human leukemia by inhibiting Akt activity associated with activation of caspases-9 and -3 as well as PARP cleavage (Zhong *et al*., 2006).

Apoptosis as a programmed cell death plays an important role in the regulation of various physiological conditions as well as pathological states (Lockshin and Zakeri, 2007). In cancer, apoptosis has been regarded as a therapeutic target because apoptosis is typically disturbed in human cancer (Fulda, 2015). Thus, many currently used anticancer therapies utilize apoptosis signaling pathways to exert their antitumor activities (Fulda, 2015).

In this study, we evaluates the apoptotic effect of silymarin

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in human colorectal cancer cells and elucidates the mechanism of ATF3 regulation as the molecular target for the induction of apoptosis by silymarin.

Materials and Methods

Reagents

Cell culture media, Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). PD98059, SB203580, SP600125, SB216763, BAY11-7082 and silymarin were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against ATF3, p-JNK, total-JNK, p-IκKα, total-IκKα, cleaved PARP, Bc-2, Bax and β-actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

Human colorectal cancer cell lines such as HCT116, SW480, Lovo and HT-29 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fatal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The cells were maintained at 37℃ under a humidified atmosphere of 5% CO_2. Silymarin was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

Cell viability

Cell viability was measured using MTT assay system. Briefly, cells were plated onto 96-well plated and grown overnight. The cells were treated with 0, 25, 50 and 100 μM of silymarin for 24 h. Then, the cells were incubated with 50 μl of MTT solution (1 ㎎/㎖) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 ㎚.

SDS-PAGE and Western blot

After silymarin treatment, cells were washed with 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 15,000 × g for 10 min at 4℃. After determining protein concentration by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), the proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4℃ overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After silymarin treatment, total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and 1 μg of total RNA was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was performed using PCR Master Mix Kit (Promega, Madison, WI, USA) with human primers for cyclin D1 and GAPDH as followed : human ATF3: 5′-gtttgaggattttgctaacctgac-3′, and reverse 5′-agctgcaatcttatttctttctcgt-3′ ; human GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

Transient transfections

Transient transfections were performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction. The cells were plated in 12-well plates at a concentration of 2×10^5 cells/well. After growth overnight, plasmid mixtures containing 1 ㎍ of ATF3 promoter linked to luciferase and 0.1 ㎍ of pRL-null vector were transfected for 24 h. The transfected cells were cultured in the absence or presence of silymarin for 24 h. The cells were then harvested in 1 × luciferase lysis

buffer, and luciferase activity was normalized to the pRL-null luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI, USA).

Expression vector

ATF3 expression vector was provided from Addgene (Cambridge, MA, USA). Transient transfection of the vector was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

Statistical analysis

All the data are shown as mean ± SEM (standard error of mean). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with *P <0.05 were considered statistically significant.

Results

Effect of silymarin on cell viability and apoptosis in human colorectal cancer cells

To evaluate whether silymarin decreases the viability of human colorectal cancer cells, HCT116 and SW480 cells were treated with silymarin at the different concentrations and cell viability was measured by MTT assay. As a result (Fig. 1A), silymarin decreased the cell viability by 16% at 25 μM, 27% at 50 μM and 61% at 100 μM of silymarin in HCT116. The viability of SW480 cells was reduced by 15% at 50 μM and 37% at 100 μM of silymarin. In addition, the viability of HT-29 and Lovo cells was decreased by 33% and 21% at 100 μM of silymarin, respectively (Fig. 1B). Next, we tested cleaved PARP using Western blot to evaluate whether silymarin-mediated decrease of the cell viability results from

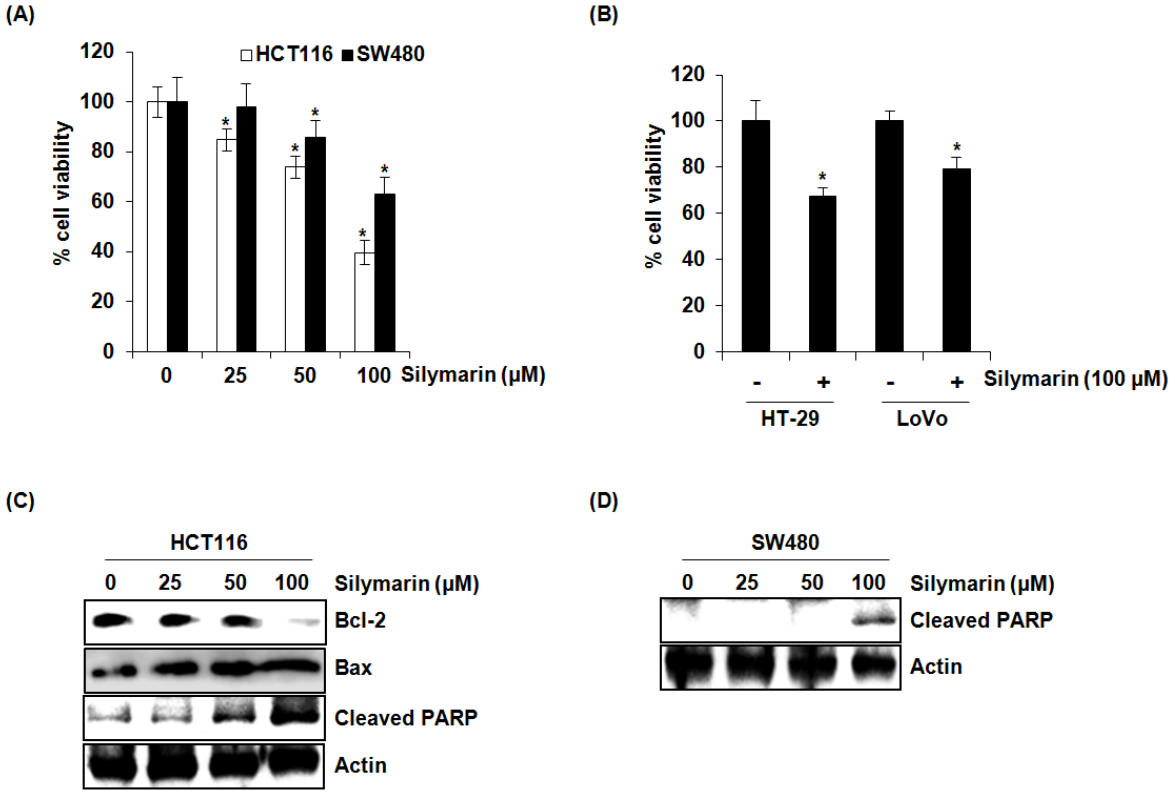

Fig. 1. Effect of silymarin on the cell viability and apoptosis. (A) HCT116 and SW480 cells were treated with silymarin at the indicated concentrations for 24 h. (B) HT-29 and LoVo cells were treated with 100 μM of silymarin for 24 h. Cell viability was measured using MTT assay system and expressed as % cell viability. *p<0.05 compared to cells without silymarin. (C, D) HCT116 and SW480 cells were treated with silymarin at the indicated concentrations for 24 h. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against cleaved PARP, Bcl-2, Bax and actin.

apoptosis. As shown in Fig. 1C, silymarin induced the cleavage of PARP and the expression of Bax as proapoptotic protein, and decreased the expression of bcl-2 as antiapoptotic protein at 50-100 μM of silymarin in HCT116. In addition, the cleavage PARP was observed in SW480 cells treated with 100 μM of silymarin (Fig. 1D).

Effect of silymarin on ATF3 expression in human colorectal cancer cells

To investigate whether silymarin affects ATF3 expression in human colorectal cancer cells, HCT116 cells were treated with silymarin (0, 25, 50 and 100 μM) for 24 h and ATF3 protein was measured using Western blot analysis. As shown in Fig. 2A, silymarin dose-dependently increased the protein level of ATF3. In time-course experiment, ATF3 protein was slightly upregulated at 1 h and increased at 24 h after silymarin treatment (Fig. 2B). Furthermore, silymarin activated ATF3 expression in other human colorectal cancer cell lines, SW480, LoVo and HT-29 (Fig. 2C). Next, HCT116 cells were transfected with Cont- or ATF3 expression vector to evaluate whether ATF3 expression contributes to silymarin-mediated apoptosis. As a result (Fig. 2D), increased cleavage of PARP by silymarin was observed in the cell transfected with an ATF3 expression vector compared with cells transfected with a control vector. These findings indicate that ATF3 may be a molecular target of silymarin to accelerate the induction of apoptosis.

Silymarin-mediated ATF3 expression is associated with transcriptional regulation in human colorectal cancer cells

To investigate whether transcriptional activation of ATF3 by silymarin contributes to the increase of ATF3 protein, ATF3 mRNA was measured by RT-PCR after silymarin treatment. As shown in Fig. 3A, dose-dependent increase of ATF3 mRNA was observed in silymarin-treated HCT116 cells. In addition, silymarin activated the expression of ATF3 mRNA in SW480, LoVo and HT-29 cells similar to the protein expression pattern (Fig. 3B). To confirm the effect on the upregulation of ATF3 transcription, we investigated ATF3 promoter activity. As a result, silymarin activated

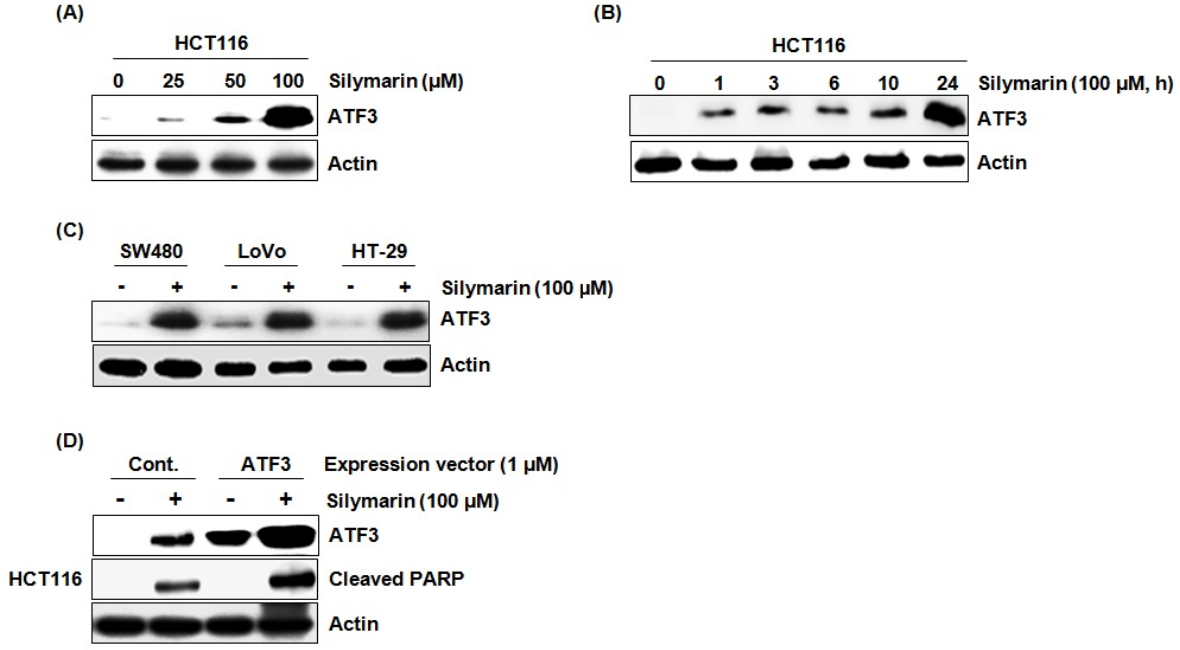

Fig. 2. Effect of ATF3 expression on silymarin-mediated apoptosis. (A) HCT116 cells were treated with silymarin at the indicated concentrations for 24 h. (B) HCT116 cells were treated with 100 μM of silymarin for the indicated times. (C) SW480, HT-29 and LoVo cells were treated with 100 μM of silymarin for 24 h. (D) HCT116 cells was transfected with empty- or ATF3 expression vector for 24 h and then treated with 100 μM of silymarin for 24 h. All cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against ATF3, cleaved PARP or actin.

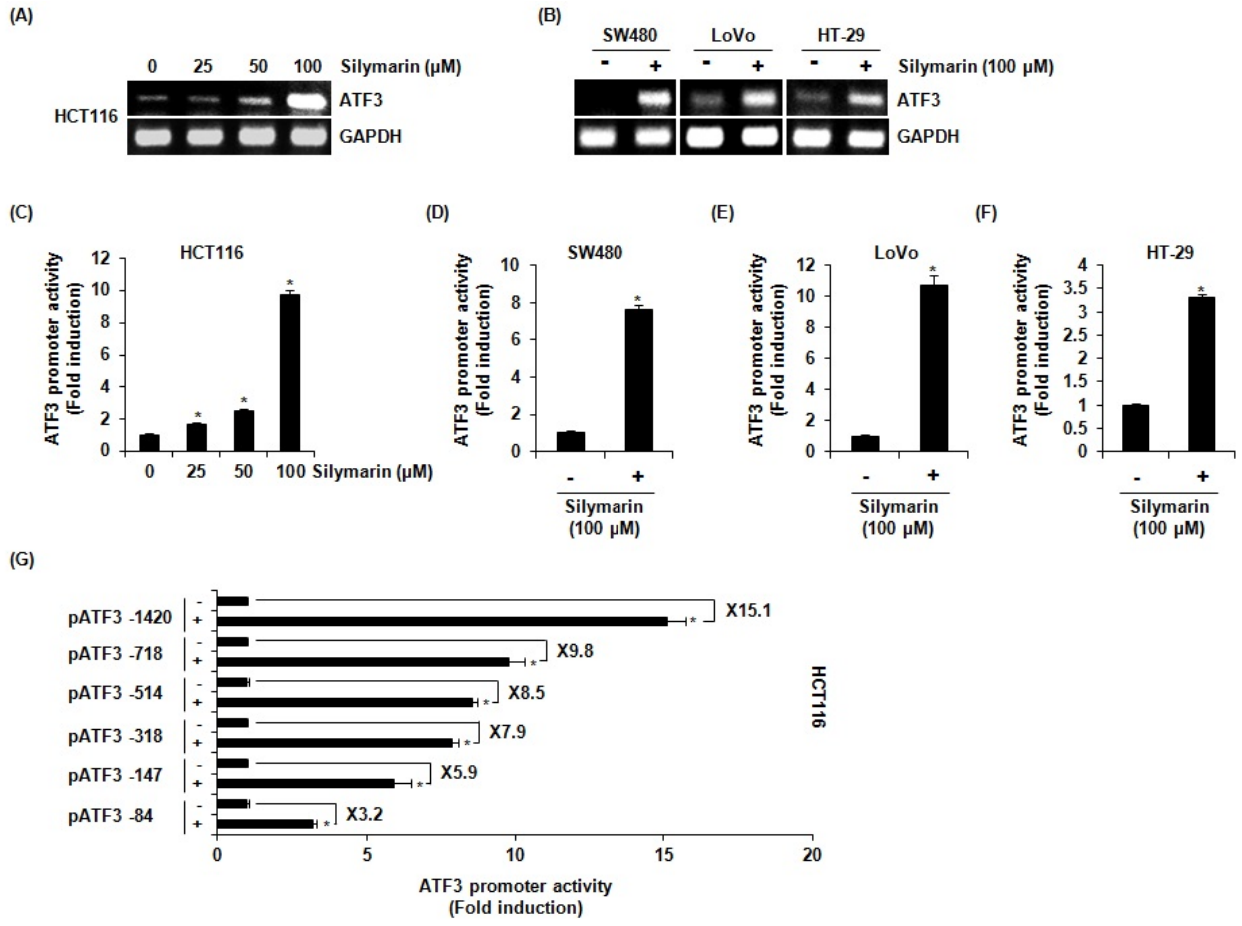

Fig. 3. Effect of silymarin on ATF3 transcriptional regulation. (A, B) HCT116, SW480, LoVo and HT-29 cells were treated with silymarin at the indicated concentrations for 24 h. Total RNA was isolated and RT-PCR was performed. (C-F) The pATF3-1420/+34 construct (1 μg) was co-transfected with pRL-null vector (0.1 μg). The cells were treated with silymarin at the indicated concentrations for 24 h and then luciferase activity was measured. *$p<0.05$ compared to cells without silymarin. (G) HCT116 cells were transfected with indicated ATF3 deletion promoter constructs (1 μg) with pRL-null vector (0.1 μg). The cells were treated with DMSO or 100 μM of silymarin for 24 h and luciferase activity was measured. *$p<0.05$ compared to cells without silymarin.

ATF3 promoter activity in HCT116, SW480, LoVo and HT-29 cells (Fig. 3C-3F).

To elucidate the ATF3 promoter region responsible for the activation of ATF3 promoter activity, HCT116 cells were transfected with serially deleted ATF3 promoter constructs such as pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34 and pATF3-84/+34, and then ATF3 promoter activity was measured after silymarin treatment. Silymarin increased ATF3 promoter by 15.1, 9.8, 8.5, 7.9, 5.9 and 3.2 fold in HCT116 cells transfected in pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34 and pATF3-84/+34, respectively (Fig. 3G). These findings indicate that silymarin-mediated increase of ATF3 expression may result from ATF3 transcriptional upregulation.

Silymarin-mediated ATF3 activation is dependent on JNK and NF-κB

To determine the upstream kinases associated with silymarin-mediated ATF3 expression, HCT116 cells were pretreated with PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), SB216763 (GSK3β inhibitor) or BAY 11-7082 (NF-κB inhibitor) and then co-treated with silymarin. As shown in Fig. 4A-4E, inhibition of

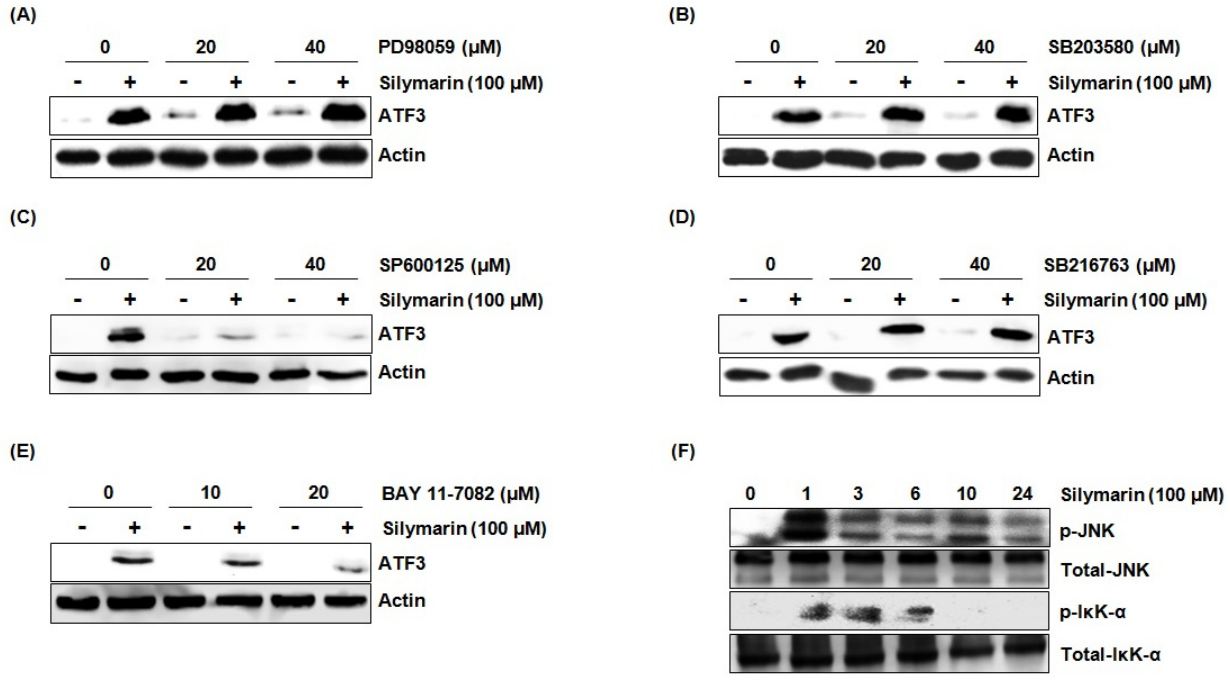

Fig. 4. Dependency of JNK and IκK-α in silymarin-mediated ATF3 activation (A-E) HCT116 cells were pretreated with PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), SB216763 (GSK3β inhibitor) or BAY11-7082 (IκK-α inhibitor) for 2 h and then co-treated with 100 μM of silymarin. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against ATF3 or actin. (F) HCT116 cells were treated with 100 μM of silymarin for the indicated times. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against p-JNK, total-JNK, p-IκKα, total-IκKα or actin.

ERK1/2, p38 or GSK3β did not block ATF3 expression by silymarin. However, silymarin-mediated ATF3 expression was attenuated when JNK and NF-κB were blocked. In addition, we observed that silymarin induces the phosphorylation of JNK and IκK-α as an active form (Fig. 4F). These results indicate that JNK and NF-κB may be important upstream kinases for silymarin-mediated ATF3 expression.

Discussion

Consumption of the phytochemicals has been known to reduce the risk of human cancer (Pan *et al*., 2008; Pan and Ho, 2008). One of the mechanisms for the natural anti-cancer phytochemicals is through the apoptosis (Pan *et al*., 2008). ATF3 mediates sensitization of human colorectal cancer cells to TRAIL-mediated apoptosis through ER stress (Edagawa *et al*., 2014; Taketani *et al*., 2012) and regulated synergistic anticancer activity of a HDAC inhibitor and anti-DR5 antibody in human colon cancer cells (Liu *et al*., 2014). From growing evidence, ATF3 may be one of the important molecular targets for chemoprevention of human colorectal cancer.

In this study, silymarin decreased cell viability in human colorectal cell lines, HCT116, SW480, LoVo and HT-29 cells. Furthermore, silymarin treatment increased PARP cleavage as the apoptotic marker and ATF3 overexpression enhanced silymarin-mediated cleavage of PARP in human colorectal cancer cells. These findings suggest that ATF3 may be one of the molecular targets for silymarin-mediated apoptosis in human colorectal cancer cells. There is growing evidence that ATF3 decreases Bcl-2 expression as anti-apoptotic protein and increases Bak as pro-apoptotic protein in human colorectal cancer cells (Jiang *et al*., 2016).

Silymarin-mediated increase of ATF3 protein resulted from transcriptional regulation by evaluating the level of ATF3 mRNA and ATF3 promoter activity. In searching for a specific promoter region responsible for silymarin-mediated ATF3 activation, we found that the transfection of serially deleted ATF3 promoter constructs (pATF3-718/+34, pATF3-

514/+34, pATF3-318/34, pATF3-147/+34 and pATF3-84/+34) decreased ATF3 promoter activity by silymarin compared to pATF3-1420/+34. ATF3 promoter has been reported to contain a variety of response elements such as E2F, AP1 and Myc/Max (Liang *et al*., 1996). Although we did not determine specific ATF3 promoter region responsible for silymarin-mediated ATF3 activation, various response elements may be important for ATF3 activation by silymarin. Thus, the further mechanistic study for elucidation of the promoter region responsible for ATF3 activation by silymarin will be demanded.

There is growing evidence that ATF3 expression is regulated by a variety of the upstream kinases (Cai *et al*., 2000; Baek *et al*., 2004). Thus, we examined whether silymarin-mediated ATF3 activation is associated with the activation of ERK1/2, p38, JNK, IκK-α or GSK3β, and found that ATF3 expression by silymarin was suppressed in the inhibition of JNK and IκK-α, but not in the inhibition of other kinases such as ERK1/2, p38 and GSK3β. Furthermore, silymarin activated JNK and IκK-α, which indicates that JNK and IκK-α may be an important upstream kinase for silymarin-mediated increase of ATF3 expression.

Taken together, our findings indicate that silymarin increases ATF3 transcription and protein level through JNK and IκK-α activation, and ATF3 expression enhances silymarin-mediated apoptosis in human colorectal cancer cells. Therefore, these findings can provide information on the apoptotic effect and the potential molecular mechanism of silymarin.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1 A1A2053448).

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(Received 7 March 2016 ; Revised 5 April 2016 ; Accepted 20 April 2016)