Food Sci. Biotechnol. Vol. 17, No. 5, pp. 1079 ~ 1085 (2008)

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

Up-regulation of Aldo-keto Reductase 1C3 Expression in Sulforaphane-treated MCF-7 Breast Cancer Cells

Sang-Han Lee

Department of Biochemistry, College of Medicine, Soonchunhyang University, Cheonan, Chungnam 330-090, Korea

Abstract The chemopreventive activity of sulforaphane (SFN) occurs through its inhibition of carcinogen-activating enzymes and its induction of detoxification enzymes. However, the exact mechanisms by which SFN exerts its anti-carcinogenic effects are not fully understood. Therefore, the mechanisms underlying the cytoprotective effects of SFN were examined in MCF-7 breast cancer cells. Exposure of cells to SFN (10 μM) induced a transcriptional change in the AKR1C3 gene, which is one of aldo-keto reductases (AKRs) family that is associated with detoxification and antioxidant response. Further analysis revealed that SFN elicited a dose- and time-dependent increase in the expression of both the NRF2 and AKR1C3 proteins. Moreover, this up-regulation of AKR1C3 was inhibited by pretreatment with antioxidant, *N*-acetyl-L-cysteine (NAC), which suggests that the up-regulation of AKR1C3 expression induced by SFN involves reactive oxygen species (ROS) signaling. Furthermore, pretreatment of cells with LY294002, a pharmacologic inhibitor of phosphatidylinositol 3-kinase (PI3K), suppressed the SFN-augmented Nrf2 activation and AKR1C3 expression; however, inhibition of PKC or MEK1/2 signaling with Gö6976 or PD98059, respectively, did not alter SFN-induced AKR1C3 expression. Collectively, these data suggest that SFN can modulate the expression of the AKR1C3 in MCF-7 cells by activation of PI3K via the generation of ROS.

Keywords: sulforaphane, AKR1C3, cytoprotection, phosphatidylinositol 3-kinase, reactive oxygen species

Introduction

The ability of cruciferous vegetables to protect against neoplastic disease has been attributed to the fact they contain a relatively high content of glucosinolates. One of the most commonly studied glucosinolates is glucoraphanin, a 4-methylsulfinylbutyl-containing glucosinolates, and their hydrolysis results in the generation of sulforaphane (SFN) responsible for anticarcinogenic activity (1). SFN [1-isothiocyanato-4-(methylsulfinyl)-butane], a major isothiocyanate (ITC) abundant in broccoli, brussel sprouts, and cabbage, has been reported as a chemopreventive agent because of its anticancer effects on the different stages that lead to the development of malignant tumors (2). SFN has been shown to induce apoptosis and cell cycle inhibition in many types of cancer cells (3,4), as well as to inhibit the progression of benign tumors to malignant tumors, angiogenesis and endothelial cell functions, and the metastatic process (5). The induction of phase II carcinogen detoxification enzymes such as glutathione transferase, UDP-glucuronyltransferase, and NAD(P)H:quinone oxidoreductase I is an important mechanism of chemoprevention (6), in which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they cause damage to critical cellular macromolecules.

SFN has received a great deal of attention because of its ability to induce antioxidant response element (ARE)-regulated enzymes that provide defense against cancer-causing chemicals (7). Specific regions of the promoters of these genes, the AREs, are tightly regulated by a basic leucine zipper transcription factor, NF-E2-related factor 2 (Nrf2). Consequently, genes for the several phase II enzymes regulated by the ARE encode proteins that help control cellular redox potential and that rescue cells from oxidative damage. Therefore, activation of the Nrf2 pathway in cells plays a central role in enhancing the antioxidative capacity. Induction of cytoprotective genes by SFN is primarily mediated by transcriptional activation through the Nrf2. The chemopreventive efficacy of SFN is lost in Nrf2-deficient mice (8), which indicates that activator of Nrf2 signaling such as SFN protects cells and animals against xenobiotic toxicities. Molecular epidemiological studies have demonstrated a strong inverse relationship between consumption of dietary ITCs and the risk of various types of malignancies (9,10). In addition, more recent epidemiologic studies have shown that dietary intake of ITC-containing cruciferous vegetables may be protective against breast cancer (11). Thus, identification of cytoprotective genes that are directly or indirectly dependent on Nrf2 in response to SFN will facilitate the understanding of the molecular mechanisms and targets of the chemopreventive effects.

In the present study, differential expression of genes in SFN-treated MCF-7 cells in comparison with non-treated cells was examined by a nonradioactive reverse transcriptase-polymerase chain reaction (RT-PCR)-based mRNA differential display analysis. The SFN-induced novel mRNA and protein expression of AKR1C3, which is suggestive of an Nrf2-associated gene, were confirmed by RT-PCR and Western blot analyses.

*Corresponding author: Tel:+82-41-570-2442; Fax: +82-41-577-0450
E-mail: m1037624@sch.ac.kr
Received April 14, 2008; Revised June 17, 2008;
Accepted June 25, 2008

Materials and Methods

Reagents and cell culture SFN was purchased from LKT Laboratories (St. Paul, MN, USA). Antibodies to

AKR1C3 and β-actin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrozolum bromide (MTT), and *N*-acetyl-L-cysteine were obtained from Sigma-Aldrich (St. Louis, MO, USA). LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], PD98059 (2'-amino-3'-methoxyflavone), U0126, and Gö6976 [12-(2-cyanoethyl)-tetrahydro-13-methyl-oxoindolopyrrolocarbazole] were obtained from Calbiochem. (La Jolla, CA, USA). Nrf2 antibody, goat anti-rabbit IgG-HRP antibody, and an enhanced chemiluminescence (ECL) system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol reagent, cell culture media and reagents were purchased from Life Technologies (Grand Island, NY, USA). The human mammary adenocarcinoma cell line, MCF-7, was obtained from Korean Cell Line Bank (Seoul, Korea) and used throughout the study. The cells grew in a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 1 mM glutamine, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. Cells were grown to 70% confluence, and the medium was changed to phenol red-free DMEM with 5% charcoal-stripped calf serum (CCS). Cells were maintained in this medium for 24 hr before treatment.

MTT assay MCF-7 cells were seeded in 24-well plates in DMEM supplemented with 5% FCS. Cells were grown to 70% confluence, after which the medium was changed to phenol red-free DMEM with 5% CCS. The cells were then maintained in this medium for 24 hr and followed by SFN treatment for 24 hr. MTT (final 0.1 mg/mL) was then added to the culture medium, after which the cells were incubated for additional 4 hr. After removal of the medium, the formazan crystals formed by the reduction of MTT by mitochondrial dehydrogenases in living cells were solubilized in 500 μL of DMSO and measured spectrophotometrically at 555 nm using a Glomax multi detection system (Promega, Madison, WI, USA). The results were expressed as a percentage based on the ratio of the absorbances of treated cells to that of controls (100%).

Differential display Total RNA obtained from cultured cells was purified using Trizol reagent (Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Differential display analysis was conducted as described previously (12). Briefly, 400 ng of DNA-free total RNA was reverse-transcribed using T_{11}VV primers (Display System Biotechnology, Vista, CA, USA) where V represents degenerative mixture of dA, dG, and dC. Amplication was performed in 20 μL volume containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate (dNTP), 0.5 μM of primer, and 1 unit of Stoffel fragment (Perkin Elmer, Norwalk, CT, USA). The PCR conditions were as follows: initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, 40°C for 40 sec, and 72°C for 60 sec. The PCR products were then analyzed by electrophoresis in nondenaturing 10% polyacrylamide mini-gels. Next, the samples were visualized by silver staining, after which PCR bands of interest ranging 150 to 600 bp were recovered from gel, reamplified, and cloned for sequencing. The DNA sequences were then compared to those in the GenBank databases utilizing the Blast program available at http://www.ncbi.nlm.nih.gov.

RT-PCR Genes that were of interest as a result of expression differences observed in the differential display analysis were further examined by RT-PCR. Briefly, 1 μg of the total RNA was converted to cDNA using the oligo$(dT)_{12-18}$ primer and Superscript III reverse transcriptase (Invitrogen, Eugene, OR, USA). Each single-stranded cDNA was then diluted and subjected to PCR amplification using Ex Taq DNA polymerase (Takara, Otsu, Japan). The PCR conditions were as follows: initial denaturation at 94°C for 2 min followed by 27 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. The following primers were used to amplify AKR1C3: forward 5'-ATTTGGCACCTAT GCACCTC-3' and reverse 5'-CACACTGCCATCTGCAA TCT-3', β-actin: forward 5'-GCTATCCCTGTACGCCTC TG-3' and reverse 5'-ACATCTGCTGGAAGGTGGAC-3'. The PCR products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide and bands were visualized under ultraviolet (UV) light.

Western blotting for AKR1C3 expression Cell lysates were prepared using RIPA buffer [1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 μg/mL phenylmetanesulfonylfluoride (PMSF)]. Proteins (40 to 50 μg/lane) were separated on 10% Tris-glycine SDS-polyacrylamide gel and electrophoretically transferred to Immuno-Blot PVDF membrane. The membrane was incubated for 1 hr at room temperature with 1:500 diluted rabbit anti-AKR1C3 monoclonal antibody. The horseradish peroxidase-conjugated secondary antibody was then applied at a dilution of 1:8,000. Anti-β-actin antibody (Sigma-Aldrich) was used as a loading control. The signal was visualized using an ECL detection kit (Santa Cruz, Biotechnology).

Statistical analysis The differences between treated and untreated groups were determined using the Student's *t*-test. The results were expressed as the mean±standard error mean (SEM).

Results and Discussion

SFN-treated MCF cells differentially express AKR1C3 To determine the appropriate concentration range of SFN required for the gene expression study, MCF-7 cells were treated with different doses of SFN for 24 hr, after which the viability was estimated by an MTT assay. As shown in Fig. 1, exposure of MCF-7 cells to 1, 10, 25, and 50 μM doses of SFN reduced the cell viability to 103±2.8, 98±3.8, 89±2.2, and 81±3.4% of the control, respectively. No apparent influence on cell viability was observed in samples that were treated with 10 μM SFN. Therefore, the 10 μM dose of SFN was considered as nontoxic dose and used for mRNA differential display analysis.

Nrf2 has been shown to be sequestrated by its cytoplasmic partner, Kelch-like ECH-associated protein 1 (Keap1) (13). Modification of specific SH groups in Keap1 by oxidation, alkylation, or arylation results in the dissociation of the Keap1/Nrf2 complex and translocation of Nrf2 to the nucleus, where it binds to the ARE and induces a series of detoxification enzymes. SFN facilitates the release of Nrf2

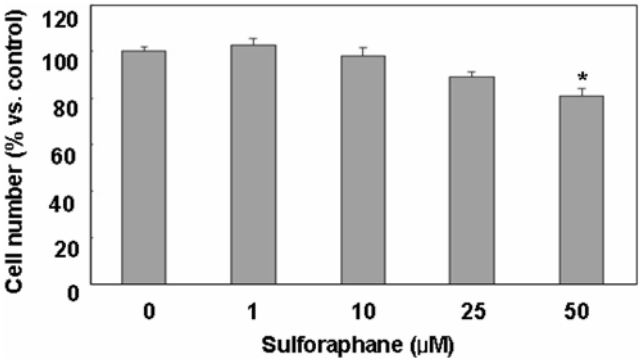

Fig. 1. Inhibition of MCF-7 cell proliferation by sulforaphane. MCF-7 cells were plated in DMEM containing 5% CCS for 24 hr, followed by treatment with vehicle (media) or SFN (1-50 μM) for 24 hr, and then harvested for viability using an MTT assay. Error bars represent the mean±SEM for 3 independent experiments (*p<0.05 vs. untreated control).

from Keap1 and subsequent translocation to the nucleus, thereby triggering the induction of ARE-bearing genes. However, because many potential cytoprotective genes are up-regulated through Nrf2 signaling, it is not clear to what extent any of these contributes to protection against oxidative stress. As an initial approach to addressing this issue, the mRNA expression patterns of MCF-7 cells treated with 10 μM SFN were compared to those of untreated cells using a nonradioactive mRNA differential display PCR. Utilizing the application of differential primer sets, lots of differentially expressed cDNA fragments were detected and excised from the gels for DNA sequence analysis. Out of these, AKR1C3, which is one of AKRs that play an important role in the cellular response to osmotic, electrophilic, and oxidative stress (14), was selected for further evaluation (Fig. 2A). Specific primers were designed for the gene and the specificity of differential expression of mRNA was assessed by RT-PCR using β-actin as internal standard. As shown in Fig. 2B, SFN slightly increased the level of AKR1C3 transcription in a dose- and time-dependent manner. When cells were treated with 10 μM SFN, an increase in AKR1C3 mRNA level was detected at 2 hr, and this increase reached a maximum of nearly 2-fold at 4 hr, after which it gradually declined until 24 hr. A slight induction of AKR1C3 mRNA was obtained in response to treatment with 1 to 20 μM SFN for 24 hr.

Next, to confirm that the increase in AKR1C3 mRNA resulted in an elevated protein level, the level of AKR1C3 protein in SFN-treated MCF-7 cells was determined by Western blot analysis. As shown in Fig. 3A, the levels of AKR1C3 increased to approximately above 2 times those of the control levels following treatment with 10 μM SFN over 4 hr. Dose response studies revealed that approximately 1 μM SFN was sufficient to significantly increase the level of AKR1C3 (Fig. 3B). Taken together, these findings indicate that the SFN-induced increase in AKR1C3 mRNA was followed by an increase in the protein level in MCF-7 cells, but the absolute magnitudes of the differences were lower; the changes of AKR1C3 mRNA levels in RT-PCR analysis were not as strong as those of protein levels (Fig. 2B). This discrepancy between the mRNA and protein levels is likely the result of lower mRNA stability. In addition, nonspecific amplification by RT-PCR may have

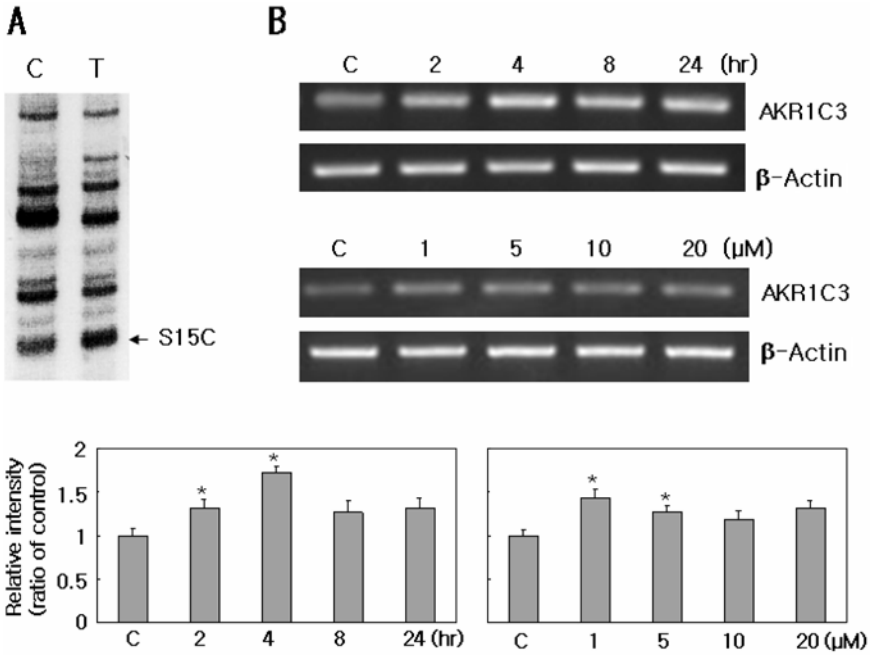

Fig. 2. Sulforaphane-induced AKR1C3 mRNA expression. (A) MCF-7 cells were treated with vehicle (media) or SFN (10 μM) for 24 hr prior to the extraction of total RNA for a non-radioactive differential display analysis. S15C indicates differentially expressed AKR1C3 mRNA. C, control cells; T, SFN-treated cells. (B) Cells were treated with vehicle (media) or SFN (10 μM) for the indicated times or with the indicated concentrations of SFN for 24 hr, after which the total RNA was extracted and subjected to RT-PCR analysis. β-Actin primers were used to amplify as an internal standard. Error bars represent the mean±SEM for 3 independent experiments (*p<0.01 vs. untreated control).

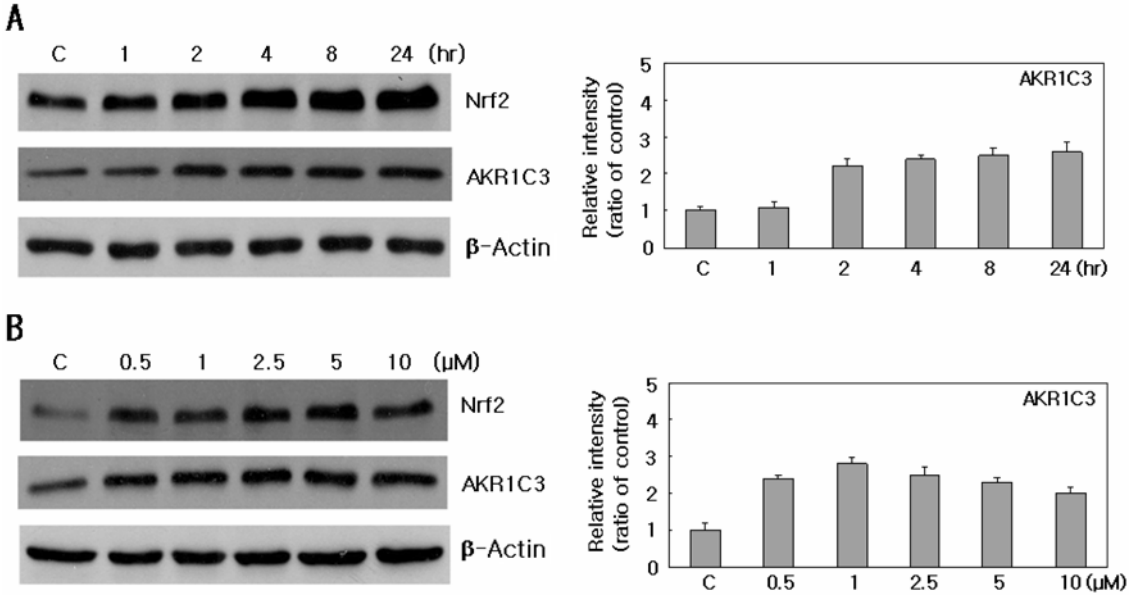

Fig. 3. Sulforaphane-induced up-regulation of the AKR1C3 protein. MCF-7 cells were treated with vehicle (media) or SFN (10 μM) for the indicated times (A) or with increasing concentrations of SFN for 24 hr (B). Cell lysates (40 μg) were then analyzed by Western blot analysis to determine the level of AKR1C3 and Nrf2 proteins. β-Actin was used as a loading control. Error bars represent the mean±SEM for 2 independent experiments.

influenced the results. However, further investigation is required to better understand the reasons for these differences. Taken together, these results indicated that SFN, at least partly, controls the AKR1C3 contents at the transcription level. The up-regulation of AKR1C3 expression in this study was striking, and to the best of my knowledge, differential expression of this gene product in cells treated with SFN has not been previously reported.

Human AKR1C3 belongs to the AKR superfamily. Although the physiological functions of AKRs have not yet been fully resolved, it is well documented that AKRs catalyze the reductive detoxification of reactive aldehydes and ketones, which are produced as a result of oxidative stress (15). This finding suggests that reactive aldehydes and ketones formed from exogenous toxicants, endogenous toxicants, and those formed from the breakdown of lipid peroxides are detoxified, in part, by this enzyme. In addition to its cytoprotective role, AKR1C3 has been regarded as a toxication enzyme because oxidation by the enzyme yields reactive and redox-active *O*-quinones and reaction oxygen species (ROS) (16). This pleiotropic action of AKR1C3 could be due in part to its broad substrate specificity for carbonyl compounds generated in the cells (15,17), however, it still remains to be further investigated. The AKR1C3 gene contains an ARE sequences at -1.4 and -6.8 kb upstream from the transcription start (18). Nrf2 functions as an important mediator in the expression of several phase II enzyme genes through interaction with the ARE sequence. Thus, I wanted to see whether AKR1C3 transcription induced by SFN is dependent on the level of Nrf2. As shown in Fig. 3, Nrf2 protein expression levels showed an initial increase at 1 hr and were maintained until 24 hr after treatment. The simultaneous induction of the Nrf2 and AKR1C3 protein expression in both a time course and dose-response experiments implies that the AKR1C3 may be another member of the antioxidant response element-inducible battery of genes. However, until now there is no evidence about the expression and inducibility of AKR1C3 gene is regulated by Nrf2 in human cells, whereas the finding that Nrf2 elicits up-regulation of AKR1C1 and AKR1C2 was already elucidated (19).

SFN up-regulates the expression of AKR1C3 in a ROS- and PI3K/AKT-dependent manner Nrf2, which is one of the transcription factors that is regulated by the oxidative status of the cells, is known to be involved in the regulation of major cell processes such as antioxidative response, xenobiotic detoxification, and proteome maintenance (20). In addition, SFN is known to induce the generation of ROS, which plays a pivotal role in the initiation of SFN-triggered apoptotic death in human prostate cancer cells (21). To clarify whether the up-regulation of AKR1C3 protein after treatment with SFN occurred as a result of changes in intracellular oxidative status, the effects of free radical scavengers on activation of Nrf2 and AKR1C3 expression were examined. As shown in Fig. 4, pretreatment with 10 mM NAC rapidly reduced the levels of Nrf2 and AKR1C3 proteins at 2 hr after SFN exposure, and this blocking effect was sustained for up to 24 hr. These findings suggest that ROS regulates the activation of Nrf2, which may be involved in the up-regulation of AKR1C3 expression following SFN treatment. At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest, to senescence, to cell death. However, low levels of ROS, whether they are generated intracellularly through a variety of physiological processes or derived from exogeneous sources, are known to serve important regulatory functions, such as mitogenic signaling or the defense systems against oxidative stress (22). Several studies have shown that stimulation of diverse receptor systems, including tumor necrosis factor-

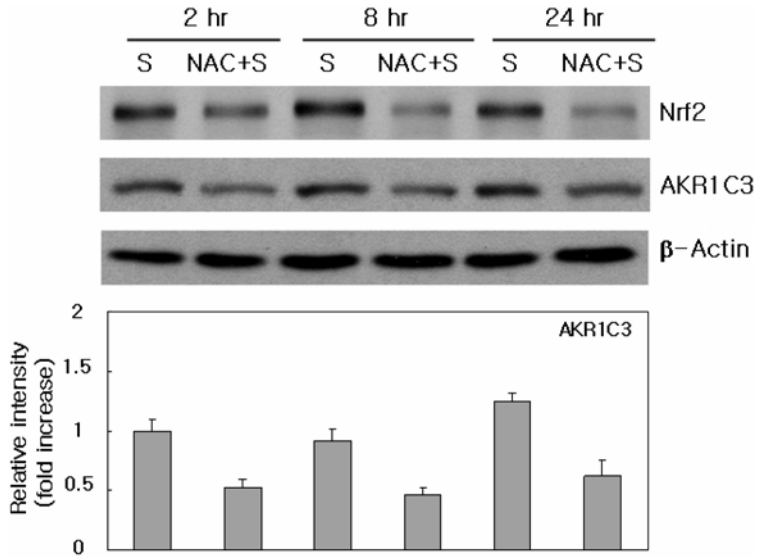

Fig. 4. Inhibition of sulforaphane-induced AKR1C3 expression by the antioxidant, NAC. MCF-7 cells were treated with vehicle (media) or NAC (10 mM) for 1 hr prior to incubation with SFN (10 μM) for the indicated times. Cell lysates (40 μg) were then analyzed for the level of AKR1C3 and Nrf2 proteins by Western blot analysis. β-Actin was used as a loading control. Error bars represent the mean±SEM for 2 independent experiments.

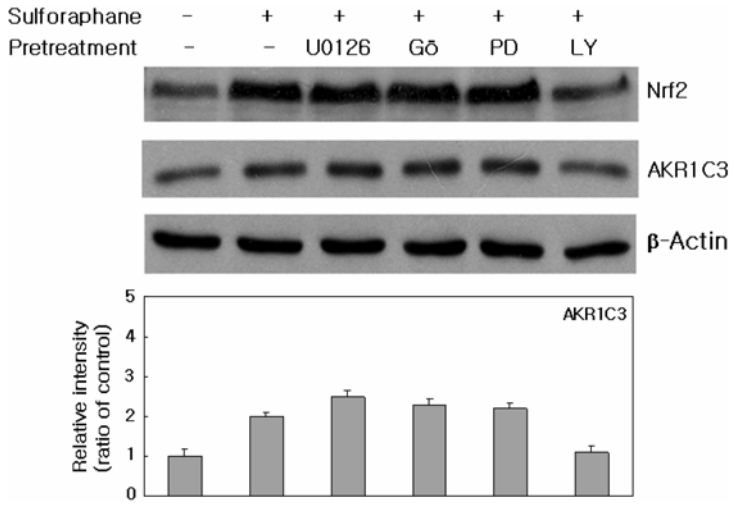

Fig. 5. Effects of various chemical inhibitors on the AKR1C3 level. MCF-7 cells were treated with vehicle (media) or with U0126 (20 μM), Gö6976 (2 μM), PD98059 (50 μM), or LY294002 (25 μM) for 1 hr prior to overnight incubation with SFN (10 μM). Cell lysates (40 μg) were then analyzed for the level of AKR1C3 and Nrf2 proteins by Western blot analysis. β-Actin was used as a loading control. Error bars represent the mean±SEM for 2 independent experiments.

α (23), platelet-derived growth factor (24), and insulin (25), induces ROS, which may serve as requisite second messengers.

In searching for the potential role of different signaling pathways in SFN-mediated AKR1C3 induction, MCF-7 cells were treated with the following inhibitors that block specific signaling pathways prior to incubation with SFN; LY294002 for PI3K, PD98059 or U0126 for MEK1/2, and Gö6976 for PKC. As shown in Fig. 5, LY294002 strongly inhibited the induction of SFN-induced AKR1C3 expression, which suggests that signaling mediated by PI3K plays a significant role in AKR1C3 induction. On the other hand, Gö6976, PD98059, and U0126 had no significant effect on AKR1C3 induction by SFN, which suggests that PKC or MEK1/2 signaling is not involved in its expression. Several previous studies have reported that pharmacologic inhibition of PI3K in both human renal and colon adenocarcinoma cells inhibited ARE-mediated transcriptional activation of target genes (26,27). The results of this study demonstrate that the activation of Nrf2 by SFN was, at least partly, mediated by the PI3K signaling pathway (Fig. 5). Indeed, accumulating evidence indicates that Nrf2 is activated by phosphorylation via several protein kinase pathways, including mitogen-activated protein kinases, protein kinase C, and phosphatidylinositol 3-kinase (28,29), although the role of each pathway in the regulation of Nrf2 and its molecular targets is controversial and probably specific to a given gene and cell type. Furthermore,

the finding that both Nrf2 and AKR1C3 expression are commonly blocked by treatment with a combination of PI3K inhibitor and SFN demonstrates that PI3K signaling is necessary for the induction of AKR1C3 in response to SFN. Recently, the role of the PI3K/Akt pathway in the regulation of Nrf2 has been the source of much interest and several studies have shown that PI3K plays a role in Nrf2 activation in cells exposed to *tert*-butylhydroquinone (30) and hemin (31). Akt is a serine/threonine kinase that is activated in response to growth factors and other extracellular responses via a PI3K pathway, and activation of the PI3K/Akt signaling pathway is known to promote a number of cellular processes including cell growth, proliferation, and survival. Therefore, it would be rational to assume that low level of ROS induced by SFN serves as a signal that warns cells of the oxidative status, thereby triggering up-regulation of AKR1C3 expression as part of a survival mechanisms in MCF-7 cells.

To the best of my knowledge, the effects of SFN on AKR1C3 expression have not yet been reported. In addition, there is limited information regarding the signaling mechanisms that modulate AKR1C3 levels. Nevertheless, it can be postulated that SFN, by inducing Nrf2, down-regulates the oxidative stress in the cell, which could indirectly affect signaling pathways that regulate AKR1C3. Alternatively, SFN up-regulates AKR1C3 expression, which can be induced by ROS and coupled to PI3K/Akt signaling in an Nrf2-independent manner. Although the precise mechanism by which SFN regulates AKR1C3 expression remains to be defined, the results presented here offer a novel role for SFN as possibly a part of the defense mechanisms of the cells under conditions of local oxidative stress. However, further characterization of this molecular pathway is needed to clarify the molecular mechanisms and targets of the chemopreventive effects of SFN.

References

1. Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wailing MA, Jeffery EH. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. J. Agr. Food Chem. 47: 1541-1548 (1999)
2. Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH. Sulforaphane inhibits histone deacetylase *in vivo* and suppresses tumorigenesis in Apc-minus mice. FASEB J. 20: 506-508 (2006)
3. Chuang LT, Moqattash ST, Gretz HF, Nezhat F, Rahaman J, Chiao JW. Sulforaphane induces growth arrest and apoptosis in human ovarian cancer cells. Acta Obstet. Gyn. Sca. 16: 1-6 (2007)
4. Matsui TA, Murata H, Sakabe T, Sowa Y, Horie N, Nakanishi R, Sakai T, Kubo T. Sulforaphane induces cell cycle arrest and apoptosis in murine osteosarcoma cells *in vitro* and inhibits tumor growth *in vivo*. Oncol. Rep. 18: 1263-1268 (2007)
5. Conaway CC, Wang CX, Pittman B, Yang YM, Schwartz JE, Tian D, McIntee EJ, Hecht SS, Chung FL. Phenethylisothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. Cancer Res. 65: 8548-8557 (2005)
6. Lee SB, Lee JY, Song DG, Pan CH, Nho CW, Kim MC, Lee EH, Jung SH, Kim HS, Kim YS, Um BH. Cancer chemopreventive effects Korean seaweed extracts. Food Sci. Biotechnol. 17: 613-622 (2008)
7. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. P. Natl. Acad. Sci. USA 89: 2399-2403 (1992)
8. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW. Sensitivity to carcinogenesis is increased and chemopreventive efficacy of enzyme inducers is lost in Nrf2 transcription factor-deficient mice. P. Natl. Acad. Sci. USA 98: 3410-3415 (2001)
9. Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, van den Brandt PA. Epidemiological studies on *brassica* vegetables and cancer risk. Cancer Epidem. Biomar. 5: 733-748 (1996)
10. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. J. Natl. Cancer Inst. 92: 61-68 (2000)
11. Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J. Nutr. 134: 1134-1138 (2004)
12. Lee SH, Shiao YH, Kasprzak KS. Nonradioactive mRNA differential display in polyacrylamide mini-gels. Res. Co. Mol. Path. 106: 108-114 (1999)
13. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap 1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Gene Dev. 13: 76-86 (1999)
14. Jin Y, Penning TM. Aldo-keto reductases and bioactivation/detoxication. Annu. Rev. Pharmacol. 47: 263-292 (2007)
15. Ellis EM. Reactive carbonyls and oxidative stress: Potential for therapeutic intervention. Pharmacol. Therapeut. 115: 13-24 (2007)
16. Penning TM. Aldo-keto reductases and formation of polycyclic aromatic hydrocarbon *O*-quinones. Methods Enzymol. 378: 31-67 (2004)
17. Hyndman D, Bauman DR, Heredia VV, Penning TM. The aldo-keto reductase superfamily homepage. Chem. Biol. Interact. 143-144: 499-525 (2003)
18. Burczynski ME, Lin HK, Penning TM. Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: Implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. Cancer Res. 59: 607-614 (1999)
19. Wang XJ, Hayes JD, Wolf CR. Generation of a stable antioxidant response element-driven reporter gene cell line and its use to show redox-dependent activation of nrf2 by cancer chemotherapeutic agents. Cancer Res. 66: 10983-10994 (2006)
20. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. 47: 89-116 (2007)
21. Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, Zeng Y, Watkins SC, Johnson CS, Trump DL, Lee YJ, Xiao H, Herman-Antosiewicz A. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J. Biol. Chem. 280: 19911-19924 (2005)
22. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. Nature 401: 79-82 (1999)
23. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120: 649-661 (2005)
24. Chen KC, Zhou Y, Zhang W, Lou MF. Control of PDGF-induced reactive oxygen species (ROS) generation and signal transduction in human lens epithelial cells. Mol. Vis. 13: 374-387 (2007)
25. Biswas S, Gupta MK, Chattopadhyay D, Mukhopadhyay CK. Insulin-induced activation of hypoxia-inducible factor-1 requires generation of reactive oxygen species by NADPH oxidase. Am. J. Physiol. -Heart C. 292: H758-H766 (2007)
26. Harrison EM, McNally SJ, Devey L, Garden OJ, Ross JA, Wigmore SJ. Insulin induces heme oxygenase-1 through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in renal cells. FEBS J. 273: 2345-2356 (2006)
27. Jakubíková J, Sedlák J, Mithen R, Bao Y. Role of PI3K/Akt and MEK/ERK signaling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription, G2/M arrest, and cell death in Caco-2 cells. Biochem. Pharmacol. 69: 1543-1552 (2005)
28. Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tan TH, Kong AN. Activation of mitogen-activated protein kinase pathways induces

antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. J. Biol. Chem. 275: 39907-39913 (2000)
29. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer 3: 768-780 (2003)
30. Gharavi N, Haggarty S, El-Kadi AO. Chemoprotective and carcinogenic effects of *tert*-butylhydroquinone and its metabolites. Curr. Drug Metab. 8: 1-7 (2007)
31. Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K, Nakashima K. PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. FEBS Lett. 546: 181-184 (2003)