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Chinese Cabbage Extracts and Sulforaphane Can Protect H_2O_2-Induced Inhibition of Gap Junctional Intercellular Communication through the Inactivation of ERK1/2 and p38 MAP Kinases

JAE-WOONG HWANG,[†] JOON-SUK PARK,[⊥] EUN-HYE JO,[†] SUN-JUNG KIM,[†] BYOUNG-SU YOON,[‡] SUNG-HOON KIM,[§] YONG-SOON LEE,[⊥] AND KYUNG-SUN KANG*,[†]

Laboratory of Stem Cell and Tumor Biology, Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Department of Veterinary, Public Health, College of Veterinary Medicine, and School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Department of Biology, College of Natural Science, Kyonggi University, Suwon 442-760, and Graduate School of East-West Medical Science, Kyunghee University, Yongin 449-701, Korea

The cruciferous vegetables such as Chinese cabbages and broccoli are known to have anticancer phytochemicals, and the consumption of cruciferous vegetables has been proposed to protect against various cancers. The anticarcinogenic properties of some Chinese cabbage extracts and sulforaphane glucosinolate (SFN) were assessed by examining their ability to prevent the inhibition of gap junctional intercellular communication (GJIC) induced by hydrogen peroxide (H_2O_2) in WB-F344 normal rat liver epithelial cells. The cells were preincubated with Chinese cabbage extracts and SFN for 24 h followed by cotreatment with cells and H_2O_2 (750 μM) for 1 h. Chinese cabbage extracts and SFN prevented the inhibition of GJIC and phosphorylation of gap junction protein connexin43 (Cx43) by H_2O_2 treatment. Chinese cabbage extracts and SFN were able to prevent the inhibition of GJIC through the blocking of Cx43 phosphorylaton and inactivation of ERK 1/2 and p38 MAP kinase. The results suggest that cruciferous vegetables and their components, SFN, may exert the anticancer effect by targeting the GJIC as a functional dietary chemopreventive agent.

KEYWORDS: Chinese cabbage; sulforaphane; gap junction; MAP kinases; chemoprevention

INTRODUCTION

The consumption of cruciferous vegetables, particularly those of the Brassica genus (broccoli, cabbage, cauliflower, radish, mustard, turnip, rutabaga, etc.), has been suggested to reduce the risk of cancers (*1*, *2*). The cruciferous vegetable is the most frequently eaten vegetable in Asia, often as a freshly cooked vegetable or processed as a brined product or in pickles such as Kim-chi in Korea. Sulforaphane glucosinolate (SFN), a major isothiocyanate found in cruciferous vegetables, is a metabolite of the glucosinolate-glucoraphainin (*2*, *3*). The possible anticarcinogenic activity of SFN is accounted for by its ability to induce phase II detoxication enzymes (*1*). These enzymes may afford protection against certain carcinogens and other toxic electrophiles, including reactive oxygen species. SFN significantly reduces the incidence, the multiplicity, and the rate of development of chemically induced mammary tumors in rats (*4*). SFN has a role of detoxification of carcinogens, and thus, it might have the ability to protect against a variety of cancers (*1*, *5*, *6*).

Cells receive many different stimuli from their environment that influence their metabolic rate, interaction with other cells, survival and proliferative potential, and other cellular processes involved in homeostasis and health of the organism. Cells can respond to different stimuli with the activation of a specific mitogen-activated protein kinases (MAPKs) pathway (*7*). MAPKs, which include extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 kinase, are important regulatory proteins through which various extracellular signals are transduced into intracellular events.

Gap junction channels are structures in the plasma membranes of most cell types. They direct cell to cell pathways for the movement of molecular information through exchange of small molecules and ions (*8*, *9*) in a process referred to as gap junctional intercellular communication (GJIC) and are a function of homeostasis, cell growth and differentiation, and many other physiological processes (*10*, *11*). Inhibition of GJIC by either

* To whom correspondence should be addressed. Tel: +82-2-880-1246. Fax: +82-2-876-7610. E-mail: kangpub@snu.ac.kr.
[†] College of Veterinary Medicine, Seoul National University.
[⊥] College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University.
[‡] Kyonggi University.
[§] Kyunghee University.

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chemical tumor promoters or oncogenes is suspected to be involved in the mechanism of tumor promotion and progression (*12*, *13*). Gap junction channel proteins are called connexins, and connexin 43 (Cx43) is a major connexin present in many tissues, including WB-F344 rat liver epithelial cells (WB cells) (*14*). The extent of GJIC directly results from the number and functionality of these connexin proteins (*15*). The phosphorylation of Cx43 appears to influence GJIC in both a positive and a negative manner (*16*). It has also been reported that many tumor promoters disrupt GJIC (*17*, *18*).

It is well-known that oxidative stress plays a crucial role under various pathological conditions. Oxidative stress is also strongly implicated in tumor promotion by epigenetic mechanisms (*19*, *20*). Oxidative stress is one of the regulating factors of GJIC (*21*). Hydrogen peroxide (H_2O_2) inhibits GJIC in some cell types (*22*, *23*). In WB-F344 (WB) rat liver epithelial cells, it also inhibits GJIC by modification of a protein (*21*). ERK1/2 and p38 kinase activation might be considered the primary mechanisms of GJIC inhibition by H_2O_2 treatment (*24*).

This study examined whether several Chinese cabbage extracts could prevent the blockage of GJIC in tumor promoter-treated cells. In addition, we investigated if SFN, which was one of the active functional materials in Chinese cabbage, could also prevent H_2O_2-induced GJIC inhibition in WB cells.

MATERIALS AND METHODS

Chinese Cabbage Extraction. Chinese cabbage, *Brassica pekinensis*, used in the present study was collected in the DongBu Hannong Co. Ltd. (An-sung, Korea). DF1 was extracted from general *B. pekinensis*, whereas DF2 was extracted from *Agrobacterium*-resistant *B. pekinensis*. Three grams of leaves (roots) was extracted with methanol for 3 days at room temperature. After filtration, the filtrate was concentrated using a rotary evaporator (Eyela, Tokyo, Japan) to obtain the methanol extract of *B. pekinensis*. These methanol fractions from each cabbage were diluted with dimethyl sulfoxide (DMSO) at 10 mL and used in this study.

Chemicals. H_2O_2, Lucifer yellow CH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, and monoclonal β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Sulforaphane was from Alexis Biochemicals. Mouse anti-Cx43 monoclonal antibody was from Chemicon International, anti-active MAPK pAb and anti-active p38 pAb were from Promega (Madison, CA), and p-JNK, JNK1, and p38 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-Map Kinase, horseradish peroxidase (HRP) goat anti-mouse IgG conjugate, and HRP goat anti-rabbit conjugate antibody were all from Zymed Laboratories (San Francisco, CA).

Cell Cultures and Chemical Treatment. WB-F344 rat liver epithelial cells were kindly provided by Dr. J. E. Trosko at Michigan State University (MI). Cells were cultured in D-media (formula no. 78-5470EB, Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (Gibco BRL) and penicillin−streptomycin−neomycin mixture (Gibco BRL). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were grown in 75 tissue culture plates, and the culture medium was changed every other day. Passage 8-22 cells were used in all experiments. The cells were pretreated with Chinese cabbage extracts or SFN for 24 h before being treated with 750 μM H_2O_2 for 1 h.

Bioassay of Cytotoxicity. To evaluate the effect of Chinese cabbage extracts and SFN treatment on cytotoxicity using the MTT assay as previously described (*25*), the cells were seeded in 96 well plates (5×10^4 cell/well). After 24 h of incubation, the cells were treated with the cabbage extracts and SFN for 24 h. Following the treatment, the cells were exposed for 4 h to 10% MTT solution, which was dissolved in PBS at 5 mg/mL. After they were removed from the media, 200 μL of DMSO was added to each well and mixed to dissolve the MTT formazan crystals formed by viable cells. The optical density was measured at 540 nm wavelength in an enzyme-linked immunosorbent assay plate reader (EL800, Bio-Tek Instruments. Inc., VT).

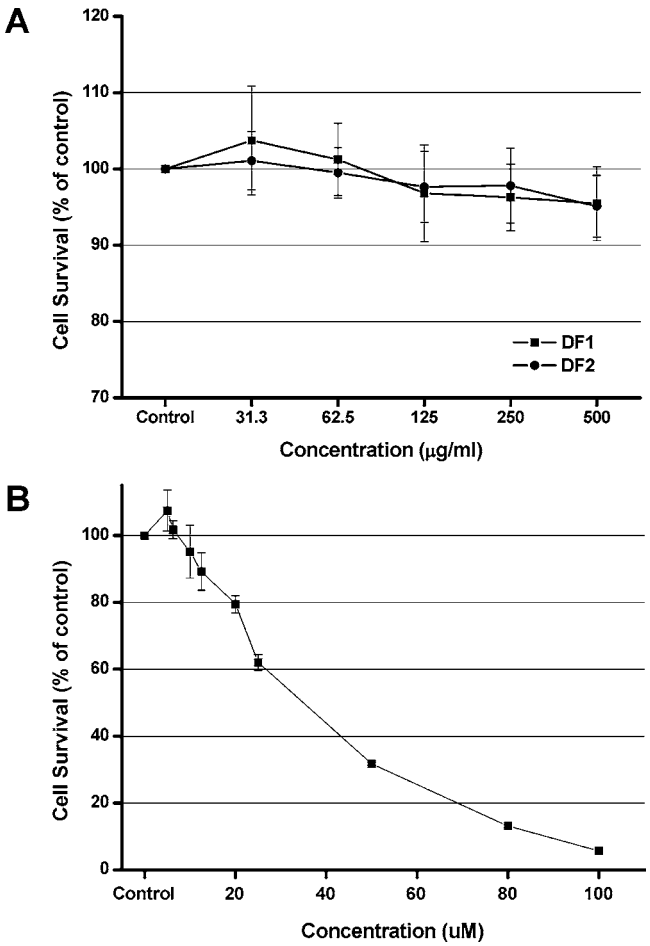

Figure 1. Cytotoxic effect of Chinese cabbage extacts (DF1 and DF2) and SFN on WB cells. Cells were treated with (**A**) DF1 and DF2 and (**B**) SFN for 24 h. The cell survival rate was determined by MTT assay. Each value represents the average and the standard deviation.

Determination of GJIC. GJIC was determined by the scrape loading/dye transfer (SL/DT) technique as previously described (*26*). The SL/DT assay is the way in which a cell's ability to perform GJIC can be determined. This assay was conducted at noncytotoxic dose levels of the samples, as determined by the MTT assay. Cells were pretreated with each Chinese cabbage extract or SFN for 24 h prior to the addition of H_2O_2 for 1 h. Following incubation, the cells were gently scraped with a scalpel blade in the presence of Lucifer yellow. The dye enters cells through wounds created by the scalpel blade. If cells contain functional gap junctions, the Lucifer yellow dye travels through the gap junctions away from the scrape line into neighboring cells. After cells were fixed with 10% neutral formalin, the distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted fluorescence microscope (Olympus IX70, Okaya, Japan).

Western Blot Analysis. Cells were grown in a 100 tissue culture dish (Nunc, Rochester, NY) to the same confluency as in the SL/DT assay. The cells were then treated with each test in the same way as described in the SL/DT assay. Western blot analysis of Cx43 was performed, as described previously (*27*). Proteins were extracted with 20% sodium dodecyl sulfate (SDS) solution containing 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate, and 5 mM sodium fluoride. The protein concentration was determined using the DC assay kit (Bio-Rad, Hercules, CA) and separated on 12% SDS−polyacrylamide gel electrophoresis. They were then transferred to nitrocellulose membranes at 100 V and 350 mA for 1 h. All antibodies were used according to the manufacturer's instructions, and protein bands were detected using an ECL detection kit (Amersham, Piscataway, NJ).

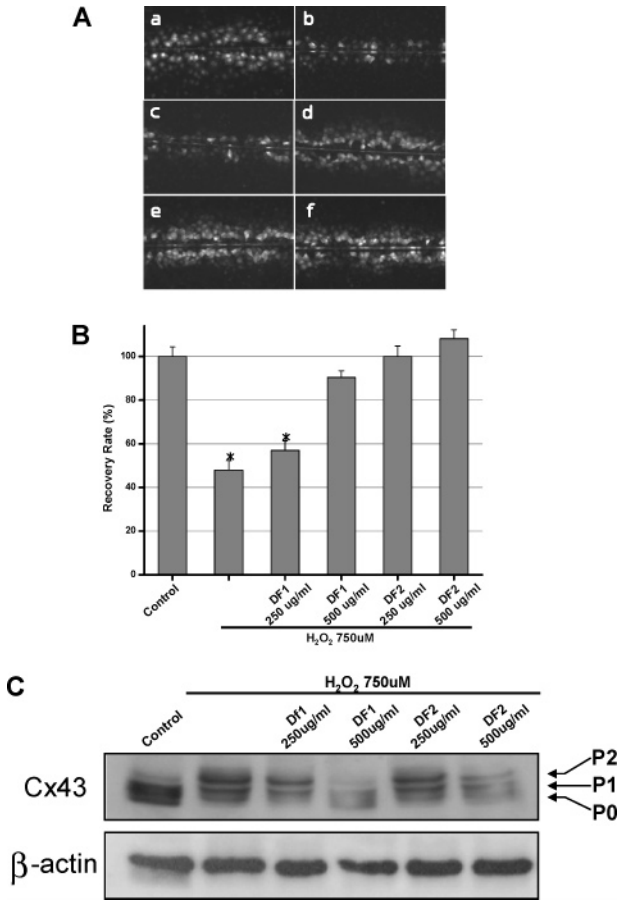

Figure 2. Recovery effect of Chinese cabbage extracts (DF1 and DF2) on the inhibition of GJIC by H_2O_2, as determined by SL/DT assay and Western blotting. (**A**) Cells were treated without cabbage extracts (a and b) or with 250 μg/mL DF1 (c), 500 μg/mL DF1 (d), 250 μg/mL DF2 (e), and 250 μg/mL DF2 (f) for 24 h prior to the addition of H_2O_2 for 1 h. Then, cells were cultured without (a) or with 750 μM H_2O_2 for 1 h and scrape loaded (b–f). Each picture is representative of two independent experiments. (**B**) Recovery rate was counted under an inverted fluorescent microscope. Each value represents the average and the standard deviation determined from six measurements of scrape loads of two culture dishes. *Significantly different with control ($P < 0.05$ by Student's t-test). (**C**) Effect of Chinese cabbage extracts (DF1 and DF2) on the phosphorylation pattern of Cx43 was measured. Total cellular protein extracts were prepared, and Western blot analysis was performed with 20 proteins using antibody specific for Cx43. β-Actin was the control for protein loading. This is a representative blot from two independent experiments.

RESULTS

Determination of Noncytotoxic Doses of Chinese Cabbage Extracts and SFN. To select the appropriate concentrations of Chinese cabbage extracts and SFN, cytotoxicities were evaluated by MTT assay in WB rat liver epithelial cells. The highest noncytotoxic concentrations of cabbage extracts (500 μg/mL for DF1 and DF2) were determined in cells treated with those extracts for 24 h (**Figure 1A**). Up to 20 μM, SFN showed no cytotoxic effects in the cells (**Figure 1B**). Therefore, we performed the following experiments using dose levels of chemicals $\leq$500 μg/mL DF1 and DF2 and $\leq$20 μM SFN. The optimal concentrations of H_2O_2 (750 μM) were obtained by time- and dose-dependent GJIC assays using SL/DT assay (data not shown) and also were proven to be noncytotoxic by our previous studies (*24*, *28*).

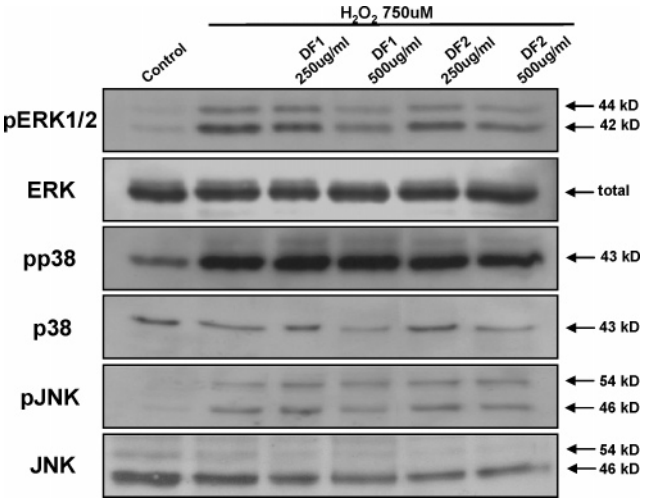

Figure 3. Effect of Chinese cabbage extracts on H_2O_2-induced ERK1/2, p38, and JNK phosphorylation. Total cellular protein extracts were prepared, and Western blot analysis was performed with 20 proteins using antibody specific for phosphor-ERK or total ERK, phopho-p38 or total p38, and phosphor-JNK or total JNK. These are representative blots from two independent experiments.

Effect of Chinese Cabbage Extracts on GJIC. The effects of Chinese cabbage extracts on GJIC in WB cells are shown in **Figure 2**. Communicating cells were visualized by fluorescence microscopy (**Figure 2A**). The GJIC of nontreated cells was not decreased during the experimental incubation period. The cells exposed to 750 μM H_2O_2 were shown about 50% inhibition of GJIC (**Figure 2B**). Pretreatment cells with DF1 (500 μg/mL) and DF2 (250 and 500 μg/mL) for 24 h was shown to recover GJIC against blockage of the inhibition by H_2O_2 exposure for 1 h. It is known that GJIC may be regulated by phosphorylaton of Cxs, including Cx43, the prominent Cx in WB cells (*29*). The phosphorylaton patterns of the gap junction proteins Cx43 were assessed by Western blot analysis (**Figure 2C**). The extent of phosphorylaton of the gap junction protein Cx43 is directly correlated to functional GJIC (*30*). H_2O_2 treatment caused the P0 band, the nonphosphorylated form of Cx43, to disappear and induced increasing of the P2 band, the hyperphosphorylation form of Cx43. Treatment of DF1 (500 μg/mL) or DF2 (500 μg/mL) in combination with H_2O_2 inhibited the hyperphosphorylaton of Cx43 induced by H_2O_2.

Chinese Cabbage Extract Can Prevent H_2O_2-Induced Cx43 Hyperphosphorylaton by Inactivation of ERK1/2. It has been documented that MAPKs play an important role in the GJIC in several kinds of cell lines (*31*, *32*). A previous study suggested that the Cx43 gap junction protein may be a target of activated MAPK and that MAPK may regulate Cx43 function (*33*). Thus, ERK1/2, JNK, and p38 kinases were examined using Western blot analysis to identify the protective action of cabbage extracts on the inhibition of GJIC. As previously described (*24*), we also observed that H_2O_2 could activate p38 kinase, ERK1/2, and JNK. A 500 μg/mL amount of DF1 and DF2 remarkably decreased the phosphorylated ERK1/2, even though the proteins were constitutively expressed (**Figure 3**). However, pretreatment of these effective cabbage extracts did not inhibit H_2O_2-induced activation of the p38 or JNK pathway.

SFN Prevents H_2O_2-Induced Cx43 Phosphorylaton by Inactivation of ERK 1/2 and p38. The SL/DT assay to measure recovery ability as a simple way of quantifying the effects of SFN treatment on GJIC was performed by treating cells with different concentrations of SFN. As shown in **Figure 4A**,**B**, SFN prevented the inhibition of GJIC induced by H_2O_2 in the same

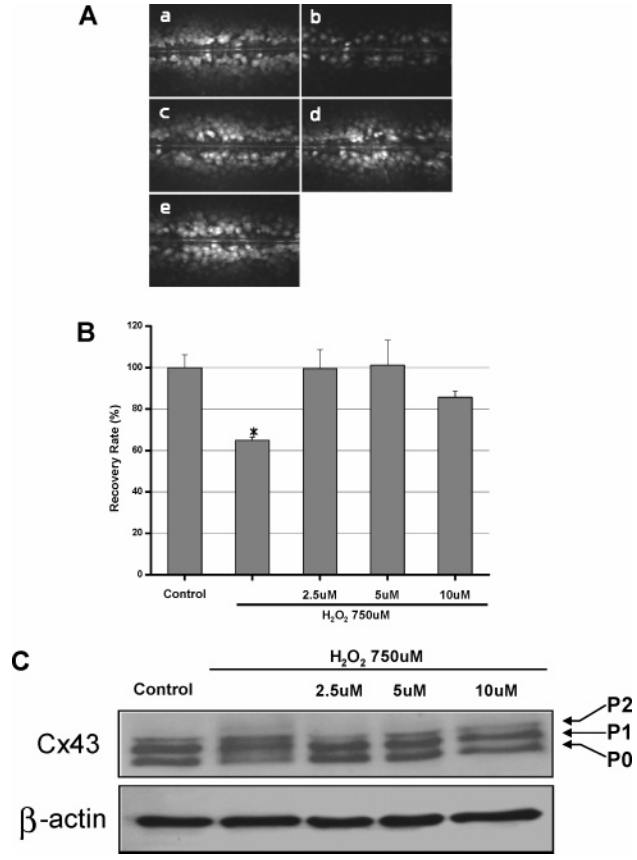

Figure 4. Recovery effect of SFN on the inhibition of GJIC by H_2O_2, as determined by SL/DT assay and Western blotting. (**A**) Cells were treated without SFN (a and b) or with 2.5 (c), 5 (d), and 10 (e) μM SFN for 24 h prior to the addition of H_2O_2 for 1 h. Then, cells were cultured without (a) or with 750 μM H_2O_2 for 1 h and scrape loaded (b–e). Each picture is representative of two independent experiments. (**B**) Recovery rate was counted under an inverted fluorescent microscope. Each value represents the average and the standard deviation determined from six measurements of scrape loads of two culture dishes. *Significantly different with control ($P < 0.05$ by Student's *t*-test). (**C**) Effect of SFN on the phosphorylation pattern of Cx43 was measured. Total cellular protein extracts were prepared, and Western blot analysis was performed with 20 proteins using antibody specific for Cx43. β-Actin was the control for protein loading. This is a representative blot from two independent experiments.

manner as the Chinese cabbage extracts did. In Western blot analyses, H_2O_2 induced hyperphosphorylaton of Cx43 protein with loss of the P0 band and shift to the P2 band, while the P0 band was recovered and normal phosphorylaton pattern of Cx43 protein (**Figure 4C**). These data support a significant association between Chinese cabbage extracts and SFN. Thus, we investigated ERK1/2 kinase activation in accordance with results shown in **Figure 3**, when cells were treated with different concentrations of SFN. As expected, H_2O_2 increased phosphorylated ERK1/2 protein kinase but this increased phosphorylated ERK1/2 protein kinase was markedly reduced in SFN-treated cells (**Figure 5**). p38 and JNK kinase could not inhibit H_2O_2-induced activation of the p38 or JNK pathway in **Figure 3**. As shown in **Figure 5**, pretreatment of WB cells with SFN for 24 h before exposure of 750 μM H_2O_2 inhibited p38 activation, significantly. In the case of JNK kinase, there was no change at the protein level. Therefore, activation of JNK kinase did not seem to influence H_2O_2-induced phosphorylaton of Cx protein in WB cells.

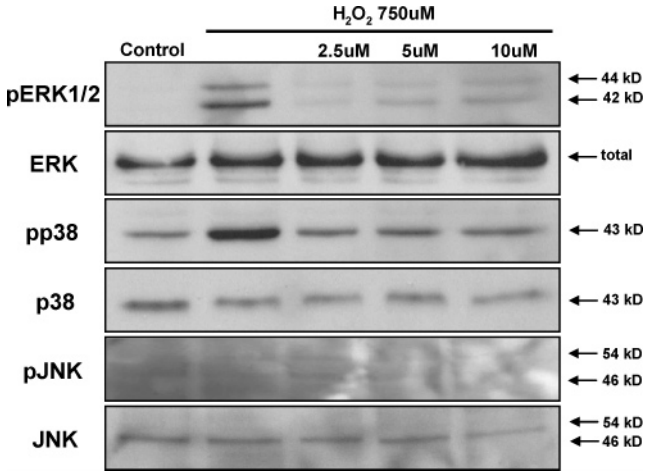

Figure 5. Effect of SFN on H_2O_2-induced ERK1/2, p38, and JNK phosphorylation. Total cellular protein extracts were prepared, and Western blot analysis was performed with 20 proteins using antibody specific for phosphor-ERK or total ERK, phopho-p38 or total p38, and phosphor-JNK or total JNK. These are representative blots from two independent experiments.

DISCUSSION

There is increasing interest in the potent cancer protective or preventive properties of natural compounds present in diet. Natural compounds present in cruciferous vegetable such as broccoli and cabbage are strong suppressors of cancer in in vivo and in vitro model systems (*34*−*36*). Despite a wealth of data about the chemopreventive properties of cruciferous vegetables and SFN, their effects on GJIC-related multistep process of carcinogenesis (*28*) have not been studied. It was therefore important to investigate the anticarcinogenic effect of Chinese cabbage extracts and SFN on GJIC of WB cells after treatment with H_2O_2.

Significant prevention against the H_2O_2-induced inhibition of GJIC was achieved with Chinese cabbage extracts DF1 and DF2 as assessed by SL/DT assay. Furthermore, treatment of DF1 and DF2 500 μg/mL was able to reduce the hyperphosphoryled P2 band and recovered P0 band of Cx43. In WB cells, opening and closure of gap junctions between cells are dependent, in part, on the level of phosphorylation of the gap junction protein Cx43 (*37*). Chinese cabbage extracts prevented the phosphorylation of Cx43 protein due to H_2O_2 treatment, indicating that Chinese cabbage extracts maintained GJIC by regulating the gating of gap junction channels.

Many studies have described the abilities of diverse stimuli to activate MAPK and induce Cx43 phosphorylation (*24*, *29*, *32*). MAPKs are part of phosphor-relay system, which is regulated by phosphorylation (*7*). These MAPKs include the ERK, the JNK, and the p38 kinase. ERK is important in the regulation of mitogenesis and differentiation, while activation of JNK and p38 kinase is related to the stress response, growth arrest, and apoptosis. ERK1/2 and p38 kinase activation might be the primary mechanisms of GJIC inhibition by H_2O_2 treatment (*24*). That the Chinese cabbage extracts DF1 and DF2 increased GJIC and prevented the inhibition of GJIC by H_2O_2 through inhibition of ERK activation might be an important mechanism whereby the Chinese cabbage extracts protect against tumor promotion.

It is well-known that SFN is abundant in cruciferous vegetables, especially Chinese cabbage and broccoli, and is used as a chemopreventive and/or chemotherapeutic agent. Thus, SFN might contribute to THE recovery of H_2O_2-induced GJIC

inhibition. On investigating the GJIC and Cx43 phosphorylation in H_2O_2-treated WB cells, it became clear that SFN could prevent the inhibition of GJIC by H_2O_2. The recovery mechanism of H_2O_2-induced GJIC inhibition between SFN and Chinese cabbage extracts was shared by ERK1/2 MAPK pathway. Clearing the inhibition of GJIC by H_2O_2 was involved in hyperphosphorylation of Cx43 via p38 and ERK 1/2, but not JNK activation in WB cells (*24*) and H-ras WB cells (*38*). It is interesting that the disruption of GJIC in SK-Hep-1 human hepatoma cells was involved in the activation of p38 and ERK 1/2, but not JNK, activation (*38*). The recovery of H_2O_2-induced GJIC inhibition by SFN may be involved in p38 MAPK inactivation, whereas treatment of Chinese cabbage extracts did not affect the activation of p38 kinase. Because Chinese cabbage extracts did not block p38 kinase activation, they could conceivably inhibit phosphorylation of gap junction protein connexin43 at higher concentrations as compared to SFN. The consensus from the data presented point to the fact that SFN was more efficient than the whole extract in terms of cancer prevention.

The recovery effect of cruciferous vegetable and SFN on the inhibition of GJIC by oxidative stress was apparent in the data presented here, thus suggesting that the chemopreventive effect of Chinese cabbage extracts and SFN may be beneficial for the preservation of differentiated functions in the liver under oxidative stress.

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