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Dynamics of Nrf2 and Keap1 in ARE-Mediated *NQO1* Expression by Wasabi 6-(Methylsulfinyl)hexyl Isothiocyanate

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ABSTRACT: 6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC) is a bioactive ingredient present in wasabi, a popular pungent spice in Japan. Previous studies have revealed the cytoprotective and cancer chemopreventive effects of 6-MSITC. This study aims to clarify the molecular mechanisms by investigating the action of 6-MSITC on the Nrf2/Keap1 system. 6-MSITC up-regulated the expression of NAD(P)H:quinone oxidoreductase 1 (NQO1) by increasing the Nrf2 level. Treatment with 6-MSITC extended the half-life ($t_{1/2}$) of Nrf2 protein from 11.5 to 35.2 min, approximately three times longer. Moreover, 6-MSITC suppressed the ubiquitination of Nrf2 but not Keap1. Alternatively, a modified Keap1 was observed in 6-MSITC-treated cells accompanying reduction of normal Keap1 protein. The results from cellular fractionation and immunocytochemistry assay revealed that Nrf2 was primarily accumulated in nucleus. EMSA and the reporter gene assay further demonstrated that 6-MSITC augmented Nrf2-ARE binding and transcription activity. Silencing Nrf2 using Nrf2 siRNA markedly reduced the Nrf2 level and ARE-driven activity under both baseline and 6-MSITC-induced conditions. Our data revealed that 6-MSITC enhanced Nrf2/ARE-driven NQO1 expression by stabilizing Nrf2 that was accomplished by modifying Keap1 with consequent inhibition of the ubiquitination and proteasomal turnover of Nrf2. The findings provide an insight into the mechanisms underlying 6-MSITC in cytoprotection and cancer chemoprevention.

KEYWORDS: 6-MSITC, NQO1, Nrf2, Keap1, ubiquitination, modification

INTRODUCTION

Wasabi [*Wasabia japonica* (Miq.) Matsumura], called Japanese horseradish, is a member of the brassicaceae family of vegetables. Its rhizome is a very popular pungent spice in Japan, widely used in Japanese cuisine to garnish traditional dishes such as sushi and sashimi. The bioactive components in wasabi have been identified as a series of isothiocyanate analogues,[1,2] of which 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC or 6-MITC) is a major component in wasabi.[3,4] In our previous studies, we have found that 6-MSITC significantly induced mRNA expression of nicotinamide adenine dinucleotide phosphate (NADP): quinone oxidoreductase 1 (NQO1). The report gene assay have revealed that NQO1 induction was regulated at the transcription level through *cis*-antioxidant response element (ARE) of the NQO1 gene.[5,6] Our previous data suggest that 6-MSITC is a potential candidate for NQO1 induction in the nontoxic concentration.[5] However, the molecular mechanisms underlying how 6-MSITC mediates ARE activation is not clear yet.

Accumulating data have shown that ARE activation contributes to the protection of cells against carcinogens and oxidative stress. Several molecules, such as nuclear factor E2-related factor 2 (Nrf2), c-Jun, ATF2, and ATF4, have been proposed as potential modulators of ARE.[7−9] Of these, Nrf2, a member of the CNC family of bZIP proteins, is extensively proven to be a strong activator of ARE-mediated gene expression.[10,11] Under unstimulated conditions, Nrf2 is sequestered in the cytoplasm, where it is associated with Kelchlike ECH-associating protein 1 (Keap1), an actin-binding protein.[7] The presence of a stimulus leads to the disruption of the Keap1-Nrf2 complex, nuclear translocation of Nrf2, and binding to the ARE with small Maf proteins.[10,12,13] Keap1 protein is a repressor of Nrf2-mediated ARE activity.[14,15] Ubiquitination of Nrf2 is provided by the Cul3-Rbx1 E3 ubiquitin ligase complex, which binds to the N-terminal BTB domain and central linker region of Keap1.[16−18] Thus, Nrf2 is a labile protein. Stabilization of Nrf2 by some active compounds is considered to be important to maintain the cellular defense system, which is likely dependent on the status of the Nrf2-Keap1 complex. Although our previous study showed that 6-MSITC could induce ARE activation, the precise mechanism by which 6-MSITC influences the Nrf2-Keap1 complex to activate ARE is poorly understood.

We here investigated the effects of 6-MSITC on Nrf2/Keap1 system, showing that 6-MSITC enhanced Nrf2/ARE-mediated NQO1 activity by stabilizing Nrf2, which was accomplished not only by inhibiting the ubiquitination and proteasomal turnover of Nrf2 but also by modifying Keap1. The findings provide an insight into the mechanisms underlying 6-MSITC in cytoprotection and cancer chemoprevention.

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MATERIALS AND METHODS

Materials and Cell Culture. 6-MSITC was purified from wasabi, and the purity was 99.3% gas chromatography.[5] MG132 and actinomycin D were from Calbiochem (Nottingham, United Kingdom). The antibodies against Nrf2 (C-20), Keap1 (E-20), ubiquitin (P4D1), α-tubulin (B-7), lamin B (C-20), rabbit IgG, and horseradish peroxidase conjugated antigoat secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) was from Equitech-Bio (Kerrville, TX). Horseradish peroxidase-conjugated antirabbit and antimouse secondary antibodies were from Cell Signaling Technology (Beverly, MA). Human hepatoblastoma HepG2 cells were obtained from the Cancer Cell Repository, Tohoku University, Japan, and cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

Transient Transfection and Luciferase Reporter Gene Assay. Transient transfection experiments were performed as described previously.[5] A pGL2-hNQO41 luciferase reporter plasmid containing the 41-bp ARE/EpRE of human NQO1 was constructed as described previously.[19] The mammalian expression plasmid pcDNA3 bearing rat Nrf2 was kindly provided by Dr. Truyen Nguyen (Schering-Plough Research Institute, Kenilworth, NJ).[11] HepG2 (1×10^5) cells were plated into each well of 12-well plates and cultured for 24 h. The cells were then cotransfected with 0.1 μg of NQO1 promoter-encoding firefly luciferase plasmid and 0.1 μg of pGL4-TK-encoding Renilla luciferase plasmid (Promega, Madison, WI) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). For overexpression of Nrf2, cells were cotransfected with 0.05 μg of pcDNA3-rNrf2 as described above. The total amount of transfected DNA was kept constant at 0.25 μg/well by the addition of pcDNA3 control vector to the DNA mixture in Opti-MEM culture medium for 5 h. The cells were then placed in DMEM medium for 24 h and then stimulated with 10 μM 6-MSITC for an additional 12 h in 0.1% DMSO or 0.1% DMSO alone as a control. The activities of firefly and Renilla luciferase were measured in a luminometer (Berthold Technologies, Bad Wildbad, Germany) with the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's recommendations. Luciferase activity values were normalized to transfection efficiency monitored by Renilla expression, and the NQO1 transcription activity was expressed as fold induction relative to the control cells.

Transfection of Small Interfering RNA (siRNA). Predesigned siRNA against human Nrf2 (Catalog #115762) and control scrambled siRNA (Catalog #4611) were purchased from Ambion (Austin, TX). Transfection of siRNA was performed as described previously.[20] Briefly, HepG2 cells were plated at a density of 3×10^5 cells per 60 mm dish. Cells were transfected with 100 nM siRNA against Nrf2 or scrambled duplex using Lipofectamine 2000 (Invitrogen). After 24 h of incubation, fresh medium was added, and the cells were cultured for another 24 h. The cells were then treated with 10 μM 6-MSITC for an additional 12 h for the reporter gene assay and 6 h for the Western blotting assay.

Immunocytochemistry. Immunocytochemistry assay was performed as described.[20] Briefly, HepG2 cells were seeded on poly-L-lysine (Sigma)-coated glass slides, which were cultured in 30 mm dishes. Cells were treated with or without 10 μM 6-MSITC for 6 h. After they were washed with phosphate-buffered saline (PBS), the cells were fixed in paraformaldehyde (4%, 15 min), permeabilized with Triton X-100 (1%, 15 min), and blocked with bovine serum albumin (0.5%, 30 min) in PBS. Cells were incubated with anti-Nrf2 antibody for 1 h, followed by incubation with Alexa Fluor 568-conjugated secondary antibody for 1 h. The glass slides were mounted on slides using Prolong Gold antifade reagent with DAPI (Invitrogen). Fluorescent images were obtained by laser scanning confocal microscopy (Keyence Co., Osaka, Japan).

Cell Fractionation. Nuclear and cytosolic protein extracts were prepared according to the method as described.[20] Briefly, cells were cultured on 100 mm dishes to 90% confluence and treated with 6-MSITC for 6 h. After they were washed, cells were harvested by scraping in ice-cold PBS and collected by centrifugation at 500*g* for 5 min. Cells were lysed with buffer A [10 mM Hepes-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride] on ice for 20 min and then centrifuged at 14000*g* for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM Hepes, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14000*g* for 15 min at 4 °C. One part of the cytosolic and nuclear fractions was subjected to immunoblot analysis using anti-Nrf2, antilamin B, or anti-α-tubulin antibody. The rest of the nuclear extract was used for electrophilic mobility shift assay (EMSA). The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

EMSA. The 5′-end labeling of *NQO1*-ARE oligonucleotide (5-TTT-TATGCTGTGTCATGG- TT-3′) was performed using T4 polynucleotide kinase (Takara Bio, Siga, Japan) with 10 pmol of double-stranded oligonucleotide and 50 μCi of [γ-^{32}P]ATP(5000 Ci/mmol; Amersham Biosciences).[21] The labeled oligonucleotides were purified using a Sephadex G-25 spin column (Amersham Biosciences). Five micrograms of nuclear extract was incubated at 25 °C for 30 min with labeled or unlabeled competitor oligonucleotides in binding buffer [25 mM Tris-HCl, pH 7.5, 75 mM KCl, 0.3% Nonidet P-40, 7.5% glycerol, 2.5 mM dithiothreitol, 1 mg/mL bovine serum albumin, and 1 μg of poly(dI)·poly(dC)]. For supershift assays, the nuclear extracts were combined with the probes for 20 min at 25 °C and then incubated with Nrf2 antibody or rabbit IgG for 30 min at 25 °C. The products were resolved at 4 °C on a 5% nondenaturing polyacrylamide gel in 0.5 × Tris borate/EDTA buffer. After electrophoresis, the gel was dried on 3 MM chromatography paper (Whatman, Maidstone, United Kingdom). The paper was exposed to a radioactive imaging plate and then detected with a FLA-2000 machine (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation and Western Blot Analysis. After treatment with 6-MSITC, HepG2 (3×10^6) cells were lysed with modified RIPA buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1% deoxycholate, 50 mM sodium fluoride, 50 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Nacarai Tesque, Kyoto, Japan). The lysates were homogenized in an ultrasonicator for 10 s twice and incubated on ice for 30 min. The homogenates were centrifuged at 14000*g* for 15 min at 4 °C. The supernatants were collected, and the protein concentration was determined by protein assay kit (Bio-Rad Laboratories). For immunoprecipitation, whole-cell lysates containing 1 mg of proteins were precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1 h and incubated with 2 μg of anti-Nrf2 or anti-Keap1 antibody for 4 h. Immunoprecipitated complexes were washed five times with RIPA buffer and then boiled in SDS sample buffer for 5 min. Either the immunoprecipitation products or the whole-cell lysates containing 40 μg of proteins were run on 8% SDS-PAGE and electrophoretically transferred to PVDF membrane (Amersham Pharmacia Biotech). After blotting, the membrane was incubated with specific antibody overnight at 4 °C and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected using the ECL system, and the relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager soft ware (Taitec Co.).

Statistical Analyses. Differences among the treatments and the control were analyzed by analysis of variance tests. Means with differently lettered superscripts differ significantly at the probability of $P < 0.05$.

RESULTS

6-MSITC Enhances the Protein Level of Nrf2 and NQO1. To investigate whether 6-MSITC induces expression of antioxidant

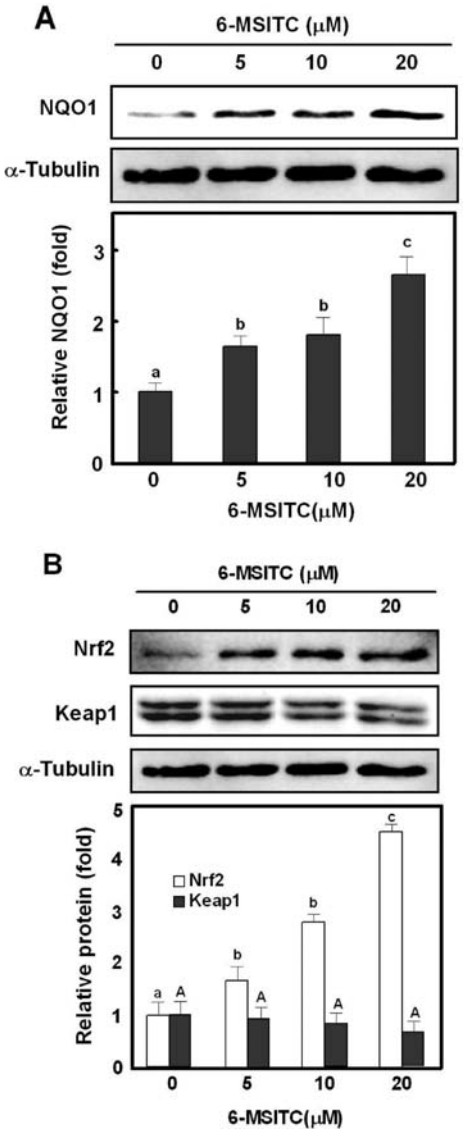

Figure 1. Effects of 6-MSITC on the levels of NQO1 (A), Nrf2, and Keap1 (B). HepG2 cells were treated with the indicated concentrations of 6-MSITC for 6 h. Whole cell lysates were used to detect NQO1, Nrf2, Keap1, and α-tubulin by Western blot analysis with their respective antibodies. Densitometry of the blots was performed using the LumiVision Imager software. Histograms show the densitometric analysis of NQO1, Nrf2, or Keap1 normalized to α-tubulin. The average value from triplicate tests with differently lettered superscripts (small for Nrf2 and capital for Keap1 in panel B) differs significantly at $p < 0.05$.

enzymes, HepG2 cells were treated for 6 h with 6-MSITC at 0−20 μM. As shown in Figure 1A, 6-MSITC caused a dose-dependent increase of NQO1. As a control, there was no change in α-tubulin. It has been well-known that Nrf2/Keap1 system plays an important role on the expression of antioxidant and detoxification enzymes; therefore, we further examined the levels of Nrf2 and Keap1 in these cell lysates. As shown in Figure 1B, 6-MSITC also caused a dose-dependent increase of Nrf2 but a decrease of the Keap1 level under the same treatment. These data suggest that the 6-MSITC might induce Nrf2/Keap1-mediated expressions of antioxidant enzyme genes.

6-MSITC Enhances Nrf2 Level through Stabilizing Nrf2. Nrf2 is a labile protein and is dependent on the status of the

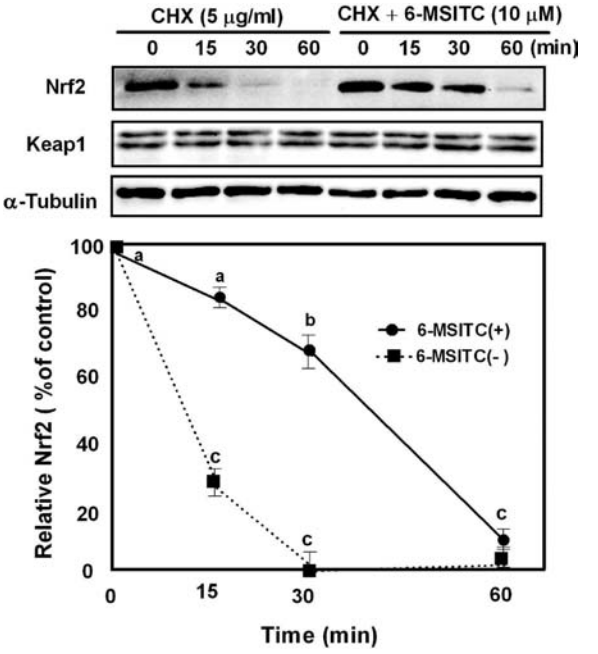

Figure 2. 6-MSITC increases stabilization of Nrf2. HepG2 cells were treated with 5 μg/mL CHX or pretreated with 10 μM 6-MSITC for 1.5 h and then treated with CHX for the indicated times. Nrf2, Keap1, and α-tubulin were detected by Western blot analysis with their respective antibodies. Histograms show the densitometric analysis of Nrf2 as compared with control. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$.

Nrf2/Keap1 system.[22,23] We speculate that the Nrf2 protein level increased by 6-MSITC may be due to the status change of the Nrf2/Keap1 system. Thus, we examined the influence of 6-MSITC on Nrf2 and Keap1 at different times after treatment with the protein synthesis inhibitor cycloheximide (CHX)[24] and then calculated the half-reduction time ($t_{1/2}$) from protein decay experiments. Treatment with 6-MSITC extended the half-life ($t_{1/2}$) of Nrf2 protein from 11.5 to 35.2 min, approximately three times longer (Figure 2). Additionally, no difference was observed in Keap1 under the same treatment. The results suggest that 6-MSITC might increase Nrf2 protein level by inhibiting the turnover of Nrf2, rather than by stimulating Keap1 degradation.

6-MSITC Inhibits Ubiquitination of Nrf2 and Modifies Keap1. Recent studies revealed that Nrf2 is rapidly degraded through the ubiquitin-dependent proteasome pathway under homeostatic conditions.[22,25] To determine whether the up-regulated ratio of Nrf2 by 6-MSITC is due to the suppression of Nrf2 ubiquitination, we examined the ubiquitination of Nrf2 by immunoprecipitation after treatment with proteasome and protease inhibitor, MG132, in the presence or absence of 6-MSITC. As shown in Figure 3A, the Nrf2 protein level was enhanced after treatment with 6-MSITC or MG132 alone or in combination, while the Keap1 protein level was reduced by such treatment. At the same time, treatment of MG132 caused an increase in ubiqutination, which is reduced by cotreatment with 6-MSITC. After immunoprecipitation with anti-Nrf2 or anti-Keap1 antibodies, a marked reduction of ubiquitination of Nrf2 (Figure 3B), but not Keap1 (data not shown), was observed in the cells cotreated with 6-MSITC and MG132. These results indicate that the 6-MSITC enhanced Nrf2 is, partially at least, due to an inhibitory effect of 6-MSITC on the ubiquitination of Nrf2. On the other hand,

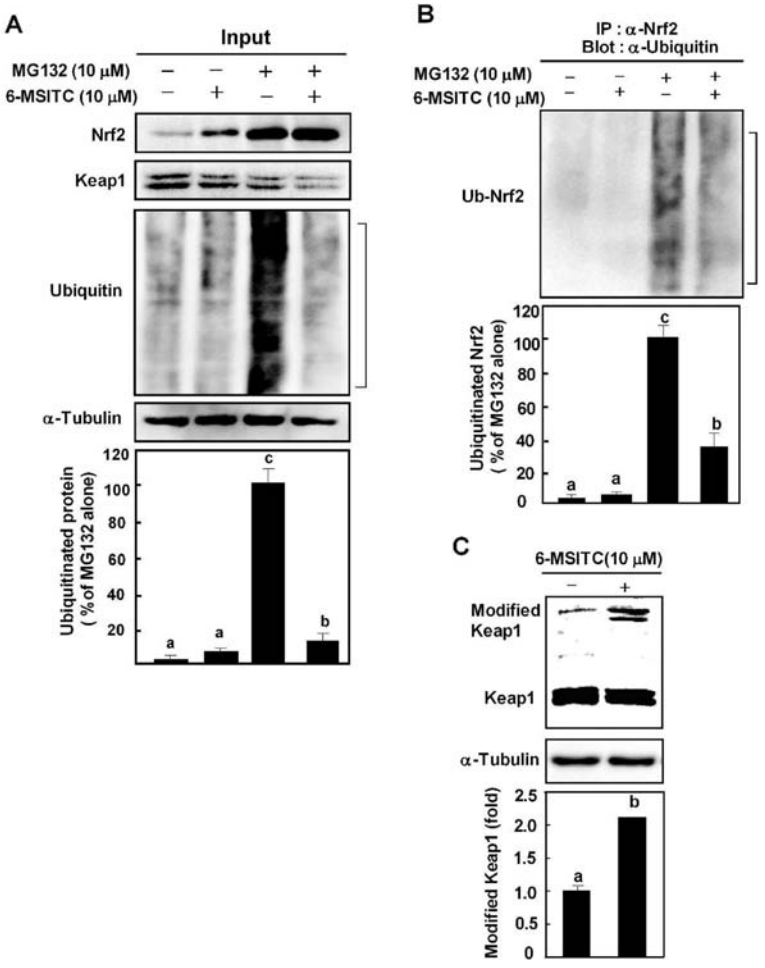

Figure 3. Effects of 6-MSITC on Nrf2 ubiquitination and Keap1 modification. (A) Western blot analysis of endogenous Nrf2, Keap1, and ubiquitin. HepG2 cells were treated with 10 μM MG132 for 1 h and then treated with or without 10 μM 6-MSITC for 6 h. Whole-cell lysates were used to detect Nrf2, Keap1, and ubiquitin with their antibodies. Densitometry of the blots was performed using the LumiVision Imager software. Histograms show the densitometric analysis of ubiquitin normalized to α-tubulin. (B) Effects of 6-MSITC on ubiquitination of Nrf2. Equivalent amounts of proteins were immunoprecipitated with Nrf2 antibody and visualized by Western blot analysis with ubiquitin antibody. The marker shows the ubiquitinated protein. Histograms show the densitometric analysis of the ubiquitinated Nrf2. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$. (C) Modification of Keap1 by 6-MSITC. HepG2 cells were treated with 10 μM 6-MSITC for 6 h and analyzed by Western blot analysis with Keap1 antibody. Densitometry of the blots was performed using the LumiVision Imager software. Histograms show the densitometric analysis of modified Keap1 normalized to α-tubulin. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$.

several lines of studies have revealed that some chemicals such as tBHQ,[26] sulforaphane,[27] quercetin,[20] and ebselen[28] induced the formation of modified Keap1 protein, which is greater than 150 kDa with null function of Keap1. Thus, we investigated whether 6-MSITC modify Keap 1 protein. As shown in Figure 3C, an approximate 150 kDa Keap1 protein was clearly detected in 6-MSITC-treated cells, suggesting that 6-MSITC-caused Keap1 decrease might be due to the formation of modified Keap1 protein.

6-MSITC Enhances Nuclear Accumulation of Nrf2 and Nrf2-Mediated ARE Activation. To further confirm whether Nrf2 stabilized by 6-MSITC is accumulated in nucleus to stimulate Nrf2-mediated ARE transcription, we examined the localization of Nrf2 in the cells treated with or without 6-MSITC for 6 h by Western blotting. As shown in Figure 4, 6-MSITC increased significantly the amount of nuclear Nrf2 at 10 μM. On the other hand, little Nrf2 was detected in the cytosol of control cells. The integrity of the cytosolic and nuclear fractions was confirmed by the analysis of the compartment-specific cytosolic α-tubulin and nuclear lamin B proteins. Moreover, the localization of Nrf2 in the same treatment was also further observed by immunocytochemical assay under microscope. The cells treated with 6-MSITC revealed stronger signal of Nrf2 in nucleus (Figure 5).

To demonstrate whether Nrf2 accumulated in the nucleus by 6-MSITC actually enhances the binding with ARE, we performed EMSA, using [γ-^{32}P]ATP-labeled *NQO1*-ARE oligonucleotides. As shown in Figure 6A, 6-MSITC caused a notable increase in binding complex of ARE (lane 2) than that in the cell lysates without 6-MSITC treatment (lane 1). Addition with 10-fold excess cold wild ARE oligonucleotides completely blocked the binding complex (lane 3), while the addition of 10-fold excess mutant ARE oligonucleotides did not block the binding complex (lane 4). Moreover, an increased supershift bind for Nrf2 was observed when Nrf2 antibody was added into the binding reaction mixer with cell lysates treated by 6-MSITC (lane 8). As a negative control, no supershift bind was observed in the addition of rabbit IgG antibody (lanes 5 and 6). These results indicated that

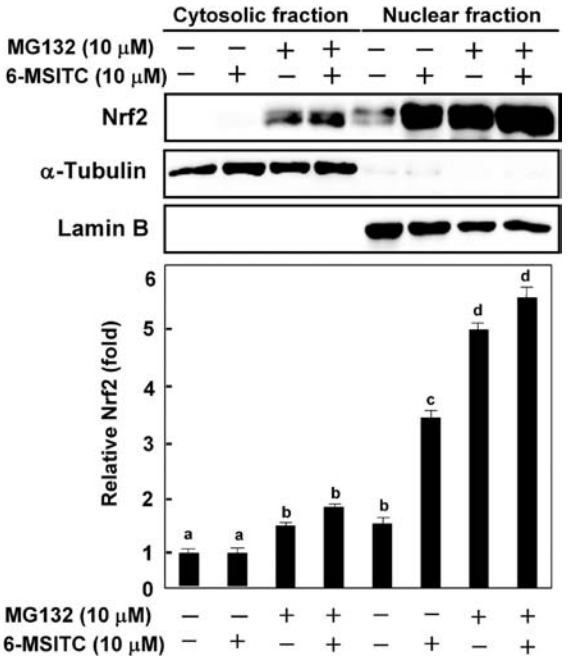

Figure 4. Western blot analysis of cytosolic and nucleus Nrf2. HepG2 cells were pretreated with 10 μM MG132 for 1 h and then treated with or without 10 μM 6-MSITC for 6 h. Cell fractionations were done as described in the Materials and Methods. The nuclear and cytosolic extracts containing 40 μg of proteins were subjected to Western blot analysis with the indicated antibodies. α-Tubulin and lamin B antibodies were used as markers for the cytosolic and nuclear extracts, respectively. Densitometry of the blots was performed using the LumiVision Imager software. Histograms show the densitometric analysis of Nrf2 normalized to α-tubulin or lamin B. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$.

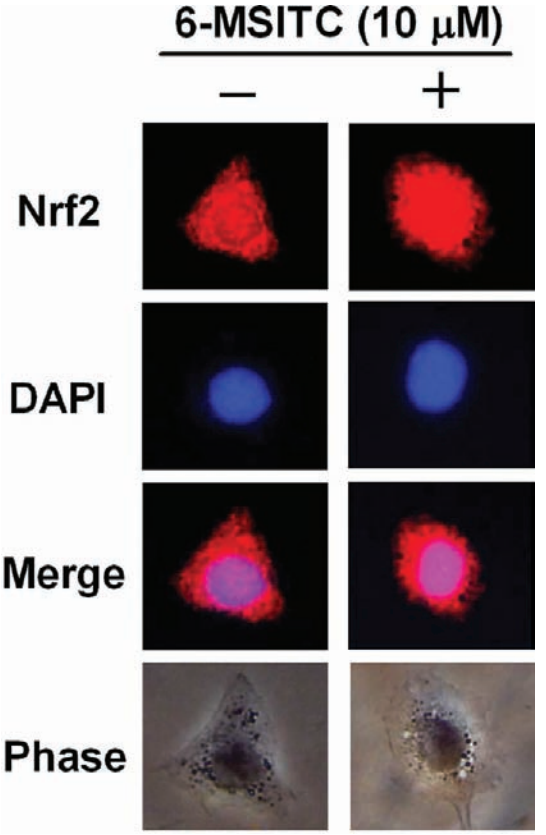

Figure 5. 6-MSITC increases the localization of Nrf2 in nucleus. HepG2 cells were untreated (left panel) or treated with 10 μM 6-MSITC for 6 h (right panel) and then subjected to immunohistochemical staining with Nrf2 antibody followed by incubation with Alexa Fluor 568-conjugated secondary antibodies. For each condition, an image of the cell nucleus stained with DAPI specific for DNA and a phase-contrast image (Phase) are also presented. Nuclear localization of Nrf2 protein is indicated by the presence of violet in the merged images.

6-MSITC increased the Nrf2-ARE binding complex. To further demonstrate whether the increased Nrf2-ARE binding complex causes ARE-driven transcription, we performed a promoter activity assay of the *NQO1* gene with a core ARE promoter-luciferase plasmid (−327/+59).[29] As shown in Figure 6B, 6-MSITC induced a dose-dependent ARE promoter-driven luciferase activity in the concentration range of 1−20 μM. These results suggest that Nrf2-mediated ARE activation is involved in 6-MSITC-induced *NQO1* gene expression.

Silencing Nrf2 Aborts 6-MSITC-Induced ARE Activation. To investigate whether the up-regulation of Nrf2 is essential for 6-MSITC-induced ARE transcriptional activity, we co-transfected Nrf2 siRNA with ARE-luciferase reporter plasmid into HepG2 cells. As shown in Figure 7A, treatment with Nrf2 siRNA reduced the basal ARE activity (lane 5) as well as 6-MSITC-induced ARE activity (lane 6). Scrambled siRNAs showed no significant effect on ARE activity (lanes 3 and 4) as compared with the control (lanes 2 and 2). These data were further supported by Western blot data that Nrf2 siRNA significantly reduced the protein level of Nrf2 and NQO1 induced by 6-MSITC (Figure 7B, lane 6). As a control, scrambled siRNAs showed no significant effect on the protein level of Nrf2 and NQO1 (Figure 7B, lane 4). These results demonstrate that the up-regulation of Nrf2 is essential for 6-MSITC-induced ARE transcriptional activity and NQO1 expression.

DISCUSSION

Several lines of studies have revealed that the status of Nrf2/Keap1 determines Nrf2-mediated ARE activity.[12,13,26] In homeostatic status, Nrf2 located in the cytoplasm is rapidly degraded through the ubiquitin-26S proteasome pathway.[13,14,22] During this process, Keap1 has been proposed to function as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase that targets lysine residues within the Neh2 domain of Nrf2 for ubiquitin conjugation.[14] Thus, Nrf2 releasing from Keap1 is considered to be the critical step for Nrf2-driven gene transcription.[14] However, the molecular events governing the activation of Nrf2 by various exogenous inducers remain unclear. In particular, how the exogenous inducers affect the interaction of Nrf2 with Keap1 during Nrf2 activation has not been clearly addressed. Recent views speculate that oxidative signals inhibit the ubiquitination activity of Keap1-Cul3, but not the association activity of Keap1 with Nrf2, thus allowing newly synthesized Nrf2 to bypass Keap1-Cul3 and accumulate in the nucleus.[14,15,18] Alternatively, the interaction of Keap1 with oxidative inducers through its thiol groups triggers the dissociation of Nrf2 from the Nrf2-Keap1-Cul3 complex in the cytoplasm, thus keeping Nrf2 from ubiquitination by Keap1-Cul3 to activate ARE.[29,30] In the present study, our data showed that 6-MSITC, an analogue of sulforaphane,

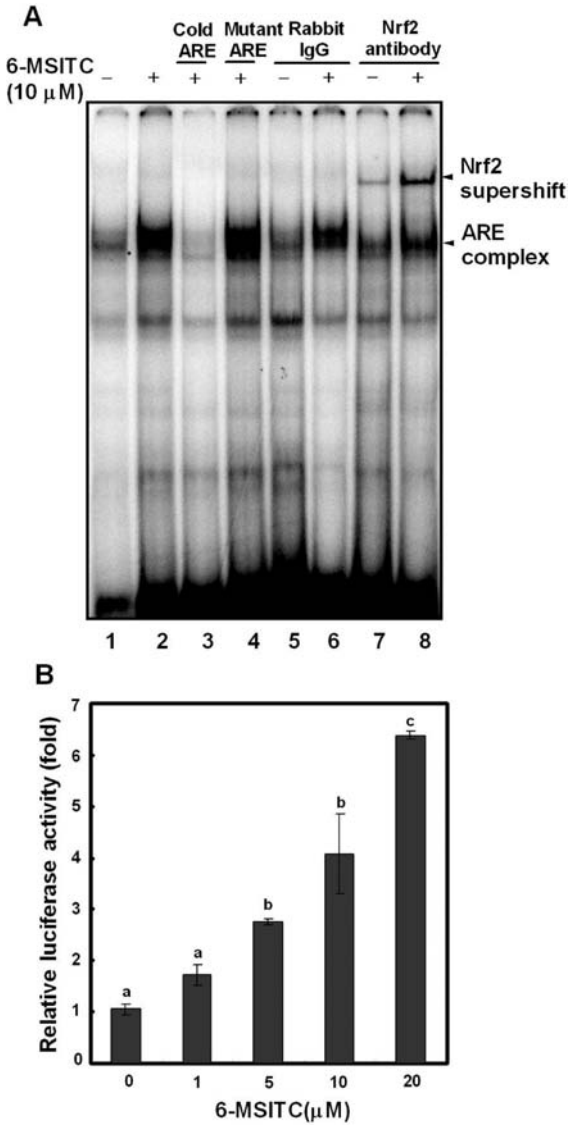

Figure 6. 6-MSITC stimulates Nrf2-mediated ARE activity. (A) EMSA of ARE-binding complex with Nrf2 antibody. HepG2 cells were treated with 10 μM 6-MSITC for 6 h, and nuclear proteins were then extracted. EMSA were carried out as described in the Materials and Methods. The nuclear extracts were reacted with γ-^{32}P-labeled oligonucleotide probe of *NQO1*-ARE for 20 min at 25 °C and then incubated with Nrf2 antibody or rabbit IgG for another 30 min at 25 °C. (B) Reporter gene assay of Nrf2-mediated ARE activity by 6-MSITC. HepG2 cells were cotransfected with 0.2 μg of pGL2-ARE-luciferase and 0.05 μg of the Nrf2 expression plasmids in Opti-MEM culture medium for 5 h. The cells were then placed in DMEM medium for 24 h and then stimulated by 10 μM 6-MSITC for an additional 12 h. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$.

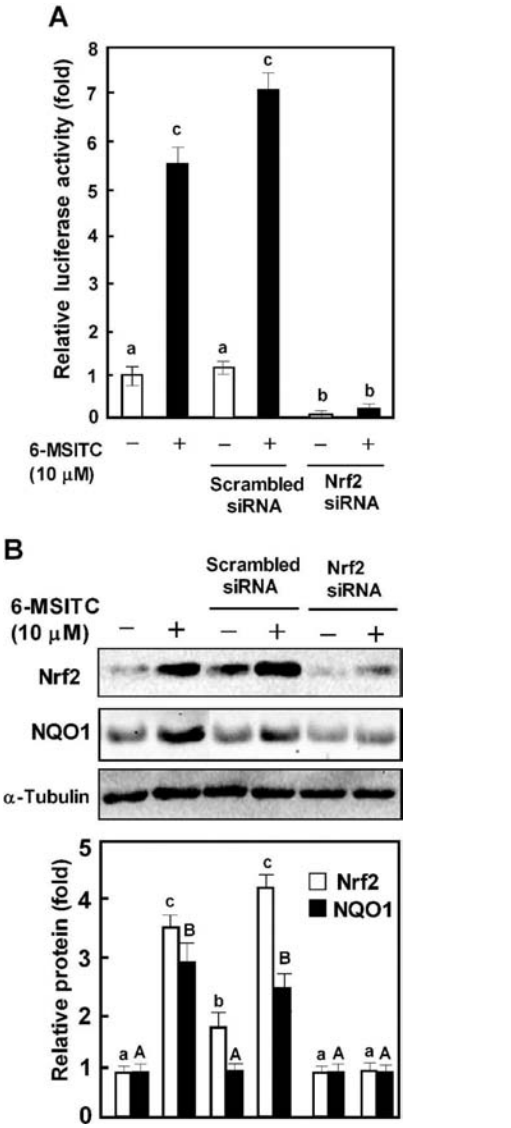

Figure 7. Effects of 6-MSITC on ARE activity in Nrf2- or Keap1-deficient cells. (A) Report gene assay. HepG2 cells were cotransfected with pGL2-ARE luciferase construct, pGL4-TK-Renilla, and 100 nM siRNA against Nrf2 or scrambled duplex in Opti-MEM culture medium for 12 h. The cells were then placed in DMEM medium for 24 h and then stimulated by 10 μM 6-MSITC for an additional 12 h. The cells were lysed and assayed for firefly and Renilla luciferase activities. The average value from triplicate tests with differently lettered superscripts differs significantly at $p < 0.05$. (B) Western blot analysis. HepG2 cells were transfected with 100 nM siRNA against Nrf2 or scrambled duplex in Opti-MEM culture medium for 5 h. The cells were further placed in DMEM medium for 24 h and then stimulated with 10 μM 6-MSITC for an additional 6 h. Whole cell lysates were used to detect Nrf2, NQO1, and α-tubulin by Western blot analysis with their respective antibodies. Densitometry of the blots was performed using the LumiVision Imager software. Histograms show the densitometric analysis of Nrf2 or NQO1 normalized to α-tubulin. The average value from triplicate tests with differently lettered superscripts (small for Nrf2 and capital for NQO1 in panel B) differs significantly at $p < 0.05$.

activated Nrf2/ARE-mediated *NQO1* expression principally through modifying Keap1 protein (Figure 3C) but not ubiquiting Keap1 (data not shown). As shown in Figures 1B and 3A, treatment with 6-MSITC reduced the Keap1 protein level accompanying a modified form of Keap1 (Figure 3C). Therefore, Nrf2 stabilization through the inhibition of Nrf2 ubiquitination (Figure 3B) and protein turnover (Figure 2) are conclusively due to the modification of Keap1 by 6-MSITC. There are some reports on different actions of exogenous inducers on Nrf2-Keap2 system. Phenolic compounds such as *t*BHQ[26] and quercetin[20] also inhibited Nrf2 ubiquitination and protein turnover by modifying Keap1[20] but not by inhibiting ubiquitination activity of Keap1-Cul3. On the other hand, the toxic compound, arsenic, inhibited

Nrf2 ubiquitination possibly by inhibiting ubiquitination activity of Keap1-Cul3.[31] These data suggest that the down-regulation of Keap1 by 6-MSITC is mediated by modifying Keap1, a nonubiquitination/26S proteasomal pathway. It has been reported that sulforaphane, an analogue of 6-MSITC, modified Keap1 by reacting with thiols to form thionoacyl adducts. The map of modification sites by LC-MS-MS method indicated that sulforaphane displays a pattern of Keap1 modification distinctly different from some ARE inducers that modify Keap1 by alkylation. Moreover, the modification of Keap1 in vivo by sulforaphane is not accompanied by Keap1 ubiquitination.[26] Therefore, it is possible that 6-MSITC also stabilizes Nrf2 by 6-MSITC-Keap1 thionoacyl adduct formation that is required to be confirmed in our coming studies. Finally, silencing Nrf2 with its siRNA dramatically affected 6-MSITC-induced protein level of Nrf2 and NQO1 (Figure 7B) as well as ARE activity (Figure 7A), suggesting that Nrf2 is essential factor for 6-MSITC-induced ARE transcriptional activity and NQO1 expression.

Several lines of studies have revealed that isothiocyanates including 6-phenethyl sothiocyanate and sulforaphane, analogues of 6-MSITC, are rapidly accumulated to very high levels in a variety of culture cell types, and the intracellular concentration can be reach millimoar levels. These isothiocyanates were taken into cells almost entirely by conjugation with cellular GSH.[32] Animal experiments have demonstrated that 6-MSITC was rapidly absorbed and entered the circulatory system as is and/or as its GSH conjugate in rats after oral administration. The plasma concentration reached a maximum within 30 min after the administration and began to fall within 1 h but can be detected in the plasma even 4 h.[33] Pharmacokinetic studies in both rats and humans also support that sulforaphane can be distributed in the body and reach micromoar concentrations in the blood. In rats, following a 50 μM gavage of sulforaphane, detectable sulforaphane was evident after 1 h and peaked at ~20 μM at 4 h, with a half-life of approximately 2.2 h.[34] In human subjects given single doses of 200 μM broccoli sprouts isothiocyanate preparation, isothiocyanate plasma concentrations peaked between 0.943 and 2.27 μM/L 1 h after feeding, with half-life times of 1.77 $\pm$ 0.13 h.[35] Once sulforaphane is distributed, there is evidence that it can accumulate in tissues and produce chemopreventive effects.[35] Collectively, the published data indicate that 6-MSITC and its analogues can be absorbed, reach micromolar concentrations in the blood, accumulate in tissues, and be maintained to achieve the chemopreventive effects.[36]

In summary, we demonstrated that wasabi 6-MSITC, an analogue of sulforaphane, regulates Nrf2-mediated ARE activation by targeting Nrf2 and Keap1 factors at molecular level. 6-MSITC reduced the level of Keap1 by modifying Keap1 and enhanced the level of Nrf2 by inhibiting Nrf2 ubiquitination and turnover. These actions finally resulted in a high ratio of Nrf2/Keap1, the surplus Nrf2 comparing with Keap1, might bypass Keap1-Cul3 and accumulate in the nucleus to mediate ARE-driven activation. The findings provide novel mechanisms underlying 6-MSITC in cytoprotection and cancer chemoprevention.

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ABBREVIATIONS USED

ARE, antioxidant-responsive element; CHX, cycloheximide; EMSA, electrophilic mobility shift assay; Keap1, Kelchlike ECH-associating protein 1; 6-MSITC, 6-(methylsulfinyl)hexyl isothiocyanate; NQO1, nicotinamide adenine dinucleotide phosphate (NADP): quinone oxidoreductase 1; Nrf2, nuclear factor E2-related factor 2; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA

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