

Involvement of AMP-activated Protein Kinase α / Nuclear Factor (Erythroid-derived 2) Like 2-initiated Signaling Pathway in Cytoprotective Effects of Wasabi 6-(Methylsulfinyl) Hexyl Isothiocyanate

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6-(Methylsulfinyl) hexyl isothiocyanate (6-MSITC) is an active ingredient present in Wasabi, which is a popular pungent spice used in Japanese cuisine. Our previous studies suggested that the primary antioxidant activity of 6-MSITC may link to other biological activity. This study aimed to clarify how the antioxidant activity of 6-MSITC contributes to preventing overloaded lipid stress in hepatic cell model. HepG2 cells were treated with 6-MSITC at defined concentrations and times in normal medium or in combined fatty acids (CFA) medium, and the targeted proteins were detected by Western blotting. The kinetic data revealed that 6-MSITC activated AMP-activated protein kinase α (AMPK α) and nuclear factor (erythroid-derived 2) like 2 (Nrf2), and then enhanced the protein expression of Forkhead box protein O1 (FOXO1) and Sirtuin1 as well as that of the Nrf2 target proteins, NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase (HO-1). Furthermore, lipid metabolic stress was mimicked in HepG2 cells by overloading CFA. 6-MSITC significantly alleviated CFA-induced formation of thiobarbituric acid reactive substances and fat accumulation. Signaling analysis data revealed that 6-MSITC enhanced phosphorylation of AMPK α , upregulated the expression of Nrf2, NQO1, heme oxygenase 1, FOXO1, and Sirtuin1, and downregulated the expression of PPAR α . Taken together, our results suggested that the AMPK α /Nrf2-mediated signaling pathways might be involved in the cytoprotective effects of Wasabi 6-MSITC against metabolic lipid stress.

Key Words Wasabia 6-(methylsulfinyl) hexyl isothiocyanates, Cytoprotection effect, AMP-activated protein kinase, Nrf2, Sirtuin1

INTRODUCTION

Wasabi (*Eutrema japonicum* [Miq.] Koidz) is a famous spice in Japan. 6-(Methylsulfinyl) hexyl isothiocyanate (6-MSITC) is a pungent ingredient of Wasabi. Many physiological functions of 6-MSITC including detoxification [1], antioxidation [2-4], anti-inflammation [5-7], blood flow improvement [1], anticancer [8], and protection of neuronal cells [9] have been reported. Recently, 6-MSITC has been shown to improve the outcomes in the mouse model of Parkinson's and Alzheimer's diseases [10,11]. Of these functions, the antioxidant activity of 6-MSITC is of prime importance. We had found that 6-MSITC enhanced nuclear factor (erythroid-derived 2) like 2 (Nrf2)-mediated expression of NAD(P)H quinone dehydroge-

nase 1 (NQO1) [2] and heme oxygenase 1 (HO-1) [3].

Nrf2 is a key antioxidant transcription factor, which transactivates antioxidant response element (ARE) and regulates the expression of many antioxidant-enzyme genes. Nrf2 can be activated in various pathological circumstances associated with oxidative stress [12]. It is known that oxygen free radicals damage cells, leading to a wide range of diseases and symptoms [13]. Supplementation of natural Nrf2 activators derived from our diet is considered as an important way to prevent oxidative stress-induced disorders. How the antioxidant activity of Wasabi 6-MSITC regulates the energy homeostasis remains unclear [14].

Sirtuin family proteins include seven enzymes that play a role in regulating oxidative stress-related processes and

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redox signaling [15]. Of these, Sirtuin1 is extremely sensitive to cellular redox and nutritional status for regulating systemic energy production and preventing cellular dysfunction and metabolic disorders [16,17].

Moreover, cellular oxidative stress is also derived from common foods [18]. Over intake of high fat food is reported to enhance mitochondrial beta oxidation and to overproduce reactive oxygen species (ROS) [19]. AMP-activated protein kinase α (AMPK α) and Nrf2 have been reported to play important roles in maintaining/restoring the redox balance which is prone to disruption by high fat diet [20,21].

Based on the effects of AMPK α , Nrf2, and Sirtuin1 on oxidative response, and the antioxidant activity of 6-MSITC, in this study, we first investigated the basic signaling transduction mediated by AMPK α , Nrf2, Forkhead box protein O1 (FOXO1) and Sirtuin1 in 6-MSITC-treated HepG2 cells. We then attempted to clarify how the antioxidant activity of 6-MSITC protects against metabolic lipid stress mimicked by overloaded fatty acid and sugar.

MATERIALS AND METHODS

Reagents and antibodies

6-MSITC with 99% purity was obtained from Kinjirushi (Nagoya, Japan). MTT and β -actin antibody were purchased from Sigma-Aldrich LLC (St. Louis, MO, USA). The antibodies against Kelch-like ECH-associated protein 1 (Keap1), NQO1, HO-1, FOXO1, p-FOXO1, Sirtuin1, PPAR α and horseradish peroxidase conjugated anti-goat secondary antibody were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), whereas antibodies against AMPK α , Nrf2, p-AMPK α and horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies were from Cell Signaling Technology (CST, Beverly, MA, USA).

Cell culture

Human hepatoblastoma HepG2 cells were obtained from the Cancer Cell Repository, Tohoku University, Japan, and cultured at 37°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, which was from EquitechBio (Kerrville, TX, USA), and 1% of penicillin-streptomycin glutamine. In vitro overloaded lipid condition was induced by exposing HepG2 cells to a long-chain mixture (combined fatty acids, CFA medium) of 0.4 mM oleic acid, 0.8 mM palmitic acid, 10 mM glucose, and 15 mM fructose at a final concentration for 24 hours after seeding [22].

MTT assay

The MTT colorimetric assay was used to assess the survival of HepG2 cells to 6-MSITC treatment as described [23]. HepG2 cells (3×10^4 cells in 100 μ L medium) were plated into each well of 96-well microtiter plates. After 24-hour preincubation, the cells were treated with defined concentrations of

6-MSITC in 0.1% dimethyl sulfoxide (DMSO) for 12, 24, or 48 hours. The control cells were treated with 0.1% DMSO alone. MTT solution was added to each well and the cells were incubated for another 4 hours. The resulting MTT-formazan product was dissolved in 100 μ L of 0.04 N HCl-isopropanol. The amount of formazan was determined by measuring the absorbance at 595 nm with a microplate reader (Bio-Rad, Model 550; Bio-Rad Laboratories, Hercules, CA, USA). The results are expressed as the optical density ratio of the treatment to the control.

Western blotting analysis

Western blotting was performed as described previously [24,25]. Briefly, the cells were lysed with modified RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenyl-methylsulfonyl fluoride, and proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were homogenized three times in an ultrasonicator for 10 seconds and incubated on ice for 30 minutes. The homogenates were centrifuged at 14,000 rpm for 30 minutes and the supernatants were collected. The protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories). Equal amounts of lysate protein were run on SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membrane (GE Healthcare UK Ltd., Amersham, UK). The membrane was first blocked with TBST buffer (500 mM NaCl, 20 mM Tris-HCl [pH7.4], and 0.1% Tween-20) containing 5% nonfat dry milk, and then incubated with specific antibody overnight at 4°C and horseradish peroxidase-conjugated secondary antibody for another 1 hour. Bound antibodies were detected with enhanced chemiluminescence (ECL) system, and relative amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager software (TAITEC, Saitama, Japan).

Measurement of thiobarbituric acid reactive substances (TBARS)

Formation of TBARS was measured as described previously [26]. HepG2 cells (3×10^4 cells in 100 μ L medium) were plated into each well of 96-well microtiter plate. After preincubation for 24 hours, cell lysates were homogenized in 1.15% KCl. The reaction mixture containing 0.1 mL of cell lysates, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution and 1.5 mL of 0.8% aqueous solution of TBA was filled up to 4.0 mL with distilled water, and then heated at 95°C for 60 minutes. The absorbance of the organic layer was measured at 532 nm after centrifuging at 4,000 rpm for 10 minutes. The result was expressed as the optical density ratio of the treatment to the control.

Oil red O staining

HepG2 cells (1.9×10^5 cells/well) were plated into each

well of the 24-well microtiter plate. After 24-hour preincubation, the cells were cultured in CFA medium with 10 μM of 6-MSITC for 12, 24, and 48 hours. The control was treated with DMEM medium alone. After treatments, cells were fixed in 40% formaldehyde in PBS for 30 minutes, subsequently washed with 70% isopropanol, and then stained with 60% Oil red O dye (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 minutes at room temperature. The cells were rinsed with distilled water, stained with hematoxylin and eosin (HE, Muto Pure Chemicals Co., Ltd., Sigma-Aldrich) for 2 minutes, and washed with distilled water. The cover slip was mounted with Fluoromount (Sigma-Aldrich) and stained cells were visualized under a microscope (Olympus IX71; Olympus, Tokyo, Japan) [27]. To quantify Oil red O content, DMSO was added to each sample. After shaking at room temperature for 5 minutes, the density of samples was read at 510 nm on a spectrophotometer [28].

Statistical analyses

The results were represented as the mean \pm standard deviation. All data were statistically analyzed by one-way ANOVA, followed by Tukey's test as a post hoc test with the SPSS statistical program (version 19.0; IBM Corp., Armonk, NY, USA). Means with differently lettered superscripts differ significantly at the probability of $P < 0.05$.

RESULTS

Determination of an appropriate concentration range of 6-MSITC for HepG2 cells

To determine the appropriate concentration range of 6-MSITC for HepG2 cells, the cytotoxicity of 6-MSITC was measured by the MTT assay. As shown in Figure 1, no cytotoxicity was observed at up to 20 μM of 6-MSITC treated for 24 hours; however, a significant inhibitory effect of 19.6% was observed

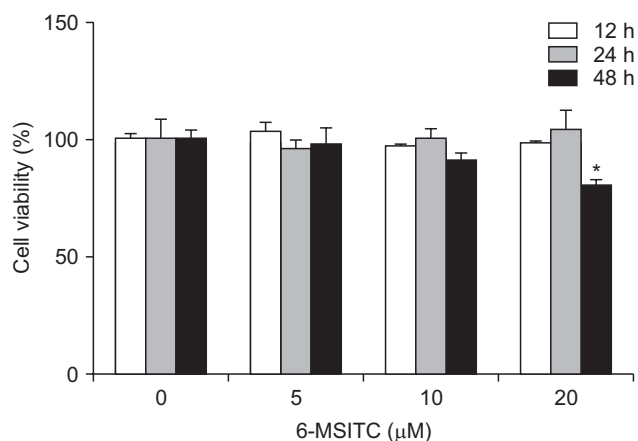


Figure 1. The cytotoxicity of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) in HepG2 cells. The cells (3×10^4 cells/100 μL) were grown for 24 hours, and then exposed to the indicated concentrations of 6-MSITC for 12, 24, or 48 hours. The cells density was assessed calorimetrically after staining with MTT. The values are means \pm standard deviation of eight data. * $P < 0.05$ vs. control.

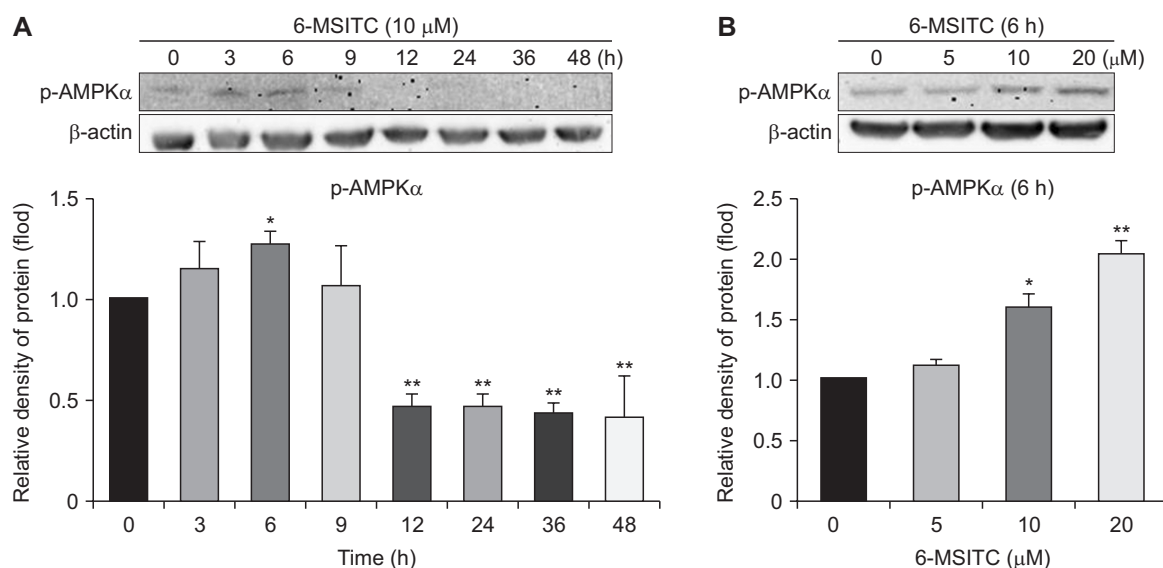


Figure 2. The activation of AMP-activated protein kinase α (AMPK α) by 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC). HepG2 cells were treated with 10 μM of 6-MSITC for 3, 6, 9, 12, 24, 36, and 48 hours (A) or with 5–20 μM of 6-MSITC for 6 hours (B). The whole-cell lysates were used to measure p-AMPK α and β -actin by Western blot analysis with their respective antibodies. Densitometry of the blots was performed using the Lumi-Vision Imager software. Histograms show the densitometric analysis of p-AMPK α normalized to β -actin. The data were from triplicate tests. * $P < 0.05$, ** $P < 0.05$.

at 48 hours. Therefore, we treated HepG2 cells with 6-MSITC at the range of 0 to 20 μ M within 24 hours in all subsequent experiments in this study except control.

6-MSITC activated AMPK α

To determine whether 6-MSITC could activate AMPK α , HepG2 cells were treated for 3, 6, 9, 12, 24, 36, or 48 hours with 10 μ M of 6-MSITC. As shown in Figure 2A, the phosphorylation of AMPK α was observed from 3 to 9 hours of treatment, and the significant induction occurred at 6 hours. As shown in Figure 2B, 6-MSITC significantly enhanced the phosphorylation of AMPK α at 6 hours in the 10 to 20 μ M range.

6-MSITC enhanced the expression of Nrf2, NQO1 and HO-1

HepG2 cells were treated for 3, 6, 9, 12, 24, 36, and 48 hours with 10 μ M of 6-MSITC or treated for 6 and 9 hours with 5 to 20 μ M of 6-MSITC. As shown in Figure 3A, 10 μ M of 6-MSITC upregulated the expression of Nrf2 from 3 to 9 hours and expression of NQO1 and HO-1 from 6 to 12 hours, with a concomitant decrease in the Keap1 level. We further conducted a concentration-dependent experiment with 5 to 20 μ M of 6-MSITC to confirm the results. As shown in Figure 3B, 6-MSITC enhanced the expression of Nrf2, NQO1 and HO-1 at 10 and 20 μ M, accompanying a decrease in the Keap1 level at 20 μ M. These data suggest that the Nrf2/Keap1 pathway is involved in 6-MSITC-induced expression of NQO1 and HO-1 antioxidant enzymes.

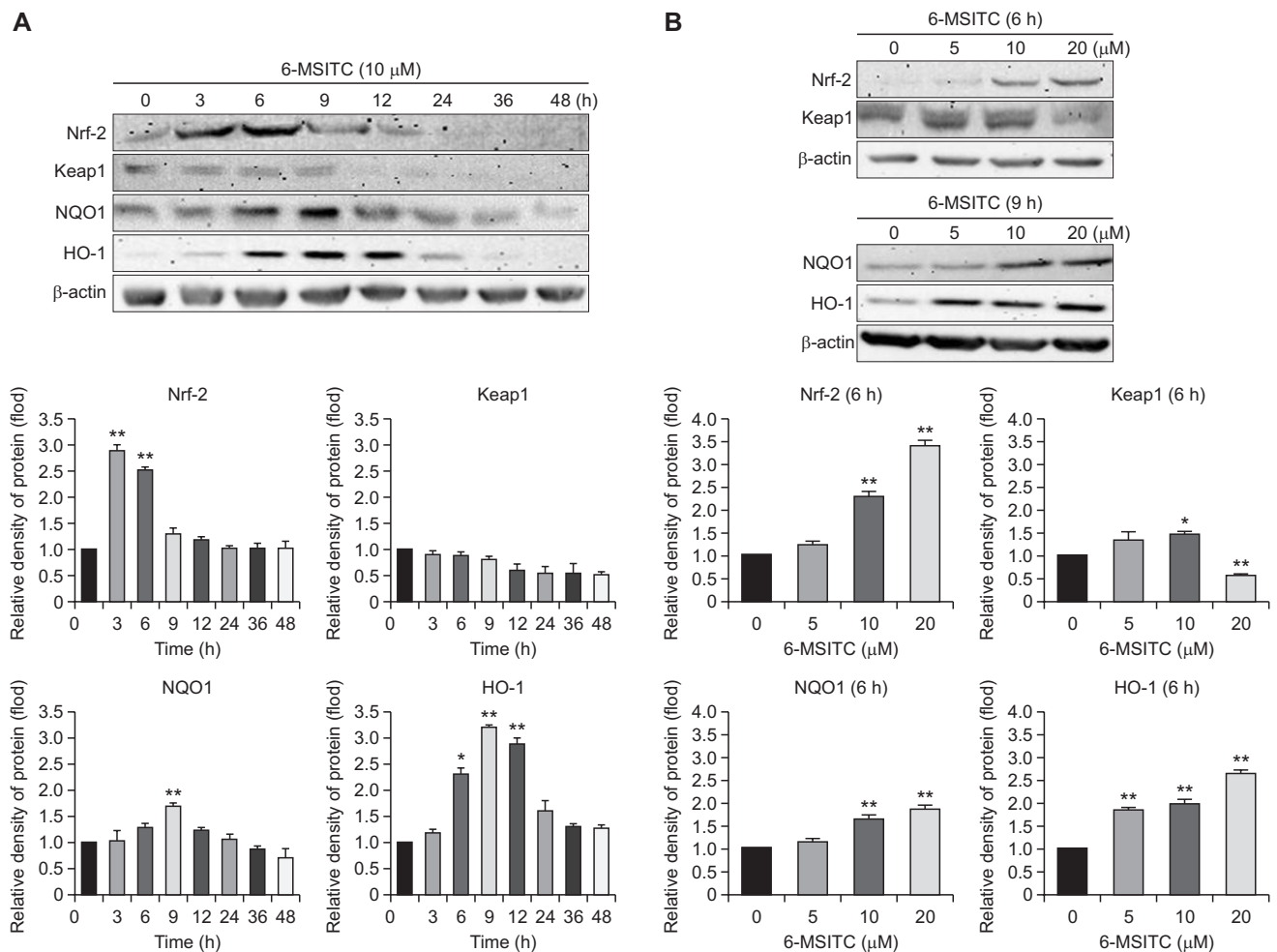


Figure 3. Effect of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) on the expression of nuclear factor (erythroid-derived 2) like 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1), NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase 1 (HO-1). HepG2 cells were treated with 10 μ M of 6-MSITC for 3, 6, 9, 12, 24, 36, or 48 hours (A), or with indicated concentrations of 6-MSITC for 6 and 9 hours (B). The whole-cell lysates were used to detect Nrf2, Keap1, NQO1, HO-1 and β -actin 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, Keap1, NQO1 or HO-1 normalized to β -actin. The data were from triplicate tests. * $P < 0.05$, ** $P < 0.05$.

6-MSITC activated FOXO1 and increased Sirtuin1 expression

To explore the effect of 6-MSITC on FOXO1 activation and Sirtuin1 expression, HepG2 cells were treated for 3, 6, 9, 12, 24, 36, and 48 hours with 10 μM of 6-MSITC, or treated for 12 and 24 hours with 5 to 20 μM of 6-MSITC. As shown in Figure 4A, the phosphorylation of FOXO1 was significantly enhanced by 6-MSITC from 6 to 24 hours, and the highest level of phosphorylation occurred at 12 hours. Meanwhile, the same expression fashion of Sirtuin1 was also observed from 6 to 48 hours with the highest expression at 24 hours. Moreover, we treated HepG2 cells for 12 or 24 hours with 5 to 20 μM of 6-MSITC to confirm the concentration-dependent effect. The significant increase in both FOXO1 phosphorylation and Sirtuin1 expression was observed at 10 and 20 μM (Fig. 4B).

6-MSITC attenuated CFA-induced lipid peroxidation

The overload of fatty acid and sugar can induce overproduction of ROS in mitochondria, finally causing DNA damage and cell death [29]. In this study, we used CFA medium to mimic this condition. The cells were cultured in normal medium, or CFA medium with or without 10 μM of 6-MSITC. The cellular lipid peroxidation was measured by measuring TBARS. There were no significant changes in the TBARS level among three groups at 24 hours of culture (Fig. 5). However, the TBARS level was significantly increased in CFA medium, and this was reduced by 6-MSITC treatment at 48 hours.

6-MSITC reduced lipid accumulation in CFA-treated HepG2 cells

In order to clarify whether the lipid peroxidation is due to lipid accumulation in the CFA medium, the lipid accumulation in HepG2 cells was detected by Oil red O staining after 48 hours. The typical photomicrographs are depicted in Figure

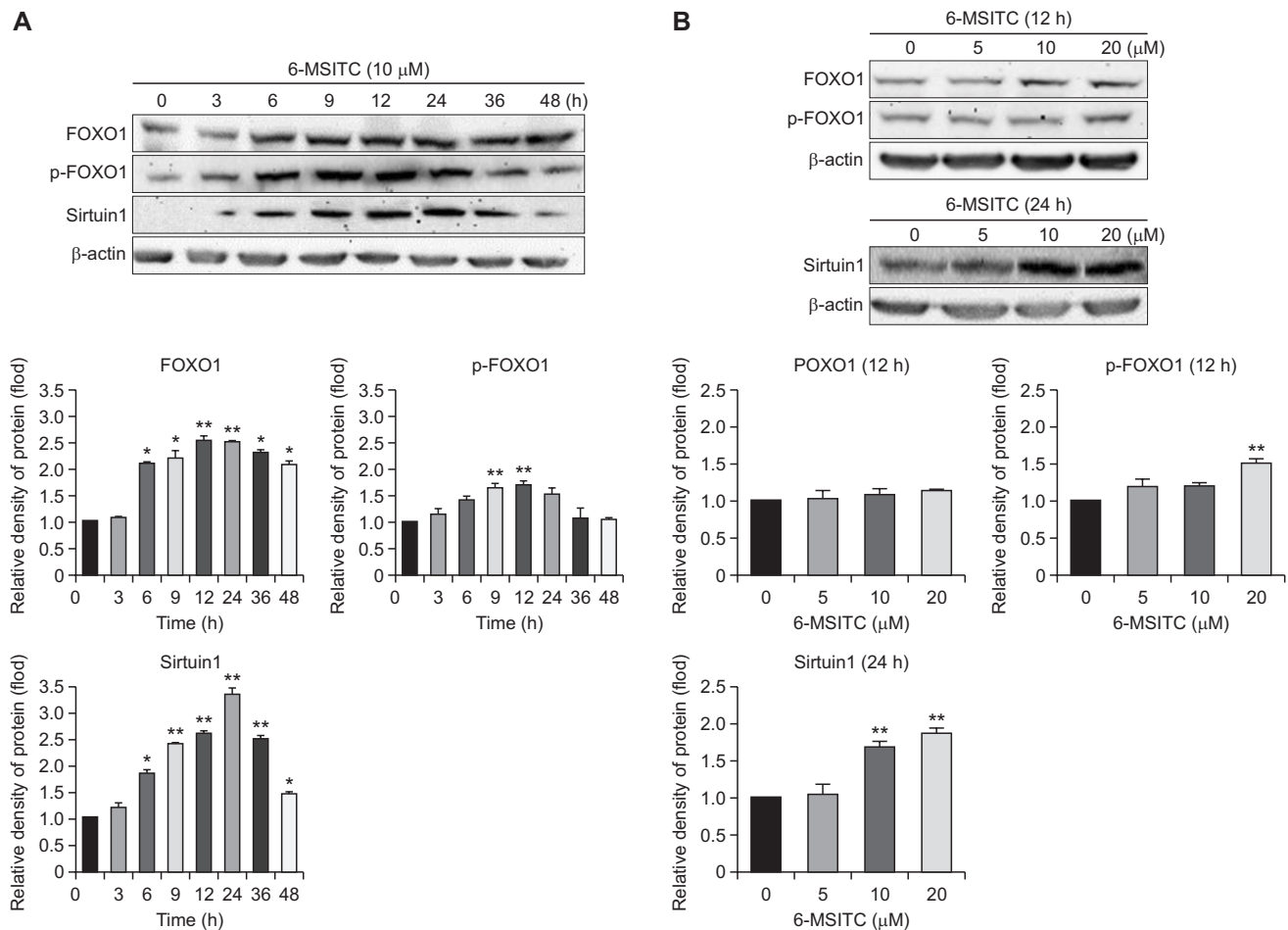


Figure 4. Effect of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) on the expression of Forkhead box protein O1 (FOXO1) and Sirtuin1 (B). HepG2 cells were treated with 10 μM of 6-MSITC for 3, 6, 9, 12, 24, 36, and 48 hours (A), or with indicated concentrations of 6-MSITC for 12 and 24 hours (B). The whole-cell lysates were used to detect FOXO1, p-FOXO1, Sirtuin1 and β -actin 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 FOXO1, p-FOXO1 and Sirtuin1 normalized to β -actin. The data were from triplicate tests. * $P < 0.05$, ** $P < 0.05$.

6A and 6B, and the quantitative results are shown in Figure 6C. The cellular lipid accumulation was increased obviously in CFA medium after 48 hours and alleviated significantly by 10 μ M of 6-MSITC treatment. These data demonstrated that 6-MSITC had a protective effect against CFA-induced lipid accumulation.

Involvement of signaling pathways in the prevention of lipid peroxidation by 6-MSITC

To examine how 6-MSITC prevented oxidative toxicity from overload fatty acid and sugar, we investigated the status of some key factors involved in cellular antioxidant signaling. HepG2 cells were cultured with or without 6-MSITC in both control medium and CFA medium for 6, 12, or 24 hours. As

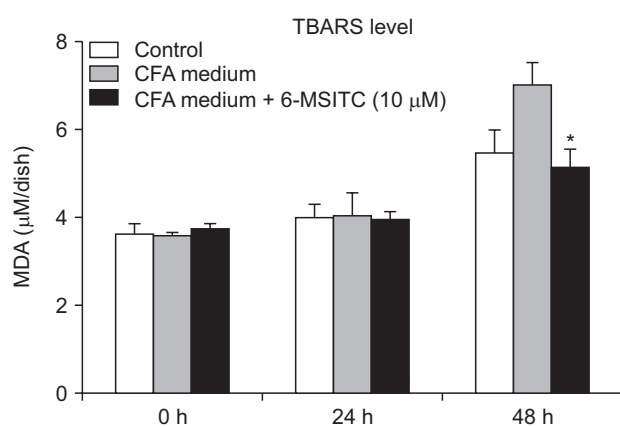


Figure 5. Thiobarbituric acid reactive substances (TBARS) level in HepG2 cells. HepG2 cells (3×10^4 cells/100 μ L) were cultured in normal medium, combined fatty acids (CFA) medium or CFA plus 10 μ M of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) medium. After 12, 24, and 48 hours incubation, the cellular TBARS levels were measured as described in Material and Methods. The data were from triplicate tests. * $P < 0.05$.

shown in Figure 7, 10 μ M of 6-MSITC enhanced the phosphorylation of AMPK α as well as expression of Nrf2 and HO-1 in both medium (Fig. 7A). 6-MSITC also upregulated significantly the phosphorylation of FOXO1 (Fig. 7B) as well as Sirtuin 1 (Fig. 7C) in CFA medium. A concentration-dependent experiment with 5 to 20 μ M of 6-MSITC also confirmed these results (Fig. 8). Thus, 6-MSITC at 10 and 20 μ M range significantly upregulated the phosphorylation of AMPK α and the expression of HO-1, p-FOXO1, and Sirtuin1, accompanying downregulated PPAR α expression (Fig. 8B). AMPK is reported as a positive upstream regulator of Nrf2 [30], whilst FOXO1 was known as a major factor the regulation of in hepatic microsomal triglyceride transfer protein regulation [31] and PPAR α is related to fatty acid metabolism. Therefore, these results suggested that the antioxidant signaling (AMPK α -Nrf2-HO-1) and lipid peroxidation (AMPK α -PPAR α /FOXO1-Sirtuin1) were involved in the inhibitory effect of 6-MSITC on CFA-induced lipid peroxidation.

DISCUSSION

Our previous studies [32,33] reported that the Wasabi 6-MSITC stimulated Nrf2-mediated ARE signaling to exert the antioxidant activity. Meanwhile, 6-MSITC was also found to activate the AMPK, which enables the cells to recover energy homeostasis by enhancing fuel oxidation [34-36]. To clarify the crosstalk between these events induced by 6-MSITC, in this study, we first investigated the time-dependent activations of AMPK, Nrf2 and Sirtuin1 in 6-MSITC-treated HepG2 cells. The data revealed that 6-MSITC induced the activation of AMPK α from 3 to 9 hours in a concentration-dependent manner. Nrf2 was upregulated from 3 to 9 hours, and expression of Nrf2-mediated antioxidant enzymes, NQO1 and HO-1, increased from 6 to 12 hours. These data showed that 6-MSITC might upregulate Nrf2/Keap1 pathway coordinated

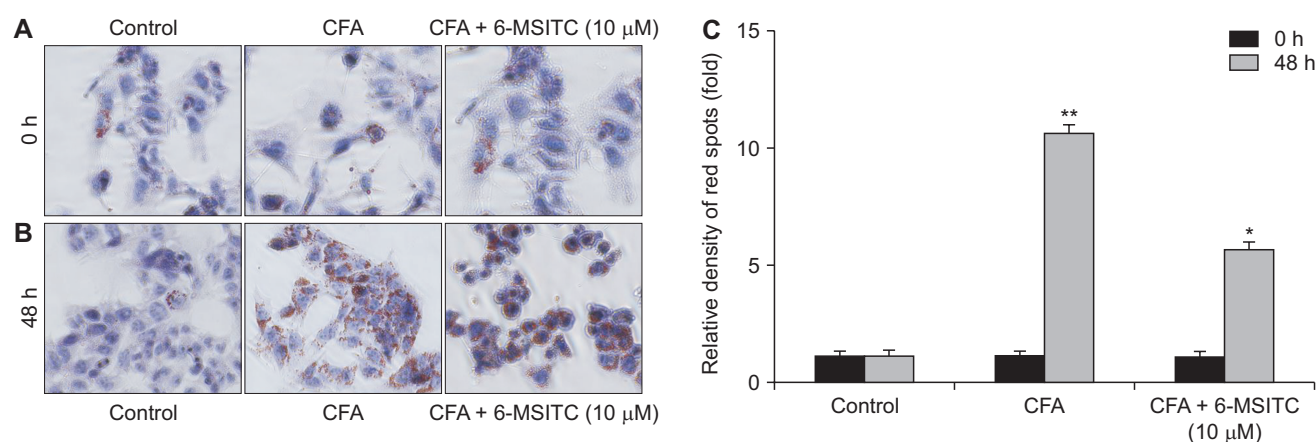


Figure 6. The lipid contents of HepG2 cells after stained by Oil red O dye and hematoxylin. HepG2 cells (1.9×10^5 cells/well) seeded in a 24-well plate. After preculture for 24 hours, the cells were cultured in normal medium, combined fatty acids (CFA) medium and 10 μ M of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC)-added CFA medium for 0 hour (A) and 48 hours (B). The magnification is 200 \times (A). The cells were stained with 60 % Oil red O dye and hematoxylin. The red spots in the figures represent lipid. Histogram shows the densitometric analysis of red spots normalized to control medium. The data represent relative fold to respective control (C). The data were from triplicate tests. * $P < 0.05$, ** $P < 0.05$.

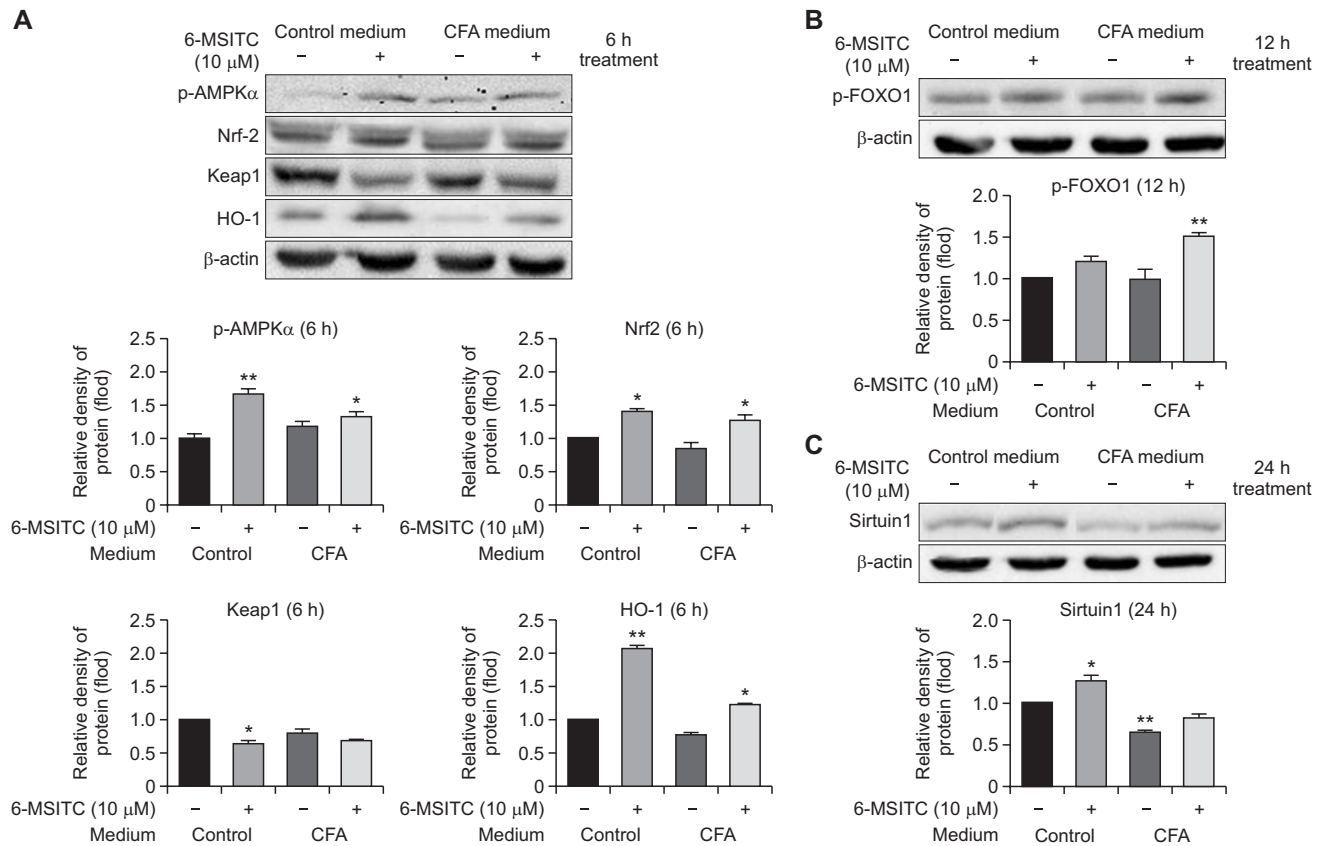


Figure 7. The effect of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) on activation of AMPK, Nrf2, and FOXO1 as well as Sirtuin1 expression in control and combined fatty acids (CFA) medium. HepG2 cells were treated with 6-MSITC (10 μ M) in both control and CFA medium. Six hours culture was used to detect the expression of phosphorylation of AMP-activated protein kinase α (p-AMPK α), nuclear factor (erythroid-derived 2) like 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap1) and heme oxygenase 1 (HO-1) (A). Twelve hours culture was used to detect the expression of p-Forkhead box protein O1 (p-FOXO1) (B), and 24 hours culture was used to detect the expression of Sirtuin1 expression (C). The whole-cell lysates were used to detect the mentioned proteins 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 the mentioned proteins normalized to β -actin. The data were from triplicate tests. * $P < 0.05$, ** $P < 0.05$.

with AMPK [14] to induce Nrf2/Keap1-mediated expression of antioxidant enzyme genes. On the other hands, FOXO1 and Sirtuin1 were upregulated from 6 to 24 hours and from 6 to 36 hours, respectively. It has been reported that the activation of AMPK α could induce the expression of FOXO1 [37] and Sirtuin1 [38]. By combining our kinetic data with previous reports, we can suggest that 6-MSITC induces AMPK α /Nrf2-initiated signaling pathways to enhance the expression of antioxidant enzymes, FOXO1 and Sirtuin1.

Secondly, we investigated whether 6-MSITC protects cells from fat metabolic stress. The cells were treated with high fatty acid and high sugar containing medium (CFA medium) and accumulation of TBARS, and lipid was monitored. The TBARS level was significantly increased in CFA medium and attenuated with 6-MSITC treatment. Meanwhile, the cellular lipid spots in CFA medium were also significantly increased and this was alleviated after 6-MSITC treatment. To further clarify the probable molecular mechanism how 6-MSITC

protects cells from fat metabolic stress, AMPK α /Nrf2-induced signaling pathways and their downstream molecules have also been investigated in both control and CFA medium with or without 6-MSITC (Fig. 7 and 8). The data revealed that 6-MSITC activated the AMPK α /Nrf2-initiated signaling pathways and regulated the expression of their downstream molecules, such as FOXO1, Sirtuin1 and PPAR α . These data supported that 6-MSITC had preventive effects against cellular lipid stress, and AMPK might be a key molecule to control the effect of 6-MSITC on such reduction. We are planning to use siRNA against AMPK to directly prove the significant role of AMPK on the effect of 6-MSITC. Moreover, AMPK activation by 6-MSITC occurs as early as 6 hours, while the TBARS level was changed after 48 hours (Fig. 5). It will be worthwhile investigating the association between AMPK activation and lipid peroxidation prevention by 6-MSITC in our future research.

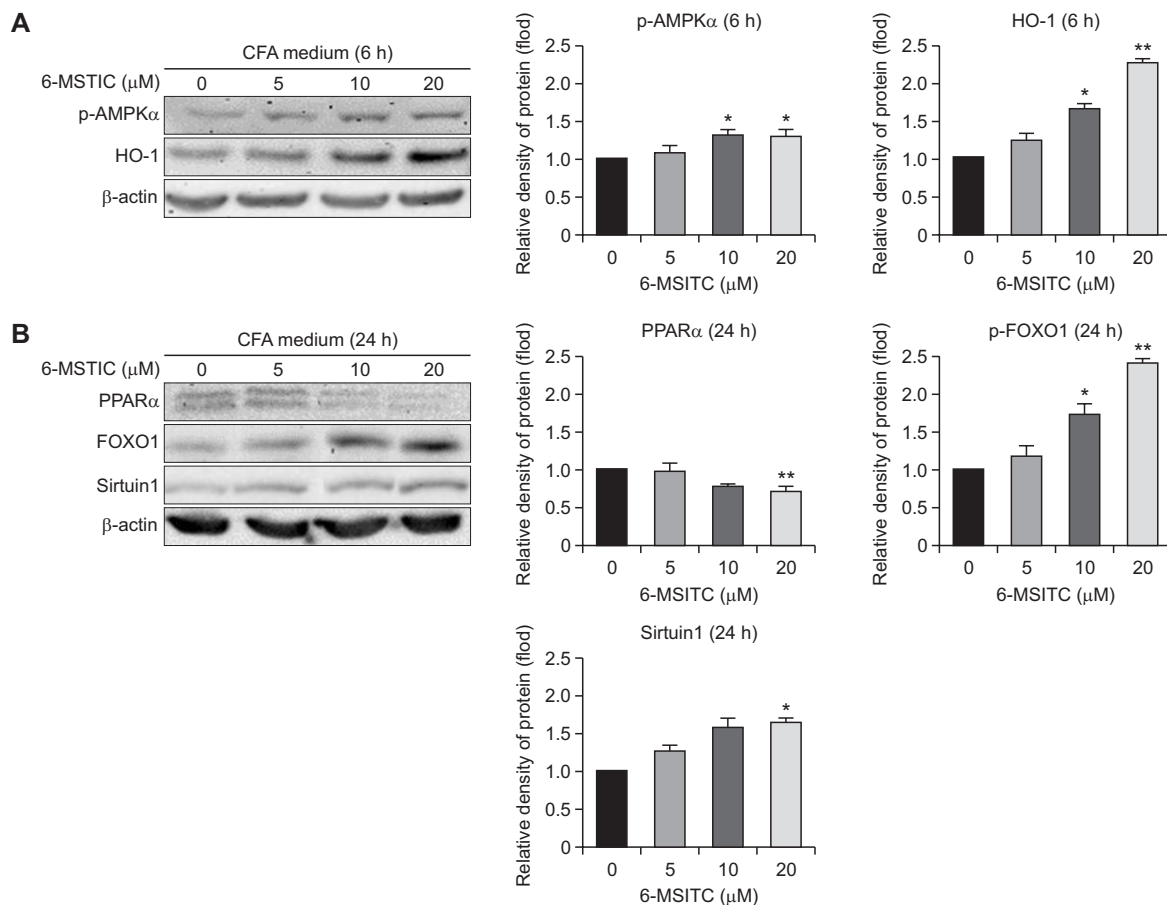


Figure 8. The effect of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) on phosphorylation of AMPK1 and FOXO1 and expression of HO-1, PPAR α , and Sirtuin1 in combined fatty acids (CFA) medium. HepG2 cells were treated with 5, 10, or 20 μ M of 6-MSITC in CFA medium. Six hours culture was used to detect the phosphorylation of AMP-activated protein kinase α (p-AMPK α) and expression of heme oxygenase 1 (HO-1) (A), and 24 hours culture was used to detect the expressions of PPAR α , p-Forkhead box protein O1 (p-FOXO1) and Sirtuin1 (B). The whole-cell lysates were used to detect the mentioned proteins 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 the mentioned proteins normalized to β -actin. The data were from triplicate analysis. * $P < 0.05$, ** $P < 0.01$.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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