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# Fraxetin Induces Heme Oxygenase-1 Expression by Activation of Akt/Nrf2 or AMP-activated Protein Kinase α/Nrf2 Pathway in HaCaT Cells

ORIGINAL ARTICLE

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**Background:** Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a coumarin derivative, has been reported to possess antioxidative, anti-inflammatory and neuroprotective effects. A number of recent observations suggest that the induction of heme oxygenase-1 (HO-1) inhibits inflammation and tumorigenesis. In the present study, we determined the effect of fraxetin on HO-1 expression in HaCaT human keratinocytes and investigated its underlying molecular mechanisms.
**Methods:** Reverse transcriptase-PCR and Western blot analysis were performed to detect HO-1 mRNA and protein expression, respectively. Cell viability was measured by the MTS test. The induction of intracellular reactive oxygen species (ROS) by fraxetin was evaluated by 2',7'-dichlorofluorescin diacetate staining.
**Results:** Fraxetin upregulated mRNA and protein expression of HO-1. Incubation with fraxetin induced the localization of nuclear factor-erythroid-2-related factor-2 (Nrf2) in the nucleus and increased the antioxidant response element-reporter gene activity. Fraxetin also induced the phosphorylation of Akt and AMP-activated protein kinase (AMPK)α and diminished the expression of phosphatase and tensin homolog, a negative regulator of Akt. Pharmacological inhibition of Akt and AMPKα abrogated fraxetin-induced expression of HO-1 and nuclear localization of Nrf2. Furthermore, fraxetin generated ROS in a concentration-dependent manner.
**Conclusions:** Fraxetin induces HO-1 expression through activation of Akt/Nrf2 or AMPKα/Nrf2 pathway in HaCaT cells.
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**Key Words:** Fraxetin, Keratinocytes, Heme oxygenase-1, Nrf2

## INTRODUCTION

Oxidative stress is known as an imbalance between the production of free radicals and their elimination by protective mechanisms.[1] Reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide, are generated by ultraviolet (UV) light, cigarette smoke, or aging. Excessive production of ROS causes oxidative damage of cellular macromolecules, such as DNA, proteins and lipids, thereby inducing neoplastic transformation of cells.[2] Skin is constantly exposed to oxidative stress from exogenous and endogenous sources. UV-generated ROS in the skin develop oxidative stress when formation of ROS exceeds the antioxidant defense ability. Therefore, antioxidant systems are important to maintain cellular homeostasis and the cellular redox system consists of a series of enzymatic and nonenzymatic antioxidants in skin.[3]

Heme oxygenase-1 (HO-1) is a representative cytoprotective enzyme that catalyzes the rate limiting steps in heme degradation. Multiple lines of evidence have revealed that HO-1 attenuates skin inflammation[4] and atopic dermatitis-like skin lesions.[5] Topical treatment of mouse skin with hemin, a chemical inducer of HO-1, reduces 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion.[6,7] Moreover, wild type mice having endogenous HO-1 are less susceptible to TPA-induced skin

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tumor formation in the early stage of papillomagenesis as compared to HO-1 knock-out mice.[8] Shin et al.[9] reported that zerumbone, a sesquiterpene derived from tropical ginger, induces HO-1 expression through activation of nuclear factor erythroid-2-related factor-2 (Nrf2) in JB6 cells and mouse skin, which may partly account for its previously reported inhibitory effects on mouse skin carcinogenesis. Thus, fortification of skin tissue with elevated HO-1 confers protection against cancer.

The expression of HO-1 is known to be regulated through the activation of a redox-sensitive transcription factor, Nrf2.[10,11] Under basal conditions, Nrf2 is constitutively sequestered in the cytoplasm by its repressor Kelch-like ECH-associated protein (Keap1), which causes proteasomal degradation of Nrf2.[12-15] However, under condition of oxidative stress, Keap1 undergoes oxidative or covalent modifications of cysteine residues,[16] thereby allowing stabilization of Nrf2 through its dissociation from Keap1. Thus, Nrf2 migrates to the nucleus and activates the antioxidant response element (ARE) within the *ho-1* gene promoter region.[17,18] Besides oxidative modification of Keap1 cysteine thiols, dissociation of Nrf2 from Keap1 can be promoted upon phosphorylation at specific serine or threonine residues of Nrf2 by kinases, such as phosphatidylionositol-3-kinase (PI3K)/Akt,[19] AMP-activated protein kinase (AMPK),[20] mitogen-activated protein (MAP) kinases,[21,22] and protein kinase C.[23] In our previous study, we demonstrated that thymoquinone, an active constituent of *Nigella sativa*, increases HO-1 expression by activating Nrf2 via ROS-mediated phosphorylation of Akt and AMPKα in human keratinocytes (HaCaT).[24]

*Fraxinus rhynchophylla*, belonging to the Oleaceae family, possesses several biological activities, including anti-oxidative,[25] anti-inflammatory[26] and anti-toxoplasmosis[27] effects. Fraxetin (6-metoxy-7,8-dihydroxycoumarin), a major dihydroxycoumarin found in bark of *F. rhynchophylla*, has been shown to have strong anti-oxidant,[28-30] anti-inflammatory,[31] neuroprotective[32] and anti-tumor[33] properties (Fig. 1A). In this study, we investigated whether fraxetin could induce HO-1 expression in HaCaT cells and elucidated its underlying mechanisms with special focus on its role in modulating Nrf2 signaling.

## MATERIALS AND METHODS

### 1. Chemicals and reagents

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise indicated. Cell culture reagents were purchased from Gibco BRL (Rockville, MD, USA). Antibodies against HO-1, p-Akt, Akt, AMPKα, p-AMPKα and Akt inhibitor LY294002 were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against each of Nrf2 and phosphatase and tensin homolog and horse-raddish peroxidase-conjugated secondary antibodies were purchased from SantaCruz Biotechnology (SantaCruz, CA, USA). The 2',7'-dichlorofluorescin diacetate (DCF-DA) was purchased from Invitrogen (Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) was purchased from Meditech (Herndon, VA, USA).

### 2. Cell culture and treatment

HaCaT cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell types were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS containing 100 U/mL of penicillin and 100 μg/mL of streptomycin and 5% $CO_2$ at 37°C. For all experiments, early passage cells were grown to 80% to 90% confluence. All chemicals were dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration was less than 0.1%.

### 3. Cytotoxicity assay

Cytotoxicity effect of fraxetin against HaCaT cells was measured by using a solution of tetrazolium compound (MTS) (Promega, Madison, WI, USA). Briefly, the cells were plated in 96-well culture plates at a density of $2 \times 10^3$ cells/well in DMEM and allowed to attach for 24 hours. After incubation, the medium was discarded and replaced with 100 μL of new medium containing various concentrations of fraxetin. After 24 hours or 48 hours of culture, 20 μL MTS reagent was added to each well, followed by 1 hour incubation. The optical density was measured at 550 nm in a microplate reader (Tecan Trading AG, Männedorf, Switzerland). Cell viabilities were presented as the percentage of the optical density of samples to that of the control.

### 4. Western blot analysis

Cells were harvested by scraping the cells from cultured dishes using a cell scraper. Cellular lysates were prepared using RIPA cell lysis buffer. The cells were disrupted and extracted at 4°C for 30 minutes. After centrifugation at 16,000 ×*g* for 15 minutes, the supernatant was obtained as the cell lysate. Protein concentrations were measured using a protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Cellular proteins (30 μg) were subjected to 10% SDS-PAGE. The resolved proteins were transferred to an Immobilon-P-membrane and allowed to react with a specific antibody. The detection of specific proteins was carried out by Super-signal pico-chemiluminescent substrate or dura-luminol substrate (Thermo Scientific, Waltham, MA, USA) accor-

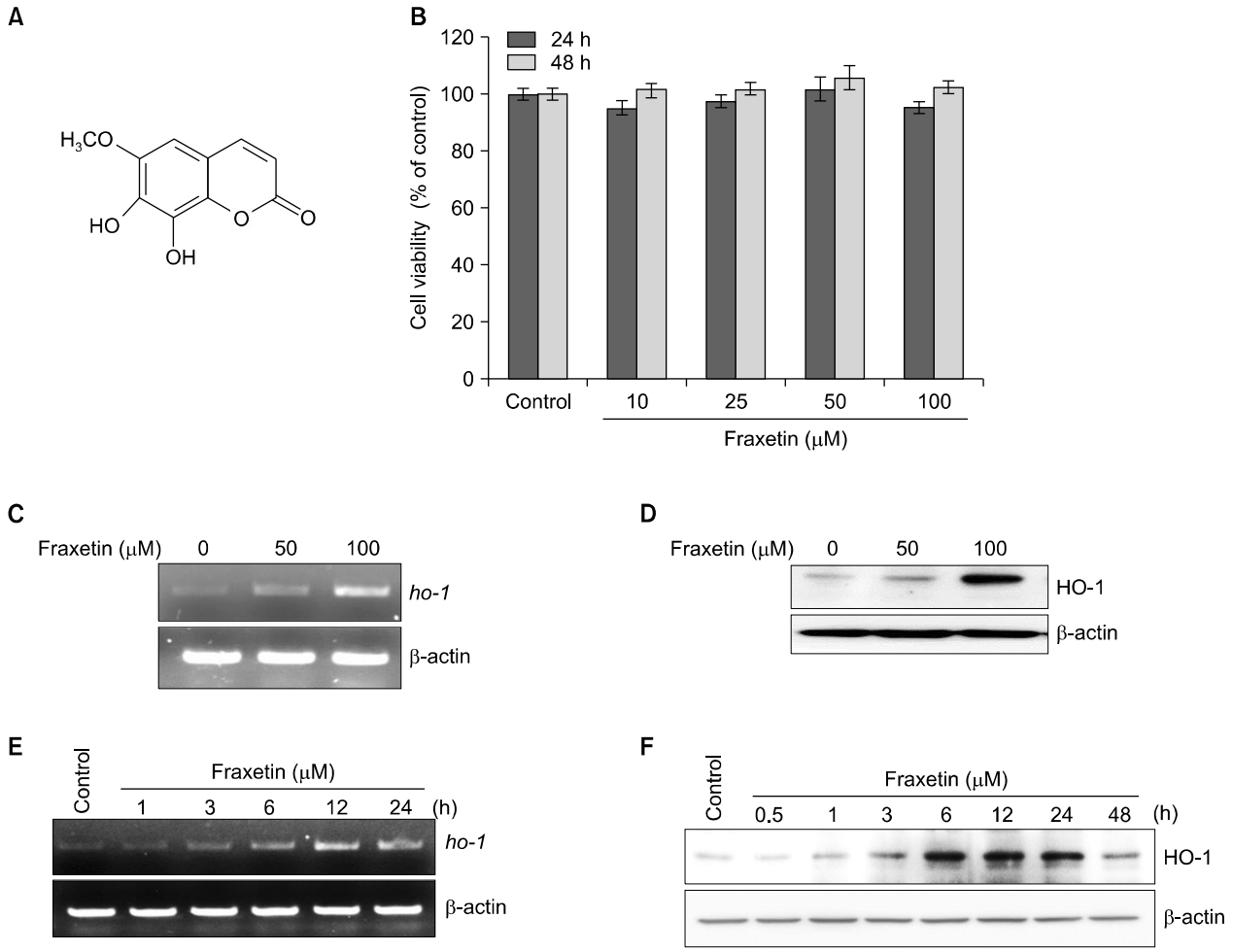


**Figure 1.** Effect of fraxetin on heme oxygenase-1 expression in HaCaT cells. (A) Chemical structure of fraxetin. (B) HaCaT cells were treated with indicated concentration of fraxetin for 24 hours and 48 hours, and cell viability was evaluated by the MTS assay. The results are presented as means ± SD (n = 3). (C, D) Cell were treated with indicated concentration of fraxetin for 6 hours. (E, F) Cell were treated with fraxetin (100 μM) for indicated time. (C, E) Expression of heme oxygenase-1 (HO-1) mRNA (*ho-1*) was detected by reverse transcriptase-PCR and (D, F) protein level of HO-1 was determined by Western blot analysis.

ding to manufacturer's instruction and visualized with image-quant LAS 4000 (Fujifilm Life Science, Tokyo, Japan). Loading differences were normalized using anti-β-actin antibody.

## 5. Reverse transcriptase-PCR

Total RNA was isolated using TRIzol (Invitrogen). Total RNA (1 μg) was used for the complementary DNA synthesis using random primers. PCR conditions for *ho-1* was as follows: *ho-1*, 35 cycles for 95°C for 2 minutes, 54.2°C for 40 seconds and 72°C for 1 minute. All primers were synthesized from Bioneer (Seoul, Korea). The primers used for PCR amplification are shown as follows: 5' CAGGCAGAGAATGCTGAGTTC-3', 5'-GATGTTGAG CGCAGGAACGCAGT-3' (*ho-1*), as well as 5'-GCCATGTACGTTGC ATC-3', 5'-CTCCTTAATGTCACGCAC-3' (β-actin). Amplification products were resolved by 1.5% agarose gel electrophoresis, stained with safe dye and photographed by ImageQuant LAS 4000.

## 6. Antioxidant response element-luciferase reporter gene assay

Cells were subcultured into 12-well plates at a density of $5 \times 10^4$ cells per well prior to transfection. Cells were transfected with pARE-luc or pCMV-β-galactosidase using Genefectin transfection reagent (Genetrone Biotech, Gwangmyeong, Korea). The constructs for pARE-luc or pCMV-β-galactosidase were the kindly gift from Professor Young-Joon Surh (College of Pharmacy, Seoul National University, Seoul, Korea). Briefly, pARE-luc and pCMV-β-galactosidase were added to the optimem (serum free medium) containing Genefectin reagent and incubated for 20 minutes. After 24 hours of transfection, cells were treated with

fraxetin (50 or 100 μM) for additional 6 hours and cell lysis was carried out with × 1 reporter lysis buffer. After preparing cell lysates, the luciferase assay was performed as previously described.[24]

### 7. Preparation of cytosolic and nuclear extracts

The cytosolic and nuclear extracts were prepared by using NE–PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific). Pellets were suspended in 50 μL of CERI (Cytoplasmic Extraction Reagent I) for 15 minutes and added CERII (Cytoplasmic Extraction Reagent II) for additional 2 minutes. The mixture was centrifuged for 10 minutes at 16,000 ×*g*. The supernatant is cytosolic extract. The pellets were washed with NER (Nuclear Extraction Reagent), incubated on ice for 1 hour and centrifuged at 16,000 ×*g* for 15 minutes. The supernatant is nuclear proteins. Prepared nuclear fraction was analyzed by Western blotting.

### 8. Determination of reactive oxygen species production

ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe, DCF-DA. Briefly, cells were plated in 6-well culture plates at a density of $2 \times 10^5$ cells/well and treated with DMSO or fraxetin. After treatment, cells were incubated with 25 μM DCF-DA in PBS at 37°C for 30 minutes, washed twice with HBSS solution, suspended in the complete media and examined under a fluorescence microscope or fluorescence activated cell sorter to detect the intracellular accumulation of ROS.

### 9. Statistical analysis

Data were expressed as mean ± SD of three independent replicate experiments. Significant differences among groups were determined using Student's *t*-test. A value of $P < 0.05$ was considered as statistically significant.

## RESULTS

### 1. Effect of fraxetin on heme oxygenase-1 mRNA and protein expression in HaCaT cells

We first examined whether fraxetin induces any cytotoxicity in HaCaT cells by MTS assay. Incubation of HaCaT cells with fraxetin at indicated concentration did not affect the cell viability (Fig. 1B). Treatment of cells with fraxetin (50 or 100 μM) for 6 hours induced the expression of *ho-1* mRNA (Fig. 1C) and protein (Fig. 1D) in a concentration-dependent manner. Moreover, treatment with fraxetin (100 μM) for indicated time periods resulted in elevated expression of *ho-1* mRNA (Fig. 1E) and protein (Fig. 1F) in a time-dependent manner.

### 2. Effect of fraxetin on nuclear factor-erythroid-2-related factor-2 and antioxidant response element activation in HaCaT cells

Nrf2 is one of the transcription factors that regulates *ho-1* expression. Thus, we investigated whether Nrf2 is involved in fraxetin-induced HO-1 expression. As illustrated in Figure 2A, fraxetin treatment for 1 and 3 hours caused Nrf2 accumulation in the nucleus with corresponding decrease in cytosolic fraction. Since Nrf2 binds to the ARE sequences located in the promoter region of *ho-1*, we examined the effect of fraxetin on the ARE-

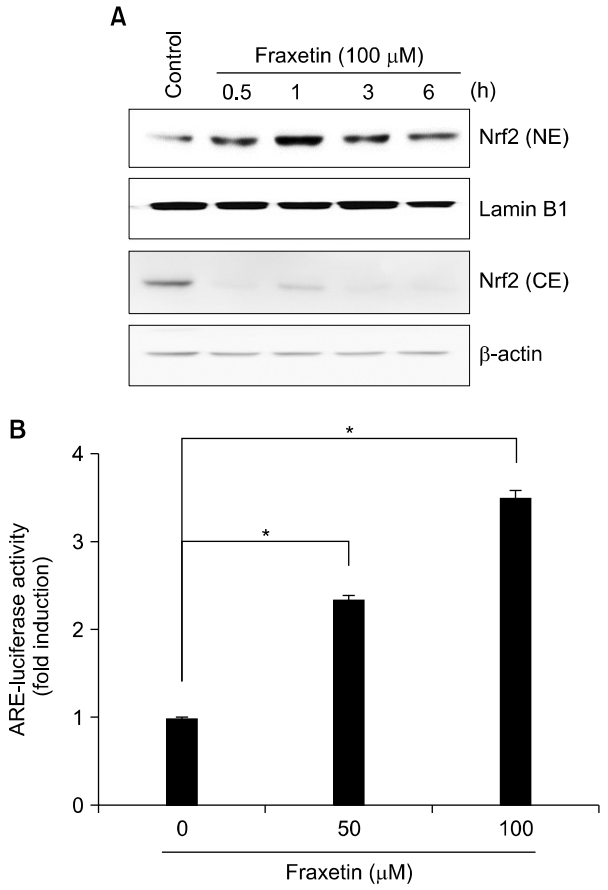


**Figure 2.** Effect of fraxetin on nuclear factor-erythroid-2-related factor-2 and antioxidant response element activation. (A) HaCaT cells were treated with fraxetin (100 μM) for indicated periods and nuclear and cytosolic levels of nuclear factor-erythroid-2-related factor-2 (Nrf2) were determined by Western blot analysis. (B) Cells transiently transfected with the antioxidant response element (ARE)-luciferase construct or control vector were treated with fraxetin (50 and 100 μM) for 6 hours and the ARE-luciferase activity was examined. The experiment was done in triplicate and the data are presented as mean ± SD. NE, nuclear extract; CE, cytosolic extract. *$P < 0.01$.

reporter gene activity. Treatment with fraxetin increased the ARE luciferase activity in a concentration-dependent manner (Fig. 2B).

### 3. Effect of fraxetin on phosphorylation of Akt and AMP-activated protein kinase α in HaCaT cells

Activation of several upstream kinases has been reported to induce Nrf2 and subsequently increase the expression of HO-1.[34,35] To explore the upstream signaling pathways involved in fraxetin-induced Nrf2 activation and HO-1 expression, we tested the effects of fraxetin on the activation of Akt and AMPKα in HaCaT cells. Treatment with fraxetin significantly increased the phosphorylation of Akt and AMPKα at 1 hour and 6 hours, respectively (Fig. 3A). Expression of PTEN, which is known as a negative regulator of Akt, was decreased at early time (0.5, 1, and 3 hours) and then returned to the basal level at 6 hours in fraxetin-treated HaCaT cells (Fig. 3A). Next, we incubated HaCaT cells with LY294002 (a pharmacological inhibitor of Akt) and Compound C (an inhibitor of AMPKα) for 1 hour before treatment with fraxetin and investigated the effect of these inhibitors on expression of HO-1. The fraxetin-induced expression of HO-1 was reversed by LY294002 or Compound C pretreatment (Fig. 3B and 3C). In our previous study, we found that thymoquinone induces HO-1 expression by Nrf2 activation through phosphorylation of Akt and AMPKα in HaCaT cells and Akt plays the role of an upstream kinase to AMPKα.[24] Therefore, we investigated the possible cross regulation between fraxetin- induced phosphorylation of Akt and AMPKα. However, in fraxetin-treated HaCaT cells, the pharmacological inhibition of Akt did not affect fraxetin-induced phosphorylation of AMPKα, suggesting Akt is not involved in fraxetin-induced AMPKα activation (Fig. 3B).

### 4. Role of Akt and AMP-activated protein kinase α in fraxetin-induced nuclear factor-erythroid-2-related factor-2 activation in HaCaT cells

We then investigated the role of Akt or AMPKα in fraxetin-induced Nrf2 activation. As illustrated in Figure 4, pharmacological inhibition of Akt and AMPKα attenuated fraxetin-induced nuclear localization of Nrf2, respectively. These results suggest that fraxetin induces HO-1 via activation of Akt/Nrf2 or AMPKα/Nrf2 pathway.

### 5. Reactive oxygen species generation by fraxetin in HaCaT cells

Cytoprotective proteins are induced as an adaptive response to mild oxidative stress as a safe guard against excessive ROS-induced cellular damage.[4] Thus, a wide variety of natural

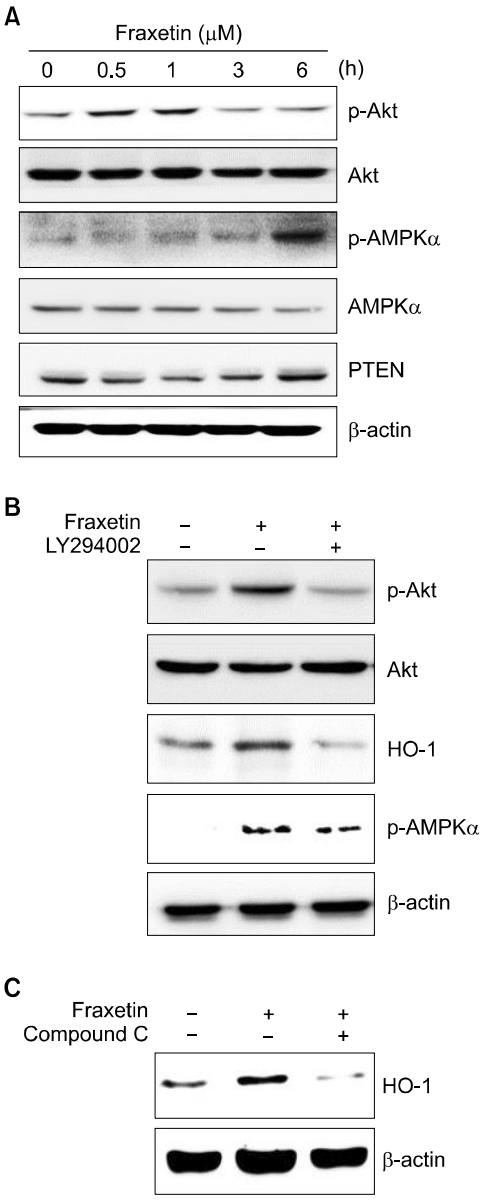


**Figure 3.** Effect of fraxetin on the activation of Akt and AMP-activated protein kinase α and expression of PTEN in HaCaT cells. (A) HaCaT cells were treated with fraxetin (100 μM) for indicated periods. Cells were pretreated with (B) Akt inhibitor LY294002 (10 μM) and (C) AMP-activated protein kinase (AMPK)α inhibitor compound C (10 μM) for 1 hour prior to incubation with fraxetin (100 μM) for 1 hour. Levels of Akt and heme oxygenase-1 (HO-1) were examined by Western blot analysis. PTEN, phosphatase and tensin homolog.

products capable of activating Nrf2 are shown to function as pro-oxidants capable of generating ROS. We examined whether fraxetin can generate ROS as a mechanism underlying its effect on Nrf2 activation and HO-1 expression. Treatment with fraxetin for 1 hour concentration-dependently increased ROS generation

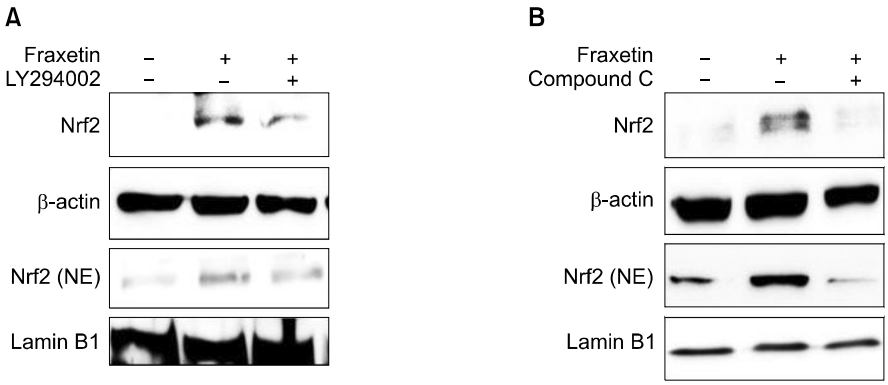


**Figure 4.** Roles of Akt and AMP-activated protein kinase α in fraxetin-induced nuclear factor-erythroid-2-related factor-2 activation and heme oxygenase-1 expression in HaCaT cells. Cells were pretreated with LY294002 (10 μM) (A) and compound C (10 μM) (B) for 1 hour prior to incubation with fraxetin (100 μM) for 1 hour. Expression of nuclear factor-erythroid-2-related factor-2 (Nrf2) was examined by Western blot analysis. Data are representative of three different experiments. NE, nuclear extract.

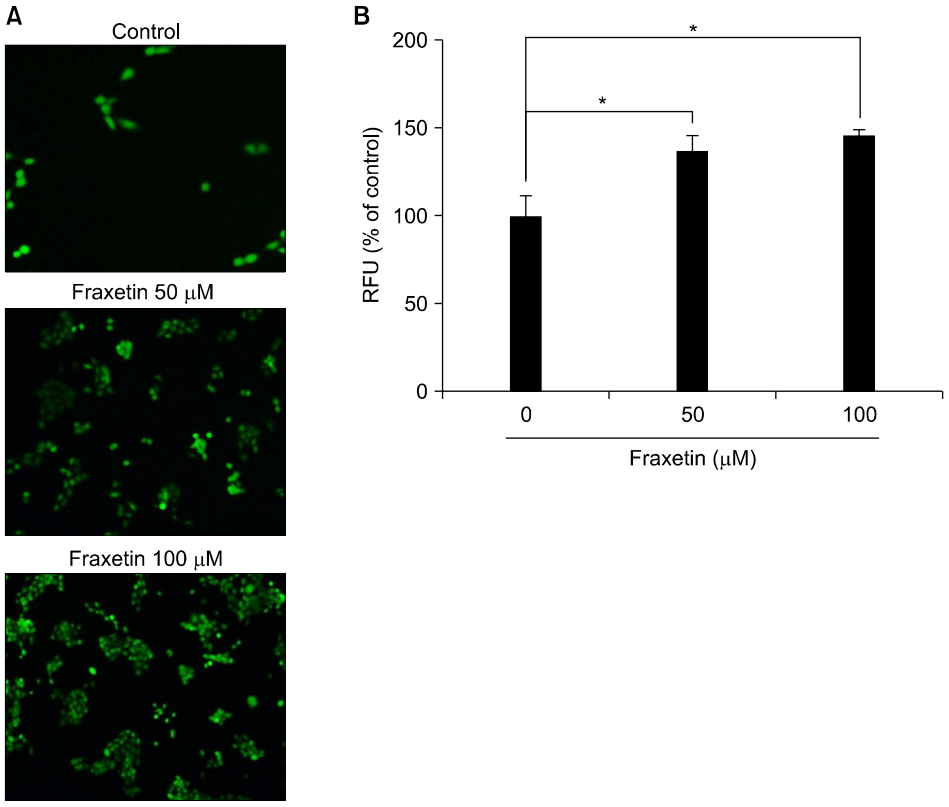


**Figure 5.** Effect of fraxetin on generation of reactive oxygen species in HaCaT cells. Cells were treated with indicated concentration of fraxetin for 1 hour and then examined for the intracellular accumulation of reactive oxygen species (ROS) (A) under the fluorescence microscope using 2'-,7'-dichlorofluorescin diacetate fluorescence staining method (× 200) and (B) ELISA plate reader. The experiment was done in triplicate and the data are presented as mean ± SD. RFU, relative fluorescence unit. $*P < 0.01$.

in HaCaT cells (Fig. 5). This finding suggests that fraxetin may function as a pro-oxidant that elicits an adaptive response to activate Nrf2 signaling and subsequent HO-1 expression in HaCaT cells.

## DISCUSSION

The incidence of skin cancer is increasing worldwide. Exposure to solar UV radiation is the major etiologic factors for skin cancer. The generation of excessive ROS by UV induces oxidative modifications of cellular macromolecules, such as nucleic acids, proteins and lipids.[36] ROS is eliminated by phase II detoxifying and antioxidant enzymes, such as HO-1, glutathione-S-transferase and NAD(P)H:quinone oxidoructase before they attack cellular macromolecules.[37] Therefore, natural products capable of inducing cytoprotective proteins have been nominated

as potential candidates for the chemoprevention of skin carcinogenesis.[4]

Fraxetin is an O-methylated coumarin isolated from *F. rhynchophylla*. Fraxetin has been reported to exert antioxidative,[28-30] anti-inflammatory,[31] neuroprotective[32] and antitumor[33] effects. For examples, fraxetin inhibits 5-lipoxygenase activity in polymorphonuclear leukocytes[31] and also inhibits tumor growth and metastasis to the lung or liver in highly metastatic osteosarcoma LM8 cell-bearing mice.[33] Furthermore, neuroprotective effect of fraxetin on rotenone-induced cytotoxicity also has been well demonstrated.[38,39] However, any skin protective effect of fraxetin has not been investigated yet. Therefore, in this study, we examined the skin cytoprotective effect of fraxetin.

HO-1 is a stress response protein that catalyzes the degradation of heme to iron, billiverdin and carbon monoxide. Expression of HO-1 is highly induced by a wide variety of oxidants in many types of cells.[40] The induction of HO-1 expression has been regarded as a crucial marker for oxidative stress, and it is involved in an adaptive protective response against oxidative damage.[40] A recent study by Was et al.[8] demonstrated the protective role of HO-1 in mouse skin carcinogenesis. According to this study, HO-1 knock out mice are more susceptible to develop skin papillomas as compared with wild type animals.[8] Shin et al.[9] showed that overexpression of HO-1 remarkably decreases TPA-induced ROS production and transformation of JB6 cells. These data suggest the possible protective role of HO-1 in skin tumorigenesis. HO-1 expression is induced at the transcription level through the ARE located in the promoter region of *Hmox1* which includes multiple copies of ARE sequences necessary for gene transcription by various inducers.[41] These ARE sequences are binding sites for the Nrf2. Thus, Nrf2 acts as the major switch in transcriptional activation of *ho-1* gene.[11] Recent studies demonstrated that Nrf2 plays a pivotal role in the protection of the skin against UV irradiation in mouse fibroblasts,[42] human skin fibroblasts[43] and ketatinocytes.[44] Therefore, Nrf2/ARE pathway activation protects the skin from UV-induced damage and has been suggested as an important therapeutic strategy for skin cancer. In this study, we demonstrated that fraxetin induces HO-1 expression and modulates Nrf2 signaling in HaCaT cells. This finding is in good agreement with the previous study which has demonstrated that fraxetin induces expression of HO-1 by activation of Nrf2 in vascular smooth muscle cells.[25]

In resting cells, Nrf2 resides in the cytoplasm by forming an inactive complex with its inhibitory protein Keap-1, which causes proteasomal degradation of Nrf2. Under mild oxidative condition, Nrf2 is liberated from Keap1 through oxidative or covalent modification of critical cysteine residues of Keap1.[13,16] On the other hand, Nrf2 can be dissociated from Keap1 upon phosphorylation at specific serine or threonine residues of Nrf2 by multiple upstream kinases, such as PI3K/Akt,[19,45] and AMPK[20] and MAP kinases.[21] In our previous study, we found that thymoquinone, an active constituent of black cumin, induces HO-1 expression by activating Nrf2 through phosphorylation of AMPKα and Akt, which plays the role of an upstream kinase to AMPKα in HaCaT cells.[24] In fraxetin-treated HaCaT cells, the compound also induced Nrf2 activation by phosphorylation of both Akt and AMPKα. However, our study revealed that Akt is not involved in fraxetin-induced AMPKα activation. The reasons for these differences are unclear. Further studies are needed to investigate the possible crosstalk of these pathways in Nrf2/ARE activation by various phytochemicals.

Since HO-1 can be induced by various oxidative stresses, we speculated that ROS may be involved in fraxetin-induced HO-1 expression in HaCaT cells. As we expected, fraxetin induced ROS generation in a concentration-dependent manner. In our previous study, ROS played a critical role in HO-1 expression in HaCaT cells.[24] These data suggest that the ability of fraxetin to induce HO-1 expression may be dependent on intracellular ROS production.

In conclusion, HaCaT cells incubated with fraxetin showed activation of Akt and AMPKα, which appears to be responsible for nuclear translocation of Nrf2 and its subsequent binding to ARE, thereby upregulating HO-1 expression.

## ACKNOWLEDGMENTS

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## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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