Mangiferin increases Nrf2 protein stability by inhibiting its ubiquitination and degradation in human HL60 myeloid leukemia cells

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Abstract. The nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant signaling pathway is a key target for cancer chemoprevention. Recent studies have that Nrf2 activation may be the result of an increase in Nrf2 protein stability. Mangiferin (MA), a compound monomer extracted from the mango plant, has antioxidant and cytoprotective activities. Our previous study demonstrated that MA increased Nrf2 expression and activated Nrf2 signaling in hematopoietic cells. Thus, in the present study, we aimed to investigate the mechanisms by which MA increases Nrf2 expression in human HL60 myeloid leukemia cells *in vitro*. Our western blot analysis results revealed that MA markedly increased Nrf2 expression in dose- and time-dependent manner. However treatment with MA did not affect the Nrf2 mRNA level. The results of cycloheximide (CHX)-chase analysis demonstrated that the Nrf2 protein half-life was prolonged to 58 min when the HL60 cells were pre-incubated with 50 μM MA for 4 h, whereas its half-life was only 20 min in the non-MA treated control cells. Further experiments revealed that MA mainly enhanced non-ubiquitinated Nrf2 protein levels when increasing Nrf2 protein stability; these effects differed from those induced by the proteasome inhibitor, MG132. Subsequent immunoprecipitation experiments confirmed that MA inhibited Nrf2 ubiquitination in HL60 cells. These results provide evidence that MA increases Nrf2 protein stability by inhibiting its ubiquitination and degradation in hematopoietic cells. This may be one of the mechanisms through which MA activates the Nrf2-mediated antioxidant response and exerts cytoprotective effects.

Introduction

The nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated signaling pathway is a major cellular defense pathway against oxidative or electrophilic stress (1). Nrf2, a cap 'n' collar (CNC) basic leucine zipper transcription factor, can bind to antioxidant response element (ARE) in the nucleus, subsequently initiating the expression of genes encoding antioxidant and phase II drug-metabolizing enzymes (2-4). These genes include glutathione S-transferase (GST) (5,6), NAD(P)H: quinine reductases (NQO1) (7,8) and heme oxygenase (HO-1) (9,10), which contribute to detoxification and elimination of reactive oxidants and electrophilic agents. It has been documented that certain synthetic or natural compounds can activate the Nrf2-mediated signaling pathway and provide protection against environmental or chemical insults (11-14). The knockout of Nrf2 in mice has been shown to reduce not only basal, but also the inducible expression of antioxidant and phase II drug-metabolizing enzymes (15,16). Silencing of the Nrf2 gene increases susceptibility to various oxidative stress-related pathologies, including chemical carcinogenesis, acetaminophen toxicity and hyperoxia (17-19). Therefore, Nrf2 has also been considered as a potential target for preventing inflammation-associated and chemical-induced carcinogenesis (20,21).

However, the regulatory mechanisms involved in Nrf2 activation are not yet fully understood. Although it is well established that Nrf2 activity is controlled, at least in part, by the cytosolic protein, Kelch-like ECH-associated protein 1 (Keap1), the mechanisms by which Keap1 acts to repress Nrf2 activity remain to be fully characterized. Previously, it was considered that most of the Nrf2 proteins were bound to Keap1 and sequestered in cytoplasm under homeostatic conditions (22,23). In response to oxidative stress or Nrf2 inducers/activators, the Nrf2 protein can dissociate from Keap1, transfer from the cytoplasm to the nucleus, bind to ARE and lead to the subsequent transcription of ARE-regulated genes (24,25). Thus, Nrf2 activation seems to be the result of its nuclear translocation. Nevertheless, a number of studies have suggested a different possible mechanism. Nrf2 was

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found to be primarily a nuclear protein. Under homeostatic conditions, Keap1 transiently shuttles the Nrf2 protein from the nucleus to the cytoplasm, prompts Nrf2 protein ubiquitination and degradation in the cytoplasm, maintains the intracellular Nrf2 protein at a basal level, thus repressing its activity (26). Oxidative stress or Nrf2 inducers/activators regulate the interaction between Nrf2 and Keap1, inhibiting Nrf2 protein degradation and increasing its stability, which leads to Nrf2 protein accumulation in stressed cells. Therefore, Nrf2 activation has been suggested to be dependent on increasing Nrf2 protein stability (1,14,27).

Mangiferin (MA), 2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone, is a compound monomer extracted from certain plants of the Anacardiaceae and Gentianaceae families, including *Mangifera indica* L. (mango), particularly in their leaves and bark (28). MA is widely used as a nutritional supplement, and as a cosmetic and phytomedicine in Southeast Asia and South America (29). This natural xanthone derivative has been reported to have various bioactivities, such as antioxidant, anti-tumor, anti-viral, anti-diabetic, anti-inflammatory, anti-allergic and immunomodulatory activities (28). Among these pharmacological activities, the antioxidant and cytoprotective properties of MA have been well elucidated. MA presents cardio-, hepato- and neuroprotective activities, as well as radioprotective activities against radiation (14,28,29).

Previous stuides have revealed that MA induces Nrf2-mediated antioxidant response, which provides an explanation for its antioxidant activity (29,30). It has been reported that MA enhances the expression of several detoxification and antioxidant enzymes, including NQO1, GST, HO-1, superoxide dismutase (SOD) and uridin 5'-diphosphate-glucuronosyl transferase (UDP-GT) (33-36). Furthermore, MA has been shown to increase Nrf2 expression in D-galactosamine intoxicated rat liver (29). Moreover, in our previous study, we found that MA activated Nrf2-ARE signaling in human HL60 myeloid leukemia cells (30). However, to the best of our knowledge, the mechanisms by which MA increases Nrf2 expression have not been documented to date.

In this study, we demonstrate that MA increases Nrf2 expression, but not transcription in human HL60 myeloid leukemia cells. We provide evidence that MA prolongs the half-life of the Nrf2 protein by inhibiting its ubiquitination and degradation.

Materials and methods

Reagents. MA ($C_{19}H_{18}O_{11}$; molecular weight, 422.34) and cycloheximide (CHX, C15H23NO4; molecular weight, 281.4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG132 ($C_{26}H_{42}N_3O_5$; molecular weight, 475.6) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). MA and MG132 were initially dissolved in dimethyl sulfoxide (DMSO), stored at -20°C and thawed on ice prior to use. CHX was dissolved in ultrapure water, stored at -80°C and thawed on ice prior to use. RNAiso plus, PrimeScript RT Master Mix and SYBR Premix Ex Taq were purchased from Takara Bio, Inc. (Otsu, Japan). The primers used for real-time PCR were designed and synthesized by Takara Bio, Inc.. Rabbit polyclonal antibody against human Nrf2 (C-20) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse

monoclonal antibody against human ubiquitin was obtained from Merck Millipore (Billerica, MA, USA). The BCA protein assay kit was from Pierce Biotechnology, Inc. (Rockford, IL, USA). The Image Lab enhanced chemiluminescence (ECL) detection system was from Bio-Rad (Hercules, CA, USA). Cell culture medium, RPMI-1640 and fetal abovine serum (FBS) were from HyClone (Logan, UT, USA).

Cell culture. The human HL60 myeloid leukemia cell line was kindly provided by Professor Jianfeng Zhou (Cancer Biology Research Center, Tongji Hospital, Wuhan, China), and then maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO_2 in air.

Real-time PCR. Total RNA was isolated using RNAiso Plus according to the manufacturer's instructions. Total RNA was isolated using RNAiso Plus, and then 400 ng total RNA were reverse transcribed into cDNA. Subsequently, 2 μl cDNA were amplified with SYBR-Green Universal PCR Master mix in triplicate on a real-time PCR system (CFX96, Bio-Rad). Nrf2 mRNA levels related to β-actin levels were calculated using the ΔCt (cycle threshold) method. The primer sequences for human Nrf2 were: forward, 5'-ACTCCGGCATTTCAC TAAACACAAG-3' and reverse, 5'-CTGAGGCCAAGTAGT GTGTCTCCA-3'. The primer sequences for human β-actin were: forward, 5'-GCCCAGTCCTCTCCCAAGTC-3' and reverse, 5'-GGCACGAAGGTCATCATTC-3'. Control cells were processed in an identical manner apart from MA treatment.

Western blot analysis. For immunoblotting, whole-cell lysates were prepared using lysis buffer for 30 min on ice. Supernatants were collected as samples following centrifuged at 15,000 rpm for 10 min at 4°C. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Inc.). Following denaturation, equal amounts of the protein extracts were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h at room temperature. It was subsequently incubated with anti-Nrf2 antibody (dilution 1:1,000) at 4°C overnight, followed by washing and treatment with HRP-labeled secondary antibody (Pierce Biotechnology, Inc.) for 2 h at room temperature. The blots were incubated with ECL reagent for 5 min, and the signals were then detected with a chemiluminescence detection system (Bio-Rad). After stripping, the membrane was re-probed with human anti-β-actin antibody as a control for equal protein loading and protein integrity.

CHX-chase analysis. Nrf2 protein degradation was analyzed by CHX-chase analysis. The HL60 cells were pre-incubated with or without 50 μM MA for 4 h. Subsequently, 100 μg/ml CHX were added to inhibit protein synthesis. The cells were collected at 0, 5, 15, 30, 45 and 60 min after following treatment with CHX, as previously described (31). Total protein (60 μg) was fractionated on SDS-PAGE and blotted with anti-Nrf2 and anti-β-actin antibodies. The results from western blot analysis were quantified by densitometry. Nrf2 protein half-lives were calculated from the slope of the semi-
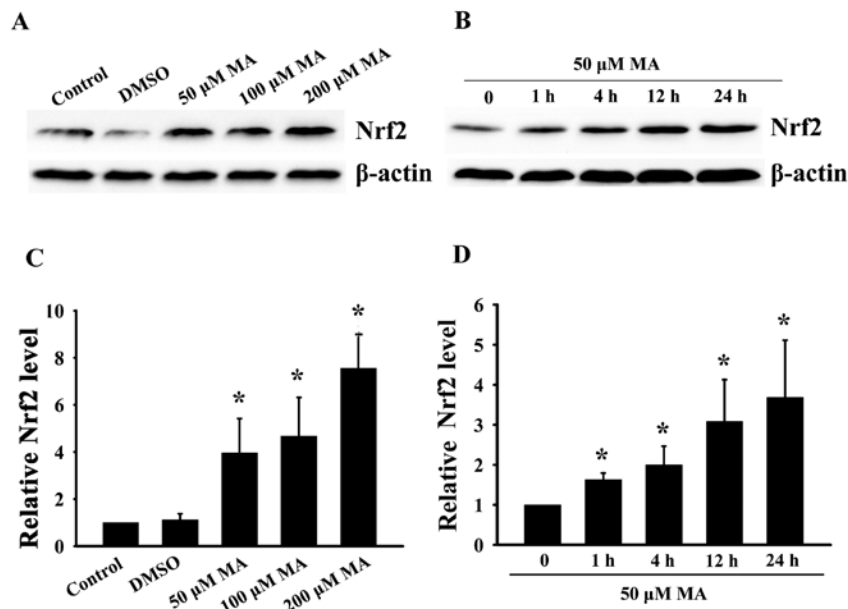
Figure 1. Mangiferin (MA) increases Nrf2 expression in HL60 cells. (A) Cells were treated with 0, 50, 100, 200 μM MA and DMSO for 24 h. (B) Cells were treated with 50 μM MA for 0, 1, 4, 12, 24 h. Total cell lysates were subjected to western blot analysis with anti-Nrf2 and anti-β-actin antibodies. β-actin was used as the control for equal protein loading and protein integrity. (C) The results in (A) were quantified by densitometry. (D) The results in (B) were quantified by densitometry. Nrf2 values were plotted after normalization to those of β-actin. Data represent the means ± SD of 3 independent experiments (*P<0.05, compared with the untreated control group).

logarithmically transformed best fit line. The decay curves were analyzed individually using linear regression of protein amount, and expressed as a percentage of protein remaining vs. time, as previously described (39).

Immunoprecipitation (IP). The HL60 cells were treated with 50 μM MA, 10 μM MG132, or a combination of 50 μM MA and 10 μM MG132 for 4 h. The cells were washed twice with ice-cold PBS. The cells were then prepared in lysis/IP buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate and 1 mM okadaic acid] on ice for 30 min, then centrifuged at 14,000 rpm for 15 min. Cell lysates were incubated with anti-Nrf2 antibody at 4°C for 16 h. The immune complexes were then precipitated with protein A-Sepharose beads at 4°C for an additional 2 h. Subsequently, the precipitates were washed extensively with IP buffer, fractionated by SDS-PAGE and immunoblotted with anti-ubiquitin antibody, as previously described (27). Control cells were processed in an identical manner apart from treatment with MA or/and MG132.

Statistical analysis. Data are expressed as the mean ± SD of at least 3 independent experiments and processed by SPSS 17.0 statistical software for Windows. One-way ANOVA and Student-Newman-Keuls tests were applied for comparisons between each group. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

MA markedly increases Nrf2 expression in HL60 cells. To determine the effects of MA on Nrf2 expression, the HL60 cells were treated with MA in dose-and time-course experiments. The Nrf2 protein levels in whole cell lysate were then detected by western blot analysis. As shown in Fig. 1, the HL60 cells were treated with 50, 100 or 200 μM MA for 24 h. These treatments resulted in a dose-dependent increase in the Nrf2 protein level. The Nrf2 protein level increased to 3.95-fold of the basal value following treatment with 50 μM MA, and increased to nearly 7.56-fold of the basal level following treating with 200 μM MA for 24 h. In the time-course experiments, Nrf2 expression also markedly increased in a time-dependent manner when the HL60 cells were treated with 50 μM MA for 1, 4, 12 or 24 h.

MA does not affect Nrf2 transcription in HL60 cells. To determine the effects of MA on Nrf2 transcription, the HL60 cells were treated with MA in dose-and time-course experiments as described above, and the Nrf2 mRNA levels were then detected by real-time PCR. As shown in Fig. 2, the Nrf2 mRNA levels did not differ significantly following treatment with MA, neither in a dose-dependent nor in a time-dependent manner.

MA prolongs the half-life of Nrf2 protein in HL60 cells. To determine the effects of MA on Nrf2 protein stability, the half-life of Nrf2 protein was calculated in the MA-treated and non-MA-treated HL60 cells. As shown in Fig. 3, the Nrf2 protein level decreased by ~50% within 20 min in the non-MA-treated HL60 cells. Its half-life was only 20 min. However, the Nrf2 protein level decreased by ~50% after 50 min in the MA-treated cells. Its half-life was significantly prolonged to 58 min. These data indicate that MA increases Nrf2 protein stability and prolongs its half-life.

MA increases Nrf2 stability by interfering with the ubiquitin-proteasome protein degradation pathway. To investigate the mechanisms by which MA increases Nrf2 protein stability, the HL60 cells were treated with 50 μM MA, 10 μM of the
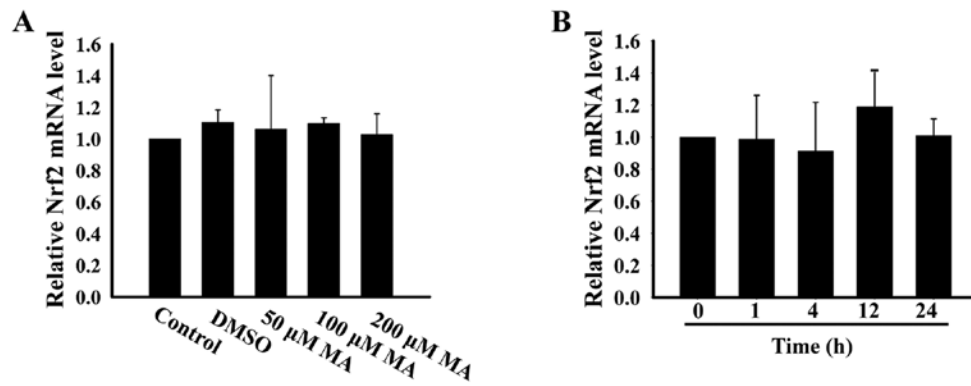
Figure 2. Mangiferin (MA) does not affect Nrf2 transcription in HL60 cells. (A) Cells were treated with 0, 50, 100, 200 μM MA and DMSO for 24 h. (B) Cells were treated with 50 μM MA for 0, 1, 4, 12, 24 h. Total RNA was extracted and reverse-transcribed into cDNA, then cDNA was subjected to real-time PCR for the detection of Nrf2 mRNA. Data represent the means ± SD of 3 independent experiments.
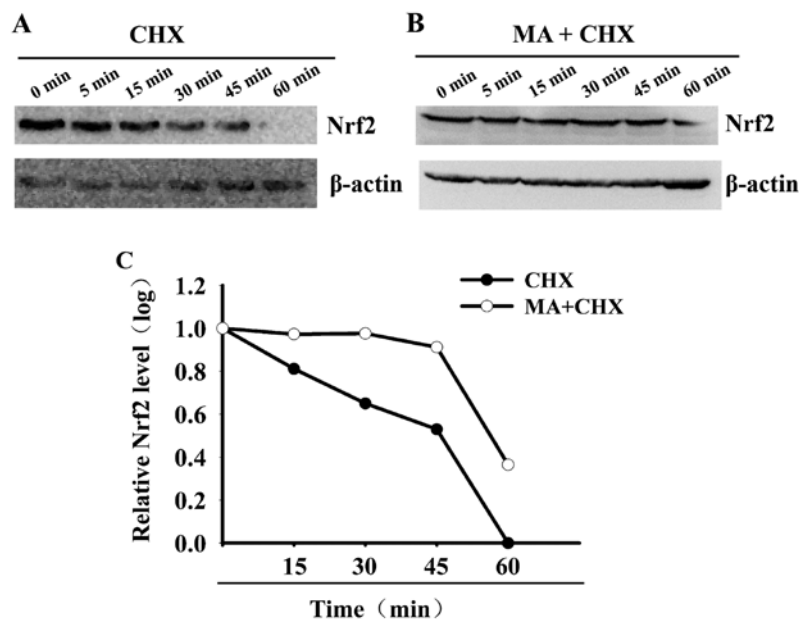

Figure 3. Mangiferin (MA) prolongs Nrf2 protein half-life in HL60 cells. (A) Cells were treated with 100 μg/ml cycloheximide (CHX) over a 1-h time period. (B) Cells were treated with 50 μM MA for 4 h followed by 100 μg/ml CHX over a 1 h time period. Cells were lysed at the indicated time points. Cell lysates were subjected to western blot analysis with anti-Nrf2 and anti-β-actin antibodies. β-actin was used as the control for equal protein loading and protein integrity. (C) The results in (A and B) were quantified by densitometry and plotted on a semi-log graph. The value of cells not treated with CHX was set as 1. Data represent the means ± SD of 3 independent experiments.

proteasome inhibitor, MG132, or a combination of 50 μM MA and 10 μM MG132 for 4 h. The cells were then prepared for the analysis of the Nrf2 level by western blot analysis. The molecular weights of Nrf2 and poly-ubiquitinated Nrf2 are 57 and 100 kDa, respectively, according to the Nrf2 antibody data sheet (Santa Cruz Biotechnology). As shown in Fig. 4, the Nrf2/ poly-ubiquitinated Nrf2 protein level significantly increased to 1.88-, 3.81- and 3.82-fold of that of the control cells following treatment with MA, MG132 or the combination treatment, respectively. The total Nrf2 level following treatment with MG132 alone did not differ significantly from that observed following combined treatment with MA and MG132. Of note, MA mainly increased the non-ubiquitinated Nrf2 protein level. However, MG132 enhanced both the non-ubiquitinated and poly-ubiquitinated Nrf2 protein levels. These results indi-

cate that MA increases Nrf2 protein stability by interfering with the ubiquitin-proteasome protein degradation pathway. However, the mechanisms involved differ from those of the proteasome inhibitor, MG132. MA inhibits ubiquitination, while MG132 suppresses proteasome activity.

MA inhibits Nrf2 ubiquitination in HL60 cells. To investigate whether MA suppresses Nrf2 ubiquitination, we performed IP experiments to pull down Nrf2 from the cell lysis of HL60 cells, and then identified ubiquitinated Nrf2 by immunoblot analysis with anti-ubiquitin monoclonal antibody. As shown in Fig. 5, the 10 kDa band represents ubiquitinated Nrf2; the levels of ubiquitinated Nrf2 decreased when the HL60 cells were treated with MA. On the contrary, these conjugated proteins significantly accumulated when the cells were incu-
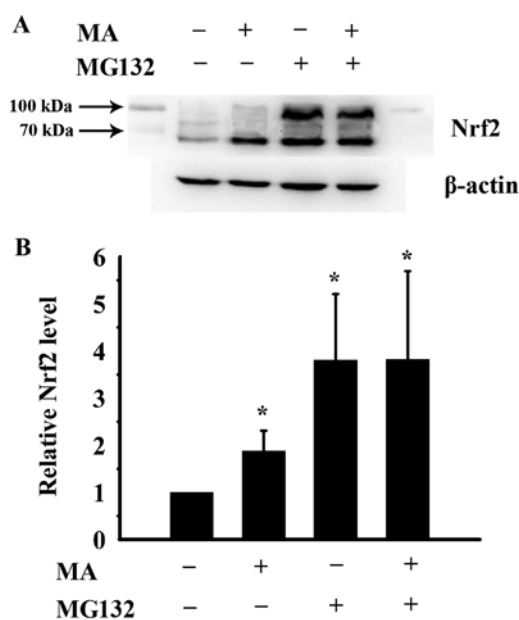
Figure 4. Mangiferin (MA) interferes with the ubiquitin-proteasome degradation of Nrf2 protein in HL60 cells. (A) Cells were treated with 50 μM MA, 10 μM MG132, or a combination of MA and MG132 for 4 h. Total cell lysates were subjected to western blot analysis with anti-Nrf2 and anti-β-actin antibodies. β-actin was used as the control for equal protein loading and protein integrity. (B) The results in (A) were quantified by densitometry. Nrf2 values were plotted after normalization to those of β-actin. Data represent the means ± SD of 3 independent experiments (*P<0.05, compared with the non-treated control group).
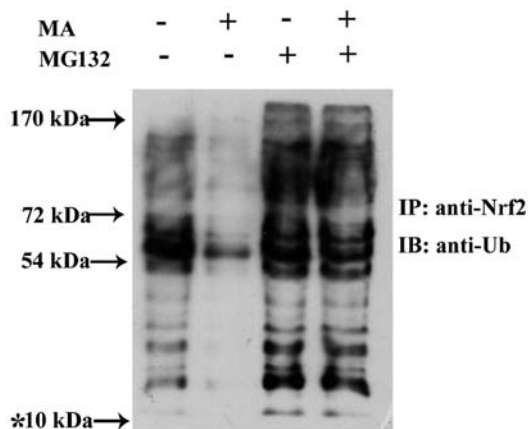

Figure 5. Mangiferin (MA) inhibits Nrf2 ubiquitination in HL60 cells. Cells were treated with 50 μM MA, 10 μM MG132, or a combination of MA and MG132 for 4 h. Total cell lysates were immunoprecipitated with anti-Nrf2 antibody, and then immunoblotted with anti-ubiquitin antibody. *Represented ubiquitinated Nrf2.

bated with the proteasome inhibitor, MG132. These results suggest that MA decreases Nrf2 degradation by inhibiting its ubiquitination.

Discussion

The present study demonstrates that the natural antioxidant, mangiferin (MA), increases Nrf2 expression through post-translational mechanisms in hematopoietic cells. MA enhances

the Nrf2 protein level, but does not affect its transcription. MA reduces protein degradation and prolongs the half-life of Nrf2 by inhibiting its ubiquitination, which leads to its intracellular accumulation. These results provide evidence that MA enhances Nrf2 expression by interfering with the ubiquitin-proteasome protein degradation pathway and increasing its protein stability. As mentioned above, MA activates Nrf2 and this activation may result from the increased protein stability and subsequent intracellular accumulation (1,27). Therefore, our data suggest that MA activates the Nrf2-mediated signaling pathway by increasing Nrf2 stability.

Previous studies have confirmed that Nrf2 is targeted for rapid degradation by the ubiquitin-proteasome pathway. Nrf2 is a highly unstable protein and its half-life is only 15-30 min in unstressed cells (32-36). In our study, the Nrf2 half-life was ~20 min in the HL60 cells and MA increased its half-life to 58 min, which suggests that MA increases Nrf2 protein stability. Under homeostatic conditions, Nrf2 binds to its repressor, Keap1. Keap1 brings Nrf2 to the Cul3-E3 ubiquitin ligase and targets the 26S proteasome for protein degradation (26). However, oxidative stress can antagonize the Keap1-Nrf2 interaction, increasing Nrf2 stability, leading to Nrf2 accumulation within cells (32,33,35). Therefore, an increase in Nrf2 stability can be a result of the interference with the Keap1-Nrf2 interaction, inhibiting Nrf2 ubiquitination or reducing 26S proteasome activity.

In the present study, MA markedly inhibited Nrf2 ubiquitination, which may explain the increase in Nrf2 stability. Certain natural or synthetic Nrf2 inducers/activators can also increase Nrf2 protein stability; however, the mechanisms involved are not completely similar to those of MA (11-14,43). Treatment with tert-butylhydroquinone (tBHQ) has been shown to prolong the half-life of Nrf2 protein in human neural stem cells. However, tBHQ increases ubiquitinated Nrf2, bu MA decreases ubiquitinated Nrf2 (14). This Nrf2 activator seems to increase Nrf2 protein stability by stabilizing ubiquitinated Nrf2, which differs from MA. Oridonin, a diterpenoid purified from the Chinese medicinal herb, *Rabdosia rubescens*, was found to suppress Nrf2 ubiquitination and enhance Keap1 ubiquitination in human MDAMB-231 breast carcinoma cells (12). Thus, oridonin may induce a shift in ubiquitination from the substrate, Nrf2, to the substrate adaptor, Keap1. Ajoene, a stable garlic by-product, has been shown to inhibit the Nrf2-Keap1 interaction and decrease Nrf2 ubiquitination in HepG2 cells (13). The latter two Nrf2 activators increase Nrf2 stability through at least partly similar mechanisms to those of MA. Another Nrf2 activator 1, 2-dithiole-3-thione (D3T) has also been shown to significantly reduce the degradation of Nrf2 protein in PC12 cells. Nevertheless, it was unexplored as to which step of the ubiquitin-proteasome degradation of Nrf2 protein was affected by D3T (11). Therefore, Nrf2 activators can increase Nrf2 protein stability by interfering with the ubiquitin-proteasome pathway; however, the specific mechanisms involved may differ. The mechanisms through which MA inhibits Nrf2 ubiquitinationt require further investigation.

As a newly identified Nrf2 activator, MA may be a potential cytoprotective agent for hematopoietic cells, as well as a chemopreventive agent against leukemia. It has been well documented that Nrf2 activators/inducers exert cytoprotective effects through antioxidant mechanisms (37-39). Our

previous study also demonstrated that MA relieved etoposide-induced DNA damage by activating Nrf2-mediated signaling and increasing NQO1 expression in mononuclear human umbilical cord blood (MNC hUCB) cells (Li S, et al, ASH Annual Meeting Abstracts 118: abs. 4626, 2011). This suggests that MA protects hematopoietic cells against injury induced by chemotherapy. Moreover, Nrf2 is a key target for chemoprevention against carcinogenesis (40-42). One major molecular mechanism is the induction of detoxification cytoprotective enzymes by Nrf2 activation (2). NQO1, a well-known detoxification and cytoprotective enzyme, is subject to a genetic polymorphism (C609T) leading to the impairment of intermediate NQO1 activity. It has been reported that the NQO1 C609T polymorphism significantly increases the risk of treatment-related leukemia and myelodysplastic leukemia (43,44). It has also been noted that oxidative stress is strongly associated with the relapse of acute myeloid leukemia and a poor prognosis (45,46). Therefore, there is a possibility that MA may protect hematopoietic cells from leukemia genesis and relapse by activating the Nrf2-mediated antioxidant response; this, however, requires further investigation.

In conclusion, our study confirms that MA inhibits Nrf2 ubiquitination and increases its stability. This may be one of the mechanims through which it induces Nrf2 cellular accumulation and activates Nrf2-mediated signaling. MA may be a potential cytoprotective agent for hematopoietic cells and a chemopreventive agent against leukemia, which warrants further study.

Acknowledgements

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