Microarray and pathway analysis highlight Nrf2/ARE-mediated expression profiling by polyphenolic myricetin

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**Scope:** Myricetin is a dietary flavonol and widely distributed in many edible plants. It has been reported to have many bioactivities and considered as a promising chemopreventive compound. The present study aimed to investigate the influences of myricetin on gene expressions in genome-wide and underlying mechanisms.

**Methods and results:** Among total 44K gene probes, myricetin treatment upregulated the signals of 143 gene probes (0.33% of total probes) and downregulated signals of 476 gene probes (1.08% of total probes) by greater than or equal to twofold in HepG2 cells. The network pathway analysis revealed that nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated antioxidant response element (ARE) activation is involved in myricetin-induced genes expressions. Molecular data revealed that myricetin activated Nrf2-ARE pathway by inhibiting Nrf2 ubiquitination and protein turnover, stimulating Nrf2 expression and kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 modification. All of these events finally increased nuclear Nrf2 accumulation and ARE-binding activity to enhance ARE-mediated genes expressions. Additionally, treatment with Nrf2 small interfering RNA attenuated the myricetin-induced ARE activity and gene expression.

**Conclusion:** An Nrf2-mediated ARE activation is involved in myricetin-induced expression profiling in hepatic cells.

**Keywords:** Keap1 modification / Myricetin / Microarray / Nrf2-ARE pathway / Nrf2 ubiquitination

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1 Introduction

Accumulating evidences have strongly indicated the beneficial effects of consumption of polyphenol-rich fruits and vegetables in prevention against cancer, cardiovascular as well as other chronic diseases, which are proven to be associated with their antioxidant activity, at least partly [1]. To understand the antioxidant mechanisms of polyphenolic compounds at cellular and molecular level, we chose myricetin as subject of research because myricetin is a typical polyphenol with hydroxyl substitutions at the 3,5,7,3',4', and 5' positions and widely distributed in many grapes, berries, fruits, vegetables, herbs as well as red wine with many bioactivities such as antioxidant, anti-inflammation, anticancer, antidiabetes, and antimutation [2, 3]. For example, myricetin could inhibit the growth of A549 cells through inhibition of thioredoxin reductase expression [4], neoplastic cell transformation by targeting MEK [5], and UVB-induced skin cancer by targeting Fyn [6]. Myricetin also induced apoptosis in human...
leukemia HL-60 cells via mitochondrial-dependent way [7], and blocked metastatic process by inhibiting MMP-2 expression [8] and multidrug resistance proteins (MRPs)-mediated resistance to anticancer drug vinricistine in transfected MDRKII cells [9]. Moreover, myricetin protected cells by repairing DNA damage [10, 11], reduced oxidative stress [12, 13], inhibited hyperglycemia and glucose uptake [14–16], modulated Ca\(^{2+}\) transport activity [17–19], and inhibited inflammation [20–22]. These data suggest that myricetin may influence expressions of many genes to exert its multiple bioactivities.

Oral administration of myricetin 3-rhamnoside in rat revealed that intestinal microorganisms can cleave glycosidic bonds into aglycone myricetin, which resulted in the urinary excretion of 3,5-dihydroxyphenylacetic acid [23] suggesting that myricetin would be metabolized in liver although there was no report on its detail metabolism. The bioavailability and bioactivity of quercetin, a similar compound to myricetin, are reported to be dependent on modulation of the drug-metabolizing enzymes of hepatocytes [24]. Drug-metabolizing enzymes primarily include phase I, phase II metabolizing enzymes and phase III transporters, which play central roles in the metabolism, elimination, and detoxification of xenobiotics and drugs [25]. In brief, phase I enzymes modify the structure of xenobiotics, then phase II enzymes increase the aqueous solubility and conjugate them with glutathione or glucuronate, and finally phase III enzymes either take them up from blood to hepatocytes (SLC family) or efflux them into bile and blood (ABC transporters). Examples of these enzymes include NQO1, GSR, HO-1, SRXN1, GCLM, AKR1C2, and TXNRD1 [26]. Several nuclear factors including the aryl hydrocarbon receptor [27, 28], orphan nuclear receptors [29, 30], and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) have been identified as mediators to regulate the gene expressions of phase I, II metabolizing enzymes and phase III transporters in drug-induced changes [27, 31].

Of which, Nrf2 is a key mediator to regulate expressions of drug-metabolizing enzyme genes since Nrf2 binds to the antioxidant-responsive element (ARE) with the consensus sequence 5′-TA/CANNA/GTGAC/TNNNGCA/G-3′ in promoter region of many drug-metabolizing enzyme genes [26, 32, 33]. Under unstimulated conditions, Nrf2 is sequestered in the cytoplasm, where it is associated with kelch-like ECH-associated protein 1 (Keap1), an actin-binding protein [34]. Keap1 is critical regulator although there are many factors modulating the activation of Nrf2-ARE [35, 36]. Nrf2 can be released from Keap1 to enter nuclear through Keap1 dissociation and Cul3-Rbx1 ubiquitination, Keap1 hinge and latch, and Keap1 ubiquitination, in which, Keap1 cysteine modification is a critical event [37]. Thus, Nrf2 is a labile protein. Stabilizing Nrf2 is considered to be important to maintain the cellular defense system, which is likely dependent on the status of the Nrf2–Keap1 complex. Accumulated data revealed that some phytochemical compounds could induce Nrf2-mediated ARE activation [38–40]. The effects and mechanisms of myricetin on Nrf2-mediated ARE activation are poorly understood.

To explore the influence of myricetin on gene expressions of hepatocytes in genome-wide, we first profiled gene expressions in myricetin-treated HepG2 cells by DNA microarray, and then performed network analysis of gene expressions by pathway analysis in the present study. The results suggested that an Nrf2-mediated ARE activation is involved in myricetin-induced expression profiling. Molecular data demonstrated that myricetin increased Nrf2 stabilization, Keap1 modification, Nrf2 nuclear accumulation, and ARE-binding activity to enhance ARE-mediated gene expressions.

2 Materials and methods

2.1 Materials, cell culture, and cytotoxicity

Myricetin purified by HPLC was obtained from Extrasynthese (Lyon Nord, Genay, France). 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 DMEM containing 10% fetal bovine serum. The antibodies against Nrf2 (C-20), Keap1 (E-20), HO-1, α-tubulin (B-7), rabbit IgG, and horseradish peroxidase-conjugated antigen secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated antirabbit and antimouse secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA).

2.2 Microarray, data analysis, and real-time PCR validation

HepG2 cells were precultured in dishes for 24 h and then treated with 20 μM of myricetin in 0.1% DMSO or 0.1% DMSO alone for 9 h. Total RNA extraction, amplification, labeling, hybridization, scanning, and real-time PCR were done as our previous study [40]. Briefly, Affymatrix Gene Chip Human U133 plus 2.0 Array containing over 44K oligonucleotides was used for microarray following manufacture’s protocol. Network pathways were analyzed by ingenuity pathway analysis (IPA) System (http://www.ingenuity.com). Gene accession numbers, the fold change upon myricetin treatment versus the control cells, and the t-test (p-value) were imported into the IPA software. IPA was carried out with p < 0.002 as the cut-off point. The genes were categorized according to the molecular functions using the software. Reverse transcription and real-time PCR were performed with DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit (Finzymes Oy., Espoo, Finland) according to the manufacturer’s manual. Briefly, RNA (200 ng) was reverse-transcribed to cDNA using Oligo-dT and M-MuLV RNase at 37°C for 30 min, and the reaction was then terminated at 85°C for 5 min. The
sequences of PCR primers and other reaction conditions used in the present study were described in detail in our previous study [40]. The result was represented by the relative expression level normalized with control cells.

2.3 Luciferase reporter gene assay
The pGL2-hQR41 luciferase reporter plasmid containing human ARE, and the expression plasmid pcDNA3-rNrf2 were described previously [38]. In brief, HepG2 cells were plated into each well of 12-well plate at the concentration of $1 \times 10^5$ and precultured for 24 h in DMEM plus 10% fetal bovine serum. The cells were then cotransfected with 0.1 μg of ARE promoter-encoding firefly luciferase plasmid and 0.1 μg of pGL4-TK-encoding renilla luciferase plasmid (Promega, Madison, WI, USA) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). For over-expression of Nrf2, HepG2 cells were cotransfected with 0.1 μg of pcDNA3-rNrf2. 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. After 24-h incubation, the cells were treated with 10–40 μM of myricetin in 0.1% DMSO, or 0.1% DMSO alone as a control, and further incubated for 24 h. The activities of firefly and renilla luciferase were measured in ARVO™SX multilabel counter (Perkin Elmer, MA, USA) with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Luciferase activity values were normalized to transfection efficiency monitored by renilla expression, and ARE transcription activity was expressed as fold induction relative to the control cells.

2.4 Cell fractionation
Nuclear and cytosolic proteins were prepared according to the modified method as described previously [39]. In brief, cells were cultured on 100-mm dishes to 90% confluence and treated with myricetin for 9 h. After being 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 DTT, 0.5 mM PMSF) on ice for 20 min and then centrifuged at 14 000 × $g$ for 15 min at 4°C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were combined with biotin-labeled DNA probes (5-′TTTTATGCTGTGTCATGGTT-3′) which was immediately cross-linked for 10 min with the membrane face down on a transilluminator equipped with 312 nm bulbs. The biotin-labeled complexes were detected by chemiluminescence (TAITEC, Saitama, Japan).

2.5 EMSA
In vitro protein–DNA interaction was examined using the Gelshift™ Chemiluminescent EMSA kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, ARE-specific DNA probes (5-′TTTTATGCTGTGTCATGGTT-3′) were synthesized, and then labeled with 5′-biotin after gel purification. Unlabeled ARE-specific probes were used as competitor. Nuclear extract (4 μg) was combined with biotin-labeled DNA probes (20 fmol) in 20 μL binding buffer (50 ng/μL poly (dl-dC), 2.5% glycerol, 5 mM MgCl$_2$, 0.05% NP-40) at room temperature for 20 min. The molar excess of unlabelled ARE-specific probe (4 pmol) was added to the binding reaction in competition experiments. Products of the binding reaction were run in 6% polyacrylamide gel with 0.5 × TBE buffer, and then transferred to a nylon membrane, which was immediately cross-linked for 10 min with the membrane face down on a transilluminator equipped with 312 nm bulbs. The biotin-labeled complexes were detected by chemiluminescence (TAITEC, Saitama, Japan).

2.6 Immunoprecipitation and Western blotting
HepG2 ($3 \times 10^6$) cells were precultured in 100-mm dish for 24 h, and treated with myricetin (40 μM) for additional 9 h. After that, 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 PMSF, 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 14 000 × $g$ for 15 min at 4°C. The supernatants were collected and protein concentration was determined by protein assay kit (Bio-Rad Laboratories). For immunoprecipitation, whole-cell lysates containing 0.5 mg of proteins were precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1 h and incubated with 1 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 20 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 horseradish peroxidase (HRP)-conjugated secondary antibody. Bound antibodies were detected using the enhanced chemiluminescence (ECL) system and the relative...
amounts of proteins associated with specific antibody were quantified using Lumi Vision Imager software (TAITEC Co.).

2.7 Transfection of small interfering RNA (siRNA)

Predesigned siRNA against human Nrf2 (Catalog No. 115762) and control scrambled siRNA (Catalog No. 4611) were purchased from Ambion (Austin, TX, USA). HepG2 cells were plated at a density of $7 \times 10^5$ cells per 60-mm dish. Cells were transfected with 100 nM siRNA against Nrf2 or 50 nM scrambled duplex by using LipofectAMINE 2000 (Invitrogen). After 24-h incubation, fresh medium was added and the cells were cultured for another 48 h. The cells were then treated with 40 μM myricetin for an additional 6 h and lysed for Western blotting.

2.8 Statistical analysis

All the experimental data shown were repeated at least three times, unless otherwise indicated. Differences between treatments and the control were analyzed by the Student’s t-test. A statistical probability of $p < 0.05$ was considered significant.

3 Results

3.1 Gene expression profiling by myricetin

According to the results of our initial experiments, HepG2 cells were treated with or without 20 μM myricetin for 9 h. Under these conditions, myricetin did not show cytotoxicity in HepG2 cells but with increased Nrf2 protein (data not shown). Cellular mRNA was prepared and processed for hybridization to the human oligonucleotide DNA microarray. Comparing the hybridization signals from myricetin-treated mRNA with those of the control mRNA revealed that the expressions of 21 genes were changed by greater than or equal to fourfold, of which, expressions of 11 genes were upregulated while ten genes were downregulated. The expressions of 598 genes were changed between twofold and fourfold, of which, expressions of 132 genes were upregulated and 466 genes were downregulated. The expressions of 2878 genes were changed between 1.5-fold and twofold, of which, expressions of 961 genes were upregulated and 1917 genes were downregulated. Taken together, there were expressions of 3497 genes out of the total 44K genes (7.95%) showing fold changes above 1.5-fold (Supporting Information Table S1).

To know the influence of myricetin on hepatocytes and to predict its metabolism in liver, we investigated the gene expression changes of drug-metabolizing enzymes and transporters in myricetin-treated cells. As shown in Supporting Information Table S2, the gene expression changes of 76 drug-metabolizing enzymes and transporters were observed. Among them, 23 genes were associated with phase I, 31 genes associated with phase II, and 23 genes associated with phase III. The genes that were upregulated more than twofold included ALDH1A1, CYP1A1, CYP24A1, and HERPUD1 in phase I, GCLC, GCLM, AKR1C1/2, SQSTM1, CBR1, HO-1, and TXNRD1 in phase II, and SLC2A14 in phase III (Supporting Information Table S2).

3.2 Canonical pathway analysis by IPA

To know the molecular mechanism of gene expressions by myricetin, signaling pathway analysis was done by IPA system. As shown in Supporting Information Table S3, top ten canonical pathways were identified to have significant changes in gene expressions. Among them, Nrf2-mediated pathway was identified as the third category with the $p$-value of $2.69 \times 10^{-10}$ in the canonical pathways, and the expressions of ten genes in this pathway were upregulated above twofold out of the total 185 genes (the ratio = 0.054). Next, we listed out these genes with respective fold change as shown in Fig. 1A: chaperone and stress response proteins such as HERPUD1 and HSPA1A; phase I and II metabolizing enzymes such as AKR1C2, CBR1, CYP1A1, GCLC, and GCLM; antioxidant proteins such as HO-1, SQSTM1, and TXNRD1. To confirm the results, the expressions of genes such as AKR1C2, HO-1, TXNRD1, GCLM, and GCLC were further detected by real-time PCR. The results exhibited a similar expression pattern with that of the DNA microarray (Fig. 1B), suggesting the DNA microarray data obtained in the present study is valid. For example, the expression of GCLC increased 4.89-fold in real-time PCR, and 3.09-fold in DNA microarray, comparing with the control (Fig. 1B).

3.3 Myricetin stimulates Nrf2-mediated ARE activation

Signaling pathway analysis of DNA microarray data by IPA suggested that Nrf2-mediated ARE transcriptional activation is involved in myricetin-induced gene expression. To verify this, we performed reporter gene assay by transfecting an ARE-luciferase reporter plasmid into HepG2 cells. As shown in Fig. 2A, myricetin-induced ARE-driven activity in a dose-dependent manner. Moreover, myricetin enhanced an Nrf2-dependent ARE activation when Nrf2 expression plasmid was cotransfected with ARE-luciferase plasmid in HepG2 cells (Fig. 2B). To further confirm the results at protein level, we treated HepG2 cells with indicated concentrations of myricetin for 9 h and then detected the protein levels by Western blotting. As shown in Fig. 2C, myricetin treatment increased the protein level of Nrf2, HO-1, AKR1C2, but increased Keap1 level significantly. These results indicated that myricetin activated the Nrf2-ARE pathway by targeting Nrf2/Keap1 system.
3.4 Myricetin promotes Nrf2 nuclear translocation and ARE-binding ability

The nuclear accumulation and ARE-binding ability of Nrf2 are essential primary actions during Nrf2-mediated ARE activation [41, 42]. To further clarify the effects of myricetin on these actions, we first examined the localization of Nrf2 in the cells treated with or without myricetin for 9 h by Western blotting. As shown in Fig. 3A, myricetin increased the amount of nuclear Nrf2 in a dose-dependent manner. 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. Furthermore, we pretreated the cells with 26S proteasome inhibitor MG132, and found that a clear Nrf2 band was also detected in the cytoplasm of MG132-treated cells, and myricetin further enhanced the Nrf2 level (Fig. 3B). These data indicated that Nrf2 sequestered in the cytoplasm is rapidly degraded by ubiquitin-proteasome under homeostatic conditions.

To demonstrate whether Nrf2 accumulated in the nucleus by myricetin actually binds to the ARE, we examined the ARE-binding complexes by chemiluminescent EMSA. The result revealed that myricetin enhanced ARE-binding complex in a dose-dependent manner (Fig. 3C, lane 1–4), while cotreated with 4 pmol of unlabeled human ARE oligonucleotides markedly blocked the formation of this DNA–protein complex (Fig. 3C, lane 5). These results demonstrated that myricetin-induced Nrf2-mediated ARE activation by enhancing Nrf2 nuclear accumulation and the binding to the human ARE oligonucleotides.

3.5 Myricetin inhibits ubiquitination of Nrf2 and modifies Keap1

To further investigate the factors that affect Nrf2 nuclear accumulation, we examined the status of Nrf2 ubiquitination and Keap1 modification by myricetin. After treatment with 26S proteasome-specific inhibitor MG132 or myricetin,
Figure 2. Myricetin stimulates Nrf2-mediated ARE transcription activity in HepG2 cells. (A) Effects of myricetin on the transcriptional activity of ARE in HepG2 cells. HepG2 cells were cotransfected with the pGL2-ARE-firefly and pGL4-TK-renilla luciferase plasmids for normalization. After 5 h, cells were maintained in 10% serum medium for 20 h and then stimulated with 0–40 μM myricetin for an additional 24 h. Cells were lysed and analyzed for firefly and renilla luciferase activities. (B) Effects of myricetin on Nrf2-mediated ARE activity. HepG2 cells were cotransfected with 0.5 μg of pGL2-ARE-luciferase and 0.1 μg of the Nrf2 expression plasmid. Other steps are the same as shown in (A). (C) Myricetin induces the expressions of typical antioxidant proteins. HepG2 cells were treated with 0–40 μM myricetin for 9 h. Nrf2, Keap1, HO-1, AKR1C2, NQO1, and α-tubulin were detected by Western blot analysis with their respective antibodies. Each value represents the mean ± SD of three separate experiments, *p < 0.05; **p < 0.01 versus control, respectively.

cellular Nrf2 or Keap1 was immunoprecipitated with its antibody, and ubiquitinated Nrf2 or Keap1 was then detected by ubiquitin antibody, respectively. As shown in Fig. 4A, the Nrf2 protein level was enhanced after treatment with myricetin (lane 2) or MG132 alone (lane 3) or their combination (lane 4) while Keap1 protein level was reduced in such treatment. Simultaneously, a significant reduction of ubiquitination of Nrf2 (Fig. 4A), but not Keap1 (Fig. 4B), was observed in the cells cotreated with myricetin and MG132. These results indicate that the myricetin-enhanced Nrf2 is, partially at least, due to an inhibitory effect of myricetin on the ubiquitination of Nrf2 while myricetin-reduced Keap1 is not due to Keap1 ubiquitination. Recently, several studies revealed that some phytochemicals such as tBHQ [37], sulforaphane [43], quercetin [38], and 6-MSITC [39] induced formation of modified Keap1 protein, which is greater than 150 kDa. The formation of modified Keap1 protein caused a relative reduction to an approximately 70-kDa Keap1 band. Thus, we next examined whether myricetin modifies Keap1. As shown in Fig. 4C, a more than 150-kDa Keap1 protein was detected in the cells treated by 10–40 μM myricetin. Thus, myricetin-caused Keap1 reduction might be due to the formation of modified Keap1 protein, rather than 26S proteasome-dependent degradation.

3.6 Myricetin enhances Nrf2 transcription and reduces Nrf2 turnover

The significant induction of Nrf2 protein (Fig. 2C) by myricetin treatment suggest a possibility that myricetin may regulate Nrf2 expression at the transcriptional level. We used...
Figure 3. Myricetin promotes Nrf2 nuclear accumulation and ARE-binding ability. (A) Myricetin increases the amount of endogenous nuclear Nrf2 in a dose-dependent manner. HepG2 cells were treated with the indicated concentrations of myricetin for 9 h. Cell fractionation was done as described in Section 2. The nuclear and cytosolic extracts containing 30 μg of proteins were prepared and subjected to Western blot analysis with indicated antibodies. (B) Myricetin increases Nrf2 in both cytoplasm and nucleus. HepG2 cells were pretreated with 10 μM MG132 for 1 h and then treated with or without 40 μM myricetin for 9 h. (C) Myricetin enhances the ARE-binding activity in a dose-dependent manner. Chemiluminescent EMSA was carried out as described under Section 2 using biotin-labeled or not labeled doubled-stranded human ARE oligonucleotide and nuclear extracts from HepG2 cells treated with 0–40 μM for 9 h. Data represent means ± SD of three independent experiments. *p < 0.05; **p < 0.01 versus control, respectively.

actinomycin D, an inhibitor of transcription, to examine the expressions of Nrf2 and Keap1. As shown in Fig. 5A, treatment with myricetin significantly increased the protein level of Nrf2, but not Keap1 (lane 3). Cotreatment with actinomycin D and myricetin significantly reduced myricetin-enhanced Nrf2 level (lane 4). These results indicate that myricetin regulated Nrf2, but not Keap1, at the transcriptional level.

On the other hand, the significant increase of Nrf2 protein (Fig. 4A) by cotreatment with myricetin and MG132 suggests that myricetin may reduce proteasome turnover of Nrf2. We next examined the steady state of Keap1 and Nrf2 protein at different times after treatment with the protein synthesis inhibitor cycloheximide and 40 μM myricetin and then calculated the half-reduction time (t1/2) from protein decay experiments. Treatment with myricetin extended the half-life (t1/2) of Nrf2 protein from 17.8 to 63.7 min, almost four times longer (Fig. 5B), but the t1/2 of Keap1 protein has no significant change. The results indicate that myricetin increased Nrf2 protein also by inhibiting the turnover of Nrf2 at posttranscriptional levels.

3.7 siRNA of Nrf2 interrupts myricetin-induced ARE activation

To confirm whether Nrf2 enhanced is essential for myricetin-induced Nrf2-mediated ARE activity, we transfected siRNA molecules against Nrf2 into HepG2 cells. As shown in Fig. 6A, siRNA of Nrf2 reduced the basal as well as myricetin-induced Nrf2 and HO-1 proteins, and scrambled siRNA showed no such effect, compared with control. To further determine whether the increased Nrf2 stimulates Nrf2-mediated ARE activation, ARE-luciferase reporter plasmid was cotransfected with or without siRNA against Nrf2. As shown in Fig. 6B, treatment with siRNA of Nrf2 reduced the basal as well as myricetin-induced ARE activity while scrambled siRNA showed no effect on the activity, compared with control. These results demonstrate that an increase in Nrf2 is critical event for myricetin-induced ARE activity.

4 Discussion

Although several lines of studies have showed that myricetin, as a typical polyphenol, has a number of biological activities [2, 4], the effect of myricetin on drug-metabolizing enzymes especially the Nrf2-mediated ARE pathway is still not clear. In the present study, we investigated gene expressions and regulation in myricetin-treated HepG2 cells by DNA microarray and pathway analysis, accompanied with molecular data.

The status of Nrf2/Keap1 determines Nrf2-mediated ARE activity [38, 39]. In the present study, we found that myricetin can affect status of Nrf2/Keap1 through multiple pathways. First, treatment with myricetin reduced the steady-state level of Keap1 (Fig. 2C), alternatively, a modified Keap1 was observed in such treatment (Fig. 4C). This result suggests that myricetin might downregulate the steady-state level of Keap1 by modifying Keap1 protein to allow Nrf2 escaping from ubiquitination (Fig. 4A). Similar actions were also observed in other antioxidant agents such as tBHQ [37], sulforaphane [43], quercetin [38], and 6-MTITC [39]. Second, the inhibition on Nrf2 ubiquitination was observed in the cotreatment with myricetin and MG132 after immunoprecipitation with anti-Nrf2 antibody (Fig. 4B), and the t1/2 reduction time of Nrf2 protein was extended from 17.8 to 63.7 min in protein decay.

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Figure 4. Myricetin inhibits Nrf2 ubiquitination and modifies Keap1. (A) Western blot analysis of endogenous Nrf2, Keap1, and ubiquitinated Nrf2. HepG2 cells were pretreated with 10 μM MG132 for 1 h and then treated with or without 40 μM myricetin for 9 h. One part of the whole-cell lysates was analyzed by Western blot analysis with the indicated antibodies, the rest for immunoprecipitation. Equivalent amounts of proteins were immunoprecipitated with anti-Nrf2 antibody and visualized by Western blot analysis with ubiquitin antibody. Each arrow shows reduced ubiquitinated protein. *p < 0.05. (B) Effects of myricetin on ubiquitination of Keap1. Equivalent amounts of proteins were immunoprecipitated with anti-Keap1 antibody and visualized by Western blot analysis with ubiquitin antibody. (C) Modification of Keap1 by myricetin. HepG2 cells were treated with 0–40 μM myricetin for 9 h and analyzed by Western blot analysis with Keap1 antibody. Data represent means ± SD of three independent experiments. *p < 0.05 versus control.

experiment (Fig. 5B). These data suggest that myricetin increased the steady-state level of Nrf2 also by stabilizing Nrf2 protein through inhibition of Nrf2 ubiquitination and protein turnover at the posttranscriptional level. Third, pretreatment with actinomycin D suppressed myricetin-induced Nrf2 expression, suggesting that myricetin-induced Nrf2 expression was at transcriptional levels. These actions caused by myricetin finally resulted in a high ratio of Nrf2/Keap1, the surplus Nrf2, compared with Keap1, might bypass Keap1-Cul3 and accumulate in the nucleus to mediate ARE activation.

In vitro data have indicated that the antioxidant property of a flavonoid is determined by its chemical structure, such as the number, positions and types of substitutions on the basic flavan nucleus, which influences radical scavenging and chelating activity [44]. Multiple hydroxyl groups seem to confer upon the flavonoid substantial antioxidant properties through their reducing capacities or other possible influences on intracellular redox status [45]. Especially, the di-OH substitution at 3′ and 4′ in ring B is particularly important and essential to the peroxyl radical absorbing activity of a flavonoid, which as indicated by the higher potency of luteolin (with two hydroxyls at 3′ and 4′ in ring B) than that of apigenin (with one hydroxyl at 4′ in ring B) and chrysin (without any hydroxyl in ring B) [46,47]. However, the above hydrogen-donating antioxidant activity is unlikely to be the sole explanation for Nrf2 activation in cell model. For example, a recent study indicated that chrysin, apigenin as well as luteolin could activate Nrf2 with the same concentration and treatment time in the order of chrysin > luteolin > apigenin [48]. Several lines of studies have revealed that different types of flavonoid have ability to induce Nrf2 activation, such as quercetin in flavonol [38], cyanidin in anthocyanidin [49], genistein in isoflavone [50], chrysin in flavone [48], and epicatechin in flavanol [51]. Based on these reports with our data, the basic flavan structure seems to be the key component of flavonoid to activate Nrf2, and flavonoids with basic flavan structure might exert their chemopreventive effect through Nrf2 pathway, at least partly.

Microarray data showed that myricetin could significantly affect gene expressions of HepG2 cells with enhanced 1104 gene signals (2.51% of total genes), and reduced 2393 gene signals (5.44% of total) by greater than or equal to 1.5-fold (Supporting Information Table S1). These changes could be classified into ten canonical pathways (Supporting Information Table S3) by signal pathway analyses with IPA software. Nrf2-mediated pathway was listed up at the 3rd, suggesting that Nrf2-mediated ARE pathway (Fig. 1) was linked to the antioxidant property of myricetin [10–13]. We also noted that the metabolism of xenobiotics by cytochrome P450 was listed up as the first canonical pathway for myricetin-induced gene
Myricetin enhances Nrf2 at both transcriptional and posttranscriptional levels. (A) Actinomycin D suppresses Nrf2 protein expression. HepG2 cells were treated with 5 μg/mL actinomycin D for 1 h and then treated with 40 μM myricetin for 9 h. *p < 0.05 versus control, respectively. (B) Myricetin stabilizes Nrf2 protein. HepG2 cells were treated with 5 μg/mL CHX or pretreated with 40 μM quercetin for 2 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. Each value represents the mean ± SD of three or four separate experiments.

expression, and the expressions of CYP1A1 and CYP24A1 were upregulated by myricetin (Supporting Information Table S2). These data suggest that myricetin also acted as a xenobiotic to cause expressions of drug-metabolizing enzyme genes. Other canonical pathway including bile acid biosynthesis, c21-steroid hormone metabolism, glycerophospholipid metabolism, glycerolipid metabolism, and IGF-1 signaling were also involved in myricetin-induced gene expressions, suggesting that myricetin may play important role in lipid metabolism and insulin resistance, which in keeping with the antidiabetic function of myricetin such as inhibition of hyperglycemia and glucose uptake [14–16]. Involvement of VDR/RXR pathway in myricetin-induced gene expressions

Figure 5. Myricetin enhances Nrf2 at both transcriptional and posttranscriptional levels. (A) Actinomycin D suppresses Nrf2 protein expression. HepG2 cells were treated with 5 μg/mL actinomycin D for 1 h and then treated with 40 μM myricetin for 9 h. *p < 0.05 versus control, respectively. (B) Myricetin stabilizes Nrf2 protein. HepG2 cells were treated with 5 μg/mL CHX or pretreated with 40 μM quercetin for 2 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. Each value represents the mean ± SD of three or four separate experiments.

Figure 6. siRNA of Nrf2 interrupts myricetin-induced ARE activation. (A) HepG2 cells were transfected with 100 nM siRNA of Nrf2 or 50 nM scrambled duplex. After transfection, cells were harvested as described under Section 2. Nrf2, HO-1, and α-tubulin were detected by Western blot analysis with their respective antibodies. (B) HepG2 cells were cotransfected with pGL2-ARE-luciferase construct, pGL4-TK-Renilla, and 100 nM siRNA of Nrf2 or 50 nM scrambled duplex. After 24 h, cells were placed in 5% serum medium for 24 h and then stimulated with 40 μM myricetin for an additional 24 h. Cells were lysed and analyzed for firefly and renilla luciferase activities. Each value represents the mean ± SD of three or four separate experiments. *p < 0.05 versus control, respectively.
may partially support the anticancer property [4–6] since VDR/RXR plays a crucial role in the regulation/metabolism of calcium and phosphorus involving in immune function, tumor suppression, growth regulation, and parathyroid hormone secretion [52].

It has been reported that myricetin acts on a variety of signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis, and metastasis [53]. Moreover, Nrf2-ARE signaling pathway has been reported to contribute to chemoprevention against many human chronic diseases by modulating expressions of genes including detoxifying enzymes, drug transporters, and cellular redox regulators [54–58], and many polyphenolic compounds are proven to enhance ARE activation [38, 40, 58, 59]. Myricetin is a typical polyphenolic compound, thus, is considered to modulate Nrf2-ARE signaling pathway to exert its chemopreventive effect although there is limited data regarding this. Our data from DNA microarray and signaling pathway analyses will be useful for future studies on chemopreventive effect of myricetin and underlying molecular mechanisms.

The absorption and metabolism of flavonoids play critical roles in maintenance of their bioactivities. Dietary flavonoid glycosides can be transformed to aglycones in gastrointestinal tract, and only the aglycones of flavonoids can be absorbed in intestinal with the form of passing through the gut wall and entering into the plasma [60]. Thus, we directly added the aglycone of myricetin into culture cells to mimic the in vivo situation of flavonoids. A recent study, that myricetin glycosides (myricetin-3-O-galactoside and myricetin-3-O-rhamnoside) were added into human chronic myelogenous leukemia cells, was carried to investigate the expression patterns of oxidative stress genes, using DNA microarray with only 21 probes [61]. Although the gene expressions of some antioxidant proteins such as TXNRD1, GPX1, TXN, and SOD1 were increased, the concentrations (∼66 µM/mL for myricetin for 72 h had no significant effect on HepG2 cell growth [62]. We also confirmed that treatment with 20 µM of myricetin for 48 h showed no cytotoxicity, but with significant induction effect of several antioxidant proteins (data not shown here). That is why we chose 20 µM of myricetin in our study.

It is also worth of noting that two bands were observed for Nrf2 (Fig. 4A) and Keap1 (Figs. 2C, 4A, and 5) in our Western blot results although the predicted molecular mass are ∼66 and ∼69 kDa, respectively. Although the exact reason still remained unknown, one previous study had identified the two bands of Nrf2 as phosphorylated forms with the aid of protein kinase CK2 [63]. Another recent paper provided solid evidence that the biologically relevant molecular weight of Nrf2 is ∼95–110 kDa, not the predicted ∼66 kDa based on its 2-kb ORF [64]. Moreover, our previous studies also found two bands of Keap1 [38–40], this may be caused by binding to other proteins that can induce higher order oligomeric forms of Keap1 [65].

In conclusion, DNA microarray allowed us to obtain gene expression profiling and pathway networks in HepG2 cell by myricetin. Molecular data demonstrated myricetin inhibited Nrf2 ubiquitination and protein turnover, and stimulated Keap1 modification. All of these events finally increased nuclear Nrf2 accumulation and ARE-binding activity to enhance Nrf2/ARE-mediated gene expressions. Our study provides a comprehensive data for understanding bioactivities and the molecular mechanisms of myricetin in hepatic metabolism and chemoprevention.

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5 References


