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Antioxidant and Anti-Inflammatory Potential of Hesperetin Metabolites Obtained from Hesperetin-Administered Rat Serum: An Ex Vivo Approach

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ABSTRACT: In recent years much attention has been focused on the pharmaceutical relevance of bioflavonoids, especially hesperidin and its aglycon hesperetin in terms of their antioxidant and anti-inflammatory actions. However, the bioactivity of their metabolites, the real molecules in vivo hesperetin glucuronides/sulfates produced after ingestion, has been poorly understood. Thus, the study using an ex vivo approach is aimed to compare the antioxidant and anti-inflammatory activities of hesperidin/hesperetin or hesperetin metabolites derived from hesperetin-administered rat serum. We found that hesperetin metabolites (2.5−20 μM) showed higher antioxidant activity against various oxidative systems, including superoxide anion scavenging, reducing power, and metal chelating effects, than that of hesperidin or hesperetin. The data also showed that pretreatment of hesperetin metabolites (1−10 μM) within the range of physiological concentrations, compared to hesperetin, significantly inhibited nitric oxide (NO) and prostaglandin E_2 (PGE_2) production, as evidenced by the inhibition of their precursors, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels without appreciable cytotoxicity on LPS-activated RAW264.7 macrophages or A7r5 smooth muscle cells. Concomitantly, hesperetin metabolites dose-dependently inhibited LPS-induced intracellular reactive oxygen species (ROS). Furthermore, hesperetin metabolites significantly downregulate LPS-induced nuclear factor-κB (NF-κB) activation followed by the suppression of inhibitor-κB (I-κB) degradation and phosphorylation of c-Jun N-terminal kinase1/2 (JNK1/2) and p38 MAPKs after challenge with LPS. Hesperetin metabolites ex vivo showed potent antioxidant and anti-inflammatory activity in comparison with hesperidin/hesperetin.

KEYWORDS: antioxidant, anti-inflammation, hesperetin metabolites, reactive oxygen species, nitric oxide synthase, cyclooxygenase-2

INTRODUCTION

In recent decades, there has been growing interest in the pharmacological intervention of bioflavonoids. Hesperidin (4′-methoxy-7-*O*-rutinosyl-3′,5-dihydroxyflavanone), a naturally occurring flavanone glycoside, is predominant in citrus fruits.[1] Dietary hesperidin was hydrolyzed by gut microflora to give hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone), the aglycon form of hesperidin.[2] It has been well documented that hesperidin and hesperetin showed a wide range of pharmacological effects including antioxidant,[3] anti-inflammatory,[4] neuroprotective,[3] anticancer,[4] and antiviral[5] activities.

During inflammation, activated macrophages and/or smooth muscle cells produce a vast amount of proinflammatory molecules including NO, PGE_2, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β).[6,7] Continual production of these molecules involves a variety of diseases, such as rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis, and cancer.[8] iNOS and COX-2 are responsible for the catalysis of NO and PGE_2 production, these mediators playing a pivotal role in further onset of inflammation.[9] NF-κB, a transcription factor, plays a fundamental role in inflammation. The expression of iNOS and COX-2 is directly coupled with upregulation of NF-κB.[6] In normal physiological conditions NF-κB is localized in the cytoplasm and tethered with its inhibitor protein called I-κB. Upon activation by a variety of external stimuli including bacterial lipopolysaccharide (LPS), I-κB phosphorylated and degraded via proteosomal degradation. This action further leads to release of NF-κB, which then translocates to the nucleus and binds to its promoter region (κB binding site), transcribing a number of genes including iNOS and COX-2.[6] The MAPKs such as p38, JNK1/2, and ERK1/2 are part of the inflammatory signal transduction pathways that also regulate iNOS and COX-2 expression in macrophage cells through the activation of NF-κB.[7,10] A number of lines evidenced that reactive oxygen species (ROS) participate in inflammation. LPS-induced ROS generation in various in vitro and in vivo systems was widely studied.[11] In biological systems, free radicals can be generated in the form of ROS, such as superoxide anion and hydroxyl radicals, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxynitrite. These ROS cause destructive and irreversible damage to cellular components, such as lipids, proteins, and DNA.[12]

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Investigation has showed that hesperidin and hesperetin possess antioxidant and anti-inflammatory activities and inhibit the expression of iNOS and COX-2 expression in macrophage cells.[13] However, a human study has demonstrated that the citrus flavanones from orange juice were mainly present in the form of hesperetin glucuronides and sulfoglucuronides.[14] We have characterized the serum of rats administered with hesperidin and hesperetin.[1] Notwithstanding various bioactivities of hesperidin and hesperetin having been reported,[3−5] limited information regarding the bioactivities of hesperetin metabolites, the real molecules in vivo based on pharmacokinetic studies, is available. As a consequence of this observation, hesperetin metabolites obtained from rat serum potentially inhibit LPS-induced oxidative stress and inflammation as evidenced by downregulation of ROS generation, NO and PGE_2 production, followed by the inhibition of iNOS and COX-2 expression in macrophage and/or smooth muscle cells. Results obtained from various antioxidative systems supports that hesperetin metabolites exert an antioxidative agent in terms of free and superoxide anion radical scavenging, reducing power, and metal chelating activity in vitro.

MATERIALS AND METHODS

Reagents. Hesperidin, hesperetin (purity 97%), β-glucuronidase (type B-1 from bovine liver), sulfatase (type H-1, from *Helix pomotia*), lipopolyssacharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2′,7′-dihydrofluorescein diacetate ($DCFH_2$-DA) were purchased from Sigma-Aldrich (St. Louis, MO). Cell-culture medium (RPMI-1640 and DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco/BRL Life Technologies Inc. (Grand Island, NY). Anti-COX-2, anti-iNOS, and I-κBα antibodies were purchased from Santa Cruz (Heidelberg, Germany). Phos-JNK, phos-p38, and phos-ERK1/2 antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA). All other chemicals were reagent grade or HPLC grade and supplied by either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Preparation and Characterization of Hesperetin Metabolites from Rat Serum. Hesperetin glucuronides/sulfates (hesperetin metabolites) were prepared from the serum of rats administered hesperetin, and the concentration of hesperetin glucuronides/sulfates was determined by HPLC after hydrolysis with sulfatase and glucuronidase as described in our previous paper.[1] Sprague−Dawley (SD) rats (450−500 g) were purchased from BioLASCO (Taipei, Taiwan) and maintained in the Animal Center of China Medical University with a 12/12 h light/dark cycle and free access to food and water. All animal experiments adhered to "The Guidebook for the Care and Use of Laboratory Animals" published by the Chinese Society of Animal Science, Taiwan. The animal protocol was approved by the Institutional Animal Care and Use Committee of China Medical University. Rats were fasted overnight, and 50 mg/kg of hesperetin was administered orally by gastric intubation twice daily for seven doses and blood collected at 15 min after the final dose of hesperetin administration. The collected serum was deproteinized with 4-fold methanol, which was centrifuged, and the methanolic solution was evaporated under vacuum. The residue was reconstituted with an appropriate volume of water to afford 10-fold serum concentration. For the determination of total hesperetin including parent form and its glucuronides/sulfates, serum sample (300 μL) was added with 200 μL of β-glucuronidase (β-glucuronidase 110.4 units/mL, sulfatase 4.2 units/mL in pH 5 buffer) and 90 μL of ascorbic acid (75 mg/mL). The vials were wrapped with aluminum foil. Incubation was conducted in a water bath shaker at 37 °C for 4 h. After incubation, each sample was treated as a calibrator prior to HPLC analysis. The concentration of hesperetin sulfates/glucuronides in serum was determined to be 55 μM, of which the final concentrations of hesperetin sulfates and hesperetin glucuronides were 44 and 11 μM, respectively.

Superoxide Anion Scavenging Activity. Measurement of the superoxide anion scavenging activity of hesperidin, hesperetin, and hesperetin metabolites was based on the method described by Robak and Gryglewski, with slight modification.[15] Superoxide radicals were generated in phenazine methosulfate (PMS)−nicotinamide adenine dinucleotide (NADH) systems by NADH oxidation and assayed by nitroblue tetrazolium (NBT) reduction. In this experiment, generated superoxide radicals were incubated with various concentrations of samples hesperidin (40−100 μM), hesperetin (40−100 μM), or hesperetin metabolites (2.5−20 μM). The reaction was started by adding 0.5 mL of PMS solution (120 μM) to the mixtures. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples using a spectrophotometer.

Reducing Power. The reducing power of hesperidin, hesperetin, and hesperetin metabolites was determined using the method proposed by Oyaizu.[16] Various concentrations of hesperidin, hesperetin, and hesperetin metabolites were mixed with phosphate buffer (pH 6.6) and potassium ferric cyanide [$K_3Fe(CN)_6$]. The mixture was then incubated at 50 °C for 20 min. A portion of trichloroacetic acid was added to the mixture, which was then centrifuged for 10 min at 1000*g*. The upper layer of solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL) for 10 min, and then the absorbance was measured at 700 nm in a spectrophotometer, with higher absorbance indicating greater reducing power.

Metal Chelating Activity. The chelation of ferrous ions by hesperidin, hesperetin, and hesperetin metabolites was estimated using the method of Dinis et al.[17] Briefly, 0.94 mL of various concentrations of hesperidin, hesperetin, or hesperetin metabolites was added to a solution which contained 0.02 mL of $FeCl_2$ (2 mM). The reaction was initiated by the addition of 0.04 mL of ferrozine (5 mM), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, the absorbance of the solution was measured spectrophotometrically at 562 nm.

DPPH Radical Scavenging Activity. The free radical scavenging activity (hydrogen donation) of hesperidin, hesperetin, and hesperetin metabolites was measured by DPPH assay.[18] Briefly, a solution of DPPH in methanol (200 μM) was prepared. Then, 1 mL of DPPH solution was added to 0.4 mL of hesperidin, hesperetin, or hesperetin metabolites at different doses. The mixture was then shaken vigorously and allowed to stand at room temperature for 30 min, with the absorbance measured at 517 nm in a spectrophotometer against blank samples.

Cell Culture and Sample Treatment. The murine RAW264.7 macrophages were cultured in RPMI-1640 containing 1% glutamine and 10% FBS. Rat aortic smooth muscle A7r5 cells were grown in DMEM medium supplemented with 10% FBS, 100 μg/mL penicillin, and 1 μg/mL streptomycin, grown at 37 °C with 5% CO_2 in humidified condition. Cells were pretreated with hesperetin or hesperetin metabolites for 30 min and then stimulated by 1 μg/mL of LPS for RAW 264.7 cells (4×10^5 cells/mL) or 0.1 μg/mL of LPS for A7r5 cells (2×10^5 cells/mL) in 0.5 to 18 h.

Determination of Cell Viability. Cell viability was monitored by the MTT assay. Briefly, cells were incubated with hesperetin or hesperetin metabolites (1−10 μM) for 30 min in a 24 well plate prior to the addition of LPS (18 h for RAW 264.7 cells or 8 h for A7r5 cells). The culture supernatant was removed, and 400 μL of 0.5 mg/mL MTT in PBS was added to each well and incubated 37 °C for 4 h. The MTT formazan crystals were dissolved in 400 μL of isopropanol, and the absorbance was measured at 540 nm (A_{540}). Cell viability (%) was calculated as (A_{540} of treated cells/A_{540} of untreated cells) × 100.

Measurement of ROS Generation. The production of intracellular ROS was detected by flow cytometry using $DCFH_2$-DA as described previously.[19] RAW264.7 and A7r5 cells (4×10^5 cells/mL)

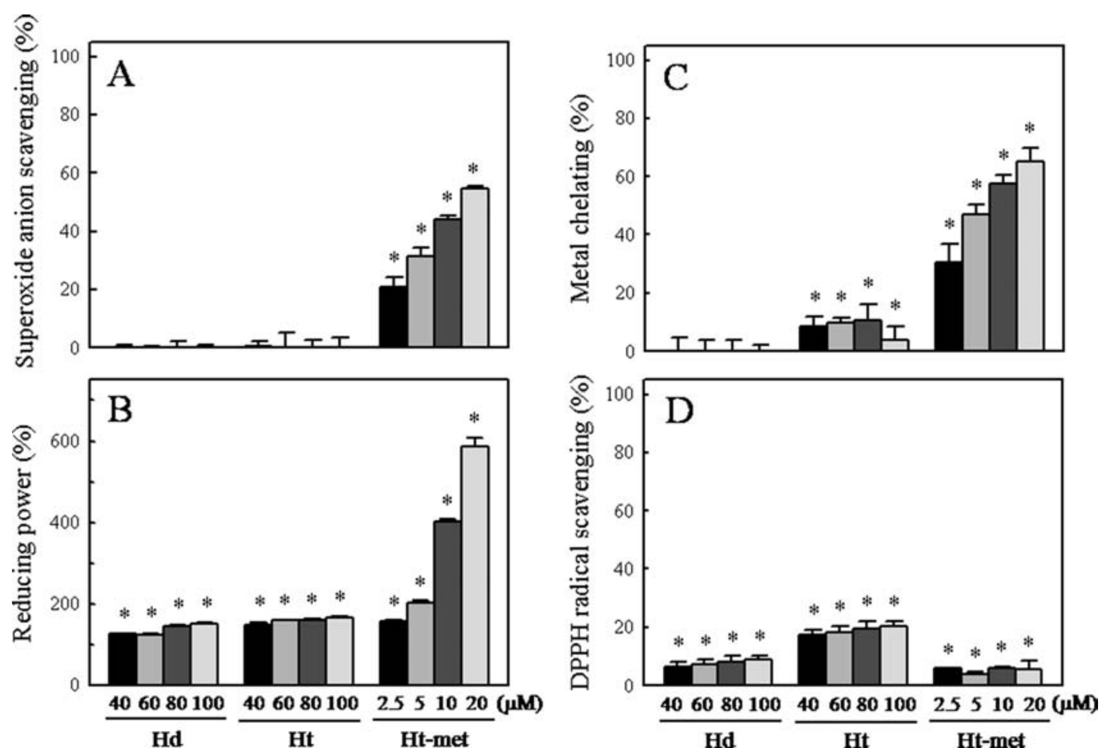

Figure 1. Antioxidant activity of hesperidin, hesperetin, and hesperetin metabolites (at the indicated concentrations) against various oxidative systems. (A) Superoxide anion radical scavenging activity of hesperidin, hesperetin, and hesperetin metabolites using the PMS-NADH-NBT method. (B) Reducing power of hesperidin, hesperetin, and hesperetin metabolites from spectrophotometric detection of the $Fe^{3+}-Fe^{2+}$ transformation; higher absorbance indicates greater reducing power. (C) The metal chelating effect of hesperidin, hesperetin, and hesperetin metabolites on ferrous ions was used. (D) Free radical scavenging activity of hesperidin, hesperetin, and hesperetin metabolites on DPPH radicals. Values are expressed as mean ± SD (n = 3−6 experiments). * indicates significant difference in comparison to control group ($p < 0.05$). Hd, hesperidin; Ht, hesperetin; Ht-met, hesperetin metabolites.

were incubated with hesperetin or hesperetin metabolites (1−10 μM) for 30 min prior to the addition of LPS. Then 10 μM $DCFH_2$-DA was added in culture medium and incubated at 37 °C for 30 min. After incubation, cells were washed with warm PBS buffer, and the ROS production was measured by changes in fluorescence due to intracellular production of DCF caused by oxidation of $DCFH_2$. The intracellular ROS level (percentage of control), as indicated by DCF fluorescence, was measured by fluorescence microscopy (200× magnification) (Olympus, Center Valley, PA).

Protein Isolation and Western Blot Analysis. RAW264.7 and A7r5 cells (3 × 10^6 cells/mL) were preincubated with hesperetin or hesperetin metabolites (1−10 μM) for 30 min prior to the addition of LPS. Cells were lysed in 100 μL of lysis buffer (10 mM Tris-HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol, and 1 mM phenylmethyl sulfonyl fluoride). The suspension was incubated on ice for 20 min and then centrifuged at 16000g for 20 min at 4 °C. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, and separated by 8−12% SDS PAGE, followed by transfer of the proteins to polyvinylidene difluoride (PVDF) membranes overnight. Transferred membranes were reacted with primary antibodies against COX-2, COX-1, iNOS, phos-JNK1/2, phos-p38, and phos-ERK1/2, or I-κBα for 2 h. They were then incubated with an HRP-conjugated secondary antibody for 2 h. The blots were detected by ImageQuant LAS 4000 mini (Fujifilm) with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, IL).

Determination of PGE_2 Production. The PGE_2 concentration of the culture media was determined using ELISA assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. RAW264.7 and A7r5 cells (4 × 10^5 cells/mL) were incubated with hesperetin or hesperetin metabolites (1−10 μM) for 30 min prior to the addition of LPS. Culture supernatant (100 μL) was collected and PGE_2 concentration determined using an ELISA microplate reader.

Determination of Nitric Oxide Production. The concentration of NO in the culture supernatant was determined by the accumulated nitrite, a major stable product of NO, using the Griess reagent colorimetric assay. RAW264.7 and A7r5 cells (4 × 10^5 cells/mL) were incubated with hesperetin or hesperetin metabolites (1−10 μM) for 30 min prior to the addition of LPS. Culture supernatant (100 μL) was mixed with the same volume of Griess reagents, and the absorbance of the mixture at 540 nm was determined using an ELISA microplate reader. A standard curve was constructed using known concentrations of sodium nitrite.

NF-κB Activation Assay. RAW264.7 cells were grown in RPMI-1640 medium containing 10% FBS, 4 × 10^5 cells/well in a 12 well plate. After an overnight incubation, pNF-κB-SEAP was cotransfected with the pIRES-hrGFP-1a expression vector (10:1) into cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA). After 6 h post-transfection, the cells were resuspended in serum-free medium and then incubated with 1−10 μM of hesperetin or hesperetin metabolites for 3−12 h, followed by stimulation with LPS. SEAP activity in the medium was evaluated by using the Phospha-Light system according to the protocol of the manufacture (Applied Biosystems, Bedford, MA). Relative SEAP activity was determined to reflect the transcriptional activity of NF-κB, and expressed as fold increases relative to the activity of untreated controls.

Statistics. Mean data values are presented with their deviation (mean ± SD). Analysis of variance (ANOVA) was used for all data analysis, followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $p < 0.05$.

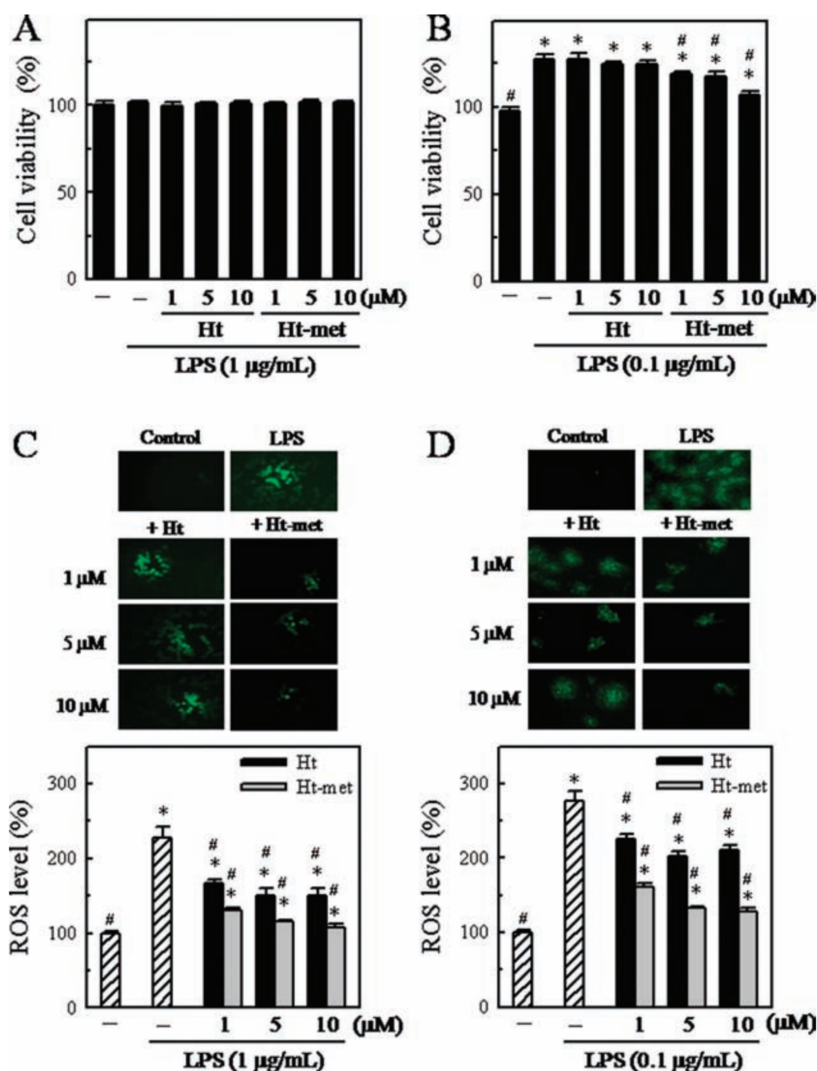

Figure 2. Effects of hesperetin and hesperetin metabolites on RAW 264.7 and A7r5 cells' viability and intracellular ROS levels. (A, B) Cells were treated with 0, 1, 5, and 10 μM hesperetin and hesperetin metabolites for 18 h (RAW 264.7 cells) or for 8 h (A7r5 cells). Cultures were harvested, and cell viability was determined via MTT assay. (C, D) Cells were treated with 0, 1, 5, and 10 μM hesperetin and hesperetin metabolites in the presence or absence of LPS (1 or 0.1 μg/mL) for 90 min for RAW 264.7 cells or 5 min for A7r5 cells. The nonfluorescent cell membrane-permeable probe DCFH2-DA was added to the culture medium at a final concentration of 10 μM. DCFH2 was reacted with cellular ROS and metabolized into fluorescent DCF. The intracellular ROS level (as a percentage of the control), as indicated by DCF fluorescence, was measured by fluorescence microscopy (200× magnification). Results are the mean ± SD of three assays. $^{*,\#}$ indicate significant difference in comparison to LPS-activated and control group ($p < 0.05$). Ht, hesperetin; Ht-met, hesperetin metabolites.

RESULTS

Antioxidative Effects of Hesperidin, Hesperetin, and Hesperetin Metabolites. The preliminary investigations were performed to compare the antioxidative activity of hesperidin (40−80 μM), hesperetin (40−80 μM), and hesperetin metabolites (2.5−20 μM) using different antioxidative models including superoxide anion scavenging, reducing power, metal chelating effect, and DPPH radical scavenging effect. The percentage inhibition of superoxide radical generation by hesperidin, hesperetin, and hesperetin metabolites is shown in Figure 1A. Hesperetin metabolites showed significant ($p < 0.05$) superoxide radical scavenging effect in a dose-dependent manner and stronger than that of hesperidin or hesperetin. Furthermore, the reducing power of hesperidin, hesperetin, and hesperetin metabolites was measured by the method of Oyaizu.[16] The $Fe^{3+}-Fe^{2+}$ transformation in the presence of hesperidin, hesperetin, and hesperetin metabolites was monitored. Evidenced from Figure 1B, the greater ($p < 0.05$) reducing power of hesperetin metabolites, compared to hesperidin and hesperetin, correlates well with its marked antioxidative action. The reducing power decreased in the order hesperetin metabolites > hesperidin > hesperetin.

Furthermore, to emphasize the antioxidant activity of hesperidin, hesperetin, and hesperetin metabolites, a metal chelating assay was performed. In this study, the chelation of ferrous ions by hesperidin, hesperetin, and hesperetin metabolites was estimated using the method of Dinis et al.[17] Chelating agents may inhibit lipid oxidation by stabilizing transition metals. Ferrozine can quantitatively form complexes with Fe^{2+}. As shown in Figure 1C, formation of $Fe^{2+}$and ferrozine complex was completed in the presence of hesperetin metabolities, indicating that hesperetin metabolites have significant ($p < 0.05$) chelating

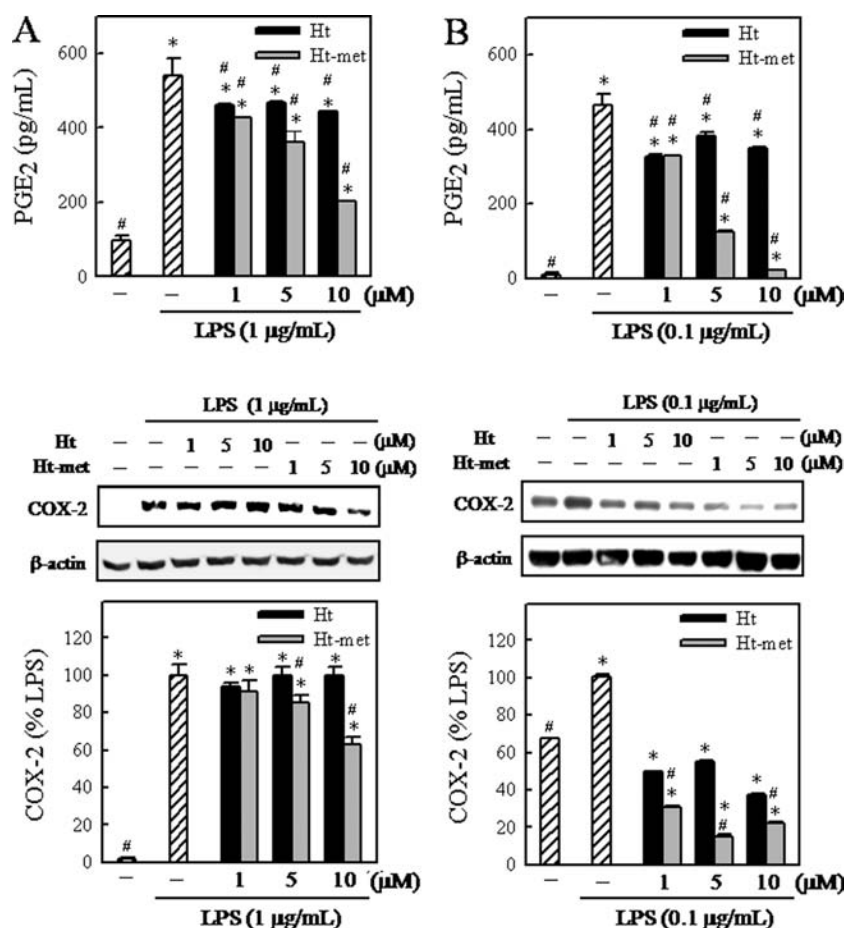

Figure 3. Hesperetin and hesperetin metabolites suppress PGE2 production through the downregulation of COX-2 expression in RAW 264.7 (A) and A7r5 (B) cells. RAW 264.7 or A7r5 cells were incubated with hesperetin and hesperetin metabolites (0, 1, 5, and 10 μM) in the presence or absence of LPS (1 or 0.1 μg/mL) for 18 h for RAW 264.7 cells or 8 h for A7r5 cells. PGE_2 production was measured using commercially available EIA kit as described in Materials and Methods. Protein (50 μg) from each sample was resolved in 8% SDS−PAGE, and Western blotting was performed. Results are the mean ± SD of three assays. *,# indicate significant difference in comparison to LPS-activated and control group ($p < 0.05$). Ht, hesperetin; Ht-met, hesperetin metabolites.

activity and capture ferrous ion. The inhibition of the Fe^{2+}−ferrozine complex was dose-dependently inhibited by hesperetin metabolites. The metal scavenging effect (%) decreased in the order hesperetin metabolites > hesperetin > hesperidin respectively. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The model of scavenging the stable DPPH radical widely used for relatively rapid evaluation of antioxidant activities compared to other methods.[12] The reduction capability of the DPPH radical is determined by its absorbance decrease at 517 nm, as induced by natural antioxidants. According to the DPPH assay, hesperidin and hesperetin metabolites have a slight observed free radical scavenging effect ($p < 0.05$). However, hesperetin showed moderate ($p < 0.05$) free radical scavenging activity (Figure 1D). These results indicate effective antioxidant activity of hesperetin metabolites in various oxidative systems, including the superoxide anion scavenging, reducing power, and metal chelation.

Effects of Hesperetin and Hesperetin Metabolites on Cell Viability in LPS-Activated RAW 264.7 and A7r5 Cells. Prior to the in vitro anti-inflammatory study, initially we determined whether hesperetin or hesperetin metabolites (1−10 μM) within the range of physiological concentrations affect RAW264.7 and A7r5 cell viability using the MTT assay. Results showed that the RAW 264.7 cell number was not affected when stimulated with LPS (1 μg/mL) and hesperetin or hesperetin metabolites for 18 h, proving to be noncytotoxic to macrophages (Figure 2A). However, significant proliferation was observed in A7r5 cells, when stimulated with LPS (0.1 μg/mL) for 8 h. In addition, compared to hesperetin, hesperetin metabolites (5 and 10 μM) showed slight ($p < 0.05$) cytotoxicity to A7r5 cells (Figure 2B).

Hesperetin and Hesperetin Metabolites Inhibit LPS-Induced ROS Generation in RAW264.7 and A7r5 Cells. The LPS-induced intracellular ROS accumulation being monitored in vitro using $DCFH_2$-DA fluorescence microscopic analysis. As shown in Figure 2C and Figure 2D, incubation of RAW 264.7 or A7r5 cells with LPS caused a significant increase in intracellular ROS, with a maximum ROS increase observed after 90 min in RAW 264.7 cells and 5 min in A7r5 cells. Cotreatment of cells with hesperetin or hesperetin metabolites (1−10 μM) and LPS resulted in a significant ($p < 0.05$) reduction of ROS accumulation; the reduction was more pronounced in hesperetin metabolite treated cells than in hesperetin treated cells (Figure 2C and Figure 2D). When the cells were incubated with hesperetin or hesperetin metabolites alone, the concentration of ROS was maintained at a background level similar to that in unstimulated cells (data not shown). We concluded that hesperetin metabolites,

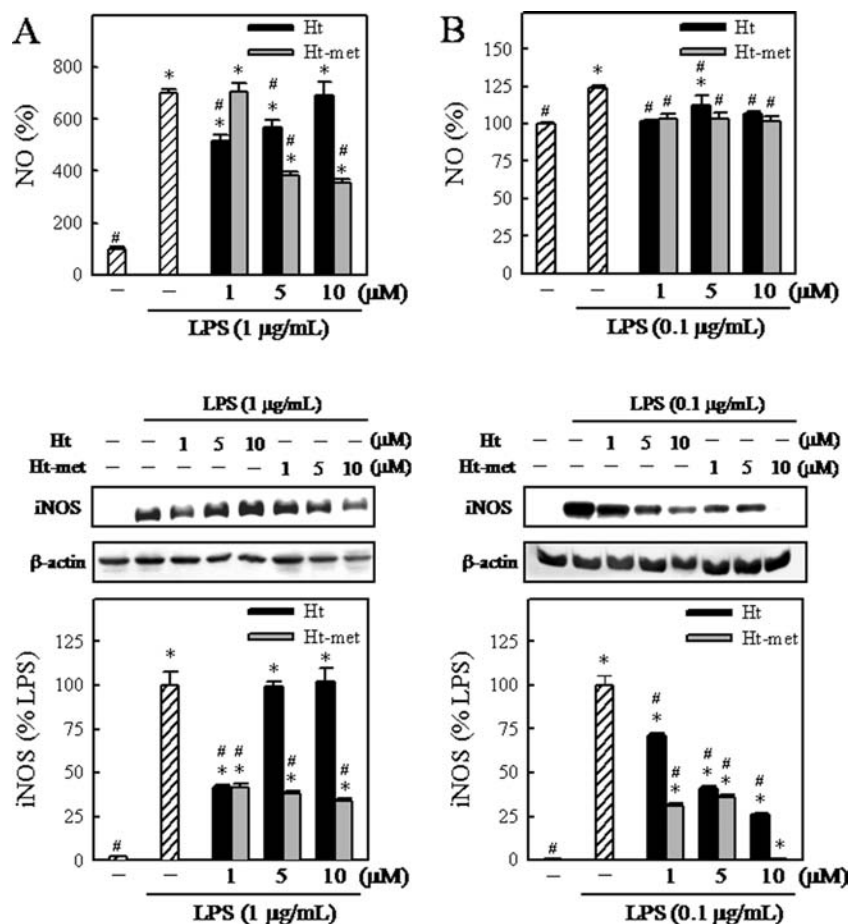

Figure 4. Hesperetin and hesperetin metabolites suppress NO production through the downregulation of iNOS expression in RAW 264.7 (A) and A7r5 (B) cells. Cells were incubated with hesperetin and hesperetin metabolites (0, 1, 5, and 10 μM) in the presence or absence of LPS (1 or 0.1 μg/mL) for 18 h for RAW 264.7 and 8 h for A7r5 cells. NO production was then determined by measuring the formation of nitrite, the stable end-metabolite of NO. Protein (50 μg) from each sample was resolved on 8% SDS−PAGE and Western blotting performed. Results are the mean ± SD of three assays. *,# indicate significant difference in comparison to LPS-activated and control group ($p < 0.05$). Ht, hesperetin; Ht-met, hesperetin metabolites.

compared to hesperetin, significantly suppressed LPS-induced ROS generation in RAW 264.7 and A7r5 cells.

Hesperetin and Hesperetin Metabolites Downregulate LPS-Induced PGE2 Production and COX-2 Expression in RAW 264.7 and A7r5 Cells. PGE2 represents the most important inflammatory product of COX-2 activity, and, thus, it was quantified in cell-free culture supernatant.[20] As shown in Figure 3A and Figure 3B, cells stimulated with LPS alone raise a significant amount of PGE_2 in RAW264.7 and A7r5 cells. PGE_2 production was markedly ($p < 0.05$) attenuated in cells pretreated with hesperetin or hesperetin metabolites (1−10 μM) (Figure 3A,B). Notably, the pronounced inhibition of PGE_2 was observed in hesperetin metabolites when compared with hesperetin treatment. However, for cells incubated with hesperetin or hesperetin metabolites alone, the concentration of PGE2 in the medium was maintained at a background level similar to that in the unstimulated samples (data not shown). In order to confirm this effect, furthermore, we examined COX-2 protein expression level using immunoblotting. Western blot analysis showed that LPS treatment significantly augmented COX-2 protein expression in RAW 264.7 and A7r5 cells (Figure 3A,B). These expressions were markedly ($p < 0.05$) attenuated in cells pretreated with hesperetin or hesperetin metabolites (1−10 μM) (Figure 3A,B), and the COX-2 inhibition was more pronounced in hesperetin metabolite treated cells than in hesperetin treated cells. It is noteworthy that cells treated with hesperetin significantly inhibited COX-2 expression in A7r5 smooth muscle cells compared to that in RAW264.7 macrophages (Figure 3A,B).

Hesperetin or Hesperetin Metabolites Downregulate LPS-Induced NO Production and iNOS Expression in RAW 264.7 and A7r5 Cells. RAW 264.7 or A7r5 cells stimulated with LPS elevated NO levels in culture medium. However, cells pretreated with hesperetin metabolites (1−10 μM) resulted in a significant ($p < 0.05$) reduction of NO production (Figure 4A,B). In contrast, low concentration of hesperetin significantly inhibits NO production, whereas, in higher concentration, the inhibition was markedly reversed in RAW264.7 cells (Figure 4A). Both hesperetin or hesperetin metabolite (1−10 μM) treatment also slightly ($p < 0.05$) inhibited NO production in A7r5 cells (Figure 4B). Further, we hypothesized that the inhibition of NO production may associate with its catalytic enzyme iNOS. Therefore, we examined the inhibitory effect of hesperetin or hesperetin metabolites (1−10 μM) on LPS-induced expression of iNOS protein in RAW264.7 and A7r5 cells. The iNOS protein expression was significantly increased when cells were treated with LPS alone compared to the control (Figure 4A,B). However, the increased iNOS expression was significantly ($p < 0.05$) suppressed by hesperetin metabolites in RAW264.7 and A7r5 cells

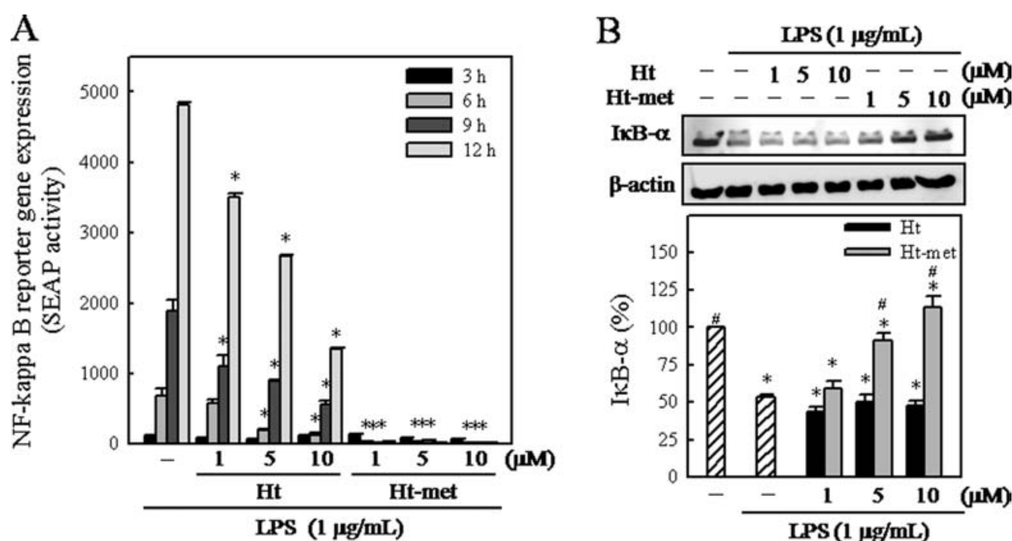

Figure 5. Hesperetin and hesperetin metabolites suppressed NF-κB transcriptional activation and IκB degradation in LPS-activated RAW 264.7 macrophages. (A) The SEAP activity was measured after treatment of hesperetin and hesperetin metabolites (0, 1, 5, and 10 μM) in the presence or absence of LPS (1 μg/mL) for 3, 6, 9, and 12 h. (B) Western blotting was performed for analysis of IκB protein expression level. Cells were treated with the indicated concentrations of hesperetin and hesperetin metabolites in the presence or absence of LPS (1 μg/mL) for 45 min. Results are the mean ± SD of three assays. *,# indicate significant difference in comparison to LPS-activated and control group ($p < 0.05$). Ht, hesperetin; Ht-met, hesperetin metabolites.

(Figure 4A,B). In addition, hesperetin treatment also dose-dependently inhibited iNOS expression in A7r5 cells, whereas hesperetin treatment dose-dependently enhanced iNOS expression in RAW264.7 cells (Figure 4A,B).

Hesperetin and Hesperetin Metabolites Suppressed NF-κB Activation and I-κBα Degradation in LPS-Activated RAW 264.7 Cells. NF-κB activation is a critical event for the LPS-induced activation of several inflammatory genes, including iNOS and COX-2.[6] Thereby, we examined the effect of hesperetin or hesperetin metabolites (1−10 μM) on LPS-induced activation of NF-κB in macrophage cells. NF-κB activation was detected by measuring NF-κB-dependent transcription in macrophages stably transfected with luciferace reporter construct. Cells incubated with LPS alone found remarkable increases of NF-κB activity. However, the decreased activity was dose- and time-dependently affected by hesperetin, and completely blocked by hesperetin metabolite treatment ($p < 0.05$) (Figure 5A). Furthermore, the LPS-induced NF-κB activation requires degradation of its inhibitor I-κB.[9] Thereby, further we examined the effect of hesperetin or hesperetin metabolites on LPS-induced NF-κB activation in RAW264.7 cells via detecting I-κBα protein stability by Western blotting. Upon exposure of the cells to LPS, the amount of I-κBα protein level was remarkably reduced (Figure 5B). However, hesperetin metabolite treatment significantly ($p < 0.05$) reversed LPS-induced I-κBα degradation in a dose-dependent manner, whereas hesperetin treatment abortive to block I-κBα degradation in RAW264.7 cells (Figure 5B).

Hesperetin and Hesperetin Metabolites Attenuate LPS-Induced MAPK Activation in RAW 264.7 Cells. It is well-established that the MAPK pathways play a major role in LPS-stimulated iNOS and COX-2 expression in macrophage cells.[10] Moreover MAPKs also involved activation of NF-κB.[6−9] To examine whether the inhibition of NF-κB activation by hesperetin and hesperetin metabolites (1−10 μM) was mediated through the MAPK pathway, we investigated LPS-induced phosphorylation of MAPK family proteins, especially JNK1/2, p38 MAPK, and ERK1/2 in RAW264.7 cells using immunoblotting. As shown in Figure 6A,B, hesperetin and hesperetin metabolites significantly ($p < 0.05$) suppressed LPS-induced JNK1/2 and p38 phosphorylation in RAW264.7 cells. However, the LPS-induced phosphorylation of ERK1/2 was unaffected by either hesperetin or hesperetin metabolite treatment (Figure 6A,B).

■ DISCUSSION

Citrus products have recently received much attention due to their potential therapeutic properties associated with high content of flavonoids, which showed antioxidant, anti-inflammatory, and anticancer activities.[21] Citrus flavonoids, including flavanones, flavonone glycosides, and polymethoxyflavones, rarely occur in non-citrus fruits.[22,23] The well-abundant flavone glycosides in citrus fruits are hesperidin and its aglycon hesperetin in orange and grapefruit, respectively. Citrus fruit hesperidin is hydrolyzed by gut microflora into aglycon form hesperetin and then conjugated mainly into glucuronides. Our study showed that hesperetin glucuronides/sulfates are more available than hesperetin. Moreover, the free form of hesperetin was almost undetectable in rat serum. In this study, we found that the hesperetin metabolites possess more effective antioxidant activity against various oxidative systems in vitro, including superoxide anion scavenging, reducing power, and metal chelating effects, than that of hesperidin or hesperetin. Previous in vivo studies also demonstrate that hesperetin and hesperidin pretreatment significantly protects mice from LPS-induced sepsis shock through the suppression of LPS and TNF-α levels in circulation.[24,25] Following in vitro study also exhibits that hesperidin and its metabolite heperetin potently inhibit LPS-induced expression of NO, PGE_2, and COX-2 gene in RAW264.7 cells.[26,27] However, the anti-inflammatory effects of hesperetin metabolities were poorly understood. Therefore, the present study demonstrated the ex vivo inhibitory effects of hesperetin metabolites in terms of LPS-induced nitric oxide (NO) and prostaglandin E2 production,

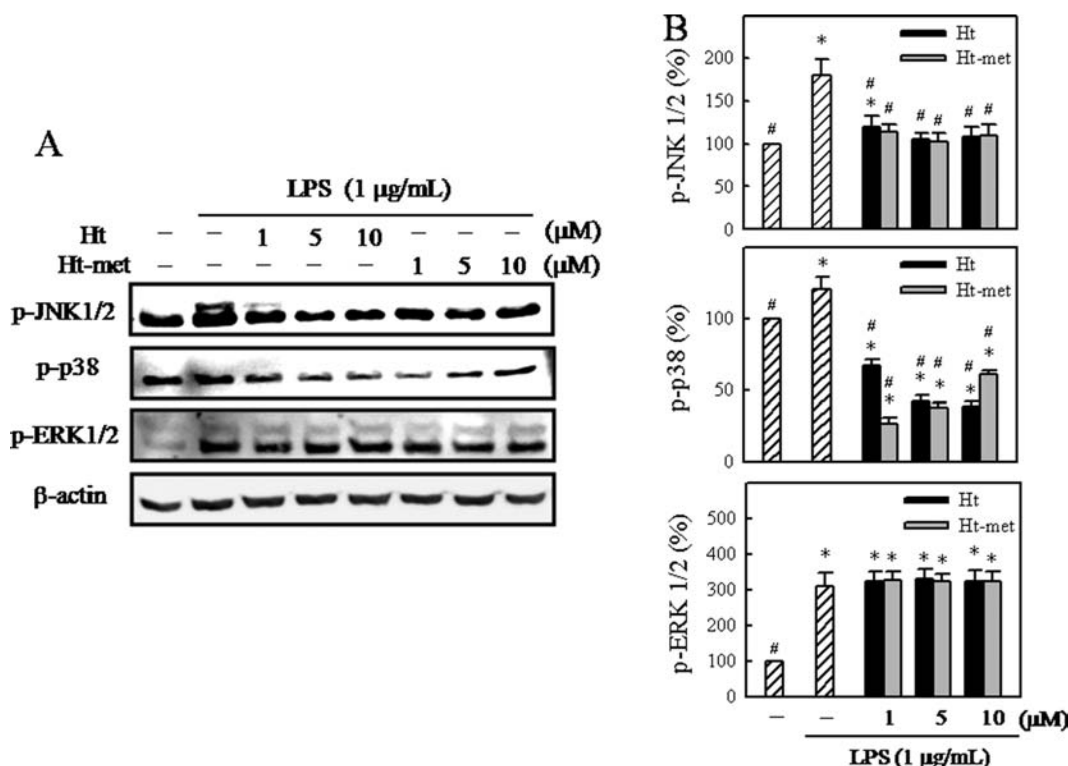

Figure 6. Hesperetin and hesperetin metabolites attenuate the MAPK signaling pathway in LPS-induced RAW264.7 cells. The phosphorylation of JNK1/2, p38 MAPK, and ERK1/2 was determined using Western blot analysis. Cells were treated with hesperetin and hesperetin metabolites (0, 1, 5, and 10 μM) in the presence or absence of LPS (1 μg/mL) for 15 min. Protein (50 μg) from each sample was resolved on 12% SDS−PAGE and Western blotting performed. The levels of indicated proteins in the cell lysates were analyzed by using specific antibodies, and the amounts of β-actin were used as internal controls for sample loading. Ht, hesperetin; Ht-met, hesperetin metabolites.

and iNOS and COX-2 expressions through blocking NF-κB activation, without appreciable cytotoxicity toward the RAW 264.7 macrophages or vascular smooth muscle A7r5 cells. We report here the first confirmation of hesperetin metabolites in vivo showing potential antioxidant and anti-inflammatory activity in comparison with hesperidin and/or hesperetin. In this study, LPS-stimulated smooth muscle cell growth was slightly inhibited by higher concentrations of hesperetin metabolites (5 and 10 μM), but not affected by lower concentration of hesperetin metabolites; various concentrations of hesperetin and LPS treatments do not alter cell viability in RAW264.7 macrophage cell, suggesting that the anti-inflammatory effects of hesperetin and hesperetin metabolites were not due to cell death. A previous report demonstrated that the bioavailability of hesperidin was improved by the enzymatic removal of the rhamnose sugar group to yield hesperetin-7-glucoside in human subjects.[28] Later, it was confirmed that hesperetin-7-glucoside was more bioavailable than hesperidin and significantly prevented bone loss in OVX rats.[29] Moreover, hesperetin-7-*O*-glucuronide, the circulating metabolite of hesperidin, regulates osteoblast differentiation through the up-regulation of Runx2 and Osterix in primary osteoblast cells.[30]

Several reports have demonstrated that the production of ROS, including superoxide anion radical (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and ROS, induces DNA or tissue damage in inflamed tissues.[13] Previous reports indicate that the antioxidant activity of citrus flavonoids is mainly due to their redox properties, which make them reducing agents, hydrogen donors, and metal chelators.[31,32] Thereby, in the present study we used a variety of cell or cell-free in vitro assays to demonstrate the antioxidant potential of hesperidin, hesperetin, and hesperetin metabolites. It is noted that hesperetin metabolites comparatively have strong superoxide radical scavenging ability, greater reducing power of iron ions, and remarkable metal chelating effects relative to their parent compounds, hesperidin and hesperetin. However, further investigation of the mechanism of the antioxidant and actions of hesperetin metabolities in vivo is necessary to permit full exploitation of its promise. Several biological activities including antioxidant, anti-inflammatory, and lipid lowering effects have been attributed to hesperidin and its metabolites.[33] Previous studies demonstrated that hesperetin metabolites lowered total cholesterol and triglyceride concentration through the inhibition of HMG-CoA and ACAT activities in high-cholesterol fed rats,[34] and exerted hypolipidemic and enhanced antioxidant efficacy in hypercholesterolemic hamsters.[35] These findings well correlated with the previous report that demonstrates the antioxidant capacity of hesperetin metabolites comparatively higher than that of hesperetin.

ROS are created by variety of cellular processes as part of a cellular signaling event. The role of ROS in LPS-induced inflammation was extensively studied in various in vitro and in vivo systems.[36] In this study, we also observed that LPS treatment significantly augmented ROS generation in RAW264.7 and A7r5 cells. Interestingly, the LPS-induced ROS production was remarkably reduced by hesperetin metabolites and this reduction was comparatively higher than that observed with hesperetin treatment. This data is consistent with the previous report,[37] which showed that hesperetin potentially inhibits ROS accumulation in cultured mononuclear cells. The LPS-induced ROS

generation in vascular smooth cells is still in debate, whereas leptin and angiotensin II induce intracellular ROS accumulation in aortic and vascular smooth muscle cells, respectively.[38,39] In this study, we observed that LPS treatment significantly elevated intracellular ROS accumulation in aortic smooth muscle (A7r5) cells and the elevated ROS generation was significantly inhibited by hesperetin metabolites comparatively higher than with hesperetin treatment.

It has been shown that several natural antioxidant compounds directly inhibit the expression of NF-κB-dependent cytokines, iNOS and COX-2 and, thus, reduce inflammation.[11,40] The suppressive effects of these antioxidant compounds on the production of the associated inflammatory mediators are associated with their antioxidant activities. The antioxidant NF-κB inhibitors restrict production of inflammatory mediators through suppression of their gene expression and also prevent inflammatory diseases. Macrophages and vascular smooth muscle cells exposed to inflammation stimulators such as LPS release several proinflammatory cytokines and other inflammatory products including NO, PGE_2, TNF-α, and IL-1β.[10,41] In order to validate the anti-inflammatory effect of hesperetin and hesperetin metabolites, we examined their effects on the production of NO and PGE_2 in LPS-induced RAW264.7 macrophage and A7r5 smooth muscle cells. We found that hesperetin metabolites compared to hesperitin significantly inhibit PGE_2 and NO production in both RAW264.7 and A7r5 cells. iNOS and COX-2 enzymatic products (NO and PGE_2, respectively) serve as key mediators of inflammation; thus agents that inhibit these mediators have therapeutic potential for inflammatory diseases.[6,9] Among the results from this study, we observed that LPS-induced iNOS and COX-2 protein expression was significantly downregulated by hesperetin metabolites compared to hesperitin.

The well-known transcription factor NF-κB regulates cell survival and coordinates the expression of proinflammatory mediators such as iNOS and COX-2.[9] However, the activation of NF-κB is dependent on phosphorylation and proteosomal degradation of its inhibitor I-κBα. The heteromeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, known as IκB, upon stimulation by endotoxins such as LPS that induce IκB proteosomal degradation, which leads to activation and nuclear translocation of NF-κB.[6] These findings suggest that hesperetin metabolites compared to hesperetin significantly downregulated LPS-stimulated NF-κB activity in RAW264.7 cells, followed by the inhibition of I-κBα phosphorylation/degradation. In this study, we describe anti-inflammatory mechanisms mediated by hesperetin metabolites, which are based on the inhibition of LPS-mediated activation of NF-κB.

On the other hand, MAPKs also transiently activate expression of iNOS and COX-2 genes in LPS-induced macrophages mediated by NF-κB activation.[9] The anti-inflammatory activity through the suppression of NF-κB activation was well correlated with other previously categorized citrus flavonoids and their metabolites, including hesperidin,[4] quercetin,[42] naringin,[43] nobiletin,[44] and nobiletin metabolites.[45] Lee et al. demonstrated that hesperidin suppressed TPA-induced MMP-9 protein expression through the inhibition of NF-κB activity followed by the downregulation of I-κB, JNK1/2, and p38 MAPK, whereas ERK1/2 was not altered by hesperidin treatment in HepG2 cells.[4] Notably, naringin also showed a parallel effect that downregulated LPS-induced NF-κB activation in macrophage cells.[43] The well-known flavonoid quercetin, but not quercitrin, significantly inhibits NF-κB activity through the suppression of I-κB protein phosphorylation, without affecting MAPKs in BMDM cells and rodent experimental model.[42] Choi and his co-workers reported that nobiletin inhibited neither LPS-induced phosphorylation/degradation of I-κB nor LPS-induced NF-κB nuclear translocation. Interestingly, nobiletin treatment inhibited NF-κB DNA binding activity in a concentration-dependent manner.[44] Additionally, nobiletin metabolites isolated from the urine of nobiletin fed mice possess more potent anti-inflammatory activity than their parent compound (nobiletin) in LPS-induced macrophage cells.[45] Downregulation of the NF-κB pathway through the suppression of I-κB phosphorylation/degradation and inhibition of MAPK activation by hesperetin metabolites in this study was similar to that in previous reports.[42−45] It is worth noting that hesperetin metabolites showed significant anti-inflammatory activity compared to their parent compound, concomitantly with a previous report by nobiletin metabolites in LPS-induced macrophage cells.[42]

In conclusion, we have demonstrated that hesperetin metabolites compared to their parent compounds (hesperidin and hesperetin) possessed effective antioxidant activity, which includes superoxide anion scavenging, reducing power, and metal chelation effects. To the best of our knowledge this is the first ex vivo report demonstrating that hesperetin metabolites compared to hesperetin within the range of physiological concentrations are effective inhibitors of LPS-induced expression of iNOS and COX-2 through the suppression of NF-κB activation in macrophages or smooth muscle cells. Based on these ex vivo approaches, hesperetin metabolites showed a great potential to be a novel chemopreventive agent for the treatment of a variety of inflammatory disorders. Therefore, hesperetin metabolites can be used as an accessible source of natural antioxidants and a possible food supplement, with potential application in the pharmaceutical industry.

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ABBREVIATIONS USED

ROS, reactive oxygen species; NO, nitric oxide; PGE_2, prostaglandin E_2COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; IκB, inhibitor κB; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase.

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