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Hesperetin: A Potent Antioxidant Against Peroxynitrite

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Peroxynitrite ($ONOO^-$) is a reactive oxidant formed from superoxide ($\cdot O_2^-$) and nitric oxide (·NO), that can oxidize several cellular components, including essential protein, non-protein thiols, DNA, low-density lipoproteins (LDL), and membrane phospholipids. $ONOO^-$ has contributed to the pathogenesis of diseases such as stroke, heart disease, Alzheimer's disease, and atherosclerosis. Because of the lack of endogenous enzymes to thwart $ONOO^-$ activation, developing a specific $ONOO^-$ scavenger is remarkably important. In this study, the ability of hesperetin (3′,5,7-trihydroxy-4-methoxyflavanone) to scavenge $ONOO^-$ and to protect cells against $ONOO^-$ and ROS was investigated. The data gained show that hesperetin can efficiently scavenge authentic $ONOO^-$. In spectrophotometric analysis, the data revealed that hesperetin led to declined $ONOO^-$-mediated nitration of tyrosine through electron donation. Hesperetin exhibited significant inhibition on the nitration of bovine serum albumin (BSA) by $ONOO^-$ in a dose-dependent manner. Hesperetin also manifested cytoprotection from cell damage induced by $ONOO^-$ and ROS. The present study suggests that hesperetin is a powerful $ONOO^-$ scavenger and promotes cellular defense activity in the protection against $ONOO^-$ involved diseases.

Keywords: Hesperetin (3′,5,7-trihydroxy-4-methoxyflavanone); Nitration; Nitrotyrosine; Peroxynitrite; Cytoprotection

INTRODUCTION

Flavonoids are a large group of dietary polyphenolic compounds that generally consist of flavanols, flavonols, flavonones, isoflavones, and catechins. Among these compounds, flavonones are widely distributed in the citrus species. Hesperidin, one of the most abundant flavonoids found in citrus fruits, such as lemons, oranges, and grapefruits,[1,2] has been exhibited to exert pharmacological effects, including capillary permeability[3] and anti-carcinogenic, anti-tumor, anti-inflammatory, and anti-oxidant effects.[4–7] Hesperetin is derived from the hydrolysis of aglycone, hesperidin (hesperetin 7-rhammnoglucoside).[8]

Reactive oxygen and nitrogen species (ROS/RNS) generated from various cells, including macrophages and neutrophils, have been reported to be effective materials against various infectious pathogens.[9] In turn, superoxide ($\cdot O_2^-$) may dismutate, either spontaneously or catalyzed by superoxide dismutases (SOD), to form hydrogen peroxide, which then possibly generates other ROS.[10] Nitric oxide (·NO) is a unique diffusible molecular messenger in the vascular and nervous systems, and is produced by a family of enzymes called nitric oxide synthase (NOS) through the enzymatic oxidation of the guanidine group of L-arginine.[11]

Concurrent generation of ·NO and $\cdot O_2^-$ can produce a cytotoxic reaction product, peroxynitrite ($ONOO^-$).[12–14] $ONOO^-$ is a strong oxidant and nitrating species, the formation of which may be beneficial in inflammatory reactions in terms of oxidative destruction of intruding microorganisms.[15] Steady-state concentrations of $ONOO^-$ may be significant in the vicinity of activated macrophages that concomitantly generate $\cdot O_2^-$ and ·NO.[10] Despite its non-radical nature, $ONOO^-$ is more reactive than its parent molecules. $ONOO^-$ initiates lipid peroxidation, causes DNA breakage, and reacts with thiols.[16] $ONOO^-$ induced modification of proteins has been connected with

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the development of several diseases, including Alzheimer's disease, Huntington's Chorea, and Parkinson's disease.[17] $ONOO^-$ induced protein modifications include protein oxidation (on methionine, cysteine, tryptophane, or tyrosine residues) and nitration (of tyrosine or tryptophane residues).[16] The nitration of tyrosyl residues in proteins may interfere with signaling pathways relying on tyrosine phosphorylation/dephosphorylation, which regulate cellular proliferation, apoptosis,[18] and oxidation. Also, the nitration of proteins mediated by $ONOO^-$ may disrupt the tertiary structure of proteins and, at higher degrees of damage, may render them inaccessible for degradation by the proteasome.[19] Moreover, $ONOO^-$ ability to modify low-density lipoproteins (LDL) may initiate cellular signals that can lead to inflammation, mitosis or cholesterol accumulation, thereby resulting in atherosclerosis.[20] Because endogenous scavenging enzymes that can inactive $ONOO^-$ are lacking, investigating specific scavengers to $ONOO^-$ is considerably important. The selenium-containing compounds, D-(−)penicillamine[21] and ebselen,[22] have been shown to be a powerful scavenger against $ONOO^-$. Recently, there is an increasing interest in screening natural products for possible $ONOO^-$ scavengers.

The goal of this study is to define the effectiveness of hesperetin as an antioxidant. In doing this, the possible regulation of $ONOO^-$ and its major production pathway, by hesperetin was checked using several *in vitro* models and *tert*-butylhydroperoxide (*t*-BHP)-stimulated YPEN-1 cells. Our results suggested that hesperetin may be a selective regulator of $ONOO^-$-mediated diseases via its direct scavenging activity.

MATERIALS AND METHODS

Materials

Hesperetin (3′,5,7-trihydroxy-4-methoxyflavanone) (Fig. 1), DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), bovine serum albumin (BSA), and *t*-BHP were obtained from Sigma Chemical Co. (St Louis, MO, USA). Trolox (6-hydroxy-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Dihydrorhodamine 123 (DHR 123) and 2,7-di-chlorodihydrofluorescein diacetate (H_2DCFDA) were from Molecular Probes (Eugene, OR, USA) and $ONOO^-$ was from Calbiochem Co. (San Diego, CA, USA). 4,5-Di-aminofluorescein (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Corp. (Billelica, MA, USA), and the chemiluminescence detection system was

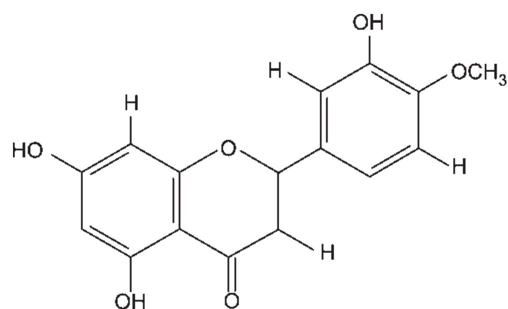

FIGURE 1 Chemical structure of hesperetin.

from Amersham Life Sciences, Inc. (Arlington Heights, IL, USA). Antibody to nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY, USA). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science (Buckinghamshire, UK). All other chemicals were of the highest purity available from either Sigma Chemical Co. (St Louis, MO, USA) or Junsei Chemical Co. (Tokyo, Japan).

Measurement of $ONOO^-$ Scavenging Activity

$ONOO^-$ scavenging was measured by monitoring the oxidation of DHR 123 by modifying the method of Kooy *et al*.[23] A stock solution of 5 mM DHR 123 in dimetyhlformamide was purged with nitrogen and stored at −20°C. Working solution with 5 μM DHR 123 diluted from the stock solution was placed on ice in the dark immediately before the experiment. The buffer (90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride) was purged with nitrogen and placed on ice before use. Just before use, 5 mM diethylenetriaminepentaacetic acid (DTPA) was added. The $ONOO^-$ scavenging ability, by the oxidation of DHR 123, was measured at room temperature on a microplate fluorescence Genious (Tecan, Austria) with excitation and emission wavelengths of 485 and 535 nm, respectively. The background and final fluorescent intensities were determined 5 min after treatment with or without authentic 10 μM $ONOO^-$ in 0.3 N sodium hydroxide (NaOH). Authentic $ONOO^-$ easily oxidized DHR 123 with its final fluorescent intensity being stable over time. Penicillamine was used as a positive control.

Measurement of $\cdot O_2^-$ Scavenging Activity

H_2DCFDA (f.c. 2.5 mM) mixed with esterase (f.c. 1.5 units/ml) was incubated at 37°C for 20 min and placed on ice in the dark until prior to the study. H_2DCFDA was oxidized to fluorescent 2,7-dichlorofluorescein (DCF) by $\cdot O_2^-$. A phosphate buffer (50 mM) at pH 7.4 was used. The fluorescence intensity of DCF was measured for 1 h by using

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microplate fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively, with or without the addition of menadione (50 mM) as an $\cdot O_2^-$ source.

Measurement of ·NO Scavenging Activity

·NO scavenging activity was determined by monitoring DAF-2 by modifying the method of Chung *et al.*[24] DAF-2, as a specific ·NO indicator, selectively traps ·NO between two amino groups in its molecule, and yields triazolofluorescein, which emits green fluorescence when excited at 490–495 nm.[25] In brief, 1 mg DAF-2 in 0.55 ml of dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400 fold.

A ·NO donor, 2 mM sodium nitroprusside (SNP), and 3.14 μM DAF-2 were added to a 96-well black microplate. The fluorescence intensity was dependent on the amount of ·NO trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with ·NO was measured for 30 min by the fluorescence at excitation and emission wavelengths of 495 and 515 nm, respectively.

Inhibition of $ONOO^-$-mediated Tyrosine Nitration by Hesperetin

The ability of hesperetin to inhibit the formation of 3-nitrotyrosine was quantified as the index of hesperetin's inhibition of tyrosine nitration utilizing spectrophotometric and Western blot analyses.

Spectrophotometric Analysis of Nitrated Proteins

The spectral change in the visible region of hesperetin in the presence of $ONOO^-$ was monitored at 430 nm to detect the existence of nitration. $ONOO^-$ (200 μM) in 0.3 N NaOH was added to a solution containing a sample and tyrosine (200 μM) in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 ml. Each mixed solution was scanned between 190 and 600 nm on an Ultraspec 2000 UV-visible spectrophotometer (Pharmacia-Biotech, UK).

Western Blotting Analysis

A 2.5 μl aliquot of hesperetin dissolved in 10% v/v EtOH was added to 95 μl of BSA (0.5 mg of protein/ml) with 2.5 μl of $ONOO^-$ (100 μM). The mixed samples were incubated with shaking at 37°C for 20 min.

The prepared samples in gel buffer, pH 6.8 (12.5 mM *tris*[hydroxymethyl]aminomethane, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue) in a ratio of 1:1 were boiled for 5 min. Total protein equivalents for each sample were separated on 8% SDS-polyacrylamide minigel at 100 V and transferred to a PVDF membrane at 100 V for 90 min in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed into a blocking solution (5% w/v skim milk powder in TBS-Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM Tween-20, pH 7.4) at room temperature for 1 h. The membrane was washed in TBS-Tween buffer for 30 min and then incubated with a monoclonal anti-nitrotyrosine antibody (1% w/v skim milk, diluted 1:2000 in TBS-Tween buffer) at room temperature for 3 h. After three 10 min washing in TBS-Tween buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (1% w/v skim milk, diluted 1:2000 in TBS-Tween buffer) at room temperature for 2 h. After three 10 min washing in TBS-Tween buffer, antibody labeling was detected using ECL and exposed to radiographic film. Prestained blue protein markers were used for molecular-weight determination.

Cells and Cell Culture Conditions

YPEN-1 cells, rat prostatic endothelial cells, were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). YPEN cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Nissui Co., Tokyo, Japan) supplemented with 5% heat-inactivated (56°C for 30 min) fetal bovine serum (Gibco, Grand Island, NY), 233.6 mg/ml glutamine, 72 μg/ml penicillin–streptomycin, and 0.25 μg/ml amphotericin B, and adjusted to pH 7.4.–7.6 with $NaHCO_3$ in an atmosphere of 5% CO_2. The fresh medium was replaced after one day to remove non-adherent cells or cell debris.

Determination of Intracellular Free Radical Scavenging Activity

Inhibition of Intracellular ROS

YPEN-1 cells in a 96-well plate were pre-incubated for 24 h. After one day, the medium was changed to fresh serum free medium. The cells were treated with or without hesperetin and incubated for 2 h. After treatment with *t*-BHP (20 μM) for 1 h, the medium was replaced with fresh serum free medium and H_2DCFDA (125 μM) was added. The fluorescence intensity of DCF was measured for 1 h using the microplate fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively.

For intracellular ROS generation on endothelial cells, various oxygen species, including $\cdot O_2^-$, hydrogen peroxide, hydroxyl radicals and lipid hydroxides, were observed via a fluorescence microscope at ×120 magnification using the probe,

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DCFDA. Cell seeded in a 6-well plate adhered overnight, and then the culture medium was replaced with fresh serum free medium. Cells were exposed to hesperetin for 2 h and then treated with *t*-BHP (20 μM) for 1 h. After treatment, the medium was removed, and cells were added DCFDA (10 μM). Then cells were observed via a fluorescence microscope Axiovert 100 (Zeiss, Germany).

Inhibition of Intracellular $ONOO^-$

YPEN-1 cells in a 96-well plate were pre-incubated for 24 h. After pre-incubation with or without hesperetin for 2 h, cells were treated with 20 μM *t*-BHP for 1 h. And then the cells were added to the rhodamine solution (50 mM sodium phosphate buffer, 90 mM sodium chloride, 5 mM potassium chloride, 5 mM DTPA, and DHR 123). The $ONOO^-$ scavenging ability by the oxidation of DHR 123 was determined on the microplate fluorescence for 1 h with excitation and emission wavelengths of 485 and 535 nm, respectively.

Intracellular $ONOO^-$ generation on endothelial cells was observed via a fluorescence microscope at × 120 magnification using the probe, DHR 123. Cells seeded in a 6-well plate were incubated overnight, and the culture medium was changed to a fresh, serum-free medium. Cells were exposed to hesperetin for 2 h and then treated with *t*-BHP (20 μM) for 1 h. After treatment, medium was removed and cells were added to DHR 123 (10 μM). Then cells were observed via a fluorescence microscope.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) of three or five experiments. Statistical analysis was confirmed by Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

The $ONOO^-$ Scavenging Activity of Hesperetin

The ability of hesperetin to scavenge $ONOO^-$ was investigated using DHR 123 as shown in Table I. The results showed that hesperetin has similar scavenging activity to penicillamine, a well-known, strong $ONOO^-$ scavenger.

TABLE I Effect of IC_{50} (μM) hesperetin on $ONOO^-$, $\cdot O_2^-$, and ·NO scavenging activities

	$ONOO^-$	$\cdot O_2^-$	·NO
Hesperetin	4.67 ± 0.16	7.47 ± 1.58	18.57 ± 2.47
Penicillamine*	4.04 ± 0.03		
Trolox*		9.80 ± 0.51	
Carboxy–PTIO*			5.38 ± 0.85

*Used as a positive control. IC_{50}: 50% inhibition concentration. Abbreviations: $ONOO^-$, peroxynitrite; $\cdot O_2^-$, superoxide; ·NO, nitric oxide.

Scavenging Ability of Hesperetin Against $\cdot O_2^-$ and ·NO

Table I shows IC_{50} values of hesperetin for $\cdot O_2^-$ and ·NO scavenging abilities. Because $ONOO^-$ can be formed from reaction of ·NO with $\cdot O_2^-$, we thought it is interesting to compare the active components of $ONOO^-$, $\cdot O_2^-$ and ·NO. Compared with trolox (IC_{50}: 9.80 ± 0.51 mM), a well-known antioxidant $\cdot O_2^-$ scavenger, hesperetin showed higher scavenging activity levels (IC_{50}: 7.47 ± 1.58 mM). In addition, hesperetin inhibitory action was slight (IC_{50}: 18.57 ± 2.47 mM) in ·NO scavenging ability, compared with a ·NO scavenging standard carboxy–PTIO (IC_{50}: 5.38 ± 0.85 mM).

Reaction of Hesperetin to $ONOO^-$

To explore the scavenging mechanisms of hesperetin by which it undergoes either nitration reaction or electron donation after the addition of $ONOO^-$, spectrophotometric analysis at 400–450 nm was employed. Figure 2A,B show that tyrosine undergoes nitration by $ONOO^-$, because there is a peak around 430 nm. Hesperetin alone did not produce the peak around 430 nm (Fig. 2C), and in contrast to tyrosine, after interaction with $ONOO^-$, hesperetin did not display any spectrometric change around 430 nm (Fig. 2D), strongly indicating that electron donation, but not nitration.

Effect of Hesperetin on BSA Nitration

Through Western blot analysis, we tested the ability of hesperetin to inhibit $ONOO^-$-mediated tyrosine nitration in common biological materials, such as BSA, using anti-3-nitrotyrosine antibody. The results indicated that hesperetin at concentrations of 2.5, 5, and 10 μM attenuated the nitration of BSA in a dose-dependent fashion, as shown in Fig. 3.

Effects of Hesperetin on the Inhibition of Intracellular ROS Generation

As shown in Fig. 4A, the results revealed that preincubation of hesperetin at concentrations of 5, 15, and 50 μM decreased the intracellular ROS generation induced *t*-BHP (20 μM) in a dose-dependent manner (Fig. 4A). As shown in Fig. 4B, compare of untreated control (Fig. 4B(a)), *t*-BHP (20 μM) and treated cells showed the generation ROS (Fig. 4B(b)). Pretreatment with hesperetin at concentrations of 5, 15, 50, and 200 μM showed suppressed dose-dependent ROS generation (Fig. 4B(c–f)).

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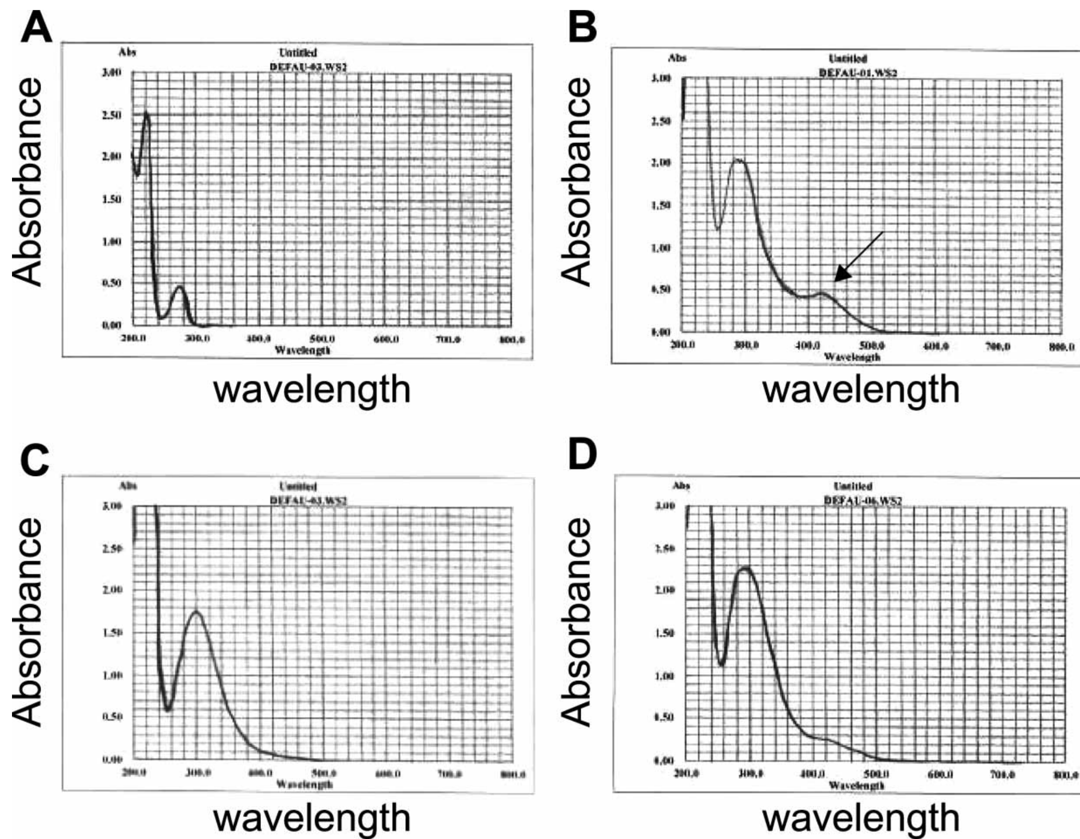

FIGURE 2 Interaction of hesperetin with ONOO$^-$. Tyrosine (200 μM) was incubated without (A) or with ONOO$^-$ (200 μM) (B) in 50 mM phosphate buffer at pH 7.0 at room temperature for 10 min. Hesperetin (100 μM) was incubated without (C) or with (D) ONOO$^-$ (200 μM) at 37°C for 10 min. The spectrophotometric analysis was performed as described in "Materials and methods" section. The arrow indicates a nitration peak at around 400–450 nm.

Ability of Hesperetin to Inhibit Intracellular ONOO$^-$ Generation

As shown in Fig. 5A, the results revealed that pre-incubation of hesperetin at concentrations with 5, 15, and 50 μM that intracellular ONOO$^-$ generation induced by *t*-BHP (20 μM) decreased in a dose-dependent manner (Fig. 5A). As shown in Fig. 5B, *t*-BHP (20 μM) treated cells generated significantly higher ONOO$^-$ levels than the untreated group (Fig. 5B(a,b)). Pretreated with hesperetin at concentrations of 5, 15, 50, and 200 μM decreased the generation of ONOO$^-$ in a dose-dependent manner (Fig. 5B(c–f)).

FIGURE 3 Effect of hesperetin on the nitration of BSA by ONOO$^-$. Hesperetin and ONOO$^-$ were added to BSA. The reaction samples were incubated with shaking at 37°C for 20 min, which was resolved in 8% polyacrylamide-gel electrophoresis.

DISCUSSION

ONOO$^-$ is an important contributor to inflammatory conditions, including arthritis, endotoxic or septic shock, which is widely considered a systemic inflammatory disease, as well as being suspect in a variety of other models of inflammation.[26] Moreover, ONOO$^-$ can nitrate tyrosine, a process specific to this species and not triggered by the hydroxyl radicals, ·NO or $\cdot O_2^-$.[27] These are a variety of additive or synergistic cytotoxic processes triggered by ONOO$^-$, the combination of which can lead to acute and delayed cytotoxicity.[28] Plant food-derived antioxidants and active principles, such as flavonoids, hydrocinnamates (chlorogenic acid, ferulic acid, vanillin, etc.), β-carotene, and other carotenoids, vitamin E, vitamin C, rosemary, sage, and tea are important dietary antioxidant substances[29] that may provide efficient ONOO$^-$ scavenger activities. Hesperetin is a widely known component of the plant kingdom that has been reported to have an antioxidant property.[30]

Our study shows that hesperetin is not only a strong ONOO$^-$ scavenger, but also effectively

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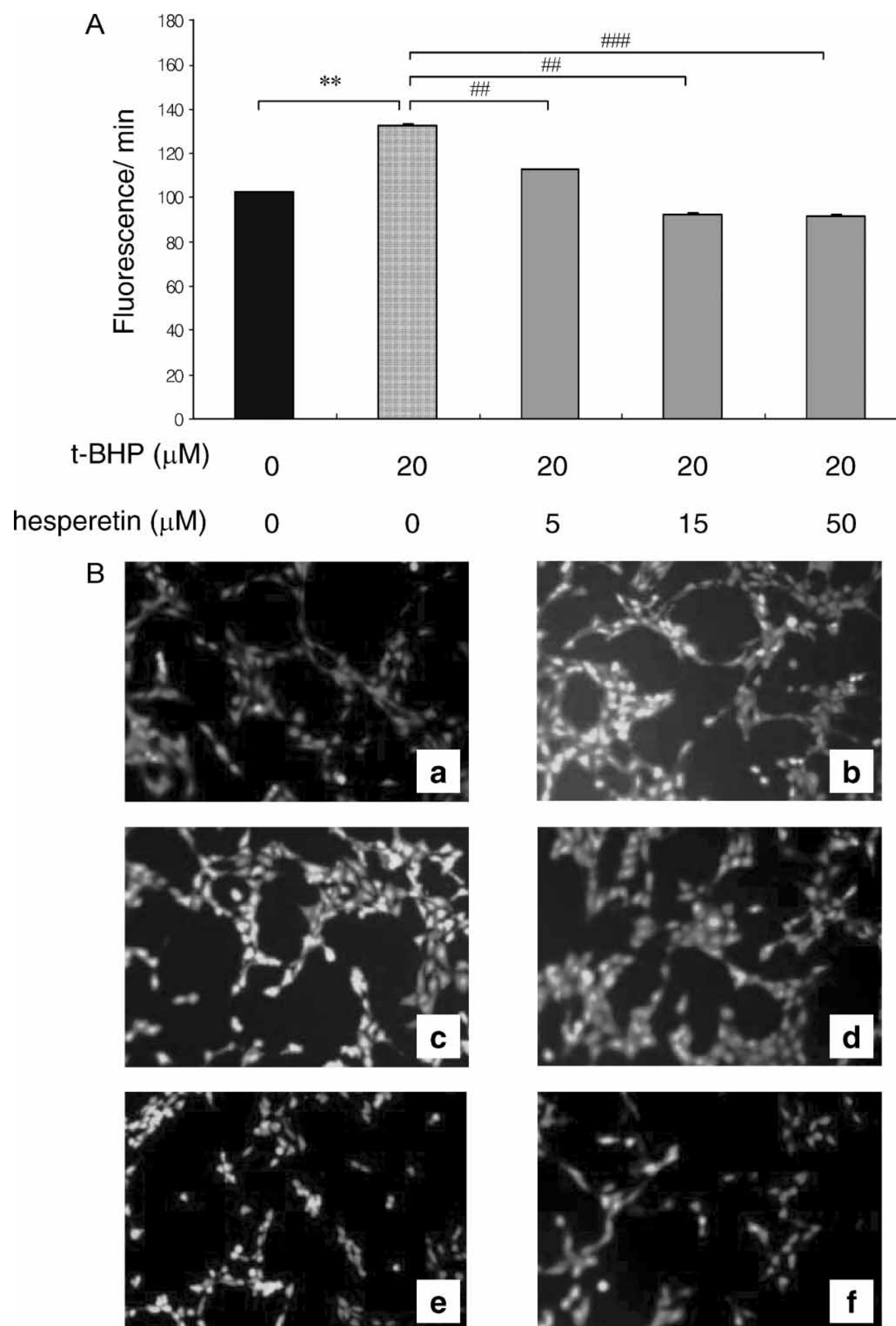

FIGURE 4 Hesperetin inhibited intracellular ROS generation induced *t*-BHP. Cells were pre-treated with various concentrations of hesperetin for 2 h and further treated with *t*-BHP (20 μM) for 1 h. (A) Detection of intracellular ROS by staining with fluorometer using DCFDA. Each value is the mean ± SEM. Statistical results of one-factor ANOVA: $^{**}p < 0.01$ vs. not treated *t*-BHP, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. treated *t*-BHP. (B) The levels of intracellular ROS were detected by DCFDA using fluorescence microscope. (a) untreated control, (b) 20 μM *t*-BHP treated alone, (c) 20 μM *t*-BHP and 5 μM hesperetin, (d) 20 μM *t*-BHP and 15 μM hesperetin, (e) 20 μM *t*-BHP and 50 μM hesperetin, (f) 20 μM *t*-BHP and 200 μM hesperetin.

inhibits intracellular free radical activity. It would seem reasonable to use the well-known $ONOO^-$, ·NO, and $\cdot O_2^-$ scavenging agents penicillamine,[31] carboxy–PTIO,[32] and trolox[33] in a comparison study against the scavenging activity of hesperetin. The most important findings from this present study are that the active component, hesperetin, not only directly enabled $ONOO^-$ scavenging, but also was involved in the inhibition of $\cdot O_2^-$ and ·NO radical formation. These phenomena might be explained in terms of chemical structures. Based on hesperetin's structural similarity with other known antioxidants, including hydroxyl groups, hesperetin's hydroxyl structure may give the clue to its $ONOO^-$ scavenging ability.

The minute mechanisms of $ONOO^-$ scavenging actions by active components are not yet fully defined; so far, two possible pathways, nitration or

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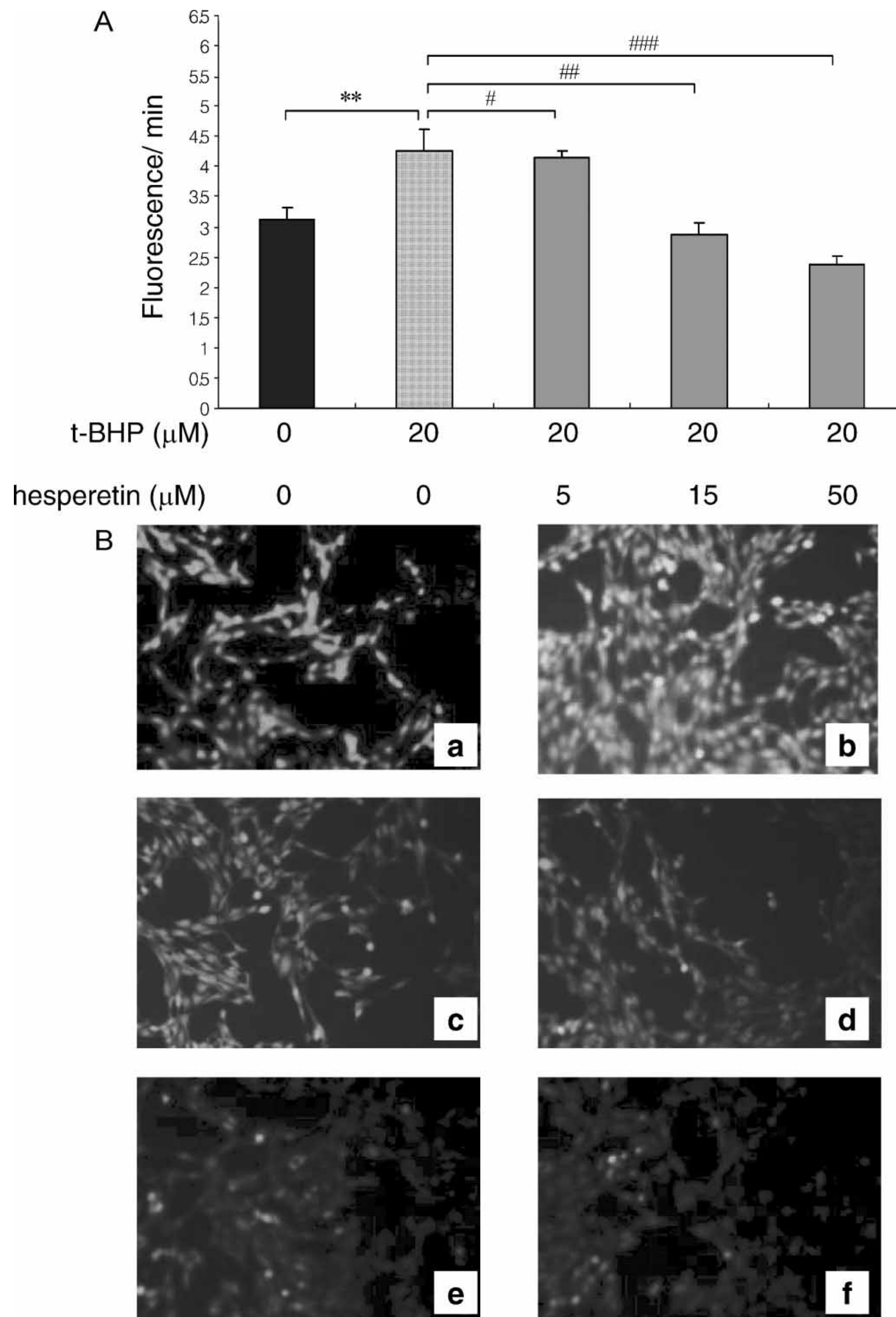

FIGURE 5 Effect of hesperetin on *t*-BHP mediated ONOO$^-$ generation. Cells were pre- treated with various concentrations of hesperetin for 2 h and then treated with *t*-BHP (20 μM) for 1 h. (A) Detection of intracellular ONOO$^-$ by staining with fluoremeter using DHR 123. Each value is the mean ± SEM. Statistical results of one-factor ANOVA: $^{**}p < 0.01$ vs. not treated *t*-BHP, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. treated *t*-BHP. (B) The levels of intracellular ONOO$^-$ were detected by DHR 123 using fluorescence microscope. (a) untreated control, (b) 20 μM *t*-BHP treated alone, (c) 20 μM *t*-BHP and 5 μM hesperetin, (d) 20 μM *t*-BHP and 15 μM hesperetin, (e) 20 μM *t*-BHP and 50 μM hesperetin, (f) 20 μM *t*-BHP and 200 μM hesperetin.

electron donation, have been suggested as a phenolic ONOO$^-$ scavenger interaction with ONOO$^-$.[34] The phenolic compound, especially with a mono-hydroxyl group such as the phenolic amino acid, tyrosine, is preferentially nitrated by ONOO$^-$. Whether nitration is derived from the breakdown of ONOO$^-$ to nitrogen dioxide radical ($\cdot NO_2$) or to the nitronium ion ($\cdot NO_2^+$) has not clarified.[31]

In the present study, we exposed hesperetin to ONOO$^-$ to determine whether hesperetin undergoes the nitration reaction. The addition of ONOO$^-$ revealed no spectral change in the visible region, which proposed that the nitration of the aromatic ring did not occur. Furthermore, incubation of tyrosine and hesperetin with ONOO$^-$ caused a decreased peak at 430 nm, which gave further

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evidence supporting the possibility of an electron donation reaction between hesperetin and $ONOO^-$. Although the intermediate form of hesperetin after electron donation is not fully identified, it seems that the intermediate form was not toxic, because hesperetin treatment up to 200 μM did not change YPEN-1 cell viability (data not shown).

The inhibitory action of hesperetin was further tested using additional physiological substances such as BSA. We found that $ONOO^-$ could induce a functional damage in some biological molecules, such as BSA and LDL, via nitrotyrosine.[24] Protein tyrosine nitration by $ONOO^-$ may interfere with phosphrylation/dephsphorylation signaling pathways and alter enzyme functions.[35–37] Nitrotyrosine has been reported in various hypertensive disorders, neurologic disorders, and chronic renal disease as a footprint of $ONOO^-$.[38–40] In the current study, evidence from Western blot analysis showed that hesperetin, even at a concentration as low as 2.5 μM, could markedly reduce the nitrotyrosine present in BSA. With increases in hesperetin concentrations, BSA nitration decreased, further suggesting the putative anti-$ONOO^-$ action *in vivo*.

It has been reported that *t*-BHP stimulates the redox status of the cell and has been extensively utilized to elucidate the nature of oxidative stress in cellular physiology and biochemistry.[41] *t*-BHP generates ROS that are responsible for lipid peroxidation, DNA adduct formation, the induction of apoptosis.[42] Therefore, in our study, cells were treated with *t*-BHP to induce oxidative stress. When treated with hesperetin, we found it inhibited the generation of ROS and $ONOO^-$ in a dose-dependent manner.

In summary, the present study suggested that the active bioflavonoid component, hesperetin, can scavenge $ONOO^-$ efficiently. Hesperetin treatment led to an inhibition of $ONOO^-$-mediated nitration of tyrosine through electron donation, and it also showed significant dose-dependent inhibition of BSA nitration from ONOO. Hesperetin was shown to *t*-BHP-induced free radical equally well. In conclusion, the current results demonstrated that hesperetin is an efficient $ONOO^-$ scavenger and therefore potentially useful in the prevention of the $ONOO^-$-related diseases, such as Alzheimer's disease, rheumatoid arthritis, cancer, atherosclerosis, and other inflammatory conditions.

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