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Neuroprotective Effects of the Citrus Flavanones against H_2O_2-Induced Cytotoxicity in PC12 Cells

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The citrus flavanones hesperidin, hesperetin, and neohesperidin are known to exhibit antioxidant activities and could traverse the blood-brain barrier. H_2O_2 formation induces cellular oxidative stress associated with neurodegenerative diseases. In this study, protective effects of pretreatments (6 h) with hesperidin, hesperetin, and neohesperidin (0.8, 4, 20, and 50 μM) on H_2O_2-induced (400 μM, 16 h) neurotoxicity in PC12 cells were evaluated. The results showed that hesperetin, hesperidin, and neohesperidin, at all test concentrations, significantly ($p < 0.05$) inhibited the decrease of cell viability (MTT reduction), prevented membrane damage (LDH release), scavenged ROS formation, increased catalase activity, and attenuated the elevation of intracellular free Ca^{2+}, the decrease of mitochondrial membrane potential (except those of 0.8 μM neohesperidin-treated cells) and the increase of caspase-3 activity in H_2O_2-induced PC12 cells. Meanwhile, hesperidin and hesperetin attenuated decreases of glutathione peroxidase and glutathione reductase activities and decreased DNA damage in H_2O_2-induced PC12 cells. These results first demonstrate that the citrus flavanones hesperidin, hesperetin, and neohesperidin, even at physiological concentrations, have neuroprotective effects against H_2O_2-induced cytotoxicity in PC12 cells. These dietary antioxidants are potential candidates for use in the intervention for neurodegenerative diseases.

KEYWORDS: Flavanones; hesperidin; hesperetin; neohesperidin; H_2O_2; PC12 cells; ROS; caspase-3

INTRODUCTION

Oxidative stress is considered a risk factor in the incidence and progression of cognitive declines that occur during normal cerebral aging and dementia. In addition, oxidative stress likely plays a critical role in neurodegenerative processes, such as Alzheimer's disease (AD) (*1*). Recently, food-derived antioxidants, such as vitamins and phytochemicals, have received growing attention for their function as chemopreventive agents against oxidative damage. However, the need for antioxidants to penetrate the blood-brain barrier (BBB) is a mandatory prerequisite for them to be considered as a potential neuroprotective treatment in acute or chronic neurological disorders (*2*). Therefore, natural flavonoids with more of a lipophilic chemical structure and antioxidant properties are promising candidates for neurodegenerative intervention.

Citrus fruits and products are widely consumed around the world. Recently, the health effects of citrus flavonoids have been attracting attention. Flavanones are widely distributed in citrus species. Among them, hesperidin and neohesperidin are the abundant flavonoids and are each found mainly in oranges, tangerines, and lemons, and grapefruits, respectively (*3, 4*). Additionally, hesperetin is a metabolite of hesperidin (*3*). These three flavanones are methoxylated and are thus more lipophilic than other nonmethoxylated citrus counterparts. Their chemical structures are illustrated in **Figure 1**. Further, hesperidin, hesperetin, and neohesperidin are known to exhibit radical scavenging activity (*5*). Of them, hesperetin was proved to possess intracellular peroxynitrite scavenging activity in fibroblasts (*6*). However, the intracellular ROS scavenging activities of these flavanones have not been tested. Importantly, studies (*7, 8*) have suggested that hesperidin and hesperetin can cross the BBB and together with neoheperidin each act on the central nervous system (*7, 9, 10*). In addition, hesperidin and hesperetin are bioavailable from diets (*11, 12*). We think that hesperidin, hesperetin, and neoheperidin show potential for use in neurodegenerative intervention and thus evaluate their neuroprotection against oxidative damage here.

Hydrogen peroxide (H_2O_2) is generated during β-amyloid aggregation (*13*), dopamine oxidation (*14*), and brain ischemia/reperfusion (*15*). Thus, formed H_2O_2 is readily converted into highly toxic hydroxyl radical by Fenton chemistry and further damages lipids, proteins, and DNA. These oxidative damages could lead to mitochrondrial dysfunction, calcium imbalance, and apoptosis in neuronal cells (*16, 17*). The rat pheochromocytoma line PC12 provides a useful model system for neurological and neurochemical studies (*18*). Recent studies (*19, 20*)

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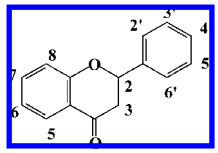

Figure 1. Structures of the flavanones. Hesperidin: 7, rutinose, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose; 3′,5, OH; 4′, OCH_3. Neohesperidin: 7, neohesperidose, 2-*O*-α-rhamnopyranosyl-β-D-glucopyranose; 3′,5, OH; 4′, OCH_3. Hesperitin: 3′,5,7, OH; 4′, OCH_3.

also showed that H_2O_2 induces cytotoxicity on PC12 cells including membrane and nuclear damage, decreases in mitochondrial membrane potential and antioxidant enzyme activities, such as those of catalase (CAT) and glutathione peroxidase (GSH-Px) accumulation of cytosolic cytochrome *c*, caspase-3 activation, increases in reactive oxygen species level, and depletion of glutathione.

In this study, we evaluated protective effects of hesperidin, hesperetin, and neoheperidin against H_2O_2-induced cytotoxicity in PC12 cells.

MATERIALS AND METHODS

Materials. Hesperidin, hesperetin, neohesperidin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), D-glucose, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), hydrogen peroxide, sodium bicarbonate, and sodium pyruvate were purchased from Sigma-Aldrich Co. (St. Louis, MO). RPMI-1640 medium was obtained from HyClone (Logan, UT). Fetal bovine serum was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Horse serum was a product of Gibco (Carlsbad, CA).

Cell Culture and Treatment. Rat pheochromocytoma PC12 cells were purchased from the Bioresource Collection and Research Center (FIRDI, Hsinchu, Taiwan). Cells were maintained in RPMI supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in humidified 5% CO_2/95% air at 37 °C. All cells were cultured in collagen coated culture dishes or flasks. The medium was changed every other day. Before treatment, cells were plated at an appropriate density on culture plates or dishes according to each experimental scale and cultured for 24 h.

Cells were pretreated for 6 h with various concentrations of samples. Then, the medium was refreshed without adding sample, and the cells were exposed to 400 μM H_2O_2 for another 16 h.

Measurement of Cell Viability. PC12 cells were plated at a density of 2.5×10^4 cells/100 μL in 96-well plates, and the cell viability was determined by MTT reduction assay. MTT, a tetrazolium salt, is cleaved to formazan by an active enzyme for the mitochondrial respiratory chain in live cells, succinate dehydrogenase. After incubation, cells were treated with the MTT solution (final concentration, 0.5 mg/mL) for 3 h. The dark blue formazan crystals formed in intact cells were solubilized with dimethylsulfoxide (DMSO), and their optical density of absorbance at 570 nm was measured using a FLUOstar Galaxy spectrophotometer (BMG Labtechnologies GmbH, Inc., Offenburg, Germany).

Lactate Dehydrogenase (LDH) Release Assay. The LDH assay was used to measure cell membrane damage as a function of the amount of cytoplasmic LDH released into the medium. The LDH assay is based on the reduction of NAD by the action of LDH. The resulting NADH is utilized in the stoichiometric conversion of a tetrazolium dye. The LDH activity can be used as an indicator of relative cell viability as a function of membrane integrity. Briefly, PC12 cells were spun down; 100 μL of the supernatants was transferred into new wells; and LDH was determined using the Takara LDH cytotoxicity detection kit (Takara Bio, Inc., Shiga, Japan). LDH release was calculated as the percentage of LDH in the medium versus total LDH activity (the amount of maximum release).

Determination of Intracellular Reactive Oxygen Species (ROS). The DCFH-DA method was used to measure intracellular ROS production (*21*). After the cells were cultured with or without treatments, they were collected, resuspended in phosphate-buffered saline (PBS), and incubated with DCFH-DA at a final concentration of 20 μM for 30 min at 37 °C. Then, cells were washed with PBS and harvested for FACS analysis. These cells were excited with a 488 nm argon ion laser in a flow cytometer (FACS200, Becton-Dickinson Immunocytometry Systems, San Jose, CA). DCF emission was recorded at 525 nm. A minimum of 10 000 events was collected for each data set.

Intracellular Ca^{2+} Levels Detection. Intracellular Ca^{2+} levels were measured by flow cytometry (*22*). Briefly, cultured cells with or without treatments were stained with Fluo3-AM (Sigma-Aldrich) at a final concentration of 5 μg/mL for 30 min at 37 °C. Then, cells were washed and harvested with PBS for FACS analysis. Relative intracellular levels were assessed as mean fluorescence (at 488 nm excitation and 530 nm emission), with the intensity in the FL1 channel calculated by the distribution histogram with cell counts on the vertical axis and fluorescence intensity on a log scale on the horizontal axis.

Measurement of Mitochrondrial Membrane Potential. Mitochondrial membrane potential was determined using the MitoPTTM 100 test kit (Immunochemistry Technologies, Bloomington, MN). Briefly, PC12 cells were seeded at a density of 6.6×10^4 cells/mL in 6-well plates and cultured for 24 h. After treatments, cells were changed to 100 μL of stain buffer and incubated at 37 °C for 15 min in a humidified 5% CO_2 incubator. Then, cells were collected and washed with 200 μL of assay buffer. After the cells were centrifuged (1000 rpm, 5 min), they were resuspended in 200 μL of assay buffer and analyzed by a FLUOstar Galaxy fluorescence plate reader (BMG Laboratory Technology, GmbH, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 590 nm for fluorescence measurement.

Assay for Antioxidant Enzymes. PC12 cells (1×10^5 cells/mL) were plated in culture plates and cultured for 24 h. After experimental treatments, cells were lysed on ice and centrifuged (12 000 rpm, 10 min) at 4 °C. The supernatants were collected for an enzyme activity assay. The catalase activity was assessed according to the method described by Cohen et al. (*23*). Briefly, a mixture of 75 mM phosphate buffer (pH 7.0), 50 mM H_2O_2, and cell lysate in a final volume of 3 mL was incubated at 30 °C for 2 min. The decrease in absorbance was recorded at 240 nm for 2 min. The glutathione peroxidase activity was determined according to the method of Mohandas et al. (*24*). The cell lysate (100 μL) was incubated at 37 °C for 5 min with 800 μL of 100 mM phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium azide, 1 mM glutathione (GSH), 1 unit/mL glutathione reductase, and 0.2 mM NADPH. After 100 μL of 2.5 mM hydrogen peroxide was added, the decrease in absorbance at 340 nm was recorded for 5 min. The glutathione reductase activity was determined using the method of Bellomo et al. (*25*). The cell lysate (100 μL) was added with 900 μL sodium phosphate buffer (100 mM, pH 7.0) containing 1.1 mM magnesium chloride, 5 mM GSSG, and 0.1 mM NADPH, and the absorbance at 340 nm was measured for 5 min. The activities of the catalase and glutathione-related enzymes were calculated from the standard curves and expressed as μmol min^{-1} (mg protein)$^{-1}$ and nmol min^{-1} (mg protein)$^{-1}$, respectively.

Measurement of Caspase-3 Activation. Caspase-3 activity was determined using a caspase-3 activity detection assay kit (Upstate, Lake Placid, NY). Briefly, cultured PC12 cells were lysed for 20 min on ice, and the lysates were centrifuged (12 000 rpm) for 10 min at 4 °C. The supernatant and the reaction buffer (containing caspase-3 substrate) were mixed and incubated for 1 h at 37 °C. Fluorescence intensity (380 nm excitation and 460 nm emission) was measured using a FLUOstar Galaxy spectrophotometer (BMG Labtechnologies GmbH, Inc., Offenburg, Germany).

Measurement of DNA Damage. DNA damage was measured by the comet assay according to the method of Colognato et al. (*26*). After treatment, PC12 cells were resuspended at 10^4 cells/100 μL in 1.0% low-melting agarose in a phosphate buffer containing 5 mM EDTA and immediately pipetted onto agarose-coated slides. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% *N*-lauroylsarcosinate, 1% Triton X-100, and 10% DMSO) for 1 h at 4 °C. The slides were then placed in an electrophoretic tray with an alkaline buffer (0.3 M NaOH and 1 mM EDTA) for 20 min to allow the DNA to unwind; electrophoresis was

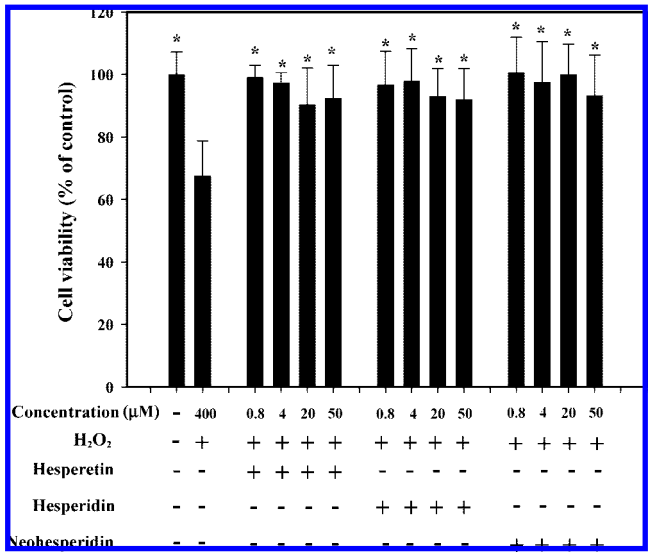

Figure 2. Cell-protective effect of citrus flavanones on H_2O_2-induced cytotoxicity in PC12 cells. The viability of control cells was defined as 100%. Data shown are means ± SD ($n = 6$) from two independent experiments. (∗) ($p < 0.05$) compared to H_2O_2-treated cells.

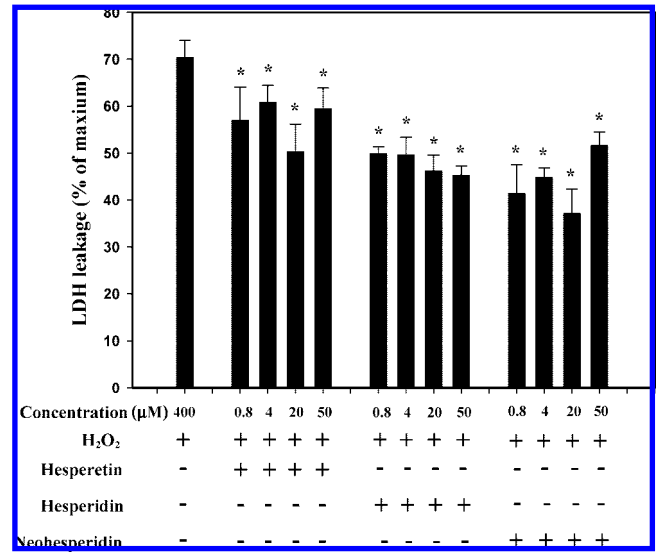

Figure 3. Inhibition of LDH release of hesperetin, hesperidin, and neohesperidin on H_2O_2-induced membrane damage in PC12 cells. LDH activity was measured using a colorimetric LDH assay kit. All data are presented as means ± SD ($n = 3$) obtained from three separate cultures. (∗) ($p < 0.05$) compared to H_2O_2-treated cells.

then performed at 300 mA for 20 min in the same alkaline buffer at 4 °C. The slides were then washed and stained with ethidium bromide. The DNA was inspected under a fluorescence microscope (EFD-3, Nikon, Tokyo, Japan) with a magnification of 200×. A total of 25 randomly selected cells on slides from duplicated treatments were analyzed using Komet 3.1 imaging software (Kinetic Imaging Ltd., Liverpool, U.K.). Results were reported as the percentage of tail DNA. A higher percentage for tail DNA represents a higher level of DNA damage.

Protein Determination. The protein concentration of cell extracts was measured by using a protein quantitation kit (Gene Research Laboratory, Taipei, Taiwan) with bovine serum albumin as the standard.

Statistical Analysis. The results are presented as means ± standard deviation (SD). Data were evaluated for significance with the one-way analysis of variation (ANOVA) procedure followed by Duncan's multiple range test by the Statistical Analysis System.

RESULTS

Flavanones Protect PC12 Cells against H_2O_2-Induced Neurotoxicity. To evaluate activities of these flavanones at physiological levels and higher concentrations without risk of inducing cytotoxicity in normal cells, we tested the samples with a concentration in the range of 0.8−50 μM. The cell viability of PC12 cells, measured by MTT reduction assay, was decreased to 68% of controls after cells were exposed to 400 μM H_2O_2 for 16 h. However, pretreatment of PC12 cells with hesperetin, hesperidin, and neohesperidin (0.8, 4, 20, and 50 μM) for 6 h significantly ($p < 0.05$) elevated the cell viability of PC12 cells to a range of 90–100% (**Figure 2**). The results clearly indicated that H_2O_2-indued cytotoxicity in PC12 cells was suppressed with hesperetin, hesperidin, and neohesperidin, even at physiological concentrations (0.8 and 4 μM).

To examine the membrane damage in H_2O_2-induced PC12 cells, an LDH release assay was used to measure LDH release in the medium and assess the protective effects of hesperetin, hesperidin, and neohesperidin against the LDH cytotoxicity. The results showed that treatment with 400 μM H_2O_2 for 16 h caused an LDH cytotoxicity (percentage of maximum release) of 70% and preincubation with hesperetin, hesperidin, and neohesperidin (0.8, 4, 20, and 50 μM) for 6 h significantly ($p < 0.05$) decreased the LDH release to 37–61% in PC12 cells (**Figure 3**).

Flavanones Inhibit H_2O_2-Induced Intracellular ROS Increase in PC12 Cells. The effects of hesperetin, hesperidin, and neohesperidin on the intracellular ROS level of PC12 cells were examined by using a flow cytometric DCFH-DA assay for measuring 2′,7′-dichlorofluorescein (DCF) fluorescence. DCFH diacetate can traverse cell membranes and be hydrolyzed to DCFH by intracellular esterase. Then, DCFH reacts with peroxides to form fluorescent DCF. Exposure of the cells to 400 μM H_2O_2 for 16 h significantly increased the intracellular ROS level to 133% of the control (**Table 1**). Such intracellular ROS accumulation was significantly ($p < 0.05$) eliminated by pretreatment (6 h) with these flavanones. Hesperetin and neohesperidin reduced the level of ROS by 16–24%, while hesperidin reduced the level of ROS by 32–48% in H_2O_2-indued PC12 cells.

Flavanones Inhibit H_2O_2-Induced Increase of Intracellular Calcium Concentration ($[Ca^{2+}]_i$) in PC12 Cells. Exposure of cells to H_2O_2 (400 μM) for 16 h resulted in an increase of $[Ca^{2+}]_i$ by 1.9-fold as compared to the control (**Table 1**). Preincubation of cells with flavanones for 6 h, at both 0.8 and 50 μM, caused significant ($p < 0.05$) reductions of the elevated $[Ca^{2+}]_i$ by 14−38% and 46−59%, respectively. Thus, the neuroprotective action of hesperetin, hesperidin, and neohesperidin on PC12 cells includes the reduction of H_2O_2-induced $[Ca^{2+}]_i$ elevation.

Flavanones Attenuate H_2O_2-Induced Decrease of MMP in PC12 Cells. Exposure of PC12 cells to H_2O_2 (400 μM) for 16 h led to a significant ($p < 0.05$) drop, with 75% reduction in MMP relative to the control. Pretreatment with hesperetin, hesperidin at 0.8–50 μM, and neohesperidin at 50 μM significantly ($p < 0.05$) elevated the decrease in MMP from 1 to 2.5-fold (**Table 1**). Hesperetin (20–50 μM), in particular, was almost able to completely inhibit the decreased MMP induced by H_2O_2.

Effects of Flavanones on Antioxidant Enzyme Activities. Besides antioxidative nutrients, antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase (GSH-Px), and glutathione reductase (GR) are the primary defense mechanisms to protect living systems from oxidative damage. As shown in **Figure 4**, exposure of PC12 cells to H_2O_2 (400 μM) for 16 h significantly ($p < 0.05$) reduced the activities of catalase (**Figure 4A**), GSH-Px (**Figure 4B**), and GR (**Figure**

Table 1. Effects of Citrus Flavanones on the Change of ROS Level, $[Ca^{2+}]_i$, Mitochondrial Membrane Potential (MMP), and Caspase-3 Activity in H_2O_2-Induced PC12 Cells[a]

treatment	ROS (percentage of control)	$[Ca^{2+}]_i$ (percentage of control)	MMP (percentage of control)	caspase-3 activity (fold of control)
control	100.0 ± 2.8 c[b]	100.0 ± 2.5 e	100.0 ± 16.5 a	1.00 ± 0.14 cd
H_2O_2	133.2 ± 3.7 a	191.0 ± 11.1 a	25.0 ± 2.5 d	2.75 ± 0.20 a
Htn (0.8 μM) + H_2O_2	109.6 ± 12.6 bc	156.0 ± 4.2 c	60.9 ± 8.6 c	1.31 ± 0.18 bc
Htn (50 μM) + H_2O_2	118.0 ± 5.6 b	148.4 ± 11.1 cd	87.2 ± 9.4 ab	1.50 ± 0.09 b
Hdn (0.8 μM) + H_2O_2	101.6 ± 10.4 c	172.0 ± 19.5 b	51.2 ± 7.8 c	0.90 ± 0.07 d
Hdn (50 μM) + H_2O_2	85.8 ± 6.6 d	137.3 ± 5.2 d	55.3 ± 4.9 c	1.23 ± 0.18 cb
Ndn (0.8 μM) + H_2O_2	112.1 ± 15.5 bc	178.5 ± 6.8 b	34.1 ± 13.8 d	0.68 ± 0.09 d
Ndn (50 μM) + H_2O_2	117.7 ± 19.2 b	149.2 ± 7.7 cd	49.3 ± 5.7 c	0.71 ± 0.18 d

[a] PC12 cells were pretreated with the flavanones for 6 h and then exposed to 400 μM H_2O_2 for additional 16 h. Htn, hesperetin; Hdn, hesperidin; Ndn, neohesperidin. [b] Data are represented as means ± SD ($n = 3-6$). Means in a column followed by different letters are significantly at a level of $p < 0.05$ using ANOVA and Duncan's test.

4C) by 59, 30, and 42%, respectively, as compared to controls. Pretreatment of PC12 cells with all flavanones tested for 6 h significantly ($p < 0.05$) inhibited the decrease of catalase activity induced by H_2O_2 while the decreasing activities of GSH-Px and GR were significantly ($p < 0.05$) attenuated in hesperetin- or hesperidin-treated cells only.

Flavanones Inhibit H_2O_2-Induced Elevation of Caspase-3 Activity in PC12 Cells. H_2O_2 (50–500 μM) was reported to elevate caspase-3 activity and cause apoptosis in PC12 cells (*20, 27*). As shown in **Table 1**, we confirmed that exposure of PC12 cells to H_2O_2 (400 μM) for 16 h led to a significant ($p < 0.05$) elevation of caspase-3 activity of 2.8-fold relative to the control. Pretreatment of PC12 cells with flavanones tested for 6 h significantly ($p < 0.05$) inhibited the elevation of caspase-3 activity induced by H_2O_2. Moreover, the inhibitory activity of hesperidin (0.8 μM) and neohesperidin (0.8–50 μM) on caspase-3 activity in H_2O_2-induced PC12 cells was significantly ($p < 0.05$) greater than that of hesperetin (0.8–50 μM).

Effects of Flavanones on the Attenuation of DNA Damage in H_2O_2-Induced PC12 Cells. Accumulating hydrogen peroxides are known to interact with metal ions in cells to generate hydroxyl radicals via Fenton reaction. These hydroxyl radicals can attack DNA and cause strand breaks. As shown in **Figure 5**, exposure of PC12 cells to H_2O_2 (400 μM) for 16 h resulted in a significant elevation of DNA damage, as compared to the control. Pretreatment of PC12 cells with hesperetin or hesperidin at 0.8 to 50 μM and neohesperidin at 0.8 to 4 μM significantly ($p < 0.05$) attenuated DNA damage induced by H_2O_2. Furthermore, the attenuating effects of these flavanones on DNA damage in H_2O_2-induced PC12 cells were almost in a reverse dose-dependent manner.

DISCUSSION

In the present study, we have demonstrated that citrus flavanones, such as hesperidin, hesperetin, and neohesperidin exhibit neuroprotection against H_2O_2-induced cytotoxicity in PC12 cells. Our data show that neuroprotective effects of the flavanones are at least attributed to their antioxidant properties, calcium ion regulation, and the attenuation of caspase-3 activity. Moreover, the physiologically relevant low concentration of hesperetin used in this study is readily available (0.6–3.8 μM) through the consumption of orange juice (*11*).

Hesperidin, hesperetin, and neohesperidin are known to exhibit radical/ROS-scavenging activity in vitro (*5, 28*). Our present study shows that the flavanones have obvious intracellular ROS scavenging activity in H_2O_2-induced PC12 cells (**Table 1**). Hence, we suggest that the protective effects of these flavanones on H_2O_2-induced cellular death are at least attributed to their ROS scavenging activities. Moreover, in terms of their lower antioxidant activity compared to that of hesperetins (*5*), the higher intracellular ROS scavenging activity of hesperidins are attributed to their chemical structure with hydrophilic glucoside, which makes them reside more in the cytosolic compartment of cells. In contrast, the more lipophilic compounds of hesperetin may preferentially associate with the plasma membranes at the cellular surface. Because our samples were preincubated with the cells, their activities only took place intracellularly.

Ca^{2+} participates in the survival and death of neural cells (*13*). Neurotoxic Aβ and 6-hydroxydopamine (6-OHDA) disrupt cellular calcium homeostasis by elevating $[Ca^{2+}]_i$ and generating H_2O_2. In addition, ROS causes mitochondrial oxidative stress, leading to Ca^{2+} accumulation, ROS formation, and membrane damage, resulting in the disruption of calcium homeostasis and cytochrome *c* release (*13, 29*). Our present results (**Table 1**) and data from previous studies (*20*) show that H_2O_2 exposure causes $[Ca^{2+}]_i$ elevation in PC12 cells. Flavanones used in our present study could attenuate the elevation of $[Ca^{2+}]_i$ induced by H_2O_2. The activity of the stabilizing Ca^{2+} homeostasis of these flavanones contributes to their neuroprotection against H_2O_2-induced cytotoxicity in PC12 cells and may also be attributed to their intracellular ROS scavenging activities, which contribute to a decrease in membrane-associated oxidative stresses that lead to $[Ca^{2+}]_i$ changes.

Mitochondria dysfunction includes a decrease of MMP and reduced ATP generation (*17*). Abnormal calcium uptake, accompanied by oxidative stress, into mitochondria promotes the opening of the mitochondrial permeability transition pore that causes a collapse of MMP and leads to the cytochrome *c* release preceding caspase-3 activation and apoptosis (*30*). In this study, treatment with H_2O_2 led to a dramatic loss of MMP in PC12 cells. Accordingly, it was thought that H_2O_2 steadily diffused into mitochondria and caused oxidative stress as well as calcium accumulation, resulting in the loss of MMP. We suggested that attenuating effects of flavanones on H_2O_2-induced MMP loss in this study were partly attributed to their antioxidant properties. Consequently, they might suppress a mitochondrion-mediated apoptotic pathway in the H_2O_2-induced PC12 cells via MMP maintenance.

H_2O_2 accumulation during many neurotoxic insults leads to cellular oxidative stress. Therefore, the elimination of H_2O_2 is critical for reducing oxidative stress. The pivotal cellular enzymes for eliminating H_2O_2 are catalase and GSH-Px. In the present study, decreases in cell viability and catalase activity were suppressed in cells preincubated with all flavanones used, while the decreased activities of GSH-Px and GR were only suppressed in hesperidin- and hesperetin-pretreated cells. The results indicate that suppression of the decrease of CAT activity in H_2O_2-induced PC12 cells alone can contribute to the

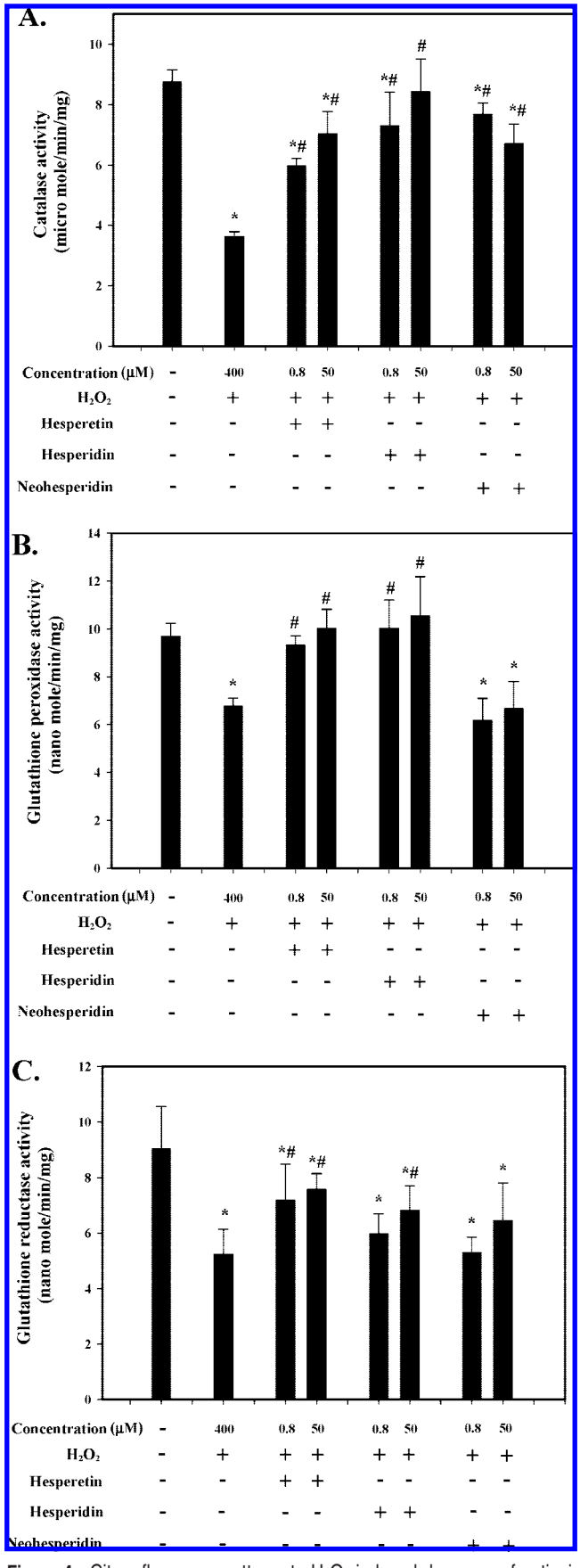

Figure 4. Citrus flavanones attenuate H_2O_2-induced decrease of antioxidant activities in PC12 cells. (A) catalase activity, (B) glutathione peroxidase activity, and (C) glutathione reductase activity. All data represent means ± SD ($n = 4$) from two independent experiments. (∗) ($p < 0.05$) compared to control cells. (#) ($p < 0.05$) compared to H_2O_2-treated cells.

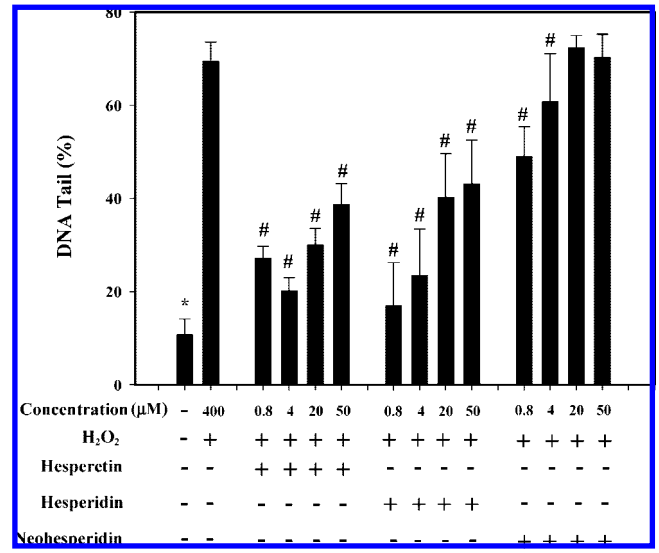

Figure 5. Effect of citrus flavanones on H_2O_2-induced DNA damage in PC12 cells. DNA damage was measured by Comet assay. Data represent means ± SD ($n = 25$) from two independent experiments. Significant difference (∗, $p < 0.05$) was observed at control cells compared to other groups. (#) ($p < 0.05$) compared to H_2O_2-treated cells.

prevention decreases in cell viability. However, the low activities of GSH-Px and GR of H_2O_2-induced PC12 cells with neohesperidin pretreatment might lead the cells with weaker protection from oxidative damage, because the suppression of GR activity leads to glutathione depletion and could result in DNA damage during cellular oxidative stress.

Caspase-3 acts as an apoptotic executor and can be activated by both H_2O_2 and the disruption of intracellular homeostasis of Ca^{2+}. In this study, activities of caspase-3 with hesperidin or neohesperidin-treated cells were clearly lower than those of hesperetin-pretreated cells. This could be attributed to the difference between their chemical structures. Because Chen et al. (*31*) have demonstrated that rutinoside at C7 in flavonoids prevents the caspase-3-mediated apoptosis. However, the casapse-3 activities of high concentration hesperetin- or hesperidin-pretreated cells were higher than those of the related low concentration-treated cells. These results are in accordance with the increasing levels of DNA damage seen in the higher concentration flavanone-treated cells (**Figure 5**). Similarly, Zeng et al. (*32*) have shown that genistein inhibits the increase of casapse-3 activity with a reverse dose dependency in amyloid-induced hippocampal cells. Accordingly, the present results indicated that there might be more mechanisms underlying the inhibition of flavanones (low concentration) on H_2O_2-triggered activation of casapse-3 besides their antioxidant properties and $[Ca^{2+}]_i$ regulation.

Hydrogen-peroxide-associated ROS-mediated DNA damage takes place via direct attack on chromosome or mitochondria pathways, leading to necrosis or apoptosis (*14, 33*). Oxidative stress-induced cellular death can be prevented by blocking DNA damage. Our present results suggest that the flavanones protect H_2O_2-induced PC12 cells from both apoptosis and necrosis by blocking DNA damage. Additionally, our data that low concentration of flavanones have greater capacity to suppress DNA damage is in accordance with former studies (*32, 34*). They demonstrated that physiological concentrations (0.1−100 nM) of genistein show neuroprotection in neural cells from amyloid-induced cytotoxicity by blocking DNA damage via the estrogen receptor-mediated pathway, in addition to the antioxidant properties.

In summary, hesperidin, hesperetin, and neohesperidin have neuroprotective effects against H_2O_2-induced cytotoxicity in PC12 by diverse mechanisms, suggesting that these flavanones may be useful in more complete intervention for neural disorders. However, mechanisms underlying the neuroprotective activity of these flavanones against oxidative stress at physiological concentrations other than those mentioned in the present study need further investigation. Moreover, the present data suggest that citrus fruits have potential as functional foods for neuroprotection.

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