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Modulation of Akt, JNK, and p38 Activation Is Involved in Citrus Flavonoid-Mediated Cytoprotection of PC12 Cells Challenged by Hydrogen Peroxide

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The physiological benefits of dietary flavonoids have been attributed to their antioxidant and signaling properties. Our previous study revealed that hesperetin exhibits neuroprotection in PC12 cells by diverse mechanisms. Biological activities of flavonoids might be determined by their chemical structures. Here, we further studied the effects of hesperetin and its structural counterparts, isorhamnetin and isosakuranetin, on kinases related to survival signaling as well as other cytoprotective actions. Pretreatment with flavonoids (0.8 or 50 μM) increased cell viability and catalase activity (CA) and decreased membrane damage, reactive oxygen species (ROS) generation, intracellular calcium level ($[Ca^{2+}]_i$), and caspase-3 activity in H_2O_2-treated PC12 cells. Increased CA, $[Ca^{2+}]_i$, and ROS levels, but lower caspase-3 activities, were obtained upon treatment with 50 μM isorhamnetin or isosakuranetin. Based on their structural differences and the concentrations used, these flavonoids differentially activated pro-survival signaling molecules, including Akt/protein kinase B, p38 mitogen-activated protein kinase, and inhibited the activation of c-jun N-terminal kinase, which triggers apoptosis. Our results demonstrate that signaling actions of thses flavonoids are involved in their neuroprotection against oxidative stress and that they act more as signaling molecules than antioxidants.

KEYWORDS: Neuroprotection; hesperetin; isorhamnetin; isosakuranetin; signaling molecules; antioxidant; Akt

INTRODUCTION

Oxidative stress likely plays a critical role in various neurodegenerative processes, such as Alzheimer's and Parkinson's diseases, as well as in brain ischemia/reperfusion injury (*1*−*3*). Oxidative stress causes damage to lipids, proteins, and DNA in neural cells in addition to altering intracellular calcium homeostasis, which leads to apoptosis, neurotoxicity, and neurodegenerative disorders (*2*). Hence, approaches to intervene in neurodegenerative disorders have increasingly proposed the use of antioxidants.

Dietary flavonoids have received great attention as potential anticancer agents, cardioprotectants, and inhibitors of neurodegeneration because of their antioxidant properties, bioavailability, and, most importantly, their modulation of the activation of kinase signaling pathways (*4*). Flavonoid modulation of regulatory and signaling pathways not only triggers pro-cell survival (protection) and pro-apoptotic (chemoprevention) responses, but also elevates endogenous antioxidant levels (*4*−*6*). Signaling properties of flavonoids might be derived from electrophilic actions after hydrogen donation as well as their structural affinity for proteins, which can alter the phosphorylation of target molecules related to regulatory pathways and modulation of gene expression. However, flavonoids may also primarily affect signaling or regulatory pathways via calcium homeostasis regulation and mitochrondrial maintenance (*4*).

Hydrogen peroxide-mediated oxidative stress occurs in neurotoxic β-amyloid aggregation (*7*), dopamine oxidation (*8*), and brain ischemia/reperfusion (*3*). Such oxidative damage could lead to mitochrondrial dysfunction, calcium imbalance and apoptosis in neuronal cells (*9*, *10*). Our previous study (*11*) showed that the citrus flavanones hesperidin, hesperetin, and neohesperidin have neuroprotective activity against H_2O_2-induced cytotoxicity in PC12 cells by diverse mechanisms including antioxidant activity, regulation of intracellular calcium ions, and inhibition of caspase-3 activity. We also suggested the involvement of other mechanisms underlying neuroprotection of the flavanones against oxidative damage. In the present study, we further evaluated the molecular signaling properties of hesperetin, a bioavailable (0.6−3.8 μM) metabolite of hesperidin by consumption of orange juice (*12*). We specifically focused on the activation of kinases such as the serine/threonine

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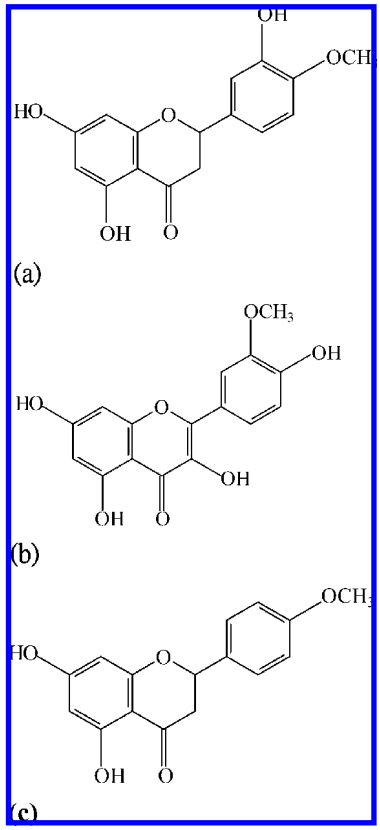

Figure 1. Structures of flavonoids: (**a**) hesperetin, (**b**) isorhamnetin, (**c**) isosakuranetin.

kinase Akt/PKB and members of the mitogen-activated protein kinase (MAPK) family, p38 and c-Jun N-terminal kinase (JNK). Because Akt/PKB is known to activate pro-survival signaling pathways (*13*), whereas flavonoid modulation of MAPK signaling may lead to cellular survival responses or apoptosis (*14*).

The chemical structures of flavonoids can determine their bioactivity, especially in terms of the number and substitution of hydroxyl groups on the B ring and degree of unsaturation of the C2−C3 bond (*4*). In this study, neuroprotection by other citrus flavonoids, such as isorhamnetin and isosakuranetin, with different chemical structures at positions mentioned above were also evaluated; these structures are illustrated in **Figure 1**. Isorhamnetin and isosakuranetin are metabolites of quercetin and naringin, respectively (*15*, *16*). This study provides evidence for a clear understanding of mechanisms of these flavonoids, either as antioxidants or modulators of cell signaling.

MATERIALS AND METHODS

Materials. Materials for cell culture, hesperetin, 30% hydrogen peroxide (w/w) solution, and antiphospho-Akt/PKB, antiphospho-JNK, antiphospho-p38, and anti-α-tubulin antibodies were purchased from Sigma-Aldrich Co. (St. Louis, MO). Isorhamnetin and isosakuranetin were obtained from Extrasynthase (Z.I. Lyon Nord, Genay Cedex, France).

Cell Culture and Treatment. PC12 cells were purchased from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were maintained in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated horse serum (Gibco, Carlsbad, CA) and 5% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) in humidified 5% CO_2 /95% air at 37 °C. The medium was changed every other day. Before treatment, cells were plated at an appropriate density on culture plates or dishes and cultured for 24 h. In all experiments, cells were pretreated for 6 h with 0.8 or 50 μM flavonoids in low serum (one-third of full serum) medium. The medium was then refreshed without addition of flavonoids and supplemented with 400 μM H_2O_2 for another 16 h, except where indicated.

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, succinate dehydrogenase, in the mitochondrial respiratory chain of live cells. After incubation, cells were treated with MTT solution (final concentration, 0.5 mg/mL) for 3 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and their optical absorbance at 570 nm was measured using a FLUO star galaxy spectrophotometer (BMG Labtechnologies GmbH Inc., Offenburg, Germany).

Lactate Dehydrogenase (LDH) Release Assay. The LDH assay is 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 generated NADH is utilized for stoichiometric conversion of tetrazolium dye. 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 were transferred into new wells, and LDH content 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 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) method described by Takahashi et al. (*17*) was used to measure intracellular ROS production. After culture with or without treatments, cells were collected, resuspended in PBS, and incubated with DCFH-DA at a final concentration of 20 μM for 30 min at 37 °C. Then cells were washed once with PBS and harvested for fluorescence activated cell sorter (FACS) analysis. These cells were excited with a 488 nm argon ion laser in a flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). DCF emission was recorded at 525 nm. Data were collected with at least 10,000 events.

Intracellular Ca^{2+} Level Detection. Intracellular Ca^{2+} levels were measured by the method of Chen et al. (*18*) using flow cytometry. Briefly, cultured cells with or without treatments were stained with Fluo3-AM (Sigma) 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) intensity in the FL1 channel as calculated by the distribution histogram with cell counts on the vertical axis and fluorescence intensity on a log scale on the horizontal axis.

Assay for Catalase Activity. PC12 cells (1×10^5 cells/mL) were plated and cultured for 24 h. After experimental treatments, cells were lysed on ice and centrifuged (12000*g*, 10 min) at 4 °C. The supernatants were collected for enzyme activity assay. Briefly, catalase activity was assessed according to the method of Cohen et al. (*19*). 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. Catalase activity was calculated from the standard curve and expressed as μmol/min/mg protein.

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 (12000*g*) 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 FLUO star galaxy spectrophotometer (BMG Labtechnologies GmbH Inc., Offenburg, Germany).

Western Blotting. Cells were washed with PBS, then incubated for 30 min at 4 °C in lysis buffer (RIPA, Millipore, Bedford, MA), preceding centrifugation at 12000*g* for 10 min at 4 °C. Western blotting was performed according to the method described by Maiti and Chen (*20*) with some modifications. Briefly, supernatant samples were boiled for 10 min in sample buffer (0.3 M Tris-HCl (pH 6.8), 25%

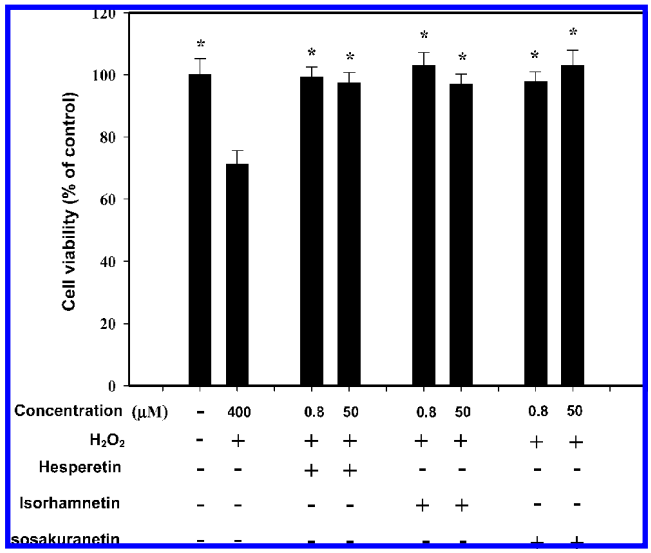

Figure 2. Cell-protective effect of citrus flavonoids on H_2O_2-induced cytotoxicity in PC12 cells. The viability of control cells was defined as 100%. Data shown are means ± SD ($n = 6$). * ($P < 0.05$) compared with H_2O_2-treated cells.

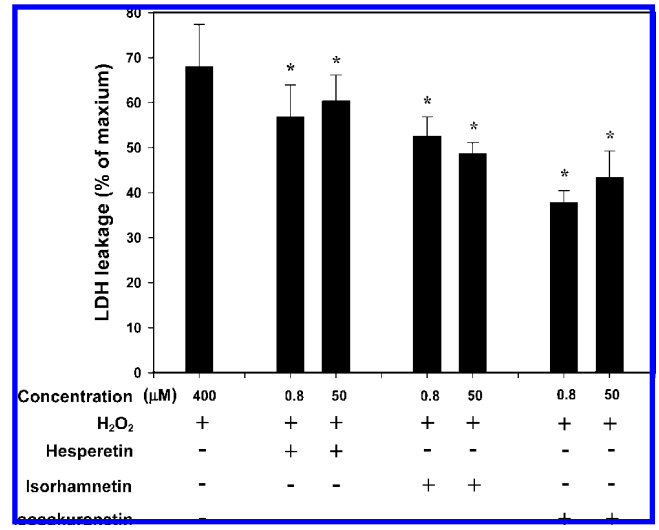

Figure 3. Inhibition of LDH release by citrus flavonoids in an H_2O_2-induced model of membrane damage in PC12 cells. LDH activity was measured using a colorimetric LDH assay kit. All data are presented as means ± SD ($n = 6$). * ($P < 0.05$) compared with H_2O_2-treated cells.

2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol and 0.1% bromophenol blue), then subjected to 10% SDS−polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (PVDF; Millipore, Bedford, MA). The membranes were then blocked in PBS with 0.1% Tween 20 containing 5% nonfat dried milk for 1 h at ambient temperature and immunoblotted at 4 °C overnight with antibodies against p-Akt/PKB, p-JNK and p-p38 MAPKs, and α-tubulin. After incubation with HRP-labeled secondary antibody for 1 h at room temperature, blots were developed using ECL (Enhance Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) and densitometric measurements of the bands were performed using LabWorks 4.5 (UVP, Upland, CA).

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 ± SD. Data were evaluated for significance with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the Statistical Analysis System.

RESULTS

Flavonoids Protect PC12 Cells against H_2O_2-Induced Cytotoxicity. The viability of PC12 cells significantly ($p < 0.05$) decreased to 71% of controls after cells were exposed to 400 μM H_2O_2 for 16 h. Pretreatment with hesperetin, isorhamnetin, and isosakuranetin (0.8 or 50 μM, 6 h) significantly ($p < 0.05$) inhibited this decrease (**Figure 2**). With respect to membrane damage, treatment with 400 μM H_2O_2 for 16 h caused LDH cytotoxicity (percentage of maximum release) of 68% and pretreatment with flavonoids significantly ($p < 0.05$) decreased the LDH release to 38−60% (**Figure 3**). Our present results show that flavonoids can suppress H_2O_2-indued cytotoxicity in PC12 cells at the physiologically relevant dose of 0.8 μM.

Flavonoids Inhibit H_2O_2-Induced Intracellular ROS Formation. Exposure of the cells to 400 μM H_2O_2 for 16 h significantly ($p < 0.05$) increased the intracellular ROS level to 137% of the control (**Table 1**). Such elevation of intracellular ROS was inhibited by pretreatment (6 h) with flavonoids. Hesperetin and isosakuranetin treatment reduced the level of ROS by 18−37%, while isorhamnetin reduced the level of ROS by 28−39% in H_2O_2-induced cells. Isorhamnetin and isosakuranetin showed greater ROS scavenging activity than hesperetin at low concentrations. Interestingly, higher levels of ROS were found in cells treated with high concentrations of isorhamnetin ($p > 0.05$) or isosakuranetin ($p < 0.05$).

Flavonoids Attenuate H_2O_2-Induced Increase of Intracellular Calcium Concentration ($[Ca^{2+}]_i$). Exposure of PC12 cells to 400 μM H_2O_2 for 16 h resulted in a 170% increase of $[Ca^{2+}]_i$ as compared to controls (**Table 1**). Pretreatment of cells with flavonoids for 6 h, at both 0.8 and 50 μM, significantly ($p < 0.05$) reduced the elevation of $[Ca^{2+}]_i$ by 15-71% and 34-38%, respectively. There was no dose−response effect of hesperetin on the reduction of elevated $[Ca^{2+}]_i$, although there was a negative dose−response effect found in isorhamnetin- or isosakuranetin-treated cells, and the high dose of isorhamnetin had no effect on the reduction of elevated $[Ca^{2+}]_i$.

Flavonoids Inhibit H_2O_2-Induced Decreases in Catalase Activity. Exposure of PC12 cells to 400 μM H_2O_2 for 16 h significantly ($p < 0.05$) reduced catalase activity by 55% as compared to the control (**Table 1**). Pretreatment of PC12 cells with flavonoids for 6 h significantly ($p < 0.05$) suppressed this decrease. Higher values of CA were found in cells treated with 50 μM flavonoids, but were not significantly ($p > 0.05$) different from values for 0.8 μM flavonoid-treated cells.

Flavonoids Inhibit H_2O_2-Induced Elevation of Caspase-3 Activity. After PC12 cells were exposed to 400 μM H_2O_2 for 16 h, their caspase-3 activity was significantly ($p < 0.05$) elevated by 245% relative to the control (**Table 1**). Pretreatment of cells with flavonoids for 6 h significantly ($p < 0.05$) inhibited the elevation of caspase-3 activity. Effects of hisperetin were not concentration-dependent, but cells treated with 50 μM isorhamnetin or isosakuranetin showed low caspase-3 activity.

Flavonoids Affect p38 Activation in H_2O_2-Treated PC12 Cells. As shown in **Figure 4**, exposure of PC12 cells to 400 μM H_2O_2 for 1 h significantly ($p < 0.05$) increased phosphorylation of p38 by 4-fold as compared to controls and such activation of p38 decreased to near the same level of controls at 3 h. Pretreatment of cells with 0.8 μM hesperetin or isorhamnetin for 6 h significantly ($p < 0.05$) inhibited the H_2O_2-induced activation of p38 observed at 1 h, especially in cells treated with hesperetin. Meanwhile, pretreatment of cells with

Table 1. Effects of Citrus Flavonoids on Intracellular ROS Level, $[Ca^{2+}]_i$, and Catalase and Caspase-3 Activities in H_2O_2-Treated PC12 Cells[a]

treatment	ROS (% of control)	$[Ca^{2+}]_i$ (% of control)	CA (μmol/min/mg)	caspase-3 activity (% of control)
control	100.0 ± 4.1 c[b]	100.0 ± 2.6 d	8.83 ± 0.84 a	100.0 ± 2.5 bc
H_2O_2	137.0 ± 14.0 a	167.8 ± 14.6 a	4.01 ± 0.71 c	245.4 ± 18.3 a
Htn (0.8 μM) + H_2O_2	116.7 ± 12.0 b	137.5 ± 16.3 c	6.10 ± 0.52 b	117.3 ± 16.3 b
Htn (50 μM) + H_2O_2	107.8 ± 15.0 bc	130.1 ± 11.1 c	6.83 ± 0.79 ab	113.9 ± 34.6 b
Ish (0.8 μM) + H_2O_2	98.3 ± 6.4 c	153.1 ± 7.4 b	7.82 ± 1.15 ab	88.3 ± 8.3 bcd
Ish (50 μM) + H_2O_2	108.7 ± 14.4 bc	173.6 ± 17.5 a	8.69 ± 1.14 a	66.0 ± 15.8 d
Isk (0.8 μM) + H_2O_2	99.7 ± 3.5 c	96.8 ± 13.5 d	5.93 ± 0.61 b	112.3 ± 7.9 b
Isk (50 μM) + H_2O_2	119.2 ± 10.1 b	134.4 ± 1.9 c	7.96 ± 1.07 ab	73.3 ± 17.6 cd

[a] PC12 cells were pretreated for 6 h with the flavonoids, then exposed to 400 μM H_2O_2 for an additional 16 h. CA, catalase activity; Htn, hesperetin; Ish, isorhamnetin; Isk, isosakuranetin. [b] Data are represented as means ± SD (n = 3−6). Means in a column followed by different letters are significantly different at a level of $P < 0.05$ using ANOVA and Duncan's test.

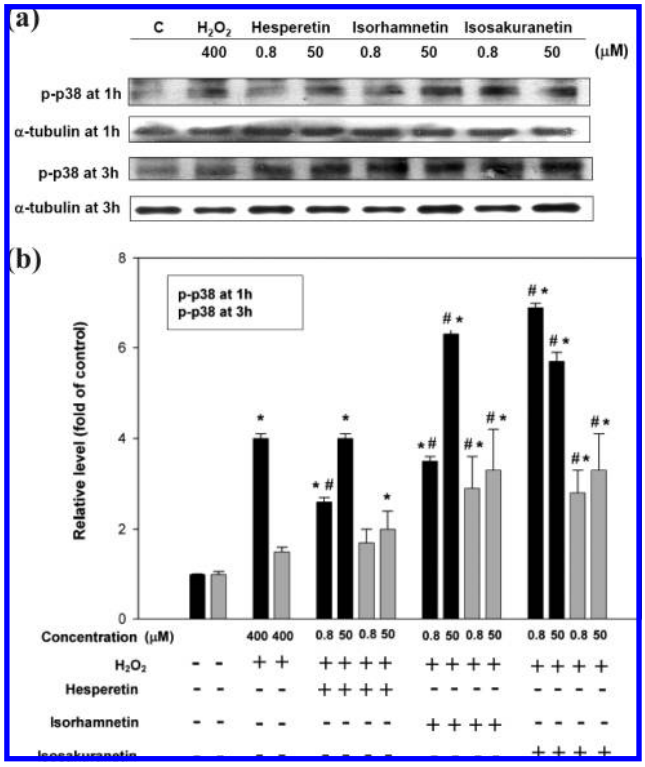

Figure 4. Effects of citrus flavonoids on p38 activation in H_2O_2-treated PC12 cells. Cells were pretreated for 6 h with 0.8 or 50 μM samples. Then, the medium was refreshed and cells were exposed to 400 μM H_2O_2 for 1 and 3 h. Cells were lysed, and the level of phosphorylated-p38 (p-p38) was determined by Western blotting. (**a**) Representative images from three independent experiments are shown; (**b**) the relative change in p-p38 level is expressed as fold change over the control, normalized against α-tubulin (loading control), and shown as mean ± SD (n = 3). * ($p < 0.05$) compared with control cells. # ($p < 0.05$) compared with H_2O_2-treated cells.

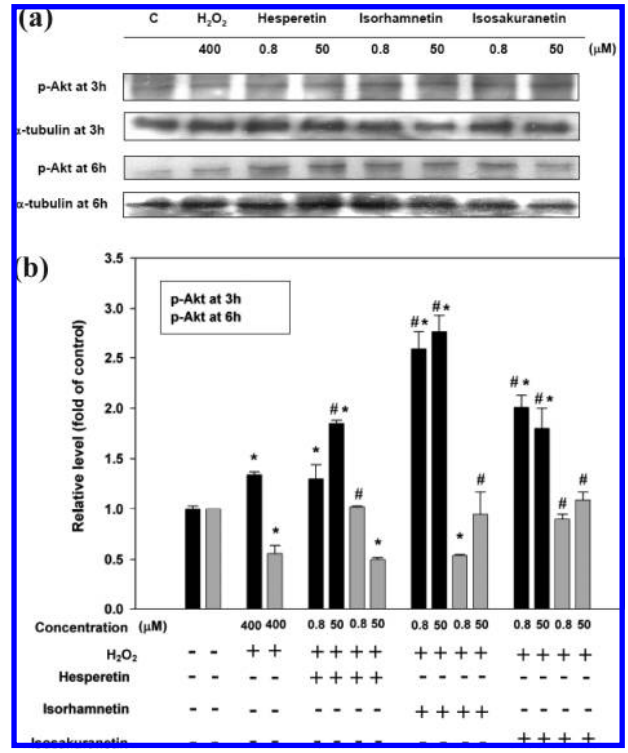

Figure 5. Citrus flavonoids affect Akt activation in H_2O_2-treated PC12 cells. Cells were pretreated for 6 h with 0.8 or 50 μM samples. The medium was refreshed and cells were exposed to 400 μM H_2O_2 for 3 and 6 h. Cells were lysed, and levels of phosphorylated-Akt (p-Akt) were determined by Western blotting. (**a**) Representative images from three independent experiments are shown; (**b**) the relative change in p-Akt level is expressed as fold change over the control, normalized against α-tubulin (loading control), and shown as mean ± SD (n = 3). * ($p < 0.05$) compared with control cells. # ($p < 0.05$) compared with H_2O_2-treated cells.

isosakuranetin or 50 μM isorhamnetin dramatically increased the elevation of phosphorylated-p38 (p-p38) in exposure to H_2O_2. Additionally, after exposure to H_2O_2 for 3 h, cells pretreated with isorhamnetin or isosakuranetin still exhibited obvious ($p < 0.05$) activation of p38 as compared to H_2O_2-treated cells.

Flavonoids Modulate Akt Activation in H_2O_2-Induced PC12 Cells. In accordance with a previous study (*21*), phosphorylation of Akt was stimulated by H_2O_2 to a level near 2-fold over the control at 1 h (data not shown). As shown in **Figure 5**, exposure of the cells to H_2O_2 for 3 h the level of phosphorylated-Akt (p-Akt) increased to 1.3-fold over that of controls ($p < 0.05$), and the level of p-Akt ($p < 0.05$) was clearly lower than that of controls at 6 h. Pretreatment of cells with 50 μM hesperetin or both concentrations of isorhamnetin or isosakuranetin resulted in a significant elevation of p-Akt in H_2O_2-treated PC12 cells at 3 h. Meanwhile, isorhamnetin exhibited a clearly stronger effect on Akt phosphorylation. In contrast to pretreatment with 0.8 μM hesperetin, 50 μM hesperetin did not prevent the decrease of Akt activation in H_2O_2-treated PC12 cells at 6 h. However, treatment with isosakuranetin or 50 μM isorhamnetin inhibited the loss of Akt activation.

Flavonoids Inhibit JNK Activation in H_2O_2-Induced PC12 Cells. After stimulation with H_2O_2 for 16 h, the level of phosphorylated JNK (p-JNK) was significantly ($p < 0.05$) elevated to 3-fold over that of the control (**Figure 6**). Pretreatment with flavonoids effectively ($p < 0.05$) suppressed JNK

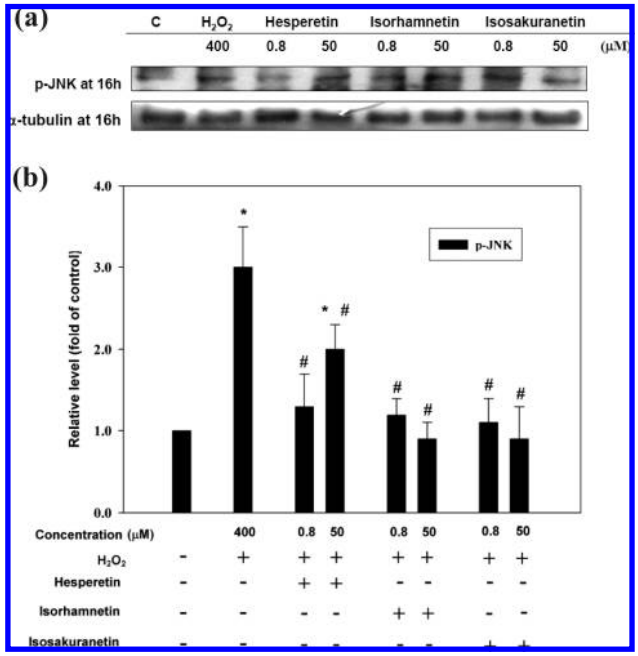

Figure 6. Citrus flavonoids inhibit JNK activation in H_2O_2-treated PC12 cells. Cells were pretreated for 6 h with 0.8 or 50 μM samples. Medium was refreshed and cells were exposed to 400 μM H_2O_2 for 16 h. Cells were lysed, and levels of phosphorylated-JNK (p-JNK, p54) were determined by Western blotting. (**a**) Representative images from three independent experiments are shown; (**b**) the relative change in p-JNK level is expressed as fold change over the control, normalized against α-tubulin (loading control), and shown as mean ± SD ($n = 3$). * ($p < 0.05$) compared with control cells. # ($p < 0.05$) compared with H_2O_2-treated cells.

activation. It is interesting that hesperetin showed an inverse relationship between concentration and inhibition of JNK activation at 16 h.

DISCUSSION

This study demonstrated that modulation of the activation of signaling kinases such as Akt/PKB, JNK and p38 is involved in cytoprotection by hesperetin, isorhamnetin, and isosakuranetin in hydrogen peroxide-treated PC12 cells. Such modulation presents an additional mechanism of action for these flavonoids in addition to their antioxidant properties, calcium homeostasis regulation and inhibition of caspase-3 activity. Our present data also suggest that these flavonoids act as more signaling molecules than antioxidants in this study.

Hydrogen peroxide-induced oxidative stress can lead PC12 cells and neural cells to apoptosis (*21*, *22*). Our present results show that these flavonoids inhibit ROS formation in H_2O_2-treated PC12 cells; this activity contributes to their neuroprotective effect against oxidative damage. Additionally, it is interesting that ROS scavenging activities of the flavonoids at low concentration were obviously different between hesperetin and isorhamnetin as well as between hesperetin and isosakuranetin. However, there were no significant differences ($p > 0.05$) in intracellular ROS levels after treatment with high concentration of the different flavonoids. Besides scavenging activity of ROS, flavonoids are known to elevate endogenous antioxidant expressions (*6*). We thus suggest that the chemical structure of these flavonoids might determine their bioactivity at low concentration.

$[Ca^{2+}]_i$ is a primary indicator of cell activation and function (*23*). The elevation of $[Ca^{2+}]_i$ during oxidative stress in neural cells has been well documented (*13*, *24*, *25*). Altered intracellular Ca^{2+} homeostasis can also lead to oxidative stress, apoptosis, and neurotoxicity (*2*). Our present study showed that elevation of $[Ca^{2+}]_i$ in H_2O_2-treated PC12 cells was inhibited by hesperetin, isorhamnetin, and isosakuranetin. Thus, $[Ca^{2+}]_i$ regulation also contributes to the neuroprotective effects of these flavonoids against oxidative damage. Moreover, an obvious difference in the inhibition of the $[Ca^{2+}]_i$ increase was found between cells treated with low concentration of the different flavonoids. This indicates that other mechanisms besides ROS scavenging activities were involved in $[Ca^{2+}]_i$ regulation, and that chemical structure might influence this activity at low concentrations.

Caspase-3 is an apoptotic executor and activated by both H_2O_2 and disruption of $[Ca^{2+}]_i$ (*26*, *27*). A previous study (*28*) showed that general inhibition of caspases and inducible catalase expression completely attenuated cytotoxicity in H_2O_2-treated PC12 cells. Our present study demonstrated that flavonoids suppressed the increased caspase-3 activity increase and maintained CA levels in H_2O_2-treated PC12 cells. Furthermore, based on the lack of obvious differences in ROS and $[Ca^{2+}]_i$ levels as well as caspase-3 activity after hesperetin treatment and high ROS and $[Ca^{2+}]_i$ levels but low caspase-3 activity with high concentration of isorhamnetin or isosakuranetin, we suggest the possibility of different mechanisms involved in flavonoid regulation of caspase-3 activity other than antioxidant properties and $[Ca^{2+}]_i$ regulation. Recently, Vauzour et al. (*22*) demonstrated that hesperetin can activate pro-survival signaling pathways and inhibit hydrogen peroxide-induced caspase-3 activity in cortical neurons. In this regard, our present data suggest that the flavonoids function more as signaling molecules than antioxidants.

Oxidative stress can stimulate MAPK signaling pathways, resulting in cell survival or death. Generally, the activation of ERK cascades sustains cell viability, whereas those of p38 and JNK promote apoptosis. However, contrasting effects of these kinases have been reported depending on cell types and severity of the activations involved (*14*, *29*). Crossthwaite et al. (*30*) demonstrated that ERK activation did not influence neuronal viability under challenge with H_2O_2 at concentration over 300 μM. Here we only evaluated the effects of flavonoids on the activation of p38 and JNK in H_2O_2-treated PC12 cells. In contrast to isosakuranetin, hesperetin inhibited (or did not affect) the activation of p38 (**Figure 4**). These findings suggest that slight differences in the chemical structures of these flavonoids could give rise to their diverse array of bioactivity. Low concentration of hesperetin protected cells from H_2O_2-induced cytotoxicity by inhibiting the activation of p38, which triggers apoptosis signaling. However, isosakuranetin and isorhamnetin might improve cell survival via p38-dependent homeostatic mechanisms. The involvement of p38 in protection and survival mechanisms as well as triggering of such activities by relatively low concentrations of flavonoids was previously reported (*14*, *31*). Recently, it has been demonstrated that p38 is involved in a signaling pathway protecting against mitochondrial stress related to Parkinson's disease (*32*). In our previous study (*33*), we also showed that gallic acid attenuates oxidative damage through a pathway that requires p38 activation.

Akt/PKB activation triggers regulatory and signaling events that promote cell survival and prevent apoptosis (*34*). Akt/PKB is important for neuronal survival and activated during challenge by hydrogen peroxide and amyloid beta peptide (*21*, *35*). Our present results suggest that flavonoids also prevented H_2O_2-treated PC12 cells from apoptosis by enhancing Akt/PKB activation and inhibiting its oxidative degradation (**Figure 5**).

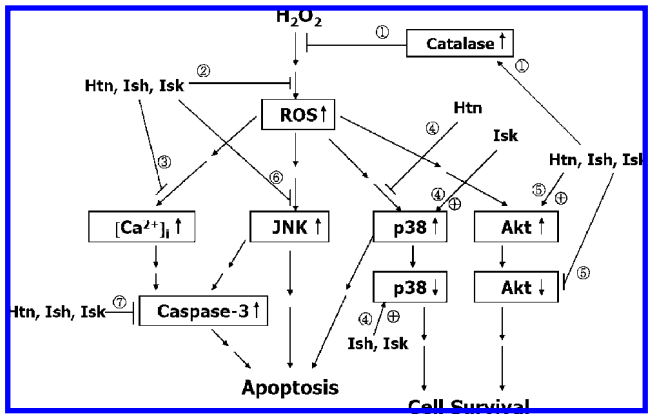

Figure 7. Possible targets of citrus flavonoid-mediated neuroprotection against H_2O_2-induced cytotoxicity in PC12 cells: (**1**) increased catalase activity to scavenge H_2O_2; (**2**) direct scavenging of ROS; (**3**) suppressed accumulation of intracellular free Ca^{2+}; (**4**) inhibition of pro-apoptotic p38 activation by low concentration of Htn, and activation of pro-survival p38 function by Ish and Isk; (**5**) modulation of Akt activity; (**6**) suppression of JNK activity; and (**7**) inhibition of caspase-3 activity.

Martín et al. (*21*) demonstrated that proteolysis of Akt/PKB occurs in H_2O_2-treated PC12 cells and reinforces the prerequisite inactivation of Akt/PKB, resulting in an irreversible commitment to apoptosis. Additionally, the activation of Akt/PKB is dependent on phosphatidylinositol 3-kinase (PI3-K), whose phosphorylation might be modulated by Ca^{2+} and receptor signaling (*13*, *21*, *36*). In this respect, Akt activation by the flavonoids in H_2O_2-treated PC12 cells might occur via regulation of calcium and interaction with related proteins such as kinases and receptors. Moreover, the obviously different levels of p-Akt observed between cells treated with high and low concentrations of hesperetin suggest different mechanisms underlying Akt activation.

Activation of JNK has been strongly linked to neural apoptosis in oxidative insults (*4*). Our present study demonstrated that flavonoids suppressed the activation of JNK in H_2O_2-treated PC12 cells (**Figure 6**). Hydrogen peroxide-mediated phosphorylation of JNK is dependent on Ca^{2+} in cortical neurons (*30*). Hence, the regulation of $[Ca^{2+}]_i$ could contribute prevention of JNK activation. However, our present results also suggest that these flavonoids might interact with key regulatory pathways, leading to JNK inactivation. It is known that Akt/PKB can inhibit the activation of apoptosis signal-regulating kinase-1 (ASK1), which triggers JNK activation (*22*). Thus, the high levels of p-JNK observed at 16 h are in accord with the low levels of p-Akt at 6 h (**Figure 5**, **6**). Furthermore, high levels of $[Ca^{2+}]_i$ were obtained in H_2O_2-induced PC12 cells treated with high concentration of isorhamnetin or isosakuranetin; however, these cells showed JNK inactivation.

In conclusion, the modulation of Akt/PKB, JNK and p38 activation is involved in neuroprotection by citrus flavonoids against oxidative stress in PC12 cells. Possible cytoprotective targets of citrus flavonoids are illustrated in **Figure 7**. The flavonoids acted more as signaling molecules than antioxidants in this study. Specific cell receptors might have allowed lower concentrations of hesperetin to exhibited stronger modulation of Akt and JNK activation, as flavonoids are known to exhibit high affinity receptor agonist-like actions at low concentrations (*37*, *38*). Such receptor-mediated actions are under investigation.

ABBREVIATIONS USED

CA, catalase activity; Htn, hesperetin; Ish, isorhamnetin; Isk, isosakuranetin; JNK, c-jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; p-Akt, phosphorylated-Akt; p-JNK, phosphorylated-JNK; p-p38, phosphorylated-p38; PKB, protein kinase B; ROS, reactive oxygen species.

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