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Effect of Hesperetin against Oxidative Stress via ER- and TrkA-Mediated Actions in PC12 Cells

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ABSTRACT: Hesperetin is known to activate estrogen receptors (ERs). Estrogen-mediated neuroprotection could be via both ER and tyrosine kinase receptor (Trk) signaling. This study tested whether hesperetin protected PC12 cells from hydrogen peroxide induced oxidative damage via ER- and/or TrkA-mediated actions. Hesperetin (0.1, 1, and 50 μM) inhibited cell viability decreases and reactive oxygen species, intracellular calcium level, and caspase-3 activity increases in H_2O_2-induced PC12 cells. Such actions were significantly ($p < 0.05$) suppressed by ICI 182,780 (an ER antagonist) or K252a (a TrkA antagonist) at low concentrations (0.1 or 1 μM) only. Hesperetin also stimulated the activation of Akt, ERK, and CREB as well as induced brain-derived neurotrophic factor, PPARγ coactivator 1α (PGC-1α), and seladin-1 (selective Alzheimer's disease indicator-1) via both ER and TrkA in the cells. This study demonstrates that the neuroprotective effects of hesperetin, at low concentrations, are attributed to its stimulation on receptor signaling. Moreover, ER and TrkA are known to be expressed in most Alzheimer's disease (AD) vulnerable brain regions. This study thus suggests that hesperetin might have potential for intervention in neurodegenerative disorders, particularly for AD.

KEYWORDS: neuroprotection, hesperetin, receptor, ER, TrkA, PC12 cells

INTRODUCTION

Oxidative stress plays a critical role in many neuronal disorders. It causes damage to lipids, proteins, and DNA as well as intracellular calcium imbalance, resulting in apoptosis and neurotoxicity. Hydrogen peroxide-mediated oxidative stress can be induced by β-amyloid (Aβ) aggregation, dopamine oxidation, and brain ischemia/reperfusion.[1−3] Our previous study[4] had shown that neuroprotection of hesperetin, a bioavailable citrus flavonoid,[5] against H_2O_2-induced oxidative stress in PC12 cells is by diverse mechanisms including antioxidant properties and modulation of kinase activations for cell survival response. It is of interest to find that hesperetin exhibits more functions acting as a signaling modulator than antioxidant.

Cellular signals could be transduced via signaling molecules, receptors, and proteins related to intracellular signal pathways. Hesperetin might act on receptors and modulate the activation of Akt/protein kinase B, extracellular signal-regulated kinase (ERK), and c-jun N-terminal kinase (JNK) for pro-survival signaling responses.[4,6] Hesperetin also possesses estrogenic activity and exerts an antiatherogenic effect via estrogen receptor (ER)-mediated actions.[7] It is known that estrogen could act on membrane receptors such as ER and tyrosine kinase receptors (Trks) to trigger signals for neural survival responses and synaptic plasticity. Estrogen also has a direct genomic effect on neuroprotection via intracellular ERs.[8] It is evidenced that PC12 cells naturally express membrane and intracellular ERs.[9,10] Moreover, neuroprotection of estrogen in PC12 cells is via membrane ER-mediated serine/threonine kinase, Akt, activation.[9]

Brain-derived neurotrophic factor (BDNF) is widely expressed in the brain and essential for neural cell survival, differentiation, and synaptic activity.[11] BDNF also has been shown to exhibit neuroprotection in neural cells against apoptosis.[12] In addition, cAMP response element-binding protein (CREB) induces BDNF gene transcription[13] and could be activated via ER- and TrkA-mediated pathways.[8] Recently, PPARγ coactivator 1α (PGC-1α) has been shown to suppress reactive oxygen species (ROS) and neurodegeneration in neural cells challenged by oxidative stressor-mediated death.[14] PGC-1α is required for the expression of many antioxidant enzymes and regulated by CREB in the presence of H_2O_2. Another recently discovered defensive protein conferring resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress in neural cells is selective Alzheimer's disease indicator-1 (seladin-1).[15] It is found that seladin-1 scavenges H_2O_2, inhibits caspase-3 activity, and is a fundamental mediator of neuroprotection of estrogen.[16,17]

In the present study we hypothesized that ER and TrkA are involved in hesperetin-triggered survival responses in H_2O_2-treated PC12 cells. We tested the hypothesis by assessing effects of hesperetin with or without ICI 182,780, a high-affinity ER antagonist, or K252a, a TrkA antagonist, on cell viability, intracellular ROS and calcium levels, caspase-3 activity, and activation or expression of proteins associated with cell survival response such as Akt, BDNF, ERK, CREB, PGC-1α, and seladin-1.

MATERIALS AND METHODS

Materials. Materials for cell culture, hesperetin, K252a, and 30% hydrogen peroxide (w/w) solution, as well as anti-seladin-1 and anti-α-tubulin antibodies, were purchased from Sigma-Aldrich Co. (St. Louis, MO). ICI 182,780 was purchased from Tocris Bioscience (Elliville, MO). Anti-Akt/PKB, anti-phospho-Akt/PKB, anti-BDNF, anti-CREB, anti-phospho-CREB,

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anti-ERK1/2, anti-phospho-ERK1/2, and anti-PGC-1α antibodies were obtained from Cell Signaling Technology (Beverly, MA).

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), 5% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 5 mg/L penicillin, 5 mg/L streptomycin, and 10 mg/L neomycin, which was defined as full-serum medium, 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 treatments, cells were plated at an appropriate density on culture plates or dishes according to each experimental scale and cultured for 24 h. ICI 182,780 or K252a was added into the medium and maintained for 1 h before hesperetin was added. In measurement of cell viability, PC12 cells were pretreated for 6 h with 0.1, 1, or 50 μM hesperetin in N2-defined medium (N2 supplement, Gibco Products, Invitrogen Corp., Grand Island, NY), whereas in other experiments, the cells were pretreated the same way in low-serum (one-third of full serum) medium. Then, the medium was refreshed without hesperetin added and exposed to 400 μM H_2O_2 for another 16 h except as indicated. The doses of hesperetin used were based on previous studies.[4,6] In a range of 0.01−3 μM, the doses (0.1−1 μM) of hesperetin exhibit higher protective effects against H_2O_2-induced neuronal injury than others.[6] For comparing the difference of protective effects of hesperetin between its low and high doses, a high dose of 50 μM was selected; 50 μM hesperetin also protects cells against H_2O_2-induced cellular injury.[4] To further investigate receptor-mediated actions by hesperetin under the same oxidative stressing condition as our previous study,[4] a dose of 400 μM H_2O_2 was used.

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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT, a tetrazolium salt, is cleaved to formazan by an active enzyme, succinate dehydrogenase, for mitochondrial respiratory chain in live cells. 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 dimethyl sulfoxide (DMSO), and their optical density of absorbance at 570 nm was measured using a FLUO star galaxy spectrophotometer (BMG Labtechnologies GmbH Inc., Offenburg, Germany).

Determination of Intracellular Reactive Oxygen Species (ROS). The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) method[18] was used to measure intracellular ROS production. After culturing with or without treatments, cells 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 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 10000 events.

Intracellular Ca^{2+} Level ($[Ca^{2+}]_i$) Detection. $[Ca^{2+}]_i$ was measured according to a previously described method using a flow cytometry.[19] 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 level was assessed as mean fluorescence (at 488 nm excitation and 530 nm emission) 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. Data were collected with at least 10000 events.

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).

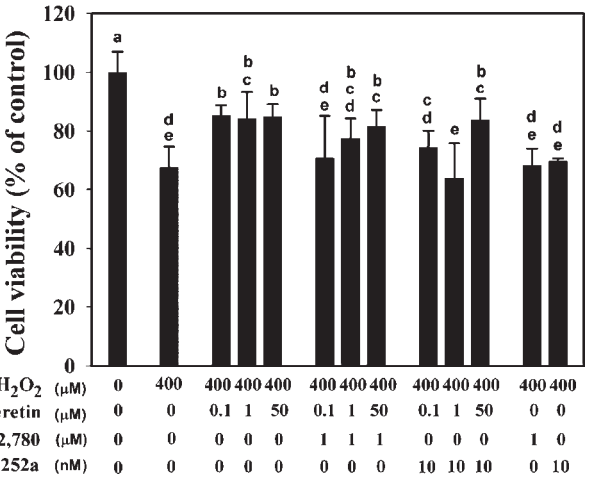

Figure 1. Hesperetin inhibits the H_2O_2-induced decrease of cell viability in PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 16 h. The viability of control cells was defined as 100%. Data shown are the mean ± SD ($n = 6$). Significant ($p < 0.05$) differences of cell viability between treatments are indicated by different letters.

Western Blotting. Cells were washed with PBS and 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 as described previously[20] with modification. Briefly, supernatant sample was boiled for 10 min in sample buffer (0.3 M Tris-HCl (pH 6.8), 25% 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 a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was 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 for Akt/PKB, p-Akt/PKB, BDNF, CREB, p-CREB, ERK1/2, p-ERK1/2, PGC-1α, seladin-1, or α-tubulin. After incubation with HRP-labeled secondary antibody for 1 h at room temperature, blots were developed using enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech, Uppsala, Sweden), and densitometric measurements of the bands were performed using the software LabWorks 4.5 (UVP, Upland, CA).

Protein Determination. The protein concentration of cell extracts was measured by using a protein assay kit (Bio-Rad Protein Assay Kit II, Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Statistical Analysis. All experiments were performed in triplicate at least. The results are presented as the mean ± SD. Data were evaluated for significance with the one-way ANOVA procedure followed by Duncan's multiple-range test using the Statistical Analysis System. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Hesperetin Differently Protects PC12 Cells against H_2O_2-Induced Cytotoxicity between Its Low- and High-Concentration Treatments. To evaluate whether neuroprotective effects of hesperetin against oxidative stress are via receptor-mediated actions,

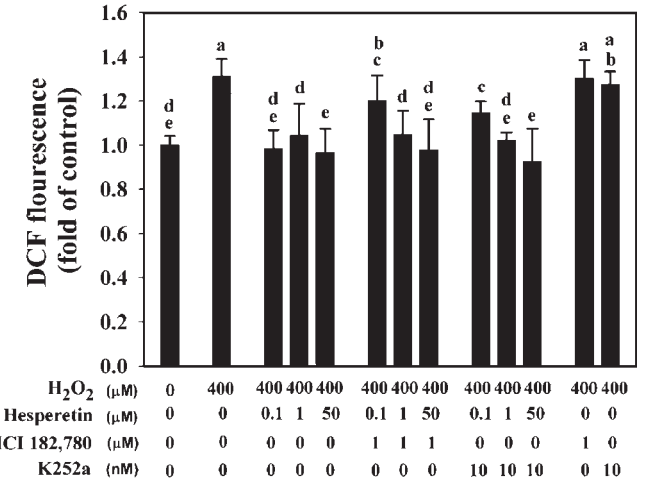

Figure 2. Hesperetin suppresses the H_2O_2-induced ROS increase in PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 16 h. Data shown are the mean ± SD (n = 6). Significant (p < 0.05) differences of ROS level between treatments are indicated by different letters.

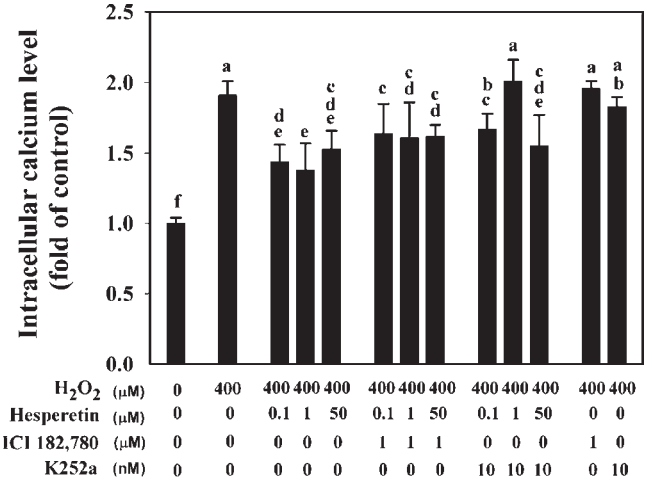

Figure 3. Hesperetin attenuates the H_2O_2-induced elevation of intracellular calcium level in PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 16 h. Data shown are the mean ± SD (n = 4). Significant (p < 0.05) differences of intracellular calcium level between treatments are indicated by different letters.

PC12 cells were pretreated with 0.1, 1, or 50 μM hesperetin for 6 h in the presence of specific receptor antagonists or alone. The cells were then washed and exposed to 400 μM H_2O_2 for an additional 16 h. As shown in Figure 1, the viability of PC12 cells, determined by using the MTT assay, significantly decreased to 68% of control after cells were exposed to H_2O_2. All pretreatments with hesperetin significantly inhibited decreases of the viability of H_2O_2-induced PC12 cells by nearly 50%. However, the protective effect of hesperetin was apparently attenuated by ICI 180,782 (1 μM), an antagonist of estrogen receptor (ER), or K252a (10 nM), an antagonist of tyrosine kinase receptor A (Trk A), at 0.1 or 1 μM, but not at 50 μM.

Receptor Antagonists Block the Suppression of ROS Increase in H_2O_2-Induced PC12 Cells Only in Treatments with Low Concentrations of Hesperetin. To determine the antioxidant effects of hesperetin, the amounts of ROS in PC12 cells, stimulated by 400 μM H_2O_2 for 16 h after treatment with 0.1, 1, or 50 μM hesperetin for 6 h in the presence of ICI 182,780 or K252a or alone, were measured by the DCFH-DA method. Figure 2 shows that H_2O_2 treatment significantly increased the ROS levels. All concentrations of hesperetin effectively (p < 0.05) suppressed the increase of ROS level in H_2O_2-induced PC12 cells. Moreover, the elevation of the ROS level occurred only in H_2O_2-induced PC12 cells pretreated with 0.1 μM hesperetin plus ICI 182,780 or K252a, but not in those pretreated with 1 or 50 μM hesperetin plus ICI 182,780 or K252a.

Receptor Antagonists Suppress the Attenuation of $[Ca^{2+}]_i$ Increase in H_2O_2-Induced PC12 Cells Only in Treatments with Low Concentrations of Hesperetin. To examine whether hesperetin affected $[Ca^{2+}]_i$ via ER or TrkA in PC12 cells under oxidative stress, $[Ca^{2+}]_i$ of the cells treated by 400 μM H_2O_2 for 16 h after treatment with 0.1, 1, or 50 μM hesperetin for 6 h in the presence of ICI 182,780 or K252a or alone was measured by Fluo3-AM using the FACS analysis. As shown in Figure 3, the H_2O_2 treatment significantly increased $[Ca^{2+}]_i$. Hesperetin pretreatments significantly reduced the elevated $[Ca^{2+}]_i$ in H_2O_2-induced PC12 cells. ICI 182,780 and K252a only attenuated such inhibition on the $[Ca^{2+}]_i$ increase in cells treated with 0.1 or 1 μM hesperetin. Particularly, the elevated $[Ca^{2+}]_i$ was obviously greater in cells treated with K252a plus 1 μM hesperetin than in other treatments of antagonist plus hesperetin.

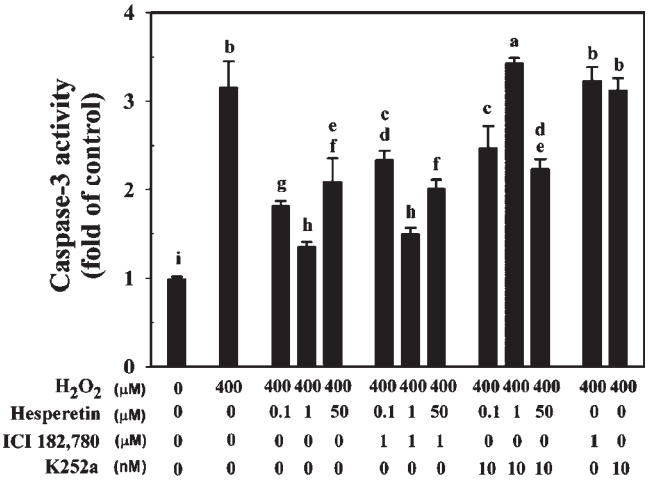

Figure 4. Hesperetin inhibits the H_2O_2-induced elevation of caspase-3 activity in PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 16 h. Data shown are the mean ± SD (n = 6). Significant (p < 0.05) differences of caspase-3 activity between treatments are indicated by different letters.

Receptor Antagonists Suppress the Inhibition of Casapse-3 Activity Increase in H_2O_2-Induced PC12 Cells Only in Treatments with Low Concentrations of Hesperetin. Caspase-3 acts as an apoptotic executor in apoptosis and can be activated by both H_2O_2 and disruption of $[Ca^{2+}]_i$. To further investigate the inhibitive effects of hesperetin on caspase-3 activation in PC12 cells under oxidative stress, the caspase-3 activity of the cells

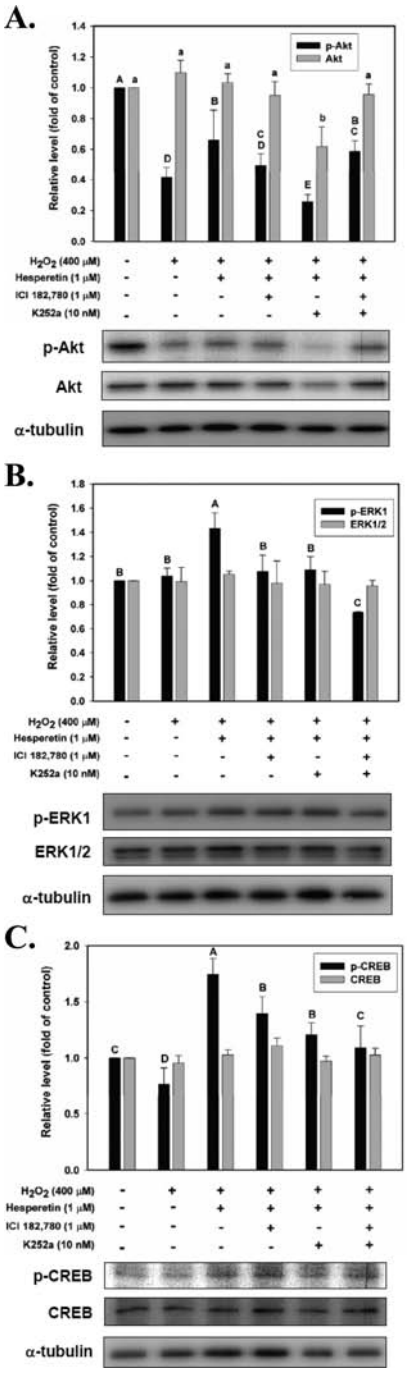

Figure 5. Hesperetin activates Akt, ERK, and CREB via ER and TrkA in H_2O_2-treated PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 6 h. Then the cells were lysed and supernatant was analyzed by Western blotting with antibody to phospho-Akt (p-Akt), total Akt, phospho-ERK1/2 (p-ERK1/2), total ERK1/2, phospho-CREB (p-CREB), and total CREB or α-tubulin. Western blots and relative levels of the proteins: (A) p-Akt and Akt, (B) p-ERK1 and ERK1/2, (C) p-CREB and CREB. The relative level of protein is expressed as fold change over the control, normalized against α-tubulin (loading control). Values are the mean ± SD (n = 3). Significant ($p < 0.05$) differences between treatments are indicated by corresponding upper or lower case letters.

stimulated by 400 μM H_2O_2 for 16 h after treatment with 0.1, 1, or 50 μM hesperetin for 6 h in the presence of ICI 182,780 or K252a or alone was determined. H_2O_2 dramatically increased the caspase-3 activity of cells by 3-fold as compared to that of control cells. Hesperetin pretreatments significantly suppressed caspase-3 activity increase in H_2O_2-induced PC12 cells (Figure 4). ICI 182,780 and K252a only attenuated such activity in cells treated with 0.1 or 1 μM hesperetin, respectively. Additionally, the H_2O_2-induced caspase-3 activity increase was apparently greater in cells treated with K252a plus 1 μM hesperetin than in those treated with K252a plus 0.1 μM hesperetin.

Hesperetin Stimulates the Activation of Akt, ERK, and CREB via ER and TrkA in H_2O_2-Induced PC12 Cells. To understand whether hesperetin affected intracellular signals via ER or TrkA in PC12 cells, we determined the activation of Akt, ERK, and CREB (proteins involved in neural cell survival) by receptor antagonist treatments and immunoblotting assay. After PC12 cells had been treated with 1 μM hesperetin for 6 h in the presence of ICI 182,780 or K252a or alone, the cells were washed and exposed to 400 μM H_2O_2 for an additional 6 h. Then PC12 cell homogenates were immunoblotted. As shown in Figure 5A, hesperetin attenuated the dramatic decrease of phosphorylated-Akt (p-Akt) level in H_2O_2-induced PC12 cells. Such action was suppressed in the cells pretreated with ICI 182,780 or K252a. Particularly, both p-Akt and Akt in the cells pretreated with K252a plus hesperetin apparently decreased to lower levels than those in other treatments. Hesperetin also significantly stimulated the activation of ERK in H_2O_2-induced PC12 cells, and its action was effectively inhibited by ICI 182,780 or K252a (Figure 5B). Additionally, the phosphorylated-ERK1 (p-ERK1) level in cells treated with both ICI 182,780 and K252a was clearly lower than that of cells treated with ICI 182,780 or K252a alone. Moreover, hesperetin significantly elevated the phosphorylated CREB (p-CREB) level in H_2O_2-induced PC12 cells. Such action of hesperetin was attenuated by ICI 182,780 or K252a (Figure 5C).The p-CREB level of cells treated with both ICI 182,780 and K252a was also lower than that of cells treated with ICI 182,780 or K252a alone.

Hesperetin Induces the Expression of BDNF, PGC-1α, and Seladin-1 via ER and TrkA in H_2O_2-Treated PC12 Cells. To further examine the effect of hesperetin on BDNF, PGC-1α, and seladin-1 (proteins involved in neural cells against apoptosis), PC12 cell homogenates obtained from the cells exposed to 400 μM H_2O_2 for 16 h after treatment with 1 μM hesperetin for 6 h in the presence of ICI 182,780 or K252a or alone were immunoblotted. The expressions of BDNF, PGC-1α, and seladin-1 were apparently decreased to levels of 0.62−0.74-fold of control in H_2O_2-induced PC12 cells (Figure 6). Pretreatment of cells with hesperetin significantly inhibited these decreases, and it even increased the level of BDNF to 1.25-fold over the control. Nevertheless, such elevation of BDNF, PGC-1α, or seladin-1 was apparently inhibited by ICI 182,780 or K252a. In addition, levels of BDNF and PGC-1α in H_2O_2-treated cells preincubated with K252a plus hesperetin were lower than those in cells preincubated with ICI 182,780 plus hesperetin, respectively (Figure 6A,B). Unlike K252a, ICI 182,780 effectively suppressed the stimulation of hesperetin on seladin-1 expression in H_2O_2-treated PC12 cells. However, all expressions of the proteins in cells preincubated with both ICI 182,780 and K252a were apparently lower than those in cells preincubated with either receptor antagonist alone (Figure 6C).

DISCUSSION

In this study, we have demonstrated that mechanisms underlying neuroprotection of hesperetin against H_2O_2-induced

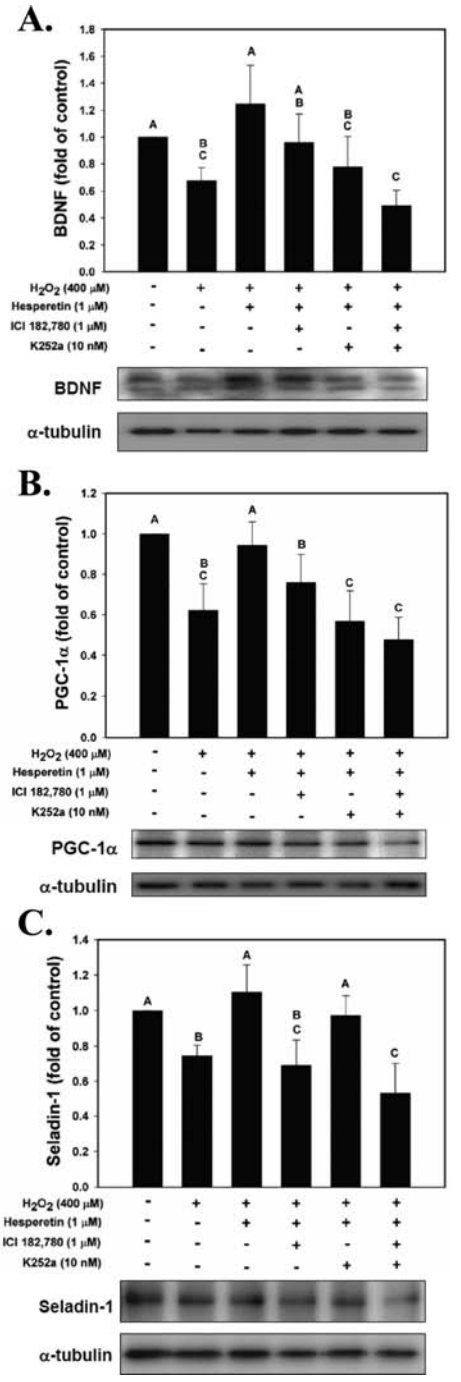

Figure 6. Hesperetin regulates protein expressions of BDNF, PGC-1α, and seladin-1 via ER and TrkA in H_2O_2-treated PC12 cells. Cells were pretreated with ICI 182,780 and/or K252a for 1 h, then hesperetin was added into the medium, and cells were incubated for an additional 6 h. Afterward, medium was refreshed without hesperetin added, and cells were exposed to 400 μM H_2O_2 for another 16 h. Then the cells were lysed, and supernatant was analyzed by Western blotting with antibody to BDNF, PGC-1α, seladin-1, or α-tubulin. Western blots and relative levels of the protein: (A) BDNF, (B) PGC-1α, (C) seladin-1. The relative level of protein is expressed as fold change over the control, normalized against α-tubulin (loading control). Values are the mean ± SD ($n = 3$). Significant differences between treatments are indicated by different letters.

cytotoxicity in PC12 cells, such as antioxidant properties, calcium ion regulation, and attenuation of caspase-3 activity, are via ER- and TrkA-mediated actions at low concentrations (0.1 and/or 1 μM). Hesperetin also activates Akt, ERK, and CREB as well as induces the expression of BDNF, PGC-1α, and seladin-1 via both ER and TrkA in the cells under oxidative stress. To our knowledge, the present study reveals for the first time that ER- and TrkA-mediated neuroprotective actions can be simultaneously induced by a bioavailable food flavonoid.

Antioxidant properties of flavonoids found in cells under oxidative stress could be attributed to their ROS scavenging or antioxidant enzyme up-regulating activities.[21] Our present data show that the ROS scavenging activity of hesperetin is via ER- and TrkA-mediated actions only at low concentration (0.1 μM) (Figure 2). It is known that ER up-regulates antioxidant enzymes, such as glutathione peroxidase and catalase.[22] We had reported that both low (0.8 μM) and high concentrations (50 μM) of hesperetin inhibited decreases of glutathione peroxidase, glutathione reductase, and catalase activities in H_2O_2-treated PC12 cells.[4,23] It is reasonable that hesperetin inhibits ROS formation without a dose dependency in the H_2O_2-treated cells. Previous studies also showed that TrkA-mediated signaling pathways led to phosphorylation of CREB, which up-regulates antioxidant enzymes including catalase, heme oxygenase-1 (HO-1), and manganous superoxide dismutase.[13,24] Hence, we suggest that the cellular ROS scavenging activity of hesperetin, at 0.1 μM, might be attributed to ER- and TrkA-mediated induction of antioxidant enzymes. The exact enzymes up-regulated need further confirmation. Nevertheless, the ROS scavenging activity of hesperetin might be mediated by its antioxidant property at 50 μM.

Ca^{2+} participates in the fundamental functions as well as cell survival and death of neurons. Evidence suggested that $[Ca^{2+}]_i$ maintenance is capable of retarding or preventing acute and chronic neurodegeneration.[25] To enhance endogenous Ca^{2+} removal, buffer mechanisms are promising for Ca^{2+} homeostasis without compromising normal functions of neurons in long-term treatment. It is known that hesperetin triggers PI-3K/Akt/ASK1 and ERK/Bad pathways, leading to the protection of the outer mitochondrial membrane against hydrogen peroxide injury.[6] These actions also result in inhibiting Bax to aggregate and locate on mitochondria and consequently prevent the calcium store release.[26] Furthermore, the activation of trophic factors/Akt, mitogen-activated protein kinase (MAPK)/CREB pathways are also beneficial for endogenous Ca^{2+} removal and buffering in cells.[25] Our present data reveal that hesperetin exhibits the activity of $[Ca^{2+}]_i$ regulation in cells under oxidative stress (Figure 3). Besides its antioxidant property, hesperetin might maintain $[Ca^{2+}]_i$ by endogenous mechanisms such as ER- and TrkA-signaling regulations (Figure 5).

It is known that oxidative stress and $[Ca^{2+}]_i$ elevation activate ASK1/JNK pathways, resulting in cytochrome *c* release.[6,27] Cytochrome *c* and caspase-9 initiate caspase-3 activation, which can be inhibited by Bcl-2 in H_2O_2-treated PC12 cells.[28] Furthermore, CREB regulates the expression of Bcl-2.[13] Growth factors can well activate Akt/GSK-3β signaling,[29,30] which leads to caspase-3 inactivation. Genistein was shown to activate ERs and up-regulate BDNF, which also led to caspase-3 inactivation.[31,32] Moreover, it was reported that receptor-mediated activation of pro-survival Akt and ERK pathways underlies anti-apoptotic effect of hesperetin in cortical neurons.[6] Hence, our data together with these findings suggest that the inhibitive effect of hesperetin, at 50 μM, on caspase-3 activation in H_2O_2-induced PC12 cells might be mainly by its actions of antioxidant and $[Ca^{2+}]_i$ regulation (Figures 2−4).

Besides these, mechanisms underlying inhibition of hesperetin on caspase-3 activation, at physiologically relevant concentrations (0.1 and 1 μM), might be mainly via ER- and TrkA-mediated actions including Akt, ERK, and CREB activations (Figure 5) as well as the induction of BDNF at least (Figure 6A). Additionally, it is suggested that hesperetin has its optimal physiological levels (0.03−1 μM) to trigger receptor-mediated signaling in neurons.[6] Hesperetin might have higher affinity to TrkA at 1 μM rather than at 0.1 μM to exert actions of caspase-3 inactivation and $[Ca^{2+}]_i$ homeostasis in our present study. It is known that the protection afforded by hesperetin at physiological levels is not reflected by its antioxidant potential.[6] Hence, it is also conceivable that hesperetin could exhibit no dose-dependent responses to cell viability, intracellular calcium level, and caspase-3 activity in the H_2O_2-induced PC12 cells. Genistein has been shown to exert more effects on suppressing caspase-3 activity and DNA fragmentation at 0.1 μM than at 40 μM.[32]

Nerve growth factor (NGF) triggers neural survival PI-3K/Akt and MAPK pathways, which converge on CREB to inhibit apoptosis.[1] NGF can trigger sustained activation of ERK for hours in PC12 cells.[33,34] Also, estrogen activates membrane ER-mediated pro-survival Akt/PKB, Src/MEK/ERK, and MAPK/ERK pathways, leading to CREB activation and NFκB nuclear translocation for pro-survival responses in PC12 cells.[35] In endothelial cells, estrogen is known to trigger rapid activation of Akt initiated at the membrane and lasts for 6 h.[36] Our present results suggest that hesperetin might trigger both membrane ER- and TrkA-mediated Akt and ERK signaling pathways, which together induce CREB activation at 6 h (Figure 5). Nevertheless, we observed that Akt degraded only in the cells pretreated with K252a plus hesperetin under oxidative stress. This indicated that TrkA-mediated action was involved in protecting Akt against oxidative challenge. It had been shown that strong oxidants led to Akt proteolysis in PC12 cells.[37] However, the induction of p-Akt and Akt in the H_2O_2-induced cells pretreated with K252a and ICI 182,780 plus hesperetin illustrated clearly that other mechanisms underlie the regulation of Akt during oxidative stress.

BDNF promotes the survival of basal forebrain cholinergic neurons, hippocampus, and parietal cortex, which are severely injured in Alzheimer's disease brains.[38] BDNF also activates Akt and MAPK/ERK pathways for cell survival responses.[30] PGC-1α plays a key role in ROS homeostasis and increases mitochondrial biogenesis. It is highly inducible in most tissues and responses to common calcium and cyclic AMP signaling.[14] Hence, PGC-1α is an almost ideal protector against mitochondrial dysfunction-associated damage seen in Parkinson's and Alzheimer's diseases. Moreover, seladin-1 exerts protection of cells against Aβ toxicity and oxidative stress and is down-regulated in AD-vulnerable brain regions.[15] Our present data show that hesperetin induces BDNF, PGC-1α, and seladin-1 via ER- and TrkA-mediated actions in H_2O_2-treated cells. These endogenously defensive actions broaden the way for hesperetin to exert its neuroprotective effects. In addition, the induction of BDNF and PGC-1α by hesperetin is via TrkA more than ER; Hesperetin upregulates seladin-1 mainly by ER-mediated action (Figure 6). However, both ER and TrkA-mediated pathways are involved in regulating these proteins. Because PGC-1α, a known product of CREB-regulated gene, is regulated by ER in this study, K252a has an effect on seladin-1 induction. It is also known that both Akt and ERK signaling pathways lead to intracellular ER activation.[39,40]

In conclusion, this study suggests that the neuroprotective effects of hesperetin against oxidative damage, at physiological concentrations, are attributed to its receptor-signaling molecule property. Proposed hesperetin-associated ER and TrkA signaling in PC12 cells against oxidative damage is illustrated in Figure 7.

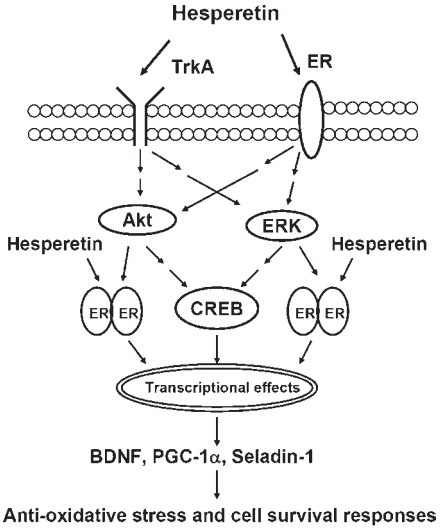

Figure 7. Proposed ER and TrkA pathways for antioxidative damage and cell survival responses triggered by hesperetin in H_2O_2-treated PC12 cells.

Because ER and TrkA are expressed in most AD-vulnerable brain regions, our findings also provide molecular bases of evidence that hesperetin might have potential for intervention in neurodegenerative disorders, particularly for AD.

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ABBREVIATIONS USED

Aβ, β-amyloid; AD, Alzheimer's disease; Akt, Ak transforming oncogene also called; PKB, protein kinase B; ASK1, apoptosis signal-regulating kinase 1; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding protein; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase-3; HO-1, heme oxygenase-1; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase or extracellular signal-regulated kinase kinase; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; PGC-1α, PPAR-gamma coactivator 1 alpha; PI-3K, phosphatidylinositol 3-kinase; Src, cellular homologue of transforming gene of Rous sarcoma virus; ROS, reactive oxygen species; seladin-1, selective Alzheimer's disease indicator-1; Trk, tyrosine kinase receptor.

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