

## Antioxidant and Neuroprotective Effects of Hesperidin and its Aglycone Hesperetin

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The present study evaluated antioxidant and neuroprotective activities of hesperidin, a flavanone mainly isolated from citrus fruits, and its aglycone hesperetin using cell-free bioassay system and primary cultured rat cortical cells. Both hesperidin and hesperetin exhibited similar patterns of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities. While hesperidin was inactive, hesperetin was found to be a potent antioxidant, inhibiting lipid peroxidation initiated in rat brain homogenates by  $\text{Fe}^{2+}$  and L-ascorbic acid. In consistence with these findings, hesperetin protected primary cultured cortical cells against the oxidative neuronal damage induced by  $\text{H}_2\text{O}_2$  or xanthine and xanthine oxidase. In addition, it was shown to attenuate the excitotoxic neuronal damage induced by excess glutamate in the cortical cultures. When the excitotoxicity was induced by the glutamate receptor subtype-selective ligands, only the N-methyl-D-aspartic acid-induced toxicity was selectively and markedly inhibited by hesperetin. Furthermore, hesperetin protected cultured cells against the  $\text{A}_{\beta(25-35)}$ -induced neuronal damage. Hesperidin, however, exerted minimal or no protective effects on the neuronal damage tested in this study. Taken together, these results demonstrate potent antioxidant and neuroprotective effects of hesperetin, implying its potential role in protecting neurons against various types of insults associated with many neurodegenerative diseases.

**Key words:** Hesperetin, Hesperidin, Neuroprotection, Antioxidant, Excitotoxicity, Beta-amyloid ( $\text{A}_{\beta}$ )

### INTRODUCTION

Flavonoids are a large group of polyphenolic compounds found ubiquitously in plants including herbal medicines, fruits and vegetables. They have been reported to exhibit a wide variety of biological effects such as antiinflammatory, antioxidant, antiviral, antibacterial, cardiovascular and chemopreventive activities (Middleton *et al.*, 2000). Hesperidin (3',5,7-trihydroxy-4'-methoxy-flavanone-7-rhamnoglucoside) is a member of the flavanone group of flavonoids mainly isolated from citrus fruits. A number of pharmacological properties of hesperidin have been reported to date (Garg *et al.*, 2001). For example, hesperidin exhibited antihypercholesterolemic activity (Bok *et al.*, 1999), antihypertensive and diuretic effects (Galati *et al.*, 1996), antiinflammatory and analgesic activities (Galati *et*

*al.*, 1994) and anti-allergic action (Matsuda *et al.*, 1991).

Hesperidin is known to be partly deglycosylated in the gut to its aglycone hesperetin (3',5,7-trihydroxy-4'-methoxy-flavanone) by intestinal microflora (Kim *et al.*, 1998). A number of studies have examined the antioxidant and radical scavenging properties of hesperidin and hesperetin using a variety of assay systems (Ratty and Das, 1988, Yuting *et al.*, 1990, Miyake *et al.*, 1998, Kim *et al.*, 2004). Although the results from different studies varied considerably depending on the assay system employed and the concentrations tested in their studies, most studies found that hesperidin is inactive or only moderately active (Garg *et al.*, 2001; Choi *et al.*, 2002; Wilmsen *et al.*, 2005). In contrast, hesperetin was shown to be a potent antioxidant (Hirata *et al.*, 2005), inhibiting  $\text{Fe}^{2+}$ -induced linoleate peroxidation and autooxidation of rat cerebral membranes (Saija *et al.*, 1995), scavenging peroxynitrite (Kim *et al.*, 2004) and inhibiting generation of reactive oxygen species (ROS) including hydroxyl radical (Jung *et al.*, 2003). In consistence with these findings, hesperetin exhibited cytoprotection against cell damage induced by peroxynitrite

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and ROS in cultured rat prostatic endothelial cells (Kim *et al.*, 2004).

Oxidative stress has also been implicated in the pathogenesis of a number of acute and chronic neurodegenerative disorders in the brain (Halliwell, 1992; Behl and Moosmann, 2002). Many flavonoids, such as epigallocatechin gallate, wogonin, and gossypin, were found to exhibit neuroprotective actions alleviating oxidative stress-induced brain damage (Mandel and Youdim, 2004; Cho and Lee, 2004; Yoon *et al.*, 2004). To examine and compare pharmacological properties of hesperidin and hesperetin in the central nervous system (CNS), the present study evaluated their antioxidant activities using cell-free bioassay system and further investigated their effects on the various types of oxidative stress-induced neuronal damage in primary cultured rat cortical cells. Additionally, we also examined their effects on the neuronal damage induced by excitotoxic amino acids and  $\beta$ -amyloid ( $A_{\beta}$ ), the well-known causative factors associated with neurodegenerative disorders including Alzheimer's disease.

## MATERIALS AND METHODS

### Materials

Materials used for cell cultures including minimum essential medium (MEM, with Earle's salts), fetal calf serum, and horse serum were obtained from Gibco BRL (Gaithersburg, U.S.A.). Hesperidin (80% purity), hesperetin (95% purity), laminin, xanthine (X), xanthine oxidase (XO),  $H_2O_2$ , 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-glutamate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, U.S.A.).  $A_{\beta(25-35)}$  was obtained from Bachem (Merseyside, UK), and N-methyl-D-aspartic acid (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate were from Tocris Cookson Ltd (Bristol, UK). All other chemicals were reagent grade or better.

Timed-pregnant Sprague-Dawley (SD) rats for primary cortical cell cultures and male SD rats for preparations of brain homogenates were obtained from Daehan Biolink (Chungbuk, Korea). Animals were maintained with Purina laboratory chow and water *ad libitum* in our animal facility with a 12 h light cycle at a controlled temperature ( $22 \pm 2^\circ\text{C}$ ) until used.

### Primary cultures of rat cortical cells

Cortical cell cultures containing neuronal and non-neuronal cells were prepared from the cerebral cortices of SD rat embryos at 16-18 days of gestation and maintained as previously described (Cho *et al.*, 2001). In brief, cortices were dissected and mechanically dissociated into single cells by triturations through fire-polished Pasteur

pipettes. Cells were plated at a density of  $4-5 \times 10^5$  cells per well on 24-well culture plates coated with poly-L-lysine and laminin. Cultures were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 95% air/5%  $\text{CO}_2$  in a medium consisting of MEM supplemented with glucose (final concentration, 25 mM), glutamine (2 mM), fetal calf serum (5%), and horse serum (5%). Proliferation of non-neuronal cells was arrested by the addition of 10  $\mu\text{M}$  cytosine arabinoside at 7 days after plating (Cho *et al.*, 2000; 2001). All experiments were performed at 10-13 days after plating.

### Experimental treatments of the cultured cells and the assessment of cell damage

Oxidative neuronal damage was induced by the exposure of the cultures to  $H_2O_2$  (100  $\mu\text{M}$ ) for 5 min or X (0.5 mM)/XO (10 mU/mL) for 10 min in HEPES-buffered salt solution (HBSS) as described (Jung *et al.*, 2002; Dok-Go *et al.*, 2003). Excitotoxic neuronal damage was induced by the exposure to L-glutamate (100  $\mu\text{M}$ ) or NMDA (300  $\mu\text{M}$ ) for 15 min in  $\text{Mg}^{2+}$ -free HBSS as described (Cho *et al.*, 2001; 2002). The cultures were then washed with HBSS and maintained at  $37^\circ\text{C}$  for 18-24 h in MEM supplemented with 21 mM glucose. The AMPA- or kainate-induced excitotoxicity and the  $A_{\beta}$ -induced toxicity were respectively induced by the exposure of cultures for 24 h at  $37^\circ\text{C}$  to AMPA or kainate (100  $\mu\text{M}$ ) or  $A_{\beta(25-35)}$  (40  $\mu\text{M}$ ) in MEM supplemented with glucose (Cho and Lee, 2004; Cho *et al.*, 2005).

To evaluate the effects of hesperidin and hesperetin on the neuronal cell damage induced as described above, the cultures were simultaneously exposed to various concentrations of hesperidin or hesperetin during the respective insults. The stock solution of the test sample was prepared in 100% dimethyl sulfoxide (DMSO) at 200-fold the highest concentration tested and then serially diluted to the desired concentrations. For the sham-treated controls, sister cultures were exposed to 0.5% DMSO, which showed no effects on cell viability as previously reported (Cho *et al.*, 2000).

Following the treatment of cells, cell viability was assessed using MTT reduction assays as previously described (Cho *et al.*, 2005). In brief, MTT was added to each well at the final concentration of 0.5 mg/mL, and the cells were incubated at  $37^\circ\text{C}$  for 3 h. The media were then removed and the formazan crystals produced in the wells were dissolved by the addition of DMSO. The absorbance was measured at 550 nm using a VERSA<sub>max</sub> microplate reader (Molecular Devices, U.S.A.). Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = 100 \times \frac{(\text{Abs}_{\text{insult + sample}} - \text{Abs}_{\text{insult}})}{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{insult}})}$$

In some experiments, the activities of lactate dehydrogenase (LDH) released into the culture media were also measured according to the methods described elsewhere (Cho *et al.*, 2000; 2001; 2002; Dok-Go *et al.*, 2003) to validate the results of the MTT reduction assay. We found that the cell viability assessed by MTT reduction and LDH assays exhibited comparable results, as previously reported (Xie *et al.*, 2001; Cho *et al.*, 2005).

### Assay of lipid peroxidation in the rat brain homogenates

Lipid peroxidation was initiated by 10  $\mu\text{M}$   $\text{Fe}^{2+}$  and 100  $\mu\text{M}$  ascorbic acid in the rat brain homogenates, and assayed as previously described (Cho and Lee, 2004). Briefly, the reaction mixture was incubated at 37°C for 1 h in the presence of various concentrations of hesperidin or hesperetin. The reaction was stopped by the addition of trichloroacetic acid (28% w/v) and thiobarbituric acid (1% w/v) in succession, and the mixture was then heated at 100°C for 15 min. After centrifugation to remove precipitates, absorbance was measured at 532 nm using VERSA<sub>max</sub> microplate reader (Molecular Devices, Sunnyvale, U.S.A.). The percent inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 100 \times (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}$$

### Assay for DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method previously described (Cho and Lee, 2004). In brief, reaction mixture containing various concentrations of hesperidin or hesperetin and 150  $\mu\text{M}$  DPPH methanolic solution was incubated at 37°C for 30 min and absorbance was measured at 520 nm. The percent scavenging activity was calculated using the above formula.

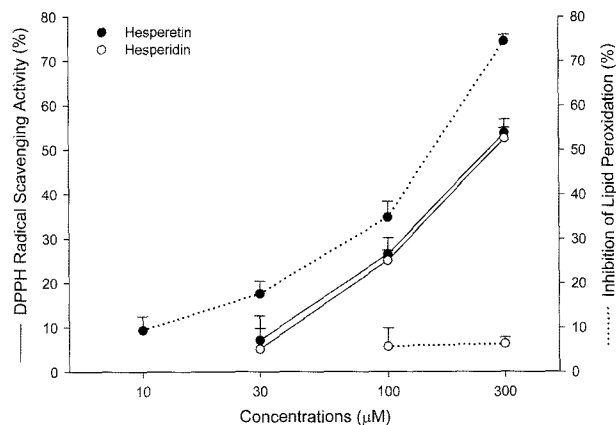
### Data calculation

All experiments were performed at least three times in duplicate. Data are expressed as means  $\pm$  S.E.M. The concentrations exerting 50% maximal inhibition,  $\text{IC}_{50}$  values, were determined by non-linear regression of the mean values using Prism (GraphPad Software Inc., U.S.A.). Statistical analysis was performed by Student's *t*-test with a *P* value of less than 0.05 being considered statistically significant.

## RESULTS

### DPPH radical scavenging activities of hesperidin and hesperetin

To evaluate and compare antioxidative properties of hesperidin and its aglycone hesperetin, we first deter-



**Fig. 1.** Antioxidant properties of hesperidin and hesperetin. DPPH radical scavenging activity and lipid peroxidation initiated in rat brain homogenates by  $\text{Fe}^{2+}$  and L-ascorbic acid were assessed as described in the Materials and methods in the absence or presence of the indicated concentrations of hesperidin or hesperetin. Each point represents the mean  $\pm$  SEM from 3 measurements performed in duplicate.

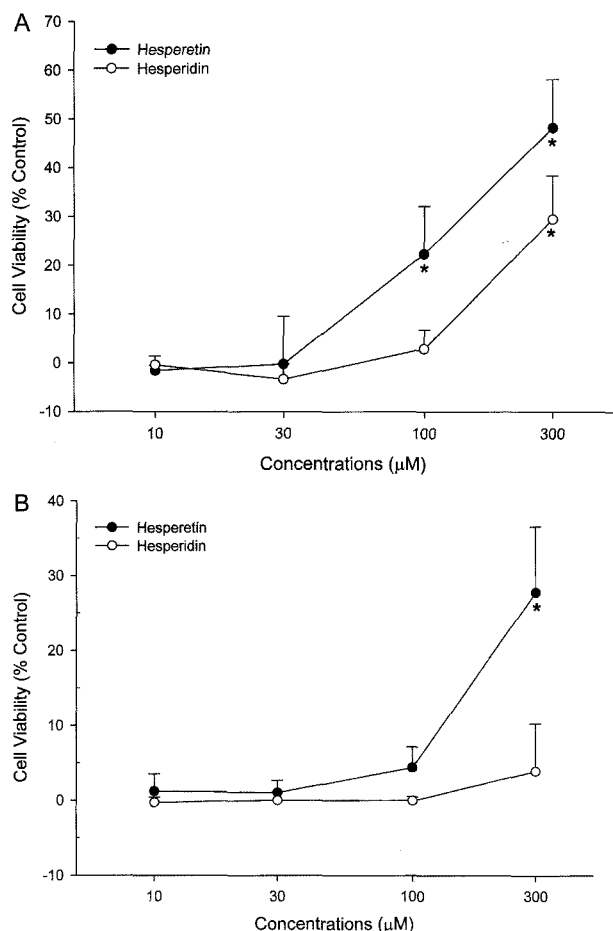
mined their radical scavenging activities using stable free radicals generated from DPPH. As shown in Fig. 1, both hesperidin and hesperetin exhibited similar patterns of radical scavenging activities, although hesperetin appeared to be slightly more potent than hesperidin. The concentrations of hesperidin and hesperetin scavenging 50% DPPH radicals were 340.0 and 252.2  $\mu\text{M}$ , respectively.

### Effects of hesperidin and hesperetin on lipid peroxidation in the rat brain homogenates

As illustrated in Fig. 1, hesperidin had negligible effect on lipid peroxidation initiated in rat brain homogenates by  $\text{Fe}^{2+}$  and L-ascorbic acid. In contrast, hesperetin was found to dramatically inhibit lipid peroxidation (Fig. 1), with the  $\text{IC}_{50}$  value of 179.1  $\mu\text{M}$ .

### Effects of hesperidin and hesperetin on the oxidative stress-induced neuronal damage

Primary cultures of rat cortical cells containing neuronal and non-neuronal cells were employed to study neuroprotective effects of hesperidin and hesperetin. We first examined if the two flavanones protect cultured cells against oxidative neuronal cell damage induced by the exposure to  $\text{H}_2\text{O}_2$  or X/XO. As described in our previous reports (Cho and Lee, 2004; Cho *et al.*, 2005), approximately 80-90% of cells were damaged by these oxidative insults after 18-20 h of treatment. Both hesperidin and hesperetin significantly attenuated the  $\text{H}_2\text{O}_2$ -induced oxidative damage (Fig. 2A). However, in agreement with our finding from Fig. 1, hesperetin exhibited more potent and efficacious protection against the  $\text{H}_2\text{O}_2$ -induced damage. Similarly, hesperetin inhibited the oxidative

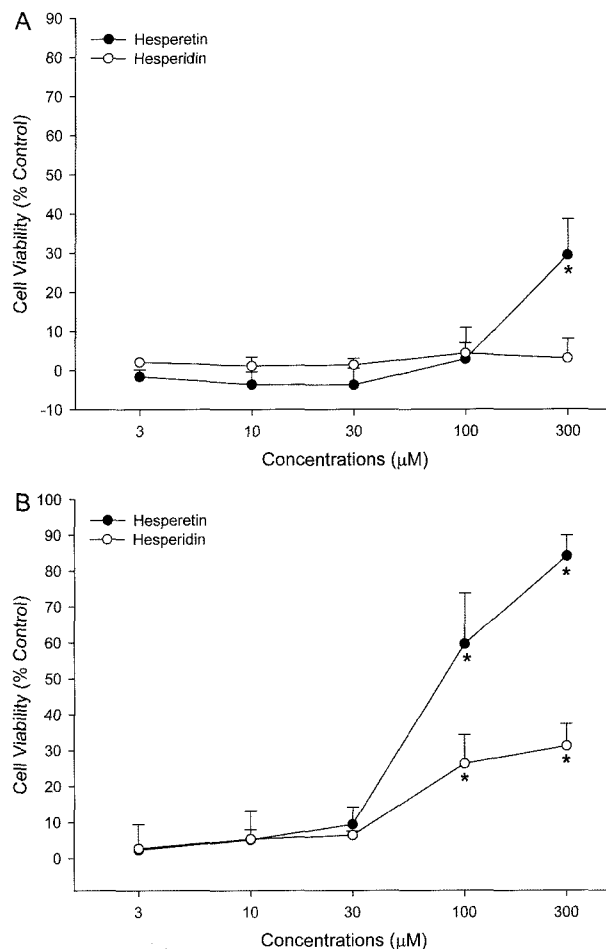


**Fig. 2.** Effects of hesperidin and hesperetin on the oxidative stress-induced neuronal damage. Primary cultured rat cortical cells (10–13 days *in vitro*) were exposed to 100 μM H<sub>2</sub>O<sub>2</sub> for 5 min (A) or 0.5 mM xanthine and 10 mU/ml xanthine oxidase for 10 min (B) in the absence or presence of the indicated concentrations of hesperidin or hesperetin. Cell viability was determined by MTT reduction assays at 18–20 h after the exposure. Data were calculated by the formula given in the Materials and methods and presented as percentages of control MTT reduction measured in the absence of hesperidin or hesperetin. Each point represents the mean ± SEM from at least 3 measurements performed in duplicate (\*,  $p < 0.05$ ).

neuronal damage induced by X/XO, whereas hesperidin was inactive (Fig. 2B).

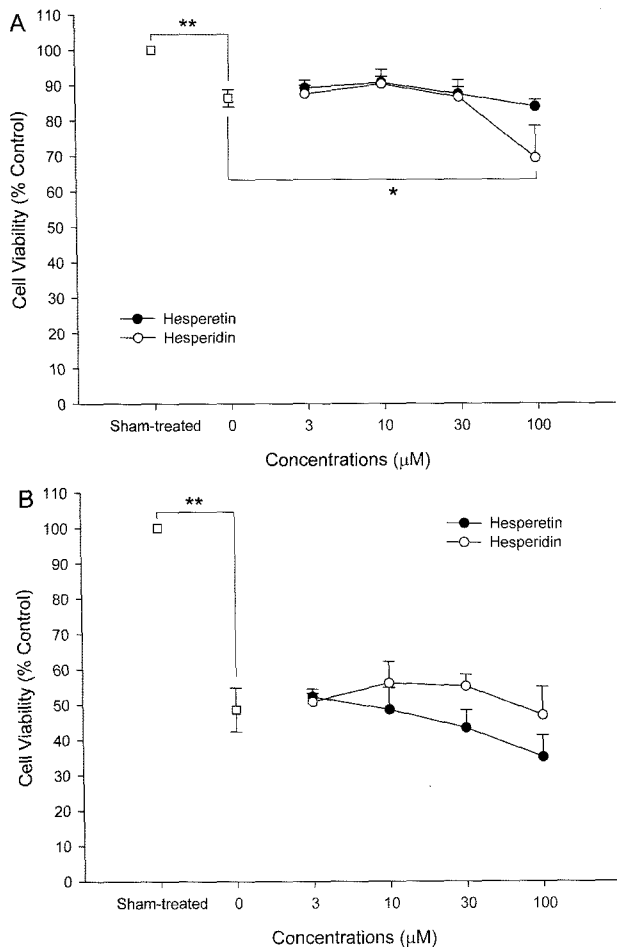
### Effects of hesperidin and hesperetin on the excitotoxic neuronal damage

To elucidate additional pharmacological properties of the two flavanones in the CNS, we investigated their effects on the excitotoxic neuronal cell damage. The excitotoxic damage induced by glutamate exposure of the cultured cortical cells was mildly attenuated by hesperetin (Fig. 3A). To further investigate which pathway of glutamate receptor subtype is involved in the protective action, cultured cells were exposed to receptor subtype-selective



**Fig. 3.** Effects of hesperidin and hesperetin on the glutamate- or NMDA-induced excitotoxic neuronal damage. Primary cultured rat cortical cells (10–12 days *in vitro*) were exposed to 100 μM glutamate (A) or 300 μM NMDA (B) for 15 min in the absence or presence of the indicated concentrations of hesperidin or hesperetin. Cell viability was determined by MTT reduction assays at 20–24 h after the exposure and presented as described in the Fig. 2 legend. Each point represents the mean ± SEM from at least 3 measurements performed in duplicate (\*,  $p < 0.05$ ).

ligands, NMDA, AMPA or kainate. As shown in Fig. 3B, hesperetin dramatically inhibited the NMDA-induced excitotoxic damage, with the IC<sub>50</sub> value of 92.1 μM. When the cultures were exposed to AMPA or kainate, significant excitotoxic damage was produced as compared to the sham-treated control cultures (Figs. 4A and 4B). However, the AMPA- or kainate-induced damage was not alleviated by hesperetin (Figs. 4A and 4B). Hesperidin, in contrast, exhibited only mild but significant inhibition of the NMDA-induced damage (Fig. 3B). The excitotoxic damage induced by glutamate, AMPA or kainate was not reduced by hesperidin (Figs. 3A, 4A and 4B). Instead, hesperidin was found to aggravate the AMPA-induced damage at the concentration of 100 μM (Fig. 4A).



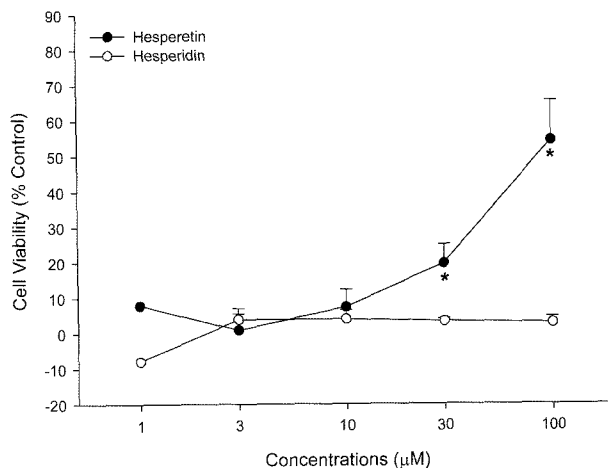
**Fig. 4.** Effects of hesperidin and hesperetin on the AMPA- or kainate-induced excitotoxic neuronal damage. Primary cultured rat cortical cells (10-13 days *in vitro*) were exposed to 300 μM AMPA (A) or 100 μM kainate (B) for 24 h and the cell viability was determined by MTT reduction assays as described in the Materials and methods. Data were expressed as percentages of control MTT reduction measured in the sham-treated sister cultures. Each point represents the mean ± SEM from at least 3 measurements performed in duplicate. (\*\*,  $p < 0.05$ , sham-treated control vs AMPA- or kainate-treated cultures; \*,  $p < 0.05$ , AMPA-treated cultures vs cultures treated with AMPA and 100 μM hesperidin).

**Effects of hesperidin and hesperetin on the neuronal damage induced by  $A_{\beta(25-35)}$**

Finally, we examined the effects of the two flavanones on the  $A_{\beta(25-35)}$ -induced neuronal damage. As shown in Fig. 5, hesperetin was found to protect cultured cortical cells against the  $A_{\beta(25-35)}$ -induced neuronal damage, while hesperidin was inactive. The  $IC_{50}$  value of hesperetin was 97.2 μM.

**DISCUSSION**

The present study evaluated antioxidant and neuroprotective activities of hesperidin and compared with the



**Fig. 5.** Effects of hesperidin and hesperetin on the  $A_{\beta(25-35)}$ -induced neuronal damage. Primary cultured rat cortical cells (10 days *in vitro*) were exposed to 40 μM  $A_{\beta(25-35)}$  for 24 h in the absence or presence of the indicated concentrations of hesperidin or hesperetin. Cell viability was determined by MTT reduction assays and presented as described in the Fig. 2 legend. Each point represents the mean ± SEM from at least 3 measurements performed in duplicate (\*,  $p < 0.05$ ).

activities of its aglycone hesperetin using cell-free bioassay system and primary cultured rat cortical cells. Both hesperidin and hesperetin showed similar patterns of DPPH radical scavenging activity. However, hesperetin was found to be a more potent antioxidant than hesperidin, markedly inhibiting lipid peroxidation in brain homogenates. In addition, hesperetin exerted significant neuroprotective actions, preventing neurons from the oxidative damage as well as excitotoxic and  $A_{\beta(25-35)}$ -induced damage in the primary cultured rat cortical cells. Hesperidin, in contrast, exhibited minimal or negligible inhibition of lipid peroxidation and protective actions against the neuronal damage tested in this study at the concentration ranges of 3-300 μM.

From a number of studies evaluated the antioxidant and radical scavenging properties of hesperidin using a variety of *in vitro* assay systems, it has been recognized to be inactive or possess only moderate antioxidant properties depending on the assay systems employed and its concentrations examined (Garg *et al.*, 2001; Choi *et al.*, 2002; Kim *et al.*, 2004; Wilmsen *et al.*, 2005). In consistence with these previous reports, we also observed in this study only moderate DPPH radical scavenging activity of hesperidin at the concentration ranges of 30-300 μM (Fig. 1). Hesperetin exhibited similar patterns of radical scavenging activity, but it appeared to be slightly more potent than hesperidin (Fig. 1). However, the two flavanones exhibited different pharmacological effects on lipid peroxidation. While hesperidin had no effect, hesperetin exhibited prominent inhibition of lipid peroxidation (Fig. 1), demonstrating its potent antioxidant action. Other studies have

also demonstrated stronger antioxidant activities of hesperetin than hesperidin using different assay systems (Saija *et al.*, 1995; Jung *et al.*, 2003; Kim *et al.*, 2004; Hirata *et al.*, 2005).

We next tested if hesperetin inhibits various types of oxidative stress-induced neuronal damage in the primary cultured rat cortical cells. Hesperetin was found to significantly attenuate the H<sub>2</sub>O<sub>2</sub>-induced oxidative neuronal damage (Fig. 2A). The antioxidant as well as hydroxyl radical scavenging activities of hesperetin (Jung *et al.*, 2003) may have contributed to its protective action against the H<sub>2</sub>O<sub>2</sub>-induced damage. We also observed that hesperetin reduced the X/XO-induced oxidative damage (Fig. 2B). It has been previously reported that either hesperetin or hesperidin does not possess superoxide radical scavenging activity (Orallo *et al.*, 2004). However, hesperetin was shown to inhibit XO activity (Nagao *et al.*, 1999; Dew *et al.*, 2005). Thus, direct inhibition of XO may be one of the mechanisms to protect against the X/XO-induced damage. In contrast to hesperetin, hesperidin exhibited only mild protective action against the H<sub>2</sub>O<sub>2</sub>-induced oxidative damage at the highest concentration tested in this study (300  $\mu$ M). Moreover, it showed no inhibition of the X/XO-induced damage.

Excessive release of glutamate is known to be the major cause for neuronal damage in many acute and chronic neurodegenerative disorders including cerebral ischemia, epilepsy, and Alzheimer's disease (Sauer and Fagg, 1992). Overstimulation of glutamatergic system results in a massive influx of Ca<sup>2+</sup>, which activates neurotoxic mechanisms including the generation of ROS such as superoxide radicals and hydrogen peroxide (Dugan *et al.*, 1995; Reynolds and Hastings, 1995; Sengpiel *et al.*, 1998). We found in this study that hesperetin attenuated the excitotoxicity induced by excessive glutamate in the cultured cortical cells (Fig. 3A). When the toxicity was induced by the receptor subtype-selective ligands, NMDA, AMPA, or kainate, only the NMDA-induced toxicity was selectively and markedly inhibited (Figs. 3B, 4A and 4B). Hesperetin inhibited the NMDA toxicity more potently and efficaciously than the glutamate toxicity (Fig. 3A vs Fig. 3B). Based on these findings, we suggest that the NMDA receptor pathway may be the target site of hesperetin to exert neuroprotective action against excitotoxicity. Since the generation of ROS is known to be involved in excitotoxicity, the strong antioxidant properties of hesperetin may also contribute to the inhibition of excitotoxicity.

One of the major factors causing neurodegenerative processes in Alzheimer's disease is believed to be  $\beta$ -amyloid (A $\beta$ ) peptides. Although the precise mechanisms mediating A $\beta$  toxicity remain to be fully elucidated, A $\beta$  toxicity is also suggested to be associated with increases in ROS, which may in turn initiate neurotoxic events (Behl

*et al.*, 1994; Harris *et al.*, 1995; Smith *et al.*, 1996). Considering that hesperetin is a strong antioxidant, we then examined if it is capable of reducing the A $\beta$  toxicity induced in the cultured cortical cells by A $\beta$ <sub>(25-35)</sub>, an active fragment of A $\beta$  peptide. Hesperetin was found to dramatically inhibit the A $\beta$ <sub>(25-35)</sub>-induced toxicity (Fig. 5). Interestingly, we found that hesperetin inhibited the NMDA and A $\beta$  toxicity more potently (the respective IC<sub>50</sub> values = 92.1 and 97.2  $\mu$ M) than it scavenged DPPH radicals or inhibited the oxidative neuronal damage and lipid peroxidation (the respective IC<sub>50</sub> values = 252.2  $\mu$ M, not determined, and 179.1  $\mu$ M). These results strongly suggest that other mechanisms as well as its antioxidant actions may contribute to the neuroprotective effects of hesperetin. Further investigation is required to elucidate the exact action mechanisms by which hesperetin exerts neuroprotective effects.

Taken together, this study demonstrated that the pharmacological properties of hesperetin in the CNS are markedly different from those of its rhamnoglucoside hesperidin. Hesperetin is a more active antioxidant and neuroprotectant than hesperidin, protecting neurons against oxidative damage as well as excitotoxicity and A $\beta$ <sub>(25-35)</sub>-induced toxicity. Based on its multiple pharmacological actions demonstrated in this study, hesperetin may provide a useful therapeutic potential for the prevention or treatment of neurodegenerative disorders associated with various types of insults such as oxidative stress, excitotoxicity, and A $\beta$ .

## ACKNOWLEDGEMENTS

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