

Comparable Attenuation of $A\beta_{25-35}$ -Induced Neurotoxicity by Quercitrin and 17β -Estradiol in Cultured Rat Hippocampal Neurons

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Accepted: 17 April 2010 / Published online: 15 May 2010
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Abstract In the present work, potential protective effects of quercitrin (a phytoestrogen) on $A\beta$ -induced neurotoxicity in cultured rat hippocampal neurons were investigated in comparison with 17β -estradiol. Cell viability, oxidative status, and antioxidative potentials were used as comparative parameters. Co-exposure of cultured neurons to $A\beta_{25-35}$ with either quercitrin or 17β -estradiol (50–100 μ M) for 72 h attenuated $A\beta_{25-35}$ -induced neurotoxicity and lipid peroxidation, but not $A\beta_{25-35}$ -induced ROS accumulation. However, only 17β -estradiol counteracted a reduction in glutathione content and only quercitrin counteracted a reduction in glutathione peroxidase activity. Both compounds displayed no effects on superoxide dismutase activity. A specific estrogen receptor antagonist, ICI 182780, did not abolish neuroprotective effects of quercitrin and 17β -estradiol. These findings suggested that quercitrin and 17β -estradiol attenuated $A\beta_{25-35}$ -induced neurotoxicity in a comparable manner. Underlying neuroprotective mechanisms of both compounds were probably not related to estrogen receptor-mediated genomic mechanisms but might involve with their antioxidant and free radical scavenging properties.

Keywords Quercitrin · 17β -estradiol · $A\beta_{25-35}$ · Neurotoxicity · Neuroprotection · Hippocampal neurons

Introduction

Alzheimer's disease (AD) is characterized by two pathologic hallmarks, extracellular plaques of amyloid- β ($A\beta$) peptide aggregates and intracellular neurofibrillary tangles [1, 2]. It has been demonstrated that $A\beta$ is toxic to neurons either in vitro [3, 4] or in vivo [3, 5]. Several synthetic $A\beta$ peptides have been used to study the mechanisms of their toxicity in various cell lines [6] and primary neuronal cell cultures [7]. Among $A\beta$ fragments, the $A\beta_{25-35}$ peptide represents the shortest fragment of $A\beta$ processed in vivo by brain proteases and exhibits significant levels of molecular aggregation, retaining toxicity of the full length peptide [7]. Recently, a study of $A\beta_{25-35}$ in organotypic hippocampal slice cultures revealed that this peptide induced neural injury in a similar pattern to $A\beta_{1-42}$ [8], thereby reinforcing the use of $A\beta_{25-35}$ as a convenient tool for the investigation of neurotoxic mechanisms involved in AD [4].

Several mechanisms by which $A\beta$ and its aggregates mediated toxicity have been proposed such as the disruption of calcium homeostasis [9], consequently inducing the accumulation of free radicals and/or reactive oxygen species (ROS), and eventually leading to lipid and protein oxidation. After the exposure of rat PC12 and primary cortical cultures to $A\beta_{25-35}$, a marked reduction in cell survival and activities of glutathione peroxidase (GPx) and catalase was observed along with an increase of malondialdehyde (MDA) production [10]. Additionally, $A\beta_{25-35}$ significantly decreased superoxide dismutase (SOD) and increased GPx activities in astrocyte cultures [11], decreased glutathione (GSH) levels in mixed hippocampal cultures [12], decreased SOD and GSH with increasing lipid peroxidation in peripheral lymphocytes [13] and decreased SOD and GPx as well as MDA in hippocampal cultures [14]. These findings suggest that $A\beta_{25-35}$ is not

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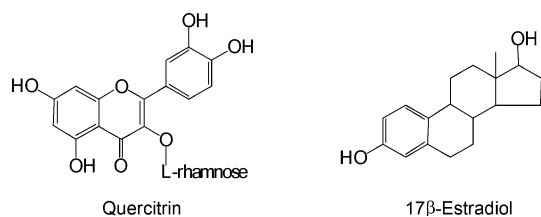


Fig. 1 Chemical structures of quercitrin and 17β-estradiol

only a potent inducer of lipid peroxide, but also alterations of the antioxidant enzyme activity.

Neurotoxic effects of $A\beta$ are at least in part mediated by free radicals, and some antioxidants such as vitamin E [15] and estradiol [16] have been proved to rescue cells from $A\beta$ -mediated apoptosis. Therefore, therapeutic efforts aimed at scavenging oxygen free radicals or reducing their formation may be beneficial in AD. There has been a substantial amount of clinical research showing that estrogen replacement therapy (ERT) could reduce the risk and delay the onset of AD. However, the use of estrogen, especially long-term use, as a treatment for AD is limited. For this reason, other estrogenic agents with fewer side effects, such as phytoestrogens, have received considerable attention as potential alternatives to ERT. Recently, there has been considerable interest in neuroprotective effects of flavonoids, especially with respect to their mode of action as antioxidants. Neuroprotective effects of several phytoestrogens on $A\beta$ -induced toxicity have been reported such as kaempferol in PC12 neuroblastoma cells and rat cortical neurons [17, 18], apigenin in rat cortical neurons [18], genistein in hippocampal neurons [19] and SH-SY5Y human neuroblastoma cells [20].

Among a wide variety of phytoestrogens, quercitrin, a glycoside form of quercetin (Fig. 1) exhibited a scavenging property and lipid peroxidation inhibition in rat brain homogenate [21] but not in microglial cells [22]. Quercitrin might be more potent antioxidant and neuroprotective agent than quercetin due to its high bioavailability in the digestive track [23, 24]. Therefore, the purpose of this study is to investigate potential neuroprotective effects of quercitrin on $A\beta$ -induced neurotoxicity in cultured hippocampal neurons, as well as its possible mechanisms that mediate the in vitro neuroprotection. A well recognized estrogenic compound, 17β-estradiol, was tested in parallel as a comparative agent.

Materials and Methods

Materials

Chemicals

Equine serum and fetal bovine serum (FBS) were purchased from Hyclone (Utah, USA). Superoxide dismutase

assay kit and glutathione peroxidase assay kit were purchased from Cayman Chemical Co. (USA). Estrogen receptor antagonist (ICI 182780) was purchased from Tocris Cookson, Inc. (Ellisville, MO, USA). All other chemicals, tissue culture media, supplements, and assay kits were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental Animals

Timed-pregnant Wistar albino rats of gestation day 14 were purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. They were individually housed in plastic cages with wood shavings as bedding in the animal room of Faculty of Pharmaceutical Sciences, Chulalongkorn University, under controlled environmental conditions (room temperature $25 \pm 1^\circ\text{C}$ with 12-h light/dark cycle, humidity of approximately 60%). All pregnant rats were fed with commercial rodent chow and tap water ad libitum until the operation day.

Methods

Neuronal Cell Culture

Primary cultures of hippocampal neurons were prepared from 18-day-old Wistar rat fetuses as previously described [25]. They were cultured at 37°C for 24 h and the medium was changed to serum-free medium with chemically defined supplements and then used in the experiments. Our control cultures at 4 DIV were approximately 90% neuronal cells and the remaining cells (about 10%) were mostly astrocytes as determined by immunohistochemical techniques (unpublished data). Animal handling and experimental protocols with animals were approved by the Animal Ethics Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand, No. 134/2004).

$A\beta_{25-35}$ -Induced Neurocytotoxicity

$A\beta_{25-35}$ stock solution was made in sterile distilled water at a concentration of 1 mg/ml and stored at -20°C . An aliquot of the stock solution was incubated at 37°C for 72 h to aggregate the peptide before use [26]. Cultured hippocampal neurons were exposed to various concentrations of $A\beta_{25-35}$ (1–20 μM) for 72 h. After exposure, cell survival and cell death was analyzed by MTT reduction and LDH release assays.

Attenuation of $A\beta_{25-35}$ -Induced Neurocytotoxicity by Quercitrin and 17β-Estradiol

Quercitrin and 17β-estradiol were dissolved in dimethylsulfoxide (DMSO) and diluted with sterile distilled water

as stock solutions. Cultured hippocampal neurons were exposed to the combination of $A\beta_{25-35}$ (5 μM) and either quercitrin or 17β -estradiol (0.1, 1, 10, 50 and 100 μM) for 72 h. Thereafter, assessment of neurocytotoxicity was carried out.

Involvement of estrogenic effects was testified by using ICI 182780, a specific estrogen receptor antagonist. Cultured hippocampal neurons were pretreated with 1 μM ICI 182780 for 2 h before the addition of $A\beta_{25-35}$ with either quercitrin or 17β -estradiol as previously mentioned. Assessment of neurocytotoxicity was carried out as well.

Assessment of Neurocytotoxicity

Cell viability. Mitochondrial metabolic activity, an indicator for cell viability, was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay which was a modified colorimetric method from that of Mosmann [27]. Briefly, MTT stock solution (5 mg/ml) was added to the culture medium (a final concentration of 100 $\mu\text{g/ml}$). After 1 h of incubation at 37°C, the medium was aspirated, and 200 μl of DMSO was added to dissolve the dark blue formazan crystal. The absorbance was measured at 570/620 nm using an Anthos Labtec HT2 microplate reader. Results were expressed as the percentage of MTT reduction.

Cell death. Lactate dehydrogenase (LDH) release, an indicator of cell death, was determined by an In Vitro Toxicity Assay Kit with a procedure according to the manufacturer's instructions. Briefly, medium LDH was assayed in 100 μl aliquots of culture medium and cellular LDH was measured in 100 μl aliquots of cell lysate. The reaction was initiated by adding 50 μl of assay mixture, left at room temperature for 30 min, and then terminated by adding 50 μl of 0.5 N HCl. The absorbance was determined at 490/690 nm using an Anthos Labtec HT2 microplate reader. LDH release into the medium was expressed as the percentage of total LDH.

Lipid peroxidation. Malondialdehyde (MDA), a by-product of lipid peroxidation, was quantitated using a modified thiobarbituric acid reactive substance (TBARS) procedure from that of Ohkawa et al. [28]. Cultured neurons were lysed with 160 μl of 2% sodium dodecyl sulfate for 30 min. The lysates from two culture wells were pooled and adjusted with 2% sodium dodecyl sulfate to 1 ml and serially added with 50 μl of 4% butylated hydroxytoluene in ethanol, 1 ml of 10% phosphotungstic acids in 0.5 M sulfuric acid and 1.5 ml of 0.7% thiobarbituric acid. The reaction mixtures were boiled at 95°C for 60 min. After cooling, the mixtures were extracted with *n*-butanol and centrifuged at 3,500 rpm for 10 min. The fluorescence of *n*-butanol layer was measured at the excitation wavelength of 515 nm and the emission wavelength of 553 nm using

Jasco Model FS 777 spectrofluorometer. In this experiment, 1,1,3,3-tetraethoxypropane (TEP) was used as an external standard. Lipid peroxidation was expressed in term of MDA equivalence (1 M TEP = 1 M MDA).

Levels of reactive oxygen species (ROS). The accumulation of ROS was determined by analyzing 2',7'-dichlorofluorescein (DCF) fluorescence as described previously [29]. Briefly, cells were washed twice with cooled PBS and incubated with 50 μM of DCFH-DA at 37°C for 45 min. Loaded cells were washed with cooled PBS and subsequently scraped into PBS. The cell suspension was pelleted by centrifugation at 5,000 $\times g$ at 4°C and then the cell pellet was resuspended in cooled PBS. The fluorescent intensity was quantified with a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader using excitation and emission wavelengths of 485 and 535 nm, respectively.

Glutathione content. Total glutathione (GSH) was determined by using enzymatic cycling method that modified from that of Tietz [30]. In brief, pooled cultured neurons from two culture wells were extracted with 300 μl of 1% (w/v) sulfosalicylic acid and centrifuged. Twenty microlitre of the supernatant was transferred to a 96-well plate and then volume was adjusted to 100 μl . After that, 100 μl of reaction mixture was added to each well. The formation of 2-nitro-5 benzoic acid was monitored at the wavelength of 405 nm every 10 s for 2 min using an Anthos Labtec HT2 microplate reader. The slope of initial rate of reaction was used for calculating GSH content from a standard curve derived from known amounts of GSH.

Superoxide dismutase activity. Superoxide dismutase (SOD) was determined by using the Cayman Chemical SOD assay kit. Briefly, pooled cultured neurons from two culture wells were harvested and lysed in cold buffer and then centrifuged at 1,500 $\times g$ for 5 min at 4°C. The assay of SOD activity in the supernatant was performed according to the manufacturer's protocol. The xanthine/xanthine oxidase system was used to generate the superoxide anion which reduced a tetrazolium salt to formazan dye. The SOD activity removed the superoxide anion and produced an inhibition of the tetrazolium salt reduction which was monitored at 450 nm with a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader. One unit of SOD activity was defined as the amount of enzyme need to exhibit 50% dismutation of superoxide radical. The specific activity was expressed as units per milligram protein.

Glutathione peroxidase activity. Glutathione peroxidase (GPx) was determined by using the Cayman Chemical GPx assay kit. In brief, pooled cultured neurons from two culture wells were harvested and lysed in cold buffer and then centrifuged at 10,000 $\times g$ for 15 min at 4°C. The assay of GPx activity in the supernatant was performed according to the manufacturer's protocol. GPx activity was measured indirectly by a coupled reaction with glutathione reductase

(GR). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm which was monitored every minute at 340 nm using a Perkin–Elmer VICTOR3 Wallac 1420 microplate reader. One unit of GPx activity was defined as the amount of enzyme that catalyzed the oxidation of 1.0 nmol of NADPH to NADP⁺/min at 25°C and specific activity is represented as units per milligram protein.

Protein content. Protein content was determined by Bradford reagent [31] using bovine serum albumin as a protein standard.

Statistical Analysis

Data were expressed as mean \pm SEM of values obtained from at least three separate independent cultures, each performed in duplicate. Differences among mean values of various groups were tested using one-way analysis of variance (ANOVA) followed by pairwise comparisons between groups with Scheffe's post hoc analysis. *P* values less than 0.05 were considered to be significant.

Results

A β_{25-35} -Induced Neurocytotoxicity

Exposure to 1–20 μ M of A β_{25-35} for 72 h was apparently cytotoxic to cultured hippocampal neurons in a concentration-dependent manner as considered from analyses of cell viability and cell death (data not shown). Neurons with typical appearances of irregular shape and fragmented neurites were clearly seen after exposure to higher concentrations of A β_{25-35} . Approximately 50% of cell viability was seen after an exposure to 5 μ M A β_{25-35} for 72 h. Therefore, this experimental setting was arbitrarily adopted as the neurotoxic insult in our in vitro cellular model of neurodegeneration.

Attenuation of A β_{25-35} -Induced Neurocytotoxicity by Quercitrin and 17 β -Estradiol

After the exposure of cultured hippocampal neurons to either quercitrin or 17 β -estradiol at increasing concentrations up to 100 μ M for 72 h, both compounds at all concentrations tested did not induce significant changes in the viability of cultured neurons (data not shown). We then employed concentrations of 0.1–100 μ M as a nontoxic concentration range of quercitrin and 17 β -estradiol in the study of their neuroprotective effects.

Cultured hippocampal neurons were exposed to 5 μ M A β_{25-35} for 72 h in combination with various concentrations of either quercitrin or 17 β -estradiol prior to determination of cell viability. As shown in Fig. 2, co-exposure with higher concentrations of quercitrin, 50 and 100 μ M, increased the percentage of cell viability (estimated by MTT reduction) from 55.62 \pm 2.98% in only A β_{25-35} -treated controls to 71.43 \pm 2.26 and 76.09 \pm 2.81%, respectively (*P* < 0.01). In a similar manner, co-exposure with 50 and 100 μ M of 17 β -estradiol also rescued cell viability from 54.15 \pm 3.38% in only A β_{25-35} -treated controls to 78.27 \pm 5.07 and 81.83 \pm 4.71%, respectively (*P* < 0.05).

Potential neuroprotective effects of quercitrin and 17 β -estradiol at higher concentrations were convincingly supported by the analysis of cell death (Fig. 3). In all experimental conditions, the magnitude of cell death was correlated with the percentage of cell viability in a complementary manner.

Possible Mechanism(s) of Neuroprotective Effects of Quercitrin and 17 β -Estradiol

Antioxidant-Related Mechanisms

As shown in Fig. 4, exposure to 5 μ M A β_{25-35} alone for 72 h increased cellular lipid peroxidation to 148.22 \pm 3.31% of controls (*P* < 0.001). Co-exposure with 50 and 100 μ M of either quercitrin or 17 β -estradiol significantly reduced that increment in a concentration-related manner. Exposure with

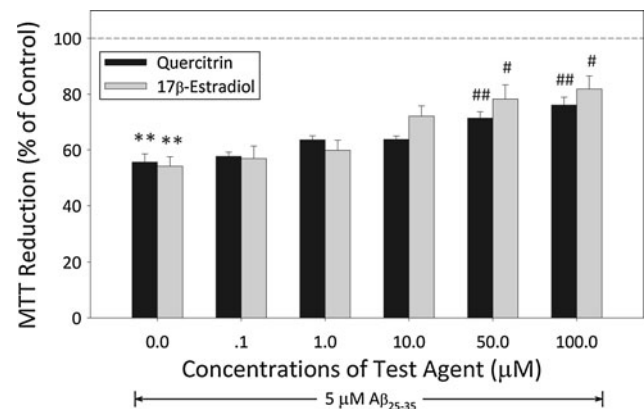


Fig. 2 Protective effects of co-exposure with quercitrin or 17 β -estradiol on A β_{25-35} -induced decrease in cell viability in cultured hippocampal neurons. Cultured neurons were treated with 5 μ M A β_{25-35} in combination with different concentrations (0.1–100 μ M) of quercitrin or 17 β -estradiol for 72 h. After co-exposure, cell viability was determined by MTT reduction assay. Data are expressed as mean \pm SEM of values from four independent experiments. ** *P* < 0.01 compared with control cultures, # *P* < 0.05, ## *P* < 0.01 compared with only A β_{25-35} -treated cultures. There are no significant differences in neuroprotection between treatment with quercitrin and treatment with 17 β -estradiol (independent sample Student *t*-test)

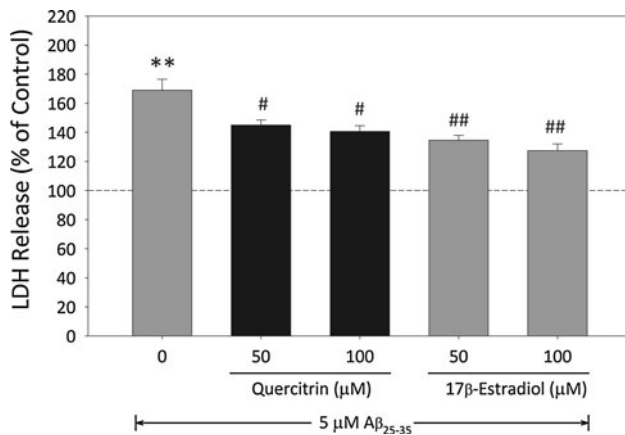


Fig. 3 Protective effects of co-exposure with quercitrin or 17 β -estradiol on A β_{25-35} -induced cell death in cultured hippocampal neurons. Cultured neurons were treated with 5 μ M A β_{25-35} in combination with quercitrin or 17 β -estradiol (50 and 100 μ M) for 72 h. After co-exposure, cell death was determined by LDH release assay. Data are expressed as mean \pm SEM of values from three independent experiments. ** $P < 0.01$ compared with control cultures, # $P < 0.05$, ## $P < 0.01$ compared with only A β_{25-35} -treated cultures. There are no significant differences in neuroprotection between treatment with quercitrin and treatment with 17 β -estradiol (independent sample Student t -test)

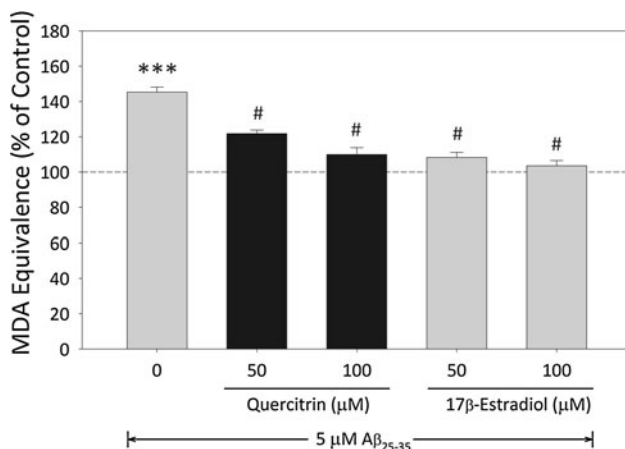


Fig. 4 Beneficial effects of co-exposure with quercitrin or 17 β -estradiol on A β_{25-35} -induced lipid peroxidation in cultured hippocampal neurons. Cultured neurons were treated with 5 μ M A β_{25-35} in combination with quercitrin or 17 β -estradiol (50 and 100 μ M) for 72 h. After co-exposure, levels of lipid peroxidation were determined by TBARS assay. Data are expressed as mean \pm SEM of values from four independent experiments. *** $P < 0.001$ compared with control cultures, # $P < 0.05$ compared with only A β_{25-35} -treated cultures

either of these two test compounds alone did not cause significant changes in cellular lipid peroxidation (data not shown). Therefore, attenuation of A β_{25-35} -induced neurotoxicity by higher concentrations of quercitrin and 17 β -estradiol was apparently associated with decreased generation of lipid peroxidation in cultured neurons.

Exposure of cultured neurons to 5 μ M A β_{25-35} gradually increased intracellular ROS levels with a peak level around 18 h of exposure ($165.71 \pm 13.19\%$ of controls, $P < 0.01$) and apparently declined thereafter. However, both quercitrin and 17 β -estradiol at concentrations of 50 and 100 μ M had no significant effects on A β_{25-35} -induced ROS accumulation (Fig. 5). Exposure with either of these two test compounds alone did not cause significant changes in ROS levels (data not shown). These results revealed that apparent neuroprotective effects of quercitrin and 17 β -estradiol were not associated with a decrease in ROS generation.

Due to a long exposure time, quercitrin and 17 β -estradiol might alter intracellular antioxidant defense systems of cultured hippocampal neurons including GSH, SOD and GPx. Exposure to 5 μ M A β_{25-35} alone for 72 h caused a marked reduction of cellular GSH contents to $34.58 \pm 5.53\%$ of controls ($P < 0.001$) (Fig. 6). Co-exposure to A β_{25-35} in combination with 50 and 100 μ M 17 β -estradiol augmented cellular GSH contents to 58.4 ± 4.7 and $64.69 \pm 5.42\%$ of controls, respectively, which were significantly different from that of only A β_{25-35} -treated groups ($P < 0.05$ and $P < 0.01$, respectively). Exposure with either of quercitrin or 17 β -estradiol alone did not cause significant changes in GSH contents (data not shown). Co-exposure with quercitrin, however, marginally and insignificantly recovered GSH contents.

Regarding the SOD activity, exposure of cultured hippocampal neurons to 5 μ M A β_{25-35} for 72 h slightly and insignificantly decreased SOD activity as compared to

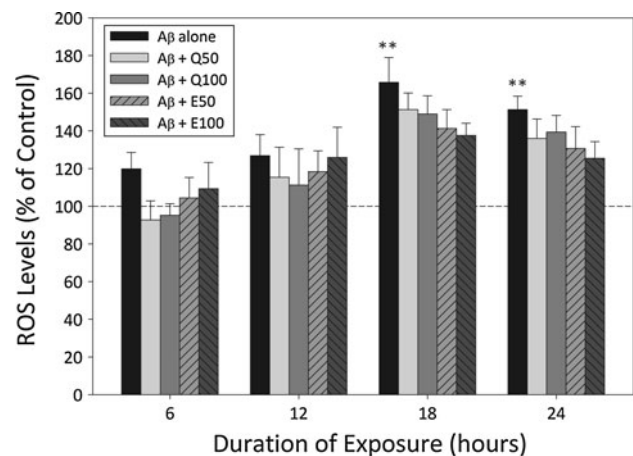


Fig. 5 Effects of co-exposure with quercitrin or 17 β -estradiol on A β_{25-35} -induced ROS accumulation in cultured hippocampal neurons. Cultured neurons were treated with 5 μ M A β_{25-35} (A β) in combination with 50 and 100 μ M of quercitrin (Q50, Q100) or 17 β -estradiol (E50, E100) for different durations (6, 12, 18 and 24 h). After co-exposure, cellular ROS level was quantified by using DCFH-DA and a spectrofluorometer. Data are expressed as mean \pm SEM of values from three independent experiments. ** $P < 0.01$ compared with control cultures

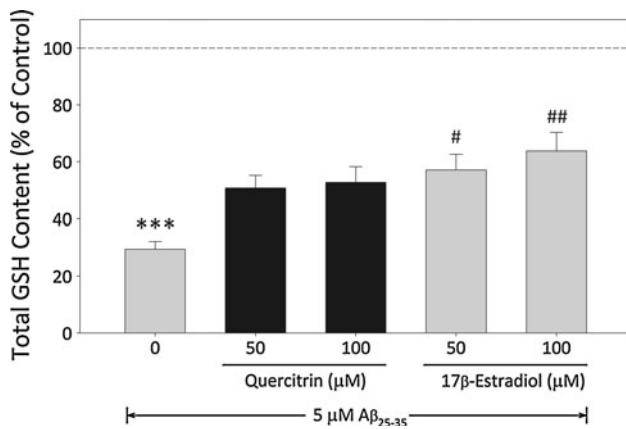


Fig. 6 Effects of co-exposure with quercitrin or 17β-estradiol on Aβ_{25–35}-induced diminution in GSH contents in cultured hippocampal neurons. Cultured neurons were treated with 5 µM Aβ_{25–35} in combination with quercitrin or 17β-estradiol (50 and 100 µM) for 72 h. After co-exposure, total cellular GSH content was measured. Data are expressed as mean ± SEM of values from four independent experiments. *** *P* < 0.001 compared with control cultures, #*P* < 0.05, ##*P* < 0.01 compared with only Aβ_{25–35}-treated cultures

controls (data not shown). In addition, no significant changes were found in all co-exposure conditions of Aβ_{25–35} with either quercitrin or 17β-estradiol.

Regarding the GPx activity, exposure to 5 µM Aβ_{25–35} alone for 72 h significantly decreased GPx activity by approximately 20% of controls (Fig. 7). Co-exposure with 50 and 100 µM of quercitrin induced significant increases in GPx activity from 136.35 ± 4.75 U/mg protein in only Aβ_{25–35}-treated cultures to 167.88 ± 6.13 U/mg protein

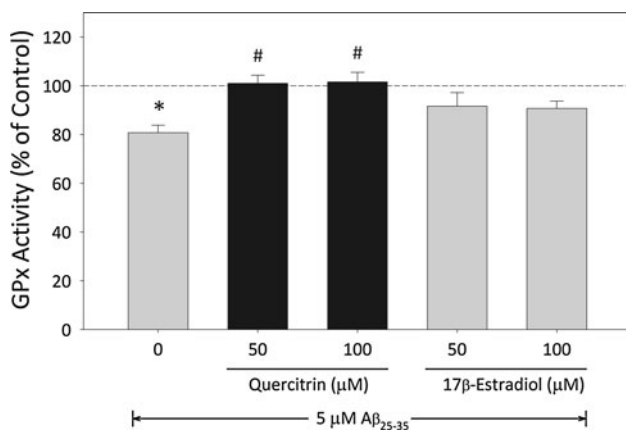


Fig. 7 Effects of co-exposure with quercitrin or 17β-estradiol on Aβ_{25–35}-induced changes in glutathione peroxidase activity in cultured hippocampal neurons. Cultured neurons were treated with 5 µM Aβ_{25–35} in combination with quercitrin or 17β-estradiol (50 and 100 µM) for 72 h. After co-exposure, cellular GPx activity was measured. Data are expressed as mean ± SEM of values from four independent experiments. **P* < 0.05 compared with control cultures, #*P* < 0.05 compared with only Aβ_{25–35}-treated cultures

and 170.32 ± 4.61 U/mg protein, respectively (*P* < 0.05). However, no significant changes in the activity of GPx enzyme were found when cultured neurons were co-exposed to Aβ_{25–35} and 17β-estradiol. Exposure with either of quercitrin or 17β-estradiol alone did not cause significant changes in the GPx activity as well (data not shown).

Estrogen Receptor-Related Mechanisms

Neuroprotective effects of quercitrin and 17β-estradiol might mediate through estrogen receptor-dependent mechanisms. This hypothesis was tested by pre-incubating cultured neurons with ICI 182780, a specific estrogen receptor antagonist, for 2 h prior to the addition of Aβ_{25–35} in combination with either quercitrin or 17β-estradiol and further incubated for 72 h. Exposure to 5 µM Aβ_{25–35} alone significantly decreased cell viability to approximately 63% of controls. Quercitrin and 17β-estradiol at a concentration of 50 µM recovered this decreased cell viability up to approximately 81 and 83% of controls, respectively (*P* < 0.01). It was notable that ICI 182780 at 1 µM, a concentration that effectively blocked estrogen receptors without affecting cell viability, was unable to antagonize neuroprotective effects of both quercitrin and 17β-estradiol (Fig. 8). These results suggested that protective efficacy of quercitrin and 17β-estradiol against Aβ_{25–35}-induced neurocytotoxicity, under our experimental conditions, might not be dependent on activation of conventional estrogen receptors.

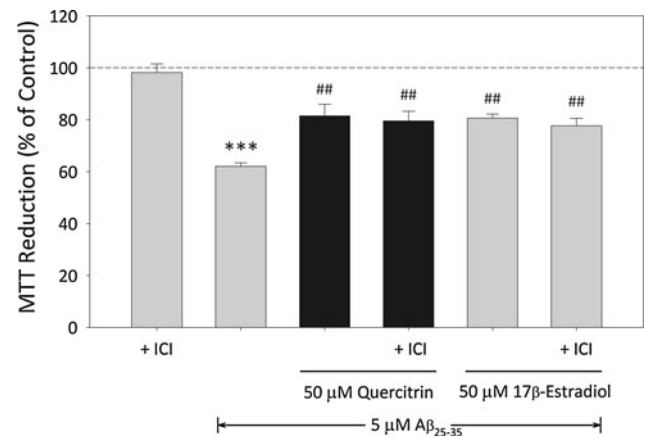


Fig. 8 Ineffectiveness of a specific estrogen receptor antagonist to block protective effect of quercitrin or 17β-estradiol against Aβ_{25–35}-induced neurotoxicity in cultured hippocampal neurons. Cultured neurons were pretreated with 1 µM of ICI 182780 (ICI) for 2 h and further treated with 5 µM Aβ_{25–35} in combination with quercitrin or 17β-estradiol (50 and 100 µM) for another 72 h. After co-exposure, cell viability was measured by MTT reduction assay. Data are expressed as mean ± SEM of values from four independent experiments. *** *P* < 0.001 compared with control cultures, ##*P* < 0.01 compared with only Aβ_{25–35}-treated cultures

Discussion

In the present study, we employed immature cultured hippocampal neurons exposed to $A\beta_{25-35}$ as an in vitro neurodegenerative paradigm because of its higher sensitivity and vulnerability to oxidative damages and the relevance to the pathogenesis of AD [32, 33]. Our results showed that $A\beta_{25-35}$ decreased the viability of cultured hippocampal neurons in a concentration-dependent manner which are consistent with previous studies [34, 35].

Our study revealed that quercitrin which is a member of phytoestrogens, at a higher concentration range of 50–100 μ M, attenuated $A\beta_{25-35}$ -induced neurocytotoxicity in comparable to a natural estrogen, 17 β -estradiol. There have been several lines of evidence supporting neuroprotective effects of 17 β -estradiol against $A\beta$ -induced toxicity [3, 16, 36, 37] whereas supporting evidence for neuroprotective effects of quercitrin has been inadequate. It is most likely that our work offers the first in vitro experimental evidence on neuroprotective effects of quercitrin against $A\beta$ -induced neurotoxicity in cultured hippocampal neurons.

Cellular injury during AD may result from ROS as well as from impaired cellular repair mechanisms following oxidative injury. Several mechanisms have been proposed whereby $A\beta$ may increase ROS generation. Apart from generating ROS directly in solution, $A\beta$ may also interact with a number of biological systems and increase the rate of ROS production through modification or stimulation of intrinsic pathways [38], e.g., causing damage to the mitochondrial respiratory chain [39]. In our study, these mechanisms might induce a gradual increase in intracellular ROS levels with a peak level around 18 h of exposure. With sustained increase of ROS generation, various cellular defense mechanisms came into play thereby lessening ROS levels. In spite of attenuating the increased lipid peroxidation induced by 72 h exposure to $A\beta_{25-35}$, quercitrin and 17 β -estradiol showed marginal benefit on intracellular ROS increment during 24 h exposure to $A\beta_{25-35}$. As considered from the nature of assay methods used, quercitrin and 17 β -estradiol seem to be more effective against membrane-derived nonpolar free radicals (TBARS assay) than cytosol-derived polar free radicals (DCF fluorescence assay). Alternatively, this discrepancy may be due to the difference in exposure time of cultured neurons to $A\beta_{25-35}$.

A role of oxidative stress in AD is supported by increased levels of thiobarbituric acid-reactive substance, a measure of lipid peroxidation [40, 41]. The neurotoxicity of $A\beta_{25-35}$ is mediated through lipid peroxidation, leading to oxidative damage of synaptic terminals and eventually to neuronal death [42]. It has been demonstrated in vitro that 17 β -estradiol protected cells from lipid peroxidation

caused by $A\beta_{25-35}$ [43]. Recently, quercitrin was found to prevent lipid peroxidation induced by pro-oxidant agents in rat brain homogenates, suggesting that quercitrin exhibited a scavenger and antioxidant role involving the negative modulation of the Fenton reaction [21]. In accordance with previous studies, the present study suggested that $A\beta_{25-35}$ -induced neurocytotoxicity by increasing cellular lipid peroxidation. After co-incubation, both quercitrin and 17 β -estradiol showed protective effects against $A\beta_{25-35}$ -induced lipid peroxidation via their antioxidant and free radical scavenging properties.

Cells possess a variety of defense mechanisms and repair systems against ROS. This can sometimes be inadequate, leading to oxidative stress in which the production of ROS overwhelms antioxidant defenses of the organism. Quercitrin and 17 β -estradiol may facilitate cellular defense systems (such as GSH levels, SOD and GPx activities) to counteract increased ROS levels. In this study, $A\beta_{25-35}$ alone or $A\beta_{25-35}$ in combination with either quercitrin or 17 β -estradiol had no apparent effects on SOD enzyme activity in cultured hippocampal neurons. One major index of oxidative stress is the GSH level which has been described in a variety of model systems in AD brains and cells exposed to $A\beta$ [44, 45]. However, the total brain levels of GSH appeared to be unaffected in AD [45], whereas GPx and glutathione reductase were found to be elevated in different brain regions [40] or unchanged [41]. In our study, $A\beta_{25-35}$ significantly decreased the GSH content and GPx activity in cultured hippocampal neurons, in line with a previous report that exposure to $A\beta$ for 24 h caused GSH depletion in hippocampal neurons [12]. However, co-incubation with 17 β -estradiol attenuated $A\beta_{25-35}$ -induced GSH depletion whereas quercitrin did not show this beneficial effect. Meanwhile, co-incubation with quercitrin, but not 17 β -estradiol, revealed a significant increase in GPx activity. Based on these findings, quercitrin might induce upregulation of antioxidant enzymes including GPx. However, there has been no published report on the induction effect of quercitrin on this antioxidant enzyme.

One of the defense mechanisms that protect the cell from oxidative damage is the dismutation of superoxide anion radical to hydrogen peroxide by SOD which is further converted to water by GPx, using GSH as a substrate. The generated hydrogen peroxide can be converted in spontaneous reaction catalyzed by metals such as iron (Fenton reaction) to the highly reactive hydroxyl radical, reacting with various biological macromolecules including polyunsaturated fatty acids by which initiating the process of lipid peroxidation [46]. In our study, a clear-cut enhancement of GPx activity that caused by quercitrin may conceal an expected increase in GSH levels. As a result, this facilitated defense mechanism shifts the conversion of

hydrogen peroxide to water thereby reducing the formation of highly reactive hydroxyl radical, and eventually leading to decreased lipid peroxidation.

Estrogens coordinate cellular mechanisms involved in the development and differentiation of various neuronal populations, modulation of synaptic plasticity and neuronal excitability, induction of neuronal survival and axonal outgrowth, and neurogenesis in adult [47]. Increasing evidence supports the role of estrogen as neuroprotective compound that can act dependently or independently of estrogen receptors. Estrogen-mediated neuroprotection may involve the activation of intracellular estrogen receptors [36, 48, 49]. The exact role of estrogen receptors in estrogen-induced neuroprotection is still controversial. It was reported that ICI 182780, a specific estrogen receptors antagonist, antagonized estrogen effects in cortical neurons and 5H-SY5Y cells [50, 51] but not in cortical, hippocampal and SK-N-SH cells [43, 52, 53]. However, our findings demonstrated that ICI 182780 did not block quercitrin- or 17 β -estradiol-mediated neuroprotection against A β _{25–35}, suggesting that neuroprotective effects of both compounds may not involve cytoplasmic or nuclear estrogen receptor-mediated mechanisms. In accordance with our study, high concentrations of 17 β -estradiol necessary for cellular protection in some studies suggest a hormone receptor-independent effect, probably mediated by the antioxidant activity [43, 54].

The endoplasmic reticulum (ER) plays a leading role in lipid and protein synthesis. In parallel with protein synthesis at ER-bounded ribosomes, membrane-spanning or water soluble proteins are folded by a chaperone in ER. However, under many insulting conditions, e.g., heat shock, dysregulation of calcium dynamics or cellular redox control, proteins are misfolded or unfolded (ER stress) which leads to the aggregation of unusual proteins and apoptosis [55]. ER stress has been attracting considerable attention because it relates to many disorders including neurodegenerative diseases, diabetes, arteriosclerosis, inflammation, and liver or kidney damage [56].

In cultured hippocampal neurons and organotypic hippocampal slice cultures, A β -induced neurotoxicity might interact with ER stress resulting in potentiated neuronal cell death which could be protected by S-allyl-L-cysteine, an organosulfur compound purified from aged garlic extract [8, 57]. Recently, it was found that quercetin, a typical flavonoid ubiquitously present in fruits and vegetables, suppressed the ER stress caused by calcium dynamics dysregulation in cultured intestinal epithelial cells via the inhibition of PI3K [58]. It is conceivable that quercitrin, a closely structure-related flavonoid to quercetin, might share this particular property which contributes to its neuroprotective activity in cultured neurons. However, there has been no direct supporting evidence available so far.

It may be apprehensive that we employed a high concentration range of test compounds which may be unreasonable in practical situations. The key point of this study is the experimental finding of potential neuroprotective effects of quercitrin in an *in vitro* model of neurodegeneration. Nevertheless, the relevance of such finding to therapeutic applications is comprehensible because various pharmacological aspects, in addition to drug concentrations or doses, determine the efficacy and safety of drug therapy in humans. Incidentally, more detailed pharmacokinetic and pharmacodynamic studies of quercitrin are needed to justify this crucial application.

In conclusion, we firstly illustrate the potential neuroprotective effect of quercitrin, a member of phytoestrogens, on a neuronal cell culture model of neurodegeneration. This beneficial effect may involve inhibition of lipid peroxidation and induction of GPx activity. Underlying neuroprotective mechanisms of quercitrin might not be related to estrogen receptor-mediated genomic mechanisms, but apparently may involve with its antioxidant and free radical scavenging properties. Currently, the exact mechanisms of quercitrin-induced neuroprotection and its potential therapeutic applications in neurodegenerative disorders are still unclear and warrant further investigation.

Acknowledgments This study was supported partly by the Graduate Research Funds from the Graduate School, Chulalongkorn University.

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