CHAPTER V

DISCUSSION AND CONCLUSION

The main objectives of the present study were to (1) characterize an *in vitro* model of Aβ-induced neurodegeneration in rat primary hippocampal neuronal cultures; (2) investigate the neuroprotective effects of quercitrin on Aβ-induced neurotoxicity in cultured neurons; and (3) elucidate the possible mechanisms of quercitrin that mediate neuroprotection in cultured neurons.

In order to fulfill our goals, we used an exposure to $A\beta_{25-35}$ as an *in vitro* neurodegenerative paradigm because it has been well studied and related to the pathogenesis of AD. We employed immature hippocampal neurons in primary culture as the *in vitro* model of neurodegeneration due to their higher sensitivity and vulnerability to oxidative damages (Koshy et al., 1998; Jiang et al., 2004; Shirai et al., 2006). Moreover, hippocampus is an important brain area afflicted in AD. Neuronal cell injury and death were measured by using MTT reduction and LDH release assays. To investigate the contribution of prooxidant and antioxidant mechanisms in oxidative stress-induced neuronal cell injury and death after $A\beta_{25-35}$, we studied the production of ROS and lipid peroxidation levels, intracellular GSH levels, SOD and GPx activities. To investigate the contribution of apoptotic cell death mechanisms, we studied caspase-3 activity, Bcl-2 and Bax protein contents, and cytochrome c release. Quercitrin, a member of polyphenol flavonols that resembles phytoestrogens, was tested for its potential neuroprotective effects with this *in vitro* neurodegenerative model. Estrogen receptor antagonist, MEK and PI3K inhibitors, were also used to investigate the probable routes of quercitrin's neuroprotective action.

In summary, we found that exposure to $A\beta_{25-35}$ induced a concentration- and exposure time-dependent injury and death to cultured hippocampal neurons as considered from mitochondrial activity (MTT reduction) and plasma membrane damage (cytoplasmic LDH release). Nearly 50% of cultured neurons died after exposure to 5 μM $A\beta_{25-35}$ for 72 hr and this condition was later adopted as a standard neurotoxic insult in further experiments on neuroprotective effects of quercitrin.

Neither exposure to quercitrin nor exposure to 17β-estradiol at a concentration range of 0.001 - 100 μM for 72 hr induced cytotoxic effects to cultured hippocampal

neurons. Instead, both compounds displayed a certain degree of neuroprotective effect as considered from the following evidence. Co-exposure but not pre-exposure with quercitrin or 17β-estradiol (50-100 μM) attenuated $A\beta_{25-35}$-induced neuronal injury and death (increased MTT reduction and decreased LDH release), prooxidative status (decreased lipid peroxidation without significant changes in ROS generation). Regarding antioxidative status, co-exposure with 17β-estradiol but not quercitrin significantly improved $A\beta_{25-35}$-induced reduction in cellular GSH content whereas co-exposure with quercitrin but not 17β-estradiol significantly improved $A\beta_{25-35}$-induced reduction in GPx activity. However, co-exposure to both compounds displayed no effects on SOD activity.

Neuroprotective effects of quercitrin and 17β-estradiol might involve inhibition of apoptotic cell death mechanisms because both compounds could attenuate $A\beta_{25-35}$-induced increases in cytochrome c release, caspase-3 activity and Bax protein expression. Moreover, both quercitrin and 17β-estradiol could counteract $A\beta_{25-35}$-induced decrease in Bcl-2 expression.

The possible mechanisms of quercitrin or estradiol-mediated neuroprotection in rat primary hippocampal neuronal cultures are shown in Figure 29.

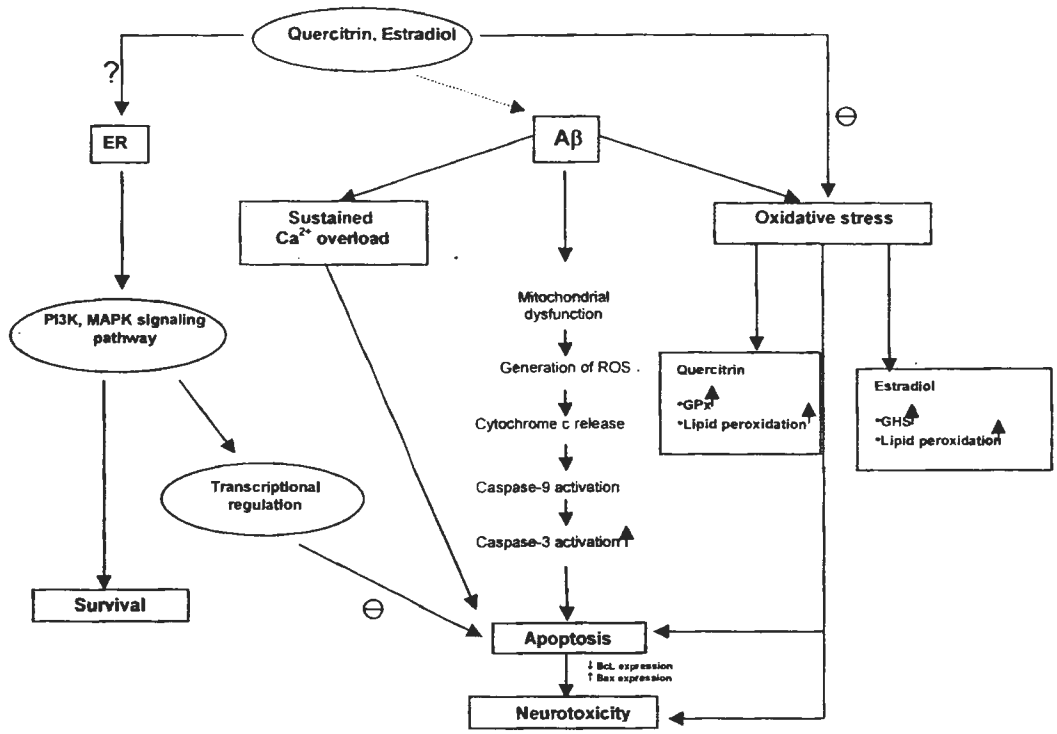

Figure 32 Schematic diagram of the possible mechanisms of quercitrin or estradiol-mediated neuroprotection in rat primary hippocampal neuronal cultures.

The pathology of AD includes vascular amyloid deposits, senile plaques, and neurofibrillary tangles. Moreover, a profound loss of neurons is an invariant feature of AD. Neuronal death may be caused by multiple insults present in the AD brain, including oxidative stress, loss of growth factors, inflammatory responses, and Aβ, a 39- to 43-amino-acid peptide which is the principle component of senile plaques. Accumulating evidence suggests that neuronal death in AD is apoptotic, although cell death in AD has also been proposed to occur through necrosis. Apoptosis, a regulated program of cell death that may be activated in disease, is characterized morphologically by plasma membrane blebbing, somal shrinkage, chromatin condensation, fragmentation of the nucleus, and eventual fragmentation of the cell into apoptotic bodies. The biochemical events that underlie apoptosis have begun to be elucidated and include activation of caspase and calpain serine proteases and exposure of phosphatidylserine on the cell surface.

Aβ causes apoptotic death of neurons in culture. Apoptotic death of neurons can also result from growth factor withdrawal and from oxidative stress. Additionally, apoptosis mediated by plasma membrane receptors, referred to as activation-induced apoptosis, occurs in cerebellar granule cells exposed to TGF_{β} and in cortical neurons treated with concanavalin A.

Aβ neurotoxicity is mediated by various mechanisms ranging from direct neurotoxic interactions with the neuronal membrane to effects of Aβ on membrane receptors and intracellular signaling. Interestingly, it was found that Aβ needs to be aggregated to exert toxic activities and Aβ aggregates then directly interact with the cell membrane. It was proposed that this interaction is not random, but is rather mediated by the receptor for advanced glycation end products (RAGE). Recent studies have revealed that the degree of neurotoxicity can be demonstrated with pre-fibrillar Aβ aggregates including Aβ oligomer. Although these studies suggest that neurotoxicity and neuronal changes associated with Aβ may stem from non-fibrillar Aβ intermediate, an important question that remains to be answered is whether this is also the case in the AD brain. There is another activity of Aβ that may endanger neuronal survival. Aβ aggregates on the nerve cell membrane have been reported to induce a sequence of events that lead to the intracellular accumulation of ROS. Micromolar concentrations of Aβ cause the oxidation of the nonsaturated carbohydrate side chains of membrane lipids, disintegration of the neuronal membrane and, ultimately, cell lysis. Various additional studies by different laboratories have supported the view that oxidative stress may be central to the Aβ-driven neurodegeneration.

In both *in vitro* and in animal studies, there are many data supporting a neuroprotective role of estrogen. 17β-estradiol has been shown to reduce cell loss in response to a variety of insults such as Aβ, glutamate and experimental stroke. Several clinical and preclinical evidence has supported the benefit effects of estrogen replacement therapy. Estrogen such as 17β-estradiol might exert multiple neuroprotective functions by the followings. First, estrogen might activate ER which translocate to the nucleus and bind to ER-response elements on the genomic DNA. There, ER might induce the transcription of modulators of neuroprotection. Second, the phenolic lead structure of estrogen might represent an intrinsic antioxidant activity that is independent of ER activation. Third, estrogen might modulate the function of

membrane receptors that might modulate neuronal transmission. Finally, estrogen might cross-talk and might interact with various intracellular signaling pathways such as MAPK, CREB, ERK1/2 and NF-κB which then indirectly have genomic effects.

However, the use of estrogen in menopausal women is associated with increased risks of uterine cancer and potentially an increase in neoplasm of the breast. On the contrary, several reports have indicated that phytostrogens did not promote neoplasm of the breast and uterus, but they might have protective effect against certain forms of cancers, cardiovascular disease and oxidative stress. In addition, they might prevent undesired menopausal symptoms. As the result of these beneficial effects, phytostrogens have received much attention as potential alternative therapy instead of estrogen. It was speculated that phytoestrogens which mimic the actions of estrogens may exert neuroprotective effects and underlying intracellular mechanisms similar to those of estrogens. However, the supporting evidence for neuroprotection of phytostrogens and underlying mechanisms are still undefined and need to be further investigated.

Aβ-induced apoptosis in cultured hippocampal neurons has been used as an *in vitro* neurodegenerative model for the screening or study of potential neuroprotective agents. In the present study, $A\beta_{25-35}$ was selected as a neurotoxic agent because it plays important role in degenerative disease associated AD. The stock solution of $A\beta_{25-35}$ was aged at 37 °C for 3 days to aggragate the peptide before using in the experiment.

In the present study, cultured rat hippocampal neurons were used to investigate the potentially beneficial effects of quercitrin (a related member of phytoestrogens) on Aβ-induced neuronal cell injury and death. Cultured hippocampal neurons have been widely used as an *in vitro* test model for neurotoxic/neuroprotective agents due to their well characterized features and involvement in cognitive functions. We selectively employed immature hippocampal neurons in primary culture as the *in vitro* model of neurodegeneration due to their higher sensitivity and vulnerability to oxidative damages.

Recently, it has been hypothesized that hippocampal neurons are capable of *de novo* estradiol synthesis. Prange-Kiel et al. (2003) established a pure neuronal hippocampal dispersion culture from adult rats cultivated under serum- and steroid-

free conditions. Subsequent radioimmunoassay revealed the presence of estradiol in the medium, indicating the ability of hippocampal cell cultures to synthesize and release estradiol. Subsequent experiments with hippocampal slice cultures and hippocampal dispersion cultures obtained from postnatal rats confirmed these findings (Kretz et al., 2004). 17β-Estradiol must be synthesized *de novo* from cholesterol, the precursor of all steroid hormones, since the medium of the dispersion cultures contained neither serum nor steroids.

It is widely accepted that estrogen, as other steroids, acts via genomic receptors. Two ER isoforms are described, ER_α and ER_β. Binding of the ligand leads to an activation cascade, finally resulting in the translocation of the receptor to the nucleus, where it functions as a ligand-dependent transcription factor (Klinge, 2001).

Hippocampal neurons in tissue sections as well as in slice cultures and dispersion cultures express ER_α and ER_β mRNA, as demonstrated by *in situ* hybridization (Wehrenberg et al., 2001; Prange-Kiel et al., 2003). Immunohisto-chemical staining with specific antibodies against ER_α and ER_β revealed the presence of these proteins in the nuclei and the cytoplasm of hippocampal neurons.

The fact that hippocampal neurons are capable to produce estrogens by themselves raises the question of the functional significance of this endogenous steroid. With respect to this issue we used a specific ER receptor antagonist to rule out the contribution of endogenous and exogenous estrogen through ER-mediated neuroprotection in our culture conditions. We found that ER-mediated signaling might not involve in neuroprotective effects of quercitrin and 17β-estradiol tested with our *in vitro* neurodegeneration model of immature hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then incubated with $A\beta_{25-35}$ at different concentrations and durations. Results showed that $A\beta_{25-35}$ decreased cell viability of hippocampal neurons in a concentration-and exposure time-dependent manner. These results are similar to many previous studies (Iversen et al., 1995; Yankner, 1996; Mattson, 1997; Behl, 1997). An important feature of Aβ toxicity is that it requires the peptide to be present in the form of an amyloid aggregate. Because 50% of cells die after exposure to $A\beta_{25-35}$ at the concentration of 5 μM for 72 h, this indicated time and duration was used as toxic condition through this experimental study.

Innitially, we did study the effects of the prototype flavonols (kaempferol and quercetin) and quercetin glycoside (quercitrin), flavones (apigenin) and isoflavones (genistein) on cell survival after 72 hr of co-incubation with $A\beta_{25-35}$. The results demonstrated that these phytoestrogens including kaempferol, quercetin, apigenin and genistein did not prevent hippocampal neuronal injury and death induced by $A\beta_{25-35}$, whereas quercitrin at the concentration of 50 and 100 μM significantly reduced hippocampal neuronal cell injury and death induced by this toxin in comparison with its vehicle. Similar results were obtained when we co-treated 17β-estradiol at the same concentrations with $A\beta_{25-35}$. These results suggested that quercitrin and 17β-estradiol had comparable neuroprotective properties against $A\beta_{25-35}$-induced neurotoxicity in primary hippocampal neurons.

There are several lines of evidence supporting neuroprotective effects of 17β-estradiol. Estrogen-mediated neuroprotection has been widely reported in Aβ-induced toxicity (Behl et al., 1995,1997; Goodman et al., 1996; Green et al., 1996; Gredley et al., 1997, 1998; Mattson, 1997; Bonnefont et al., 1998; Pike, 1999; Roth et al., 1999). On the contrary, there is no previous report on neuroprotection of quercitrin. This study is the first to report the neuroprotective effect of quercitrin against Aβ-induced neurotoxicity.

Quercetin is the most abundant bioflavonoid found in vegetable and fruits with high concentration found in onions, apples and tea. This compound is mainly present in the glycoside form, for example, as quercitrin. Knownledge on the pharmacokinetics and the bioavailability of quercetin in humans and animals is still limited. There are only a few publications indicated that the relative bioavailability of quercetin glycosides may be greater than of the aglycones. Previous study has demonstrated that the absorption of quercetin glycosides contained in onions was higher (52%) than that of quercetin aglycones (24%) (Hollman et al., 1995). Another study reported the 3-O-β-glucoside of quercetin was better absorbed than quercetin (Morand et al., 2000). Flavonoid glycosides are also considered to be the efficacious compounds of phytomedicines used in the therapy of infections of lower urinary tract as frequently used in Western European countries (De Smet and Brouwers, 1997). However, the majority of studies have been carried out with the aglycone form and

little known about the biological properties of glycoside forms, due to the lack of commercial standards (Scalbert et al., 2002).

Recently *in vitro* study, Wagner et al., (2006) investigated the antioxidant action of quercitrin in rat brain lipid peroxidation induced by different agents including Fe^{2+}, Fe^{2+} plus EDTA, quinolinic acid and sodium nitroprusside. Additionally, the effect of quercitrin on the Fenton reaction was investigated. These results indicated that quercitrin exhibits a scavenger and antioxidant role and these effects probable might mediated via different mechanisms involving the negative modulation of the Fenton reaction and NMDA receptor. Based on these data, the glycoside form of quercetin was able to prevent lipid peroxidation in the presence of different inducers. These results were interesting due to flavonoids are widely found in human foods and predominantly as beverages. *In vivo* study, quercitrin can be more important antioxidant and neuroprotective agent than quercetin because of its high bioavailability in the digestive track. However, there were a few *in vivo* studies with this glycoside (Camuesco et al., 2004; Comalada et al., 2005)

Regarding amyloid toxicity and oxidative stress, it has been reported that Aβ can induce lipid peroxidation, protein oxidation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation (Butterfield and Stadman, 1997; Varadarajan et al., 2000; Butterfield and Lauderback, 2002). This oxidative damage or membrane modification, resulting from reaction of the lipid peroxidation products 4-hydroxy-2-nonenal or arolein with enzymatic, transport, structural proteins, alters synaptic membranes that can lead to oxidative damage of synaptic terminals and eventually, lead to the death of the neurons (Butterfield and Stadtman, 1997; Butterfield and Lauderback, 2002). Aβ has also been shown using a variety of assays to cause increase production of ROS or impaired antioxidant defenses in a number of model systems. Similarly, considerable evidence suggested that antioxidant strategies may protect cell model systems from Aβ-induced toxicity (Varadarajan et al., 2000).

In this study $A\beta_{25-35}$ likely mediated its neurotoxicity by increased cellular lipid peroxidation. After co-incubation for 3 days, higher concentrations of quercitrin or 17β-estradiol (50 and 100 μM) showed protective effect against $A\beta_{25-35}$-induced lipid peroxidation in hippocampal neuronal cultures. These results suggested that neuroprotective effects of quercitrin or 17β-estradiol may be related to their

antioxidant property. In accordance to our study, the neurotoxicity of $A\beta_{25-35}$ had been reported to be mediated through lipid peroxidation (Butterfield and Stadman, 1997; Varadarajan et al., 2000; Butterfield and Lauderback, 2002). In the *in vitro* studies, estrogen had been shown to protect cells from the toxicity of various Aβ peptides (Goodman et al., 1996; Green et al., 1996; Gridley et al., 1997; Mattson et al., 1997; Mook-Jung et al., 1997 Bonnefont et al., 1998; Bae et al., 2000; Hosoda et al., 2001; Kim et al., 2001; Fitzpatrick et al., 2002). In the case of quercitrin, there has been no published report in term of neuroproptective effects.

With respect to its chemical structure the female sex hormone estrogen (estradiol) is a steroid hormone but also a monophenolic compound similar to vitamin E. The direct structural comparison of 17β-estradiol and α-tocopherol demonstrates that both molecules contain a phenolic radical scavenging moiety and a lipophilic carbohydrate moiety. In general, phenolic A ring estrogens are powerful inhibitors of lipid peroxidations in various cell free test tube models (Sugioka et al., 1987; Moosmann and Behl, 1999). Similar to estrogen, the flavonoid quercetin prevent membrane lipid peroxidation and protect neuronal cells against oxidative cell death *in vitro* (Moosmann et al.,1997; Skaper et al., 1997; Moosmann and Behl, 1999). In the present study quercitrin, a quercetin glycoside, apparently exhibited a scavenger and antioxidant role therefore it neuroprotective effects might probably mediated via different mechanisms involving the negative modulation of the Fenton reaction and NMDA receptor (Wagner et al., 2006).

One major index of oxidative stress is the level of reduced GSH and oxidation of GSH has been described repeatedly in a variety of model systems in AD brains and other tissues of AD patients with familial forms of AD and models in which cells have been exposed to Aβ (Muller et al.,1997; Cecchi et al., 1999; Abramov et al., 2003). Loss of GSH and oxidative damage have been suggested to constitute early, possibly signaling events in apoptotic cell death (Sato et al., 1995).

GSH is one of the major antioxidant systems in the CNS. Using confocal imaging of GSH in mixed hippocampal culture, It was found that exposure to Aβ for 24 hr caused depletion of GSH both in astrocytes and in neurons (Keelan et al., 2001; Abramov et al., 2003). GSH depletion was Ca^{2+} dependent, suggesting that alterations in Ca^{2+} homeostasis might lie upstream in the cascade of injury caused by Aβ and that

the oxidative injury might itself be Ca^{2+} dependent. Strong evidence reported by Li et al., (1997) suggested that glutathione depletion causes death in cell culture studies.

In the present study, we found that $A\beta_{25-35}$ may mediate its neurotoxicity through GSH depletion. After co-incubation for 3 days, it was evident that 17β-estradiol at the only dose of 100 μM possessed a protective effect against β_{25-35}-induced GSH depletion in hippocampal neuronal cultures whereas quercitrin did not show this beneficial effect. There has been no clear explanation but the possibility is that we measured total cellular glutathione contents (GSH + GSSG) which may not reflect the real intracellular equilibrium of this important endogenous antioxidant.

In the case of ROS generation, several mechanisms have been proposed whereby Aβ may increase ROS generation. Aβ alone may apparently generate oxygen radicals directly in solution but may also interact with a number of biological systems to increase the rate of radical production through modification or stimulation of intrinsic pathways. It may be activation of endogenous radical generating systems in microglia and possible other cell types in the CNS by activation of the flavoprotein linked enzyme system NADPH oxidase. The action of Aβ on mitochondria, it has also been suggested that Aβ may increase the production of ROS from mitochondria by causing damage to the mitochondrial respiratory chain (Benzi and Moretti. 1995).

In our study, $A\beta_{25-35}$ significantly increased intracellular ROS at 18 and 24 hr. Co-incubation with quercitrin or 17β-estradiol at concentrations of 50 and 100 μM for 18 to 24 hr did not reduce an accumulation of ROS caused by $A\beta_{25-35}$. These results indicated that neuroprotective effects of both compounds did not involve with ROS accumulation.

It is well known that method of defense against ROS includes detoxification of the superoxide anion and H_2O_2 catalysed by intracellular SOD, catalase and GPx enzyme activities. In our study, we examined the effects of quercitrin or 17β-estradiol on antioxidant enzymes of cultured hippocampal neurons after co-treatment with these compounds. In the present study, the SOD activity of hippocampal neurons tended to decrease after an exposure to 5 μM $A\beta_{25-35}$ but not statistically significant and no protective effects were found with any of the compounds tested when co-treated with $A\beta_{25-35}$. Our results indicate that $A\beta_{25-35}$ alone or $A\beta_{25-35}$ in combination with

quercitrin or 17β-estradiol has no apparent effects on SOD enzyme activity in cultured hippocampal neurons.

In the case of GPx activity, it was shown that 5 μM $A\beta_{25-35}$ alone significantly decreased GPx activity. Meanwhile, co-incubation with quercitrin at concentrations of 50 and 100 μM, but not 17β-estradiol, revealed a significant increase in GPx activity when compared with $A\beta_{25-35}$-treated cultures. These results suggest that $A\beta_{25-35}$-induced neurotoxicity may involve a decrease in GPx levels and the protective effect of quercitrin is related to the enhancement of GPx enzyme activity.

It is now well known that Aβ is neurotoxic and kills neuronal cells via an apoptotic process. Substantial evidence indicates that Aβ contributes significantly to be the pathological cascades in AD by various mechanisms such as by generating ROS, elevating intracellular free $Ca^{2+}$and other cytotoxic stimuli. It is documented that $A\beta_{25-35}$ induces massive Ca^{2+} influx and free radical production that eventually lead to apoptosis. Activation of caspases is now well accepted to be responsible for causing apoptotic cell death (Matsuzawa and Ichijo, 2001). In particular, caspase-3 is a major executioner of the apoptotic signals.

Caspase-3 is a member of the caspase family proteins that is activated in response to apoptotic signals coming either from the mitochondria through caspase-9 or from a mitochondrial-independent apoptotic pathway through caspase-8 as well as through other pro-apoptotic pathways inside the cell (Cohen, 1997; Porter and Janicke, 1999). The protein level of caspase-3 is increased in AD brain (Shimohama et al., 1999). It has been reported that caspase-3 is activated by $A\beta_{25-35}$ (Harada and Sugimotor, 1999). In addition, caspase-3 is activated by ROS and the disruption of intracellular homeostasis of Ca^{2+}. Furthermore, Singh (2001) has reported that estradiol activates Akt/protein kinase B which can mediate the anti-apoptotic signaling pathway through increased expression of anti-apoptotic protein Bcl-2.

In this study, we found that caspase-3 activity was activated in hippocampal neurons by $A\beta_{25-35}$. This result suggested that $A\beta_{25-35}$ likely generates apoptotic signals through activation of caspase-3. Interestingly, both quercitrin and 17β-estradiol inhibited the increase in caspase-3 activity that induced by $A\beta_{25-35}$ thereby indicating that they may prevent hippocampal neuronal apoptotic cell death.

Different apoptotic stimuli can stimulate mechanistically distinct cell death pathways such as caspase-independent pathways and pathways that involve direct caspase activation. Bax and Bcl-2 are found in various cell types as pro and anti-apoptotic members that initiate or hinder the caspase cascade. In particular, Bcl-2 has a central role in preventing adaptors from activating caspases. On the contrary, Bax is a Bcl-2 homologue that counteracts the protective action of Bcl-2.

Cytochrome c is an essential component of the mitochondrial electron transport chain where it shuttles electrons from complex III (cytochrome c reductase) to complex IV (cytochrome c oxidase). When cytochrome c is present in the cytosol, it drives the assembly of a high molecular weight caspase activating complex termed the apoptosome complex. This complex composes of released cytochrome c, Apoptotic Activation Factor-1 (Apar-1) and procaspase-9 which in turn results in the conversion of pro-caspase-9 to actvated caspase-9. Caspase-9 cleaves and activates other caspases such as caspase-3 resulting in cell apoptosis (Lemasters et al., 1998; Luo et al., 1998).

In the present study, $A\beta_{25-35}$ likely mediated its neurotoxicity by induction of cytochrome c release and upregulation of Bcl-2 protein as well as down regulation of Bax protein. In this connection, both quercitrin and 17β-estradiol tended to attenuate the intracellular processes of $A\beta_{25-35}$-induced neuronal apoptosis.

The recent discovery of the ER_β has spurred intense interest in its potential role in estrogen signaling. It was reported that phytoestrogens exhibit greater affinity to ERβ relative to ERα (Kuiper et al., 1998) The finding is consistent with the fact that ERβ has a higher level of expression than than ERα in brain regions critical to memory function and vulnerable to AD such as the basal forebrain, hippocampus and cerebral cortex (Stahl et al., 1998).

Estrogen neuroprotection may involve the activation of intracellular ER (Singer et al., 1996; Dubal et al., 1998; Sawada et al., 2000). In studies of ER involvement in estrogen-induced neuroprotection, it has been reported that ICI 182,780 antagonized estrogen effects (Singer et al., 1998, 1999; Honda et al., 2000; Sawada and Shimohama, 2000; Wilson et al., 2000; Honda et al., 2001) that ICI 182780 did not antagonize estrogen effects (Briton et al., 1997; Gridley et al., 1998; Gotz et al., 1999; Bae et al., 2000; Howard et al., 2001) and also reported ICI 182,780

alone had a protective effect (Nakamizo et al., 2000; Gridley et al., 1998; Regan and Gue, 1997).

In the present study, we determined whether the protective effects of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurodegeneration were dependent on estrogen receptor. The results showed that addition of ICI 182780, a specific estrogen receptor antagonist, at the concentration of 1 μM did not affect cell survival thereby suggesting that ER antagonism did not prevent quercitrin or 17β-estradiol-mediated neuroprotection against $A\beta_{25-35}$. It is essentially suggestive that neuroprotective effects of quercitrin or 17β-estradiol in our model do not involve with cytoplasmic or nuclear ER-mediated mechanisms.

MAPK pathways transduce extracellular and intracellular stimuli into cellular responses. In general, MAPK signaling motifs are highly conserved throughout evoluation and appear to be essential signal transduction systems in yeast, higher eukaryotes as well as in plants. These responses consist of phosphorylation of cytosolic or nuclear target proteins and activation of transcription factors, which consequently modulate gene expression. Mammals express at least three distinctly regulated groups of MAPKS which may exist in different isoforms including ERK, JNK and p38 kinase (Chang and Karin, 2001)

Active MAPKs function as modulators for differentiation, proliferation, cell death and survival. Commonly, the activation of ERK1/2 has been linked to cell survival, whereas that of JNK and p38, also called the stress-activated protein kinase has been associated with apoptosis. This perspective is an oversimplification and the actual roles are highly dependent on the cell type, the state of cell development, the kind of stimulus and the context of stimulation. Numerous studies have demonstrated that estrogen can rapidly influence cellular physiology in many different cell types of reproductive and nonreproductive tissues through the activation of a diverse array of intracellular signaling mechanisms.

Recently, there is much evidence concerning rapid non-genomic actions of estrogen in the CNS. It was suggested that these rapid effects are of major important for normal development and function of the brain (Kelly and Wagner, 1999; Toran-Allerand et al., 1999; Woolley, 1999). Estrogen has also been shown to influence signaling pathways and results in increased neuronal survival. In cultured cortical

neurons, estrogen can rapidly activated PI3-K (Honda et al., 2000) and MAPK signaling pathway (Singer et al., 1999; Singh et al., 1999). Both estrogen-activated mechanisms have been shown to increase cell survival and to protect cortical neurons from excitotoxic cell death. Additionally, estrogen rapidly protects hippocampal neurons from excitotoxicity through mechanism that requires MAPK activation (Bi et al., 2000) and can rapidly activate MAPK signaling in developing cerebellar neurons (Wong and Belcher, 2000).

In our study, we determined whether protective effects of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurodegeneration mediated via MAPK or PI3K signaling pathway. Inhibitors of intracellular signaling cascade including MEK inhibitor (PD 98059) and PI3K inhibitor (LY 294002) were used in this study. The results demonstrated that 2 hr pretreatment with PD 98059 at the concentration of 30 μM or LY 294002 at the concentration of 40 μM did not abolish quercitrin or 17β-estradiol-mediated neuroprotection against $A\beta_{25-35}$. Therefore, it was essentially suggestive that neuroprotective effects of quercitrin or 17β-estradiol may not involve MAPK- and PI3P-dependent signaling pathway.

However, 2 hr pretreatment with ER antagonist, MEK inhibitor and PI3K inhibitor used in the present study may not be sufficient to elicit desired antagonistic effects due to the omission of those antagonists during the co-exposure of quercitrin or 17β-estradiol with $A\beta_{25-35}$. It is reasonable that an antagonist should be concurrently present with an agonist in order to render maximum antagonism. Therefore, the clearcut contribution of ER-dependent mechanisms, MAPK and PI3K signaling pathways, in quercitrin- and 17β-estradiol-mediated neuroprotection requires further thoroughly designed elucidative studies. At present, the exact mechanisms of quercitrin-mediated neuroprotective effects are still unclear.

In conclusion, we firstly illustrate the potential neuroprotective effect of quercitrin, a related member of phytoestrogens, on a neuronal cell culture model of neurodegeneration. This beneficial effect may involve inhibition of ongoing processes of apoptotic cell death. The underlying neuroprotective mechanisms of quercitrin are unlikely related to estrogen receptor-mediated mechanisms but, apparently, may involve with its antioxidant and free radical scavenging properties. Nevertheless, the exact mechanisms of quercitrin-induced neuroprotection and its

potential therapeutic applications in neurodegenerative disorders are still unclear and warrant further investigation.