CHAPTER I

INTRODUCTION

Senile plaque and neurofibrillary tangles are the hallmarks of Alzheimer's disease (AD) which is one of the major neurodegenerative disorders. Amyloid β protein (Aβ), the major protein component of senile plaque, has been suggested to play an important role in pathogenesis of AD (Selkoe, 1996). However, the precise mechanisms by which Aβ mediates neurotoxicity remain to be fully established. It has been postulated that Aβ-induced neurotoxicity is associated with the disturbance in calcium homeostasis, the accumulation of reactive oxygen species (ROS), as well as increased vulnerability to excitotoxicity (Mattson, 1997; Behl, 1999; Varadarajan et al., 2001). These mechanisms may converge to the same targets and induce cell death in a concert action.

Increasing evidence suggests that a loss in estrogen can increase a woman's risk of developing AD (Sherwin, 2000). Several epidemiological studies have indicated that estrogen replacement therapy could reduce the risk and delay the onset of AD (Paganini-Hill and Henderson, 1996; Tang et al., 1996; Baldereschi et al., 1998; Brinton, 2001; Polo-Kantola and Eekkola, 2001). *In vitro* studies have demonstrated neuroprotective effects of estrogens against Aβ-induced neurotoxicity in several cell lines and in immature primary neuronal cultures (Goodman et al., 1996; Green et al., 1997a; Gridley et al., 1997, 1998; Green and Simpkins, 2000; Kim et al., 2001; Zhang et al., 2001). However, the mechanisms by which estrogen protects against Aβ-induced neurotoxicity are not completely understood. It has been postulated that estrogen protects against fibrillar Aβ-induced neurodegeneration in those cell types by its antioxidant effects, by regulating the metabolism of amyloid precursor protein (APP) and/or by favoring uptake of Aβ by microglia (Goodman et al., 1996; Gridley et al., 1997; Green and Simpkins, 2000; Brinton, 2001).

The mechanism underlying estrogen-related attenuation of Aβ-induced neurotoxicity may involve estrogen receptors (ER) or may be ER-independent (Garcia-Segura et al., 2001). Estrogen may directly affect cell survival or prevent cell death by acting on cell death cascades. It may promote cell survival by induction of axonal sprouting, augmenting regeneration and promoting synaptic transmission

(Garcia-Segura et al., 2001). In addition, estrogens may protect neurodegeneration that mediated by apoptosis and ascendant cholinergic pathways via regulation of apoptotic inhibitor Bcl-XL (Pike, 1999). Furthermore, it has been concerned that rapid non-genomic actions activated by estrogen in the brain including phosphatidylinositol 3-phosphate kinase (PI3-K) (Honda et al., 2000), mitogen activated protein kinase (MAPK) signaling pathway (Singer et al., 1999; Singh et al, 1999), and the Akt/protein kinase B (Akt/PKB) (Singh, 2001) are the major importance for normal development and function of the brain.

Phytoestrogens are a group of natural compounds that exhibit some estrogen like-properties (Mäkelä et al., 1995; Stahl et al., 1998) and also act as partial ER antagonists (Bowers et al., 2000). Phytoestrogens may have protective effects against certain forms of cancer, cardiovascular disease and osteoporosis, and may also prevent undesirable menopausal symptoms (Kurzer and Xu, 1997; Murkies et al., 1998; Tham et al., 1998). As the result of these potentially beneficial effects, phytoestrogens, especially soy isoflavones, have received considerable attention as potential alternatives to estrogen-based hormone replacement therapy (HRT). In addition, it is anticipated that phytoestrogens may have the potential to influence rapid E_2-induced mechanisms in the nervous system that may result in modified brain functions (Belcher and Zsarnovszky, 2001). The above signaling events were shown to be activated by estradiol, the same mechanism might be activated by phytoestrogens. Taken together, phytoestrogens which mimic the actions of estrogens may exert neuroprotective effects and underlying intracellular mechanisms similar to those of estrogens. Among a wide variety of phytoestrogens, flavonoids have been the class of great interest.

Flavonoids comprise a large group of compounds occurring widely throughout the plant kingdom. Daily flavonoid intake (typically present in onion, apple, grape, wine, herbs and spices) in the human diet is highly variable, with estimations ranging from 23 mg/day (Hertog et al., 1993) to more than 500 mg/day (Manach et al., 1996). Flavonoids exert several biological activities, which are mainly related to their ability to inhibit enzymes and/or to their antioxidant properties, and are able to regulate the immune response (Hollman et al., 1995). These activities may explain the beneficial effects that flavonoid intake exerts in different human pathologies, including hypertension, inflammatory conditions and even cancer (Middleton et al., 2000).

Among flavonoids, quercetin is the most common flavonoid in nature, and it is mainly present as its glycosylated forms such as quercitrin (3-rhamnosylquercetin) or rutin (3-rhamnosy-glucosyl quercetin) (Hertog et al., 1993). In vitro studies have clearly shown that quercetin acts as a potent pleiotropic modulator in several physiological functions, showing different activities such as anti-proliferative effect in numerous cell lines (Nair et al., 2004; Fan et al., 2003), pro-apoptotic effect in lung carcinoma cell lines (Nguyen et al., 2004), and inhibitory effect of osteoclastic differentiation (Wattel et al., 2004). It is important to note that when the glycosylated forms of quercetin are assayed, there is usually a loss of activity in these effects in comparison with those obtained with the aglycone, due to the presence of the sugar moiety in the flavonoid structure (Shen et al., 2003). On the contrary, both glycosides, quercitrin and rutin, have shown to exert intestinal anti-inflammatory effects in experimental models of rat colitis (Cruz et al., 1998; Camuesco et al., 2004), whereas no clear effects have been demonstrated for the aglycone form.

Therefore, the purpose of this study is to determine whether quercitrin, the glycoside form of quercetin, could exert neuroprotective effects against Aβ-induced neurotoxicity and whether the neuroprotection conferred by quercitrin is mediated by modulating signaling pathways in survival/apoptotic cascades or by a scavenging antioxidant mechanism. Proposed diagram of neuroprotective effect of quercitrin in Aβ-induced neurotoxicity was shown in Figure 1.

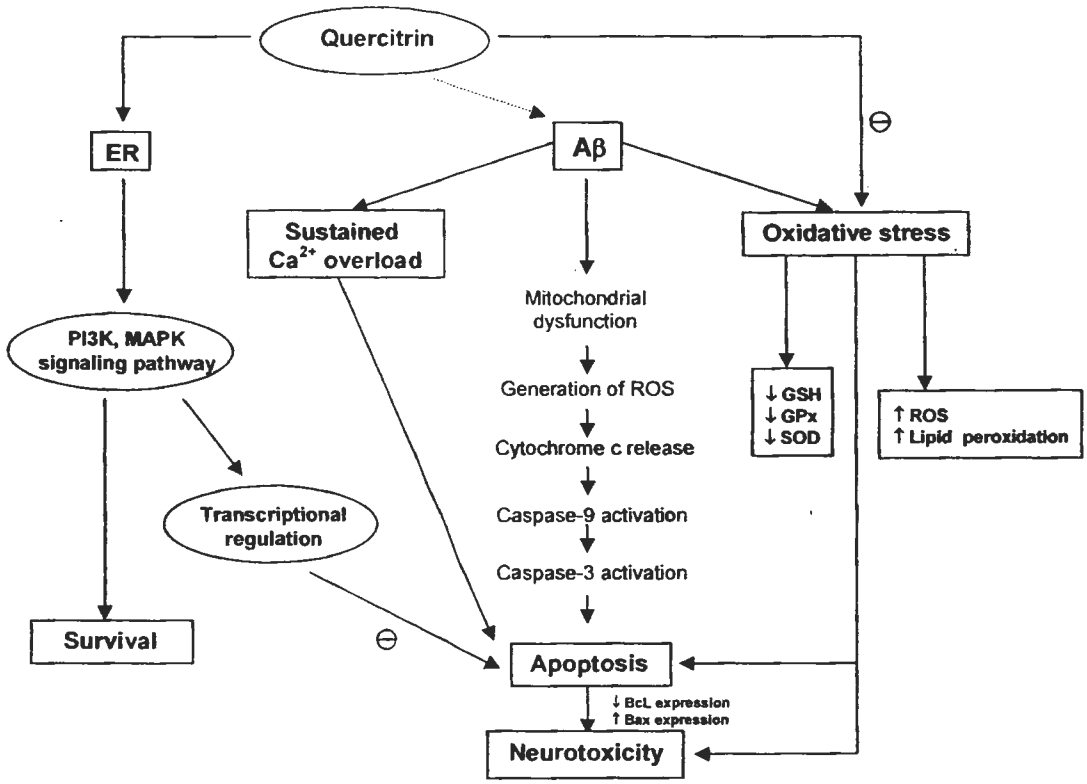

Figure 1 Proposed diagram of neuroprotective effect of quercitrin in Aβ-induced neurotoxicity.

Aβ is the major protein component of senile plaques that plays an important role in pathogenesis of AD. Many brain areas are affected by senile plaques including hippocampus and prefrontal cortex. 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.

Conceivably, Aβ can induce alterations in Ca^{2+} homeostasis that directly or indirectly lead to cell death via apoptotic process. Elevation of cytoplasmic Ca^{2+} levels can promote mitochondrial superoxide production, membrane dysfunction and lead to mitochondrial dysfunction. In addition, Aβ may be directly toxic to mitochondria and cause mitochondrial dysfunction. Damages to mitochondrial membranes enhance the production of ROS and release cytochrome c as pro-apoptotic molecule into the cytosol. There, together with ATP, a complex is formed with

apoptosis protease activating factor-1 and procaspase-9 which leads to the formation of active caspase-9 as the initiator caspase and subsequently activates caspase-3 as the effector caspase. This process leads to an amplification of the cascade and destruction of the cell. Furthermore, Aβ may cause an imbalance of ROS generation and antioxidant systems which leads to overwhelming oxidative stress. A variety of critical biomolecules including lipids, protein and DNA can be damaged by oxidative stress and this oxidative damage can eventually lead to neuronal death.

Quercitrin, by mimicking estrogenic actions, may reverse the neurotoxicity of Aβ by lowering ROS and lipid peroxidation, increasing antioxidant agents and enzymes, e.g., GSH, GPx and SOD, as well as decreasing apoptotic signaling, e.g., cytochrome c and caspase activity. In addition, they may decrease the apoptotic protein expression such as Bax, and increase the anti-apoptotic protein expression such as Bcl-2.

Objectives

1. To characterize an *in vitro* model of Aβ-induced neurodegeneration in rat primary hippocampal neuronal cultures.
2. To investigate the neuroprotective effects of quercitrin on Aβ-induced neurotoxicity in rat primary hippocampal neuronal cultures.
3. To elucidate the possible mechanisms of quercitrin that mediate neuroprotection in cultured neurons.

Scope of study

In this study, primary neuronal cultures from rat hippocampus were used as a sample population to test neuroprotective effects of quercitrin on Aβ-induced neurotoxicity and possible mechanisms of quercitrin that mediate neuroprotection. The signaling pathway involved neuroprotection via oxidative stress (lipid peroxide and ROS generation), antioxidant system (GSH, GPx and SOD), ER, MAPK and PI3K as well as apoptotic signals (cytochrome c release, Bcl-2 and Bax proteins) were evaluated.

Experimental design

To investigate the neuroprotective effects of quercitrin on Aβ-induced neurotoxicity, rat primary hippocampal neuronal cultures were used as a test model. The evaluations of study results were designed as the followings. Cell viability was measured by MTT reduction assay and LDH release assay. Lipid peroxide and ROS production were investigated by spectrofluorometor using thiobarbituric acid reactive substance (TBARS) and 2',7'-dichlorofluorescein diacetate (DCFH-DA). Antioxidant status was evaluated by measurement of GSH, GPx and SOD. Studies on ER-dependent mechanism, MAPK and PI3K signaling pathways were done by using ER antagonist, MEK1 and PI3K inhibitor. Apoptotic signals were determined by measurement of caspase-3 activity and assessment of cytochrome c release, as well as Bcl-2 and Bax protein levels using Western blot analysis.

Contributions of the study

1. Understanding the role of quercitrin on neuroprotection against Aβ-induced neurotoxicity in cultured rat hippocampal neurons and the possible mechanisms involving its neuroprotection.
2. Information about *in vitro* neuroprotective effects and mechanisms of quercitrin would provide more understanding to the role of quercitrin on *in vivo* neuroprotection which may lead to appropriate designs for further animal or human studies and may serve as the new therapeutic approach for the prevention or treatment of neurodegenerative disorders.