Research article

Anti-inflammatory effects of daidzein on primary astroglial cell culture

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Introduction: Alzheimer's disease is the common cause of dementia in old people. The pathological hallmarks of Alzheimer's disease include neuronal loss, deposition of amyloid-β, and presence of neurofibrillary tangles. The endogenous steroid estrogen has been shown to affect neuronal growth, differentiation and survival, while isoflavones also have a neuroprotective effect on human cortical neurons. Daidzein, however, has a superior neuron-protective effect to other isoflavones. The present study is to determine whether daidzein is able to inhibit the production of pro-inflammatory mediators under amyloid-β and lipopolysaccharide stimulation.
Materials and methods: Astrocyte cells were stimulated with amyloid-β or lipopolysaccharide in the absence and presence of diadzein. Nitric oxide released into the culture media was determined using the Griess reaction, and concentrations of IL-1, IL-6, TNF-α and estrogen receptor gene expression were measured by semi-quantitative real-time polymerase chain reaction assay.
Results: Diadzein-treatment increases astrocyte cell counts and attains its maximal effect at the 10^{-12}M concentration. The addition of 20 μM amyloid-β or 10^{-6} g/ml LPS can significantly decrease the viability of astrocytes, up-regulated IL-1, IL-6, TNF-α mRNA and estrogen receptor expression; in addition, 1-h daidzein pre-treatment can restore the decreased viability of astrocytes induced by amyloid-β or lipopolysaccharide as well as down-regulate their mRNA expression.
Conclusions: It seems that this response is estrogen receptor-mediated. These results further increase the possibility that daidzein may have potential to ameliorate the inflammatory process and also alleviate the risk of Alzheimer's disease progression.

Keywords: neuroprotective effect, diadzein, amyloid beta-peptide, lipopolysaccharide

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Received 17 Oct. 2008; revised manuscript accepted 12 Jan. 2009

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder characterized by excessive deposition of amyloid-β peptides in the brain. One of the earliest neuropathological changes in Alzheimer's disease is the accumulation of astrocytes at sites of amyloid-β

DOI 10.1179/147683009X423274

deposition. There has been a novel mechanism for the accumulation of astrocytes around amyloid-β deposits, indicating a direct role for astrocytes in degradation of amyloid-β and implicate deficits in astroglial clearance of amyloid-β in the pathogenesis of Alzheimer's disease.[1] The characteristic brain pathology of Alzheimer's disease includes intra-neuronal neurofibrillary tangles as well as extracellular neuritic plaques containing fibrillar amyloid-β and diffuse plaques with soluble non-fibrillar amyloid-β that are evidence of apoptosis and neuronal death, and generalized cortical atrophy.[2]

Astrocytes are the major non-neuronal cells in the brain and they have been implicated in the segregation, maintenance, and support of neurons. Astrocytes are glial cells constituting part of the blood–brain barrier and capable of performing a variety of functions within the central nervous system. Astrocyte expression of pro-inflammatory cytokines,[3] major histocompatibility complex class II antigen,[4] and antigen presentation[5] have been demonstrated both *in vivo* and *in vitro*. There is emerging evidence that astrocytes are an important source of neuro-active substances such as growth factors, eicosanoids, and neurosteroids such as 3α,5α-tetra-hydroprogesterone (3α,5α-THP) and progesterone, which may subsequently influence neuronal development, survival, and neurosecretion.[6] Thus astrocyte– neuronal interactions have important implications for all areas of the brain. Recently, the importance has been recognized in the form of inflammatory mechanisms in the pathogenesis of a variety of neurodegenerative diseases, such as Alzheimer's disease.[7] However, the mechanisms initiating deleterious neuro-inflammation in neurodegenerative disease are still poorly understood.

Lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, damages neurons only in the presence of microglia.[8] Furthermore, LPS activation of microglia both *in vivo* and *in vitro* causes the progressive and cumulative loss of neurons over time.[9] Bacterial LPS, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) have been shown to trigger astrocytes to up-regulate chemokine expression *in vitro*.[10]

Multiple epidemiological studies indicate that estrogen/hormone replacement therapy can significantly reduce the risk of Alzheimer's disease.[11] These data, which were rapidly conveyed to the clinical and lay communities, stand as a unique and profoundly important series of observations. It has also been suggested that estradiol may delay the onset and ameliorate the symptoms of Alzheimer's disease, although some controversy exists on the topic.[12] In spite of potential benefits, the use of estradiol is limited in clinical situations because its effects on the breast and the uterus have been associated with an increased risk of cancer in these tissues. Thus, selective estrogen receptor modulators (SERMs) such as tamoxifen that can exhibit tissue-specific estrogen-like agonistic or estrogen-antagonistic effects are being used in clinical practice.[13] The endogenous steroid estrogen has been shown to affect neuronal growth, differentiation and survival, while isoflavones also have a neuron-protective effect on human cortical neurons.[14] These agonist/antagonist profiles for individual SERMs may differ also among brain areas.[15] Genistein, daidzein and other isoflavones have been shown to mimic the pharmacological actions of the gonadal steroid estrogen with which they have structural similarities. Several studies have looked at the effect of isoflavones in the brain. In 2008, Chen *et al*.[16] showed that daidzein had a superior neuron-protective effect on lipopolysaccharide-induced dopaminergic neurodegeneration than other isoflavones. In the present study, we used amyloid-β and LPS to simulate chronic glial cell inflammation and analyzed the changes in the inflammatory cytokine, reactive oxygen species (ROS), and evaluated the effects of a selective estrogen receptor modulator, daidzein, on inflammatory responses of primary astroglial cell cultures.

Materials and methods

Primary cell culture

This study received prior approval of the National Yang-Ming University Investigation Research Board Committee. Enriched astrocyte cultures were derived from the ICR mice neonatal cortex (3–4-day-old) using a technique modified from McCarthy and de Vellis[17] that favors the survival and proliferation of astrocytes over neurons. Briefly, glial cells dissociated with papain were grown in DMEM/F12 medium supplemented with 10% fetal calf serum (HyClone; Utah, USA). By assessment with astrocyte immunocytochemical markers (galactocerebroside and glial fibrillary acidic protein [GFAP]), the purity of the cell culture was found to be more than 95% astrocytes as previously described.[18] For each study, the cultures were grown for 7 days until confluence. The medium was removed and cells were washed twice with 5 ml EDTA-trypsin solution (2.5% trypsin), and then re-suspended in 8 ml DMEM + 1% fetal calf serum. Before each experiment, all cells were incubated for 2 days in the same media. In order to minimize any

difference in cellular growth between the controls and treated cultures, cells from the first passage of the same batch were used as controls and treated cultures in each experiment. Also, the same batch of serum was used and reduced to 1% during test procedures to minimize the possible growth-enhancing effect derived from the fetal calf serum in all experiments.

Colorimetric formazan assay for cell viability

To assess the stimulatory effects of various medications on the astrocytes, a series of cultures was stimulated and the MTT (tetrazolium) assay for cell viability was performed on the 2nd, 4th, and 9th days of culture. The mitochondrial activity of the astrocytes after exposure was determined by colorimetric assay which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; catalog no. M2128, Sigma Co., St Louis, MO, USA) to formazan. For the assay, 6250 cells/well were incubated (5% CO_2, 37ºC) in 96-well culture plate in DMEM + 1% fetal calf serum for 2 days. Before the experiment, the cells were pre-treated with different concentrations of daidzein (10^{-9}–10^{-13} M daidzein dissolved in ethanol; Biotic Chemical Co., Ltd, Taipei County, Taiwan, ROC) for 1 h, and then 20 μM amyloid-β or 10^{-6} g/ml LPS was added to the culture. The samples tested in the presence of amyloid-β or LPS were defined as positive neurotoxic control groups; the samples tested in the absence of neurotoxic medication and diadzein were defined as negative control groups. At the end of each time interval, the supernatant was removed, 100 μl per well of MTT solution (1 mg/ml in test medium) was added and the wells were incubated at 37ºC for 4 h to allow the formation of formazan. The supernatant was then removed and acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to dissolve the dark blue crystals. The supernatant was then read on a Micro Elisa reader (Emax Science Corp., Sunnyvale, CA, USA), using a test wavelength of 570 nm against a reference wavelength of 690 nm. Plates were normally read within 1 h of adding the isopropanol.

Nitrite assay

For the assay, 2×10^5 cells/well were incubated (DMEM + 1% fetal calf serum, 5% CO_2, 37ºC) in a 6-well culture plate for 2 days. Before the experiment, cells were pre-treated with different concentrations of daidzein for 1 h, and then 20 μM amyloid-β or 10^{-6} g/ml LPS was added to the culture. The production of NO was assessed by measurement of NO_2^- (an oxidation product of NO) accumulation in the cell-free supernatants. Following the incubation, a 100 μl aliquot of the culture medium was mixed with 100 μl of Griess reagent (1% sulphanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrocloride, 2.5% H_3PO_4), a colorimetric indicator for the presence of nitrite.[19] The samples were incubated in the dark at room temperature for 10 min, and the absorbance was measured at 570 nm in a microplate reader (TiterTek MultiSkan MCC, Alabama, USA). Fresh culture medium served as the blank in all experiments. Solutions of sodium nitrite diluted in culture medium were used as standards. The protein content was determined by a BCA Protein Assay Reagent (Pierce, Illinois, USA) with bovine serum albumin as the standard.

RT-PCR analysis

All experiments were performed in quadruplicate. The total amount of RNA was isolated from cell cultures

Table 1 Polymerase chain reaction primer sequences

Gene name GeneBank access number	Primer sequence (upper – sense primer) (lower – antisense primer)	PCR product (bp)
β-Microglobulin NM009735.3	F: 5′-TTCAGTGTGAGCCAGGATATAGAAA-3′ R: 5′-GAAGCCGAACATACTGAACTGCT-3′	153
IL-1β NM8361	F: 5′-GTGTGTGACGTTCCCATTAGA-3′ R: 5′-AGGTGGAGAGCTTTCAGCTCA-3′	101
IL-6 X54542	F: 5′-CAAGTCGGAGGCTTAAAC-3′ R: 5′-AAGTGCATCATCGTTGTTCAT-3′	101
TNF-α NM013693.1	F: 5′-TCTCTACCTTGTTGCCTCCTCTTTT-3′ R: 5′-TGTAGGGCAATTACAGTCACGG-3′	150
ER-α M38651	F: 5′-TGTACACATTTCTATCCAGCACCTT-3′ R: 5′-GCCATCAAGTGGATCAAAGTGTC-3′	244
ER-β U81451	F: 5′-GATTCTGGAAATCTTTGACATGCTC-3′ R: 5′-ACATACTGGAGTTGAGGAGGATCAT-3′	203

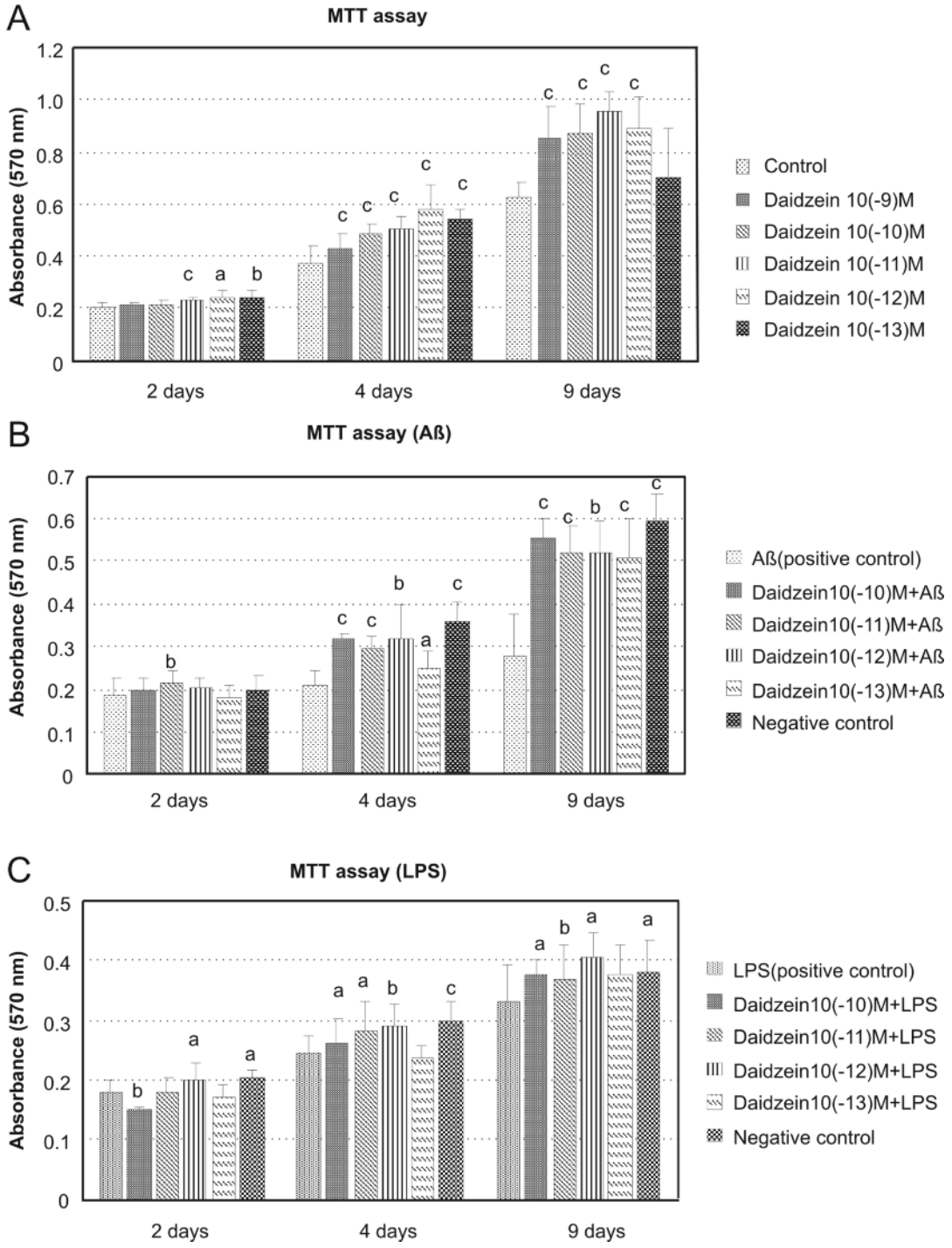

Figure 1 Effect of daidzein on astrocyte proliferation and viability. The astrocyte cell number of the control sample kept increasing during 9 days of culture. When the cells were treated with daidzein in concentrations within the range of 10^{-13} M to 10^{-9} M, the proliferation activity of the astrocyte culture was always higher than that of the control samples, with its maximal effect attained at 10^{-12} M (A). The addition of 20 μM amyloid-β (B) or 10^{-6} g/ml LPS (C) can significantly decrease the viability of astrocytes, while 1 h of daidzein pre-treatment can restore the decreased viability of the astrocytes originally induced by amyloid-β or LPS. (n = 8; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ when compared with control or positive control)

using the ABI-Nucleic acid purification kit with the ABI PRISM 6100 Nucleic Acid PrepStation (ABI, Foster City, CA, USA) following the manufacturer's protocol. Reverse transcription, PCR, and real-time PCR reagents were supplied by Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed using

an ABI Prism 7000 Sequence Detector (Applied Biosystems). Primers were designed and synthesized by Applied Biosystems (Table 1). Quantitative PCR conditions were: (stage 1) 95ºC for 10 min and (stage 2) 40 cycles at 95ºC for 15 s, 60ºC for 30 s, and 72ºC for 2 min. The final cycle (stage 3) heated products slowly to 95ºC while fluorescence measurements were used to generate a dissociation curve utilizing the software program v1.Ob1 provided by Applied Biosystems. From such melting curves, the degree of product purity was assessed. This was accomplished by confirming the presence of a single peak at the known product melting temperature and the absence of any primer dimers that may generate a peak at a lower temperature.[20] In addition to real-time and melting curve analysis of the reactions, amplified products were separated electrophoretically in 4% agarose gels with ethidium bromide and visualized and photographed under UV light to confirm the proper size as well as the absence of non-specific products.

Statistical analysis

Results are expressed as mean ± SD and two-way ANOVA with *post hoc* Bonferroni-corrected *t*-tests was used to examine inter- and intragroup differences for MTT assay and nitrite assay. For the RT-PCR analysis, statistical comparisons were made using the Kruskal–Wallis test to examine differences between individual data points. In all cases, a level of 5% was considered as statistically significant.

Results

Effect of daidzein on astrocyte proliferation and viability

In this study, the astrocyte cell number of the control sample kept increasing during the 9 days of culture. With daidzein treatment, the proliferation activity was always higher than the control samples and attained its maximal effect at 10^{-12} M concentration. At 10^{-12} M diadzein, the viability of astrocytes was increased to 116.59% ($P < 0.05$), 153.31% ($P < 0.001$), and 143.09% ($P < 0.001$) of the control samples on the 2nd, 4th and 9th day of culture (Fig. 1A). In this study, we used a 10^{-12} M concentration of diadzein for all subsequent assays.

The addition of 20 μM amyloid-β or 10^{-6} g/ml LPS can significantly decrease the viability of astrocytes, while a 1-h daidzein pre-treatment (10^{-10}–10^{-13} M daidzein dissolved in ethanol) can restore the decreased viability of the astrocytes induced by amyloid-β (Fig. 1B) or LPS (Fig. 1C).

In the presence of 20 μM amyloid-β, the viability of astrocytes was decreased to 58.10% and 46.31% of the control sample on the 4th and 9th day of culture, respectively. With a 1-h 10^{-12} M daidzein pre-treatment, the decreased viability of the astrocytes was restored to 89.53% ($P < 0.001$ when compared with positive control) and 87.65% ($P < 0.001$ when compared with positive control) of the control sample on the 4th and 9th day of culture, respectively (Fig. 1B).

When astrocytes were cultured with 10^{-6} g/ml LPS for more than 5 days, the viability of astrocytes decreased significantly; thus, we limited our tests to within 4 days of culture. In the presence of 10^{-6} g/ml LPS, the viability of astrocytes was decreased to 88.15%, 81.73% and 86.92% of the control sample on the 2nd, 3rd, and 4th days of culture, respectively. With a 1-h 10^{-12} M daidzein pre-treatment, the decreased viability of the astrocytes were restored to 99.44% ($P < 0.05$ when compared with positive control), 97.25% ($P < 0.01$ when compared with positive control) and 106.14% ($P < 0.05$ when compared with positive control) of the control sample on the 2nd, 3rd, and 4th days of culture, respectively (Fig. 1C).

Effect of daidzein on the astrocyte nitric oxide synthesis

Astrocyte cells treated with daidzein showed a significant decrease in the synthesis of nitric oxide. Amyloid-β did not enhance NO synthesis by astrocytes, but the LPS did increase the NO synthesis significantly (Fig. 2).

In this study, astrocyte cells treated with daidzein can significantly decrease the synthesis of nitric oxide. In the presence of 10^{-12} M diadzein, the synthesis of nitric oxide by astrocytes on the 2nd day of culture was decreased to 87.40% ($P < 0.001$) of the original control samples (Fig. 2A).

NO synthesis by astrocytes was not enhanced by the presence of 20 μM amyloid-β, and the effect of pre-treatment of daidzein in decreasing the NO synthesis was also not so obvious; pre-treatment of daidzein only significantly decreased the NO synthesis on the 4th day of culture (Fig. 2B).

When astrocytes were cultured with 10^{-6} g/ml LPS, the synthesis of nitric oxide were increased to 116.53%, 132.38% and 145.93% of the control sample on the 2nd, 3rd, and 4th days of culture, respectively. Pre-treatment of daidzein significantly decreased the NO synthesis induced by LPS (Fig. 2C).

Effect of daidzein on astrocyte cytokines and estrogen receptor gene expression

After induction for 4 days in the culture conditions, the highest mRNA activity of various cytokines and estrogen receptors was measured. The relative mRNA

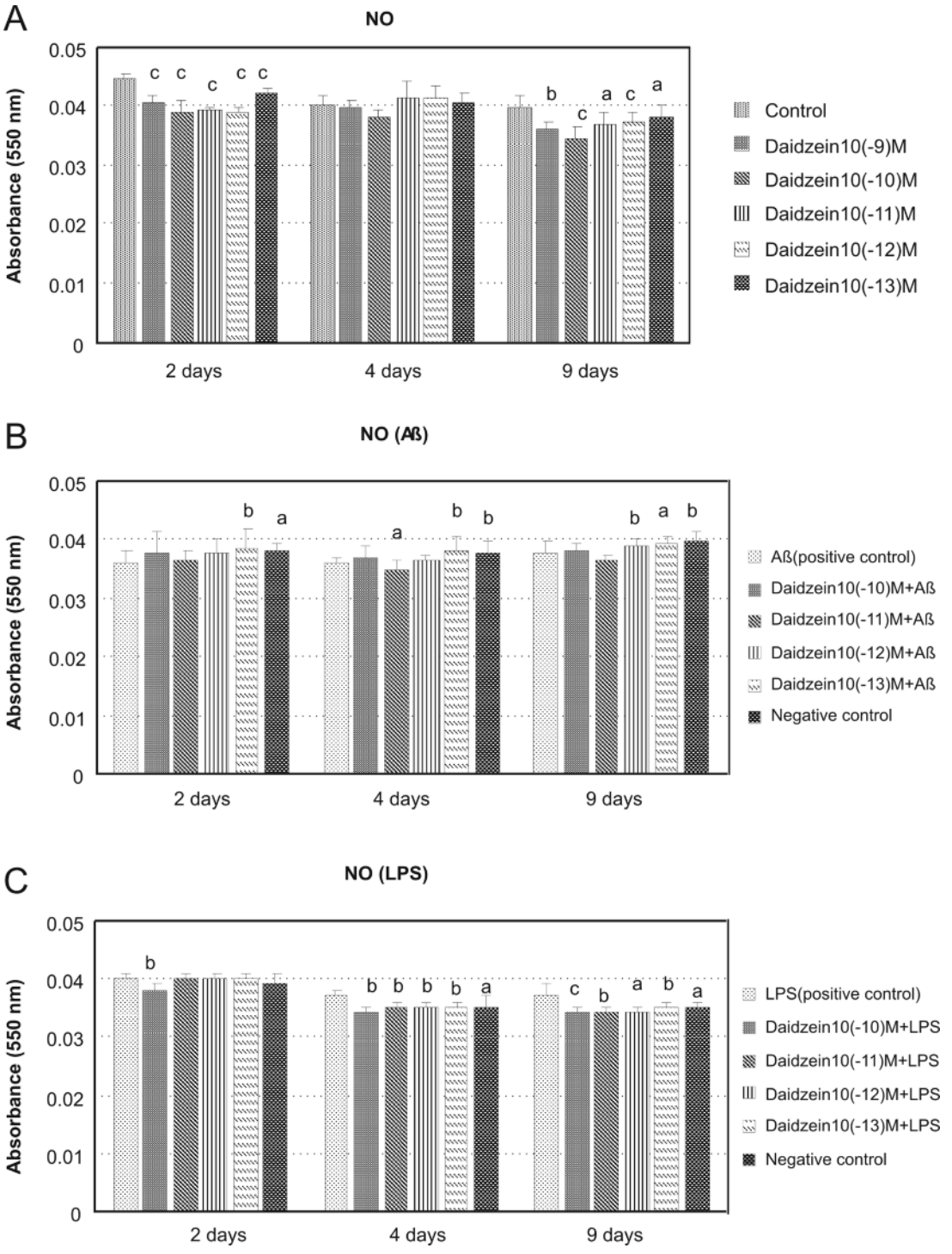

Figure 2 Effect of daidzein on astrocyte nitric oxide synthesis. Astrocyte cells treated with daidzein could significantly decrease the synthesis of nitric oxide (A). The NO synthesis of astrocytes was not enhanced by the presence of 20 μM amyloid-β; pre-treatment of daidzein only significantly decreased the NO synthesis on the 4th day of culture (B). While 10^{-6} g/ml LPS did increase the NO synthesis significantly, pre-treatment of daidzein significantly decreased the NO synthesis induced by LPS (C). ($n = 8$; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ when compared with control or positive control)

expression (ΔCt) represents the difference in threshold cycles for the target gene and the house-keeping gene. The lower the ΔCt value shown, the earlier the target gene expressed. The 2^(–ΔΔCt) method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. As expected from the gene expression results, the addition of amyloid-β and LPS consistently showed the earlier

Table 2 The relative mRNA expression (ΔCt) of different cytokines and estrogen receptor inductions

	ΔCt value (mean ± SD)				
Target gene	IL-1	IL-6	TNF-α	ER-α	ER-β
Control	9.78 ± 0.731	14.36 ± 0.847	18.36 ± 0.795	10.62 ± 0.486	7.19 ± 0.835
Daidzein (10^{-12} M) + Aβ	12.60 ± 2.747	9.21 ± 0.216	17.96 ± 2.160	9.90 ± 0.422	6.91 ± 0.975
Estradiol (10^{-12} M) + A	11.40 ± 2.152	7.65 ± 1.387	19.46 ± 3.159	9.92 ± 1.949	5.10 ± 1.592
A	6.97 ± 4.658	5.53 ± 4.888	16.36 ± 3.800	11.83 ± 4.904	4.83 ± 1.247
Control	15.02 ± 0.967	15.03 ± 0.707	17.89 ± 2.303	15.93 ± 2.241	5.08 ± 1.304
Daidzein (10^{-12} M) + LPS	8.68 ± 0.748	8.41 ± 0.459	15.09 ± 1.420	17.12 ± 1.354	6.93 ± 2.117
Estradiol (10^{-12} M) + LPS	8.88 ± 0.756	7.88 ± 0.765	15.28 ± 1.716	18.55 ± 4.236	6.09 ± 0.718
LPS	8.24 ± 0.658	8.18 ± 0.708	15.08 ± 2.648	12.49 ± 7.580	4.67 ± 1.057

Aβ, amyloid-β.
Measured by real-time PCR detection system at 4 days in different culture conditions.
Statistically significant differences ($P < 0.05$) existed between groups by one-way ANOVA test ($n = 6$).

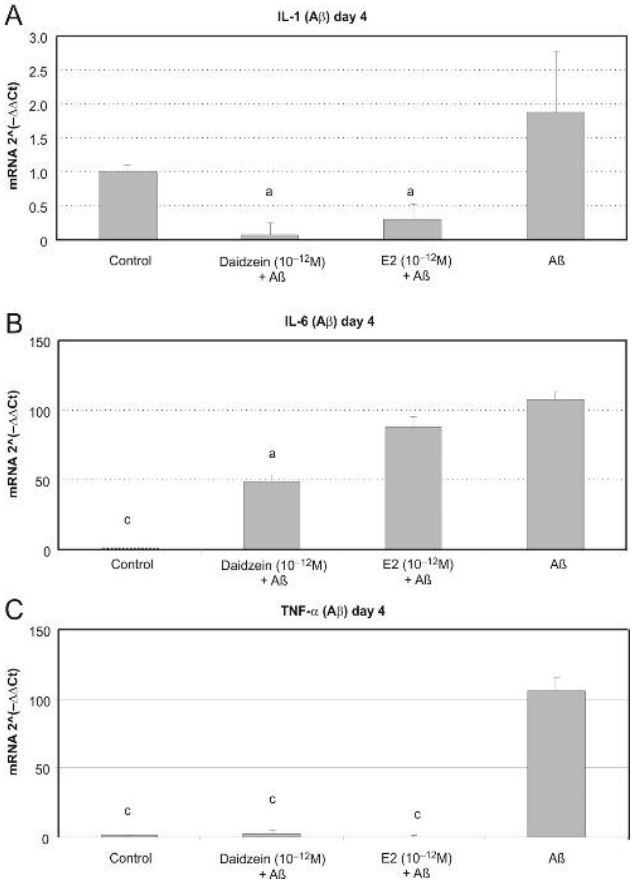

Figure 3 Effect of daidzein on amyloid-β induced inflammatory cytokine synthesis. When astrocytes were cultured in the presence of amyloid-β, the expressions of IL-1, IL-6 and TNF-α mRNA were up-regulated as compared to that of control samples. Pre-treatment with diadzein reduced mRNA expression. In IL-1 (A) and IL-6 (B) mRNA expression, the effect of diadzein was better than estradiol; for TNF-α synthesis (C), the effect of estradiol was better than that of diadzein. ($n = 4$; [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ when compared with positive control)

inflammatory mRNA gene expression, including IL-1, IL-6, TNF-α and ER-β (Table 2).

Effect of daidzein on amyloid-β and LPS-induced inflammatory cytokine synthesis

In this study, astrocytes were pre-treated with 10^{-12} M diadzein and then treated with 20 μM amyloid-β to simulate the cytotoxic effect of Alzheimer's disease. A similar concentration of estradiol (10^{-12} M) was also tested for comparison. When astrocytes were cultured in the presence of amyloid-β or LPS, the expression of IL-1, IL-6 and TNF-α genes were up-regulated as compared to that of control samples (Figs 3 and 4).

When astrocytes were cultured in the presence of amyloid-β, the expression of IL-1 mRNA was up-regulated as compared to that of control samples. Pre-treatment with diadzein can reduce IL-1 mRNA expression on the 4th day down to 4% of the positive control, while pre-treatment with estradiol can reduce the IL-1 mRNA expression on the 4th day down to 16% of the positive control (Fig. 3A). Similar results were observed for IL-6 and TNF-α synthesis, where pre-treatment with diadzein can reduce the IL-6 mRNA expression on the 4th day down to 45% of the positive control and pre-treatment with estradiol can reduce the IL-6 mRNA expression on the 4th day down to 82% of the positive control (Fig. 3B). For TNF-α mRNA expression, pre-treatment with diadzein can down-regulate its expression to 2.4% of the positive control on the 4th day and pre-treatment with estradiol can down-regulate the TNF-α mRNA expression on the 4th day to 0.1% of the positive control (Fig. 3C).

When astrocytes were pre-treated with 10^{-12} M diadzein and then stimulated with 10^{-6} g/ml of LPS to simulate the cellular inflammation response of Alzheimer's disease, the expression of IL-1, IL-6 and TNF-α mRNA were up-regulated as compared to

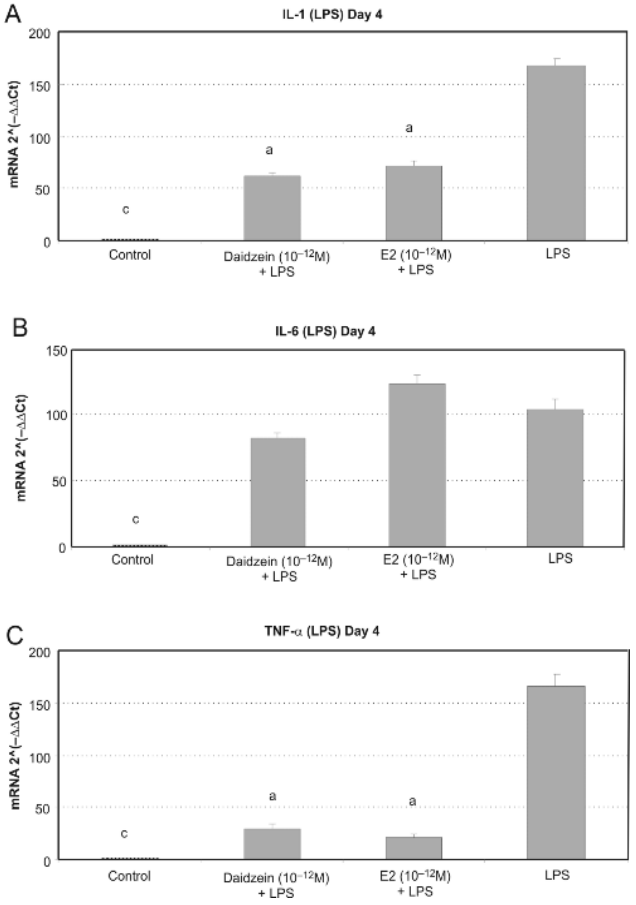

Figure 4 Effect of daidzein on LPS-induced inflammatory cytokine synthesis. When astrocytes were cultured in the presence of LPS, the expression of IL-1, IL-6 and TNF-α mRNA was up-regulated as compared to that of control samples. Pre-treatment with diadzein reduced mRNA expression. In IL-1 (A) and IL-6 (B) mRNA expression, the effect of diadzein was better than estradiol, whereas for TNF-α synthesis (C), the effect of estradiol was better than that of diadzein. (*n* = 4; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ when compared with positive control)

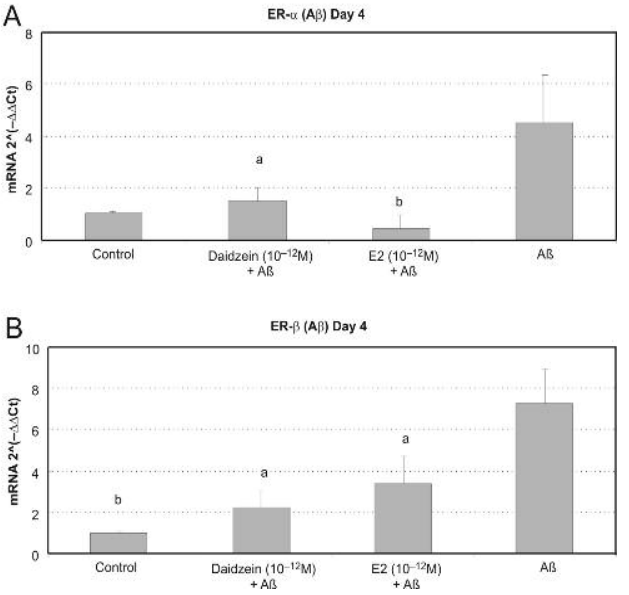

Figure 5 Effect of daidzein on amyloid-β induced estrogen receptor gene expression. When astrocytes were cultured in the presence of amyloid-β, the expression of ER-α (A) and ER-β (B) mRNA was up-regulated as compared to that of control samples. Pre-treatment with diadzein reduced mRNA expression. (*n* = 6; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ when compared with positive control)

control samples. Pre-treatment with diadzein can down-regulate their mRNA expression. For IL-1 and IL-6 mRNA expression, the effect of diadzein was more obvious than estradiol; for the TNF-α synthesis, the effect of estradiol was more obvious than that of diadzein (Fig. 4).

Effect of daidzein on amyloid-β and LPS-induced estrogen receptor gene expression

When astrocytes were cultured in the presence of amyloid-β or LPS, the expression of ER-α and ER-β mRNA were up-regulated as compared to control samples. Pre-treatment with diadzein can down-regulate their mRNA expression (Figs 5 and 6).

When astrocytes were cultured in the presence of amyloid-β, the expression of ER-α mRNA was up-regulated as compared to that of control samples. Pre-treatment with diadzein can down-regulate ER-α mRNA expression to 32% of the positive control on the 4th day, while pre-treatment with estradiol can down-regulate ER-β mRNA expression to 4% of the positive control on the 4th day (Fig. 5A). Pre-treatment with diadzein can down-regulate ER-β mRNA expression to 30% of the positive control on the 4th day, while pre-treatment with estradiol can down-regulate ER-β mRNA expression to 47% of the positive control on the 4th day (Fig. 5B).

When astrocytes were cultured in the presence of LPS, the expression of ER-α and ER-β mRNA were up-regulated as compared to control samples. Pre-treatment with diadzein can down-regulate their mRNA expression (Fig. 6).

Discussion

The ovarian steroid hormone, 17β-estradiol (E2), exerts many diverse effects throughout the body

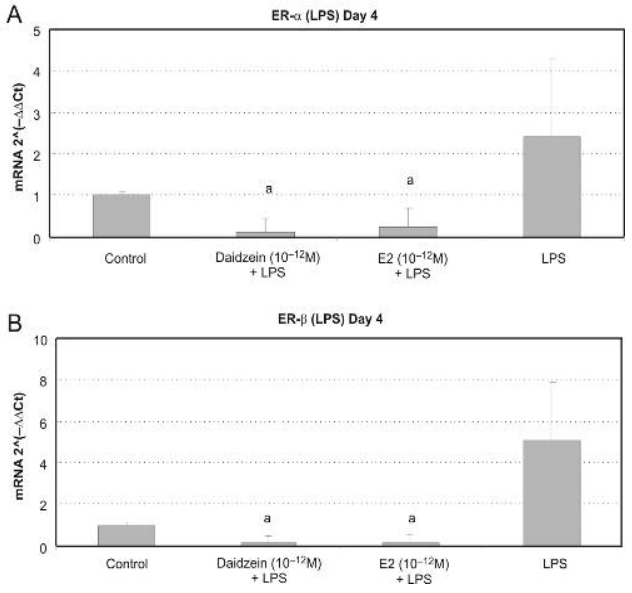

Figure 6 Effect of daidzein on LPS-induced estrogen receptor gene expression. When astrocytes were cultured in the presence of LPS, the expression of ER-α (A) and ER-β (B) mRNA was up-regulated as compared to that of control samples. Pre-treatment with diadzein reduced mRNA expression. ($n = 6$; $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ when compared with positive control)

including regulation of reproduction, control of breast and uterine growth and maintenance of bone density. Over the past decade, significant attention has been focused on the ability of E2 to regulate non-reproductive actions in the central nervous system, such as regulation of synaptic plasticity and protection of the brain against injury.[21,22] Particularly, evidence has emerged suggesting a protective role for E2 against various neurodegenerative diseases including ischemic stroke, Alzheimer's disease and Parkinson's disease. Epidemiological evidence supports a role for estrogen replacement therapy in the reducing of Alzheimer's disease in postmenopausal women,[23] while several small clinical trials indicate that estrogen therapy improves cognitive functioning in Alzheimer's disease patients.[24,25]

In recent years, studies have confirmed this theory of inflammation, with an array of inflammatory proteins including complement factors, acute-phase proteins and pro-inflammatory cytokines to be present in Alzheimer's disease brains.[26] These pro-inflammatory substances, including the cytokines IL-1, IL-6, macrophage-colony stimulating factor and TNF-α, along with prostaglandins and free radicals[27,28] are secreted by microglia and astrocytes. Although the significance and contribution of inflammation to the progression of Alzheimer's disease has been a topic of debate,[26,29] recent clinical and epidemiological research into the use of anti-inflammatory medications indicates a significant correlation between inflammation and neurodegeneration.[30]

In vivo, diadzein can stimulate skeletal tissue growth and differentiation and is more effective in reversing menopausal changes than any of the other isolated phytoestrogens.[31] The efficacy and bioavailability of diadzein administered in different formulations were also evaluated in recent clinical studies both in animal and human subjects.[32–34] The isoflavones had been demonstrated to have a neuroprotective effect on human cortical neurons.[14] Chen *et al.*[16] showed that daidzein had a superior neuron-protective effect on LPS-induced dopaminergic neurodegeneration than other isoflavones. In this study, we evaluated the effects of the selective estrogen receptor modulator daidzein on inflammatory responses of primary astroglial cell cultures. The results showed that the proliferation activity of diadzein-treated astrocyte cultures were always higher than the control samples. Indeed, the addition of 20 μM amyloid-β or 10^{-6} g/ml LPS can significantly decrease the viability of astrocytes; however, this deleterious effect can be alleviated by 1 h of daidzein pre-treatment (Fig. 1).

Glial cells normally provide trophic support to neurons in the developing and adult brain. It is postulated that abnormal glial function leads to the release of free radicals and glutamate, and these toxic substances can cause cell death or render neurons vulnerable to other toxic insults.[35] In this study, our results showed that LPS enhanced nitric oxide synthesis by astrocytes, yet amyloid-β did not (Fig. 2). This result is consistent with previous studies showing that activation of astrocytes or microglia by LPS or cytokines can cause neuronal death by a mechanism involving glial iNOS mRNA expression, as well as the production and release of NO from glial cells.[36] Our results also showed that astrocyte cells treated with daidzein can significantly decrease the synthesis of nitric oxide; however, pre-treatment with daidzein protected astrocytes from the effect of increased NO synthesis induced by LPS (Fig. 2). Our study also demonstrated that the presence of amyloid-β did not significantly enhance NO synthesis by glial cells (Fig. 2). This fact implied that some other mechanisms should be sought before we can make a definite conclusion saying that Alzheimer's disease is mediated by oxidative free radicals.

Nearly all the cytokines and chemokines that have been studied in Alzheimer's disease, including IL-1β, IL-6, TNF-α, IL-8, TGF-β and macrophage

inflammatory protein-1a (MIP-1a) seem to be up-regulated in Alzheimer's disease when compared with control individuals.[26] Recent studies have suggested the astrocyte to be a key cell in mediating the inflammatory process in the brain and in expressing adhesion molecules and chemokines; IL-1 is a key inducer of the expression of these molecules in astrocytes.[37] The over-expression of IL-1 is cytotoxic to astrocytes and will induce cellular degeneration and death.[38] In the brain, the expression of the pleiotropic cytokine IL-6 is enhanced in various chronic or acute central nervous system disorders. In both mixed and pure neuronal cultures, IL-6 has a dose-dependent protective effect on neurons that was blocked by a competitive inhibitor of IL-6.[39] However, the significance of IL-6 production in such neuropathological states remains controversial. In this study, when astrocytes were cultured in the presence of amyloid-β or LPS, the expression of IL-1, IL-6 and TNF-α mRNA was up-regulated as compared with that of control samples. Pre-treatment with diadzein can reduce their mRNA expression (Figs 3 and 4). It is reported that neurodegenerative conditions such as Alzheimer's disease are accompanied by reactive changes in astrocytes. This reactivity is associated with an increase in the cellular expression of astrocyte-specific proteins including glial fibrillary acidic protein (GFAP) and S100B. TNF-α would increase the intracellular expression of both proteins as well as the extracellular concentrations of these proteins, yet not affect astrocyte viability.[40] From the results of our study, we suggest that the cytotoxicity of amyloid-β or LPS was possibly mediated by IL-1, IL-6 and TNF-α.

Many, although not all, of the physiological effects of estrogens are mediated through estrogen receptors.[41] There is now abundant evidence indicating that estrogens may have direct neuroprotective effects on neurons in cultures and in animal models that are mediated by ER-α and/or ER-β.[41] Astrocytes are an essential component of normal neural function and of the response of neural tissue to injury. Astrocytes appear to have a critical role in the protection and survival of neurons, shown when ablation of astrocytes *in vivo* results in a significant decrease in neuronal survival.[42] Recent studies have shown that estrogen may inhibit astrocytosis and the accumulation of reactive astrocytes after injury,[43] as well as regulate the production of cytokines by astrocytes that may act directly on neurons[44,45] Since ER has been detected in astrocytes *in vitro*[46,47] and *in vivo*,[48] the effect of estrogen on astrocytes may be mediated through this receptor. It has been hypothesized that estrogen may exert protective effects on neurons in Alzheimer's disease, through both a direct effect on neurons and an indirect protective effect mediated by astrocytes.[49] In this study, when astrocytes were cultured in the presence of amyloid-β or LPS, the expression of ER-α and ER-β mRNA was up-regulated as compared to control samples. Pre-treatment with diadzein can reduce their mRNA expression (Figs 5 and 6). This means that when neuron injuries occurred, the estrogen receptors (both ER-α and ER-β) will be up-regulated.[50,51] In other words, in the presence of astrocyte cytotoxic effects, the endogenous neuroprotective property of estrogen will be activated and the up-regulation of estrogen receptors will occur.[52] The pre-treatment of diadzein has a neuroprotective effect which will alleviate the cytotoxic effect of amyloid-β and LPS, and then cause the down-regulation of estrogen receptors (Figs 5 and 6).

Pathologically, Alzheimer's disease is manifested by selective oxidative stress-induced neuronal cell death, the deposition of amyloid-β peptide in senile plaques in the extracellular space, and the formation of neurofibrillary tangles inside the neuron.[53,54] Estrogen has been shown to be effective against oxidative stress and amyloid-β-induced toxicity in neurons.[55] The current data support a possible role for astrocytes in the mediation of neuroprotection by diadzein. An estrogen receptor may underlie some of the protective effects of diadzein by activating cellular signaling pathways, such as extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt. A possible indirect pathway involving astrocytes may act in concert with the proposed direct pathway to down-regulate the expression of IL-1, IL-6, and the TNF-α cytotoxic effect to achieve a wide-spread, global protection of both ER positive and negative neurons.[56]

Conclusions

An increasing body of evidence suggests that estrogen exerts significant neuroprotective effects against a variety of neurodegenerative pathologies. However, there is a risk of tumor development associated with estrogen and progestin replacement therapy. Here, we demonstrated the selective estrogen receptor modulator diadzein in the treatment of neuro-degenerative diseases. The results indicate that daidzein is able to reduce NO production of astrocytes and also down-regulate IL-1,IL-6 and TNF-α mRNA expression after amyloid-β and LPS treatment. Diadzein can also promote astrocyte proliferation and protect astrocytes from amyloid-β and LPS damage. These results suggest the possibility that the positive

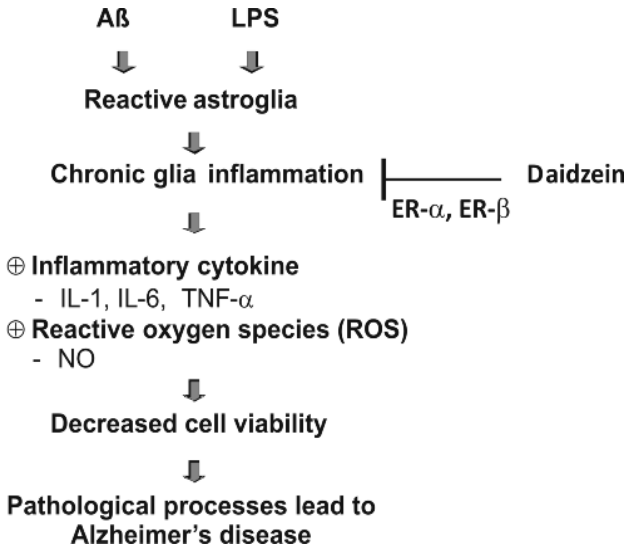

Figure 7 Neuroprotective mechanism of daidzein

effects of daidzein may ameliorate the process of neural inflammation and alleviate the progression of Alzheimer's disease, although the molecular mechanisms underlying the beneficial actions of diadzein in the brain are not completely understood. The ER-dependent events have been proved to occur (Fig. 7), thus further studies are needed to characterize better the modes of action of diadzein in the brain. This can range from their cellular targets to their downstream molecular effectors, in order to provide a rationale for their pharmacological exploitation as effective neuroprotective agents.

Acknowledgements

The authors sincerely thank the National Health Research Institutes (ROC) for financial support and the staff of the Second Core Lab, Department of Medical Research, National Taiwan University Hospital for technical support during the study. The authors also thank Miss Margaret Man-Ger Sun for her help in the editing and preparation of this manuscript.

References

1. Wyss-Coray T, Loike JD, Brionne TC *et al*. Adult mouse astrocytes degrade amyloid-β *in vitro* and *in situ*. *Nat Med* 2003; **9**: 453–457.
2. Petrella JR, Coleman RE, Doraiswamy PM. Neuroimaging and early diagnosis of Alzheimer disease: a look to the future. *Radiology* 2003; **226**: 315–336.
3. Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J Clin Invest* 1991; **87**: 949–954.
4. Traugott U. Multiple sclerosis: relevance of class I and class II MHC-expressing cells to lesion development. *J Neuroimmunol* 1987; **16**: 283–302.
5. Fontana A, Fiers W, Wekerle H. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature* 1984; **307**: 273–276.
6. Araque A, Carmignoto G, Haydon PG. Dynamic signaling between astrocytes and neurons. *Annu Rev Physiol* 2001; **63**: 795–813.
7. Vehmas AK, Kawas CH, Stewart WF, Troncoso JC. Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. *Neurobiol Aging* 2003; **24**: 321–331.
8. Gibbons HM, Dragunow M. Microglia induce neural cell death via a proximity-dependent mechanism involving nitric oxide. *Brain Res* 2006; **1084**: 1–15.
9. Ling Z, Zhu Y, Tong CW, Snyder JA, Lipton JW, Carvey PM. Progressive dopamine neuron loss following supra-nigral lipopolysaccharide (LPS) infusion into rats exposed to LPS prenatally. *Exp Neurol* 2006; **199**: 499–512.
10. Peterson PK, Hu S, Salak-Johnson J, Molitor TW, Chao CC. Differential production of and migratory response to beta chemokines by human microglia and astrocytes. *J Infect Dis* 1997; **175**: 478–481.
11. Yaffe K, Sawaya G, Lieberburg I, Grady D. Estrogen therapy in postmenopausal women: effects on cognitive function and dementia. *JAMA* 1998; **279**: 688–695.
12. Cholerton B, Gleason CE, Baker LD, Asthana S. Estrogen and Alzheimer's disease: the story so far. *Drugs Aging* 2002; **19**: 405–407.
13. Bernardi F, Pluchino N, Stomati M, Pieri M, Genazzani AR. CNS: sex steroids and SERMs. *Ann NY Acad Sci* 2003; **997**: 378–388.
14. Occhiuto F, Zangla G, Samperi S *et al*. The phytoestrogenic isoflavones from *Trifolium pratense* L. (Red clover) protects human cortical neurons from glutamate toxicity. *Phytomedicine* 2008; **15**: 676–682.
15. Zhou W, Koldzic-Zivanovic N, Clarke CH *et al*. Selective estrogen receptor modulator effects in the rat brain. *Neuroendocrinology* 2002; **75**: 24–33.
16. Chen HQ, Wang XJ, Jin ZY, Xu XM, Zhao JW, Xie ZJ. Protective effect of isoflavones from *Trifolium pratense* on dopaminergic neurons. *Neurosci Res* 2008; **62**: 123–130.
17. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 1980; **85**: 890–902.
18. Tiwari-Woodruff SK, Buznikov AG, Vu TQ *et al*. OSP/claudin-11 forms a complex with a novel member of the tetraspanin super family and beta1 integrin and regulates proliferation and migration of oligodendrocytes. *J Cell Biol* 2001; **153**: 295–305.
19. Okuda Y, Nakatsuji Y, Fujimura H *et al*. Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis. *J Neuroimmunol* 1995; **62**: 103–112.
20. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 1997; **245**: 154–160.
21. McEwen B, Akama K, Alves S *et al*. Tracking the estrogen receptor in neurons: implications for estrogen-induced synapse formation. *Proc Natl Acad Sci USA* 2001; **98**: 7093–7100.
22. Wise PM, Dubal DB, Wilson ME, Rau SW, Liu Y. Estrogens: trophic and protective factors in the adult brain. *Front Neuroendocrinol* 2001; **22**: 33–66.
23. Waring SC, Rocca WA, Peterson RC, Kokmen E. Postmenopausal estrogen replacement therapy and Alzheimer's disease: a population-based study in Rochester, Minnesota. *Neurology* 1997; **485**: A79–A83.
24. Fillit H, Weinreb H, Cholst I *et al*. Observations in a preliminary open trial of estradiol therapy for senile dementia-Alzheimer's type. *Psychoneuroendocrinology* 1986; **11**: 337–345.
25. Ohkura T, Isse K, Akazawa K, Hamamoto M, Yaoi Y, Hagino N. Evaluation of estrogen treatment in female patients with dementia of the Alzheimer's type. *Endocrine J* 1994; **41**: 361–371.
26. Akiyama H, Barger S, Barnum S *et al*. Inflammation and Alzheimer's disease. *Neurobiol Aging* 2000; **21**: 383–421.
27. Meda L, Baron P, Scarlato G. Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins. *Neurobiol Aging* 2001; **22**: 885–893.
28. Pachter J. Inflammatory mechanisms in Alzheimer's disease – the role of beta-amyloid-glial interactions. *Mol Psychiatry* 1997; **2**: 91–95.

29. McGeer P, Rogers J. Medical hypothesis: antiinflammatory agents as a therapeutic approach to Alzheimer's disease. *Neurology* 1992; **42**: 447–449.
30. Stuchbury G, Münch G. Alzheimer's associated inflammation, potential drug targets and future therapies. *J Neural Transm* 2005; **112**: 429–453.
31. Somjen D, Katzburg S, Kohen F, Gayer B, Livne E. Daidzein but not other phytoestrogens preserves bone architecture in ovariectomized female rats *in vivo*. *J Cell Biochem* 2008; **103**: 1826–1832.
32. Urpi-Sarda M, Morand C, Besson C *et al*. Tissue distribution of isoflavones in ewes after consumption of red clover silage. *Arch Biochem Biophys* 2008; **476**: 205–210.
33. He J, Gu S, Zhang Q, Feng E, Li X, Xu G. Bioequivalence of two formulations of daidzein administered in a single dose to healthy Chinese volunteers. *Arzneimittelforschung* 2008; **58**: 211–214.
34. Rüfer CE, Bub A, Möseneder J, Winterhalter P, Stürtz M, Kulling SE. Pharmacokinetics of the soybean isoflavone daidzein in its aglycone and glucoside form: a randomized, double-blind, crossover study. *Am J Clin Nutr* 2008; **87**: 1314–1323.
35. McNaught KS, Jenner P. Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced toxicity in astrocytic/ventral mesencephalic co-cultures. *J Neurochem* 1999; **73**: 2469–2476.
36. Chao CC, Hu S, Sheng WS, Bu D, Bukrinsky MI, Peterson PK. Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. *Glia* 1996; **16**: 276–284.
37. Moynagh PN. The interleukin-1 signalling pathway in astrocytes: a key contributor to inflammation in the brain. *J Anat* 2005; **207**: 265–269.
38. Acarin L, González B, Castellano B. STAT3 and NF kappa B activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei. *J Neuropathol Exp Neurol* 2000; **59**: 151–163.
39. Ali C, Nicole O, Docagne F *et al*. Ischemia-induced interleukin-6 as a potential endogenous neuroprotective cytokine against NMDA receptor-mediated excitotoxicity in the brain. *J Cereb Blood Flow Metab* 2000; **20**: 956–966.
40. Edwards MM, Robinson SR. TNF alpha affects the expression of GFAP and S100B: implications for Alzheimer's disease. *J Neural Transm* 2006; **113**: 1709–1715.
41. Garcia-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by estradiol. *Prog Neurobiol* 2001; **63**: 29–60.
42. Cui W, Allen ND, Skynner M, Gusterson B, Clark AJ. Inducible ablation of astrocytes shows that these cells are required for neuronal survival in the adult brain. *Glia* 2001; **34**: 272–282.
43. Garcia-Estrada J, Luquin S, Fernandez A, Garcia-Segura LM. Dehydroepiandrosterone, pregnenolone and sex steroids down-regulate reactive astroglia in the male rat brain after a penetrating brain injury. *Int J Dev Neurosci* 1999; **17**: 145–151.
44. Azcoita I, Garcia-Ovejero D, Chowen JA, Garcia-Segura LM. Astroglia play a key role in the neuroprotective actions of estrogen. *Prog Brain Res* 2001; **132**: 469–478.
45. McCarthy JB, Barker-Gibb AL, Alves SE, Milner TA. TrkA immunoreactive astrocytes in dendritic fields of the hippocampal formation across estrous. *Glia* 2002; **38**: 36–44.
46. Su JD, Qiu J, Zhong YP, Li XY, Wang JW, Chen YZ. Expression of estrogen receptor (ER)-α and -β immunoreactivity in hippocampal cell cultures with special attention to GABAergic neurons. *J Neurosci Res* 2001; **65**: 396–402.
47. Zwain IH, Arroyo A, Amato P, Yen SS. A role for hypothalamic astrocytes in dehydroepiandrosterone and estradiol regulation of gonadotropin-releasing hormone (GnRH) release by GnRH neurons. *Neuroendocrinology* 2002; **75**: 375–383.
48. Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, Alves SE. Ultrastructural evidence that hippocampal estrogen receptors are located at extranuclear sites. *J Comp Neurol* 2001; **429**: 355–371.
49. Dhandapani KM, Brann DW. Protective effects of estrogen and selective estrogen receptor modulators in the brain. *Biol Reprod* 2002; **67**: 1379–1385.
50. Garcia-Ovejero D, Garcia-Segura LM, Doncarlos LL. Glial expression of estrogen and androgen receptors after rat brain injury. *J Comp Neurol* 2002; **450**: 256–271.
51. Sortino MA, Merlo S, Vancheri C *et al*. Glia mediates the neuroprotective action of estradiol on beta-amyloid-induced neuronal death. *Endocrinology* 2004; **145**: 5080–5086.
52. Amantea D, Bagetta G, Corasaniti MT. From clinical evidence to molecular mechanisms underlying neuroprotection afforded by estrogens. *Pharmacol Res* 2005; **52**: 119–132.
53. Zhu X, Raina AK, Lee HG, Casadesus G, Smith MA, Perry G. Oxidative stress signalling in Alzheimer's disease. *Brain Res* 2004; **1000**: 32–39.
54. Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* 2005; **120**: 545–555.
55. Goodenough S, Schafer M, Behl C. Estrogen-induced cell signaling in a cellular model of Alzheimer's disease. *J Steroid Biochem Mol Biol* 2003; **84**: 301–305.
56. Dhandapani KM, Brann DW. Role of astrocytes in estrogen-mediated neuroprotection. *Exp Gerontol* 2007; **42**: 70–75.