Effects of S-2474, a novel nonsteroidal anti-inflammatory drug, on amyloid β protein-induced neuronal cell death

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1 The accumulation of amyloid β protein ($A\beta$) in the brain is a characteristic feature of Alzheimer's disease (AD). Clinical trials of AD patients with nonsteroidal anti-inflammatory drugs (NSAIDs) indicate a clinical benefit. NSAIDs are presumed to act by suppressing inhibiting chronic inflammation in the brain of AD patients.

2 In the present study, we investigated effects of S-2474 on $A\beta$ -induced cell death in primary cultures of rat cortical neurons. S-2474 is a novel NSAID, which inhibits cyclo-oxygenase-2 (COX-2) and contains the di-*tert*-butylphenol antioxidant moiety.

3 S-2474 significantly prevented neurons from $A\beta(25-35)$ - and $A\beta(1-40)$ -induced cell death. S-2474 ameliorated $A\beta$ -induced apoptotic features such as the condensation of chromatin and the fragmentation of DNA completely.

4 Prior to cell death, $A\beta(25-35)$ generated prostaglandin D_2 (PGD_2) and free radicals from neurons. PGD_2 is a product of cyclo-oxygenase (COX), and caused neuronal cell death.

5 S-2474 significantly inhibited the $A\beta(25-35)$ -induced generation of PGD_2 and free radicals.

6 The present cortical cultures contained little non-neuronal cells, indicating that S-2474 affected neuronal survival directly, but not indirectly *via* non-neuronal cells. Both an inhibitory effect of COX-2 and an antioxidant effect might contribute to the neuroprotective effects of S-2474.

7 In conclusion, S-2474 exhibits protective effects against neurotoxicity of $A\beta$. Furthermore, the present study suggests that S-2474 may possess therapeutic potential for AD *via* ameliorating degeneration in neurons as well as suppressing chronic inflammation in non-neuronal cells.

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Abbreviations: AA, arachidonic acid; $A\beta$, amyloid β protein; AD, Alzheimer's disease; ANOVA, analysis of variance; $[Ca^{2+}]_i$, intracellular Ca^{2+} levels; COX, cyclo-oxygenase; DCFDA, 2',7'-dichlorofluorescein diacetate; $15d\text{-}\Delta^{12,14}\text{-PGJ}_2$, $15\text{-deoxy-}\Delta^{12,14}\text{-prostaglandin J}_2$; GFAP, glial fibrillary acidic protein; IL, interleukin; 5-LO, 5-lipoxygenase; LT, leukotriene; MAP-2, microtubule-associated protein 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PLA_2 , phospholipase A_2 ; PPAR γ , peroxysome proliferator-activated receptor γ ; TUNEL, TdT-mediated dUTP-biotin nick end-labelling; TXA_2 , thromboxane A_2

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed in drug therapy for inflammatory diseases such as Alzheimer's disease (AD) (McGeer *et al.*, 1996; Stewart *et al.*, 1997). Currently available NSAIDs have dual inhibitory activities against cyclo-oxygenase (COX) and 5-lipoxygenase (5-LO) (Vane & Botting, 1998; Hidaka *et al.*, 1984; Ikuta *et al.*, 1987). COX is classified into two distinct isoforms, a constitutive form, COX-1, and a mitogen-inducible form, COX-2. By COX and 5-LO, arachidonic acid (AA) is metabolized to prostaglandins (PGs) and leukotrienes (LTs), respectively. S-2474 is a novel NSAID containing the γ -sultam skeleton and the di-*tert*-butylphenol antioxidant moiety (Inagaki *et al.*, 2000). This compound very strongly suppresses the COX-2 pathway ($IC_{50}=0.2-$

3.6 nM), and inhibits production of the multiple inflammatory mediators such as LTs, interleukin (IL)-1, IL-6, IL-8 and NO ($IC_{50}=0.5-30 \mu\text{M}$) (Matsumoto *et al.*, 1997). On the other hand, it does not affect the COX-1 pathway ($IC_{50}>10 \mu\text{M}$) (Inagaki *et al.*, 2000). COX-1 is responsible for the synthesis of cytoprotective prostaglandins (PGs) in the gastrointestinal tract and its inhibition of COX-1 leads to adverse effects, i.e. gastrointestinal ulceration (Miller, 1983). Oral S-2474 exerts anti-inflammatory and analgesic action without side effects, gastrointestinal ulceration (Jyoyama *et al.*, 1997). Thus, S-2474 is thought to offer anti-inflammatory therapeutic effects *via* inhibition of COX-2, 5-LO, IL production and radical generation.

Alzheimer's disease (AD) is characterized clinically by progressive dementia and pathologically by cortical atrophy, neuronal loss, neurofibrillary tangles, senile plaques, and deposits of amyloid β protein ($A\beta$) in the various region of brain such as cerebral cortex and hippocampus (Selkoe,

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1991). Aggregated deposits of A β are generally assumed to have a causative role in neurodegeneration and development of AD (Selkoe, 1994; Cummings & Cotman, 1995). A β is a 39 to 43-amino-acid hydrophobic peptide, and causes neuronal cell death in primary cultures (Pike *et al.*, 1991; Ueda *et al.*, 1994). A β -induced neuronal cell death is typified by several characteristic features of apoptosis, such as formation of cell surface blebs, chromatin condensation, and DNA fragmentation (Forloni *et al.*, 1993; Ueda *et al.*, 1996). Therefore, neuroprotective compounds against A β toxicity are drug candidates for the clinical management of AD.

A β causes peroxidation of plasma membrane (Behl *et al.*, 1994; Ueda *et al.*, 1997a). A β neurotoxicity is attenuated by a number of antioxidants, e.g., vitamin E (Behl *et al.*, 1994; Ueda *et al.*, 1997a). A β -induced lipid peroxidation impairs plasma membrane ion-motive ATPases, leading to depolarization and the activation of L-type voltage-dependent calcium channels (Weiss *et al.*, 1994; Ueda *et al.*, 1997a). Subsequently, intracellular Ca²⁺ levels ([Ca²⁺]_i) are elevated (Mattson *et al.*, 1992; Ueda *et al.*, 1997b). The resulting increase in [Ca²⁺]_i activates phospholipase A₂ (PLA₂), which releases AA from the membrane. Cytosolic PLA₂ immunoreactivity is increased in AD brain (Stephenson *et al.*, 1996). However, it has not yet been clearly demonstrated how AA metabolites are involved in A β neurotoxicity and AD.

A clinical trial of AD patients with a COX inhibitor, indomethacin, indicated a beneficial effect (Rogers *et al.*, 1993; McGeer, 2000). In the brain, both COX-1 and COX-2 are expressed (Yasojima *et al.*, 1999). COX-2 is up-regulated in AD brain and in A β -treated SH-SY5Y neuroblastoma cells (Pasinetti & Aisen, 1998), suggesting the involvement of COX-2 in AD. Among PGs, the formation of PGD₂ is significantly increased in AD brain (Iwamoto *et al.*, 1989). On the other hand, 5-LO inhibitors protect hippocampal neurons against A β -toxicity (Goodman *et al.*, 1994), suggesting the association of LTs with AD. Recently, NSAIDs are reported to reduce apoptotic neuronal cell death *via* activation of peroxysome proliferator-activated receptor γ (PPAR γ) in rat cerebella granule cells. (Heneka *et al.*, 2000). However, an endogenous ligand for PPAR γ , 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d- $\Delta^{12,14}$ -PGJ₂) induces apoptosis in rat cortical neurons (Rohn *et al.*, 2001). Thus, it is not yet understood how NSAIDs exhibit clinically beneficial effects on AD.

A β -induced neuronal cell death is established as *in vitro* model of AD (Pike *et al.*, 1991). Using this model, we found neuroprotective effects of S-2474 against A β neurotoxicity in the primary culture of rat cortical neurons. In the present study, we examined how S-2474 exhibits neuroprotective effects in this model.

Methods

Materials

S-2474 ((E)-(5)-(3,5-di-tert-butyl-4-hydroxybenzylidene)-2-ethyl-1,2-isothiazolidine-1,1-dioxide) and U-46619 (1,5,5-hydroxy-11 α ,9 α -(epoxymethano)prosta-5Z,13E-dienoic acid), a stable agonist for thromboxane A₂ (TXA₂) receptor, were synthesized in our laboratory, and its stock solution was prepared by solution at 10 mM in DMSO (Inagaki *et al.*,

2000). A β (25–35) and A β (1–40) were purchased from Bachem AG (Bubendorf, Switzerland). Their scrambled and reversed forms were obtained from Takara (Shiga, Japan). A stock solution of A β was prepared by solution of the peptide at 1 mM in deionized water and was incubated at 37°C for 2 days to aggregate the peptide (Ueda *et al.*, 1994). AA, PGD₂, PGE₂, 9 α -11 β -PGF₂, PGF₂ α , PGI₂, 15d- $\Delta^{12,14}$ -PGJ₂, LTB₄, LTC₄, LTD₄ were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Their solvents are evaporated under a gentle stream of nitrogen and the residues are redissolved in ethanol. They are stored at –20°C. Hoechst 33258 fluorescent dye was purchased from Molecular Probes (Eugene, OR, U.S.A.). 2',7'-Dichlorofluorescein diacetate (DCFDA) was purchased from Kodak (Tokyo, Japan). Anti-microtubule-associated protein 2 (anti-MAP2) and anti-gial fibrillary acidic protein (anti-GFAP) were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-microglial antigen (OX-42) was purchased from BMA biomedical AG (Augst, Switzerland). Reversed phase (C18) Sep-Pack cartridges was purchased from Waters (Bedford, MA., U.S.A.). PGD₂ [³H]-assay system was purchased from Amersham (Buckinghamshire, U.K.). PGE₂ [¹²⁵I]-RIA kit and LTB₄ [³H]-RIA kit were purchased from DuPont NEN (Boston, MA, U.S.A.).

Animals

All experiments were carried out according to the guidelines of the European Community's Council for Animal Experiments. The following experimental procedures were approved by the Institutional Animal Care and Use Committee at the Discovery Research Laboratories of Shionogi & Co., Ltd., and all efforts were made to minimize the number of animals used and their suffering. Pregnant Sprague-Dawley rats were used. The rats were individually housed in macrolon cages with free access to food and water and maintained on a 12-h light/dark cycle, at 25°C room temperature.

Tissue culture

Neuronal cell cultures were prepared from cerebral cortices of day-19 Sprague-Dawley rat embryos as previously reported (Mattson *et al.*, 1995; Ueda *et al.*, 1996). Cells were plated at a density of 2.5×10^5 cells cm^{–2} on poly-L-lysine-coated dishes in conditioning medium, Leibovitz's L-15 medium supplemented with 5% foetal bovine serum and 5% horse serum at 37°C. Cultures were treated with 0.1 μ M arabinosylcytosine C only on day 1 and used for experiments on day 2 after plating. The compositions of neurons, astrocytes and microglia in cortical cultures were determined by use of antibodies for MAP2, GFAP and microglial antigen, which are specific for neurons, astrocytes and microglia, respectively. Most of the cells (more than 95%) were stained by anti-MAP2 antibody, whereas there were few anti-GFAP-positive cells (less than 4%) and anti-microglial antigen positive cells (less than 3%). Thus, the present culture contained primarily neurons and few non-neuronal cells.

Analysis of neuronal survival

Experiments were principally performed in the two conditions as follows. (i) Neurons (2.5×10^5 cells cm^{–2}) were treated with

10 μ M A β (25–35) or A β (1–40) in the presence or absence of S-2474 at 37°C. Vehicle controls were treated with culture medium containing 1% deionized water and 0.1% DMSO. A β controls were treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. (ii) Neurons (2.5×10^5 cells cm^{-2}) were treated with eicosanoids at 37°C. Vehicle controls were treated with culture medium containing 0.1% ethanol.

Two different methods were employed for assessment of neurotoxicity of A β , as previously reported (Ueda *et al.*, 1996). First, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed. Second, residual cells were counted according to morphologic criteria; neurons with intact neurites and a smooth, round soma were considered viable, whereas those with degenerated neurites and an irregular soma were considered nonviable.

Fluorescent microscopy

Assessment of condensation of chromatin, an index of apoptosis (Deckwerth & Jonson, 1993), was performed as previously described (Ueda *et al.*, 1996). Neurons (2.5×10^5 cells cm^{-2}) were treated with 10 μ M A β (25–35) in the presence or absence of 10 μ M S-2474 at 37°C for 48 h. Vehicle controls were treated with culture medium containing 1% deionized water and 0.1% DMSO. A β controls were treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. At this time, culture medium was exchanged with PBS containing 10 μ M Hoechst 33258 fluorescent dye (Molecular Probes, Eugene, OR, U.S.A.). Cells were incubated for 10 min at 37°C in the dark and washed with PBS. Stained nuclei were categorized as follows: (i) normal nuclei, homogeneously stained chromatin; (ii) intact nuclei with condensed chromatin, crescent-shaped areas of condensed chromatin often located near the periphery of the nucleus; and (iii) fragmented nuclei, more than two condensed micronuclei within the area of a neuron.

In situ labelling of nuclear DNA fragments

Neurons (2.5×10^5 cells cm^{-2}) were treated with 10 μ M A β (25–35) in the presence or absence of 10 μ M S-2474 at 37°C for 48 h. Vehicle controls were treated with culture medium containing 1% deionized water and 0.1% DMSO. A β controls were treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. Cortical cell cultures were stained by the TUNEL technique (TdT-mediated dUTP-biotin nick end-labelling), as described (Gavrieli *et al.*, 1992). Apoptotic cells could be distinguished morphologically from necrotic cells by the presence of condensed brown nuclei.

Measurement of PGD₂, PGE₂ and LTB₄

Neurons (2.5×10^5 cells cm^{-2}) were treated with 10 μ M A β (25–35) in the presence or absence of 10 μ M S-2474 at 37°C. Vehicle controls were treated with culture medium containing 1% deionized water and 0.1% DMSO. A β controls were treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. At the indicated times

in Figure 4 or 5, eicosanoids were extracted according to the method of previous report (Powell, 1980). Supernatants of culture medium (1 ml) was mixed homogeneously with cold ethanol (4 ml). The mixture was centrifuged at $1500 \times g$ at 4°C for 10 min to remove the particulate matter. Supernatants were diluted with an appropriate volume of distilled water to yield a final concentration of 10% ethanol, and the pH was adjusted to 3.5–4.0. Samples were loaded onto reversed phase (C18) Sep-Pack cartridges, which had been prepared by washing with ethanol, followed by distilled water. Samples were washed onto the Sep-Pak with 15 ml of 10% aqueous ethanol, followed by 15 ml of petroleum ether. Samples were extracted with 5 ml of methyl formate. The methyl formate effluents were pooled and evaporated with a heating module and dissolved in RIA buffer (50 mM phosphate buffer, pH 7.3, with 0.1% gelatin and 0.1% azide). The samples were stored frozen until RIA analysis for PG D₂, PGE₂ and LTB₄. PG D₂, PGE₂ and LTB₄ were measured with their RIA kits (duplicate/sample). The cross reactivity of PGJ₂ with the PGD₂ RIA kit is 7%, whereas that of other PGs such as PGA₂, PGE₁, PGE₂, PGF_{1 α} , PGF_{2 α} , 6-keto PGE₁, 6-keto PGF_{1 α} and TXB₂ is less than 1%. The cross-reactivity of PGE₁ with the PGE₂ RIA kit was 30%, and that of other PGs such as AA, PGA₁, PGA₂, PGB₂, PGD₂, DHKPGE₂, PGF_{1 α} , 6-keto PGF_{1 α} , PGF_{2 α} , DHKPGF_{2 α} and 6-keto linoleic acid was less than 1%. The cross reactivity of 20-OH-LTB₄, 6-trans LTB₄, 5,12-DiHETE with the LTB₄ RIA kit are 1.3%, 1.0% and 3.6%, respectively. On the other hand, that of other eicosanoids such as AA, ETYA, 5S,6S-DiHETE, 5S,6R-DiHETE, 5S,12S-DiHETE, 5-HETE, 5,12-HETE, 12-HETE, 20-COOH-LTB₄, 6-trans, 12-epi-LTB₄, 12-epi-LTB₄, 20-COOH-LTB₄, LTC₄, PGA₁, PGA₂, PGD₂, PGE₁, PGE₂, PGF_{1 α} , PGF_{2 α} , 13,14 dihydro, 15-keto PGF_{2 α} , NDGA and TXB₂ was less than 1%.

Measurement of reactive oxygen species

Neurons (2.5×10^5 cells cm^{-2}) were treated with 10 μ M A β (25–35) in the presence or absence of 10 μ M S-2474 at 37°C for 24 h later. Vehicle controls were treated with culture medium containing 1% deionized water and 0.1% DMSO. A β controls were treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. Intracellular reactive oxygen species were measured by the 2',7'-dichlorofluorescein diacetate (DCFDA) assay (Chacon & Acosta, 1991). In brief, cultures were loaded with 1 μ M DCFDA for 20 min, and then washed twice with 1 ml of PBS. One ml of deoxycholate (1%) was added to lyse the cells. The lysates were transferred to a new tube and chilled on ice. The dishes were washed with 1 ml of distilled water, and the liquid used for washing was added to the lysate. The fluorescent intensity of the lysate was determined with a spectrometer using excitation and emission wavelengths of 488 and 525 nm, respectively. Data are given as percentage of DCFDA fluorescence of corresponding vehicle-treated values.

Statistical analysis

Data are given as means $\pm$ s.e.mean (n = numbers of observations). We performed two experiments at least on different days, and confirmed their reproductivity. We analysed

observations obtained on the same day, and presented the typical experimental results among independent ones on different days to minimize experimental errors. Comparison tests were performed by two-way ANOVA followed by Dunnett's test. IC_{50} values were calculated by Microsoft Excel Fit as previously reported (Asakura *et al.*, 1999).

Results

Effects of S-2474 on $A\beta(25-35)$ - and $A\beta(1-40)$ -induced neuronal cell death

Primary cultures of dissociated cortical neurons were exposed to $A\beta$ -related peptides for 48 h, and their toxicity was quantified by the MTT reduction assay (Table 1). $A\beta(25-35)$, the toxic fragment of $A\beta$ (Yanker *et al.*, 1990), showed neurotoxicity in a concentration-dependent manner ($LD_{50} = 3.4 \pm 0.4 \mu M$). At $10 \mu M$, $A\beta(25-35)$ decreased 70% of MTT-reducing activity markedly [$F(3,36) = 8.797$, $P < 0.001$]. The application of $10 \mu M$ $A\beta(1-40)$, a native form of $A\beta$, resulted in a 34% decrement of the MTT-reducing activity [$F(3,36) = 4.154$, $P = 0.001$]. There was no statistical difference in MTT-reducing activity between vehicle-treated and scrambled $A\beta(25-35)$ [$F(3,36) = 0.778$, $P < 0.389$] or $A\beta(35-25)$, the reversed sequence of $A\beta(25-35)$ [$F(3,36) = 0.156$, $P = 0.197$].

Next, we investigated effects of S-2474, a novel NSAID, on the $A\beta(25-35)$ -induced neuronal cell death (Figure 1 and Table 1). S-2474 prevented neurons from $A\beta$ -induced cell death significantly in a concentration-dependent manner ($IC_{50} = 26 \pm 12$ nM). At $10 \mu M$, S-2474 completely inhibited $A\beta(25-35)$ -induced neuronal cell death [$F(3,36) = 11.276$, $P < 0.001$]. Moreover, S-2474 also showed neuroprotective effects in the $A\beta(1-40)$ -induced neuronal cell death (Table 1) [$F(3,36) = 6.356$, $P < 0.001$]. As compared to vehicle-treated neurons, there was a slight but statistical difference in S-2474-treated [$F(3,36) = 2.310$, $P = 0.014$], $10 \mu M$ S-2474-added

Figure 1 Effects of S-2474 on $A\beta(25-35)$ -induced neuronal cell death. Cortical neurons were treated with S-2474 at the indicated concentrations in the presence (open circles) or absence (closed circles) of $10 \mu M$ $A\beta(25-35)$. MTT reducing activity was determined 48 h after $A\beta(25-35)$ treatment. Data are expressed as mean $\pm$ s.e.mean values ($n = 4$). $**P < 0.01$, compared with $A\beta(25-35)$ control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. $A\beta$ control is treated with culture medium containing $10 \mu M$ $A\beta(25-35)$ and 0.1% DMSO.

$A\beta(25-35)$ -treated [$F(3,36) = 2.147$, $P = 0.015$] and S-2474-added $A\beta(1-40)$ -treated neurons [$F(3,36) = 2.066$, $P = 0.017$].

Effects of S-2474 on $A\beta(25-35)$ -induced morphological changes of neurons

The compositions of neurons, astrocytes and microglia in cortical cultures were determined by use of antibodies for MAP2, GFAP and OX-42, which are specific for neurons, astrocytes and microglia, respectively. Approximately 95% of the cells were stained by anti-MAP2 antibody, whereas there were few anti-GFAP-positive cells (less than 4%) and anti-microglial antigen-positive ones (less than 3%) (Figure 2A). Examination of cultures treated with $A\beta(25-35)$ by light microscopy showed disruption of neurites at 48 h. Some cell bodies shrank and lost their bright phase-contrast appearance. There were markedly fewer cells, and extensive debris was seen attached to the substratum. The morphologic disruption in $A\beta(25-35)$ -treated neurons was suppressed completely by S-2474 (Figure 2B).

Effects of S-2474 on $A\beta(25-35)$ -induced apoptotic features of neurons

Previously, we reported that $A\beta(25-35)$ -induced neuronal cell death was accompanied with characteristic features of apoptosis, such as chromatin condensation and DNA fragmentation (Ueda *et al.*, 1996). Therefore, chromatin condensation was examined with Hoechst 33258 fluorescent dye (Figure 3A and Table 2). In vehicle-treated cultures, cells showed little fluorescence in the nucleus. On the other hand, condensed and fragmented chromatin was clearly observed in $A\beta(25-35)$ -treated cultures [$F(3,36) = 8.550$, $P = 0.001$]. The amount of condensed chromatin in $A\beta(25-35)$ -treated neurons

Table 1 Effects of amyloid-related peptides on neuronal cell survival

Treatment	Concentration	MTT-reducing activity (% of control)
Control		100.0 $\pm$ 4.5
$A\beta(25-35)$	0.1 μM	96.5 $\pm$ 6.4
	1 μM	70.5 $\pm$ 7.1*
	10 μM	29.5 $\pm$ 1.1**
$A\beta(1-40)$	10 μM	66.7 $\pm$ 2.5*
Scrambled $A\beta(25-35)$	10 μM	106.2 $\pm$ 5.0
Reversed $A\beta(25-35)$	10 μM	101.2 $\pm$ 12.3
S-2474	10 μM	119.5 $\pm$ 3.5
$A\beta(25-35)$ + S-2474	10 μM , 10 μM	117.2 $\pm$ 2.5###
$A\beta(1-40)$ + S-2474	10 μM , 10 μM	116.6 $\pm$ 2.4###

Rat cortical neurons were treated with various amyloid-related peptides. MTT-reducing activity was determined 48 h later. Data are expressed as mean $\pm$ s.e.mean values ($n = 4$). * $P < 0.05$ and ** $P < 0.01$, compared with vehicle controls, and ### $P < 0.01$, compared with $A\beta$ controls by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. $A\beta$ controls are treated with culture medium containing $10 \mu M$ $A\beta(25-35)$ or $10 \mu M$ $A\beta(1-40)$ and 0.1% DMSO.

Figure 2 Morphologic changes in cortical neurons by $A\beta(25-35)$. Immunocytochemical analysis for anti-MAP2 (A), anti-GFAP (B) or OX-42 (C) was performed on the present cortical cultures. Magnification, $\times 95$. Cortical neurons were treated with vehicle control (D), $10 \mu M$ $A\beta(25-35)$ (E), or $10 \mu M$ $A\beta(25-35)$ + $10 \mu M$ S-2474 (F). Neurons were examined by phase-contrast microscopy 48 h later. Magnification, $\times 95$. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. $A\beta$ control is treated with culture medium containing $10 \mu M$ $A\beta(25-35)$ and 0.1% DMSO.

was decreased significantly by S-2474 [$F(3,36)=8.382$, $P<0.01$]. There was no statistical difference in MTT-reducing activity between vehicle-treated and $10 \mu M$ S-2474-added $A\beta(25-35)$ -treated neurons [$F(3,36)=0.168$, $P=0.293$].

Fragmentation of DNA was also estimated with the TUNEL technique (Figure 3B and Table 2). After neurons were incubated with or without $A\beta(25-35)$ for 48 h, the number of TUNEL-positive nuclei was increased significantly in $A\beta(25-35)$ -treated neurons as compared to vehicle-treated ones [$F(3,36)=3.949$, $P=0.004$]. The number of TUNEL-positive nuclei in $A\beta(25-35)$ -treated neurons was decreased significantly by S-2474 [$F(3,36)=4.584$, $P=0.003$]. There was no statistical difference in MTT-reducing activity between vehicle-treated and $10 \mu M$ S-2474-added $A\beta(25-35)$ -treated neurons [$F(3,36)=0.635$, $P=0.107$]. Thus, S-2474 ameliorated $A\beta$ -induced apoptotic features.

$A\beta(25-35)$ -induced generation of AA metabolites

Activation of PLA_2 contributes to the neurotoxicity of $A\beta(25-35)$ (Stephenson *et al.*, 1996), and level of PGD_2 among AA metabolites is significantly elevated in AD (Iwamoto *et al.*, 1989). We examined the association of

Figure 3 Apoptotic features of cortical neurons induced by $A\beta(25-35)$. Cortical neurons were treated with vehicle control (A and D), $10 \mu M$ $A\beta(25-35)$ control (B and E) or $10 \mu M$ $A\beta(25-35)$ + $10 \mu M$ S-2474 (C and F). Neurons were stained with $1 \mu M$ Hoechst 33258 for 10 min 48 h later (A, B, and C). Neurons were fixed with 4% paraformaldehyde, washed twice with PBS, and stained by the TUNEL technique 48 h later (D, E, and F). Magnification, $\times 95$. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. $A\beta$ control is treated with culture medium containing $10 \mu M$ $A\beta(25-35)$ and 0.1% DMSO.

Table 2 Effects of S-2474 on $A\beta$ -induced Hoechst 33258 and TUNEL-positive neurons (cells/cm²)

Treatment	Hoechst 33258	TUNEL
Control	634.9 $\pm$ 84.0	253.8 $\pm$ 6.7
$A\beta(25-35)$	7253.4 $\pm$ 681.3**	485.3 $\pm$ 51.5**
$A\beta(25-35)$ + S-2474	765.1 $\pm$ 75.3###	216.7 $\pm$ 18.5###

Cortical neurons were treated in the absence (control) or presence of $10 \mu M$ $A\beta(25-35)$ or $10 \mu M$ $A\beta(25-35)$ + $10 \mu M$ S-2474. Hoechst 33258- and TUNEL-positive neurons were detected 36 h later. Data are expressed as mean $\pm$ s.e. mean values ($n=4$). ** $P<0.01$, compared with vehicle control, ### $P<0.01$, compared with $A\beta(25-35)$ control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. $A\beta$ controls are treated with culture medium containing $10 \mu M$ $A\beta(25-35)$ and 0.1% DMSO.

eicosanoids generation with $A\beta(25-35)$ -induced neuronal cell death (Figure 4). There was no statistical difference in MTT-reducing activity between vehicle-treated and $A\beta(25-35)$ -treated neurons at 24 h [$F(3,36)=0.354$, $P=0.483$]. After 24 h, $10 \mu M$ $A\beta(25-35)$ decreased MTT-reducing activity in a time-dependent manner, and killed 70% of neurons at 48 h later [$F(3,36)=9.009$, $P=0.001$] (Figure 4A).

Figure 4 $A\beta(25-35)$ -induced neuronal cell death and generation of eicosanoids from Neurons. Cortical neurons were treated with $10 \mu\text{M}$ $A\beta(25-35)$. MTT-reducing activity (A) and eicosanoids (B) were measured at the indicated time points after $A\beta$ treatment. PGD_2 (circles), PGE_2 (triangles) and LTB_4 (squares) were measured with their RIA kits. The control level of PGD_2 , PGE_2 or LTB_4 was 73 ± 3 , 198 ± 20 or 752 ± 38 pg/ml, respectively. Data are expressed as mean $\pm$ s.e.mean values ($n=4$). ** $P<0.01$, compared with vehicle control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO.

Next, we examined generation of eicosanoids from neurons after $A\beta$ -treatment. The level of PGD_2 in vehicle controls was not altered throughout the culture. When neurons were exposed to $A\beta(25-35)$, there was no statistical difference in generation of PGD_2 between vehicle-treated and $A\beta(25-35)$ -treated neurons at 20 h [$F(3,36)=0.073$, $P=0.810$]. Then, PGD_2 was increased transiently at 29 h [$F(3,36)=5.803$, $P=0.004$] and decreased thereafter [$F(3,36)=0.444$, $P=0.019$] (Figure 4B). On the other hand, there was no statistical difference in generation of PGE_2 [$F(3,36)=0.908$, $P=0.409$] and LTB_4 [$F(3,12)=1.490$, $P=0.187$] between vehicle-treated and $A\beta(25-35)$ -treated neurons at 29 h (Figure 4B). These results indicated that $A\beta$ significantly generated PGD_2 , but not PGE_2 nor LTB_4 , from neurons prior to neuronal cell death.

Effects of PGD_2 on neuronal cell survival

To ascertain whether PGD_2 possesses neurotoxicity, we examined effect of PGD_2 on neuronal survival (Figure 5

and Table 3). As shown in Table 3, PGD_2 caused neuronal cell death in a concentration-dependent manner ($\text{LD}_{50}=7.9 \pm 0.8 \mu\text{M}$). MTT reducing activity was decreased to 34% of control neurons by $10 \mu\text{M}$ PGD_2 [$F(3,56)=6.531$, $P<0.001$] at 24 h. At 6 h, there was no statistical difference in MTT-reducing activity between vehicle-treated and $10 \mu\text{M}$ PGD_2 -treated neurons [$F(3,56)=0.633$, $P=0.072$]. Then, PGD_2 displayed significant neurotoxicity at 9 h [$F(3,56)=2.897$, $P=0.001$] and killed 80% of neurons at 24 h [$F(3,52)=7.250$, $P<0.001$] (Figure 5A). Moreover, $10 \mu\text{M}$ $15d-\Delta^{12,14}\text{-PGJ}_2$ caused neuronal cell death more potently than PGD_2 [$F(3,56)=8.197$, $P<0.001$] at 24 h (Table 3). As compared to vehicle controls, however, no statistical difference was detected in other eicosanoids such as AA [$F(3,56)=1.207$, $P=0.388$], PGE_2 [$F(3,56)=0.268$, $P=0.739$], 9α , $11\beta\text{-PGF}_2$ [$F(3,56)=0.525$, $P=0.328$],

Figure 5 Effect of S-2474 on $A\beta(25-35)$ -induced generation of PGD_2 from Neurons. (A) PGD_2 -induced neuronal cell death: Cortical neurons were treated with $10 \mu\text{M}$ PGD_2 . MTT-reducing activity was determined at the indicated times after the PG treatment. * $P<0.05$, ** $P<0.01$, compared with vehicle control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 0.1% ethanol. (B) Effect of S-2474 on $A\beta(25-35)$ -induced generation of PGD_2 from Neurons: Cortical neurons were treated with S-2474 at the indicated concentration in the presence of $10 \mu\text{M}$ $A\beta(25-35)$. Generation of PGD_2 was determined 29 h later. Data are expressed as mean $\pm$ s.e.mean values ($n=4$). ** $P<0.01$, compared with $A\beta$ control by ANOVA followed by Dunnett's test. $A\beta$ control is treated with culture medium containing $10 \mu\text{M}$ $A\beta(25-35)$ and 0.1% DMSO.

Table 3 Effects of eicosanoids on neuronal cell survival

Eicosanoids	Concentration	MTT-reducing activity (% of control)
Control		100.0 $\pm$ 4.0
AA	30 μ M	88.3 $\pm$ 11.9
PGD ₂	1 μ M	97.9 $\pm$ 2.5
	3 μ M	90.7 $\pm$ 2.7
	6 μ M	71.5 $\pm$ 8.2
	10 μ M	36.9 $\pm$ 3.1**
15d- $\Delta^{12,14}$ -PGJ ₂	10 μ M	20.8 $\pm$ 3.2**
PGE ₂	10 μ M	102.6 $\pm$ 6.4
9 α , 11 β -PGF ₂	10 μ M	105.1 $\pm$ 2.7
PGF ₂ α	10 μ M	107.3 $\pm$ 1.3
PGI ₂	10 μ M	104.1 $\pm$ 2.5
U-46619	10 μ M	96.5 $\pm$ 4.7
LTB ₄	10 μ M	90.3 $\pm$ 2.4
LTC ₄	10 μ M	95.3 $\pm$ 4.1
LTD ₄	10 μ M	97.8 $\pm$ 3.6

Rat cortical neurons were treated with various PGs and LTs. MTT-reducing activity was determined 24 h later. Data are expressed as mean $\pm$ s.e.mean values ($n=4$). ** $P<0.01$, compared with vehicle control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 0.1% ethanol.

PGF₂ α [F(3,56)=0.746, $P=0.132$], PGI₂ [F(3,56)=0.418, $P=0.416$], U-46619 [F(3,56)=0.363, $P=0.588$], LTB₄ [F(3,56)=1.003, $P=0.083$], LTC₄ [F(3,56)=0.490, $P=0.437$] and LTD₄ [F(3,56)=0.200, $P=0.661$].

Next, we examined effect of S-2474, a COX-2 inhibitor, on A β -induced PGD₂ generation. As shown in Figure 5B, S-2474 inhibited the PGD₂ generation in a concentration dependent manner (IC₅₀=69.8 $\pm$ 21.9 nM). At 10 μ M, S-2474 lowered the elevated level of PGD₂ significantly [F(3,36)=4.285, $P=0.012$]. There was a slight but statistical difference in PGD₂ generation between vehicle-treated and 10 μ M S-2474-added A β (25–35)-treated neurons [F(3,36)=1.286, $P=0.015$]. Thus, S-2474 suppressed generation of PGD₂ from A β -treated neurons significantly, although not completely.

A β (25–35)-induced generation of free radicals

S-2474 contains the di-*tert*-butylphenol antioxidant moiety (Inagaki *et al.*, 2000). To examine effects of S-2474 on A β (25–35)-induced generation of free radicals, we measured the intracellular reactive oxygen species by the DCFDA assay (Figure 6). Application of A β (25–35) for 24 h markedly increased the intracellular reactive oxygen species [F(3,24)=8.599, $P<0.001$]. The radical scavenger, vitamin E (100 μ M), potently reduced accumulation of reactive oxygen species [F(3,24)=8.378, $P<0.001$]. There was no statistical difference in generation of free radicals between vehicle-treated and 100 μ M vitamin E-added A β (25–35)-treated neurons [F(3,24)=0.384, $P=0.488$]. As well as vitamin E, S-2474 significantly decreased the elevated level of free radicals in a concentration-dependent manner (IC₅₀=601 $\pm$ 75 nM) (Figure 6A). At 10 μ M, S-2474 significantly reduced radicals from A β (25–35)-treated neurons [F(3,24)=5.883, $P=0.003$]. There was no statistical difference in generation of free radicals between vehicle-treated and 10 μ M S-2474-added A β (25–35)-treated neurons [F(3,24)=2.291, $P=0.051$].

Figure 6 Involvement of free radicals in A β (25–35) neurotoxicity. (A) Level of free radicals measured by DCFDA assay: Cortical neurons were treated with 10 μ M A β (25–35) in the presence or absence of S-2474 or 100 μ M vitamin E, and the DCFDA assay was performed 24 h later. Data are expressed as mean $\pm$ s.e.mean ($n=4$), per cent of control cultures, which were incubated for the same amount of time as experimental cultures ($n=4$). ** $P<0.01$, compared with vehicle control by ANOVA followed by Dunnett's test. ## $P<0.01$, compared with A β control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. A β control is treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO. (B) Effect of S-2474 on A β (25–35)-induced neuronal cell death. Cortical neurons were treated with S-2474 at the indicated concentration or 100 μ M vitamin E in the presence of 10 μ M A β (25–35). Forty-eight hours later, cell viability was measured according to the morphological criteria. Data are expressed as mean $\pm$ s.e.mean values ($n=4$). ** $P<0.01$, compared with vehicle control by ANOVA followed by Dunnett's test. # $P<0.05$, ## $P<0.01$, compared with A β control by ANOVA followed by Dunnett's test. Vehicle control is treated with culture medium containing 1% deionized water and 0.1% DMSO. A β control is treated with culture medium containing 10 μ M A β (25–35) and 0.1% DMSO.

To examine the effects of anti-oxidants on A β neurotoxicity, we performed morphometric cell counting to quantify the magnitude of cell death according to the previous report (Ueda *et al.*, 1997a). The presence of anti-oxidants may affect the MTT reduction assay (Behl *et al.*, 1992). Therefore, neurotoxicity was measured morphologically (Figure 2). S-2474 or vitamin E was added to cortical cultures simultaneously with A β (25–35). Morphometric cell counting was then performed

48 h later (Figure 6B). In accordance with the results using MTT-reducing activity, A β (25–35) induced morphologic neurodegeneration [F(3,24)=19.403, $P<0.001$]. Vitamin E significantly ameliorated A β (25–35)-induced neurodegeneration [F(3,24)=16.818, $P<0.001$]. S-2474 also exhibited neuroprotective effects in a concentration-dependent manner (IC₅₀=43±19 nM). At 10 μ M, S-2474 rescued neurons from A β (25–35)-induced neurodegeneration [F(3,24)=16.594, $P<0.001$]. There was no statistical difference in morphometric cell viability between vehicle-treated and vitamin E-added A β (25–35)-treated neurons [F(3,24)=2.585, $P=0.048$]. There was a slight but statistical difference in morphometric cell viability between vehicle-treated and 10 μ M S-2474-added A β (25–35)-treated neurons [F(3,24)=2.810, $P=0.030$]. Thus, S-2474 ameliorated A β -induced morphologic neurodegeneration significantly, although not completely.

Discussion

In the present study, we demonstrated that S-2474 prevented neurons from A β -induced cell death completely. S-2474 also ameliorated A β -induced morphological changes and apoptotic features such as the condensation of chromatin and the fragmentation of DNA significantly. The present cortical cultures contained little non-neuronal cells, indicating that S-2474 exhibited neuroprotective effect directly, but not indirectly *via* non-neuronal cells.

S-2474 is reported to inhibit COX-2 and 5-LO (Inagaki *et al.*, 2000). COX-2 is up-regulated in AD brain and in A β -treated SH-SY5Y neuroblastoma cells (Pasinetti & Aisen, 1998). Among PGs, the formation of PGD₂ is significantly increased in AD brain (Iwamoto *et al.*, 1989). In the present culture of rat cortical neurons, changes in COX-2 expression could not be detected after A β -treatment (data not shown). However, PGD₂ was transiently increased prior to A β -induced neuronal cell death. PGD₂ induced neuronal cell death in a concentration- and time-dependent manner. Neuronal cell survival was not affected by other eicosanoids such as PGE₂, 9 α -11 β -PGF₂, PGF_{2 α} , PGI₂ and U-46619, a stable agonist for thromboxane A₂ receptor. Among eicosanoids tested, only PGD₂ showed neurotoxicity. Furthermore, S-2474 decreased significantly the PGD₂. On the other hand, LTs including LTB₄, C₄, and D₄ were not altered, and did not kill neurons. Thus, S-2474 appears to rescue neurons from A β -induced cell death *via* inhibiting generation of PGD₂.

PGD₂ is the major PG in the brain of the rat and other mammalian species (Abdel-Halim *et al.*, 1977), and PGD₂ levels were significantly increased in AD brain (Iwamoto *et al.*, 1989), suggesting involvement in neurodegeneration. Indeed, PGD₂ caused neuronal cell death in our cortical cultures, indicating a possible involvement of PGD₂ in the

apoptosis of neurons induced by A β . Interpretation of PGD₂ as an apoptosis inducer requires circumspection for several reasons, however. First, the LD₅₀ value of PGD₂ is high as compared to the affinity for its receptors. Second, there is a latent period for PGD₂ to induce neuronal cell death. Third, it has not been clarified sufficiently how PGD₂ is associated with inflammatory diseases including AD. Therefore, further studies are necessary for determining the pathologic role of PGD₂ in AD.

There was a close correlation between MTT-reducing activity and morphologic viability. A comparison of the former activity with the latter viability in detail gave rise to a small question, however. In the presence of 10 μ M S-2474, the former activity of A β -treated neurons was significantly higher than that of vehicle controls. On the other hand, in the presence of 10 μ M S-2474, the latter viability of A β -treated neurons was lower than that of control neurons. S-2474 contains the di-*tert*-butylphenol antioxidant moiety (Inagaki *et al.*, 2000), and scavenged free radicals from A β -treated neurons. The presence of anti-oxidants may affect the MTT reduction assay (Behl *et al.*, 1992), and vitamin E increased the MTT-reducing activity regardless of the presence of A β (data not shown). Thus, S-2474 appears to increase MTT-reducing activity *via* an antioxidant effect at high concentrations.

Besides COX-2, expression of peroxysome proliferator-activated receptor- γ (PPAR γ) is also increased in AD brains (Kitamura *et al.*, 1999). PPAR γ is activated by NSAIDs such as indomethacine (Kitamura *et al.*, 1999; Klegeris *et al.*, 1999). Recent studies have demonstrated that NSAIDs with PPAR γ agonist effects reduce apoptosis in cerebellar granule cells (Heneka *et al.*, 2000). However, an endogenous PPAR γ agonist, 15d- $\Delta^{12,14}$ -PGJ₂, induces apoptosis in rat cortical neurons (Rohn *et al.*, 2001). In the present study, we confirmed the latter report. Thus, it is unlikely that PPAR γ contributes to the neuroprotective effect of S-2474.

The combination of the present study with our previous reports gave rise to therapeutic potentials of S-2474 for AD. First, S-2474 causes little adverse effect such as gastrointestinal ulceration (Jyoyama *et al.*, 1997), because it is a weak inhibitor for COX-1 (Inagaki *et al.*, 2000). Second, S-2474 possesses inhibitory activities against the production of inflammatory factors (Inagaki *et al.*, 2000), including IL-1, IL-6 and NO, which are elevated in the AD brain (Minster *et al.*, 2000). It suggests a suppressive effect of S-2474 on the inflammation in AD brain. Third, S-2474 appears to exhibit neuroprotective effect as a COX-2 inhibitor and an anti-oxidant. Thus, we conclude that S-2474 has neuroprotective effect against A β toxicity. Furthermore, we demonstrate that S-2474 possesses therapeutic potentials for AD *via* a direct neuroprotective effect as well as an indirect anti-inflammatory effect.

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