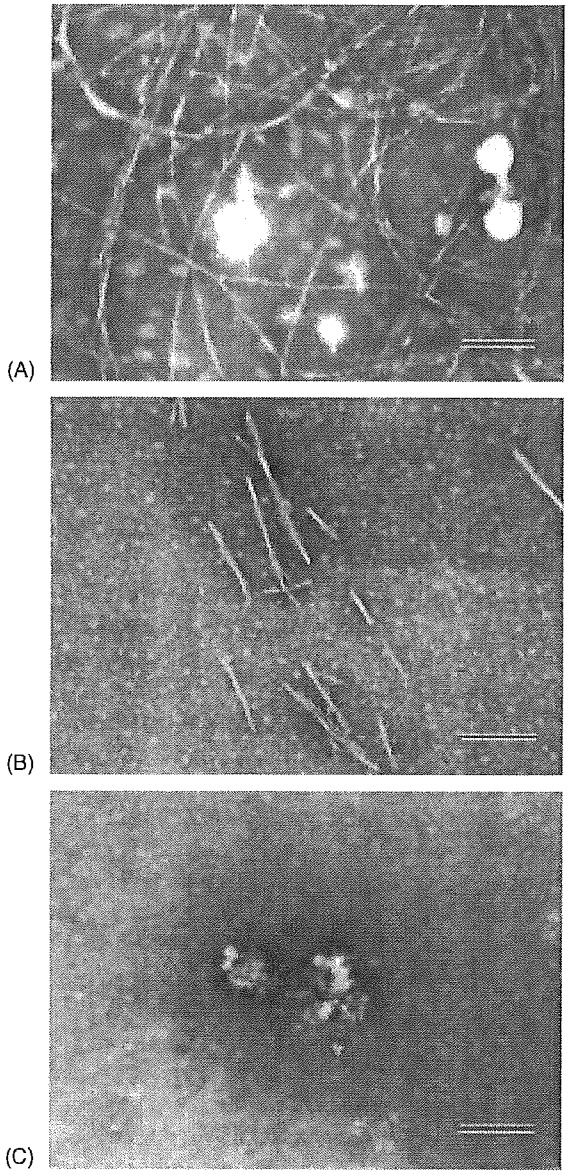

Fig. 5. Electron micrographs of destabilized fAβ(1-40). The reaction mixture containing 25 μM fAβ(1-40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 50 μM dopamine was incubated at 37 °C for 0 h (A), 1 h (B) or 6 h (C). Scale bars indicate a length of 250 nm.

the concentrations to destabilize fAβs to 50% of the control value, by the sigmoidal curve fitting of the data as shown in Figs. 1E, 2F and 4E (Table 1). For all molecules examined, the EC_{50} value for inhibiting the formation or extension of fAβs was similar to the EC_{50} value for destabilizing fAβs. As shown in Fig. 6, the EC_{50} to inhibit the formation of fAβ(1-40) and fAβ(1-42) showed a significant positive correlation with the EC_{50} to destabilize fAβs ($r = 0.885$, $p < 0.001$ in Fig. 6A; $r = 0.863$, $p < 0.001$ in Fig. 6B). All data presented in Table 1 show that the anti-amyloidogenic and fibril-destabilizing activity of the molecules examined in this study are in the order of: dopamine > Sel = NDGA > L-Dopa = Per > Bro = RIF.

Table 1
The effective concentrations (EC_{50})[a] of NDGA, RIF and anti-Parkinsonian agents for the formation, extension and destabilization of fAβ(1-40) and fAβ(1-42)

Compounds	Formation[b]		Extension[c]		Destabilization[d]	
	fAβ (1-40)	fAβ (1-42)	fAβ (1-40)	fAβ (1-42)	fAβ (1-40)	fAβ (1-42)
NDGA (μM)	0.16	0.88	0.23	0.09	11	0.82
RIF	9.7	9.1	7.7	10	22	31
Dopamine	0.01	0.04	0.03	0.06	0.21	0.23
L-Dopa	5.0	1.7	18	8.3	2.5	2.2
Per	0.89	2.0	7.1	9.1	2.5	2.2
Bro	14	20	47	40	5.8	5.3
Sel	0.68	0.72	0.15	0.13	1.6	1.2

[a] EC_{50} (μM) were defined as the concentrations of NDGA, RIF or anti-Parkinsonian agents to inhibit the formation or extension of fAβs to 50% of the control value, or the concentrations to destabilize fAβs to 50% of the control value. EC_{50} were calculated by the sigmoidal curve fitting of the data as shown in Figs. 1E, 2F and 4E, using Igor Pro ver.4 (WaveMetrics Inc., Lake Oswego, OR, USA).

[b] The reaction mixtures containing 50 μM Aβ(1-40) or 25 μM Aβ(1-42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM NDGA, RIF, dopamine, L-Dopa, Per, Bro, or Sel were incubated at 37 °C for 7 days and 24 h, respectively.

[c] The reaction mixtures containing 10 μg/mL (2.3 μM) sonicated fAβ(1-40) or fAβ(1-42), 50 μM Aβ (1-40) or Aβ(1-42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM NDGA, RIF, dopamine, L-Dopa, Per, Bro, or Sel were incubated at 37 °C for 1 h.

[d] The reaction mixtures containing 25 μM fAβ(1-40) or fAβ(1-42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM NDGA, RIF, dopamine, L-Dopa, Per, Bro, or Sel were incubated at 37 °C for 6 h.

4. Discussion

In the present study, the anti-Parkinsonian agents, dopamine, L-Dopa, Per, Bro and Sel dose-dependently inhibited fAβ formation from fresh Aβ, and destabilized preformed fAβ, while Tri did not exhibit anti-amyloidogenic and fibril-destabilizing effects in vitro. Additionally, we analyzed the real dose dependence of these agents. When we used NDGA and RIF as standard molecules, the anti-amyloidogenic and fibril-destabilizing activity of the molecules examined in this study was in the order of: dopamine > Sel = NDGA > L-Dopa = Per > Bro = RIF (see Table 1, Fig. 6). In particular, dopamine exhibited potent anti-amyloidogenic and fibril-destabilizing effects because EC_{50} of dopamine for the formation, extension and destabilization of fAβs were in the order of 0.01–0.10 μM. Because the estimated dopamine concentration in striatal nerve terminals after administration is ≫50 μM (Spencer et al., 1996), the concentration used in our experiments is physiologically relevant. Very recently, Li et al. (2004) reported that 10–100 μM of dopamine and L-Dopa inhibit the formation of amyloid fibrils from 1 mg/mL of Aβ(1-40) and α-synuclein in vitro. In their study of fAβ formation from Aβ(1-40), the mixture containing 230 μM (1 mg/mL) Aβ in 25 mM Tris, 100 mM NaCl buffer were stirred or shaken for 0–40 h at 37 °C. On the other hand, in our polymerization and

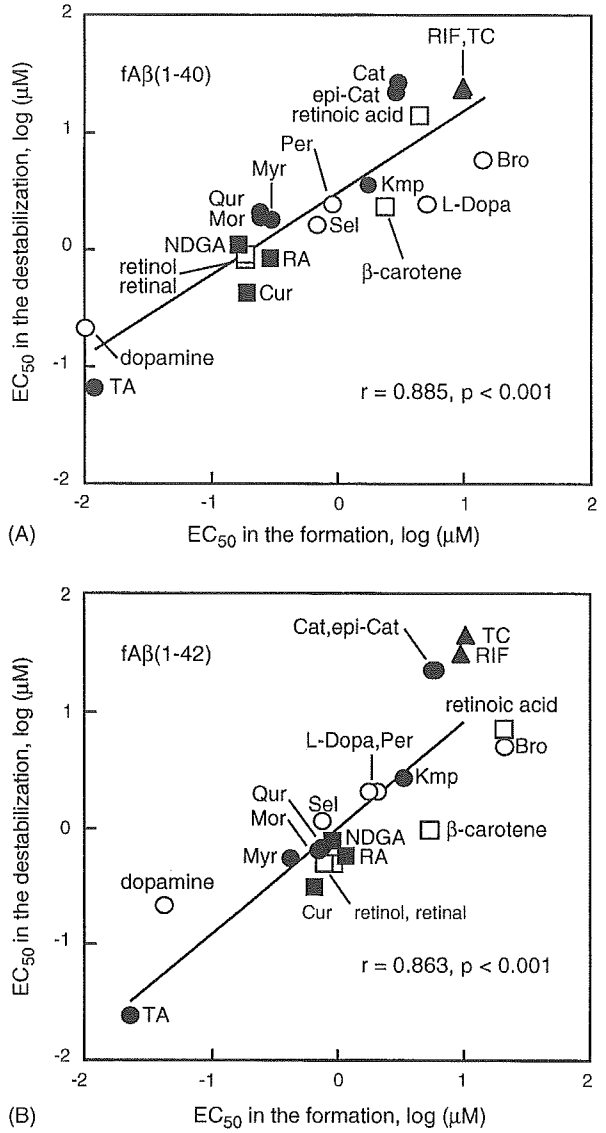

Fig. 6. Correlation of the effective concentrations (EC_{50}) of wine-related polyphenols (TA, Myr, Mor, Qur, Kmp, Cat and epi-Cat) (●), anti-Parkinsonian agents (dopamine, L-Dopa, Per, Bro and Sel) (○), NDGA, Cur and RA (■), Vitamin A (retinol, retinal, retinoic acid) and β-carotene (□) or RIF and TC (▲) for the formation and destabilization of fAβ(1-40) (A) and fAβ(1-42) (B). In both cases, significant positive correlations were observed between the EC_{50} for the formation of fAβ and the EC_{50} for the destabilization of fAβ ($r = 0.885$, $p < 0.001$ in panel A, $r = 0.863$, $p < 0.001$ in panel B, Pearson's correlation test).

destabilization experiments, the concentrations of Aβs or fAβs present in the mixture were 25–50 μM, about one order less than those used by Li et al. Moreover, in our destabilization study, we obtained fresh and non-aggregated fAβ(1-40) by extending sonicated fAβ(1-40) with fresh Aβ(1-40) solutions just before the destabilization reaction. Thus, the discrepancy of the effective concentrations between us and Li et al. is likely to mainly stem from the differences in the peptide concentrations, the incubation condition and the procedure of fAβ preparation.

As shown in Fig. 6, based on our present and previous findings, we classified the overall activity of the anti-amyloidogenic molecules into four groups (Ono et al., 2002a,b, 2003, 2004a,b,c). Dopamine and tannic acid (TA) were classified into the strongest anti-amyloidogenic group, Sel, NDGA, Cur, RA, myricetin (Myr), morin (Mor), quercetin (Qur), retinol and retinal into the second group, L-Dopa, Per, β-carotene and kaempferol (Kmp) into the third group, and Bro, catechin (Cat), epicatechin (epi-Cat), retinoic acid, RIF and tetracycline (TC) into the fourth group. Poly(vinylsulfonic acid, sodium salt), 1,3-propanedisulfonic acid, disodium salt, β-sheet breaker peptide and nicotine exhibited anti-amyloidogenic activity in a mM order. The inhibitory activities of RIF and TC for the formation of fAβ(1-40) from Aβ(1-40) were similar to those reported by other research groups (Tomiyama et al., 1996; Forloni et al., 2001), which may additionally support the validity of the data in Fig. 6.

What is the mechanism by which the anti-Parkinsonian agents other than Tri dose-dependently inhibit fAβ formation and destabilize preformed fAβ in vitro? From the results of NDGA, Cur and RA, we speculated that the 3,4-dihydroxyphenyl ring of these molecules might be quite suitable for specifically binding to free Aβ and subsequently inhibiting the polymerization of Aβ into fAβ, or might be suitable for specific binding to fAβ and subsequent destabilization of the β-sheet rich conformation of Aβ molecules in fAβ (Ono et al., 2002b). Like these molecules, dopamine and L-Dopa have a 3,4-dihydroxyphenyl ring (Fig. 7). From the results obtained on wine-related polyphenols and TA, we speculated that the difference in the three-dimensional structure and the numbers of hydroxyl groups in the polyphenol rings would affect greatly the anti-amyloidogenic and fibril-destabilizing activity (Ono et al., 2003; Ono et al., 2004b). Yen and Hsieh (1997) suggested that a phenol compound with hydoxyl groups, especially in the 3,4 position can be a good antioxidant. Although Per, Bro, and Sel have no 3,4-dihydroxyphenyl ring (Fig. 7), all of these agents have high antioxidant activity (Wu et al., 1993; Ogawa et al., 1994; Gómez-Vargas et al., 1998). The free radicals are trapped on either acetylenic carbon of the active site of Sel (Nakai and Yoneda, 2000) (Fig. 7), but the details of the antioxidant activities of Per and Bro are unknown. Interestingly, both RIF and nicotine have been reported to have antioxidant activity (Tomiyama et al., 1996; Linert et al., 1999), and the anti-amyloidogenic activity of them may be related to the propensity to bind to the specific sites of Aβ (Tomiyama et al., 1996; Zeng et al., 2001). Thus, anti-Parkinsonian agents other than Tri with potent antioxidant motifs may bind to Aβ and/or fAβ, inhibit fAβ formation and/or destabilize preformed fAβ through mechanisms yet unknown.

Dopamine did not extend the length of the lag phase in the formation of fAβs from Aβs (Fig. 1). Moreover, it did not extend the time to proceed to equilibrium in the extension reaction (Fig. 2). As shown in Fig. 2E, the extension of fAβ(1-40) followed a first-order kinetic model even in the presence of dopamine. The net rate of fAβ(1-40) extension is the sum of the rates of polymerization and depolymerization (Naiki and Nakakuki, 1996; Hasegawa et al., 2002). Thus, one possible explanation for the extension in Fig. 2E may be that dopamine

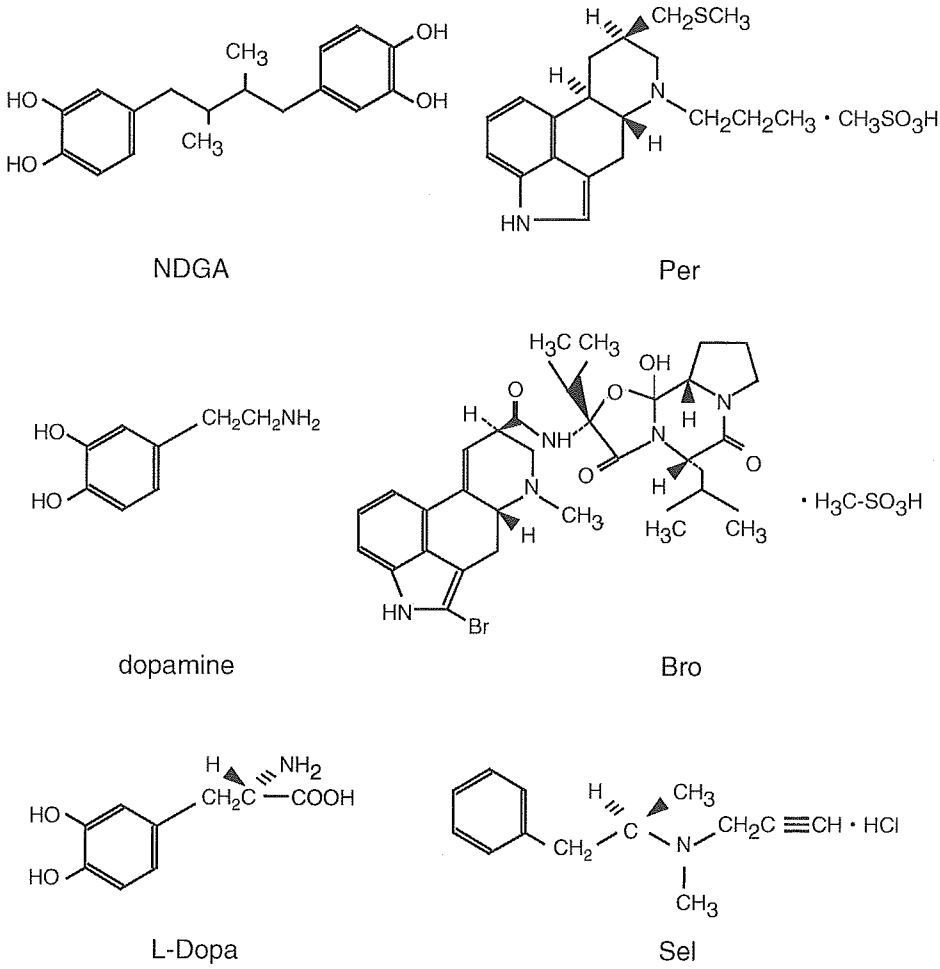

Fig. 7. Structure of NDGA, dopamine, L-Dopa, Per, Bro and Sel.

bound to the ends of extending fAβ(1-40) and increased the rate of depolymerization by destabilizing the conformation of Aβ(1-40), which has just been incorporated into the fibril ends. Alternatively, dopamine would bind to Aβ(1-40) and consequently decrease the rate of polymerization. Previously, Harper et al. (1999) analyzed the process of in vitro Aβ assembly using an atomic force microscope at a fine resolution. They reported that protofibrils, transient species of Aβ assembly, were formed during the first week of incubation of Aβ40 before mature fibrils were generated. This model of Aβ assembly was supported by a recent study by Nichols et al. (2002). In the present study, both the formation of fAβs from Aβs and the fAβs extension were inhibited by all anti-Parkinsonian agents other than Tri. In the electron microscopic study, we observed mature fibrils in Aβ(1-40) solutions incubated with fAβ(1-40) at 50 μM and 37 °C for 6 h, but few protofibrils (Fig. 3B). This discrepancy may be due to the differences in incubation period and peptide concentrations. Further studies are essential to clarify the extent to which each anti-Parkinsonian agent inhibits formation of protofibrils.

Lewy bodies are usually present in the substantia nigra, locus ceruleus, nucleus basalis of Meynert, dorsal motor nucleus of the vagus, and hypothalamus, and often at other sites of predilection in PD. Lewy bodies also occur in 10–40% of individuals with AD and other diseases (Hansen and Samuel, 1997), suggesting that formation of Lewy bodies may not represent PD-specific underlying pathophysiological mechanisms. Similarly, many PD and dementia with Lewy bodies have AD-type pathologic findings (Aβ plaques and neurofibrillary tangles) (Hansen and Samuel, 1997). Although Tri did not accelerate nor inhibit fAβ formation in our in vitro study, Perry et al. (2003) suggested that anti-muscarinic drugs, such as Tri might promote β amyloidosis by impairing neurotrophin function because anti-muscarinic receptor activation facilitates amyloid precursor protein secretion promoted by nerve growth factor. They also suggested that exacerbation of AD pathology depends on long-term receptor antagonism (Perry et al., 2003), but they did not examine the relationships between the Aβ deposition and the dose or duration of medication with L-Dopa, dopamine agonists and MAO-B inhibitors. Our data suggest that the PD patients taking anti-Parkinsonian agents such as L-Dopa, dopamine agonists, and MAO-B inhibitors, might bear a smaller Aβ burden than the patients treated mainly with Tri. Further studies using animal

AD models and human postmortem studies are needed to explore whether anti-Parkinsonian agents inhibit Aβ deposition in vivo.

Our results suggest that anti-Parkinsonian agents may prevent the development of AD, not only through scavenging reactive oxygen species, but also through directly inhibiting the deposition of fAβ in the brain. Although the exact mechanism of the anti-amyloidogenic activity of these agents is unclear, these and other structurally related compounds could be key molecules for the development of therapeutics for AD and other conformation diseases.

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Non-steroidal anti-inflammatory drugs have anti-amyloidogenic effects for Alzheimer's β-amyloid fibrils in vitro

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Abstract

The pathogenesis of Alzheimer's disease (AD) is characterized by cerebral deposits of amyloid β-peptides (Aβ) and neurofibrillary tangles which are surrounded by inflammatory cells. Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD and delays the onset of the disease. In the present study, we used fluorescence spectroscopy with thioflavin T and electron microscopy to examine the effects of NSAIDs such as ibuprofen, aspirin, meclofenamic acid sodium salt, diclofenac sodium salt, ketoprofen, flurbiprofen, naproxen, sulindac sulfide and indomethacin on the formation, extension, and destabilization of β-amyloid fibrils (fAβ) at pH 7.5 at 37 °C in vitro. All examined NSAIDs dose-dependently inhibited formation of fAβ from fresh Aβ(1–40) and Aβ(1–42), as well as their extension. Moreover, these NSAIDs dose-dependently destabilized preformed fAβs. The overall activity of the molecules examined was in the following order: ibuprofen ≈ sulindac sulfide ≥ meclofenamic acid sodium salt > aspirin ≈ ketoprofen ≥ flurbiprofen ≈ diclofenac sodium salt > naproxen ≈ indomethacin. Although the mechanisms by which these NSAIDs inhibit fAβ formation from Aβ, and destabilize preformed fAβ in vitro are still unclear, NSAIDs may be promising for the prevention and treatment of AD.

Keywords: Alzheimer's disease; β-Amyloid fibrils; Electron microscopy; Non-steroidal anti-inflammatory drugs; Thioflavin T

1. Introduction

In the pathogenesis of Alzheimer's disease (AD), the abundance of intraneural neurofibrillary tangles and the extracellular deposition of the amyloid β-peptide (Aβ) as amyloid plaques and vascular amyloid are seminal events (Selkoe, 2001). The aggregation of Aβ and β-sheet formation are considered to be critical events that render these peptides neurotoxic (Pike et al., 1995). The presence of chronic neuroinflammation also contributes to the protracted degenerative course of AD (McGeer and McGeer, 1995), and it is also common to other neurodegenerative disorders, such as Parkinson's disease and Creutzfelt–Jacob disease (Eikelenboom et al., 2002; Gao et al., 2003). A chronic inflammatory response characterized by activated microglia, reactive astrocytes, complement factors, and increased inflammatory cytokine expression associated with Aβ deposits has been described in the brain of AD patients (Rogers et al., 1996). A number of epidemiological studies have demonstrated a reduced risk for AD in population with long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) (McGeer et al., 1996; in 't Veld et al., 1998, 2001; Akiyama et al., 2000).

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These epidemiological findings have been supported by experimental studies. First, chronic ibuprofen (Ibu) treatment significantly diminished amyloid deposition (Lim et al., 2000), and improved behavioral impairment in the APPsw transgenic mouse (Tg2576) (Lim et al., 2001). Second, a subset of NSAIDs such as Ibu, sulindac sulfide (SSide), indomethacin (Ind) (Weggen et al., 2001) and flurbiprofen (Flu) (Eriksen et al., 2003) selectively decreased the secretion of Aβ(1–42) from cultured cells independently of cyclooxygenase (COX) activity and lowered the amount of soluble Aβ(1–42) in the brains of Tg2576 mouse. Third, SSide and enantiomers of Flu have been reported to target γ-secretase and preferentially reduce Aβ(1–42) generation (Takahashi et al., 2003; Eriksen et al., 2003). These data suggest that some of the NSAIDs may affect the pathogenetic process of AD directly. However, except the preliminary study where Thomas et al. (2001) used the non-physiological short peptide Aβ(25–35), the effects of NSAIDs on the formation and destabilization of Alzheimer's β-amyloid fibrils (fAβ) in vitro have not been studied in detail.

Using a nucleation-dependent polymerization model explaining the mechanism of fAβ formation in vitro (Jarrett and Lansbury, 1993; Naiki and Gejyo, 1999), we previously found that nordihydroguaiaretic acid (NDGA) and rifampicin (Rif) inhibit fAβ formation from Aβ and fAβ extension dose-dependently (Naiki et al., 1998). Moreover, we reported that they also destabilize fAβ(1–40) and fAβ(1–42) in a concentration-dependent manner within a few hours at pH 7.5 at 37 °C, based on fluorescence spectroscopic analysis with thioflavin T (ThT) and electron microscopic studies (Ono et al., 2002b).

Here, we examined the effects of the major NSAIDs on the formation, extension, and destabilization of fAβ(1–40) and fAβ(1–42) at pH 7.5 and 37 °C in vitro, using fluorescence spectroscopy with ThT and electron microscopy.

2. Methods

2.1. Preparation of Aβ and fAβ solutions

Aβ(1–40) (trifluoroacetate salt, lot number 540111 and 530108, Peptide Institute Inc., Osaka, Japan) and Aβ(1–42) (trifluoroacetate salt, lot number 540127 and 530914, Peptide Institute Inc.) were dissolved by brief vortexing in 0.02% ammonia solution at a concentration of 500 μM (2.2 mg/mL) and 250 μM, respectively, in a 4 °C room and stored at −80 °C before assaying (fresh Aβ(1–40) and Aβ(1–42) solutions). fAβ(1–40) and fAβ(1–42) were formed from the fresh Aβ(1–40) and Aβ(1–42) solutions, respectively, sonicated, and stored at 4 °C as described elsewhere (Hasegawa et al., 1999).

Fresh, non-aggregated fAβ(1–40) and fAβ(1–42) were obtained by extending sonicated fAβ(1–40) or fAβ(1–42) with fresh Aβ(1–40) or Aβ(1–42) solutions, respectively, just before the destabilization reaction (Ono et al., 2002a,b). The reaction mixture was 600 μL and contained 10 μg/mL (2.3 μM) fAβ(1–40) or fAβ(1–42), 50 μM Aβ(1–40) or Aβ(1–42), 50 mM phosphate buffer, pH 7.5, and 100 mM NaCl. After incubation at 37 °C for 3–6 h under non-agitated conditions, the extension reaction proceed to equilibrium as measured by the fluorescence of ThT. In the following experiment, the concentration of fAβ(1–40) and fAβ(1–42) in the final reaction mixture was regarded as 50 μM.

2.2. Fluorescence spectroscopy, electron microscopy, and polarized light microscopy

A fluorescence spectroscopic study was performed as described by Naiki and Nakakuki (1996) on a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan). Optimum fluorescence measurement of fAβ(1–40) and fAβ(1–42) were obtained at the excitation and emission wavelengths of 445 and 490 nm, respectively, with the reaction mixture containing 5 μM ThT (Wako Pure Chemical Industries Ltd, Osaka, Japan) and 50 mM of glycine–NaOH buffer, pH 8.5. An electron microscopic study and polarized light microscopic study of the reaction mixtures were performed as described elsewhere (Hasegawa et al., 1999).

2.3. Polymerization assay

Polymerization of Aβ with or without fAβ added as seeds was assayed as described elsewhere (Naiki et al., 1998). The reaction mixture contained 50 μM Aβ(1–40), or 25 or 50 μM Aβ(1–42), 0 or 10 μg/mL fAβ(1–40) or fAβ(1–42), 0, 0.01, 0.1, 1, 10, or 50 μM NSAIDs [Ibu, aspirin (Asp), meclofenamic acid sodium salt (Mec), diclofenac sodium salt (Dic), ketoprofen (Ket), Flu, naproxen (Nap), SSide, Ind], NDGA, Rif, or nicotine (Sigma Chemical Co., St. Louis, MO, USA), 1% dimethyl sulfoxide (DMSO) (Nacakai Tesque, Inc., Kyoto, Japan), 50 mM phosphate buffer, pH 7.5, and 100 mM NaCl. NSAIDs dissolved in DMSO at concentrations of 1, 10, 100 μM, 1 mM and 5 mM, were added to the reaction mixture to make the final concentration 0.01, 0.1, 1, 10 μM and50 μM, respectively.

Aliquots (30 μL) of the mixture were put into oil-free PCR tubes (size: 0.5 mL, code number: 9046; Takara Shuzo Co. Ltd, Otsu, Japan). The reaction tubes were then put into a DNA thermal cycler (PJ480; Perkin Elmer Cetus, Emeryville, CA, USA). The plate temperature was elevated at maximal speed, starting at 4 °C, to 37 °C. Incubation times ranged between 0 and 8 days (as indicated in each figure), and the reaction was stopped by

placing the tubes on ice. The reaction tubes were not agitated during the reaction. From each reaction tube, triplicate 5 μL aliquots were removed, then subjected to fluorescence spectroscopy and the mean of each triplicate determined. In the ThT solution, the concentration of NSAIDs examined in this study was diluted up to 1/200 of that in the reaction mixture. We confirmed that these compounds did not quench ThT fluorescence at the diluted concentration (data not shown).

2.4. Destabilization assay

Destabilization of fAβ was assayed as described elsewhere (Ono et al., 2002b). Briefly, the reaction mixture contained 25 μM fresh fAβ(1–40) or fAβ(1–42), 0, 0.01, 0.1, 1, 10 or 50 μM NSAIDs, NDGA, Rif, or nicotine, 1% DMSO, 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 1% (w/v) polyvinyl alcohol (Wako Pure Chemical Industries Ltd) to avoid the aggregation of fAβ and the adsorption of fAβ onto the inner wall of the tube during the reaction.

After being mixed by pipetting, triplicate 5 μL aliquots were subjected to fluorescence spectroscopy and 30 μL aliquots were put into oil-free PCR tubes (Takara Shuzo). The reaction tubes were then transferred into a DNA thermal cycler (Perkin Elmer Cetus). Starting at 4 °C, the plate temperature was elevated at maximal speed to 37 °C. Incubation times ranged from 0 to 24 h (as indicated in each figure), and the reaction was stopped by placing the tubes on ice. The reaction tubes were not agitated during the reaction. From each reaction tube, 5 μL aliquots in triplicate were subjected to fluorescence spectroscopy and the mean of the three measurements was determined. ThT fluorescence did not change significantly when fAβ were incubated with 0–50 μM of NSAIDs at either 4 °C or 37 °C for 1 min, then subjected to ThT assay, indicating that these compounds did not compete with ThT for fAβ (data not shown).

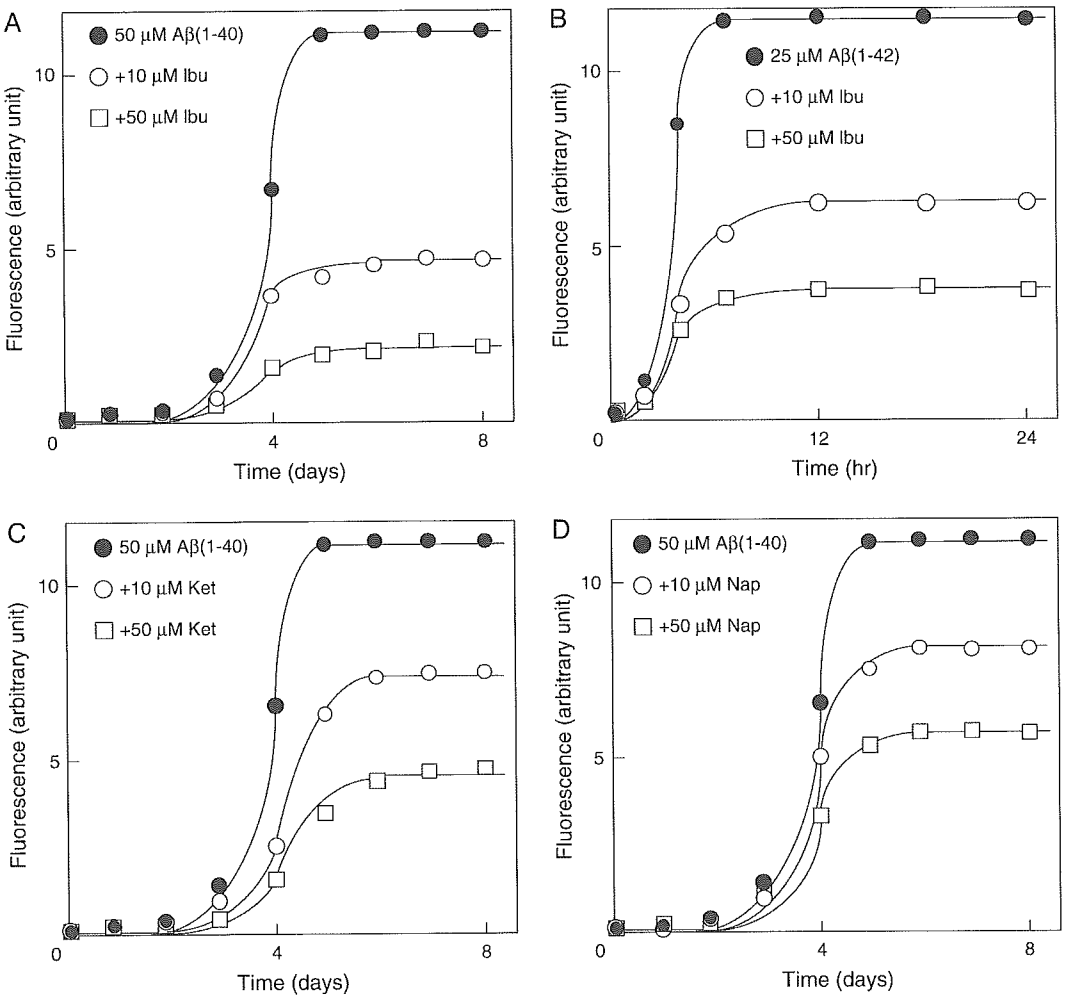

Fig. 1. Effects of Ibu (A,B), Ket (C), and Nap (D) on the kinetics of fAβ(1–40) (A,C,D) and fAβ(1–42) (B) formation from fresh Aβ(1–40) and Aβ(1–42), respectively. The reaction mixtures containing 50 μM Aβ(1–40) (A,C,D) or 25 μM Aβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○), or 50 μM (□) of Ibu (A,B), Ket (C), or Nap (D), were incubated at 37 °C for the indicated times. Each point represents the mean of three 5 μL aliquots from the same sample. Each figure is a representative pattern of three independent experiments.

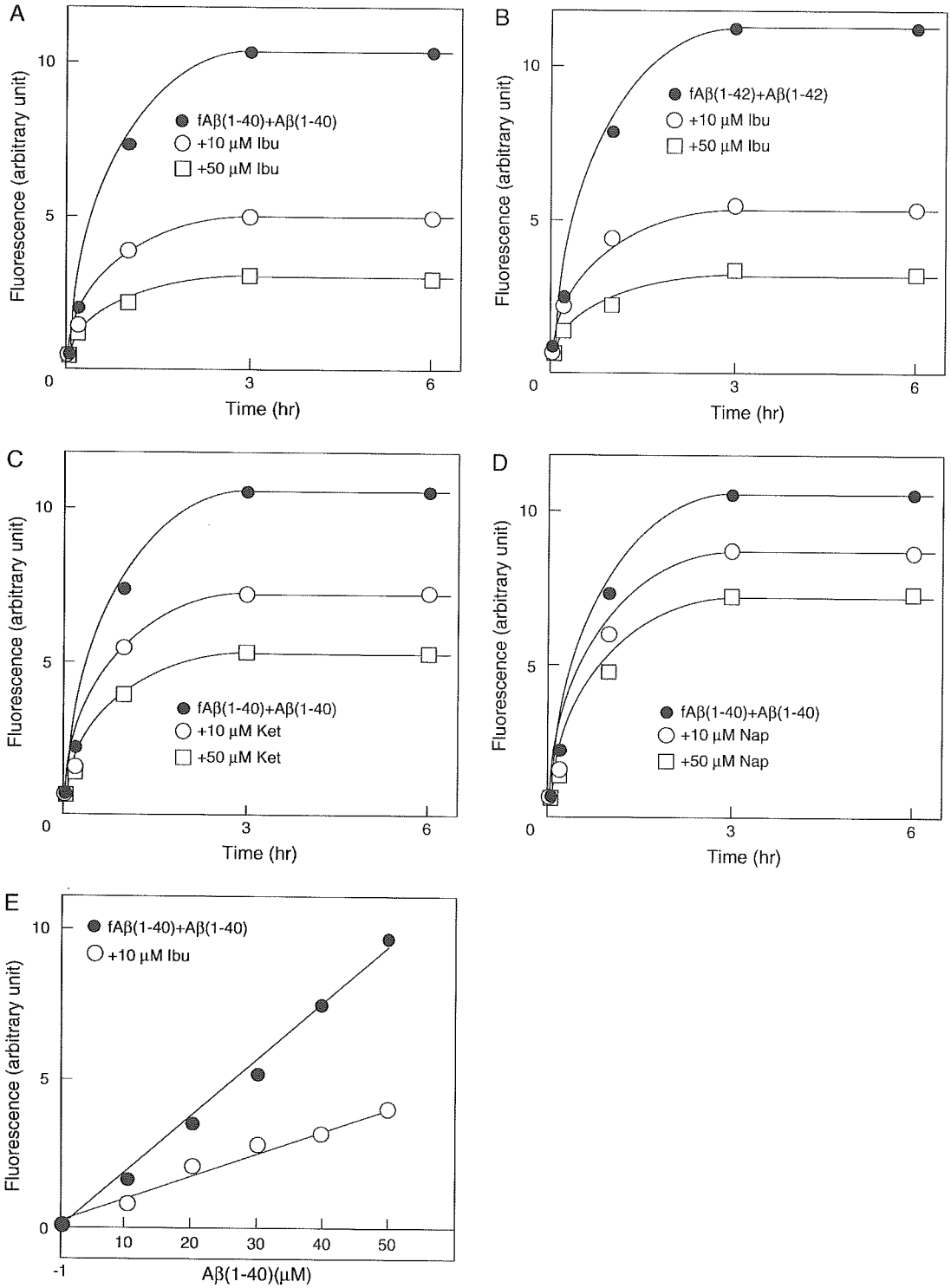

Fig. 2. Effects of Ibu (A,B), Ket (C), and Nap (D) on the kinetics of fAβ(1–40) (A,C,D) and fAβ(1–42) (B) extension. The reaction mixtures containing 10 μg/mL (2.3 μM) sonicated fAβ(1–40) (A,C,D) or fAβ(1–42) (B), 50 μM Aβ(1–40) (A,C,D) or Aβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○) or 50 μM (□) of Ibu (A,B), Ket (C), or Nap (D), were incubated at 37 °C for the indicated times. Each point represents the mean of three 5 μL aliquots from the same sample. Each figure is a representative pattern of three independent experiments. (E) Effect of Aβ(1–40) concentration on the initial rate of fAβ(1–40) extension in the presence (○) and absence (●) of Ibu. The reaction mixtures containing 10 μg/mL (2.3 μM) sonicated fAβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, 0 (●) or 10 μM (○) Ibu, and 0, 10, 20, 30, 40, and 50 μM Aβ(1–40), were incubated at 37 °C for 1 h. Points represent means of three independent experiments. In all points, standard errors were within symbols. Liner least-square fit was performed for each straight line (R^2 = 0.971 and 0.959 for ○ and ●, respectively).

2.5. Other analytical procedures

Protein concentrations of Aβs and fAβs in the reaction mixtures were determined by the method of Bradford (1976) with a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). The Aβ(1–40) solution quantified by amino acid analysis was used as the standard. The linear least squares fit and one-way analysis of variance, post hoc test by Scheffe were used for statistical analysis.

3. Results

3.1. Effect of NSAIDs on the kinetics of fAβ polymerization

As shown in Fig. 1A–D, the fluorescence of ThT followed a characteristic sigmoidal curve when fresh Aβ(1–40) or Aβ(1–42) was incubated at 37 °C. This curve is consistent with a nucleation-dependent polymerization model (Jarrett and Lansbury, 1993; Naiki and Gejyo, 1999). Fresh fAβ(1–40) assumed a non-branched, helical filament structure of approximately 10 nm width and exhibited a helical periodicity of approximately 220 nm. In the fresh fAβ(1–42) solution, two types of filaments of approximately 8 and 12 nm width were observed. Although the Aβ batches used here were different from those in our previous experiments, these morphologies of fAβs were similar to those reported previously (Naiki and Nakakuki, 1996; Naiki et al., 1998). Both fAβ(1–40) and fAβ(1–42) stained with Congo red showed typical orange-green birefringence under polarized light. The final equilibrium level of Aβ(1–40) or Aβ(1–42) was lowered dose-dependently by incubation with 10 and 50 μM Ibu, Ket or Nap (Fig. 1A–D). Other NSAIDs (Asp, Mec, Dic, Flu, SSide and Ind) also significantly inhibited the polymerization of Aβ(1–40) and Aβ(1–42) (Fig. 6A,B).

As shown in Fig. 2A–D, the fluorescence increased hyperbolically without a lag phase and proceeded to equilibrium much more rapidly by incubation of fresh Aβ(1–40) or Aβ(1–42) with fAβ(1–40) or fAβ(1–42), respectively, at 37 °C, than that without seeds (compare Fig. 1A,B, and 2A,B). This curve is consistent with a first-order kinetic model (Naiki and Nakakuki, 1996). The final equilibrium level was dose-dependently decreased by incubation of Aβ(1–40) or Aβ(1–42) with fAβ(1–40) or Aβ(1–42), respectively, with 10 or 50 μM Ibu, Ket or Nap (Fig. 2A–D). Other NSAIDs (Asp, Mec, Dic, Flu, SSide and Ind) also significantly inhibited the extension of fAβ(1–40) and fAβ(1–42) (data not shown).

As shown in Fig. 2E, at a constant fAβ(1–40) concentration, a good linearity was observed between the Aβ(1–40) concentration and the initial rate of fAβ(1–40) extension both in the presence and absence of Ibu. This linearity is again consistent with a first-order kinetic model and indicates that at each Aβ(1–40) concentration, the net rate of fAβ(1–40) extension is the sum of the rates of polymerization and depolymerization (Naiki and Nakakuki, 1996; Hasegawa et al., 2002). In the presence of 10 μM Ibu, the slope of the straight line decreased to about 36%.

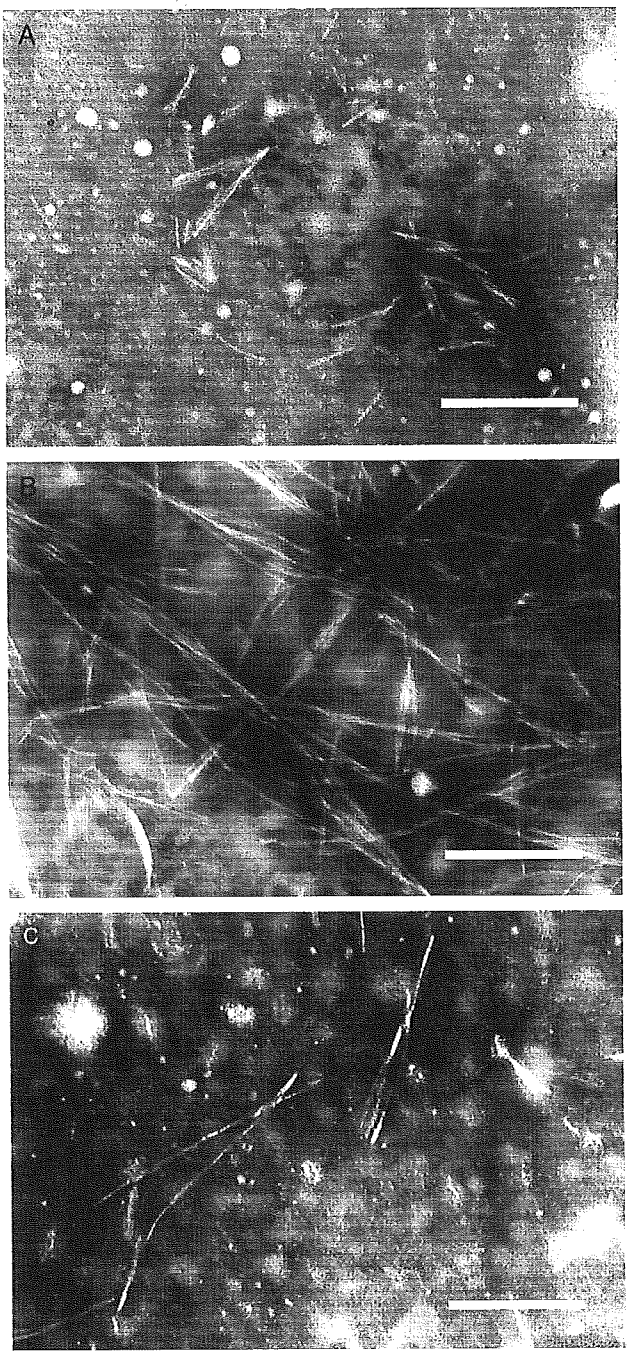

Fig. 3. Electron micrographs of extended fAβ(1–40). The reaction mixtures containing 10 μg/mL (2.3 μM) fAβ(1–40), 50 μM Aβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (B) or 50 μM Ibu (A,C), were incubated at 37 °C for 0 (A), or 6 h (B,C). Scale bars indicate a length of 250 nm.

As shown in Fig. 3B, clear fibril extension was observed by electron microscopy when fresh Aβ(1–40) was incubated with fAβ(1–40) at 37 °C. However, 50 μM Ibu completely inhibited the extension of sonicated fAβ(1–40) (Fig. 3A,C). Ibu inhibited the extension of fAβ(1–42) and other NSAIDs (Asp, Mec, Dic, Ket, Flu, Nap, SSide and Ind) also inhibited the extension of fAβ(1–40) and fAβ(1–42) (data not shown).

3.2. Effect of NSAIDs on the kinetics of fAβ destabilization

As shown in Fig. 4A–D, the fluorescence of ThT was almost unchanged during the incubation of fresh fAβ(1–40) or fAβ(1–42) at 37 °C without additional molecules. On the other hand, the ThT fluorescence decreased immediately after addition of NSAIDs to the reaction mixture. As shown in Fig. 4A–D, the ThT fluorescence of fAβ(1–40) and fAβ(1–42) decreased immediately after addition of Ibu, Ket, Nap, in a concentration-dependent manner. Other NSAIDs (Asp, Mec, Dic, Flu, SSide and Ind) also significantly destabilized fAβ(1–40) and fAβ(1–42) (Fig. 6C,D).

After incubation of 25 μM fresh fAβ(1–40) with 50 μM Ibu for 1 h, many short, sheared fibrils were observed (Fig. 5B). At 6 h, the number of fibrils was markedly decreased, and small amorphous aggregates were occasionally observed (Fig. 5C). Ibu destabilized fresh fAβ(1–42) and other NSAIDs (Asp, Mec, Dic, Ket, Flu, Nap, SSide and Ind) also destabilized preformed fAβ(1–40) and fAβ(1–42) (data not shown).

As shown in Fig. 6, statistical analysis revealed a significant difference in the potency of 50 μM NSAIDs for the anti-polymerization effect (Fig. 6A,B) and for the fibril destabilizing effect (Fig. 6C,D). In both the cases of fAβ(1–40) and fAβ(1–42), the potency of NSAIDs for the anti-polymerization effect was similar to that for the

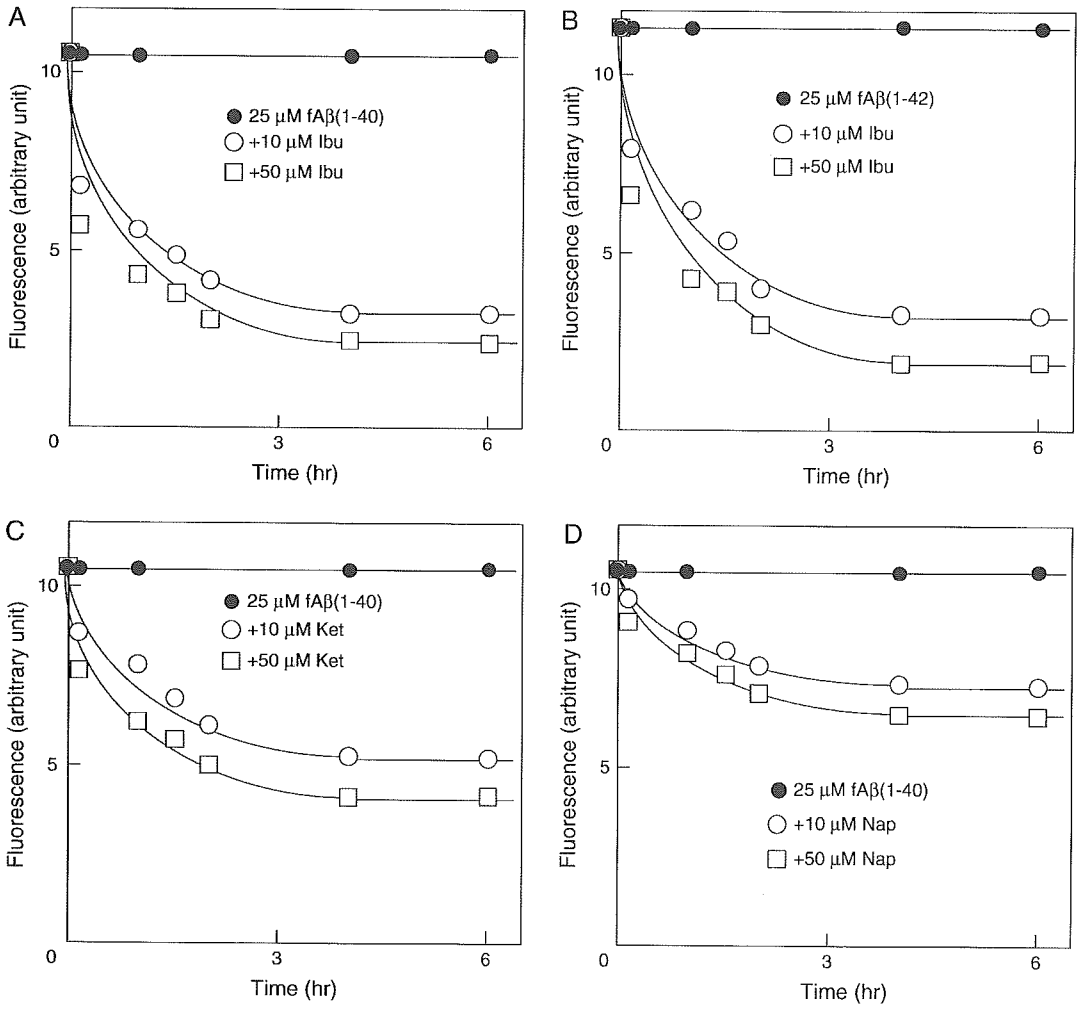

Fig. 4. Effects of Ibu (A,B), Ket (C), and Nap (D) on the kinetics of fAβ(1–40) (A,C,D) and fAβ(1–42) (B) destabilization. The reaction mixtures containing 25 μM fAβ(1–40) (A,C,D) or fAβ(1–42) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (●), 10 (○), or 50 μM (□) of Ibu (A,B), Ket (C), or Nap (D), were incubated at 37 °C for the indicated times. Each point represents the mean of three 5 μL aliquots from the same sample. Each figure is a representative pattern of three independent experiments.

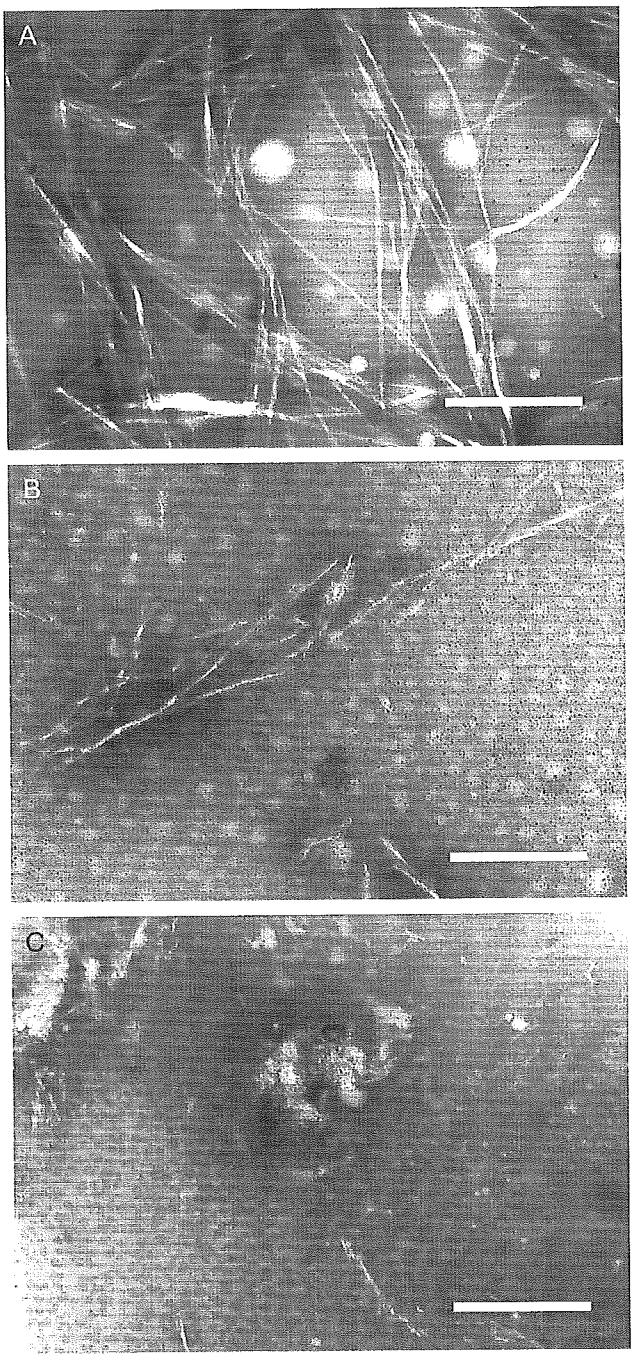

Fig. 5. Electron micrographs of destabilized fAβ(1–40). The reaction mixtures containing 25 μM fAβ(1–40), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 50 μM Ibu was incubated at 37 °C for 0 (A), 1 (B), or 6 h (C). Scale bars indicate a length of 250 nm.

fibril-destabilizing effect. Additionally, similar anti-polymerization and fibril-destabilizing effects were observed for both fAβs. The overall activity of the molecules examined was in the order of: Ibu ≈ SSide ≥ Mec > Asp ≈ Ket ≥ Flu ≈ Dic > Nap ≈ Ind. We finally compared the anti-amyloidogenic and fibril-destabilizing activity of SSide and Ibu with that of NDGA, Rif, and nicotine (Fig. 7). Although NDGA exhibited the potent anti-amyloidogenic and fibril-destabilizing activity dose-dependently, the activity of nicotine was weak or negligible. Rif, SSide and Ibu exhibited similar dose-dependent activity.

After incubation with 50 μM Ibu for 4 h, fAβ(1–40) and fAβ(1–42) were stained with Congo red much more weakly than fresh fAβ(1–40) and fAβ(1–42), but they all showed orange-green birefringence under polarized light. This means that a significant amount of intact fAβ(1–40) and fAβ(1–42) still remains in the mixture after the reaction, as shown in Fig. 5C. No proteins were detected by the Bradford assay in the supernatant after centrifugation at 4 °C for 2 h at 1.6×10^4 g. This implies that although Ibu could destabilize fAβ(1–40) and fAβ(1–42) to visible aggregates (Fig. 5C), they could not depolymerize fAβ(1–40) and fAβ(1–42) to monomers or oligomers of Aβ(1–40) and Aβ(1–42). When fresh 50 μM Aβ(1–40) or Aβ(1–42) was incubated with 10 μg/mL of the pellet at 37 °C, no increase in the fluorescence was observed for 6 h. This implies that destabilized fAβ(1–40) and fAβ(1–42) could not function as seeds.

4. Discussion

In this study, we demonstrated that various NSAIDs dose-dependently inhibited formation of fAβ from fresh Aβ(1–40) and Aβ(1–42), as well as their extension in vitro. Moreover, these NSAIDs dose-dependently destabilized preformed fAβs.

Thomas et al. (2001) suggested that the anti-aggregation effect of NSAIDs may be due to their interaction with epitope 3–6 and/or 25–35 in Aβ, which are considered crucial to Aβ aggregation and neurotoxicity. As shown in Fig. 2E, the extension of fAβ(1–40) followed a first-order kinetic model even in the presence of Ibu. The net rate of fAβ(1–40) extension is the sum of the rates of polymerization and depolymerization (Naiki and Nakakuki, 1996; Hasegawa et al., 2002). Thus, one possible explanation for the finding in Fig. 2E may be that Ibu bound to the ends of extending fAβ(1–40) and increased the rate of depolymerization by destabilizing the conformation of Aβ(1–40) which has just been incorporated into the fibril ends. Alternatively, Ibu would bind to Aβ(1–40) and consequently decrease the rate of polymerization. Tomiyama et al. (1996) suggested that Rif binds to Aβ by hydrophobic interactions between its lipophilic ansa chain and the hydrophobic region of Aβ, thus blocking associations between Aβ molecules leading to fAβ formation. The anti-amyloidogenic and fibril-destabilizing activity of tetracycline (Tc), small-molecule anionic sulfonates or sulfates, melatonin, β-sheet breaker peptides (iAβ5) and nicotine may also be related to the propensity to bind to the specific sites of Aβ (Kisilevsky et al., 1995; Soto et al., 1996; Pappolla et al., 1998; Forloni

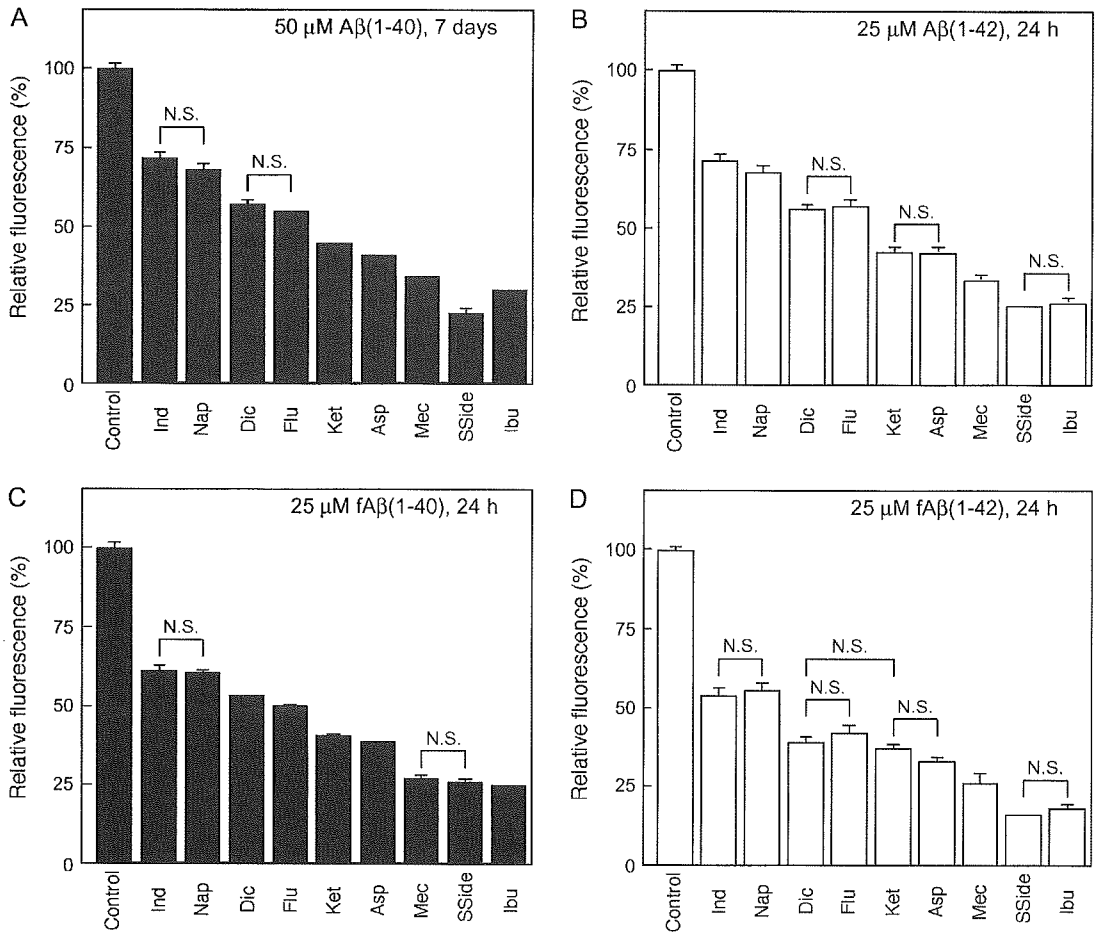

Fig. 6. Effects of NSAIDs on the formation of fAβ(1–40) (A) and fAβ(1–42) (B) from fresh Aβ(1–40) and Aβ(1–42), respectively, and the destabilization of fAβ(1–40) (C) and fAβ(1–42) (D). In (A) and (B), the reaction mixture containing 50 μM Aβ(1–40) or 25 μM Aβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 50 μM NSAIDs was incubated at 37 °C for 7 days and 24 h, respectively. In (C) and (D), the reaction mixture containing 25 μM fAβ(1–40) or fAβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 50 μM NSAIDs was incubated at 37 °C for 24 h. Each column represents the average of three independent experiments. SD is indicated by bars. N.S. denotes not significant. Significant difference ($P < 0.05$) was observed in all other combinations (one-way analysis of variance, post hoc test by Scheffe).

et al., 2001; Zeng et al., 2001). Thus, it may be reasonable to consider that NSAIDs could exhibit their anti-amyloidogenic and fibril-destabilizing effects by directly binding to Aβs and/or fAβs.

Taking into consideration the findings we obtained previously by systematic in vitro studies, we judged the overall activity of the anti-amyloidogenic molecules to be in the order of: tannic acid >> NDGA = wine-related polyphenols (myricetin, morin, quercetin) >> Rif = Tc > poly(vinylsulfonic acid, sodium salt) = 1,3-propanedisulfonic acid, disodium salt > iAβ5 > nicotine (Ono et al., 2002a,b, 2003, 2004a). In this study, we observed that the activity of SSide and Ibu is similar to that of Rif (Fig. 7).

Next, we considered the structure–activity relationships of NSAIDs and other anti-amyloidogenic compounds. The antioxidant compounds with hydroxyphenyl rings are suggested to bind specifically to Aβ and/or fAβ, inhibit fAβ formation and/or destabilize preformed fAβ (Ono et al., 2002b, 2003, 2004a,b; Li et al., 2004). On the other hand, the NSAIDs examined in this study have no hydroxyphenyl rings (Fig. 8). In general, these NSAIDs have an aromatic-based hydrophobic structure with some fused ring structures and methyl and/or carboxyl groups. This structure might be quite suitable for specifically binding to free Aβ and subsequently inhibiting the polymerization of Aβ into fAβ. Alternatively, this structure might be suitable for a specific binding to fAβ and subsequent destabilization of the β-sheet rich conformation of Aβ molecules in fAβ. Further studies, such as nuclear magnetic resonance experiments, are essential to reveal the exact structure–activity relationships for NSAIDs and other organic compounds that exhibit anti-amyloidogenic and fibril-destabilizing effects in vitro.

Clinically, Ibu has been used in the treatment of nerve-root compression pain. Although their penetration into the cerebrospinal fluid (CSF) is 0.9–1.5% of the plasma level, the CSF level of Ibu derivatives is the range 595–1241 ng h/mL in the patients taking oral

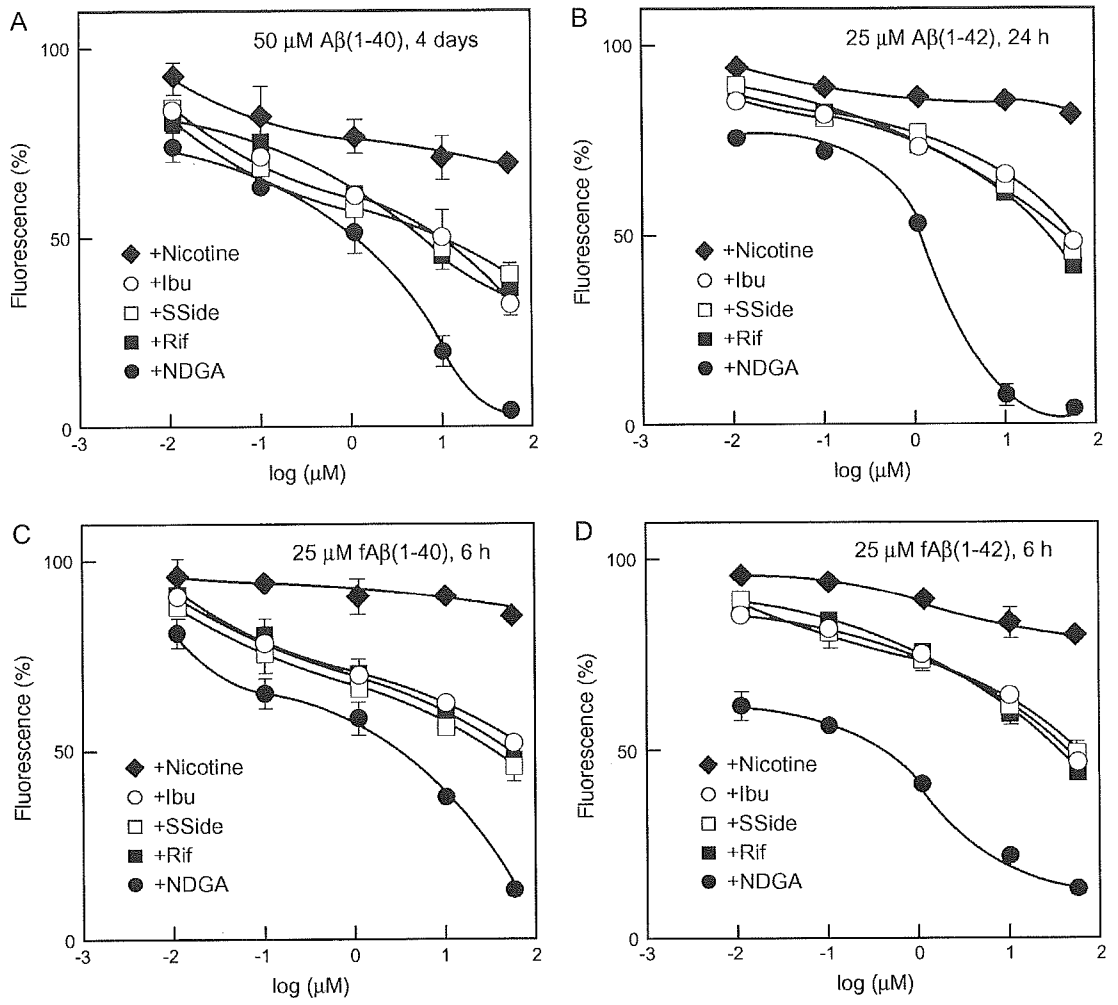

Fig. 7. Dose-dependent inhibition of the formation of fAβ(1–40) (A) and fAβ(1–42) (B) from fresh Aβ(1–40) and Aβ(1–42), respectively, and dose-dependent destabilization of fAβ(1–40) (C) and fAβ(1–42) (D). In (A) and (B), the reaction mixture containing 50 μM Aβ(1–40) or 25 μM Aβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM NDGA (●), Rif (■), SSide (□), Ibu (○), or nicotine (◆) were incubated at 37 °C for 4 days or 24 h, respectively. In (C) and (D), the reaction mixture containing 25 μM fAβ(1–40) or fAβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0, 0.01, 0.1, 1, 10 and 50 μM NDGA (●), Rif (■), SSide (□), Ibu (○), or nicotine (◆) were incubated at 37 °C for 6 h. Each point represents the average of three independent experiments. SD is indicated by bars. The average without compounds was regarded as 100%. Note that the final equilibrium levels in the presence of 50 μM SSide and Ibu were different from those in Fig. 6 because different lots of Aβ(1–40) and Aβ(1–42) (530108 and 530914, respectively) were used in these experiments.

doses of 800 mg Ibu (Bannwarth et al., 1995). This corresponds to 2884–6016 μM h of Ibu, which were higher than the concentration used in the present study. It is therefore conceivable that therapeutic doses of Ibu could provide a concentration sufficient to achieve the anti-amyloidogenic effects.

Recent epidemiological studies have revealed that long-term and/or short-term use of some NSAIDs may protect against AD (McGeer et al., 1996; in 't Veld et al., 1998, 2001; Akiyama et al., 2000). Our study and several reports on the effects of NSAIDs may well explain this correlation. First, the major therapeutic effect of NSAIDs is believed to be due to their inhibition of COX-1 and COX-2, leading to the suppression of prostaglandin synthesis and chronic neuroinflammation contributing to the protracted degenerative course of AD (McGeer and McGeer, 1995; Akiyama et al., 2000). Second, Weggen et al. (2001) recently suggested that a subset of NSAIDs including Ibu, SSide, Ind and *R*-Flu directly affect amyloid pathology in the brains of transgenic mouse by reducing the amount of soluble Aβ(1–42) peptide levels independently of COX activity. They also suggested that NSAIDs subtly alter γ-secretase activity without significantly perturbing other amyloid precursor protein (APP) pathways or Notch cleavage. Some NSAIDs were reported to lower Aβ(1–42) levels selectively both in cultured cell and broken cell assays, suggesting that these compounds interact directly with the γ-secretase complex itself that generates Aβ from human APP (Sagi et al., 2003; Eriksen et al., 2003). In addition, a subset of NSAIDs can reduce Aβ(1–42) through inhibition of Rho activity, which may regulate

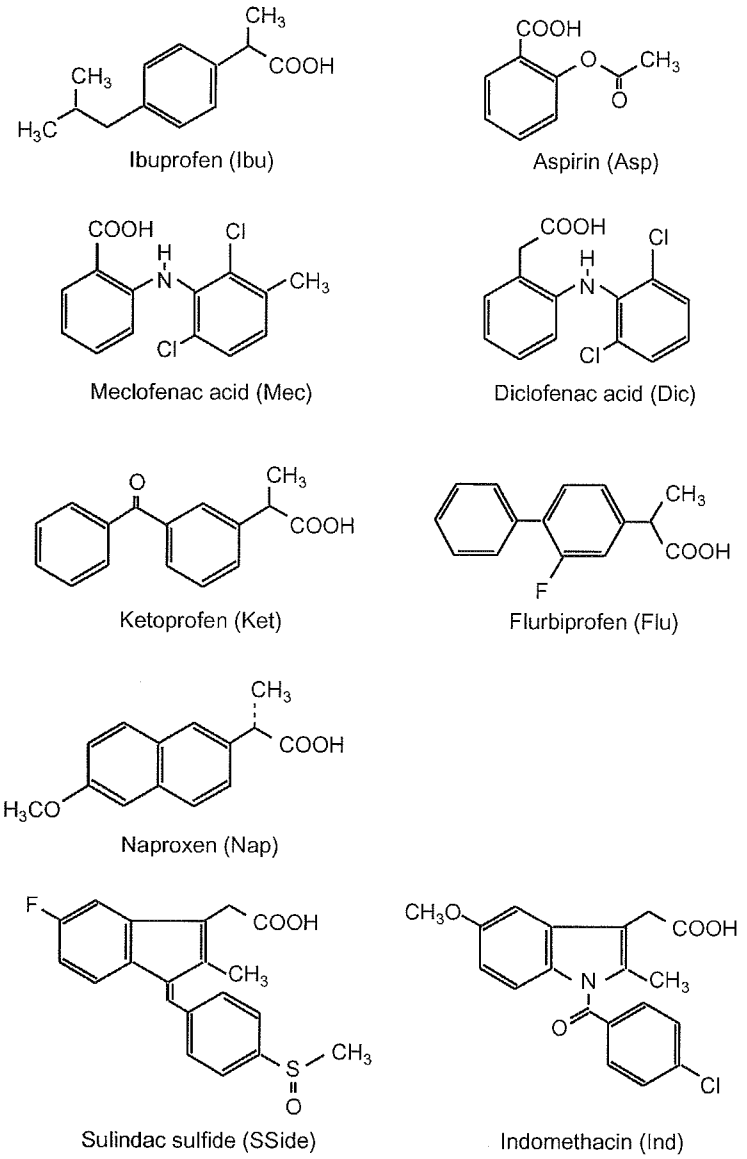

Fig. 8. Structures of NSAIDs examined in this study.

APP processing in a cell culture (Zhou et al., 2003). Finally, as shown in this paper, NSAIDs dose-dependently inhibit fAβ formation from fresh Aβ and their extension, as well as destabilize preformed fAβ in vitro. Thus, it may be reasonable to speculate that NSAIDs could prevent the development of AD, not only through suppressing chronic neuroinflammation and reducing the amount of soluble Aβ(1–42) peptide levels, but also through directly inhibiting the deposition of fAβ in the brain. In relation to the latter scenario, it may be essential to evaluate the effect of NSAIDs on the Aβ oligomer formation in a future study, because the Aβ oligomer has been reported to be the most neurotoxic form of all the Aβ aggregates (Gong et al., 2003). Yang et al. (2005) recently reported that curcumin prevented Aβ42 oligomer formation and toxicity between 0.1 and 1.0 μM, which is consistent with our EC_{50} values of curcumin for the anti-amyloidogenic and fibril-destabilizing activity (Ono et al., 2004b). Although the exact mechanisms of anti-amyloidogenic and fibril-destabilizing activity of NSAIDs are unclear, the careful and safe use of them could contribute the prevention and therapy of AD and other kinds of human amyloidosis.

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Cerebrospinal fluid of Alzheimer patients promotes β-amyloid fibril formation in vitro

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Cerebral deposition of amyloid β-peptide (Aβ) is an invariant feature of Alzheimer's disease (AD). To answer why soluble Aβ does not aggregate to β-amyloid fibrils (fAβ) in the brain of normal humans, we examined the influence of cerebrospinal fluid (CSF) obtained from AD and non-AD patients on the formation of fAβ(1–40) and fAβ(1–42) in vitro, by using fluorescence spectroscopy with thioflavin T and electron microscopy. Although the CSF obtained from both groups inhibited the formation of both fAβ(1–40) and fAβ(1–42), the CSF from non-AD patients inhibited the formation of fAβs more strongly than that from AD patients. In AD patients, the final levels of fAβs formation showed a significant negative correlation with the Aβ(1–42) level in CSF. These results indicate that fAβ deposition in the brain of AD may be enhanced by the decrease of specific inhibitory factors and/or by the increase of specific accelerating factors in CSF.

Keywords: Alzheimer's disease; CSF; β-amyloid fibrils; Thioflavin T; Electron microscopy

Introduction

Alzheimer's disease (AD) is the most common cause of progressive mental failure in aged humans and is a major cause of disability and death in the developed world (Ewbank, 1999). Two neuropathological lesions characterize the disease: the abundance of intraneuronal neurofibrillary tangles, composed of the tau protein, and the extracellular amyloid plaques, composed primarily of the amyloid β-peptide (Aβ) (Selkoe, 2001). The accumulation of Aβ is thought to cause the progression of AD (Kang et al., 1987). Aβ is a normal byproduct of cellular/neuronal metabolism and can be detected as a circulating peptide in the plasma and cerebrospinal fluid (CSF) of healthy humans (Haass et al., 1992). In AD, it has been postulated that increased production and/or decreased metabolism/clearance of Aβ may be primary events that lead to amyloid plaque deposition and subsequently to the cascade of other neuropathological changes associated with the disease (Du et al., 2003). In vitro studies using synthetic Aβ peptide(s) have shown that neurotoxicity is dependent on Aβ being fibrillar and predominantly present in a β-pleated sheet conformation (Beyreuther and Masters, 1997). Then what keeps soluble Aβ from aggregating to β-amyloid fibrils (fAβ) in normal humans? The specific factors accelerating fAβ formation may be increased in the CSF of AD patients, or the specific inhibitors of fAβ formation may be decreased in the CSF of AD patients. Recent studies including our reports showed that various molecules in CSF, such as apolipoprotein E (apoE) (Naiki et al., 1997, 1998), apolipoprotein J (apoJ) (Calero et al., 2000), serum amyloid P component (SAP) (Janciauskiene et al., 1995), transthyretin (TTR) (Schwarzman et al., 1994), α1-antichymotrypsin (ACT) (Eriksson et al., 1995), and α2-macroglobulin (α2M) (Du et al., 1998) inhibited fAβ formation in vitro. Very recently, Du et al. (2003) showed that naturally occurring human anti-Aβ antibodies block fAβ formation and prevent Aβ-induced neurotoxicity in vitro. On the other hand, apoE, especially apoE4 and ACT have been found to promote the assembly of Aβ into filaments in vitro, and the apoE4 allele to have a strong association with increased vascular and plaque Aβ deposits (Ma et al., 1994; Schmechel et al., 1993).

A nucleation-dependent polymerization model has been used to explain the mechanisms of fAβ formation in vitro (Jarrett and Lansbury, 1993; Lomakin et al., 1997; Naiki and Gejyo, 1999; Naiki et al., 1997). This model consists of two phases, i.e., nucleation and extension phases. Nucleus formation requires a series of association steps of monomers that are thermodynamically unfavorable, representing the rate-limiting step in amyloid fibril formation. Once the nucleus has been formed, further addition of monomers becomes thermodynamically favorable,

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resulting in the rapid extension of amyloid fibrils. We and other groups have independently developed a first-order kinetic model of fAβ extension in vitro and confirmed that extension of fAβ proceeds via the consecutive association of Aβ onto the end of existing fibrils (Esler et al., 1996; Lomakin et al., 1997; Naiki and Nakakuki, 1996). Although this model is based on the assumption that Aβ is monomeric in the reaction mixture, the protofibrils suggested by Walsh et al. (1997, 1999) and Hartley et al. (1999) would also be consistent with this model. A characteristic sigmoidal time-course curve of fAβ formation from Aβ at a physiological pH is widely believed to represent the essence of a nucleation-dependent polymerization model; i.e., the initial lag phase represents the thermodynamically unfavorable nucleus formation and the subsequent growing phase represents the extension reaction (Jarrett and Lansbury, 1993; Naiki and Gejyo, 1999; Naiki et al., 1997).

By using fluorescence spectroscopy with thioflavin T (ThT) and electron microscopy, we examined the effect of the CSF obtained from both AD and non-AD patients on fAβ(1–40) and fAβ(1–42) formation from fresh Aβs at pH 7.5 at 37°C in vitro. Additionally, we examined the degree of fAβ formation after incubation with AD-CSF in relation to the levels of Aβ(1–42), tau, total protein, and IgG in AD-CSF, as well as to the clinical course, mini-mental state examination (MMSE) score (Folstein et al., 1975), clinical dementia rating (CDR) (Hughes et al., 1982), and apoE phenotype of AD patients.

Material and methods

AD patients

We investigated 22 Japanese women and 18 Japanese men with a median age of 71.7 years (range; 60–86), who visited our clinics in 2000–2003 and were serially registered in this study without selection if their CSF was available. For the diagnosis of AD, patients had to fulfill the criteria of Diagnostic and Statistical Manual-IV and the NINCDS-ADRDA criteria published by McKhann et al. (1984). Patients with the genetic-linkage were excluded. Patients with mild cognitive impairment (CDR = 0.5) were included when they later fulfilled the AD criteria after progression. The clinical course was defined as the period from the onset to the lumbar puncture. The degree of dementia was expressed by the MMSE score (Folstein et al., 1975), and the severity by CDR (Hughes et al., 1982).

Non-AD patients

We investigated 17 Japanese women and 23 Japanese men with a median age of 70.1 years (range; 60–83), who visited our clinics in 2000–2003 and whose CSF was available. They were serially registered in this study without selection. They were diagnosed with cerebral infarction ($n = 1$), Parkinson's disease ($n = 1$), corticobasal degeneration ($n = 7$), progressive supranuclear palsy ($n = 3$), diffuse Lewy body disease ($n = 2$), Creutzfeldt–Jakob disease ($n = 1$), multiple system atrophy ($n = 2$), motor neuron disease ($n = 6$) including amyotrophic lateral sclerosis, multiple sclerosis ($n = 1$), myasthenia gravis ($n = 1$), meningitis ($n = 2$), muscle pain ($n = 3$), epilepsy ($n = 1$), hepatic encephalopathy ($n = 1$), syndrome of inappropriate secretion of antidiuretic hormone ($n = 1$), malignant lymphoma ($n = 1$), or peripheral neuropathy ($n = 6$).

CSF from AD and non-AD patients

The human subjects committee of our university hospital approved this study. CSF was taken by routine lumbar puncture within 1 month after first consult, after written informed consent was obtained from the patient or a family member. After CSF was collected and centrifuged at 1500 rpm for 10 min, the samples were aliquoted and stored at −80°C until analysis.

ApoE phenotype

ApoE phenotyping was done in 34 of 40 AD patients after written informed consent was obtained from the patient or a family member. The phenotype was determined from serum samples with isoelectric focusing and immunoblotting techniques by using commercial antibodies (Incstar, Stillwater, MN), as described by Kataoka et al. (1994) previously.

Enzyme-linked immunosorbent assay of Aβ(1–42) and tau

The levels of Aβ(1–42) in CSF were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using a monoclonal antibody (Mab), 21F12 specific for the C-terminus of Aβ(1–42) as the capturing agent, and a biotinylated monoclonal anti-Aβ(1–42) N-terminal antibody, 3D6 for detection [INNOTEST β-amyloid(1–42); Innogenetics, Gent, Belgium] (Andreasen et al., 1999; Vanderstichele et al., 1998). There was no cross-reactivity with Aβ(1–40). The CSF samples and the standards were assayed in duplicate.

The total tau concentration in CSF was determined by a sensitive sandwich ELISA using a Mab, AT120, as the capturing antibody, and 2 Mabs, HT7 and BT2, as detection antibodies, recognizing different epitopes (INNOTEST hTAU-ag; Innogenetics, Gent, Belgium) (Blennow et al., 1995; Vandermeeren et al., 1993). The CSF samples and the standards were assayed in duplicate.

Polymerization assay

Aβ(1–40) (lot number 530108, Peptide Institute, Inc., Osaka, Japan) and Aβ(1–42) (lot number 521205, Peptide Institute) were dissolved by brief vortexing in a 0.02% ammonia solution at a concentration of 500 μM (2.2 mg/mL) and 250 μM, respectively, in a 4°C room and stored at −80°C before assaying [fresh Aβ(1–40) and Aβ(1–42) solutions].

The polymerization assay was performed as described elsewhere (Naiki et al., 1998). Briefly, the reaction mixture contained 50 μM Aβ(1–40) or 25 μM Aβ(1–42), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 or 78% (vol/vol) CSF. Thirty-μL aliquots of the mixture were put into oil-free PCR tubes (size: 0.5 mL, code number: 9046; Takara Shuzo Co. Ltd., Otsu, Japan). The reaction tubes were put into a DNA thermal cycler (PJ480, Perkin Elmer Cetus, Emeryville, California). Starting at 4°C, the plate temperature was elevated at maximal speed, to 37°C. Then, they were incubated for 0–9 days as indicated in each figure, and the reaction was stopped by placing the tubes on ice. The reaction tubes were not agitated. From each reaction tube, triplicate 5-μL aliquots were removed, then subjected to fluorescence spectroscopy, and the mean of each triplicate was determined.

Fluorescence spectroscopy, electron microscopy, and polarized light microscopy

A fluorescence spectroscopic study was performed on a Hitachi F-2500 fluorescence spectrophotometer as described elsewhere (Naiki and Nakakuki, 1996). Optimum fluorescence of fAβ(1–40) and fAβ(1–42) was measured at the excitation and emission wavelengths of 445 and 490 nm, respectively, with the reaction mixture containing 5 μM ThT (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mM of glycine-NaOH buffer, pH 8.5. Electron microscopy and polarized light microscopy of the reaction mixtures were performed as described elsewhere (Hasegawa et al., 1999).

Other analytical procedures

The total protein concentration in CSF was measured by using the color reaction with pyrocatechol violet–molybdenum (VI) complex (Sysmex Co., Kobe, Japan) (Fujita et al., 1984). The IgG level in CSF was determined by the latex agglutination photometric immunoassay (Eiken Chemical Co., Ltd., Tokyo, Japan) (Fritz and Rivers, 1972).

Statistical analysis

The comparison of ThT fluorescence level between AD and non-AD patients in the study population was based on unpaired *t* test with Welch's correction. Multiple comparisons are made between the final level of fAβ formation and the levels of Aβ(1–42), tau, total protein, IgG in CSF, and the clinical course by Stepwise regression. Correlation analysis was performed according to Pearson's or Spearman correlation test. Pearson's correlation coefficients were calculated to determine the relationship between the final level of fAβ formation and the levels of Aβ(1–42) in CSF. Spearman correlation coefficients were calculated to determine the relationship between the final level of fAβ formation and MMSE score, apoE phenotype. Association between the final level of fAβ formation and CDR, as well as association between the levels of Aβ(1–42) in CSF and CDR were analyzed with the Kruskal–Wallis test. A P value less than 0.05 was considered significant.

Results

Inhibitory effects of CSF obtained from AD and non-AD patients on the formation of fAβ from fresh Aβ

As shown in Figs. 1A and B, the fluorescence of ThT after incubation of fresh Aβ(1–40) or Aβ(1–42) at 37°C showed a characteristic sigmoidal curve. This curve is consistent with the nucleation-dependent polymerization model (Jarrett and Lansbury, 1993; Naiki et al., 1997). fAβ(1–40) and fAβ(1–42) stained with Congo red showed typical orange–green birefringence under polarized light (data not shown). The CSF obtained from both AD and non-AD patients decreased the final levels of fAβ(1–40) formation from fresh Aβ(1–40) (Fig. 1A). A similar inhibitory effect of CSF was observed on fAβ(1–42) formation (Fig. 1B). Moreover, the CSF from non-AD patients (non-AD-CSF) inhibited the formation of both fAβs more strongly than did the CSF from AD patients (AD-CSF) (Figs. 1A and B). We confirmed that the

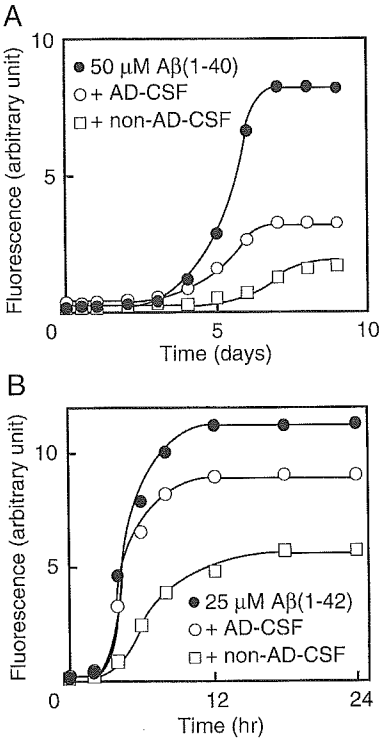

Fig. 1. Effects of the CSF obtained from AD patients and non-AD patients on the kinetics of fAβ(1–40) (A) and fAβ(1–42) formation (B) from fresh Aβ(1–40) and Aβ(1–42), respectively. The reaction mixtures containing 50 μM Aβ(1–40) (A) or 25 μM Aβ(142) (B), 50 mM phosphate buffer, pH 7.5, 100 mM NaCl, and 0 (□) (n = 10) or 78% (vol/vol) CSF obtained from AD (○) (n = 40) or non-AD patients (□) (n = 40), were incubated at 37°C for the indicated times. Each symbol represents the mean value. At all points, standard errors are within the diameter of symbols. Each figure is a representative pattern of 3 independent experiments.

reaction mixtures containing AD- or non-AD-CSF with no Aβs exhibited no significant ThT fluorescence (0.04 ± 0.01 vs. 0.03 ± 0.04 for AD- and non-AD-CSF, respectively; mean ± SD, n = 3 each, the same arbitrary unit as in Fig. 1).

As shown in Fig. 2A, incubation with AD-CSF gave a significantly higher final level of fAβ(1–40) formation than that with non-AD-CSF [3.25 ± 1.04 vs. 1.63 ± 0.27 (mean ± SD), P < 0.001]. Similarly, incubation with AD-CSF gave a significantly higher final level of fAβ(1–42) formation than that with non-AD-CSF (9.00 ± 1.55 vs. 5.69 ± 1.02, P < 0.001) (Fig. 2B). In both cases, incubation with AD-CSF gave a significantly lower final levels of fAβ(1–40) and fAβ(1–42) formation than the incubation without CSF [3.25 ± 1.04 vs. 8.19 ± 0.16 in fAβ(1–40) formation, 9.00 ± 1.55 vs. 11.25 ± 0.86 in fAβ(1–42) formation, P < 0.001 in both cases].

Incubation of fresh Aβ(1–40) without CSF gave clear fibril formation electron-microscopically (Fig. 3A). fAβ formed from fresh Aβ(1–40) assumed the nonbranched, helical filament structure of approximately 7 nm width and exhibited a helical periodicity of approximately 220 nm, as described previously (Naiki and Nakakuki, 1996). Protofibrils with a diameter of about 4 nm (Harper et al., 1999) were not observed in the reaction mixture. On the other hand, many shorter, sheared fibrils were observed after incubation with the CSF obtained from an AD patient (Figs. 2 and 3B). Fibrils were rarely found