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Nordihydroguaiaretic Acid Does Not Disaggregate β-Amyloid(1–40) Protofibrils but Does Inhibit Growth Arising from Direct Protofibril Association

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ABSTRACT

Nordihydroguaiaretic acid (NDGA) was observed by Ono et al. (*J Neurochem* **87:**172–181, 2002) to decrease the fluorescence of thioflavin T associated with freshly extended amyloid β-protein (Aβ) fibrils. They concluded that NDGA could disaggregate Aβ fibrils into aggregates that were larger than monomers or oligomers and did not bind thioflavin T. Such an effect could be of therapeutic importance in the treatment of Alzheimer's disease. In the current study, we confirmed that NDGA induces a decrease in the fluorescence of thioflavin T associated with Aβ(1–40) fibrils and extended this observation to Aβ(1–40) protofibrils. However, attempts to identify protofibril disaggregation products using dynamic light scattering, electron microscopy, and size exclusion chromatography failed to demonstrate any decrease in aggregate size or concentration or a parallel increase in Aβ monomers or small oligomers when protofibrils were incubated with excess NDGA. We propose instead that the decreases in thioflavin T fluorescence resulted from either displacement or conformational alteration of thioflavin T upon the binding of NDGA to these aggregates. In fact, the same equilibrium fluorescence values were observed regardless of the order in which NDGA, thioflavin T, and Aβ protofibrils were added to the incubation. Although NDGA failed to disaggregate Aβ protofibrils, it did inhibit direct protofibril-protofibril association but did not alter protofibril elongation by monomer addition. These results suggest that NDGA might bind along the lateral surface of Aβ protofibrils. In addition, the binding of NDGA to Aβ protofibrils increased their nonspecific adherence to Superdex 75 resin and diminished their effects on cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Alzheimer's disease (AD) is characterized by the presence of senile plaques in the brain. These plaques are composed primarily of the fibrillar form of the amyloid β protein (Aβ). Although the relationship between amyloid plaques and AD remains unclear, multiple lines of evidence suggest a role for Aβ in disease progression. Mutations that give rise to inherited forms of early onset AD, which occur in both the amyloid precursor protein and the presenilin genes (PS1 and PS2), all result in an elevated production of Aβ or an increase in the relative amount of the longer, more fibrillogenic form of Aβ (Duff et al., 1996; Hsiao et al., 1996). Overexpression of these mutant genes in transgenic mice causes an age-dependent development of Aβ fibril deposition (Hsiao et al., 1996; Kawarabayashi et al., 2001). Furthermore, aged Aβ preparations that have had the opportunity to aggregate into fibrils in vitro evoke neurotoxicity in culture, whereas freshly dissolved, monomeric Aβ is inert (Yankner, 1996a). These observations have contributed to a growing support for the amyloid hypothesis, which proposes that accumulation of fibrillar Aβ in the brain initiates a cascade of events that results in neuronal cell death and leads to cognitive decline (Yankner, 1996b). Therefore, the development of compounds that inhibit Aβ fibrillogenesis has emerged as one therapeutic strategy for AD.

The primary components of senile plaques are 40- and 42-residue peptides, denoted Aβ(1–40) and Aβ(1–42) (Glenner and Wong, 1984; Miller et al., 1993). The self-association of synthetic Aβ monomers to fibrils in vitro has provided an assay to identify compounds that inhibit fibril formation. Among these compounds are nordihydroguaiaretic acid (NDGA) (Naiki et al., 1998), nicotine (Salomon et al., 1996; Ono et al., 2002a), melatonin (Pappolla et al., 1998), laminin (Monji et al., 1999; Castillo et al., 2000), tetracycline (Forloni et al., 2001), sulfonated dyes (Pollack et al., 1995), the rifa-

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ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid β-protein; NDGA, nordihydroguaiaretic acid; DLS, dynamic light scattering; EM, electron microscopy; SEC, size exclusion chromatograph; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; R_H, hydrodynamic radius.

mycin series (Tomiyama et al., 1996), porphyrins (Howlett et al., 1997), benzofurans (Howlett et al., 1999), monoclonal antibodies raised against the Aβ peptide (Solomon et al., 1996), and peptide fragments homologous to the hydrophobic region of Aβ (Ghanta et al., 1996; Soto et al., 1996; Tjernberg et al., 1996; Findeis et al., 1999). Some of these compounds have also been reported to disassemble preformed Aβ aggregates (Solomon et al., 1997; Forloni et al., 2001; Kiuchi et al., 2002; Ono et al., 2002a,b). In particular, Naiki and colleagues presented evidence that NDGA (structure in Fig. 1A) inhibited Aβ fibril formation (Naiki et al., 1998) and, in a later study, found that NDGA disaggregated preformed Aβ fibrils more effectively than other compounds (Ono et al., 2002b). The primary evidence for this finding involved thioflavin T (structure in Fig. 1B), a fluorophore that shows greatly enhanced fluorescence on binding to amyloid fibrils (LeVine, 1993). A significant decrease in thioflavin T fluorescence was observed when freshly extended Aβ fibrils were incubated in the presence of excess NDGA. The authors speculated that NDGA was breaking Aβ fibrils into smaller aggregates, but the mechanism of this fibril-breaking activity and the size of the resulting aggregates were not determined.

The potential importance of these results prompted us to examine the effect of NDGA on processes of Aβ protofibril formation and growth that we have defined previously (Nichols et al., 2002) and to explore more specifically changes in Aβ protofibril size. Dynamic light scattering (DLS), electron microscopy (EM), and size exclusion chromatography (SEC) were used to probe specifically for decreases in Aβ protofibril size and concentration, as well as parallel increases in Aβ monomers or oligomers, in the presence of NDGA. Furthermore, this study investigates the consequences that NDGA binding to Aβ protofibrils might have on cellular activity as well as protofibril growth.

Materials and Methods

Materials. The 40-residue Aβ(1–40) peptide was obtained from QCB (Hopkinton, MA). [^{3}H]HCHO (80–85.5 mCi/mmol) and scintillation cocktail (Ultima Gold) were from PerkinElmer Life Sciences (Boston, MA). Nordihydroguaiaretic acid (NDGA), bovine serum albumin, horse serum, and thioflavin T were from Sigma (St. Louis, MO). RPMI 1640 media, fetal bovine serum, penicillin/streptomycin/glutamine, and phosphate-buffered saline were from Invitrogen (Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Molecular Probes (Eugene, OR).

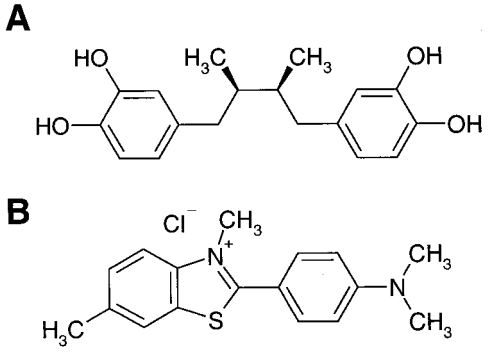

Fig. 1. A, structure of nordihydroguaiaretic acid (1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane). B, structure of thioflavin T (2-(4-dimethylamino-phenyl)-3,6-dimethyl-benzothiazol-3-ium chloride).

Preparation of Aβ Peptides. Aβ(1–40) was obtained in lyophilized form and stored at −20°C desiccated until reconstitution in deionized water (Nichols et al., 2002). Before use in aggregation or elongation assays, any pre-existing aggregates were removed by size exclusion chromatography (SEC) on a 1 × 30-cm Superdex 75 HR 10/30 column (Amersham Biosciences, Piscataway, NJ). For some experiments, Aβ(1–40) was radiolabeled by reductive methylation with [^{3}H]HCHO and $NaCNBH_3$ under conditions that insure full methylation with two methyl groups at each of the three primary amines in the peptide (Nichols et al., 2002), and the radiomethylated Aβ was purified by SEC (specific activity, 245-1060 dpm/pmol). Concentrations of low molecular weight Aβ obtained from SEC were determined with an extinction coefficient of 1450 cm^{-1} M^{-1} at 276 nm (Nichols et al., 2002). The monomeric nature of this Aβ was confirmed by multiangle light scattering in tandem with SEC. This analysis was conducted with radiomethylated Aβ for better quantitation and indicated a molecular weight of 4670 ± 90 (calculated molecular weight was 4415 for the hexamethylated 1–40 peptide) (M. R. Nichols, M. A. Moss, D. K. Reed, J. H. Hoh, and T. L. Rosenberry, submitted for publication). The monomeric assignment was in agreement with translational diffusion measurements by NMR (Tseng et al., 1999).

Fluorescence Determinations of the Binding of Thioflavin T to Aβ Amyloid Aggregates. Thioflavin T fluorescence measurements were made as described previously (LeVine, 1993; Walsh et al., 1999). Fluorescence of solutions containing Aβ and thioflavin T was monitored at 23°C on an LS-50B luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA) with excitation at 450 nm, emission at 470 to 500 nm, and slits of 10 nm (Nichols et al., 2002). The area under the emission curve was obtained by integration and expressed as area units. Time drive experiments were conducted in a similar fashion with continuous monitoring of 480 nm fluorescence emission. Fluorescence values (F) are reported as the fluorescence relative to control samples containing no NDGA.

Preparation of Aβ(1–40) Protofibrils and Fibrils. Aβ(1–40) protofibrils and fibrils were prepared as described previously (Nichols et al., 2002). In brief, unlabeled or radiomethylated Aβ(1–40) monomer (final concentration, 70–120 μM), freshly isolated by SEC on Superdex 75 in 0.5 to 1 ml of 5 or 50 mM Tris-HCl, 5 mM EDTA-NaOH, pH 8.0 (denoted 5 or 50 mM Tris-EDTA) at room temperature, was agitated vigorously by continuous vortexing to promote aggregation in the presence of 0 to 150 mM NaCl. Aggregation was monitored by thioflavin T fluorescence until a fluorescence increase, estimated to be 20 to 100% of the maximum final fluorescence, was observed. The sample was spun for 10 min in a tabletop Microfuge (Beckman Coulter, Fullerton, CA) at 18,000*g*. The pellet obtained from this centrifugation was defined as the fibril fraction. The supernatant was fractionated by SEC on Superdex 75, and Aβ eluting in the void volume was defined as the protofibril fraction. Unlabeled fibril and protofibril concentrations were estimated by UV absorbance at 280 nm corrected for light scattering as described previously (Nichols et al., 2002). Protofibril and fibril concentrations are expressed in monomer concentration units. Fibrils and protofibrils were used immediately or stored at 4°C for up to several days. This storage had no apparent effects on fibril or protofibril behavior in the assays discussed below.

Preparation of NDGA. NDGA was dissolved at 100 mM in DMSO and stored at −20°C. Stock solutions of NDGA were diluted to 1 mM in 10% DMSO and 5 or 50 mM Tris-EDTA or 50 mM Tris-HCl, pH 8.0. For light-scattering experiments, 1 mM solutions were passed through a 0.22-μm polyethersulfone membrane filter to yield a clean light-scattering signal. The concentration of filtered NDGA solutions was determined using an extinction coefficient of 5800 cm^{-1} M^{-1} at 281 nm (estimated from NDGA concentrations measured by dry weight). Reactions with diluted NDGA contained no greater than 1% DMSO, and control reactions contained equivalent amounts of DMSO.

Protofibril Association Assay. Aβ(1–40) protofibrils isolated on Superdex 75 were diluted to 2 to 4 μM in 5 or 50 mM Tris-EDTA with 0 to 100 μM filtered NDGA and incubated without agitation at room temperature for 30 min. Concentrated NaCl was added such that final reaction concentrations were 1 to 2 μM protofibril, 0 to 50 μM NDGA, and 150 mM NaCl. Association reactions were incubated without agitation at room temperature, and DLS intensity and aggregate size as measured by the intensity averaged hydrodynamic radius (R_H) were monitored as described previously (Nichols et al., 2002) with a DynaPro MSX instrument (Protein Solutions, Inc., Piscataway, NJ).

Protofibril Elongation Assay. Isolated Aβ(1–40) protofibrils were diluted to a final concentration of 4 μM in 5 or 50 mM Tris-EDTA with 0 to 260 μM filtered NDGA and incubated without agitation at room temperature for 30 min. Elongation was induced by addition of freshly isolated Aβ(1–40) monomer such that final concentrations were 2 μM protofibril, 0 to 130 μM NDGA, and 20 μM monomer. Elongation reactions were incubated without agitation at room temperature, and light scattering intensity and aggregate size were followed using DLS.

Protofibril Disaggregation Assay. Aβ(1–40) protofibrils isolated on Superdex 75 were diluted to 2 μM in 50 mM Tris-EDTA with 30 μM freshly isolated Aβ(1–40) monomer, 100 mM NaCl, and 5 μM thioflavin T and incubated without agitation at room temperature for several days. To test the ability of NDGA to induce disaggregation, these elongated protofibrils were diluted by a factor of 2 into 0 to 100 μM NDGA with 100 mM NaCl and 5 μM thioflavin T. The reactions were incubated without agitation at room temperature, and thioflavin T fluorescence was continuously monitored in situ. Single fluorescence values reported for a given NDGA concentration are those observed at a constant final steady state. To monitor aggregate size, Aβ(1–40) protofibrils isolated on Superdex 75 were diluted to 5 μM in 50 mM Tris-HCl, pH 8.0, with or without 50 μM filtered NDGA. Reactions were incubated without agitation at room temperature, and light scattering intensity and aggregate size were followed using DLS. Reactions were also monitored by fluorescence after periodic dilution of aliquots into 5 μM thioflavin T.

Electron Microscopy (EM). Sample aliquots were applied to 200 mesh Formvar-coated copper grids (Ernest F. Fullam, Inc., Latham, NY) as described previously (Nichols et al., 2002). Samples were fixed using 0.5% glutaraldehyde (Sigma), washed, stained with 2% uranyl acetate (Polysciences, Inc., Warrington, PA), and air-dried. Grids were visualized in a Philips EM208S transmission electron microscope.

MTT Reduction Assay. Rat PC-12 pheochromocytoma cells were grown on polystyrene 75-cm^2 tissue culture flasks (Corning Glassworks, Corning, NY) in RPMI medium supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml glutamine. Cells were maintained in a humidified incubator that provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37°C. The MTT reduction assay was performed as described previously (Moss et al., 2003). In brief, cells were plated onto 96-well flat-bottomed tissue culture-treated plates (Costar, Cambridge, MA) at a density of approximately 6000 cells/well (100 μl/well) and incubated at 37°C for 24 h. Aβ(1–40) preparations in 50 mM Tris-HCl, pH 8.0, were diluted to a final concentration of 200 nM in cell culture media without phenol red and added to wells. Each experiment included a positive control containing Aβ(1–40) alone and a negative control containing medium and buffer, as well as samples containing both Aβ(1–40) and NDGA. Each treatment was performed in six replications, and values are presented as the mean ± S.E. Plates were incubated with Aβ(1–40) at 37°C for 24 h, MTT was added at 0.5 mg/ml for 4 h, and 10% SDS in 0.01 M HCl was added to solubilize the formazan product. The absorbance of the formazan product of MTT reduction was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA) with background subtraction. Values were reported as percentage negative control [(100%)(Abs$_{sample}$ − Abs$_{background}$)/(Abs$_{negative\ control}$ − Abs$_{background}$)].

Results

Incubation of Aβ Protofibrils with NDGA Decreased Thioflavin T Fluorescence. Ono et al. (2002b) previously demonstrated that sonicated Aβ fibrils extended with freshly dissolved Aβ and incubated in the presence of NDGA exhibited a substantial decrease in thioflavin T fluorescence. To replicate these results, we generated Aβ(1–40) protofibrils and elongated them via addition of freshly isolated Aβ(1–40) monomer. The elongated protofibril product was then incubated with excess NDGA in the presence of thioflavin T. As shown in Fig. 2A, the thioflavin T fluorescence initially decreased rapidly and eventually reached a steady-state value. In contrast, elongated protofibrils incubated without NDGA exhibited no change in the thioflavin T fluorescence signal. The extent of the decrease in thioflavin T fluorescence depended on the NDGA concentration, with greater decreases observed at higher NDGA concentrations (Fig. 2B). At comparable ratios of NDGA to Aβ, similar decreases in thioflavin T fluorescence were observed for purified Aβ(1–40) protofibrils that had not been elongated, Aβ(1–40) fibrils, and un-

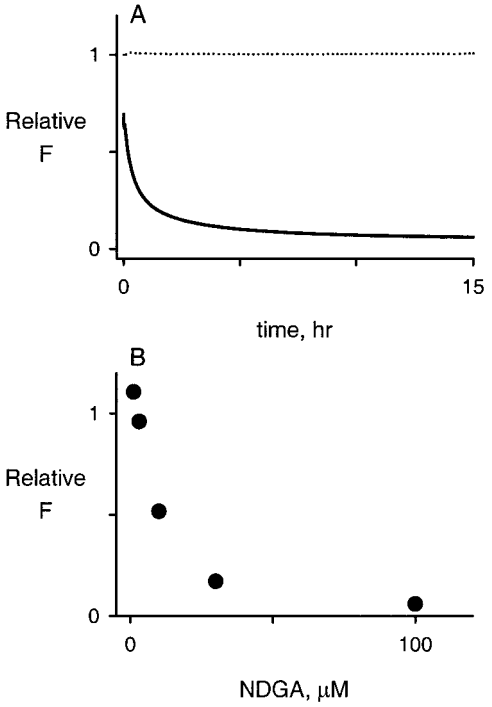

Fig. 2. Elongated Aβ protofibrils exhibited a decrease in thioflavin T fluorescence when incubated with excess NDGA. Isolated Aβ(1–40) protofibrils were diluted to 2 μM in 50 mM Tris-EDTA and 100 mM NaCl and elongated at room temperature in the presence of 30 μM Aβ(1–40) monomer for 1 day (B) or 8 days (A). The elongated protofibrils were diluted 2-fold and incubated with 0 to 100 μM NDGA at room temperature until a constant fluorescence value was reached. Thioflavin T fluorescence (F) was monitored in situ by inclusion of 5 μM thioflavin T in the reaction mixture and is expressed as the fluorescence relative to control samples containing no NDGA. When NDGA and thioflavin T were incubated together and in the absence of Aβ(1–40) protofibrils, fluorescence did not exceed background levels. A, time course for thioflavin T fluorescence in the absence (Control, dashed line) or presence of 100 μM NDGA (solid line). Results are representative of nine experiments. B, the effect of NDGA on the final steady-state thioflavin T fluorescence values was dose-dependent. Results are representative of three experiments.

purified Aβ(1–40) aggregation reactions that contained a mixture of Aβ(1–40) monomer, protofibril, and fibril (data not shown). Thus, our results replicated the observations of Ono et al. (2002b) and extended these observations to Aβ protofibrils.

NDGA Failed to Reduce Aβ Protofibril Size or Concentration. As suggested by Ono et al. (2002b), the observed decrease in thioflavin T fluorescence in the presence of excess NDGA might indicate a decrease in Aβ(1–40) protofibril concentration resulting from disaggregation into monomers or small oligomers that do not bind thioflavin T. To investigate this possibility, Aβ(1–40) protofibrils were incubated in the presence of excess NDGA and changes in aggregate size and concentration were monitored by DLS (Fig. 3). The Aβ protofibrils used in these experiments were isolated by SEC and previously shown by atomic force microscopy to consist of either globular or short rigid rod (<1 μm length) structures with a peak height of about 3 nm (Nichols et al., 2002). The R_H value for the protofibrils in Fig. 3 was approximately 155 nm. This value was slightly higher than values observed previously (Nichols et al., 2002) because a low concentration of NaCl was present during this protofibril preparation. These protofibrils were incubated in the absence of Aβ monomer to maintain their initial size, and both R_H and the light scattering intensity (LS) remained unchanged over a period of 45 h (Fig. 3, B and C). A similar trend was observed for Aβ(1–40) protofibrils incubated without NDGA. Concurrent thioflavin T fluorescence for Aβ(1–40) protofibrils incubated in the presence of NDGA decreased by more than 80%, whereas the fluorescence for control protofibrils remained relatively unchanged (Fig. 3A). Thus, DLS results indicated that the decrease in thioflavin T fluorescence was not accompanied by a decrease in aggregate size or concentration. This trend was not unique to Aβ(1–40). Incubation of NDGA with insulin fibrils also resulted in a decrease in thioflavin T fluorescence, yet light scattering intensity was unchanged and the fibrils remained sedimentable (data not shown).

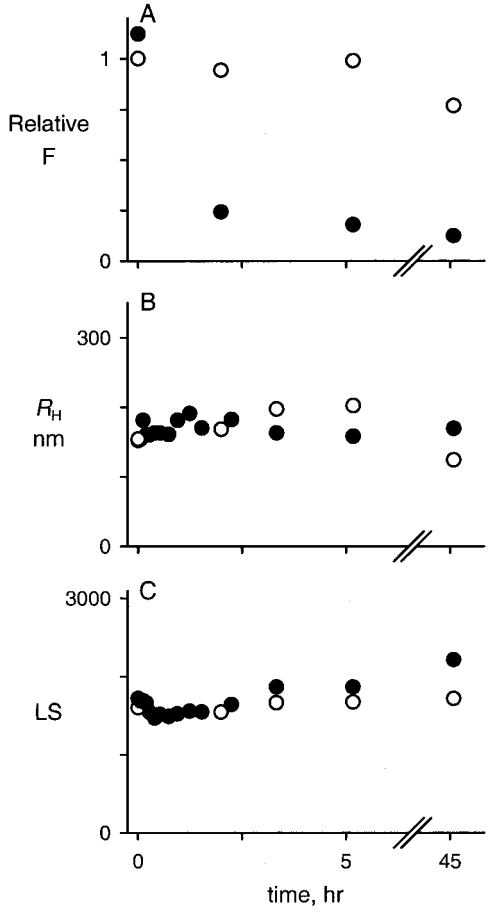

Fig. 3. NDGA failed to reduce Aβ protofibril size or concentration. Isolated Aβ(1–40) protofibrils were diluted to 5 μM in 50 mM Tris-HCl, pH 8.0, and incubated alone (control, ○) or with 50 μM NDGA (●) at room temperature. Aliquots were periodically diluted into 5 μM thioflavin T for fluorescence (F) measurements, expressed here relative to the initial control value (A). Changes in intensity-averaged R_H (B) and light scattering intensity (LS) (C) were monitored by DLS.

To corroborate DLS results with a second analysis technique, EM images were obtained. Isolated Aβ(1–40) protofibrils were incubated in the presence and absence of NDGA, and monomer was added to allow elongation to proceed. As shown in Fig. 4, NDGA failed to induce any change in the morphology of elongated Aβ protofibrils. EM images of these samples displayed a network of filamentous or ribbonlike structures composed of both single and multiple longitudinal strands. These structures were also comparable with those previously observed for elongated Aβ(1–40) protofibrils, which displayed long wispy tendrils extending to lengths of several micrometers (Nichols et al., 2002). In addition, no changes in aggregate size or morphology were noted when Aβ(1–40) protofibrils were elongated via monomer addition before incubation with excess NDGA.

Incubation of Aβ Protofibrils with NDGA Failed to Increase Monomer Content. Although DLS and EM results indicated that Aβ protofibril size and concentration were unchanged after incubation with excess NDGA, these techniques cannot directly assess Aβ monomer content. If Aβ(1–40) protofibrils were disaggregated in the presence of NDGA, a parallel increase in the quantity of Aβ(1–40) monomer would be anticipated. To probe for any increase in monomer composition induced by NDGA, Aβ(1–40) protofibrils were radiomethylated to facilitate quantitation of Aβ after chromatography. Unlabeled and radiomethylated Aβ(1–40) have been found to react similarly in monomer aggregation and protofibril growth and stability assays, except that rates of aggregation and growth are slower with the radiomethylated Aβ (Nichols et al., 2002) and radiomethylated protofi-

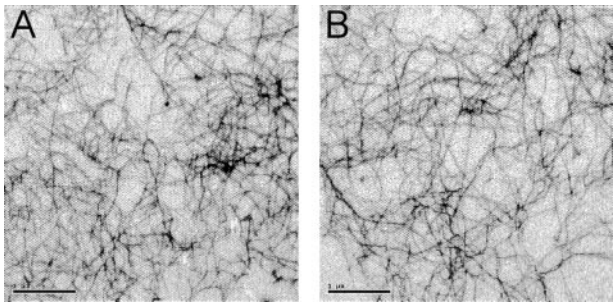

Fig. 4. NDGA failed to alter Aβ protofibril size or morphology. Aβ(1–40) protofibrils isolated on Superdex 75 were diluted to 4 μM in 5 mM Tris-EDTA and incubated alone (Control, A) or with 100 μM NDGA (B) at room temperature for 30 min. Aβ(1–40) monomer was then added such that final concentrations were 2 μM protofibril, 0 or 50 μM NDGA, and 20 μM monomer, and elongation was allowed to proceed for 19 h at room temperature. Aliquots were then applied to a grid without dilution, and grids were analyzed by EM as described under *Materials and Methods*. Images are shown relative to a calibration bar of 1 μm.

brils are more prone to disaggregation (M. R. Nichols, M. A. Moss, D. K. Reed, J. H. Hoh, and T. L. Rosenberry, submitted for publication). Radiomethylated protofibrils were isolated by SEC, incubated in the presence or absence of excess NDGA, and chromatographed on Superdex 75 to separate resulting monomer from protofibril. The effects of NDGA on thioflavin T fluorescence and protofibril size were the same with labeled and unlabeled Aβ. After 2 h of incubation, a time period over which thioflavin T fluorescence was reduced by 60%, the quantity of radiomethylated Aβ(1–40) monomer eluting from Superdex 75 remained unchanged in the presence or absence of NDGA (Fig. 5). Thus, SEC provided no evidence that NDGA could disaggregate Aβ protofibrils into monomers or small oligomers. However, as Fig. 5 indicates, NDGA induced an 85% decrease in protofibril radioactivity eluting in the void peak. In the absence of a parallel increase in the monomer peak, this decrease is unlikely to reflect a disaggregation of Aβ protofibrils. Instead, the decrease in overall column recovery suggested a loss of material during SEC. We regularly observe a slight loss of Aβ monomers and somewhat larger losses of Aβ protofibrils by monitoring radioactivity during aggregation of radiomethylated Aβ(1–40) and SEC isolation of the protofibrils, presumably because of protofibril adsorbance to the resin. In these experiments, interaction of Aβ protofibrils with NDGA enhanced this loss to an unusual extent.

NDGA Altered the Interaction of Fluorescent Thioflavin T with Aβ Protofibrils. DLS, EM, and SEC collectively failed to demonstrate that NDGA could disaggregate Aβ protofibrils. We postulated instead that the decrease in thioflavin T fluorescence might result from either displacement or conformational alteration of thioflavin T by NDGA. Our previous measurements had monitored thioflavin T fluorescence changes after addition of NDGA to Aβ protofibril-thioflavin T mixtures and revealed an initially high value of fluorescence that decreased with time to a steady level. We also monitored the fluorescence of Aβ protofibrils that had been incubated with NDGA before addition of thioflavin T. As shown in Fig. 6A, preincubation of Aβ protofibrils with 0.2 μM NDGA resulted in a lower fluorescence that remained constant over time. Furthermore, this decreased fluorescence was equivalent to the final steady-state fluorescence observed after addition of 0.2 μM NDGA to Aβ protofibrils incubated first with thioflavin T. When a 10-fold higher concentration of NDGA was used, comparable results were observed with the predictable exception that the steady-state fluorescence was lower (Fig. 6B). Thus, the same equilibrium fluorescence was reached regardless of the order in which Aβ protofibrils, NDGA, and thioflavin T were added.

NDGA Inhibited Aβ Protofibril Growth by Association. The experimental evidence that NDGA bound to Aβ protofibrils led us to investigate any effects that NDGA might have on Aβ protofibril growth. Previously, we distinguished between two modes of protofibril growth: direct protofibril-protofibril association and elongation by monomer deposition. These growth mechanisms could be resolved by varying NaCl and Aβ monomer concentration (Nichols et al., 2002). Aβ protofibrils grow by direct protofibril-protofibril association on addition of NaCl in the absence of monomer (Nichols et al., 2002). Isolated Aβ(1–40) protofibrils were incubated in the presence or absence of NDGA, and association was initiated by addition of NaCl to 150 mM and monitored by an increase in light scattering intensity and aggregate size. As shown in Fig. 7A, NDGA inhibited Aβ protofibril association. Thirty minutes after association was initiated, the R_H for control protofibrils increased more than 8-fold, whereas the R_H for protofibrils first incubated with NDGA increased only 2-fold. A parallel increase in light scattering intensity was similarly inhibited. Inhibition of Aβ protofibril association required preincubation of protofibrils with NDGA and was dependent upon the concentration of NDGA.

We have demonstrated previously that Aβ protofibril asso-

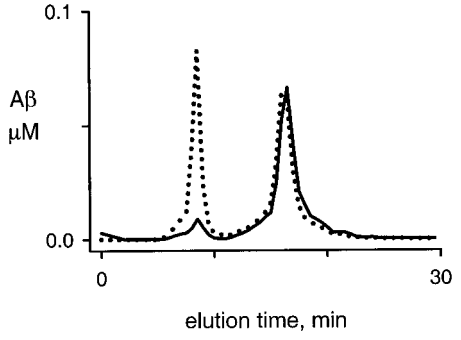

Fig. 5. NDGA failed to convert Aβ protofibrils to Aβ monomer. Isolated ^{3}H-radiomethylated Aβ(1–40) protofibrils were diluted to 1 μM in 50 mM Tris-EDTA and incubated alone (Control, dotted line) or with 50 μM NDGA (solid line) at room temperature for 2 h. Incubations were centrifuged (18,000*g*, 10 min) and the supernatants were chromatographed on Superdex 75. Loss of radioactivity in the supernatants after centrifugation was 8% in the absence and 12% in the presence of NDGA, indicating an insignificant difference in sedimentable material. Profiles correspond to radioactivity in eluted fractions. Recoveries of input radioactivity were 82% in the absence and 55% in the presence of NDGA.

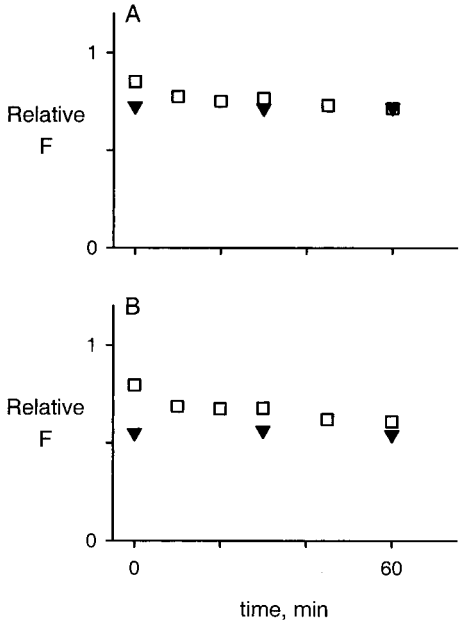

Fig. 6. Identical thioflavin T fluorescence was obtained regardless of the order in which Aβ protofibrils, NDGA, and thioflavin T were added to the incubation. Aβ(1–40) protofibrils isolated on Superdex 75 were diluted to 0.3 μM in 5 mM Tris-EDTA with either 5 μM thioflavin T (□) or NDGA (▼) and incubated without agitation at room temperature for 2 h. NDGA (□) or 5 μM thioflavin T (▼) was then added at zero time, and thioflavin T fluorescence (F) was subsequently monitored. A control incubation of Aβ protofibrils with thioflavin T in the absence of NDGA displayed little change in fluorescence, similar to results shown in Fig. 2. A, incubations with 0.2 μM NDGA. B, incubations with 2 μM NDGA.

ciation is induced by addition of the peptide KLVFF-K_6 (Moss et al., 2003). This association was significantly faster than that induced by 150 mM NaCl and involved binding of KLVFF-K_6 to Aβ protofibrils. Preincubation of Aβ(1–40) protofibrils with NDGA was unable to inhibit Aβ protofibril association induced by KLVFF-K_6, even when NDGA was present at concentrations 100-fold higher than KLVFF-K_6 (data not shown).

A second mode of Aβ protofibril growth, elongation by monomer addition, can be induced by incubation of isolated Aβ protofibrils in the presence of excess monomer and in the absence of NaCl (Nichols et al., 2002). Elongation typically is followed by an increase in thioflavin T fluorescence with time. Because NDGA was found to interfere with thioflavin T fluorescence, elongation was instead followed by DLS as an increase in light scattering intensity and aggregate size after addition of Aβ monomer. Isolated Aβ(1–40) protofibrils were incubated in the presence or absence of excess NDGA, and Aβ(1–40) monomer was subsequently added to initiate growth. Elongation was indicated by a 2-fold increase in R_H that was accompanied by a parallel increase in light scattering intensity. These changes were equivalent for both control and NDGA samples, suggesting no effect of NDGA on protofibril elongation by monomer addition (Fig. 7B). This was true even at NDGA concentrations as high as 130 μM.

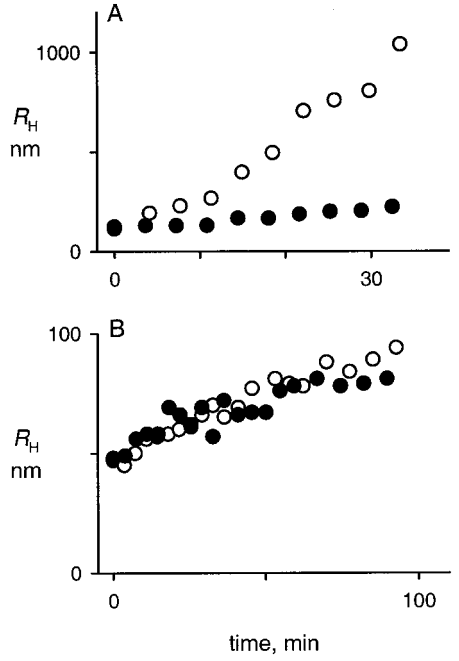

Fig. 7. NDGA inhibited Aβ protofibril association promoted by high ionic strength. A, direct Aβ protofibril-protofibril association. Isolated Aβ(1–40) protofibrils exhibiting an R_H of 124 nm were diluted to 4 μM in 5 mM Tris-EDTA and incubated alone (Control, ○) or with 40 μM filtered NDGA (●) at room temperature for 30 min. Concentrated NaCl was added such that final concentrations were 2 μM protofibril, 0 or 20 μM NDGA, and 150 mM NaCl. The R_H was followed by DLS, and increases in R_H were paralleled by increases in light scattering intensity. Aβ protofibrils incubated in the absence of salt showed no change in R_H or light scattering intensity. Results are representative of four experiments. B, elongation by monomer deposition. Isolated [^{3}H]Aβ(1–40) protofibrils exhibiting an R_H of 47 nm were diluted to 4 μM in 50 mM Tris-EDTA and incubated alone (Control, ○) or with 170 μM filtered NDGA (●) at room temperature for 30 min. [^{3}H]Aβ(1–40) monomer was added such that final concentrations were 2 μM protofibril, 0 or 85 μM NDGA, and 20 μM monomer, and R_H was followed by DLS. Increases in R_H were paralleled by increases in light scattering intensity. Aβ protofibrils incubated in the absence of monomer showed no change in R_H or light scattering intensity. Results are representative of five experiments.

NDGA Diminished the Effects of Aβ Protofibrils on Cellular Reduction of MTT. Ono et al. (2002b) reported that freshly elongated fibrils incubated in the presence of NDGA displayed reduced cytotoxicity in human embryonic kidney 293 cell cultures, as measured by an MTT reduction assay. We used a similar MTT reduction assay to investigate whether interaction of NDGA with Aβ(1–40) protofibrils inhibits activity of Aβ in cultured rat PC-12 pheochromocytoma cells. The MTT reduction assay is not a direct measure of cell survival but instead indicates changes in cellular redox activity that may correlate with cell viability (Shearman et al., 1994). Because decreases in MTT reduction can be observed at Aβ concentrations below those that compromise cell survival (Shearman et al., 1994), this assay has been widely used to investigate the neurotoxicity of Aβ fibrils, protofibrils, and oligomers (El-Agnaf et al., 2000; Ward et al., 2000). However, concerns about the correlation of MTT reduction with cell viability, as well as direct interactions between Aβ aggregates and MTT, should be considered when interpreting MTT results (Shearman et al., 1994; Liu and Schubert, 1997; Moss et al., 2003).

As we observed previously (Moss et al., 2003), treatment of PC-12 cells with Aβ(1–40) protofibrils incubated without NDGA resulted in a decrease of ~65% in cellular reduction of MTT (Fig. 8B). Protofibrils incubated for 24 h in the presence of 50-fold excess NDGA exhibited a 90% decrease in thioflavin T fluorescence, and treatment of PC-12 cells with these aggregates decreased cellular reduction of MTT by only 30% (Fig. 8B). As the ratio of NDGA to protofibril was decreased, MTT reduction approached levels observed for protofibrils incubated in the absence of NDGA. Thus, NDGA diminished the effect of Aβ(1–40) protofibrils on cellular reduction of MTT in a dose-dependent manner.

Discussion

In a recent report, Ono et al. (2002b) concluded that NDGA could disaggregate preformed Aβ fibrils based largely on observations that thioflavin T fluorescence was reduced after exposure of freshly extended Aβ fibrils to NDGA. In the current study, we have confirmed the decrease in thioflavin T fluorescence for Aβ fibrils incubated in the presence of NDGA and extended this observation to include Aβ protofibrils. A similar trend has been noted for the related phenolic compound curcumin (data not shown), as well as for other polyphenols (Ono et al., 2003). However, we establish here that the NDGA-induced decrease in thioflavin T fluorescence was not accompanied by a reduction in Aβ aggregate size or quantity. DLS showed no change in Aβ protofibril size or concentration, and SEC failed to indicate any increase in the quantities of Aβ monomer or small oligomer after incubation of Aβ(1–40) protofibrils with NDGA. In addition, EM images revealed no differences in size or morphology for Aβ(1–40) protofibrils incubated in the presence of NDGA. Although Ono et al. (2002b) presented EM images implying that freshly extended Aβ fibrils were fewer in number after 4 h of incubation in the presence of NDGA, our observations suggest that this decrease might have resulted from a nonspecific loss of Aβ aggregates during sample manipulation (e.g., Fig. 5). Furthermore, these workers conducted protein deter-

minations to show that both their NDGA-treated and untreated Aβ aggregates sedimented completely after centrifugation at 16,000*g* for 2 h. These NDGA-treated Aβ aggregates should have been evident by EM, in that even Aβ protofibrils and oligomers that remain soluble after centrifugation at 16,000*g* have been detected using EM (Walsh et al., 1997, 1999). In short, the observations of Ono et al. (2002b) are largely consistent with our SEC results, which indicated no increase in Aβ monomer or small oligomer content after incubation of Aβ protofibrils with NDGA.

In the absence of evidence that NDGA can disaggregate Aβ protofibrils or fibrils, we propose instead that changes in thioflavin T fluorescence can be explained by either displacement or conformational alteration of thioflavin T upon the binding of NDGA to these aggregates. Although simple competition of thioflavin T and NDGA for a common binding site cannot be ruled out by our data, the slow approach to equilibrium when NDGA is added to pre-equilibrated Aβ protofibrils and thioflavin T suggests a more complicated process. If NDGA and thioflavin T interact simultaneously with Aβ protofibrils at distinct sites, the binding of NDGA could slightly alter the environment of bound thioflavin T. The fluorescence of thioflavin T has been shown to depend on solvent viscosity (Friedhoff et al., 1998) with a relationship described previously for a class of fluorescent dyes called molecular rotors (Loutfy and Arnold, 1982; Kung and Reed, 1986). These dyes show increased fluorescence when introduced into high-viscosity media because of a decreased torsional relaxation. Binding of NDGA to an adjacent site could alter the conformation of the thioflavin T site to increase the torsional mobility of thioflavin T with a consequent quenching of its fluorescence. On the other hand, binding of NDGA to Aβ protofibrils could result in a conformational change in the β-sheet structure that results in the dissociation of thioflavin T. However, the circular dichroism spectra of Aβ(1–40) protofibrils incubated in the presence of excess NDGA for up to 21 h failed to indicate any change in β-sheet content (data not shown). Regardless of its mechanistic basis, a reduction of thioflavin T fluorescence on addition of NDGA is not restricted to fibrillar Aβ. As noted under *Results*, NDGA also bound to insulin fibrils and decreased associated thioflavin T fluorescence. In addition, NDGA has been observed to decrease the fluorescence of thioflavin T associated with elongating light chain amyloid fibrils (Takahashi et al., 2002).

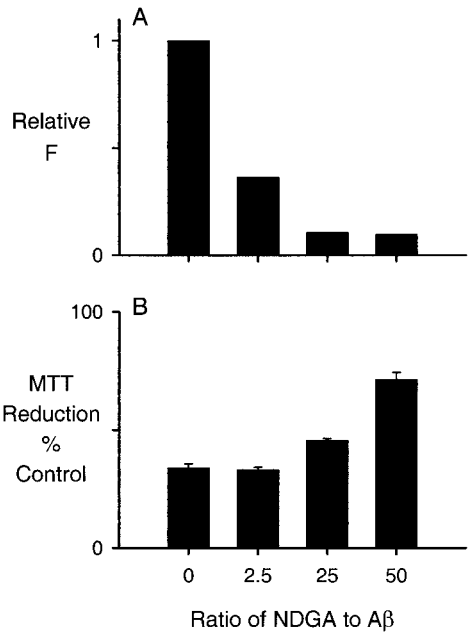

Fig. 8. NDGA diminished the effect of Aβ protofibrils on cellular reduction of MTT. Isolated Aβ(1–40) protofibrils were diluted to 2 μM in 50 mM Tris-HCl, pH 8.0, and incubated with 0 (Control), 5, 50, or 100 μM NDGA at room temperature for 19 h. Reaction products were diluted 10-fold into cell culture media to give a final Aβ concentration of 200 nM. Diluted solutions were applied to PC-12 cells for MTT reduction assays as outlined under *Materials and Methods*. A, relative thioflavin T fluorescence for Aβ(1–40) protofibrils incubated in the presence of NDGA. Measurements were taken after 19 h incubation and before dilution of Aβ(1–40) protofibrils for MTT reduction assay. B, cellular reduction of MTT after treatment of PC-12 cells with Aβ(1–40) protofibrils incubated in the presence of NDGA. Results are expressed as percentage MTT reduction compared with negative control wells containing an equivalent dilution of buffer into media. Error bars indicate standard error ($n = 6$). No change in cellular reduction of MTT was observed when cells were treated with NDGA alone.

The question of whether NDGA inhibits Aβ monomer aggregation remains interesting. Naiki et al. (1998) showed that NDGA prevented an increase in thioflavin T fluorescence when both were added to freshly dissolved Aβ solutions, but this could have resulted from interaction between thioflavin T and NDGA rather than from inhibition of aggregate formation. It is difficult to monitor NDGA inhibition of Aβ monomer aggregation by light scattering. We used DLS to monitor the aggregation of SEC isolated Aβ(1–40) monomer (50 μM) in the presence and absence of equimolar NDGA and observed only a modest 2-fold decrease in aggregation rate with NDGA (data not shown). However, these measurements were technically challenging because the DLS signal increases substantially with particle size, making it difficult to detect small particles such as Aβ monomers and small oligomers.

Although NDGA did not disaggregate Aβ protofibrils, it did inhibit direct protofibril-protofibril association induced by 150 mM NaCl. Preincubation of Aβ protofibrils with NDGA was necessary for this inhibition of protofibril association, suggesting that NDGA must first bind Aβ protofibrils. If NDGA binds along the length of Aβ protofibrils, lateral interactions might be prevented. Thioflavin T, which also binds Aβ(1–40) protofibrils, failed to inhibit protofibril association (data not shown). These contrasting results could be the consequence of differences in binding orientation, binding affinity, or structure between thioflavin T and NDGA. In contrast, protofibril elongation was unaffected by NDGA, suggesting that NDGA is not likely to bind protofibril ends where Aβ monomer is incorporated. Together, results from protofibril association and elongation studies suggested that NDGA might bind to the lateral surface of Aβ protofibrils, where interference with protofibril-protofibril association would be expected but interference with protofibril elongation would not.

We observed that incubation of Aβ(1–40) protofibrils with NDGA also diminished the effects of the protofibrils on cellular reduction of MTT. This result was consistent with previous observations that NDGA reduced cellular activity of Aβ when incubated with preformed Aβ fibrils before cell treatment (Ono et al., 2002b) or when added simultaneously with freshly dissolved Aβ to cell cultures (Goodman et al., 1994). Although we obtained no evidence that protofibril or fibril disaggregation could account for these effects, an alteration

in the protofibril or fibril surface resulting from NDGA binding may be involved. The decreased recovery of NDGA-treated protofibrils during SEC separation suggested that interaction of NDGA with Aβ aggregates enhanced nonspecific adherence of Aβ. A similar loss of Aβ aggregates during sample manipulation before and during cell treatment could provide a rather trivial explanation for the decreased cellular effect of protofibrils incubated with NDGA. On the other hand, the ability of NDGA to inhibit Aβ protofibril association might contribute to the decreased cellular activity. However, any therapeutic benefit of inhibiting Aβ protofibril growth by association remains uncertain. Increasing evidence suggests that soluble Aβ aggregates may be the more toxic Aβ species, whereas larger, insoluble fibrils may, in fact, be protective (Kirkitadze et al., 2002). Slowing the progression of small, soluble aggregates into large, insoluble fibrils thus might increase Aβ toxicity, although we found no difference between control Aβ protofibrils and protofibrils associated with the 11-residue peptide KLVFF-K_6 on cellular MTT reduction (Moss et al., 2003). Another possibility is that NDGA binding to Aβ protofibrils could curtail specific interactions of Aβ with the cell surface. Sulfonated dyes, including Congo red and Chrysamine-G, inhibited toxicity of preformed Aβ fibrils (Lorenzo and Yankner, 1994; Pollack et al., 1995; Klunk et al., 1998). This inhibition was specific for compounds containing sulfonate groups capable of interacting with β-sheet structure (Pollack et al., 1995). Furthermore, for Chrysamine-G, this inhibition required binding to Aβ fibrils (Klunk et al., 1998). However, it is unclear whether this interaction blocked Aβ recognition by the cells or if the inhibition of cellular activity was a consequence of other properties of Chrysamine-G. Likewise, other properties of NDGA might contribute to the observed decrease in Aβ protofibril effects on cellular MTT reduction, even though we found no effects of NDGA alone in this assay (see legend to Fig. 8). NDGA is an antioxidant, and accumulation of reactive oxygen species induced by either Aβ or other sources, such as iron, can be suppressed by NDGA (Goodman et al., 1994). NDGA has also been shown to reduce levels of intercellular free calcium that are stimulated by Aβ (Goodman et al., 1994). In addition, NDGA can act as a lipoxygenase inhibitor (Goodman et al., 1994) and can inhibit Aβ stimulation of phospholipase D (Singh et al., 1997).

In summary, whereas the explanation for NDGA inhibition of Aβ protofibril effects on cellular MTT reduction remains unclear, the data presented here show that this inhibition does not result from disaggregation of Aβ aggregates into monomers or small oligomers. Instead, the decrease in thioflavin T fluorescence induced by incubation of Aβ fibrils or protofibrils with NDGA can be explained by either displacement or conformational alteration of thioflavin T upon the binding of NDGA to these aggregates. Whereas NDGA did not disaggregate Aβ protofibrils, interaction of NDGA with protofibrils did alter protofibril properties. NDGA binding to Aβ protofibrils inhibited protofibril association and increased nonspecific loss of Aβ aggregates. Future experiments will be needed to determine whether interactions between NDGA and Aβ protofibrils might have therapeutic benefits.

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