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Glycated tau protein in Alzheimer disease: A mechanism for induction of oxidant stress

(glycation/reactive oxygen intermediate/neuron)

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ABSTRACT **The stability of proteins that constitute the neurofibrillary tangles and senile plaques of Alzheimer disease suggests that they would be ideal substrates for nonenzymatic glycation, a process that occurs over long times, even at normal levels of glucose, ultimately resulting in the formation of advanced glycation end products (AGEs). AGE-modified proteins aggregate, and they generate reactive oxygen intermediates. Using monospecific antibody to AGEs, we have colocalized these AGEs with paired helical filament tau in neurofibrillary tangles in sporadic Alzheimer disease. Such neurons also exhibited evidence of oxidant stress: induction of malondialdehyde epitopes and heme oxygenase 1 antigen. AGE-recombinant tau generated reactive oxygen intermediates and, when introduced into the cytoplasm of SH-SY5Y neuroblastoma cells, induced oxidant stress. We propose that in Alzheimer disease, AGEs in paired helical filament tau can induce oxidant stress, thereby promoting neuronal dysfunction.**

Proteins or lipids exposed to reducing sugars undergo nonenzymatic glycation and oxidation, initially with formation of Schiff bases and Amadori products on free amino groups, which ultimately undergo molecular rearrangement, to form irreversible advanced glycation end products (AGEs; refs. 1–5). The AGEs are heterogeneous compounds of yellow-brown color and characteristic fluorescence (1–5). Accumulation of AGEs occurs on both intra- and extracellular structures, especially those whose turnover is prolonged. Although the formation of AGEs is accelerated in diabetes, it also occurs in normal aging. Proteins with many free amino groups (i.e., with high lysine content) are most readily glycated. AGE-modified proteins form crosslinks which result in aggregation and insolubility; they are also a continuing source of potentially damaging reactive oxygen intermediates (ROIs) and, when present extracellularly, interact with a distinct class of receptors (1–9). In cells, we have found that AGEs impart an oxidant stress manifested in endothelium by induction of heme oxygenase, activation of the transcription factor NF-κB, and formation of malondialdehyde epitopes of lipid peroxidation products (9). These perturbations, which result in changes in a spectrum of cellular properties (e.g., cell adherence, proliferation), were not accompanied by diminished cell viability (in short-term experiments), in keeping with a role for low levels of ROIs in signal transduction.

The longstanding protein aggregates in Alzheimer disease (AD), such as paired helical filament (PHF) tau and amyloid β-protein (10–12), could form AGEs and contribute to the development of neuronal dysfunction. Our results indicate that PHF tau bears AGEs: tau-1 and AGE antigens are colocalized in neurofibrillary tangles, and neurons bearing these also exhibit malondialdehyde epitopes and display increased heme oxygenase, two indicators of oxidant stress. Introduction of nonenzymatically glycated recombinant tau into neuroblastoma (SH-SY5Y) cells causes induction of these markers of oxidant stress. Taken together, our data suggest that AGEs in PHF tau can generate ROIs sufficient to cause cellular oxidant stress, thereby contributing to eventual neuronal dysfunction and death.

METHODS

AGE ELISA, Immunoblotting, and Immunohistochemistry. AGE antigen was determined by using affinity-purified antibody to AGEs (9, 13). This antibody selectively recognizes AGE forms of multiple proteins, but not the nonglycated counterparts (9) or formylated, maleylated, oxidized, or acetylated protein (9). To assay for AGE antigen (9), an ELISA was established by coating plates with brain homogenates/PHF tau (10–100 μg/ml) overnight at 4°C, blocking with normal goat serum (5%) for 2 hr at 37°C, and then adding anti-AGE IgG (1 μg/ml). A standard curve was generated over a wide range of AGE albumin concentrations (25 pg/ml to 250 μg/ml). Binding of anti-AGE antibody was quantitated by incubation with peroxidase-conjugated goat anti-guinea pig IgG followed by *o*-phenylenediamine (Sigma), with measurement of the absorbance of the reaction product at 490 nm. The limit of detection in the ELISA was 50 pg/ml. Homogenates of temporal lobe from AD patients were prepared as described (14), treated with 1% SDS, and boiled. PHF-derived tau protein was prepared as described (15). These samples were from AD patients, all of whom had the characteristic clinical and anatomical features of AD, and age-matched, apparently normal controls.

Immunoblotting of PHF tau from AD temporal lobe and tau from age-matched normal controls was performed with anti-tau-1 IgG (specific for nonphosphorylated tau) and affinity-purified anti-AGE IgG. The tau preparations (22 μg per lane), made as described above, were subjected to reducing SDS/10% PAGE (16), electrophoretic transfer to nitrocellulose membranes, and dephosphorylation by exposure to alkaline phosphatase (100 units for 2 hr at 37°C; Sigma). Immunoreactive proteins on the nitrocellulose blots were visualized by the avidin/biotin method using either anti-tau-1 IgG (15, 17) or anti-AGE IgG (0.8 μg/ml).

Immunohistologic studies were performed on paraffin sections of formalin-fixed temporal lobe from AD and age-

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Abbreviations: AGE, advanced glycation end product; ROI, reactive oxygen intermediate; AD, Alzheimer disease; PHF, paired helical filament.

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matched controls. Every specimen had been fixed for 28 days before processing for embedment. After treatment with alkaline phosphatase (130 units for 2.5 hr at 34°C), sections were stained with anti-tau-1 IgG (0.5 μg/ml; Boehringer Mannheim) (18–20) or anti-AGE IgG (2 μg/ml), and sites of binding of primary antibody were visualized by the avidin/biotin peroxidase method. The 100,000 × *g* P-enriched fraction (15) was placed on Formvar-coated grids for immunoelectron microscopy, blocked with 10% donkey serum, reacted with anti-AGE IgG (2 μg/ml), and then negatively stained with phosphotungstic acid (1%). Sites of primary antibody binding were localized with 12-nm-gold-conjugated donkey anti-guinea pig IgG. Controls employed nonimmune guinea pig IgG in place of guinea pig anti-AGE IgG. Adjacent paraffin sections of temporal lobe from AD or age-matched controls were also stained with anti-malondialdehyde IgG (25 μg/ml; refs. 9 and 21) and anti-hemoxygenase 1 immune serum (1:100; StressGen Biotechnologies, Victoria, BC, Canada) (22).

Preparation and Glycation of Recombinant tau. Recombinant tau, the isoform having four repeats (21), was prepared in the baculovirus system as described (21, 23). On SDS/PAGE, this form of tau migrates as several bands of 45–60 kDa (21) and contains at least seven phosphorylatable groups (23). Recombinant tau was glycated by incubation with 0.5 M ribose in phosphate-buffered saline (pH 7.2) containing 1 mM EDTA and 1.5 mM phenylmethylsulfonyl fluoride for 4 weeks at 37°C. Samples were withdrawn for AGE ELISA and reducing SDS/10% PAGE. Controls employed recombinant tau incubated in the same buffer in the absence of ribose.

Liposomes were prepared by incubating AGE tau or nonglycated tau (25 μg/ml) with Lipofectin (10 μg/ml; GIBCO) (24). Generation of ROIs by AGE tau was studied with the cytochrome *c* assay (reduction of ferricytochrome *c* by superoxide): AGE tau was incubated in 10 mM phosphate buffer (pH 7.8) with 10 μM cytochrome *c* (Sigma) and 100 μM EDTA for 10 min at 25°C, and absorption at 550 nm was determined as described (7, 8). As a control, 100 units of superoxide dismutase (Sigma) was added just before cytochrome *c*.

Introduction of Recombinant tau into SH-SY5Y Neuroblastoma Cells and Induction of Oxidant Stress. AGE tau, or nonglycated tau, was introduced into SH-SY5Y cells (2.5 × 10^5 per well) with liposomes [prepared as described above with Lipofectin (10 μg; GIBCO)] containing tau (25 μg/ml) for 18 hr at 37°C in Opti-MEM (GIBCO). Cells were then immunostained with anti-AGE IgG (2 μg/ml), anti-malondialdehyde IgG (10 μg/ml), or anti-heme oxygenase immune serum (1:100). Some cultures were also exposed to probucol for 16 hr or to *N*-acetylcysteine for 1 hr before and during exposure to AGE tau. SH-SY5Y cells loaded with AGE tau were compared with cells into which nonglycated tau had been introduced. Generation of thiobarbituric acid-reactive substances (indicative of oxidant stress) was studied by the method of Dennery *et al.* (25).

RESULTS

AGEs Are Present on PHF tau in Neurofibrillary Tangles. Homogenates of temporal cortex from patients with AD (n = 13) and age-matched control subjects (n = 6) were studied for AGE content by ELISA using affinity-purified antibodies to AGEs (9); AD extracts had ≈3 times more AGE immunoreactivity (Fig. 1*A*). PHF tau isolated from six AD patients contained immunoreactive AGEs, whereas tau from normal individuals did not (Fig. 1*B*; pooled data is shown in each case). The positive signal in this ELISA was completely blocked by preabsorption of the antibody with soluble AGEs, indicating specific recognition of AGEs. Immunoblotting of tau preparations from normal individuals revealed a series of bands spanning a range of apparent molecular mass, 30–70 kDa (Fig. 1*C*, lane 1), as described previously (15, 17), whereas three major bands corresponding to 60–68 kDa were observed in PHF tau preparations (Fig. 1*C*, lane 2). Although the anti-AGE antibody was nonreactive with normal tau (Fig. 1*C*, lane 3), multiple bands were observed in PHF tau preparations, including those corresponding to 60–68 kDa and even more prominently other more rapidly migrating bands (Fig. 1*C*, lane 4; AGE albumin in lane 5 is a positive control). The latter bands could represent various forms of tau, as previously observed in these PHF preparations (18–20, 26), or associated AGE-modified proteins.

Immunohistology of temporal lobe using anti-tau-1 IgG demonstrated the characteristic neurofibrillary tangles of AD patients (Fig. 2*A*). The adjacent section stained with anti-AGE IgG showed immunoreactivity with the same distribution (Fig. 2*B*; *C* shows the same section as in *B*, double stained to reveal tau, validating the use of double staining). Virtually every neuron bearing neurofibrillary tangles stained similarly with anti-AGE IgG. Nonimmune IgG or anti-AGE preabsorbed with soluble AGE did not stain, and sections from age-matched controls showed only very weak staining

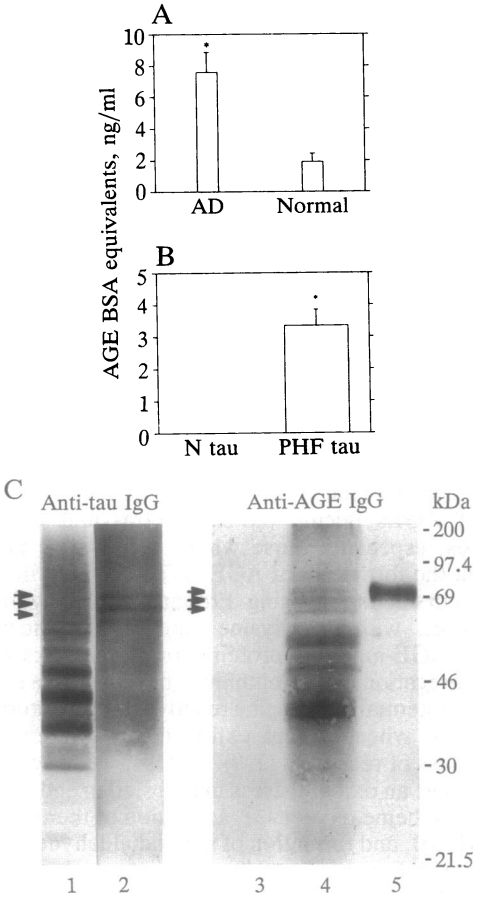

FIG. 1. Increased levels of AGEs [measured as AGE-modified bovine serum albumin (BSA) equivalents] are present in brain tissue from AD patients and are associated with PHF tau. (*A*) ELISA for AGEs on extracts of temporal lobe of AD subjects (n = 13) and age-matched normal subjects (n = 6). *, $P < 0.01$. (*B*) Comparison of AGE content of PHF tau (n = 6) and normal (N) tau (n = 3) by ELISA. *, $P < 0.01$. PHF tau was isolated as described, and heat-soluble PHF tau was analyzed in the AGE ELISA. (*C*) PHF tau (lanes 2 and 4), normal tau (lanes 1 and 3), and AGE albumin (2.5 μg; lane 5) were subjected to SDS/10% PAGE under reducing conditions, followed by immunoblot analysis with anti-tau-1 IgG (lanes 1 and 2) or anti-AGE IgG (lanes 3–5). Migration of simultaneously run marker proteins is indicated at right, and the three closely spaced arrows correspond to bands at 60–68 kDa.

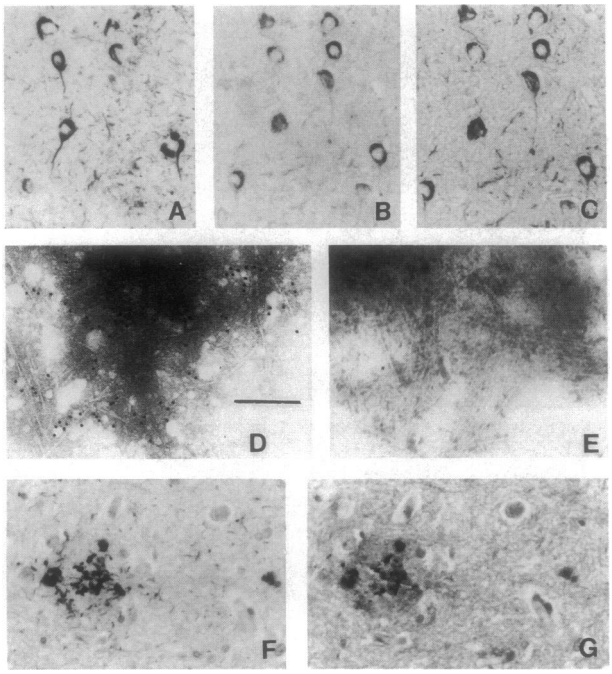

FIG. 2. Colocalization of tau and AGE immunoreactivity in neurofibrillary tangles from AD brain tissue. (*A–C*) Immunostaining of AD-affected temporal lobe for tau and AGE antigen. Adjacent sections were stained with anti-tau-1 IgG (0.5 μg/ml) (*A* and *C*) and anti-AGE IgG (2 μg/ml) (*B*). *A* and *B* are adjacent sections stained by the avidin/biotin peroxidase method in which 3-amino-9-ethylcarbazole was chromophore (brown-red); *B* and *C* are the same section, sequentially double immunostained with anti-AGE IgG and then, after decolorization, with anti-tau IgG by the conjugated alkaline phosphatase method in which fast red TR/naphthol ASMX was chromophore (bright pink). Sections were photographed through a Leitz Dialux microscope using appropriate contrast filters. (*D*) Immunoelectron microscopic localization of AGEs in PHFs. The 100,000 × *g* pellet fraction of PHFs was prepared and reacted with anti-AGE IgG (2 μg/ml). Note the association of immunogold particles with PHF. (*E*) As in *D*, but with nonimmune IgG substituted for anti-AGE IgG. (*F* and *G*) Colocalization of tau and AGE in neuritic plaque, using anti-tau-1 IgG and anti-AGE IgG, respectively. Sequential double immunostaining with anti-tau IgG or anti-AGE IgG was performed as above. (*A–C* and *F–G*, ×100; *D* and *E*, bar = 200 nm.)

with anti-AGE in occasional neurons (data not shown; note that there is an age-related increase in AGE immunoreactivity in whole brain tissue due to AGEs in the vasculature). By immunoelectron microscopy of PHF tau preparations, AGE determinants were localized in PHF with anti-AGE IgG (Fig. 2*D*), whereas nonimmune IgG did not stain PHF (Fig. 2*E*). Gold particles were aligned with filaments in a pattern resembling that visualized with anti-tau IgG (27). Within a few senile plaques the foci stained by anti-AGE IgG were the entrapped dystrophic neurites or tangles from defunct neurons, immunostained with anti-tau-1 IgG and anti-AGE IgG (Fig. 2 *F* and *G*, respectively).

Because AGEs generate ROIs (2) and because of previous suggestions that oxidant stress occurred in AD (28–32), we examined neurons affected by AD for evidence of oxidant stress—i.e., malondialdehyde determinants and heme oxygenase—induced in the cellular response to ROIs (9, 33, 34). Immunoreactive malondialdehyde were present in AD neurons (Fig. 3*A*), which invariably also contained PHF tau (Fig. 3*B*); these epitopes were diffusely distributed in the perikarya of neurofibrillary tangle-bearing neurons, as would be expected of multiple (e.g., membranous) cytoplasmic targets of ROIs presumably originating in glycated tau deposits. In contrast, there was no significant anti-malondialdehyde staining of section from normal individuals (Fig. 3*C*). Increased expression of heme oxygenase 1 antigen was also demonstrable in AD brain (Fig. 3*D*), colocalized with anti-tau-1-stained neurofibrillary tangles and neuritic plaques (Fig. 3*E*); age-matched controls had no significant staining (Fig. 3*F*). All cases of AD showed this evidence of oxidant stress, but the intensity of staining and numbers of involved neurons varied somewhat from case to case.

Glycation of Recombinant tau. Tau should be an avid substrate for glycation because of its considerable lysine content (10) and its exposure to high levels of intracellular aldose or aldose phosphate derivatives. Recombinant human tau incubated with ribose underwent time-dependent glycation to AGEs, observable by ELISA (Fig. 4*A*); AGE antigen was evident by day 14, increasing to higher levels after 21 and 28 days. SDS/PAGE of recombinant human tau incubated for 4 weeks without ribose showed characteristic bands at 50–60 kDa (3) (Fig. 4*B*, lane 1). But tau glycated for 4 weeks demonstrated a predominant more slowly migrating species, as well as even higher molecular mass aggregates (Fig. 4*B*, lane 2, arrowheads). AGE tau in SDS/polyacrylamide gels

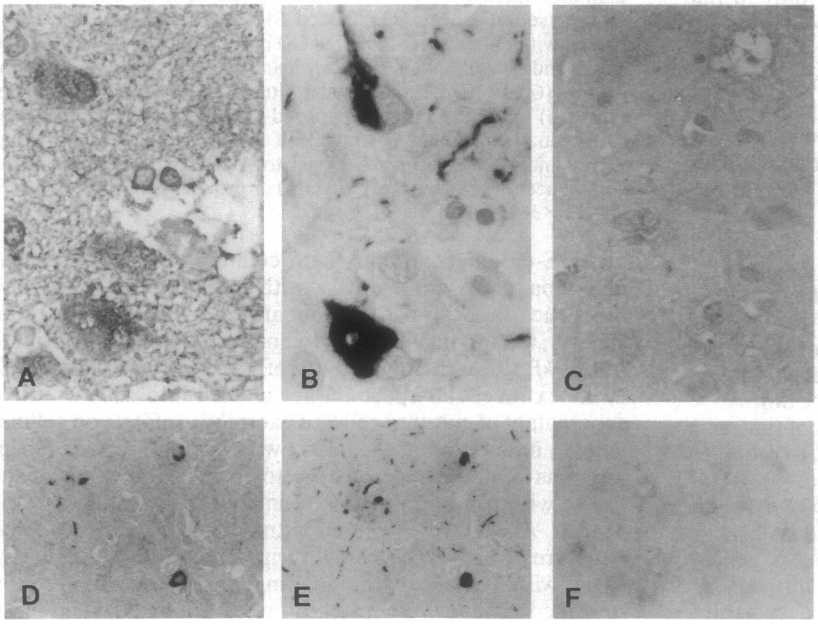

FIG. 3. Immunohistologic evidence of oxidant stress in AD. Adjacent paraffin sections of temporal lobe from AD were stained with anti-malondialdehyde IgG (25 μg/ml) (*A*) and anti-tau-1 IgG (*B*) or normal (age-matched) temporal lobe was stained with anti-malondialdehyde IgG (*C*) and localization of primary antibodies was accomplished by the avidin/biotin/alkaline phosphatase system. Nuclei were visualized by counterstaining with hematoxylin. In *A* and *B*, three affected neurons are shown adjacent to a perivascular space, at right: the identity of the two malondialdehyde-reactive cells abutting this space is not known; in general, most glial cells do not stain for malondialdehyde antigen. Adjacent paraffin sections of temporal lobe from AD were stained with anti-heme oxygenase 1 (*D*) and anti-tau-1 IgG (*E*), and temporal lobe from a normal individual was stained with anti-heme oxygenase 1 IgG (*F*). (*A* and *B*, ×390; *C*, ×200; *D–F*, ×100.)

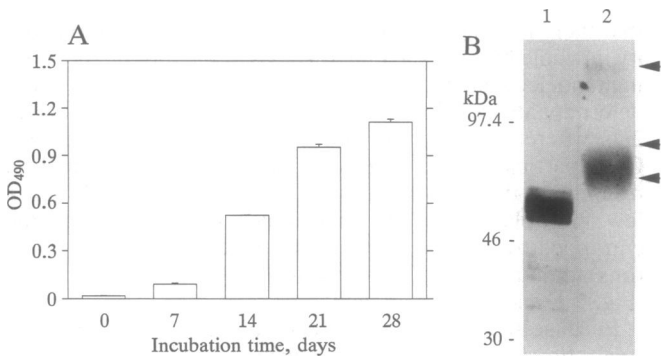

FIG. 4. AGE modification of tau promotes crosslinking. (*A*) Exposure of recombinant human tau to ribose leads to AGE formation. Recombinant tau was produced in Sf9 cells transfected with baculovirus, purified, and incubated for the indicated times with ribose, and samples were assayed in the AGE ELISA. (*B*) SDS/10% PAGE of reduced samples of recombinant tau glycated *in vitro*. tau preparations incubated without (lane 1) or with (lane 2) ribose for 4 weeks were subjected to SDS/PAGE followed by silver staining. The migration of simultaneously run standard proteins is indicated at left.

does not display the more rapidly migrating species present in PHF tau preparations (Fig. 1*C*, lane 4), perhaps due to proteolysis that is operative *in vivo*.

Introduction of AGE-Modified Recombinant tau into SH-SY5Y Neuroblastoma Cells Induces Oxidant Stress. AGE-modified recombinant tau was loaded with liposomes into cultured SH-SY5Y human neuroblastoma cells to determine whether oxidant stress would ensue. Immunoreactive AGE antigen was distributed in cytoplasm of cells loaded with AGE-modified recombinant tau, but not in cells treated with nonglycated recombinant tau [Fig. 5 *A* and *B*, respectively; uptake of tau was comparable in each case (data not shown)]. AGE tau-loaded SH-SY5Y cells stained for malondialdehyde determinants and heme oxygenase 1 antigen (Fig. 5 *C* and *E*, respectively); cells loaded with nonglycated tau were negative (Fig. 5 *D* and *F*, respectively). In addition, AGE tau-loaded cultures had increased levels of thiobarbituric acid-reactive substances, reflective of oxidant stress, as their induction was prevented by the antioxidants *N*-acetylcysteine and probucol (Fig. 5*G*); these same antioxidants also blocked induction of heme oxygenase antigen and malondialdehyde epitopes in SH-SY5Y cells exposed to AGE-modified recombinant tau (data not shown). Viability of the AGE tau-loaded SH-SY5Y cells over the course of these experiments (1–4 days) was not affected by AGEs, as judged by continued exclusion of trypan blue and lack of lactate dehydrogenase release. These findings, which suggested that AGEs were generating intracellular ROIs, were corroborated in the cytochrome *c* reduction assay, where the positive signal with AGE tau was prevented in large part by addition of superoxide dismutase (data not shown).

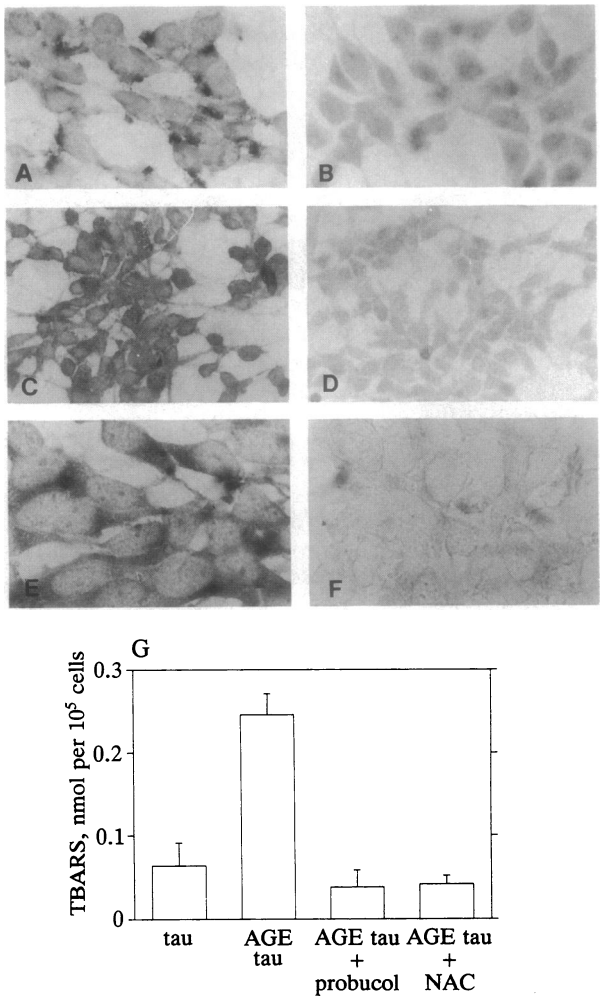

FIG. 5. Effect of AGE tau prepared *in vitro* on neuroblastoma cells. (*A* and *B*) AGE-modified (*A*) or nonglycated (*B*) recombinant human tau was introduced into SH-SY5Y cells (10^5 per well) by incubation for 18 hr with Lipofectin/nonglycated tau or lipofectin/AGE tau and then immunostained with anti-AGE IgG (2 μg/ml). Anti-AGE IgG staining is observed only in cells in which AGE-modified tau was introduced. (*C* and *D*) Malondialdehyde epitopes in AGE-loaded SH-SY5Y cells. Cultures were loaded with either AGE-modified (*C*) or nonglycated (*D*) recombinant tau and then immunostained with anti-malondialdehyde IgG. (*E* and *F*) Heme oxygenase antigen in AGE-loaded SH-SY5Y cells. Cultures were loaded with either AGE-modified (*E*) or nonglycated (*F*) tau as in *C* and *D* and then immunostained with anti-heme oxygenase 1 immune serum. (*G*) Generation of thiobarbituric acid-reactive substances (TBARS) in SH-SY5Y cells exposed to AGE-modified or nonglycated tau. Note that AGE tau itself is nonreactive in this assay. Where indicated, cultures exposed to AGE tau were coincubated with *N*-acetylcysteine (NAC, 30 mM) or probucol (50 μM). (*A*, *B*, *E*, and *F*, ×340; *C* and *D*, ×170.)

DISCUSSION

Intensive efforts have been focused on features of aggregated tau and amyloid β-protein in AD (10, 11). The presence of these protein deposits in brain could have important functional effects on neuronal activity and the extracellular deposits could incite an inflammatory response (35, 36).

AGE-modified proteins form over long periods of time and have been shown to affect monocytes (13, 37, 38), to promote protein aggregation (1–5), and to generate ROIs (6–9). ROIs contribute to the cellular response to certain cytokines, such as tumor necrosis factor (39, 40), are associated with apoptosis (41, 42), and result in activation of a number of genes, in part through mediation of the transcription factor NF-κB (43–45). A previous study demonstrated that the association of AGE-modified proteins with cellular surfaces led to the induction of oxidant stress, with perturbation of cellular properties, appearance of thiobarbituric acid-reactive substances, induction of heme oxygenase mRNA, and activation of NF-κB (9), signaling activity of ROIs. In order for AGEs in the extracellular space (either infused into mice or in the supernatant of cultured cells) to exert these effects on cellular targets, interaction of the ligand with specific surface receptors was required, presumably to facilitate docking of the AGEs with generation ROIs in immediate proximity to the cell. If AGEs were formed intracellularly, especially on structures which have a slow turnover, they could generate ROIs within the cell, modulating cellular functions in a sustained fashion. The steady release of ROIs in close

proximity to vital intracellular targets would also render less likely their interception by cellular antioxidant mechanisms. AD, with its insoluble deposits in neurofibrillary tangles, provided an ideal situation in which to test this hypothesis.

The current study suggests that nonenzymatic glycation of PHF tau could contribute to its stabilization and also allow intracellular generation of ROIs, providing a molecular mechanism linking oxidant stress to the pathogenesis of AD (28–32). Aberrantly phosphorylated tau protein laid down in PHFs in enduring structures would be eminently susceptible to glycation because of its high lysine content and the high intraneuronal concentrations of aldose/aldose phosphate. These findings are consistent with a recent report demonstrating the presence of AGEs in AD (46) and suggest a reason for presence of heme oxygenase in AD brain tissue (47). We postulate the role of AGEs in AD to be one of a "progression factor," superimposed on a preexisting pathologic state in which PHF tau is deposited and then glycated to AGEs, an event that can accelerate and perhaps determine the course of the neuronal disease. These data do not exclude the possibility that other proteins associated with PHFs in neurofibrillary tangles may also be glycated to AGEs. The recognition that AGEs may be involved in disorders such as AD contributes to understanding their pathogenesis: since AGEs generate reactive oxygen intermediates, a mechanism is set up for inducing oxidant stress leading to neuronal dysfunction (28–32). This suggests possible strategies aimed at preventing AGE or ROI formation and its consequences for cellular integrity.

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