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Protective Effect of Transforming Growth Factor-β1 on β-Amyloid Neurotoxicity in Rat Hippocampal Neurons

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SUMMARY

Neurodegeneration associated with Alzheimer's disease is believed to involve toxicity to β-amyloid (Aβ) and related peptides. Treatment of cultured rat hippocampal neurons with Aβ 1–40 (1 μM) or the active fragment Aβ 25–35 (1 μM) for 5 days led to a ~40–50% decrease in neuronal viability. The hydrophilic antioxidant ascorbic acid (300 μM) and the lipophilic antioxidant 2-mercaptoethanol (10 μM) both protected significantly against Aβ neurotoxicity. Despite the protective effects of these antioxidants, both acute and chronic treatments with Aβ 25–35 did not increase production of superoxide anions, as monitored with the fluorescent probe hydroethidine. Similarly, overexpression of Cu/Zn-superoxide dismutase using adenovirus-mediated gene transfer did not protect against Aβ neurotoxicity. Aβ neurotoxicity, however, was prevented in cultures infected with a recombinant, replication-defective adenovirus overexpressing the Ca^{2+} binding protein calbindin D_{28k}. Transforming growth factor-β1 (TGF-β1) has been shown to protect neurons against both Ca^{2+}- and free radical-mediated neuronal degeneration. We found that Aβ neurotoxicity was significantly attenuated by single treatments with TGF-β1 (0.1–10 ng/ml) and prevented by repetitive treatments (10 ng/ml/day). The protective effects of TGF-β1 were associated with a preservation of mitochondrial potential and function, as determined with rhodamine-123-based microfluorimetry. Because both increased oxidative stress and pathophysiological Ca^{2+} fluxes can impair mitochondrial function, preservation of mitochondrial potential by TGF-β1 could be directly associated with its protection against Aβ neurotoxicity. The ability of TGF-β1 to increase the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L is discussed in this context.

Abnormal processing of the amyloid precursor protein, leading to the increased formation of 39–43-amino acid-derived Aβs, is believed to be a key step in the pathophysiology of Alzheimer's disease (1, 2). Accumulation of Aβ within neurons, cerebral vessels, and "plaques" is a hallmark of Alzheimer's disease (1–3). These peptides have been shown to be toxic to neurons in culture and *in vivo* (4–8), suggesting a causal relationship between the accumulation of Aβ and the neurodegeneration found in Alzheimer's disease.

The neurotoxic effects of the Aβ have been linked to its ability to aggregate and form insoluble β-sheets in aqueous solutions (9, 10). Less clear, however, are the precise cellular events that underlie Aβ neurotoxicity. In this regard, it has been demonstrated that high concentrations of Aβ are able to render central neurons more vulnerable to glutamate neurotoxicity and glutamate-induced Ca^{2+} overloading (11, 12). Recently, evidence has been presented that Aβ may also produce its toxic effects by generating reactive oxygen species such as hydrogen peroxide or other toxic free radicals, leading to an increased oxidative stress (13–17). Indeed, this may be a mechanism shared by several neurotoxic peptides (18).

It is also not clear from previous reports in the literature whether Aβ neurotoxicity involves a "necrotic" or an "apoptotic" pathway. There is evidence for both mechanisms (8, 19–22). There is great interest in developing neuroprotective agents that can inhibit Aβ toxicity. Substances that influence

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ABBREVIATIONS: Aβ, β-amyloid peptide; $[Ca^{2+}]_i$, internal free calcium concentration; DMSO, dimethylsulfoxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEt, hydroethidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; R-123, rhodamine-123; TGF-β1, transforming growth factor-β1; TTX, tetrodotoxin; Ad-CABP-1, replication-defective calbindin D_{28k}-expressing adenovirus; Ad-SOD-1, replication-defective Cu/Zn-superoxide dismutase-expressing adenovirus; AdLacZ, replication-defective β-galactosidase-expressing adenovirus; SOD, superoxide dismutase; DCF, dichlorofluorescein.

pathophysiological processes involved in both necrotic and apoptotic types of neuronal injury may be of particular interest. We have recently shown that the multifunctional cytokine TGF-β1 is able to prevent NMDA-induced Ca^{2+} overloading and neurotoxicity in hippocampal neurons and to protect against oxidative injury and apoptosis resulting from trophic factor deprivation (23, 24). We were therefore interested in determining whether and, if so, by what mechanisms TGF-β1 was able to reduce Aβ neurotoxicity. Our results demonstrate that TGF-β1 is very effective at protecting against Aβ-induced neurotoxicity and further define the roles of Ca^{2+}, reactive oxygen species, and mitochondrial function in this process.

Experimental Procedures

Materials. Aβ 25–35 was purchased from Sigma Chemical Co. (St. Louis, MO). Aβ 1–40 and Aβ 35–25 were gifts from Bayer (West Haven, CT). Aβs were dissolved as 1000× stocks in DMSO (12). No "aging" of Aβ solutions was necessary to produce acute effects or for cell mortality. Recombinant human TGF-β1 was obtained from R & D Systems (Minneapolis, MN) and was prepared as a 1000 ng/ml stock in PBS containing 1 mg/ml ovalbumin and 4 mM HCl. Dizocilpine, 6-cyano-7-nitroquinoxaline-2,3-dione, NMDA, and TTX were obtained from RBI (Natick, MA); nimodipine was from Bayer; and 2-mercaptoethanol, FCCP, and ascorbic acid were from Sigma. R-123 and HEt were purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies specific for Bcl-2 and Bax oncoproteins have been described in detail elsewhere (25, 26). Rabbit polyclonal antibody specific for Bcl-x has been described by Gottschalk *et al.* (27). The specificity of the antisera was confirmed by immunoblot, immunoprecipitation, immunocytochemical, and peptide competition assays.

Cell culture. Primary hippocampal neurons were dissociated from E17 Holtzman rat embryos as described previously (28). The neurons were plated onto poly-L-lysine-coated, 15-mm glass coverslips and were maintained above a layer of secondary astrocytes in serum-free, N2.1-supplemented minimal essential medium. Cells were cultured in a humidified atmosphere of 5% carbon dioxide/95% air at 37°. Non-neuronal proliferation was halted by the addition of 10 μM cytosine arabinofuranoside after 3–4 days of cultivation. Animal care was performed according to university guide lines.

Aβ neurotoxicity. Aβ neurotoxicity was induced in neurons after 5 days in culture by adding Aβs into the culture medium, followed by gentle agitation. Controls were treated with the vehicle (DMSO; 0.1% v/v). Cell viability was determined 5 days later by two different methods: morphology and Trypan blue staining. Morphologically, viable neurons were identified as having round-to-oval, phase-bright cell bodies; smooth appearance; and intact neurites. Damaged neurons were identified as having shrunken, irregularly shaped cell bodies; rough appearance; and dystrophic neurites with "blebs." For Trypan blue exclusion, the cultures were incubated in 0.4% Trypan blue in HBS for 5 min. Blue-stained neurons were considered to be damaged. A total of 300–600 neurons were counted on three or four randomized subfields per coverslip. After exposure of the hippocampal cultures to Aβ 25–35 (1 μM) for 5 days, we found that in a direct comparison the two methods yielded comparable results (cell viability of 48.1 ± 2.2% by morphology compared with 52.4 ± 2.1% by Trypan blue exclusion; $p > 0.1$; four experiments). For comparison of the data, cell viability shown in Tables 1–3 and Figs. 1 and 2 was determined by the dye exclusion methods and is expressed relative to cell viability of DMSO-treated controls. Cell viability of DMSO-treated controls ranged from ~80% to 90%. Each toxicity experiment was repeated at least twice on cells from a different plating.

Drug treatments were performed either as a single treatment concurrent with the addition of Aβ or as repetitive treatments. For the latter, drugs were added concurrently with the addition of Aβ, as well as 24, 48, 72, and 96 hr afterward, without replacement of the culture medium. Controls were treated with the respective vehicle. All coverslips were turned face-up for these experiments.

Construction of replication-defective Ad-CABP-1 or human Ad-SOD-1 and infection protocol. The construction of Ad-CABP-1 has been reported previously (29). Briefly, the full-length chicken calbindin D_{28k} cDNA was obtained from Dr. D. Dowd (St. Louis University Medical Center, St. Louis, MO), and a blunt-ended *Ban*II/*Spe*I fragment of calbindin D_{28k} cDNA was inserted into the *Eco*RV

TABLE 1

Aβ neurotoxicity in cultured rat hippocampal neurons: concentration-toxicity relationship

Aβ neurotoxicity was induced in cultured rat hippocampal neurons after 5 days *in vitro* by exposure to Aβ 25–35 (0.01–10 μM). As controls, cells were exposed for 5 days to the vehicle (DMSO) or to Aβ 35–25 or received no treatment. Five day slater, cell viability was determined by Trypan blue exclusion. For comparison of data, cell viability is expressed relative to cell viability of vehicle-treated controls (DMSO; 0.1% v/v). Data are mean ± standard error from *n* coverslips.

Treatment	Concentration	Cell viability	*n*
		%	
No treatment		100.2 ± 3.2	4
DMSO	0.1% (v/v)	100.0 ± 1.9	8
Aβ 35–25	1 μM	99.6 ± 1.2	4
Aβ 25–35	0.01 μM	95.2 ± 0.9	4
Aβ 25–35	0.1 μM	72.2 ± 3.5[a]	4
Aβ 25–35	1 μM	58.6 ± 2.1[b]	4
Aβ 25–35	10 μM	57.5 ± 2.7[b]	8

[a] $p < 0.01$, [b] $p < 0.001$, different from DMSO-treated controls (analysis of variance and Tukey's test). Experiment was performed in duplicate with similar results.

TABLE 2

Aβ neurotoxicity in cultured rat hippocampal neurons: time-response relationship

Aβ neurotoxicity was induced in cultured rat hippocampal neurons by an exposure to Aβ 25–35 (1 μM) for 24, 48, or 120 hr. Controls were exposed to the vehicle (DMSO). In each case, cell viability was determined by Trypan blue exclusion at a culture age of 10 days *in vitro*. Data are mean ± standard error from *n* coverslips.

Treatment	Time of treatment	Cell viability	*n*
	hr	%	
Control (DMSO)	24	100.0 ± 0.9	4
Aβ 25–35	24	88.7 ± 0.4[a]	4
Control (DMSO)	48	100.0 ± 1.4	4
Aβ 25–35	48	79.6 ± 3.0[b]	4
Control (DMSO)	120	100.0 ± 1.2	4
Aβ 25–35	120	52.5 ± 2.7[b]	4

[a] $p < 0.01$, [b] $p < 0.001$, different from respective DMSO-treated controls (*t* test). Experiments were performed in duplicate with similar results.

TABLE 3

Pharmacology of Aβ neurotoxicity

Aβ neurotoxicity was induced in cultured rat hippocampal neurons after 5 days *in vitro* by a 5-day exposure to 1 μM Aβ 25–35. Data are mean ± standard error from *n* coverslips in three separate experiments.

Treatment	Concentration	Cell viability	*n*
		%	
Control (DMSO)	0.1% (v/v)	100.0 ± 1.9	12
Aβ 25–35	1 μM	57.2 ± 2.5[a]	12
Aβ 25–35 + ascorbic acid	300 μM/day	86.0 ± 3.6[d]	4
Aβ 25–35 + 2-mercaptoethanol	10 μM/day	93.9 ± 1.6[d]	4
Aβ 25–35 + dizocilpine	1 μM	36.1 ± 3.0[b]	4
Aβ 25–35+ 6-cyano-7-nitroquinoxaline-2,3-dione	1 μM	63.7 ± 1.8	4
Aβ 25–35+ TTX	0.5 μM	45.5 ± 1.2[b]	4
Aβ 25–35+ nimodipine	1 μM	82.8 ± 2.4[c]	4

[a] $p < 0.001$ different from DMSO-treated controls, [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.001$, different from Aβ 25–35-treated cells (analysis of variance and Tukey's test).

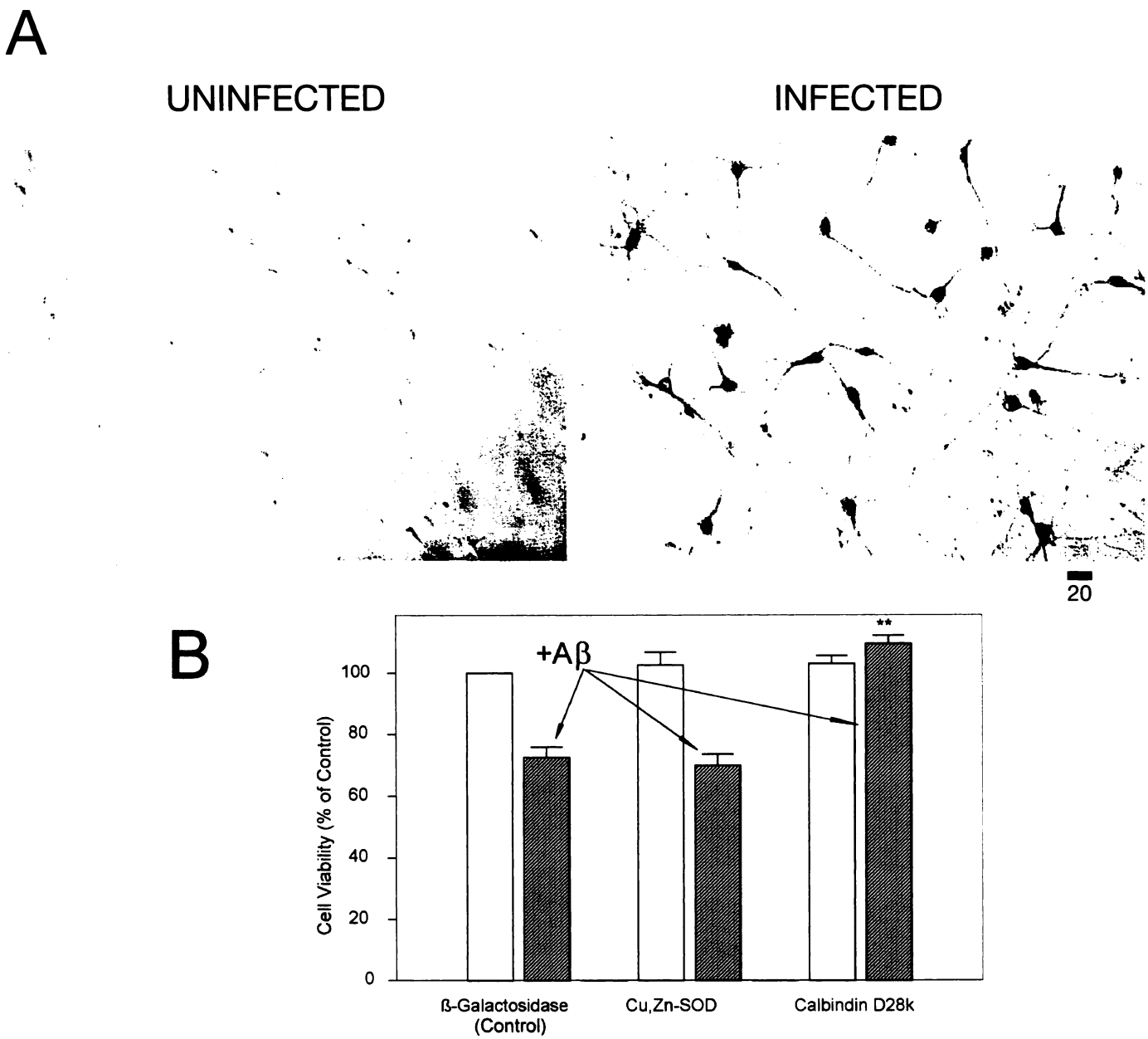

Fig. 1. Effect of calbindin D_{28k} and Cu/Zn-SOD overexpression on neurotoxicity induced by Aβ 25–35 in rat hippocampal neurons. A, Immunocytochemical demonstration of human Cu/Zn-SOD overexpression in cultured rat hippocampal neurons with the use of adenovirus-mediated gene transfer. Cells were infected with 100 M multiplicities of infection of the virus and stained for protein expression 48 hr later. Left, lack of staining in uninfected cultures. Right, positive staining for human Cu/Zn-SOD in ~100% of the hippocampal neurons. Similar levels of calbindin D_{28k} and β-galactosidase overexpression were achieved as previously reported (29). *Scale bar*, 20 μm. B, Effect of β-galactosidase (*Control*), Cu/Zn-SOD, and calbindin D_{28k} overexpression on Aβ neurotoxicity induced by a 5-day exposure to Aβ 25–35 (1 μM). Cultures were infected at 3 days *in vitro* with 100 multiplicities of infection of the viruses. At 48 hr later, cultures were exposed to Aβ 25–35. Data are mean ± standard error from six coverslips. **, Different from control (AdLac*Z*-infected, Aβ-treated) cultures ($p < 0.01$). Experiment was performed in triplicate on cells from different platings and yielded similar results.

site of transfer vector pAdKN downstream from elongation factor 1α and upstream from the cellular heavy chain enhancer and bovine growth hormone polyadenylation site. The pAdKN plasmid also contained 0–1 and 9–16 map units of DNA sequence of adenovirus 5. For construction of the virus, the plasmid was linearized and cotransfected with replication-defective adenovirus type 5 (sub 360) DNA into human embryonic kidney cells (HEK 293 cells), a *trans*-complementing cell line for E1 function (30), using the calcium phosphate precipitation method. Purification of the virus was performed as described previously (29).

Construction of Ad-SOD-1 was similar to construction of Ad-CABP-1 and has been reported by Jordan *et al.* (31). Ad-SOD-1 contained a blunt-ended *PstI/NheI* fragment of a human Cu/Zn-SOD cDNA clone obtained from American Type Culture Collection (32). As a control, we used AdLac*Z* (for a description, see Ref. 33).

Cultured rat hippocampal neurons were infected after 3 days of cultivation, similar to the procedure of Chard *et al.* (29). The coverslips were removed from the astrocyte feeding layer and placed into a 60-mm tissue culture dish in astrocyte-conditioned, N2.1-supplemented minimal essential medium. An aliquot (typically 1 μl for Ad-CABP-1 and Ad-SOD-1) of high titer virus yielding a multiplicity of infection of 100 was then added to the culture medium. The dish

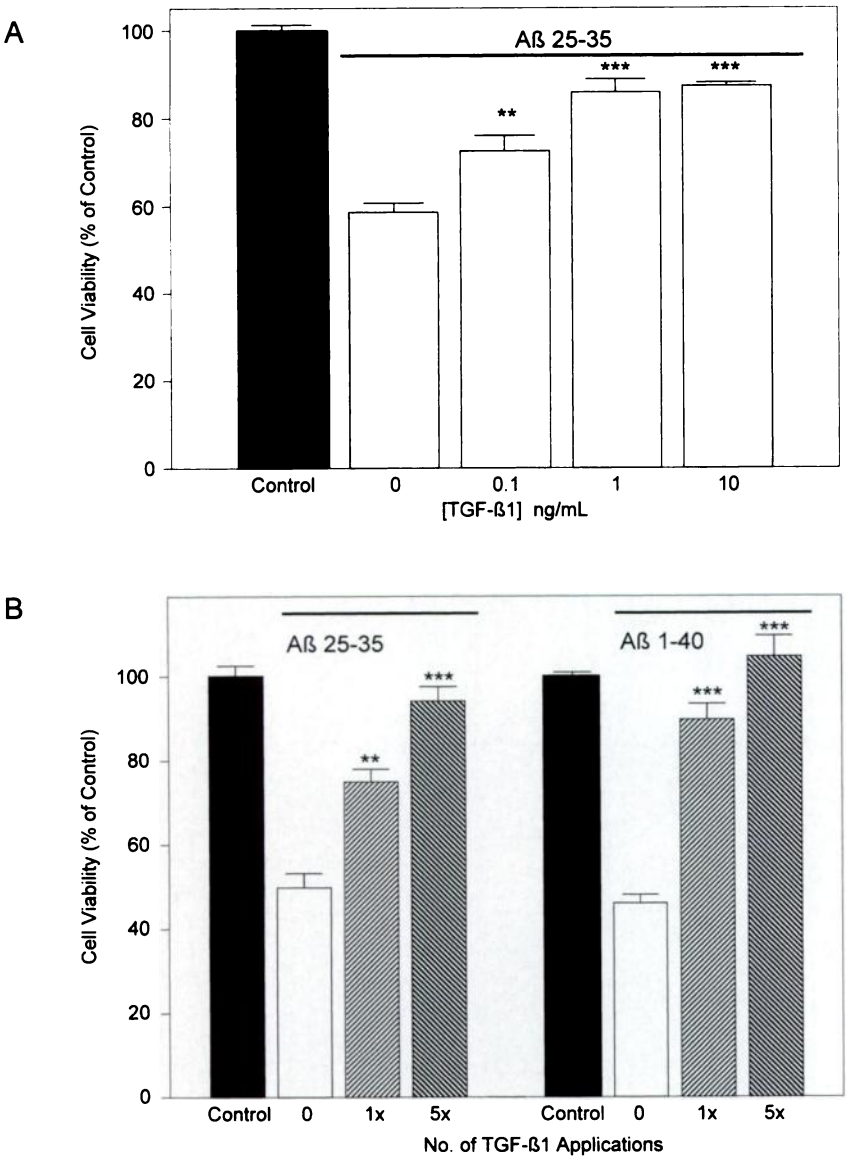

Fig. 2. TGF-β1 protects against Aβ neurotoxicity in cultured rat hippocampal neurons. A, Concentration-protection relationship. After 5 days *in vitro*, cultured rat hippocampal neurons were exposed for 5 days to the toxic fragment of Aβ, Aβ 25–35 (1 μM), or the vehicle DMSO (*Control*). TGF-β1-treated cultures received a single dose of TGF-β1 (0.1–10 ng/ml) concurrent with the exposure to Aβ. Data are mean ± standard error from four coverslips. **, $p < 0.01$; ***, $p < 0.001$ (analysis of variance and Tukey's test), different from Aβ 25–35-treated controls. Experiment was performed in duplicate on cultures from different platings and yielded similar results. B, Repetitive treatments with TGF-β1 (10 ng/ml/day) prevented neurotoxicity in rat hippocampal cultures induced by exposure to Aβ 25–35 or Aβ 1–40. Aβ neurotoxicity was induced by a 5-day exposure to either Aβ 25–35 (1 μM) or Aβ 1–40 (1 μM). Treatment with TGF-β1 was performed either as a bolus treatment (1 × 10 ng/ml), concurrent with the addition of Aβs, or as a repetitive treatment (5 × 10 ng/ml/day). Data are mean ± standard error from four coverslips. Experiment was repeated twice on cultures from different platings performed with similar results. **, $p < 0.01$; ***, $p < 0.001$ (analysis of variance and Tukey's test), different from Aβ-exposed controls.

was gently agitated and placed into an incubator for 2 hr. Then, the coverslips were returned to the original tissue culture dish; the cells were used 48 hr later.

In each toxicity experiment, successful expression of the respective proteins was verified by immunocytochemistry (one coverslip per experimental condition). For this purpose, cultures were fixed with 4% paraformaldehyde in culture medium at 37° for 15 min. After three washes in PBS, cells were permeabilized with 0.1% Triton X-100 (Eastman Kodak, Rochester, NY) in PBS for 2.5 min. The coverslips were then incubated for 1 hr in blocking medium (0.1% Tween-20, 4% bovine serum albumin in PBS) at room temperature. Incubation with primary antibodies (Sigma) was performed overnight at 4° with murine monoclonal antibodies specific for human Cu/Zn-SOD (1:300), calbindin D_{28k} (1:1000), and β-galactosidase (1:1000) diluted in blocking medium. Primary antibodies were detected using anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in blocking medium, followed by Vectastain ABC Kit (Vector, Burlingame, CA). Peroxidase was visualized using diaminobenzidine as the chromogenic substrate.

We previously demonstrated the expression of the respective recombinant proteins by protein gel electrophoresis after adenovirus infection of cultured rat hippocampal neurons (calbindin D_{28k}; Ref. 29) and in cultured rat sympathetic neurons (human Cu/Zn-SOD; Ref. 31). We have also shown that the expressed proteins were functional with the use of a $^{45}Ca^{2+}$-binding assay of cell lysates (calbindin D_{28k}; Ref. 29) and SOD activity gels with the use of the Nitroblue tetrazolium method (human Cu/Zn-SOD; Ref. 31).

Microfluorimetry. Digital videomicrofluorimetry was performed as described previously (34). Mitochondrial potential was monitored through measurement of R-123 fluorescence and according to methods described by Duchen (35; see also Ref. 24). Cells were loaded for 2 min with 10 μg/ml R-123 and washed for 5 min with HBS before monitoring of fluorescence intensity. The formation of reactive oxygen species was monitored with the probe HEt, which appears to selectively detect superoxide anion production in cells (36, 36a). Under assay conditions, the dye was not sensitive to H_2O_2, nitrogen radicals, hydroxyl radical, or perchlorate. HEt was dissolved in degassed DMSO as a 10× stock and stored at −70° packed under N_2 gas. The dye was added to the extracellular solutions (HBS) at a concentration of 1 μg/ml during the entire experiment. The increase in fluorescence intensity of its oxidized form, ethidium, was determined with the use of imaging microfluorimetry of the cell somas under rhodamine optics. Slopes for fluorescence intensity increases in each neuron were fitted by linear regression for each treatment. Experiments were conducted at room temperature.

Protein gel electrophoresis. Hippocampal neurons were plated onto plastic culture dishes, and perforated plastic coverslips containing a layer of secondary astrocytes were placed into the dish so that the astrocytes directly opposed the neurons. After a particular treatment, the coverslips containing the astrocytes were removed, the cultures were washed twice in ice-cold PBS, and whole-cell extracts were prepared in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (10 μM). After protein quantification (Pierce Protein Assay, Pierce, IL), extracts were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon PVDF membranes. Nonspecific protein binding was blocked in PBS containing 2% bovine serum albumin, 2% nonfat dry milk, and 0.1% Tween-20 for 2 hr. Bcl-2, Bax, and Bcl-x oncoproteins were detected using purified, polyclonal rabbit antisera specific for the respective proteins (1:1000 in blocking solution). After being washed with PBS, membranes were incubated with a secondary antibody (peroxidase-labeled goat anti-rabbit IgG, Promega, Madison, WI; 1:5000) in blocking solution, and the signal was detected using an Amersham enhanced chemiluminescence detection kit (Little Chalfont, England).

Results

Aβ toxicity to cultured rat hippocampal neurons. Exposure of rat hippocampal neurons for 5 days to a toxic fragment of Aβ (Aβ 25–35) produced a concentration-dependent decrease in neuronal viability that was maximal at concentrations of 1–10 μM (Table 1). In a study of the time course of Aβ toxicity in this system, we found that treatment of the hippocampal cultures with 1 μM Aβ 25–35 for 24 hr led to small, yet statistically significant, decreases in neuronal viability (Table 2). Aβ-induced degeneration was further enhanced after 48 and 120 hr of exposure, demonstrating that Aβ-neurotoxicity on cultured rat hippocampal neurons is a delayed, yet progressive type of degeneration. In contrast, exposure to the reverse peptide Aβ (35–25) or vehicle (DMSO) did not induce neuronal degeneration (Table 1).

Morphologically, neuronal injury was characterized by shrunken, irregularly shaped cell bodies; nuclear condensation; and cytoplasmic vacuolization (see also Refs. 19 and 22). With the use of terminal deoxynucleotidyl transferase-based labeling of double-stranded DNA breaks with digoxigenin/dUTP and detection of the reaction with an anti-digoxigenin antibody coupled to peroxidase, we could detect internucleosomal-type DNA fragmentation in 61.0 ± 6.7% of the neurons exposed for 5 days to 1 μM Aβ 25–35 (four experiments).

Antioxidants, but not modulators of synaptic transmission, protect against Aβ neurotoxicity. Previous reports have demonstrated that antioxidants have the capacity to protect neurons against Aβ-induced toxicity (13, 14, 16). We found that daily treatments with both the hydrophilic antioxidant ascorbic acid (300 μM) and the lipophilic antioxidant 2-mercaptoethanol (10 μM) exerted clear neuroprotection in hippocampal cultures concurrently exposed for 5 days to 1 μM Aβ 25–35 (Table 3).

In contrast, neither the selective NMDA receptor antagonist dizocilpine, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate /kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione, nor the Na^+ channel blocker TTX reduced Aβ neurotoxicity (Table 3).

The rate of production of superoxide anions is not increased by Aβ. The protective effects of antioxidants against Aβ neurotoxicity suggested that increased formation of free radicals may be a crucial event leading to the death of the neurons exposed to Aβ (see also Ref. 14). Reactive oxygen species such as hydrogen peroxide and extremely toxic hydroxyl radicals are frequently derived from the superoxide anion radical (37). Superoxide anions are generated by single electron reduction of molecular oxygen, mainly from enzymes of the mitochondrial respiratory chain, and are detoxified in animal cells by the activity of enzymes of the SOD family (37).

To determine whether superoxide anions are the basis of an increased oxidative stress in Aβ neurotoxicity, we monitored superoxide anion production with HEt-based microfluorimetry. However, we could not detect any difference in the basal rate of superoxide anion-dependent conversion of HEt to its fluorescent product ethidium between control cultures and cultures treated for 1, 2, 4, or 24 hr with Aβ 25–35 (1 μM) (Table 4). In contrast, exposure of the neurons to the glutamatergic agonist NMDA produced a 3–4-fold increase in superoxide production (Fig. 3; see also Ref. 38). In addition, NMDA-induced increases in the rate of superoxide production were not potentiated in cultures pretreated with Aβ 25–35 (Fig. 3 and Table 4).

The delayed, yet progressive degeneration of the hippocampal neurons after exposure to Aβ (Table 2) did not allow us to monitor the effects of longer periods of Aβ exposure (e.g., 48 or 120 hr) on superoxide anion production. Measurements after such longer treatments preferentially monitored the response of the Aβ-resistant neurons (data not shown).

Overexpression of Cu/Zn-SOD does not protect against Aβ neurotoxicity. In a second approach to determine whether an increase in the production of superoxide anions was associated with Aβ neurotoxicity, we infected cultured rat hippocampal neurons with Ad-SOD-1. The infection of the cultures with 100 multiplicities of infection of the recombinant virus led to a robust expression of human Cu/Zn-SOD, as detected immunocytochemically (Fig. 1A and Ref. 31). This method increased SOD activity (31). Expres-

TABLE 4

No increase in production of superoxide anions after treatment with Aβ

Cultures were treated with Aβ 25–35 (1 μM) for 1, 2, 4 or 24 hr. Then, the rate of superoxide anion production was monitored using HEt-based microfluorimetry (see Experimental Procedures). Data shown are slopes for the conversion of HEt (1 μg/ml in HBS) to its fluorescent, oxidized form, ethidium, and are given as intensity units/min. NMDA-stimulated superoxide anion production was performed in Mg^{2+}-free HBS containing 30 μM NMDA. HEt was present during the entire experiments at a concentration of 1 μg/ml. Data are means ± standard error from 11–25 neurons. Experiments were performed in duplicate (1-, 2-, and 4-hr treatments) or triplicate (24-hr treatments) on cultures from different platings with similar results. One-way analysis of variance did not yield a statistical significance in each experiment.

Treatment	Rate of fluorescence intensity increase	
	Baseline	NMDA
	arbitrary units/min	
Protocol 1: 1- 2-hr treatments		
Control (vehicle)	0.33 ± .03	0.56 ± 0.06
Aβ 25–35, 1 μM (1 hr)	0.28 ± .06	0.67 ± 0.1
Aβ 25–35, 1 μM (2 hr)	0.34 ± .03	0.54 ± 0.05
Protocol 2: 4-hr treatment		
Control (vehicle)	1.6 ± 0.2	6.8 ± 1.1
Aβ 25–35, 1 μM	1.5 ± 0.2	7.4 ± 1.7
Protocol 3: 24-hr treatment		
Control (vehicle)	2.4 ± 0.6	6.3 ± 1.7
Aβ 25–35, 1 μM	1.7 ± 0.2	4.2 ± 1.2

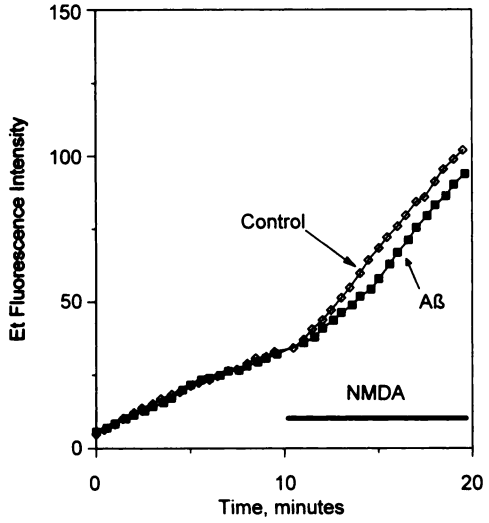

Fig. 3. HEt oxidation rates are not altered by Aβ 25–35. The selective oxidation of HEt to ethidium by superoxide anions was monitored in individual neurons with the use of digital imaging microfluorimetry. Mean basal rates in this example (9–11 experiments) were not significantly altered by 24-hr pretreatment with Aβ 25–35 (1 μM) ($p = 0.64$; t test). Applications of NMDA (30 μM in Mg-free HBS) increased the HEt oxidation rate by 2.8 ± 0.1-fold above the basal rate. This rate was not affected by Aβ 25–35 pretreatment ($p = 0.83$). HEt was present during the entire experiment at a concentration of 1 μg/ml. Fluorescence intensities are given as arbitrary units.

sion of human Cu/Zn-SOD could be obtained in ~100% of the neurons and was stable up to 16 days after infection, the longest time point examined (data not shown). In agreement with the lack of effect of Aβ on superoxide production, Cu/Zn-SOD-overexpressing neurons exposed to Aβ 25–35 (1 μM, 5 days) did not exhibit increased viability compared with control cultures infected with AdLac*Z* (Fig. 1B).

Overexpression of calbindin D_{28k} and nimodipine protect against Aβ neurotoxicity. Infection of the hippocampal cultures with another recombinant, replication-defective adenovirus was used to produce overexpression of the Ca^{2+} binding protein calbindin D_{28k} in ~100% of cells (29). Resting Ca^{2+} levels were not significantly affected by calbindin overexpression (data not shown). In contrast to the lack of protection afforded by SOD overexpression, expression of calbindin D_{28k} completely ameliorated Aβ neurotoxicity (Fig. 1B). This result suggested that Aβ involved disruption of Ca^{2+} homeostasis. This interpretation was further supported by the finding that the L-type Ca^{2+} channel antagonist nimodipine (1 μM) showed a neuroprotective effect against Aβ toxicity (Table 3).

TGF-β1 protects against Aβ toxicity. The previous data suggested a role for both oxidative stress and pathophysiological Ca^{2+} fluxes in Aβ neurotoxicity. TGF-β1 has previously been shown to protect central neurons against free radical- and Ca^{2+}-mediated degeneration (23, 24). A single treatment with the cytokine, concurrent with the initial addition of Aβ 25–35, significantly reduced the degree of subsequent neuronal degeneration (Fig. 2A). Studies of the dose-response relationship for TGF-β1 revealed that even concentrations of TGF-β1 as low as 0.1 ng/ml significantly protected against Aβ toxicity.

In contrast to single treatments, repetitive treatments with TGF-β1 (10 ng/ml/day) completely protected the hippocampal neurons against Aβ neurotoxicity (Fig. 2B). Similar results were obtained when neurotoxicity was induced with Aβ 1–40 (1 μM) (Fig. 2B).

Loss of mitochondrial potential on exposure to Aβ is prevented by TGF-β1. There is evidence from the literature that an early loss of mitochondrial function is involved in Aβ neurotoxicity (39, 40). With R-123-based microfluorimetry, we detected a significant loss in mitochondrial potential in cultures treated with 1 μM Aβ 25–35 for 24 hr (Table 5). Exposure to Aβ 25–35 also affected the ability of the mitochondria to recover from a metabolic challenge induced by exposure to the uncoupling agent FCCP (Fig. 4). The effects of Aβ 25–35 on mitochondrial potential and recovery from FCCP intoxication were completely reversed by treatment with TGF-β1 (10 ng/ml) (Table 5 and Fig. 4).

TGF-β1 induced up-regulation of Bcl-2 and Bcl-x oncoprotein expression. Neuronal cell death, especially if associated with programmed cell death or apoptosis, has frequently been shown to be regulated by a set of genes, including *Bcl-2*, *Bax*, and *Bcl-x* (41). We have previously shown that cultured rat hippocampal neurons express the Bcl-2 oncoprotein and that its expression is regulated by TGF-β1 (24). Rat hippocampal neurons in culture also expressed Bcl-x and Bax oncoproteins (Fig. 5). In the case of Bcl-x, we found that the long version of the protein, $Bcl\text{-}x_L$, was predominantly expressed, whereas little $Bcl\text{-}x_\beta$ and no $Bcl\text{-}x_s$ could be detected by Western blotting (data not shown). Treatment of rat hippocampal cultures with TGF-β1 (0.1–10 ng/ml) for 24 hr led to a pronounced increase in expression of the Bcl-2 and $Bcl\text{-}x_L$ oncoproteins (Fig. 5). Maximal effects of TGF-β1 on protein expression were observed at concentrations of 1–10 ng/ml (Bcl-2) or 0.1–1 ng/ml (Bcl-xL). In contrast, the expression of Bax protein, which inhibits the protective action of Bcl-2 (42, 43), remained unchanged on treatment with TGF-β1 (Fig. 5). In fact, TGF-β1 had no effect on Bax oncoprotein expression over a wide dose range of TGF-β1 that was tested (0.01–10 ng/ml) or under conditions of prolonged and repetitive treatments (data not shown).

Discussion

Several studies both in culture and *in vivo* have demonstrated the neurotoxic properties of Aβ and related peptides (4–8). In contrast, the molecular events that lead to Aβ neurotoxicity are not fully understood. We provide new evi-

TABLE 5

Exposure of rat hippocampal to Aβ results in a decline in R-123 fluorescence that is prevented by TGF-β1

Cultured rat hippocampal neurons were treated with vehicle (DMSO, control), vehicle plus TGF-β1 (10 ng/ml), Aβ 25–35 (1 μM), or Aβ 25–35 plus TGF-β1 for a period of 24 hr. After loading with R-123 (10 μg/ml; 2 min) and extensive washing, basal fluorescence intensity was monitored. Data are mean ± standard error from 12 separate experiments on cells from three different platings and are indicative of mean basal transmembrane potential within mitochondria.

Treatment	Fluorescence intensity	*n*
	arbitrary units	
Control (DMSO)	70.1 ± 2.1	110
Control (DMSO) + TGF-β1 (10 ng/ml)	93.6 ± 5.9[b]	25
Aβ 25–35 (1 μM)	57.5 ± 1.9[a]	136
Aβ 25–35 (1 μM) + TGF-β1 (10 ng/ml)	73.8 ± 3.1[b]	56

[a] $p < 0.001$, difference between Aβ25–35 and DMSO control. [b] $p < 0.001$, difference between TGF-β1-treated cultures and respective controls (Kruskal-Wallis H test and Dunn's test for nonparametric data).

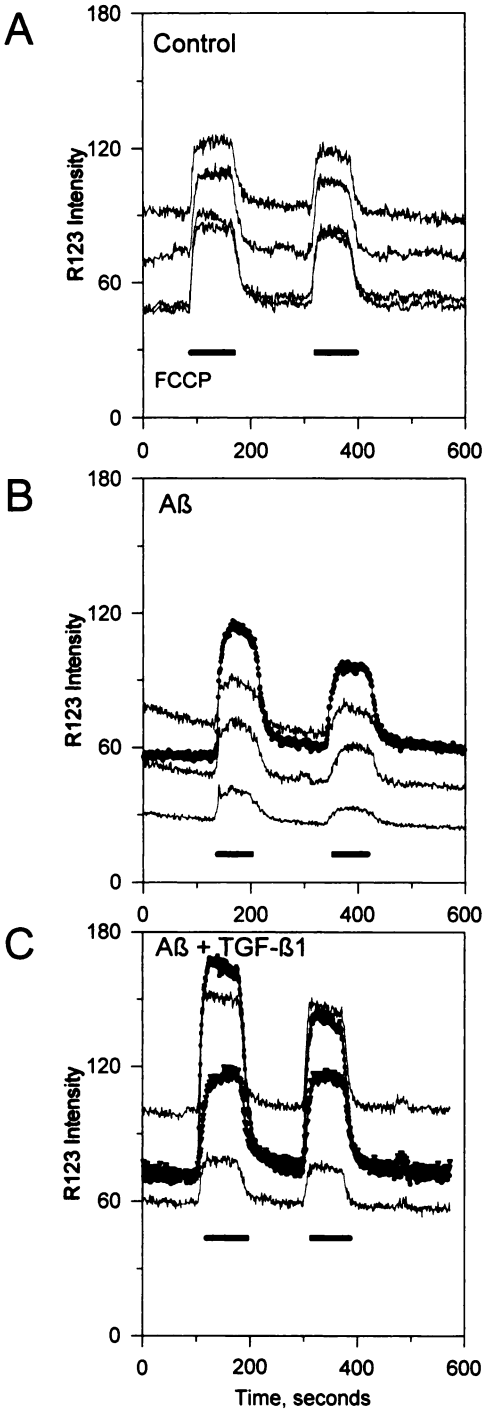

Fig. 4. Exposure to Aβ affects the ability of hippocampal neurons to recover from exposure to the mitochondrial uncoupler FCCP. A, Traces representative of four control neurons that have been exposed to the vehicle (DMSO) for 24 hr. Cells were loaded with R-123 and washed as described in Experimental Procedures. Under control conditions, R-123 is retained in mitochondria as indicated by a stable base-line. Note the increase in fluorescence intensity on exposure to FCCP (1 μM) due to depolarization of the mitochondrial membrane and release of R-123 ("unquenching"). A second exposure induces a similar, albeit smaller, increase in fluorescence. B, Traces representative of four neurons that have been exposed to Aβ 25–35 (1 μM) for 24 hr. Exposure to Aβ lowered basal R-123 fluorescence (see also Table 5). Note that two cells exhibit a decline in R-123 fluorescence base-line, indicating the loss of ability to retain the dye. Characteristically, exposure to FCCP leads to a lower release of R-123 compared with control cultures ($p < 0.01$ for both first and second FCCP exposures; Mann-Whitney *U* test for nonparametric data; 32 control neurons and 77 Aβ-treated neurons). Of note, the ratio of the peak fluorescence intensity during the first and second FCCP exposures (peak 2/peak 1) is significantly smaller in Aβ-treated cells than in controls ($p = 0.01$; *U* test), suggesting a significant loss of mitochondrial function caused by exposure to Aβ. C, Neurons exposed for 24 hr to Aβ plus TGF-β1 (10 ng/ml) showed stable base-lines and complete preservation of mitochondrial function after exposure to FCCP ($p < 0.01$ for release of R-123 during first and second FCCP exposures and for peak 2/peak 1 ratio compared with Aβ-treated neurons; 77 Aβ-treated and 56 Aβ/TGF-β1-treated neurons; *U* test). Traces of four typical neurons are shown.

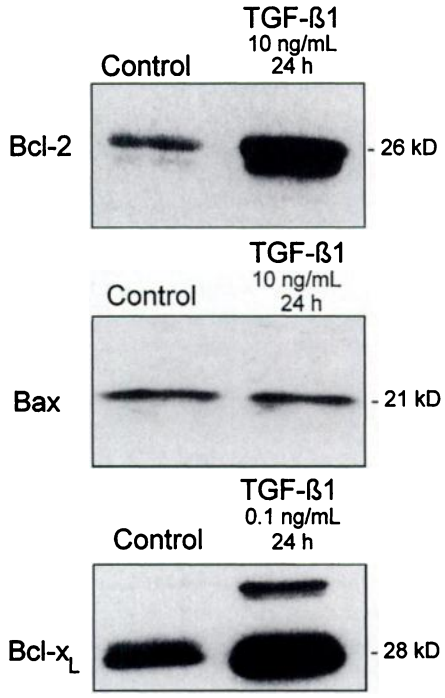

Fig. 5. TGF-β1 induces Bcl-2 and Bcl-x_L oncoprotein expression, whereas the level of Bax oncoprotein remains unchanged. Cultured rat hippocampal neurons were treated with TGF-β1 or vehicle for 24 hr. Western blots demonstrate that TGF-β1 increases the expression of both Bcl-2 and of the long-form of Bcl-x, Bcl-x_L, but not of Bax oncoprotein. Triplicate experiments yielded similar results.

dence that free radicals and Ca^{2+} ions are involved in Aβ neurotoxicity and that Aβ neurotoxicity is ameliorated by the protective actions of TGF-β1. In addition to the up-regulation of Bcl-2 protein expression, TGF-β1 led to increased Bcl-x_L expression and no change in Bax expression. TGF-β1 therefore increased the relative ratio of proteins opposing programmed cell death to those promoting it.

Involvement of reactive oxygen species in Aβ neurotoxicity. There is good evidence from a variety of sources that reactive oxygen species are involved in Aβ neurotoxicity. First, Aβ has been shown to generate peptidyl radicals and reactive oxygen species in aqueous solutions (15, 17). Second, Aβ neurotoxicity is associated with increased oxidative stress in neural cells (14, 16). Third, Aβ (25–35) can initiate lipid peroxidation via peptide free radicals (15). Finally, antioxidants have been shown to protect against Aβ neurotoxicity (13, 14, 16, 44, and present study; see also Ref. 45).

Less clear, however, are the precise biochemical reactions and cellular events involved in Aβ-induced oxidative stress. As mentioned above, reactive oxygen species, including hydrogen peroxide, are frequently derived from the superoxide

anion radical, which is primarily produced in mitochondria (37). Despite the protective effects of the antioxidants used in the present study suggesting free radical involvement in Aβ toxicity, we could not detect an increase in superoxide radical production in cells treated acutely for 1, 2, 4, or 24 hr with Aβ with HEt as a selective indicator. Aβ also did not potentiate NMDA-mediated superoxide production (Fig. 3). The lack of a protective effect of Cu/Zn-SOD overexpression further suggests that superoxide anions play a minor role in Aβ neurotoxicity. In this regard, it should be pointed out that overexpression of Cu/Zn-SOD using the same adenovirus construct strongly inhibited the death of cultured rat hippocampal neurons exposed to ionizing radiation[2] and the death of cultured rat sympathetic neurons on withdrawal of nerve growth factor (31). It therefore appears unlikely that in our system, Aβ-induced oxidative stress is caused by an increased production of superoxide anions (and eventually hydrogen peroxide; Ref. 37).

Behl *et al.* (14) reported an increased production of hydrogen peroxide or related peroxides on treatment of PC12 cells or cortical neurons with Aβ, primarily based on the interpretation of DCF fluorescence. DCF and similar fluorescein-based derivatives are commonly believed to be selectively oxidized by hydroperoxides, but other effective oxidants include lipid peroxides and hydroxyl radicals (46, 47). The indicator is also a high affinity substrate for xanthine oxidase (48). Very recent research shows that the oxyradicals produced by Aβ are not affected by SOD yet were reduced by catalase (49). It is therefore likely that the Aβ-mediated increase in DCF accumulation observed by Behl *et al.* was due to peroxyl radicals arising without intermediate superoxide production (14, 15). Other radical species may be formed in cells exposed to Aβ. In this context, it should be mentioned that Aβ binds with high affinity to lipids, has been shown to incorporate into lipid bilayers, and to initiate lipid peroxidation (15, 50, 51). However, it appears that the increase in oxidative damage observed on exposure of cells to Aβ is derived from sources other than superoxide anions.

Ca^{2+} and Aβ neurotoxicity. Acute treatment with Aβ has been shown to increase synaptic activity and $[Ca^{2+}]_i$ in cultured central neurons (12, 52, 53). However, these Aβ-induced, activity-dependent increases in $[Ca^{2+}]_i$ are not clearly associated with the neuronal degeneration reported here. In our system, the acute effects of Aβ on $[Ca^{2+}]_i$ are quite effectively blocked by inhibitors of glutamatergic synaptic transmission (53), whereas glutamate antagonists and TTX were ineffective in reducing Aβ neurotoxicity in the present study (Table 3; see also Ref. 54). Nevertheless, considering the protective effect of nimodipine on Aβ neurotoxicity reported here and elsewhere (55, 56), a role cannot be excluded for Ca^{2+} influx through voltage-sensitive Ca^{2+} channels or other Ca^{2+}-permeable channels in Aβ neurotoxicity (50). Although Ca^{2+} imaging studies with the fluorescent dye Fura-2 provided no evidence of deregulation of neuronal Ca^{2+} homeostasis in cultures pretreated with Aβ 25–35 (1 μM) for 24 or 48 hr,[2] it is possible that changes in subcellular Ca^{2+} store distribution were not recognized. Furthermore, an important role for Ca^{2+} in Aβ neurotoxicity was suggested by the protective effects of calbindin D_{28k} overexpression. The protection afforded by calbindin D_{28k} overexpression may not be solely associated with buffering of extracellular Ca^{2+} influx; it could be linked to a suppression of a phenomenon such as mitochondrial Ca^{2+} cycling, as described for certain forms of apoptotic cell death (57, 58).

Interestingly, recent reports have demonstrated that neurons containing a related Ca^{2+} binding protein, calretinin, are also more viable in Alzheimer's disease (59, 60). These results are also consistent with the observed sparing of GABAergic hippocampal interneurons in Aβ neurotoxicity and Alzheimer's disease, a neuronal population known to be rich in several Ca^{2+} binding proteins, including calbindin D_{28k} (61–63). Normally, 10–20% of the neurons in our culture system contain immunocytochemical staining for calbindin. Their presence may partially explain the apparent maximum observed in Aβ neurotoxicity dose-response assays (Table 1). Also, the physical state of the peptide has been shown to be a critical factor in inducing toxicity. It is possible that this state is affected by the peptide concentration or the amount of vehicle.

Effects of TGF-β1 on Aβ neurotoxicity. We recently demonstrated that the cytokine TGF-β1, which is synthesized in the brain in response to various insults (e.g., Ref. 64), can protect different kinds of neurons from Ca^{2+}-mediated excitotoxic injury, oxidative injury, and apoptosis after the removal of trophic factors (23, 24, 31). The protective effects of TGF-β1 were associated with stabilization of Ca^{2+} homeostasis and a large increase in Bcl-2 oncoprotein expression (24). In the present study, we demonstrated that Aβ neurotoxicity could be inhibited by single and prevented by repetitive treatments with the cytokine TGF-β1. Similar protective effects were recently reported for human fetal neurons (44). This protection may be due to TGF-β actions on Ca^{2+} homeostasis, on regulation of gene products associated with control of apoptosis (Bcl-2, Bcl-x), and on mitochondrial function.

Recent studies have suggested that the protective action of Bcl-2 depends on the concomitant level of Bax expression. Bax is a Bcl-2 homologue that counteracts the protective action of Bcl-2 (42, 43). We observed that in contrast to Bcl-2, the levels of Bax remained unchanged on treatment with TGF-β1. It is therefore conceivable that TGF-β1 affords protection against cell death, e.g., Aβ neurotoxicity, by increasing the Bcl-2/Bax ratio in hippocampal neurons (43). Although effects of TGF-β1 other than or in combination with those on Bcl-2 expression (and Bcl-x_L expression; see below) may contribute to its protective action, our observations are consistent with other recent evidence that Bcl-2 overexpression protects neurons against Aβ toxicity. Cortical cultures derived from transgenic mice overexpressing the human *Bcl-2* gene were significantly more resistant toward Aβ-induced neuronal degeneration than were cultures derived from control animals (65).

Bcl-x, a homologue of the *Bcl-2* gene, is highly expressed in the nervous system and has recently been cloned (66). Recent studies have demonstrated a key role for Bcl-x expression in the normal development of the nervous system (67). We have shown that TGF-β1 also up-regulates the expression of this protein. Treatment of rat hippocampal neurons with the cytokine increases the level of the long form of Bcl-x (Bcl-x_L), which has been reported to have a cytoprotective and neuroprotective effect as well (66, 68). Increased expression of both

[2] J. Jordán, M. F. Galindo, G. D. Ghadge, R. P. Roos, J. H. M. Prehn, and R. J. Miller, unpublished observations.

Bcl-2 and Bcl-x_L forms, changing the Bcl/Bax ratio, may be a mechanism by which TGF-β1 protects neurons from Aβ.

Of note, we observed a decrease in the mitochondrial potential of the hippocampal neurons on treatment with Aβ. Furthermore, mitochondria challenged with the uncoupler FCCP recovered less quickly (lower ratio of peak fluorescence for first and second FCCP applications; Fig. 4) in Aβ-pretreated cells, indicating decreased mitochondrial function. These data are consistent with the finding that Aβ causes a decrease in mitochondrial dehydrogenase activity in PC12 cells as determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-based redox activity assay (40). Indeed, both lipid peroxidation and pathophysiological Ca^{2+} fluxes are known to impair mitochondrial function (58). Immunocytochemical studies have demonstrated that Bcl-2 and Bcl-x_L are membrane proteins found in mitochondria and other cell organelles (25, 69, 70). Interestingly, cells overexpressing Bcl-2 have previously been shown to have an increased mitochondrial potential (71). Consistent with this idea, we observed an increased mitochondrial potential in hippocampal neurons treated with TGF-β1. TGF-β1 may thus afford protection against Aβ neurotoxicity to central neurons by increasing mitochondrial potential and function (Table 5 and Fig. 4).

Finally, it is interesting to note that TGF-β1 has been found to be associated with plaques in Alzheimer's brains (72). Furthermore, Bcl-2 protein expression in Alzheimer's brains was increased in neurons surrounding those that have died.[3] One attractive hypothesis, therefore, is that a diffusible factor is secreted in the course of neuronal degeneration that helps to preserve surrounding cells. Clearly, TGF-β1 could be one such factor.

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