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Temporal Relations Among Amyloid β-Peptide-Induced Free-Radical Oxidative Stress, Neuronal Toxicity, and Neuronal Defensive Responses

Servet M. Yatin,[1] Marina Aksenova,[2] Michael Aksenov,[3] William R. Markesbery,[3] Timothy Aulick,[1] and D. Allan Butterfield*[,1–3]

[1]Department of Chemistry and Center of Membrane Sciences, [2]Department of Pharmacology, and [3]Sanders-Brown Center on Aging, 121 Chemistry-Physics Bldg., University of Kentucky, Lexington, KY 40506-0055

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Abstract

Amyloid β-peptide (Aβ), the main constituent of senile plaques in Alzheimer's disease (AD) brain, is hypothesized to be a key factor in the neurodegeneration seen in AD. Recently it has been shown that the neurotoxicity of Aβ occurs in conjunction with free-radical oxidative stress associated with the peptide. In the present study, we investigated the temporal relations among the formation of Aβ-associated free radicals, the oxidative damage to, and the activation of antioxidant defense mechanisms in rat embryonic hippocampal neuronal culture subjected to toxic Aβ(25–35). Temporal electron paramagnetic resonance (EPR) spectroscopy results show that synthetic Aβ(25–35) forms free radicals rapidly after solubilization with a high signal intensity at initial time points. At those time points, neuronal toxicity and oxidative stress gradually increase as assessed by reduction of 3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide, trypan blue exclusion, formation of reactive oxygen species, and detection of protein carbonyl levels. The latter occurs before neurotoxicity. When the EPR signal intensity of Aβ solution decreases at later time points, neuronal toxicity levels off and remains the same until the end of the experiment. The oxidative-sensitive enzyme creatine kinase (CK) (brain isoform) (CK-BB) content increases at initial points of the Aβ treatment in correlation with the EPR signal to keep the CK activity constant, presumably to overcome the Aβ-induced oxidative insult. CK-BB content returns to normal levels by the end of the experiment. CK activity normalized to CK content implies the presence of inactivated CK molecules during the treatment. Both Mn SOD and Cu / Zn superoxide dismutase (SOD) mRNA levels show robust increases initially, which later return to control level with decreasing oxidative insult. These results are consistent

*Author to whom all correspondence and reprint requests should be addressed.

with the notion that Aβ(25–35) promotes a rapid free-radical oxidative stress to neurons, which respond by modulating various oxidative stress-handling genes.

Index Entries: Amyloid β-peptide; creatine kinase; free radical; oxidative stress; protein carbonyl.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles (Pearson et al., 1985; Perry et al., 1993), extracellular deposits of insoluble amyloid that form senile plaques (SPs) (Selkoe, 1989), and neuronal and synapse loss (Davies et al., 1987; DeKosky and Scheff 1990). Amyloid β-protein (Aβ) is a 39–43 amino acid long peptide derived from proteolytic processing of amyloid precursor protein (APP), a transmembrane glycoprotein that is expressed during normal cellular metabolism (Haass et al., 1992). An increasing amount of experimental and genetic evidence points to an essential role of Aβ in AD. Down's syndrome patients, who carry three copies of chromosome 21, which encodes APP, show AD pathology (Wisniewski et al., 1985). Mutations on APP encoding genes (chromosomes 19 and 21) are found in early onset AD patients (Goate et al., 1991; Murrell et al., 1991). Mutations in presenilin 1 and presenilin 2, which lead to early-onset AD, are associated with excess Aβ deposition and oxidative stress (Selkoe, 1996; Guo et al., 1998). Transgenic animals overexpressing APP also show increased Aβ content in the brain and exhibit oxidative stress (Games et al., 1995; Selkoe, 1996; Smith et al., 1998). Direct neurotoxicity of Aβ can be related to its ability to associate with the plasma membrane (Behl et al., 1992; Mattson et al., 1993; Butterfield et al., 1994; Etcheberrigaray et al., 1994; Butterfield, 1997; Koppal et al., 1998; Subramaniam et al., 1998). Ca^{2+} influx and disrupted cell membrane functions are found following Aβ addition to neuronal culture (Mattson et al., 1993, 1997a; Goodman and Mattson, 1994; Harris et al., 1995; Mark et al., 1995). Lipid peroxidation is induced by Aβ (Butterfield et al., 1994; Gridley et al., 1997; Koppal et al., 1998), as is protein oxidation (Harris et al., 1995; Subramaniam et al., 1998; Yatin et al., 1999). Cytoskeletal proteins are altered (Refolo et al., 1991), glutamate toxicity to neurons is enhanced (Yankner et al., 1990; Mattson et al., 1992), and the activity of several oxidatively sensitive enzymes is decreased (Chauhan et al., 1991; Smith et al., 1991,1994; Hensley et al., 1993, 1994a; Wallace, 1994; Yatin et al., 1998) by Aβ. All these effects are modulated by antioxidants (reviewed in Butterfield [1997]), consistent with Aβ-associated free-radical oxidative stress.

In vitro studies using synthetic peptide homologs of Aβ and its fragments showed that Aβ(25–35) retained the biological activity of Aβ(1–40) and Aβ(1–42) (Yankner et al., 1990; Abe and Kimura, 1996; Yan et al., 1996). Although the mechanisms responsible for neuronal loss in AD remain uncertain, recent evidence points out the involvement of oxidative stress (Smith et al., 1991; Friedlich and Butcher, 1993; Harman, 1993; Hensley et al., 1995b; Butterfield, 1997; Markesbery, 1997) in neurodegeneration in AD. Evidence for involvement of free radicals in AD includes the presence of elevated levels of protein oxidation (Hensley et al., 1995b; Schubert et al., 1995), lipid peroxidation products (Richardson, 1994; Lovell et al., 1997; Markesbery and Lovell, 1998), and oxidative damage to mitochondria (Sims et al., 1987; Beal, 1992,1995) in AD brains. Our laboratory reported enhanced protein oxidation in Aβ containing the SP-rich region of AD brain but not in SP-poor cerebellum (Hensley et al., 1995b). The ability of toxic Aβs to induce protein oxidation and inhibit the activity of the oxidation-sensitive enzymes is consistent with the concept that Aβ can act like a prooxidant (Hensley et al., 1994; Aksenov et al., 1997; Butterfield, 1997; Butterfield et al., 1997; Yatin et al., 1998). In addition to numerous reports supporting the free-radical hypothesis in Aβ toxicity, a sizable body of evidence demonstrates involvement of free radicals indirectly. For example, antioxidants and free-radical scavengers, such as propylgallate, t-butyl-α-phenylnitrone (PBN), vitamin E, and EUK-8, protect Aβ-treated cells from

cytotoxicity (Behl et al., 1992, 1994; Dyrks et al., 1992; Harris et al., 1995; Mark et al., 1995; Bruce et al., 1996; Davis, 1996; Ueda et al., 1997; Yatin et al., 1999).

As a defense mechanism, cells have evolved specific enzymes, one class of which is superoxide dismutase (SOD), for the detoxification of reactive oxygen species (ROS). In eukaryotic cells, there are two different intracellular SODs: cytosolic Cu/Zn SOD and mitochondrial Mn SOD (McCord, 1979). Although the toxic effects of free-radical-associated Aβ are well known, the temporal relation between the ability of Aβ to produce free radicals and the development of oxidative damage in neuronal cells is not well established. Evidence exists that cultured neurons can respond to Aβ toxicity by activation of a pattern of neuroprotective mechanisms (Barger et al., 1996; Behl and Sagara, 1997; Aksenov et al., 1998; Sagara et al., 1998). Recently, we reported that cultured rat hippocampal neurons respond to Aβ(1–40) oxidative impact by a temporal increase of Mn-SOD gene expression and an increase of CK-BB production (Aksenov et al., 1998). CK catalyzes the reversible transfer of phosphoryl groups between phosphocreatine pools and adenosine diphosphate (ADP), which buffers adenosine triphosphate (ATP) concentration and enables a constant supply of high-energy phosphoester bonds. The addition of Aβ to neuronal culture causes decreased ATP production (Zang et al., 1996). Energy-starved cells may be more susceptible to oxidative insult or less capable of repairing oxidative damage. Although the oxidative stress was shown to underlie the toxicity of different Aβ fragments (Davis, 1996; Mattson et al., 1997b), synthetic Aβ peptides are known to have different aggregation properties and different free-radical generating abilities (Hensley et al., 1995a; Atwood et al., 1998; Bush et al., 1998). Thus, the cellular responses to different synthetic Aβ can vary depending on their ability to generate free radicals. Although Aβ(25–35) is not found in vivo, insight into oxidative stress associated with the longer peptides Aβ(1–40) and Aβ(1–42) may be gained by study of the simpler 11-mer. Indeed, previous electron paramagnetic resonance (EPR) studies showed that Aβ(1–40) (Hensley et al., 1994) and Aβ(1–42) (Butterfield et al., 1998) generated free radicals.

In the present study, we investigated the temporal relation between free-radical formation in Aβ(25–35) solution by using EPR spectroscopy and peptide-mediated toxicity in terms of neuronal survival and markers of oxidative stress (level of protein carbonyls and formation of intracellular ROS). We also investigated the temporal cellular response to Aβ(25–35)-mediated oxidative stress in relation to free-radical formation by measuring the mRNA levels of the oxidative stress-handling enzymes Cu/Zn-SOD and Mn SOD, and of CK expression in a rat embryonic hippocampal culture system.

Materials and Methods

Peptides, Enzymes, and Other Chemicals

Synthetic Aβ(25–35) was purchased from AnaSpec (San Jose, CA) lot no. 5375. Peptides were stored in the dry state at 4°C. The protein carbonyl Oxyblot kit was purchased from Oncor, cat. no. S7150 (Gaithersburg, MD), and the CK activity kit was purchased from CIBA Corning (Palo Alto, CA). All other chemicals used were at the highest purity.

Neuronal Cultures

Neuronal cultures were prepared from 18-d-old Sprague-Dawley rat fetuses. Rat hippocampi were dissected and incubated for 15 min in a solution of 2 mg/mL of trypsin in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) buffered with 10 m*M* HEPES (Gibco, Grand Island, NY). The tissue was then exposed for 2 min to soybean trypsin inhibitor (1 mg/mL in HBSS) and rinsed three times in HBSS. Cells were dissociated by trituration and distributed to 60-mm^2 polyethlyleneimine-coated plastic culture dishes (Costar, Cambridge, MA). Initial plating densities were 75–100 cells/mm^2. At the time of plating, the culture dishes contained 2 mL of Eagle's minimum essential medium (MEM; Gibco) supplemented with 100 mL/L of fetal bovine serum (Sigma, St. Louis, MO), 1 m*M* L-glutamine, 20 m*M* KCl, 1 m*M* pyruvate, and 40 m*M* glucose. After a 5-h period to allow cell attachment, the original medium was removed and replaced with 1.6 mL of fresh medium of the same composition. After a 24-h period, 1.2 mL of

MEM was replaced with 1.0 mL of Neurobasal medium (Gibco) supplemented with 2% (v/v) B-27 (Gibco), 2 m*M* L-glutamine (Gibco), and 0.5% (w/v) D-(+) glucose. On the fifth day, two-thirds of the Neurobasal medium was replaced with freshly prepared Neurobasal medium of the same composition. Cultures were maintained at 37°C in a 5% CO_2/95% room air-humidified incubator at all times. Cultures were used for the experiments for between 9 and 11 d.

Experimental Treatment of Cultures

Synthetic Aβ(25–35) was dissolved in double-distilled water immediately before use at a concentration of 1 mg/mL. This stock solution was added to cultures to produce a final concentration of 15 μ*M*. The same volume of sterile deionized water was added to control cultures. Neuronal cultures were treated with Aβ(25–35) for 0, 1, 3, 6, 12, and 24 h.

EPR Spectroscopy and Spin Trapping

Aβ peptide was solubilized to a concentration of 1 mg/mL by addition of 50 m*M* phosphate-buffered saline (PBN)/150 m*M* PBS at pH 7.4. The peptide solution was placed into a 300-μL quartz EPR flat cell and incubated at 37°C for up to 24 h. EPR analysis was performed at the desired time points, including 0 h, and EPR spectra were obtained using a Burker (Billerica, MA) 300 EPR spectrometer equipped with computerized data acquisition software. EPR parameters were microwave power, 20 mW; modulation amplitude, 0.96 G; gain, 1×10^5, and conversion time, 10.28 ms.

Assessment of Neuronal Survival

Mitochondrial function and neuronal survival were evaluated by the [3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) reduction assay (Shearman et al., 1995) and trypan blue exclusion, respectively. MTT is reduced to blue formazan by mitochondrial dehydrogenases by living cells but not by dead cells (Mosmann, 1983; Green et al., 1984). Cells were incubated with MTT at 37°C for 4 h. After incubation the unreacted dye and the medium were removed by inverting the plate. Into each well 1 mL of 0.04 *M* HCl in isopropanol was added to solubilize MTT formazan. Absorbance was measured by UV-VIS spectrophotometer at a wavelength of 570 nm and a reference wavelength of 630 nm.

Trypan blue exclusion was performed by counting the neurons that internalized the dye. Cells were rinsed three times with 1 mL of PBS (pH 7.4). Trypan blue (0.4%, Sigma) was added to cells along with 300 μL of PBS and incubated for 10 min. Sixteen different microscopic areas were counted for uptake of trypan blue, an index of cell death. Data are given as percentages of corresponding vehicle-treated values.

Protein Carbonyl Assay (Oxyblot Method)

To determine the level of protein oxidation, an Oxidized Protein Detection Kit (Oxyblot, cat. no. S7150-Kit; Oncor) was used. This kit is based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990,1994).

The samples were treated with 20 m*M* DNPH in 10% trifluoroacetic acid and derivatization-control solution and incubated for 15–30 min. After derivatization and neutralization with 2 *M* Tris/30% glycerol (neutralization solution, Oxyblot Kit) and 19% 2-mercaptoethanol, cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Oxidized bovine serum albumin (BSA) with known concentration of carbonyls (20 nmol of carbonyls/mg of protein) were treated with DNPH and loaded as a standard (1 pmol of protein carbonyl per lane) with each set of the samples. For the standard preparation, the BSA (Standard for Gel Filtration Chromatography, cat. no. A3581; Sigma) was dissolved in double-deionized water at 2 mg/mL and oxidized by Fe^{2+}/H_2O_2 (100 μ*M*/1 m*M*) for 2 h at 37°C. The reaction was stopped with deferoxamine mesylate, and the small molecular weight substances were removed from the protein passage through a Sephadex G-25 desalting column. The concentration of carbonyl groups per milligram of protein was determined by colorimetric carbonyl assay (Levine et al., 1994). SDS-PAGE was performed in minislabs ($0.75 \times 60 \times 70$ mm, 12% total acrylamide) according to the method of Laemmli (1970). Following electrophoresis, proteins were transferred on nitrocellulose for further immunoblotting analysis. Western blotting was performed according to the procedure adapted from Glenney (1986). The transfer of

proteins on nitrocellulose after SDS-PAGE was completed in 2 h. The transfer buffer was Tris-Glycine pH 8.5 with 20% methanol. After the transfer, membranes were blocked in 3% BSA (in PBS with sodium azide 0.01% and Tween-20 0.2%) for 1 h at room temperature. Rabbit anti-DNP antibody from the Oncor Oxyblot Kit (1:150 working dilution) was used as a primary antibody. Secondary antibodies (anti-Rabbit IgG conjugated with alkaline phosphatase; Sigma) were diluted in the blocking solution 1:15,000 and incubated with the membrane for 1 h at 37°C. Membranes were washed after every step in washing buffer (PBS with 0.01% sodium azide and 0.2% Tween-20) for 10 min at room temperature. Washed membranes were developed using BCIP-NBT solution (SigmaFast tablets; Sigma). Western blots were analyzed using computer-assisted imaging (MCID/M4 software supplied by Imaging Research [St. Catharines, Ontario, Canada]). Data are given as percentages of corresponding vehicle-treated values.

ROS Measurement

Intracellular ROS were measured by a 2',7'-dicholorofluorescin diacetate (DCF-DA) assay (Chacon and Acosta, 1991) with some modifications to adapt it to fluorescence microscopy (Bass et al., 1983; LeBel et al., 1992). Cells were loaded with DCFH-DA (Molecular Probes, Eugene, OR) by incubating in the non-CO_2 incubator for 50 min in the presence 100 m*M* of the dye in an HBSS containing of 10 μ*M* HEPES and 10m*M* glucose. Once inside the cell, nonpolar DCF-DA was converted by esterases to the negatively charged 2,7-dichlorofluorescein, which because it is an anion, was trapped within the cell. Cells were washed three times with warm MSF buffer (140 m*M* NaCl, 4 m*M* KCl, 2.5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* HEPES, 10 m*M* glucose) and fluorescence visualization was accomplished using a confocal laser-scanning microscope (Molecular Dynamics, [Sunnyvale, CA] Sarastro 2000) coupled to an inver-ted microscope (Nikon, Tokyo, Japan). Fluorescence was excited at 488 nm and emission filtered using a 510-nm barrier filter. Cells were chosen randomly for scanning.

CK Activity

The CK-BB activity was detected by enzyme activity staining following nondenaturating gel electrophoresis (CIBA Corning, Palo Alto, CA). One microliter of crude cell extract (1 mg/mL total protein) was applied to each lane of 1.0% agarose Corning Multitrac CK Isoenzyme Gel/8 and electrophoresed in CK Isoenzyme Buffer (Corning) at 90 V for 20 min. The enzyme pattern was developed by layering the plates with CK reagent (Corning). The gel was incubated at 370C for 20 min, and the enzyme bands were visualized with ultraviolet light. The CK band was digitized, and data for Aβ-treated cultures were compared with data of control cultures.

Immunoreactive BCK Measurement

Primary polyclonal antihuman CK-BB antibodies (Burbaeva et al., 1992) were diluted in blocking solution and incubated with the membrane for 1 h at 37°C. The working dilution for the anti-BCK antibodies was 1:1000. Secondary antibodies (antirabbit IgG conjugated with alkaline phosphatase, Sigma) were diluted 1:30,000 in blocking solution and incubated for 1 h at 37°C. Membranes were washed after every step in washing buffer (PBS with 0.01% sodium azide and 0.2% Tween-20) for 10 min at room temperature. The washed membranes were developed using BCIP-NBT solution (SigmaFast tablets; Sigma).

RNA Isolation and Reverse Transcriptase-PCR Analysis for Cu/Zn SOD and Mn SOD mRNA

Total RNA was isolated from 500,000 cells using TRIzol (Gibco Life Technology) following the method of Chromczynski and Sacchi (1987). RNA concentration was determined by 260/280 absorbance measurements.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the Access RT-PCR kit (Promega, Madison, WI). Stock PCR reaction mixtures were prepared on ice and contained 1X AMV/Tfl reaction buffer, 1 m*M* $MgSO_4$, 0.2 m*M* of each dNTP, 50 pmol of each primer, and 5 U of AMV Reverse Transcriptase, 5 U of Tfl DNA polymerase. The stock solutions were separated into 24-μL aliquots, 1 μL (0.1 μg) of total RNA was added to each reaction tube, and samples were subjected to reverse transcription followed by various cycles of PCR. The use of multiple cycles

allowed us to determine the minimum number of cycles necessary to detect PCR product and thereby remain in the linear region of PCR. Negative control (no AMV reverse transcriptase added) was always performed to ensure the absence of DNA contamination. The sequences of the primers used in this study were as follows:

For Cu / Zn SOD
Sense primer 5'-GTGATCTCACTCTCAGGAGA-3'
Antisense primer 5'-TCATTTCCACCTTTGCCCAA-3'
Product size: 88 bp
For Mn SOD
Sense primer: 5'-AGATCATGCAGCTGCACCACA-3'
Antisense primer: 5'-GTTCTCCACCACCGTTAGGGC-3'
Product size: 204 bp

The optimized program for RT-PCR included the cDNA first-strand synthesis step (45 min at 48°C, 2 min at 94°C) and 22 cycles of amplification (90°C, 30 s of melting; 56°C, 1 min of annealing; 68°C, 2 min of elongation). Ten microliter aliquots of each RT-PCR reaction were analyzed by 12% PAGE. Gels were stained with SYBR Green I (Molecular Probes) and used for imaging analysis. The yield of specific products obtained using standard amounts (100 ng / 25 μL reaction) of total RNA isolated from Aβ(25–35)-treated cultures was compared with yields of specific products obtained using the same amounts of total RNA from control cultures.

Statistical Analysis

Statistical comparisons were made using analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons.

Results

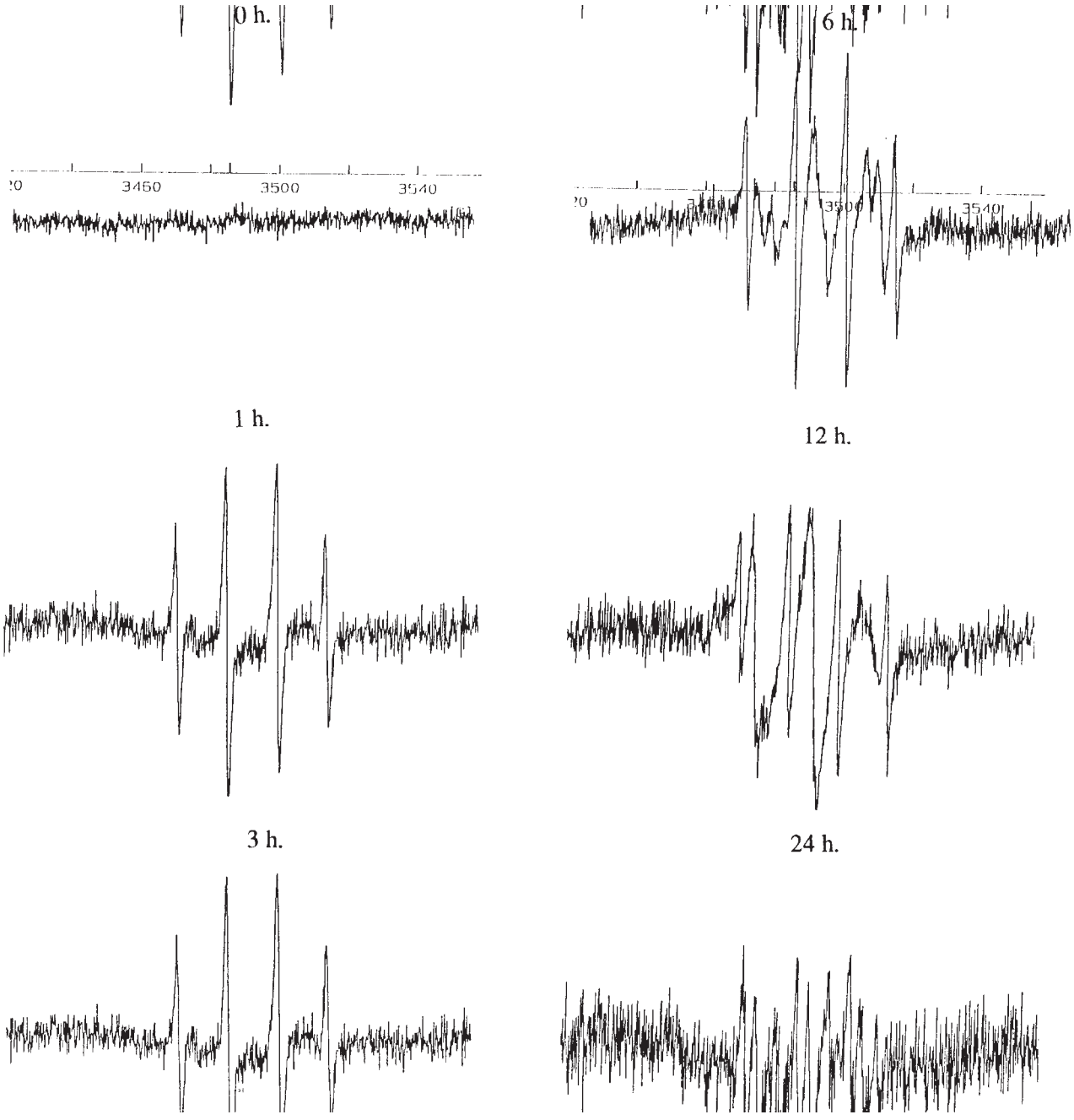
Spin-Trapping Studies

Because the only direct way to detect the presence of free radicals is EPR spectroscopy, we performed an EPR time-course spin-trapping study with Aβ(25-35) in the presence of the metal chelator deferoxamine. In spin trapping, a transient free radical reacts with a nonparamagnetic nitrone (PBN) to form a paramagnetic spin adduct (Butterfield, 1997). The presence of an EPR spectrum is *prima facia* evidence that a free radical was present (Butterfield, 1982). Toxic amyloid β-peptide gives either a three- or four-line EPR spectrum with PBN (Hensley et al., 1994, 1995a; Tomiyama et al., 1996). Figure 1 demonstrates the temporal progression of EPR spectra of Aβ(25–35) in PBN / PBS solution. The EPR spectrum of the spin trap PBN alone is EPR silent, i.e., gives no signal (data not shown), indicating that the radical originates from the peptide. Figure 1A demonstrates the spectrum of Aβ(25–35) at 0 h showing no signal. The first EPR signal appears after 1 h of incubation (Fig. 1B), suggesting that in contrast to longer times necessary for Aβ(1–40) or Aβ(1–42), Aβ(25–35) rapidly forms free radicals. The high-intensity four-line spectrum is conserved up to 6 h (Fig. 1C,D). However, starting from 6 h both the signal intensity and nature of the spectra change (Fig. 1D), and at 12 h the signal begins to diminish (Fig. 1E). At 24 h the EPR signal disappears almost completely (Fig. 1F), suggesting that the peptide is losing its ability to produce free radicals after 6 h for this specific peptide lot.

Neurotoxicity

To evaluate the peptide toxicity in relation to free-radical formation, the same time points used for EPR studies were used for the measurement of MTT reduction, which is an indicator of mitochondrial respiration, and trypan blue exclusion, which indicates cell membrane integrity. Both MTT reduction and trypan blue exclusion began to decrease after 1 h of treatment, reaching a maximum of 40% at 6 h and remaining the same afterward up to 24 h of treatment (Fig. 2). The absence of further neuronal death coincides with the diminishing EPR signal after 12 h. This result suggests, like the findings for Aβ(1–40) (Harris et al., 1995), that the peptide's ability to form free radicals is directly related to its neurotoxicity.

Fig. 1. *(opposite page)* Temporal EPR spectra of Aβ(25–35) PBN adducts: **(A)** Aβ(25–35) immediately after solubilization; **(B)** Aβ(25–35) after 1 h of incubation; **(C)** Aβ(25–35) after 3 h of incubation; **(D)** Aβ(25–35) after 6 h of incubation; **(E)** Aβ(25–35) after 12 h of incubation; **(F)** Aβ(25–35) after 24 h of incubation. All the incubations were in PBS solution containing deferoxamine.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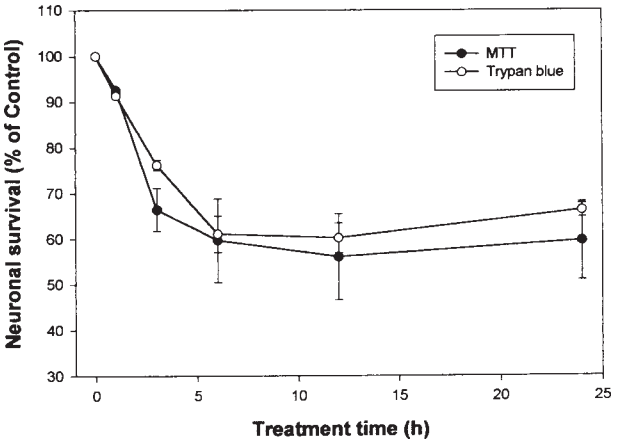

Fig. 2. Temporal neuronal survival after Aβ(25–35) treatment. Survival was assessed by the MTT reduction and trypan blue exclusion assays and expressed as a percentage of control. Statistical comparisons were made using ANOVA followed by Dunnett's test. Error bars represent SEM values. Each point is the average of three to four measurements.

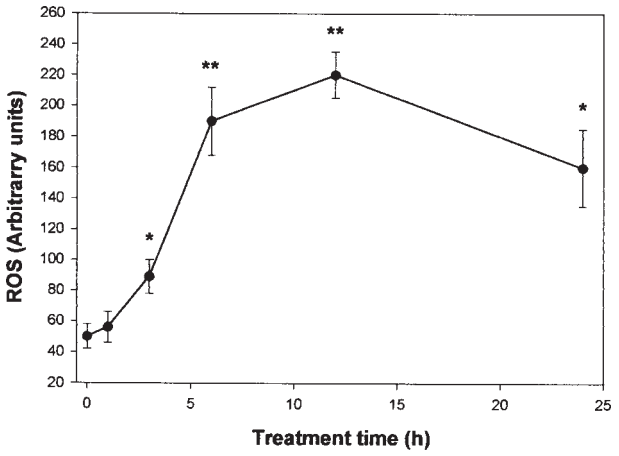

Fig. 3. Temporal formation of ROS, detected by the conversion of 2,7 dichlorofluorescin to 2,7-dichlorofluorescein (DCF), in neurons treated with Aβ(25–35). Statistical comparisons were made using ANOVA followed by Dunnett's test. Error bars represent standard error of the mean standard error of the mean (SEM) values. Significance at $*p < 0.0005$ and $**p < 0.005$ ($n = 3$; each n is the average of 9–11 neurons).

ROS Generation

ROS production for our study was assessed by the DCF-DA assay in cultures treated with 15 μ*M* Aβ(25–35). ROS began to increase after 1 h of treatment, reaching a significant twofold increase at 3 h and approaching a maximum increase of four times that of control at 12 h. Although after 12 h of incubation the Aβ peptide-derived EPR signal begins to diminish, surviving cells still have high amounts of ROS. However, at 24 h the ROS level is significantly lower than that of 12 h, consistent with the notion that 60% of the surviving neurons have increased ability to resist decreasing amounts of free radicals (Fig. 3).

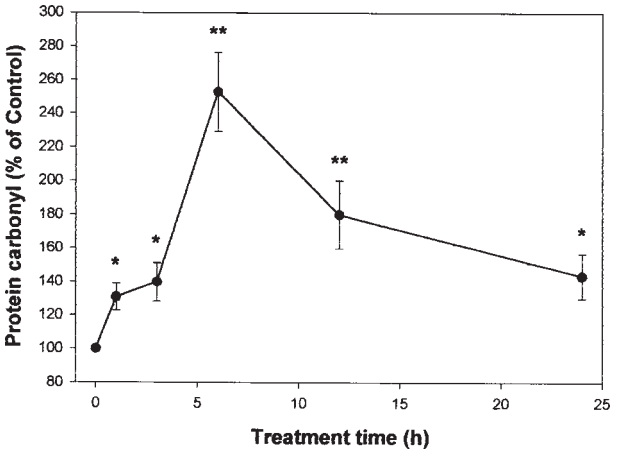

Fig. 4. The relative changes of protein carbonyl content in rat embryonic hippocampal neuronal culture treated with Aβ(25–35). Results are presented as an average for three different trials. For each trial, anti-DNP staining was performed three times and averaged $*p < 0.001$; $**p < 0.01$.

Protein Carbonyl Levels

Protein carbonyl levels began to increase soon after the addition of peptide to cell cultures (Fig. 4). After 6 h of treatment, the protein carbonyl level was 2.5 times greater than that of control, and 12 h of treatment, the protein carbonyl level was approximately twice the value of control. The control value was less than the control value at 6 h but still significantly higher than the control value of 3 h. At 24 h the protein carbonyl level decreased to 1.5 times greater than the control value. At 24 h neuronal survival was 60%, suggesting that survivor neurons maintain their protein carbonyl content. Both a decrease in neuronal survival and an increase in protein carbonyl began after about 1 h

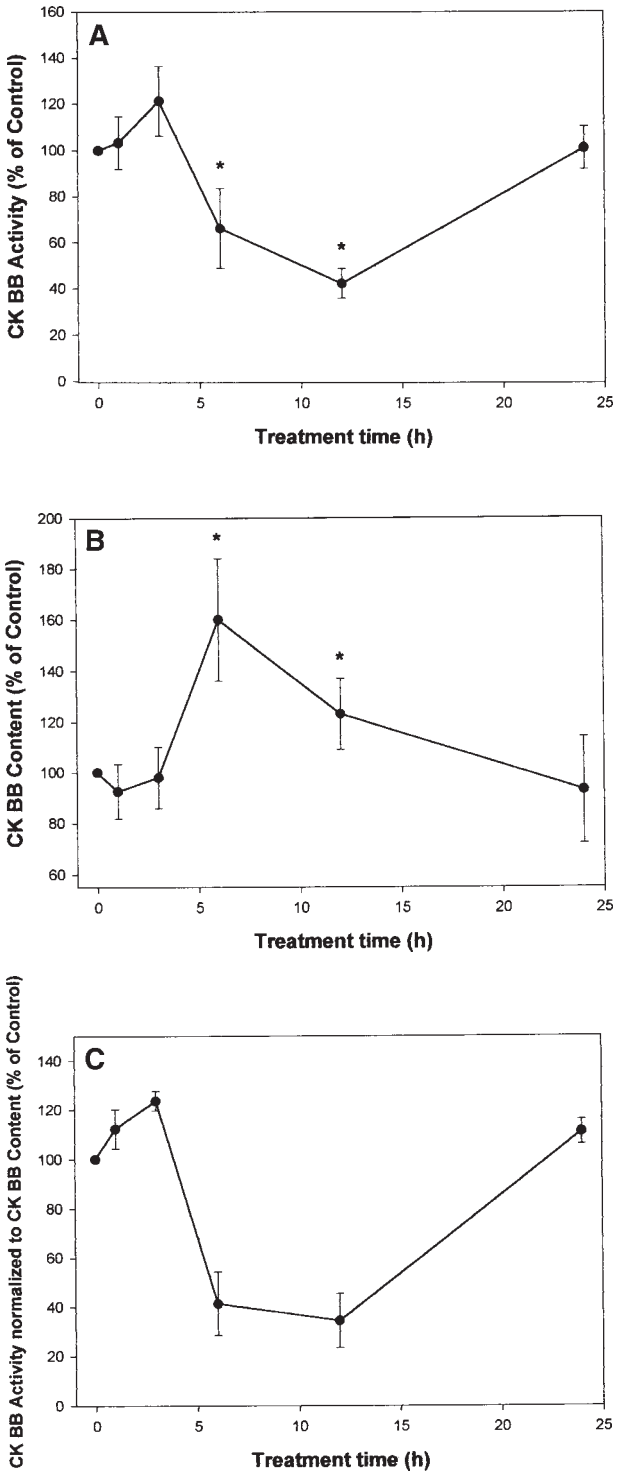

Fig. 5. **(A)** Temporal changes of CK-BB activity during Aβ(25–35) treatment. Results are presented as an average for three to four different trials. For each trial, fluorescent staining was performed twice and averaged. Statistical comparisons were performed using ANOVA followed by Dunnett's test. Error bars represent SEM values; *$p < 0.005$. **(B)** Relative temporal change of immunoreactive CK-BB content during Aβ(25–35) treatment. Results are expressed as a percentage of control ± SEM from four separate experiments; *$p < 0.001$. **(C)** CK-BB activity normalized for immunoreactive CK-BB content. Results are expressed as a percentage of control. Error bars represent ± SEM from 4 different trials.

of Aβ treatment, suggesting that Aβ(25–35) is rapidly and strongly toxic to cells, which correlates with the appearance of the EPR signal.

CK Studies

Because CK is a sulfhydryl-containing enzyme, it is susceptible to oxidative inactivation (McCord and Russell, 1988). Inactivation of thiol enzymes is considered to play an important role in oxidative cell injury. In our study, we measured the activity and immunoreactive content of the CK-BB. Figure 5A shows the change in CK-BB activity in neurons treated with Aβ(25–35). The CK activity gradually decreases to 40% of the control value by 12 h of exposure and then begins to increase, reaching the control value at 24 h. Figure 5B shows the immunoreactive CK-BB content. The CK-BB content increases gradually, reaching 220% of the control at 6 h and then decreases to the control level by the end of the experiment. When CK-BB activity is normalized to immunoreactive CK-BB content, the ratio found shows that inactive CK-BB is present in the cells during the exposure to the Aβ (Fig. 5C).

SOD Message Analysis

A sharp increase in both Mn SOD mRNA (Fig. 6A) and Cu / Zn SOD mRNA (Fig. 6B) expressions, reaching to 2.5 times that of control in 3 h of treatment, was observed. Mn- and Cu / Zn-SOD gene expressions returned to control levels after 6 h of treatment and stayed the same thereafter.

Discussion

This study found that the temporal responses to Aβ-generated free radicals in cultured neurons are related to the degree of free-radical produc-

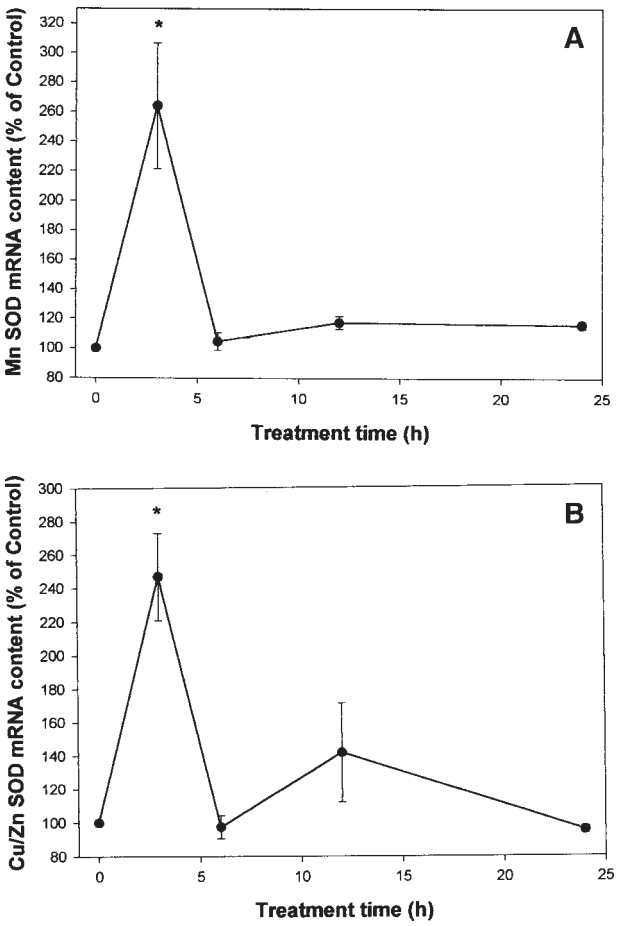

Fig. 6. Temporal change in the levels of **(A)** Mn SOD mRNA and **(B)** Cu/Zn SOD mRNA. Message was assessed by RT-PCR in Aβ(25–35)-treated cultured rat embryonic hippocampal neurons. Data show mean values (±SEM) from a single, representative experiment; *$p < 0.001$ vs control.

tion, as monitored by EPR signal intensity. Our results also suggest that cellular antioxidant defense mechanisms are activated in an attempt to overcome oxidative stress and that these defense mechanisms are time dependent. Previously it has been reported by us that the time course and intensity of the EPR signal produced by Aβ(1–40) correlate with the time course and magnitude of cell death and induction of antioxidant defense enzymes in hippocampal neuronal cultures (Harris et al., 1995; Aksenov et al., 1998). The time course for EPR signal and neuronal changes induced by Aβ(25–35) seen here follows the same trend as Aβ(1–40) (Hensley et al., 1994; Harris et al., 1995; Aksenov et al., 1998) but is faster, i.e., Aβ(25–35) aggregates immediately after solubilization and gives EPR signal as early as 1 h after solubilization. Differences in the mechanism of interaction with cell membranes between Aβ(25–35) and longer Aβ have been reported by Mattson et al. (1997b). It is as though the slower oxidative stress of the larger peptide may provide time for cellular responses to resist the stress. A similar consideration may apply to our studies relative to that of Aβ(1–40) (Aksenov et al., 1998) and as a function of time with Aβ(25–35). That is, with Aβ(25–35), a rapid, strong oxidative stress conceivably leads to rapid protein oxidation, ROS production, changes in CK activity and expression, and SOD expression. After sufficient time, ROS are still being generated, but the cell has time to respond to the oxidative stress in the surviving cells and less free-radical production is occurring. Such a scenario is consistent with Figs. 1–6.

The toxic properties of synthetic Aβ-peptides show lot-to-lot variation (May et al., 1992), as do EPR and glutamine synthetase (GS) toxicity properties (Hensley et al., 1995a). We classified synthetic Aβ-peptides into highly toxic three-line EPR spectral generating species; average toxic, four-line EPR generating species; and nontoxic, non-EPR generating species (Hensley et al., 1995a). The peptide used in the current study belonged to the middle group and showed a strong four-line EPR signal up to 6 h, which is well correlated with neuronal survival. With the diminishing EPR spectra at 12 and 24 h, cell survival remains the same as it was at 6 h, consistent with the foregoing concept.

ROS are highly reactive free-radical and non-free-radical molecules. The latter can easily lead to free-radical reactions. Our results demonstrate that an increased production of ROS continues to exist in neurons that survived the Aβ-induced oxidative injury. However, it seems likely that activation of compensatory mechanisms in neuronal survivors allows these neurons to maintain and gradually decrease the ROS production to normal level.

Oxidative stress can also be detected by measurement of protein carbonyl content, because protein carbonyl groups increase following oxidative insult by several mechanisms (Stadtman, 1992; Butterfield and Stadtman, 1997). ROS gener-

ated by Aβ-peptide can attack amino acid residues in proteins to produce carbonyl functionalities. Increased protein oxidation was found in regions of AD brain in which neurodegeneration is found (Smith et al., 1991; Hensley et al., 1995b). In the current study, neuronal protein carbonyls measured immunochemically show that protein oxidation begins as soon as cells are exposed to Aβ-peptide. Although the decrease in cell survival is not yet significant, the protein carbonyl level is significantly higher than that of control after 1 h of Aβ treatment, suggesting that protein oxidation is correlated with the presence of free radicals and that this measure of oxidative stress occurs even before cell death. Protein carbonyl levels began to decline after 6 h of treatment, paralleling the time point after which the EPR signal intensity also declines. Although neuronal survival remained the same after 6 h, protein carbonyl level continued to decrease, suggesting that surviving neurons may have successfully increased their antioxidant defense system, consistent with the foregoing notion. It is conceivable that this declining level might have returned to the control level had the experiment continued further.

In cultured neurons, CK-BB is the dominant isoform; in adult brain almost 90% of the total CK activity is associated with CK-BB. It has been demonstrated that CK is sensitive to oxidative modification (Oliver et al., 1990; Banerjee et al., 1991), and AD patients have decreased levels of phosphocreatine and ADP and an increased oxidative metabolic rate (Pettegrew et al., 1994). Increased protein oxidation and decreased oxidatively sensitive enzyme activities have been reported in aging and in AD (Smith et al., 1991; Hensley et al., 1995b). CK-BB activity gradually decreases following Aβ(25–35) exposure, reaching a minimum at 12 h, and then returns to control level by the end of the experiment. CK activity normalized to immunoreactive CK-BB content shows the presence of inactive CK-BB molecules during exposure to Aβ. Increased CK-BB content in response to the peptide helps restore the CK activity to overcome Aβ-mediated oxidative stress.

Overexpression of Cu/Zn SOD in transgenic mice confers resistance to ischemic brain injury (Kinouchi et al., 1991). We observed induction of Mn SOD and Cu/Zn SOD gene expressions at the early time points of treatment. Induction of expression of those antioxidant genes may be a cellular defensive response to Aβ-associated free-radical-induced oxidative insult. Tumor necrosis factor (TNF) protects hippocampal neurons against Aβ toxicity (Barger et al., 1995). TNF induces the expression of Mn SOD through a nuclear factor-κB (NF-kB) dependent mechanism (Cheng et al., 1994), and the induction of Mn SOD in hippocampal neurons may contribute to NF-κB-mediated protection against Aβ-peptide.

Our observations imply that oxidative injury is caused by Aβ-peptide-associated free radicals and/or ROS in neuronal culture. Increased protein oxidation, decreased neuronal survival, and the presence of inactive CK-BB molecules are related to the intensity of the formation of free radicals. Neurons respond by increasing CK-BB expression and Mn SOD and Cu/Zn SOD mRNA levels, which helps keep the amounts of protein carbonyl and CK activity at their normal levels. Our results point out the importance of antioxidant gene expression and the antioxidant enzyme mechanism in the protection of rat hippocampal neurons from Aβ(25–35)-associated free-radical oxidative insult. The implications to potential therapeutic intervention in AD need to be explored further.

We conclude that Aβ(25–35) as well as Aβ(1–40) (Aksenov et al., 1998) induce the activation of protective mechanisms in rat hippocampal neurons, which allow a significant part of neuronal population to adapt to Aβ-mediated oxidative stress. Following Aβ(25–35)-mediated oxidative insult stemming from Aβ-associated free radicals, neurons demonstrate an increase of protein oxidation and an increased intracellular ROS generation, a temporal decrease in CK-BB activity, and an induction of SOD gene expression. These findings are consistent with the notion that Aβ-associated free-radical oxidative stress is important in AD neurotoxicity (Butterfield et al., 1997).

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