Published in final edited form as:

Neurobiol Aging. 2008 December ; 29(12): 1815–1822. doi:10.1016/j.neurobiolaging.2007.05.001.

Neuroglobin protects PC12 cells against β -amyloid-induced cell injury

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Abstract

Excessive accumulation of amyloid beta (A β) has been proposed as a pivotal event in the pathogenesis of Alzheimer's disease. Possible mechanisms underlying A β -induced neuronal cytotoxicity include excess production of reactive oxidative species (ROS) and apoptosis. Neuroglobin (Ngb), a newly discovered globin in vertebrates that exhibits neuroprotective functions, may have a potential role in scavenging ROS. To examine the potential protective role of Ngb in A β -induced cytotoxicity, PC-12 cells were treated with A β (1–42 fragment) for 24 hrs. A β treatments increased ROS production in PC-12 cells. Overexpression of Ngb but not Ngb mutant in the PC-12 cells significantly attenuated A β -induced ROS production and lipids peroxidation. Furthermore, overexpression of Ngb also attenuated A β -induced mitochondrial dysfunction and apoptosis, and promoted cell survival. in PC-12 cells. Therefore, Ngb may act as an intracellular ROS scavenger, and such antioxidant properties may play a protective role against A β -induced cell injury.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder (Forman et al., 2004; Nordberg, 2004). Excessive accumulation of amyloid-beta (A β) peptide has been proposed as a pivotal event in the pathogenesis of AD (Hardy and Selkoe, 2002; Zheng et al., 2002), although the precise mechanism by which A β induces neuronal death is still unknown. Furthermore, the possibility has been raised that apoptosis may not play a mechanistic role in AD progression (Raina et al., 2001; Zhu et al., 2006). Possible mechanisms include excess production of reactive oxidative species (ROS) (Behl et al., 1994; Butterfield et al., 2002; Floyd, 1999; Hensley et al., 1994) and apoptosis (Caricasole et al., 2003; Martin et al., 2001). Several studies suggest that the oxidative stress play a key role in A β -mediated neuronal cytotoxicity by triggering or facilitating neurodegeneration through a wide range of molecular events which eventually lead to neuronal cell loss (Barkats et al., 2000; Coyle and Puttfarcken, 1993; Crack et al., 2006; Huang et al., 1999). Furthermore, it is well established that oxidative stress is involved in an apoptotic mechanisms by which excessive ROS

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Disclosure Statement: All authors declare no conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) our work.

production could lead to neuronal apoptosis in neurodegenerative disorders, such as A β -induced neuronal apoptosis (Fukui et al., 2005; Kadowaki et al., 2005; Li et al., 2004). Antioxidants have been shown a beneficial effect in neurodegenerative disorders (Calabrese et al., 2006; Coyle and Puttfarcken, 1993; Yu and Yang, 1996) and A β -induced neurotoxicity (Floyd, 1999; Koh et al., 2005; Sultana et al., 2004). Therefore, Antioxidants may merge as one of therapeutic strategies to treat A β -induced neurotoxicity and improve neurological outcome in AD.

Neuroglobin (Ngb) is a newly discovered vertebrate heme protein that is expressed in the brain and other neural tissues and can reversibly bind oxygen (Burmester et al., 2000; Moens and Dewilde, 2000; Trent, III et al., 2001). Ngb is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus, and cerebellum of the rat brain (Geuens et al., 2003; Reuss et al., 2002; Wystub et al., 2003). Recently, it has been suggested that Ngb plays a role in neuronal protection in response to hypoxia and ischemia (Khan et al., 2006; Sun et al., 2001; Sun et al., 2003; Venis, 2001). Ngb expression was reported to increase in response to neuronal hypoxia and after focal cerebral ischemia in vivo. However, the mechanism of this neuroprotection remains unclear. Moreover, recent study has point to that Ngb may act as a scavenger of toxic species, such as nitrogen monoxide, peroxynitrite and hydrogen peroxide (Herold et al., 2004). Since oxidative stress is involved in the neurodegenerative process in AD, and thus raise the possibility that Ngb may play a role in the cellular defense against oxidative stress induced by A β .

Therefore, we hypothesized that overexpression of Ngb will prevent or at least markedly attenuate A β -induced excess production of ROS/RNS and protect neuronal cells from oxidative stress-induced cell injury in AD.

2. Materials and Methods

2.1. Amyloid- β preparation

A β 1–42 peptide was purchased from Bachem (Torrance, California) and dissolved in 0.1 ml ammonium hydroxide (0.4 M). The peptide was further diluted in 10 mM sodium phosphate buffer to a final concentration of 0.1 mg/ml. This solution was stored as 50 μ l aliquots at –80 °C until needed. Prior to use each aliquot was placed at 37 °C for 24 h to induce soluble aggregates of the a- β peptide.

2.2. Cell Culture

PC-12 cells that were originally obtained from American Tissue Type Cell Collection (ATCC) were cultured on 100 mm plates coated with collagen. The collagen was diluted in 30% ethanol (1:10 dilution). The cells were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, and 1% antibiotics (penicillin/streptomycin). One day before the experiment, the cells were seeded in 6-well culture dishes (10⁶ per well). Following the transfection of Ngb and treatment of A β , cells were subject to ROS and MDA measurement, JC-1 assessment and MTT assay.

2.3. Ngb Plasmid Construct

To achieve high expression, a cDNA encoded with human wild type Ngb was synthesized using a modification of recursive PCR strategy. Ngb cDNA was subcloned into expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) with a cytomegalovirus promoter. The C-terminal was tagged with an in-frame V5 epitope for easy detection of Ngb fusion protein. The ligation products were introduced into E. coli strain XL-10 Gold ultracompetent cells (Stratagene, La Jolla, CA). The QuikChange system (Stratagene, La Jolla, CA) was used to introduce mutations into the Ngb coding sequence in pcDNA3.1-Ngb plasmid, which directed

the expression of Ngb with H64V/H96A mutation. These mutations were selected based on conformational role that they seem to play in their binding to heme iron (Uno et al. 2004). The inserted Ngb DNA and point mutations were verified by DNA sequencing. The functional overexpression of Ngb was confirmed by western blotting or immunohistochemistry using specific anti-Ngb or anti-V5 antibody. Ngb plasmid DNA was then transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection efficiency was determined by use of a plasmid encoding the β -galactosidase (pcDNA3.1-LacZ) compared with an empty vector control (pcDNA3.1).

2.4. Quantitative real-time RT-PCR

Total RNA was prepared from cortical tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Aliquots of total RNA (1 μ g) were reverse transcribed using random primers and Superscript II-Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA equivalent to 20 ng of total RNA were subjected to real-time PCR analysis (MX4000, Stratagene, La Jolla, CA) with the following primers and probes: Ngb: forward primer, 5'-GCCGCCAGTTCTCCAGTCC-3', reverse primer, 5'-CCTCCAGTGAAGACAGGTCCTC-3'; Taqman probe, 5'-ACAGCAGCATCAATCACAAGCA-3'; β -actin: forward primer, 5'-ACCCTGAAGTACCCCATG-3'; reverse primer 5'-TACGACCAGAGGCATACAG-3'; Taqman probe, 5'-ACGGCATTGTCACCAACTGGGA-3'. Standard curves for the target gene of interest (Ngb) and the housekeeping gene (β -actin) were performed for each assay. We have previously shown that β -actin mRNA expression is not significantly altered by either IH or SH exposures. Briefly, ten fold serial dilutions of control cDNA of target gene and house keeping gene were amplified by the MX-4000 PCR machine (Stratagene, La Jolla, CA). C_T Value (threshold cycle number) of each standard dilution was plotted against standard cDNA copy numbers. Based on the standard curves for each gene, the sample cDNA copy number was calculated according to the sample C_T value. Finally, each of the calculated copy numbers for Ngb was normalized against the corresponding β -actin copy numbers. Standard curves and RNA expression were analyzed using MX4000 software (Stratagene, La Jolla, CA).

2.5. Western Blotting

Transfected PC-12 cells were homogenized by standard procedures. Protein concentrations were determined using the Bradford protein assay. Homogenate proteins (50 μ g) were heated for ten minutes at 90°C and then loaded on 18% gradient PAGE gels, then transferred electrophoretically onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and incubated overnight at 4°C with the Ngb polyclonal antibody (1:2500, Biovador, Heidelberg, Germany). Ngb protein bands were detected with horseradish peroxidase-conjugated anti-chicken secondary antibodies and visualized by ECL reagents. Densitometric analysis was performed with a gel scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

2.6. Immunohistochemistry

PC-12 cells were fixed for 30 min in 10% neutral-buffered formalin solution at room temperature. The cells will be blocked for 1 hour at room temperature in fresh blocking buffer (5% normal goat serum in TBST). Following the blocking, the cells were incubated with Ngb polyclonal antibody (1:500, Biovador, Heidelberg, Germany) in TBS with 3% bovine serum albumin (BSA). After three washes with TBST, the cells were incubated with FITC-conjugated secondary antibodies in TBS with 3% BSA for 1 hour at room temperature and examined under a microscope. The immunostaining were assessed using a Nikon Ellipse E800 microscope, and the digital images were acquired using a SPOT digital camera.

2.7. Measurement of ROS/RNS Production

The fluorescent dye CM-H₂DCFDA and DHR 123 (Molecular Probe) were used to estimate total level of ROS/RNS production in PC-12 cells. PC-12 cells were loaded with CM-H₂DCFDA or DHR 123. After incubating with fluorescent probes for 30 min, the cells were treated with A β . The ROS or RNS production will be determined by measurement of fluorescence derived from the fluorescent dye. The fluorescence was read with a spectrofluorometer (Victor3 1420, Perkin Elmer, Boston, MA) with appropriate excitation and emission wavelengths. Appropriate positive and negative controls will be included in each measurement.

2.8. Lipid peroxidation assay

Malonyldialdehyde (MDA), an index of lipid peroxidation, was measured by using a commercial assay Kit (OxisResearch, Portland, OR). In brief, following 24 h A β treatment, cells were washed three times with 1x PBS and homogenized in 20 mM phosphate buffer (pH 7.4) containing 0.5 mM butylated hydroxytoluene to prevent sample oxidation. The lysate was centrifuged at 1000 $\times$ g for 10 min, and a 200 μ L aliquot of the supernatant was used to measure MDA levels according to the instructions of the manufacturer. This assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA at 45°C, and measures specifically MDA and not other lipid peroxidation products. An MDA standard curve was used to determine the absolute concentration of MDA in the samples using the stable MDA precursor tetramethoxypropane. Values were standardized to micrograms of protein.

2.9. Caspase-3/7 activity

The caspase-3/7 activity was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega), according to the manufacturer's instructions. Briefly, PC12 cells were rinsed twice with PBS and collected in a flash tube. The cells were lysed in 50 μ L of Homogeneous Caspase-3/7 Buffer containing the caspase-3 substrate, Z-DEVD-rhodamine 110, and the cell lysates were incubated for 14 h at room temperature. After incubation, the fluorescence (excitation, 480 nm and emission, 535 nm) of cell lysates (50 μ L) was measured using a spectrofluorometer (Victor3 1420, Perkin Elmer).

2.10. Mitochondrial Membrane Potential

The mitochondrial membrane potential was measured by using JC-1 fluorescent dye (Molecular Probe). PC12 cells were incubated with JC-1 dye for 30 min. After incubation, cells were rinsed with PBS. The emission signals at 590 and 527 nm elicited by excitation at 485 nm was measured with a spectrofluorometer (Victor3 1420, Perkin Elmer). The ratio of the signal at 590 nm over that at 527 nm (red/green ratio) was then calculated.

2.11. Cell Viability Assays

Cell viability was assessed by measuring formazan produced by the reduction of MTT. Transfected PC-12 cells in 6-well culture dishes were treated with a- β or H₂O₂ and incubated for six hours at 37°C. MTT was added (5mg/ml), and the cells were incubated an additional hour. After this, the medium was removed and the cells were solubilized with dimethylsulfoxide and transferred to a 96-well plate. The formazan reduction product was measured by reading absorbance at 540nm in a plate reader.

2.12. Data analysis

Data in text and figures are expressed as mean $\pm$ SE. Two group comparisons were evaluated by paired or unpaired *t* tests, as appropriate. Multiple comparisons were analyzed by ANOVA

and Tukey's or Newman Keuls post-hoc tests. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Transient Ngf transfection induces overexpression of Ngf mRNA and protein in PC-12 cells

Ngf overexpression was achieved by transient transfections with Ngf plasmid DNA. PC-12 cells were transfected with 0.5 μ g, 1.0 μ g and 2.0 μ g/per well (6-well plates) of Ngf DNA or vector DNA for 48 h. Following the transfection, Ngf mRNA and protein expression were determined by quantitative RT-PCR and western blotting, respectively. A dose-dependent increase in Ngf mRNA expression was induced by transfection of wild type or mutant Ngf (0.5, 1.0 and 2.0 μ g/per well, $n=6$) in PC-12 cells ($*P < 0.01$ vs. vector, Fig 1A). Ngf protein expression was also elevated in a similar pattern following transfection of wild type Ngf ($*P < 0.01$ vs. vector, Fig 1B). Ngf immunoreactivity increased in the cytoplasm of PC-12 cells following wild type Ngf (1.0 μ g/per well) transfection (Fig 1C). Thus, transient Ngf transfections induce overexpression of Ngf mRNA and protein in PC-12 cells.

3.2. Overexpression of Ngf significantly attenuates A β -induced ROS/RNS production

ROS/RNS production, as measured by the fluorescent dyes CM-H₂DCFDA and DHR 123, was significantly increased following either 50 μ M or 100 μ M A β treatments ($*P < 0.01$ vs. vector, Fig 2A, 2B). The lower dose of A β (25 μ M) also increased CM-H₂DCFDA and DHR 123 fluorescence, but changes did not achieve statistical significance. These findings indicate that A β treatment induces the accumulation of ROS/RNS in PC12 cells. Furthermore, the transfection of wildtype Ngf (0.5, 1.0 and 2.0 μ g/per well, $n=6$) was associated with dose-dependent reductions in the CM-H₂DCFDA fluorescence induced by 100 μ M A β treatments. In contrast, transfection of mutant Ngf DNA did not affect CM-H₂DCFDA fluorescence increases as induced by 100 μ M A β treatments ($*P < 0.01$ vs. vector, Fig 2C). In addition, transfection of wildtype Ngf also attenuated A β -induced DHR123 fluorescence in PC12 cells in a dose dependent manner. In contrast, the transfection of mutant Ngf mutant did not show any effect on the increased DHR 123 fluorescence induced by 100 μ M A β treatments ($*P < 0.01$ vs. vector, Fig 2D). These findings suggest that overexpression of wild type Ngf, but not of mutant Ngf, reduces A β -induced accumulation of ROS/RNS in PC-12 cells.

3.3. Overexpression of Ngf improves mitochondrial membrane potential and reduces A β -induced lipid peroxidation in PC-12 cells

To determine the effects of overexpression of wildtype or mutant Ngf on A β -induced lipid peroxidation and mitochondrial membrane potential in PC-12 cells, cells were transfected with either wild type or mutant Ngf DNA (1.0 μ g/per well) for 48 h and treated with A β for 24 h. Following Ngf transfection and A β treatment, lipid peroxidation and mitochondrial membrane potential were assessed using MDA and JC-1 assays, respectively. In the vector alone transfected cells, A β treatment induced marked increases in MDA formation and decreased JC-1 ratio ($n=6$, $*P < 0.01$ vs. Vector, Fig 3A, 3B), indicating that A β treatments induce lipid oxidation and mitochondrial dysfunction as evidenced by decreased mitochondrial membrane potentials. Transfection of wild type Ngf significantly decreased A β -induced MDA formation, while transfection of mutant Ngf had no such effect ($n=6$, $*P < 0.01$ vs. Vector, $\#P < 0.01$ vs. control, Fig 3A). Transfection of wild type Ngf also improved A β -induced impairments in mitochondrial membrane potential, while mutant Ngf had no such effect ($n=6$, $*P < 0.01$ vs. Vector, $\#P < 0.01$ vs. control, Fig 3B). These results suggest that Ngf overexpression attenuates A β -induced lipid peroxidation and mitochondrial dysfunction, and that such effects could play an important role in protecting PC-12 cells against A β -induced cytotoxicity.

3.4. Overexpression of Ngf protects PC-12 cells against A β -induced apoptosis and promotes cell survival

To further examine whether overexpression of Ngf could attenuate A β -induced apoptosis and improve cell viability, cellular responses after A β treatment were assessed using caspase 3/7 activity and MTT assay respectively. In cells transfected with vector, A β treatment significantly increased caspase 3/7 activity and decreased cell survival ($n=6$, $*P<0.01$ vs. Vector, Fig 3C, 3D). Transfection of wild type Ngf significantly decreased A β -induced caspase 3/7 activity while no significant changes occurred in Ngf mutant transfected cells ($n=6$, $*P<0.01$ vs. Vector, $\#P<0.01$ vs. control, Fig 3C). Transfection of wild type Ngf also improved cell survival, while no significant changes developed with mutant Ngf transfections ($n=6$, $*P<0.01$ vs. Vector, $\#P<0.01$ vs. control, Fig 3D). Thus, Ngf over-expression will decrease A β -induced apoptosis and improve overall cell survival.

4. Discussion

This study shows that exposure to A β leads to increased accumulation of ROS/RNS and lipid peroxidation, ultimately leading to mitochondrial dysfunction, apoptosis, and cell death. In addition, transient Ngf transfection leading to increased expression of Ngf mRNA and protein, not only suppressed A β -induced ROS/RNS production, but also decreased A β induced lipid peroxidation, mitochondrial dysfunction, and thereby improving overall cell survival. The rat pheochromocytoma (PC)-12 cell line exhibits unique sensitivity to changes in O $_2$ availability, and is frequently used as a cellular model to study neuronal vulnerability to hypoxia (Gozal et al., 2005; Seta et al., 2002). PC-12 cells have also been widely used as an experimental model to study cellular toxicity (Koh et al., 2005; Zhang and Jope, 1999). For example, studies in PC-12 cells have shown that A β not only induced cytotoxicity, but also elicited excessive ROS production (Gao and Tang, 2006; Koh et al., 2005), mitochondrial dysfunction (Gao and Tang, 2006), apoptosis and cell death (Lin et al., 2006; Martin et al., 2001). Therefore, we inferred that PC-12 cells as used in current experiments would provide a suitable approach to determine whether Ngf affords protection against A β -induced cytotoxicity.

Ngf was initially discovered in human and murine brain, and since then it has been identified in a wide range of vertebrate species (Burmester et al., 2000; Moens and Dewilde, 2000; Trent, III et al., 2001). In mouse and rat, Ngf is expressed in most neurons, but is apparently absent in glial cells (Geuens et al., 2003; Reuss et al., 2002; Wystub et al., 2003). Ngf is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum of brain (Geuens et al., 2003; Reuss et al., 2002; Wystub et al., 2003). At the subcellular level, Ngf is located in the cytosolic compartment, and is primarily concentrated in sub-cellular regions rich in mitochondria, i.e. the apical region of the inner segments and synapses in the plexiform layers (Schmidt et al., 2003). The sub-cellular distribution pattern of Ngf suggested that the major functional roles of Ngf may correlate with mitochondrial function, i.e., oxygen consumption and energy generation, and ROS generation and scavenging. As a corollary of such studies, Ngf has recently been found to play a role in neuronal cell protection to hypoxic and ischemic insults (Khan et al., 2006; Sun et al., 2001; Sun et al., 2003; Venis, 2001). However, the mechanism(s) underlying Ngf protection of neurons under hypoxic/ischemic stress remain unclear. Evidence does not support a role for Ngf as a cellular oxygen delivery system in neurons (Fago et al., 2004). Notwithstanding, it is conceivable that Ngf operates as a ROS/RNS scavenger, considering its *in vitro* properties against toxic reactive species, such as nitrogen monoxide, peroxynitrite and hydrogen peroxide (Herold et al., 2004). Since oxidative stress is involved in the neurodegenerative process of AD, the possibility exists that Ngf may play a role in the cellular defense mechanisms against the oxidative stress induced by A β .

Oxidative stress reflects a situation in which ROS is overproduced and exceeds the capacity of endogenous antioxidant defense systems. A β significantly increases superoxide and peroxynitrite production (Behl et al., 1994; Butterfield et al., 2002; Hensley et al., 1994; Huang et al., 1999), and enhances membrane lipid peroxidation (Mark et al., 1997) before apoptotic cell death ensues. Indeed, increased peroxynitrite formation and membrane lipid peroxidation are directly associated with degenerating neurons in AD patients (Behl et al., 1994; Cash et al., 2002), suggesting that peroxynitrite-induced lipid peroxidation may play a key role in the cell death process induced by A β in AD. Therefore, antioxidant therapies may enable delayed occurrence of apoptosis or prevention of apoptosis altogether in AD. In the present study, we have shown that the presence of A β in cell culture not only elicited ROS/RNS overproduction, but was also associated with increased lipid peroxidation in PC-12 cell, all of which are consistent with previous studies. Furthermore, overexpression of wild type Ngb decreased ROS/RNS overproduction as well as lipid peroxidation in PC-12 cells. However, mutated Ngb with H64V/H96A mutation lost its antioxidant capacity, indicating that the H64 and H96 residues in the Ngb molecule are critically important for its antioxidant function. Since Ngb is predominantly expressed in the CNS, the antioxidant properties of this globin may indeed provide a novel and effective neuroprotective approach against the oxidative injury induced by A β particularly when considering that antioxidants attenuate A β -induced oxidative injury (Floyd, 1999; Sultana et al., 2004). Thus, Ngb may play a role in the cellular defense pathways recruited against oxidative stress during neurodegenerative process in AD.

We found that A β treatment not only induced excess ROS/RNS but also caused mitochondrial dysfunction, apoptosis and cell death in PC-12 cells. Considering the strong correlation between A β -induced oxidative stress and cytotoxicity (Behl et al., 1994; Butterfield et al., 2002; Hensley et al., 1994), the decreases in mitochondrial membrane potential found in the present study further indicate disruption of mitochondrial membrane integrity, whereby ROS produced in mitochondria may then leak to the cytoplasm, lead to oxidative stress, and initiate apoptosis via activation of apoptosis signaling (Budihardjo et al., 1999). We found that A β treatment significantly increased caspase 3/7 activity and that transfection of wild type Ngb significantly decreased A β -induced caspase 3/7 activity while no significant change was observed in the Ngb mutant transfected cells, suggesting that overexpression of functional Ngb is able to decrease A β -induced apoptosis. Taken together, Ngb associated decreases in A β -induced ROS production, we postulate that Ngb anti-apoptotic role may be mediated by its antioxidant properties.

Several studies have demonstrated that ROS are involved in the apoptotic mechanisms triggered in the process of A β -mediated neurotoxicity, and may therefore contribute to the increased apoptosis found in some models of AD (Ferreiro et al., 2006; Fukui et al., 2005; Kadowaki et al., 2005). Indeed, dead neurons in AD may exhibit typical features of apoptosis, such as chromatin condensation, and DNA fragmentation (Budihardjo et al., 1999; Nicholson and Thornberry, 1997), although such findings are not consistently identified in brains from AD patients (Raina et al., 2001; Zhu et al., 2006). Apoptosis is associated with the activation of caspases, in which activation of pro-caspase-3 to caspase-3 is a central event in the execution phase of apoptosis (Budihardjo et al., 1999; Nicholson and Thornberry, 1997). The A β peptide has been shown to induce apoptosis in neurons (Kadowaki et al., 2005; Ran et al., 2004), including PC12 cells (Gao and Tang, 2006; Martin et al., 2001), and as such has been proposed as an important mediator of neuronal death in AD. Several studies have now reported that antioxidant treatment provides a protective effect against A β -induced neurotoxicity (Barkats et al., 2000; Sultana et al., 2004). Conversely, inhibition of endogenous antioxidant scavenger capacity leads to exacerbation of A β -induced neurotoxicity (Crack et al., 2006). We now show that overexpression of Ngb decreases A β -induced cell death most likely through reduction of oxidant stress.

In conclusion, Ngb not only decreases A β -induced ROS/RNS overproduction and lipid peroxidation, but also attenuates A β -induced mitochondrial dysfunction, apoptosis and cell death. Therefore, Ngb may act as an intracellular ROS/RNS scavenger, and such antioxidant properties may play a protective role against A β -induced cell injury. Further exploration of Ngb antioxidant properties may provide opportunities for novel pharmacological interventions aiming at preventing or palliating the consequences of AD.

Acknowledgments

We thank Kenneth R. Brittan and Heather B. Clair for their expert technical assistance in cell culture and immunocytochemistry. This study was supported by National Institutes of Health grants SCOR 2P50-HL-60296 (Project 2) and RO1-HL-69932, The Children's Foundation Endowment for Sleep Research, and by the Commonwealth of Kentucky Challenge for Excellence Trust Fund.

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Figure 1. Ngf mRNA and protein expression in PC-12 cells following transfection
 PC-12 cells were transfected with 0.5 μ g, 1.0 μ g and 2.0 μ g/ 10^6 cells of Ngf, Ngf mutant or vector DNA for 48 h. Following the transfection, Ngf mRNA and protein expression was determined by quantitative RT-PCR and western blotting, respectively. **1A**, Ngf mRNA expression. after Ngf transfection. Data were expressed as a fold increase over vector transfection (n=6/group, * P<0.01 vs.vector). **1B**, Immunoblots of Ngf protein expression following Ngf transfection. **1C**. Ngf immunoreactivity after Ngf transfection in PC-12 cells.

Figure 2. Effect of Ngf overexpression on Aβ-induced ROS/RNS production

ROS/RNS production was measured with the fluorescent dyes CM-H₂DCFDA and DHR 123.

2A. DCFDA fluorescence after Aβ treatment. Data were expressed as a fold increase over control (n=6/group, * P<0.01 vs.vector). **2B.** DHR123 fluorescence after Aβ treatment. Data were expressed as fold increase over control (n=6/group, * P<0.01 vs.vector). **2C.** Effect of Ngf overexpression on Aβ-induced DCFDA fluorescence. Data were expressed as the percentage of vector transfection (n=6/group, * P<0.01 vs.vector). **2D.** Effect of Ngf overexpression on Aβ-induced DHR123 fluorescence. Data were expressed as the percentage of vector transfection (n=6/group, * P<0.01 vs.vector).

Figure 3. Effect of Ngf transfection on Aβ-induced lipid peroxidation, mitochondrial membrane potential, Caspase 3/7 activity, and cell viability in PC-12 cells

PC-12 cells were transfected with vector, wild type or mutant Ngf DNA ($1.0 \mu\text{g}/10^6$ cell) for 48 h and treated with Aβ for 24 h. **3A.** Effect of Ngf transfection on Aβ-induced MDA formation. Data are expressed as percentage of control ($n=6/\text{group}$, * $P<0.01$ vs. control, # $P<0.01$ vs. vector). **3B.** Effect of Ngf transfection on Aβ-induced mitochondrial membrane potential. Data are expressed as percentage of control ($n=6/\text{group}$, * $P<0.01$ vs. control, # $P<0.01$ vs. vector). **3C.** Effect of Ngf transfection on Aβ-induced caspase 3/7 activity. Data are expressed as percentage of control ($n=6/\text{group}$, * $P<0.01$ vs. control, # $P<0.01$ vs. vector). **3D.** Effect of Ngf transfection on Aβ-induced cell death. Data are expressed as absolute OD values (570 nm OD) ($n=6/\text{group}$, * * $P<0.01$ vs. control, # $P<0.01$ vs. vector).