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Ginkgolide B Revamps Neuroprotective Role of Apurinic/Apyrimidinic Endonuclease 1 and Mitochondrial Oxidative Phosphorylation Against $A\beta_{25–35}$-Induced Neurotoxicity in Human Neuroblastoma Cells

Navrattan Kaur,[1] Monisha Dhiman,[2] J. Regino Perez-Polo,[3] and Anil K. Mantha[1,3]*

[1]Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Punjab, India
[2]Centre for Genetic Diseases and Molecular Medicine, School of Emerging Life Science Technologies, Central University of Punjab, Bathinda, Punjab, India
[3]Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas

Accumulating evidence points to roles for oxidative stress, amyloid beta (Aβ), and mitochondrial dysfunction in the pathogenesis of Alzheimer's disease (AD). In neurons, the base excision repair pathway is the predominant DNA repair (BER) pathway for repairing oxidized base lesions. Apurinic/apyrimidinic endonuclease 1 (APE1), a multifunctional enzyme with DNA repair and reduction–oxidation activities, has been shown to enhance neuronal survival after oxidative stress. This study seeks to determine 1) the effect of $A\beta_{25–35}$ on reactive oxygen species (ROS)/reactive nitrogen species (RNS) levels, 2) the activities of respiratory complexes (I, III, and IV), 3) the role of APE1 by ectopic expression, and 4) the neuromodulatory role of ginkgolide B (GB; from the leaves of *Ginkgo biloba*). The prooxidant $A\beta_{25–35}$ peptide treatment increased the levels of ROS/RNS in human neuroblastoma IMR-32 and SH-SY5Y cells, which were decreased after pretreatment with GB. Furthermore, the mitochondrial APE1 level was found to be decreased after treatment with $A\beta_{25–35}$ up to 48 hr, and the level was increased significantly in cells pretreated with GB. The oxidative phosphorylation (OXPHOS; activities of complexes I, III, and IV) indicated that $A\beta_{25–35}$ treatment decreased activities of complexes I and IV, and pretreatment with GB and ectopic APE1 expression enhanced these activities significantly compared with $A\beta_{25–35}$ treatment. Our results indicate that ectopic expression of APE1 potentiates neuronal cells to overcome the oxidative damage caused by $A\beta_{25–35}$. In addition, GB has been shown to modulate the mitochondrial OXPHOS against $A\beta_{25–35}$-induced oxidative stress and also to regulate the levels of ROS/RNS in the presence of ectopic APE1. This study presents findings from a new point of view to improve therapeutic potential for AD via the synergistic neuroprotective role played by APE1 in combination with the phytochemical GB.

Key words: Alzheimer's disease; amyloid beta; $A\beta_{25–35}$; OXPHOS; APE1; Ref-1; ginkgolide B

Mitochondria have a central role in age-related neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (Lin et al., 2006; Dhillon and Fenech, 2014). Extensive studies in recent years have linked many neurodegenerative diseases with altered DNA repair mechanisms, i.e., base excision repair (BER), double strand break (DSB) repair, single strand break (SSB) repair, mitochondrial defects, and oxidative stress (Martin, 2008; Ciccone et al., 2013; Akbari et al., 2014). Although the presence of the nucleotide excision repair (NER) pathway in mitochondria has

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*Correspondence to: Dr. Anil K. Mantha, PhD, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Pin Code: 151 001, Punjab, India. E-mail: anilmantha@gmail.com; Anil.kumar@cup.ac.in

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not yet been clarified, emerging evidence reveals the existence of mismatch repair and BER pathways as reviewed in (Hegde et al., 2012). Oxidative DNA damage repair is the major mechanism observed in neurodegenerative diseases and the best known DNA repair mechanism studied in mitochondria. The BER pathway is known to be the predominant pathway in mitochondria for removal of the oxidized base lesions generated in the presence of oxidative stress. Apurinic/apyrimidinic endonuclease 1 (APE1) is the central enzyme of the BER pathway, acting on apurinic/apyrimidinic sites and exerting its reduction–oxidation (redox) modification activity on the majority of transcription factors (TFs), including activator protein 1 (AP-1), nuclear factor-κ light-chain enhancer of activated B cells (NF-κB), and tumor protein p53, regulating their DNA-binding activity as summarized in (Izumi et al., 2003; Tell et al., 2005; Bhakat et al., 2009; Hegde et al., 2012). APE1 plays a central role in the cellular responses to oxidative stress. Attempts have been made to link increased levels of APE1 with protection in neuronal cells. One study has shown that there is an increased nuclear expression of APE1 in cerebral cortical regions of AD patients (Marcon et al., 2009). Another study showed the colocalization of APE1 with amyloid beta (Aβ) in the senile plaques in AD hippocampus (Tan et al., 2009). Also, APE1 immunostaining was found to be relatively low in control AD brain sections but was observed to be increased in regions of neuronal injury (Tan et al., 1998). An earlier study demonstrated that there is an inhibition of APE1 expression in the hippocampus but not in other brain areas after 6 hr of reperfusion following an induced ischemic injury (Edwards et al., 1998a). In line with this, it has been shown that isobaric hyperoxia, which induces chronic oxidative stress, stimulates APE1 expression in the hippocampus and basal forebrain of young rats, whereas aged rats show no significant changes in APE1 protein levels after hyperoxia (Edwards et al., 1998b). Thus, this difference in expression of APE1 may be linked to degeneration associated with the aging process. These studies indicate that stresses such as ischemia and hyperoxia lead to oxidative damage that activates the expression of DNA repair enzymes such as APE1 and suggest that overexpression of APE1 in injured neurons is part of a response to oxidative stress and an attempt to repair damaged DNA in various human etiologies.

Many lines of evidences have suggested that oxidative damage in association with mitochondrial dysfunction plays a major role in AD progression and development which are reviewed in (Lin et al., 2006; Hegde et al., 2012; Mantha et al., 2014; Kaur et al., 2015). In relation to this, a decline in mitochondrial function with advancing age resulting from accumulation of somatic mutations in mtDNA has been reported (Wei and Lee, 2002). Various evidences point to the alteration of DNA repair mechanisms in age-dependent neurodegenerative diseases (Imam et al., 2006; Fishel et al., 2007; Weissman et al., 2007), but the mechanistic link among oxidative damage, altered DNA repair mechanisms, and mitochondrial dysfunction in AD development and progression is still unclear. To address this issue, the current study is designed to understand how ectopic expression of APE1, the major BER pathway enzyme, supports the neuronal cells in overcoming the oxidative stress induced by $A\beta_{25–35}$ in vitro by using neuroblastoma (SH-SY5Y and IMR-32) cell lines. In addition, the current study seeks to determine whether the phytochemical ginkgolide B (GB; from the leaves of *G. biloba*) helps in modulating/protecting APE1's functions in reducing oxidative stress and Aβ-induced dysfunction of mitochondrial oxidative phosphorylation (OXPHOS) of the neuronal cells in maintaining cellular energy demands.

MATERIALS AND METHODS

Cell Culture and Transfection

Human neuroblastoma SH-SY5Y cells (a gift from Dr. Pankaj Seth, National Brain Research Centre, Manesar, India) were grown according to the protocol of Mantha et al. (2012). Briefly, SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with 10% fetal bovine serum (FBS), 10% horse serum, and 1% penicillin–streptomycin (PS) at 37°C under 5% CO_2 atmospheric pressure. The IMR-32 neuroblastoma cells procured from the National Centre for Cell Science (Pune, India) were grown in DMEM containing 10% FBS and 1% PS and were maintained at 37°C in a 5% CO_2 humidified incubator as described above. The SH-SY5Y and IMR-32 cells grown overnight in 96-well plates were transiently transfected with 0.2 μg plasmid DNA per well containing C-terminally FLAG-tagged full-length APE1 or empty vector with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol in serum-free Opti-MEM medium. The cells, in 100-mm^2 dishes, were transiently transfected with 2 μg plasmid DNA per plate containing C-terminally FLAG-tagged full-length APE1 empty vector per plate with Lipofectamine 2000 as described above (Mantha et al., 2012). At 6 hr after transfection, the cells were transferred to complete medium for 18 hr, followed by cell treatments.

Cell Treatments

The SH-SY5Y and IMR-32 cells were seeded on 96-well plates at a density of 1×10^5 cells per well in complete media as described elsewhere (Mantha et al., 2012) for treatment with oxidant $A\beta_{25–35}$ and the phytochemical GB.

Preparation and Treatment With $A\beta_{25–35}$

$A\beta_{25–35}$ peptide (GenScript, Piscataway, NJ) stock solutions were freshly prepared before each treatment at 1 mM in double-distilled deionized water, considered the soluble form (Mantha et al., 2012). The cells were then treated with $A\beta_{25–35}$ peptide in a range of 0–80 μM in serum-free medium containing 1% PS for 24 hr. Then, the cells were incubated at 37°C in a humidified and sterile atmosphere containing 5% CO_2 for 24, 48, and 72 hr.

Preparation and Treatment With GB

A dose–response test for GB was performed by treating with GB (Sigma, St. Louis, MO) in the range of 0–100 μM in

the corresponding serum-free medium in both SH-SY5Y and IMR-32 cells (Ahlemeyer ans Krieglstein, 2003). The plates were then incubated at 37°C in a humidified and sterile atmosphere containing 5% CO_2 for 12, 24, 48, and 72 hr. The SH-SY5Y and IMR-32 cells were pretreated with GB for 3 hr before treatment with $A\beta_{25-35}$ in further experiments.

Measurement of Intracellular Reactive Oxygen Species

The SH-SY5Y and IMR-32 cells were seeded 1 day before in 96-well plates at a density of 1×10^5. To measure the intracellular reactive oxygen species (ROS) levels, cells were equilibrated in phosphate-buffered saline (PBS) and incubated in the dark for 30 min with 100 μM H_2DCF-DA (Invitrogen; $Ex_{478\ nm}/Em_{518\ nm}$). After cells had been washed twice with PBS, fluorescence was read at the excitation wavelength of 478 nm and emission wavelength of 518 nm with a Bio-Tek (Winooski, VT) microplate reader according to the protocol described by Carrière et al. (2004) and Dhiman and Garg (2011).

Measurement of Intracellular Reactive Nitrogen Species

SH-SY5Y and IMR-32 cells were seeded at a density of 1×10^5 and grown overnight in 96-well plates. To measure intracellular nitric oxide (NO) levels, cells were equilibrated in PBS and incubated in the dark for 30 min with 20 μM DAF-FM (Invitrogen; $Ex_{478\ nm}/Em_{515\ nm}$). After cells had been washed twice with PBS, fluorescence was read at the excitation wavelength of 478 nm and emission wavelength of 515 nm with a Bio-Tek microplate reader according to the protocol described by Keil et al. (2004).

Preparation of Mitochondria

The control and treated SH-SY5Y and IMR-32 cells seeded in 100-mm^2 dishes were trypsinized and centrifuged at 2,000 rpm for 10 min. The resulting cell pellet was resuspended in ice-cold lysis buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, and 0.05% digitonin and protease inhibitor cocktail (Sigma) for 30 min on ice with vortexing every 5 min. The cell suspension was centrifuged at 800*g* for 10 min at 4°C. The pellet was discarded, and supernatant was again centrifuged at 20,000*g* for 15 min at 4°C. The resulting supernatant contained the cytosolic fraction, and the pellet contained the mitochondria. The mitochondrial pellet was resuspended in PBS and stored at 4°C, followed by determination of protein content by the Bradford method (Rhein et al., 2010).

Measurement of Endogenous APE1 Levels by ELISA

A 96-well microtiter plate was coated with 10 μg mitochondrial protein for 16 hr at 4°C. Wells were washed and incubated with blocking buffer (PBS with 2% bovine serum albumin [BSA] and 0.1% Tween 20) for 1 hr at 37°C. After the washing, APE1 antibody (anti-mouse, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated for 2 hr at 37°C. The wells were washed, and secondary antibody (anti-mouse IgG horseradish peroxidase, 1:1,000) was added and incubated for another 1 hr at 37°C. Blue coloration was observed after addition of 3,3′,5,5′-tetramethylbenzidine as a chromogenic substrate. The reaction was terminated after 10 min with 2 M H_2SO_4, and the absorbance was read at 450 nm with a Bio-Tek microplate reader (Dhiman et al., 2012).

Determination of Mitochondrial OXPHOS Capacity

Complex I activity. A total of 20 μg mitochondrial protein was used to estimate the NADH–ubiquinone oxidoreductase activity. First, the mitochondrial protein was incubated with 800 μl H_2O and incubated for 2 min at 37°C. Then, 200 μl of 50 mM Tris-HCl (pH 8.0) medium supplemented with 5 mg/ml BSA, 800 μM NADH as donor, 1.2 mM KCN, and 2 μM antimycin was added. The basal activity was measured at 340 nm for 3 min. The reaction was started by the addition of 50 μM of the acceptor decylubiquonone. The activity was measured at 30°C by following the decrease in absorbance at 340 nm resulting from the oxidation of NADH for 3 min with a Bio-Tek microplate reader. The extinction coefficient of NADH is 6.1 $mM^{-1} \cdot cm^{-1}$ (Barrientos et al., 2009; Rhein et al., 2010).

Complex III activity. A total of 10 μg mitochondrial protein was used to estimate the ubiquinol cytochrome c reductase activity. Decylubiquinol was freshly prepared by adding crystals of sodium borohydride to 10 mM decylubiquinone in ethanol and mixed by pipetting until the solution became transparent. Five to ten microliters of concentrated HCl was added to eliminate excess sodium borohydride, and the solution was adjusted to pH 2.0. The assay was carried out at 30°C in a medium containing 80 μM decylubiquinol as the donor, 240 μM KCN, 4 μM rotenone, 200 μM ATP, and 0.6 mM dodecyl β-D-maltoside. This was followed by incubation for 10 min at 30°C. Then, the reaction was started by addition of 40 μM oxidized cytochrome c. The activity was measured at 550 nm for 3 min with a Bio-Tek microplate reader. The extinction coefficient of cytochrome c is 18.5 $mM^{-1} \cdot cm^{-1}$ (Barrientos et al., 2009; Rhein et al., 2010).

Complex IV activity. A total of 10 μg of mitochondrial protein was used to measure the cytochrome c oxidase activity. Briefly, cytochrome c (0.22 mM) was reduced by using 5 μl of 0.1 M dithiothreitol. After a 15-min incubation, the color change from dark orange to pale purple was measured by recording absorbance at 550 nm and 565 nm. The ratio of 550/565 was estimated to be between 10 and 20, indicating reduction of cytochrome c. The assay was carried out with 10 mM phosphate buffer (pH 6.5), 0.25 M sucrose, 1 mg/ml BSA, and 2.5 mM dodecyl β-D-maltoside to permeabilize the outer membrane to cytochrome c. Then, the reaction was started by adding 10 μg of mitochondrial protein. The absorbance was measured at 550 nm for 3 min with a Bio-Tek microplate reader. The extinction coefficient of cytochrome c is 18.5 $mM^{-1} \cdot cm^{-1}$ (Barrientos et al., 2009).

Statistical Analysis

Data are mean ± SD. Results were analyzed by paired Student's *t*-test and ANOVA for statistical evaluation of mean values for experimental and control samples. $P < 0.05$ was considered statistically significant across the treatment groups.

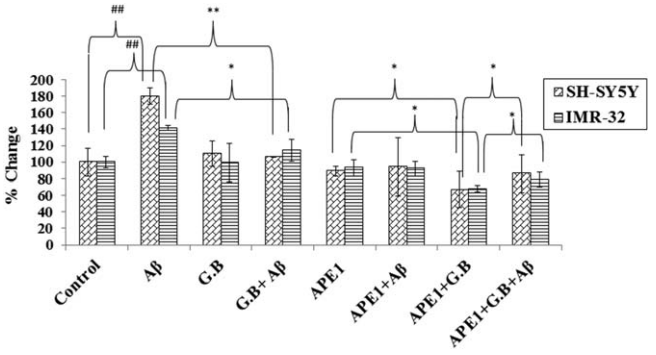

Fig. 1. Intracellular ROS produced after $A\beta_{25–35}$-induced oxidative stress in SH-SY5Y and IMR-32 cells with and without ectopic APE1 expression by H_2DCF-DA and phytochemical modulation by GB. Student's *t*-test was performed to evaluate the significance of the results. $^{\star}P \leq 0.05$, $^{\star\star}P \leq 0.005$, GB/GB + Aβ-treated cells compared with respective control; $^{\#\#}P \leq 0.005$, Aβ-treated cells compared with respective control. Results are mean ± SD (n = 3).

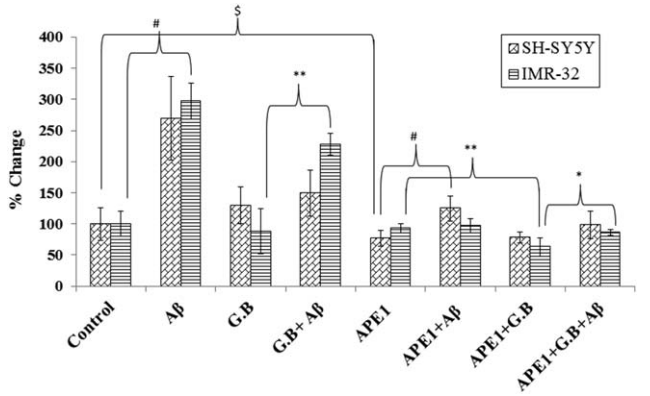

Fig. 2. Intracellular RNS produced after $A\beta_{25–35}$-induced oxidative stress in SH-SY5Y and IMR-32 cells with and without ectopic APE1 expression by DAF-FM and phytochemical modulation by GB. Student's *t*-test was performed to evaluate the significance of the results. $^{\$}P \leq 0.05$, untreated cells compared with untreated cells with ectopic APE1 expression; $^{\star}P \leq 0.05$, $^{\star\star}P \leq 0.005$, GB/GB + Aβ-treated cells compared with control; $^{\#}P \leq 0.05$, Aβ-treated cells compared with control. Results are mean ± SD (n = 3).

RESULTS

$A\beta_{25–35}$ Induces Oxidative Stress and Varies Cell Viability

To induce oxidative stress, SH-SY5Y and IMR-32 cells were treated with various concentrations of $A\beta_{25–35}$ in a range of 0–80 μM for 24, 48, and 72 hr. The results showed a decrease in cell viability at higher concentrations of $A\beta_{25–35}$ and as treatment time increased in both SH-SY5Y and IMR-32 cells (data not shown). $A\beta_{25–35}$ below a 20 μM concentration did not show any significant change in cell viability in either SH-SY5Y or IMR-32 cell lines. From our results and the literature, 20 μM $A\beta_{25–35}$ was chosen as the optimal concentration for further experimentation.

ROS Levels

The intracellular ROS levels in the cells were measured with H_2DCF-DA, a fluorescent dye, in the presence of oxidative stress induced by 20 μM $A\beta_{25–35}$. The results demonstrated that treatment with $A\beta_{25–35}$ for 24 hr had increased the ROS levels significantly by 80% and 41% compared with the untreated SH-SY5Y and IMR-32 cells, respectively (Fig. 1), which is consistent with other studies showing $A\beta_{25–35}$ to be a ROS generator, which is further associated with neurotoxicity (Barger et al., 1995; Jang et al., 2005; Wang et al., 2009; Zhang et al., 2009).

Reactive Nitrogen Species Levels

The fluorescent DAF-FM assay was used to measure the reactive nitrogen species (RNS) levels in SH-SY5Y and IMR-32 cells after treatment with 20 μM $A\beta_{25–35}$ for 24 hr. This study examined whether $A\beta_{25–35}$ induces NO within the cells by using a fluorescence probe, DAF-FM, which can directly detect NO. Treatment with 20 μM $A\beta_{25–35}$ showed an increase in RNS levels of 169% and 197% in SH-SY5Y and IMR-32 cells, respectively, compared with control cells (Fig. 2). This indicates the overproduction of NO intracellularly in SH-SY5Y and IMR-32 cells in the presence of oxidant $A\beta_{25–35}$.

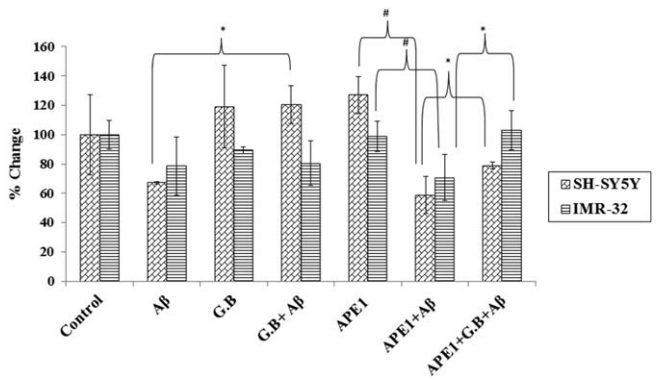

Fig. 3. Complex I activity in SH-SY5Y and IMR-32 cells treated with the oxidant $A\beta_{25–35}$ and the phytochemical GB with and without ectopic APE1 expression. Student's *t*-test was performed to evaluate the significance of the results. $^{\star}P \leq 0.05$, GB/GB + Aβ-treated cells compared with respective control; $^{\#}P \leq 0.05$, Aβ-treated cells compared with respective control. Results are mean ± SD (n = 3).

OXPHOS Capacity

To delineate the effect of $A\beta_{25–35}$ at the mitochondrial OXPHOS performance, we determined electron transport chain (ETC) activities of complexes I, III, and IV in response to oxidative stress induced by $A\beta_{25–35}$. The results showed that complex I activity in the mitochondria was reduced by 33% and 22% in SH-SY5Y and IMR-32 cells, respectively, after $A\beta_{25–35}$-induced oxidative stress (Fig. 3). There was increased complex III activity of 52% and 33% in SH-

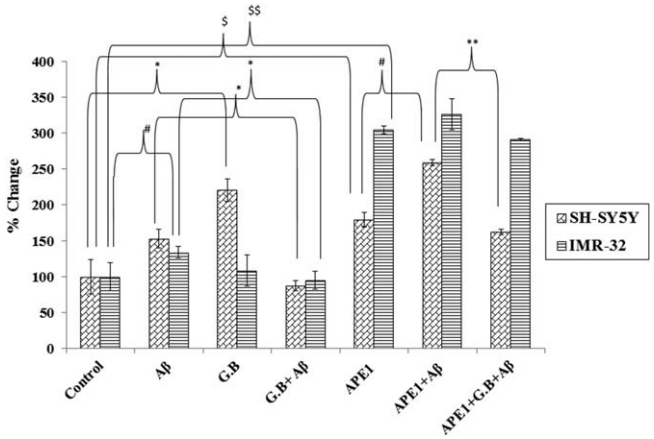

Fig. 4. Complex III activity in SH-SY5Y and IMR-32 cells treated with the oxidant $A\beta_{25–35}$ and the phytochemical GB with and without ectopic APE1 expression. Student's *t*-test was performed to evaluate the significance of the results. $^{\$}P \leq 0.05$, $^{\$\$}P \leq 0.005$, untreated cells compared with untreated cells with ectopic APE1 expression; $^{\star}P \leq 0.05$, $^{\star\star}P \leq 0.005$, GB/GB + Aβ-treated cells compared with respective control; $^{\#}P \leq 0.05$ Aβ-treated cells compared with respective control. Results are mean ± SD (n = 3).

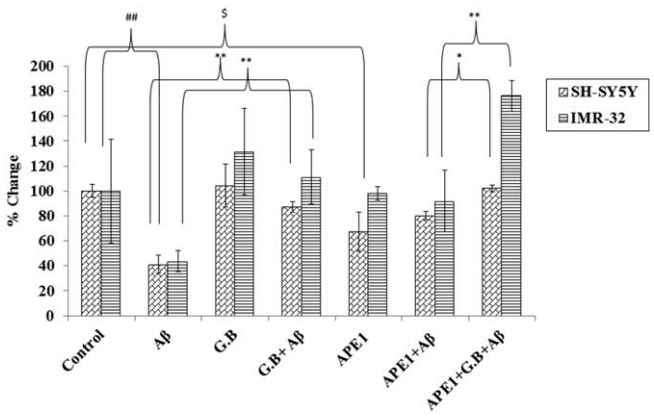

Fig. 5. Complex IV activity in SH-SY5Y and IMR-32 cells treated with the oxidant $A\beta_{25–35}$ and the phytochemical GB with and without ectopic APE1 expression. Student's *t*-test was performed to evaluate the significance of the results. $^{\$}P \leq 0.05$, untreated cells compared with untreated cells with ectopic APE1 expression; $^{\star}P \leq 0.05$, $^{\star\star}P \leq 0.005$, GB/GB + Aβ-treated cells compared with respective control; $^{\#\#}P \leq 0.005$, Aβ-treated cells compared with respective control. Results are mean ± SD (n = 3).

SY5Y and IMR-32 cells, respectively, treated with $A\beta_{25–35}$ for 24 hr (Fig. 4). The activity of complex IV was found to be reduced significantly by 59% in the presence of $A\beta_{25–35}$-induced stress responses in SH-SY5Y cells (Fig. 5). A similar trend of reduction of 57% of activity of complex IV was also seen in IMR-32 cells (Fig. 5). Thus, our results demonstrate that $A\beta_{25–35}$ interferes with mitochondrial function as shown by the decline in complex I and IV activities in the presence of 20 μM $A\beta_{25–35}$.

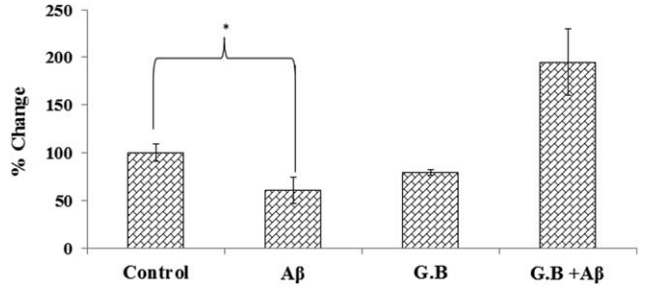

Fig. 6. Level of endogenous APE1 in mitochondria of SH-SY5Y cells treated with $A\beta_{25–35}$, GB, and GB + $A\beta_{25–35}$ in SH-SY5Y cells by ELISA and phytochemical modulation by GB. Student's *t*-test was performed to evaluate the significance of the results. $^{\star}P \leq 0.05$, GB/GB + Aβ-treated cells compared with control. Results are mean ± SD (n = 3).

Phytochemical GB Modulates Oxidative Stress Parameters Against $A\beta_{25–35}$ Treatment

There is a growing body of evidence encouraging phytochemical-based therapeutic interventions for various human diseases and health. To discover the beneficial effects shown by GB, SH-SY5Y and IMR-32 cells were treated with different concentrations of GB in a concentration range of 0–80 μM for 12, 24, 48, and 72 hr. Our results showed 20 μM GB to be beneficial, with minimum cytotoxicity and better capacity in countering ROS and RNS produced after $A\beta_{25–35}$-induced oxidative stress. This concentration, which had been used in various studies (Chan and Hsuuw, 2007; Shi et al., 2009), was chosen for further assays. The effect of GB, the terpene lactone component of standardized extract EGb 761, on the intracellular ROS levels altered by $A\beta_{25–35}$ in both cell lines showed significant attenuation of ROS production of 41% and 19% in GB + $A\beta_{25–35}$-treated SH-SY5Y and IMR-32 cells, respectively, after 48 hr of total experimental procedure (Fig. 1). An increase in intracellular NO levels in response to $A\beta_{25–35}$ in both SH-SY5Y and IMR-32 cells was decreased to a greater extent by pretreating the cells with GB (Fig. 2).

Alteration in APE1 expression during $A\beta_{25–35}$-induced oxidative stress was studied by ELISA to measure mitochondrial APE1 levels in SH-SY5Y cells. It was found that $A\beta_{25–35}$-treated cells showed a significant decrease in APE1 levels of 39% compared with untreated control cells. Treatment with GB showed a decrease of 20% compared with untreated control SH-SY5Y cells. Pretreatment with GB along with exposure to $A\beta_{25–35}$ caused an increase of 95% of APE1 levels in GB + $A\beta_{25–35}$-treated SH-SY5Y cells (Fig. 6). This indicates a role for APE1 in modulating the oxidative stress in neuronal cells that is induced by the treatment of $A\beta_{25–35}$ and synergistic modulation in the presence of GB.

Ectopic APE1 Expression Passably Alters Cellular Parameters Against $A\beta_{25–35}$

APE1 in neuronal cells is under the influence of constant oxidative stress induced by various forms of Aβ

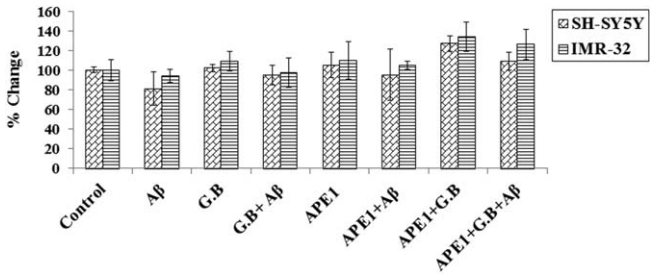

Fig. 7. Cell viability after $A\beta_{25–35}$-induced oxidative stress in SH-SY5Y and IMR-32 cells with and without ectopic APE1 expression and phytochemical modulation by GB. Results are mean ± SD (n = 3).

during AD. To enhance the neuronal survival rate, both SH-SY5Y and IMR-32 cells were transfected with mammalian expression vector with human APE1. Production of ROS/RNS was noted in both SH-SY5Y and IMR-32 cells with the treatment of $A\beta_{25–35}$ (Figs. 1, 2). In comparison with cells without ectopic APE1 expression, APE1 expression led to attenuation of ROS levels after treatment with 20 μM $A\beta_{25–35}$ in both SH-SY5Y and IMR-32 cells. NO production was decreased in $A\beta_{25–35}$-treated APE1-expressing cells compared with the corresponding $A\beta_{25–35}$-treated SH-SY5Y and IMR-32 cells without APE1 expression (Figs. 1, 2). These results indicate a neuroprotective role for APE1 in regulating cellular oxidative stress induced by Aβ during AD.

Pretreatment With GB in Association With Ectopic APE1 Synergistically Functions Against $A\beta_{25–35}$

The neuromodulatory effect of GB on mitochondrial OXPHOS capacity after $A\beta_{25–35}$-induced oxidative stress in SH-SY5Y and IMR-32 cells with ectopic APE1 expression was studied. Treatment with GB alone led to a moderate increase in neuronal cell survival and presence of ectopic APE1 synergistically enhanced ETC in both SH-SY5Y and IMR-32 cells. When pretreatment with GB was performed prior to the treatment with $A\beta_{25–35}$, increased cell survival was observed (Fig. 7) in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression compared with enhanced cell survival in corresponding cells without APE1 expression given the same treatment. This indicates that pretreatment with GB might have a therapeutic potential by modulating the activities of APE1 or restoring the APE1 functions, resulting in neuroprotection of neuronal cells from the oxidative damage caused by Aβ in AD.

ROS and RNS Levels

Subsequently, regulation of intracellular ROS/RNS levels by ectopic APE1 in the presence of GB in neuronal cells after $A\beta_{25–35}$-induced oxidative stress was analyzed. The results showed that pretreatment with GB prior to the treatment with $A\beta_{25–35}$ caused attenuation of ROS levels in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression compared with the corresponding cells without ectopic APE1 expression (Fig. 1). A similar trend of lowered RNS levels in GB + $A\beta_{25–35}$-treated cells was seen in both SH-SY5Y and IMR-32 cells ectopically expressing APE1 (Fig. 2). These results further indicate a neuroprotective role for APE1 in regulating neurotoxicity induced by $A\beta_{25–35}$ in coordination with phytochemical GB.

OXPHOS Capacity

Complex I activity. After ectopic expression of APE1, the activity of complex I was found to be enhanced compared with that of untreated control SH-SY5Y cells without ectopic APE1 expression, indicating a neuroprotective effect of APE1 in these cells. However, complex I activity in IMR-32 cells with ectopic APE1 expression was found to be unaltered compared with cells without ectopic APE1 expression. After pretreatment with GB, a decrease in the activity of complex I in the presence of 20 μM $A\beta_{25–35}$ was found to be increased significantly by 34% and 46% in both SH-SY5Y and IMR-32 cells, respectively, with ectopic APE1 expression (Fig. 3), indicating a modulatory effect of APE1 on complex I activity in bringing the activity of complex I to the basal level in the presence of GB.

Complex III activity. In relation to complex III activity, there was a significant increase in activity of 79% and 203% in ectopic APE1-expressing SH-SY5Y and IMR-32 cells, respectively (Fig. 4). Furthermore, an increase in activity was seen in the presence of $A\beta_{25–35}$ in both cell lines studied with ectopic APE1 expression as observed earlier when cells without ectopic APE1 expression were given the same treatment. Pretreatment with GB prior to treatment with $A\beta_{25–35}$ showed a significant decrease of 37% and 11% in ectopic APE1-expressing SH-SY5Y and IMR-32 cells, respectively (Fig. 4), which was much more than the basal level activity. This indicates the protection of complex III activity by APE1 in the presence of GB after increased ROS.

Complex IV activity. In addition, the combined effect of APE1 and GB on the mitochondrial complex IV activity was determined. An increase in the complex IV activity was observed in ectopically APE1-expressing and $A\beta_{25–35}$-treated SH-SY5Y and IMR-32 cells compared with corresponding $A\beta_{25–35}$-treated cells without ectopic APE1 expression (Fig. 5). Furthermore, a significant increase in activity of 27% and 92% was seen in ectopic APE1-expressing SH-SY5Y and IMR-32 cells, respectively, that were treated with GB + $A\beta_{25–35}$, which was found to be close to the basal level in SH-SY5Y cells and much higher than the basal level in IMR-32 cells (Fig. 5). This suggests that GB together with APE1 ameliorates the complex IV activity in response to oxidative stress induced by $A\beta_{25–35}$. Thus, the phytochemical GB together with APE1 has the potential to modulate $A\beta_{25–35}$-induced oxidative stress and restore OXPHOS and eventually might lead to neuronal cell survival against Aβ during the onset of AD.

DISCUSSION

The current study seeks to provide insight into the pathogenesis of AD, linking Aβ-induced oxidative stress, mitochondrial dysfunction, the role of APE1, and to determine how ectopic APE1 expression affects the neuronal cells in overcoming the oxidative stress induced by $A\beta_{25–35}$ in human neuroblastoma cells. In addition, modulation/protection of APE1's activities by the phytochemical GB was examined. Various studies have attempted to find a link between oxidative stress-related neurodegeneration and decreased mitochondrial DNA repair capacity in neuronal cells (Harrison et al., 2005; Mao and Reddy, 2011; Ciccone et al., 2013). Extensive studies in recent years have linked many neurodegenerative processes to altered DNA repair mechanisms, i.e., BER, SSB, and DSB repair pathways; mitochondrial defects; and oxidative stress, which have been systematically reviewed in recent publications (Hegde et al., 2012; Mantha et al., 2014; Thakur et al., 2014; Kaur et al., 2015). APE1 is the central enzyme of the BER pathway, acting on apurinic/apyrimidinic sites and exerting its redox modification activity on key TFs, including AP-1, NF-κB, and p53, thus regulating their DNA-binding activity as reviewed in (Tell et al., 2005, 2009; Bhakat et al., 2009; Hegde et al., 2012).

Recent studies provide evidence that Aβ oligomers are a more toxic species than insoluble fibrillary deposits relevant to AD pathology (Ferreira et al., 2007; Sakono and Zako, 2010; Benilova et al., 2012). The signature region of $A\beta_{1–42}$, $A\beta_{25–35}$, has been shown to be neurotoxic (Yankner et al., 1990; Pike et al., 1993). The current study has shown that, in the presence of $A\beta_{25–35}$, there is a significant increase in intracellular ROS levels in SH-SY5Y cells. A similar trend was seen in intracellular ROS levels in IMR-32 cells after exposure to $A\beta_{25–35}$. These results are consistent with previously published studies showing $A\beta_{25–35}$ to be an ROS generator and a neurotoxic agent (Barger et al., 1995; Kang et al., 2001; Jang et al., 2005). In relation to RNS levels, there was increased NO production in the presence of $A\beta_{25–35}$ in SH-SY5Y and IMR-32 cells. Studies have shown that an overproduction of NO results from upregulation of inducible nitric oxide synthase expression in neurons and glia as a result of various inflammatory responses in the brain in a variety of neurodegenerative diseases, such as AD and PD (Heneka and Feinstein, 2001; Togo et al., 2004; Mander and Brown, 2005).

Studies have indicated that polyphenols and flavonoids act as antioxidants and have the ability to scavenge ROS (Chen et al., 2006; Lagoa et al., 2011; Lin et al., 2012). *G. biloba* extracts have been reported to protect neurons against oxidative stress, but the underlying mechanisms are not fully understood (Maclennan et al., 2002; Ahlemeyer and Krieglstein, 2003). In the current study, treatment with GB, a terpene lactone component of standardized extract EGb 761, showed attenuation of ROS production in GB + $A\beta_{25–35}$-treated SH-SY5Y and IMR-32 cells. Our results are in line with the findings of Shi et al. (2009) showing the neuroprotective effect of GB against $A\beta_{25–35}$-induced neurotoxicity, leading to inhibition of ROS accumulation in SH-SY5Y cells. The increase in NO levels caused by $A\beta_{25–35}$ in both SH-SY5Y and IMR-32 cells was decreased to a greater extent after pretreating the cells with GB. Similarly, an earlier study showed that EGb 761 significantly reduced gentamycin-induced NO production in human cochlear cells (Yang et al., 2011).

APE1 plays a central role in the cellular response to oxidative stress. Attempts have been made to link increased levels of APE1 with protection in neuronal cells. APE1 is a dually targeted protein with preferred location in the nucleus and conditional distribution in the mitochondria (Li et al., 2010). With regard to the importance of mitochondria in cellular responses to oxidative stress, studies have been conducted to determine the role of APE1 in mitochondria. Recent studies have confirmed the existence of APE1 in mitochondria (Chattopadhyay et al., 2006; Li et al., 2010). Under normal cellular conditions the level of APE1 is minimal, but it increases in the presence of oxidative stress in the neuronal as well as the nonneuronal cells. An earlier study showed that APE1 rapidly relocalizes into mitochondria following H_2O_2 activation and might exert a protective function (Frossi et al., 2002). In the current study, modulation of APE1 expression during $A\beta_{25–35}$-induced oxidative stress in human neuroblastoma SH-SY5Y cell line was studied. An earlier study conducted by Tan et al. (2009) showed that high concentrations of $A\beta_{1–42}$ caused a reduction in APE1 expression leading to neuronal cell death, whereas lower concentrations of $A\beta_{1–42}$ transiently induced APE1 expression and were associated with prolonged neuronal survival. The current study shows that 20 μM $A\beta_{25–35}$ caused a significant decrease in endogenous mitochondrial APE1 levels in SH-SY5Y cells. Pretreatment with GB along with exposure to $A\beta_{25–35}$ caused an increase in APE1 levels in the mitochondria as determined by ELISA. In support of this finding, a recent article described a decrease in APE1 levels against $AlCl_3$-induced neurotoxicity that was upregulated after treatment with the phytochemical resveratrol, providing protection against neuroinflammation induced by $AlCl_3$ (Zaky et al., 2013). This is in agreement with our observations and points to the modulation of APE1 by GB in the presence of $A\beta_{25–35}$-induced oxidative stress.

Although studies have demonstrated a reduction in the expression of APE1 in neural tissue after oxidative insult, the role of APE1 in regulating neurotoxicity remains to be identified. An earlier study showed that overexpression of wild-type APE1 in hippocampal and sensory neuronal cells resulted in a significant increase in cell viability after exposure to various concentrations of H_2O_2 (Vasko et al., 2005). In the current study, pretreatment with GB prior to treatment with $A\beta_{25–35}$ increased cell survival in both SH-SY5Y and IMR-32 cells with ectopic APE1 expression. This shows that pretreatment with GB might modulate the activities or restore both repair and redox functions of APE1, providing

neuroprotection to cells from the damage caused by $A\beta_{25–35}$, leading to increased neuronal cell survival. In both SH-SY5Y and IMR-32 cells, there was an attenuation of ROS/RNS levels in cells with ectopic APE1 expression after exposure to $A\beta_{25–35}$. Pretreatment with GB prior to treatment with $A\beta_{25–35}$ caused a decrease in ROS/RNS levels in cells with ectopic APE1 expression compared with cells without ectopic APE1 expression. These results indicate a neuroprotective role for APE1 in regulating neurotoxicity induced by $A\beta_{25–35}$ and a synergistic role in the presence of the phytochemical GB.

A general decline in CNS function is associated with normal aging. In relation to this, certain mutations in mitochondrial genes have been implicated in the etiology of various age-related neurodegenerative disorders such as AD. The pathogenesis resulting from mtDNA mutations is believed to be involved in impaired OXPHOS, with a concomitant increase in ROS production. To study the effect of $A\beta_{25–35}$ at the mitochondrial level via OXPHOS performance, the current study was designed to observe the modulation of ETC activities of complexes I, III, and IV by APE1 in the presence of GB. Numerous studies have shown that GB protects against aging-associated mitochondrial dysfunction and reduces oxidative stress in the mitochondria (Abdel-Kader et al., 2007; Shi et al., 2009). The current study shows that APE1 has a neuromodulatory effect on complex I activity in bringing the activity of complex I to the basal level in the presence of GB and protecting the cellular energy levels through OXPHOS. The GB + $A\beta_{25–35}$-treated cells with ectopic APE1 expression showed a rise in complex III activity in both IMR-32 and SH-SY5Y cells, which could be a compensatory/remunerative internal mechanism in response to the toxic effect of $A\beta_{25–35}$, which was much more than the basal level activity (Rhein et al., 2010). This indicates the protection of complex III activity by APE1 in the presence of increased ROS in the presence of GB, although our study does not provide a direct link for this support. The activity of complex IV was found to be significantly reduced in the presence of $A\beta_{25–35}$-induced oxidative stress responses in SH-SY5Y and IMR-32 cells compared with the untreated control cells. This is in agreement with earlier studies showing a decline in complex IV activity after exposure to Aβ (Canevari et al., 1999; Rhein et al., 2010). Furthermore, a significant upregulation of complex IV activity was seen in GB + $A\beta_{25–35}$-treated IMR-32 cells with ectopic APE1 expression, whereas the activity was found to be close to the basal level in SH-SY5Y cells, showing the combined effect of GB and APE1 in restoration of activity of complex IV.

Taken together, our data suggest that ectopic APE1 expression helps in overcoming the oxidative stress induced by $A\beta_{25–35}$ in human neuroblastoma cells. Phytochemical GB has been shown to modulate the ROS/RNS levels in the presence of APE1. The current study demonstrates that $A\beta_{25–35}$ interferes with mitochondrial function as seen from the decline in complex I and IV activities in the presence of $A\beta_{25–35}$. GB may act as an antioxidant, reducing ROS/RNS generated by electron leakage at complexes I and III and restoring OXPHOS. Thus, pretreatment with GB might have therapeutic potential by modulating the activities of APE1 and providing neuroprotection from the oxidative damage caused by Aβ in AD, leading to increased neuronal cell survival. The precise mechanism of the mode of action of APE1 along with GB on OXPHOS performance requires further investigation.

CONCLUSIONS

Our results suggest that ectopic expression of APE1 enhances the capacity of human neuronal cells (SH-SY5Y and IMR-32) to overcome the oxidative damage caused by $A\beta_{25–35}$. The data show that $A\beta_{25–35}$ interferes with mitochondrial function, as seen from the decline in complex I and IV activities in the presence of $A\beta_{25–35}$. A noteworthy observation is that phytochemical GB together with APE1 modulates the $A\beta_{25–35}$-induced oxidative stress and restores OXPHOS. The current study also shows a decrease in APE1 levels in mitochondria after treatment with $A\beta_{25–35}$, which was restored and increased by pretreatment with GB. Thus, this study provides new insight into how the phytochemical GB helps in modulating/protecting APE1 functions by reducing mitochondrial oxidative stress and further helps in countering Aβ-induced dysfunction of the mitochondrial respiratory system, serving as a useful therapeutic tool for the treatment of AD in combination with phytochemical modulation. These observations apply directly in understanding the precise mechanism of the mode of action of GB on OXPHOS performance. The current study also suggests that APE1-mediated mitochondria-targeted therapies might be effective in AD therapeutics. Thus, therapies targeting the basic mitochondrial processes, such as energy metabolism, free radical generation, and restoring/protecting functions of APE1, hold great promise for AD treatment.

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