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Glutathione Cycle Impairment Mediates Aβ-induced Cell Toxicity

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Alzheimer's disease is widely held to be associated with oxidative stress due, in part, to the action of amyloid β-peptide (Aβ). We observed that Aβ 25–35 induced an increase in reactive oxygen species (ROS) in NT2 ρ^+ cells, leading to protein and lipid oxidation. This oxidative status was partially prevented by the antioxidants, vitamin E, reduced glutathione, and by melatonin. However, NT2 ρ^0 cells (that lack mitochondrial DNA) in the absence of Aβ showed an increase in ROS production, lipid and protein oxidation, as compared with parental ρ^+ cells. Upon Aβ 25–35 treatment, in ρ^+ cells, a decrease in glutathione reductase activity and in GSH levels was observed, whereas glutathione peroxidase activity was shown to be increased. In NT2 ρ^0 cells, in the absence of Aβ, GSH levels were maintained, whereas glutathione reductase and peroxidase activities were increased. The exposure of Aβ to ρ^0 cells did not induce any change in these parameters. We observed that melatonin prevented caspase activation and DNA fragmentation in ρ^+ cells treated with Aβ. Considering the evidence presented, we argue that the glutathione cycle impairment is a key event in Aβ-induced cell toxicity.

Keywords: Alzheimer' disease; Amyloid β-peptide; Reactive oxygen species; Reduced glutathione; Glutathione peroxidase; Glutathione reductase

INTRODUCTION

Senile plaques and neurofibrillary tangles are the hallmarks of Alzheimer's disease (AD)[1], which is the most common neurodegenerative disease of late life. Amyloid β-peptide (Aβ), the major component of senile plaques, has been suggested to play an important role in AD pathogenesis[1,2]. The sequence corresponding to amino-acids 25–35 of Aβ (Aβ 25–35) forms fibrils and has been shown to contribute to neurodegeneration in a variety of *in vitro* studies[3,4]. Nevertheless, the mechanisms by which Aβ elicits its toxic effect on neurons remain to be established.

Extensive studies have been shown that Aβ-induced neurotoxicity may be mediated by induction of oxidative stress[5–7]. The accumulation of reactive oxygen species (ROS) can initiate lipid[8] and protein[9] oxidation, initiating a positive feedback loop. Also, Aβ renders cells vulnerable to apoptosis, indicating that caspase activation may play an important role in Aβ-induced neurotoxicity. Several caspases involved in apoptosis have been described to be activated by Aβ[10–12]. However, the mechanisms by which Aβ activates caspases cascade are subject of discussion.

Several agents have been shown to be neuroprotective *in vitro* by targeting to specific pathways responsible for Aβ-induced toxicity. These agents include antioxidants or free radical scavengers[13–15], thus suggesting the involvement of oxidative stress and ROS generation in Aβ-induced neurotoxicity.

In the present study, we demonstrate that Aβ-induced neurotoxicity by caspase activation involves GSH depletion levels. We observed that ρ^0 cells (mtDNA depleted NT2 cells that lack functional mitochondria) were in a higher oxidative status, as compared with parental ρ^+ cells. We also explored

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the regulation of glutathione levels, one of the more important antioxidant defences in mitochondria, by Aβ 25–25. We found that despite Aβ had no effect on ρ^0 cells, it decreased GSH levels and glutathione reductase activity in ρ^+ cells, and induced an over activation of glutathione peroxidase activity. In untreated ρ^0 cells an over activation of both glutathione reductase and peroxidase activities and similar GSH levels to those of ρ^+ cells were observed. Melatonin was the antioxidant tested which was efficient in preventing caspase activation and DNA fragmentation by Aβ 25–35 on ρ^+ cells.

MATERIALS AND METHODS

Chemicals

Amyloid β-peptide fragments (Aβ 25–35) were obtained from Bachem (Bubendorf, Germany). Vitamin E (α-tocopherol succinate), GSH ethyl ester, and melatonin were purchased from Sigma Chemical Company (St. Louis, MO). Fetal calf serum was obtained from Biochrom KG (Germany). Dihydrorhodamine 123 was purchased from Molecular Probes (Eugene, OR). TUNEL kit was obtained from Promega (Madison, WI).

Cell Culture

Stock cultures of NT2 (human teratocarcinome) ρ^+ cells were purchased from Stratagene (La Jolla, CA). Obtention of the NT2 ρ^0 cell line used in these experiments was previously described[16]. Cells were grown routinely in 75 cm^2 tissue culture flasks in Optimen Medium that was supplemented with 10% heat inactivated fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Uridine (50 μg/ml) and pyruvate (200 μg/ml) were also added to ρ^0 cell growth medium. Both cell lines were grown and maintained at 37° C in a humidified incubator containing 95% air and 5% CO_2. The cells were plated at 0.6×10^6/ml for ROS determinations, carbonyl groups quantification, and for determination of GSH levels, and at 2×10^6/ml for glutathione reductase, peroxidase, caspase activity and TBARS quantification.

Twenty-four hours after seeding of cells the medium was refreshed and 10 μM of Aβ 25–35 added, during 24 h from a 1 mM stock solution of Aβ 25–35 (water solution). For antioxidant experiments, vitamin E was prepared in ethanol and melatonin in DMSO. The final concentration of ethanol and DMSO was 0.1% (v/v), which had no effects by itself. Cells were pre-incubated with vitamin E (13 μM), and melatonin (83 μM) for 22 h and then incubated for an additional 24 h in the presence of Aβ 25–35. For GSH experiments, GSH (2 mM) was incubated simultaneously for 24 h in the presence of Aβ 25–35. For all conditions tested, control experiments were performed in which Aβ was not added, keeping all other incubation parameters unchanged.

Monitorization of ROS Generation

ROS were measured according to the method of Royall and Ischinopoulos[17], following the oxidation of dihydrorhodamine-123 to fluorescent rhodamine, which detects the formation of intracellular peroxides, peroxidase activity and can also reflect reduced glutathione concentration. After exposure to Aβ 25–35 or antioxidants, the cells were incubated for 15 min in the dark, with 5 μM dihydrorhodamine-123 in a saline buffer, pH 7.4, at 37° C. After washing the cells, fluorescence was measured during 10 min with excitation at 500 nm and emission at 536 nm.

Quantification of Lipid Peroxidation

The extent of lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS), which include malondialdehyde, using the Thiobarbituric Acid Test according to a modified procedure described by Ernster and Nordenbrand[18]. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}$/cm and expressed as nmol TBARS/mg of protein.

Protein Oxidation Determination

Protein carbonyl content was determined as described by Levine *et al.*[19], with slight modifications. Cell extracts were incubated with 0.5 ml of 10 mM dinitrophenylhydrazine in 2 N HCl (or 2 N HCl alone for the blanks), for 1 h at room temperature. The protein hydrazone derivatives were precipitated with 0.5 ml of 20% trichloroacetic acid and the precipitates were washed three times with 1 ml ethanol/ethylacetate (1:1). During each washing, the homogenized pellet was vortexed and left in the washing solution for 10 min at room temperature before centrifugation. The final pellet was resuspended in 6 M guanidine HCl, and incubated during 15 min at 37° C. The carbonyl content was determined spectrophotometrically at 360 nm on the basis of molar absorbance coefficient of 22,000 M^{-1}/cm, and expressed as nmol/mg of protein.

Glutathione Peroxidase Assay

The activity of glutathione peroxidase was measured using a method described by Plagia and Valentine[20]. Cells were washed twice in PBS and collected in a buffer containing 25 mM HEPES, pH 7.5, 1 mM

EDTA, 1 mM EGTA, 2 mM $MgCl_2$. Cells were scraped, homogenized on ice, and frozen three times on liquid nitrogen. The activity of this enzyme was measured indirectly, by determining the oxidation of NADPH at 340 nm, induced by the action of glutathione reductase. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, supplemented with 500 μM EDTA, 10 μl triton X-100, 1 mM GSH, 1 U glutathione reductase and the cellular extract. The reaction, performed at 30° C, was initiated by the addition of 70 μM H_2O_2 and 280 μM NADPH. Results were expressed as U/mg protein.

Glutathione Reductase Assay

The activity of glutathione reductase was measured, by following the oxidation of NADPH at 340 nm, using a method described by Goldberg and Richard[21]. Cells were washed twice in PBS and collected in a buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$. Cells were scraped, homogenized on ice, and frozen three times on liquid nitrogen. The reaction was initiated by the addition of 170 μM NADPH to the reaction mixture containing 100 mM potassium phosphate buffer, pH 7.4, supplemented with 500 μM EDTA, 2.2 mM oxidized glutathione and the cellular extract, at 30° C. Results were expressed as U/mg protein.

Determination of Total Cellular Glutathione

After incubation with Aβ 25–35 or antioxidants, the medium was removed and the cells were extracted, in ice, with 0.3 M perchloric acid. The cells were scraped from the wells and centrifuged at 15,800*g* for 5 min. The resulting pellets were solubilized with 1 M NaOH and analysed for total protein content by the Sedmak method[22], using bovine serum albumin as standard. The supernatants were neutralized with 10 M KOH and 5 M Tris and centrifuged at 15,800*g* for 5 min. The resulting supernatants were assayed for cellular reduced glutathione by reverse phase equipped with a Nova-pack C-18 column (75 × 3.9 mm internal diameter, particle size 10 μm), using a linear gradient elution system consisting of an eluent A (30 mM sodium acetate, pH 6.8) from 100 to 50%, and an eluent B (methanol) from 0 to 50%, with a flow rate of 1 ml/min during 30 min. Reduced glutathione (GSH) was detected as fluorescent derivates at excitation and emission wavelengths of 360 and 450 nm, with a Gilson fluorescent detector (model 121), after precolumn derivatization with *o*-phthaldialdehyde/2-mercaptoethanol (OPA/MCE), according to the method described by Sitges *et al.*[23]. The GSH content was determined by quantification of peak areas compared to external standards, and was expressed as nmol/mg of protein.

Ac-DEVD-pNA Cleavage (Caspase Activity) Assay

After treatment with Aβ 25–35 or antioxidants, cells were harvested for assays of caspase activity, by the method described by Cregan *et al.*[24]. Cells were washed twice in PBS and collected in a buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1:100 dilution of a protease inhibitor cocktail. Cells were scraped, homogenized on ice, and frozen three times on liquid nitrogen. The lysate was centrifuged at 15,800*g* for 10 min, and the resulting supernatant was stored at −80° C. Lysates (50 μg of protein) were incubated at 37° C, during 90 min, in 25 mM HEPES, pH 7.5, containing 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol, and 40 μM DEVD-pNA to determine Ac-DEVD-pNA cleavage. Substrate cleavage was detected in a Ph L Mediators Luminometer (Vienna, Austria) at 405 nm.

TUNEL Assay

The modified terminal deoxynucleotidyltransferase dUTP nick end labelling (TUNEL) assays were performed using the DeadEnd™ colorimetric apoptosis detection system (Promega) according to the protocol provided by the manufacture. The cells in the poly-lysine pre-coated slides were fixed in 4% paraformaldehyde for 30 min. After washing twice with PBS, the slides were immersed in 0.2% triton X-100 for 5 min to permeabilize cells. The slides were incubated with biotinylated nucleotide and terminal deoxynucleotidyl-transferase in 100 μl of equilibration buffer at 37° C for 1 h inside a humidified chamber to allow the end labelling reactions to occur. The reaction was stopped by immersing slides in 150 μM sodium chloride, 15 mM sodium citrate, pH 7.4, for 15 min followed by immersion in PBS for 15 min (2 times). Thereafter, the endogenous peroxidases were blocked by immersing the slides in 0.3% H_2O_2 for 5 min. The slides were treated with 100 μl of horseradish peroxidase-labelled streptavidin solution and incubated for 30 min at room temperature. Finally, the slides were developed using the peroxidase substrate, H_2O_2, and the stable chromogen, diaminobenzidine, for 15 min. The slides were rinsed with water and examined under a light microscope. The photographs were taken at × 40 magnification.

Data Analysis

Data were expressed as mean ± SEM of the indicated number of determinations, from at least three independent experiments. Statistical significance analysis was determined using the two-tailed

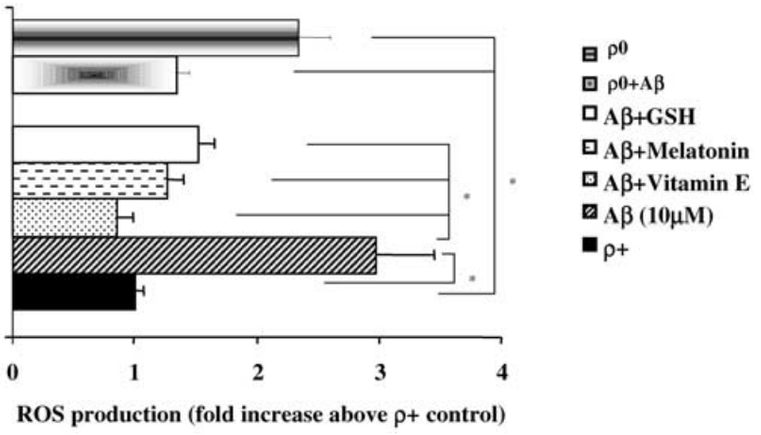

FIGURE 1 Effect of Aβ 25–35 on ROS production in NT2 ρ^+ and ρ^0 cells and role of antioxidants. NT2 cells were incubated, during 24 h, with 10 μM of Aβ 25–35. NT2 ρ^+ cells were incubated with Aβ 25–35 in the presence of vitamin E (13 μM), melatonin (83 μM), and GSH (2 mM). ROS formation was detected using dihydrorhodamine 123 oxidation and fluorescence. Results, expressed as the increase above of ρ^+ control values, are the mean ± SEM of duplicated determinations for four to ten independent experiments. *$p < 0.05$, significantly different.

Student's *t*-test (a p value <0.05 was considered significant).

RESULTS

Oxidative Stress Involvement in Aβ-mediated Toxicity

As shown in Fig. 1 native ρ^+ cells, that have a normal mitochondrial electron transport chain, an increase of 2.5 fold in ROS production above control values was observed after Aβ 25–35 (10 μM) treatment (1.00 ± 0.08 in ρ^+ control and 2.96 ± 0.48 in ρ^+ Aβ). To confirm the involvement of oxygen radicals in the Aβ-induced toxicity in this model, we tested the effect of the antioxidants vitamin E, melatonin and GSH on ROS production. All compounds tested prevented the increase in free radicals production upon Aβ treatment (Fig. 1).

In NT2 ρ^0, ROS production was increased 1.7 fold as compared to parental ρ^+ cells (2.34 ± 0.35 in ρ^0 cells). These results were probably due to the lack of functional mitochondria the abnormalities in mitochondria respiratory chain enzymes activity, leading to an abnormal free radical production. However, Aβ 25–35 treatment did not increase ROS formation in ρ^0 cells (1.33 ± 0.14 in ρ^0 cells treated with Aβ).

Effect of Aβ 25–35 on Lipid and Protein Oxidation: Role of Antioxidants

Previous studies showed that Aβ could induce lipid and protein oxidation in PC12 cells[25]. Exposure of NT2 ρ^+ cells to Aβ 25–35 (10 μM) resulted in an increase of 1.6 fold in TBARS formation (0.61 ± 0.02 nmol/mg in control cells and 1.02 ± 0.07 nmol/mg in Aβ treated cells) (Fig. 2A). All the antioxidants tested prevented an increase in lipid peroxidation. As we can observe in Fig. 2A, ρ^0 cells show a higher lipid oxidation than ρ^+ cells (for about 1.2 fold) (0.74 ± 0.05 nmol/mg in ρ^0 cells), probably due to the increase in ROS production in ρ^0 cells. However, Aβ 25–35 treatment did not increase lipid oxidation in ρ^0 cells (0.78 ± 0.01 nmol/mg in Aβ treated ρ^0 cells).

NT2 ρ^+ cells treated with Aβ 25–35 (10 μM) showed an about 2 fold increase formation of carbonyl groups, as compared to untreated cells (3.18 ± 0.57 nmol/mg in controls and 6.27 ± 1.00 nmol/mg in Aβ treated cells) (Fig. 2B). The carbonyl content in ρ^+ cells pre-incubated with vitamin E, melatonin and GSH, decreased as compared to ρ^+ cells treated with Aβ 25–35. However, an about 2 fold increase in carbonyl groups occurred in ρ^0 cells (6.48 ± 0.89 nmol/mg), in comparison to parental ρ^+ cells, but no alteration was observed with Aβ 25–35 (4.93 ± 1.02 nmol/mg).

GSH Levels, Glutathione Reductase and Peroxidase Activities Determined in the Presence of Aβ: Effect of Antioxidants

The intracellular levels of GSH were determined in NT2 ρ^+ and ρ^0 cells, being identical in both cell lines (23.62 ± 0.60 nmol/mg in control ρ^+ cells and 24.02 ± 1.70 nmol/mg in control ρ^0 cells). However, only in ρ^+ cells Aβ 25–35 (10 μM) induced a decrease in GSH levels of 33% (15.68 ± 1.44 nmol/mg) (Fig. 3A). Treatment of the cells with vitamin E or melatonin completely protected ρ^+ cells against the Aβ-induced depletion of GSH levels (Fig. 3A).

Aβ significantly inhibited glutathione reductase activity, in ρ^+ cells, the enzyme responsible for

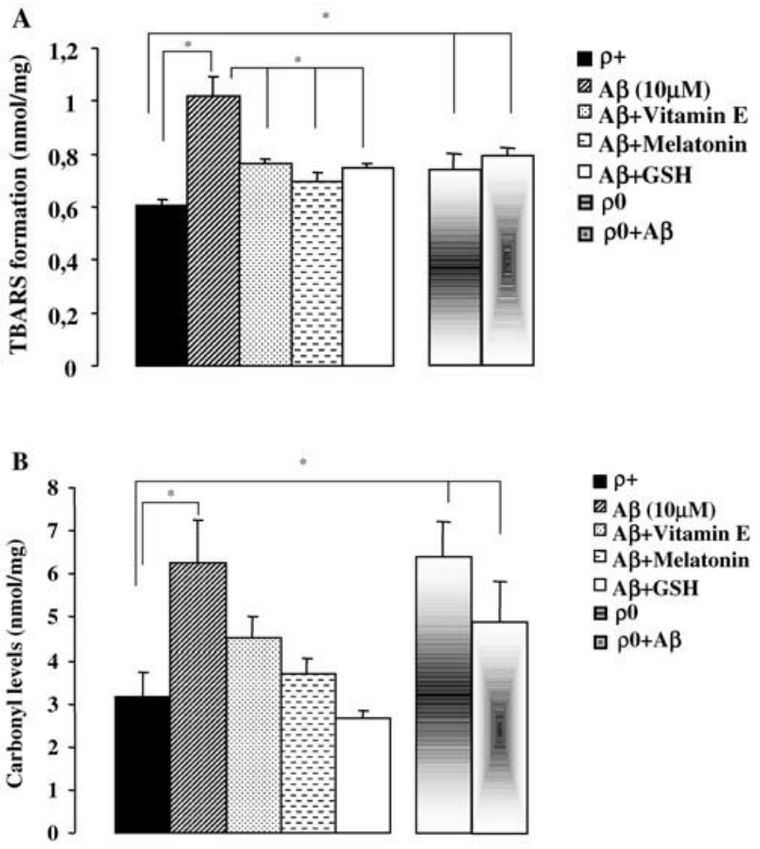

FIGURE 2 Effect of Aβ 25–35 on lipid and protein oxidation: role of antioxidants. NT2 ρ^+ cells were incubated with Aβ 25–35 in the presence of vitamin E (13 μM), melatonin (83 μM), and GSH (2 mM). (A) Lipid peroxidation was determined by TBARS formation, and (B) protein oxidation detected by the presence of carbonyl groups. Data, expressed in nmol/mg of protein, are the mean ± SEM values of duplicated determinations derived from three to five independent determinations. *$p < 0.05$, significantly different.

the conversion of oxidized glutathione into GSH (Fig. 3B). After incubation of ρ^+ cells with Aβ 25–35 (10 μM), glutathione reductase activity was reduced by 30% (5.24 ± 0.47 U/mg in ρ^+ untreated cells and 3.60 ± 0.26 U/mg in Aβ treated cells). The simultaneous addition of GSH to the cell culture protected ρ^+ cells against Aβ-induced reduction of glutathione reductase activity. Also, glutathione reductase activity was observed to be increased for about 1.4 fold in ρ^0 cells as compared to parental ρ^+ cells (7.43 ± 0.58 U/mg in untreated ρ^0 cells) (Fig. 3B). Aβ did not alter the activity of glutathione reductase in ρ^0 cells (6.98 ± 0.55 U/mg).

The activity of glutathione peroxidase was also studied, because this enzyme is responsible for the conversion of hydrogen peroxide into water, at GSH expenses. The activity of glutathione peroxidase was largely increased in ρ^0 cells as compared to ρ^+ cells (for about 4 fold) (13.65 ± 0.80 U/mg in control ρ^+ cells and 54.65 ± 7.00 U/mg in control ρ^0 cells), probably because ρ^0 cells show an increase in ROS formation but maintained GSH levels similar to ρ^+ cells (Fig. 3C). Aβ 25–35 (10 μM) had no effect on ρ^0 cells (66.15 ± 8.00 U/mg), but increased glutathione peroxidase activity on ρ^+ cells (17.24 ± 1.10 U/mg) (Fig. 3C). These results seem to indicate that ρ^+ cells are responding to Aβ-induced toxicity, by regulating glutathione peroxidase activity (Fig. 3C). Vitamin E, melatonin or GSH were able to decrease ROS formation (Fig. 1) and, consequently, prevented the need to the cellular increase of glutathione peroxidase activity (Fig. 3C).

Caspase Activation and DNA Fragmentation Upon Aβ Treatment: The Role of Melatonin

Recently, it has been described[10–12] that Aβ mediates cell toxicity by the activation of several caspases. In order to determine if oxidative stress is involved in the apoptotic signalling induced by Aβ, the effect of several antioxidants on Aβ-mediated caspase activation was tested (Fig. 4). Aβ 25–35 (10 μM) induced Ac-DEVD-pNA cleavage for about 3 fold in ρ^+ cells, melatonin being the only antioxidant tested capable of preventing

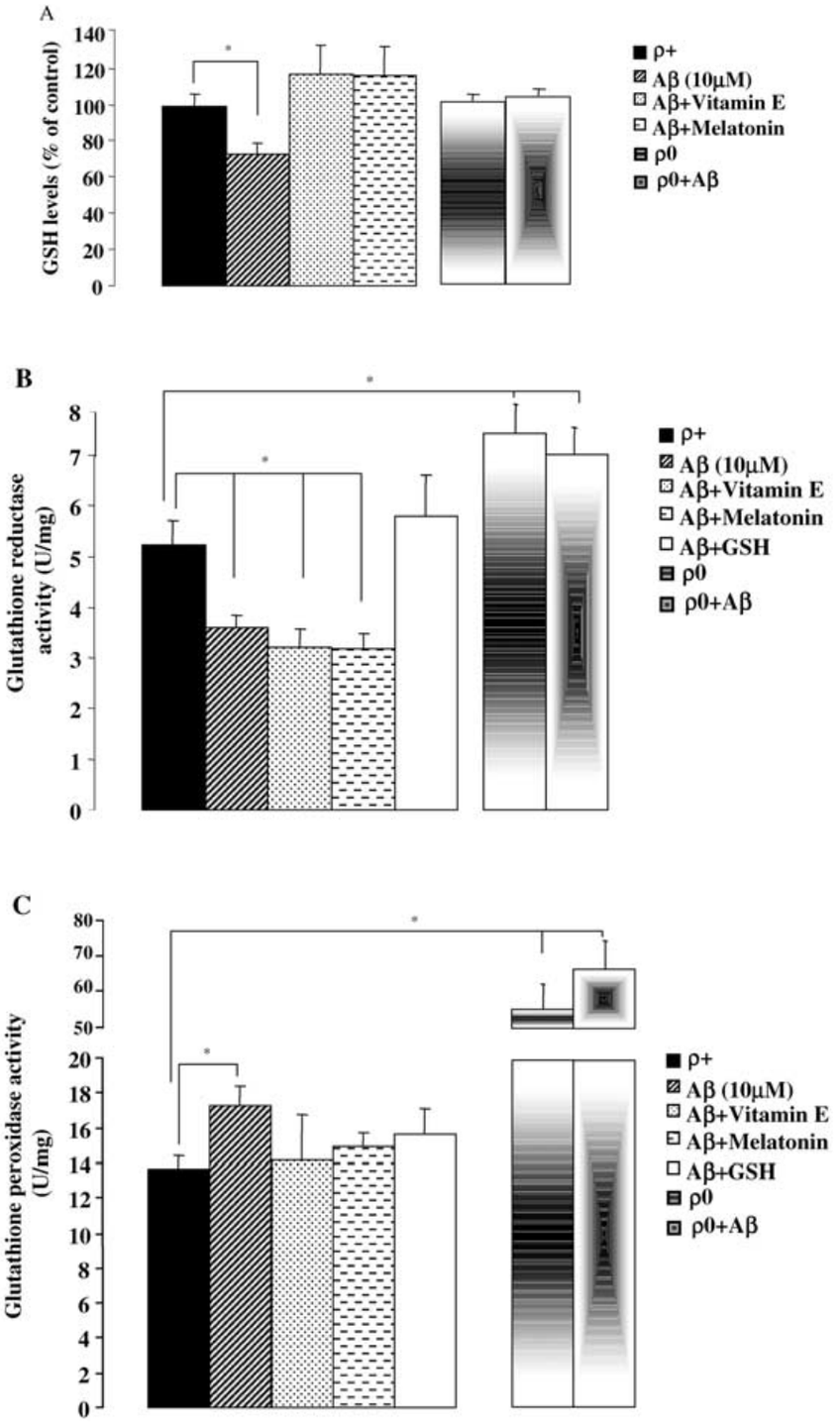

FIGURE 3 Effect of Aβ 25–35 peptide on the glutathione cycle: role of antioxidants. NT2 cells were incubated, during 24 h, with 10 μM of Aβ 25–35. NT2 ρ^+ cells were incubated with Aβ 25–35 in the presence of vitamin E (13 μM), melatonin (83 μM), and GSH (2 mM). (A) GSH levels, (B) glutathione reductase activity and (C) glutathione peroxidase activity were determined in both cell lines. Data expressed % of control for GSH levels and in U/mg of protein for the enzymatic activities, are means ± SEM values of duplicated determinations of three to five independent experiments. *$p < 0.05$, significantly different.

this activation. DNA fragmentation was detected after a 96 h treatment with Aβ 25–35 (10 μM) (Fig. 4B). Melatonin was able to prevent DNA fragmentation.

DISCUSSION

The present results show that Aβ 25–35 (10 μM) decreased glutathione reductase activity and GSH

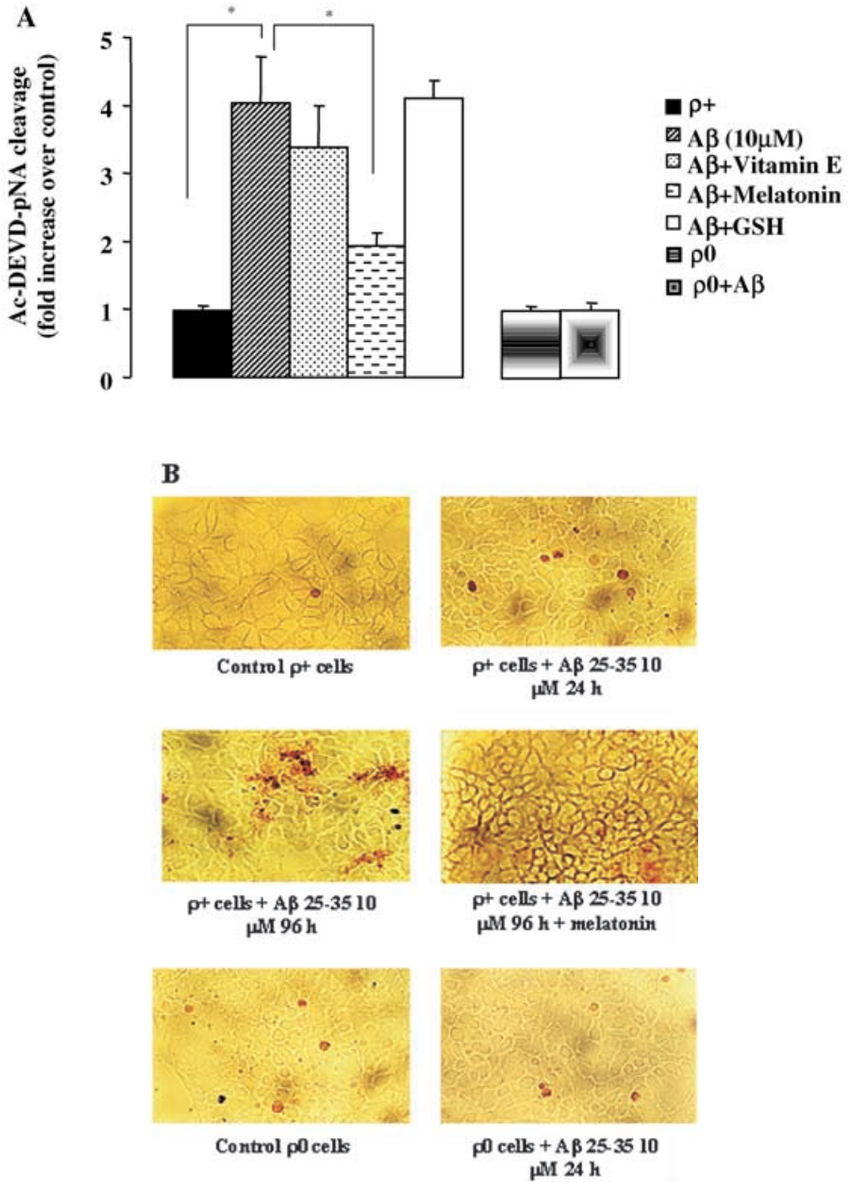

FIGURE 4 Effect of Aβ 25–35 peptide and antioxidants on caspase activation and DNA fragmentation. NT2 cells were incubated, during 24 h, with 10 μM of Aβ 25–35. NT2 ρ^+ cells were incubated with 10 μM Aβ 25–35, during 24 h in the presence of vitamin E (13 μM), melatonin (83 μM), and GSH (2 mM). (A) Ac-DEVD-pNA cleavage. Results, expressed as the increase above of control values, are the mean ± SEM of duplicated determinations of three independent. *$p < 0.05$, significantly different. (B) DNA fragmentation was analyzed by optical microscopy, identifed by TUNEL positive cells (dark brown), and shows representative images from 2 independent experiments.

levels, but induced an increase in glutathione peroxidase activity in ρ^+ cells. We observed that glutathione reductase and peroxidase activities were increased in untreated ρ^0 cells, GSH levels being similar to those found in ρ^+ cells. In our previous work, we clearly demonstrated that Aβ induces neurotoxicity by acting on mitochondria[26], being described that mitochondria are the principal source of ROS generation. The purpose of the present work was to clarify the involvement of the glutathione cycle on Aβ-induced cell toxicity by using NT2 (ρ^+) and mitochondrial DNA depleted NT2 (ρ^0) cell lines.

We observed that Aβ 25–35 increased ROS formation (Fig. 1), this finding being consistent with previous data from our laboratory and others[14,27–29]. Despite the increased ROS production observed in untreated ρ^0 cells, no further increase was observed upon Aβ 25–35 treatment (Fig. 1). The increase in dihydrorhodamine oxidation in ρ^0 cells could be explained by one hand due to the lack of mtDNA, leading to a non functional electron

transport chain, as 13 protein subunits of the mitochondrial respiratory chain are mtDNA encoded[30,31], or due to the great peroxidase activity that is observed in ρ^0 cells. Aβ 25–35 treatment in ρ^0 cells showed that ROS levels returned to parental resting levels, indicating that this peptide may act at peroxidase level. In line with the oxidative hypothesis, we found that lipid and protein oxidation was also occurring in ρ^0 untreated, and ρ^+ treated cells (Fig. 2A,B). Increasing evidence demonstrates that oxidative stress causes cell dysfunction in age-related disorders such as AD[28,29]. The nervous tissue is very susceptible to oxidative stress, due to its high rate of oxygen consumption, relatively low levels of antioxidant defences, and a high content of polyunsaturated fatty acids that serve as substrates for lipid peroxidation[28,29]. Aβ enhancement of oxidative stress can be facilitated further by its interaction with mitochondria[26], since mitochondria constitute the greatest source of ROS[32]. On the basis of the oxidative hypothesis, antioxidants such as vitamin E, GSH and melatonin[26] were tested to explore oxidative stress involvement in Aβ-mediated toxicity. Pre-incubation of NT2 ρ^+ cells with vitamin E and melatonin ameliorated Aβ-induced oxidative stress. Reduced glutathione was also shown to be effective in preventing ROS formation, lipid and protein oxidation in ρ^+ cells induced by Aβ 25–35 (Figs. 1 and 2A,B).

Since GSH is the major cellular thiol participating in cellular redox reaction and thioether formation[33], and is the substrate of glutathione peroxidase, an antioxidant enzyme that inactivates peroxides, we studied Aβ 25–35 action on the glutathione cycle. Although, ρ^0 cells were more sensitive to oxidation, they had similar GSH levels as parental ρ^+ cells (Fig. 3). Aβ 25–35 peptide decreased GSH levels only in ρ^+ cells, this effect being reverted by melatonin and vitamin E action. Furthermore, Aβ induced a decrease in glutathione reductase activity thus enhancing the decrease in GSH levels (Fig. 3A,B). GSH was more effective than other compounds in preserving glutathione reductase activity in Aβ-treated ρ^+ cells (Fig. 3B). We also observed an increase in glutathione peroxidase activity upon Aβ-treatment in ρ^+ cells, probably by a tight regulation to compensate the increase of peroxides inside the cell (Fig. 3C). Despite the fact that ROS production was found to be increased in ρ^0 cells, GSH levels were similar to those found in control ρ^+ cells, probably due to the large increase in glutathione reductase in untreated ρ^0 cells (Fig. 3A,B). Glutathione peroxidase activity was increased in ρ^0 cells, suggesting that ρ^0 cells found a way to prevent oxidative stress.

In previous reports[10–12], it has been suggested that Aβ induced cell death through an apoptotic pathway. In order to demonstrate if ROS have a role in apoptosis signalling, the effect of several antioxidants on caspase activation induced by Aβ 25–35 was tested. As shown in Fig. 4A, melatonin was the only antioxidant tested that protected cells against Aβ-induced toxicity by preventing ROS production and Ac-DEVD-pNA cleavage. Although melatonin

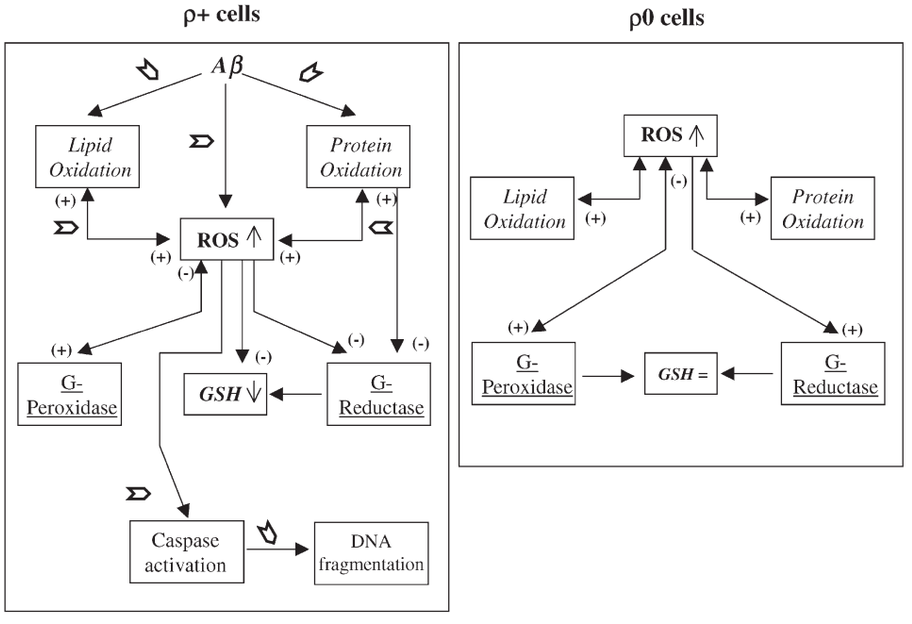

FIGURE 5 Proposed model for role of ROS in Aβ 25–35 induced neurotoxicity. Schematic illustration describes a possible pathway contributing to cell toxicity by Aβ 25–35 and the cellular antioxidants regulation. The opened arrowhead indicates the target site for antioxidants.

mechanism of action is at present unclear, it is known to bind heavy metals[34] and to increase intracellular GSH levels through an up-regulation of GSH-synthesizing enzymes[35]. It has been reported that antioxidants that act on preventing caspase activation could be the next therapeutical approach to AD[36,37]. Furthermore, Aβ treatment showed DNA fragmentation only in ρ^+ cells, melatonin being effective in preventing apoptosis (Fig. 4B). Taken together, the results presented provide a possible mechanism by which Aβ provokes cell neurodegeneration and/or death. In this model (Fig. 5), Aβ-mediates cell toxicity by acting on mitochondrial electron transport chain leading to free radical generation and, consequently, to lipid and protein oxidation. In an acute insult like Aβ-treatment, GSH levels decreased and caspase was activated leading to DNA fragmentation. In untreated ρ^0 cells a fine regulation of antioxidant enzymes allowed the maintenance of GSH levels, and cell viability. Antioxidants can ameliorate Aβ toxicity, melatonin being the most effective antioxidant in preserving cell viability.

In conclusion, our data suggest that the inhibition of caspase activation and the maintenance of GSH levels can be proposed as neuroprotective strategies in AD. The development of compounds such the pineal indoleamine melatonin, that combines potent antioxidant and caspase inhibitory properties, may prevent the incidence of AD or retard the progression of the this disease. Thus, our findings point melatonin as a potential therapeutic agent in AD.

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