Functional mitochondria are required for amyloid β-mediated neurotoxicity

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ABSTRACT

The role of mitochondria in amyloid βpeptide (Aβ)-induced cytotoxicity is unclear. We therefore exposed NT2 cells, a clonal human teratocarcinoma cell line capable of differentiation into terminal neurons, to Aβ 25–35 or to Aβ 1–42 to evaluate cell viability and altered mitochondrial function. A 24-h incubation of native NT2 cells (ρ+ cells) with Aβ 25–35 or with Aβ 1–42 produced a dose-dependent decline in MTT reduction. Aβ 1–42 was shown to be more toxic compared with Aβ 25–35. Aβ 25–35 toxicity was prevented or diminished by a 22-h preincubation with antioxidants (vitamin E, melatonin, and idebenone), as well as by simultaneous incubation with GSH or the nicotinic receptor agonist nicotine. Aβ 25–35 exposure was also associated with (1) inhibition of mitochondrial respiratory chain complexes (I, NADH-ubiquinone oxidoreductase; II/III, succinate-cytochrome *c* oxidoreductase; and IV, cytochrome *c* oxidase), (2) ATP depletion, and (3) reduction of the mitochondrial membrane potential. In contrast, NT2 cells rendered incapable of oxidative phosphorylation via depletion of their mitochondrial DNA (ρ0 cells) were unaffected by exposure to Aβ 25–35 or Aβ 1–42. These data indicate that Aβ can disrupt mitochondrial function and that such disruption causes oxidative stress. It is further suggested that a functional mitochondrial respiratory chain is required for Aβ toxicity.

Key Words: Alzheimer's disease • mitochondria • β-amyloid, ρ0 cells

Alzheimer's disease (AD) is the most common neurodegenerative disease of late life. Clinically, AD is characterized by insidious, chronic, and progressive memory impairment. Histopathologically, accumulation of neurofibrillary tangles and neuritic plaques in selective brain regions is observed (1, 2). Various biochemical defects occur, including mitochondrial dysfunction.

The amyloid β-peptide, the major component of neuritic plaques, is a 40 to 42 amino acid proteolytic fragment of the amyloid precursor protein (3). The sequence corresponding to amino acids 25–35 of Aβ (Aβ 25–35) forms fibrils and is perhaps responsible for *in vitro* toxicity

observed in Aβ (4, 5). Despite this knowledge, the mechanisms by which neurodegeneration occurs in AD and the ways in which Aβ might influence neurodegeneration are poorly understood. Other data suggest that bioenergetic failure resulting from depressed cytochrome c oxidase activity and reactive oxygen species (ROS) overproduction could contribute to neuronal death in AD (6, 7, 8). It is therefore tempting to try associating Aβ with these events. For instance, it appears that extracellular Aβ accumulation as dense core plaques is associated with protein oxidation (9), lipid peroxidation (10), and advanced glycation end products (11) in AD brain. Aβ neurotoxicity is attenuated by antioxidants (12, 13) and exacerbated by ROS generation (7). More recently, studies in our laboratory and others (7, 14) showed that Aβ 25–35 and 1–40 peptides decrease the activity of mitochondrial respiratory chain complexes. However, data demonstrating that a functional mitochondrial respiratory chain is required for Aβ toxicity is still lacking.

One potential strategy for establishing whether Aβ toxicity proceeds through disruption of mitochondrial respiratory function involves the use of cells depleted of mitochondrial DNA (mtDNA). Such "ρ0 cells" lack critical catalytic subunits of the mitochondrial respiratory chain (15). A ρ0 cell line created from NT2 cells—a clonal human teratocarcinoma cell line that differentiates to form neurons, exhibits an excitotoxic response—and secretes amyloid (16, 17) previously described (18). This cell line may therefore serve as an appropriate resource for studying the role of mitochondria in a number of neurodegeneration-relevant events.

In the present work, to explore the connection between mitochondria and Aβ–neurotoxicity, we compared the effects of Aβ 25–35 or Aβ 1–42 on native NT2 (ρ+ cells) and NT2 ρ0 cells. We found that in ρ+ cells, Aβ 25–35 affected cell viability and caused inhibition of the mitochondrial respiratory chain (MRC) enzymes complex I (NADH-ubiquinone oxidoreductase), II/III (succinate-cytochrome c oxidoreductase), and IV (cytochrome c oxidase) and depletion of ATP levels, as well as a decrease in mitochondrial membrane potential (mt $\Delta\psi$). These toxic effects could be prevented or ameliorated by the antioxidants vitamin E, idebenone, melatonin, and GSH, which suggests an important role for ROS as toxic intermediaries. Nicotine, which agonizes nicotinic cholinergic receptors, also exerted a neuroprotective effect. In contrast, Aβ did not affect cell viability, alter ATP levels, or change the mitochondrial membrane potential of ρ0 cells, which suggests that Aβ toxicity is dependent on the presence of a functional MRC.

MATERIALS AND METHODS

Chemicals

Amyloid β-peptide fragments (25–35 and 1–42) were obtained from Bachem (Bubendorf, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), vitamin E (α-tocopherol succinate), GSH ethyl ester, melatonin, nicotine, and ubiquinone-1 were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was obtained from Biochrom KG (Berlin, Germany). Rhodamine 123 was purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Idebenone was a generous gift from Seber (Lisbon, Portugal).

Cell culture

Stock cultures of NT2 ρ+ cells were purchased from Stratatgene (La Jolla, CA). Creation of the NT2 ρ0 cell line used in these experiments was described previously (18). 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 ρ0 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 0.1×10^6 cells/ml for toxicity experiments, 0.6×10^6/ml for ATP measurements, 0.15×10^6/ml for membrane potential determinations, and 2×10^6/ml for determination of mitochondrial respiratory chain complex activities.

Cytotoxicity Assays

Twenty-four hours after seeding the cells, the medium was refreshed and the desired amount of amyloid β-peptide fragment was added from a 1-mM stock solution for Aβ 25–35 or 276.9 µM for Aβ 1–42, prepared in sterile water, and left to age 72 h at 37ºC. Whenever tested, the reverse sequences of Aβ peptides were prepared under the same conditions. Staurosporine was prepared in DMSO (1 µM) and was incubated for 6 h. Hydrogen peroxide (H_2O_2) was prepared in sterile water (100 µM) and was incubated for 15 min. For antioxidant experiments, vitamin E and idebenone were prepared in ethanol and melatonin in DMSO. The final concentration of ethanol and DMSO was 0.1% (v/v), which had no protective or toxic effect by itself. Cells were preincubated with Vitamin E (13 µM), idebenone (1 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 and nicotine experiments, GSH (2 mM) and nicotine (5 µM) were 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; all other incubation parameters were unchanged.

We evaluated Aβ-peptide-induced cytotoxicity by measuring MTT reduction according to the method of Mosmann (19). Briefly, after cells were incubated under their specified conditions, the medium was aspirated and 0.5 ml of MTT-containing medium was added. After an additional 3-h incubation at 37°C, 0.5 ml isopropanol/HCl was added to each well and the absorbance at 570 nm was measured.

Cell viability was also assessed by monitoring the activity of the cytoplasmic enzyme LDH in the extracelular incubation medium. The LDH activity was measured spectrophotometrically according to the method of Bergmeyer and Brent (20), by following the rate of conversion of NADH to NAD^+, at 340 nm. LDH leakage was expressed as a percentage of total LDH activity, which corresponds to the activity determined in the supernatant plus that determined after incubation in a hypotonic solution containing 15 mM tris, pH 7.4.

Fluorometric analysis of membrane potential (Δψ)

We estimated changes in cell membrane potential by using the fluorescent cationic dye rhodamine 123 according to Palmeira et al. (21). At the end of the Aβ 25–35 exposures,

experiments were terminated by addition of Triton X-100, which disrupted cells and allowed for optimal retention of the dye. Cells were subsequently incubated with 1 μM rhodamine 123 at 37°C for 10 min, washed, and transferred to a clean cuvette, and the amount of dye within the cells was determined fluorometrically at 37°C with excitation and emission wavelengths of 490 and 515 nm.

NADH-Ubiquinone oxidoreductase (complex I) assay

Mitochondrial complex I activity was measured by modifying the method of Ragan et al. (22), which determines the decrease in NADH absorbance at 340 nm that occurs when ubiquinone (CoQ_1) is reduced to ubiquinol. We initiated the reaction by adding CoQ_1 (50 μM) to the reaction mixture containing 20 mM potassium phosphate, pH 7.2, 10 mM $MgCl_2$, 0.15 mM NADH, 2.5 mg BSA-FFA, 1 mM KCN, and the mitochondrial sample at 30°C. After 5 min, rotenone (10 μM) was added and the reaction was followed for another 5 min. The activity of complex I was determined by using the rotenone-sensitive rate, and for the Aβ experiments this activity is expressed as a percentage of the control rate.

Succinate cytochrome *c* oxidoreductase (complex II/III) assay

Complex II/III activity was measured according to the method of King (23), which measures the appearance of reduced cytochrome *c* at 550 nm. The reaction mixture contained 0.1 M potassium phosphate, pH 7.4, 0.3 mM di-K EDTA, and 0.1 mM cytochrome *c*. The mitochondrial sample was preincubated at 30°C for 5 min with 20 mM succinate and 1 mM KCN to activate the enzyme and was then added to the reaction mixture. The reaction was performed at 30°C. Antimycin A (0.02 mM) was subsequently added to inhibit complex II/III. Activities were thus determined as the antimycin A sensitive rate and are reported relative to the activity measured under control conditions.

Cytochrome *c* oxidase (complex IV) assay

Complex IV activity was measured according to the method of Wharton and Tzagoloff (23) by measuring the decrease in absorbance at 550 nm that occurs as reduced cytochrome *c* is oxidized. Cytochrome *c* reduction was performed initially by adding ascorbate crystals to cytochrome *c* and placing the mixture in a dialysis membrane for 18–24 h against 0.01 M phosphate buffer, pH 7.0, at 4°C. Reduced cytochrome *c* concentration was then determined with 0.1 M ferricyanide. The reaction mixture contained 0.01 M potassium phosphate; pH 7.0, and 50 μM reduced cytochrome *c*. The reaction was initiated by adding the mitochondrial sample at 30°C. The pseudo first-order rate constant K was calculated, because the reaction is of first-order with respect to cytochrome *c*. Results are expressed as a percentage of the control condition.

Analysis of adenine nucleotides

After the incubation period, the medium was removed. Cells were extracted, on 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 were analyzed for total protein content by the Sedmak method (25), by using bovine serum albumin as standard. The supernatants were

neutralized with 10 M KOH in 5 M Tris and centrifuged at 15,800 g for 5 min. The resulting supernatants were assayed for adenine nucleotides (ATP, ADP, and AMP) by separation in a reverse-phase HPLC, as described by Stocchi et al. (26). The chromatographic apparatus we used was a Beckman System Gold (Fullerton, CA), consisting of a 126 Binary Pump Model and a 166 Variable UV detector controlled by computer. The column was a Lichrospher 100 RP-18 (5 μm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mM KH_2PO_4 buffer at pH 7.4 and 1% methanol was performed at a flow rate of 1.2 ml/min. The adenine nucleotides were detected at 254 nm for 6 min.

Data analysis

Data are expressed as means ± SE of the indicated number of determinations and are derived from at least three independent experiments. Statistical analyses were performed by two-tailed Student's *t*-test, with a *P* value < 0.05 considered significant.

RESULTS

Toxic effects of Aβ 25–35 or Aβ 1–42 on NT2 ρ+ and ρ0 cells

Amyloid β-peptide toxicity was evaluated by MTT reduction assay and by LDH leakage test. When NT2 ρ+ cells were incubated with the active peptide Aβ 25–35 or Aβ 1–42, a dose-dependent decrease in cellular viability was observed by MTT reduction (Fig. 1A, B). Incubation of NT2 ρ+ cells with increasing concentrations of Aβ reverse sequence (35–25) did not affect cell viability as determined by MTT reduction, although Aβ 42–1 peptide affected cell viability for concentrations above 1 μM.

Exposure of NT2 ρ+ cells to Aβ 25–35 peptide (10 μM) for 24 h resulted in a 1.65-fold reduction of cell viability (from 101.9 ± 1.5% in controls to 62.1 ± 2.0% in the presence of the peptide). Viability of NT2 ρ0 cells was not affected by 10 μM Aβ 25–35 (50.9 ± 2.1% in control cells, 46.6 ± 3.1% in the presence of the peptide) (Fig. 2). In fact, Aβ 25–35 concentrations as high as 100 μM did not influence MTT reduction in ρ0 cells (data not shown). Incubation of ρ+ cells with Aβ 1–42 peptide (1 μM) induced a 1.71-fold reduction of cell viability (from 101.9 ± 1.5% in controls to 59.5 ± 6.3% in the presence of the peptide). Aβ 1–42 (1 μM) also did not affected ρ0 cells viability (50.9 ± 2.1% in control cells, 44.7 ± 2.5% in the presence of the peptide) (Fig. 2). Exposure of ρ+ or ρ0 cells to H_2O_2 (100 μM) for 15 min resulted in a reduction of both cell lines' viability (43.8 ± 7.3% in ρ+ cells, 28.8 ± 5.3% in ρ0 cells). Figure 2 also shows that baseline MTT reduction in ρ0 cells is lower than that of ρ+ cells (50.9 ± 2.0% vs. 101.9 ± 1.5%).

To confirm these data, amyloid β-peptide toxicity was also determined by LDH leakage. As shown in Fig. 3, Aβ 25–35 and Aβ 1–42 increased LDH leakage only in ρ+ cells (9.5 ± 1.0% and 11.3 ± 1.0%, respectively) compared with untreated cells (4.8 ± 0.3%). Nevertheless, amyloid β-peptides (25–35 and 1–42) did not reduce cell viability in ρ0 cells. STS promoted an increase in LDH leakage in both cell lines (11.1 ± 1.5% in ρ+ cells and 10.9 ± 0.7% in ρ0 cells).

The amyloid β reverse sequences (35–25 and 42–1) did not affect cell viability as determined by LDH leakage.

We tested the protective effect of vitamin E, idebenone, GSH, melatonin, and nicotine on Aβ 25–35 (10 μM) toxicity (Fig. 4). Idebenone completely protected NT2 ρ+ cells from cell damage induced by Aβ 25–35 (from 62.1 ± 2.0% MTT reduction in the absence of idebenone to 94.2 ± 5.5% in the presence of the compound), whereas partial protective effects for vitamin E, GSH, melatonin, and nicotine were observed (79.2 ± 1.1%, 77.5 ± 2.8%, 71.9 ± 4.5%, and 77.9 ± 2.3%, respectively).

Effect of Aβ 25–35 on mitochondrial membrane potential

As shown in Figure 3, treatment of NT2 ρ+ cells with 10 μM Aβ 25–35 led to a significant twofold decrease in rhodamine 123 retention (from 98.8 ± 4.8% in control cells to 50.6 ± 4.8% in the presence of the peptide). This retention reflects the status of the mitochondrial membrane potential and declines as the mitochondrial membrane potential is dissipated. Nicotine, melatonin, and vitamin E could ameliorate this Aβ-induced decline of mitochondrial membrane potential in ρ+ cells. In contrast, the mitochondrial membrane potential of ρ0 cells—although reduced relative to that of ρ+ cells at baseline—was unaffected by exposure to Aβ 25–35 (89 ± 5.1% and 87.2 ± 15.7% of ρ+ cells, in the absence and presence of the peptide, respectively).

Effect of Aβ 25–35 on mitochondrial electron transport chain function in NT2 ρ+ and NT2 ρ0 cells

After incubation of NT2 ρ+ cells with Aβ 25–35, MRC enzyme activities were evaluated, NADH-ubiquinone oxidoreductase (complex I) activity was halved by exposure to the peptide (48.8 ± 8.2% the activity of untreated cells) (Fig. 6A). The activity of complex II/III decreased significantly (79.1 ± 5.15% of the control cell activity) (Fig. 6B). Cytochrome c oxidase activity was also inhibited after Aβ 25–35 peptide treatment (60.9 ± 10.7% compared with control cells) (Fig. 6C). In ρ0 cells, the activity of the mitochondrial respiratory chain complexes (I, II/III, and IV) was virtually undetectable at baseline.

As the data in Figure 6A demonstrate, melatonin completely prevented Aβ-induced inhibition of complex I activity, whereas vitamin E, idebenone, GSH, and nicotine partially prevented inhibition of this enzyme complex. Inhibition of complex II/III activity in ρ+ cells was minimized with melatonin, GSH, and vitamin E. Idebenone and nicotine did not show any protective effect (Fig. 6B). Preincubation with idebenone and vitamin E partially prevented complex IV activity inhibition by Aβ 25–35, whereas melatonin, nicotine, and GSH were ineffective (Fig. 6C).

Effect of Aβ 25–35 peptide on adenine nucleotide levels

ATP levels in NT2 ρ+ cells exposed to Aβ 25–35 (10μM) for 24 h were approximately half that of unexposed cells (48.3 ± 2.7% the control level) (Fig. 7). Neuroprotective agents helped protect against Aβ-induced reduction of ATP levels. Vitamin E, melatonin, and nicotine were more

effective than idebenone and GSH in this regard. In ρ0 cells, ATP levels were similar to those measured in NT2 ρ+ cells (100.3 ± 8.8% in ρ0 cells vs. 100 ± 6.4% in ρ+ cells). In ρ0 cells, Aβ 25–35 did not induce a statistically significant decline in ATP levels in these cells (86.1 ± 3.6%).

DISCUSSION

The objective of this work was to explore the role mitochondria play in Aβ-induced cellular toxicity and to analyze how mitochondrial respiratory chain dysfunction is induced by this protein. For this purpose, we investigated the effects of the neurotoxic Aβ peptides on NT2 ρ+ and ρ0 cell lines. Our results show that Aβ 25–35 or Aβ 1–42 peptide is toxic to ρ+ cells with functional mitochondria, but it does not adversely affect ρ0 cells that do not contain a functional electron transport chain.

Treatment of NT2 ρ+ cells for 24 h with increasing concentrations of the amyloid β-peptides resulted in a dose–dependent decrease in cell survival as determined by the MTT reduction assay (Fig. 1A, B). This finding is consistent with previous data from our laboratory and others (7, 14, 26, 27). The finding that ρ+ cells exposed to equal amounts of the reverse Aβ peptide (Aβ 35–25 or Aβ 42–1) maintain total viability (Fig. 1A, B) suggests that Aβ 25–35 or Aβ 1–42 toxicity relates to its ability to form insoluble fibrils *in vitro* (5).

Although it is widely assumed that MTT reduction primarily occurs in mitochondria, cellular MTT reduction also proceeds in other cellular compartments as well (28). In addition to showing that functional mitochondria are required for Aβ 25–35 or Aβ 1–42 toxicity (Fig. 2), the fact that some MTT reduction did occur in ρ0 cells corroborates the conclusion of Liu et al (28) that extra-mitochondrial dehydrogenases can reduce limited amounts of MTT. Nevertheless, when ρ0 cells were exposed to Aβ 25–35 or Aβ 1–42 peptides no further decrease in MTT reduction was observed. Aβ also did not appear toxic to ρ0 cells when evaluated by LDH leakage assay (Fig. 3). These results strongly suggest that Aβ-mediated cell toxicity requires functional mitochondria and is mediated through disruption of mitochondrial function. To exclude the possibility for an inappropriate selection for ρ0 cells, we tested for cell viability following the exposure to an oxidative insult, H_2O_2 (Fig. 2) and to the apoptotic inductor, staurosporine (Fig. 3).

NT2 ρ+ cells treated with Aβ 25–35 peptide demonstrated decreased mitochondrial membrane potentials (Fig. 5) and respiratory chain enzyme activities (Fig. 6). This finding was associated with an overall impairment of cellular energy metabolism, which was manifested by diminished ATP levels. This observation extends our previous one that, in cell culture, Aβ 25–35 reduces activities of complex I, II/III, and IV (but not complex II and V) (14, 26).

ρ0 cells lack mtDNA and cannot produce a functional electron transport chain, as 13 protein subunits of the mitochondrial respiratory apparatus are mtDNA encoded (7 subunits of NADH-Q oxidoreductase, 1 subunit of cytochrome c reductase, 3 subunits of cytochrome *c* oxidase, and 2 subunits of ATP synthase) (29, 30). Mitochondrial DNA also encodes 2 rRNAs and 22 tRNAs that are necessary for the expression of the 13 structural mtDNA genes (18). Therefore, ρ0 cells rely on anaerobic glycolysis for ATP production. Lack of mitochondrial electron transport leads to a high $NADH/NAD^+$ ratio, and so pyruvate is required to regenerate reducing equivalents

(18). Our results show that in NT2 ρ0 cells ATP levels are maintained (Fig. 7) despite a virtual absence of mitochondrial respiratory chain enzyme activities (Fig. 6). The maintenance of a mitochondrial membrane potential in our ρ0 cells (Fig. 5) likely arises from transport of protons out of the mitochondrial matrix, a phenomenon previously shown to occur in 143B-87 ρ0 cells in which a "reversal" of ATP synthase activity took place (31). In ρ0 cells, nuclear-encoded ATP synthase subunits assemble and can maintain a proton gradient across the mitochondrial membrane (32). The finding that Aβ peptide did not reduce ATP levels and mitochondrial membrane potential in ρ0 cells, further supports the hypothesis that Aβ peptide toxicity is mediated through disruption of normal mitochondrial function.

In this study, the neuroprotective effects of various antioxidants (vitamin E, idebenone, GSH, and melatonin) and of the cholinergic agonist nicotine were tested to explore the importance of oxidative stress in Aβ-mediated toxicity. Preincubation of NT2 ρ+ cells with idebenone prevented alteration of MTT reduction and, by extension, eliminated Aβ 25–35 toxicity. By the same criterion vitamin E, GSH, and melatonin partially ameliorated Aβ toxicity (Fig. 4). Melatonin was more effective than the other compounds in preserving mitochondrial membrane potential in Aβ-treated ρ+ cells. Melatonin also prevented Aβ-induced inhibition of complex I and complex II/III, but not complex IV inhibition (Fig. 6). Idebenone and vitamin E, however, could preserve complex IV activity (Fig. 6C). Nicotine preserved complex II/III activity (Fig. 6B). These data indicate that mitochondrial-mediated Aβ 25–35 toxicity is reduced by agents that protect proton pumps (melatonin and idebenone) or reduce oxidative stress (Fig. 7).

Idebenone is a lipophilic antioxidant that can also act to reduce oxidative stress by serving as an electron carrier (33). Vitamin E, one of the major lipophilic antioxidants in biological systems (34), incorporates into membranes and interferes with the propagation of lipid peroxidation chain reactions (35). GSH, the substrate of glutathione peroxidase, inactivates peroxides. In addition, it prevents s-thiolation of proteins (36). Melatonin, a pineal neurohormone, is an antioxidant that is perhaps a better scavenger of hydroxyl and peroxide radicals than vitamin E and glutathione. It is quite lipophilic and therefore incorporates into cell membranes where it prevents both initiating and chain reaction peroxidation events (37, 38). Nicotine and other nicotinic receptor agonists appear to exert anti-apoptotic effects in several neuronal cell lines, including differentiated PC12 cells and primary cortical neurons (39, 40). This property is dependent on nicotinic receptor-mediated Ca^{++} influx and subsequent modulation of protein kinase C-dependent intracellular signalling pathways (41, 42). In clinical studies of AD, nicotinic receptor stimulation was associated with improvements in the acquisition and retention of both verbal and nonverbal information (43, 44, 45). Overall, our results support the contention that generation of oxidative stress and probably alteration of Ca^{++}-dependent signaling pathways are important in Aβ cell toxicity (46, 47).

In conclusion, these experiments demonstrate that Aβ peptide toxicity requires the presence of functional mitochondria, which are both a principal site of reactive oxygen species generation, as well as a major target for their deleterious effects. Reduction of mitochondrial respiratory chain enzyme activities, possibly due to protein and/or lipid oxidation, also occurs. It is conceivable that oxidative damage of mtDNA might also arise (18), which could perpetuate electron transport chain dysfunction and contribute to additional oxidative stress. Whether Aβ production

represents primary or secondary pathology in AD, its lack of toxicity in a ρ0 cell system emphasizes the importance of mitochondria in this disease and illustrates the importance of defining their role in neurodegenerative pathways.

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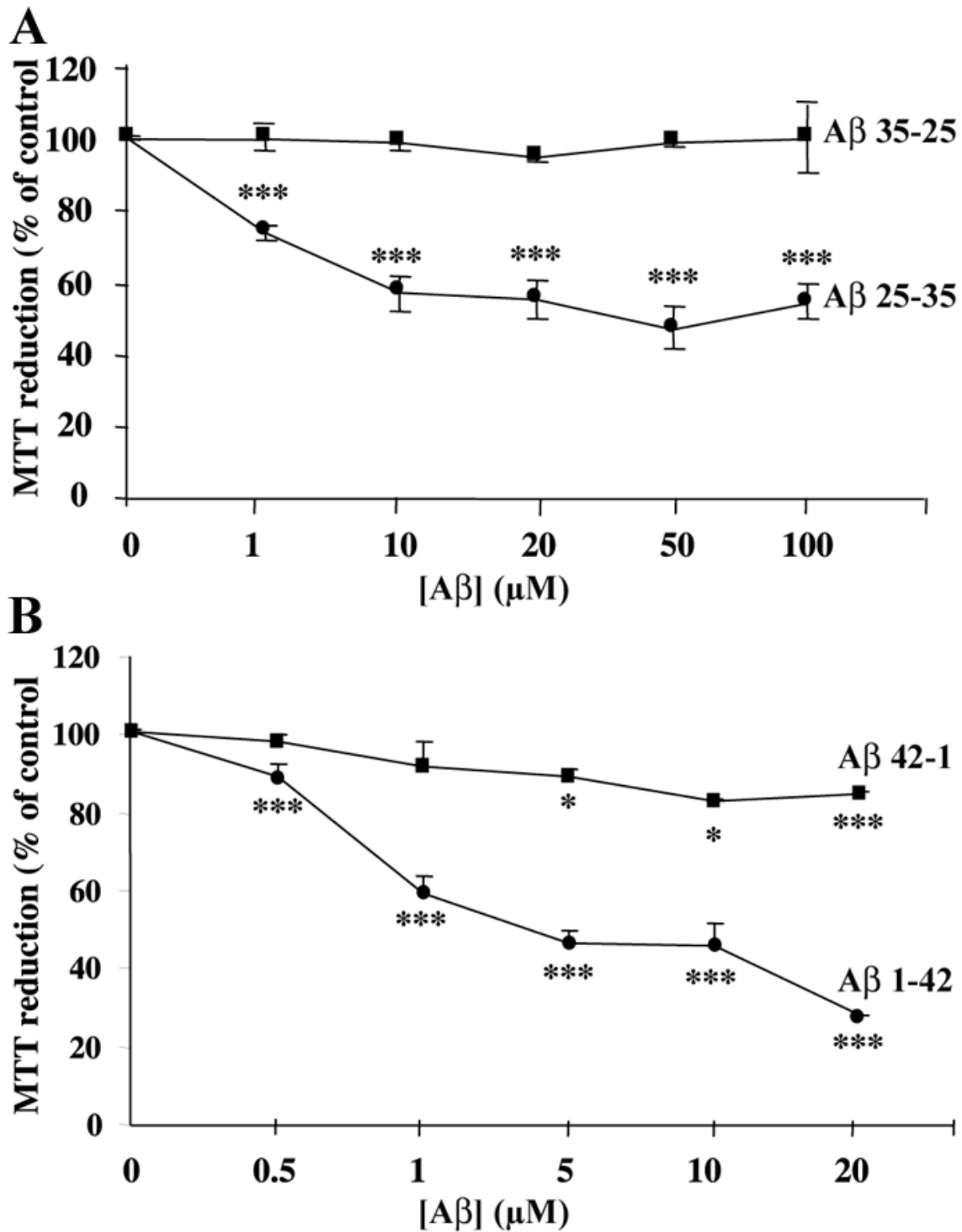
Fig. 1

Figure 1. Dose-response curves of the toxic effect of amyloid β-peptides. NT2 ρ+ cells were incubated in the presence of increasing concentrations of Aβ 25-35 and Aβ 35-25 peptides **(A)** or of Aβ 1-42 and Aβ 42-1 peptides **(B)**. Aβ toxicity was evaluated by the reduction of the tetrazolium salt MTT, as described in Materials and Methods. Data are expressed as the percentage of control values, with the mean ± SE derived from 5 to 13 independent determinations. * $P < 0.05$, *** $P < 0.001$, significantly different compared with untreated cells.

Fig. 2
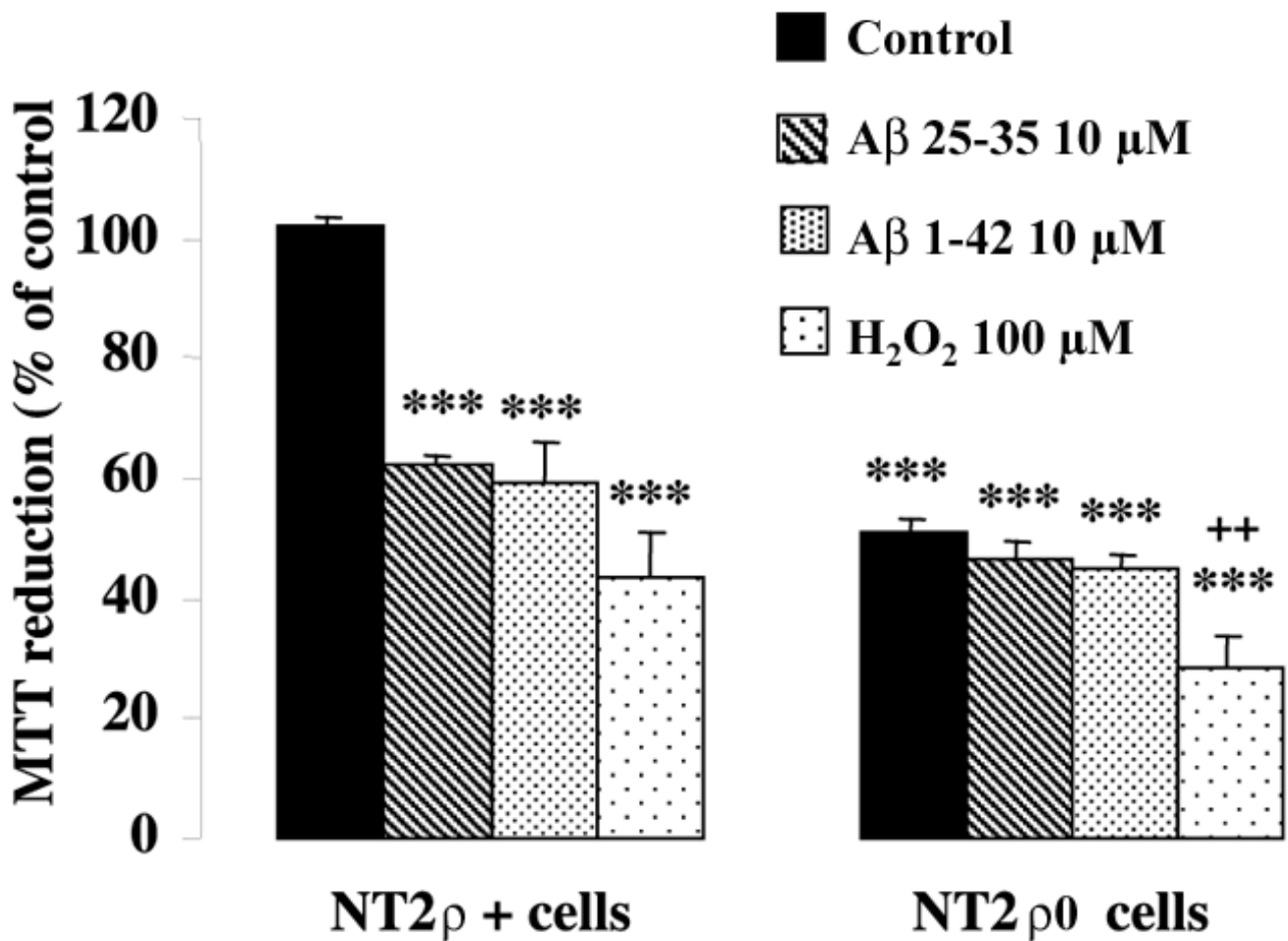
Figure 2. **Effect of Aβ peptide or H₂O₂ on cells lacking functional mitochondria.** NT2 ρ+ and ρ0 cells were incubated in the presence of 10 μM Aβ 25-35 peptide, 1 μM Aβ 1-42, and 100 μM H₂O₂. Cell toxicity was evaluated by the reduction of the tetrazolium salt MTT, as described in Materials and Methods. Data are expressed as a percentage of control values, with the mean ± SE derived from 5 to 13 independent determinations. *** $P < 0.001$, significantly different compared with untreated ρ+ cells; ++ $P < 0.01$, significantly different compared with untreated ρ0 cells.

Fig. 3
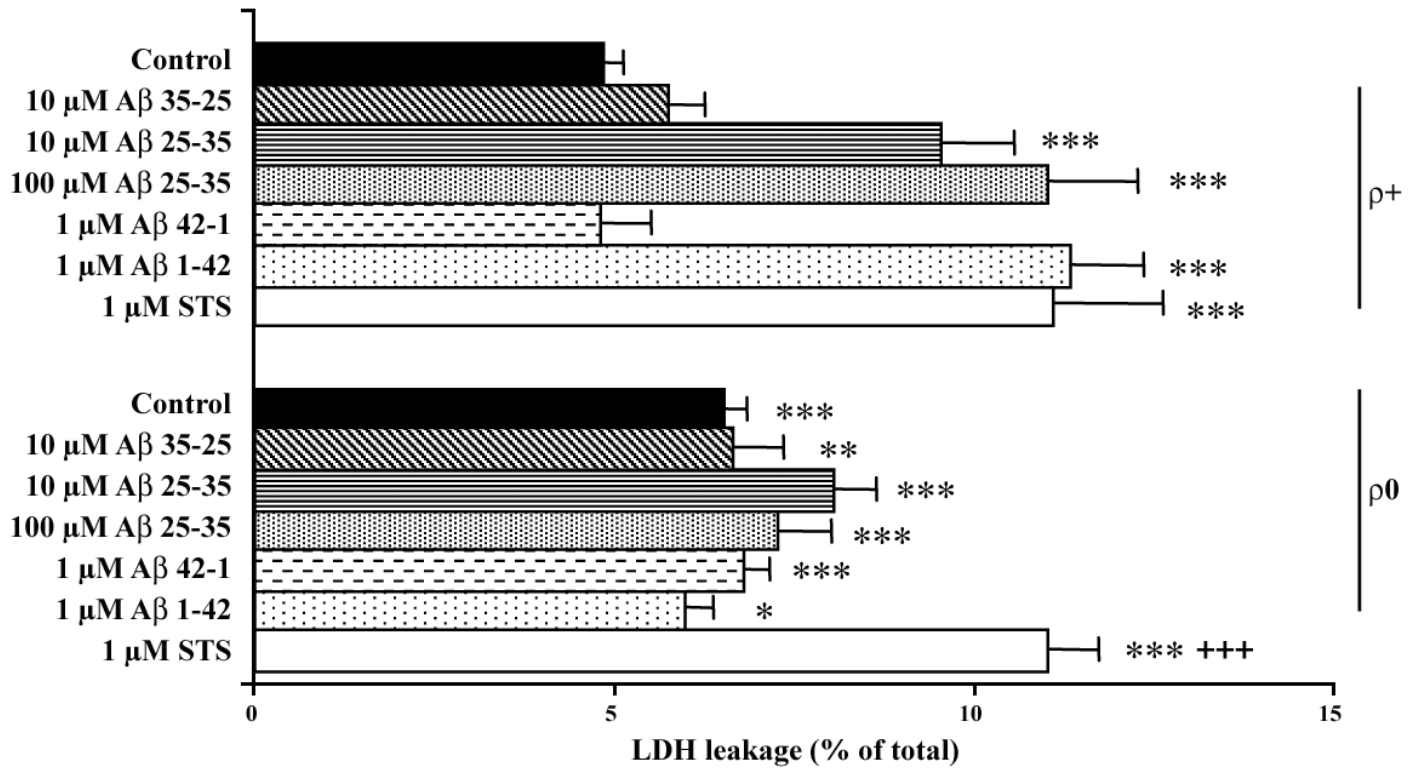
Figure 3. Effect of Aβ peptides or staurosporine on cell viability. NT2 ρ+ and ρ0 cells were incubated in the presence of 10 μM and 100 μM of Aβ 25-35 peptide, 1 μM of Aβ 1-42, and 1 μM of staurosporine (STS). Cell toxicity was evaluated by the LDH leakage test, as described in Materials and Methods. Data are expressed as a percentage of total, with the mean ± SE derived from 5 to 8 independent determinations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different compared with untreated ρ+ cells, +++ $P < 0.001$, significantly different compared with ρ0 cells treated with Aβ 25-35 (10 μM).
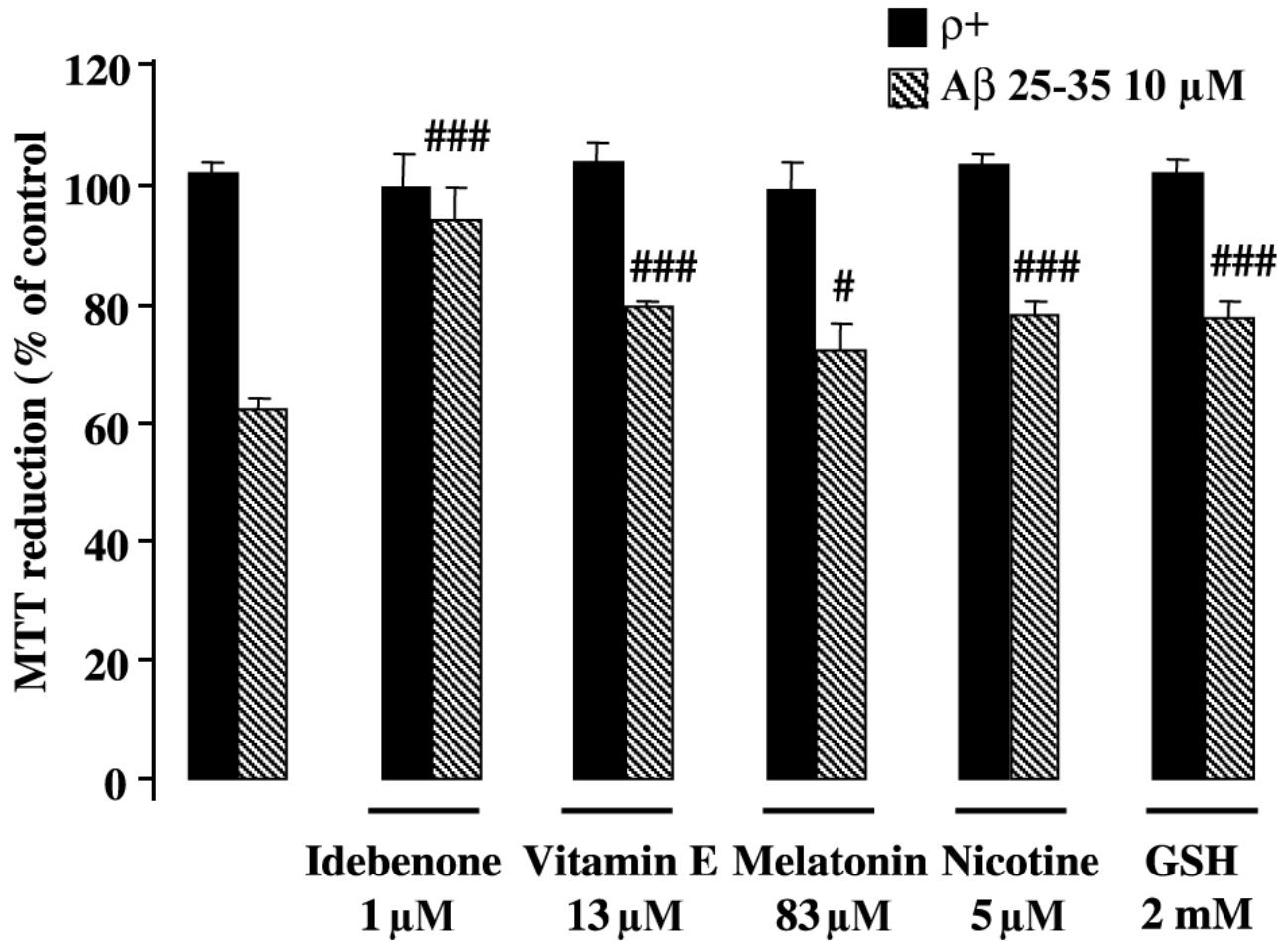
Fig. 4

Figure 4. Effect of antioxidants and nicotine on amyloid β-peptide toxicity. NT2 ρ+ cells were incubated with Aβ 25-35 in the presence of idebenone (1 µM), vitamin E (13 µM), melatonin (83 µM), nicotine (5 µM), and GSH (2 mM). MTT reduction was measured as described in Materials and Methods. Data are expressed as a percentage of control values, with the mean ± SE derived from four to five independent determinations. *** $P < 0.001$, significantly different compared with untreated ρ+ cells. # $P < 0.05$; ### $P < 0.001$, significantly different compared with NT2 ρ+ cells treated with Aβ 25-35.
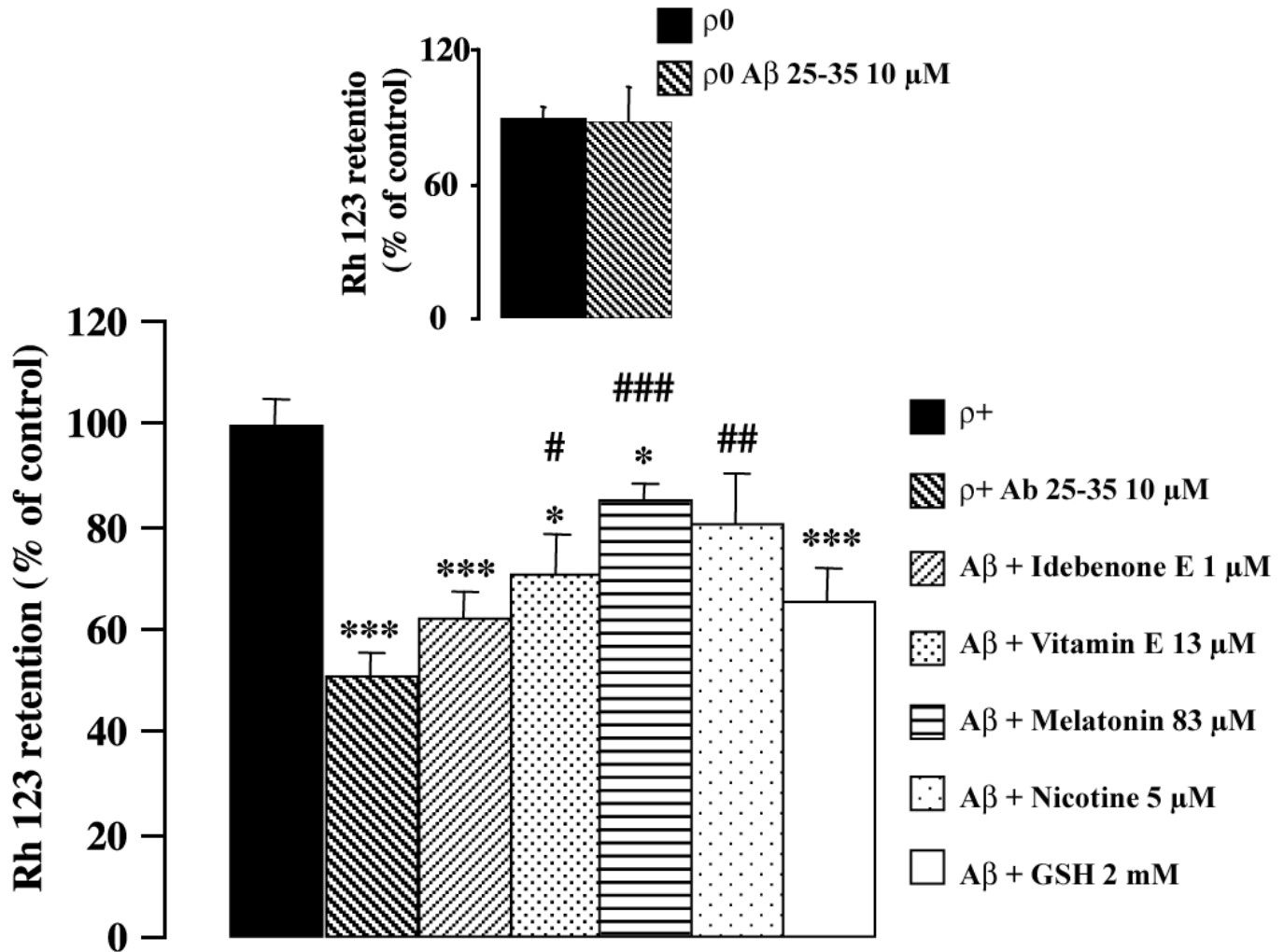
Fig. 5

Figure 5. Mitochondrial membrane potential in cells exposed to Aβ peptide. Δψmt was determined in NT2 ρ+ and ρ0 cell lines. NT2 ρ+ cells were incubated with Aβ 25-35 in the absence or presence of idebenone (1 μM), vitamin E (13 μM), melatonin (83 μM), nicotine (5 μM), and GSH (2 mM). Δψmt was measured as described in Materials and Methods. Data are expressed as the percentage of control values, with the mean ± SE derived from four to five independent determinations. * $P < 0.05$, significantly different compared with NT2 ρ+ control cells.
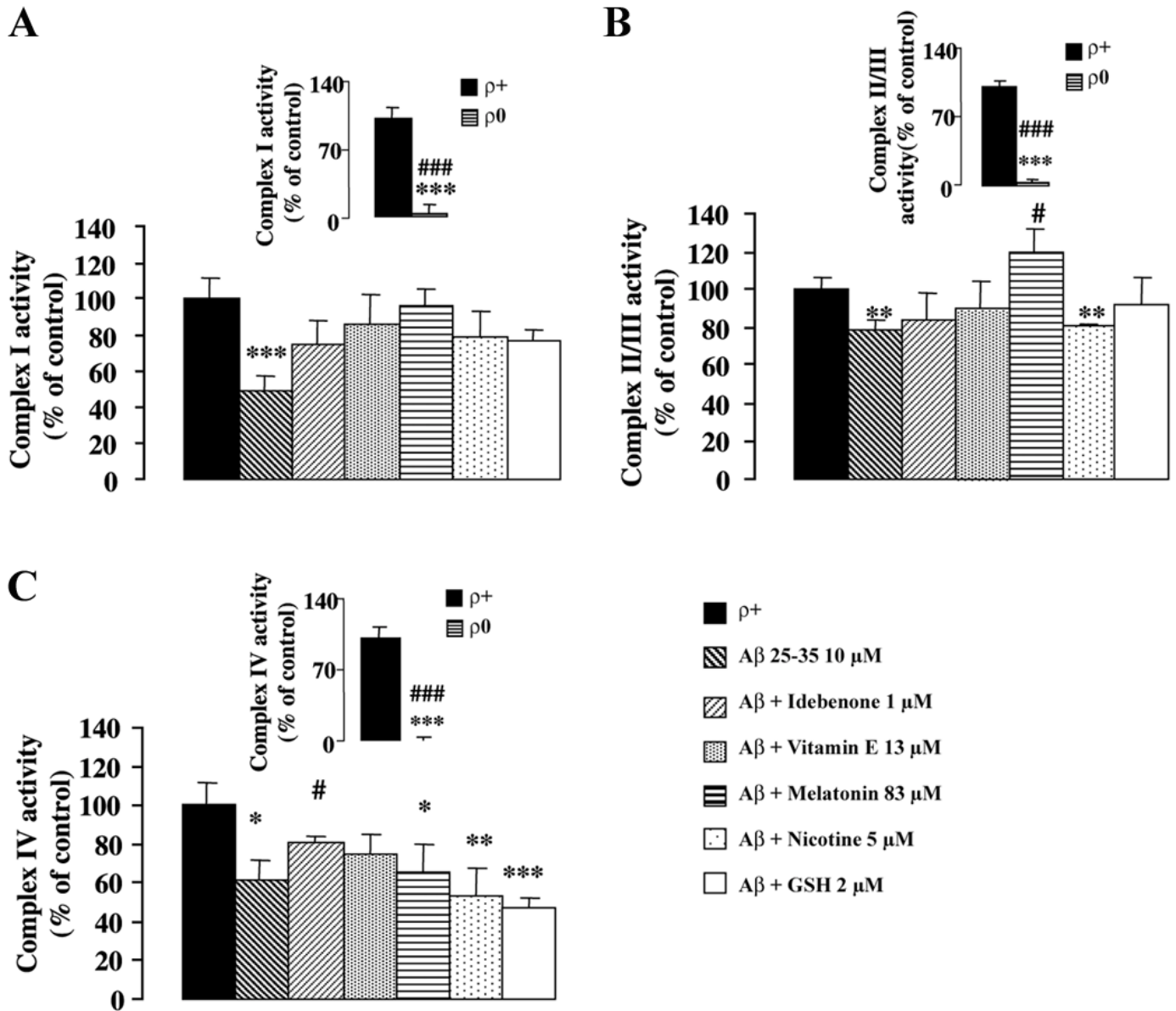
Fig. 6

Figure 6. Effect of amyloid β-peptide, antioxidants, and nicotine on the activity of mitochondrial respiratory chain complexes. Complex I **(A)**, complex II/II **(B)**, and COX **(C)** activities were determined in NT2 ρ+ and ρ0 cell lines. NT2 ρ+ cells were incubated with Aβ 25-35 in the presence of idebenone (1 μM), vitamin E (13 μM), melatonin (83 μM), nicotine (5 μM), and GSH (2 mM), and MRC activities were measured as described in Materials and Methods. Data are expressed as the percentage of control values, with the mean ± SE derived from four to six independent determinations. * $P < 0.05$; *** $P < 0.001$, significantly different compared with NT2 ρ+ control cells. # $P < 0.05$; ### $P < 0.001$, significantly different compared with NT2 ρ+ cells treated with Aβ 25-35.
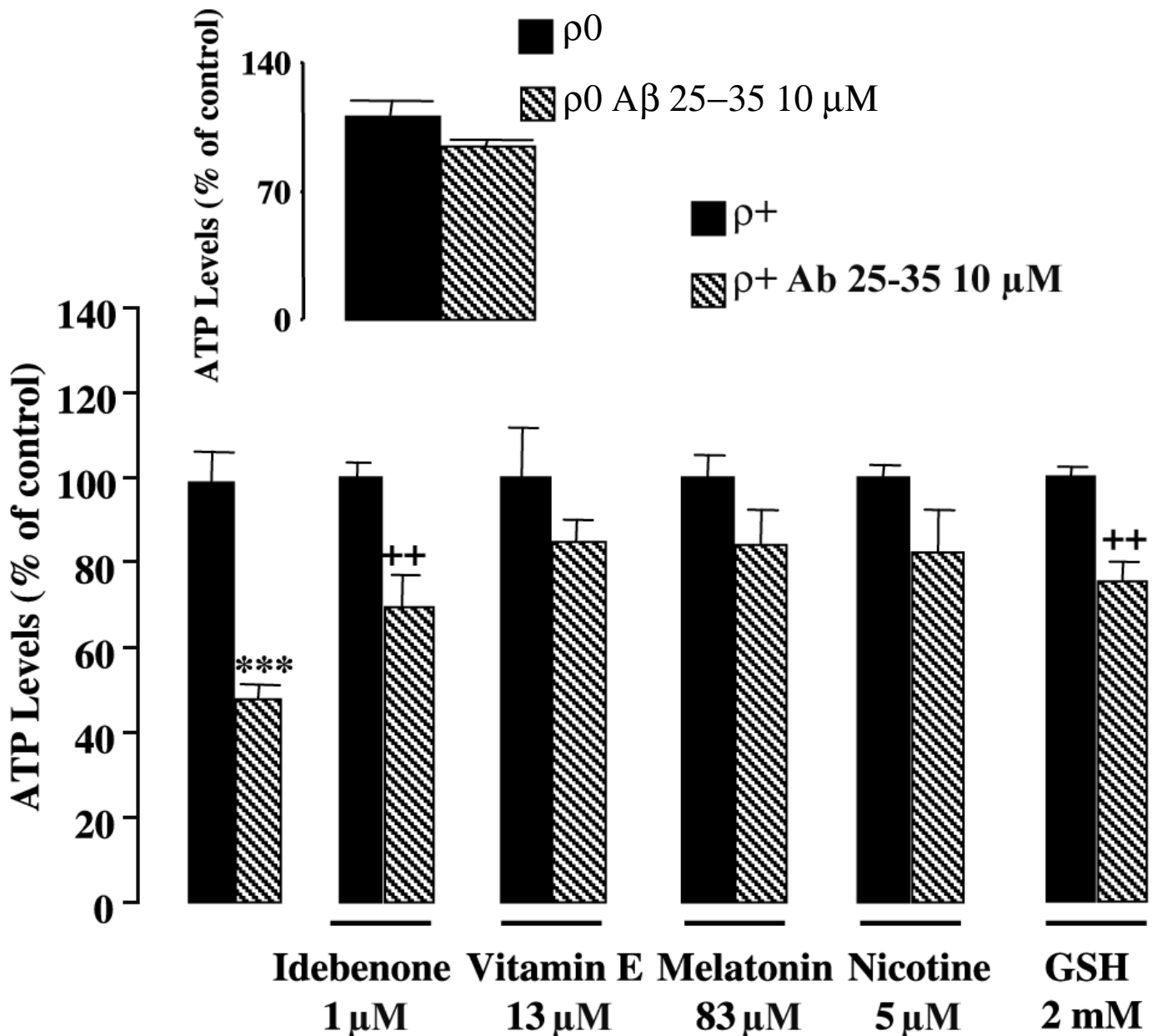
Fig. 7

Figure 7. Effect of amyloid β-peptide, antioxidants, and nicotine on adenine nucleotide levels. ATP levels were determined in NT2 ρ+ and ρ0 cell lines. NT2 ρ+ cells were incubated with Aβ 25-35 in the absence or presence of idebenone (1 μM), vitamin E (13 μM), melatonin (83 μM), nicotine (5 μM), and GSH (2 mM). ATP levels were measured as described in Materials and Methods. Data are expressed as the percentage of control values, with the mean SE derived from three to ten independent determinations. *** $P < 0.001$, significantly different compared with NT2 ρ+ control cells incubated in the absence of Aβ 25-35. ### $P < 0.001$, significantly different compared with NT2 ρ+ cells treated with Aβ 25-35. ++ $P < 0.01$, significantly different compared with NT2 ρ+ control cells treated with these drugs in the absence of Aβ 25-35.