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Oxidative Stress Modulates Tyrosine Kinase Receptor A and p75 Receptor (Low-Affinity Nerve Growth Factor Receptor) Expression in SHSY5Y Neuroblastoma Cells

■ The interaction of neurotrophins and their tyrosine kinase receptors (trks) is essential for differentiation and survival of brain cells. In Alzheimer's disease (AD), the number of neurotrophins and receptors is markedly decreased. The cause of this reduction is unclear, but the role of β-amyloid (Aβ) seems central in understanding the mechanisms controlling neurotrophin and trk expression. In the study reported here, we exposed SHSY5Y neuroblastoma cells to Aβ or hydrogen peroxide (H_2O_2) and measured the expression of trk-A and p75 at the protein and molecular levels. Both Aβ and H_2O_2 induced oxidative stress (measured by a decrease in cellular glutathione), which decreased trk-A levels and increased p75 levels, decreased messenger RNA (mRNA) levels of both receptors, and increased nerve growth factor (NGF) secretion. Pretreatment of cells with the antioxidant melatonin returned levels of protein expression, mRNA, and NGF secretion to normal. These results are significant, as they can help in the planning and implementation of AD treatment strategies involving neurotrophins. ■

Keywords: tyrosine kinase receptor A (trk-A), p75, β-amyloid (Aβ), melatonin, Alzheimer's disease (AD), oxidative stress, SHSY5Y neuroblastoma cells

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Several authors have suggested that Alzheimer's disease (AD) pathology involves deficits in neurotrophin expression and/or tyrosine kinase receptor (trk) expression (Appel, 1981; Lucidi-Phillipi and Gage, 1993; Rylett and Williams, 1994; Connor et al, 1996; Savaskan et al, 2000). Recently, Mufson et al (1995) proposed that neurodegeneration associated with AD may be due to impaired retrograde transport of nerve growth factor (NGF), which is caused by deficits in the production and/or use of the neurotrophin receptor trk-A. Hock et al (1998) found that trk-A expression in the cholinergic regions of brains with AD is significantly reduced compared with that of age-matched control brains. Interestingly, p75 is up-regulated in cholinergic neurons of the nucleus basalis of Meynert in patients with AD (Ernfors et al, 1990; Mufson and Kordower, 1992). Coupled to the loss of trophic support in neurodegeneration is the involvement of neurotrophin–trk interactions in oxidative stress. NGF treatment reduces reactive oxygen species (ROS) synthesis in neuronal cell cultures by activating the mitogen-activated protein kinase pathway (Dugan et al, 1997) and increases the activity of oxygen free-radical scavengers (eg, glutathione peroxidase, catalase) in rats (Goss et al, 1997). NGF pretreatment further protects PC12 cells from oxidative stress induced by hydrogen peroxide (H_2O_2) by increasing levels of cellular glutathione (GSH) and by increasing the activity of γ-glutamylcysteine synthetase, a rate-limiting enzyme for GSH synthesis (Pan and Perez-Polo, 1993; Satoh et al, 1999).

β-Amyloid (Aβ), characteristically found in the plaques of brains with AD, is derived from the cleavage of amyloid precursor protein (Selkoe 1994). Aβ destabilizes neurons and leads to cell death through induction of oxidative stress (Pacifici and Davies, 1991; del Rio et al, 1992; Benzi and Moretti, 1995; Martin et al, 1996). Oxidative stress, in turn, seems to mediate Aβ toxicity by producing free radicals and elevating cellular H_2O_2 (Behl et al, 1994; Butterfield et al, 1994; Hensley et al, 1994; Behl, 1997). Aβ interacts with trks. Yaar et al (1997) found that Aβ specifically binds to p75 receptors in rat cortical neurons and in NIH3T3 cells engineered to stably express p75 receptors; similarly, Kuner et al (1998) found that human neuroblastoma cell lines exposed to Aβ enter apoptotic cell death through a mechanism involving both direct binding of Aβ to p75 receptor and activation of nuclear factor–κB (NF-κB). In both studies, NGF administration prevented the induction of apoptosis, presumably by NGF–Aβ competition for the trk (Rabizadeh et al, 1994; Yaar et al, 1997; Kuner et al, 1998). Molecules that function as antioxidants (Reiter, 1995) or that promote cellular antioxidant enzyme activity (Liu and Ng, 2000)—melatonin, for example—are neuroprotective

against Aβ toxicity (Pappolla et al, 2000). Interestingly, melatonin levels are reduced in the brain of patients with AD (Reiter, 1995).

AD progression is accompanied by increases in Aβ synthesis and release, accumulation of Aβ into plaques, induction of oxidative stress, and decreased neurotrophic support caused by a decrease in the number of neurotrophins and receptors (Kaplan and Miller, 2000; Pappolla et al, 2000). This course of events raises the question of the possible influence or role of oxidative stress in the modulation of trk-A and p75 expression. The significance of this question lies in the central role of neurotrophins and their receptors in the maintenance and survival of neuronal cells in the brain. In the study reported here, we investigated the effects of oxidative stress, induced by Aβ or H_2O_2, on the expression of trk-A and p75. We also investigated the neuroprotective influences of the pineal hormone melatonin.

Materials and Methods

Cell Culture

SHSY5Y neuroblastoma cells were grown in complete minimum essential medium (MEM) in a humidified air/5% carbon dioxide chamber at 37°C. Sixteen hours before treatment, cells were washed free of medium containing fetal calf serum (FCS) and further incubated in FCS-free MEM containing neuroblastoma growth supplement N2. All cell culture reagents were from Gibco (Life Technologies, Paisley, UK), and plastic ware was from Nunc (Roskilde, Denmark).

GSH Assay

Cellular GSH was measured according to manufacturer instructions (ApoAlert GSH Detection Kit; Clontech, Palo Alto, Calif), except that FCS-free medium, containing either 1 μM of Aβ1–42 or 50 μM of H_2O_2 in the presence or absence of 12-hour preincubation with 1 μM of melatonin, was added to the cells for 24 hours. All GSH assays were performed in quadruplicate.

MTT Reduction Assay

SHSY5Y cells were seeded into 96 well culture-plates and were allowed to attach. FCS-free medium, containing either 1 μM of Aβ1–42 or 50 μM of H_2O_2 in the presence or absence of 12-hour preincubation with 1 μM of melatonin, was added to the cells for 24 hours. MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, St. Louis, Mo), was added to all wells and was allowed to incubate in the dark at 37°C for 5 hours; cells were lysed, and 590-nm spectrophotometric measurement was performed on a Labsystems Multiskan RC plate reader using Genesis software (Labsystems, Helsinki, Finland). All MTT assays were performed in triplicate.

NGF ELISA

Cell-culture supernatant levels of NGF were measured with enzyme-linked immunosorbent assay (ELISA) using monoclonal anti-β (2.5S, 7s) NGF antibodies (clone 27/21; Roche Diagnostics, Mannheim, Germany) according to Weskamp and Otten (1987). The detection limit was 0.5 pg/mL; cross-reactivity with other neurotrophins at 10 ng/mL was less than 2%, and the ELISA was linear over a range of 0.5 to 500 pg/mL. All NGF ELISA assays were performed in triplicate.

Western Analysis of Cell Membranes

SHSY5Y neuroblastoma cell membranes were isolated according to Walter et al (1987) with some modifications. Briefly, cells were washed free of culture medium, scraped off culture flasks in homogenizing buffer (10 mM of tris-HCI, pH 7.4, containing 1.5 mM of $CaCl_2$, 1 mM of spermidine, and protease inhibitors), transferred to Eppendorf tubes, and sonicated for 20 seconds. Homogenates were centrifuged at 1000*g* for 5 minutes to pellet, large-particulate matter. The resultant supernatant was carefully placed in a bilayer of 5% sucrose on top, 50% sucrose in homogenization buffer. After centrifugation in a Sorvall RC 28S (Newtown, Conn) at 40,000*g* for 30 minutes, the turbid layer containing the membranes, at the boundary between the 5%- and 50%-sucrose layers, was retained. All procedures were performed at 4°C. Protein concentration was normalized with Bradford's protein assay and 12 μg of total protein loaded onto 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels (Laemmli, 1970; Baek et al, 1996). After separation, the proteins were transferred to nitrocellulose membranes, blocked overnight, and probed with N-terminal–recognizing anti–trk-A receptor antibodies (Upstate Biotech, Lake Placid, NY) and anti-p75 (NGF receptor [clone NGFR5]; NeoMarkers, Fremont, Calif). Secondary peroxidase POD labeled antibodies (antirabbit POD, antimouse POD; Roche Diagnostics, Mannheim, Germany) directed against the primary antibodies and then exposed to SuperSignal chemiluminescent substrate (Pierce, Rockford, Ill) allowed visualization of the bands. Images of the bands were digitally captured using the Eagle Eye II still video system (Stratagene, La Jolla, Calif) and were analyzed using ImageMaster software (Pharmacia Biotech, Uppsala, Sweden). The results represent the mean ± SEM of 3 independent experiments and are presented as percent of the zero value.

RNA Extraction and RT-PCR

Total RNA was extracted from equivalent numbers of cells and was treated as was done in the MTT and GSH assays but using the RNeasy protocol (Qiagen, Valencia, Calif). One microgram of total RNA ($\sim 3 \times 10^5$ cells) was used for reverse transcription (RT) with oligodeoxythymidine primers and Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc, Piscataway, NJ). Primers specific for trk-A (sense, 5′-CCATCGTGAAGAGTGGTCTC-3′; antisense, 5′-GGTGACATTGGCCAGGGTCA-3′) and p75 (sense, 5′-AGCCAACCAGACCGTGTGTG-3′; antisense, 5′-TTGCAGCTGTTCCACCTCTT-3′) were used for polymerase chain reaction (PCR) amplification. The internal control gene glyceraldehyde-6-phosphate dehydrogenase (sense, 5′-G-GTGAAGGTCGGAGTCAACGG-3′; antisense, 5′-GGTCATGAGTCCTTCCACGAT-3′) was used to normalize for the amount of RNA in the initial reverse transcriptase reaction. Controls using RNA without RT or controls without RNA were used to demonstrate absence of contaminating DNA. Conditions used (initial denaturation, 95°C, 4 minutes; denaturation, 95°C, 30 seconds; annealing, 55°C, 30 seconds; extension, 72°C, 60 seconds; final extension, 72°C, 7 minutes; 40 cycles) were chosen to allow the PCR to proceed in a linear range according to the FastStart protocol (Roche Biochemicals, Mannheim, Germany).

PCR products were separated on 1.4% agarose gels containing ethidium bromide, were recorded using the Eagle Eye II still video system (Stratagene, La Jolla, Calif), and were quantitated using ImageMaster software (Pharmacia Biotech, Uppsala, Sweden). The amount of trk-A messenger RNA (mRNA) or p75 mRNA over the assay period is expressed as a percent of the mRNA from the zero time point.

NGF Blocking Assay

To exclude and neutralize the influence of endogenously secreted NGF within the cell culture, 1 μg/mL of anti-NGF β-antibodies (clone 25623.1; Sigma, St. Louis, Mo) was added. The specificity of the antibody for this purpose was demonstrated by Kitamura et al (1989). trk-A and p75 were then assayed for as was done using the Western analysis protocol.

Statistical Analysis

Statistical analysis was performed using the Kruskal-Wallis test and the Dunn multiple-comparisons test. Statistical significance was assumed at $P < .05$.

Results

Over 24 hours, 1 μM of Aβ and 50 μM of H_2O_2 both induced significant cell cytotoxicity and oxidative stress, as measured by decreased MTT metabolism and decreased cellular GSH levels, respectively (Table 1). Twelve-hour preincubation of cells with 1 μM of melatonin prevented the deleterious effects of both Aβ and H_2O_2 (Table 1).

Western analysis of cell membranes following exposure to Aβ showed a significant loss of trk-A (Fig. 1A) and an increase of p75 (Fig. 1C) at the cell surface over time. Pretreatment of cells with melatonin restored p75 (Fig. 1D) to control levels and partially restored trk-A (Fig. 1B) to control levels. H_2O_2 treatment of cells produced similar results to those observed with Aβ namely, significantly reduced trk-A levels (Fig. 1A) and significantly increased p75 levels, over time (Fig. 1C), which was reversed by melatonin pretreatment (Figs. 1B, 1D).

To exclude the effects of endogenous NGF, cells were exposed first to antibodies that specifically bind and inactivate NGF and then to the stress agent Aβ or H_2O_2. Western analysis showed that, compared with the results in untreated control cells, anti-NGF antibodies alone affected trk-A only slightly (Fig. 2A) and did not alter p75 (Fig. 2C) in cell membranes. The presence of blocking antibody and melatonin did not affect receptor levels (Fig. 2). With NGF blocking in effect, both Aβ and H_2O_2 decreased trk-A in the cell membrane (Fig. 2A); this result is similar to that found in experiments without NGF blocking in effect (Fig. 1A). In both Aβ- and H_2O_2-stressed cells, melatonin pretreatment restored trk-A to control levels (Fig. 2B); this change was significant. Compared with control cells, H_2O_2-stressed cells treated with NGF blocking showed a significant increase in p75

Table 1 Cell Cytotoxicity (MTT Assay) and Oxidative Stress (GSH Assay) in SHSY5Y Cells*

		MTT (Absorbance at 590 nm)		*Cellular GSH (% of Control)*
Control		0.39 ± 0.02		100
Melatonin		0.38 ± 0.08		93 ± 5
Aβ1–42	–	0.17 ± 0.04†	–	70 ± 12†
	+	0.40 ± 0.06	+	94 ± 7
H_2O_2	–	0.21 ± 0.06†	–	40 ± 12†
	+	0.37 ± 0.09	+	89 ± 9

*MTT indicates 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, St. Louis, Mo); GSH, glutathione; Aβ1–42, β-amyloid; and H_2O_2, hydrogen peroxide. The table shows the effects of 1 μM of Aβ1–42 or 50 μM of H_2O_2 after 24-hour exposure in the presence (+) or absence (–) of 12-hour preincubation with 1 μM of melatonin. Results are the mean ± SEM of 9 observations (triplicate independent assays) for the MTT assay and of 13 observations (quadruplicate independent assays) for the GSH assay. †Significant difference ($P < .005$) from the control and melatonin groups (Kruskal-Wallis test, Dunn multiple-comparisons test).

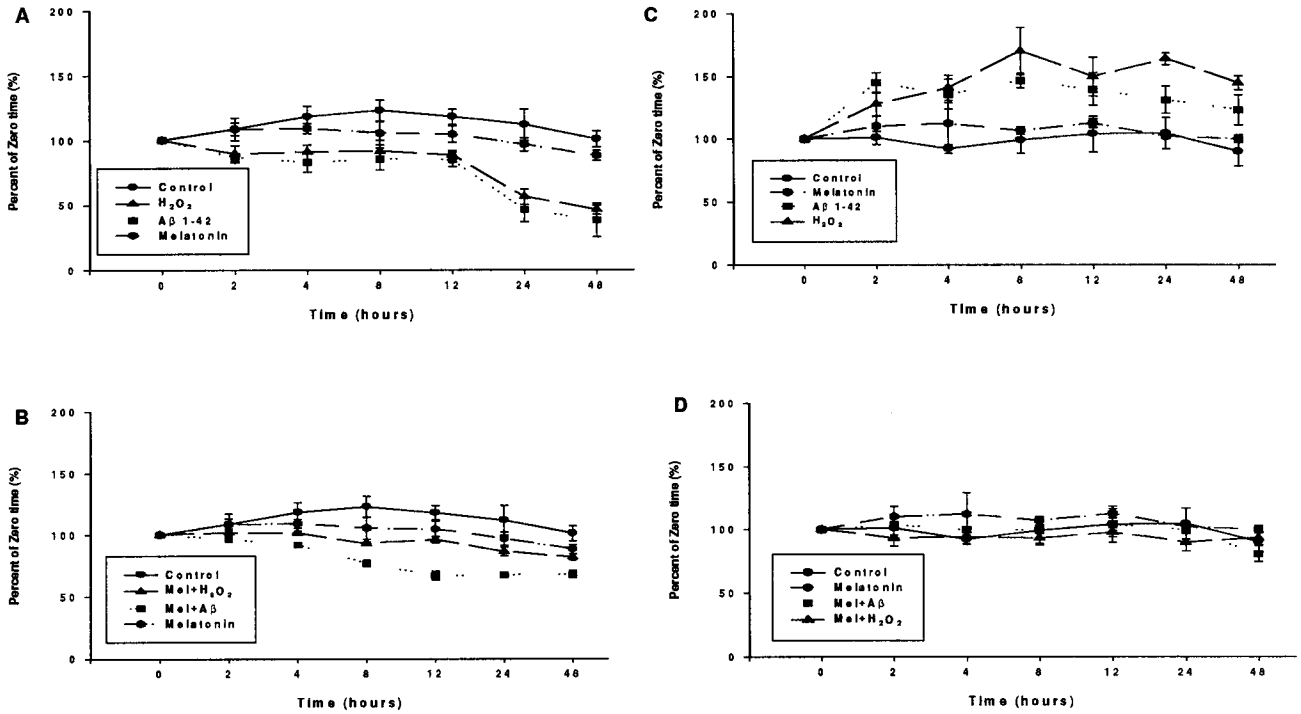

Figure 1 Time course of the effect of 50 μM of hydrogen peroxide (H_2O_2) or 1 μM of β-amyloid (Aβ) on tyrosine kinase receptor A (trk-A) and p75 expression at the cell surface in SHSY5Y neuroblastoma cells in the presence or absence of 12-hour preincubation with 1 μM of melatonin. (A) Compared with untreated control cells, cells exposed to H_2O_2 or Aβ showed a significant ($P < .001$) decrease in trk-A over the entire experimental period. Melatonin alone did not alter trk-A expression. (B) Melatonin pretreatment significantly reduced Aβ- and H_2O_2-induced decreases in trk-A in study cells compared with control cells, with the exception of 8 to 12 hours ($P < .005$) for Aβ-stressed cells. (C) Compared with untreated control cells, cells exposed to H_2O_2 or Aβ showed a significant ($P < .005$) increase in p75 over the entire experimental period. Melatonin alone did not alter p75 expression. (D) Melatonin pretreatment restored Aβ- and H_2O_2-induced increases in p75 to control levels; this change was significant. Results represent the mean ± SEM of 9 observations (triplicate independent experiments).

(Fig. 2C); this increase was eliminated with melatonin pretreatment, which restored p75 to control levels (Fig. 2D).

NGF levels in the culture medium of SHSY5Y cells exposed to Aβ or H_2O_2 were assayed by ELISA. Over time, Aβ markedly increased the release of NGF from the neuroblastoma cells compared with the untreated control cells (Fig. 3A). Melatonin treatment and H_2O_2 exposure produced a slight decrease in the release of NGF, but this change was not significantly different from what occurred in control cells (Fig. 3A). Melatonin pretreatment strongly attenuated the Aβ-induced increase in NGF to levels similar to those obtained with melatonin alone (Fig. 3B). Similarly, NGF levels in cells pretreated with melatonin and then exposed to H_2O_2 were also below control levels at 24 and 48 hours (Fig. 3B). ELISAs for cells treated with the NGF-blocking antibody could not be performed, as the antibody interferes with ELISAs. Immunoprecipitation studies of culture medium with the blocking antibody verified NGF secretion into the culture medium and its capture by this antibody (data not shown).

trk-A mRNA and p75 mRNA were also affected by the stress agents used in this study. Aβ and H_2O_2 progressively decreased trk-A mRNA over the entire experimental period (Fig. 4A) and significantly decreased p75 mRNA after 24 hours of exposure, with p75 mRNA returning almost to control levels after 48 hours (Fig. 4C). Melatonin pretreatment significantly protected cells against Aβ- and H_2O_2-induced decreases in both trk-A mRNA and p75 mRNA, with levels approaching those of control cells (Figs. 4B, 4D). Melatonin alone did not affect mRNA levels.

Discussion

In this study, both Aβ and H_2O_2 induced significant cell cytotoxicity and oxidative stress in SHSY5Y cells. Aβ seems to induce toxicity through several mechanisms, including induction of ROS and resultant oxidative stress (Pacifici and Davies, 1991; del Rio et al, 1992; Benzi and Moretti, 1995; Martin et al, 1996), induction of lipid peroxidation (Behl et al, 1994), activation of NF-κB (Behl et al, 1994; Yang et al, 1995), and Aβ–p75 interaction leading to apoptosis (Kuner

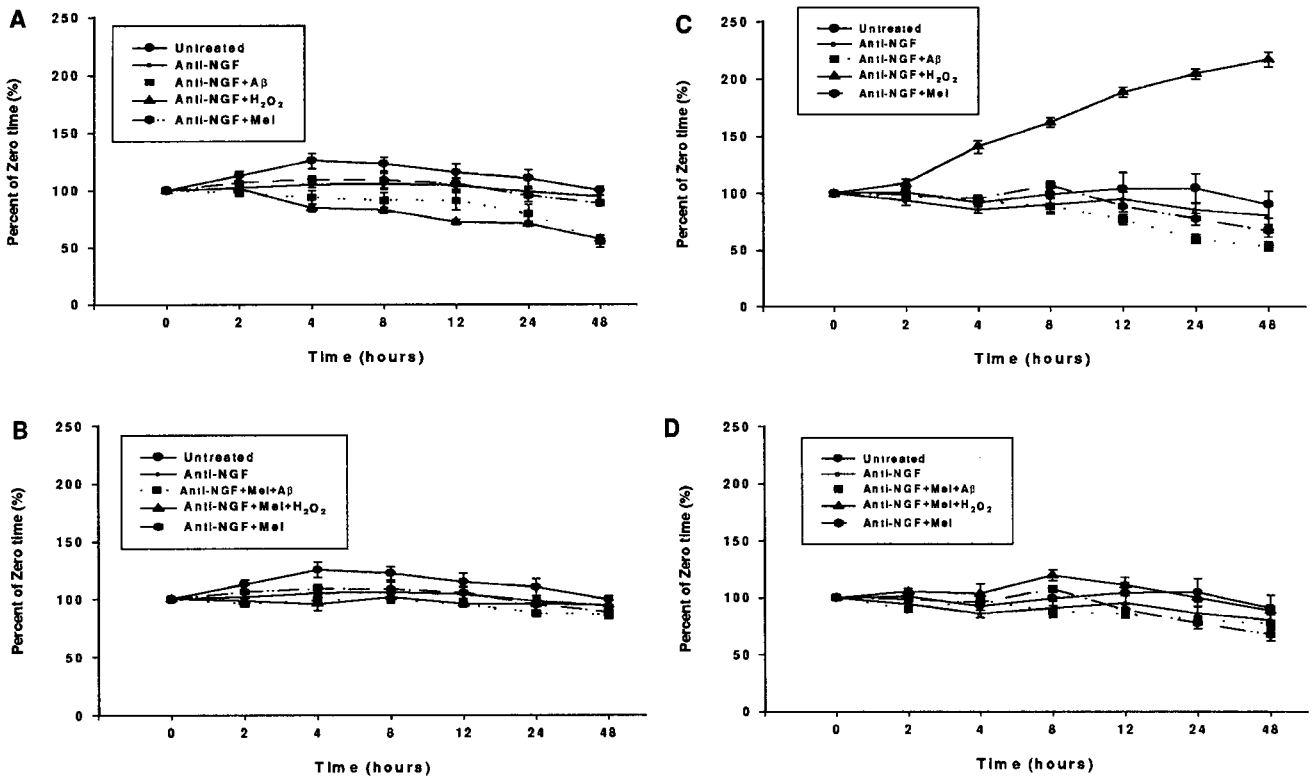

Figure 2 To exclude the effects of endogenous nerve growth factor (NGF), SHSY5Y neuroblastoma cells were exposed to anti-NGF antibodies. Then, the effect of either 50 μM of hydrogen peroxide (H_2O_2) or 1 μM of β-amyloid (Aβ) on tyrosine kinase receptor A (trk-A) and p75 expression at the cell surface in the presence or absence of 12-hour preincubation with 1 μM of melatonin was investigated. (A) Treatment with anti-NGF antibodies did not alter trk-A expression. Compared with untreated control cells, cells exposed to H_2O_2 or Aβ showed a significant ($P < .001$) decrease in trk-A from 4 hours of exposure until the end of the assay. Melatonin alone did not alter trk-A expression. (B) Melatonin pretreatment restored trk-A to control levels. (C) Treatment with anti-NGF antibodies did not alter p75 expression. Compared with all other groups of cells, cells exposed to H_2O_2 showed a significant ($P < .001$) increase in p75 expression over the entire experimental period. Melatonin alone did not alter p75 expression. (D) Melatonin pretreatment restored p75 to control levels. Results represent the mean ± SEM of 9 observations (triplicate independent experiments).

et al, 1998). Incubation of cells with melatonin was used to block the deleterious actions of Aβ and H_2O_2. Apparently exerting its neuroprotective effects through activation of the GSH system, melatonin increases synthesis of GSH-synthesizing enzymes such as γ-glutamylcysteine synthetase (Todoroki et al, 1998; Urata et al, 1999); increases the activity of antioxidant enzymes superoxide dismutase, catalase, and GSH reductase (Liu and Ng, 2000); inhibits Aβ-induced lipid peroxidation (Daniels et al, 1998); and acts as a general antioxidant (Reiter, 1995; Sewerynek et al, 1995; Pappolla et al, 2000).

Exposure of cells to Aβ and H_2O_2 produced a marked decrease in trk-A and an increase in p75. Many authors have speculated that trk-A is involved in cell differentiation and survival (Kuner and Hertel, 1998; Kaplan and Miller, 2000) and that p75 is involved in cell apoptosis (Kuner and Hertel, 1998; Kaplan and Miller, 2000). Further, the ratio of receptors relative to each other also seems important in regulating their cellular effects (Twiss et al, 1998). In the brain of patients with AD, Hock et al (1998) found down-regulation of trk-A, and Ernfors et al (1990) found up-regulation of p75. A combination of trk-A decrease and p75 increase could push the cells into an apoptotic pathway, which seems to be the case during exposure of cells to toxic Aβ and H_2O_2. In addition, Kuner et al (1998) found that Aβ can directly bind p75 and induce apoptosis through a mechanism involving NF-κB. As a result, Aβ-induced trk-A decrease and p75 increase, combined with Aβ–p75 binding, could prove fatal to neuronal cells—a situation not unlike that found in the brain of patients with AD.

To ascertain the neuroprotective effects of the pineal indolamine melatonin within the cell culture system, a specific anti-NGF antibody was added to inactivate the NGF secreted by the neuroblastoma cells.

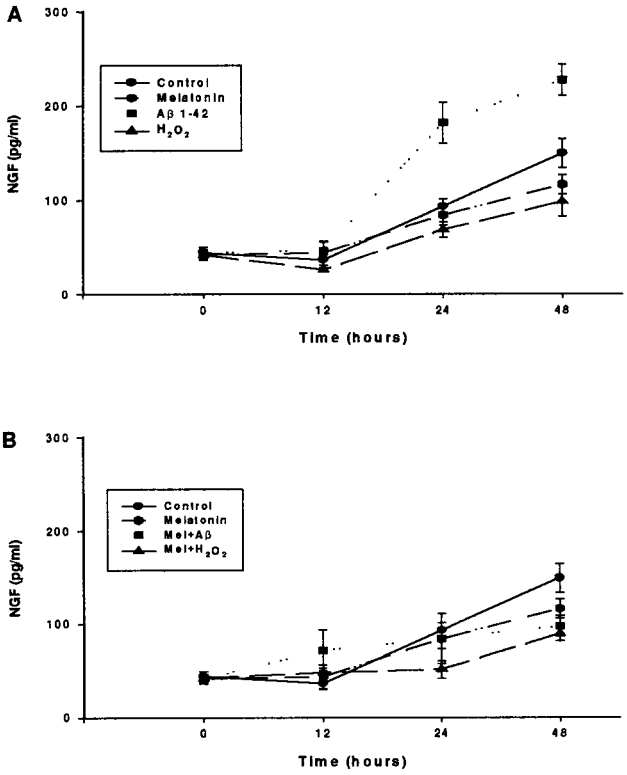

Figure 3 Time course of the effect of 50 μM of hydrogen peroxide (H_2O_2) or 1 μM of β-amyloid (Aβ) on nerve growth factor (NGF) release from SHSY5Y neuroblastoma cells in the presence or absence of 12-hour preincubation with 1 μM of melatonin. (A) Compared with untreated control cells, cells exposed to Aβ showed a significant ($P < .001$) increase in NGF release after 12 hours of exposure. Melatonin pretreatment and H_2O_2 exposure did not alter NGF release. (B) Melatonin pretreatment restored the Aβ-induced increase in NGF to control levels; this change was significant. Results represent the mean ± SEM of 9 observations (triplicate independent experiments).

In numerous systems, NGF protects cells from oxidative stress (Dugan et al, 1997), promotes cell differentiation and survival (Kaplan and Miller, 2000), and affects cellular antioxidant enzymes (Goss et al, 1997). Results of the blocking experiments were similar to those not using the anti-NGF antibodies, except that, in the blocking experiments, Aβ lost its ability to increase p75 levels. This exception is significant, as it suggests NGF involvement in Aβ-induced p75 receptor modulation—a possibility strengthened by results showing an Aβ-induced increase in NGF release from SHSY5Y cells. trk-A mRNA and p75 mRNA were also decreased in the presence of the stress agents. The decrease in p75 mRNA is surprising, as an increase in p75 mRNA was expected to take into account the increased expression of receptor protein. The result suggests that p75 mobilizes on the membrane surface from some sort of storage area or that p75 exposes epitopes that under normal conditions are not available, thus allowing detection of p75 on the membrane surface. This idea, however, is purely speculative.

Melatonin pretreatment reversed the deleterious effects of both Aβ and H_2O_2—including returning trk-A and p75 to control levels, returning mRNA to control levels, and returning NGF to normal levels. Melatonin's mechanism of action remains unclear however, melatonin has been shown to be a strong scavanger of free radicals (Reiter, 1995; Sewerynek et al, 1995; Pappolla et al, 2000), inducer of cellular antioxidant enzymes (Liu and Ng, 2000), and neuroprotective against Aβ(reducing Aβ fibrillogenesis, β-sheet formation, and Aβ-induced oxidative stress/ROS) (Pappolla et al, 1998). In light of these effects, we hypothesize that the primary action of melatonin is that of blocking trk-A and p75 modulation caused by oxidative stress induced by Aβ.

In conclusion, we have found that oxidative stress induced by Aβ and H_2O_2 can modulate trk-A and p75 receptor expression. Further, we have found that the antioxidant melatonin can protect neuroblastoma cells from these deleterious effects. These results are particularly important in that they can be used in designing AD treatment strategies involving NGF. For example, using only NGF in a cellular environment similar to one affected by AD can be deleterious, but coadministering NGF and melatonin to counteract the cellular redox imbalance can allow NGF to function purely as a neuroprotective agent.

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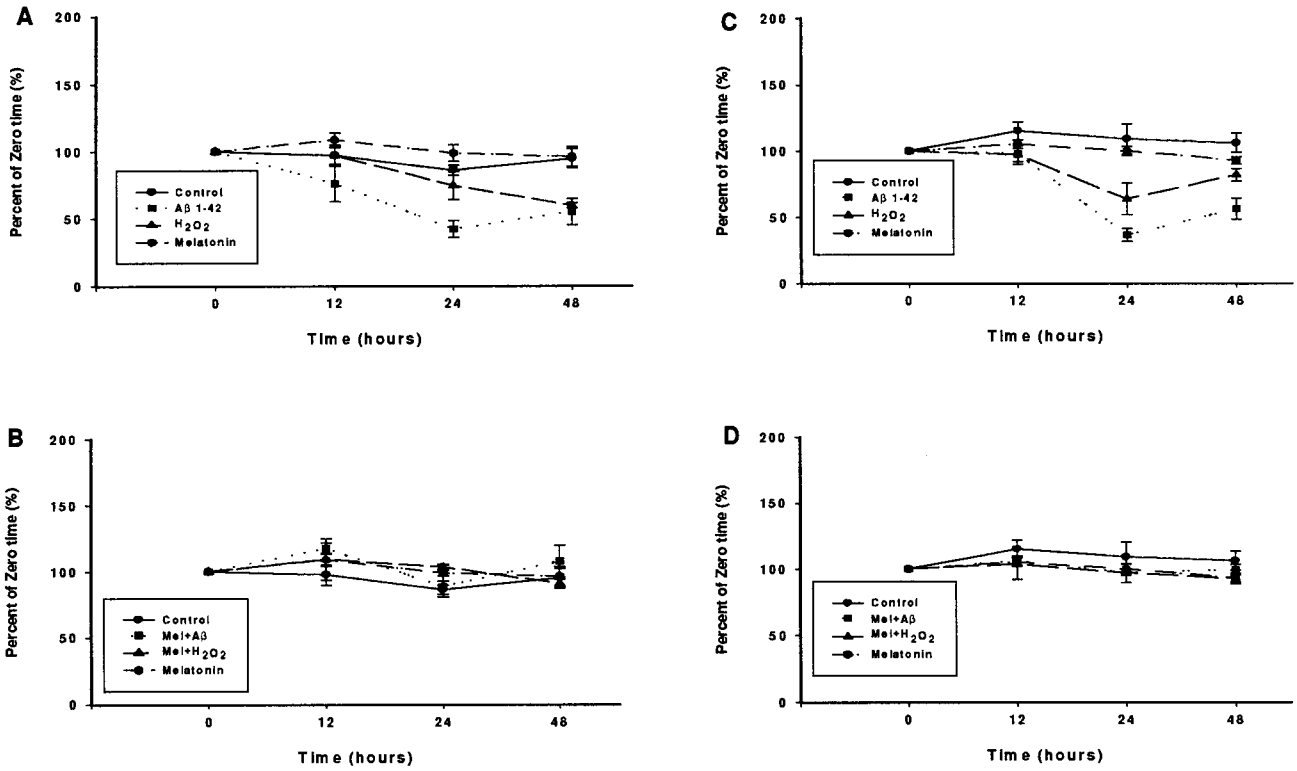

FIGURE 4 Time course of the effect of 50 μM of hydrogen peroxide (H_2O_2) or 1 μM of β-amyloid (Aβ) on tyrosine kinase receptor A (trk-A) messenger RNA (mRNA) and p75 mRNA from SHSY5Y neuroblastoma cells in the presence or absence of 12-hour preincubation with 1 μM of melatonin. (A) Compared with untreated control cells, cells exposed to H_2O_2 or Aβ showed a significant ($P < .004$) decrease in trk-A mRNA over the entire experimental period, with the exception of the 12-hour time point for H_2O_2. Melatonin alone did not alter trk-A mRNA levels. (B) Melatonin pretreatment restored Aβ- and H_2O_2-induced decreases in trk-A mRNA to control levels; this change was significant. (C) Compared with untreated control cells, cells exposed to H_2O_2 or Aβ showed a significant ($P < .001$) decrease in p75 mRNA over the entire experimental period, with the exception of the 12-hour time point for both. Melatonin alone did not alter p75 mRNA levels. (D) Melatonin pretreatment restored Aβ- and H_2O_2-induced decreases in p75 mRNA to control levels; this change was significant. Results represent the mean ± SEM of 9 observations (triplicate independent experiments).

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