Amitriptyline and fluoxetine protect PC12 cells from cell death induced by hydrogen peroxide

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Objective: To investigate the potential protective effects of amitriptyline and fluoxetine in a catecholamine cell model. Methods: Cultured rat pheochromocytoma (PC12) cells were pretreated with amitriptyline or fluoxetine for 24 or 48 hours and were then subjected to neurotoxic insult (200 µmol/L hydrogen peroxide). Cell viability was determined by measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The enzyme activity of superoxide dismutase (SOD) was determined by a commercial SOD assay kit. Results: The decrease in cell viability induced by hydrogen peroxide was attenuated in PC12 cells pretreated with 100 µmol/L amitriptyline for 24 hours or with 50 µmol/L amitriptyline or 50 µmol/L fluoxetine for 48 hours. Pretreatment with either amitriptyline or fluoxetine was associated with increased SOD activity in PC12 cells. Inhibition of SOD activity with diethyldithiocarbamic acid reduced the cytoprotective action of fluoxetine. Conclusions: These data suggest that the neuroprotective actions of some antidepressants include the upregulation of SOD activity.

Introduction

Neuroanatomic studies suggest that impairment of neuroplasticity and cellular resilience may be involved in the pathophysiology of major depressive disorder. Familial studies have highlighted the importance of genetic factors in the development of mood disorders, and it may be that depression occurs when neuronal systems lack the ability to exhibit adaptive plasticity in response to external stressors. Defects in these adaptive responses may be involved in the pathogenesis of depression, making vulnerable individuals more susceptible to mood disorders. Although a common mechanism of action of antidepressant therapies has eluded researchers for many years, recent evidence suggests that antidepressants and other mood stabilizers influence important signalling pathways that regulate neuroplasticity and cell survival. The clinical utility of antidepressant therapy may therefore be related to the downstream effects of these drugs.

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which are ultimately responsible for reversing dysfunctional adaptive responses and stimulating adaptive neuronal plasticity in patients with depression.2

One potential target of antidepressant regulation is the enzyme copper-zinc superoxide dismutase (SOD1, EC 1.15.1.1). This homodimeric metalloenzyme neutralizes the reactive oxygen species superoxide anion.2 Although SOD1 is found in all aerobic cells, it occurs in higher concentrations in the large pyramidal neurons of the hippocampus and neocortex of the mammalian brain.2,3 This endogenous antioxidant enzyme functions to reduce the oxidative stress of a cell and to prevent premature aging or neuronal death.2 Various neuroprotective factors have been shown to upregulate the transcription or enzyme activity of SOD1, including ginsenosides,4 neurotrophins5 and L-deprenyl.6 SOD1 is also upregulated by brain-derived neurotrophic factor (BDNF) and nerve growth factor.7 Furthermore, in vivo studies have established that upregulation of this enzyme is associated with neuroprotective capabilities in both ischemia8 and glutamate toxicity;9 whereas its downregulation induces apoptotic cell death of cultured neurons10 and PC12 cells.11 Glucocorticoids also cause downregulation of SOD1, in addition to their being implicated in the development of major depressive disorder.12 If episodes of clinical depression are accompanied by progressive hippocampal atrophy throughout the duration of the disease, antidepressant therapy or other forms of treatment that upregulate SOD1 gene expression may prevent worsening of affective symptoms that are either directly or indirectly related to hippocampal degeneration.13

The objectives of the present investigation involved testing the ability of the tricyclic antidepressant amitriptyline (a classic nonselective reuptake inhibitor) and of fluoxetine (a classic selective serotonin selective reuptake inhibitor) to protect PC12 cells from cell death when exposed to neurotoxic insult induced by hydrogen peroxide, as well as to assess the levels of SOD enzyme activity in these cell cultures.

Methods

Cell culture

The PC12 rat pheochromocytoma cell line was obtained from the American Type Culture Collection (Rockville, Md.) and cultured in RPMI 1640 medium (Media Laboratory, College of Veterinary Medicine, University of Saskatchewan, Saskatoon) supplemented with 0.03% glutamine, 5% fetal calf serum and 10% horse serum plus 100 IU/mL penicillin G sodium salt, as outlined in protocols provided by the supplier. Cells were grown in collagen-coated flasks at 37°C in 95% humidified air with 5% carbon dioxide. When the cells were 80% confluent, they were dislodged from the flask surface with a flow of medium from a pipette and then dispersed through a 22-gauge needle. The dispersed cells were plated onto collagen-coated 96-well plates at a density of 3 × 10^4 cells/well and were cultured under various combinations of times and drug regimens. The cells were cultured for either 24 or 48 hours in the presence of amitriptyline or fluoxetine and then cultured for an additional 4 hours in the presence of 200 μmol/L H_2O_2. Control cells were cultured with neither drug nor H_2O_2 treatment. All drugs were purchased from commercial sources.

Determination of cell viability

After incubation of the PC12 cells with the experimental reagents for the indicated time periods, cell viability was determined by colorimetric measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenybezolium bromide (MTT). The original medium was removed from the 96-well plates, and the cells were incubated for 4 hours at 37°C in the presence of RPMI 1640 medium with 1% fetal bovine serum containing 0.5 mg/mL MTT. A 100-μL aliquot of acid-isopropanol (0.04 mol/L hydrochloric acid) was then added to each well, and the plates were incubated at 37°C overnight to dissolve the formazan that had formed in the wells. Reduced MTT was measured by means of a kinetic microplate reader (Molecular Devices, Palo Alto, Calif.) at a wavelength of 570 nm. The protein contents of cell lysates were measured using a bicinchoninic acid protein assay reagent kit (Fierce Inc., Rockford, Ill.). The value for each treatment group was converted to the percentage of control.

Determination of SOD enzyme activity

The level of SOD enzyme activity in PC12 cells was measured using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc., Gaithersburg, Md.) according to the protocol previously described by Wei et al.2 After incubation of the PC12 cells with the experimental reagents for the indicated time periods, the original medium was removed from the 96-well plates, and the PC12 cells were lysed with Nonidet P-40 lysis buffer (1% NP-40, 50 mmol/L Tris-HCl [pH 7.5], 0.05 mmol/L ethylenediamine tetra-acetate) for 20 minutes at 4°C. The lysates were centrifuged at 300g for 10 minutes, and 20 μL of this sample solution was used for determination of SOD enzyme activity according to the manufacturer’s instructions. The value for each treatment group was converted to the percentage of control.

Statistical analysis

All data are presented as mean (and standard error of the mean). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Scheffé’s post hoc comparison.

Results

Effects of antidepressants on viability of PC12 cells exposed to H_2O_2

Cell viability was reduced significantly after treatment for 24 hours with 200 μmol/L of fluoxetine or 400 μmol/L of amitriptyline (Fig. 1A) but was not affected by lower concentrations of these drugs. Further reduction in cell viability was observed after incubation for 48 hours (Fig. 1B). One-way
ANOVA revealed that the viability of PC12 cells treated for 24 hours with 100 µmol/L amitriptyline and then exposed to 200 µmol/L H₂O₂ was significantly greater than the viability of PC12 cells exposed only to the H₂O₂ insult ($F = 36.9$, df = 3,28, $p < 0.001$; Fig. 2A). Statistical analysis also demonstrated that cells treated with 50 µmol/L amitriptyline or fluoxetine for 48 hours and subsequently exposed to H₂O₂ had significantly higher viability than untreated PC12 cells exposed to the same H₂O₂ insult ($F = 84.2$, df = 3,28, $p < 0.001$; Fig. 2B). When the cells were cotreated with 50 µmol/L fluoxetine and 1.0 mmol/L diethyldithiocarbamic acid (DETC, a metal-chelating agent that inhibits SOD1 activity) for 48 hours, the protective effect of fluoxetine was attenuated (Fig. 2C).

Effects of antidepressants on SOD activity of PC12 cells

SOD activity increased with increasing concentrations of amitriptyline, reaching its highest level with incubation at 100 µmol/L for 24 hours (Fig. 1C), although cell viability was about the same as control at that concentration. No significant increase in SOD activity was observed after treatment with fluoxetine (Fig. 1C). SOD activity after treatment with amitriptyline or fluoxetine for 48 hours was highest at drug concentrations of 50 µmol/L (Fig. 1D), whereas cell viability was the same as control under these conditions. One-way ANOVA revealed that the SOD activity of PC12 cells treated with 100 µmol/L amitriptyline for 24 hours was significantly greater than SOD activity in control cells (Fig. 3A). No significant increase in SOD activity was observed in the cells treated with 100 µmol/L fluoxetine for 24 hours. Levels of SOD activity were significantly higher in PC12 cells treated with either 50 µmol/L amitriptyline or 50 µmol/L fluoxetine for 48 hours than in control cells (Fig. 3B). When the cells were treated with DETC, SOD activity decreased, and fluoxetine did not reverse the decrease caused by DETC (Fig. 3C).

Discussion

Endogenous H₂O₂ that is formed as a natural byproduct of enzymatic oxidase action or as a product of the dismutation of superoxide anion catalyzed by SOD1 contributes to the background level of cellular oxidative stress. It is generally

![Fig. 1: Effects of antidepressants on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction and superoxide dismutase (SOD) activity of PC12 cells. Cells were treated with amitriptyline (AMI) or fluoxetine (FLU) for 24 hours (A, C) or 48 hours (B, D). MTT reduction assay (A, B) and test for SOD activity (C, D) were carried out according to the Methods. Data are presented as mean (and standard error of the mean), from 4 independent experiments for the MTT assay and 3 independent experiments for SOD activity.](image-url)
accepted that the antioxidant enzymes catalase and glutathione peroxidase can protect cells from the effects of basal H$_2$O$_2$ production. However, the administration of exogenous H$_2$O$_2$ can elevate oxidative stress beyond the protective capacities of endogenous antioxidant defense systems, resulting in apoptosis or necrosis of cultured neurons and PC12 cells. There is good evidence from in vitro studies to suggest that astrocytes respond to oxidative insults by upregulating protective molecules, including SOD1. Other researchers

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
have observed an increase in SOD1 in activated astrocytes following addition of the cytotoxic agent quinolinic acid. Upregulation of SOD1 and similar antioxidative enzymes may protect these cells from apoptosis or necrosis upon exposure to a range of neurotoxins.

PC12 cells have been widely employed to investigate the mechanisms involved in neurotoxicity, neuroprotection and neuronal repair, and previous research has implicated SOD1 in the action of antidepressants on PC12 cells. Where a decrease in SOD1 corresponds with an increase in apoptotic cell death, an increase in endogenous SOD1 activity has been shown to protect neurons from apoptosis induced by staurosporine. However, the effects of SOD1 may become deleterious when this enzyme is overexpressed and the resulting H2O2 overwhelms catalase or glutathione peroxidase detoxification mechanisms. Some researchers have even suggested that overexpression of SOD1 leading to excessive H2O2 production may be partly responsible for the brain injury observed after perinatal hypoxia–ischemia.

The results of the investigation reported here indicate that some antidepressants increase SOD1 activity in PC12 cells. Our finding that fluoxetine could not reverse the inhibition of SOD1 activity caused by DETC (i.e., DETC attenuates the protective effect of this drug) supports the hypothesis that increased SOD1 activity may be involved in the mechanisms by which fluoxetine and amitriptyline provide cytoprotective action to PC12 cells. However, the underlying mechanisms by which these drugs increase SOD1 activity are still unknown.

Although the SOD1 activity was approximately 10%–20% higher in cells treated with subcytotoxic concentrations of fluoxetine or amitriptyline than in control cells, there were no differences in cell viability between the 2 groups. Moreover, no obvious signs of cell death were observed in the treated cells. Other researchers have similarly reported that subcytotoxic concentrations of these antidepressants increase levels of reduced glutathione in C6 cells, but that higher concentrations of fluoxetine and amitriptyline decrease glutathione levels. In our experiment, treating the PC12 cells with antidepressants increased the levels of SOD1 activity, which ultimately led to the production of more endogenous H2O2. Because the viability of cells treated with subcytotoxic concentrations of antidepressants was essentially identical with that of control cells, we propose that catalase or glutathione peroxidase or both were upregulated to participate in the cellular defence against H2O2 produced by the activation of SOD1. Thus, when the challenge H2O2 was added, the defence system had already been induced. This hypothesis is consistent with the fact that the viability of cells pretreated with antidepressants and subsequently subjected to exogenous H2O2 was significantly greater than the viability of untreated cells subjected to the same neurotoxic insult. Our results suggest that subcytotoxic concentrations of antidepressants may provide protection to PC12 cells exposed to H2O2 by increasing levels of the antioxidative enzyme SOD1; future studies should attempt to determine whether antidepressants increase production of catalase or glutathione peroxidase. Further work will also be necessary to elucidate the mechanisms by which different doses and exposure times initiate reactive responses and whether these doses approach or fall within the therapeutic range for achieving remission from depression in humans. A major limitation of an in vitro model is the impossibility of accurately comparing concentrations that produce changes in the culture dish with those to which the endogenous cells may be exposed in the central nervous system. At present there are no relevant data, so caution must be exercised in extrapolation of results from a culture system to an in vivo situation.

Although major depressive disorder is currently considered a heterogeneous disease, both stress and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis have been implicated in the origin and exacerbation of this disease. Stress and glucocorticoid injections have also been shown to initiate dendritic atrophy in the hippocampus and to reduce cellular resilience, making neurons in this region more vulnerable to injury from other insults. However, the findings that repeated administration of selective norepinephrine or serotonin reuptake inhibitors increase the rate of hippocampal cell proliferation and that long-term, but not short-term, treatment of antidepressants enhances neurogenesis provide an explanation as to why these drugs may help reverse or prevent the symptoms of depression in most patients. Given that glucocorticoids have been shown to decrease BDNF messenger RNA, one of the key mediators in the therapeutic response of antidepressant medications, further research should be directed at investigating the feasibility of upregulation of SOD1 as a protective measure against glucocorticoid toxicity and an important mechanism whereby antidepressants could protect against further cell death or atrophy caused by HPA dysregulation. In those patients whose depression is caused, or accompanied, by stress or glucocorticoid-induced neurotoxicity, continued efforts aimed at maintaining or increasing levels of neuroprotective enzymes such as SOD1 may prove extremely beneficial. In short, increased levels of SOD1 activity may account, at least in part, for the observed clinical effects of certain antidepressant drugs used in the treatment of depression.

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