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Clozapine protects PC-12 cells from death due to oxidative stress induced by hydrogen peroxide via a cell-type specific mechanism involving inhibition of extracellular signal-regulated kinase phosphorylation

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Abstract

Recent evidence suggests that some atypical antipsychotic drugs may protect against oxidative stress and consequent neurodegeneration by mechanisms that remain unclear. Using the neuron-like rat pheochromocytoma (PC-12) cell line, Clozapine and N-desmethylozapine were tested for their ability to protect against cell death due to oxidative stress induced by hydrogen peroxide (H₂O₂). These drugs demonstrated significant protection of PC-12 cells, as measured by both the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) and Alamar Blue cell viability assays. However, neither viability assay detected a protective effect of Clozapine on human embryonic kidney (HEK293), rat primary cortical neurons, or human neuroblastoma (SHSY5Y) exposed to H₂O₂ treatment. The mechanism of protection involves a PC-12 cell-specific differential response to H₂O₂ treatment vs. the other cell lines. Pre-treatment with 250 μM or 125 μM diethyldithiocarbamate (DETC), a superoxide dismutase (SOD) inhibitor, unexpectedly showed protection of the PC-12 cells from H₂O₂ treatment. Western blots revealed that Clozapine, N-desmethylozapine, and DETC reduce the phosphorylation of extracellular signal-regulated kinase (ERK) that is caused by H₂O₂ exposure in PC-12 cells. In both HEK293 and SH-SY5Y cells, H₂O₂ exposure did not increase ERK phosphorylation over control, demonstrating a different response to H₂O₂ vs. PC-12 cells, and explaining why Clozapine could not protect these cells. Also, U0126, a specific MEK inhibitor, was able to protect PC-12 cells from H₂O₂ exposure, showing that inhibiting ERK phosphorylation is sufficient to provide protection. Cumulatively, these results indicate that Clozapine, N-desmethylozapine, DETC, and U0126 protect PC-12 cells by blocking the cell-type specific H₂O₂ induced increase in ERK phosphorylation.

Keywords

PC-12 cell; Clozapine; Oxidative stress; Hydrogen peroxide; ERK; N-desmethylozapine; SH-SY5Y cell; HEK293 cell; U0126; Neuroprotection; Schizophrenia

1. Introduction

Schizophrenia is a severe chronic debilitating disease characterized by positive symptoms (hallucinations, delusions), negative symptoms (socially withdrawn behavior, flat emotional expression), and cognitive deficits. A growing body of evidence suggests that oxidative stress may play a major role in the neurodegeneration and disease progression of schizophrenia (Carter 2006; Fendri et al., 2006; Sarandol et al., 2007; Young et al., 2007; Ng et al., 2008). Pre-clinically, chronic administration of MK-801 in adult male rats mimics psychosis through NMDA. This model shows significant increases over controls in malondialdehyde, protein carbonyl, and nitric oxide levels, as well as superoxide dismutase, glutathione peroxidase, xanthine oxidase, and adenosine deaminase enzyme activities in the prefrontal cortex, and co-administration of MK-801 with caffeic acid phenethyl ester (a powerful antioxidant) showed significant reductions in all of these (Ozyurt et al., 2007). In patients, the earlier treatment with atypical antipsychotics begins, lesser degeneration and disease progression are observed (Lieberman et al., 2001; Schaffner and McGorry, 2001; Mahadik et al., 2006). One possibility for this effect is that, in addition to improving positive and negative symptoms, atypical antipsychotics provide some degree of neuroprotection, specifically against oxidative stress, by mechanisms that have begun to be unraveled, but largely remain unknown (Krebs et al., 2006).

The rat pheochromocytoma (PC-12) cell line provides an established neuron-like system that can be used as a model for cell death due to oxidative stress. In addition, several drug classes and mechanisms have been reported to reduce this death. Propofol protects PC-12 cells from hydrogen peroxide (H_2O_2) induced oxidative stress by inhibiting the p38 phosphorylation caused by H_2O_2 (Wu et al., 2007). Preconditioning PC-12 cells with low doses of H_2O_2 activates JAK2, and reduces PC-12 cell death. Pre-treatment with the JAK inhibitor, AG-490, blocks the protective effects of preconditioning the cells with low doses of H_2O_2 (Yu et al., 2006). More relevantly, the atypical antipsychotic drugs Clozapine, Olanzapine, Quetiapine, and Risperidone have been shown to protect PC-12 cells from N-methyl-4-phenylpyridinium ion challenge (Qing et al., 2003). Pre-treatment with Olanzapine also protects PC-12 cells from death due to oxidative stress induced by H_2O_2 (Wei et al., 2003). In both cases, the possible mechanism of protection appears to be altering the expression levels of copper/zinc superoxide dismutase (SOD-1) mRNA expression.

The purpose of the present study was to look for neuroprotective properties of Clozapine and N-desmethylozapine by testing their ability to protect PC-12 cells from death caused by oxidative stress induced by H_2O_2 . The cellular mechanism of this protection would then be determined. Since apoptosis due to oxidative stress is a highly conserved pathway involving the mitochondria (Ryter et al., 2007), Clozapine would be tested for its ability to protect HEK293 cells, SH-SY5Y cells, and rat primary cortical neurons from death due to oxidative stress induced by H_2O_2 to demonstrate whether its mechanism of protection is conserved. If protection is shown in all cell lines, then a correlation could be drawn as to a possible additional benefit provided by these drugs at the cellular level in the brain.

2. Results

Briefly, all of the following experiments were conducted by seeding cells into plates on Day 1, treating with drug or equivalent amounts of DMSO on Day 2 (allowing for overnight exposure to the drugs), and treating with H_2O_2 for 2 h to cause oxidative stress-induced cell death on Day 3. The only exceptions to this were experiments conducted with rat primary cortical neurons, which were seeded 15 days prior to drug exposure, and generation of cell lysates for western blots, which were only treated with H_2O_2 for 20 min on Day 3.

2.1. Experiment 1: Does Clozapine protect PC-12 cells from death caused by H₂O₂ induced oxidative stress?

PC-12 cells were treated with H₂O₂ dose response curves for 2 h, followed by both the MTT and Alamar Blue cell viability assay methods to determine appropriate experimental conditions (Fig. 1). Due to variability among assays, compound plates were run at 200 μ M H₂O₂ or 300 μ M H₂O₂ to target a cell survival range of 10–50% of the control in both assays. Clozapine was tested at multiple doses for its ability to protect PC-12 cells from death caused by H₂O₂ induced oxidative stress. Pre-treatment of PC-12 cells with Clozapine overnight (~20 h) significantly increased cell viability vs. 2-hour treatment with 200 μ M or 300 μ M H₂O₂ the next day in both the MTT and Alamar Blue assays. Average viability increases of 25.9% and 8.7% were seen with 100 and 50 μ M Clozapine, respectively, in the MTT assay (Fig. 2A), and average increases of 12.5%, 9.9%, and 8.5% were seen with 100, 50, and 25 μ M Clozapine in the Alamar Blue assay (Fig. 2B). There was a 12.4% decrease in cell viability with 100 μ M Clozapine without H₂O₂ in the Alamar Blue assay. Pre-treatment with 200 μ M Clozapine was also tested, however the viability of the PC-12 cells without H₂O₂ was reduced to 61% in the MTT assay and 42% in the Alamar Blue assay, and no protection vs. H₂O₂ was seen, indicating a maximum dose threshold to the protective benefits of Clozapine before drug toxicity takes over.

Next, N-desmethylozapine, the major active metabolite of Clozapine, was assessed for its ability to protect PC-12 cells from death due to H₂O₂ induced oxidative stress. Pre-treatment of PC-12 cells with 50 μ M or 25 μ M N-desmethylozapine overnight (~20 h) significantly increased cell viability vs. 2-hour treatment with 200 μ M or 300 μ M H₂O₂ the next day by 15.1% (50 μ M) and 16.0% (25 μ M) in the Alamar Blue assay, while 50 μ M N-desmethylozapine significantly increased viability by 24.6% in the MTT assay (Figs. 3A, B). Pre-treatment with 100 μ M N-desmethylozapine was also tested, however the viability of the PC-12 cells without H₂O₂ was reduced to 34% in the MTT assay and 23% in the Alamar Blue assay, and no protection vs. H₂O₂ was seen, indicating toxicity more potent than that of Clozapine.

2.2. Experiment 2: Does Clozapine protect rat primary cortical neurons, HEK293, or SH-SY5Y cells from death caused by H₂O₂ induced oxidative stress?

Since apoptosis due to oxidative stress is a highly conserved pathway, Clozapine was tested for its ability to protect other cell types from death due to H₂O₂ induced oxidative stress. H₂O₂ dose response curves were established with 2-hour treatments for rat primary cortical neurons, HEK293 cells, and SH-SY5Y cells (Figs. 4A–C). Interestingly, H₂O₂ stress did not produce complete cell death in HEK293 cells or SH-SY5Y cells. The inflection point of H₂O₂ induced cell death was 556 μ M for HEK293 cells, with the bottom of the curve leveling off at ~54% viability. For SH-SY5Y cells, H₂O₂ produced kill curves with inflection points of 85 μ M and 94 μ M, and the bottoms of the curves leveling off at ~35% and ~34% viability, for the MTT and Alamar Blue assays respectively.

Overnight pre-treatment with 100, 50, or 25 μ M Clozapine was tested for protection vs. 2-hour treatments of 750 μ M H₂O₂ in HEK293 cells, 125 μ M H₂O₂ in SH-SY5Y cells, and 200 μ M H₂O₂ in rat primary cortical neurons (100 and 50 μ M Clozapine only, Alamar Blue assay only). In all cases, Clozapine did not protect these cells from H₂O₂ induced cell death (Figs. 5A–E). 100 μ M Clozapine did demonstrate significant toxicity in rat primary cortical neurons and HEK293 cells that had not been treated with H₂O₂, as well as significantly increase cell death in the presence of H₂O₂ in all three cell types vs. H₂O₂ without Clozapine.

2.3. Experiment 3: Does the mechanism of Clozapine's protection of PC-12 cells from death caused by H₂O₂ induced oxidative stress involve superoxide dismutase?

Previous studies have reported that treatment of PC-12 cells with atypical antipsychotics or antidepressants causes an upregulation of Cu/Zn superoxide dismutase (SOD-1) gene expression and/or enzyme activity (Bai et al., 2002; Wei et al., 2003; Kolla et al., 2005). To pharmacologically test if Clozapine protects PC-12 cells through the SOD pathway, PC-12 cells were pre-treated overnight with 100, 50, 25, or 0 μ M Clozapine $\pm$ 1 mM DETC (a SOD inhibitor). Following 2-hour H₂O₂ treatment the next day, the results of the cell viability assays indicated that pre-treatment with both Clozapine and DETC did not show any protection of the PC-12 cells, and appeared to increase toxicity with increasing doses of Clozapine, vs. 1 mM DETC without Clozapine (data not shown). The caveat to these data was that 1 mM DETC in the absence of H₂O₂ was extremely toxic to the cells, reducing the experimental window to the point that any increase over 1 mM DETC in the presence of H₂O₂ would not have been significant. Therefore, PC-12 cells were pre-treated with a titration of DETC overnight, and then exposed to 2-hour treatments with or without H₂O₂ the next day to find a suitable dose of DETC for future studies. Surprisingly, several lower doses of DETC significantly increased cell viability vs. 2-hour treatment with 200 μ M or 300 μ M H₂O₂ in both the MTT and Alamar Blue assays. Average viability increases of 26.9% and 41.0% were seen with 250 μ M and 125 μ M DETC in the MTT assay, and increases of 29.5%, 22.9%, and 20.0% were seen with 250 μ M, 125 μ M, and 25 μ M in the Alamar Blue assay (Figs. 6A, B). DETC was tested for its ability to protect HEK293 cells or SH-SY5Y cells (Figs. 6C–F). Only toxicity, not protection, was observed in a dose dependent manner in HEK293 cells, and at the single concentration of 125 μ M DETC in SH-SY5Y cells, for both the MTT and Alamar Blue assays.

2.4. Experiment 4: Does the mechanism of Clozapine's protection of PC-12 cells from death caused by H₂O₂ induced oxidative stress involve extracellular signal-regulated kinase?

The mitogen-activated protein (MAP) kinase signaling pathways also play a role in the cytotoxicity associated with oxidative stress. H₂O₂ treatment of PC-12 cells causes significant increases in ERK, JNK, and p38 phosphorylation within 10 min of treatment (Wu et al., 2007). Western blotting was used to examine the phosphorylation state of ERK in PC-12 cells that were treated with 300 μ M H₂O₂ for 20 min following overnight pre-treatment with 100 μ M or 50 μ M Clozapine, 125 μ M DETC, or 0.1% DMSO (Fig. 7, for quantification see Supplemental Figs. 2A, B). H₂O₂ treatment increased ERK phosphorylation in PC-12 cells over the control. This activation of both ERK1 (p44) and ERK2 (p42) was attenuated by pre-treatment with DETC and both concentrations of Clozapine. To test if this inhibition of ERK phosphorylation is sufficient to protect the PC-12 cells from H₂O₂ induced oxidative stress, PC-12 cells were pre-treated overnight with 100, 30, 10, or 0 μ M U0126 (a specific MAP/ERK kinase (MEK) inhibitor which prevents ERK phosphorylation). Following 2-hour treatment with 200 μ M or 300 μ M H₂O₂, U0126 significantly protected the PC-12 cells by 28.1% (100 μ M), 21.8% (30 μ M), and 27.1% (10 μ M) in the Alamar Blue assay, and by 33.9% (100 μ M) and 27.2% (30 μ M) in the MTT assay (Figs. 8A, B). Western blotting was again used to examine the phosphorylation state of ERK in PC-12, HEK293, and SH-SY5Y cells that were treated with 300 μ M, 750 μ M, or 200 μ M H₂O₂ respectively for 20 min following overnight pre-treatment with 100 μ M or 50 μ M Clozapine, 30 μ M U0126, 50 μ M N-desmethylozapine, 125 μ M DETC, or 0.1% DMSO (Figs. 9A–C, for quantification see Supplemental Figs. 3A–F). While H₂O₂ treatment increased ERK phosphorylation in PC-12 cells over the control, this increase was not seen in HEK293 or SH-SY5Y cells. In PC-12 cells, U0126, Clozapine, and N-desmethylozapine all attenuated the phosphorylation of ERK1 (p44), and Clozapine attenuated the phosphorylation of ERK2 (p42). However, N-desmethylozapine did not reduce the phosphorylation of ERK2, and U0126 caused an increase in phosphorylation of ERK2. In HEK293 cells, no ERK phosphorylation was seen in any of the samples. In the SHSY5Y cells, basal ERK phosphorylation was observed in the controls, and the level of

phosphorylation was slightly reduced in the presence of H₂O₂ treatment. This basal phosphorylation was almost eliminated with 30 μ M U0126 plus H₂O₂ treatment, and with 50 μ M N-desmethylozapine plus H₂O₂ treatment.

3. Discussion

These studies have shown that Clozapine (an atypical anti-psychotic), N-desmethylozapine (Clozapine's major active metabolite), DETC (a superoxide dismutase inhibitor), and U0126 (a specific MEK inhibitor), all protect PC-12 cells from death caused by acute (2 h) H₂O₂ induced oxidative stress. Given the specificity of U0126 for the immediate upstream kinase of ERK, and the above results showing that A) H₂O₂ exposure increases ERK phosphorylation in PC-12 cells, and B) Clozapine, N-desmethylozapine, DETC, and U0126 significantly reduce ERK 1 phosphorylation in all cases, and ERK 2 phosphorylation when using Clozapine or DETC, the mechanism of protection for all of the above agents is the inhibition of ERK phosphorylation.

This is not the first time that Clozapine has been implicated in modulating the activation state of ERK, however, whether Clozapine increases or decreases this activation is subject to debate. In vivo studies dosing Sprague–Dawley rats with Clozapine, followed by tissue harvesting for western blotting, have shown increases ERK phosphorylation in the prefrontal cortex and the ventrolateral region of the rostral caudate-putamen (Ahmed et al., 2008). Similar experiments have not only shown that Clozapine increases ERK phosphorylation in the prefrontal cortex of Sprague-Dawley rats, but that this increase can be reduced by administration of (+/-)-2,5-dimethoxy-4-iodoamphetamine (DOI, a 5-HT_{2A/2C} agonist) (Browning et al., 2005). In contrast to these findings, administration of Clozapine to C57BL/6 mice, followed by tissue extraction, homogenization, and western blotting, resulted in a decrease of ERK phosphorylation in the dorsal striatum which became more significant over time, out to 1 h (Pozzi et al., 2003). In vitro studies have also been done to profile Clozapine's ability to modulate ERK phosphorylation. Clozapine has been shown to increase ERK phosphorylation in CHO cells stably expressing human serotonin 5-HT_{1A} receptors, and decrease ERK phosphorylation in CHO cells stably expressing human dopamine D_{2S} receptors (Bruins Slot et al., 2006). Therefore, the answer may lie in the serotonergic or dopaminergic tone of the region of interest. Accordingly, PC-12 cells have been shown to express native dopamine receptors (Courtney et al., 1991; Esteve et al., 1995; Pothos et al., 1998). Therefore, the current findings that Clozapine inhibits ERK phosphorylation are in agreement with previous work, and this decrease is most likely due to Clozapine's direct interaction with the PC-12 cell's dopamine receptors.

The current experiments have revealed a differential response to H₂O₂ induced oxidative stress between cell types. In PC-12 cells, H₂O₂ increased ERK phosphorylation above the control, and was able to completely kill the cells at high concentrations (1 mM), as shown by the dose response curves in Fig. 1. This response was unlike those of HEK293 and SH-SY5Y cells. H₂O₂ did not cause any detectable ERK phosphorylation in HEK293 cells, and there were no detectable levels of ERK phosphorylation in the controls. Also, high concentrations of H₂O₂ (up to 1.5 mM tested) were not able to achieve complete cell death in HEK293 cells, as cell viability did not drop below 54% in the Alamar Blue assay. For SH-SY5Y cells, basal levels of ERK phosphorylation were seen in the control, and H₂O₂ slightly reduced these basal levels. Once again, high concentrations of H₂O₂ (up to 1.5 mM tested) were not able to achieve complete cell death, as the H₂O₂ dose response curve bottomed out at 34–35% in the Alamar Blue and MTT assays. Perhaps the inability of H₂O₂ to completely kill either HEK293 or SH-SY5Y cells is due to its inability to activate ERK in these cell lines. These differences between cell lines in the theoretically conserved mechanism of cytotoxicity due to oxidative stress point

out that caution must be exercised when attempting to translate results from one model system to another.

The above results with SH-SY5Y cells are contrary to previous work which showed ERK phosphorylation increases in SH-SY5Y cells at 5, 10, and 20 min after H₂O₂ exposure (Ruffels et al., 2004), however, these studies used 500–1000 μ M H₂O₂. The same authors showed that 30-minute pre-treatment with PD98059 (another MEK inhibitor) afforded protection from overnight exposure to 1000 μ M H₂O₂, implying that ERK phosphorylation leads to cell death. Others have reported that ERK phosphorylation is increased at 3 and 6 h after exposure to 300 μ M H₂O₂, but not at 1 or 9 h after exposure, and that this increase in phosphorylation is associated with cell survival (Kim et al., 2005). These authors show that MAP Kinase Phosphatase-1 (MKP-1) reduces ERK phosphorylation, leading to increased SH-SY5Y cell death. This makes sense with the data from the current experiments. Fig. 9C shows that H₂O₂ causes a reduction of basal ERK phosphorylation, and that none of the following agents, U0126, Clozapine, or N-desmethyloclozapine, could rescue the cells from this basal reduction (Fig. 9C). Since SH-SY5Y cells are dopaminergic (Chetsawang et al., 2006; Chen et al., 2008), Clozapine could exert its inhibition of ERK phosphorylation through these receptors. However, there is no increase in phosphorylation to inhibit, and thus, Clozapine does not protect SH-SY5Y cells from death caused by H₂O₂ induced oxidative stress. These results emphasize the differential response to H₂O₂ between SH-SY5Y cells and PC-12 cells.

In summary, Clozapine and N-desmethyloclozapine, along with DETC and U0126, protect PC-12 cells from death due to H₂O₂ induced oxidative stress by inhibiting ERK phosphorylation. The differential responses to H₂O₂ among cell lines, and the lack of protection by Clozapine in HEK293 cells, SH-SY5Y cells, and rat primary cortical neurons, add another layer of complication when trying to translate in vitro systems to what is actually going on in the neurons of the human brain.

4. Experimental procedures

4.1. General experimental design

All experiments involved seeding cells of various lines (PC-12, HEK293, SH-SY5Y, and rat primary cortical neurons, as detailed below) into plates on Day 1, adding compounds or equivalent amounts of DMSO for overnight pre-treatment on Day 2, and exposing the cells to H₂O₂ for 2 h on Day 3 to cause death induced by oxidative stress. The only exception to the above design was that rat primary cortical neurons were seeded into plates two weeks prior to the assay, so compound addition occurred on Day 15 and H₂O₂ exposure was on Day 16 for these cells. After H₂O₂ exposure, two independent measures of cell viability were used to quantitate oxidative stress. These measures of cell viability permitted testing for potential protection afforded by the drugs in question. Subsequently, the mechanism of protection was investigated pharmacologically in the cell viability assays, and by the use of western blotting techniques that measured the expression of phospho-ERK and total ERK following manipulation of cell lines with 20-minute exposure to H₂O₂. Details of each experimental protocol are presented below.

4.2. Cells and culture conditions

PC-12 cells (ATCC #CRL-1721) were grown at 37 °C in 5% CO₂ in RPMI 1640 media (Invitrogen #11875) supplemented with 10% Horse Serum (ATCC #30-2040), 5% FBS (ATCC #30-2021), and 1% Pen/Strep (Invitrogen #15070) in Collagen I coated flasks (NUNC #132707 or #132708). Cells were plated by scraping into 10 mL of fresh media and diluting to 200,000 cells/mL. 100 μ L was seeded into each well (except E1-H1) of a clear, flat-bottom, collagen I

coated 96-well plate (Beckton Dickinson #35-6698) for 20,000 cells/well, and the plates were allowed to incubate at 37 °C in 5% CO₂ overnight.

HEK293 cells (ATCC #CRL-1573) were grown at 37 °C in 5% CO₂ in DMEM (ATCC #30-2002) supplemented with 10% FBS (ATCC #30-2021), Sodium Pyruvate (Invitrogen #11360) and 1% Pen/Strep (Invitrogen #15070) in tissue culture treated flasks (NUNC #178883). Cells were plated at 200,000 cells/mL by seeding 100 µL into each well (except E1-H1) of a clear, flat-bottom, tissue culture treated 96-well plate (Beckton Dickinson #35-3072) for 20,000 cells/well. The plates were allowed to incubate at 37 °C in 5% CO₂ overnight.

SH-SY5Y cells (ATCC #CRL-2266) were grown at 37 °C in 5% CO₂ in DMEM/F12 media (Invitrogen #11039) supplemented with 10% FBS (Invitrogen #16000), 1× Non-essential amino acids (Invitrogen #11120) and 1% Pen/Strep (Invitrogen #15070) in Collagen I coated flasks (NUNC #132707 or #132708). Cells were plated at 200,000 cells/mL by seeding 100 µL into each well (except E1-H1) of a clear, flat-bottom, collagen I coated 96-well plate (Beckton Dickinson #35-6698) for 20,000 cells/well. The plates were allowed to incubate at 37 °C in 5% CO₂ overnight.

Rat primary cortical neurons were isolated from E18 Sprague–Dawley rat pups in accordance with the Merck West Point Institutional Animal Care and Use Committee (IACUC). Rat dams were obtained from Taconic and were anesthetized by carbon dioxide inhalation and decapitated prior to rat pup removal. Rat pups were decapitated, and the brains were harvested. Cortical neurons were dissociated with 0.05% trypsin, and plated at 75,000 cells in 100 µL per well onto PDL coated, 96-well clear-bottom black plates in Complete Minimum Eagle's Media (Minimum Eagle's Media supplemented with 10% FBS, 5% Horse serum, 2 mM L-glutamine, and 1% Pen/Strep). Plates were allowed to incubate at 37 °C in 90–95% humidity and 5% CO₂. After 24 h, media were switched to Neurobasal media (Invitrogen #21103)+2 mM L-glutamine (Invitrogen #25030) + B27 Supplement (Invitrogen #17504), and allowed to grow in the incubator for 14 more days, with one media change at day 7 in vitro. Cells were not seeded in the outer wells of the plate.

4.3. Pre-treatment with compounds

Clozapine (#0444), N-desmethylozapine (#1007), and U0126 (#1144) were obtained from Tocris Bioscience (Ellisville, MO). Sodium Diethyldithiocarbamate Trihydrate (DETC, #S287-100) was obtained from Fisher Scientific. The compounds were solubilized in dimethyl sulfoxide (DMSO) to make 100 mM or 10 mM stocks.

For cell lines: the day after plating cells, compounds were diluted appropriately in RPMI 1640 media for 10× stocks of the desired concentration, maintaining 1% DMSO concentration. 11 µL of the diluted compounds were added directly to the wells containing 100 µL of cells for 1× compound and 0.1% final DMSO concentrations. The plates were allowed to incubate at 37 °C in 5% CO₂ overnight.

For rat primary cortical neurons: on day 15, compounds were prepared at the desired concentration, 0.1% final DMSO concentration, in Neurobasal media (Invitrogen #21103) +B27 Supplement without Antioxidants (Invitrogen #10889). The media were aspirated from the wells and replaced with 100 µL of media containing the 1× compound and 0.1% final DMSO concentrations. The plates were allowed to incubate at 37 °C in 5% CO₂ overnight.

4.4. Apply oxidative stress with H₂O₂

30% H₂O₂ stabilized in water (8.82 M, Acros #41188-5000) was diluted 1:882 in RPMI 1640 media (or Neurobasal media+B27 Supplement without Antioxidants for rat primary cortical neurons) for a 10 mM working stock. This stock was then diluted again to obtain 10× of the

desired concentration in the assay. For example, if 200 μM H_2O_2 was the desired final concentration, the 10 mM stock would have been diluted 1:5 for 2 mM stock. 11 μL of the 10 $\times$ stock was then added to the 110 μL in each well, and the plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 for 2 h.

4.5. Measure cell viability

4.5.1. MTT assay—50 mg of MTT reagent (Reagent A) (Chemicon MTT Cell Growth Kit, #CT01) was dissolved in 10 mL of Solution B (PBS). 1.1 mL of reagent was added to 11 mL of cell-type specific growth media for each plate to be assayed. The 2-hour H_2O_2 stress was stopped by aspirating the treatment media from each well. 110 μL of growth media containing MTT reagent was added to each well, and the plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 for 4 h. After 4 h, 100 μL of Solution C (isopropanol +0.04 N HCl) was added to each well of the plates, and they were allowed to shake on a plate shaker to mix for about 5 min. The plates were sealed and returned to the incubator overnight to allow formazan crystals to dissolve completely. The plates were read the following day on the Molecular Devices SPECTRAMax Plus using SoftMax Pro software with the Basic Endpoint protocol at 570 nm. Raw OD readings were converted to % viability by first subtracting the assay blanks (wells E1-H1, which contained no cells) and then dividing by the average of the control wells, which received 1% DMSO in RPMI 1640 media (0.1% final concentration) at the time of compound treatment, and RPMI 1640 media at the time of H_2O_2 treatment. There were a minimum of four replicate wells for each condition on each plate. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Significance was determined by oneway ANOVA, followed by Newman–Keul's post-hoc comparisons.

4.5.2. Alamar Blue assay—1.1 mL of Alamar Blue reagent (Invitrogen #DAL1100) was added to 11 mL of cell-type specific growth media (or Neurobasal media+B27 Supplement without Antioxidants for rat primary cortical neurons) for each plate to be assayed. The 2-hour H_2O_2 stress was stopped by aspirating the treatment media from each well. 110 μL of growth media containing Alamar Blue reagent was added to each well, and the plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 for 2 to 5 h. The plates were then read on the Tecan Safire2 with 530 nm excitation and 590 nm emission wavelengths. Raw fluorescent counts were converted to % viability, and statistical significance was assessed by the same calculations used for the MTT assay.

4.6. Western blotting

4.6.1. Lysate preparation—PC-12, HEK293, and SH-SY5Y cells were seeded onto 6-well plates (Becton Dickinson #353046) at 200,000 cells/mL for PC-12 and HEK293 cells, or 500,000 cells/mL for SH-SY5Y cells, 3 mL/well. For the PC-12 and SH-SY5Y cells, the plates were coated with Collagen type I (Becton Dickinson #354236), washed with H_2O , and allowed to dry in the hood for 30 min before seeding. The plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 overnight. The day after plating cells, compounds were diluted appropriately in RPMI 1640 media for 10 $\times$ stocks of the desired concentration, maintaining 1% DMSO concentration. 330 μL of the diluted compounds were added directly to the wells containing 3 mL of cells for 1 $\times$ compound and 0.1% final DMSO concentrations. The plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 overnight. 30% H_2O_2 stabilized in water was diluted 1:882 in RPMI 1640 media for a 10 mM working stock. This stock was then diluted again to obtain 7.5 mM (for HEK293), 3 mM (for PC-12), or 2 mM (for SH-SY5Y) stocks (10 $\times$ of the desired concentration in the assay). 330 μL of RPMI control or the appropriate H_2O_2 stock was added to each well for 750 mM (for HEK293), 300 mM (for PC-12), or 200 mM (for SH-SY5Y) final concentration, and the plates were allowed to incubate at 37 $^\circ\text{C}$ in 5% CO_2 for 20 min. After 20 min, the media were aspirated completely from each well, the plates were placed on ice, and 1 mL (PC-12 and HEK293 cells) or 0.25 mL (SH-SY5Y cells) of cold RIPA Buffer (10

mM Tris HCl, 150 mM NaCl, 1% Deoxycholic acid, 1% Triton X-100, 0.1% SDS, 20 mM NaF, 1 mM Na₃VO₄ (activated), 1 mM Pefabloc (Roche #1429876), 1 mM PMSF, and Complete protease inhibitor (Roche #1697498, 1 tablet/50 mL) was added to each well. After a 5-minute incubation on the ice, whole lysates were collected, and protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad #500-0006) according to the manufacturer's instructions.

4.6.2. Western blotting—The samples were adjusted for protein concentration by diluting in RIPA buffer, and then further prepared by combining 65% sample, 25% Nupage sample buffer (Invitrogen #NP0007), and 10% reducing agent (Invitrogen #NP0004). The samples were heated to 70 °C for 15 min. 15 µL of each sample, 10 µL of Bench Mark standard (Invitrogen #10748), or 10 µL of 30% Licor standard (Licor #928-40000) was loaded onto Nupage 4–12% Bis-Tris 15-well 1.0 mM gels (Invitrogen #NP0323Box). 500 µL of Antioxidant (Invitrogen #NP0005) was added to the chamber between the gels after loading the samples. The gels were run in MOPS Buffer (Invitrogen #NP0001) at 200 V constant for 50 min. Proteins were transferred onto PVDF membranes (Invitrogen #IB4010-02) using the Invitrogen iBlot dry transfer method. Membranes were placed directly into Licor Odyssey Block (Licor #927-40000), and allowed to block with gentle rocking at room temperature for 1 h. Phospho-p44/42 MAP Kinase (Thr202/Tyr204) rabbit polyclonal antibody (Cell Signaling #9101) (P-ERK Ab) was diluted 1:1000 and p44/42 MAP Kinase (3A7) mouse monoclonal antibody (Cell Signaling #9107) (total ERK Ab) was diluted 1:2000 in the same tube of Odyssey Block. The membranes were allowed to incubate in the combined primary antibody solution with gentle rocking at 4 °C overnight. The next day, membranes were washed with Tris-Buffered Saline (TBS) with gentle rocking at room temperature for 1 h. For PC-12 or HEK293 samples, goat anti-rabbit IR Dye 700 conjugated antibody (Rockland #611-130-122) was diluted 1:5000 and goat anti-mouse IR Dye 800 conjugated antibody (Rockland #610-132-121) was diluted 1:20000 in the same tube of Odyssey Block. For SH-SY5Y samples, donkey anti-rabbit IR Dye 800 conjugated antibody (Licor #926-32213) was diluted 1:15000 and donkey anti-mouse IR Dye 680 conjugated antibody (Licor #926-32222) was diluted 1:15000 in the same tube of Odyssey Block. The membranes were allowed to incubate in the combined secondary antibody solution with gentle rocking at room temperature for 50 min. The membranes were washed with TBS+0.05% Tween with gentle rocking at room temperature for 1 h. The membranes were then rinsed with TBS and washed an additional 10 min with TBS to remove detergent before imaging. The membranes were imaged with the Licor Odyssey, simultaneously detecting both P-ERK and total ERK in each blot. All images were converted to .jpeg format and bands were quantified using ImageJ free software from the NIH website (<http://rsb.info.nih.gov/ij/download.html>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DETC	diethyldithiocarbamate
DMSO	dimethyl sulfoxide
ERK	extracellular signal-regulated kinase

H2O2	hydrogen peroxide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
NMDA	N-methyl-d-aspartate
SOD	superoxide dismutase
U0126	1,4-diamino-2,3-dicyano1,4-bis[2-aminophenylthio]butadiene

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Fig. 1. PC-12 cells treated with H₂O₂ dose response curves for 2 h followed by cell viability determinations with both the Alamar Blue and MTT assays. H₂O₂ produced a kill curve with an EC₅₀ of 245 μM in the Alamar Blue assay and 189 μM in the MTT assay (cumulative results from 4 experiments for both methods).

Fig. 2.

Overnight pre-treatment with Clozapine protects PC-12 cells from 2-hour treatment with 200 μM or 300 μM H₂O₂ in both the Alamar Blue and MTT assays. Cumulative results for 100 μM, 50 μM, and 25 μM Clozapine or 0.1% DMSO (indicated as 0 μM Clozapine) vs. H₂O₂ (+) or no H₂O₂ (-) in the A) MTT assay or B) Alamar Blue assay. These are cumulative results from 10 experiments for each assay method. For the MTT assay method, 6 experiments were conducted with 200 μM H₂O₂ and 4 experiments were conducted with 300 μM H₂O₂. For the Alamar Blue assay method, 4 experiments were conducted with 200 μM H₂O₂ and 6 experiments were conducted with 300 μM H₂O₂. ****P*<0.001, compared to the untreated control. #*P*<0.05, ###*P*<0.001, compared to H₂O₂ without Clozapine.

Fig. 3. Overnight pre-treatment with N-desmethylozapine (N-DMC) protects PC-12 cells from 2-hour treatment with 200 μ M or 300 μ M H₂O₂ in both the Alamar Blue and MTT assays. Cumulative results for 50 μ M and 25 μ M N-desmethylozapine, or 0.1% DMSO (indicated as 0 μ M N-DMC) vs. H₂O₂ (+) or no H₂O₂ (-) in the A) MTT assay or B) Alamar Blue assay (cumulative results from 4 experiments for both assay methods). *** P <0.001, compared to the untreated control. ### P <0.001, compared to H₂O₂ without N-desmethylozapine.

Fig. 4.

H₂O₂ dose response curves for 2 h followed by cell viability determinations. A) In rat primary cortical neurons, H₂O₂ produced a kill curve with an EC₅₀ of 148 μM in the Alamar Blue assay. B) In SH-SY5Y cells, H₂O₂ produced a kill curves with EC₅₀ values of 85 μM in the Alamar Blue assay and 94 μM in the MTT assay. C) In HEK293 cells, H₂O₂ produced a kill curve with an EC₅₀ of 556 μM in the Alamar Blue assay.

Fig. 5.

Overnight pre-treatment with Clozapine did not protect other cell types from 2-hour treatment with H_2O_2 . Cumulative results for 100 μM , 50 μM , and/or 25 μM Clozapine or 0.1% DMSO (indicated as 0 μM Clozapine) vs. no H_2O_2 (-) or A) 200 μM H_2O_2 (+) in rat primary cortical neurons with the Alamar Blue assay B) 750 μM H_2O_2 (+) in HEK293 cells with the MTT assay C) 750 μM H_2O_2 (+) in HEK293 cells with the Alamar Blue assay D) 125 μM H_2O_2 (+) in SH-SY5Y cells with the MTT assay and E) 125 μM H_2O_2 (+) in SH-SY5Y cells with the Alamar Blue assay. * P <0.05, *** P <0.001, compared to the untreated control for each cell type. # P <0.05, ## P <0.01, ### P <0.001, compared to H_2O_2 without Clozapine for each cell type.

Fig. 6.

Overnight pre-treatment with diethyldithiocarbamate (DETC) protects PC-12 cells, but not HEK293 cells or SH-SY5Y cells, from 2-hour treatment with H_2O_2 . Cumulative results for 1000 μM , 500 μM , 250 μM , 125 μM , 25 μM , and 5 μM DETC or 0.1% DMSO (indicated as 0 μM DETC) vs. no H_2O_2 (-) or A) 200 or 300 μM H_2O_2 (+) in PC-12 cells with the MTT assay, B) 200 or 300 μM H_2O_2 (+) in PC-12 cells with the Alamar Blue assay C) 750 μM H_2O_2 (+) in HEK293 cells with the MTT assay D) 750 μM H_2O_2 (+) in HEK293 cells with the Alamar Blue assay E) 125 μM H_2O_2 (+) in SH-SY5Y cells with the MTT assay and F) 125 μM H_2O_2 (+) in SH-SY5Y cells with the Alamar Blue assay. SH-SY5Y cells were only tested with 125 μM DETC. ** P <0.01, *** P <0.001, compared to the untreated control for each cell type. # P <0.05, ### P <0.001, compared to H_2O_2 without DETC for each cell type.

Fig. 7.

Western blot of PC-12 samples shows ERK phosphorylation increased by H_2O_2 and reduced by pre-treatment with Clozapine or DETC prior to H_2O_2 exposure. Extracts were prepared after overnight pre-treatment with 100 μM or 50 μM Clozapine, 125 μM DETC, or 0.1% DMSO, followed by a 20-minute exposure to 300 μM H_2O_2 . The representative blot shown was simultaneously probed with primary antibodies for phosphorylated ERK (p44/p42) and total ERK, followed by simultaneous addition of secondary antibodies containing either 700 nm or 800 nm dye. The blot was imaged using the Licor Odyssey, and the overlaying colored layers were separated and converted to black and white for ease of visualization and analysis.

Fig. 8.

Overnight pre-treatment with U0126 protects PC-12 cells from 2-hour treatment with 200 μM or 300 μM H₂O₂ in both the Alamar Blue and MTT assays. Cumulative results for 100 μM, 30 μM and 10 μM U0126, or 0.1% DMSO (indicated as 0 μM N-DMC) vs. H₂O₂ (+) or no H₂O₂ (-) in the A) MTT assay or B) Alamar Blue assay (cumulative results from 3 experiments for both assay methods). ****P*<0.001, compared to the untreated control. ##*P*<0.01, ###*P*<0.001, compared to H₂O₂ without U0126.

Fig. 9.

Western blots of PC-12 cells, HEK293 cells, and SH-SY5Y cells reveal differential activation of ERK due to H_2O_2 . All extracts were prepared after overnight pre-treatment with 100 μM or 50 μM Clozapine, 50 μM N-desmethylozapine (N-DMC), 30 μM U0126, 125 μM DETC, or 0.1% DMSO, followed by a 20-minute exposure to H_2O_2 : A) 300 μM H_2O_2 for PC-12 cells, B) 750 μM H_2O_2 for HEK293 cells, or C) 200 μM H_2O_2 for SH-SY5Y cells. The blot for each cell line was simultaneously probed with primary antibodies for phosphorylated ERK (p44/p42) and total ERK, followed by simultaneous addition of secondary antibodies containing either 700 nm or 800 nm dye. The blots were imaged using the Licor Odyssey, and the overlaying colored layers were separated and converted to black and white for ease of

visualization and analysis. Note that the 100 μ M Clozapine sample in the HEK293 western was not normalized for protein concentration due to cell toxicity resulting in extremely low protein yield of the sample.