Biology

Expression of BID in SH-SY5Y Cells After Acute Exposure to Rotenone

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Introduction

Parkinson's Disease (PD), a chronic neurodegenerative disease, affects as many as 1-2% of the global population aging 60 and over, and has been noted to have a higher incidence in rural areas of the world.1,12 In recent years, efforts have been focused on identifying the cause of Parkinsonian symptoms which include rigidity, resting tremor and instability of gait and posture.4 Studies have revealed that diminished mitochondrial complex I activity resulting in cellular apoptosis, or programmed cell death, is common among PD patients.1,2,9

Rotenone, classified as a General Use Pesticide, is both a naturally occurring toxin and a botanical insecticide used in home gardens for insect control, for lice and tick control on pets, and for fish eradications in water body management. Depending on its formulation it is classified as either mildly or highly toxic (Class I or III) by the EPA.4, 10

Along with its toxicity, rotenone is a chemical of potential hazard because it has recently been identified as one of the most relevant neurotoxins that can induce parksinonian symptoms. Evidence indicates that rotenone inhibits mitochondrial complex I.1 Therefore, it is likely that exposure to rotenone contributes to the development of PD through inhibition of this mitochondrial complex.

The activation of caspase proteins, which are proteases possessing the ability to cleave their substrate, also contribute to apoptosis in the cell.11 One signaling pathway activated by caspases involves the Bcl-2 family protein, Bid. Cleaving of Bid begins upon the binding of a cell death receptor on the membrane of a malfunctioning cell. When the Bid protein is cleaved by caspase-8, the carboxylic acid group cleaved from the Bid protein, termed tBid, migrates to the cell's mitochondria and causes the release of Cytochrome-c.5,8,11 This results in the relay of a signal to the cell membrane that causes blebbing of the membrane and consequent apoptosis.8

Therefore, the concentration of Bid after acute exposure to rotenone is a potential target in order to understand the mechanism of rotenone's contribution to PD. I hypothesize that there is a direct correlation of both the degree of cellular apoptosis and Bid expression to the concentration and length of exposure to rotenone.

Materials and Methods

Cell Culture

Human neuroblastoma cells of the SH-SY5Y lineage (passages 35-37) were cultured in 75 cm2 flasks maintained at 37° C with 5% CO2 in a humidified incubator. Cells were grown in HAM-12 media supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic solution and 1% L-glutamine (v/v) (Atlantic Biological, Atlanta, GA). Approximately 12mL of media was replaced every 3-5 days. At 80% confluency, cells were differentiated for 3 days using 10-7 M retinoic acid in fresh medium.

Rotenone was dissolved in DMSO to achieve three different concentrations: 1µM, 2.5 µM, and 5 µM; the control of .05% DMSO in fresh medium was used as well. Cells were harvested at 24 and 48 hours post-rotenone exposure by treating with trypsin-EDTA for 2 minutes at 37°C to lift the cells from the flasks' surfaces. Trypsin was neutralized with 10mL of fresh media. There were 3 replicates for each treatment at each time point.

The trypsinzed cellular material was placed into sterile 15mL tubes and centrifuged at 1200 RPM for 7 minutes at room temperature. The cell pellet was resuspended with cold PBS and again centrifuged at 1200 RPM for 7 minutes at room temperature. Cell lysis was then performed using a lysis buffer [150 mM NaCl, 20mM Tris-HCl, 10% glycerol (v:v), 1% Triton X-100 (v:v), 1mM EDTA, 1mM NaF, 1mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail Set III]. Cell membranes were further sheared by passing the lysate through a 21 gauge needle three times. The lysate was placed on ice and rocked gently for 30 minutes. The lysate was then centrifuged at 10,000*g* for 10 minutes at 4°C. The lysate was transferred to sterile, cold 1.7 mL tubes and stored at -20°C.

Cell lysate was thawed and a standard BCA protein curve from Fisher Scientific (Auburn, AL) was generated to determine the concentration of total protein in each sample of lysate. A series of standard dilutions ranging from 0 µg/mL-2000 µg/mL were used to generate a standard curve. Absorbance of each sample was read at 562.0 nm by a spectrophotometer. Cell lysate was diluted 1:1 with sample buffer, boiled at 95°C for 5 minutes, and immediately cooled on ice. Samples were stored at -20°C.

Western Blot Analysis

Cell lysates were thawed and boiled for five minutes followed by vortex. Samples were immediately placed on ice to cool before gel electrophoresis using 7.5% SDS-PAGE gels from Bio-Rad (Hercules, CA) was performed. A total of 5.25 µg of protein from each sample was loaded in each well. Membranes were treated with 3% Tween-Milk in 1XPBST for one hour on a gentle rotator to block any unbound protein. Membranes were rinsed in PBST. The membranes were then incubated in anti-Bid primary antibody (1:200 in Tween-milk; Santa Cruz) for 1 hour

at room temperature on rocker. Upon rinsing in PBST, membranes were treated in secondary antibody (1:5000 in Tween-milk; Santa Cruz) for one hour at room temperature on rocker. The membrane was rinsed with PBST and the signal amplified with BAR (Bio-Rad) for 10 minutes on rocker. The membrane washed 3 times in 20% DMSO/PBST at room temperature for 5 minutes. Following a final rinse in PBST for five minutes the membrane was exposed to streptavidin-HRP (1:1000 in Tween-Milk; Bio-Rad) at room temperature for 30 minutes on gentle rocker. The membrane was then washed two times in PBST for 5 minutes and treated with Opti-4CN (Bio-Rad) for 30 minutes until colorimetric detection observed. The membranes were then washed in dH2O for 15 minutes. The membranes were photographed and wrapped in plastic wrap for storage.

Results

Protein was detected in all samples. The detectable protein concentration ranged from 31.38 µg/mL -1038.61µg/mL. Cells exposed to 1µM rotenone for 24 hours expressed an average of 1200µg/mL (+/- 20µg) of total protein whereas cells exposed to 2.5 µM and 5 µM of rotenone expressed an average of 2400 µg/mL (+/- 25 µg) and 3000µg/mL (+/- 35 µg), respectively (Figure 2).

Cells treated with rotenone for 24 hours had a similar Bid expression level to each other as indicated by the density of the band on the membrane. Cells treated for 48 hours had lower concentrations of Bid than cells treated for 24 hours as indicated by lighter bands (Figure 3).

Discussion

As hypothesized, there was a direct correlation between cell death and the concentration of rotenone, as well as cell death and the length of exposure. There was a significant decrease in the total protein concentration of cells treated for 48 hours compared to 24 hours (Figure 2), suggesting decreased cell survival with extended exposure. Furthermore, total protein concentration decreased for all rotenone concentrations (1µM, 2.5µM, and 5µM) compared to control. These data demonstrate that cell death is elevated in rotenone-treated cells.

The results of the Western blot suggest an indirect correlation between the expression of Bid and the concentration of rotenone. As the concentration of rotenone increased, the expression of intact Bid protein concentration decreased (Figure 3). While the results did not support the hypothesis, they do provide insight into the mechanisms of rotenone-induced apoptosis and PD. It is possible that tBid is inducing apoptosis in the rotenone-exposed cells. Since these experiments used an antibody to intact Bid rather than the cleaved form of tBid, it is intact Bid that is present in control cells and in cells treated with 1µM and 2.5µM rotenone for 24 hours. This indicates that the apoptotic pathway has not yet been triggered in these cells.

Further experimentation using a primary antibody to tBid would be useful to determine if tBid is present in cells exposed to high concentrations of rotenone. If tBid is present, this suggests that the ratio of Bid:tBid in rotenone-induced PD contributes to cell death.

These findings support the suggestion that the Bid protein and tBid contribute to cellular apoptosis in rotenone-treated cells. It is critical to understand the role of Bid in apoptotic cell death as a contributor to the development of PD after exposure to rotenone. Enhancing the expression of Bid or preventing the formation of tBid may be a target in preventing the onset of environmentally-induced PD after exposure to rotenone.

References

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