Expression of Caspase-8 in SH-SY5Y Cells After Acute Exposure to Chlorpyrifos

Biology Department

Faculty Sponsor: Dr. Melinda Pomeroy-Black

Introduction

Parkinson's Disease (PD) is a neurological disorder that results in the degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Viswanath et al., 2001). The substantia nigra is involved with motor control of the body. It is suspected that when roughly 50-80% of dopamine cell death is obtained, symptoms begin to appear. Patients will have slow-to-absent movement, change in posture, rigidity, and tremors (Chang et al., 2003). Genetic and environmental factors, such as pesticides, play a role in the premature death of dopamine cells. Chlorpyrifos (CPF), an organophosphate compound, is a widely used pesticide. Chlorpyrifos is used for agricultural and domestic pest control. Contact with the pesticide is most commonly through manufacture, storage, and spraying (Tuite, 2016). Researchers found that organophosphorus insecticides affect dopaminergic neurotransmission (Karen et al., 2001). Low concentrations of CPF can induce oxidative stress, disturb neurotransmission, inhibit mitosis of the nervous system, and induce apoptosis in neuroblastoma cells. Chlorpyrifos induces acute and chronic neurotoxicity by also inhibiting cholinesterase activity (Raszewski et al., 2015). Acetylcholinesterase, a member of the cholinesterase family, catalyzes the hydrolysis of acetylcholine (ACh) into choline and acetic acid. Acetylcholinesterase (AChE) is found in various conducting tissues and has a higher activity in motor neurons. Inhibition of AChE leads to an accumulation of ACh, resulting in muscle weakness, muscle fasciculations, agitation, hypersalivation, and sweating. Muscle weakness and muscle fasciculations are similar symptoms found in PD patients (Čolović et al., 2013). While several studies have examined the role of AChE in chlorpyrifos toxicity, intracellular signaling cascades are also under investigation. For example, death receptors in the tumor necrosis factor receptor (TNFR) superfamily mediate an apoptotic pathway in mammalian cells. When bound by a ligand, the receptors recruit procaspase-8 and procraspase-3, inactive proteases, that when cleaved initiate the apoptotic process (Chang et al., 2003). Procaspase-8 undergoes two cleavage events to form caspase-8, which activates downstream caspases to complete cell death. Chlorpyrifos is known to increase the expression of cleaved caspase 3, suggesting that chlorpyrifos causes cell death via acaspase pathway (Raszewiski et al., 2015).

This study examined the role of caspase-8 in chlorpyrifos-mediated cell death. It was hypothesized that acute exposure to chlorpyrifos would have a dose-and time-dependent effect on the activation of caspase-8.

Materials and Methods

Human neuroblastoma cells of SH-SY5Y lineage were cultured in 75 cm3 flasks maintained at 37° C in an incubator. The HAMs 12 media with heat-inactivated FBS in the flasks was replaced every three days. At 80% confluency, the cells were differentiated for three days with 10-7 M retinoic acid in fresh media. Chlorpyrifos was diluted in media + FBS to make concentrations of 10 µM, 25 µM, and 50 µM. Cells were exposed to each concentration for either 1 hour or 3 hours. The control cells were exposed only to media + FBS. At one or three-hours, the cells were rinsed with 1X PBS and treated with 2 mL of 0.25% trypsin for two minutes in the 37° C incubator to lift the cells from the flask surface. The trypsin was neutralized with 10 mL media + FBS. Excess cells on the flask surface were scraped off before they were aliquoted to 15 mL centrifuge tubes. The cells were centrifuged at 1200 RPM for seven minutes at room temperature. The cell pellets were resuspended in 5 mL of 1X PBS twenty times then centrifuged at 1200 RPM for seven minutes at room temperature. The pelleted cells were stored at -20° C. After thawing, the pellets were resuspended in 1 mL RIPA lysis buffer (25 µl Protease inhibitor to 5mL lysis buffer). The lysate was put on ice for thirty minutes. The lysate was then centrifuged at 8,000xg for ten minutes at 4° C. The lysate was transferred to cold, sterile 1.7 mL tubes and stored at -20° C.

A standard BCA protein curve was generated to determine the concentration of total protein in each sample of lysate. Standard dilutions ranging from 0 µg/mL-2000 µg/mL were used to generate a standard curve. The absorbance of each sample was read at 562.0 nm by a multi-well plate reader. The cell lysate was then diluted 1:1 with sample buffer, boiled at 95° C for five minutes, and put on ice. The samples were stored at -20° C. The cell lysates in sample buffer were thawed and put on ice. A total of 9.88 µg of protein from each lysate was loaded in each well of a 10% Tris-HCL gel (Bio-Rad). Gel electrophoresis at room temperature for 40 minutes. Gel, nitrocellulose membrane and filter papers soaked in Transfer Buffer for 10 minutes. After a wet transfer for 1 hour at 100V, membranes were rinsed with 1X TBS for two minutes at room temperature on a rocker then dried in the hood. Next, the cells were treated with 1:11X TBS/Odyssey Blocking Buffer (LiCor) for one hour on a gentle rotator to block unbound protein. Membranes were incubated with dilution of anti-caspase 8 (Cell Signaling Technology, 9746) in 0.2% Tween-Blocking Buffer at 4°C overnight on the rocker. Membranes were rinsed with 0.2% TBST (3 times for 5 minutes each on rocker) before incubation with secondary antibody (1:5000, LiCor) in 0.2% Tween Blocking Buffer for an hour at room temperature on gentle rocker. The membranes were washed three times, each with TBST at room temperature for five minutes on gentle rocker. The membranes were washed for five minutes with 1X TBS on gentle rocker once to remove the Tween. The membranes were then placed between two pieces of thin filter paper to dry. The membranes were scanned in the F Odyssey (LiCor) to detect proteins.

Results

Protein was detected in all samples in the BCA assay. The protein concentration ranged

from 439.32 µg/mL – 1168 µg/mL. The gels were loaded with protein from controls and chlorpyrifos concentrations of 10 µM, 25 µM, and 50 µM. Each well received 9.88 µg of protein. After exposure to the antibodies, there was no appearance of protein bands on all the membranes.

Discussion

The results of the experiment did not support the hypothesis that there will be a dose- and time-dependent effect on the activation of caspase-8 from acute chlorpyrifos exposure in human neuroblastoma cells. There were no protein bands present on the membranes to make an accurate conclusion on the effect of chlorpyrifos on caspase-8. The concentration of protein loaded in each well was low (9.88 µg). It is suspected that decreasing the primary antibody dilution (1:10,000) at the final concentration at 0.2% Tween may put more primary antibodies on the membranes to bind to the proteins. Decreasing the secondary antibody concentration from 1:5,000 to 1:10,000 could prevent the level of background that was observed on the membranes. Another factor that may have contributed to the lack of protein bands could be if the membranes had expired. Membranes from a second container were used for second replicate of gel electrophoresis. Expired membranes would cause the proteins to not transfer from the gel onto the membrane. For future experimental usage, these factors could be modified in order to observe protein bands on the membranes.

Works Cited

Chang, D. W., Xing, Z., Capacio, V. L., Peter, M. E., Yang, X.

Interdimer processing mechanism of procaspase-8 activation. The EMBO Journal [Internet] 2003 [cited 2018 Feb 17]; 22: 4132-4142. Available from http://emboj.embopress.org/content/22/16/4132#page

Čolović, M. B., Krstić, D. Z., Lazarević-Pašti, T. D., Bondžić, A. M., Vasić, V. M. 2013.

Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. Current Neuropharmacology. 11 (3): 315-335.

Karen, D. J., Li, W., Harp, P. R., Gillette, J. S., Bloomquist, J. R. 2001.

Striatal Dopaminergic Pathways as a Target for the Insecticides Permethrin and Chlorpyrifos. Elsevier. 22: 811-817.

Raszewski, G., Lemieszek, M. K., Lukawski, K., Juszczak, M., Rzeski, W. 2015.

Chlorpyrifos and Cypermethrin Induce Apoptosis in Human Neuroblastoma Cell Line SH- SY5Y. Basic & Clinical Pharmacology & Toxicology. 116 (2): 158-167.

Tuite, P.

Parkinson's Disease. [Internet]. 2016 [cited 2018 Feb 17]. McGraw-Hill Education. Available from http://www.accessscience.com.relay.lagrange.edu/content/parkinson-s-disease/489900

Viswanath, V., Wu, Y., Boonplueang, R., Chen, S., Stevenson, F. F., Yantiri, F., Yang, L.,

Beal, M. F., Anderson, J. K.

Caspase-9 Activation Results in Downstream Caspase-8 Activation and Bid Cleavage in 1- Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Induced Parkinson's Disease. Journal of Neuroscience [Internet] 2001 Dec 15 [cited 2018 Feb 17]; 21 (24): 9519-9528. Available from http://www.jneurosci.org/content/21/24/9519