Inhibition of Acetylcholinesterase in the *Anaxyrus cognatus* Liver by Chlorpyrifos Oxon

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Abstract

This study investigated the effects of a commonly used organophosphate pesticide, chlorpyrifos, on the liver of the Great Plains toad, *Anaxyrus cognatus*. The activity of acetylcholinesterase in the liver of the toad was evaluated by the Ellman method using a plate reader, and its in vitro sensitivity to a range of concentrations of chlorpyrifos oxon, the active metabolite of chlorpyrifos. At chlorpyrifos oxon concentrations from 100 nM to 100 µM, concentration-dependent inhibition was noted (IC50 [concentration that inhibited 50% of the enzyme activity] = 1.8 µM). This concentration is about 100-fold higher than published for mammalian brain acetylcholinesterase inhibition; thus, the data suggest the toad may be resistant to acute toxicity of this pesticide. The findings suggest that organophosphate pesticides may have lesser acute toxicity potential in the Great Plains toad compared to mammals.

Keywords: Acetylcholinesterase, *Anaxyrus cognatus*, Chlorpyrifos oxon

Introduction

In today's world, we use pesticides all over for agricultural and domestic uses. Approximately 5 billion lbs. of pesticides were used in the years 2000 and 2001 in the United States alone (Kiely *et al*. 2004). The most common ways of spreading pesticides are by spraying, fumigating and baiting the target areas. The target species (usually insects, but also fungi and plant pathogens) take in these pesticides in different ways. 'Contact' pesticides must enter the organism through the external surface, whereas some other pesticides are systemic and are transferred from where they are applied to another place where they can reach their target, such as when a pesticide is applied to the leaves of a plant to exterminate the worms at its roots. One of the most common and most toxic pesticides used today is Organophosphate (OP) pesticide. OP pesticides make up 70% of the pesticides used in the US today. Although they are in the process of being phased out by the EPA in the United States, they are still used worldwide. According to Dana Boyd Barr, an exposure scientist from Emory University, OP have been developed into chemical warfare agents similar to nerve gases such as sarin (Than 2013).

OP pesticides are known to block the inhibition of Acetylcholine (ACh) by Acetylcholinesterase (AChE). This makes OPs significant, because the inhibition of ACh is vital to the nervous System, which plays an important role in muscle and neuron function. ACh is released by a neuron stimulating another neuron or cell. Overstimulation by ACh is associated with depression, while under-stimulation is associated with dementia. If AChE does not inhibit enough ACh, then ACh will build up. If ACh builds up in the synapses and neuromuscular junctions, it can lead to hyper-excitability, paralysis, tremors, muscle contractions, cardiac/respiratory distress, and death. (Blaustein and Wake 1995; Wake 1991)

Some AChE inhibitors have been used in acts of chemical warfare and terrorism, as well as a therapeutic agent (Woltjer and Milatovic 2006; Pope *et al*. 2005; Watson *et al*. 2006). A leading OP pesticide around the world, despite its decline in the US, is chlorpyrifos. (Mehta *et al*. 2008). OP pesticides bind to AChE in the nervous system preventing them from hydrolyzing Acetylcholine (ACh) in its corresponding synapses in which the enzyme is important (Ecobichon 1991, Kwong 2002). This causes a disturbance in the cholinergic signaling by creating a surplus of ACh, which causes signs of toxicity (Antunes-Madeira and Madeira 1979). The appearance of toxicity in non-target species that have been in areas where OPs exist, suggests that the pesticides are causing harm to them. Many researchers have even associated the pesticide applications with the decline of the of the amphibian population (Blaustein and Wake 1995; Wake 1991) There has been a wide variety of research done on

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pesticides looking for DNA damage, gene mutation, and chromosomal aberrations (Bolognesi 2003)

Amphibians are susceptible to OP pesticides for a variety of reasons, the first since tadpole skin is highly permeable for toxic substances. Studies show that the skin of tadpoles is more permeable to soluble solutions, such as OP pesticides (Dreamer *et al*. 1999). Frogs also lay eggs in spring, which is the primary time that agricultural society applies pesticides, because it is the best time of year to grow and when most pests come out. Exogenous compounds can interrupt the metamorphosis stage in their growth. This is dangerous, because this is the same time in which the liver is remodeled. When the tadpoles metamorphose to frogs, they change to urea excretion taking place in the liver and increasing the regulation of its enzyme (Brown and Cai 2007). There has been research conducted showing a relation between the decline in the amphibian population and the use of pesticides. (Lien *et al*. 1997; Harris *et al*. 1998; Gillilland *et al*. 2001; Karen *et al*. 2001; Sahu and Ghatak 2002). Contorted posture, abnormal notochord, and abnormal tail flexure in frog embryo have all been connected to exposure to OPs. These deformities were also observed in studies over the *Xenopus laevis* larvae when exposed to OPs (Snawder and Chambers 1989; Snawder and Chambers 1990; Snawder 1993; Richards and Kendall 2002; Richards and Kendall 2003; Bonfanti *et al*. 2004). Since these studies have been mostly on larvae and tadpoles, our research on adult toads could further expand the field.

Because of the negative effects that could possibly be caused by OPs towards the AChE inhibition, we will be looking at the *Anaxyrus cognatus*. *A. cognatus* is a great model to use as a non-target species affected by OP pesticides, because they commonly inhabit agricultural communities and other places where pesticides are commonly spread. The liver is likely being affected by CPO since it functions as a detoxifier for different compounds. The liver is crucial to the body, because it filters the toad's blood. It helps nutrients spread throughout the toad's body, as well as send unwanted things like toxins out of the body (Snawder and Chambers 1989; Snawder and Chambers 1990; Snawder 1993, Richards and Kendall 2002; Richards and Kendall 2003; Bonfanti *et al*. 2004). I would expect that toads would have a low tolerance to the CPO and that they would be negatively affected by it.

Methods

Throughout this experiment, we measured AChE activity in the toad liver. First, we collected liver tissues from the Great Plains toad (GPT15-5). We euthanized the toads by immersing them in buffered 0.5% Ms-222. We then collected and homogenized liver tissues 1:25 in PBS (purchased commercially), using a Polytron Homogenizer at 22,000 rpm for 20 seconds. This homogenate was stored at -70° C. To determine AChE activity, we used a modified Ellman's photometric assay. We diluted the homogenate further with PBS. In a 96-well plate, we added the homogenized tissue and the 76.2 mM Acetylthiolcholine Iodide. We used PBS as a plate blank in equal amount as the homogenate. To prepare the assay we first set the "control temperature" on the Spectramax software for the plate reader to 37° C. We then placed a 96-well plate on a piece of glass lying on top of ice to keep the tissues cold. The next step was to set up the wells and the plate reader. In the first eight wells, we added 25 µL PBS as no-enzyme plate blanks. This plate blank was to determine how much yellow was already in the PBS so that the program would not take that amount of yellow into consideration when evaluating the other plates. Then we put 25 µL of the homogenized tissue to each experimental well and ran each concentration of tissue in octuplet. We made a DTNB-Substrate Cocktail by first making 7.62 mM ACh. We then thawed one vial of 76.2 mM substrate and added it to 3.6 ml Tris-E in a 12×75 plastic culture tube, and capped it with parafilm, keeping it in ice. Then, in a 20 mL scintillation vial, we combined 14.15 mL Tris/E, 2 mL of 1.143 mM DTNB, and 2.85 ml of 7.62 mM substrate. We put this solution in the plastic reagent reservoir. Using the multi-channel micropipette, we added 175 µL of the DTNB-substrate cocktail to each well. We immediately placed the plate in the plate reader and press "Read."

We then run it through a photometric plate reader, reading at a wavelength of 412 nm for 15 min. This gave us the V_{max} results for standard curve and unknowns and compared the activity. We then were able to interpret the data by taking the mean V_{max} value and dividing by 70.75 (derived from Beer's Law) to give us nmole of substrate hydrolyzed by AChE per min (activity). Then, by dividing all of these by the control and multiplying by 100, we found the percent of AChE activity. We ran a wide range of toad liver homogenate concentrations, from 1:25 to 1:500, to generate a standard activity curve. That helped us characterize the toad's AChE activity. We found that the liver concentration at a 1:100 concentration had the most activity.

Once this was established, the next step was to determine how sensitive toad AChE was to inhibition by chlorpyrifos oxon. We characterized inhibition kinetics by doing the same activity assay,

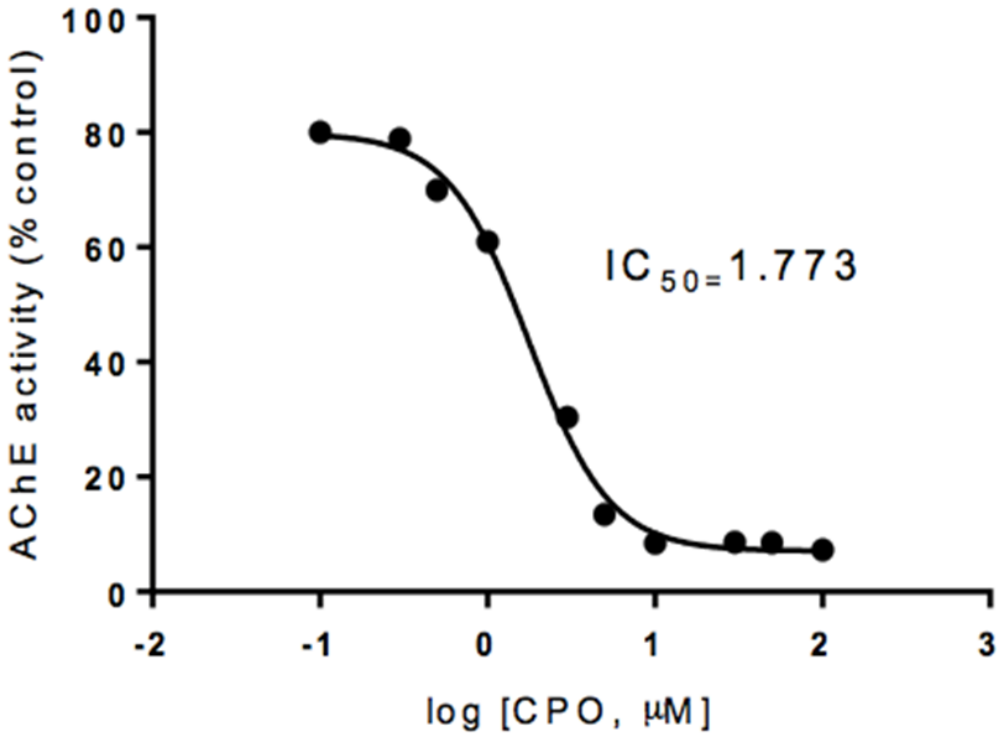

Figure 1 *- This figure models a standard activity curve showing the activity of AChE compared to the concentration of CPO. The figure also shows the IC50 found where the activity is 50%. (Illustration by Dominique Davis)*

but adding various concentrations of the chlorpyrifos oxon to the homogenate with a pre-incubation. To do this we mixed a range of chlorpyrifos oxon in ethanol concentrations, ranging from 100 nM to 100 µM, 1:1 with the 1:100 homogenate concentration (dependent on results of preliminary assay) in plastic culture tubes. These were then incubated for 20 mins at 37° C in a water bath. In increments of eight, 25 µL was then taken out of each tube and put in wells. Then we added 25 µL PBS as no-enzyme blank to 8 of the wells to serve as the plate blank. We mixed the same substrate as before and added 175 µL of it, using the micropipette, to every well. Then we placed the well plate in the plate reader and ran it as before.

This gave us an IC50, which indicates how much OPs are needed to inhibit the enzyme AChE. This also informed us of a rate of inhibition constant, which helped us determine how high Chlorpyrifos oxon's affinity for toad AChE is. The purpose of doing this experiment was to lead us to a better understanding of whether toads are more susceptible to OP pesticides, and what their potential risk of exposure is.

Results

When measuring the AChE activity of the Great Plains Toad, it has resulted that the toads are rather resistant to the CPO. At an extremely high concentration of 100 µM, there is less than 10% of enzyme activity, but also at a high concentration of 100 nM there is about 80% enzyme activity. This results in a toad IC50 of about 1.773 as seen in Figure 1.

Discussion

The data suggests that toads are resistant to the chlorpyrifos oxon. This could show that the reason that OP pesticides including chlorpyrifos oxon are so popular around the world is that they mainly affect their target species. Compared to the mouse, the resistance to chlorpyrifos oxon in the toad is rather high. With the strongest legal concentration of CPO spread, the toad still would have high levels of AChE activity in their liver. There is no realistic threat to the toads directly because of their strong tolerance.

This combats our hypothesis, but matches other research done in the field. In a study of the effects of CPO on the *X. laevis*, the "most sensitive

effect would not have a high probability (≤4.2%) of occurring in the environment" (Richards and Kendall 2002). This matches the data showing that toads are not likely affected in realistic situations. This may be because they are exposed to a habitat they are built to withstand. Still some studies show differently such as the study of the effects of CPO on *Duttaphrynus melanostictus* larve. In this study the effects were rather detrimental to the toads (Wijesinghe *et al*. 2011)

To make this study more applicable in society it would be useful to run this study many more times; this could turn out the most accurate data. In addition, testing this on others organs in the body of the toad could show different amounts of tolerance, possibly showing how the toad would be affected if not through the liver. Even running this experiment for an extended amount of time to possibly find that their tolerance increases or decreases over time would possibly bring about different results. You could take the idea from this research and test it on other non-target species such as some birds and reptiles to see if it applies. It would also be highly effective to look into how the toad's consumption of insects exposed to the CPO affects them. In that case, the concentration of CPO could be amplified higher than the legal amount and possibly reach a concentration that would actually negatively affect the toad.

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