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Induction of Drosophila melanogaster Cyp6a8 promoter by different xenobiotic compounds

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ABSTRACT

Research is a learning process, one that is ever changing and growing. The following paper highlights not only scientific findings, but also the progression of experimentation.

Background Story

One of the most important aspects of research and experimentation in a laboratory is trial and error. Oftentimes, no results provide just as much information as a positive result does. A negative result could cause the researcher to change a variable in the experiment or pursue a completely new direction. Either way, this leads to a progression of experimentation, enabling an eventual discovery or new idea.

I decided to pursue this area of research based on a previous experience working with *Drosophila melanogaster*. In the laboratory of Dr. Ranjan Ganguly, a large amount of research has been performed to discover the fact that caffeine increases activity of the promoter of Cyp6a8, a gene that codes for one of many cytochrome P450 monooxygenase enzymes or CYPs. CYPs comprise a superfamily of enzymes. Besides their normal metabolic functions, CYPs are also responsible for the metabolism of various xenobiotics (foreign compounds) that could be potentially harmful to the organism. By doing this, CYPs are an integral part of conferring metabolic resistance in insects to different insecticides and pesticides.

Several studies have shown that caffeine is responsible for the increased expression of several CYP genes. Since caffeine is a stimulus that causes increased gene expression, my goal was to discover if other xenobiotics would have the same effect on the Cyp6a8 promoter. The following paper shows not only the scientific experiments performed using different xenobiotics, but also indicates the progression and alteration of experimental procedures.

Induction and *Drosophila melanogaster* Cyp6a8 promoter by different xenobiotic compounds

1. Background and Objectives

Cytochrome P450 monooxygenases or CYPs form a family of enzymes that are found in almost all living organisms. CYPs are responsible for the metabolism of xenobiotics, foreign chemicals that an organism could be exposed to on a regular basis. These xenobiotics could range from drugs and carcinogens, to hormones and environmental pollutants (Maitra et al., 2002). Based on the studies by Maitra et al. (2002), there is a positive correlation between levels of CYP genes and insecticide resistance. There is the same relationship between CYP levels and tolerance of other toxic chemicals that an organism may come into contact with.

This relationship has been studied extensively in mammals and plants, but limited studies have been performed in insects. Studies by Maitra et al. (2002) and Bhaskara et al. (2006) have identified that caffeine is an inducer of the Cyp6a8 promoter and increases its activity, thereby increasing the transcription of the Cyp6a8 gene. The objective of this research described in this paper has been to test the effects of several different xenobiotic compounds on the expression of the Cyp6a8 gene. We hypothesize that the application of these chemicals and hormones will result in an increased activity of the Cyp6a8 promoter, thereby increasing the transcription of the Cyp6a8 gene.

2. Experimental Design

In each separate experiment, a different chemical will be used. The chemicals utilized are gibberellic acid, progesterone, and barbital. Batches of flies or larvae will be treated with each of these chemicals and other batches will be left untreated to serve as a control. After treatment, luciferase activity will be measured using a luciferase assay kit to determine if there is an increase in activity. In order to ensure that this activity is due to the chemical and not due to an increase in the amount of protein, protein content will be determined using a BCA assay kit. The specific activity of the luciferase enzyme in treated and untreated flies will then be compared.

2.1 Fly strain and fly culture

Genetically engineered transgenic fruit flies, *Drosophila melanogaster*, carrying the luciferase gene, which is responsible for bioluminescence in fireflies, were used for these experiments. Maitra et al. (2002) is responsible for creating this transgenic fruit fly strain, called 0.8luc/A8, for experimentation. The luciferase gene in these fruit flies is under the control of the 0.8-kb upstream promoter DNA of the Cyp6a8 gene. Flies were raised at room temperature on standard fruit fly medium made of corn meal, agar, yeast, molasses, and corn syrup.

2.2 *The effect of gibberellic acid on adult female flies and larvae*

Adult female flies from the 0.8luc/A8 strain were treated with 10 μM gibberellic acid (GA) for 24 hours. Water served as the control. Female flies were sorted and placed in a vial containing instant fly food saturated in either 10

µM GA or water. The vial was kept in a cool quiet environment for approximately 24 hours. After treatment, the flies were placed in microcentrifuge tubes in groups of 10 females per tube and processed for extract preparation. The same procedure was followed for the next experiment with an increased concentration of GA of 1 mM and increased treatment times of 24, 48, or 72 hours.

To treat the larvae, flies were allowed to lay eggs in a dish containing fly food for approximately 12 hours. Approximately 48 hours after egg laying, the larvae were transferred to food containing 1 mM gibberellic acid. Food made with water served as control. Following a 24-hour treatment, larvae were transferred to microcentrifuge tubes in groups of 15-20 larvae per tube. Extracts were prepared from these larvae for luciferase activity.

~~2.2~~2.3 *The effect of 1µg/mL progesterone on larvae*

Adult female flies of the 0.8luc/A8 strain were allowed to lay eggs for 3 hours on glass dishes containing normal fly food. The adults were then removed and the larvae were allowed to hatch and grow for 2 days. On the third day, the larvae were transferred to new dishes containing a treatment of 1µg/mL progesterone and a control of water. These larvae were treated for 24 hours. After treatment, groups of 15-20 larvae were placed in 3 microcentrifuge tubes and extracts were prepared. These extracts were examined using a luciferase assay.

~~2.3~~2.4 The effect of 8 mM barbital on larvae

Adult female flies of the 0.8luc/A8 strain were allowed to lay eggs overnight on glass dishes containing normal fly food. The adults were removed and the larvae were allowed to hatch and grow for 2 days. On the third day, the larvae were transferred to new dishes containing a treatment of 8 mM barbital and a control of water. The larvae were treated for 24 hours. After treatment, larvae were transferred to microcentrifuge tubes in groups of 10-15 larvae per tube. Extracts were prepared from these larvae and examined through a luciferase assay and a protein assay.

2.5 Preparation of fly or larval extract for luciferase assay.

To prepare extracts, 1X CCLR buffer was added to each microcentrifuge tube in which the adult flies or larvae were stored. The volume of buffer varied between 10 -20 µL per adult fly or larva. The adult flies or larvae were homogenized for approximately 30 seconds with a hand-held homogenizer and kept on ice. The homogenate was then centrifuged at 13000 RPM for 8 minutes in a microcentrifuge at 4^0C. The supernatant was carefully transferred to fresh tube and centrifuged again. About 50-60 µL of the supernatant was collected and stored in individual vials at -20^0C.

2.6 Luciferase assay

To assay luciferase activity, the extracts were first brought to room temperature. In a 1.5 mL Eppendorf tube, 25 µL of luciferase reagent and 5 µL of fly or larval extract was mixed and immediately placed into a luminometer. Relative light units (RLU) were measured for 15 seconds and recorded. For

comparison between samples, protein concentration of each extract was measured and the final results were expressed as RLU/ µg of protein after determining protein concentration.

2.7 Protein assay

For the protein assay, a BCA protein assay kit was used. Commercially available Bovine serum albumin (BSA) was used as standard. In individual glass test tubes, different amounts of BSA in 50 µL 0.5X CCLR were taken. For experimental samples, 5 µL of fly or larval extracts adjusted to a final volume of 50 µL with 0.5X CCLR were individually placed in glass test tubes. One mL of BCA reagent was added to all the test tubes and incubated at 37^0C for 30 min. The samples were cooled to room temperature and absorbance at 595 nm was measured using a spectrophotometer. Duplicate assays were done for the standards and experimental sample.

3. Results and Discussion

Several different combinations of variables were tested in these experiments, finally resulting in the idea that of the three xenobiotics tested, barbital is the most likely to have an inducing effect on the Cyp6a8 gene, while the other xenobiotics show little or no effect at all. The values obtained from the luciferase assays and BCA protein assays are shown in Table 1 and Table 2 for the larvae treated with 1 mM GA. Although the RLU results showed that GA treatment gives higher activity (Table 1), no significant difference was found between the treated and control after the results were normalized by protein concentration (Table 2; Figure 1).

Luciferase Assay of Larvae Treated with 1 mM GA			
Sample	**Treatment**	**RLU/5µL**	**Mean RLU/5µL**
1	H_2O	9,804,584	9,665,195
2	H_2O	8,827,624	
3	H_2O	10,363,378	
4	GA	11,298,434	12,812,814
5	GA	14,164,244	
6	GA	12,975,764	

Table 1. Luminometer readings (in RLU/s) from the Luciferase Assay of 0.8lucA8 larvae treated with 1 mM gibberellic acid and using water as a control. Each treatment was performed in triplicate and the mean RLU value was calculated from the readings of the three samples in each set.

BCA Protein Assay of Larvae Treated with 1 mM GA					
Sample	**Tube**	**Protein µg/mL**	**Mean µg/mL**	**RLU/µg**	**Average RLU/µg**
1	1	23.8	25.27	387993.035	527083.94
	2	26.74			
2	3	16.23	15.33	575839.791	
	4	14.43			
3	5	16.65	16.79	617419.005	
	6	16.92			
4	7	30.48	30.04	376112.983	559943.69
	8	29.6			
5	9	22.98	20.82	680482.537	
	10	18.65			
6	11	21.13	20.82	623235.543	
	12	20.51			

Table 2. Readings from the spectrophotometer (in µg/mL) as a result of the BCA Protein Assay performed on 0.8lucA8 larvae treated with 1 mM gibberellic acid and using water as a control. Each sample of the triplicate was run as a duplicate, resulting in 12 total samples. The mean µg/mL was calculated from the duplicate test for each sample. The resulting mean was used, along with the RLU values for each sample, to calculate the RLU/µg for each sample. The mean RLU/µg was calculated from the values of the three samples in each set.

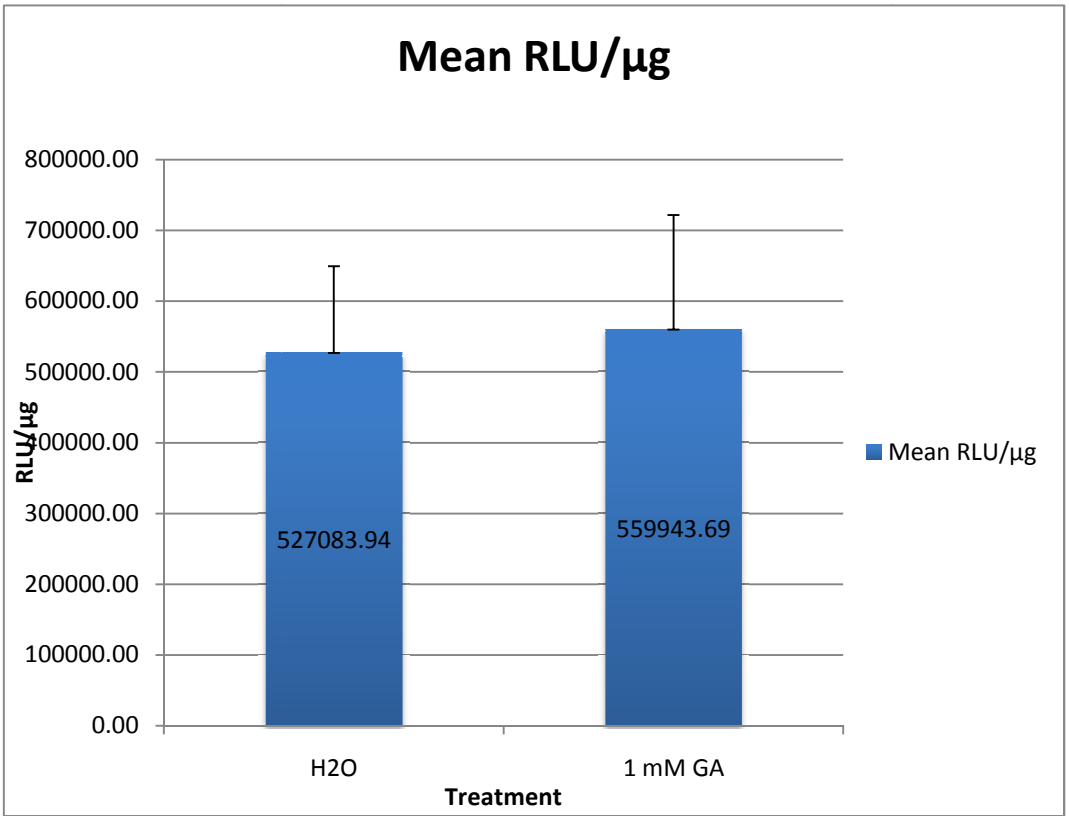

Figure 1. Mean RLU/µg measurements from water (control) and 1 mM GA treatment of 0.8lucA8 larvae.

Table 3 and Table 4 represent the values obtained from luciferase and BCA protein assay of larvae treated with 8 mM barbital. The RLU results show that barbital causes a significant increase in luciferase activity (Table 3). Also, when the test and control results were normalized with protein concentration, the difference remained and alludes to the fact that barbital could be an inducer of the Cyp6a8 gene. These results are graphically represented in Figure 6. (Note: Some values in Table 4 are represented by a "-". These values were not readable by the spectrophotometer because their absorbance value was less than the absorbance of the standard.

Luciferase Assay of Larvae Treated with 8 mM Barbital			
Sample	**Treatment**	**RLU/5µL**	**Mean RLU/5µL**
1	H_2O	509,008	465,210
2	H_2O	449,961	
3	H_2O	436,660	
4	Barbital	8,054,285	7,888,380
5	Barbital	9,085,210	
6	Barbital	6,525,644	

Table 3. Luminometer readings (in RLU/s) from the Luciferase Assay of 0.8lucA8 larvae treated with 8 mM barbital and using water as a control. Each treatment was performed in triplicate and the mean RLU value was calculated from the readings of the three samples in each set.

BCA Protein Assay of Larvae Treated with 8 mM Barbital					
Sample	**Tube**	**Protein µg/mL**	**Mean µg/mL**	**RLU/µg**	**Average RLU/µg**
1	1	2.247	2.247	226527.81	331118.09
	2	-			
2	3	1.241	1.051	428330.32	
	4	0.86			
3	5	1.29	1.29	338496.12	
	6	-			
4	7	0.813	0.813	9906869.62	11042717.65
	8	-			
5	9	0.746	0.746	12178565.68	
	10	-			
6	11	-	-	-	
	12	-			

Table 4. Readings from the spectrophotometer (in µg/mL) as a result of the BCA Protein Assay performed on 0.8lucA8 larvae treated with 8 mM barbital and using water as a control. Each sample of the triplicate was run as a duplicate, resulting in 12 total samples. The mean µg/mL was calculated from the duplicate test for each sample. The resulting mean was used, along with the RLU values for each sample, to calculate the RLU/µg for each sample. The mean RLU/µg was calculated from the values of the three samples in each set.

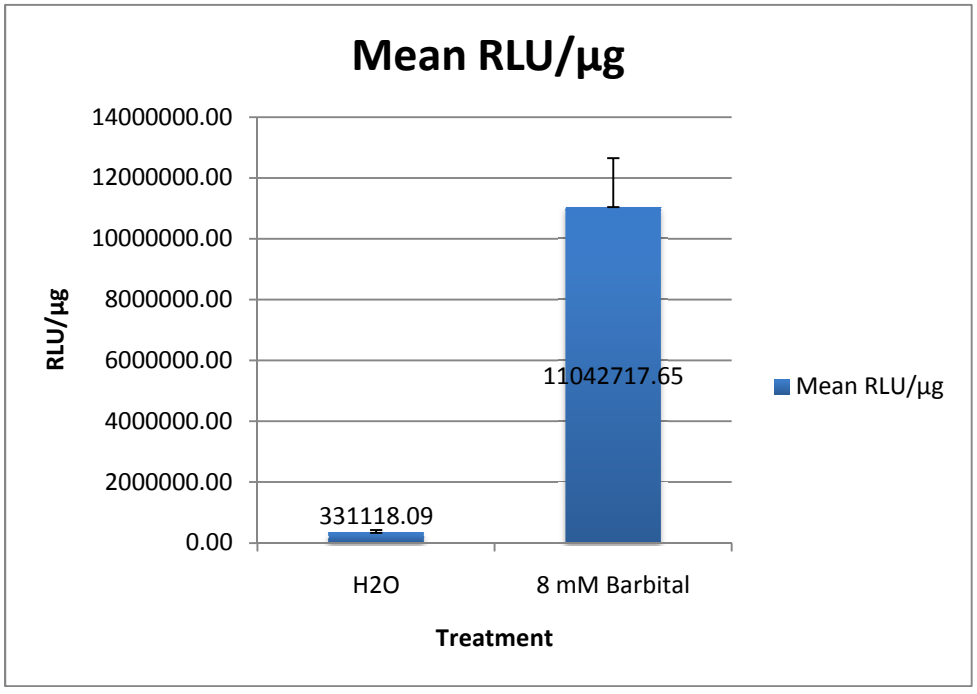

Figure 2. Mean RLU/µg measurements from water (control) and 8 mM barbital treatment of 0.8lucA8 larvae.

The data collected in the previous experiments showed that there was a slight increase in gene activity as a result of treatment with 1 mM GA. This suggests that gibberellic acid could potentially be a xenobiotic that induces the expression of the Cyp6a8 gene in *D. melanogaster*. However, as seen in the graph (Figure 3), the effect is minimal while comparing the mean RLU/µg values.

On the other hand, there seems to be a considerable increase in Cyp6a8 gene activity as a result of treatment with 8 mM barbital. This is seen not only through the large increase in luciferase activity, but also when comparing the difference in the graph (Figure 6). There is approximately a three-fold increase in the mean RLU/µg values between the control and barbital.

While the results look promising for barbital, there is also a potential for gibberellic acid as an inducer of the Cyp6a8 gene. More extensive experiments and tests would need to be pursued in order to pinpoint these xenobiotics and their precise effects on the Cyp6a8 promoter in *Drosophila melanogaster*.

Implications

Through these experiments, I learned the value of trial and error. Not everything in life can be achieved through trial and error, but the beauty of this research is that, you can always order more supplies or equipment, and there are more than enough fruit flies to go around if you mess up a few times.

Another benefit that I came across while participating in research is the idea that it is ok for an experiment to fail. If the results you get are negative or inconclusive, that is sometimes just as good as a positive result. The negative results are those that lead you to the next discovery. For example, the initial concentration of gibberellic acid had little effect on the flies, so the concentration was increased. Also, the length of treatment was increased to see if that would increase the effectiveness of the xenobiotic. When all concentrations of GA had little effect on adult flies, I performed the same experiment on the larvae because they hatch and grow in the treated food along with eating it. This doubles the chance of the xenobiotic having an effect on gene activity since the food and xenobiotic can be digested or absorbed through the "skin." This produced slightly positive, though inconclusive results, so I switched to another xenobiotic, progesterone. When that had barely any effect, I tried barbital, and to great success!

In conclusion, learning the inner workings of a laboratory and the important research that takes place there was a fascinating and enlightening learning experience. I not only learned how to perform experiments but also enhanced my appreciation of dedication and determination. If something did not succeed, I tried and tried again, in hopes of someday achieving my goal. This is a lesson that is

applied not only in the lab, but also in the pursuance of my professional goals. I never would have believed that I could learn so much from fruit flies.

References

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