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Tea Induction of Cyp6a8 Promoter in Drosophila melanogaster

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Abstract

Cytochrome P450 monooxygenases (CYPs) are a superfamily of enzymes found in all living organisms that are vital in many biosynthesis pathways as well as metabolizing foreign compounds. In *Drosophila melanogaster*, CYPs have been shown to be involved in development of insecticide resistance. Insecticide resistant strains have been found to have overexpression of certain CYPs. For example, in *Drosophila*, resistant strains have higher levels of expression of the *Cyp6a8* gene. Studying the expression of specific CYPs in *Drosophila* can help shed light on how these mechanisms of resistance work. Previous studies have shown that caffeine is an inducer of the *Cyp6a8* promoter in *Drosophila.* In this investigation, I examined the effects of black tea and herbal tea on *Cyp6a8* promoter activity. To do this, the *0.8 luc 110 (H-ry)* transgenic reporter strain carrying firefly *luciferase* reporter gene under the control of *Cyp6a8* promoter was used. Female flies were allowed to feed on food containing black tea or herbal tea for 72 hours, after which fly extracts were prepared and luciferase activity was measured. The results showed that black tea caused a 2.2- to 3.6-fold induction of the *Cyp6a8* promoter, while the herbal tea did not show any induction compared to the control group. These results indicate that the ingredients within the caffeine-free herbal tea (chamomile, hibiscus, peppermint leaves, rose blossoms, spearmint leaves, spice, and orange blossoms) do not induce expression of *Cyp6a8*. The caffeine present in the black tea was likely the cause of induction in black tea fed flies.

Introduction

Cytochrome P450 monooxygenases are a superfamily of hemoprotein enzymes that are present in all living organisms. The name P450 comes from the observations that these enzymes show peak absorbance at 450 nm when their reduced form is combined with carbon monoxide (Scott 2001). The number of CYPs found in each species varies from as little as one to over one hundred. These enzymes are used in many biosynthetic pathways for synthesis of prostaglandins, plant pigments, steroid hormones, and much more. However, CYPs are most widely known for their important role in the detoxification and metabolism of many foreign toxins and drugs (xenobiotics) that organisms are exposed to on a regular basis (Bhaskara 2006). In eukaryotes, most of these enzymes are typically found within the endoplasmic reticulum. The overall metabolic reaction that CYPs catalyze is as follows, with RH being the substrate:

$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$ (Scott 2001).

CYPs are named based on amino acid sequence similarities; all enzymes in this superfamily are given the CYP prefix, which is followed by a number, indicating the family (>40% amino acids identical). After this is a letter, indicating the subfamily (>55% amino acids identical), and all enzymes within a subfamily are also given a number (Scott 2001).

In insects, CYPs have been found to detoxify various insecticides and make the insects resistant to different types of insecticides, e.g. pyrethroids, DDT, permethrin, deltamethrin, and neonicotinoids (Feyereisen 2012). It has been observed in many species of insects that the insecticide resistant strains have overexpression of one or more CYP genes. This has been found in *Drosophila, Tribolium castaneum*, *Myzus persicae*, *Anopheles,* and many other insects (Feyereisen 2012). However, the mechanisms of insecticide resistance and overexpression of multiple CYP genes in resistant insects are not fully understood. To better understand these mechanisms, *Drosophila melanogaster* has been used as a model insect.

Drosophila strains resistant to DDT (dichlorodiphenyltrichloroethane) are known to show much higher expression of multiple CYP genes, including *Cyp6a8* and *Cyp6a2*, as compared to susceptible strains (Bhaskara 2006). Researching the mechanisms of resistance in insects (i.e. CYP enzyme regulation) could open up possibilities for manipulating these systems in order to restore insecticide susceptibility over time (Hemingway 2000).

To explore how regulation of CYPs works, different xenobiotic compounds, such as caffeine, insecticides, and phenobarbital, have been used. Caffeine is a xenobiotic compound that most people are exposed to on a daily basis because it is found naturally in many plants used for food and drink, such as berries, coffee and cocoa beans, and tea leaves (Bhaskara 2006). Using a transgenic luciferase reporter gene assay system, Bhaskara et al. (2006) demonstrated that the promoter of the *Cyp6a8* gene of *Drosophila* is significantly induced by caffeine. In the present investigation, I planned to examine how a common beverage, tea, can affect the promoter of the *Cyp6a8* gene. For this purpose I have used the same transgenic luciferase reporter strain (*0.8 luc 110 (H-ry))* used by Bhaskara et al (2006).

Materials and Methods

Fly Strains and Culture Conditions

The strain of *Drosophila melanogaster* used in this experiment was *0.8 luc 110 (H-ry)*, which was available in the laboratory. This transgenic strain contains a transgene that has the firefly luciferase reporter under the control of 0.8-kb (-11/-761) upstream promoter DNA of the *Cyp6a8* gene. This strain is homozygous for the transgene and the chromosomes of the ry^{506} host strain (Bhaskara 2006). Since the *luciferase* or *luc* reporter gene is under the control of the *Cyp6a8* gene promoter, luciferase enzyme activity in the fly extract reflects the activity of the *Cyp6a8* promoter.

Flies were cultured at about 23°C. The medium used in the fly cultures was made using the following ingredients: 2 g agar, 7.5 g dextrose, 2.5 g sucrose, 16.5 g cornmeal, 4.5 g yeast flakes, 6 mL Tegosept, and 250 mL water. Ingredients were combined in a 500 mL Erlenmeyer flask, heated in a microwave until boiling, stirred, allowed to heat another one to two minutes with occasional stirring, and then distributed into culture vials.

Treatment of *0.8 luc 110 (H-ry)* Strain with Different Types of Tea

For the tea treatment, black tea (Bigelow English Teatime ®) and herbal tea (Bigelow Sweet Dreams ®) were used. The only ingredient listed for the black tea is black tea leaves, and it contains 30-60 mg of caffeine per tea bag ("English Teatime" 2014). The herbal tea ingredients are chamomile, hibiscus, peppermint leaves, rose blossoms, spearmint leaves, spice, and orange blossoms, and it contains 0 mg of caffeine per tea bag ("Sweet Dreams Herbal Tea" 2014).

The teas were both brewed under the same conditions. Water (100 mL) was brought to boiling in the microwave, and then one tea bag was allowed to brew in the hot water for

two and a half minutes. The medium for fly treatment was instant *Drosophila* medium from Carolina Biological Supply Company, and the teas were added to the food by pipetting approximately 4-5 mL of tea onto the dry food. Control vials were also prepared using water instead of tea. Approximately 20 adult female *0.8 luc 110 (H-ry)* flies were placed in each vial: one containing black tea, one with herbal tea, and one with water. They were allowed to feed in the dark for approximately 72 hours before being removed for extract preparation.

Extract Preparation

After the flies were allowed to feed on the tea or water containing food for 72 hours, they were etherized and sorted into individual 1.5 mL Eppendorf tubes as groups of three flies. To each Eppendorf tube, 120 μL of 1X Cell Culture Lysis Reagent (CCLR, Promega) was added, and the flies were homogenized thoroughly with a hand-held homogenizer. The homogenates were then centrifuged at 13,000 RPM for 8 minutes at room temperature. After centrifugation, 100 μL of the clear supernatant from each tube was removed and placed into new 1.5 mL Eppendorf tubes, leaving behind the cell debris and lipid layer to be discarded. The extracts were then centrifuged again at 13,000 RPM for 8 minutes at room temperature. Then 50 μL was removed from each of the extracts, placed in new 1.5 mL Eppendorf tubes, and stored at -20°C until they could be used for luciferase and protein assays.

Luciferase Assay

Firefly Luciferase Assay Reagent (LAR) from Promega Corporation was used to measure luciferase activity of all fly extracts. LAR was stored in 1.5 mL Eppendorf tubes at -80°C until used. The luciferase assay was performed under defused light. Fly extracts and LAR were taken out of the freezer, thawed, and kept on ice. To assay the luc activity in each extract, 25 μL of LAR was rapidly mixed with 5 μL of the fly extract in the bottom of an

Eppendorf tube. The tube was closed and quickly placed in the well of a luminometer (Zylux) for 15 seconds. The relative light units or RLU displayed by the machine was recorded, and the procedure was repeated until all extracts were assayed.

BCA Protein Assay

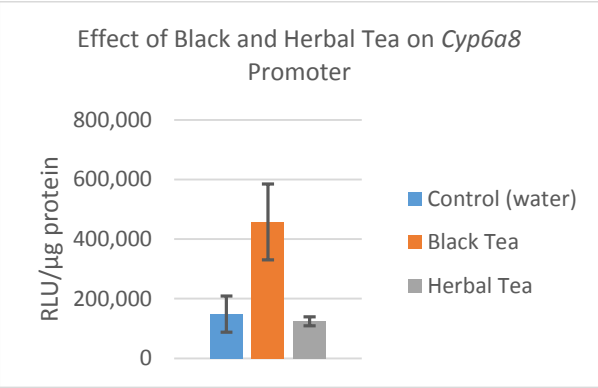
The Pierce ® BCA Protein Assay kit was used to determine the amount of protein present in the fly extracts that had been used in the luciferase assay. A 2 mg/mL stock Bovine Serum Albumin (BSA, Thermo Scientific) was diluted with equal volume of 1X CCLR buffer to give it a concentration of 1 mg/mL in 0.5X CCLR. This was used in making the protein standards for the assay. To make these standards (done in duplicates), ten glass test tubes were used, with the following concentrations of protein: 0 µL BSA with 50 µL 0.5X CCLR, 12.5 µL BSA with 37.5 0.5X CCLR, 37.5 µL BSA with 12.5 µL 0.5X CCLR, and 50 µL BSA with 0 µL 0.5X CCLR. To assay the fly extracts, 5 µL of each extract was added to 45 µL 0.5X CCLR in a glass test tube. For each extract this was done in duplicate. After standard BSA and fly extract tubes were prepared, 1 mL of BCA Reagent was added to all the test tubes (including the standards). This reagent was prepared by mixing BCA Protein Assay Reagent A with BCA Protein Assay Reagent B in a 50:1 ratio. After adding the BCA Reagent to all the samples, they were placed in an incubator at 37°C for 30 minutes for color development. A BioMate 3 spectrophotometer was then used to measure the absorbance of the standards and extract samples at 595 nm. After the determination of the protein concentration, data for the extracts were converted to RLU/µg protein.

Results and Discussion

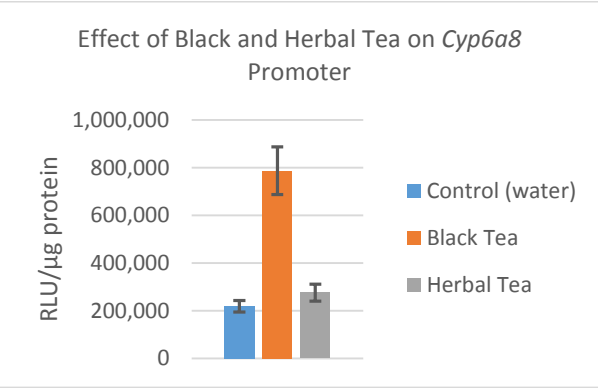
To investigate the effects of different teas on *Cyp6a8* promoter activity, adult females of *0.8 luc 110 (H-ry)* strain were allowed to feed for 72 hours on food prepared in black tea or herbal tea. The black tea contained 30-60 mg of caffeine per tea bag, and caffeine has been shown to be an inducer of this gene in previous studies (Bhaskara 2006). The herbal tea claimed to be caffeine-free but contained chamomile, hibiscus, peppermint leaves, rose blossoms, spearmint leaves, spice, and orange blossoms. Therefore, this experiment could help determine if any of these ingredients would have any effect on *Cyp6a8* promoter activity. After 72 hours of feeding on black or herbal tea containing food, fly extracts were prepared to determine luciferase activity and protein concentration.

All three trials of this experiment had similar results, with black tea causing significant induction and herbal tea more or less having the same effect as the water (control) on the expression of *Cyp6a8* (Figure 1). The average fold induction by black tea ranged from 2.2 to 3.6, whereas average fold induction by herbal tea was 0.7 to 1.3 (Table 1). These results indicate that the black tea had a significant effect on the increased expression of *Cyp6a8*, most likely explained by the presence of caffeine, a known inducer of *Cyp6a8*. The herbal tea results indicate that the claim for this herbal tea being caffeine-free is likely correct considering no induction was observed. Also, the ingredients of the herbal tea can be ruled out as inducers of the *Cyp6a8* gene.

Experiment 1



Experiment 2



Experiment 3

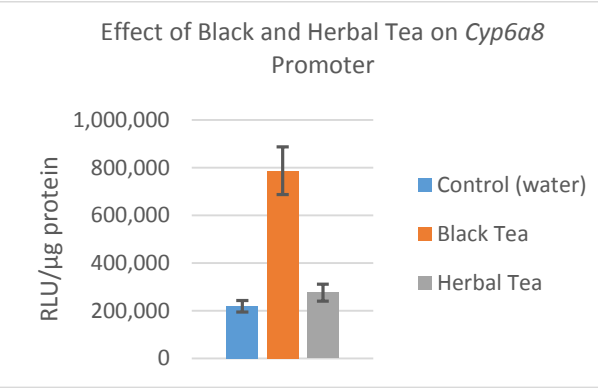


Figure 1. Flies were treated for 72 hours as described in methods. Extracts prepared using 1X CCLR buffer, and luciferase activity measured by using luciferase assay reagent (LAR) by Promega. Protein concentration in the extracts was used to normalize the luciferase activity. Each bar represents average of triplicate determination.

Table 1. Results of *Cyp6a8* induction in female *0.8 luc 110 (H-ry) Drosophila* exposed to black tea, herbal tea, and water for 72 hours. Average RLU/µg protein calculated from data collected from luciferase assay and BCA protein assay.

	RLU/µg protein*			**Fold Induction**	
Experiment	**Water**	**Herbal tea**	**Black tea**	**Herbal Tea**	**Black Tea**
1	148,623	123,977	458,161	0.8	3.1
2	285,296	190,950	620,806	0.7	2.2
3	218,155	275,383	787,183	1.3	3.6

*Mean of triplicate determination

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