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Genome-wide analysis of phenobarbital-inducible genes in *Drosophila melanogaster*

W. Sun*†, V. M. Margam*, L. Sun*, G. Buczkowski*†, G. W. Bennett*†, B. Schemerhorn*§, W. M. Muir‡ and B. R. Pittendrigh*

**Department of Entomology, Room 100, 1158 Smith Hall, Purdue University, West Lafayette, IN, USA; †Center for Urban and Industrial Pest Management – Department of Entomology, Purdue University, West Lafayette, IN, USA; ‡Department of Animal Sciences, Lilly Hall, Purdue University, West Lafayette, IN, USA; §USDA-ARS, Department of Entomology, Smith Hall, Purdue University, 901 W. State Street, West Lafayette, IN, USA*

Abstract

An oligoarray analysis was conducted to determine the differential expression of genes due to phenobarbital exposure in *Drosophila melanogaster* (w^{1118} strain) third instar larvae. Seventeen genes were observed to be induced with increased expression by a statistical analysis of microarrays approach with a $q \leq 0.05$. At $q \leq 0.12$, four more genes (*Cyp12d1, DmGstd4*, and two genes with unknown function) were found to be up-regulated, and 11 genes with unknown function were found to be down-regulated. Fifteen of these genes, *Cyp4d14, Cyp6a2*, *Cyp6a8*, *Cyp12d1*, *Cyp6d5*, *Cyp6w1*, CG2065, *DmGstd6, DmGstd7*, Amy-p/Amy-d, *Ugt86Dd*, GC5724, Jheh1, Jheh2 and CG11893, were verified using quantitative real time polymerase chain reaction. Some of these genes have been shown to be over-transcribed in metabolically DDT-resistant *Drosophila* strains.

Keywords: P450, GST, carbohydrate metabolism, oxido-reductase.

Introduction

Phenobarbital (PB) treatment has a variety of effects on mammals: (1) reduces frequency of epileptic seizures (Baumann, 2001; Hernandez *et al*., 2002; Kwan & Brodie, 2004; Zhong *et al*., 2004); (2) induces resistance/tolerance to insecticides in rodents, cows and humans (Fries *et al*., 1971; Zavon, 1971; Vitarius *et al*., 1995); and (3) causes liver cancer (Henry, 1997; Ferko *et al*., 2003; Calvisi *et al*., 2004; Hokaiwado *et al*., 2004). PB induction of P450s has also been demonstrated across insect species, including *Drosophila hydei* (Danielson *et al*., 1997), *Helicoverpa zea* (Li *et al*., 2002), *Blatella germanica* (Brown *et al*., 2003), *D. melanogaster* (Amichot *et al*., 1994), *Aedes aegypti* (Sousa-Polezzi & Bicudo, 2004) and across multiple P450 families (Danielson *et al*., 1998). In insects, the best-studied P450 response element for plant allelochemicals has been the xenobiotic response element to xanthotoxin (XRE-xan) (Petersen *et al*., 2003; McDonnell *et al*., 2004) and this element has been identified as an important component in the induction of the *Cyp6b1v3* gene in the black swallowtail butterfly (*Papilio polyxenes*).

In *Drosophila*, treatment with PB induces multiple cytochrome P450 genes, including *Cyp6a2* and *Cyp6a8* (Amichot *et al*., 1994; Dunkov *et al*., 1997; Maitra *et al*., 2000). PB causes a 15-fold increase in expression of *Cyp6a2* in the DDT-susceptible strain Canton-S (Brun *et al*., 1996). Numerous DDT laboratory-selected (e.g. 91R) and field-collected DDT-resistant strains (e.g. Hikone-R and Wisconsin) have been shown to constitutively over-transcribe several P450s, including *Cyp6a2* and *Cyp6a8* (Brun *et al*., 1996; Pedra *et al*., 2004). PB treatment of normally pesticide-susceptible (Canton-S) *Drosophila* also (1) induces increases in cytochrome P450 enzymatic activity, and (2) causes these flies to become more resistant to pesticides such as DDT and certain pyrethroids (Amichot *et al*., 1994, 1998). Thus, evidence exists that PB treatment of *Drosophila* results in increased expression of multiple P450 genes, some of which may be associated with metabolic pesticide resistance.

In house-fly (*Musca domestica*), PB has been shown to induce expression of several P450s. While using a cDNA expression library from PB-induced house-fly, Feyereisen *et al.* (1989) isolated a P450 gene with similarity to mammalian P450III family of proteins. PB increased the expression of Cyp6d3 in both insecticide resistant and susceptible strains by 16-fold and 1.6-fold, respectively (Kasai & Scott, 2001). PB treatment increased total P450 proteins in the susceptible house-fly adult and larvae (Scott *et al*., 1996). There was a 4.2-fold increase in *Cyp6d1* mRNA expression

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Received 14 October 2005; accepted after revision 13 March 2006. Correspondence: Barry R. Pittendrigh, Department of Entomology, Purdue University, 1158 Smith Hall, West Lafayette, IN 47907-1158, USA. Tel.: 765 494 7730; fax: 765 494 0535; e-mail: pittendr@purdue.edu

in susceptible strain treated with PB (Scott *et al*., 1996). A *trans*-acting factor of PB inducible element has been hypothesized to exist for *Cyp6d1* gene (Liu & Scott, 1997*b*). These results are in keeping with the hypothesis that PB induction and P450 mediated metabolic resistance to insecticides share some common regulatory pathways.

Metabolic resistance is a common mechanism by which insects, including lepidopteran, coleopteran and dipteran species, develop resistance to insecticides (Hemingway *et al*., 1991; Ottea *et al*., 1995; Rose *et al*., 1997; Chandre *et al*., 1998; Kasai *et al*., 1998; Stuart *et al*., 1998; Feyereisen, 1999; Scott, 1999; Kasai *et al*., 2000; Li *et al*., 2000; Scharf *et al*., 2000). Additionally, metabolic pesticide resistance in insects is (1) typically polygenic, and (2) associated with over-transcription of detoxification enzymes, including cytochrome P450s and GSTs (Houpt *et al*., 1988; Daly & Fisk, 1992; Heckel *et al*., 1998; Scott, 1999; Maitra *et al*., 2000; Tang *et al*., 2000; Kranthi *et al*., 2001; Rajurkar *et al*., 2003).

The cloning of numerous P450 and GST genes that are over-transcribed in resistant insects, but which fail to map back to a major resistance locus, has led workers in the field to hypothesize that there is a PB-sensitive *trans*-regulatory protein that, in the absence of PB, represses expression of these detoxification enzymes (Plapp, 1984; Terriere, 1984; Grant & Hammock, 1992; Liu & Scott, 1997*b*; Dombrowski *et al*., 1998; Scott & Kasai, 2004). It has also been hypothesized that mutations, in the genes that code for these regulatory proteins, reduce the regulatory proteins' ability to suppress transcription of the P450 genes, resulting in insects that constitutively over-transcribe these cytochrome P450s (Plapp, 1984; Terriere, 1984; Grant & Hammock, 1992; Carino *et al*., 1994; Maitra *et al*., 1996; Liu & Scott, 1997*a*; Dombrowski *et al*., 1998; Kasai *et al*., 1998; Scott & Kasai, 2004). To date, no such *trans*-acting PB-sensitive repressor has been identified in insects. Additionally, in bacterial systems this repressor-mechanism hypothesis of PB induction has been challenged (Shaw *et al*., 1998). Regardless of the exact mechanism of regulation, strong evidence suggests a relationship between constitutive over-transcription of normally PB inducible gene(s) and metabolic resistance to pesticide. Although we know the complement of array of genes over-transcribe in some DDT-resistant *Drosophila* strains (Pedra *et al*., 2004), we do not know the complement of genes in *Drosophila* that are induced in response to PB.

In the current study we used the Affymetrix *Drosophila* Genome 2.0 Arrays to investigate differential expression of genes in PB-untreated and PB-treated w^{1118} third instar *Drosophila*. This allowed us to define if there is an overlap between the array of the genes constitutively over-transcribed in metabolic DDT-resistant strains and PB inducible genes in a DDT-susceptible strain. We also used quantitative real-time PCR to verify the differential transcription of a set of these candidate genes. Our transcriptome approach identified a manageable number of genes to investigate further the molecular response of *Drosophila* to PB treatment. Here we show that PB induces genes associated with detoxification, development, and carbohydrate metabolism.

Results

The statistical analysis of microarrays (SAM) of the oligoarrays data revealed 17 putative differentially expressed genes between the PB and control treatments at the 95% level ($q \leq 0.05$) (Table 1). All 17 genes were over-transcribed in the PB treatment, of which 15 genes have known functions. When the false discovery rate (FDR) was relaxed to 88% ($q \leq 0.12$) five additional putative genes were identified as being over-transcribed and 11 genes as under-transcribed (Table 1). The over-transcribed genes included those coding for cytochrome P450s, oxido-reductase, glutathione S-transferases, steroid metabolism, calcium ion binding, α-amylase, juvenile hormone epoxide hydrolases, and genes that code for proteins with unknown function. Interestingly, all the genes observed to be under-transcribed (at the $q \leq 0.12$ level) in the PB-treated flies have currently unknown functions (Table 1).

We performed quantitative real-time PCR (qRT–PCR) on 17 of the genes described in Table 1. Fifteen of the 17 genes were confirmed to be differentially transcribed in the PB treatment (Table 2, Fig. 1a,b). From each of the following categories, we verified one or more genes to be over-transcribed in the presence of PB: cytochrome P450s, oxido-reductase, glutathione S-transferases, steroid metabolism, calcium ion binding, α-amylase, juvenile hormone epoxide hydrolases, and a gene that codes for a protein with unknown function.

Even though *Cyp6g1* was not identified in the initial oligoarrays experiments, at the FDR level of $q \leq 0.12$, we elected to determine if PB treatment of *Drosophila* resulted in differential transcription of *Cyp6g1*, as this gene has been implicated as one of the two P450s associated with a major metabolic pesticide resistance locus (loci) in *Drosophila*, known as *Rst(2)DDT* (Tang *et al*., 2000; Brandt *et al*., 2002; Daborn *et al*., 2002; Pedra *et al*., 2004, 2005; Festucci-Buselli *et al*., 2005). Using qRT–PCR we observed a 5.82-fold increase in transcription of *Cyp6g1* in third instars treated with PB as compared with the control (Table 2). These data suggest that PB does induce *Cyp6g1*, but not at levels equivalent to other genes investigated in this study.

We also investigated the impact of PB on expression of several P450 genes, *Cyp12d1*, *Cyp6a2*, and *Cyp6g1*, at the adult stage of development with qRT–PCR (Fig. 2). Both *Cyp12d1* and *Cyp6a2* were highly over-transcribed in response to PB treatment at the adult stage, whereas *Cyp6g1* was only 3.6-fold over-transcribed (Fig. 2).

Table 1. Genes that were differentially expressed in the oligoarray experiments at the $q = 95\%$ or $q = 88\%$ levels in *Drosophila* after a 24 h exposure to 0.1% phenobarbital in the diet of third-instar larvae as compared with larvae reared on control diet

Gene category/name	Probe Id	Ratio†	*q*-value	Cytogenetic map
Monooxygenase activity (GO:0004497)				
Cyp4d14	1627180_at	11.31	0.05	2E1
Cyp6w1	1634143_at	89.07	0.05	42A13
Cyp6a2	1626401_at	39.60	0.05	47D4
Cyp6a8	1640755_at	79.28	0.05	51D1
Cyp6d5	1638562_a_at	20.44	0.05	88A4
Cyp12d1	1633401_s_at	32.67	0.12	47D4
Oxidoreductase activity (GO:0016491)				
CG2065	1630212_at	27.11	0.05	43E9
Cytochrome b5-related	1632623_at	9.20	0.05	36B1
Glutatione S Transferases (GO:0004364)				
DmGstd6	1626136_at	57.06	0.05	87B8
DmGstd7	1624793_at	27.90	0.05	87B8
DmGstd9	1636174_at	9.10	0.05	87B8
DmGstd4	1626253_at	15.71	0.12	87B8
Steroid metabolism (GO:0008202)				
Ugt86Dd	1641481_at	14.43	0.05	86D5
CG5724	1638903_at	17.17	0.05	87C8
Alpha-amylase activity (GO:0004556)				
Amy-p/Amy-d	1639374_s_at	35.76	0.05	53F12
Alpha-glucosidase activity				
CG8693	1631806_at	12.79	0.12	44D1
Epoxide hydrolase activity (GO:0004301)				
Juvenile hormone epoxide hydrolase 1 (Jheh1)	1626414_at	15.00	0.05	55F7
Juvenile hormone epoxide hydrolase 2 (Jheh2)	1627773_a_at	10.60	0.05	55F8
Nucleotide phosphatase activity (GO:0019204)				
CG5150	1637660_at	6.68	0.12	64D5
Genes that code for proteins with unknown function				
CG11893	1635512_at	6.62	0.05	
X01472	1626392_s_at	6.62	0.05	
CG6908	1622946_at	15.50	0.12	
CG30043	1624823_at	0.09	0.12	
CG10513	1623265_at	0.08	0.12	
CG10514	1638424_at	0.10	0.12	
CG11892	1624892_s_at	0.14	0.12	
CG31745	1638743_at	0.14	0.12	
CG15919	1637672_at	0.18	0.12	
CG14129	1623779_at	0.15	0.12	
1624823_at	1624823_at	0.09	0.12	
1638347_at	1638347_at	0.14	0.12	
1635966_at	1635966_at	0.10	0.12	
1630583_at	1630583_at	0.13	0.12	

†Ratio of >1 indicates upregulation of mRNA in the presence of PB; and a ratio of <1 indicates downregulation of mRNA in the presence of PB.

Discussion

Interestingly, some of the genes observed to be over-transcribed (in the PB treatment) in the top 88% ($q \leq 0.12$), and hence inclusive of the 95% ($q \leq 0.05$) probability level using SAM, have previously been implicated in metabolic pesticide resistance both in *Drosophila* and other insects (Houpt *et al*., 1988; Daly & Fisk, 1992; Liu & Scott, 1996; Heckel *et al*., 1998; Tang *et al*., 2000; Kranthi *et al*., 2001; Rajurkar *et al*., 2003). These included cytochrome P450s, cytochrome b5-related, and glutathione S-transferases. Additionally, we observed differential expression of genes coding for proteins involved in epoxide hydrolase activity, carbohydrate/lipid steroid metabolism, along with a series of genes with a currently unknown functions. All of the under-transcribed genes were in the unknown category, making it difficult at this time to hypothesize how (or if) their differential expression may play a part in altering the biology of the treated flies.

Several of the genes induced by PB, in this study, have previously been associated with metabolic DDT-resistance phenotype in *Drosophila*: *Cyp6a2* (Waters *et al*., 1992; Dombrowski *et al*., 1998; Maitra *et al*., 2000), *Cyp6a8* (Maitra *et al*., 1996, 2000), *Cyp6a9* (Maitra *et al*., 1996), *Cyp6g1* (Daborn *et al*., 2001, 2002; Brandt *et al*., 2002) and *Cyp12d1* (Brandt *et al*., 2002; Le Goff *et al*., 2003; Pedra *et al*., 2004; Festucci-Buselli *et al*., 2005). Interestingly, *Cyp6g1*, which has been implicated as one of many genes putatively involved in the polygenic DDT resistance phenotype, was not observed in the 5% or 12% FDR

Table 2. Genes that were differentially expressed, based on qRT–PCR, in *Drosophila* after a 24 h exposure to 0.1% phenobarbital in the diet of third instar larvae as compared with larvae reared on control diet

Gene category/name	ΔCT* (SE)	*P*-value
Monooxygenase activity (GO:0004497)		
Cyp4d14	3.75 (0.33)	< 0.0001
Cyp6w1	5.92 (0.23)	< 0.0001
Cyp6a2	7.37 (0.26)	< 0.0001
Cyp6a8	9.04 (0.23)	< 0.0001
Cyp6d5	6.40 (0.18)	< 0.0001
Cyp12d1	5.03 (0.33)	< 0.0001
†*Cyp6g1*	2.54 (0.23)	< 0.0001
Oxidoreductase activity (GO:0016491)		
CG2065	5.49 (0.35)	< 0.0001
Cytochrome b5-related	3.93 (0.29)	< 0.0001
Glutatione S transferases (GO:0004364)		
DmGstd6	6.85 (0.39)	< 0.0001
DmGstd7	7.56 (0.52)	< 0.0001
DmGstd9	−0.02 (0.58)	0.977
Steroid metabolism (GO:0008202)		
Ugt86Dd	4.10 (0.30)	< 0.0001
GC5724	3.54 (0.58)	< 0.0001
Alpha-amylase activity (GO: 0004556)		
Amy-p/Amy-d	7.08 (0.40)	< 0.0001
Epoxide hydrolase activity (GO:0004301)		
Juvenile hormone epoxide hydrolase 1 (Jheh1)	3.89 (0.28)	< 0.0001
Juvenile hormone epoxide hydrolase 2 (Jheh2)	4.52 (0.22)	< 0.0001
Genes that code for proteins with unknown function		
CG11893	3.20 (0.39)	< 0.0001
1626392_s_at	−0.02 (0.24)	0.923

*ΔCT is extra number of PCR cycles needed for control sample to reach same level of amplifications as PB-treated samples.
†Not identified in the oligoarrays experiment (Table 1), but previously shown to be associated with metabolic resistance to pesticides in *Drosophila* (Brandt *et al*., 2002; Daborn *et al*., 2002; Pedra *et al*., 2004).

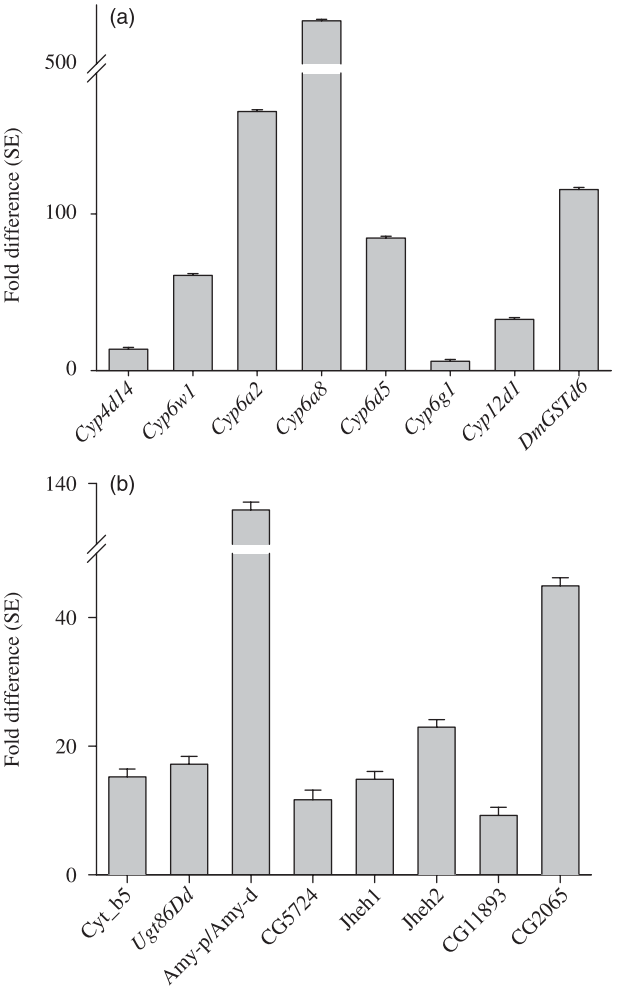

Figure 1. Fold induction of genes in third instars treated with phenobarbital for a 24 h interval as compared with larvae not treated. The expression level of the genes in the control group was 1 for all the genes and hence is not shown. Quantitative real-time PCR was used to distinguish between non-treated (control) and treated (0.1% phenobarbital in the diet) larvae. In (a) differential expression of cytochrome P450s and glutathione S-transferases are given. In (b) are given differential expression of genes associated with oxido-reductase, epoxide hydrolases (Jheh1 and Jheh2), Alpha-amylase activity/steroid metabolism (Amy-p/Amy-d, *Ugt86Dd*, and GC5724), and genes that code for proteins with unknown function. All genes displayed significant increases in expression due to phenobarbital treatment (Table 2).

groups. Subsequent qRT–PCR analysis revealed 5.8-fold and 3.6-fold levels of induction, respectively, in the larval and adult stages of development.

PB treatment of *Drosophila* has previously been shown to cause a rapid increase in both *Cyp6a2* mRNA and protein level (Dunkov *et al*., 1997). *Cyp6a2 has* been shown to catalyse the metabolism of several insecticides including aldrin and heptachlor (Dunkov *et al*., 1997). As well, *Cyp6a8* oxidizes the endogenous substrate lauric acid (C12:0), a short-chain unsaturated fatty acid (Helvig *et al*., 2004). PB inducible cytochrome P450s involved in lauric acid metabolism has been identified across a diverse set of species ranging from rats to wheat (Tanaka *et al*., 1990; Forthoffer *et al*., 2001).

Increases in *Cyp6a2* mRNA (and the resultant *Cyp6a2* protein) have been associated with metabolic pesticide resistance both in terms of over-transcription of this gene and in terms of changes in catalytic properties. *Cyp6a2* expression levels in pesticide resistant and susceptible strains of *Drosophila* have been measured using western blots (Bride *et al*., 1997) and a correlation was observed between *Cyp6a2* titre and resistance (Bride *et al*., 1997). However, *Cyp6a2* expression level was not a predictor of resistance level. *Cyp6a2* has also been sequenced in RDDT^R strain. Using heterologous expression Amichot *et al*. (2004) demonstrated that several point mutations in this gene account for an increase in DDT metabolism by the *Cyp6a2* protein. Thus, resistance is not only associated with increased P450 expression levels, but also with qualitative changes in the enzyme that results in increased metabolism.

Oligoarray analysis revealed that third instar *Drosophila* treated with PB over-transcribed multiple glutathione S-transferases as compared with the untreated larvae. We also verified, through qRT–PCR, that *DmGSTd6* and *DmGSTd7* were over-transcribed in the PB treated third instars. These GSTs belong to the insect specific delta

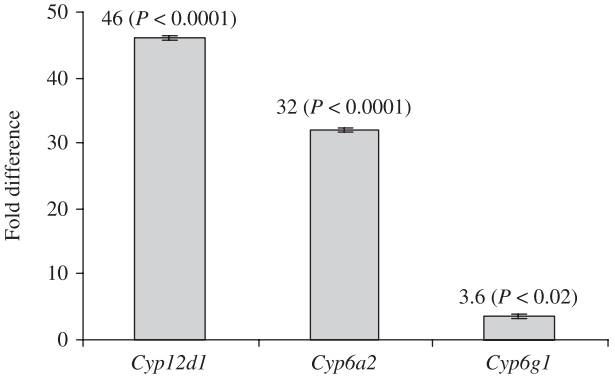

Figure 2. Fold induction of genes in adult *Drosophila* treated with phenobarbital for a 24-h interval as compared with adults not treated. The expression level of the genes in the control group was 1 for all the genes and hence is not shown. Quantitative real time PCR (qRT–PCR) was used to distinguish between non-treated (control – apple juice adjusted to pH 7 with extra 5% sucrose added) and treated (5 mg/ml of phenobarbital in apple juice adjusted to pH 7 with extra 5% sucrose added) adults. All three genes were cytochrome P450s previously associated with metabolic pesticide resistance in adult *Drosophila*. Fold-levels of induction and significance levels for each gene are given.

class (Ding *et al*., 2003) and all are in the same general cytological region (87B8). Over-transcription of insect specific delta and epsilon classes of glutathione S-transferases has been shown to be associated with metabolic pesticide resistance in a variety of insect species (Clark & Shamaan, 1984; Ku *et al*., 1995; Ranson *et al*., 2001; Vontas *et al*., 2001; Pedra *et al*., 2004; Ranson & Hemingway, 2005). In *Anopheles gambiae*, GSTs play an important role in conferring insecticide resistance (Ding *et al*., 2003). Additionally, glutathione S-transferases have also been shown to be PB inducible in both insects and mammals (Sasaki *et al*., 1989; Pinkus *et al*., 1993; Tang & Tu, 1995).

DmGSTd7 was originally thought to be a pseudogene, but recent work by Sawicki *et al*. (2003) has revealed that *DmGSTd7* does code for a functional protein. Sawicki *et al*. (2003) also observed that *DmGSTd7* is effective at metabolizing 4-hydroxynonenal (4-HNE). 4-HNE is a highly reactive aldehyde derived from other reactive oxygen species (ROS) and is associated with oxidative stress (Sawicki *et al*., 2003). 4-HNE-protein adducts have been observed both in *Drosophila* and house-flies (Yan & Sohal, 1998; Singh *et al*., 2001). The major route for 4-HNE metabolism in mammals is through glutathione conjugation (Alin *et al*., 1985; Videla *et al*., 2000). Additionally, PB has been shown to have an impact on oxidative stress response in mammals. Findings by Sawicki *et al*. (2003) suggest a potential role of *DmGSTd7* in oxidative stress in *Drosophila*.

Third instar *Drosophila* treated with PB over-transcribed both juvenile hormone epoxide hydrolase 1 and 2 (Jheh1 and Jheh2) as compared with untreated larvae. Epoxide hydrolases have been implicated in juvenile hormone degradation in insects (Anspaugh & Roe, 2004). PB has been shown to increase expression of juvenile hormone epoxide hydrolases both in house-flies, *Musca domestica* (Yu & Terriere, 1978) and in the cabbage looper, *Trichoplusia ni* (Anspaugh & Roe, 2004). Epoxide hydrolases have been shown to be inducible by a diversity of xenobiotics, including compounds with and without epoxides, leading Anspaugh & Roe (2004) to hypothesize that these enzymes may play a role in xenobiotic metabolism.

We also observed over-transcription of four genes associated with lipid/steroid and carbohydrate metabolism in the PB-treated flies (Table 1). Using qRT–PCR we verified differential expression of three of these genes: Amy-p/Amy-d, *Ugt86Dd*, and GC5724 (Table 2). PB has been shown to influence carbohydrate metabolism levels in rat liver (Schaub & Freksa, 1976). Total glycogen synthetase was also increased by PB treatment in rats during prenatal development, but this activity decreased during postnatal development (Schaub & Freksa, 1976). Both the active and the inactive forms of phosphorylase were increased significantly by PB during the prenatal period (Schaub & Freksa, 1976). In the postnatal period the active form of phosphorylase was influenced to a greater extent, as compared with the inactive form, by PB (Schaub & Freksa, 1976). PB treatment also increased α-glucosidase activity during the prenatal period (Schaub & Freksa, 1976). The activity of Fructose-6-P-kinase (F-6-PK) and 6-phosphogluconate dehydrogenase (6-PGDH) were also decreased by PB during the prenatal periods, as was Glucose-6-phosphate dehydrogenase (G-6-PDH) activity during both pre- and postnatal periods (Schaub & Freksa, 1976).

Metabolic pesticide resistance is also associated with differential expression of genes and proteins associated with carbohydrate and lipid metabolism. Pedra *et al*. (2004) observed over-transcription of genes associated with carbohydrate and lipid/steroid metabolism in metabolically DDT-resistant strains of *Drosophila*, including *Ugt86Dd*, which we also observed in the current study to be induced by PB (Tables 1 and 2, Fig. 1b). Additionally, two-dimensional gel comparisons between DDT resistant and susceptible *Drosophila* protein patterns revealed differential expression of proteins associated with carbohydrate and lipid metabolism (Pedra *et al*., 2005). In insects DDT metabolism has been shown to be associated with glucose utilization (Plapp, 1970; Maltseva & Golovleva, 1982) and in mammals DDT has also been shown to affect β-oxidation of fatty acids (Hinton *et al*., 1978; Oda *et al*., 1994). Maltseva & Golovleva (1982) observed that the activity of isocitrate and glucose-6-phosphate dehydrogenases was increased in *Pseudomonas aeruginosa* strains capable of metabolizing DDT.

Rencurel *et al*. (2005) have recently shown that in hepatocytes, and in a newly derived human hepatoma cell line, PB influences AMP-activated protein kinase (AMPK), which activates CAR through phosphorylation. CAR then binds with the NR1 site of PBREM (PB Responsive Enhancer

Module), which in turn is responsible for activating increases in P450 expression (Rencurel *et al*., 2005). The AMPK is important for regulating energy levels in the cell and AMPK is activated when cells have experienced energy depletion. Constitutive over-expression of AMPK in mammalian cells results in constitutive over-expression of P450s and loss of function of AMPK inhibits induction of P450s by PB. AMPK is important for regulation of glucose uptake in skeletal muscle cells during exercise and has been proposed to be a target site for antidiabetic drugs (Zangar & Novak, 1997). In *Drosophila*, DDT resistance is associated with constitutive over-expression of genes and proteins involved in regulating energy levels in the cell (Pedra *et al*., 2004, 2005) and metabolically resistant insects have a greater metabolic rate than susceptible insects (Plapp, 1970).

Although considerable work has been done in vertebrates regarding PB inducible response elements and genes, we know very little about such regulatory mechanisms in insects. PB alters gene expression of mammalian P450s by activating the pregnane X receptor (PXR) and constitutive androstane receptor (CAR). In non-mammalian animals only one xenosensor gene has been found (e.g. *Drosophila*, *Caenorhabditis elegans* and chicken) and sequence-wise this xenosensor is about equally related to the mammalian PXRs and CARs (Handschin *et al*., 2004). Interestingly, chicken xenobiotic receptor and mammalian PXR and CAR can be freely interchanged in *trans*-activation and electrophoretic mobility shift assays suggesting evolutionary conservation of the fundamental drug-induction mechanisms from birds to mammals (Handschin *et al*., 2001). Of the 18 nuclear receptors in the *Drosophila* genome only DHR96 bears some similarity to the non-mammalian xenosensors, but it remains to be determined whether DHR96 plays a role in PB induction (Handschin *et al*., 2004).

In conclusion, PB induces differential expression of genes associated with detoxification, metabolism, oxidative stress, juvenile hormone esterases, as well as genes that code for proteins with unknown function. Interestingly, metabolic pesticide resistance, which can be induced by PB, is also associated with increases in genes associated with both detoxification and metabolism. These results support the hypothesis that constitutive over-expression of certain detoxification enzymes and metabolic proteins in pesticide-resistant *Drosophila* are associated with perturbations of regulatory mechanisms associated with PB induction.

Experimental procedures

Experimental design

The experiment was designed as two treatments (control vs. PB) with three biological replicates for each treatment. The bioassays were conducted as pairs (control vs. PB) on separate days. The six separate RNA samples were processed on the same day and were later used in the oligoarray and qRT–PCR analyses.

Bioassays, RNA extraction and Affymetrix experiments

The w^{1118} strain used in this experiment was obtained from Dr Misha Ludwig (University of Chicago). The larvae were reared on Formula 24® *Drosophila* diet (Carolina Biological Supply, Burlington, NC, USA) at room temperature (≈ 23 °C and 60–70% R.H) until they became third instar larvae. The third-instar w^{1118} were transferred to Formula 24® *Drosophila* diet containing apple juice (Great Value®, WalMart, Bentonville, AR, USA), in order to assure larval feeding. The larvae were equally split between one of two treatments: (1) no additional chemicals (control), and (2) 0.1% PB (Sigma-Aldrich, St Louis, MO, USA). The concentration of PB in diet (0.1%) was the maximum dose that did not retard the growth of the larvae, which we determined in dose–response experiment (data not shown). All larvae were 120–122 h of age (recorded from the time the eggs were laid) at the time of transfer. After transfer the larvae were allowed to feed on the media for 24 h. At the end of the 24 h exposure the larvae were removed from the media, immediately placed in RNA later, then flash-frozen in liquid nitrogen and stored at –80 °C.

For each treatment of each biological replicate 30 larvae were combined for a single RNA extraction. For the adult experiments, adult flies were first starved (with no water either) overnight (12 h), then fed with pH adjusted (pH 7.0) apple juice (with extra 5% sucrose added) containing 0 or 5 mg/ml of PB for 24 h. The dose (5 mg/ml) for PB was the maximum non-lethal dose that we determined in a dose–response experiment for w^{1118} adults (data not shown). Thirty adults were used for each control and treatment replicate. For both larval and adult experiments, a total of three biological replicates were performed for both the control and the PB treatments. At the end of 24-h period, the flies were flash frozen in liquid nitrogen and stored at –80 °C until total RNA extraction.

Messenger RNA was extracted and Affymetrix oligoarray experiments were performed as described in Pedra *et al*. (2004), with the exception that we used the Affymetrix *Drosophila* Genome 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). Image data were quantified by using genechip-operating software (GCOS 1.2). The identification of the informative probe sets were performed by using default settings (α1, 0.05; α, 0.065; δ, 0.015; scale factor, 1.0; normalization factor, 1.0).

Statistical analysis of oligoarrays

The data were analysed using the SAM permutation based statistical analysis software (Tusher *et al.,* 2001). A list of genes with associated *q*-values (Storey & Tibshirani, 2003) was generated. The *q*-value gives the minimum value for the FDR at which that gene will be declared significant.

Quantitative real-time polymerase chain reaction

For each treatment and replicate RNA was extracted from 30 flies using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) with an on column DNase digestion procedure. The first stranded cDNA was synthesized by using 1 μg of total RNA with iScript cDNA kit from Bio-Rad (Hercules, CA, USA) in a 20 μl reaction volume. A 25-fold dilution was made to each first stranded cDNA for quantitative PCR. qRT–PCR was performed with iQ SYBR Green Supermix from Bio-Rad with SYBR Green dye on an iCycler Thermal Cycler. For each cDNA, three qRT–PCR reactions were conducted. To minimize the positional effect on PCR reactions due to variations of block temperature, PCR reactions for target gene and reference gene were placed on the cycler with a randomized block design.

Table 3. Primer sequences for qRT–PCR

Gene name	Accession no.	Primer sequence (5′–3′)
rp49	U92431	CGGTTACGGATCGAACAAGCG
		TTGGCGCGCTCGACAATCT
Cyp12d1	NM_206090	CAACGAGGGTATCTGGCCACGT
		GGCATCCACACCAGCGAATAGG
Cyp6g1	NM_136899	AACTCCTTTGGGATGCACCTATCG
		GGTTGTGGAACCGATTGAAGTCCT
1626392	X01472	CCTACCAGATTGGCAATGTGGAG
		AGGCTCCAATGCAGCGCA
Amy-p/Amy-d	NM_080421	CAGGTCTCCCCTGTGAACGAGAA
		GGCGGCCATGTGGTTGAA
CG11893	NM_143118	GCGACTCACATTTGTTCTCCACC
		TGCGGCACCAAATCCTCG
CG2065	NM_136465	CCTGCTCACCCACTTGCTGC
		GGCCAATTCCCGGGTGAA
CG5724	NM_141971	GAATGTCCTTCCGACAGCGCT
		TGCGTGGGAGGCGAAGAA
Cyp4d14	NM_143763	AGATGTTGGGCGAAAGTGGCT
		TGATCTTCCTGCGGCGGA
Cyp6a2	NM_078904	TTCACCACCGATGTGATTGGC
		TCGGGCATCATGCGCATT
Cyp6a8	NM_079025	ACGAACTGGCAAAGAATCCGGAC
		TGGCGGCCATTCGATCCA
Cyp6d5	NM_169567	GTGAAAATCGGCTGGAAACAGG
		TGCTTTCCGCCGACCAAA
Cyp6w1	NM_136362	AGCCCATAGGGTTCTAGCCGG
		CCATCGCCTCGGCATCAA
Cyt-b5	NM_057806	GGACGATGGAGCCGAAGGA
		TTTCGGGAGCCGTGGTCA
DmGstd6	NM_080176	ACCCGGGCCGTTATGATGA
		TGGCCCGAGTTTCCCAAA
DmGstd7	NM_080375	AGGCTTTGGGTCTGGAGCTGA
		TGGGGAATCGGGTTTGCC
DmGstd9	NM_141924	TATATGCTCTACTCGGCACCTTGC
		TACTCGCAGCCCTCCCAGTT
Jheh1	NM_137541	GTCGTGGAGTACTGGCGCGA
		GGCCATCCGTGGAGCAGC
Jheh2	NM_137542	CCAAATGGAGCGAACGGGA
		ATTCGCGAACGGTTCCCG
Ugt86Dd	NM_144367	GCCTGGAAAGCCAGCTAATGTG
		GGCGCGCTGCACATTCAT

The threshold cycle (CT) was calculated by the iCycler IQ software. The relative expression levels were calculated as given in Pfaffl *et al.* (2002), and the statistical analysis of the relative gene expression level were performed using SAS (SAS Institute Inc., Cary, NC, USA). *Rp49* was used as the reference gene. The primers used for qRT–PCR and gene bank accession numbers were listed in the Table 3. Statistical analysis followed a General Linear Model (with log transformation for linearization and normalization) and planned contrasts between the control and PB treatments.

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