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RESEARCH ARTICLE

Interactions between detoxification mechanisms and excretion in Malpighian tubules of *Drosophila melanogaster*

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SUMMARY

Insects have long been known to excrete toxins *via* the Malpighian (renal) tubules. In addition, exposure to natural or synthetic toxins is commonly associated with increases in the activity of detoxification enzymes such as the P450 monoxygenases (P450s) and the glutathione-S-transferases (GSTs). We examined the links between mechanisms for detoxification and excretion in adult *Drosophila melanogaster* using functional assays and measurements of changes in gene expression by quantitative reverse transcriptase PCR in response to dietary exposure to compounds known to alter activity or gene expression of P450s and GSTs. Dietary exposure to phenol, which alters gene expression for multiple GSTs after seven to 10 generations, was also associated with an increase (more than twofold) in secretion of the organic anion methotrexate (MTX) by isolated tubules. Dietary exposure to the insecticide synergist piperonyl butoxide (PBO) was associated with reduced expression of two P450 genes (Cyp4e2, Cyp4p1) and two GST genes (GstD1, GstD5) in the tubules, as well as increased expression of Cyp12d1 and GstE1. Thin layer chromatographic analysis of fluid secreted by isolated tubules indicated that dietary exposure to PBO resulted in increased levels of an MTX metabolite. In addition, exposure to PBO altered the expression of transporter genes in the tubules, including a *Drosophila* multidrug resistance-associated protein, and was associated with a 73% increase in MTX secretion by isolated tubules. The results suggest that exposure of *Drosophila* to toxins evokes a coordinated response by the Malpighian tubules, involving both alterations in detoxification pathways as well as enhanced transport.

Key words: Malpighian tubule, P450 enzyme, glutathione-S-transferase, toxin transport.

INTRODUCTION

Exposure of insects to plant-derived or anthropogenic toxins is often associated with increased activity of phase I and phase II detoxification mechanisms. Phase I enzymes introduce reactive and polar groups into their substrates through oxidation, hydrolysis or reduction. Prominent among the phase I enzymes are the P450 monoxygenases (P450s), which are well known for their role in the metabolism of natural and synthetic pesticides by insects (Feyereisen, 1999). Following phase I, the activated metabolites of xenobiotics are conjugated with compounds such as glutathione, sulphate or glucuronate in phase II reactions. The glutathione-S-transferases (GSTs) are among the best known of the phase II enzymes, and increases in their levels are associated with resistance to all major classes of insecticides (Ranson and Hemingway, 2005).

Phase I and phase II pathways can be altered by exposure of insects to a variety of organic compounds. Piperonyl butoxide (PBO) is an insecticide synergist that inhibits the P450 detoxification system but also increases the expression of twelve P450 genes and five GST genes in *Drosophila* (Willoughby et al., 2007). The Cyp genes Cyp6a2, Cyp6a8 and Cyp12d1, which are most strongly induced by PBO in adult male *Drosophila*, are also among the 12 Cyp genes associated with insecticide resistance (Willoughby et al., 2007; Giraudo et al., 2010). Previous studies have also shown that rearing *Drosophila* for multiple generations on diets containing 0.3% phenol is associated with an increase in GST enzymatic activity but with no change in the levels of mRNA for GstD1, GstD5, GstD8 and GstE1 and with a reduction in the levels for GstD4 and GstD7 (Shen et al., 2003; Ding et al., 2005).

Following phase I and phase II detoxification, a variety of transporters, including members of the ATP-binding cassette superfamily of transporters, are involved in phase III elimination of the products of phase I and phase II reactions. We have previously studied the roles of the gut and the Malpighian tubules in the elimination of organic cations (Bijelic et al., 2005; Rheault et al., 2006) and organic anions (Ruiz-Sanchez and O'Donnell, 2007; Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). The organic anion methotrexate (MTX) is secreted by the Malpighian tubules of larval and adult *Drosophila*, and chronic exposure of the larvae to dietary MTX increases both the rate of MTX secretion by the tubules and the expression of multiple transporter genes (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). MTX was used in these studies because it is a well-characterized substrate of multidrug resistance-associated proteins (MRPs) (e.g. Vlaming et al., 2009) and because its availability in tritiated form facilitates measurement of its rate of secretion by isolated Malpighian tubules.

There are several reasons to believe that phase I and phase II pathways are likely to interact with phase III elimination mechanisms. Firstly, P-glycoproteins, which are the product of multidrug resistance (MDR) genes, are a group of ABC transporters that share many substrates with P450 enzymes (Bard, 2000; Abu-Qare et al., 2003). P-glycoproteins may thus transport moderately hydrophobic xenobiotics before or after the action of P450 enzymes. Secondly, the genes for several P450 enzymes

and GSTs are enriched in the Malpighian (renal) tubules (Dow and Davies, 2006). The importance of the tubules in xenobotic metabolism has been emphasized by a recent study that showed that manipulation of a single P450 gene (Cyp6g1) in the tubules alters the survival of the whole fly during exposure to dichlorodiphenyltrichloroethane (DDT) (Yang et al., 2007). Thirdly, there are well-described links between MRP transporters and GST activity (Cole and Deeley, 2006) and there are MRP-like transporters in *Drosophila* Malpighian tubules (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010).

Given that phase I and phase II enzymes can be induced or altered by exposure to specific chemicals, the present study tests the hypothesis that such chemicals will also lead to a coordinated upregulation of phase III elimination pathways. The rationale behind this hypothesis is that the actions of phase I and phase II do not produce products that are without toxicity themselves. Indeed, the products of some P450 reactions can be more toxic than the parent compound, a finding that has been exploited in the development of bioactivated pesticides such as the phosphorothioates (Feyereisen, 1999). In such instances, phase III elimination pathways may act as a primary line of defense towards these organic toxins.

MATERIALS AND METHODS

Insects and diet preparation

Drosophila melanogaster (Oregon R strain) were raised on standard artificial diet and maintained at 21–23°C in laboratory culture. The standard diet was prepared as previously described (Roberts and Stander, 1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept, Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol were added to the mixture. Diets enriched in organic anions or compounds known to alter the activity and/or expression of P450 enzymes or GSTs were prepared by addition of MTX (0.1 mmol l^{-1}), PBO (1 mmol l^{-1}), both MTX and PBO, phenol (0.3% w/v) or both MTX and phenol.

Chemicals

[^{3}H]-methotrexate ([^{3}H]MTX) (50.8 Ci mmol l^{-1}) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich. Stock solutions of chemicals were prepared in control saline or ethanol. The final concentration of ethanol in the experiments did not exceed 0.5%. Ethanol at concentrations <1% has no effect on fluid secretion rate (O'Donnell et al., 1996; Linton and O'Donnell, 1999).

Malpighian tubule dissection and Ramsay fluid secretion assay

Pairs of Malpighian tubules were dissected from adult females reared on standard or experimental diets 7 days post-eclosion. The dissection technique is described by Dow et al. (Dow et al., 1994).

Ramsay assays were performed as described by O'Donnell and Rheault (O'Donnell and Rheault, 2005). Briefly, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that the main (fluid-secreting) segment of one tubule was in the bathing droplet containing [^{3}H]MTX and the other was wrapped around a steel pin positioned approximately 1–2 mm away from the bathing droplet. Preliminary experiments showed that differences in MTX secretion by Malpighian tubules from flies reared on control *versus* experimental diets were most apparent at high concentrations of MTX (≥100 µmol l^{-1}). Therefore, we used a concentration of 400 µmol l^{-1} MTX in the bathing saline. Secreted fluid droplets formed at the ureter and were collected after 60 min using a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (d) of the secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Fluid secretion rates (nl min^{-1}) were calculated by dividing the secreted droplet volume by the time over which it formed. The concentration of [^{3}H]MTX in secreted droplets was measured by placing the droplets in vials containing 4 ml of scintillation fluid and counting β-radiation in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, MA, USA). Transepithelial flux of MTX (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and MTX concentration (µmol l^{-1}).

Thin layer chromatography

Three 4.5 µl samples from stock [^{3}H]MTX (500 µmol l^{-1}) or secreted fluid collected from tubules set up in Ramsay assays and bathed in saline containing 400 µmol l^{-1} [^{3}H]MTX were chromatographed on 250 µm thickness silica gel thin layer chromatography (TLC) plates (EDM Chemicals Inc., Gibstown, NJ, USA) using 0.02 mol l^{-1} HEPES/0.1 mol l^{-1} NaCl (pH 7) as the mobile phase (Henderson and Zevely, 1985). Chromatographic plates were subsequently exposed to X-ray film for 4–7 days at –80°C before being developed in a Kodak X-OMAT 2000A automatic X-ray film processor (Kodak, Rochester, NY, USA). The corresponding retention factors (*Rf*=distance migrated by the compound/distance migrated by the solvent) were determined for samples secreted by tubules of flies reared on the standard or PBO-enriched diet. The relative percentage of each methotrexate metabolite or parent compound was determined by spot densitometry using a FluorChem™ 800 digital imaging system and Alpha EaseFC™ software (Alpha Innotech Corporation, San Leandro, CA, USA).

RNA extraction and reverse-transcriptase PCR amplification

Malpighian tubules were dissected from adult females maintained for 7 days from the time of eclosion on standard diet or on diets enriched with MTX, PBO or PBO plus MTX. For diets containing phenol or phenol plus MTX, the adult females were collected from the F10 generation. Total RNA was pooled in groups of 200 Malpighian tubules using TRIzol (Invitrogen). RNA was extracted as described by Nawata and Wood (Nawata and Wood, 2008). Briefly, RNA concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. cDNA was prepared from six to 12 independent RNA samples. One microgram of RNA was used per sample for cDNA synthesis, after first treating with DNase I (Invitrogen) to prevent any genomic DNA contamination. First-strand cDNA was synthesized using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at –70°C.

Table 1. Primer list

Primer	GenBank accession no.	Forward/reverse sequence (5′–3′)
GAPDH1	CG12055	tgaagggaatcctgggctac/accgaactcgttgtcgtacc
MET	CG30344	cctgctgacaacttttacgg/gtaatcaaggcgcaagttcc
dMRP	CG6214	actttacgccctgctttgag/tcacgttcagcttgttccac
OATP	CG3380	tcgaagcctccaagtttctg/catgtgagcagtcgcaaatc
Cyp4e2	CG2060	gccggagaagagtgcttaac/tgatgccaccaggagaaaac
Cyp4p1	CG10842	ccctaagtgccctgctctac/ctatgggagcgatgatct
Cyp6a2	CG9438	ccacacgatgctcctcacc/cgctcttccggtacttgttg
Cyp6a8	CG10248	gccctactggcgatcttaac/cctccatgtttcccctgatg
Cyp12d1	CG30489	atttacgtgggtcccgttc/gcggaatctcatcgtaggtc
GstD1	CG10045	tcctgaacaccttcctggag/cttgctgatctcgaatttgg
GstD5	CG12242	tattactcgccccgtggaag/tggtgtgctatggattgagc
GstE1	CG5164	gaggaccgtcaaacttaccc/gtaccgtgtgttggggattc

Detoxification and transporter gene expression (quantitative real-time PCR)

Gene expression in Malpighian tubules from flies raised on the experimental diets were compared with tubules from flies raised on a standard diet by quantitative real-time PCR (qPCR) using the cDNA prepared above. The primers and GenBank accession numbers for each gene are listed in Table 1.

Specific transporter genes were chosen based on their putative function as solute transporters, their enrichment in Malpighian tubules (Chintapalli et al., 2007; Wang et al., 2004) and our previous studies of organic anion transport (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). The mRNA expression of the multidrug efflux transporter (MET), *Drosophila* multidrug resistance-like protein (dMRP) and organic anion transporting polypeptide (OATP) increase >3500-fold, >1200-fold and >23-fold, respectively, in tubules of adult flies reared on diets containing 0.1 mmol l^{-1} MTX, relative to controls (Chahine and O'Donnell, 2009).

The large numbers of P450 genes (89) and GST genes (37) (Willoughby et al., 2007) in *Drosophila* precluded an exhaustive analysis of their levels of expression using qPCR. Specific genes encoding P450 and GST enzymes (Table 1) were chosen, therefore, based on their enrichment in Malpighian tubules as specified in FlyAtlas (Chintapalli et al., 2007) and by Yang et al. (Yang et al., 2007), and also on the basis of changes in their expression in adult *Drosophila* after PBO treatment (Willoughby et al., 2007) or exposure to dietary phenol (Ding et al., 2005).

Each 20 µl reaction contained 4 µl of cDNA, 4 pmol of each primer and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The reactions were performed at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt-curve analysis verified the production of a single product. Non-reverse-transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. For each gene, a standard curve was performed by serial dilution of one randomly selected experimental sample (Malpighian tubules of flies raised on 0.1 mmol l^{-1} MTX-enriched diet) to ensure that qPCR amplification efficiency was above 95% with respect to the appropriate primer pair. The specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured the expression of five potential reference genes, including the ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha tubulin (alphaTub84B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH1). Calculations of relative mRNA expression by the standard curve method used GAPDH1 as the endogenous standard because it had the most stable expression across samples.

Data analysis

Values from all experiments were expressed as means ± s.e.m. for the indicated numbers of samples. Graphing and statistical analyses were performed using GraphPad Prism and InStat 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Significant differences were determined using paired sample or two-sample *t*-tests assuming either equal or unequal variance, according to the outcome of a two-sample *F*-test. Differences were considered significant if $P<0.05$.

RESULTS

Effects of addition of PBO and MTX to the diet on tubule secretion of MTX

We first examined the effects of PBO added to the diet, alone or in combination with methotrexate. There was no effect of either experimental diet on the rates of fluid secretion by isolated Malpighian tubules bathed in saline containing 400 µmol l^{-1} MTX relative to tubules isolated from flies reared on the control diet (Fig. 1A).

By contrast, the concentration of MTX in the fluid secreted by tubules from flies reared on diets containing PBO or both PBO and MTX was more than twice that of the corresponding controls (Fig. 1B). As a result, secretion of MTX by tubules from flies reared on the diets containing PBO or both PBO and MTX increased by 73 and 136%, respectively, relative to the controls (Fig. 1C).

Effects of dietary exposure to phenol for multiple generations on tubule secretion of MTX

GST activities increase in *Drosophila* reared for more than seven generations on diet containing 0.3% phenol (Shen et al., 2003). We therefore assessed whether increases in GST activity were correlated with an increase in secretion of the MRP substrate methotrexate.

Fluid secretion rates, secreted fluid methotrexate concentration and methotrexate secretion rates of tubules isolated from flies reared on the phenol-enriched diet were similar to those of the control groups in the F0, F1 and F4 generations (Fig. 2). However, there was an increase in fluid secretion rate of 46 and 65% for tubules from the F7 and F10 generations, respectively, of flies reared on the phenol-enriched diet relative to the controls (Fig. 2A). There was also an increase of 43 and 50% in the methotrexate concentration in the fluid secreted by the tubules from flies of the F7 and F10 generations, respectively, on the phenol-enriched diet (Fig. 2B). As a consequence of the increases in both fluid secretion rate and secreted fluid methotrexate concentration, tubules from flies of the F7 and F10 generations on the phenol-enriched diet secreted MTX at more than double the rate of the corresponding controls (Fig. 2C).

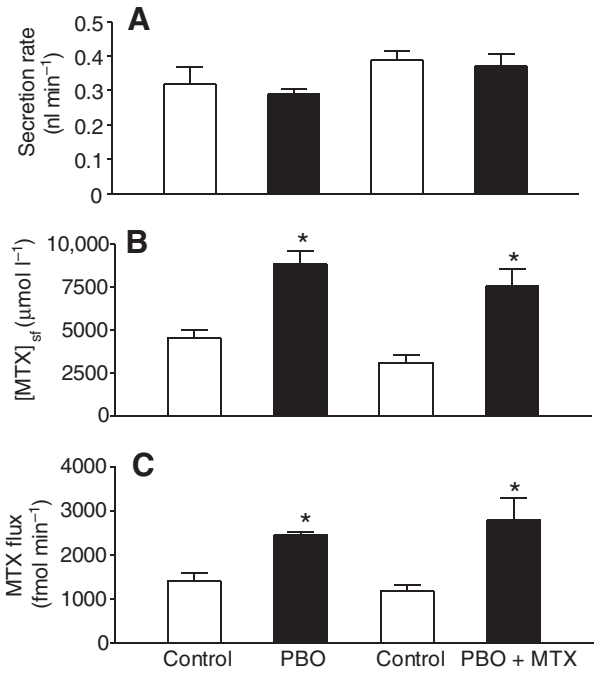

Fig. 1. The effects of chronic exposure to dietary piperonyl butoxide (PBO; 1 mmol l^{-1}) alone or in combination with methotrexate (MTX; 0.1 mmol l^{-1}) on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ([MTX]$_{sf}$) and (C) transepithelial flux of MTX. Isolated Malpighian tubules were set up in a Ramsay assay containing 400 μmol l^{-1} [^{3}H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences between means from flies reared on a standard diet (control; open bars) and on experimental diets (solid bars) are indicated by asterisks (*P<0.05, paired t-test, N=8–12). Error bars are +s.e.m.

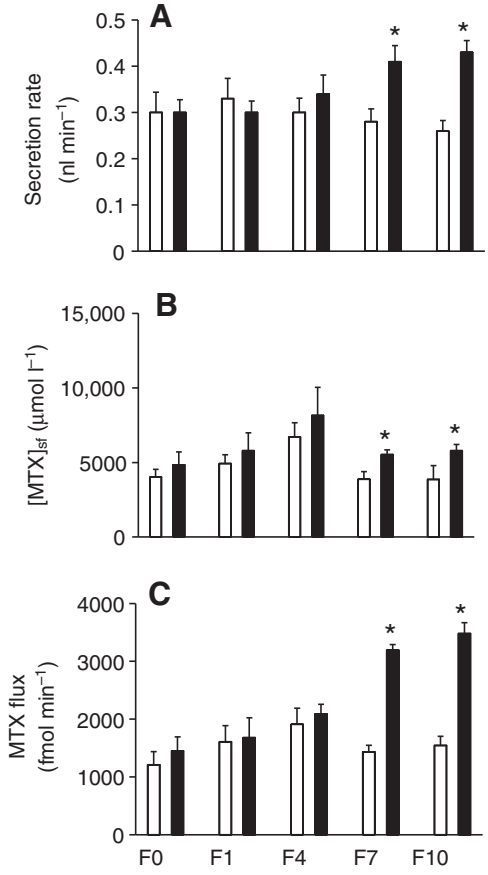

Fig. 2. Effects of dietary exposure to phenol (0.3%) for multiple generations (F0–F10) on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ([MTX]$_{sf}$) and (C) transepithelial flux of MTX. Isolated Malpighian tubules were set up in a Ramsay assay containing 400 μmol l^{-1} [^{3}H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences between means for control (open bars) and experimental groups (solid bars) are indicated by asterisks (*P<0.05, paired t-test, N=8–12). Error bars are +s.e.m.

Effects of PBO on MTX metabolism by the Malpighian tubules

Analysis of secreted fluid by TLC indicated that there is a single metabolite of MTX; *Rf*s for MTX and the metabolite were 0.41 and 0.79, respectively. The *Rf* values for samples of fluid secreted by tubules from flies reared on control and PBO-enriched diets were the same, indicating that dietary PBO does not alter the number of metabolites. However, spot densitometry indicated that, whereas 24.5% of the MTX was metabolized to the compound with an *Rf* of 0.79 in control tubules (Table 2), 38.2% of MTX was metabolized to this compound in tubules of flies reared on the PBO-enriched diet.

Effects of PBO and MTX on expression of P450 genes

Dietary exposure to MTX was associated with a significant reduction in relative mRNA expression for five P450 genes in the Malpighian tubules in comparison with tubules from flies reared on the control diet (Fig. 3A). Dietary exposure to PBO was associated with a reduction in the expression of Cyp4e2 and Cyp4p1, no significant change in expression of Cyp6a2 and Cyp6a8 and a fourfold increase in expression of Cyp12d1 in comparison with the controls (Fig. 3B). Exposure to both MTX and PBO was associated with no change in Cyp4e2 and Cyp6a2, a reduction in Cyp4p1 expression and a 10- to 18-fold increase in the expression of Cyp6a8 and Cyp12d1 (Fig. 3C).

Effects of MTX, PBO and phenol on expression of GST genes

Chronic exposure to MTX in the diet was associated with a reduction in relative expression of GstD1 and GstE1 (Fig. 4A). Chronic exposure to PBO or both PBO and MTX in the diet was associated with a reduction in GstD1 expression and either a reduction (PBO) or no change (PBO + MTX) for GstD5 (Fig. 4B,C). Expression of GstE1 increased 1.5-fold in response to dietary PBO (Fig. 4B) and there was no significant change (P>0.1), relative to controls, in GstE1 expression in response to both PBO and MTX (Fig. 4C).

When added to the diet on its own or in combination with MTX for 10 generations, phenol was associated with reductions in GstD1 expression in the Malpighian tubules (Fig. 5A,B). Phenol was

Table 2. Thin layer chromatography of samples of secreted fluid by the Malpighian tubules of *Drosophila* flies raised on different diets

Sample	Retention factor	Spot density (% total)
Control [^{3}H] methotrexate	0.41	100
Flies raised on control diet	0.41	75.5±3.0
	0.79	24.5±3.0
Flies exposed to PBO diet	0.41	61.8±2.4*
	0.79	38.2±2.3*

Asterisks indicate significant differences (P<0.05) between the percent of total spot density for control and PBO-enriched diets, based on t-tests of arcsin-transformed data. Spot density values are means ± s.d.

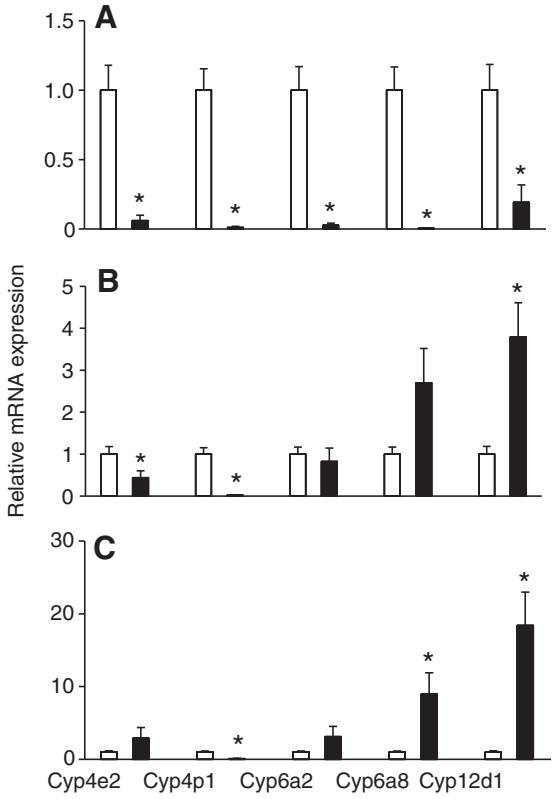

Fig. 3. mRNA expression of five cytochrome P450 genes relative to GAPDH1 expression in adult Malpighian tubules isolated from flies reared on standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.1 mmol l^{-1} MTX, (B) 1 mmol l^{-1} PBO or (C) both PBO and MTX. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6–12). Error bars are +s.e.m.

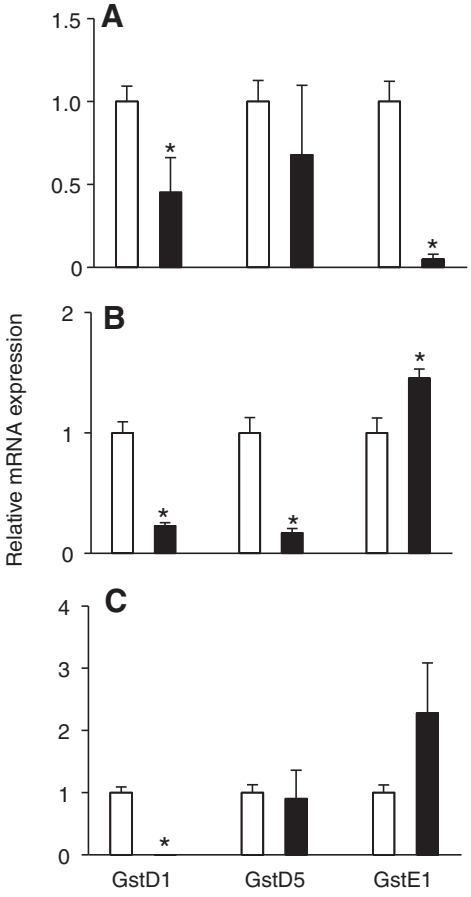

Fig. 4. mRNA expression of three glutathione-S-transferase (GST) genes relative to GAPDH1 expression in Malpighian tubules isolated from adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.1 mmol l^{-1} MTX, (B) 1 mmol l^{-1} PBO, (C) both PBO and MTX. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6–8 for A and B, N=4–8 for C). Error bars are +s.e.m.

associated with a reduction in GstD5 and no change in GstE1 expression (Fig. 5A). By contrast, dietary exposure to both MTX and phenol for 10 generations resulted in no change in GstD5 expression but a fourfold increase in GstE1 expression (Fig. 5B).

Effects of PBO and MTX on transporter gene expression

Chronic exposure to PBO was associated with a 123-fold increase in MET expression and a 27-fold increase in dMRP expression, but no change in OATP expression (Fig. 6A). Chronic exposure to both PBO and MTX in the diet resulted in increases in expression of all three genes in the Malpighian tubules: 93-fold for MET, 267-fold for dMRP and 50-fold for OATP (Fig. 6B).

DISCUSSION

The major finding of this study is that treatments that alter detoxification pathways in the Malpighian tubules result in increases both in the secretion of the organic anion MTX by the tubules and in the expression of genes for several organic anion transporters. Some of the treatments that alter MTX secretion also result in increases in fluid secretion rate.

Piperonyl butoxide increases MTX secretion and alters expression of both detoxification and organic anion transporter genes

Our results show that, whereas dietary MTX dramatically reduces the expression of five Cyp genes in the Malpighian tubules, dietary PBO increases Cyp12d1 expression and dietary MTX plus PBO increases expression of both Cyp12d1 and Cyp6a8. Cyp12d1 stands out as a gene that is induced by a broad spectrum of compounds, including PBO (Le Goff et al., 2006), caffeine, DDT (Willoughby et al., 2007), pyrethrum and piperamides (Jensen et al., 2006) and atrazine (Le Goff et al., 2006). Cyp6a8 is also induced by at least four compounds and its constitutive overexpression has been causally linked to resistance to phenobarbital, PBO, DDT or atrazine (Le Goff et al., 2006). In whole adult male flies, PBO results in increases in expression of 12 P450 genes, including three genes that were reduced or unchanged in the tubules in our studies (Willoughby et al., 2007). Dietary PBO is also associated with increases in the expression of GstE1 in the tubules. This gene is one of five whose expression increases in adult males after PBO treatment (Willoughby et al., 2007). Expression of both MET (CG30344) and dMRP (CG6214) was also increased by dietary PBO. Although dietary PBO was not associated with an increase in OATP expression, levels of this gene increased more (50-fold) in Malpighian tubules of flies reared on diets containing PBO plus MTX relative to tubules of flies reared on diet containing MTX alone (23-fold) (Chahine and

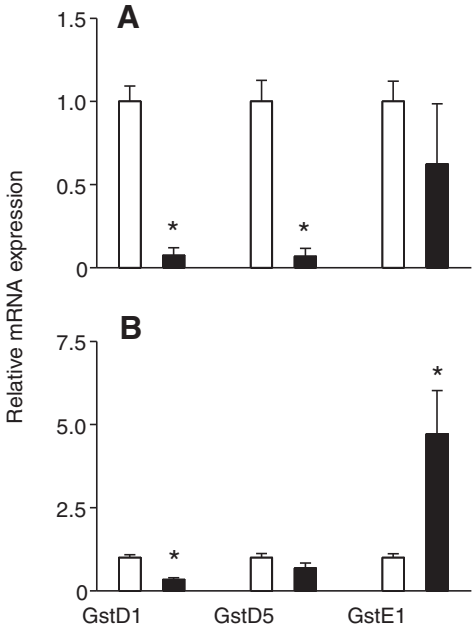

Fig. 5. mRNA expression of three GST genes relative to GAPDH1 expression in Malpighian tubules isolated from adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.3% phenol or (B) both phenol and MTX. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6–8). Error bars are +s.e.m.

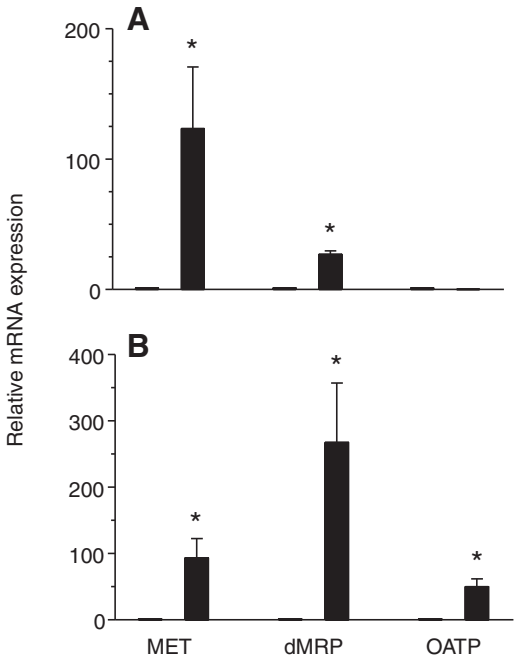

Fig. 6. mRNA expression of three transporter genes relative to GAPDH1 expression in Malpighian tubules of adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 1 mmol l^{-1} PBO or (B) both PBO and MTX (0.1 mmol l^{-1}). Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6–12). Error bars are +s.e.m.

O'Donnell, 2009). By contrast, the 93-fold and 267-fold increases in the expression of MET and dMRP, respectively, were much lower in tubules of flies on the MTX plus PBO diet than in tubules of flies reared on MTX alone, where increases of 3500-fold and 1280-fold are seen for MET and dMRP, respectively (Chahine and O'Donnell, 2009). Taken together, the results of the present study indicate that PBO induces an alteration in expression of both detoxification genes and transporter genes. Increases in the expression of MET and dMRP in the latter may contribute to the doubling in MTX secretion seen in our functional studies.

In mammals, the main metabolite of MTX is 7-hydroxymethotrexate and both compounds are transported by MRP2 (Vlaming et al., 2009). In human liver, MTX may be metabolized by aldehyde oxidase and xanthine oxidase, but there is no evidence for interactions of MTX with P450 enzymes (Chládek et al., 1997). Our results show that MTX is metabolized within the tubules, and exposure to dietary PBO is associated with an increase in the proportion of the metabolite in the secreted fluid. This increase could reflect either an increase in the activity of enzymes that metabolize MTX and that are increased by PBO, or it could result from enhanced transport of MTX metabolites, given that PBO also alters the expression of MET and dMRP.

Treatments that alter GST gene expression or activity also increase MTX secretion

Our results indicate a reduction in the levels of gene expression for GstD1 and GstD5 in tubules of flies reared on a phenol-enriched diet for 10 generations. Relative to controls, there is either a reduction or no change in the levels of GstE1 in tubules of flies reared on MTX or phenol. However, when both MTX and phenol are present in the diet, the levels of GstE1 mRNA increase more than fourfold. This finding indicates that the response to the two toxins combined can be very different than the response to either toxin on its own. Our results also emphasize that the responses to PBO can be very different in whole flies relative to the tubules. Microarray studies show that topical PBO application is associated with 2.1- to 4.3-fold increases in gene expression for GstD1, GstD5 and GstE1 in whole flies (e.g. Willoughby et al., 2007), whereas dietary PBO is associated with decreases in expression of GstD1 and GstD5 in the tubules and a 1.5-fold increase in GstE1. It is important to exercise caution in inferring changes in enzymatic actvity of GSTs in the tubules because whole-fly studies indicate that phenol exposure may result in increases in GST enzymatic activity but no change in the levels of mRNA for GstD1, GstD5, GstD8 and GstE1 and a reduction in the levels of mRNA for GstD4 and GstD7 (Shen et al., 2003; Ding et al., 2005).

Our functional studies also showed that MTX secretion more than doubled in tubules of flies reared on the phenol-enriched diet for seven to 10 generations relative to controls. It thus appears that treatments that are known to stimulate GST activity in whole flies and alter GST gene expression in whole flies and in the tubules are also associated with increases in the secretion of the type II organic anion MTX.

At present, we do not know whether this increase in MTX secretion and that seen in tubules of flies reared on phenol-enriched diet reflects increases in the activity or expression of organic anion transporters or if the products of the actions of GSTs or other enzymes on MTX are better substrates for transporters such as dMRP and MET. In mammals, MRPs are well known as export pumps for xenobiotics conjugated to glutathione, glucuronate or sulfate (Homolya et al., 2003).

Alterations of fluid secretion rate

Exposure of flies to phenol for multiple generations alters not only the rate of MTX secretion but also the rate of fluid secretion. Previous studies have shown that acute or chronic exposure to the type I organic anions salicylate or fluorescein or chronic exposure to the type II organic anion MTX is also associated with increases in the secretion rates of both fluid and MTX (Chahine and O'Donnell, 2010). Type I organic anions are typically small (<400 Da), hydrophilic and univalent, whereas type II organic anions are larger (>400 Da), amphiphilic and often polyvalent (Wright and Dantzler, 2004). It was first proposed for *Calliphora erythrocepha*, whose tubules have a high permeability to solutes, that an increase in tubule fluid secretion rate could increase transepithelial organic anion transport by minimizing passive backflux (Maddrell et al., 1974). In essence, the increase in fluid secretion rate tends to lower the concentration of the organic anion in the tubule lumen, so that the concentration difference driving backflux from lumen to bath is reduced. Increases in fluid secretion rate indicate that dietary exposure to organic anions must also lead to increases in rates of transport of inorganic ions (Na^+, K^+ and Cl^-), which drive the flow of osmotically obliged water. These increases may result from increases in stellate cell chloride permeability and/or increases in the activity of the V-type H^+-ATPase that energizes active transport of alkali cations. Increases in fluid secretion rate in tubules isolated from flies reared on diets enriched in salicylate do not appear to be due to the effects of circulating diuretic factors or changes in the basal level of intracellular second messengers such as cAMP (Ruiz-Sanchez and O'Donnell, 2007).

In this study, the increase in fluid secretion rate in response to dietary exposure to phenol for seven to 10 generations suggests that enhancement of detoxification pathways in the Malpighian tubules may also result in increases in fluid secretion rates. By contrast, dietary exposure to PBO was not associated with an increase in fluid secretion rate, although MTX secretion does increase in response to dietary PBO. Nonetheless, the results suggest that induction of detoxification pathways may lead to a coordinated increase in MTX secretion, possibly through increases in the activity of organic anion transporters such as MET, dMRP and OATP, and at the same time may result in reduced diffusive backflux of organic anions from lumen to bath through an increase in fluid secretion rate.

Recent studies of mammalian systems provide clues to the possible mechanisms by which compounds such as PBO and phenol could regulate phase I and phase II detoxification pathways as well as phase III transporters. The so-called 'orphan' nuclear receptors such as the pregnane-X-receptor (PXR) and the constitutive androstane receptor (CAR) are known to regulate expression of P450 genes as well as genes for the transporters MDR1, MRP3 and OATP2 in mice (Staudinger et al., 2003; Wang and LeCluyse, 2003; Xu et al., 2005). In *Drosophila* tubules, one possible means by which xenobiotics could alter expression of genes for P450 enzymes, GSTs and transporters is through DHR96, the *Drosophila* ortholog of the vertebrate PXR/CAR family of nuclear receptors (LeGoff et al., 2006). Further studies of the regulation of detoxification and excretion pathways in *Drosophila* tubules will contribute to our understanding of how such processes are linked in other animals and may also aid development of novel control strategies for pest species of insect.

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REFERENCES

Abu-Qare, A. W., Elmasry, E. and Abou-Donia, M. B. (2003). A role for P-glycoprotein in environmental toxicology. *J. Toxicol. Environ. Health B Crit. Rev.* **6**, 279-288.
Bard, S. M. (2000). Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* **48**, 357-389.
Bijelic, G., Kim, N. and O'Donnell, M. J. (2005). Effects of dietary or injected organic cations on larval *Drosophila melanogaster*: Mortality and elimination of tetraethylammonium from the hemolymph. *Arch. Insect Biochem. Physiol.* **60**, 93-103.
Chahine, S. and O'Donnell, M. J. (2009). Physiological and molecular characterization of methotrexate transport by Malpighian tubules of adult *Drosophila melanogaster*. *J. Insect Physiol.* **55**, 927-935.
Chahine, S. and O'Donnell, M. J. (2010). Effects of acute or chronic exposure to dietary organic anions on secretion of methotrexate and salicylate by Malpighian tubules of *Drosophila melanogaster* larvae. *Arch. Insect Biochem. Physiol.* **73**, 128-147.
Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007). Using FlyAtlas to identify better *Drosophila* models of human disease. *Nat. Genet.* **39**, 715-720.
Chládek, J., Martínková, J. and Sispera, L. (1997). An *in vitro* study on methotrexate hydroxylation in rat and human liver. *Physiol. Res.* **46**, 371-379.
Cole, S. P. and Deeley, R. G. (2006). Transport of glutathione and glutathione conjugates byMRP1. *Trends Pharmacol. Sci.* **27**, 438-446.
Ding, K., Chien, Y. and Chien, C. (2005). Reducing the expression of glutathione transferase D mRNA in *Drosophila melanogaster* exposed to phenol and aniline. *Environ. Toxicol.* **20**, 507-512.
Dow, J. A. and Davies, S. A. (2006). The Malpighian tubule: rapid insights from post-genomic biology. *J. Insect Physiol.* **52**, 365-378.
Dow, J. A. T., Maddrell, S. H. P., Gortz, A., Skaer, N. J. V., Brogan, S. and Kaiser, K. (1994). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421-428.
Feyereisen, R. (1999). Insect P450 enzymes. *Annu. Rev. Entomol.* **44**, 507-533.
Giraudo, M., Unnithan, G. C., Le Goff, G. and Feyereisen, R. (2010). Regulation of cytochrome P450 expression in *Drosophila*: genomic insights. *Pestic. Biochem. Physiol.* **97**, 115-122.
Henderson, G. B. and Zevely, E. M. (1985). Characterization of the multiple transport routes for methotrexate in L1210 cells using phthalate as a model anion substrate. *J. Membr. Biol.* **85**, 263-268.
Homolya, L., Váradi, A. and Sarkadi, B. (2003). Multidrug resistance-associated proteins: export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors* **17**, 103-114.
Jensen, H. R., Scott, I. M., Sims, S, Trudeau, V. L. and Arnason, J. T. (2006). Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper nigrum*. *J. Agric. Food Chem.* **54**, 1289-1295.
LeGoff, G., Hilliou, F., Siegfried, B. D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R. H. and Feyereisen, R. (2006). Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. *Insect Biochem. Mol. Biol.* **36**, 674-682.
Linton, S. M. and O'Donnell, M. J. (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 1561-1570.
Maddrell, S. H. P., Gardiner, B. O. C., Pilcher, D. E. M. and Reynolds, S. E. (1974). Active transport by insect Malpighian tubules of acidic dyes and of acylamides. *J. Exp. Biol.* **61**, 357-377.
Nawata, C. M. and Wood, C. M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* **211**, 3226-3236.
O'Donnell, M. J. and Rheault, M. R. (2005). Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules and gut of *Drosophila melanogaster*. *J. Exp. Biol.* **208**, 93-104.
O'Donnell, M. J., Dow, J. A. T., Huesmann, G. R., Tublitz, N. J. and Maddrell, S. H. (1996) Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **199**, 1163–1175.
Ranson, H. and Hemingway, J. (2005). Mosquito glutathione transferases. *Methods Enzymol.* **401**, 226-241.
Rheault, M. R., Plaumann, J. S. and O'Donnell, M. J. (2006). TEA and nicotine transport by the Malpighian tubules of insects. *J. Insect Physiol.* **52**, 487-498.
Roberts, D. B. and Stander, G. N. (1998). *Drosophila: A Practical Approach*. Oxford: Oxford University Press.
Ruiz-Sanchez, E. and O'Donnell, M. J. (2007). Effects of chronic exposure to dietary salicylate on elimination and renal excretion of salicylate by *Drosophila melanogaster* larvae. *J. Exp. Biol.* **210**, 2464-2471.
Shen, S., Chien, Y. and Chien, C. (2003). Induction of glutathione S-transferases activities in *Drosophila melanogaster* exposed to phenol. *Arch. Insect Biochem. Physiol.* **53**, 80-91.
Staudinger, J. L., Madan, A., Carol, K. M. and Parkinson, A. (2003). Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab. Dispos.* **31**, 523-527.
Vlaming, M. L., van Esch, A., Pala, Z., Wagenaar, E., van de Wetering, K., van Tellingen, O. and Schinkel, A. H. (2009). Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. *Mol. Cancer Ther.* **8**, 3350-3359.
Wang, H. and LeCluyse, E. L. (2003). Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin. Pharmacokinet.* **42**, 331-357.
Wang, J., Kean, L., Yang, J., Allan, A. K., Davies, S. A., Herzyk, P. and Dow, J. A. (2004). Function-informed transcriptome analysis of *Drosophila* renal tubule. *Genome Biol.* **5**, R69.
Willoughby, L., Batterham, P. and Daborn, P. J. (2007). Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Manag. Sci.* **63**, 803-808.
Wright, S. H. and Dantzler, W. H. (2004). Molecular and cellular physiology of renal organic cation and anion transport. *Physiol. Rev.* **84**, 987-1049.
Xu, C., Li, C. Y. and Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* **28**, 249-268.
Yang, J., McCart, C., Woods, D. J., Terhzaz, S., Greenwood, K. G., ffrench-Constant, R. H. and Dow, J. A. (2007). A *Drosophila* systems approach to xenobiotic metabolism. *Physiol. Genomics.* **30**, 223-231.