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A novel role of *Drosophila* cytochrome *P450-4e3* in permethrin insecticide tolerance

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ABSTRACT

The exposure of insects to xenobiotics, such as insecticides, triggers a complex defence response necessary for survival. This response includes the induction of genes that encode key Cytochrome P450 monooxygenase detoxification enzymes. *Drosophila melanogaster* Malpighian (renal) tubules are critical organs in the detoxification and elimination of these foreign compounds, so the tubule response induced by dietary exposure to the insecticide permethrin was examined. We found that expression of the gene encoding *Cytochrome P450-4e3* (*Cyp4e3*) is significantly up-regulated by *Drosophila* fed on permethrin and that manipulation of *Cyp4e3* levels, specifically in the principal cells of the Malpighian tubules, impacts significantly on the survival of permethrin-fed flies. Both dietary exposure to permethrin and *Cyp4e3* knockdown cause a significant elevation of oxidative stress-associated markers in the tubules, including H_2O_2 and lipid peroxidation byproduct, HNE (4-hydroxynonenal). Thus, *Cyp4e3* may play an important role in regulating H_2O_2 levels in the endoplasmic reticulum (ER) where it resides, and its absence triggers a JAK/STAT and NF-κB-mediated stress response, similar to that observed in cells under ER stress. This work increases our understanding of the molecular mechanisms of insecticide detoxification and provides further evidence of the oxidative stress responses induced by permethrin metabolism.

1. Introduction

The evolution of insecticide resistance is a continuing problem in the control of insect pest species. One major mechanism of insecticide resistance involves transcriptional induction of detoxification enzymes that are capable of metabolizing insecticides, i.e., the P450 monooxygenases (P450s), the glutathione-S-transferases (GSTs) and the carboxylesterases (Feyereisen, 1995; Daborn et al., 2002; Enayati et al., 2005; Heidari et al., 2005). Increased activity of members from all these families has been associated with pyrethroid resistance in different insect species (Jamroz et al., 2000; Kasai and Scott, 2000; Vontas et al., 2001).

The synthetic pyrethroids are among the most commonly used pesticides for controlling agricultural and insect pests. Distinct classes of pyrethroids have been identified, based on different behavioural, neuropsychological and biochemical properties (Soderlund and Bloomquist, 1989; Shafer et al., 2005; Breckenridge et al., 2009). In addition to its functions as a neurotoxin, the induction of oxidative stress conditions in different tissues following pyrethroid exposure has been proposed as the major cause of cytotoxicity and ecotoxicity (Hill, 1989; Banerjee et al., 1999).

The cytochrome P450s (monooxygenases) compose a large enzyme superfamily involved in the detoxification of many xenobiotics (Feyereisen, 1999) and several studies have shown that increased cytochrome P450s catalysed metabolism is an important mechanism of pyrethroid resistance in many insect species (Tomita et al., 1995; Maitra et al., 1996; Pittendrigh et al., 1997). *Drosophila* Malpighian (renal) tubules are highly enriched for many P450 enzymes, GSTs, esterases and multidrug resistance-associated proteins (MRPs) (Dow and Davies, 2006; Chintapalli et al., 2007; Chahine and O'Donnell, 2009), and the importance of the tubules in xenobiotic metabolism has previously been demonstrated, by manipulation of a single P450 gene (*Cyp6g1*) in the tubules, which altered the survival of the whole fly following exposure to dichlorodiphenyltrichloroethane (DDT) (Yang et al., 2007).

Recent studies have shown increased reactive oxygen species (ROS) production upon permethrin (PM) exposure in rat adrenal pheochromocytoma cells or cardiac myocytes (Hu et al., 2010; Vadhana et al., 2011). Thus, we examined if the oxidative effects induced by PM metabolism are recapitulated in an organotypic context, the Malpighian (renal) tubule, an important epithelial tissue for xenobiotic handling. In the present study, *Drosophila melanogaster* was used as a simple genetic model to investigate the role of Cytochrome *P450-4e3* (*Cyp4e3*) in oxidative stress responses. Here, we show that manipulation of *Cyp4e3* in the Malpighian tubule results in a continuous production of ROS, ultimately causing lipid peroxidation, ER stress, and death upon PM exposure.

2. Materials and methods

2.1. Drosophila stocks

All lines were maintained on a standard *Drosophila* diet at 22 °C, 55% humidity with a 12:12 h light:dark photoperiod. Wild-type Canton-S (CS), 'cantonised' *white*[1118] and the *UAS-Catalase RNAi* (TRIP line) were obtained from the Bloomington Stock Center (Bloomington, IN). The *UAS-cyto-roGFP2-Orp1*, a cytosolic hydrogen peroxide reporter line was obtained from the German Cancer Research Centre, Heidelberg, Germany (Albrecht et al., 2011). The *capaR-GAL4* driver, which drives expression in adult tubule principal cells, was previously generated in-house (Terhzaz et al., 2012). The *c564-GAL4* driver specific to the fat body (Takehana et al., 2004) was a kind gift of Prof. Shoichiro Kurata, Tohoku University, Japan.

2.2. Generation of UAS-Cyp4e3 RNAi and UAS-Cyp4e3-eYFP(Venus) transgenic lines

To generate the construct for heritable RNAi of the *Cyp4e3* gene, an inverted repeat of a 270-base pair fragment of *Cyp4e3* was generated by PCR using the primers described in Table S1 and cloned in opposite orientations separated by a ftz intron into the P-element vector pRISE (Kondo et al., 2006). To generate the construct for overexpression of the *Cyp4e3* gene, the coding sequence of *Drosophila Cyp4e3* (*CG4105*) was amplified by PCR using DreamTaq green PCR master mix (Thermo Scientific) with the primer pairs listed in Table S1. The product was cloned into a pENTR donor vector (Invitrogen) and transferred to the pTWV destination vector (DGRC) using Gateway LR Clonase II Enzyme mix according to the manufacturer's instructions. The sequence integrity for each construct was confirmed by GATC Biotech, and transgenic lines were generated using standard methods for P-element-mediated germline transformation (BestGene Inc, USA).

2.3. Quantitative RT-PCR

For validation of whole fly or tubule mRNA expression, qRT-PCR was performed using an Opticon DNA engine 4 (Bio-Rad Technologies UK) with Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent, UK) and primer pairs described in Table S1. Data was normalized against the *rp49* standard and expressed as fold change compared to controls ± SEM (n = 3).

2.4. Immunofluorescence

Adult Malpighian tubules were dissected in Schneider's medium (Invitrogen, UK) and fixed with 4% (wt/vol) paraformaldehyde (PFA) for 20 min at room temperature. Mouse anti-GFP primary antibody (1:500, Zymed) and the rabbit anti-PDI (1:1000, Abcam) were used. Incubations in the primary antibodies were performed overnight. FITC or Texas red-conjugated affinity-purified goat anti-mouse or anti-rabbit antibodies (Jackson Immunologicals) were used in a dilution of 1:1000 for visualization of the primary antiserum. Samples were mounted on poly-L-Lysine (0.1% w/vol in H_2O, Sigma−Aldrich) covered 35 mm glass bottom dishes (MatTek Corporation, MA, USA) in Vectashield (Vector Laboratories, Burlingame, CA). Confocal images were taken by using a Zeiss LSM META 510 microscope and processed with LSM 510 image examiner and Adobe Photoshop CS 5.1.

2.5. Measurement of hydrogen peroxide and peroxidase activity

For tubule quantification of hydrogen peroxide (H_2O_2) levels or peroxidase activity, 20 pairs of rapidly dissected tubules from *D. melanogaster* wild-type male flies fed on PM or *Cyp4e3* transgenic flies were added to 60 μl of 5 mM sodium phosphate (pH 7.4, Invitrogen), ruptured by brief sonication to release cellular contents, and spun down briefly at 5000 g at 4 °C; 50 μl of the supernatant was used immediately to measure either H_2O_2 levels or
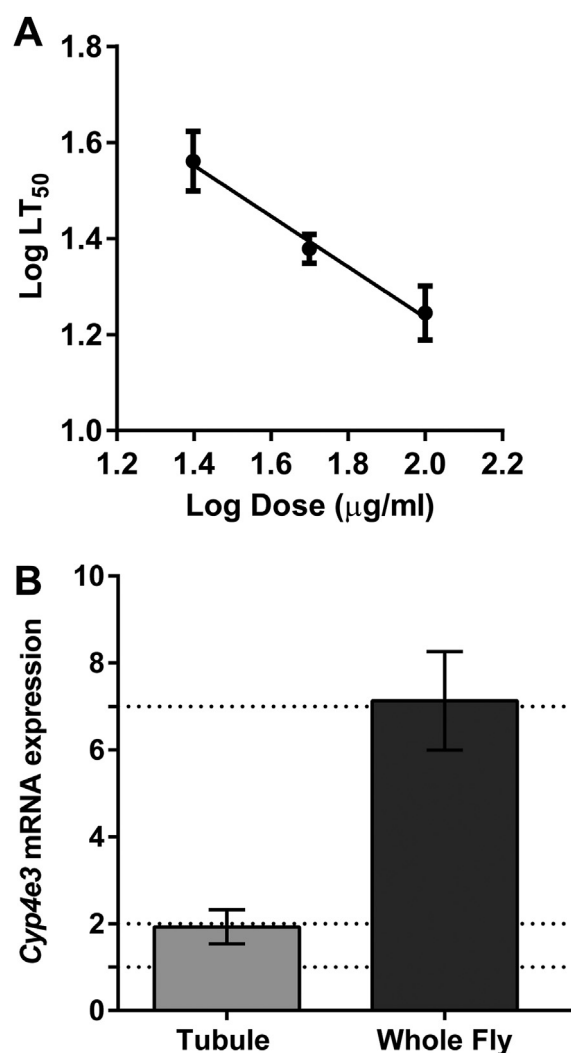
Fig. 1. PM toxicity assay and up-regulation of *Cyp4e3* mRNA levels. (A) Wild-type Canton-S male flies were exposed to increasing concentrations of PM, and survival recorded until 100% mortality was reached. Log of the median lethal time (LT_{50}) was plotted against the log of the dose (μg/ml), and error bars show 95% confidence intervals (n = 120–130 male adult wild-type flies for each concentration tested). (B) Relative tubule and whole animal *Cyp4e3* mRNA expression after 24 h feeding with either sucrose alone (control) or sucrose supplemented with 75 μg/ml PM. Data are expressed as fold change compared to non-treated control ± SEM (n = 3).

peroxidase activity using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) as previously described (Terhzaz et al., 2010). Total H_2O_2 levels and peroxidase activity were normalized to protein amounts in each homogenate using a Bradford assay (Bio-Rad).

For organotypic imaging, live and PFA fixed intact transgenic tubules expressing roGFP2 probes were allowed to adhere to glass-bottomed dishes coated with poly-L-lysine. To conserve the redox state of the roGFP2-based probes and prevent post-dissection oxidation, 20 mM of the thiol-alkylating agent N-Ethylmaleimide (NEM, Sigma–Aldrich) was added to Schneider's medium and fixation solution, with the exception for the live imaging experiments. The cytosolic hydrogen peroxide sensing reporter was assessed by treatment with H_2O_2 (25 μM, Sigma–Aldrich) or dithiothreitol (DTT, 10 mM, Invitrogen) by imaging on a Zeiss 510 meta confocal system coupled to an inverted Zeiss microscope. The reporter was excited at 405 nm and 488 nm, and the emission was filtered through a 505–530 nm band pass filter. Real time images of tubule-expressed roGFP2 were captured, and average fluorescence values for specified regions of interest were calculated for each frame. Images were saved as 16-bit tiff files and processed using ImageJ. Background was subtracted using the function "disable smoothing". Images were then converted to a 32-bit format and displayed in false colours using the lookup table "fire".

2.6. Measurement of oxidative damage to lipids

For each sample, 20 pairs of tubules from male wild-type flies fed on PM or Cyp4e3 transgenic flies were added to 60 μl of 1X phosphate buffered saline (pH 7.4, Gibco), ruptured by brief sonication to release cellular contents, and spun down briefly at 5000 *g* at 4 °C. Protein amounts in each homogenate were quantified using a Bradford assay (Bio-Rad) and 10 μg/ml input protein were used. Triplicate samples were generated for each genotype. The lipid peroxidation byproduct, HNE (4-hydroxynonenal)-Histidine protein adduct, was measured using the OxiSelect HNE-His adduct ELISA Kit (Cell Biolabs) according to the manufacturer's protocol. The signals generated by the colorimetric assays were detected at 450 nm on a Mithras LB 940 plate reader luminometer (Berthold technologies).

2.7. Xenobiotic survival assays

For feeding assays, *D. melanogaster* wild-type or Cyp4e3 transgenic 7-day-old flies were briefly anesthetized on CO_2, starved for 4–6 h and then placed in 30-ml cellulose-capped vials containing 5 ml of 1% aqueous agarose/1% sucrose medium (control) or fed with either 1% H_2O_2 or specified concentration of permethrin or fenvalerate (all Sigma–Aldrich). The vials were placed in an incubator at 22 °C, 55% humidity with 12:12 h light:dark photoperiod and checked for dead flies every 3–6 h until no living flies were left. All experiments were run in triplicate with at least 30 flies in each run of a specified genotype. For topical application, *D. melanogaster* wild-type or transgenic 7-day-old male flies were briefly immobilised on CO_2, and a 69 nl volume of permethrin (0, 2, 5, 6, 8, 10, 12.5, 15, 17.5 and 20 ng/fly doses which were then converted to ng/mg by dividing the dose by the average weight, n = 25 flies with at least 3 biological replicates) was applied to the thoracic notum using a microinjector (NanojectII, Drummond Scientific Company). Treated flies were placed in an inverted food vial to prevent mortality through adherence to the food and vials were placed in the same incubator at 22 °C (55% humidity with 12:12 light:dark h photoperiod) because pyrethroids have a temperature dependent toxicity. Mortality data was recorded 24 h after application.

2.8. Data analysis

A two-tailed Student's *t*-test (for two independent groups: non treated vs treated), or one-way ANOVA (for three independent groups: *capaR-Gal4/ +* vs *Cyp4e3 RNAi/ +* or *Cyp4e3/ +* vs *capaR-Gal4>Cyp4e3 RNAi* or *capaR-Gal4>Cyp4e3*) was used to compare whole fly or tubule mRNA expression, H_2O_2 levels, peroxidase activity and lipid peroxidation in wild-type Canton-S and transgenic flies. For ANOVAs, P-values were adjusted with the Sidak multiple comparisons test. For insecticide feeding assays, significance was assessed by the LogRank (Mantel–Cox) test. Data was plotted using GraphPad Prism 6.0 software (GraphPad Software Inc., USA). For topical application assays, data was corrected using Microsoft Excel for control mortality using Abbott's formula before using Probit analysis to linearize the cumulative Gaussian distribution. Probits were plotted against the log of the dose per mg body weight using Graphpad Prism 6.0 software. A linear regression analysis was performed and the 50% lethal dose (LD_{50}) determined. Statistical significance between LD_{50}s of different genotypes was determined using the Litchfield & Wilcoxon method.

3. Results

### 3.1. Cyp4e genes expression are enriched in Malpighian tubules

All three *Cyp4e* genes encoded by the *D. melanogaster* genome show highly abundant expression in adult Malpighian tubules and fat body (Chintapalli et al., 2007) (Supplementary Fig. S1A and B), which are tissues acknowledged to have functions analogous to the human kidney and liver respectively. All three *Cyp4e* genes encode monooxygenases which catalyse many reactions involved in xenobiotic metabolism. The genes *Cyp4e1* (*CG2062*) and *Cyp4e2* (*CG2060*) are very similar as they share 79% identity at the AA sequence level, and their close proximity at the genomic level could imply that these genes may have been derived from a DNA-based tandem duplication. As well as being expressed in the canonical detoxification tissues, *Cyp4e1* also has strong expression in accessory glands, and could potentially metabolize endogenous compounds such as hormones, fatty acids, and steroids. Based on the tissue distributions, high expression levels and gene function, we focused our attention on the functional role of the *Cyp4e3* (*CG4105*) gene in Malpighian tubules.

Recently, a *Drosophila* Malpighian tubule microarray study showed the gender-specific expression of several defence genes in tubules (Chintapalli et al., 2012). For example, males preferentially express a series of cytochrome P450s, including the cardinal insecticide resistance locus, *Cyp6g1* (2.5x enrichment). In contrast, female-enriched genes include the detoxification enzyme cytochrome P450 *Cyp4e3* (6.5x enrichment). This finding was confirmed by qRT-PCR, where wild-type female tubule *Cyp4e3* mRNA levels were 7-fold higher compared to males (P = 0.0016, Supplementary Fig. S1C). The gender-specific expression of several defence genes in tubules implies that males and females meet differing defence challenges.

3.2. Permethrin toxicity in D. melanogaster and Cyp4e3 response

We first tested PM toxicity using feeding survival assays. Wild-type male flies were fed on increasing concentrations of PM and the LD_{50} after 24 h exposure was established at a concentration of approximately 75 μg/ml (Fig. 1A).

Exposure of insects to insecticides triggers a defence response, inducing genes that encode key detoxification enzymes. We therefore determined if dietary exposure to PM could induce changes in *Cyp4e3* mRNA levels. Wild-type male flies were treated
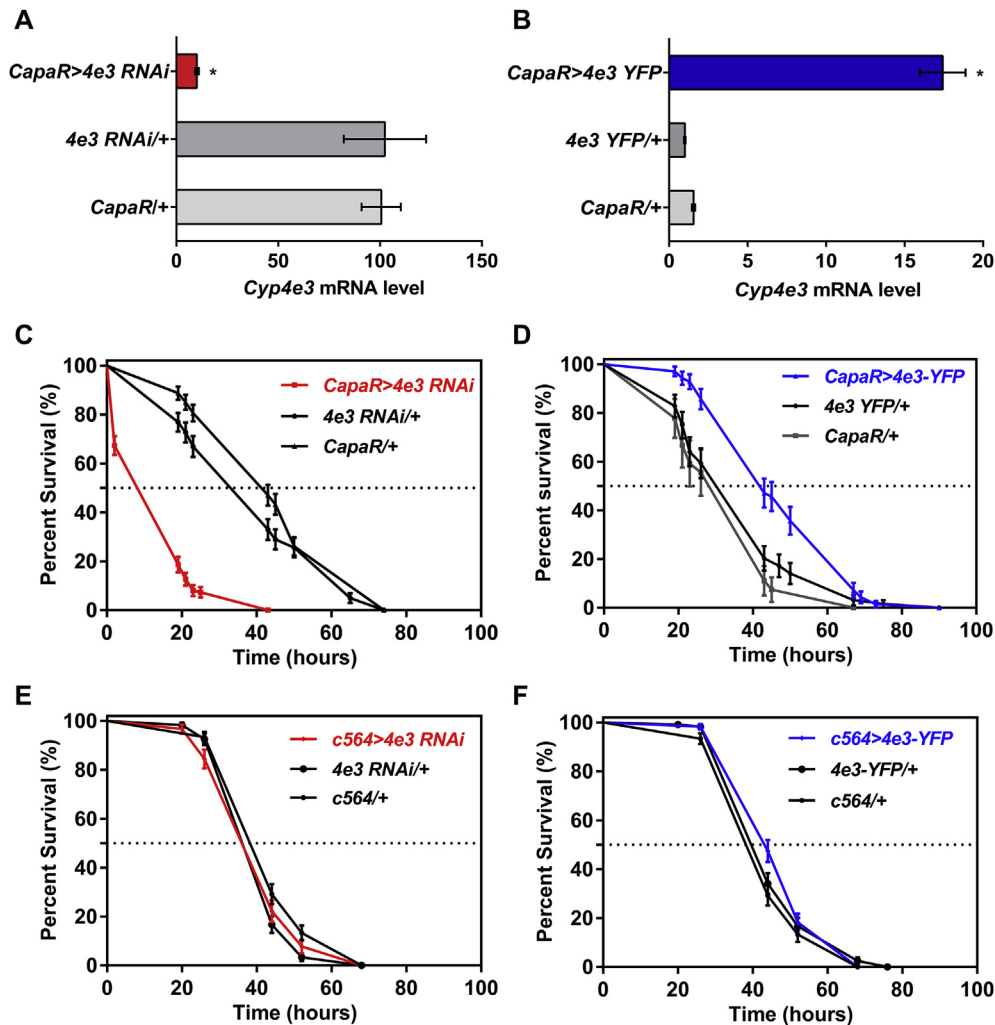
Fig. 2. Consequence of loss- and gain-of-function of the *Cyp4e3* gene upon PM exposure. (A—B) Down- and up-regulation of *Cyp4e3* in principal cells compared to both *Cyp4e3 RNAi/+* and *capaR/+* parental lines (*P < 0.05). The observed difference in sample quantity corresponds to (A) an approximately 90% decrease in *Cyp4e3* mRNA levels and (B) an approximately 17-fold increase of *Cyp4e3* mRNA expression. Data are expressed as (A) percentage and (B) fold change compared to parental controls ± SEM (n = 3). Effect of *Cyp4e3* manipulation has on PM dietary exposure (75 μg/ml). (C—D) Survival was significantly reduced after knockdown of *Cyp4e3* in just the principal cells of Malpighian tubules (C) while over-expression of *Cyp4e3* significantly increased survival compared to parental controls (D). In C and D, P < 0.001 was used as the significance level; Log-rank test; n = 100—130 male flies for the different genotypes. (E—F) Selective knockdown (E) or overexpression (F) of *Cyp4e3* in fat body using the *c564-GAL4* driver does not change sensitivity to PM (n > 210 male flies for the different genotypes).

for 24 h with either sucrose alone or sucrose supplemented with 75 μg/ml PM. Whole fly and tubule RNA was extracted from these animals and *Cyp4e3* mRNA expression determined by qRT-PCR. Dietary exposure to PM elicited significant increases in *Cyp4e3* mRNA levels (2- and 7-fold in tubule and whole fly, respectively) compared to untreated controls (Fig. 1B). This suggests that C*yp4e3* enrichment in adult tubules, fat body, carcass by 10.2, 8.9 and 5.7-fold respectively compared to the rest of the fly (Flyatlas.org) may explain the increases of *Cyp4e3* mRNA levels in the whole fly compared to that observed in tubules. Although only a small subset of tissues have been tested, these data suggest that *Cyp4e3* is an insecticide-responsive gene and that several detoxification tissues, including tubule, fat body and carcass, might also be involved in the PM response.

3.3. Manipulation of Cyp4e3 expression alters survival to permethrin

The *GAL4/UAS* binary expression system allows the knockdown or overexpression of any genes in specific cells within an otherwise

normal organism, under control of the appropriate GAL4 driver lines. We therefore generated *UAS-dsRNA-* and *eYFP*-tagged- *Cyp4e3* transgenic fly lines and drove expression of both constructs in tubule principal cells using the tubule principal cell specific *capaR-Gal4* line. The *UAS-Cyp4e3 RNAi* line produced a strong knockdown (~90%) while the *UAS-Cyp4e3-eYFP* showed an ~17-fold increase in overall tubule expression of *Cyp4e3* mRNA when driven in principal cells compared to parental control lines (Fig. 2A,B).

Given the induction of *Cyp4e3* mRNA levels by PM exposure, we investigated the role of the *Cyp4e3* gene in organismal insecticide survival. Targeted knockdown of *Cyp4e3* specifically to tubule principal cells in males resulted in significantly reduced survival under PM dietary exposure (median life span of 19 h, as compared with the median control life span of 43 h) (Fig. 2C). Conversely, male flies with increased *Cyp4e3* levels exhibited significantly extended survival compared to parental controls (Fig. 2D) (in C and D, P < 0.001; Logrank test, Mantel—Cox). These results clearly show that the whole insect tolerance to insecticide is related to the expression level of *Cyp4e3* in the metabolically active tubule principal cells. A similar pattern in PM survival was observed in females
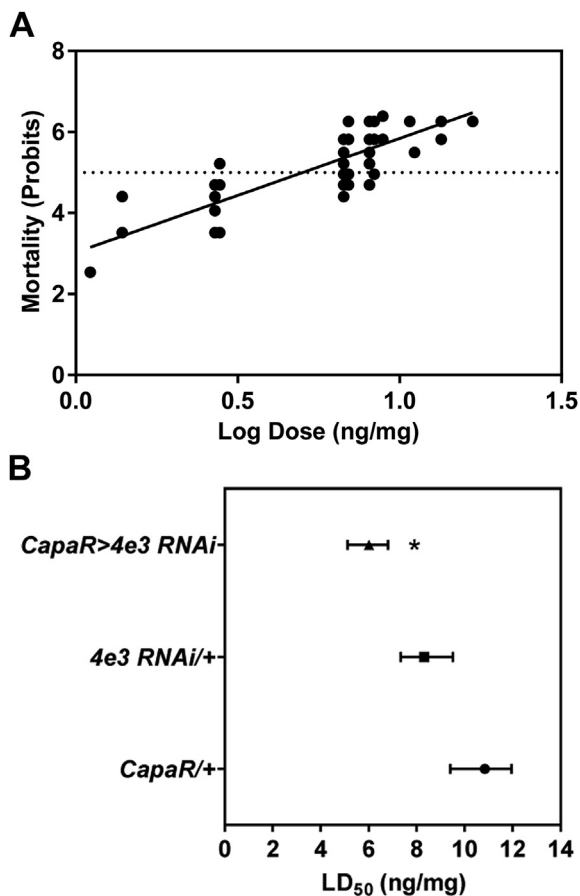
Fig. 3. PM topical application and the effect on *Cyp4e3* knockdown flies. (A) Wild-type Canton-S male flies were exposed to PM, and mortality measured at 24 h. Probits were plotted against the Log of the dose per mg body weight (n = 150 male adult wild-type flies for each concentrations tested). (B) For the different genotypes, a linear regression analysis was performed to determine the dose at which 50% mortality was observed (LD$_{50}$). Significant change in LD$_{50}$ was tested using the Litchfield & Wilcoxon method. Knockdown of *Cyp4e3* in principal cells of Malpighian tubules significantly reduced the PM LD$_{50}$ dose compared to both parental controls (*P < 0.001; n > 380 male flies for the different genotypes).

(Supplementary Fig. S2A and B). The enrichment of *Cyp4e3* mRNA in the fat body prompted us to assess if the manipulation of *Cyp4e3* levels, specifically in this tissue, impacted on the survival of PM-fed flies. We show that selective knockdown or overexpression of *Cyp4e3* in the fat body (Supplementary Fig. S3) does not change sensitivity to PM (Fig. 2E and F). These data are consistent with those obtained for the DDT insecticide resistance gene, *Cyp6g1* (Yang et al., 2007), and suggest that the Malpighian tubules constitute the key tissue for insecticide metabolism.

We next examined if the survival phenotype observed in the *Cyp4e3* knockdown flies could be recapitulated using a member of the type II synthetic pyrethroid insecticide family. Fenvalerate, characterised by the presence of an alpha-cyano group, is known to be more resistant to degradation and thus more potent than the type I PM. We therefore tested if manipulation of *Cyp4e3* expression alters survival to dietary fenvalerate at a concentration ~15-fold lower than PM. As expected, manipulation of *Cyp4e3* expression, specifically in the principal cells of the Malpighian tubule, impacts significantly on fly survival upon fenvalerate exposure (Supplementary Fig. S2C and D).

In addition to the "kidney" tissue, the carcass expresses high *Cyp4e3* mRNA levels, so we performed PM topical application survival assays. Wild-type Canton-S male flies were treated with different PM doses and the mortality after 24 h exposure was determined (Fig. 3A). Although the carcass and fat body tissue can also impact on the topical application of insecticides, the tubules are more likely to handle topically applied agents that appear in the hemocoel. Selective knockdown of *Cyp4e3* in the principal cells of the Malpighian tubule significantly increases PM susceptibility of the whole organism (P < 0.001 against both parental controls) and demonstrate a major role of the tubule in insecticide metabolism (Fig. 3B).

3.4. Cyp4e3 knockdown flies display increased tubule oxidative stress

Which mechanisms contribute to the deleterious effects on survival observed in the *Cyp4e3* knockdown flies on PM diet? Previous studies have shown an increase in ROS production upon PM exposure (Hu et al., 2010; Vadhana et al., 2011). To determine if the oxidative effects induced by the insecticide metabolism is recapitulated in wild-type flies fed with PM, we measured tubule H$_2$O$_2$ levels. Dietary exposure to PM was associated with a significant increase in H$_2$O$_2$ levels in the Malpighian tubules in comparison with tubules from flies reared on the control diet (Fig. 4A). We next investigated if *Cyp4e3* knockdown flies displayed a significant elevation in oxidative stress, leading to reduced survival on PM exposure. Data shown in Fig. 4A revealed that H$_2$O$_2$ production in isolated *Cyp4e3* knockdown tubules is significantly increased compared to either parental line, suggesting that *Cyp4e3* knockdown mediates increased H$_2$O$_2$ production in tubules principal cells. Therefore, *Cyp4e3* and PM each directly modulate tubule H$_2$O$_2$ production and these effects could be additive. This increased H$_2$O$_2$ production in tubules, presumably by the mitochondria, can either result from Superoxide Dismutase actions on superoxide anions (Miwa et al., 2003; Murphy, 2009) or could be the consequence of decreased tubule peroxidase activity. Data shown in Fig. 4B revealed that, indeed, flies fed on PM or *cyp4e3* knockdown flies had significantly decreased peroxidase activity compared to normally-fed flies and parental lines respectively. These data suggest that PM exposure or the absence of *Cyp4e3* plays an important role in regulating tubule antioxidant function leading to oxidative stress. Interestingly, we found that tubules dissected from flies fed with H$_2$O$_2$ or the superoxide generator paraquat elicited significant increases in *Cyp4e3* mRNA levels (more than fourfold compared to controls) (Supplementary Fig. S4A), and manipulation of *Cyp4e3* in the tubules alters the survival of the whole fly during exposure to H$_2$O$_2$ (Supplementary Fig. S4B and C), suggesting that *Drosophila Cyp4e3* may play a role in the regulation of cellular redox balance.

To further validate these results, we performed *in vivo* imaging using redox sensitive GFPs (roGFPs) (Dooley et al., 2004) that have been converted into specific probes for H$_2$O$_2$ by coupling them to the microbial H$_2$O$_2$ sensor oxidant receptor peroxidase 1 (Orp1) (Gutscher et al., 2009). The cytosolic *roGFP2-Orp1* probe was targeted to Malpighian tubules principal cells and the fluorescence of the reporter imaged in intact live tubules to determine H$_2$O$_2$ levels. Intact tubules exhibited higher levels of H$_2$O$_2$ compared to other tissues as evidenced by the stronger level of oxidation of the H$_2$O$_2$ probe, confirming the intense metabolic activity occurring within the tubule principal cells. Next, we tested the H$_2$O$_2$ probe for *in situ* responsiveness, and Supplementary Fig. S5A and B show that, as expected, exogenous application of DTT and H$_2$O$_2$ elicited complete probe reduction and oxidation respectively, which permitted calibration of the probe and therefore the quantification of H$_2$O$_2$ levels.

We also explored the possibilities of combining redox imaging with reverse genetics. For this purpose, we created a fly line expressing the H$_2$O$_2$ probe stably and specifically in the tubule principal cells that was used together with dsRNA constructs for
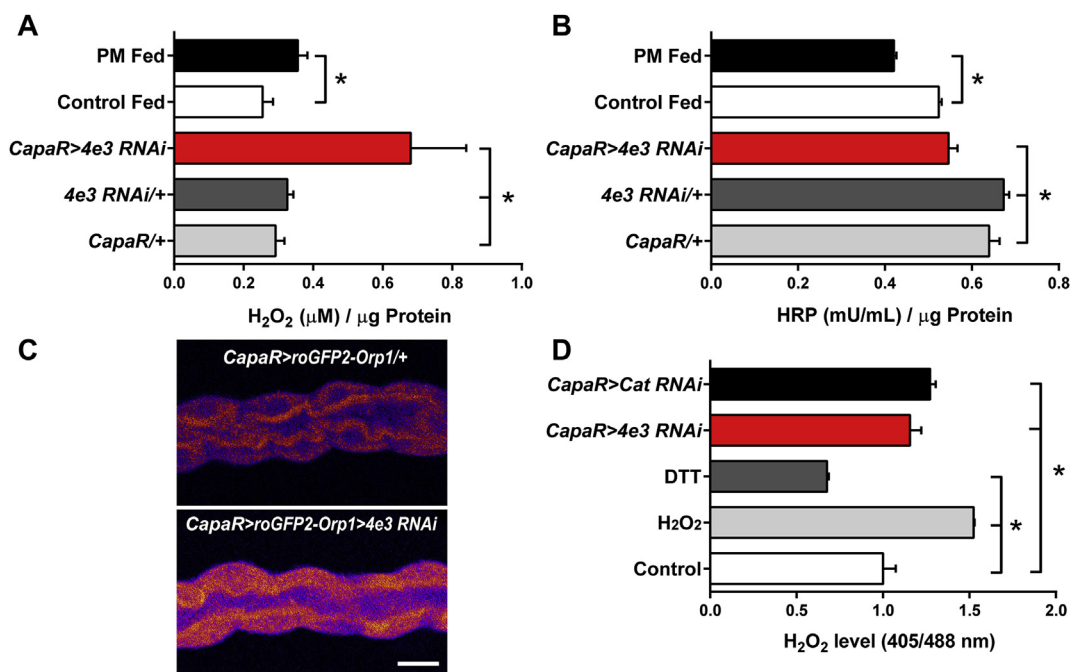
Fig. 4. PM fed and *Cyp4e3* knockdown flies display increased levels of H_2O_2 and decreased peroxidase activity. Tubules from wild-type Canton-S male flies fed for 24 h with either sucrose alone (control) or sucrose supplemented with 75 µg/ml PM and from *Cyp4e3* knockdown or parental controls were collected and homogenates assayed for (A) H_2O_2 levels and (B) peroxidase activity. Error bars represent SEM, n ≥ 3, independent samples of 20 pairs of tubules of male flies each in triplicate; *P < 0.05. (C) *Ex vivo* imaging of the cytosolic *roGFP2-Orp1* probe targeted to Malpighian tubule principal cells. Silencing of *Cyp4e3* leads to increased cytosolic H_2O_2 levels as evidenced by the higher oxidation of the specific H_2O_2 probe. Scale bar = 25 µm. (D) Redox state of the cytosolic *roGFP2-Orp1* probe targeted to Malpighian tubule principal cells in control (Schneider's medium), challenged with 25 mM H_2O_2, 10 mM DTT, or targeted *Cyp4e3* and *Catalase* knockdowns. Data from different tubule principal cells are expressed as the relative H_2O_2 level (405/488 nm) ± SEM (n ≥ 6), where *P < 0.05.

antioxidant genes. The Malpighian tubules constitute a major sources of ROS in the organism due to the high rates of metabolic activity, and play a significant role in the organismal response to oxidative stress (Terhzaz et al., 2010). Interestingly, the tubules have specific adaptations to counter the high production of ROS as the expression of genes encoding antioxidant enzymes are enriched in tubules compared to the rest of the fly. For example, the *Catalase* (*Cat*) gene, which is an important H_2O_2 scavenger, by catalysing the decomposition of hydrogen peroxide to water and oxygen, is enriched in tubules by 4.7-fold compared to the rest of the fly (Flyatlas.org). As expected, gene silencing of *Cat* led to significantly increased H_2O_2 levels in the cytosol compared to parental control lines (Supplementary Fig. S5C and D). Moreover, when *Cyp4e3* was silenced, we also observed a cytosolic increase in H_2O_2 levels in the Malpighian tubules (Fig. 4C,D). This suggests that silencing of *Cyp4e3* increases steady-state H_2O_2 levels in tubule principal cells and that *Cyp4e3* mediates production of H_2O_2 by potentially regulating enzyme activity, i.e. peroxidases.

3.5. Cyp4e3 knockdown flies display ER stress

Cytochrome P450s are membrane-bound enzymes commonly located in the endoplasmic reticulum (ER), the inner mitochondrial membrane and peroxisomes. Targeted expression of the eYFP-tagged *Cyp4e3* construct in the tubule principal cells enabled determination of the subcellular localization of the CYP4E3 protein. The eYFP fluorescence was seen throughout the cytosol with a clear reticular pattern (Fig. 5A), characteristic of ER, as demonstrated by co-localization with the antibody for the ER protein disulfide isomerase (PDI). As endogenous CYP4E3 is localized to the ER, and may play an important role in regulating H_2O_2 levels in the ER, we investigated the possible effect of targeted knockdown of *Cyp4e3* or PM dietary exposure on ER stress. ER redox status has to be

maintained as high levels of H_2O_2 may affect protein-folding capacity and lipid peroxidation which is the formation of unsaturated lipid oxidation products that occur as a result of oxidative damage. Samples prepared from flies fed on PM and *Cyp4e3* knockdown flies were immunoassayed for the detection and quantification of HNE-His adduct. HNE (4-Hydroxynonenal), a natural catabolite of lipid peroxidation, is capable of covalently binding to proteins and forming stable adducts, causing both structural and functional changes. Data shown in Fig. 5B revealed that the production of HNE-His adduct in isolated *Cyp4e3* knockdown tubules or PM fed tubules is significantly increased compared to either parental line and normally-fed flies, suggesting that *Cyp4e3* knockdown and PM dietary exposure mediates increased H_2O_2 and HNE production in tubule principal cells.

A number of conditions affecting ER function have been observed to cause the activation of the NF-κB pathways and JAK/STAT stress response pathways (Pahl and Baeuerle, 1997). This prompted an assessment of both immune response (Imd) and stress response (JAK/STAT) pathways in the *Cyp4e3* knockdown or PM fed flies to detect any disruption in homeostasis as revealed by the expression of "readout" genes that are specific for each pathway. The *D. melanogaster* orthologue of NF-κB transcription factor, Relish (Stoven et al., 2003), is associated with the expression of anti-microbial peptide (AMP) genes like *Diptericin* (Lemaitre et al., 1997). Interestingly, *Cyp4e3* knockdown flies exhibited a several fold elevation in the expression of *Diptericin* (Fig. 5C), clearly indicating the activation of the NF-κB pathway. We found that the expression of a downstream target of the JAK/STAT pathway, the cytokine-like protein *Turandot A* (*TotA*) (Ekengren and Hultmark, 2001), was also induced in these flies as well as in PM fed flies (Fig. 5C). The JAK/STAT signaling leads to activation of the transcription factor Stat92E, which then translocates to the nucleus and triggers the expression of *TotA*. Interestingly, it has been shown
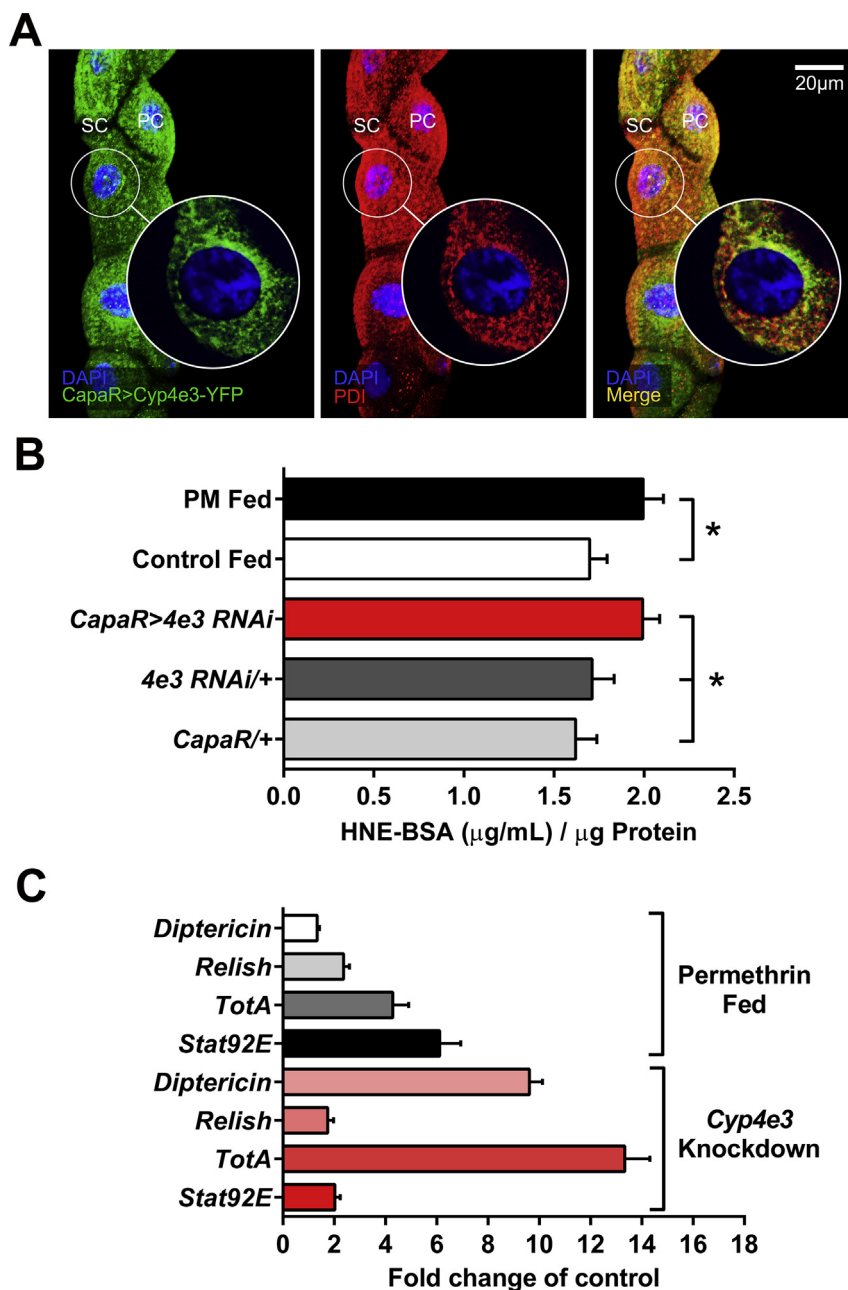
Fig. 5. PM fed and *Cyp4e3* knockdown flies display ER stress and increased immune and stress response genes. (A) Endoplasmic reticulum localization of CYP4E3. Tubule principal cells with eYFP fluorescence (green, left) and the antibody for the ER protein disulfide isomerase (PDI, red, middle) shows co-localization (yellow, merge, right). Maximum projection of confocal z-series with inserts showing single focal plane images of selected regions. Scale bar = 20 μm. (B) Tubules from wild-type Canton-S male flies fed for 24 h with 75 μg/ml PM and *Cyp4e3* knockdown flies and respective controls were collected and homogenates assayed for HNE-His adduct levels. Error bars represent SEM, n = 3, independent samples of 20 pairs of tubules each in triplicate; *P < 0.05. (C) Immune and stress genes were up-regulated in adult wild-type males fed for 24 h with 75 μg/ml PM and *Cyp4e3* knockdown flies. Data are expressed as fold change compared to controls ± SEM (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that Relish can also regulate the expression of *TotA* (Agaisse et al., 2003), so the high levels of *TotA* expression may be the result of both a JAK/STAT and NF-κB-mediated response to stress.

4. Discussion

An important mechanism of pyrethroid resistance involves increased expression of enzymes involved in insecticide metabolism. Insect Cytochrome P450 genes compose a large superfamily with approximately 90 members in the *D. melanogaster* genome.

Deciphering the individual enzymes responsible for insecticide metabolism is therefore a major challenge. Although several P450 genes and multiple tissues might impinge on insecticide detoxification, we have shown that targeted, cell specific manipulation of a single gene, *Cyp4e3*, in the Malpighian tubule principal cells of adult *Drosophila* alters the survival of the insect following pyrethroid feeding or topical application. A previous study has demonstrated that *Cyp4e3* is significantly up-regulated in a *Drosophila* strain resistant to the neonicotinoid insecticide Imidacloprid (Kalajdzic et al., 2012), and here we show that *Cyp4e3* is a

pyrethroid insecticide-responsive gene.

D. melanogaster is a useful model for the functional validation of genes involved in insecticide resistance. We have shown that gain- and loss-of-function of *Cyp4e3* significantly impacts on fly survival when fed on PM. The overexpression of *Cyp4e3* in tubules conferred an increased survival, while *Cyp4e3* knockdown a dramatically reduced survival. We found that both dietary exposure to PM and *Cyp4e3* knockdown causes a significant elevation in oxidative stress with increased H_2O_2 levels and lipid peroxidation in tubules. These findings corroborate a recent study describing the effect of pyrethroids on the oxidative stress response of *Helicoverpa armigera* larvae, along with their neurotoxic effects (Akbar et al., 2012) and further confirms that oxidative stress is one of the main mechanisms of pyrethroid action. Given the induction of *Cyp4e3* mRNA levels by the *in vivo* ROS generators paraquat, H_2O_2 and by PM dietary exposure, *Drosophila Cyp4e3* is likely to play a role in ROS metabolism. Silencing *Cyp4e3* in the tubule may result in a continuous production of ROS causing lipid peroxidation and reduced survival, while overexpression of *Cyp4e3* demonstrated a protective function on exposure to oxidants and PM.

Endogenous CYP4E3 localizes to the ER, and so may impact on the ER redox state by regulation of H_2O_2 levels causing a JAK/STAT and NF-κB-mediated stress response, similar to that observed in cells under ER stress (Radyuk et al., 2013). In agreement with regulation by the transcription factors Sta92E and Relish, cytokine-like protein *TotA* was induced in the *Cyp4e3* knockdown flies as well as in PM fed flies. The *Turandot* gene is induced in response to a variety of stressors, including heat, mechanical stress, ROS, and bacteria (Ekengren and Hultmark, 2001) and although *TotA* is known to be mainly expressed in the fat body, our data suggest that *TotA* plays a novel role in mediating stress responses in the Malpighian (renal) tubule. Furthermore, ER stress is known to cause an induction of immunity-related genes from the NF-κB pathway that is regulated through activation of the NF-κB transcription factor Relish. The up-regulation of this pathway in *Cyp4e3* knockdown flies is confirmed by the observed increase in tubule levels of *diptericin*. Ultimately, it would be interesting to manipulate both NF-κB and JAK/STAT stress response pathways in the whole fly during survival under PM exposure.

The main function of the monooxygenase system is the oxygenation of exogenous compounds, and xenobiotics undergo deactivation by Cytochrome P450 enzyme, either directly or by facilitated excretion from the body. Therefore, one might expect that the *Cyp4e3* gene would participate in the metabolism and excretion of PM by the tubules and thus, the effects on PM insecticide tolerance could be explained by variation in the effective dose experienced under each of the *Cyp4e3* transgenic manipulations. Further insight in to this possible mechanism could be obtained by assessment of either levels of PM in the fed flies or examining metabolite profiles of PM in the *Cyp4e3* transgenic flies. Now that an important Cytochrome P450 enzyme (*Cyp4e3*) involved in PM insecticide metabolism has been identified, determining if the *D. melanogaster* CYP4E3 protein is capable of catalysing the detoxification of pyrethroid insecticides might help in designing new strategies for the control of insect pest species.

Author contributions

S.T., J.A.T.D. and S.-A.D. designed the research. S.T., P.C., R.A.B. and K.A.H. performed the experiments. S.T., P.C., R.A.B., K.A.H. and S.-A.D analysed and interpreted the data, S.T. wrote the manuscript, and all authors revised the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.06.002.

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