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Research paper

Identification of proteasome subunit beta type 2 associated with deltamethrin detoxification in *Drosophila* Kc cells by cDNA microarray analysis and bioassay analyses

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ABSTRACT

Insecticide deltamethrin resistance has presented a difficult obstacle for pest control and the resistance development is complex and associated with many genes. To better understand the possible molecular mechanisms involved in DM stress, in this study, cDNA microarray analysis was employed. 448 differentially expressed genes with at least a 2-fold expression difference were identified in *Drosophila* cells after DM exposure. Moreover, some genes were confirmed with qPCR, which yielded results consistent with the microarray analysis. Three members of the ubiquitin–proteasome system were significantly elevated in DM-stressed cells, suggesting that the ubiquitin–proteasome pathway may play an important role in DM detoxification. The proteasome beta2 subunit (*Prosbeta2*) is a member of 20S proteasome subunit family, which forms the proteolytic core of 26S proteasome. Whether *Prosbeta2* participates in DM detoxification requires further study. RNAi and heterologous expression were conducted to investigate the contribution of *Prosbeta2* in DM detoxification. The results revealed *Prosbeta2* knockdown significantly reduce the level of DM detoxification in RNAi-treated cells after 48 h. Overexpression of *Prosbeta2* increased cellular viability. These detoxification results represent the first evidence that *Prosbeta2* plays a role in the detoxification of DM, which may provide new idea and target for studying the molecular mechanisms of insect resistance.

1. Introduction

Deltamethrin (DM), a representative synthetic pyrethroid insecticide, is widely used for pest control due to high potency and low persistence of its residues (Sayeed et al., 2003; Soderlund et al., 2002; Yang et al., 2008). Unfortunately, widespread and improper use of insecticides has induced the development of insecticide resistance (OMS, 1992; Casimiro et al., 2006) which presents a difficult obstacle for pest control (Denholm et al., 2002; Hemingway et al., 2002; Wilding et al., 2012).

Environmental exposure to xenobiotics may result in a widespread transcriptional response that activates the expression of the cellular detoxification machinery (Misra et al., 2011), causing a massive and rapid reprogramming of gene expression in response to xenobiotic challenge (Perry et al., 2011). It has been reported that pesticide resistance was multigenic in diamondback moth (Sayyed and Wright, 2004) and *Drosophila melanogaster* MEIG (Peyronnet et al., 1994). The *P450* and *GST* genes, which are regulated by xenobiotics, have been shown to be involved in DM resistance (Le et al., 2006).

cDNA microarray technology is one of the most powerful techniques in biology, which contributes to bridging the gap between sequence information and functional genomics (Deyholos and Galbraith, 2001). The extensive knowledge of *Drosophila* genetics has made this organism a model for genetic studies in toxicity and genotoxicity research. Large-scale transcriptional gene expression profiling based on cDNA microarray studies were carried out in this study to identify DM detoxification-associated genes in *Drosophila* Kc cells. *Prosbeta2* was significantly elevated.

Prosbeta2 belongs to the 20S subunit family and mediates the trypsin-like (T-L) activity (Heinemeyer et al., 1997). The proteasome 20S core is a multi-subunit protein complex composed of two α-rings and two β-rings, which have regulatory and proteolytic activity, respectively (Dafonseca et al., 2012). Ubiquitin-tagged proteins are degraded

Abbreviations: DM, Deltamethrin; DMSO, Dimethyl sulphoxide; es, Elution solution; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PCR, Polymerase chain reaction; qPCR, Quantitative real-time PCR; dsRNA, Double-stranded RNA; RNAi, RNA interference; CCK-8, Cell Counting Kit-8.

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by the 26S proteasome, which is involved in cellular apoptosis, DNA repair, endocytosis, cell cycle regulation (Smalle and Vierstra, 2004; Kerscher et al., 2006; Ravid and Hochstrasser et al., 2008).

Therefore, the current study was aimed to isolate differentially expressed genes identified by cDNA microarray analysis after DM treatment and to study the contribution of *Prosbeta2* in *Drosophila* cells DM detoxification.

2. Materials and methods

2.1. Drosophila Kc cells and cell culture

Drosophila Kc cells were generously provided by Prof. Junhai Han from the Institute of Life Sciences, Southeast University, China. Cells were cultured at 28 °C in serum-free insect cell culture medium (Thermo, USA) that was placed every 3–4 days. In medium-changing process, the supernatant culture medium was extracted, and new medium was added with repeated gently blowing to minimize the chemical and mechanical damage to the cells as much as possible.

2.2. Cell viability analysis

Stably grown cell suspensions were pipetted (100 μl per well) into a 96-well plate and incubated at 28 °C for 24 h. The cells were then treated with various concentrations of DM in DMSO (final DM concentrations: final DM concentrations: 0, $10^{0.5}$, 10^{1}, $10^{1.5}$, 10^{2}, $10^{2.5}$ μg/ml in 0.5% DMSO), after 24 h DM treatment, a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) was used to determine the cell viability. After 24 h DM treatment, 10 μl of CCK-8 solution was added to each well and the plates were incubated for another 3 h. The absorbance was measured at 450 nm using a microplate reader. In addition, to ensure the accuracy of the measurements, bubble is avoided in the process of adding.

2.3. Total RNA extraction and cDNA synthesis

Based on the results of the cytotoxicity assay after DM stimulation, we chose cell treated with $10^{1.5}$ μg/ml DM for 48 h as experimental group and normal Kc cells and DMSO-exposed cells as control groups. Total RNA of the three groups was isolated using the TriZol Rreagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was then purified with the Qiagen RNeasy®Mini Kit (Qiagen, Germany). RNA integrity and purity were monitored by 1% agarose gel electrophoresis and a NanoDrop Spectrophotometer (Thermo, USA). First-strand cDNA was reverse transcribed using the PrimeScript® RT Reagent Kit (Takara, Japan) according to the manufacturer's protocol and used as templates for qPCR.

2.4. Preparation of cDNA probes

Aliquots of the same RNA samples were also used for preparation of cDNA probes. Total RNA (0.2 μg) was first amplified with a One-Color RNA Spike-In Kit (Agilent, USA) according to the manufacturer's instructions. Target production for microarray hybridization was performed by incorporating the fluorescent nucleotide analog Cy3-dCTP. The fluorescently labeled RNA samples were purified with the Qiagen RNeasy®Mini Kit (Qiagen, Germany) before hybridization.

2.5. Microarray hybridization and data analysis

Drosophila cDNA microarrays (4 × 44 K, G2519F, 4 GeneChips, multiple 60mer oligonucleotide probe sets) have 43,803 *Drosophila* probes. *Drosophila* transcripts were constructed by Agilent. Following the Agilent Gene Expression Hybridization Kit instructions, purified Cy3-cRNA was fragmented using fragmentation buffer at 60 °C for 30 min, immediately cooled on ice for 1 min, then hybridized with GeneChips for 17 h at 65 °C at 10 rpm. After hybridization, the cocktail was removed from the GeneChips, which were washed using a Gene Expression Wash Pack (Agilent, USA), and then scanned with an Agilent scanner. The fluorescence intensity of Cy3 was measured to determine the relative gene expression level. Data were extracted and quantitatively analyzed using Agilent Feature Extraction Software. Genes were considered differentially expressed if the change in gene expression was greater than 2-fold. Venn analysis eliminated genes that showed expression changes in the Kc/DMSO-exposed controls, and pathway and gene ontology analysis were then performed.

2.6. qPCR verification

In order to confirm the accuracy of the results of microarray hybridization, 10 genes were detected and verified by qPCR. Amplification was performed with SYBRGreen I (Takara, Japan) in 20 μl reactions containing cDNA, specific forward and reverse primers according to the manufacturer's instructions. The primer sequences used for qPCR are shown in Table 1. A melting curve was generated to check the specificity. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. All analyses were based on normalized Ct values, which were read automatically with the included software. The relative gene expression levels were calculated through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression levels of selected genes without DM treatment were designated as the background levels and normalized to 1. The results are expressed in arbitrary units and are representative of three independent experiments.

2.7. Western immunoblot analysis

A total protein extraction kit (Shenggong, China) was used to extract total protein from $10^{1.5}$ μg/ml DM-exposed cells, normal Kc cells (control) according to the manufacturer's instructions. The BCA Protein Assay Kit (Generay, China) was used to determine the concentration of the extracted protein. Protein samples were resolved on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane.

Table 1
List of primers used in real-time RT-PCR to confirm the differentially expressed genes.

Gene	Forward primer	Reverse primer
Heat shock gene (Hsp26)	GCTCACCGTCAGTATTCC	CCTCGCTTTCATTTGCCTTA
Peptidoglycan recognition protein LD (PGRP-LD)	GCTGCCCTACAACTTTCT	CCATCACCAACGAGTCTATT
Calmodulin (Cam)	AGCAACAAGAACAACAAGTC	ATCTTTCGGCAATACTGGT
Eukaryotic initiation factor 4B (eIF-4B)	GTAGATACAGCAGCAAGGGA	ACTAACTGTTGCACTTAGACT
Proteasome beta2 subunit (Prosbeta2)	CCCGATGACTTCGTTGTG	CCCGATGACTTCGTTGTG
Smad on X (Smox)	TGGTGTTCCATCTCCTACTATACTAT	ATTGGAGGGATCGG TAAAGTAAAG
Chitinase-like (Chit)	GTGAACTCATCCCTGTTCTAC	GTCGGCAACTTCGGGATTA
Nitric oxidesynthase (Nos)	TTTGGACGCGCCTTA TCGAA	TGTTAGACTCACCTGTGCATTGA
CytochromeP450-6a8 (Cyp6a8)	GGCCAAGCAAATACTGATTAAG	CACCGTCCAGATTGAAGAG
Heme oxygenase-1 (HO-1)	ATGTCAGCGAGCGAAGAAAC	GTCATCAGAAAGGGCAAGTG
GAPDH	CGGCTACCACATCTAAGGAA	GCTGGAATTACCGCGGCT