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Effects of phoxim on nutrient metabolism and insulin signaling pathway in silkworm midgut

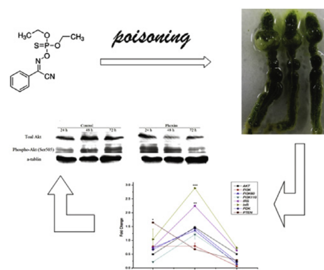
Fanchi Li [a, 1], Jingsheng Hu [a, 1], Jianghai Tian [a, 1], Kaizun Xu [a], Min Ni [a], Binbin Wang [a], Weide Shen [a, b], Bing Li [a, b, *]

[a] School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu 215123, PR China
[b] National Engineering Laboratory for Modern Silk, Soochow University, Suzhou, Jiangsu 215123, PR China

HIGHLIGHTS

- Digital gene expression profiling was used to analysis of differentially expressed genes.
- Phoxim dysregulated activity changes in digestive enzymes.
- Phoxim dysregulated the expression of IIS pathway genes in silkworm midgut.
- Phoxim dysregulated the levels of toal Akt and phospho-Akt.

GRAPHICAL ABSTRACT



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ABSTRACT

Silkworm (*Bombyx mori*) is an important economic insect. Each year, poisoning caused by phoxim pesticide leads to huge economic losses in sericulture in China. Silkworm midgut is the major organ for food digestion and nutrient absorption. In this study, we found that the activity and expression of nutrition metabolism-related enzymes were dysregulated in midgut by phoxim exposure. DGE analysis revealed that 40 nutrition metabolism-related genes were differentially expressed. qRT-PCR results indicated that the expression levels of insulin/insulin growth factor signaling (IIS) pathway genes Akt, PI3K, PI3K60, PI3K110, IRS and PDK were reduced, whereas PTEN's expression was significantly increased in the midgut at 24 h after phoxim treatment. However, the transcription levels of Akt, PI3K60, PI3K110, IRS, InR and PDK were elevated and reached the peaks at 48 h, which were 1.48-, 1.35-, 1.21-, 2.24-, 2.89-, and 1.44-fold of those of the control, respectively. At 72 h, the transcription of these genes was reduced. Akt phosphorylation level was increasing along with the growth of silkworms in the control group. However, phoxim treatment led to increased Akt phosphorylation that surged at 24 h but gradually decreased at 48 h and 72 h. The results indicated that phoxim dysregulated the expression of IIS pathway genes and induced abnormal nutrient metabolism in silkworm midgut, which may be the reason of the slow growth of silkworms.

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1. Introduction

Silkworm belongs to Lepidoptera and is an important economic insect. Sericulture plays an important role in human life and culture (Li et al., 2010; Ma et al., 2013). China's raw silk production accounts

* Corresponding author. School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu, 215123, PR China. Tel./fax: +86 512 65880262.
E-mail address: lib@suda.edu.cn (B. Li).
[1] These authors contributed equally to this study.

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for more than 80% of the world-wide output. However, silkworm's resistance to adverse environments, such as ultraviolet light, heat, and pesticides, has become increasing weak due to long-term directional selection (Shen et al., 2003). Pesticide pollution of mulberry leaves, a result of irrational agricultural production and disconnect between planning and production, has caused serious damages to sericulture (Nath, 1993; Zhang et al., 2011a,b; Peng et al., 2011). O, O-diethyl O-(alpha-cyanobenzylideneamino) phosphorothioate (phoxim) is an effective organic phosphorus pesticide, widely used in the prevention and treatment of lepidopteran pests and other harmful organisms in agriculture and forestry (Wang et al., 2013). Phoxim exposure mainly inhibits the activity of acetylcholinesterase, which leads to the accumulation of acetylcholine in postsynaptic membrane and the eventual death of pests (Shang et al., 2007). Phoxim affects silkworm's growth, reproduction, and oxidative stress response by reducing feeding rate and digest rate and inducing the production of reactive oxygen in silk gland and nervous system (Li et al., 2012).

The midgut is the main organ of silkworm for food digestion and nutrient absorption (Zhang et al., 2011a,b). Intestinal epithelial cells secrete digestive enzymes, such as protease, carbohydrate and lipid esterase that degrade proteins, carbohydrates and fats. Seventeen cytochrome P450 family members are highly expressed in silkworm midgut (Xia et al., 2007). Phoxim exposure inhibits the gene expression of Toll and IMD immune signaling pathways, leading to apoptosis and the release of cytochrome C from mitochondria into the cytoplasm (Su et al., 2014; Gu et al., 2013). Overall, the midgut is not only a vital organ for pesticide metabolism but also a recipient organ of pesticide damages.

The insect insulin/insulin growth factor signaling (IIS) pathway is particularly interesting as a highly conserved pathway in the animal kingdom. In *Drosophila*, the IIS pathway regulates the rates of nutrition-dependent growth (Brogiolo et al., 2011; Oldham and Hafen, 2003; Edgar, 2006; Engelman et al., 2006). Akt is the most important effector molecule in the IIS pathway by activating downstream target proteins. Akt phosphorylates glucose transporters and glycogen synthase kinase to promote glucose absorption and glycogen synthesis (Edwards et al., 2005; Taniguchi et al., 2006a,b). Akt also activates the TOR pathway by phosphorylating the tumor suppressor Tuberous Sclerosis Complex (TSC). The TSC–TOR pathway has been shown to play important roles in metabolism, cell growth, proliferation, survival, and differentiation (Bhaskar and Hay, 2007). However, the effects of phoxim on the nutrient metabolism and insulin signaling pathway of *Bombyx mori* midgut have been rarely reported.

In order to clarify the mechanism of phoxim's effects on nutrient metabolism and insulin signaling pathway in silkworm midgut, DGE analysis was used in this study to profile the gene expression in silkworm midgut after phoxim exposure, which was examined by qRT-PCR, Western blotting, and enzyme activity assays.

2. Materials and methods

2.1. Insect strain and chemicals

The larvae of *B. mori* (Jingsong × Haoyue strain) were maintained in our laboratory and reared on mulberry leaves under a 12-h light/dark cycle.

Phoxim was purchased from Sigma–Aldrich (USA).

2.2. Phoxim treatment and total RNA extraction

The lethal concentration 50 (LC_{50}) of phoxim treatment at 24 h for *B. mori* is 7.86 μg/mL (Peng et al., 2011; Wang et al., 2013). No death was observed 24 h after treatment with 4.0 μg/mL phoxim, thus 4.0 μg/mL phoxim was used as the concentration for further experiments. 100 mg phoxim was dissolved in 1 mL acetone as stock solution, which was further diluted with double distilled water as working solution (4.0 μg/mL). Mulberry leaves were dipped in 200 mL working solution for 1 min before being air-dried. Phoxim-treated leaves were used to rear *B. mori* larvae which were fed with these leaves three times a day on the third day of the fifth-instar, respectively. Mulberry leaves treated with water were used as control. Fifth instar larvae were fed with phoxim-treated leaves that were replaced with fresh leaves 24 h later.

At 24, 48 and 72 h after treatment, 50 fifth-instar larvae were randomly selected from each group. The larval midguts were quickly removed, placed on ice, dissected with intestinal contents being removed, and washed with cold PBS (phosphate buffer saline, PH 7.4). Total RNA was extracted from the midgut samples using Trizol reagent (Invitrogen, USA). The DNA was removed from the total RNA (5 μg) of each sample using DNase (CapitalBio, China). The DNA-free total RNA (0.5 mg/sample) was reverse-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis kit (Takara, Japan) and a random hexamer primer following the manufacturer's instructions. The quality of RNA was assessed by formaldehyde agarose gel electrophoresis and was quantified spectrophotometrically.

2.3. Digestive juice collection

After 24, 48 and 72 h treatment, 10 instar larvae were selected randomly from each group for the collection of larval digestive juice from peritrophic matrix. 0.2 mL digestive juice was extracted from each larva, and pooled hemolymph from each treatment was used for biochemical measurements. After centrifuged for 10 min at 10,000 rpm, the supernatant was transferred to new tubes and kept at −80 °C for further experiments.

2.4. Assay of digestive enzymes in digestive juice

The activities of digestive enzymes in midgut digestive juice were measured using respective assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The enzyme activities were determined at least 3 times with 3 replicates for each sample. All the data were defined as the amounts of enzymes required for 1 mg sample and are presented as means ± SD. These procedures were performed according to the manufacturer's instructions.

2.5. Quantitative real-time PCR analysis

Quantitative real-time PCR was used to measure the transcription of P450 genes. qRT-PCR primers were designed using the Primer 5.0 software (Table 1) with the actin3 gene as the reference gene. Primers were synthesized by Shanghai Sangon Biological Technology and Services Co., Ltd. Each qRT-PCR reaction (25 μL final volume) contained 1 × SYBR Green master mix, 1 μL cDNA, and a P450 gene specific primer pair (Table S1) at a final concentration of 3–5 μM. All samples, including the 'no-template' negative control, were assayed in triplicates. The reaction cycle consisted of a melting step of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All data were normalized with the actin3 expression level. Data represent fold change of RQ (relative quantification) in treated vs. control samples.

2.6. DGE library preparation, sequencing and tag mapping

For RNA library construction and deep sequencing, equal quantities of midgut RNA samples (n = 3) were pooled for the control group and the treatment group. Approximately 6 μg of RNA

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