Inhibition of oocyte development by a trehalase inhibitor, validoxylamine A, in *Periplaneta americana*

Yoshiaki Kono\(^1\), Masakazu TakaHashi\(^2\), Kazuhiro Matsushita\(^3\), Masami Nishina\(^3\) and Yukihiko Kameda\(^4\)

\(^1\)Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, 305-8572 Japan
\(^2\)National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo, 162-8640 Japan
\(^3\)Saitama Medical School, Moroyama, Irumagun, Saitama, 350-0451 Japan
\(^4\)School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, 920-1148 Japan

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**Abstract:** Injection of validoxylamine A (VAA), a specific trehalase inhibitor, suppressed oocyte development in the American cockroach, *Periplaneta americana*. Trehalases of the ovary, accessory gland and fat body in the cockroach were strongly inhibited by the injection of VAA. Elevation of hemolymph trehalose concentration was observed indicating inhibition of trehalose metabolism in the tissues. In control cockroaches, vitellin accumulated in the ovary in parallel with oocyte development, and the amount of vitelligenin in the hemolymph changed showing negative correlation with the uptake of vitelligenin to the ovary. In VAA-injected cockroaches, vitelligenin production was suppressed in the early stage of oocyte development; uptake of vitelligenin by the ovary reduced in the late stage and, consequently, vitelligenin accumulated in the hemolymph. It is concluded from these results that the primary effect of VAA occurs in the vitelligenin production and the vitelligenin intake for the oocyte development.

**INTRODUCTION**

Validoxylamine A (VAA) is an aglycon of validamycin A, a product of *Streptomyces hygroscopicus* var. *limoneus* (Iwasa et al., 1971), and shows potent and specific inhibitory activity against trehalases of various organisms (Kameda et al., 1987; Asano et al., 1987; 1990; Salleh and Honek, 1990; Kyosseva et al., 1995). It is well known, on the other hand, that insect hemolymph contains a high concentration of trehalose which is utilized as an energy source in insect tissues. Therefore, the trehalase is an important enzyme for carbohydrate metabolism and inhibition of the trehalase is thought to lead to abnormal physiological function in insects. Administration of VAA, in fact, evoked various kinds of physiological alterations in insects. Injection of VAA into pupae induced a change in egg diapause determination (Takeda et al., 1988), and prevented glutinous material production (Takeda et al., 1990; Yao et al., 1991) in the silkworm, *Bombyx mori*. VAA also showed lethal activity caused by incomplete metamorphosis in certain lepidopteran species, *B. mori* (Kono et al., 1993), *Spodoptera litura* (Asano et al., 1990; Kono et al., 1994b), and *Mamestra brassicae* (Kono et al., 1995). Flight was suppressed by an injection of VAA to *Periplaneta americana*, and reproduction was inhibited in *P. americana* (Kono et al., 1997) and *Locusta migratoria* (Tanaka et al., 1998). In the present paper, inhibitory effect of VAA on the oocyte
development was analyzed through the changes in hemolymph components, including vitellogenin, in the developmental cycle of oocyte of *P. americana*.

**MATERIALS AND METHODS**

*Insects.* The stock culture of *P. americana* was maintained with rabbit food and water under long day conditions (25°C, LD 16:8) in the laboratory of National Institute of Infectious Diseases. Adults of 20–40 days after emergence were used for the experiments.

*Administration of VAA.* Five microliters of an aqueous solution including 50 μg VAA was injected into the dorsal body cavity of the female cockroach on the day of ootheca deposition. The same volume of water was injected into the control cockroaches. Five cockroaches were used for each plot of observation of oocyte and measurement of hemolymph components.

*Observation of VAA effect on oocyte development.* Female adults of treated and control groups were confined individually with a non-treated male in a plastic cup containing food and water, and the oocyte development was observed every other day following administration of VAA. The development of the oocyte was categorized into five stages according to yolk deposition and egg sheath formation of the terminal oocyte as in a previous paper (Kono et al., 1997); stage 1: a small amount of yolk deposited, stage 2: the yolk occupying about 50% of oocyte, stage 3: the yolk occupying most of oocyte, stage 4:

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![Fig. 1. 1H-NMR spectrum of the hemolymph from control (a) and VAA treated (b) females at 1 day after injection. AA: acetic acid, Ch: choline, G: glycine, Gl: glucose, PCh: phosphoryl choline, Tr: trehalose, TSP: see text.](image-url)
maturated oocyte, stage 5: with ootheca.

**Measurement of trehalase activity.** Trehalase activities of the ovary, accessory gland and fat body dissected out on ice from control and treated female cockroaches were measured by the glucose oxidase method (Glucose B-test Wako, Wako Chemical Industries Ltd.; Kono et al., 1997) at 1 and 7 days following VAA administration. The activity was expressed as international unit (U) per mg fresh weight.

**NMR analysis.** Five microliters of hemolymph was collected from the coxa of a severed hind leg and centrifuged with a small amount of phenylthiourea at 2,000 g for 10 min at 4 °C. The supernatant was diluted to 500 μl with heavy water (D2O) and stored at −20 °C until nuclear magnetic resonance spectroscopy (NMR) analysis. Four-hundred and fifty microliter of the supernatant, to which 50 μl of 1mM 3-trimethylsilyl2,2,3,3-tetradeteropropionate (TSP) was added as an internal standard, was subjected to 1H-NMR analysis. On 1H-NMR spectrograms shown in Fig. 1, several major constituents of hemolymph could be assigned in both control and VAA-treated cockroaches. The hemolymph constituent concentrations were determined by the ratio of signal strength belonging to each compound to that of TSP signal at 0.00 ppm based on the calibration curve of each compound (Kono et al., 1994a).

**SDS polyacrylamide gel electrophoresis (SDS-PAGE).** Hemolymph (1 μl/lane) and ovary homogenate supernatant (1/130 equivalent of ovary /lane) were electrophoresed on 5–20% SDS gradient polyacrylamide gel. Vitellogenin and vitellin were identified by staining with coomassie brilliant blue and their Rf values (Kim et al., 1992), and the amounts were graded from 0 to 5 by the direct observation of the colored density of their bands about 100 kDa.

**RESULTS**

**Effect of VAA on the oocyte development.** In the control cockroach without VAA injection, terminal oocytes in the ovary grew gradually and matured in 6–8 days after the treatment, and then they were arranged in the ootheca (Table 1). When

<table>
<thead>
<tr>
<th>Stage of oocyte</th>
<th>Number of females in the stage of oocyte development days after treatment</th>
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<tbody>
<tr>
<td>Control</td>
<td>0 2 4 6 8 10 12 14</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2 3 2</td>
</tr>
<tr>
<td>3</td>
<td>2 3 3</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>0 2 4 6 8 10 12 14</td>
</tr>
<tr>
<td>1</td>
<td>3 3</td>
</tr>
<tr>
<td>2</td>
<td>1 1</td>
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<td>3</td>
<td>1 1</td>
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<td>4</td>
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<td>5</td>
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</table>

50 μg/female VAA was injected into the abdominal body cavity of the female on the day of ootheca deposition. Treated and control females were reared individually with a non-treated male. Figure in parenthesis indicates number of females with oocytes degenerated.

1) Remaining individuals (two and three females 8 and 10 days after treatment, respectively) deposited the ootheca.

2) One female died accidentally.
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Table 2. Inhibition of trehalase in ovary, accessory gland and fat body by VAA injection.

<table>
<thead>
<tr>
<th></th>
<th>1 DAT</th>
<th></th>
<th>7 DAT</th>
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<tbody>
<tr>
<td></td>
<td>Activity of control U/mg weight</td>
<td>Inhibition (%)</td>
<td>Activity of control U/mg weight</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Ovary</td>
<td>1.3</td>
<td>96.2</td>
<td>1.6</td>
<td>96.9</td>
</tr>
<tr>
<td>Accessory gland</td>
<td>0.72</td>
<td>67.8</td>
<td>0.72</td>
<td>86.8</td>
</tr>
<tr>
<td>Fat body</td>
<td>1.7</td>
<td>95.9</td>
<td>1.7</td>
<td>88.7</td>
</tr>
</tbody>
</table>

50 μg VAA in 5 μl distilled water was injected to each female cockroach. The control cockroach was injected with 5 μl distilled water only. All experiments were triplicated, and values were the mean of the experiments.

DAT: days after injection, weight: fresh weight.

cockroaches were injected with VAA on the day of ootheca deposition, the oocyte development was strongly suppressed and rarely reached the mature stage. The degenerating features of the oocyte were observed in every stage after several days of VAA administration. Consequently, the ootheca was not formed (Table 1).

Inhibitory effect of VAA on trehalase in ovary, accessory gland and fat body. Trehalase activity of the female reproductive organs, the ovary and accessory glands, and fat body was measured at 1 and 7 days after VAA injection. As shown in Table 2, the trehalase activity was inhibited by the VAA injection in both reproductive organs, and the inhibition lasted for more than 7 days. The trehalase activity was inhibited stronger in the ovary (96.2% and 96.9%, 1 and 7 days after injection, respectively) than in the accessory gland (67.8% and 86.8%, 1 and 7 days after injection, respectively). Inhibition of trehalase in the fat body was similar to that in the ovary.

Effect of VAA on hemolymph sugar level and other components. The hemolymph was sampled every other day after VAA injection and analyzed by ¹H-NMR. Trehalose, glucose, acetate, choline, phosphoryl choline and glycine were detected, and their concentrations were determined (Fig. 1). Glucose levels were maintained at higher level than trehalose concentration in the control cockroach (Fig. 2). In the treated cockroaches, trehalose concentration was steeply raised in 4 days following the VAA injection and increased gradually thereafter; however, glucose was not detected in the hemolymph.

In other components, the concentration of choline increased temporarily and the concentration of phosphoryl choline, to the contrary, decreased to less than half at the initial level by the injection of VAA after the 6th day (Fig. 3a). Acetate concentration gradually elevated after VAA treatment. Glycine concentration decreased until the 8th day after the treatment (Fig. 3b).
Changes of hemolymph vitellogenin and ovary vitellin. Proteins in the hemolymph and ovary were analyzed by SDS polyacrylamide gel electrophoresis (Fig. 4) and the amounts of vitellogenin and vitellin were estimated by the density of stained bands at ca 100 kDa. Changes in vitellogenin and vitellin were shown in Fig. 5. In the control cockroach, the amount of vitellin in the ovary increased in parallel with the oocyte development. The amount of vitellogenin in the hemolymph increased at the middle stage of oocyte development and then decreased in the control cockroach. To the contrary, the amount of vitellin in VAA-injected cockroaches hardly increased and remained at the second-day level of the control female until the 8th day and then steeply decreased. The amount of vitellogenin in the hemolymph of the treated cockroach gradually increased at lower level than in the control. Steep increase of vitellogenin between the 2nd to 4th day in the control
disappeared in the treated cockroach but the level at the 12th day exceeded the maximum level of the control cockroach.

**DISCUSSION**

In the previous study (Kono et al., 1997), malformed oothecae were deposited for several days after VAA administration to the female cockroaches. However, the present study showed that no ootheca formation was observed when VAA was administered on the day of ootheca deposition (Table 1). The result indicates that the VAA administration taken place at an early stage of the oocyte developmental cycle suppresses the growth of oocyte and inhibits further events such as chorion formation and ootheca formation. Furthermore, degeneration of the terminal oocyte occurred.

In the hemolymph of the female developing oocyte, glucose concentration was higher than that of trehalose (Fig. 1a), while trehalose concentration is much higher than that of glucose in the male hemolymph (Kono et al., 1994b). The trehalose concentration rose steeply to more than 50 mM in four days, and glucose diminished from the hemolymph when VAA was administered to the female (Fig. 1b). These observations supported that the trehalases in tissues and organs, including hemocyte, were inhibited by VAA administration. The glucose in the hemolymph was obviously the product of hydrolyzing trehalose. In fact, measurement of the trehalase activity in the ovaries and accessory glands indicated the inhibition of the enzyme by VAA administration for more than 7 days (Table 2). The trehalase was inhibited by VAA in other tissues, muscles and fat body (Kono et al., 1998; Table 2).

Four subunits with molecular weights of ca. 102, 81, 49, 40 kDa were isolated from vitellogenin of *P. americana* (Kim et al., 1992). Two vitellin molecules in the ovary of *P. americana* were shown to include ca. 105 kDa subunit which shows common immunological reactivity to ca. 102 kDa vitellogenin subunit (Storella et al., 1985). In the present study, the amounts of ca. 100 kDa subunits of vitellogenin and vitellin on the electrophoretogram, the same subunit mentioned in the previous papers as 105 kDa and 102 kDa subunits (Kim et al., 1992), were estimated. Judging from the changes in vitellin amount in the cockroach injected with VAA, suppression of the intake of vitellogenin by the oocyte is a main cause of inhibition of oocyte maturation. The amount of hemolymph vitellogenin and oocyte vitellin of the VAA-injected cockroach maintained at a lower level than that of the control one for 8 days suggests that vitellogenin synthesis in the fat body also is suppressed by VAA. However, a higher level of vitellogenin comparing with the control cockroach measured at the end of experimental period in the VAA-injected cockroach indicates that the gradual production occurred at the later period of the experiment in such insect. The steep decline of oocyte vitellin between the 8th day and the 10th day in VAA-injected cockroach coincided with the oocyte degeneration. Similar results were obtained in the migratory locust, *L. migratoria* (Tanaka et al., 1998). Protein synthesis, including vitellogenin synthesis in fat body, and uptake of vitellogenin by ovary were suppressed by the injection of VAA in this species. Furthermore, in the locust, synthesis of JH which stimulates the vitellogenin synthesis was also inhibited by the application of VAA (Tanaka et al., 1998).

In *P. americana*, trehalase in all tissues and organs; fat body, ovary, accessory gland and muscles, was strongly inhibited for an extended period by the injection of VAA. Therefore, hormone synthesis like JH and production of ootheca protein(s) seems to be suppressed by shortage of energy along with inhibition of the vitellogenin synthesis in the fat body and uptake of vitellogenin by the oocyte.
References


摘 要

トレハラーゼ阻害剤バリドキシルアミン A によるワモンゴキブリ卵巣発育の抑制

河野義明1) 高橋正和2) 松下和弘3) 仁科正美4) 亀田幸彦4)

1)筑波大学農林学系
（〒305-8572 つくば市天王台）
2)国立感染症研究所昆虫医学部
（〒162-8640 東京都新宿区戸山1-23-1）
3)埼玉医科大学動植物学教室
（〒354-0451 埼玉県入間郡毛呂山町）
4)北陸大学薬学部
（〒920-1148 金沢市金川町）

トレハラーゼの特異的阻害剤バリドキシルアミンA (VAA) をワモンゴキブリに注射すると (50μg/虫), 卵巣中の卵発育抑制と卵の進化は見られず、卵鞘が形成されなかった。卵巣を発育に属し、脂質体のトレハラーゼ活性は VAA の注射により7日以上阻害された。トレハラーゼ阻害による体組織でのトレハロース利用阻害のために、体液中のトレハロース濃度は著しく上昇し、他の体液成成分濃度に変化が見られた。電気泳動による体
液中卵黄前駆蛋白ビテロン（Vg）、卵巣中卵黄蛋白ビテリンの変化の観察から、VAA 投与が脂肪体での Vg 合成、卵巣へのその取り込みを抑制することが明らかになり、これらが卵巣発育障害の原因であると考えられた。