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Localization of an Aldo-Keto Reductase (AKR2E4) in the Silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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Subject Editor: Guy Smagghe

Abstract

The aldo-keto reductase AKR2E4 reduces 3-dehydroecdysone to ecdysone in the silkworm *Bombyx mori* L. In this study, a quantitative polymerase chain reaction analysis revealed that the level of *AKR2E4* mRNA was higher in the testes than in other tissues, and a western immunoblot analysis revealed that the AKR2E4 content in the testes was stage-specific from the fifth larval instar to the pupal stage. Immunohistochemical analysis showed that the AKR2E4 protein was present in cyst cells associated with sperm cells and spermatocytes. These results indicate that AKR2E4 plays an important role in 3-dehydroecdysone conversion to ecdysone and spermatogenesis in silkworm testes.

Key words: Aldo-keto reductase, Lepidoptera, testes

In insects, ecdysteroids affect molting, metamorphosis, and reproduction (Gilbert et al. 2002). The ecdysteroid, ecdysone, was first isolated in 1954 (Karlson 1996) and shown to be produced through the reduction of 3-dehydroecdysone (3DE) by a 3DE 3β-reductase (Sakurai et al. 1989); the ecdysone is then hydroxylated at the 20 position to form the active steroid hormone, 20-hydroxyecdysone. Thus, close regulation of the 20-hydroxyecdysone titer through production and inactivation is needed to mediate precise molting in insects.

Previously, we identified a 3DE 3β-reductase in the silkworm *Bombyx mori* L. and determined its X-ray structure (Yamamoto and Wilson 2013). The 3DE 3β-reductase of *B. mori* belongs to the aldo-keto reductase (AKR) family 2, and the enzyme was named AKR2E4. This enzyme catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of ketones and aldehydes, and reduces 3DE to ecdysone in the presence of NADPH. The structure of AKR2E4 in a complex with an NADP+ co-substrate has been determined (Yamamoto and Wilson 2013). The amount of AKR2E4 in the silkworm hemolymph is likely to change in response to the hormonal activities of ecdysteroids (Yamamoto and Wilson 2013).

Ecdysteroids stimulate spermatogenesis in insect testes (Kambysellis and Williams 1972, Dumser and Davey 1975). *B. mori* testes contain ecdysteroids, the concentrations of which fluctuate during development (Fugo et al. 1995). Although the mechanisms involved in ecdysteroid regulation in *B. mori* ovaries have been established (Ohnishi 1990), those in the testes remain uncertain; we initiated the present study to investigate these mechanisms through an immunohistochemical analysis of AKR2E4 in the testes.

Materials and Methods

Insect Culture and Tissue Dissection

B. mori larvae (p50T strain) were reared at 25°C and fed on mulberry leaves. For a real-time polymerase chain reaction (PCR) analysis, Day 3 fifth-instar larvae were dissected on ice, and the fat body, midgut, silk gland, ovaries, and testes were stored at –80°C until use. For western blot analysis, Days 1, 3, 5, 7, 9, and 10, fifth-instar larvae were dissected on ice, and the testes were stored at –80°C until use.

Quantitative PCR Analysis

Total RNA was extracted from the dissected tissues using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNAs were prepared as described by Yamamoto and Wilson (2013). qPCR primer sets for *AKR2E4* and *B. mori* ribosomal protein 49 (*Bmrp49*) were designed. The primer sequences were as follows.

AKR2E4F (forward), 5′-CCGAAATCCACAAACAAGCA-3′, and AKR2E4R (reverse), 5′-TGGCCGCTACCTCTTCAAAC-3′; Bmrp49F (forward), 5′-GATGTGTTTTATATTC-3′, and Bmrp49R (reverse), 5′-GCATCATCAAGATTTCCAGCTC-3′. qPCR was performed on a Dice Real Time System TP-800 Thermal Cycler (Takara, Shiga, Japan) using SYBR *Premix Ex Taq* (Takara). PCR amplification was initiated with a 10-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 20 s, and an extension at 72°C for 20 s. The samples were analyzed in triplicate, and *AKR2E4* levels were normalized against corresponding *Bmrp49* levels and expressed as the *AKR2E4/Bmrp49* ratio.

Immunoblot Analysis

Recombinant AKR2E4 and anti-AKR2E4 polyclonal primary antibodies were produced previously (Yamamoto and Wilson 2013). The testes were homogenized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and centrifuged at $12,000 \times g$ for 15 min at 4°C. The supernatant was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using a 15% polyacrylamide slab gel containing 0.1% SDS (Laemmli). The proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After blocking for 1 h in 5% fat-free milk in PBS, the membrane was incubated with anti-AKR2E4 polyclonal primary antibody (1:8000) in 5% fat-free milk in PBS for 1 h. The membrane was then washed (3×) with PBS, followed by incubation with goat anti-rabbit IgG secondary antibody (Cappel, Solon, OH) (1:8000) in 5% fat-free milk in PBS for 1 h. After the membrane was washed (3×) with PBS, detection was performed using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Chalfont St. Giles, United Kingdom or Piscataway, NJ).

Immunohistochemistry

The testicular envelope and contents were placed on a slide, dried for 2 min, and incubated in 0.2 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) containing 4% (v/v) paraformaldehyde for 1 h. The tissue was then incubated in methanol for 10 min, blocked in PBS-BT (PBS containing 10%, v/v, bovine serum albumin, 12 mM NaN_3, and 0.1%, v/v, Tween-20), and incubated overnight at 4°C in PBS-Tr (PBS-BT with the primary antibody, anti-rabbit serum [1:1000]). After rinsing (3 × 10 min, room temperature) with PBS-Tr, the preparations were incubated (1 h, room temperature) with 7.5 µg/ml of the secondary antibody, goat anti-rabbit IgG (H+L)-CF 488A. After washing in PBS-Tr, the stained preparations were mounted in Aqua-Poly/Mount medium and examined using a BX50 microscope (Olympus, Tokyo, Japan) equipped with BX-FLA reflected light fluorescence and WIG and NIBA mirror/filter units (Olympus). Between three and five individuals were studied in each immunocytochemical experiment. The excitation and emission ranges of the NIBA mirror/filter unit were 470–490 and 515–550 nm, respectively. In control experiments, the primary antibodies were replaced with pre-immune rabbit serum. Controls did not show significant staining above background levels.

Results and Discussion

Tissue Distribution of AKR2E4 mRNA

Knowledge of the tissue distribution of *AKR2E4* gene expression can provide insight into its function. Fig. 1 shows the presence of *AKR2E4* mRNA in larval tissues that were examined by qPCR. The measurements of relative levels of *AKR2E4* expression used *Bmrp49* expression as the internal control. *AKR2E4* mRNA was detected in all tested tissues (Fig. 1), although the relative levels varied. Expression of *AKR2E4* in the testes was ~10-fold higher than that in the midgut and silk gland. These results support the hypothesis that *AKR2E4* plays a key role in ecdysone metabolism in silkworm testes.

Testes AKR2E4 Content

As shown in Fig. 2, western immunoblots for AKR2E4 revealed a single band at ~33 kDa in the testes extracts at Days 1, 3, and 10 (Fig. 2), whereas no bands were detected at Days 5, 7, and 9 (Fig. 2). The 33 kDa band is in agreement with the theoretical molecular weight of AKR2E4. The amount of AKR2E4 increased at Day 3 (in the middle of the fifth-instar stage) and at Day 10 (just after pupation).
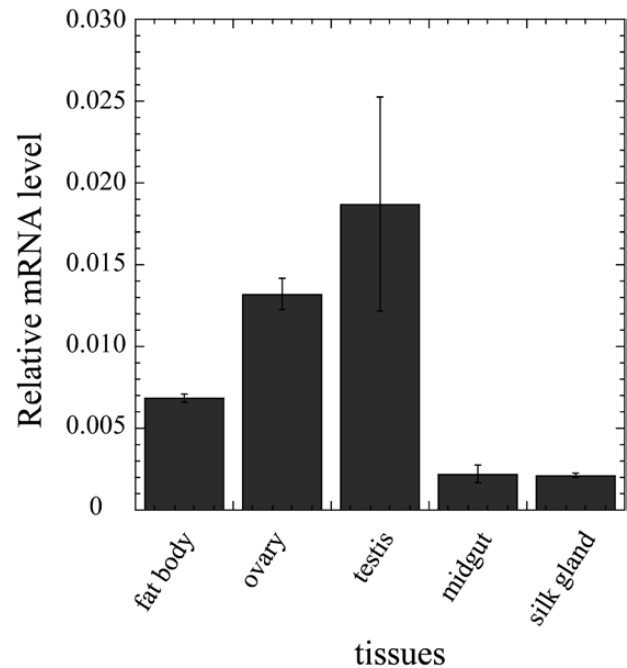

Fig. 1. Localization of AKR2E4 transcripts. Error bars denote the standard deviations (SDs) obtained from three experiments.
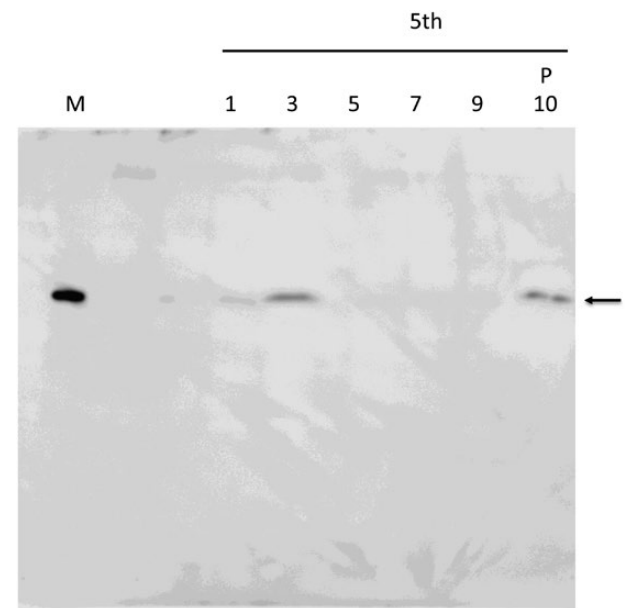

Fig. 2. Immunoblot analysis of AKR2E4. M and P indicate purified AKR2E4 and pupation, respectively. The amounts of purified AKR2E4 and crude protein in each lane were 0.07 and 50 µg, respectively.

Immunohistochemistry of AKR2E4 in Testes

The AKR2E4 protein was present in cyst cells around sperm and spermatocytes (Fig. 3A and B). AKR2E4 localization did not change between Days 3 and 10 of the fifth-instar stage (Figs. 3 and 4). The function of cyst cells in the silkworm testes is unclear; in *Drosophila melanogaster*, testicular cyst cells are crucial for the correct progression of the germline to meiosis (Schulz et al. 2002, Fabrizio et al. 2003, Shields et al. 2014, Fairchild et al. 2015). Therefore, AKR2E4 might play an important role in meiosis in silkworm testes.
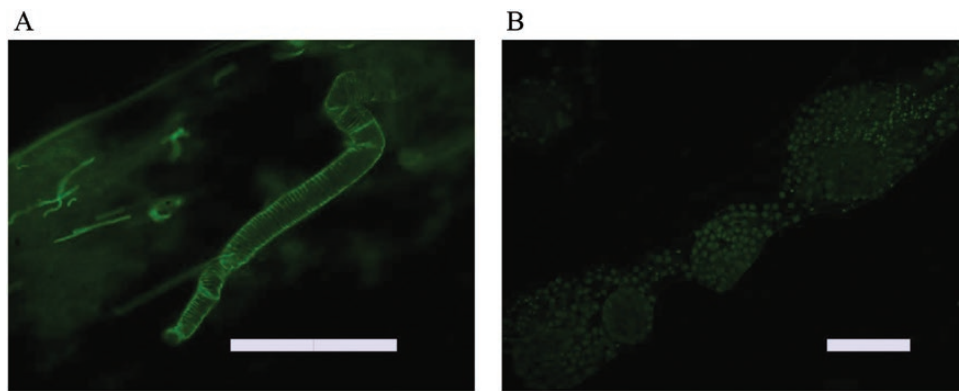
Fig. 3. Immunohistochemistry of AKR2E4 in a testicular sperm cell (A) and spermatocyte (B) on Day 3 of the fifth-instar stage. AKR2E4 was visualized using CF 488A (a green fluorophore). Bar indicates 100 μm.
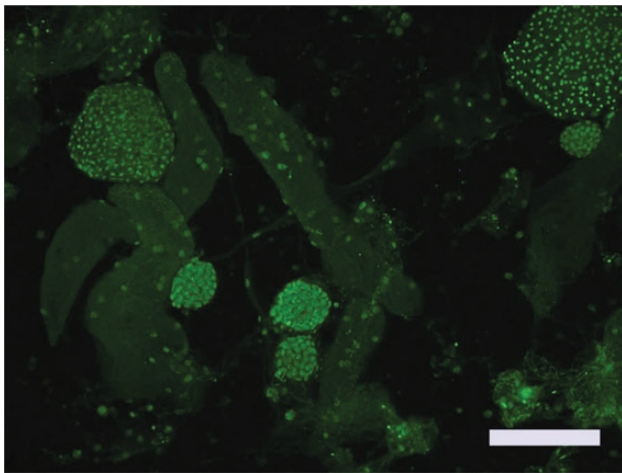

Fig. 4. Localization of AKR2E4 in testicular sperm cells and spermatocytes on Day 10 of the fifth-instar stage. AKR2E4 was detected by CF 488A (a green fluorophore). Bar indicates 100 μm.

In some insects, ecdysteroids influences spermatogenesis (Mitsui et al. 1976). Our observation that AKR2E4 is located in the testes of *B. mori* suggests that it may also have an effect on spermatogenesis. Silkworm testes contain ecdysteroids and the amounts of these hormones vary at different times after fourth ecdysis (Fugo et al. 1995). The ecdysteroid titer shows two major peaks in testes, namely at Days 8 and 12 after fourth ecdysis (Fugo et al. 1995). Our results here showed that before these two peaks, *AKR2E4* mRNA was abundant in the middle of the fifth-instar stage and just after pupation (Fig. 2). Based on AKR2E4-mediated catalysis of ecdysone production, our results support the hypothesis that AKR2E4 plays a role in regulation of ecdysteroid titer in testes during silkworm development. Currently, we are examining the amount of 3DE in silkworm testes. We are also producing knock-in silkworm mutants with AKR2E4 by genome editing. These will be of value for investigating the physiological role of AKR2E4 in spermatogenesis of *B. mori* in the future.

Acknowledgments

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