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Ovarian 3-hydroxy-3-methylglutaryl-CoA reductase in *Blattella germanica* (L.): pattern of expression and critical role in embryogenesis

R. Zapata, M.-D. Piulachs, X. Bellés *

Department of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona (CID, CSIC), Jordi Girona 18, Barcelona 08034, Spain

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Abstract

In the ovary of adult *Blattella germanica*, the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) is highly expressed in mid-late vitellogenesis, suggesting a functional link of the mevalonate pathway with choriogenesis. The inhibitor of HMG-CoA reductase, fluvastatin, applied in females in late vitellogenesis, inhibits the activity of the enzyme in the ovary and in the developing embryos within the ootheca. This does not affect choriogenesis or ootheca formation but reduces the number of larvae per ootheca. Our results suggest that fluvastatin is incorporated into the oocytes and has delayed inhibitory effects on the oviposited eggs. HMG-CoA reductase is essential for embryogenesis, but not for chorion formation.

Keywords: *Blattella germanica*; Cockroach; HMG-CoA reductase; Ovary; Embryogenesis

1. Introduction

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (EC 1.1.1.34) plays a key role in isoprenoid biosynthesis (Goldstein and Brown, 1990), as it catalyses the formation of mevalonate, which is the common precursor for a number of essential compounds, including dolichols, ubiquinones and sterols like cholesterol, the most emblematic final product of the pathway. Insects do not synthesise cholesterol de novo (Beenakers et al., 1985), but, as a genuine trait, they produce juvenile hormones, a family of compounds that regulate embryonic development, repress metamorphosis and induce the synthesis of vitellogenin in most insect species (Nijhout, 1994). These peculiarities make insects an interesting model to study the mevalonate pathway and HMG-CoA reductase.

HMG-CoA reductase has been cloned in the fruitfly *Drosophila melanogaster* (Gertler et al., 1988), the German cockroach *Blattella germanica* (Martínez-González et al., 1993) and the moth *Agrotis ipsilon* (Duportets et al., 2000). In addition, a partial sequence of the enzyme has been described in the bark beetle *Ips paraconfusus* (Tittiger et al., 1999). The case of *B. germanica* is especially interesting because it is a pseudovoviviparous cockroach: the vitellogenic cycle, which lasts 7 days, is followed by oocyte choriogenesis and oviposition in an egg-case, or ootheca, which is carried by the female attached to the genital atrium during the 15 days that lasts the embryogenesis. In this cockroach, molecular information derived from enzyme cloning has prompted expression and functional studies focused on HMG-CoA reductase, especially in the fat body, where this enzyme plays key roles in reproduction associated with the glycosylation of vitellogenin (Casals et al., 1996, 1997; Martín et al., 1996; Zapata et al., 2002). However, HMG-CoA reductase is expressed not only in the fat body but also in other tissues such as the ovary, where it is highly expressed (Martínez-González et al., 1993) following a particular pattern along the first reproductive cycle (Buesa et al., 1994). These authors showed that

* Corresponding author. Tel.: +34-9340-06124; fax: +34-9320-45904.

E-mail address: xbragr@cid.csic.es (X. Bellés).

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HMG-CoA reductase is strongly expressed in the ovaries during full vitellogenesis, whereas maximal enzymatic activity is reached in late vitellogenesis, which points to the involvement of HMG-CoA reductase in choriogenesis (Buesa et al., 1994).

In the present work we used fluvastatin, a well-known inhibitor of HMG-CoA reductase in mammals (Endo and Hasumi, 1989; Farnier, 1999) and insects (Debernard et al., 1994; Zapata et al., 2002), to study the function of HMG-CoA reductase in the ovary of *B. germanica*. The results suggest that ovarian HMG-CoA reductase is essential for embryogenesis, but not for chorion formation.

2. Materials and methods

2.1. Insects

Adult *B. germanica* (L.) (Dictyoptera, Blattellidae) females were obtained from a colony reared in the dark at 30 ± 1°C and 60–70% r.h. Freshly moulted virgin females were isolated and used at the appropriate ages.

2.2. Dissections and treatments

Ovaries were dissected from CO_2-anaesthetised specimens and preserved at −70 °C until use. Fluvastatin was applied topically, on the dorsal part of the abdomen, in acetone solution. The maximal volume used in treatments was 2 μl, and the same volume of acetone was applied to controls. In short-term assays, virgin females were treated on day 5 of adult life and ovaries were dissected 24 h later. To study the delayed effects on embryogenesis, virgin females were treated on day 5 of adult life and the ootheca and its contents were studied on day 3 of oothecal transport (the carrying of the ootheca). The term ootheca used throughout the article always refers to the egg case plus the eggs or developing embryos packed inside. In long-term assays to study ootheca formation and larval production, 5-day-old virgin females were treated with fluvastatin, placed in the presence of adult males and left until the eggs of the first and the second ootheca had hatched.

2.3. Reagents

HMG-CoA, DL-3-[glutaryl-3-^{14}C] was obtained from American Radiolabeled Chemicals (ARC), and ^{32}PdCTP from Amersham. Fluvastatin was a gift from Novartis.

2.4. Enzymatic activity measurements

To measure HMG-CoA reductase activity, individual ovary pairs or ootheca (with the eggs inside) samples were homogenised with a plastic pestle in 200 μl of a buffer composed of 100 mM sucrose, 40 mM K_2HPO_4 pH 7.2, 30 mM EDTA, 50 mM KCl, 0.5 mM PMSF, 0.25% (v/v) Triton X-100 and 10 mM DTT. Protein contents in homogenates were measured by the method of Bradford (1976). Two aliquots were assayed in parallel for each determination of enzymatic activity following the method of Goldstein et al. (1983). The assay was performed with 100 μg of total ovary or ootheca protein for 40 min. In these conditions, the assay was linear and the substrate consumed was less than 5%.

2.5. Western blot analysis

For protein extraction, ovaries or oothecae were homogenised as described for enzymatic activity measurements. An amount of 100 μg of ootheca total proteins and a variable amount of ovary equivalents were electrophoresed in 7.5% SDS-PAGE gels, transferred to a nitrocellulose membrane (Amersham) and processed for ECL Western blotting using a kit from Amersham, following the manufacturers' guidelines. The antibody used was that described and characterised by Zapata et al. (2002). It was obtained using the amino acid sequence 828–841 (GHLVKSHMRHNRSS) of *B. germanica* HMG-CoA reductase (see the entire sequence in Martínez-González et al., 1993) as antigen. In the present experiment the antibody was used at 1:400. Densitometry of gels was carried out with a Molecular Dynamics computing densitometer, and results were expressed in arbitrary units.

2.6. Northern blot analysis

Ovary and ootheca RNA was isolated using RNeasy kit of Quiagen. Both ovaries or the ootheca from the experimental female were used for each determination, and three determinations were carried out daily during the study. A total of 30 μg of RNA from each sample was fractionated in 1.2% agarose/formaldehyde gels, transferred to Hybond-N$^+$ membranes (Amersham) and UV cross-linked. A fragment of 0.7 kb of cDNA from HMG-CoA reductase was amplified by PCR and used as a probe. The oligonucleotides used in the PCR were 5′CACTTGCAACAACTGAGGGC3′ as forward and 5′GAAGGCATGGTGCAGGATAC3′ as reverse. Hybridations and washes were performed at 42 °C following the procedure of Sambrook et al. (1989).

3. Results

3.1. Expression of ovarian HMG-CoA reductase during the first gonadotropic cycle

The enzymatic activity increased steadily from day 2 to 4, remained high on day 5 and decreased slightly on

days 6 and 7 (Fig. 1A). Western blot analysis revealed two bands of molecular masses of 58 and 66 kDa (Fig. 1B), although from day 4 to 7 the band of 58 kDa molecular mass became undetectable. Protein levels increased sharply on day 5 and decreased steadily thereafter (Fig. 1B and C). The levels of mRNA increased from day 2 to 3, maintained relatively high values until day 5, and then decreased on days 6 and 7 (Fig. 1D).

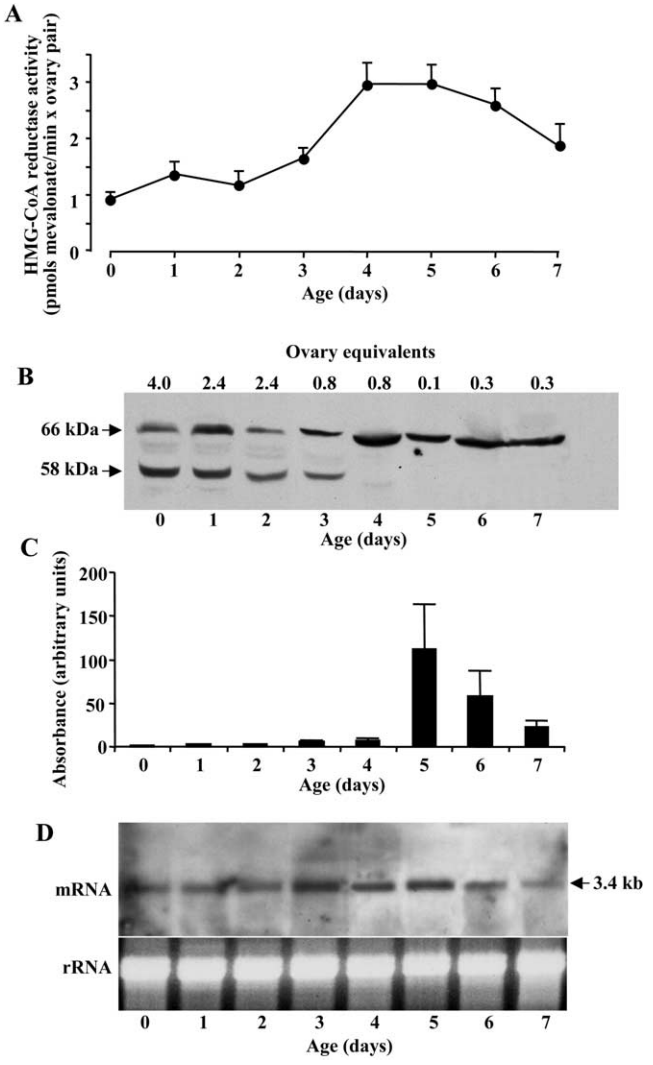

Fig. 1. Expression and activity of HMG-CoA reductase in the ovary of *B. germanica* during the first gonadotropic cycle. A. Enzymatic activity; values are expressed as the mean±SEM ($n = 10$). B. Western blot analysis of HMG-CoA reductase levels; the blot is representative of three replicates; note the various ovary equivalents loaded depending on the age. C. Densitometry of the bands corresponding to HMG-CoA reductase (panel B) in the three blots studied; values have been corrected to represent one ovary equivalent in all cases and are expressed as the mean±SEM. D. Northern blot analysis of HMG-CoA reductase mRNA levels; the portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading (lower panel); the blot is representative of three replicates.

3.2. Effect of fluvastatin on HMG-CoA enzymatic activity and expression

Given that the highest levels of HMG-CoA reductase in terms of mRNA, protein and enzymatic activity were observed around day 5 (Fig. 1), short-term effects of fluvastatin were studied on females treated with various doses of the compound (1, 10 and 50 μg) on day 5 and analysed 24 h later. Fluvastatin significantly inhibited the enzymatic activity in a dose-dependent fashion (Fig. 2A), inducing 58, 74 and 83% inhibition at 1, 10 and 50 μg, respectively. Conversely, Western (Fig. 2B) and Northern (Fig. 2C) blot analyses indicated that the treatment with 50 μg of fluvastatin did not affect protein or mRNA levels, respectively.

3.3. Effect of fluvastatin on choriogenesis, ootheca formation and larval production

We applied a separate treatment of 50 μg of fluvastatin on day 5 of adult life to study its effects on chorion formation 36 h later. Results showed that 72% of controls (10 out of 14) and 78% of treated females (14 out of 18) had well-chorionated oocytes, with the characteristic nipple morphology of the apical pole, as described by Pascual et al. (1992). Conversely, fluvastatin induced clear-cut effects on larval production (Table 1). The mortality observed in the treated adults (10–20%) was similar to that of controls (10%). All the survivors formed ootheca but none of the embryos from 10 and 50 μg treatments was viable (Table 1). The other parameters studied (time elapsed until the formation of the first ootheca, duration of embryogenesis and number of larvae emerging from viable ootheca) were similar to those obtained in controls (Table 1). The females surviving these experiments were observed throughout a second reproductive cycle. The groups of females that had been treated with 1 or 10 μg of fluvastatin formed a number of oothecae similar to that of the controls. In contrast, the group of females that had been treated with 50 μg of fluvastatin formed fewer viable oothecae. All these second-cycle oothecae, formed by females treated with 1, 10 or 50 μg of fluvastatin, or formed by control females, yielded a similar number of live larvae (Table 1).

3.4. Expression of HMG-CoA reductase during embryogenesis

The results described in Section 3.3 rule out the participation of HMG-CoA reductase in choriogenesis and point to its key role in embryogenesis. In addition, the effect of fluvastatin on larvae production suggests that the treated female incorporated the compound into the oocytes, and it exerted a delayed inhibitory effect on the embryo. To test this hypothesis we first studied the

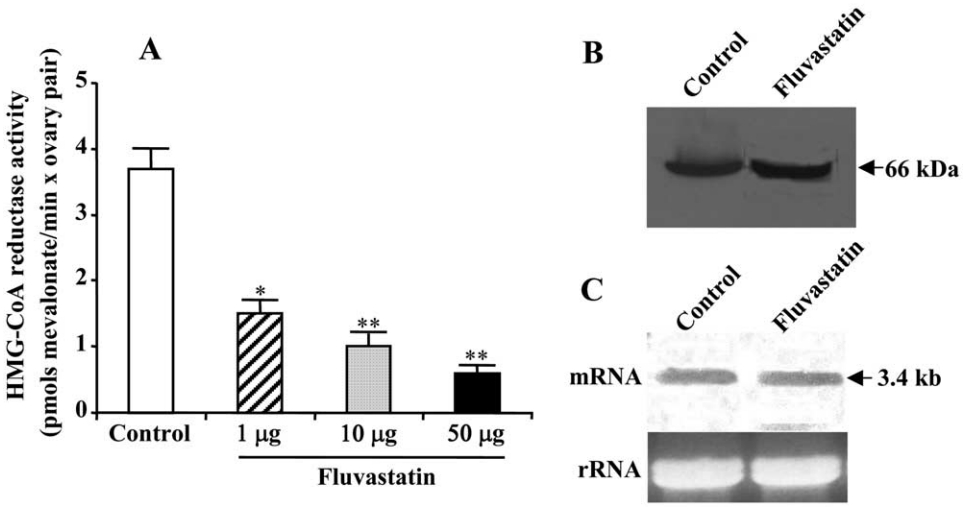

Fig. 2. Effects of fluvastatin on HMG-CoA reductase in the ovary of *B. germanica*. Adult females were treated with fluvastatin on day 5 of adult life and studied 24 h later. A. Enzymatic activity; values are expressed as the mean±SEM ($n = 6$–10) and the asterisks indicate significant differences with respect to controls (*t*-test, $*p < 0.01$, $**p < 0.0001$). B. Western blot analysis of HMG-CoA reductase levels; the dose used was 50 µg of fluvastatin, and the blot is representative of four replicates. C. Northern blot analysis of HMG-CoA reductase mRNA levels; the portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading (lower panel); the dose used was 50 µg of fluvastatin, and the blot is representative of three replicates.

Table 1
Effect of fluvastatin on ootheca formation and larval production in *B. germanica*. Adult females were treated with selected doses of fluvastatin or with acetone on day 5 of adult life. Values are expressed in absolute terms or as the mean±SEM

	Control	Fluvastatin (µg)		
		1	10	50
Number of females used	10	10	10	10
Females that died	1	1	2	1
Females that formed the first ootheca	9	9	9[a]	9
Days until the formation of the first ootheca	7.33±0.17	7.44±0.24	7.44±0.18	7.67±0.17
Number of viable first ootheca	9	9	0	0
Duration of embryogenesis (days)	16.3±0.24	16.4±0.24	–	–
Number of larvae emerging from the first viable ootheca	36.9±0.5	33.1±1.4	–	–
Females that formed a second ootheca	7	7	6	5
Number of viable second ootheca	7	7	6	2
Number of larvae emerging from the second viable ootheca	42.0±2.4	34.7±0.6	34.0±3.8	44.0±1.4

[a] One female died just after the formation of the ootheca.

expression pattern of HMG-CoA reductase in the ootheca during embryogenesis.

Enzymatic activity peaked on day 3 (Fig. 3A), decreased on day 4 and remained relatively low until egg hatching. In terms of protein, HMG-CoA reductase showed the highest levels on days 0 and 1, the levels decreased on days 2 and 3, and became undetectable thereafter (Fig. 3B). Finally, Northern blot analysis (Fig. 3C) revealed that HMG-CoA reductase mRNA levels were high on the first days of embryogenesis (until day 6) and then decreased. The levels of mRNA were not determined between days 0 and 2 because we were not able to isolate RNA from these samples, which was possibly due to the low number of cells and the high amounts of vitellin present.

3.5. Effect of fluvastatin on HMG-CoA enzymatic activity and expression in oothecae

We next studied whether fluvastatin administered to the female inhibited HMG-CoA reductase in the embryo. Given that the highest levels of enzymatic activity were observed on day 3 after the formation of the ootheca (Fig. 3A), fluvastatin was applied on day 5 of adult life and the delayed effects were analysed in the ootheca on day 3 after its formation. Fluvastatin significantly inhibited the enzymatic activity in a dose-dependent fashion (Fig. 4A) by 58, 85 and 93% at 1, 10 and 50 µg, respectively. Conversely, Western blot analysis (Fig. 4B) indicated that the treatment did not affect HMG-CoA reductase in terms of protein.

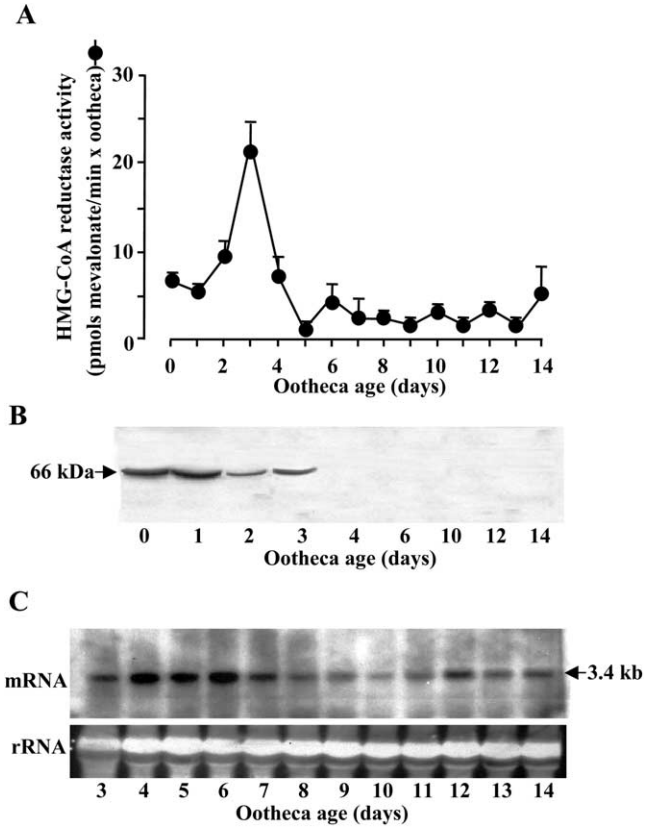

Fig. 3. Expression and activity of HMG-CoA reductase in the ootheca of *B. germanica*. A. Enzymatic activity and total protein levels; values are expressed as the mean±SEM ($n = 4-16$). B. Western blot analysis of HMG-CoA reductase levels; the blot is representative of three replicates. C. Northern blot analysis of HMG-CoA reductase mRNA levels; the portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading (lower panel); the blot is representative of three replicates; days 0–2 were not determined owing to the low number of cells and the large quantity of vitellin which hindered the isolation of RNA.

4. Discussion

In the ovary of *B. germanica*, the expression of HMG-CoA reductase in terms of mRNA, protein and enzymatic activity was high around mid-late vitellogenesis. At the beginning of the first reproductive cycle, the three parameters were low, then increased on day 4, reached top values on day 5 and decreased slightly but steadily afterwards. The timing of enzymatic activity differs from that reported by Buesa et al. (1994), who found the peak of activity on days 6 and 7, whereas we found the peak on days 4 and 5. However, the difference may be merely as a result of the different duration of the reproductive cycle in the specimens of *B. germanica* used in the respective studies (7 days in our specimens, 8–10 days in the specimens used in the previous study).

In most Western blot analyses, the antibody inmunodetected two bands corresponding to proteins of molecular masses of 58 and 66 kDa, both being proteolytic fragments of the native *B. germanica* HMG-CoA reductase (Casals et al., 1996; Zapata et al., 2002). This fragmentation is similar to the well-documented proteolysis of mammalian native HMG-CoA reductase (Edwards et al., 1980; Liscum et al., 1985), whose fragments of 62 and 53 kDa show enzymatic activity. The absence of the 58 kDa band in fully vitellogenic oocytes and eggs is interesting but difficult to explain. Perhaps the conditions of extraction in fully vitellogenic oocytes and eggs, which present the highest amounts of vitellin, impair the proteolysis at the site that generates the 58 kDa fragment.

Short-term experiments, applying fluvastatin on 5-day-old females to examine its effects upon ovarian HMG-CoA reductase 24 h later, showed that this statin reduced enzymatic activity in a dose-dependent fashion. However, protein and mRNA levels were not altered. These results differ from those reported for the fat body of *B. germanica* (Zapata et al., 2002), where fluvastatin

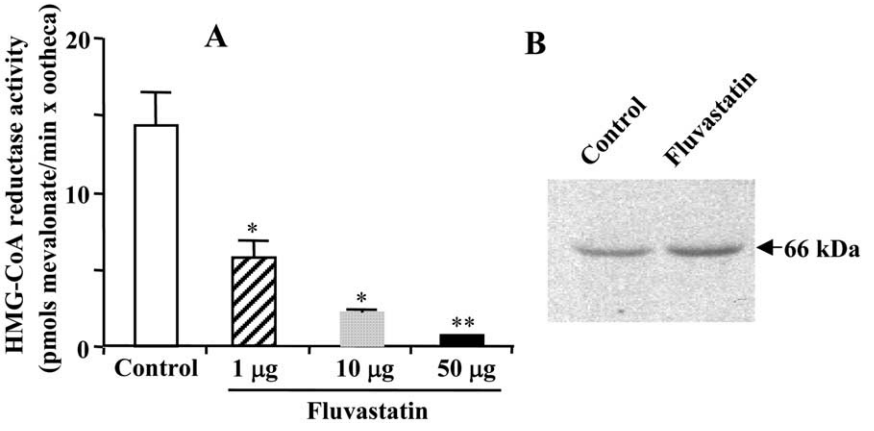

Fig. 4. Effects of fluvastatin on HMG-CoA reductase in the ootheca of *B. germanica*. Adult females were treated with fluvastatin on day 5 of adult life and activity was studied in the ootheca 3 days after its formation. A. Enzymatic activity; values are expressed as the mean±SEM ($n =$ 4–7) and the asterisks indicate significant differences with respect to controls (*t*-test, $*p < 0.01, **p < 0.001$). B. Western blot analysis of HMG-CoA reductase levels; the dose used was 50 µg of fluvastatin, and the blot is representative of three replicates.

reduced both the enzymatic activity and protein levels, possibly by altering the structure of the enzyme and increasing its vulnerability to proteases. This hypothesis was based on data reported by Istvan et al. (2000) and Istvan and Deisenhofer (2001), who showed that several statins complex with HMG-CoA reductase and modify the conformation of the enzyme, which increases its vulnerability to proteases. Accordingly, the lack of effects on ovarian HMG-CoA reductase in terms of protein, may be merely due to the fact that the ovary is metabolically much less active than the fat body. The cysteine-proteases involved in the first steps of HMG-CoA reductase degradation may be less abundant, or less active, in the ovary than in the fat body.

Concerning the functions of the mevalonate pathway in the ovary, the high levels of expression and activity of HMG-CoA reductase in mid-late vitellogenesis had suggested that this enzyme could play a significant role in choriogenesis (Buesa et al., 1994). However, our present results are not consistent with this hypothesis, given that females treated with effective inhibitory doses of fluvastatin produced normally chorionated oocytes that were oviposited into correctly formed oothecae.

Treatment of the female resulted in a delayed effect on the viability of embryos, as shown by the long-term experiments. We thus studied HMG-CoA reductase in the ootheca. Its expression and activity were high on the first days of embryogenesis, and the pattern of enzymatic activity, with a peak on day 3, did not parallel those of mRNA and protein. The contrast between low protein levels and high enzymatic activity, or vice versa, that occur between days 0 and 3 might be explained by mechanisms regulating the activity by phosphorylation and dephosphorylation of the enzyme (Goldstein and Brown, 1990). But what is especially striking is that the protein was undetectable from day 4 thereafter, when mRNA and enzymatic activity levels are still well apparent. The well-quantifiable levels of enzymatic activity indicate that there must be HMG-CoA reductase from day 4 thereafter. The fact that it is not detected with our Western blot method may be due to the generation of new proteolytic fragments in this mid- and late-embryogenesis period that are not recognised by our antibody.

The high expression and activity of HMG-CoA reductase in early embryogenesis is not surprising, as on these days the germ cell line migrates towards the embryo poles, followed by cellular division and organogenesis (Tanaka, 1976), while HMG-CoA reductase is involved in all these processes. In *D. melanogaster*, maternal HMG-CoA reductase is required for early embryonic development (Perrimon et al., 1996) and to guide primordial germ cells to the somatic gonad (van Doren et al., 1998), which makes HMG-CoA reductase mutant embryos inviable. In addition, the critical role of HMG-CoA reductase in cellular proliferation is widely documented (Leonard et al., 1990; Dricu et al., 1997; Elson et al., 1999).

The inhibition of HMG-CoA reductase activity in 3-day-old embryos, induced by topical treatment of the female with fluvastatin, indicates that the compound was incorporated into the growing oocytes and exerted its inhibitory effect on the egg and embryo. At effective doses (10 and 50 μg), fluvastatin blocked embryogenesis and induced 100% sterility in the first reproductive cycle. The results of long-term experiments are consistent with those obtained by Zapata et al. (2002) who studied HMG-CoA reductase in the fat body. In this study, fluvastatin administered to 3-day-old adult females induced 50 and 100% sterility at doses of 10 and 50 μg, respectively. However, our present results indicate that the sterilising effects of fluvastatin do not persist in the second reproductive cycle, in which the treated females produce a number of larvae per viable ootheca similar to that of controls.

Acknowledgements

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