Effects of Hypocholesterolaemic Agents on the Expression and Activity of 3-Hydroxy-3-Methylglutaryl-CoA Reductase in the Fat Body of the German Cockroach

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In the fat body of adult *Blattella germanica* females, the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) during the first reproductive cycle is parallel to that of vitellogenin, suggesting a functional link between the mevalonate pathway, and vitellogenesis and reproduction. We have studied the effects of compactin and fluvastatin, two inhibitors of HMG-CoA reductase, on the expression and activity of the enzyme in the fat body, and on the ootheca formation, ootheca viability, and number of larvae per viable ootheca. Short-term assays showed that both compounds reduce the protein levels and enzymatic activity of HMG-CoA reductase, and long-term experiments revealed that fluvastatin impairs embryo development. Arch. Insect Biochem. Physiol. 49:177–186, 2002.   © 2002 Wiley-Liss, Inc.

INTRODUCTION

In vertebrates, the main final product of the mevalonate pathway is cholesterol, and the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) plays a key role in its regulation (Goldstein and Brown, 1990). In contrast, insects do not synthesize cholesterol de novo (Beenakers et al., 1985) and the mevalonate pathway mainly leads to ubiquinone, dolichol, and juvenile hormone. Ubiquinone is involved in the electron transport chain, dolichol behaves as a donor of oligosaccharide residues in the glycosylation of proteins, and juvenile hormone regulates embryonic development, represses metamorphosis, and induces the synthesis of vitellogenin in most insect species (Nijhout, 1994). By analogy with vertebrate animals, HMG-CoA reductase has been postulated as a key enzyme in the regulation of the mevalonate pathway in insects (Feyereisen, 1985), and it has been studied at the molecular scale in the fruitfly *Drosophila melanogaster* (Gertler et al., 1988), the German cockroach *Blattella germanica* (Martínez-González et al., 1993), the moth *Agrotis ipsilon* (Duportets et al., 2000), and the bark beetle *Ips paraconfusus* (Tittiger et al., 1999).

In *B. germanica*, Casals et al. (1996) reported the pattern of expression of HMG-CoA reductase in the fat body during the reproductive cycle of the adult female, in terms of mRNA, protein, and enzymatic activity. Protein levels and enzymatic activity peak in the middle of the cycle, which is

paralleled by an increase of vitellogenin levels, as described by Martín et al. (1995). This suggested a functional link between the mevalonate pathway and vitellogenin production. A study by Martín et al. (1996) on the inhibitory effects of allatostatin peptides (Bellés et al., 1994) on vitellogenin glycosylation showed that mevalonolactone counteracts such effects, thus suggesting that the mevalonate pathway in the fat body of the adult female is involved in the glycosylation of vitellogenin through dolichol intermediates. Another paper by Casals et al. (1997) on the effect of allatectomy and ovariectomy upon HMG-CoA expression in the fat body also supported this hypothesis.

We, thus, aimed to analyze the effects of hypocholesterolaemic agents that inhibit HMG-CoA reductase upon the expression and activity of the enzyme in the fat body and the reproductive capabilities of *B. germanica*. As hypocholesterolaemic agents, we used compactin and fluvastatin, two well-known statins that are efficient HMG-CoA inhibitors not only in vertebrates (Endo and Hasumi, 1989; Farnier, 1999) but also in insects. For example, compactin and fluvastatin inhibit juvenile hormone biosynthesis in *B. germanica* (Bellés et al., 1988) and in *Locusta migratoria* (Debernard et al., 1994), respectively. The results obtained in the present work showed that both statins have a short-term inhibitory effect upon HMG-CoA reductase in the fat body of *B. germanica* and fluvastatin shows long-lasting effects that impair fertility.

MATERIALS AND METHODS

Insects, Dissections, and Treatments

Adult *Blattella 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. Fat bodies, brain, and ovaries were dissected from CO_2-anaesthetized specimens and preserved at –70°C until use. Haemolymph was collected with a calibrated micropipette inserted into the femur. Fluvastatin and compactin were applied topically, on the dorsal part of the abdomen, in acetone solution. The maximal volume used in treatments was 2 μl and so the same volume of acetone was applied to controls. In short-term assays, virgin females were treated on day 2 of adult life and tissues were dissected 24 h later. To study the effects on ovarian vitellin, females were treated on day 2 and ovarian vitellin was determined 4 days later. In long-term assays to study ootheca formation and larval production, 2-day-old virgin females were treated with the compounds, placed in the presence of adult males, and left until the eggs of the first ootheca had hatched.

Reagents

3-Hydroxy-3-methylglutaryl coenzyme A, DL-3-[glutaryl-3-^{14}C] was obtained from American Radiolabeled Chemicals (ARC), and [32]PdCTP from Amersham. Fluvastatin was a gift from Novartis and compactin was provided by Sigma (St. Louis, MO).

RNA Blot Analysis

Fat body RNA was isolated using an RNeasy kit (Quiagen). Fat bodies from 3 to 5 females were pooled 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 PCR were 5′CACTTGCAACAACTGAGG-GC3′ as forward and 5′GAAGGCATGGTGCAGGAT AC3′ as reverse. Hybridizations and washes were performed at 42°C following Sambrook et al. (1989). Densitometry of gels was carried out with a Molecular Dynamics computing densitometer, and results were expressed in arbitrary units.

Preparation of Conjugates and Production of Antibodies

In the study by Casals et al. (1996), the protein pattern of HMG-CoA reductase was obtained

with an antibody raised against the rat enzyme. Here, we generated a homologous antibody. To this end, a peptide corresponding to amino acid sequence 828-841 (GHLVKSHMRHNRSS) of *B. germanica* HMG-CoA reductase (see the entire sequence in Martínez-González et al., 1993) was conjugated to keyhole-limpet hemocyanin (KLH) (Sigma) in accordance with Sambrook et al. (1989). The antibody was raised in male New Zealand white rabbits as described in Vilaplana et al. (1999) with minor modifications. Analogously, an antibody against the HMG-CoA synthase 2 of *B. germanica* was obtained using the amino acid sequence 286-300 (GVKLEDTYFDREVEK) of the corresponding enzyme (see the entire sequence in Buesa et al., 1994), which was used in control experiments.

Western Blot Analysis

For protein extraction, tissues were homogenized 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). Eighty micrograms of total proteins 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 manufacturer's guidelines. To detect HMG-CoA reductase and HMG-CoA synthase, the antibodies were used at 1:400 and 1:800, respectively.

Enzymatic Activity Measurements

To measure HMG-CoA reductase activity, individual fat body samples were homogenized as described for Western blot analysis. Two aliquots were assayed in parallel for each determination of enzymatic activity following Goldstein et al. (1983). The assay was performed with 100 µg of total fat body protein for 40 min. In these conditions, the assay was linear and the substrate consumed was less than 5%.

ELISA Quantification of Vitellogenic Proteins

The procedure and materials were as described by Martín et al. (1995). Vitellogenic proteins from haemolymph and ovary samples were dissolved in carbonate buffer (0.05 M, pH 9.6), and the resulting solutions (100 µl) were absorbed to 96-well ELISA microplates (NUNC-Immuno Plate Maxisorp 96F) by incubation at 4°C overnight. ELISA was conducted using secondary peroxidase labeling developed with 3,3',5,5'-tetramethylbenzidine (Sigma) and the antiserum against vitellogenin-vitellin reported by Martín et al. (1995). Absorbance was read at 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems).

RESULTS

Validation of the HMG-CoA Reductase Antibody

To confirm that the HMG-CoA reductase antibody was specific for the *B. germanica* enzyme, immunoinactivation experiments based on enzymatic activity were carried out. Aliquots of fat body extracts from 3-day-old females were incubated with either the HMG-CoA reductase antibody or pre-immune serum as a negative control. Increasing amounts of the HMG-CoA reductase antibody reduced the enzymatic activity in a dose-dependent manner (up to 55–60% reduction), whereas no detectable effect was observed with the pre-immune serum (Fig. 1A). Thereafter, we examined the occurrence of the enzyme in a number of tissues of *B. germanica* using this HMG-CoA reductase antiserum. The antibody immunodetected two proteins of molecular masses of 58 and 66 kD in brain, ovarian, and fat body tissues (Fig. 1B), which are proteolytic fragments of *B. germanica* HMG-CoA reductase (see Discussion).

Expression of Fat Body HMG-CoA Reductase During the Gonadotropic Cycle

Once the newly raised antibody for *B. germanica* HMG-CoA reductase had been validated, we studied the developmental pattern in the adult fat body
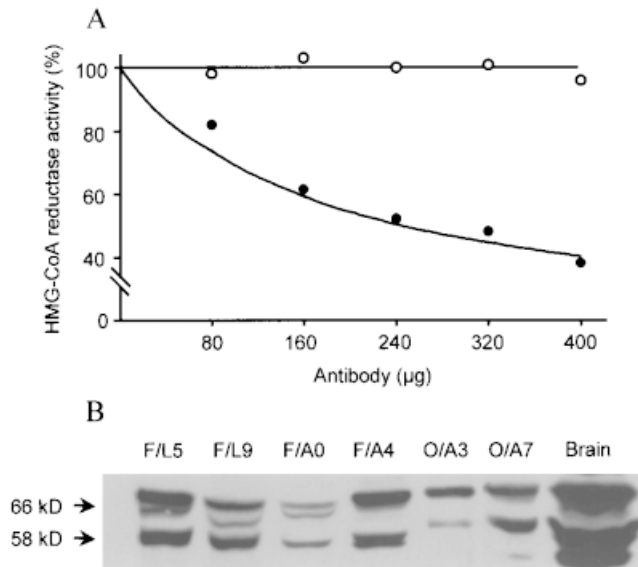
A

B

Fig. 1.   **A:** Inactivation of HMG-CoA reductase of *Blattella germanica* by the antibody described in the present paper. Fat body homogenates were incubated for 40 min at 37°C with increasing amounts of HMG-CoA reductase antibody (closed circles) or pre-immune serum (open circles). After incubation, the enzymatic activity was measured in duplicate; the activity is expressed as a percentage of the value obtained in the absence of antibody. **B:** Western blot analysis of HMG-CoA reductase in various tissues of *Blattella germanica*. F/L5: Fat body from 5-day-old last instar larvae. F/L9: Fat body from 9-day-old last instar larvae. F/A0: Fat body from 0-day-old adult female. F/A4: Fat body from 4-day-old adult female. O/A3: Ovary from 3-day-old adult female. O/A7: Ovary from 7-day-old adult female, with chorionated oocytes. Brain: brain from a 4-day-old adult female. The blot is representative of 3 replicates.

during vitellogenesis. Protein levels (Fig. 2A) increased steadily until day 3 and remained relatively high on days 4 and 5. Conversely, the levels of mRNA (Fig. 2B) were high just after the imaginal molt and then decreased progressively. The enzymatic activity (Fig. 2C) showed a clear peak on day 3 of the cycle.

Effects of Fluvastatin and Compactin on HMG-CoA Enzymatic Activity and Expression in the Fat Body

Given that the most significant increase of enzymatic activity was observed between days 2 and 3 (Fig. 2C), short-term effects of fluvastatin and compactin were studied on females treated with 50 μg of the corresponding compound on day 2 and analyzed 24 h later. Both compounds significantly inhibited approximately 25% the enzymatic activity (Fig. 3A) and the protein (Fig. 3B and D). Conversely, Northern blot analysis (Fig. 3E) indicated that the treatment did not affect mRNA levels. The effect of fluvastatin and compactin seems specific for HMG-CoA reductase, at least when studied in terms of protein, given that HMG-CoA synthase levels were not affected (Fig. 3C).

Effects of Fluvastatin and Compactin on Haemolymph Vitellogenin and Ovarian Vitellin

The same treatment was applied to study the effects of fluvastatin and compactin upon haemolymph vitellogenin and ovarian vitellin in 2-day-old females. Twenty-four hours after the treatment, the vitellogenin content in the haemolymph of treated insects was similar to that of controls (Fig. 4A). Given that these negative results could be due to the high turnover of vitellogenin in the haemolymph, and in order to obtain clear-cut cumulative effects, we studied the vitellin content in the ovary 4 days after the treatment. Results (Fig. 4B) showed that the ovarian vitellin content was sig-

Fig. 3.   Effects of fluvastatin and compactin on HMG-CoA reductase activity and expression in the fat body of *Blattella germanica*. Adult females were treated with 50 μg of fluvastatin or compactin on day 2 of adult life and studied 24 h later. **A:** Enzymatic activity. **B:** Western blot analysis of HMG-CoA reductase levels. The blot is representative of 14 replicates. **C:** Western blot analysis of HMG-CoA synthase 2 levels. The blot is representative of 3 replicates. **D:** Densitometry of the bands corresponding to HMG-CoA reductase (B) in the 14 blots studied. **E:** Northern blot analysis of mRNA levels. The portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading (bottom). The blot is representative of 3 replicates. In the histograms, values are expressed as the mean ± SEM (n = 9–14) and the asterisks indicate significant differences with respect to controls (*t*-test, $*P < 0.05$, $**P < 0.01$).
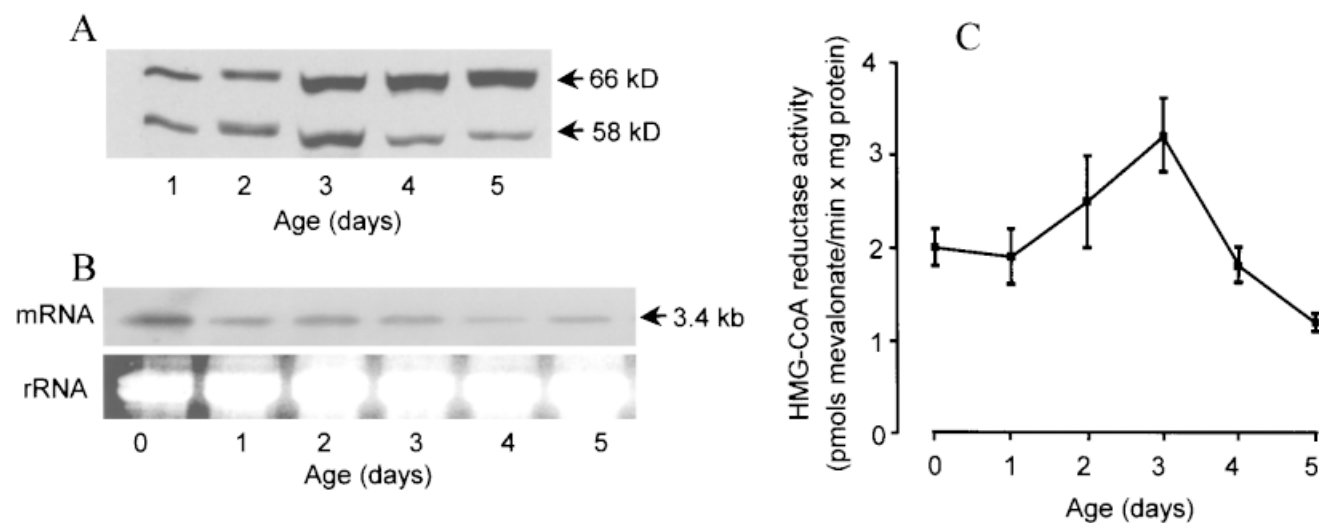
Fig. 2.   Expression and activity of HMG-CoA reductase in the fat body of *Blattella germanica* during the first days of the gonadotropic cycle. **A:** Western blot analysis of HMG-CoA reductase levels. The blot is representative of 3 replicates. **B:** Northern blot analysis of HMG-CoA reductase mRNA levels. The portion of the gel containing rRNA was stained with ethidium bromide to control for equivalent sample loading (bottom panel). The blot is representative of 3 replicates. **C:** Enzymatic activity. Values are expressed as the mean ± SEM (n = 7).
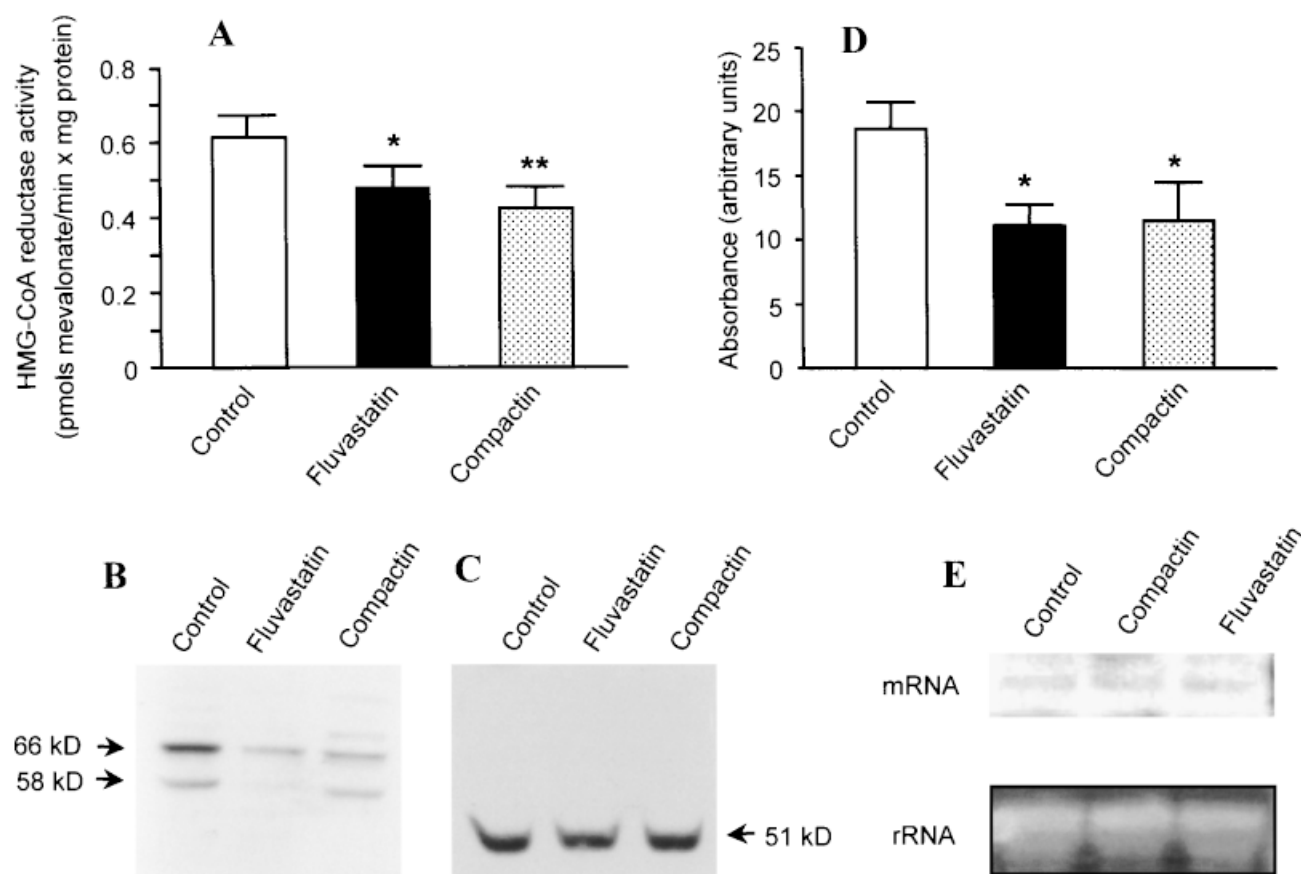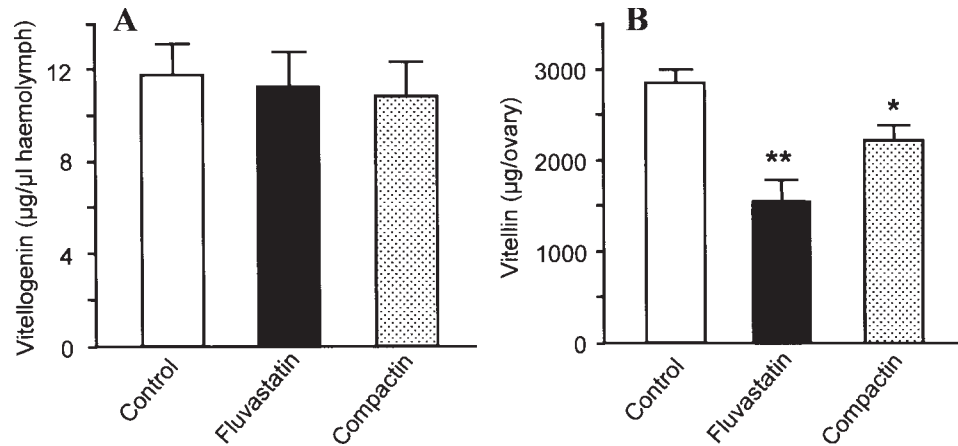

Fig. 3.

Fig. 4.   Effects of fluvastatin and compactin on haemolymph vitellogenin and ovarian vitellin in *Blattella germanica*. Adult females were treated with 50 µg of fluvastatin or compactin on day 2 of adult life and checked 24 h later for haemolymph vitellogenin (**A**), or 4 days later for ovarian vitellin (**B**). Values are expressed as the mean ± SEM (n = 10–16) and the asterisks indicate significant differences with respect to controls (*t*-test, *$P <$ 0.05, **$P < 0.001$).

nificantly lower. The average reduction of vitellin accumulation induced by fluvastatin in the ovary was 46%, which is significantly higher than that caused by compactin (22%).

Effects of Compactin and Fluvastatin on Ootheca Formation and Larval Production

The inhibited accumulation of vitellin in the ovary observed 4 days after the treatment suggested that the effect of the compounds is long-lasting and that it conditioned the reproductive capabilities of the insect. Therefore, we applied a new treatment with fluvastatin or compactin on day 2 of adult life and studied its effects on ootheca formation and larval production. At 50 µg, fluvastatin induced 58% mortality, usually during the formation of the ootheca. All the survivors formed ootheca but none of them was viable (Fig. 5B). In addition, the time elapsed until the formation of the ootheca was slightly higher than that of controls (Fig. 5A). We thus tested fluvastatin at 10 µg, which did not induce mortality or affect ootheca extrusion, but halved the number of viable oothecae (Fig. 5B), slightly lengthened embryogenesis (Fig. 5C), and reduced the number of larvae emerging per viable ootheca by 34% (Fig. 5D). Concern-

ing compactin, no mortality was observed at 50 µg and the other parameters studied gave results similar to those found in controls (Fig. 5A–D).

DISCUSSION

In previous papers (Casals et al., 1996, 1997), we studied the pattern of expression of HMG-CoA reductase in *B. germanica* using a heterologous antibody raised against the rat enzyme (Casals et al., 1996). In the present study, before investigating the effects of hypocholesterolaemic agents, a homologous antibody against *B. germanica* HMG-CoA reductase was raised. The antibody was validated by immunoactivation and tested on various *B. germanica* tissues, revealing two main proteolytic fragments of HMG-CoA reductase of molecular masses 58 and 66 kD, identical to those detected by the rat antibody (see Casals et al., 1996). For control purposes, we also raised a homologous antibody for *B. germanica* HMG-CoA synthase 2 (Buesa et al., 1994), which showed a 51-kD band, which corresponds to the expected molecular weight of the enzyme.

The new antibody of HMG-CoA reductase was then used to study the expression of the enzyme on fat body tissues during the 5 first days of the
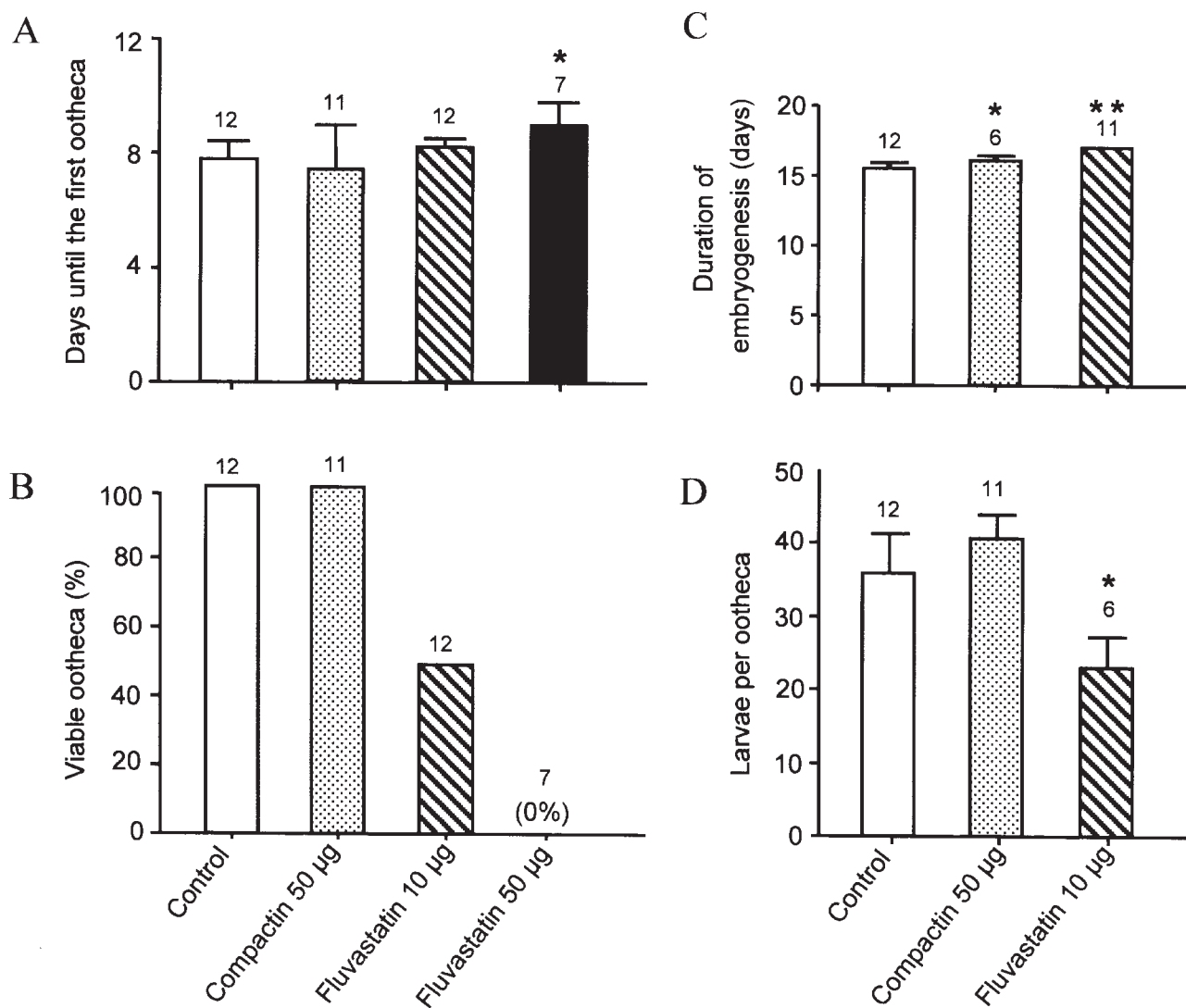
Fig. 5. Effects of fluvastatin and compactin on ootheca formation and larval production in *Blattella germanica*. Adult females were treated with fluvastatin or compactin on day 2 of adult life and monitored until the eclosion of the first ootheca eggs. **A:** Days until the formation of the first ootheca. **B:** Percentage of viable oothecae. **C:** Dura-tion of embryogenesis. **D:** Larvae emerging per ootheca. The number of replicates is indicated at the top of col-umns. In A, C, and D, values are expressed as the mean ± SEM and the asterisks indicate significant differences with respect to controls (*t*-test, *$P < 0.05$, **$P < 0.001$).

gonadotropic cycle of *B. germanica*. Enzymatic ac-tivity and mRNA levels were also determined. Pro-tein levels and enzymatic activity increased steadily until day 3, whereas mRNA levels were the high-est just after adult emergence and then decreased progressively. In general, the patterns obtained were similar to those reported by Casals et al. (1996).

Short-term experiments, i.e., fluvastatin and compactin administered on 2-day-old females and

determination of the effects upon fat body HMG-CoA reductase 24 h later, showed that both statins reduced approximately 25% protein and enzymatic activity. This suggests that the decreased enzymatic activity is due to the lower levels of protein. Con-versely, none of the two compounds affected mRNA levels. The lower levels of protein observed in treated specimens could suggest that the two statins inhibited translation. However, comparison

of the protein and mRNA patterns during the gonadotropic cycle indicates that the increase in protein is paralleled by a decrease in mRNA levels, suggesting that the inhibition of translation would be reflected by an accumulation of mRNA. As this is not the case, the treatment may decrease the HMG-CoA reductase stability. In this sense, Cappel and Gilbert (1989) suggested that mevilonin (=lovastatin), a statin structurally similar to compactin, alters the structure of HMG-CoA reductase, impairing the formation of disulfide bridges and facilitating degradation. More recently, Istvan et al. (2000) have described the crystal structure of the catalytical domain of human HMG-CoA reductase, and Istvan and Deisenhofer (2001) have determined this domain complexed with six different statins, including compactin and fluvastatin. These studies indicate that the statins modify the conformation of the enzyme, which may increase its vulnerability to proteases and hence its lability.

The coincidence between the expression patterns of HMG-CoA reductase (Casals et al., 1996) and vitellogenin (Martín et al., 1995) in the fat body of *B. germanica* suggests that the cycle of the enzyme is associated with the production of the protein. Martín et al. (1996) reported that the mevalonate pathway in the fat body is involved in the glycosylation and export of vitellogenin. These data led us to study the effects of fluvastatin and compactin on haemolymph vitellogenin and ovarian vitellin. No effect was detected on haemolymph vitellogenin, which may be due to the high turnover of vitellogenin in this tissue, which hinders the comparison between treated and controls. Conversely, vitellin contents in the ovary were clearly reduced in treated insects, especially in those treated with fluvastatin, which may be because the effects were cumulative in this case. In these experiments, fluvastatin was more active than compactin, in agreement with the higher potency and long-lasting effects of the former in vertebrates (Endo and Hasumi, 1989; Farnier, 1999). In this context, it is worth noting that vitellogenesis in *B. germanica* is strictly dependent on juvenile hormone (Comas et al., 1999, 2001) and that both statins inhibit juvenile hormone biosynthesis, as

reported in *B. germanica* (Bellés et al., 1988) and *L. migratoria* (Debernard et al., 1994). Therefore, the inhibition of ovarian vitellin may be due not only to direct impairment of vitellogenin production, but also to disruption of juvenile hormone synthesis.

Long-term studies on the effects on ootheca formation and larval production showed that fluvastatin at 50 μg induces high mortality and total inviability of the ootheca formed by the survivors, whereas compactin shows practically no effects at the same dose, which supports that fluvastatin is more potent in long-term assays. At 10 μg, fluvastatin did not affect ootheca extrusion but reduced ootheca viability and the number of larvae emerging per viable ootheca. This indicates that this statin does not affect ootheca formation, but rather embryogenesis. 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. Therefore, if HMG-CoA reductase is also crucial for the embryogenesis of *B. germanica*, the inhibition of embryo viability by fluvastatin could be due to the persistent inhibition of HMG-CoA reductase in treated females and/or to the transference of fluvastatin to the growing oocyte and the delayed effects of it on the embryos.

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