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Production of vitellogenin *in vitro* by the periovaric fat body of *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

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Summary

The fat body produces vitellogenic proteins, which are released to the haemolymph and incorporated into developing oocytes. In the cockroach *Blattella germanica*, endocrine and other physiological cues regulate vitellogenesis in a complex homeostatic pattern, which is not fully understood. The *in vitro* approach may be useful to study this pattern, and the present paper describes an experimental set-up based on the incubation of the periovaric portion of the fat body and the measurement of vitellogenin production with an EIA method. The results correspond fairly well with equivalent data obtained *in vivo* and suggest that the periovaric fat body is an appropriate anatomical unit to study vitellogenin production *in vitro*.

Key words: Fat body, vitellogenin, EIA, cockroach, *Blattella germanica*

Introduction

During vitellogenesis, the fat body is a key organ since it produces the vitellogenic proteins which are further incorporated into developing oocytes (Keeley, 1985). In turn, the production of vitellogenin by the fat body is regulated by endocrine effectors and other physiological cues in a complex homeostatic pattern (Engelmann, 1983; Valle, 1993). This pattern is not yet fully understood, and studies *in vitro* would surely afford valuable data to analyze the mechanisms of action of those factors which affect vitellogenin synthesis and secretion, with the advantage of working with controlled concentrations of experimental compounds (see Keeley et al., 1995).

Blattella germanica (L.) (Dictyoptera, Blattellidae) is an ovoviviparous cockroach in which vitellogenesis is induced by juvenile hormone and proceeds in discrete cycles separated by periods of ootheca transport (Bellés et al., 1987; Martín et al., 1995). Therefore, it offers a good model to study the endocrine modulation of vitellogenesis, and recent studies *in vivo* have provided some cues in this direction. For example, Maestro et al. (1994) have shown that ovariectomized specimens of *B. germanica* produce low levels of juvenile hormone during the first 9 days of imaginal life, whereas Martín et al. (in press) described that ovariectomized females produce huge amounts of vitellogenin which accumulate in the haemolymph (see also Kunkel,

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1981). All the data suggest that the juvenile hormone cycle (Bellés et al., 1987; Maestro et al., 1994) does not modulate that of vitellogenesis (Martín et al., 1995), and that a supplementary factor, possibly coming from the ovary, may be involved in terminating the synthesis of vitellogenin at the end of the cycle.

An approach *in vitro* may be useful to test this hypothesis, but the fat body of insects is a rather ungrateful material to be studied *in vitro*, especially because it is morphologically diverse (and disperse in most species), and functionally polyvalent (see Dean and Locke, 1985; Keeley, 1985). In this context, the fat body of *B. germanica* appears to be a typical example of dispersed (ungrateful) organ to be incubated. To overcome this difficulty, we have developed an *in vitro* experimental set-up based on the incubation of the periovaric portion of the fat body. The results show that the periovaric fat body is an appropriate anatomical unit to study vitellogenin production *in vitro*.

Materials and Methods

Insects

Specimens of *B. germanica* were obtained from a colony fed on Panlab dog chow and water and reared in the dark at 30±1°C and 60–70% r.h. Freshly moulted virgin females were isolated and used at the appropriate physiological ages, which were assessed by measuring the basal oocyte length (Bellés et al., 1987). All dissections and tissue samplings were carried out on carbon dioxide-anaesthetized specimens.

Fat body incubations

Incubations were carried out in Grace's medium, with L-glutamine and without insect haemolymph (Whittaker Bioproducts, Inc. Walkersville, MD, USA). For the incubations we selected the periovaric fat body, i.e., three well defined and connected lobes surrounding each ovary, containing the three characteristic cell types of *B. germanica* fat body: trophocytes, bacteriocytes and urate cells (De Piceis Polver et al., 1986). A single periovaric fat body was used for each incubation. It was dissected out and washed for 10 min in Ringer's saline, pre-incubated for 30 min in Grace's medium, and then transferred to 300 µl of fresh medium where the experimental incubation was carried out for the desired time (in the dark, at 30°C and with gentle shaking). To study the influence of incubation duration, 5-day-old virgin females were used, and periods of 1, 3, 5, 7 and 9 h were compared. A period of 7 h was found adequate and was used as the standard period to study the production of vitellogenin in relation to the gonadotropic cycle. Vitellogenin content in the medium and in the periovaric fat body was measured by EIA (see below) at the end of the incubation.

Tissue extraction for protein studies

Fat body tissues (freshly explanted, as a reference, or incubated *in vitro*) were thoroughly rinsed with Ringer's solution and homogenized in TRIS-buffer (0.4 M NaCl, 50 mM Tris-HCl pH:7.3, 1 mM EDTA, 1 mM PMSF) with a plastic pestle. Homogenates were centrifuged at 10,000 rpm for 20 min, and the resulting supernatant, lying between the pellet and a lipid plug, was recovered for EIA studies (see below) or total protein quantification according to Bradford (1976).

Quantification of vitellogenin by enzyme immunoassay (EIA)

An aliquot accounting for 400–500 ng of total soluble proteins from the extract of fat body tissues was used. It was dissolved in carbonate buffer (0.05 M, pH 9.6) and absorbed to 96-well ELISA microplates (NUNC, Roskilde, Denmark) by incubation overnight at room temperature, with gentle shaking. The subsequent steps, namely incubation with antibodies and revealing procedures, were as previously described (Martín et al., 1995). To analyze the incubation medium, a slight modification of our EIA method (Martín et al., 1995) was introduced. Appropriate aliquots of medium were dissolved in fresh Grace's medium (instead of carbonate buffer) and absorbed to microplate wells at 4°C overnight. To validate this modification a sensitivity standard curve was obtained with carbonate buffer (Martín et al., 1995) and compared with that obtained in parallel with samples diluted in Grace's medium. The two sigmoidal curves (Fig. 1) were equivalent, thus indicating that absorption of extracts in Grace's medium was operative. In addition, standard curves subjected to a logit transformation (Tijsen, 1986) showed that the optimal sensitivity was slightly better for Grace's medium (22.6 ng) than for carbonate (33.6 ng) (Fig. 1, inset).

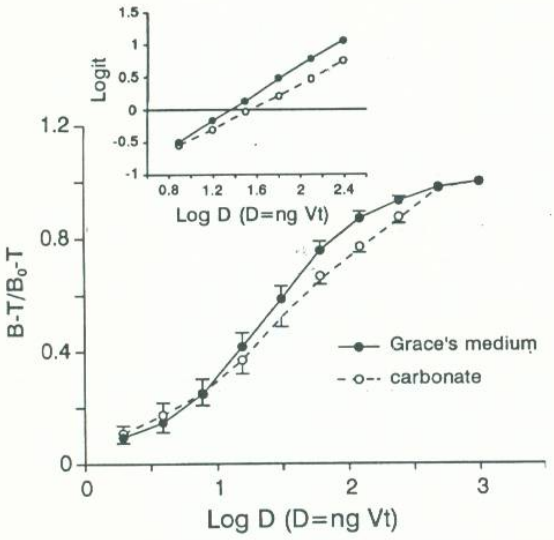

Fig. 1. Sensitivity curve of *B. germanica* anti-vitellin serum (diluted 1:60,000) obtained with increasing amounts of vitellin (Vt) (from 1.9 to 1000 ng), and comparing carbonate buffer and Grace's medium as absortion agent. To homogenize the results, the ratio $B-T/B_0-T$ was used, where B_0 is the maximal OD corresponding to a saturating concentration of antigen, B is the OD corresponding to lower concentrations of antigen, and T is the OD of non-specific binding. The inset shows the logit transformation of both curves. Optimal sensitivity value was 22.6 ng for Grace's medium, and 33.6 ng for carbonate buffer. All values are expressed as the mean ±SEM (n=8-9).

Western blot analysis

Electrophoresis (SDS-PAGE) of vitellogenin was carried out as previously described (Martín et al., 1995). A pool of two media of incubation (600 µl) was transferred to a Microcon-30 microconcentrator (Amicon Inc., Bevery, MA, USA), following the manufacturer's protocol. The resulting protein concentrate and appropriate aliquots of homogenized individual periovaric fat bodies were applied onto a 7.5% SDS-PAGE gel, and the bands were visualized with silver stain. Protein bands were transferred to a nitrocellulose membrane using a Hoefer semi-dry system (Hoefer Scientific Instruments, San Francisco, CA, USA). After the transfer, proteins were visualized by Ponceau S stain, and the molecular weight standard proteins were located and marked. The primary polyclonal antibody against vitellin-vitellogenin (Martín et al., 1995) was used at a dilution of 1:20,000. ECL Western blotting (Amersham Inc., Bucks, UK) was used as a detection system, following the manufacturer's protocol. Chemiluminescent immunoblots were exposed to X-ray film for 10 min. Bands corresponding to vitellogenin were identified according to previously reported criteria (Wojchowski et al., 1986; Purcell et al., 1988; Martín et al., 1995). Four replicates were obtained for each Western blot analysis.

Results

Symmetry of left and right periovaric fat body

Left and right periovaric fat bodies do not exhibit significant morphological differences. However, before starting systematic studies *in vitro*, we were interested in ascertaining whether left and right periovaric fat bodies were also functionally symmetrical.

Therefore, comparing left and right periovaric fat bodies in 4-day-old virgin females, we measured total protein contents when freshly explanted, vitellogenin released to the medium after 3 h of incubation, and vitellogenin in the tissue after that period. For each of these three parameters, the statistical analysis of data (either using a paired or unpaired t-test) showed no significant differences between left and right periovaric fat bodies. In addition, the three sets of data fit the corresponding linear regressions with fairly good coefficients of correlation (Fig. 2).

Protein content of periovaric fat body during the first gonadotropic cycle

We were then interested in ensuring that the periovaric fat body is an anatomical unit representative of the whole organ. Therefore, we measured the protein content of the periovaric fat body, in comparison with that corresponding to abdominal fat body (which represents c. 90% of the whole organ), during the first reproductive cycle.

The results (Fig. 3) showed that the pattern was identical in both cases, although with logical differences in magnitude. Protein contents showed a clear increase from day 2 to day 4, and steadily decreased thereafter until day 7, just before the formation of the ootheca.

Production of vitellogenin in relation to incubation time

The first step in the development of the incubation method was to study the influence of incubation

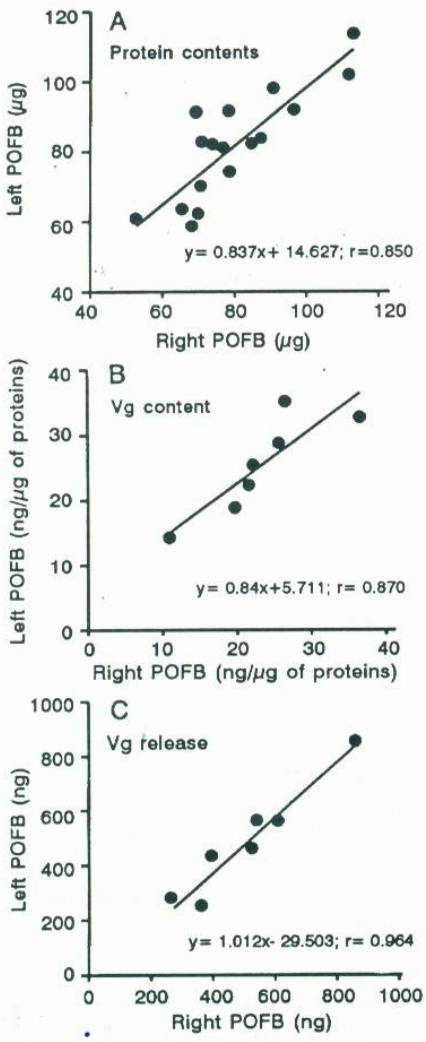

Fig. 2. Symmetry of left and right periovaric fat body (POFB) in 4-day-old virgin females of *B. germanica*. A. Protein content (µg) of a freshly explanted POFB. B. Vitellogenin (Vg) content (ng of Vg/µg of POFB proteins) after 3 h of incubation. C. Vitellogenin (Vg) release (ng) after 3 h of incubation.

time on the production of vitellogenin. This would allow us to define the optimal period of incubation, and to ascertain whether vitellogenin levels present in the medium and also in the incubated tissue would be sufficient for routine quantification. For this purpose vitellogenin levels in both tissue and medium were measured after periods of 1, 3, 5, 7 and 9 h of incubation of periovaric fat body from 5-day-old females.

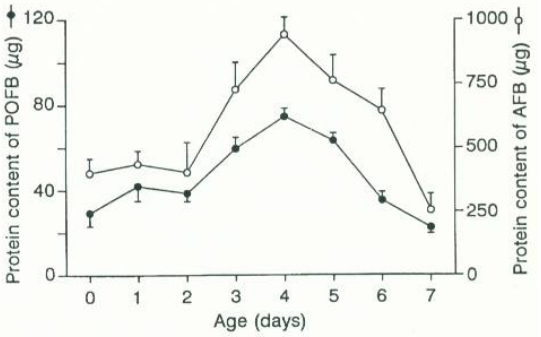

Fig. 3. Total protein contents of a periovaric fat body (POFB), compared with those of the whole abdominal fat body (AFB), during the first gonadotropic cycle in *B. germanica*. Values are expressed as the mean ±SEM (n= 8–23).

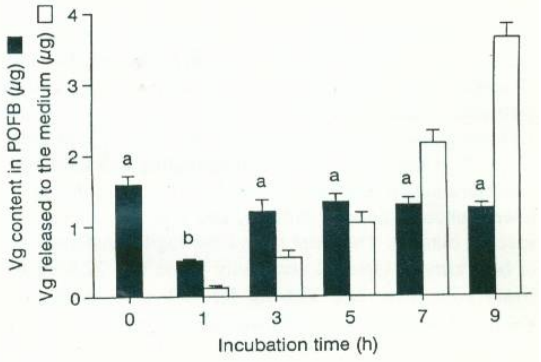

Fig. 4. Production *in vitro* of vitellogenin (Vg) by periovaric fat body (POFB) from 5-day-old virgin females of *B. germanica* in relation to the incubation time. Values are expressed as the mean ±SEM (n=7–15). Freshly explanted POFB (incubation time = 0 h) were used as reference for Vg content. Different letters (a, b) at the top of black columns indicate significant differences (Student's t-test) at p=0.01.

The results (Fig. 4) showed that the periovaric fat body reached and maintained the levels of vitellogenin content observed *in vivo* after 3 h of incubation. It was also observed that the amount of vitellogenin released to the medium was incubation time-dependent, and that from 5 to 9 h of incubation (i.e., when the vitellogenin content of the tissue was similar to that of *in vivo* conditions), the release rates were close to linearity.

Western blotting analyses of vitellogenin

In order to assess the correct functioning of the

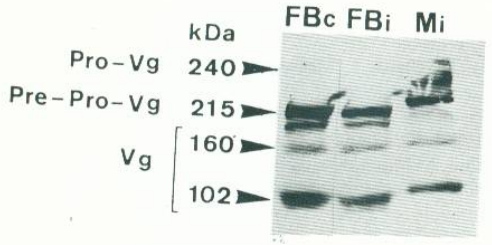

Fig. 5. Immunodetection on ECL Western-blots of vitellogenin produced by the periovaric fat body of 5-day-old virgin females of *B. germanica* incubated *in vitro* for 7 h. Lane FBc: a periovaric fat body freshly explanted (control). Lane FBi: a periovaric fat body after 7 h of incubation. Lane Mi: medium of incubation (pool of two media). The antiserum reveals the bands corresponding to pre-provitellogenin (Pre-Pro-Vg, 215 kDa), to pro-vitellogenin (Pro-Vg, 240 kDa) and to the subunits of vitellogenin (Vg, 160 and 102 kDa). Note that lane Mi eluted somewhat inclined with respect to both FB lanes.

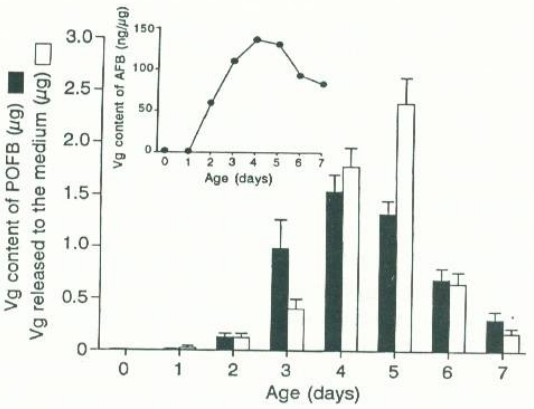

Fig. 6. Vitellogenin (Vg) production *in vitro* by periovaric fat body (POFB) during the first gonadotropic cycle in *B. germanica*. Data are expressed as the mean ±SEM (n=7–14). The inset shows the dynamics of vitellogenin content in the abdominal fat body (AFB) *in vivo* according to Martín et al. (1995).

incubated periovaric fat body, and the vitellogenic nature of the released proteins, SDS-PAGE and ECL western blot analysis were used. SDS-PAGE demonstrated that the protein pattern of periovaric fat body from 5-day-old females which had been incubated *in vitro* for 7 h was identical to that from freshly explanted control tissue (not shown).

Concerning the vitellogenin pattern, ECL-western blot (Fig. 5) confirmed that there were no differences between freshly explanted and *in vitro* incubated periovaric fat bodies. In both cases, the antibody revealed the double band of 215 kDa, corresponding to pre-pro-vitellogenin, and two additional bands of 160 and 102 kDa, corresponding to the subunits of processed pro-vitellogenin. In the medium, the antibody again revealed the bands corresponding to the subunits of 160 and 102 kDa, those which are exported from the organ in *in vivo* conditions. In addition, we also observed the bands corresponding to pre-pro-vitellogenin (215 kDa) and pro-vitellogenin (240 kDa).

Production of vitellogenin in relation to the gonadotropic cycle

The ability of periovaric fat body to synthesize vitellogenin *in vitro* was measured during the first gonadotropic cycle (Fig. 6). Vitellogenin released to the medium was almost undetectable on days 0 and 1. However, from day 2 a remarkable increase in vitellogenin release was observed, up to maximal levels on day 5 (some 2.5 µg of vitellogenin released in 7 h). Then the levels sharply decreased towards days 6 and 7.

Values corresponding to vitellogenin content in the incubated periovaric fat body showed a similar profile to that of vitellogenin release. However, maximal values were reached on day 4 and decreased progressively thereafter until day 7. Interestingly, the pattern was parallel to that previously found in abdominal fat body *in vivo* (Fig. 6, inset).

Discussion

The purpose of the present study was to develop an *in vitro* system for physiological studies on the fat body of *B. germanica* in relation to vitellogenin production.

As the anatomical unit, we chose the periovaric fat body, a portion of the whole organ consisting of three well-defined, connected lobes surrounding each ovary. It was shown that left and right periovaric fat bodies are symmetrical, not only from a morphological point of view but also in functional terms. This allows us to choose either at random in the same specimen for experimental purposes. In addition, the parallel between total protein content of a periovaric fat body and that of abdominal fat body during the first reproductive cycle suggests that the anatomical unit chosen is representative of the whole periviscer-al organ.

The study of the influence of incubation time indicated that a minimum of 3 h is needed before the

incubated periovaric fat body reach and maintains the levels of vitellogenin content usually found *in vivo*, and that release rates were close to linearity between 5 and 9 h of incubation. According to these results, a period of 7 h of incubation was established to study vitellogenin production in relation to the first gonadotropic cycle. This experiment showed the pattern of vitellogenin production *in vitro* and that of vitellogenin content in the incubated periovaric fat body during oogenesis, and it was found that both patterns are similar to those reported by Martín et al. (1995) in abdominal fat body *in vivo*.

The identity of vitellogenin produced *in vitro* was assessed by electrophoresis and ECL western blot analysis, and the bands corresponding to pre-pro-vitellogenin (215 kDa), pro-vitellogenin (240 kDa) and to processed pro-vitellogenin (160 and 102 kDa) were recognized. The occurrence of pre-vitellogenin and pro-vitellogenin in the medium may be due to leaking from the cutting area of the incubated tissue. Even in samples of fat body, pro-vitellogenin is seldom observed due to its fast turnover (see Wojchowski et al., 1986; Purcell et al., 1988; Martín et al., 1995).

The periovaric fat body was found to be an appropriate anatomical unit to study the physiology of fat body *in vitro*, especially in relation to vitellogenin production. Results obtained are reliable and agree fairly well with equivalent data observed *in vivo*. Jointly with the EIA for quantification of vitellogenin (Martín et al., 1995), the *in vitro* system described herein would provide a good standardized method to test possible stimulatory or inhibitory effectors of vitellogenin production. Some of the questions arising in our previous studies (Maestro et al., 1994; Martín et al., 1995), such as the occurrence of a factor inhibiting vitellogenin production at the end of the reproductive cycle, can now be investigated following this new approach. In the end, an appropriately chosen part of the fat body of *B. germanica* has proven not to be such ungrateful tissue for *in vitro* studies.

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