Treatment of Immature Mice with Gonadotrophins

THE INFLUENCE OF MOUSE AGE ON THE RESPONSE OF OVARIAN ALKALINE PHOSPHATASE ACTIVITIES TO GONADOTROPHINS

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Treatment of mice aged 23–25 days with chorionic gonadotrophin induced large amounts of an ovarian alkaline phosphatase activity (phosphatase Ib) kinetically distinct from that of untreated ovaries (phosphatase I). The activities of alkaline phosphatase I and Ib varied with age in untreated mice. Phosphatase Ib appeared when serum luteinizing hormone concentrations increased (days 4–10 and days 35–45), and disappeared when concentrations were low (days 11–35). Injection of human chorionic gonadotrophin induced progressively larger amounts of phosphatase Ib activity between day 19 and day 29. However, gonadotrophin treatment failed to induce this activity on days 10–18 and 30–35. Nevertheless, during the latter period, human chorionic gonadotrophin induced especially large increases in uterine weight. Treatment at different ages with sheep luteinizing hormone plus human pituitary follicle-stimulating hormone induced a pattern of response identical with that induced by human chorionic gonadotrophin, although sheep luteinizing hormone alone was ineffective before 35 days. In contrast, human luteinizing hormone induced a response in the absence of exogenous follicle-stimulating hormone.

Homogenates prepared from the ovaries of normal pre-pubertal mice possess a weak alkaline phosphatase activity with a low K_m (approx. 0.45 mM). Treatment of similar mice with human chorionic gonadotrophin induces a large increase in activity per mg of protein and a highly significant shift in K_m (Bramley, 1974). Although human pituitary follicle-stimulating hormone alone fails to induce a response, it synergistically augments the effects of chorionic gonadotrophin in a dose-dependent manner. However, a latent period of 24 h elapses before this augmentation is fully expressed. In contrast with chorionic gonadotrophin and human luteinizing hormone, sheep luteinizing hormone alone fails to induce any response, although in the presence of follicle-stimulating hormone a full response is induced (Bramley, 1974).

The present paper examines further the changes induced by gonadotrophins and describes the variations with mouse age both of control alkaline phosphatase activity and of its responsiveness to exogenous gonadotrophins.

Materials and Methods

Mice

Mice of an outbred CFW strain were reared from birth in standard foster litters of ten females, the day of birth being termed day 0. Each foster litter was

weaned at 21 days and subsequently maintained in an individual box. Outbred LACA mice were purchased from Olac Northern, King's Norton, Birmingham 30, U.K.

Materials

Substrates and fine chemicals were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. All reagents were of the highest grade obtainable. Human chorionic gonadotrophin (Pregnyl) was obtained from Organon Laboratories, Redhill, Surrey, U.K. Human pituitary follicle-stimulating hormone was prepared in this laboratory. It had a potency of 560 i.u. of follicle-stimulating hormone per mg and a follicle-stimulating hormone/luteinizing hormone ratio of 4:1, in terms of the Second International Reference preparation of human menopausal gonadotrophin (Butt *et al.*, 1961). The preparation of sheep luteinizing hormone was NIH-LH-S₁₂, obtained from Dr. S. S. Lynch, Birmingham and Midland Hospital for Women. The preparation of human luteinizing hormone (DEAE-IRC-2) was a gift from the Medical Research Council and contained 3200 i.u. of luteinizing hormone per mg (by bioassay) and less than 7 i.u. of follicle-stimulating hormone per mg (by radioimmunoassay). Gonadotrophins were dissolved in 0.15 M-NaCl–0.01 M-Tris–HCl, pH 7.4, containing 1% (w/v) bovine serum albumin, and were administered by subcutaneous injection at the doses

Fig. 1. DEAE-cellulose column chromatography of partially purified ovarian alkaline phosphatase preparations

Mice (23–25 days of age) received daily subcutaneous injections of saline-bovine serum albumin alone (untreated) or saline medium containing a total dose of 30 i.u. of human chorionic gonadotrophin plus 2 i.u. of human pituitary follicle-stimulating hormone in three equal daily injections (gonadotrophin-treated). The mice were killed on the morning of the fourth day, ovarian homogenates were prepared, and alkaline phosphatase was extracted and partially purified as described in the Materials and Methods section. Portions (2 ml) of each extract were applied to a column (25 cm × 1 cm) of DEAE-cellulose equilibrated with 1 mM-magnesium acetate–10 mM-Tris-HCl, pH 8.5. The column was washed with magnesium-Tris buffer, then eluted with buffer containing first 40 mM-KCl (arrow 1), then 150 mM-KCl (arrow 2). Fractions (2 ml) were collected at a flow rate of 10 ml/h. The E_{280} of all fractions was measured, and 0.1 ml portions of each fraction were assayed for alkaline phosphatase activity. Fractions 26–30 (peak B) and 34–36 (peak C) were pooled from the gonadotrophin-treated material (a); fractions 27–30 and 34–37 were pooled from the untreated material (b). Shaded areas denote alkaline phosphatase activity. Unshaded areas denote E_{280} .

(i.u./10 g body weight) indicated in the legends to the appropriate experiments. Controls received saline-bovine serum albumin only.

Preparation of homogenates

Mice were killed by chloroform anaesthesia 24 h after injection. The ovaries were removed, blotted, freed of fat and connective tissue, pooled and stored

Table 1. K_m and specific activities of alkaline phosphatase in DEAE-cellulose fractions

Peaks B and C from DEAE-cellulose chromatography of partially purified alkaline phosphatase extracts were isolated as described in the legend to Fig. 1 and pooled. V_{max} and K_m were determined as described in the Materials and Methods section, and V_{max} was adjusted for the protein content of the appropriate pool measured by the E_{280} with a 1 cm light-path. It was assumed that an absorbance of 1.0 was equivalent to a 1 mg/ml solution of bovine serum albumin.

	V_{max} , (nmol/min per mg of protein)	K_m (mM)
Untreated ovaries		
Peak B	978	0.39
Peak C	55	1.00
Gonadotrophin-treated ovaries		
Peak B	282	0.45
Peak C	5770	1.10

Fig. 2. Lineweaver-Burk plots of DEAE-cellulose fractions

The rate of hydrolysis of *p*-nitrophenyl phosphate was measured over a range of substrate concentrations from 0.15 to 3.0 mM by using material from peak C of Fig. 1(a) (●) and peak B of Fig. 1(b) (○), and $1/v$ was plotted against $1/[S]$.

in ice-cold 0.3 M-sucrose–0.01 M-Tris-HCl, pH 7.4. Where required, uteri and oviducts were carefully dissected free of surrounding connective tissue and

Fig. 3. Specific activity and K_m of ovarian alkaline phosphatase in control mice of different ages

CFW mice were reared in foster litters of ten females and weaned on day 21. Mice were injected with saline-bovine serum albumin on the day indicated and killed 24h later. Ovarian homogenates were prepared as described in the Materials and Methods section; each homogenate contained ovaries from five to ten mice. Alkaline phosphatase activity was measured at at least six substrate concentrations (0.15–3.0mM), and V_{max} and K_m were calculated from Lineweaver-Burk plots. Values of V_{max} were adjusted for the protein content of the appropriate homogenate. Points represent means of data from 3–18 homogenates at each age. The limits define ± 1 s.d. from the mean. (a) V_{max} . (b) K_m .

blood vessels, blotted firmly, and weighed on a torsion balance. The pooled ovaries were homogenized by hand in an all-glass Potter homogenizer in sufficient sucrose-Tris medium to give approx. 5ml of medium per 0.1 g of tissue. Homogenates were filtered through glass wool and stored at -20°C until required.

Extraction of ovarian alkaline phosphatases

All operations were performed at 4°C . The ovarian homogenates were centrifuged at 4000 rev./min for 10 min to remove inactive nuclei, erythrocytes and cell debris, which interfere with the subsequent extraction. A 10ml portion of the supernatant, containing approx. 2mg of protein/ml, was diluted with 20ml of ice-cold 1mM-magnesium acetate-10mM-Tris-acetic acid, pH4.8, and homogenized vigorously with 10ml of ice-cold butan-1-ol in a loose all-glass Potter homogenizer. The mixture was stirred for 1h, then centrifuged at 4000 rev./min for 15 min. The lower aqueous layer was carefully removed, the pH adjusted to 8.5 by the addition of dilute NaOH, and 12ml of ice-cold ethanol added. The mixture was allowed to stand for 2h, then centrifuged at 4000 rev./min for 15 min. The active precipitate was redissolved

in 1mM-magnesium acetate-10mM-Tris-HCl, pH8.5, left overnight, then centrifuged again at 4000 rev./min for 15 min to remove inactive insoluble material. The slightly opaque solution was dialysed against 1mM-magnesium acetate-10mM-Tris-HCl, pH8.5, for 24h, and the inactive sediment was removed by centrifugation. This procedure resulted in a 20–30-fold purification of phosphatase activity. The pH had a marked influence on the butanol extraction step, which was much less efficient in alkaline conditions.

Previous studies (Bramley, 1974) have shown that homogenates of pre-pubertal mouse ovaries possess both an alkaline phosphatase and a nucleotidase activity. These two enzymes differ with regard to substrate specificity, pH optimum and sensitivity to inhibitors (T. A. Bramley & J. Kent, unpublished work). The conditions described for the efficient extraction of ovarian alkaline phosphatase activity do not cause extraction of nucleotidase activity; moreover, butanol extraction appears to inactivate the latter.

Assay methods

All assays were performed at least in duplicate with the appropriate controls. Ovarian alkaline phos-

Fig. 4. Effects of chorionic gonadotrophin on the specific activity and K_m of ovarian alkaline phosphatase in mice of different ages

CFW mice of different ages were injected with saline-bovine serum albumin containing 5 i.u. of human chorionic gonadotrophin per 10 g body weight. Injections were given on the day indicated and animals were killed 24 h later. Ovarian homogenates were prepared and V_{max} and K_m were measured as described. Points with no limits represent single determinations. Points with limits represent the means from 3–14 separate determinations. The limits define ± 1 s.d. from the mean. Shaded limits are for the data from chorionic-gonadotrophin-treated mice; unshaded limits are for control mice, from Fig. 3(a). (a) V_{max} . (b) K_m .

phatase activity was measured as described previously (Bramley, 1974) with *p*-nitrophenyl phosphate as the substrate. When comparison of reaction velocity between homogenates was necessary, reaction rates were adjusted for the protein contents of the homogenates. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Sigma, A4378, no. 436–8120) as a standard.

Statistical analysis

The standard error of the estimation of V_{max} and K_m was computed for single experiments, and means $\pm$ s.d. of V_{max} and K_m were calculated where sufficient similar experiments had been performed. Student's *t* test was used for the estimation of significance.

Results

DEAE-cellulose chromatography of ovarian alkaline phosphatase extracts

Alkaline phosphatase was extracted and partially purified from both untreated and gonadotrophin-

treated mouse ovaries as described in the Materials and Methods section, and the extracts were subjected to chromatography on DEAE-cellulose. Stepwise elution of an extract of gonadotrophin-treated ovaries with 1 mM-magnesium acetate–10 mM-Tris–HCl, pH 8.5, containing 40 mM- and 150 mM-KCl, resulted in the appearance of two peaks possessing alkaline phosphatase activity (Fig. 1a). The activity of the minor peak (B) had a K_m of 0.39 mM, and the K_m of the major peak (C) was 1.10 mM (Table 1, Fig. 2).

When a partially purified extract of untreated mouse ovaries was similarly eluted (Fig. 1b), two peaks containing alkaline phosphatase activity were again resolved in identical positions. Further, the K_m values of the two peaks were similar to the values obtained for the gonadotrophin-treated extract (Table 1, Fig. 2). However, whereas 87% of the alkaline phosphatase activity of the gonadotrophin-treated extract was eluted in peak C and only 13% in peak B, in the unstimulated ovarian extract only 7% of the alkaline phosphatase was eluted in peak C and 93% in peak B.

log [Dose of human chorionic gonadotrophin (i.u. per 10g body weight)]

Fig. 5. Dose-related responses of ovarian alkaline phosphatase to chorionic gonadotrophin at different ages

CFW mice of different ages were injected with saline-bovine serum albumin or with saline medium containing chorionic gonadotrophin at different doses corrected for body weight. They were killed 24 h later and ovarian homogenates were prepared as described. Alkaline phosphatase activity was measured at a substrate concentration of 3 mM and adjusted for protein content. Five to ten mice were used for each ovarian homogenate. Open symbols represent single determinations; solid symbols represent means of four separate experiments, and vertical bars represent 1 S.D. Δ , $\triangle$, 22–24-day-old mice; $\bullet$, $\circ$, 26–28-day-old mice; $\square$, 29–30-day-old mice; ∇ , 32–34-day-old mice.

Thus the major alkaline phosphatase activity of the unstimulated immature ovary was the low- K_m form of peak B (phosphatase I, K_m about 0.4 mM). Gonadotrophin treatment *in vivo* induced large amounts of a distinct high- K_m form of alkaline phosphatase (phosphatase Ib, K_m about 1.0 mM) which was eluted in peak C. Since the K_m values of the two forms are of the same order of magnitude, curved Lineweaver–Burk plots would be expected from a crude mixture of the two enzymes. However, no curvature was observed unless substrate concentrations of less than 0.1 mM were used. Therefore K_m was measured with a range of substrate concentrations from 0.15 to 3.0 mM.

Alkaline phosphatase activities of untreated mice of different ages

To investigate the responsiveness of the immature mouse ovary to gonadotrophins at different ages, the activity per mg of protein and K_m were measured in homogenates prepared from the ovaries of untreated and gonadotrophin-treated mice.

The activity per mg of protein of alkaline phosphatase increased from the earliest observation on day 4 to a peak at days 10–11, then declined to a minimum between days 15 and 18 (Fig. 3a). K_m also increased sharply, reached a plateau at about 1.0 mM between days 6 and 13, then fell back to approx. 0.4 mM between days 15 and 18 (Fig. 3b). The simultaneous increase in both K_m and activity per mg of protein indicates a rapid increase in alkaline phosphatase Ib activity between day 4 and day 10, followed by a decline to minimal values. A further brief rise in activity was observed on day 20, but subsequently there was no further significant change in either V_{max} or K_m until about day 35, when both parameters increased, reaching maximal values at about day 45. The mice are sexually mature by 40–45 days of age, when corpora lutea first appear. The changes in K_m and activity per mg of protein after day 20 were in good agreement with values published previously (Bramley, 1974).

Effects of human chorionic gonadotrophin on ovarian alkaline phosphatase

Mice of different ages were injected with 5 i.u. of human chorionic gonadotrophin per 10g body weight, a dose which induces a maximal response in 23–25-day-old mice (Bramley, 1974). Fig. 4 shows that the response varied greatly with age. The hormone failed to induce a significant increase either in activity per mg of protein (Fig. 4a) or in K_m (Fig. 4b) before 18 days. Thereafter, the response in activity rose sharply with increasing mouse age to a maximum at day 29. Simultaneously, a highly significant ($P < 0.001$) increase in K_m was observed (Fig. 4b), indicating the induction of the kinetically distinct alkaline phosphatase Ib activity. The response to chorionic gonadotrophin suddenly diminished between 30 and 36 days, as judged by a sharp fall, in treated mice, in both activity per mg of protein and K_m . Although the gonadotrophin induced a significant increase in K_m at this age ($0.001 < P < 0.01$), it was very much smaller than that in 21–29 day-old animals ($P < 0.001$). Finally, after 35 days the response to human chorionic gonadotrophin increased once more.

Since the increasing response of ovarian alkaline phosphatase activity between 19 and 29 days of age might be due to increasing tissue sensitivity, the effects of different doses of chorionic gonadotrophin were examined in mice of different ages (Fig. 5). In all age groups, a significant stimulation was first observed

Fig. 6. Effects of sheep luteinizing hormone and human follicle-stimulating hormone, alone and in combination, on the response of ovarian alkaline phosphatase activity at different ages

CFW mice were injected on the day indicated with saline medium containing, per 10g body weight, 10 μ g of sheep luteinizing hormone alone ($\circ$), 5.6 i.u. of follicle-stimulating hormone alone (Δ), or 10 μ g of sheep luteinizing hormone + 5.6 i.u. of follicle-stimulating hormone ($\bullet$). Animals were killed 24h later. Ovarian homogenates were prepared and V_{max} and K_m were measured as described. All points represent single determinations. The shaded and unshaded limits are from Figs. 3 and 4 for comparison. (a) V_{max} . (b) K_m .

by between 0.2 and 0.5 i.u. per 10g body weight, indicating a similar threshold of sensitivity, although the slopes of the curves and the levels of stimulation achieved were proportionally greater in the more responsive age groups.

Effects of sheep luteinizing hormone plus follicle-stimulating hormone on ovarian alkaline phosphatase

Fig. 6 shows the effects of mouse age on the response to a single injection of sheep luteinizing hormone, of human pituitary follicle-stimulating hormone and of both hormones together. The changes in activity induced by administration of both gonadotrophins together were identical with those induced by chorionic gonadotrophin (Fig. 6a). There was no response before day 18, a pronounced stimulation between 19 and 29 days, followed by a fall between 30 and 35 days and a further increase thereafter. The same dose of sheep luteinizing hormone or follicle-stimulating hormone alone failed to increase the specific activity

of alkaline phosphatase before 35 days, confirming previous observations in 23–25-day-old mice (Bramley, 1974). After 35 days, however, either hormone alone induced a response. The changes in K_m induced by sheep luteinizing hormone plus follicle-stimulating hormone were also identical with those induced by chorionic gonadotrophin (Fig. 6b). Again neither hormone alone modified K_m until after 35 days.

Effects of human luteinizing hormone on ovarian alkaline phosphatase activity

Human luteinizing hormone induced a dose-dependent increase in ovarian alkaline phosphatase activity (in 28-day-old mice) even in the absence of exogenous follicle-stimulating hormone (Fig. 7). In the presence of 10 μ g of human pituitary follicle-stimulating hormone per 10g body weight, the dose-response curve was shifted to the left by an amount corresponding to approx. 3.0 μ g of human luteinizing

Fig. 7. Effects of human luteinizing hormone, alone or with follicle-stimulating hormone, on specific activity of mouse ovarian alkaline phosphatase

LACA mice (28 days of age) were injected subcutaneously with saline-bovine serum albumin containing, per 10g body weight, various doses of human luteinizing hormone alone (●) or with 10μg of human follicle-stimulating hormone (■). They were killed 24h later and ovarian homogenates were prepared. The specific activity of alkaline phosphatase was measured as described in the Materials and Methods section. Points represent means and vertical bars represent $\pm$ s.d. of three separate experiments. Points with no bars represent single determinations.

Fig. 8. Effects of mouse age on uterine weight response induced by human chorionic gonadotrophin

LACA mice of different ages were injected subcutaneously with saline-bovine serum albumin containing human chorionic gonadotrophin at the doses indicated and were killed 24h later. The uteri and oviducts were carefully dissected out, blotted firmly and weighed on a torsion balance. Points represent means of several determinations. The limits shown define $\pm$ 1 s.d. for all points of all age groups except 30–33 days, for which the vertical bars represent $\pm$ 1 s.d. *n* indicates the number of mice used per point. ○, 23 $\pm$ 1-day-old mice (*n* = 7); □, 25 $\pm$ 1-day-old mice (*n* = 8); △, 27 $\pm$ 1-day-old mice (*n* = 7); ▽, 34 $\pm$ 1-day-old mice (*n* = 5); ●, 30 $\pm$ 1- and 32 $\pm$ 1-day-old mice (*n* = 14).

hormone. This value correlated well with the known contamination of this preparation by human luteinizing hormone. Thus the effect of human luteinizing hormone resembled that of chorionic gonadotrophin, but differed from that of sheep luteinizing hormone.

Changes in uterine responsiveness to gonadotrophins in mice of different ages

Chorionic gonadotrophin injected into animals aged 22–29 days increased uterine weight in a dose-dependent manner up to a maximum of approx. 100% (Fig. 8), presumably owing to enhanced ovarian oestrogen secretion. However, a dramatic

change in the dose-response curve was observed between 30 and 33 days of age. Low doses of chorionic gonadotrophin induced very large (>300%) increases in uterine weight, indicating a marked increase in oestrogen secretion during this period. No age-related changes in uterine responsiveness to exogenous oestrogens were observed (T. A. Bramley, unpublished work).

Discussion

DEAE-cellulose chromatography of partially purified extracts of alkaline phosphatase demonstrated that the ovaries of normal pre-pubertal mice possess large amounts of a weak low- K_m alkaline phosphatase activity, phosphatase I, and very small amounts of a distinct high- K_m activity, phosphatase Ib. Treatment with chorionic gonadotrophin *in vivo* greatly increased the activity of phosphatase Ib. However, this activity could be induced by exogenous gonadotrophin only at certain ages, depending critically on the stage of sexual development reached by the mouse.

Endogenous serum luteinizing-hormone concentrations were measured in CFW mice by Dullaart *et al.* (1975). Luteinizing-hormone concentrations rose from day 4 to day 9, then fell sharply, probably as a result of negative feedback due to rising ovarian steroidogenesis (Kent, 1975), and reached low values by day 13. These low concentrations persisted (except for a transient rise at day 17–18) up to day 35, then began to increase sporadically until the age of first ovulation. Thus the age-related fluctuations in circulating luteinizing-hormone concentration regularly preceded, by about 24h, the changes in ovarian alkaline phosphatase Ib activity seen in normal mice. This interval equals that required for the full induction of the enzyme by injected gonadotrophin (Bramley, 1974).

Injections of chorionic gonadotrophin failed to enhance alkaline phosphatase activity between days 5 and 10, perhaps initially because the ovary was already maximally stimulated by endogenous gonadotrophins. After day 9, however, although both serum luteinizing hormone and ovarian alkaline phosphatase Ib declined, there was still no response to injected gonadotrophin. The ovary itself appeared to be refractory.

After day 18, a sharp rise in activity per mg of protein occurred in control ovaries in the absence of a marked change in K_m , indicating increased activity of phosphatase I. Simultaneously, sensitivity to chorionic gonadotrophin was recovered, as judged by the dramatic increase in alkaline phosphatase Ib induced between day 19 and day 29. From about day 30 to day 35, however, the response was much less. This period of diminished alkaline phosphatase response coincided with the period of unusual sensitivity to gonadotrophins in terms of oestrogen secretion.

After day 35, phosphatase Ib activity again rose in the control animals as endogenous gonadotrophin secretion increased before the onset of puberty. Moreover, a large increase in phosphatase Ib could then be induced either by sheep luteinizing hormone or by follicle-stimulating hormone alone, perhaps because sufficient endogenous gonadotrophin was by this time available to synergize with the injected material. Sheep luteinizing hormone plus follicle-stimulating hormone also induced alkaline phosphatase Ib activity between 19 and 29 days, although at this age neither hormone alone was effective. Follicle-stimulating and luteinizing hormones are known to affect synergistically various ovarian processes, but this is the first confirmed report of synergism at the enzyme level.

In contrast with sheep luteinizing hormone, human luteinizing hormone (like chorionic gonadotrophin) was effective alone. This contrast cannot be accounted for by different rates of removal from the circulation, nor by contamination of the chorionic gonadotrophin preparation by follicle-stimulating hormone (Bramley, 1974). A major difference in the interaction of the two luteinizing hormones with ovarian cells is indicated. Since the response to the human material was apparently unaffected by an excess of follicle-stimulating hormone, this system may offer a rapid and convenient bioassay for human luteinizing hormone. Attention should meanwhile be drawn to the relevance of the age-related changes in response to the bioassay of gonadotrophins in general.

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