Identification, purification and immunohistochemical detection of the inhibitor from porcine ovarian follicular fluid to compensatory ovarian hypertrophy in mice

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Summary. An inhibitor to compensatory ovarian hypertrophy in mice was detected in porcine follicular fluid. Compensatory hypertrophy was inhibited by treatment with 0·6 ml whole follicular fluid, 0·2 ml of the non-dialysable fraction, or a fraction of the fluid salted out by ammonium sulphate at a saturation of 14·5–18·5%. The salted-out fraction was separable into two peaks by Sephadex G-200 column chromatography and the second peak, detectable as a single band by polyacrylamide gel disc electrophoresis, contained all the inhibitory activity. Specific fluorescence of an antiserum to the second peak was demonstrated at the granulosa cells.

Introduction

It has been reported that bovine and porcine follicular fluid from which steroids have been removed are able to reduce peripheral blood concentrations of FSH in the rat (de Jong & Sharpe, 1976; Hopkinson et al., 1977; Welschen, Hermans, Dullaart & de Jong, 1977; Marder, Channing & Schwartz, 1977). Follicular growth in post-natal mice can also be inhibited by injection of bovine follicular fluid (Peters, Byskov & Faber, 1973). Sato & Ishibashi (1977) have demonstrated the presence of an inhibitor in the non-dialysable fraction of bovine follicular fluid which occurs in mice.

The present experiments were to examine the presence of this inhibitor in porcine follicular fluid, to attempt purification and to determine its localization in the ovarian tissue.

Materials and Methods

The ovaries were obtained from sows at a local slaughterhouse, and transported to the laboratory in physiological saline (0·154 M-NaCl) at 4°C within 3 h. Follicular contents were aspirated from follicles of 2–6 mm in diameter within 5 h of death, centrifuged at 10 000 g for 20 min at 4°C. The supernatant, i.e. the follicular fluid, was kept at −20°C until use. The follicular fluid was then dialysed with Visking tube (seamless cellulose tubing, type 8/32) in 0·154 M-NaCl for 12 h at 4°C and the non-dialysable fraction was centrifuged at room temperature for 15 min at 2000 g. Determination of protein was made by the method of Lowry, Rosebrough, Farr & Randall (1951).

Purification of the inhibitor

Selective precipitation. Fractionation of the follicular fluid into 4 fractions, A, B, C and D, was accomplished by salting out with ammonium sulphate at saturations of 14·5, 18·5, 22·5 and 40·0% (w/v). The follicular fluid saturated at each concentration of ammonium sulphate was kept for 2 h at 4°C, then centrifuged and the precipitate was separated. The separated fractions were dissolved in distilled water and dialysed to remove the ammonium sulphate. The small particles precipitated during dialysis were removed, and the supernatant was freeze dried. The fractions were extremely soluble and their biological activities were retained well in distilled water.

Gel chromatography. The greatest inhibitor activity was detected in Fraction B of the 4 fractions obtained by the above procedure. Fraction B was dissolved in 1 ml distilled water and filled into a column of Sephadex G-200 (5 × 100 cm, height of bed 95 cm). Elution was carried out with distilled
water and the concentration of the eluate was determined electrophotometrically by measuring the absorbance at 280 nm. Gel filtration was performed at 4°C and a flow rate of 4.5 ml/h. The volume of each fraction was 1.85 ml, and the separated fractions were freeze dried. Polyacrylamide gel disc electrophoresis was performed according to the method described by Smith (1968).

**Examination of compensatory ovarian hypertrophy in mice**

Young adult mice of the JCL-ICR strain were kept in an air-conditioned room in 13 h light/24 h. The mice were anaesthetized with ether and unilaterally ovariotomized between 09:00 and 11:00 h on the day of dioestrus. The left ovaries were weighed immediately after removal on a torsion balance. The whole and fractionated samples from follicular fluid to be tested were injected into experimental mice, while the control mice received 0.154 M NaCl: 5 mice/group were used in each experiment and each experiment was replicated once.

The animals were killed 3 days after the operation and the right ovaries were weighed. The degree of compensatory hypertrophy was calculated and expressed as the percentage increase in the weight of the right ovary (ovarian weight at death) to that of the left (ovarian weight at hemi-spaying).

**Immunohistochemical detection of the inhibitor**

Japanese white rabbits (KBL) weighing 3–4 kg were immunized with the freeze-dried substance from the second peak obtained by gel filtration. The initial subcutaneous injection was made with 600 µg of the substance mixed with Freund’s complete adjuvant and booster injections, each of 200 µg of the substance, were given 2 and 3 weeks later. On the 21st day after the last injection, the rabbits were bled from the jugular vein and the serum was separated and stored at −20°C until use. All serum samples were salted out at 4°C with 40% ammonium sulphate by addition of 1 volume 0.154 M NaCl and 1:33 volumes of cold saturated ammonium sulphate. The separated precipitate, which contained approximately 530 mg protein, was washed once with 50% ammonium sulphate. The precipitated protein was centrifuged, suspended in 0.154 M NaCl, dialysed for 8 h against 0.45% NaCl solution. The globulin fraction was then conjugated with fluorescein isothiocyanate (FITC); the globulin fraction was diluted with 0.15 M NaCl and sodium carbonate–bicarbonate buffer (0.5 M, pH 9.0), so that the final solution contained 10 mg protein/ml. After cooling to 4°C, 0.05 mg FITC was added for each mg protein. The mixture of globulin and FITC was shaken by a rotary shaker for 6 h at 4°C. The conjugated globulin fraction was placed in a Sephadex G-25 column (3.5 × 50 cm). Elution was carried out with 0.1 M NaCl and NaHPO₄ buffer (0.005 M, pH 7.0), and the FITC-conjugated globulin was purified on a diethylaminoethyl (DEAE) cellulose column according to the method of McDevitt, Peter Pollard, Harter & Coons (1963).

Pig ovaries were frozen immediately after removal by immersion in solid CO₂–isopentane. Sections (10 µm thick) were prepared in a cryostat at −20°C and were fixed in 95% ethanol for 5 min. The slides were placed in a moist chamber and the preparations were overlaid with a drop of FITC-conjugated globulin and kept for about 12 h at 4°C. After 3 washes, each for 10 min, with cold phosphate-buffered saline (pH 7.1), the slides were removed and a coverslip was applied and sealed with a drop of 90% glycerol in buffered saline. The sections were examined by fluorescence microscopy.

**Results**

**Effects of whole follicular fluid and fractions from follicular fluid on compensatory ovarian hypertrophy**

As shown in Table 1, the hypertrophy of the remaining ovary was significantly suppressed in the mice injected with 0.6 ml whole follicular fluid or with 0.2 or 0.6 ml of the non-dialysable fraction of follicular fluid.

The effects of Fractions A–D are summarized in Table 2: the greatest suppression of compensatory hypertrophy occurred with Fraction B. Gel chromatography of Fraction B gave two peaks (Text-fig. 1): no protein was detected in the fractions eluted earlier than Peak I. The freeze-dried material of only the second peak (Fractions 85–92) caused suppression of compensatory hypertrophy in the mice (Table 3). The material from Peak I (Fractions 71–79) had no suppressive effect.
Fig. 1. Patterns of polyacrylamide gel disc electrophoresis in 7-5% gel at pH 8.3 of (a) whole follicular fluid and (b) Peak II (Fractions 85–92 from Fraction B, see Text-fig. 1), arrowed.

Fig. 2. Mouse ovarian tissue treated with antiserum to the inhibitor conjugated with fluorescein isothiocyanate. Specific fluorescence is detected at the granulosa cells. A, antrum; G, granulosa cells; T, thecal cells. ×200.
Table 1. Effects (mean ± s.e.m.) of whole porcine follicular fluid and its non-dialysable fraction on compensatory ovarian hypertrophy in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (ml/mouse)</th>
<th>No. of mice treated</th>
<th>Wt of left ovary (mg)</th>
<th>% hypertrophy of right ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>0.6</td>
<td>10</td>
<td>5.1 ± 0.5</td>
<td>51.0 ± 3.7</td>
</tr>
<tr>
<td>Whole follicular</td>
<td>0.2</td>
<td>10</td>
<td>4.6 ± 0.4</td>
<td>47.4 ± 3.2</td>
</tr>
<tr>
<td>fluid</td>
<td>0.6</td>
<td>10</td>
<td>4.9 ± 0.3</td>
<td>35.9 ± 5.5*</td>
</tr>
<tr>
<td>Non-dialysable</td>
<td>0.2</td>
<td>10</td>
<td>4.7 ± 0.4</td>
<td>21.6 ± 2.3†</td>
</tr>
<tr>
<td>fraction</td>
<td>0.6</td>
<td>10</td>
<td>4.8 ± 0.4</td>
<td>-7.8 ± 4.1†</td>
</tr>
</tbody>
</table>

* Significantly different from the control value (P < 0.05, Student's t test).
† Significantly different from the control value (P < 0.01), and from the equivalent whole follicular fluid value (P < 0.01, Student's t test).

As shown in Pl. 1, Fig. 1, a concentrated sample from Peak II was detected as a single band, preceding those of the albumin fractions of the follicular fluid, when studied by polyacrylamide gel disc electrophoresis.

Table 2. Effects (mean ± s.e.m.) of the porcine follicular fluid fractions obtained by ammonium sulphate precipitation on compensatory ovarian hypertrophy in mice

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Saturation of ammonium sulphate (%)</th>
<th>Dose (µg/mouse)</th>
<th>No. of mice treated</th>
<th>Wt of left ovary (mg)</th>
<th>% hypertrophy of right ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>4.9 ± 0.3</td>
<td>48.3 ± 6.9</td>
</tr>
<tr>
<td>A</td>
<td>14.5</td>
<td>400</td>
<td>10</td>
<td>5.1 ± 0.4</td>
<td>43.1 ± 4.9</td>
</tr>
<tr>
<td>B</td>
<td>18.5</td>
<td>400</td>
<td>10</td>
<td>4.9 ± 0.3</td>
<td>16.2 ± 3.9*</td>
</tr>
<tr>
<td>C</td>
<td>22.5</td>
<td>400</td>
<td>10</td>
<td>4.6 ± 0.5</td>
<td>41.7 ± 5.4</td>
</tr>
<tr>
<td>D</td>
<td>40.0</td>
<td>400</td>
<td>10</td>
<td>4.8 ± 0.4</td>
<td>45.1 ± 7.4</td>
</tr>
</tbody>
</table>

* Significantly different from the control value (P < 0.01, Student's t test).

Table 3. Effects (mean ± s.e.m.) of the fractions separated from Fraction B by column chromatography on compensatory ovarian hypertrophy in mice

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Dose (µg/mouse)</th>
<th>No. of mice treated</th>
<th>Wt of left ovary (mg)</th>
<th>% hypertrophy of right ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>—</td>
<td>10</td>
<td>4.7 ± 0.4</td>
<td>53.1 ± 5.3</td>
</tr>
<tr>
<td>Peak I</td>
<td>100</td>
<td>10</td>
<td>4.9 ± 0.3</td>
<td>52.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10</td>
<td>4.7 ± 0.5</td>
<td>54.2 ± 6.3</td>
</tr>
<tr>
<td>Peak II</td>
<td>100</td>
<td>10</td>
<td>4.8 ± 0.3</td>
<td>31.1 ± 3.8*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10</td>
<td>4.6 ± 0.5</td>
<td>19.2 ± 6.7†</td>
</tr>
</tbody>
</table>

* Significantly different from the control value (P < 0.05, Student's t test).
† Significantly different from the control value (P < 0.01, Student's t test).

**Immunohistochemical localization of the inhibitor in the ovarian tissue**

The preparation from porcine ovary stained with antisera conjugated with FITC fluoresced brightly at the granulosa cells, but not at the theca cells. Ovaries treated with normal serum and conjugate alone did not show any specific fluorescence.
Text-fig. 1. Chromatogram of the Fraction B salted out at 14·5 to 18·5% ammonium sulphate on Sephadex G-200 (5 × 95 cm). Each fraction contained 1·85 ml of the effluent. Peak I is represented by Fractions 71-79, and Peak II by Fractions 85-92.

Discussion

Injection of 0·2 and 0·6 ml of the non-dialysable fraction separated from the follicular fluid partly or completely suppressed the compensatory ovarian hypertrophy in the mice, but the suppression after injection of whole follicular fluid was significantly less, suggesting that follicular fluid may contain a 'masking factor'.

Oestrogen and progesterone are able to suppress compensatory ovarian hypertrophy in rats (Benson, Sorrentino & Evans, 1969). The concentrations of these hormones in porcine follicular fluid are 15·5 ng oestrogen/ml and 203 ng progesterone/ml (Marder et al., 1977) and the amounts in the 0·6 ml volume of follicular fluid used in the present experiments were therefore 9·3 ng oestrogen and 121·8 ng progesterone. These quantities might have been too small to suppress the compensatory hypertrophy because Benson et al. (1969) reported that a daily dose of 25 µg oestrogen and 1·0 mg progesterone for 6 days was required for adequate suppression. It is also possible that these steroids might have been dialysed with the Visking tube during the present experiment. It is therefore suggested that the suppression of compensatory ovarian hypertrophy in mice by the non-dialysable fraction from whole pig follicular fluid may be due to a factor other than steroids. The retention of activity in the substance obtained after purification supports this suggestion. The inhibitor isolated from porcine follicular was similar to that obtained from bovine follicular fluid (Sato & Ishibashi, 1977, 1978), although the single band detected by electrophoresis was clearer with the bovine material. Benson et al. (1969) considered that the increase in FSH concentrations plays an important role in the mechanism of compensatory ovarian hypertrophy. The inhibitory factor from follicular fluid may therefore act by depressing FSH activity at the ovary or by decreasing plasma FSH values.

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References


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